Euphytica (2011) 181:341–356
DOI 10.1007/s10681-011-0445-7

Allele mining and haplotype discovery in barley candidate genes for drought tolerance

András Cseri · Mátyás Cserháti · Maria von Korff · Bettina Nagy · Gábor V. Horváth · András Palágyi · János Pauk · Dénes Dudits · Ottó Törjék

Received: 17 January 2011 / Accepted: 26 April 2011 / Published online: 7 May 2011

Abstract In the present study, allele mining was conducted on a panel of drought related candidate genes in a set of 96 barley genotypes using EcoTILLING, which is a variant of the targeting induced local lesions in genomes (TILLING) technology. Analyzing approximately 1.5 million basepairs in barley a total number of 94 verified unique haplotypes were identified in 18 amplicons designed for 9 genes. Overall, 185 single nucleotide polymorphisms (SNPs) and 46 insertions/deletions (INDELs) were detected with a mean of 1SNP/92 bp and 1INDEL/372 bp genomic sequence. Based on overlapping haplotype sequences, markers were developed for four candidate genes (*HvARH1*, *HvSRG6*, *HvDRF1*, *HVA1*), which allows distinguishing between the main haplotypes showing either differences in amino acid sequence or which have larger INDELs in the promoter region. As "proof of concept", the *HvARH1* and *HvSRG6* haplotypes were tested for the level of abscisic acid-induced gene expression in subsets of genotypes belonging to different haplotype categories. An integrated database was developed to contain information about the genes, genotypes, and haplotypes analyzed in this study. The database supplies profound information about the natural variation in the tested drought related candidate genes providing a significant asset for further mapping studies dealing with this highly polygenic trait.

Keywords Allele mining · EcoTILLING · Drought tolerance · Candidate genes · Barley

András Cseri and Mátyás Cserháti are contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s10681-011-0445-7) contains supplementary material, which is available to authorized users.

A. Cseri (✉) · M. Cserháti · B. Nagy · G. V. Horváth · D. Dudits · O. Törjék
Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, Temesvári krt. 62, Szeged 6726, Hungary
e-mail: cseria@brc.hu

M. von Korff
Max Planck Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, 50829 Köln, Germany

A. Palágyi · J. Pauk
Cereal Research Non-Profit Company, Alsó kikötő sor 9, Szeged 6726, Hungary

Present Address:
O. Törjék
KWS SAAT AG, Grimsehlstrasse 31, 37555 Einbeck, Germany

Introduction

Drought is one of the main factors limiting yield in cereals. Plants respond to drought by altering the

expression of a large number of genes, thereby modifying cellular, physiological and biochemical processes. Recently, a number of potential candidate genes (CGs) have been identified by transcriptome and transgenic approaches involved in the adaptive responses to drought in cereals including barley (*Hordeum vulgare* L.) (Guo et al. 2009; Talamè et al. 2007; Marzin et al. 2008). However, the function of only very few CGs has been tested and no clear evidence exists today for the real contribution of these genes to drought tolerance. In addition, according to our knowledge the allelic variation in CGs has not been systematically examined yet. The exploration of genetic variation in genes involved in drought response can be an important prerequisite towards a better understanding of stress mechanisms. This knowledge is also of great relevance to plant breeders.

Drought-related CGs and their identified products recently can be conceptually classified into two groups (Shinozaki and Yamaguchi-Shinozaki 2007): (a) functional proteins (e.g. water channel proteins, sugar and proline transporters, detoxification enzymes, late embryogenesis abundant (LEA) proteins, enzymes for osmolyte biosynthesis, proteases etc.); and b) regulatory proteins (e.g.: various transcription factors, protein kinases, protein phosphatases, enzymes involved in phospholipid metabolism, signaling molecules, etc.).

In barley, several drought-related CG families and CGs were analyzed more extensively. The drought-inducible LEA (Late Embryogenesis Abundant) proteins belong to a small multigene family. Dehydrin genes (*Dhn*, LEA Dll family) are expressed in seeds during late embryogenesis and also in seedlings in response to drought, high salt, low temperature or ABA application (Close 1997; Choi et al. 1999). Barley *Dhn* genes have been identified and characterized by Choi et al. (1999), Park et al. (2006) and Rodriguez et al. (2005).

Recently, missense mutations were identified in a barley TILLING (Targeting Induced Local Lesions IN Genomes, Colbert et al. 2001) population for *Dhn12* and *Dhn13* genes, which could be an interesting step towards a better understanding of the role of these genes (Lababidi et al. 2009). Transgenic studies with *HVA1* (Bahieldin et al. 2005), a barley group 3 LEA protein, conferred tolerance to water deficit and other stress conditions in wheat and rice (Sivamani et al. 2000; Rohila et al. 2002). von Korff et al. (2008) suggest that this drought-related candidate gene may be involved in adaptation to drought stress. This gene was identified through QTL analysis in a Tadmor and ER/Apm RIL (Recombinant Inbreed Line) population and a high number of cross-over interactions for yield and yield components were mapped close to the *HVA1* gene. The hydrophilic character of the LEA proteins suggests an osmoprotective role during cell dehydration (Jinyou et al. 2004). Other genes such as *HvNHX1* and *HVP1* may enhance osmotic adjustment through re-establishing homeostasis under stress (Shi et al. 2002; Gaxiola et al. 2001). Fukuda et al. (2004) reported increased *HVP1* and *HvNHX1* transcript levels under osmotic stress. Overexpressing wheat *TNHX1* and *TVP1* in *Arabidopsis* improved salt- and drought-stress tolerance (Brini et al. 2007). A further strategy of plant adaptation to abiotic stresses involves preventing oxidative stress or reducing the level of reactive molecules (Bartels 2001; Oberschall et al. 2000). Early studies on the function of barley *HvARH1* protein linked increased accumulation in response to desiccation stress emphasizing its protective role during drought stress (Bartels et al. 1991).

Several drought-related transcription factors have been described in barley. Xu et al. (2009) found that expression levels of *HvDREB1* (*H. vulgare* dehydration-responsive element binding protein 1) were significantly increased in barley leaves grown under salt, drought, and low-temperature treatments. Overexpression of this gene in transgenic *Arabidopsis* plants increased tolerance to salt stress. *HvDRF1* (*H. vulgare dehydration*-responsive factor-1) belongs to the CBF/DREB subfamily of the ERF/AP2 family (Taketa et al. 2008) and it is known as an upstream activator of the *HVA1* gene (Xue and Loveridge 2004). Alternatively spliced *HvDRF1* transcripts were observed in both barley and wheat (Egawa et al. 2006; Xue and Loveridge 2004).

More recently, several investigations were directed in functional testing of drought-related, disease resistance genes (Silvar et al. 2010) in a more systematic manner. Marzin et al. (2008) tested dehydration stress-related CGs of barley by using a transient induced gene silencing (TIGS) assay system. In a study of von Korff et al. (2009) 30 genes putatively involved in stress response were analyzed for asymmetric allele-specific expression in five barley hybrids and their reciprocals

assessing the frequency of cis-acting regulatory variation. In the past years surveys of diversity in barley have focused mainly on SSRs (e.g.: Kota et al. 2001; Maestri et al. 2002; Matus and Hayes 2002). Nucleotide diversity within expressed genes was estimated for a smaller number of genes by sequencing multiple barley genotypes (e.g.: Russell et al. 2004; Cronin et al. 2007; Morrell et al. 2005).

A number of different techniques can be used for the analysis of nucleotide variation in CGs and their regulatory sequences, but all have their own limitations. Sequencing is the most accurate alternative, but is relatively expensive when applied to multiple loci in large numbers of individuals. EcoTILLING (Comai et al. 2004), is a variant of TILLING, based on certain PCR steps, such as the formation of heteroduplexes, nuclease-cutting of DNA mismatches and detection. It is a cost-effective technology and allows both SNP discovery and haplotyping through the sequencing of unique haplotypes. In barley allelic variation was examined and identified by using this method in *mlo* and *Mla* resistance genes (Mejlhede et al. 2006).

In the present pilot study, the EcoTILLING technology was used as a tool for allele mining in a panel of barley CGs representing a range of diverse genes involved in the abiotic stress response pathways. Haplotype and sequence variation (Single Nucleotide Polymorphism—SNP; INDEL—Insertion/Deletion) was examined at nine loci (*HvARH1*, *HvSRG6*, *HvDRF1*, *HVA1*, *HvDREB1*, *HvNHX1*, *HVP1*, *HvNud* and *HvPPRPX*) in a set of 96 barley cultivars and landraces collected worldwide and containing drought tolerant and sensitive genotypes. The analysis comprised sequence information of approximately 1.5 million basepairs in barley and the identified haplotypes and polymorphisms were recorded in a web-based database named BAHADAS (“BArley HAplotype DAtabaSe for drought-related candidate genes”). Based on overlapping haplotype sequences, markers were developed for four CGs (*HvARH1*, *HvSRG6*, *HvDRF1*, *HVA1*), which allows distinguishing the main haplotypes showing either differences in amino acid sequence or which have larger INDELs in the promoter region. As “proof of concept”, the *HvARH1* and *HvSRG6* haplotypes were tested for the level of abscisic acid-induced gene expression in subsets of genotypes belonging to different haplotype categories.

Materials and methods

Plant material

A set of 96 barley cultivars and landraces collected worldwide were analyzed in this study. Thirty-eight genotypes were selected based on the literature or based on available passport data showing that these genotypes were drought tolerant (in the field and or under control conditions). We included almost all genotypes (20 genotypes) available in the European Barley Database (Knüpffer 1988) for which we found the remark “drought tolerant” in the passport card. Seven interesting genotypes characterized for the trait “yield under drought” were taken from the Spanish Barley Core Collection (Igartua et al. 1998). Eleven barley lines were selected based on literature data dealing with drought tolerance (references in Supplementary Table S1 in Online Resource 1). Furthermore 5 high yielding commercial barley cultivars were also included in the analysis. This initial set of 43 genotypes was finally completed with 53 diverse genotypes obtained from Nils Stein (IPK Gatersleben). To provide the greatest potential for identifying genetic variation, these 53 genotypes were selected from different geographic locations. EcoTILLING analysis was performed on 92 genotypes (96 set minus No.13b-ISR42-8, No.73b-SCA239, No.83b-SCA118 and No.93-Keel). Regenotyping was done in all of the 96 genotypes. A detailed overview of the selected genotypes is summarized in Supplementary Table S1.

DNA Isolation and sample preparation for EcoTILLING

Genomic DNA was extracted from 10- to 14-day-old seedlings (1 seedling/genotype) according to the protocol described by Törjék et al. (2006). Following extraction, aliquots of genomic DNA were separated on 1.5% agarose gels to check for yield and variability of the DNA preparation. Concentrations were adjusted to 100 ng/µl in $T_{10}E_1$ (10 mM Tris–HCl pH 8, 1 mM EDTA) and mixed with an equal amount of No.53-GK Rezi DNA (reference DNA).

PCR amplification and EcoTILLING assays

PCR amplification was carried out in 12 µl of reaction mixture containing 1 µl genomic DNA

(100 ng/μl), 1 μl primer mix containing forward and reverse primers in equal amounts (stock solution: 20 μM for each forward and reverse primer) and 10 μl of pre-prepared MasterMix containing 810 μl autoclaved distilled water, 125 μl 10× PCR Buffer, 50 mM $MgCl_2$, 50 μl of 5 mM dNTPs (GeneCraft, Köln, Germany) containing dTTP and Flu-12-dUTP (GeneCraft, Köln, Germany) in a 9:1 ratio, 15 μl Taq DNA Polymerase either from Roboklon (catalogue number: E2600) or GeneCraft (catalogue number: GC-045-1000 or GC-002-1000) with their respective components. PCR reactions and heteroduplex formation were performed using a PTC-200 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) as follows: heat denaturation at 94°C for 2 min (10 min activation step for BioThermStar Hot Start Taq DNA Polymerase) followed by 40 polymerization cycles (denaturation: 94°C for 10 s, annealing: 55–60°C for 30 s, extension: 72°C for 1–3 min) and finally extension, denaturation and reannealing steps (72°C for 5 min, 99°C for 10 s, 70°C for 20 s, followed by 70 cycles of 70°C for 20 s decreasing 0.3°C per cycle). After PCR amplification, samples were digested either with CelI enzyme (provided by Georg Strompen, University of Potsdam, Germany) according to the protocol described by Törjék et al. (2008) or with ENDO-1 (Serial Genetics, Evry, France) following the manufacturer's recommendations. After this step samples were precipitated with 15 μl isopropanol (centrifuged for 15 min at 4,000 rpm.), washed with 20 μl of 70% ethanol (centrifuged for 15 min at 4,000 rpm.) and finally resuspended in 2 μl formamide loading buffer (33% deionised formamide, 10 mM Tris, pH 7.5, 1 mM EDTA and 0.02% bromphenol blue) and denatured (95°C for 4 min and 4°C for 5 min). Samples (0.5 μl) were loaded onto 100-tooth membrane combs (LI-COR Biotechnology, Lincoln, USA) then electrophoresed on ABI Prism 377 Sequencer (Applied Biosystems, Lincoln, USA) through a 16 cm long polyacrylamide (6.5% KB Plus, LI-COR) gel in 1× TBE running buffer at 1,400 V/60 mA/50°C for 2–4 h.

Candidate gene selection and primer design

Drought related CGs were selected based on their putative role in molecular drought response. The list of 9 genes is shown in Table 1 and includes 8 candidates for which a role in drought response has been demonstrated based on expression data or by transgenic approaches (for references, see Table 1). *HvPRPX* was used for the establishment of the EcoTILLING technology (this gene was also used as negative control in the study of Marzin et al. 2008). Based on available genomic DNA sequences 4, 4, 3 and 2 overlapping amplicons were designed for the CGs *HvARH1*, *HvSRG6*, *HvDRF1* and *HVA1*, respectively, which made possible the analysis of the sequence diversity for nearly the complete gene. In the case of *HvNud* and three further CGs (*HvDREB1*, *HvNHX1* and *HVP1*) for which only mRNA or coding sequences were accessible, only one amplicon was designed and analyzed. The 18 amplicons, their sizes and the created primers are shown in Supplementary Table S2. All primers used in this study were designed using the software Primer3 (Rozen and Skaletsky 2000).

Haplotype identification and sequencing

Analyses of the gel images were carried out manually using the PhotoFiltre 6.2.7 software (freeware version, http://www.photofiltre.com/). Genotypes were grouped into putative haplotype categories based on the cleaved Cel1 or ENDO-1 products in evaluated gel-frames. Afterward one representative of each unique haplotype was reamplified without fluorescently labeled nucleotides and the purified PCR product was sequenced on both strands to confirm the polymorphisms (sequencing was performed by Bio Basic Inc. Markham, Canada and particularly by the BRC sequencing unit, Szeged, Hungary). Sequences were analyzed using the Chromas 1.45 (McCarthy 1998) and CLC Free Workbench (www.clcbio.com) softwares. All sequence data from this study have been deposited in NCBI under accession numbers: GU108387–GU108455.

Regenotyping

Selected INDELs were regenotyped using the same reaction conditions as for EcoTILLING incorporating Flu-12-dUTPs into the amplicons. CAPS assays for the detection of selected SNPs were labeled also with Flu-12-dUTPs and the enzymatic digestion was done using MspI (MBI Fermentas, Vilnius, Lithuania) following the manufacturer's recommendations. A PCR reaction for detecting SNPs called Duplex SNaPshot assays (Applied Biosystems, Lincoln, USA) was carried out as described previously (Törjék et al. 2003).

Table 1 List of genes used for ecoTILLING

No.	Candidate gene, abbreviation	Available molecule type	Locus	Reference
1	*H. vulgare* AR-h gene for aldose reductase, HvARH1	Genomic DNA	Z48360	Roncarati et al. (1995), Oberschall et al. (2000)
2	*Hordeum vulgare* srg6 gene for stress responsive gene protein 6, SRG6	Genomic DNA	AJ300144	Malatrasi et al. (2002)
3	*Hordeum vulgare* AP2 transcriptional activator (DRF1) gene, HvDRF1	Genomic DNA	AY223807	Xu and Loveridge (2004)
4	*H. vulgare* HVA1 gene, HVA1	Genomic DNA	X78205	Bahieldin et al. (2005)
5	*Hordeum vulgare* subsp. vulgare dehydration responsive element binding protein 1 (DREB1) mRNA, HvDREB1	mRNA	DQ012941	Xu et al. (2009)
6	*Hordeum vulgare* HvNHX1 mRNA for sodium/proton antiporter, HvNHX1	mRNA	AB089197	Fukuda et al. (2004)
7	*Hordeum vulgare* HVP1 mRNA for vacuolar proton-inorganic pyrophosphatase, HVP1	mRNA	AB032839	Fukuda et al. (2004)
8	Barley fungal pathogen induced mRNA for pathogen-related protein, HvPPRPX	mRNA	X16648	Marzin et al. (2008)
9	NUD putative ethylene-responsive transcription factor, HvNUD	Genomic DNA	AP009567	Taketa et al. (2008)

Statistical analysis and visualization

Genetic distances of genotypes based on polymorphisms in the *HvARH1* gene were calculated and visualized using the GGT 2.0 software (van Berloo 1999) and SplitsTree 4.10 (Huson and Bryant 2006). Representative alleles identified by EcoTILLING were sequenced in a subset of barley genotypes, and in silico sequences were generated for all other individuals. Nucleotide diversity per gene (P_i), Tajima's D, Haplotype diversity (H_d) and the ratio of the number of non-synonymous substitutions (K_a) to the number of synonymous substitutions (K_s) was calculated using DnaSP v 5.0 (Librado and Rozas 2009). For statistical analysis the INDELs were treated as single site polymorphisms (P_i, Tajima's D and H_d). Average nucleotide and haplotype diversity was the average of all P_i and H_d values.

Analysis of abscisic acid-induced gene expression changes of selected candidate genes

Plant treatment: Seeds of selected genotypes representing different haplotype groups were grown in greenhouse at 18/16°C day/night temperature with a 12-h photoperiod and ambient relative humidity. Shoot samples were collected from 6 week old plants which were immersed in 50 µM abscisic acid (ABA) solution in Petri dishes. Shoots were harvested after 24 h long ABA treatment. Water-treated shoots served as negative controls in these experiments. For the expression analysis of *HvSRG6* gene the ABA treatment protocol of Malatrasi et al. (2002) was followed. Seeds were germinated on moist filter paper in sterile Petri dishes and grown at room temperature for 7 days. Treated seedling roots were bathed in a 10 mM ABA solution for 24 h and the control samples were immersed in water. After 1 day, shoots were cut off, rapidly frozen in liquid nitrogen, and stored at −80°C.

RNA isolation and quantitative Real-Time PCR analysis: For qRT-PCR, total RNA was extracted from control and stress-treated samples. According to the AGPC (acid guanidium thiocyanate-phenol chloroform) method (Chomcyznski and Sacchi 1987) using Tri reagent the RNA samples were treated with RNase-free DNase I (Fermentas). The concentration of RNA was determined with a Nanodrop ND-1000 spectrophotometer (NanoDrop Products, Wilmington, DE, USA). The RNA samples were quantified by measuring the absorbance at 260 nm and purities were assessed by the 260/280 nm ratio and on a 1% agarose gel stained with ethidium bromide. First-strand cDNA synthesis of 2 µg total RNA in a final volume of 20 µl was performed with RevertAid

M-MuLV Reverse Transcriptase (MBI Fermentas, Vilnius, Lithuania) according to the supplier's protocol using random hexamers. The real time quantitative PCR analyses were performed using the ABI Prism 7000 Sequence Detection System. For the real-time qPCR, 1 µl cDNA were mixed with 10 µl Power SYBR Green PCR Master Mix (Applied Biosystems, Lincoln, USA), 1.0 µl gene-specific 10 µM PCR primer pair stock and 8 µl ddH_2O in a final volume of 20 µl in three replicates. Controls containing no template molecules were included. Amplification conditions were: 45 cycles at 95°C for 15 s, followed by 60°C for 1 min. Specificity of the amplifications was verified at the end of the PCR run using ABI Prism Dissociation Curve Analysis Software. The expression level of the *HvARH1* and *HvSRG6* gene was normalized using *18S rRNA* as a housekeeping gene and the relative transcript levels were obtained by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The forward and reverse primers used for expression analysis are shown in Supplementary Table S2.

Results

Summary of allele mining in drought-related CGs

Natural variation was analyzed in eighteen amplicons designed for 8 putative drought related CGs and the *HvPRPX* gene by EcoTILLING screens in a set of 92 barley genotypes. An example of an EcoTILLING screen is shown in Fig. 1. Altogether, approximately, 1.5 million basepairs were analyzed via EcoTILLING. A total of 94 verified unique haplotypes were identified for 18 amplicons including the reference sequence variants from NCBI. The primer sets yielded products ranging from 571 to 1,374 bp. The number of identified haplotypes varied between 2 (*HvDREB1*) and 8 (*HVP1*). Haplotype frequencies in the analyzed population were determined for 15 amplicons. In the case of the remaining three amplicons (*HvSRG6*-0, *HVA1-F* and *HvDRF1-AA*) genotypes were not classified into haplotype categories because of the ambiguous EcoTILLING patterns (numerous missing and fuzzy lanes or too many cleaved fragments in lanes). For these 3 amplicons all distinguishable lanes (genotypes) were sequenced afterwards.

When taking into account the 15 amplicons with an average EcoTILLING call rate of 92.5%, we found on average of 4.5 haplotypes per amplicon. As displayed in Fig. 2, for most genes a major haplotype (average frequency = 0.65) was detected, with the average cumulative frequency of the first three haplotypes being 0.9, followed by a series of minor haplotypes. Relative haplotype frequencies found in the EcoTILLED population are shown in Fig. 3.

One representative genotype for each unique haplotype was sequenced on both strands. Overall, 185 SNPs and 46 INDELs were detected with a mean of 1 SNP/92 bp and 1 INDEL/372 bp genomic sequence. A total number of 22 replacement SNPs (causing changes in the amino acid sequence) and

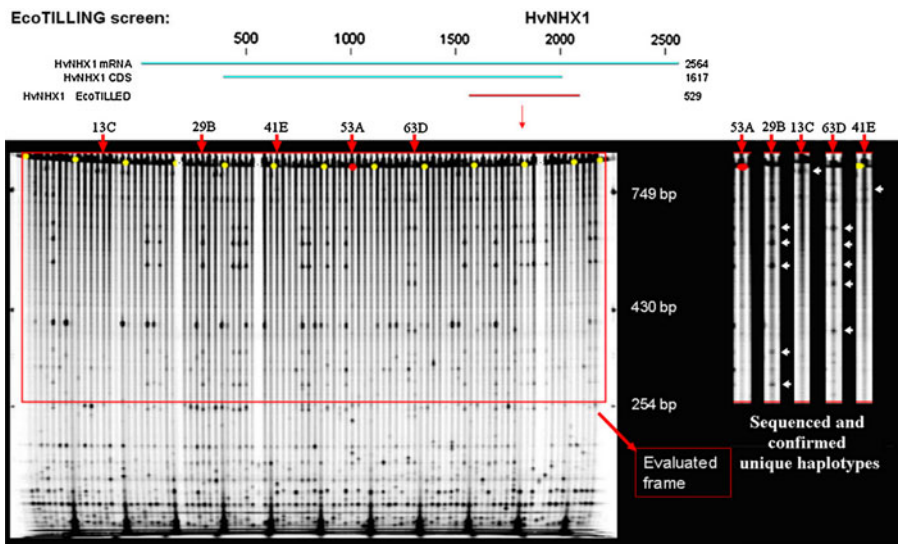


Fig. 1 EcoTILLING image obtained for HvNHX1. Variant haplotypes were discovered in the evaluated gel-frame contained in the *red box*. *White arrows* show the cleaved fragments indicating polymorphisms in the amplicon. *Red arrows* show the genotypes selected for sequencing, which belongs to different unique haplotype groups

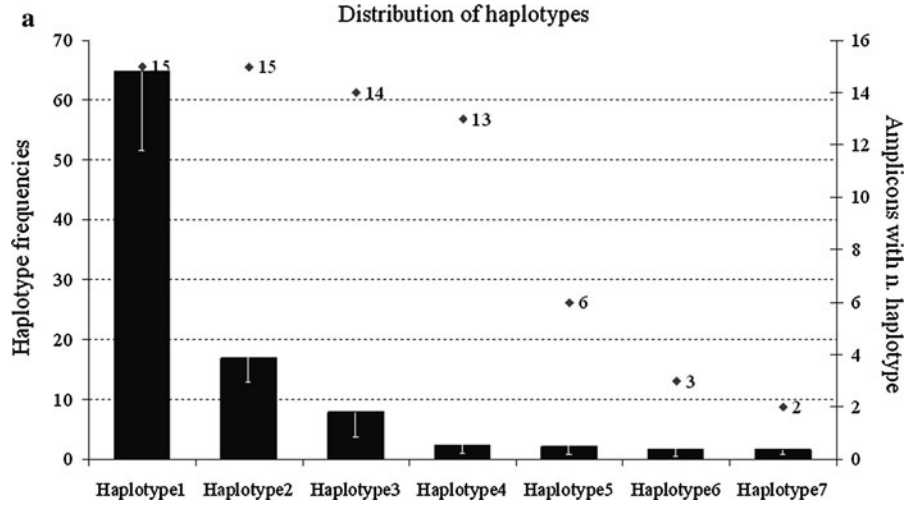


Fig. 2 Distribution of haplotype frequencies in 15 amplicons based on EcoTILLING patterns. Haplotype frequencies sorted in decreasing order. Class "1" corresponds to the most frequent haplotypes, Class "2" to the second most frequent haplotype and so on consequetively

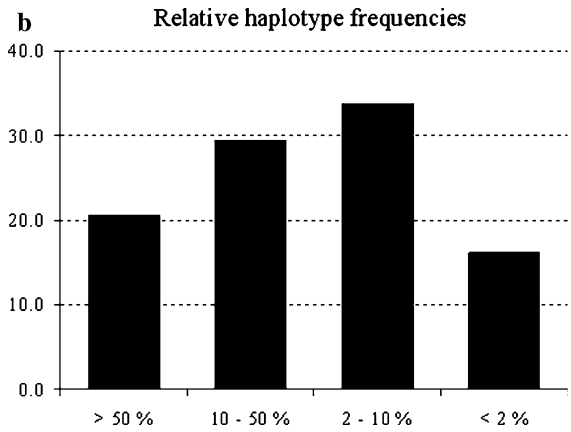


Fig. 3 Overall relative haplotype frequencies. Most of the haplotypes had an allele frequency between 2 and 10% in the analyzed population

five INDELs were found in coding regions**.** The range of nucleotide diversity spanned from Pi = 0.00039 for HvNud (only two SNPs and one INDEL was observed) to Pi = 0.01220 for HVA1-L (16 SNPs and 10 INDELs). The average level of haplotype diversity was 0.465. An overview of the EcoTILLING analysis and haplotype sequencing is summarized in Table 2. All polymorphisms are available on the Web interface created in the frame of this project.

Development of haplotype specific markers, validation of the EcoTILLING haplotypes by regenotyping

One of the goals of our project was to develop molecular markers that can be used for the distinction of the genetic variants. Haplotype specific marker sets were developed for four genes, which were screened with overlapping EcoTILLING amplicons. To this end single haplotypes determined for each amplicon of a given gene were arranged into combined haplotype sequences. Based on these assembled "whole-gene haplotypes", subsets of polymorphisms were selected for regenotyping.

In the case of *HvARH1* four amplicons were screened covering 3,346 bp of the gene (Supplementary Fig. 1a in Online Resource 2). For the individual amplicons 3, 5, 4, and 4 haplotypes were identified by EcoTILLING respectively (with an average call rate of 93.5%). After arranging the individual haplotype sequences a total number of 77 polymorphisms (56 SNPs and 21 INDELs) and 9 potential combined haplotypes were determined including the sequence from NCBI (for further details see the website or Supplementary Table 3 in Online Resource 4). In the panel of 92 barley lines eight combined haplotypes (named *HvARH1*-Z1–*HvARH1*-Z8) were deduced. Most of the genotypes were grouped into one of the three main haplotypes: *HvARH1*-Z1, *HvARH1*-Z4 or *HvARH1*-Z6. *HvARH1*-Z2 (corresponding to genotype No. 80-Claret), *HvARH1*-Z5 (corresponding to genotype No.46-Natasha), *HvARH1*-Z7 (corresponding to genotype No. 88-BCC197), *HvARH1*-Z8 (corresponding to genotype No. 20-BCC195) were determined as individual recombinant haplotypes and finally *HvARH1*-Z3 (corresponding to genotype No. 86-Fengtien Black) was determined as a unique haplotype differing from *HvARH1*-Z1 in the 21st SNP allele (with

Table 2 Overview of allele mining in barley candidate genes

Candidate genes	Amplicons	Length (bp)	No. of haplo-types	No. of SNPs	Coding SNPs	Noncoding SNPs	Replace-ment SNPs	SNP/bp genomic sequence	No. of INDELs	Coding INDELs	INDEL/bp genomic sequence	Nucleotide diversity, P_i[a]	Haplotype diversity, H_d[a]	Tajima's D	K_a/K_s
HvARH1[b]	HvARH1-F	1205	3 + 1[c]	18	1	16	1	67	6	0	201	0.00512	0.433	0.85555 (NS)	NA
	HvARH1-A	863	5 + 1[c]	17	2	15	0	51	3	0	288	0.01045	0.465	1.91651 (NS)	NA
	HvARH1-B	842	4 + 1[c]	12	3	9	1	70	3	0	281	0.00436	0.449	−0.34215 (NS)	5.239
	HvARH1-L	780	4 + 1[c]	7	4	3	2	111	8	0	97.5	0.00102	0.429	−0.52127 (NS)	NA
HvSRG6[b]	HvSRG6-0	927	5	20	0	20	0	46	2	0	463.5	–	–	–	–
	HvSRG6-1	945	4 + 1[c]	0	0	0	0	0	3	0	315	0.00056	0.341	−0.49432 (NS)	NA
	HvSRG6-2	933	6 + 1[c]	11[d](2)	4	7[d](2)	1	85	1	0	933	0.00256	0.375	0.05344 (NS)	0.0310
	HvSRG6-3	821	5 + 1[c]	18[d](2)	2	16[d](2)	1	46	1	0	821	0.00300	0.344	−0.28799(NS)	0.0851
HvDRF1[b]	HvDRF1-AA	1059	3 + 1[c]	2	0	2	0	529	1	1	1059	–	–	–	–
	HvDRF1-P	1340	4 + 1[c]	5	0	5	0	268	1	0	1340	0.00091	0.550	0.36070 (NS)	0.0115
	HvDRF1-C	1252	4 + 1[c]	9	9	0	8	139	2	1	626	0.00209	0.543	0.31985 (NS)	2.3184
HVA1[b]	HVA1-F	919	5	12[d](2)	6[d](1)	6	5	77	2	2[d](1)	459.5	–	–	–	–
	HVA1-L	709	7	16[d](2)	1[d](1)	15	1	44	10	1[d](1)	71	0.01220	0.738	0.52262 (NS)	NA
HvDREB1	HvDREB1	620	2	1	0	1	0	620	0	0	0	0.00070	0.376	1.06245 (NS)	NA
HvNHX1	HvNHX1B	904	5	11	7	4	0	82	0	0	0	0.00281	0.640	0.26479 (NS)	NA
HVP1	HvP1	1374	7 + 1[c]	18	8	10	1	72	2	0	687	0.00299	0.595	−0.45677 (NS)	NA
HvPPRPX[e]	HvPPRPX	571	4 + 1[c]	8	5	3	2	71	1	0	571	0.00386	0.388	0.53311 (NS)	0.2771
HvNUD	HvNUD	1034	4	2	1	1	0	517	1	1	1034	0.00039	0.310	−0.76083 (NS)	NA
Total/ average:	18	17098/ 950	946 5.2	185	53	132	22	92.4	46	5	371.7	0.03535	0.465		

NA not applicable, *NS* not significant

[a] Representative alleles identified by Ecotilling were sequenced in a subset of barley genotypes, and in silico sequences were generated for all other individuals, thus greatly reducing the amount of sequencing required for genotype identification. For statistical purposes the indels were treated as single site polymorphisms along with the other SNPs in our analysis

[b] The whole set of 96 barley genotypes was regenotyped using the developed markers

[c] EcoTILLING analysis was repeated

[d] The same number of polymorphisms within *parentheses* were detected in overlapping amplicons in this amplicon are the same as the last 2 SNPs from the adjacent overlapping amplicon

[e] +1 Means the available databank sequence represents a plus haplotype

a T instead of a G). Seven polymorphisms (2 INDELs and 5 SNPs) were selected for marker development and regenotyping, which gave the opportunity for the confirmation of all 8 deduced combined haplotypes (Fig. 4). The two INDELs were analyzed in a duplex SSLP reaction. A duplex SNaPshot reaction allowed the analysis of the 21st and 56th SNPs. The 51st SNP was analyzed by a CAPS reaction and SNPs 41 and 42 were interrogated using ENDO-1 enzyme (see Supplementary Fig. 1b in Online Resource 2). Regenotyping of all the 96 genotypes showed only two discrepancies in comparison to the results determined based on the four EcoTILLING screens. Two of the four potential recombinant haplotypes were not confirmed (*HvARH1-Z5* was refitted to *HvARH1-Z4* and *HvARH1-Z7* to *HvARH1-Z6*). None of the genotypes were heterozygous for the *HvARH1* gene.

In the case of *HvDRF1*, *HVA1* and *HvSRG6* 3, 2 and 3 informative polymorphisms were converted into easily detectable genetic markers, respectively (Table 2), which allow the discrimination of the main haplotypes and/or those haplotypes which showed differences on the amino acid level or contained large INDELs in the promoter region. All regenotyping results can be found on the subjoined website.

Expression analysis of the main *HvARH1* and *HvSRG6* haplotypes—a proof of concept

Transcription of *HvARH1* and *HvSRG6* genes is known to be regulated by abscisic acid; moreover the induction of these genes can be linked to desiccation tolerance (Bartels et al. 1991; Malatrasi et al. 2002). To test the genotype-dependent transcriptional

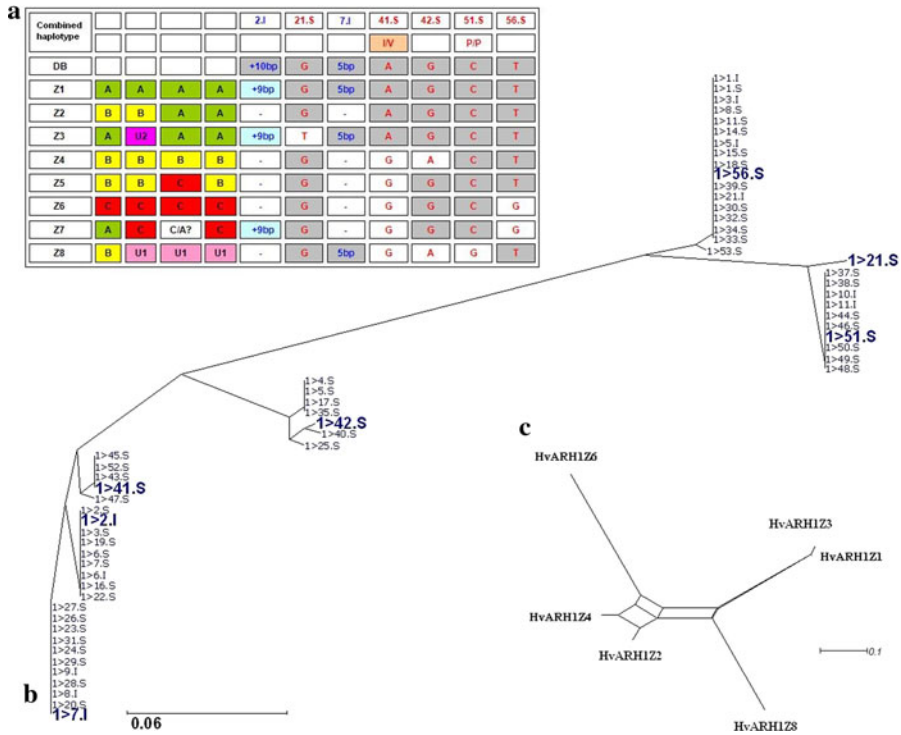


Fig. 4 Subset of polymorphisms in the HvARH1 gene selected for regenotyping. Information for each of eight combined regenotyped haplotypes Z1–Z8. **a** Detection of 2 Indels (*columns in blue*) and 5 SNPs (*columns in red*) allows the discrimination of all potential HvARH1 haplotypes. *Letters* in the *first four columns* denote which haplotypes make up the combined haplotype. Table values denote length of INDEL or specific SNP. **b** Genetic distances were calculated among polymorphisms using the GGT software, and a NJ (neighbor joining) dendrogram was created based on simple matching similarity coefficients. Selected polymorphisms for regenotyping are highlighted shown in **a**. **c** Regenotyping confirmed the three main haplotype categories (HvARH1-Z1, HvARH1-Z4 and HvARH1-Z6) one recombinant haplotype (HvARH1-Z2, No.80-Claret) and the two unique ones: HvARH1-Z3 (No.86-Fengtien Black) and HvARH1-Z8 (genotype: No.20-BCC195). The tree was visualized using the Software SplitsTree version 4.10

response of these genes, ABA-treated leaves of plants belonging to the major haplotype groups were tested by quantitative RT-PCR in order to analyze the effect of SNPs and INDELs found inside the genomic sequence of the promoter and the coding regions. In the case of the *HvARH1* gene three representatives of the three major haplotypes (*HvARH1*-Z1, *HvARH1*-Z4 and *HvARH1*-Z6), respectively were tested for ABA inducibility. According to the results shown on Fig. 5a the transcript level increased significantly following the 24 h long treatment of young leaves with 50 μM ABA solution in all cases, and differences in gene induction were detected between the main haplotype groups. The abscisic acid response of gene expression was the most pronounced in the case of genotypes belonging to the *HvARH1*Z1 haplotype group. In this case the gene induction reached a 20–60-fold increase compared to the water-treated control. Plants belonging to the *HvARH1*-Z4 and *HvARH1*-Z6 haplotype groups exhibited a lower (approx. 10-fold) induction, however, an extremely high value was measured in the case of a genotype (No. 73a-Compana) belonging to the *HvARH1*-Z4 haplotype group demonstrated the importance of individual genomic characteristics and the necessity of the statistical analysis of data derived from multiple samples.

Testing the ABA-induced expression changes of the *HvSRG6* gene revealed that in spite of the applied very high concentration of abscisic acid (10 mM vs. the 50 μM in the previous experiment), thus following strictly the experimental setup of Malatrasi et al. (2002), we could not detect a significant induction of gene expression. Nevertheless it demonstrated that the loss of a significant portion (196 basepairs) of the *cis*-acting promoter did not change the expression of *HvSRG6* gene in cultivars No.18-Otis, No.23-Chilga Arpa and No.61-Diamond (Fig. 5b).

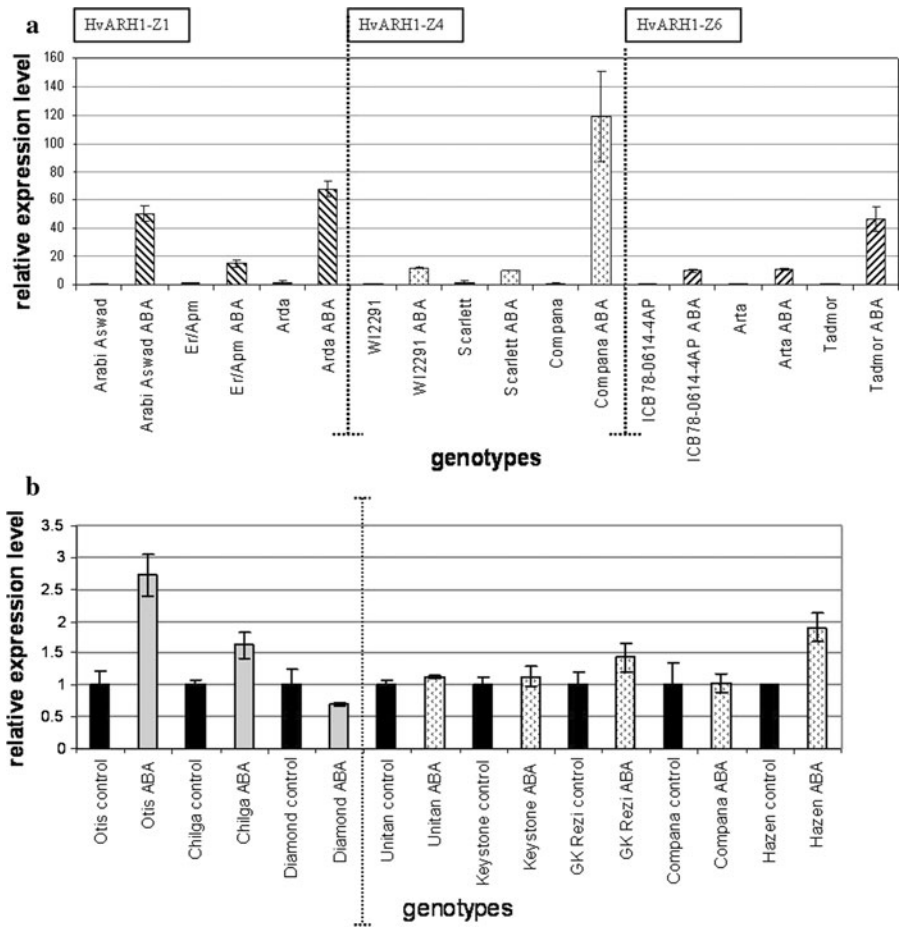
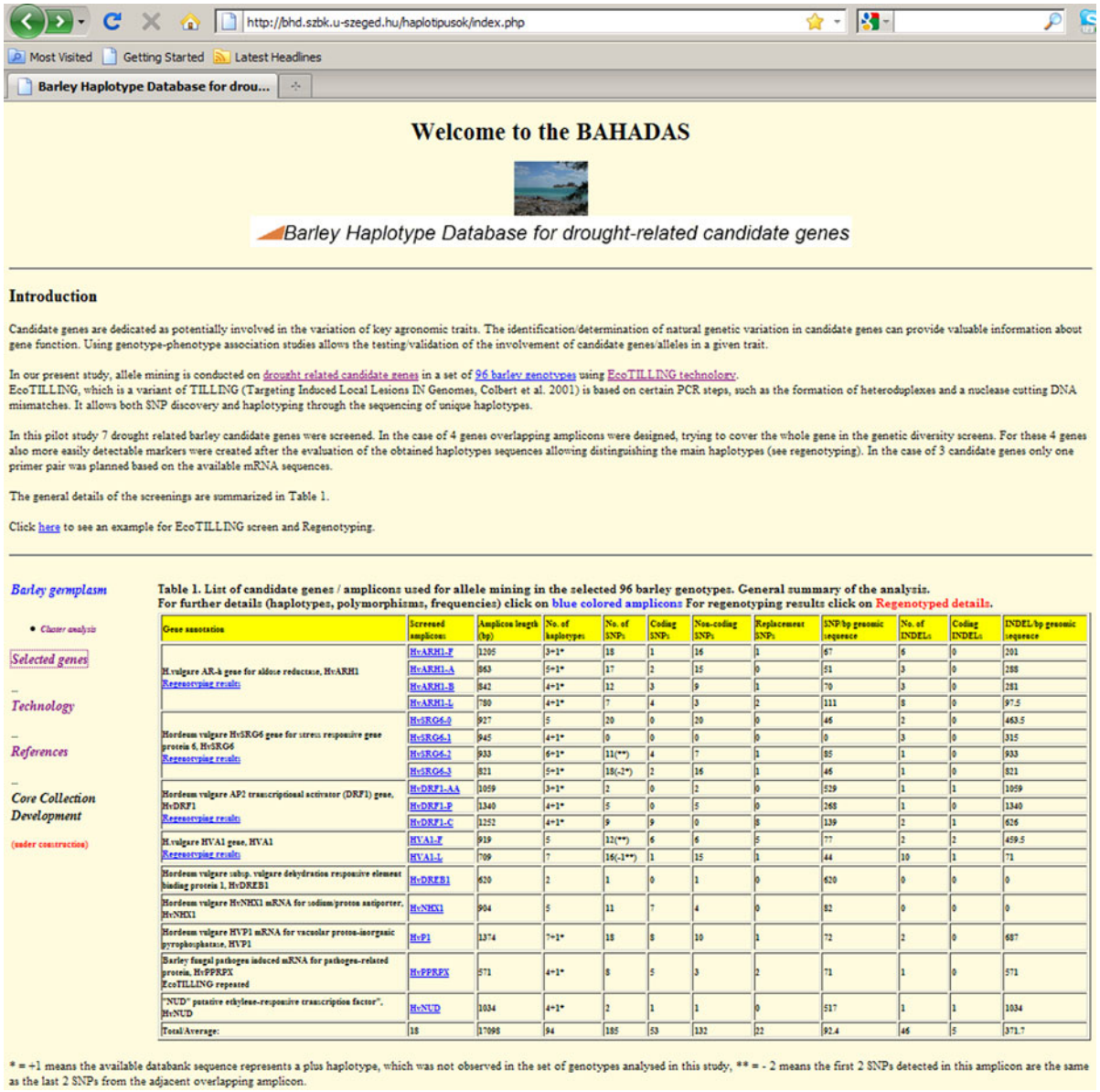


Fig. 5 Expression analysis of the main HvARH1 and HvSRG6 haplotypes. **a** Expression of the three main HvARH1 haplotypes after abscisic acid treatment (three genotypes were tested for each of the main HvARH1 haplotypes). **b** Abscisic acid induction of SRG6 gene in barley genotypes containing full length version (No.62-Unitan, No.3-Keystone, No.53-GK Rezi, No.73a-Compana, No.8-Hazen) or 196 bp long insertion-containing version (No.18-Otis, No.23-Chilga, No.61-Diamond) of the promoter

Barley Haplotype Database for drought-related candidate genes (BAHADAS)

To provide public access to haplotypes and polymorphisms generated in this study, a Web interface was created that can be accessed at http://bhd.szbk.u-szeged.hu/haplotipusok/index.php. This searchable database contains a set of information obtained for the analyzed CGs' haplotype sequences, frequencies and multiple alignments with sequence polymorphisms. In the case of four gene targets regenotyping data are available. The Web interface provides additional information about the used genotypes and about the technology and the primers used. The integrated "core collection" allows the building of flexible genotype subsets with maximized genetic diversity based on the analyzed candidate genes. The database will be freely available upon publication. A screenshot of the developed database is shown in Fig. 6.



Gene annotation	Screened amplicon	Amplicon length (bp)	No. of haplotypes	No. of SNPs	Coding SNPs	Non-coding SNPs	Replacement SNPs	SNP/bp genomic sequence	No. of INDELs	Coding INDELs	INDEL/bp genomic sequence
H.vulgare AR-k gene for aldose reductase, HvARH1 Regenotyping results:	HvARH1-F	1205	3+1*	18	1	16	1	67	6	0	201
	HvARH1-A	863	5+1*	17	2	15	0	51	3	0	288
	HvARH1-B	842	4+1*	12	3	9	1	70	3	0	281
	HvARH1-L	780	4+1*	7	4	3	2	111	8	0	97.5
Hordeum vulgare HvSRG6 gene for stress responsive gene protein 6, HvSRG6 Regenotyping results:	HvSRG6-0	927	5	20	0	20	0	46	2	0	463.5
	HvSRG6-1	945	4+1*	0	0	0	0	0	3	0	315
	HvSRG6-2	933	6+1*	11(**)	4	7	1	85	1	0	933
	HvSRG6-3	821	5+1*	18(-2*)	2	16	1	46	1	0	821
Hordeum vulgare AP2 transcriptional activator (DRF1) gene, HvDRF1 Regenotyping results:	HvDRF1-AA	1059	3+1*	2	0	2	0	529	1	1	1059
	HvDRF1-P	1340	4+1*	5	0	5	0	268	1	0	1340
	HvDRF1-C	1252	4+1*	9	9	0	8	139	2	1	626
H.vulgare HVA1 gene, HVA1 Regenotyping results:	HVA1-F	919	5	12(**)	6	6	5	77	2	2	459.5
	HVA1-L	709	7	16(-1**)	1	15	1	44	10	1	71
Hordeum vulgare subsp. vulgare dehydration responsive element binding protein 1, HvDREB1	HvDREB1	620	2	1	0	1	0	620	0	0	0
Hordeum vulgare HvNHX1 mRNA for sodium/proton antiporter, HvNHX1	HvNHX1	904	5	11	7	4	0	82	0	0	0
Hordeum vulgare HVP1 mRNA for vacuolar proton-inorganic pyrophosphatase, HVP1	HvP1	1374	7+1*	18	8	10	1	72	2	0	687
Barley fungal pathogen induced mRNA for pathogen-related protein, HvPPRPX EcoTILLING repeated	HvPPRPX	571	4+1*	8	5	3	2	71	1	0	571
"NUD" putative ethylene-responsive transcription factor", HvNUD	HvNUD	1034	4+1*	2	1	1	0	517	1	1	1034
Total/Average:	18	17098	94	185	53	132	22	92.4	46	5	371.7

Fig. 6 Screenshot of the Web interface "BAHADAS"

Discussion

Natural variation in the screened population

Our primary goal was to characterize the extent of genetic variation in drought related barley CGs. To this end we assembled a collection of barley genotypes for allele mining. This set contains almost all the known barley genotypes from the literature and/or from different databases (based on genotype passport cards), which showed a marked response to drought. This initial set of 43 genotypes was supplemented with 53 diverse genotypes (provided by Nils Stein, IPK Gatersleben) from different geographic location with the aim of providing a high potential for identifying genetic variation in the analyzed genes. Approximately 1.5 million basepairs were analyzed via EcoTILLING, which revealed 94 verified unique haplotypes for the 18 studied amplicons with a mean length of 950 bp/amplicon in the studied barley collection. On average we found 5.2 haplotypes per amplicon taking also the NCBI sequences into account (4.5 haplotypes/amplicon were detected in the collected set of barley genotypes). The average frequency of polymorphisms was 1 SNP/92.4 bp and 1 INDEL/371.7 bp genomic sequence, including the database sequences. We found nucleotide diversity values ranging between $Pi = 0.000390$ and $Pi = -0.1220$. The mean level of haplotype diversity was 0.465. Test for selection did not provide significant Tajima's D values (Table 2), although high value of 1.91651 was detected for HvARH1-A, which may be an indication for balancing selection. In case of the HvARH1B amplicon also the Ka/Ks ratio (5.239) indicates for a positive selection. In barley, polymorphism frequency estimates showed a wide range in different studies: a. 1SNP/27 bp, 1INDEL/94 bp (Bundock and Henry 2004); b. 1SNP/78 bp, 1INDEL/680 bp (Russell et al. 2004); c. 1SNP/200 bp, INDELs not examined (Rostoks et al. 2005). Comparable levels of nucleotide diversity (from 0.0021 to 0.0189) and haplotype diversity (0.518) were found in work of Russell et al. (2004). The frequency estimates are largely dependent on the selected germplasm (landraces, wild barley accessions, cultivated germplasm) and its size as well as the analyzed genomic regions (coding, non-coding genomic regions). Analyzing the haplotype structure in seven barley genes Kilian et al. (2006) found on average 10 haplotypes per locus in wild lines, but only 2.43 haplotypes in domesticated genotypes. Our barley collection was assembled first of all with the aim to include all (as many as possible) of the barley genotypes which showed a differentiable drought response. Based on the large number of identified polymorphisms and haplotypes, we can assume that a large extent of the natural variation is present in the analyzed population. We think that our population and the data obtained in this study can provide a good starting point for further large scale projects aiming to analyze the natural variation in drought related CGs. By applying core collection algorithms flexible research project targeted genotype sets can be defined eliminating genetic redundancies and maximizing allele-mining efficiency.

Use of EcoTILLING technology as a tool for allele mining in drought related CGs

The main goal of allele mining is to identify and isolate unknown and valuable alleles within genetic resource collections at known loci that are candidates for conferring essential traits. One of the most powerful strategies for allele mining that has been developed in recent years is EcoTILLING. This technology in combination with sequencing allows the detection and identification of multiple types of polymorphisms across germplasm collections (Comai et al. 2004). Until now EcoTILLING has been used in plants to examine DNA variation in *Arabidopsis* (Comai et al. 2004), in black cottonwood (Gilchrist et al. 2006), in mung bean (Barkley et al. 2008), in melon (Nieto et al. 2007) and in barley in *mlo* and *Mla* resistance genes (Mejlhede et al. 2006).

In the present pilot study we have adopted and set up the EcoTILLING technology for detecting natural variation in barley CGs for drought tolerance according to the protocol described by Törjék et al. (2008), which is based on the incorporation of fluorescently labeled dNTPs into the PCR products. An advantage of this kind of labeling is that fluorescently labeled primers are not required, making this approach more cost-effective. We have found that the robustness of PCR-reactions with fluorescently labeled dNTPs is the same as using normal dNTPs and so the robustness is notably higher, than using labeled primers. A disadvantage of dNTP labeling (labeling of both PCR strands—one channel detection

possible) in comparison to the end labeling of amplicons with two different dyes (two channel detection) is that potential polymorphic sites can not be confirmed in one step based on summing up the cleaved fragments detected on the two different wavelengths (the sum should equal the full length PCR product). Nevertheless, in the case of multiple polymorphisms both of the labeling methods lead to very complicated patterns and a haplotype validation should be done by sequencing of the potential haplotypes.

The correctness of the EcoTILLING-based genotype classification was verified by regenotyping of the *HvARH1* gene using 7 polymorphisms which had the capacity to distinguish between all of the deduced *HvARH1* combined haplotypes. Regenotyping confirmed nearly all the data obtained by EcoTILING and we found discrepancies only in two cases (two of the four potential recombinant haplotypes were not affirmed). Haplotype specific markers were developed also for three further candidate genes EcoTILLED with overlapping amplicons: *HvDRF1* (3 markers), *HVA1* (2 markers) and *HvSRG6* (3 markers). After reanalyzing the whole 96 barley genotype collection the same low levels of inconsistencies were observed between the EcoTILLING-based and the regenotyping results (with a maximum of two discrepancies/gene). These last three marker sets allow the discrimination between the most frequent haplotypes and those ones which were different at the amino acid level or contained large INDELs in the promoter region. As potential "trait-in markers" they can be verified in association and QTL mapping studies. Further details of marker development (selected polymorphisms, details of the regenotyping) can be obtained from the web interface at http://bhd.szbk.u-szeged.hu/haplotipusok/index.php.

Abscisic acid-induced expression of genes involved in drought stress response in selected haplotypes

Plant aldo–keto reductases, among other enzymes, have been shown to be effective in the detoxification of lipid peroxidation-derived reactive aldehydes generated under a wide range of environmental stresses (Mundree et al. 2000; Oberschall et al. 2000; Hideg et al. 2003). The first identified aldose reductase homologue gene from barley, *HvARH1*, was shown to be constitutively expressed during embryo maturation and was modulated by the plant hormones abscisic acid and gibberellic acid. The expression of a barley gene homologous to aldose reductase is restricted to the embryo and temporally correlated with its acquisition of desiccation tolerance. (Bartels et al. 1991; Roncarati et al. 1995).

In our experiments the induction of the *HvARH1* gene expression by abscisic acid has shown considerable differences in the three haplotypes tested. The highest average transcript levels were measured in genotypes belonged to the *HvARH1*-Z1 haplotype. Except for one genotype of haplotypes *HvARH1*-Z4 and *HvARH1*-Z6 their characteristic *HvARH1* transcript levels were only moderately increased (approximately 10-fold). The comparison of the promoter regions revealed that the most pronounced differences between the haplotypes are two INDELs: the presence of a 6 bp long deletion in the *HvARH1*-Z4 and *HvARH1*-Z6 promoter region 71 basepairs upstream of the ATG triplet and a 9 bp long insertion in the *HvARH1*-Z1 promoter 735 bp upstream of the translational start. Their importance in the gene's ABA inducibility and the genotype-specific influence of *trans*-acting factors on gene induction should be the target of future experiments.

The *HvSRG6* (Stress-Responsive Gene), was mapped in a barley doubled haploid mapping population to chromosome 7H within a region that previously has been linked to osmotic adaptation in barley and other grass genomes. The *HvSRG6* gene expression was shown to be induced under drought stress or upon exposure to ABA, thus suggesting it to be a promising candidate gene for a conserved determinant of drought tolerance (Malatrasi et al. 2002). Despite the reported abscisic acid inducibility of this gene, we observed only a very moderate response in *HvSRG6* expression that ranged from a maximal 2.7-fold induction to a 0.7-fold repression. These minor changes in transcript levels did not correlate with a very pronounced change in the gene's promoter: in genotypes No.18-Otis, No.23-Chilga and No.61-Diamond a 196 bp long INDEL can be found 158 bp upstream to the translational start site. PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare) analysis of the promoter showed that none of the promoter elements responsible for ABA or drought responsive gene expression could be localized to the deleted region (data not shown).

In conclusion, we have presented an efficient way for allele mining in drought related barley CGs by using the EcoTILLING technology and haplotype sequencing. In the frame of this pilot project we analyzed 8 drought related candidate gene loci for natural variation. The set of 185 identified SNPs and 46 INDELs organized into 94 unique haplotypes were collected in a publicly accessible queryable database. Haplotype specific marker sets were developed for four genes. In addition, proof of concept was obtained for haplotype expression in the case of two CGs.

Acknowledgments This work was funded by the OMFB (Országos Műszaki Fejlesztési Bizottság) 445733—00514/2007 NAP BIO and OTKA (Országos Tudományos Kutatási Alapprogramok) 72366. We are grateful to Krisztina G. Talpas for excellent technical assistance. We also thank Ernő Homolya and Gábor Németh for help in the initialization of the website. We thank Georg Strompen (University of Potsdam, Germany) for Cel1 aliquots.

Conflict of interest None.

References

Bahieldin A, Mahfouz HT, Eissa HF, Saleh OM, Ramadan AM, Ahmed IA, Dyer WE, El-Itriby HA, Madkour MA (2005) Field evaluation of transgenic wheat plants stably expressing the HVA1 gene for drought tolerance. Physiol Plant 123:421–427

Barkley NA, Wang ML, Gillaspie AG, Dean RE, Pederson GA, Jenkins TM (2008) Discovering and verifying DNA polymorphisms in a mung bean [*V. radiata* (L.) R. Wilczek] collection by EcoTILLING and sequencing. BMC Res Notes 1:28

Bartels D (2001) Targeting detoxification pathways: an efficient approach to obtain plants with multiple stress tolerance? Trends Plant Sci 6:284–286

Bartels D, Engelhardt K, Roncarati R, Schneider K, Rotter M, Salamini F (1991) An ABA and GA modulated gene expressed in the barley embryo encodes an aldose reductase related protein. EMBO J 10(5):1037–1043

Brini F, Hanin M, Mezghani I, Berkowitz GA, Masmoudi K (2007) Overexpression of wheat Na^+/H^+ antiporter TNHX1 and H^+-pyrophosphatase TVP1 improve salt- and drought-stress tolerance in *Arabidopsis thaliana* plants. J Exp Bot 58:301–308

Bundock PC, Henry RJ (2004) Single nucleotide polymorphism, haplotype diversity and recombination in the Isa gene of barley. Theor Appl Genet 109:543–551

Choi DW, Zhu B, Close TJ (1999) The barley (*Hordeum vulgare* L.) dehydrin multigene family: sequences, allele types, chromosome assignments, and expression characteristics of 11 Dhn genes of cv Dicktoo. Theor Appl Genet 98:1234–1247

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159

Close TJ (1997) Dehydrins: a commonalty in the response of plants to dehydration and low temperature. Physiol Plant 100:291–296

Colbert T, Till BJ, Tompa R, Reynolds S, Steine MN, Yeung AT, McCallum CM, Comai L, Henikoff S (2001) High-throughput screening for induced point mutations. Plant Physiol 26:480–484

Comai L, Young K, Reynolds SH, Codomo C, Enns L, Johnson J, Burtner C, Henikoff JG, Greene EA, Till BJ, Henikoff S (2004) Efficient discovery of nucleotide polymorphisms in populations by ecotilling. Plant J 37:778–786

Cronin JK, Bundock PC, Henry RJ, Nevo E (2007) Adaptive climatic molecular evolution in wild barley at the Isa defense locus. Proc Natl Acad Sci USA 104:2773–2778

Egawa C, Kobayashi F, Ishibashi M, Nakamura T, Nakamura C, Takumi S (2006) Differential regulation of transcript accumulation and alternative splicing of a *DREB2* homolog under abiotic stress conditions in common wheat. Genes Genet Syst 81:77–91

Fukuda A, Chiba K, Maeda M, Nakamura A, Maeshima M, Tanaka Y (2004) Effect of salt and osmotic stresses on the expression of genes for the vacuolar H^+-pyrophosphatase, H^+-ATPase subunit A, and Na^+/H^+ antiporter from barley. J Exp Bot 55:585–594

Gaxiola RA, Li J, Undurraga S, Dang LM, Allen GJ, Alper SL, Fink GR (2001) Drought- and salt-tolerant plants result from overexpression of the AVP1 H^+-pump. Proc Natl Acad Sci USA 98:11444–11449

Gilchrist EJ, Haughn GW, Ying CC, Otto SP, Zhuang J, Cheung D, Hamberger B, Aboutorabi F, Kalynyak T, Johnson L, Bohlmann J, Ellis BE, Douglas CJ, Cronk QCB (2006) Use of EcoTILLING as an efficient SNP discovery tool to survey genetic variation in wild populations of *Populus trichocarpa*. Mol Ecol 15:1367–1378

Guo P, Baum M, Grando S, Ceccarelli S, Bai G, Li R, von Korff M, Varshney KR, Graner A, Valkoun J (2009) Differentially expressed genes between drought-tolerant and drought-sensitive barley genotypes in response to drought stress during the reproductive stage. J Exp Bot. doi:10.1093/jxb/erp194

Hideg É, Nagy T, Oberschall A, Dudits D, Vass I (2003) Detoxification function of aldose/aldehyde reductase during drought and ultraviolet-B (280–320 nm) stresses. Plant Cell Environ 26:513–522

Huson DH, Bryant D (2006) Application of phylogenetic networks in evolutionary studies. Mol Biol Evol 23:254–267

Igartua E, Gracia MP, Lasa JM, Medina B, Molina-Cano JL, Montoya JL, Romagosa I (1998a) The Spanish barley core collection. Genet Resour Crop Evol 45:475–482

Igartua E, Gracia MP, Lasa JM, Medina B, Molina-Cano JL, Montoya JL, Romagosa I (1998b) The Spanish barley core collection. J Hered 90:328–329

Jinyou D, Xiaoyang C, Wei L, Qiong G (2004) Osmoregulation mechanism of drought stress and genetic engineering strategies for improving drought resistance in plants. For Stud China 6:56–62

Kilian B, Ozkan H, Kohl J, von Haeseler A, Barale F et al (2006) Haplotype structure at seven barley genes: relevance to gene pool bottlenecks, phylogeny of ear type and

site of barley domestication. Mol Genet Genomics 276:230–241

Knüpffer H (1988) The European Barley Database of the ECP/GR: an introduction. Kulturpflanze 36:135–162

Kota R, Varshney RK, Thiel T, Dehmer KJ, Graner A (2001) Generation and comparison of EST-derived SSRs and SNPs in barley (*Hordeum vulgare* L.). Hereditas 135:145–151

Lababidi S, Mejlhede N, Rasmussen SK, Backes G, Al-Said W, Baum M, Jahoor A (2009) Identification of barley mutants in the cultivar 'Lux' at the Dhn loci through TILLING. Plant Breed 128:332–336. doi:10.1111/j.1439-0523.2009.01640.x

Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$. Methods 25(4):402–408

Maestri E, Malcevschi A, Massari A, Marmiroli N (2002) Genomic analysis of cultivated barley (*Hordeum vulgare*) using sequence-tagged molecular markers. Estimates of divergence based on RFLP and PCR markers derived from stress-responsive genes, and simple-sequence repeats (SSRs). Mol Genet Genomics 267:186–201

Malatrasi M, Close TJ, Marmiroli M (2002) Identification and mapping of a putative stress response regulator gene in barley. Plant Mol Biol 50:143–152

Marzin S, Mihaly R, Pauk J, Schweizer P (2008) A transient assay system for the assessment of cell autonomous gene function in dehydration-stressed barley. J Exp Bot 59:3359–3369

Matus IA, Hayes PM (2002) Genetic diversity in three groups of barley germplasm assessed by simple sequence repeats. Genome 45:1095–1106

McCarthy C (1998) Chromas 1.45. Technelysium Pty. Ltd., Helensvale

Mejlhede N, Kyjovska Z, Backes G, Burhenne K, Rasmussen SK, Jahoor A (2006) EcoTILLING for the identification of allelic variation in the powdery mildew resistance genes *mlo* and *Mla* of barley. Plant Breed 125:461–467

Morrell PL, Toleno DM, Lundy KE, Clegg MT (2005) Low levels of linkage disequilibrium in wild barley (*Hordeum vulgare* ssp. *spontaneum*) despite high rates of self-fertilization. Proc Natl Acad Sci USA 102:2442–2447

Mundree SG, Whittaker A, Thomson JA, Farrant JM (2000) An aldose reductase homolog from the resurrection plant *Xerophyta viscosa* Baker. Planta 211:693–700

Nieto C, Piron F, Dalmais M, Marco CF, Moriones E, Gómez-Guillamón ML, Truniger V, Gómez P, Garcia-Mas J, Aranda MA, Bendahmane A (2007) EcoTILLING for the identification of allelic variants of melon eIF4E, a factor that controls virus susceptibility. BMC Plant Biol 21:7–34

Oberschall A, Deák M, Török K, Sass L, Vass I, Kovács I, Fehér A, Dudits D, Horváth GV (2000) A novel aldose/aldehyde reductase protects transgenic plants against lipid peroxidation under chemical and drought stress. Plant J 24:437–446

Park SY, Noh KJ, Yoo JH, Yu JW, Lee BW, Kim JG, Seo HS, Paek NC (2006) Rapid upregulation of Dehyrin3 and Dehydrin4 in response to dehydration is a characteristic of drought tolerant genotypes in barley. J Plant Biol 49:455–462

Rodriguez EM, Svensson JT, Malatrasi M, Choi DW, Close TJ (2005) Barley Dhn13 encodes a KS-type dehydrin with constitutive and stress responsive expression. Theor Appl Genet 110:852–858

Rohila JS, Jain RK, Wu R (2002) Genetic improvement of Basmati rice for salt and drought tolerance by regulated expression of a barley Hva1 cDNA. Plant Sci 163:525–532

Roncarati R, Salamini F, Bartels D (1995) An aldose reductase homologous gene from barley: regulation and function. Plant J 7:809–822

Rostoks N, Mudie S, Cardle L, Russell J, Ramsay L et al (2005) Genome-wide SNP discovery and linkage analysis in barley based on genes responsive to abiotic stress. Mol Genet Genomics 274:515–527

Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132:365–386

Russell J, Booth A, Fuller J, Harrower B, Hedley P, Machray G, Powell W (2004) A comparison of sequence-based polymorphism and haplotype content in transcribed and anonymous regions of the barley genome. Genome 47:389–398

Shi HZ, Lee BH, Wu SJ, Zhu JK (2002) Overexpression of a plasma membrane Na^+/H^+ antiporter gene improves salt tolerance in *Arabidopsis thaliana*. Nat Biotechnol 21:81–85

Shinozaki K, Yamaguchi-Shinozaki K (2007) Gene networks involved in drought stress response and tolerance. J Exp Bot 58:221–227

Silvar C, Casas AM, Kopahnke D, Habekuß A, Schweizer G, Gracia MP, Lasa JM, Ciudad FJ, Molina-Cano J, Igartua E, Ordon F (2010) Screening the Spanish Barley Core Collection for disease resistance. Plant Breed 129(8):45–52

Sivamani E, Bahieldin A, Wraith JM, Al-Niemi T, Dyer WE, Ho TD, Qu R (2000) Improved biomass productivity and water use efficiency under water deficit conditions in transgenic wheat constitutively expressing the barley *HVA1* gene. Plant Sci 155:1–9

Taketa S, Amano S, Tsujino Y, Sato T, Saisho D, Kakeda K, Nomura M, Suzuki T, Matsumoto T, Sato K et al (2008) Barley grain with adhering hulls is controlled by an ERF family transcription factor gene regulating a lipid biosynthesis pathway. Proc Natl Acad Sci USA 105:4062–4067

Talamè V, Ozturk NZ, Bohnert HJ, Tuberosa R (2007) Barley transcript profiles under dehydration shock and drought stress treatments treatments: a comparative analysis. J Exp Bot 58:229–240

Törjék O, Berger D, Meyer R, Müssig C, Schmid K, Rosleff-Sörensen T, Weisshaar B, Mitchell-Olds T, Altmann T (2003) Establishment of a high-efficiency SNP-based framework marker set for *Arabidopsis*. Plant J 36:122–140

Törjék O, Witucka-Wall H, Meyer R, von Korff M, Kusterer B, Rautengarten C, Altmann T (2006) Segregation distortion in *Arabidopsis* C24/Col-0 and Col-0/C24 recombinant inbred line populations is due to reduced fertility caused by epistatic interaction of two loci. Theor Appl Genet 113:1551–1561

Törjék O, Meyer RC, Zehnsdorf M, Teltow M, Strompen G, Witucka-Wall H, Blacha A, Altmann T (2008) Construction and analysis of 2 reciprocal *Arabidopsis* introgression line populations. J Hered 99:396–406

van Berloo R (1999) GGT: software for the display of graphical genotypes

von Korff M, Grando S, This D, Baum M, Ceccarelli S (2008) Quantitative trait loci (QTL) associated with agronomic performance of barley under drought. Theor Appl Genet 117:653–669

von Korff M, Radovic S, Choumane W, Stamati K, Udupa SM, Grando S, Ceccarelli S, Mackay I, Powell W, Baum M, Morgante M (2009) Asymmetric allele-specific expression in relation to developmental variation and drought stress in barley hybrids. Plant J 59(1):14–26. doi:10.1111/j.1365-313X.2009.03848.x

Xu ZS, Ni ZY, Li ZY, Li LC, Chen M, Gao DY, Yu XD, Liu P, Ma YZ (2009) Isolation and functional characterization of HvDREB1—a gene encoding a dehydration-responsive element binding protein in *Hordeum vulgare*. J Plant Res 122:121–130

Xue GP, Loveridge CW (2004) HvDRF1 is involved in abscisic acid-mediated gene regulation in barley and produces two forms of AP2 transcriptional activators, interacting preferably with a CT-rich element. Plant J 37:326–339