Activation tagging identifies Arabidopsis transcription factor AtMYB68 for heat and drought tolerance at yield determining reproductive stages

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SUMMARY

Heat stress occurring at reproductive stages can result in significant and permanent damage to crop yields. However, previous genetic studies in understanding heat stress response and signaling were performed mostly on seedling and plants at early vegetative stages. Here we identify, using a developmentally defined, gain-of-function genetic screen with approximately 18 000 *Arabidopsis thaliana* activation-tagged lines, a mutant that maintained productive seed set post-severe heat stress during flowering. Genome walking indicated this phenotype was caused by the insertion of 35S enhancers adjacent to a nuclear localized transcription factor AtMYB68. Subsequent overexpression analysis confirmed that *AtMYB68* was responsible for the reproductive heat tolerance of the mutant. Furthermore, these transgenic Arabidopsis plants exhibited enhanced abscisic acid sensitivity at and post-germination, reduced transpirational water loss during a drought treatment, and enhanced seed yield under combined heat and drought stress during flowering. Ectopic expression of *AtMYB68* in *Brassica napus* driven either by 35S or by heat-inducible promoter recapitulated the enhanced reproductive heat stress and drought tolerance phenotypes observed in the transgenic Arabidopsis. The improvement to heat stress is likely due to enhanced pollen viability observed in the transgenic plants. More importantly, the transgenic canola showed significant yield advantages over the non-transgenic controls in multiple locations, multiple season field trials under various drought and heat stress conditions. Together these results suggest that *AtMYB68* regulate plant stress tolerance at the most important yield determining stage of plant development, and is an effective target for crop yield protection under current global climate volatility.

Keywords: genetic screen, reproductive development, heat and drought tolerance, R2R3-MYB transcription factors, AtMYB68 (accession At5G65790), field trial, *Arabidopsis thaliana*, *Brassica napus*.

INTRODUCTION

Agricultural management against abiotic stresses, such as heat and drought, is essential for the mass production of both food and biomass crops as they are grown in open fields. For example, corn and soybean production throughout the United States for the period 1982–1998 showed a 17% decrease in yield for each degree increase in growing season temperature (Lobell and Asner, 2003). Recent estimates, based on extensive historical studies from maize in Africa, indicated that each one degree increase above 30°C a day resulted in a 1% yield loss under optimal rain-fed

conditions, and 1.7% yield loss under drought conditions (Lobell *et al.*, 2011). Although these examples show the depth of the problem across various climate zones, the situation will become even more precarious with the advent of global climate change, which makes it difficult to predict weather pattern changes (Mazdiyasni and AghaKouchak, 2015). Nevertheless, under the slowest and fastest global warming scenarios, area-weighted average yields are predicted to decrease by 20–40% and 63–82% respectively, before the end of the 21st century (Battisti and Naylor, 2009; Schlenker and Roberts, 2009).

These and many other studies have broadly framed the urgent issue and the impact of heat and drought on plant productivity (Savage and Jacobson, 1935; Heyne and Brunson, 1940; Craufurd and Peacock, 1993; Savin and Nicolas, 1996; Jiang and Huang, 2001; Wang and Huang, 2004; Mittler, 2006; Lamaoui *et al.*, 2018). Abiotic stresses are usually episodic in nature and occur randomly over a growing season, which means a plant experiences various stresses at different times of its life cycle. However, our understanding of how plants deal with abiotic stresses is mostly limited to the experimentally tractable developmental stages in model genetic systems (Hong and Vierling, 2000; Suzuki *et al.*, 2005; Kurek *et al.*, 2007). Therefore, most mechanistic information is limited to vegetative tissues such as leaves and roots because genetic analysis is mostly performed on seedlings. However, under heat stress, distinct transcriptomic responses are found in the vegetative and reproductive tissues of Arabidopsis (Zhang *et al.*, 2017), and a particularly sensitive developmental stage to both heat and drought stress with respect to crop productivity is at reproductive development. For example, detrimental effects of both heat and drought have been reported on flowering time, male and female reproductive development and the production of gametes in a variety of crops (for reviews, see Barnabás *et al.*, 2008; Zinn *et al.*, 2010). Previous studies focused on understanding thermal tolerance at seedling development stages were able to unearth a series of heat stress responsive genes in multiple signaling pathways, including those mediated by heat shock proteins and heat stress factors (Larkindale *et al.*, 2005; Kotak *et al.*, 2007). Unfortunately, there is limited success on manipulation of these genes for increased heat tolerance, presumably due to their genetic redundancy and regulatory complexity. Therefore, even though genetic screens involving reproductive tissues are much more laborious and tedious, they should be the preferred method for the identification of gene targets that may provide direct impact on seed set and yield upon stresses. Recently, using silique length in the main inflorescence as a phenotypic marker, multiple Arabidopsis quantitative trait loci have been found to be associated with fertility reduction upon heat stress (Bac-Molenaar *et al.*, 2015). However, subsequent loss-of-function mutational analysis on some candidate genes within these quantitative trait loci regions does not provide specific gene targets for crop breeding and improvement of heat tolerance.

To address this issue further, in this study we carried out a gain-of-function, forward genetic screen using an activation tagged population of Arabidopsis to identify heat stress tolerant mutants directly, at the yield determining reproductive stages. One heat-tolerant mutant was found able to maintain productive seed set after severe heat stress during flowering, and this phenotype was caused by transcriptional activation of a R2R3 MYB transcription factor, AtMYB68. Spatial and temporal expression analysis indicate that this nuclear localized transcription factor expresses at low levels in most tissues during plant growth and development, with relatively higher levels in roots and flower buds. In addition, the expression of *AtMYB68* is not inducible by either heat or drought treatments. We further demonstrated that ectopic expression of *AtMYB68* in Arabidopsis resulted in enhanced seed yield against heat or heat and drought combined stress during flowering, and the improved tolerance to drought stress might be mediated through the increased abscisic acid (ABA) sensitivity observed at germination and early seedling development of the transgenic Arabidopsis plants. The enhanced heat and drought tolerance phenotypes were further recapitulated in transgenic canola when *AtMYB68* was driven either by CaMV 35S or a heat-inducible promoter. Physiological analysis indicated the observed increased heat tolerance at the reproductive stages might be a direct result of enhanced pollen viability of the transgenic canola. Most importantly, the transgenic canola showed significant yield advantages over the non-transgenic controls in multiple field trials under various drought and heat stress conditions. Together these results show that *AtMYB68* identified in Arabidopsis can be used directly for crop yield improvement in the field, and such a genetic discovery system is an effective platform for the further identification of useful targets to address the need of crop protection under the current global climate variability.

RESULTS

Activation tagging identifies Arabidopsis AtMYB68 for heat stress tolerance during flowering

In many plants, including Arabidopsis, occurrence of heat stress during reproductive development results in a dramatically reduced silique length, and consequently there is a reduction in overall seed production (Zinn *et al.*, 2010). Fortunately, Arabidopsis continually produces siliques so a transient heat stress during flowering results in aborted or shortened siliques only in a limited section of the main inflorescence (Figure 1a). Once plants are removed from the stress, newly produced siliques will develop normally so seeds can be obtained for further propagation and genetic analysis that may underline regulatory elements responsible for reproductive heat tolerance phenotypes. Using this assay, we screened a collection of approximately 18 000 Arabidopsis T-DNA activation tagged lines (Weigel *et al.*, 2000) that showed good silique growth after heat stress. One mutant line, *h138*, was identified in which the silique length was not dramatically affected by the heat stress (Figure 1a), and it was able to maintain relatively constant amounts of seeds while the Columbia wild-type (WT) showed significant yield reduction after 2-, 3- and 4-h treatments at 45°C during flowering (Figure 1b). Genome walking revealed that the 4× 35S enhancers from the activation tagging vector were inserted in an intergenic region

Figure 1. Activation tagging identifies Arabidopsis *AtMYB68* for heat stress tolerance during flowering.
(a) Silique formation of Columbia wild-type (WT) and the heat stress tolerant mutant *h138* after heat stress at 45°C for 30 min. Pictures were taken 6 days after heat treatment. White brackets indicate the regions of the main inflorescence affected by heat treatment.
(b) Relative seed yield of WT and mutant *h138* after heat stress treatments at 45°C for 2, 3 and 4 h during flowering, presented as percentage of their yields obtained under optimal growth conditions. Error bars represent standard errors ($n = 10$), and the levels of significant differences from the WT control are indicated by asterisks (Student *t*-test at $*P < 0.05$ and $**P < 0.01$).
(c) Insertion of the activation tag with the T-DNA containing $4\times$ 35S enhancers on chromosome 5 of mutant *h138*. Three protein coding genes (*AtMYB68, AtACS5* and *AtCGR3*) are shown flanking the T-DNA insertion site.
(d) Seed yield protection of six homozygous transgenic lines of 35S-*AtMYB68* (lines 8, 20, 22, 35, 88 and 104) in comparison with WT after heat stress treatment during flowering at 45°C for a total of 7.5 h (1.5 h day^{-1} over 5 consecutive days). Seed yields after heat stress for transgenic lines are expressed in percentages of their yields under optimal growth conditions, which are further normalized over that of WT. Lower panels indicated transcript levels of *AtMYB68* and loading control *YLS8* in the six transgenic lines and WT, determined by Northern blot hybridization.
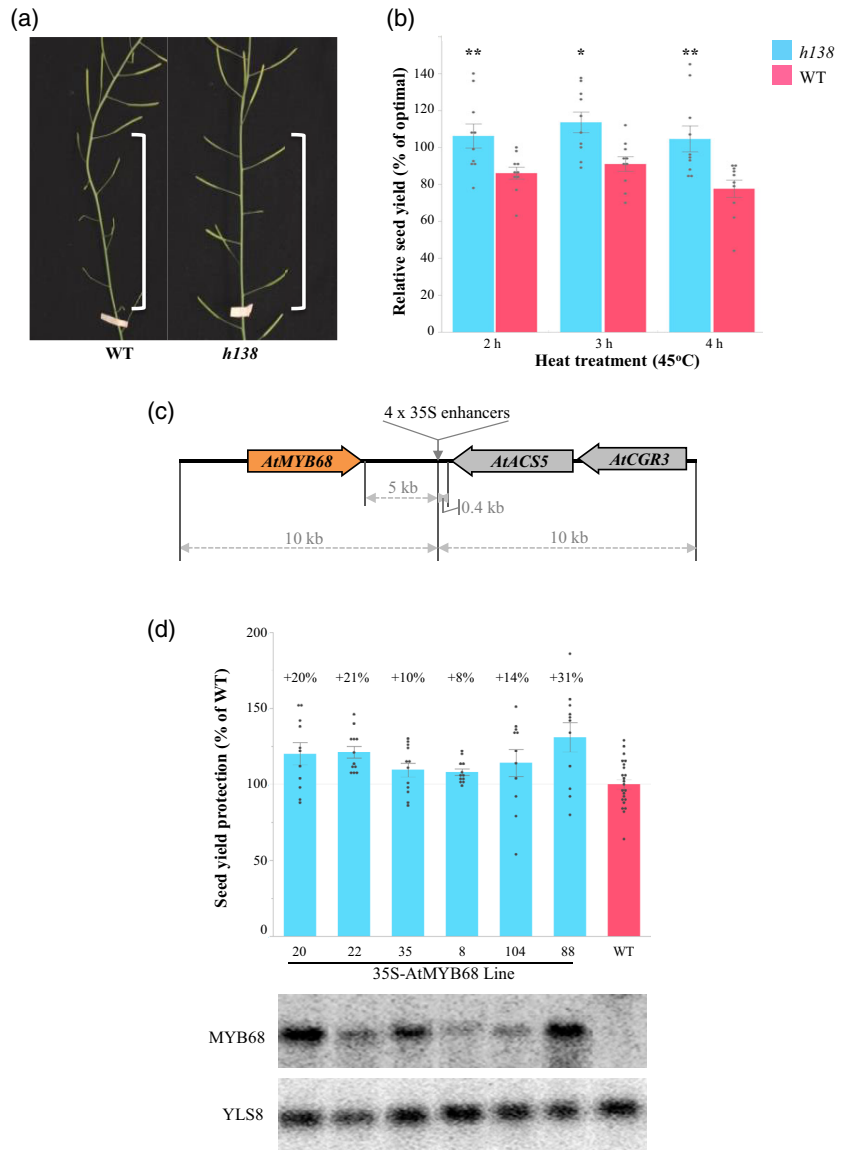
on chromosome 5 containing three genes within the 10-kb regions flanking the insertion site (Figure 1c): At5G65790 (*AtMYB68*), At5G65800 (*AtACS5*) and At5G65810 (*AtCGR3*). Transcript profiling of 2-week-old *h138* seedlings showed the expression of *AtACS5* and *AtMYB68* increased by 2.8- and 21.3-fold respectively over the WT, while *AtCGR3* gene expression was not affected. To determine which of the two genes with elevated expression contributed to the heat tolerance phenotype, we overexpressed the complementary (c)DNAs of *AtMYB68*, *AtACS5* and *ETO2* (mutated *AtACS5* at C-terminus to have constitutive activation of AtACS5) in Arabidopsis using the 35S promoter. From this collection of homozygous transgenic plants, we found that only the overexpression of *AtMYB68* resulted in a heat-tolerant silique phenotype. As shown in Figure 1(d), the six independent transgenic lines with elevated levels of

AtMYB68 transcripts exhibit 8–31% better seed yield protection against heat stress during flowering than the WT. When the 11-day-old seedlings of four of the homozygous transgenic lines were subjected to heat stress treatment at 42°C for three consecutive days, their shoot biomass was reduced by approximately 40% in comparison with the plants grown under optimal conditions. However, the level of biomass reduction observed in the transgenic plants is similar to that of WT (Figure S1), suggesting a role of *AtMYB68* in specific regulation of heat tolerance in the reproductive tissues rather than the vegetative tissues.

Primary structure, expression and subcellular localization of AtMYB68

AtMYB68 is a member of subgroup 14 of R2R3 MYB transcription factors in Arabidopsis (Kranz *et al.*, 1998;

Figure S2). In this subgroup 14, AtMYB68 shows highest amino acid sequence homology with AtMYB84 (67%) while sharing 43–47% homology with other more distant members (AtMYB36, 37, 38 and 87). Using AtMYB68 as the query to perform a blast search in the NCBI crop genome database, a series of putative homologs were identified from *Brassica napus*, cotton, soybean, *Brachypodium*, sorghum, corn and rice. Among them, the homolog from *B. napus* shows the highest identity (90%) in protein sequence, while homologs from the other crops show a range of identity between 46% and 51%, with the highly conserved regions distributed in the R2R3 domains (Figure S3). Several members of the subgroup 14 MYB genes have been implicated in the control of lateral meristem initiation and formation (Schmitz *et al.*, 2002; Müller *et al.*, 2006; Keller *et al.*, 2006). Transcripts of five other members in the *AtMYB68* sub-family: *AtMYB36, AtMYB37, AtMYB38, AtMYB84* and *AtMYB87* were detected by reverse transcription–polymerase chain reaction (RT-PCR) in various tissues, including shoot tip, internode, leaf, flower bud, open flower and roots (Müller *et al.*, 2006; Keller *et al.*, 2006). Our data confirmed that *AtMYB68* was predominantly expressed in the vascular tissues of roots and shoots (Figure 2a). Further analysis by quantitative RT-PCR (RT-qPCR) validated the relatively high expression of AtMYB68 in root tissues and lower expression in buds, open flowers and leaves (Figure 2b). It appears that the native *AtMYB68* is expressed predominantly in roots. However, the heat-tolerant phenotype observed in the reproductive stages is likely the result of constitutive activation of the gene by the 35S enhancers and the 35S promoter.

Previous AtMYB68 promoter-GUS study suggested that the expression of AtMYB68 was inducible by higher temperature (Feng *et al.*, 2004). However, this observation was based solely on GUS staining results without checking the actual changes in *AtMYB68* transcripts. To validate this, the tissue-specific expression of the gene was examined under a time course of either drought or heat treatments, using archived microarray data for Arabidopsis (Toufighi *et al.*, 2005). As shown in Table S1, the levels of *AtMYB68* expression in shoots and roots remained rather constant with stress treatments ranging from 15 min to 24 h, indicating that its expression is not inducible by drought or heat stress.

The subcellular localization of AtMYB68 was examined using the *GFP-AtMYB68* overexpression lines in Arabidopsis, and the results indicated that AtMYB68 localizes in the nucleus of the root cells (Figure 2c), consistent with that of a typical transcription factor.

Increased ABA sensitivity, reduced transpirational water loss and enhanced drought and heat tolerance by overexpression of *AtMYB68* in Arabidopsis

To examine further, whether *AtMYB68* is involved in the regulation of cross stress tolerance, we examined the ABA response with two independent, single-locus insertion homozygous Arabidopsis lines (lines 22 and 104) of the 35S-*AtMYB68* construct. In the absence of exogenous ABA in the 0.5× Murashige and Skoog growth medium, seeds of the two transgenic lines germinated at the same rate as Columbia WT, reaching 100% germination 1 day after imbibition (Figure 3a). However, in the presence of 0.5, 0.75 or 1 μM ABA in the media, the germination rates of the transgenic lines decreased much more than that of WT (Figure 3a). Similarly, in the absence of exogenous ABA, the curves of cotyledon and first true leaf emergence followed that of WT very closely over the 5 days post-imbibition (Figure 3b,c). At ABA concentrations of ≥0.5 μM, cotyledon emergence and leaf development of the transgenic lines were much more delayed and inhibited than the WT. These results indicate that overexpression of *AtMYB68* results in increased ABA sensitivity at germination and during early seedling development.

The enhanced ABA response phenotypes prompted us to examine if these transgenic plants would have reduced transpirational water loss under water deficit conditions. In this experiment, multiple replicates of Arabidopsis transgenic plants (lines 22 and 104) along with Columbia WT were grown under optimal watering conditions in covered 3-inch pots to prevent surface evaporation. At the start of flowering, irrigation was withheld for 4 consecutive days and each pot was weighed to determine the amount of water loss through transpiration. To account for any difference in soil water loss due to the potential difference in plant size, the total water loss in the 4 days of water deficit treatment was divided by the total shoot biomass at the fourth day. The results indicated that over the course of water stress, transgenic lines 22 and 104 lost 10% and 19% less water loss per unit of shoot weight than that of the control, which are statistically significant ($P < 0.05$ and $P < 0.001$, Figure 4a). These data collectively suggest that enhanced ABA sensitivity would result in reduced transpirational water loss in the transgenic plants under drought stress.

The productivity of transgenic line 104 was further tested by measuring final seed yield under four different combined heat and water stress conditions during flowering. In these experiments, plants were subject to 45°C heat treatments for a total of either 6.25 h (H1) or 8.25 h (H2) when soil water content was reduced to 30% (D1) or 40% (D2), and seeds were harvested upon maturity. Under these conditions, transgenic line 104 showed significantly better seed yield than the WT post-stress treatments (by 18–59%, Figure 4b). The level of yield reductions in WT correlate well with the severity of the stress treatment, whereas the highest yield reduction was observed after the longer heat treatment under the lower soil water content (H2D1). Thus, our data suggest that upregulation of *AtMYB68* improved plant's tolerance against simultaneous heat and drought

(a)

PAtMYB68-GUS

(b)

(c)

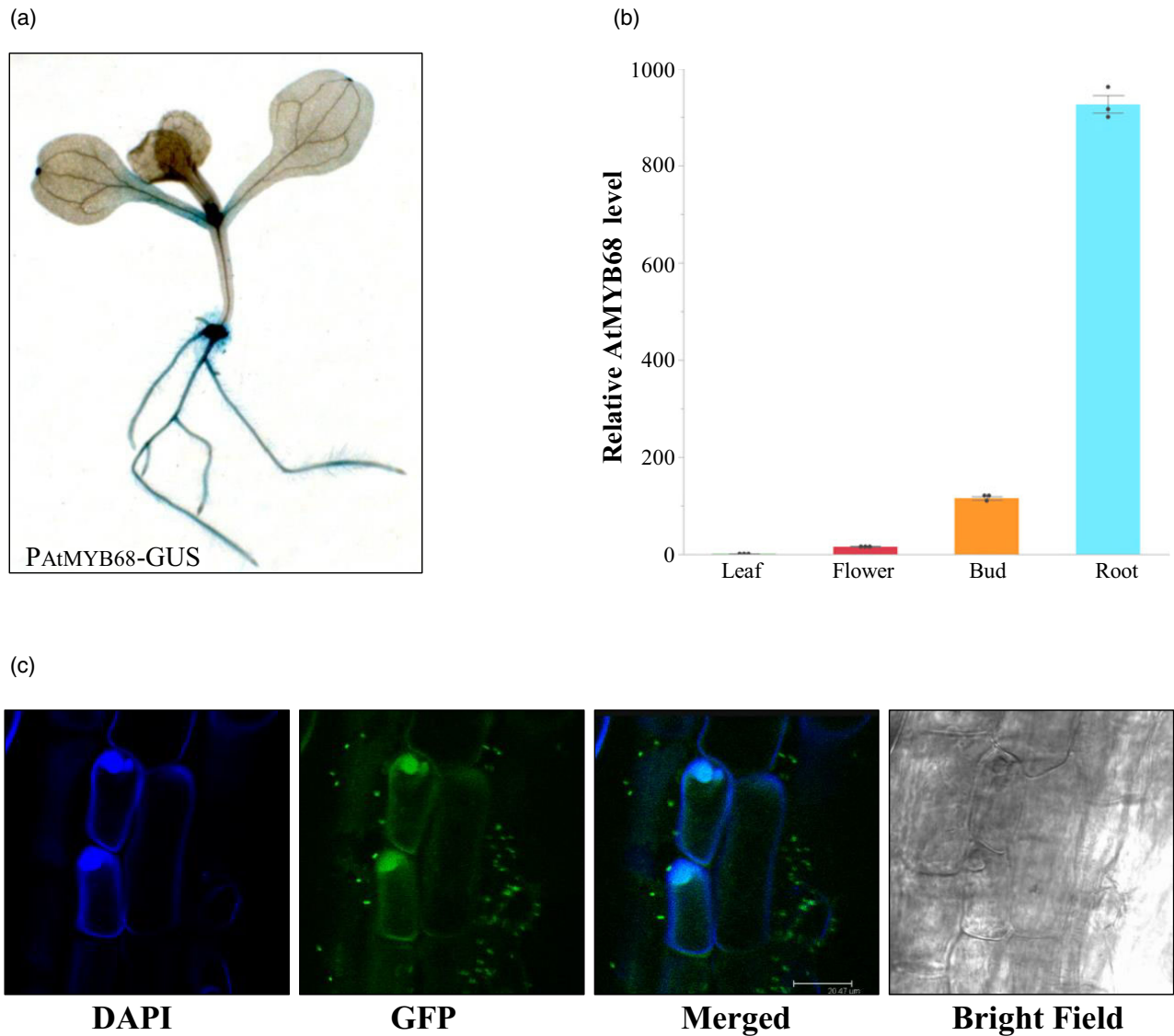**DAPI** **GFP** **Merged** **Bright Field**

Figure 2. Expression and nuclear localization of *AtMYB68* in Arabidopsis.
(a) GUS staining of 2-week-old T3 homozygous seedlings of P*AtMYB68*-GUS construct.
(b) Relative levels of *AtMYB68* transcript in various tissues of Columbia. Values were determined by quantitative polymerase chain reaction and were normalized using *AtPP2AA3* as an internal control. Error bars represent standard errors ($n = 3$).
(c) Confocal microscopy of a section of 1-week-old root tissue of the transgenic line. green fluorescent protein (GFP)-AtMYB68 is localized in the nucleus by the nuclear marker 4′,6-diamidino-2-phenylindole (DAPI), GFP and the DAPI/GFP merged images. Overall cell structure in this root tissue is also shown under the bright field setting.

stresses at the yield determining reproductive stages of Arabidopsis development.

Ectopic expression of *AtMYB68* in canola results in increased heat tolerance during flowering and reduced vegetative water loss during drought stress

The promising results in Arabidopsis prompted us to see if the heat and drought tolerance phenotypes could be translated directly to a crop species. Blast search using AtMYB68 identified a highly homologous sequence from *B. napus,* showing 90% identity in nucleotide and amino acid sequences. Furthermore, quantitative transcriptome analysis indicates the expression pattern of this homologous *B. napus* gene is very similar to that of *AtMYB68* in Arabidopsis, with extremely low expression in leaves and relatively high in roots (Duke *et al.*, 2017; Haddad *et al.*, 2019). This suggests that the ortholog of *AtMYB68* in canola may function similarly as in Arabidopsis. We introduced *AtMYB68* into *DH12075,* a doubled haploid canola line, under the control of either the strong constitutive 35S
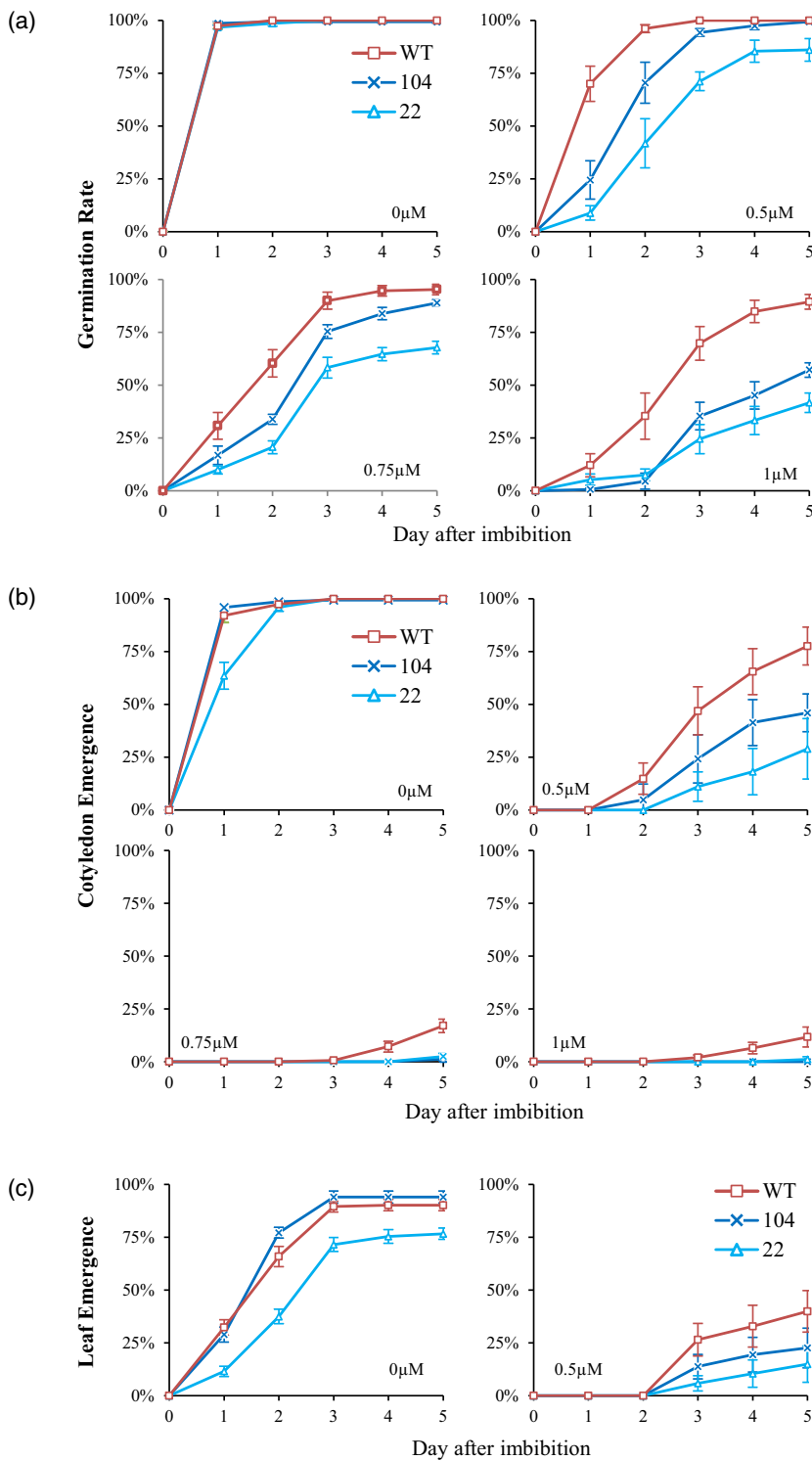
Figure 3. Overexpression of *AtMYB68* in Arabidopsis resulted in increased abscisic acid sensitivity upon germination and during early seedling development.

Seeds of wild-type (WT) Columbia and two independent, homoz8ygous 35S-AtMYB68 transgenic lines (22 and 104) were plated in the 0.5× Murashige and Skoog media containing 0, 0.5, 0.75 and 1 μM of abscisic acid. Percentages of (a) seed germination rates, (b) cotyledon emergence and (c) true leaf emergence during the first 5 days of post-imbibition are shown. Error bars represent standard errors (*n* = 3).

promoter or P*81.1*, the promoter of Arabidopsis heat shock protein *AtHSP81.1* (Schmid *et al.*, 2005; Ueda *et al.*, 1996; and Yabe *et al.*, 1994). P*81.1* appeared to be a leaky promoter with a detectable basal level of activities at 22°C, and it drove a much higher expression at 40°C when measured by the relative levels of *AtMYB68* transcripts in the tissues of homozygous transgenic lines 1–5 of P*81.1*-AtMYB68, or by GUS staining with various tissues harvested from transgenic lines of the P*81.1*-GUS construct (Figure 5a,b and Figure S4). Similar to our Arabidopsis
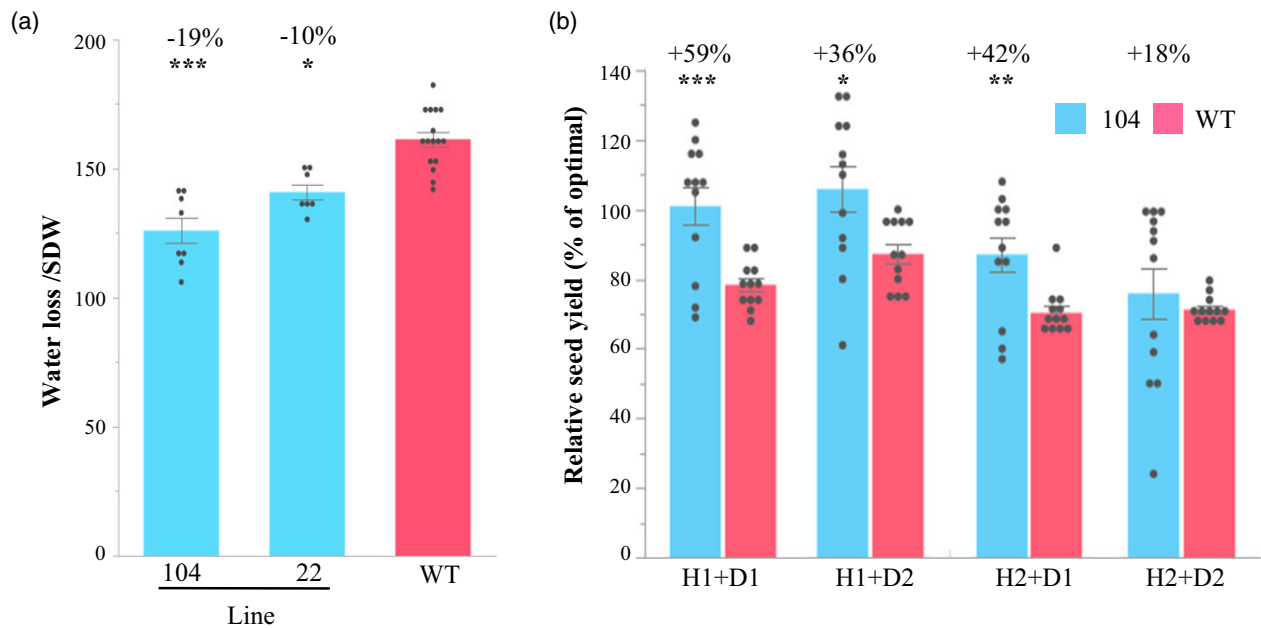
Figure 4. Overexpression of *AtMYB68* in Arabidopsis resulted in reduced transpirational water loss and enhanced tolerance to simultaneous drought and heat stress.

(a) Transpirational water loss in the 4 days of water-withholding treatment divided by shoot dry weight (SDW) during flowering in wild-type (WT) Columbia and two independent, homozygous 35S-*AtMYB68* transgenic lines (22 and 104). Error bars represent standard errors ($n = 8$), and asterisks above a bar indicated a significant difference from Columbia WT (*$P < 0.05$ and ***$P < 0.001$).

(b) Relative seed yield of Columbia WT and transgenic line 104 after simultaneous treatments of 45°C for a total of either 6.25 h (H1) or 8.25 h (H2) at soil water content of either 30% (D1) or 40% (D2) during flowering. Values of relative seed yields are determined on a percentage basis of yield under stress conditions over that obtained under optimal growth conditions. Error bars represent standard errors ($n = 12$), and asterisks above a bar indicated a significant difference from Columbia WT (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

results, the heterozygous 35S-*AtMYB68* transgenic canola plants exhibited improved silique formation under heat stress (Figure S5a) and significant reduction in water loss under drought stress compared with the DH12075 (Figure S5b). However, the homozygous plants of 35S-*AtMYB68* were developmentally compromised, showing delayed growth, reduced overall plant size and seed yield. By contrast, when the heat-inducible P*81.1* promoter was used to drive *AtMYB68* expression, the homozygous transgenic canola plants showed normal growth in all developmental stages. With heat treatment during flowering, the homozygous transgenic lines of P*81.1*-*AtMYB68* produced significantly fewer aborted siliques than that of DH12075 (Figure 5c,d).

Conditional expression of *AtMYB68* in canola leads to enhanced pollen viability against heat stress

Canola is a temperate crop with a critical threshold tolerance to a temperature of 29°C (Morrison and Stewart, 2002), and moderate heat stress at 35°C during flowering would result in a reduction of pollen viability and gametophyte fertility (Young *et al.*, 2004). To gain insight into the observed reproductive heat tolerance of the P*81.1*-*AtMYB68* transgenic canola, we carried out a series of

pollen viability assays with pollens harvested from transgenic line 5 before, after and the recovery phases of heat stress at 43°C during flowering. Pollen viability was scored by the percentage of pollens that germinated successfully with subsequent pollen tube elongation in the growth medium. The transgenic plants had similar pollen viability in comparison with the control under optimal growth conditions. However, their pollen germination rate was significantly higher than that of DH12075 (by 88%) when pollens were collected immediately after the 2-h heat treatment and by 143% when pollens were collected after the 2-h recovery post-heat treatment, respectively (Figure 6). Thus, conditional expression of *AtMYB68* in canola enhances pollen viability, which may be the main contributing factor for the improved silique formation under heat stress.

Conditional expression of AtMYB68 in canola improves seed yield in the fields under different water and heat conditions

To assess the performance of the P*81.1*-*AtMYB68* transgenic canola in the field, the same five homozygous canola lines (lines 1–5) that showed an increased heat tolerance in the growth chamber experiments were subjected to standard single row and full-scale field trials in multiple
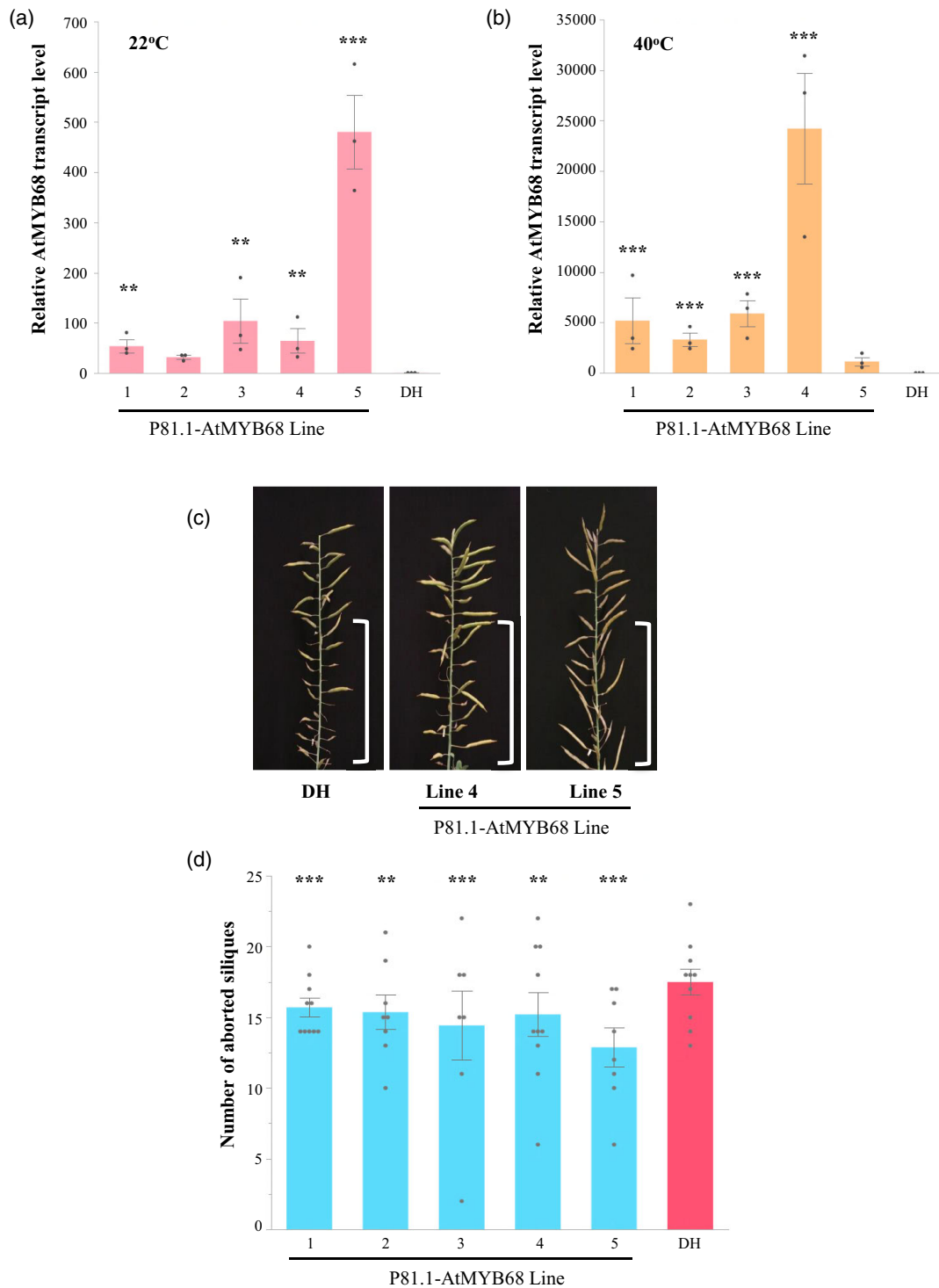
Figure 5. Conditional expression of *AtMYB68* in canola resulted in increased heat tolerance during flowering.
Relative levels of *AtMYB68* transcript in the 2-week-old seedlings of DH12075 (DH) and five independent, homozygous P*81.1-AtMYB68* transgenic lines at (a) 22°C and (b) 40°C. *AtMYB68* transcript levels were determined by qPCR and normalized using *BnPP2AA3* as an internal control. Error bars represent standard errors ($n = 3$), and asterisks above a bar indicated a significant difference from DH (**$P < 0.01$ and ***$P < 0.001$).
(c) Silique phenotypes at the raceme of homozygous transgenic plants of P*81.1-AtMYB68* (lines 4 and 5) in comparison with DH, photographically captured 2 weeks after heat stress treatment of 4 h at 40°C during flowering. White brackets indicate the regions of the main inflorescence affected by heat treatment.
(d) Silique abortion of homozygous P*81.1-AtMYB68* transgenic lines (lines 1–5) in comparison with DH, counted 2 weeks after heat stress treatment of 4 h at 40°C during flowering. Error bars represent standard errors ($n = 10$), and asterisks above a bar indicated a significant difference from DH (**$P < 0.01$ and ***$P < 0.001$).
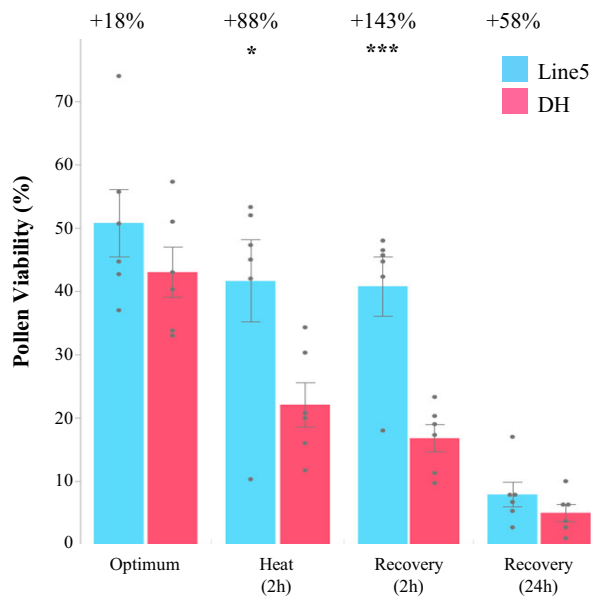
Figure 6. Conditional expression of *AtMYB68* in canola enhanced pollen viability against heat stress. Fresh pollens were collected from the DH12075 control (DH) and homozygous transgenic plants of P*81.1-AtMYB68* (line 5) before heat treatment (Optimum), 2 h after heat treatment at 43°C (Heat 2 h), 2 h after recovery (Recovery 2 h), and 24 h after recovery (Recovery 24 h). Pollen was placed in culture medium and pollen viability was scored by the percentage of pollens that germinated and initiated pollen tube elongation. Error bars represent standard errors (*n* = 6), and asterisks above a bar indicated a significant difference from DH (**P* < 0.05 and ****P* < 0.001).

environments. Single row trials (*n* = 4; 90 seeds/row) were seeded in Taber (Alberta) and two locations in Okanagan Falls (British Columbia). The seed yield data indicated a strong positive trend where lines 1, 4 and 5 consistently performed better than the DH12075 control in all three trials (by 3–39%) and lines 2 and 3 yielded higher than the control in two of the three trials (Figure 7a). These results were further confirmed in the full-scale field trials (*n* = 6; 1800 seeds/replicate) at four different locations: Taber and Oliver of Canada, and Nancagua and Pirque of Chile, where higher temperatures during flowering were observed (Figure S6 and Table S2). Similar to the single row trials, lines 1, 4 and 5 yielded higher than that of the control in all four locations, while yields of lines 2 and 3 were higher in three of the four locations in these large-scale trials (Figure 7b). To account for the variation within each trial and to allow for more direct comparisons between the trials, the yield for each transgenic line in each plot was expressed as a percentage of the control within that same block. When data for all locations was combined for analysis, location effect was not significant (*P* = 0.1226) and therefore, combined data were used for further comparison analysis (Figure 7c). All five homozygous transgenic lines had greater seed yield (ranging from 106% to 121%) than the control. It has been indicated that

the threshold high temperature for canola is about 29°C and flower abortion occurs when exceeding this temperature (Morrison and Stewart, 2002; Wahid *et al.*, 2007). In our field trials, canola plants experienced different severities of heat stress during flowering at the four sites with moderate heat stress at Taber (7 days at ≥28°C) and Pirque (maximum day time temperatures during the flowering were ranging between 25°C and 35°C with an average of 30°C), medium heat stress at Oliver (22 days of ≥28°C with 8 consecutive days of maximum temperature 38°C at early flowering) and severe heat stress at Nancagua (maximum daily temperature averaging from low to mid 30s reaching high 30s). Thus, the homozygous *AtMYB68* transgenic canola outperformed the DH12075 controls under various degrees of heat stress in the field. Furthermore, additional agronomic data collected from all field trial locations showed no overall significant differences in plant vigor, stand, lodging or height (Table S3).

In the Chilean-based canola field experiments, all field sites were irrigated throughout the growth season except for one site at Pirque where irrigation was reduced during flowering (*n* = 6; 1800 seeds/replicate). In this reduced irrigation site, the treatment with regular irrigations (approximately 40 mm/irrigation once a week for 20 weeks) was referred to as the heat stress only group; the treatment with two missed irrigations during the first 2 weeks of flowering and three missed irrigations right after flowering was referred to as the heat and water stressed group. When the yields of the transgenic events were compared with the control within each block, lines 1, 3 and 5 yielded 6%, 15% and 6% higher under heat stress only and 16%, 27% and 8% higher under heat plus water stresses (Figure 7d). Based on these results, conditional expression of *AtMYB68* using the P*81.1* promoter in canola consistently improved plant tolerance under various levels of heat and drought stress conditions during yield determining flowering stages of development.

DISCUSSION

Reproductive development in crops is one of the most sensitive stages to environmental stresses such as heat and drought, and consequently a single or combined stress at this developmental stage often leads to profound reductions in crop yield (Wang *et al.*, 2005; Wan *et al.*, 2009; Lobell *et al.*, 2011; Guo *et al.*, 2016; Chao *et al.*, 2017). The lack of direct genetic screens for stress tolerant mutants at the reproductive stages is somewhat surprising given our long-standing knowledge in this area (Zinn *et al*, 2010). This may be partly due to the added space and time constraints that are required in assessing flowering phenotypes and seed yield. In addition, as these screens cannot be done on Petri plates, they are vulnerable to the nuances of growth room conditions. However, even with these constraints, we were able to screen >18 000 activation-tagged
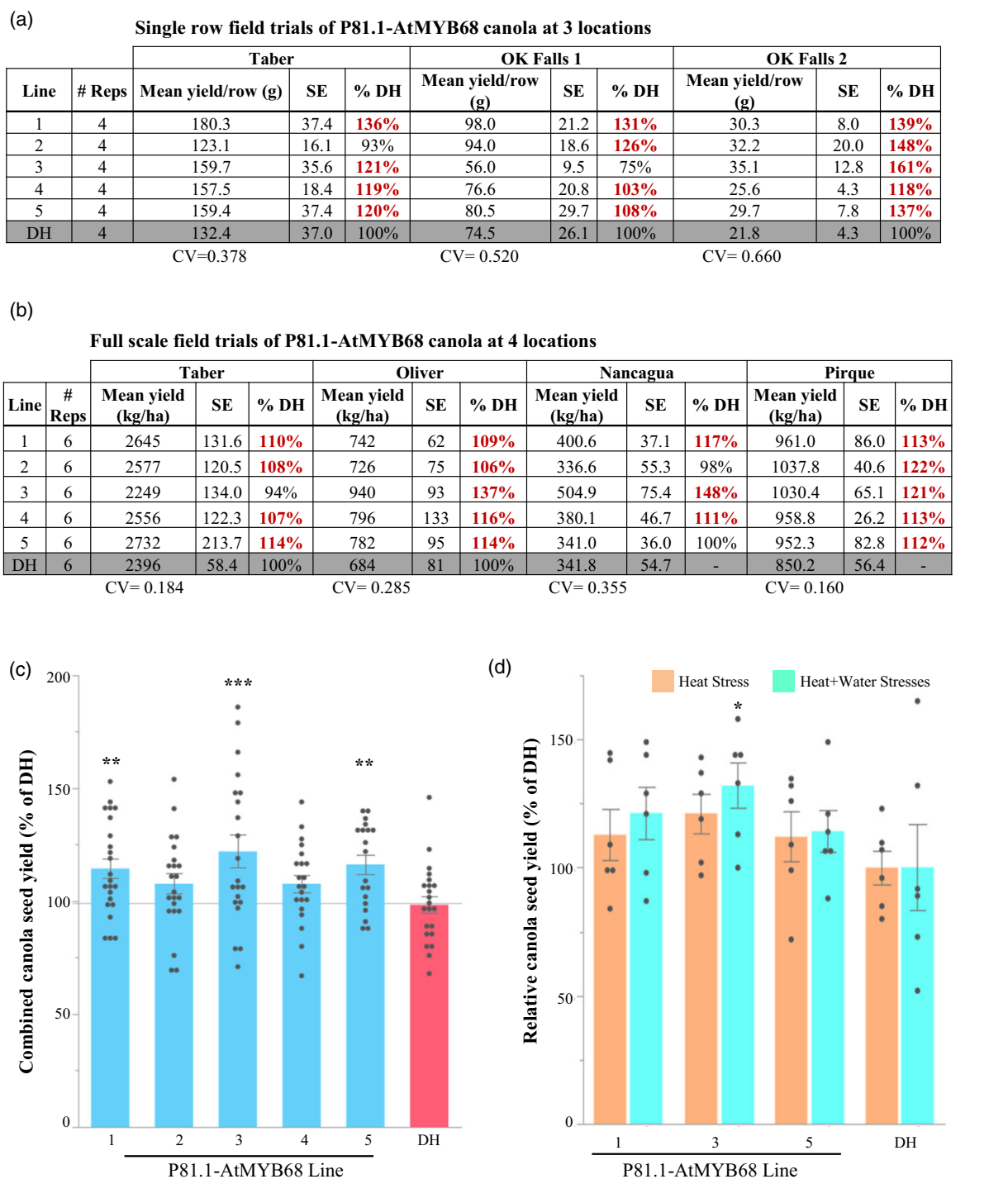
(a)

Single row field trials of P81.1-AtMYB68 canola at 3 locations

Line	# Reps	Taber			OK Falls 1			OK Falls 2		
		Mean yield/row (g)	SE	% DH	Mean yield/row (g)	SE	% DH	Mean yield/row (g)	SE	% DH
1	4	180.3	37.4	**136%**	98.0	21.2	**131%**	30.3	8.0	**139%**
2	4	123.1	16.1	93%	94.0	18.6	**126%**	32.2	20.0	**148%**
3	4	159.7	35.6	**121%**	56.0	9.5	75%	35.1	12.8	**161%**
4	4	157.5	18.4	**119%**	76.6	20.8	**103%**	25.6	4.3	**118%**
5	4	159.4	37.4	**120%**	80.5	29.7	**108%**	29.7	7.8	**137%**
DH	4	132.4	37.0	100%	74.5	26.1	100%	21.8	4.3	100%
		CV=0.378			CV= 0.520			CV= 0.660		

(b)

Full scale field trials of P81.1-AtMYB68 canola at 4 locations

Line	# Reps	Taber			Oliver			Nancagua			Pirque		
		Mean yield (kg/ha)	SE	% DH	Mean yield (kg/ha)	SE	% DH	Mean yield (kg/ha)	SE	% DH	Mean yield (kg/ha)	SE	% DH
1	6	2645	131.6	**110%**	742	62	**109%**	400.6	37.1	**117%**	961.0	86.0	**113%**
2	6	2577	120.5	**108%**	726	75	**106%**	336.6	55.3	98%	1037.8	40.6	**122%**
3	6	2249	134.0	94%	940	93	**137%**	504.9	75.4	**148%**	1030.4	65.1	**121%**
4	6	2556	122.3	**107%**	796	133	**116%**	380.1	46.7	**111%**	958.8	26.2	**113%**
5	6	2732	213.7	**114%**	782	95	**114%**	341.0	36.0	100%	952.3	82.8	**112%**
DH	6	2396	58.4	100%	684	81	100%	341.8	54.7	-	850.2	56.4	-
		CV= 0.184			CV= 0.285			CV= 0.355			CV= 0.160		

(c)

(d)

Figure 7. Seed yields of DH12075 controls (DH) and five *P81.1-AtMYB68* transgenic canola lines in multiple location, confined field trials.
(a) Yields of single row trials (*n* = 4) in three Canadian locations: Taber, Okanagan (OK) Falls 1 and OK Falls 2. Yields of transgenic lines that were higher than DH are marked in red fonts. CV, coefficient of variation; Reps, replicates.
(b) Yields of full-scale trials (*n* = 6) in two Canadian locations (Taber and Oliver) and two Chilean locations (Nancagua and Pirque). Yields of transgenic lines that were higher than DH are marked in red fonts.
(c) Combined seed yields of *P81.1-AtMyb68* homozygous plants from the full-scale field trials at the four locations are compared with that of DH. Error bars represent standard errors (SEs; *n* = 24).
(d) Seed yield of *P81.1-AtMyb68* transgenic canola (lines 1, 3 and 5) in comparison with that of DH in the irrigation-controlled field site of Pirque (Chile) where daily maximum temperatures during flowering were in the 25–35°C range. Error bars represent SEs (*n* = 6). Significant differences between the transgenic lines and DH are indicated by asterisks (Student *t*-tests at *P < 0.1; **P < 0.05; ***P < 0.01).

lines and found one line, *h138*, which showed reproducible improved heat tolerance during flowering and seed set. The low frequency of mutants most likely reflects the source of mutagenized material, which was an activation tagged T-DNA population. Unlike chemical mutagenesis, T-DNA-based populations only contain one or a few mutations per plant. Even with this limitation, a gain-of-function strategy versus a loss-of-function approach has certain advantages. First, activation tagging should reduce issues of genetic redundancy that might mask interesting phenotypes (Weigel *et al.*, 2000; Bac-Molenaar *et al.*, 2015). Secondly, there are many clear examples where overexpression of genes confers protection against environmental stresses such as drought, salt and cold (Kasuga *et al.*, 1999; Thomashow, 1999, 2001; Zhu, 2001; Yang *et al.*, 2009). It will be interesting in the future to screen ethyl methanesulfonate or other mutagenized populations using our silique-based phenotype to identify additional genes that can confer improved reproductive heat tolerance.

By defining our screening to reproductive development, we first discovered that activation of an Arabidopsis transcriptional factor, AtMYB68 in mutant *h138* background resulted in enhanced tolerance to severe heat stress, as the mutant was able to maintain its seed set over 4 h at 45°C while the same treatment would cause significant reduction in seed yield for the WT control. We followed up to produce heat stress tolerant, transgenic Arabidopsis plants through constitutive expression of *AtMYB68*, thereby validating the gene's direct involvement in regulating heat stress tolerance. Our results from cross stress tolerance tests showed that the *AtMYB68* overexpressing plants lost less water through transpiration in the water-deficit treatment and produced relatively higher seed yield than the control under simultaneous heat and drought stress conditions. As these transgenic plants were hypersensitive to ABA at germination and during early vegetative growth, the observed enhanced drought stress tolerance maybe mediated through the ABA sensing pathway, as in the case of many other ABA hypersensitive mutants (Yang *et al.*, 2010). Most importantly, we provided laboratory and large-scale field trial data to show that the advantageous stress tolerance and yield protection phenotypes observed in Arabidopsis could be translated into canola through different ways of ectopic expression of *AtMYB68*.

Although *AtMYB68* is predominantly located in the root, misexpression of the gene using ubiquitous promoters improved heat and drought tolerance of the whole plant. Unrestricted overexpression of *AtMYB68* using a constitutive promoter such as 35S in Arabidopsis did not cause a drag on the overall growth and development of the plant. In the case of overexpressing *AtMYB68* in canola, the use of an inducible promoter such as P*81.1* avoided some pleotropic phenotypes from the use of a strong constitutive 35S promoter, while maintaining the enhanced water and heat stress tolerance phenotypes. At this stage, it is not yet clear whether improving expression in specific tissue such as roots and flowers can improve the tolerance.

AtMYB68 is a member of a large family of R2R3-MYB transcription factors that have been implicated in a variety of hormonal and environmental responses (Kranz *et al.*, 1998; Stracke *et al.*, 2001). *AtMYB68* knock-out Arabidopsis mutant did not show quantifiable change in phenotype when grown under optimal conditions. However, under elevated temperatures, the mutant showed some contradicted developmental phenotypes: significant reduction in leaf growth but increase in root growth when comparing with the WT control (Feng *et al.*, 2004). Hence, it was not clear how and what roles *AtMYB68* might play in response to environmental stress. As the expression levels of *AtMYB68* in leaves and reproductive organs are very low, loss-of-function mutations of the gene may not result in obvious phenotypes related to stress responses. Overexpression of *AtMYB37*, another transcription factor in the Arabidopsis R2R3 subgroup 14 of MYB genes, has been found to enhance ABA sensitivity, drought tolerance and seed production (Yu *et al.*, 2016). These results suggest that some closely related members in this subgroup 14 of *MYB* genes might play overlapping roles in regulating a plant's response to ABA and in water stress tolerance.

Previous studies suggest that thermotolerance in plants may be regulated through multiple signaling pathways, and heat stress tolerance observed in vegetative tissues had little or no relationship with tolerance in reproductive tissues (Larkindale *et al.*, 2005; Salem *et al.*, 2007). Our current results are in good agreement with these observations, as the transgenic Arabidopsis lines of 35S-*AtMYB68* displayed enhanced heat stress tolerance in the reproductive organs but showed similar response to heat stress as WT during an early vegetative growth stage (Figure 1 and Figure S1). Therefore, a genetic screen based solely on vegetative response to heat stress would be unlikely to identify the involvement of *AtMYB68* for reproductive heat stress tolerance. Hence, it is critical to test pollen fitness directly when the tolerance is measured by eventual seed yield, which is the most important agronomic parameter for agricultural productivity (Walbot, 2011). In this study, we observed better seed set and silique formation for the *AtMYB68* transgenic plants under heat stress, which may largely be a result of enhanced pollen viability (Figure 6). Together, the data suggest that *AtMYB68* may promote the pollination and seed formation processes, resulting in better seed yields under various heat stresses. On the other hand, these transgenic plants showed reduced transpirational water loss in drought stress conditions, suggesting that like *AtMYB37*, the enhanced drought tolerance of these transgenic plants may be through a different physiological mechanism acting at the stomatal level in the leaves. As the drought stress experiments were carried out

over a period of days, whereas the heat stress is over hours, it is reasonable that the observed drought and heat tolerances are through different physiological mechanisms – one mediated through ABA and the other through pollen fitness. Further research is required to find the missing links and the possible downstream targets of *AtMYB68* so that we can gain more insight into the molecular and cellular regulatory mechanism mediated by this transcriptional factor for heat and drought tolerance.

It is important that the present study demonstrated the success of the transgenic canola in improving dual tolerance to heat and drought in the field, as this paves the road for broader application in other crop species. In the field, crops often suffer from simultaneous heat and drought stresses, and the yield losses from those combined stresses are significantly higher than any single stress (Mittler, 2006). It has become increasingly clear that central controlling mechanisms for multiple stresses must be identified to cope with crops grown in natural agricultural settings (Sewelam *et al.*, 2014; Thoen *et al.*, 2017; Zhang and Sonnewald, 2017). Stress tolerance technologies, particularly those based on Arabidopsis gene studies, have recently been criticized because these technologies are often assessed under artificial growth environments (Skirycz *et al.*, 2011). As most environmental stresses are episodic, this makes these technologies impractical. Consistent with this notion, our manipulations of *AtMYB68* show specific tailoring through conditional expression is required for translation of this technology to the field. Therefore, it appears that future broad-based functional screens for environmental stress protection using Arabidopsis may miss many potentially useful targets. However, with the myriad of resources available in this model system it should be possible to develop screens that are more refined, to uncover new stress tolerance mechanisms.

EXPERIMENTAL PROCEDURES

Plant growth conditions

The Columbia ecotype of *Arabidopsis thaliana* (WT) along with various transgenic plants were grown in 3-inch pots containing Sunshine soil mix in a growth chamber (Biochambers Inc., Winnipeg, Manitoba, Canada) at 22°C, 16 h light (200 μE)/8 h dark cycle at 70% relative humidity. Canola (cv. DH12075) was used as the WT control and they were grown along with various transgenic plants in 6-inch pots containing Sunshine soilless media in a greenhouse or a growth chamber at 22°C, 16 h light (450 μE)/8 h dark cycle at 70% relative humidity.

Genetic screen for reduced silique abortion under heat stress

In total, 71 pools of about 18 000 activation-tagged T-DNA lines obtained from the Arabidopsis Biological Resource Center (ABRC, Columbus, Ohio, USA) were used for this study. These lines were generated by transformation of WT *A. thaliana* (Columbia) plants with the pSKI15 with 4× 35S enhancers (Weigel *et al*, 2000). T4 seeds were bulked for the mutant screen, and approximately 1350–1400 plants were screened per pool. Seeds sown on wet soil in flats were placed at 4°C for 4 days before being transferred to a growth chamber set at optimal growth conditions. Plants on the fifth day of flowering were heat shocked at 45°C for 60 min, and subsequently examined for aborted siliques. Putative heat stress tolerant mutants identified by the reduction of silique abortion were transferred to 3-inch pots and allowed to continue to seed. Mutant *h138* was identified through primary and secondary screens based on its reproducible heat-resistant silique phenotype.

PCR-based genome walking to localize the T-DNA insertion

Genomic DNA was purified by phenol/chloroform extraction using 10-day-old seedlings of mutant *h138*. The isolated DNA was subsequently digested by restriction enzymes (*Eco*RV, *Pvu*II, *Nru*I or *Stu*I) to generate DNA fragments with blunt ends. The resultant DNA fragments from each digestion were ligated to an adaptor that was annealed by two oligos: Adaptor 1 (5′-GTA ATA CGA CTC ACT ATA GGG CAC GCG TGG TCG ACG GCC CGG GCT GGT-3′), and Adaptor 2 (5′-PO₄-ACC AGC CC-N₂H-3′). The addition of the adaptor to the DNA fragments enables PCR amplifications using primers specific to the adaptor and the T-DNA insert. Two rounds of PCR were usually needed to generate DNA fragments for sequencing analysis. Primer HeatL1 (specific to the T-DNA left border, 5′-AGG TCA AAC CTT GAC AGT GAC GAC AAA TC-3′) and primer CAP1 (specific to the adaptor, 5′-GTA ATA CGA CTC ACT ATA GGG C-3′) were used for the first round of PCR. The resultant PCR products were diluted 50-fold to serve as templates for the second round of PCR. A confirmed DNA fragment was then amplified by two nested primers HeatL2 (5′-TGG ACG TGA ATG TAG ACA CGT CGA AAT AA-3′) and CAP2 (5′-ACT ATA GGG CAC GCG TGG T-3′). All PCR was carried out using Ex-Taq as DNA polymerase and Biometra® thermocycler.

Constructs and transgenic plant materials

For the 35S-*AtMYB68* and P81.1-*AtMYB68* constructs, a 1.1 kb of *AtMYB68* cDNA fragment was cloned into a pBI121 vector (without GUS) driven either by 35S or AtHSP81.1 promoters, respectively. cDNAs (1.4 kb) encoding *AtACS5* and *ETO2* were cloned into the same binary vector driven by 35S promoter. For the P*AtMYB68-GUS* construct, the promoter of AtMYB68 was cloned in front of the GUS gene in the pBI121 vector. For the 35S-GFP-*AtMYB68* construct, a 1.4 kb of *AtMYB68* genomic DNA fragment including 83 bp of 3′-UTR was cloned into pEGAD vector. These constructs were introduced into *Agrobacterium tumefaciens* GV3101 and subsequently transformed into *A. thaliana* (Columbia ecotype) by the floral dip method (Clough and Bent, 1998) or into *B. napus* (canola cv. DH12075) as described (Moloney *et al.*, 1989).

RNA analyses

For RT-qPCR, total RNA was extracted from three replicated plant tissues using the RNeasy Plant Mini Kit (Qiagen, Mississauga, Ontario, Canada). Genomic DNA was removed by in-column DNase digestion before RNA elution. cDNA was synthesized from 1 to 5 μg of total RNA using oligo-dT primer and SSII RT (Invitrogen, Burlington, Ontario, Canada). *AtPP2AA3* was used as an internal reference gene for Arabidopsis experiments, and *BnPP2AA3* was used for canola experiments. *PP2AA3*-specific

TaqMan probes were synthesized with FAM reporter dye at 5′ end and BHQ quencher dye at the 3′ end. Similarly, probes specific to target genes were also synthesized for expression quantification. The total qPCR reaction mix of 25 μl contained 2 μl of cDNA, 12.5 μl of 2× Qiagen Multiplex Master Mix, 1 μl of forward and reverse primers specific for each gene: TaqManF and TaqManR at 5 μM, and 1 μl of TaqMan probe (2 μM). qPCR was run on Rotor-Gene platform (Corbett, Mortlake, NSW, Australia) using 36-well rotor. PCR cycles were set to start with one holding at 95°C for 15 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. For each sample, three technique repeats and three biological repeats were analyzed with both probes of the reference gene and target gene. For data analysis, the C_t value for each sample was determined using the threshold line at which standard curve yields maximum amplification efficiency (>97%). Using the standard curve, the C_t value was converted to template cDNA concentration. Gene expression in a given sample was calculated as the template cDNA concentration of tested gene relative to that of *PP2AA3* in the same sample. Northern blot analysis was carried out using AtMYB68 cDNA as the probe for hybridization, and the blots were subsequently striped and reprobed with cDNA of Yellow Leaf Specific Gene 8 (YLS8, At5g08290) as the loading control.

Microscopy

Histochemical GUS staining was performed with various tissues of homozygous transgenic Arabidopsis and T2 transgenic canola along with their WT controls. The plant samples were incubated in the GUS stain solution (50 mM $NaPO_4$, pH 7.0, 10 mM EDTA; 0.1% v/v Triton X-100; 0.5 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-glucuronic acid) at 37°C for 16 h. The samples were fixed in 3.7% formaldehyde and 50% ethanol for 30 min before further chlorophyll clearing by incubation in 50–100% ethanol. Localization of the blue precipitate in these tissues was observed using a Leica MZ 12.5 stereo microscope (Wetzlar, Germany). Subcellular localization of AtMYB68 was determined by staining the roots of 1-week-old Arabidopsis seedlings of the 35S-GFP-AtMYB68 construct with 10 μM 4′,6-diamidino-2-phenylindole (DAPI) solution. The DAPI and GFP signals were visualized by fluorescence microscopy using a Carl Zeiss LSM510 confocal laser microscope (Zeiss, Munich, Germany).

ABA sensitivity test

Seeds (30–80 seeds/plate) were place on 0.5× Murashige and Skoog plates and chilled for 3 days with or without (+/−) ABA (Sigma, St. Louis, MO, USA). They were subsequently transferred to room temperature under continuous light for up to 2 weeks. Germination was scored by full penetration of a radical tip through the seed coat, and cotyledon and first pair of true leaves were recorded.

Heat stress treatment

Arabidopsis plants were grown under optimal conditions in a growth chamber until 3–4 days into flowering. At this stage, the last open flower was tagged on the main meristem of each plant to define the assessment region and then plants were exposed to heat stresses of various durations at 45°C. Plants were returned to optimal conditions to grow until maturity, and final seed yield was determined for both the heat-treated group and the optimal group. Canola plants were grown under optimal conditions in a growth chamber until 2 days into flowering. The last unfertilized flower was tagged to define the assessment region and then plants were exposed to 4 h at 40°C (including a 1-h ramp up from 22 to 40°C). Two weeks later the region of the raceme that was exposed to heat treatment was assessed for abortion by counting aborted siliques.

Drought stress treatment

Five Arabidopsis seedlings were grown under optimal conditions (watered daily) in each pre-weighed 3-inch pot that had the soil surface covered to prevent surface evaporation. At first open flower, pots were irrigated up to an optimal weight and further irrigation was stopped for 4 consecutive days. Each pot was weighed daily and at the end of the 4-day drought treatment, shoot biomass was harvested on the fourth day. Overall water loss relative to shoot dry weight was calculated. For canola experiments, plants were maintained in 6-inch pots under optimal growth conditions until the emergence of the fourth leaf. Before drought treatment, the pots were watered to a set weight and covered with foil to prevent surface evaporation. Water loss was monitored daily by weighing the pots and at the end of the drought period (day 7), plants were harvested to determine shoot dry weight.

Combined heat and drought treatment

Arabidopsis plants were grown under optimal conditions in a growth chamber until early flowering and the soil water content was maintained at either 30% (D1) or 40% (D2). The two groups of plants were then subjected to five consecutive days of treatment at 45°C treatments for either 1.25 h per day (H1) or 1.65 h per day (H2). The plants were then returned to optimal conditions until maturity for seed harvests.

Pollen viability test

Canola was grown under optimal condition until flowering. Pollens from flowers of different plants at the same developmental stage were harvested before a heat treatment of 2 h at 43°C, and 2 and 24 h after heat treatment. These pollens were transferred to pollen culture medium containing 20% sucrose, 300 ppm $CaCl_2$, 100 ppm H_3BO_3, 1 mM Tris–HCl pH7, 100 ppm KNO_3 and 0.7% agar as described (Roberts *et al.*, 1983). Germination and pollen tube elongation per 100 pollen grains in each sample were recorded.

Canola field trials

The single row canola trials were conducted in three locations in the summer of 2009 in Canada: one site in Taber (Alberta) and two sites in Okanagan Falls (British Columbia). Five independent, homozygous events of P81.1-*AtMyb68* construct along with the *DH12075* control were tested in these trials, which were arranged in a randomized complete block design with four replicates per entry. Each replicate consisted of a single row with 90 seeds. Standard farming practices were used with irrigation applied as needed and plots were harvested by hand.

Full-scale canola field trials of the same five independent events of P81.1-*AtMyb68* were first conducted in the summer of 2009 in two locations: Taber (Alberta) and Oliver (British Columbia). The trials were arranged in a randomized complete block design with six replicate plots per entry, and each replicated plot was 6.5 × 1.5 m in size containing 1800 seeds. Standard farming practices were used with irrigation applied as needed and plots harvested by direct combining. Temperatures and rainfalls at the field trial locations were recorded. The transgenic events along with DH12075 were further tested in three different locations in the fall of 2009 in Nancagua and Pirque, Chile. The trials of six replicates per entry were carried out with the same field design. The trial in

Nancagua received regular irrigation (approximately 40 mm/irrigation once a week for 20 weeks), whereas the trails in Pirque received regular as well as reduced irrigation during the flowering period (two less irrigations) and after flowering finished (three less irrigations), respectively. Maximum and minimum daily temperatures during the entire growth season in the field trial locations were recorded.

Statistical analysis

All measured parameters were analyzed statistically using JMP software (2007, JMP release 7; SAS Institute Inc., Cary, NC USA). Data for each parameter were checked for normality and equal variance and a one-way analysis of variance (ANOVA) was done only if the assumptions of normality and variance were met. Variations among transgenic plants and their controls subjected to different treatments were compared using ANOVA. Statistically significant differences between transgenic and control entries were determined using a Student *t*-test at levels of $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ for all indoor experiments, and $*P < 0.1$, $**P < 0.05$ and $***P < 0.01$ for all field trials. In the case of non-normality or unequal variance, data were checked for outliers, transformed or ranked to improve data. If assumptions of the ANOVA could not be satisfied, non-parametric tests were conducted, and in that case, comparisons were not possible.

AUTHOR CONTRIBUTIONS

JW and YH designed and supervised the overall study; JW, YH and PM prepared the manuscript with input from other authors; MD, MC, LT, YW, SY, XT, GT and RG contributed to the genetic, molecular and bioinformatics work; MK, AS, KJ and JY were responsible for the physiological assessments; HW managed and conducted the canola transformation; PM and DT provided technical advice.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the manuscript and its supporting materials.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Effect of heat stress on vegetative biomass accumulation of wild-type and 35S-*AtMYB68* transgenic Arabidopsis. Shoot dry weight (SDW) of 14-day-old seedlings of 4 homozygous transgenic lines of 35S-*AtMYB68* (lines 20, 22, 35 and 104) along with wild-type (WT) was measured after 3 consecutive days of heat stress treatment at 42°C for a total of 12 h (4 h day^{-1}). Relative accumulation of SDW for each entry was expressed in percentage of its SDW grown under optimal conditions. Error bars represent standard errors ($n = 8$).

Figure S2. Amino acid sequence comparison of AtMYB68 protein with five closely related members in the subgroup 14 of the Arabidopsis R2R3-MYB family. The sequence alignment was generated using the Clustal 2.1 program. Residues that are identical in all six sequences are marked with an asterisk, and conservative residues with strongly similar properties are marked with a colon, and the ones with weak similarity are marked with a dot. Hydrophobic, acidic, basic and other amino acid residues are in red, blue, magenta and green fonts, respectively. R2 and R3 MYB domains are shown.

Figure S3. Amino acid sequence comparison of AtMYB68 protein with putative orthologs identified from *B. napus, Gossypium raimondii, Glycine max, Brachypodium distachyon, Sorghum bicolor, Zea mays* and *Oryza sativa*, respectively. The sequence alignment was generated using the Clustal 2.1 program. Residues that are identical in all eight sequences are marked with an asterisk, and conservative residues with strongly similar properties are marked with a colon, and the ones with weak similarity are marked with a dot. Hydrophobic, acidic, basic and other amino acid residues are in red, blue, magenta and green fonts, respectively. R2 and R3 MYB domains are shown.

Figure S4. Histochemical staining of GUS in various T2 transgenic canola tissues of the P*81.1-AtMYB68* construct at 22°C and 40°C.

Figure S5. Phenotypic analysis of heterozygous 35S-*AtMYB68* transgenic canola under heat and water stresses. (a) Seed set of transgenic line 3 in comparison with the DH12075 control (DH), photographed 2 weeks after heat stress treatment of 4 h at 40°C at early flowering. (b) Water loss during the 7 days of water-withholding treatment divided by shoot dry weight (SDW) in DH12075 (DH) and two transgenic lines (lines 3 and 4). Plants were maintained in 6-inch pots under optimal growth conditions until the emergency of the 4th leaf before subjecting to the water deficit treatment. Error bars represent standard errors ($n = 8$), and three asterisks above a bar indicated significant difference between the transgenic line and DH ($***P < 0.001$).

Figure S6. Temperature records at the canola field trial sites. (a) Recorded daily maximum temperature during flowering and seed filling period at Taber (Alberta); (b) Recorded daily maximum temperature during flowering and seed filling period at Oliver, Okanagan Falls (British Columbia); (c) Recorded daily minimum and maximum temperatures during the entire growth season at Nancagua (Chile); (d) Recorded daily minimum and maximum temperatures during the entire growth season at (Pirque, Chile).

Table S1. Normalized expression levels of *AtMyb68* in shoots and roots at different time points post drought and heat treatments. Data were extracted from the Arabidopsis eFP Browser at http://bar.utoronto.ca/ (Toufighi *et al.*, 2005).

Table S2. Seasonal normal and actual average temperatures and total rainfalls at the Canadian field trial sites over the growth season.

Table S3. Agronomic parameters of P*81.1-AtMYB68* transgenic canola in comparison with the DH12075 control in field trials of 4 different locations (Taber, Oliver, Nancagua, and Pirque). Identified significant differences between the transgenic lines and DH12075 are indicated by asterisk (Student *t*-test at $*P < 0.1$).

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