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Effect of heat shock treatment on *Hordeum vulgare* protoplast transformation mediated by polyethylene glycol

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Abstract

The stable transformation of barley protoplasts has been observed in cv. Dissa by PEG-mediated pGL2 plasmid DNA uptake containing hygromycin phosphotransferase (*hph*) gene under the control of 35S transcript of CaMV. Hygromycin resistant colonies were obtained, when protoplasts were incubated with hygromycin B (25 μg cm^{-3}) at 3 - 4 cell stage. Heat shock treatment, prior to polyethylene glycol (PEG) treatment, markedly increased the protoplast transformation frequency. The presence of active *hph* gene product, and integration of *hph* gene was confirmed by hygromycin assay and Southern blotting, respectively. Analysis indicated that rearrangement had possibly occurred during the integration of plasmid DNA into genomic DNA of barley callus. These transformed calli showed no morphogenic response onto the regeneration medium.

Additional key words: barley, gene transfer, hygromycin phosphotransferase resistance.

Introduction

Gene transfer has been well documented in *Solanaceae* plant species using *Agrobacterium tumefaciens* and *A. rhizogenes* (Hänisch *et al.* 1987). The integration of foreign genes into the genomic DNA of cereals have been achieved by the methods of direct gene transfer (Paszkowski *et al.* 1984, Potrykus 1990, Lazzeri *et al.* 1991) and *A. tumefaciens*-mediated transfer (Tingay *et al.* 1997, Cheng *et al.* 1997). In barley, transient expression of *npt* II and *cat* genes have been reported by using cell suspension-derived protoplasts (Junker *et al.* 1987), mesophyll-derived protoplasts (Teeri *et al.* 1989) and aleurone-derived protoplasts (Lee *et al.* 1989). Transient DNA uptake was chemically or electrically stimulated. Mendel *et al.* (1989) transformed suspension cells with a *npt* II/*gus* gene construct and Kartha *et al.* (1989) transformed immature zygotic embryos with a *cat* gene construct, both by particle bombardment. Creissen *et al.* (1990) successfully transferred plasmid DNA containing tandem dimers of wheat dwarf virus into microspores of barley. Other transient transformation techniques have also been reported, such as DNA uptake into wheat embryos by imbibition (Töpfer *et al.* 1989) and electrophoresis of DNA into germinating seeds (Ahokas 1989). Lazzeri *et al.* (1991) obtained stably transformed callus through chemically mediated direct gene uptake of an *npt* II/*gus* gene construct. Recently, fertile transgenic plants of barley have been reported by Ritala *et al.* (1994), Wan and Lemaux (1994). In our earlier report (Zhang *et al.* 1995) we reported the influence of various parameters on the transient and stable transformation of barley protoplasts.

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Abbreviations: PEG - polyethylene glycol; *hph* - hygromycin phosphotransferase; CaMV35S - cauliflower mosaic virus promoter; TLC - thin-layer chromotography; ATP - adenine triphosphate; 2,4-D - dichlorophenoxyacetic acid; NAA - 1-naphthyl acetic acid; MES - methyl ethyl sulphonate; CPW 13M - cell and protoplast washing medium containing 13 % (m/v) mannitol.

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In this paper we describe the selection of hygromycin resistant cells derived from suspension protoplasts and effect of heat-shock followed by cooling, on the absolute transformation frequency and assess response of transformed calli onto the regeneration medium.

Materials and methods

Immature embryos-derived embryogenic callus of barley (*Hordeum vulgare* L.) spring cultivar Dissa was used to initiate embryogenic cell suspension line DDKD 2.2. The maintenance of cell suspension, protoplast isolation, purification, viability of protoplasts were performed as previously described by Tiwari (1992). Hygromycin B (5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 μg cm^{-3}) was added to cultured protoplasts at 3 - 4 and 8 - 10 cell stage. Protoplasts (6.0×10^5 cm^{-3}) were cultured in KM8P medium (Kao and Michaylul 1975) modified by Gilmour *et al.* (1989) and solidified with *Sea-plaque* agarose (1.2 % m/v) and incubated in the dark at 26 ± 2 °C. 2 cm^3 of the same liquid medium was added after making sectors. After 2 weeks, sectors were transferred to 5 cm Petri dishes with 4 cm^3 of liquid medium per Petri dish. The KM8P liquid medium was replaced weekly with same volume of fresh medium containing hygromycin (5 - 50 μg cm^{-3}) to the respective Petri dishes. The observations were recorded after 4 weeks of culture of protoplasts.

The plasmid pGL2 was constructed by Datta *et al.* (1990) contained the coding region of the hygromycin phosphotransferase gene (*hph*) under the control of a CaMV 35S promoter and polyadenylation site, in the polylinker sequence of plasmid pDH 51. The selectable marker gene (*hph*) shows resistance to hygromycin B. Plasmid was isolated by using *Qiagen 41011 Plasmid Isolation Tips* (*Hybaid Ltd.*, Teddington. UK). After purification of protoplasts, resuspended at a density of 2×10^6 cm^{-3} in CPW 13M (Frearson *et al.* 1973) and heat-shocked at 45 °C for 5 min followed by 10-s cooling on ice (Thompson *et al.* 1987). The PEG-mediated DNA uptake procedure was used as previously described (Krens *et al.* 1982). 0.5 cm^3 of protoplast suspension were mixed with 50 μg of *Bgl* I digested pGL2 plasmid DNA. After adding DNA, 1.0 cm^3 of 40 % (m/v) PEG 1500 (*Merck*) solution in F medium (Wullems *et al.* 1980) was added dropwise, with occasional shaking. The mixture was incubated at 22 °C for 20 min, after which 10 cm^3 F medium was added step-wise in 2.0 cm^3 aliquots at 5 min intervals with occasional shaking. The protoplast suspension was centrifuged (80 *g* for 5 min) and washed two times in CPW 13M.

Treated protoplasts were resuspended at a density of 5×10^5 cm^{-3} in L1D2 medium (Jähne *et al.* 1991) and cultured in agarose-layer method. At 3 - 4 cell stage of development, agarose in which colonies were embedded, was cut into 4 sectors and L1D2 medium containing 25 μg cm^{-3} hygromycin B (*Sigma*, St. Louis, USA) was added. Each week, old medium was replaced by fresh medium. The transformation frequency of calli were calculated as previously described (Tiwari 1992) and morphogenic capacity of developed calli were tested when transferred onto MS medium containing 3 % (m/v) glucose and 0.5 mg dm^{-3} kinetin (previously identified for calli differentiation into plantlets; Tiwari 1992).

The enzymatic assay was carried out according to the published procedure of Datta *et al.* (1990) with some modifications. Calli were frozen in liquid nitrogen and ground in a pestle and mortar to a fine powder. 0.1 - 0.2 cm^3 of extraction buffer (0.05 M Tris HCl, pH 7.0, 10 % glycerol, 0.1 mM phenylmethyl sulphonyl fluoride) was added to 50 - 100 mg of tissue. The suspensions were transferred to 1.5 cm^3 Eppendorf tubes and centrifuged at at 4 °C for 10 min. The assay reaction was performed in 5 mm^3 volume containing 50 mM Tris-maleate pH 7.0, 50 mM $CaCl_2$, 0.05 mM ATP, 0.2 mm^3 γ-labelled ^{32}P ATP (3.7×10^5 Bq mm^{-3}), 31 μg hygromycin B and 1 - 3 mm^3 of crude extract. Reactions were performed with and without hygromycin B. Samples were mixed and incubated at 37 °C for 45 min. The reaction was stopped by cooling on ice for 2 min. Two mm^3 aliquots of reaction mixture were spotted onto a PEI cellulose TLC plate (*Merck*) and spots were air-dried and developed in 100 cm^3 of 50 mM sodium formic acid (pH 5.4). The plate was air dried and exposed to *Kodak XAR-5* film at -70 °C for 24 h.

Callus tissues (≤ 0.5 g) were frozen in liquid nitrogen and ground to a fine powder. DNA extraction was performed as described by Saghai-Maroof *et al.* (1984). Five μg of total DNA isolated from each callus line was digested with *Bam* HI restriction enzyme, which cuts a 1.0 kb fragment containing *hph* gene from pGL2 plasmid transformed tissue. DNA was electrophoresed, on a 0.8 % (m/v) agarose gel and transferred onto nylon filter (*Amersham*) according to the procedure reported by Maniatis *et al.* (1982). The 1.0 kb *hph* gene fragment of pGL2 was isolated by electrophoresis. This fragment was used for the preparation of radiolabelled probe using the multiprime labelling kit (*Amersham*). Prehybridization and hybridization of the *Hybond N* membrane were performed according to the instructions supplied by the manufacturers.

Results and discussion

Protoplasts treated with PEG and DNA behaved differently than non-treated protoplasts. They expanded in size to 80 - 120 μm in diameter and 10 - 20 % protoplasts had burst by day 5 after culture. In contrast, non-treated protoplasts remained small (20 - 30 μm in diameter) and did not burst. First division of non-treated protoplasts had been observed at day 10, after culture, whereas, treated protoplasts showed first division after 14 - 18 d of culture. Eventually, protoplasts led to the formation of cluster of cells. The number of colonies have been reduced as the concentration of hygromycin B increases and no colony development occurred at the concentration of 25 μg cm^{-3} when incubated at 3 - 4 cell development stage. Whereas, no effect of hygromycin concentration (5 - 50 μg cm^{-3}) was observed when added at 8 - 10 cell development stage. Thus, protoplasts-derived cells remained viable and formed calli. Datta *et al.* (1990) have also used 25 μg cm^{-3} hygromycin B to select hygromycin resistant cells derived from cell suspension protoplasts of rice. In this case, hygromycin B was added to the medium at 14 d after protoplast culture.

A large number of non-DNA treated protoplasts failed to develop when cultured in medium containing hygromycin B at 25 μg cm^{-3}. Whereas, at lower concentration of hygromycin B protoplasts divide and formed colonies. It is believed that cell development stage and concentration of antibiotic are crucial factors to obtain maximum transformed cells. The development of hygromycin resistant colonies occurred when protoplasts were treated with DNA and PEG and cultured in an agarose-solidified medium containing 25 μg cm^{-3} hygromycin. Whereas, more colonies development occurred in the non-treated protoplasts. This could be due to the protoplast plating density and stimulating effect of heat shock treatment in the cell division. In the absence of PEG and DNA protoplasts failed to form colonies with antibiotic selection. In contrast, higher protoplast density and cumulative effect of heat shock treatment and DNA and toxic effect of PEG on protoplast reduced the colony formation in the absence of hygromycin. Whereas, heat shock treatment of protoplasts before the addition of plasmid and PEG was found to have a stimulatory effect on the protoplasts with respect to the integration of foreign gene into the protoplasts. Similar results have been reported by Zhang *et al.* (1988). Kao and Michayluk *et al.* (1974) have also reported the toxic effect of PEG on protoplasts. Significantly, higher relative and absolute transformation frequencies were observed for heat-shocked protoplasts than for non-heat shocked protoplasts (Table 1). Thus, heat shock treatment had an effect on these colonies proliferation (Fig. 1*b*). Matsuki *et al.* (1989) and Datta *et al.* (1990) successfully obtained hygromycin resistant colonies in rice, but, failed to provide an evidence that the *hph* gene had been integrated into the genome and was only able to see these colonies proliferated (Fig. 1*b*). Matsuki *et al.* (1989) and Datta *et al.* (1990) successfully obtained hygromycin resistant colonies in rice, but, failed to provide an evidence that the *hph* gene had been integrated into the genome and was only able to see enhancing effect on transformation frequencies. Hence, our transformation frequency was higher than the relative and absolute transformation frequencies obtained by Lazzeri *et al.* (1991) in barley and Toriyama *et al.* (1988) in rice. Similar findings were

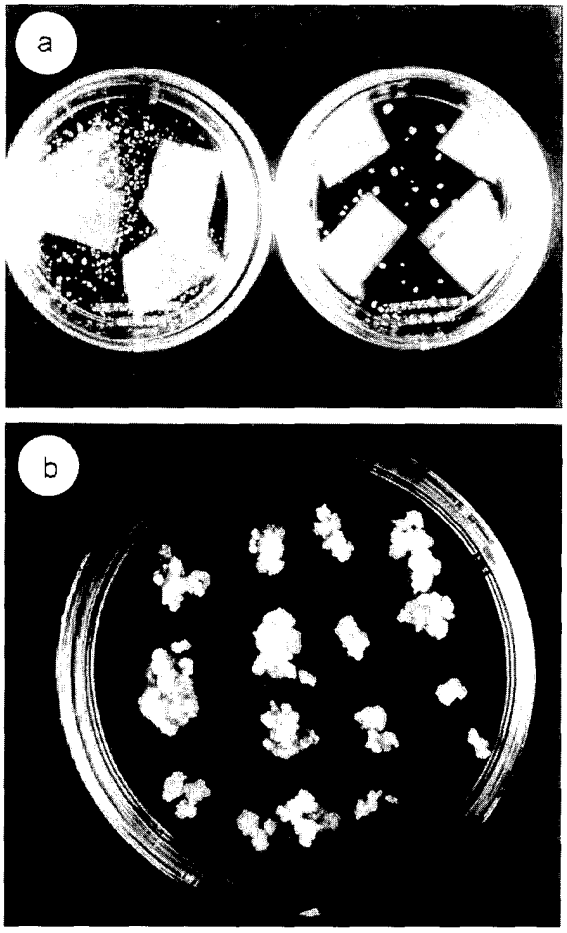

Fig. 1. Selection for protoplast-derived hygromycin B resistant colonies. Protoplasts non-treated with pGL2 and cultured in L1D2 agarose-solidified medium (control, *a* - *left*). Protoplasts treated with pGL2 in the presence of PEG and cultured in L1D2 medium containing 25 μg cm^{-3} hygromycin containing medium (*a* - *right*). Observations were recorded after 4 weeks of culture. 18-week-old hygromycin resistant calli on hygromycin-free CC1 medium (*b*).

reported by Peng *et al.* (1990) and Zhang *et al.* (1988) in rice and Shillito *et al.* (1985) in tobacco. In contrary, transformation frequencies were higher than ours as reported by Armstrong *et al.* (1990) and Zhang *et al.* (1995) in barley, and Zhang *et al.* (1988), Lyznik *et al.* (1989a), Peng *et al.* (1990) and Datta *et al.* (1992) in rice. Hygromycin resistant colonies were transferred from L1D2 liquid medium containing hygromycin to hygromycin free CC1 medium (CC medium of Potrykus *et al.* 1979 containing 1.0 g dm^{-3} casein hydrolysate), where these colonies proliferated (Fig.1*b*). Matsuki *et al.* (1989) and Datta *et al.* (1990) successfully obtained hygromycin resistant colonies in rice, but, failed to provide an evidence that the *hph* gene had been integrated into the genome and was only able to see the expressed *gus* reporter gene in the transformed cells. In another

Table 1. Effect of heat-shock treatment on colony formation and transformation frequency of line DDKD 2.2 initiated from immature embryo-derived callus cv. Dissa. H.F.M. - hygromycin B free medium, H.C.M. - hygromycin B (25 μg cm^{-3}) containing medium. Experiment was repeated twice with 2 replicates. Means ± SE; * - significant at $P < 0.05$.

	Mean number of protoplasts treated [$\times 10^6$]	Mean number of colonies obtained after 7 weeks of treatment		Transformation frequencies	
		H.F.M.	H.C.M.	[%][a]	[$\times 10^{-5}$][b]
Heat shock	4.0	1140	0	0	0
Without heat shock	4.0	954	0	0	0
Heat shock	4.3	420	20	4.76 ± 0.80*	0.47 ± 0.13*
Without heat shock	4.3	660	16	2.42 ± 0.45*	0.37 ± 0.16*

[a] - relative transfromation frequency (the ratio of number of transformed colonies to number of untransformed colonies × 100); [b] - absolute transformation frequency (the ratio of number of transformed protoplast-derived colonies to number of protoplasts treated with PEG and DNA).

study, Meyer *et al.* (1985) have reported that synchronous tobacco protoplasts significantly increased stable transformation frequency by using PEG-mediated DNA uptake. It is believed that foreign DNA is introduced into cells at dividing stage at metaphase. In the present study, DNA was introduced into protoplasts isolated from exponential growth phase of cell suspension. Although, protoplasts divide continuously in culture, the transformation frequency might be reduced. Gene transfer into protoplasts has been influenced by the nature of plasmid, its concentration, concentration of PEG and carrier DNA. In this study, we have used 25 % of the final concentration of PEG. Whereas, Krens *et al.* (1982) have used 13.3 % (m/v) PEG concentration for optimum transformation frequencies in rice. The highest transient *cat* gene expression have protoplasts in barley. Negrutiu *et al.* (1987) have shown that PEG concentration affect transformation frequencies. We believe that stable transformation frequencies affected by concentration of PEG, integration of plasmid DNA into protoplasts is dependent on source of protoplasts and culture conditions.

Five hygromycin resistant colonies were randomly selected in order to confirm *hph* gene product in the transformed calli. Their crude extract was used in assay reactions in the presence and absence of hygromycin B. Extract containing the *hph* gene product detoxified hygromycin by phosphorylation and phosphorylated product was visualized by autoradiography. Phosphorylated product was observed for transformed tissue in the presence of hygromycin (Fig. 2). No phosphorylated product was observed for transformed tissue in the absence of hygromycin and also in the presence and absence of hygromycin in the extract of untransformed callus. Therefore, all 5 detected calli contained active *hph* gene product. It indicated that *hph* gene has been integrated to the genome of barley. Similar results were

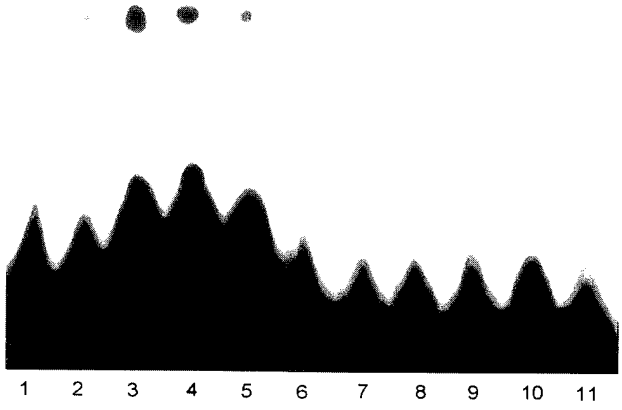

Fig. 2. Enzymatic assay for hygromycin-phosphotransferase in transformed barley callus. The autoradiogram of the TLC plate showed labelled ATP in all lanes. *Lanes 1 - 5* contain extract from selected transformed calli incubated without hygromycin. *Lanes 6* and *7* contain extract from selected transformed callus incubated without hygromycin B. *Lanes 8* and *9* contain extract from untransformed callus incubated with hygromycin. *Lanes 10* and *11* contain extract from untransformed callus incubated without hygromycin.

reported by Datta *et al.* (1990) in rice.

Five putative transformed calli were randomly selected for DNA isolation and Southern blot analysis. Two hundred to 400 µg DNA was isolated from 0.5 g of callus tissue. The hybridisation results indicated that 4 calli contained 1.0 kb *hph* gene fragment of plasmid pGL2 (Fig. 3). Extra bands were observed in all transformed calli (Fig. 3). These bands indicated that possible rearrangement of the transforming DNA occurred during its integration into the genomic DNA. No hybridization was observed for DNA from non-transformed callus tissue (Fig. 3). The existence of the additional hybridization bands may be the result of rearrangements of the plasmid sequence in the transformed cells, especially since some of the bands appeared common in certain clones (Fig. 3). However, the additional hybridization bands were widely observed in the transformed cells of many species reported previously (Fromm *et al.* 1985, Paszkowski *et al.* 1985, Uchimiyia *et al.* 1986, Neuhaus *et al.* 1986, Neuhaus *et al.* 1987, Horn *et al.* 1988b, Rhodes *et al.* 1988, Toriyama *et al.* 1988, Lyznik *et al.* 1989) and in the offsprings of transformed plants reported by Datta *et al.* (1990). The copy number of the 4 transformed calli was ranged from 5 to 40 copies of the *hph* gene per haploid genome in the transformed tissues. In contrast, Datta *et al.* (1990) obtained an estimate of 50 - 100 copies of plasmid pGL2 in transformed rice plant tissues. Above results confirmed the findings of stable transformation as previously reported by Lazzeri *et al.* (1991) in barley.

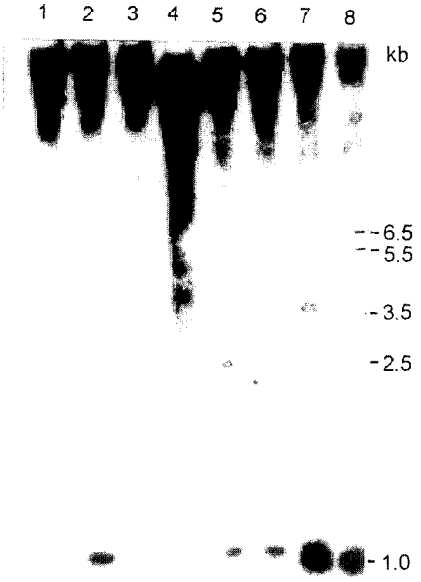

Fig. 3. Southern blot analysis of hygromycin resistant calli DNA digested with *Bam* HI and probed with 1.0 kb *hph* fragement of pGL2. *Lanes 1* and *2* represent 1× and 10× reconstructions of the 1.0 kb *Bam* HI fragment *hph* gene. *Lane 3* contains DNA from untransformed callus digested with *Bam* HI. *Lanes 4 - 8* contain DNA from selected transformed calli digested with *Bam* HI.

In the present study, we were able to integrate plasmid pGL2 containing *hph* gene into barley cell suspension derived protoplasts of barley, followed by heat-treatment and cooling, which led to the formation of callus. Hence, it provide a unique method to achieve a well defined modification which is not possible by conventional breeding methods. Therefore, the development of direct gene transfer into barley, is of great importance in conjunction with protoplast to plant regeneration protocols as previously reported by Lazzeri and Lörz (1990), Yan *et al.* (1990), Jähne *et al.* (1991), Funatsuki *et al.* (1992) and Golds *et al.* (1994) in order to obtain stable transformed barley plants.

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