Forward Genetic Stomatal CO_2 Response Screen in Grass *Brachypodium distachyon* Reveals Central Function of a MAP Kinase in Early Stomatal CO_2 Signal Transduction

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BNKL Development of methods, plant growth, forward genetic screens, plant crosses, molecular and physiological analyses, data interpretation, writing; PHOC Conceptualization, establishment of forward genetic screen pipeline, conduct screen and mutant isolation, development of methods, gas exchange analysis, data interpretation, co-supervision; BNKL and PHOC contributed equally to this study. FR, MS & ES Plant growth, support with forward genetic screens; KK Bulk segregant analysis; YT Protein phosphorylation analyses; LZ Plant crosses; RS & DL NaN line generation; CS & JAM Structural modeling; DPW & JV Establishment of EMS lines, seed bank establishment; DPW crossing assistance; JIS Conceptualization, method design and planning, data discussion and interpretation, supervision, funding acquisition, writing.

Abstract

Plants respond to increased CO2 concentrations through rapid stomatal closure which can contribute to increased water use efficiency. Grasses display faster stomatal responses than dicots due to dumbbell-shaped guard cells flanked by subsidiary cells working in opposition. However, forward genetic screening for stomatal CO2 signaling mutants in grasses has not been reported. The grass model Brachypodium distachyon is closely related to agronomically important cereal crops, sharing largely collinear genomes. To gain insights into CO2 control mechanisms of stomatal movements in grasses, we developed a forward genetics screen on an EMS-mutagenized Brachypodium distachyon M5 population using infrared imaging to identify plants with altered canopy leaf temperature at elevated CO2. A "chill1" mutant was isolated, exhibiting consistently cooler leaf temperatures than wildtype control plants after exposure to increased [CO2]. chill1 plants showed strongly impaired high CO2-induced stomatal closure, despite retaining a robust response to abscisic acid. Through bulked segregant whole-genome-sequencing analyses and generation and characterization of CRISPR-cas9 mutants, chill1 was mapped to a protein kinase, BdMPK5. The chill1 mutation impaired bdmpk5 protein-mediated CO2/HCO3- sensing in-vitro consistent with AlphaFold2-directed structural modeling. BdMPK5 is a key signaling component involved in CO2-induced stomatal movements, potentially functioning as a component of the CO2 sensor in grasses.

Keywords:

ABA, Bicarbonate sensor, Carbon dioxide, CO_2 sensing, MAP kinase, Graminacea, Guard cell, Stomata

Introduction

The atmospheric CO_2 concentration has continuously increased since the industrial revolution and has long since surpassed the highest levels recorded in human history; continuing to rise at a high rate (Headquarters, 2014; Stocker et al., 2013). The rise in CO_2 levels will have a profound impact on the growth and development of plants, including crops. Some models predict a net increase in plant growth worldwide due to effects of CO_2 fertilization under well-watered conditions (Zhu et al., 2016). However, crop productivity is negatively impacted by rising CO_2 concentrations as it increases the risk of frequency, duration, and intensity of heat waves due to the heat-trapping nature of greenhouse emissions (Asseng et al., 2015; Wahid et al., 2007, Zheng et al., 2019). Drought stress is expected to increase in both occurrence and severity in response to climate change (Sherrard & Maherali, 2006). Abiotic stressors such as salinity and drought are estimated to contribute to over 50% of agricultural yield losses globally (Ashraf et al., 2012). These circumstances highlight the need for an increased understanding of how water use efficiency (WUE) of important cereal crops is regulated.

Stomatal pores adjust in response to environmental conditions to regulate CO_2 uptake and water loss. CO_2 induced reduction in stomatal apertures can contribute to an increase in water use efficiency in grasses by limiting transpiration, while maintaining CO_2 assimilation (Allen et al., 2011, De Souza et al., 2008; Wang et al., 2015). Stomata are present on leaf surfaces and facilitate gas exchange in response to diverse stimuli (Bertolino et al., 2019; Hetherington & Woodward, 2003; Scavo et al., 2018). Stomata are composed of a pair of guard cells regulating stomatal movements by adjusting relative turgor pressure of the guard cells (Willmer and Fricker, 1996). Turgor pressure is mediated by water and ion movement across guard cell membranes (Kanemoto et al. 2009; Xu et al. 2016; Zhang et al., 2018). When exposed to high CO_2 levels, stomatal pores close, leading to a reduced stomatal

conductance and limited evapotranspiration, resulting in an increase in canopy leaf temperature (Hetherington and Woodward 2003; Harrison et al. 2020; Xie et al., 2006; Zhang et al., 2018).

In darkness, respiration causes an increase in leaf CO_2 concentrations, which leads to stomatal closure in C3 and C4 plants (Zhang et al, 2018). Stomatal closure induced by elevated CO_2 levels is initiated as CO_2 enters guard cells and is converted into HCO_3- (bicarbonate) by carbonic anhydrases (Hu et al., 2010; Hu et al., 2015; Kolbe et al,. 2018; Qiao et al., 2014; Wang et al., 2015; Zhang et al., 2018). MAP kinases have been shown to play an important role in CO_2-induced stomatal closing in eudicots (Marten et al., 2008; Jakobson et al., 2016; Toldsepp et al., 2018; Takahashi et al., 2022). Increased CO_2 is detected by Mitogen Activated Protein Kinase 4 and 12 (MPK4/12) with the High Temperature 1 (HT1) Raf-like protein kinase which in combination serve as stomatal CO_2 sensor in *A. thaliana* (Takahashi et al., 2022). High CO_2 -induced interaction of MPK4/12 with HT1 causes inhibition of the HT1 protein kinase activity, preventing downstream activation of CBC1/2 (Convergence of Blue Light and CO_2) protein kinases (Takahashi et al., 2022). Thus, high CO_2 is modeled to prevent CBC1/2 and HT1 from inhibiting S-type anion channels, which contribute to stomatal closure (Hiyama et al., 2017, Takahashi et al., 2022). Conversely, active HT1 protein kinase leads to downstream activation of the CBC1 protein kinase, which leads to initiation of stomatal opening mechanisms (Hiyama et al., 2017).

Monocots possess stomata composed of dumbbell-shaped guard cells flanked by functional subsidiary cells unlike the kidney-shaped guard cells found in eudicots. This difference is thought to account for the faster stomatal response observed in monocots when exposed to stimuli (Fahad et al. 2017; Hetherington and Woodward 2003; Lawson and Vialet-Chabrand, 2019; Raschke and Fellows 1971). However, forward genetic screens for stomatal

movements have been difficult to perform in grasses including cereal crops; and to date no forward genetic screens have been reported on the stomatal CO_2 responses in grasses.

Brachypodium distachyon is emerging as a model organism for monocots as opposed to the widely investigated eudicot model *Arabidopsis thaliana*, which has been shown to have very limited genomic collinearity to staple cereal crops (Brkljacic et al., 2011; Keller & Feuillet, 2000; Scholthof et al., 2018; Vogel and Bragg., 2009). In contrast to *A. thaliana*, *B. distachyon* is much more closely related to cereal crops with a high degree of evolutionary collinearity (Hasterok et al., 2022; Sun et al., 2017). *B. distachyon* compared to other monocotyledonous models has a smaller size, compact genome, and short lifespan; making it favorable for high throughput screening (Brkljacic et al. 2011; Huo et al., 2009; Raissig and Woods, 2022; Scholthof et al. 2018).

In the present study, we screened over 1000 M5 generation EMS-mutagenized *B. distachyon* lines for impairments in the stomatal CO_2 response. We report isolation of a mutant, *chill1*, that strongly impairs high CO_2-induced stomatal closure and show that this mutant on average has a higher stomatal conductance and a disrupted response to increased CO_2 while retaining robust abscisic acid (ABA)-induced stomatal closing. Data suggest that *chill1* encodes a central component of CO_2 regulation of stomatal movements in grasses.

Results:

Rapid stomatal closure is induced by exposure to elevated CO_2, reducing stomatal conductance and evapotranspiration. This in turn causes higher leaf temperatures which can be measured through infrared thermal imaging (Mustilli et al., 2002; Hauser et al., 2019). Infrared imaging was applied to a population of individually stored EMS mutagenized M5 generation *B. distachyon* to screen for plants with reduced sensitivity to elevated CO_2 by selecting plants appearing cooler than the wildtype control (Bd21-3).

Through an infrared leaf thermal imaging screen conducted using high and low CO_2 concentrations (approximately 900ppm and 150ppm) of 1,075 individually stored M5 generation EMS mutagenized *B. distachyon* lines, we isolated 50 putative "chill" mutants with leaf temperatures cooler than the WT parent (Bd21-3). After re-screening five plants from each of the individually stored M5 seed stock in a secondary screen, 28 of the 50 putative mutants showed cool leaves at elevated CO_2 (900 ppm). In a tertiary screen in the following (M6) generation, 7 of the 28 mutants could not be confirmed as markedly different from WT controls through infrared imaging analyses and were removed as candidates. The remaining 21 mutants were then named the *chill* mutant lines, and reconfirmation for these 21 mutants was pursued by rescreening in the M6 generation.

Of the mutants identified in this screen, we initially focused on a mutant that we have named *chill1* (Figure 1A), which consistently and reliably appears cooler than WT (Bd 21-3) controls in infrared thermal imaging following exposure to increased CO_2 (900ppm) (Figure 1A). To determine whether the difference observed in canopy leaf temperature was related to stomatal development or stomatal movements, stomatal index, and density analyses were conducted comparing *chill1* to the parental WT control (Bd21-3). No significant difference could be observed in stomatal index and density (Figure 1B and 1C).

Stomatal conductance analyses were pursued (Figure 1D) wherein *chill1* shows a dramatically reduced response to elevated CO_2 when compared to WT controls. We next investigated stomatal closure in response to abscisic acid. Interestingly, *chill1* leaves responded similarly to WT following the addition of 2 μM abscisic acid to the transpiration stream of 5-week-old leaves (Figure 1E).

To determine inheritance patterns, *chill1* plants were backcrossed to the parental strain Bd21-3 (see Methods). The F1 *chill1* x Bd21-3 population was initially analyzed by infrared

thermal imaging experiments following exposure to increased (1000 ppm) CO_2. F1 crossed *chill1* X Bd21-3 plants exhibited a phenotype more similar to that of WT (Bd21-3 controls) (Figure 2A). These data were consistent across four individual *chill1* x Bd21-3 F1 plants. F1 plants were also analyzed in stomatal conductance analyses to quantify real time responses to shifts in CO_2 concentrations. The F1 backcrossed plants exhibited a WT-like response to high CO_2 (900 ppm) consistently across three biological replicates (Figure 2B).

Tillers were created from these F1 plants (O'Connor et al. 2017; Woods and Amasino, 2015) to generate large numbers of seeds for an F2 mapping population (Figure S1). The 4-week-old F2 mapping population of approximately 560 individual F2 plants was screened following 1000 ppm CO_2 exposure. Screening was done with a maximum of three F2 plants alongside a WT plant (Bd21-3) as a control for all plants that were screened (Figure 3). In an initial analysis of all infrared thermal images of F2 plants, 116 of approximately 560 plants screened showed a *chill1*-like phenotype (21%). Analyses of F1 and F2 generations suggest that the *chill1* phenotype is due to a single locus recessive mutation. All images were also independently reanalyzed by three laboratory members in a very conservative manner to reduce false positive selection for bioinformatic mapping analyses. Of the plants screened, 57 plants (10%) were individually selected by all members as clearly showing a *chill1*-like phenotype and 50 plants (9%) were selected to be strongly wildtype-like to be used as a point of comparison in bioinformatic analyses. The 57 plants selected as *chill1*-like were named M-pool (mutant pool) and were screened a second time for those which exhibited the strongest phenotype. A smaller mutant pool of 25 individuals with the strongest *chill* leaf canopy phenotype was named SM-pool (Strongest Mutant Pool).

DNA from F2 plants selected as *chill1*-like or WT-like was pooled and submitted for whole genome sequencing (WGS) to be utilized in Bulked Segregant Analyses (BSA) (Michelmore and Kesseli; 1991). Quality checks were performed on sequence reads before

use in further analyses (Figure S2). QTLseqr (Mansfeld and Grumet, 2018) was used to analyze files and generate visual representations of the -$\log_{10}$(p-value) derived from G' values (Magwene et al 2011) (Figure 4) (see Methods), which are generated based on SNPs called during analysis (Takagi et al. 2013). BSA analysis of both the mutant (M)-pool and the SM-pool show likely heterozygous and homozygous variants on chromosome 3 (Bd3) with some smaller potential peaks on other chromosomes (Figure 4). Most of the called variants were heterozygous. We filtered the variant data for homozygous variants and compared the called variants in the M-pool and SM-pool vs. the wildtype-like pool. Called homozygous variants, from short read WGS in both the M-pool and SM-pool were analyzed for variants predicted to cause impactful mutations, were compiled into a single list via SnpEff and QTLseqR (Cingolani et al., 2012; Mansfeld and Grumet, 2018). Heterozygous variants were excluded from further analyses on the basis that *chill1* behaves like a recessive mutant as determined by the F1 generation phenotype and F2 generation segregation. After variants were filtered for homozygosity four variants were called for the mutant (M) pool and three for the strongest mutant (SM) pool. Variants in two genes (BdiBd21-3.3G0222500 and 3G0296900) were called in both pools leading to a total compiled list of five possible candidate genes (Figure 5A).

To reduce the number of potential candidates, additional *chill1* F2 plants were screened and those selected as cooler than WT controls were backcrossed to WT Bd21-3 a second time. Plants were taken into the F4 generation and were rescreened by infrared thermal imaging. DNA was extracted from those plants selected individually by two different laboratory members to be cooler than WT (Bd21-3) controls. DNA from selected F4 plants was used to confirm the presence of called variants via Sanger sequencing. Of the remaining five candidate genes, four were excluded from further analyses based on the absence of the called variant or heterozygosity at the called loci, confirmed via Sanger sequencing. The only

candidate which could not be excluded was in the BdiBd21-3.3G0222500 gene. The called variant in exon 2 was confirmed by Sanger sequencing and found to be homozygous in 11 individually analyzed *chill1*-like F4 plants but was not found in three WT (Bd21-3) controls nor in a F4 plant selected as WT-like (Figure 5B). These analyses suggested that the variant in BdiBd21-3.3G0222500 may be responsible for the *chill1* phenotype.

To further test this hypothesis, we searched the Phytozome (phytozome-next.jgi.doe.gov) database for sequence-indexed sodium azide (NaN) mutagenized lines (Dalmais et al, 2013) predicted to contain a mutation in BdiBd21-3.3G0222500. The NaN0770 line was obtained and was sequenced to confirm the presence of the predicted T to C point mutation sustained at position 14916843 on chromosome Bd3 via Sanger sequencing. NaN0770 plants in which the predicted mutation was homozygous and confirmed were analyzed by infrared thermal imaging alongside the original *chill1* mutant allele, WT controls and F1 crossed plants of the NaN0770 line and WT Bd 21-3 plants (Figure 6A and 6B). Infrared imaging of the NaN0770 line showed canopy temperatures that were more similar to *chill1* than to WT control plants (Figure 6A). However, the NaN0770 x wildtype F1 backcrossed line appeared more similar to WT controls (Figure 6A). NaN0770 was also used in an allelism cross with *chill1* wherein the F1 cross appeared cooler than WT (Figure 6C and 6D). Canopy temperatures in the NaN0770 line and in the F1 cross to *chill1* were cooler than WT (Bd 21-3) controls but less pronounced than *chill1*. These data indicated that the NaN0770 line has a less severe mutant phenotype compared to *chill1* (Figure 6C and D).

Taken together, the above data provide evidence that the *chill1* variant in BdiBd21-3.3G0222500 may be responsible for the cool leaf canopy temperature. To further test this hypothesis, CRISPR-cas9 lines were generated using a guide RNA targeting BdiBd21-3.3G0222500, which lies in a gene annotated as *BdMPK5* (Figure 7A). *BdMPK5* was amplified in both WT and CRISPR-cas9 lines and aligned for comparison to determine the

presence of mutations sustained within the gene of interest leading to identification of individual CRISPR alleles in in *BdiBd21-3.3G0222500*. Individual T2 generation CRISPR plants were also genotyped to detect the presence or absence of cas9. T2 CRISPR plants were additionally genotyped to confirm mutations sustained within *BdMPK5*. Only plants which were confirmed to be cas9-free (Figure S3) and to have sustained predicted impactful mutations in *BdMPK5* were taken into the following generation. Two independent CRISPR alleles were isolated and confirmed via Sanger Sequencing (Figure 7B; CRISPR #1 and CRISPR #2).

Stomatal conductance analyses were pursued in T3 CRISPR plants wherein both alleles exhibited a constitutively high stomatal conductance which did not decrease in response to shifts to high CO_2 conditions (800 ppm) (Figure 8A). In further stomatal conductance analyses, 2 μM ABA was added to the transpiration stream and a steep decline in conductance was observed in T3 CRISPR lines (Figure 8B). Genotype blinded stomatal index and density analyses were pursued for these lines, and while the average stomatal density of CRISPR #1 line appeared lower when compared to WT parent controls, a significant difference could not be discerned with a significance cutoff of $p \leq 0.05$ for a 95% confidence interval (Figure 8C and 8D).

Blast analysis against *A. thaliana* genes suggested that the mapped BdiBd21-3.3G0222500 (*BdMPK5*) gene encodes a close *B. distachyon* ortholog of the *A. thaliana* AtMPK4 and AtMPK12 proteins (AT4G01370 and AT2G46070 respectively). A recent study showed that the *A. thaliana* AtMPK12 and AtMPK4 proteins function as part of the primary CO_2/bicarbonate sensor together with the HT1 protein kinase, that mediates CO_2 control of stomatal movements (Takahashi et al., 2022). Recent findings showed that HT1 activates the downstream protein kinase CBC1 by phosphorylating CBC1 at defined residues (Takahashi et al., 2022). Furthermore, this activation of CBC1 is inhibited by elevated CO_2/bicarbonate

only when HT1, CBC1 and MPK4 or MPK12 are included in the reconstituted reaction (Takahashi et al., 2022). *In-vitro* protein kinase assays were pursued using the recombinant BdMPK5 protein to investigate whether BdMPK5 shows a CO_2 response together with HT1 and CBC1. HT1 and CBC1 together with the BdMPK5 protein showed a high CO_2/bicarbonate-induced down-regulation of the CBC1 protein kinase activity (Figure 9A, Figure S4; n = 2). The high CO_2/bicarbonate response was similar to high CO_2 responses found for AtMPK4 and AtMPK12, but not for the close homolog AtMPK11 (Takahashi et al., 2022).

We next investigated whether the BdMPK5-D90N variant identified in *chill1* has an effect on CO_2-mediated downregulation of CBC1 phosphorylation. The BdMPK5-D90N variant protein abrogated the high CO_2/bicarbonate-mediated downregulation of CBC1 phosphorylation (Figure 9A, Figure S4; n = 2). These analyses showed that BdMPK5 can replace AtMPK4 and AtMPK12 in the reconstitution of this CO_2 response and that the *chill1* variant in BdMPK5 cannot mediate downstream regulation of CBC1 protein phosphorylation (Figure 9A).

To gain insight into the mechanism by which the BdMPK5-D90N protein disrupts CO_2-induced stomatal closing *in vivo* (Figures 1,3, 8) and reconstitution of the CO_2 sensing core *in vitro* (Figure 9A), we pursued AlphaFold2 modeling (Jumper et al., 2021; Evans et al., 2022) to predict the BdMPK5–HT1 protein complex structure (Figure 9B), using methods previously developed from the *A. thaliana* MPK12/MPK4–HT1 sensor complex (Takahashi et al., 2022). Apart from the flexible loop regions, the predicted HT1 structure of the mutant BdMPK5-HT1 complex, with the BdMPK5-D90N mutation, is similar to HT1 in the MPK12/MPK4–HT1 complexes predicted previously (Takahashi et al, 2022), displaying the reproducibility of these methods. Interestingly, the mutant residue BdMPK5-D90N is predicted to lie directly at the interaction surface of BdMPK5 and HT1 (Figure 9B).

DISCUSSION

CO_2 regulated stomatal closure can increase water use efficiency (WUE) in grasses by reducing water loss through transpiration (Allen et al., 2011; De Souza et al., 2008; Wang et al., 2015). However, to our knowledge, no forward genetic screens of stomatal responses to increased CO_2 in grasses have been reported to date. In the present study, we have developed an unbiased forward genetic screen in the grass *B. distachyon* to identify mutants showing altered canopy leaf temperature under elevated CO_2. Mutant lines were isolated and confirmed throughout three individual stages of infrared screening. Subsequently isolated mutants were screened using real-time stomatal conductance assays. The *chill1* mutant was then taken into the following (M6) generation and investigated in blinded stomatal index and density analyses which showed that the impairments observed were due to stomatal movement responses as opposed to stomatal development. Bulked segregant analyses, resequencing of candidate genes in individual segregating F4 plants, and analysis of CRISPR/cas9-generated *bdmpk5* alleles further confirmed mapping of the mutant locus *chill1* to the *BdMPK5* gene.

Although *A. thaliana* is the most widely used eudicot model organism, *B. distachyon* is an emerging model organism for monocots due to the high evolutionary collinearity to important cereal crops such as wheat (Brkljacic et al., 2011, Scholthof et al., 2018, Vogel et al., 2010). Some components of the CO_2 signaling cascade appear to be conserved across monocots and eudicots such as β−carbonic anhydrases which have been documented to perform similar functions in stomatal CO_2 responses in *A. thaliana*, rice, and maize (Chen et al., 2017; Hu et al., 2010; Hu et al., 2015; Kolbe et al,. 2018) and the functions of the SLAC1 anion channel in CO_2 regulation of stomatal conductance in *A. thaliana* and rice (Kusumi et al., 2012; Negi et al., 2008; Vahisalu et al., 2008). However, stomatal CO_2 sensing mechanisms and the underlying signal transduction pathway remain unknown in grasses. A

recent study identified AtMPK4/12 (Des Marais et al., 2014; Jakobson et al, 2016; Tõldsepp et al., 2018) and AtHT1 (Hashimoto et al., 2006) as the two components that together function as primary biosensor for stomatal CO_2 signal transduction controlling stomatal movements in *A. thaliana* (Takahashi et al., 2022). Our protein sequence analyses show that BdMPK5 is the closest homologue to AtMPK4 and AtMPK12 in *A. thaliana*, further suggesting a level of conservation between CO_2 signaling in both species. This hypothesis is further supported by findings that BdMPK5 could replace AtMPK4/12 in *in-vitro* reconstitution of the "MPK-HT1-CBC1" CO_2 sensing core (Figure 9A, Figure S4). Interestingly, the *chill1* mutant was mapped to a SNP that causes the BdMPK5- D90N variant. AlphaFold2 modeling predicts that this variant lies at the interface of BdMPK5 and HT1 (Figure 9B), which may contribute to the observed disruption of CO_2 signaling. Considering that AlphaFold2 has sometimes been able to correctly predict the effects of point mutations in some cases (Ivanov et al., 2022; Keskin Karakoyun et al., 2023; Wayment-Stelle et al., 2022; McBride et al., 2023) but not in others (Buel and Walters, 2022; Pak et al., 2023; Zhang et al., 2021) these results should be treated with caution and will require future structural resolution.

Grass stomata are composed of guard cells flanked by lateral subsidiary cells which shrink as turgor increases within guard cells (Franks and Farquhar., 2007; Lawson and Vialet-Chabrand, 2019; McAusland et al., 2016; Nunes et al; 2020; Raschke and Fellows, 1971). This arrangement has been shown to contribute to the increased speed at which grass stomata are able to respond to stimuli, thereby improving WUE (Des Marais et al., 2016; Franks and Farquhar., 2007; Lawson and Vialet-Chabrand, 2019; McAusland et al., 2016; Nunes et al; 2020, Raissig et al., 2017). High CO_2 triggers stomatal closure facilitated by ionic efflux out of guard cells leading to a loss of turgor pressure, while subsidiary cells function in direct opposition and increase turgor pressure (Raschke and Fellows; 1971) assisting in the speed

and effectivity of stomatal responses (Dubeaux et al., 2021; Franks and Farquhar., 2007; Lawson and Vialet-Chabrand, 2019; McAusland et al., 2016; Raschke 1972).

Brachypodium distachyon has a high level of evolutionary similarity to other monocot model species, including maize, wherein the closest homologue, *ZmMPK12*, has been predicted to be expressed in both guard cells and subsidiary cells, based on leaf single cell transcriptomics (Sun et al., 2022). The same study indicates that transcripts of both of the most closely related orthologues of *AtHT1* were expressed in maize guard cells but may not be clearly expressed in subsidiary cells (Sun et al,. 2022). Due to the increased collinearity between maize and *B. distachyon* it is possible that the expression patterns may resemble that of maize. Future protein expression studies of BdMPK5 as well as FRET-FLIM analyses in *B. distachyon* will be needed to determine potential CO_2-dependent interactions between BdMPK5 and BdHT1. This gives rise to the questions for future studies (1) whether BdHT1 is present only in *B. distachyon* guard cells or in both guard and subsidiary cell types. (2) Does BdMPK5 contribute to the CO_2 response in both guard cells and subsidiary cells or only in guard cells?

In summary, development of a forward genetics CO_2 response screen in *B. distachyon* has led to isolation of stomatal CO_2 response mutants. The *chill1* mutant is strongly impaired in stomatal CO_2 responses but shows robust abscisic acid-induced stomatal closure. Mapping of the *chill1* mutant to the *BdMPK5* gene, generation, and phenotyping of *BdMPK5* CRISPR-cas9 alleles and *in-vitro* reconstitution of the CO_2 sensing core with BdMPK5 suggest that *chill1* encodes a component of the CO_2 sensor in grasses. Future mapping of additional mutants and characterization of BdMPK5 in guard cells and subsidiary cells will further dissect stomatal CO_2 sensing and signaling mechanisms in grasses.

Methods:

Plant Growth Conditions:

The M5 and subsequent generations of an Ethyl methyl sulfonate (EMS) mutagenized *Brachypodium distachyon* seed library obtained from the Joint Genome Institute (JGI) were grown alongside the wildtype parent control (Bd21-3). All seeds were cold-treated for a minimum of 5 days at 4°C prior to sowing. Seeds were sown in soil containing a mixture of perlite, vermiculite, and Osmocote™ following manufacturer's instructions (3:1:1 soil: perlite: vermiculite). Trays were covered with transparent domes which were removed 7 days following emergence of first true leaves. Plants were grown under ambient CO_2 concentrations and 16/8 light/dark conditions under a minimum 200 $\mu E\ m^{-2}\ s^{-1}$ light intensity which has been shown to improve growth conditions. Sequence-indexed sodium azide (NaN) mutagenized lines were grown under the same conditions alongside appropriate controls and the wildtype Bd21-3 parent line.

Infrared Thermal Imaging Analyses:

Four to five-week-old well-watered *B. distachyon* plants were imaged using an infrared FLIR Thermal Imaging camera T650sc (FLIR Systems, Inc. Wilsonville, OR 97070 USA). Ambient CO_2 imaging was performed at approximately 450 ppm CO_2 within a plant growth room. Infrared thermal imaging at elevated CO_2 was conducted at a minimum of 900 ppm by placing plants within Percival, E-36HO high CO_2 chambers. Imaging was conducted immediately following removal of plants from high CO_2 chambers before plants were able to equilibrate. Plants were removed from the chamber in small, staggered batches and arranged in groups containing wildtype (Bd21-3) for comparative analysis. All captured infrared and corresponding parallel bright field images were analyzed individually off-line by at least two laboratory members to reduce bias.

Stomatal Conductance Analyses:

Healthy five to six-week-old plants were selected for gas exchange analyses to measure stomatal conductance (gs) using Licor gas exchange analyzers (LI-6800, LI-COR, Lincoln, NE, USA, or LI-6400XT, LI-COR, Lincoln, NE, USA). Prior to analysis, 4 leaves were bound together using micropore tape (3M), abaxial side downwards (Ceciliato et al., 2019). Leaves were allowed to equilibrate within the analyzer for 1 hour under ambient CO_2 of 400 ppm, 65% humidity, with a light intensity of 250 µmol m^{-2} s^{-1}. Data are representative of average data of 3 experiments each using 4 leaves per experiment (minimum total =12 leaves per genotype).

Microscopy:

Stomatal imaging was performed by creating an imprint of the 4th true leaf from healthy five to six-week-old plants. Leaves were glued onto slides using quick dry super glue to mount the abaxial side to the slide which was then peeled off approximately 45 minutes later once the glue was dry. The resulting imprint was imaged via a compound fluorescence microscope and attached camera. A total of four images were taken per leaf at 40x magnification with 3 biological replicates per experiment for a total of 12 images per genotype. Stomatal imaging was done by capturing 2 images above the main vein and 2 below to account for uneven dispersal of stomata. Counting of stomata and pavement cells was done using the "cell-counter" function of ImageJ. Confocal imaging was done on the 4th true leaf after leaves were exposed to a fixing solution of 1:5 35% formaldehyde: ethanol for <2 hours (Sultana et al., 2021). Leaves were then rinsed and stained with 10 µg/mL propidium iodide (PI) for a minimum of 5 minutes before leaves were mounted on slides with water to be imaged using a Leica Confocal Microscope (Raissig 2017). Stomatal apertures were measured manually using FIJI software.

Brachypodium distachyon Crossing:

Crossing *B. distachyon* plants was performed over a 2-day period in five to six-week-old plants. Individual spikelets containing mature stigma but that had not yet developed mature anthers were used in day 1 and each spikelet was then emasculated. Flowers were taped at the base of the flower with micropore tape (3M) and both anthers were removed using fine pointed forceps without damaging stigma or rupturing anthers. All other flowers were removed from the inflorescence following emasculation. The following day mature anthers were harvested and placed onto a slide inside of a closed plate with a wet paper towel to increase humidity and promote dehiscence. Only anthers that dehisce without manual rupturing are to be used for pollination and were applied to stigma of the emasculated plants from the previous day that did not show any signs of wilting. Plants were then returned to the growth room and kept under well-watered conditions. Seed development should be visible after approximately 4 days.

Generation of Tillers & Growth Conditions:

F1 progeny generated by backcrossing to WT (Bd21-3) were grown under short day (8 hours light/16 hours dark) to promote large growth of plants and aerial root formation. Once aerial root formation could be observed, tillers were created by using a razor blade to make a cut below the node from which the roots grew (Supplemental Figure 1). Tillers were immediately placed into Falcon Tubes containing a mix of water and 1X fertilizing solution (Technigro ®, Agawam, MA, USA) and grown under short day conditions (8 hours light/16 hours dark). After a minimum of 7 days, cuts were transferred to soil and remained covered with domes to maintain increased humidity for approximately 2 weeks (Supplemental Figure 1). Once plants were established in soil, domes were removed, and light conditions were switched to long day (22 hours light/2 hours dark) which promotes flowering. F1 tiller populations were allowed to self-cross. F2 seeds were harvested and grown in staggered batches under short day to be used for infrared thermal imaging and subsequent DNA extraction.

F2 Selection

Four to five-week-old F2 *chill 1* lines backcrossed with the Bd21-3 parent line were analyzed by infrared thermal imaging alongside Bd 21-3 (wildtype) plants following exposure to 1000 ppm CO_2 for 2 hours. Approximately 560 individual plants were screened and images were analyzed independently by three persons. Plants were sorted based on selections made by laboratory members and were placed into groups to keep only those appearing to have the most robust phenotype. F2 plants selected independently by all 3 persons as showing wildtype-like canopy temperature formed the "wildtype-pool" to be used for comparison to plants with *chill1-like* phenotypes for Bulked Segregant Analyses (BSA) (Michelmore and Kesseli; 1991). To reduce selection of false positive chill1-like plants all images were screened in a very conservative manner by three independent lab members. Only plants that were selected to be clearly *chill1*-like by all 3 persons would become the "M" or 'mutant' pool. A subset of this pool (SM-pool) selected to be the strongest phenotypically was created by one laboratory member by reanalyzing only those plants which had already been designated as *chill1*- like by all three persons after individual analyses had been compiled. DNA was extracted from all selected plants using the DNeasy Plant Min Kit (Qiagen, 2016), pooled, and sent out for sequencing via Novogene (Davis, CA).

Bulked Segregant Analyses

Sequence data files obtained from Novogene short read whole genome sequencing were first run through quality checks (Ewing and Green, 1998) before proceeding with analysis and all pools were aligned to a *B. distachyon* reference sequence obtained via Phytozome (https://phytozome-next.jgi.doe.gov/). Sequences were converted from SAM file format to BAM before being sorted and indexed using samtools (Li et al, 2009). GATK (Van der Auwera and O'Connor, 2020) was then used to mark duplicate sequences, add headers to

columns, as well create a BAM index. Before proceeding the reference genome also needed to be prepared before it could be utilized within GATK. Following this, variants were called using the haplotypecaller function of GATK for both M and SM pools and SNPs and indels were separated into 2 separate files for each pool. VariantFiltration was used on all four generated files which were then exported using VariantsToTable to be used in QTLseqr. Variants were annotated using snpEff. The R package QTLseqr was used to import SNP data from GATK, verify total read depth, check reference allele frequency and the SNP-index as well as filter SNPs. QTLseqr was also used for running the QTLseq analysis as well as the G' analysis and generating plots and producing summary tables (Takagi et al, 2013; Magwene et al, 2011).

Creation of CRISPR Lines

CRISPR lines were designed to induce small deletions in BdMPK5 by generating gRNA with the following oligos: Chill1_CRISPR_Fw (ACTTGTATCAACGTGCAGACTCGCG) and Chill1_CRISPR_Rv (AAACCGCGAGTCTGCACGTTGATAC). Oligos were phosphorylated and utilized in a Golden Gate Reaction to be ligated into the CRISPR destination vector JD633 (Addgene). JD633-*chill1* plasmid was used to transform *E. coli* (TOP10) and screened with kanamycin containing media. The destination vector JD633 contains hygromycin resistance as required for transformation by the Boyce Thompson Institute (BTI). Assembled and sequence confirmed plasmids were sent to BTI for transformation in the Bd 21-3 parent background. Lines were screened on plates for hygromycin resistance before shipping. Lines received from BTI were screened again via imaging using the FLIR infrared thermal imaging camera as well as by genotyping to confirm the presence of mutations in the target site. Lines were also genotyped in T2 to confirm the absence of active cas9 to prevent further sustained off target mutations. All sequencing was done via Sanger Sequencing (Retrogen, San Diego, CA).

In vitro phosphorylation assays

In-vitro phosphorylation assays were performed as described previously (Takahashi et al. 2022). In brief, 0.5 µg GST-AtCBC1, 0.01 µg His-AtHT1 and 0.5 µg His-BdMPK5 or His-BdMPK5(D90N) recombinant proteins were incubated in reaction buffer [50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 0.1% Triton X-100, and 1 mM dithiothreitol (DTT)] with 20 mM NaHCO3 or 20 mM NaCl (control) for 30 min, and then phosphorylation reactions were carried out for 30 min with 200 µM ATP and 1 µCi [γ-32P]-ATP.

Structural Predictions of BdMPK5–HT1

The BdMPK5–HT1 complex structure was predicted with AlphaFold2, version 2.2.2 using the multimer functionality (Evans et al., 2022; Jumper et al., 2021). The source code was downloaded from the AlphaFold2 Github page (https://github.com/deepmind/alphafold). The sequences of BdMPK5-D90N and HT1 were used for structure predictions. We predicted the complex of the long form of HT1 (Uniprot ID: Q2MHE4) with BdMPK5 (BdiBd21-3.3G0222500). The maximum template release date we used was from May 14, 2020. We used the full genetic database configuration and included a final relaxation step on all predicted models. For complex prediction, we created five BdMPK5–HT1 complex models (e.g., Takahashi et al., 2022), each starting with a random seed, and for each of these models, made five structure predictions. We then ranked all 25 predictions and used the top ranked prediction as our final model. This ranking system uses the predicted template modeling (pTM) score, which may produce a different set of rankings than if one uses the predicted local difference distance test (pLDDT) score to rank structures. However, apart from the manually set maximum template release date, all of these settings were the default ones provided by AlphaFold2. This workflow was similar to previous predictions of MPK–HT1 complexes (Takahashi et al., 2022).

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon request or included as per journal requirements. *Brachypodium distachyon chill1* mutant seeds will be provided upon request.

Figure 1 *chill 1* mutant shows impaired stomatal response to changes in CO_2 but remains responsive to exogenous ABA.
(A) 4 to 5-week-old *Brachypodium distachyon* plants were imaged following exposure to high CO_2 Pseudo-colored temperature scale is on the left. (top infrared thermal image, bottom photo of the same plants).
(B) Stomatal density and (C) stomatal index were determined using leaf imprints created of the 4th true leaf of plants with 3 independent replicates plants per genotype with 4 images per plant. Circles represent counts per each image while diamonds are the average per each plant. Error bars represent SEM while p-values were obtained from two-tailed t-tests comparing mutant to WT.
(D) Stomatal conductance was quantified using a gas exchange analyzer. Data shown are the average of n=3 plants per genotype $\pm$ SEM using 4 leaves per plant.
(E) Stomatal conductance was recorded prior to and following addition of 2 μM ABA. Data shown are the average of n= 3 $\pm$ SEM plants per genotype using 1 leaf per experiment and genotype.

Figure 2 Characterization of *chill1* F1 backcross.
(A) 5 to 6-week-old backcrossed *chill1 Brachypodium distachyon* plants were analyzed (right) by infrared thermal imaging following 2-hour exposure to 1000 ppm CO_2 alongside

parent line Bd 21-3 (WT) and the *chill1* mutant. Pseudo-colored temperature scale is on the right.
(B) Stomatal conductance response of the F1 cross of the parent line Bd21-3 with the *chill1* mutant was analyzed using a gas exchange analyzer. Data shown are the average of n=3 $\pm$ SEM experiments using 4 leaves per genotype in each experiment.

Figure 3 *chill1* x Bd21-3 parent F2 infrared thermal imaging selection.
(A) 4 to 5-week-old *chill1* x Bd 21-3 F2 backcrossed plants were exposed to 1000 ppm CO_2 for 2 hours then immediately imaged using the infrared thermal imaging camera. The left most plant is the Bd21-3 parent line. The blue ellipse highlights a plant showing a *chill1-like* lower temperature. Pseudo-colored temperature scale is on the right.
(B) Color images were taken at the same time for plant identification.

Figure 4 QTLseqr output for bulk-segregant analysis for M- and SM-Pool respectively.

Bulk-segregant analysis was performed with two different data sets. The (left) M (mutant)-pool (57 *chill1*-like individuals) was used as the mutant pool and (right) the SM (strong phenotype mutant)-pool (37 *chill1*-like individuals with the most robust infrared thermal image phenotypes) were used as the mutant pools. In both analyses the WT-like pool (50 WT-like F2 individuals) was used as the reference. X-axis shows the chromosome number and genomic position. The Y-axis represents the -log10(p-value) derived from the G' value (see Methods). The genome-wide false discovery rate of 0.01 is indicated by the red line.

Figure 5 Candidate Genes Derived from BSA.
(A) Variants in genes called as homozygous via Bulked Segregant analyses for the SM and M pools were compiled to create a list of called homozygous candidate mutations (see Results). Results of targeted Sanger sequencing are shown in the right column (het: heterozygous).
(B) The called homozygous mutation within the 2nd exon of BdMPK5 (BdiBd21-3.3G0222500) is shown by the circled nucleotides and has been confirmed via sequencing.

Figure 6 Sodium Azide Line 0770 crosses.
(A) 4 to 5-week-old *chill1*, sequence indexed and confirmed sodium azide (NaN) line and the Bd21-3 X *chill1* F1 backcross were exposed to 1000 ppm CO_2 for 2 hours alongside the Bd21-3 parent (WT) control.
(B) Color images were taken at the same time for identification of plants. Plants from left to right are labeled Bd21-3 (WT), *chill1*, NaN0770, and the F1 NaN0770 backcross.
(C) The sodium azide mutagenized NaN0770 line was crossed to *chill1* and the F1 plants were used for infrared thermal imaging following exposure to high (1000 ppm) CO_2.
(D) Color image of the same plants. Plants from left to right are Bd21-3, *chill1*, NaN0770 and the *chill1*x NaN0770 F1 cross. Pseudo-colored temperature scale is on the right in (A) and (C).

Figure 7 Design and Sequencing for isolation of *chill1* CRISPR plants.
(A) Guide RNA design used in generation of CRISPR plants in the Bd21-3 parent background (highlighted in pink) and followed by the PAM sequence (highlighted in yellow).
(B) Plants generated using this gRNA were sequenced and two of the alleles generated are

shown aligned to the parent sequence. Alignment mis-matches between the CRISPR alleles and parent line are highlighted in red showing several small deletions near the PAM sequence.

Figure 8 *Bdmpk5* CRISPR Alleles Show Strong CO_2 insensitivity but functional ABA responses.
(A, B) 5 to 6-week-old plants were analyzed in time-resolved stomatal conductance analyses.
(A) CRISPR alleles show strongly impaired stomatal responses to CO_2 shifts. Data shown are the average of n=3 $\pm$ SEM experiments using 4 leaves per genotype in each experiment.
(B) CRISPR leaves show functional ABA-induced stomatal closing, despite the large stomatal conductance. Stomatal conductance was recorded for 10 minutes prior to addition of 2 μM ABA. Data shown are the average of n= 3 $\pm$ SEM experiments using one leaf per experiment and genotype.
(C, D) Stomatal density (C) and stomatal index (D) were determined using leaf imprints created of the 4th true leaf with 3 replicate plants and genotype. Circles represent counts per each image while diamonds are the average per each plant. Error bars represent SEM while p-values were obtained from two-tailed t-tests comparing mutant to the Bd21-3 parent line ("WT").

Figure 9. BdMPK5 functions together with *Arabidopsis* HT1 and CBC1 in high CO_2/bicarbonate-mediated down-regulation of CBC1 protein kinase phosphorylation and structural prediction of BdMPK5–HT1 complex.
(A) In contrast to wildtype BdMPK5 protein, the *chill1* BdMPK5(D90N) variant isoform does not mediate CO_2/HCO_3^--dependent downregulation of CBC1 kinase phosphorylation. *In vitro* phosphorylation assays were performed using GST-CBC1, His-HT1 and His-BdMPK5 (WT or D90N) recombinant proteins with or without 20 mM $NaHCO_3$. Phosphorylation levels of CBC1 (top) and Coomassie brilliant blue (CBB)-stained gels (bottom) are shown.
(B) AlphaFold2-predicted complex of BdMPK5-D90N with HT1. BdMPK5 is shown in red, while HT1 is shown in blue. The BdMPK5-N90 residue, which is mutant in *chill1*, is colored green, and is predicted, based on the wildtype BdMPK5 D90N–HT1 simulation, to lie at the interface of BdMPK5 and HT1.

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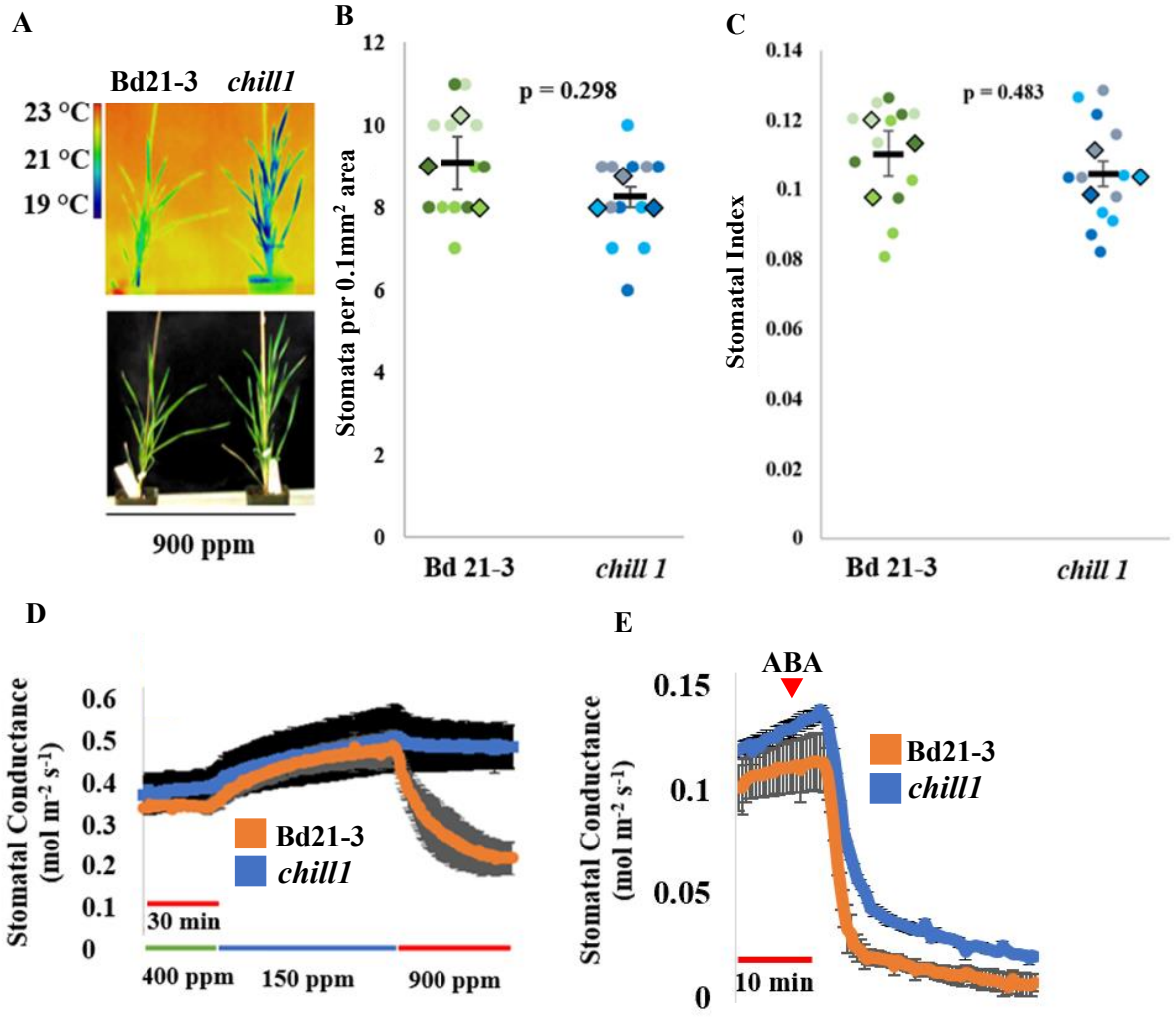

A
Bd21-3
chill1
23 °C
21 °C
19 °C
900 ppm
B
Stomata per 0.1mm² area
p = 0.298
Bd 21-3
chill 1
C
Stomatal Index
p = 0.483
Bd 21-3
chill 1
D
Stomatal Conductance (mol m⁻² s⁻¹)
Bd21-3
chill1
30 min
400 ppm
150 ppm
900 ppm
E
ABA
Stomatal Conductance (mol m⁻² s⁻¹)
Bd21-3
chill1
10 min

Figure 1

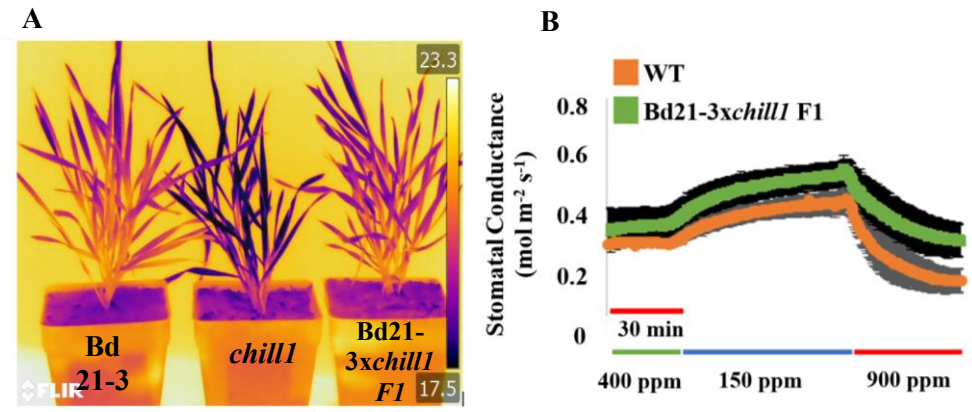

A
23.3
17.5
Bd
21-3
chill1
Bd21-
3xchill1
F1
B
WT
Bd21-3xchill1 F1
Stomatal Conductance
(mol m-2 s-1)
0.8
0.6
0.4
0.2
0
30 min
400 ppm
150 ppm
900 ppm

Figure 2

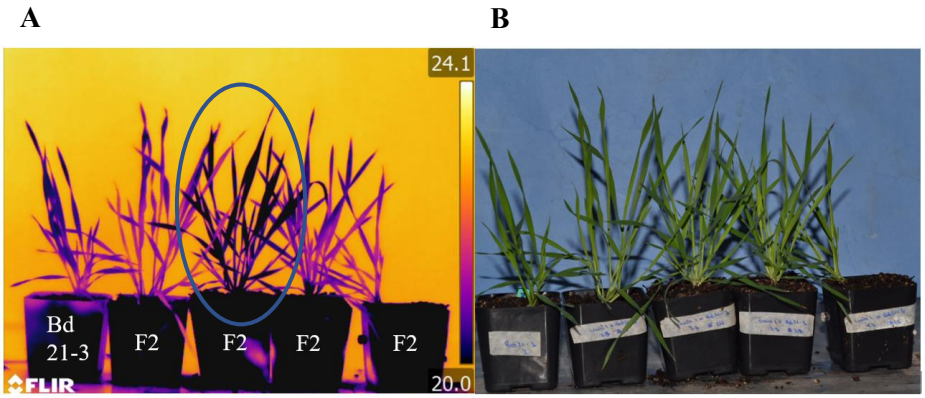


Figure 3

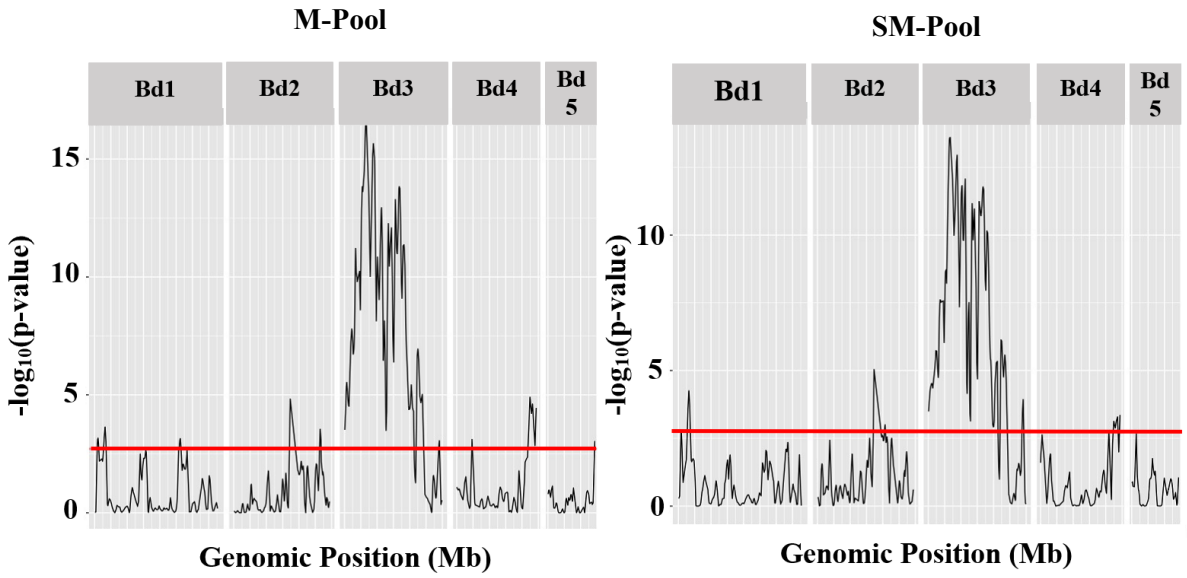

M-Pool
Bd1
Bd2
Bd3
Bd4
Bd 5
15
10
5
0
-log10(p-value)
Genomic Position (Mb)
SM-Pool
Bd1
Bd2
Bd3
Bd4
Bd 5
10
5
0
-log10(p-value)
Genomic Position (Mb)

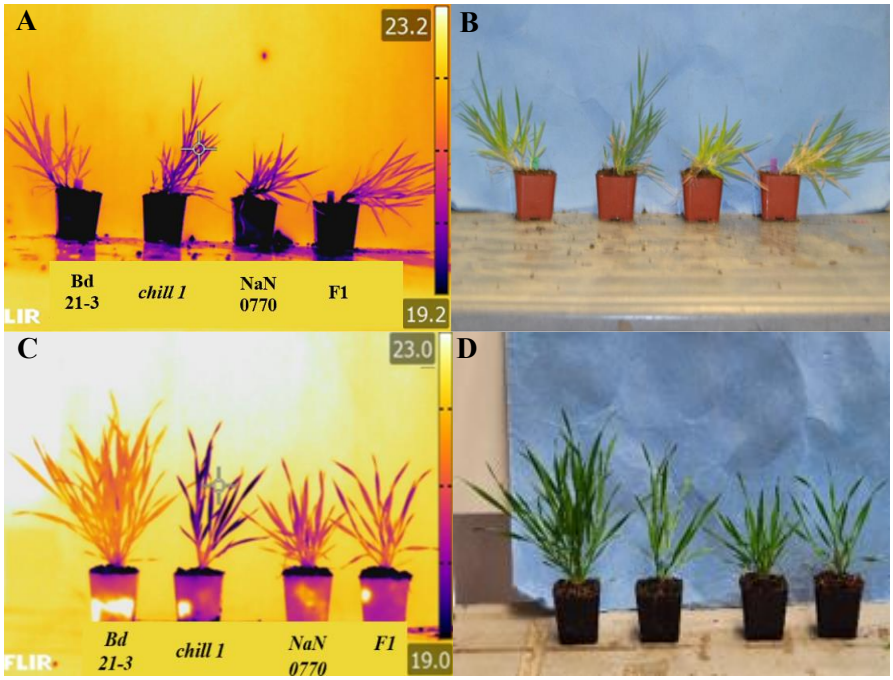
Figure 4

A

Gene ID	Annotation	Status
BdiBd21-3.3G0222500	***MPK5	Found in all cool plants in *chill1* F4 Could not be excluded
BdiBd21-3.3G0296900	DUF4283	3 of the F4 plants were het at predicted mutation site Excluded
BdiBd21-3.3G0336900	unknown	Short read-called mutation was also found in WT Excluded
BdiBd21-3.3G0407500	Mitochondrial import receptor	2 of the F4 plants were het at predicted mutation site Excluded
BdiBd21-3.3G0296100	Reverse transcriptase	2 of the F4 plants were het at predicted mutation site Excluded

B

Figure 5


A
23.2
19.2
Bd
21-3
chill 1
NaN
0770
F1
B
C
23.0
19.0
Bd
21-3
chill 1
NaN
0770
F1
D
Figure 6

A

TGCTGCTATCAACGTGCAGACTCGCGAGGAGGTCGCCATCAAGAAGATCGGCAACGCGTTCGACAACCA
GATCGACGCCAAGCGCACTTTGCGAGAAGTGAAGCTGCTTCGACACATGAATCATGAGAAT

PAM sequence

gRNA: TGCTGCTATCAACGTGCAGACTCGCG**AGG**AGGTCG

B

```
Bd 21-3:     TGCTGCTATCAACGTGCAGACTCGCGAGGAGGTCGCCATCAAGAAGATCGGCAACGCGTTCGACAACCAGATCGACG
             |||||||||||||||||||||||| |         | # |      |                |         |    ||||
CRISPR #1:   TGCTGCTATCAACGTGCAGACTC-C--------C-N-A-----A----------------T--------A----GACG

Bd 21-3:     TGCTGCTATCAACGTGCAGACTCGCGAGGA-G--GTCGCCATCAAGAAGATCGGCAACGCGTTCGACAACCAGATCGACG
             |||||||||||||||||||||| #| ||||| |  |||| |  | | | #|||#|||||||||||||##|||#|||#||#
CRISPR #2:   TGCTGCTATCAACGTGCAGAC-AG-GAGGACGACGTCG-C--C-A-A-CATCAGCAACGCGTTCGACTTCCACATCCACA
```

Figure 7

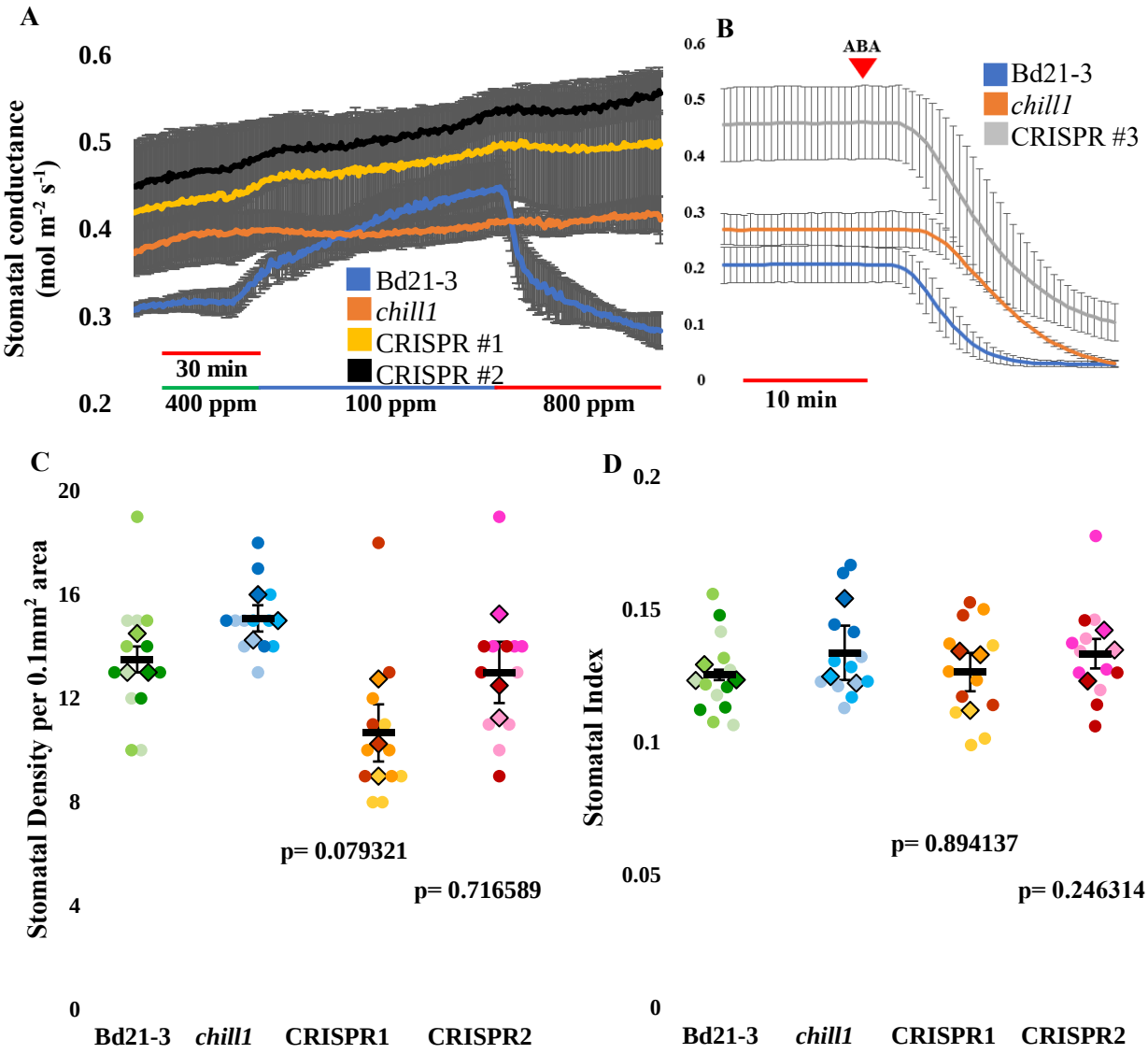

A
Stomatal conductance (mol m-2 s-1)
0.6
0.5
0.4
0.3
0.2
Bd21-3
chill1
CRISPR #1
CRISPR #2
30 min
400 ppm
100 ppm
800 ppm
B
ABA
Bd21-3
chill1
CRISPR #3
10 min
C
Stomatal Density per 0.1mm2 area
20
16
12
8
4
0
p= 0.079321
p= 0.716589
Bd21-3
chill1
CRISPR1
CRISPR2
D
Stomatal Index
0.2
0.15
0.1
0.05
0
p= 0.894137
p= 0.246314
Bd21-3
chill1
CRISPR1
CRISPR2

Figure 8

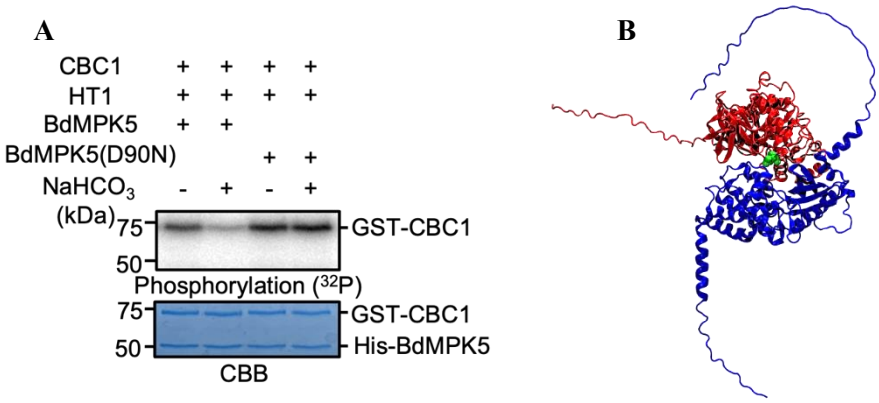
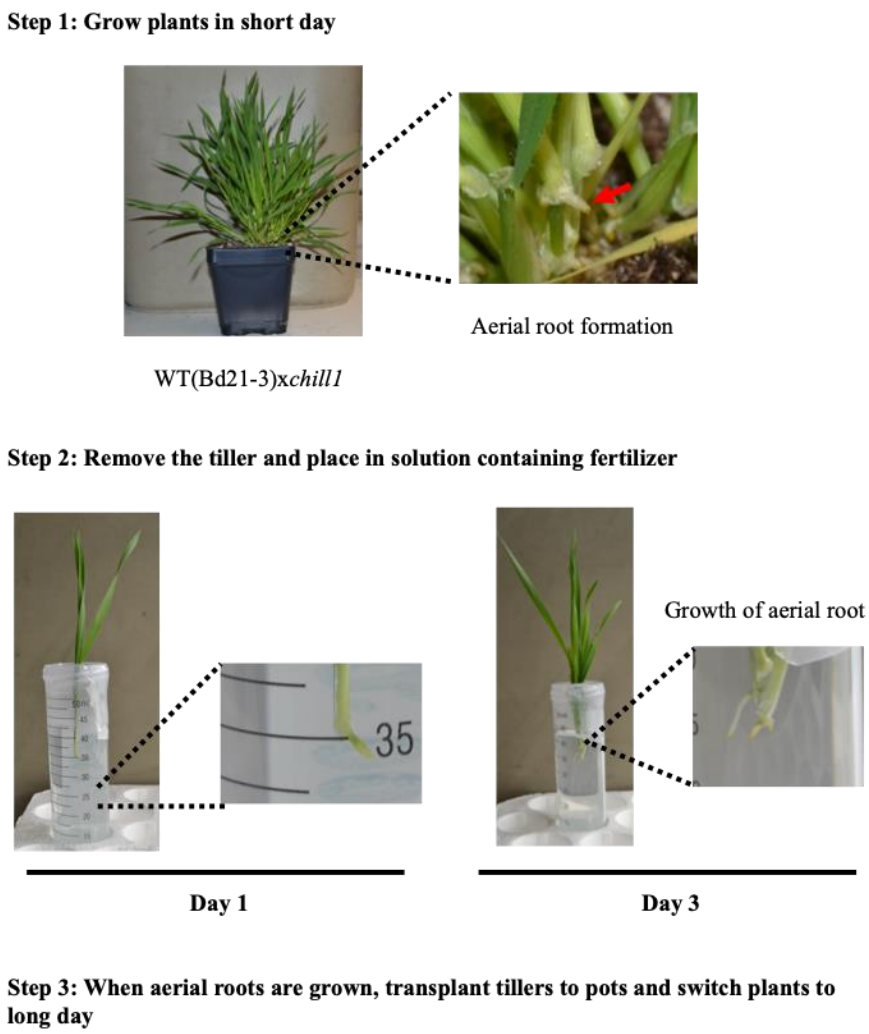
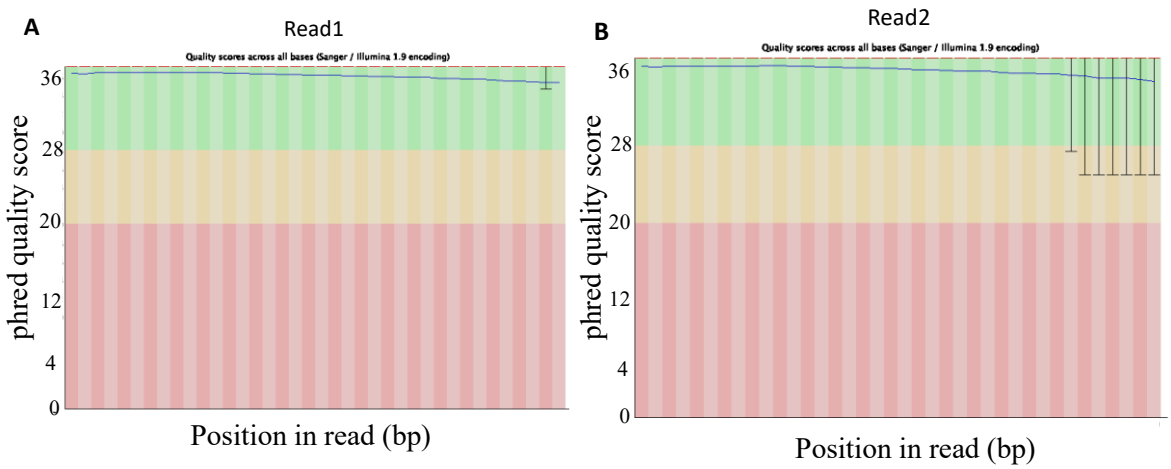

A
CBC1 + + + +
HT1 + + + +
BdMPK5 + +
BdMPK5(D90N) + +
NaHCO3 - + - +
(kDa)
75
50
GST-CBC1
Phosphorylation (32P)
75
50
GST-CBC1
His-BdMPK5
CBB
B

Figure 9

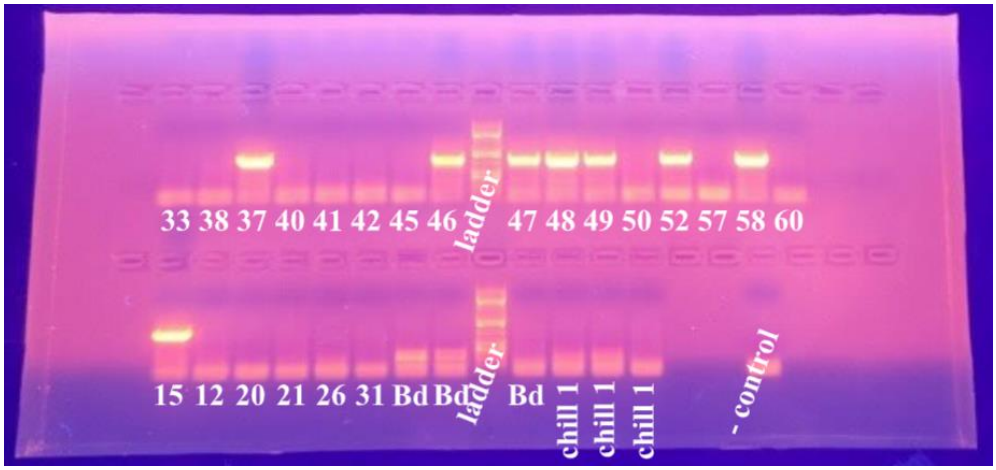
Supplement:



Supplemental Figure 1 Tiller harvesting and growth methods for F1 backcrossed plants enables generation of large number of F2 seeds:
Step1 (top): Backcrossed F1 plants were grown under short day (8L:16D) until visible aerial root formation could be observed. Step 1 of tiller creation involves growing plants under short (8D: 16 L) day until aerial root formation can be observed.
Step 2 (left): Tillers were removed below the aerial root and placed into a nutrient containing solution (see Methods).
Step 2 (right) shows aerial root growth prior to transfer to soil.



Supplemental Figure 2: Per base sequence quality of read one (A) and read two (B) for the raw sequencing data of the *chill1*-linked pool consisting of 57 *chill1* BC1F2 individuals exhibiting a cooler leaf phenotype. Paired end whole genome sequencing outputs were created in two files for each sample, because all DNA fragments were sequenced in two directions (forward and reverse complement orientation) referred to as read 1 (forward, A) and read 2 (reverse-complement, B). Y-axis represents the phred quality score. Phred quality score is a measure of the quality of base calling generated by automated DNA sequencing. The higher the Phred quality score the more accurate the base calling is. A quality phred score above 28 indicates very high quality (green), between 28 to 20 good quality (orange). A phred quality score below 20 (corresponding to 99% accuracy) is considered to be the cutoff for good sequencing quality (red). The thin dark line (top of A and B) represents the mean quality score at each base position/window. The inner-quartile range for 25th to 75th percentile was so high that range boxes were mostly not visible, with exception of right most read positions. The upper and lower whiskers represent the 10th and 90th percentile scores.



Supplemental Figure 3 Genotyping of CRISPR plants to detect presence of Cas9

T2 CRISPR plants were genotyped to detect the presence of Cas9 using primers designed around the first 800 BP of the Cas9 sequence used in the trasnformation vector JD633 (Addgene). Untransformed wildtype Bd21-3 plants were also used as controls as well as water as a negative control. Bands indicate presence of Cas9 within T2 plants.

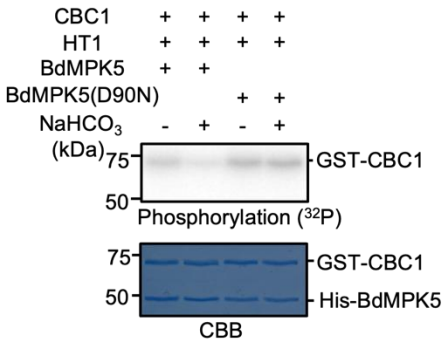


Figure S4 Replicate example of in vitro phosphorylation experiments using the BdMPK5 and the BdMPK5(D90N) isoforms.

In vitro phosphorylation assays were performed using GST-CBC1, His-HT1 and His-BdMPK5 (WT or D90N) recombinant proteins with or without 20 mM $NaHCO_3$. Phosphorylation levels of CBC1 (top) and Coomassie brilliant blue (CBB)-stained gels (bottom) are shown. See Figure 9A for an independent experiment.