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Assessment of barley *(Hordeum vulgare L.)* mature embryos for *Agrobacterium*-mediated transformation

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Abstract

This study describes a protocol for *Agrobacterium*-mediated transformation of barley mature embryos. Cultured embryos were co-cultivated by EHA 105 strain carrying pCambia2301 binary vector and assayed for transient GUS expression. After 3 days of transformation, GUS expressing spots have been observed over 20.3% of total embryos. Among the tested several pre-treatments, infiltration in a vacuum chamber (400 mm Hg for 20 min) and infection by AGL-1 strain instead of EHA 105 increased the numbers of embryos with GUS staining significantly ($p<0.05$). PCR and Southern analyses showed that transformation occured in some of the T_0 plants which were also tolerant to selective conditions.

Key words: *Hordeum vulgare* L., *Agrobacterium*-mediated transformation, mature embryo

INTRODUCTION

Hordeum vulgare L. (barley) is an important cereal crop and excellent model plant for biochemists, physiologists, geneticists and molecular biologists [1]. Barley also provides a reference to the genomes of other *Triticeae* crops such as wheat, rye and some forage grasses due to its true diploid nature (2n=2x=14). Transformed barley is an ideal tool for searching specific traits and over-expression or silencing of genes in a cereal crop plant. Stable transformation has been reported for *Hordeum vulgare* L. cv. Golden promise at early 1990s using biolistics to introduce DNA to immature embryos [2]. *Agrobacterium*-mediated transformation has also been proven to be an efficient method in gene transfer to barley tissue cultures [3]. To date, 0.3 to 86.7 transgenic plants per 100 explants have been produced in certain barley genotypes transformed by *Agrobacterium* [4]. In these reports, initial explants were immature embryos which can be obtained in only limited numbers from the green-house plants requiring additional resource and labour. On the other side, the late embryos are abundantly available throughout a year, easy to handle and can be useful for optimization studies. Rapid germination of embryos eliminate the problem of somaclonal variations frequently seen in long regeneration process from callus tissues [5]. *Agrobacterium*-mediated transformation of mature embryos as early differentiated tissues have not been reported in the literature.

In this study, we have used two days grown mature embryos (MEs) directly for *Agrobacterium* transformation. Several pre-treatments such as using cell-wall degrading enzymes, vacuum infiltration and electroporation were assessed in increasing *Agrobacterium* attachment to embryo cells.

MATERIALS AND METHODS

Tissue culture conditions

Dry seeds of *Hordeum vulgare* L. cvs. Tokak 157/37 (also known as “Tokak”) and Golden promise were obtained from Aegean Agricultural Research Institute, İzmir (Turkey) and Minnesota University (USA), respectively. Mature embryos were surface sterilized and cultured as described previously [6]. Basal salts of Murashige and Skoog (MS) were supplemented with 3% (w/v) sucrose, 1ml of MS vitamin mixture and 0.8% agar with a pH of 5.75. All cultures were kept in a growth chamber (Heraeaus, Vötsch) with standard growth conditions [25± oC, 16/8-h day/night photoperiod with fluorescent lights at 7000 lux] and transformations were performed on the 2 day of culture period.

Agrobacterium-mediated transformation

A. tumefaciens EHA 105 containing a modified binary vector (pCambia2301) was used for transformations. This vector contains *gusA* gene between the SVBV –a viral

promoter isolated from strawberry vein banding virus- [7] and *A. tumefaciens* nopaline synthase (nos) terminator. The plasmid also contains neomycin phosphotransferase II (*npt II*) gene inserted between the cauliflower mosaic virus 35S promoter and the nos terminator. pCambia2301 vector was transferred into AGL-1 cells by a procedure described by Tzfira et al. [8] in our work. Bacterial cells were grown in Luria-Bertani medium (1% tryptone, 0.5% yeast extract and 1% NaCl, pH 7.0) containing 50 mg/L kanamycin and 50 mg/L rifampicin for EHA 105 and 50 mg/L kanamycin and 200mg/L carbenicillin for AGL-1, overnight at 28^{0}C by shaking (180 rpm). When the cultures reached to the log phase (OD_{600} = 0.8), bacteria were harvested and resuspended in liquid MS medium containing 10mM MES, 200µM acetosyringone, 2mM $CaCl_2$, 1gr/L pluronic F-68 and 10 g/L glucose (inoculation medium) to a final concentration of OD_{600} =1.0. The inoculation medium was kept at 22^{0}C at dark for 1 hour and then used for embryo transformations. Batches of 60 embryos of Tokak 157/37 variety were pre-treated as the following; First group of the embryos were directly immersed in inoculation medium for 30 min, second group was pre-treated with 3% macerase and 0.5 % cellulase for 30 min; third group was placed in a glass vacuum chamber and kept in pressure at 200 mmHg, 400 mmHg and 560 mmHg for 20 min and fourth group was electroporated with a single pulse of 220 µsec, 1000 V/cm and 10 µFd capacitance by an electroporator (Eppendorf, Multiporator®). Following the pre-treatments, the embryos were washed with sterile distilled water and transferred to MS medium with 500µM acetosyringone and 800mg/L L-cyctein for co-cultivation at 25^{0}C, 2-3 days at dark. After co-cultivation, the explants were transferred to cefotaxime solution (500 mg/L in dH_2O) and washed for 1h by shaking 120 rpm. Half of the explants were used in GUS assays while the resting transferred to selection medium (MS) containing 200 mg/L cefotaxime and 25 mg/L geneticin (G418) for organogenesis. Pre-treatment experiments were repeated 3 times and ANOVA (Analysis of variance) was performed for each teratments by using Statistical Package for Social Sciences (SPSS).

Evaluation of transformations

Histochemical assay was performed according to Jefferson et al. [9] and GUS staining were observed under a stereomicroscope (Olympus SZX7). Each GUS expressing spots were scored as one regardless of size. Plant genomic DNA was isolated from leaf tissue of individual regenerants by CTAB method [10]. For conventional PCR, the *gusA* gene was amplified with the following primers of GUS-1 5' CAT TAC GCT GCG ATG GAT TCC 3' and GUS-2 5' CAC ATC TTT GCA GCA GAT GTG 3' which would produce a 1.2-kb fragment. Primers for *npt II* gene were NPT-1 5' ATC GGG AGC GGC GAT ACC GTA 3' and NPT-2 5'GAG GCT ATT CGG CTA TGA CTG 3'. PCR was performed in 25 µl volume, containing 50 ng template DNA, 2.0 mM $MgCl_2$, 100 µM of each dNTP, 10 pmoles of each primer, 1 x PCR buffer and 0.5 Unit of Taq DNA polymerase (Fermentas EP0402). For Southern blot analyses, five micrograms of genomic DNA was digested with NcoI, separated by 1.2 % (w/v) agarose gel and transferred to a nylon membrane by capillary blotting [11]. A DNA fragment corresponding to the *npt II* gene (0.785 kb) was amplified by PCR and labelled with a DIG- DNA labelling and detection kit (Roche 11093657910). The blots were prehybridized at 68^{0}C for 1 h and then hybridized at the same temperature overnight. Filters were washed at high stringency and hybridized prob was detected using a chemilimunescent detection system according to the manufacturer's instructions.

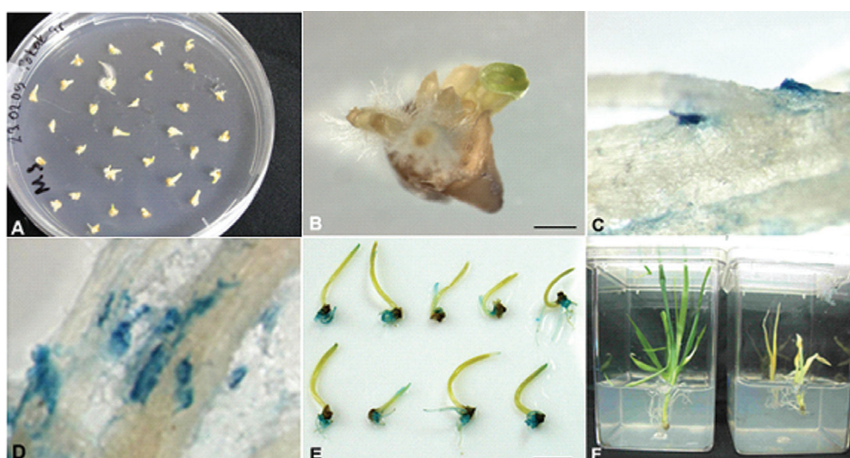

Figure 1. A-B: Tokak 157/37 embryos on the 2nd day of culture (bar= 2mm) **C-D.** GUS expression on embryo parts 3 days after transformation (C: Non-pre-treated embryo and D: 400 mmHg vacuum infiltrated embryo) Mag. 100x **E.** GUS expression observed in roots of embryos co-cultivated by AGL-1/pCambia2301 bar=1cm **F.** Golden promise plants grown on MS+25mg/L G418).

Table 1 Effects of different pre-teratments prior to embryo co-cultivation with *Agrobacterium* EHA 105 strain carrying pCambia2301.

Experiment No.	Pre-treatment Type	% MEs with GUS Activity*
1	-	20.3a
2	Enzyme application	29.6a
3	Electroporation	25.3a
4	Vacuum infiltration at 200 mmHg	22.1a
5	Vacuum infiltration at 400 mmHg	33,0a
6	Vacuum infiltration at 560 mmHg	26.6a

*Means followed by same letters in the column differ significantly at p<0.05

RESULTS

Agrobacterium transformation

Total 720 embryos of Tokak 157/37 were cultured and transformed for pre-treatments GUS expressing spots with variable sizes were observed at microscobic level on growing embryos 3 days after transformation (Fig 1C, D). Among the pre-treatments, enzyme treatment and vacuum infiltration at 400mm Hg increased the number of MEs with GUS activity (33%) compared to non-pretreated ones and other applications (Table 1). Use of AGL-1 strain in transformation increased the numbers of MEs with GUS staining two fold higher than EHA105 in a separate experiment in which 400mm Hg vacuum infiltration was applied to embryos. GUS expressing spots were (% ± SD) 58.9 ± 0.88 in AGL-1 and 27.6 ± 0.8819 in EHA105 transformed embryos. GUS activity was appearantly observed in embryonic roots after co-cultivation by AGL-1 (Fig 1 E). Standard procedure with 400mm Hg vacuum infiltration were applied to 276 and 155 embryos from Tokak 157/37 and Golden promise, respectively. Transformants induced shoots on selection media were phenotypically normal in both Tokak 157/37 and Golden promise varieties while control plants were killed in 40 days (Fig 1F). In three months period, 16.6% of Tokak 157/37 and 10.3% of Golden promise T_0 plants were grown on G418 media.

Molecular analysis of T_0 plants

Conventional PCR of Tokak 157/37 and G. promise plants were produced DNA bands (Fig 2 A) as following numbers: 24 plants were positive for *gusA*; 15 were *npt II* positive and 4 were positive for both *gusA* and *npt II* genes in Tokak 157/37. In G. promise; 8 plants were positive for *gusA;* 7 were *npt II* positive and 6 were positive for both *gusA and npt II genes. Genomic DNA* from 10 Tokak 157/37 plants were used in Southern blot analysis. Two bands hybridized with labeled *npt II* gene probes were observed in 2 transformed plants (Fig. 2 B).

DISCUSSION

Chimeric expression of GUS was observed in 20.3 % of the barley MEs co-cultivated by Agrobacterium without any pre-treatments. In addition of vacuum infiltration to the procedure enhanced the number of explants with GUS expression as well as the amount of GUS staining in some embryos (Fig 1D). Usefulness of vacuum infiltration have also been demonstrated in lentil cotyledonary nodes [12] and barley immature embryo-derived cultures [13]. Cell wall degrading enzymes are commonly used in protoplast cultures and do not have adverse effects on cell viability. Incubation of embryos with cellulase and macerase enzymes might have positive effects on T-DNA transfer to wounded tissues by increasing the rate of

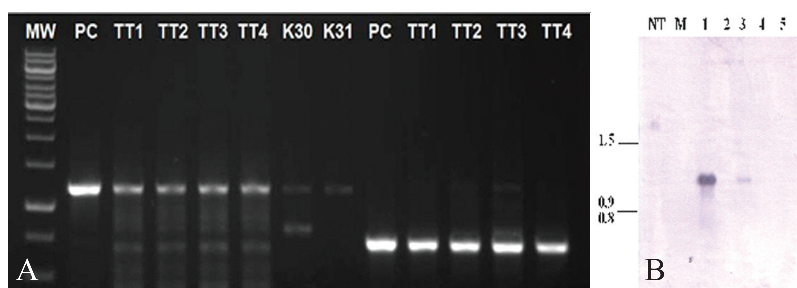

Figure 2. A. PCR amplification of *gusA* (1.2 kb) and *npt II* (0.7 kb) genes in transformed plants by A. *tumefaciens.* MW: 1 kb DNA marker; PC: pCambia2301 as positive control; TT1-TT4, K30, K31: primary generation of transformed elite Tokak 157/37 variety B. Southern hybridization of *npt II* probe to genomic DNAs from one untransformed (NT) control and two T_0 plants.

transformed embryos. T-DNA delivery may be affected from many conditions such as pre-culture duration time, use of different combinated inoculation media and length of the inoculation time as recently tested in wheat [14]. Our data shows that different effects of treatments on T-DNA delivery to barley cells can be analysed by using mature embryos.

Roughly 10% of T_o plants derived from transformed embryos were survived on selection media in extended periods. Likely, mature embryos which have been electroporated in our previous work, yielded 10% of germinated plants however those explants were insufficient for further growth [6]. Tolerance of transformed plants to geneticin in this work, showed maintained expression of *npt II* gene during the germination. RT-PCR of *npt II* gene in 18 barley plants resulted with amplification in 5 individuals (unpublished data) also confirmed the expression of this gene in mature plants. However, transgene integration occurs in a low frequency according to Southern analysis despite efficient DNA uptake based upon GUS assays.

Our results demonstrated the expression and integration of the introduced genes into barley genome, therefore the procedure described in this study has the potential to search T-DNA delivery conditions and to obtain high levels of transformation in mature embryos.

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