TISSUE CULTURE AND GENE TRANSFER IN BARLEY

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ABSTRACT

Advances have been made in barley tissue culture for haploid production, somatic embryogenesis, and protoplast isolation and regeneration. Haploids are produced by culture of anthers, ovaries, and isolated microspores, and by chromosome elimination. However, albinism occurs frequently in plantlets obtained by anther culture, and barley protoplasts are recalcitrant for plant regeneration. Somatic embryogenesis is readily induced in barley apical meristems, mature and immature embryos, and somatic embryoids are regenerable into plants. A wide range of dominant selectable markers have been developed for use in dicots and monocots. These new techniques (electroporation, electroinjection, pollen and ovary transformation, liposomes and spheroplasts) and tissue culture should facilitate genetic engineering for improving barley. However, these new technologies are important additional tools for plant breeders in crop improvement.

INTRODUCTION

CULTIVATED barley (*Hordeum vulgare* L.) is one of the oldest cereals known to mankind. It ranks fourth in world's cereal production. Barley is a diploid ($2n = 2x = 14$), self-pollinating annual and is grown as a spring or winter crop. Traditionally, barley has been used in human diet, as an animal feed and in the production of beer. Tissue culture and gene transfer technologies are additional tools for plant breeders and have great potential for improvement of this commercially important crop.

The basic requirement for the practical use of tissue culture is the ability to regenerate plants. Generally, dicotyledonous plants readily induce callus and regenerate plants more readily than monocotyledons. The capability for plant regeneration via tissue culture in barley is lower than in other economically-important cereal crops[1]. Plants can now be regenerated from tissue culture in most of the important species of cereals and grasses. Recently, several review articles have addressed problems associated with cereal tissue culture[2–6]. Barley plant regeneration through tissue culture[7–12] indicates that it can be used to improve malting quality[13], nutrition[14], and disease resistance[15], and to induce mutations[16].

TISSUE CULTURE

Recent progress in barley plant regeneration from anthers[17–19], isolated pollen[20,21], ovaries[22], endosperm[23], immature embryos (obtained after crossing between *H. vulgare* and *H. bulbosum*.)[24–26], somatic embryogenesis[12,27–30] will enable researchers to develop more effective genetic engineering programs. However, more research is required before plants can be regenerated from protoplasts[27]. Genotype and culture medium greatly influence callus induction and plant regeneration in barley[31,32]. Callus can be easily induced by culturing different seedling explants[32] and using immature embryos[33–35]. Cell suspension cultures have been initiated by inoculating friable callus into a liquid medium, maintained on a rotary shaker. The fine cell suspension is then plated on a solidified culture medium containing cytokinins and auxins for organogenesis and somatic embryogenesis. Cell suspension and callus are applicable for the isolation of mutants[36], induction of somaclonal variation[35,37,38] and protoplast isolation[5,6,39]. Limitations of callus and cell suspension cultures are chromosomal variation[40,41] and maintenance by frequent subculturing. Plant tissue culture has been used in barley extensively for: a) induction of ha-

ploidy, b) somatic embryogenesis, and c) isolation of protoplasts.

a) *Induction of haploidy*

Haploid plants are sporophytes containing the gametic chromosome numbers, and their utility has been realized for a long time in crop improvement. Haploids are applicable for the induction of mutations and rapid production of genetically homozygous lines in large numbers[42,43]. A natural haploid plant in barley was first discovered by Johansen[44]. Conventional methods applied by plant breeders are not efficient for producing large numbers of haploids in a short time[45]. Guha and Maheshwari[46,47] first reported induction of haploids by culturing excised anthers of *Datura innoxia*. Since then, many reports have appeared on the successful use of anther culture for the induction of haploids in other species[48]. Haploid barley plants have been produced by the bulbosum method of chromosome elimination[49], from cultured anthers[50,51] ovules[52,53], isolated pollen[54] and by the haploidy initiator gene[55]. Problems of poor regeneration and albinism resulting from microspore callus need to be overcome; otherwise, chromosome elimination will remain in use as the principal method for haploid production in barley[56]. However, these two techniques may be combined in a doubled haploid production program in barley[57]. Direct regeneration of plants via pollen embryogenesis would reduce plastid mutation and chimerism[21].

1) *Chromosome elimination*

1a. *Interspecific crossing of H. vulgare and H. bulbosum*

Haploid plants in cultivated barley are recovered due to selective chromosome elimination of *H. bulbosum* following the interspecific cross between *H. vulgare* and *H. bulbosum*[49,57–60]. The mechanism of chromosome elimination in interspecific crosses is not known, but it has been shown that the elimination of *H. bulbosum* chromosomes is genetically-controlled by chromosomes two and three of *H. vulgare*[42,61]. Chromosome elimination occurs at a higher rate in spike primordia than in root meristems of the hybrids, suggesting that it is a tissue-dependent phenomenon[62,63]

Cytological observations have shown that the chromosome number of callus cells derived from spike primordia increased rather than decreased[64]. However, cell cycle time does not relate to chromosome elimination[65]. Disturbance in protein metabolism of hybrid cells is caused by abnormalities in genetic regulation, which affect the function or formation of the spindle at mitosis, and consequently, chromosomes are eliminated[65,66]. The abnormal interaction between fiber and kinetochore causes chromosome elimination in hybrids between *H. vulgare* and *H. bulbosum*[67]. Furthermore, a high rate of chromosome elimination occurs in tissues having high physiological activity, especially for differentiated cells such as the embryo, endosperm and spike primordia. Low physiological activity of callus may be related to the decreasing degree of disharmony in the gene regulating system in hybrid cells which reduces the rate of chromosome elimination[64]. Chromosome elimination phenomena leading to haploid production have been observed from other *Hordeum* interspecific crosses[68–73]

1b. *Use of intergeneric crosses*

Haploids have been recovered from intergeneric crosses between species. With chromosome doubling, haploids make it possible to enhance development of desirable true breeding lines. Haploid wheat from intergeneric crosses between *Triticum aestivum* × *H. bulbosum*[74] and haploid barley from intergeneric crosses of *H. vulgare* × *Psathyrostachys fragilis* have been produced[75]. Frequency of haploid production is dependent on seed set. It has been observed that crossability of wheat is genetically-controlled by genes located on chromosomes 5B and 5A[76,77] and the age of cultured immature embryos[78].

Embryo rescue technique has often produced intergeneric hybrids, but in low frequency. Viable hybrids have been obtained amongst

the genera *Triticum*, *Hordeum* and *Secale*[79] The intergeneric hybrids are self-sterile, and fertile amphiploids have rarely been produced between either *Triticum* and *Hordeum* or *Secale* and *Hordeum*.

Fedak and Nakamura[80] recovered viable intergeneric hybrids between hexaploid *T. crassum* (of *Aegilops crassa*) and diploid *H. vulgare* through the use of the embryo rescue technique. Nakamura[81] regenerated plants from totipotent callus, obtained by culturing infloresence tissue of a *T. crassum* and *H. vulgare* intergeneric hybrid. Hybrid regenerants had a somatic chromosome number of 28, but all *T. crassum* regenerants had 35 chromosomes rather than the original expected number of 42. This type of chromosomal instability was reported in *Triticum*[82,83], *Hordeum* species[72,84,85], and somaclones of *T. crassum* × *H. vulgare* hybrid[86]. Intergeneric hybrids between *H. vulgare* and *T. timopheevi*[87] and embryogenic callus in cultured young inflorescence tissue of *H. vulgare* and *T. aestivum* hybrids[88] have been produced. Regenerated plants and embryoids had chromosome numbers similar to the original hybrid plants.

2) *Anther culture*

Anther culture has allowed plant breeders and geneticists to improve crop plants and develop new plant varieties[19], but it is dependent on the availability of techniques for doubling chromosome numbers of haploid plants. Colchicine has been successfully used for doubling the chromosome numbers in barley[89], rice[90], soybean[91] and petunia[92].

The success of haploid induction by anther culture in the Gramminae has not been as great as in Solanaceous plants. Haploid plants have been recovered in rice[93,94], triticale[95], wheat[96,97], corn[98], aegilops[99] and rye[100]. Clapham[51] induced green and albino plantlets from the pollen of *H. vulgare* anthers. The green plants were haploid, diploid and tetraploid. Generally, differentiation of haploid barley plantlets from anther culture has been low[101].

Factors contributing to the success of androgenesis are genotype[57,102,103], developmental stage of the pollen at the time of culture[20,104], high anther density[105], addition of ficoll to the liquid medium[21,106], growth hormones[107–109] and preconditioning of anthers[110–112]. Cold treatment of anthers increases frequency of pollen callus formation in barley[21,113,114] and in rice[19].

The orientation of barley anthers on the culture medium has a marked effect on their response to the induction of callus formation[115] and embryoid production[17,18]. Normally, embryoids develop in the upper lobes of the cultured barley anthers with the lobe touching the medium producing no embryoids.

The effect of sucrose in stabilizing and inducing germination of mature pollen is well known[116], and it may play a similar role in inducing embryogenesis in immature pollen. High sucrose concentration is necessary only for induction of embryogenesis, but not for subsequent embryo development[117]. High sucrose levels are necessary for barley anther culture[118]. Levels of sucrose as high as 9%[119] to 12%[107] in the culture medium cause induction of androgenesis in barley anther culture, and may also be responsible for albinism[51]. Albino plants occur frequently in cultured barley anthers[54,120] as well as other cereal plants.

3) *Ovary/ovule culture*

There are a few reports on successful use of ovary culture for the haploid induction. Haploids have been produced from cultured ovules of four cereals: barley, rice, wheat and maize[22,121] San Noeum[52,53] first produced haploid plants by culturing unfertilized ovaries of *H. vulgare*; this was repeated by Gu and Zheng[8].

4) *Culture of isolated microspores*

Normally, haploid plants are regenerated through androgenesis with anther culture. Nevertheless, there are certain limitations to this method. Anther walls may inhibit the

induction of androgenic embryoids and callus. This can be overcome by manipulation of the culture medium and growing conditions of the plants[92] and by culture of isolated microspores in liquid medium. Cell cluster formation has been reported from isolated pollen grains of *Brassica oleracea*[122], plant regeneration via somatic embryogenesis in *T. aestivum*[123], and embryogenesis in *Solanum tuberosum*[124].

Attempts have been made to culture isolated pollen grains for the induction of haploids in cereals such as rye[125], barley[105,126,127], rice[128] and wheat[129]. In all cases, callus was induced in the cultured pollen, but plants could not be regenerated. However, plants have been regenerated by direct culture of isolated barley pollen grains[11,130]. The efficiency of callus induction by culturing pollen grains can be increased by improving precultural and cultural methods[18,114,131].

b) *Somatic embryogenesis*

Regeneration from barley tissue culture has been achieved either by organogenesis or by somatic (non-zygotic) embryogenesis[31]. Somatic embryogenesis and plant regeneration have been reported in other cereal plants[5,6,132–138]. Norstog[139] recovered embryo-like structures in cultured, excised, immature barley embryos, and plants can now be regenerated via somatic embryogenesis[12,27,29]. It has been shown that somatic embryogenesis can be induced from barley apical meristems[28] and mature embryos[140].

Somatic embryoids may be used for artificial seed production[141,142] and cryopreservation[143]. Since they arise from single cells[2], the regenerated plants are uniform and have less chromosomal variation[144,145].

c) *Isolation and regeneration of protoplasts*

There is no evidence for regeneration of plants from barley protoplasts although callus has been obtained[27,146]. Recent successes of plant regeneration have been reported from rice[147], sugar cane[148], and pearl millet[149] protoplasts. Genotype and ammonium and ferric ions are critical for plant regeneration from rice protoplasts[147]. The rate of rice protoplast division is enhanced by heat shock treatment[150]. It has been postulated that enzymes presently used to isolate cereal protoplasts are not optimal[151].

Although regeneration of complete plants from many cereal protoplasts is still an obstacle, callus can be induced from protoplast culture of wheat[152], triticale[153] as well as barley[27]. The fact that callus[154] and somatic embryoids can be formed from maize protoplasts[155] and that plants can be regenerated from rice protoplasts[156,157], indicates that cereal protoplasts are totipotent.

Barley protoplasts may be applicable for the transfer of herbicide resistance[158,159], protovariation[160], electroporation[161], DNA microinjection[162], transformation by spheroplasts of *Agrobacterium*[163], intergeneric gene transfer[164],organelle uptake[165], restoration of male fertility by fusion of two different cybrids[166], cytoplasmic male sterility transfer[167], and mutation[16,168]. Recently, *Petunia hybrida* protoplasts have been genetically-transformed via microinjection with *P. alpicola* chromosomes[169]. However, there are two limitations in using protoplasts: first, a reliable plant regeneration system is needed and second, chromosomal variation may occur[41].

GENE TRANSFER IN BARLEY

Gene transfer technologies have been well developed for Solanaceous plant species using the soil bacteria *Agrobacterium tumefaciens* and *A. rhizogenes*[170–172]. These bacteria do not readily infect monocotyledons, therefore it has been difficult to utilize genetic engineering in cereals. In the past, cereal plants were recalcitrant in terms of *in vitro* culture and genetic manipulation. However, due to recent developments in plant regeneration from rice protoplasts[147,173] and in several other species of grasses[5,6], it is now possible to establish gene transfer in other cereals[174]. Until now, only a small number of isolated genes were available for cereal breeding programs. Therefore, more collaboration between plant breeders, physiologists, pathologists, molecular and cellular

biologists is needed in order to: (i) define the genes of interest, (ii) isolate and modify them and reintroduce them into the recipient plants, and finally, (iii) breed genetically engineered cereal crops[175] and other economic plants[176].

METHODS OF GENE TRANSFER

a) *Electroporation*

Electrical impulses of high field strength reversibly permeabilize biomembranes[177] and thus have two important applications: firstly, the introduction of exogenous DNA in animal cells[178,179] and secondly, induction of cell fusion[180-182]. Fromm[183] developed an electroporation method for the introduction of exogenous DNA into plant cells. Gene transfer efficiency increased with the DNA concentration, and was affected by amplitude, duration of electric pulses and the medium in both monocot and dicot plants. Shillito[184] increased the efficiency of direct gene transfer to tobacco protoplasts with treatments of high voltage electric pulse, polyethylene glycol (PEG), and heat shock. Recently, stable transformation has been recovered in maize[185], tobacco[186], rice[187], wheat[187], sorghum[187] and carrot[188].

Electroporation has been used for optimizing gene transfer efficiency and transient gene expression[182]. By this method, it is simpler and more efficient to transfer exogenous DNA in plant protoplasts compared to microinjection. Furthermore, electroporation is not as laborious as microinjection[189].

Although transformation frequency by electroporation is high, the pores in the plasma membrane created by the electric pulses are likely to cause the loss of intracellular macromolecules and metabolites.

b) *Electroinjection*

Electroinjection has been used to tranform yeast cells[190] and to introduce tobacco mosaic virus RNA directly into mesophyll cells of *N. tabacum*[191]. The main difference between electroporation and electroinjection is that electroporation is used for the introduction of exogenous DNA or RNA into protoplasts, whereas electroinjection is performed with intact cells without removing the cell wall.

In barley, it is possible to regenerate plants from both embryogenic and non-embryogenic cell supensions. Therefore, electroinjection is ideal for the introduction of exogenous DNA into barley cell suspension cultures which can be induced with somatic embryogenesis to regenerate plants with less variation. If barley cells are not permeable enough for electroinjection, cell walls may be partially digested with cellulase before application of the electric pulse. However, there may be a leakage of metabolites and nucleic acids from the cell membrane pores.

c) *Microinjection of DNA in multicellular structures*

The lack of success of plant regeneration from protoplasts has limited genetic transformation in barley. However, plants are easily recovered via somatic embryogenesis and organogenesis in barley and other cereals. Therefore, alternative methods for genetic transformation in barley independent of protoplasts should be developed (microinjection of DNA into: morphogenetic calli, somatic embryos, unfertilized and fertilized egg cells and zygotic embryos). If one can microinject isolated DNA directly into immature barley embryos and induce somatic embryogenesis from selected transformed calli, then subsequent plant regeneration will result with less variation. It may also be possible to regenerate plants directly from mature embryos that have been injected with exogenous DNA.

It has been shown that archesporial cells of rye are susceptible to exogenously applied caffeine and colchicine about 2 weeks before metaphase I[192,193]. During this developmental stage[175], researchers have injected plasmid DNA coding for kanamycin resistance into the developing rye intlorescences, and have recovered kanamycin-resistant plants. Similarly, kanamycin-resistant plants have been recovered by co-cultivation of germinating seeds of *Arabidopsis thaliana* with *A. tumefaciens*[194]. With these technologies, gene transfer systems may be developed without using tissue culture.

d) *Transformation of pollen and ovary*

In normal plant fertilization, pollen applied to the stigma of the recipient flowering plants produces a tube that penetrates the stigma and style and finally reaches the micropylar canal. The pollen tube enters the micropylar canal and discharges two male nuclei. One of the two nuclei fuses with the egg nucleus to form a zygote and the other male nucleus fuses with two polar nuclei to form a primary endosperm. The zygote and primary endosperm divide repeatedly to form an embryo and endosperm, respectively. Both male and female cells do not have cell walls at the time of cell fusion and this fusion is similar to fusion of two protoplasts. If exogenous DNA containing selective markers and cloned genes is injected directly into the ovary, it may be taken up by the egg cell and possibly by the polar cell also. Therefore, expression of the introduced gene may be tested both in embryos and the endosperm. Haploid plants have been regenerated from unpollinated ovaries of barley[8,195] and induced callus obtained by culturing immature barley endosperm[23]. Chapman[196] suggested that exogenous DNA may be directly applied to pollen as a possible strategy for a directed genetic change. Newly germinated pollen tubes briefly lack a wall which can then readily adsorb DNA into the tube. A high efficiency of genetic transformation in maize has been obtained by pollination of the recipient plants along with DNA of the donor by applying it to silks in a pollen/DNA mixture[197].

e) *Use of spheroplasts*

It has been difficult to transfer genes by *A. tumefaciens* in cereal monocots due to lack of *Agrobacterium* attachment site(s) which prevents crown gall tumor formation in monocots[198]. It may also be due to insensitivity of monocots to increased phytohormones induced by *Agrobacterium* infection. However, Ti-plasmid gene expression without tumors, have been reported in monocots[199,200]. In order to bypass the dependence on *Agrobacterium* infection process for transformation in cereal cells, other methods will have to be developed.

E. coli spheroplasts have been successfully used for gene transfer in yeast[201,202] and in mammalian cells[203]. Furthermore, Hasezawa[204] developed a method of transformation with *Vinca rosea* protoplasts by treating with *A. tumefaciens* spheroplasts and then confirming by detection of nopaline synthase[205] and octopine synthase[206]. The process of transformation of plant protoplasts by spheroplasts is due to endocytosis[207–209]. Earlier, tranformation of Gramineae protoplasts of *Triticum*[210] and *Lolium*[211] was accomplished with naked DNA uptake into protoplasts with PEG treatment. Transformation of rice protoplasts has recently been mediated by *A. tumefaciens* spheroplasts[212].

f) *Use of liposomes*

Liposomes (artificial lipid vesicles) have been used as a vehicle to transfer biologically active molecules into animal cells[213,214] and into plant protoplasts[215,216]. The mechanism of uptake of liposome-DNA by protoplasts is probably due to adsorption of liposome-DNA on the surface of protoplasts or via fusion between liposomes and the plasma membrane[217]. In contrast, liposomes enter plant protoplasts via endocytosis in the presence of PEG and the nucleic acids are released when the endocytosed liposomes fuse with the endosomal membrane[218]. When plasmid DNA is transferred via liposomes into carrot protoplasts, variations have been found in DNA–uptake. The gradual disappearance of plasmid molecules suggests instability of plasmid DNA in prolonged cell culture[219]. However, the substantial portion of donor DNA becomes associated with nucleic acids and chromatin[220]. Wang *et al*[221] demonstrated that pH-sensitive liposomes dramatically release their contents into the cytoplasm of plant protoplasts at a pH less than or equal to 6.0.

SELECTION OF TRANSFORMANTS

It is essential to have a good selection marker for testing the expression of introduced genes in transformed cells and plants. *A. tumefaciens* with a defined DNA sequence has been used in gene transfer into the genome of

many dicotyledonous plants[222–224]. Several workers have used chimeric genes which combine the promoter sequences of the nopaline synthase (nos) with coding sequences of bacterial antibiotic-resistance genes, and have shown expression of such chimeric genes in plant cells. The selectable dominant bacterial antibiotic resistance genes, which confer resistance to several antibiotics (neomycin phosphotransferase (NPT II) — for kanamycin (neomycin and G 418); dihydrofolate reductase (DHFR) — for methotrexate; chloramphenicol acetyltransferase (CAT) — for chloramphenicol) have been used mainly in dicots[225]. The NPT II gene from transposon Tn5, for stable transformation, has been used in maize[185], rye[175], wheat[204], rice[226] and barley[175]. Another gene which codes for CAT has been used in rice[187], wheat[187], sorghum[187] and maize[183]. A new selectable marker, bleomycin[227], has been tried in *N. plumbaginifolia* using *A. tumefaciens*. The antibiotic bleomycin is a glycopeptide which interacts with DNA, resulting in single and double stranded breaks. It is used as cytotoxic drug in human cancer therapy. Plant cells are sensitive to bleomycin. Lloyd *et al*[228] have used a new selective marker, hygromycin resistance in transformation of *A. thaliana* with *A. tumefaciens*. Hygromycin B is an aminocyclitol antibiotic that inhibits protein synthesis in prokaryotic and eukaryotic cells.

The luciferase gene from the firefly, *Photinus pyralis*, is used routinely as a reporter of gene expression by light production in transferred plant cells and transgenic plants[229], and is 100 times more sensitive than a standard CAT assay. Recently, another reporter enzyme has been developed and tested which is expressed by the *E. coli* B-glucuronidase gene (GUS)[224]

CONCLUSIONS

Barley plants can be regenerated from isolated immature embryos, anthers, endosperm, unpollinated and pollinated ovaries, pollen and by somatic embryogenesis. Still, it has not been possible to regenerate plants from barley protoplasts. More emphasis should be given for the development of a reliable system for protoplast regeneration. Competent cell selection, genotype, cultural medium and conditions are important factors to be considered for plant regeneration from cereal protoplasts.

Chromosome elimination and anther culture methods may be combined for barley haploid production. However, more research is needed for improving poor plant regeneration and decreasing albinism resulting from microspore callus.

A reliable method should be developed for gene transfer in barley cells and protoplasts without affecting their capability for plant regeneration. Exogenous DNA can be microinjected into the multicellular structures such as immature embryos, somatic embryoids, ovaries (both pollinated and unpollinated) and developing inflorescences. Tissue culture may be avoided if exogenous DNA is directly injected into developing inflorescences.

The markers, NPT II and CAT, have been commonly used in gene transfer for the selection of transformed cereal calli. New selective gene markers like bleomycin, hygromycin B, luciferase and GUS can now be used.

Recent developments in barley plant regeneration by tissue culture, progress in the development of new vectors, and the utilization of selection markers and gene transfer technologies, suggest a great potential in improving barley and other cereal crops by genetic engineering. However, tissue culture and gene transfer are two important additional tools for plant breeders in crop improvement programs.

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