Theor Appl Genet (2014) 127:1935–1948
DOI 10.1007/s00122-014-2350-7

ORIGINAL PAPER

Genetic dissection of temperature-dependent sorghum growth during juvenile development

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Received: 26 October 2013 / Accepted: 17 June 2014 / Published online: 15 July 2014
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Abstract
Key message **Promising genome regions for improving cold tolerance of sorghum were identified on chromosomes SBI-01, SBI-03, SBI-07, and SBI-10. Chlorophyll fluorescence had no major effect on growth rates at low temperatures.**
Abstract Developing fast growing sorghum seedlings is an important breeding goal for temperate climates since low springtime temperatures are resulting in a prolonged juvenile development. The adaptation of sorghum to tropical and subtropical highlands gives hint for certain genetic variation. The goals of the present study were to detect marker-trait associations for leaf and dry matter growth rate and for chlorophyll fluorescence and content (SPAD) in relation to temperature. A diversity set comprising 194 genotypes was tested in eight controlled environments with temperatures ranging from 9.4 to 20.8 °C. Significant marker-trait associations ($p < 0.05$) were identified for each individual temperature regime and on the parameters of regression analyses describing the responses of growth or chlorophyll related traits to temperatures. The diversity set was fingerprinted with 171 diversity array technology (DArT) and 31 simple-sequence repeat (SSR) markers. SSRs were used to analyze the population structure while association studies were performed on DArT markers. Promising marker-trait associations for growth rates in relation to temperature were detected on chromosomes SBI-01, SBI-03, SBI-07, and SBI-10. Many promising loci were also significantly associated to the results obtained in individual low-temperature environments. Marker-trait associations for chlorophyll content and fluorescence did occasionally co-locate to those for growth during juvenile development but there was no evidence supporting our hypothesis that seedling growth at low temperatures is largely influenced by SPAD or fluorescence.

Communicated by Hai-Chun Jing.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-014-2350-7) contains supplementary material, which is available to authorized users.

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Introduction

Improving sorghum cold tolerance is an important issue for breeders in order to provide farmers an alternative crop to maize for bioenergy production in temperate regions. Sorghum shows small early stage growth rates at low springtime temperatures and has high base temperatures for DMGR and LGR (Lafarge et al. 1998). However, the adaptation of sorghum to tropical and subtropical highlands gives hint for certain genetic variation in cold tolerance during juvenile development. Genotypes with high dry matter accumulation at low temperatures have early soil coverage and canopy closure (Richards 2000) which improves competitiveness with weeds, reduces water losses

due to evaporation and may increase the vegetation period at the same time. Significant genotypic differences in dry weight of sorghum hybrids at early development stages were found in growth chamber experiments carried out at different temperatures (Yu and Tuinstra 2001). The authors suggested the selection of cold tolerant genotypes from growth chamber experiments since results obtained were highly correlated to field evaluation data. However, Knoll et al. (2008) found only low correlations between germination under controlled conditions and field-based emergence and speculated that both traits could be under separate genetic control, which would make direct selection in the field more relevant.

High biomass accumulation is driven by high photosynthetic rates and rapid leaf growth, which may result from both high leaf appearance rates (LAR) and LGR. In general, LAR increases linearly from the base (T_b) to optimum temperature. T_b is the temperature below which no growth or development takes place and temperatures above the optimum do not lead to a further increment in development rates per time unit i.e., the temperature optimum equals to the maximum growth or development rate. Genotype specific T_b of maize varies between 2.9 and 5.0 °C and the phyllochron, the inverse of the regression slope of leaf number plotted against thermal time, ranges between 48.6 and 65.5°Cd (Padilla and Otegui 2005). Superiority of exotic maize cultivars in LAR compared to European germplasm was observed until the third leaf stage but was lost at later development stages (Soldati et al. 1999). Genotypic differences in LAR were also observed for sorghum (Kumar et al. 2009). In contrast to LAR, DMGR at early growth stages generally increases exponentially with increasing temperatures (Thornley and Johnson 1990). A rapid leaf area development enhances light harvesting to maximize assimilate production. Hund et al. (2008) found a high correlation between dry weight and leaf area at warm temperatures. At low temperatures, dry weight was closest related to the operating efficiency of photosystem II (Φ_{PSII}). Under cold stress, photosynthetic rates may decrease due to a reduction in the membrane fluidity (Steponkus 1984), photoinhibition (Foyer et al. 2002) and changes in enzyme activities (Kocova et al. 2009). Photoinhibition affects mainly the photosystem II (PSII) while the effect on PSI is small (Krause 1988; Savitch et al. 2011). Chlorophyll fluorescence, as an indicator for the efficiency of the PSII in using photons for carbon fixation, and SPAD, which is closely related to the chlorophyll content and photosynthesis rate per unit leaf area, are useful traits to describe the photosynthetic performance of a crop under suboptimal conditions. Fluorescence was successfully used as a selection tool for cold tolerance in maize (Fracheboud et al. 1999). Trachsel et al. (2010) assumed that stage specific genetic regulation seems to play an important role since maize QTL for chlorophyll content detected during different growth stages did not co-locate. A major QTL for photosynthetic performance of maize identified only at low temperatures co-localized to a QTL for shoot dry matter accumulation, suggesting that the genetic control for photosynthesis differs depending on the temperature regime (Fracheboud et al. 2004).

Dealing with GxE interactions occurring in association studies on complex traits is important since some QTL can be found over a broad range of environments while many seem to be environment specific. Maccaferri et al. (2011) found only one stable grain yield QTL in durum wheat lines tested in environments with different soil water availability. Since the number of significant associations decreased with increasing drought stress conditions they concluded that there is limited effectiveness of association mapping under extreme conditions. Promising tools to overcome this problem are, (1) to integrate GxE interactions into the statistical framework or (2) to combine crop models with QTL analysis (Collins et al. 2008). QTL for crop model parameters were identified in bi-parental populations for maize leaf elongation rate (Reymond et al. 2003) for flowering time in barley (Yin et al. 2005) and leaf senescence in potato (Malosetti et al. 2006). QTL for parameters describing the adaptability across different temperature regimes and QTL for mean genotype performance enable to distinguish between genome regions responsible for temperature dependent control of a trait and the trait itself (Via et al. 1995; van Euwijk et al. 2010). QTL for the genotype specific response to the environment might be an important step in developing stable markers for marker-assisted selection.

The objectives of the present study were: (1) to identify marker-trait associations for SPAD, chlorophyll fluorescence, and traits directly related to juvenile growth and development in eight individual environments and for regression parameters describing the adaptation to different temperature conditions, (2) to identify genetic links between chlorophyll content and fluorescence related traits and crop growth during juvenile development, (3) and to compare the results obtained by analyzing each environment separately to those of the joint analysis through regression parameters.

Materials and methods

Plant material

The study was carried out on 194 biomass sorghum lines. The diversity set includes *Sorghum bicolor* and *S. bicolor sudanense* genotypes. DNA was extracted from leaf tips using the cetyl trimethylammonium bromide (CTAB) method. The genotypes were fingerprinted with 688 polymorphic DArT

markers. Marker positions were taken from Mace et al. (2008). Unmapped markers, completely linked markers, and markers with frequencies of one allele <5 % were removed. The final map comprised 171 informative DArT markers.

Experimental design

An experiment with eight temperature regimes was conducted in climate chambers at the Leibniz Universität Hannover. The diversity set was sown in pots with a diameter of 7 cm, and filled with 50 % Klasmann Potgrond P (Klasmann-Deilmann, Groß-Hesepe, Germany) and 50 % loamy sand. Three seeds per pot and genotype were sown at 10 mm depth. All plants were grown at an optimal temperature of 25/22 °C (day/night) until most plants were in the three-leaf stage (6 days). After thinning to one plant per pot, plants were moved to different climate chambers representing the eight temperature treatments (Table S1). Each single temperature treatment was designed as randomized complete block design with three replications of the diversity set within one climate chamber. Air temperature was measured every 5 min directly above the pots using TinyTag View 2 data loggers (Gemini Ltd., West Sussex, U.K.) during the duration of the study. Plants were grown at a photoperiod of 12 h with 10 h full light (455 µmol m^{-2} s^{-1}) and 1 h twilight in the morning and evening.

Determination of growth rates and physiological traits

The number of leaves of every plant was counted at the beginning and end of each temperature treatment. Leaf appearance rate (LAR) was calculated as follows:

$$\mathrm{LAR} = (\mathrm{LN_{dn}} - \mathrm{LN_{d6}})/n \quad (1)$$

where LN_{d6} is the number of leaves 6 days after sowing, LN_{dn} is the number of leaves at the end of the experiment, and n is the number of days of the temperature treatment.

The dry matter growth rate (DMGR) was estimated as follows:

$$\mathrm{DMGR} = (\mathrm{DM_{dn}} - \mathrm{DM_{d6}})/n \quad (2)$$

where DM_{dn} is the dry weight at the end of the experiment, DM_{d6} is the dry weight 6 days after sowing and n represents the number of days of temperature treatments. DM_{d6} was recorded in an additional set of plants harvested 6 days after sowing. The dry weight of leaves and stems was measured after drying at 105 °C.

Leaf area was measured at the end of temperature treatments with a leaf area meter (LICOR 3100, USA). LGR was estimated using the following equation:

$$\mathrm{LGR} = (\mathrm{LA_{dn}} - \mathrm{LA_{d6}})/n, \quad (3)$$

where LA_{dn} is the leaf area at the end of the experiment, LA_{d6} is the leaf area 6 days after sowing and n is the number of days of temperature treatments. An LA_{d6} of 2.2 cm^2 was assumed for all genotypes of the diversity set.

The greenness of the fourth leaf was recorded as mean of three measuring points using a SPAD-502 plus chlorophyll meter (Konica Minolta Sensing Inc., Osaka, Japan). Chlorophyll fluorescence was measured at the fourth leaf of light adapted plants with an LI-6400 instrument equipped with the LI-6400-40 pulse amplitude modulation fluorometer (LICOR, Lincoln, NE, USA) using a modified measuring protocol from Fracheboud et al. (1999). The temperature in the measurement chamber was kept at the corresponding temperature inside the growth chamber. Steady-state fluorescence (F'_s) was recorded when the rate of change in fluorescence in relation to temperature (dF/dt) was <5, indicating a stable signal. In order to obtain the maximum fluorescence (F'_m) a saturation flash of >8,000 µmol m^{-2} s^{-1} was applied for 1 *s*. Actinic light was turned off and leaves were illuminated with far red light to measure the ground fluorescence of light adapted leaves (F'_0). The fraction of absorbed photons used in photochemistry (Φ_{PSII}) was calculated as $(F'_m - F'_s)/F'_m$ (Genty et al. 1989). The efficiency of energy harvesting of the oxidized PSII (F'_v/F'_m) was calculated as $(F'_m - F'_0)/F'_m$.

Data analysis

Coefficients of variation (CV_g) were determined for the regression parameters and for each trait in every environment to describe the variation among genotypes. In addition to that, mean CVs within genotypes (CV_e) were computed based on the replications as an indicator for the error. Variance components were estimated using SAS 9.2 and broad sense heritability (h^2) was calculated according to Hill et al. (1998):

$$h^2 = \frac{\sigma_G^2}{\sigma_G^2 + \sigma_{G\times E}^2 \frac{1}{n} + \sigma^2 \frac{1}{rn}}, \quad (4)$$

where σ_G^2 is the genotypic variance, $\sigma_{G\times E}^2$ is the genotype × environment interaction variance, σ^2 is the error variance, r is the number of replications, and n is the number of environments. Analysis of variance (ANOVA) was carried out using the following model with $i = 1, 2, 3,\ldots$, a genotypes, $j = 1, 2, 3,\ldots, n$ environments and $k = 1, 2, 3,\ldots, b$ genotype × environment interactions:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_{ij} + \varepsilon_{ijk}, \quad (5)$$

where μ is the overall mean, α_i is the effect of the ith genotype, β_j is the effect of the jth environment, γ_{ij} is the

genotype × environment interaction, and ε_{ijk} is the random error.

Linear regression analysis was carried out on LAR, SPAD, F'_v/F'_m and Φ_{PSII} data from the eight temperature regimes using the following model:

$$y_{ij} = \beta_i + sl_i x_j + \varepsilon_{ij}, \quad (6)$$

where y_{ij} is trait value of the *i*th genotype in the *j*th temperature regime, β_i is the estimated intercept and sl_i the regression slope of the *i*th genotype, x_j is the temperature of the *j*th environment and ε_{ij} is a random error. Base temperature (T_b) was estimated by linear extrapolation to define the theoretical temperature below which LAR, SPAD, F'_v/F'_m, and Φ_{PSII} become 0:

$$T_{bi} = -\beta_i / sl_i, \quad (7)$$

An exponential function was used to describe the relation between DMGR and LGR, respectively, and temperature:

$$GR_{ij} = GR_{0i} e^{[a\,(T - T_0)]}, \quad (8)$$

where GR_{ij} is the growth rate of the *i*th genotype in the *j*th environment, GR_0 is the estimated GR in the lowest temperature environment T_0 (9.4 °C for DMGR and 13.5 °C for LGR), *a* is the exponent and *T* the temperature. Pearson's correlation coefficients were calculated between the regression parameters (i.e. the linear slopes $LAR_{(sl)}$, $SPAD_{(sl)}$, $F'_v/F'_{m(sl)}$, and $\Phi_{PSII(sl)}$, the exponents $DMGR_{(a)}$ and $LGR_{(a)}$, base temperatures $LAR_{(Tb)}$, $SPAD_{(Tb)}$, $F'_v/F'_{m(Tb)}$, and $\Phi_{PSII(Tb)}$, and initial growth rates $DMGR_0$ and LGR_0) and the respective trait values of the individual environments and between regression parameters and across environment means of the traits using SAS 9.2.

171 DArT markers and 31 SSR markers were used to analyze the population structure of 194 individuals with the software package STRUCTURE assuming an admixture model (Pritchard et al. 2000) and using a burn-in phase of 10,000 iterations followed by 10,000 Markov chain Monte Carlo iterations in order to detect the ''true'' number of *K* groups in the range of $K = 1$–20 possible groups. d*K* was calculated according to Evanno et al. (2005). Prior to association mapping data were arcsine-square root transformed in order to achieve approximately normal distribution. For identifying significant associations between 171 DArT markers and the traits Tassel 2.1 was used (Bradbury et al. 2007). The SSR based Q-matrix and a kinship matrix were used in a mixed linear model (MLM) (Zhang et al. 2010). Association studies were carried out for all traits in each individual environment, for mean genotype performance across all environments, and for regression parameters. A marker-trait association was considered to be significant at $p < 0.05$.

Results

LAR, SPAD, F'_v/F'_m and Φ_{PSII} increase linearly while DMGR and LGR increase exponentially with increasing temperatures within the range of environmental conditions used in the experiments (Fig. 1). DMGR of the population mean across all environments was 0.009 g day^{-1} and ranged between 0.0003 at 9.4 °C and 0.037 g day^{-1} at 20.8 °C. LGR of the best performing genotype was on an average over all environments 6.6 cm^2 day^{-1} while LGR of the worst performing genotype was 1.09 cm^2 day^{-1} (Fig. 1). At 20.8 °C population mean for LAR was 0.32 d^{-1}. No increase in leaf number was observed at 9.4 °C (Table 1).

Estimations for T_b of LAR [$LAR_{(Tb)}$] varied between 9.2 and 10.9 °C. SPAD of the population mean averaged over the environments was 15.8 and ranged between 11.2 and 19.4. Mean F'_v/F'_m and Φ_{PSII} across treatments were 0.47 and 0.32, respectively. CV_g of the estimated DMGR at the lowest temperature ($DMGR_0$) was 36 %, and CV_g of *a* of DMGR [$DMGR_{(a)}$] was 11.8 % (Table 1). CV_e of $DMGR_{(a)}$ and *a* of LGR [$LGR_{(a)}$] were also relatively low, while the comparatively high CV_g for $DMGR_0$ and for the estimated LGR at the lowest temperature (LGR_0) corresponded to a high CV_e.

Analysis of variance (ANOVA) revealed that both environment and genotype effects were significant for all analyzed traits ($p < 0.05$, Table 2). Genotype × environment interaction effects were highly significant for DMGR, LGR, LAR, SPAD and F'_v/F'_m ($p < 0.001$) but not significant for Φ_{PSII} ($p = 0.06$). Estimated h^2 was lowest for Φ_{PSII} (0.34). For all other traits h^2 ranged between 0.46 and 0.67.

Pearson's correlation coefficients between regression parameters and trait values of single temperature regimes are presented in Table 3. Highest correlation coefficients were found between mean DMGR [$DMGR_{(mean)}$] and mean LGR [$LGR_{(mean)}$] and DMGR and LGR at 20.8 °C. $DMGR_0$ and LGR_0 were highly correlated with DMGR and LGR at 9.4 or 13.5 °C, respectively. $DMGR_{(a)}$ and $LGR_{(a)}$ were negatively correlated to DMGR and LGR at low temperature regimes. In case of LAR and SPAD, highest correlations were found between the slopes of LAR [$LAR_{(sl)}$] and SPAD [$SPAD_{(sl)}$] and the respective trait values at 20.8 °C. Highly negative correlations were observed between T_b and the low temperature environment trait values of LAR, SPAD, F'_v/F'_m, and Φ_{PSII}. Pearson`s correlation coefficients between across environment means of the traits revealed that DMGR and LGR were highly correlated while both traits were not significantly correlated to LAR ($p < 0.05$, Table S2). Mean SPAD was significantly correlated to all other traits except $LAR_{(mean)}$ and $LAR_{(Tb)}$. The genotype with the highest DMGR across all environments had a much higher LGR, SPAD and Φ_{PSII} in comparison to the

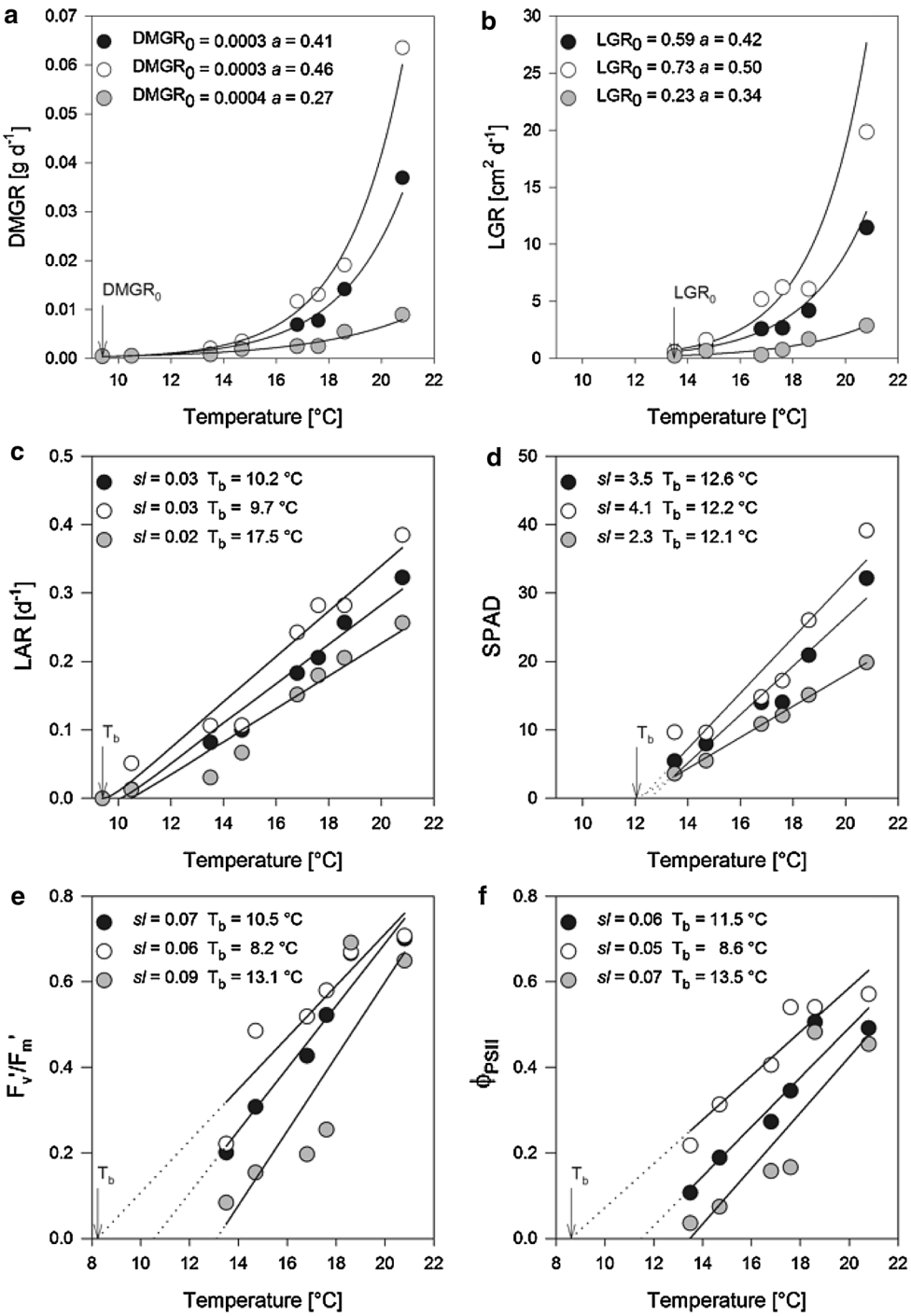

Fig. 1 Relationship between dry matter growth rates (DMGR) (**a**), leaf growth rates (LGR) (**b**), leaf appearance rates (LAR) (**c**), chlorophyll contents (SPAD) (**d**), fluorescence [Φ_{PSII} (**e**) and F'_v/F'_m (**f**)] and temperature for calculating the exponent (*a*), initial growth rates ($DMGR_0$, LGR_0), temperature effects (*sl*) and base temperatures (T_b). *Black circles* indicate population means, *unfilled circles* represent the best and *grey circles* the worst performing genotype. Selection criterion was mean across environments

population mean, while LAR and F'_v/F'_m were only slightly increased (Fig. 2).

There was for all traits a strong negative correlation between temperature and CV_g (Table 4). The correlation between temperature and CV_e was always negative as well and there was a strong correlation between CV_g and CV_e ($R = 0.96$).

The population of 194 individuals consists of two distinct groups. The estimated population structure based on 31 SSR or 171 DArT markers, respectively, is shown in Fig. 3. According to DArT, 140 lines (72 %) belong to group 1 while 54 lines (28 %) belong to group 2. Using SSRs, 131 genotypes (68 %) were considered to belong to group 1 while 61 genotypes (32 %) were part of group 2.

A total of 138 significant marker-trait associations ($p < 0.05$) were identified for the regression parameters and 449 QTL were detected in the individual environments (Table 5). The highest number of significant marker-trait

Table 1 Genotype means, ranges and coefficients of variation among (CV_g) and within genotypes (CV_e) for averages across all environments, the parameters regression slope (*sl*) and base temperature (T_b) of leaf appearance rate (LAR), chlorophyll content (SPAD), and chlorophyll fluorescence (F'_v/F'_m and Φ_{PSII}), and for exponents (*a*) and initial growth rates ($DMGR_0$ or LGR_0) of dry weight growth rate (DMGR) and leaf area growth rate (LGR). Mean R^2 values of regression analyses are shown

	Genotype mean, min and max across all environments					*sl* or *a*					T_b or LGR_0 or $DMGR_0$					R^2
	Mean	Min	Max	CV_g (%)	CV_e (%)		Mean	Min	Max	CV_g (%)	CV_e (%)		Mean	Min	Max	CV_g (%)	CV_e (%)	
DMGR	0.009	0.003	0.014	25.3	16.5	$DMGR_{(a)}$	0.41	0.27	0.56	11.8	11.2	$DMGR_0$	0.0003	0.0001	0.0007	36.1	35.8	0.97
LGR	3.78	1.09	6.59	23.8	13.5	$LGR_{(a)}$	0.44	0.26	0.77	15.4	19.5	LGR_0	0.57	0.09	1.24	36.7	38.5	0.94
LAR	0.15	0.11	0.18	9.0	6.4	$LAR_{(sl)}$	0.03	0.02	0.04	8.6	10.4	$LAR_{(Tb)}$	10.2	9.2	10.9	2.8	4.8	0.95
SPAD	15.76	11.17	19.41	9.9	9.0	$SPAD_{(sl)}$	3.55	2.05	4.94	16.7	17.2	$SPAD_{(Tb)}$	12.5	9.4	13.7	5.7	7.9	0.90
F'_v/F'_m	0.47	0.34	0.58	9.3	6.2	$F'_v/F'_{m(sl)}$	0.07	0.04	0.09	11.4	12.2	$F'_v/F'_{m(Tb)}$	10.5	8.0	13.1	9.6	12.3	0.89
Φ_{PSII}	0.32	0.23	0.45	11.3	21.5	$\Phi_{PSII(sl)}$	0.06	0.03	0.09	17.4	13.0	$\Phi_{PSII(Tb)}$	11.3	8.0	14.0	9.7	10.2	0.79

associations was found for DMGR; 22 marker-trait associations for DMGR were found at 13.5 °C. The number of significant marker-trait associations for regression parameters ranged between four [$\Phi_{PSII(sl)}$] and 18 ($DMGR_0$). Table S3 summarizes positions, *p* values, and differences in allelic means of all significant marker-trait associations.

The marker with the highest number of significant associations to the traits ($p < 0.05$) was sPb-4874 on SBI-07 (Fig. 4; Table S3). The marker was significantly associated with DMGR in seven of the eight environments while 32 marker-trait associations for DMGR were found in only one environment. Marker-trait associations found in only one environment were rarely co-located with QTL for regression parameters. 29 marker-trait associations for DMGR in individual environments were co-located with QTL for $DMGR_{(mean)}$ while only 12 QTL for LAR in the different temperature regimes coincided with QTL for $LAR_{(mean)}$.

QTL for $DMGR_{(a)}$ were found at the same positions of markers significantly associated ($p < 0.05$) with DMGR at low temperature regimes. Nine marker-trait associations for $DMGR_0$ co-located with QTL for DMGR at 9.4 °C. The highest number of co-localizations of *sl* and trait QTL detected in only one environment was observed for SPAD. Eight $SPAD_{(sl)}$ marker-trait associations co-located with QTL for SPAD at 20.8 °C. Likewise, QTL for $LAR_{(sl)}$ often co-located with QTL for LAR obtained in the high temperature treatments. Most of the significant marker-trait associations for T_b of LAR, SPAD, F'_v/F'_m and Φ_{PSII} coincided with QTL for the same traits at low-temperature regimes.

Significant marker-trait associations ($p < 0.05$) for DMGR and LGR were co-localized on all the chromosomes. Promising regions were identified on SBI-01 between 74 and 107 cm and on SBI-03 between 30 and 56 cm. The region on SBI-03 carries also QTL for LAR and F'_v/F'_m. Another interesting region was identified on SBI-07. sPb-4874 was associated with DMGR in many temperatures and with $SPAD_{(sl)}$ and $SPAD_{(Tb)}$. Further co-localizations between QTL for DMGR and SPAD regression parameters were detected on SBI-10 between 42 and 46 cm.

Discussion

Multi-environment data in association mapping

For analyzing juvenile development, a certain leaf number is often the harvest time criterion, which makes results comparable. Testing many different lines in different environments makes the use of a fixed leaf number as harvest time criterion nearly impossible. Working with growth rates, as done in the present study, has the advantage of

being independent of exactly identical harvest times, if the goal is to compare results or analyze them together. Plant growth rates change during different development stages (El-Lithy et al. 2004). However, within short periods during certain development stages as the juvenile phase or later pre-flowering development stages, growth rates can be constant. Relating growth rates to temperature enabled us to dissect the genetic basis of processes regulated by temperature, and parameterizing simple functions allowed us to characterize the genotype specific temperature response of sorghum during juvenile development.

For marker-assisted selection, the identification of QTL, which are stable across environments, is required (Burow et al. 2011) but the detection of stable QTL across experimental conditions is difficult even in controlled experiments, varying only in one environmental factor. In the present study, one QTL on SBI-07 for DMGR was found in seven of eight environments while many marker-trait associations were environment specific. Maccaferri et al. (2011) suggested that the lack of stable marker-trait associations is due to similar phenotypes of genotypes, which have different physiological mechanisms to cope with

Table 2 Variance components and heritability for dry matter growth rate (DMGR), leaf growth rate (LGR), leaf appearance rate (LAR), chlorophyll content (SPAD), and chlorophyll fluorescence (Φ_{PSII} and F'_v/F'_m)

	Variance components[a]				Heritability
	σ^2_E	σ^2_G	$\sigma^2_{G\times E}$	σ^2	h^2
DMGR (g day^{-1})	0.00017***	0.000003***	0.000017***	0.000003	0.60
LGR (cm^2 day^{-1})	16.09***	0.56***	1.03***	1.81	0.67
LAR (day^{-1})	0.01166***	0.00014***	0.00013***	0.00117	0.62
SPAD	94.42***	1.37***	1.75***	12.57	0.58
F'_v/F'_m	0.0453***	0.0005***	0.0027***	0.0003	0.53
Φ_{PSII}	0.0293***	0.0004***	0.0013 n.s.	0.0056	0.46

***, **, * Significant at the 0.001, 0.01, or 0.05 probability level

σ^2_E, σ^2_G, $\sigma^2_{G\times E}$ and σ^2 are environment, genotype, genotype × environment interaction and error variances

Table 3 Pearson's correlation coefficients between the traits dry weight growth rate (DMGR), leaf growth rate (LGR), leaf appearance rate (LAR), chlorophyll content (SPAD) and chlorophyll fluorescence (F'_v/F'_m and Φ_{PSII}) measured in each temperature regime and means across environments, exponents (*a*), initial growth rates ($DMGR_0$ and LGR_0), temperature effects (*sl*) and base temperatures (T_b)

Temperature (°C)	DMGR			LGR			LAR		
	Mean	*a*	$DMGR_0$	Mean	*a*	LGR_0	Mean	*sl*	T_b
20.8	0.95***	0.38***	0.22**	0.94***	0.15*	0.43***	0.72***	0.82***	0.21*
18.6	0.82***	0.29***	0.27***	0.79***	0.09 n.s.	0.42***	0.62***	0.60***	−0.02 n.s.
17.6	0.77***	0.24***	0.37***	0.78***	−0.05 n.s.	0.56***	0.52***	0.48***	−0.04 n.s.
16.8	0.75***	0.14*	0.46***	0.77***	−0.04 n.s.	0.55***	0.63***	0.47***	−0.21**
14.7	0.57***	0.01 n.s.	0.46***	0.65***	−0.41***	0.74***	0.47***	0.25**	−0.32***
13.5	0.47***	0.02 n.s.	0.45***	0.47***	−0.74***	0.92***	0.60***	0.23**	−0.56***
10.5	0.26***	−0.47***	0.70***				0.24***	−0.20**	−0.68***
9.4	0.34***	−0.61***	0.86***						
Temperature (°C)	SPAD			F'_v/F'_m			Φ_{PSII}		
	Mean	*sl*	T_b	Mean	*sl*	T_b	Mean	*sl*	T_b
20.8	0.74***	0.85***	0.49***	0.25***	0.16*	0.02 n.s.	0.43***	0.77***	0.47***
18.6	0.73***	0.65***	0.27***	0.25***	0.13 n.s.	−0.01 n.s.	0.37***	0.18*	−0.07 n.s.
17.6	0.65***	0.31***	−0.08 n.s.	0.71***	−0.09 n.s.	−0.35***	0.68***	0.11 n.s.	−0.29***
16.8	0.55***	0.23***	−0.09 n.s.	0.76***	−0.21**	−0.45***	0.54***	−0.08 n.s.	−0.36***
14.7	0.29***	−0.32***	−0.55***	0.58***	−0.71***	−0.76***	0.52***	−0.40***	−0.67***
13.5	0.33***	−0.45***	−0.74***	0.48***	−0.69***	−0.67***	0.38***	−0.32***	−0.48***

***, **, * Significant at the 0.001, 0.01, and 0.05 probability level

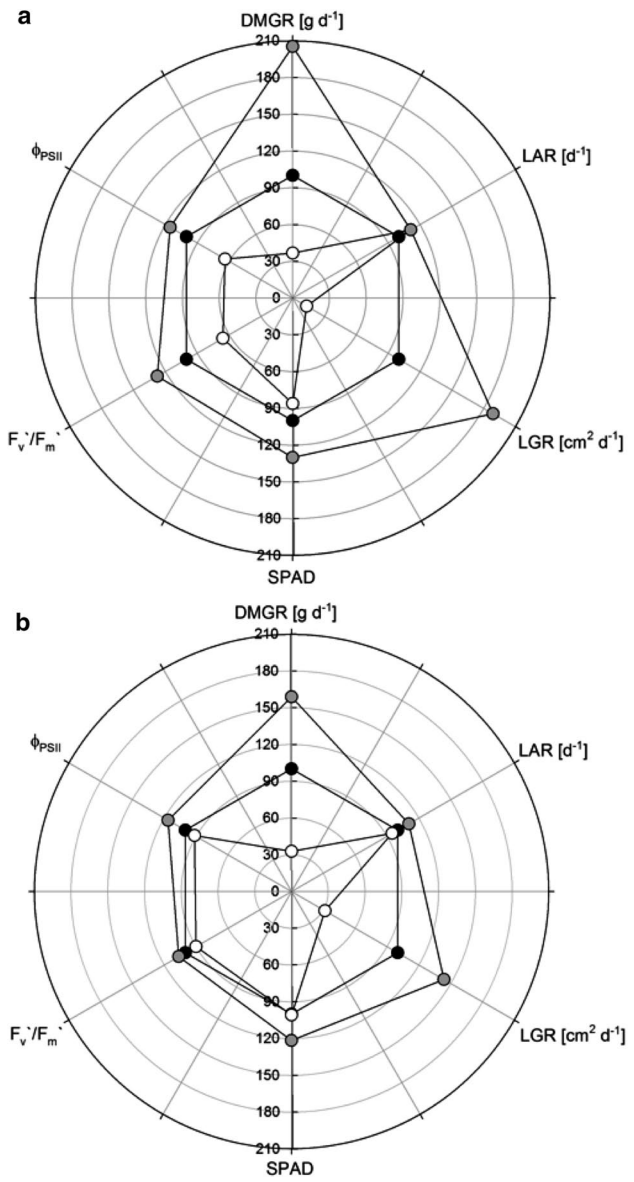

Fig. 2 Percentage deviations for the best (*grey circle*) and worst (*unfilled circle*) performing genotype compared to the population mean (*filled circle*) for the traits dry matter growth rate (DMGR), leaf appearance rate (LAR), leaf growth rate (LGR), chlorophyll content (SPAD), and fluorescence (Φ_{PSII} and F'_v/F'_m). Selection criterions were highest or lowest DMGR at 13.5 °C (**a**) and across all environments (**b**)

stress, if complex traits like grain yield are analyzed in diverse populations. They detected fewer QTL under more stressful situations. The plant material of the present study shows strong variation in the adaptation to low temperatures and more similar phenotypes under optimum growing conditions. We observed a strong linear increase in the number of marker-trait associations with decreasing temperatures if we take the total number of marker–trait associations of all traits at temperatures between 13.5 and 20.8 °C into account (only two traits were measured at temperatures below 13.5 °C). However, the situation is different for each trait. The highest number of marker–trait associations was observed at the lowest temperature only for LGR. Highest coefficients of variation between genotypes were always found at lowest temperatures. Coefficients of variation within genotypes increased also with decreasing temperatures. Since the chance for the detection of a QTL is highest due to high phenotypic variation, the increasing error or variation within genotypes in the more stressful environment made the circumstances for the identification of a marker–trait association sub-optimal. Consequently, it might be useful to find the optimum compromise between the variation within and among genotypes for each trait or to increase the number of observations if stress increases.

It was suggested that QTL mapping approaches using repeated measurements on growth curves and functions describing the adaptation to environmental factors, provide maximum information about QTL effects and positions and reduce random errors (Ma et al. 2002, Reymond et al. 2003, Uptmoor et al. 2009). Our results show a very similar trend for the regression parameters *sl*, *a*, T_b, $DMGR_0$, and LGR_0: A high CV_g, which is advantageous for the detection of significant marker-trait associations ($p < 0.05$), came always along with an increasing CV_e. Using more observations from extreme environments as carried out by Fiedler et al. (2012) may increase the accuracy of parameter estimations if linearity can be assumed. We often found non-significant correlations between *a* or *sl* and the trait values at intermediate temperature regimes, suggesting that mainly the high and low temperature environments contributed to the parameter estimation.

Sadok et al. (2007) found no co-localization of QTL for regression parameters and QTL detected in stress environments and concluded that trait QTL, which were detected in stress environments, might have another genetic network than QTL for regression parameters. We found co-localizations between low-temperature and response curve QTL especially for LGR but also for other traits. Most co-localizations were found between QTL for treatments at low temperatures and T_b or $DMGR_0$ and LGR_0, respectively. These parameters are closely correlated to *sl* or *a*, i.e., a small *sl* or *a* leads to a low T_b, $DMGR_0$, or LGR_0 and co-localizations between QTL for the parameters and/or low temperature QTL are likely to occur.

Identification of physiological mechanisms, which promote growth at low temperatures

We analyzed plant growth and several chlorophyll content and fluorescence related traits and LAR in order to see if these traits may have positive impacts on crop performance under low temperatures. We assumed high correlations

Table 4 Coefficients of variation among genotypes (CV_g) and within genotypes (CV_e) for dry matter growth rate (DMGR), leaf growth rate (LGR), leaf appearance rate (LAR), chlorophyll content (SPAD), and chlorophyll fluorescence (Φ_{PSII} and F'_v/F'_m) in different temperature regimes

Temperature	DMGR		LGR		LAR		SPAD		F'_v/F'_m		Φ_{PSII}	
	CV_g	CV_e	CV_g	CV_e	CV_g	CV_e	CV_g	CV_e	CV_g	CV_e	CV_g	CV_e
20.8	29.5	23.5	28.0	22.6	10.5	8.5	11.5	10.2	11.5	1.7	14.8	18.7
18.6	28.9	28.1	28.9	30.7	14.2	13.4	14.9	17.9	14.9	2.4	12.0	12.0
17.6	27.7	27.2	29.6	28.4	13.0	15.6	19.4	23.7	19.4	13.5	26.5	25.4
16.8	32.7	25.8	31.0	28.5	12.6	11.9	16.6	21.1	16.6	16.0	30.3	31.0
14.7	33.8	40.4	33.5	39.6	16.6	21.4	22.8	30.7	22.8	27.4	34.6	34.8
13.5	37.5	47.2	45.4	53.3	27.6	30.2	43.3	63.2	43.3	33.9	32.8	33.7
10.5	31.9	44.0			106.1	117.7						
9.4	51.7	37.2										
R	−0.74	−0.79	−0.84	−0.90	−0.80	−0.82	−0.85	−0.87	−0.94	−0.97	−0.86	−0.82

R Pearson's correlation coefficient between temperature and CV

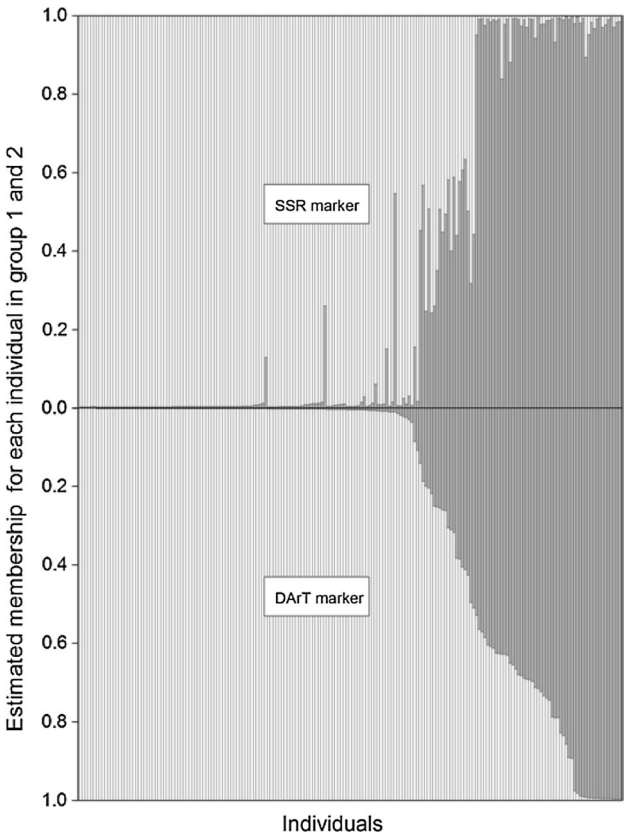

Fig. 3 Estimated population structure for 194 individuals of a diversity set using 31 SSR markers and 171 DArT markers. Both marker systems distinguish between group 1 (*grey area*) and group 2 (*dark grey area*)

between these traits and plant growth and that the co-localization of marker-trait associations are strong indicators for significant influences on cold tolerance. Across environment means of SPAD values were significantly correlated ($p < 0.05$) with DMGR and LGR. QTL for mean SPAD and DMGR co-localized on SBI-01 and SBI-07. In both cases the reduced mean SPAD value of one marker allele was associated with a smaller mean DMGR, i.e. higher chlorophyll contents may have improved photosynthesis and growth. However, there was no strong evidence that the preservation of high chlorophyll contents under unfavorable conditions promoted growth at low temperatures since both $SPAD_{(sl)}$ and $SPAD_{(Tb)}$ were positively correlated to mean DMGR, i.e., a strong increase in SPAD with increasing temperatures was correlated with high $DMGR_{(mean)}$. As mentioned before, the marker-trait association for DMGR on SBI-07 was found to be significant ($p < 0.05$) in seven environments, while a marker-trait association for SPAD was detected at the same locus only in the two environments with highest temperatures. Fracheboud et al. (2004) identified overlapping positions of SPAD and carbon exchange rate QTL but no co-localizations of QTL for SPAD and shoot dry-matter in maize.

T_b and *sl* of F'_v/F'_m were negatively correlated with $DMGR_0$, i.e., higher energy harvesting efficiencies at low temperatures may promote growth in low temperature environments. However, at sPb-1631 on SBI-02, the only locus at which QTL for T_b and *sl* of F'_v/F'_m and $DMGR_0$ were co-located, the same allele was associated with increasing $DMGR_0$ and T_b as well as *sl* of F'_v/F'_m. $SPAD_{(Tb)}$ was significantly correlated ($p < 0.05$) with $F'_v/F'_{m\ (Tb)}$. Accordingly, high chlorophyll contents at low temperatures may improve the efficiency of PSII. The marker alleles on SBI-10, which were associated with a decreasing $SPAD_{(Tb)}$ were also associated with decreasing $F'_v/F'_{m\ (Tb)}$ or $\Phi_{PSII(Tb)}$, respectively. However, one of the alleles was also associated with an increasing $DMGR_0$ (Table S3). Trachsel et al. (2010) found a QTL allele with positive effects on $\Phi_{PSII(Tb)}$ close to a QTL allele with negative effects on shoot dry

Table 5 Number of significant marker-trait associations ($p < 0.05$) for dry weight growth rate (DMGR), leaf growth rate (LGR), leaf appearance rate (LAR), chlorophyll content (SPAD), and chlorophyll fluorescence (F'_v/F'_m and Φ_{PSII}) in each environment and number of marker-trait associations for means across environments, exponents (*a*), initial growth rates ($DMGR_0$ and LGR_0), temperature effects (*sl*), and base temperatures (T_b)

Temperature (°C)	Number of marker-trait associations					
	DMGR	LGR	LAR	SPAD	F'_v/F'_m	Φ_{PSII}
20.8	5	8	8	14	15	3
18.6	10	10	10	12	10	13
17.6	9	7	7	13	15	12
16.8	11	11	8	17	12	11
14.7	15	14	8	11	14	15
13.5	22	15	11	13	8	12
10.5	18		6			
9.4	16					
Mean	11	9	9	14	12	12
a or *sl*	11	16	6	13	15	4
$T_b/DMGR_0/LGR_0$	18	15	9	12	10	9

weight in maize. Pleiotropic effects of a single gene seem less likely than the occurrence of multiple genes that affect growth, SPAD and fluorescence, and are associated to the same marker loci on SBI-10.

Comparison of results to earlier QTL studies

Rami et al. (1998) and Ritter et al. (2008) found QTL for plant height on SBI-01. Mace and Jordan (2010) integrated the flanking markers of QTL from different studies into a consensus map. txp-37, a flanking marker of the mentioned QTL was mapped within the genomic region spanning from 71 to 107 cm, where nine marker-trait associations for DMGR were identified. Shiringani et al. (2010) detected a QTL for plant height on SBI-08 in the region where sPb-0325 was mapped. sPb-0325 showed significant marker-trait associations ($p < 0.05$) with DMGR in four environments of the present study. Since plant height is closely correlated to biomass, the same genetic mechanisms may regulate growth during early and later development stages.

Stay green is closely related to traits like SPAD and fluorescence, which are relevant for photosynthesis (Thomas and Howarth 2000). Marker-trait associations for SPAD and fluorescence of the present study were detected in genomic regions where stay green QTL were found in earlier studies. We detected marker-trait associations for SPAD in five environments, for $SPAD_{(mean)}$ and $SPAD_{(sl)}$ on SBI-04 between 71 and 85 cm. A stay green QTL was found by Kebede et al. (2001) in the same region. The authors found another QTL for stay green on SBI-05. The flanking markers were mapped close to sPb-6855 (Mace and Jordan 2010), a significant locus for F'_v/F'_m and Φ_{PSII}. A QTL for stay green found by Subudhi et al. (2000) was mapped in the region of sPb-6518 on SBI-07 (Mace and Jordan 2010), which was associated with Φ_{PSII} in two and with LGR in four temperature regimes. The same genetic mechanisms may have effects on leaf growth at early development stages and on a delayed senescence. Between 43 and 46 cm on SBI-10, four QTL for Φ_{PSII}, five for SPAD and six for F'_v/F'_m were identified. Tao et al. (2000) found a QTL for stay green. The QTL for Φ_{PSII}, SPAD, and F'_v/F'_m co-localized with marker-trait associations for DMGR and LGR in the present study. However, as mentioned before, situations are less clear at this locus. The alleles associated with increased photon harvesting efficiencies were also associated to decreased growth rates (Table S3).

Recently, physical positions of several DArT markers became available (Bouchet et al. 2012). sPb-2583, which was significantly associated to SPAD as well as to $LAR_{(Tb)}$ ($p < 0.05$), final emergence percentage and base temperature of median emergence time (Fiedler et al. 2012) on SBI-01, mapped in the same region as *Sb01g007395* (NCBI nr. XP_002466413). The gene is an interesting candidate since it encodes a cytochrome P450 enzyme, which is involved in the biosynthesis of plant hormones, lipids and secondary metabolites (Werck-Reichert et al. 2000). According to Bekele et al. (2014), the QTL hotspots on SBI-01 contain several other genes involved in abiotic stress stimuli.

Sugars play an important role in the cold acclimation of plants (Stitt and Hurry 2002). sPb-3311 was mapped in a region where *Sb01g033060* (NCBI nr. EER92159), annotated as "similar to sucrose synthase 2" is located. The DArT marker was significantly associated to DMGR and LGR ($p < 0.05$) at low temperatures. A QTL for sucrose content was mapped in the same region by Ritter et al. 2008). sPb-0319 was also associated with DMGR, LGR, and SPAD at low temperatures. However, the allele, which was associated with higher SPAD values in our study, was associated with smaller growth rates (Table S3). In the region on SBI-03, where sPb-0319 was mapped (Mace and Jordan 2010), QTL for glucose content were detected by Shiringani et al. (2010).

Regions, which carry marker-trait associations on SBI-03, contain several genes encoding cold-acclimation proteins and a gene belonging to the fatty acid hydroxylase

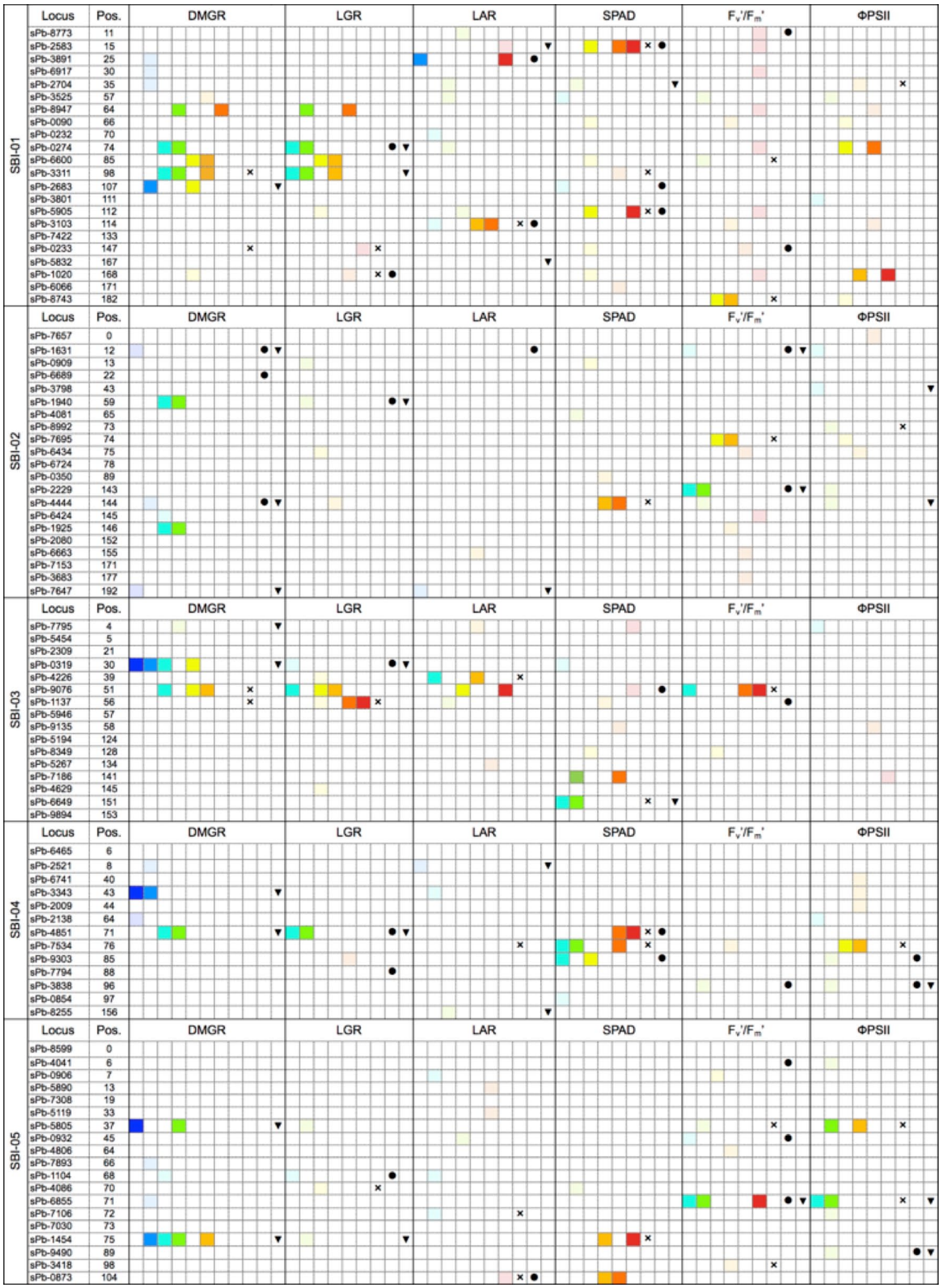

Fig. 4 Significant marker-trait associations ($p < 0.05$) for dry matter growth rate (DMGR), leaf growth rate (LGR), leaf appearance rate (LAR), chlorophyll content (SPAD), and fluorescence (F_v'/F_m' and Φ_{PSII}) in eight different temperature regimes (9.4 | 10.5 | 13.5 | 14.7 | 16.8 | 17.6 | 18.6 | 20.8 °C) and for means across environments (*cross symbol*), exponents or temperature effects (*filled circle*) and initial growth rates or base temperatures (*filled inverted triangle*). *Pale colors* were used for marker-trait associations significant in only one environment (color figure online)

Fig. 4 continued

superfamily, which may be important since the ratio of unsaturated to saturated fatty acids in plasma membranes affects their fluidity, and a high proportion of saturated acids would have negative effects on membrane function at low temperatures (Steponkus 1984). However, high-resolution SNP maps allowing regional association studies are needed for a more efficient candidate gene selection.

Conclusions

Several loci with effects on sorghum growth at low temperatures were identified. Most marker-trait associations for $DMGR_0$ did co-locate with those for DMGR at low temperatures, so that association studies carried out on a regression parameter like $DMGR_0$, LGR_0, and T_b for LAR,

SPAD and fluorescence might be advantageous only if the response to an environmental factor is more important than the development in an extreme environment itself. An important application for marker-trait association based modeling approaches may arise if the behavior of progenies in response to environmental factors can be predicted by parameter estimates of their parental lines. While DMGR and LGR were highly correlated and marker-trait associations for the traits often co-localized, marker-trait associations for chlorophyll content and fluorescence co-localized only occasionally with those for plant growth during juvenile development and gave no hint for a major direct contribution to dry matter and leaf area accumulation. Since earlier studies on maize described the influence of these traits on carbon exchange rates, it has to be verified if high leaf greenness and the efficiency of PSII positively influence seedling survival in the field.

Acknowledgments We thank Katharina Meyer for excellent technical assistance and gratefully acknowledge the German Federal Ministry of Education and Research (BMBF) for funding the project (BioEnergie 2021, Project No. 03154211).

Conflict of interest The authors declare that they have no conflict of interests.

Ethical standards The experiments comply with the current German laws.

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