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TRANSFORMATION OF TWO PLASMIDS INTO MAIZE CALLUS USING PARTICLE BOMBARDMENT

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ABSTRACT

Particle bombardment offers a simple method for the introduction of DNA into maize callus. Two separate plasmids (co- transformation) were used in this study to improve maize transformation. Anthranilate synthase (AS) is a key enzyme in tryptophan biosynthesis. ASA2 -NOS-ASB (tobacco feedback insensitive anthranilate synthase).gene and bar gene were used to increase the Trp synthesis and herbicide resistance in transformed lines. 6- Methyl-DL-tryptophan) 6MT (75-125) µm and PPT (3-5) mg/ml were used as selective agents to inhibit untransformed callus. 36 plants came from transformation and tested via PCR and southern blot to verify the presence of introduced plasmid DNAs. These results demonstrate that 100 µm 6MT and 5 mg/l PPT were the best concentrations to get real transformed plants according to molecular analysis.

INTRODUCTION

Maize is one of the most important food crops in the world. It is widely grown annual crops cultivated world wide in the USA alone, maize production reached nearly 300 million metric tones in 2003 (Gadob et al., 2006).

Genetic engineering has the potential to improve the economic value of maize by introducing genes to improve its adaptability and agronomic characteristics. Transformation of cereal plants was

successfully achieved after the development of the particle bombardment technology that uses high velocity particles to deliver DNA directly into the cell nucleus.

In this study, two plasmids were used , the first one is PAHC25 which contains both a selectable marker (bar) gene and its derived from the soil bacterium *Streptomyces hygroscopicus* (Thompson *et al*., 1987) and encodes phosphinothrycin acetyltransferase that catalyzes the transformation of L-phosphiothricin (PPT) to acetyl- phosphiothricin and confers herbicide resistance in transgenic plant (Zhao, 2007) and UidA gene codifies the enzyme β- glucuronidase (gene reporter GUS). The problem of field littering would be effectively solved by using herbicides, but in case of maize cultivation this process becomes multistage and too labor- intensive due to the fact that this culture has a high sensitivity to most herbicides. However, this problem would be solved by gene engineering methods that would allow the introduction to the traditional selection process of maize genotypes which would have the effect of resistance to herbicides.

The bar gene from *Streptomyces hygroscopicus* (De Block *et al.*,1987) has been widely used as a selectable marker for transformation and to provide resistance to the broad-spectrum herbicide ammoniu glufosinate { ammonium-DL- homoalanine-4- methyl phosphinate} also called L-phosphinothricin. This is a herbicide with a very short half-life in soil as it is quickly degraded through microbiological activity (Go¨tz et al. 1983).

Among available herbicide- based selection systems, those that are based on resistance to phosphinothricin (PPT) or the related compounds bialaphos and ammonium glufosinate (GA) are particularly attractive. PPT is a glutamate analogue which irreversibly binds glutamine synthetase, leadind to a disruption of nitrogen metabolism in plant tissues (D'Halluin *et al*.,1992), and this disruption indirectly inhibits photosynthesis and leads to chlorosis in green tissues (Hess, 2000). PPT is produced for agricultural use in two forms: the free chemical synthate ammonium glufosinate or a component of the natural tripeptide bialaphos.

T he selectable marker bar, which was isolated and *S. hygroscopicus*, respectively (Strauch *et al*.,1988), encode enzyme that detoxify PPT.

The second plasmid is C2ASA2-NOS-ASB 16.6 kb (tobacco feedback insensitive anthranilate synthase). Anthranilate synthase (AS) catalyzes the first committed step in the sequence of reactions which lead to the biosynthesis of tryptophan from chorismate.

Anthranilate synthyase (AS) catalyzes the conversion of chorismate into anthranilate, the first reaction leading from the common aromatic amino acid (shikmic acid) pathway towards the biosynthesis of Trp in both microorganisms and plants, and is feedback inhibited by the end product, Trp. (Ishikawa *et al*., 2003)

Tryptophane (Trp) is an essential amino acid for animal growth and cereal crops such as wheat, maize and rice exhibits relatively low content of Trp in seed proteins. In higher plants, the Trp biosynthetic pathway provides an amino acid for protein synthesis as well as precursors for secondary metabolites such as the phytohoromone indole-3- acetic acid and phytoalexins (Normanly *et al*., 1995, Radwanski and Last 1995, and Tsuji *et al*., 1993)

Toxic tryptophan (Trp) analogs have been used to select many resistant plant cell cultures and plants that in most cases derive their resistance from the presence of a Feedback insensitive form of the tryptophan control enzyme, anthranilate synthase (Inaba *et al*., 2007).

The aim of this study was the optimization particle bombardment transformation parameters and the creation of transgenic lines of maize expressing bar gene and anthranilate synthase gene. This work has done in Illinois University and two plasmids are used from Widholm's lab.

MATERIALS AND METHODS

1. Plasmids

1.1. Plasmid pAHC25 (fig, 1) includes a selectable marker (bar gene) and a screenable marker β- glucuronidase (uidA)

1.2. Plasmid C2ASA2-NOS-ASB (fig, 2) contains a selectable marker (ASA2 α & β) the coding region of kanamycin, gusA, under control of the cauliflower mosaic virus (CAMV). ANOS polyA (nopline synthase) terminator sequence

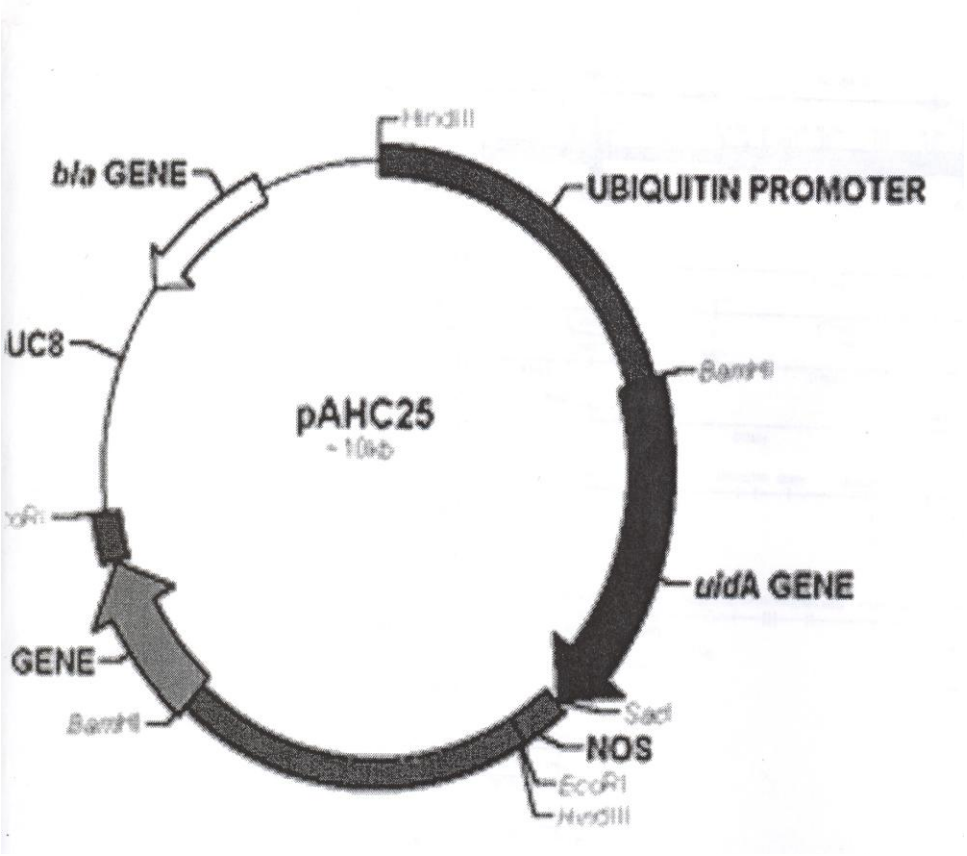


Figure 1- Schematic drawing of the plasmid pAHC25 used to produce transgenic maize plants. The plasmid 10 kb consists of the uidA, and bar expression cassettes.

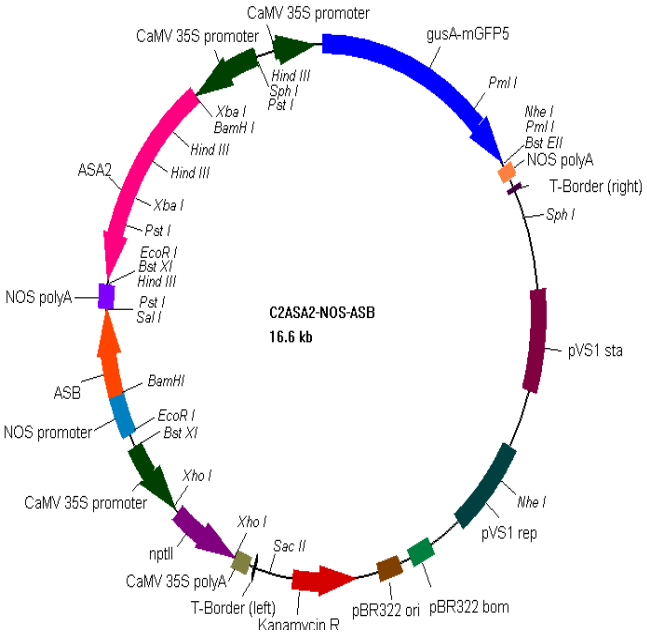


Figure 2- Schematic drawing of the plasmid C2ASA2-NOS-ASB.The plasmid 16.6 kb consists of ASA2,ASB, CaMV35S : cauliflower mosaic virus 35 S promoter and 3'NOS : the polyadenylation signal of nopaline synthase.

2. Plant materials and transformation

Calluses derived from immature embryos of HiII were used for bombardment, Calluses were subcultured on N6E medium (callus initiation), N6 salt, plus 1ml/L(1000X) N6 vitamin stock, 2 mg/l 2, 4-D, 3% sucrose, 100 mg/L myo-inositol, 2.76 g/Lproline,100 mg/L casein hydrolysate and 2.5% gelrite. , pH5.8, filter sterilized silver nitrate (25µM) added after autoclaving (Chu *et al*., 1975)

3. Particle Coating

The gold particles were coated with both plasmids with concentration1µg (ratio 6:1 for ASA2: bar gene) using $CaCl_2$ /spermidine.Gold particles (6-10) mg was washed with 1 ml of ethanol

(from the freezer). The particles were rinsed with 1 ml of cold sterile distilled water.

For DNA preparation 220 µl 2.5 M $CaCl_2$, 1µg of plasmid DNA and 50 µl 0.1 M spermidine (freshly prepared) were mixed in a microfuge tube by vortexing after each addition. The mixture was kept on ice for 5 min and vortexes for 5 min, micro centrifuged at 5000 rpm for 1 min. The supernatant was removed and the pellet washed with 250 µl of EtOH, microfuge tube were shacked with hand and left on ice for 5 min, micro centrifuged at 5000 rpm for 1 min, once with absolute ethanol, and pelleted. The pellet was resuspended in 40 µl of absolute EtOH and briefly sonicated before shooting. 10 µl of the DNA –coated particles were pipetted onto each macrocarrier (washed in absolute EtOH, dried before uses).

4. Bombardment optimization experiments

Before bombardment, calluses were centered on N6OSM (osmotic medium) supplemented with 1ml/L N6 vitamin stock , 2 mg/L2,4-D, 100 mg/Lmyo-inositol, 0.69 g/Lproline , 30 g/L sucrose , 100 mg/Casein hydrolysate, 36.4 g/L sorbitol, 36.4 g/L mannitol, 2.5 g/L gelrite , pH5.8 , filter sterilized silver nitrate (25µM) added after autoclaving 4 hours prior to bombardment, each plate was bombarded once with a rupture disk pressure (1100 psi) Bio-Rad . Target cells were bombardment.

5. Selection for stable transformation

After bombardment calli were kept in their respective plates for 1 hour and then transferred off the osmatic medium to callus initiation medium. After 7-10 days calli were transferred to N6S (selection medium) which was similar to callus initiation medium but without proline and casein hydrolysate, filter sterilized silver nitrate (5μM) added after autoclaving.

Every experiment were divided into two equal plates, the half one was contained 6- Methyl-DL-tryptophan) 6MT to select for ASA2, the second half was selected for bar gene using PPT. Three concentrations were used for 6MT (75,100 and 125mg/ml). But for PPT (3, 4 and 5mg/L) were used.

6. Regeneration of transgenic plants

After 2 rounds of selection, the surviving embryogenic calli (friable, yellowish) were regenerated on MS mediumI (Murashige and Skoog 1962)supplemented with 1ml/L(1000X) MS vitamin stock, 100mg/Lmyo-inositol, 60 g/Sucrose , 3g/Lgelrite, pH5.8 ,after autoclaving 6MT and PPTwere added.

The calluses were incubated for 2 weeks at 25^{o}C in the dark. After 2 weeks, transformed green calluses were transferred to the light on Regeneration medium (II) which is the same for MSI except MSII supplemented with 30 g/L sucrose.

7. Histochemical detection of GUS activity

After bombardment calli were incubated for 24h-48h in the dark at 28^{o}C. The best substrate available for histochemical localization of β-glucuronidase activity in calli is 5-bromo-4- chloro-3- indolyl glucuronide (X-Gluc). Some samples of tissue and cells were put in wells of microtiter plate. X-Glue solution were added which contains 380 μl IM Na_2HPO_4, 620 μl 1M NaH_2PO_4, 200 μl 0.5 mM EDTA,1000 μl 0.5 mM K-ferricyanide,1000 μl 0.5 mM K-ferrocyanide, X-Glue (10 mg in 40 μl of DMF) and 6760 μl water.Vaccuum for 5-10 min, avoid air bubbles and floating samples, incubate on 37^{o}C over night, seal with parafilm.

The next day, samples were washed with 70% ETOH several times. Under binocular blue spots were appeared. Photographs were taken using Leitz stereomicroscope.

8. DNA extraction

The CTAB method based on Doyle and Doyle (1987) was used for DNA extraction from maize transgenic leaves. Samples were dried and frizzed over night. Samples were ground in fast prep (FP 120) for 20 sec. CTAB buffer (100Mm Tris, 1.4 M NaCl, 20 Mm EDTA, 2% (w/v) CTAB (hexa- decyltrimethylammonium bromide) and 1% -Mercaptoethanol) were added to samples (incubated in water bath 65^{o}C prior to use). Samples were put in water bath for 30 min. After that, 1 volume of Phenol: Chloroform: Isoamylalcol (PCI) was added, mix by inverting the tubes gently, centrifuge at 12000 rpm at 22^{o}C for 10 min, the upper layer were taken in new tubes, 1 volume Chloroform:Isomamylalcol (24:1 v/v) was added , mix the tubes gently, centrifuge with the last condition. The upper layer were collected in a fresh eppendorf, 2/3 volume of ice cold Isopropanol and 1/10 volume of 5M ammonium acetate was added. Samples were left in the fridge for 1h to overnight.

Samples were centrifuged for 15 min at 4^{o}C (12,000 rpm), screw the supernatant, 70% ETOH were added to pellet, centrifuged for 2 min. Samples were kept in the incubator until there was no ETOH , 50 µl of water were added and kept on the incubator for 10 min to dissolve all DNA in the water. 1 µl of RNase were added to all tube. Samples were stored at – 20^{o}C.

9. Molecular analysis

Enzymatic amplification by PCR was performed using Taq DNA polymerase. The primers used for amplification are listed in table (1).
The 20 µl reaction mixture consisted of 2 µl of Taq buffer 10X, 0.5 µl of dNTPS 10 mM, 1 µl of each primer, and 0.3 µl of Taq polymerase.

Table (1) Primers and PCR programmers for four gene.

Gene	5'Forward primer-3'	5'-Reverse primer-3'	PCR conditions
Bar	TGCACCATCGTCAA CCACTA	ACAGCGACCACGCTCTT GAA	1cycle at 94^{o}c for 5 min, 30 cycles at 94^{o}cfor 45s,annealing at 57^{o}cfor 30s ,72^{o}cfor 60s and a final extension at 72^{o}c for 7 min
ASA2	TCTGTACACTTCAAAT GGGTCAGC	CTAAAAGCGGGAACTTG ATTCCGC	1cycle at 94^{o}c for 5 min, 35cycles at 94^{o}cfor 30s,annealing at 57^{o}cfor 45s ,72^{o}cfor 60s and a final extension at 72^{o}c for 7 min
ASB	TGTCCAAGATCCCATG ACGATTCC	CAGAAATCCACAGAACC GGGAGAT	1cycle at 94^{o}c for 5 min, 35cycles at 94^{o}cfor 45s,annealing at 57^{o}cfor 45s ,72^{o}cfor 60s and a final extension at 72^{o}c for 7 min
Gus	GGTATCAGCGCGAAGT CTTT	TCGGTGATGATAATCGG CTG	1cycle at 94^{o}c for 5 min, 35 cycles at 94^{o}cfor 45s,annealing at 54.6^{o}cfor 45s ,72^{o}cfor 60s and a final extension at 72^{o}c for 7 min

10. Southern blotting analysis

Genomic DNA was isolated from transgenic leaves .Aliquots of DNA (5 µg) were digested overnight with BamH1, fractionated by 1.0 % agarose gel electrophoresis and blotted on to Nylon membranes. Probe were extracted ,purified using QIAEXII and labeling with ^{32}P by

sequence random primer labeling kit (Usb)according to the manufacture's instructions. Both prehybridization and hybridization were carried out in 6x of 20 x SSC, 5 x Denhardt's solutions, 0.5 % SDS, distilled water and 100 µg/ml salmon sperm DNA (boil 5 min before use) over night. After hybridization, membranes were washed several times depend on the activity of radioactive at 65^{o}C with 6x SSC (low stringency), 2x SSC/0.1 % SDS (medium stringency) and 0.1 x SSC/0.1 % SDS (high stringency). Membrane was photographed and stored at -80^{o}C.

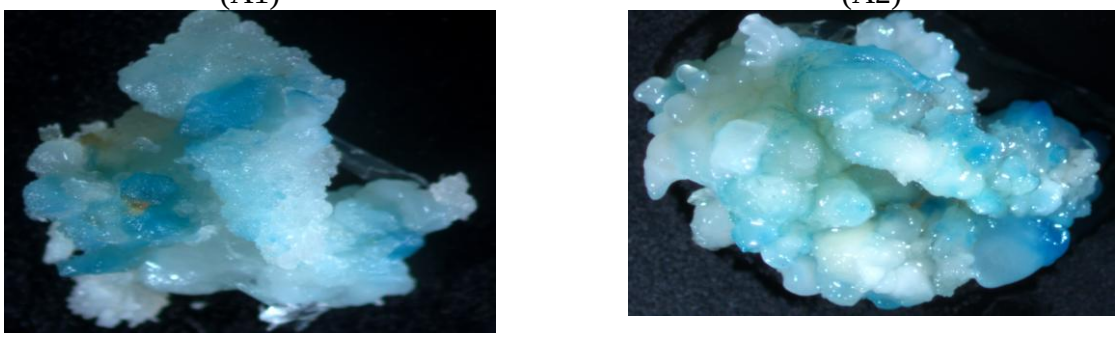
RESULTS

1. Tissue culture and transformation

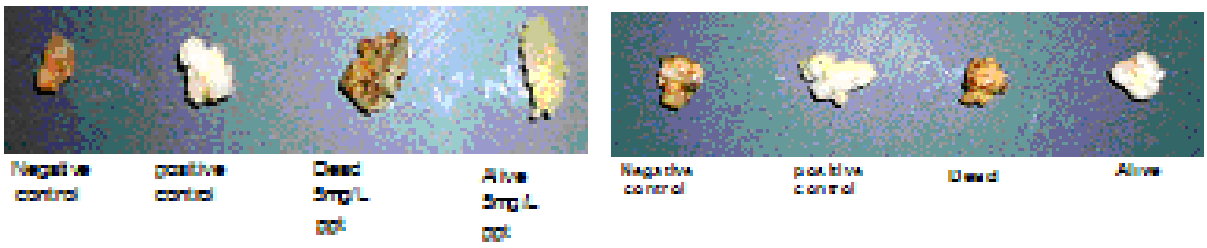
For particle bombardment, two constructions were used together with ratio 6 ASA2-NOS-ASB: 1 pAHC25 and final concentration 1 µg of DNA. The transformed calluses became morphologically distinguishable from nontransformed calluses after the second times of selection. While negative control (calluses were put on selection medium without bombardment) (no transformed) died on selection medium. The positive calluses were put on medium to show the growth of calluses and ability to regenerate. (Fig. 3)

Two selection media were used PPT for PAHC25 and 6MT for ASA2-NOS-ASB. Three concentrations for PPT were used and the number of calluses decreased with the increasing of PPT. (Table 2). Also, three concentrations for 6MT were used and 125 mg/ml increased the death of calluses and turned most of calluses to brown. For 75 and 100 mg/ml were succeed to get selection from them but when PCR was used ,75 mg/ml 6MT was got negative results, so 100 mg/ml 6MT was the best concentration to get selection and positive results for PCR (Table 3).

(A1) (A2)



(B) (C)



(D) (E)

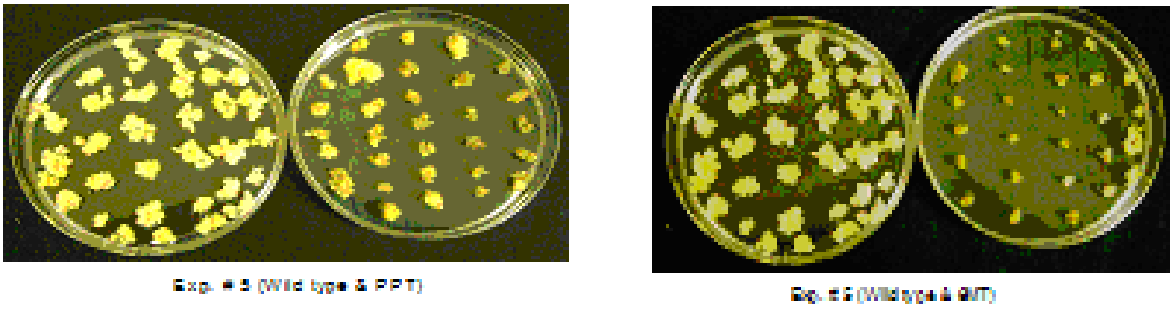


Figure (3): Gus assay activity (A1and A2),the effect of PPT and 6MT on calli after the first selection (B and C), the comparison between the calli on

PPT and control (D) and the calli on 6MT and its control after the second selection.(E)

Table (2): Number of calli after each selection until regeneration medium ,the number of calli decrease by increasing the concentration of PPT.

	first selection		second selection		RI		RII	
PPT	alive	dead	alive	dead	alive	dead	alive	dead
3mg/l	430	395	225	385	190	249	89	69
control	20	30	16	29	9	21	6	18
4mg/l	683	335	428	201	170	177	85	149
control	15	40	11	30	7	23	4	8
5mg/l	270	244	277	315	167	290	46	208
control	19	35	14	25	10	23	9	10

Table (3): Number of calli on selection medium containing three concentration of 6MT.

	first selection		second selection		RI		RII	
6MT	alive	dead	alive	dead	alive	dead	alive	dead
75 mg/ml	282	455	206	435	176	167	30	58
control	32	112	15	59	6	30	4	14
100mg/ml	360	873	358	882	188	291	149	266
control	37	86	25	68	19	18	3	6
125mg/ml	69	259	70	256	39	197	40	65
control	29	81	19	40	13	18	2	13

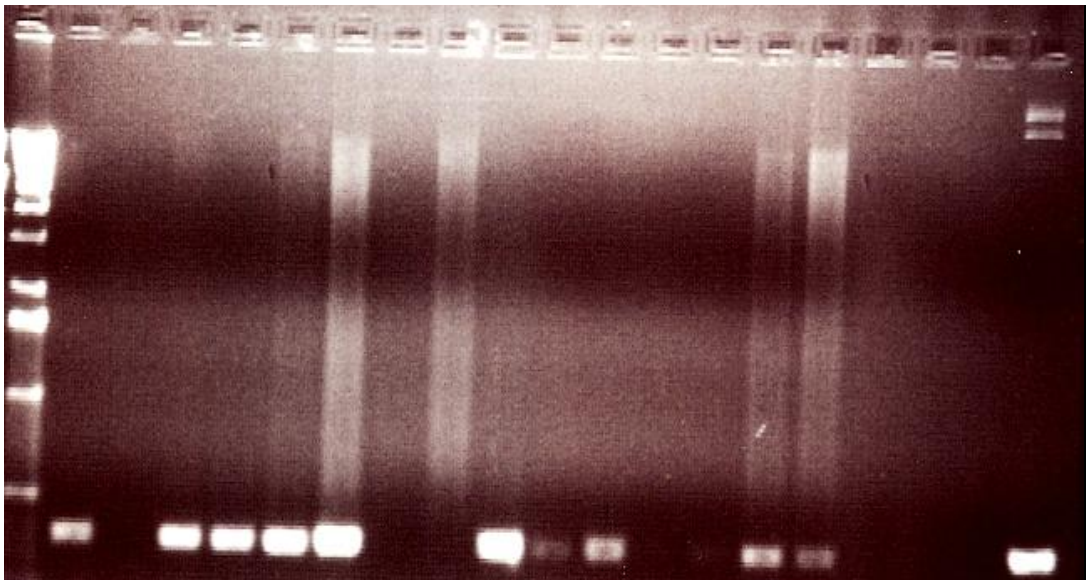
2. Genomic analysis of transgenic plants and Gus activity

Gus expression was detected after 48 h in bombardment calluses. The blue spots were appeared to confirm the insertion of the constructs (Fig. 3). Those lines selected with 75- 100 mg/ml 6MT and 3-5 mg/l PPT were confirmed by PCR.These results clearly demonstrate that the use of the ASA2-NOS-ASB vector and 100 µm6MT selection allows for identification of transformants in a manner similar to that in which 5mg/l PPT was used for detection of transformants. However, no transformants were selected using 75 µm 6MT and 3mg/l PPT. One plant came from herbicide resistant contained bar cDNA as determined by PCR analysis of genomic DNA using bar primers to produce a 311 bp fragment (Fig. 4a).

Also, 36 plants ASA2 α & β resistant contained ASA2 and ASB cDNA as detected by PCR analysis using ASA2 primers to get 815 bp (Fig. 4b), ASB primers to produce 800 bp fragment (Fig. 4c) and Gus primers (Fig. 4d). To analyze transgenic copy numbers in the selected lines, DNA gel blots using a ASA2 probe were conducted. Southern blot hybridization detected one band in all transgenic lines indicating integration one copy of the transgenic (Fig. 4e)

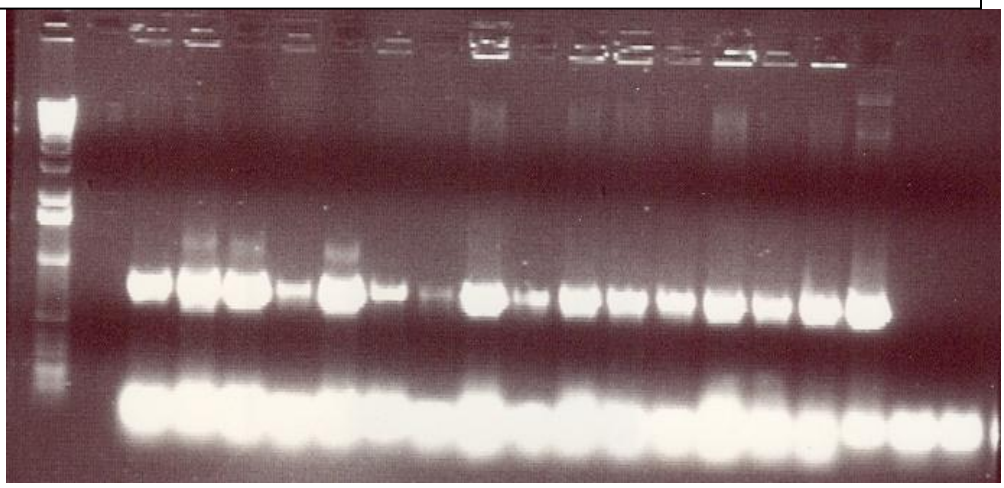
(A)

M, 1t , 1s , 2 , 3t , 4 , 5 , 6 ,7 ,8t ,8s , 9 , 10 , 11 , 12 , 13 , wt , w , p



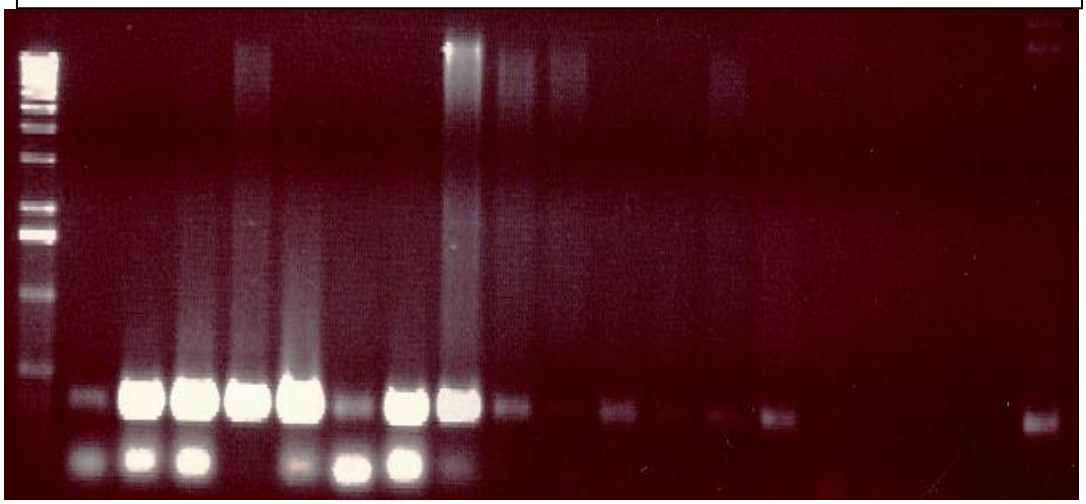
(B)

M, 1, 2, 3, 4 , 5, 6, 7 , 8, 9 , 10, 11, 12 , 13, 14 , 15, 16 , wt, w

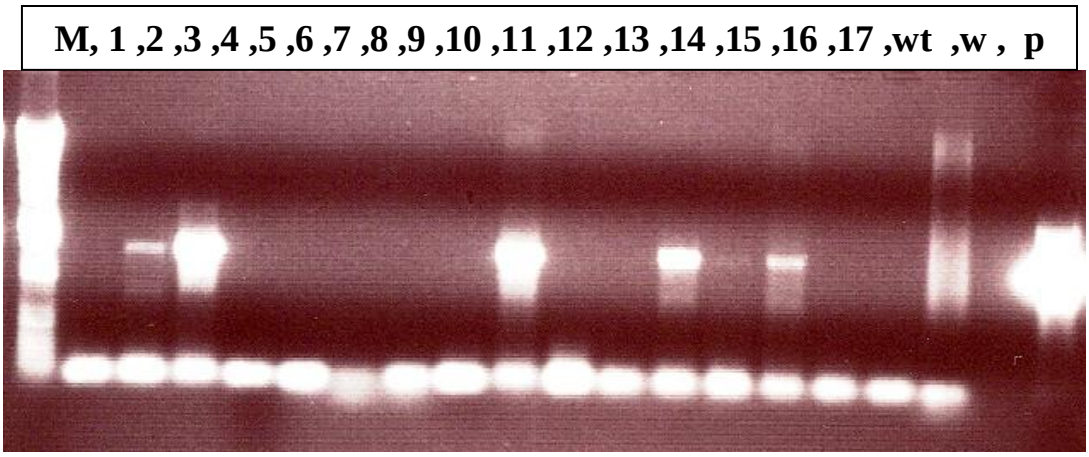


(C)

M, 18, 19 ,20 ,21 ,22 ,23 ,24 ,25 ,26 ,27 ,28 ,29 ,30 ,31 ,32 ,33 ,wt, w, p



(D)



(E)

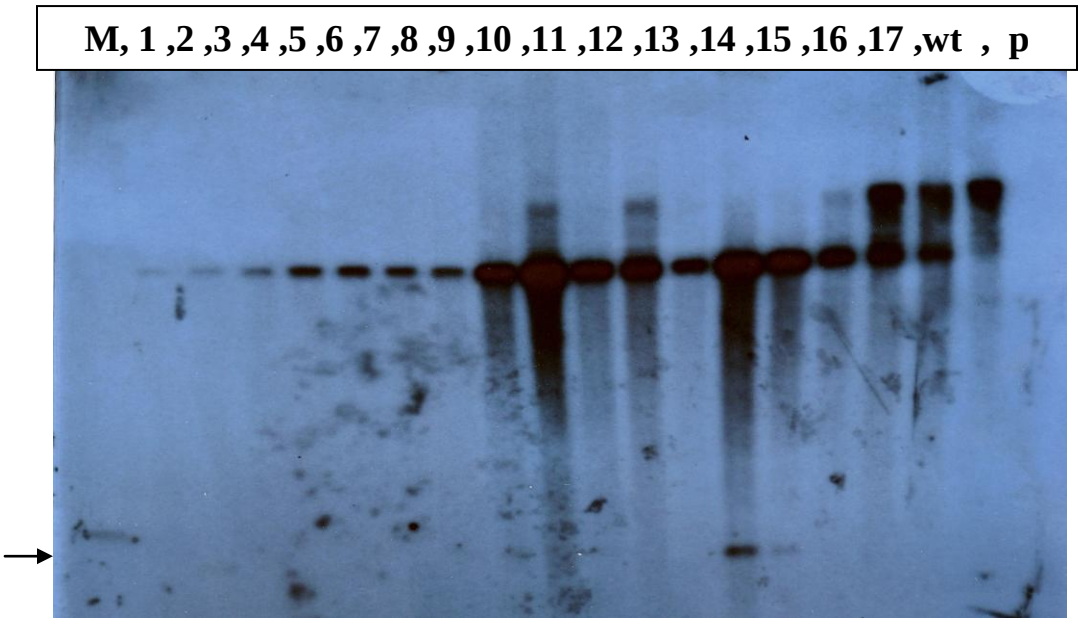


Figure (4) PCR for specific genes (A) bar gene which indicate 311 bp, (B) ASA2 gene with 815 bp, (C) ASB with 800 bp , (D) Gus gene and southern for ASA2 gene 815 bp. (E).

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DISCUSSION

1. Plant material and growth condition

Maize is a cereal crop with superiority hybrid lines and a complex genetic background. The development of improved genetic transformation technologies for recalcitrant species like cereals would benefit greatly from a better understanding of the molecular basis of plant regeneration (Zhang and Lemaux 2004), the identification of regeneration genes (Nishimura *et al.*2005) and the successful molecular manipulation of the underlying pathways (Gordon- Kamm *et al*., 2002)

2. Improving the Co- transformation efficiency

In this paper, we have co- transformed a feed back-insensitive anthranilate synthase gene (ASA2α& β) and a bialaphos resistance gene (bar) gene into maize calluses by particle bombardment. Co-transformation efficiency is defined as the ratio of the transgenic plants with two genes. Wakita et ai (1998) reported 28% co-transformation efficiency in rice for the bialaphos resistance gene (bar) and the tobacco fatty acid desaturase gene (NtFAD). In the work present here, the average co-transformation efficiency was very low (36 plants) However, many factors could affect the co-transformation efficiency and one of these is the plasmid ratio (Chen et al (1998). In our study, the ASA2 α& β : bar was 6:1 compared to the co-transformation efficiency in other crops, our co- transformation efficiency was not high, and future studies will need to explore the optimal ratio for co-transformation of two genes in maize. Inaba et al. (2007) found that the ASA2 gene was not expressed highly in most of the soybean plants transformed with the same promoter ASA2 combination but selected with another selectable marker gene.

Optimizing culture conditions such as increasing the selection agent concentration from 75 μm to 100 μm depending on the plant species might be required in order to increase the selection efficiency in both systems and decrease the number of escapes.

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3. Effect of Particle bombardment

There have been several studies concerning recovery of marker free transgenic plants. Substantial progress has been made in recent years with the development of transformation with two independent T-DNAs using a standard *Agrobacterium* transformation vector. This has led to recovery of marker – free transgenic tobacco (Mc Cormac *et al*., 2001), barley (Matthews et al., 2001 and maize Miller et al., 2002). However, Particle bombardment is still the method of choice for multiple gene co-transformation because the procedure is highly efficient particularly for monocot species and is convenient to use for large numbers of samples.

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الملخص العربي

التحول الوراثي في كالس الذرة بإستخدام نوعين من البلازميدات بواسطة قاذف الجينات.

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يقدم جهاز قاذف الجينات طريقة بسيطة لادخال الجين إلي كالس الذرة. تم استخدام اثنين من البلازميدات لانتاج نباتات محولة وراثيا يحتوي البلازميد الأول علي جين خاص بتخليق الحمض الأميني التربتوفان من خلال تغذية رجعية لتخليق ال anthranilate والبلازميد الثاني يحتوي علي جين المقاومة للمبيدات الحشرية.

بعد عملية قذف الجينات يوضع الكالس علي بيئة اختيارية حيث تم تقسيم الكالس إلي قسمين:- القسم الأول تم استخدام 6MT (75,100 and 125µm) والقسم الثاني تم استخدام PPT (3, 4 and 5 mg/L) وتم الحصول علي 36 نبات محول وراثيا وتم التأكد من ذلك من خلال ال PCR and Southern blotting وأوضحت النتائج أن أفضل التركيزات المستخدمة للمثبطات هي 6MT 100 µm, PPT 5mg/L.