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RESEARCH PAPER

High temperature stress and its effect on pollen development and morphological components of harvest index in the C_3 model grass *Brachypodium distachyon*

Jeffrey Harsant, Lazar Pavlovic, Greta Chiu, Stefanie Sultmanis and Tammy L. Sage*

Department of Ecology and Evolutionary Biology, University of Toronto, 25 Willcocks Street, Toronto, Ontario, Canada, M5S 3B2

* To whom correspondence should be addressed. Email: tammy.sage@utoronto.ca

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Abstract

The effect of high temperatures on harvest index (HI) and morphological components that contribute to HI was investigated in two lines (Bd21 and Bd21-3) of *Brachypodium distachyon*, a C_3 grass recognized as a tractable plant, to address critical issues associated with enhancing cereal crop yields in the presence of global climate change. The results demonstrated that temperatures ≥32 °C eliminated HI. Reductions in yield at 32 °C were due primarily to declines in pollen viability, retention of pollen in anthers, and pollen germination, while abortion of microspores by the uninucleate stage that was correlated with abnormal tapetal development resulted in yield failure at 36 °C. Increasing temperatures from 24 to 32 °C resulted in reductions in tiller numbers but had no impact on axillary branch numbers per tiller. Grain developed at 24 and 28 °C primarily in tiller spikes, although spikes on axillary branches also formed grain. Grain quantity decreased in tiller spikes but increased in axillary branch spikes as temperatures rose from 24 to 28 °C. Differential patterns of axillary branching and floret development within spikelets between Bd21 and Bd21-3 resulted in higher grain yield in axillary branches of Bd21-3 at 28 °C. The response of male reproductive development and tiller branching patterns in *B. distachyon* to increasing temperatures mirrors that in other cereal crops, providing support for the use of this C_3 grass in assessing the molecular control of HI in the presence of global warming.

Key words: anther dehiscence, *Brachypodium distachyon*, harvest index, heat stress, pollen.

Introduction

Global agriculture has reached a time of crisis. Increases in crop yields facilitated by the 'Green Revolution' are approaching their maximum potential, resulting in a slowing and ultimately a levelling off of the growth of agricultural productivity (Mitchell and Sheehy, 2006). Rice, wheat, and corn provide roughly 50% of calories in less-developed countries (Reynolds *et al*., 2012). To keep up with expanding populations and maintain global food security, cereal production will have to improve substantially (Reynolds *et al*., 2012). This observation is alarming, given that yields in rice, wheat, corn, and other cereal crops decline with increasing temperatures and that yield enhancements predicted by increases in CO_2 are either not realized or are negated by rising temperatures (Allen *et al*., 1995; Peng *et al*., 2004; Prasad *et al*., 2006*a*). Rice grain production drops 10% for each 1 °C increase in minimum growing season temperature (Peng *et al*., 2004). Estimates for wheat indicate that for every 1 °C increase in temperature above 15 °C there is a reduction of 3–4% in yield (Wardlaw *et al*., 1989) and declines of 1% in maize yield occur for every day spent above 30 °C (Lobell *et al*., 2011).

The unfavourable influence of high temperature (HT) on cereal crop yields results from the negative impact on development of morphological units that contribute to harvest index (HI), and responses vary with the timing, duration, and severity of the heat stress (Barnabas *et al*., 2008). HTs result in reductions in tillers, and thus spike and floret numbers per

Abbreviations: ANOVA, analysis of variance; ATS, Alexander's triple stain; CI, confidence interval; CWI, cell wall invertases; HI, harvest index; HT, high temperature.

plant, as well as spikelets per spike (Dawson and Wardlaw, 1989; Allen *et al.*, 1995; Prasad *et al.*, 2006*a*). Within a floret, anthers and pollen are more sensitive to HT than ovules, and floret sterility at temperatures ≥30 °C has been correlated with diminished anther dehiscence (Saini and Aspinall, 1982; Matsui *et al.*, 2000), production of fewer pollen grains (Prasad *et al.*, 2006*a*,*b*), pollen sterility (Saini and Aspinall, 1982; Saini *et al.*, 1984; Sakata *et al.*, 2000; Prasad *et al.*, 2006*a*), and reduced *in vivo* pollen germination (Jagadish *et al.*, 2010). Yield components contributing to HI that are negatively affected by HT after fertilization include grain numbers and weight (Prasad *et al.*, 2006*a*,*b*; Farooq *et al.*, 2011). Notably, increasing night temperatures exacerbates the HT response on morphological components that influence yield (Saini and Aspinall, 1982; Ziska and Manalo, 1996; Prasad and Djanaguiraman, 2011). In spite of the importance of the negative effect of HT on cereal crop yields, molecular mechanisms regulating reductions in yield at HT in grasses have not been fully elucidated (Barnabas *et al.*, 2008; Hedhly, 2011; Parish *et al.*, 2012).

Two species within the Poaceae, *Brachypodium distachyon* (L.) Beauv. and *Setaria viridis* (L.) Beauv. have recently been recognized as model monocotyledonous plants to address critical issues associated with enhancing cereal crop yields in the presence of global climate change (Draper *et al.*, 2001; Li and Brutnell, 2011). A member of the PACMAD clade, *S. viridis* functions as a model for C_4 photosynthetic grasses of agronomic importance that include maize, *Sorghum*, and millets (Li and Brutnell, 2011). The C_3 species *B. distachyon* belongs to the BEP clade and therefore serves as a model for a group of related grasses of economic significance that include wheat, barley, rye, rice, and forage crops (Draper *et al.*, 2001; Vogel *et al.*, 2010). Both *B. distachyon* and *S. viridis* share features that make *Arabidopsis* a tractable system. They are small in stature, have quick generation times and diploid ecotypes, and are self-fertile (Draper *et al.*, 2001; Vogel *et al.*, 2010; Li and Brutnell, 2011). The genomes of both species have recently been sequenced and a wide array of resources are available to facilitate the use of these species as model systems for grasses (Vogel *et al.*, 2010; Bennetzen *et al.*, 2012).

The importance of *B. distachyon* as a tractable model plant for identifying molecular mechanisms regulating HT tolerance or intolerance of crop yields in cereals remains unclear. It has recently been proposed that this species may not be a suitable model to study the effect of abiotic stress on yields because, as a non-domesticated grass, *B. distachyon* may withstand abiotic stress more so than domesticated cereals (Dolferus *et al.*, 2011). The purpose of the present study was to determine whether chronic HT following initiation of the reproductive meristem had an impact on yield in *B. distachyon*, and if so, whether the effect was similar to what has been observed for domesticated grasses. The specific questions addressed were: (i) What impact does increasing levels of HT, including higher night temperatures, have on HI and morphological yield components that contribute to HI, and is there a temperature where HI collapses? (ii) Does HT stress affect pollen development, pollen deposition, and pollen germination as noted for other crop species, and if so, at what temperature(s) are these processes impacted and how do they influence HI? (iii) If pollen development is impaired at HT, what stage is affected? We used two lines of *B. distachyon*, Bd21 and Bd21-3, to address our questions. These inbred lines generated from accessions collected in Turkey have been demonstrated to be closely related but genetically distinct (Vogel and Hill, 2008; Vogel *et al.*, 2009). Any variation in the HT response between these two lines may assist in future characterizations examining the molecular and genetic basis of HT tolerance or susceptibility during reproduction in cereal crops.

Materials and methods

Plant material and growth conditions

B. distachyon seeds (lines Bd21 and Bd21-3 provided by J. Vogel) were sown in 0.3 l pots containing 75% potting soil and 25% sand. Seeds were vernalized at 4 °C for 7 d and transferred to growth chambers (GC-20; Enconair, Winnipeg, Manitoba, Canada), with a 20 h photoperiod at 400 µmol photons m^{-2} s^{-1} (Vogel and Hill, 2008), at day/night temperatures of 24/18 °C. Dissections indicated that the reproductive meristem was initiated when the third leaf was fully expanded. Therefore, when the third leaf was fully expanded, plants were heat shocked for 1 h at one of three day temperature treatments (28, 32, or 36 °C), returned to growth conditions described above for 24 h, and subsequently grown until grain maturation was complete at either 24/18, 24/23, 28/18, 28/23, 32/18, 32/23, 36/18, or 36/23 °C. Plants were watered with full-strength Hoagland's. Temperature treatments were replicated a minimum of three times and replicates were rotated between chambers. No chamber or replicate effects were observed; therefore, data collected from different replicates and chambers for each treatment were pooled.

Quantification of HI and HI yield components

Above-ground dry weight and total grain dry weight were used to calculate HI after grain maturation (Donald, 1962). Tillers originated from meristems at the base of the grass culm, and axillary branches developed from meristems in the leaf axils of each tiller (Doust, 2007; Supplementary Fig. S1 at *JXB* online). To determine the effect of temperature on branching patterns in *B. distachyon*, tiller and axillary branch numbers per plant were tallied for each temperature treatment on plants that had completed grain maturation. To determine the impact of temperature on additional morphological components that contribute to yield, the following traits were quantified after grain maturation ended: number of spikelets per tiller spike, florets per spikelet, florets per plant, grains per plant, grains per tiller spike, grains per tiller spikelet, axillary branched grain per plant, and grains per axillary spike. The position of mature grains, aborted grains, and unfertilized ovules (characterized by failure of anther elongation and dehiscence) within a spikelet were also recorded to calculate the probability of grain set at each floret position.

Anther and pollen development, viability, and germination

All florets within a spike were dissected and anthers were classified as either dehisced or not dehisced to determine the impact of HT on anther and pollen development on plants developing in each treatment. Florets were then digitally photographed (Infinity Capture v5.0.2 software; Lumenera Corp., Ottawa, ON, Canada) and either mounted in Alexander's triple stain (ATS; Alexander, 1969) or fixed in glutaraldehyde and prepared for light microscopy (Sage and Williams, 1995). Both ATS-mounted anthers and sectioned anthers were used to stage pollen development. Some anthers were

also stained with fluorescein diacetate and iodine–potassium iodide to quantify pollen viability and starch content, respectively, at the time of anther dehiscence (O'Brien and McCully, 1981). Anther and ovary length were quantified with Image J (National Institutes of Health, Bethesda, MD, USA) from digital floral images to assess whether a correlation existed between anther length and stage of pollen development, as observed in rice (Raghavan, 1988), and whether or not there was an impact of HT on anther and pollen development and anther dehiscence. Stigmas from flowers with dehisced anthers were also stained with aniline blue to quantify pollen deposition and pollen germination on stigmas (Sage and Williams, 1995).

Data analysis and statistics

For HI and morphological yield components, comparisons between treatments (day temperature, night temperature, and line) were first assessed using three-way analysis of variance (ANOVA). If no effect of night temperature was revealed in these analyses, data from all day versus night temperatures were pooled, and the three-way ANOVAs were reduced to two-way ANOVAs. Trait values recorded for Bd21 and Bd21-3 were not significantly different unless noted. A two-way ANOVA was also used to analyse positional effects of grain produced per tiller spikelet by spikelet position. Similarly, unless noted, interaction effects between line and day temperature were not significant. Statistical analyses were conducted in SPSS 17.0 (IBM).

Results

Effect of HT on HI and morphological yield components

Increasing temperatures resulted in decreased growth of both lines (Fig. 1). Harvest index was eliminated at 32 and 36 °C [Fig. 1A, $F(3, 43)=257.7$, $P<0.001$]. There was a significant interaction effect between day temperature and line at 24 and 28 °C [$F(3, 43)=4.5$, $P=0.008$]; the HI of Bd21 dropped at 28 °C, whereas the HI of Bd21-3 increased and was significantly higher than that of Bd21 (Fig. 1A). The higher HI in Bd21-3 at 28 °C was not associated with greater shoot or grain weight per plant relative to 24 °C but to alterations in partitioning of dry weight between shoot and grain dry weight (Fig. 1B, C). While total grain weight per plant declined at 28 °C relative to 24 °C in both lines, Bd21-3 maintained the same individual grain weight (Fig. 1C, D). In contrast, Bd21 exhibited a decline in individual grain weight at 28 °C with significant temperature, line, and interaction effects [Fig. 1D; $F(1, 23)=6.26$, $P=0.02$; $F(1, 23)=52.03$, $P<0.001$; $F(1, 23)=20.82$, $P<0.001$, respectively]. Grain weight was eliminated at 32 °C in line Bd21 because no grains developed and, although grains were produced in Bd21-3 (see below), grain weight in this line declined (Fig. 1C, D).

The quantity of tillers declined in response to temperature in a linear pattern from 24 to 36 °C at a rate of approximately one tiller for every 1.7 °C increase [Fig. 2A; $F(3, 51)=94.3$, $P<0.001$]. The mean number of axillary branches per tiller was stable between 24 and 32 °C but dropped significantly at 36 °C [Fig. 2B; $F(3, 334)=28.5$, $P<0.001$]. Line Bd21-3 had more axillary branches per tiller than Bd21 from 24 to 32 °C [Fig. 2B; $F(1, 334)=17.92$, $P<0.001$]. Tillers terminated in a spike composed of spikelets, and axillary branches terminated in a spike with one spikelet (Supplementary Fig. S1). The number of spikelets per spike on tillers did not differ between temperature treatments and remained constant at 2.7 spikelets per spike [Fig. 2C; $F(3, 47)=2.2$, $P=0.098$]. Line Bd21 had more florets per spikelet than Bd21-3 at all temperatures [Fig. 2D; $F(1, 1594)=262.2$, $P<0.001$; Supplementary Fig. S1], and the average number of florets per spikelet was unaffected by temperature except for a decline at 36 °C [Fig. 2D; $F(3, 1594)=110.5$, $P<0.001$].

The total number of florets per plant did not vary between lines even though there were differences between lines in the number of axillary branches and florets per spikelet [Fig. 3A; $F(1, 42)=0.112$, $P=0.739$]. Floret numbers declined linearly with increasing temperatures at a rate of approximately 41 florets $°C^{-1}$ [Fig. 3A; $F(3, 42)=100.4$, $P<0.001$]. Similarly, grain quantity per plant declined from 24 to 32 °C at a rate of approximately 23 grains $°C^{-1}$ [Fig. 3B; $F(3, 43)=121.8$, $P<0.001$], at which point grain set was eliminated in Bd21 and severely reduced to less than four grains per plant in Bd21-3 (Fig. 3B, C, F). Higher temperatures caused a significant grain reduction in tiller spikes [Fig. 3C; $F(3, 363)=117.95$, $P<0.001$], and there was no influence of spikelet position within a spike on grain set in a tiller [Fig. 3D; $F(3, 956)=0.287$, $P=0.84$]. There was a significant line effect on grain yield in a tiller with increasing temperature such that grain number was greater in Bd21 than Bd21-3 at 24 and 28 °C [Fig. 3C; $F(1, 363)=3.97$, $P=0.04$]. Although grain number per plant was not different between lines, more grains were produced on axillary branches at 28 than at 24 °C on a plant and individual axillary branch basis [Fig. 3E, F; $F(3, 43)=56.16$, $P<0.001$; and $F(1, 594)=96.88$; $P<0.001$, respectively]. Line Bd21-3 produced more grain on axillary branches at the plant and spike level [Fig. 3E, F; $F(1, 43)=49.75$, $P<0.001$; and $F(1, 594)=30.53$, $P<0.001$, respectively]. The probability of grain set as a function of floret position within a spikelet declined towards the apex of the spikelet (Supplementary Fig. S2 at *JXB* online).

Effect of temperature on pollen and anther development

The anatomy of anther wall and pollen development in *B. distachyon* at 24 °C, illustrated in Figs 4 and 5, was similar to that of other grasses (Saini *et al.*, 1984; Raghavan, 1988). Microspore mother cells (Fig. 4A) underwent meiosis to give rise to microspores (Figs 4B and 5A). Uninucleate microspores became vacuolate (Figs 4C and 5B) prior to the first mitotic division and were also vacuolate after the second mitotic division (Figs 4D and 5C). Starch grains, were present only in bicellular and tricellular pollen (Figs 4D, E and 5D, E). The endothecium was fully formed by the bicellular stage (Fig. 4D), and the tapetum was completely degenerated by the tricellular stage (Fig. 4E).

Anther length and stage of pollen development were correlated [Fig. 6A; $t(329)=11.64$, $P<0.001$, $R^2=0.54$]. Increasing temperature had a significant effect on the relationship between anther length and stage of pollen development [$F(4, 327)=10.4$, $P<0.001$]. Anthers developing at 32 and 36 °C had a mean final length of 0.66 ± 0.04 and

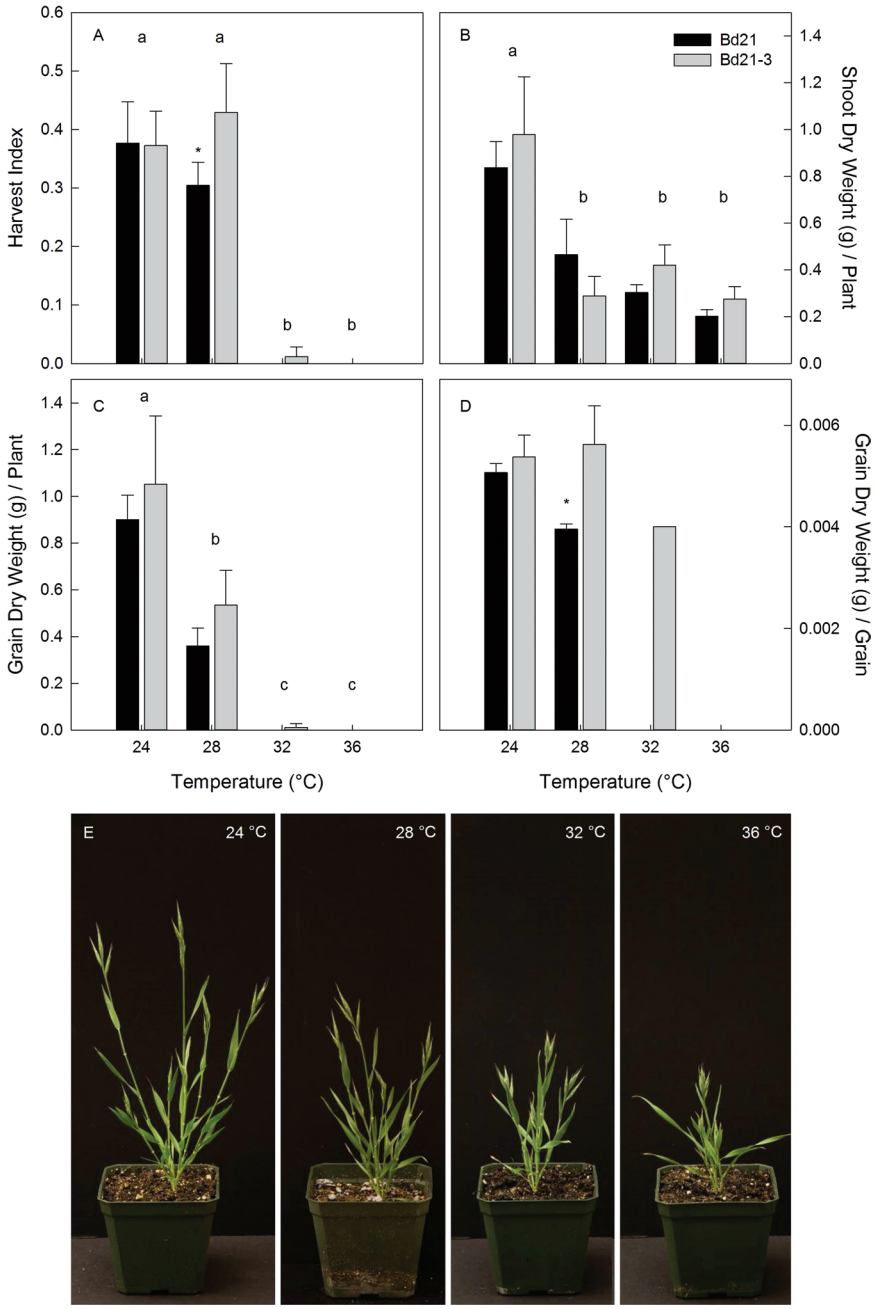

Fig. 1. Impact of temperature on vegetative and reproductive growth of *B. distachyon*. (A) HI; (B) shoot dry weight; (C) grain dry weight per plant; (D) individual grain dry weight; (E) plant growth. Different lower-case letters in (A)–(D) indicate statistical significance between temperature treatments. Asterisks denote statistical differences between lines. Statistics have not been added for 32 °C in (D) because fewer than four seeds per plant developed in Bd21-3 and no seed developed in Bd21. Error bars indicate 95% confidence interval (CI). (This figure is available in colour at *JXB* online.)

0.60 ± 0.03, respectively, whereas anthers from 24 °C averaged a final size of 0.80 ± 0.01 mm (Fig. 6A). Anthers of Bd21-3 contained fewer pollen grains than Bd21 at all temperatures except 36/18 and 36/23 °C (Supplementary Table S1A at *JXB* online). Increasing daytime temperatures resulted in a decrease in pollen grain numbers per anther. High night temperatures compounded reductions in pollen grain numbers, with Bd21-3 more affected than Bd21 (Supplementary Table S1A). Pollen grain abortion showed similar trends and abortion was virtually complete at 36 °C (Supplementary Table S1B). Pollen development at 36 °C ceased almost exclusively at the uninucleate stage (Figs 4F and 5H, I) and this corresponded to the mean final length of the anthers. Aborting pollen was swollen and misshapen (Figs 4F and 5I). Tapetal cells in aborting anthers were enlarged and frequently ruptured (Fig. 4F). An endothecium developed in anthers with aborted pollen (Fig. 5I). A very small number of anthers had locules that lacked evidence of meiosis but contained densely stained cells indicative of microspore mother cell differentiation (data not shown).

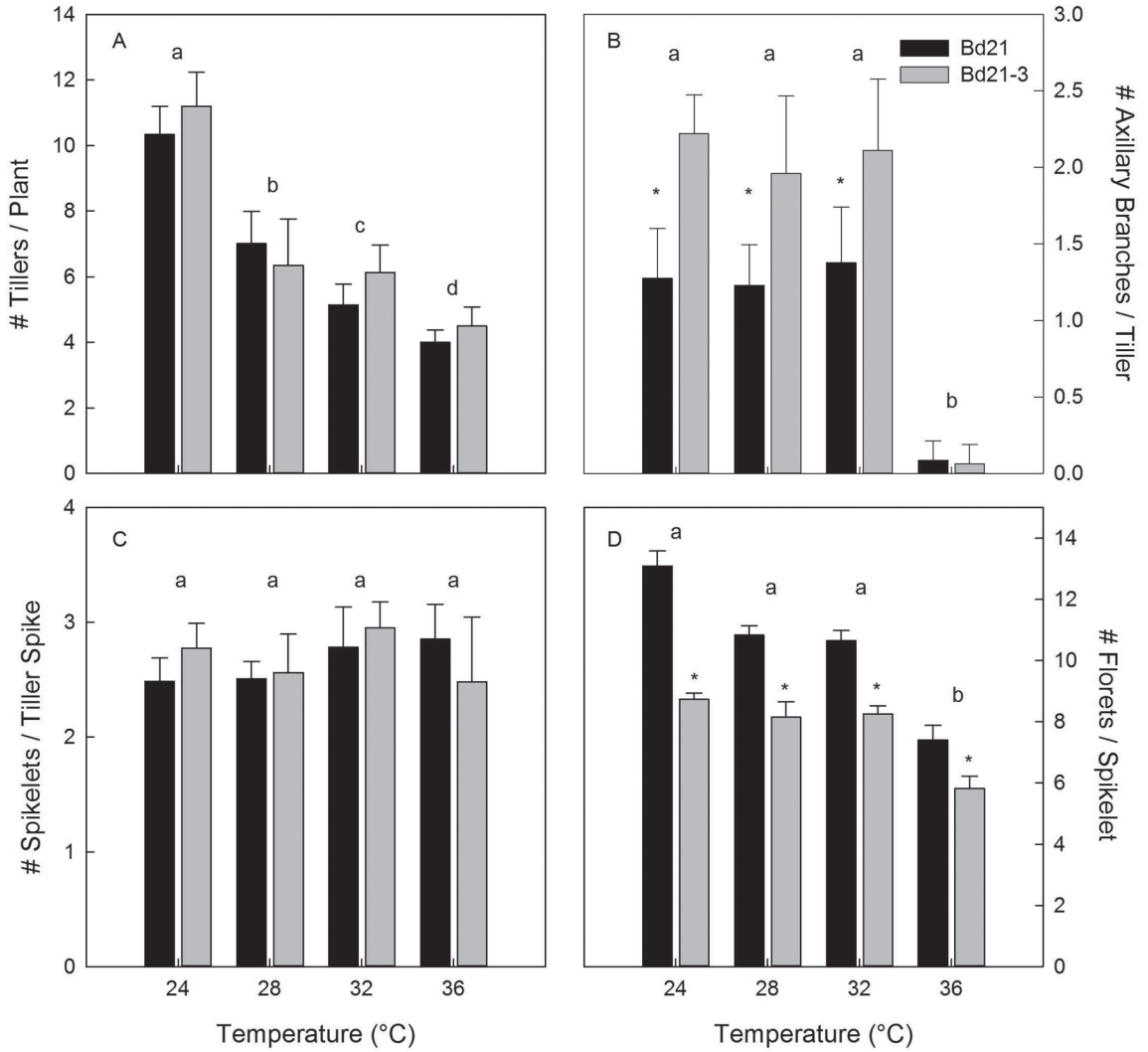

Fig. 2. Effect of increasing temperatures on morphological yield components of *B. distachyon*. (A) number of tillers per plant; (B) numbr of axillary branches per tiller; (C) number of spikelets per tiller spike; (D) number of florets per spikelet. Different lower-case letters indicate statistical significance between temperature treatments. Asterisks denote statistical differences between lines. Error bars indicate 95% CI.

HT effect on anther dehiscence

A χ^2 test of independence examined the relationship between anther dehiscence and day temperature. The relation between these variables was significant [χ^2 (3, N=302, 302)=61.77, P <0.001]. Anther dehiscence at 24 °C occurred when ovaries were 1.6mm (Supplementary Fig. S3 at *JXB* online). Anther indehiscence was negligible at 24 and 28 °C above this length, whereas it rose to 23.8 and 100% at 32 and 36 °C, respectively (Supplementary Fig. S3). Dehisced anthers from florets grown at 24 °C released almost all of their pollen (Fig. 5F). If anthers did not dehisce at 24 and 28 °C, they were positioned at the terminal regions of a spikelet, contained fully mature pollen, and filaments did not go through the final stages of elongation. Indehiscent anthers from florets grown at 32 °C developed an endothecium and exhibited septum separation. Dehisced anthers with elongated filaments from florets grown at 32 °C retained most of their pollen (Figs 5G and 6G).

HT effect on pollen viability, deposition, and germination on the stigma

Anthers that developed at 24 and 32 °C contained viable and non-viable pollen at anther dehiscence (Fig. 6B–D). However, the percentage of viable pollen at dehiscence at 24 °C was significantly greater than that at 32 °C (Fig. 6B). Viable and non-viable pollen grains at 32 °C contained starch grains (Fig. 6E). The number of pollen grains deposited on stigmas at 32 °C [15 ± 7 (95% CI)], was significantly less (df=2, H=20.24, P 0.001) than at 24 [47 ± 16 (95% CI)] and 28 °C [54 ± 12 (95% CI)]. Pollen deposited on stigmas at 32 °C was clumped relative to that at 24 and 28 °C (Fig. 6G and H versus F). Pollen deposition was eliminated at 36 °C because anthers did not dehisce. The percentage pollen germination at 24 °C was greater than that at 32 °C (Fig. 6B). Germinated pollen grains at 32 °C failed to grow beyond the diameter of the grain.

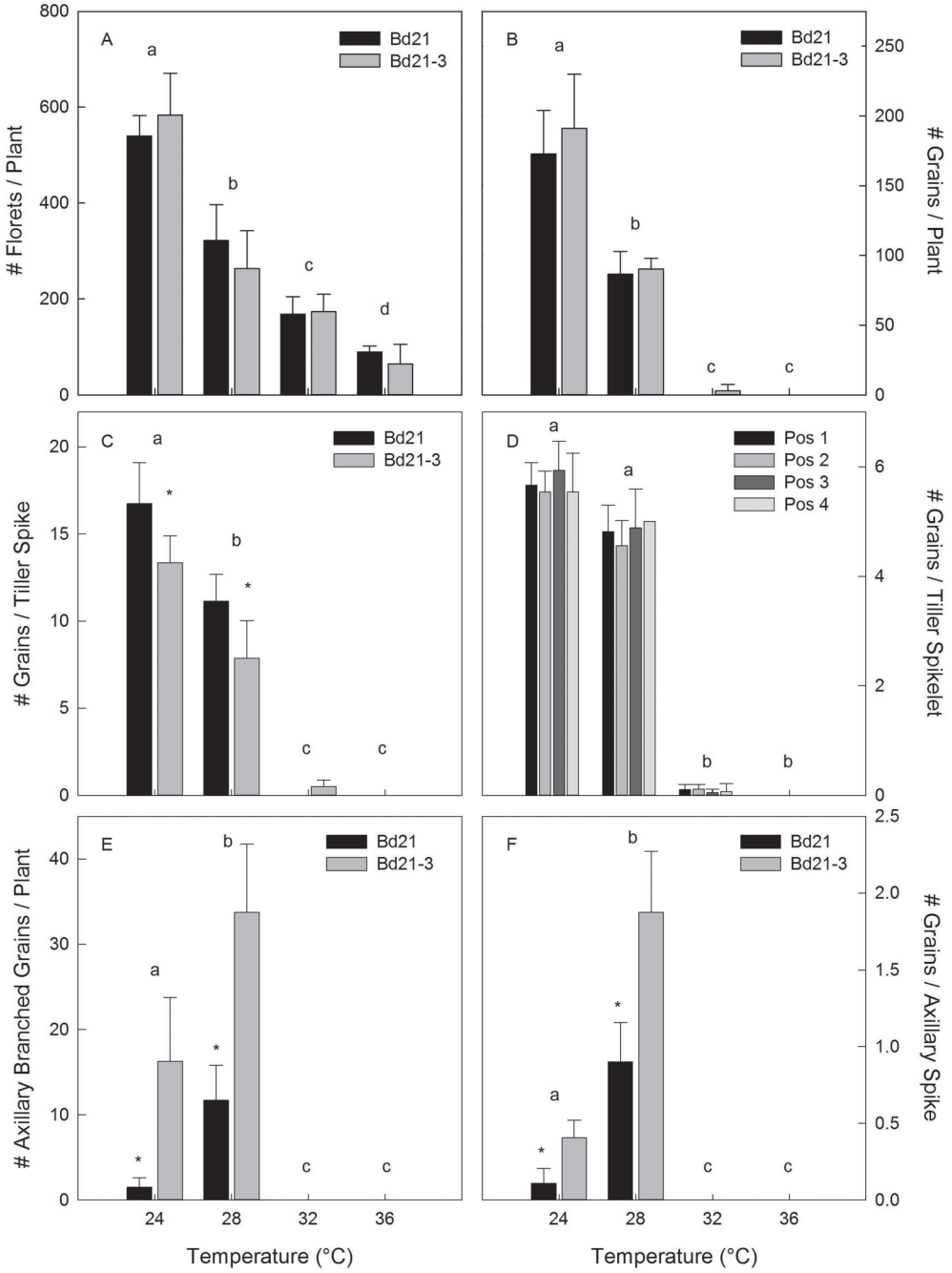

Fig. 3. Effect of increasing temperatures on production of morphological yield components of *B. distachyon*. (A) number of florets per plant; (B) number of grains per plant; (C, D) number of grains per tiller spike and tiller spikelet; (E) number of axillary branched grains per plant; (F) number of grains per axillary spike. Pos 1, 2, 3, and 4 indicate the spikelet positions from the base to the tip of a spike. Different lower-case letters indicate statistical significance between temperature treatments. Asterisks denote statistical differences between lines. Error bars indicate 95% CI.

Discussion

The principal finding of this study was that exposure of *B. distachyon* to chronic temperatures ≥32 °C during the reproductive stage eliminated HI. The precipitous impact on HI as temperatures increased from 28 to 32 °C indicates that this species has a narrow range of thermal sensitivity. The diminished HI at 32 °C in *B. distachyon* was due to a lack of viability in 90% of mature, starch-filled pollen at anther dehiscence that was compounded by a 70 and 97% reduction in stigmatic pollen deposition and germination, respectively. The decline in pollen deposition resulted primarily from pollen retention in dehisced anthers. Non-viable pollen was also produced at 36 °C in *B. distachyon*. However, the lack of pollen viability at this temperature was due to arrested pollen development almost exclusively at the uninucleate vacuolate stage of microsporogenesis prior to starch deposition. Abnormal tapetal development was also

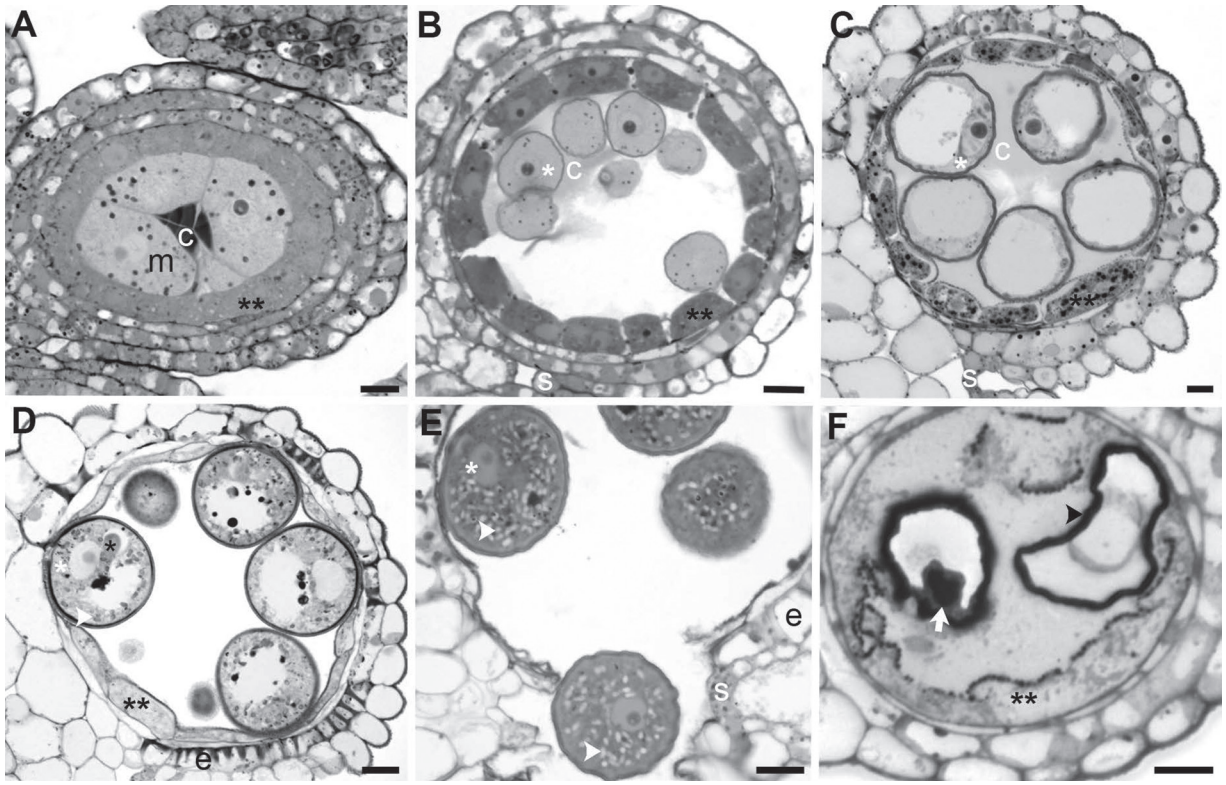

Fig. 4. Anther and pollen development of *B. distachyon* at 24 °C (A–E) and 36 °C (F). (A) Microspore mother cells; (B) uninucleate, non-vacuolate microspores; (C) uninucleate, vacuolated microspores; (D) bicellular pollen; (E) tricellular pollen prior to dehiscence; (F) aborted uninucleate, vacuolated microspore in anther locule with swollen and ruptured tapetal cells. The single white asterisk sits adjacent to the vegetative nucleus, the single black asterisk denotes the generative nucleus, the double black asterisks label the tapetum, the white arrowhead indicates starch, and the white arrow marks the nucleus in aborted pollen grain. c, callose; e, endothecium; m, microspore mother cells; s, stomium. Bars, 10 μm

observed at 36 °C. Increasing temperatures resulted in a decline in pollen numbers that was exacerbated by a higher night temperature primarily in line Bd21-3. In addition to the negative impact on anther and pollen development, the present study also demonstrated that increasing temperatures caused a decrease in tiller numbers and consequently total spikes and florets per plant. Mature grain developed primarily in tiller spikes, although axillary branches also formed grain at 24 and 28 °C, the two temperature treatments that yielded grain. Unlike tiller quantities, axillary branch numbers per tiller were not affected by temperatures below 36 °C and, while grain amounts per tiller spike declined when temperatures were elevated from 24 to 28 °C, yields unexpectedly increased in axillary branch spikes. Although high night temperatures have resulted in reductions in HI in other crop species (Saini and Aspinall, 1982; Peng *et al*., 2004; Prasad and Djanaguiraman, 2011), a 5 °C increase in night temperature did not have an additional influence on HI in *B. distachyon* at 24 or 28 °C. The absence of an impact of increased night temperature on *B. distachyon* HI may be related to a limited duration of exposure to high night temperatures used in the present study associated with the photoperiod determined previously to promote flowering (Vogel and Hill, 2008).

Anther and pollen development at HT

The reduction in viable mature pollen at the time of anther dehiscence, retention of pollen in anthers, and decrease in pollen germination on stigmas as observed in *B. distachyon* following chronic exposure to the more moderate HT of 32 °C also contributes to the failure in yield in other cereal and eudicot crop species (Barnabas *et al*., 2008). HT-induced declines in mature pollen viability and germination in rice have been posited to occur from decreased iron uptake by microspores or pollen tubes, as well as reductions in ribosome assembly, protein synthesis, and expression of heat- and cold-shock proteins deemed important for heat tolerance (Jagadish *et al*., 2010). Inviability of mature pollen in *Sorghum* exposed to high night temperatures has been associated with enhanced production of reactive oxygen species and alterations in composition of phospholipids that enable pollen viability and germination (Prasad and Djanaguiraman, 2011). Pollen retention in anthers leading to diminished deposition on stigmas is proposed to result from incomplete endothecial wall thickening or cell separation in the septum (Rudich *et al*., 1977; Porch and Jahn, 2001; Matsui and Omasa, 2002), an increase in anther cell wall rigidity arising from modifications in lignin biosynthesis (Jagadish *et al*., 2010), or a failure of

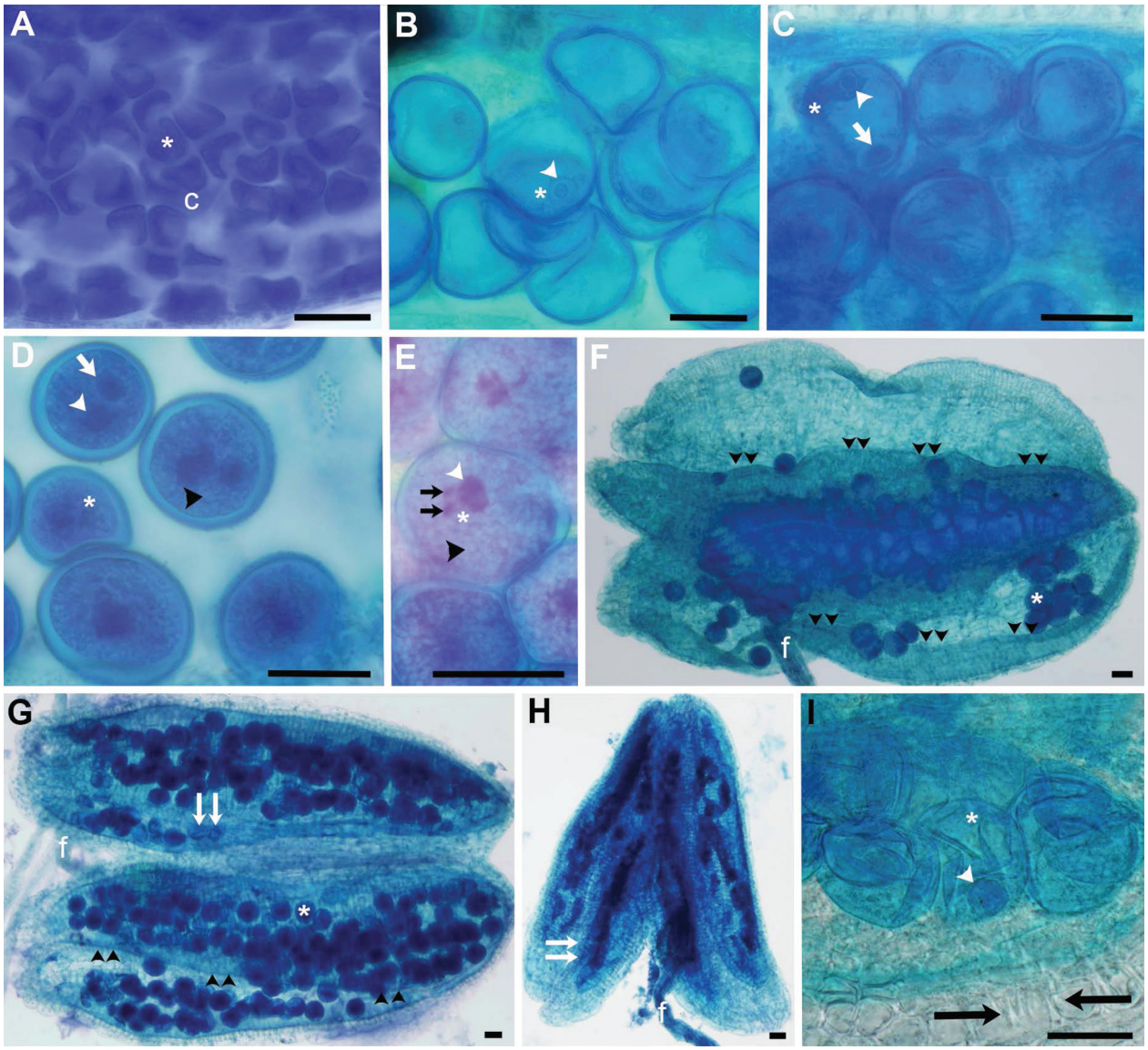

Fig. 5. Anthers and pollen of *B. distachyon* stained with ATS illustrating development at 24 °C (A–F), 32 °C (G), and 36 °C (H, I). (A) Microspore mother cells; (B) uninucleate, non-vacuolate microspores; (C) uninucleate, vacuolate microspores; (D) bicellular microspore; (E) tricellular microspore; (F) dehisced anther with minimal pollen grain retention; (G) dehisced anther with abundant pollen grain retention; (H) indehiscent anther with aborted uninucleate microspores; (I) aborted uninucleate microspore. The single asterisk denotes the microspore, the white arrowhead marks the vegetative nucleus, the black arrowhead labels starch, the double black arrowheads indicate the line of anther dehiscence, the single white arrow denotes the generative nucleus, the single black arrow indicates the endothecium wall, the double black arrows label sperm cells, and the double white arrows highlight the aborted uninucleate microspores. c, callose; f, filament. Bars, 20 μm.

pollen swelling required for final septum separation (Matsui *et al.*, 2000). Notably, microscopic observations from the present study on *B. distachyon* indicated that the few anthers remaining indehiscent at 32 °C completed septum separation and endothecium development. Reductions in the efficacy of pollen release from dehisced anthers has also been noted to occur in rice from drought stress-induced adherence of pollen grains to one another (Liu *et al.*, 2006), and it has been suggested that this trait may also prevent pollen release at HT from dehisced rice anthers (Jagadish *et al.*, 2010). Observations from the present study indicate that pollen retention in dehisced anthers of *B. distachyon* at 32 °C may also result from adherence of grains to one another as well as the anther locule. Importantly, pollen grains deposited on *B. distachyon* stigmas at 32 °C were clumped. This phenotype was not observed at lower temperatures. Retention of pollen within anthers and clumping on the stigma might arise from the negative effect of abiotic stress, to include HT, on tapetal components deposited onto the pollen grain exine, alterations in exine morphology, or inefficient degradation of cell wall matrices during tetrad separation (Porch and Jahn, 2001; Jagadish *et al.*, 2010; Prasad and Djanaguiraman, 2011).

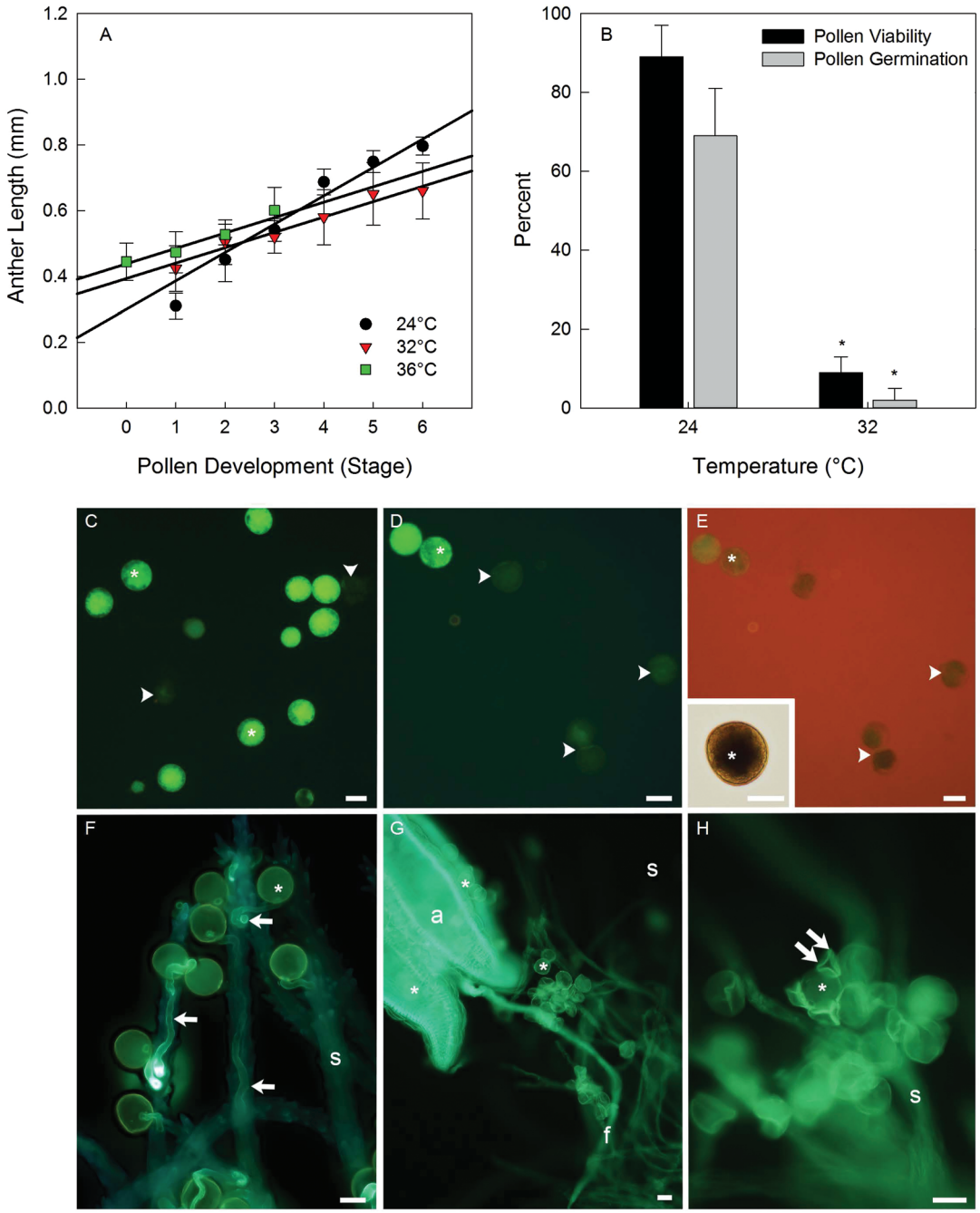

Fig. 6. Anther development and pollen viability, germination, and deposition on stigmas in *B. distachyon*. (A) Anther length versus pollen development. Pollen stages: 0, no meiosis; 1, meiosis; 2, uninucleate; 3, uninucleate vacuolate; 4, bicellular; 5, bicellular plus starch; 6, tricellular plus starch. (B) Percentage pollen viability and pollen germination. (C–E) Fluorescein diacetate staining for pollen viability at 24 °C (C) and 32 °C (D, E). Positive fluorescence indicates viable pollen. (E) The same field of view as in (D) with background lighting to illustrate starch in the non-viable pollen. Inset, iodine–potassium iodide staining for starch. Black staining indicates starch. (F–H) Aniline blue fluorescence detection of pollen germination on stigmas at 24 °C (F) and 32 °C (G, H). Note the absence of pollen grain germination on stigma, clumped deposition of pollen on stigma, and retention of pollen grains in dehisced anther (G, H). The white asterisks indicate the pollen grain, the white arrowheads mark non-viable pollen grains, the single white arrows label pollen tubes, and the double white arrows indicate an aborted pollen grain. a, anther; f, filament; s, stigma. Bars, 20 μm. Black asterisks denote statistical differences between temperature treatments. Error bars indicate 95% CI.

Impaired germination may result from similar modifications of the tapetal pollen coating and cell wall matrices of both the pollen and stigma (Endo *et al.*, 2009).

Microscopic studies on the impact of chronic HT stress on anther and pollen development in cereal and eudicot crops indicate that, as observed for *B. distachyon* at 36 °C, HT stress

negatively influences the early stages of anther development such that pollen grains do not progress beyond the uninucleate stage of microsporogenesis and tapetal development is altered (Barnabas *et al*., 2008; Hedhly, 2011; Parish *et al*., 2012). Similar effects on pollen and tapetum development occur when various crop species experience cold and drought (Parish *et al*., 2012), leading investigators to conclude that this early stage of male reproductive development represents 'an Achilles tendon' (Dolferus *et al*., 2011). The ephemeral tapetal cell layer that surrounds developing pollen plays a central role in partitioning of assimilates from the anther wall to the anther locule (Parish *et al*., 2012). Tapetal cell wall invertases (CWIs) are posited to function in sucrose unloading from the anther wall, as well as provisioning of hexoses to the symplastically isolated tapetum and pollen at this critical stage of development (Parish *et al*., 2012). Drought and cold stress during meiosis in wheat and rice result in a downregulation of CWIs and monosaccharide transporter gene expression in the anther and tapetum, thereby limiting hexose availability for pollen development (Dorion *et al*., 1996; Sheoran and Saini, 1996; Ji *et al*., 2011). HT results in changes in carbohydrate metabolism in tomato (Pressman *et al*., 2002), *Capsicum* (Aloni *et al*., 2001), and *Sorghum* anthers that includes reduced expression of CWIs and sugar transport and starch synthesis genes in *Sorghum* tapetum and anther (Jain *et al*., 2007, 2010) and reduced CWI activity in pollen of *Capsicum* (Aloni *et al*., 2001). Physiological similarities between the HT response of *Sorghum*, *Capsicum*, and tomato and the drought and cold stress response in wheat and rice suggest that mechanisms inducing pollen abortion may be the same across abiotic stresses (Jain *et al*., 2010; Parish *et al*., 2012). However, transcription profiles of barley anthers experiencing chronic HT during meiosis and the uninucleate stage of pollen development did not reveal any differential expression of genes involved in carbohydrate metabolism (Oshino *et al*., 2007). Rather, HT resulted primarily in repression of cell proliferation genes within anthers and premature expression of genes regulating the progression of meiosis and programmed cell death of the tapetum (Oshino *et al*., 2007). The apparent differences in the HT responses between barley and *Sorghum* may be due to a 4 °C difference in experimental regimes. Alternatively, molecular mechanisms leading to early male reproductive failure during chronic HT stress may vary among species.

Abiotic stress, including HT, is well known to have an impact on cell division and elongation (Potters *et al*., 2007). The negative influence of HT on cell proliferation genes within the early stages of anther development reported for barley (Oshino *et al*., 2007) is probably a common response, as indicated by declines in pollen grain numbers in *B. distachyon* and other crop species exposed to high day and night temperatures (Prasad *et al*., 2006*a*; Devasirvatham *et al*., 2012). A question of interest is whether repression in cell proliferation genes at HT operates to prevent uninucleate microspores of *B. distachyon* and other species from progressing beyond the first mitotic division. Results from the present study on *B. distachyon* correlating anther length and pollen differentiation at both NT and HT provide a blueprint for the critical stages to sample to answer this question and dissect additional features of the molecular regulation of the HT response.

HI, branching patterns, and grain production at HT

Branching patterns in cereal crops control the spatial array of morphological components that contribute to HI. Tillers develop from meristems at the base of the plant, and axillary branches arise from meristems on tillers (Doust, 2007). Domesticated and wild grasses within the BEP clade are noted to have many tillers and no axillary branches, a phenotype posited to reflect grazing pressures and other factors of ecological importance (Doust, 2007). Thus, as a member of the BEP clade, *B. distachyon* is typical of other cereal crops because of the production of many tillers with HT exerting a negative impact on tiller and therefore total floret and grain numbers (Dawson and Wardlaw, 1989; Allen *et al*., 1995; Prasad *et al*., 2006*b*). However, *B. distachyon* does not fit the BEP clade branching model due to additional formation of axillary branches on tillers. Tiller and axillary branching have been demonstrated to be under different genetic control in *Setaria* and *Pennisetum* (Poncet *et al*., 2000; Doust *et al*., 2004), and data indicate that tiller meristems, in contrast to axillary meristems, exhibit plasticity in response to shading in grasses (Lukens and Doebley, 1999; Takeda *et al*., 2003). The negative impact of HT on tiller development and absence of a response by axillary branches to temperatures lower than 36 °C in *B. distachyon* may arise from separate mechanisms of genetic control regulating development of each meristem type under HT.

The neutral response of axillary branch numbers per tiller to increasing temperatures was crucial for grain production and thus maintenance of HI at 28 °C in *B. distachyon*. Importantly, grains from axillary branch spikes contributed to roughly 13 and 33% of total grain produced per plant at 28 °C in lines Bd21 and Bd21-3, respectively, which was up from 1 and 7% at 24 °C. Higher grain numbers in Bd21-3 reflect the development of more axillary branches per tiller in addition to a greater number of grains per axillary spike. High numbers of spikelets in a spike have been correlated with high grain yield within a tiller (Farooq *et al*., 2011), and HT can result in a decrease in number of spikelets per spike in tillers (Dawson and Wardlaw, 1989). In addition, HT has been noted to exacerbate a trend wherein distally located florets contribute less to grain production within a spikelet (Dawson and Wardlaw, 1989). Notably, HT did not influence spikelet number in either tiller or axillary branch spikes of *B. distachyon* at 28 °C. Enhanced grain production in axillary branch spikes was due to more grains maturing within the individual spikelet, and this corresponded to a higher probability of grain set at more distal positions within the spikelet. Experimental manipulations that remove all but primary tillers in wheat alter assimilate partitioning patterns and result in more grains per tiller spike at HT in comparison with controls with tillers left intact (Wardlaw, 1994). The reduction in tiller numbers in *B. distachyon* as temperatures increase from 24 to 28 °C parallels experimental manipulations removing tillers in wheat (Wardlaw, 1994). However, in *B. distachyon*, reductions in tiller number altered partitioning that subsequently

resulted in increased grain production in spikes on axillary branches versus tillers. In addition, given that Bd21-3 develops fewer florets per axillary spike than Bd21, enhanced numbers of grains per axillary spike in Bd21-3 indicates that more assimilates were available for partitioning to grain versus floret production in this line. Differential assimilate partitioning within axillary branches between the two lines further amplified grain yields by enabling the production of heavier individual grains in Bd21-3 at 28 °C. Stem reserves are considered an important source of carbon for grain development under stress conditions (Barnabas *et al*., 2008) and may have contributed more to yield in Bd21-3.

Conclusion

The Wheat Yield Consortium has recently emphasized the need to refocus research activities to increase yields (Reynolds *et al*., 2012). One goal is to improve grain production by modifying the sensitivity of developmental processes to environmental cues prior to and after fertilization. A second goal is to optimize partitioning among organs. The present research on *B. distachyon* has provided critical information regarding the sensitivity of floret fertility and tiller and axillary branching to HT. This study has established that (i) declines in pollen viability, retention of pollen in anthers, and reduced pollen germination at 32 °C, (ii) abortion of uninucleate microspores at 36 °C, and (iii) reductions in tiller numbers with increasing temperatures form pre-zygotic barriers to grain production. These processes are susceptible to HT in wheat and other cereal crop species, indicating that *B. distachyon* is an ideal model system for identification of the molecular and genetic basis of the sensitivity of critical developmental stages to HT. The phenotypic variation exhibited between the two very closely related lines, Bd21 and Bd21-3, in response to HT, demonstrates the genotypic potential of *B. distachyon* to further enhance such studies.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table S1. The effect of day and night temperatures on pollen number and abortion in *B. distachyon*, lines Bd21 and Bd21-3.

Supplementary Fig. S1. Vegetative and reproductive morphology of *B. distachyon*, lines Bd21 and Bd21-3.

Supplementary Fig. S2. The effect of 24 and 28 °C on the probability of grain production at each floret position within a spikelet in *B. distachyon*, lines Bd21 and Bd21-3.

Supplementary Fig. S3. The effect of increasing temperatures on anther dehiscence versus ovary length in *B. distachyon*.

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