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Pioneering Biotechnological Works on *Hordeum vulgare* L. cvs Performed in Collaboration with the İstanbul University Biology Department and the TÜBİTAK Research Institute for Genetic Engineering and Biotechnology

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Abstract: *Hordeum vulgare* L. (barley) is an important cereal crop and is also an excellent model organism for biochemists, physiologists, geneticists and molecular biologists. *H. vulgare* cvs. have been used as a model system for almost 30 years at the Biology Department of İstanbul University, İstanbul-Turkey. The first studies on experimental mutagenesis were followed by tissue culture, gene transfers, DNA marker applications and finally DNA arrays which, progressed further after the 1990 when collaboration was established with the Plant Biotechnology group at the TÜBİTAK Research Institute for Genetic Engineering and Biotechnology in Gebze, Kocaeli-Turkey. This review article outlines the results of original research into Turkish barley cultivars and wild types with the intention of contributing to barley-breeding programmes with recent biotechnological techniques.

Key Words: barley, mutation, tissue culture, gene transfer, DNA array

İstanbul Üniversitesi Biyoloji Bölümü ve TÜBİTAK Gen Mühendisliği ve Biyoteknoloji Araştırma Enstitüsü İşbirliği ile *Hordeum vulgare* L. cvs.'de Gerçekleştirilen Öncü Biyoteknolojik Çalışmalar

Özet: *Hordeum vulgare* L. (arpa) önemli bir tahıl bitkisidir, biyokimyacılar, fizyologlar, genetikçiler ve moleküler biyologlar için çok etkin bir model organizmadır. *Hordeum vulgare* cvs. yaklaşık 30 yıldır İstanbul Üniversitesi Biyoloji Bölümü'ndeki genetik temelli çalışmalarda model sistem olarak kullanılmaktadır. İlk deneysel mutasyon çalışmaları 1990'lı yıllarda TÜBİTAK, Gen Mühendisliği ve Biyoteknoloji Araştırma Enstitüsü, Bitki Biyoteknolojisi Grubu işbirliği ile doku kültürü, gen transferleri, DNA marker uygulamaları ve DNA "array" çalışmaları ile sürdürülmüştür. Bu derleme makalede Türk kültür ve yabancı arpa varyeteleri ile yapılan özgün çalışmalar arpa yetiştiriciliğine güncel biyoteknolojik yöntemlerle katkılar sağlanmak amacı ile özetlenmiştir.

Anahtar Sözcükler: arpa, mutasyon, doku kültürü, gen aktarımı, moleküler markırlar, DNA-"array"

Introduction

Cultivated barley (*Hordeum vulgare* L.) is the second-most important cereal crop for Turkey after wheat, and is consumed as feed for livestock and, food for humans and, most importantly, is also used for brewing malts. Barley is also an excellent model plant for biochemists, physiologists, geneticists and molecular biologists (Shewry, 1992). According to world statistics, it is cultivated on 53,827,895 hectares with a 25,723 Hg/Ha world yield of which Turkey's contribution is 3,550,000 hectares with an 18,592 Hg/Ha yield (FAO, 2001). Barley is a self-pollinating diploid with $2n = 2x = 14$

chromosomes. Moreover, it has two-rowed and six-rowed types, according to spike morphology (Bothmer et al., 1991). The genome size of barley is 5.5 picogram/haploid nucleus and is equivalent to approximately 5.3×10^9 bp (Bennet & Smith, 1976), and 50-60% of the genome consists of repeated sequences (Rimpau et al., 1980). Copia-like retrotransposon BARE-1 comprises almost 7% of the barley genome (Manninen & Schulman, 1993). Ease of growth under laboratory conditions facilitates the development of molecular markers for the construction of genetic maps (Williams et al., 2001). The barley genome project and production of

barley ESTs are in progress with contributions from various organisations (Michalek et al., 2002). Recently, the first functional genomic studies were carried out for stress tolerance (Öztürk et al., 2002) and tissue-specific differential expression (Sreenivasulu et al., 2002) in barley.

The main objective of barley breeding programmes is mainly to increase yield and grain quality. Improvement efforts are also concentrated on producing varieties resistant to biotic (pathogens, fungal, viral and other organisms) and abiotic stresses (e.g. drought, salt, cold and heat) (Dunwell, 1986). During conventional breeding programmes via hybridisations between high-yielding cultivars and wild barley, specific traits may be introgressed in back-crossing programmes (Nevo, 1992). Mutation breeding is also important for widening variation. Radiation and chemical mutagenesis have been used to increase the numbers and varieties of barley which might have desirable traits. For example, one of the most popular malting barleys, "Golden promise", was produced in 1957 using radiation mutagenesis (Milne Marsters Co., 1970).

Professor Emine Bilge performed the first basic genetic experiments in which barley was used at the Biology Department of İstanbul University in the framework of the project "Basic Genetic Studies for Obtaining High Quality Barley Lines" Grant No.162, TÜBİTAK, TOAG. In this study, Zafer 160 barley seeds were treated before sowing with X and gamma rays, ethyl alcohol, streptomycin, terramycin, penicillin G, sodium cyanide and ethylmethane sulphonate solutions. In addition to chlorophyll deficient types, large-eared, high-yielding, thick-stemmed, dwarf and early-heading mutants were obtained in M₁ and succeeding generations of the treated material. As a result of seed irradiation with 16,000 rad doses of X-rays, a mutant barley called KA/14 was obtained. The ear shape of this mutant resembled that of the hooded type, and, the number of tillers and the yield were higher than the control. After artificial pollination of Zafer 160 females with 1000 rad gamma irradiated pollen, short-stemmed and early mutants appeared in the F₂ generation. Their heading time was 23 days earlier than that of the control. Meiosis was studied in the anthers, and the following abnormalities were observed in the treated material: breaking and sticking together of the chromosomes, chromatin bridges, translocations, micronucleus

formation, spindle splitting and non-synchronised phases in the second division etc. (Bilge et al., 1981 a,b). The effects of X and gamma rays on mitotic cell division and the protein content of the irradiated seeds were also investigated (Olgun, 1985). The adaptation efficiency and micro yields of these mutant types were studied in the framework of "Studies on Agricultural Applications of Experimental Mutations Induced on Native Barley Variety Zafer 160" TÜBİTAK, TOAG Grant No.162.

Mutation studies were continued on two projects, one of which was supported by TÜBİTAK-TBAG Grant No. 515 and the other by İstanbul University Research Foundation Grant No. 212/030186, on tissue cultured material. In the framework of the first project the effects of pesticides were studied. The effects of two commercial pesticide preparations, 2,4-dichlorophenoxyacetic acid isooctylester (2,4-D) and phenylmercury acetate (PMA) on different organisms were investigated. These reagents did not produce numerical and structural changes in the mitotic chromosomes of *Hordeum vulgare* embryo cultures (Oraler et al., 1984). In the second project the effects of X and gamma rays on calli cultures were studied. Mature embryo parts were used for callus formation and plant regeneration was achieved on Murashige-Skoog (MS) medium (Gözükırmızı & Ekmekçiler 1987; Arı 1994).

Gene transfer technologies offer a suitable alternative for improving desirable gene(s) in a directed manner without the undesirable insertion of DNA fragments. The establishment of stable and regenerative tissue culture systems is a prerequisite for barley transformation. Different explants, immature embryos (Breiman, 1985), mature embryos (Lupotto, 1984), apical meristems (Chen & Smith 1975), anthers (Kao & Horn 1982), microspores (Köhler & Wenzel 1985), cell suspensions (Kott & Kasha 1984) and protoplasts (Lazzeri & Lörz 1990) have been used for this purpose.

In 1987, under a grant from NATO-TU-BIOTECH I, No. 842 in subproject 1.2.2 entitled "Callus Induction, Plant Regeneration and Chromosomal Variations in Barley" callus cultures were induced on mature embryo mesocotyl explants in Zafer 160 barley. The callus induction ratio was 54% in MS medium supplemented with 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). After transfer at 22, 45, 360 and 540 days of culture to MS medium, containing lower concentrations of or lacking 2,4-D, only the 45-day-old callus showed somatic

embryogenesis (Fig. 1). Abnormalities in both the number and structure of chromosomes increased with the age of the calli. This phenomenon might be related to the loss of regeneration ability in 540-day-old calli. In vitro regenerated plantlets gave rise to normal-looking plants after their transfer to soil. Regenerated plants had the normal diploid chromosome number in their root tips (Gözükırmızı et al., 1990). Anther and microspore cultures of the same variety were also established (Arı et al., 1992).

Plant transformation was achieved using the electrophoresis of germinating seeds (Ahokas, 1989) or the incubation of embryos in a DNA solution (Töpfer et al., 1989), PEG and electroporation-mediated protoplast transformation (Junker et al., 1987; Teeri et al., 1989), microspores electroporation (Joersbo et al., 1990), particule bombardment (Wan & Lemaux 1994), macro-injections (Mendel et al., 1990) and micro-injections (Olsen, 1991) in barley.

The Plant Biotechnology Group was organised in 1992 at TÜBİTAK's, Marmara Research Centre, and biotechnological research on barley was supported as a strategic project until 2000. Transformation was performed using both biolistic and tissue electroporation techniques at TÜBİTAK laboratories. In general, the second technology was used successfully for the first time in the literature (Gürel & Gözükırmızı, 2000). This study was conducted to detect the optimum conditions for DNA transfer into mature embryos via electroporation. Cultured mature barley embryos were directly electroporated in the presence of the pBI 121 vector carrying both the glucuronidase and neomycin phosphotransferase genes. It was found that 500 v/cm and 500 Fd capacitance was the optimum combination

for the healthy germination of transformed plants from mature electroporated embryos. Gene transfer performed on 3-day-old cultures resulted in the highest germination frequencies. Transgenesis was confirmed by PCR and Southern hybridisation analyses (Gürel & Gözükırmızı, 2003).

A variety of molecular markers have become available in recent years (Mohans et al., 1997; Gupta et al., 1999), and efforts are also being made to identify the most efficient and cost-effective markers that can be used by practicing plant breeders. In addition to their use in plant breeding, molecular markers have been put to several other uses, including genome mapping (Kleinhofs et al., 1993; Han et al., 1993), DNA fingerprinting (Faccioli et al., 1999) and the study of genetic diversity (Baum et al., 1997).

In 1992, the plant biotechnology group in TÜBİTAK started investigating molecular markers using RAPD techniques. Tissue culture regenerated plantlets were tested for stability (Gözükırmızı et al., 1992), methods were developed for hybrid selection from wild lines and cultivars of barley (*Hordeum vulgare* cvs. Kaya, Quantum, Tokak, Yerçil and Cumhuriyet) and these hybrids were characterised by random amplified polymorphic DNA assay. DNA isolated from parents and F₁ hybrids was amplified using 10 base long primers. Hybrids giving selective banding patterns from both the cultivars and wild parents were taken as real hybrids. This technique is convenient for plant breeders since it is rapid, sensitive and inexpensive (Arı et al., 1995). At this time wild type barleys originating in Turkey were being obtained from gene banks, and using these seeds a DNA bank was established and DNA fingerprinting studies were performed for the first time in Turkey (Gürel & Gözükırmızı, 1998; Albayrak & Gözükırmızı 1999).



Figure 1. Tissue culture stages of Zafer 160 barley. (A) Embryogenic callus (B) Somatic embryo (C) Plantlet regeneration (Gözükırmızı et al., 1990).

In 1997, our group became a partner in the EUREKA-1322 Cerealsstressol Project. We aimed to investigate physiological and molecular markers related to drought tolerance. We investigated the correlation between the drought-associated traits of two F_2 populations derived from the crosses made between drought-tolerant and drought-sensitive barley and wheat parental genotypes. The parental genotypes of these crosses also differed by at least three other traits: paraquat tolerance, leaf size and relative water content. These three traits were scored in two F_2 populations of 80 individuals for each barley and wheat cross. Analysis of the results indicated that enhanced tolerance to paraquat correlated with water stress phenotypes of the drought-tolerant barley and wheat parents. Our results suggested that selection based on paraquat tolerance is technically less demanding and thus useful for rapid screening for enhanced drought-tolerance in segregating populations (Altinkut et al., 2001). Using the same material, some promising drought-related 'amplified fragment length polymorphisms' (AFLP) (Altinkut et al., 2003) (Fig. 2) and 'simple sequence repeats' (SSRs) markers were also found, not only in barley (unpublished results), but also in wheat (Altinkut & Gözükmızı, 2003).

Which genes are expressed in different cell types under different conditions will allow the prediction of gene expression networks, thereby uncovering the logic of transcriptional control. Such analyses at the transcriptional level will be accompanied by similar analyses at the protein expression level, leading to the development of an integrated model of cellular gene and protein expression dynamics. In the new millennium we aim to establish the DNA array technique and facilities at

the TÜBİTAK laboratories. To this end one of the PhD. student's has joined Professor Bohnerts' group at Arizona University to learn the technology. During this project responses to drought and salinity in barley (*H. vulgare* cv. Tokak) were for the first time monitored by the micro-array hybridisation of 1463 DNA elements derived from cDNA libraries of 6 h and 10 h drought-stressed plants. Functional identities indicated many cDNAs in these libraries associated with drought stress. Approximately 38% of the transcripts were novel and functionally unknown. Hybridisation experiments were analysed for drought- and salinity-regulated sequences. A significant change was defined as a deviation from the control exceeding 2.5-fold. Transcript responses showed stress-dependent expression patterns and time courses. Nearly 15% of all transcripts were either up- or down-regulated under drought stress, while NaCl led to changes in 5% of the transcripts (24 h, 150 mM NaCl). The transcripts that showed significant up-regulation under drought stress were exemplified by jasmonate-responsive, metallothionein-like, late-embryogenesis-abundant (LEA) and ABA-responsive proteins. The most drastic down-regulation was observed for the photosynthesis-related function category. Up-regulation under both drought and salt stress was restricted to ESTs for metallothionein-like and LEA proteins, while increases in ubiquitin-related transcripts characterised salt stress. A number of functionally unknown transcripts from cDNA libraries of drought-stressed plants showed up-regulation by drought but down-regulation by salt stress, demonstrating how precisely transcript profiles describe different growth conditions and environments (Öztürk et al., 2002).

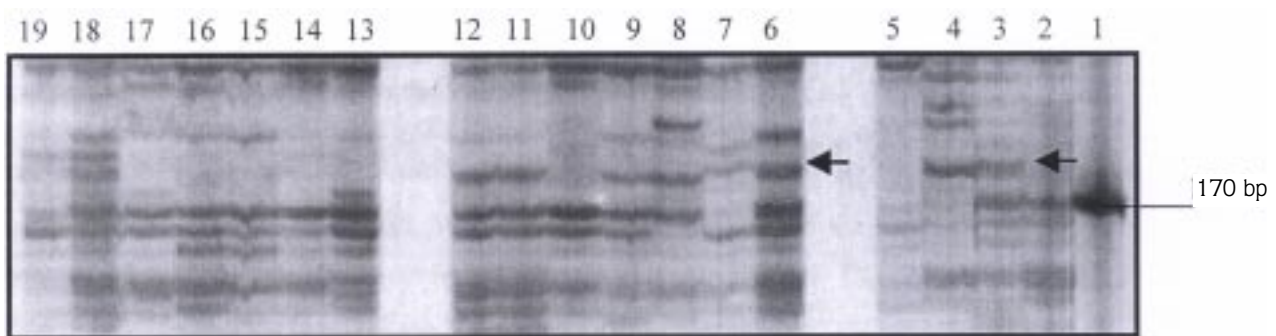


Figure 2. Amplification of drought-related AFLP markers on 6% polyacrylamide gel containing 7.5 M urea. 1-10 bp ladder, amplification from sensitive parent ST5819 (2), tolerant parent Tokak (3), tolerant bulk (4), sensitive bulk (5). 6-12: tolerant F_2 individuals, 13-19: sensitive F_2 individuals. AFLP marker is indicated with an arrow (Altinkut et al., 2003).

Except for the last data, all the investigations were carried out using facilities in Turkey. At the moment we are investigating stress tolerance markers using both molecular markers, in situ hybridisation and cDNA-AFLP profile technologies, in the framework of the grants from İstanbul University Research Foundation Grant No.1676. With regard to barley as a model organism we always tried to keep up with recent applications. One MSc and five PhD theses were completed as part of these investigations, and one more is still continuing. The valuable contributions by these scientists comprise the majority of the articles cited in the references. While carrying out the studies mentioned above we were in close collaboration with agricultural faculties and agricultural research institutions all over the country. Their contributions are gratefully acknowledged, especially with regard to the selection of materials and field test experiments. Future measures for the next 10 years will most probably be at the protein level. A

proteomic assay was recently announced on rice (Salekdeh et al., 2002) under stress conditions. "Protein arrays", "proteomics" investigations and metabolomics will become involved not only for improvement studies but also for product safety analyses for GMO (genetically modified organisms) barleys. The methods developed during these studies could easily be adapted to other important plant species with the intention of understanding how to manipulate plant genomes successfully, which will be one of the main milestones of the 21st century.

This review article is a tribute to the memory of Prof. Emine Bilge (1926-1978) my former PhD supervisor. She was a great scientist and mentor who contributed with unflinching dedication to the development of genetic applications in plant breeding. She has always been with us in our hearts and minds over the last 25 years of research on barley genetics and biotechnology.

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