

Monitoring drought responses of barley genotypes with semi-robotic phenotyping platform and association analysis between recorded traits and allelic variants of some stress genes

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Abstract

Genetic improvement of complex traits such as drought adaptation can be advanced by the combination of genomic and phenomic approaches. Semi-robotic phenotyping platform was used for computer-controlled watering, digital and thermal imaging of barley plants grown in greenhouse. The tested barley variants showed 0–76% reduction in green pixel-based shoot surface area in soil with 20% water content, compared to well-watered plants grown in soil with 60% water content. The barley *HvA1* gene encoding the group 3 LEA (Late Embryogenesis Abundant) protein exhibited four (A–D) haplotypes as identified by the EcoTILLING and subsequent DNA sequencing. The green pixel mean value of genotypes with haplotype D was higher than the mean value of the remaining haplotypes, indicating a pivotal role of haplotype D in optimizing the green biomass production under drought condition. In water limitation, the canopy temperature of a highly sensitive genotype was 18.0°C, as opposed to 16.9°C of leaves from a tolerant genotype as measured by thermal imaging. Drought-induced changes in leaf temperature showed moderate correlation with the water use efficiency ($r^2 = 0.431$). The haplotype/trait association analysis based on the *t*-test has revealed a positive effect of a haplotype B (SNPs:GCCCCTGC) in a gene encoding the barley fungal pathogen induced mRNA for pathogen-related protein (*HvPPRPX*), on harvest index, thousand grain weight, water use efficiency and grain yield. The presented pilot study established a basic methodology for the integrated use of phenotyping and haplotyping data in characterization of genotype-dependent drought responses in barley.

Keywords: Digital imaging, leaf temperature, EcoTILLING, allelic, correlation.

Abbreviations: D_ deletion, GY_ grain yield, I_ insertion, LEA_ late embryogenesis abundant, RGB_ red, green, blue, SNP_ single nucleotide polymorphisms, TGW_ thousand grain weight, TILLING_ Targeting Induced Local Lesions In Genomes, WUE_ water use efficiency.

Introduction

Plant breeding is an art that monitors phenotypic traits, primarily yield performance of genotypes to improve crop productivity. Conventional screening technologies have limitations in non-invasive evaluation of organ morphology or structure, growth rate, biomass, physiological parameters or stress responses. Recent progresses in phenomics as an independent discipline offer new possibilities for plant science by acquisition and interpretation of high-dimensional phenomic data from different fields such as morphology, physiological state and molecular scale (Houle et al., 2010; Fabre et al., 2011; Salekdeh et al., 2009). Fully automatic high-throughput plant growth and phenotyping platforms extensively use imaging technologies to capture spatial or temporal data in intensive details (Hartmann et al., 2011). Crop productivity is largely depends on water use efficiency of plant cultivars. Breeding for drought tolerance is one of the central objectives in efforts to attain yield stability (Condon et al., 2004). A prime target for the recent development of automated phenotyping infrastructures is to provide complex technologies for screening genotypes under limited water supply. Phenotyping facilities in the greenhouse allow the control of the severity of drought stress and the testing of different watering protocols. The

monitoring growth parameters can be accomplished by digital imaging of shoot traits or leaf area (Fabre et al., 2011). Golzarian et al. (2011) reported a method to estimate the biomass of individual cereal plants from the projected shoot area on two-dimensional images. Leaf growth, leaf area and physiology are the main determinants of photosynthesis through light interception and biomass production both in optimal and sub-optimal environments. The net exchange between water and CO₂ in leaves is the basic process for the production of biomass. The yield penalty under low water availability can be reduced through different strategies of drought resistance such as dehydration avoidance or tolerance (Berger et al., 2010). Stomatal closure is an indicator of plant drought stress response. Without transpirational cooling, leaf canopy temperature can be increased to air temperature. Therefore phenotyping platforms for drought response tests are generally equipped with thermal imaging facilities (Morison et al., 2008; Munns et al., 2010; Berger et al., 2010). Phenomic-level data may efficiently support the progress of plant breeding especially if they can be related to genomic variations. As reviewed by Salekdeh et al. (2009), phenotyping is expected to become an integrated component in gene discovery and practical

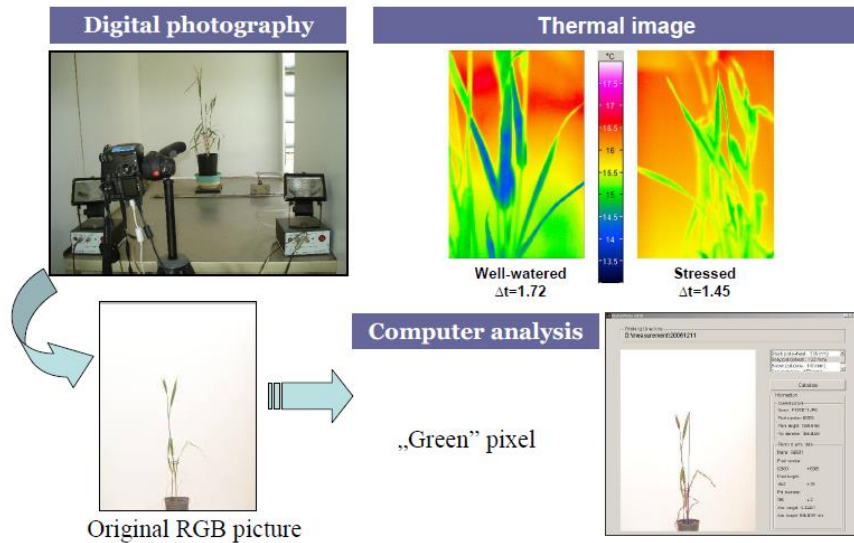


Fig 1. Complex Stress Diagnostic System as phenotyping platform in greenhouse.

breeding for drought improvement. Among others, the technique known as Targeting Induced Local Lesions In Genomes (TILLING) offers an efficient tool for the identification of mutant allelic series in selected genes in relation to altered traits in plants (McCallum et al., 2000). The same technique can be used for rapid screening of germplasm accessions (EcoTILLING) for single nucleotide polymorphisms (SNPs) and haplotyping (Comai et al., 2004). In barley, allelic variation was examined and identified by this method in *mLo* and *MLa* resistance genes (Mejlhede et al., 2006).

The objective of the present work was the establishment of a phenotyping facility that can serve as a Complex Stress Diagnostic System for cereal species (Fig. 1). The tested semi-automatic workstation includes digital and thermal imaging of barley plants grown in pots with optimal and sub-optimal water supply in greenhouse. The stress responses of individual plants from 23 genotypes were recorded by monitoring computed surface area of shoot, and leaf temperature. These traits are generally considered as primary indicators for the drought-induced damages. Seed production parameters, yield potential and water use efficiency were used for ranking of genotypes. We have developed an informatic system for image analysis based on digital RGB parameters for projection of the shoot area or storage of environmental and plant watering data. Our goals extended to search for possible relation between defined phenotypic traits and specific allelic variants of selected genes, previously shown to be involved in the drought response of cereal plants.

Results

Variation in phenotypic parameters of barley genotypes recorded by the Complex Stress Diagnostic System under water limitation in greenhouse

Based on published data, a collection of 23 barley genotypes representing both drought resistant and sensitive variants was established and tested in the present phenotyping study. The Complex Stress Diagnostic System functions was developed as a semi-automated phenotyping platform that

includes computer-controlled watering, digital and thermal imaging. Barley plants were grown in soil with 20% or 60% water content. The applied water limitation caused significant growth retardation of barley plants cultivated in greenhouse. The computer-controlled watering program ensured the re-supply of water that was used by each barley plant to keep the preset soil water content. In the control combination, the average water consumption of all genotypes was 2.3 L, whereas the stressed plants utilized only 0.8 L during the whole growing period. Fig. 2 presents characteristic differences in water use between a resistant (No. 1: Albacete) and a sensitive genotype (No. 87: Arda) differing in reduction of the grain yield (Fig. 6). With optimal water supply, the sensitive variant evaporated more water than the tolerant one. In soil with 20% water content the overall water use was significantly reduced and the tolerant genotype consumed more water than the sensitive counterpart.

The quantification of morphological and physiological characteristics was based on digital photography and thermal imaging (Fig. 1). In the present study, the number of pixels inside the plant region was determined from the mean value of eleven orthogonal views (Golzarian et al., 2011). We have developed image-processing algorithms to extract information from the plant RGB images. Fig. 3 presents green pixel-based shoot surface area data for the analyzed genotypes at the time of flowering. Genotypes (No. 51: Seco; No. 60: Arta; No. 86: Fengtien Black) showed less than 20% reduction in shoot surface area under stress form a minority group. High green mass is characteristic for the Fengtien Black cultivar (No. 86). This spring type Chinese genotype shows significant capacity for the efficient use of limited water during vegetative growth. In this test the most sensitive genotypes (No. 56: Monlón; No. 58: ICB79-0583-2AP) produced less than 40% of computed shoot surface area under water limitation as compared to plants grown with normal water supply. Digital imaging allowed monitoring individual plants during the whole growing period. As representative examples, Fig. 4 shows growth profiles for a sensitive (No. 56: Monlón) and a tolerant (No. 86: Fengtien Black) genotype. Under optimal growth conditions the higher computed green shoot surface area

production of plants of the Fengtien Black cultivar can be detected from the early growth phase. The differences in growth responses between these genotypes were enlarged in soil with 20% water content. The growth of plants of the Fengtien Black cultivar continued during the whole cultivation period. In contrast, plants of the Monlón cultivar were retarded from the early phase and they practically stopped growing after 9 weeks.

Under water limitation the avoidance reaction can ensure the maintenance of tissue water potential by reducing stomata aperture and leaf surface. Since transpiring leaves are cooler than leaves with closed stomata, leaf temperature can serve as indicator for the water status of stressed plants. The temperature differences between plants grown with normal or suboptimal water supply are demonstrated by thermal imaging as shown in Fig. 1. At heading time under well-watered condition, the cooling effect of evaporation resulted in lower leaf temperature than in leaves of stressed plants. This trend is characteristic for the majority of the genotypes tested. Fig. 5 presents actual temperature values for each genotype under the two growing conditions. Two genotypes (No. 8: Hazen and No. 1: Albacete) represent exceptions with cooler leaves in soils with 20% water content than under normal condition. A set of genotypes (No. 75: Rihane-01, No. 60: Arta, No. 18: Otis) exhibited small differences between temperature of leaves at the two water regimes. We identified genotype No. 99 with relative cooler leaves under both growing conditions. We searched for a potential link between leaf temperature and shoot surface area or water use efficiency (WUE). Our data failed to indicate correlation between these parameters ($r^2=0.008$). Moderate correlation was detected between leaf temperature and WUE ($r^2=0.431$). At the time of harvesting the plant height data were measured for characterization of the tested genotypes. The final growth retardation by drought ranged between 0-40% (Supplementary Fig. 1). If we rank the tested genotypes according to the reduction in plant height and in the shoot surface area production (Fig. 3) we are not able to recognize a significant overlap between resistant or

sensitive variants. Correlation was not detected between these traits ($r^2=0.087$).

Since yield stability is the primary target in breeding programs, the yield parameters such as grain yield (GY), thousand grain weight (TGW), harvest index (HI), and water use efficiency (WUE), were also evaluated for plants grown in soil with different water supply. As shown in Fig. 6, the tested genotypes were divided into three categories according to the seed production. Variants with less than 48% grain reduction showed a tolerant response with low overall yield under both well-watered and stressed conditions (I). Among the higher yielding genotypes, there is a stress-sensitive category with 18-37% grain production in soil with 20% water content (III). This screening could identify stocks (No. 24: Hex.Wh. Barley; No. 75: Rihane-01; No. 8: Hazen) with considerable seed production under both optimal and sub-optimal soil conditions (II). These genotypes may have agronomic significance. Although both shoot surface area production and seed yield are important parameters, they did not show any correlation in barley lines investigated in the present study ($r^2=0.249$). Changes in thousand grain weight (TGW) exhibited a large variation that is reflected by either increase or decrease in drought-exposed plants from defined genotypes (Fig. 7). Plants of variants as No. 101: SCA239; No. 66: Scarlett; No. 1: Albacete showed 10-30% increase in the seed size. These genotypes belong to the resistant but low yielding category I as the seed production data are concerned (Fig. 6). The yield loss of sensitive variants such as No. 56 Monlón; No. 75: Rihane-01; No. 7: AZ8501 can derive from the drought-induced reduction in thousand grain weight. Analysis of the harvest index (HI) with water limitation indicated genotypes with increased (No. 1: Albacete; No. 48: WI2291; No. 58: ICB79-0583-2AP) or with reduced values under drought (No. 87: Arda; No. 74: ICB77-0091, No. 8: Hazen) in our tests (Supplementary Fig. 2). The improvement of water use efficiency (WUE) is an important goal in cereal breeding. The grain weight produced by a unit of water is a key parameter for genotypes in characterization of drought

Table 1. Nucleotide sequence of 4 unique haplotypes of *HvPPRPX* gene and nucleotide sequence of 7 unique haplotypes of *HvPI1* gene.

A HvPPRPX									
	1.S	2.S	3.S	4.S	5.S	6.S	1.I	7.S	8.S
Database sequence	C	G	C	?	?	T	?	?	G
A haplotype	C	G	A	T	T	C	..	A	G
B haplotype	G	C	C	C	C	T	..	G	C
C haplotype	C	G	A	T	T	T	TATA	G	G
D haplotype	?	G	C	T	T	C	..	G	G
Base pair mutation	C/G	G/C	C/A	T/C	T/C	T/C	-TATA	-G/A	G/C
Exon/intron	E	E	E	I	I	E	I	I	E
Amino acid mutation	L/L	K/N	L/M	-	-	R/R	-	-	V/V

B HvPI1																				
	1.S	2.S	3.S	4.S	5.S	6.S	1.I	7.S	8.S	9.S	10.S	11.S	12.S	13.S	14.S	2.I	15.S	16.S	17.S	18.S
Database sequence	?	?	C	C	C	-	-	G	A	G	T	?	G	?	?	?	?	?	?	A
A haplotype	G	G	C	C	C	C	T	G	A	G	T	G	G	C	G	..	C	G	T	G
B haplotype	T	G	T	T	C	T	T	G	A	G	C	C	A	C	A	..	C	C	C	A
C haplotype	G	G	C	C	C	C	T	A	A	G	T	G	G	?	?	?	?	?	?	?
D haplotype	G	G	C	C	T	C	T	G	A	G	T	G	G	C	G	..	T	G	T	A
E haplotype	G	G	C	C	C	C	-	G	A	G	T	G	G	T	A	..	C	G	T	A
U1 haplotype	G	C	C	C	C	C	T	G	A	T	T	G	G	C	A	GA	C	G	T	A
U3 haplotype	G	G	C	C	C	C	T	G	G	G	T	G	G	C	A	..	C	G	T	A
Base pair mutation	G/T	C/G	C/T	C/T	C/T	T/-	G/A	A/G	G/T	T/C	G/C	G/A	C/T	A/G	-G/A	C/T	G/C	T/C	A/G	
Exon/intron	E	E	E	E	E	E	I	E	E	E	E	I	E	I	I	I	I	I	I	E
Amino acid mutation	-	V/V	N/N	L/L	-	-	K/K	I/V	L/L	G/G	-	-	K/K	-	-	-	-	-	-	-

Table 2. T-test based gene-haplotype/trait association analysis. This analysis shows the positive effect of haplotype B of gene encoding the barley fungal pathogen induced mRNA for pathogen-related protein (*HvPPRPX*) on harvest index (HI), thousand grain weight (TGW), water use efficiency (WUE) and grain yield (GY). The haplotype B of the vacuolar proton-inorganic pyrophosphatase (*HvP1*) gene has positive effect on WUE trait.

Gene	Haplotype/ Trait combination	p-value	Haplotype effect	Trait mean (associated haplotype)	Sample No.	St. Dev.	Trait mean (remaining haplotypes)	Sample No.	St. Dev.	Mean difference (%)	Significance
<i>HvPPRPX</i>	HaplotypeB/ HI	0.0054	Positive	127.96	4	26,4	89.91	17	20,9	38,05	***
<i>HvPPRPX</i>	HaplotypeB/ TGW	0.0208	Positive	113.09	4	18,2	93.99	17	12,6	5.56	**
<i>HvP1</i>	HaplotypeB/ WUE	0.0339	Positive	189.91	4	75,3	123.42	16	45,7	29.66	**
<i>HvPPRPX</i>	HaplotypeB/ WUE	0.0452	Positive	185.18	4	74,8	123.37	17	46,4	5.56	**
<i>HvPPRPX</i>	HaplotypeB/ GY	0.0504	Positive	62.24	4	23,2	44.23	17	13,6	19,10	*
<i>HvDRF1C</i>	HaplotypeB/ GY	0.0713	Positive	59.41	5	10,6	43.98	16	16,9	15.43	
<i>HvP1</i>	HaplotypeB/ GY	0.1055	Positive	60.25	4	21,8	44.63	16	15,1	15.63	

responses. The phenotyping platform provided the water utilization data for individual plants on tested genotypes. As shown by Fig. 8 majority of genotypes exhibited better WUE in drought than under normal water supply. The highest WUE values were characteristic for four variants such as genotype No. 75: Rihane-01; No. 51: Seco; No. 24: Hex. Wh. Barley and No. 70: Tadmor under limited water supply. As the yield potential is concerned, these genotypes belong to category II with good yield potential and moderate drought tolerance (Fig. 6). Genotype No. 8: Hazen and No. 87: Arda produced the highest grain yield with 1 L of water in well-watered soil. Both cultivars showed significant reduction of WUE in drought condition while their sensitive response is indicated by lower yield potential (Fig. 6). The linear regression analysis revealed a strong correlation ($r^2=0.8066$) between WUE and grain yield.

Haplotypes of barley group 3 LEA protein *HvA1* gene differentially influence shoot surface area under drought stress

The *HvA1* gene was described by Xu et al. (1996) as a drought responsive gene that was included into the EcoTILLING-based allele discovery analysis (Cseri et al., 2011). In the present study, we have tested whether nucleotide sequence changes in this gene could be linked with observed drought responsive phenotypes. Fig. 9A represents the nucleotide sequences of the four (A-D) haplotypes identified EcoTILLING analysis and subsequent sequencing of haplotypes, including both the intron and exon regions of the *HvA1* gene. This characterization revealed 12 single nucleotide sequence polymorphisms (SNPs), and 2 INDELS. The five SNPs and the two INDELS identified in the exon region can cause alterations in amino acid sequences in the tested haplotypes. Fig. 9B provides the average of the relative green pixel values as indicators for

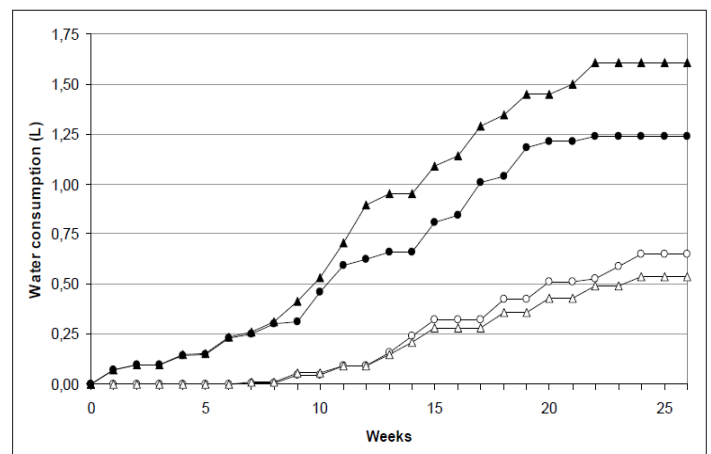


Fig 2. Genotype- and water supply-dependent water use of barley plants. Tolerant genotype (No. 1: Albacete) 20% water -○-; 60% water-●-. Sensitive genotype (No. 87: Arda) 20% water -△-; 60% water -▲-.

shoot surface area of genotypes with defined haplotypes in soil with 20% water content. According to the *t*-test this difference was not significant. Considering the enlarged variation caused by the extreme high green pixel values of the most tolerant (No. 86: Fengtien Black) cultivar (Fig. 3) the *t*-test was also carried out without the parameters of Fengtien Black variant. In the latter case one can detect a low level of significance (Fig. 9C). Despite the fact that the present analysis relies on a relatively small sample size, the detected differences support a potential relationship between haplotype D of the *HvA1* gene and the shoot surface area as an indicator for the biomass productivity.

Search for relation between haplotypes of selected drought responsive genes and phenotypic parameters of yield components

Fig. 10 provides an example for the EcoTILLING analysis of the barley *HvPPRPX* gene encoding the barley fungal pathogen induced mRNA protein. The analyzed amplicon was PCR amplified and after *Cel I* enzyme digestion the EcoTILLING image was obtained. The cleaved fragments indicate polymorphisms and four haplotypes could be recognized with the highest frequency of haplotype A. Table 1 presents the characteristic nucleotide sequence motives of haplotypes of *HvPPRPX* gene. Haplotype B carries several single nucleotide sequence polymorphisms (SNPs) both in exon and intron regions. For haplotype/trait association analysis a *t*-test was conducted. To this end the calculated relative trait units (stressed/control $\times 100$) for 5 different traits (GY, TGW, WUE, PH, HI) were associated with the gene-haplotypes. Statistically significant *p* values ($p \leq 0.05$) were found in 6 cases. The top 7 associations shown in Table 2, revealed a positive effect of haplotype B of *HvPPRPX* gene on harvest index (HI), thousand grain weight (TGW), water use efficiency (WUE) and grain yield (GY). Because of the small sample size, it is possible that not all the real associations were detected or some of the *p* values may be biased, nevertheless the differences between the favorite and non-favorite haplotype trait means are very high (Fig. 11) and the favorite groups in almost each case contain drought tolerant genotypes (Fig. 6). As indicated in haplotype B of the vacuolar proton-inorganic, pyrophosphatase (*HvPI*) gene has positive effect on WUE trait. Table 1 lists the characteristic sequence motives for allelic variants of the *HvPI* gene. The phenotypic levels measured for the different allelic variants of the *HvPI* gene are also presented in Fig. 11.

Discussion

Development of the Complex Stress Diagnostic System

The present study aims to combine phenomics and genomics approaches to analyze drought responses and to search for potential link between phenotypic traits and allelic variants of known stress genes. Here, we report quantitative data on genotype-dependent drought reaction of barley plants using a semi-automatic phenotyping platform. Availability of high-throughput phenotyping platforms facilitate automatic imaging, watering and weighing technologies which are preferentially suitable for monitoring abiotic stress responses (Munns et al., 2010). Because of the complexity and multigenic nature of drought adaptive traits, the phenotypic characterization of genomic variants requires data acquisition for a high number of variables including morphological, physiological and yield parameters. These measurements can be accomplished most accurately and efficiently using non-destructive imaging technologies. Our phenotyping facility, as a stress diagnostic system, was used for the analysis of barley plants in greenhouse. This methodology can serve as a complementary approach to the field studies in the characterization of cereal plants. It should be considered as a pre-screen and the selected variants need to grow in the field as well. Under these growing conditions, the severity of water stress can be tightly controlled as a key limiting factor. In the present experiments, the control/reference plants were grown in soil with 60% water content, whereas the stressed plants were exposed to 20% soil water

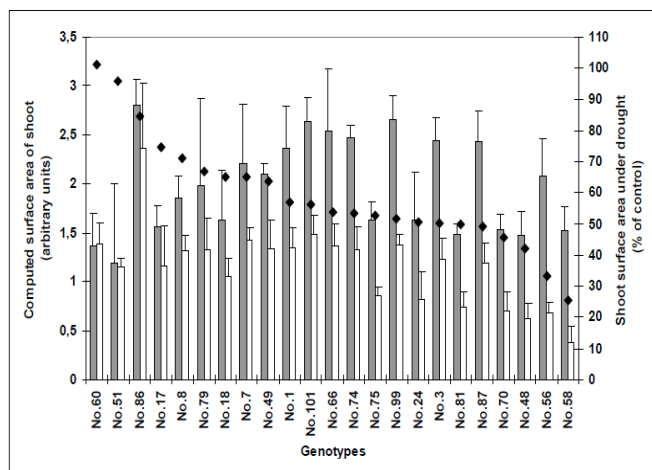


Fig 3. Genotype-dependent variation in green pixel-based shoot surface area at flowering stage under normal and water-scarce conditions: ■ 60% water content; □ 20% water content. ♦ represents the drought value as % of green pixel-based shoot surface area at normal soil water content. The green pixel-based shoot surface area was calculated by normalizing plant related pixels to an internal reference (see Materials and Methods).

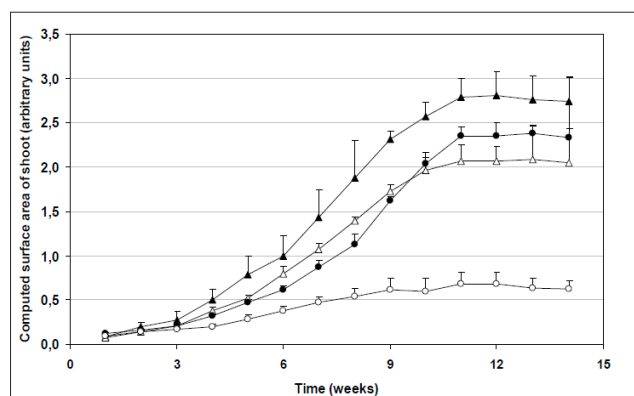


Fig 4. Growth of the green pixel-based shoot surface area during development depends on soil water status and genotype. Tolerant genotype (No. 86: Fengtien Black cultivar) 60% water-▲-; 20% water-●-. Sensitive genotypes (No. 56: Monlón); 60% water-△-; 20% water-○-. The green pixel-based shoot surface area was calculated by normalizing plant related pixels to an internal reference (see Materials and Methods).

content. The later growth condition can be considered as a strong stress exposure that is indicated by significant growth inhibition (Fig. 3 and Supplementary Fig. 1). Skirycz et al. (2011) reported that transgenic *Arabidopsis* plants with stress tolerance genes showed improved survival under severe drought, whereas these plants failed to grow under mild drought conditions. This observation emphasizes the need for precise regulation of the water status of the soil that translates to different degrees of stress. Since the computer-controlled water supply ensured the maintenance of stable water status during the whole growing period, the applied stress exposure provides advantages for genotypes with the capability of long-term adaption and efficient use of limited water. Out of the analyzed set of variants, the genotype No. 86: Fengtien Black may represent this type of drought

adaptation. The stress diagnostic system recorded the water use of each individual plant and these data are essential for the interpretation of green biomass and leaf temperature information. Under greenhouse conditions, the lower light intensity and relative higher humidity did not interfere with the test of water stress response. Because of the controlled environment drought was not combined with other extreme effects, such as temperature or wind that can have a modifying function in the field. The co-occurrence of drought and heat stress can have a significantly greater detrimental effect on productivity than the different stresses applied separately (Savin and Nicolas, 1996).

Phenotyping of green biomass under drought stress

In the pilot phase of this operation, the primary goal was the optimization of methodological details based on the characterization of a smaller collection of barley genotypes with a wide range of origin. Biomass prediction on the basis of RGB image analysis is one of the basic components of plant phenotyping (Golzarian et al., 2011; Hartmann et al., 2011). The above-ground biomass of the individual plants was defined as the shoot surface area covered by green plant pixels. The lower degree of growth retardation can be a primary indicator for the adaptation or tolerance capability of plants that may originate from the maintenance of cell proliferation and expansion under water scarce condition (Skirycz and Inzé, 2010). Green biomass is an integrative phenotypic characteristic determined by morphological traits such as plant height, leaf and head size or by physiological parameters including photosynthetic capacity. Therefore, it can serve as a basic parameter for genotype-dependent stress response. The genotypes tested show considerable variation in the computed shoot surface area in soil with optimal or suboptimal water content. The Chinese Fengtien Black genotype (No. 86), which is a Manchurian landrace with black seeds, is exceptional with high biomass production under drought condition. Although these plants belong to the low grain yield category, they represent a drought tolerant variant regarding biomass (Fig. 3) and grain yield (Fig. 6). The accumulation of green biomass under limited water availability can be a prerequisite for seed productivity. However, the seed yield is also influenced by the functionality of reproductive organs representing another level of control. This can be concluded from the lack of direct correlation between the shoot surface area production and seed yield ($r^2=0.249$). Therefore, if the target is grain yield optimization the seed production needs to be analyzed as integrated element of the phenotyping program. The seed parameters are influenced by the cultivation method when the plants are grown in pots at greenhouse condition. This is an artificial growing condition; therefore, field experiments are required for the final assessment of agronomic values and yield potential of genotypes in drought environment.

Thermal imaging in characterization of water stress responses

Green biomass production is primarily depends on the actual water status of crop plants that can be monitored by a variety of non-destructive screening techniques, including thermal imaging (Berger et al., 2010). In agreement with the general view, Fig. 1 shows that if water is available, the transpiring leaves are cooler than leaves with reduced water use under drought. As concluded from the canopy temperature data, the response of stomata function to water

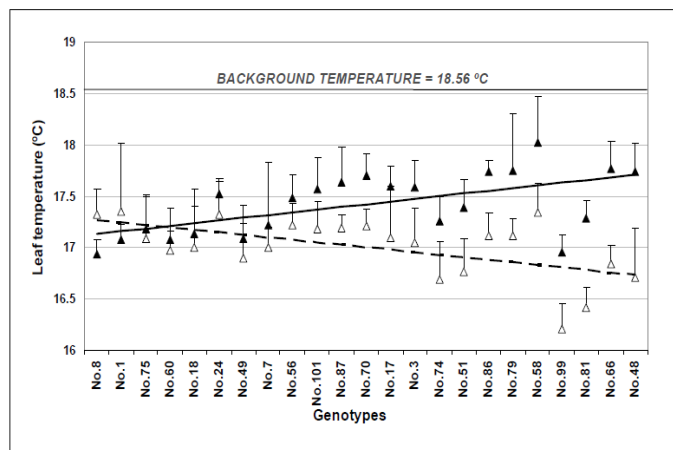


Fig 5. Genotype-dependent differences in canopy temperatures between well watered and drought stressed conditions as an indicator for evaporation by barley plants.

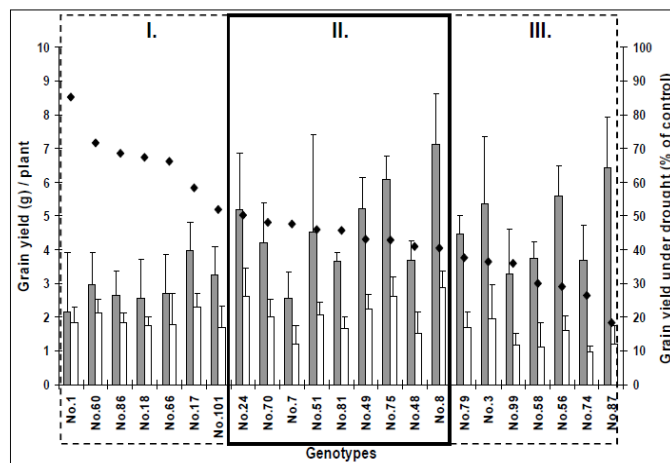


Fig 6. Genotype-dependent variation in the grain yield (GY) under normal and water-scarce conditions: ■ 60% water content; □ 20% water content; ♦ represents the drought value as % of grain yield at normal soil water content.

limitation shows large variation among different cultivars (Fig. 5). Genotypes (No. 18: Otis, No. 60: Arta) were identified with smaller leaf temperature differences between the control and stressed conditions and these variants may belong to the tolerant category with less than 40% reduction in shoot surface area (Fig. 3). The temperature variation from leaf to leaf of individual plants from the same genotype may contribute to the failure in detection of a correlation between the leaf temperature and the biomass production ($r^2=0.008$). Only in selected cases we observed relationship between these traits. Genotype No. 58: ICB-790583-2AP showed the most severe reduction in shoot surface area under drought stress, whereas genotype No. 60: Arta represents a drought tolerance variant with no biomass reduction in drought (Fig. 3). Leaves of sensitive plants (No. 58) were found to be warmer (+0.90°C) than leaves from this tolerant variant (No. 60). In the future, use of thermal imaging protocols will assist characterization of genotype-dependent stress responses. The present system is expected to be improved by combining the information derived from thermal and visible images (Möller et al., 2007). Based on the fact that drought tolerance can be generated by the variety of molecular and cellular mechanisms the

phenotyping technologies will require improvement with introduction of additional methodologies.

Role of allelic variants of stress responsive genes in biomass production

Control versus stressed pixel-based shoot surface area parameters of genotypes defined a rank ranging from the resistant to the sensitive categories (Fig. 3). Having this phenotypic categorization, it became possible to search for characteristic haplotypes of drought responsive genes that can be related to defined allelic variants. As regards the selection of candidate drought stress-related genes we can rely on transcript profiling and transgenic data. Shinozaki and Yamaguchi-Shinozaki (2007) suggested two major categories of these genes encoding either functional proteins (e.g. late embryogenesis abundant (LEA) proteins, and detoxification enzymes, water channel proteins, sugar and proline transporters, enzymes for osmolyte biosynthesis, proteases etc.) or regulatory proteins (e.g. protein kinases-phosphatases, transcription factors, enzymes of phospholipid metabolism, signaling molecules, etc.). Transcript profiling or characterization of transgenic plants or transient assay highlighted a significant number of barley (*Hordeum vulgare* L.) genes that are involved in drought responses (Guo et al., 2009, Marzin et al., 2008). Based on published information, we have previously selected nine candidate genes for the EcoTILLING analysis (Cseri et al., 2011). Among them, we identified the barley gene *HvA1*, a member of group 3 late embryogenesis abundant (LEA) protein genes exhibiting alleles with differential effect on pixel-based shoot surface area (Fig. 9). The *HvA1*-haplotype *D* carrying a unique *cca* insertion (position +740) and a unique replacement G-C SNP (position +270) was linked with higher values compared to other three variants (Fig.9/D). The functional role of the *HvA1* gene in green biomass accumulation has been demonstrated by production of transgenic plants overproducing this LEA protein (Xu et al., 1996; Sivamani et al., 2000). These data were sufficient to select this gene. The pivotal role of *HvA1* gene, responding to biomass, may be concluded from the fact that the analysis of alleles of the *HvARH1*, *HvSRG6*, *HvDRF1*, *HvDREB1*, *HvNXH1*, *HvP1* genes failed to exhibit specific variants for sensitive or tolerant genotypes. At the present stage of the work, we can propose a correlation at low statistical significance between the haplotype frequencies and the phenotypic categories because of the limited number (23) of the analyzed barley genotypes (Fig.9/C): Potential correlation may also be biased by population structure (mainly due to geographic origin) therefore additional genotypes need to be involved in this type of analysis.

Allelic variants behind seed parameters

In cereal cultivation, grain productivity is the number one trait that is also the primary target in breeding for stress tolerance. In commercial breeding programs, a complex set of testing systems support the selection of tolerant variants that can include the growing plants in controlled environments such as greenhouse, but the screen for agronomic yield potential requires field testing. Considering the limits of information from greenhouse-based analysis of grain production, in the present phenotyping study, we provide a rank of tested genotypes according to yield parameters as grain yield (GY), thousand grain weight (TGW), harvest index (HI), and water use efficiency (WUE), under both optimal and sub-optimal water supply

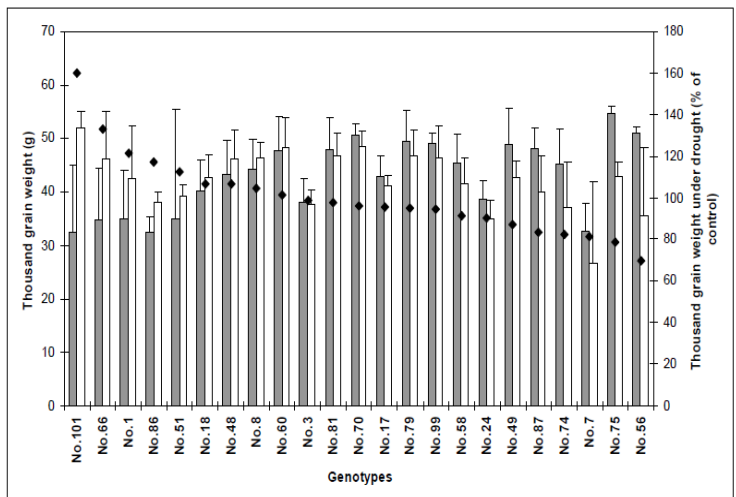


Fig 7. Genotype-dependent variation in the thousand grain weight (TGW) under normal and water-scarce conditions: ■ 60% water content; □ 20% water content; ◆ represents the drought value as % of grain yield at normal soil water content.

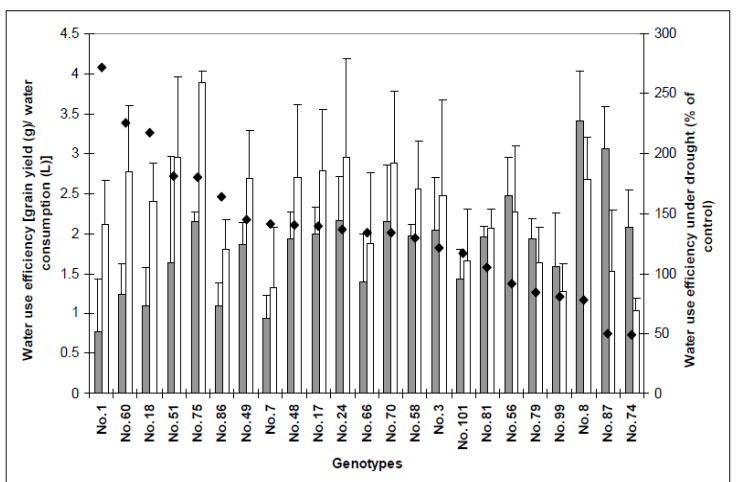


Fig 8. Genotype-dependent variation in the water use efficiency (WUE) under normal and water-scarce conditions: ■ 60% water content; □ 20% water content; ◆ represents the drought value as % of the water use efficiency at normal soil water content.

(Fig. 6, Fig. 7, Fig. 8 and Supplementary Fig. 2). The grain yield data indicated a basic contradiction between yield potential and high level of drought tolerance. As exception, we identified genotype No. 17 Arabi Aswad with average yield in the resistant category. Identification of sensitive and resistant genotype categories were supported by other barley studies. Guo et al., (2009) considered genotypes Martin and HS41 as tolerant variants with 26.6% and 16.3% yield reduction, respectively, whereas the drought-sensitive genotype Moroc9-75 showed 56.8% yield loss. Having the haplotype profile of nine barley drought responsive genes (Cseri et al., 2011), and the presented values of yield components we searched for any haplotype/trait association. As a novel finding, we identified the haplotype B of *HvPPRPX* gene encoding the barley fungal pathogen induced mRNA for pathogen-related protein that showed a strong influence on harvest index, thousand grain weight

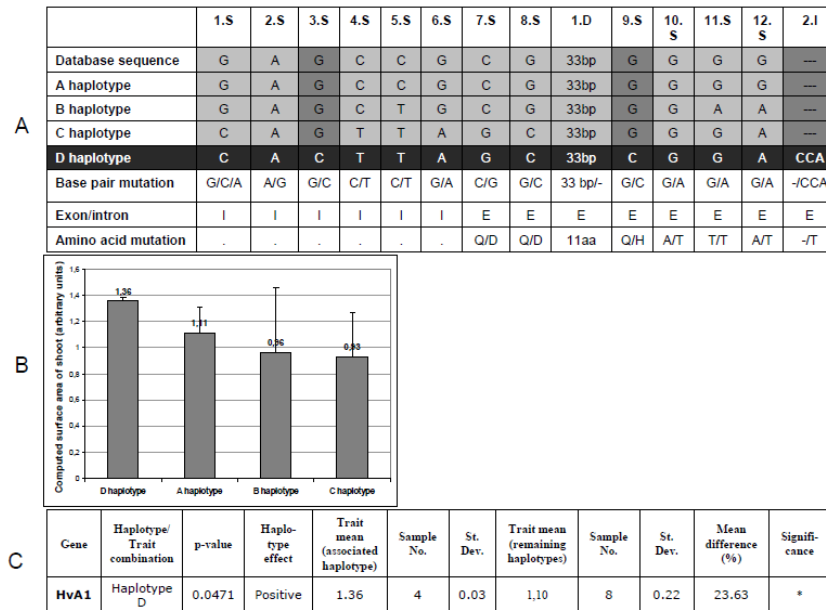


Fig 9. The haplotype D of the barley group 3 LEA protein *HvA1* gene can positively influence the green pixel-based shoot surface area. A: nucleotide sequence of 4 unique haplotypes of *HvA1* gene with the following characteristics: amplicon length: 919 bp; number of SNPs: 12; coding SNPs: 6; replacements: 5; number of insertion: 1; number of deletion: 1; coding indels: 2. B: average of green pixel-based shoot surface area data belonging to the four haplotypes C: Significance level of difference between the average of green pixel-based shoot surface area of genotypes carrying the haplotype D and the remaining haplotypes

and water use efficiency as supported by the high level of significance. This allelic variant had positive effect on the grain yield, but with lower significance level. The present observation that haplotype B can positively influence several high priority traits in seed yield production opens a way to generate molecular markers for additional verification of the described haplotype/trait association using bigger genotype collection or segregating populations. The water use efficiency (WUE) trait gains special significance not only under dry environment but also in irrigated plant production. The improvement of water use efficiency has been proposed to be achieved by establishing genetic potential for increased transpiration of water in limited supply, more efficient exchange of transpired water for CO₂ in producing biomass, and conversion of more of the biomass into grain or other harvestable products (Morison et al., 2008). The continuation of biomass accumulation under soil water deficit depends on the maintenance of growth, photosynthesis and high stomatal conductance. Based on WUE data, we can conclude that WUE is higher under drought than with optimal water supply. Only in a few exceptional cases the selected variants showed reduction under water stress (Fig. 8). The Syrian cultivar Rihane-01 (No. 75) was identified as an outstanding genotype in water use efficiency. Its yield potential was also good under optimal water supply (Fig. 6). As shown in Table 2, the WUE was improved in those genotypes that carry B type allelic variants of two tested genes such as *HvPPRPX* and *HvPI*. The later vacuolar proton-inorganic pyrophosphatase gene is involved in abiotic stress reactions as it was shown by transgenic plants (Bao et al., 2009). The present attempts for integrating phenotyping with EcoTILLING approach are in line with the recent publication reporting the combination of haplotyping, linkage mapping and expression profiling for a set of core drought-regulated barley genes (Worch et al., 2011). The

phenotypic and haplotype dataset created in the frame of this study provides a good starting point to design large scale QTL or association studies aiming to validate the interesting candidate gene variants. These further studies will require a larger phenotyping capacity.

Materials and methods

Collection of the tested barley genotypes

To test drought responses and identify physiological and agronomic parameters using the Complex Stress Diagnostic System, we have established a genotype collection. Barley genotypes were selected from different gene banks using literature and available passport data to ensure the involvement of both drought tolerant and sensitive genotypes. The present study is based on 23 barley genotypes that were a sub-collection of the set of 96 barley genotypes previously studied with EcoTILLING analysis of drought-related candidate genes (Cseri et al., 2011). The barley cultivars and landraces were obtained from various sources. The list of plant material is provided in Supplementary Table 1.

Plant growth conditions and experimental treatment

Ten-day-old seedlings of a sub-collection of 23 barley genotypes (Supplementary Table 1), were vernalised and phenologically synchronized at 3-4°C through 35 days in continuous dim light. After this treatment, the seedlings were planted into pots, containing the 1:1 mixture of sand (1340 g pot⁻¹) and peat soil (526 g pot⁻¹). The Substral Osmocote® fertilizer was mixed (4 g pot⁻¹) into the soil as

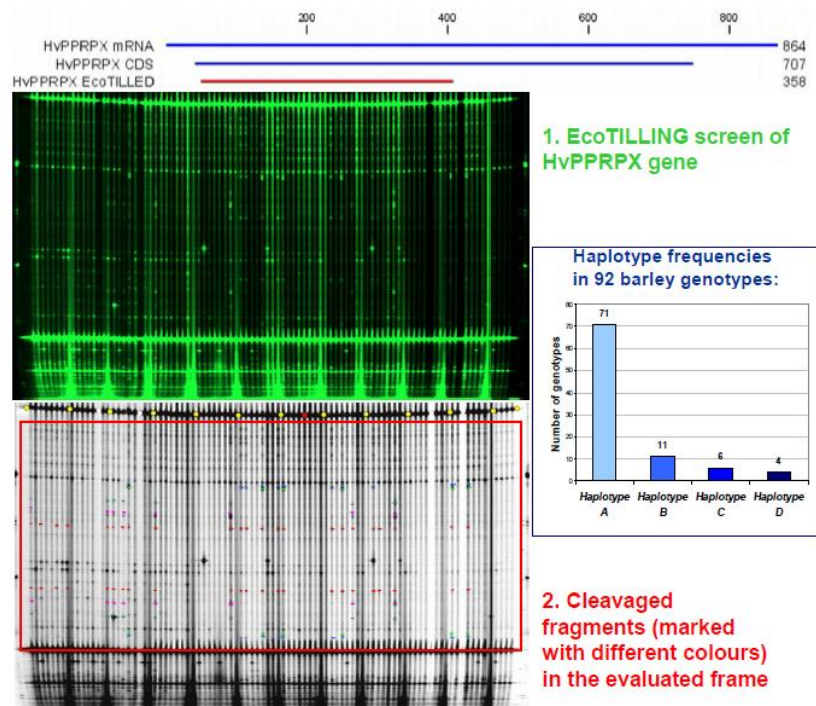


Fig 10. Allelic variants detected by EcoTILLING profile of the amplicon of *HvPPRPX* gene encoding the barley fungal pathogen induced mRNA protein. Cleaved fragments (marked with different colours) in the evaluated frame.

suggested by producer. Pot volume was 2L with a 13cm diameter. The 1.866 g soil was condensed into a 18cm deep column.

The temperature in the greenhouse was maintained according to the developmental stage of the plants following a standard greenhouse small grain cereal growing protocol. The daytime temperatures were 8-15°C for young plants at seedling stage, 16-22°C for the growing phase, and 23-28°C after heading. The night time temperatures were cca 5°C lower than the daytime minimum temperatures, but did not drop below 5°C even in case of the young plants at seedling stage. The relative humidity was kept at 60-65%. Watering and the digital imaging were performed twice and once a week during the whole life cycle, respectively. Thermal images were taken at the time of seed setting. After harvesting, several agronomic traits and parameters were determined. Here, we present data of grain yield, thousand grain weight and harvest index.

Controlled watering of plants

The analyzed plants were grown either under optimal water supply (60% soil water content during the whole life cycle) or under water scarcity (20% soil water content during the whole life cycle). The amount of water, which corresponds to 100% soil water content, was obtained from the weight difference of air dry soil and water saturated soil, by gradual watering until the point when it could not uptake more water. The amounts of water required to keep the 20 or 60% soil water content were added to the pots. In the drought test, each genotype was represented by five pots with a single plant and exposed to low water stress and five others served as controls with optimal water supply. At the time of potting, the barley plants were watered with 100 mL per pot in order to ensure the adaptation of seedlings. In case of stress treatment cca. five weeks were required to reach the

20% soil water content level. This situation is mimicking the conditions, which occurs under field conditions, when the soil has sufficient amount of water at the time of sowing in the spring season. Plants were weighed by a computer-controlled balance (GSE model 350, 6.000±1 g), and amount of used water was resupplied to maintain the required soil water status. The calculated amounts of water were added by using a peristaltic pump (Gilson Minipuls 3), while the pot was still on the balance. As these pots had a radiofrequency identifier (RF proximity TAG), watering data could be stored automatically by the computer.

Digital imaging of plants for green biomass analysis

Development of barley plants was monitored by digital photography (Fig. 1). Individual plants, planted into radio-tagged pots, were photographed by an Olympus C-7070WZ digital camera from 11 different sideways positions, produced by 32-33° step rotation of the pot. Monitoring of plant growth was performed during the whole growth period once a week. According to the data sheet of the camera, it did not show a significant barrel distortion of the picture. In order to minimize this effect further, we always used the same zoom setting, and placed the camera relatively far from the plants (22° angle of view). Therefore, there was no danger that a cluster with the same number of pixels located in different parts of the picture would represent different areas. Plant-related pixels were determined by separating the pot and background from the plant in each photograph using a home developed image analysis software tool. The shoot and leaf surface that corresponds to the plant-related pixel number was calibrated by dividing it with the square of the upper diameter of the pot, which was used as an internal calibration surface. We present green pixel values reflecting leaf/shoot surface area, which is considered to be proportional with green mass.

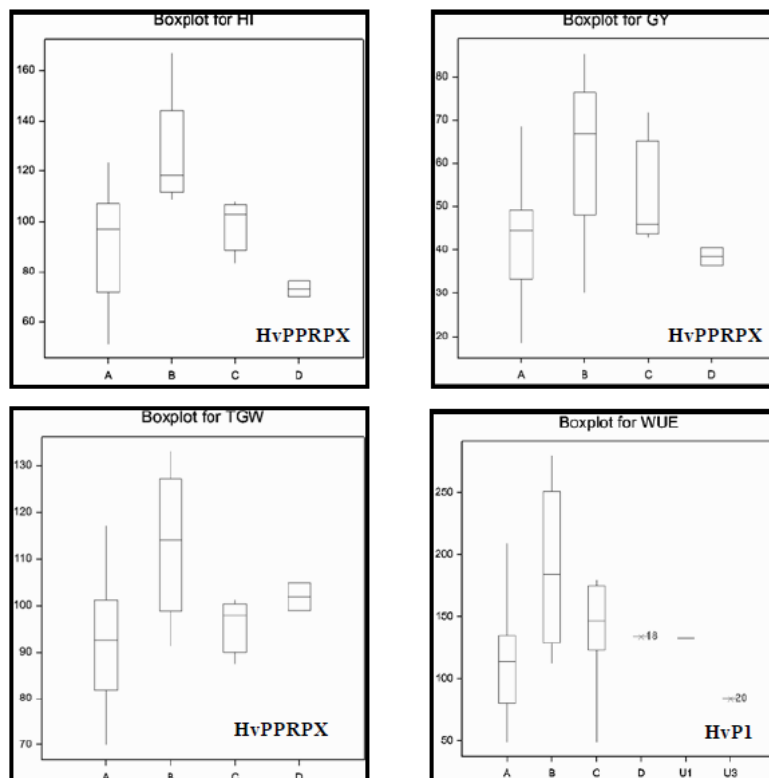


Fig 11. Relative trait unit levels measured in haplotype groups: HI/*HvPPRPX*, GY/*HvPPRPX*, TGW/*HvPPRPX* and WUE/*HvPI*. Box-plots illustrate the maximum and minimum values as well as the first and third quartiles plus the medians for the different haplotypes. In all 4 cases the most significant p values were found for the B haplotypes (see Table 2). HI=harvest index; GY= grain yield; TGW= thousand grain weight; WUE= water use efficiency.

Thermal imaging of plants

The efficiency of leaf evaporation was assessed by measuring leaf temperature relative to the surrounding air using a sensitive thermo camera (VarioSCAN 3021 ST, 0.03°C temperature resolution). The leaf temperature measurements were performed in a closed chamber within the greenhouse. Air temperature was obtained from a reference surface, which is in thermal equilibrium with air in the background of a plant. Different cultivars were measured under the same conditions. The temperature of the plant leaves was measured during the morning hours. The relative humidity was cca. 60-65%, and the air temperature was 18.56°C during the analysis presented in Fig. 5. The water pressure deficit (VPD) values were in the 0.79-0.92 kPa range. These measurements were carried out in the winter season under cloudy conditions without artificial illumination.

Data management

Data analysis was performed by a home constructed software package, which is based on Matlab software tools (version 2008b) with the Image Processing Toolbox™ (The MathWorks Inc., Natick, MA, USA). For quantification of the total green area of plants, we used ‘plant pixels’ that were determined by subtracting the homogenous background from the image. The height of the plants was obtained from the distance of top and bottom pixels on the digital image. The temperature of the leaf surface was calculated from the difference between the average of

thermal values of ‘plant pixels’ and of the background. Data collected by the measuring stations, containing the instruments for controlled watering and imaging, and was transferred via internet connection to the server computers, which perform data storage and analysis via an internet-based access.

EcoTILLING – haplotyping

In our previous study, we have tested the natural variations of drought-related candidate genes in barley genotypes (Cseri et al., 2011) using the EcoTILLING technology as a polymorphism discovery tool. The haplotype/trait association analysis was based on the *t*-test. In the present phenotyping work, we established a set of tolerant and sensitive genotypes on the basis of the reduction in green biomass or grain yield and we searched characteristic differences in the frequencies of the previously identified haplotypes.

Conclusion

The used semi-robotic phenotyping platform opened the way for the detailed characterization of the drought response of barley genotypes. We described resistant and sensitive variants based on the reduction in green pixel-based shoot surface area and alterations in canopy temperature. We provided data to support the pivotal role of haplotype D of the *HvAI* gene encoding the group 3 LEA protein in optimization of green biomass. The haplotype/trait association study supported the positive effect of the

haplotype B of the gene encoding the barley fungal pathogen induced mRNA for pathogen-related protein (*HvPPRPX*) on harvest index, thousand grain weight, water use efficiency and grain yield. The present work outlines the potential in the integration of genomic and phenomic approaches for characterization of barley genotypes under water limitation.

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Supplementary data are available online

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