Genetic transformation of Egyptian maize lines using the late embryogenesis abundant protein gene, *HVA1*, from barley

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ABSTRACT

Maize genotype specificity has been one of the constraints limiting the transformation of many tropical and subtropical maize lines with desirable genes. In the present study callus induction and regeneration ability from immature embryos of four elite Egyptian maize lines were examined. Two media were assayed to investigate the effect of 2,4-D and dicamba on type II callus production and regeneration. Dicamba promoted fast differentiation in all inbred lines that led to increasing the number of shoots in comparison to 2,4-D.Inbreds Gz 650 and Sd 34 gave significantly high regeneration frequencies when maintained on callus induction medium containing dicamba. The late embryogenesis abundant (LEA) protein coding gene, HVA1, from barley (Hordeum vulgare L.) for abiotic stress tolerance along with the bar gene for herbicide resistance were introduced in three of these inbred lines using the biolistic mediated transformation method and independent transgenic events were obtained. Putative transgenic events have been tested by herbicide application. Moreover, molecular analysis using PCR and Southern blot hybridization proved the presence and integration of the transgenes in the genome of the putatively transgenic plants. The copy number of the transgenes ranged between 10 and 15 copies in individual events. This study suggests that LEA genes could hold considerable potential for use as molecular tools for genetic crop improvement toward stress tolerance.

Key words : *maize , immature embryos , embryogenic callus , plant regeneration , transformation , salt stresses , drought , hva1 gene .*

INTRODUCTION

Plants being sessile, their growth and yield are strongly influenced by abiotic stress such as drought, high salt content and temperature change. Environmental stress presents a major challenge in our quest for sustainable food production as it reduces the potential yields as high as 70% in crop plants. Water stress imparted by drought and temperature severity is the most prevalent abiotic stress that limits plant growth and productivity. Plants respond and adapt to these conditions with an array of biochemical and physiological alterations (Pradeep *et al.*, 2006).

Like other cereal crops, the yield of maize (*Zea mays* L) is affected by several biotic and abiotic factors. Drought and salt cause 24 million tons of yield loss in maize annually (Boyer, 1982; Heisey and Edmeades, 1999 and Halsey *et al.*, 2005).

It has been also recorded by Hu and Schmidhalte (2007) that salinity inhibits leaf growth in association with changes in cell size of wheat leaves As irrigation water sources have become scarce, development of crop cultivars with improved adaptation to drought is a major goal in many crop breeding programs (Siviamani *et al.*, 2000 and Shou *et al.*, 2004). Accumulation of compatible, low-molecular-weight osmol-ytes, such as sugar alcohols, special amino acids, and glycine betaine, have been suggested as major mechanisms that may underlie the adaptation or tolerance of plants to osmotic stresses (Xu *et al.*, 1996).

Current advances in plant biotechnology offer opportunities to solve abiotic stress problems in a short period of time as compared to conventional breeding (Rafiq *et al.*, 2005). Various transformation systems have been established to circumvent environmental stresses using various genes. One of these systems is to constitutively over-express certain plant proteins, such as late embryogenic abundant (LEA) proteins that accumulate during seed desiccation and in vegetative tissues when plants experience water deficit.

*HVA*1, is a group–3 late embryogenesis abundant (LEA) stress-related protein coding gene from barley (*Hordeum vulgare* L.), which is specifically expressed in the aleurone layer and the embryos during late seed development (Hong *et al.*, 1988 and Babu *et al.*, 2004). Expression of the *HVA1* gene is rapidly induced in young seedling under several stress conditions, such as dehydration, salt, drought and extreme temperatures or by absisic acid (ABA) treatment (Hong *et al.*, 1992). The function of *HVA*1 protein in stress protection has been investigated using a transgenic approach in rice (Xu *et al.*, 1996), barley (Martila *et al.*, 1996), wheat (Siviamani *et al.*, 2000) and Oat (Maqbool *et al.*, 2002).

The present investigation has been carried out to achieve three main objectives:(1) To investigate the regeneration capacity of four Egyptian maize inbred lines of commercial importance using different callus induction and regeneration media;(2) To introduce the *HVA*1 gene into three of these inbred lines by bombarding the scutellum tissues of maize embryos using the biolistic bombardment method and (3) To confirm the integration of the *HVA*1 gene in the genome of T_0 plants by different molecular biology tests.

MATERIALS AND METHODS

Plant material

Four elite Egyptian maize (*Zea mays L.*) inbred lines, namely, Sd 63 ,Sd 34 ,Gz 643 and Gz 650 ,were used in this investigation .These inbred lines were produced by the Maize Department, Field Crops Research Institute, ARC, Giza, Egypt.

Callus induction and Regeneration

Ears were harvested from field grown plants of the four maize inbred lines after 10-15 days from self pollination and sterilized for 20 min with 50% chlorox solution containing 0.1% Tween 20 .Ears were then washed 2-3 times with autoclaved distilled water under sterilized condition. Thirty immature embryos (1.0 – 1.5 mm long) were aseptically removed from the kernels and placed scutellum side up on callus induction media in each Petri dish (100 x 15 mm). The total number of cultured immature embryos was 2400.

Two N6 based culture media namely MI (Bohorova *et al.*, 1995) and MII (Carvalho *et*

al., 1997) were used to initiate and maintain callus cultures from immature zygotic embryos. Both media (MI and MII) contained 2.0 mg/l glycine, 1.0 mg/l Thiamine HCl, 0.5 mg/l pyridoxine HCl and 0.5 mg/l Nicotinic acid. In addition, medium MI contained 2.302 gm/l of L-proline, 2.0 mg/l dicamba and 200 mg/l Casein hydrolysate. While, MII medium contained 2.0 mg/l 2,4-dichlorophenoxy acetic acid, 2.88 g/l of L-proline, 8.5 mg/l silver nitrate and 100 mg/l casein hydrolysate .The sucrose concentration in both media was 3% and the pH was adjusted to 5.8. The cultures were incubated in darkness at 28°C with 16/8 hour photoperiod from cool-white fluorescent lights with an intensity of 2000 lux and embryogenic tissues were subcultured every 14 days.

After 8 weeks on callus induction media, plantlets were regenerated from embryogenic calli by transferring callus to magenta boxes containing MS based regeneration media (RMI, RMII, RMIII). The regeneration medium RMI contained 0.5 mg/l IAA (Indole acetic acid), 1 mg/l 6-BAP (6-benzyl amino purine) and 2% sucrose. Medium RMII contained 0.25 mg/l 2,4-D and 6% sucrose. While, medium RMIII contained 1 mg/l NAA (Naphthaline acetic acid), 1.0 gm/l Myo-inositol and 6% sucrose. The pH was adjusted to 5.8 for the three media.

The regenerated plantlets were transferred to the greenhouse into an aquarium containing Hoagland solution, then transferred to soil medium composed of 1/3 soil, 1/3 sand and 1/3 peat moss.

Plant Expression Vector

The plasmid pAB1 (kindly provided by Prof.Dr. Ahmed Bahieldin, Faculty of Agriculture, Ain Shams University and AGERI) has been used for the transformation of maize tissues. Plasmid map of transformation vector pABI is shown in Fig.(1). The plasmid contains the barley *HVA1* gene driven by the maize ubil promoter region (including the first exon and intron) and terminated by the *Nos* gene 3' non-translated region. The plasmid also contains the *bar* gene (under the control of the 35S promoter and Nos terminator) which has been used as a selective marker. The *bar* gene encodes the enzyme phosphinothricin acetyl transferase (PAT) which inactivates phosphinothricin, the active ingredient of the herbicide bialaphos.

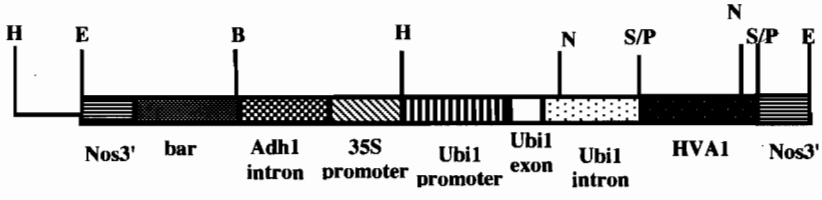

Fig. (1): Plasmid map of the transformation vector pAB1.
Abbreviations for the restriction sites: B: BamHI, P: PstI, S: SalI, E: EcoRI. H: HindIII and N: NcoI.

Maize transformation

Transformation has been carried out using the biolistic particle acceleration device (PDS 1000/He, Bio-Rad). For microprojectile bombardment, plasmid DNA (1μg/μl) was precipitated onto gold particles (1.0 μm in diameter) following the procedure described in the Bio-Rad instruction Manual. Each plate of maize tissues was bombarded once at a rupture pressure of 1100 psi with 5μl of particle suspension mixture per bombardment.

Transformation experiments were performed on only three of the maize

inbred lines, i.e., Sd 34,Sd 63 and Gz 650 . Thirty immature embryos (aseptically removed from maize caryopses and precultured on callus induction medium for 4 days) were placed on each petri plates (100x 15mm) with 10ml of MI or MII. The embryos were arranged in a circle of about 2 cm in diameter in the center of the plates and exposed to an osmotic treatment of 45.4 gm/l of sorbitol and 45.4 gm/l mannitol for 4h before and 16h after bombardment.

Selection and recovery of transgenic calli

Bombarded tissues were incubated on the callus induction media for one week after bombardment. Then, selection of transformed cells was carried out by transferring the transformed calli to (MI) or (MII) media containing 1.5 mg/l bialaphos for 2 weeks. The calli were, then, transferred onto fresh selective media containing 3mg/l bialaphos, with continuous subcultures every 2 weeks. All cultures were kept at 28°C in a dark growth chamber.

Regeneration and recovery of transgenic plants

Bialaphos-resistant calli, which grew uniformly on the selection media, were transferred to the regeneration medium supplemented with 3 mg/l bialaphos. Somatic embryogenic calli capable of developing into green shoots within 2-4 weeks were classified as putative transformants. The selected plantlets were transferred to the RMIII medium (Bohorova *et al.*, 1995) supplemented with 1mg/l bialaphos. The developed putatively transgenic events were transferred into Hoagland solution for 4-6 days and then transferred to soil in environmentally controlled growth chambers and bioconta-inment greenhouse conditions.

Evaluation of transgenic events

Herbicide testing

The activity of PAT, the *bar* gene product, was assayed indirectly by the resistance of transgenic plants to herbicide application. The herbicide basta (Hoechst, Germany) containing 200 g/l glufosinate ammonium was used for leaf painting of the putatively transgenic plants. Basta application was performed by painting approximately 5-10 cm leaf sectors near the tip of the youngest fully-extended leaf at the four-and eight-leaf stage with a 1% solution of the herbicide containing 0.1% (v/v) Tween 20.

Molecular Analysis

Genomic DNA extraction

Genomic DNA was isolated from leaf tissues of each putatively transformed plant as well as from untransformed plants (control) using the CTAB method (Roger and Bendich, 1985).

Polymerase chain reaction analysis

Two sets of primers were used to detect the *bar* and *HVAl* genes by PCR The sequence of the specific primers for the *HVA*1gene were: HVA1a (5'- GGA GAT CTA ACA ATG GCC TCC AAC CAG AAC CAG GGG -3') and HVA1b (5'- GGG ATA TCT AGT GAT TCC TGG TGG TGG TGG TG -3'). While, the sequence of the primers for the *bar* gene were: bar-1 (5'-TGC CAC CGA GGG GAC ATG CCG GC-3') and bar-2 (5'-CCT GAA GTG GAG GCC ATG GGG-3').The PCR reaction was carried out in a 25μl reaction volume containing 50 ng genomic DNA ,25 pmole /ml primers ,200μM each of dATP , dCTP,dGTP and DTTP ,50 μM KCl, 10 mM Tris-HCl, 0.2

mM $MgCl_2$ and one unit of Taq polymerase .

The PCR temperature profile used for the amplification consisted of an initial denaturation cycle at 94°C for 5 min followed by 35 cycles of 94°C (1 min), 55°C (2 min), 72°C (2 min) and a terminal extension cycle at 72°C (8 min) for the *bar* gene, while, for the *HVA1* gene the PCR temperature profile was 94°C (4 min),followed by 35 cycles of 94°C (1 min), 58 °C (45 sec), 72 °C (2 min) and a final cycle at 72°C (7 min). The PCR products were resolved by electrophoresis on 1% agarose gel.

Southern blot hybridization

Genomic DNA(10μg) isolated from leaves of each putative transformed maize plant was digested with *Pst*I restriction enzyme, electrophoresed in 0.8% agarose gels and transferred to a Hybond N^+ nylon membrane (Roche) and then cross-linked to the membrane by UV irradiation. The hybridization probes were prepared by digesting the plasmid pAB1 with *Pst*I to liberate the *bar* and *HVA*I DNA. The desired inserts were labeled using random priming DNA labeling and detection kit (Roche). Further processing, prehybridization, hybridization and washes were carried out mainly according to the protocol described by Kreike *et al.* (1990). The hybridization signals were detected by exposure of the membrane to X-ray films.

Statistical analysis

Statistical analysis were performed according to Steel and Torrie (1980) using the SAS computer software (version 5) with associated least significant differences (LSD) function. Experiments were designed as factorial experiments in completely randomized design.

RESULTS AND DISCUSSION

Maize regeneration

The regeneration frequency of maize immature embryos maintained on two different callus induction media containing two different auxins, i.e.2,4-D and dicamba, was investigated for four Egyptian maize inbred lines (Gz650, Gz643, Sd34 and Sd63).

Table (1): Regeneration Frequency of the four maize inbred lines maintained on two different callus induction media.

	Regeneration Frequency (%)		
Genotype	MI	MII	Mean
Gz 650	10.67a	3.33bc	7.00A
Sd 34	9.33a	0.67c	5.00AB
Sd 63	4.00bc	2.67c	3.33B
Gz 643	8.00ab	2.67c	5.33AB

The results presented in Table (1) indicated that the highest regeneration frequency was recorded by lines Gz 650 and Sd 34 (10.67 and 9.33, respectively) by maintaining the calli on callus induction medium MI containing dicamba. While, the lowest regeneration frequency (0.67) was revealed by line Sd 34 on medium MII containing 2,4-D and $AgNO_3$ .All the tested inbred lines showed better response in embryogenic calli formation when maintained on callus induction medium containing dicamba as compared to the medium supplemented with 2,4-D.In addition, the results clearly revealed that the components of medium MI

(specially dicamba) greatly influenced the quality and quantity of embryogenic calli formed by lines Gz650 and Sd34 as compared to $AgNO_3$ and 2,4-D in MII medium (Fig.2Aand B).

Although, more embryogenic calli were formed by maize lines maintained on dicamba medium, most of these calli failed to form root systems on regeneration media RMI and RMII. While, shooted calli transferred to RMIII were able to produce very good rooting systems (Fig.2C and D).Therefore, it was deduced that dicamba promoted fast differentiation in all tested inbred lines that led to increasing the number of shoots and the regeneration medium RMIII is more suitable for rooting of regenerated shoots than media RMI and RMII (Fig.2E).These results indicate that the presence of dicamba in callus induction medium is beneficial for the initiation of friable embryogenic callus. The promoting effect of dicamba has been also reported by Bohorova *et al.*, (1995). Moreover, dicamba has been reported to increase somatic embryoge-nesis in some Gramineae such as maize (Duncan *et al.*, 1985 *and* Bohorova *et al.*, 1995), wheat (Hunsinger *and* Schauz 1987) and barley (Hazman, 2006).

On the other hand many groups have demonstrated the effect of $AgNO_3$ on the enhancement of type II callus production and the promotion of maize regeneration (Vain *et al.*, 1989a and b; Songstad *et al.*, 1991 and 1996). The Ag NO_3 effect has been observed in some maize genotypes (Vain *et al.*, 1989a and b; Songstad *et al.*, 1991). However, in most of the experiments, Ag NO_3 was used with 2,4-D.

Table (2): The average number of embryogenic calli formed by maize lines on selective media and their transformation efficiency.

Genotype	Average No. of embryogenic calli			Transformation frequency %		
	MI	MII	Mean	MI	MII	Mean
Sd 34	33.00a	4.00c	18.50A	28.67a	5.00b	16.83A
Sd 63	10.67bc	6.67bc	8.67B	5.67b	2.67b	4.17B
Gz 650	7.00bc	13.00b	10.00B	13.33b	10.00b	11.67AB

Maize transformation

Transgenic maize plants were achieved by particle bombardment using plasmid pAB-1 harboring the *HVA1* gene .The transformation experiments were performed on three inbred lines (Sd 34, Sd 63 and Gz 650) and the frequency of putatively transgenic embryogenic calli and the transformation efficiency of these lines were studied. The putatively transgenic calli were selected on media MI or MII containing bialaphos as a selective agent. Medium RMIII was used for regeneration of the putatively transgenic plants (Fig.3 A, B and C).

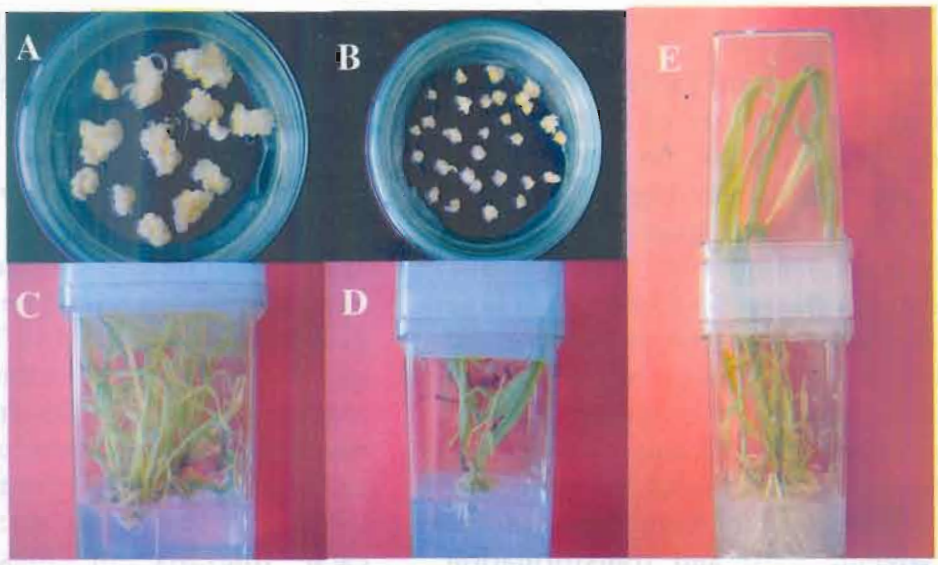

Fig.(2):Maize regeneration:(A) Type II callus showing somatic embryogenesis on MI medium. (B)Somatic embryos on MII medium. (C and D) Regenerated maize plantlets on RMI and RMII medium. (E) Regenerated maize plantlet on RMIII.

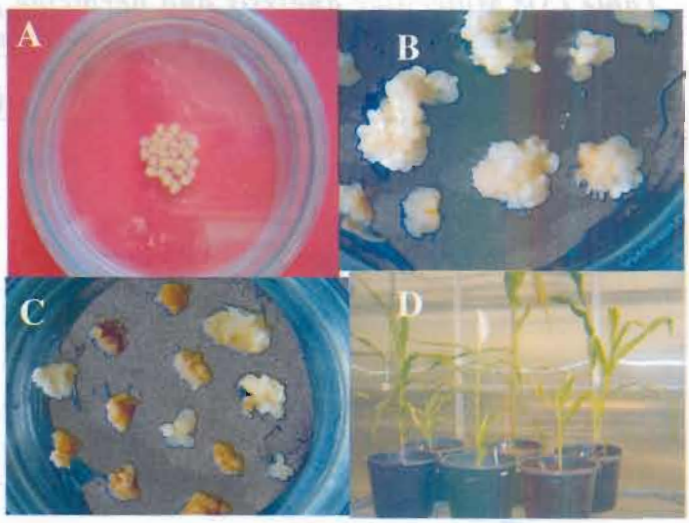

Fig. (3) Maize transformation :(A) Immature embryos after bombardment. (B and C)Callus on MI medium containing 1.5 mg/l bialaphos and 3 mg/l bialaphos, respectively. (D)Fertile regenerated maize plants.

Fig. (4): Leaves of transformed plant (T) and non transformed control plant (C) painted with BASTA™.

Data presented in Table (2) showed that, the average number of embryogenic calli formed by Sd 34 on both media (18.50) was significantly higher than that formed by the other lines .While, the lowest average number of embryogenic calli was revealed by Sd 63 (8.67). These results have been reflected on the average transformation frequency of the lines under investigation, where the highest mean frequency (16.83) was obtained by Sd 34.Moreover, the results revealed that medium MI resulted in higher percentage of embryogenic calli and transformation frequency than medium MII. This may indicate that the presence of dicamba in the callus maintenance and selection media promoted the formation of embryogenic calli and improved the transformation efficiency of some maize lines.

The results of the transformation experiments of maize lines with *bar*/*HVA*1 genes are summarized in Table (3). A total of fourteen independent events were regenerated (Fig.3 .D) from the transformation experiments out of 1800 immature embryos bombarded from the three lines (300 embryos from each treatment for each line).Six of these events were positive as indicated by leaf painting assay for the *bar* gene (Fig.4).

Table (3): Molecular analysis and herbicide painting of the putatively transgenic events recovered out of the transformation experiments.

Plant #	Genotype	PCR		Southern blotting		Leaf painting
		Bar	*HVA*1	*bar*	*HVA*1	
1	Sd34	+	+	+	+	+ +
2	Sd34	-	-	-	-	-
3	Sd63	+	+	+	+	+ +
4	Gz650	+	+	+	+	+ + +
5	Gz650	-	-	-	-	-
6	Gz650	+	+	+	+	+ + +
7	Sd34	-	-	-	-	-
8	Sd34	-	-	-	-	-
9	Sd34	-	-	-	-	-
10	Gz650	+	+	+	+	+ +
11	Sd63	-	-	-	-	-
12	Gz650	-	-	-	-	-
13	Gz650	-	-	-	-	-
14	Sd34	+	+	+	+	+++

The PCR assay confirmed the presence of both the *bar* and *HVA*1 genes in the DNA extracted from these plantlets, (Fig. 5.A and B). Putatively transgenic plants were subjected to Southern blot analysis to confirm the integration of the transgenes (*bar* and *HVA*1) in their genome.

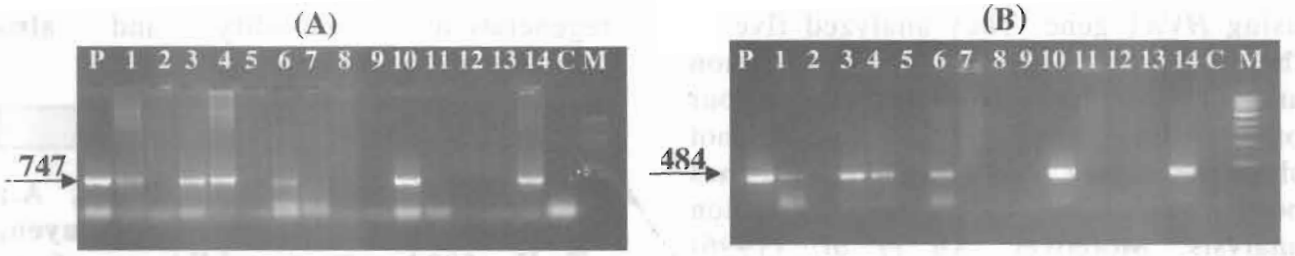

Fig. (5): PCR amplified DNA using the HVA1 gene specific primers (A)and the bar gene primers (B) from pABI(P), untransformed (C) and putatively transformed maize plants (1-14), (M) molecular weight size marker.

As illustrated in Fig. (6.A and B) the estimated copy number of the intact fragment of each gene varied from approximately 10 to 15 for these independently produced transgenic plants as compared to the positive control. Moreover, Fig. (6.A and B) illustrates that the Southern blot hybridization analysis of the genomic DNA of the putatively transgenic plants exhibited the desired gene fragments corresponding to the intact *HVA*1 and *bar* genes (1.0 Kb and 2.0 Kb, respectively) in six plants.

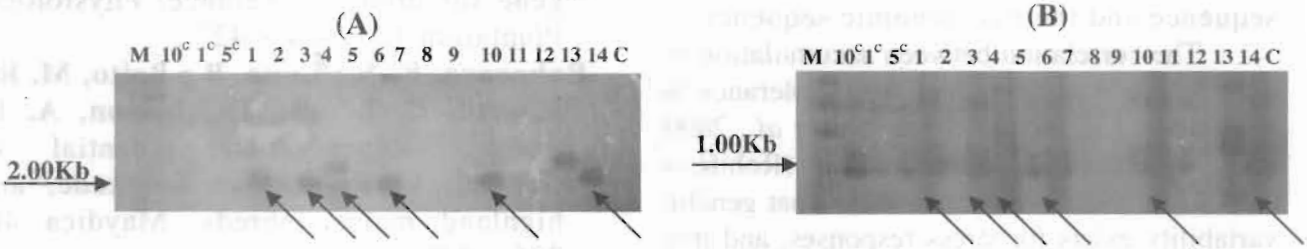

Fig. (6): DNA hybridization analysis of putatively transgenic maize plants for the bar gene (A) and the HVA1gene (B).Lanes (10^c, 1^c and 5^c):pAB1-DNA represents 10, 1 and 5 copies, respectively. Lanes (1-14): putatively transgenic plants .Lane (C): negative control untransformed plant. (M): DNA molecular weight size marker.

In the present investigation, three maize inbred lines were transformed by particle bombardment method and transgenic events were recovered. PCR analysis and leaf painting with herbicide Basta proved the presence of the transgenes (*bar* and *HVA*1) in the genome of these events. Southern blot analysis proved the integration of the transgenes in the genome of these plants. The six positive samples in the leaf painting and PCR test showed the correct size of *Pst*1 digested *HVA*1 fragment (1kb). Sample number (13) showed a fragment which did not correspond to the correct size of *Pst*1 fragment (Fig.6).This indicates that this line did not contain the intact copy of *HVA*1 transgene. This could suggest that the gene might be expressed in all of the PCR and Basta positive transgenic plants except this line and these *HVA*1 expressing plants could tolerate abiotic stresses. Other hybridizing fragments were also observed.In this respect, Siviamani *et al.* (2000) recovered ten transgenic wheat lines out of transformation experiments

using *HVA*1 gene. They analyzed five of these lines by Southern blot hybridization and found the transgene integrated in four of these lines while the fifth line did not show the correct *HVA*1 fragment. This has been confirmed by gene expression analysis. Moreover, Xu *et al.* (1996) introduced *HVA*1 gene into rice suspension cells and generated a large number of independent transgenic rice plants. Digestion of genomic DNA of these plants with *Hind*III (single site on the plasmid) and with combination of *EcoR*I, *BamH*I probe proved the integration of *HVA*I gene in the genome of these plants by the presence of 1kb fragment in *Hind*III digested samples and the fusion fragments containing *HVA*1 sequence and the rice genomic sequence.

The correlation between accumulation of LEA group 3 proteins and stress tolerance is well studied in wheat (Siviamani *et al.*, 2000 and Bahieldin *et al.*, 2005) and rice (Rohila *et al.*, 2002). Moreover, it is evident that genetic variability exists for stress responses, and this could be due to the differential expression and regulation of stress responsive genes such as *HVA*1 gene when the plants are exposed to stress (Oraby *et al* 2005). On the basis of our experimental results, the presence of *HVA*1 gene in the genome of transgenic plants under the control of the ubi 1 promoter suggests that constitutive expression of *HVA*1 gene can improve growth performance in transgenic plants under salinity stress conditions, however, the exact function of LEA proteins remains uncertain.

Results of this study showed that a reproducible regeneration system for four Egyptian maize inbred lines was successfully developed using the immature embryo explants. Among the lines studied, Gz 650 and Sd34 possess the highest regeneration potentiality and also transformation frequency.

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الملخص العربي

التحول الوراثي لبعض سلالات الذرة الشامية المصرية بإستخدام جين الـ*HVA1* المعزول من الشعير

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يعتبر التركيب الوراثي من العوامل المحددة في عمليات التحول الوراثي للعديد من سلالات الذرة الشامية الاستوائية والتحت استوائية . وفي هذا البحث تم دراسة إمكانية تكوين الكالس وإعادة التمايز من الأجنة الغير ناضجة لأربعة أصناف مصرية من الذرة الشامية . ولذلك تم اختبار نوعين من البيئات الصناعية لدراسة تأثير كلاً من dicamba & 2,4-D على استحداث الكالس وإعادة التمايز . وقد أظهرت النتائج أن الـ dicamba أدت إلي زيادة كفاءة استحداث عملية إنتاج الكالس وإعادة التمايز في جميع السلالات مقارنة بالـ 2,4-D . كما أظهرت السلالتين Gz 650 و Sd34 زيادة معنوية في إعادة التمايز عند زراعتها علي البيئة المحتوية علي dicamba وفي محاولة لإنتاج نباتات مقاومة للظروف البيئية المعاكسة تم نقل جيني الـ *HVA1* (late embryogenesis abundant protein coding gene) المعزول من الشعير وكذلك جين الـ *bar* الخاص بمقاومة الحشائش كجين انتخابي وذلك في ثلاث سلالات من السلالات الأربعة من الذرة الشامية باستخدام قاذفة الجينات وتم الحصول علي نباتات محوله وراثياً أجري عليها اختبار المقاومة لمبيد الحشائش Basta . ثم تم تأكيد اندماج الجينين المنقولين في جينوم النباتات المحولة باستخدام الـ PCR و Southern Blotting وأظهرت النتائج أن عدد نسخ الجينين يتراوح ما بين ١٠ – ١٥ نسخة في النباتات المحولة المختلفة والنتائج توضح أن جينات الـ LEA يمكن أن توفر أداة جزيئية مفيدة في التحسين الوراثي للنباتات المقاومة للظروف البيئية المعاكسة .