Journal of Applied Botany and Food Quality 80, 171 - 178 (2006)

Institute of Plant Molecular Physiology and Biotechnology, University of Bonn, Germany

Particle bombardment as a strategy for the production of transgenic high oleic sunflower (*Helianthus annuus* L.)

Sherin Mohamed, Robert Boehm, Heide Schnabl*

(Received August 2, 2006)

Summary

In order to develop an efficient and reproducible protocol for genetic engineering of high oleic *Helianthus annuus* L. genotypes (cv. capella and SWSR2 inbred line) important parameters of a particle bombardment strategy have been optimized, such as gold particle size, particle acceleration pressure, distance between macrocarrier assembly and target plate, pre-culture period of the explant and number of bombardments per explant. These parameters were evaluated on the basis of resulting GUS activity coupled with regeneration frequency and efficiency as well as plant cell vitality. Split shoot apices were used as explants. The maximum GUS activity was observed at 1550 psi acceleration pressure combined with 6 cm target distance and 1.6 µm gold particle size. A pre-culture of one day prior to bombardment gave the best results. In addition, two subsequent bombardments increased the GUS activity of cv.capella and SWSR2 inbred line 1.6 and 2.1 fold, respectively, compared to explants bombarded once. The optimized bombardment conditions were applied for estimating the transformation frequency which reached 3.1 and 4.5 % for high oleic cv.capella and SWSR2 inbred line, respectively. This frequency was calculated on the basis of positive PCR results of putative transgenic plants and in relation to the total number of bombarded explants.

Abbreviations: MUG – 4-methylumbelliferyl-ß-glucuronide; 4-MU – 4-methylumbelliferone; BAP – 6-benzylaminopurine; gus – β-glucuronidase; MS – Murashige and Skoog; nos – nopaline synthase gene; nptII – neomycin phosphotransferase gene; psi – pounds per square inch; SE – standard error; SIM – shoot induction medium

Introduction

Sunflower (*Helianthus annuus* L.) is one of the three most important annual oil-bearing crops world-wide following soybean (*Glycine max* L.) and rapeseed (*Brassica napus* L.) (WEBER et al., 2003). High oleic sunflower oil can also be used as food oil or deep-frying fat (FICK and MILER, 1997; DORREL and VICK, 1997). Its high oxidative performance of oleic acid and its very low content of polyunsaturated fatty acids combined with low content of stearic acid make them suitable for industrial applications like cosmetics, pharmaceuticals, detergents, lubricants, metal working fluids, surfactants or for chemical synthesis. Sunflower is known as one of the most recalcitrant species for tissue culture and genetic transformation. Therefore, progress in sunflower transformation has been restricted for many years by the limitations of an available regeneration system and problems in combining regeneration and transformation within the same cells (POTRYKUS, 1990). Particle bombardment is a popular method of direct gene delivery into cells, tissues and organs. This technique uses pressurized helium to accelerate sub-cellular size microprojectiles of tungsten or gold coated with DNA (or other biological material) into cells over range of velocities necessary to optimally transform many different cell types (BATHNAGAR et al., 2002). In the first report on the introduction of a foreign gene by particle bombardment of sunflower meristem explants, the regenerated plants showed *gus* expression sectors indicating that chimeric plants had been produced (BIDNEY, 1990). Transient expression of the *gus* gene has been induced in sunflower cotyledonary explants and immature zygotic embryos at different developmental stages after microprojectile bombardment (HUNOLD et al., 1995). Small embryos of approximately 1.5-2.0 mm in diameter were the most suitable for efficient transient *gus* expression (LAPARRA et al., 1995; HUNOLD et al., 1995) and multiple shoot formation, but the conversion rate of transient to stable transformation was shown to be very low (HUNOLD et al., 1995). The limited success of DNA transfer into sunflower cotyledons by microprojectile bombardment is likely due to the strong cuticle (HUNOLD et al., 1995). Several factors have been described to influence the applicability and efficiency of biolistic gene transfer such as genotype (KOPREK et al., 1996), particle size (BHAT et al., 2001), pre-culture period prior to gene transfer (RASCO-GAUNT et al., 1999), acceleration pressure (KOPREK et al., 1996; BHATNAGAR et al., 2002; TADESSE et al., 2003), the adjustable distances between rupture disc and target plate (BHAT et al., 2001; RASCO-GAUNT et al., 1999) and number of bombardments (LONSDALE et al., 1990). The aim of this study was to optimize the physical and biological parameters in order to work out the highest transformation efficiency without compromising the plant cell vitality and regeneration frequency and to deliver a strategy for obtaining stable transformants of high oleic sunflower, a hybrid (cv. capella) and an inbred line (SWSR2).

Material and methods

Plant material and explant preparation

Seeds of high oleic sunflower (*Helianthus annuus* L.), cv. capella and inbred line SWSR2, kindly provided by Südwestsaat (Rastatt, Germany), were surface sterilized for one min with 70 % (v/v) ethanol, rinsed in a 6 % (v/v) sodium hypochlorite solution plus one drop tween-20 for one hour and washed three times with sterile water. Seeds germinated on a MS-based medium (MURASHIGE and SKOOG, 1962) containing MS salts 2.3 g l^{-1}, sucrose 2 % (w/v), 2-(n-morpholino) ethanesulfonic acid (MES) 3.2 mM, phytoagar 7.5 g l^{-1} and pH adjusted to 5.7 with NaOH (1 M). Seedlings grew for 10 days in growth chamber at 25 ± 1°C and a light period of 12 h (115 µ E m^{-2} s^{-1}). After 10 days, aseptic shoot apices (4-5 mm length) were bisected longitudinally according to MALONE-SCHONEBURG et al. (1994) and used as explants in this study.

Plasmid isolation

Preparation of plasmid DNA from *E. coli DH5α* carrying the plasmid pBI121 was performed as described by SAMBROOK and RUSSEL (2001) using the alkaline lysis method. This plasmid containes the *gus* gene under the transcriptional control of the CaMV35S promoter

* Corresponding author.

and the selectable *nptII* marker gene under the control of *nos* promoter.

Preparation of the gold particles and coating with DNA

Preparation of the gold particles was performed according to the method of SANFORD et al. (1993) and stored at -20°C at a final concentration of 60 mg ml^{-1}. The particle suspension was thawed when required and vortexed vigorously to resuspend the particles. 50 µl of microcarriers were taken in an eppendorf tube and while vortexing continuously (for uniform DNA precipitation onto microcarriers) the following was added sequentially: 5 µl DNA (1 µg $µl^{-1}$), 50 µl 2.5 M $CaCl_2$ and 20 µl 0.1 M spermidine. Contents were vortexed for 5-6 min, microcarriers were allowed to settle for 1 min and pelleted by spinning for 2 sec at 14,000 rpm. The liquid was removed and replaced by 140 µl of 70 % (v/v) ethanol for washing. The first washing was followed by washing with 100 % (v/v) ethanol and finally particles were resuspended in 48 µl of 100 % ethanol. These coated particles were kept at 4 ºC, used within 1 h of preparation and 6 µl of the coated particle suspension were loaded onto the macrocarrier membrane which was allowed to dry for 10 min prior to use.

Particle bombardment and plant regeneration

Various physical and biological parameters were applied in a single or multifactorial way using pBI121 coated gold particles for cv. capella hybrid and SWSR2 inbred line. These tested parameters included different gold particle size (1 and 1.6 µm), particle acceleration pressures (0, 450, 900, 1550 and 1800 psi), distance between macrocarrier assembly and target plate (6 and 9 cm), preculture of the explant (0, 1 and 2 days) and number of bombardments per explant (1 and 2 shots). Split shoot apices were grouped in the center of 4 cm Petri dishes on 2 % (w/v) autoclaved agarose gel (the cut surface facing up). The bombardment was performed according to SANFORD et al. (1993) using a Biolistic® PDS-1000/He particle delivery system (Biorad, Germany). Bombarded explants were cultured on shoot induction medium (SIM2, MOHAMED et al., 2003) containing MS salts 4.3 g l^{-1}, *myo*-inositol 0.6 mM, thiamine-HCl 0.3 µM, glycine 26.6 µM, nicotinic acid 4.1 µM, pyridoxine-HCl 2.4 µM, sucrose 3 % (w/v), BAP 0.4 µM, pH 5.7 and plant-agar 6 g l^{-1} without any selecting agent. Culture conditions were 25 ± 1°C and a light period of 12 h (115 µ E m^{-2} s^{-1}). After three weeks, explants were sub-cultured on fresh SIM2 and incubated under the same conditions. Under these conditions, multiple-shoot regeneration could be observed and the biggest regenerant of each explant was used for further molecular analysis.

Measurement of plant cell vitality

Regenerated shoots from the bombardment were chosen randomly to measure the vitality by using a pulse-amplitude modulated fluorescence measurement system (PAM 2000 fluorometer, Waltz, Germany) as described by SCHREIBER and BILGER (1993). Vitality was measured as a yield, which represents the essence of fluorescence quenching analysis by the saturation pulse method and calculated according to the equation

$$Yield = \frac{Fm' - Ft}{Fm'}$$

Ft = the parameter which represents the basic fluorescence yield at any given time.
Fm′ = the parameter defined as the maximal fluorescence yield reached in a pulse of saturating light with an illuminated sample.

Histochemical GUS activity assay

ß-glucuronidase (GUS) activity was assayed according to JEFFERSON et al. (1987). Regenerated shoots (five weeks after bombardment in the transformation experiments testing different parameters and 8 weeks after bombardment in the final representative transformation experiment) were immersed in GUS staining solution (0.1M Na_2HPO_4; pH 7.0, 10 mM NaEDTA, 0.5 mM K-ferricyanide, 0.5 mM K-ferrocyanide, 0.1 % (v/v) Triton-X-100, 1 mM X-Gluc (5 bromo-4-chloro-3-indolyl glucuronide) and 20 % (v/v) methanol) (KUSOGI et al., 1990) and vacuum infiltration was applied at 200 mbar for 10 min, then incubated overnight in the dark at 37°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. By this assay qualitative data concerning the specificity of the *gus* gene expression in the tissue were obtained. After the histochemical assay, positive plants were used for PCR analysis.

Fluorometric GUS activity assay

The fluorometric GUS activity assay was performed according to JEFFERSON et al. (1987). For testing different parameters, the assay was performed four weeks after bombardment. In the final representative transformation experiment it was performed 8 weeks after bombardment. Leaf tissue from transformed and non-transformed regenerants was ground to homogeneity with a pestle and mortar in the presence of liquid nitrogen. Tissue was extracted in microcentrifuge tubes with extraction buffer (2 ml g^{-1} ground tissue) containing 50 mM NaH_2PO_4, pH 7.0, 10 mM EDTA, 0.2 % (v/v) Triton X-100 and 10 mM ß-mercaptoethanol, centrifuged to pellet the debris and the supernatant was collected. Protein content of this crude extract was quantified according to BRADFORD (1976), mixed with 500 µl MUG solution (1 mM 4-methylumbelliferyl-ß-glucuronide in 20 % (v/v) methanol). The reaction was carried out in the dark at 37°C for 1 h. and stopped with 400 µl 0.2 M Na_2CO_3. The assays were analyzed in a spectro fluorometer (Fluoro-Max, Spex, Germany) and the fluorescence was recorded at an excitation wavelength of 365 nm and an emission wavelength of 455 nm. The readings were compared to readings of 4-MU standards of varying concentrations and were plotted against time to determine the amount of 4-MU produced. GUS activity was calculated as micromoles of 4-MU formed per mg protein and min.

Plant DNA extraction and polymerase chain reaction analysis

DNA from transformed (positive GUS activity assay), putatively transformed and non transformed plants was extracted according to the CTAB method (DOYLE and DOYLE, 1987; CULLINGS, 1992) and digested with *Eco*RI. Detection of *gus* and *nptII* in these samples was conducted using PCR with the following primers: *gus* primers 5´-ATG TTA CGT CCT GTA GAA AC-3´ and 5´-CTT CAC TGC CAC TGA CCG GA-3´, which were designed to amplify an approximately 830 bp DNA fragment of the *gus* gene and *nptII* primers 5´-ACA AGA TGG ATT GCA AGG-3´and 5´-AAC TCG TCA AGA CGA TAG-3´ which amplify an approximately 800 bp DNA fragment of the *nptII* gene. PCR reaction was performed in 50 µl reaction mix containing 50-100 ng *Eco*RI digested genomic DNA, 5 µl 1.5 mM $MgCl_2$ 5 µl 10 x Taq DNA polymerase buffer, 0.75 µl 0.2 mM dNTP, 12.5 µl 0.25 µM of each primer and 2 U Taq DNA polymerase. As a positive control, the corresponding plasmid was used as a template. DNA samples were denatured for 5 min at 95°C and amplified during 32 cycles, denaturation for 1 min at 95°C, annealing for 1 min at 64°C for *gus* and at 58°C for *nptII*, extension for 1 min at 72°C. Cycling was closed with a final extension step for

10 min at 72°C. Amplified products were separated on a 0.8 % (w/v) agarose gel and the DNA was visualized with ethidium-bromide on a UV transilluminator (SAMBROOK and RUSSEL, 2001) at 305 nm.

Results

Evaluation of different physical and biological parameters of bombardment

Two microcarrier gold particle sizes (1 and 1.6 µm) were used at four particle acceleration pressures (0, 450, 900, 1550 and 1800 psi) in combination with 6 and 9 cm target distance. Moreover, pre-culture durations of the explant (0, 1 and 2 days) and number of bombardments per explant (1 and 2 shots) were studied to support the introduction of DNA with minimal tissue damage or interference with the regeneration potential. Evaluation of these parameters was based on histochemical and fluorometric GUS activity assays coupled with shoot induction frequency (%), which was calculated as number of regenerated explants per total number of explants, and plant cell vitality. These experiments were laid out as a complete randomized design and each treatment was based on four replicates of forty explants.

Effect of gold particle size, particle acceleration pressure and target distance on GUS activity

Fig. 1 and 2 show that the general pattern of response was similar in both tested genotypes (cv. capella and SWSR2) with respect to GUS activity. The highest fluorometric activity value resulted by using 1.6 µm particle size regardless of helium pressure and target distance used. Moreover, there was a direct correlation between the fluorometric GUS activity and the helium pressure up to 1550 psi. When the helium pressure exceeded this value, the fluorometric GUS activity dramatically decreased.

Increasing the target distance to 9 cm induced a reduction in the fluorometric GUS activity with respect to the particle size and the helium pressure used. In comparison, the fluorometric GUS activity of cv. capella and SWSR2 inbred line plants which were bombarded with 1.6 µm particle size and 1550 psi at 9 cm was 3.7 and 2.9 times lower than those bombarded with the same particle size and helium pressure at 6 cm, respectively (Fig. 1A and 2A). Increasing the target distance to 9 cm could not be compensated by the elevation of acceleration pressure with the use of any particle size.

A helium pressure of 1550 psi in combination with a target distance of 6 cm and 1.6 µm particle size resulted in the highest *gus* expression frequency which amounted to 33.3 and 30.8 % for cv. capella and SWSR2 inbred line, respectively (Fig. 1B and 2B). There was no *gus* expression observed in the plants which were bombarded at 9 cm and 450-900 psi using either particle size of both genotypes.

Effect of gold particle size, particle acceleration pressure and target distance on plant regeneration and cell vitality

The cell vitality of the bombarded tissues appeared to be inversely related to the helium pressure at either of the two target distances (Fig. 1C and 2C). At the highest helium pressure (1800 psi) the bombarded tissues with 1.6 µm particle size at 6 cm distance were extensively damaged in both genotypes.

Shoot induction frequency was also affected by varying helium pressure. This effect was more pronounced at 6 cm target distance with 1.6 µm particle size. Using a combination of 1.6 µm particle size, 6 cm target distance and 1800 psi the shoot induction frequency was decreased to 12.5 and 7.5 % of cv. capella and SWSR2 inbred line, respectively (Fig. 1D and 2D).

Considering the overall effects of acceleration pressure on the GUS activity as well as the cell vitality and the tissue culture response, a helium pressure of 1550 psi, combined with 6 cm target distance and 1.6 µm particle size was found to be acceptable for bombardment of sunflower split shoot apices.

Effect of pre-culture duration and number of shots per explant on GUS activity

All the hitherto optimized parameters (shown above) were applied in subsequent transformation experiments. These experiments were designed to find out the effect of pre-culture duration and number of shots per explant on the transformation events as well as cell vitality and tissue culture response.

As presented in Fig. 1E and 2E, the bombardment of the explants for two times resulted in the highest levels of GUS activity in the fluorometric assay. In comparison, when the explants were bombarded twice after one day of pre-culture, an increase in GUS activity of 1.6 and 2.1 fold was achieved in cv. capella and SWSR2 inbred line, respectively, compared to explants bombarded one time.

Moreover, pre-culture duration also affected GUS activity. When explants were cultured for one day prior to bombardment, higher values in the fluorometric assay were obtained compared to 0 and 2 days of culture.

In terms of the histochemical GUS activity assay, the *gus* expression decreased by19.6 and 53 % with increasing the pre-culture duration to 2 days in cv. capella and SWSR2 inbred line, respectively, when explants were bombarded one time. Meanwhile, there was a variation in the general pattern of *gus* expression between the two genotypes when the explants were bombarded twice. The lowest *gus* expression frequency was 12.5 % in cv. capella and resulted from pre-culture of the explants for one day prior to the double bombardment. Conversely, the highest *gus* expression frequency was 25 % in SWSR2 inbred line and resulted from the same condition (Fig. 1E and 2E).

Effect of pre-culture duration and number of shots per explant on plant regeneration and cell vitality

Influence of pre-culture duration and number of shots per explant on the shoot induction and the cell vitality is illustrated in Fig. 1F and 2F. Shoot induction frequency and the cell vitality increased in dependency of pre-culture duration. Number of bombardment per explant affected shoot induction frequency as well as the cell vitality. Cv. capella was highly affected by the number of shots per explant compared to SWSR2 inbred line.

In conclusion, on the basis of the results obtained during the optimization experiments, the optimized bombardment conditions are: 1550 psi of acceleration pressure in combination with 6 cm target distance, 1.6 µm gold particle size and pre-culture the explants for one day prior to a double bombardment.

Estimation of transformation frequency

All the resulting optimal parameters were applied in final representative transformation experiments using 95 split shoot apices of cv. capella and 110 of SWSR2 inbred line. Regenerated plantlets 8 weeks after bombardment were subjected to histochemical and fluorometrical GUS activity assay as well as to molecular analysis. The transformation frequency was calculated on the basis of PCR analysis and recorded as a percentage of the total number of co-cultivated explants.

As shown in Tab. 1 the recorded number of *gus* expressing plants was 1 and 4 in cv.capella and SWSR2 inbred line, respectively. *gus*

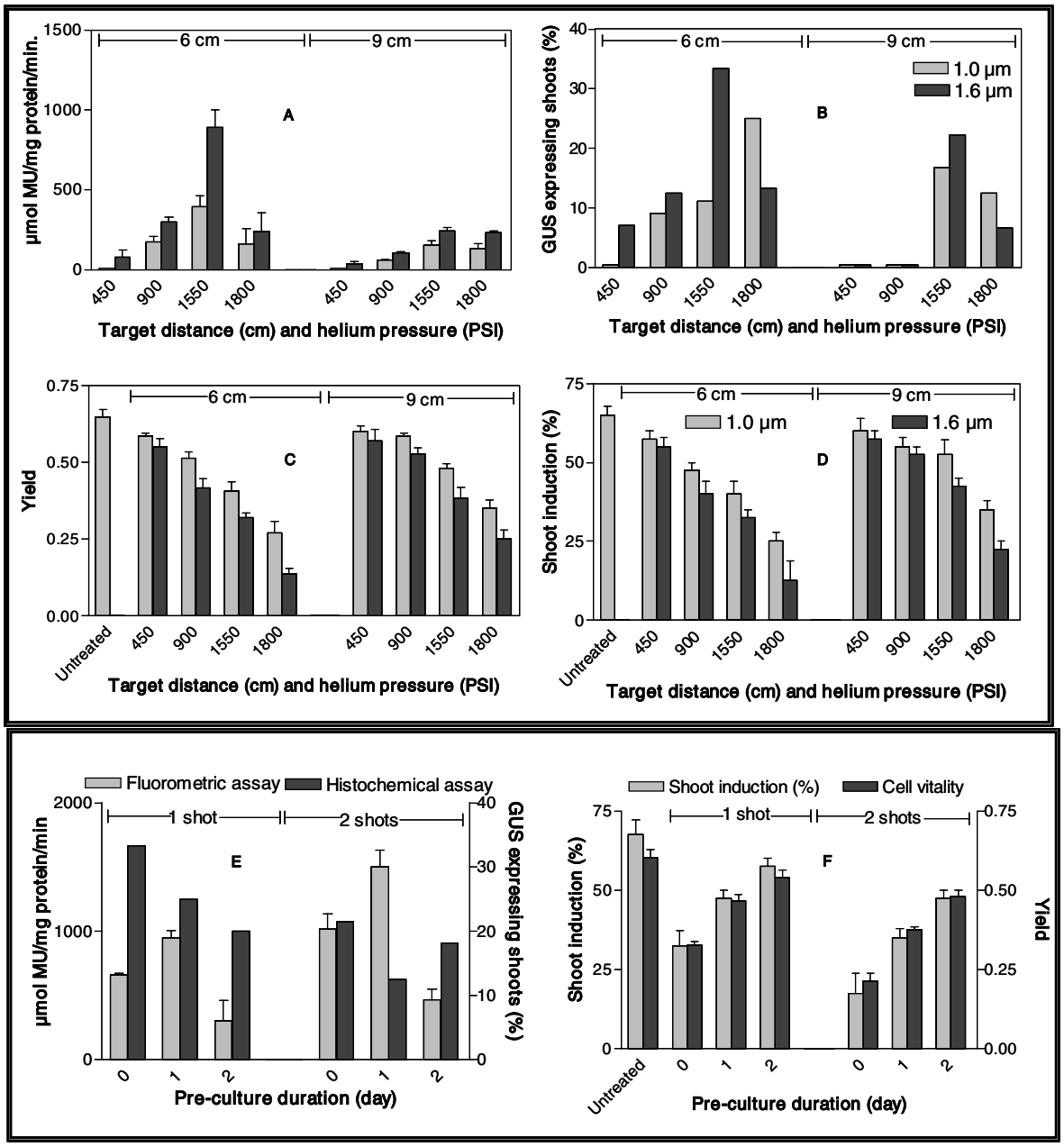

Fig. 1: Assessment of different particle bombardment parameters affecting the transformation efficiency of split shoot apices for high oleic *H. annuus* L. cv. capella. A - D: different target distances, different helium pressures and different gold particle sizes and its effect on (A) *gus* expression level, (B) GUS activity in the histochemical assay, (C) vitality and (D) shoot induction frequency. The bombardment were applied without pre-culture of the explants and a single shot per plate. E - F: different pre-culture durations and numbers of shots per plate and its effect on (E) GUS activity and (F) shoot induction frequency and vitality. Here, bombardments were applied under 6 cm distance between macrocarrier assembly and target plate using 1.6 µm gold particles and 1550 psi helium pressure. Results are data of at least three replicates and error bars represent the SE. For protocol details see materials and methods.

expression was complete and uniform in the transformed plants, whereas no expression was detected in the untreated plants (control) (see Fig. 3).

The fluorometric GUS activity assay results reflect that SWSR2 inbred line had a slightly higher average *gus* expression than the cv. capella. The fluorometric value of SWSR2 was 1723.2 µmol MU mg protein^{-1} min^{-1}. whereas the corresponding value recorded from cv. capella was 1509.6 µmol MU mg protein^{-1} min^{-1} (Tab. 1).

The specific amplified fragments of 830 and 800 bp for *gus* and *nptII*, respectively, were amplified in the transformed regenerated plantlets of cv. capella and SWSR2 inbred line (Fig. 4), whereas no amplified band was detected in the non-transformed plants. From 30 and 40 tested PCR plants 3 and 5 plants were positive with either of the two primers of cv. capella and SWSR2 inbred line, respectively (Tab. 1). This indicates that some of the PCR positive plants did not express the *gus* gene. The transformation frequency of cv. capella was 3.1 %, while the corresponding frequency of SWSR2 was 4.5 % in relation to the total number of explants used in the experiment.

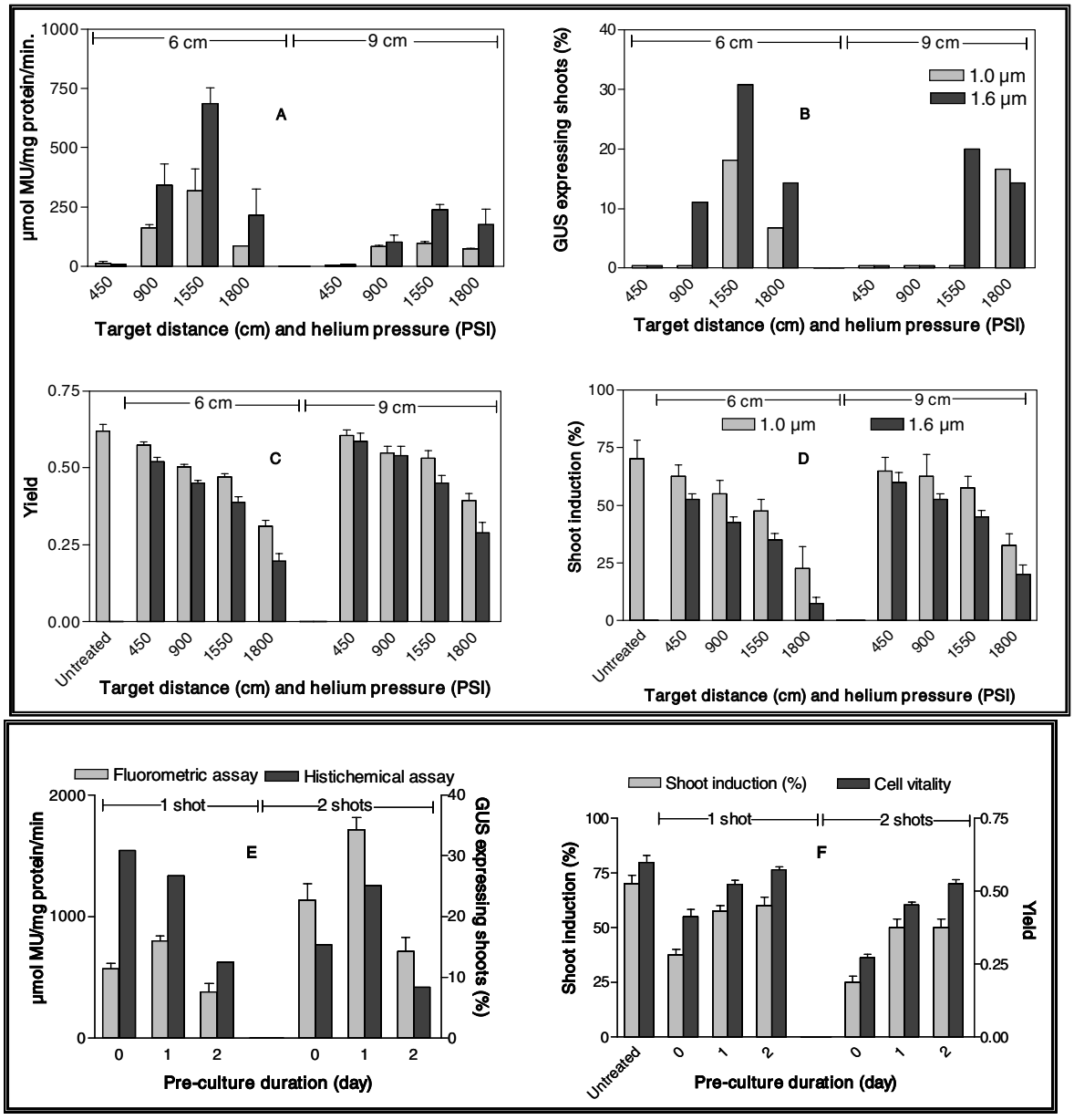

Fig. 2: Assessment of different particle bombardment parameters affecting the transformation efficiency of split shoot apices for high oleic *H. annuus* L. SWSR2 inbed line. A - D: different target distances, different helium pressures and different gold particle sizes and its effect on (A) *gus* expression level, (B) GUS activity in the histochemical assay, (C) vitality and (D) shoot induction frequency. The bombardments were applied without pre-culture of the explants and a single shot per plate. E - F: different pre-culture durations and numbers of shots per plate and its effect on (E) GUS activity and (F) shoot induction frequency and vitality. Here, bombardments were applied under 6 cm distance between macrocarrier assembly and target plate using 1.6 µm gold particles and 1550 psi helium pressure. Results are data of at least three replicates and error bars represent the SE. For protocol details see materials and methods.

Discussion

The ability to accelerate DNA-coated particles directly into intact tissue by particle bombardment technique has expanded the range of organisms that can be genetically transformed. However, the efficiency of microparticle bombardment depends on a large number of physical, biological and environmental factors (CHRISTOU, 1992; SANFORD et al., 1993; KOPREK et al., 1996; RASCO-GAUNT et al., 1999; BHAT et al., 2001; BHATNAGAR et al., 2002; TADESSE et al., 2003), which have to be optimized for every plant tissue.

In the present investigation different physical and biological factors were optimized to obtain an efficient biolistic gene transfer protocol for high oleic sunflower genotypes. The results showed that the highest *gus* expression values resulted from using 1.6 µm gold particle size in both tested genotypes. The same size of gold particle size was successfully employed in the transformation of sunflower cotyledons (VISCHI et al., 1999), buffel grass calli (BHAT et al., 2001) and sorghum shoot tip and immature embryo (TADESSE et al., 2003). Changes in helium pressure were found to affect the level of the gene expression. There was a direct proportion between the *gus* expression and the helium pressure up to 1550 psi. This could be related to high penetration rate of the gold particles. On other hand,

Tab. 1: Summary of transformation results of split shoot apices from high oleic *H. annuus* L. genotypes cv. capella and SWSR2 using biolistic gene transfer method.

Genotype	cv. capella	SWSR2
Total number of used explants	95	110
Number of positive plants in fluorometric GUS activity assay	1	4
Gus frequency* (%)	1,1	3.6
Activity in the fluorometric assay (µmol MU mg protein^{-1} min^{-1}). Mean ± SE	1509.6±130.8	1723.2±101.3
Number of plants tested with PCR	30	40
Number of PCR-positive plants	3	5
Transformation frequency* (%)	3.1	4.5

(*) The percentage was calculated in relation to the total number of bombarded explants and PCR was performed with *gus* and *nptII* primers 14-16 weeks after bombardment. Transformation frequency was calculated on the basis of positive PCR plants.

a higher pressure (1800 psi) caused a dramatically reduction in the *gus* expression due to the decreased vitality of the explants.
Eventually, the cell vitality was affected by varying helium pressure and was inversely related to it. These results were related to the penetration of those heavy metal particles into intact cells or tissues which may provoke various levels of tissue wounding that can have various effects on subsequent plant regeneration (TADESSE et al., 2003). The data explain that the enhanced cell damage with increasing the helium pressure resulted in decrease of the *gus* expression. Our results and explanation were in accordance with GAUNT et al. (1999), BHAT et al. (2001), BHATNAGAR et al. (2002) and TADESSE et al. (2003).
In general, increase of the target distance to 9 cm induced a reduction in the GUS activity regardless to the particle size and the helium pressure used. Similarly, TADESSE et al. (2003) bombarded different types of sorghum explants at 6 cm target distance and reported that increasing the target distance could not be compensated by the elevation of acceleration pressure in any of the explants. Thus, a helium pressure of 1550 psi in combination with a target distance 6 cm and 1.6 µm particle size resulted in the highest *gus* transformation frequency.

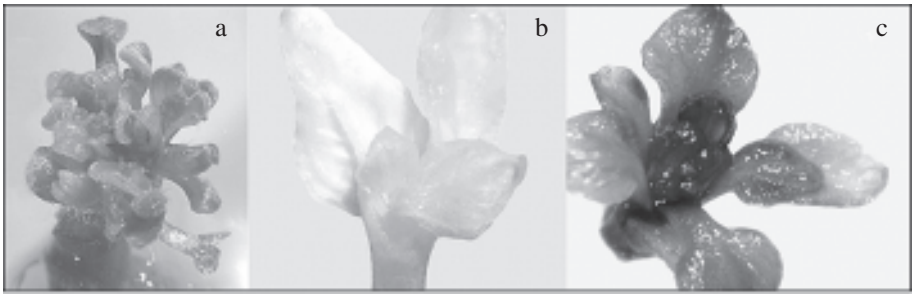

Fig. 3: Histochemical GUS assay of two high oleic sunflower genotypes, cv. capella and SWSR2 inbred line. (a) Multiple shoots from direct shoot apices regeneration on SIM2 medium, (b) Untreated shoot and (c) GUS expressing shoot after GUS staining assay.

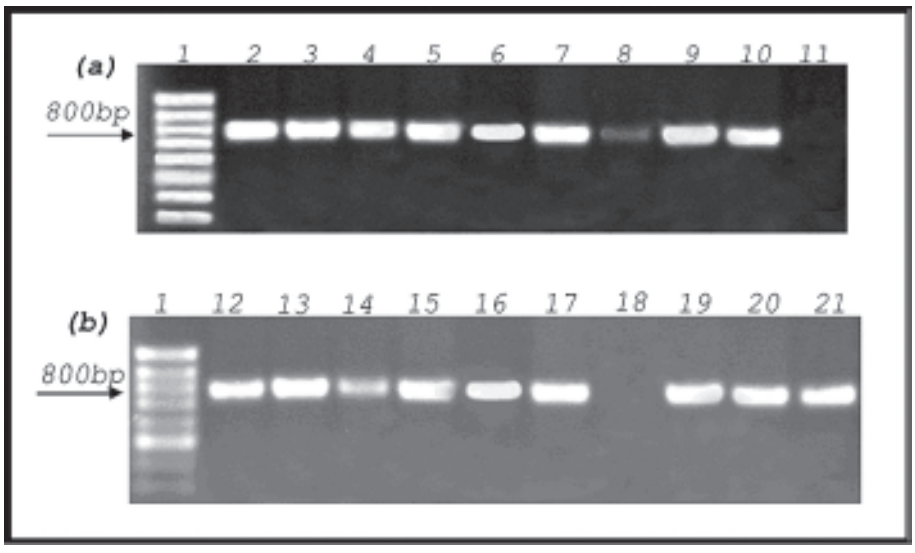

Fig. 4: PCR analysis of transformed plants of high oleic *H. annuus* L. genotypes, cv. capella and SWSR2 inbred line, 14-16 weeks after co-cultivation. 100 ng of *EcoRI* digested genomic DNA was amplified with specific primers to *gus* and *nptII* genes. (a) with *gus* primers and (b) with *nptII* primers, lane (1) molecular marker DNA, lane (2-6 and 12-16) transformed SWSR2 inbred line plants, lane (7-9 and 19-21) transformed cv. capella plants, lane (10, 17) positive control (plasmid DNA) and lane (11, 18) negative control (untreated plants).

In an evaluation of the number of shots per explant, the results show that bombarding the explants two times resulted in the highest levels of GUS activity. These results were in agreement with PEREIRA and ERICKSON (1995), SCHÖPKE et al. (1997) and BHATNAGAR et al. (2002) who revealed that double bombardment of the same tissue increased the number of transformed explants.

Moreover, our results clearly demonstrated the importance of pre-culture phase which seems to be a general feature with the current technology (HARWOOD et al., 1995; HUNOLD et al., 1995; PEREIRA and ERICKSON, 1995; NEHLIN et al., 2000). When explants were cultured for one day prior to bombardment, the highest *gus* expression frequency was obtained compared to 0 and 2 days of culture. We hyopothesize that the increase of the *gus* expression after 1 day of pre-culture is due to the reduction of bombardment shock and, consequently, to tissue injury. FOLLING and OLESEN (2002) suggested that the positive effect of pre-culture on transformation efficiency of wheat could be related to the easiness by which plasmid DNA reaches the chromosomes as well as to the damaging effect of the bombardment.

Therefore, a helium pressure of 1550 psi in combination with a target distance of 6 cm, 1.6 µm gold particle size, bombardment the explants twice and pre-culture the explants for one day prior to bombardment were found to be a compromise between cell vitality and *gus* expression frequency.

For an estimation of the resulting transformation frequency the optimized parameters were applied on split shoot apices of cv. capella and SWSR2 inbred line and the plants were subjected to histochemical, fluorometric and molecular analysis.

With regard to histochemical analysis, two plants of cv. capella and one plant of SWSR2 inbred line did not express the *gus* gene despite of their positive PCR results. We suggest that gene silencing took place which possibly resulted from the interaction among the multiple integrated copies of transgene (SANFORD, 1990; KUMPATLA et al., 1997) or when additional copies of an endogenous gene are expressed ectopically involves homology dependent gene silencing (HDGS) (REDDY et al., 2003) or DNA methylation (AL-KAFF et al., 2000; REDDY et al., 2003). Similar results were previously observed in potato (OTTAVIANI et al., 1993), pearl millet (LAMBÉ et al., 1995) and soybean (REDDY et al., 2003).

The fluorometric GUS activity and the transformation frequency results reflected that the SWSR2 inbred line showed a higher response than cv. capella for the present transformation, since the transformation frequency amounted to 3.1 and 4.5 % for cv. capella and SWSR2 inbred line, respectively. In accordance, stable transformation was achieved via particle bombardment for different plant species such as alfalfa (PEREIRA and ERICKSON, 1995), cassava (ZHANG et al., 2000), potato (ROMANO et al., 2001), barley (MANOHARAN and DAHLEEN, 2002), wheat (CHUGH and KHURANA, 2003), orchid (MEN et al., 2003), soybean (REDDY et al., 2003), sorghum (TADESSE et al., 2003) and rice (CHO et al., 2004). Additionally, particle bombardment appears to be the best technique for gene transfer into conifers (HUMARA et al., 1999).

Finally, the results showed for the first time that the biolistic method can be successfully combined with direct regeneration system (avoiding dedifferentiated cell stages) in producing high oleic *H. annuus* L. plants.

Acknowledgements

The authors want to thank Mrs. Gröhne (Südwestsaat) for the provision of seeds of cv. capella and SWSR2 inbred line. This project was supported by the egyptian gouvernment (grants to Sh. M.) which is greatfully acknowledged.

References

AL-KAFF, N.S., KREIKE, M.M., COVEY, S.N., PITCHER, R., PAGE, A.M., DALE, P.J., 2000: Plants rendered herbicide-susceptible by cauliflower mosic virus-elicited suspension of a 35S promoter-regulated transgene. Nat. Biotechnol. 18, 995-999.

BHAT, V., DALTON, S.J., KUMAR, S., BHAT, B.V., GUPTA, M.G., MORRIS, P., 2001: Particle-inflow-gun-mediated genetic transformation of buffel grass (*Cenchrus ciliaris* L.): optimizing biological and physiological parameters. J. Appl. Genet. 42, 405-412.

BHATNAGAR, S., KAPUR, A., KHURANA, P., 2002: Evaluation of parameters for high efficiency gene transfer via particle bombardment in Indian mulberry. Indian J. Exp. Biol. 40, 1387-1392.

BIDNEY, D., 1990: Expression of ß-glucuronidase in sunflower apical meristems following microprojectile bombardment. In: Abstracts 7th International Congress on Plant Tissue and Cell Culture, Amsterdam.

BRADFORD, M.M., 1976: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

CHO, M.J., YANO, H., OKAMOTO, D., KIM, H.K., JUNG, H.R., NEWCOMB, K., LE, V.K., YOO, H.S., LANGHAM, R., BUCHANAN, B.B., LEMAUX, P.G., 2004: Stable transformation of rice (*Oryza sativum* L.) via microprojectile bombardment of highly regenerative green tissues derived from mature seed. Plant Cell Rep. 22, 483-489.

CHRISTOU, P., FORD, T.L., KOFRON, M., 1992: The development of a variety-independent gene-transfer method for rice. Trends Biotech. 10, 239-246.

CHUGH, A., KHURANA, P., 2003: Regeneration via somatic embryogenesis from leaf basal segments and genetic transformation of bread and emmer wheat by particle bombardment. Plant Cell Tiss. Organ Cult. 74, 151-161.

CULLINGS, K.W., 1992: Design and testing of a plant-specific PCR primer for ecological and evolutionary studies. Mol. Ecol. 1, 233-240.

DORRELL, G., VICK, A., 1997: Properties and processing of oilseed sunflower. In: Schneiter, A.A. (ed.), Sunflower Technology and Production Agronomy, 709-745. Madison, Wisconsin, USA.

DOYLE, J.J., DOYLE, J.L., 1987: A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bul. 19, 11-15.

FICK, M., MILLER, F., 1997: Sunflower Breeding In: Schneiter, A.A. (ed.), Sunflower Technology and Production Agronomy, 395-495. Madison, Wiscosin, USA.

FOLLING, L., OLESEN, A., 2002: Transformation of wheat (*Triticum aestivum* L.) microspore-drived callus and microspores by particle bombardment. Plant Cell Rep. 20, 1098-1105.

HARWOOD, W.A., BEAN, S.J., CHEN, D.F., MULLINEAUX, P.M., SNAPE, J.W., 1995: Transformation studies in *Hordum vulgare* using a highly regenerable microspore system. Euphytica 85, 113-118.

HUMARA, J.M., LÓPEZ, M., ORDAS, R.J., 1999: *Agrobacterium tumefaciens*-mediated transformation of *Pinus pinea* L. cotyledons: an assessment of factors influencing the efficiency of *uidA* gene transfer. Plant Cell Rep. 19, 51-58.

HUNOLD, R., BURRUS, M., BRONNER, R., DURET, J., HAHNE, G., 1995: Transient gene expression in sunflower (*Helianthus annuus* L.) following microprojectile bombardment. Plant Sci. 105, 95-109.

JEFFERSON, R.A., KAVANAGH, T.A., BEVAN, M.W., 1987: GUS fusions: ß-glucuronidase as sensitive and versatile gene fusion marker in higher plants. EMBO J. 6, 3901-3907.

KOPREK, T., HÄNSCH, R., NERLICH, A., MENDEL, R.R., SCHULZE, J., 1996: Fertile transgenic barley of different cultivars obtained by adjustment of bombardment conditions to tissue response. Plant Sci. 119, 79-91.

KUMPATLA, S.P., TENG, W., BUCHHOLZ, W.G., HALL, T.C., 1997: Epigenetic transcriptional silencing and 5-azacytidine-mediated reactivation of complex transgene in rice. Plant Physiol. 115, 361-373.

KUSOGI, S., OHASHI, Y., NAKAJIMA, K., ARAI, Y., 1990: An improved assay for ß-glucuronidase in transformed cells: Methanol almost compeletly suppresses a putative endogenous ß-glucuronidase activity. Plant Sci. 70, 133-140.

Lambé, P., Dinant, M., Matagne, R., 1995: Differential long-term expression and methylation of the hygromycin phosphotransferase (hph) and β-glucuronidase (GUS) genes in transgenic pearl millet (*Pennisetum glaucum*) callus. Plant Sci. 108, 51-62.

Laparra, H., Burrus, M., Hunold, R., Damm, B., Bravo-Angel, A.M., Bronner, R., Hahne, G., 1995: Expression of foreign genes in sunflower (*Helianthus annuus* L.) evaluation of three gene transfer methods. Euphytica 85, 63-74.

Lonsdale, D., Ontec, S., Cuming, A., 1990: Transient expression of exogenous DNA in intact viable wheat embryos following particle bombardment. J. Exp. Bot. 41, 1161-1165.

Malone-Schoneberg, J., Scelonge, C.J., Burrus, M., Bidney, L., 1994: Stable transformation of sunflower using *Agrobacterium* and split embryonic axis explants. Plant Sci. 103, 199-207.

Manoharan, M., Dahleen, L.S., 2002: Genetic transformation of the commercial barley (*Hordeum vulgare L.*). Plant Cell Rep. 21, 76-80.

Men, S., Ming, X., Wang, Y., Lui, R., Wei, W., Li, Y., 2003: Genetic transformation of two species of orchid by biolistic bombardment. Plant Cell Rep. 21, 592-598.

Mohamed, Sh., Binsfeld, P.C., Cerboncini, C., Schnabl, H., 2003: Regeneration systems at high frequency from high oleic *Helianthus annuus* L. genotypes. J. App. Bot. 77, 85-89.

Murashige, T., Skoog, F., 1962: A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15, 473-497.

Nehlin, L., Müller, C., Bergman, P., Glimelius, K., 2000: Transient *gus* and *gfp* gene expression and viability analysis of micro-projectile bombarded microspores of *Brassica napus* L. J. Plant Physiol. 156, 175-183.

Ottaviani, M.P., Smits, T., Hänisch, C.H., 1993: Differential methylation and expression of the β-glucuronidase and neomycin phosphotransferase genes in transgenic plants of potato cv Bintje. Plant Sci. 88, 73-81.

Pereira, L.F., Erickson, L., 1995: Stable transformation of alfalfa (*Medicago sativa* L.). Plant Cell Rep. 14, 290-293.

Potrykus, I., 1990: Gene transfer to cereals: an assessment. Biotechnol. 8, 535-542.

Rasco-Gaunt, S., Riley, A., Barcelo, P., Lazzeri, P.A., 1999: Analysis of particle bombardment parameters to optimize DNA delivery into wheat tissues. Plant Cell Rep. 19, 118-127.

Reddy, M.S., Dinkins, R.D., Collins, G.B., 2003: Gene silencing in transgenic soybean plants transformed via particle bombardment. Plant Cell Rep. 21, 676-683.

Romano, A., Raemakers, K., Visser, R., 2001: Transformation of potato (*Solanum tuberosum)* using particle bombardment. Plant Cell Rep. 20, 198-204.

Sambrook, J., Russell, D.W., 2001: Molecular cloning – a laboratory manual. Cold Spring Harbor Laboratory Press, New York.

Sanford, J.C., 1990: Biolistic plant transformation. Physiol. Plant. 79, 206-209.

Sanford, J.C., Smith, F.D., Russell, J.A., 1993: Optimizing the biolistic process for different biological application. Methods Enzymol. 217, 483-509.

Schöpke, C., Taylor, N.J., Cárcamo, R., Beachy, R.N., Fauquet, C., 1997: Optimization of parameters for particle bombardment of embryonic suspension cultures of cassava (*Manihot esculenta* Crantz) using computer image analysis. Plant Cell Rep. 16, 526-530.

Schreiber, U., Bilger, W., 1993: Progress in chlorophyll fluorescence research: Major developments during the past years in retrospect. In: Lüttge, U., Ziegler, H. (eds.), Progress in Botany 54, 151-153. Springer Verlag, Berlin.

Tadesse, Y., Sági, L., Swennen, R., Jacobs, M., 2003: Optimisation of transformation conditions and production of transgenic sorghum (*Sorghum bicolor*) via particle bombardment. Plant Cell Tiss. Organ Cult. 75, 1-18.

Vischi, M., Marchetti, G., Quagliaro, O., Olivieri, A.M., 1999: A focusing device for biolistic transformation of sunflower (*Helianthus annuus* L.) cotyledons. Helia 22, 71-80.

Weber, S., Friedt, W., Landes, N., Molinier, J., 2003: Improved *Agrobacterium*-mediated transformation of sunflower (*Helianthus annuus* L.): assessment of macerating enzymes and sonication. Plant Cell Rep. 21, 475-482.

Zhang, F.L., Takahata, Y., Watanabe, M., Xu, J.B., 2000: *Agrobacterium*-mediated transformtion of cotyledonary explants of Chinese cabbage (*Brassica campestris* L. ssp. *Pekinensis*). Plant Cell Rep. 19, 569-575.

Address of the authors:
Institut für Molekulare Physiologie und Biotechnologie der Pflanzen, Universität Bonn, Karlrobert-Kreiten-Straße 13, D-53115 Bonn