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Research Article

Evaluation of Various Sunflower (*Helianthus annuus* L.) Genotypes for *Agrobacterium tumefaciens*-mediated Gene Transfer

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Abstract: In this work, 10 sunflower (*Helianthus annuus* L.) genotypes were evaluated for their suitability for *Agrobacterium tumefaciens*-mediated gene transfer based on detection of a reported gene expression (GUS). A number of factors including the type of shoot-tip explant (split vs. intact), bacterial strain/binary vector combinations and wounding intact shoot-tips by particle bombardment were examined. Genotypes showed significant differences in their transformation efficiencies ranging from 0.0 to 82.7% GUS positive explants, hybrid genotypes being more responsive to *Agrobacterium* infection than inbred lines. Use of split shoot-tip explants did not increase the transformation efficiency over intact explants but the AGL-1/pKIWI strain/vector combination was more effective than the LBA4404/pTOK233 combination. Wounding explants by particle bombardment prior to inoculations with *Agrobacterium* had no positive effect on transformation.

Key Words: Sunflower, gene transfer, *Agrobacterium tumefaciens*, genotypes.

Çeşitli Ayçiçeği (*Helianthus annuus* L.) Genotiplerinin *Agrobacterium tumefaciens* Aracılığıyla Gen Transferi Açısından Değerlendirilmesi

Özet: Bu çalışmada, 10 ayçiçeği (*Helianthus annuus* L.) genotipinin, reporter bir genin (GUS) ekspresyonunun belirlenmesine dayanan *Agdobacterium tumefaciens* aracılığı ile gen transferine uygunluğu değerlendirilmiştir. Sürgün-ucu eksplant tipi (bölünmüş/split ve bölünmemiş/intact), bakteri suşu/binary vektör kombinasyonu ve bölünmemiş sürgün-ucu eksplantlarının partikülbombardımanı ile yaralanması gibi faktörler incelenmiştir. Transformasyon etkinligi bakımından genotipler arasında önemli farklılıklar gözlenmiş, ortalama %GUS pozitif eksplant oranları %0.0 ile %82.7 arasında değişmiştir. Hibrit genotipler, kendilenmiş hatlara göre *Agrobacterium* enfeksiyonuna karşı daha duyarlı olmuşlardır. Bölünmemiş (intact) eksplantlarla karşılaştırıldığında, bölünmüş (split) sürgün-ucu explantlarının kullanılması, transformasyon etkinliğini artırmazken AGL-1/pKIWI suş/vektör kombinasyonunun, LBA4404/pTOK233 kombinasyonuna göre daha etkili olduğu gözlenmiştir. Eksplantların, *Agrobacterium* ile inoküle edilmeden önce partikül bombardımanı ile yaralanmasının ise transformasyon üzerine herhangi bir olumlu etkisi görülmemiştir.

Anahtar Sözcükler: Ayçiçeği, gen transferi, *Agrobacterium tumefaciens*, genotipler.

Introduction

Increased disease resistance and oil content have been the main goals for the improvement of sunflower (*Helianthus annuus* L.) and achievement of these has been mainly restricted to conventional breeding methods. Because natural variations is a limiting factor in sunflower breeding, successful application of gene transfer techniques for the improvement of technological and agricultural qualities of sunflower varieties would be of great value. Development of a reliable gene transfer system, however, requires optimization of a number of variables. First, an efficient plant regeneration system from the target cells following transformation must be developed. There are several reports describing plant regeneration from different explants of sunflower including immature embryos (1, 2), cotyledons (3, 4) and thin cell layers from hypocotyls (5) but none of these systems has been conclusively shown to be applicable for transformation studies. Furthermore, the efficiency of plant regeneration has been hampered by the genotypic variation (6, 7).

A number of other factors affecting *Agrobacterium tumefaciens*-mediated transformation of sunflower have been studied. These included the physiological age of the explants (8), wounding explants prior to inoculations (9-11), *Agrobacterium* strain/vector combinations (9), co-cultivation period (8), hormonal composition of culture medium (12), and the type and the concentration of the

selection agents (13, 14) as well as the different gene transfer methods (15, 16). Use of shoot-tip meristems appears to be the only efficient method currently applied for genetic transformation of sunflower genotypes (12, 16, 17). This is simply because of the ease of regeneration of plants from the meristematic tissue in which shoot and leaf primordia already exist. Wounding of the shoot-tip meristems by microprojectiles (9, 10) or glass-beads (11) was also reported to increase the transformation efficiency. A more recent work, however, demonstrated that wounding immature zygotic embryos by microprojectile bombardment prior to bacterial inoculation had no effect on the transformation frequency of sunflower inbred lines (12). Although it has not been extensively studied, genotype dependency also appears to be a factor affecting the success of genetic transformation studies in sunflower (10). Therefore, the determination of the most responsive sunflower genotypes may be necessary for the establishment of an efficient gene transfer system.

In an effort to faciliate the transfer of genes encoding novel antimicrobial peptides into sunflower, we aimed to identify material which was most responsive to *Agrobacterium* infection. Using a subset of diverse sunflower genotypes (18), we examined the effects of the type of shoot-tip explant (split vs. intact), *Agrobacterium* strain/binary vector combinations and wounding of intact shoot-tips by particle bombardment on transformation efficiency.

Materials and Methods

Plant Material and Explant Preparation

Four commercial hybrids (Hysun 25, Hysun 36, Hysun 45 and Hyoleic 31) and six public inbred lines (HA 89, HA 341, RHA 271, DL 9542, DL 9546 and DL 9548) were used. Before sterilization, seeds were rinsed twice with sterile distilled water and then soaked in sterile distilled water for 2 h at room temperature. Seeds were washed in 70% ethanol for 2 min, dehulled and then rinsed in 70% ethanol for another 2 min followed by surface sterilisation in 2.5% sodium hypochlorite for 10 min under vacuum infiltration. Seeds were then rinsed again in 70% ethanol for 2 another min and finally rinsed in sterile distilled water 3-4 times followed by 5-6 hours imbibition in sterile distilled water at room temperature. Seed coats were removed and the seeds were cultured on MS-A medium, which contained full-strength MS salts and vitamins (19), 0.1 mg/l BAP (benzyladenine), 500 mg/l casamino acid (Difco), 30 g/l sucrose, 8 g/l bacto agar (Difco) at pH 5.7. After two days incubation in dark at 28°C, the cotyledons and radicles were removed and the explants were transferred back to the same medium for another two days in dark at 28°C. At the end of the 4 day's incubation (Figure 1A), the first two levels were removed to encourage the development of younger leaf primordia, and the remaining parts of the cotyledons and hypocotyls were trimmed down. Immediately after explant preparation, half of the explants were wounded by bombarding once with a particle inflow gun (20) using 5 µl of 0.5-1.2 µm size sterile tungsten particles (100 mg/ml) at 16 cm distance at 70 psi He pressure before bacterial inoculation and the other half of the explants were inoculated without wounding. Immediately after bombardment, one half of both the wounded and non-wounded explants were inoculated with the LBA4404/pTOK233 and the other half with the AGL-1/pKIWI strain/plasmid combinations. Also, to compare split explants with intact shoot-tips, split explants were prepared by cutting intact shoot-tips into two longitudinal halves before inoculations with LBA4404/pTOK233 or AGL-1/pKIWI combination.

Bacterial Strains/Binary Vectors and Co-cultivation

Two binary vectors, pTOK233 (21) kindly provided by T. Komari (Japan Tobacco Co., Japan) and pKIWI (22) by R. Gardner (University of Auckland, New Zealand), were used in combination with *A. tumefaciens* strains LBA4404 (23) and AGL-1 (24), respectively. The T-DNA region of both pTOK233 and pKIWI contained the *uid*A gene encoding GUS (β-glucuronidase), driven by 35S promoter of cauliflower mosaic virus. Presence of an intron in the *uid*A coding sequence in pTOK233 and the lack of ribosome binding site required by bacterial expression in pKIWI ensured that GUS expression could only be detected upon transfer of T-DNA into plant cells.

Overnight cultures of LBA4404/pTOK233 and AGL-1/pKIWI (OD_{600}=1.65) were grown in liquid YEP medium (25) with 50 mg/l kanamycin, 50 mg/l rifampicin and 200 µM acetosyringone by shaking in dark at 28°C at 180 rpm. The cultures were centrifuged at 4000 g for 10 min at 25°C and then resuspended in 10 mM $MgSO_4$, and used in co-cultivation. Wounded and non-wounded explants were immersed in bacterial suspension (diluted 10 times) for 45 mins and co-cultivated on MS-A medium for 3 days under 16/8 hours of light/dark regime at 25/20°C day/night temperatures. Following co-cultivation, explants were washed once in full-strength hormone-free MS liquid medium containing 30 g/l sucrose at pH 5.7 and then transferred to MS-B medium, which contained full-strength MS salts and vitamins, 0.5 mg/l BAP, 0.25 mg/l IAA (indole acetic acid), 0.1 mg/l

GA3 (giberellic acid), 500 mg/l cefotaxime, 50 mg/l kanamycin and 200 μM acetosyringone.

GUS Assay and Data Evaluation

After 5 days' incubation on MS-B medium, the explants were stained for GUS activity by immersing and vacuum infiltrating in GUS staining solution for 15 min and incubated overnight in dark at 37°C. The GUS staining solution was modified from Kosigo et al. (26) and contained 0.1% Triton x-100, 50 mM Na_2PO_4 at pH 7.0, 10 mM EDTA, 2 mM X-Gluc and 10% methanol. Chlorophyll from the stained tissue was removed by 70% ethanol.

Because the size of the isolated transformed tissue varied geratly, ranging from a very tiny sector consisting of a small group of cells to a large sector or a complete coverage of the leaf or hypocotyl, we decided to express the scoring in three different ways; i) mean number of GUS positive sectors per explant, ii) mean % of GUS positive area per explant and iii) % of explants showing GUS activity. In each treatment, 45-60 explants were used, and all of the explants used were taken into account when calculating the means. SE (standard error of the mean) values were also calculated for the parameters (i) and (ii).

Results and Discussion

Comparison of Split and Intact Shoot-tip Explants

Previously, it was shown that the split shoot-tip explants were more effective in the production of transgenic sunflower plants (10). We, therefore, first compared the split and intact explants of Hysun 36 following inoculations with either LBA4404/pTOK233 or AGL-1/pKIWI combination. Our results suggested that transformation efficiency was not changed with the use of split explants (Table 1). This was consistent with the recent work of Burrus et al. (12) who found that split and intact meristem explants produced comparable transformation rates in public and experimental inbred lines. We also observed that shoot development was considerably reduced as a result of splitting and most of the split explants produced only callus which failed to regenerate into shoots. This might be due to the damage to the shoot-tip meristems caused by cutting which then appeared to provoke necrosis after co-cultivation with *Agrobacterium,* as also reported by others (16). This finding is, however, contrary to the previous work of Knittel et al. (10) who reported that a longitudinal section through the apical meristem favoured multiple shoot induction and subsequent gene transfer into the cut region. Our results suggested that the type of shoot-tip explant (split or intact) may not be very important while the existence of rapidly dividing cells in the meristem, which are potential targets for the *Agrobacterium,* is probably a more critical factor. However, it may be important that other tissues surrounding the meristematic region be removed to facilitate full exposure of the meristematic cells to to bacteria.

Comparison of Different Genotypes

Ten sunflower genotypes, which contained a reasonable diversity based on genetic similarity values previously assessed by AFLP analysis (18), were compared for their transformation efficiency. The method we used to evaluate different genotypes assessed the transformation efficiency based on whether the *gus* reporter gene had been successfully transferred into young explants. Although successful recovery of fertile transgenic plants at reasonable frequencies should be a key criterion for evaluating the effectiveness of a gene transfer protocol, our method represented an easy and convenient way of evaluating the responsiveness of each genotype to the transformation conditions employed.

Our results showed that genotypes differed significantly in terms of all three parameters assessed (Table 2). Hysun 45 was the most responsive genotype with the highest number of GUS positive sectors and the largest % of transformed area per explant when compared in terms of the mean of the four treatments. However, when individual treatments were compared, the responses were varying among cvs. Hysun 25, Hysun 36

Table 1. Comparison of split and intact shoot-tip explants from cv. Hysun 36 following inoculation with LBA4404/pTOK233 or AGL-1/pKIWI. Values are the mean±SE (45-60 explants per treatment).

Parameters	Treatments			
	Split/LBA4404	Intact/LBA4404	Split/AGL-1	Intact/AGL-1
Mean number of GUS positive sectors per explant	3.6±0.18	3.5±0.26	3.4±0.21	3.6±0.22
Mean % of GUS positive area per explant	2.4±0.22	2.0±0.13	2.7±0.17	3.8±0.31
% of GUS positive explants	72.1	62.8	68.6	78.0

Table 2. Comparison of transformation efficiencies of 10 sunflower genotypes after inoculations with LBA4404/pTOK233 (L) or AGL-1/pKIWI (A) with (+) or without (-) wounding with a particle inflow gun. Values are the mean±SE (45-60 explants per treatment).

	Mean number of GUS positive sectors per explant					Mean % of GUS positive area per explant					% of GUS positive explants				
Genotypes	L/+	L/-	A/+	A/-	Mean	L/+	L/-	A/+	A/-	Mean	L/+	L/-	A/+	A/-	Mean
Hysun 25	1.7±0.2	2.5±0.3	3.8±0.6	3.1±0.4	**2.8±0.4**	5.7±0.4	2.0±0.4	1.9±0.3	3.1±0.2	**3.2±0.3**	77.8	87.4	62.5	77.2	**76.2**
Hysun 36	5.2±0.7	3.8±0.4	4.8±0.5	1.8±0.3	**3.9±0.5**	1.5±0.1	1.6±0.1	4.4±0.4	2.6±0.3	**2.5±0.2**	59.3	81.2	83.4	79.6	**75.9**
Hysun 45	4.9±0.5	3.3±0.3	6.2±0.7	6.7±1.0	**5.3±0.6**	5.4±0.8	2.6±0.7	2.7±0.4	14.2±2.1	**6.2±1.0**	83.4	55.4	91.6	100	**82.7**
Hyoleic 31	2.4±0.3	2.9±0.5	3.8±0.2	4.9±0.5	**3.5±0.4**	1.2±0.2	1.9±0.4	1.4±0.4	4.0±0.7	**2.1±0.4**	54.5	90.0	72.0	73.0	**72.4**
HA 89	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0	**0.1±0.1**	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0	**0.1±0.1**	9.1	0.00	0.00	0.00	**2.3**
Ha 341	1.0±0.2	1.1±0.2	0.8±0.1	1.4±0.3	**1.1±0.2**	0.6±0.1	1.6±0.3	0.6±0.2	1.0±0.3	**1.0±0.3**	50.0	42.8	25.0	57.0	43.7
RHA 271	0.9±0.3	2.3±0.5	0.5±0.2	0.0±0.0	**1.0±0.3**	1.2±0.1	3.4±0.3	0.3±0.1	0.0±0.0	**1.2±0.1**	38.5	75.0	23.0	0.00	**34.2**
DL 9542	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	**0.0±0.0**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	**0.0±0.0**	0.00	0.00	0.00	0.00	**0.00**
DL 9546	0.6±0.2	0.7±0.2	1.2±0.3	2.2±0.6	**1.2±0.3**	1.0±0.1	0.5±0.1	0.9±0.1	1.3±0.2	**0.9±0.1**	37.5	28.6	54.8	47.0	**42.0**
DL 9548	1.5±0.3	2.4±0.4	6.6±0.8	2.6±0.4	**3.3±0.5**	1.3±0.2	0.9±0.1	1.8±0.5	2.2±0.3	**1.6±0.3**	46.4	56.2	83.4	55.0	**60.3**

and Hysun 45. The hybrid genotypes, together with DL 9548 inbred line, were distincively better than the rest of the genotypes. The percentage of GUS positive explants in our hybrid genotypes was similar to those found by Knittel et al. (10) for the shoot-tip meristem explants. Seeds from hybrid genotypes germinated more vigorously than those from other genotypes and this suggests that there may be a relationship between the vigorous growth and the higher transformation efficiencies of these genotypes. These results indicated that the capacity of individual genotypes for transformation is determined in large part by their genotypes. Therefore, testing the capability of a given genotype or breeding material of sunglower for *Agrobacterium* infection should be a prerequisite when optimizing a transformation system.

After co-cultivation with *Agrobacterium,* GUS expressing sectors of varying sizes were found on leaves, hypocotyls and meristematic region (Figure 1B-I). GUS positive sectors either appeared at the distal end of the lamina only (Figure 1B) or were scattered all over the leaf lamina (Figure 1C), and in some cases, covered the whole stem (Figure 1H) or leaf (Figure 1I). Most of the small GUS positive sectors could be related to the infection of the particle-wounded cells by *Agrobacterium.* The transformed cells located at the leaf tip or in small clusters of cells in the lamina appear to be origination from the cells in the leaf primordium which were present at the time of bombardment and/or inoculation. Occasionally, GUS staining was associated with the vascular tissue as sectors along the leaf midrib (Figure 1G), meristematic region (Figure 1D, E) or cell lineages from the petiole base to the lamina tip (Figure 1F). The sectors running the length of the leaf could be interpreted as arising from transformation of a meristematic cell proior to the initiation of the leaf primordium. The expression was excepter to be stable as GUS positive areas on the explants were observed even 20-30 days after co-cultivation. It is possible that such transformation events will give rise to plants which are usually chimeric for the transferred gene. However, fully transformed individuals can be isolated in the next generation from the progeny of chimeric plants. This can be done by germinating the seeds collected from chimeric plants (primary transformants) on a medium containing kenemycin resistant indivuduals can later be confirmed by GUS assays and other molecular analyses (i.e. southern blot analysis).

Effect of Bacterial Strain/Binary Vector Combination

We also compared the effect of two different *Agrobacterium* strain/binary plasmid vector combinations on the transformation efficiency. The LBA4404/pTOK233 has been successful in *Agrobacterium*-mediated transformation of monocotyledons such as rice (21) and maize (27). The AGL-1 strain is known to be a 'super virulent' on a number of plant species (24). Although the two bacterial strains we used are not directly comparable since they carry different binary vectors, our results suggested that the AGL-1/pKIWI was more effective than the LBA4404/pTOK233, producing approximately 35% more GUS positive sectors (Figure 2A) and 30% larger GUS positive area (Figure 2B). Both combination, however, produced similar proportions of GUS positive shoots (Figure 1C). The slightly better transformation efficiency of AGL-1/pKIWI might be due to higher virulence of this strain on sunflower as the other nopaline type *A. tumefaciens* strains, such as EHA101, were also

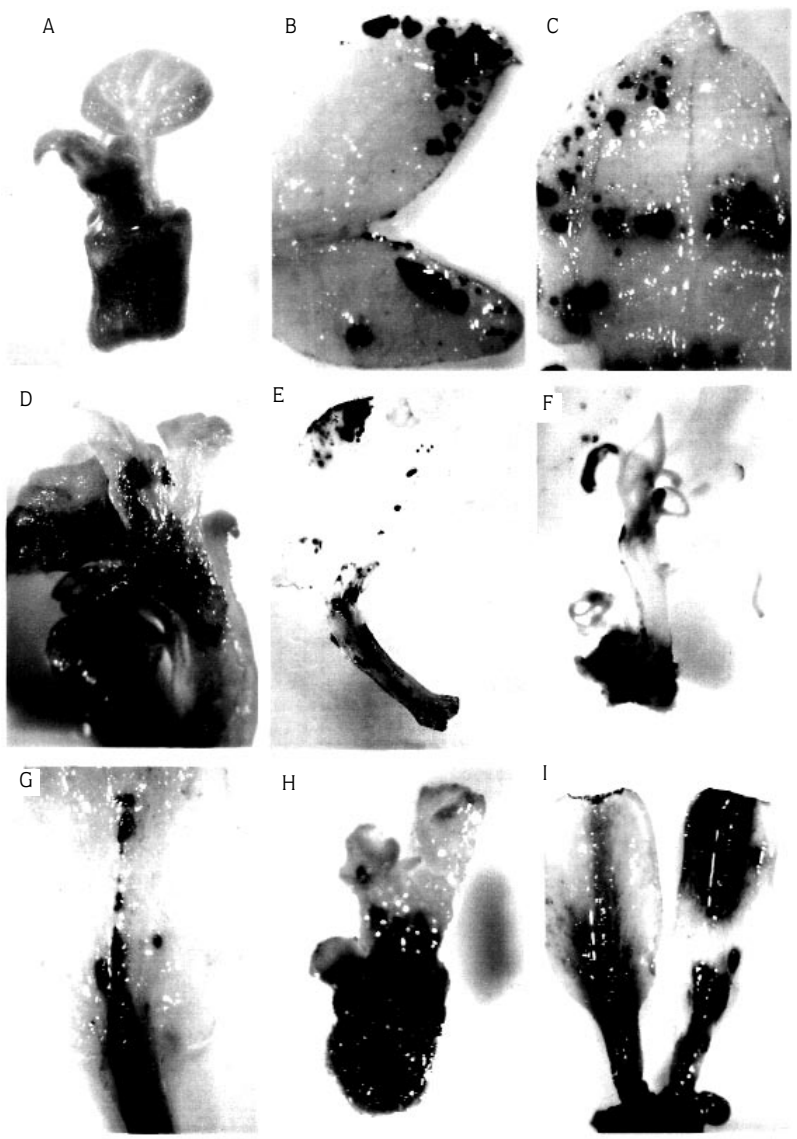

Figure 1. a) A typical sunflower explant after the removal of cotyledons and radicle (2 days), and before removal of first two leaves (4 days). Inoculation with or without particle bombardment was performed immediately before leaves were removed b) GUS expression located mainly at the distal end or c) scattered all over the lamina. d, e) Explants showing GUS expression on the meristematic region as well as on the leaves and hypocotyl. f) Transformed cell lineages running from the hypocotyl base to the lamina tip or g) associated with the vascular tissue as sectors along the leaf midrib. h) GUS expression on the hypocotyl and i) leaves.

reported to be more effective for sunflower transformation (9).

Effect of Wounding by Particle Bombartment

Wounding the meristematic region by particle bombardment or treatment with glass-beads has been reported to be critical for the recovery of transgenic shoots in sunflower (9-11). We, therefore, compared the transformation efficiencies of wounded and non-wounded intact shoot-tip explants of the same 10 genotypes using a single He pressure at 70 psi. Our results showed that the wounded explants had slightly higher numbers of GUS positive sectors than the non-wounded explants, 2.29 sectors per explant compared to 2.10 (Figure 2A). However, when mean % GUS positive area per explart is cmopared, non-wounded explants had approximately 35% more GUS positive tissue than the wounded explants (Figure 2B). In a later experiment, we also compared the effects of a range of bombardment presssures (0, 40, 70 and 100 psi) using two of the most responsive genotypes (Hysun 45 and Hysun 36) and observed that increased bombardment pressures reduced both the number of GUS positive sectors and % of transformed area irrespective of the bacterial strain/plasmid combinations (data not given). These findings are different from the results of Bidney *et al.* (9)

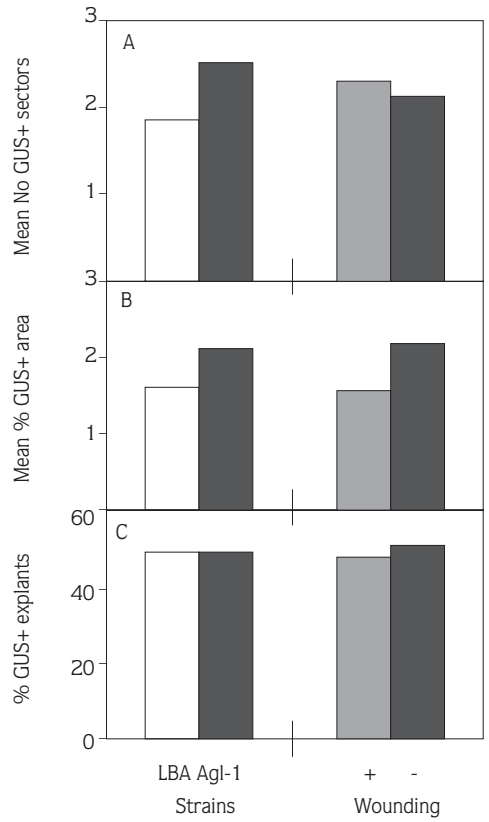

Figure 2. Comparison of the LBA4404/pTOK233 and AGL-1/pKIWI strain/binary vector combinations, and the wounded and non-wounded shoot-tip explants in terms of the mean number of GUS positive sectors per explant (A), mean % of GUS positive area per explant (B) and mean % of GUS positive explants (C). The results are the means of 10 genotypes.

and Knittel *et al.* (10) who found a marked increase in transformation frequency after wounding sunflower shoot-tip meristems. In the latter work, 41% of the explants showed GUS activity when wounded whereas only 11% showed activity in the non-wounded explants (10). In our experiments, both wounding and non-wounding treatments resulted in the production of similar proportions of GUS positive explants, 47.7 and 50.3% (Figure 2C). This is consistent with a recent work by Burrus et al. (12) in which wounding by bombardment before *Agrobacterium* inoculation did not increase the transformation efficiency.

Conclusions

Our results suggest that the proper selectin of genotype and the *Agrobacterium* strain/vector combination may be critical in sunflower transformation. Wounding by particle bombardment or the use of split explants did not have any positive effect on the overall efficiency of transformation in those experiments. However, whether these factors will be important in obtaining stably transformed sunflower plants still awaits further investigation.

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