Journal of Agricultural Science and Technology A 7 (2017) 246-257
doi: 10.17265/2161-6256/2017.04.003

Transgenic Date Palm Containing Endotoxin *Cry3Aa* Gene

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Abstract: Date palm, like all other crops, is very sensitive to the injury by many insect pests, which may lead to the death of the affected plant and causes decrease in yield. In the present study, an efficient *Agrobacterium* for genetic transformation was successfully achieved for well known date palm (*Phoenix dactylifera* L.) cv. Medjool and Khalas using callus as explant. Embryogenic callus were recorded 100% mortality when cultured on MS medium containing 100 mg/L kanamycin with different cultivars, thus it was chosen for the selection of transformed explants. Embryogenic callus of Medjool and Khalas were incubated with *Agrobacterium tumefaciens* strain LBA 4404 for 0.5, 1, 2, 4 and 24 h on LB medium. After the incubation periods, embryogenic callus was transferred to MS medium with 0.1 mg/L NAA, 0.05 mg/L BA, 250 mg/L carbenicillin and 100 mg/L kanamycin for detection of transgenic embryogenic callus. Polymerase chain reaction (PCR) was used for the rapid screening of *Cry3Aa* gene. For screening, total genomic DNA was isolated from transformants. Using primer specific to *Cry3Aa* gene (forward and reverse), a PCR product with a size of about 2,000 bp was amplified when all nucleic acid from the transformants were utilized as templates. PCR analysis confirmed the appearance of the transgene of 2,000 bp in one individual plantlet. Presence and integration of foreign *Cry3Aa* gene in regenerated kanamycin resistant embryogenic callus was also confirmed by Southern blot hybridization. It was found that one transgenic embryogenic callus for both Medjool and Khalas showed a single copy of gene integration. These results signify the successful transfer of *Cry3Aa* gene into date palm plant.

Key words: Date palm, *Agrobacterium tumefaciens*, *Cry3Aa* gene, Southern blot analysis, transformation.

1. Introduction

Date palm tree has a large socioeconomic value and is repeatedly mentioned with appreciation in the Bible and Quran. The tree plays a key role in the Arabic countries and increases exceptional consideration from Arabic governments and scientists. The tree of the date palm is the most suitable tree for cultivation in arid and semi-arid regions of the world. Regrettably, there are many biotic stresses that hinder expansion of date palm cultivation, and accordingly date palm productivity and revenue [1].

Unluckiness, the development of cultivation and yield of date palm can not be achieved, because there are many biotic stresses that faced this tree. The fruit of date palm is known as a date. According to FAOSTAT report in 2012, the world's largest date-producing countries are Egypt, followed by Iran, Saudi Arabia, Algeria, Iraq, Pakistan, Sudan, Oman, United Arab Emirates, Tunisia and Libya [2]. Egypt is topped the list of date palm in date production and cultivation. In Egypt, date palm cultivation includes the whole area from the Mediterranean up to Aswan. The cultivated area of palm in Egypt was increased to more than 100% since 1993 and currently has an estimated 15,582,000 date palm trees. There are about 12,261,651 female palm trees [3]. Every year, Egypt produces approximately 1,373,570 tons of dates. The region of palm trees presently is 73.653 thousand acres, or around 6.32% of the total cultivated fruit area [2].

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This equals a little over more than 17% of worldwide date production, but only 3% of world exports. Thus, dates are regarded one of the promising fruits for local consumption, shipping as well as for agriculture expansion in desert areas and reclamation areas.

The low cultivars quality, neglecting of post-harvest processes, pests and diseases, the weakness of marketing services and insufficiently appropriate research are the main constraints of the date palm in Egypt. Furthermore, Egyptian date palm producers faces many obstacles which hinder fill the unmet domestic and international demand due to low quality in combination with low-grade packaging provides a considerable challenge [4]. The tree is the most susceptive for many pests and diseases, so it is important to converge on its *in vitro* propagation and genetic engineering to succeed some of these problems. The dreaded pest of palm, *viz.*, the red palm weevil, *Rhynchophorus ferruginous* (Olivier), has attained a key pest status since the early nineties of the last century after first being reported in Egypt during the mid-eighties. The red palm weevil *Rhynchophorus ferrugineus* (Olivier), a concealed tissue borer, is a lethal pest of palms and is reported to attack 17 palm species worldwide and continues to spread among and within date palm plantation countries. On the worldwide scale, *Rhynchophorus ferruginous* has the widest geographical range in the genus of *Rhynchophorus* causing destruction of palms [5]. Furthermore, several obstacles faced the improvement of Egyptian date palm cultivars by using conventional breeding, due to the long production period and heterozygosity of date palm cultivars [6].

The use of tissue culture technique give some advantages over traditional methods of propagation for a rapid and large scale proliferation of important plants under *in vitro* tissue culture conditions regardless of the season with conservation of time and space [7]. *Agrobacterium*-mediated transformation is a widely-used technique in plant biotechnology. The infection mechanism of *Agrobacterium* owns to control the transfer of DNA among host plant and *Agrobacterium*. For survival, the target plant must excrete a group of a mixture, like acetosyringone, when the plant is wounded [8]. *Agrobacterium*-mediated transformation and genetic engineering offer some advantages over traditional methods of breeding of genes [9, 10]. The lack of suitable improvement systems, low cell competence and requisite wound responses is usually faced the development of monocots by genetic engineering. Although these obstacles, *Agrobacterium* transformation method has been used with high efficiency to produce various transgenic monocots by adjusting the parameters that govern effective delivery and integration of transgene(s) into the plant genome [11]. Confirmed the prescience, integration and copy number of foreign gene in regeneration plants by using Southern blot hybridization, is the effective tool for determining transgenic copy number of insertion, but the physical location of the transgene insert can not be indicated [12]. In this study, a simple transformation method was reported for transgenic date palm development, which was targeted at the embryogenic callus.

2. Materials and Methods

This study was carried out during 2013-2016 at the Laboratory of Tissue Culture Center, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Egypt.

2.1 Plant Materials and Preparation

Two to three-year-old offshoots of date palm cv. Medjool and Khalas were kindly provided by Prof. Ibrahim Abdel-Maksoud Ibrahim. For excising the shoot tip explant, the outer leaves and fiber sheath were removed from offshoots acropetally with a hatchet and sharp knife (always sprayed with ethyl alcohol 70%) until they reached 3.0-4.0 cm in width and 6.0-8.0 cm in length. Then, the explants were soaked in a cold sterilized antioxidant solution containing citric and ascorbic acids each at the

concentration of 150 mg/L, and kept in the refrigerator until the surface sterilization procedure is performed.

2.2 Surface Sterilization of Explant Materials

Under aseptic conditions, plant materials were surface sterilized twice by soaking in a commercial disinfectant Clorox (5.25% NaOCl) solution 2% for 30 min, and two drops/100 mL solution of Tween 20 (polyoxyethylenesorbitan monolaurate) as wetting agent were used. Then, they were washed with sterile distilled water, followed by being immersed in sterilized mercuric chloride solution (0.1%) for 10 min. It was then rinsed five times with sterile distilled water and finally soaked in a filter sterilized antioxidant solution including citric and ascorbic acids each at the concentration of 150 mg/L to avoid browning. The primordial leaves were carefully removed one by one, and shoot tip explants were about 0.5-1.0 cm in length and consisted of the apical meristem, 4-6 primordial leaves, cut longitudinally into four sections.

2.3 Callus Initiation

The prepared shoot tip explants were inoculated on MS basal medium [13] supplemented with 40 mg/L adenine-sulfate, 10 mg/L 2,4-diclorophenoxy acetic acid (2,4-D), 3 mg/L isopenetyl adenine (2iP), 170 mg/L NaH_2PO_4, 30 g/L sucrose, 2 g/L activated charcoal (AC) and 8 g/L agar [14].

Data were calculated in every treatment at the end of each subculture for six subcultures (six weeks for each one). Data were taken as the average per explant for callus induction degree value. The average of callus induction degree and average of swelling degree were scored visually as follows, according to Ref. [15]: negative results (-) = 1; below average results (+) = 2; average results (++) = 3; good results (+++) = 4; excellent results (++++) = 5.

2.4 Sensitivity of Embryogenic Calli (Non-transformed) to Kanamycin

The sensitivity test of kanamycin was carried out to find the inhibitory concentration which arrests embryogenic callus growth. The sensitive nature of embryogenic callus to kanamycin was determined by culturing the explants in regeneration MS medium enriched with 0.1 mg/L NAA and 0.05 BA with different kanamycin concentrations (0.0, 25, 50, 100, 125 and 150 mg/L). Kanamycin was sterilized by filtration through 0.22 um disposable filter and incorporated into pre-cooled (45-50 °C) autoclaved medium. The number of survival explants was recorded after eight weeks from cultivation. After four weeks, the germinating embryogenic callus was transferred onto fresh medium containing the same concentrations of antibiotic. Each treatment contained five replicates, and each replicate consisted of 50 explants. The minimum inhibitory concentration of the selection marker was used throughout the selection procedure of selection transformed explants.

2.5 Bacterial Strain and Plasmid Vector

Agrobacterium tumefaciens strain LBA4404, harbouring a T-DNA, a pCambia2300 plasmid containing a kanamycin resistance (*npt-II*) gene for bacterial selection and the plasmid including insect resistance (*Cry3Aa*) and *npt-II* genes driven by CaMV and NOS promoters, were kindly provided separately by Prof. Silvio Alejandro López-Pazos of Institute of Biotechnology, National University of Colombia, under material transfer agreement.

2.6 Preparation of Agrobacterium Cells

A single colony of *Agrobacterium* strain LBA4404, was suspended in 5 mL yeast extract and peptone (YEP) medium [16] containing 150 mg/L kanamycin. Bacterial cell density was measured using a U-2001 spectrophotometer (Hitachi, Japan) and adjusted to the final working concentration by diluting it with washing medium. Cultures were pelleted at 5,000 rpm at 28 °C, and the pellet was resuspended in *Agrobacterium* minimal medium (pH 5.6) to a final density of 108 cells/mL culture.

2.7 Optimization of an Agrobacterium-Mediated Transformation System for Date Palm

Embryogenic callus (1 cm size) of date palm cv. Medjool and Khalas were injured slightly by pricking 4-5 times with a sterile hypodermic needle, and the injured explants were immersed in *Agrobacterium* suspension which contains 200 mg/L of L-cysteine and 60 μM of silver nitrate [17]. The co-cultivation was performed for 0.5, 1, 2, 4 and 24 h, along with or without acetosyringone (100 mM) (Sigma). After co-cultivation, the explants were rinsed three times with sterile distilled water containing filter-sterilized carbenicillin (250 mg/L), blotted dry and subjected to selection.

2.8 Culture Selection and Explant Regeneration of Putative Transformants

Explants were cultured in MS medium supplied with 0.1 mg/L NAA, 0.05 mg/L BA and 250 mg/L carbenicillin along with kanamycin (150 mg/L) for tested transformed explants. Two subcultures were done on the same medium. The medium of the same structure was changed once in 10 d. Each treatment consisted of a total of 50 explants.

2.9 Determination of the Presence of Transgenes by PCR Technique

The PCR is a sensitive technique allowing single-copy genes to be amplified and extracted out of a complex mixture of genomic sequence. Amplified DNA is visualized as distinct bands on agarose gels. PCR amplification was used to confirm the presence of the T-DNA in kanamycin-resistant embryogenic callus. Genomic DNA was isolated from embryogenic callus of both non-transformed plants and transformed kanamycin-resistant embryogenic callus as described by Akashi et al. [18]. The DNA was stored at -20 °C. Specific primer was designed based on the nucleotide sequence of *Cry3Aa* gene. The forward sequence of *Cry3Aa* gene was 5′-CTGGATCCATGAATCCGAACAATCGA-3′ and the reverse sequence was 5′-TAGTCGACTTAATTCACTGGAATAAA-3′. The designed primer was then used to amplify a fragment of *Cry* gene with 2,000 bp. PCR amplification reaction was used in a final volume of 25 μL containing 10× PCR buffer (10 mM Tris-HCl, 25 mM $MgCl_2$ and pH 7.5), 1.5 mM dNTPs, 10 μM for each primer, 50 ng of template DNA and 0.5 U of Taq polymerase. Reactions were performed in a thermocycler (Eppendorf, Germany). The PCR conditions were as follow: one cycle of 94 °C for 4 min (denaturation), 35 cycles of 94 °C for 30 s, 58 °C for 30 s (annealing), 72 °C for 1 min (extension), and the last extension of 10 min at 72 °C. PCR products were analyzed using 1% agarose gel electrophoresis. They were stained with 0.5 μg/mL ethidium bromide and visualized (probably in a trans-illuminator). The sizes of the fragments were determined based on a DNA ladder of 3,000 bp.

2.10 Southern Blot Analysis

To confirm T-DNA integration into the date palm genome, Southern blot analysis was performed on total genomic DNA isolated from the embryogenic callus of *Cry3Aa*-resistant and non-transformed control plants. Genomic DNA was extracted from embryogenic callus of transgenic and untransformed control date palm plants by using the modified DNA extraction methods described by Akashi et al. [18]. Then genomic DNA (50 μg per reaction) from each plant was digested with the Ncol restriction enzyme for 16 h. Next, the DNA was transferred by capillary transfer [19] onto a positively charged membrane. The PCR amplified gene probe (900 bp) of *Cry3Aa* labeled with DIG-dUTP was used in the hybridization. Pre-hybridization and hybridization were done by using the standard protocol [19]. After 16 h of hybridization, the membrane was washed with different concentrations of saline sodium citrate (SSC) buffer (2×, 1× and 0.5× SSC). The membrane was transferred into a plastic bag containing 15 mL

blocking solution (10× blocking reagent: maleic acid buffer = 1:9) with 1 μL of anti-digoxigenin-AP (0.75 U/μL) Fab fragments, sealed and shaken for 30 min. Then, the membrane was washed twice with washing buffer (1× maleic acid and 0.3% Tween 20) for 30 min. After rinsing, the membrane was exposed to X-ray film (Kodak medical X-ray film, USA) for 15 min. The film was transferred to a box containing developing solution for 1-2 min. When the DNA band was visible, the film was immediately transferred to a box containing fixing solution for 1-2 min and air dry the film for 10-15 min at room temperature.

2.11 Statistical Analysis

This experiment was carried in a completely randomized design (CRD) with three replications. The data were analyzed by one-way analysis of variance, and the mean values were separated using the Fisher's least significant difference test (LSD test at 5%) [20].

3. Results and Discussion

3.1 Swelling Degree and Callus Induction Degree Value

Swelling degree was responded differently to the different subcultures used (Table 1). In this concern, swelling degree ranged from 1.00 to 5.00. Concerning to the effect of cultivars on swelling of callus, data showed that Khalas cultivar produced the higher mean swelling degree value of callus (2.55) than Medjool cultivar (2.50), but without significant differences between them. On the other hand, the highest mean swelling degree value (5.00) was achieved in subculture No. 2. On the interaction between cultivar type and subcultures number, data revealed that subculture No. 2 of Medjool and Khalas cultivars gave the highest significant swelling degree of callus (5.00) without significant differences between them. The increase of swelling degree is the first indicator of growth and tissue response. The growth is the most complex physiological processes known so far; it is one of the remarkable features of the attributes of living matter. The current obtained results clearly showed that there was an increase in swelling degree from the first subculture to the second subculture, but there was the gradual increase in swelling degree from the third subculture to the six subculture. The first mode of action of the known natural growth regulating is probably their act on membrane systems and control of ion fluxes. That, in turn, may lead to many of the biochemical changes known to induce the response in specific ways [21]. In this regard, Zayed et al. [22] showed that flower swelling (the first sign of callusing) could be observed after 4-6 weeks of culturing. Then, callus emergence was noticed from

Table 1 Effect of cultivar type and subculture number on induction degree of callus and swelling degree of date palm cv. Medjool and Khalas.

Subcultures No.	Swelling degree			Induction degree of callus		
	Cultivar		Mean	Cultivar		Mean
	Mejdool	Khalas		Mejdool	Khalas	
1	4.00	4.00	4.00	1.13	1.23	1.18
2	5.00	5.00	5.00	1.70	1.86	1.78
3	3.00	3.33	3.16	2.06	2.33	2.20
4	1.00	1.00	1.00	2.83	3.06	2.95
5	1.00	1.00	1.00	3.06	3.53	3.30
6	1.00	1.00	1.00	2.53	3.03	2.78
Mean	2.50	2.55		2.22	2.51	
LSD at 5% for A	0.11			0.04		
LSD at 5% for B	0.19			0.07		
LSD at 5% for A × B	0.27			0.10		

A = subculture number; B = cultivar type.

Fig. 1 Formation of embryogenic callus on MS medium contained 10 mg/L 2,4-D and 3 mg/L 2iP after six subcultures.

the swelled carpels. Recently, Ali et al. [23] found that swelling degree was significantly enhanced at the end of the second subculture.

Moreover, the effect of subculture number and cultivar type on induction of callus of date palm cv. Medjool and Khalas was clearly shown in Table 1 and Fig. 1. Referring the effect of cultivar type, data revealed that Khalas cultivar produced the higher mean value of callus induction (2.51), followed by Medjool cultivar (2.22). Concerning the effect of subcultures number, data clearly found that the highest callus induction degree was observed in subculture No. 5 (3.30), followed by the value of callus induction degree in subculture No. 4 (2.95) with significant differences between them. While, callus induction degree in subculture No. 1 showed the lowest value (1.18). The interaction between subculture number and cultivar type found that the highest significant value of callus induction degree was observed for Khalas in subculture No. 5 (3.53). The similar results for callus induction were reported by other researchers [24-27], who reported that the induction of date palm explants for callus formation were found in small amounts for the first subculture and gradually increased in the last subculture No.4. Furthermore, Bouguedoura et al. [28] showed that callus grew up to 2.5 cm after six months. The ability for callogenesis in Deglet Nur cultivar was higher compared to Takerbucht. The rates of callus formation were higher (more intense) in the explants containing young primordia, such as vegetative axillary buds and apex fragments.

3.2 Sensitivity of Embryogenic Callus (Non-transformed) to Kanamycin

Kanamycin resistance is the most generally utilized as the selectable marker for transformation of plants. Survival percentage was 20.17% when using embryogenic callus of Medjool as explants, higher than Khalas as explant (18.50%), as shown in Table 2. Matured somatic embryos were germinated. In the kanamycin free medium (control), the matured somatic embryos after five weeks of inoculation started the germination. Meanwhile, the highest survival number was recorded in control (50.00), while the lowest survival number was recorded when adding 100 mg/L kanamycin in the medium. The interaction between cultivar and kanamycin concentration was significant at 5% level. The highest survival was achieved with control (50.00%) when using the embryogenic callus of both cultivars Medjool and Khalas. No survival (100% mortality) was observed when adding 100 mg/L kanamycin to the medium for embryogenic callus of both cultivars (Table 2 and Fig. 2). However, at the concentration of 100 mg/L or higher, development of the embryogenic callus was fully retarded or inhibited (Fig. 2). Kanamycin acts by involving the synthesis of protein.

Table 2 Effect of kanamycin concentrations on the survival of non-transformed embryogenic callus.

Kanamycin concentration (mg/L)	Survival numbers (%)		Mean
	Medjool	Khalas	
25	50.00	50.00	50.00
50	40.00	40.00	40.00
75	31.00	21.00	26.00
100	0.00	0.00	0.00
125	0.00	0.00	0.00
150	0.00	0.00	0.00
Mean	20.17	18.50	
LSD at 5% for A	2.98		
LSD at 5% for B	5.17		
LSD at 5% for A × B	7.32		

A = kanamycin concentration; B = cultivar type.

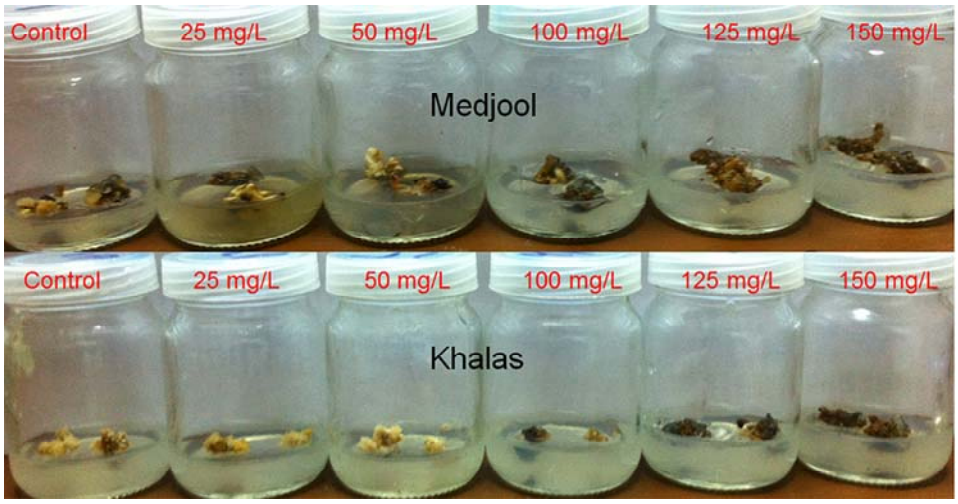

Fig. 2 Effect of kanamycin concentrations on non-transformed embryogenic calli.

Kanamycin binds the bacterial ribosome through sub unit 30S. These results are not true with the mRNA, and finally lead to a misread that causes the incorrect amino acid to be placed into the peptide, thus leading to nonfunctional peptide chains. These results are in agreement with Aslam et al. [29], who showed that matured somatic embryos were inoculated with *Agrobacterium tumefaciens* and plantlet conversion MS medium containing BA (0.75 mg/L) + kanamycin (100 mg/L). Also, Dhumale et al. [30] found that explant when cultured in MS contained, kanamycin at 25 mg/L led to 30.8% explants showing necrosis in AKS 207 and 22.8% in PKV Pink after four weeks from culture initiation. At higher concentrations, necrosis appeared on explants within 4-8 d of culture. Recently, Kayani et al. [31] showed that putative transformants were selected on kanamycin 100 mg/L.

3.3 Culture Selection and Explant Regeneration of Putative Transformants

In both monocots and dicots, *Agrobacterium tumefaciens* is one such strategy which is well known for enabling efficient for gene transfer. Due to its efficiency, this system had many advancements, including developed *in vitro* plant propagation system, incubation and selection methods, and use of hyper-virulent strains of *Agrobacterium tumefaciens* [32]. The transformation efficiency has also been enhanced during this investigation by adding acetosyringone to induce the activity of *vir* genes.

Concerning to the main effect of acetosyringone, the addition of acetosyringone gave the higher

transformation frequency (0.09 for Medjool and 0.04 for Khalas). Transformation experiments were designed to optimize the effective *Agrobacterium tumefaciens* co-cultivation period and the superior cultivar type for transformation. During this investigation, incubation time was assessed for each hour from 0.5 h to 24 h. Embryogenic callus was co-cultivated with *Agrobacterium* at different hours interval, and thereafter, transferred to the selection medium (MS solid medium containing 100 mg/L kanamycin). In this experiment, the best time for transformation was recorded after 1 h for Medjool and Khalas embryogenic callus from incubation with *Agrobacterium tumefaciens*. So, 1 h co-cultivation was found as an optimal for Medjool and Khalas cultivars and led to the production of a significantly higher rate of resistance frequency as compared to other periods (Table 3 and Fig. 3). As regard to the interaction between the addition of acetosyringone and co-cultivation periods, it was clear that 1 h with the addition of acetosyrengone produced the highest resistance expression (0.40 and 0.20 for Medjool and Khalas, respectively) than other treatments. The mean of embryogenic callus growing well on the selective medium was calculated as transformation frequency. Table 3 shows growth performance of embryogenic callus after successful selection on germination and plantlet conversion medium. Several factors influenced on transformation activity, including strain of *Agrobacterium tumefaciens*, the addition of phenolic compounds (e.g., acetosyringone) in the co-cultivation medium, wounding treatment of target

Table 3 Transformation efficiency of different date palm cultivars after incubation with *Agobacterium tumefaciens*.

Treatments	Transformation efficiency					
	Mejdool			Khalas		
	With Acetosyringon	Without Acetosyringon	Mean	With Acetosyringon	Without Acetosyringon	Mean
0.5 h	0.00	0.00	0.00	0.00	0.00	0.00
1 h	0.40	0.00	0.10	0.20	0.00	0.10
2 h	0.00	0.00	0.00	0.00	0.00	0.00
4 h	0.00	0.00	0.00	0.00	0.00	0.00
24 h	0.00	0.00	0.00	0.00	0.00	0.00
Mean	0.09	0.00		0.04	0.00	
LSD at 5% for A	0.09			0.08		
LSD at 5% for B	0.15			0.12		
LSD at 5% for A × B	0.22			0.17		

A = co-cultivation time; B = cultivar type.

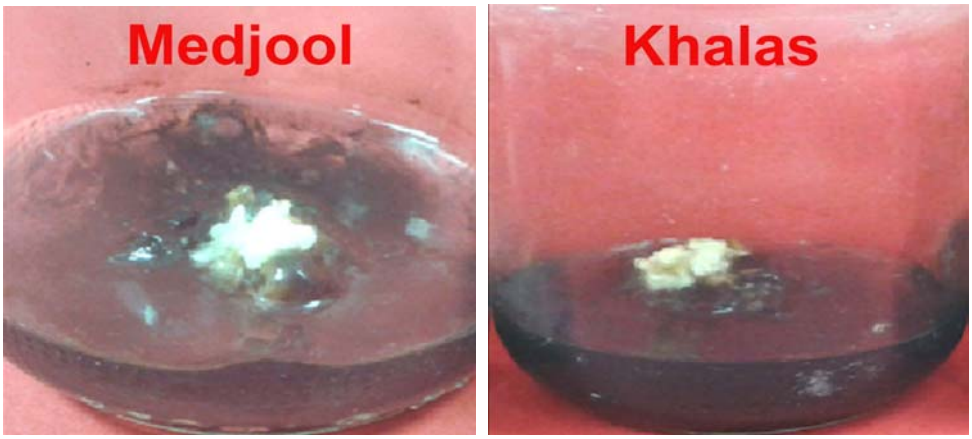

Fig. 3 Selection of kanamycin resistant after incubation of embryogenic callus with *Agobacterium tumefaciens*.

tissue and appropriate selection of transformed cells or tissue from all untransformed tissue [33]. Some reports propose that transformation efficiency can be enhanced by pre-induction of *Agrobacterium* and/or inclusion of acetosyringone in the incubation medium [34-36]. Furthermore, El-Rakashy et al. [37] showed that the transgenic date palm expressing endotoxin *Cry3Aa* gene was in field evaluated for red palm weevil resistance, yield and fruit quality. The higher transformation efficiency has been achieving by the use of super-binary vectors with the hyper-virulent strain [32]. Choice of a powerful promoter that is constitutively expressed is important to ensure success and good constitutive expression of the foreign gene in a new environment, i.e., in a transgenic plant. Selection for an effective promoter was carried out, since the effectiveness of promoters is sometimes plant species dependent. There is another drawback for *Agrobacterium tumefaciens* for transformation of monocots crop, because monocots are not natural host for *Agrobacterium*. But a little numbers of wider host range hyper-virulent strains (LBA4404, EHA101) or their derivatives (EHA105, AGL0, AGL1) have been widely used in cereal transformation. However, the use of standard binary vector with super-virulent strains or super-binary vector in the regular strain of *Agrobacterium* has also resulted in the successful transformation in cereals [38]. L-cysteine is an amino acid with a thiol side chain, an important component of antioxidant glutathione. L-cysteine acts as antioxidant and has effect on *Agrobacterium*-mediated transformation. Similar to L-cysteine, silver nitrate compound is known to inhibit ethylene production from the *in vitro* culture, which affects plant cell growth mechanisms.

3.4 Determination of the Presence of Transgenes by PCR Technique

Many techniques have been developed to detect transgenic plants. These include PCR-based methods, followed by gel electrophoresis and detection, and they are routinely used to determine transformation plants [39, 40]. Molecular beacon assays [41] and fluorescence in situ hybridization analysis (FISH) [42] can be utilized. However, this was not for some late developing plants, like date palm, where detection of transgenes has always been a major problem. During this investigation, the first analyses on transgenic date palm were carried out by PCR analyses. The PCR is a sensitive method allowing single-copy genes to be amplified and extracted out of a complex mixture of genomic sequence. Magnified DNA is visualized on agarose gels as distinct bands.

The trials were repeated three times and the same result was observed, indicating that the foreign gene was successfully transferred into date palm. Genomic DNA extracted from non-transformed embryogenic callus (negative control) were used as templates and also extracted from putative transformed embryogenic callus. In the situation of transformed embryogenic callus, the expected 2,000 bp bands were presented in the sample tested (Fig. 4), indicating the presence of *Cry3Aa* utilized as non-transformed and DNA of

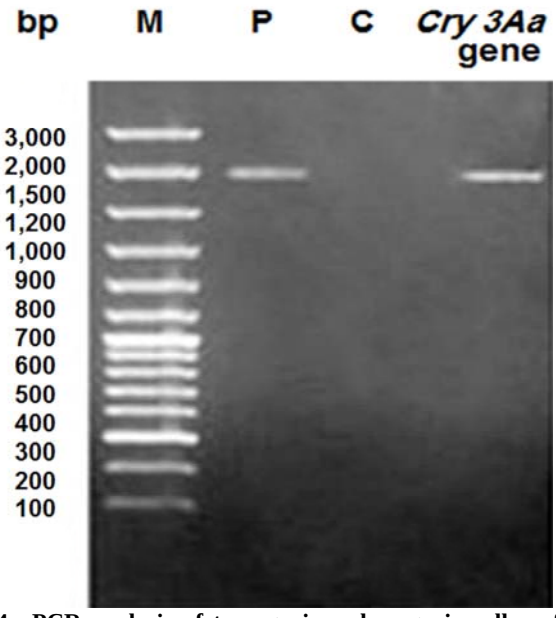

Fig. 4 PCR analysis of transgenic embryogenic callus of date palm.
Lane M = 3 kb DNA marker; P = transforming plasmid (pCambia2300); C = nontransformed control; *Cry3Aa* = putative transformed date palm plants.

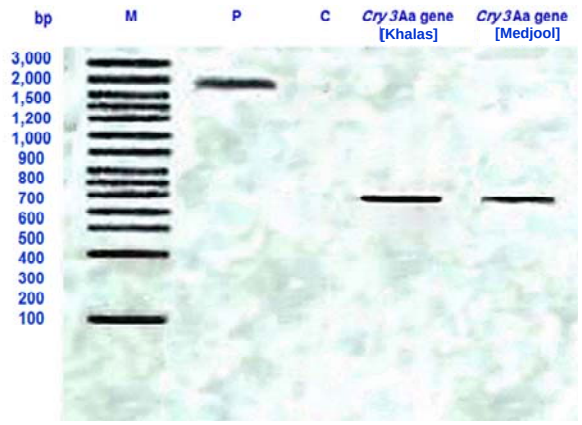

Fig. 5 Southern blot analysis of transgenic date palm embryogenic callus.
Lan M = 3 kb DNA marker; P = positive control (pCambia2300); C = non-transformed control; *Cry3Aa* = putative transformed date palm embryogenic callus**.**

pCambia2300 was taken as a positive control as presented in Fig. 4. However, the two primers CRY-F and CRY-R arranged based on the coding sequence of *Cry3Aa* from plasmid pCambia2300 with a PCR product of 2,000 bp were used in PCR in the genomic DNA of putatively transformed embryoenic callus. In contrast, all control samples did not show any band. Transformation efficiency (0.1%) observed following PCR of *Cry3Aa* was the same to those observed before from kanamycin resistance define using the same sample. Therefore, this further confirmed successful transfer of *Cry3Aa* gene into the tissues of date palm. DNA of pCambia2300 was used as a positive control and DNA of non-transformed embryogenic callus was used as a negative control. Nevertheless, the presence of *Cry3Aa* in putatively transformed embryogenic callus is not evidence on its role in its new environment.

3.5 Analysis Using Southern Blot

Analysis by using Southern Blot was done in three *Cry3Aa* PCR positive embryogenic callus. The genomic DNA (50 µg) of four PCR positive plants, untransformed control plant and pCambia2300 were digested with Ncol restriction enzyme. A *Cry3Aa* probe (900 bp) was used for detection of the transgenic embryogenic callus. It was found that transgenic embryogenic callus for both Medjool and Khalas showed a single copy of gene integration (Fig. 5). The bands indicate the integration of *Cry3Aa* gene into the genome of the transformed date palm embryogenic callus, whereas the untransformed embryogenic callus did not show any integration of *Cry3Aa* gene. In sugarcane, using *Agrobacterium*-mediated transformation method, one copy of transgene integration was found by Kalunke et et al. [43]. Recently, Islam et al. [44] reported that three transgenic plants showed two copies of the gene integration and one transgenic plant showed a single copy of gene integration.

4. Conclusions

The results of this research have provided an insight into the effect of the cultivar type on the transformation of date palm cv. Medjool and Khalas using *Agrobacterium tumefaciens*. Acetosyringone as a chemical was recommended in most of the transformation protocols of date palm. Incubation of explants with *Agrobacterium tumefaciens* with the addition of acetosyringone for 1 h was found effective in the production of the transgenic plant for both cultivars Medjool and Khalas. Using primer specific to *Cry3Aa* gene (forward and reverse), a PCR result with a size of about 2,000 bp was magnified when the all nucleic acid from the transformants were utilized as templates. PCR analysis confirmed the appearance of transgene of 2,000 bp in one individual plantlet. These results signify the successful introduction of *Cry3Aa* gene into date palm plant. The presence and integration of foreign *Cry3Aa* gene in regenerated kanamycin resistant embryogenic callus were also confirmed by Southern blot hybridization.

Acknowledgments

This investigation was carried out through the project entitled “development of a system for commercial production and genetic improvement of

some date palm cultivars in New Valley" under the program of the Science and Technology Development Fund (STDF), Ministry of Scientific Research, Egypt.

References

[1] Saker, M. M. 2011. "Transgenic Date Palm." In *Date Palm Biotechnology,* edited by Jain, S. M., Al-Khayri, J. M., and Johnson, D. V. Dordrecht: Springer, 631-50.

[2] FAOSTAT. 2012. "Food and Agricultural Organization Corporate Statistical Database." Crop Production, Statistics Division, Food and Agriculture Organization of the United Nations (FAO).

[3] Food and Agricultural Organization of the United Nations (FAO). 2012. *Date Palm Cultivation.* Rome: FAO.

[4] Bekheet, S., and El-Sharabasy, S. F. 2015. "Date Palm Status and Perspective in Egypt." In *Date Palm Genetic Resources and Utilization,* 1st ed., edited by Al-Khayri, J. M., Jain, S. M., and Johnson, D. V. Netherlands: Springer, 75-123.

[5] Al-Dosary, N. M., Al-Dobai, S., and Faleiro, J. R. 2016. "Review on the Management of Red Palm Weevil *Rhynchophorus ferrugineus* Olivier in Date Palm *Phoenix dactylifera* L.." *Emirates J. Food Agri.* 28 (1): 34-44.

[6] Bekheet, S. 2013. "Date Palm Biotechnology in Egypt: A Review." *App. Sci. Report.* 3 (3): 144-52.

[7] Nehra, S. A., and Kartha, K. K. 1994. "Meristem and Shoot Tip Culture: Requirements and Applications." In *Plant Cell and Tissue Culture,* edited by Vasil, I. K., and Thorpe, T. A. Dordrecht, Netherlands: Kluwer Academic Publishers, 37-70.

[8] Tzfira, T., and Citovsky, V. 2006. "*Agrobacterium*-Mediated Genetic Transformation of Plants: Biology and Biotechnology." *Curr. Opin. Biotech.* 17 (2): 147-54.

[9] Shingote, P. R., Kawar, P. G., Pagariya, M. C., Kuhikar, R. S., Thorat, A. S., and Babu, K. H. 2015. "SoMYB18, a Sugarcane MYB Transcription Factor Improves Salt and Dehydration Tolerance in Tobacco." *Acta Physiol. Plantarum* 37: 217.

[10] Kharte, S. B., Watharkar, A. S., Shingote, P. R., Sharanya, C., Kawar, P. G., Pagariya, M. C., and Govindwar, S. P. 2016. "Functional Characterization and Expression Study of Sugarcane MYB Transcription Factor Gene *PEaMYBAS1* Promoter from *Erianthus arundinaceus* That Confers Abiotic Stress Tolerance in Tobacco." *The Royal Society of Chemistry* 6: 19576-86.

[11] Sood, P., Bhattacharya, A., and Sood, A. 2011. "Problems and Possibilities of Monocot Transformation." *Biologia Plantarum* 55 (1): 1-15.

[12] Van Buren, R., and Ming, R. 2014. "Sequencing and Assembly of the Transgenic Papaya Genome." In *Genetics and Genomics of Papaya,* edited by Ming, R., and Moore, P. H. New York: Springer, 187-203.

[13] Murashige, T., and Skoog, F. 1962. "A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures." *Physiol. Plant.* 15 (3): 473-97.

[14] Ibrahim, A. I., Hassan, M. M., and Taha, R. A. 2012. "Partial Desiccation Improves Plant Regeneration of Date Palm *in Vitro* Cultures." *Wudpecker J. Agri. Res.* 1 (6): 208-14.

[15] Bottino, B. G. 1981. *Methods in Plant Tissue Culture.* MD, USA: University of Maryland, 8-29.

[16] Van-Larebeke, N., Genetello, C. H., Hernalsteens, J. P., De Picker, A., ZaenenI, I., Messens, E., Van Montagu, M., and Schell, J. 1977. "Transfer of Ti Plasmids between *Agrobacterium* Strains by Mobilization with Conjugative Plasmid." *Mol. Gen. Genet.* 152 (2): 119-24.

[17] Subramaniam, S., and Rathinam, X. 2010. "Emerging Factors that Influence Efficiency of T-DNA Gene Transfer into *Phalaenopsis violacea* Orchid via *Agrobacterium tumefaciens*–Mediated Transformation System." *International Journal of Biology* 2 (2): 64-73.

[18] Akashi, K., Nishimura, N., Ishida, Y., and Yokota, A. 2004. "Potent Hydroxyl Radical-Scavenging Activity of Drought-Induced Type-2 Metallothionein in Wild Watermelon." *Biochem. Biophys. Res. Commun.* 323 (1): 72-8.

[19] Sambrook, J., and Russell, D. W. 2001. "Southern Hybridization." In *Molecular Cloning: A Laboratory Manual,* 3rd ed.. NY: Cold Spring Harbor Laboratory Press, 633-64.

[20] Steel, R. G. D., and Torrie, J. H. 1980. *Principles and Procedures of Statistics: A Biometrical Approach,* 2nd ed.. New York: McGraw-Hill Book Co., 137-77.

[21] Sharp, W. R., Sondahl, M. R., Caldas, L. S., and Maraffa, S. B. 1980. "The Physiology of *in Vitro* Asexual Embryogenesis." *Horticultural Reviews* 2: 268-310.

[22] Zayed, E. M. M., Zein El Din, A. F. M., Manaf, H. H., and Abdelbar, O. H. 2016. "Floral Reversion of Mature Inflorescence of Date Palm *in Vitro*." *Ann. Agri. Sci.* 61 (1): 125-33.

[23] Ali, K. M. S., Sabbour, A. M., Khalil, M. K., Aly, A. S., and Zein El Din, A. F. M. 2017. "*In Vitro* Morphogenesis of Direct Organs in Date Palm (*Phoenix dactylifera* L.) cv. Siwy." *Int. J. Adv. Agri. Sci. Technol.* 4 (2): 1-25.

[24] Dixon, R. A., and Gonzales, R. A. 1994. *Plant Cell Culture: A Practical Approach.* Oxford, USA: Oxford University Press, 415-25.

[25] Zaid, Z. E. 2003. "Comparative Studies on the Production of Date Palm Cultivars via Tissue Culture Technique." Ph.D. thesis, Department of Pomology, Faculty of Agriculture, Cairo University, Egypt.

[26] Zaid, Z. E., Hassan, M. M., and Sidky, R. A. Y. 2006.

"Role of Mannitol in Inducing Direct Somatic Embryogenesis of Date Palm." *Assiut J. Agri. Sci.* 37 (2): 103-12.

[27] Zein El Din, A. F. M. 2004. "Physiological Studies on Vegetative Propagation of Some Date Palm Cultivars Using the Tissue Culture Technique." M.Sc. thesis, Department of Plant Physiology, Faculty of Agriculture, Ain Shams University, Egypt.

[28] Bouguedoura, N., Si-Dehbi, F., Fergani, K., Arban, A., and Chabane, D. 2017. "Somatic Embryogenesis in Date Palm (*Phoenix dactylifera* L.) 'Deglet Nur' and 'Takerbucht' Cultivars." *Int. J. Plant Reprod. Biol.* 9 (1): 43-8.

[29] Aslam, J., Khan, S. A., and Azad, M. A. 2015. "Agrobacterium-Mediated Genetic Transformation of Date Palm (*Phoenix dactylifera* L.) Cultivar Khalasah via Somatic Embryogenesis." *Plant Science Today* 2 (3): 93-101.

[30] Dhumale, D. R., Shingote, P. R., Dudhare, M. S., Jadhav, P. V., and Kale, P. B. 2016. "Parameters Influencing *Agrobacterium*-Mediated Transformation System in Safflower Genotypes AKS-207 and PKV Pink." *3 Biotech* 6 (2): 1-8.

[31] Kayani, W. K., Fattahi, M., Palazòn, J., Cusidò, R. M., and Mirza, B. 2016. "Comprehensive Screening of Influential Factors in the *Agrobacterium tumefaciens*-Mediated Transformation of the Himalayan Elixir: *Ajuga bracteosa* Wall. ex. Benth.." *J. Appl. Res. Med. Aromatic Plants* 3 (4): 151-9.

[32] Singh, R. K., and Prasad, M. 2016. "Advances in *Agrobacterium tumefaciens*-Mediated Genetic Transformation of Graminaceous Crops." *Protoplasma* 253 (3): 691-707.

[33] Afolabi-Balogun, N. B., Inuwa, H. M., Ishiyaku, M. F., Bakare-Odunola, M. T., Nok, A. J., and Adebola, P. A. 2014. "Effect of Acetosyringone on *Agrobacterium*-Mediated Transformation of Cotton." *RPN J. Agri. Biol. Sci.* 9 (8): 284-6.

[34] Yao, S. 2001. "Optimization of *Agrobacterium*-Mediated Genetic Transformation of Soybean Using Glufosinate as a Selection Agent." Ph.D. thesis, Louisiana State University and Agricultural and Mechanical College.

[35] Samuels, M. N. 2001. "Optimization of Apex-Mediated DNA Transformation in Rice." M.Sc. thesis, Louisiana State University and Agricultural and Mechanical College.

[36] Sunikumar G., Vijayachandra, K., and Veluthambi, K. 1999. "Pre-incubation of Cut Tobacco Leaf Explants Promotes *Agrobacterium*-Mediated Transformation by Increasing *vir* Gene Induction." *Plant Sci.* 141: 51-8.

[37] El-Rakashy, S., Martin, D., and Johanson, C. 2011. "Field Evaluation of Transgenic Date Palm That Was Genetically Engineered to Resist Red Palm Weevil through Expressing of the *Bacillus thuringiensis* Coleopteran-Specific Endotoxin *Cry3Aa* Transgene." Presented at the First Scientific Conference for the Development of the Date Palm and Date Sector in the Arab World, December 4-7, 2011, Riyadh.

[38] Shrawat, A. K., and Lörz, H. 2006. "*Agrobacterium*-Mediated Transformation of Cereals: A Promising Approach Crossing Barriers." *Plant Biotechnology Journal* 4 (6): 575-603.

[39] Higuchi, R., Dollinger, G., Walsh, P. S., and Griffith, R. 1992. "Simultaneous Amplification and Detection of Specific DNA Sequences." *Bio-Technology* 10: 413-7.

[40] Abedinia, M., Henry, R. J., Blakeney, A. B., and Lewin, L. 1997. "An Efficient Transformation System for the Australian Rice Cultivar, Jarrah." *Australian Journal of Plant Physiology* 24 (2): 133-41.

[41] Kota, R., Holton, T. A., and Henry, R. J. 1999. "Detection of Transgenes in Crop Plants Using Molecular Beacon Assays." *Plant Molecular Biology Reporter* 17 (4): 363-70.

[42] Jin, W. W., Li, Z. Y., Fang, Q., Altosaar, I., Liu, L. H., and Songm, Y. C. 2002. "Flourescence *in Situ* Hybridization Analysis of Alien Genes in *Agrobacterium*-Mediated *Cry1A* (b)-Transformed Rice." *Annals of Botany* 90 (1): 31-6.

[43] Kalunke, M. R., Archana, M. K., Babu, K. H., and Prasad, D. T. 2009. "*Agrobacterium*-Mediated Transformation of Sugarcane for Borer Resistance Using *Cry1Aa3* Gene and One-Step Regeneration of Transgenic Plants." *Sugar Tech.* 11 (4): 355-9.

[44] Islam, N., Laksana, C., and Chanprame, S. 2016. "*Agrobacterium*-Mediated Transformation and Expression of *Bt* Gene in Transgenic Sugarcane." *J. ISSAAS* 22 (1): 84-95.