II.6 Barley

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1 Introduction

Barley (*Hordeum vulgare* L.), one of the earliest crops to be domesticated, is genetically suited to improvement through the use of biotechnology since it is a self-pollinated diploid ($2n = 2x = 14$) that does not cross-hybridize with species outside of its primary gene pool. Barley has been cultivated since 8000 BC and possibly as far back as 17,000 BC. Germplasm collections are numerous, as barley has been adapted to a wide variety of environments around the world.

The book "*Diversity in Barley*" edited by von Bothmer et al. (2003) is an excellent source on the history of the crop. It covers the distribution of barley from its origins in the Fertile Crescent of Asia to various parts of the world. This book also details the wide diversity of mutants in barley (Lundqvist and Frankowiak 2003), as this species was a major crop in the nineteenth century for both mutation and genetic research. "*Diversity in Barley*" also contains details of various other types of diversity, such as good cytogenetic and molecular tools, biotic and abiotic stress resistance or tolerance, as well as details of the "Barley Core Collection" germplasm.

Cultivated barley is considered to have evolved from two ancestral subspecies, *Hordeum vulgare* ssp. *vulgare* and ssp. *spontaneum*, that today also serve as the primary gene pool for germplasm diversity. Of the other 30 *Hordeum* species (von Bothmer et al. 2003), the only one with which barley might hybridize in nature is *H. bulbosum*; and it serves as the secondary genepool. *H. bulbosum* exists in both diploid and autotetraploid forms in nature and is out-crossing, enhanced by a self-incompatability system. With the aid of embryo culture, this interspecific hybrid of *H. bulbosum* and barley was shown to produce haploids of barley through preferential *H. bulbosum* chromosome elimination after hybridization (Kasha and Kao 1970; Subrahmanyam and Kasha 1973). The genomes of the other *Hordeum* species are different from those of *H. vulgare and H. bulbosum*, although some polyploids may contain the I genome of barley.

The karyotype and chromosome numbering for barley has changed over the years and this knowledge is important for biotechnological research, par-

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ticularly for molecular mapping and transformation. The original karyotype numbering followed the traditional system so that chromosome 1 was the longest, chromosome 2 the second longest and this pattern continued except for the satellited chromosomes which were placed last (6 and 7 in barley). However, using the C-banding technology on trisomic lines for each chromosome, Noda and Kasha (1978) demonstrated that the linkage groups assigned to each chromosome, as originally numbered, were not correct for the three longest chromosomes. Thus, the karyotype was rearranged to fit the linkage groups; and chromosome 3 with the most median centromere became 1, the longest chromosome (1) became chromosome 2 and the original chromosome 2 became chromosome 3. Linde-Laursen et al. (1997) proposed that the barley genome chromosomes be numbered with the symbol H to be consistent with the homoeologous chromosomes within the Triticeae genomes. Thus, in the literature published most recently, the symbol H is used with the number and chromosomes 1, 5 and 7 are changed in order, becoming 7H, 1H and 5H, respectively. The numbers of the other four chromosomes remain unchanged and are referred to as 2H, 3H, 4H and 6H. The Seventh International Barley Genetics Symposium adopted this system. Often the relationship between the two systems is pointed out when gene linkage or molecular markers are assigned to chromosomes.

Barley has one large genome with about 5.6×10^9 bp of DNA. About 80% of the genome consists of highly repetitive DNA which is mostly retrotransposons (Schulman et al. 2004).

In this review, papers from the Proceedings of the Ninth International Barley Genetics Symposium held in Brno, Czech Republic, in 2004 and from other recent reviews are cited for information in order to reduce the large numbers of references that could be cited for this broad topic. This, unfortunately, means that many of the original papers on topics are not cited, but may be traced through the citations used. The paper on transformation in barley by Lemaux et al. (1999) is an extensive and excellent review of the early literature on this topic, particularly when only transient gene expression occurred. This area will not be discussed here. The papers of Varshney et al. (2005a, b; 2006) should also be consulted for a review of molecular markers and maps of barley, although it will be briefly reviewed here.

Within the topic of biotechnology are included both tissue culture and molecular marker developments, as they are basic to the bioengineering of barley. Tissue culture procedures are essential, as totipotent cells are the targets for gene delivery and for the regeneration of transgenic plants. Low regeneration from tissue culture of most genotypes has been a problem for barley transformation using *Agrobacterium tumefaciens*. Molecular markers have revolutionized gene identification, tagging and isolation and are used for mapping and as tags for marking and selecting genes of importance in the improvement of barley.

2 Economic Importance of Barley

Barley ranks fourth in the world in cereal production, behind wheat, rice and maize. In 2004/2005, it was grown on approximately 56×10^6 ha (USDA-FAS 2005). About 85% of the barley grown around the world is used for animal feed and most of the remainder is used as malt for brewing and distilling, or as foods. While barley has decreased in world acreage in recent years, it appears to have levelled out in 2004/2005. The advent of molecular tools for biotechnology may stop this decline, as barley becomes improved and more acceptable as livestock feed and human health food. Genes that can improve barley for poultry feed and for human consumption have been added and are being tested, as are genes for disease resistance (von Wettstein 2004). Recently, barley was designated as a "health food" in North America similar to oats for human consumption. This should lead to further markets and increased production of barley.

3 Current Research and Development

This topic is discussed in three sections in the order of tissue culture, molecular markers and genetic transformation. As the transformation techniques rely to a large extent on the first two topics, they are summarized in that order.

3.1 Cell and Tissue Culture

Barley transformation has been lagging behind some of the other cereals because of difficulties in the regeneration of plants of most genotypes using cell and tissue culture. However, recent studies would indicate that this situation may be improving (for reviews, see Cheng et al. 2004; Murray et al. 2004; Tiidema and Truve 2004). The tissues or cells used for barley transformation are embryos (immature or mature), shoot meristems, microspores and protoplasts. Initially, immature embryos were the most suitable targets.

To date, the systems for the culture of anthers and isolated microspores for haploid production in barley are more advanced than in other cereals. These systems are also effective in breeding new improved cultivars of barley (Thomas et al. 2003; Devaux and Pickering 2005). Transformation of single-cell microspores has been successful in barley only through particle bombardment (Jähne et al. 1994; Yao et al. 1997). *Agrobacterium* does not appear to be able to penetrate the microspore cell wall and has not been compatible with uninucleate microspore survival. However, recent reports indicate that, once the microspore wall is broken, *Agrobacterium* can effectively produce transgenics from these multicellular structures, which may be haploid or doubled haploids (DHs; Kumlehn et al. 2006). Regeneration from callus, or through secondary embryogenesis produced from embryos, has also been difficult, with only a few

genotypes responding (Lemaux et al. 1999). However, as mentioned earlier, recent studies suggest that this genotype problem may be overcome through the use of mature embryo or shoot meristem explants. The culture of protoplasts has also been extensively studied for purposes of gene insertion or cytoplasmic organelle transfer between species or lines (Shillito 1999). With isolated protoplasts, electroporation or polyethylene glycol (PEG) systems are suitable for transformation. However, the predominant methods of transformation in barley today are the bombardment of microspores and embryos or co-culture of embryos or 7- to 8-day-old cultured microspores with *Agrobacterium*.

Embryo culture has been a tool used for many years in cereals following wide hybridization where small embryos have been rescued by culturing them in nutrient media. The use of this approach in barley, hybridization with *H. bulbosum*, led to a system of haploid production in barley (Kasha and Kao 1970) that worked across genotypes with sufficient frequencies to make it efficient for barley breeding programs. Thus far, about 60 cultivars of barley have been produced around the world by this wide hybridization method (Thomas et al. 2003; Devaux and Pickering 2005). More recently, most haploid-derived barley cultivars have been produced by anther or isolated microspore culture (IMC). In total, more than 100 barley cultivars have been produced through time-saving haploid systems, more than in any other crop species. In addition, the DH lines have been used extensively in mutation and cell culture selection and are basic for collaborative molecular marker mapping and map construction.

Anther and IMC culture protocols have also been developed that work well for haploid production in barley, although there are slightly more problems with genotype response limitations, albinism and linkage disequilibrium. However, genotype differences and albinism are not much of a problem today because of the large numbers of haploids produced through IMC. It is recommended that donor plants of the anthers or microspores be grown under a controlled environment, such as in growth rooms or glasshouses in order to obtain consistent results. Any stress to the donor plants through nutrition problems, pests or temperatures can reduce the response in culture (Devaux and Pickering 2005). Various pretreatments of the anthers or microspores at the uninucleate stage are required to induce a high frequency of microspores to enter the embryogenic pathway. In barley, this is usually a mannitol and/or cold pretreatment, although other stresses can also be successful. Various protocols for the induction, culture and regeneration of plants from anthers or IMC are presented in the book edited by Maluszynski et al. (2003). One of the advantages of IMC in barley is that about 80% of the progeny become DHs from processes that occur during the first (PMI) or second (PMII) mitotic division in the microspores. Kasha et al. (2001) demonstrated that failure of cell wall formation occurs due to the induction pretreatment and can lead to nuclear fusion, resulting in a plant that is completely DH and fertile. Nuclear fusion as the main pathway to doubling the chromosomes has been verified cytologically (Gonzalez-Melendi et al. 2005; Shim et al. 2006). The use of anti-microtubule agents during the initial stage of anther or microspore culture can also produce

high frequencies of chromosome doubling (see Kasha 2005). Giménez-Albián et al. (2004) described how the microtubule cycle during mitosis in binucleate mitotic cells could be responsible for nuclear ploidy; and this may be one way that anti-microtubule agents increase ploidy as well as induce embryogenesis.

Regeneration from barley cell cultures for purposes of obtaining transgenic plants has been a problem and has limited the genotypes that can be used in transformation procedures. The main problems are maintaining the regeneration potential of the culture over a three to four month period and preventing the increase of albino plant formation during this same culture period. Efforts to overcome these problems have centered on the evaluation of culture media components (Bregitzer et al. 1998) and the source of somatic cells. Immature embryos, and their scutellum in particular, have been the main target for transformation and regeneration. Most barley cultivars now can be regenerated from these tissues (Rikiishi et al. 2003). Ganeshan et al. (2003) used four commercial barley cultivars and compared plant regeneration from immature embryos, mature embryos and leaf base apical meristems. It was observed that, using the plant growth regulator thidiazuron, direct shoot regeneration from mature embryos and leaf base apical meristems was feasible, with the best being the mature embryos. In the search for systems to obtain better shoot regeneration and overcome genotype effects, Zhang et al. (1999) were successful in barley transformation using in vitro shoot meristematic cultures from germinated seedlings. The use of shoot apical meristem cultures for cereal transformation has been reviewed by Sticklen and Oraby (2005). Both mature embryos from seeds and shoot apical meristems should widen the range of genotypes that can be used for barley transformation. Direct embryogenesis can avoid somaclonal variation that can be induced in callus cultures (Karp and Lazzeri 1992). Using the correct balance of nutrients and hormones in the media (Ziauddin and Kasha 1990; Ziauddin et al. 1992) can also reduce such variation.

A number of improvements in procedures have also led to a wider range of genotypes that can be used in transformation (Cho et al. 1998; Nuutila et al. 2000; Roussy et al. 2001; Manoharan and Dahleen 2002; Chernobrovkina et al. 2004; Murray et al. 2004; Sharma et al. 2004; Tiidema and Truve 2004). However, the first cultivar Golden Promise to respond to *Agrobacterium*-mediated transformation methods still tends to provide higher transgenic frequencies than other cultivars.

3.2 Development of Molecular Markers

Molecular markers are now used routinely in many barley breeding programs (Varshney et al. 2005b). They are used to track important agronomic loci through the selection of parents for crossing and through segregating progeny from crosses. They are also helpful in locating genes on the chromosome regions, thus reducing the need to isolate genes or to use transformation.

Molecular markers have also been used to develop maps for the chromosomes and to identify quantitative trait loci (QTLs) for many agronomic traits, pest or stress resistance and quality traits. The web site http://barleyworld.org will lead readers to most of the molecular maps and mapping projects around the world. Within that site, GrainGenes will provide the molecular mapping of various cereal species including barley. Lörz and Wenzel (2005) have edited review papers on all aspects of molecular markers in many crops. The introductory chapter by Langridge and Chalmers (2005) in that book provides an overview of the identification and application of molecular markers.

The types of molecular markers developed have evolved rapidly over the past 20 years. They can be divided into two classes: those molecular markers closely linked to important genes and those that result from expressed gene sequences. Over time, the molecular markers have been refined to enable more rapid handling and have become smaller in size. The first molecular markers mapped in barley were restriction fragment length polymorphism (RFLPs; Graner et al. 1991; Heun et al. 1991; Kleinhofs et al. 1993). Next to be developed in barley were the randomly amplified polymorphic DNA (RAPDs); and these were quickly followed by simple sequence repeats (SSRs) or microsatellites. La Rota et al. (2005) observed the non-random distribution and genome frequencies of microsatellite markers derived from expressed sequence tags (ESTs) in rice, wheat and barley. Varshney et al. (2005a) showed the interspecific transferability and comparative mapping of barley EST-SSR markers with those in wheat, rice and rye. Varshney et al. (2006) assigned EST-derived SSR markers to BACs and among the most abundant recent markers are single-nucleotide polymorphisms (SNPs) that are developed from expressed barley sequences. Bundock et al. (2006) demonstrated an allele-specific PCR procedure with three primers to produce robust SNP markers with a system that does not require sophisticated equipment. However, ESTs that usually are the partial cDNA sequences produced by reverse transcriptase PCR of mRNA have become the most valuable tools for gene identification, isolation and mapping.

The original linkage maps in barley were based upon genes for morphological traits and biochemical markers such as isozymes. It had taken many years of work to develop these maps. However, with the advent of molecular markers, new molecular marker maps quickly surpassed the old gene maps in both density and coverage of the chromosomes. The integration of the many molecular maps that developed with various types of molecular markers has been difficult but achievable (Varshney et al. 2005a). The first RFLP molecular marker maps were made using populations of DHs produced from the F_1 of crosses between good cultivars. Since each DH produced is genetically homozygous for a different combination of alleles of genes, the DHs could be maintained eternally and the DNA extracted from them could be sent to many collaborators working together on developing molecular maps. These same DH lines could be grown in replicated field trials at various locations to identify and locate QTLs for various agronomic traits such as yield, pest and

stress resistance, as well as for quality traits. One of many such collaborative groups was the North American Barley Genome Mapping Project (NABGMP), consisting of 49 researchers from the United States and Canada, with Kleinhofs et al. (1993) providing the first of many map publications.

For use in breeding, the mapping of QTLs also meant that they could be tagged with molecular markers located close to each QTL. The molecular markers could be assigned to a physical location on the chromosomes by in situ hybridization (ISH) and, subsequently, by their relationship to chromosomal interchange breakpoints (Sorokin et al. 1994; Kunzel et al. 2000). Such studies have confirmed the uneven distribution of recombination frequency and more precisely located the molecular markers or genes along the chromosomes, with the majority being located in the distal regions of the barley chromosomes. Varshney et al. (2005b) concluded that 4.9% of the physical map developed from the Igri × Franka cross contained high recombination rates and 47.3% of 429 markers assigned to the map.

The eventual goal of mapping is to identify the genes and to sequence them. For this purpose, large insert DNA libraries have been constructed, both as bacterial artificial chromosome (BAC) and yeast artificial chromosome (YAC) contigs (Varshney et al. 2005b). A number of additional BAC libraries are under construction or completed (Kleinhofs 2004). In addition, a collaborative approach has produced a large number of ESTs that have been placed in a common database (http://www.ucbi.nih.gov/UniGene/). It lists 344,352 ESTs, as of March 2005. These ESTs are thought to include about 40,000 distinct genes or about 85% of the gene compliment of barley (Varshney et al. 2005b). The aim is to develop a high-density transcript map using about 1000 ESTs that would complement the existing molecular marker consensus map. The latter consists of 510 RFLP, 234 SNP and 185 SSR markers, with at least 100 markers in total on each of the seven chromosomes. Another valuable crop EST data base (CR-EST) has been established online with public access by Kunne et al. (2005) and it contains over 200,000 ESTs. It provides online access to sequence, classification, clustering and annotation data derived from cDNA libraries of four species: barley, wheat, pea and potato. About one-third of these ESTs are derived from barley. Sequences are clustered in species-specific projects and have generated a non-redundant set of about 22,600 consensus sequences and about 17,200 singleton sequences.

Comparative mapping of marker and gene maps is extremely useful in determining synteny between different cereal and grass species and thus, in the study of their evolution. Moore (1995) proposed that the grass genomes are made up of conserved segments and all are derived from a common ancestor. The early molecular markers such as RFLPs permitted the rough assignment of homoeologous chromosome regions between species with various different basic numbers (x) of chromosomes (Moore 1995).

The number of mapped genes in barley exceeds 1100 and these markers are preferred over random DNA markers such as RFLP and SSR, as genes are more conserved in evolution and useful in crop improvement. The ESTs are also being

developed in other related species and this helps in identifying more genes in all species (Waugh et al. 2004). In rice, for example, there are 6591 ESTs placed on a physical contig map (Wu et al. 2002). When comparing mapped barley ESTs against this rice database, sequence identity was more than 80% (Varshney et al. 2005b) and there were more than 30 syntenic genes on each barley chromosome. Thus, ESTs represent a means for rapidly identifying genes in all cereal and grass species. La Rota et al. (2005) found a non-random distribution of EST-derived microsatellite markers in cereals. This might be expected since the genes appear to be in small clusters and located more distally than centrally on the chromosomes. More recently, Varshney et al. (2006) attempted to map EST-derived SSR markers on a BAC library in barley. They found a non-uniform distribution of genes in the barley genome, concluding that the barley genome contains gene-rich and gene-poor regions.

An important collaborative project (Close et al. 2004) has been the development of a micro-array chip (Barley 1 GeneChip) fabricated by Affeymetrix, which contains about 22 000 unique 25-mer 3′ end ESTs screened from over 400 000 ESTs in barley. This chip will greatly facilitate the identification and location of various genes by their expression on the matrix. It, as well as the database of Kunne et al. (2005) will be extremely useful for comparing identified ESTs from other cereal species and we can expect a very rapid advance in numbers of known genes in barley.

A number of other tools are being used to assist in assigning genes and gene function in barley. Koprek et al. (2000) introduced Ds transposable elements into barley to help detect genes and gene function. Doležel et al. (2004) used flow cytometry chromosome sorting to develop libraries for individual chromosomes or parts of chromosomes. In barley, only chromosome 1H could be distinguished by sorting, but through the sorting of unequal chromosomal interchange stocks, it was possible to sort segments of the chromosomes ranging from 5% to 9% of the total genome. Because of the numerous markers mapped and those yet to be mapped in barley, Kleinhofs and Graner (2001) proposed that the chromosome regions be divided into 10 cM (centi-Morgan) "bins", with a distinct marker at each end of a "bin". Newbigins et al. (2004) discussed functional genomics that requires the technology to identify large sets of genes that influence a particular biological process. Such research includes genomics, proteomics and metabolomics and would require high-throughput data collection and analysis of gene function and structure. Barley DNA micro-arrays, high-density genetic maps and barley EST and mutant libraries provide critical support for this research. In the future, the development of procedures to measure gene activity by monitoring and assessing the amount of mRNA produced from such genes in a related group will provide insight into the expression of quantitative traits of agronomic importance and biochemical processes (Waugh et al. 2004). Recent reports on using proteomics to analyze traits in barley include those of Corrado et al. (2005), working on the photosynthetic apparatus, Maeda et al. (2005) and Wong et al. (2002), working on thioredoxin h relative to seed proteins, and

Finnie et al. (2004) working on stress and fungal resistance as well as malting quality.

3.3 Transformation in Barley

Genetic transformation of barley, like many other grass or cereal species, has been hindered by the lack of a system that is easy to handle and that will produce sufficient numbers of transgenic plants. Many systems for transformation have been tested in barley (Mannonen et al. 1994; Lemaux et al. 1999; Murray et al. 2004). However, only three systems have produced transgenic plants. The first reports with these systems are the electroporation of protoplasts (Mannonen et al. 1994), particle bombardment of immature embryos (Wan and Lemaux 1994; Ritala et al. 1994), microspores (Jähne et al. 1994) or shoot meristems (Zhang et al. 1999) and the inoculation of immature embryos with *Agrobacterium* (Tingay et al. 1997).

There are many reasons why barley transformation has lagged behind other cereals. The slow development of efficient tissue culture systems for plant regeneration after three to four months in culture is one; and this is associated with increased albino plant production with length of time in culture. High frequencies of somaclonal variation were indicative that improved embryogenic response from cultures was required. The first report with *A. tumefaciens* (Tingay et al. 1997) used the cv. Golden Promise and this cultivar remains the best for regeneration of transgenic plants. Recently, Wang et al. (2001) and Murray et al. (2004) were able to obtain very low frequencies of transgenic plants with three Australian cultivars using the *Agrobacterium* system. Tiidema and Truve (2004) reported improved regeneration with some Nordic cultivars, while Roussy et al. (2001) evaluated the transformation and regeneration capacities of five Nordic barley elite cultivars. The limitations of using electroporation of protoplasts are similar to those of using *Agrobacterium*, namely the difficulty in regeneration of plants from protoplasts and the frequencies of the production of albino plants.

The majority of barley transformation successes, to date, have been from particle bombardment of immature embryos, or the scutellum from such embryos, because success with *Agrobacterium* has been limited by strong genotype dependency. Southgate et al. (1995) and Klein and Jones (1999) reviewed the factors affecting transformation of plants by microprojectiles. Microprojectile bombardment has a few desirable attributes, such as being highly versatile and adaptable to a wide range of tissues and cells. It is simple to utilize and is widely used for the study of gene expression and to test the efficiency of different gene promoters.

Although the *Agrobacterium* system has had limited success, it has some advantages. One is that the majority of transgenic plants have a single intact gene copy inserted at one site. In contrast, particle bombardment tends to introduce more than one copy at a site and some of the copies may not be com-

plete and transgene instability is higher (Harwood et al. 2004; Travella et al. 2005). Svitashev and Somers (2001) showed through fiber-FISH (fluorescence in situ hybridization) studies that the multiple inserts are found at one site and are interspersed with host genomic fragments of various sizes following bombardment. However, methods to select plants with a single transgene copy and greater stability following bombardment are available and such lines are currently under field evaluation (von Wettstein 2004). Fang et al. (2002) studied the sequences of the T-DNA inserted by *Agrobacterium* and found the right T-DNA ends were highly conserved, while the left ends were more variable. They showed the lack of transmission of the vector sequences, suggesting a mode of molecular T-DNA transfer similar to that in dicotyledonous plants. Another advantage of *Agrobacterium* transformation is that, with double-cassette vectors, it is possible to remove the selectable marker cassette by selection in segregating generations following transformation (Matthews et al. 2001; Stahl et al. 2002).

Koprek et al. (2001) demonstrated a method to produce single transgene copies using transposons delivered via particle bombardment. Taking the Ac/Ds system from maize, the gene of interest was placed between inverted-repeat Ds ends and transformed into the host cells. Regenerated plants were then crossed with a plant containing the Ac element that activated the movement of Ds. Thus, many plants that had a single copy of the gene of interest were selected among the F_2 progeny. Such plants were shown to be highly stable in the expression of the inserted gene, allowing one to obtain stable transgenic plants following transformation by particle bombardment. Cooper et al. (2004) were able to map the Ds insertions in barley using a sequence-based approach. von Wettstein (2004) and colleagues successfully used this system in transforming barley with genes valuable for malting, non-ruminant feed and disease resistance.

The bombardment of isolated immature microspores offers the potential to obtain DH plants homozygous for the transgene. Jähne et al. (1994) obtained homozygous transgenic DH plants when using a long 28-day cold (4 °C) pretreatment of spikes with immature microspores before bombardment, whereas Yao et al. (1997) obtained only hemizygous transgenic plants when they pretreated by incubating in 0.3 M mannitol at room temperature for four days. Lemaux et al. (1999) suggested that the difference between the results of Jähne et al. (1994) and Yao et al. (1997) was that the microspores treated only in mannitol at room temperature would be at an older stage when bombarded and, therefore, would be hemizygous for the transgene. Shim and Kasha (2003) demonstrated that, during the pretreatment in mannitol for only four days at room temperature, most of the microspores would have completed PMI (first post-meiotic mitosis) without cell wall formation. This permitted nuclear fusion (Kasha et al. 2001) and a few nuclei had entered PMII before bombardment. However, when using a cold plus mannitol pretreatment, the microspores were held at the uninucleate microspore stage, whereas after a 21-day cold pretreatment most microspores had completed the S phase of the cell cycle and many

had gone through PMI. Thus, it was not clear why Jähne et al. (1994) had obtained homozygous transgenic DH plants. However, González-Melendi et al. (2005) cytologically showed that chromosome doubling in barley microspores can occur after the subsequent mitotic divisions by nuclear fusion of multinucleate microspores. Therefore, the transgene could be incorporated during the S phase in these subsequent nuclear divisions prior to nuclear fusion. Other advantages of barley transformation using the culture of isolated microspores are that success can be obtained across genotypes and a high proportion of regenerates are derived by direct embryogenesis, reducing the potential for somaclonal variation. While the frequency of transgene-expressing microspores is very low, large numbers of microspores are available from which can be obtained adequate numbers of transgenic plants.

The treatment of uninucleate barley microspores with *A. tumefaciens* has not been successful to date because the microspores do not survived the 2-day *Agrobacterium* treatment (Kasha and Gu, unpublished data). When the barley microspores become multicellular after 6–8 days in culture, they can survive *Agrobacterium* treatment, but the goals of obtaining completely fertile DHs that are homozygous for the transgene and free from chimeric sectors are lost. However, Kumlehn et al. (2006) showed that, once the microspore wall has been disrupted by the multicellular structures within, the *Agrobacterium* can induce transformation and large numbers of transgenic plants can be selected and regenerated. The transgenic haploid barley plants can then be treated with anti-microtubule agents to induce chromosome doubling and to produce plants homozygous for the transgene. Such plants will only be partially doubled but will produce sufficient seed to obtain the homozygous transgenic line.

Trifonova et al. (2001) studied a number of factors involved in *Agrobacterium*-mediated transformation using immature embryos of the barley cv. Golden Promise. Using 12 different procedures, they obtained transgenic plants with five of these procedures; and the frequency of success ranged from 1.7% to 6.3%. Southern analysis proved transgene integration with a copy number from one to six, but most had a single integration with the expected Mendelian inheritance patterns.

Travella et al. (2005) compared the production of transgenic barley lines by particle bombardment with *Agrobacterium*-mediated techniques on immature embryos. They compared transformation efficiency, transgene copy number, expression, inheritance and location of the transgenes. The efficiency of *Agrobacterium* transformation was 2%, whereas that following bombardment was 1%. The *Agrobacterium*-mediated transgenic plants had from one to three copies of the gene, while 60% of the plants produced following bombardment had more than eight copies. Relative to inheritance pattern, all six lines tested from the *Agrobacterium* system showed the expected inheritance patterns, while only three of nine lines from bombardment showed the expected inheritance pattern. A high frequency of gene silencing was observed after DNA delivery by bombardment, which is consistent with the earlier study of Bregitzer and Tonks (2003). This study strongly suggests that *Agrobacterium*

is more effective for transformation using immature embryos as targets in barley. However, the limitation of only a few responding genotypes needs to be resolved and this might be achieved by using microspore culture and transformation, as demonstrated by Kumlehn et al. (2006).

Through the use of in situ hybridization, a number of studies examined the sites on the barley chromosomes where the transgenes were inserted (Pedersen et al. 1997; Salvo-Garrido et al. 2001, 2004; Choi et al. 2002). Some inserts appeared to be at random sites amongst the seven chromosomes, while other studies showed a more limited distribution. Large numbers were not examined in any study, but the general conclusion is that inserts tend to be towards the ends of the chromosomes and, more often than expected, in regions of active genes. For example, about 75–80% of the barley genome is made up of highly repeated retrotransposons; and von Wettstein (2004) mentioned that, when chromosome sites were determined, only 12 of 46 transgenic sites were located in the retrotransposons. There is a trend in the findings of all studies showing that the locations of transgenes are distributed more in the telomere or subtelomere regions of the chromosomes (Choi et al. 2002; Salvo-Garrido et al. 2004). This is consistent with the reports of von Wettstein (2004) and Varshney et al. (2005a), showing that transgenes and molecular markers, respectively, are located more often in small gene clusters.

In attempts to improve the efficiency of transformation in barley, many factors have been examined. Cheng et al. (2004) reviewed the factors influencing *Agrobacterium*-mediated transformation of monocotyledonous species. These factors include plant genotype, explant type, *Agrobacterium* strain, plasmid construction and the use of binary vectors. For the *Agrobacterium* infection stage, the recovery of plants is influenced by osmotic pretreatment, antioxidants and bactericides, desiccation of the target explants and the inoculation and co-culture media composition. For example, Y.S. Shim (unpublished data) found improved transgenic plant recovery when adding arabinogalactan protein (AGP) to the culture media following bombardment. AGP has been shown to improve the viability of microspores in culture and improve the frequencies of recovered plants from isolated microspore culture of wheat (Letarte et al. 2006).

Many types of selectable markers have been used and various promoters attached to them have been studied. Antibiotics and herbicide resistance have been used extensively as selectable markers in cell cultures, while green fluorescent protein (GFP; Sheen et al. 1995; Pang et al. 1996) and the firefly luciferase gene (Harwood et al. 2002) are the main markers used to follow the stability of transgene expression. Because of the undesirability of having selectable marker genes and other foreign DNA in food products, transformation systems have been developed for producing marker-free transgenic barley (Xue et al. 2003; von Wettstein 2004). Xue et al. (2003) used binary vectors with the marker gene on a separate vector from the transgene of interest, so that the marker could be removed in subsequent segregating generations. Hensel and Kumlehn (2004)

reported a protocol for *Agrobacterium* transformation on immature barley embryos.

The stability of transgene expression in subsequent generations is a concern and must be examined for a number of generations following the selection of the transgenic plant. The copy number of insert is a major factor, as is the construction of the plasmid/gene constructs used, relative to stability. *Agrobacterium* is favored over other methods used for introducing genes as it tends to have fewer copy numbers inserted into the plant host. This would reduce the number of transgenic plants required in order to obtain stable transgenic plants. The promoters used for the gene to be inserted have also been found to influence the stability of the transgene in the host plant; and tissue-specific promoters are also valuable in some instances (Furtado and Henry 2005). Cho et al. (2002) and Choi et al. (2003) observed that the barley endosperm-specific hordein promoters driving *uidA* or *sgfp* marker genes were much more stable through the T4 and later generations than the maize ubiquitin promoter. Schunmann et al. (2004) studied the promoters from the phosphate transporter genes (*Pht1*) in barley and found a 20-fold increase in marker gene expression when an intron was utilized, supporting the concept that introns incorporated into gene constructs are important for expression. They concluded that the *Pht1* promoters were ideally suited for driving the expression of foreign genes associated with nutrient uptake. Petersen et al. (2002) demonstrated that including matrix attachment regions (MARs) in the plasmid/gene construct both enhanced transformation frequencies and improved transgene expression in barley.

4 Practical Applications of Biotechnology in Barley

The applications of transformation in barley have, to this point in time, concentrated on inserting disease, stress and pest resistance genes. There is also interest in using transformation to improve the quality and malting characteristics of barley and, in the future, in developing new foods from barley with improved or new quality traits. Because barley is grown around the world for feeds and food, there has been little or no interest in using barley as a host species for the production of pharmaceuticals.

While regeneration from somatic cell and tissue culture has been problematic in barley, the production of haploids from wide crosses or microspore culture has been exemplary (Thomas et al. 2003; Devaux and Pickering 2005), with well over 100 DH-derived cultivars being released world-wide. The extensive development of molecular marker maps in barley (Varshney et al. 2005a) has been accompanied by the tagging of genes and QTLs. That has then led to the quite extensive use of molecular marker-assisted selection (MMAS, or more simply MMS). Ullrich et al. (2004) reviewed some of the North American

research on MMAS and listed web sites for different areas of the world where the studies are posted. Results with MMAS are encouraging and many breeders now have the facilities to exploit this approach. The markers are shifting from random DNA molecular markers to genes, facilitated by ESTs that also simplify locating and sequencing genes (Sato et al. 2004). The ESTs are also used to select markers like SNPs and SSRs (Kota et al. 2003) that can then be used to mark the genes themselves. The Barley 1 GeneChip disc will also greatly facilitate gene identification and sequencing (Close et al. 2004), as will the CR-EST resource (Kunne et al. 2005).

Transformation of barley is expected to progress more rapidly now, with the identification in many cereals of genes through the available ESTs. At present, a number of genes for disease resistance have been isolated and transformed into susceptible barley cultivars (von Wettstein 2004). These include the *Rpg1* stem rust resistance (Horvath et al. 2003; Rostoks et al. 2004), the endochitenase gene (*ThEn42*) from the fungus *Trichoderma harziamun* that can confer resistance to one or more *Rhizoctonia* root rot organisms (von Wettstein 2004), the *mlo* gene for powdery mildew resistance (Bieri et al. 2004) and virus resistance (Wang et al. 2001; Stein et al. 2005). Genes for stress tolerance and malting quality are also of interest, the latter more specifically for food processing. To convert barley from a low nutritional value to a high nutritional value for poultry, a gene (1,3-1,4)-β-glucanase from *Bacillus* was transformed into barley (von Wettstein 2004) which improved the nutritive value to that of maize. In tests with chickens, the addition of 0.02% transgenic grain to barley feed achieved as high a nutritive value as adding commercial enzymes to barley feed for non-ruminant animals. Xue et al. (2003) increased cellulose production in barley by inserting a hybrid cellulose gene. Matthews et al. (2002) studied both α-amylase production and the transport of gibberellin in malting grain by inserting a marker tagged α-amylase gene into barley. Kim et al. (2003) found that thioredoxin h over-expression enhanced selenite resistance and uptake during germination, while Maeda et al. (2005) found it was involved in the regulation of protein in barley seeds. Wong et al. (2002) observed that thioredoxin h influenced communication between the embryo and aleurone. Antisense constructs of limit dextrinase inhibitor (LDI) protein in barley modulates quality factors such as starch granule size, amylopectin structure and starch composition (Stahl et al. 2004). The evaluation of transgene stability under field conditions and in breeding programs is also necessary (Horvath et al. 2001). However, an expressed inserted gene may not always improve the trait desired, as Rae et al. (2004) observed with a high-affinity phosphate transporter gene in barley.

Delhaize et al. (2004) engineered high aluminum tolerance in barley by inserting a gene (ALMT1) from wheat that is associated with malate efflux and aluminum tolerance. The barley *Lem 1* gene promoter drives expression in outer floret organs in wheat and may be useful in engineering organ-specific *Fusarium* resistance (Somleva and Blechl 2005).

5 Conclusions and Future Challenges

The production of haploids in barley through wide hybridization and microspore culture for the development of new improved cultivars has been very successful, with more cultivars produced than in any other crops. Haploid-produced populations have also been essential for much of the molecular marker mapping in barley, as the DHs are eternal populations of homozygous genetic lines. Such lines are also valuable for mutation and selection research (Szarejko 2003). Further exploration of the potential to obtain fertile DHs that are homozygous for transgenes is warranted, although Kumlehn et al. (2006) provided evidence for a method using 7- to 8-day-old microspore cultures.

The development of molecular marker maps and, more recently, gene maps through the use of ESTs, has accelerated the identification of genes in barley. This will be further enhanced by similar work in related species because of the synteny of genes and chromosomes. Gene isolation in barley through DNA sequencing aided by BAC and YAC libraries and the use of transposable elements will also continue to be valuable. In the near future, we can expect the identification of genes involved in many traits. For example, through the combination of QTL and EST studies, we can expect many of the genes involved in grain quality to be identified and examined for their roles. The ability to identify the genes involved in a process (functional genomics) is feasible and this will open up a much more precise monitoring of plant physiology and gene expression in the development of traits (Newbigins et al. 2004). This will enable biotechnology to change genes for the improvement of feed and food quality. The study of proteomics (proteins produced by known genes and their interactions) will become a major area of research.

For transformation, research on identifying the genes and their locations is important for the incorporation of transgenes by homologous recombination, leading to more stable transgenics (Monostori et al. (2003). Much more research on the methods of producing transgenics in barley and all cereals is required. Gene constructs with tissue-specific promoters and their delivery into and selection among totipotent cells are also in need of improvement.

Barley has the potential to be classed as a health food like oats and this will open new markets for barley producers.

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