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Transient Eexpression of β-glucuronidase Reporter Gene in Sainfoin (*Onobrychis viciifolia* Scop.) Cotyledons Via Microprojectile Bombardment

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Abstract: Transient β-glucuronidase reporter gene (GUS) expression was visualised in whole cotyledons of sainfoin (*Onobrychis viciifolia* Scop.) after microprojectile bombardment with pBI221.23 DNA. Optimum rupture disk pressure and sample plate distance were determined to be 1350 psi and 6 cm, respectively. Mean gold particle diameter of 1.0 µm and 48 h post-bombardment histochemical GUS assay was found to be superior to 1.6 µm gold particles and 120 h post-bombardment GUS assay. Microscopic analysis revealed the localisation of the gold particles predominantly in the epidermal cell layer, whereas less than 1% of the particles were localised in sub-epidermal cells, as deep as three cell layers. Availability of a regeneration protocol from sainfoin cotyledons and the potential of the microprojectile bombardment recommend future applications for obtaining transgenic sainfoin plants.

Key Words: *Onobrychis viciifolia* Scop., sainfoin, transient gene expression, biolistic, particle bombardment

Mikroprojektil Bombardımanı ile Korunga (*Onobrychis viciifolia* Scop.) Bitkisinde Geçici β-glukuronidaz Reportör gen İfadesi

Özet: Tüm korunga (*Onobrychis viciifolia* Scop.) kotiledonlarına pBI221.23 plazmid DNA'sı ile gerçekleştirilen mikroprojektil bombardımanı sonrasında geçici β-glukuronidaz (GUS) reportör gen ifadesi gözlemlendi. Optimum basınç ve örnek-tabla mesafesi 1350 psi ve 6 cm olarak belirlendi. Ortalama altın partikül çapı olarak 1.0 µm ve bombardımandan 48 saat sonra yapılan histokimyasal GUS incelemesinin 1.6 µm altın çapı ve 120 saat sonra yapılan incelemeden daha iyi sonuç verdiği bulunmuştur. Mikroskopik incelemeler altın partiküllerinin yoğunlukla epidermal hücre katmanında bulunduğunu fakat % 1'den daha az sayıda altın partikülünün, en fazla üç hücre katmanı derinlikte, sub-epidermal katmanda bulunduğunu göstermiştir. Korunga kotiledonları için bir rejenerasyon protokolünün varlığı ve mikroprojektil bombardımanının içerdiği potansiyel gelecekteki uygulamalar ile transgenik korunga bitkilerinin elde edilmesinin mümkün olacağını göstermektedir.

Anahtar Sözcükler: *Onobrychis viciifolia* Scop., korunga, geçici gen ifadesi, biolistik, partikül bombardımanı

Introduction

For the genetic engineering of legume family members a considerable amount of effort has been dedicated to species such as the soybean (1,2), pea (3), chickpea (4), and common bean (5,6). Sainfoin (*Onobrychis viciifolia* Scop.), being a bloat-safe perennial forage legume that has well adapted to poor soils and dry areas, has received very little attention for genetic engineering-based improvement, although 2 stem crown boring insects namely *Bembecia scopigera* and *Sphenoptera carceli* were known to cause considerable crop losses and necessitating the need for such improvements. The only study published so far utilised wild-type *Agrobacterium tumefaciens*-mediated gene transfer but failed to produce transgenic plants (7). Therefore, it is imperative to employ genetic engineering techniques to improve sainfoin.

Starting with the first demonstration in onion (8), microprojectile bombardment proved to be a versatile approach for transformation of living cells, regardless of species (9-11). However, being a physical approach, optimisation of a number of parameters for efficient transgenic plant recovery is mandatory for this technique (12).

In the present study, we show transient expression of β-GUS reporter gene in sainfoin and a number of bombardment parameters were optimised for this species.

Materials and Methods

Preparation of sainfoin cotyledons for bombardment

Seeds of a sainfoin ecotype widely cultivated in Turkey were surface-disinfected in 70% ethanol for 5 min, then in commercial bleach for 30 min and finally rinsed 6 times with sterile distilled water. The seeds were cultured on MS medium (13) supplemented with 3% sucrose and 0.8% agar. Eight days post-germination, cotyledons were excised, ca. 10 cotyledons / plate, under aseptic conditions and placed, adaxial side up, in the central 4 cm area of petri dishes containing the medium, and incubated 24 h at 26ºC in darkness prior to bombardment.

Microprojectile bombardment parameters

All bombardments were carried out with the Bio-Rad Biolistic® PDS 1000/He particle delivery system according to the manufacturer's protocol. Bombardments were made under a partial vacuum of 25" Hg pressure and unless stated otherwise, gold particles, 1.0 μm mean diameter, were coated with 5 μg of pBI221.23 DNA (14) as described in the manufacturer's protocol.

Two rupture disk pressures (1100 and 1350 psi) and 2 sample plate distances (6 and 9 cm) were used and for every parameter combination a total of 3 plates were bombarded as triplicates and this 12-plate bombardment experiment was repeated again 2 months later.

GUS assays and photomicroscopy

Cotyledons were subjected to histochemical GUS assay 48 h after bombardment, unless stated otherwise. The assay solution consisted of 0.25 mg / ml X-gluc, 0.1 M $NaPO_4$ buffer, 0.01 M Na_2EDTA, 0.1 % Triton X-100, 0.5 mM $K_4Fe(CN)_6$.3H2O and 0.5 mM $K_3Fe(CN)_6$ all

at pH 7.0 for 24 h at 37ºC. Transient gene expression events were counted using a dissecting microscope. Cotyledons displaying GUS activity were paraffin embedded, essentially as described by Meyerowitz (15). Ten micron serial sections were cut and visualised by light and polarised microscopy.

Results

Transient expression of GUS in sainfoin cotyledons

Figures 1A and 1B show results typical of transient GUS expression in sainfoin cotyledons. The average number of GUS positive spots (GUPS) per petri plate as well as per cotyledon in 2 independent experiments are summarised in Table 1. Sainfoin cotyledons bombarded with plasmid DNA lacking the GUS gene and with uncoated gold particles were observed to be GUS-negative (data not shown).

From 125 cotyledons bombarded in experiment I, only 6 were tested GUS-negative (4.8 %) and regardless of rupture disk pressures all were noticed to be bombarded at 9 cm sample plate distance. However, 13/147 cotyledons (8.8 %) in experiment II were found to be GUS-negative. The minimum and maximum number of GUPS per cotyledon were 0-245 in experiment I and 0-180 in experiment II. While 47 cotyledons (37.6 %) were observed to be showing over 100 GUPS in experiment I, the percentage dropped to 12.2 % (18/147) in experiment II.

Figures 1C and 1D show the localisation of the gold particles, predominantly in the epidermal cells of the sainfoin cotyledons. However, less than 1 % of the examined gold particles were found to be localised in subepidermal cells, as deep as 3 cell layers. The localisation of the gold particles was also reconfirmed by polarising microscopy.

Effects of particle size and assay time

For the determination of the possible effects of gold particle size and GUS assay time on transient expression of GUS, 4 petri plates were bombarded supplementary to experiment II. Gold particles, 1.0 or 1.6 βm mean diameter were used for bombardment and the cotyledons were subjected to GUS assay 120 h post-bombardment. Results of this experiment are summarised in Table 2.

When compared with the data summarised in Table 1 (experiment II), increasing the time of the GUS assay from 48 h to 120 h resulted in a slight reduction in the average number of GUPS per cotyledon. However, increasing the gold particle size from 1.0 to 1.6 μm resulted in approximately 2.5-fold reduction in the average number of GUPS per cotyledon. This reduction might be due to particle size rather than assay time.

Discussion

This report is the first demonstration of microprojectile bombardment-mediated DNA transfer and expression in sainfoin. From the two rupture disk pressures and the two sample plate

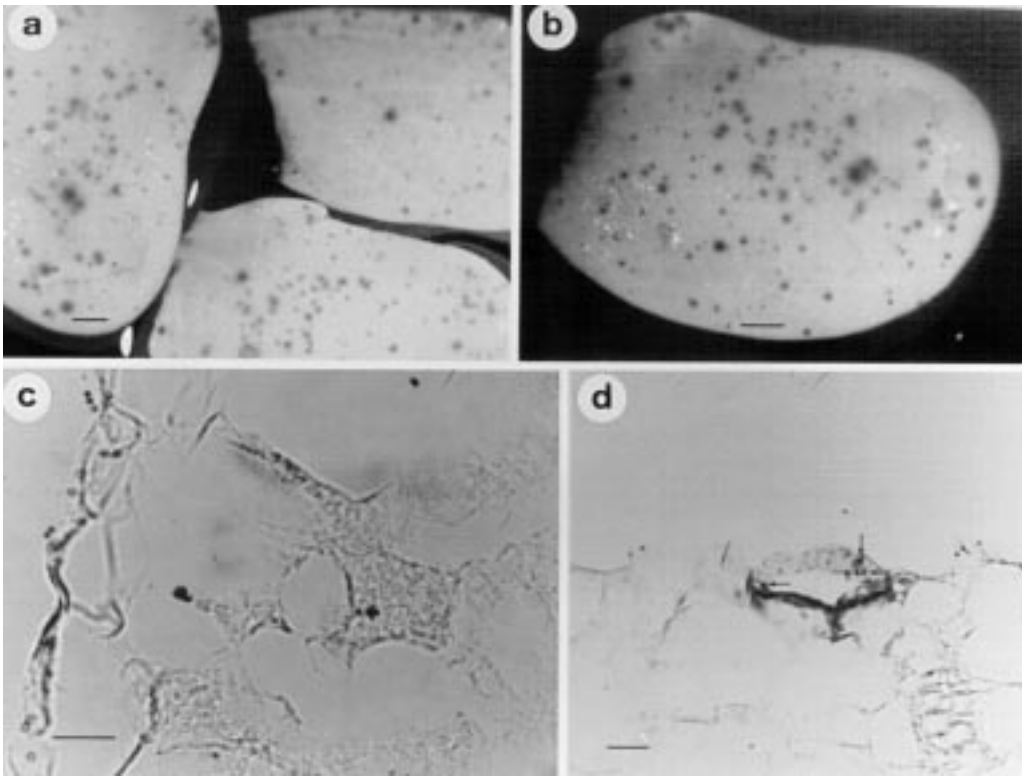

Figure 1. Transient GUS expression in sainfoin cotyledons. (a) Sainfoin cotyledons bombarded 24 h after excision and stained 48 h after bombardment. GUPS are clearly visible. (b) Higher magnification of individual GUS positive events. (c) Localisation of the gold particles in epidermal cell layer, arrow indicates a gold particle localised in subepidermal cell. (d) An epidermal cell displaying GUS activity, arrows indicate gold particles. Bars: 1 mm for a and b and 10 µm for c and d

distances tested, 1350 psi pressure and 6 cm sample plate distance were found to be optimum for sainfoin cotyledons and yielded the highest GUPS per cotyledon. A significant effect of the gold particle size on the average number of GUPS per cotyledon was noted which was demonstrated by nearly 2.5-fold reduction observed when 1.6 βm gold particles were used. However, the average number of GUPS per cotyledon did not decrease significantly as a function of time after bombardment, averaging approximately equal to 48 h post-bombardment by 120 h. In histochemical GUS assays, half of the recommended X-gluc concentration of 0.5 mg/l was found to be sufficient to detect GUPS within 6 h. However, for convenience the incubation time was extended to 24 h with no visible detrimental effects.

Table 1. Effects of rupture disk pressures and sample plate distances on the number of GUPS in sainfoin cotyledons[1].

Pressure / Distance (psi/cm)	Experiment I	Experiment II
1100 / 6	1057.7 ± 213.1 (104.2 ± 19.1)	659.3 β 31.5 (52.80 β 4.71)
1100 / 9	427.0 ± 106.5 (40.2 ± 9.9)	244.7 β 139.3 (19.5 ± 10.0)
1350 / 6	1498.0 ± 105.1 (141.7 ± 14.5)	645.0 ± 87.5 (53.6 ± 4.8)
1350 / 9	518.0 ± 427.6 (48.1 ± 38.5)	437.0 ± 110.0 (35.2 ± 8.6)

[1]Average of three petri plates ± standard error of means. Numbers in parentheses indicate the average number of GUPS per cotyledon ± standard error of means.

Table 2. Effects of gold particle size and GUS assay time on the average number of GUPS in sainfoin cotyledons[1].

Pressure / Distance (psi/cm)	Particle size / Assay Time (μm/h)	
	1.0 / 120	1.6 / 120
1100 / 6	45.8 ± 8.0	17.7 ± 5.4
1350 / 6	48 ± 14.9	19.7 ± 8.4

[1]Average number of GUPS per cotyledon ± standard error of means.

In our experiments, we also experienced one of the major dilemmas associated with the microprojectile bombardment, "shot-to-shot variation" (12). This problem was reflected in the high standard error values we obtained in our experiments. However, at least in 1 case (experiment I, 1350 psi/9 cm parameter combination, final replicate petri plate), the high standard error value is attributed to poor particle load on the particular capton disk.

Analysis of the bombarded cotyledons by microscopy revealed the localisation of the gold particles predominantly in the epidermal cell layer with less than 1 % of the particles being localised in the subepidermal cells as deep as 3 cell layers. These cell layers are the potential sources for adventitious shoot regeneration and a practical method for regenerating sainfoin from cotyledonary explants was published (16).

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