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PRODUCTION OF TRANSGENIC BANANA PLANTS CONFERRING TOLERANCE TO SALT STRESS

[19]

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ABSTRACT

Production of bananas is limited in areas that have soils with excess sodium. In this study, a transformation system in banana Grand Nain cultivar was established using the apical meristem explant and plasmid pAB6 containing the herbicide-resistant gene (*bar*) as a selectable marker and *gus* reporter gene. The microprojectile bombardment transformation system using 650 psi was successfully used for introducing the studied genes in banana explants. The expression of the introduced genes was detected using leaf painting and GUS histochemical tests, respectively. The present results showed that among the selection stage, 36.5% of the bombarded explants survived on the BI3 medium supplemented with 3 mg/L bialaphos, while, 26.6% of the tested explants showed a positive reaction in the GUS assay. To detect the presence of *bar* and *gus* genes the PCR was successfully used. These results encourage the idea of possibility of banana crop improvement using *in vitro* technique through microprojectile bombardment. Therefore, the plasmid pNM1 that carries the *bar* and *P5CS* (Δ1-pyrroline-5-carboxylate synthetase for proline accumulation) genes was introduced in banana Grand Nain cultivar to produce transgenic plants expressing the salt tolerance gene. Results showed that the majority of herbicide-resistant banana plantlets were successfully acclimatized. In studying the effects of different salt concentrations on the produced transgenic banana plants, results showed lower decrease in the percentage of survived plants, pseudostem diameter and leaf area with an increase of salt concentrations in case of transgenic plants compared with the controls.

Key words: Banana, Transformation, *gus* gene, *bar* gene, Leaf painting, Microprojectile bombardment, Salt tolerance

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INTRODUCTION

In Egypt, the cultivated area of bananas increased to 60000 Feddans with total production of 1032000 tones **(Anonymous, 2004).**

Drought and high salinity are two major environmental factors that cause osmotic stress and dramatically limit plant growth and crop productivity (**Boyer, 1982**). Production of bananas is limited in areas with salinized soils or with excess sodium. These problems are increasing seriously in arid and semi-arid regions of the world (**Waked *et al* 2002**). Soil salinity reduces banana growth and its productivity because of the reduced osmotic pressure in the soil and the increase in certain ions to concentrations that are toxic to plants. These factors interfere in physiological processes as well as causing imbalances in the water and/or ionic equilibrium of the plant (**Richards 1992 and Bohra & Doerffling, 1993**). Methods to restore saline soils are generally slow and expensive. The use of cultivars of banana which tolerate saline stress may provide a solution which is technically and economically viable. The selection of banana genotypes in soils that are naturally saline is difficult because of the large spatial and temporal fluctuations in the ionic concentration of soil. In order to avoid such natural variation at the first stage of selection, it is possible to use nutrient solutions with added salinity to control ionic concentration (**Rawson *et al* 1998**).

Several investigators (**Sagi *et al* 1995; May *et al* 1995 and Ismail 2003**) encouraged the idea the improvement of banana crop *via* genetic transformation.

The main objectives of the present investigation is to establish a transformation system in the banana Grand Nain cultivar through introduce a salt tolerance gene in the banana cultivar under investigation, and to evaluate the transformed banana plants for their salt tolerance under greenhouse conditions.

MATERIAL AND METHODS

Preparation of plasmid DNA for transformation

The plasmid pAB6 (9.45 kbp) containing both *gus* reporter gene and the herbicide-resistance *bar* gene was used to establish the banana transformation system. The plasmid pNM1 (8.18 kbp) containing the *P5CS* gene controlled by 35S promoter and *nos* terminator and the *bar* gene as a selectable marker were used. These plasmids were kindly provided by Environmental Stress Laboratory (ESL), AGERI, ARC, Giza, Egypt.

Regeneration condition

All regeneration treatments were carried out using the BI3 medium (inorganic salt MS medium, **Murashige and Skoog, 1962**), containing 30 g/L sucrose and solidified with 2.8 g/L phytagel). The pH was adjusted to 5.8 before autoclaving. Plant materials were maintained at 28°C±2 for 16 h light cycle with fluorescent light of 330-350 Footcandles.

Banana transformation

Banana plants Grand Nain cultivar (6-month-old) were used as a source of explants, for banana transformation. The outer leaves, leaf bases and corm tissues of selected explants were trimmed to 2.5 X 2.5 X 5 cm and shoot tips were soaked

in 30% sodium hypochlorite solution (Clorox) for 20 min followed by soaking in mercuric chloride solution (1 g/L) for 25 min and 40% Clorox for 30 min. After washing with sterile distilled water (d.H_2O) three times for 5 min each, they were kept under sterile conditions. The apical meristems were then trimmed to the approximate size, cultured on the BI3 medium containing kinetin (1.5 mg/L) and incubated for 20 days under low light density conditions. The explants were then divided into 2-3 parts based on its original size. For 4-5 days, the explants were cultured on osmotic medium (BI3 + 0.4 M sorbitol and 0.4 M mannitol) and incubated for 5 h before bombardment.

A volume of 5 ml LB medium was inoculated with a single colony of *E. coli* DH5α strain containing the plasmid pAB6 or pNM1 and incubated overnight at 37°C with moderate shaking (250 rpm). Plasmids were prepared using High Pure Plasmid Isolation Kit (Promega Inc., Cat. # A1470).

Using the Biolistics Particle Delivery System (Bio-Rad PDS/1000/He Gene Gun), the prepared explants were bombarded under the following conditions: microcarrier (gold), 1.1 μM (60 mg/ml d.H_2O); rupture disk (650 psi) macrocarrier (6 mm), microcarrier travel distance (9 cm); and chamber vacuum (25 inch Hg).

After bombardment, the explants were incubated at 28°C in a dark incubator for 24 h in semisolid BI3 medium with 100 mg ascorbic acid and 100 mg citric acid. Then, they were transferred onto a selective medium (BI3 medium + 3 mg/L bialaphos) and incubated for 3 weeks. The selected explants were subcultured on shoot formation and root formation media as mentioned.

Evaluation of putative transgenic banana plants

GUS histochemical assay

Twenty four hours post bombardment samples, i.e., parts of apical meristem explants were histochemically tested for the expression of *gus* gene following the protocol of **Jefferson *et al* (1987).**

Leaf painting

Leaves of the transformed banana plants were painted with BASTA herbicide (2 g/L) to detect the expression of *bar* gene. The leaves that gave dark brown color (necrosis) were considered negative, while others that appeared in the normal green color were considered as positive.

PCR detection

In this experiment, six different oligonucleotides (Table, 1) were used as primers for PCR detection of the introduced genes. The primers specific for *gus*, *bar* and *P5CS* genes were obtained from ESL, AGERI, ARC, Giza, Egypt.

DNeasy™ Plant Mini Kit (Qiagen Inc., Cat. # 69104) was used for DNA isolation as described by the Manufacturer from putative banana plant materials. PCR was conducted in a volume of 50 μl (**Sadik *et al* 1999**) containing two specific oligonucleotides as primers. A PCR mixture without any DNA was used as a negative control. PCR amplification was performed in a Perkin-Elmer (Gene Amp PCR System 2400) for 35 cycles after initial denaturation for 4 min at 94°C. Each cycle consisted of denaturation at 95°C for 1 min, annealing at 60°C

Table 1. Nucleotide sequences of three pairs of primers specific for *gus*, *bar* and *P5CS* genes

Genes	Primers	Size (nt)	Sequences (5'----------3')	Expected PCR products (bp)
gus	P1	31	CCA GAT CTA ACA ATG CGC GGT GGT CAG TCC C	1800
	P2	32	CCA GAT CTA TTC ATT GTT TGC CTC CCT GCT GC	
bar	P3	30	AAA AGC TTC CAC CAT GAG CCC AGA ACG ACG	540
	P4	25	AAG GAT CCT CAG ATC TCG GTG ACG G	

(for *bar* or *gus* gene) for 1 min and extension at 72°C for 2 min. The primer extension was done for 7 min at 72°C in the final cycle. The PCR amplified product was analyzed by electrophoresis on 1.0% agarose gel in 1X TAE buffer at 80 volts for 1 h (Sambrook *et al* 1989). The DNA was visualized by staining gel in ethidium bromide (0.5 mg/mL) and photographed under UN transilluminator using a Polaroid camera.

Salt tolerance evaluation of transgenic banana plants

To evaluate the salt tolerance of the transgenic banana plants Garnd Nain cultivar expressing the *P5CS* gene, 60 T0 plants were divided into four groups, each of 15 plants. The first group was left without any treatment as a control. Groups 2, 3 and 4 were subjected to irrigation with saline solutions (1000, 1500 and 2000 ppm) containing 3 NaCl+1(3 $CaCl_2$+1 $MgCl_2$). As a control, similar number of non-transgenic banana plants Grand Nain cultivar was subjected to the same treatments. After 21 days, three parameters were determined, i.e., number of survived plants, leaf area (**Moreira, 1987**), and pseudostems-base diameter. The data were statistically analyzed using the analysis of variance as outlined by **Gomez and Gomez (1984)** using MSTATC program. The differences between means were compared using Duncan multiple range test (**Duncan, 1955**).

RESULTS AND DISCUSSION

Establishment of transformation system in banana Grand Nain cultivar

The transformation system of banana Grand Nain cultivar was established. The obtained results (Table 2) showed that a transformation frequency of 36.5% (38/104) was obtained. The selection stage of the putative transgenic banana explants and their regenerations were shown in Figures (1) and (2), respectively.

Furthermore, the transformed plantlets were subjected to leaf painting after acclimatization.

Table 2. Transformation frequency based on number of survived explants on the selection medium

Replicates	NUE	NBP	SP	
			No.	%
R1	16	32	11	34.3
R2	16	32	9	28.1
R3	20	40	18	45.0
Total	52	104	38	36.5

NUE : Number of used explants.
NBP: Number of bombarded pieces.
SP: Survived pieces.

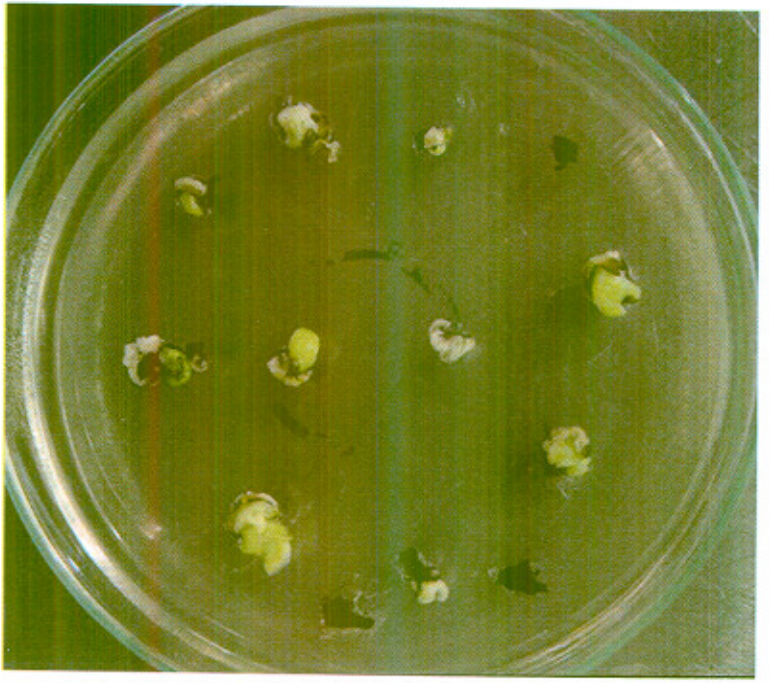

Fig. 1. Selection stage of putative transgenic banana explants bombarded with plasmid pAB6 carrying *bar* and *gus* genes. The explants were grown on a selection medium supplemented with 3 mg/L bialaphos.

Fig. 2. Regeneration of transformed banana plant materials. a) Excised explants; b) Bombarded pieces; c) Selected explants; d) Shoot formation; e) Shoot elongation and f) Root formation.

Detection of *gus* gene

The expression of *gus* gene was detected in the parts of the banana bombarded explants by GUS assay as mentioned before. Data in Table (3) as illustrated in Figure (3) showed that 26.6% of the tested explants showed a positive reaction indicating the expression of the *gus* gene in banana meristem tissues. To detect the presence of *gus* gene in transformed banana plant materials, the DNA was extracted from 18 parts of the bombarded explants (the eight GUS-positive parts and 10 GUS-negative parts). Data in Figure (4) showed the amplification of a fragment with a size of about 1800 bp. This result proved the data obtained from GUS assay.

Table 3. Detection of *gus* gene expression in transgenic banana plant material bombarded with plasmid pAB6

Replicates	NTE	Positives	
		No.	%
R1	10	3	30
R2	10	2	20
R3	10	3	30
Total	30	8	26.6

NTE: Number of tested explants.

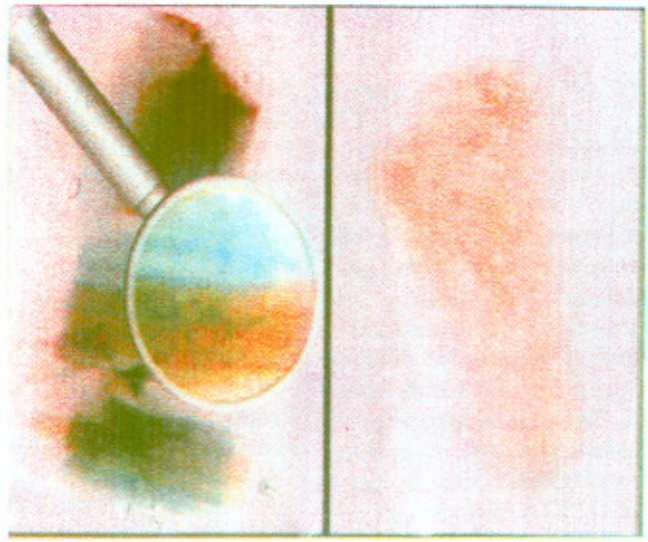

Fig. 3. GUS assay of bombarded meristem explants of banana Grand Nain cultivar bombarded with plasmid pAB6.

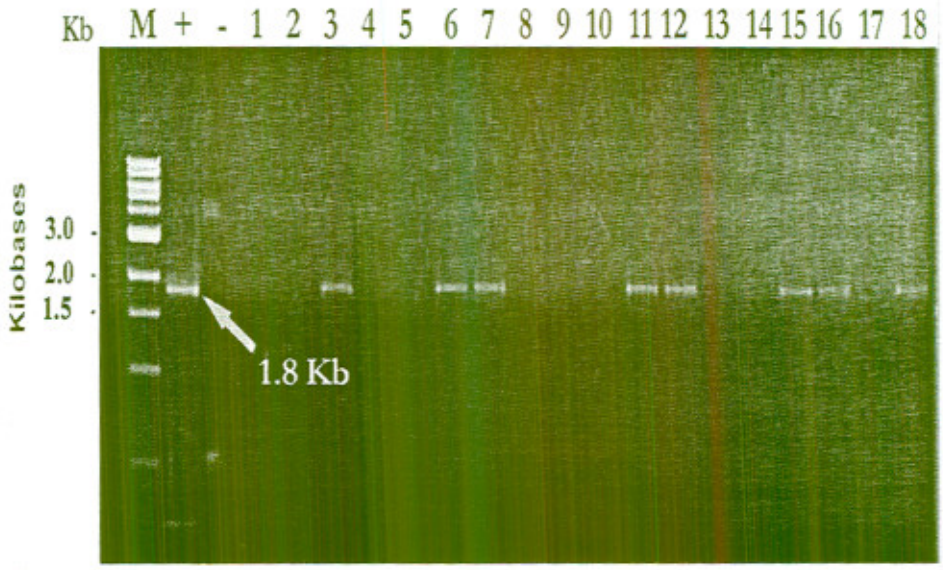

Fig. 4. PCR detection of *gus* gene in 18 apical meristem explants of the banana Grand Nain cultivar bombarded with plasmid pAB6.

Detection of *bar* gene

PCR was successfully used for detecting the presence of *bar* gene in the regenerated eighteen GUS-assayed lines. Results shown in Figure (5) confirmed the presence of *bar* gene into the banana genome in 8 transgenic banana lines.

Herbicide resistance of putative transgenics was tested by painting the middle green parts of the plant leaves from both sides with 2 g/L BASTA. The experimental results showed that the transgenic plants under investigation varied in their tolerance to the BASTA herbicide as illustrated in Fig (6). Therefore, 6 tolerance levels, i.e., +++++, ++++, +++, ++, + and -, were suggested to evaluate the expression of *bar* gene in the obtained transgenic plants. It worth to mention that in case of highly tolerant plant, its leaf stayed green, while leaves of the other plants showed a different color that ranged from light yellow color to complete necrosis of the cells of the painted area. On the other hand, the control non-transgenic plant leaves were turned yellow and the cells died within two days. This result demonstrated the expression of *bar* gene in the genomic background of banana plants.

Production of transgenic banana plants expressing the *P5CS* gene

Data in Table (4) showed that three transformation experiments with plasmid pNM1 replicated 3 times were carried out. After selection on BI3 medium supplemented with 3 mg/L bialaphos, a set of 110 out of the 180 explants were survived. Out of this number, 90 explants gave shoots, and then 72 of them gave

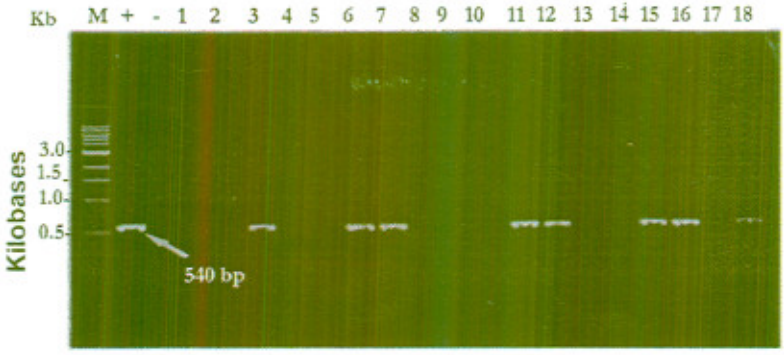

Fig. 5. PCR detection of *bar* gene (540 bp) in 18 transgenic lines of the banana Grand Nain cultivar.

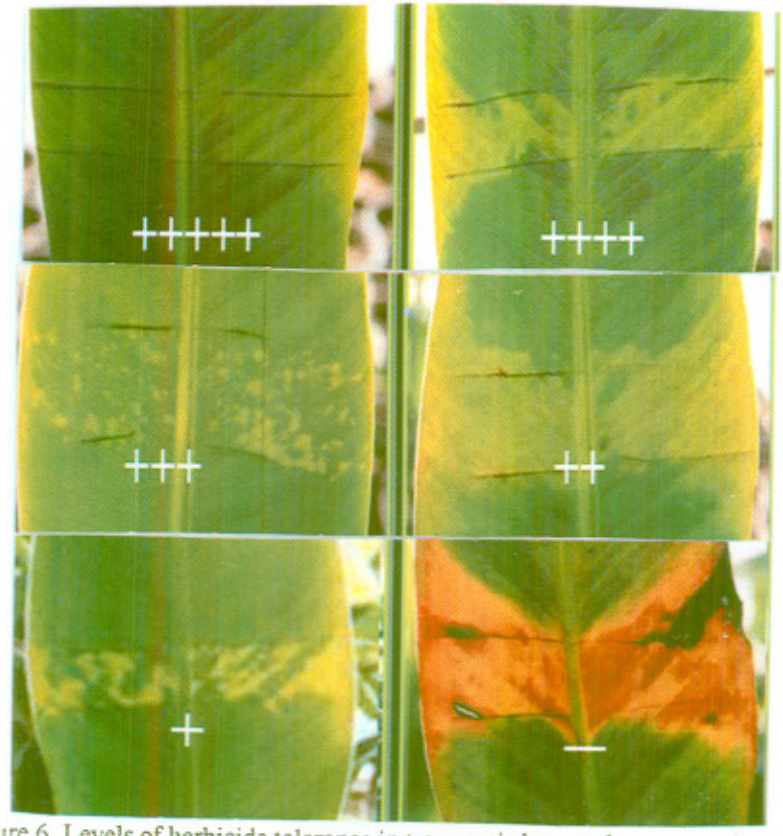

Figure 6. Levels of herbicide tolerance in transgenic banana leaves carrying the plasmid pAB6 containing *bar* and *gus* genes 5 days post painting with BASTA herbicide (2 g/L). Levels of tolerance: +++++, ++++, +++, ++, +, -.

Table 4. Banana Grand Nain cultivar transformed with plasmid pNM1 carrying the *P5CS* and *bar* genes and regeneration status of the selected plant materials

Exper.	Rep.	NUE	SE		PS		RS	
			No.	%	No.	%	No.	%
1	R1	20	13	65	11	84.6	9	81.8
	R2	20	12	60	10	83.3	8	80.0
	R3	20	13	65	10	76.9	8	80.0
	Total	60	38	63.3	31	81.5	25	80.6
2	R1	20	11	55	10	90.9	7	70
	R2	20	12	60	9	75	8	88.8
	R3	20	12	60	10	83.3	8	80
	Total	60	35	58.3	29	82.8	23	79.3
3	R1	20	12	60	9	75	8	88.8
	R2	20	14	70	11	78.5	7	63.6
	R3	20	11	55	10	90.9	9	90
	Total	60	37	61.6	30	81.1	24	80.0
Total		180	110	61.1	90	81.8	72	80.0

NUE = Number of used explants. SE = Survived explants. PS: Produced shoots. RS = Rooted shoots.

roots. These plantlets were then acclimatized under a controlled greenhouse (Biocontainment) conditions and 65 of them successfully acclimatized.

Evaluation of transgenic banana plants containing the *P5CS* gene

A number of 120 banana plants (60 transgenic and 60 non-transgenic plants) with 8-10 leaves were used. Each was divided into four groups (15 plants for each group). The groups were irrigated with three different concentrations (1000, 1500 or 2000 ppm) salt mixture twice per week for 3 weeks. Then, three parameters, i.e., percentage of survived plants, pseudostem diameter and leaf area were recorded. Results in Table (5) showed the percentage of survived (tolerant) plants which were higher in the case of transgenic banana plants (53.3, 40 and 40) comparing with the non-transgenic plants (26.6, 20 and 6.6) after irrigating with 1000, 1500 and 2000 ppm salt mixtures, respectively. A negative correlation between the salt concentrations and survived plants, diameter of pseudostem and leaf area was noted. The result reflected the integration of the *P5CS* gene into the banana genome and its expression that varied from plant to another. Results illustrated in Figure (8) showed stress symptoms such as chlorosis and necrosis of the margin of the leaf lamina which were more severe up to the time of leaf death in the salt sensitive plants compared with the transgenic plants. In addition, the expression of *bar* gene was also confirmed in the salt-tolerance lines (Figure 7).

Table 5. Evaluation of transgenic banana plants cv. Grand Nain containing the *P5CS* salt tolerance gene.

Salt mixture concentrations (ppm)	Transgenic plants			Non-transgenic plants		
	SP (No./15)	PsD (cm)	LA (cm^2)	SP (No./15)	PsD (cm)	LA (cm^2)
0000	13 a	9.84a	195.9 a	14 a	9.64 a	188.1 a
1000	8.0 b	7.32a	182.3 ab	6.0 d	5.89 b	151.5cd
1500	7.0 c	4.54b	163.7 bc	4.0 d	4.09 b	139.1 d
2000	6.0 c	4.20b	143.6 d	3.0 d	3.77 b	132.0 e

SP: Survived plants. PsD: Pseudostem diameter. LA: Average number of leaf area.
Means having the same letter (s) in a column or line are not significantly differ at 5% level.

Figure 7. A transgenic banana leaf with the plasmid pNM1 containing *bar* and *P5CS* genes 5 days post painting with BASTA herbicide (2 g/L) (Left). Right: Control, non-transgenic banana Grand Nain cv. leaf showing high sensitivity to BASTA.

Figure 8. Non-transgenic (Left) and Transgenic (Right) banana plants evaluated for their tolerance to salt mixture (2000 ppm) under greenhouse condition.

DISCUSSION

The use of banana (*Musa* spp.) plant production by tissue culture is increasing (**Mendes *et al* 1996**). Bananas and plantains have not received as much attention as many other smaller crops. Therefore, this investigation aimed to establish a transformation system in the banana Grand Nain cultivar and producing transgenic banana plants having the ability to tolerate salinity.

Successful foreign gene transfer to a plant was first reported by **Zupan *et al*** (1996) using genetically manipulated strains of *Agrobacterium tumefaciens.* Since this different approaches have developed for producing transgenic plants worldwide.

Biolistics Particle Delivery System using 650 psi helium pressure was successfully used for introducing the plasmid pAB6 that carries the *gus* reporter and *bar* selectable marker genes in the apical meristem explants of banana. Banana transformation *via* biolistic gun system was reported with **Sagi *et al* (1995); Remy *et al* (1998)** and **Becker *et al* (2000).** Results showed that 36.5% of the bombarded pieces survived on the BI3 selection medium that contains 3 mg/L bialaphos. The presence and expression of *gus* gene was detected in the banana bombarded plant materials, using PCR and histochemical-GUS assay, respectively. In addition, the herbicide BASTA at the recommended dose (2 g/L) was used for detecting the expression of *bar*

gene in some transgenic banana plants. Leaf painting results showed variation in the expression of *bar* gene in the banana cells of the tested lines. Similarly, the presence of *bar* gene into the banana genome of the 8 herbicide-tolerant transgenic banana lines was confirmed by PCR.

In Egypt, **Ismail (2003)** used the same plasmid to establish the transformation system of the banana Williams cultivar *via* microprojectile bombardment transformation system. The author also stated that *gus* and *bar* genes were successfully expressed in the transformed plant materials.

In addition, **May *et al* (1995)** developed an *Agrobacterium*-mediated plant transformation system for the generation of transgenic banana (*Musa* spp. var. Grand Nain). They reported that the cut surfaces of corm slices or bisected apical meristems were bombarded with gold microparticles (not coated with DNA) to cause wounding of cells underlying the cut surfaces. The bombarded tissues were then maintained in culture for three days to maximize biochemical wounding responses before co-cultivation with *Agrobacterium*. They also demonstrated conditions for the recovery of genetically transformed banana (var. Grand Nain) using kanamycin as a selective agent following introduction of the *npt II* gene. Analyses of roots and shoots of each individual transformant showing GUS activity in both tissues. Other investigators (**Khayat *et al* 1998; Perez-Hernandez *et al* 1999; Bosque-Perez *et al* 2000 and Ganapathi *et al* 2001**) discussed the successive use of *Agrobacterium*-mediated transformation system in banana.

At the present time, the dominant cultivar used for the production of export bananas is Grand Nain (**Anonymous, 1992 and May *et al* 1995**). Saline or excess sodium problems in the soil reduce crop growth and productivity because of the reduced osmotic pressure in the soil and the increase in certain ions to concentrations that are toxic to plants (**Richards 1992 and Bohra and Doerffling, 1993**). The negative effects of saline in soils on banana were discussed by **Jeyabaskaran and Sundararaju (2000)**. One of the objectives of this work was producing transgenic banana plants tolerant to salt stress *via* microprojectile bombardment transformation system.

The gene encoding Δ^1 pyrroline-5-carboxylate synthetase (P5CS) a bifunctional enzyme, has been shown to be transcriptionally regulated by osmotic stress and abscissic acid (ABA) treatments (**Hu *et al* 1992 and Yoshiba *et al* 1995**). It has been also shown that P5CS is the rate-limiting enzyme of the proline synthesis pathway under stress conditions (**LaRosa *et al* 1991 and Sozke *et al* 1992**) and the overexpression of P5CS has been shown to increase proline levels both in unstressed and osmotically stressed plants.

Therefore, the plasmid pNM1 that carries the *bar* and *P5CS* genes was introduced in the banana Grand Nain cultivar. Results showed that after selection on BI3 medium supplemented with 3 mg/L bialaphos, 65 plants were successfully acclimatized under a controlled greenhouse (Biocontainment) conditions. The obtained transgenic banana plants were subjected to leaf painting and results were positive.

The expression of *P5CS* gene for proline accumulation was detected in 45 transgenic banana lines by evaluating their salt tolerance by irrigation with

three concentrations of salt mixtures (1000, 1500 and 2000 ppm). Three parameters, i.e., rate of survival, diameter of pseudostem, and leaf area were determined.

The experimental results showed that percentage of survived (tolerant) plants were higher in case of transgenic banana plants compared with the non-transgenic plants. Among the three salt concentrations, it was found that the increase in salt concentrations was accompanied with a high decrease in the values of three parameters in the controls (Non-transgenic plants) compared with the transgenic plants.

Gomes *et al* (2002) showed that increasing NaCl concentration in the nutrient solution resulted in a reduction in leaf area and dry weight for most banana genotypes. They also reported that stress symptoms such as chlorosis and necrosis of the margin of the leaf lamina were more severe in the diploid 'Calcutta 4' up to the time of leaf death. The symptoms reduced the photosynthetically active area and markedly reduced growth.

The reduction in leaf growth was partially due to a reduction in the net assimilation rate of CO_2 (**Akita and Cabuslay, 1990**) as a result of the closure of the stomata in response to the low water potential in the soil.

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إنتاج نباتات موز معدلة وراثيا متحمله للإجهاد الملحي

[١٩]

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يتأثر إنتاج الموز في المناطق التي تتصف فيها التربة بالملوحة أو زيادة الصوديوم. وفي هذه الدراسة تم تأسيس نظام التحول الوراثي في نبات الموز صنف جراندنان باستخدام القمة المرستيمية كمنفصل نباتي (جزء نباتي) وبلازميد الـ pAB6 المحتوي علي جين الـ *bar* والمسئول عن المقاومة لمبيد الحشائش كجين كاشف وجين الـ *gus* كجين دال. وتم استخدام تقنية القذف الجيني بنجاح عند ضغط ٦٥٠ psi لإدخال الجينات موضع الدراسة في المنفصلات النباتية للموز. تلي ذلك الكشف عن تعبير الجينات التي تم إدخالها عن طريق دهان أوراق النباتات بمبيد الحشائش الباستا وكذلك الاختبار اللوني للكشف عن جين الـ *gus* . وقد أظهرت النتائج أن ٣٦,٥% من بين المنفصلات النباتية المقذوفة والتي تم تنميتها علي بيئة BI3 المحتوية علي ٣ ملجم/ليتر مادة الـ bialaphos قد تم انتخابها نظرا لقدرتها علي البقاء حية ومقاومتها للـ bialaphos ، كما أن ٢٦,٦% من المنفصلات النباتية المقذوفة قد أعطت نتيجة موجبة عند اختبارها بالاختبار اللوني للكشف عن الـ *gus*. كما تم بنجاح استخدام تقنية الـ PCR في الكشف عن وجود الجينات موضع الدراسة باستخدام بادئات متخصصة لكل منهما. وقد أيدت هذه النتائج التفكير في احتمالية تحسين الموز بالتقنيات المعملية من خلال تقنية القذف الجيني. وبناء علي ذلك فقد تم إدخال بلازميد الـ pNM1 والمحتوي علي جينات الـ *gus* والـ p5CS المسئول عن إكساب النباتات صفة تحمل الملوحه وذلك في صنف الجراندنان من نبات الموز. وقد تم أقلمة ٦٥ نبات متحمل لمبيد الحشائش من الـ ٧٢ نبتة المتحصل عليهم بنجاح. وبتقييم مجموعة من تلك النباتات المعدلة وراثيا لدراسة تأثير الملوحة عليها أظهرت النتائج انخفاض أقل في مساحة الورقة ومعدل البقاء حيا ومتوسط قطر الساق الكاذبة بزيادة تركيز الملوحة في النباتات المعدلة وراثيا مقارنة بالنباتات غير المعدلة وراثيا.

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