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The genetic basis of heat tolerance in temperate maize (*Zea mays L.*)

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1. General Introduction

Heat tolerance in maize

Predictions on the progress of climate change suggest that the global temperature and variance of the worlds' climate are expected to increase in the future (IPCC, 2013). For temperate Europe, Della-Marta et al. (2007), Semenov (2007) and Semenov & Halford (2009) expected an increase of the frequency and duration of heat waves. Plants are not able to escape from unfavourable climate conditions as animals can do. They can, thus, be damaged, when exposed to heat stress situations.

The morphological alterations plants show in presence of heat stress range from leaf scorching, which causes a reduction of photosynthetic tissue, over growth inhibition to a reduction of yield with respect to crop species (Wahid et al., 2007). Lobell & Field (2007) observed a negative correlation between the yield of maize (*Zea mays L.*) and increasing global mean temperature, despite maize is a rather heat tolerant crop (Sage et al., 2011). The yield of US maize germplasm was heavily reduced at temperatures above 30°C (Schlenker & Roberts, 2009) and yield decrease caused by heat stress was observed as well in maize genotypes of temperate regions (Giaveno & Ferrero, 2003). The intensity of damage caused by heat stress is strongly dependent on the stage of plant development in which it occurs. During flowering and the maturity stage maize plants are critically susceptible to heat stress (Barnabás et al., 2008). High temperatures during and before flowering induce problems in the synchronisation of male and female flowering and impair the pollination capacity of maize pollen (Barnabás et al., 2008). Furthermore, high temperatures during early corn filling lead to yield reductions (Wilhelm et al., 1999).

Due to early sowing, until recently, maize was not affected by heat stress during the seedling stage in temperate Europe. Biogas production gained increasing importance in temperate Europe, for which maize is the most important supply crop (Deutsches Maiskomitee, 2013). For this use, maize plants are harvested prematurely. As full maturation is not required, the sowing can be postponed until the harvest of the winter cereals in early summer to fill the cropping gap until the sowing of the following winter cereal generation. In this case, maize seedlings are potentially objected to high temperatures (Reimer et al., 2013). Therewith, molecular processes, plant development and consequently yield can be impaired (Collins et al., 2008).

The damages caused by heat stress under field conditions are frequently caused by a combination of heat and drought stress. Thus, it would be reasonable to study the tolerance of maize to both abiotic stresses. Furthermore, natural variation and the molecular mechanisms of drought tolerance were widely studied (Bruce et al., 2002; Pennisi, 2008). However, drought tolerance is genetically the most complex abiotic stress tolerance (Collins et al., 2008) and it is still not fully understood. In contrast, the genetic mechanisms of heat tolerance are expected to be easier to dissect and the use of molecular markers is more promising (Collins et al., 2008). Furthermore, the study of the reactions of maize to a combination of heat and drought impedes the dissection of the molecular mechanisms associated with single stresses and is, thus, not the aim of this present study.
Apart from the genetic mechanisms, heat tolerance in maize was investigated in terms of yield potential at field conditions by Chen et al. (2012), Cairns et al. (2013) and Rattalino Edreira & Otegui (2013). However, none of the mentioned papers used natural variation to genetically dissect the trait heat tolerance. Further, the previously mentioned studies were focussed on North American, tropical and subtropical germplasm, or hybrids of temperate and tropical lines, respectively. I am not aware of studies, where heat tolerance was assessed in vivo with temperate European material and as different heterotic pools contain different alleles the transfer of results collected from one type of germplasm to the other is limited.

The molecular response of plants to heat stress

Avoidance and tolerance are two strategies of plants to mitigate heat stress (Hasanuzzaman et al., 2013). Avoidance includes changing of leaf orientation and leaf rolling (Sarieva et al., 2010), stomatal closure, transpirational cooling and early maturity (Wahid et al., 2007). The molecular tolerance mechanisms in reaction upon heat stress include the increase of stress signalling, the control of transcription, the expression of heat shock genes (HSG) and of genes which are involved in the response to oxidative (Almeselmani et al., 2006) and osmotic stress (Hasanuzzaman et al., 2013). The elimination of harmful reactive oxygen species is achieved by increased expression genes coding for antioxidants (Xu et al., 2006; Sairam et al., 2000), which is correlated with heat tolerance in different crops (Almeselmani et al., 2006; Chakraborty & Pradhan, 2011). Furthermore, plants increase the expression of pathways accumulating compatible osmolytes e.g. sugars (Sakamoto & Murata, 2002; Hare et al., 1998) to maintain osmotic pressure. Heat shock genes (HSGs) are transcribed and translated to heat shock proteins. They are increasingly expressed under heat stress due to binding of heat shock transcription factors to conserved heat shock elements included in promotor regions of the latter genes (Nover et al., 2001). The expression of HSGs is an important module of plant response to cope with heat stress and the corresponding proteins serve as chaperones, conserving stability and function of target proteins (Baniwal et al., 2004) and membranes (Török et al., 2001).

Beat the heat – How to adapt maize cultivation to increased temperatures

The increase of temperatures due to climate change might lead to a strong decrease of crop production in the future (Bita & Gerats, 2013). Several strategies to face this challange are conceivable. Farmers could plant heat tolerant maize cultivars from Southern Europe. In biomass production systems, farmers could further use other, more heat tolerant crop species, e.g. sorghum. However, in comparison to exotic maize germplasm and to sorghum, local maize cultivars experienced adaptation to temperate European conditions over a long period of time since the introduction of maize germplasm from the Americas. They possess first, an appropriate short time to maturity, second, resistances to patogens present in temperate Europe. And third, Flint maize types, which were the predominant germplasm in temperate Europe before the introduction of hybrid breeding, contributed an increased chilling tolerance (Hallauer, 1990). To ensure economically reasonable maize cultivation under unfavourable conditions, the development of new cultivars, thus, becomes necessary. This could be achieved by introgressing exotic germplasm into local cultivars, as described by Giaveno & Ferrero (2003). This introgression, however, has the potential to include deleterious alleles into existing breeding pools. The most promising approach to tackle reduced maize yields with the progress of climate change is the assessment of heat tolerance variation in local germplasm and to enhance heat tolerance of existing cultivars by increasing the frequency of present positive alleles. Up to my knowledge, information on the phenotypic and genotypic variation for heat tolerance in temperate European maize does not exist in the literature.

Tools to understand the inheritance of heat tolerance and the molecular responses to heat stress in maize

Development of molecular markers for heat tolerance Marker-assisted selection (MAS) is increasingly utilized in plant breeding programs to support phenotypic selection. This is reasonable for traits, where phenotyping requires increasing effort of time and money (Schön et al., 2004). Heat waves are of increasing importance, however, in Central Europe they still do not appear on an annual basis. Increased effort is, thus, associated with the assessment of heat tolerance. Phenotyping under controlled conditions, as an alternative for field experiments, is possible, however, it is not practical for high-throughput needs of modern plant breeding (Wahid et al., 2007). An increase of the heat tolerance of maize by MAS, in contrast, is affordable and economically reasonable, if molecular markers are available, which are explain a high percentage of the variance for heat tolerance in the respective germplasm. The development of molecular markers for MAS is embedded in linkage mapping, which is a

promising tool to understand the molecular basis of heat tolerance taking into account the association of whole genome regions with the quantitative trait of heat tolerance. Linkage mapping makes use of recombination events in segregating biparental populations and serves to identify molecular markers, which are linked to the trait of interest.

Expression profiling Molecular pathways associated with heat tolerance can be studied by expression profiling experiments on a single gene basis across the whole genome. Expression profiling was widely used to understand molecular pathways of plants in response to internal or external stimulus. Sekhon et al. (2011) revealed the transcriptional patterns of maize across developmental stages and organs, Fu et al. (2010) and Fu et al. (2012) dissected grain yield pathways and studied the prediction of hybrid performance by means of transcriptome profiling of maize lines. The transcriptomic response of rice plants to high temperature was studied by Zhang et al. (2013) and Jung & An (2013). In the previously mentioned publications, transcriptomic variation was revealed by microarray experiments. RNA-Seq, a recently developed and already widely used technology to analyse transcriptomic variation, has higher sensitivity and lower technical variation compared to microarray experiments (Wang et al., 2009; Oshlack et al., 2010; Marguerat & Bähler, 2010). It allows to survey the whole transcriptome and to assess expression differences between multiple genotypes across diverse environmental conditions. Kakumanu et al. (2012) revealed the effects of drought stress, which is often linked to heat stress, on certain maize tissues of the maize variety B73 by RNA-Seq. Despite the high number of studies examining the molecular response of plants upon heat stress, most of the studies focused on the heat response of one or few genotypes and, thus, results are based on a narrow genetic background. Furthermore, to the best of my knowledge, all previous studies compared one standard condition with one heat level, but information about the behaviour of genotypes across a gradient of heat levels is missing. Thus, there is a lack of studies on, first, the response of plants in general to a quantitatively increasing temperature, second, the response of maize upon heat stress and, third, the natural variation for heat tolerance in temperate maize.

Objectives

The goals of my thesis research were to contribute to unravel the inheritance of heat tolerance in temperate maize and to lay the foundation for a genetic improvement of heat tolerance in temperate maize by marker assisted selection. In order to achieve these goals, I combined the detection of single genes associated with the reaction upon heat stress with the detection of genome regions associated with heat tolerance by linkage mapping.

In particular, the objectives were to

1. assess phenotypic variation for heat tolerance at seedling stage of temperate European Flint and Dent maize inbred lines and of a set of six connected segregating Dent and Flint populations;
2. assess phenotypic variation for heat tolerance with respect to agronomic traits of a set of six connected segregating Dent and Flint populations;
3. propose a measure for heat tolerance which integrates observations from multiple levels of heat stress on a multi-trait level;
4. investigate the transcriptomic response of temperate maize to linearly increasing heat levels;
5. identify QTL and candidate genes for heat tolerance during seedling stage;
6. identify QTL and candidate genes for heat tolerance during adult stage.

Bibliography

Almeselmani M., Deshmukh P.S., Sairam R.K., Kushwaha S.R. & Singh T.P. (2006) Protective role of antioxidant enzymes under high temperature stress. *Plant Science* 171, 382–8.

Baniwal S.K., Bharti K., Chan K.Y., Fauth M., Ganguli A., Kotak S., Mishra S.K., Nover L. et al. (2004) Heat stress response in plants: a complex game with chaperones and more than twenty heat stress transcription factors. *Journal of Biosciences* 29, 471–87.

Barnabás B., Jäger K. & Fehér A. (2008) The effect of drought and heat stress on reproductive processes in cereals. *Plant, Cell and Environment* 31, 11–38.

Bita C.E. & Gerats T. (2013) Plant tolerance to high temperature in a changing environment: scientific fundamentals and production of heat stress-tolerant crops. *Frontiers in Plant Science* 4, 273.

Bruce W.B., Edmeades G.O. & Barker T.C. (2002) Molecular and physiological approaches to maize improvement for drought tolerance. *Journal of Experimental Botany* 53, 13–25.

Cairns J.E., Crossa J., Zaidi P.H., Grudloyma P., Sanchez C., Araus J.L., Thaitad S., Makumbi D. et al. (2013) Identification of Drought, Heat, and Combined Drought and Heat Tolerant Donors in Maize. *Crop Science* 53, 1335.

Chakraborty U. & Pradhan D. (2011) High temperature-induced oxidative stress in Lens culinaris, role of antioxidants and amelioration of stress by chemical pre-treatments. *Journal of Plant Interactions* 6, 43–52.

Chen J., Xu W., Velten J., Xin Z. & Stout J. (2012) Characterization of maize inbred lines for drought and heat tolerance. *Journal of Soil and Water Conservation* 67, 354–364.

Collins N.C., Tardieu F. & Tuberosa R. (2008) Quantitative trait loci and crop performance under abiotic stress: where do we stand? *Plant Physiology* 147, 469–86.

Della-Marta P.M., Haylock M.R., Luterbacher J. & Wanner H. (2007) Doubled length of western European summer heat waves since 1880. *Journal of Geophysical Research* 112, D15103.

Deutsches Maiskomitee (2013) http://www.maiskomitee.de/web/public/ Fakten.aspx/Statistik/Deutschland/Statistik_Biogas. Accessed 19. November 2013.

Fu J., Falke K.C., Thiemann A., Schrag T.A., Melchinger A.E., Scholten S. & Frisch M. (2012) Partial least squares regression, support vector machine regression, and transcriptome-based distances for prediction of maize hybrid performance with gene expression data. *Theoretical and Applied Genetics* 124, 825–33.

Fu J., Thiemann A., Schrag T.A., Melchinger A.E., Scholten S. & Frisch M. (2010) Dissecting grain yield pathways and their interactions with grain dry matter content by a two-step correlation approach with maize seedling transcriptome. *BMC Plant Biology* 10, 63.

Giaveno C. & Ferrero J. (2003) Introduction of tropical maize genotypes to increase silage production in the central area of Santa Fe, Argentina. *Crop Breeding and Applied Biotechnology* 3, 89–94.

Hallauer A.R. (1990) Methods used in developing maize inbreds. *Maydica* 35, 1–16.

Hare P.D., Cress W.A. & Van Staden J. (1998) Dissecting the roles of osmolyte accumulation during stress. *Plant, Cell and Environment* 21, 535–553.

Hasanuzzaman M., Nahar K., Alam M.M., Roychowdhury R. & Fujita M. (2013) Physiological, biochemical, and molecular mechanisms of heat stress tolerance in plants. *International Journal of Molecular Sciences* 14, 9643–84.

IPCC (2013) Summary for policymakers. In *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* (eds. T.F. Stocker, D. Qin, G.K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex & P. Midgley). Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Jung K.H. & An G. (2013) Application of MapMan and RiceNet drives systematic analyses of the early heat stress transcriptome in rice seedlings. *Journal of Plant Biology* 55, 436–449.

Kakumanu A., Ambavaram M.M.R., Klumas C., Krishnan A., Batlang U., Myers E., Grene R. & Pereira A. (2012) Effects of Drought on Gene Expression in Maize Reproductive and Leaf Meristem Tissue Revealed by RNA-Seq. *Plant Physiology* 160, 846–67.

Lobell D.B. & Field C.B. (2007) Global scale climate-crop yield relationships and the impacts of recent warming. *Environmental Research Letters* 2, 014002.

Marguerat S. & Bähler J. (2010) RNA-seq: from technology to biology. *Cellular and Molecular Life Sciences* 67, 569–79.

Nover L., Bharti K., Döring P., Mishra S.K., Ganguli A. & Scharf K.D. (2001) Arabidopsis and the heat stress transcription factor world: how many heat stress transcription factors do we need? *Cell Stress & Chaperones* 6, 177–89.

Oshlack A., Robinson M.D. & Young M.D. (2010) From RNA-seq reads to differential expression results. *Genome Biology* 11, 220.

Pennisi E. (2008) The Blue Revolution, Drop By Drop, Gene by Gene. *Science* 320, 171–173.

Rattalino Edreira J. & Otegui M. (2013) Heat stress in temperate and tropical maize hybrids: A novel approach for assessing sources of kernel loss in field conditions. *Field Crops Research* 142, 58–67.

Reimer R., Stich B., Melchinger A.E., Schrag T.A., Sørensen A.P., Stamp P. & Hund A. (2013) Root response to temperature extremes: association mapping of temperate maize (Zea mays L). *Maydica* 58, 156–168.

Sage R.F., Kocacinar F. & Kubien D.S. (2011) C4 photosynthesis and temperature. In *C4 Photosynthesis and Related CO2 Concentrating Mechanisms* (eds. A.S. Raghavendra & R.F. Sage), chap. 10, pp. 161–195. Springer, Dordrecht, The Netherlands.

Sairam R., Srivastava G. & Saxena D. (2000) Increased antioxidant activity under elevated temperatures: a mechanism of heat stress tolerance in wheat genotypes. *Biologia Plantarum* 43, 245–251.

Sakamoto A. & Murata N. (2002) The role of glycine betaine in the protection of plants from stress: clues from transgenic plants. *Plant, Cell and Environment* 25, 163–171.

Sarieva G.E., Kenzhebaeva S.S. & Lichtenthaler H.K. (2010) Adaptation potential of photosynthesis in wheat cultivars with a capability of leaf rolling under high temperature conditions. *Russian Journal of Plant Physiology* 57, 28–36.

Schlenker W. & Roberts M. (2009) Nonlinear temperature effects indicate severe damages to US crop yields under climate change. *Proceedings of the National Academy of Sciences of the United States of America* 106, 15594–15598.

Schön C.C., Utz H.F., Groh S., Truberg B., Openshaw S. & Melchinger A.E. (2004) Quantitative trait locus mapping based on resampling in a vast maize testcross experiment and its relevance to quantitative genetics for complex traits. *Genetics* 167, 485–98.

Sekhon R.S., Lin H., Childs K.L., Hansey C.N., Buell C.R., de Leon N. & Kaeppler S.M. (2011) Genome-wide atlas of transcription during maize development. *The Plant Journal* 66, 553–63.

Semenov M.A. (2007) Development of high-resolution UKCIP02-based climate change scenarios in the UK. *Agricultural and Forest Meteorology* 144, 127–138.

Semenov M.A. & Halford N.G. (2009) Identifying target traits and molecular mechanisms for wheat breeding under a changing climate. *Journal of Experimental Botany* 60, 2791–804.

Török Z., Goloubinoff P., Horváth I., Tsvetkova N.M., Glatz A., Balogh G., Varvasovszki V., Los D.A. et al. (2001) Synechocystis HSP17 is an amphitropic protein that stabilizes heat-stressed membranes and binds denatured proteins for subsequent chaperone-mediated refolding. *Proceedings of the National Academy of Sciences of the United States of America* 98, 3098–3103.

Wahid a., Gelani S., Ashraf M. & Foolad M. (2007) Heat tolerance in plants: An overview. *Environmental and Experimental Botany* 61, 199–223.

Wang Z., Gerstein M. & Snyder M. (2009) RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics* 10, 57–63.

Wilhelm E.P., Mullen R.E., Keeling P.L. & Singletary G.W. (1999) Heat Stress during Grain Filling in Maize: Effects on Kernel Growth and Metabolism. *Crop Science* 39, 1733–1741.

Xu S., Li J., Zhang X., Wei H. & Cui L. (2006) Effects of heat acclimation pretreatment on changes of membrane lipid peroxidation, antioxidant metabolites, and ultrastructure of chloroplasts in two cool-season turfgrass species under heat stress. *Environmental and Experimental Botany* 56, 274–285.

Zhang X., Rerksiri W., Liu A., Zhou X., Xiong H., Xiang J., Chen X. & Xiong X. (2013) Transcriptome profile reveals heat response mechanism at molecular and metabolic levels in rice flag leaf. *Gene* 530, 185–92.

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BMC Genomics

RESEARCH ARTICLE Open Access

Genome-wide expression profiling and phenotypic evaluation of European maize inbreds at seedling stage in response to heat stress

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Abstract

Background: Climate change will lead in the future to an occurrence of heat waves with a higher frequency and duration than observed today, which has the potential to cause severe damage to seedlings of temperate maize genotypes. In this study, we aimed to (I) assess phenotypic variation for heat tolerance of temperate European Flint and Dent maize inbred lines, (II) investigate the transcriptomic response of temperate maize to linearly increasing heat levels and, (III) identify genes associated with heat tolerance in a set of genotypes with contrasting heat tolerance behaviour.

Results: Strong phenotypic differences with respect to heat tolerance were observed between the examined maize inbred lines on a multi-trait level. We identified 607 heat responsive genes as well as 39 heat tolerance genes.

Conclusion: Our findings indicate that individual inbred lines developed different genetic mechanisms in response to heat stress. We applied a novel statistical approach enabling the integration of multiple genotypes and stress levels in the analysis of abiotic stress expression studies.

Keywords: Climate change, Zea mays, Heat tolerance, Genetic variation, Transcriptome, Natural phenotypic diversity

Background

Silage maize (*Zea mays L.*) is of increasing importance [1] as predominantly used biogas substrate in Germany [2]. Sowing in early summer after cereals leads to an exposure of the seedlings to high temperature and potentially heat stress [3]. Temperate maize genotypes are severely damaged when temperature rises over an optimum level [4] and yields of maize are heavily reduced at temperatures above 30°C, which was shown for US maize germplasm [5]. Besides the seedling stage, heat stress during flowering and corn filling as well has severe impacts on maize cultivation [6].

Climate predictions suggest that the mean global temperature and variance of the temperature are expected to increase in the future [7]. This will cause globally in the future an occurrence of heat waves with a higher frequency and duration than observed today [8]. This in turn leads in the future to an increase of the duration and intensity of heat stress situations in cropping systems.

In response to heat stress, plants show various symptoms, including scorching (burning) of leaves as well as growth inhibition and reduction of yield [9], which also has been reported for maize in temperate regions [4]. Improving maize genotypes to be able to cope with high temperatures leads to high reduction of yield losses due to climate change [10]. In this respect, the development of heat tolerant varieties is a major challenge for plant scientists and is of crucial importance for future maize cropping in temperate regions. The latter can be facilitated by gaining knowledge of the molecular basis of heat response and tolerance in maize. Furthermore, knowledge on the heat tolerance of European

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Flint and Dent lines is rare and highly valuable for plant breeding.

Recently, the understanding of the molecular response upon heat stress in plants in general and in maize in particular has increased (see reviews of [9,11] and [12]). The primarily major adverse effects of heat stress on plants are the decreased stability of membranes [13] and proteins, the excessive production of reactive oxygen species, a loss of cellular water, and an alteration of enzymatic reactions [12]. These changes lead especially to oxidative stress, impairment of metabolite synthesis, disturbed osmotic potential, and cell organization, to leaf burning, premature senescence, reduced growth, and cell death [12]. To cope with these adverse effects, plants developed several heat tolerance mechanisms (reviewed by [14]). They include the alteration of signaling cascades and transcriptional control, increasing production of antioxidants [15-17] and osmoprotectants, as well as the expression of stress proteins [12], especially heat shock proteins. We hypothesize that increasing heat stress is followed by a strong common transcriptomic response across different maize genotypes and that, however, certain genes exist, which are differentially regulated between genotypes with different heat tolerance.

Despite the high number of studies examining the molecular response of plants upon heat stress, most of the studies focused on the heat response of one or few genotypes and, thus, results are based on a narrow genetic background. Furthermore, to the best of our knowledge, all previous studies compared one standard condition with one heat level, but information about the behaviour of genotypes across a gradient of heat conditions is missing.

The objectives of this study were to (I) assess phenotypic variation for heat tolerance of temperate European Flint and Dent maize inbred lines, (II) investigate the transcriptomic response of temperate maize to linearly increasing heat levels and, (III) identify genes associated with heat tolerance in a set of genotypes with contrasting heat tolerance behaviour.

Methods

Plant material

This study was based on four Dent (S058, S067, S070, P040) and four Flint (L043, L017, L023, L012) maize inbred lines from the University of Hohenheim, Germany. These inbreds have been selected from an experiment studying the phenotypic reaction of 74 European maize inbreds upon low and high temperature conditions during seedling stage [3]. Out of this set, we selected four heat tolerant (S058, S067, L043, L012) and four heat susceptible (L023, L017, S070, P040) (each two dent and two flint) inbreds for our study.

Phenotypic evaluation

Experimental conditions and assessed traits

Seeds were sown in soil (50% ED73, 50% Mini Tray (Einheitserde- und Humuswerke, Gebr. Patzer GmbH & Co. KG, Sinntal-Altengronau, Germany)) in single pots (9 cm edge length) with $n = 10$ replications. The experimental design was a randomized complete block design. The plants were grown at 25°C during a 16h light period and at 20°C during a 8h dark period for a total of three weeks in a walk-in growth chamber (Bronson Incubator Services B.V., Nieuwkuijk, Netherlands). Relative humidity was set to 60%. Photosynthetic active radiation, emitted by fluorescent tubes, was between 270 - 280 μmol $m^{-2}s^{-1}$ in the canopy of the plants to avoid any type of radiation stress, which could be observed with higher light intensities, especially for the Flint germplasm of our study. Watering was conducted every morning to avoid drought stress.

Leaf growth rate was calculated as follows: the length of the fourth leaf from the shoot base to the leaf tip was measured daily for a period of three days during the stage of linear growth. The slope of a linear trendline of leaf length measurements vs. time represented the leaf growth rate. Twenty days after sowing, leaf greenness (SPAD-502, Minolta Corporation, Ramsey, NJ, USA) was assessed as the average value of four readings on the leaf blade of the latest fully developed leaf. Further, the leaf temperature was assessed with an infrared thermometer (Optris LaserSight, Optris GmbH, Berlin, Germany). The plant height from the shoot base to the point where the youngest leaf detached from the older leaf's sheath and the number of leaves per plant with visible leaf ligule were recorded. A total of 21 days after sowing, shoot dry weight was determined. The above outlined experiment was repeated at two further heat levels, where the temperature was increased after six days to induce heat stress. The mild heat level was at 32°C at day and 27°C at night, the strong heat level was at 38°C at day and 33°C at night. The studied heat levels were chosen such that similar levels of heat stress can be expected in field experiments in Europe.

Data analysis

Adjusted entry means for each inbred line - trait - heat level combination were calculated as best linear unbiased estimates using the mixed model

$$Y_{ik} = \mu + I_i + R_k + e_{ik}\,, \qquad (1)$$

separately for each heat level, where Y_{ik} was the observed value for the i^{th} inbred in the k^{th} replication, μ the general mean I_i the effect of the i^{th} inbred line, R_k the effect of the k^{th} replication, and e_{ik} the residual error. The replications can be seen as a sample of total number of possible replications and, thus, R_k was considered as random factor.

The inbred lines were selected specifically for this project and, thus, I_i was considered as a fixed effect.

A principal component (PC) analysis of the adjusted means of the six traits of the eight inbred lines at three heat levels was performed to characterize the overall reaction of the inbred lines at different heat levels. Correlations between the trait means and the first PC (PC1) of the 24 inbred line - heat level combinations were calculated as described by [18]. As a measure of heat susceptibility, the heat susceptibility index (HSI) was defined as the slope of a linear trendline of the loading of an inbred line on PC1 versus the three studied heat levels. Heat susceptible inbred lines were characterized by a high HSI, where heat tolerant inbreds had a low HSI.

To estimate the genotypic variance σ_I^2 and the residual error variance σ_e^2 of the experiment, a further analysis was conducted using model (1) with the genotype effect I_i as random. For each trait, the repeatability H^2 of the results at the three heat levels was calculated using the formula

$$H^2 = \frac{\sigma_I^2}{\sigma_I^2 + \frac{\sigma_e^2}{n}}\,. \qquad (2)$$

To check the significance of the effects of the inbred lines, heat levels and the interaction of inbreds and heat levels, a combined model across all heat levels

$$Y_{ijk} = \mu + I_i + H_j + (IH)_{ij} + R_{jk} + e_{ijk} \qquad (3)$$

was fitted, where Y_{ijk} was the observed value for the i^{th} inbred in the k^{th} replication in the j^{th} heat level, H_j was the effect of the j^{th} heat level, $(IH)_{ij}$ the effect of the interaction between the i^{th} inbred line and the j^{th} heat level, R_{jk} the effect of replication k nested in heat level j, and e_{ijk} the residual error. The heat level, inbred line and the interaction effect were set as fixed effects, whereas the replication effect was set as random. All mixed model analyses were performed using the software ASReml [19].

Transcriptome sequencing

Sample preparation and RNA sequencing

At the end of the previously described growing period, leaf samples of the inbred lines were collected at the three heat levels with $n = 10$ replications at each of the three heat levels. A sample of about 0.5 cm^2 was cut from the centre of the latest fully developed leaf of each plant, immediately frozen in liquid nitrogen, and stored at −80°C. The leaf tissue of five replications was pooled to a total of two replications for each genotype - heat level combination to reduce biological variation for the following RNA sequencing. This resulted in a total of 47 samples (the sample for one replication of a genotype - heat level combination was missing). Total RNA was isolated using the RNeasy Plant Kit (Qiagen, Hilden, Germany). RNA quantity was assessed and quality control was performed using a Qubit fluorometer (Life Technologies, Darmstadt, Germany) and the 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany). DNA was removed using the TURBO DNA free Kit (Ambion, Kaufungen, Germany) and the solution was purified using the RNeasy® MinElute® Cleanup spin columns (Qiagen). rRNA was depleted prior to sequencing using the RiboMinus™Plant Kit (Invitrogen, Life Technologies, Darmstadt, Germany). Library preparation and RNA sequencing were performed at the Max Planck Genome Centre Cologne using an Illumina HiSeq2000 sequencing machine (Illumina, Inc., San Diego, CA USA). The 47 samples were combined to eight 100-bp single-end Illumina sequencing libraries with each six (one with five) individually barcoded samples. Each library was sequenced on one lane of the sequencing machine.

Data analysis

Outcoming single-end sequence reads were cleaned for reads containing primer or adaptor sequences. Sequencing reads with more than 30% of bases with a Phred quality score of ≤20 were excluded from the following analyses (cf. [20]). High quality reads were aligned to the B73 reference sequence (AGPv3 release 20) using TopHat (Version 2.0.3, [21]). We used the R package easyRNASeq (Version 1.6.2, [22]) to filter the aligned reads for protein-coding genes located on the nuclear chromosomes and counted transcript reads per gene model in the 47 samples. As there is no purpose in analysing genes, which are not expressed at a reasonable level in none of the inbred line - heat level combinations, we excluded poorly expressed genes which did not show at least two counts per million reads in at least two samples (cf. [23]). The biological coefficient of variation (BCV) was calculated according to [24] from the square root of the common dispersion using the R package EdgeR (Version 3.2.4, [23]). The easyRNASeq table of counts was subject to a PC analysis to assess transcriptomic variation in the 47 samples and identify clustering of inbred lines, heat levels, and heterotic pools using the R package DeSeq (Version 1.10.1, [25]).

To identify first, genes involved in heat response and second, heat tolerance related genes, we selected three sets of candidate genes. (i) Genes with differential regulation upon increasing heat stress, where the eight inbred lines were considered as replications of one average genotype. These genes are designated in the following as overall heat responsive genes. (ii) Genes with differential expression in every single inbred line, where the individual inbreds were considered and the number of overlapping differentially expressed genes between the inbreds was assessed. These genes are designated in the following as common heat responsive genes. (iii) Genes, where differential regulation upon increasing heat stress was a

function of the phenotypically assessed heat tolerance of each inbred line. These genes are designated in the following as heat tolerance genes.

For establishing the set of overall heat responsive genes, expression of each gene across all inbred lines at a heat level was explained by the metric value of the respective heat level using the linear regression model in EdgeR:

$$Y_{ijk} = \mu + x_j\beta + e_{ijk}\,, \tag{4}$$

where Y_{ijk} was the expression of the respective gene of inbred i at heat level j in replication k. μ was the y-intercept and β the slope of the linear regression respectively. x_j defined the j^{th} heat level, where the heat levels 25°C and 38°C were assigned the metric values 0 and 1. Correspondingly the 32°C heat level was assigned $x = 7/13$. e_{ijk} was the residual error term. β was estimated to obtain the expression change across all inbred - replication combinations across the three heat levels. The data samples were normalized with EdgeR's internal normalization procedure for library size and dispersions between biological replications were calculated genewisely.

In this study, genes with a false discovery rate (FDR) [26] of < 0.05 and $|log_2(\beta)| > 2$ were considered as significantly differently expressed genes. MAPMAN (Version 3.6.0RC1, [27]) was used to classify the overall heat responsive genes by biological function and to graphically illustrate them in a custom created overview of involved molecular processes (mapping file version ZM_B73_5b_FGS_cds_2012). For the same set of genes, information on genome position and gene description (www.uniprot.org) was accessed via the R package bioMart (Version 2.16.0, [28]). Gene ontology (GO) terms were assigned to each of the overall heat responsive genes and a GO term enrichment analysis was carried out [29] using the Zea mays ssp maize genome locus reference (maizesequence.org). To determine significantly enriched GO terms between the heat responsive genes within the RNA-Seq approach and the reference a hypergeometric test with FDR< 0.05 was applied for the upregulated and downregulated genes, separately.

In a next step, we identified heat responsive genes for each inbred line by using the model:

$$Y_{ijk} = \mu + x_j\gamma_i + e_{ijk}\,, \tag{5}$$

to calculated the expression change of each gene for one inbred line. In this model, γ_i represented the slope of the linear regression, and thus the expression change, for each inbred line i. Genes with an FDR < 0.05 and $|log_2(\gamma)| > 2$ were considered as significantly differently expressed genes for each inbred line. The overlapping differentially expressed genes among all inbred lines was examined to define the set of common heat responsive genes.

The set of heat tolerance genes was established applying the following linear model,

$$\gamma_i = \mu + h_i\delta + e_i\,, \tag{6}$$

where γ_i was the expression change for the i^{th} inbred, estimated with model (5) and h_i the HSI of the i^{th} inbred. δ was the slope of the linear regression for the respective gene across all inbreds which represented the magnitude of differential regulation between heat tolerant and heat susceptible inbred lines. The heat tolerance genes showed a significant ($P < 0.05$) association between γ_i and the HSI and a slope of $|\delta| > 2$ across inbred lines. The heat tolerance genes were included in a heatmap with $\log_2$ fold change ($\log_2\gamma_i$) of each inbred line over heat levels 0, 7/13 and 1, i.e. 25°C, 32°C and 38°C, where genes were clustered by their differential reaction across the inbreds lines. Information on biological processes of the heat tolerance genes was obtained using the R package biomaRt with dataset zmays_eg_gene and from MaizeGDB (www.maizegdb.org).

To validate RNA sequencing results, quantitative real-time PCR (qRT-PCR) was conducted using DyNAmo SYBR Green 2-Step qRT-PCR Kit (Thermo Scientific, Bremen, Germany). Primers were developed using Primer3web interface (primer3.ut.ee, Version 4.0.0, [30]) for 11 genes, randomly selected from the total set of detected genes, excluding poorly expressed genes, and for *Actin*1 (gene GRMZM2G126010) as a reference gene. RNA extraction and DNAse treatment were carried out as described previously and the RNA of ten replications was pooled. A total of 24 RNA samples (inbred line - heat level combinations) each with $2.5\mu g$ was reversely transcribed using SuperScript™First-Strand Synthesis System for RT-PCR (Invitrogen, Life Technologies). The PCR protocol was replicated three times as follows: Initially 96°C for 2 minutes was followed by 40 cycles of each 96°C for 30 seconds, 55°C for 45 seconds and 72°C for 90 seconds. The last step at 72°C lasted 5 minutes. To determine the correlation of sequencing and qRT-PCR, the relative $\log_2$ fold expression changes for the mentioned 11 genes to *Actin*1 was calculated for the data obtained by sequencing and by qRT-PCR for each inbred line - heat level combination according to [31].

Results

Repeatabilities of the assessed traits at each of the three studied heat levels were high with values between 0.59 and 0.93 (Table 1). All measured traits were monotonic increasing or decreasing with increasing heat level, except the leaf elongation rate, which showed a maximum at 32°C. Effects of inbred lines and heat levels as well as the interaction between both were significant ($P < 0.001$) for all studied traits. The first two PCs of the PC analysis of the phenotypic data (Figure 1) explained 78 and 12% of

Table 1 Repeatability, mean trait value, and correlation of traits with PC1 across eight inbred lines examined at three heat levels

	Repeatability at			Mean value at			Correlation
Trait	25°C	32°C	38°C	25°C	32°C	38°C	with PC1
Growth rate [cm/hour]	0.80	0.85	0.88	0.24	0.29	0.19	-0.67***
Dry weight [g]	0.71	0.77	0.93	2.02	1.55	0.62	-0.99***
Plant height [cm]	0.78	0.88	0.89	21.9	20.6	12.5	-0.93***
Number of leaves	0.91	0.90	0.89	3.5	4.4	4.6	0.59**
Leaf temperature [°C]	0.71	0.59	0.89	24.7	31.8	36.4	0.81***
Leaf greenness [SPAD value]	0.90	0.90	0.93	47.1	34.2	29.4	-0.68***

**Significant with $P < 0.01$, ***Significant with $P < 0.001$.

the total variance of the inbred line-by-heat level means (Additional file 1). PC1 correlated significantly ($P < 0.01$) with all measured traits with $|r|$ between 0.59 and 0.99. We observed a clustering of the samples with respect to the heat levels, whereas no clustering of inbred lines or heterotic pools was observed. The HSI ranked the inbred lines in the order from tolerant to susceptible: S058, S067, L043, L012, L023, L017, S070 and P040 (Figure 2). Dent inbreds were the two most heat tolerant and the two most heat susceptible genotypes.

RNA sequencing resulted in a total of 1,461,089,891 single end sequence reads across all 47 samples. The BCV was 0.26 in this experiment. A total of 19 and 13% of the variation of the high-quality protein-coding chromosomal transcripts was explained by the first two PCs of the PC analysis of the transcriptomic data (Figure 3). PC1 and PC2 separated six clusters, where PC1 separated the three heat levels and PC2 separated mainly the pools Flint and Dent. We identified 17,905 genes with at least two counts per million in at least two samples. A total of 567,485,727 transcript reads accounting for previously described genes were used for the following analyses.

We identified across all inbred lines 607 overall heat responsive genes, of which 460 were up- and 147 downregulated, when considering increasing heat levels. A total of 594 of these genes a biological function could be assigned by MAPMAN (Figure 4, Additional files 2 and 3). Our data indicated the involvement of 53 types of biological functions in the overall heat responsive genes. The biological function of heat response, containing 14 heat shock genes, included exclusively upregulated genes (Additional file 3). Furthermore, genes involved in the regulation of transcription, DNA replication, and posttranslational modification of proteins were, except for two genes, upregulated with increasing heat levels. The GO terms analysis resulted in 26

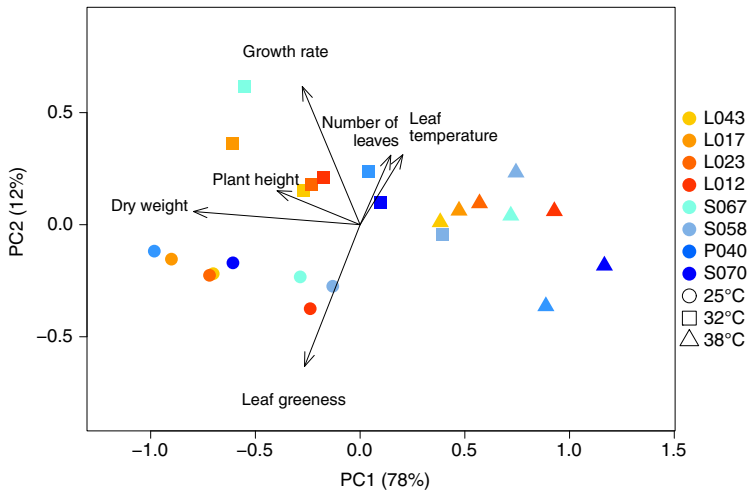

Figure 1 PCA of phenotypic data. Biplot of principal component (PC) analysis of inbred - heat level means for six traits of eight inbred lines examined at three heat levels. Numbers in parentheses represent the percentage of total variance explained by the first and second PC. Yellow and red colors represent Flint, and blue represents Dent inbred lines. The shapes (circle, square, triangle) represent the studied heat levels (25, 32 and 38°C).

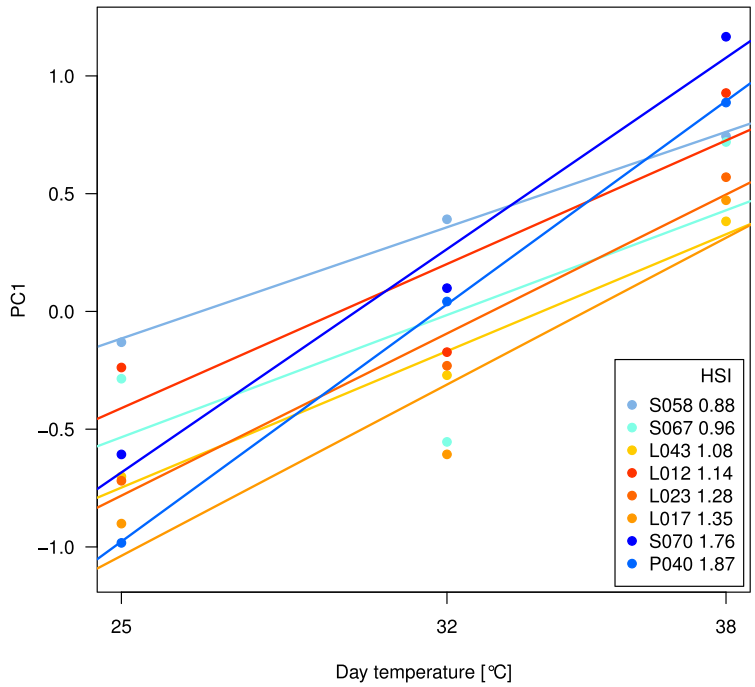

Figure 2 Linear regression of PC1. First PC of the PC analysis with six phenotypic traits of each inbred line plotted over three heat levels. The slope of the linear regression represents the heat susceptibility index (HSI).

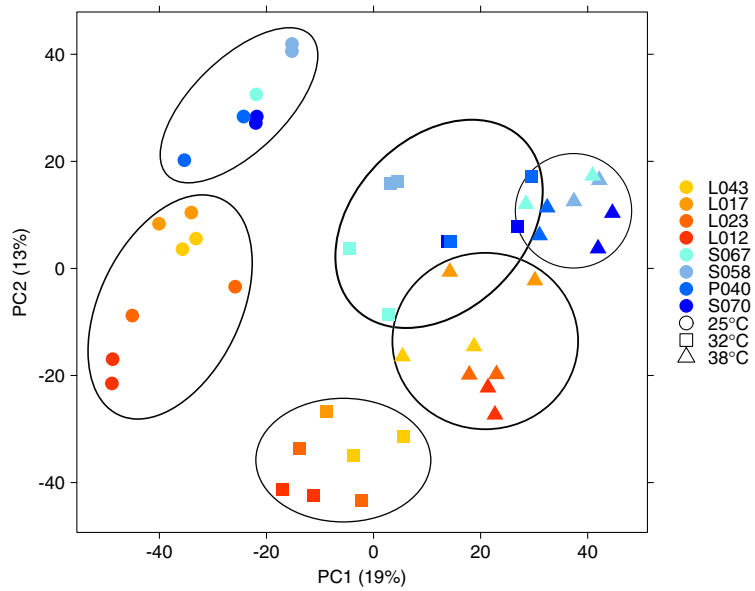

Figure 3 PCA of transcriptomic data. Principal component (PC) analysis from *DeSeq* of the gene counts of eight inbred lines examined at three heat levels and two replications. Numbers in parentheses represent the percentage of total variance explained by the first and second PC. Yellow and red represent Flint, and blue represents Dent inbred lines. The shapes (circle, square, triangle) represent the studied heat levels. Circles represent the six pool-by-heat level clusters.

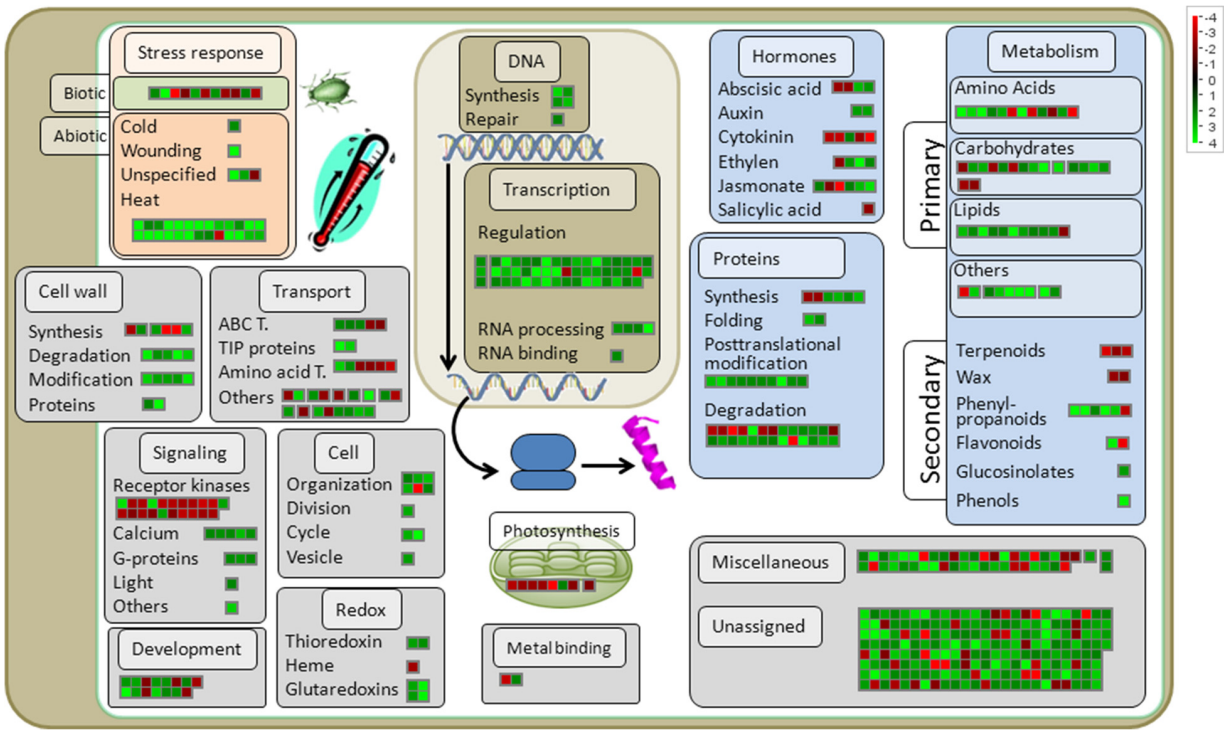

Figure 4 Heat responsive genes. Biological functions of overall heat responsive genes (FDR < 0.05 and $|log_2(\beta)| > 2$ with increasing heat levels across all inbred lines). Colours represent log_2 fold changes higher (green) and lower (red) than 0.

enriched GO terms in the upregulated overall heat responsive genes and 9 enriched GO terms in the downregulated heat responsive genes (Additional file 4) with an up to 8 fold GO enrichment (Figure 5). The overrepresented cellular component GOs were related to the apoplast and the extracellular region (Additional file 4). Within the GOs associated with biological processes, responses to external stimulus, the amino acid and protein metabolism, as well as to the carbohydrate metabolism were enriched. Concerning the GOs related to molecular function, these can be roughly grouped to catalytic activities, enzyme regulation and tetrapyrrole binding.

The number of highly differentially regulated genes for each inbred line (identified using model (5)) was between 227 and 695 (Table 2), where the number of upregulated genes was generally higher than the number of downregulated genes. The number of genes that were commonly differentially expressed in all inbred lines was 14, where 7 genes were upregulated and 7 genes were downregulated (Table 3). The 7 commonly upregulated genes included three heat shock genes and two genes previously characterized as being heat responsive. The 7 commonly downregulated genes, in contrast, did not include genes which were described as heat responsive, but included diverse classes of genes.

By explaining the gene expression change of each inbred line by its phenotypic HSI, we identified 39 heat tolerance genes (Table 4). These heat tolerance genes were divided into two major clusters of genes by their expression in heat susceptible inbreds (Figure 6). The first class included 28 genes with strong upregulation of gene expression with increasing heat levels in heat susceptible inbreds compared to heat tolerant inbreds, whereas the second class of 11 genes showed the contrary regulation pattern. Seven of the heat tolerance genes were also overall heat responsive genes. The heat tolerance genes were of a variety of biological functions. Amongst others, we found that the heat tolerance genes have a predicted biological function of protein folding and biosynthesis, cell wall modification, and calcium signalling.

Validation of the sequencing data by qRT-PCR resulted in a highly significant correlation of $p < 0.001$, $r = 0.68$ between the relative expression changes of 11 genes for 24 inbred line - by heat level combinations, obtained by RNA sequencing and by qRT-PCR (Additional file 5).

Discussion

Phenotypic variation for heat tolerance of European Flint and Dent maize inbred lines

We observed a high repeatability for all evaluated phenotypic traits at the three examined heat levels (Table 1).

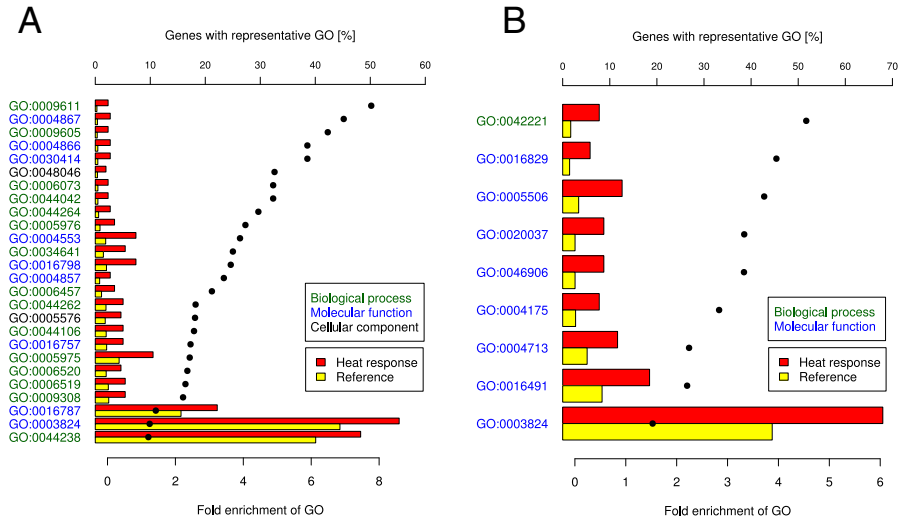

Figure 5 Enriched GO terms in the set of heat responsive genes. Significantly (FDR< 0.05) enriched GO terms for biological process, molecular function, and cellular component in the upregulated **(A)** and downregulated **(B)** heat responsive genes are plotted according to increasing enrichment (dots) of the percentage of genes in the RNA-Seq gene set (red bars) compared to that of the maize reference set (yellow bars). Mentioned GO are described in Additional file 4.

This was in accordance with results of [39], who detected similar levels of repeatability for the traits leaf greenness and plant dry weight under optimal (27/25°C) and chilling conditions (16/13°C) in a set of Flint and Dent inbred lines in growth chamber experiments. The true trait means for the genotypes at the studied heat levels could, thus, be estimated reliably in our study and therewith are a good basis for the following genome-wide expression profiling experiment.

The low and strong reduction of mean dry weight at 32 and 38°C, respectively, compared to 25°C, indicates that we were successful in setting the appropriate temperatures of the medium and the strong heat level. The trait means across inbred lines for leaf greenness and dry weight per plant showed different alteration with increasing heat levels, depending on the severity of heat stress. Compared to 25°C, at the medium heat level (32°C), the mean leaf greenness across all inbreds was reduced notably, where the mean dry weight was only slightly decreased. At the high heat level (38°C) in turn, leaf greenness did not show further notable decrease in comparison with 32°C, where dry weight was decreased substantially (Table 1). Chlorophyll content, which is correlated with leaf greenness [40], was reduced in wheat at high temperature (38°C in average) compared to control temperature (26°C in average) in a study of [41]. Generally, growth reduction in plants upon high temperature stress may be due to reduced photosynthesis, which is associated with leaf greenness, caused by an injury of the photosynthetic system [42]. Fokar 1998, [41] found a negative (although not significant, $P \geq 0.05$) association between chlorophyll retention and grain filling, as a measure for plant performance, and stated that grain filling could even be promoted by fast leaf senescence i.e. leaf greenness reduction, as metabolites might be transported from senescent tissue to the grain. This effect could be similar in our study, where at 32°C, where leaf greenness was highly reduced, metabolites could sustain plant growth and development. Another effect sustaining plant growth is the increased development speed at increased heat level, which was observed from the

Table 2 Number of heat responsive genes for each inbred line, identified with model (5), which were differently expressed (FDR < 0.05 and expression change $|log_2(\gamma)| > 2$) with increasing heat levels and the overlapping genes between the eight inbred lines (common heat responsive genes), between Flint and Dent inbreds, respectively and between heat tolerant and susceptible inbreds, respectively

Inbred line	S058	S067	L043	L012	L023	L017	S070	P040	Overlap	Flint	Dent	Tolerant*	Susceptible**
Upregulated	395	284	133	515	225	290	289	177	7	13	28	21	17
Downregulated	248	130	94	180	108	152	202	141	7	11	22	17	17

*Tolerant inbreds S058, S067, L043, L012.
**Susceptible inbreds L023, L017, S070, P040.

Table 3 List of the common heat responsive genes, which were differentially expressed (FDR < 0.05 and $|log_2(\gamma)| > 2$) with increasing heat levels in each inbred line, with mean $\log_2(\gamma)$, the mean expression change of the respective gene with increasing heat levels across all inbred lines

Gene	Mean $\log_2(\gamma)$	Gene description
GRMZM5G833699	8.29	Heat shock protein
GRMZM2G149647	8.26	Heat shock protein 26; Small heat shock protein
GRMZM2G366532	7.51	Heat response
GRMZM2G007729	5.92	Heat response
GRMZM2G158394	4.92	Extracellular ribonuclease
AC209784.3_FG007	4.10	Heat shock protein 70, MreB/Mbl protein
GRMZM2G111014	2.41	DNA.synthesis/chromatin structure
GRMZM2G057611	-2.81	Peptides transport protein
GRMZM2G147819	-3.15	Uncharacterized protein
GRMZM2G439195	-3.27	Nicotianamine synthase (metal handling)
GRMZM2G009189	-4.26	Uncharacterized protein
GRMZM2G114588	-4.34	Isoflavone reductase (secondary metabolism)
GRMZM2G125314	-4.99	LOL3 (protein.degradation)
GRMZM2G173710	-6.77	Cytokinin, signal transduction

increased number of leaves per plant at heat stress. The increased growth rate at 32°C compared to the lower as well as higher temperature regime was probably due to this increased speed of development at increased temperature, where plants were still not greatly damaged by heat stress.

To obtain a description of total plant performance across the three examined heat levels, we performed a PC analysis integrating all observed traits, i.e. leaf growth rate, shoot dry weight, plant height, the number of leaves, leaf temperature, and leaf greenness. PC1 explained with 78% a high proportion of the total variance and was correlated significantly ($\alpha < 0.01$) with each observed trait (Table 1). Therewith, in our study, PC1 was sufficient as a unique integrative trait to explain plant performance. In order to quantify heat tolerance in maize seedlings, several morphological and physiological traits were studied by [43]. In this paper, the shoot fresh and dry mass, shoot length, leaf area, growth rate, increase in leaf area, and the assimilation rate were used as single traits to quantify the reaction of maize seedlings upon strong heat stress (38°C day temperature). Our integrative plant performance trait, i.e. PC1, has the advantage that it represents each of the observed traits and gives a broad picture of plant performance under stress conditions with one single value for each genotype - heat level combination.

To quantify heat tolerance on a multi-trait level, the HSI was calculated as the slope of a linear regression of PC1 over the three examined heat levels. We observed a strong difference in heat tolerance between the eight inbred lines with HSI ranging from 0.88 up to 1.87 (Figure 2). Two of the inbred lines (S070 and P040) showed a high HSI (Figure 2) and were therewith considered as heat susceptible. In contrast, the other six inbreds showed lower HSIs and were therewith considered as more heat tolerant than the before mentioned two inbreds. This finding was associated with a significant ($\alpha < 0.001$) inbred line - heat level interaction, which was observed for all examined traits. We have, thus, a very divers set of inbreds, which indicates that our study is appropriate to investigate heat response and elucidate the molecular mechanisms of heat tolerance in diverse genetic backgrounds.

Despite observing diverse heat tolerance reactions for the eight examined inbreds, we found that neither Flint nor Dent inbreds showed systematically higher or lower heat tolerance (Figure 1). The same trend was observed for the adaptation to low temperatures, where [39] showed that both European Flints and European Dents showed chilling tolerance. Besides this finding, we observed that Dent inbreds were the most heat tolerant and the most heat susceptible inbreds, suggesting that the Dent pool shows more variability in terms of tolerance to high temperature during seedling stage.

Transcriptomic variation

The validation of the transcriptome sequencing results by qRT-PCR resulted in an r of 0.68 (Additional file 5), and therewith were in the order of magnitude of results of another RNASeq study in maize [31]. This finding indicated that our RNA sequencing results were reliable.

We observed in our study that the biological coefficient of variation (BCV) of the transcriptomic data across all observed genes and inbred - heat level combinations was

Table 4 List of the heat tolerance genes, with significant ($P < 0.05$) association between γ and the heat susceptibility index and a slope of $|\delta| > 2$ across inbred lines

Gene identifier	δ	Biological function (Possible function found for *Oryza sativa L.* or *Arabidopsis thaliana L.* orthologs)
GRMZM2G385925	3.39	Kinesin heavy chain-like protein
GRMZM2G013478	2.41	Nucleoside diphosphate kinase
GRMZM2G076544	2.72	Peptidyl-prolyl cis-trans isomerase
GRMZM2G018027	5.62	OXIDATIVE STRESS 3 (ATOXS3, *A. thaliana* best hit)
GRMZM2G179473	4.28	Inositol-tetrakisphosphate 1-kinase 3
GRMZM2G436710	7.09	Unknown
GRMZM2G074017	4.43	ATPase inhibitor
GRMZM2G430362	3.71	ATP-dependent RNA helicase SUV3
GRMZM2G537291	2.82	Uncharacterized protein
GRMZM2G324886	4.31	Unknown
GRMZM2G100403	2.80	Ribosomal protein L4/L1 family
GRMZM2G157019	2.16	Nucleosome/chromatin assembly factor A
GRMZM2G140609	3.47	40S ribosomal protein S23
GRMZM2G010743	3.59	Tim17/Tim22/Tim23/Pmp24 family
GRMZM2G148998	2.23	Unknown
GRMZM2G347808	2.02	RNA cap guanine-N2 methyltransferase
GRMZM2G060726	3.53	Transcriptional regulator
GRMZM2G173734	5.74	Protein phosphatase
GRMZM2G175019	3.39	Unknown
GRMZM2G384884	7.31	Cytochrome P450 (Phenol stress [32])
GRMZM2G460617	12.88	Unknown
GRMZM2G115658	10.01	Unknown
GRMZM2G094990	14.66	Rare lipoprotein A (RlpA)-like double-psi beta-barrel
GRMZM2G371793	7.58	Uncharacterized protein
GRMZM2G175867	2.47	Putative DEAD-box ATP-dependent RNA helicase family protein
GRMZM2G035063	10.50	Chaperonin (Heat stress [33])
GRMZM2G316030	22.34	UDP-glucoronosyl and UDP-glucosyl transferase (Salinity stress [34])
GRMZM2G004036	35.26	Short-chain dehydrogenase (Metals and oxidizing chemicals and reduction of superoxide radicals [35], *Striga hermonthica (Del.) Benth* infection [36], defoliation [37])
GRMZM2G041527	-2.84	Ribonucleases P/MRP protein subunit POP1
GRMZM2G099425	-3.44	Calcium-dependent protein kinase, isoform AK1
GRMZM2G036543	-2.81	Histidine biosynthesis protein
GRMZM2G051012	-9.69	Unknown
GRMZM2G110553	-11.06	Unknown
GRMZM2G136072	-3.29	Glyoxylate reductase
GRMZM2G172451	-2.56	Plant organelle RNA recognition domain
GRMZM2G024180	-2.19	RNI-like superfamily protein
GRMZM2G122277	-2.40	Cellulose synthase (locus rs129668732) (Salt stress [38])
GRMZM2G079281	-3.99	Unknown
GRMZM2G056407	-2.16	MYB family transcription factor

0.26 and was therewith in the range of previously reported values [44].

The number of read counts per sample in our study was between 5 and 19 million (Table 5). The mean library size was in accordance with results of [31], who observed a median library size of 7.6 million reads. In the current study, we observed a remarkable variation of library size between the three examined heat levels. Tarazona 2011, [45] advised a balanced sequencing depth between conditions for differential expression analyses to support accurate statistical analyses. As the number of read counts was at the minimum 5 million for one sample in our study, however, we do not expect an impact on the power to detect differentially expressed genes.

The genes, for which the detected transcript reads accounted, showed a strong expression variation between inbred lines (Figure 3). Consistent with the PCA of the phenotypic data (Figure 1), we observed a clustering of the heat levels in the PCA of the transcriptomic data. Furthermore, we observed a clustering of the inbreds with respect to transcriptomic variation according to their heterotic pool assignment, which was not observed for the phenotypic data. This finding was in good agreement to the findings of clustering of heterotic groups with respect to genotypic variation in previous studies [39,46,47]. The differentiation we observed based on the genome-wide expression data between the Flint and Dent pool was stronger than the differentiation between heat tolerant and heat susceptible inbreds. This finding can be

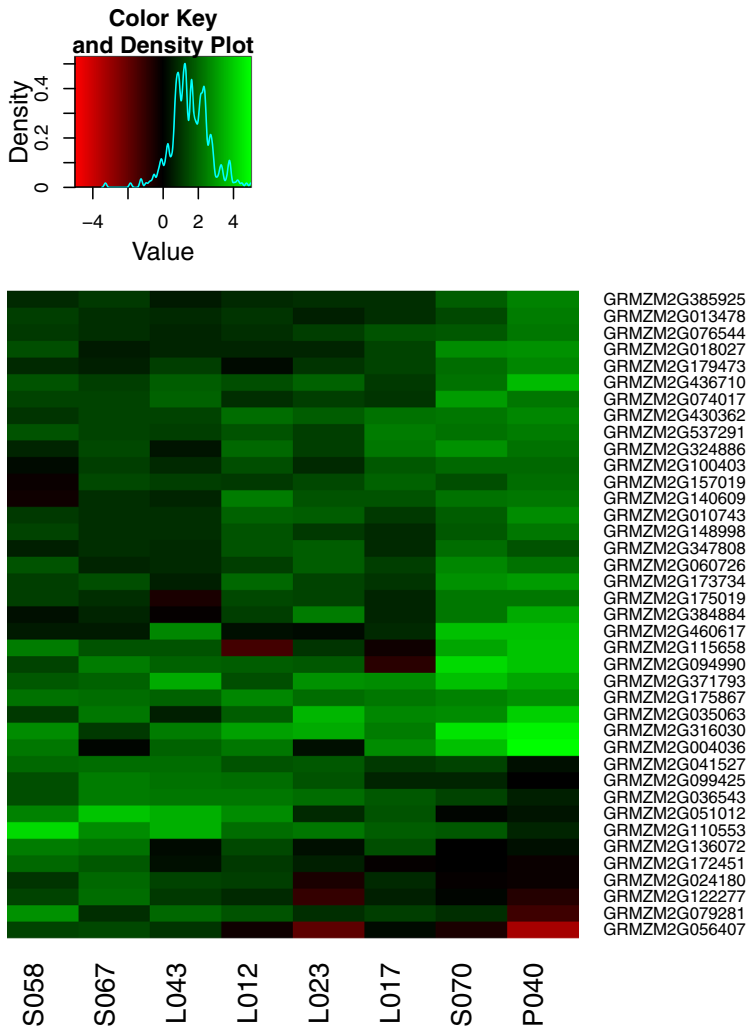

Figure 6 Heat tolerance genes. Differential regulation of the heat tolerance genes, which have been identified with significant ($P < 0.05$) linear association between γ and the heat susceptibility index with a slope of $|\delta| > 2$, in eight inbred lines. Colours represent $\log_2$ upregulation (green) and downregulation (red) with increasing heat levels.

Table 5 Mean number of high quality reads aligned to protein-coding chromosomal genes of RNA sequencing results of eight inbred lines growing at three heat levels

Inbred line	25°C	32°C	38°C
L012	12,339,774	14,851,918	13,329,198
L017	13,769,342	8,072,699	8,376,793
L023	11,568,570	11,703,940	8,505,396
L043	19,314,142	11,881,390	10,475,758
P040	15,637,596	9,184,952	7,922,566
S058	14,812,251	14,107,242	7,922,258
S067	19,073,758	12,920,434	4,874,014
S070	15,755,252	11,228,139	10,151,830

explained by the separate breeding history of Flints and Dents, based on their introduction into Europe from the Americas [48] and the pools show, thus, strong genetic and transcriptomic differences.

Approaches to identify heat responsive genes

In this study, two different approaches for identifying heat responsive genes were applied in order to cover the different aspects. Firstly, we identified overall heat responsive genes as genes with differential regulation upon increasing heat levels, where the eight inbreds were considered as replications of one average genotype. The set of overall heat responsive genes can help to understand molecular defence mechanisms against heat stress of maize. This set represents genes showing the general response of temperate maize to heat stress and may not be essential for each inbred lines' heat response, but include all strategies to cope with heat stress used by the studied maize inbreds. In our study a broad set of maize inbreds were studied, whereas in previous papers mostly two contrasting genotypes were included to study the transcriptomic response upon abiotic stress. Therefore we expect that based on our results generally applicable statements on transcriptomic heat response of European Flint and Dent inbreds are possible.

In addition to the overall heat responsive genes, we identified the common heat responsive genes by overlapping the heat responsive genes of each inbred line. These genes account for heat responsive mechanisms shared by all inbred lines. The common heat responsive genes represent a small set of genes, which are, as they are differentially expressed in each inbred line likewise, absolutely necessary, i.e. indispensable key genes for the heat response as discussed for drought stress in maize by [49].

Molecular response of temperate maize upon increasing heat levels

In the set of 607 overall heat responsive genes, three GO terms, associated with the response to external stress (GO:0009611, GO:0009605, GO:0042221), were enriched (Figure 5 and Additional file 4). This suggests a strong connection of the response to heat stress with other types of external stress response.

Furthermore, we observed an upregulation upon increasing heat levels of seven calcium-dependent signalling genes (Figure 4). As membrane fluidity is increased with increasing temperature, this results in an increased calcium-ion influx in the cells [9], serving as messenger for stress signalling [50]. Our results are in accordance with the previously reported finding that calcium-dependent signalling genes play essential roles in plant response to abiotic stress [51].

Stress signalling pathways, e.g. calcium signalling, in turn, trigger the regulation of transcriptional factors [52]. Transcription regulation genes were the most prominent group of heat responsive genes in our study with 40 upregulated genes. They have the potential to activate further stress responsive mechanisms to re-establish cell homeostasis, to protect, as well as repair proteins and membranes [52].

Coping with the damages produced by oxidative stress is viable for plant survival at heat stress. The binding of tetrapyrrole, which was found to be associated with oxidative stress and cell death in plants [53], is associated with three GO terms (GO:0005506, GO:0020037, GO:0046906), enriched in the heat responsive gene set (Figure 5). We observed, further, an upregulation of six antioxidant genes (Thioredoxins and Glutaredoxins) and six cytochrome P450 related genes in the set of overall heat responsive genes (Figure 4). These genes are known to be involved in the antioxidant defence of plants [54] and act in the detoxification of damages due to oxidative stress [55,56].

Further we found an increased expression of 11 heat responsive genes associated with the lipid metabolism. Plants try to change membrane composition as an adaptive mechanism to heat stress [9,57]. The identified lipid metabolism genes could be involved in phospholipid changes of the membrane composition to protect and recover damaged cell membranes. However this requires further research.

We observed that with 44 a high number of the 607 overall heat responsive genes were involved in the protein metabolism. Furthermore, we identified 14 heat shock genes, which act as chaperones and are involved in protein-folding [52]. This finding was supported by 5 GO terms associated with protein folding (GO:0006457) and amino acid metabolism (GO:0044106, GO:0006520, GO:0006519, GO:0009308), which were enriched in the upregulated heat responsive genes (Figure 5). This illustrates that protection of proteins against oxidative stress is another key component of the response to heat stress in maize.

Several GO terms were enriched in the upregulated heat responsive genes (Additional file 4), which are associated with carbohydrate metabolism (GO:0006073, GO:0044042, GO:0044264, GO:0005976, GO:0044262, GO:0005975) and could play a role in a modification of starch synthesis at heat stress.

The set of overall heat responsive genes illustrates that heat stress response in temperate maize involves a multitude of biological processes (Figure 4). The enrichment of GO terms in the heat responsive genes (Figure 5), which revealed the involvement of numerous biological functions and molecular processes in the heat response was in agreement with this statement.

The overlap of the heat responsive genes of each inbred between inbred lines, designated as the common heat responsive genes (Table 2), was very small, representing 1% total of genes detected as heat responsive in one of the eight inbreds. Our finding indicated that individual inbred lines developed different genetic mechanisms in response to environmental stress, which overlap only to a small degree between genotypes. Therefore it is advisable to include a variety of genotypes with different genetic backgrounds and origin in abiotic stress expression studies in order to combine different genetic strategies to cope with heat stress.

Identification of heat tolerance genes

We used a new approach to identify heat tolerance genes, which is characterized by the inclusion of phenotypic and environmental variation in the statistical analysis. The traditional approach to select stress tolerance candidate genes is to compare two groups of genotypes with contrasting stress tolerance, as outlined in several studies, discussed previously in this paper in the context of heat response. The inclusion of a diverse set of inbred lines with high variation for heat tolerance in our study, has the advantages of, first, considering the continuous distribution of the values of quantitative traits, and second, considering phenotypically intermediate genotypes without focussing only on the extremes.

Further, we included a linear regression model across three heat levels to identify differentially expressed genes over a temperature gradient, which is rarely used in other abiotic stress tolerance studies. [49] considered three levels of stress intensity (drought), but compared gene expression of pairs of stress levels instead of evaluating linear dependency of gene expression across stress conditions. This results in an increased number of statistical tests. Our approach consisted in one statistical test, including all stress levels, which leads to a reduction of the multiple-test problem. Furthermore, our approach has the advantage of considering the effect of a linear increase in temperature more independently of the actual studied heat levels. We could, thus, identify heat tolerance candidate genes for a stress intensity range between the examined heat levels in our study, i.e. from 25°C to 38°C. These two particularities can be reasons that none of the heat tolerance genes, identified in our study, was previously described in literature to be involved in heat tolerance in maize.

In this study, the p-value, which states the significance of the linear dependency between the expression change for each inbred line and the HSI of the respective inbred, was not adjusted for multiple testing to not lower even more the low power to detect heat tolerance genes. This low power comes from the consideration of phenotypic variation for heat tolerance in the gene identification method, which is comparable with an association mapping approach. In typical association mapping studies in maize, the number of genotypes in a population ranges between around 100 and 500 individuals [58]. We conclude that, in a study to identify candidate genes using transcriptome profiling, which includes phenotypic variation, it is indispensable to use a higher number of genotypes, comparable to those of association mapping studies. Nevertheless, for reasons of completeness, we discuss the identified heat tolerance genes.

The heat tolerance genes identified in this study were upregulated in most of the inbred lines and there was rarely downregulation in one of the inbreds (Figure 6). This indicated that genes which are differentially regulated between inbred lines based on phenotypic heat tolerance are typically genes, which are generally upregulated with increasing heat levels. Genes, which are downregulated with increasing heat levels are typically not differentially regulated between inbred lines. This may be partly explained due to the finding that, in general, more genes are upregulated than downregulated at heat stress, as it comes obvious from the set of overall heat responsive genes (Figure 4). Nevertheless, there must be a further, still elusive, physiological or statistical explanation for the almost absence of downregulated genes in the set of heat tolerance genes.

For 6 of the total of 39 heat tolerance genes, earlier studies indicated a mechanistic involvement in different abiotic stress responses including salt, heat and oxidative stress. This finding could be explained by interconnection between the molecular responses to different kinds of abiotic stresses, which similarly produce osmotic and oxidative stress on the cellular level [52].

To further validate, if the identified 39 heat tolerance genes explain phenotypic variation for heat tolerance, further studies have to be performed e.g. with segregating populations derived from crosses of heat tolerant and heat susceptible inbred lines presented in this study. In such experiments with a similar experimental design as the present study, the expression change with increasing heat

levels of the 39 heat tolerance candidate genes could be detected using RNA sequencing or qRT-PCR and correlated with the phenotypic heat tolerance of each genotype from the segregating populations. Another validation approach could be to overexpress or inhibited the expression of the heat tolerance candidate genes in selected inbred lines. Comparing the phenotypic heat tolerance of the modified genotype with non-transformed genotypes can evidence a possible heat tolerance function of the respective gene. In a subsequent experiment, thus transformed genotypes and previously mentioned segregating populations could be tested at heat stress conditions in field experiments to examine if the selected candidate genes have a heat tolerance function in a natural environment as well as in the adult stage. Validated genes could then be used in a molecular breeding approach, in order to obtain heat tolerant varieties.

Conclusion

In this study, we found a high variation for heat tolerance during seedling stage in a set of European maize inbred lines, which is not dependent on heterotic pools, but comes with different molecular strategies of single inbred lines to cope with increasing heat levels. We could, further, support and expand knowledge of the heat response pathways in maize and plants in general (Figure 4). Finally, we identified 39 heat tolerance candidate genes (Figure 6), whose molecular function for heat tolerance and adaptation is unknown and should be clarified using functional studies. We suggest further the performance of transcriptome profiling experiments with populations of inbred genotypes derived from biparental crosses in order to improve the power to detect significance of detected heat tolerance genes.

Availability of supporting data

The data sets supporting the results of this article are included within the article and its additional files.

Additional files

Additional file 1: Adjusted entry means for all assessed traits for eight inbred lines examined at three heat levels.

Additional file 2: MAPMAN bins of heat responsive genes. MAPMAN bins of overall heat responsive genes, shown in figure 4, which were significantly (FDR < 0.05 and $|log_2(\beta)| > 2$) differently expressed across all inbred lines with increasing heat levels, as well as the number of genes, that were allocated to each bin.

Additional file 3: Description of heat responsive genes. Overall heat responsive genes with MAPMAN bins shown in figure 4, which were significantly (FDR < 0.05 and $|log_2(\beta)| > 2$) differently expressed across all inbred lines with increasing heat levels, with their bin description, log_2 fold change ($log_2(\beta)$), log_2 counts per million, gene name, chromosome, as well as start and end position in the genome.

Additional file 4: Significantly (FDR< 0.05) enriched GO terms in the set of heat responsive genes.

Additional file 5: Correlation between RNA sequencing and qRT-PCR. Log_2 fold expression changes (FC) between 25°C and 32°C, 25°C and 38°C, as well as 32°C and 38°C of 11 genes determined by RNA sequencing and qRT-PCR.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

FPF carried out the experiments and the statistical analyses, participated in the design and coordination and drafted the manuscript. CU participated in the quality control and alignment of the sequencing data as well as in the identification of candidate genes and helped to draft the manuscript. BH and RR carried out library preparation and RNA sequencing and participated in short read quality control. BS conceived the study, participated in its design and coordination, participated in the statistical analyses and drafted the manuscript. All authors read and approved the final manuscript.

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References

1. Deutsches Maiskomitee, Anbaufläche Silomais. 2013. [http://www.maiskomitee.de/web/public/Fakten.aspx/Statistik/Deutschland/Anbaufl%C3%A4che_Silomais]
2. Deutsches Maiskomitee, Statistik Biogas. 2013. [http://www.maiskomitee.de/web/public/Fakten.aspx/Statistik/Deutschland/Statistik_Biogas]
3. Reimer R, Stich B, Melchinger AE, Schrag TA, Sørensen AP, Stamp P, Hund A. Root response to temperature extremes: association mapping of temperate maize (Zea mays L). Maydica. 2013;58:156–68.
4. Giaveno C, Ferrero J. Introduction of tropical maize genotypes to increase silage production in the central area of Santa Fe, Argentina. Crop Breeding Appl Biotechnol. 2003;3(2):89–94.
5. Schlenker W, Roberts M. Nonlinear temperature effects indicate severe damages to US crop yields under climate change. Proc Nat Acad Sci USA. 2009;106(37):15594–8.
6. Barnabás B, Jäger K, Fehér A. The effect of drought and heat stress on reproductive processes in cereals. Plant, Cell Environ. 2008;31:11–38.
7. Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change In: Stocker TF, Qin D, Plattner GK, Tignor M, Allen SK, Boschung J, Nauels A, Xia Y, Bex V, Midgley P, editors. United Kingdom and New York. NY, USA: Cambridge University Press, Cambridge; 2013.
8. Della-Marta PM, Haylock MR, Luterbacher J, Wanner H. Doubled length of western European summer heat waves since 1880. J Geophys Res. 2007;112(D15):D15103.
9. Wahid A, Gelani S, Ashraf M, Foolad M. Heat tolerance in plants: An overview. Environ Exp Bot. 2007;61(3):199–223.
10. Butler EE, Huybers P. Adaptation of US maize to temperature variations. Nat Climate Change. 2013;3:68–72.
11. Kotak S, Larkindale J, Lee U, von Koskull-Döring P, Vierling E, Scharf KD. Complexity of the heat stress response in plants. Curr Opin Plant Biol. 2007;10(3):310–6.
12. Hasanuzzaman M, Nahar K, Alam MM, Roychowdhury R, Fujita M. Physiological, biochemical, and molecular mechanisms of heat stress tolerance in plants. Int J Mol Sci. 2013;14(5):9643–84.

13. Savchenko GE, Klyuchareva EA, Abramchik LM, Serdyuchenko EV. Effect of periodic heat shock on the inner membrane system of etioplasts. Russ J Plant Physiol. 2002;49(3):349–59.
14. Sung DY, Kaplan F, Lee KJ, Guy CL. Acquired tolerance to temperature extremes. Trends Plant Sci. 2003;8(4):179–87.
15. Almeselmani M, Deshmukh PS, Sairam RK, Kushwaha SR, Singh TP. Protective role of antioxidant enzymes under high temperature stress. Plant Sci. 2006;171(3):382–8.
16. Xu S, Li J, Zhang X, Wei H, Cui L. Effects of heat acclimation pretreatment on changes of membrane lipid peroxidation, antioxidant metabolites, and ultrastructure of chloroplasts in two cool-season turfgrass species under heat stress. Environ Exp Bot. 2006;56(3):274–85.
17. Sairam R, Srivastava G, Saxena D. Increased antioxidant activity under elevated temperatures: a mechanism of heat stress tolerance in wheat genotypes. Biologia Plant. 2000;43(2):245–51.
18. Holland SM. Principal components analysis (PCA). 2008. [http://strata.uga.edu/software/pdf/pcaTutorial.pdf].
19. Gilmour A, Gogel B, Cullis B, Thompson R. ASReml User Guide Release 2.0. VSN International Ltd, Hemel Hempstead, HP1 1ES, UK. 2006.
20. Garg R, Patel RK, Tyagi AK, Jain M. De novo assembly of chickpea transcriptome using short reads for gene discovery and marker identification. DNA Res. 2011;18:53–63.
21. Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics. 2009;25(9):1105–11.
22. Delhomme N, Padioleau I, Furlong EE, Steinmetz LM. easyRNASeq: a bioconductor package for processing RNA-Seq data. Bioinformatics. 2012;28(19):2532–3.
23. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010;26:139–40.
24. Robinson MD, McCarthy DJ, Chen Y, Smyth GK. edgeR: differential expression analysis of digital gene expression data. 2014. [http://www.bioconductor.org/packages/release/bioc/vignettes/edgeR/inst/doc/edgeRUsersGuide.pdf].
25. Anders S, Huber W. Differential expression analysis for sequence count data. Genome Biol. 2010;11(10):R106.
26. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc. Ser B (methodological). 1995;57:289–300.
27. Thimm O, Bläsing O, Gibon Y, Nagel A, Meyer S, Krüger P, et al. Mapman: a User-Driven Tool To Display Genomics Data Sets Onto Diagrams of Metabolic Pathways and Other Biological Processes. Plant J. 2004;37(6):914–39.
28. Durinck S, Spellman PT, Birney E, Huber W. Mapping identifiers for the integration of genomic datasets with the R/Bioconductor package biomaRt. Nat Protoc. 2009;4(8):1184–91.
29. Du Z, Zhou X, Ling Y, Zhang Z, Su Z. agriGO: a GO analysis toolkit for the agricultural community. Nucleic Acids Res. 2010;38(Web Server issue): W64–70.
30. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3–new capabilities and interfaces. Nucleic Acids Res. 2012;40(15):e115.
31. Urbany C, Benke A, Marsian J, Huettel B, Reinhardt R, Stich B. Ups and downs of a transcriptional landscape shape iron deficiency associated chlorosis of the maize inbreds B73 and Mo17. BMC Plant Biol. 2013;13:213.
32. Xu J, Su ZH, Chen C, Han HJ, Zhu B, Fu XY, et al. Stress responses to phenol in Arabidopsis and transcriptional changes revealed by microarray analysis. Planta. 2012;235(2):399–410.
33. Jung KH, An G. Application of MapMan and RiceNet drives systematic analyses of the early heat stress transcriptome in rice seedlings. J Plant Biol. 2013;55(6):436–49.
34. Walia H, Wilson C, Zeng L, Ismail AM, Condamine P, Close TJ. Genome-wide transcriptional analysis of salinity stressed japonica and indica rice genotypes during panicle initiation stage. Plant Mol Biol. 2007;63(5):609–23.
35. Blanvillain R, Kim JH, Wu S, Lima A, Ow DW. OXIDATIVE STRESS 3 is a chromatin-associated factor involved in tolerance to heavy metals and oxidative stress. Plant J. 2009;57(4):654–65.
36. Swarbrick PJ, Huang K, Liu G, Slate J, Press MC, Scholes JD. Global patterns of gene expression in rice cultivars undergoing a susceptible or resistant interaction with the parasitic plant Striga hermonthica. The New Phytologist. 2008;179(2):515–29.
37. Chen S, Li XQ, Zhao A, Wang L, Li X, Shi Q, et al. Genes and pathways induced in early response to defoliation in rice seedlings. Curr Issues Mol Biol. 2009;11(2):81–100.
38. Hamann T, Osborne E, Youngs HL, Misson J, Nussaume L, Somerville C. Global expression analysis of CESA and CSL genes in Arabidopsis. Cellulose. 2004;11(3/4):279–86.
39. Strigens A, Freitag NM, Gilbert X, Grieder C, Riedelsheimer C, Schrag TA, et al. Association mapping for chilling tolerance in elite flint and dent maize inbred lines evaluated in growth chamber and field experiments. Plant, Cell Environ. 2013;36(10):1871–87.
40. Dwyer L, Tollenaar M, Houwing L. A nondestructive method to monitor leaf greenness in corn. Can J. Plant Sci. 1991;71:505–9.
41. Fokar M, Blum A, Nguyen HT. Heat tolerance in spring wheat. II. Grain filling. Euphytica. 1998;104:9–15.
42. Berry J, Björkman O. Photosynthetic response and adaptation to temperature in higher plants. Annu Rev Plant Physiol. 1980;98(673): 491–543.
43. Ashraf M, Hafeez M. Thermotolerance of Pearl Millet and Maize at Early Growth Stages: Growth and Nutrient Relations. Biologia Plant. 2004;48: 81–6.
44. McCarthy DJ, Chen Y, Smyth GK. Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. Nucleic Acids Res. 2012;40(10):4288–97.
45. Tarazona S, García-Alcalde F, Dopazo J, Ferrer A, Conesa A. Differential expression in RNA-seq: a matter of depth. Genome Res. 2011;21(12): 2213–23.
46. Stich B, Melchinger AE, Frisch M, Maurer HP, Heckenberger M, Reif JC. Linkage disequilibrium in European elite maize germplasm investigated with SSRs. Theor Appl Genet. 2005;111(4):723–30.
47. Van Inghelandt D, Melchinger AE, Lebreton C, Stich B. Population structure and genetic diversity in a commercial maize breeding program assessed with SSR and SNP markers. Theor Appl Genet. 2010;120(7): 1289–99.
48. Messmer MM, Melchinger AE, Boppenmaier J, Brunklaus-Jung E, Herrmann RG. Relationships among Early European Maize Inbreds: I. Genetic Diversity among Flint and Dent Lines Revealed by RFLPs. Crop Sci. 1992;32(6):1301.
49. Zheng J, Fu J, Gou M, Huai J, Yunjun L, Jian M, et al. Genome-wide transcriptome analysis of two maize inbred lines under drought stress. Plant Mol Biol. 2010;72:407–21.
50. Reddy ASN, Ali GS, Celesnik H, Day IS. Coping with stresses: roles of calcium- and calcium/calmodulin-regulated gene expression. The Plant Cell. 2011;23(6):2010–32.
51. Jiang S, Zhang D, Wang L, Pan J, Liu Y, Kong X, et al. A maize calcium-dependent protein kinase gene, ZmCPK4, positively regulated abscisic acid signaling and enhanced drought stress tolerance in transgenic Arabidopsis. Plant Physiol Biochem. 2013;71:112–20.
52. Wang W, Vinocur B, Altman A. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta. 2003;218:1–14.
53. Tanaka R, Tanaka A. Tetrapyrrole biosynthesis in higher plants. Annu Rev Plant Biol. 2007;58:321–46.
54. Meyer Y, Siala W, Bashandy T, Riondet C, Vignols F, Reichheld JP. Glutaredoxins and thioredoxins in plants. Biochim Biophys Acta. 2008;1783(4):589–600.
55. Casati P, Walbot V. Rapid transcriptome responses of maize (Zea mays) to UV-B in irradiated and shielded tissues. Genome Biol. 2004;5(3):R16.
56. Li Yf, Wang Y, Tang Y, Kakani VG, Mahalingam R. Transcriptome analysis of heat stress response in switchgrass (Panicum virgatum L.) BMC Plant Biol. 2013;13:153.
57. Maestri E, Klueva N, Perrotta C, Gulli M, Nguyen HT, Marmiroli N. Molecular genetics of heat tolerance and heat shock proteins in cereals. Plant Mol Biol. 2002;48(5-6):667–81.
58. Zhu C, Gore M, Buckler ES, Yu J. Status and Prospects of Association Mapping in Plants. The Plant Genome. 2008;1:5–20.

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ORIGINAL ARTICLE

First steps to understand heat tolerance of temperate maize at adult stage: identification of QTL across multiple environments with connected segregating populations

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Abstract
Key message **Dents were more heat tolerant than Flints. QTL for heat tolerancewith respect to grain yield at field conditions were identified considering multiplepopulations and environments.**
Abstract High temperatures have the potential to cause severe damages to maize production. This study aims to elucidate the genetic mechanisms of heat tolerance under field conditions in maize and the genome regions contributing to natural variation. In our study, heat tolerance was assessed on a multi-environment level under non-controlled field conditions for a set of connected intra- and interpool Dent and Flint populations. Our findings indicate that Dent are more heat tolerant during adult stage than Flint genotypes. We identified 11 quantitative trait loci (QTL) including 2 loci for heat tolerance with respect to grain yield. Furthermore, we identified six heat-tolerance and 112 heat-responsive candidate genes colocating with the previously mentioned QTL. To investigate their contribution to the response to heat stress and heat tolerance, differential expression and sequence variation of the identified candidate genes should be subjected to further research.

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Introduction

Maize (*Zea mays* L.) was grown on 184 million hectares in 2013 and was, thus, the second most widely cultivated crop after wheat (FAOSTAT 2014). In temperate regions of Europe, maize is of increasing importance as fodder for animal production and, lately, for biogas production (Deutsches Maiskomitee 2013).

With the progress of climate change, the global mean temperature and variance are expected to increase in the future (IPCC 2013). Lobell and Field (2007) observed a negative correlation of the yields of major crops, including maize, and an increasing global mean temperature. The effects of heat stress on plants are yield losses, growth inhibition and leaf scorching (Wahid et al. 2007), which was also reported for maize in temperate regions (Giaveno and Ferrero 2003). Especially during flowering and grain filling, heat stress has severe impacts on maize plants (Barnabás et al. 2008). Thus, breeding heat-tolerant cultivars is crucial to sustain crop production in the future (Chen et al. 2012).

Two complementary approaches are conceivable to increase heat tolerance in European maize germplasm. One possibility is to introgress exotic germplasm as described by Giaveno and Ferrero (2003). The second approach, which is described in this present study, has the potential to reduce the introgression of alleles which are associated with non-adaptedness to a temperate climate.

It consists in assessing heat-tolerance variation in local germplasm and enhancing the frequency of the present positive alleles.

The molecular and physiological basis of heat tolerance in maize was studied intensively by Crafts-Brandner and Salvucci (2002), Ashraf and Hafeez (2004) and Sinsawat et al. (2004). Further, Ottaviano et al. (1991), Frova and Sari-Gorla (1994), Reimer et al. (2013) and Frey et al. (2015) investigated this question with a focus on natural variation. All these mentioned studies examined the heat tolerance of seedlings or pollen grains grown under controlled conditions. Nevertheless, experiments on seedlings can never substitute experiments on adult plants grown under field conditions (Roy et al. 2011) and can only be an auxiliary means to study the phenotypic and genotypic response to heat stress. Chen et al. (2012), Cairns et al. (2013) and Rattalino Edreira and Otegui (2013) examined heat tolerance of maize in adult stage and measured yield potential under field conditions. However, to the best of our knowledge, no previous study has used natural variation to genetically dissect heat tolerance under field conditions.

Earlier studies used different approaches to quantify the effect of a certain level of heat stress on the occurrence of phenotypic heat stress symptoms. Chen et al. (2012) and Cairns et al. (2013) described the heat tolerance of a genotype as the performance at high temperature conditions, without considering the relation of the performance at heat conditions to a control environment. Fokar et al. (1998) estimated heat tolerance in wheat by the reduction of trait values at heat conditions compared to a control condition. A more advanced approach was pursued by Mason et al. (2010) and Paliwal et al. (2012), who calculated heat susceptibility for wheat on a one-trait basis for yield components, relating the trait value of plants grown under heat conditions with their trait value at control conditions, taking into account the stress intensity at the heat conditions across all genotypes. However, to the best of our knowledge, no previous approach has been described, which includes more than two contrasting environments in the calculation of heat susceptibility.

The objectives of this study were to (I) propose a measure for heat tolerance which integrates observations from multiple levels of heat stress and assess the heat tolerance of a set of six connected segregating Dent and Flint populations for several traits and on a multi-trait level, (II) identify QTL for heat tolerance with the previously mentioned populations and (III) identify heat-tolerance candidate genes in these QTL regions.

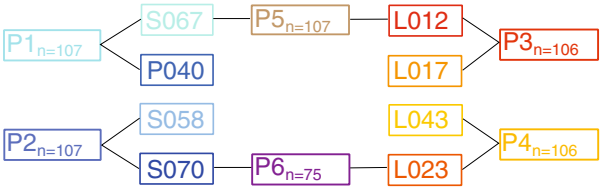

Fig. 1 Crossing scheme used to create six segregating populations (P1–6) with number of genotypes (*N*), derived from four Dent (S067, P040, S058 and S070, in *blue*) and four Flint (L012, L017, L043 and L023, in *red*) inbred lines

Material and methods

Experimental conditions

Plant material and field experiments

This study was based on segregating populations derived from pairwise crosses of four Dent (S058, S067, S070, P040) and four Flint (L043, L017, L023, L012) maize inbred lines from the University of Hohenheim (Andersen et al. 2005). The eight inbred lines have been selected from an experiment with 74 European maize inbreds in hydroponic culture by their tolerant and susceptible phenotypic reaction upon high temperatures during seedling stage (Reimer et al. 2013) and were in detail characterized for their heat tolerance during seedling stage by Frey et al. (2015). The inbreds have been crossed pairwisely to create two Dent × Dent, two Flint × Flint and two Dent × Flint F_1 genotypes (Fig. 1). The F_1 genotypes were further self-pollinated resulting in six segregating populations comprising between 75 and 107 $F_{3:5}$ genotypes and with a total of $N = 608$ genotypes.

The genotypes were grown in field trials in summer 2012 at four locations, supervised by the plant breeding companies Limagrain (Chappes, France) and the KWS Saat AG (Einbeck, Germany), comprising two locations with standard conditions in Germany, namely Greven and Einbeck, and two locations with heat conditions, namely Zsombó (Hungary) and Monselice (Italy) (Table 1). The trials at each location were replicated twice, where each replication comprised six neighbouring subexperiments, which were outlined in alpha lattices. Each segregating population was assigned to one subexperiment. The genotypes were planted in two-row plots with 65–110 seeds per plot and a plot area between 9 and 10.5 m^2. The eight parental inbred lines were included as standards, one time in each subexperiment. At the locations with heat conditions, plots were irrigated upon necessity by drip irrigation at Monselice and

Table 1 Experimental conditions at four field locations

Condition	Standard		Heat	
Location	Einbeck	Greven	Monselice	Zsombó
Breeding company	KWS	Limagrain	KWS	Limagrain
GPS coordinates	51°49′N, 9°52′E	52°6′N, 7°36′E	45°13′N, 11°45′E	46°19′N, 19°58′E
Meter above see level (m)	112	45	9	75
Seeds per plot	110	105	80	65
Plot area [m^2]	9	10	9.3	10.5
Sowing date	30 April	2 May	26 April	9 May
Growing degree days	899	1136	1588	1390
Mean flowering time	3 August	2 August	3 July	14 July
Hours above 35 °C during flowering[a]	0	0	76	34

[a] 1 week before until 1 week after mean flowering

by spray irrigation at Zsombó to avoid drought stress. Further agronomic field treatments were done similarly at all locations. Air temperature and relative air humidity were recorded at 1.50 m height in all experimental fields.

The number of days after planting when 50 % of the plants of a plot showed male (MF) and female flowering (FF), respectively, were assessed. Furthermore, data for leaf scorching (LS) of young leaves before flowering from 1 (weak damage) to 9 (strong damage) were collected. Total grain fresh yield (FY) was assessed by machine harvesting at physiological maturity, where grain moisture (GM) was measured by near infrared spectroscopy. MF, FF, GM and FY were determined by the respective plant breeding company, LS was assessed by the author of this paper. Grain dry yield per hectare (DY) at 15 % grain moisture was calculated. The anthesis silking interval (ASI) was calculated with FF − MF. Growing degree days (GDD) at each location were calculated using the model of McMaster and Wilhelm (1997):

$$\mathrm{GDD} = \frac{(T_{\max} + T_{\min})}{2} - T_{\mathrm{base}}, \quad (1)$$

where $T_{\max}$ and $T_{\min}$ were the minimum and maximum day temperature, respectively, and T_{base} the base temperature 10 °C.

Genotyping

The parental inbred lines of the populations were genotyped with a set of 56,110 single nucleotide polymorphism (SNP) markers using a 50K SNP array (Ganal et al. 2011). Out of these SNPs, a total of 161 SNP markers were selected to genotype the individuals of the six segregating populations. For each population, between 47 and 77 markers were chosen (60 for population 1, 47 for population 2, 75 for population 3, 64 for population 4, 67 for population 5 and 77 for population 6) being polymorphic between the two parents of each population and not showing heterozygosity in either parental line. SNP marker selection was optimized for equal distribution across the physical map (due to the unavailability of a genetic map at that time) and the overlapping of markers between populations. The selected SNP markers were genotyped using KASP marker technology by TraitGenetics GmbH (Gatersleben, Germany) in the respective populations.

Statistical analysis

Phenotypic data

Adjusted entry means calculation To estimate the environmental error effect present in each subexperiment, we used mixed model (2) with data of each trait collected for the standard genotypes, i.e. the parental inbreds, at each of the four locations separately:

$$Y_{bprs} = \mu + S_s + R_r + P_{pr} + B_{bpr} + e_{bprs}, \quad (2)$$

where Y_{bprs} was the phenotypic observation of the sth standard in the rth replication, the pth subexperiment, and the bth incomplete block, μ the general mean, S_s the effect of the sth standard, R_r the effect of the rth replication, P_{pr} the effect of the pth subexperiment nested in the rth replication, B_{bpr} the effect of the bth incomplete block, nested in the pth subexperiment nested in the rth replication and e_{bprs} the residual error term. The standard factor S_s was not of primary interest in this analysis and was considered as a random term, just as the block effect B_{bpr}. The replication effect R_r was set as fixed, because of the small numbers of replications per location. P_{pr} was planned to be estimated and considered as a fixed effect. The estimated subexperiment effect $\hat{P}_{pr}$ was subtracted from the phenotypic observations of all genotypes in the corresponding subexperiment.

To calculate adjusted entry means (AEM) for each trait of the genotypes at each location, the above-mentioned adjusted phenotypic observations were analysed with model (3) at each location separately,

$$Y_{bipr} = \mu + G_i + R_r + B_{bpr} + e_{bipr}, \quad (3)$$

where Y_{bipr} was the adjusted phenotypic observation for the ith genotype in the bth block of the pth subexperiment within the rth replication. G_i denoted the fixed effect of the ith genotype and e_{bipr} the residual error term.

AEM across locations with the same condition, i.e. standard and heat, were estimated using model (4),

$$Y_{bijpr} = \mu + L_j + R_{jr} + B_{bjpr} + G_i + e_{bijpr}, \quad (4)$$

where Y_{bijpr} was the adjusted phenotypic observation for the ith genotype in the bth block of the pth subexperiment within the rth replication at the jth location. L_j was the effect of the jth location within the respective condition, namely Einbeck and Greven for standard conditions, Monselice and Zsombó for heat conditions, respectively. R_{jr} was the effect of the rth replication nested in the jth location, B_{bjpr} was the effect of the bth block nested in the pth subexperiment nested in rth replication nested in the jth location. G_i was the effect of the ith genotype, which was estimated to receive AEM for the genotypes in each condition. e_{bijpr} was designated as the residual error term. G_i, L_j and R_{jr} were set as fixed and the block effect B_{bjpr} was regarded as random.

To calculate AEM of the traits for each location across genotypes and to assess the significance of the condition effect (standard vs. heat conditions), model (5) was used,

$$Y_{bcijpr} = \mu + C_c + L_{cj} + R_{cjr} + B_{bcjpr} + G_i + (G.L)_{cij} + (C.G)_{ci} + e_{bcijpr}, \quad (5)$$

where Y_{bcijpr} was the adjusted phenotypic observation of the ith genotype in the bth block of the pth subexperiment within the rth replication nested in the jth location in the cth condition. C_c was the effect of the cth condition, L_{cj} was the effect of the jth location in the cth condition, R_{cjr} was the effect of the rth replication nested in the jth location in the cth condition, B_{bcjpr} was the effect of the bth block of the pth subexperiment within the rth replication nested in the jth location in the cth condition, $(G.L)_{cij}$ was the interaction between the ith genotype and the jth location in the cth condition and $(C.G)_{ci}$ was the interaction between the ith genotype and the cth condition. e_{bcijpr} was designated as the residual error term. C_c and L_{cj} were regarded as fixed, while all other effects were regarded as random. AEM for L_{cj} were estimated. Traits with a significant C_c effect were regarded as heat-dependent traits.

Heritability Genotypic σ^2_{gj} and error σ^2_{ej} variance components for each location j were calculated using model (3) with a random genotype G_i effect. For each trait, the broad sense heritability (H^2_j) (cf. Becker 2011; Hallauer et al. 2010) of the observations of each location j was calculated considering the number of replications per location (2).

Modifying the genotype model term G_i of model (3) enabled the calculation of specific genotypic $\sigma^2_{g\,jp}$ and error $\sigma^2_{e\,jp}$ variance components for each population p and location j. Therefore, the G_i effect of model (3), was substituted with a $(G.P)_{ip}$ interaction effect of the ith genotype and the pth population (cf. Horn et al. 2013), which was set as random. The broad sense heritability for population p and location j (H^2_{jp}) was calculated based on the population-specific $\sigma^2_{g\,jp}$ and $\sigma^2_{e\,jp}$.

To calculate genotypic $\sigma^2_{g\,cp}$, genotype–location interaction $\sigma^2_{gl\,cp}$ and error variance components $\sigma^2_{e\,cp}$ for each condition c and population p, model (4) was extended by a random genotype–location interaction effect $(G.L)_{ij}$ and the G_i effect was regarded as random. Further, a random $(G.P)_{ip}$ and a random $(G.L.P)_{ijp}$ effect were added to the model, analogously as described previously. Broad sense heritability for each condition c and population p (H^2_{cp}) was calculated for each trait with the following model:

$$H^2_{cp} = \frac{\sigma^2_{g\,cp}}{\sigma^2_{g\,cp} + \frac{\sigma^2_{gl\,cp}}{U} + \frac{\sigma^2_{e\,cp}}{E*U}}, \quad (6)$$

where U was the number of locations per condition (2) and E the number of replications per locations (2). All mixed model analyses were performed using the software ASReml (Gilmour et al. 2006).

Heat tolerance A heat susceptibility index (HSI) was calculated in two steps for each heat-dependent trait (DY, FF, LS, MF and GM) times genotype combination. In the first step, the AEM of the genotypes at each location and the AEM of each location were adjusted by calculating the ratios r_{ij} for genotype i and location j with

$$r_{ij} = \frac{\mathrm{AEM_{ij}}}{\mathrm{AEM_{iEinbeck}}}, \quad (7)$$

and the ratios r_j for each location j across all genotypes with

$$r_j = \frac{L_{cj}}{L_{\mathrm{Einbeck}}}, \quad (8)$$

where AEM_{ij} was the AEM of genotype i at location j, calculated with model (3) and $\mathrm{AEM_{iEinbeck}}$ the AEM for genotype i at the location Einbeck. L_{cj} was the AEM for location j in condition c across all genotypes and L_{Einbeck} was the AEM for location Einbeck across all genotypes, calculated with model (5).

The second step consisted in a stability analysis (cf. Finlay and Wilkinson 1963). For each trait–genotype combination, a linear regression of r_{ij} over r_j was calculated:

$$r_{ij} = \mathrm{HSI_i} \times \mathrm{r_j} + \mathrm{y_i} + \mathrm{e_{ij}}, \quad (9)$$

where HSI_i and y_i were the slope and the y-intercept of the linear regression for genotype i and e_{ij} the residual error term. Heat susceptibility of genotype i for the respective trait, was defined by the HSI_i. Pairwise Pearson correlation coefficients were calculated between the HSI of all heat-dependent traits across all genotypes. A secondary HSI was calculated, where the HSI for DY (HSI_{DY}) was adjusted with the HSI for FF (HSI_{FF}) as a cofactor using a linear regression. The residuals of the regression represented the HSI for the adjusted dry yield (HSI_{DYA}).

For a multi-trait approach, the first two principal components (PC1 and PC2) of a principal component analysis (PCA) considering the previously calculated HSI of the traits DY (HSI_{DY}), LS (HSI_{LS}), GM (HSI_{GM}), MF (HSI_{MF}) and FF (HSI_{FF}) for all genotypes were used as multi-trait measures for heat susceptibility.

Genotypic data

Genetic map creation SNP markers with a significant ($P < 0.001$) deviation of that observed from the expected allele frequency were excluded from the analysis. To improve the mapping of markers, marker information of five segregating populations, which have been genotyped with the same set of molecular markers in a companion study (Horn et al. 2015), was included in the map creation. A consensus genetic linkage map was calculated chromosome-wise using the software CarthaGène (de Givry et al. 2005).

QTL analysis QTL for the assessed phenotypic data were detected using an iterative composite interval mapping approach (iQTLm) (Charcosset et al. 2001), implemented in the software MCQTL (cf. Bardol et al. 2013), making use of the above-described consensus linkage map. QTL analyses were conducted for PC1 and PC2 as well as for the HSI of the individual traits, HSI_{DY}, HSI_{LS}, HSI_{GM}, HSI_{MF}, HSI_{FF} and HSI_{DYA}.

The analyses were performed across all populations (cf. the multipopulation analyses described in Bardol et al. 2013; Blanc et al. 2006). We took into account connections between populations through shared parental inbred lines using a kinship matrix specifying the parents of the six populations. We considered the additive effects of the eight parental inbred lines. Since the included biparental $F_{3:5}$ populations showed a supposed heterozygosity of 25 %, the QTL analyses included further dominance effects between parental alleles of each biparental population. Genotypic probabilities were computed every 5 cM, taking into account information from neighbouring markers. F thresholds for each trait to detect QTL were determined by 1000 permutation tests, to correspond to a global type I risk of 5 % across populations and across the entire genome. F thresholds used to select cofactors were fixed at 90 % of the F threshold values for QTL detection, as suggested by the MCQTL software during the cofactor selection process. SNP markers associated with the respective trait were selected as cofactors by forward regression, where the minimal distance between two cofactors was 10 cM. At the end of the detection process, confidence intervals [logarithmic odds ratio drop regions (LOD)] were estimated on the basis of a 1.5 LOD unit fall.

To test if the dominance effects of each population on the respective QTL were significantly different from 0, significance ($\alpha = 0.05$) was calculated a posteriori from a normal distribution using a two-sided test (personal communication, Mangin, August 2014). The difference between the additive effects of pairs of parental alleles on the respective QTL was tested a posteriori using a multicomparison t test (Tukey) with $\alpha = 0.05$.

Candidate gene search To identify candidate genes for heat tolerance in terms of the assessed traits, we mined genes, which were identified to be associated with the response and the tolerance to heat stress in a previous study (Frey et al. 2015) Therefore, we determined the genomic position on our QTL map of the previously mentioned genes by linear regression with information of the nearest two SNP markers. Candidate genes mapping in the identified QTL confidence intervals were designated in the following as heat-tolerance and heat-responsive candidate genes.

Results

The growing degree days (GDD) from sowing until maturity were between 1400 and 1600 at locations with heat condition and between 900 and 1100 at the two locations with standard conditions (Table 1). Temperatures of the above 35 °C were observed at the locations with heat conditions during flowering (1 week before until 1 week after mean flowering) for a period of 76 and 34 h, respectively, whereas temperatures did not reach 35 °C during flowering at the locations with standard conditions.

We observed a significant ($P < 0.001$) condition effect across populations for the traits LS, DY, FF, MF and GM (Table 3), where it was not significant for ASI. Despite the general increase of LS and decrease of DY at the location with heat compared to locations with standard conditions, we observed that Dent × Dent populations (populations 1 and 2) showed a lower increase and decrease of LS and DY, respectively, compared to Flint × Flint populations (populations 3 and 4). The decrease in DY of Dent × Flint populations (populations 5 and 6) was in between the decrease of the intra-pool (Dent × Dent and Flint × Flint) populations.

Broad sense heritability of the four locations across populations (H_j^2, Table 2, upper left) was high (0.60 –0.79) to very high (>0.80) for the traits MF and FF and

Table 2 Broad sense heritability of the anthesis silking interval (ASI), leaf scorching (LS), dry grain yield (DY), time to female (FF) and male flowering (MF) and grain moisture (GM) for each location *j* across the six populations (H^2_j, upper left), for each location *j* and population *p* (H^2_{jp}, bottom), and for each condition *c* and population *p* (H^2_{cp}, upper right)

Population	Across populations						1	2	3	4	5	6	1	2	3	4	5	6
Trait	Einbeck	Greven	Monselice	Zsombó			Standard (Einbeck and Greven)						Heat (Monselice and Zsombó)					
ASI	0.64	0.56	0.89	0.59			0.36	0.12	0.61	0.33	0.51	0.17	0.36	0.33	0.31	0.33	0.29	0.25
LS	0.34	0.06	0.59	0.56			0.11	0.00	0.00	0.00	0.00	0.00	0.36	0.13	0.23	0.29	0.52	0.50
DY	0.92	0.81	0.67	0.49			0.71	0.42	0.66	0.68	0.65	0.73	0.44	0.05	0.40	0.25	0.03	0.45
FF	0.91	0.82	0.82	0.79			0.73	0.43	0.87	0.68	0.88	0.77	0.68	0.67	0.75	0.70	0.80	0.47
MF	0.88	0.78	0.80	0.82			0.70	0.46	0.81	0.63	0.67	0.63	0.64	0.55	0.77	0.60	0.72	0.63
GM	0.95	0.83	0.69	0.55			0.77	0.42	0.87	0.79	0.74	0.88	0.18	0.00	0.02	0.22	0.14	0.22
Population	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
Trait	Einbeck						Greven						Monselice					
ASI	0.61	0.55	0.74	0.76	0.68	0.16	0.57	0.15	0.77	0.58	0.76	0.52	0.93	0.91	0.90	0.96	0.93	0.87
LS	0.11	0.20	0.75	0.27	0.14	0.34	†	†	†	†	†	†	0.20	0.04	0.50	0.48	0.68	0.74
DY	0.95	0.92	0.93	0.92	0.93	0.88	0.86	0.43	0.85	0.85	0.86	0.90	0.78	0.54	0.69	0.63	0.67	0.68
FF	0.87	0.89	0.92	0.94	0.93	0.91	0.88	0.47	0.94	0.79	0.93	0.90	0.82	0.85	0.83	0.77	0.89	0.79
MF	0.84	0.86	0.95	0.95	0.80	0.83	0.89	0.38	0.93	0.62	0.82	0.82	0.79	0.80	0.83	0.76	0.85	0.80
GM	0.95	0.95	0.98	0.93	0.94	0.95	0.85	0.38	0.91	0.90	0.88	0.94	0.83	0.80	0.64	0.57	0.54	0.91

Population	1	2	3	4	5	6
Trait	Zsombó					
ASI	0.48	0.57	0.42	0.64	0.66	0.59
LS	0.62	0.33	0.39	0.33	0.71	0.77
DY	0.7 0	0.00	0.41	†	0.12	0.72
FF	0.73	0.74	0.84	0.86	0.85	0.76
MF	0.81	0.68	0.89	0.83	0.88	0.81
GM	0.36	0.00	0.88	†	0.71	0.65

† Insufficient data collected

medium (0.40–0.59) to very high for ASI. H_j^2 was high or very high for DY and GM at Einbeck, Greven and Monselice, whereas it was medium at Zsombó. H_j^2 for LS was medium at locations with heat stress and low (0.20–0.39) to very low (<0.19) at locations with standard conditions. The heritability across locations with the same condition, calculated for the individual populations (H_{cp}^2, Table 2, upper right) was lower at heat conditions compared to standard conditions for all examined traits except LS. The heritability of population 2 (H_{cp}^2) was lower compared to that of the other populations for the traits ASI, DY, FF, MF and GM at locations with standard conditions and for the traits LS, MF and GM at locations with heat conditions.

The first two PCs of the PCA (Fig. 2) explained 41 and 21 % of the total variance of all five HSI (linear regression to calculate HSI_{DY} and HSI_{LS} of the parental inbreds, cf. Fig. 3). PC1 captured heat susceptibility with respect to yield and flowering time, with main loadings for HSI_{DY} in the negative range and for HSI_{FF} and HSI_{MF} in the positive range. PC2 had high loadings for HSI_{GM} and an intermediate high loading for HSI_{LS}. In agreement with the loadings for the HSI in the PCA, we observed significant ($\alpha = 0.05$) negative correlations of HSI_{DY} with HSI_{MF} and HSI_{FF} (Fig. 4), and the correlations of HSI_{DY} with HSI_{LS} and HSI_{GM} were negligably low (<0.3) although they were significant. With respect to PC1 and PC2, only overlapping clusters of Dent × Dent types (populations 1 and 2), the Flint × Flint types (populations 3 and 4) and the Dent × Flint types (populations 5 and 6) were observed (Fig. 2).

The consensus genetic linkage map (Fig. 5) had a total length of 1 823.5 centiMorgan (cM). The average distance was 11.3 cM and the maximum distance 83.2 cM between two markers, where markers were condensed at the centromeres of the chromosomes. Of the total of 161 markers, 21 were situated on chromosome 1, 19 on chromosome 2, 18 on chromosome 3, 19 on chromosome 4, 18 on chromosome 5, 13 on chromosome 6, 15 on chromosome 7, 14 on chromosome 8, 12 on chromosome 9 and 12 on chromosome 10.

We identified a total of 11 QTL (Table 4), each explaining between 7 and 13 % of the variance (R^2) of the respective HSI or PC. With simultaneous fits across all QTL detected for each HSI or PC with several QTL, 19, 17, 19 and 18 % of the variance could be explained for HSI_{DY}, HSI_{DYA}, HSI_{MF} and PC1, respectively. The highest additive effects on QTL for HSI_{DY} and HSI_{DYA} ($Q_{HSI:DYa}$ and $Q_{HSI:DYb}$ as well as $Q_{HSI:DYAa}$ and $Q_{HSI:DYAb}$) were observed for the parental alleles of inbreds P040 and S067, which were the parental inbred lines of population 1. At the genomic position of $Q_{HSI:DYa}$ and $Q_{HSI:DYAa}$, the S067 allele had a negative additive effect, whereas at position of $Q_{HSI:DYb}$ and $Q_{HSI:DYAb}$, the P040 allele showed a negative additive effect. We observed further a highly significant dominance effect in population 1 for the previously mentioned four QTL, which was negative at $Q_{HSI:DYa}$ and $Q_{HSI:DYAa}$ and positive at $Q_{HSI:DYb}$ and $Q_{HSI:DYAb}$. A total of 6 heat-tolerance genes and 112 heat-responsive genes, identified by Frey et al. (2015), were found in the 11 QTL confidence intervals (Table 5 and Supplementary material—Table 2). Overlapping the QTL confidence intervals resulted in 5 QTL hot spots (Fig. 6), where two were located on chromosome 2 and one on chromosomes 3, 5 and 9.

Table 3 Population-wise means of the adjusted entry means of the genotypes under heat conditions relative to the performance under standard conditions

Heterot ic group	Dent × Dent		Flint × Flint		Dent × Flint		Condition effect
Population	1	2	3	4	5	6	
ASI	$116^{*\ BC}$	$135^{***\ BC}$	$67^{***\ A}$	$136^{***\ C}$	$96^{ns\ B}$	$308^{***\ D}$	ns
LS	$178^{***\ A}$	$161^{***\ A}$	$222^{***\ B}$	$240^{***\ B}$	$236^{***\ B}$	$238^{***\ B}$	***
DY	$57^{***\ D}$	$52^{***\ C}$	$45^{***\ AB}$	$43^{***\ A}$	kg $49^{***\ BC}$	$50^{***\ BC}$	***
FF	$71^{***\ B}$	$70^{***\ A}$	$71^{***\ B}$	$72^{***\ C}$	$71^{***\ B}$	$72^{***\ C}$	***
MF	$70^{***\ B}$	$69^{***\ A}$	$71^{***\ C}$	$71^{***\ C}$	$70^{***\ B}$	$70^{***\ B}$	***
GM	$44^{***\ C}$	$37^{***\ A}$	$48^{***\ D}$	$36^{***\ A}$	$52^{***\ E}$	$41^{***\ B}$	***

Asterisks illustrate the significance level of a pairwise *t* test, examining the difference between heat and standard conditions per population. Letters illustrate non-paired Tukey tests between the relative heat-standard differences of the six populations. In the last column, the significance of the condition (standard and heat) effect for each trait across all populations calculated with model (5) is given. For details see "Material and methods"

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

ns not significant

^{A}, ^{B}, ^{C}, ^{D} Relative differences between heat and standard conditions of populations with the same letters are not significantly ($\alpha = 0.05$) different from each other

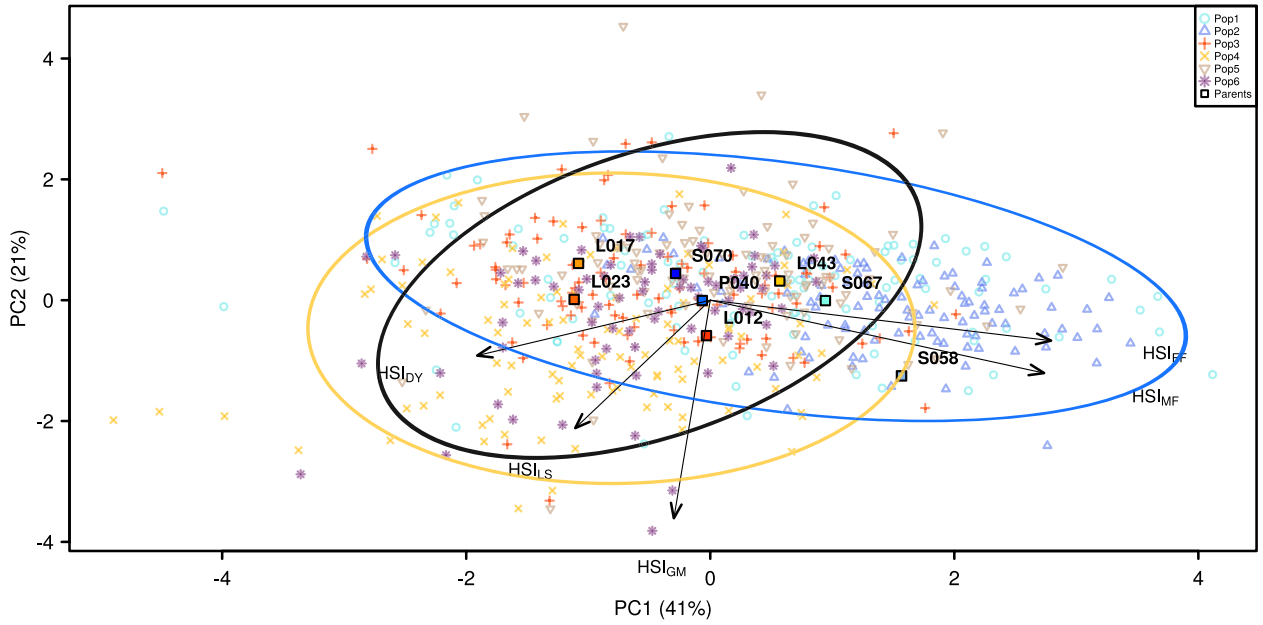

Fig. 2 Plot of the first two principal components (PC1 and PC2) of a principal component analysis with the heat susceptibility indexes (HSI) of the time to female (FF) and male flowering (MF), leaf scorching (LS), grain moisture (GM) and dry yield (DY). The *numbers in brackets* denote the proportion of the explained variance of the respective PC of the total variance across all HSI. The *circles* represent Dent × Dent (*blue*, populations 1 and 2), Flint × Flint (*yellow*, populations 3 and 4) and Dent × Flint (*black*, populations 5 and 6) cluster

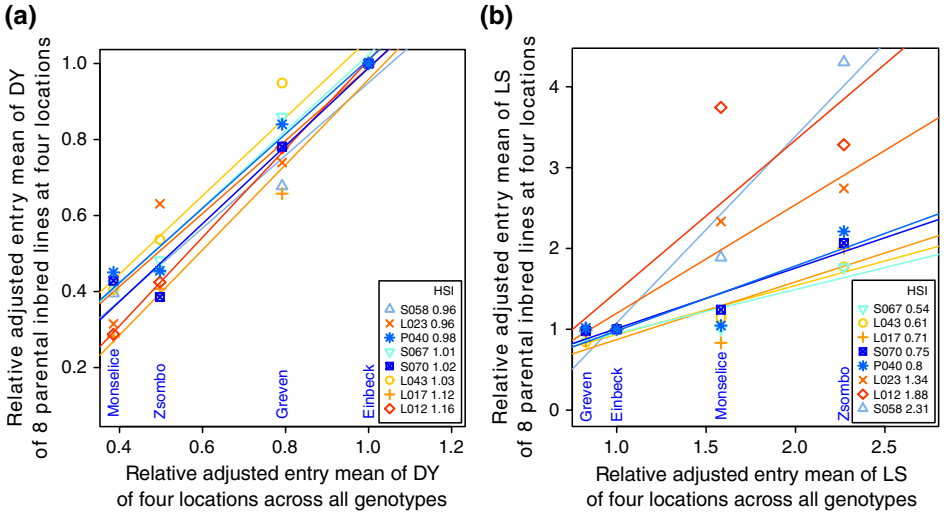

Fig. 3 Stability analysis of the adjusted entry means (AEM) relative to that of Einbeck of **a** dry yield (DY) and **b** the leaf scorching (LS) for the parental inbred lines over the AEM of four locations across all genotypes for calculation of the heat susceptibility index (HSI)

Discussion

In our experiments, the maximum daily temperatures were constantly higher at the locations with heat conditions (Monselice and Zsombó) compared to the locations with standard conditions (Einbeck and Greven), except for a heat wave in Germany in late July (Table 7). One week before until one week after the mean flowering time, temperatures exceeded 35 °C, a total of 76 and 34 h at the locations with heat conditions, Monselice and

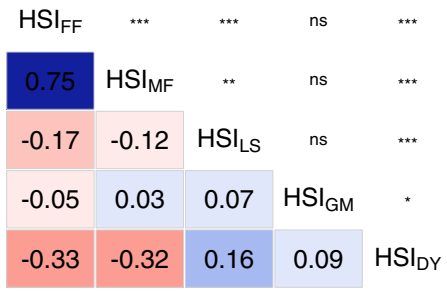

Fig. 4 Correlations of heat susceptibility indexes (HSI) of the heat-dependent traits of female flowering (FF), male flowering (MF), leaf scorching (LS), grain moisture (GM) and dry yield (DY) with significance level (* 0.05, ** 0.01, *** 0.001, ns not significant) across all genotypes

Zsombó, respectively, whereas the temperature did not exceed 35 °C at the locations with standard conditions, Einbeck and Greven (Table 1). Temperatures of 35 °C during the reproductive stage of maize were stated to produce heat-related yield reduction (Hasanuzzaman et al. 2013). Maximum daily temperatures of 35 °C and above during reproductive development of maize were associated with heat conditions (Cairns et al. 2013). In our experiments, during 15 days around flowering, we observed 0 days of maximum temperatures above 35 °C at the locations with standard conditions and a total of 14 and 7 days of maximum temperatures above 35 °C at the locations with heat conditions, Monselice and Zsombó, respectively. Thus, strong heat stress was present at the two locations in southern Europe in comparison to the locations in Germany and heat tolerance was successfully assessed in the year when the experiments were conducted. Besides heat stress, there might be further factors, which differed between the locations with standard conditions and the locations with heat conditions that we did not include in our analysis. We, thus, did not measure only heat tolerance but heat tolerance confounded with other factors. However, to our knowledge, the difference in temperature between the standard and the heat location were the most striking factors between them (cf. Fig. 7).

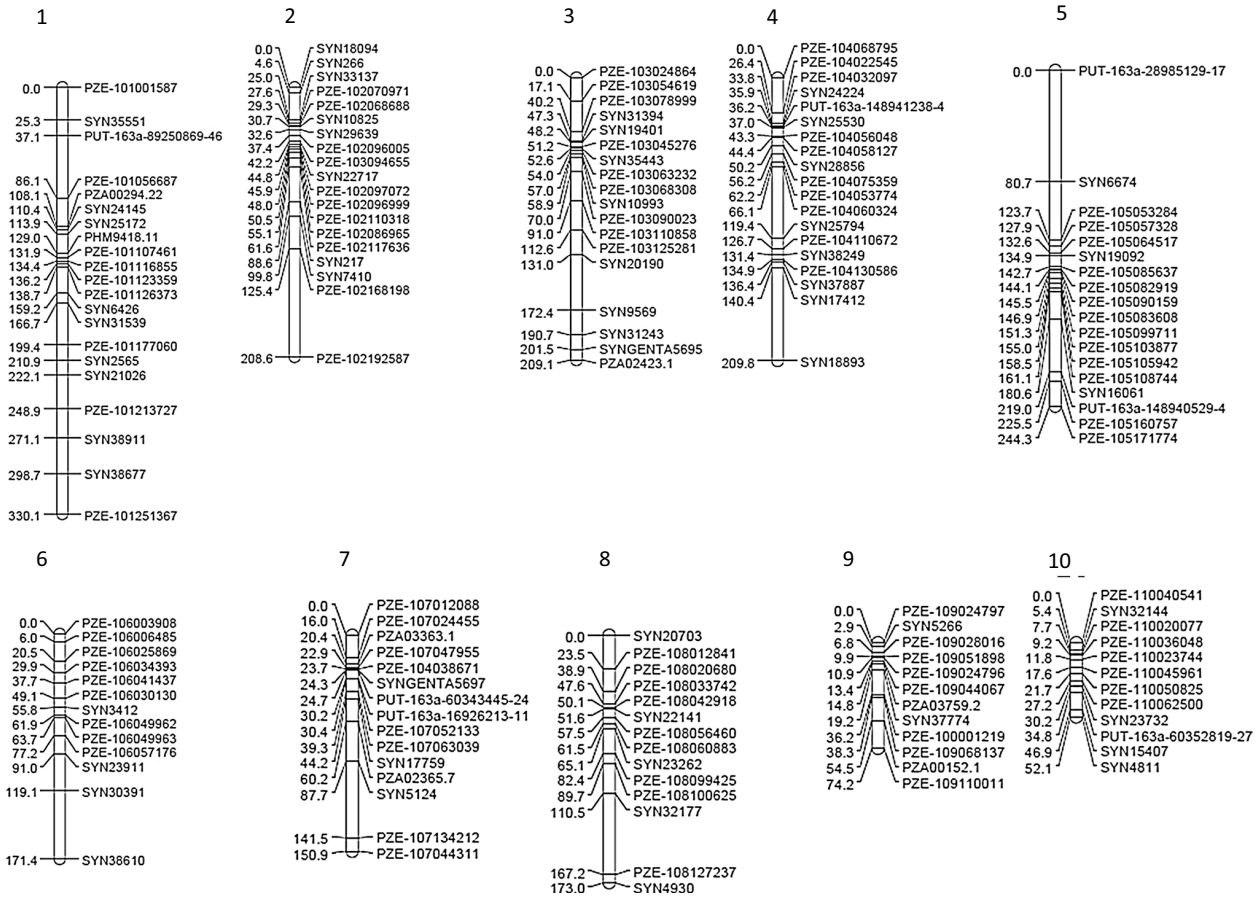

Fig. 5 Consensus genetic linkage map with the positions of the molecular markers in cM

Table 4 Quantitative trait loci (QTL) detected for each trait at a significance level of $\alpha = 0.05$, with chromosome, genetic map position (cM), logarithmic odds ratio (LOD) support interval, proportion of explained variance (R^2) (%), additive effects of each parent (L043, S058, L017, L023, L012, S067, S070, P040) and dominance effects of the six (1–6) populations (1 P040xS067, 2 S070xS058, 3 L012xL017, 4 L043xL023, 5 S067xL012, 6 S070xL023)

Trait	QTL	Chr	Pos	LOD interval	R^2	Additive effect of parent								Dominance effect of population					
						L043	S058	L017	L023	L012	S067	S070	P040	1	2	3	4	5	6
HSI_{DY}	Q_{HSIDYa}	2	45	33-50	12	-0.03^{ABC}	-0.00^{ABC}	-0.01^{ABCD}	0.00^{AB}	-0.03^{BC}	-0.06^{C}	0.03^{AD}	0.10^{D}	-0.15^{***}	-0.01^{ns}	0.00^{ns}	0.04^{ns}	0.02^{ns}	0.06^{ns}
	Q_{HSIDYb}	3	131	104-140	9	-0.01^{AB}	0.05^{AB}	0.04^{AB}	-0.03^{A}	0.07^{B}	0.07^{B}	-0.00^{AB}	-0.18^{C}	0.61^{***}	0.15^{ns}	-0.08^{ns}	-0.37^{ns}	0.15^{ns}	-0.01^{ns}
	Simultaneous fit				19														
HSI_{DYA}	$Q_{HSIDYAa}$	2	45	33-55	9	-0.03^{BC}	-0.01^{ABC}	0.01^{ABC}	0.00^{ABC}	-0.03^{B}	-0.05^{B}	0.04^{AC}	0.07^{A}	-0.15^{***}	-0.01^{ns}	-0.02^{ns}	0.03^{ns}	-0.00^{ns}	0.08^{ns}
	$Q_{HSIDYAb}$	3	131	118-141	9	-0.01^{AB}	0.05^{AB}	0.04^{AB}	-0.04^{A}	0.06^{B}	0.06^{B}	-0.01^{AB}	-0.17^{C}	0.62^{***}	0.17^{ns}	-0.12^{ns}	-0.32^{ns}	0.11^{ns}	-0.01^{ns}
	Simultaneous fit				17														
HSI_{FF}	Q_{HSIFF}	2	15	0-28	9	0.00^{A}	-0.00^{AB}	0.01^{A}	-0.00^{A}	0.00^{A}	0.01^{A}	0.00^{A}	-0.03^{B}	0.03^{*}	-0.04^{*}	-0.06^{***}	-0.00^{ns}	-0.02^{ns}	0.16^{ns}
HSI_{LS}	Q_{HSILS}	9	64	0-74	7	-0.04^{ABC}	-0.04^{ABC}	-0.22^{ABC}	0.20^{AB}	0.51^{B}	0.29^{AB}	-0.13^{AC}	-0.57^{C}	-0.47^{ns}	-0.33^{ns}	1.21^{ns}	-0.09^{ns}	-3.21^{ns}	-0.96^{ns}
HSI_{MF}	Q_{HSIMFa}	2	15	0-26	9	0.01^{A}	-0.01^{AB}	0.00^{AB}	-0.00^{AB}	0.01^{AB}	0.01^{A}	-0.00^{AB}	-0.02^{B}	0.05^{***}	-0.03^{ns}	-0.05^{***}	-0.00^{ns}	-0.02^{ns}	-0.01^{ns}
	Q_{HSIMFb}	5	140	83-146	8	0.01^{CD}	-0.00^{ABCD}	-0.01^{ABCD}	-0.00^{AB}	0.00^{ABCD}	-0.01^{B}	-0.01^{BC}	0.01^{AD}	0.02^{ns}	-0.01^{ns}	0.02^{ns}	0.02^{ns}	0.01^{ns}	0.00^{ns}
	Q_{HSIMFc}	9	13	0-17	7	-0.00^{AB}	-0.00^{A}	-0.01^{A}	0.00^{AB}	-0.01^{A}	0.01^{B}	0.00^{AB}	-0.00^{AB}	0.02^{ns}	0.02^{ns}	-0.02^{ns}	-0.00^{ns}	-0.01^{ns}	-0.02^{ns}
	Simultaneous fit				19														
PC1	Q_{PC1a}	2	10	0-22	13	0.28^{AB}	-0.12^{A}	0.28^{AB}	0.01^{A}	0.22^{AB}	0.53^{B}	-0.16^{A}	-1.03^{C}	1.59^{***}	-0.55^{ns}	-1.57^{***}	0.08^{ns}	-0.46^{ns}	2.96^{ns}
	Q_{PC1b}	5	140	91-156	7	0.27^{BC}	0.12^{ABC}	-0.16^{ABC}	-0.20^{A}	0.05^{ABC}	-0.21^{A}	-0.19^{AB}	0.33^{C}	0.69^{*}	-0.12^{ns}	0.26^{ns}	0.41^{ns}	0.35^{ns}	-0.04^{ns}
	Simultaneous fit				18														

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

^{A} ^{B} ^{C} ^{D} Additive effects of parents with same letters are not significantly different from each other

^{ns} Not significant

Table 5 Heat-tolerance candidate genes within QTL confidence intervals

Gene	Chr	QTL	Description
GRMZM2G148998	2	Q_{PC1a}, $Q_{HSI:FF}$, $Q_{HSI:MFa}$	Uncharacterized protein
GRMZM2G115658	2	$Q_{HSI:DYAa}$	Uncharacterized protein
GRMZM2G537291	2	$Q_{HSI:DYAa}$	Uncharacterized protein
GRMZM2G324886	3	$Q_{HSI:DYb}$, $Q_{HSI:DYAb}$	Calcyclin-binding protein, uncharacterized protein
GRMZM2G436710	5	$Q_{HSI:MFb}$, Q_{PC1b}	Uncharacterized protein
GRMZM2G094990	9	$Q_{HSI:LS}$	Beta-expansin 1a, rare lipoprotein A (RlpA)-like double-psi beta-barrel

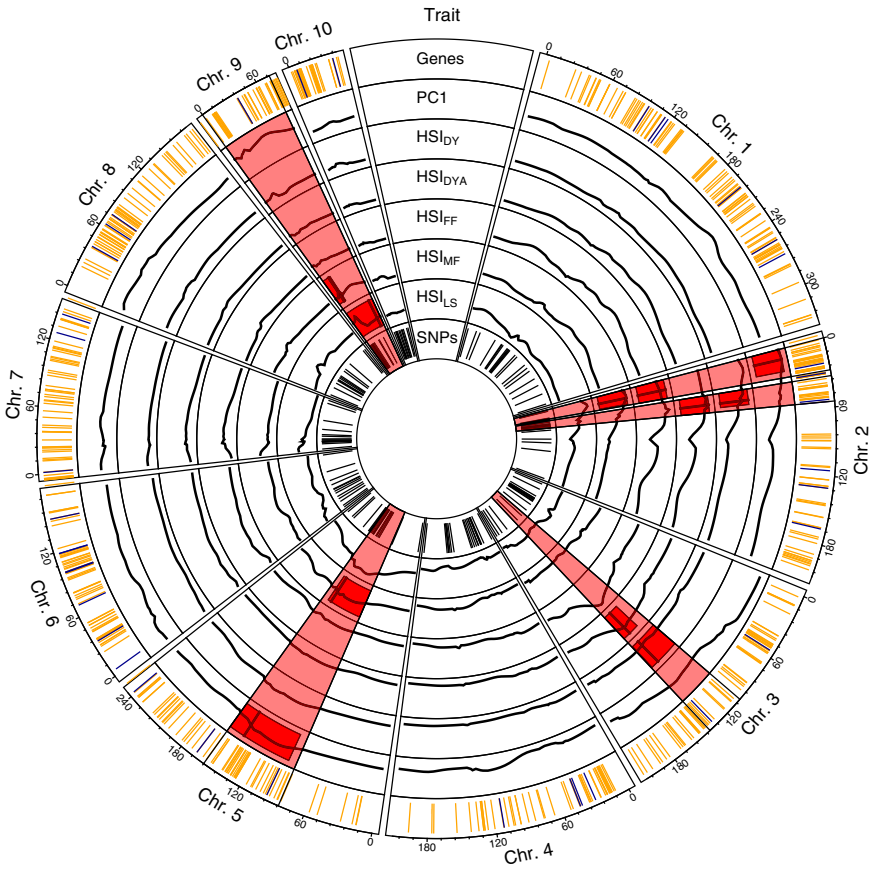

Fig. 6 Genetic positions of heat-tolerance (*black*) and heat-responsive (*orange*) candidate genes in the quantitative trait loci (QTL) confidence intervals and flanking markers (*black*) of the QTL hot spot regions in the first track. Tracks 2–7 show logarithmic odds ratio (LOD) scores (*circumferential black*), detected QTL positions (*radial black*) and confidence intervals (*red*) of the QTL analyses for which QTL have been detected: principal component 1 (PC1) and the heat susceptibility indexes (HSI) of the traits dry yield (DY), adjusted dry yield (DYA), the time to female (FF) and male flowering (MF) and the leaf scorching (LS). QTL hot spots are denoted in *transparent red*. Genetic positions of SNP markers are shown in the *most inner circle*

Novel approach to assess heat susceptibility A novel approach to calculate heat susceptibility was applied in our study to combine two characteristics of each genotype, which are involved in its response to heat stress in multiple environments. First, heat susceptibility of each genotype was defined as the difference between observations collected at heat conditions and those collected at standard conditions (Paliwal et al. 2012; Mason et al. 2010). Second, environmental stability was assessed across multiple locations (Finlay and Wilkinson 1963).

In detail, to calculate the heat susceptibility index (HSI), we first related the adjusted entry means (AEM) calculated for each genotype at each location to the AEM at the location with least heat stress, i.e. lowest temperature during the entire growing period, in this case the location Einbeck, with 899 of GDD (Table 1). With this adjustment, we removed the effect of the growth potential at optimal conditions from the observation for each genotype–location combination to account only for the relative effect of heat stress, which was the main interest of this study. The second part of the calculation of the HSI was derived from the stability analysis approach described by Finlay and Wilkinson (1963), where the stability of a genotype across environmental conditions was calculated on the basis of the performance in multiple environments. By means of these steps, we were able to combine phenotypic variation

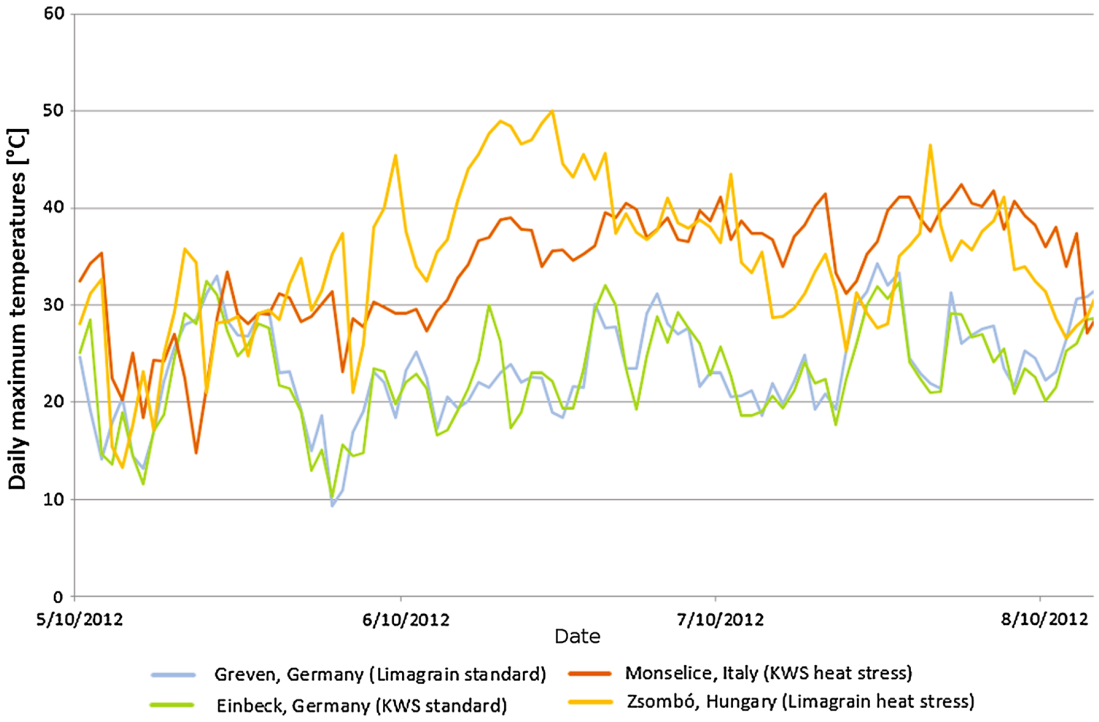

Fig. 7 Daily maximum temperatures at four field locations during the growing period

for heat tolerance in multiple environments to one index, the HSI, which can be further extrapolated to predict the performance of genotypes in other potential environments. Our approach could be appropriate to quantify the tolerance to abiotic stresses in general and could have a wide application in plant breeding experiments. However, validation with further datasets should be performed. Further, conclusions drawn from the results have to consider the data adjustment and calculations mentioned above which served to reduce the complexity of the data set.

Heritability and assessment of traits The heritabilities observed at the location level (H_j^2, Table 2, above left) were between 0.49 and 0.95 for all traits, except LS at the locations Einbeck and Greven. In another study on maize at 15 field locations with drought, heat and without stress conditions (Cairns et al. 2013), heritabilities were between 0.32 and 0.80 for grain yield, between 0.55 and 0.95 for male flowering, between 0.12 and 0.76 for the anthesis silking interval and between 0.26 and 0.90 for plant height. The moderate to very high heritabilities in our study suggested that in the current study a reliable estimation of adjusted entry means was achieved which served for calculating heat susceptibility and the detection of QTL.

We observed lower heritabilities (H_j^2 and H_{cp}^2) of DY at locations with heat conditions compared to heritabilities at locations with standard conditions. Cairns et al. (2013) observed the mean heritabilities of 0.84 at control, 0.64 at drought, as well as 0.50 at drought combined with heat conditions. This lower heritability at stress conditions rise from much higher error and genotype-by-location interaction variance components compared to genotypic variance components (Cairns et al. 2013). For abiotic stress studies, there is, thus, an extra need for an increased number of environments to reliably assess stress tolerance of genotypes and to study natural variation. This was achieved, in our study, with two locations in different regions of Southern Europe. The insecurity of the yield assessment of non-adapted genotypes at heat conditions strengthen furthermore the need for the application of molecular markers in the assessment of heat tolerance.

We observed heritabilities (H_j^2, above left, and H_{cp}^2, above right, Table 2) for LS between 0.00 and 0.34 at locations with standard conditions, whereas they were between 0.13 and 0.59 at locations with heat conditions. The lower heritability of LS at locations with standard conditions in comparison with heritabilities at locations with standard conditions for all other traits (0.12–0.95) was due to the fact that LS was rarely observed at locations without heat stress.

Besides the previously described general trends, the heritability for DY across populations (H_j^2, above left, Table 2) was lower at Zsombó (0.49) in comparison to the other locations (0.67–0.92). We concluded a certain insecurity of the assessment of DY at the location Zsombó. DY was calculated from the FY using GM which was assessed by

near infrared spectroscopy, which demands a minimum plot yield. In Zsombó, only 65 seeds were sown per plot, in contrast to 80–110 at the other locations. FY decreased, thus, below the range, necessary for successful assessment of GM in more than 50 % of the plots, which led to missing observations. Nevertheless, the medium heritability of DY at Zsombó was sufficient to include data from this location in the analysis. Furthermore, missing data of Zsombó was compensated with data assessed at Monselice, the other location with heat conditions.

The heritability of population 2 (H^2_{cp}) was lower compared to that of the other populations for the traits ASI, DY, FF, MF and GM at locations with standard conditions (0.37 on average for population 2 and 0.67 on average for populations 1, 3, 4, 5 and 6) and for the traits LS, MF and GM at locations with heat conditions (0.23 on average for population 2 and 0.33 on average for populations 1, 3, 4, 5 and 6). This reduced heritability of population 2 was due to an especially low genotype variance component of this population (low variability between individuals) (supplementary material—Table 1). The low genotypic variance of population 2 was balanced using multiple populations which lead to a wide variation between individuals as a basis for the QTL analysis.

Relation between anthesis silking interval and heat stress In contrast to the traits LS, DY, FF, MF and GM, which were considered as heat stress dependent, we did not find a significant ($P < 0.05$) condition effect for ASI (Table 3) and, thus, no relation of the ASI and heat stress across populations. This was in contrast to other experiments (Agrama and Moussa 1996; Bolaños and Edmeades 1996; Tuberosa et al. 2002), where the ASI was strongly increased upon drought stress. This implies that in the examined plant material in our study, a selection for flowering synchrony, i.e. reduced ASI, does not lead to increased heat tolerance.

Influence of the reduction of the time to flowering on yield loss upon heat stress We observed significant ($P < 0.001$) negative correlations between HSI_{DY} and HSI_{FF} as well as HSI_{MF} (Fig. 4). Note that the correlations shown here do not state a negative correlation between the absolute values of dry yield and flowering time. Rather, genotypes which show later flowering at heat conditions compared to standard conditions also have higher yield losses at heat conditions. The analysis regarded differences between heat and standard conditions without considering absolute performance. As an avoidance mechanism, many crop plants escape heat stress by pre-maturation which is connected with preponed flowering (Hasanuzzaman et al. 2013). With our phenotypic analysis, we confirmed that preponed flowering is strongly correlated (with about 30 %) with reduced yield losses due to heat stress, which was stated previously by Hasanuzzaman et al. (2013). Thus, breeding for preponed flowering under heat conditions can help to ensure yield potential at unfavourable conditions. Hybrid testing should be performed to verify if this statement is also true in advanced breeding material. Nevertheless, earlier maturation is generally correlated with lower yields due to a shorter time to accumulate photosynthetic products. We introduced the HSI_{DYA}, where the HSI_{DY} was adjusted with HSI_{FF} as a cofactor. The HSI_{DYA} can be applied to assess heat tolerance with respect to grain yield independently from the reduction of the time to flowering.

Leaf scorching as a phenotypic marker The correlation between HSI_{DY} and HSI_{LS} was neglegibly low (0.16) (Fig. 4). This could be explained by the low heritability of LS (Table 2) and, thus, high error of the LS assessment. Furthermore, we observed no collocation of QTL for HSI_{LS} and HSI_{DY}, where a QTL for HSI_{LS} was on chromosome 9 and QTL for HSI_{DY} were on chromosomes 2 and 3. These results indicate that genetic mechanisms for LS and DY were not located at the same genomic positions. LS has, thus, limited usability as a phenotypic marker for heat tolerance in terms of yield in our populations and environments.

Multi-trait measure for heat tolerance The first principal component (PC) of the PC analysis represented a multi-trait measure which combined several HSI to one trait (Fig. 2). PC1 explained 41 % of the total variance and covered heat susceptibility in terms of the time to flowering and heat tolerance in terms of grain yield, as well as of leaf scorching. The QTL which were detected to be associated with PC1 (Q_{PC1a} and Q_{PC1b}; Table 4) explained 18 % of the total variance of PC1 in a simultaneous fit across QTL and across populations. The highest positive influence on the PC1 was contributed by the allele of parent S067 at QTL Q_{PC1a} and by the allele of P040 at QTL Q_{PC1b}. Homozygosity for allele S067 at locus Q_{PC1a} increased PC1 by a value of 0.53 and homozygosity for allele P040 at locus Q_{PC1b} increased PC1 by 0.33. Combining alleles of S067 at locus Q_{PC1a} and alleles of P040 at locus Q_{PC1b}, PC1 would be increased by a total of 0.86. Furthermore, high dominance effects (significant with $\alpha < 0.001$ at Q_{PC1a} and $\alpha < 0.05$ at Q_{PC1b}) were observed at both QTL associated with PC1 in population 1, where the parental inbreds were S067 and P040. Heterozygosity for alleles P040 and S067 at position Q_{PC1a} and Q_{PC1b} resulted in a combined dominance effect of $1.59 + 0.69 = 2.28$. Comparing homozygosity and heterozygosity at the loci associated with PC1 led to the assumption that strong heterosis for heat tolerance was present in the genetic material used in our study. As the calculation of heat tolerance in our study included phenotypic stability across conditions, the higher heat tolerance of heterozygous individuals could be attributed to higher stability across temperatures. This effect was detected previously by McWilliam and Griffing (1965), who related increased heterosis of maize hybrids at high temperatures compared

to optimal growth conditions with their increased stability across growth conditions. A selection on heterozygosity with the alleles S067 and P040 at Q_{PC1a} and Q_{PC1b} would improve heat tolerance in terms of grain yield, lower leaf damages produced by heat stress and lead to an increased speed of development enabling plants to escape the strongest summer heat waves.

Heat tolerance of Flint and Dent heterotic pools We observed a lower yield loss and a lower increase of leaf scorching at locations with heat conditions of genotypes derived from Dent × Dent crosses in comparison with genotypes derived from Flint × Flint crosses (Table 3). Dent genotypes showed, thus, a higher heat tolerance with respect to yield and leaf scorching. Genotypes derived from interpool crosses (Dent × Flint) showed an intermediate heat tolerance with respect to the mentioned traits. To the best of our knowledge, the heat tolerance under field conditions of genotypes of the European Dent and Flint pools was not quantified previously. In a study on heat tolerance during seedling stage under controlled conditions with the inbred lines which served as parents of the populations in our study (Frey et al. 2015), no pool effect was detected. However, the low number of four Dent and four Flint inbred lines, which were phenotyped by Frey et al. (2015), did not allow a reliable conclusion on the presence of a pool effect. In the present study, heat tolerance of a total of 608 Flint, Dent and Flint × Dent genotypes from six populations was assessed. Thus, the effect on the heat tolerance of a genotype which is associated with the affiliation to a certain heterotic pool was quantified more reliably, although the genetic basis of the 608 genotypes was only eight parental inbred lines. The knowledge that genotypes derived from Dent × Dent crosses are more heat tolerant than those derived from Flint × Flint crosses is very valuable in the context of the suitability of different breeding pools for a selection on heat tolerance by plant breeders. The results were assessed with inbred lines and may be different in hybrids. However, testing inbred lines is a first step in commercial breeding programs as heritabilities are expected to be higher.

We observed significant differences between populations for heat tolerance in terms of the time to flowering (Table 3). However, those differences were not associated with the affiliation to heterotic pools. That means that, besides the reduction of the time to flowering at heat stress in general, there was no pool-specific response related to this trait. The higher heat tolerance in terms of yield of Dent genotypes compared to that of Flint genotypes might, thus, not be based on stronger reduction of the time to flowering. A possible explanation for this difference in heat tolerance is that the photosynthetically active leaf surface of Dent genotypes was less reduced by leaf scorching at heat stress compared to Flint genotypes (Table 1). As, however, the detected loci, associated with heat tolerance in terms of grain yield and in terms of leaf scorching were not overlapping (Fig. 6), the main genetic mechanisms underlying heat tolerance in terms of yield must be different. To elucidate these, we advise fine mapping of the detected QTL and functional gene studies of the candidate genes, which were located in the genome regions associated with heat tolerance (Table 5; Supplementary material—Table 2).

Genetic linkage map The genetic map was constructed based on molecular marker information of six segregating populations of this study and five populations of a companion study (Horn et al. 2015). This multi-population approach improved the quality of the genetic map due to a higher possibility of two markers segregating in the same population. The total length of the genetic map (1 823.5 cM) was similar to the properties of genetic maps in earlier studies in maize (e.g. Blanc et al. 2006). The average distance between molecular markers was 11.3 cM. With intervals between markers of <15 cM, any QTL is closely linked to a molecular marker, which is necessary to detect QTL and to not underestimate the magnitude of their effects (Tanksley 1993). We observed a condensation of molecular markers at the centromeres of the chromosomes on the genetic map. This was in contrast to the fact that markers were selected to be distributed evenly across the genome by physical distance. As the construction of a genetic map is always based on the probability of recombinations between loci, genetic and physical distances can vary greatly. The condensation of markers can, thus, be explained by lower recombination rates at the centromeres. This effect was described previously by Payseur and Nachman (2000). The order of the markers by their genetic positions, however, was consistent with the physical order of markers on the chromosomes.

QTL for heat tolerance As outlined above, heat tolerance was confounded with other envirnmental factors, but the difference in temperature between the standard and the heat locations was the most striking factor. Thus, the detected QTL represent mostly heat tolerance. Two QTL hot spots for heat tolerance with respect to grain yield (HSI_{DY} and HSI_{DYA}) were identified, one on chromosome 2 and one on chromosome 3 (Fig. 6). To the best of our knowledge, QTL for heat tolerance in maize in vivo were not reported in previous studies. The latest reports on molecular markers or QTL associated with thermotolerance of maize were published in 1991 and 1994 (Ottaviano et al. 1991; Frova and Sari-Gorla 1994) and focussed on the relation of single physiological mechanisms with heat stress, i.e. the cellular membrane stability and the germination of pollen grains under heat conditions. A cluster of RFLP markers, which were associated with the injury of the pollen grain germinability and the pollen tube growth (Frova and Sari-Gorla 1994) were located at the center of chromosome 3,

putatively collocating with the loci $Q_{HSI:DYb}$ and $Q_{HSI:DYAb}$, identified in the present study. Pollen viability is a critical mechanism involved in pollination and, consequently, seed growth. The genetic mechanisms of pollen viability, at heat stress could, thus, be a part of the reaction of maize upon heat stress with respect to grain yield. To investigate this question, the genotypes studied in this paper could be phenotyped for pollen viability traits.

Even though QTL for heat tolerance with respect to grain yield assessed on the field level were not reported previously, we observed an overlapping of the confidence intervals of the QTL detected in our study with QTL for other abiotic stresses than heat stress. The above-mentioned QTL hot spot on chromosome 2, including QTL for HSI_{DY} and HSI_{DYA}, overlapped with a QTL for cold tolerance found in a meta-analysis across multiple QTL studies (Rodríguez et al. 2013) and with a QTL associated with the shoot and root dry weight and the leaf area under water stress conditions (Ruta et al. 2010). This suggests that the mentioned genomic regions might be associated with a general tolerance to abiotic stresses in maize. This, however, requires further research.

Each QTL, associated with heat tolerance with respect to different traits, detected in our study, explained between 7 and 13 % of the variance of the respective HSI or PC (Table 4). This was in accordance with the explained variances of QTL associated with abiotic stress in maize identified by Rodríguez et al. (2013) and Messmer et al. (2011). The low variance explained by single QTL in this study revealed the multigenic inheritance of heat tolerance in maize. However, with a simultaneous fit, we could explain 19 and 17 % of the total variance for HSI_{DY} and HSI_{DYA}, respectively, with each of two QTL, which are located between 33 and 55 cM on chromosome 2 ($Q_{HSI:DYa}$ and $Q_{HSI:DYAa}$) and between 104 and 141 cM on chromosome 3 ($Q_{HSI:DYb}$ and $Q_{HSI:DYAb}$) (Table 4). As the statistical analyses presented in this study were based on six segregating populations, a wide genetic variation was considered. This increased the validity of the detected QTL. After validation of the genome regions in another set of environments and/or a different set of plant material, it may be profitable to invest in MAS on the previously mentioned QTL as an additional means to a traditional breeding approach.

The average absolute additive effects of the alleles of the parental inbreds P040 and S067 at the QTL for HSI_{DYA} ($Q_{HSI:DYAa}$ and $Q_{HSI:DYAa}$) were with 0.06 and 0.17 higher than for the other parental alleles (Table 4). to fine-map the detected QTL, i.e. to reduce their confidence intervals, we recommend performing QTL mapping including a segregating population with a higher number of progeny derived from the inbreds P040 and S067.

Candidate genes for heat tolerance and heat response

Frey et al. (2015) identified 607 and 39 genes which were associated with the tolerance and the response upon heat stress during seedling stage under controlled conditions. To unravel the genetic mechanisms underlying heat tolerance of maize under field conditions, we examined the presence of heat-tolerance and heat-responsive genes identified by Frey et al. (2015) in seedling leaves within the QTL confidence intervals of the present study. We found that a total of 3 heat-tolerance genes and 23 heat-responsive genes were situated in the QTL regions for HSI_{DY} and HSI_{DYA} ($Q_{HSI:DYa}$, $Q_{HSI:DYb}$, $Q_{HSI:DYa}$ and $Q_{HSI:DYb}$) (Table 5; Supplementary material—Table 2). As they appear in the present study as well as in Frey et al. (2015), these genes represent genetic mechanisms which are associated with heat tolerance and heat response during both adult and seedling stage. They may, thus, be key factors for heat-related pathways in general. The heat-tolerance gene GRMZM2G324886 is of particular interest, as it was the only heat-tolerance gene, which was found in a QTL for both HSI_{DY} and HSI_{DYA} and it was already described to code for a calcicyclin-binding protein, which may be involved in calcium signalling as a response to external stress. An ortholog of this gene in rice is Os01g0757500, which was described as an HSP20-like chaperone domain containing protein and is, thus, involved in the response to heat shock. Beside its potential functional relationship with heat tolerance, our study suggests that it might be also involved in explaining phenotypic variation. This, however, needs to be studied further as follows. Due to the consideration of phenotypic variation resulting in low power to detect heat-tolerance candidate genes (Frey et al. 2015), the differential expression of GRMZM2G324886 should be verified by replicating the experiment described by Frey et al. (2015). The expression of the previously mentioned candidate gene at different heat levels could be quantified in such an experiment by quantitative real-time PCR with specific primer combination in the eight parental inbreds or even in genotypes derived from the populations used in the present study, which showed contrasting heat tolerance. After validating that GRMZM2G324886 is involved in heat tolerance, its gene sequence could be investigated with respect to polymorphisms (e.g. SNPs) between the sequences present in heat-tolerant and heat-susceptible lines, respectively, which could be the cause of differential expression. If polymorphisms are detected in the gene of interest, they would be genetically very close to the the actual QTL position detected in this study. MAS could be applied on the basis of such polymorphisms to select more heat-tolerant genotypes instead of using the flanking markers of the QTL confidence interval, which were tested in the present study and, thus, reducing the probability of recombinations between marker and QTL position in tested plants.

Conclusion

Compared to other abiotic stresses associated with climate change (e.g. drought stress), relatively little research has been conducted on heat stress in maize. Existing studies on heat tolerance in maize focussed on a limited number of genotypes with short artificial heat stress events, rather than on the response to heat under field conditions (Cairns et al. 2013). Despite the similarities of drought and heat stress response in plants, we found that the ASI is, in contrast to drought tolerance, not related to heat tolerance. We presented a method to describe heat susceptibility without accounting for the growth potential, and which can use data of multiple environments. This approach can also be applied in studies on other abiotic stresses with multiple environments. Further, there was a lack of knowledge about the heat tolerance of either European Flint or Dent pool. This paper is a first step towards studying this point. However, a bigger set of inbred genotypes should be tested concerning their heat tolerance to verify that Dent genotypes are more heat tolerant than Flint genotypes. A further important step towards breeding of more heat-tolerant varieties is the investigation of the reaction upon heat stress of hybrid genotypes. Marker-assisted selection for heat tolerance with respect to grain yield is of great importance due to the lack of highly heritable phenotypic markers and the difficult nature of the assessment of heat tolerance (multi-environment field trials). The experiments underlying this paper can help to design experiments to further develop markers for heat tolerance in the future.

Author contribution statement FPF participated in the design and coordination of the study, carried out the statistical analysis and the QTL mapping and drafted the manuscript. TP, PL and AO participated in the design of and conducted the field experiments. BS conceived the study, participated in its design and coordination as well as in the statistical analysis and drafted the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

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

References

Agrama H, Moussa M (1996) Mapping QTLs in breeding for drought tolerance in maize (*Zea mays* L.). Euphytica 91:89–97

Andersen JR, Schrag T, Melchinger AE, Zein I, Lübberstedt T (2005) Validation of Dwarf8 polymorphisms associated with flowering time in elite European inbred lines of maize (*Zea mays* L.). Theor Appl Genet 111(2):206–217

Ashraf M, Hafeez M (2004) Thermotolerance of pearl millet and maize at early growth stages: growth and nutrient relations. Biol Plant 48(1):81–86

Bardol N, Ventelon M, Mangin B, Jasson S, Loywick V, Couton F, Derue C, Blanchard P, Charcosset A, Moreau L (2013) Combined linkage and linkage disequilibrium QTL mapping in multiple families of maize (*Zea mays* L.) line crosses highlights complementarities between models based on parental haplotype and single locus polymorphism. Theor Appl Genet 126(11):2717–2736

Barnabás B, Jäger K, Fehér A (2008) The effect of drought and heat stress on reproductive processes in cereals. Plant Cell Environ 31(1):11–38

Becker H (2011) Pflanzenzüchtung, 2nd edn. Verlag Eugen Ulmer, Stuttgart

Blanc G, Charcosset A, Mangin B, Gallais A, Moreau L (2006) Connected populations for detecting quantitative trait loci and testing for epistasis: an application in maize. Theor Appl Genet 113(2):206–224

Bolaños J, Edmeades G (1996) The importance of the anthesis-silking interval in breeding for drought tolerance in tropical maize. Field Crops Res 48:65–80

Cairns JE, Crossa J, Zaidi PH, Grudloyma P, Sanchez C, Araus JL, Thaitad S, Makumbi D, Magorokosho C, Bänziger M, Menkir A, Hearne S, Atlin GN (2013) Identification of drought, heat, and combined drought and heat tolerant donors in maize. Crop Sci 53(4):1335–1346

Charcosset A, Mangin B, Moreau L, Combes L, Jourjon MF, Gallais A (2001) Heterosis in maize investigated using connected RIL populations. In: Quantitative genetics and breeding methods : the way ahead. Les colloques no. 96. INRA, Paris, pp 89–98

Chen J, Xu W, Velten J, Xin Z, Stout J (2012) Characterization of maize inbred lines for drought and heat tolerance. J Soil Water Conserv 67(5):354–364

Crafts-Brandner S, Salvucci M (2002) Sensitivity of photosynthesis in a C4 plant, maize, to heat stress. Plant Physiol 129(August):1773–1780

de Givry S, Bouchez M, Chabrier P, Milan D, Schiex T (2005) CARTHAGENE: multipopulation integrated genetic and radiation hybrid mapping. Bioinformatics 21(8):1703–4

Deutsches Maiskomitee (2013) http://www.maiskomitee.de/web/public/Fakten.aspx/Statistik/Deutschland/Statistik_Biogas. Accessed 19 November 2013

FAOSTAT (2014) http://faostat3.fao.org. Accessed 16 October 2014

Finlay K, Wilkinson G (1963) The analysis of adaptation in a plant-breeding programme. Aust J Agric Res 14:742–754

Fokar M, Blum A, Nguyen HT (1998) Heat tolerance in spring wheat II. Grain filling. Euphytica 104:9–15

Frey FP, Urbany C, Hüttel B, Reinhardt R, Stich B (2015) Genome-wide expression profiling and phenotypic evaluation of European maize inbreds at seedling stage in response to heat stress. BMC Genom 16(1):123

Frova C, Sari-Gorla M (1994) Quantitative trait loci (QTLs) for pollen thermotolerance detected in maize. Mol General Genet 245(4):424–430

Ganal MW, Durstewitz G, Polley A, Bérard A, Buckler ES, Charcosset A, Clarke JD, Graner EM, Hansen M, Joets J, Le Paslier MC, McMullen MD, Montalent P, Rose M, Schön CC, Sun Q, Walter H, Martin OC, Falque M (2011) A large maize (*Zea mays* L.) SNP genotyping array: development and germplasm genotyping, and genetic mapping to compare with the B73 reference genome. PloS One 6(12):e28334

Giaveno C, Ferrero J (2003) Introduction of tropical maize genotypes to increase silage production in the central area of Santa Fe, Argentina. Crop Breed Appl Biotechnol 3(2):89–94

Gilmour A, Gogel B, Cullis B, Thompson R (2006) ASReml user guide release 2.0. VSN International Ltd, Hemel Hempstead

Hallauer AR, Carena MJ, Miranda Filho JB (2010) Quantitative genetics in maize breeding. Springer, New York

Hasanuzzaman M, Nahar K, Alam MM, Roychowdhury R, Fujita M (2013) Physiological, biochemical, and molecular mechanisms of heat stress tolerance in plants. Int J Mol Sci 14(5):9643–9684

Horn F, Habekuß A, Stich B (2013) Natural variation for BYDV resistance in maize. Maydica 58:173–181

Horn F, Habekuß A, Stich B (2015) Linkage mapping of barley yellow dwarf virus resistance in connected populations of maize. BMC Plant Biol 15(1):29

IPCC (2013) Summary for policymakers. In: Stocker TF, Qin D, Plattner GK, Tignor M, Allen SK, Boschung J, Nauels A, Xia Y, Bex V, Midgley P (eds) (2013) Climate change 2013: the physical science basis. Contribution of working group I to the fifth assessment report of the intergovernmental panel on climate change. Cambridge University Press, Cambridge

Lobell DB, Field CB (2007) Global scale climate-crop yield relationships and the impacts of recent warming. Environ Res Lett 2(1):014002

Mason RE, Mondal S, Beecher FW, Pacheco A, Jampala B, Ibrahim AMH, Hays DB (2010) QTL associated with heat susceptibility index in wheat (*Triticum aestivum* L.) under short-term reproductive stage heat stress. Euphytica 174(3):423–436

McMaster G, Wilhelm W (1997) Growing degree-days: one equation, two interpretations. Agric For Meteorol 87(1):291–300

McWilliam JR, Griffing B (1965) Temperature-dependent heterosis in maize. Aust J Biol Sci 18:569–583

Messmer R, Fracheboud Y, Bänziger M, Stamp P, Ribaut JM (2011) Drought stress and tropical maize: QTLs for leaf greenness, plant senescence, and root capacitance. Field Crops Res 124(1):93–103

Ottaviano E, Sari Gorla M, Pè E, Frova C (1991) Molecular markers (RFLPs and HSPs) for the genetic dissection of thermotolerance in maize. Theor Appl Genet 81(6):713–719

Paliwal R, Röder MS, Kumar U, Srivastava JP, Joshi AK (2012) QTL mapping of terminal heat tolerance in hexaploid wheat (*T.aestivum* L.). Theor Appl Genet 125(3):561–575

Payseur BA, Nachman MW (2000) Microsatellite variation and recombination rate in the human genome. Genetics 156:1285–1298

Rattalino Edreira J, Otegui M (2013) Heat stress in temperate and tropical maize hybrids: a novel approach for assessing sources of kernel loss in field conditions. Field Crops Res 142:58–67

Reimer R, Stich B, Melchinger AE, Schrag TA, Sørensen AP, Stamp P, Hund A (2013) Root response to temperature extremes: association mapping of temperate maize (*Zea mays* L.). Maydica 58:156–168

Rodríguez VM, Butrón A, Rady MOA, Soengas P, Revilla P (2013) Identification of quantitative trait loci involved in the response to cold stress in maize (*Zea mays* L.). Mol Breed 33(2):363–371

Roy SJ, Tucker EJ, Tester M (2011) Genetic analysis of abiotic stress tolerance in crops. Curr Opin Plant Biol 14(3):232–239

Ruta N, Stamp P, Liedgens M, Fracheboud Y, Hund A (2010) Collocations of QTLs for seedling traits and yield components of tropical maize under water stress conditions. Crop Sci 50(4):1385–1392

Sinsawat V, Leipner J, Stamp P, Fracheboud Y (2004) Effect of heat stress on the photosynthetic apparatus in maize (*Zea mays* L.) grown at control or high temperature. Environ Exp Bot 52(2):123–129

Tanksley SD (1993) Mapping polygenes. Annu Rev Genet 27:205–233

Tuberosa R, Salvi S, Sanguineti MC, Landi P, Maccaferri M, Conti S (2002) Mapping QTLs regulating morpho-physiological traits and yield: case studies, shortcomings and perspectives in drought-stressed maize. Ann Bot 89(7):941–963

Wahid A, Gelani S, Ashraf M, Foolad M (2007) Heat tolerance in plants: an overview. Environ Exp Bot 61(3):199–223

Heat tolerance in temperate maize

Identification of QTL during seedling stage with multiple connected populations

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Abstract

Climate change has the potential to lead to increasing heat stress during summer in temperate regions, which can affect late planted maize. We assessed heat tolerance during seedling stage on a multi-trait level in a set of connected intra- and interpool Dent and Flint populations. Four quantitative trait loci were associated with heat tolerance with respect to two principal components, which represented a combination of nine seedling traits. Heat tolerance during different developmental stages were loosely correlated with each other, where genotypes derived from Flint lines were more heat tolerant during seedling stage and genotypes derived from Dent lines were more heat tolerant during adult stage. Further, we detected eight heat tolerance candidate genes, which need to be functionally studied to reveal their potential role in the tolerance upon heat stress. One of them was described previously and points out the importance of Calcium signaling in the response to heat stress.

Key-words: QTL analysis, heat stress, Zea mays L., climate change, genetic variation, seedling stage

Introduction

Maize (*Zea mays L.*) is, compared to other crop species growing in temperate Europe, heat tolerant due to its C4 metabolism and its tropical origin (Sage et al., 2011). Nevertheless, temperate maize cultivars can suffer substantial damages when opposed to heat stress (Giaveno & Ferrero, 2003). In temperate Europe, maize is the most important biogas crop (Deutsches Maiskomitee, 2013) due to its short vegetation period. The sowing can, thus, be postponed until the harvest of the winter cereals in early summer. With this cropping system, sensitive maize seedlings are exposed to heat stress (Reimer et al., 2013).

In general, heat stress is expected to become, in the future, a more critical threat to crop cultivation (Lobell & Field, 2007) as the mean temperature and the severity of heat events will rise due to climate change (IPCC, 2013). Damages in maize caused by heat stress during adult stage include a reduction in the time to flowering (Frey et al., 2015a), a reduction of photosynthetic tissue due to leaf scorching and a reduction of yield of grains and total plant matter (Wahid et al., 2007).

The identification of heat tolerant genotypes and knowledge of the genetic mechanisms of heat tolerance in maize are, thus, of crucial importance for future crop production. The tolerance to heat stress in maize was studied on a molecular level with a focus on natural variation by Ottaviano et al. (1991), Frova & Sari-Gorla (1994) and Reimer et al. (2013). The previous mentioned studies focussed on isolated plant characteristics like the cellular membrane stability, pollen germination and root architecture, respectively, where heat tolerance in this present study referred to whole maize seedlings. Heat tolerance during adult stage under field conditions with respect to whole plants was studied by Frey et al. (2015a) In this present study in contrast, we aimed to identify quantitative trait loci (QTL) for heat tolerance during seedling stage under controlled conditions.

The objectives of this study were to (I) describe the variation for heat tolerance of a set of six segregating connected Flint and Dent populations with respect to seedling traits and on a multi-trait level under controlled conditions, (II) identify QTL for heat tolerance during seedling stage, and (III) overlap genomic regions associated with heat tolerance during adult stage and during seedling stage and positions of candidate genes for heat tolerance and for heat response to an overall picture of heat tolerance in maize.

Material and methods

Experimental conditions

Plant material and field experiments

This study was based on six segregating populations derived from pairwise crosses of four Dent (S058, S067, S070, P040) and four Flint (L043, L017, L023, L012) maize inbred lines from the University of Hohenheim (Andersen et al., 2005). The eight inbred lines were in detail characterized for their heat tolerance during seedling stage by Frey et al. (2015b). The inbreds have been crossed pairwisely to create two Dent x Dent, two Flint x Flint and two Dent x Flint F1 genotypes (Figure 1). The F1 genotypes were further self pollinated resulting in six segregating populations comprising between 75 and 107 $F_{3:4}$ genotypes and with a total of $N = 608$ genotypes. The mentioned genotypes were used in the $F_{3:5}$ generation in an experiment on heat tolerance during adult stage (Frey et al., 2015a).
Seeds were sown in soil (50% ED73, 50% Mini Tray (Einheitserde- und Humuswerke, Gebr. Patzer GmbH & Co. KG, Sinntal-Altengronau, Germany)) in single pots (9 cm edge length) as described by Frey et al. (2015b). The experiment was replicated three times. The experimental design was a lattice design with 32 incomplete blocks per replication, which were distributed on four tables in a walk-in growth chamber (Bronson Incubator Services B.V., Nieuwkuijk, Netherlands). The parental inbred lines were included once on each table. The plants were grown at 25°C during a 16h light period and at 20°C during a 8h dark period for a total of three weeks in the growth chamber. Relative humidity was set to 60%. Photosynthetic active radiation, emitted by fluorescent tubes, was between 270 - 280 µmol $m^{-2}s^{-1}$ in the canopy of the plants to avoid any type of radiation stress, which could be observed with higher light intensities. Watering was conducted every morning with an automatic irrigation system (Itec DC station Multi Program, I.T.Systems Ltd., Bazra, Israel) to avoid drought stress.
The leaf growth rate was calculated as follows: the length of the fourth leaf from the shoot base to the leaf tip was measured daily for a period of three days during the stage of linear growth. The slope of a linear trendline of leaf length measurements vs. time represented the leaf growth rate (LR). Twenty days after sowing, leaf greenness (SD) (SPAD-502, Minolta Corporation, Ramsey, NJ, USA) was assessed as the maximum value of four readings on the leaf blade of the latest fully developed leaf. Furthermore, leaf scorching of young leaves (SC) and leaf senescence of old leaves (SN) were recorded from 1 (weak damage) to 9 (strong damage). The length of the fourth fully developed leaf (LL), the plant height (PH) from the shoot base to the point where the youngest leaf detached from the older leaf's sheath and the number of leaves (NL) per plant with visible leaf ligule were recorded. A total of 21 days after sowing, shoot dry weight (DW) and the shoot water content (WC)of the fresh material were determined. The above outlined experiment was repeated at a further heat level, where the temperature was increased after six days to 38°C at day and 33°C at night to induce heat stress for a two-week period. The study was, thus, based on two experiments with different heat levels, i.e. standard and heat conditions.

Genotyping

The parental inbred lines of the populations were genotyped with a set of 56 110 single nucleotide polymorphism (SNP) markers using a 50K SNP array (Ganal et al., 2011). Out of these SNPs, 161 SNP markers were selected to genotype the individuals of the six segregating populations. For each population, between 47 and 77 markers were chosen (60 for population 1, 47 for population 2, 75 for population 3, 64 for population 4, 67 for population 5 and 77 for population 6) being polymorphic between the two parents of each population and not showing heterozygosity in neither parental line. SNP marker selection was optimized for equal distribution across the physical map (due to the unavailability of a genetic map at that time) and the overlapping of markers between populations. The selected SNP markers were genotyped using KASP marker technology by TraitGenetics GmbH (Gatersleben, Germany) in the respective populations.

Statistical analyses

Phenotypic data

Adjusted entry means calculation: To calculate adjusted entry means (AEM) for each assessed trait of the genotypes at each heat level, the phenotypic observations were analysed with the following model at each heat level separately:

$$Y_{bir} = \mu + G_i + R_r + B_{br} + e_{bir} \ , \qquad (1)$$

where Y_{bir} was the phenotypic observation of the i^{th} genotype in the r^{th} replication and the b^{th} incomplete

block, μ the general mean, G_i the effect of the i^{th} genotype, R_r the effect of the r^{th} replication, $B_{br}$the effect of the b^{th} incomplete block nested in the r^{th} replication, and e_{bir} the residual error term. The genotype factor G_i was of primary interest in this analysis and was considered as a fixed term. R_r was set as fixed and B_{br} as random.

To calculate AEM for the assessed traits of each heat level across genotypes and to assess significance of the heat level effect, the following model was used:

$$Y_{bcir} = \mu + C_c + R_{cr} + B_{bcr} + G_i + (G.C)_{ci} + e_{bcir} \ , \quad (2)$$

where Y_{bcir} was the phenotypic observation of the i^{th} genotype in the b^{th} block within the r^{th} replication nested in the c^{th} heat level. C_c was the effect of the c^{th} heat level, R_{cr} was the effect of the r^{th} replication nested in the c^{th} heat level, B_{bcr} was the effect of the b^{th} block in the r^{th} replication nested in the c^{th} heat level and $(C.G)_{ci}$ was the interaction between the i^{th} genotype and the c^{th} heat level. e_{bcir} designated the residual error term. C_c was regarded as fixed, all other effects were set as random. Traits with a significant C_c effect were regarded as heat dependent traits. AEM of each heat level across genotypes were estimated.

Heritability: Genotypic σ^2_g and error σ^2_e variance components for each heat level were calculated using model (1) with a random G_i effect. For each trait, the broad sense heritability (H^2) (Hallauer et al., 2010) of the observations at each heat level was calculated considering the number of replications. All mixed model analyses were performed using the software ASReml (Gilmour et al., 2006).

Heat tolerance: A heat susceptibility index (HSI) for each trait and each genotype was calculated according to the formula of Mason et al. (2010).

$$\mathrm{HSI}_i = \frac{1 - \mathrm{AEM}_{i\mathrm{H}}/\mathrm{AEM}_{i\mathrm{S}}}{1 - \mathrm{AEM}_H/\mathrm{AEM}_S}, \quad (3)$$

where HSI_i was the HSI for genotype i, $\mathrm{AEM}_{i\mathrm{H}}$ the AEM for genotype i at heat conditions and $\mathrm{AEM}_{i\mathrm{S}}$ the AEM for genotype i at standard conditions, calculated with model (1). $\mathrm{AEM}_{\mathrm{H}}$ was the AEM at heat conditions and $\mathrm{AEM}_{\mathrm{S}}$ the AEM at standard conditions across all genotypes. Pairwise Pearson correlations were calculated between the HSI of all assessed traits across genotypes. Further, HSI of this study were correlated with HSI assessed during adult stage under field conditions (Frey et al., 2015a). For a multi-trait analysis, we used the first two principal components (PC) of a PC analysis considering the previously calculated HSI for all genotypes of all traits, assessed in this study, as it was done for field traits by Frey et al. (2015a).

Genetic map creation

SNP markers with a significant ($P< 0.001$) deviation of the observed from the expected allele frequency were excluded from the analysis (Benke et al., 2014). To improve the mapping of markers, marker information of five segregating populations, which have been genotyped with the same set of molecular markers in a companion study (Horn et al., 2015), was included in the map creation. A consensus genetic linkage map was calculated chromosome-wise using the software CarthaGène (de Givry et al., 2005).

QTL analyses

QTL associated with heat tolerance were detected using an iterative composite interval mapping approach (iQTLm) (Charcosset et al., 2001), implemented in the software MCQTL (cf. Bardol et al., 2013), making use of the above described consensus linkage map. QTL analyses were conducted for PC1 and PC2 as well as for the HSI of the individual traits, $\mathrm{HSI}_{\mathrm{LR}}$, $\mathrm{HSI}_{\mathrm{SD}}$, $\mathrm{HSI}_{\mathrm{SC}}$, $\mathrm{HSI}_{\mathrm{SN}}$, $\mathrm{HSI}_{\mathrm{LL}}$, $\mathrm{HSI}_{\mathrm{PH}}$, $\mathrm{HSI}_{\mathrm{NL}}$, $\mathrm{HSI}_{\mathrm{DW}}$ and $\mathrm{HSI}_{\mathrm{WC}}$.

The analyses were performed across all six populations (cf. the multipopulation analyses described by Bardol et al., 2013 and Blanc et al., 2006). We took into account connections between populations through shared parental inbred lines using a kinship matrix specifying the parents of the six populations. We considered additive effects of the eight parental inbred lines. Since the included biparental $F_{3:4}$ populations showed a supposed heterozygosity of 25%, the QTL analyses included, further, dominance effects between parental alleles of each biparental population. Genotypic probabilities were computed every 5 cM, taking into account information from neighbouring markers. F thresholds for each trait to detect QTL were determined by 1 000 permutation tests, to correspond to a global type I risk of 5% across populations and across the entire genome. F thresholds used to select cofactors were fixed at 90% of the F threshold values for QTL detection, as suggested by the MCQTL software during the cofactor selection process. SNP markers associated with the respective HSI or PC were selected as cofactors by forward regression, where the minimal distance between two cofactors was 10 cM. At the end

of the detection process, confidence regions (logarithmic odds ratio (LOD) drop regions) were estimated on the basis of a 1.5 LOD unit fall.
To test, if the dominance effect of each population on the respective QTL was significantly different from 0, significance ($\alpha = 0.05$) was calculated a posteriori from a normal distribution using a two-sided test (personal communication, B. Mangin Aug. 2014). The difference between the additive effects of pairs of parental alleles on the respective QTL was tested a posteriori using a multicomparison t-test (Tukey) with $\alpha = 0.05$.
A multi-variate QTL analysis was conducted including the HSI_{LR}, HSI_{SD}, HSI_{SC}, HSI_{SN}, HSI_{LL}, HSI_{PH}, HSI_{NL}, HSI_{DW} and HSI_{WC}. Therefore, a pleiotropic QTL test, described in Mangin et al. (2012) (cf. Mangin et al., 1998), was applied. Genomic regions of particular interest were specified as regions comprising one or multiple QTL confidence intervals. Overlaps of confidence intervals of QTL for heat tolerance during seedling stage with QTL for heat tolerance during adult stage were specified as QTL hot spots.
Candidate gene search: To identify candidate genes for heat tolerance in terms of the assessed traits, we mined candidate genes, which were identified in a previous study (Frey et al., 2015b) as heat responsive or heat tolerance genes. Therefore, we determined the position of these genes on the previously described genetic map by linear regression with information of the nearest two SNP markers. Candidate genes mapping in the identified QTL confidence intervals were designated in the following as heat tolerance and heat responsive candidate genes.

Results

At the two studied heat levels, broad sense heritability (H^2) across populations was medium to high ($0.50 - 0.83$) for all assessed traits (Table 1). H^2 was higher at heat compared to standard conditions for all traits except NL and SD. We observed a significant heat level effect across populations for all assessed traits (Table 2). The adjusted entry means (AEM) across all genotypes for DW, LL, PH, SD and WC at heat were lower compared to standard conditions, whereas the AEM of NL, SC and SN were higher at heat compared to standard conditions.
The AEM for LR was significantly ($\alpha = 0.05$) lower at heat compared to standard conditions across genotypes of populations 1 and 2 (Dent x Dent), whereas it was not significantly different between the heat levels across genotypes of populations 4, 5 and 6 and it was higher at heat conditions across genotypes of population 3. For the traits DW, LR, LL and SC, we observed higher differences between heat and standard conditions of the AEM across genotypes of Dent x Dent compared to genotypes of Flint x Flint and Dent x Flint populations, whereas the differences between heat levels for SD was lower in Dent x Dent compared to Flint x Flint populations. An obvious pool effect was not observed for the traits PH, SN, WC and NL, i.e. differences between heat levels of populations from different pools did not represent separate significance clusters (Table 2).
The first two PCs of the PC analysis (Figure 2) explained 45 and 14% of the total variance. PC1 was significantly ($\alpha = 0.01$) correlated with each HSI, where the correlation was low ($< |0.25|$) with HSI_{SD} and between 0.36 and 0.85 with the other HSI (Figure 3). PC2 was significantly correlated with the HSI of each trait except HSI_{NL} and HSI_{WC}, where the correlation was low ($< |0.25|$) with all HSI except with HSI_{SC} and HSI_{SD}. PC1 had positive loadings for HSI_{SC}, HSI_{LR}, HSI_{WC}, HSI_{DW}, HSI_{LL}, HSI_{PH}, HSI_{SN} and a negative loading for HSI_{NL} (Figure 2). PC2 had high loadings for HSI_{SC} and HSI_{SD}, where the loading of the former HSI was in the positive and the loading of the latter mentioned in the negative range. PC1 and PC2 separated three overlapping clusters, which represented the Dent x Dent types (populations 1 and 2), the Flint x Flint types (populations 3 and 4) and the Dent x Flint types (populations 5 and 6) (Figure 2).
We observed significant ($\alpha = 0.01$) correlations between heat susceptibility during the seedling stage on a multi-trait level (PC1) and heat susceptibility during adult stage with respect to the HSI for the time to male (HSI_{MF}^{Field}) and female flowering (HSI_{FF}^{Field}), leaf scorching (HSI_{SC}^{Field}) and adjusted dry grain yield (HSI_{DYA}^{Field}) assessed at field conditions (Frey et al., 2015a; Figure 3). PC1 was positively correlated with HSI_{MF}^{Field} and HSI_{FF}^{Field} and negatively correlated with HSI_{DYA}^{Field} and HSI_{LS}^{Field}.
The consensus genetic linkage map had a total length of 1 823.5 centiMorgan (cM) with an average distance of 11.3 cM and a maximum distance of 83.2 cM between two adjacent markers.
For all HSI, PCs, and the multi-trait analysis in this study, we identified a total of 22 QTL (Table 3), each explaining between 6 and 9% of the variance (R^2) of the respective HSI or PC. With simultaneous fits across all QTL detected for each HSI and PC, 13, 14 and 12% of the variance could be explained for HSI_{LL}, PC1 and

PC2, respectively. We observed 7 genomic regions of particular interest which include all detected QTL with confidence intervals (Figure 4). From a set of candidate genes associated with heat tolerance and heat response, identified by Frey et al. (2015b), a total of 8 heat tolerance and 134 heat responsive candidate genes were found within the 22 QTL confidence intervals detected in this present study (Table 4 and Supplementary material Table 5). A total of four hot spots were found, overlapping QTL detected in this study and by Frey et al. (2015a) (Figure 5).

Discussion

Heritability and multi-trait heat susceptibility The broad sense heritability (H^2) for the assessed traits was medium to high. H^2 was comparable with heritability of traits observed by Frey et al. (2015b). This observation suggested that we achieved a reliable estimation of the adjusted entry means (AEM) for each genotype, which is the prerequisite for a successful detection of QTL.

We observed higher H^2 values for most traits at heat compared to standard conditions. This difference between conditions of H^2 was due to an increased variance between genotypes at heat conditions while the error variance was not notably increased (data not shown). This was in contrast to studies under field conditions, where the heritability was generally lower at heat compared to standard conditions due to increased error variances in relation to the genotypic variance (Frey et al., 2015a; Cairns et al., 2013). This difference can be explained by inferring environmental factors which become more important under heat conditions, i.e. soil heterogeneity, are generally not present under controlled conditions.

With PC1 and 2, two multi-trait measures for heat susceptibility were presented. These were significantly ($\alpha = 0.05$) correlated with each HSI, with the exception of non significant correlations of PC2 with HSI_{NL} and HSI_{WC} (Figure 3). A low PC1 implies heat tolerance with respect to most seedling traits, where PC1 explained 45% of the total variance of all assessed HSI. A high PC2 means higher heat tolerance with respect to SD, SN, PH, LL and DW, whereas it means lower heat tolerance with respect to SC and LR. PC2 is, thus, in contrast to PC1, not a clearly defined multi-trait measure for heat tolerance. The use of PC1 enables an easy classification of heat tolerant and heat susceptible genotypes across multiple phenotypic seedling characters with one single value.

Relationship between heat tolerance during seedling and adult stage Multi-trait heat tolerance in terms of seedling performance (PC1 and PC2) was significantly ($\alpha = 0.05$) negatively correlated with heat tolerance with respect to grain yield and leaf scorching under field conditions (Frey et al., 2015a; Figure 3). Reduced performance during seedling stage of genotypes with higher heat tolerance during adult stage could be an indication of not yet defined developmental adaptation to heat stress leading to reduced yield loss. This, however, requires further detailed research on phenotypic growth components of seedlings and adults plants. Nevertheless, the correlation was low ($<|0.25|$), which was expected as plant performance in young stages may have limited implications on plant performance at maturity.

Despite negative correlation of heat tolerance during seedling and adult stage, we observed with L043 and S067 that genotypes exist which show heat tolerance in both stages (Figure 2). Both types of heat tolerance in one genotype can be achieved by a selection on a combination of QTL associated with both, heat tolerance during seedling and during adult stage (Figure 5). A selection on heat tolerance during both developmental stages is possible, where QTL do not fall into the same genomic region, e.g. the QTL for HSI^{Field}_{DYA} and for PC1 on chromosome 3. In cases of overlapping of QTL between heat tolerance during seedling and during adult stage, we observed contrary effects of the parental alleles, e.g. the additive effect of the allele of parental inbred line P040 on the QTL for HSI^{Field}_{LS} was -0.57, whereas it was 0.50 at the QTL associated with HSI_{SC}, where the QTL are at the same location. This observation could be a pleitropic effect of the same genome region causing a different phenotype in different developmental stages. In such cases it is not possible to select on heat tolerance during seedling stage without deteriorating heat tolerance during adult stage and vice versa. To verify if pleiotropy is present fine mapping of the genome region should be performed.

Further genomic hot spots, where QTL for heat tolerance during seedling and adult stage overlapped were located at the beginning of chromosome 2, in the middle of chromosome 5 and on chromosome 9. At these locations we assume important genetic mechanisms associated with heat tolerance during seedling and during adult stage. To verify, if the QTL for heat tolerance during different developmental stages are linked

and, thus, pleiotropic needs further fine mapping, as mentioned previously. However, as important aspects of heat stress may be overlooked in experiments under controlled conditions (Roy et al., 2011), we encourage the verification of the results of this present study with experiments at field conditions according to Frey et al. (2015a).

Heat tolerance of Flint and Dent heterotic pools We observed higher differences of the AEM for LR, DW, LL and SC between heat and standard conditions of genotypes derived from Dent x Dent crosses in comparison with genotypes derived from Flint x Flint crosses. This suggests higher heat tolerance during seedling stage of the latter. From a statement of Hallauer (1990) that Flints contributed chilling tolerance to cultivars bred for temperate Europe, we infer that genotypes derived from the Flint pool exhibit increased tolerance to low temperatures in contrast to genotypes derived from the Dent pool. Higher tolerance to temperature extremes during seedling stage of genotypes derived from the Flint pool compared to genotypes derived from the Dent pool has already been reported for root growth by Reimer et al. (2013). The reduced heat tolerance during seedling stage of genotypes derived from Dent x Dent crosses is in contrast to their higher heat tolerance during adult stage at field conditions (Frey et al., 2015a). Due to heat tolerance in different developmental stages of Dent and Flint types, both heterotic pools should be considered to increase heat tolerance across developmental stages.

Genetic linkage map The genetic map was constructed based on marker information of six segregating populations mentioned above and five additional populations of a companion study (Horn et al., 2015). This multi-population approach improved the creation of the genetic map due to a higher marker density and increased information for each marker. The map length and the distances between adjacent markers were similar to the properties of the genetic map in another study in maize (Blanc et al., 2006), where the map length was 1 794 cM and the average marker distance was 7cM. We observed a condensation of markers at the centromeres of the chromosomes by genetic position in comparison to the distribution of markers by physical position, which was the basis of the selection of markers for this study. This discrepancy between genetic and physical position was due to lower recombination rates at the centromeres, which was described previously by Payseur & Nachman (2000). Nevertheless, the order of the markers by their genetic positions was consistent with the physical order of markers on the chromosomes and a sufficient marker density was present across the whole genome to establish a genetic linkage map.

Detected QTL and candidate genes Each of the detected QTL explained a relatively small part of the variance of the respective trait (<10%). This was comparable with the proportion of variance explained by QTL observed in other papers studying heat (Frey et al., 2015a) and other abiotic stresses in maize (Rodríguez et al., 2013, Messmer et al., 2011). This finding suggests that the inheritance of the assessed seedling traits is of quantitative nature.
Besides overlapping of QTL confidence intervals for heat tolerance during seedling and during adult stage, we also observed overlapping of the detected QTL confidence intervals in this study with QTL regions associated with other abiotic stresses. We identified an overlapping region of the confidence intervals of QTL for HSI_{LL} and PC2 on chromosome 2 with, first, a QTL associated with cold tolerance, which was identified by a meta-analysis across QTL studies (Rodríguez et al., 2013), second, a QTL, which was identified for the shoot and root dry weight and the leaf area under drought stress conditions (Ruta et al., 2010) and, third, a QTL for the leaf chlorophyll content at drought stress, which was identified by Messmer et al. (2011), in the first half of chromosome 2. Thus, different abiotic stresses might have similar genetic regulation mechanisms, as outlined by Frey et al. (2015a) and the previously mentioned region may be a genetic hotspot for abiotic stress tolerance. In future studies, the mentioned region should be fine mapped. Furthermore, functional studies on candidate genes in the respective region, which are discussed in the following, could clarify the involved genes and pathways in heat tolerance and in the tolerance to abiotic stresses in general. The candidate genes, located in the identified QTL confidence intervals detected in this study (Tables 4 and Supplementary material Table 5), can help to explain the molecular basis of heat tolerance and response mechanisms. Six out of a total of eight heat tolerance genes were found in the QTL confidence intervals on chromosome 2. This overrepresentation of heat tolerance genes in a particular region, colocated with several QTL for heat tolerance, show the importance of genetic mechanisms for heat tolerance, located on this chromosome, as outlined previously. One of the six heat

tolerance genes, GRMZM2G099425, was already described to be a calcium-dependent protein kinase and may be involved in calcium signalling, which is one major factor of the heat tolerance in maize (Frey et al., 2015b). To narrow down further the set of candidate genes associated with the reaction upon heat stress and with heat tolerance, fine mapping of the QTL regions should be conducted, as outlined by Frey et al. (2015a) and functional studies of the candidate genes should be applied.

Conclusion

Genotypes derived from crosses of temperate Flint lines were more heat tolerant during seedling stage than genotypes derived from the temperate Dent pool, during adult stage (Frey et al., 2015a) this observation was vice versa. We detected each two QTL for two principal components which represented 59% of the heat tolerance with respect to nine seedling traits. For each principal component, in a simultaneous fit, the detected QTL explained a total of 14 and 12%, respectively. With this study, we provided a base for marker assisted selection, allowing plant breeders to prescreen genotypes for a selection on heat tolerance during seedling stage. Pleiotropy between heat tolerance during seedling and during adult stage was observed in genomic hot spot regions, especially on chromosome 2. This, however requires further research.

Acknowledgements

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References

Andersen J.R., Schrag T., Melchinger A.E., Zein I. & Lübberstedt T. (2005) Validation of Dwarf8 polymorphisms associated with flowering time in elite European inbred lines of maize (Zea mays L.). *Theoretical and Applied Genetics* 111, 206–17.

Bardol N., Ventelon M., Mangin B., Jasson S., Loywick V., Couton F., Derue C., Blanchard P. et al. (2013) Combined linkage and linkage disequilibrium QTL mapping in multiple families of maize (Zea mays L.) line crosses highlights complementarities between models based on parental haplotype and single locus polymorphism. *Theoretical and Applied Genetics* 126, 2717–36.

Benke A., Urbany C., Marsian J., Shi R., Wirén N.V. & Stich B. (2014) The genetic basis of natural variation for iron homeostasis in the maize IBM population. *BMC plant biology* 14, 12.

Blanc G., Charcosset A., Mangin B., Gallais A. & Moreau L. (2006) Connected populations for detecting quantitative trait loci and testing for epistasis: an application in maize. *Theoretical and Applied Genetics* 113, 206–24.

Cairns J.E., Crossa J., Zaidi P.H., Grudloyma P., Sanchez C., Araus J.L., Thaitad S., Makumbi D. et al. (2013) Identification of Drought, Heat, and Combined Drought and Heat Tolerant Donors in Maize. *Crop Science* 53, 1335.

Charcosset A., Mangin B., Moreau L., Combes L., Jourjon M.F. & Gallais A. (2001) Heterosis in maize investigated using connected RIL populations. In *Quantitative genetics and breeding methods : the way ahead. Les colloques no 96*, pp. 89–98. INRA, Paris, France.

de Givry S., Bouchez M., Chabrier P., Milan D. & Schiex T. (2005) CARTHAGENE: multipopulation integrated genetic and radiation hybrid mapping. *Bioinformatics* 21, 1703–4.

Deutsches Maiskomitee (2013) http://www.maiskomitee.de/web/public/Fakten.aspx/Statistik/Deutschland/Statistik_Biogas. Accessed 19. November 2013.

Frey F.P., Presterl T., Lecoq P., Orlik A. & Stich B. (2015a) Heat tolerance in temperate maize - Identification of QTL across multiple environments with connected

segregating populations. *submitted to Theoretical and Applied Genetics* .

Frey F.P., Urbany C., Hüttel B., Reinhardt R. & Stich B. (2015b) Genome-wide expression profiling and phenotypic evaluation of European maize inbreds at seedling stage in response to heat stress. *BMC Genomics* 16, 123.

Frova C. & Sari-Gorla M. (1994) Quantitative trait loci (QTLs) for pollen thermotolerance detected in maize. *Molecular and General Genetics* 245, 424–30.

Ganal M.W., Durstewitz G., Polley A., Bérard A., Buckler E.S., Charcosset A., Clarke J.D., Graner E.M. et al. (2011) A large maize (Zea mays L.) SNP genotyping array: development and germplasm genotyping, and genetic mapping to compare with the B73 reference genome. *PloS one* 6, e28334.

Giaveno C. & Ferrero J. (2003) Introduction of tropical maize genotypes to increase silage production in the central area of Santa Fe, Argentina. *Crop Breeding and Applied Biotechnology* 3, 89–94.

Gilmour A., Gogel B., Cullis B. & Thompson R. (2006) *ASReml User Guide Release 2.0*. VSN International Ltd, Hemel Hempstead, HP1 1ES, UK.

Hallauer A.R. (1990) Methods used in developing maize inbreds. *Maydica* 35, 1–16.

Hallauer A.R., Carena M.J. & Miranda Filho J.B. (2010) *Quantitative Genetics in Maize Breeding*. Springer New York, New York, NY.

Horn F., Habekuß A. & Stich B. (2015) Linkage mapping of barley yellow dwarf virus resistance in connected populations of maize. *BMC Plant Biology* 15, 29.

IPCC (2013) Summary for policymakers. In *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* (eds. T.F. Stocker, D. Qin, G.K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex & P. Midgley). Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Lobell D.B. & Field C.B. (2007) Global scale climate-crop yield relationships and the impacts of recent warming. *Environmental Research Letters* 2, 014002.

Mangin B., Cathelin R., Delannoy D., Escalière B., Lambert S., Marcel J., Ngom B., Jourjon M.f. et al. (2012) MCQTL : A reference manual.

Mangin B., Thoquet P. & Grimsley N. (1998) Pleiotropic QTL Analysis. *Biometrics* 54, 88–99.

Mason R.E., Mondal S., Beecher F.W., Pacheco A., Jampala B., Ibrahim A.M.H. & Hays D.B. (2010) QTL associated with heat susceptibility index in wheat (Triticum aestivum L.) under short-term reproductive stage heat stress. *Euphytica* 174, 423–436.

Messmer R., Fracheboud Y., Bänziger M., Stamp P. & Ribaut J.M. (2011) Drought stress and tropical maize: QTLs for leaf greenness, plant senescence, and root capacitance. *Field Crops Research* 124, 93–103.

Ottaviano E., Sari Gorla M., Pè E. & Frova C. (1991) Molecular markers (RFLPs and HSPs) for the genetic dissection of thermotolerance in maize. *Theoretical and Applied Genetics* 81, 713–719.

Payseur B.A. & Nachman M.W. (2000) Microsatellite variation and recombination rate in the human genome. *Genetics* 156, 1285–1298.

Reimer R., Stich B., Melchinger A.E., Schrag T.A., Sørensen A.P., Stamp P. & Hund A. (2013) Root response to temperature extremes: association mapping of temperate maize (Zea mays L). *Maydica* 58, 156–168.

Rodríguez V.M., Butrón A., Rady M.O.a., Soengas P. & Revilla P. (2013) Identification of quantitative trait loci involved in the response to cold stress in maize (Zea mays L.). *Molecular Breeding* 33, 363–371.

Roy S.J., Tucker E.J. & Tester M. (2011) Genetic analysis of abiotic stress tolerance in crops. *Current Opinion in Plant Biology* 14, 232–9.

Ruta N., Stamp P., Liedgens M., Fracheboud Y. & Hund A. (2010) Collocations of QTLs for Seedling Traits and Yield Components of Tropical Maize under Water Stress Conditions. *Crop Science* 50, 1385.

Sage R.F., Kocacinar F. & Kubien D.S. (2011) C4 photosynthesis and temperature. In *C4 Photosynthesis and Related CO2 Concentrating Mechanisms* (eds. A.S. Raghavendra & R.F. Sage), chap. 10, pp. 161–195. Springer, Dordrecht, The Netherlands.

Wahid a., Gelani S., Ashraf M. & Foolad M. (2007) Heat tolerance in plants: An overview. *Environmental and Experimental Botany* 61, 199–223.

Tables & Figures

Table 1: Broad sense heritability (H^2) of the studied traits for each condition across the six populations.

Trait	25°C	38°C
Leaf length (LL)	0.73	0.79
Plant height (PH)	0.58	0.73
Number of leaves (NL)	0.71	0.63
Leaf scorching (SC)	0.49	0.82
Leaf senescence (SN)	0.57	0.70
Leaf greenness (SD)	0.83	0.67
Shoot dry weight (DW)	0.69	0.76
Shoot water content (WC)	0.54	0.59
Leaf growth rate (LR)	0.50	0.70

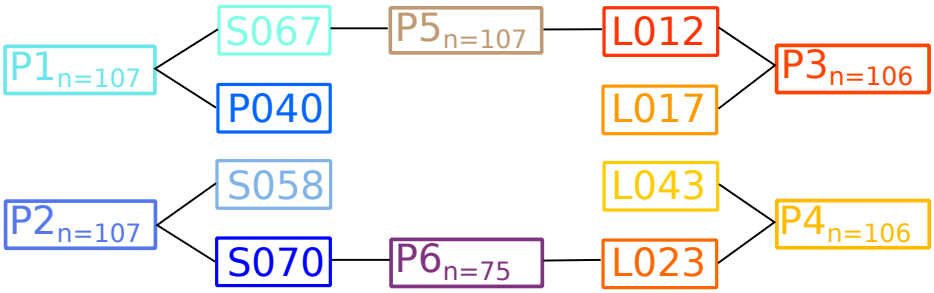

Figure 1: Crossing scheme used to create six segregating populations (P1-6) with number of genotypes (n), derived from four Dent (S067, P040, S058 and S070, in blue) and four Flint (L012, L017, L043 and L023, in red) inbred lines.

Table 2: Population-wise means of the adjusted entry means of the genotypes under heat conditions relative to the performance under standard conditions. Asterisks illustrate the significance level of a pairwise t-test, examining the difference between heat and standard conditions per population. Letters illustrate non-paired Tukey tests between the relative heat-standard differences of the six population. In the last column, the significance of the condition (standard and heat) effect for each trait across all population calculated with model (2) is given. For details see the material and methods section.

Trait / Population	1	2	3	4	5	6	Condition
Shoot dry weight (DW)	72^{***} B	54^{***} A	91^{***} DE	81^{***} CD	92^{**} E	76^{***} BC	***
Leaf growth rate (LR)	81^{***} B	59^{***} A	106^{***} C	98^{ns} C	98^{ns} C	98^{ns} C	***
Leaf length (LL)	67^{***} B	62^{***} A	77^{***} D	71^{***} C	71^{***} BC	74^{***} CD	***
Number of leaves (NL)	130^{***} B	116^{***} A	132^{***} B	134^{***} B	136^{***} B	131^{***} B	***
Plant height (PH)	72^{***} BC	62^{***} A	73^{***} C	67^{***} B	75^{***} C	72^{***} C	**
Leaf scorching (SC)	446^{***} C	553^{***} D	216^{***} A	248^{***} AB	204^{***} A	290^{***} B	***
Leaf greenness (SD)	84^{***} D	83^{***} D	73^{***} B	77^{***} C	69^{***} A	82^{***} D	***
Leaf senescence (SN)	192^{***} B	233^{***} C	182^{***} B	157^{***} A	176^{***} AB	172^{***} AB	***
Shoot water content (WC)	97^{***} C	95^{***} A	97^{***} C	97^{***} C	97^{***} C	96^{***} B	***

**, *** Significant at the 0.01 and 0.001 probability level, respectively.

^{ns} Not significant.

^{A}, ^{B}, ^{C}, ^{D}, ^{E} Relative differences between heat and standard conditions of populations with same letters are not significantly ($\alpha = 0.05$) different from each other.

Table 3: Quantitative trait loci (QTL) detected at a significance level of $\alpha = 0.05$, with genetic map position [cM], logarithmic odds ratio (LOD) support interval, proportion of explained variance [%], additive effects of each parent and dominance effects of the six (1-6) populations (1 P040xS067, 2 S070xS058, 3 L012xL017, 4 L043xL023, 5 S067xL012, 6 S070xL023).

						Additive effect of parent								Dominance effect of population					
Trait	QTL	Chr	Pos	Interval	R^2	L043	S058	L017	L023	L012	S067	S070	P040	1‡	2	3	4	5	6
HSI_{LR}	$Q_{HSI:LR}$	2	82	60-141	7	0.13^{AB}	0.12^{AB}	0.73^{AB}	-0.19^{A}	1.00^{B}	-0.24^{AB}	-0.06^{AB}	-1.49^{C}	0.99^{ns}	-3.63^{ns}	0.24^{ns}	-0.69^{ns}	-0.87^{ns}	0.66^{ns}
HSI_{SC}	$Q_{HSI:SC}$	9	74	0-74	7	-0.02^{A}	-0.02^{A}	0.18^{A}	0.01^{A}	-0.35^{A}	-0.33^{A}	0.03^{A}	0.50^{A}	1.34^{***}	1.05^{**}	-1.19^{ns}	0.29^{ns}	-2.51^{ns}	-0.28^{ns}
HSI_{LL}	$Q_{HSI:LLa}$	2	37	0-42	7	0.03^{ABC}	0.07^{AB}	0.01^{ABC}	-0.04^{AC}	0.02^{ABC}	0.08^{B}	-0.06^{AC}	-0.11^{C}	0.00^{ns}	-0.23^{ns}	-0.02^{ns}	-4.40^{ns}	-0.03^{ns}	0.78^{ns}
	$Q_{HSI:LLb}$	10	30	14-38	7	0.06^{AB}	-0.03^{AB}	0.02^{AB}	0.07^{A}	0.03^{A}	0.00^{AB}	-0.10^{B}	-0.05^{AB}	0.00^{ns}	-0.06^{ns}	-0.19^{**}	0.05^{ns}	-0.07^{ns}	0.29^{ns}
	Simultaneous fit				13														
Multi-trait	Q_{MTa}	1	136	123-144															
	Q_{MTb}	2	82	60-141															
	Q_{MTc}	2	115	106-133															
	Q_{MTd}	2	10	0-17															
	Q_{MTe}	3	80	68-94															
	Q_{MTf}	5	50	34-115															
	Q_{MTg}	5	101	70-151															
	Q_{MTh}	5	151	144-159															
	Q_{MTi}	5	158	0-167															
	Q_{MTj}	9	0	0-20															
	Q_{MTk}	9	0	0-17															
	Q_{MTl}	10	17	14-52															
PC1	Q_{PC1a}	3	80	57-92	8	-0.16^{ABC}	0.20^{ABC}	-0.42^{AB}	-0.52^{B}	0.18^{AC}	0.23^{AC}	0.48^{C}	0.02^{ABC}	0.50^{ns}	-3.30^{***}	0.32^{ns}	-1.12^{ns}	1.27^{ns}	-0.85^{*}
	Q_{PC1b}	9	0	0-19	8	-0.12^{ABC}	0.43^{A}	0.18^{ABC}	0.15^{AB}	-0.01^{ABC}	-0.44^{C}	-0.46^{BC}	0.26^{ABC}	0.64^{ns}	-0.32^{ns}	-0.09^{ns}	0.72^{ns}	0.15^{ns}	0.46^{ns}
	Simultaneous fit				14														
PC2	Q_{PC2a}	2	25	0-58	6	-0.03^{AB}	0.06^{AB}	-0.15^{AB}	-0.13^{A}	-0.16^{A}	-0.15^{A}	0.11^{AB}	0.47^{B}	0.15^{ns}	0.44^{ns}	0.61^{*}	0.28^{ns}	0.44^{ns}	-0.77^{*}
	Q_{PC2b}	5	147	68-161	7	-0.32^{CD}	0.45^{A}	-0.03^{ABCD}	-0.33^{C}	-0.09^{BCD}	-0.18^{C}	0.19^{ABD}	0.30^{AB}	-0.19^{ns}	0.34^{ns}	-0.16^{ns}	0.03^{ns}	0.37^{ns}	0.74^{**}
	Simultaneous fit				12														
HSI_{PH}	$Q_{HSI:PH}$	9	3	0-17	9	0.01^{ABCD}	0.10^{A}	0.05^{AB}	0.03^{AC}	0.05^{A}	-0.11^{BD}	-0.13^{CD}	0.01^{ABCD}	0.10^{ns}	0.00^{ns}	0.12^{ns}	0.20^{ns}	0.05^{ns}	0.15^{ns}
HSI_{SD}	$Q_{HSI:SD}$	5	101	70-151	8	0.26^{B}	-0.22^{A}	0.18^{B}	0.19^{B}	-0.01^{AB}	0.10^{B}	-0.23^{A}	-0.27^{A}	-0.17^{ns}	0.13^{ns}	-0.16^{ns}	-0.58^{ns}	0.00^{ns}	-0.11^{ns}

$^{A, B, C, D}$ Additive effects of parents with same letters are not significantly different from each other.
$^{*, **, ***}$ Significant at the 0.05, 0.01 and 0.001 probability level, respectively.
^{ns} Not significant.

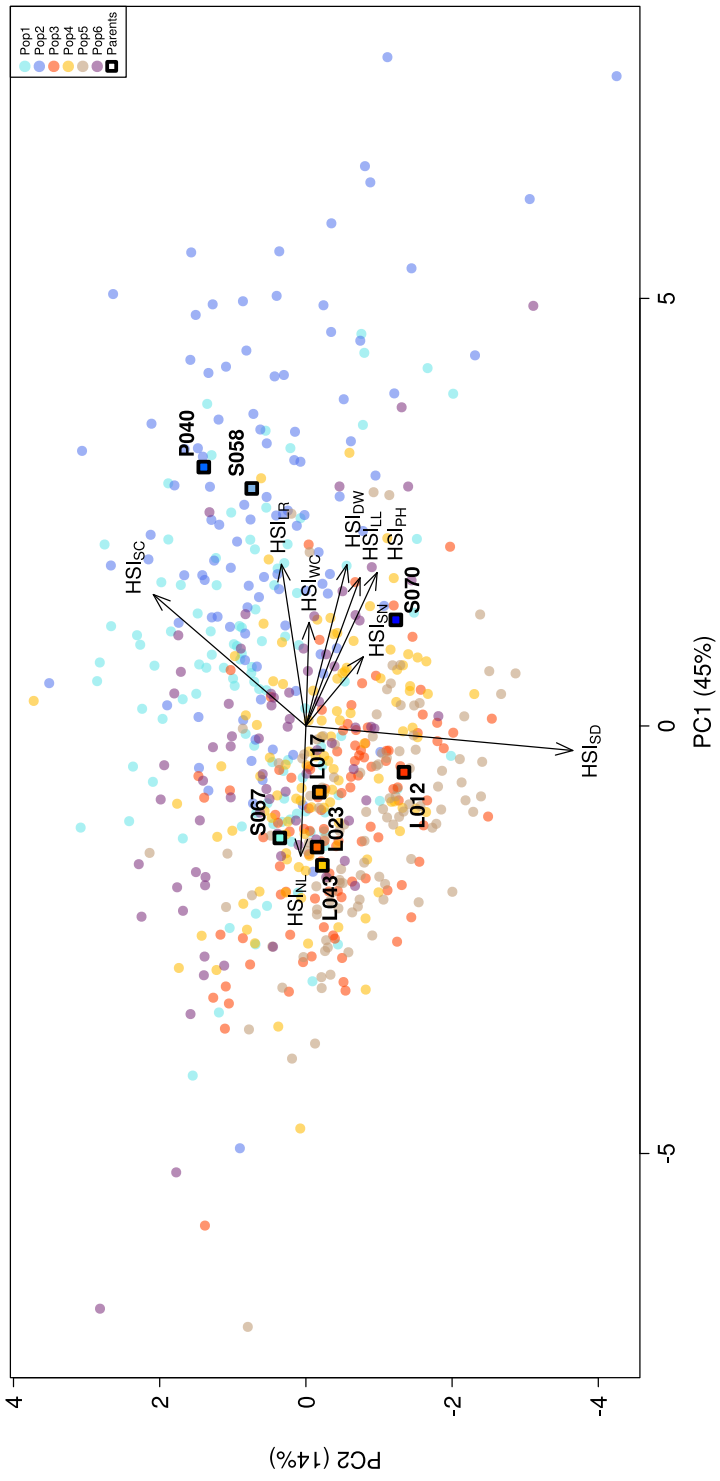

Figure 2: Plot of the first two principal components (PC1 and PC2) of a principal component analysis with the heat susceptibility indexes (HSI) of all assessed seedling traits (leaf length (LL), plant height (PH), number of leaves (NL), leaf scorching (SC), leaf senescence (SN), leaf greenness (SD), shoot dry weight (DW), shoot water content (WC) and leaf growth rate (LR)). The numbers in brackets denote the proportion of explained variance of the respective PC.

Table 4: Heat tolerance candidate genes within QTL confidence intervals.

Gene	Chr	QTL	Description
GRMZM2G115658	2	Q_{PC2a}	Uncharacterized protein
GRMZM2G148998	2	Q_{PC2a}, $Q_{HSI:LLa}$	Uncharacterized protein
GRMZM2G430362	2	Q_{PC2a}, $Q_{HSI:LLa}$	ATP-dependent RNA helicase SUV3
GRMZM2G537291	2	Q_{PC2a}	Uncharacterized protein
GRMZM2G035063	2	$Q_{HSI:LR}$, Q_{MTb}, Q_{MTc}	Chaperonin
GRMZM2G099425	2	$Q_{HSI:LR}$, Q_{MTb}, Q_{MTc}	Calcium-dependent protein kinase, isoform AK1; Uncharacterized protein
GRMZM2G436710	5	Q_{MTf}, $Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	Uncharacterized protein
GRMZM2G074017	5	Q_{MTi}	ATPase inhibitor

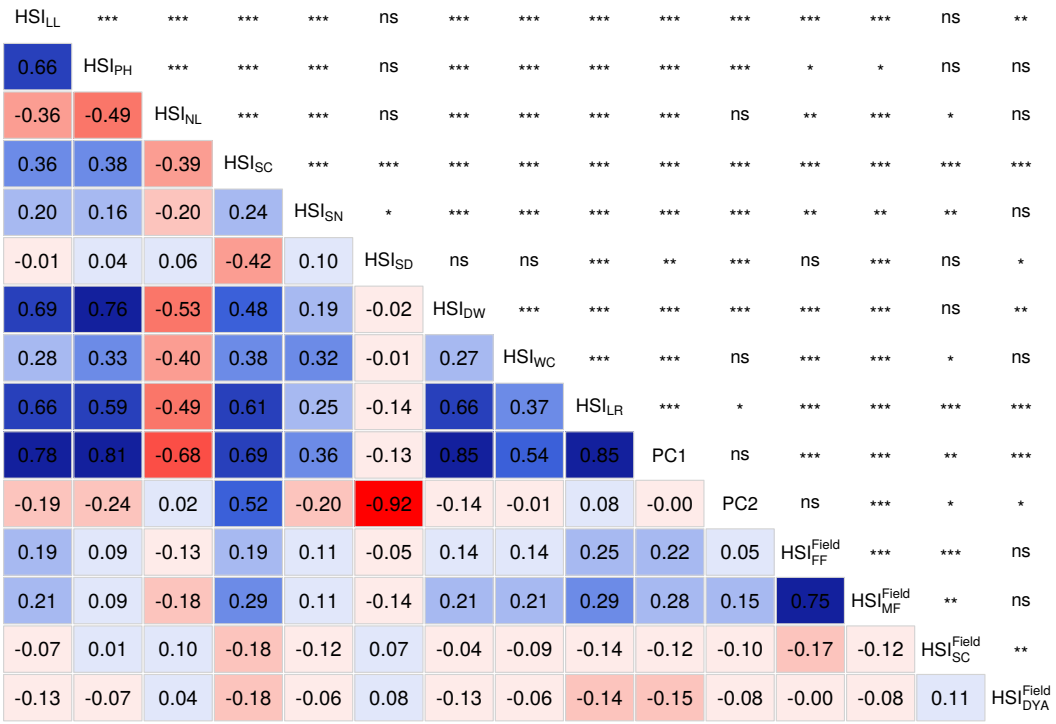

Figure 3: Correlations between the heat susceptibility indexes (HSI) of all seedling traits (leaf length (LL), plant height (PH), number of leaves (NL), leaf scorching (SC), leaf senescence (SN), leaf greenness (SD), shoot dry weight (DW), shoot water content (WC) and leaf growth rate (LR)), the first two principal components (PC) of the PC analysis (PC1 and PC2) and the HSI of traits, assessed during adult stage under field conditions (Frey et al., 2015a) (time to female (FF) and male flowering (MF), leaf scorching (SC) and dry grain yield adjusted with the time to female flowering (DYA)) across all genotypes and parental inbred lines. Significance of correlations is denoted with *, **, *** or ns for significant with $\alpha = 0.05$, $\alpha = 0.01$, $\alpha = 0.001$, or not significant, respectively.

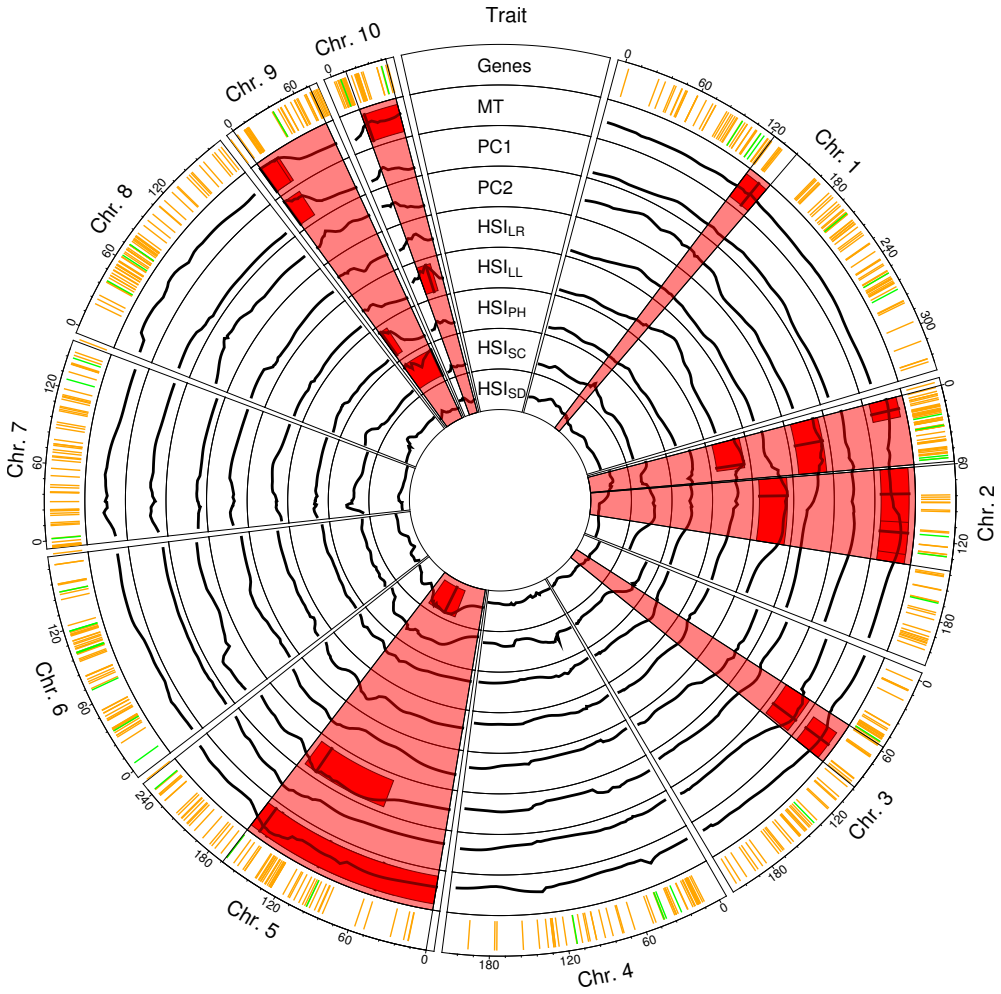

Figure 4: Heat tolerance (green) and heat responsive (orange) candidate genes and borders of the genomic regions of particular interest spanning the confidence intervals of all detected QTL (black) are represented in the first track. Tracks 2-8 show logarithmic odds ratio (LOD) scores (black), detected QTL and confidence intervals (red) of the QTL analyses for traits for which QTL have been detected (the multi-trait analysis (MT), principal component (PC) 1, PC2, and the heat susceptibility indexes (HSI) of the traits leaf elongation rate (LR), leaf length (LL), plant height (PH), leaf scorching (SC) and leaf greenness (SD). Genomic regions of particular interest are denoted in transparent red.

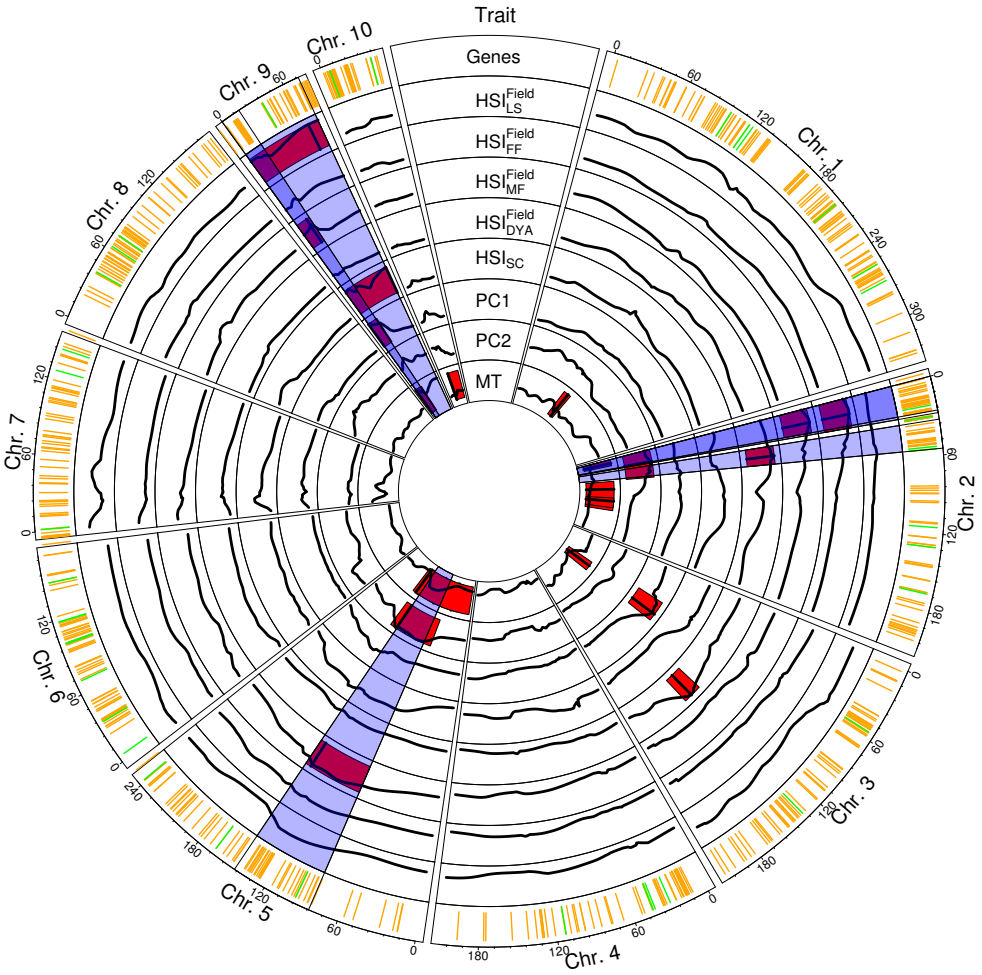

Figure 5: Heat tolerance (green) and heat responsive (orange) candidate genes and borders of the genomic hot spots, where QTL for seedling traits overlap with QTL for traits assessed with adult plants detected by Frey et al. (2015a) (black) are represented in the first track. Tracks 2-7 show logarithmic odds ratio (LOD) scores (black), detected QTL and confidence intervals regions (red) of the QTL analyses for the heat susceptibility indexes (HSI) of leaf scorching (LS), the time to female (FF) and male flowering (MF) and dry yield adjusted with the time to flowering (DYA), assessed during adult stage under field conditions by Frey et al. (2015a), leaf scorching (SC), principal component (PC) 1, PC2 and the multi-trait analysis (MT) assessed during seedling stage in this present study. Genomic hot spots are denoted in transparent blue.

Supplementary material

Table 5: Heat responsive candidate genes within QTL confidence intervals.

Gene	Chr	QTL	Description
GRMZM2G141526	1	Q_{MTa}	No information
GRMZM2G175351	1	Q_{MTa}	No information
GRMZM2G352248	1	Q_{MTa}	Uncharacterized protein
GRMZM2G430710	1	Q_{MTa}	No information
GRMZM2G464885	1	Q_{MTa}	Uncharacterized protein
GRMZM2G474602	1	Q_{MTa}	No information
AC212835.3_FG008	2	Q_{MTd}, Q_{PC2a}, $Q_{HSI:LLa}$	No information
GRMZM2G016649	2	Q_{MTd}, Q_{PC2a}, $Q_{HSI:LLa}$	Uncharacterized protein
GRMZM2G031904	2	Q_{MTd}, Q_{PC2a}, $Q_{HSI:LLa}$	Putative uncharacterized protein
GRMZM2G125669	2	Q_{MTd}, Q_{PC2a}, $Q_{HSI:LLa}$	Alternative oxidase
GRMZM2G153378	2	Q_{MTd}, Q_{PC2a}, $Q_{HSI:LLa}$	Putative uncharacterized protein
GRMZM2G175447	2	Q_{MTd}, Q_{PC2a}, $Q_{HSI:LLa}$	No information
GRMZM2G304745	2	Q_{MTd}, Q_{PC2a}, $Q_{HSI:LLa}$	No information
GRMZM2G322819	2	Q_{MTd}, Q_{PC2a}, $Q_{HSI:LLa}$	No information
GRMZM2G362413	2	Q_{MTd}, Q_{PC2a}, $Q_{HSI:LLa}$	No information
GRMZM2G392125	2	Q_{MTd}, Q_{PC2a}, $Q_{HSI:LLa}$	Uncharacterized protein
GRMZM6G859365	2	Q_{MTd}, Q_{PC2a}, $Q_{HSI:LLa}$	Cytochrome P450 CYP87A15
GRMZM2G007256	2	Q_{PC2a}, $Q_{HSI:LLa}$	Adhesive/proline-rich protein; Uncharacterized protein
GRMZM2G015727	2	Q_{PC2a}, $Q_{HSI:LLa}$	Putative uncharacterized protein
GRMZM2G032209	2	Q_{PC2a}, $Q_{HSI:LLa}$	Uncharacterized protein
GRMZM2G037413	2	Q_{PC2a}, $Q_{HSI:LLa}$	Cellulose synthase catalytic subunit 11
GRMZM2G040858	2	Q_{PC2a}, $Q_{HSI:LLa}$	Uncharacterized protein ycf72
GRMZM2G042443	2	Q_{PC2a}, $Q_{HSI:LLa}$	RNA-dependent DNA polymerase; RNA-dependent RNA polymerase
GRMZM2G049538	2	Q_{PC2a}, $Q_{HSI:LLa}$	Acyclic sesquiterpene synthase
GRMZM2G051571	2	Q_{PC2a}, $Q_{HSI:LLa}$	Uncharacterized protein
GRMZM2G060444	2	Q_{PC2a}, $Q_{HSI:LLa}$	Homeodomain leucine zipper family IV protein
GRMZM2G077420	2	Q_{PC2a}, $Q_{HSI:LLa}$	No information
GRMZM2G082032	2	Q_{PC2a}, $Q_{HSI:LLa}$	Uncharacterized protein
GRMZM2G088778	2	Q_{PC2a}	No information
GRMZM2G089596	2	Q_{PC2a}, $Q_{HSI:LLa}$	Uncharacterized protein
GRMZM2G093526	2	Q_{PC2a}, $Q_{HSI:LLa}$	Uncharacterized protein
GRMZM2G106092	2	Q_{PC2a}	No information
GRMZM2G115705	2	Q_{PC2a}, $Q_{HSI:LLa}$	Uncharacterized protein
GRMZM2G119773	2	Q_{PC2a}, $Q_{HSI:LLa}$	Uncharacterized protein
GRMZM2G121700	2	Q_{PC2a}, $Q_{HSI:LLa}$	Uncharacterized protein
GRMZM2G135387	2	Q_{PC2a}, $Q_{HSI:LLa}$	Uncharacterized protein
GRMZM2G135990	2	Q_{PC2a}	Putative uncharacterized protein
GRMZM2G137161	2	Q_{PC2a}, $Q_{HSI:LLa}$	Amino acid/polyamine transporter II
GRMZM2G137964	2	Q_{PC2a}	Uncharacterized protein
GRMZM2G147491	2	Q_{PC2a}	Uncharacterized protein
GRMZM2G154437	2	Q_{PC2a}, $Q_{HSI:LLa}$	Uncharacterized protein
GRMZM2G154685	2	Q_{PC2a}, $Q_{HSI:LLa}$	No information
GRMZM2G157822	2	Q_{PC2a}, $Q_{HSI:LLa}$	No information
GRMZM2G160585	2	Q_{PC2a}, $Q_{HSI:LLa}$	Glycosyltransferase
GRMZM2G168985	2	Q_{PC2a}, $Q_{HSI:LLa}$	No information
GRMZM2G169013	2	Q_{PC2a}, $Q_{HSI:LLa}$	Uncharacterized protein
GRMZM2G177561	2	Q_{PC2a}, $Q_{HSI:LLa}$	No information
GRMZM2G178321	2	Q_{PC2a}	Uncharacterized protein
GRMZM2G323309	2	Q_{PC2a}, $Q_{HSI:LLa}$	No information
GRMZM2G331701	2	Q_{PC2a}, $Q_{HSI:LLa}$	Uncharacterized protein
GRMZM2G334336	2	Q_{PC2a}, $Q_{HSI:LLa}$	No information
GRMZM2G365815	2	Q_{PC2a}	Calcium-dependent protein kinase
GRMZM2G408038	2	Q_{PC2a}, $Q_{HSI:LLa}$	No information
GRMZM2G408963	2	Q_{PC2a}, $Q_{HSI:LLa}$	Peroxidase 65
GRMZM2G520811	2	Q_{PC2a}	No information
GRMZM5G829946	2	Q_{PC2a}, $Q_{HSI:LLa}$	Uncharacterized protein
GRMZM2G035063	2	$Q_{HSI:LR}$, Q_{MTb}, Q_{MTc}	Chaperonin
GRMZM2G052908	2	$Q_{HSI:LR}$, Q_{MTb}	Uncharacterized protein
GRMZM2G065665	2	$Q_{HSI:LR}$, Q_{MTb}	No information
GRMZM2G066343	2	$Q_{HSI:LR}$, Q_{MTb}, Q_{MTc}	Uncharacterized protein
GRMZM2G075244	2	$Q_{HSI:LR}$, Q_{MTb}, Q_{MTc}	Cytochrome P450 CYP709C14
GRMZM2G075461	2	$Q_{HSI:LR}$, Q_{MTb}, Q_{MTc}	Uncharacterized protein
GRMZM2G079231	2	$Q_{HSI:LR}$, Q_{MTb}	Uncharacterized protein
GRMZM2G080466	2	$Q_{HSI:LR}$, Q_{MTb}	Uncharacterized protein
GRMZM2G082487	2	$Q_{HSI:LR}$, Q_{MTb}	Protein phosphatase 2C
GRMZM2G096106	2	$Q_{HSI:LR}$, Q_{MTb}, Q_{MTc}	No information
GRMZM2G125775	2	$Q_{HSI:LR}$, Q_{MTb}	AN17
GRMZM2G125850	2	$Q_{HSI:LR}$, Q_{MTb}, Q_{MTc}	Uncharacterized protein
GRMZM2G373554	2	$Q_{HSI:LR}$, Q_{MTb}	Uncharacterized protein
GRMZM5G846916	2	$Q_{HSI:LR}$, Q_{MTb}, Q_{MTc}	No information
GRMZM2G028665	3	Q_{PC1a}	Uncharacterized protein
GRMZM2G057413	3	Q_{PC1a}, Q_{MTe}	No information
GRMZM2G060536	3	Q_{PC1a}	EMP5
GRMZM2G110195	3	Q_{PC1a}, Q_{MTe}	Uncharacterized protein
GRMZM2G149330	3	Q_{PC1a}	No information
GRMZM2G156861	3	Q_{PC1a}, Q_{MTe}	Lipoxygenase
GRMZM2G346312	3	Q_{PC1a}, Q_{MTe}	Uncharacterized protein

Continued on next page

Gene	Chr	QTL	Description
GRMZM2G426046	3	Q_{PC1a}, Q_{MTe}	No information
GRMZM2G479112	3	Q_{PC1a}, Q_{MTe}	Uncharacterized protein
GRMZM2G078667	3	Q_{MTe}	Putative uncharacterized protein
GRMZM2G066578	5	Q_{MTf}, $Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	Uncharacterized protein
GRMZM2G067306	5	Q_{MTf}, $Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	5S rRNA binding protein
GRMZM2G100412	5	Q_{MTf}, $Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	No information
GRMZM2G102862	5	Q_{MTf}, $Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	No information
GRMZM2G111475	5	Q_{MTf}, $Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	No information
GRMZM2G130053	5	Q_{MTf}, Q_{MTi}	Cysteine protease 1
GRMZM2G144420	5	Q_{MTf}, $Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	No information
GRMZM2G173596	5	Q_{MTf}, $Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	ZIM motif family protein
GRMZM2G305446	5	Q_{MTf}, $Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	Aquaporin TIP3-1
GRMZM2G317614	5	Q_{MTf}, Q_{MTi}	Nucleotide binding protein
GRMZM2G414159	5	Q_{MTf}, $Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	Uncharacterized protein
GRMZM2G436710	5	Q_{MTf}, $Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	Uncharacterized protein
GRMZM2G439195	5	Q_{MTf}, $Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	Nicotianamine synthase 3
GRMZM2G474555	5	Q_{MTf}, $Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	Putative uncharacterized protein
AC195914.2_FG003	5	$Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTh}, Q_{MTi}	Uncharacterized protein
GRMZM2G042278	5	$Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	Uncharacterized protein
GRMZM2G048672	5	$Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	Macrophage migration inhibitory factor
GRMZM2G048904	5	$Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	Alpha-L-fucosidase 2
GRMZM2G059124	5	$Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	No information
GRMZM2G064360	5	$Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	Basic endochitinase 1
GRMZM2G087495	5	$Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	Uncharacterized protein
GRMZM2G089836	5	$Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	Beta-fructofuranosidase 1; Invertase
GRMZM2G120539	5	$Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	AMP binding protein
GRMZM2G128938	5	$Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	No information
GRMZM2G133262	5	$Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	No information
GRMZM2G133434	5	$Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	Peroxidase 45
GRMZM2G162093	5	$Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	Uncharacterized protein
GRMZM2G165308	5	$Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	Uncharacterized protein
GRMZM2G168747	5	$Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	No information
GRMZM2G177863	5	$Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	Uncharacterized protein
GRMZM2G340282	5	$Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	No information
GRMZM2G375607	5	$Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	No information
GRMZM2G413006	5	$Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTi}	Xyloglucan endotransglucosylase/hydrolase protein 23
GRMZM2G419455	5	$Q_{HSI:SD}$, Q_{MTg}, Q_{PC2b}, Q_{MTh}, Q_{MTi}	No information
GRMZM2G002240	5	Q_{PC2b}, Q_{MTh}, Q_{MTi}	No information
GRMZM2G004856	5	Q_{PC2b}, Q_{MTi}	No information
GRMZM2G412604	5	Q_{PC2b}, Q_{MTh}, Q_{MTi}	Uncharacterized protein
AC159612.1_FG007	5	Q_{MTi}	No information
AC210013.4_FG006	5	Q_{MTi}	Uncharacterized protein
GRMZM2G018553	5	Q_{MTi}	Uncharacterized protein
GRMZM2G074017	5	Q_{MTi}	ATPase inhibitor
GRMZM2G336533	5	Q_{MTi}	NAC transcription factor; Uncharacterized protein
GRMZM2G019872	9	Q_{MTj}, Q_{MTk}, $Q_{HSI:PH}$	NADP-dependent oxidoreductase P2; Putative alcohol dehydrogenase superfamily protein
GRMZM2G051135	9	Q_{MTj}, Q_{MTk}, $Q_{HSI:PH}$	Uncharacterized protein
GRMZM2G056093	9	Q_{MTj}, Q_{MTk}, $Q_{HSI:PH}$	Uncharacterized protein
GRMZM2G113203	9	Q_{MTj}, Q_{MTk}, $Q_{HSI:PH}$	Uncharacterized protein
GRMZM2G126900	9	Q_{MTj}, Q_{MTk}, $Q_{HSI:PH}$	Uncharacterized protein
GRMZM2G133050	9	Q_{MTj}, Q_{MTk}, $Q_{HSI:PH}$	Uncharacterized protein
GRMZM2G145446	9	Q_{MTj}, Q_{MTk}, $Q_{HSI:PH}$	Uncharacterized protein
GRMZM2G163178	9	Q_{MTj}, Q_{MTk}, $Q_{HSI:PH}$	Uncharacterized protein
GRMZM2G166459	9	Q_{MTj}, Q_{MTk}, $Q_{HSI:PH}$	Putative MATE efflux family protein; Uncharacterized protein
GRMZM2G404603	9	Q_{MTj}, Q_{MTk}, $Q_{HSI:PH}$	Putative uncharacterized protein 9C20.6a; Uncharacterized protein
GRMZM2G704251	9	Q_{MTj}, Q_{MTk}, $Q_{HSI:PH}$	Uncharacterized protein
GRMZM5G844143	9	Q_{MTj}, Q_{MTk}, $Q_{HSI:PH}$	Photosystem Q(B) protein

5. General Discussion

Phenotypic variation for heat tolerance present in temperate European maize pools

The main goal of my thesis was to lay the foundation for the genetic improvement of heat tolerance in temperate maize by breeding, i.e. by increasing the increasing the frequency of positive alleles. Therefore, genotypic variation for heat tolerance must be present in the respective germplasm. Up to know, knowledge of the heat tolerance of temperate European Flint and Dent lines was rare and is, thus, highly valuable for plant breeding.

Statistical models used to describe heat tolerance Heat tolerance was defined in different previous studies.
Chen et al. (2012) and Cairns et al. (2013) equalized the performance of plants at high temperature with their heat tolerance. However, these authors did not consider the relation of the performance at high temperature to a performance at standard conditions. This approach is useful to identify genotypes with high performance at heat conditions, but it results in an overestimation of the heat tolerance of genotypes which perform high at any condition.
Another measure for heat tolerance, proposed by Fokar et al. (1998), considered the relation between observations at heat and at standard conditions. The authors estimated heat tolerance in wheat by the reduction of trait values at heat conditions compared to a standard condition. This was adopted in the frame of my thesis by Frey & Stich (2015), where heat susceptibility of maize seedlings was assessed. To achieve that, the general performance of a genotype at standard conditions was considered in the calculation, so that heat tolerance perse of a genotype could be assessed in an experiment with a heat and a standard environment. However, multiple environments beyond two could not be considered in such an approach.
Frey et al. (2015) and Frey et al. (2016), in contrast, calculated heat tolerance including observations at multiple heat levels.
In the study of Frey et al. (2015), heat susceptibility in a controlled experiment was defined by regression of observations of maize seedlings at three heat levels over the temperature which

was set during the experiments. This approach is appropriate to describe stress tolerance in experiments with multiple stress levels which are metrically defined. However, it is not flexible enough to account for variable environmental conditions as they are generally observed in field experiments.

In Frey et al. (2016), heat tolerance of a genotype was described by regression of observations over the mean performance across genotypes at each experimental location. In this context, an approach described by Mason et al. (2010), Paliwal et al. (2012) and Fischer & Maurer (1978) was adopted, who calculated a heat susceptibility index for each genotype as following: First the quotient of the observation of the respective genotype at heat conditions and at standard conditions was calculated and subtracted from 1. The result was divided by 1 - the quotient of the mean of the observations of all genotypes of the experiment at heat and at standard conditions. Frey et al. (2016) combined the previously described approach with a stability analysis (Finlay & Wilkinson, 1963), where stability of a genotype across environments was calculated on the basis of performance in multiple environments. This combined approach was, up to my knowledge, not presented previously and is appropriate to describe all kinds of stress tolerances of genotypes on a multi-environmental level, especially with a focus on field experiments where environmental conditions are highly variable and multiple abiotic stresses can ocur simultaneously.

In the frame of my thesis, heat tolerance was calculated on a single-trait basis for multiple agronomically important traits. To make an assumption on the heat tolerance of a genotype, plant breeders, thus, have to draw multiple traits into consideration. However, usually multiple assessed traits are correlated with each other, as it was observed by Weller et al. (1996), Frey et al. (2015), Frey et al. (2016) and Frey & Stich (2015). To facilitate the selection process, Weller et al. (1996) proposed, in the context of QTL mapping, to combine a set of correlated traits to uncorrelated principal components. With this multi-trait approach, Frey & Stich (2015) combined heat tolerance with respect to nine seedling traits in two principal components which explained 59% of the total variance (45 and 14%, each). The principal components are especially useful for plant breeders, as they enable the selection of heat tolerant genotypes across several correlated seedling traits with one or two criteria.

Phenotypic variation for heat tolerance In the studies which were the basis of my thesis, reduced plant performance of maize seedlings as well as of adult maize plants was observed at heat stress (Frey et al., 2016; Frey & Stich, 2015). The shoot dry weight of seedlings was reduced by 20% on average (ranging from a reduction of 88% to an increase of 170%) and leaf scorching of seedlings was increased by 239% on average (ranging from a 58% decrease to 819% increase) at heat conditions. Adult plants showed on average 114% more leaf scorching (ranging from a 50% decrease to a 718% increase) and an average reduction of 50% of grain yield (ranging from a 83% decrease to a 11% increase) at heat stress. In both developmental stages, heat stress, thus, harmed temperate maize plants severely. Consequently, maize cultivars must be improved to face the probably increasing number of heat events in the future, as

predicted by IPCC (2013). The previously presented effects of heat stress not only show the sensitivity of temperate maize to heat stress but also show that there is a considerable variation for heat tolerance between genotypes. This fact opens the possibility for plant breeders to select genotypes with advantageous alleles and to cross and introgress these alleles in their cultivars.
In the frame of my thesis, heat tolerance was assessed at two developmental stages in which heat stress can damage maize plants, during seedling and during adult stage. The correlations between heat tolerance during the two stages was rather low, with correlation coefficients below 0.30 (Frey & Stich, 2015), although in cases correlations were significant. Similarly low correlation between seedling and agronomic traits were observed in other species, e.g. *Brassica napus* (Körber et al., 2012). This fact impedes indirect selection, as discussed in the following section. Furthermore, it becomes obvious that heat tolerance during different developmental stages is based on different genetic mechanisms.

Methods to genetically improve heat tolerance in maize

Yield losses due to climate change can be reduced by the development of maize genotypes which are able to maintain their yield potential at high temperatures (Butler & Huybers, 2013). Direct selection of more heat tolerant genotypes with respect to grain yield is expensive and time-consuming, as, due to the low heritability of grain yield at heat conditions (Frey et al., 2016), yield experiments have to be performed at multiple locations. Heat tolerance during seedling stage is easier to assess than heat tolerance with respect to grain yield during adult stage, as the time to assess seedling traits is shorter compared to the complete growing period which is necessary to assess grain yield. Additionally, seedlings can be evaluated under controlled conditions in growth chambers, as their evaluation requires less space. However, the investment to evaluate heat tolerance during seedling stage, either in multi-environmental experiments or in costly growth chambers, is likely to be not cost-effective for breeding companies. To facilitate the selection process in order to develop more heat tolerant cultivars, breeder could make use of indirect selection and marker assisted selection (MAS).

Indirect selection Indirect selection can be profitable, if secondary traits exist which are easier to assess and more heritable than the trait of interest and which are, furthermore, highly correlated with it (Becker, 2011). The benefits and constraints of indirect selection were reviewed by Becker & Léon (1988).
I am not aware of traits which could be easier assessed and used as indirect selection traits for heat tolerance during seedling stage. However, indirect selection criteria for heat tolerance with respect to grain yield are imaginable. The assessment of leaf scorching during flowering can be conducted earlier during plant development and it is easier to determine compared

to the assessment of grain yield. An indirect selection on leaf scorching would, thus, reduce the effort to assess heat tolerance considerably. However,the correlation between the heat tolerance with respect to leaf scorching and with respect to grain yield was relatively low, with a correlation coefficient 0.16 (Frey et al., 2016). Heat tolerance with respect to the time to flowering, in contrast, was correlated comparably higher with heat tolerance in terms of grain yield with a correlation coefficient of -0.33 (Frey et al., 2016). This means that genotypes which reduced their time to flowering upon heat stress showed reduced yield losses. A selection on the time to flowering, nevertheless, is not desirable, as earlier maturation is generally correlated with lower yields due to a shorter time to accumulate photosynthetic products. Previous publications associated the interval between the time to male and female flowering, the anthesis-silking-interval (ASI), with the tolerance to drought in maize. Tuberosa & Salvi (2006) stated that a selection on small ASI improved the performance of maize at severe drought stress. Frey et al. (2016) investigated, if the relation between ASI and performance might be true with respect to heat stress as well. They observed a significant correlation between low ASI and high grain yield at heat stress, but the coefficient of correlation was again low (0.16). Furthermore, heat stress had no significant effect on the ASI across genotypes. The ASI is, thus, for the genetic material examined in my thesis, not an appropriate selection criterion for heat tolerance although it might be for drought tolerance.

Marker assisted selection In order to preselect heat tolerant genotypes without even planting field trials nor performing controlled experiments, one can make use of MAS as proposed and discussed in depth by Collard & Mackill (2008). It consists in a selection of genotypes carrying specific marker alleles in genome regions which were associated with heat tolerance. These genome regions are denominated quantitative trait loci (QTL). With this preselection, field capacities in the course of variety development can be reduced considerably and breeding success can be increased. The QTL which can be used in the process of MAS were identified by linkage mapping (Frey et al., 2016; Frey & Stich, 2015) in order to select heat tolerant genotypes.

QTL associated with heat tolerance in maize

Linkage mapping with multiple connected populations In the frame of my thesis (Frey et al., 2016; Frey & Stich, 2015), linkage mapping approaches were used to detect QTL associated with heat tolerance during adult and seedling stage. For this, six segregating populations were used, which shared parental inbred lines. The benefit of the use of such multiple connected populations in comparison with a single population was that it allowed the consideration of multiple alleles originating from different genetic backgrounds and increasing the possibility of a marker being polymorphic in at least one populations. Thus, the probability to

detect alleles associated with increased heat tolerance was higher (Bardol et al., 2013; Blanc et al., 2006). As the QTL were detected in six populations, the validity of the detected QTL was higher than for QTL which are detected in a single population. Thus, the probability that the identified QTL are as well present in pools of breeding companies is increased.
Several QTL associated with heat tolerance were identified in the frame of my thesis. Heat tolerance with respect to grain yield was associated with two QTL with confidence intervals with a length of 22 and 23 cM, respectively. They explained 19% of the total variance in a simultaneous fit (Frey et al., 2016). Each two QTL with confidence intervals with a length of between 19 and 93 cM were associated with the first two principal components representing heat tolerance during seedling stage. They explained together 14 and 12% of the variance of the first and the second principal component, respectively, in simultaneous fits across QTL (Frey & Stich, 2015). The explained variances of the detected QTL were comparable with the explained variances of QTL for heat tolerance described in previous publications, e.g. Paliwal et al. (2012), who observed QTL which explained between 8 and 13% of the variance of heat tolerance with respect to grain yield in wheat. The explained variances are, however, relatively small to exclusively rely on a MAS approach in the selection of heat tolerant genotypes. But as phenotypic selection on heat tolerance is laborious, it might be worthwhile to perform a marker-assisted preselection based on the results of Frey et al. (2016) and Frey & Stich (2015) to reduce the field capacities in the following phenotypic selection step.
To increase the benefit of a marker-assisted preselection, the variance explained by QTL could be increased in a follow-up project. For this, the experiments described by Frey et al. (2016) and Frey & Stich (2015) could be repeated at a higher number of locations and in multiple years. This would increase the heritability of the assessed traits (Becker, 2011) and, thus, increase the power of the QTL detection. According to Schön et al. (2004), the most efficient way to raise the variance explained by QTL is to increase the population size. For grain yield, which is a polygenic trait like heat tolerance, Schön et al. (2004) observed that QTL explained up to 42.9% of the total variance. I expect, thus, that with an increased population size and an improved experimental design a higher percentage of the total variance for heat tolerance could be explained by QTL and MAS could, thus, be used more efficiently.
Besides the maximization of the proportion of the variance explained by QTL, the success of MAS is increased by a reduction of the confidence intervals of the detected QTL. The confidence intervals of the QTL, identified in the frame of this thesis were between 19 and 93 cM, as mentioned previously. To efficiently use MAS in plant breeding, Collard & Mackill (2008) stated that distances between the flanking markers of a QTL of 9 cM would be advantageous. As the average distance between markers in Frey et al. (2016) and Frey & Stich (2015) was 7cM, the QTL locations could be defined more accurately with the same set of markers. Therefore, fine mapping with a segregating population with an increased number of individuals could be performed. The increased population size in a QTL experiment would ensure an increased number of recombinations within the confidence intervals of the detected QTL. This would subsequently increase the precision of the QTL locations. The respective

population could be derived from a cross of individuals which show highly divergent heat tolerance in the experiments of Frey et al. (2016) and Frey & Stich (2015).
The described fine-mapping approach could be performed in a follow-up project and would, first, increase the proportion of the variance explained by QTL and, second, reduce the confidence intervals, which would enable plant breeders to employ MAS more efficiently.

Transcriptomic variation associated with heat stress

To complete the rather descriptive information about the inheritance of heat tolerance in temperate maize obtained by linkage mapping with an additional method, Frey et al. (2015) applied transcriptome profiling in the frame of my thesis. The latter mentioned method was used to provide a description of the response of maize to heat stress on a single gene level.

Transciptomic response of temperate maize to heat stress The pathways associated with the general response of temperate maize to heat stress were described by Frey et al. (2015), by relating differential gene expression of eight inbred lines to a temperature gradient. By including the linear increase of the temperature, the number of statistical tests was reduced from three (comparison of three heat levels with each other) to one, which increases the power to detect differentially expressed genes. The response of temperate maize to heat stress included 53 biological processes, which were described to be involved in plant stress responses in multiple previous studies. A number of the processes, identified by Frey et al. (2015), were described by Wahid et al. (2007) and Li et al. (2013) to be involved in the heat stress response of several plant species. Some of the biological processes described by Frey et al. (2015) have been further connected with the response to drought (Jiang et al., 2013), oxidative stress (Tanaka & Tanaka, 2007; Casati & Walbot, 2004) and several types of abiotic stresses (Wang et al., 2003; Reddy et al., 2011). The by Frey et al. (2015) described genetic mechanisms might, besides enhancing the understanding of pathways and genes involved in heat response in maize, be advantageous for the understanding of the responses to abiotic stresses in plants in general and the molecular relations between them. Especially, the knowledge of the response to drought stress in plants can profit from the studies presented in this thesis,, as the morphological and genetic responses to heat and drought are similar. From an agricultural point of view, the exact delimitation of heat and drought stress is less relevant, as both types of stresses often appear simultaneously. However, to understand the plant responses to both stresses, it is essential to distinguish the specific transcriptomic responses to heat stress and the response to drought. Therefore, an experiment could be designed, which compares gene expression under heat stress with gene expression under drought stress in a combined experimental setup.

Heat tolerance candidate genes In the previous paragraph, the general response of temperate maize to heat stress was described. The detection of differentially expressed genes across inbred lines, mentioned there, was not appropriate to identify genes which were associated with the tolerance to heat stress, as it did not consider transcriptomic differences between heat tolerant and heat susceptible genotypes. Natural variation, however, was considered in another analysis described by Frey et al. (2015), where genes associated with heat tolerance were identified. There, in comparison to the previously described approach, an additional linear regression of the expression change of each gene in each genotype over its phenotypic heat tolerance was performed. This linear regression, and the previously mentioned consideration of linear temperature dependency, which was included in both analyses, reduced the theoretical number of statistical tests to compare eight genotypes (28 possibilities) at three heat levels ($28 * 3 = 84$ possibilities) for each gene, to only one single statistical test. However, the fact that natural variation was included in the analysis caused a highly reduced power in order to detect differentially expressed genes. To increase the power and significance to detect genes associated with heat tolerance, transcriptome profiling with bigger populations is necessary, similar to those used for association mapping. In the here described expression study, the number of genotypes which were tested was held down to reduce the high costs of whole transcriptome profiling. However, the costs of RNA sequencing will be reduced, as they already decreased dramatically in the past few years (Chen et al., 2014). In the future, expression profiling of populations with several hundreds of individuals could, thus, be performed to understand in depth the transcriptomic variation for heat tolerance in maize.
A total of 39 genes were detected as significantly associated with heat tolerance during seedling stage with the analysis described previously. Two of these genes, GRMZM2G115658 and GRMZM2G537291, were located in a genome region between 33 and 55 cM on chromosome 2, where confidence intervals of QTL for heat tolerance with respect to grain yield and with respect to the second principal component associated with seedling traits overlapped (Frey & Stich, 2015). These two candidate genes might, thus, be important heat tolerance genes with respect to both stages. The effect of a differential expression of these genes on the phenotypic heat tolerance of temperate maize, should be further confirmed. Therefore, the expression of the two candidate genes could be measured by quantitative real-time PCR in a validation experiment with genotypes, which showed contrasting heat tolerance in the experiment conducted by Frey et al. (2016) and Frey & Stich (2015). To investigate the genes' functions, publicly available accessions carrying mutations in the respective candidate gene could be compared to wild type plants with respect to their heat tolerance, as it was done by Horn et al. (2014) for the resistance of maize against barley yellow dwarf virus. The function of the candidate genes in maize could further be studied by over-expression or knock-out techniques and by comparative genomics. Therefore, sequence homologs in other plants, e.g. *Arabidopis thaliana* or rice, with known molecular function, can be compared.

Differences between temperate European Dent and Flint genotypes

Frey & Stich (2015) stated a higher heat tolerance during seedling stage of genotypes derived from Flint x Flint crosses compared to genotypes derived from Dent x Dent crosses. Higher tolerance during seedling stage of Flints to both heat and cold tolerance was observed already by Reimer et al. (2013) for root growth. This increased stress tolerance in early stages in general is a prerequisite of maize cultivars bred for Central Europe, as environmental conditions during spring can be highly variable, especially concerning cold stress, even if the probability of heat extremes will rise in the future.

Genotypes derived from Dent x Dent crosses, in contrast, showed higher heat tolerance during adult stage compared to genotypes derived from Flint x Flint crosses (Frey et al., 2016). This reduced yield loss of Dents under heat conditions could be associated with a higher yield potential, which is currently exploited with Dent x Dent hybrids in Southern Europe (Reif et al., 2010), where the growing period is longer compared to Central Europe. To exploit the advantages of Dents and Flints, it would be advantageous for plant breeders to include both types of heterotic groups in order to develop heat tolerant cultivars. A separation of the pools, however, is necessary to exploit heterosis. Thus, a possible breeding strategy, to achieve heat tolerant hybrids, arising from the results of this thesis is, to maintain a Flint pool with high heat tolerance during seedling stage and a Dent pool with high heat tolerance during adult stage. Hybrids derived from crosses between these pools would possess high heterosis and would combine heat tolerance during both developmental stages. To test this hypothesis, the performance at multiple locations of testcrosses between Flint genotypes with high heat tolerance during seedling stage and Dent genotypes with high heat tolerance during adult stage could be evaluated.

Complementary to the phenotypic differences between Flints and Dents concerning heat tolerance during different developmental stages, the two pools showed two separated clusters with respect to principal components calculated on the basis of their gene expression during seedling stage, irrespectively of the degree of heat stress, which was present during plant growth (Frey et al., 2015). However, with respect to a comparison of gene expression at different heat levels, inbred lines did not share more significantly differentially expressed genes with inbreds from their pool than with inbreds from the other pool. Increased heat tolerance during seedling stage is, thus, not based on a single genetic mechanism, which is present in Flints and not present in Dents. The genetic differences between Flints and Dents could be investigated in detail with an experimental design, proposed previously, which consists in transcriptome profiling of a high number of individuals.

Conclusion and Outlook

In my thesis, I observed high genotypic variation for heat tolerance in temperate maize, during seedling stage as well as during adult stage. The two heterotic pools, used for hybrid breeding in temperate Europe, Flint and Dent, possess contrasting heat tolerances. Flints resulted to be more heat tolerant during early, Dents more heat tolerant during late developmental stages. Plant breeders could exploit the advantages of both pools to develop heat tolerant cultivars by crossing an early heat tolerant Flint parent with a late heat tolerant Dent parent. To verify this hypothesis, testcrosses could be performed on the basis of the results of my thesis. To select and further improve parents of heat tolerant hybrids from proprietary genetic material of breeding companies, marker assisted selection could be employed with information on molecular makers, flanking the QTL detected in my thesis. To define these QTL and increase the explained variance, I advise to perform linkage mapping experiments with larger populations at multiple locations. However, selection of more heat tolerant genotypes by MAS should be followed by a verification of the increased heat tolerance by phenotypically comparing plants with advantageous and disadvantageous allele combinations. If the alleles, which are advantageous for heat tolerance are not present in the respective breeding pool, these alleles could be introgressed from external origins. The successful application of MAS leads finally to a quicker inbred line development and variety release.

The findings about the association of regions of the maize genome which are associated with its heat tolerance were complemented, in my thesis, with the investigation of the transcriptomic response of temperate maize to heat stress. Therewith, I identified two candidate genes associated with heat tolerance, which require further validation and functional investigation. The knowledge of the expression networks presented here are useful for the investigation of plant responses to other abiotic stresses, e.g. drought. To distinguish the responses of maize to heat and drought, I proposed a combined transcriptome profiling experiment.

In the course of transcriptome profiling, a new statistical approach was presented to identify candidate genes for heat tolerance, which considered the natural phenotypic variation for heat tolerance. To take full advantage of this analysis, I advise transcriptomic profiling of a larger set of individuals. The presented approach enables researchers to investigate the transcriptomic response of multiple genotypes to changing conditions across several experiments, considering their natural variation for a quantitative trait.

References

Bardol N., Ventelon M., Mangin B., Jasson S., Loywick V., Couton F., Derue C., Blanchard P. et al. (2013) Combined linkage and linkage disequilibrium QTL mapping in multiple families of maize (Zea mays L.) line crosses highlights complementarities between models based on parental haplotype and single locus polymorphism. *Theoretical and Applied Genetics* 126, 2717–36.

Becker H. (2011) *Pflanzenzüchtung*. Verlag Eugen Ulmer, Stuttgart, 2 edn.

Becker H.C. & Léon J. (1988) Stability Analysis in Plant Breeding. *Plant Breeding* 101, 1–23.

Blanc G., Charcosset A., Mangin B., Gallais A. & Moreau L. (2006) Connected populations for detecting quantitative trait loci and testing for epistasis: an application in maize. *Theoretical and Applied Genetics* 113, 206–24.

Butler E.E. & Huybers P. (2013) Adaptation of US maize to temperature variations. *Nature Climate Change* 3, 68–72.

Cairns J.E., Crossa J., Zaidi P.H., Grudloyma P., Sanchez C., Araus J.L., Thaitad S., Makumbi D. et al. (2013) Identification of Drought, Heat, and Combined Drought and Heat Tolerant Donors in Maize. *Crop Science* 53, 1335.

Casati P. & Walbot V. (2004) Rapid transcriptome responses of maize (Zea mays) to UV-B in irradiated and shielded tissues. *Genome Biology* 5, R16.

Chen J., Xu W., Velten J., Xin Z. & Stout J. (2012) Characterization of maize inbred lines for drought and heat tolerance. *Journal of Soil and Water Conservation* 67, 354–364.

Chen Y., Lun A.T.L. & Smyth G.K. (2014) Differential Expression Analysis of Complex RNA-seq Experiments Using edgeR. In *Statistical Analysis of Next Generation Sequencing Data* (eds. S. Datta & D. Nettleton), chap. 3, pp. 51–73. Springer International Publishing, Cham.

Collard B.C.Y. & Mackill D.J. (2008) Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Philosophical Transactions of the Royal Society of London Series B, Biological sciences* 363, 557–72.

Finlay K. & Wilkinson G. (1963) The analysis of adaptation in a plant-breeding programme. *Australian Journal of Agricultural Research* 14, 742–54.

Fischer R.A. & Maurer R. (1978) Drought resistance in spring wheat cultivars. I. Grain yield responses. *Australian Journal of Agricultural Research* 29, 897–912.

Fokar M., Blum A. & Nguyen H.T. (1998) Heat tolerance in spring wheat. II. Grain filling. *Euphytica* 104, 9–15.

Frey F.P., Presterl T., Lecoq P., Orlik A. & Stich B. (2016) First steps to understand heat tolerance of temperate maize at adult stage: identification of QTL across multiple environments with connected segregating populations. *Theoretical and Applied Genetics* 129, 945–961.

Frey F.P. & Stich B. (2015) Heat tolerance in temperate maize: Identification of QTL during seedling stage with multiple connected populations. *submitted to PLOS Biology* .

Frey F.P., Urbany C., Hüttel B., Reinhardt R. & Stich B. (2015) Genome-wide expression profiling and phenotypic evaluation of European maize inbreds at seedling stage in response to heat stress. *BMC Genomics* 16, 123.

Horn F., Habekuß A. & Stich B. (2014) Genes involved in barley yellow dwarf virus resistance of maize. *Theoretical and Applied Genetics* 127, 2575–84.

IPCC (2013) Summary for policymakers. In *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* (eds. T.F. Stocker, D. Qin, G.K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex & P. Midgley). Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Jiang S., Zhang D., Wang L., Pan J., Liu Y., Kong X., Zhou Y. & Li D. (2013) A maize calcium-dependent protein kinase gene, ZmCPK4, positively regulated abscisic acid signaling and enhanced drought stress tolerance in transgenic Arabidopsis. *Plant Physiology and Biochemistry* 71, 112–20.

Körber N., Wittkop B., Bus A., Friedt W., Snowdon R.J. & Stich B. (2012) Seedling development in a Brassica napus diversity set and its relationship to agronomic performance. *Theoretical and Applied Genetics* 125, 1275–1287.

Li Y.f., Wang Y., Tang Y., Kakani V.G. & Mahalingam R. (2013) Transcriptome analysis of heat stress response in switchgrass (Panicum virgatum L.). *BMC Plant Biology* 13, 153.

Mason R.E., Mondal S., Beecher F.W., Pacheco A., Jampala B., Ibrahim A.M.H. & Hays D.B. (2010) QTL associated with heat susceptibility index in wheat (Triticum aestivum L.) under short-term reproductive stage heat stress. *Euphytica* 174, 423–436.

Paliwal R., Röder M.S., Kumar U., Srivastava J.P. & Joshi A.K. (2012) QTL mapping of terminal heat tolerance in hexaploid wheat (T. aestivum L.). *Theoretical and Applied Genetics* 125, 561–75.

Reddy A.S.N., Ali G.S., Celesnik H. & Day I.S. (2011) Coping with stresses: roles of calcium- and calcium/calmodulin-regulated gene expression. *The Plant Cell* 23, 2010–32.

Reif J.C., Fischer S., Schrag T.A., Lamkey K.R., Klein D., Dhillon B.S., Utz H.F. & Melchinger A.E. (2010) Broadening the genetic base of European maize heterotic pools with US Cornbelt germplasm using field and molecular marker data. *Theoretical and Applied Genetics* 120, 301–10.

Reimer R., Stich B., Melchinger A.E., Schrag T.A., Sørensen A.P., Stamp P. & Hund A. (2013) Root response to temperature extremes: association mapping of temperate maize (Zea mays L). *Maydica* 58, 156–168.

Schön C.C., Utz H.F., Groh S., Truberg B., Openshaw S. & Melchinger A.E. (2004) Quantitative trait locus mapping based on resampling in a vast maize testcross experiment and its relevance to quantitative genetics for complex traits. *Genetics* 167, 485–98.

Tanaka R. & Tanaka A. (2007) Tetrapyrrole biosynthesis in higher plants. *Annual Review of Plant Biology* 58, 321–46.

Tuberosa R. & Salvi S. (2006) Genomics-based approaches to improve drought tolerance of crops. *Trends in Plant Science* 11, 405–12.

Wahid a., Gelani S., Ashraf M. & Foolad M. (2007) Heat tolerance in plants: An overview. *Environmental and Experimental Botany* 61, 199–223.

Wang W., Vinocur B. & Altman A. (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta* 218, 1–14.

Weller J.I., Wiggans G.R., VanRaden P.M. & Ron M. (1996) Application of a canonical transformation to detection of quantitative trait loci with the aid of genetic markers in a multi-trait experiment. *Theoretical and Applied Genetics* 92, 998–1002.

6. Summary

The global mean temperature and probability of heat waves are expected to increase in the future, which has the potential to cause severe damages to maize production. To elucidate the genetic mechanisms of the response of temperate maize to heat stress and for the tolerance to heat stress, in a first experiment I applied gene expression profiling. Therewith, I investigated the transcriptomic response of temperate maize to linearly increasing heat levels. Further, I identified genes associated with heat tolerance in a set of eight genotypes with contrasting heat tolerance behavior. I identified 607 heat responsive genes, which elucidate the genetic pathways behind the response of maize to heat stress and can help to expand the knowledge of plant responses to other abiotic stresses. Further, I identified 39 genes which were differentially regulated between heat tolerant and heat susceptible inbreds and, thus, are putative heat tolerance candidate genes. Two of these candidate genes were located in genome regions which were associated with heat tolerance during seedling and adult stage that have been detected in QTL studies in the frame of this thesis. Their exact molecular functions, however, are still unknown. The statistical approach to identify heat tolerance genes, presented in my thesis, enables researchers to investigate the transcriptomic response of multiple genotypes to changing conditions across several experiments, considering their natural variation for a quantitative trait.

In order to develop more heat tolerant cultivars, knowledge of natural variation for heat tolerance in temperate maize is indispensable. Therefore, heat tolerance was assessed in a set of intra- and interpool Dent and Flint populations on a multi-environment level. Usually, heat stress in temperate Europe occurs during the adult stage of maize. However, as maize is of increasing importance as a biogas crop, farmers can reduce the growth period by postponed sowing after the harvest of the winter cereals in early summer and, thus, sensitive maize seedlings can be exposed to heat stress. Therefore, I aimed to assess heat tolerance in six connected segregating Dent and Flint populations during both developmental stages considering besides multiple environments also multiple traits. At heat stress, I observed an average decrease of 20% of the shoot dry weight during seedling stage and an average of 50% of yield loss, when heat stress was present during adult stage. At the heat locations heat stress was present in the year, when the experiments were conducted as temperatures exceeded 32°C there for more than 400 hours during the growing period in contrast to less than 30 hours at

the standard locations. This emphasizes that maize crop production can suffer with the increasing number and intensity of summer heat waves. Furthermore, the study revealed strong differences between genotypes, which was indispensable to differentiate between heat tolerant and heat susceptible inbred lines. The tested genotypes originating from the Flint pool turned out to possess higher heat tolerance during seedling stage, whereas the genotypes derived from the Dent pool possessed higher heat tolerance during adult stage. This fact could be exploited by the maintenance of two pools with contrasting heat tolerance and could be beneficial for hybrid breeding.

A direct selection of more heat tolerant genotypes in terms of grain yield is expensive and time-consuming. To facilitate the selection process in order to develop more heat tolerant cultivars, breeders could make use of marker assisted selection. To lay the foundation for this technique, in my thesis, QTL for heat tolerance during adult and during seedling stage were identified with the previously mentioned populations. Two QTL explained 19% of the total variance for heat tolerance with respect to grain yield in a simultaneous fit. Furthermore each two QTL were identified for two principal components, which accounted for heat tolerance during seedling stage. They explained 14 and 12% of the respective variance. The results can be used by breeding companies to develop marker assays in order to select heat tolerant genotypes from their proprietary genetic material during both stages in an initial screening. This would reduce the field capacities considerably, which are needed to test heat tolerance on a field level.

7. Zusammenfassung

Es wird erwartet, dass der Klimawandel global zu einer Erhöhung der Temperatur als auch der Wahrscheinlichkeit von Hitzewellen führt. Um die genetischen Mechanismen der Reaktion von Mais auf Hitzestress aufzuklären, untersuchte ich die Genexpressionsänderung von Mais mit steigendem Hitzestress in acht Inzuchtlinien mit gegensätzlicher Hitzetoleranz. Um Gene zu identifizieren, die mit der Hitzetoleranz von Mais in Verbindung stehen, wurde außerdem die natürliche phänotypische Variation dieser Inzuchtlinien bezüglich ihrer Hitzetoleranz in die statistische Analyse miteinbezogen. In meiner Studie wurden 607 Gene identifiziert, die ein Bild der Stoffwechselveränderungen ergaben, die in Mais mit steigendem Hitzestress stattfinden. Die Ergebnisse können des Weiteren hilfreich sein, um genetische Mechanismen von Pflanzen in Reaktion auf andere Arten abiotischen Stresses zu erklären. Insgesamt 39 Kandidatengene wurden identifiziert, die in hitzetoleranten und hitzeempfindichen Genotypen unterschiedliche Expressionsänderung mit steigendem Hitzestress erfuhren. Zwei dieser Kandidatengene für Hitzetoleranz wurden Genomregionen zugeordnet, die im Zuge von QTL-Studien mit der Hitzetoleranz im Jungpflanzen- und adulten Stadium assoziiert wurden. Die genaue molekulare Funktion dieser beiden Kandidatengene ist bisher unbekannt. Der neue statistische Ansatz, mit dem Hitzetoleranzgene in meiner Studie ermittelt wurden, erlaubt es Wissenschaftlern die Genexpression von multiplen Genotypen unter sich verändernden Bedingungen über mehrere Experimente hin zu untersuchen und dabei die natürliche Variation der Genotypen bezüglich eines quantitativen Merkmals in die Analyse miteinzubeziehen.

Hitzetolerante Maissorten für mitteleuropäische Bedingungen können nur dann entwickelt werden, wenn natürliche Variation für Hitzetoleranz in lokalem genetischem Matrial vorhanden ist. Um dies zu ermitteln, wurde Hitzetoleranz in einem mehrortigen Versuch mit sechs, durch gemeinsame Elternlinien verbundene, Populationen erhoben, die aus den europäischen Flint und Dent Pools stammen. In Mitteleuropa treten Hitzewellen normalerweise während des adulten Stadiums von Maispflanzen auf. Dadurch dass Mais immer öfter als Biomassepflanze genutzt wird und damit kürzere Wachstumsphasen benötigt, kann die Aussaat auf den Zeitpunkt nach der Ernte der Wintergetreide hinausgezögert werden. Somit können Maiskeimlinge starkem Hitzestress im Frühsommer ausgesetzt werden. Deswegen war mein Ziel, die Hitzetoleranz der Populationen während beider Entwicklungsstadien zu testen, wobei ich bei der Bewertung der Hitzetoleranz neben mehreren Orten auch mehrere Merkmale in Betracht

zog. In den Versuchen beobachtete ich bei Hitzestress einen durchschnittlichen 20-prozentigen Verlust an Gesamtsprossmasse im Jungpflanzenstadium sowie einen durchschnittlichen Ertragsverlust von 50%, wenn Hitzestress im adulten Stadium auftrat. Hitzestress trat an den Hitzestandorten im Durchführungszeitraum auf, was dadurch verdeutlicht wurde, dass Temperaturen über 32°C dort für mehr als 400 Stunden auftraten, im Vergleich zu weniger als 30 Stunden im gleichen Zeitraum an den Kontrollstandorten. Diese Ergebnisse unterstreichen die Problematik, dass der Anbau von Mais unter Hitzewellen leidet. Es wurde außerdem beobachtet, dass es starke Unterschiede zwischen den getesteten Genotypen bezüglich ihrer Hitzetoleranz gab, was es erst ermöglichte effektiv zwischen hitzetoleranten und hitzeanfälligen Genotypen unterscheiden zu können. Ein Vergleich der heterotischen Pools ergab, dass Flint-Linien hitzetoleranter im Jungpflanzen, jedoch Dent-Linien hitzetoleranter im adulten Stadium waren. Diese Tatsache kann ausgenutzt werden, indem zwei Pools mit gegensätzlicher Hitzetoleranz erhalten werden, was sich als vorteilhaft für die Erzeugung von Hybriden erweisen könnte.

Eine direkte Selektion hitzetoleranterer Genotypen anhand deren Ertragsverlust bei Hitzestress ist teuer und zeitaufwändig. Um die Selektion im Zuge der Entwicklung von hitzetoleranteren Sorten zu vereinfachen, können Züchter auf markergestützte Selektion zurückgreifen. Um die Grundlage für die Markerentwicklung zu legen, wurden in meiner Studie QTL für Hitzetoleranz im Jungpflanzen- und adulten Stadium mithilfe der letztgenannten Populationen identifiziert. Zwei QTL für Hitzetoleranz mit Hinblick auf den Kornertrag wurden ermittelt, die zusammen 19% der Gesamtvarianz erklärten. Außerdem wurden je zwei QTL für zwei Hauptkomponenten identifiziert, die für die Hitzetoleranz im Jungpflanzenstadium stehen. Diese erklärten 14 und 12% der jeweiligen Gesamtvarianz. Die Ergebnisse dieser Studien können durch Züchtungsunternehmen verwendet werden, um ihr genetisches Material mithilfe von Markeranalysen auf deren Hitzetoleranz während beider Entwicklungsstadien zu untersuchen. Diese Vorgehensweise könnte einen ersten Selektionsschritt darstellen und die benötigten Feldkapazitäten einer anschließende phänotypische Selektion auf Hitzetoleranz erheblich reduzieren.

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