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Transient expression of β-glucuronidase reporter gene in date palm (*Phoenix dactylifera* L.) embryogenic calli and somatic embryos via microprojectile bombardment

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Abstract

The date palm, *Phoenix dactylifera* L. 'Khorma' (Persian), is an example of a tree crop that can benefit immediately from the applications of the modern biotechnology. Despite the importance as an economic plant, there is no report on its genetic transformation. Palm tree is the target for several pests and diseases, so it is necessary to focus on its *in vitro* propagation and genetic engineering technology (date palm transformation) to overcome some of these problems. The objective of this study was to identify the major parameters controlling DNA delivery by particle bombardment to date palm embryogenic calli and somatic embryos. The main factors studied were osmotic conditioning of explants before and after bombardment (osmotic and non-osmotic), type of explants (embryogenic callus and somatic embryo) and different bombardment parameters like acceleration pressure (900 and 1100 psi), bombardment distance (6 and 9 cm) and gold particle size (1 and 0.6 μm). Efficiency of DNA (*gus* gene) delivery was assessed by scoring transient GUS expression in bombarded tissues. Statistical analysis of blue spots produced by GUS histochemical assay showed that all parameters significantly affected the transient GUS expression. Results of mean comparison showed that GUS transient expression increased when 1.0 μm gold particle, 1100-psi rupture disk, 6 cm bombardment distance, osmotic medium and mature somatic embryos were used.

Key words: Particle bombardment, DNA delivery, histochemical assay.

Introduction

The date palm (*Phoenix dactylifera* L.) is a long-lived dioecious species and has great socioeconomic importance, especially in North Africa and the Middle East. In addition to its valuable fruit, the tree is cultivated for fuel, fiber and as shelter for ground crops[9]. Palms are much neglected plant group in terms of application of biotechnology and genetic engineering in their improvement. It can benefit immediately from applications of recent biotechnologies of plant tissue culture and plant molecular biology. Slow growth, dioecy, the slow offshoot-based propagation system, and the problems involved in prediction of adult characteristics in seedlings have limited improvement of this ancient tree crop. Different pests and diseases [3, 4] attack the date palm. Biotechnology, one of the newest tools in plant breeding, can be helpful for breeders and palm producers to improve the qualities of date palm. At the same time, *in vitro* manipulation of cell and tissue cultures are essential to improve existing cultivars [9]. Despite of the date palm importance as a strategic plant, there is no report on its genetic transformation. Palm tree can be used as a target for application of genetic engineering to improve resistance to pests and diseases. Hence, there is a need for a reliable and efficient date palm transformation technology for general application in a range of genotypes. Genetic improvement of date palm is extremely slow, thus making date palm breeding a very long process. As in the case of most monocots, the introduction of foreign genes into date palm is limited by the lack of efficient, reliable and rapid regeneration system. However, the ability to regenerate whole plants from apical meristem has made date palm amenable to genetic manipulation for the incorporation of foreign genes into any targeted plant genome. One routine technique is by using *Aagrobacterium* spp. Unfortunately, until recently the hosts for *Agrobacterium* have been limited to many dicots and a few monocots. On the other hand, using particle bombardment, it is now possible to transfer any gene to any tissues or cell types. This ability would benefit genetic transformation of recalcitrant and perennial crops, like date palm. The main prerequisite for an efficient transformation system is the ability to produce whole plants from treated target tissues. Unlike other crops, date palm tissue culture is a very slow process. On average, at least 18 months are required to produce whole plants from callus-derived apical meristem of date palm offsets. We are now at the beginning of research on genetic engineering of date palm. Several projects should be done with the aim of introducing foreign genes into date palm, in order to improve date palm yield, quality and its resistance to pests and diseases. Chowdhury *et al.* [21] have recently published preliminary work on the oil palm, but there have been no reported studies on genetic transformation of date palm. The objective of this study was to identify the major parameters controlling DNA delivery by particle bombardment to date palm embryogenic calli and embryos. The main factors studied were type of explants, osmotic conditioning of tissues, gold particle size, acceleration pressure and bombardment distance.

Materials and Methods

Plant materials and preparation: Embryogenic calli and somatic embryos were used as explants. To determine the effect of medium osmolarity on transient expression, we tested two concentrations of sucrose (3 and 6%) for both explant types. Explants were placed on media 4-8 hours before bombardment and 20 hours after bombardment.

Plasmid and microprojectile preparation: The plasmid *pCAMBIA3301* which contains the *uidA* and the *bar* genes, was used in all experiments. Plasmid was isolated from bacterial cultures using alkaline lysis method. Circular plasmid DNA was precipitated on gold particles following the protocol described by Barcelo and Lazzeri [2]. The BiolisticTM PDS/1000 Helium System (BioRad, USA) was used in this study. The biolistic device parameters analyzed were as follows: rupture disk pressure (helium pressure of 900, 1100 and 1350 psi); macrocarrier to stopping screen stopping plate to target tissue distance (6, 9, 12 cm), gold microparticles size (0.6, 1.0 µm). Each plate was bombarded twice. DNA delivery experiments were conducted using a factorial based completely randomized design on blue spots achieved by histochemical assay on embryogenic calli and embryos. For every parameter combination, three plates were bombarded as triplicates and these bombardment experiments were repeated again 2 months later.

Histochemical GUS staining: Two days after bombardment, GUS expression was examined by immersing explants in x-gluc buffer. Blue staining was assessed after incubation overnight at 37ºC. After fixation, the tissues were rinsed several times in 70% ethanol to remove x-gluc solution and were photographed under a binocular microscope (Fig. 1).

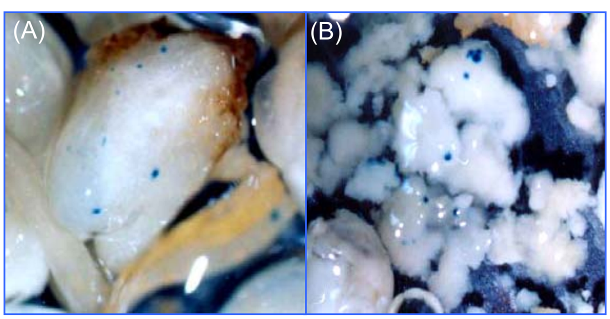

Figure 1. Transient GUS expression in embryos (A) and embryogenic calli (B) of date palm two days after bombardment.

Results

Initial analyses of parameters were conducted using two different explants, in order to test different effects of them on transient GUS expression. The result of statistical analysis indicated that type of explant, pre and after bombardment conditions of explants (3 or 6% sucrose), bombardment distance, bombardment pressure and gold particle size had significant effect on GUS transient expression (Table 1).

Table 1. Analysis of variance of different particle bombardment parameters effect on transient GUS expression.

Source	DF	Mean square	F Value
Explant type	1	0.820	117.06**
Osmotic condition	1	0.251	35.87**
Bombardment distance	1	1.918	273.98**
Bombardment pressure	1	1.099	156.93**
Gold particle size	1	2.127	303.83**

** Significant at the 1% probability levels

Fig. 2 shows that the mean of blue spots for embryos was significantly higher than for somatic embryogenic callus explants. Culture of explants on 6% (0.18 M) sucrose four hours before and twenty hours after bombardment significantly improved transient GUS expression, in comparison with tissues cultured on 3% (0.09 M) sucrose (Fig. 2).

There was a significant difference in GUS expression at pressures of 900 and 1100 psi (Fig. 3). The mean of GUS transient expression was higher at 1100 psi acceleration pressure. Decreasing the bombardment distance resulted in a significant and considerable increase in GUS transient expression. The mean of blue spots for 6 cm bombardment distance was significantly higher than for 9 cm (Fig. 3).

A significant discrepancy in GUS transient expression was observed between two sizes of particle bombardment. Bombarding with *BIO-RAD* 1 µm particles resulted in finer expression units than those obtained from *BIO-RAD* 0.6 µm particles (Fig. 4).

Discussion

Particle bombardment offers several advantages over *Agrobacterium*-mediated transformation such as simplified plasmid construction and less demanding transformation protocols, since complex bacterial/plant interrelationships are eliminated [6].

Effect of osmotic condition of explants pre and after bombardment: The effect of short-term osmotic pre-conditioning (plasmolysis) of target cells or tissues on transient and stable transformation has been reported in several studies [1, 7, 14, 16]. Short-term high osmotic treatments, typically for a few hours before and after bombardment help the targeted tissue to tolerate the damage/stress of the bombardment process. The result of present investigation showed that osmotic medium increased the transient GUS expression. Increasing osmoticum concentration may work by protecting the cell against leakage and bursting (lower turgor pressure) and may improve particle penetration itself [17]. Enhancement of transient expression by preconditioning on high osmotic medium was also observed in other species such as rice[11], tangelo [19] and immature embryos of wheat.

Effect of acceleration pressure: A helium shock wave is used to propel the plastic macrocarrier disc carrying DNA coated microparticles towards the target tissues. Ability of microprojectile to penetrate the different cell layers or tissue types is greatly dependent on propelling force of the helium gas [8]. The highest transient expression was observed at 1100 psi, consistent with observations made in rice [15, 20], cassava [17] and oil palm [13]. Bombarding using lower pressures (900 psi) did not result in any significant increase in transient GUS expression. Lower pressures could be attributed to the poor penetration capability of the microprojectiles as they moved towards the tissues.

Effect of bombardment distance: The lower transient expression at 9 cm could be due to decreased velocity of the microprojectiles with the long flight distance causing by reduced penetration force and thereby fewer cells receiving the oncoming DNA. Increasing bombardment distance resulted in reduced transient expression was also reported by Oard *et al.* [12] and Parveez *et al.* [13].

Effect of gold particle size: Two gold particle sizes (0.6 and 1.0 µm) were compared for their efficiency in delivering DNA into the target tissues. The intermediate size (1.0 µm) gave significantly ($p<0.5$) higher expression level compared to 0.6 µm size (Fig. 6). Similar results have been obtained by Parveez *et al.* [13], Schopke *et al.* [17] and Xiao and Ha [18] in oil palm, cassava and creeping bent grass, respectively. Folling and Olsen [5] reported higher damaging effect with large gold particles (>1.0 µm) in their wheat transformation.

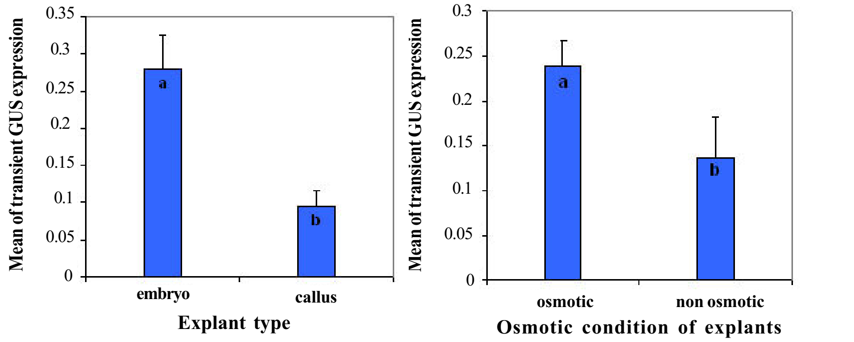

Figure 2. The effect of explants (left) and osmotic condition of explants pre and after bombardment (right) on GUS transient expression using Duncan's multiple range test ($P<0.1$).

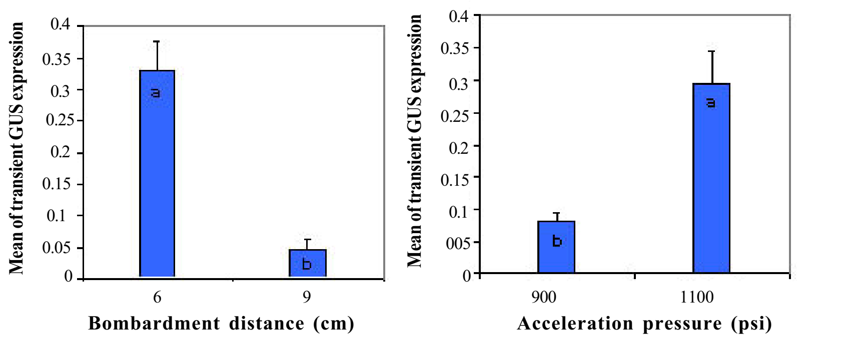

Figure 3. The effect of acceleration pressure (left) and bombardment distance of explants pre and after bombardment (right) on GUS transient expression using Duncan's multiple range test ($P<0.1$).

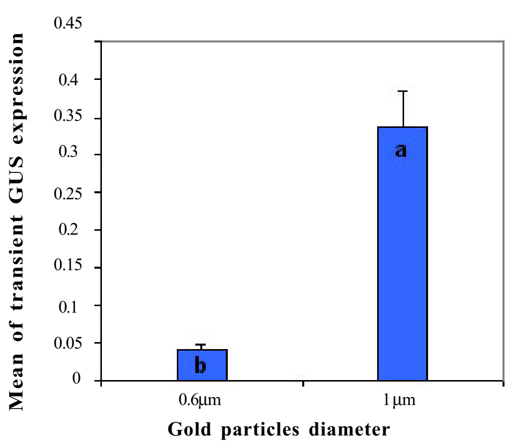

Figure 4. The effect of gold particle size on GUS transient expression using Duncan's multiple range test ($P<0.1$).

Conclusions

In some cases a corresponding increase in the efficiency of the stable transformation was shown [19], while in others the transient expression was not directly correlated with stable transformation events [1, 11]. In any case, it could be advisable that conditions, which most favored transient expression, should be used as a basic protocol for recovering stable transformants [17]. The best conditions for transient GUS expression in date palm tissues were: 1.0 µm gold particles, 1100 psi helium pressure, 6 cm bombardment distance, and pre and post culture of explants on high osmoticum medium (0.6 % sucrose) four hours prior and 20 hours after bombardment.

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