**The effects of reduced height (*Rht*) genes to the heat-stress sensitivity of hexaploid wheat: meiotic stability and fertility**

Project ID: NKFI-FK-124266

Final report

Wheat grain is the result of sexual reproduction, requiring a successful fertilisation between a female reproductive (egg) cell and a male gamete. Meiotic cell division, the key process ensuring functional gamete formation, is highly error prone and is one of the most vulnerable cellular events affecting fertility. In plants, meiosis has a strict requirement for temperature with the developmental period prior flowering being particularly sensitive to high temperature stress (De Storme & Geelen, 2020). Bread wheat, one of our most important crops, is directly exposed to climatic conditions in Nature and has been recently shown to respond with altered reproductive development and reduced productivity to the current rising global temperature trends (Asseng *et al.*, 2015; Stratonovitch & Semenov, 2015; Jacott & Boden, 2020; Zhu *et al.*, 2021). The aim of the present study was to model temperature conditions imposed by the warming climate and study the effects of elevated spring temperatures on the fertility of wheats carrying the widely adopted *Rht-B1b* (also known as *Rht1*) *and Rht-D1b* (*Rht2*) alleles. We applied a short period of elevated temperature to the early reproductive stage of near isogenic lines carrying the *Rht-B1b and Rht-D1b* mutations and we assessed the effects on plant fertility and on the progression of meiosis with a focus on synapsis and meiotic recombination, both essential for genome stability and fertility. The effects of heat stress have then been assessed on chromosome segregation and the fidelity of genome inheritance within lines carrying the *Rht* mutations and in wild type wheat. Transcriptional activation of the gibberellin biosynthetic pathway was monitored by RT qPCR and droplet digital PCR combined with direct hormone measurements by ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) revealed the levels of bioactive gibberellins (GAs) in the young developing wheat ears.

Objective 1: Study the effect of temperature to seed set and pollen viability in *Rht* RIL

Seeds of the 'Maris Huntsman' *Rht-B1b*- and *Rht-D1b*- mutants and the wild type near isogenic lines (accession numbers: W9983, W9984, W9982, respectively) were provided by the Germplasm Resources Unit of John Innes Centre (Norwich, UK). Experiments were carried out in growth cabinets at the Phytotron Facility of the Centre for Agricultural Research. Following a six-week vernalisation period (4°C, 10 h : 14 h, light : dark) plants were potted in 12 cm x 12 cm x 18 cm pots and transferred to a growth cabinet (PGR-15, Conviron, Canada) following standard spring programme (Tischner et al., 1997). Plants were transferred to a stress cabinet (PGR-15) when the main shoot entered meiotic interphase. Heat stress involved a day : night temperature of 30°C for 24 h. Seed set was determined after full maturity by counting the total number of grains per primary ear. Spikelet number was recorded for each ear analysed and spikelet fertility was determined by counting the number of grains per spikelet. Elevated temperature resulted in a significantly lower mean seed set for both *Rht-B1b* (n=60) and *Rht-D1b* (n=54) lines compared to a non-significant decrease in wild type (n=64; ANOVA, F (5,

172) = 2377.9, p < 0.001). Spikelet number did not vary with genetic background, or the temperature treatments, indicating that reduced spikelet fertility is responsible for the seed set reduction (Kruskal-Wallis, Dunn's post hoc test, H (5) = 76.829, p < 0.001). The main spikes of *Rht-B1b* and *Rht-D1b* mutants exhibited a 20% and 31% loss in spikelet fertility (Figure 1). A pronounced effect (40% loss) was detected within the basal region of *Rht-D1b* ears (Kruskal-Wallis, Dunn's post hoc test, H (5) = 66.899, p < 0.001) whereas the apical region exhibited a 22% reduction (Kruskal-Wallis, Dunn's post hoc test, H (5) = 50.292, p < 0.001). Reduction in the number of viable pollens as detected for each mutant lines supported male meiotic defects as a cause of significant infertility. Our results showed that the early reproductive stage of *Rht-B1b* and *Rht-D1b* lines exhibits a higher vulnerability to heat stress compared to the wild type (tall) line, with the basal region of *Rht-D1b* ears being particularly heat-sensitive within the single mutants.

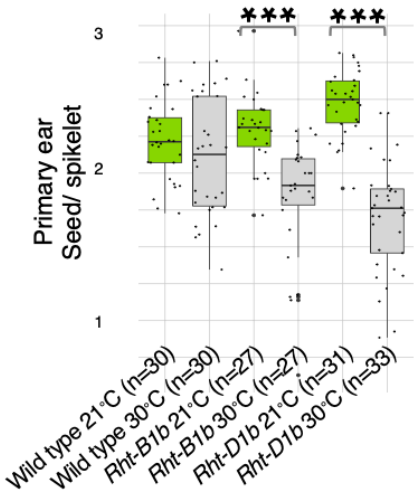


*Figure 1. Spikelet fertility of wild type Rht-B1b and Rht-D1b under optimal (21°C) and heat stressed (30°C) temperature conditions.*

Objective 2: Gene expression assay of *Rht*- and GA biosynthesis genes:

The vegetative tissues of GA insensitive *Rht* mutants exhibit an increased GA content under optimal conditions (Appleford & Lenton, 1991; Webb *et al.*, 1998). The considerable negative effect of heat stress exerted on the fertility of the GA insensitive *Rht* mutants may thus be caused by a further boost in GA production via transcriptional activation of the gibberellin biosynthesis genes (Stavang et al., 2009). We therefore sought to use RT-qPCR and droplet digital PCR (ddPCR) and investigate the transcription of GA biosynthesis genes within the young developing wheat ears. Two key enzymes, involved in the late stages of biosynthesis in wheat are *TaGA 20-oxidase* (*TaGA20ox*) and *TaGA 3-oxidase* (*TaGA3ox*) (Pearce et al., 2015; Barker et al., 2021). *TaGA20ox* catalyses multiple late reactions, whilst *TaGA3ox* controls the final step of GA biosynthesis. RT-qPCR showed relatively stable *TaGA20ox* relative transcript levels following elevated temperature treatment in each genotype (Kruskal-Wallis, H (5) = 17.5, p = 0.004, Dunn's post hoc test), implying that heat stress did not have a major effect on the transcription of *TaGA20ox*. *TaGA3ox* transcription levels however significantly increased in the ears of each genotype after elevated temperature treatments (Figure 2; Kruskal-Wallis, H (5) = 30.5, p < 0.001; Dunn's post hoc test, wild type p = 0.022, *Rht-B1b* p = < 0.001 and *Rht-*

*D1b* p = 0.005). mRNA accumulation was higher within the *Rht-B1b* and *Rht-D1b* mutants (4.7-fold and 2.3-fold increase, respectively) compared to the wild type (2.15-fold increase).

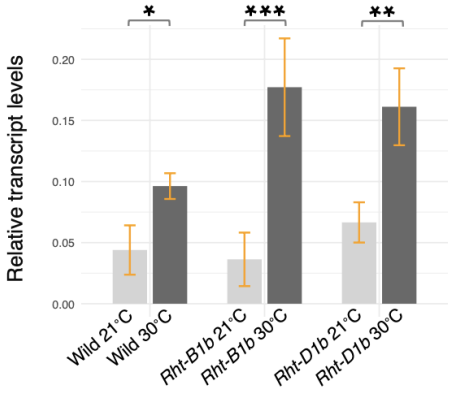


*Figure 2. Relative transcript levels of TaGA3ox gene encoding the enzyme that catalyses the final stage of bioactive gibberellin synthesis. Transcript levels increased in each genotype after heat treatment, but at a greater increase is shown in the mutants.*

To obtain a larger sample partitioning and to distinguish between transcript concentrations measured at the basal and apical regions of the ears we investigated spikelets collected selectively from the basal- and apical regions of the ears by ddPCR assay. All lines showed a steady transcription along the spike under control conditions (Kruskal-Wallis test, H (11) = 28.2, p = 0.003, Dunn's post hoc test: wild type p = 0.166, *Rht-B1b* p = 0.454, *Rht-D1b* p = 0.176). Heat stress caused a significant increase in the basal ear region of the wild type, whereas the apical region remained unaffected (p = 0.017 and 0 = 0.231, respectively). *Rht-B1b* mutants showed a significant increase in both regions at high temperature (p = 0.031 and p = 0.011, respectively) and both basal and apical regions showed an induction in *Rht-D1b* (p = 0.005 and p = 0.022, respectively), however the increase in the basal region was considerably greater (ca. 3-fold increase).

These revealed a heat-induced transcriptional bias towards the base of the spike, particularly in the *Rht-D1b* mutant and suggested an increased GA level following heat stress treatment. Although not included in the original work plan, on account of the gene expression data, we conducted direct GA hormone measurements by UPLC-MS/MS. Bioactive $GA_1$, $GA_3$ and $GA_4$, and the inactive forms $GA_{20}$ and $GA_8$ were quantified using deuterated GAs as internal standards. $GA_3$ and $GA_4$ levels remained below 0.5 ng $g^{-1}$ fresh weight (FW) in each genotype and treatment indicating that they are not predominant. A similar profile was detected in the $GA_1$ precursor $GA_{20}$ (< 0,5 ng $g^{-1}$ FW). Bioactive $GA_1$ however ranged from 2-3 ng $g^{-1}$ FW without showing any significant difference between the genotypes and treatments (Kruskal-Wallis, H (5) = 7.53, p = 0.184, wild type M = 2.1 ng $g^{-1}$ FW, *Rht-B1b* M = 3.1 ng $g^{-1}$ FW, *Rht-D1b* M = 2.2 ng $g^{-1}$ FW). The $GA_1$ metabolite, $GA_8$ was present in the ears of all genotypes and showed a minor, non-significant decline after heat treatment (Kruskal-Wallis, H (5) = 15.3, p = 0.009, Dunn's post hoc test, wild type control vs. wild type treated p = 0.06; *Rht-B1b* control vs. *Rht-B1b* treated p = 0.051, *Rht-D1b* control vs. *Rht-D1b* treated p = 0.2). These indicate that in the wheat tissues measured by our analysis neither the genetic background neither the heat treatment affected GA levels and thus a higher GA level cannot be responsible for the

significant infertility of the *Rht* mutant lines. Further studies are needed to determine the physiological background of heat-induced infertility in the *Rht* mutants.

Objective 3: Study of meiosis within the mutant and wild *Rht* lines subject to high temperature stress

We then sought to use immunofluorescence analysis of ASY1 axial element protein to investigate whether fertility losses are associated with errors observed in the meiotic chromosome axis, which is essential for synaptonemal complex (SC) formation and CO assurance (Armstrong *et al.*, 2002; Lambing *et al.*, 2020; Pochon *et al.*, 2022). The effects of heat stress on SC polymerisation were also measured in the *Rht-B1b* and *Rht-D1b* lines by using ZYP1- immunofluorescence. ZYP1 labelling revealed the nuclear localisation of the SC central element and indicated synapsed chromosomal regions (Higgins *et al.*, 2005). ASY1 loading appeared normal in each genotype after heat treatment, but depletion was defective in *Rht-B1b* and *Rht-D1b*, as observed on pachytene stage meiocytes. Heat stress thus affected the structure of the chromosome axes in heat-treated *Rht* mutant lines via an altered axis remodelling at the late stages of prophase I.

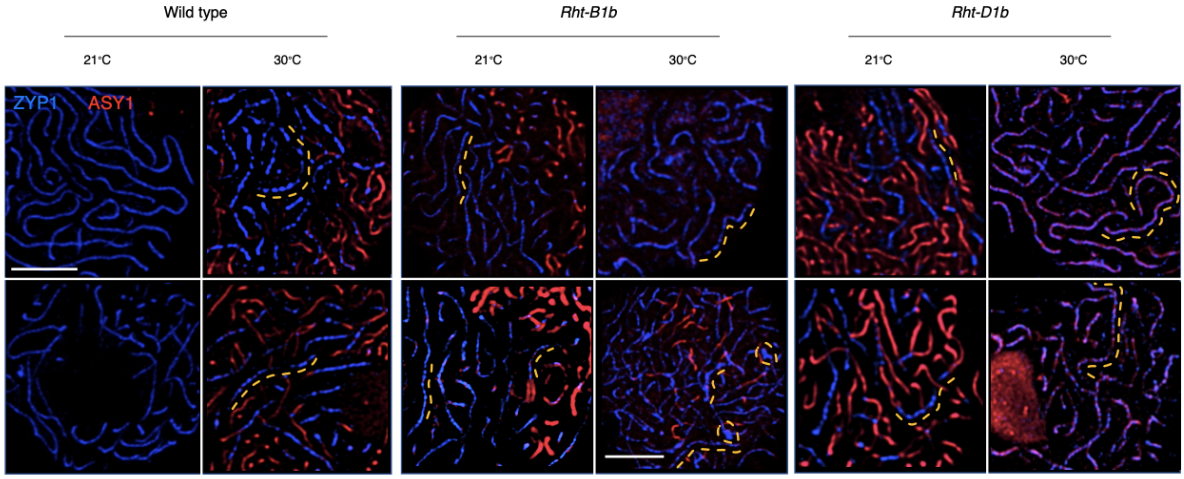


*Figure 3. Representative images of disrupted synaptonemal complex formation after heat treatment (30°C) in the wild type and the Rht-B1b and Rht-D1b mutants.*

*Rht-D1b* mutants exhibited a complete lack of the spatial asymmetry during SC polymerisation, characteristic of early meiosis in cereals. Instead, multiple SC initiation points were uniformly distributed in the nucleus, indicating a perturbed meiosis already under control conditions. Heat stress further altered SC formation in the *Rht* mutants. In *Rht-B1b* nuclei subtelomeric synapsis initiated along with enlarged ZYP1 protein aggregates (polycomplexes), disclosing a disrupted SC structure. A subset of the sampled *Rht-D1b* early-zygotene nuclei (20%) exhibited equivalent SC initiation defects to *Rht-B1b*, whereas the majority (ca. 50%) showed a dispersed SC initiation, reminiscent of the *Rht-D1b* controls. High-resolution microscopy revealed discontinuous SC structures in the heat-treated *Rht-B1b* and *Rht-D1b* mutant PMCs, and at a lesser extent (ca. 18% of the nuclei) in the wild type (Figure 3). Synaptonemal complex formation thus shows an increased heat-susceptibility in the *Rht-B1b* and *Rht-D1b* lines

compared to the wild type, most likely due to a genetic predisposition seen by meiotic alterations under optimal conditions. We next determined heat-induced changes in CO frequency in *Rht-B1b* and *Rht-D1b* mutant plants by scoring the number of chiasmata per meiotic metaphase I (MI) spread (Sybenga, 1975). Our analysis showed that heat stress significantly reduced chiasma number in all genotypes causing a 12% reduction in the wild type (M = 45, SD = 8,2, n = 17; p = 0.01), a loss of 38% in *Rht-B1b* (M = 33, SD = 6.2, n = 2 1; p<0.001) and a reduction of 21% in *Rht-D1b* (M = 42, SD = 4.6, n = 18; p < 0.001). Reduction in CO frequencies affected crossover assurance, resulting in the appearance of univalent chromosomes, which were more frequent in *Rht-B1b* and *Rht-D1b* lines ($\chi 2$ (5) = 26.452, p<0.001). Univalent chromosomes lack the obligate chiasma, essential for correct chromosome segregation in anaphase I and segregate randomly, frequently leading to lagging chromosomes and programmed chromosome elimination. Reduction in the homologous recombination frequency was also accompanied by the occurrence of non-legitimate connections between the bivalents. This was clear from poorly spread chromosome conformations connected by thin chromatin bridges, often hindering regular chromosome alignment and centromere biorientation at the equatorial plate.

To reveal the effect of heat stress on anaphase I and anaphase II chromosome segregation and on genome stability in tetrads and microspores, we combined DAPI counterstaining with CENH3 immunolabelling and followed chromosome behaviour together with centromere activity. Chromosome mis-segregation and chromatin elimination by micronuclei were detected from anaphase I to the early microspore stage in the heat-treated wild type, *Rht-B1b* and *Rht-D1b* mutant lines (Figure 4).

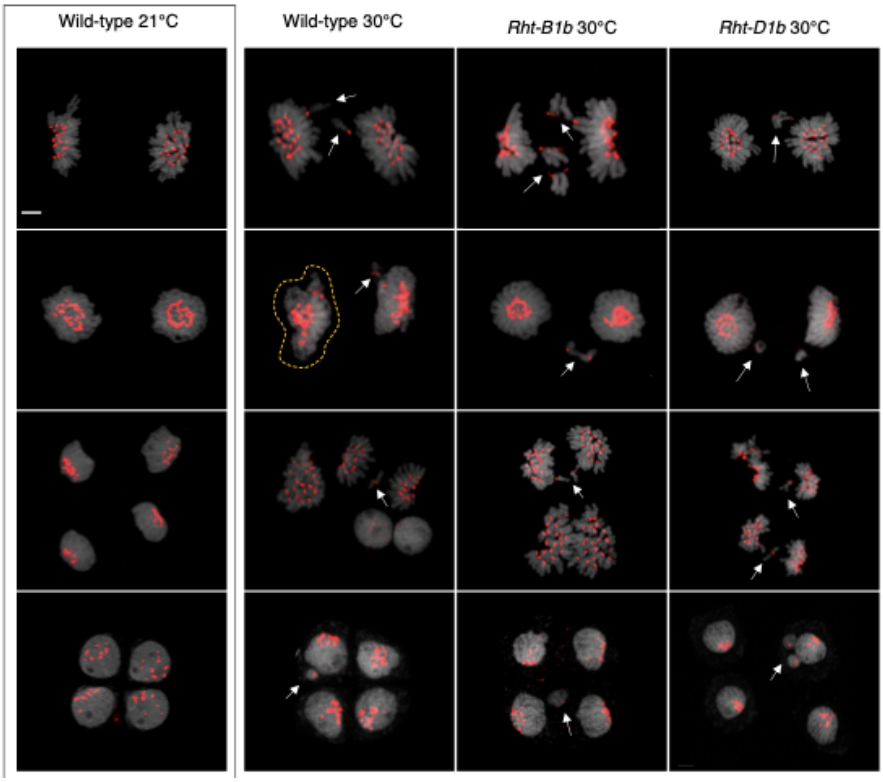


*Figure 4. Chromosome mis-segregation and chromatin elimination by micronuclei from telophase I to the tetrad stage are indicated by arrows on heat-treated wild type, Rht-B1b and Rht-D1b meiocytes.*

Unexpectedly, equivalent meiotic errors were detected in the control *Rht-B1b* and *Rht-D1b* meiocytes, although with lower frequencies than in the heat-treated cells. CENH3 immunofluorescence revealed mis-segregating chromosomes equipped with functional centromeres, although incorrect spindle attachments and random positioning around the bulk chromatin were frequent.

Analysis of contingency tables followed by Chi-squared tests showed unequal incidence of aberrant cells within the investigated wild type and *Rht* mutant genotypes and between their respective temperature treatments ($\chi 2$ (5) = 148.654, p<.001). *Rht* mutant meiocytes carried aberrant meiocytes under optimal temperature conditions and these meiotic aberrations became more frequent after heat treatment. Cytological analysis showed a higher number of aberrant microspores within the heat stressed *Rht* mutant lines compared to their controls ($\chi 2$ (5) = 37.416, p<0.001). Aberrations were manifested in deformed nuclei or as multiple micronuclei located around the main nucleus (Figure 5).

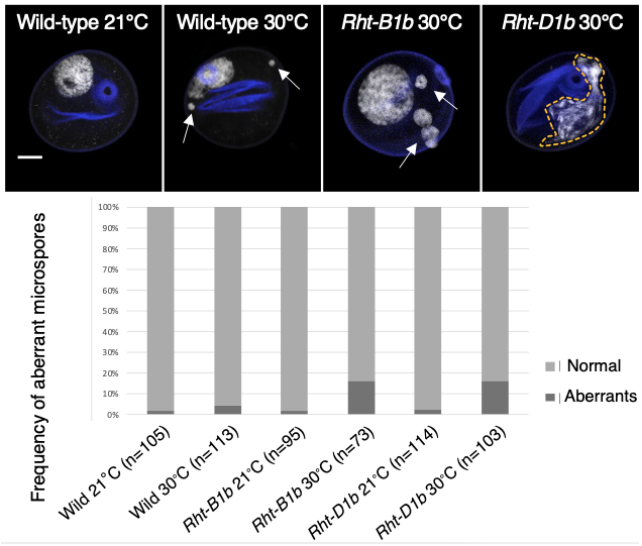


*Figure 5. Frequency of aberrant microspores in the control (21°C) and heat treated (30°C) wild type, Rht-B1b and Rht-D1b plants. Aberrant micronuclei showing chromatin elimination are highlighted by an arrow. An amorphous nucleus is circled in yellow.*

Heat-inferred meiotic errors detected during earlier meiotic stages thus resulted in unbalanced chromosome segregation and programmed chromatin elimination throughout MII where the frequency of aberrations was significantly higher in the mutants than in the wild type. These confirmed an increasingly heat-susceptible meiosis in *Rht-B1b* and *Rht-D1b* lines compared to the wild type.

Conclusions

In the present research project, the extent of heat stress was specifically selected to model the effects of a single heat wave on the meiotic cell division and fertility of wheat, by focusing on the Green Revolution semi-dwarf genetic backgrounds, which occur in most of today's wheat cultivars. We showed that wheat lines carrying the *Rht-B1b* or *Rht-D1b* Green Revolution alleles are more vulnerable to heat stress than their wild type, tall counterparts. We showed evidence that *Rht* mutants respond with a considerable degree of meiotic defects after heat

treatments, leading to a significant loss in spikelet infertility. Our study suggests, that under the warming climate, vulnerability of current crop varieties needs to be revisited and addressed globally. Among these, thermotolerance during the period of floral development needs a primary focus, due to the temperature sensitivity of the production of viable gametes. Further research on novel dwarfing genes is urgently needed for the development of new high-yielding genotypes, adaptable to the warmer temperature conditions.

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Dissemination

Currently one paper summarising the results outlined abow is submitted to a multidisciplinary international journal (New Phytologist), authors are now awaiting for the assessments.

Title: **Meiotic instability and irregular chromosome pairing underpin heat-induced infertility in bread wheat carrying the *Rht-B1b* or *Rht-D1b* Green Revolution genes.**

Authors: András Cseh, Andrea Lenykó-Thegze, Diána Makai, Fanni Szabados, Kamirán Áron Hamow, Zsolt Gulyás, Tibor Kiss, Blanka Moncsek, Edit Mihók, Adél Sepsi

Published Scientific papers:

**Adél Sepsi**, Attila Fábián, Katalin Jäger, John Seymour Heslop-Harrison, Trude Schwarzacher: **ImmunoFISH: Simultaneous Visualisation of Proteins and DNA Sequences Gives Insight Into Meiotic Processes in Nuclei of Grasses**. Frontiers in Plant Science, 2018

**Adél Sepsi**, Trude Schwarzacher: **Chromosome–nuclear envelope tethering – a process that orchestrates homologue pairing during plant meiosis?** Journal of Cell Science, Vol 133, issue 15., 2020

Andrea Lenykó-Thegze, Attila Fábián, Edit Mihók, Diána Makai, András Cseh, **Adél Sepsi**: **Pericentromeric chromatin reorganisation follows the initiation of recombination and coincides with early events of synapsis in cereals.** The Plant Journal,

Abstract:

**Sepsi Adél**, Fábián Attila, Jäger Katalin: **A női ivarsejteket kialakító meiótikus sejtosztódás vizsgálata modern sejtbiológiai módszerekkel kenyérbúzában**, XXIV Növénynemesítési Tudományos Nap, 2018

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Makai Diána, Mihók Edit, Cseh András, Kiss Tibor, Karsai Ildikó, Darkó Éva, Jäger Katalin, **Sepsi Adél**: **The Effects of RhtB1b and RhtD1b Reduced Height Alleles to the HEAT-Stress Sensitivity of Hexaploid Wheat**, PO0923, 2020

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