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Seed conservation in *ex situ* genebanks—genetic studies on longevity in barley

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Abstract Recognizing the danger due to a permanent risk of loss of the genetic variability of cultivated plants and their wild relatives in response to changing environmental conditions and cultural practices, plant *ex situ* genebank collections were created since the beginning of the last century. World-wide more than 6 million accessions have been accumulated of which more than 90% are stored as seeds. Research on seed longevity was performed in barley maintained for up to 34 years in the seed store of the German *ex situ* genebank of the Leibniz-Institute of Plant Genetics and Crop Plant Research in Gatersleben. A high intraspecific variation was detected in those natural aged accessions. In addition three doubled haploid barley mapping populations being artificial aged were investigated to study the inheritance of seed longevity. Quantitative trait locus (QTL) mapping was based on a transcript map. Major QTLs were identified on chromosomes 2H, 5H (two) and 7H explaining a phenotypic variation of up to 54%. A sequence homology search was performed to derive the putative function of the genes linked to the QTLs.

Keywords *Ex situ* collections · Germplasm · Long term seed storage · QTL mapping · Seed longevity

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Introduction

World-wide existing germplasm collections contain more than 6 million accessions of which wheat represents the biggest group with about 800,000 samples followed by barley and rice comprising about 500,000 and 420,000 accessions, respectively. A list of the ten world-wide largest germplasm collections by crop is given in Table 1 (FAO 1998).

Plant *ex situ* genebank collections comprise seed genebanks, field genebanks and in vitro collections. Species, whose seed can be dried, without damage, down to low moisture contents, can be stored in seed banks. Field genebanks and in vitro storage are used primarily for species which are either vegetatively propagated or which have recalcitrant seeds that cannot be dried and stored for long periods. In addition, perennial species, for example certain forage species, which produce small quantities of seed, and

Table 1 The ten largest world-wide germplasm collections by crop (FAO, 1998)

Crop	Genus	Accessions
Wheat	*Triticum*	788,654
Barley	*Hordeum*	486,724
Rice	*Oryza*	420,341
Bean	*Phaseolus*	268,369
Maize	*Zea*	261,584
Oat	*Avena*	223,287
Soybean	*Glycine*	176,400
Sorghum	*Sorghum*	168,550
Mustard/rape	*Brassica*	106,923
Apple	*Malus*	97,543

long-lived plants (trees) are also maintained this way. It is estimated that worldwide, less than 10% of genebank holdings are stored in vivo in the field, and less than 1% are conserved in vitro (FAO 1998).

The German ex situ genebank, located at the Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK) in Gatersleben, is one of the four largest global collections. About 150,000 accessions are maintained including cereals (65,000), legumes (28,000), vegetables (18,000), forage crops (14,000), oil crops (8,000), potatoes (6,000) and medicinal and spice plants (6,000). As on the global scale wheat (*Triticum*) and barley (*Hordeum*) are the largest groups having 28,000 and 21,000 accessions, respectively (Annonymus 2008).

Seed storage is managed in large cold chambers, maintained at 0°C or at −15°C. Seeds are kept in glass jars, covered with bags containing silica gel. The oldest material in cold storage is originated from the harvest 1974. Natural aged accessions of barley (*Hordeum vulgare* L.) being from the 1974 harvest and kept at 0°C were selected and used to study the intraspecific variability of seed longevity. In addition, three barley doubled haploid mapping populations were exploited to study the inheritance of seed longevity by performing accelerated ageing tests.

Materials and methods

Plant materials

For investigating the intraspecific variability of seed longevity 55 barley accessions comprising 45 cultivars, 6 breeding lines and 4 landraces and belonging to the subspecies/varieties *Hordeum vulgare* L. convar. *distichon* (L.) Alef. var. *nutans* (Rode) Alef., *Hordeum vulgare* L. convar *distichon* (L.) Alef. var. *erectum* (Rode) Alef., *Hordeum vulgare* L. convar. *distichon* (L.) Alef. var. *medicum* Körn., *Hordeum vulgare* L. convar. *vulgare* var. *hybernum* Vib., *Hordeum vulgare* L. convar. *vulgare* var. *rikotense* Regel, *Hordeum vulgare* L. convar. *vulgare* var. *nigrum* (Willd.) Link, *Hordeum vulgare* L. convar. *vulgare* var. *parallelum* Körn., *Hordeum vulgare* L. convar. *intermedium* (Körn.) Mansf. var. *attergergii* Körn. were used. The material, originating from 12 countries was harvested in 1974 and stored in glass jars at 0 ± 1°C and 8 ± 2% seed moisture content. Germination data were available from 1976 (initial germinability) and 1993. Together with the present investigation performed in 2008, data after 19 and 34 years of storage were available, beside the basic germination records.

For the genetic studies three different doubled haploid mapping populations were investigated. (1) The 'Steptoe' × 'Morex' (S × M) population, consisting of 150 doubled haploid (DH) lines developed by pollinating the F_1 hybrid of the cv. 'Steptoe' × cv. 'Morex' cross with *H. bulbosum* (Kleinhofs et al. 1993). 'Steptoe' is a high yielding six-rowed feed-type barley (Muir and Nilan 1973) and 'Morex', a six-rowed cultivar used as the American malting industry standard (Rasmusson and Wilcoxson 1979). Seeds of 94 DH lines used in the present study were originated from 2006 field trials. (2) The OWB mapping population, a set of 94 spring barley DH lines developed again by the *H. bulbosum* method (Costa et al. 2001). Characteristics of 'OWB_{DOM}' and 'OWB_{REC}', which were selected as dominant and recessive morphological marker stocks, are described by Wolfe and Franckowiak (1991). Seeds investigated derived from field and greenhouse multiplications in 2006. (3) The winter barley population W766, resulting from a cross between the two-rowed cultivar 'Angora' and the accession 'W704/137', a two-rowed, short-stemmed, dense-eared winter barley of Japanese origin. DH lines had been derived using in vitro culture of anthers of which 100 were used in the current investigation. Further details of the mapping population are given by Buck-Sorlin (2002). Seeds were obtained from a 2005 field multiplication.

Accelerated ageing tests

The AA test exposes seeds for short periods to the two environmental variables which cause rapid seed weakening; high temperature and high relative humidity. High vigour seed lots will withstand these extreme stress conditions and deteriorate at a slower rate tan low vigour ones (Hampton and TeKrony 1995).

Artificial Aging (AA) test: For each DH line 2 replicates of 100 seeds were placed in a stainless metal cage on a rack in glass jars containing 200 ml deionised water. The ageing was performed at 43 ± 0.5°C for 72 h.

Controlled Deterioration (CD) test: After determining the original moisture content of the seeds following the ISTA standards (ISTA 2008) it was increased to 18% by adding deionised water according to the following formula recommended by ISTA (Hampton and TeKrony 1995):

$$m_{H_2O} = \left(\frac{100 - \mathrm{smc_I}(\%)}{100 - \mathrm{smc_T}(\%)}\right) \times m_I$$

where m_{H_2O} = added water, $\mathrm{smc_I}$ (%) = initial seed moisture content (%), $\mathrm{smc_T}$ (%) = target seed moisture content (%) and m_I inital seed weight (g). After a 2 h period of equilibration and a 22 h period of relaxation at 7°C the two replications of 100 seeds per genotype were sealed in aluminium bags and aged at 44 ± 0.5°C for 72 h.

Germination test

For investigating the intraspecific variability of seed longevity, the 55 barley accessions were tested in three replications consisting of 50 seeds each. From the mapping populations two replications of 100 seeds each of all DH lines and originated from the field multiplications were tested as controls supplemented by one replication of 100 seeds of the OWB population originated from the greenhouse. After two different aging methods (AA, CD), two replications of 100 seeds per method of all DH lines divided by two were analysed, i.e. four replications with 50 seeds each, per line and aging treatment. All seeds were originated from the field multiplications mentioned above except the OWB ones used for AA method which were originated from the greenhouse.

Each replication was germinated between two layers of filter paper, formed to rolls standing on a Jacobsen apparatus (day 25 (±2)°C; night 23 (±2)°C). After a defined period of 7 days (ISTA 2008) the germination percentage was calculated from the proportion of normal appearing seedlings.

Statistical analysis

For testing the intraspecific variability of germinability the arithmetic means and standard deviations of the 55 different barley accessions were calculated. A paired *t*-test was applied.

Both absolute germinabilities obtained after accelerated ageing and relative germination gained by dividing the germinabilities of the single replicates and their mean by the mean germination of the corresponding controls were determined. QTL analyses were performed using absolute and relative germinabilities for each replicate and the means separately. Single marker and simple interval mapping options provided by QGENE software (Nelson 1997) were exploited. A LOD threshold of 3.0 was set to claim significance.

For OWB and S × M mapping populations transcript maps consisting of 586 and 312 expressed sequence tag (EST)-based markers, respectively, developed by Stein et al. (2007) are available. Markers were designated as GBR, GBM and GBS (for Gatersleben barley RFLP, microsatellite and SNP). Markers in a ± 10 cM interval of the marker detected by single marker QTL analysis and having LOD values >3 in at least one replication were considered only. Annotation of the ESTs was performed by BLASTX (Basic Local Alignment Tool) similarity search against the public non-redundant protein database, NRPEP (version from June 2008), from NCBI (National Center for Biotechnology Information). Candidate orthologs were defined as those with hits with best high scoring pair (HSP) and significant *E*-value (Expect value) of <1.0E−10. The sequence information of the barley ESTs are stored in the CR-EST database (The IPK Crop EST database, v1.5) (http://pgrc.ipk-gatersleben.de/cr-est).

Results

Intraspecific variability

The initial germination test showed high germination for all accessions having a mean of 99.06 ± 1.08%.

The average decreased after 19 and 34 years of storage to 93.60 ± 3.26% and 76.18 ± 17.36%, respectively. There was a clear increase in variation, being highly significant in the 2008 analysis (paired *t*-test). Germination after 34 years of storage ranged between 3.36% for accession HOR 2110 and 98.67% for HOR 1320 (Fig. 1). There were no differences detectable between subspecies with respect to longevity. In fact both accessions reaching highest (98.67%) and lowest (3.36%) germinabilities belong to the same subspecies and even variety *Hordeum vulgare* L. convar. *distichon* (L.) Alef. var. *nutans* (Rode) Alef.

QTL mapping

Comparing the accelerated ageing QTL mapping results obtained from the data achieved using absolute germinabilities with the relative germination a high coincidence was found for both aging methods in all three mapping populations. One example for the S × M population is given in Fig. 2. Due to this correspondence the relative germination was considered for the further assessment only.

S × M population

Applying both accelerated aging methods (AA-test, CD-test) corresponding, highly significant QTLs (LODs > 14) were associated with marker GBS0892 on chromosome 5HL explaining more than 50% of the phenotypic variation (Table 2; Fig. 2). The high longevity was contributed by the parent 'Steptoe'.

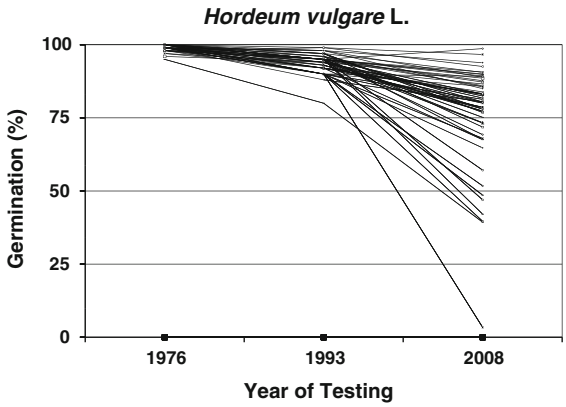

Fig. 1 Mean germination of selected barley accessions in different years of testing

No other region was discovered in that population. The locus detected by both methods was designated *QLng.ipk-5H.1*. Within a region 10 cM proximal and distal, respectively, to GBS0892 six additional markers including the gene aleurain (*Ale*) and four ESTs having LODs > 3 in at least one replicate were detected. The biological functions of the candidate EST based markers (GBR0482, GBR0613) are given in Table 3.

OWB population

Analysing the greenhouse originated population (AA-test) one significant QTL was detected in the distal region of chromosome 2H (Table 2; Fig. 3) associated with the gene locus *Zeo1* (*Zeocriton 1*). The same QTL, however, having a LOD value >3 in only one replicate was detected analysing the population grown in the field but applying the CD-test. Here, however, two more chromosomes were identified carrying QTLs. They are located on the distal part of the long arm of chromosome 5H connected with GBS800 and on 7HL associated with GBR1478 (Table 2; Fig. 3). On chromosome 7H a second peak appeared about 25 cM distal to GBR1478 reaching a significant threshold in only one replicate (Fig. 3). This region was not considered in the further analysis and discussion, because it emerged after CD-test only. The QTLs detected on chromosome 2H were contributed by parent 'OWB_{REC}', whereas the positive alleles on 5HL and 7HL came from 'OWB_{DOM}'. The QTLs explained a phenotypic variation of around 15% each. Significant (LOD > 3) markers/genes within the region of 10 cM proximal and distal to the markers associated with the QTLs are indicated in Fig. 3. Candidate ESTs and their functions are given in Table 3. On chromosome 7H the gene determining naked caryopsis (*nud*) is in the region of interest. The QTLs detected were designated *QLng.ipk-2H*, *QLng.ipk-5H.2* and *QLng.ipk-7H*.

W766 population

One chromosomal region carrying QTLs for seed longevity was discovered analysing the W766 population. Corresponding QTLs were detected with both methods on the long arm of chromosome 7H close to the centromere (Fig. 4). The position was

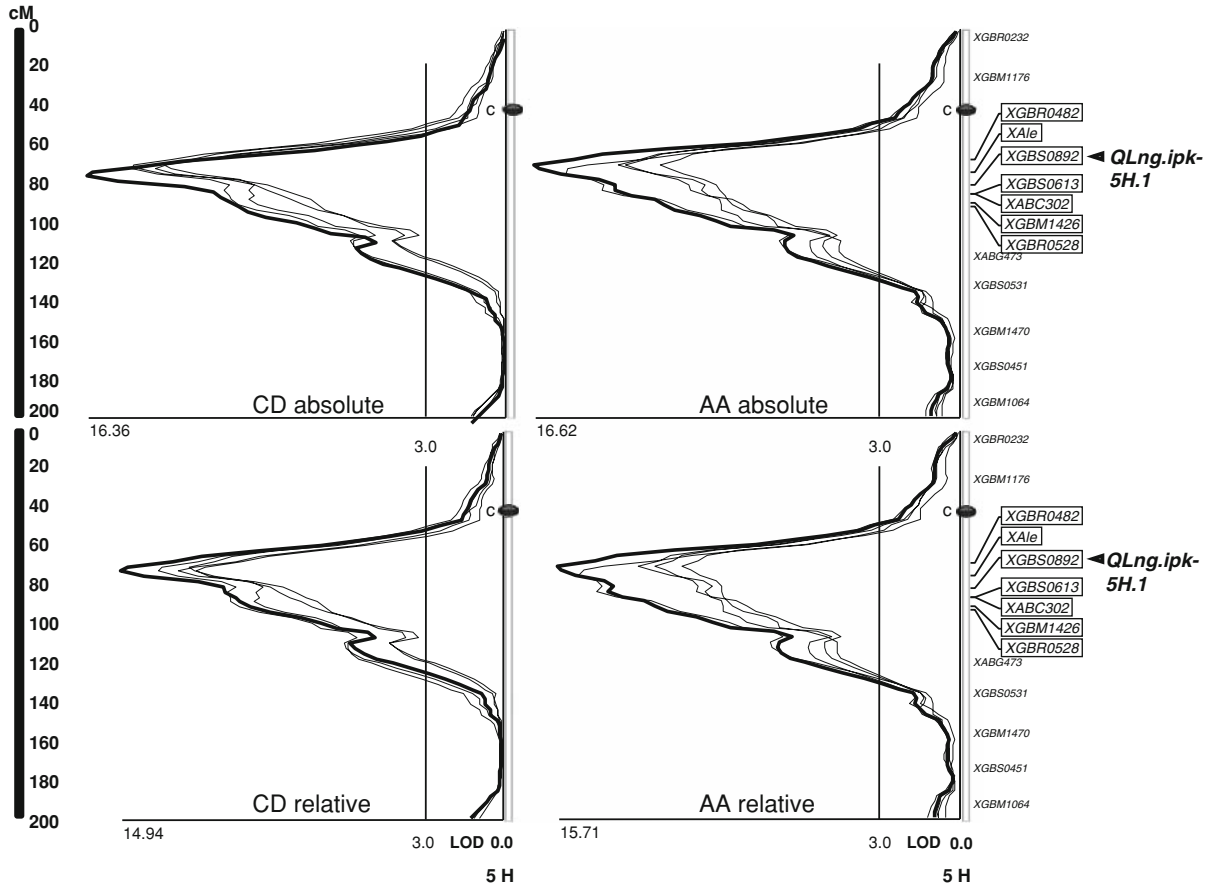

Fig. 2 QTL interval mapping results obtained for S × M population considering absolute and relative germinations in AA- and CD-tests. Skeletal map is based on data from Stein et al. (2007). Loci within a region of 10 cM proximal and distal to the marker detected by single marker QTL analysis and reaching a LOD > 3 in at least on replicate are *boxed*. c = centromere

Table 2 Single marker QTL analysis of the means of relative germination rates

Population	Treatment	Marker/gene	Chromosome	Source allele	LOD score	R^2 value
S × M	AA	GBS0892	5H	Steptoe	14.66	0.5397
	CD	GBS0892	5H	Steptoe	14.34	0.5320
OWB	AA	*Zeo1*	2H	OWB_{REC}	3.00	0.1452
	CD	GBS0800	5H	OWB_{DOM}	3.37	0.1633
		GBR1478	7H	OWB_{DOM}	3.19	0.1660
		GBM1047	2H	OWB_{REC}	2.80	0.1393
W766	AA	M38E54-320	7H	Angora	3.79	0.1633
	CD	*nud*	7H	Angora	8.14	0.3177

Loci having a LOD > 3.0 in at least one single replicate were considered

highly comparable to that detected with CD-method in the OWB mapping population (Fig. 3). The phenotypic variation explained was 16 and 32% considering the AA-method and CD-method, respectively (Table 2). The higher longevity was contributed by the 'Angora' parent. Because the population

Table 3 Biological functions of candidate ESTs having significant *E*-value (<1.0E−10)

EST-marker	Chr.	Hit_name	Functional annotation	Organism	Score	*E*-value
GBM1498	2H	gi|68518815|gb|AAY98505.1|	Dehydration responsive element binding protein	*Triticum aestivum*	92	2E-17
		gi|68303944|gb|AAY89658.1|	DREB protein	*Glycine max*	76	9E-13
GBM1164	5H	gi|50919595|ref|XP_470158.1|	Putative hydrolase	*Oryza sativa*	155	1E-36
		gi|12642902|gb|AAK00393.1|	Putative epoxide hydrolase ATsEH	*Arabidopsis thaliana*	88	2E-16
GBR170	5H	gi|56682582|gb|AAW21725.1|	Thaumatin-like protein TLP5	*Hordeum vulgare*	188	9E-47
		gi|2454602|gb|AAB71680.1|	Barperm1	*Hordeum vulgare*	166	5E-40
GBR304c	5H	gi|115456247|ref|NP_001051724.1|	Heat shock cognate 70 kDa protein 2, putative, expressed	*Oryza sativa*	153	6E-36
GBS0800	5H	gi|18476518|gb|AAL50205.1|	APETALA2-like protein	*Hordeum vulgare*	409	1E-112
		gi|53830037|gb|AAU94926.1|	Floral homeotic protein	*Triticum aestivum* subsp. *spelta*	394	1E-108
XGBR0482	5H	gi|115478929|ref|NP_001063058.1|	Putative permease 1	*Oryza sativa*	95	2E-18
XGBS0613	5H	gi|89511843|dbj|BAE86874.1|	Putative asparate aminotransferase	*Hordeum vulgare*	217	3E-55
		gi|115479507|ref|NP_001063347.1|	Putative cysteine conjugate beta-lyase	*Oryza sativa*	209	9E-53
GBR1478	7H	gi|115449079|ref|NP_001048319.1|	Ethylene-responsive transcription factor 1	*Oryza sativa*	348	1E-94
		gi|6689918|gb|AAF23899.1|AF193803_1	Transcription factor EREBP1	*Oryza sativa*	334	3E-90
		gi|145390028|gb|ABP65298.1|	Ethylene responsive element binding protein	*Oryza sativa*	277	4E-73
GBR283	7H	gi|115475922|ref|NP_001061557.1|	Putative enoyl-ACP reductase *Oryza sativa*	*Oryza sativa*	67	3E-10

was genotyped using AFLP markers no conclusions about biological functions were possible. However, the QTLs were again closely related to the gene *nud*. Because the 7H loci detected in the OWB and W766 populations are in highly comparable positions, both were designated *QLng.ipk-7H*.

Discussion

Plant genetic resources are of particular high value for mankind. Some were collected already at the beginning of the last century and are not any longer available in the original habitats. The importance of genebank accessions is undoubted because they form the basic material for the work of plant breeders, pharmacists and ecologists. For an accurate maintenance of genebank collections consisting predominantly (90%) of seeds (Börner 2006), studies on the longevity of the accessions stored are essential.

Since the investigation of seed samples discovered in the foundation stone of the 'Nürnberger Stadttheater' in 1956 (Aufhammer and Simon 1957; Steiner et al. 1997) and the evaluation of the experiments initiated by the Austrian researcher Friedrich Haberland at the 'Universität für Bodenkultur' in Vienna in 1877 but rediscovered in 1967 (Ruckenbauer 1971; Steiner and Ruckenbauer 1995) it is known that cultivated crops (cereals) can keep their germinability for more than 100 years. The longevity of seeds is species specific and formulas for the prediction of seed viability have been created for many species (e.g. see Ellis and Roberts 1980, 1981; Ellis 1988, 1989). Species dependent differences in storability of seeds were also detected in studies performed at the IPK genebank considering cereals, legumes, vegetables, oil crops and herbs (Specht et al. 1997, 1998). However, beside this interspecific variability of seed longevity variation within a species does exist, as demonstrated in the present study on barley. The accessions investigated coming from a seed multiplication performed in the same year (1974) at the same place (experimental fields, IPK Gatersleben). Furthermore they were handled in the same way during/after harvest (threshing, cleaning) and stored under identical conditions in one and the same cold chamber in glass jars. Therefore, the differences in germinability

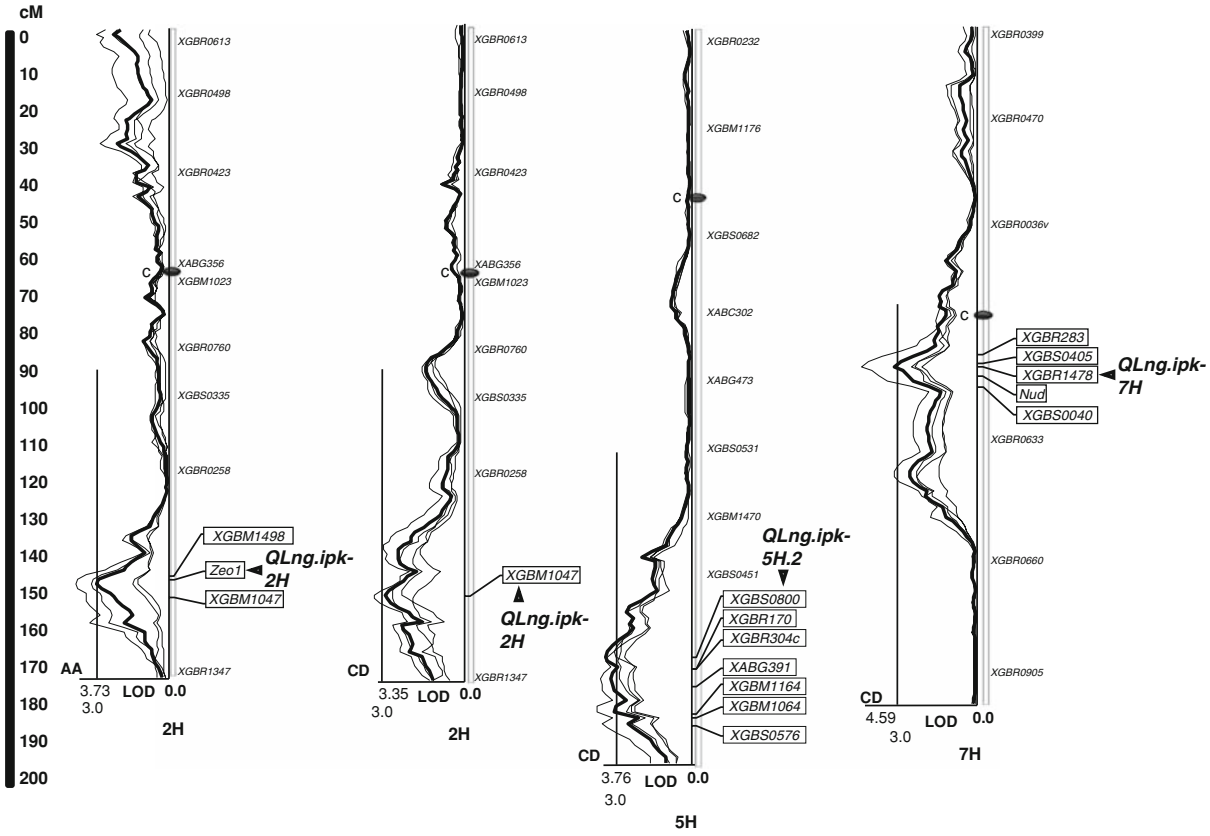

Fig. 3 QTL interval mapping results obtained for OWB population considering relative germinations in AA- and CD-tests. Skeletal map is based on data from Stein et al. (2007). Loci within a region of 10 cM proximal and distal to the marker detected by single marker QTL analysis and reaching a LOD > 3 in at least on replicate are *boxed*. c = centromere

discovered in the present study (Fig. 1) must be due to genetic variation in seed longevity. It should be mentioned here, that corresponding studies on wheat and *Sorghum* show a very similar behaviour (unpublished data).

The consequence of these findings was to perform a genetic analysis in order to identify loci responsible for the differences in seed longevity. Comparable studies in plants are rare. One mapping study was performed in the model plant Arabidopsis (Bentsink et al. 2000). In total four QTLs located on chromosomes 1 (two closely linked QTLs), 3 and 5 were identified. Other studies were performed in rice, where chromosomes 2, 4, 9 (two QTLs) 11 and 12 carry QTLs for seed longevity (Miura et al. 2002; Zeng et al. 2006; Shigemune et al. 2008). In the present study three barley mapping populations were analysed in parallel and four genomic regions associated with seed longevity QTLs were identified on chromosomes 2HL (*QLng.ipk-2H*), 5HL (*QLng.ipk-5H.1*, *QLng.ipk-5H.2*) and 7HL (*QLng.ipk-7H*). Compared to QTL studies for other traits in cereals this number is rather low, whereas the phenotypic variation explained by the loci detected is high, reaching up to 54%.

In two of the mapping populations (W766 and OWB), segregating for the character hulled/naked caryopsis QTLs closely related to the *nud* gene determining the trait (Lundqvist et al. 1997) were identified. Therefore it is very likely, that the gene itself is involved. In both populations the hulled parent contributes to the higher longevity, i.e. naked grains are disadvantageous.

The region detected on chromosome 2HL is carrying another gene, designated *Zeo1* and determining a short plant habit having very compact spikes with long awns and reduced fertility (Lundqvist et al. 1997). Here the high longevity is

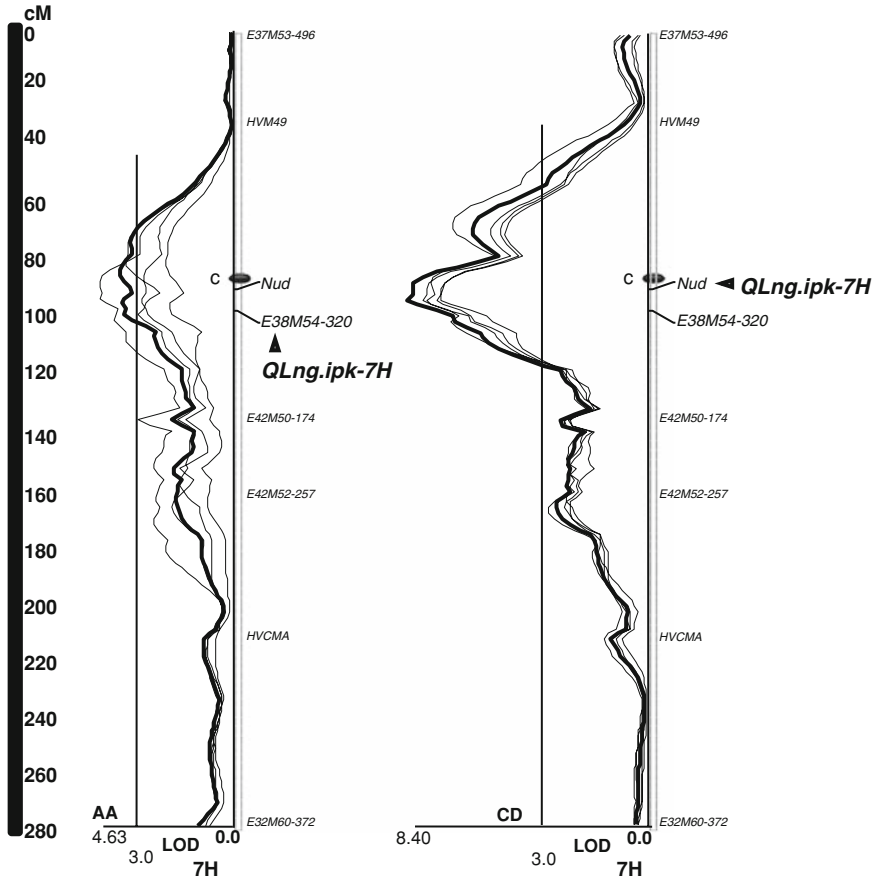

Fig. 4 QTL interval mapping results obtained for W766 population considering relative germinations in AA- and CD-tests. Skeletal map is based on data from Buck-Sorlin (2002). c = centromere

contributed by the parent 'OWB_{REC}', carrying the recessive wild-type allele determining 'normal' spikes. A negative effect due to the changed spike architecture may be postulated.

On chromosome 5H two regions were detected in two different mapping populations. The S × M population is segregating for the gene *Ale* (*Aleurain*) related to the QTL detected in the proximal region of chromosome 5HL. Aleurain is a barley vacuolar thiol protease. The aleurain gene (cDNA) was synthesized from gibberellic acid-stimulated aleurone cell mRNA (Rogers et al. 1985). The expression is regulated by the plant hormones gibberellic acid and abscisic acid, well known to be included in germination process. Interestingly, analysing wheat-barley single chromosome addition lines Li et al. (1991) localised another aleurain gene on chromosomes 7H whereas a copy of exon 3-intron 3 from the aleurain gene is present on chromosome 2H. Unfortunately no intrachromosomal mapping data are available and therefore, a comparison to the positions of the longevity loci mapped on these particular chromosomes in the present study is not possible.

The candidate ESTs identified in the longevity QTL regions have orthologs in rice, wheat, soybean and Arabidopsis or have known functions in barley itself. On chromosome 2H the marker GBM1498 has orthologs in wheat and soybean, associated with dehydration responsive element binding protein (DREB). Together with the ethylene-responsive element (ERE) binding factors (rice ortholog of GBR1478 on chromosome 7H) they belong to the APETALA2/ethylene-responsive element-binding protein family which play an important role in the regulation of abiotic and biotic stress responses, respectively. The expression of DREBs is activated by drought, cold or ethylene (Sun et al. 2008). But mainly the osmotic stress conditions such as drought and salinity lead to induction of DREB (Huang and Jin-Yuan 2006). Apart from that the ERE binding elements regulate the expression of pathogen response gene and prevent disease progression. The

ethylene pathway together with the jasmonate pathway is required for regulation of defence response genes (Lorenzo et al. 2003).

APETALA2 (AP2) itself represents wheat and barley orthologs of the marker GBS0800 on chromosome 5H. The transcription factors of the AP2 gene family implicate a wide range of plant development roles and perform in angiosperms the establishment of the floral meristem, the specification of floral organ identity, the regulation of floral homeotic gene expression, the regulation of ovule development and the growth of floral organs (Kim et al. 2006). One gene of this family called 'indeterminate spikelet 1' specifies determinate fates which lead to many types of lateral organ primordial and spikelet meristems (Chuck et al. 1998). These may contribute to an inflorescence architecture which is important for development and physical health of seeds. In a similar way the GBR283 (chromosome 7H) ortholog, a putative enoyl ACP reductase is conducive for the ripening of fruits.

Furthermore, thaumatin-like proteins like Barperm1, which is related to the sequence of GBM1164 on chromosome 5H, is responsible for an antifungal activity and indicates a possible role in defence against leaf pathogens (Zareie et al. 2002). Interestingly the investigations of Tattersall et al. (1997) show the timing of its accumulation correlates with the inability of the fungal pathogen initiate new infections. Together with DREB and ERE binding elements it contributes to withstand environmental stress and could be one hint for different shelf-life of seeds. The effect of the orthologs of the remaining markers (Table 3) on seed longevity is rather speculative and therefore not discussed further.

Finally, we compared the longevity QTL regions detected here with those described in rice (Miura et al. 2002; Zeng et al. 2006) by using the co-linearity information of Stein et al. (2007). For two of the barley QTLs co-linearity to loci in rice may be suggested. Based on the co-linearity between barley 2HL and rice Os4L the loci *QLng.IPK-2H* and *qLG-4* may represent homoeologous loci. In addition barley *QLng.IPK-5H.1* may be related to rice *qLG-9* or *qLS-9*. Ongoing studies in wheat and rye will provide more information about a possible conservation of genes for seed longevity within the Poaceae.

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