Maize Transformation with Feedback- Insensitive Anthranilate Synthase Gene by Particle Bombardment of Immature Embryo

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ABSTRACT

Anthranilate synthase (AS) is a key enzyme in the synthesis of tryptophan (Trp). Tryptophan is one of the essential amino acids and the major contributor of the indole ring for the synthesis of many important compounds including auxins, glucosinolates, nicotinic acid, phytoalexins and alkaloids. Anthranilate synthase (AS) catalyzes the first reaction in the multi-step Trp biosythesis pathway by converting chorismate to anthranilate. Particle bombardment is a popular method currently used for producing transgenic maize. Particle bombardment was carried out using a plasmid pC2ASA2-NOS-ASB with (feedback anthranilate synthase) ASA2 (α&ß) genes under control of CaMV35S promoter. The best conditions to transform maize variety Hi_{II} were obtained when immature embryos were cultivated, prior to the bombardment,in higher osmolarity for 4 hours and bombarded at an acceleration helium gas pressure of 900 and 1,100 psi single and double shout. (6methyl – D-L- tryptophan) 6 MT 100µM used on selection medium as selective agent. PCR for nptII, ASA2, ASB genes and Southern blot using ASA2 probe were used to detect insertion of gene. The results clarified that two lines showed 12-16 fold increase in the tryptophan level compared with wild type.

Keywords : nuclear transformation, immature embryo culture, 6methyl-D-L- tryptophan, plant regeneration , transformation.

INTRODUCTION

Maize is a major commodity in international agriculture and an important source of protein and energy for human and livestock nutrition; it has been one of the prime targets for genetic manipulation. However, most studies on maize transformation have utilized genotypes adapted to temperate zone. Maize (*Zea mays* L.) is the third most planted cereal crop after wheat and rice worldwide.However, the production of this crop is decreasing due to increased population, limited land, environmental and biotic stresses over the years. Conventional breeding has been used as a tool to overcome these constraints, this has resulted in development of modest increments in yields and agronomic characteristics such as disease resistance and drought adaptability to different agroecological zones (Ayaga, 2003).

Biolistic transformation of maize has become routine since the recovery of fertile transgenic plants by this method was first reported by Gordon-Kamm et al. (1990). This technology has been used to transform various maize target tissues (Gordon-Kamm et al., 1999), including immature zygotic embryos from inbred lines (Koziel et al., 1993; Dunder et al., 1995; Brettschneider et al., 1997) and Hi II germplasm (Songstad et al., 1996; Pareddy et al., 1997). Immature zygotic embryos provide an excellent transformation target because they minimize both time in tissue culture and the expertise needed for callus induction and maintenance (Brettschneider et al., 1997; Songstad et al., 1996).

The regeneration of maize in tissue culture is important for the production of transgenic maize and for crop improvement using genetic engineering approaches. The first somatic embryos in maize tissue culture were produced by (Green and Phillips 1975) The enormous progress achieved by molecular and cellular biology in recent years has generated a new understanding for many basic genetic concepts and biochemical mechanisms, allowing the emergence of improved strategies for breeding and cultivar development. Among the new generated technologies, plant transformation is an important tool for the genetic manipulation and improvement of crop species (Carneiro et al., 2000). Molecular breeding methods involving marker assisted selection and genetic transformation now provide viable alternatives in several crops, including maize (Frame et al., 2002). However, the pre-requisite for crop genetic transformation is existence of a reliable plant regeneration system.
To transfer foreign genes into the maize genome, various reports have shown that microprojectile bombardment is a successful technique (Klein et al., 1988; Brettschneider et al., 1997; Frame et al., 2000; Lorence and Verpoorte 2004; Herrera-Estrella et al., 2005).

Plant transgenic technology has undergone key changes in the past 30 years; major technological developments have been sustained by breakthrough studies by investigators all over the world. However, there are still a number of bottlenecks in the technology used to transform plant species. (Vain 2007). Tryptophan (Trp) is one of the essential amino acids and the major contributor of the indole ring for the synthesis of many important compounds including auxins, glucosinolates, nicotinic acid, phytoalexins and alkaloids (Haslam 1993). Trp is synthesized by a five-enzyme biosynthetic pathway from the branch point compound chorismic acid at the end of the shikimic acid pathway (Radwanski and Last 1995). The first step of this five-enzyme pathway is catalyzed by anthranilate

synthase (AS), which converts chorismate to anthranilate. As a branch point enzyme in aromatic amino acid biosynthesis AS has an important regulatory role through feedback inhibition by Trp (Widholm 1974; Singh and Widholm 1974). Plant AS is a heterotetramer of two a- and two b-subunits encoded by separate nuclear genes and synthesized in the cytosol, as precursor proteins that are then localized in the plastids (Poulsen et al.1993; Romero et al. 1995). The a-subunit binds the substrate chorismate and carries out its aromatization while the b-subunit transfers an amino group from the other substrate glutamine (Crawford 1989). This glutamine- dependent AS reaction requires both a- and bsubunits. The a-subunit alone can synthesize anthranilatenfrom chorismate using ammonia as the amino donor rather than glutamine, if ammonia is present in high concentrations. The AS reaction is normally subjected to feedback inhibition by Trp which binds to an allosteric site on the AS a-subunit (Bohlmann et al. 1996; Li and Last 1996). In addition to Trp, different Trp and indole analogs that can be converted to Trp analogs (Widholm 1981) can also inhibit AS and in turn plant cell growth.

The final objective of this study is to get transformed plants with feedback-anthranilate synthase and used 6-methyl-DL-tryptophan (6MT) as selection agent instead of most commonly used 5-methyltryptophan (5MT). The maize genetic transformation protocol developed in this work will possibly improve the efficiency to produce new transgenic maize lines expressing desirable biochemical pathway.

MATERIALS AND METHODS

1- Preparation of the culture

Immature embryos, between 1 and 2 mm length(14-16 days after pollination) were harvested from plants grown in the green house, Illinois University. Ears for embryo extraction were surface-sterilized using 70% alcohol for 5min then10% sodium hypochloride was used for 20 min and rinsed three-five times with sterilized distilled water. Immature embryo were isolated and cultivated scutellum-side up onto the surface of D medium (Duncan et al.,1985) for 21 days then to N6salts and N6 vitamins (Chu et al.,1975), 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 .Cultures were kept in Petri dishes at 28°C and grown in the dark for callus ignition and maintenance.

2- plasmid

The plasmid used in this work, pC2ASA2-NOS-ASB 16.6 kb (Illinois University), (fig, 1) contains a selectable marker (ASA2 α & β) the coding region of kanamycin, gusA, under control of the cauliflower mosaic virus (CAMV). ANOS polyA (nopaline synthase) terminator sequence

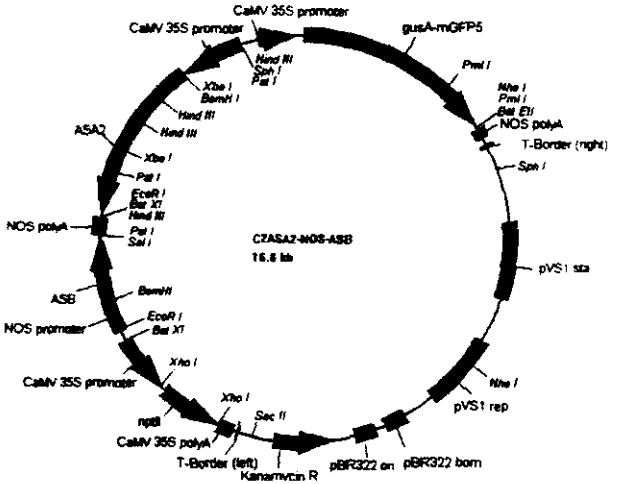


Figure 1- Schematic drawing of the plasmid C2ASA2-NOS-ASB.The plasmid 16.6 kb consists of ASA2,ASB, CaMV35S : cauliflower mosaic virus 35 S promoter and 3'NOS : the polyadenylation signal of nopaline synthase.

3- Maize transformation and regeneration

Friable callus was used for particle bombardment. Callus was transferred to osmoticum medium (N6 medium + 36.4 g/L sorbitol and 36.4 g/L mannitol) for 4 hours prior to bombardment.The gold particles (Bio-Rad) (1µg) was used to precipitate DNA onto the microparticles 1µL plasmid DNA (stock 1µg μL^{-1}). Then, 220µL(stock 2.5 M) and 50µL spermidine (stock 0.1M) were added and homogenized. The mixture was kept on ice for 5 min and vortexed for 5 min, micro centrifuged at 5000 rpm for 1 min, rinsed carefully with 250 µl of ethanol and suspended in 40 µL 100% ethanol. 10 µl of the DNA –coated particles were pipetted onto each macrocarrier (washed in absolute EtOH, dried before uses).Bombardments

were performed on Petri dishes containing friable callus clump in the middle. Different treatments were designed to test the pressure of the accelerating helium pulse (900 and 1100 psi single and double shout).Selection of putative transformed callus began 10 days after bombardment when the callus were cultured every 21 days on N6S selection medium which was similar to callus initiation medium but without proline and casein hydrolysate, filter sterilized silver nitrate (5µM) was added after autoclaving, 6- Methyl-DL-tryptophan (6MT) 100 µM was added also.

The surviving callus was transferred to regeneration medium MS medium (Murashige and Skoog 1962) supplemented with 1ml/L (1000X) MS vitamin stock, 100mg/Lmyo-inositol, 60 g/Sucrose , 3g/L gelrite, pH5.8 ,after autoclaving 100 mg/mL of 6MT was added. The calluses were incubated for 2 weeks at 25°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. For the reporter gene gusA transient expression studies, 20% of bombardment callus was incubated two days after bombardment in a solution containing 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. Vacuum for 5-10 min, avoid air bubbles and floating samples, incubate on 37°C over night, sealed with parafilm. Gus spots appeared.

4- PCR analysis and Southern blotting

For the molecular characterization of the transgenic plants generated,total genomic DNA was isolated from leaf tissue of primary leaves using a CTAB protocol described by(Doyle and Doyle 1990) and submitted to PCR and southern blot analysis. For the presence of ASB, the primers 5' TGTCCAAGATCCCATGACGATTCC 3' and 5' CAGAAATCCACAGAACCGGGAGAT 3', which amplify 800 bp were used. For the ASA2 (α&β), the primers 5'TCTGTACACTTCAAATGGGTCAGC3' and 5' CTAAAAGCGGGAACTTGATTCCGC3' which amplify 815 bp were used and for nptII the primers 5'ATCTCACCTTGCTCCTGC3' and5'ATACCGTAAAGCACGAGG3'which amplify 200bp were used. Each 20 µl amplification reactions mixture consisted of 100ng of template DNA,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. The reactions were carried out using a thermal conditions: 1cycle at 94°c for 5 min, 35 cycles at 94°cfor 45s,annealing at 54.6°cfor 45s ,72°cfor 60s and a final extension at 72°c for 7 min for GUS, 1cycle at 94°c for 5 min, 35cycles at 94°cfor 30s,annealing at 57°cfor 45s

,72°cfor 60s and a final extension at 72°c for 7 min for ASA2, and1cycle at 94°c for 5 min. 30cycles at 94°cfor 45s,annealing at 55°cfor 30s ,72°cfor45s and a final extension at 72°c for 7 min. The amplified products were separated by electrophoresis on 1% agarose gel and visualized with ethidium bromide stain under UV light.

For southern blot analysis, 15 micrograms of total genomic DNA from each T0 plant were completely digested with BamH1 at 37°C overnight, separated by electrophoresis in 1% agarose gel, and then transferred onto a Hybond- N^+ nylon membrane according to (Sambrook et al., 1989). The blot was hybridized with a ^{32}P labeled ASA2gene coding region.Hybridized membranes were exposed to Kodak film and kept at -80°C for 7 days.

The youngest expanded leaf of a shoot culture was ground into coarse powder in liquid nitrogen. The leaves powders were homogenized in 5 volumes of 0.1 N HCl by the fast prep FP120 Cell Disrupter (Savant Instruments, Holbrook, N.Y.) at 4.0 ms^{-1} for 40 sec. The supernatant was transferred to a new tube after centrifugation at 4°c and 13,000 g for 10 min. Another five volumes of 0.1 N HCl were added to the pellet, followed by homogenation and centrifugation as described above. The procedure was repeated 1 more time and the resultant were 15 volumes of supernatant was filtered with an Ultra Free-MC filter unit (Millipore, Bedford, Mass). Trp concentrations were measured by HPLC according to Cho et al. (2000).

5- Statistical analysis

Chi square test of homogeneity was used to determine the significance of differences.

RESULTS AND DISCUSSION

1- Effect of callus morphology on transformation efficiency:

The immature embryos of Hill were produced from selections of A188x B73, whose length was 1.6-2.0 mm. The embryos were selected and cultivated in D culture medium (Fig. 2a), then transferred to N6E culture medium. After 3 times of cultivation, two types of callus were produced. The present results are in accordance with those reported by (Vassil et al., 1991; Armstrong and Green, 1985) who found that Type I compact, white that failed to regenerate, Type II friable , yellowish and able to regenerate. Type II was selected and transferred for particle bombardment. (Figures 2b &2c). When cultured *in vitro* can originate Type I or Type II embryogenic calluses. Type I callus is formed by hard, compact

and yellowish tissue, usually unable to regenerate plants. Type II callus is soft, friable, highly embryogenic and able to regenerate a higher number of plants than Type I callus. Type II embryogenic callus can regenerate only. On the other hand (Brettschneider et al., 1997; Frame et al., 2006), found that both types of calluses can be used to generate transgenic plants.

2- Effect of osmotic treatment on transformation efficiency:

An important aspect in transformation via biolistics is the damage to the target tissue during microparticle penetration into the cell. To minimize this problem, the target cells are usually plasmolysed by an osmatic treatment and increase stable clone recovery (Vain et al., 1993, Brettschneider et al., 1997) so callus were put on osmoticum medium for 4 hours before bombardment (Fig.2d). For bombardment, 900 and 1100 psi were used to shot calluses (single and double shout). The results indicated that double shot were more efficient than single shot. However, the differences between treatments were not significant according to Chi square test at p0.05 (0.3327). (Table 1) Clearing by demonstrates that the number of living calluses decreased with double shot but these living calluses showed positive results for PCR and are more efficient than single shot. (El-itriby et al., 2003) concluded from the transient GUS expression that, in the co-transformation experiments, the use of osmotic pre- and post-treatment with acceleration pressure of 1100 psi and double shots per plate is most efficient treatment.

3- Transient GUS activity

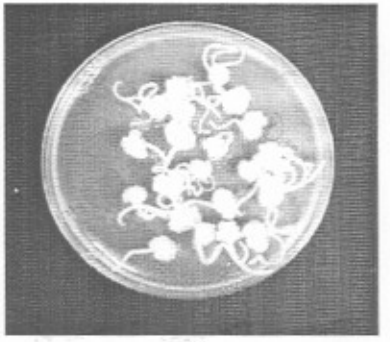
Gus expression was detected after 48 h in bombarded calluses. The blue spots appeared to confirm the insertion of the constructs (Fig. 2e). For selection medium, 6MT 100µM was used to select the transformed plants (Table 1, Fig 3a, 3b, 3c). (Abdel-Rahman and Widholm 2009) found that 6MT 100µM was the best concentration used for selection compared with 75 and 125 µM. The use of feedback-insensitive AS α-subunit genes as selectable markers with 5MT as the selective agent has been reported in maize (Anderson et al., 1997) and rice (Tozawa et al., 2001).

4- Selection of transgenic maize plants over expressing ASA2

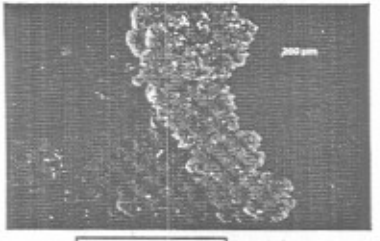
PCR analysis was conducted on all of the (133) plants regenerated from immature embryo transformation. It was observed 200 bp band of nptII gene (Fig4a), 815bp band of ASA2gene (Fig. 4b) and 800 bp of ASB gene (Fig.4c). The result showed that (11) transgenic maize were positive for nptII, (57) were positive for ASB and ASA2gene. Southern blot of total

DNA from transgenic plants digested with BamHI and probed with ASA2gene showed that feed back anthranilate synthase (high tryptophan) plants contained copies of the transgene (Fig 5) (Cho et al., 2004) showed that, the tobacco- feedback –insensitive ASA2 gene can be inserted into legume hairy roots (*A. sinicus* and soybean) and be expressed to produce feedback-insensitive AS activity that leads to increased free Trp levels and resistance to the Trp analog 5MT. The Trp analog 5MT and 6MT inhibit AS enzyme activity and plant growth. Over expression of the ASA2 gene results in resistance to 5MT in *E.coli* (Song et al., 1998) and transgenic *A. sinicus* (Cho et al., 2000).

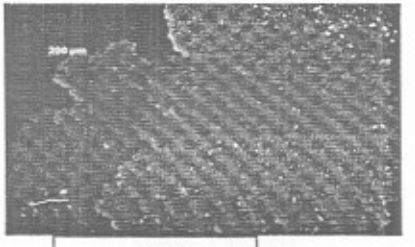
The expression of ASA2 in plant tissues leads to increased levels of free Trp, which could be desirable since Trp. is an essential amino acid required in the diets of humans and non ruminant animals. To determine the over expression of the feedback- insensitive ASA2 had on Trp. production, free Trp levels in the T0 transgenic plant leaves were measured. The high- expresser lines 9 and 12 contained > 12-16 times, line 1 show the Trp. level as wild type (Fig 6). In this report, we demonstrate that a feedback-insensitive ASA2 gene can be used as a selectable marker for the production of transgenic maize plants. Since maize expressing ASA2 resistant to 6MT, thus ASA2 may be an effective selectable marker gene for use with many different species.



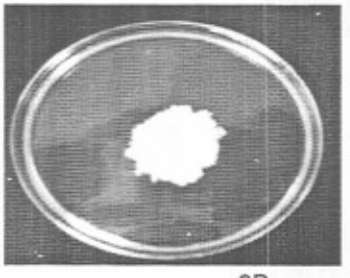
2A



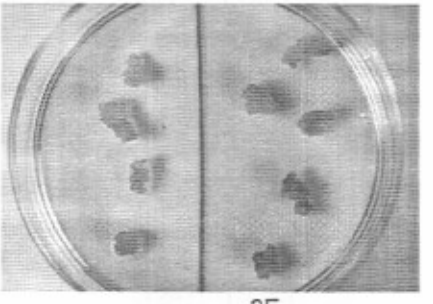
2C



2B

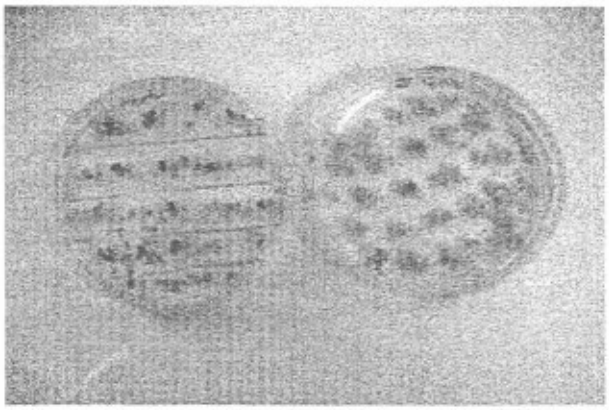


2D

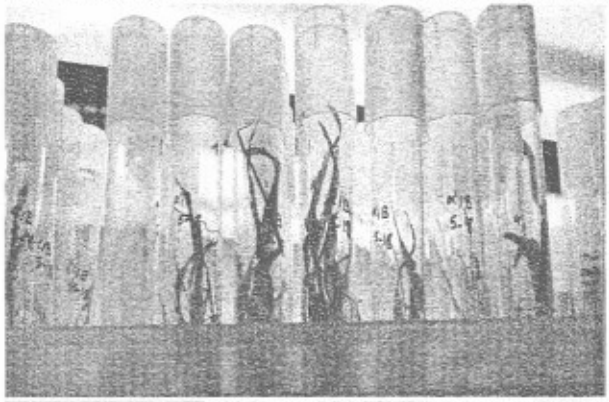


2E

Figure (2) Morphology of different types of callus (A) immature embryo on medium D. (B) compact callus, (C) friable callus(embryogenic callus)(D) callus on osmetic medium before bombardment (E)transient GUS activity48 h after bombardment.



3A



3B

3C

Figure (3): Transgenic maize plants(A) calluses on selection medium with 6MT100µM,(B) shoot and root differentiation(C) Transgenic plants in the green house.

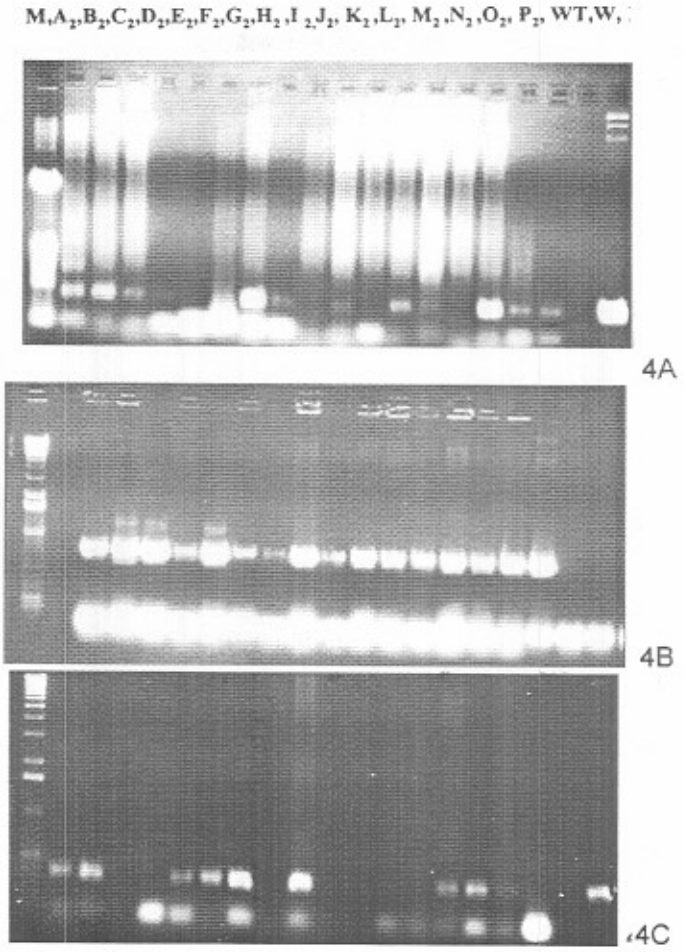


Figure (4) PCR analysis of regenerated plants. Agarose electrophoresis gels of PCR amplification products (A) co-transformed with nptII, (B) PCR products from ASA2 gene and (C) ASB gene.

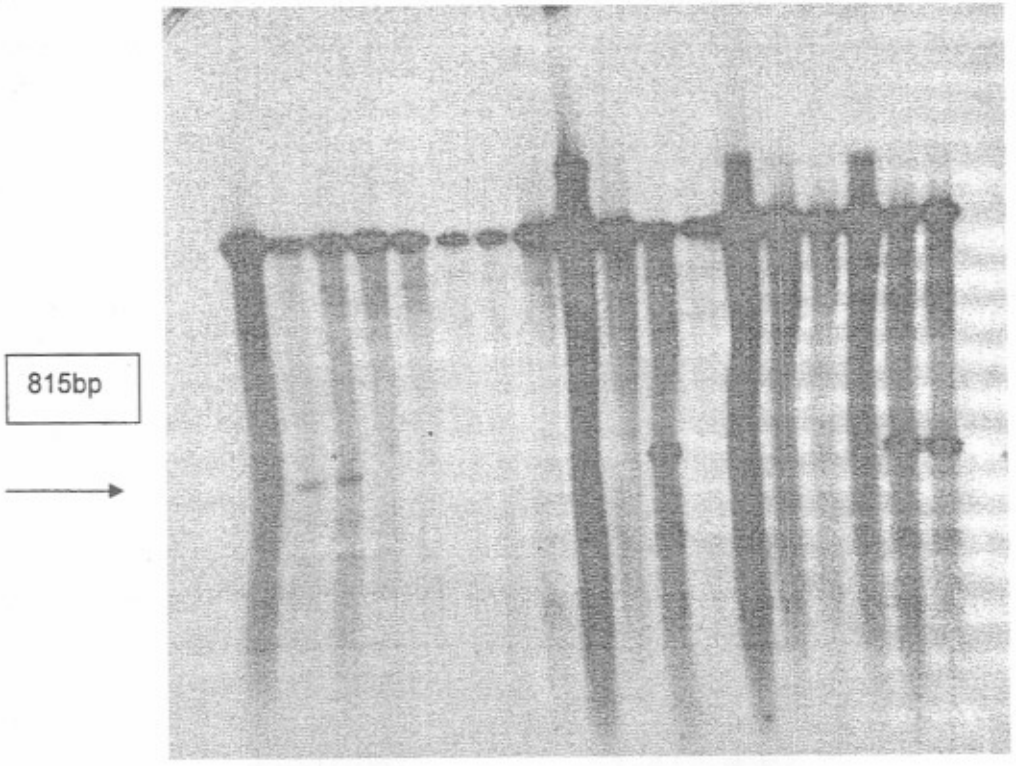


Figure (5): Southern analysis for ASA2 gene in transgenic plants

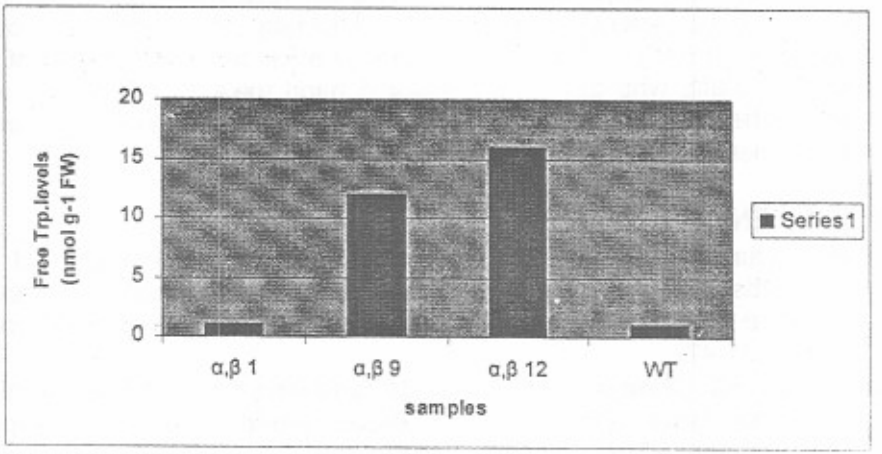


Figure (6): Free Trp levels in leaves of WT and T0 transgenic lines. Lines were selected on 100µM 6MT.

Table (1) # of calluses living and dead on selection medium N6S + 6MT 100µM

Helium acceleration pressure	Callus efficiency on selection medium						
	#of calluses	living calluses	% living calluses	Chi square	Dead calluses	% dead calluses	Ch squa
single 900	49.67±12.93	7.84±4.71	16	0.054	41.72±15.78	84	0.0
single 1100	30.33±7.49	5.67±1.38	19	0.0145	24.56±5.25	81	0.0
double 900	20.34±3.94	3.0±0.84	15	0.0312	17.28±0.35	85	0.0
double 1100	20.67±8.38	4.0±1.34	19	0.206	16.74±2.81	81	0.0

Total chi square cal. = 0.3327 , df=3 p=0.05, Chi square tab. 7.82

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REFERENCES

Abdel-Rahamn M.M. and Widholm M.J (2009) Transformation of two plasmids into maize callus using particle bombardment. Agriculture Science and Enviromental Alexandria University Damanhour branch v.8,(2) 31-47.

Anderson PC, Chomet PS, Griffor MC and Kriz AL (1997) Anthranilate synthase gene and its use thereof. World Intellectual Property Organization, 97/26336.

Armstrong, C.L.; Green, C.E. (1985) Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. Planta, v.164, p.207-214.

Ayaga G.O. (2003). Maize yield trend in Kenya in the last 20 years. Paper presented at the regional workshop on declining Maize yield trend in Trans Nzoia district organized by Moi University. Centre Consortium. KARI Kitale and Ministry of Agriculture.

Bohlmann J, Lins T, Martin W. and Eilert U. (1996) Anthranilate synthase from Ruta graveolens. Duplicated ASa genes encode tryptophansensitive and tryptophan-insensitive isoenzymes specific to amino acid and alkaloid biosynthesis. Plant Physiol 111:507–514

Brettschneider, R.; Becker, D. and Lorz, H. (1997) Efficient transformation of scutellar tissue of immature maize embryos. Theor. Appl. Genet. 94:737–748.

Carneiro, A.A.; Carneiro, N.P.; Carvalho, C.H.S.; Vasconcelos, M.J.V.; Lopes, M.A. and Paiva, E. Milho (2000) Transgênico: melhoria da qualidade nutricional do grão. Biotecnologia,v.15, p.42-46.

Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, Chu CY and Bi F (1975) Establishment of efficient medium for anther culture of rice through comparative experiments on the nitrogen source. Sci. Sinica. 18:659-668.

Cho HJ, Brotherton JE, Song H-S and Widholm JM (2000) Increasing tryptophan synthesis in a forage legume Astragalus sinicus by

expressing the tobacco feedback-insensitive anthranilate synthase (ASA2) gene. Plant Physiol 123:1069–1076.

Cho H-J, Brotherton JE and Widholm JM (2004) Use of the tobacco feedback- insensitive anthranilate synthase gene (ASA2) as a selectable marker for legume hairy root transformation. Plant Cell Reports 23:104-113.

Crawford IP (1989) Evolution of a biosynthetic pathway: the tryptophan paradigm. Annu Rev Microbiol 43:567–600.

Doyle J.J. and Doyle J.L., (1990) Isolation of plant DNA from fresh tissue. Focus 12:13-15.

Duncan D. R.; Williams M. E.; Zehr B. F. and Widholm J. M.(1985) The production of callus capable of plant regenerations from immature embryos of numerous Zea mays L. genotypes. Planta 165: 322–332.

Dunder, E.; Dawson, J.; Suttie, J. and Pace, G. (1995) Maize transformation by microprojectile bombardment of immature embryos. In: Potrykus, I.; Spangenberg, G., eds. Gene transfer to plants. Berlin: Springer- Verlag; 127–138.

El-Itribyi H., Aassem SK., Hussein E.H.A., Abdel-Galil F.M. and Madkour M.(2003) Regeneration and transformation of egyptian maize inbred lines via immature embryo culture and a biolistic particle delivery. *In Vitro* Cell Dev Biol-Plant 39:524-531.

Frame BR, Shou H, Chikwamba RK, Zhang Z, Xiang C, Fonger TM, Green CE and Phillips RL (2002). Plant regeneration from tissue culture of maize. Crop Sci. 15: 417-421.

Frame, B.R.; Mcmurray, J.M.; Fonger, T.M.; Main, M.L.; Taylor, K.W.; Torney, F.J.; Paz, M.M. and Wang, K.(2006) Improved *Agrobacterium*-mediated transformation of three maize inbred lines using MS salts. Plant Cell Reports, v.25, p.1024-1034.

Gordon-Kamm, W. J.; Spencer, T. M.; Mangano, M. L.; Adams, T.; Daines, R.; Start, W.; O'Brien, J.; Chambers, S.; Adams Jr., W.; Willetts, N.; Rice, T.; Mackey, C.; Krueger, R.; Kausch, A. and Lemaux, P.(1990) Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell 2:603–618.

Gordon-Kamm, W. J.; Baszczynski, C. L.; Bruce, W. B. and Tomes, D. T. (1999) Transgenic Cereals—*Zea mays* (maize). In: Vasil, I. K., ed. Molecular improvement of cereal crops. Great Britain: Kluwer Academic Publishers:189–253.

Green C.E. and Phillips R.L. (1975) Plant regeneration from tissue cultures of maize. Crop Sci 15: 417-420.

Haslam E. (1993) Shikimic acid: metabolism and metabolites. Wiley, Chichester.

Herrera -Estrella, L.; Simpson, J. and Martineztrujillo, M. (2005) Transgenic plants: an historical perspective.Methods of Molecular Biology, v.286, p.3-32.

Klein, T.M.; Fromm, M.E.; Weissinger, A.; Tomes, D.; Schaff, S.; Slettern, M. and Sanford, J.C.(1988) Transfer of foreign genes into intact maize cells using high velocity microprojectiles. Proceedings National Academy of Science of the United States of America, v.85, p.4305-4309, 1988

Koziel, M. G.; Beland, G. L.; Bowman, C.; Carozzi, N. B.; Crenshaw, R.; Crossland, L.; Dawson, J.; Desai, N.; Hill, M.; Kadwell, S.; Launis, K.; Lewis, K.; Maddox, D.; McPherson, K.; Meghji, M.; Merlin, E.; Rhodes, R.; Warren, G.; Wright, M. and Evola, S. (1993) Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. Bio/Technology 11:194–200.

Li J, Last RL (1996) The Arabidopsis thaliana trp5 mutant has a feedback-resistant anthranilate synthase and elevated soluble tryptophan. Plant Physiol 110:51–59

Lorence, A. and Verpoorte, R. (2004) Gene transfer and expression in plants. In: TUAN, R.S. Recombinant gene expression: reviews and protocols. 2nd ed. Totowa: Humana Press, p.329-350.

Murashige T and Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol. 15: 473-479.

Pareddy, D.; Petolino, J.; Skokut, T.; Hopkins, N.; Miller, M.; Welter, M.; Smith, K.; Clayton, D.; Pescitelli, S. and Gould, A. (1997) Maize transformation via helium blasting. Maydica 42:143–154.

Poulsen C, Bongaerts RJM and Ver poorte R (1993) Purification and characterization of anthranilate synthase from Catharanthus roseus. Eur J Biochem 212:431–440

Radwanski ER and Last RL (1995) Tryptophan biosynthesis and metabolism: biochemical and molecular genetics. Plant Cell 7:921–934

Romero RM, Roberts MF and Phillipson JD (1995) Anthranilate synthase in microorganisms and plants. Phytochemistry 39:263–276

Sambrook, J.; Fritsch E.F. and Maniatis, T.(1989) Molecular cloning: a laboratory manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press.

Singh M and Widholm JM (1974) Measurement of the five enzymes which convert chorismate to tryptophan in wheat plants (Triticum aestivum L.). Physiol Plant 32:362–366

Songstad, D. D.; Armstrong, C. L.; Peterson, W. L.; Hairston, B. and Hinchee, M. A. W.(1996) Production of transgenic maize plants and

progeny by bombardment of Hi-II immature embryos. In Vitro Cell. Dev. Biol. –Plant 32:179–183.

Tozawa Y,Hasegawa H, Terakawa T and Wakasa K (2001) Characterization of rice anthranilate synthase α –subunit genes OASA1 andOASA2. Tryptophan accumulation in transgenic rice expressing a feedback- insensitive mutant of OASA1.Plant Physiol 126: 1493-1506.

Vain, P; McMullen, M.D and Finer, J.J. (1993) Osmotic treatment enhances particle bombardment Plant Cell Report 12:84-88.

Vain P (2007) Thirty years of plant transformation technology development. Plant Biotechnol J 5:221–229

Vasil V, Brown SM, Re D, Fromm ME and Vasil IK (1991) Stably transformed callus lines from microprojectile bombardment of cell-suspension cultures of wheat. Biotechnology 9:743–747

Widholm, J.M. (1974) Control of aromatic amino acid biosynthesis in cultured plant tissues: effect of intermediates and aromatic amino acids on free levels. Physiol Plant 30:13–18

Widholm, J.M. (1981) Utilization of indole analogs by carrot and tobacco cell tryptophan synthase *in vivo* .

الملخص العربي

التحول الوراثي في الكالس الناتج من الجنين غير الناضج للذرة لإدخال جين التغذية الرجعية لتخليق انزيم Anthranilate بواسطة قاذف الجينات.

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إنزيم ال Anthranilate synthase هو الإنزيم الرئيسي في تكوين الحمض الأميني التربتوفان. ويعتبر التربتوفان واحد من الأحماض الأمينية الرئيسية والمصدر الرئيسي لحلقة الإندول اللا زمة لتخليق مركبات كثيرة ومهمة بما في ذلك الأوكسينات وحمض النيكوتينك والقلويدات والفيتوالكسينات.يحفزإنزيم ال Anthranilate synthase التفاعل الأول في المسار المتعدد الخطوات لتخليق التربتوفان عن طريق تحويل ال Chroismate إلي Anthranilate. يعد جهاز قاذف الجينات من إحدي الطرق التي تستخدم لإنتاج ذرة معدل وراثيا حيث تم إستخدام البلازميد-pC2ASA2-NOS

ASB with (feedback anthranilate synthase)ASA2&ßو لذي يحمل جينات المسئولة عن زيادة الحمض الأميني التربتوفان تحت التحكم من البادئCaMV35S .وضع الكالس علي بيئة ذات إسموزية عالية لمدة أربعة ساعات قبل قذف الكالس بالبلازميدات المحملة علي حبيبات الذهب. تم إستخدام نوعان من ضغط الهيليوم 900 رطل/ بوصة^2 ,1100 رطل/ بوصة^2 . وتم القذف إما مرة أ ومرتان متتاليتان.ثم وضع الكالس علي بيئة إختيارية تحتوي علي6MT 100ميكرومول من المشبط .تم إستخدام ال PCR , Southern blotting لتحديد الجينات ومعرفة النباتات المحورة وراثيا و تم الحصول علي نباتات محورة وراثيا والتي أظهرت زيادة في الحمض الأميني التربتوفان من 12-16 ضعف مقارنة بالنباتات غير المحورة وراثيا.كذلك ووجد أن كفاءة قذف الكالس مرتان أعلي من قذفه مرة واحدة بالبلازميد في إنتاج نباتات محورة وراثيا .