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Characterization of a segregating winter wheat population regarding abiotic stress

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Abstract

Breeding for cultivars with improved yield stability and high quality under drought stress conditions is one of the most important challenges in time of global climate change. Identification of drought/heat-related QTLs plays a central role in crop improvement through marker assisted selection. Within the Cornet Wheat Stress project a doubled haploid (DH) winter wheat population was used to detect new QTLs associated with tolerance to drought stress specific for European winter wheat. 100 DH lines were grown in the semi-controlled environment of a greenhouse in two seasons, 2009/2010 and 2010/2011. In consideration of a segregation of the used DH population in the *Ppd-D1* locus, QTLs for flag leaf senescence, chlorophyll content and thousand kernel weight were identified.

Keywords

Chlorophyll content, drought stress, flag leaf senescence (FLS), QTL mapping, *Triticum aestivum*

Introduction

The European Union is one of the most significant wheat producers in the world. Impacts of global climate change also affect winter wheat production in Central Europe and are projected to become more severe. Extreme weather events like heat waves and drought seasons are expected to become more frequent and intense. Regions most prone to an increase in drought risk are the Mediterranean Basin and widespread regions of Central and Eastern Europe. In order to continue the leading position of the European Union at the global wheat market it is necessary to develop cultivars with high yield stability and high baking quality even under unfavorable environmental conditions.

Markers associated with tolerance for a variety of environmental stresses rank as important targets for marker-assisted selection (MAS). Efforts to identify quantitative trait loci (QTL) associated with drought using molecular mapping approaches represent an important first step to achieve this goal. Using molecular maps, putative gene loci affecting traits of interest can be detected by testing for statistical associations between marker variants and traits of interest. Once a marker-trait association has been established, MAS reduces the reliance on specific environmental conditions during the selection, a major hindrance in the conventional breeding of traits influenced by drought.

Material and Methods

Plant material

A winter wheat population of 100 doubled haploid (DH) lines was used in this study. Plants derived from the cross Kerubino×Rainer whose heterozygous offspring was then crossed with SZD7916A (for details see *Figure 1*).

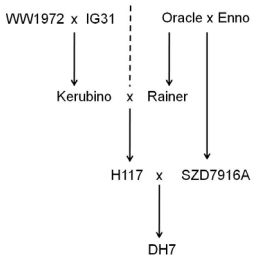

Figure 1: **Pedigree of the used DH population (produced by Saaten-Union Biotec GmbH)**

Greenhouse experimental design

120 genotypes including parents, grandparents and check lines were used in a randomized lattice design with 2 replications for both treatments. The greenhouse experiments were carried out in two seasons, 2009/2010 and 2010/2011. After sowing in boxes in late autumn, 3 week old plants were vernalized in a greenhouse for 8 to 10 weeks. After an adaptation period, at the beginning of March the boxes were placed on tables in another greenhouse. Two types of sensors were installed in the boxes: a watermark sensor for measuring soil moisture tension, and an EC5 sensor, which measures the water content (%). The sensors were tested in pilot experiments and are suitable for the used substrate. The sensors are connected to a data logger which constantly compares present and desired water content in the boxes and gives an irrigation signal if necessary. The defined treatments were a well watered variant where the EC5 sensor measures 28% water content in the soil, and a stressed variant where the water content is limited to 16%. As starting point for the drought stress application that day was defined, at which for 50% of the plants heading was noticed. Drought stress was continuously applied until harvest.

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Phenotyping

Collected phenological data were the following: heading date, flowering time and date of grain maturity. By visual scoring, the percentage of yellow flag leaf area was rated every 2 to 3 days, to estimate the progress in flag leaf senescence (FLS). In order to prevent any bias between operators influencing results, the same person for each experiment carried out the assessments. Thousand kernel weight (TKW) was recorded as yield data. Using SPAD 502 (Konica Minolta Sensing, Inc., Osaka, Japan), chlorophyll meter readings were repeatedly taken at the lower end of the upper third of flag leaves throughout the experiments. For each entry consisting of 2 plants, the average value was accumulated from 8 measures. Stomatal conductance as a measure of the maximum rate of passage of water vapour through the stomatal apperture of flag leaves was recorded with an AP4 leaf porometer (Dynamax, Inc., Houston, TX). The measurements were taken at three dates using four leaves for each entry. In order to estimate the osmotic adjustment (OA) of the different genotypes, flag leaves were sampled three times and placed in distilled water for 4 hours. After rehydration, leaves were wrapped in polyethylene bags and frozen at -20°C until analysis. After thawing at room temperature, cell sap was expressed using a manual leaf press, and the osmotic potential at full turgor (OP100) of the sap was measured by means of a PSYPRO water potential data logger and a C-52 sample chamber (Wescor, Inc., South Logan, UT). Subsequently, the osmotic adjustment was calculated as the difference of the OP100 values measured for well-watered and stressed plants (MOINUDDIN et al. 2005).

Marker analysis and map development

Diversity Array Technolgy (DArT) marker assays were performed by Triticarte Pty. Ltd. (Diversity Arrays Technology P/L-Triticarte P/L, Yarralumla, Australia). Additionally, the DH population was genotyped using 287 SSR markers. The final marker set included 465 DArT and 96 SSR markers. Genetic linkage mapping was performed with JoinMap 4.0 (VAN OOIJEN 2006). The order of molecular markers along each chromosome was determined using a LOD linkage threshold of 3.0 and recombination frequency threshold of 0.4. Map distances (cM) were calculated using the Kosambi mapping function (KOSAMBI 1944).

Statistical and QTL analysis

First, the dataset of each treatment was statistically analyzed separately to determine the significance of differences between genotypes. Estimation of correlation coefficients and analysis of variation was performed using Plabstat (University Hohenheim, Stuttgart, Germany), to identify significant genotype by environment interaction and replicate structure for all analyzed traits.Unlinked and redundant markers were eliminated from the dataset prior to interval mapping. QTL were detected and mapped on winter wheat chromosomes by performing simple interval mapping (SIM) analysis using PlabQTL (University Hohenheim, Stuttgart, Germany) with a significance value at the 0.01 confidence level defined as a minimum LOD threshold for each trait in SIM.

Results

Phenotypic and correlation analysis

Summarizing the results of the measurements, the equipment used to estimate the stomatal conductance and the osmotic potential was not suitable for our greenhouse experiments. Beside the electromagnetic susceptibility, the porometer is especially sensitive at low values for stomatal conductance which are mostly present in drought stressed plant leaves. It needs up to 10 minutes to take one measure. Finally, a repeatability of 0 to 7% between the 2 replications of the stress treatment was reached. For the psychrometer scanner, the effort to receive one value likewise is much too high. Pressing out the leaf sap, loading, measuring and subsequent cleaning of the equipment take about 5 minutes for one leaf. With 480 entries in the greenhouse 40 hours are used for one sampling day. For the osmotic potential measurement, the repeatability was between 0 and 5% between the 2 replications of the stress treatment. In consequence, we excluded the stomatal conductance and the osmotic potential from further analysis. The chlorophyll measurements were satisfying; here we received a repeatability of about 70%.

Analysis of variance showed significant effects of genotypes and treatments for all investigated traits at $P<0.001$ (*Table 1*). Also the years influenced the phenotypic traits significantly. This can be explained by the weather conditions, especially the number of sunny hours, which differed strongly between

Table 1: **Variability, variance components and heritability estimates for phenological and grain traits** (Level of significance: ** significant at $P\leq0.01$; * significant at $P\leq0.05$)

	HD[1]	DA	DGM	DGM-HD	TKW	FLS	SPAD
Minimum	97.1	100.8	141.9	29.1	20.8	8.1	22.2
Maximum	139.9	145.0	185.3	65.9	60.7	100.0	58.1
Variance components							
Genotype	62.6**	59.8**	32.5**	5.3**	26.0**	280.8**	5.6**
Treatment	119.2**	131.6**	147.1**	1.4**	16.9**	709.1**	2.5**
Year	4.9**	4.8**	0.11*	3.4**	2.3**	74.1**	0.1**
G×T×Y	3.9**	3.6**	2.5**	5.0**	5.8**	186.6**	1.3**
Heritability	97.44	97.36	94.72	69.71	88.03	80.22	77.37

[1] HD, heading date; DA, date of anthesis; DGM, date of grain maturity; DGM-HD, time between heading and grain maturity; TKW, thousand kernel weight; FLS, flag leaf senescence; SPAD, chlorophyll content

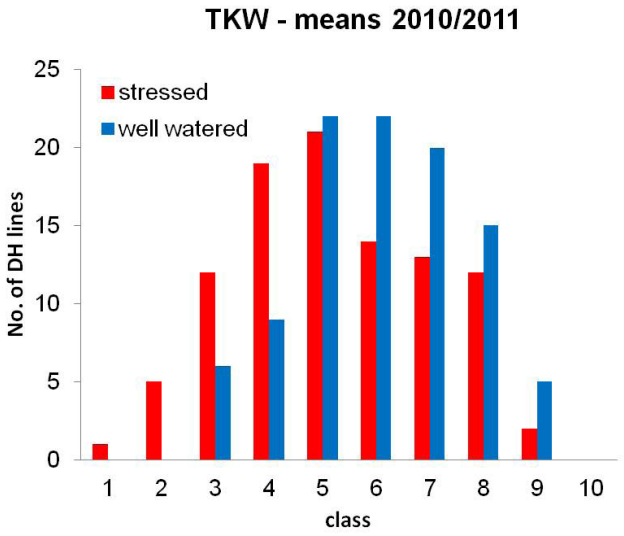

Figure 2: **Frequency distribution of thousand kernel weight for both treatment variants in two experimental years**

the years. The genotype by environment (treatment) interaction effects were significant for the phenological traits, TKW as well as chlorophyll content and FLS. Further, we observed significant interactions between genotype, treatment and year at a level of significance of $P<0.001$. Heritability for phenological data calculated across all experiments was between 0.95 and 0.97. Heritability estimates for TKW, FLS and chlorophyll content across all experiments were 0.88, 0.80 and 0.77, respectively.

The significance of the treatment effect as shown in the analysis of variance was visualized for TKW (*Figure 2*). During evaluation of the frequency distribution, mean values for the TKW of both experimental years were divided into 10 classes. The number of DH lines was counted for each TKW class, separately for both treatments. Resulting from the distributions, the TKW of the stressed variant was shifted to the left, meaning that the TKW was clearly reduced compared to the well-watered treatment.

Investigating the correlations between different phenotypic traits, we observed differences between the well watered and the stress variant (*Table 2*). The phenological traits heading date, flowering and grain maturity showed a high and positive correlation one with another in both treatments. The duration from heading to grain maturity was negatively correlated with phenological data and higher in the stress variant. That means, the later the heading occurs the shorter was the period until grain maturity. The correlation of TKW with heading date is positive, meaning that later genotypes have a higher TKW. Regarding chlorophyll content and flag leaf senescence, we found that the later the heading occurs the higher was the chlorophyll content and subsequently the lower was FLS at the same time. Furthermore, the more chlorophyll was measured for one line, the higher was the TKW at the end. And also, the stronger the senescence is in progress the lower is the TKW at the end. This last mentioned behavior was not observed for the well watered variant and corresponds to the already known stay green effect (VERMA et al. 2004).

Genetic map

In order to discover new QTLs associated with tolerance to drought stress specific for European winter wheat the DH population was genotyped using SSR and DArT markers. Altogether, 287 SSR markers were screened for polymorphism. The resulting 96 polymorphic SSRs and further 465 polymorphic DArT markers were used to genotype the 100 DH lines. Based on these marker data a genetic map was developed which contains 244 markers and encompasses 1691 cM with an average marker interval of 6.9 cM. This relatively low marker density is rooted in the selection of ancestors of the DH population (*Figure 1*). The varieties Rainer and SZD7916A are sister lines, this fact led to a low degree of polymorphism and subsequently to a low genetic resolution. Based on this map the QTL analysis was realized.

QTL analysis

At first we analyzed the whole DH population using all phenotypic data with the exception of stomatal conductance and osmotic potential. The result of QTL analysis were two single QTLs on the short arms of chromosome 2A and 2D which were identified for all investigated traits. These are the well-known QTL for flowering time were the major

Table 2: **Correlation analysis between phenological and grain traits in the well watered and stress variant 2011** (Level of significance: ** significant at $P\leq0.01$; * significant at $P\leq0.05$)

Variable	HD[1]	DA	DGM	DGM-HD	TKW	SPAD
Well watered						
DA	0.969**					
DGM	0.901**	0.914**				
DGM-HD	-0.319**	-0.226*	0.115			
TKW	0.353**	0.352**	0.376**	0.031		
SPAD	0.409**	0.396**	0.468**	0.069	0.157	
FLS	-0.752**	-0.737**	-0.763**	0.065	-0.297**	-0.513**
Drought stress						
DA	0.991**					
DGM	0.951**	0.951**				
DGM-HD	-0.747**	-0.723**	-0.508**			
TKW	0.757**	0.740**	0.687**	-0.630**		
SPAD	0.897**	0.889**	0.878**	-0.615**	0.750**	
FLS	-0.921**	-0.914**	-0.906**	0.624**	-0.751**	-0.958**

[1] abbreviations see *Table 1*

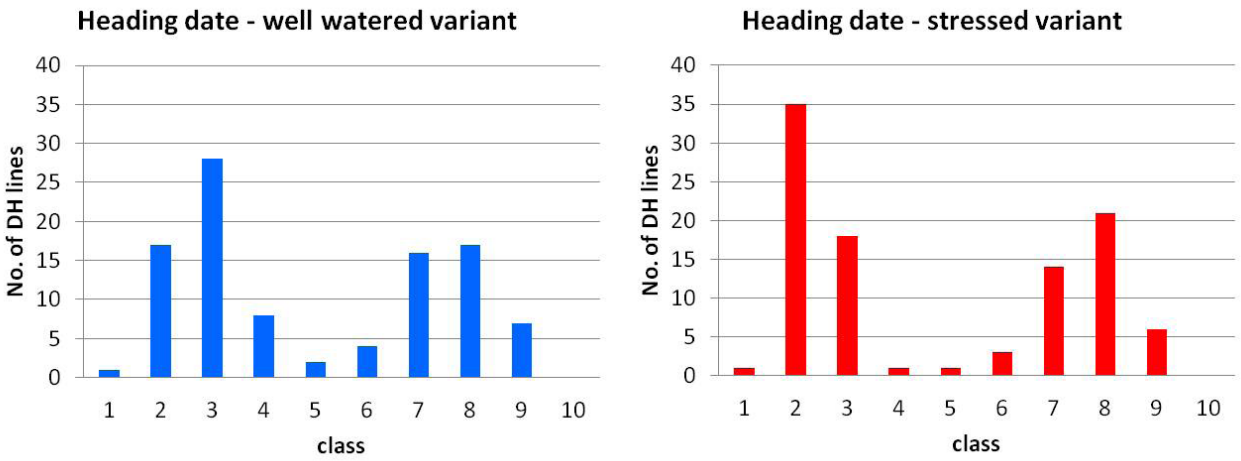

Figure 3: **Frequency distribution of the heading date for both treatment variants**

genes controlling photoperiod response, the *Ppd* genes *D1* and *A1*, are located. Apparently these genes are segregating in our DH population.

Investigating the distribution of traits, no normal distribution was observed but two peaks for the phenological parameters heading, flowering time and date of physiological grain maturity as exemplarily shown for heading date (*Figure 3*). This was the case in both experimental years for both treatments. In order to identify anyhow QTL for drought tolerance in our population we decided to use subpopulations.

Using the means of both treatments for heading date, again classes were calculated (*Figure 4*). Summarizing, the heading was spread to 27 days, with 5 days between the two peaks at which no heading for any line was noticed. The subpopulations were formed using this gap. With exception of one line, exactly the same DH lines belonged to the respective subpopulation in both experimental years. Therefore, this line was excluded from further analysis. The resulting subpopulations contain 54 and 45 lines, respectively. It is important to note, that the drought was applied when for 50% of the plants heading was noticed. Considering that drought realization needs about 1 week, the second (later) subpopulation received the drought stress already before heading and continuously during grain filling.

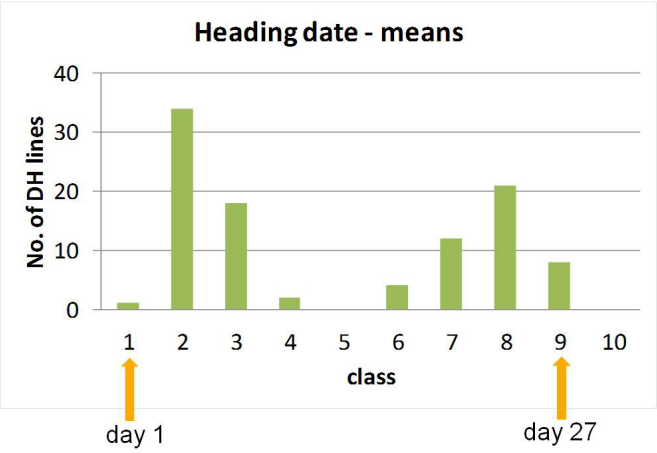

Figure 4: **Frequency distribution of the mean heading date from both treatment variants**

At a level of significance of 1%, QTL analysis (*Table 3*) of the first subpopulation (early heading) revealed one QTL for the duration from heading until grain maturity on the short arm of chromosome 1B. This QTL was identified for the well watered variant. From the stress variant a second QTL was identified for flag leaf senescence on the long arm of chromosome 5B. These putative QTL explain 39% and 30% of the phenotypic variance of duration from heading to grain maturity and flag leaf senescence, respectively.

Analyzing the second subpopulation (later heading), for the well watered variant one QTL for the duration from heading until grain maturity was detected on the long arm of chromosome 3A. For TKW one putative QTL was identified on the long arm of chromosome 1A for both treatments. Further some putative QTL for flag leaf senescence were detected, likewise for both treatments. One QTL for flag leaf senescence was identified on chromosome 2D, such a QTL associated with the SSR markers *Xgwm311* and *Xgwm382* was already described by VERMA et al. (2004). A second QTL for FLS is located on the long arm of chromosome 6B. The third QTL for flag leaf senescence was detected on the short arm of chromosome 7A and is associated with the candidate gene 6-SFT which was successfully mapped in our population. The gene encodes a 6-sucrose-fructan fructosyltransferase which is involved in fructan biosynthesis. The expression of 6-SFT is induced by drought stress, and the produced fructan serves as a carbon source for storage but also plays an important role as anti-stress agent (GAO et al. 2010). One fructan fructosyltransferase (FFT) was shown to be associated with a QTL for chlorophyll content on chromosome 6A of durum wheat (DIAB et al. 2008). These 3 QTL for flag leaf senescence explained 38%, 25% and 25% of the phenotypic variation of that trait, respectively. Furthermore, a putative QTL for chlorophyll content was detected on chromosome 7A, but with other flanking markers.

Conclusions

The drop irrigation realized for the greenhouse experiment worked well but the equipment used for measuring water potential and stomatal conductance was not suitable for the experiments. The development of the DH population from

Table 3: **QTL for phenotypic traits and associated molecular markers identified from the stress or the well watered variant or from mean of both**

Trait[1]	Marker	Chromosome	LOD	Subpopulation	Variant
HD	wpt6105	5B	4.30	1	stress
DGM-HD	wpt2019	1B	5.58	1	well-watered
DGM-HD	wpt2019	1B	5.89	1	mean of both
DGM-HD	wpt1277	3A	5.00	2	well-watered
TKW	wpt6005	1A	7.52	2	stress
TKW	wpt6005	1A	4.87	2	mean of both
FLS	wpt9797	2A	4.52	2	mean of both
FLS	wpt0071	2D	4.80	2	mean of both
FLS	wpt6105	5B	6.50	1	stress
FLS	wpt6105	5B	5.92	1	mean of both
FLS	wpt2991	6B	4.81	2	stress
FLS	wpt2991	6B	5.39	2	mean of both
FLS	6-SFT	7A	5.58	2	well-watered
FLS	6-SFT	7A	4.36	2	mean of both
SPAD	wpt7785	7A	5.06	2	well-watered
SPAD	wpt7785	7A	4.37	2	mean of both

[1] Abbreviations see *Table 1*

a backcross line led to a very low degree of polymorphism and strongly hindered the effort to develop a genetic map. As a result, molecular markers could not be mapped with high resolution. It could be shown that under dry conditions, flag leaf senescence as well as chlorophyll content have effects on TKW as yield parameter (stay green). Major genes controlling photoperiod response, the *Ppd-D1* and *Ppd-A1* are segregating in our DH population with the effect that flowering time overlaid all other traits of interest. Dividing the 100 DH lines into two subpopulations according to their heading date resulted in the successful identification of several known QTL for FLS. One new putative QTL on chromosome 7A is associated with the candidate gene 6-sucrose:fructan fructosyltransferase which might be implicated in osmotic adjustment during drought stress through the accumulation of fructan.

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