Cell Biology
Chromosomes and Gene Expression

Heat stress impairs centromere structure and segregation of meiotic chromosomes in Arabidopsis

Reviewed Preprint

Revised by authors after peer review.

About eLife's process

Reviewed preprint version 2
March 12, 2024 (this version)

Reviewed preprint version 1
September 20, 2023

Sent for peer review
July 7, 2023

Posted to preprint server
June 16, 2023

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Abstract

Heat stress is a major threat to global crop production, and understanding its impact on plant fertility is crucial for developing climate-resilient crops. Despite the known negative effects of heat stress on plant reproduction, the underlying molecular mechanisms remain poorly understood. Here, we investigated the impact of elevated temperature on centromere structure and chromosome segregation during meiosis in *Arabidopsis thaliana*. Consistent with previous studies, heat stress leads to a decline in fertility and micronuclei formation in pollen mother cells. Our results reveal that elevated temperature causes a decrease in the amount of centromeric histone and the kinetochore protein BMF1 at meiotic centromeres with increasing temperature. Furthermore, we show that heat stress increases the duration of meiotic divisions and prolongs the activity of the spindle assembly checkpoint during meiosis I, indicating an impaired efficiency of the kinetochore attachments to spindle microtubules. Our analysis of mutants with reduced levels of centromeric histone suggests that weakened centromeres sensitize plants to elevated temperature, resulting in meiotic defects and reduced fertility even at moderate temperatures. These results indicate that the structure and functionality of meiotic centromeres in *Arabidopsis* are highly sensitive to heat stress, and suggest that centromeres and kinetochores may represent a critical bottleneck in plant adaptation to increasing temperatures.

eLife assessment

This study is an **important** contribution to our insights into the impact of heat stress on sexual reproduction in plants and provides information about how centromere integrity is affected by heat stress during male meiosis in Arabidopsis thaliana. The evidence supporting the claims, specifically the dynamics of tagged proteins in meiocytes by live cell imaging is **solid**, even though a deeper mechanistic understanding is still lacking.

Introduction

Global warming impacts crop productivity with more frequent and extreme heatwaves being particularly damaging (Bras et al., 2021). The lower yields associated with heatwaves are mainly attributed to the impairment of the plant reproductive system (Lippmann et al., 2019). Thus, a fundamental understanding of how heat stress affects plant reproduction can guide breeding strategies towards generating climate change resilient crops. Heat stress impairs both male and female reproductive structures, but male gametogenesis is particularly sensitive to higher temperatures (Zinn et al., 2010). Studies in crop and non-crop species have shown that heat stress affects pollen count, morphology, viability, dehiscence, germination and pollen tube growth (De Storme and Geelen, 2014; Chaturvedi et al., 2021).

Acute heat stress applied at different stages of flower development has been found to have the most detrimental effect during microsporogenesis (Pecrix et al., 2011; Hedhly et al., 2020). Meiosis, a reductive cell division that halves number of chromosomes in two consecutive rounds of chromosome segregation, is a crucial step of microsporogenesis. It is a relatively slow process, lasting approximately 1.5 to 2 days in Arabidopsis and barley (Armstrong et al., 2003; Higgins et al., 2012; Prusicki et al., 2019; Valuchova et al., 2020). Prophase I, the longest phase of meiosis, is characterized by pairing and recombination of homologous chromosomes to form stable bivalents. It is followed by two chromosome segregation cycles that first divide homologous chromosomes and then the sister chromatids. Heat stress affects various aspects of plant meiosis. In prophase I, elevated temperature alters the rate and distribution of meiotic recombination (Higgins et al., 2012; Lloyd et al., 2018; Modliszewski et al., 2018) and a recent study in Arabidopsis has shown that heat shock response pathway directly regulates the recombination machinery (Kim et al., 2022). More severe heat stress can impair synapsis and pairing of homologous chromosomes (Loidl, 1989; De Storme and Geelen, 2020; Ning et al., 2021). This is likely due to inefficient completion of homologous recombination, which is monitored by specialized pachytene checkpoint (De Jaeger-Braet et al., 2022).

Heat stress affects the later stages of meiosis as well. Elevated temperatures disturb spindle orientation during meiotic divisions and the formation of radial microtubule arrays, resulting in aberrant cytokinesis and diploid microspores (Pecrix et al., 2011; De Storme and Geelen, 2020). Furthermore, acute heat stress leads to chromosome mis-segregation and the formation of micronuclei (Wang et al., 2017; De Storme and Geelen, 2020; De Jaeger-Braet et al., 2022). The meiotic micronuclei and chromosomal aberrations may arise as a consequence of abnormal repair of meiotic DNA breaks or homologous recombination intermediates in prophase I. However, they can also be caused by defects in chromosome segregation during meiotic divisions (Fenech et al., 2011). Proper attachment of kinetochores to the spindle microtubules before anaphase is crucial for faithful chromosome partitioning to daughter cells, and this process is controlled by spindle assembly checkpoint (SAC). Therefore, micronuclei typically occur in mutants with impaired centromere/kinetochore structure or SAC when treated with spindle inhibitors (Kalitsis et al., 2000; Lermontova et al., 2011b).

In most eukaryotes centromeres are confined to a restricted region on each chromosome that serves as a platform for assembly of the kinetochore, a multiprotein structure that connects chromosome with the spindle. Centromeres are defined epigenetically by a specialized centromeric histone H3 variant (CENH3), which forms the foundation for the recruitment of kinetochore proteins (McAinsh and Marston, 2022). In contrast to canonical histones, replicative dilution of CENH3 is not replenished immediately in S-phase, but in subsequent stages of the cell cycle (Stirpe and Heun, 2023). In plants, CENH3 is loaded on centromeres in G2 and persists there throughout the cell cycle (Talbert et al., 2002; Lermontova et al., 2006). CENH3 is a very stable component of centromeric chromatin, although its amount at centromeres gradually declines in terminally differentiated cells in plants and animals (Lermontova et al., 2011a; Swartz et al., 2019).

In this study, we have discovered that heat stress significantly reduces the amount of CENH3 on meiotic chromosomes in *Arabidopsis thaliana*. This loss of CENH3 leads to the formation of micronuclei, which in turn contributes to the decrease in pollen formation and fertility in plants exposed to heat stress. Additionally, we have found that plants with a genetic mutation that reduces the amount of centromeric histone are more sensitive to moderately elevated temperature. These results suggest that meiotic centromeres may represent a crucial point of vulnerability for plants in adaptation to raising temperatures.

Results

Pollen production and fertility decline with increasing temperature

In our previous work, we identified the *cenh3-4* allele of Arabidopsis centromeric histone *CENH3* that carries a mutation in the splicing donor site of the 3rd exon (Capitao et al., 2021). This leads to a 10-fold reduction in fully spliced *CENH3* mRNA and a decreased amount of centromeric histone. Consequently, *cenh3-4* plants have smaller centromeres and are sensitive to oryzalin (Capitao et al., 2021). Under standard conditions, *cenh3-4* mutants are barely distinguishable from wild type, but we noticed their reduced fertility when grown at an elevated temperature. To systematically assess this phenotype, we grew plants under standard conditions (21°C) until they formed four true leaves, and then continued their cultivation in growth chambers tempered to 16, 21, 26 and 30°C (Figure S1). At 16°C, plants exhibited the slowest growth, but also the highest fertility, as assessed by pollen count and silique length, which is indicative of seed yield (**Figure 1**). Fertility slightly decreased at 21°C and further declined at 26°C. In *cenh3-4* mutants, we noticed a sharp decline in pollen production and fertility at 26°C (86±48 pollen per anther), whereas the fertility of the wild type was still relatively high (213±58 pollen per anther) (**Figure 1B,C**). Both *cenh3-4* and wild type plants became infertile at 30°C. The heat-induced sterility was reversible and *cenh3-4* as well as wild type plants transferred from 30 to 21°C regained fertile flowers (Figure S1C). These data indicate that pollen production and fertility gradually decline with increasing temperature and this trend is particularly pronounced in *cenh3-4* mutants that have become almost sterile already at 26°C.

It has been reported that extreme heat stress alters chromosome segregation fidelity and the duration of Arabidopsis meiosis (De Jaeger-Braet et al., 2022). Temperatures of 34°C and above abolished chromosome pairing and synapsis and led to the formation of univalents. However, meiotic chromosomes are fully paired at 30°C (Ning et al., 2021; De Jaeger-Braet et al., 2022; Fu et al., 2022) indicating that the recombination defects are not primarily responsible for abortive pollen development at this temperature.

Heat stress delays meiotic progression and induces micronuclei

To assess the effect of temperature on meiotic progression and chromosome segregation at temperatures up to 30°C, we performed live imaging of meiotic divisions in the HTA10:RFP reporter line, which marks chromatin (Valuchova et al., 2020). We measured the duration of meiotic divisions from the end of diakinesis until the formation of haploid nuclei in telophase II (Figure S2). Meiotic divisions were slowest at 16°C and lasted, on average, 441 and 459 min in wild type and *cenh3-4*, respectively (Movies 1 and 2, **Figure 2A**). Meiosis progressed significantly faster with increasing temperature. Wild type meiotic divisions took 169 min at 21°C and 142 min at 26°C (Movies 3 and 4). Meiotic divisions were also rapid in *cenh3-4* at 21°C, lasting on average 156 min, but slowed down to 238 min at 26°C (Movies 5 and 6). This contrasts with the situation in wild type, where divisions occur at the fastest rate at 26°C. Interestingly, meiotic divisions were prolonged at 30°C in both wild type and *cenh3-4* mutants to 286 and 264 min, respectively (Movies

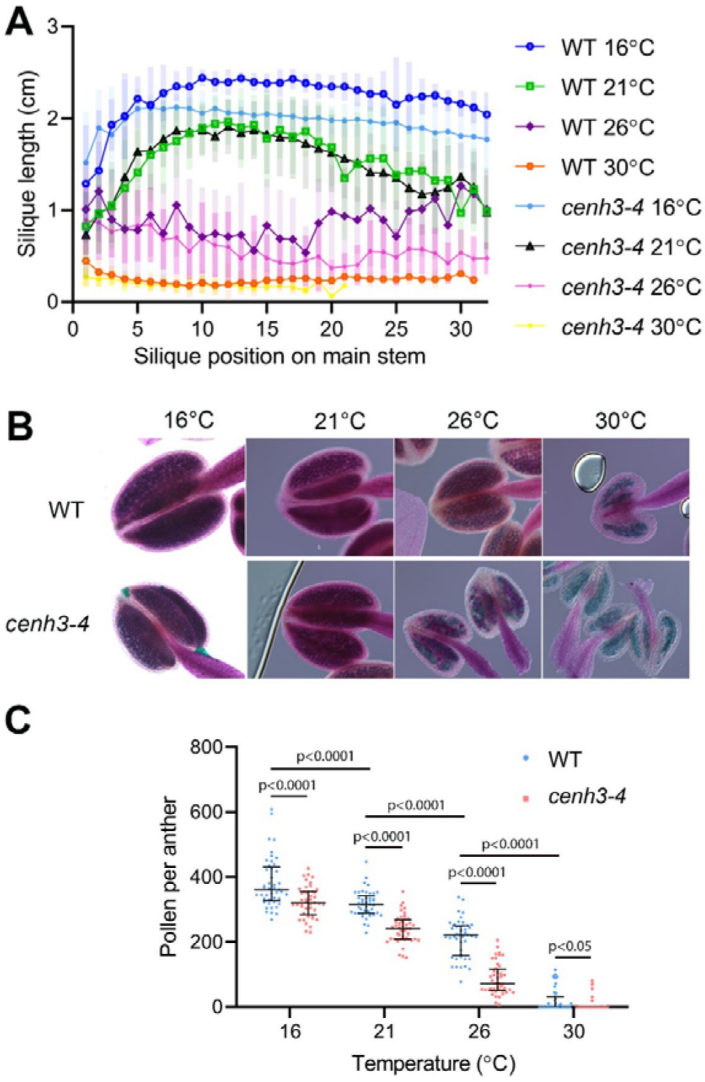

Figure 1.

Effect of temperature on fertility in wild type and *cenh3-4* plants.

(A) Analysis of silique length through the main stem of wild type (WT) grown at 16°C (n=18), 21°C (n=25), 26°C (n=13), 30°C (n=20) and *cenh3-4* mutant at 16°C (n=22), 21°C (n=20), 26°C (n=22) and 30°C (n=11). The silique position is numbered from the oldest to the youngest silique on the main stem. Error bars depict standard deviation. (B) Anthers of the abovementioned plants after Alexander staining. (C) Quantification of viable pollen per anther (n=45). Significance of the difference is counted using Two-tailed t-test.

7 and 8). The slowdown of meiosis at 26°C and 30°C in *cenh3-4*, and at 30°C in wild type coincides with the steep decline of pollen production (**Figure 1B,C**) and indicates problems with meiotic progression.

Live imaging in *cenh3-4* plants and, at 30°C, also in wild type showed formation of micronuclei that began to form during meiosis I and persisted till telophase II (Movies 7 and 8). We quantified the micronuclei in fixed anthers at the tetrad stage using confocal microscopy (**Figure 2B,C**). The micronuclei were apparent in *cenh3-4* mutants at all temperatures, which is consistent with partially impaired centromere function in these plants (Capitao et al., 2021). Nevertheless, their occurrence substantially increased at 26°C and severe defects in chromosome segregation were observed at 30°C (**Figure 2B,C**; Movies 6 and 8) resulting in very few regular tetrads. Whereas we occasionally detected micronuclei at lower temperatures also in wild type, albeit at a much lower level than in *cenh3-4*, their incidence increased 17-fold between 26°C and 30°C (**Figure 2B,C**, Movies 4 and 7). In addition, we observed dyads and polyads, which is consistent with the previous study performed at a similar temperature (De Storme and Geelen, 2020). These data show that temperature-induced fertility reduction coincides with increased occurrence of micronuclei.

Micronuclei may arise from acentric fragments and dicentric chromosomes derived from defective repair of meiotic breaks. Indeed, temperatures above 30°C were reported to cause aberrant recombination intermediates and induce pachytene checkpoint (De Storme and Geelen, 2020; De Jaeger-Braet et al., 2022). In this scenario, the absence of meiotic breaks in SPO11-deficient plants should prevent the formation of temperature-induced micronuclei. Arabidopsis *spo11-2* mutants do not form bivalents, and homologous chromosomes segregate randomly in anaphase I (Stacey et al., 2006; Hartung et al., 2007). Cytogenetic analysis in *spo11-2* plants revealed a relatively high level of micronuclei in tetrads at 21°C (**Figure 2B,C**). Because unpaired univalents cannot properly bi-orient at metaphase I spindle, these micronuclei are likely generated by chromosome mis-segregation. Importantly, number of the micronuclei almost doubled in *spo11-2* plants grown at 30°C (**Figure 2B,C**) arguing that temperature has a direct effect on chromosome segregation.

In its natural habitats, *A. thaliana* usually flowers from March to early summer under average daily temperatures lower than the ones used for cultivation in laboratories (Shindo et al., 2007; Brachi et al., 2010). Due to the day-night cycle, they never experience sustained temperatures over 30°C. Therefore, we tested whether meiotic defects observed after continuous cultivation at 30°C could also be induced under more physiological conditions that mimic a hot day. To this end, we cultivated plants in chambers tempered to 18°C overnight and increased temperature to 34°C for 6 hrs during the day (Figure S3A). We observed a drastic reduction in fertility and pollen count, as well as an increased frequency of micronuclei compared to control plants grown under the 18°C/21°C night/day regime (Figure S3B-D). These data suggest that heatwaves occurring during flowering can have a detrimental effect on Arabidopsis meiosis.

Heat stress reduces the amounts of centromeric histone on meiotic centromeres

Our phenotypic analysis indicates that *cenh3-4* mutants grown at 26°C exhibit the same behavior as wild type plants grown at 30°C. Therefore, we investigated whether increasing temperature weakens the centromere structure, which could explain the temperature sensitivity of *cenh3-4* plants with less centromeric histone. CENH3 is present on meiotic chromosomes from early prophase I (Talbert et al., 2002; Lermontova et al., 2006) and its signal is most visible in pachytene when homologous chromosomes are fully synapsed.

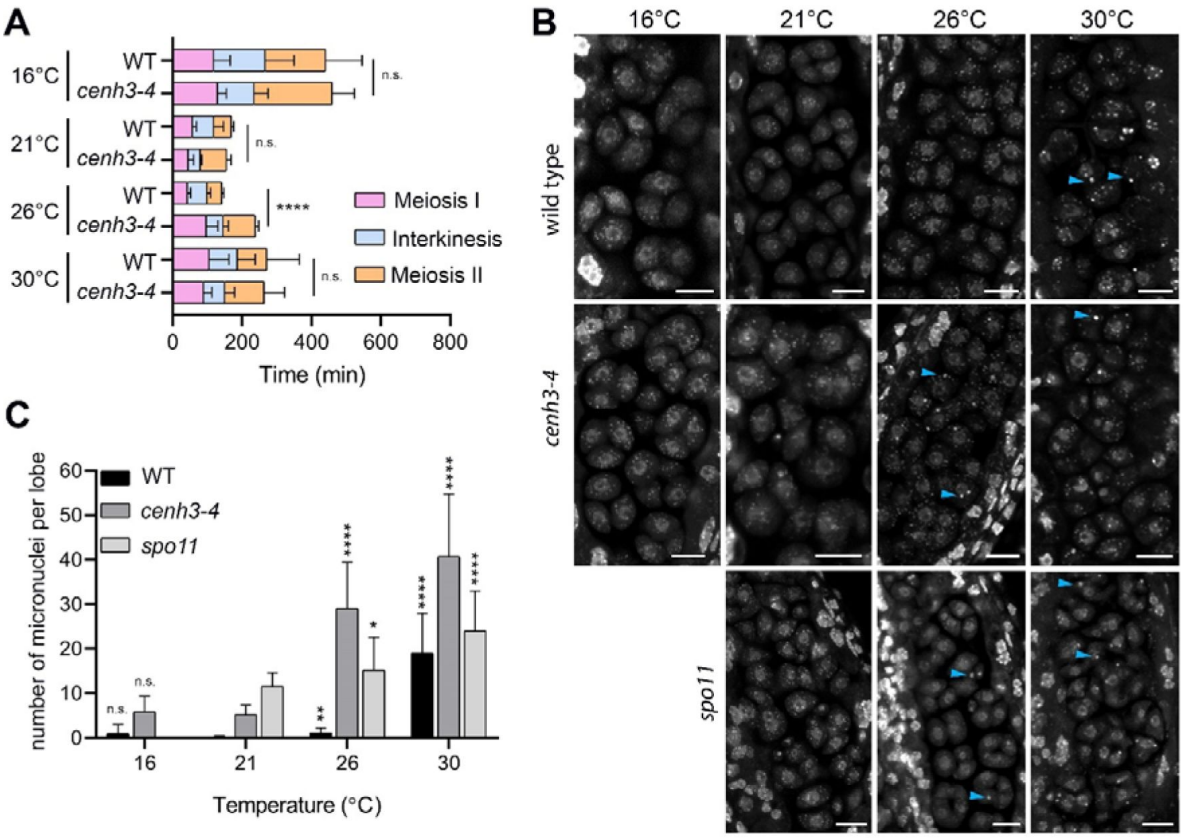

Figure 2.

Effect of temperature on meiosis duration and micronuclei formation.

(A) Graphical representation of the duration of Meiosis I (from the end of diakinesis to the end of anaphase I; Figure S2), Interkinesis, and Meiosis II (prometaphase II to telophase II) calculated from live imaging of anthers in wild type (WT) and *cenh3-4* plants grown at 16, 21, 26 and 30°C. Error bars represent standard deviation (from 16 to 30°C: in wild type n=36,36, 36,46 and *cenh3-4* n=35,24,24,4, *resp.*) Significance of the difference is indicated (two tailed t-test; **** p<0.0001). (B) Anther loculi in the tetrad stage of wild type, *cenh3-4* and *spo11-2-3* plants grown at 16, 21, 26 and 30°C. DNA was stained with DAPI. Blue arrowheads indicate examples of produced micronuclei. Scale bar=10 µm. (C) Number of micronuclei per lobe in wild type (WT, n=19,19,19,19), *cenh3-4* (n=19,19,19,19), and *spo11-2-3* (n=19,26,21) plants. Error bars represent standard deviation. Significance of the difference from plants of the corresponding genotype grown at 21°C is indicated (two tailed t-test; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001).

To assess the effect of temperature on centromeres, we analyzed the CENH3 signal on pachytene chromosomes in anthers of the Arabidopsis *eYFP:CENH3* reporter line (Le Goff et al., 2020; Demidov et al., 2022) grown at 21, 26 and 30°C. While eYFP:CENH3 was readily detectable on pachytene chromosomes at 21°C, the signal decreased significantly at 26°C and declined further at 30°C (**Figure 3A,B**). This trend was observed in three independent experiments (**Figure 3B**). In contrast, the eYFP:CENH3 signal in tapetum nuclei adjacent to pollen mother cells (PMCs) exhibited the opposite trend and increased with elevated temperature (**Figure 3A,C**). This resulted in a striking difference in signal intensity at 30°C, where the tapetum exhibited a strong eYFP:CENH3 signal, whereas the signal on pachytene chromosomes in the same field of view was barely detectable (**Figure 3A**). We validated the observation that elevated temperature reduces the level of CENH3 on pachytene chromosomes also in non-tagged wild type plants by immunodetection using a CENH3 antibody (Figure S4).

CENH3 acts as a platform for the binding of additional kinetochore and spindle assembly checkpoint proteins. BMF1 is an Arabidopsis BUB1-related protein that associates with centromeres throughout the cell cycle (Komaki and Schnittger, 2017). We generated Arabidopsis marker lines expressing *BMF1::BMF1:eYFP* and *BMF1::BMF1:TagRFP* constructs, and validated BMF1 co-localization with CENH3 from early prophase I to telophase II (Figure S5, Movie 9). The BMF1 signal was not detected in roots or in meiotic cells of *cenh3-4* mutants indicating that BMF1 loading on centromeres is CENH3 dependent (Figure S6A,B). Analysis of the BMF1:eYFP signal in wild type plants grown at elevated temperatures showed a decrease in the signal on pachytene chromosomes at 26°C, and BMF1 was undetectable at 30°C (**Figure 3D,E**). These data suggest that similar to CENH3, elevated temperature reduces the kinetochore protein BMF1 at meiotic centromeres.

Heat stress prolongs spindle assembly checkpoint in metaphase I

In our previous report, we showed that reduced amount of CENH3 and smaller centromeres prolong the biorientation of mitotic chromosomes in *cenh3-4* plants (Capitao et al., 2021). Biorientation is monitored by the SAC and its satisfaction triggers anaphase. We hypothesized that meiotic chromosomes with heat-induced reductions in CENH3 might take longer to properly attach to the spindle and satisfy the SAC. The core SAC proteins temporarily associate with the kinetochore during spindle formation and disappear just before the onset of anaphase.

BMF3 is one of the core components of the Arabidopsis SAC, which associates with the kinetochores during prometaphase and extends this association in the presence of a spindle inhibitor (Komaki and Schnittger, 2017; Lampou et al., 2023). To monitor the SAC on meiotic chromosomes, we generated an Arabidopsis line expressing *BMF3::BMF3:GFP* together with the tubulin marker TagRFP:TUB4. First, we validated the localization of BMF3 on meiotic chromosomes by live cell imaging, as BMF3 has previously only been analyzed in the context of mitosis (Komaki and Schnittger, 2017). We observed the BMF3:GFP signal during prometaphase/metaphase I, it disappeared at the onset of anaphase I and reappeared again during metaphase II, suggesting that BMF3 is a component of the meiotic SAC (**Figure 4A**, Movie 10). We then analyzed the effect of temperature on SAC duration. Since the signal was more prominent in metaphase I, we measured the duration of the active SAC during meiosis I. We observed that while the BMF3:GFP signal persisted for an average approximately 22.7 min at 21 and 26°C, its appearance was prolonged to 40.5 min at 30°C (**Figure 4B**, Movies 11 and 12). We also noticed that the intensity of the BMF3 signal appeared to decrease with increasing temperature (Figure S6C). A weaker but prolonged association of BMF3 with the meiosis I kinetochore was also observed in *cenh3-4* mutants with reduced levels of centromeric histone (Figure S6D,E). These observations are consistent with the notion that elevated temperature leads to partial depletion of CENH3 and impairment of centromere structure, which may prolong the time required for chromosome biorientation and SAC satisfaction.

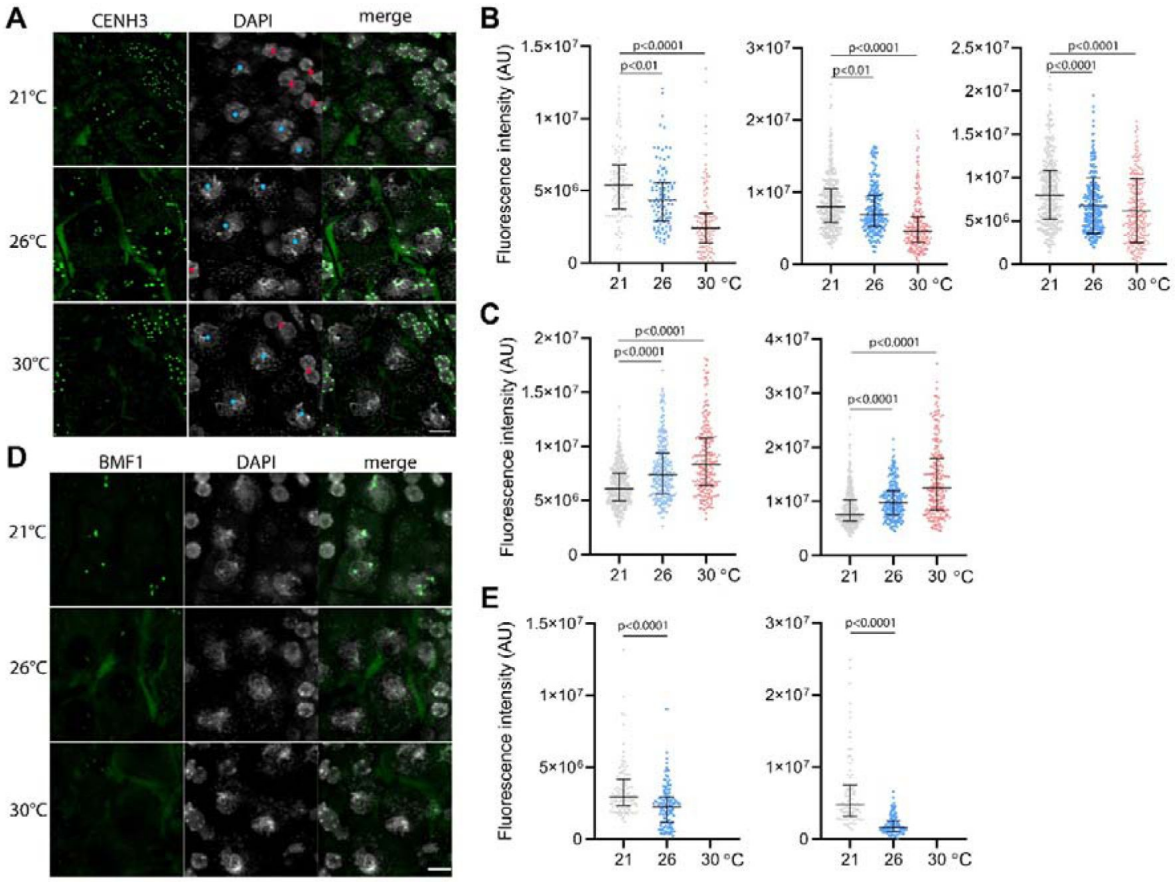

Figure 3.

Effect of high temperature on centromere structure in wild type plants.

(A) eYFP:CENH3 expression and DAPI staining of meiotic pachytene (blue dots) and mitotic tapetal cells (red dots) in wild type plants grown at 21, 26 and 30°C. Scale bar=5μm. (B) quantification of CENH3 fluorescence intensity per centromere in pachytene. Each interleaved scatter plot with median and interquartile range shows results of an independent experiment (left graph n=102,103,125, middle n=359,220,233 and right graph n=286,268,233). (C) Quantification of eYFP:CENH3 signal intensity in tapetum cells of plants grown at 21, 26 and 30°C. Each graph represents an independent experiment (left graph n=369,262,233 and right graph n=358,266,213). (D) Expression of BMF1:eYFP in pachytene in plants grown at 21, 26 and 30°C. DNA is counterstained with DAPI. Scale bar=5μm. (E) Quantification of BMF1:eYFP signal intensity per centromere in pachytene in two independent experiments; left graph n=120 and right n=100. Two-tailed t-test is used to depict the significance of the difference.

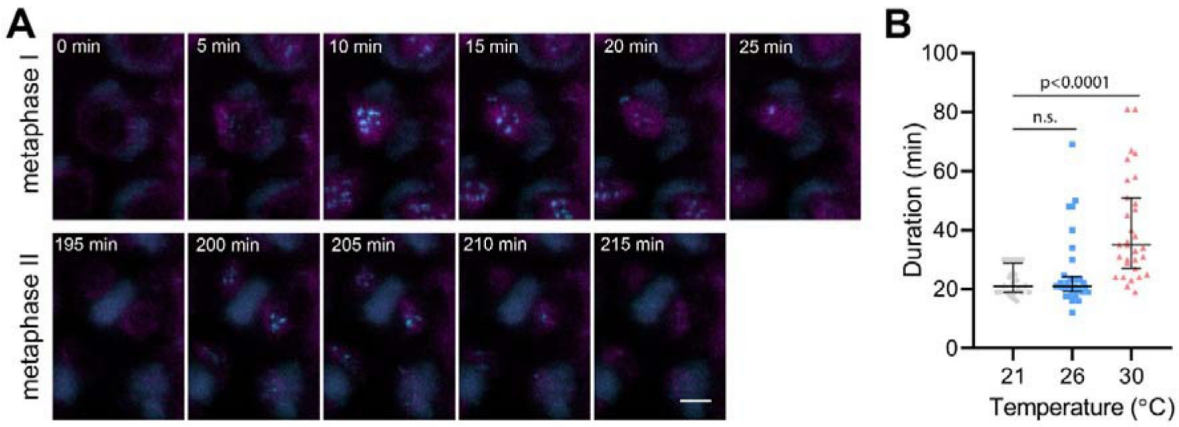

Figure 4.

Effect of high temperature on the duration of BMF3:GFP localization during wild type meiosis.

(A) Time lapse series of BMF3:GFP (cyan) and TagRFP:TUB4 (magenta) in PMC from nuclear envelope breakdown to telophase II. Scale bar=5μm. (B) Duration of BMF3:GFP signal in plants grown at 21°C (n=24), 26°C (n=32) and 30°C (n=31). Significance of the difference was calculated via Two-tailed t-test.

Discussion

In this study we investigated the impact of increased temperature on fertility and meiotic progression in Arabidopsis. We observed that *A. thaliana* (ecotype Col-0) exhibited the highest fertility when grown at 16°C, which closely resembles the average temperature during flowering in its natural habitats (Brachi et al., 2010). At this temperature, meiotic divisions took more than twice as long as at 21°C. Arabidopsis grown at 17°C was reported to exhibit slower microsporogenesis, with the entire meiosis process lasting 48 hours as opposed to 32 hours at 24°C (Zhu et al., 2020). Interestingly, the slower progression was found to be less detrimental for several mutations that affect microsporogenesis, indicating that a slower pace provides greater robustness. Overall, these observations suggest that the pace of meiosis in Arabidopsis is well optimized for its natural growth conditions, enabling the plants to achieve maximum fitness.

The process of meiosis is progressively accelerated with increasing temperature (De Jaeger-Braet et al., 2022) (**Figure 2**). However, the speed of meiotic divisions reaches its maximum at 26°C and slows down at 30°C under the conditions used in this study. At 30°C, fertility is lost and high incidence of micronuclei is observed, implying that the temperature around 30°C impairs some molecular processes involved in chromosome segregation and genome integrity. The micronuclei can represent acentric fragments derived from aberrant processing of recombination intermediates. Indeed, the impact of temperature on recombination machinery is well documented. A moderate increase in temperature leads to shift in chiasma distribution and cross-over frequency (Higgins et al., 2012; Lloyd et al., 2018; Modliszewski et al., 2018). Heat stress above 30°C severely impairs recombination and synapsis, leading to univalents and chromosome segregation defects (De Storme and Geelen, 2020; Ning et al., 2021; De Jaeger-Braet et al., 2022; Fu et al., 2022). Nevertheless, we observed an increased incidence of micronuclei also in SPO11-deficient plants that do not form meiotic DNA double-strand breaks indicating that only a portion of micronuclei is derived from aberrant recombination.

Micronuclei can also be formed by aberrant chromosome segregation during anaphase (Fenech et al., 2011). The accurate partitioning of chromosomes depends on the correct attachment of centromeres to the spindle microtubules. Recent reports have indicated that the stability of microtubules in plant meiocytes is compromised by heat stress (Wang et al., 2017; De Jaeger-Braet et al., 2022). In this study, we have demonstrated that high temperature also affects the structure and function of meiotic centromeres. Our findings suggest that elevated temperature reduces the amount of CENH3 and BMF1 on meiotic centromeres (**Figure 3**). These weakened centromeres may be less effective in establishing productive interactions with spindle microtubules. This is consistent with the prolonged spindle assembly checkpoint we observed through the longer residency of BMF3 on prometaphase I centromeres at 30°C (**Figure 4**). Taken together, our data indicate that heat stress impairs centromere function, which in turn decreases the efficiency of proper attachment of chromosomes to the meiotic spindle. As a result, these chromosomes may not be transported to their intended destination in a timely manner and, therefore, not incorporated into the newly formed nuclei. This scenario also explains the increased temperature-sensitivity of *cenh3-4* plants, which already have smaller centromeres (Capitao et al., 2021); even moderately elevated temperature may further enhance this defect.

How elevated temperature affects the amount of CENH3 on centromeres? Studies in human cells have revealed that CENH3 nucleosomes are less stable than previously thought. The amount of CENH3 at centromeres declines over time and must be actively replenished to preserve centromere identity and proliferative potential (Swartz et al., 2019). Transcription has been shown to cause the eviction of pre-existing CENH3 from the centromeres (Swartz et al., 2019). In Arabidopsis, depletion of CENH3 from centromeres has been observed in leaf cells with increasing age, indicating that this phenomenon also occurs in plants (Lermontova et al., 2011a). The Arabidopsis centromeric satellite repeat CEN180 undergoes pervasive transcription, which is largely repressed by epigenetic silencing mechanisms (May et al., 2005). However, heat stress

can alleviate the repression and induce transcription of CEN180 from silent loci (Tittel-Elmer et al., 2010). This temperature-induced transcription can increase the eviction of CENH3 nucleosomes, potentially compromising centromere structure if not replenished. Interestingly, in contrast to pollen mother cells, tapetum cells do not exhibit CENH3 loss at elevated temperatures (**Figure 3C**). This may be due to the different efficiency of CENH3 deposition to replenish the heat-induced loss of CENH3 in these cell types. Accordingly, Arabidopsis meiotic cells possess a specialized CENH3 loading mechanism that seems more stringent than the loading mechanism operating in mitotic cells (Lermontova et al., 2011b; Ravi et al., 2011; Schubert et al., 2014).

Crosses between wild type and plants with altered CENH3 can result in postzygotic loss of one set of parental chromosomes (Ravi and Chan, 2010). This is attributed to inefficient re-loading of CENH3 on the set of parental chromosomes with the altered centromeres in hybrid embryos (Marimuthu et al., 2021). Recent studies showed that heat stress applied during early embryogenesis could enhance the centromere-mediated genome elimination in Arabidopsis (Ahmadli et al., 2022; Jin et al., 2023; Wang et al., 2023). This indicates that similarly to meiocytes, elevated temperature can also destabilize centromeres in embryonic cells.

In conclusion, our study highlights the significant impact of temperature on the centromere structure and reproduction in Arabidopsis. We found that already moderate increase in temperature has a discernable effect on pollen production and silique length, and exposure to 30°C impairs the structure of centromeres and leads to plant sterility. These findings are particularly relevant in the context of climate change, where rising temperatures and more frequent weather extreme periods pose a threat to global food security by disrupting plant reproductive processes. As such, our study provides important insights into the mechanisms that contribute to the reduction of plant fertility in response to elevated temperature. Further research on molecular aspects underlying these effects may help to develop strategies to generate plants more resilient to extreme weather during their reproductive phase.

Material and Methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0), *cenh3-4* (Capitao et al., 2021) and *spo11-2-3* (Hartung et al., 2007) seeds were grown on soil in growth chambers at 21°C, 16 h/8 h light/dark cycles and 50% of humidity until the 1.04 growth stage. Plants were then transferred to different chambers with continuous 16, 21, 26, 30°C or 34°C/18°C and 21°C/18°C at 16 h/8 h light/dark cycles and 50% of humidity. Plants used for live cell imaging were generated by crossing *HTA10:RFP* (Valuchova et al., 2020) and *pRPS5A::TagRFP:TUB4* (Prusicki et al., 2019) reporter lines with *cenh3-4* mutant and *BMF3::BMF3:GFP* reporter line, respectively. *eYFP:CENH3* reporter line was described and characterized in previous studies (Le Goff et al., 2020; Demidov et al., 2022).

Generating reporter lines

To generate the BMF1 and BMF3 reporter lines, we amplified the promoter and genomic regions of BMF1 using the primers CACCTGAGTCTCCAACGTTA and CGAAGAGCATAACGAGATGCG. The PCR products were then cloned into the destination vectors pGWB659 and pGWB640, respectively, to create marker lines tagged with TagRFP and eYFP at the C-terminus. Similarly, we amplified the BMF3 promoter and genomic regions using the primers CACCATGCAGATGGTCCTCC and GAAGTCCATTGGCATTGCAAA, and subcloned them into pGWB459 and pGWB650 destination vectors using gateway cloning to generate the *BMF3::BMF3:TagRFP* and *BMF3::BMF3:GFP* lines, respectively. We then introduced these constructs into wild type and *cenh3-4* plants using Agrobacterium-mediated floral dip transformation. We used non-segregating homozygous transgenic lines for microscopic analyses.

Assessment of plant fertility

Pollen viability was determined by Alexander staining (Alexander, 1969). Anthers were imaged using Zeiss Axioscope A1 equipped with 20x/0.5 objective (Zeiss) and processed in Zeiss ZEN software. Silique length was assessed from images of main stems scanned by an Epson scanner and the siliques were measured using the Fiji Analyze/Measure function (Schindelin et al., 2012).

Cytology

To examine micronuclei, DAPI staining of PMCs was used in whole anther as described (Capitao et al., 2021). Anthers were imaged using Zeiss LSM780 confocal microscope (63X/1.4 oil objective) and image processing was done using Zeiss ZEN software (Zeiss). To quantify the fluorescence signal intensity of eYFP:CENH3 and BMF1:eYFP, anthers were DAPI stained as described above, and Z-stacks were acquired using Zeiss LSM880 confocal microscope equipped with the Fast module 32-channels Airyscan detector (63x/1.4 oil objective). The fluorescence intensity was quantified using Fiji (Schindelin et al., 2012) according to the procedure described by Shihan et al. (Shihan et al., 2021). This involved generating a SUM of signal in Z-stacks covering one nucleus, background subtraction, and measuring Raw Integral Density per one signal. This process was repeated individually in each nucleus.

Immunodetection of CENH3 was performed with a custom-made polyclonal antiserum raised against the N-terminal peptide of CENH3 (1:1000) (Capitao et al., 2021) and anti-Rabbit-CY3 (Jackson ImmunoResearch). Arabidopsis inflorescences were fixed in 1xPBS buffer supplemented with 4% formaldehyde and 0.05% tween for 15 min in vacuum and 45 min at room temperature. The floral buds were washed with 1xPBS and digested with cytohelicase (0.1g Cytohelicase, 0.25g Polyvinylpyrollidone and 0.375g sucrose in 25 ml of water) for 2 hours. Buds were washed once with 1xPBS and anthers were dissected on glass slides, squashed and frozen in liquid nitrogen. The slides were blocked with 3% BSA in 1xPBS supplemented with 0.5% Triton X-100 for 30 min at 37°C. Anti-CENH3 antibody in 1xPBS supplemented with 3% BSA was added to each slide and incubated over night at 4°C. Slides were washed with 1xPBS and anti-Rabbit-CY3 secondary antibody diluted in 1xPBS supplemented with 3% BSA was added to each slide and incubated for 1h at 37°C. Slides were washed with 1xPBS, stained with DAPI and mounted in Vectashield. Slides were observed using AxioImager.Z2 fluorescence microscope (Zeiss). Images were analyzed using ZEN (Zeiss) and Fiji (Schindelin et al., 2012). Signal intensity was measured at the peak signal of each centromere and was normalized to the background signal.

Live cell imaging

Live cell imaging of meiosis was performed by light-sheet fluorescence microscopy using Light-sheet Z.1 microscope (Zeiss) as previously described (Valuchova et al., 2020; Capitao et al., 2021). Imaging of male meiosis was conducted using 10x or 20x objectives (Detection optics 10x/0.5 or 20x/1.0), single illumination (Illumination Optics 10x/0.2) and two track imaging with 488 nm laser for GFP and eYFP, and 561 nm laser for RFP, in 5 min time increments. Imaging of BMF3:GFP and TagRFP:TUB4 markers for SAC analysis was performed using fast scanning in 1 min time increments. Images were deconvolved with a Regularized Inverse Filter and further processed in Zeiss ZEN software for Light-sheet (Zeiss). To correct occasional sample drift, the Correct 3D drift plugin in Fiji (Parslow et al., 2014) was used.

Acknowledgements

We acknowledge the support from CEITEC MU Core facilities Plant Sciences and CELLIM, supported by the Czech-Bioimaging (No. LM2018129) infrastructure project funded by MEYS CZ. This work was funded by the Czech Science Foundation (grant 21-25163J to K.R.). MK is supported by Deutsche Forschungsgemeinschaft (LE2299/5–1).

Movie 1. Live imaging of meiosis in HTA10:RFP chromatin marker in plants grown at 16°C.

Movie 2. Live imaging of meiosis in *cenh3-4* plants carrying HTA10:RFP chromatin marker grown at 16°C. Production of micronuclei could be seen in a few PMCs.

Movie 3. Live imaging of meiosis in HTA10:RFP chromatin marker in plants grown at 21°C.

Movie 4. Live imaging of meiosis in HTA10:RFP chromatin marker in plants grown at 26°C.

Movie 5. Live imaging of meiosis in *cenh3-4* plants carrying HTA10:RFP chromatin marker grown at 21°C. Production of micronuclei could be detected in some PMCs.

Movie 6. Live imaging of meiosis in *cenh3-4* plants carrying HTA10:RFP chromatin marker grown at 26°C. Production of micronuclei could be detected in most PMCs.

Movie 7. Live imaging of meiosis in HTA10:RFP chromatin marker in plants grown at 30°C. Aberrant meiotic products are detected.

Movie 8. Live imaging of meiosis in *cenh3-4* plants carrying HTA10:RFP chromatin marker grown at 30°C. Most PMCs undergo aberrant meiotic division and only a few PMCs undergo normal meiotic division resulting in forming unbalanced tetrads.

Movie 9. Live imaging of BMF1:eYFP kinetochore marker and HTA10:RFP chromatin marker during meiosis.

Movie 10. Live imaging of BMF3:GFP spindle assembly checkpoint marker and TagRFP:TUB4 tubulin marker during both meiosis.

Movie 11. Live imaging of BMF3:GFP spindle assembly checkpoint marker and TagRFP:TUB4 tubulin marker during first meiotic division of plants grown at 26°C.

Movie 12. Live imaging of BMF3:GFP spindle assembly checkpoint marker and TagRFP:TUB4 tubulin marker during the first meiotic division of plants grown at 30°C.

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Reviewer #2 (Public Review):

Summary:

Here the authors examine how increased temperature affects pollen production and fertility of Arabidopsis thaliana plants grown at selected temperature conditions ranging from 16C to 30C. They show that pollen production and fertility decline with increasing temperature. To identify the cause of reduced pollen and fertility, they resort to living cell imaging of male meiotic cells to identify that duration of meiosis increases with an increase in temperature. They also show that pollen sterility is associated with the increased presence of micronuclei likely originating from heat stress-induced impaired meiotic chromosome segregation. They correlate abnormal meiosis to weakened centromere caused by meiosis-specific defective loading of the centromere-specific histone H3 variant (CenH3) to the meiotic centromeres. Similar is the case with kinetochore-associated spindle assembly checkpoint(SAC) protein BMF1. Intriguingly, they observe a reverse trend of strong CENH3 presence in the somatic cells of the tapetum in contrast to reduced loading of CENH3 in male meiocytes with increasing temperature. In contrast to CENH3 and BMF1, the SAC protein BMF3 persists for longer periods than the WT control, based on which authors conclude that the heat stress prolongs the duration of SAC at metaphase I, which in turn extends the time of chromosome biorientation during meiosis I. This study provides insights onto the processes that affect plant reproduction with increasing temperatures which may be relevant to develop climate-resilient cultivars.

Strengths:

This study shows that the centromere function is affected under heat stress in meiotic cells by modulating the dynamics of the centromere specific histone H3 (CENH3) that in turn compromises the assembly of kinetochore complex proteins. This they have demonstrated by the way of live cell imaging of male meiocytes by tracking the loading dynamics and resident time of fluorescently tagged centromere/kinetochore proteins and spindle assembly checkpoint proteins.

Weaknesses:

Though the results presented here are interesting and solid, the current study lacks a deeper mechanistic understanding of what causes the defective loading of CenH3 to the centromeres, and why the SAC protein BMF3 persists only at meiotic centromeres to prolong the spindle

assembly checkpoint, which will be interesting to delve further to completely understand the process.

Here the authors monitor one representative centromere protein CENH3, one kinetochore-associated SAC protein BMF1, and the SAC protein BMF3 to conclude that heat stress impairs centromere/kinetochore function and prolongs SAC with increased temperatures. Centromere and its associated protein complex the kinetochores and the SAC contains a multitude of proteins, some of which are well characterized in Arabidopsis thaliana. Hence the authors could have used additional such tagged proteins to further strengthen their claim.

https://doi.org/10.7554/eLife.90253.2.sa1

Reviewer #3 (Public Review):

Summary:

Khaitova et al. report the formation of micronuclei during Arabidopsis meiosis under elevated temperature. Micronuclei form when chromosomes are not correctly collected to the cellular poles in dividing cells. This happens when whole chromosomes or fragments are not properly attached to the kinetochore microtubules. The incidence of micronuclei formation is shown to increase at elevated temperature in wild type and more so in the weak centromere histone mutant cenH3-4. The number micronuclei formation at high temperature in the recombination mutant spo11 is like that in wild type, indicating that the increased sensitivity of cenh3-4 is not related to the putative role of cenh3 in recombination. The abundance of CENH3-GFP at the centromere declines with higher temperature and correlates with a decline in spindle assembly checkpoint factor BMF1-GFP at the centromeres. The reduction in CENH3-GFP under heat is observed in meiocytes whereas CENH3-GFP abundance increases in the tapetum, suggesting there is a differential regulation of centromere loading in these two cell types. These observations are in line with previous reports on haploidization mutants and their hypersensitivity to heat stress.

Strength:

The paper shows that the kinetochore function during meiosis is sensitive to high temperature and this leads to inequivalent chromosome segregation during meiosis and reduced fertility.

Weakness:

The increased sensitivity to high temperature stress of the hypomorphic mutant cenh3-4 mutant not only reduces fertility but also growth, which is not accompanied with the formation of micronuclei as in meiosis. The impact on mitosis therefore seems to be different from that in meiosis.

https://doi.org/10.7554/eLife.90253.2.sa0

Author Response

The following is the authors' response to the original reviews.

Response to reviewers:

We would like to thank all the reviewers and the editors for their thorough and helpful feedback on our work. Before addressing specific questions and points, we would like to

make a general comment on a mechanistic aspect of this study. The reviewers correctly pointed out that our study does not reveal the molecular mechanism that leads to centromeric histone depletion specifically from meiotic chromosomes. Identifying this mechanism requires a deep and thorough understanding of how centromeric histones are loaded and centromeres are established each cell cycle, and how they are maintained over time in different cell types. To our knowledge, these mechanisms have not been described in plants. To add a further layer of complexity, it appears that the mechanisms governing CENH3 maintenance may be (partially) different in plant mitotic and meiotic cells, and the mechanistic basis of this difference is unknown. Obviously, these are interesting but also complex questions and their resolution will require considerable resources and effort, which we believe is beyond the scope of this manuscript. Nevertheless, our finding that CENH3 maintenance and centromere function in meiotic cells are sensitive to heat stress is an unexpected discovery with profound implications for plant adaptation, which provides a strong incentive for further exploration of centromere maintenance mechanisms in plants.

Furthermore, we would like to apologize to reviewers for poor quality of pictures in the original submission. It was decreased by conversion to a pdf format during submission.

> ***eLife assessment***
>
> *This important study reports how heat stress affects centromere integrity by compromising the loading of the centromere protein CENH3 and by prolonging the spindle assembly checkpoint during male meiosis in Arabidopsis thaliana. The evidence supporting the claims by live cell imaging is convincing, although deeper mechanistic insight is lacking, making the study overall somewhat preliminary in nature. This work will be of interest to a broad audience of biologists working on how chromatin states are affected by stress conditions.*
>
> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *Khaitova and co-workers present here an analysis of centromere composition and function during elevated temperatures in the plant Arabidopsis. The work relates to the ongoing climate change during which spikes in high temperatures will be found. Hence, the paper addresses a timely subject.*
>
> *The authors start by confirming earlier studies that high temperatures reduce the fertility of Arabidopsis plants. Interestingly, a hypomorphic mutant of the centromeric histone variant CENH3 (CENP-A), which was previously described by the authors, sensitizes plants to heat and results in a drop in viable pollen and silique length. The drop in fertility coincides with the formation of micronuclei in meiosis and an extension of meiotic progression as revealed by live cell imaging. Based on this finding, the authors then show that at high temperatures, the fluorescence intensity of a YFP:CENH3 declines in meiosis but remarkably not in the surrounding cells (tapetum cells). In addition, the amount of BMF1 (a Bub1 homolog and part of the spindle assembly checkpoint) also appears to decline on the kinetochores of meiocytes as judged by BMF1 reporter line. However, whether this is dependent on a decline of CENH3 or represents a separate pathway is not clear.*

We provide new data in Figure S6 showing that BMF1 loading on centromeres is substantially reduced in cenh3-4 mutants. Thus, efficient tethering of BMF1 to centromeres depends on CENH3.

> *Finally, the authors measure the duration of the spindle checkpoint and find that it is extended under high temperatures from which they conclude that the attachment of spindle fibers to kinetochores is compromised under heat.*
>
> *Strengths:*
>
> *This is an interesting and important paper as it links centromere organization/function to heat stress in plants. A major conclusion of the authors is that weakened centromeres, presumably by heat, may be less effective in establishing productive interactions with spindle microtubules.*
>
> *Weaknesses:*
>
> *The paper does not explain the molecular reason why CENH3 levels in meiocyctes are reduced or why the attachment of spindle fibers to kinetochore is less efficient at high versus low temperatures.*

While we cannot explain the molecular mechanism underlying temperature-dependent depletion of CENH3 in meiocytes, the less efficient attachment of microtubules to the kinetochores at higher temperatures is likely caused by reduced levels of CENH3, which result in smaller centromeres that are less effective in establishing productive microtubule-kinetochore attachments. Here (new Figure S6) and in our previous study (Capitao et al. 2021), we have shown that amount of centromere/kinetochore proteins is reduced at centromeres in cenh3-4 mutants, and that these plants exhibit prolonged SAC and slower chromosome biorientation.

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *This work investigates how increased temperature affects pollen production and fertility of Arabidopsis thaliana plants grown at selected temperature conditions ranging from 16C to 30C. They report that pollen production and fertility decline with increasing temperature. To identify the cause of reduced pollen and fertility, they resort to living cell imaging of male meiotic cells to identify that the duration of meiosis increases with an increase in temperature. They also show that pollen sterility is associated with the increased presence of micronuclei likely originating from heat stress-induced impaired meiotic chromosome segregation. They correlate abnormal meiosis to weakened centromere caused by meiosis-specific defective loading of the centromere-specific histone H3 variant (CenH3) to the meiotic centromeres. Similar is the case with kinetochore-associated spindle assembly checkpoint(SAC) protein BMF1. Intriguingly, they observe a reverse trend of strong CENH3 presence in the somatic cells of the tapetum in contrast to reduced loading of CENH3 in male meiocytes with increasing temperature. In contrast to CENH3 and BMF1, the SAC protein BMF3 persists for longer periods than the WT control, based on which authors conclude that the heat stress prolongs the duration of SAC at metaphase I, which in turn extends the time of chromosome biorientation during meiosis I. The study provides preliminary insights into the processes that affect plant reproduction with increasing temperatures which may be relevant to develop climate-resilient cultivars.*
>
> *Strengths:*
>
> *The authors have mastered the live cell imaging of male meiocytes which is a technically demanding exercise, which they have successfully employed to examine the time course of meiosis in Arabidopsis thaliana plants exposed to different temperature conditions. In continuation, they also monitor the loading dynamics and resident time of fluorescently*

> *tagged centromere/kinetochore proteins and spindle assembly checkpoint proteins to precisely measure the time duration of respective proteins to study their precise dynamics and function in male meiosis.*
>
> *Weaknesses:*
>
> *Here the authors use only one representative centromere protein CENH3, one kinetochore-associated SAC protein BMF1, and the SAC protein BMF3 to conclude that heat stress impairs centromere function and prolongs SAC with increased temperatures. Centromere and its associated protein complex the kinetochores and the SAC contain a multitude of proteins, some of which are well characterized in Arabidopsis thaliana. Hence the authors could have used additional such tagged proteins to further strengthen their claim.*

Indeed, several other proteins have recently been characterized as centromere/kinetochore components and could have been included in the study to further validate the results presented. To strengthen our argument, we have added new experimental data (Figure S4) showing temperature-induced depletion of CENH3 in wild-type plants by immunocytology. Thus, we convincingly show that temperature stress reduces the amount of CENH3. This is likely to affect the loading of most kinetochore and centromeric proteins. Here (new Figure S6) and in our previous study (Capitao et al., 2021), we have shown that genetic depletion of CENH3 in cenh3-4 mutants results in reduced loading of CENPC, MIS12 and BMF1 at mitotic centromeres and reduced loading of BMF3 and BMF1 at meiotic centromeres. We also attempted to assess the levels of CENPC and MIS12 on meiotic chromosomes by immunocytology, but our antibodies, which work on mitotic spreads, did not stain meiotic chromosomes.

> *Though the results presented here are interesting and solid, the study lacks a deeper mechanistic understanding of what causes the defective loading of CenH3 to the centromeres, and why the SAC protein BMF3 persists only at meiotic centromeres to prolong the spindle assembly checkpoint. Also, this observation should be interpreted in light of the fact that SAC is not that robust in plants as several null mutants of plant SAC components are known to grow as healthy as wild-type plants at normal growth conditions without any vegetative and reproductive defects.*

Thank you for raising this point. We are of the opinion that SAC operates and it is important in plants - we have added a citation to a preprint from the Schnittger lab (Lampou et al., 2023, BioRxiv) that was published while this manuscript was under review. We think this is the most comprehensive analysis of plant SAC to date, clearly showing that SAC delays progression to anaphase in the presence of spindle inhibitors, although adaptation eventually occurs and the cell cycle progresses. This is very similar to the situation in animals, which also undergo spindle adaptation in similar situations. The difference between plants and animals may be due to subsequent events, where plants are better able to tolerate genome instability and resume cell division in the presence of abnormal chromosome numbers. Robustness and redundancy may be another reason why plant mutants deficient in SAC do not show obvious growth retardation.

> *One of the immediate responses to heat stress is the production of heat shock proteins(Hsps), which act as molecular chaperones to safeguard the proteome. It will be interesting to see if the expression levels of known HsPs can be correlated with their role in stabilizing the structure of SAC proteins like BMF1 to prolong its presence at the meiotic kinetochores.*

Indeed, the heat stress response is likely to be involved in this process. We sought to investigate the role of this pathway by analyzing Arabidopsis mutants deficient in HEAT-

SHOCK FACTOR BINDING PROTEIN (HSBP), which acts as a negative regulator of the heat shock response. This experiment was prompted by the observation that hsbp mutants have reduced fertility. We expected that an unrestricted heat stress response might affect meiosis and pollen formation. However, our initial experiments did not show altered pollen viability in response to heat stress in hsbp plants and we did not pursue this line of research further.

> ***Reviewer #3 (Public Review):***
>
> *Summary:*
>
> *Khaitova et al. report the formation of micronuclei during Arabidopsis meiosis under elevated temperatures. Micronuclei form when chromosomes are not correctly collected to the cellular poles in dividing cells. This happens when whole chromosomes or fragments are not properly attached to the kinetochore microtubules. The incidence of micronuclei formation is shown to increase at elevated temperatures in wild-type and more so in the weak centromere histone mutant cenH3-4. The number of micronuclei formed at high temperatures in the recombination mutant spo11 is like that in wild-type, indicating that the increased sensitivity of cenh3-4 is not related to the putative role of cenh3 in recombination. The abundance of CENH3-GFP at the centromere declines with higher temperature and correlates with a decline in spindle assembly checkpoint factor BMF1-GFP at the centromeres. The reduction in CENH3-GFP under heat is observed in meiocytes whereas CENH3-GFP abundance increases in the tapetum, suggesting there is a differential regulation of centromere loading in these two cell types. These observations are in line with previous reports on haploidization mutants and their hypersensitivity to heat stress.*
>
> *Strengths:*
>
> *This paper is an important contribution to our insights into the impact of heat stress on sexual reproduction in plants.*
>
> *Weaknesses:*
>
> *While it is highly significant, I struggled to interpret the results because of the poor quality of the figures and the videos.*

We apologize for the poor quality of the figures. The figure resolution was drastically reduced during the conversion of the manuscript to pdf on publisher web site.

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *To complete the presented analysis, it would be great to analyze the signal strength of the here-presented BMF3 reporter at high temps, see below for further reasoning.*

Quantification of the BMF3 signal is difficult - it is only transiently associated with kinetochores and its level changes over time. Nevertheless, analysis of our movies taken under the same microscope settings indicates that the amount of BMF3 decreases with increasing temperature. This is illustrated in the new Figure S6C.

> *Conversely, how is the BMF1 and BMF3 signal strength in cenh3-4 mutants?*

We performed an analysis of BMF1 and BMF3 signal in cenh3-4 mutants and observed a reduced level of signal from both proteins (Figure S6). In the case of BMF1, no signal was detectable in either somatic or meiotic cells.

> *How do the authors explain the reduction in BMF1 signal at 26 and 30{degree sign}C versus the extension of the duration of the SAC as measured by the persistence of a BMF3 signal (line 192: "...reduces the amount of CENH3 and the kinetochore protein BMF1 on meiotic centromeres, potentially affecting their functionality..." versus line 213: "...We observed that while the BMF3:GFP signal persisted, on average, for about 22.7 min at 21 and 26{degree sign}C, its appearance was prolonged to 40.5 min at 30{degree sign}C..."). Is the BMF3 signal also reduced at high temps (see question above)?*

This is a very interesting point. While we see reduced levels of both proteins under heat stress or in cenh3-4 plants, the effect on BMF1 is much more pronounced and becomes undetectable under these conditions. This contrasts with BMF3, which appears to be reduced but is still clearly visible. These data suggest that BMF1 is more sensitive to reduced levels of CENH3 and it further corroborates the findings from the Schnittger lab that BMF1 is not the core component of SAC.

> *Line 18-20: The observation that heat stress reduces fertility has been made by several research teams before this study. I propose to write "confirm"/"support" etc. instead of "reveal" to avoid a (presumably not intended) false priority claim in the abstract.*

We apologize, this was unintentional and we cite the relevant literature in the article. We have rewritten the abstract to avoid this impression.

> *Figure 2: The panel/legend appears to be a bit mixed up. Panel C is described in legend under A. In addition, I cannot find any blue arrows in panel A (which is described as panel B). Correspondingly, the references to the panels in this figure (lines 134/135 and following) need to be updated. I am also not sure how the meiocytes in this figure were stained. The dots look like centromeres but then their intensity rather increases with increasing temperature. If correct, how can this be reconciled with the authors' statement that centromeres decrease in size at higher temps?*

We apologize for the mix up. An early version of the Figure was accidentally submitted and we now corrected it. The Panel B shows DAPI stained meiocytes at the tetrad stage and examples of micronuclei are indicated by arrowheads.

> *Line 520: Should read "genotype" not "phenotype".*

Corrected

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *(1) It is intriguing that heat stress impairs only the centromeres and segregation of meiotic chromosomes but not the mitotic chromosomes. No analysis of mitotic divisions is provided in the manuscript. As they have generated marker lines, it is reasonable to examine the mitotic time course as well by live monitoring of root tissues exposed to similar temperature conditions as done for meiotic analysis. This will help to address the effect of heat stress on mitotic centromeres and its comparison with meiosis will provide a better picture. There are two likely outcomes during mitosis:*
>
> *(a) It is possible that the heat stress also slows down mitotic progression as well as is the case in meiosis as shown in this paper and hence it is important to examine those as well to compare and contrast the CENH3/BMF1 dynamics in mitosis and meiosis.*
>
> *(b) The second scenario is that there is no effect of heat stress on the centromere integrity of mitotic chromosomes. In fact, the authors show indirect evidence in support*

> *of this wherein the eYFP: CENH3 showed a strong signal in the tapetal cells (somatic origin) surrounding the male meiocytes (generative origin). It is interesting that somatic cells of the tapetum show a strong signal whereas the meiocytes lack this. The authors should elaborate on this contrasting result.*

The effect we observed seems to be specific to meiosis. We analyzed the progression of mitosis in root cells and we see a negligible effect of temperature on mitotic progression and no micronuclei formation. Interestingly, in terms of CENH3 loading, root cells show a slight decrease in CENH3 at 30°C, in contrast to the situation in tapetum cells. These and other data suggest a tissue/cell specific behavior of centromere maintenance and deserve further analysis. We plan to publish data on mitosis and tissue-specific aspects of CENH3 loading in a separate manuscript.

> *(2) Spindle assembly checkpoint (SAC) comprises several core proteins that are recruited to the kinetochores to correct the errors during the defective cell cycle. Here the authors demonstrate the prolonged presence of BMF3 as the only proof to claim that heat stress prolongs the spindle assembly checkpoint during metaphase I. Have the authors observed the dynamics of any other SAC core components such as MAD1, MAD2, MPS1, BUB3, and the like during heat stress?*

No, we did not. We provide several independent lines of evidence that centromere structure and functionality are affected, and spindle checkpoint analysis is only one of them. At the time we designed these experiments, the only experimentally validated and well-characterized component of the SAC was BMF3, and we used only on this protein as SAC reporter because a general analysis of the SAC was not the primary goal of our study. While this paper was under review, a preprint from the Schnittger lab focusing on plant SAC was published that comprehensively analyzed these SAC components in Arabidopsis and provided a solid foundation and resources for further research in this direction. This study also uses BMF3 as a reporter for SAC in meiotic cells. It is noteworthy that despite using different microscopic methods and different plant reporter lines, our labs independently arrived at exactly the same duration of BMF3 association with the kinetochore (i.e. 22 min).

> *(3) Is BMF1 a component of SAC or the kinetochore? I understand that BMF1 is a part of the core SAC (Komaki and Schnittger, 2017) although it localizes to the kinetochore. There are well-characterized kinetochore proteins in Arabidopsis such as Mis12, NUF2, NNF1, and SPC24(MUN1) which the authors could have used as a kinetochore marker. Regardless, here the authors used it as a kinetochore marker. Being a part of SAC, one would expect the prolonged presence of BMF1 similar to BMF3 in the meiotic kinetochores but it is the other way. How to explain these contrasting results?*

As discussed in the public section of the review, BMF1 does not seem to be the core component of SAC. Furthermore, this protein localizes to centromeres/kinetochore throughout the cell cycle and therefore, it cannot be used as SAC reporter.

> *(4) Micronuclei can form as a result of chromosome missegregation as shown for spo11-1 and also due to segregation error caused by DNA repair defects. Here it is not clear what is the origin of micronuclei. It is very hard to decipher from live cell imaging. A simple meiotic spread of anthers of different treatments would address the origin of micronuclei.*

Cytology cannot easily determine the origin of micronuclei in meiotic cells. Acentric fragments produced from aberrant DNA repair will still be cytologically detectable only after metaphase I as they are tethered to the remaining chromatin via cohesion. Therefore, we took advantage of spo11 mutants that do not form any meiotic breaks, and hence cannot

generate acentric fragments by aberrant repair, to discriminate the origin of micronuclei. We reason that all micronuclei produced in spo11 plants originate from chromosome mis-segregation and their increase at elevated temperature support the notion that heat stress further impairs chromosome segregation.

> *(5) Fig.1 B The microspores are not clearly visible in the alexander-stained anthers. It is not clear which is fertile and which is sterile. A better quality picture would be ideal to appreciate the fact.*

Again, we apologize for poor quality of pictures due to manuscript conversion.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *(1) In Figure 2, it should be pointed out where the micronuclei are. I see here and there a single bright spot. In Arabidopsis, we have noticed bright spots under stress conditions that are autofluorescent signals. It needs to be shown that these spots are not observed in non-GFP lines. Better image quality may help too.*

The micronuclei in Figure 2 are visualized by DAPI staining, not with GFP. The nuclei are now indicated by arrowheads.

> *(2) It was not possible to see the centromeres in Figure 3 hence I could not verify the fluorescence intensities of CENH3 and BMF1. There is also something wrong with the color codes blue and red in fig3B, C, and D.*

Again, we apologize for poor quality of pictures due to manuscript conversion.

> *(3) Also in the videos it would help to point out where the micronuclei are seen. At what stage were these nuclei quantified? Given that meiosis progression in the cenh3-4 mutant is slower, it may be necessary to wait long enough to see established micronuclei. This information is supposed to be presented in Figure 2C. However, the X-axis shows time, not number. So I presume Fig 2C shows the duration of meiosis stages in the mutant. In Fig 2B, it shows the number of micronuclei per lobe. However, to correlate the incidence of micronuclei formation and the frequency of polyad formation (inviable microspores), one needs the quantification of the numbers of meiocytes carrying micronuclei. Then one can correlate the number of pollen per anther (shown in Fig 1c) with the incidence of micronuclei formation. The question of whether the degree of fertility reduction is due to micronuclei formation is a major issue that should be clarified.*

Then micronuclei were not quantified from the movies, but from DAPI stained whole anthers at the tetrad stage as indicated in the main text. We also apologize for confusion with the Figure 2 as we mixed up the panels in the original submission. This has been corrected in the new submission.