A modified protocol for laser- mediated gene transfer in wheat

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ABSTRACT

A modified laser-mediated setup for introducing exogenous DNA (pAB$_6$ plasmid with GUS and bar genes) into cells of embryogenic calli of the Egyptian wheat (Triticum aestivum L.) cv. Giza 164 was done. The new setup secures the transformation as high as 400,000 embryo-derived cells in less than 35 min using a homemade UV excimer laser with two dimensional translation stages, a suitable computer program and a proper optical device. Immature embryos of wheat were grown for six days on TW medium. Osmotic treatment was done by using mannitol (0.4 M) mixed with the exogenous DNA, in which laser treatment was immediately conducted. The calli were irradiated by a focused laser microbeam to puncture holes ~ 0.5 μm in the cell wall and membrane to allow uptake of the exogenous DNA. Three regenerated putative transgenic events were evaluated for the presence and expression of both genes and results indicated that this modified procedure of laser-mediated transformation can be successfully used in transforming wheat with a very high efficiency.

Keywords: *Laser microbeam, wheat transformation, immature embryo, exogenous DNA*

INTRODUCTION

Wheat (*Triticum aestivum* L.) is considered as the most important field crop worldwide. Conventional plant breeding in wheat has offered a great deal towards the improvement of flour quality and resistance to biotic and abiotic stresses (Potrykus, 1990). The accessibility of tissue culture to improve cereal characteristics through genetic engineering in wheat is limited due to the low rate of regeneration of transformed calli (Bahieldin *et al.*, 2000). Attempts to develop a novel plant transformation system to secure the least damage to transformed cells developed by other mechanical transformation devices are needed to allow for the recovery of transformed cells and the stable transgene integration and expression. Therefore, we report an effective, less damaging system for introducing exogenous DNA into cells of embryogenic wheat calli using laser microbeam cell surgery (Guo *et al.*, 1995).

MATERIALS AND METHODS

UV excimer laser system setup

A modified laser microbeam setup to that of Guo *et al.* (1995) was used in which a Lambda Physics Excimer Laser device (193 nm wavelength, 6 ns pulse duration, 13 mJ energy and repetition rate up to 200 Hz) was constructed. The mechanical system was developed with two Oriel steper motors in the X-Y directions to allow a lateral motion of 8-

40 microns with a time interval matching the laser repetition rate. A suitable software was used for controlling the mechanical system, the optical device (the inverted microscope connceted to a CCD camera) and the output parameters of the excimer laser.

Wheat transformation

Immature embryo-derived calli of the wheat cv. Giza 164 were used as recipient cells for genetic transformation with the eukaryotic expression vector pAB_6 (Figure 1) harboring the β-glucuronidase gene (GUS or *uidA*) under the control of the rice Act1 promotor and the *bar* gene as a selectable marker gene for basta herbicide resistance driven by the cauliflower mosaic virus (*CaMV*) 35S promotor, with the maize *Adh1* intron in the 5′ non-translated region. The NOS 3' terminator was used to terminate transcription of GUS as well as *bar* gene. The *bar* gene encodes the enzyme phosphinothricin acetyl transferase (PAT), which inactivates phosphinothricin, the active ingradient of the herbicide basta. Immature embryo excision, callus induction and cell regeneration and selection conditions were done as previously described (Sivamani *et al.*, 2000). Calli were transferred to liquid TW medium (Weeks *et al.* 1993) with mannitol (0.4 M) and plasmid DNA (5 ug/100 ul medium). Immediately, laser transformation was done and calli were left on the same medium over night, then transferred to solid TW medium for recovery. To allow uptake of the exogenous DNA, a laser microbeam was focused to puncture holes ~0.5 μm in the cell wall and membrane. Plantlets recovered from the regenerated transformed cells were evaluated for transgene presence and expression.

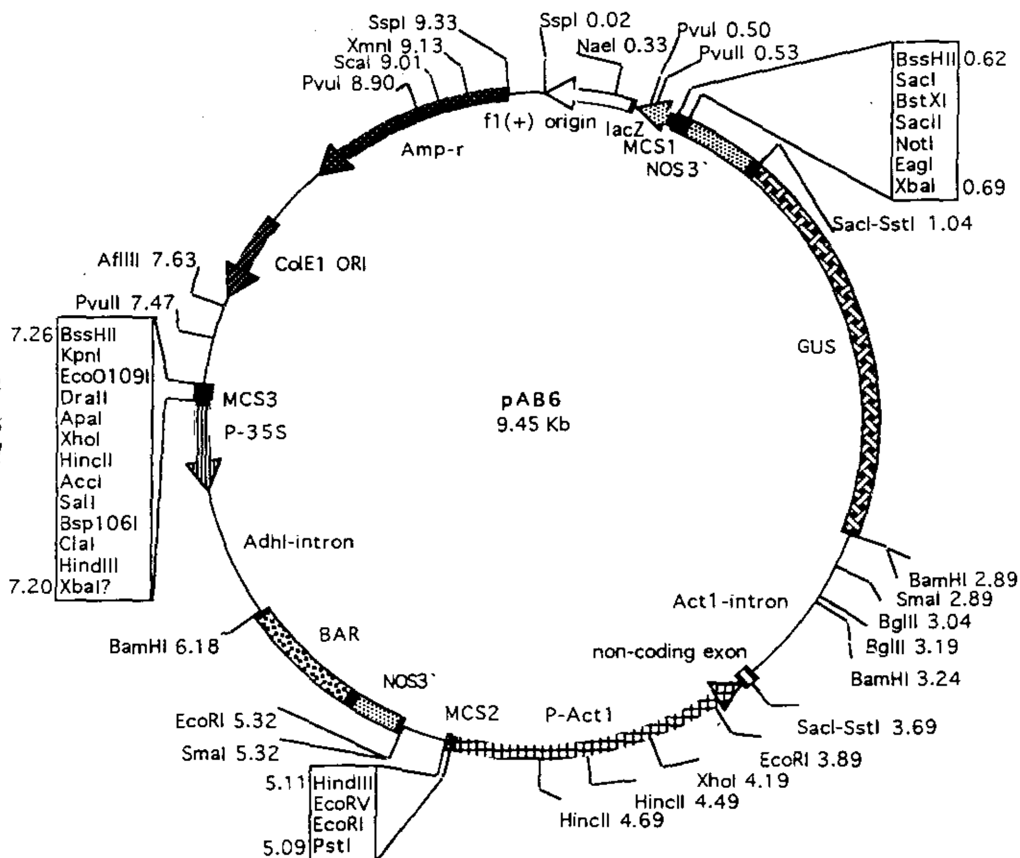

Fig. (1): Restriction map of pAB_6 with GUS and bar genes.

GUS histochemical assay

Histochemical localization of the GUS activity in the laser-irradiated tissues was performed on embryo-derived calli seven days after laser treatment according to the method described by Jefferson *et al.* (1987).

Polymerase chain reaction

DNeasy™ Plant Mini kit was used for DNA isolation as described by the manufacturer (Qiagen Inc., cat. no. 69104) from the recovered putatively transgenic plantlets. PCR reaction was conducted to ensure the presence of the genes of interest in the genomic DNA of the putatively transformed plantlets.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from putatively transformed plantlets using Total RNA Isolation System (Qiagen Inc., cat. no. 74104). RT-PCR was carried out using the Titan One Tube RT-PCR system (Roche, cat. no. 1888382). PCR and RT-PCR reactions were done using oligonucleotide primers specific for GUS and *bar* genes. M refers to 1 kb DNA ladder (Stratagen, cat. no. 201115). Primers used for either PCR or RT-PCR to recover full-length *bar* as well as GUS gene had the following sequences: bar (forward) 5' AAAAGCTTCCATGAGCCCAGAACGACG 3', bar (reverse) 5' AAGGATCCTCAGATCTCGGTGACGG 3', GUS (forward) 5' CCAGATCTAACAATGGCGGGTGGTCAG TCCC 3', GUS (reverse) and 5' CCAGATCTATTCATTGTTTGCCTCCCTG CTGC 3'.

RESULTS AND DISCUSSION

Great efforts have been made in the development of gene transfer systems in plant. Although many approaches, such as viral vectors (Ahlquist *et al.*, 1987), *Agrobacterium* (Graves and Golgmann, 1986), Liposomes (Ahokas, 1987), biolistics or particle gun (Sanford, 1990), microinjection (Neuhaus *et al.*, 1990) and electroporation (Lindsay and Jones, 1990), have been developed for gene transfer into higher plant cells or protoplasts. Considerable improvement is needed especially, for cereal species (Potrykus, 1990). Recently, the use of laser has been extended to introduce foreign DNA into cells or organelles (Guo *et al.*, 1995).

Callus GUS histochemical assay seven days after transformation was done to avoid the transient presence of the GUS gene. It was evident that transformed cells were homogenously distributed across the scutellum side of the embryo-derived calli (Figure 2). No severe mechanical damages or malformations were observed in transformed calli in which regeneration effeciency of transformed cells was comparable to that of the non transformed cells and the recovered plantlets had normal agronomic performance (data not shown). This laser microbeam puncture method has recently been utilized for the introduction of chitinase and glucanase genes for sclerotia resistance in rapeseed (*Brassica napus* L.) and results were promising (Chen *et al.*, 1999). Up to our knowledge, no more recent reports for introducing genes of economic importance in plants have been published. Our going on research activity showed prelminarly that the efficiency of transformation is very high. Fig.(2) shows that the size and homogenity of the blue coloured area indicated that most cells are transformed since there are no white areas (negative areas) of untransformed cells. The transformation done using the introduced herein system was carried out with the rate of one single laser pulse per cell for more than 120,000 cells for each treated sample containing up to 6 calli.

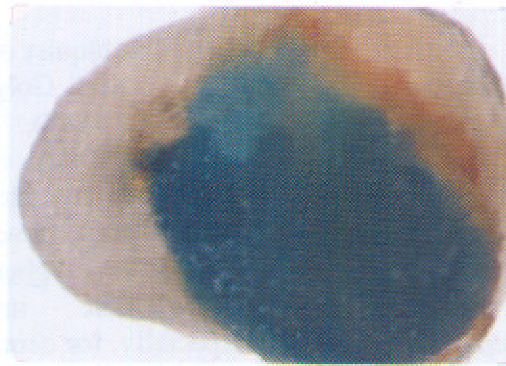

Fig. (2): GUS histochemical assay in wheat immature embryo-derived callus seven days after laser microbeam treatment.

Results of PCR and RT-PCR to confirm the presence and expression, respectively, of the two transgenes with the expected band sizes (1.8 and 0.54 kb for GUS and *bar* genes, respectively) in the the genomic background of the three putatively transformed plantlets, shown in Figures (3 and 4) respectively, indicated that both genes were expressed in two out of them. Use of PCR and RT-PCR as indicators for the presence and expression of foreign genes has been reported by many investigators (Hamill *et al.*, 1991; Bahieldin *et al.*, 2000; Wang *et al.*, 2000).

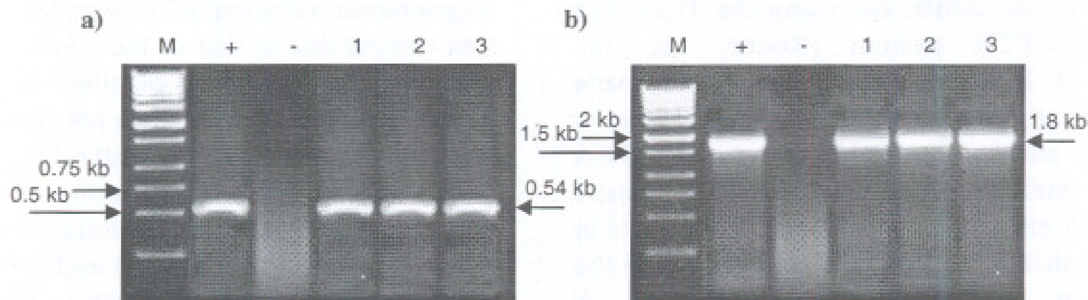

Fig. (3): PCR products for bar (0.54 kb, a) and GUS (1.8 kb, b) genes of three putatively transformed plantlets (lanes 1, 2 and 3). + = positive control, - = negative control, M = 1 kb DNA ladder (Stratagene).

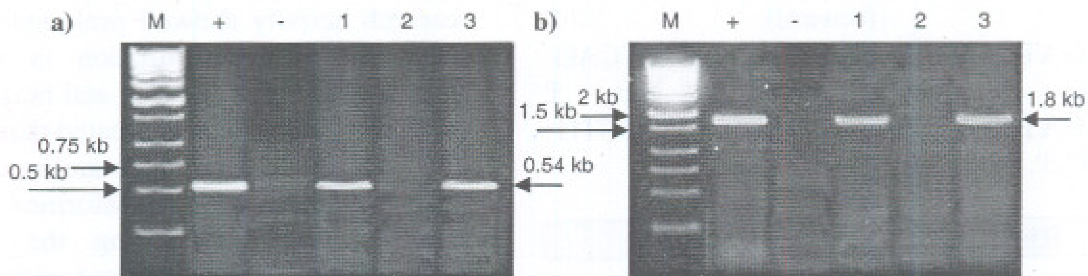

Fig. (4): RT-PCR products for bar (0.54 kb, a) and GUS (1.8 kb, b) genes of three putatively transformed plantlets (lanes 1, 2 and 3). + = positive control, - = negative control, M = 1 kb DNA ladder (Stratagene).

By applying the opto-mechanical laser setup reported herein, it was possible to introduce foreign DNA into as high as 400,000 cells in less than 35 minutes. This setup allows the handling of individual cells with atmost precision and minimal damage and the possible use of different parameters (energy/pulse, pulse duration, repetition rate (frequency Hz), wavelength, etc.) which can be optimized for different plant species as well as other organisms such as bacteria, fungi and animal cells.

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الملخص العربي

الليزر – وسيط لنقل الجينات في القمح السداسي *Triticum aestivum L.*

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تم بنجاح تطبيق نظام متكامل لنقل ألدنا البلازميدي المحتوي على جينات واسمة و منتخبة (GUS & *bar*) إلى خلايا الكالس المستولد من الأجنة غير ناضجة للقمح السداسي (الخبز) *Triticum aestivum L.* صنف جيزة 164 . باستخدام ليزر نبضي في منطقة الأشعة فوق البنفسجية بمدي زمني مقداره 6 نانو ثانية . ونبدأ العملية بانماء الاجنة غير الناضجة علي بيئة TW يليها تعريض الكالس الناتج لشعاع الليزر الميكرونى المركز لإحداث ثقوب في كل من الجدار الخلوي والغشاء البلازمي للخلايا باستخدام نظام يتكون من ميكروسكوب مقلوب ذى بعدين للانتقال في الاتجاهين X& Y ، وباتباع برنامج كومبيوتر تم تصميمه للتحكم في النظام الميكانيكي . وقد ساعد أحداث ثقوب في جدار خلايا الكالس وتدرج أسموزية الخلايا من الداخل للخارج في إدخال ألدنا البلازميدي الي الخلايا لاحداث تحول وراثى فى خلايا الكالس . وتم اختبار تعبير جين ال GUS هستوكيميائيا . وتقييم التحولات الوراثية الناتجة علي المستوي التركيبي والوظيفي للجينات محل الدراسة . وقد وجد أن شعاع الليزر فوق البنفسجي يزود ويحفز كفاءة الاستيلاد في الكالس المعامل به .
في النهاية يمكن القول انه أمكن تطويع شعاع الليزر الميكروني كطريقة محورة لنقل الجينات في القمح السداسي ذات كفاءة تحويل عالية .