

## ***Agrobacterium*-MEDIATED TRANSFORMATION OF TWO HIGH OLEIC SUNFLOWER (*Helianthus annuus* L.) GENOTYPES: ASSESSMENT AND OPTIMIZATION OF IMPORTANT PARAMETERS**

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### SUMMARY

The establishment of an efficient *Agrobacterium*-mediated transformation protocol for a recalcitrant crop like high oleic sunflower genotypes, cv. Capella and SWSR2 inbred line, is presented in this paper. The protocol requires the identification and optimization of parameters affecting T-DNA delivery and plant regeneration. The basis for identifying the most appropriate conditions for transformation was the fluorometric, histochemical GUS activity coupled with plant cell vitality. *Agrobacterium tumefaciens*-mediated transformation was performed using strain LBA4404 for cv. Capella and strain GV3101 for the inbred line. Both strains harboring the pBI121 plasmid with the *gus* gene were under the control of 35S promoter. The fluorometric GUS activity was increased in cv. Capella and SWSR2 inbred line 1.9 and 1.6 fold, respectively, relative to transformation without inducers, by optimizing the bacterial density to OD<sub>600</sub>=1.0, by splitting shoot apices and by using MS medium as a co-cultivation medium supplemented with 200 µM acetosyringone. Pre-culturing the explants for three days on SIM2 medium before the bacterial inoculation followed by co-cultivation for 72 h increased the percentage of the GUS expression to 40% and 30% in cv. Capella and SWSR2 inbred line, respectively. It also improved the regeneration percentage as well as the vitality of cells.

**Key words:** *Agrobacterium tumefaciens*,  $\beta$ -glucuronidase, genetic transformation, *Helianthus annuus* L., shoot apices

### INTRODUCTION

Plant genetic transformation is a core research tool in modern plant biology and agricultural biotechnology. *Agrobacterium*-mediated transformation repre-

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sents an effective and widely used approach to introduce desirable genes into plants, including sunflower (*H. annuus* L.), and for fundamental studies of gene expression. Sunflower is an economically important renewable raw material for industrial purposes. The high oxidative performance of oleic acid and its very low content of polyunsaturated fatty acids combined with low content of stearic acid make it suitable for industrial applications such as cosmetics, pharmaceuticals, detergents, lubricants, metal working fluids, surfactants or for chemical syntheses. High oleic sunflower oil can also be used as food oil or deep-frying fat (Fick and Miller, 1997; Dorrel and Vick, 1997). There are several reports describing plant regeneration from different explants of sunflower including cotyledons (Ceriani *et al.*, 1992; Fiore *et al.*, 1997), hypocotyls (Pelissien *et al.*, 1990) and immature embryos (Finer, 1987; Freyssinet and Freyssinet, 1988) but none of these systems has been applied to transformation studies. Use of shoot apex meristems seems to be the most efficient method currently applied for genetic transformation of sunflower genotypes (Malone *et al.*, 1994). The non-availability of general and reproducible regeneration and transformation protocols, combined with the complicated biology of *Agrobacterium tumefaciens* plant interaction and unusual sensitivity to antibiotics, have hindered the application of transformation techniques for sunflower genotypes (Molinier *et al.*, 2002; Weber *et al.*, 2003). Therefore, biotechnological improvement of sunflower (*H. annuus* L.) is limited and it requires an optimization of a number of variables affecting transformation efficiency. In the present investigation, we placed the focus on the optimization and evaluation of some transformation parameters: different *Agrobacterium tumefaciens* strains, bacterial density, type of shoot apices, co-cultivation media, virulence inducer, co-cultivation duration and pre-culture duration that enhance T-DNA transfer aiming at a more efficient transformation of meristematic shoot apices of two high oleic sunflower genotypes.

## MATERIAL AND METHODS

### Plant material

Seeds of high oleic sunflower (*Helianthus annuus* L.), cv. Capella and inbred line SWSR2, kindly provided by Südwestsaat (Rastatt, Germany), were surface sterilized with ethanol (70%) for one minute, rinsed in a sodium hypochlorite solution (6%) plus one drop of tween-20 for one hour and washed three times with sterile water. Seeds were germinated on Murashige and Skoog medium (1962), MS salts 2.3 g/l, sucrose 2%, 2-(n-morpholino) ethanesulfonic acid (MES) 3.2 mM, phytoagar 7.5 g/l and pH adjusted to 5.7 with NaOH (1 M). Seedlings grew for 10 days in a growth chamber at  $25 \pm 1^\circ\text{C}$  and a light period of 12 h ( $115 \mu\text{E m}^{-2} \text{s}^{-1}$ ).

### Explant preparation

Aseptic shoot apices were bisected longitudinally according to Malone *et al.* (1994). To compare the split explants with intact shoot apices, two types of explants were prepared: (1) intact shoot apices with the complete meristematic dome, or (2) split lengthwise in two halves with the cut passing through the apical meristem and between the reminders of leaf primordia.

### Bacterial strains and plasmids

Four *Agrobacterium tumefaciens* strains from different opine groups were used (Table 1). All bacterial strains contained the  $\beta$ -glucuronidase (*gus*) reporter gene under the transcriptional control of 35S promoter and the selectable marker neomycin phosphotransferase (*nptII*) gene under the control of nopaline synthase (*nos*) promoter except C58, which contained the *gus* gene under the control of mas promoter.

Table 1: *Agrobacterium tumefaciens* strains and plasmids used in this study

| Bacterial strain | Helper plasmid | Antibiotic resistance | Binary plasmid | Opine type | Reference                    |
|------------------|----------------|-----------------------|----------------|------------|------------------------------|
| LBA4404          | pAL4404        | Streptomycin          | pBI121         | Octopine   | Hoekema <i>et al.</i> , 1983 |
| C58              | pTiC58         | Streptomycin          | pAM194         | Nopaline   | Han <i>et al.</i> , 2000     |
| GV3101           | pMP90          | Gentamycin            | pBI121         | Nopaline   | Koncz and Schell, 1986       |
| EHA101           | pTi-EHA101     | Kanamycin             | pIBGus         | Agropine   | Hood <i>et al.</i> , 1986    |

### *Agrobacterium*-mediated transformation and regeneration

The transformation parameters were optimized for split shoot apices using the bacterial strains (GV3101 with SWSR2 and LBA4404 with cv. Capella). Parameters were tested one at a time in a sequential order. The following parameters were tested in the order stated: bacterial strains (GV3101, LBA4404, C58 and EHA101) (Table 1), density of bacterial culture (OD<sub>600</sub> 0.5, 1.0, 1.5 and 2.0), explant characteristics (split and intact shoot apices), co-cultivation media (MS and YEB), virulence inducer (200  $\mu$ M acetosyringone and 100  $\mu$ M coniferyl alcohol), co-cultivation duration (2 and 3 days) and pre-culture (0, 1, 2 and 3 days).

*Agrobacteria* were cultured overnight on YEB medium (Sambrook *et al.*, 1989) at 28°C with continuous shaking at 200 rpm under appropriate antibiotics (Table 1). Cells selected from overnight cultures were centrifuged at 4000 rpm for 15 min. at room temperature, washed once in one volume MS medium (Murashige and Skoog, 1962) and re-centrifuged under the same conditions, then re-suspended once in either MS or YEB medium to the required OD<sub>600</sub> with or without the addition of different virulence inducers tested.

For each transformation experiment, 60-100 explants were divided into groups. Each group was imbibed, directly or after 1, 2 or 3 days of pre-culture, in 2 ml *Agrobacterium* suspension for 30 min. and transferred to vacuum infiltration flask.

Vacuum (150 mbar) was applied for 1 minute, then rapidly ventilated. The infiltration procedure was repeated twice. *Agrobacterium tumefaciens* suspension was removed and the explants dried using Whatmann 3MM filter paper, then cultivated in petri dishes containing shoot induction medium (SIM2): MS salts 4.3 g/l, myo-inositol 0.56 mM, thiamine-HCl 0.30  $\mu$ M, glycine 26.64  $\mu$ M, nicotinic acid 4.1  $\mu$ M, pyridoxine-HCl 2.43  $\mu$ M, sucrose 3%, BAP 0.44  $\mu$ M and plant-agar 6 g/l. The pH was adjusted to 5.7 with NaOH (1M) before autoclaving at 121°C for 20 min. and adding the growth regulator after autoclaving (Mohamed *et al.*, 2003). Co-cultivation was done at  $25 \pm 1^\circ\text{C}$  for either 2 or 3 days and with a light period of 12 h ( $115 \mu\text{E m}^{-2}\text{s}^{-1}$ ). After co-cultivation periods, explants were transferred to SIM2 supplemented with 250mg/l to eliminate the *Agrobacteria*. No selecting antibiotic was applied.

For testing the first parameter, shoot split apices were pre-cultured on SIM medium for 2 days, transformed using different bacterial strains re-suspended in MS medium at  $\text{OD}_{600}=1.2$  without virulence inducer and co-cultivated for two days on SIM2 at  $25 \pm 1^\circ\text{C}$  and a light period of 12 h ( $115 \mu\text{E m}^{-2}\text{s}^{-1}$ ). The resulting optimal parameters were applied in the final experiment.

#### **Histological GUS-assay**

Beta-glucuronidase (GUS) activity was assayed by immersing and vacuum infiltrating the regenerated shoots (five weeks old) in GUS-staining solution (0.1M  $\text{Na}_2\text{HPO}_4$ , pH 7, 10 mM NaEDTA, 0.5mM K-Ferricyanid, 0.5mM K-Ferrocyanid, 0.1% Triton-X-100, 1mM X-Gluc (5 bromo-4-chloro-3-indolyl glucuronide) and 20% methanol) modified according to Jefferson *et al.* (1987) for 10 min. and incubated overnight in dark at  $37^\circ\text{C}$ . Before microscopic analysis, chlorophyll was removed by extraction in an ethanol series (70%, 96%) for 24 h. Untreated explants were cultured under identical conditions and served as negative control.

#### **Fluorometric assay**

The fluorometric GUS assay was performed according to Jefferson *et al.* (1987). For all the transformation experiments the fluorometric GUS assay was performed four weeks after co-cultivation except for the bacterial strains experiment, in which the GUS activity was measured after two and four weeks. Plant tissue was ground with a pestle and mortar in the presence of liquid nitrogen. Tissue was homogenized in micro centrifuge tube with extraction buffer (50 mM  $\text{NaH}_2\text{PO}_4$ , pH 7; 10 mM EDTA, pH 8; 0.2% Triton X-100 and 10 mM  $\beta$ -mercaptoethanol), centrifuged to pellet debris and the supernatant collected. Crude protein content of the extract was quantified according to Bradford (1976), mixed with MUG solution (1 mM 4-methylumbelliferyl- $\beta$ -glucoronide and 20% methanol). The reaction was carried out in the dark at  $37^\circ\text{C}$  for 1 h. The reaction was stopped using 0.2 M  $\text{Na}_2\text{CO}_3$ . Preparation was analyzed in a Fluoro-Max (Biorad, Germany) spectrofluorometer, the fluorescence was recorded at an excitation wavelength of 365 nm and an emis-

sion of 455 nm. GUS activity was calculated as micro moles of 4-MU formed/mg protein/min.

#### **Vitality measurement**

Regeneration percentages were estimated and random-treated regenerated shoots chosen to measure the vitality by using a PAM 2000 fluorometer [pulse-amplitude modulated system] (Waltz, Effeltrich, Germany) (Schreiber and Bilger, 1993). Vitality was measured as yield, which represents the essence of fluorescence quenching analysis by the saturation pulse method, calculated according to the equation:

$$Y = (Fm' - Ft) : Fm'$$

Fm' = the parameter which represents the measured fluorescence yield at any given time.

Ft = the parameter defined as the maximal fluorescence yield reached in a pulse of saturating light with an illuminated sample.

#### **Molecular analysis of transformants**

DNA from transformed and non-transformed shoots was extracted according to the CTAB method (Cullings, 1992). Detection of the *gus* gene in the samples was conducted using PCR with the following primers: *gus*-for (5'-ATG TTA CGT CCT GTA GAA AC-3') and *gus*-back (5'-CTT CAC TGC CAC TGA CCG GA-3'), which were designed to amplify a 800-bp DNA fragment of the *gus* cDNA. To test the possibility of bacterial contamination of the plant tissue, the following primers were used: *virA*-for (5'-TCT ACG GTC ATG GTC CAC TAG ACG-3') and *virA*-back (5'-TGC TGC TCA ACT GCT ACG CCA GCT-3') to amplify a 500 bp fragment of the *Agrobacterium* chromosomal *vir A* (Bond and Roose, 1998) and *picA*-for (5'-ATG CGC ATG AGG CTC GTC TTC GAG-3') and *picA*- back (5'-GAC GCA ACG CAT CCT CGA TCA GCT-3'). These primers define a 750 bp domain of the coding sequence.

PCR reaction was performed in 50 µl vol. containing 50-100 ng of *EcoRI* digested genomic DNA, 200 µM of dNTP, 0.25 µM of each primer, 2U Taq of DNA polymerase, 1.5 mM of MgCl<sub>2</sub> and 5 µl of 10 × Taq DNA polymerase buffer. DNA samples were denatured at 95°C for 5 min. and amplified during 32 cycles at 95°C for 1 minute, 64°C for 1 minute, 72°C for 1 minute, followed by a final extension step at 72°C for 10 min. The amplified samples were electrophoresed on a 1% agarose gel and stained with ethidium bromide.

## RESULTS AND DISCUSSION

### Optimization of plant transformation conditions

#### *Sensitivity to Kanamycin*

The effect of Kanamycin as a selectable agent on regeneration efficiency was studied using different concentrations (12.5-200 mg/l). All concentrations showed a detrimental effect on the regenerated transformed shoots with regard to regeneration percentage and the explant cell vitality (data not shown). These results were in agreement with those of Escandon and Hahne (1991), Müller *et al.* (2001) and Gould *et al.* (2002), who reported that Kanamycin is known to be detrimental to organogenic potential. Therefore, we avoided the use of Kanamycin in the subsequent experiments.

#### *Effect of bacterial strain/binary vector*

*Agrobacterium* strains play an important role in the transformation process, as they are responsible not only for infectivity but also for the efficiency of gene transfer (Gelvin and Liu, 1994; Bhatnagar and Khurana, 2003). In addition, virulence of *Agrobacterium* strains varies widely among plant hosts (Bush and Pueppke, 1991; Davis *et al.*, 1991), which is particularly important for the transformation of recalcitrant species.

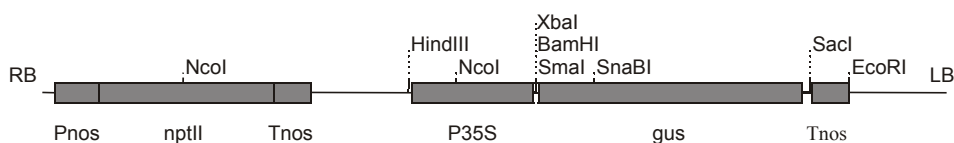


Figure 1: Schematic representation of the pBI121 plasmid used for transformation of two high oleic *H. annuus* L. genotypes (cv. Capella and SWSR2 inbred line) containing the *gus* and *nptII* genes and their promoters

We investigated the sensitivity of the two high oleic genotypes, cv. Capella and SWSR2 inbred line, to different *Agrobacterium* strains (Table 1). The different bacterial strains tested produced transformation events with different efficiency (Table 2). LBA4404 and GV3101, both carrying the plasmid pBI121 (Figure 1) (Chen *et al.*, 2003), were the most efficient strains with both genotypes. The pBI121 plasmid had also been found to be effective for *Pinus taeda* (Tang, 2001) and *Morus indica* (Bhatnagar and Khurana, 2003). Based on the percentages and the intensities of the fluorometric and histochemical assays obtained four weeks later, LBA4404 strain (octopine group) was superior and more infective than the other strains with cv. Capella, while GV3101 strain (nopaline group) was superior with SWSR2 inbred line (Table 2 and Figure 2a). Nopaline strains were also infective for other plant species such as poplars (Han *et al.*, 2000). The other nopaline strain tested in this study, C58, exhibited a low transformation efficiency. This strain harbored the plasmid pAM194 and the reporter *gus* gene under the control of *mas* promoter

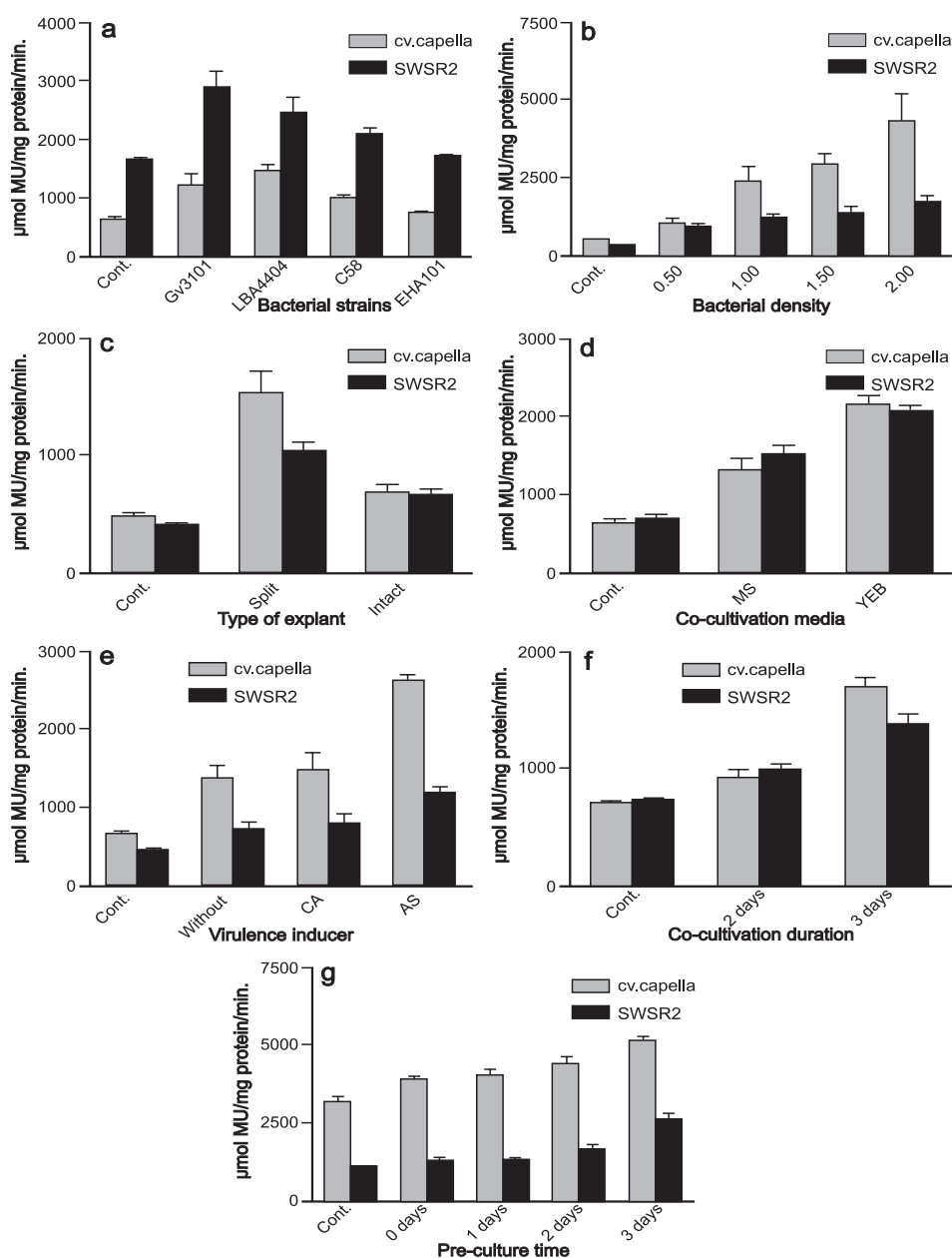


Figure 2: Assessment of different parameters enhancing the transformation efficiency of two high oleic sunflower genotypes using fluorimetric GUS activity (a) different bacterial strains, (b) different bacterial densities, (c) different types of explant, (d) different co-cultivation media, (e) different inducers, (f) different co-cultivation durations and (g) different pre-culture times.

Bars are SE,  $n = 5$ . CA= conferyl alcohol and AS= acetosyringone

(Table 1). Comparing the percentages of the fluorometric assay conducted after two and four weeks, we saw that there occurred a reduction in the GUS activity with time. This indicates that the GUS expression after two weeks was partially transient, which was also previously observed in *H. annuus* L. by Hunold *et al.* (1995) who reported that GUS expression declined with increased culture time. The expression of the *gus* gene showed variability in the number of GUS positive shoots and expression levels which is in agreement with Gürel and Kazan (1999). Generally, results of the transformation experiments showed that the transformation efficiency of the hybrid (cv. Capella) was higher than that of the inbred line (SWSR2) (Figure 2). This result was in agreement with that of Gürel and Kazan, (1999), who reported that hybrid genotypes are more responsive to *Agrobacterium* infection than inbred lines. This means that the GV3101 nopaline group and 35S promoter, widely used in plant transformation, was most effective for SWSR2 inbred line. The different bacterial strains had a slight effect on the shoot vitality as well as on the regeneration percentage, which ranged from 60-50% and 55-50% in cv. Capella and SWSR2, respectively (Table 2). Thus, the strain LBA4404 was selected for cv. Capella and GV3101 for SWSR2 inbred line for all of the following optimization experiments.

Table 2: Effect of different bacterial strains on the regeneration percentage, fluorometric and histochemical GUS assays and the vitality of two high oleic sunflower genotypes, cv. Capella and SWSR2 inbred line

| Bacterial strain | % Regeneration |       | % Fluorometric assay (two weeks after co-cultivation) |       | % Fluorometric assay (four weeks after co-cultivation) |       | % Histochemical assay (four weeks after co-cultivation) |       | Mean of the vitality <sup>c</sup> |                 |
|------------------|----------------|-------|-------------------------------------------------------|-------|--------------------------------------------------------|-------|---------------------------------------------------------|-------|-----------------------------------|-----------------|
|                  | cv. capella    | SWSR2 | cv. capella                                           | SWSR2 | cv. capella                                            | SWSR2 | cv. capella                                             | SWSR2 | cv. capella                       | SWSR2           |
| Untreated        | 60             | 55    | -                                                     | -     | -                                                      | -     | -                                                       | -     | 0.723<br>±0.018                   | 0.756<br>±0.034 |
| GV3101           | 55             | 55    | 80                                                    | 70    | 30                                                     | 40    | 40                                                      | 50    | 0.677<br>±0.019                   | 0.567<br>±0.025 |
| LBA4404          | 55             | 50    | 80                                                    | 60    | 40                                                     | 30    | 50                                                      | 30    | 0.662<br>±0.037                   | 0.569<br>±0.031 |
| C58              | 50             | 50    | 60                                                    | 30    | 20                                                     | 10    | 30                                                      | 20*   | 0.600<br>±0.034                   | 0.529<br>±0.017 |
| EHA101           | 50             | 50    | 40                                                    | 20    | 10                                                     | 10    | 20*                                                     | nd    | 0.573<br>±0.026                   | 0.477<br>±0.034 |

(\*) light blue and (nd, not detected).

The vitality was expressed as a mean of the yield (PAM 2000 fluorometer)

± SE, n = 3

#### *Effect of bacterial density*

We evaluated the effect of different bacterial densities (OD<sub>600</sub> 0.5, 1.0, 1.5 and 2.0) on the regeneration percentage, vitality, fluorometric and histochemical GUS activity (Table 3). Using the OD<sub>600</sub> at value 2.0 decreased the regeneration percentage from 60 to 40% and 55 to 35% in cv. Capella and SWSR2 inbred line, respectively, as compared with the control (Table 3). The same effect was observed for

vitality. The reduction of vitality, caused by inoculation with high concentration of bacterial OD<sub>600</sub> (1.5 and 2.0), related to a hypersensitivity response of explants to the bacteria. This was also observed in safflower transformation (Orlikowska *et al.*, 1995) and sweet orange and citrange (Changhe *et al.*, 2002). Histochemical and fluorometric GUS activity generally increased in both genotypes as the bacterial concentration increased (Table 3 and Figure 2b).

Table 3: Effect of different transformation parameters on the regeneration percentage, histochemical GUS assay and the vitality of two high oleic sunflower genotypes, cv.capella and SWSR2 inbred line

| Transformation parameter            | % regeneration |       | Mean for vitality |             | % GUS expressing shoots |       |
|-------------------------------------|----------------|-------|-------------------|-------------|-------------------------|-------|
|                                     | cv. Capella    | SWSR2 | cv. Capella       | SWSR2       | cv. Capella             | SWSR2 |
| Bacterial density OD <sub>600</sub> |                |       |                   |             |                         |       |
| Untreated                           | 60             | 55    | 0.699±0.003       | 0.694±0.011 | 0                       | 0     |
| 0.5                                 | 55             | 55    | 0.702±0.006       | 0.672±0.021 | 0                       | 0     |
| 1.0*                                | 55             | 50    | 0.670±0.017       | 0.703±0.019 | 30                      | 30    |
| 1.5                                 | 50             | 45    | 0.599±0.042       | 0.639±0.008 | 40                      | 30    |
| 2.0                                 | 40             | 35    | 0.551±0.028       | 0.569±0.012 | 40                      | 40    |
| Type of explant                     |                |       |                   |             |                         |       |
| Untreated                           | 60             | 55    | 0.580±0.015       | 0.687±0.008 | 0                       | 0     |
| Split*                              | 60             | 55    | 0.569±0.013       | 0.699±0.011 | 30                      | 30    |
| Intact                              | 50             | 50    | 0.490±0.021       | 0.572±0.025 | 10                      | 10    |
| Co-cultivation media                |                |       |                   |             |                         |       |
| Untreated                           | 60             | 55    | 0.520±0.005       | 0.531±0.009 | 0                       | 0     |
| MS*                                 | 60             | 55    | 0.470±0.015       | 0.481±0.004 | 30                      | 20    |
| YEB                                 | 40             | 40    | 0.344±0.003       | 0.254±0.010 | 20                      | 30    |
| Virulence inducers                  |                |       |                   |             |                         |       |
| Untreated                           | 60             | 55    | nm                | nm          | 0                       | 0     |
| Without                             | 55             | 60    | nm                | nm          | 20                      | 10    |
| 100µM coniferyl alcohol             | 55             | 50    | nm                | nm          | 10                      | 10    |
| 200µM acetosyringone*               | 60             | 55    | nm                | nm          | 50                      | 40    |
| Co-cultivation time                 |                |       |                   |             |                         |       |
| Untreated                           | 60             | 50    | 0.565±0.018       | 0.616±0.016 | 0                       | 0     |
| 2 days                              | 55             | 55    | 0.571±0.020       | 0.592±0.029 | 10                      | 10    |
| 3 days*                             | 55             | 55    | 0.550±0.023       | 0.582±0.010 | 40                      | 30    |
| Pre-culture time                    |                |       |                   |             |                         |       |
| Untreated                           | 60             | 55    | 0.645±0.014       | 0.581±0.011 | 0                       | 0     |
| 0 day                               | 45             | 40    | 0.417±0.008       | 0.403±0.006 | 0                       | 0     |
| 1 day                               | 55             | 50    | 0.485±0.008       | 0.504±0.012 | 10                      | 0     |
| 2 days                              | 55             | 50    | 0.616±0.006       | 0.552±0.010 | 20                      | 20    |
| 3 days                              | 60             | 55    | 0.636±0.012       | 0.591±0.028 | 40                      | 30    |

\*) Conditions chosen for subsequent experiment, (nm) not measured, the vitality was expressed as a mean of the yield (PAM 2000 fluorometer) ± SE, n = 3

At OD<sub>600</sub> value 2.0, the fluorometric GUS activity increased 4.2 and 1.8 fold in cv. Capella and SWSR2 inbred line, respectively, as compared with the values of fluorometric GUS activity at OD<sub>600</sub> value 0.5. For both genotypes, the OD<sub>600</sub> value 1.0 has been selected as the suitable bacterial density for transformation experiments. The same concentration has been often used in transformation experiments with other plant species (Gutiérrez *et al.*, 1997; Bond and Roose, 1998).

#### *Effect of type of explant*

It was shown previously that the split shoot tip explants were effective in the production of transgenic sunflower plants (Knittel *et al.*, 1994; Malone *et al.*, 1994). Therefore, we compared the split and intact explants of cv. Capella and SWSR2 inbred line. The results showed that there was an increase in the GUS activity with the use of split explants (Figure 2c and Table 3). The splitting of the explant facilitates the full exposure of the meristematic cells and the other tissues surrounding the meristematic region to the *Agrobacterium* infection. Meanwhile, the regeneration percentage was increased by longitudinal cutting of the explants and subsequent gene transfer (Table 3). In addition, longitudinal section through the apical meristem favored multiple shoot induction (Figure 3a). The obtained results agreed with those of Knittel *et al.* (1994) and Changhe *et al.* (2002), but they are in contrast to the previous work of Gürel and Kazan (1999), who reported that the type of shoot-tip explant (split or intact) may not be a very important factor while the existence of rapidly dividing cells in the meristem, which are potential targets for the *Agrobacterium*, is probably a more critical factor.

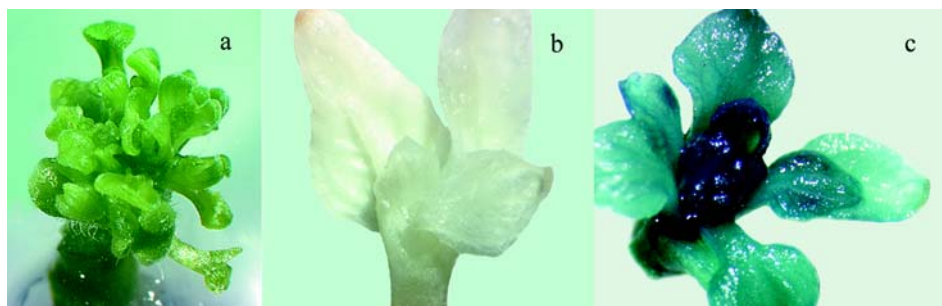


Figure 3: Histochemical GUS assay of two high oleic sunflower genotypes, cv. Capella and SWSR2 inbred line. (a) Multiple shoots from direct shoot apices regeneration on SIM2 medium, (b) Untreated shoot and (c) GUS expressing shoot after gus staining assay

#### *Effect of co-cultivation medium*

Co-cultivation medium was among the different parameters affecting the transformation efficiency. A co-cultivation medium was selected with respect to transformation efficiency, but without inhibiting the cell vitality and regeneration rate. The effect of MS and YEB media on the fluorometric GUS activity and the vitality is illus-

trated in Figure 2d and Table 3. The Use of YEB as a co-cultivation medium increased the fluorometric GUS activity 1.6 and 1.4 fold for cv. Capella and SWSR2 inbred line, respectively, as compared with the values obtained when using MS as a co-cultivation medium (Figure 2d). YEB as a co-cultivation medium had a negative effect on the vitality and regeneration percentage (Table 3) due to excessive growth of the bacterium. YEB medium is known to be suitable medium for bacterial growth. It enhances the bacterial activity and, hence, the transformation efficiency expressed as fluorometric and histochemical GUS activities. On the other hand, YEB medium is not recommended as tissue culture medium for plants. Thus, we used MS medium for co-cultivation medium, as a compromise between transformation efficiency and vitality of the explant. MS has been used often in transformation experiments (Müller *et al.*, 2001).

#### *Effect of virulence inducer*

Acetosyringone and coniferyl alcohol are phenolic compounds which, released by the wounded plant cells, are virulence inducers similar to syringaldehyde (Stachel *et al.*, 1985). These compounds play important roles in the natural infection of plants by *Agrobacterium tumefaciens* because they activate the virulence genes of the Ti-plasmid and initiate the transfer of the T-DNA region into plant cell (Tang, 2001). The addition of 200  $\mu$ M acetosyringone during the co-cultivation increased the fluorometric GUS activity 1.9 fold in cv. Capella and 1.6 fold in SWSR2 inbred line, while the addition of 100  $\mu$ M coniferyl alcohol increased the fluorometric GUS activity 1.1 fold in both genotypes compared with the transformed without inducer (Figure 2e). These results were confirmed by the histochemical GUS assay (Table 3). An addition of acetosyringone during pre-culture and co-cultivation increased the number of transformed cells in the target tissues in a number of species such as *H. annuus* L. (Müller *et al.*, 2001) and wheat (Weir *et al.*, 2001). However, the addition of the virulence inducers had little or no effect on the regeneration percentage in both genotypes (Table 3). Therefore, we used acetosyringone in our transformation protocol for all subsequent experiments.

#### *Effect of pre-culture and co-cultivation duration*

Pre-culture and co-cultivation duration are important factors affecting fluorometric GUS activity, GUS expression and regeneration percentage. Prior to inoculation with *Agrobacterium*, explants were pre-cultured on SIM2 medium for 0 to 3 days. Our results revealed that GUS expression as well as fluorometric GUS activity increased with length of pre-culture time (Table 3 and Figure 2g). Improvement in transformation frequency following the pre-culturing of explants has also been reported for other plants such as *Helianthus annuus* L. (Molinier *et al.*, 2002) and *Brassica napus* L. (Cardoza and Stewart, 2003); also, regeneration percentage and shoot vitality improved with increase in pre-culture period (Table 3), as reported by Molinier *et al.* (2002). Explants were hypersensitive to the bacterial culture without

any pre-culture. A short pre-culture period (1-3 days) increased the regeneration percentage and shoot vitality, because pre-culturing of the explants before co-cultivation supported explants to overcome the stress resulting from the co-cultivation with *Agrobacterium*. After pre-culture, the explants were transformed by co-cultivation with *Agrobacterium* for two and three days. We found that different co-cultivation times had a slight or no effect on the regeneration percentage and shoot vitality (Table 3). Co-cultivating the explants for three days increased the fluorometric GUS activity 1.8 and 1.3 fold in cv. Capella and SWSR2 inbred line, respectively, relative to the co-cultivation for two days (Figure 2f). This was also confirmed by the histochemical data (Table 3 and Figure 3c). This supports the earlier reports showing that the co-cultivation of explants for three days yielded the highest transformation frequency in a number of species, e.g., Chinese cabbage (Zhang *et al.*, 2000), *Helianthus annuus* L. (Molinier *et al.*, 2002) and wheat (Wu *et al.*, 2003). Finally, the pre-culturing the explants on the co-cultivation medium for three days followed by the co-cultivation with *Agrobacterium* for three days was found to create best conditions in our transformation protocol.

#### Molecular analysis of transformants

The PCR analysis was carried out to confirm the presence of the *gus* gene in the GUS-positive regenerated shoots. *Eco*R1-digested genomic DNA of independently random transformed and untransformed control tissues from the final representative experiment was analyzed using primers specific to the *gus* gene. The expected 800 bp band was amplified in the GUS expressing shoots from *H. annuus* L. cv. Capella and SWSR2 inbred line, whereas no amplification was detected in the non-transformed shoots (Figure 4). When *virA*- and *pica*-specific primers were used, no amplification was detected in any of the transgenic material analyzed (data not shown). This indicates that no residual *Agrobacterium* was present in the analyzed material.

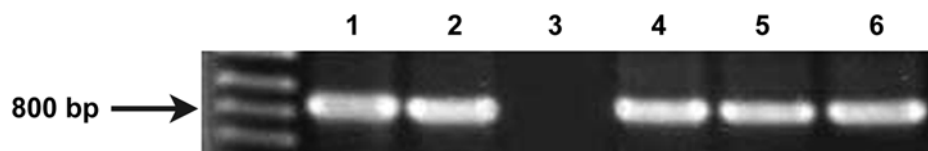


Figure 4: Internal *gus* fragments from total isolated DNA of transformed shoots of two high oleic sunflower genotypes, cv. Capella and SWSR2 inbred line. (1, 2) transformed shoots of cv. Capella, (3) DNA from untreated sunflower, (4) plasmid positive control and (5,6) transformed shoots of SWSR2 inbred line

#### CONCLUSION

Our results showed that by fine-tuning the transformation conditions, even a recalcitrant crop such as sunflower (*H. annuus* L.) can be transformed. The fluoro-

metric and histochemical GUS assays combined with the cell vitality measurement approach were found to be the easy and reliable way of establishing optimal conditions for transformation. Optimal conditions for transformation of the two high oleic sunflower genotypes, cv. Capella and SWSR2 inbred line, were created using LBA4404 strain and GV3101 strain, respectively, at OD<sub>600</sub> value 1.0, split shoot apices explants, and MS as a co-cultivation medium supplemented with 200 µM acetosyringone for the explants inoculation. Pre-culturing the explants for three days on SIM2 prior to the transformation followed by three-day co-cultivation on the same medium achieved the best results. This protocol of transformation can be used to transfer genes of agronomic importance into elite cultivars.

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### **DETERMINACIÓN Y OPTIMIZACIÓN DE PARÁMETROS QUE APOYAN LA TRANSFORMACIÓN DE DOS GENOTIPOS DE GIRASOL (*Helianthus annuus* L.), MEDIANTE *Agrobacterium***

#### **RESUMEN**

El establecimiento de un protocolo eficiente para la obtención de rendimientos altos de aceite de las líneas consanguíneas de los genotipos de girasol, cv. Capella y SWSR2, mediante transformación de *Agrobacterium*, requiere de la identificación y optimización de los factores que afectan la expresión de T-ADN y la regeneración de la planta. Las bases por identificar las condiciones más apropiadas para la transformación fueron fluoremetría y la actividad histoquímica de GUS en comparación con la vitalidad. Mediante la transformación de *Agrobacterium tumefaciens*- se utilizó la raza LBA4404 para cv. Capella y la raza GV3101 para la línea producida. Ambas Razas albergan el plásmido pBII21. Perfeccionando la densidad bacteriana para valores de 1.0, usando partes de las puntas del embrión y el medio MS, como un medio de co-cultivo suplementado con 200µM acetosyringona, aumenta la actividad fluorométrica de GUS en pliegues de 1.9 y 1.6 en las líneas producidas cv. Capella y SWSR2 respectivamente, la transformación es relativa sin inductores. Pre-cultivando las explantaten tres días en SIM2, antes de la inoculación de la *Agrobacteria*, seguidas por el co-cultivo para 72 horas, aumenta el porcentaje de la expresión de GUS a 40% y 30% en cv. Capella y SWSR2 respectivamente, y mejoró el porcentaje de la regeneración así como la vitalidad.

**TRANSFORMATION DE DEUX TOURNESOLS OLÉICS  
(*Helianthus annuus* L.) GENOTYPES VIA *Agrobacterium*  
*tumefaciens*: ÉVALUATION ET PARAMÈTRES OPTIMUM**

**RÉSUMÉ**

L'établissement d'un protocole efficace de transformation via *Agrobacterium* des plantes oléïcs supérieures : tournesols génotypes cv. cappella et SWSR2 de même lignée est ici présenté. Le protocole nécessite l'identification des paramètres optimum affectant le transfert de l'ADN-T et la régénération des plantes. Les conditions appropriées pour une transformation efficace sont basées sur l'activité fluorométrique et histochimique du GUS et la vitalité des cellules. La transformation de cv. cappella et de SWSR2 est réalisée par les souches *Agrobacterium tumefaciens* LBA 4404 et GV3101 respectivement. Les deux souches renferment le plasmide pBI 121. L'activité fluorométrique du GUS dans cv. Cappella et SWSR2 plantes est de 1,9 et 1,6 fois respectivement plus élevée que la contrôle en absence d'inducteurs et sous transformation réalisée en aspergeant les poudres avec une densité bactérienne optimale de 1.0 et en utilisant le médium MS contenant 200 µM d'acetosyregone comme milieu de co-culture. La pré-culture des explants dans un milieu SIM2, 3 jours avant l'inoculation bactérienne suivit de 72 h de co-culture augmente le pourcentage de l'expression de GUS de 40 et 30% dans cv. Cappella et SWSR2 respectivement. Cette condition améliore également le pourcentage de régénération aussi bien que la vitalité des cellules.