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Genetic transformation of barley for quality traits

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SUMMARY - Procedures have been developed for barley allowing for transformation of aleurone cells by polyethylene glycol mediated DNA uptake and for cells of the developing endosperm by particle bombardment. This permits an analysis of regulation of gene expression in these tissues by monitoring the transient expression of the introduced genes. For aleurone the studies are devoted to an analysis of cis-acting elements in genes responsive to the phytohormones gibberellic acid and abscisic acid (α-amylase and rab 16A). In endosperm cells the studies are directed towards identifying elements responsible for the tissue specific expression of the hordeins. Current strategies are described for establishing stable transformation in barley by means of protoplast transformation or particle bombardment of intact suspension cells or microspores. The potential of transformation for creating new, improved malting barley varieties is discussed.

RESUME - "Transformation génétique pour des caractères de qualité chez l'orge". Des procédés ont été développés pour l'orte, permettant la transformation des cellules des cellules de l'aleurone par absorption de l'ADN à l'aide de polyéthylène glycol et la transformation des cellules de l'endosperme en cours de développment en utilisant le bombardement avec des particules. Ceci permet d'analyser la régulation de l'expression des gènes dans ces tissus en contrôlant l'expression des gènes introduits. En ce qui concerne l'aeurone, les études portent sur une analyse des éléments qui "cis-agissent" chez les gènes réagissant aux phytohormones acide gibérellique et abscissique (α-amylase et rab 16A). Chez les cellules de l'endosperme, les études réalisées visent à identifier les éléments responsables de l'expression spécifique des hordéines pour chaque tissu. Les stratégies actuelles visent á établir une transformation stable chez l'orte au moyen de la transformation de protoplastes ou du bombardement avec des particules appliqué aux suspensions de cellules intactes on aux microspores. Le potentiel de transformation en vue de la création de nouvelles variétés améliorées d'orge de malterie est discuté.

Introduction

Barley is generally regarded as a bulk crop with a primary use as feed for livestock, while a smaller fraction is used as malt for the brewing industry. Breeding of barley varieties for malting purposes has resulted in cultivars with improved malting characteristics, and a malt barley variety can thus be considered a specialized crop which also receives a higher price than conventional feed barley, e.g., in Denmark the price difference is typically of the order of 20%.

A good malting barley is characterized by the following properties: large kernels of a uniform size, resistance to fungal attack during storage, a low dormancy and a high vitality resulting in a uniform and rapid germination, low protein content, high extract yield of starch and the ability to produce sufficient amounts of amylases, glucanases, proteases, etc. providing for a rapid conversion of the nutritional reserves in the endosperm. In addition other factors in the grain are important for color, flavour, foam and stability of the beer.

Brewing is an industry with a long tradition for monitoring and characterizing the physiological and biochemical processes required for making beer. It is thus natural for the brewing industry to explore the potentialities of molecular genetics to contribute to the production of new malting barley varieties allowing for

a faster, more reproducible and more economically way of making high quality beer.

One of the exciting possibilities for determining and improving quality characteristics in barley is transformation which permits the introduction of genes into malting barley from other pro- and eukaryotes, genes from wild barley species as well as endogenous genes, modified by site-directed mutation and recombination. In the present paper the current status for establishing techniques for transforming barley are summarized and a few processes highlighted where the molecular knowledge is sufficiently advanced to allow for genetic improvements of genetic traits.

Transient gene expression

General aspects

When exogenous DNA is introduced into a cell it may either be stably integrated in the host genome and transmitted to daughter cells/progeny or it remains in a free form in the nucleoplasm. Provided that the introduced DNA is not inactivated, it can be transcribed using the endogenous transcription machinery of the cell. While transcription of the integrated DNA may continue, the free DNA is transcribed only for a few days, i.e., transiently expressed. Current estimates from maize indicate that only 0.1-5% of the cells expressing the introduced gene has a stable integration of the gene (Spencer *et al.*, 1990; Gordon-Kamm *et al.*, 1990). The low frequency (0.1%) was found in cells with an embryogenic potential sufficient for allowing regeneration into plants while the high frequency was observed in non regenerable cell lines.

From the plant breeding point of view, stable integration and transmission is a prime objective. However, for molecular assessment transient gene expression has become an important tool since the introduced genes generally are expressed with a fidelity similar to that found *in vivo*, provided that they are introduced into the cell types where they normally function. Furthermore, an experiment using transient expression may be completed within a week, while a similar experiment using stable transformation is more laborious and requires several months for regeneration of plants, in the present context formation of seeds. Therefore the simpler procedures for transient gene expression are used to assess the performance of new genes before they are introduced in the variety by stable transformation.

Transformation of aleurone cells

Upon imbibition of water into the barley grain, the living tissues, i.e., the embryo and the aleurone layers are activated and start secreting hydrolytic enzymes into the endosperm tissue. In conventional biochemical analyses, aleurone layers are peeled off the endosperm tissue and the transcription of genes or secretion of enzymes monitored with respect to time after germination or in response to external stimuli such as hormones or Ca^{2+} (see Fincher, 1989 for review). Transformation of aleurone cells provides a molecular approach permitting analysis of regulation of gene expression at the level of the individual gene: the promoter sequence of the gene selected for study is fused to a suitable reporter gene, the construct introduced into the aleurone cells and the transient expression of the introduced gene monitored.

A technique has been established for isolating and transforming protoplasts from aleurone layers of germinating barley seeds (Olsen *et al.*, 1990). The technique is a combination of that described by Jacobsen *et al.* (1985) and Lee *et al.* (1985) and the procedures for polyethylene glycol (PEG) mediated transformation of the protoplasts are adapted from Negrutiu *et al.* (1987) and Lee *et al.* (1989). Mature grains of the huskless cultivar 'Himalaya' are deembryonated and divided longitudinally through the grove of the seed. Following a sterilization and imbibition step, the aleurone layer with adhering testa and pericarp is peeled off and transferred to a filter screen for digestion with cellulase. After 16 hours the screens are gently swirled to release the aleurone protoplasts to the surrounding medium and the protoplasts further purified by centrifugation. Approximately 1 x 10^6 protoplasts can be isolated from 50 grains using this technique. After PEG mediated transformation the protoplasts are washed and purified by centrifugation. In the present studies the CAT reporter gene (*E. coli* chloramphenicol acetyl transferase) was combined with the pea SSU E9 3' terminator (Lam *et al.*, 1989) and 5' upstream sequences of the genes selected for study were fused to the CAT gene. Initial experiments using the constitutive 35S promoter showed strong CAT expression two days after transformation, the assay used for detection of CAT activity being that of Gorman *et al.* (1982).

This procedure is currently being used to study the effects of the phytohormones abscisic acid (ABA) and gibberellic acid (GA) on gene expression in aleurone protoplasts. The work is performed in collaboration with Drs. John Mundy and Karen Skriver at the Carlsberg Research Laboratory (Olsen *et al.*, 1990). ABA controls the expression of a specific set of genes, normally expressed during seed formation, which may be involved in embryo dormancy and desiccation tolerance. GA controls the expression of several germination-specific proteins, including α-amylase. The two hormones appear to be antagonists: ABA inhibits the GA-responsive expression of alpha-amylase, while the expression of certain ABA-responsive genes is inhibited by GA. To determine the effect of the hormones on specific gene transcription, the promoters of an ABA-responsive gene

(rice rab 16A, Mundy and Chua, 1988), and two GA-responsive genes (barley alpha-amylases 1 and 2) were fused to the CAT cassette.

Recent work on the ABA-responsive expression of the rice rab 16A gene in rice protoplasts has delineated two conserved sequences which appear to be abscisic acid responsive DNA elements (Mundy *et al.*, 1990). Studies using oligonucleotides containing these sequences are currently directed towards identifying the functions of these putative abscisic acid responsive element. Similar constructions containing 5' upstream sequences of α-amylase genes showed induction by GA. This GA induction could be antagonized by equimolar concentrations of ABA.

Transformation of cells of the developing endosperm

Hordein is the major storage protein of the barley endosperm and may constitute 50% of the protein in the mature grain. The hordein polypeptides are synthesized in the developing endosperm cells, the messengers being translated on ribosomes bound to the endoplasmic reticulum with co-translational signal peptide cleavage and transport across the membrane. Subsequently hordein polypeptides are transported from the lumen of the endoplasmic reticulum to the vacuoles. Hordein polypeptides are divided into different groups (B-, C-, D-and τ-hordein) according to their size, their sulfur content and location on the chromosome. Genes encoding B-hordein, C-hordein, and τ-hordein polypeptides have been isolated and sequenced (Forde *et al.*, 1985; Brandt *et al.*, 1985; Entwistle, 1988; Cameron-Mills and Brandt, 1988). Comparison of the upstream sequences of the different hordein genes show an overall high degree of homology and a sequence of extensive homology is found approximately 300 bp 5' upstream of the initiation codon . This motif is also found in upstream sequences from other isolated storage protein genes which show endosperm specific expression in the cereals (Forde *et al.*, 1985). The importance of this motif with respect to tissue specificity has been studied in tobacco plants transformed with constructs where promoter fragments from a zein gene from maize (Matzke *et al.*, 1990) and a low molecular weight glutenin gene from wheat (Colot *et al.*, 1987) were able to drive the expression of a reporter gene in tobacco endosperm. The data indicate that a few base pairs are of major importance for the tissue specific expression of the reporter gene in the tobacco endosperm. The promoter activity is also stimulated by an increased supply of nitrogen during grain filling as addition of nitrogen promotes an increase in the total synthesis of hordein mRNA and hordein polypeptides (Giese and Hopp, 1984).

As initial studies showed that it was virtually impossible to purify protoplasts of the very large and fragile starch containing endosperm cells it was decided to use the BIOLISTIC particle gun (see Klein *et al.*, 1990 for review) for establishing a transient expression system for cells of the developing endosperm. These studies are aiming at analyzing motifs in the 5' upstream region regulating the expression of the hordein genes with respect to the developmental stage of the caryopsis, the tissue specific expression of the genes as well as the effect of environmental stimuli on transcription.

Extruded endosperms (9-19 days after pollination) were bombarded with gold or tungsten microprojectiles coated with plasmids carrying either the constitutive 35S promoter from the cauliflower mosaic virus or the tissue specific B1-hordein promoter (Hor-B1) from barley (Brandt *et al.*, 1985) in front of the ß-glucuronidase (GUS) reporter gene. Coating of the microprojectiles was performed by the procedure described by Klein *et al.* (1988) or the procedure described by Christou *et al.* (1988). Endosperms were incubated for 2-3 days on solid modified LS-media (Donovan and Lee, 1977) at 24 °C before assaying for GUS expression by addition of 5-bromo-4-chloro-3-indolyl-glucuronide (X-Gluc) (Scott *et al.*, 1988). Regions of the endosperm showing GUS expression were subjected to histological analysis by fixation in glutaraldehyde, embedding in Spurrs resin (Spurr, 1969) and sectioning to identify the cell types expressing the introduced gene. Blue cells or cell clusters were never observed in endosperms stained with X-Gluc after bombardment with a promotorless construct or a construct (CaMVI1CN) containing the gene encoding for the CAT reporter gene.

All sectioned cells expressing the construct with the Hor-B1 promotor in front of the GUS gene proved to be endosperm cells with large starch grains. On the other hand both cells with and without starch grains expressed the construct with the 35S promotor in front of the GUS gene. Occasionally brown areas adhering to the white starchy endosperm appeared during *in vitro* culture of endosperms extruded from older caryopses (17-19 days after pollination). Numerous cells expressing the GUS gene were found in this brown tissue after bombardment with the pBSGUS plasmid. Thin sections of those areas showed that the brown layer, usually two to four cell layers thick, contained cells without starch grains. Those layers presumably originate from differentiating aleurone. Expression of the pHor-B1 construct never occurred in this brown tissue but expression could be observed in starch containing cells below this tissue (Knudsen and Müller, 1990).

Stable transformation

General aspects

Unfortunately the cereals have proven to be exceedingly difficult to transform since the techniques successfully applied to a range of other dicot- and monocotyledonous plants (*Agrobacterium* mediated gene transfer or protoplast transformation followed by regeneration of plants from single transformed cells) have turned out to be ineffective in the cereals. It is now apparent (see Potrykus, 1989 for review) that these difficulties reflect a basic biological difference between the cereals and other plants, namely the poor capacity of a differentiated cereal cell to dedifferentiate and become embryogenic. In the so called non recalcitrant species cells from e.g. mesophyll, apical meristems, wound regions, leaf bases, roots, etc. may when infected with *Agrobacterium* or when subjected to proper cultivation conditions, dedifferentiate and enter an embryogenic pathway, eventually resulting in the formation of a plant from a single cell. Often such regeneration potential is maintained even when the cell wall has been removed by enzymes and the resulting protoplasts transformed by using polyethylene glycol mediated gene transfer or electroporation. The second problem in transformation of cereals by means of *Agrobacterium* is that the introduced DNA may not be integrated as shown by Grimsley *et al.* (1987) who found that although plant virus DNA (maize streak virus), inserted into the T-DNA of *Agrobacterium* could be transferred to cells of injured meristems, there was no evidence for an integration of the viral DNA into the host genome.

In order to circumvent these problems with cereals a variety of other procedures has been tried e.g., injection of large quantities of DNA into immature floral tillers (de la Peña *et al.*, 1987; Mendel *et al.*, 1990), imbibition of DNA into desiccated embryos (Töpfer *et al.*, 1989), the use of the pollen tube as a capillary for directing DNA into the newly fertilized egg cell (Luo and Wu, 1988), microinjection and particle bombardment of immature microspore derived embryoids or zygotic embryos (see Potrykus, 1989 for review) and transformation of pollen by various procedures (Heberle-Bors *et al.*, 1990). None of these procedures have yet provided conclusive evidence for stable transformation.

The present efforts in the cereals are concentrated on establishing embryogenic cell suspensions from only partly differentiated tissue such as immature zygotic embryos and microspore derived embryoids. Such suspensions can serve as a source for protoplasts, which subsequently can be transformed and regeneration attempted. Alternatively, the intact cells are transformed using the biolistic technique, where DNA coated high velocity microprojectiles are introduced through the cell wall of the cultured cells. This approach has been successful resulting in regeneration of transgenic *japonica* and *indica* varieties of rice from protoplasts (Toriyama *et al.*, 1989; Datta *et al.*, 1990) and transgenic maize plants has been regenerated from protoplasts (Donn *et al.*, 1990) or from suspension cultures transformed by electroporation (Rhodes *et al.*, 1988) or particle bombardment (Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990) . In these cases either the neomycin phosphotransferase gene conferring resistance to the antibiotic kanamycin or the PAT gene giving resistance to the herbicide Basta have been used as selectable markers.

There is, however, the inherent problem in this approach that the cells during the long term culture regimes required to establish the adapted embryogenic suspensions as well as during the subsequent regeneration phase accumulate several mutations at the gene, chromosome and genome level. This so called somaclonal variation often results in retardation of growth, albinism and sterility. In maize sterility of the transgenic regenerants has been a major problem (Rhodes *et al.*, 1988) and the first plants regenerated from protoplasts of wheat have likewise turned out to be completely sterile (Vasil *et al.*, 1990 and information presented by Vasil at the VIIth International Congress on Plant Tissue and Cell Culture). Secondly, the regeneration capacity is extremely genotype dependent and elaborate screening procedures for finding varieties with sufficient regeneration potential are therefore required and subsequent conventional crossings are necessary to transfer the introduced gene into the agronomically important varieties.

For barley there are at present only two examples of regeneration of green plants or plantlets from barley protoplasts (Quisheng *et al.*, 1990; Lazzeri and Lörz, 1990) while it is somewhat easier to regenerate green plants from intact suspension cells. Green plants have been recovered from four embryogenic cell lines established from immature zygotic embryos (Lührs and Lörz, 1987).

As outlined above, the easiest route to transformation of barley may involve particle bombardment of embryogenic cell suspensions or transformation of protoplasts from such suspensions, followed by regeneration into plants. A reasonable alternative to this approach is to transform microspores induced to undergo microspore embryogenesis, the advantage of this technique being that the regenerated plants appear to be completely normal (Olsen, 1987). Field tests of progeny from selfed regenerants of the variety 'Igri' thus showed that only 1% of the 200 lines tested deviated from seed grown material with respect to vigor and fertility.

Transformation of protoplasts from suspension cultures

Initial experiments have been aimed at establishing suspension cultures derived from immature zygotic embryos and to optimize the conditions for transformation of protoplasts isolated from calli grown on solid media or from the suspensions. Suspension cultures from cereals are normally initiated from calli derived from immature embryos. To suppress shoot and root formation, the meristems are carefully peeled off the embryos and the remaining part of the embryos cultivated on solid medium with the scutellum facing up from the medium. Differentiation of the callus is generally avoided using the auxin 2,4-D while omitting other hormones. It is thereafter necessary to select the friable type of callus (globular and yellowish callus - not translucent) prior to protoplast isolation or initiation of cell suspensions. The friable type of callus has in maize been classified as type-II callus (Kamo and Hodges, 1986).

Initial studies have been performed to establish good suspensions from barley as well as to define the conditions for transforming the protoplasts (Jensen, 1990) using a protocol developed by Lührs and Lörz (1988). Zygotic embryos, isolated 13, 16, 19, 22, 26 and 30 days after pollination were cultured to determine the optimal stage of development for induction of secondary embryogenesis. The meristems were peeled off and the embryos cultured on L2-media (Lazzeri and Lörz, 1988) solidified with 8% agarose and supplemented with 1 or 2.5 mg/l 2,4-D with either maltose, glucose or sucrose as the carbon source (30 g/l). The best calli were obtained from embryos, isolated 13-19 days after pollination and grown on media with maltose as the carbon source. In general there was no obvious difference in response from embryos cultured on 1 or 2.5 mg/l 2,4-D. A number of media were tested for the initiation of suspension but so far cell suspensions only grew on Kao8p medium (Kao and Michayluk, 1975) supplemented with 2 mg/l 2,4-D and 60 g/l maltose. Protoplasts were isolated from calli grown on solid media after at least four subcultures or from the cell suspensions. The digestion was performed 2-3 days after subculturing of suspensions and 8-13 days after subculture of calli grown on solid media. The cells were digested using the protocol described by Lührs and Lörz (1988) and following purification the viability of the protoplasts was evaluated using fluorescein diacetate staining. 2-4x10^6 protoplasts were isolated per gram of tissue. Two different transformation procedures were used, i.e., the procedure developed by Negrutiu *et al.* (1987) and later modified by Lee *et al.* (1989) and the droplet procedure described by Mass and Werr (1989). Testing of a number of PEG's with different molecular weight revealed PEG 6000 to be optimal.

Initial studies were addressed towards establishing reproducible assays for the GUS reporter gene, using the fluorometric assay for GUS activity (Scott *et al.*, 1988). 35S-CAT constructs were reproducibly expressed in the protoplasts whereas expression of the GUS gene turned out to be erratic. Similar problems with GUS have been encountered for oat aleurone protoplasts but were solved by purifying the cell extract used for the fluorometric assay for GUS activity by addition of polyvinyl pyrrolidone and Sephadex column chromatography (Hutley and Baulcombe, 1989). It is thus apparent that in extracts of protoplasts there are compounds which either inhibit the GUS enzyme or quenches the fluorescence, compounds which may originate from dead or stressed protoplasts.

Two different promoters, namely the constitutive 35S promoter and the ADh1 promoter of maize, which is induced by hypoxic conditions, were compared for their ability to drive expression of the GUS gene. The comparison also included the effect of inserting the 557 bp long intron 1 of the maize alcohol dehydrogenase gene 1 between the promoter and the reporter gene. This intron has in maize been reported to increase gene expression in transient expression studies by a factor of 2-20 (Callis *et al.*, 1987). The presence of this intron increases expression by 50% compared to the intron less 35S-GUS construct, which showed an expression comparable to that of the Adh-GUS construct. Surprisingly enough, the B1 hordein promoter-GUS construct, used for transforming endosperm cells, showed a level of expression similar to that of 35S-GUS. This apparent lack of tissue specific expression has also been reported for a zein promoter which turned out to be active in carrot protoplasts (Boston *et al.*, 1987), while other studies have documented the lack of expression of CAT driven by the zein promoter in suspension derived protoplasts (Schwall and Feix, 1988). It is thus apparent that even though suspension cells are dedifferentiated cells they should not be used uncritically for evaluating tissue specificity of promoter activity.

Microspore embryogenesis

It is now shown in a large number of species that microspores can be induced to dedifferentiate and switch from their normal gametophytic development to a sporophytic one (Fig. 1) (see Dunwell, 1985; Sangwan and Sangwan-Norreel, 1987 for review) and in some species large amounts of green plants can be regenerated from microspores. This method has been used by several plant breeders and a number of new varieties are currently being produced by this approach (Morrison and Evans, 1988; Thomes, 1990). In barley the techniques for generating pollen plants by means of microspore embryogenesis have improved substantially over the last few years and are now on their way to become routines for making pure lines in conventional breeding programs whereby time consuming inbreeding or backcrossing steps are eliminated. The improvements include

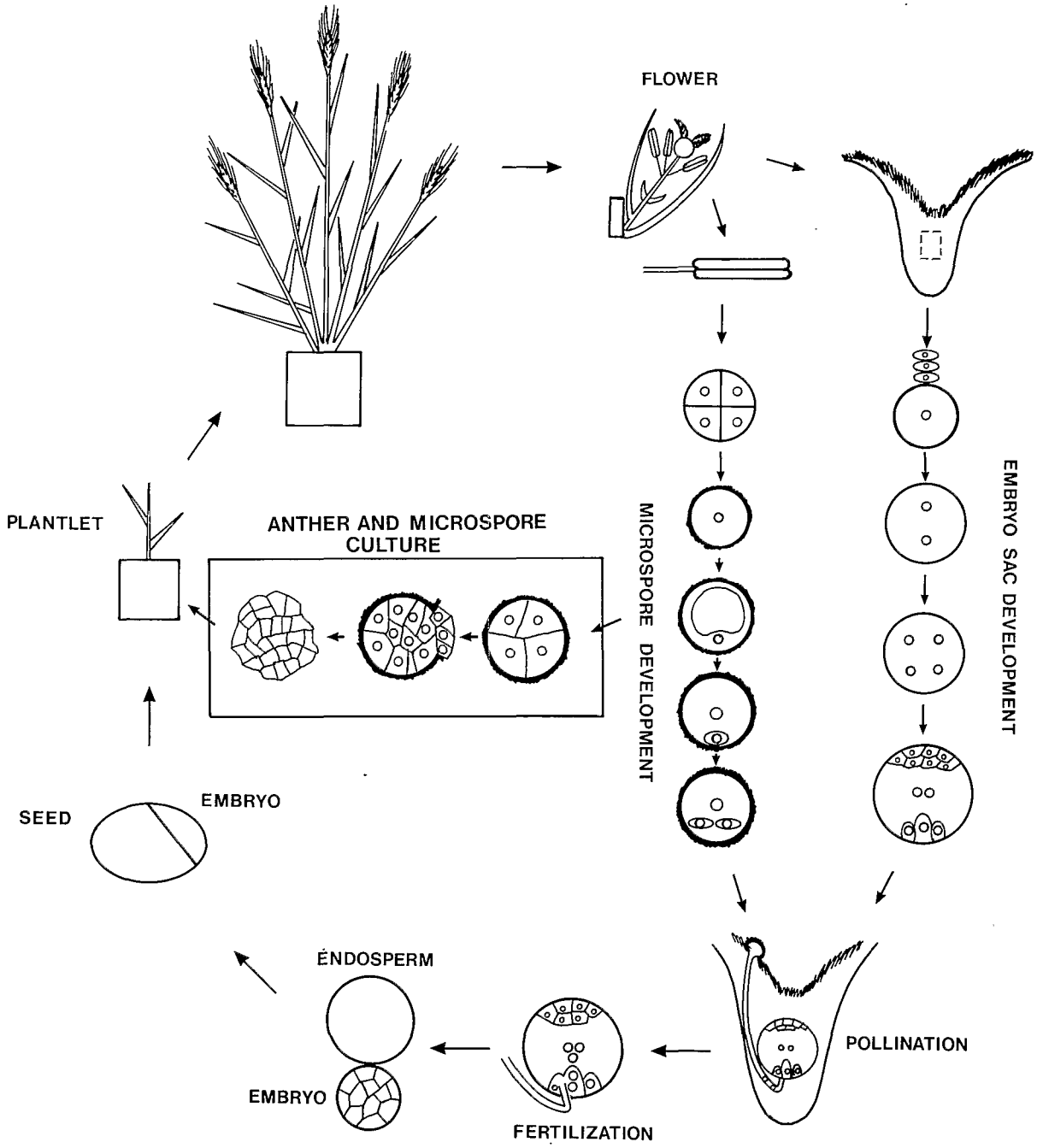

Fig. 1. The life cycle of barley. By means of microspore embryogenesis diploid, homozygous plants can be obtained directly from microspores.

optimization of the growth conditions of the donor plants, the pretreatment of the spikes and anthers and improvements of the media used, in particular the replacement of sucrose with maltose (Huang and Sunderland, 1982; Olsen, 1987; Hunter, 1987).

For the transformation approach it is necessary to have techniques for an efficient microspore isolation-cultivation-regeneration system. A microblending technique has been established for a rapid, reproducible and gentle isolation of microspores for the cultivar 'Igri' permitting a high frequency of regeneration of green plants (Olsen, in preparation). Anthers from cold pretreated spikes or anthers starved on 0.3 M mannitol for 3-4 days were blended in MS medium. The crude

microspore preparation was filtered and further purified by centrifugation whereafter the microspores were cultured in membrane supported multiwell dishes. In the following four weeks the microspores develop into embryoids which subsequently can be regenerated to plants on solid medium. This technique which was developed for microspore isolation from flower buds of *Brassica* (Coumans *et al.*, 1989) is clearly superior to the conventional technique involving maceration of the anthers with a teflon rod (Nitsch, 1974). With the microblending technique a mean of 9.4 green plants and 0.4 albino plants (a total of 2250 green and 97 albino plants) were regenerated per anther, while only a mean of 2.8 green and 0.17 albino plants (a total of 676 green and 40 albino plants) were regenerated from microspores isolated after maceration of the anthers with the teflon rod.

A series of experiments have been performed attempting to microinject single celled microspores selected from mass cultures of isolated microspores. This approach is impeded by the microspore wall which is difficult to penetrate by the injection needle and further by a very high turgor pressure of the dedifferentiating microspore resulting in the blocking of the orifice of the injection needle by cytoplasm upon penetration of the wall. It is however, possible to identify stages in the development of the dedifferentiating microspore where penetration of the wall is possible. In another experiment microinjection was performed into the cells exposed upon rupture of the microspore wall by the growing embryoid. It is apparent, however, that the cells are severely damaged by the injection needle and generally stop growing. This approach has therefore not been pursued. A more promising approach has been initiated, namely particle bombardment of the embryogenic microspores. Transient gene expression has been achieved by particle bombardment of pollen of tobacco, using a pollen specific promoter from tomato fused to the GUS reporter gene (Twell *et al.*, 1989). This illustrates that the tungsten particles indeed are able to penetrate the pollen wall without killing the cells.

Transgenic plants for the brewing industry

When a transformation system has been established for barley, transgenic barley plants resistant to various herbicides, fungi, vira, etc. may be generated as is the case for a number of other plants. In the present context, where an improvement of the barley grain is the objective, it can be envisaged that transgenic barley with improved malting characteristics, better nutritional quality or producing specific compounds can be created. This may be achieved by manipulation of endogenous genes, by introducing genes isolated from wild barley species or by using genes from other pro- and eukaryotes. Likewise grain specific barley genes may be transferred to other cereals and crops.

At present there are a number of limitations to the practical application of transformation for developing new varieties, namely: (a) So far only single genes can be introduced while the molecular knowledge on multigene traits as well as the technology for transferring them are insufficient. (b) The knowledge on the molecular biology of the barley grain is still fragmentary even though the cereal grain must be considered to one of the best characterized plant parts. Thus little is known about the molecular biology of the embryo/scutellum compared to aleurone and endosperm and there are at present only few grain specific genes available for manipulation. (c) With current transformation techniques it is not possible to perform gene targeting for a replacement of an endogenous gene by one modified by genetic engineering.

There are, however, a few processes where the molecular knowledge is sufficiently advanced to allow a manipulation of parameters important for the brewing process. These cases may be used as pilot studies for elucidating the potential of transgenic barley for the brewing industry. A detailed description of these cases is given by von Wettstein (1989) and will only be summarized here.

In modern malting and brewing the major component of the endosperm walls, the (1-3,1-4)-ß-glucans, are often insufficiently degraded as the endogenous (1-3,1-4)-ß-glucanases due to a low thermostability are inactivated during kilning and mashing. Residual ß-glucans will thus clog up the filters during filtration of worth and beer. To solve this problem a thermostable ß-glucanase gene has been constructed by combining different size fragments of ß-glucanase genes from B. *amyloliquefaciens* and *B. macerans* (Borris *et al.*, 1989; Olsen and Thomsen, 1989). Analyses of the specific activity of the hybrid enzymes at various temperatures, their thermostability and their pH optimum as well as model mashing studies revealed that e.g. an enzyme consisting of the first 16 amino acids from *B. amyloliqefaciens* and the remaining 198 amino acids from *B. macerans* as well as enzymes containing the first 107 amino acids from *B. amyloliquefaciens* and the last 107 amino acids from *B. macerans* were active under the conditions for mashing even after 15 min at 76°C. It will thus be of great theoretical as well as practical interest to transform barley with these hybrid genes under the control of a barley ß-glucanase promoter to see whether the introduced heat stable *Bacillus* ß-glucanase can prevent filtration problems.

Transformation with antisense constructs is a promising strategy for blocking molecular pathways and thus provides an alternative to conventional mutagenesis for eliminating the synthesis of unwanted compounds (see Lewis, 1989 for review). Antisense constructs to the chalcone synthase has thus been used for blocking the formation of anthocyanins in *Petunia*, resulting in the formation of new colored variants of the flower (Mol *et*

al., 1989). In barley the testa-pericarp tissue of the grain contain proanthocyanidins, which end up in the beer and precipitate protein resulting in haze during storage. Over a period of 15 years mutation breeding has resulted in the isolation of more than 700 proanthocyanidin free mutants which by complementation analysis has been assigned to six different genes in the metabolic pathway giving rise to proanthocyanidins (Jende-Strid and Kristiansen, 1987). Beer produced from such mutants has an excellent haze stability (von Wettstein *et al.*, 1985). However, in spite of intense breeding efforts it has not yet been possible to develop a proanthocyanidin free barley variety which with respect to malting quality and yield is competitive with the varieties currently used. It will thus be an interesting possibility to transform with antisense constructs to the genes involved in the production of proanthocyanidins. Thereby it can be assessed if a blocking of this pathway by antisense RNA has less side effects on germination and yield than elimination by mutation.

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