Thesis of doctoral dissertation

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INVESTIGATION OF HEAT STRESS TOLERANCE IN WINTER BARLEY (*HORDEUM VULGARE* L.) VARIETIES BASED ON PRODUCTION BIOLOGICAL AND GENETIC STUDIES

THESIS OF DOCTORAL (PHD) DISSERTATION

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1. Background and objectives

The cultivation of cereals has a history spanning millennia. Since the middle of the last century, the development of agricultural technologies and the success of plant breeding have enabled a significant increase in the yield and security of various cereal crops. Besides common wheat (Triticum aestivum L.), barley (*Hordeum vulgare* L.) is also of great importance for the brewing industry, animal feed, and increasingly for food security. The escalating trends of climate change, including globally rising temperatures, exacerbate environmental fluctuations, posing increasingly threatening challenges to cultivated crops (Lobell et al. 2011). Meeting the food demands of the world's rapidly growing population, particularly ensuring the security of cereal crops under climate change conditions, presents numerous challenges and tasks for breeders and growers (Bedő et al. 2007; Bedő et al. 2014). Numerous new research findings offer untapped opportunities for breeders to enhance abiotic stress tolerance. Success relies on leveraging modern agricultural and biotechnological methods. Studying barley's response mechanisms to heat stress is of significant importance for understanding adaptation to heat stress. Prolonged supraoptimal temperatures, where the average temperature ranges between 20°C and 28°C, can have negative indirect consequences on yield, resulting from shortened growing cycles and/or disturbances in optimal developmental patterns (Horváth et al. 2023). To thoroughly understand the heat stress tolerance of individual genotypes, it is crucial to employ combined phenotypic, physiological, and genetic methods and knowledge. Identifying Quantitative Trait Locus (QTL) regions responsible for quantitative traits, gene expression studies, and investigating molecular and biochemical mechanisms may form the basis for identifying gene variants or gene families that enable certain cultivars to achieve adequate yields even under sustained high-temperature conditions.

The objectives of the thesis during the research were as follows:

- Examination of the year-to-year effect in a multi-year field trial involving 190 barley varieties in an association panel.
- Comparative study of heat stress tolerance under controlled conditions in different barley varieties.
- Investigation of the combined effect of heat stress on plants, heat priming effect.
- The effect of heat stress on the gene expression patterns of barley varieties with different sensitivities.

2. Material and method

In the dissertation, I examined the genotypes of barley experiments from the GINOP-2.3.2-15-2016-00029 project in terms of their resistance to high temperatures using production biological, physiological, and molecular genetic research methods. Our investigations involved the BARGEN association mapping population consisting of 190 barley varieties of different geographical origins, whose production biological parameters and yield were evaluated in multi-year field trials. Based on the field phenotype results, we selected 28 genotypes from the BARGEN panel of 190 varieties for further controlled heat stress experiments. In the selection process, we also considered the spike type, with half of the 28 varieties being two-rowed and the other half six-rowed barley.

3.2. Phenotypic experiments

The field experiments were conducted for four consecutive years (2019–2022) at the same location in Martonvásár (latitude: 47° 21’ N, longitude: 18° 49’ E, altitude: 150 m), where the soil type is forest-steppe chernozem (Árendás et al. 2004). Each year, using the same experimental layout, sowing took place in mid-October. The 190 barley genotypes were sown without replication. The plot size containing each genotype, consisting of 5 rows, was 1 m × 2 m. The experiment received standard agronomic management; soil preparation and fertilization remained consistent over the four years, while pest and disease control measures and weed management were applied as needed. During the growing season, we recorded the heading date (ZD49 stage) and plant height (from soil level to the base of the spike). At full maturity, two 25 cm sections were harvested from each plot using scissors, and various yield components were measured. Parameters of the main spike were determined on six spikes typical of the sample and of similar size. These parameters included spike length, spikelet number, kernel number, and kernel weight. The remaining spikes were bulked together, and the spikelet number and weight of the lateral spikes were counted and measured. The combined data of the main and lateral spikes provided the total kernel number and total kernel weight. The latter, supplemented with data from the six main spikes, represented the quantity of grain harvested within the 25 cm. Meteorological data at daily intervals for the four years of the field experiment were also available (temperature, precipitation) through the meteorological collection station located at the Centre for Agricultural Research.

3.2.2. Heat stress tolerance experiments

The experiment consisted of three treatments: control (K), single heat stress (ZD49), and combined heat stress (ZD31+49). For each treatment, the plants were germinated in Jiffy pellets, followed by 60 days of vernalization at 4°C under low light intensity and short day length. The plants were grown at 18°C with a 16-hour photoperiod, maintaining continuous irrigation. In the stress chamber, the photoperiod and light quality and quantity were the same as in the control treatment, but the temperature profile was specific. At the onset of illumination, the temperature gradually increased from the nighttime level of 20°C to the level of heat stress, which was maintained for 8 hours during the illumination period, then gradually decreased back to 20°C at night. The stress treatments were initiated at the respective developmental stages for each variety. The temperature applied for single heat stress was 35°C, which the plants received for 10 days at the booting stage. For combined heat stress, the first treatment was applied at the end of tillering/early stem elongation (Zadoks-31), and the second treatment was applied at the spike emergence stage characteristic of the variety (Zadoks-49, Tottman and Makepeace 1979). In the combined stress treatment, we applied 30°C for 5 days at ZD31 and 35°C for 10 days at ZD49 (Balla et al. 2019, Balla et al. 2021). After completing the stress treatments, the plants were returned to the control chamber for recovery until full maturity. Three plants from each variety were regularly sampled for measurements of plant height, fully expanded leaf number, tiller number (every 5 days), and two developmental stages according to the Zadoks scale: ZD31 (first node detectable at the base of the main stem) and ZD49 (spike emerged from the tip of the flag leaf sheath), following Tottman and Makepeace (1979). Upon reaching full maturity, the plants were harvested. During the measurement of yield components, the following traits were determined: plant height, length of the last internode, length of the main spike, weight of the main spike, number of spikelets per main spike, number and weight of kernels per main spike, number of lateral spikes, weight of lateral spikes, number and weight of kernels per lateral spike, and above-ground mature plant biomass. Based on the results of the experiment with 28 varieties, we selected six barley varieties (highlighted in red in Table 1) and repeated the heat stress experiment using the same experimental layout, with one exception. In this experiment, only the control and single heat stress treatments at the booting stage were applied. Here, the emphasis was on genomic studies. Leaf samples were collected from the flag leaves of the plants at the beginning of the treatment and on the 7th day of the treatment. From these leaf samples, RNA was isolated for sequencing purposes later on. Sampling was always done at the same time, in the middle of the illumination period. For each variety and sampling point, leaf samples were collected from 9 plants, forming one biological replicate from the leaves of 3 plants.

3.3. Genome-level investigations

We isolated DNA from the leaves of each variety in the BARGEN panel using the QIAGEN DNeasy® Plant Mini Kit (Qiagen GmbH, Hilden, Germany). The DNA samples were analyzed using the 45K Infinium array by TraitGenetics GmbH (Gatersleben, Germany) (https://www.traitgenetics.com) for genetic diversity assessment. The BARGEN LD marker database consisted of a total of 30,006 SNP markers. Total RNA was isolated using the Qiagen RNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany). Short reads were aligned to the reference genome provided by the Joint Genome Institute (JGI) Phytozome 13 (The Plant Genomics Resource - Plant Genomic Database) based on *Hordeum vulgare* r1. Gene annotation for barley was performed based on the Morex v1.0 reference genome. The overlap of gene expression patterns among varieties was determined using functional genomics methods (https://bioinformatics.psb.ugent.be/webtools/Venn/).

3.4. Statictical and data analysis methods

We applied various commands of the R statistical package to analyze different morphological and yield-related parameters (R Core Team 2021). Several program packages aided in evaluating the results. Data processing and analysis were carried out using Microsoft Excel (2016) with the data analysis add-in and R software version 4.2.1 (R Core Team 2021). In Excel, basic statistics and simple graphical representations were performed, including one-way and multi-factor analysis of variance (ANOVA), descriptive statistics, and column charts. Stress tolerance index and yield stability index calculations were performed using the iPASTIC online program available at the iPASTIC website (https://manzik.com/ipastic) (Pour-Aboughadareh et al. 2019). To substitute values into the formula for the heat stress tolerance index (STI - Stress Tolerance Index), values for control and treated grain yield were required (Fernandez et al. 1992). Based on the data of 28 varieties of crops, the program generated a ranked list according to the yield stability index (YSI), where the genotype most stable against heat stress was ranked first, and the variety with the lowest stability index was ranked 28th (Bouslama and Schapaugh, 1984).

3. Results and discussion

We utilized a wide geographic diversity association panel (BARGEN) comprising 190 barley genotypes originating from 16 gene banks for multi-year field performance evaluations.

3.1. Genetic Diversity of the Barley Panel

The genotypes of the BARGEN panel were genetically characterized using the TraitGenetics 45K SNP chip (Bayer et al. 2017). A total of 30,006 polymorphic SNPs were identified across the 190 genotypes. We determined the precise positions of SNP markers and the coverage of individual chromosomes based on a marker density map. The genetic diversity of the barley population in BARGEN was analyzed using a neighbor-joining phylogenetic tree. The tree diagram provided insights into relatedness levels and groupings. The genetic diversity of the diverse panel was primarily determined by spike type, resulting in the population being divided into two major groups. Within these two groups, the extent of diversity varied, with two-rowed varieties exhibiting much greater diversity than six-rowed ones.

3.2. Effect of Years on Individual Development of Barley Genotypes

The field experiment was characterized by four years with completely different meteorological conditions. Consequently, apart from differences in the nature and intensity of abiotic stress between years, there were also variations in the individual development of barley varieties. Despite similar sowing dates each year (around October 10th), varieties reached the heading stage (ZD49) and flowering at different times. Flowering in 2019 started early and extended (beginning on April 24th), was average in 2020 (May 1st), while 2021 and 2022 were characterized by late flowering initiation (between May 8th and 12th). These annual differences in flowering time resulted in significantly different developmental stages of plants when they encountered abiotic stress. In 2019, during the critical period, i.e., heading and flowering, plants did not experience high-temperature stress, and rainfall was optimal. Stress effects were only observed after full heading during the grain filling and ripening stages. In 2020, the rainy period extended into late May and June. Daily maximum temperatures in critical developmental stages did not negatively influence yield formation in 2020. The temperature only exceeded the 30°C threshold in mid-June, and temperatures remained above 30°C for prolonged periods at the end of June. In 2021, a rainy May characterized the critical heading period. At the beginning of the flowering period, temperatures already entered the supra-optimal range, increasing the likelihood of sterility. A 10-day period of prolonged high temperatures above 30°C accelerated ripening this year. The year 2022 was characterized by extremely variable precipitation and severe drought. Temperatures were also extremely variable, with four instances of daily maximum temperatures near or above 30°C during the flowering interval, indicating an optimal temperature range.

3.2.1. Barley Yield Components as a Function of Years

The primary aim of data analysis was to identify the effects of genotype, year, and their interactions. Except for developmental stages, genotype had the strongest effect on yield-related traits and parameters related to yield components, primarily due to fundamental phenotypic differences between two- and six-rowed spike-type groups. The genotype × year interaction was most pronounced for grain yield, indicating the diversity of genotypic sensitivities to various abiotic stress combinations over the four years. The year 2019 had the highest yield, which was followed by the stress-free year 2021, the heat-stressed year 2022, and finally the drought-stressed year 2020 with the lowest yield. To focus more on the genotype × year interaction and eliminate spike type effects, all original data were transformed into ratios referenced to the 4-year averages. K-means clustering was applied to the unified data matrix of eight traits (4 years × 190 barley genotypes), with the highest probability of belonging to seven clusters. The year 2020, which was significantly affected by drought, reduced main spike weight and average thousand kernel weight to varying degrees across all seven clusters. In this scenario, members of Clu1, Clu3, and Clu4 clusters performed better in terms of yield due to increased numbers of grains developed in main and side spikes. Clu3 and Clu4 clusters exhibited relatively high kernel weight values compared to other clusters. Therefore, varieties within Clu3 and Clu4 clusters can be considered more drought-tolerant and valuable genetic resources for breeding purposes. On the other hand, the Clu5 cluster was the most sensitive to drought, exhibiting the greatest reduction in yield within this group, mainly due to a significant decrease in kernel weight.

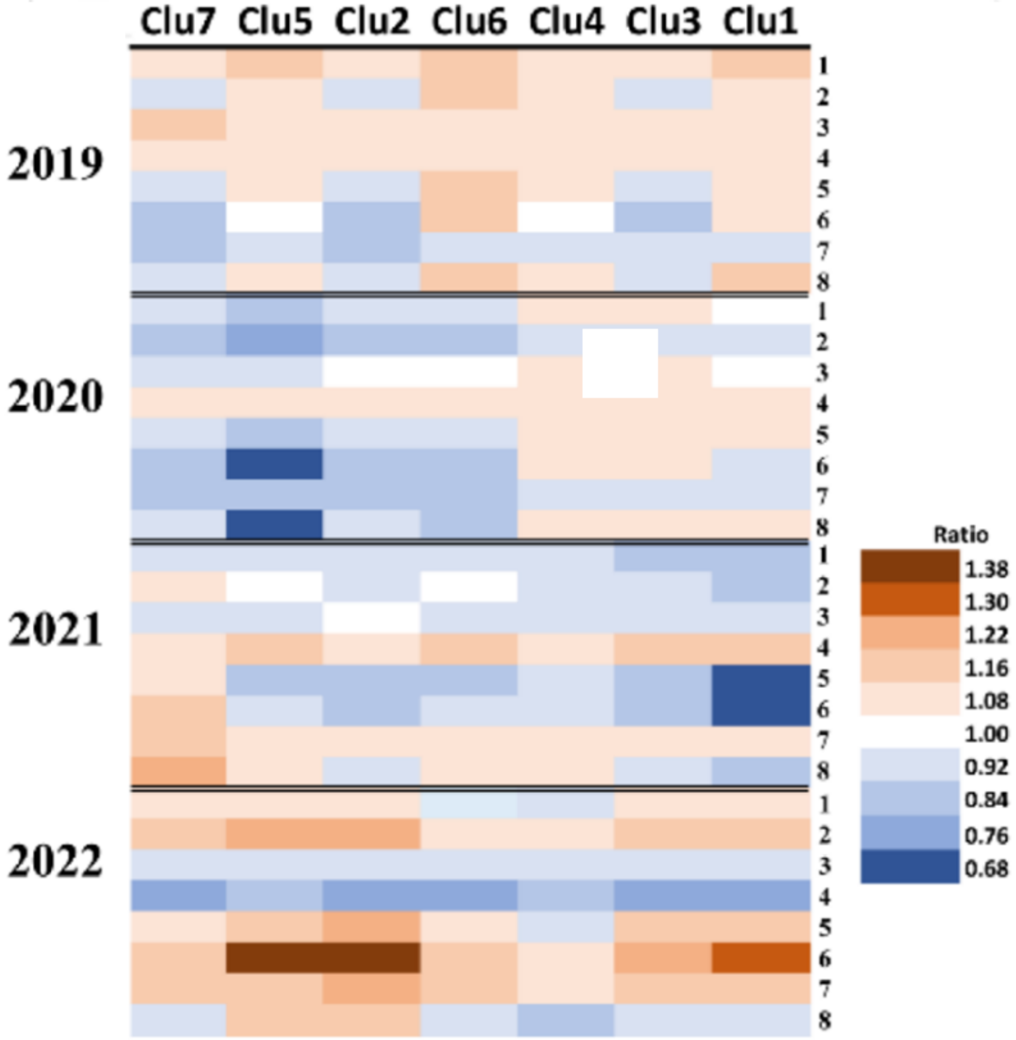

Figure 1. The role of genotype × environment interaction in clustering 190 barley varieties and their year-specific responses during the four-year field performance evaluation. Heat map of standardized traits of variety clusters across the four years, where 1 - main spike grain number, 2 - main spike grain weight, 3 - grain number per spike, 4 - productive tiller number, 5 - average grain number, 6 - average grain weight, 7 - average thousand kernel weight, 8 - grain yield.

In 2021, characterized by terminal stage heat and drought stress, a general decrease in main spike grain number and an increase in reproductive tiller number were observed across all seven clusters. The reduction in grain number might have been a consequence of the complex effect of meteorological conditions before flowering. Only Cluster 7 proved to be more tolerant in this year, maintaining main spike grain number, grain weight, and consequently average thousand kernel weight. Conversely, Cluster 1 was the most sensitive, where the decrease in grain number and weight was so severe that neither the increased reproductive tiller number nor the slightly increased thousand kernel weight could compensate for the loss, resulting in the largest yield reduction among the seven clusters.

In 2022, characterized by heat stress, there was a general trend of significant reduction in reproductive tiller number and slight decrease in fertility (number of grains per spike). Some clusters compensated for these negative effects by increasing the number of grains per main spike or showing particularly strong growth in average grain weight. This trend was most pronounced in Clusters 5 and 2, leading to higher yields compared to the other clusters. Cluster 4 was the most sensitive group in this year, as it experienced a decrease in both average grain number and weight, resulting in the greatest reduction in yield among the clusters.

3.3. Discussion of Field Experiment Results

Field crops often suffer damage from abiotic stressors, as demonstrated by the data from our four-year experiment conducted between 2019 and 2022, which included parameters related to yield and other yield components. Abiotic stress affected plant growth, development, and yield, but there were significant differences among varieties in their adaptability. In contemporary field crop production, drought and heat stress are the two main challenges due to changing climatic conditions, with their frequency increasing rapidly. Therefore, increasing tolerance to heat stress under conditions of high temperature stress during grain filling is crucial for maintaining crop security. Our results indicate that the weather over the four years of our field performance evaluation followed this climatic trend. Based on this evaluation, we divided the 190 genotypes into seven clusters, with varieties resistant to heat stress in 2022 (Clusters 2 and 5) showing significant overlap with tolerant varieties from the fitotron heat stress experiment. The year 2019 can be considered relatively neutral in terms of abiotic stress, but significant abiotic stressors in subsequent years had a substantial impact on our experimental panel. In 2020, very little rainfall occurred, which was unevenly distributed. In 2021, barley plants experienced double stress during grain filling, as both terminal heat and drought stress were present simultaneously. Then, in 2022, temperature records were broken, indicating a significant impact on barley yield and various parameters. The heatmap analysis of cluster results showed that varieties in Clusters 2 and 5 exhibited the best yield stability in the year characterized by temperature peaks in 2022. Varieties such as 'Gerlach', 'Sprite', and 'Elan', which belong to these clusters, also showed similar resistance to heat stress in the fitotron climatic chamber conditions.

3.4. Barley Varieties' Reaction to Heat Stress in Controlled Climate Chamber Experiments

Out of the 190 barley varieties, we selected 28 for heat stress experiments under controlled conditions to determine barley's sensitivity to heat stress and the extent of variability in variety reactions. When selecting the 28 varieties, we considered preserving genetic diversity and maintaining a balanced representation of two- and six-rowed spike types, selecting genotypes in equal proportions from the diverse population. The genetic diversity of the 28 varieties based on SNP markers is illustrated in Figure 10, demonstrating that the original genetic diversity of the 190-barley panel was largely preserved even in this narrower set of varieties. The response to stress is significantly influenced by the developmental stage at which plants are exposed to stress. Therefore, we subjected all varieties to stress treatments at the same growth stage. For the single heat stress treatment, this was the grain filling stage (ZD49), while for the repeated heat stress treatment, plants were exposed to the first stress treatment at the appearance of the stem node (ZD31), followed by a regeneration period before the second stress treatment, also in the grain filling stage (ZD49). Thus, we examined the effects of three treatments on the yield components, various morphological and biomass properties of 28 barley varieties, along with controls.

3.4.1. Effect of Heat Stress on Biomass, Morphological Parameters, and Yield Components

The most pronounced response of barley to heat stress was observed in grain yield per plant, affecting both two- and six-rowed spike types significantly when exposed to prolonged periods of high temperature. In two-rowed varieties, the grain yield under control conditions was approximately 5 grams on average, decreasing by 60% after a single stress treatment. For six-rowed spike types, we observed a 52% decrease in yield after a single treatment, while after the combined stress, plants produced 60% less yield on average compared to the control. It was evident that the treatments resulted in an average yield reduction of 50-60% across varieties. Due to the genetic diversity of the varieties, some exceeded the average yield under a given stress treatment, indicating to some extent the stress tolerance of varieties and providing breeders with the opportunity to select strains with more favorable traits in breeding programs.

3.4.2. Barley Varieties' Reaction to Single Heat Stress Treatments

For a more detailed evaluation of individual variety responses and to normalize differences in measurement units and magnitudes of traits, we created heat maps by expressing the measurements in each stress treatment as a percentage of the control treatment for each variety. This matrix served as the basis for generating heat maps, allowing us to simultaneously group traits and genotypes based on their response to heat stress. Heat maps were generated according to the combined dendrograms of genotypes and traits calculated using the UPGMA method. Based on a single treatment, varieties could be grouped into four clusters following stress based on yield results. The first cluster included moderately heat-tolerant varieties, the second comprised poor heat-tolerant varieties, while the third and fourth clusters contained varieties with good heat stress tolerance and moderate heat tolerance, respectively. It can be said that plants applied different strategies to the treatments. Adaptation to high temperatures was manifested by the plants producing larger green masses in response to heat stress, which increased the transpiration surface, allowing them to survive the treatment with less yield loss for some genotypes. However, significant differences in averages between groups were already evident after a single heat stress treatment, primarily in terms of individual yield components and grain yield. Groups 3 and 4 stood out in terms of yield average compared to the other two groups. Upon closer examination of yield components, it was found that different strategies were followed by varieties in response to single heat stress. Cluster 3 showed an increased number of productive tillers. However, the most significant change was that they produced significantly higher yields in the secondary spikes compared to the other clusters, compensating for the decrease in main spike grain number and weight caused by heat stress. In Cluster 4, the opposite was observed, as stress-treated main spikes produced yields close to control levels, while the number of productive tillers and grain yield in secondary spikes decreased to a greater extent. Overall, the highest grain yield was obtained from barley varieties belonging to Cluster 3. These varieties were characterized by their compensatory ability in secondary tillers after stress cessation. Cluster 4 yielded the second-highest grain yield because the varieties in this cluster were truly heat-tolerant genotypes, able to maintain main spike grain number and weight under stress but lacked compensatory ability after stress cessation.

3.4.3. Barley Variety Responses to Combined Heat Stress

We also examined how barley varieties' response to stress changes when stress occurs repeatedly. For this purpose, plants were subjected to stress treatment twice. The first stress impact occurred at a young age, at the end of tillering and the beginning of stem initiation, followed by a regeneration period, after which they received heat stress treatment during the heading stage. Based on the heat map, it can be concluded that the values of the 16 examined traits relative to the control percentage changed mostly in a negative direction in the case of repeated heat stress treatment. Compared to single treatment, we observed a stronger negative response in plants treated with double heat stress, which was reflected in both morphological and yield component data. In terms of yield components, changes in thousand kernel weight differed significantly from single stress. While the decrease in grain number was often compensated for by an increase in thousand kernel weight under single stress, this compensatory ability was largely lost under repeated stress. This is partly attributed to the fact that under repeated stress, biomass and other morphological traits promoting nutrient translocation and plant cooling through transpiration surface significantly decreased or deteriorated. Based on statistical analysis, barley varieties also divided into four groups in the combined treatment, but there was a significant difference in the number of varieties belonging to each group. It is important to note that the position, reaction type, and composition of the groups were not identical to those identified under single stress. In the combined treatment, the reactions of Groups 2, 3, and 4 were separated from the first group, which included most varieties. Group 2 (3 varieties) was distinguished by more favorable values of main spike and average spike parameters characteristic of the whole plant, but this was associated with fewer side tillers. Varieties in Group 3 (5 varieties) were least affected by repeated stress, performing best in all yield-determining parameters. In contrast, the most sensitive barley genotypes were found in Group 4 (4 varieties), as not only did parameters of the main spike decrease significantly, but those of the side spikes as well. Group 1, based on side spike data, resembled Group 4, but had better main and average spike data, representing the average yield quantity.

3.5. Application of Stress Tolerance Indices in Selection

Tolerance developed in the majority of the 28 varieties based on their response reactions, influenced by the spike type as a genetic characteristic, but according to our results, this factor did not determine the level of tolerance. Stress tolerance and yield stability indices were determined using the iPASTIC (Plant Abiotic Stress Index Calculator) program based on the substituted variable values of a given parameter for the 28 varieties. During the analysis of single stress treatment effects, the Dolphin, Mavlono, Faraday, and Bereke 54 varieties showed good resistance to prolonged high temperatures based on yield results, yield stability index, and heat stress tolerance index values. The yield of Dolphin, Mavlono, Elan, Lorena, and Sprite varieties remained the best under combined treatment. In terms of yield stability, Dolphin, Elan, Lambada, and Mavlono were outstanding. However, Lambada, fundamentally, had the smallest yield among the 28 varieties, so it produced little but had a high degree of stability. Based on statistical analysis results, Dolphin and Mavlono were identified as heat stress-tolerant two- and six-row barley varieties, and further genetic and bioinformatic analyses were conducted on these varieties. Considering the stress tolerance index calculations, Faraday, Bereke 54, and Lorena varieties can also be used as sources for improving heat stress tolerance in crossing programs.

3.6. Discussion of Morphological and Yield Component Results of 28 Barley Varieties under Heat Stress Treatment

Among the selected barley varieties, 14 two-row and 14 six-row spike types applied completely different survival and regeneration strategies against prolonged high-temperature treatments based on our results. According to various analyses and statistical analyses, the examined genotypes formed four distinct groups. Based on the hierarchical cluster analysis algorithm, the variety set can be divided into four groups based on yield reaction to treatment. Two of the four groups performed worse, while the other two contained outstanding varieties, but they separated into groups based on their good adaptive and regenerative capabilities and higher heat stress tolerance levels. The group with larger main spike grain number and weight comprised mainly varieties with better main spike yield data for both treatments, supporting that these genotypes showed greater resistance to heat stress treatment since the main spike was mainly affected by prolonged stress during the heading stage. For the other group, side spike data suggested that after stress treatment, the plant tried to compensate for yield loss by investing energy in grain filling and saturation of the side spikes following regeneration. Through the groups created based on the phenotypic results of the 28 barley varieties, it was revealed that the spike type had no effect on the tolerance level in terms of resistance to high temperatures. They applied fundamentally different strategies, as described by DODIG et al. (2018), where, for two-row types, higher thousand kernel weight was typically characteristic compared to six-row types. Furthermore, it was observed that six-row types were much more sensitive to heat and drought stress, as they dried up and shed leaves in the reproductive phase under abiotic stress. Variance analysis revealed that high-temperature stress treatment at different developmental stages had a significant effect on certain traits. The settings of the treatments have a significant impact on barley yield results, so the choice of stress timing is a key factor (Hütsch et al. 2019). Previous research and literature suggest that crops are most sensitive to heat stress during the tillering, stem initiation, heading, and pre-anthesis stages (Castro et al. 2007, Prasad and Djanaguiraman 2014, Barber et al. 2017). During the developmental interval between the ZD33 stage and full maturity (ZD94), biomass mass derived from all stem parts can decrease by 19-41% due to heat stress, and even plant height can be significantly smaller (Hütsch et al. 2019). In the fitotron experiment, there was a decrease in total biomass for both spike types compared to the average of the 28 varieties (Figure 12). In the case of combined high-temperature stress treatment, we investigated how plants can adapt if they have already received stress treatment at a young age, for a shorter period (Fan et al. 2018). For the selected barley varieties, genotypes subjected to this treatment protocol performed even worse than those under single treatment. The phenomenon of heat hardening is a real concept, but the "priming" effect mentioned in several literary sources was not observed in the vast majority of the 28 varieties (Wang et al. 2017, Balla et al. 2021). To study the effect of heat hardening, we selected the ZD31 phenophase, as at this stage, plants are still extremely vulnerable, but they have a high degree of adaptability to minor weather anomalies, as described by several research groups (Zhang et al. 2016, Mendanha et al. 2018).

3.7. Heat Stress Effects on Barley Gene Expression Patterns

Heat stress poses a serious challenge to plants, and barley (*Hordeum vulgare* L.) is no exception. The expression of the genetic code through gene expression is essential for plant adaptation and response to environmental changes. Our aim with the RNA sequencing of heat-treated varieties was to map changes in gene expression due to treatment and determine the genes whose expression changed the most during treatment. These genes may be crucial in developing heat stress tolerance. The results of the studies showed that heat stress significantly affects the barley gene expression pattern, and the pattern of change was variety-dependent. Based on samples collected at the heading stage, we determined genome-wide gene expression changes, providing further information about this critical phenophase for plants. During the bioinformatics evaluation of RNA sequencing results, we were able to evaluate and characterize the expression changes of 63,295 genes compared to the control treatment.

3.7.1. Characterization of Six Barley Varieties Included in Gene Expression Analysis

Spinner, Robur, Mascara, Dolphin, Mavlono, and Balda varieties proved to be the most versatile genotypes in terms of response to stress and genetic diversity among the six selected varieties. We applied three variables during principal component analysis. The three variables were control, single treatment, and combined heat stress treatment spike yield. Based on changes in yield data, six-row genotypes were clearly negatively affected by both stress treatments. Among six-row varieties, only Mavlono showed outstanding heat stress tolerance, with Balda also achieving better-than-average results. The response and yield reactions of the 14 varieties differed significantly among two-row varieties. In this group, differences were observed between early and late-heading varieties. Among late-heading varieties, Dolphin exhibited outstanding resistance. From the sensitive varieties, we tried to select genotypes that showed identical yield reactions to both stress conditions and had similar parameters. Based on spike type, we selected one six-row (Robur) and two two-row (Spinner, Mascara) varieties from the sensitive genotypes. From the more resistant varieties, we chose two six-row (Mavlono, Balda) and one two-row (Dolphin) variety.

3.7.2. Heat Stress Effects on Barley Gene Expression Activity

With the selected six barley varieties, we repeated the heat stress experiment, collecting flag leaf samples on the 7th day of stress treatment for varieties exposed to single heat stress. Sampling of control plants was also conducted simultaneously. RNA samples extracted from these samples were sequenced, and the genome-wide gene expression pattern was determined. The number of genes showing the greatest expression changes was depicted in a Venn diagram. The diagram determined overlaps of the top 20 genes showing the most increased or decreased expression by variety. Five genes were found in the intersection of the sets of the two-row Dolphin, and six-row Balda and Mavlono genotypes with the best resistance to high temperatures, and one gene was found in the intersection of all three more tolerant varieties, totaling six genes studied in more detail through functional genomic analysis. The physical position of these genes on barley chromosomes was determined. The REVEILLE 1 protein-like gene is located on chromosome 2H. On chromosome 3H, genes encoding the 17.5 kDa Class II heat shock protein-like and ATP-dependent Clp protease were identified. On chromosome 5H, a Small heat shock protein and a Serine carboxypeptidase-like gene were identified. At the end of the long arm of chromosome 6H, a gene encoding a 24.1 kDa heat shock protein is located.

3.8. Discussion of Gene Expression Changes Under Heat Stress

Heat shock proteins (HSPs), also known as chaperones, belong to the group of supporting proteins functionally, aiding in protein folding within cells under both normal and stress conditions (Baniwal et al., 2004; Kotak et al., 2007). HSPs are classified by their molecular weight, but their function is determined more by their structure than their size (Jacob et al., 2016). Heat shock proteins (HSPs) play important roles in proteostasis (protein homeostasis) and regulation of cell stress responses (Kaur et al., 2016). Under stress conditions such as heat, oxidative stress, toxic substances, or other environmental factors, the expression of HSPs can increase, helping cells adapt and survive these harmful effects. Our RNA sequencing data supported this, showing the highest level of gene expression changes (LFC) in the most resistant varieties (Dolphin, Mavlono). These heat shock proteins (Hsp10 (10 kDa), Hsp27 (27 kDa), Hsp40 (40 kDa), Hsp60 (60 kDa), Hsp70 (70 kDa), Hsp90 (90 kDa)) act as molecular chaperones, assisting in the correct folding and stabilization of other proteins in cells (Chaudhary et al., 2019; Sadura et al., 2020). Thus, they play an important role in maintaining cellular homeostasis and regulating stress responses. Among the heat shock proteins identified and studied in barley, the larger Hsp70 and Hsp90 proteins, are the most significant and well-studied heat shock proteins in plants (Sadura et al., 2020). These proteins are expressed more under stress conditions in barley, helping plants adapt and survive stress. In our studies, only heat shock proteins falling within the range of HSP10 and HSP27 showed significant expression activity in the two well-adapted, heat stress-tolerant barley varieties. Similar results have been reported in heat stress experiments conducted in ryegrass and potato, supporting the significant role of smaller heat shock proteins in plant defense responses (Usman et al., 2014).

The serine carboxypeptidase (SCPL) protein family genes have been described in Arabidopsis, rice, and wheat as playing a crucial role not only in plant growth and development but also in defense against abiotic stress effects (Fraser et al., 2005; Li et al., 2016; Zhu et al., 2018). Although similar results have not been published for barley as in other species, our studies showed a significant increase in the expression of serine carboxypeptidase genes under heat stress in the Mavlono, Dolphin, and Balda varieties, indicating a similar role in barley's response to abiotic stresses. The SCPL protein family contains enzymes that exhibit serine carboxypeptidase activity or have similar structural characteristics. These enzymes participate in protein metabolism and peptide breakdown (Feng et al., 2006; Mugford and Osbourn, 2010). Members of the SCPL protein family can be classified based on sequence and structural similarities. These enzymes typically appear as dimers or oligomers and contain an active site where carboxypeptidase activity occurs. SCPL enzymes may play a role in the metabolism of organic acids, phenolic compounds, and other bioactive compounds in plants (Mugford et al., 2009; Li et al., 2011). Additionally, they may play a significant role in shaping the composition and structure of the plant cell wall.

Xu et al. (2021) reported the results of their study, which was based on the groundwork laid by DAL DEGAN and colleagues in 1994, indicating that members of the SCPL protein family are involved in responses to drought and heat stress and in regulating plant heat stress tolerance. These enzymes may play a role in proteolytic processes induced by heat stress, protein degradation, and stress response signaling. For example, among the members of the SCPL protein family, the HSP81-3 (Heat Shock Protein 81-3, part of the HSP90 gene family) enzyme, also known as Hsp17.6C-CI1, has been shown to degrade heat stress-induced proteins and enhance drought and heat stress tolerance in plants (Xu et al., 2021). RVE1 (REVEILLE1) is a transcription factor that plays an important role in the development of response reactions to abiotic stress (Jang et al., 2024). The expression changes of the barley RVE1 gene under heat stress correlated with the regulation of stress response genes and tolerance. Furthermore, functional analysis of the RVE gene supported its role in heat stress tolerance in barley (Markham and Greenham, 2021). The RVE1 transcription factor plays a role in regulating the circadian rhythm of plants. The circadian rhythm represents the internal biological clock of plants, which dictates the pattern of their daily life cycle, including development, metabolic processes, and responses to environmental stimuli (Dakhiya and Green, 2023). The expression of RVE1 periodically changes with the circadian rhythm, rising and falling in accordance with the time of day in plants. Thus, it participates in the regulatory network of the circadian clock, which includes other transcription factors and genes. The RVE1 protein interacts with key components of the circadian clock, such as the CCA1 (CIRCADIAN

CLOCK ASSOCIATED 1) and LHY (LATE ELONGATED HYPOCOTYL) transcription factors, influencing the expression and activity of these genes (Rawat et al., 2009). The expression of the gene encoding ATP-dependent chloroplast protease (ATP-dependent Clp protease) significantly increased under stress in tolerant barley varieties. LI and colleagues (2021) found that this gene has a significant effect on defense against drought stress, as evidenced by its strong upregulation in barley in drought test experiments (Li et al., 2021b). Our results confirm that this enzyme also played a role in the development of a phenotype more resistant to heat stress, as we observed a significant increase in gene expression in Dolphin and Mavlono varieties.

4. Conclusions and recommendations

We compared the heat stress response of various winter barley genotypes, measured through field and controlled environmental experiments. Our results emphasized that both environments provide valuable, comparable, and, more importantly, complementary information about the extent and types of heat stress responses observed in barley varieties. Although the field experiment was conducted in one location, it was characterized by highly diverse climatic conditions over four consecutive years, the frequency and intensity of which are expected to increase due to the negative impacts of climate change. Our results highlighted that multi-year experiments conducted in a single location can complement experiments conducted at multiple sites, as the complexity of multivariate factors in the latter can be significantly reduced by combining them with weather patterns. Under controlled conditions, the scale of heat stress responses largely intensified, allowing for a more precise differentiation of various stress responses. In the combined heat stress experiment, the precise determination of the priming phenomenon was not possible, but the results showed a stronger correlation with the field experiment, indicating its closer simulation power. Heat stress was not constantly present in either environment; under such conditions, the ability to restore stress proved to be more effective in preserving yield than stress tolerance itself. Among the genotypes tested in our experiments, the Dolphin and Mavlono varieties best met this criterion, thus we recommend incorporating these varieties into new breeding programs. In general, it can be concluded that the level of tolerance is closely related to the adaptability of plants, as heat stress tolerance and subsequent regeneration greatly influence crop yield in cereals. To increase barley's heat stress tolerance, we recommend conducting a broader genetic study of a wider range of varieties and assembling an even more genetically diverse set of varieties, followed by detailed phenotypic analysis, in order to identify the best breeding partners. We believe that new breeding programs involving crosses between genetically distant but heat stress-tolerant genotypes may be the most successful in improving barley's resistance to heat stress. Using RNA sequencing results from samples in controlled heat stress experiments, we identified five genes whose expression significantly increased in tolerant varieties. Four of these genes were related to the production (17.5 kDa Class II heat shock protein, small heat shock protein, 24.1 kDa heat shock protein) or regulation (serine carboxypeptidase protein) of heat shock proteins. The use of these genes in breeding is currently only possible through breeding programs involving varieties that respond to heat stress with higher expression. However, we also consider it important to create overexpressing and knockout mutants and characterize their phenotypes more precisely to better understand the effects of these genes. RVE1 (REVEILLE1) was the only gene whose expression significantly decreased under heat stress in the more resistant varieties. The major functions of this gene are already known, and experiments have been conducted in barley confirming its significant role in the development and regulation of heat stress responses, as well as its role in regulating the circadian rhythm of plants. Exploring the conserved connections between the circadian clock and stress may help identify targets to be studied in plants. We believe that understanding the relationship between the circadian rhythm and heat stress response could be aided by conducting stress experiments using wild barley and barley landraces to identify the evolutionary processes or genetic variations that have influenced the regulation of the circadian rhythm and stress response during the selection of modern varieties.

4.1. Literature

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5. New scientific results

1. We have determined that in the case of winter barley varieties, spike type as a genetic characteristic does not influence heat stress tolerance within the variety group.

2. When evaluating the yield stability of 28 varieties against high temperatures, we created a ranking using stress tolerance and yield stability indices to identify which varieties are most resistant to stress. We recommend including the top-ranked varieties in new breeding programs to improve barley heat stress tolerance.

3. We identified various yield response types based on the distribution of main and secondary spike yield results under stress. We found significant differences in yield components depending on the stress effect. Varieties with better heat tolerance mainly showed less reduction in main spike yield. Varieties with better adaptability compensated for the lower main spike yield by increasing the yield values of secondary spikes.

4. It is noticeable that the group of genotypes compensating for secondary spike yield had better regeneration and adaptability, as they were able to restore their physiological and developmental status in the regeneration chamber after stress, significantly improving the yield of secondary spikes.

5. Among the varieties with good heat tolerance and adequate adaptability, we successfully identified heat stress tolerance-related gene expression changes in three out of three cases. The expression of the REVEILLE1 gene decreased in all three considered tolerant varieties. The expression of genes encoding the 17.5 kDa heat shock protein (HSP17), ATP-dependent chloroplast protease, 24.1 kDa heat shock protein (HSP24), small heat shock protein (sHSP), and serine carboxypeptidase protein (SCPL) significantly increased under stress, and these genes may have a strong impact on heat stress tolerance in the examined varieties.

6. The author’s publication activity

6.1. Publications in international scientific journals with IF:

HORVÁTH, Á, KISS, T, BERKI, Z, HORVÁTH, D. Á, BALLA, K, CSEH, A, VEISZ, O, KARSAI, I. (2023). Effects of genetic components of plant development on yield-related traits in wheat (Triticum aestivum L.) under stress-free conditions, *Frontiers in Plant Science,* 13 Paper: 1070410, 22 p.

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6.2. Book, Part of books:

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6.3. Proceedings:

BALLA, K., KARSAI, I., BÓNIS, P., KISS, T., BERKI, Z., **HORVÁTH, Á.**, MAYER, M., VEISZ, O. (2019). Eltérő ideig tartó hőstressz hatása búza különböző fejlődési stádiumában, In: Karsai, Ildikó (szerk.) Növénynemesítés a 21. század elején: kihívások és válaszok: XXV. Növénynemesítési Tudományos Nap 2019, Budapest, Magyarország: Magyar Tudományos Akadémia Agrártudományok Osztályának Növénynemesítési Tudományos Bizottsága, pp. 127-131., 5 p.

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6.4. Scientific lectures, posters:

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