Time saving method for protoplast isolation, transformation and transient gene expression assay in barley

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JENES, B.[1], PUOLIMATKA, M.[2], BITTENCOURT, P.[1] & PULLI, S.[2] 1994. **Time saving method for protoplast isolation, transformation and transient gene expression assay in barley**. Agricultural Science in Finland 3: 199–205. ([1]Institute for Plant Sciences, Agricultural Biotechnology Center, Gödöllö, P.O.Box 170, H-2100 Hungary and [2]Agricultural Research Centre of Finland, Institute of Crop and Soil Science, Plant Breeding Section, FIN-31600 Jokioinen, Finland.)

This study was conducted to establish a rapid method for barley (*Hordeum vulgare* L.) protoplast isolation to provide an easy-to-use procedure for the transformation and primary investigation of new gene constructs by transient gene expression assays. Protoplasts were successfully isolated from the chopped embryo and scutellum parts of mature barley seeds by digesting three hours with an enzyme mixture. Isolated protoplasts were washed in W5 washing solution, sieved through plastic meshes and then cleaned on sucrose gradient. The suitability of these directly from embryo-scutellum complexes derived protoplasts for transient gene expression studies was determined by transforming the protoplasts using the PEG (polyethylene glycol) method. Plasmid *pAct1*-F containing the rice *Act1* promoter linked with the *gus* coding sequences and the *nos* polyadenylation signal was used in the transformation. After the PEG treatment protoplasts were cultured on KPR culture medium and the transient *gus* expression was assayed 24–36 hours after transformation. Up to 6% of the transformed protoplasts showed *gus* expression after treating the protoplasts with X-gluc. The results of this study show that the protoplasts isolated directly from dissected mature barley scutellum-embryo complexes could be used to investigate transient gene expressions in barley. This procedure requires negligible time prior the transformation experiment and so can be done in a very short time compared to the protoplast system based on a suspension culture.

Key words: *Hordeum vulgare* L., plasmid, gene transfer, β-glucuronidase

Introduction

During the last decade much interest has been focused on the culture of plant protoplasts and their potential in producing transgenic plants as well as in assaying the expression of recombinant gene constructs in transformed cells. The use of protoplasts provides the advantages of easier uptake of foreign DNA by the target cell in the absence of the cell wall and also single cell origin of regenerants thus avoiding chimerism in transformants. Considerable progress has been made in protoplast culture of important monocot species and the first protoplast derived regeneration in cereals was reported in rice (*Oryza sativa* L.) by FUJIMURA and his group (1985). Succesful protoplast-plant regeneration systems have also been reported in wheat (VASIL et al. 1990, CHANG et al. 1991, YANG et al. 1993, AHMED and SÁGI 1993, PAUK et al. 1994), maize (MÓROCZ et al.

1990) and barley (JÄHNE et al. 1991). Improvements in protoplast culture and regeneration have significantly given a contribution to the genetic transformation of monocots. In rice, the transformation of protoplasts and the subsequent regeneration of transgenic rice plants have become a routine procedure (JENES et al. 1992). Protoplast transformation resulting in stable integration of foreign genes into the target cells has also been reported in other cereals such as rice (SHIMAMOTO et al. 1989), maize (MÓROCZ et al. 1990) and barley (LAZZERI and LÖRZ 1990, LAZZERI et al. 1992).

In barley, most of the reported studies in protoplast isolation and transformation have so far been based on suspension derived protoplasts (LÜHRS and LÖRZ 1988, LAZZERI and LÖRZ 1990, JÄHNE et al. 1991, LAZZERI et al. 1992). JUNKER et al. (1987) detected transient expression of NPT II (Neomycin phosphotransferase II) gene in protoplasts derived from suspension culture and transformed with PEG. The initiation and maintenance of fine suspension cultures, a prerequisite for successful protoplast isolation in barley as well as in other species, is laboriuos and may take a considerably long time from some weeks to several months. It can therefore be a limiting factor in applying barley protoplasts to transient gene expression studies. In addition, a long lasting preculture phase before the transformation may result in undesirable somaclonal variation at the level of transgenic regenerants. Therefore the use of protoplasts in transformation experiments would benifit from a procedure that would reduce the time in culture prior to the gene transfer.

DIAZ and CARBONERO (1992) investigated tissue specific transient expression of the *gus* reporter gene in transformed barley protoplasts isolated from developing endosperm. There are reports on the isolation of barley aleurone protoplasts for transient expression studies (SKRIVER et al. 1991). This isolation procedure, however, requires very specific skills. In this paper we introduce a quick method to isolate barley protoplasts directly from dissected mature scutellum-embryo complexes and the use of such protoplasts in transient gene expression studies. This isolation method was found to be equivalent in efficiency to the transformation of protoplasts derived from cell suspension but the time requirement was negligible compared to the suspension cultures.

Material and methods

Plant material

Dry seeds of barley cultivars 'Pohto', 'Kymppi', 'Prisma' and 'Igri' were provided by the Institute of Plant Breeding, Agricultural Research Centre of Finland, Jokioinen.

Protoplast isolation

Dry seeds of barley were surface sterilized in 0.01% solution of mercuric chloride and then washed and rinsed with sterile distilled water for seven times. After sterilization the seeds were imbibed in sterile distilled water and incubated in 25°C for 24–36 hours. The imbibed embryo-scutellum complexes were excised from the seeds and chopped into small species with a scalpel (Fig. 1). The following three enzyme mixtures were originally used for the digestion of protoplasts:

1) JE enzyme mixture modified after JUNKER et al. (1987) containing 1.0% Onozuka RS Cellulase, 0.5% Macerozyme R 10 (Serva), 0.05% Pectolyase Y23 (Seishin), 5 mM $CaCl_2$, 0.5 mM Na_2HPO_4, at pH 5.8,
2) KE enzyme mixture modified after AHMED and SÁGI (1993) containing 2% Onozuka RS Cellulase, 0.5% Driselase, 0.1% Pectolyase Y-23, 6.5% Glycerol, 1.0% $CaCl_2$, 0.1% $MgSO_4$ and 0.05% KH_2PO_4,
3) LLE enzyme mixture modified after LÜHRS and LÖRZ (1988) containing 1% Onozuka RS Cellulase, 0.5% Macerozyme R10, 0.1% Pectolyase Y-23 and 0.1% Casein hydrolysate.

The protoplasts were kept in the enzyme at 25°C for 3 to 6 hours. The protoplast were then

cleaned by sieving the mixture through a plastic mesh with pores of 100 µm in diameter and centrifuged at 800 rpm for 5 min. Protoplasts were resuspended in 2 ml W5 washing solution (MENCZEL et al. 1981) and the suspension was then laid on the top of 0.6 M sucrose solution. After 5 min of centrifugation at 800 rpm the protoplasts were collected from the interphase. The cleaning was finished by washing the protoplasts twice with W5 solution.

Plasmids

Plasmid *pAct1*-F (MCELROY et al. 1991) was provided by the courtesy of Professor Ray Wu, Cornell University, Ithaca, NY. This plasmid includes the rice Actin 1 gene 5' regulatory elements linked to the *gus* gene coding sequences (synonym *uidA*, codes for β-glucuronidase enzyme) from *E. coli* and the *nos* polyadenylation site from *Agrobacterium tumefaciens*.

Transformation of protoplasts

The number of protoplasts suspended in the W5 solution was estimated in a Buerker chamber and then divided into 1×10^6 aliquots in plastic Wassermann tubes. After centrifugation at 800 rpm for 5 min the supernatant was removed and the pellet was resuspended in 1 ml MgMa transformation buffer (ZHANG et al. 1991). Twenty mg of uncut *pAct1*-F plasmid and 100 µg of Herring Testis DNA (SIGMA, ruptured by sonication) were added to the protoplast suspension. After 5 min of gentle handvortexing 1 ml of 30% solution of PEG (Polyethylen Glykol, fw 3450, SIGMA) solution was added (ZHANG et al. 1991). The protoplasts were incubated with the PEG for 28 min. During this time the suspension was gently shaken by hand in every 5 min.

At the end of the incubation time the mixture was slowly diluted with W5 solution up to 10 ml within 5 min, adding the W5 solution drop by drop and mixing. The diluted mixture then was centrifuged and the protoplasts washed twice in W5 solution.

Culture of protoplasts

The washed and pelleted protoplasts were resuspended in 1 ml of KPR protoplast culture medium (THOMPSON et al. 1986) and placed into a 24 well sterile plate (CORNING) so that 250 µl of suspension was transferred into each well. The plate was sealed with PARAFILM and incubated at 25°C in dark until the GUS assay.

Detection of transient expression of *gus* gene

Samples of control and transformed protoplast cultures were placed into a 96 well sterile plate (CORNING), 100 µl of culture into each well. Forty µl of X-Gluc staining solution (JEFFERSON et al. 1986, JEFFERSON 1987) was added into each well. The plates were incubated at 29°C for 12 hours before the visual counting of the cells showing transient expression was carried out.

Results and discussion

Protoplast isolation

Successful protoplast isolation was achieved from the mature scutellum-embryo complexes of each of the four genotypes used in this study (Fig.2). In our first experiments three different digestion enzyme mixtures were tested, each of which were modified from the original references. The results showed the superiority of the JE enzyme mixture in protoplast yielding (Table 1). Conse-

Table 1. Differencies in the effectiveness of the three enzyme mixtures applied to protoplast isolation from embryo-scutellum complexes of barley 'Kymppi'.

Enzyme mixture	Yield of protoplasts / 100 embryos
JE enzyme mixture[1]	2.2×10^6
KE enzyme mixture[2]	1.2×10^6
LLE enzyme mixture[3]	0.2×10^6

[1] modified after JUNKER et al. 1987.
[2] modified after AHMED and SÁGI 1993.
[3] modified after LÜHRS and LÖRZ 1988.

Table 2. Average yields of protoplasts of the four different barley cultivars Kymppi, Prisma, Pohto and Igri. Digestion was performed by the JE enzyme mixture[1].

Name of genotype	Number of protoplasts / 100 embryos
Kymppi	2.2×10^6
Prisma	4.1×10^6
Pohto	2.5×10^6
Igri	0.6×10^6

[1] modified after JUNKER et al. 1987.

quently, this enzyme mixture was chosen for the later experiments.

The four barley genotypes tested in this study showed differences in protoplast yielding and characteristics. In the digestion experiments as much as $0.2–4 \times 10^6$ protoplasts per 100 dissected embryo-scutellum complexes were obtained. The protoplast yield seemed to be dependent on the genotype and the enzyme mixture used (Table 2). The genotypes differed also in the size of released protoplasts. When using the JE enzyme digestion, Pohto provided bigger protoplasts of 50–80 µm in diameter than any of the other three cultivars (20–30 µm in diameter).

During the digestion of embryos from the mature embryo-scutellum complexes it was visually estimated that about 80% of the protoplasts were released from the scutellar tissue. These protoplasts started cell division 3–5 days after isolation and transformation (Fig. 3) and continued further forming 20–30 celled aggregates. This observation encourages us towards our further goals to obtain regenerated transgenic plants from these transformed protoplasts. It is well known from the earlier studies that the scutellar tissue in monocot species has a great regeneration capacity (FUJIMURA et al. 1985).

Protoplast transformation and transient gene expression

Protoplasts isolated directly from scutellum-embryo complexes were succesfully transformed with the PEG method showing transient expression of the reporter gene under the control of the rice *Actin1* promoter, similarly to the results in rice transformation experiments (MCELROY et al. 1991). This became evident after treating the transformed cells with X-gluc. Some of the cells showed the typical blue colour resulting from the reaction between the enzyme and its substrate (Fig 4.). The frequency of transformed cells was estimated by visual examination. The frequency of the transformation events that showed transient expression of the *gus* gene was estimated to be in some cases up to 6% of the isolated and transformed protoplasts.

In plant molecular biology research cell suspension is the most common source of barley protoplasts used in the investigation of new gene constructs. The establishment of a suspension-protoplast system in monocots is a time consuming process (JÄHNE et al. 1991) taking three to six months of culture until the first protoplasts can be digested and transformed (JUNKER et al. 1987). To reduce this time, our aim was to set up a rapid system for producing viable protoplasts suitable for transformation and transient gene expression studies. The embryo-scutellum derived protoplast system seemed to fullfil these requirements. Only 24 hours was needed prior the protoplast isolation and transformation instead of several months of subculturing. This system was also suitable for obtaining 4.5% of the transformed cells showing transient gene expression.

The time needs of the protoplast isolation and transformation system presented in this paper is comparable to the transformation with particle bombardment but it still has all the advantages of the protoplast system. For example, as protoplast derived regenerants have a single cell origin, one can expect genetic uniformity in the whole plant after regeneration from transformed protoplast which is not always the case after regeneration from the bombarded material. Further refinary of this method to isolate barley protoplasts directly from mature embryo-scutellum complexes is needed to improve the yield of protoplasts and the induction level of cell divisions. A working and repeatable protoplast-plant regeneration system based on a quick protoplast isolation could pro-

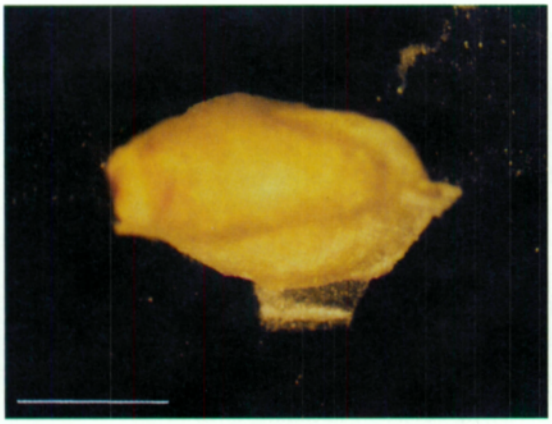

Fig. 1. Excised mature barley scutellum-embryo complex after 24 hours of imbibition in distilled, sterilized water. Scale bar = 1 mm. (Photo: Matti Puolimatka)

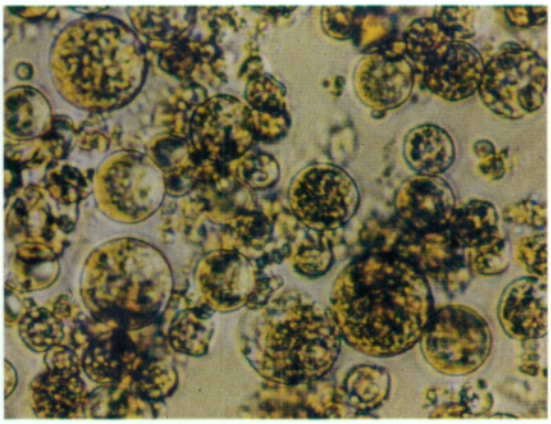

Fig. 2. Barley protoplasts isolated from the mature scutellum-embryo complexes of 'Kymppi'. (Photo: Barnabás Jenes)

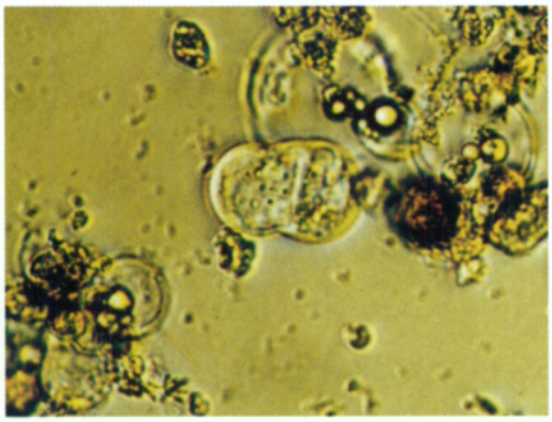

Fig. 3. Cell divisions in barley protoplasts. (Photo: Barnabás Jenes)

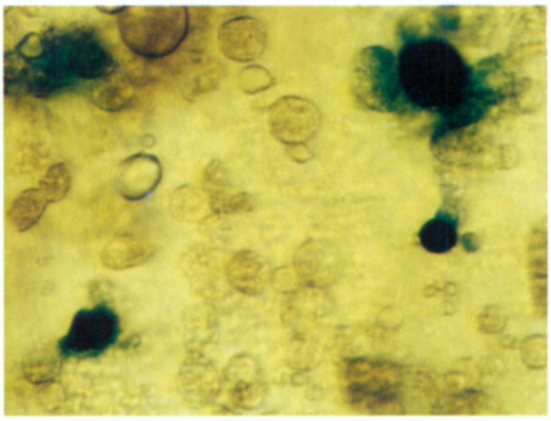

Fig. 4. Blue colour shows transient *Gus* gene activity in the transformed protoplasts of barley. (Photo: Barnabás Jenes)

vide with the ability to produce fertile transgenic plants of barley within much shorter time than through the suspension-protoplast-transgenic plant system.

Acknowledgements. The authors are grateful to the Agricultural Research Centre of Finland, Jokioinen, Finland, and Agricultural Biotechnology Centre, Gödöllö, Hungary, and to the Ministry of Agriculture and Forestry of Finland for providing the facilities and finance for this study.

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Manuscript received January 1994

SELOSTUS

Nopea menetelmä ohran protoplastien eristämiseksi ja sen soveltaminen geeninsiirtoon

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Tutkimuksessa kehitettiin menetelmä, jossa protoplasteja eristetään suoraan ohran jyvien alkion ja sirkkakilven solukosta ilman edeltävää solukkoviljelyvaihetta. Jyvät pintasteriloitiin ja niitä liuotettiin steriloidussa vedessä vuorokausi. Tämän jälkeen alkion ja sirkkakilven solukot eristettiin jyvistä ja pilkottiin pieniksi paloiksi. Protoplastien eristämiseksi kokeiltiin aluksi kolmea erilaista entsyymiliuosta, joista paras valittiin PEG (polyetyleeniglykoli)-menetelmällä tehtyihin geeninsiirtokokeisiin. Siirtokokeissa käytetty rengasmainen plasmidi-DNA *pAct1-F* sisälsi riisin *Act1*-säätelyjakson, *Gus*-geenin (koodaa β-glukuronidaasi-entsyymiä) ja *Nos*-lopetusjakson. PEG-käsittelyn jälkeen protoplasteja kasvatettiin KPR-kasvatusalustalla. Transienttinen (väliaikainen) *Gus*-geenin ilmentyminen määritettiin vuorokauden kuluttua käsittelemällä transformoituja protoplasteja X-gluc-entsyymisubstraatilla. Enimmillään noin kuudessa prosentissa transformoiduista protoplasteista havaittiin *Gus*-geenin ilmentymistä. Tulosten mukaan tämä nopea protoplastien eristystekniikka soveltuu erityisesti väliaikaisen geeni-ilmentymisen tutkimukseen, sillä ajansäästö protoplastien normaaliin suspensioviljelmään verrattuna on huomattava.