Genetica (2006) 127:87–99
DOI 10.1007/s10709-005-2484-2

Correlation between hordatine accumulation, environmental factors and genetic diversity in wild barley (*Hordeum spontaneum* C. Koch) accessions from the Near East Fertile Crescent

A.K. Batchu, D. Zimmermann, P. Schulze-Lefert & T. Koprek*
*Max-Planck-Institute for Plant Breeding Research Cologne, Carl-von-Linné Weg 10, 50829 Cologne, Germany; *Author for correspondence (Fax: +49-221-5062-353; E-mail: koprek@mpiz-koeln.mpg.de)*

Received 24 February 2005; Accepted 25 August 2005

Key words: barley, defense-related compounds, diversity, hordatine, *Hordeum vulgare*, *Hordeum spontaneum*

Abstract

Wild barley shows a large morphological and phenotypic variation, which is associated with ecogeographical factors and correlates with genotypic differences. Diversity of defense related genes and their expression in wild barley has been recognized and has led to attempts to exploit genes from *H. spontaneum* in breeding programs. The aim of this study was to determine the variation in the accumulation of hordatines, which are *Hordeum*-specific preformed secondary metabolites with strong and broad antimicrobial activity *in vitro*, in 50 accessions of *H. spontaneum* from different habitats in Israel. Differences in the accumulation of hordatines in the seedling stage were significant between different *H. spontaneum* genotypes from different regional locations and micro-sites. Variation in the hordatine accumulation within genotypes was between 9% and 45%, between genotypes from the same location between 13% and 38%, and between genotypes from different locations up to 121%. Principal component analysis showed that water related factors explain 39%, temperature related factors explain 33% and edaphic factors account for 11% of the observed variation between the populations of *H. spontaneum*. Genetic analysis of the tested accessions with LP-PCR primers that are specific for genes involved in the biosynthetic pathway of hordatines showed tight correlations between hordatine abundance and genetic diversity of these markers. Multiple regression analyses indicated associations between genetic diversity of genes directly involved in hordatine biosynthesis, ecogeographical factors and the accumulation of hordatines.

Introduction

Domestication and the application of pure breeding practices has led to a marked truncation of genetic diversity found in wild species (Plucknett et al., 1983; Nevo, 1992), reducing the individual fitness and the ability to adapt to environmental challenges. Crop species, which are derived from self-pollinating progenitors such as cereal crops are especially affected by the reduction of genetic diversity. Comparative studies of the genetic variation in wild barley (*H. spontaneum* C. Koch) found at its centre of origin and cultivated barley (*H. vulgare* L.) demonstrated that cultivated barley represents only a fraction of allelic variation present in wild populations (Brown, 1992; Nevo, 1992). Within the wild populations, the genetic diversity is the highest in populations from the most highly stressed environments (Nevo et al., 1997). A considerable degree of correlation between diversity at the molecular level and phenotypic variation influencing adaptive traits and individual fitness has been demonstrated using different molecular markers (Nevo, 1987; Pakniyat et al., 1997; Marmiroli et al., 1999; Turpeinen et al., 2001; Ellis et al., 2002; Liviero et al., 2002). The genetic basis of adaptation to environmental factors is still not understood in detail. Data suggest that variation in

stress responsive genes may affect the individual performance under environmentally unfavorable conditions (Gillespie & Turelli, 1989; Quesada et al., 2002; Ungerer et al., 2003) and may therefore be involved in the genetic manifestation of adaptation. A significant proportion of diversity at the molecular level of stress responsive genes appears to be adaptive rather than neutral (Père zdse la Vega, 1996; Favatier et al., 1997; Nevo et al., 1998). Molecular markers derived from stress responsive genes frequently exhibit a high degree of polymorphism and have therefore successfully been used to gain valuable information about correlations between phenotypic and genetic diversity (Marmiroli et al., 1998, 1999; Liviero et al., 2002).

H. spontaneum accessions from the Near East Fertile Crescent represent a genetically highly diverse gene pool and have been the subject of many studies on plant evolution, physiological adaptation and population genetics (reviewed by Nevo, 1992; Forster, 1999). In numerous studies, morphological and stress-related traits with agricultural importance were the main objective (Joergensen, 1992; Gunasekera et al., 1994; Ceccarelli & Grando, 1996; Forster et al., 1997; Lu et al., 1999; Ivandic et al., 2000, 2003; Suprunova et al., 2004; Chen et al., 2004; Verhoeven et al., 2004a and b). The analysis of immediate stress responsive genes in accessions of *H. spontaneum* from different habitats demonstrated a strong correlation between genetic variability and adaptation to different environmental stress factors (Turpeinen et al., 2001, 2003; Huang et al., 2002; Maestri et al., 2002; Suprunova et al., 2004). On the other hand, there is little information about the variation of physiological adaptations to different environmental stress factors. The occurrence and accumulation of stress-related constitutive or induced secondary compounds and genes involved in their biosynthesis or regulation have largely been neglected. For example, the analysis of the contents of other defense related secondary compounds such as DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) and DIBOA (2,4-dihydroxy-1,4-benzoxazin-3-one) in different species and accessions of *Triticum* (Niemeyer, 1988; Gianoli & Niemeyer, 1998; Quader et al., 2001) and the indole alkaloid gramine in accessions of *H. vulgare* and *H. spontaneum* (Lovett, Hoult & Christen, 1994) showed significant variation within the same species but no in depth correlative studies between phenotypic variation, genetic diversity or ecological factors were made.

In the present study, we analyzed the variation of hordatine accumulation in seedlings of different *H. spontaneum* accessions and tried to identify correlations between hordatine accumulation, environmental factors and genetic diversity. Hordatines are dimerization products of the conjugated polyamines coumaroylagmatine (hordatine A) or coumaroylagmatine and feruloylagmatine (hordatine B) and occur in *H. spontaneum* in a ratio of about 25:1 to 30:1. They directly inhibit spore germination of a variety of fungal pathogens *in vitro* (Stoessl & Unwin, 1970) and are exclusively found in some species of the genus *Hordeum* (Smith & Best, 1978) where they are abundant during the development of the young seedling. Results of several reports indicate that hordatines (A and B) are involved in defense mechanisms as preformed compounds in the seedling (Smith & Best, 1978) and that their accumulation is inducible after pathogen attack in older plants (Wei et al., 1994; von Roepenack, Parr & Schulze-Lefert, 1998; Burhenne, Kristensen & Ramussen, 2003). The concentration of hordatines A and B were measured under controlled conditions in plants of 50 different *H. spontaneum* accessions from different habitats in Israel representing a large variety of different temperatures, irradiation, rainfall and edaphic conditions. In order to evaluate the genetic diversity of specific genes involved in the hordatine biosynthesis, we employed Long Primer (LP) PCR using gene specific oligonucleotides. The LP-PCR marker system has successfully been used in similar studies and was able to discriminate genetic diversity specifically in targeted genes related to stress responses in accessions of cultivated barley and its progenitor *H. spontaneum* (Marmiroli et al., 1998; Liviero et al., 2002; Maestri et al., 2002). In this method, primers are designed on sequences corresponding to promoter elements or protein motifs, taken from well characterised genes of interest. The oligonucleotides were used as single primers in amplification reactions at moderate stringency, and thus the fragments amplified were encompassed by inverted (imperfect) repeats of the primer sequence. The length of primers (19–24 nt) ensured a more stable annealing than observed in techniques using shorter primers leading to better reproducibility of profiles and a higher proportion of targeted gene specific regions among the amplification products.

Material and methods

Plant material

Seeds of 50 accessions from eight populations of *Hordeum spontaneum* C. Koch were kindly provided by Prof. E. Nevo (University of Haifa, Israel). They were directly collected from 5 different Israeli locations reflecting macro and micro ecogeographic differences. We defined a population as a group of different accessions growing at the same location under identical environmental conditions; consequently, groups of accessions growing at the same location under different environmental conditions are two different populations. Positions of accessions and individual plants in each accession at the collection site were randomized and seed stocks from all accessions are maintained at the University of Haifa. Ecogeographic and climatic characteristics for each of the sampling locations have previously been documented by Nevo et al. (1979, 1984) and Pakniyat et al. (1997) (Table 1). Growth conditions at micro-sites are identical in all but one or two specific environmental characteristics (different microclimate and irradiation at the Evolution Canyon, different soil types at Tabigha and different soil depth/sun exposure at Neve Ya'ar). These sharply contrasting conditions allow the direct analysis of the influence of one particular ecogeographical factor on the accumulation of hordatines.

Different environmental categories (temperature means, mean water factors and geographical factors) were considered for correlation analysis. Edaphic factors were included as dummy variables for different soil types (rendzina, terra rossa, basalt, loess) (Table 1) in ascending order from light to heavy (A. Beiles, University of Haifa, personal communication). Because this procedure does not correspond to a normal distribution, non-parametric correlations were used in statistical analysis of data. Plants (five plants per accession) were grown under sterile conditions in petri dishes on moist (sterile ddH_20) filter paper with a photoperiod of 16 h/8 h (light/dark) at 20 °C. Ten days after germination the plantlets were transferred from petri dishes into soil and grown under the same growth regime.

Extraction of hordatines

Leaf material from seedlings (5 seedlings from each accession) was harvested three, 10 and 28 days after germination. Leaf samples from 10 and 28 days old plants were taken from two or three different leaves, which were combined for extraction. Soluble hydroxycinnamic acid amides were extracted after thorough grinding of the leaves in

Table 1. Ecogeographical data of *Hordeum spontaneum* collection sites in Israel

Location	Acc	Lon	Lat	Alt	Tm	Tj	Ta	Ev	Rn	Rd	Hu_{14}	Hu_{an}	So
Tabigha-Terra Rossa	5	35.53	32.90	0	24	15	32	158	436	56	45	59	2
Tabigha-Basalt	5	35.53	32.90	0	24	15	32	158	436	56	45	59	4
Neve Ya'ar rock/sun	5	35.11	32.44	100	19	10	26	160	570	43	55	61	1
Neve Ya'ar soil/shade	5	35.11	32.44	100	19	10	26	160	570	43	55	61	1
Evolution Canyon mesic	5	35.02	32.43	75	19	13	28	148	690	56	59	66	3
Evolution Canyon xeric	5	35.02	32.43	75	19	11	30	178	690	56	59	53	3
Sede Boqer	10	34.78	30.87	450	19	9	26	168	91	15	36	53	5
Maalot	10	35.27	33	500	17	8	23	150	785	55	50	64	1

Acc = Accession
Geographical variables: Lon = longitude; Lat = latitude; Alt = altitude (m).
Temperature variables: Tm = mean annual temperature (°C); Tj = mean January temperature (°C); Ta = mean August temperature (°C);
Ev = mean annual evaporation.
Water variables: Rn = mean annual rainfall (mm); Rd = mean annual number of rainy days; Hu_{14} = mean humidity at 14.00 h (%); Hu_{an} = mean annual humidity (%);
Soil variables: So = soil types: 1, rendzina; 2, terra rossa; 3, forrest mediterranean; 4, basalt; 5, loess. Soil types are in ascending order from light to heavy.

liquid nitrogen with 200 µl of methanol/acetic acid (98:2, v/v) under vigorous shaking for 1 h. The slurry was centrifuged and the pellet was extracted a second time in the same way. The unified extracts were directly used for HPLC analysis.

HPLC analysis of hordatines

Samples (20 µl) were subjected to HPLC on a Nucleosil C-18 column (EC 250/4, 120–5; Macherey & Nagel, Düren, Germany) using 0.1% trifluoroacetic acid in water (v/v) as solvent A and acetonitrile/H_2O/trifluoroacetic acid (98:1.9:0.1; v/v/v) as solvent B at a flow rate of l/min at 30 °C. The following gradient elution conditions were used for solvent A: time = 0 min/90% A; time = 30 min/70% A; time = 31 min/0% A; time = 3in/90% A; time = 40 min/0% A; time = 50 min/90% A). A dual wavelength detector (Bio-Tek, Neufahrn, Germany) was used for monitoring the phenolic profiles at 306 nm and 280 nm, and the peak area was quantified at 306 nm. In above conditions, the retention times are as follows, for *p*-coumaroylagmatine 16 min, hordatine B 24 min and hordatine A 24.5 min. All solvents were of HPLC grade purity. Purified hordatine A was used as a standard for quantification. Because the ratio of the concentration between hordatine A and hordatine B was in all experiments and at different time points relatively constant, we added both values and refer henceforth to the total hordatine concentration simply as hordatines.

Mass spectrometry

The molecular weight (M_r) and the structures of *p*-coumaroylagmatine, hordatine A and hordatine B were determined by liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) (Micromass Quattro II, Manchester, UK). The HPLC conditions used were as follows: column C18 reverse phase column Supelco, 250 × 2.1 mm with a quad column, gradient and UV detection as described above. Conditions for the ESI-MS/MS were as follows: positive mode; 3.0 kV capillary voltage; collision energy = 20 eV; collision gas, argon; collision pressure = 1.5×10^3 Torr; carrier solvent, acetonitrile/water (1:1 (v/v)); flow injection = 20 µl in 10 µl/min; injected amount = 100 µg; scan rates = 3–10 s/scan over the calibrated mass range (23–1800 Da); ESI probe voltage = 3.2–3.5 kV; cone voltage = 30 V; source temperature = 70 °C.

Isolation of genomic DNA

Genomic DNA was isolated from three leaves per plant and from two plants per accession (Cone, 1989).

LP-PCR analysis

Genomic DNA samples from two different plants per accession were analyzed independently. Nine oligonucleotide primers (21–24 nt) were designed with the software Primer3 (Rozen & Skaletsky, 2000). Different oligonucleotides were derived from cDNA sequences deposited in public databases from arginine decarboxylase (*Adc*, TC132257), agmatine coumaroyltransferase (*Act*, Burhenne, Kristensen & Rasmussen, 2003) and barley peroxidase 7 (*Prx7*, Kristensen, Bloch & Rasmussen, 1999) which are involved in the biosynthesis of hordatines A and B. In addition, primers were designed for ornithin decarboxylase (*Odc*, **AB109206**), which codes for the first committed step in polyamine biosynthesis, but is not involved in hordatine biosynthesis. As an internal control for comparison with hordatine biosynthetic genes, one primer (ABRE) was used from the promoter region (abscisic acid response) of the ABA-, cold-, heat- and drought-induced barley gene *ABA7* (Gulli et al., 1995) according to Liviero et al. (2002). Primer sequences are shown in Table 2.

LP-PCR reactions were carried out in 1 × reaction buffer containing 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1 µM primer, 1 unit *Taq* DNA polymerase (Roche) and 50 g of genomic DNA in a total volume of 25 µl. Amplifications were carried out with an initial denaturing step at 94 °C for 2 min followed by 35 cycles with 1 min at 94 °C, 30 s at 55 °C (except for primer ODC5–1 and ABRE at 50 °C and primer ADC3–2 at 52 °C) and in at 72 °C followed by a final elongation step at 72 °C for 5 min. PCR products were analyzed by gel electrophoresis on ethidium bromide stained 2% agarose gels.

The identity of the amplified fragments was confirmed for the main PCR products by sequencing the isolated fragments obtained from one accession of the Maalot region (data not shown).

Table 2. Oligonucleotides used as primers in LP-PCR

Primer Name	Sequence (5′–3′)	Gene	Strand	Position
Prx5–1	ACTAGTCCTCTTCCACGACTGTTTCC	*Prx7*	Sense	Coding region
Prx5–2	CCATGGCCTCTTCCACGACTGTTTCC	*Prx7*	Sense	Coding region
Prx3–1	GAAGGAGGAGATGAGGGTGGAATTC	*Prx7*	Antisense	Coding region
Act5–1	AGA TCA CCG TGC ACT CTT CC	*Act*	Sense	Coding region
Act5–2	CGG ATC AAC GAT GGC TAC TT	*Act*	Sense	Coding region
Act3–1	GGC AAG TGG CTA ACG TTG AT	*Act*	Antisense	Coding region
Act3–2	AGA TGA CGT TGC CGG TGT ATC	*Act*	Antisense	Coding region
Adc5–1	GAG ATA ACT CAC CTA TCA TGG CCA	*Adc*	Sense	Coding region
Adc3–1	CCG TAC TGG GTG TAC TTG ATG GCG	*Adc*	Antisense	Coding region
Odc5–1	GCG TCA AGG ACA AGA AGG TGC T	*Odc*	Sense	Coding region
Odc3–1	CCA TGT CGT CGA ACA CGA GCC A	*Odc*	Antisense	Coding region
ABRE	ACA CGT ATG ATC TTT GTA CCC AT	*ABA 7*	Sense	Promoter (ABA response)

Statistical analysis

Students *t*-test was performed to analyze the significance of variation in the hordatine content within and between the different accessions from the same location. Principal component analyses (PCA) were performed using the JMP version 5.1 software package. Populations were grouped using a correlation matrix containing all ecogeographical factors and the hordatine level as variables. A varimax rotation with Kaiser normalization of the primary results was performed in order to obtain more interpretable results.

Non-parametric and correlation coefficients (Spearman rank) and stepwise multiple regression analyses were used to reveal associations between hordatine accumulation in different accessions, LP-PCR markers and ecogeographic parameters of the collection sites.

Amplification products of the LP-PCR reactions were designated according to their primer name and the size of the generated fragment. Bands were scored as present (1) or absent (0) and used for further statistical analyses if they were reproducible in at least two out of three independent reactions. The POPGENE version 1.32 program (Yeh et al., 1997) was used to compute molecular diversity indices as suggested by Nei (1978) based on gene diversity values H_e (Nei 1973, 1978) averaged across all loci analyzed. The gene diversity coefficient H_e was used as a suitable value for typical inbred populations with a very small degree of heterozygocity but, in which different types of homozygotes may occur.

Results

Time course of hordatine accumulation

Earlier studies on cultivated barley showed a maximum of hordatine accumulation 3 days after germination, which declined progressively until day 30 after germination (Smith & Best, 1978). We conducted similar time course experiments with cultivated and wild barley accessions and we found for all of the tested accessions a maximum of hordatine accumulation between 2 and 4 days after germination with an average peak at 3 days after germination, followed by a decrease to about 50% of the maximum accumulation at day 10. At about day 28 after germination, all accessions reached a constant level of hordatines of about 10% of the maximum value (data not shown). Based on these earlier findings and in order to detect general differences in the hordatine accumulation between accessions, we conducted time course experiments for all 50 accessions of *H. spontaneum* at three time points that are characteristic for the accumulation of hordatines in young barley plants (Table 3). All accessions except from those collected at Sede Boqer had the highest hordatine concentration at day 3. Accessions from Sede Boqer had a relatively constant hordatine concentration until day 10 after germination, which decreased to a constant and low concentration at day 28 after germination. It is noteworthy that maximum hordatine concentrations in wild barley accessions were three to four times higher than in cultivated barley and the decrease of the hordatine concentration between day 3 and 10 was much more

Table 3. Time course of hordatine accumulation in genotypes from eight different *H. spontaneum* populations

Location	Hordatine concentration (μg/g FW)		
	3 days	10 days	28 days
Tabigha-Terra Rossa	527 (62)	347 (27)	82 (12)
Tabigha- Basalt	753 (122)	354 (47)	74 (10)
Neve Ya'ar rock/sun	479 (73)	395 (35)	62 (13)
Neve Ya'ar soil/shade	1200 (164)	936 (98)	109 (11)
Evolution Canyon mesic	723 (131)	543 (95)	104 (8)
Evolution Canyon xeric	612 (170)	450 (52)	106 (12)
Sede Boqer	741 (77)	736 (59)	75 (13)
Maalot	1001 (156)	799 (124)	89 (7)

Hordatine concentrations were measured 3, 10 and 28 days after germination.
Standard deviation in parenthesis.

pronounced and faster in cultivated barley than in *H. spontaneum* accessions. At all time points, the ratio of the concentrations of hordatine A and hordatine B was relatively stable between 25:1 and 30:1. In order to rule out that hordatines or mRNA of genes which are involved in the hordatine biosynthesis are stored in the dried seeds, we measured hordatines in extracts of seeds and performed RT-PCR analysis with primers for *Act* on mRNA isolated from developing caryopses. In accordance with earlier studies (Stoessl & Unwin, 1970; Smith & Best, 1978) we were unable to detect hordatines or *Act* transcripts in the seeds (data not shown).

Variation of hordatine concentrations in H. spontaneum

Hordatine concentrations in different accessions of *H. spontaneum* were compared 3 days after germination when hordatine levels reach in most accessions a maximum. Variation of hordatine levels in individuals of the same accession and between accessions from the same location (regional site as well as from the same micro-site) was in most cases low (Table 4). The highest variation within and between accessions from the same micro-site was found in populations collected at the Evolution Canyon and Neve Ya'ar. Accessions from the mesic slope of Evolution Canyon exhibited a wide variation in the accumulation of hordatines. However, comparison between accessions from contrasting slopes of the Evolution Canyon using Student's *t*-test showed that differences between these two groups had a low level of significance ($p = 0.28$, $F = 1.16$). In contrast, for other regional sites or micro-sites results of Student's *t*-test revealed significant differences in the hordatine accumulation between accessions collected at

Table 4. Variation of hordatine concentrations in *H. spontaneum* genotypes from eight different macro and micro sites

Location	Number of accessions	Hordatine A (μg/g FW), Mean (SD)	Variance component		Pairwise Student's *t*-test[a] (*p*)s
			Within accessions (%)	Between accessions (%)	
Tabigha-Terra Rossa	5	527 (62)	9.1	13.7	**3.22**
Tabigha- Basalt	5	753 (122)	10.1	17.7	(0.016)
Neve Ya'ar rock/sun	5	479 (73)	29.3	23.1	**8.90**
Neve Ya'ar soil/shade	5	1200 (164)	36.1	31.0	(0.0002)
Evolution Canyon mesic	5	723 (131)	45.2	38.1	1.16
Evolution Canyon xeric	5	612 (170)	25.3	21.7	(0.280)
Sede Boqer	10	741 (77)	14.2	15.1	**4.71**
Maalot	10	1001 (156)	33.2	27.3	(0.0004)

[a]Significant differences in pairwise Student's *t*-test between populations from contrasting environments are in bold numbers.

locations with contrasting environments. Pair-wise comparison of accessions from regional sites (i.e. Maalot and Sede Boqer) showed significant differences at a very high probability level (Table 4). Accessions from the Tabigha microsite could clearly be grouped into two categories corresponding to different edaphic factors (terra rossa and basalt). Similar results were obtained from the Neve Ya'ar microsite with two groups of populations growing either on very shallow soil with high exposure to sun or on deep soil in the shade.

Correlations between hordatine levels and ecogeographical factors

Pair-wise non-parametric regression analysis was used to calculate correlations between hordatine levels and ecogeographical factors at different sites (Table 5). Significant correlations were found between hordatine concentrations and humidity at 14:00 and the annual mean humidity (correlation coefficients 0.448 and 0.438) and between hordatine concentrations and soil types (correlation coefficient 0.433). Close negative correlations were found between hordatine concentrations and temperature related factors (annual average temperature [−0.543], average temperatures in January [−0.513] and in August [−0.55]) at very high probability levels. Weak correlations were found between hordatine accumulation and total rain fall (0.275 at $p = 0.059$) and between hordatine accumulation and evaporation (−0.283 at $p = 0.049$). Other ecogeographical factors (geographical location and altitude) did not correlate with hordatine accumulation.

Principal component analysis

Principal component analysis showed that the first three principal components explained 83% of the observed variation. Pc1 accounted for 39% of the variation and showed the largest loading values with water related factors (rain, humidity). The pc2 explained 33% of the observed variation with largest loading value on temperature (Table 6). Pc3 accounted for 11% of variation and is based on edaphic factors.

Genetic diversity

LP-PCR primers proved to be useful to amplify polymorphic PCR fragments. Only bands which were clearly reproducible in at least two out of three repetitions where used for further analysis. An example of a profile obtained with primer Prx5–1 is shown in Figure 1. The genetic diversity index (H_e) was calculated according to Nei (Nei, 1978) as an unbiased measure of gene diversity with respect to the small number of plants sampled from each location. H_e was calculated for all loci in all populations analyzed (Table 7). The highest gene diversity across all loci was observed in populations

Table 5. Spearman rank correlations between hordatine contents and ecogeographic factors

Ecogeographical factor	Spearman's Rho	Significance ($p < \|\text{Rho}\|$)
Longitude	0.165	0.259
Latitude	0.121	0.218
Altitude	0.132	0.257
Rain annual	0.275	0.057
Rainy days annual	−0.181	0.212
Humidity at 14:00	0.438	0.017
Humidity Annual mean	0.448	0.017
Evaporation	−0.283	0.049
Temperature annual mean	−0.543	<0.0001
Temperature January mean	−0.513	0.0002
Temperature august mean	−0.550	<0.0001
Soil	0.433	0.044

Table 6. Principal component analysis of environmental data

Ecogeographical factor	PCA values		
	pc1	pc2	pc3
Longitude	0.19	0.15	−0.04
Latitude	0.04	−0.13	0.12
Altitude	−0.09	−0.39	−0.26
Rain annual	0.40	−0.11	−0.11
Rainy days annual	0.42	0.11	−0.14
Humidity at 14:00	0.38	−0.14	−0.05
Humidity Annual mean	0.40	−0.12	0.07
Evaporation	−0.37	0.09	−0.15
Temperature annual mean	0.04	0.46	−0.03
Temperature january mean	0.13	0.44	−0.01
Temperature august mean	0.01	0.47	−0.08
Soil	−0.06	0.18	0.70

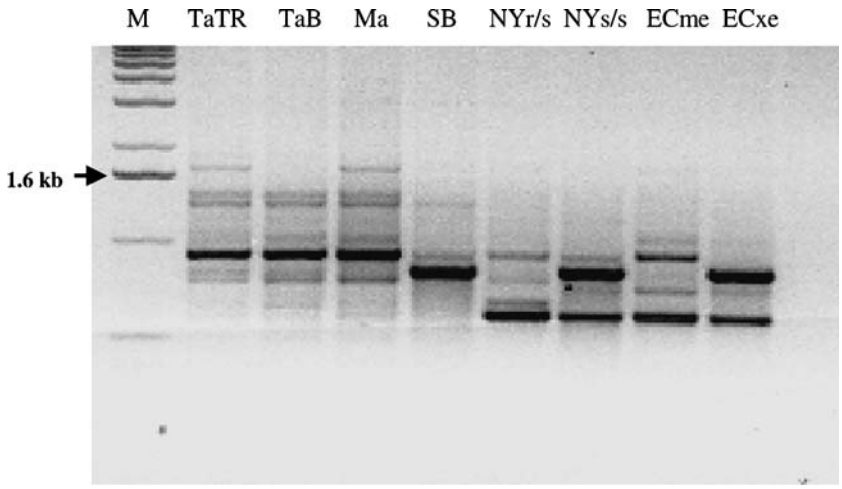

Figure 1. LP-PCR fingerprints. Example of amplification products obtained with primer Prx5–1 and 8 different genotypes from eight populations. M: 1 kb ladder (Gibco). TaTR: Tabigha terra rossa; TaB: Tabigha basalt; Ma: Maalot; SB: Sede Boqer; NYr/s: Neve Ya'ar rock/sun; NYs/s: Neve Ya'ar soil/shade; ECme: Evolution Canyon mesic; ECxe: Evolution Canyon xeric.

Table 7. Genetic indices* at 52 LP-PCR 'loci' of 49 *H. spontaneum* accessions from eight macro and micro sites from the Near East Fertile Crescent

Location	Number of accessions	Genetic indices		
		A	*P*	H_e
Tabigha-Terra Rossa	5	1.500	0.500	0.213
Tabigha-Basalt	5	1.692	0.692	0.261
Tabigha-total	*10*	*1.789*	*0.788*	*0.309*
Neve Ya'ar rock/sun	4	1.461	0.461	0.171
Neve Ya'ar soil/shade	5	1.654	0.653	0.284
Neve Ya'ar total	*9*	*1.788*	*0.788*	*0.334*
Evolution Canyon mesic	5	1.792	0.792	0.342
Evolution Canyon xeric	5	1.711	0.710	0.280
Evolution Canyon Total	*10*	*1.904*	*0.904*	*0.377*
Sede Boqer	10	1.634	0.634	0.264
Maalot	10	1.762	0.761	0.298

* A = Number of alleles per locus.
P = Proportion of polymorphic loci.
H_e = Genic diversity equivalent to the expected heterozygosity under panmixia (Nei, 1978).
The calculations include data for the internal control *ABRE*.

from Evolution Canyon (0.377), followed by populations from Neve Ya'ar (0.334) and Tabigha (0.309). The highest value for H_e in populations from contrasting environments was found in accessions from the mesic slope in Evolution Canyon; the lowest gene diversity index was found for accessions from the Neve Ya'ar rock/sun microsite. Comparison of H_e between populations from contrasting regional or micro-sites showed the highest differences between Neve Ya'ar rock/sun and Neve Ya'ar soil/shade. In all contrasting environments, H_e was lower in accessions from dry environments or soil types with low water retention capacity (e.g. Sede Boqer/Maalot and Tabigha terra rossa/Tabigha basalt).

Correlation between gene diversity index H_e and ecogeographical variables

The Spearman rank correlation analysis was performed between ecogeographical factors representing geographic and climatic variables and the genetic diversity index H_e (Table 8). The resulting correlation matrix contained 144 correlations. For six LP-PCR primers significant associations with $p < 0.05$ were established between H_e and ecogeographical factors. Positive correlations exist for mean annual temperature (Prx5–1), mean January temperature (Prx5–1), mean August temperature (Prx5–1, ABRE), annual rainfall (Act5–1), and number of rainy days (Prx5–1). Negative correlations exist for mean annual temperature (Act5–1, Odc3–1), mean January temperature (Act5–1, Odc3–1), mean August temperature (Act5–1, Odc3–1), mean number of rainy days (Prx3–1, Odc5–1), and soil type (Act3–1). No significant correlation was established between genetic diversity and

Table 8. Spearman rank correlations between gene diversity (H_e) in 11 LP-PCR loci and 12 ecogeographical variables in eight populations of *Hordeum spontaneum* in Israel

	Lon	Lat	Alt	Tm	Tj	Ta	Ev	Rn	Rd	Hu_{14}	Hu_{an}	So
Prx5–1	0.022	−0.412	−0.601	0.692*	0.731*	0.761*	0.084	0. 121	0.638*	−0.407	−0.278	0.123
Prx5–2	−0.691	−0.231	0.606	−0.541	−0.560	−0.540	0.132	−0.206	−0.523	−0.321	0.060	−0.481
Prx3–1	0.047	0.586	0.557	−0.452	−0.497	−0.454	0.060	−0.509	−0.689*	0.148	−0.097	−0.284
Act5–1	−0.186	0.427	0.578	−0.732*	−0.832**	−0.736*	0.349	0.89***	-0.424	0.222	−0.169	0.086
Act5–2	0.135	−0.083	−0.015	−0.135	−0.071	−0.245	−0.168	−0.254	−0.268	0.506	0.448	0.210
Act3–1	0.652	0.332	0.344	0.130	0.272	0.149	−0.493	0.331	0.349	0.506	0.398	−0.650*
Act3–2	0.431	0.002	−0.013	0.417	0.535	0.405	−0.048	−0.509	0.025	0.308	0.036	−0.074
Adc5–1	0.350	0.181	0.169	−0.232	−0.197	−0.270	−0.289	0.545	0.166	0.580	0.436	−0.370
Adc3–1	0.296	0.147	−0.040	−0.126	−0.093	−0.233	−0.192	−0.169	−0.229	0.555	0.375	0.308
Odc5–1	−0.099	0.486	0.273	−0.674	−0.692	−0.605	0.133	−0.195	−0.616*	0.124	−0.091	−0.441
Odc3–1	−0.253	0.104	0.607	−0.884**	−0.801*	−0.883*	−0.012	−0.254	−0.779	0.420	0.400	−0.444

For abbreviations refer to Table 1; *, **, *** Significance at $p < 0.05$, 0.01, 0.001, respectively.

geographical variables (longitude, latitude, altitude). In total, 15 (10.4%) of the 132 tested correlation coefficients between H_e of five LP-PCR primers and 12 ecogeographical variables were significant. This frequency of significant correlations exceeds the 5% probability level for correlations expected by chance (Aiken et al., 1955).

Associations of hordatine accumulation with gene diversity index (H_e) and ecogeographic factors

A test of the best predictors of hordatine levels in the eight *H. spontaneum* populations was conducted by stepwise multiple regression analysis using the hordatine level as the dependent variable, H_e values of each locus and ecogeographical variables in the form of principal component values as independent variables. The following associations between hordatine concentrations and independent variables were found to be significant ($p < 0.05$): hordatine concentration with H_e of LP-PCR marker Prx5–1 and temperature related factors ($R^2 = 0.726$; $p < 0.027$); hordatine concentration with H_e of LP-PCR marker Adc5–1 and temperature related factors ($R^2 = 0.730$; $p < 0.043$) and hordatine concentration with H_e of LP-PCR marker Act5–1 and water related factors ($R^2 = 0.562$; $p < 0.031$).

Discussion

The Fertile Crescent is considered to be the center of origin of wild barley, *Hordeum spontaneum*. This geographical region, and Israel in particular, is characterized by a wide range of different ecological habitats. Morphological, physiological and functional adaptations of *H. spontaneum* are based on an exceptionally high degree of genetic diversity across the genome which allows the growth in and the dynamic migration into primary and secondary habitats including many extremely unfavorable conditions (Nevo Beiles & Zohary, 1986). Populations of *H. spontaneum* from this area proved to be perfectly suited for the analyses of correlations between adaptive traits, genetic diversity and environmental factors (Turpeinen et al., 2001; Huang et al., 2002; Maestri, 2002).

The measurement of hordatine accumulation in *H. spontaneum* seedlings revealed a wide range of hordatine concentrations between different populations and a close correlation between hordatine levels and environmental factors. This can be expected at the collection site under natural growth conditions where limiting factors such as water or nutrients may directly influence the secondary metabolism and especially the biosynthesis of preformed antimicrobial substances (van Dam & Baldwin, 1998). Statistical analysis showed that the observed hordatine concentrations measured under controlled conditions *ex situ* correlate with environmental factors at the collection site. Therefore, the differences in hordatine accumulation seem to have a genetic manifestation rather than being directly influenced by the availability of resources such as nutrients and water. The use of marker systems which are specifically targeted at genes

involved in the biosynthetic pathway of hordatines allowed for the estimate of the genetic diversity of these genes in the populations analyzed. Earlier studies have shown that LP-PCR markers reliably discriminate different accessions and are closely correlated with environmental or phenotypic characteristics (Marmiroli et al., 1998; Liviero et al., 2002; Maestri et al., 2002). For four LP-PCR markers, which are specific for hordatine biosynthetic genes, significant correlations between genetic diversity and environmental factors have been identified. This indicates the responsiveness of genetic variation of these genes to environmental conditions. Not surprisingly, similar correlations were found between markers for *ODC* and *ABA7*, which are not involved in hordatine biosynthesis, with temperature and water related factors, as both genes are involved in stress responses. However, further statistical analysis using stepwise multiple regression analysis, revealed significant correlations between hordatine accumulation, accession and environmental factors only for genes, which are directly involved in hordatine biosynthesis, but not for the genes used as a control. This leads to the postulation that environmental conditions influence genetic diversity of specific genes, which in turn influence the phenotypic differences in hordatine accumulation between populations from different environments.

Nonetheless, there are important questions arising from our results which cannot be resolved in this study: The number of oligonucleotides used in this study is too small to rule out that the findings of the stepwise multiple regression analysis, even though they are statistically significant, are coincidental. Similar correlations could have been identified for oligonucleotides derived from genes, which are unrelated to hordatine biosynthesis if a larger number of controls had been analysed. Furthermore, it is not clear if all observed polymorphisms are due to differences in promoter or coding regions of functional genes. Therefore, different degrees of genetic variation in some of the hordatine related genes do not necessarily reflect differences in the genetic variation of functional and expressed genes. It is conceivable that the observed polymorphisms are present in non-functional copies or alleles of the gene and are not relevant for hordatine biosynthesis. It is also an important question why a higher degree of genetic diversity in some populations results in higher levels of hordatine accumulation. The relatively high frequency of polymorphisms in some populations could be explained by balancing selection models. Simple heterozygous advantage, however, is unlikely to be of importance in a highly self-fertilizing species like wild barley. Further analysis using primers for additional control genes and different independent marker systems such as target gene derived RFLPs as well as a more detailed analysis of the LP-PCR fragments will help to substantiate our observations and calculated correlations between genetic diversity, phenotypic variation and environmental factors.

It is an intriguing question what the biological function of this accession–environment–phenotype interaction is. The biological function of hordatines has not been elucidated in detail but their strong and broad antimicrobial activity suggests a role in defense responses against invading pathogens. Conjugated polyamines are believed to be involved in a hypersensitive response after pathogen infection in an incompatible interaction (Cowley & Walters, 2002) whereas hordatines and *p*-coumaroylagmatine directly inhibit spore germination of a variety of fungal pathogens *in vitro* (Stoessl & Unwin, 1970). The high abundance of hordatines in the young barley seedling (Stoessl, 1967) suggests that hordatines are preformed infection inhibitors (Stoessl & Unwin, 1970; Smith & Best, 1978). Recent studies showed that the biosynthesis of hydroxycinnamoyl agmatine derivatives, the precursors of hordatines, are strongly induced in response to fungal inocculation even in older leaves of barley (Peipp et al., 1997, von Roepenack, Parr & Schulze-Lefert, 1998).

The presumptive role of hordatines as preformed and inducible compounds in defense responses may indicate that environmental factors such as water availability and temperature do not directly influence hordatine accumulation. On the contrary, it is tempting to speculate that these environmental conditions favor the proliferation and the range of pathogens resulting in higher pathogen pressure in these environments. Evolutionary adaptive processes may then have lead to the selection of *H. spontaneum* accessions with higher contents of hordatine, which acts as an antimicrobial compound against a wide range of pathogens.

Further investigation of the biological function of hordatines *in vivo* through the isolation of

mutants defective in hordatine accumulation will shed light on the contribution of hordatines to defense mechanisms in barley. It has been shown that pathogen pressure selects functional race-specific resistance gene (*R*-gene) specificities and results in the maintenance of *R*-gene diversity (Ellis, Dodds & Pryor, 2000). The analysis of hordatine accumulation in *H. spontaneum* at the site of origin as well as the assessment of local pathogen pressure may help to elucidate the biological function of the observed relationship between environment, genotype and phenotype.

Acknowledgements

The authors wish to acknowledge the precious help of Dr. Eviatar Nevo for supplying seeds of *Hordeum spontaneum* and critical discussions and the help of Dr. Avigdor Beiles for support with statistical analysis. This work was supported by the German-Israeli-Foundation with grant 2067 to T. Koprek.

References

Aiken, H.H., J.A. Harr & R.L. Ashenhurst et al., 1955. Tables of the Cumulative Binomial Probability Distribution. Harvard University Press, Cambridge, MA.

Brown, A.H.D., 1992. Genetic Variation and Resources in Cultivated Barley and Wild Hordeum. Proceedings of the Sixth International Barley Genetics Symposium, 1991. Helsingborg, Sweden, 669–682.

Burhenne, K., B.K. Kristensen & S.K. Rasmussen, 2003. A new class of N-hydroxycinnamoyltransferases. Purification, cloning, and expression of a barley agmatine coumaroyltransferase (EC 2.3.1.64). J. Biol. Chem. 278: 13919–13927.

Ceccarelli, S. & S. Grando, 1996. Drought as a challenge for the plant breeder. Plant Growth Regul. 20: 149–155.

Chen, G., T. Suprunova, T. Krugman, T. Fahima & E. Nevo., 2004. Ecogeographic and genetic determinants of kernel weight and color of wild barley (*Hordeum spontaneum*) populations in Israel. Seed Sci. Res. 14: 137–146.

Cone, K., 1989. Yet another rapid plant DNA prep. Maize Genet. Coop. News Lett. 63: 68.

Cowley, T. & D.R. Walters, 2002. Polyamine metabolism in barley reacting hypersensitively to the powdery mildew fungus *Blumeria graminis* f. sp. Hordei. Plant Cell Environ. 25: 461–468.

Ellis, J., P. Dodds & T. Pryor, 2000. Structure, function and evolution of plant disease resistance genes. Curr. Opin. Plant Biol. 3: 278–284.

Ellis, R.P., B.P. Forster, D.C. Gordon, L.L. Handley, R. Keith, P. Lawrence, R. Meyer, W. Powell, D. Robinson, C.M. Scrimgeour, G. Young & W.T.B. Thomas, 2002. Phenotype/genotype associations for yield and salt tolerance in a barley mapping population segregating for two dwarfing genes. J. Exp. Bot. 53: 1163–1176.

Favatier, F., L. Bornman, L.E. Hightower, E. Gunther & B.S. Polla, 1997. Variation in hsp gene expression and Hsp polymorphism: do they contribute to differential disease susceptibility and stress tolerance? Cell Stress Chaperones 2: 141–155.

Forster, B.P., J.R. Russell, R.P. Ellis, L.L. Handley, D. Robinson, C.A. Hackett, E. Nevo, R. Waugh, D.C. Gordon, R. Keith & W. Powell, 1997. Locating genotypes and genes for stress tolerance in barley: a strategy using maps, markers and the wild species. New Phytol. 137: 141–147.

Forster, B.P., 1999. Studies on wild barley, Hordeum spontaneum C. Koch at the Scottish Crop Research Institute, pp. 325–341 in Evolutionary Theory and Processes: Modern Perspectives. Papers in Honour of Eviatar Nevo edited by S.P. Wasser. Kluwer Academic Publishers.

Gianoli, E. & H.M. Niemeyer, 1998. DIBOA in wild Poaceae: Sources of resistance to the Russian wheat aphid (*Diuraphis noxia*) and the greenbug (*Schizaphis graminum*). Euphytica 102: 317–321.

Gillespie, J.H. & M. Turelli, 1989. Genotype–environment interactions and the maintenance of polygenic variation. Genetics 121: 129–138.

Gulli, M., E. Maestri, H. Hartings, G. Raho, C. Perrotta, K.M. Devos & N. Marmiroli, 1995. Isolation and characterization of abscisic acid inducible genes in barley seedlings and their responsiveness to environmental stress. Plant Physiol. 14: 89–96.

Gunasekera, D., M. Santakumari, Z. Glinka & G.A. Berkowitz, 1994. Wild and cultivated barley genotypes demonstrate varying ability to acclimate to plant water deficits. Plant Sci. 99: 125–134.

Huang, Q., A. Beharev, Y. Li, V. Kirzhner & E. Nevo, 2002. Mosaic microecological differential stress causes adaptive microsatellite divergence in wild barley, *Hordeum spontaneum*, at Neve Yaar, Israel. Genome 45: 1216–1229.

Ivandic, V., C.A. Hackett, Z.J. Zhang, J.E. Staub, E. Nevo, W.T. Thomas & B.P. Forster, 2000. Phenotypic responses of wild barley to experimentally imposed water stress. J. Exp. Bot. 51: 2021–2029.

Ivandic, V., W.T.B. Thomas, E. Nevo, Z. Zhang & B.P. Forster., 2003. Associations of simple sequence repeats with quantitative trait variation including biotic and abiotic stress tolerance in *Hordeum spontaneum*. Plant Breeding 122: 300–304.

Joergensen J.H. 1992. Discovery, characterization and exploitation of *Mlo* powdery mildew resistance in barley. Euphytica 63: 141–152.

Kristensen, B.K., H. Bloch & S.K. Rasmussen, 1999. Barley coleoptile peroxidases, molecular cloning, purification and induction by pathogens. Plant Physiol. 120: 501–512.

Liviero, L., E. Maestri, M. Gulli, E. Nevo & N. Marmiroli, 2002. Ecogeographic adaptation and genetic variation in wild barley: application of molecular markers targeted to environmentally regulated genes. Genet. Res. Crop Evol. 49: 133–144.

Lovett, J.V., A.H.C. Hoult & O. Christen, 1994. Biologically activity secondary metabolites of barley: IV. Hordenine

production by different barley lines. J. Chem. Ecol. 20: 1945–1954.

Lu, Z., T. Krugman, P.M. Neumann & E. Nevo, 1999. Physiological characterization of drought tolerance in wild barley (*Hordeum spontaneum*) from the Judean Desert. Barley Genet. Newsl. 29: 36–40.

Maestri, E., A. Malcevschi, A. Massari & N. Marmiroli, 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.

Marmiroli, N., E. Maestri, L. Liviero, A. Massari, A. Malcevschi & P. Monciardini, 1999. Application of genomics in assessing biodiversity in wild and cultivated barley. Mol. Ecol. 8: 95–106.

Marmiroli, N., A. Malcevschi & E. Maestri, 1998. Application of stressresponsive genes RFLP analysis to the evaluation of genetic diversity in plants, pp. 464–470 in Molecular Tools for Screening Biodiversity edited by A. Karp, P.G. Isaac &D.S. Ingram. Chapman and HallLondon.

Nei, M., 1973. Analysis of gene diversity in subdivided populations. Proc. Natl. Acad. Sci. USA 70: 3321–3323.

Nei, M., 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89: 583–590.

Nevo, E., D. Zohary, A.H.D. Brown & M. Haber, 1979. Genetic diversity and environmental associations of wild barley, *Hordeum spontaneum*, in Israel. Evolution 33: 815–833.

Nevo, E., A. Beiles, Y. Gutterman, N. Storch & D. Kaplan, 1984. Genetic resources of wild cereals in Israel and vicinity II Phenotypic variation within and between populations of wild barley, *Hordeum spontaneum*. Euphytica 33: 737–756.

Nevo, E., A. Beiles & D. Zohary, 1986. Genetic resources of wild barley in the Near East: structure, evolution and application in breeding. Biol. J. Linn. Soc. 27: 355–380.

Nevo, E., 1987. Plant genetic resources: Prediction by isozyme markers and ecology. Isozymes current Topics in Biological and Medical Research 16: 247–267.

Nevo, E., 1992. Origin, evolution, population genetics and resources for breeding of wild barley, Hordeum spontaneum, in the Fertile Crescent, pp. 19–43 in Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology edited by R. Peter. C. A. B. InternationalShewry.

Nevo, E., I. Apelbaum-Elkaher, J. Garty & A. Beiles, 1997. Natural selection causes microscale allozyme diversity in wild barley and a lichen at 'Evolution Canyon' Mt. CarmelIsrael. Heredity 78: 373–382.

Nevo, E., B. Baum, A. Beiles & D.A. Johnson, 1998. Ecological correlates of RAPD DNA diversity of wild barley, *Hordeum spontaneum* in the Fertile Crescent. Genet. Res. Crop Evol. 45: 151–159.

Niemeyer, H.M., 1988. Hydroxamic acid content of *Triticum*-ssp. Euphytica 37: 289–294.

Pakniyat, H., W. Powell, E. Baird, L.L. Handley, D. Robinson, C.M. Scrimgeour, E. Nevo, C.A. Hackett, P.D.S. Caligari & B.P. Forster, 1997. AFLP variation in wild barley (*Hordeum spontaneum* C. Koch) with reference to salt tolerance and associated ecogeography. Genome 40: 332–341.

Peipp, H., W. Maier, J. Schmidt, V. Wray & D. Strack, 1997. Arbuscular mycorrhizal fungus-induced changes in the accumulation of secondary compounds in barley roots. Phytochemistry 44: 581–587.

Père zde la Vega, M., 1996. Plant genetic adaptedness to climatic and edaphic environment. Euphytica 92: 27–38.

Plucknett, D.L., N.J.H. Smith, J.T. Williams & N.M. Anishetty, 1983. Crop germplasm conservation and developing countries. Science 220: 163–169.

Quader, M., G. Daggard, R. Barrow, S. Walker & M.W. Sutherland, 2001. Allelopathy, DIMBOA production and genetic variability in accessions of *Triticum speltoides*. J. Chem. Ecol. 27: 747–760.

Quesada, V., S. Garcia-Martinez, P. Piqueras, M.R. Ponce & J.L. Micol, 2002. Genetic architecture of NaCl tolerance in *Arabidopsis*. Plant Physiol. 130: 951–963.

Rozen Skaletsky, S. H.J., 2000. . Primer3 on the WWW for general users and for biologist programmers, pp. 365–386 in Bioinformatics Methods and Protocols: Methods in Molecular Biology edited by S Krawetz &S Misener. Humana PressTotowa, NJ.

Smith, T.A. & G.R. Best, 1978. Distribution of the hordatines in barley. Phytochemistry 17: 1093–1098.

Stoessl, A., 1967. The antifungal factors in barley. IV. Isolation, structure, and synthesis of the hordatines. Can. J. Chem. 45: 1745–1760.

Stoessl, A. & C.H. Unwin, 1970. The antifungal factors in barley. V. Antifungal activity of the hordatines. Can. J. Bot. 48: 465–471.

Suprunova T., T. Krugman, T. Fahima, G. Chen, I. Shams, A.B. Korol & E. Nevo, Differential expression of dehydrin (Dhn) in response to water stress in resistant and sensitive wild barley (*Hordeum spontaneum*). Plant Cell Environ. 27: 297–308.

Turpeinen, T., T. Tenhola, O. Manninen, E. Nevo & E. Nissilä, 2001. Microsatellite diversity associated with ecological factors in *Hordeum spontaneum* populations in Israel. Mol. Ecol. 10: 1577–1794.

Turpeinen, T., V. Vanhala, E. Nevo & E. Nissila., 2003. AFLP genetic polymorphism in wild barley (*Hordeum spontaneum*) populations in Israel. Theor. Appl. Genet. 106: 1333–1339.

Ungerer, M.C., S.S. Halldorsdottir, M.D. Purugganan & T.F. Mackay., 2003. Genotype–environment interactions sat quantitative trait loci affecting inflorescence development in *Arabidopsis thaliana*. Genetics 165: 353–365.

van Dam, N.M. & I.T. Baldwin, 1998. Costs of jasmonate-induced responses in plants competing for limited resources. Ecol. Lett. 1: 30–33.

Verhoeven, K.J.F., A. Biere, E. Nevo & J.M.M. van Damme., 2004a. Differential selection of growth rate-related traits in wild barley, *Hordeum spontaneum*, in contrasting greenhouse nutrient environments. J. Evol. Biol. 17: 184–196.

Verhoeven, K.J.F., T.K. Vanhala, A. Biere, E. Nevo & J.M.M. van Damme., 2004. The genetic basis of adaptive population differentiation: a QTL-analysis of fitness traits in two wild barley populations from contrasting habitats. Evolution 58: 270–283.

von Roepenack, E., A. Parr & P. Schulze-Lefert, 1998. Structural analyses and dynamics of soluble and cell wall-bound

phenolics in a broad spectrum resistance to the powdery mildew fungus in barley. J. Biol. Chem. 273: 9013–9022.

Wei, Y.D., E. de Neergaard, H. Thordahl-Christensen, D.B. Collinge & V. Smedegaard-Petersen, 1994. Accumulation of a putative guanidine compound in relation to other early defence reactions in epidermal cells of barley and wheat exhibiting resistance to Erysiphe graminis f.sp. hordei Physiol. Mol. Plant Pathol. 45: 469–484.

Yeh, F.C., R.C. Yang, T.B.J. Boyle, Z.-H. Ye & J.X. Mao, 1997. POPGENE, the user-friendly shareware for population genetic analysis. Molecular Biology and Biotechnology Centre, University of Alberta, Canada.