

HEAT STRESS TOLERANCE IN SPRING WHEAT:
LEVERAGING BRASSINOSTEROID AND
ANTIOXIDANT INTERACTION

by

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ABSTRACT

Global temperatures are increasing, and this threatens crop production as heat stress damages plant growth and yields. To breed more heat tolerant crops, we must understand heat tolerance mechanisms and how plant hormones can enhance heat responses. In this research, I used the natural plant hormone, brassinosteroid (BR) to investigate spring wheat yield component heat tolerance during the reproductive development stage. Heat stress at the wheat booting stage caused significant losses across multiple yield components which was mitigated by exogenous application of BR. I compared gene expression, antioxidant activity, and photosynthetic capability of plants treated with BR or a BR inhibitor in an effort to understand trait specific heat tolerance. The consistent expression of genes, high activity of antioxidant enzymes, and regulatory response to heat by antioxidant enzymes were all correlated to BR-supported heat tolerance. I additionally examined how BR application was inducing BR-response in wheat since this has been shown to be dependent on a reactive oxygen species (ROS) signaling burst in other crops. The inhibition of ROS during BR application reduced photochemical efficiency during heat stress while treatment with the ROS-inhibitor alone had no measurable effect on photosynthetic yields. Interaction of BR and ROS during BR application correlated with improved seed development for plants experiencing heat stress during the booting stage. This research contributes to our understanding of wheat heat tolerance as directed by BR and supports ongoing global efforts to improve food production despite heat stress.

INTRODUCTION

Human populations are increasing in size and food demand is rising, thus efficient food production becomes more urgent and all-important. Climate change is destabilizing the environment, making food production more difficult and unpredictable (Dhankher and Foyer, 2018). As extreme weather events, water availability, and seasonal temperatures rapidly change, there is an increasing effort across the globe to optimize crop growth for suitability in these escalating climate extremes (Dhankher and Foyer, 2018). The rate at which severe temperatures are intensifying is beyond the rate at which plants can naturally adapt to, and this disparity results in crop loss and potential famine (Reynolds and Ortiz, 2010).

In this thesis, I contributed to the understanding of heat stress response in spring wheat. Using both exogenous application and inhibition of a natural plant hormone, brassinosteroid (BR), I investigated the mechanistic function of this hormone during heat stress. I explored genetic, molecular, and phenotypic effects by germinating the spring wheat variety ‘Vida’ (*Triticum aestivum*, (PI 642366)) in this hormone and then exposing the plants to heat stress during the reproductive development stage (Lanning et al., 2006). I uncovered genetic and molecular changes that could contribute to improving wheat heat stress tolerance and therefore increase seed production in hotter climates.

Heat Stress

Heat stress can be a devastating stressor given its broad molecular and phenotypic effects on intolerant plants (Farooq et al., 2011; Akter and Rafiqul, 2017; Hassan et al., 2020; Poudel and Poudel, 2020; Lal et al., 2022; Ullah et al., 2022). As heat increases, unregulated energy

within cells produces changes in conformation, oxidation, and association of molecular species, resulting in losses of function (Farooq et al., 2011; Hassan et al., 2020). Heat stress increases catabolic enzyme activity, lipid peroxidation, and membrane and vacuolar collapse, thus accelerating organ senescence (Lal et al., 2022). If the effects of heat proliferate beyond what a cell is able to control or reverse, homeostasis is lost, and cell function will cease such that the plant will be unable to grow, reproduce, or be economically useful to people (Hassan et al., 2020). As a cell's internal function and external structure deteriorate with heat stress, leaves, roots, and reproductive organs lose morphological organization critical for function: curled leaves are less effective at catching sunlight, shorter roots are not efficient in nutrient uptake, and warped flowers risk ability to reproduce (Akter and Rafiqul, 2017; Huang et al., 2012; Ullah et al., 2022). For organisms, including plants, form is the driver of function, and heat stress warps plant formation, which impairs plant viability.

Therefore, plants fight back against heat stress with defenses across all organisms that prevent and correct heat damage, referred to as basal thermotolerance (Kumar and Rai, 2014). Heat response genes are triggered by heat stress and encode specialized proteins and molecules that address heat damage (Wang et al., 2004). Heat shock proteins (HSPs) are involved in stabilizing protein deformation and refolding malformed proteins into their functional conformations (Wang et al., 2004). HSPs are also responsible for targeting overly damaged proteins for degradation and preventing aggregation of damaged proteins (Wang et al., 2004). Small molecules such as proline help damaged membranes maintain osmobalance and scavenge oxidizers to limit oxidative damage (Ashraf and Foolad, 2007).

However, heat stress threatens not only a plant's immediate viability, but also the effectiveness of photosynthesis and thus ability of growth and development. Specific damage to photosynthetic proteins in plant leaves prevents effective carbon assimilation, which can limit how quickly plants are able to grow. Heat stress slows the action and effectiveness of multiple crucial photo assimilation proteins including PEP carboxylase and Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Lal et al., 2022; Prywes et al., 2023). The effects of heat stress on rubisco are particularly strong as it experiences a two-sided attack (Farooq et al., 2011, Lal et al., 2022). First, heat stress slows the activity of thermolabile rubisco activase (RCA), linked to protein aggregation, when RCA typically removes the end product from rubisco's active site to allow further CO₂ assimilation (Henderson et al., 2013; Farooq et al., 2011). Secondly, increased temperatures decrease the solubility of both oxygen and carbon dioxide, but carbon dioxide is more sensitive to this, leaving higher relative concentrations of oxygen in the cells (Posch et al., 2019). As rubisco can act as an oxygenase under favorable concentrations, rubisco activity increasingly supports photorespiration more so than photosynthesis (Farooq et al., 2011).

Further, disruption of the electron transport chain (ETC) can affect energy assimilation in the chloroplast (Havaux, 1993). Failure in the ETC is mainly from the sensitivity of the PSII protein and the thylakoid membrane to heat stress; PSII is highly susceptible to deformation or detachment under extreme temperatures (> 40°C), and this is worsened by the thylakoid membrane's own propensity to lose form during high temperatures (Akter and Rafiqul, 2017; Ristic et al., 2007). The change in activity along the ETC further affects the PSI, which undergoes the Mehler reaction to produce superoxide (O₂⁻), a reactive oxygen species (ROS) (Lal et al., 2022; Mittler et al., 2022; Khorobrykh et al., 2020).

Hyperactive oxidizing molecules, ROS accumulate under heat stress and can cause destruction throughout the cell (Mittler et al., 2022). These ROS such as O_2^- or hydrogen peroxide (H_2O_2) oxidize membranes and proteins, changing or demolishing their function (Mittler et al., 2022). The oxidation of membranes compromises their structure, leading to electrolyte leakage and improper flow of key molecules (Cossani and Reynolds, 2012; Hasanuzzaman et al., 2012). For proteins, oxidation at vulnerable cysteine, arginine, lysine, threonine, and methionine residues change the within-protein amino acid interactions which can affect structure and function (Farooq et al., 2011). In the chloroplast, PSII is impeded by ROS and ROS degrade chlorophyll, reducing chlorophyll effectiveness and further inhibiting photosynthesis (Farooq et al., 2011; Guo et al., 2006). Strict reactive ROS control is thus requisite to maintaining cell viability and plant function.

Plants combat ROS effects via antioxidants which reduce and reverse oxidation damage (Mittler et al., 2022; Nolan et al., 2017; Sharma et al., 2022). Antioxidants can be small, non-enzymatic molecules, such as pigments, or specific protein enzymes -- both are crucial for stress alleviation and continued cell function under high ROS concentrations (Mittler et al., 2022). Antioxidant enzymes including superoxide dismutase (SOD) and ascorbate peroxidase (APX) are the first lines of defense against O_2^- and H_2O_2 respectively (Poudel and Poudel, 2020). In the chloroplast, SOD works to control ROS produced during ETC breakdown and chlorophyll degradation (Pilon et al., 2011). Small, non-enzymatic antioxidants, such as phenylpropanoids, glutathione, and ascorbate can also scavenge ROS, sequestering or reducing them to prevent further oxidative damage (Farooq et al., 2011).

Temperature response and tolerance are key for plant function -- even small temperature increases cost valuable resources from a plant as they divert energy to combat these effects. In Montana where this research is focused, average summer temperatures are not generally considered extreme, averaging around 29°C. Yet this average temperature is increasing year over year and most crops grown in the state achieve peak production at 5-15°C below this temperature (Pederson et al., 2010; Porter and Gawith, 1999). One such crop grown in Montana is a significant food around the world: wheat (*Triticum aestivum* L.).

Wheat

Among the many crops that will be affected by the changing climate, wheat is vital around the globe for food, fuel, and animal feed (Bentley et al., 2022). Wheat is a relatively low-water footprint crop, requiring less water and having higher water use efficiency than other grains, such as corn or rice (Hadebe et al., 2017). Therefore, should water resources become scarcer during climate change, wheat is a better option for continuing yield compared to crops which need more resources for viable production. Wheat seeds and flour also contain a higher protein and essential nutrient content than several other cereal crops and global wheat consumption was 67 kg/year per capita in 2023 (McKevith, 2004; FAO, 2024). It is critical for producers to mitigate heat stress in wheat to maximize seed count and quality to achieve total possible production (Yadav et al., 2022).

Spring wheat varieties, which are grown in the summer, are most susceptible to heat during the reproductive development phase of growth. The wheat reproductive period spans from the wheat tiller elongation and head formation within a leaf sheath (booting), through head emergence and fertilization (heading), and to grain development and filling (Acevedo et al.,

2006). Wheat heads are composed of structural rachis tissue and fertile floret tissue, the rachis elongates during the booting stage and florets develop along the rachis in an alternate distichous form (McMaster, 1997). Heat stress during the wheat reproductive phase speeds up developmental processes -- the wheat head development period, pollination window, and seed fill time are all reduced in duration with heat (Ullah et al., 2022). Each sped-up stage during reproduction accumulates to result in fewer, smaller, and lower quality seeds (Talukder et al., 2014; Modarresi et al., 2010; Bheemanahalli et al., 2019). Much research has investigated the effects of heat stress during pollination and grain development, but relatively few studies have investigated heat stress on the booting stage. Yet, the conditions under which the reproductive booting period is carried out directly affect yield, with every 1°C increase in temperature over 15°C resulting in 3-5% yield losses (Ullah et al., 2022; Gibson and Paulsen, 1999).

The booting stage in particular can have cascading yield effects, as poor growth of the wheat head can affect all aspects of yield components (Zhen et al., 2019; Zhang et al., 2023). Head size determines possible seed number on the head and heat stress results in shorter heads (Kothari et al., in preparation). Spikelet development affects fertility of the wheat flower, with heat stress injuring embryo development within the spikelets (Ullah et al., 2022). Anther development affects fertility of the wheat pollen, and heat stress shortens anthers and increases pollen mortality (Xu et al., 2022). All of these head components are established during booting, therefore heat stress during the booting stage can cause multiplicative deterioration and significantly impact yield regardless of conditions during further stages of reproduction.

Brassinosteroids

Considering the damage that heat stress can cause, many methods are being used to enhance wheat heat tolerance. Breeding of heat tolerant lines and application of protective compounds which induce heat tolerance in plants are key contributors in the race to produce more heat tolerant wheat (Ning et al., 2021; Sarkar et al., 2021). A review from 2021 of wheat breeding outcomes for heat and drought tolerance noted there were 131 publications at that time focused on identifying genetic regions, or QTLs, relating to heat tolerance (Langridge and Reynolds, 2021). At Montana State University, breeding for abiotic stress tolerance, including against heat stress, is a stated priority of spring wheat breeding programs (Lavey, 2018). However, to breed for improved heat tolerance, we must first understand the mechanisms of heat tolerance and considering its large hexaploid genome, there is still much unknown about specifics contributing to heat tolerance in spring wheat.

Therefore, many different compounds are being tested to try to induce heat tolerance without precise understanding of the mechanisms involved (Sehar et al., 2023; Buttar et al., 2020; Munir and Sharbir, 2018; Liu et al., 2021; Tao et al., 2022; Omar et al., 2023). Of the heat tolerance-inducing compounds investigated in spring wheat, brassinosteroids are of specific interest given their status as a natural plant hormone and considerable effect on plant development. Brassinosteroids (BR) are short distance plant signaling hormones which, when applied exogenously, caused a 200-fold increase in growth for *Brassica napus*, the plant in which they were first discovered (Zhuoyun and Jia, 2020; Grove et al., 1979). In 1980, BR was formally classified as a plant hormone but progress on its signal transduction pathway was not advanced until about 20 years later (Mitchell et al., 1970; Li and Chory, 1997; Choe et al., 1998).

Promoting growth and cell elongation, BR are a family of steroids with different active forms throughout the plant kingdom, found in different forms dependent on growth stage and environmental conditions (Clouse and Sasse, 1998; Janeczko, 2019). All organs of plants have natural BR, with different forms being more common based on plant identity (Clouse and Sasse, 1998; Janeczko and Swaczynová, 2010a). As with most plant hormones, there is significant crosstalk between other hormones such as ethylene, abscisic acid, and gibberellic acid (Abhinandan et al., 2018).

Transport of BR occurs acropetally within a plant, therefore effects of BR application can only be observed in regions above where the BR is applied (Nishikawa et al., 1994). In wheat, concentrations of BR increase as the plant develops, and brassinolide (BL) is the most prevalent form, with 24-epibrassinolide (24-EBR) and castasterone also present but only at about half the concentration of BL (Janeczko and Swaczynová, 2010a). Exogenous application of BR can affect the endogenous levels of BR, with 24-EBR treatment in wheat tripling the endogenous concentrations of this same BR (Janeczko and Swaczynová, 2010a).

Cellularly, BR are perceived on the cell membrane by the receptor BRI1, whereby it triggers broad gene transcription changes and alters cellular functions (Sun et al., 2010; Goda et al., 2002). When BR is not perceived, BIN2 phosphorylates BR transcription factors (BR-TFs), BES1/BZR1, and tags them for degradation by 26S proteasomes (Li et al., 2001). Upon perception of BR, BRI1 initiates a phosphorelay in the cytoplasm which ultimately results in the phosphorylation and inactivation of BIN2 by BSU1 (Nolan et al., 2017). The transcription factors are then dephosphorylated by PP2a proteins which allow the BR-TFs to accumulate and enter the nucleus (Nolan et al., 2017). Once in the nucleus, BES1 and BZR1 act both alone and

in concert with other co-transcription factors to repress and induce expression of several thousand target genes (Nolan et al., 2017). In wheat, there are 20 homologous BR-TFs which have variable expression based on developmental stage and stress conditions (Kesawat et al., 2021).

Synthesis of BR is carried out by several chemical modification steps beginning with acetyl-CoA and ending with one of several BR forms (Oklestkova et al., 2015; Zhao and Li, 2012). The rate limiting enzyme in the synthesis pathway is DWF4, and expression of this enzyme is repressed by BR perception, leading to negative feedback regulation of BR expression (Kim et al., 2006; Zhao and Li, 2012). Therefore, plant response to BR takes on a parabolic curve – application of 0.1 μ M improves wheat seedling growth, but 2 μ M can inhibit it (Janeczko and Swaczynová, 2010a).

Brassinosteroid Induced Heat Tolerance

In many instances, the inhibition of BR production and transport has improved crop yields by dwarfing, extending the Green Revolution (Vriet et al., 2012; Dockter et al., 2014). Several commercial semi-dwarf Poaceae varieties achieve increased yield by blocking endogenous BR synthesis and therefore minimizing vegetative growth to improve reproductive yields (Chono et al., 2003; Morinaka et al., 2006).

Yet, exogenous BR treatment can improve stress tolerance in plants to biotic, heavy metal, drought, cold, and, most importantly, heat stress (Sharma et al., 2022; Lv and Li, 2020; Krishna, 2003). Specifically, BR can enhance heat tolerance in several crops by increasing antioxidant activity and photosynthesis efficiency to reduce cellular damage while enriching growth. Seed germination treatment (priming) with BR improved the photosynthetic rate of

soybeans during drought stress, and increased chlorophyll content and antioxidant activity in cotton during salt stress and in rice during chromium stress (de O. Maia Júnior, 2024; Surgun et al., 2015; Basit et al., 2021). Following foliar application of BR on rice, barley, and wheat, there was protection of chlorophyll content and antioxidant activity, improvement of PSII efficiency, and improved PSII efficiency recovery in response to heat stressors (Cao and Zhao, 2008; Janeczko et al., 2011; Hairat and Khurana, 2015). For wheat seedlings subjected to an eight hour 40°C heat stress, treatment with 24-EBR delayed losses to photosynthetic capacity and improved the activity of RCA, SOD, and peroxidase (Zhao et al., 2017). Further, seed priming with 1µM 24-EBR helped heat tolerant wheat cultivars maintain chloroplast levels during late-sowing heat stress both pre- and post-anthesis (Kumari and Hemantaranjan, 2018). Kumari and Hemantaranjan (2018) also found that there was increased membrane stability and higher APX and SOD activities as long as 65 days after BR seed treatment, compared to untreated plants.

Though it has been repeatedly illustrated under several different application conditions that exogenous BR can improve heat stress tolerance in crops, the degree to which this effect can impact tangible crop yields can differ (Hirt et al., 2009). The diverse range of crops, variety within the species, application methods, and BR form and concentrations tested have given rise to widely varying, and sometimes conflicting, results (Kothari and Lachowiec, 2021). Particularly given the negative feedback of BR regulation, excess BR application can result in decreased plant growth and slower development (Roddick and Ikekawa, 1992). The inability to consistently replicate positive results from BR application exemplifies the lack of understanding of the broad molecular effects exogenous BR has on plants as determined by BR timing, concentration, and the plant species tested (Kothari and Lachowiec, 2021).

Even between different plant tissues, the changes in genetic expression following BR application may have conflicting effects. Phenotypic changes resulting from enhancement or suppression of the BR pathway may have tissue specific effects, some of which could have agronomic benefits and some of which may be detrimental (Sakamoto et al., 2006; Janeczko et al., 2010b). The broad action of BR to increase cell growth throughout the plant without specifically targeting seed development, relating to yield, can draw resources from seed production (Song et al., 2023).

Exogenous BR applications can be utilized as a tool to understand endogenous heat stress response mechanisms. Since BR definitively improves some aspects of heat tolerance in wheat, discovering relevant genes participating in this process and the way in which they function should allow targeted improvements of heat tolerance in wheat, in ways that provide more specific and robust protection than total BR response.

The Interplay of BR and ROS

An emerging understanding of BR-induced stress tolerance in crops is its reliance on ROS activity to induce transcriptional and enzymatic changes in the cell. While ROS can have a harmful effect on cells when in excess, ROS are also used as important signals throughout plant structures (Mittler et al., 2022). For instance, the relocation of heat response transcription factor, AtHSF8, from the cytoplasm to the nucleus is dependent on oxidation from ROS; even during a 40°C heat shock it will not relocate unless its cysteine residues have also been oxidized (Giesguth et al., 2015). Controlled oxidation of cell components can enhance activity or modify functions positively, allowing plants to respond quickly to stressors which produce these ROS (Mittler et al., 2022). In fact, plants specifically synthesize ROS at controlled levels to change

cell function and send signals both within and between cells (Foreman et al., 2003; Nolan et al., 2017). A particularly vital ROS, H_2O_2 is the most easily reduced which allows regulation to occur rapidly (Mittler et al., 2022). Respiratory burst oxidase homolog (RBOH) enzymes embedded in the cell membrane produce H_2O_2 directly, but H_2O_2 can also be generated from the breakdown of O_2^- via SOD (Mittler et al., 2022; Sharma et al., 2022). The stability of H_2O_2 and its ability to move through cell membranes without damage via aquaporins makes it a crucial and useful signal molecule (Rodrigues et al. 2017; Mittler et al., 2022).

There is increasing evidence that BR signaling contributes to ROS homeostasis and ROS presence in turn modulates BR response (Lei et al., 2022, Xia et al., 2014). The BR-TF BZR1 has increased binding affinity for co-transcription factors in the nucleus when oxidized by H_2O_2 (Tian et al., 2018). The expression of RBOH is elevated in BR-treated cells immediately following application, which increases cellular H_2O_2 content (Jiang et al., 2012; Nie et al., 2013; Zhou et al., 2014; Yan et al., 2015; Wang et al., 2023). Yet, ROS also participates in the negative regulation of BR signaling, with escalating ROS levels increasing BIN2 binding affinity and therefore acting as a negative regulator of BR-TFs (Song et al., 2019).

Several studies have displayed how blocking ROS proliferation during the time of BR application limits the effect of BR on both stress tolerance and enzymatic activity (Xia et al., 2009; Zhang et al., 2010; Nie et al., 2013; Zhou et al., 2014; Xia et al., 2014; Yan et al., 2015; Wang et al., 2023). This BR-ROS interaction was initially investigated by Xia et al. (2009) in cucumbers, where scavenging of ROS by N,N'-dimethylthiourea (DMTU) or prevention of ROS production by diphenyleneiodonium (DPI), which blocks RBOH function, reduced biotic stress tolerance previously induced by BR application. Further research from this same lab continued to

prove BRs reliance on ROS for signal transduction in tomatoes, with RBOH silencing in concurrence with BR application leading to muted oxidative and heat stress tolerances (Nie et al., 2013; Zhou et al., 2014). In maize and rice as well, co-treatment with BR and DMTU or DPI blocked a typical expression induction for several key stress tolerance genes including the antioxidant enzyme catalase (CAT) (Yan et al., 2015).

As mentioned earlier in this chapter, BR promotes antioxidant activity, with the activity of several antioxidant enzymes persistently increased 24 hours to eight weeks after BR application (Basit et al., 2021; Zhu et al., 2016; Kumari and Hemantaranjan, 2018). Yet, prevention of this initial ROS burst during BR application negates BR-dependent increased antioxidant activity in some crops (Nie et al., 2013; Zhou et al., 2014; Deng et al., 2016). Therefore, BR application induces short-term oxidizing effects to enhance long-term antioxidant activity and improve stress tolerance.

Heat, Wheat, BR, and ROS, All Together Now

The following chapters discuss two experiments which increase the understanding of the BR mechanism of action in wheat that contributes to improved heat tolerance during the booting stage. In our preliminary research, wheat seeds treated with BR had improved heat tolerance when undergoing heat stress four weeks later during the booting stage. Particularly, the wheat head length did not suffer from as much shortening during heat stress when the plants had been germinated in 24-EBR (Kothari et al., in preparation). I investigated tissue specific, enduring changes caused by BR application to try to narrow the contributing genetic mechanisms unique to wheat heat tolerance.

The first experiment investigates gene regulation changes induced by BR, occurring in the wheat head during booting immediately after the plant was exposed to heat. Considering literature support of BR-induced antioxidant activity, I was curious whether there would be persistent expression differences in antioxidants as much as four weeks after the hormone treatment. Since our lab observed heat tolerance indicators only for specific tissues, I also wanted to determine if BR was affecting the gene regulation of the structural and fertile tissues differently.

The second experiment explores potential ROS interplay which allows BR germination effects to persevere until the booting stage. I posited that blocking an initial ROS burst during germination in BR would affect the wheat yield component improvements seen in our preliminary research. I looked at antioxidant activity of wheat from germination until the flowering stage to confirm if BR treatment affected this, and if blocking the ROS burst would have additional consequences during heat stress. The requirement of ROS during BR treatment for induced tolerance has not been investigated in wheat, and some BR-mediated effects demonstrated in dicots have not been observed in monocot plants such as wheat (Sekimata et al., 2001).

Understanding the interplay of BR effects, ROS production, and antioxidant activity in wheat can elucidate short- and long-term heat stress tolerance mechanisms. By defining the molecular method by which BR provides heat tolerance in wheat, we can select and breed more heat tolerant varieties for improved yields. Discovering these varieties is essential for sufficient food production in the coming global environments and to agronomic growth in heat-stressed regions.

CHAPTER TWO

BRASSINOSTEROID-MEDIATED ANTIOXIDANT PRIMING
MAY INFLUENCE WHEAT HEAT STRESS TOLERANCE

Contribution of Authors and Co-Authors

Manuscript in Chapter Two

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Contributions: Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing – original draft, Writing – review & editing.

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Contributions: Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Validation, Writing – review & editing.

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Abstract

As heat stress becomes an ever-growing concern in crop production, it is imperative to understand and search for methods by which crops can increase heat tolerance. Here, we investigated heat tolerance in spring wheat as it underwent mild heat stress during the reproductive development, booting stage, and how mechanisms are affected by a germination stage brassinosteroid (BR) treatment. The persistent effect of exogenous BR treatment at germination was evident in gene expression differences as much as six weeks later both before and in response to booting heat stress. Germination in BR reduced the plants' normal expression or repression of heat response and reactive oxygen species (ROS) -homeostasis related genes. Plants not treated with BR had significant upregulation of redox related genes during heat stress whereas BR-treated plants had persistent maintenance of redox gene expression levels regardless of heat stress. We further compared reproductive tissues specific gene regulation and found wheat plants tended to upregulate redox related genes in the structural tissue containing the meristem more so than the fertile tissue during tissue development. During heat stress, BR-treated plants continued to favor redox expression in the structural tissue, which may contribute to meristematic maintenance during heat induced ROS proliferation. Here we highlight the longevity of BR treatment, its tissue specificity, and its importance to heat stress response in spring wheat through ROS-homeostasis. Improving the heat tolerance of wheat is essential to boosting production and meeting global food demands.

Introduction

The changing global climate provides both challenge and opportunity for those interested in optimizing crop yield. Stressors including increased drought, salinity, and heat can damage plant health and thus result in yield loss. Yet, investigating the mechanisms that crops use to tolerate these stressors allows plant biologists to dive deeper into the inner workings of organisms that we rely on for food. A keystone crop, wheat, can be grown in non-irrigated lands given its relative drought tolerance; however, drought susceptible areas can be additionally subjected to extreme temperatures (Mazdiyasni et al., 2015). Heat stress, specifically during the reproductive stage of wheat, results in fewer, smaller, and lower quality seed (Talukder et al., 2014; Gibson et al., 1999). Considering the prevalence of wheat consumption, 801 tons globally in 2023, optimizing wheat yields is crucial to maintaining the food system and providing essential starch and protein for consumers (FAO, 2024).

Heat stress affects multiple aspects of flowering wheat that are additive to intensify yield losses. When a wheat head is elongating and forming flower-containing spikelets, heat stress can prematurely cease elongation and thus reduce the number of flowers (Modarresi et al., 2010). Once the plant is ready for pollination, heat stress may have deformed, burst, or killed the pollen grains before they can reach the stigma (Ullah et al., 2022). Heat stress disrupting the formation and pollination of wheat flowers during reproduction has downstream negative effects seed production and yield (Bheemanahalli et al., 2019, Talukder et al., 2014).

A highly conserved, short distance signaler, brassinosteroid (BR) has primary function in cell elongation and replication (Bajguz and Tretyn, 2003; Sun et al., 2010). Overexpression of BR synthesis genes and exogenous BR applications have also been demonstrated to improve

plant tolerance to a variety of both biotic and abiotic stressors (Sahni et al., 2016; Dhaubhadel et al., 1999; Kagale et al., 2007). The application of BR has a variable effect on wheat which is dependent on application time, method, and the line it is applied to (Kothari and Lachowiec, 2021). We have previously investigated the beneficial effect of germinating wheat seeds in BR on specific yield components after the mature plant undergoes heat stress. Specifically, when ‘Vida’ (PI 642366) wheat seeds are soaked in BR solution then exposed to a mild heat stress (29°C) a month later, during the reproductive booting stage, the plants germinated in BR had longer spikes and more spikelets after senescence compared to control plants -- yield components essential to yield (Lanning et al., 2006; Kothari et al., in preparation). Sequencing RNA of the entire wheat head taken after heat stress revealed multiple heat response genes as well as many histone related genes being differentially expressed between control and BR-treated plants (Kothari et al., in preparation). We theorized that using whole spike tissue for RNA-sequencing may not have revealed the entire, detailed relationship of a BR mechanism leading to heat tolerance, considering how BR has different roles in different plant tissues (Vragović et. al., 2015). The structural part of the wheat spike – the rachis – is crucial to overall seed yield since a shorter rachis results in a smaller spike which can hold fewer spikelets and seeds. As BR plays a large role in cell development and elongation, rachis development may be strongly influenced by the BR application which could result in a longer spike overall. Therefore, we performed a second RNA sequencing experiment, partitioning the dissected tissues into rachis and florets, the reproductive “pockets” where seeds develop. Delving into tissue specific differentially expressed genes, as well as identifying those which are shared in multiple tissues,

reveals key genes and pathways manipulated by BR which are likely contributing to heat protection.

Heat stress generates excess energy in the cells of plants which leads to higher levels of reactive oxygen species (ROS) (Suzuki et al., 2012). The generation of excess ROS can cause significant damage throughout cells via oxidation and loss of function of key structural, metabolic, and reproductive components (Suzuki et al., 2012; Sharma et al. 2012). Yet ROS, including superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), also act as potent secondary messengers when present in small, controlled amounts (Mittler et al., 2022). Plants attempt to maintain a beneficial homeostasis of ROS by using antioxidants which reduce ROS and through respiratory burst oxidase homologs (RBOHs) which generate ROS as a primary process (Mittler, 2002; Sairam et al., 2000; Foreman et al., 2003). Regulation of these two opposing controls can take place via protein modification of the reduction-oxidation (redox) pathways that produce ROS or by transcriptional and translational control of oxidant/antioxidant enzymes and proteins which generate small molecule antioxidants (Mittler et al., 2022). Plants with more efficient control of ROS homeostasis are better able to tolerate ROS generating stressors, such as heat, and continue efficient signaling under excess oxidation conditions.

Application of BR affects ROS homeostasis, with reciprocal interactions on both ROS prevalence and antioxidant activity. Foliar spray of BR on *Solanum lycopersicum* and *Cucumis sativus* causes an initial ROS burst of increased H_2O_2 levels associated with NADPH-oxidase activity and increased respiratory burst oxidase gene expression (Zhu et al., 2016; Zhou et al. 2014; Xia et al. 2009). However, BR treatment also increases the activity of antioxidant enzymes including superoxide dismutase, catalase, and ascorbate peroxidase (Nie et al, 2012; Basit et al.

2021; Xia et al. 2009). Antioxidant promotion from BR has been further demonstrated in wheat cultivars yet there is a lack of understanding as to how specifically this can affect yields (Avaelbaev et al. 2024). Both the tissues affected and the longevity of the effect of BR treatment are poorly understood; understanding these aspects can reveal key genes controlling mechanisms that could reduce heat-related yield loss in wheat.

We investigated transcriptional changes at the time of heat stress to illuminate how BR application could improve wheat yield components such as spike length and spikelet number following a heat stress as much as six weeks later. By comparing genes induced and repressed by heat at the reproductive stage in control plants to those in BR-treated plants, we revealed BRs long term manipulation of ROS homeostasis. Genes involved in heat shock response were not significantly different in expression, however those involved in oxidative stress and redox pathways were consistently enriched. We discovered the tissue specificity of this redox enrichment by comparing gene expression changes between structural and reproductive tissues before and after heat stress was applied.

Methods

Plant material and experimental design

In order to achieve the analysis outlined in this project, *Triticum aestivum* plants were grown for in vivo studies (Figure 1). The variety ‘Vida’ (PI 642366), which was responsive to BR in preliminary studies, was grown in the following way: seeds were soaked in 0.4mL water per seed with either 1µM 2,4-epibrassinolide (EBR) or 1µM DMSO in a sterile petri dish covered in aluminum foil to prevent de-etiolation (Lanning et al., 2006). This germination treatment was the only time these plants were treated with BR throughout their lifespan. After 48

hours of germination in BR or control, the seeds were planted approximately 1" deep in untreated Sunshine mix soil in an 8" pot, with five seeds per pot evenly spaced. Pots were placed in growth chambers with a 16-hour photoperiod with fluorescent and LED bulbs at 27,000 Lux light intensity, kept at 22°C at all times. Plants were watered approximately every other day depending on soil moisture needs and fertilized once a week with 100 mg/L 20-20-20 Professional Peters fertilizer, 330mL per pot. Pot placement within the chamber was fully randomized. At the tetrad stage of growth on the Waddington 7.5 scale (booting), half of the plants from each treatment were moved to a growth chamber under the same light control but kept at 29°C continuously to simulate mild heat stress, while the other half of each treatment was kept at 22°C for control comparison. There were 64 total plants grown, with four plants for each seed soak, temperature, and time of sampling treatment combinations. At 0, 8, 16, and 24 hours after heat exposure, the unemerged heads from four wheat plants, acting as four biological replicates, were collected. The unemerged wheat heads were removed from the leaf sheath and further dissected into rachis and floret tissues with each tissue separately flash frozen in liquid nitrogen. All samples were stored at -70°C to keep cell contents stable from the time of sampling to processing.

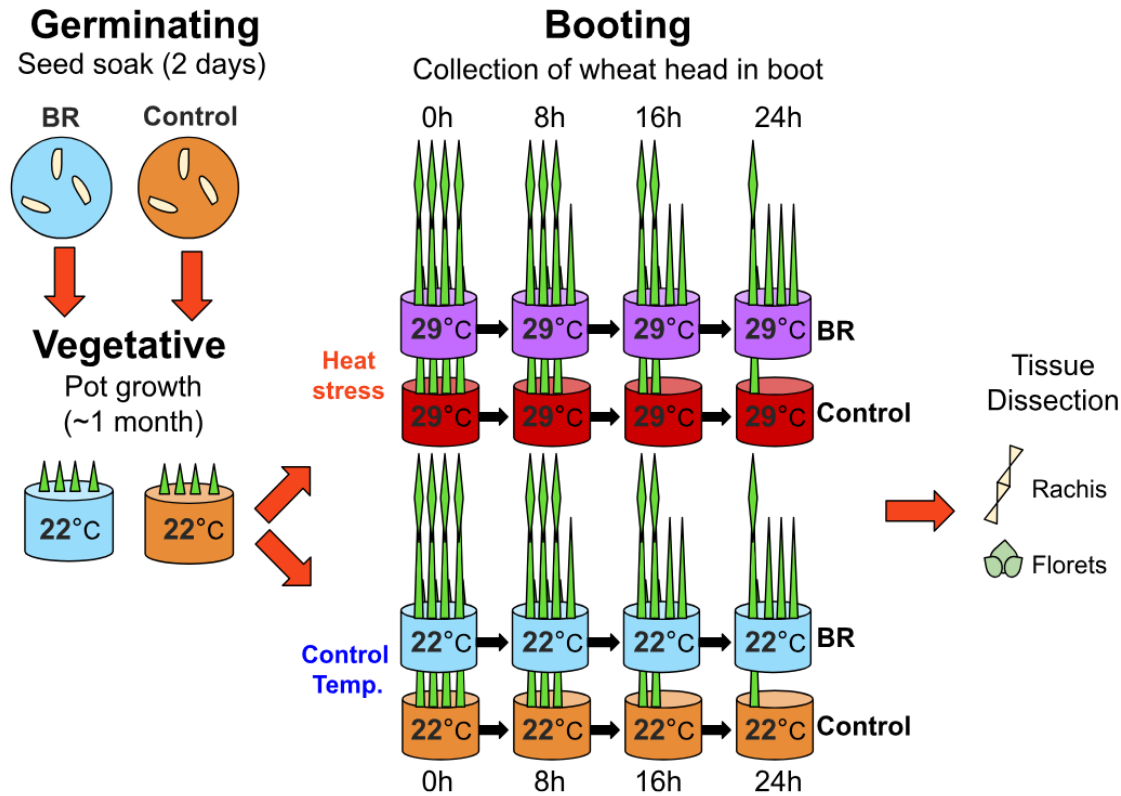


Figure 1: Experimental Design for RNAseq data collection. Visual display to distinguish hormone germination treatment, booting heat treatment, and time of sampling after heat stress. Plants were only treated with BR for two days maximum during germination and then grown under simple conditions for about four to five weeks. At the time of booting, plants were exposed to a heat stress or not, and tissue sampling was carried out every eight hours. Once the unemerged wheat heads were harvested, they were dissected into two tissues, rachis and florets, and these tissues were evaluated for sequencing separately. Four pots represented each germination and booting stage treatment combination, with one plant from each pot collected at the given time points.

RNA extraction

Once all samples were collected, the tissue was ground with 3mm sterilized glass beads in a bead beater to break cell walls and free the cell contents. RNA extraction was performed with the RNeasy Plant Mini Kit (Qiagen, Cat #74903) and Beta-mercaptoethanol. Samples were treated with DNase I (Turbo DNA-free Kit, Invitrogen AM1907) to remove any contaminating genomic DNA and assure purity for library preparation. Both RNA extraction and DNase

treatments were performed in 16 sample batches with a randomized block design of samples comprising each batch. Quality of the samples was confirmed via Bioanalyzer using the Agilent RNA 6000 Nano Kit (Agilent, 5067-1511) to ensure RNA integrity. All samples sequenced had a minimum of an eight RNA integrity number (RIN), while five samples were removed for poor or low RNA quality, leaving a minimum of three biological reps per treatment combination.

RNA sequencing and DGE analysis

RNA-sequencing was performed by Azenta Illumina paired end NGS and raw read data was received from this service. Read quality was checked with FASTQC and MULTIQC software with an average phred score of 36 (Andrews, 2010). RNA sequence trimming, mapping, and read counting was performed via the Montana State University Tempest supercomputer with Trimmomatic v.0.36, STAR, and the Rsubread package v.1.5.2 with function featureCounts, respectively (Bolger, 2014; Dobin, 2013; Liao, 2014). Reads were aligned using the *Triticum aestivum* cv. Chinese Spring IWGSC RefSeq v2.1 genome assembly (Zhu, 2021). Once read counts were assembled, genes with fewer than 10 total reads across all 123 remaining samples were removed as they were considered non-expressed. Differential gene expression analysis and read count normalization was performed with DESeq2 (Love, 2014) and two samples were removed from analysis as they clustered far away from the three other biological replicates within its treatment group during PCA comparisons. DESeq2 was additionally used to normalize read counts by length and read depth so that adjusted, normalized read counts could be extracted for further data visualization with R packages including pheatmap and ggplot2 (Kolde, 2010; Wickham, 2016).

GO enrichment was performed by comparing total expressed gene counts across all samples in given GO categories to gene counts in the same GO category for given differential expression gene sets. Using the Goseq R package, genes were normalized for length bias by generating the requisite probability weighting function (Young, 2010). A significance cut-off for GO term p-values was determined following the BH method with a 0.05 false discovery rate (Benjamini and Hochberg, 1995). GO enrichment was performed three times for each comparison, once for the full set of differentially expressed genes, and once each additionally for genes with higher expression in each given comparison category. For example, when comparing gene expression differences between zero and 24 hours for control rachis tissue, GO enrichment was assessed on all DEGs which were both down and upregulated over the 24 hours. Then, the DEGs were separated into genes with higher expression at zero hours or higher expression at 24 hours, and GO enrichment repeated independently for these two sets of separated DEGs.

H₂O₂ quantification

Wheat seedlings were germinated in 0.4mL per seed with either 1 μ M 24-EBR or 1 μ M DMSO in a sterile petri dish covered in aluminum foil to prevent de-etiolation. After three days, half the seedlings were placed in a 45°C incubator for two hours while still soaking and covered from light. Following this two hour period, whole seedlings were flash frozen in 2mL centrifuge tubes in liquid nitrogen and the tissue was ground in a thin layer of liquid nitrogen with mortar and pestle. Five mg of this ground powder was scraped into a clean, cold tube and thawed in a pH 7.4 phosphate buffer. Samples were analyzed on the day of collection and quantification was performed using the Invitrogen Amplex Red H₂O₂ assay kit (Invitrogen, Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit Cat# A22188). Fluorescence of each sample was

analyzed in 96-well plates using a microplate reader with excitation at 545 nm and fluorescence measured at 590 nm. Fluorescence results were compared to a standard H_2O_2 dilution curve from 10 μM to 0.0625 μM . Values of H_2O_2 are reported by mol divided by grams per fresh weight.

MDA - lipid peroxidation quantification

MDA generation as a detector of lipid peroxidation has been well established since the protocol by Heath and Packer (1968). The specific procedure from Sairam and Srivastava (2008) was followed, with slight modification. The same procedure for growing seedlings and exposing them to heat stress as above for the H_2O_2 quantification was followed. For MDA testing, three seedlings of approximately similar development stage were combined and total weight in grams measured. These seedlings were homogenized in 5mL of 0.1% trichloroacetic acid (TCA) with an electric homogenizer for a maximum of five minutes. The samples were centrifuged at 15,000g for 10 minutes and 2mL of the supernatant added to 4mL of 20% TCA and 0.5% of thiobarbituric acid (TBA). The mixture was incubated at 95°C in a hot water bath for 30 minutes and then cooled in an ice bath. Centrifugation at 10,000g and 4°C for 10 minutes was then performed and 200 μL of the supernatant transferred to a 96 well microplate. Absorbance at 532 and 600 nm was measured and the difference between these values divided by the molar extinction coefficient ($1.56 \text{ M}^{-1} \text{ mL}^{-1}$) and then divided by total seedling weight for quantification. Each treatment consists of four technical replicates for a total of 12 plants represented. Final concentration is reported as mol divided by milligrams of fresh weight.

Statistical analysis

All statistical analysis for H_2O_2 and MDA experiments was performed in R with one-way Anova significance of treatment analysis and linear modeling using the car and agricolae open-

source packages (Fox, 2001; de Mendiburu, 2023). Specific significant differences were calculated with Tukey's Honest Significant Difference analysis.

Results

Germination with BR Affects Wheat Gene Expression Throughout Plant Life

To understand the long-term transcriptional effects of BR seed treatment on wheat reproductive development, the wheat seeds were treated with BR only during germination, and then gene expression was evaluated about six weeks later. Evidence of the persistence of BR treatment from germination to booting is apparent in specific, differing gene expression between control plants and BR-treated plants even without heat stress. At the 0-hour time point, when plants were not yet subjected to stress, there were 130 differentially expressed genes (DEGs) between control and BR-treated floret tissue. Overall, the DEGs were enriched for six Gene Ontology (GO) categories with the most significant being “regulation of cellular respiration” (GO:0043457) and the others relating mainly to photosynthetic processes. Upon investigation of specific DEGs in the florets, there were three known peroxidases, two known heat shock proteins (HSPs), and one gene whose function is unknown in *Triticum aestivum* but whose closest ortholog in *Triticum turgidum* is an HSP (TraesCS1B03G0317200, TraesCS2B03G0307700, TraesCS3B03G1442700, TraesCS2B03G0098600, TraesCS7B03G0930300, TraesCS3D03G0238800). All three peroxidases had higher expression in the control floret while the HSPs had higher expression in the BR-treated florets prior to heat stress.

Differences between control and BR-treated plants without heat stress persisted and increased over the 24 hours of sampling. Under favorable conditions (22°C) at the 24-hour

sample time point, the number of genes differentially expressed between BR-treated and control plants increased to 7,895 genes in the rachis tissue and 2,477 in the floret tissue. Of these genes, those that were more highly expressed in the control rachis tissue were enriched for 94 GO categories including “Lactoperoxidase activity” (GO:0140825), “Cellular oxidant detoxification” (GO:0098869), “Hydrogen peroxide catabolic process” (GO:0042744), “Response to oxidative stress” (GO:0006979), “Phenylpropanoid metabolic process” (GO:0009698), and “Heat shock protein binding” (GO:0031072). Floret tissue showed a similar trend.

Disruption of Overall Heat Stress Response by BR Seed Soak

With heat stress, the durable effect of BR germination as much as six weeks post treatment is further elucidated. Principal component analysis of total gene expression for all tissue samples showed primary clustering due to tissue effects and no obvious clustering was visible by germination treatment, temperature conditions, or time of sampling (Figure 2a, Appendix A, Supplementary 1a). Yet, looking at the clustering of samples from specific tissues under heat stress showed control samples after 24 hours of heat specifically segregating in both the rachis and floret tissues (Figure 2b, Appendix A, Supplementary 1b). Conversely, the BR-treated samples after 24 hours of heat stress continued to cluster near earlier time point samples, indicating an obvious difference in gene regulation from the control samples following stress.

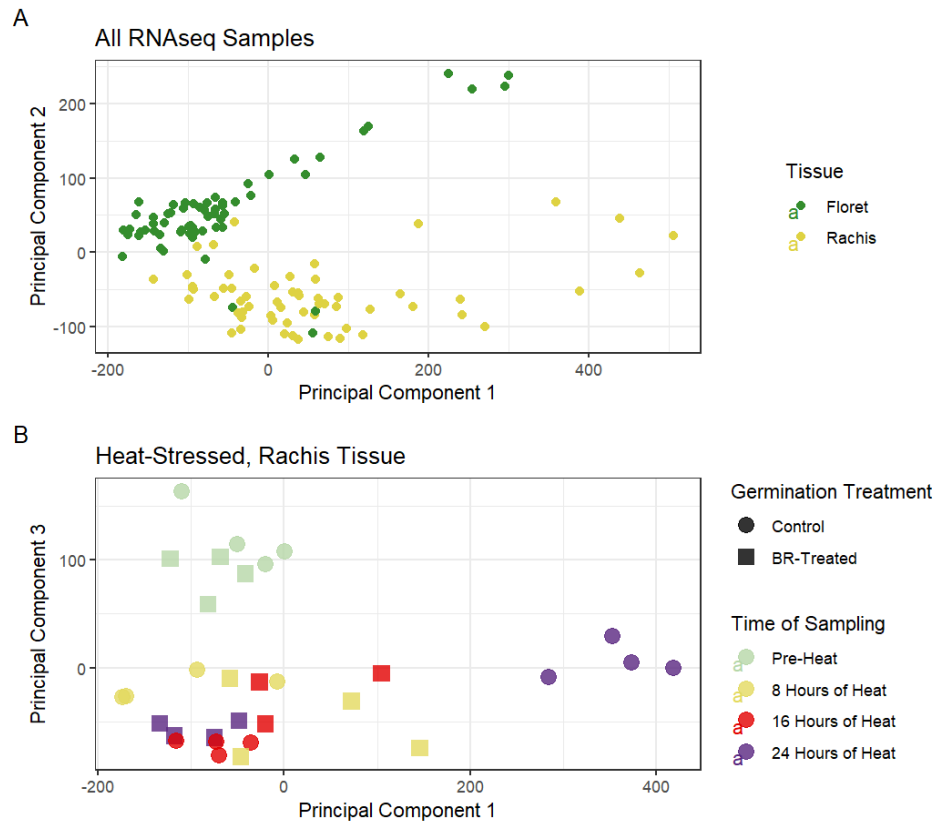


Figure 2: Principal Component Analysis of total gene expression for given samples, revealing tissue specific and germination treatment effects. A) All samples in the study plotted along PC axis one and two, colored by tissue type. B) Only rachis tissue, heat-stressed samples plotted along independently defined PC axis one and three, colored by hour of sampling after heat stress start and with shapes indicating the germination treatment.

To investigate the heat stress response differences between control and BR-treated plants, we looked at how each treatment group responded to heat over the 24 hours of sampling. Gene expression changes over time from zero hours to eight hours after heat stress shows BR-treated rachis tissue to have 12,039 DEGs, five times as many DEGs than the control rachis which had 2,417 DEGs (Figure 3a). The genes being expressed at eight hours of heat stress are very different between the BR-treated and control rachis, with DEGs being enriched for 427 GO categories. Interestingly, this relationship reversed when comparing time changes from zero to 24

hours of heat, as control plants had an average of 34,309 DEGs within both tissues while BR-treated plants had an average of only 5,826 DEGs within both tissues (Figure 3a, 3b).

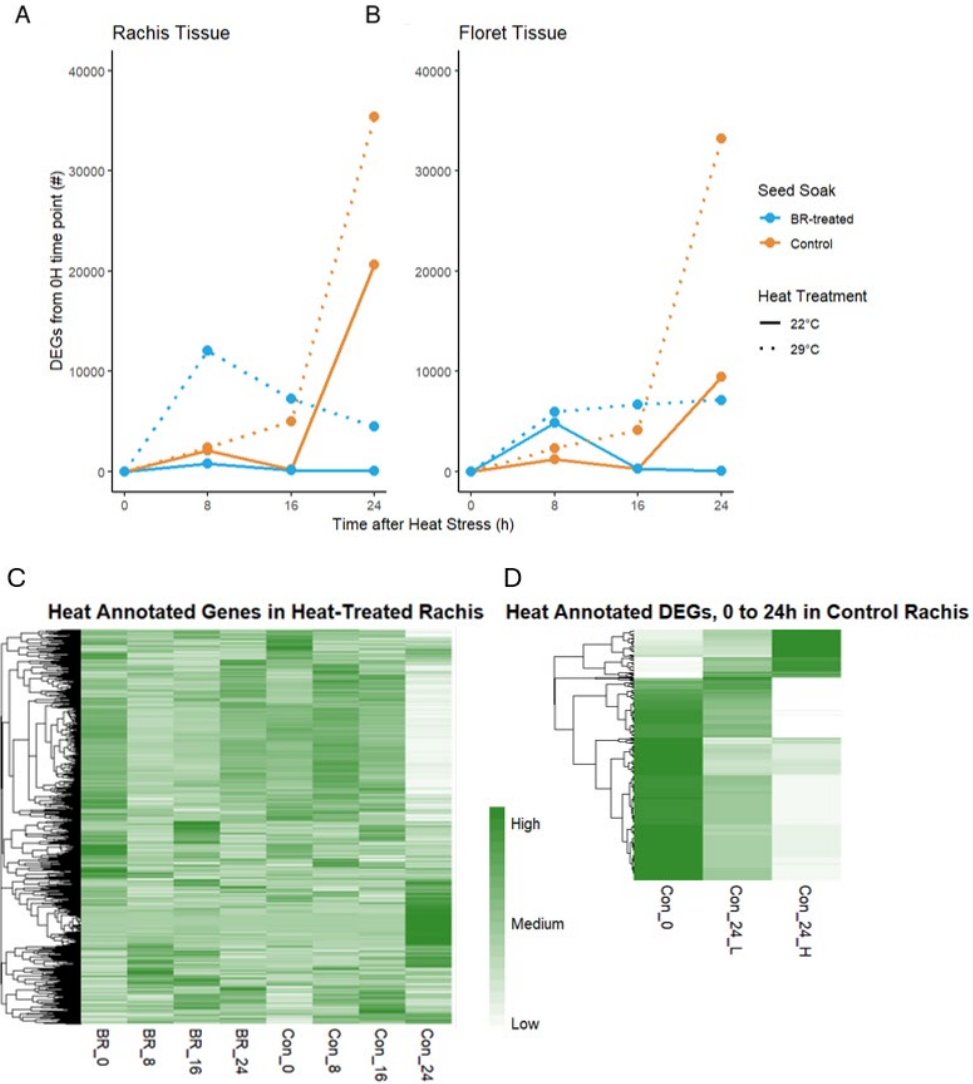


Figure 3: Difference in heat response of control and BR-treated plants. For heatmaps, each row represents a unique gene and the expression colorization is normalized within each gene or row, with increasing darkness of color relating to increasing expression. A) Quantification of the total number of significant DEGs from zero to each plotted time point in the rachis tissue. B) Quantification of the total number of significant DEGs from zero to each plotted time point in the floret tissue. C) Heatmap of all heat annotated genes sequenced and mapped; each column is labeled as seed soak treatment _ time point sampled (full gene list in Appendix A, Supplementary

2a). D) Heatmap of DEGs, between 0- and 24-hour sampling timepoints for control rachis tissue, which are heat response annotated. Columns represent average gene expression from zero hours, 24 hours without heat, and 24 hours with heat of control plants, from left to right.

While investigating relative gene expression changes over time, a distinction was observed between the number and the function of genes which are changing at given time points. Specifically, it is observed that the greatest change in gene regulation from pre-heat to heat stress, in control plants, occurs after 24 hours, yet enrichment of genes upregulated after 24 hours of heat stress does not include GO categories relating to heat stress (Table 1, Appendix A, Supplementary 3). Comparing zero to 16 hours after heat stress in control plants shows many heat related categories enriched, primarily for those genes upregulated after 16 hours of heat stress (Table 1). However, the total expression levels of all heat annotated genes in our mapped dataset still reveals the greatest evident change in expression at 24 hours after heat stress (Figure 3c). In response to 24 hours of heat stress, there is dynamic differential gene expression (DGE), with up and down regulation, while a more specific response at the 16 hour time point appears to favor primarily gene upregulation (Table 1, Figure 3d).

Conversely, BR-treated plants downregulated genes within heat response GO categories after heat stress. Gene expression comparison of zero hours to either eight or 24 hours after the start of heat stress, in both BR-treated rachis and floret tissues, shows heat responsive categories enriched primarily prior to heat stress (Table 1, Appendix A, Supplementary 4). When clustering each sample by the similarities in expression of all heat annotated genes, BR-treated rachis at eight hours after heat stress was most similar to control rachis at 24 hours with heat.

Table 1	0 versus 8 hours	0 versus 16 hours	0 versus 24 hours
Control Floret	<u>0 Hours</u> No obvious heat related categories <u>8 Hours</u> DNA repair	<u>0 Hours</u> Response to heat <u>16 Hours</u> Heat shock protein binding Chaperone-mediated protein folding Unfolded protein binding ATP-dependent protein folding chaperone Protein refolding Response to heat Cellular response to heat Protein folding chaperone Chaperone cofactor-dependent protein refolding Protein-folding chaperone binding Hsp90 protein binding Hsp70 protein binding Response to salt stress	<u>0 Hours</u> DNA repair <u>24 Hours</u> No obvious heat related categories
Control Rachis	<u>0 Hours</u> No obvious heat related categories <u>8 Hours</u> No obvious heat related categories	<u>0 Hours</u> Cellular response to heat <u>16 Hours</u> Unfolded protein binding Response to heat Protein refolding Protein-folding chaperone binding Heat shock protein binding ATP-dependent protein folding chaperone Chaperone cofactor-dependent protein refolding	<u>0 Hours</u> Hsp90 protein binding DNA repair <u>24 Hours</u> No obvious heat related categories
BR-treated Floret	<u>0 Hours</u> Unfolded protein binding Protein refolding Cellular response to heat Heat shock protein binding Hsp90 protein binding Hsp70 protein binding Post replication repair Misfolded protein binding Response to heat <u>8 Hours</u> Hsp70 protein binding Cellular response to cold	<u>0 Hours</u> No obvious heat related categories <u>16 Hours</u> Protein refolding	<u>0 Hours</u> Unfolded protein binding Response to heat Response to salt stress Cellular response to heat <u>24 Hours</u> Hsp70 protein binding
BR-treated Rachis	<u>0 Hours</u> DNA repair Unfolded protein binding Hsp90 protein binding Hsp70 protein binding Protein refolding DNA damage response Post replication repair <u>8 Hours</u> No obvious heat related categories	<u>0 Hours</u> Protein-folding chaperone binding Unfolded protein binding Response to cold <u>16 Hours</u> No obvious heat related categories	<u>0 Hours</u> Response to heat Unfolded protein binding Response to salt stress Post replication repair Chaperone cofactor-dependent protein refolding Hsp90 protein binding Heat shock protein binding Cellular response to heat Hsp70 protein binding Protein refolding Misfolded protein binding <u>24 Hours</u> No obvious heat related categories

Table 1: GO enrichment categories related to heat stress, comparing each time point to expression levels before heat stress (zero hour time point). For each time comparison, GO enrichment was performed for DEGs more highly expressed at zero hours and then separately for DEGs more highly expressed at the given time after heat. Therefore, this table does not represent the enrichment of all DEGs varying between the two times, but separately for DEGs with higher expression at the given time point subtitle. Not all GO categories shown; categories relating to heat stress were identified within total GO category enrichments.

Treatment with BR Interferes with Specific ROS-related Gene Expression during Heat Stress

Plants which were not treated with BR, the control plants, consistently utilized another method of long-term stress control: ROS homeostasis. Compared to the zero hour time point before heat, after both eight and 16 hours of heat stress control floret tissue shows enrichment for ROS homeostasis GO categories and by 24 hours of heat stress, both control tissues are enriched for these redox-related categories. Genes upregulated in control floret tissue after eight hours of heat were enriched for “Response to reactive oxygen species” (GO:0000302) and “Cellular response to oxidative stress” (GO:0034599). Following 16 hours of heat stress this redox GO enrichment continued strongly in the floret tissue while control rachis tissue upregulated genes were enriched only for “L-ascorbate peroxidase activity” (GO:0016688). Yet by 24 hours of heat stress, many more redox related GO categories including “Cellular oxidant detoxification” (GO:0098869), “Peroxidase activity” (GO:0004601), and “Response to oxidative stress” (GO:0006979) were enriched in both tissues for control plants. Expression of all redox annotated genes in our mapped dataset over time displays obvious regulation changes in control plants of these genes after 24 hours of heat stress, differing from all other heat time points in both tissues (Figure 4a, Appendix A, Supplementary 5).

Plants treated with BR do not utilize the same redox response pathway as control plants (Appendix A, Supplementary 6). Genes which are downregulated after 24 hours of heat stress in

BR-treated plants are enriched for several redox GO categories. Prior to heat stress, genes that had higher expression in BR-treated tissues were enriched for “Cell redox homeostasis” (GO:0045454), “Response to hydrogen peroxide” (GO:0042542), and “Proline metabolic process” (GO:0006560). Yet, the expression levels of all redox related genes in BR-treated plants appears more similar across all time points compared to the distinct change in control tissues (Figure 4a, Appendix A, Supplementary 5).

Direct comparison of gene expression between control and BR-treated plants at eight or 16 hours after heat stress had no enrichment of genes relating to redox homeostasis in either tissue, meaning both control and BR-treated plants had relatively similar expression of these ROS-related genes during the first 16 hours of heat stress. However, the 24-hour heat time point highlighted the branching redox responses of control and BR-treated plants. For genes with higher expression in control rachis tissues compared to BR-treated rachis tissue, several GO categories enriched are related to redox pathways (Figure 4b). This trend was similar in the floret tissue, however there were fewer categories noted overall (Appendix A, Supplementary 7).

Control and BR-treated plants do diverge in their redox gene regulation at the 24-hour time point, but it is less immediately clear that this difference arises due primarily to a heat stress response of control plants. Specifically, there was some enrichment of a few redox related categories comparing zero to 24 hours in the non-stressed control plants as well. We thus investigated whether we could differentiate changes in regulation of redox related genes for control plants which would be distinct between the control temperature (22°C) and heat stress. We compared the expression patterns for specific peroxidase genes that were DEGs in control rachis and florets from zero to 24 hours of heat stress (Figure 4c, Appendix A, Supplementary 8).

Several of these genes do increased expression over time in control rachis regardless of stress conditions, however all of them have additional increased expression at 24 hours when heat stress is added. Additionally, heatmap analysis of all redox annotated DEGs arising from zero to 24 hours of control plants undergoing heat stress shows a clear added effect of the heat stress which further upregulates the expression of these genes (Figure 4d). Finally, additional GO enrichment and LRT analysis supported that, while control plants do upregulate redox gene expression throughout development, there is an additional redox gene regulation response to heat stress in control plants, which is not evident in BR-treated tissues (Appendix A, Supplementary 9).

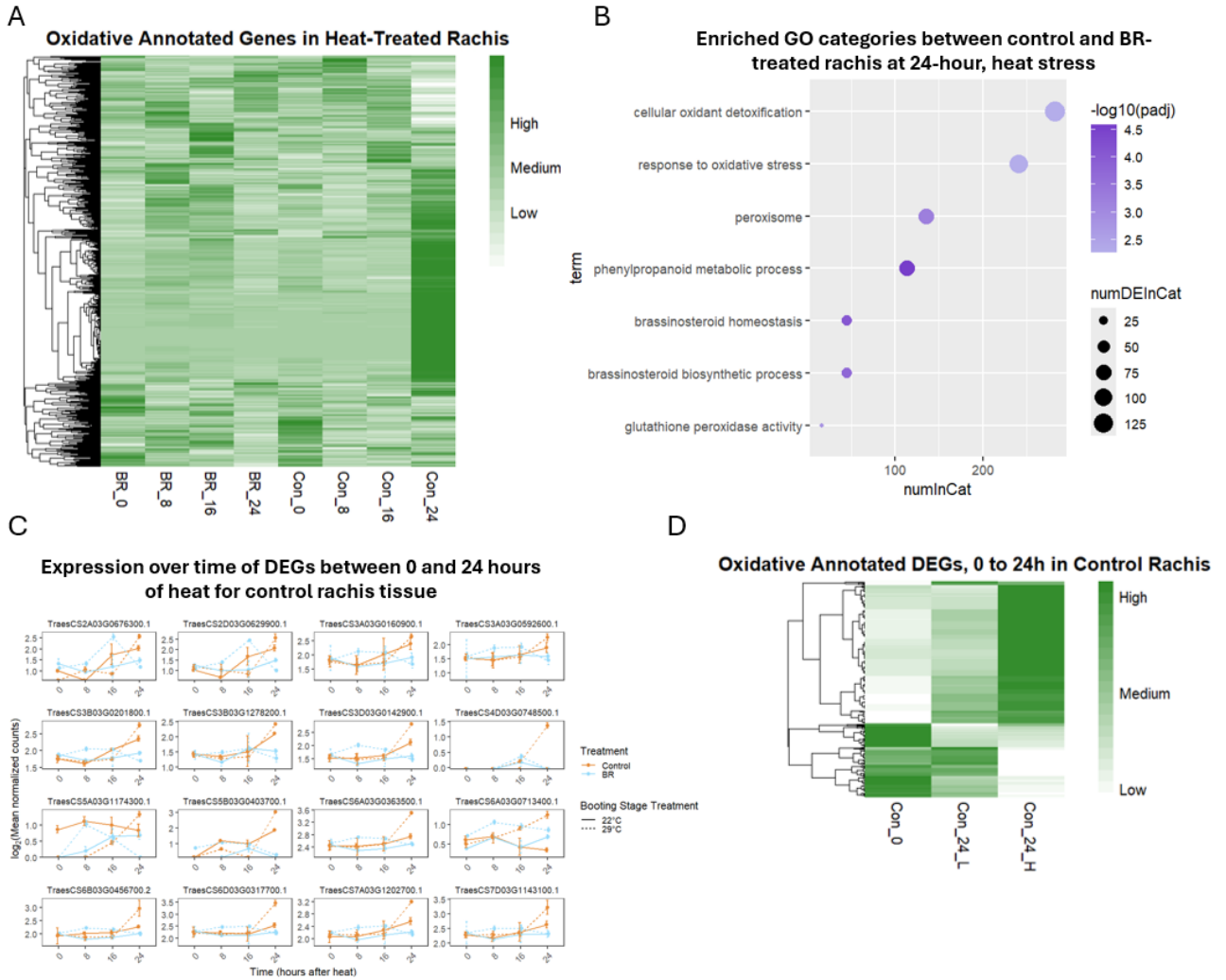


Figure 4: Patterns of differentially expressed redox related genes all for rachis tissue. For heatmaps, each row represents a unique gene and the expression coloration is normalized within each gene or row, with increasing darkness of color relating to increasing expression. A) Heatmap of all redox annotated genes sequenced and mapped. (full gene list in Appendix A, Supplementary 8). B) Select GO terms enriched between BR-treated and control plants at 24 hours of heat stress. C) Time course of expression for peroxidases with the 16 highest LFCs from zero to 24 hours in control rachis. Plotted read count for BR-treated and control rachis over each time point as averaged between each sample in the treatment group and error bars representing the standard deviation. D) Heatmap of redox annotated DEGs from zero to 24 hours of heat stress in control plants. Columns represent average gene expression from zero hours, 24 hours without heat, and 24 hours with heat of control plants from left to right.

Differing Redox Related Gene Expression
 Patterns Between Rachis and Floret Tissues
Show Tissue-Specific BR Effects

There was an overall lack of heat response and redox related gene upregulation from BR-treated plants displayed in both reproductive tissues that we sampled. Still, we felt that understanding potential specific differences between the two tissues was crucial to answering why only certain phenotypes were improved by BR treatment following heat stress. We looked at the differences in expression changes over time of rachis tissue against those of the floret tissue. From zero to 24 hours of heat stress in control rachis tissue, DEGs were enriched for “BR-homeostasis” (GO:0010268), and “BR-biosynthetic processes” (GO:0016132) while control florets were not. Comparing BR-treated and control rachis at 24 hours of heat stress additionally revealed these BR related GO categories while floret tissue comparison did not (Figure 4b). Genes differentially expressed between no stress and heat stress at eight hours in BR-treated rachis were also enriched for “BR metabolic process” (GO:0016131), which was not the case in floret tissue. Taken together with the large number of DEGs arising between zero to eight hours in BR-treated rachis and the clustering of eight-hour BR-treated rachis alongside 24-hour control rachis for heat annotated gene expression, neither of which appeared in the floret tissue, it appears that BR-treated rachis is behaving somewhat more similarly at eight hours post heat stress to control rachis tissue at 24 hours, while floret tissue is not.

We further performed GO enrichment between the rachis and floret tissues from both BR-treated and control plants. Without heat stress, analyzing control and BR-treated plants independently showed both treatments to have upregulation and enrichment of redox related pathways in the rachis tissue relative to floret tissue (Table 2). After heat stress, control plants continued to display expression differences and enrichment of redox-related genes between the

two tissues but this difference was no longer specific to genes upregulated in the rachis compared to the floret (Table 2). This may indicate that heat stress response does not require increased expression of redox genes in either specific tissue, more than in the other, but that regulation of each gene is more fluid, and each tissue may be using unique gene combinations to overcome ROS stress (Figure 5a). Contrastingly, after heat stress, BR-treated plants showed continued enrichment of redox related genes, specifically upregulated in the rachis as compared to the florets (Table 2). Overall expression levels of ROS-homeostasis related genes, which were differentially expressed between the rachis and floret of BR-treated plants, do not appear to be significantly affected by heat stress (Figure 5b).

Table 2 -- Rachis versus Floret Tissue at 24 hours		ALL DEGs	Rachis Upregulated	Rachis Downregulated
Control Plants	No Heat	Hsp90 protein binding Peroxidase activity Hydrogen peroxide catabolic process Lactoperoxidase activity Response to oxidative stress Cellular oxidant detoxification Response to endoplasmic reticulum stress Peroxisome	Response to oxidative stress Lactoperoxidase activity Peroxidase activity Cellular oxidant detoxification Hydrogen peroxide catabolic process Peroxisome	Hsp90 protein binding
Control Plants	Heat Stressed	DNA damage response Response to cold Thioredoxin peroxidase activity Hsp90 protein binding Cellular response to oxidative stress Response to endoplasmic reticulum stress Glutathione metabolic process Glutathione binding	Defense response	Glutathione metabolic process Hsp90 protein binding Response to cold
BR-Treated Plants	No Heat	Peroxidase activity Lactoperoxidase activity Hydrogen peroxide catabolic process Response to oxidative stress Cellular oxidant detoxification Response to stress Glutathione binding	Peroxidase activity Cellular oxidant detoxification Hydrogen peroxide catabolic process Lactoperoxidase activity Response to oxidative stress Response to stress	Cellular response to heat

Table 2 -- Rachis versus Floret Tissue at 24 hours		ALL DEGs	Rachis Upregulated	Rachis Downregulated
BR-Treated Plants	Heat Stressed	Regulation of cellular respiration Peroxidase activity Hydrogen peroxide catabolic process Response to oxidative stress Lactoperoxidase activity Cellular oxidant detoxification Response to stress Response to reactive oxygen species Glutathione binding Trehalose metabolism in Response to stress	Hydrogen peroxide catabolic process Peroxidase activity Lactoperoxidase activity Response to oxidative stress Cellular oxidant detoxification Systemic acquired resistance Glutathione binding Response to reactive oxygen species Phenylpropanoid biosynthetic process Response to water deprivation	Response to stress

Table 2: GO enrichment categories of DEGs as comparing rachis to floret tissue within each treatment group at the 24-hour sampling time. For each treatment group we analyzed GO enrichment for all DEGs arising between the tissues as well as by grouping DEGs by tissue specific expression. Select categories shown relating to stress and ROS homeostasis.

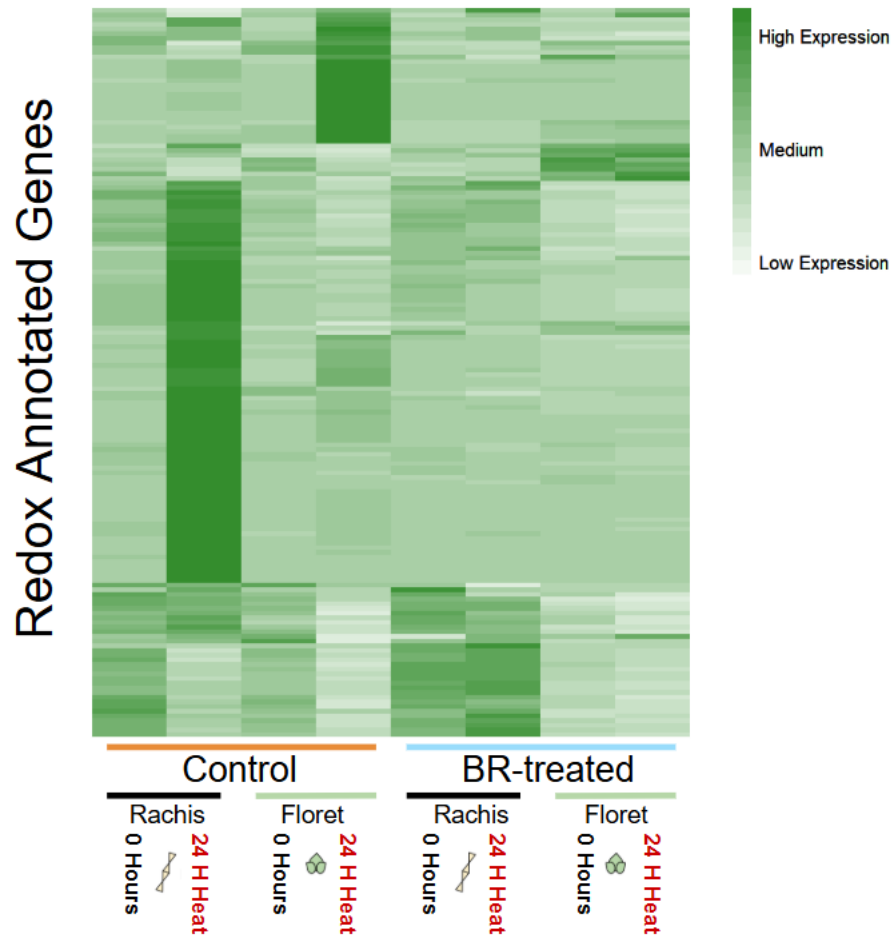


Figure 5: Heatmap of redox-annotated genes which were differentially expressed between rachis and floret tissues at 24 hours of heat stress. Genes were selected from control rachis versus floret and from BR-treated rachis versus floret and filtered for annotations related to oxidation control, then combined into one dataset for mapping. For all heatmaps, each row represents a unique gene and the expression coloration is normalized within each gene or row, with increasing darkness of color relating to increasing expression.

Seedling ROS Homeostasis is Improved by BR Treatment under Heat Stress

As these RNAseq data indicated a strong difference in the regulation of ROS-homeostasis gene expression between control plants and BR-treated plants, we wanted to determine if a difference in measurable redox control could be seen in the wheat seedlings immediately following BR application. We germinated seeds for three days in BR or a control then applied a

strong heat to these seedlings and tested ROS presence and lipid peroxidation to quantify their ability to control ROS proliferation prior to and following stress.

The most stable and prolific ROS, H_2O_2 , had lower accumulation in BR-treated seedlings after heat stress as compared to the control seedlings (ANOVA, $p < 0.001$, Figure 6a).

Accumulation of MDA, indicating lipid peroxidation, was additionally reduced in the BR-treated plants prior to heat stress (T-test, $p = 0.03$, Figure 6b). However, BR-treated seedlings did not appear as well able to limit oxidation during heat stress, as MDA content between non-stressed and heat-stressed conditions slightly increased in BR-treated seedlings, while control plants were trending to reduce MDA content (T-test, $p = 0.3$, $p = 0.8$ respectively, Figure 6b). These tests do indicate that BR-treated seedlings may have higher antioxidant capability prior to heat stress such that these seedlings have reduced ROS accumulation and overall oxidative levels when compared to the control. These tests further reveal that the difference in redox homeostasis capabilities resulting from BR treatment begins at least as early as the 3-day seedling stage. However, this redox capability may not rely on persistent gene expression of antioxidant genes until booting, as no redox GO categories are enriched when comparing control and BR-treated plants before heat stress during the booting stage.

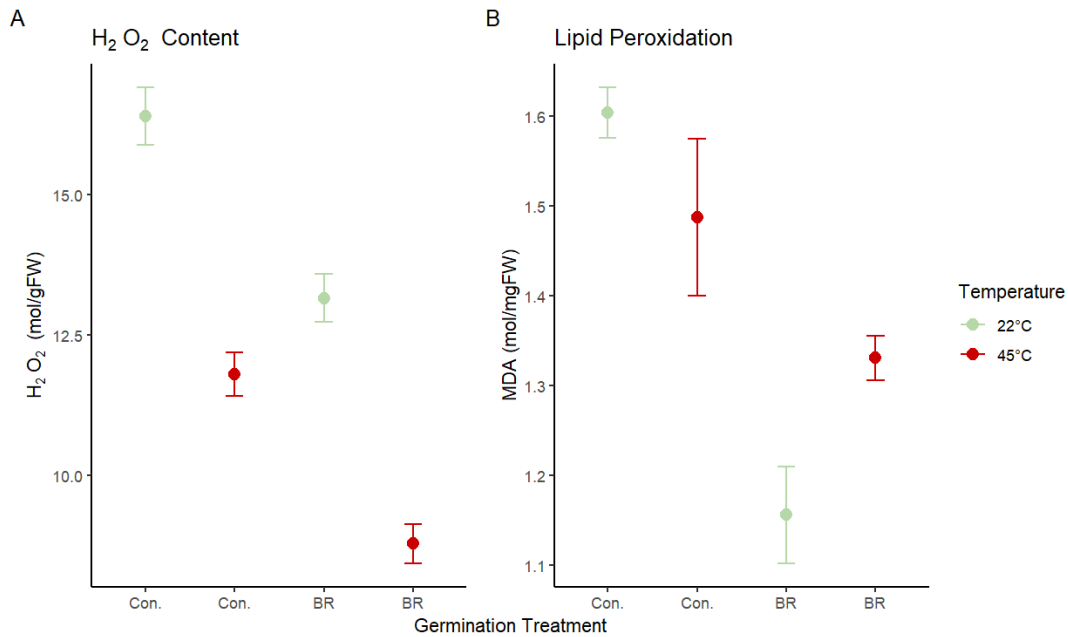


Figure 6: Three-day Seedling ROS homeostasis quantification pre- and post-heat stress after germination in either control or BR. Seedlings were exposed to the corresponding temperatures for two hours immediately prior to sampling. Points represent averages from a minimum of n=12 plants, error bars are standard error. A) Quantification of H₂O₂ in seedlings utilizing an Amplex Red assay kit. B) Lipid peroxidation quantification of seedlings utilizing the MDA oxidation method.

Discussion

In this study, we leveraged brassinosteroid (BR) hormone, known to improve crop yield and stress tolerance in inconsistent manners, to identify potential heat tolerance mechanisms in spring wheat that would support improved yield components without sacrificing vigor (Kothari and Lachowicz, 2021). For Vida wheat, germination in BR hormone provided heat stress protection for spike length and spikelet number yield components following a heat stress which began as late as the booting stage (Kothari et al, in preparation). In keeping with our previous research, we found that there was a persistent effect of BR on gene expression during the normal booting stage as much as six weeks following application. This long-term effect was discernible

by differential gene expression between control and BR-treated plants without heat stress as well as by substantial differences in each treatment group's response to heat stress. The gene expression response to heat stress was greatly diminished in BR-treated plants as compared to control plants as evidenced by much fewer DEGs arising between zero and 24 hours of heat stress. Analysis of gene regulation in heat-stressed and tissue specific samples revealed the 24-hour, heat-stressed control plants to have a very different gene expression pattern compared to all other time points. Yet, the BR-treated 24-hour heat-stressed plants continued to cluster near the earlier time point samples, show their maintenance of reduced, “unstressed” gene expression.

While investigating the difference in heat stress response between the BR-treated and control plants, we found an interesting dichotomy in how control plants regulated genes annotated as heat stress responsive. A number of these genes were in fact downregulated in response to heat stress, indicating a negative regulation of heat stress response and highlighting the variability in function of genes with only annotation related functions. Further, these genes followed gene-specific regulation patterns, with significant variability in whether each gene was up or down regulated in response to heat stress. Contrastingly, redox related genes were more consistently upregulated in control plants after 24 hours of heat stress. This finding could point to a greater reliance on ROS regulation long-term as a more global and positively regulated response to heat stress. This may also relate to the necessary control of ROS in developing reproductive tissues which becomes more pressing as heat stress generates additional ROS. We therefore investigated redox homeostasis gene expression specifically as it differed between BR-treated and control plants.

We hypothesized that BR-treated plants may upregulate redox-related genes after heat stress, more so than the control plants, since BR has an observed positive influence on antioxidant activity in several plants (Xia et al., 2009; Zhou et al. 2014; Zhu et al., 2016). However, we found that BR-treated tissue continued to have a reduced expression of redox related genes in response to heat as compared to the control plants, similarly to what we observed in the heat response annotated genes. This is a direct result of the many fewer genes changing expression in BR-treated plants from zero to 24 hours of heat stress compared to control plants, both in total number and specifically for DEGs annotated to have redox function. Yet, the positive influence of BR treatment on spike length, which most closely relates to rachis tissue elongation, led us to investigate ROS homeostasis in each tissue more closely. The difference in gene expression and enrichment between rachis and floret tissues, without heat stress, highlights an increased expression of certain ROS-homeostasis genes in the rachis over the floret tissue. Several studies have reviewed the importance of ROS maintenance for preserving meristematic identity which could account for the necessity of increased expression in rachis tissue, as this tissue contains the inflorescence meristem (Qin, 2023; Zeng et al., 2017). The ability of BR-treated plants to maintain redox related gene expression in the rachis, regardless of heat stress, could thus explain an improved spike length as meristem identity is sustained and the spike is able to elongate further. There is evidence that BR signaling is required for meristem function and growth in roots (González-García et al., 2011). Although, the BR-treated plants do have lower expression overall of these ROS homeostasis and antioxidant genes at 24 hours of heat stress when compared to the control plants. To understand this, we can consider the contrast in antioxidant activity between the BR-treated and control seedlings. It is

possible that the expression of redox related genes is not as necessary in BR-treated tissue during the booting stage, before or after heat stress, since antioxidant proteins may be already existent within the cell. We hypothesize that BR treatment is “priming” the wheat seedlings to increase antioxidant activity beginning during germination, when the hormone is applied, up until the booting stage (Figure 6).

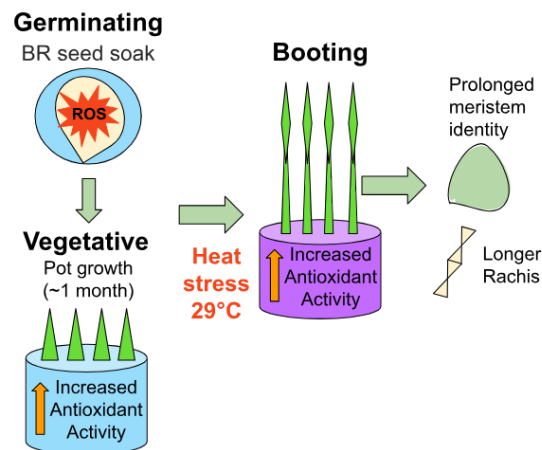


Figure 6: Proposed model for BR treatment which results in increased spike length. Treatment with BR during the germination phase causes a ROS burst which leads to increased antioxidant activity throughout the lifespan of the plant. The increased antioxidant activity protects meristem identity in the rachis under heat stress, allowing elongation beyond that of the control plants.

In addition to the differences in ROS-homeostasis which may have a stronger effect on the rachis tissue, specific differences of BR-treated rachis and floret tissue points to a greater effect of BR on the rachis tissue itself. Rachis tissue treated with BR at eight hours after heat stress was similar to control rachis after 24 hours in having significant gene expression changes, enrichment of BR related GO categories, and in expression of heat annotated genes. None of these similarities appeared between BR-treated and control floret tissue. Rachis tissue which had

been treated with BR may therefore be reacting more quickly to heat stress than the floret, in tandem or perhaps in response to its different redox gene expression course.

In our prior research, we have identified expression differences of genes regulating histone and DNA structure between control and BR-treated whole spikes under heat stress (Kothari et al., in preparation). Indeed, these differences may be the cause of the other expression differences between BR-treated and control plants identified in this paper. Investigating specific tissue responses in these plants allowed us to refine our understanding of pathways which may be contributing to the unique phenotypic changes we have observed for yield components. Considering we found tissue to be the primary cause of sample clustering in PCA analysis, and that there were many DEGs between the two tissues before and after heat stress, it is obvious that heat stress response is, in part, tissue specific. The distinct differences in rachis and floret tissue expression may have masked the findings detailed here when both tissues were sampled together.

Research into continuing crop improvement is hindered by our limited understanding and depth of effectual genes within a given organism. Wheat's large hexaploid genome has left researchers with much to explore and more to find as our knowledge of the interconnectedness of plant function expands. Therefore, to direct our search, we must consider novel investigation methods and alternative reactive pathways in gene targeting for crop improvement.

Here we utilized brassinosteroid application to manipulate existing molecular pathways in spring wheat and identify genetic regions that could be targeted for improved heat tolerance. Treatment with BR led to improved yield components after heat stress associated with redox homeostasis pathways. These redox gene expression changes were not correlated with detrimental trade-offs for total seed count, protein, or seed size -- all final factors crucial to

producers and the wheat market (Kothari et al., in preparation). It is essential we consider non-traditional tolerance mechanisms and understand the specific aspects of wheat affected by heat in order to produce spring wheat lines with improved heat tolerance. We further the understanding of mechanisms by which wheat tolerates stressors to address conditions that are arising under climate change.

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CHAPTER THREE

HEAT STRESS TOLERANCE IN WHEAT MODULATED BY
REACTIVE OXYGEN SPECIES INTERACTION WITH
BRASSINOSTEROID

Contribution of Authors and Co-Authors

Manuscript in Chapter Three

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Abstract

The production of wheat is crucial to feeding people across the globe but also simultaneously susceptible to heat stress. To improve heat stress tolerance, researchers must explore the complex regulation of tolerance mechanisms used by plants. In this study, we applied brassinosteroid (BR) hormone or a BR synthesis inhibitor, propiconazole, during spring wheat seed germination to investigate the long-term effects of initial hormonal disruption on plant heat tolerance as much as six weeks later. We tested the effects of heat stress during the reproductive, booting stage of wheat and found that BR application had an enduring effect on antioxidant activity and photosynthetic traits. Effective photochemical yield decreased, and control of superoxide dismutase activity improved from BR-treatment during heat stress compared to controls. These findings correlated with better heat tolerance of yield components and were opposite to the effects of the BR-inhibitor. Blocking an ROS burst by co-germinating plants in a NADPH-oxidase inhibitor, DPI, concurrently with the BR, exaggerated the BR effect on photosynthetic traits measured under heat stress and during heat stress recovery. These findings improve our understanding of the BR regulation of specific pathways that can contribute to wheat heat tolerance. We also offer evidence that in spring wheat, similar to other plants tested, some aspects of BR treatment response are affected by reactive oxygen species signaling at the time of BR application. These results further the knowledge of heat stress response in spring wheat and suggest BR modulation of pathways which can improve heat tolerance.

Introduction

Heat stress is a rising concern around the globe, with even small increases in temperature causing yield losses in crop species (Petersen, 2019). The mitigation of heat stress is a complex process for any plant which necessitates the balance of growth and damage control (Hassan et al., 2020). Investigating the mechanisms of plant stress alleviation and how these can promote continued growth will allow researchers to engineer heat tolerant crops and increase yields in a changing environment (Dhankher and Foyer, 2018). For wheat, which provides 67 kg/year of food per capita, understanding heat tolerance mechanisms is crucial to continue feeding the human population (FAO, 2024).

At the cellular level, heat stress affects many aspects of crops which are deleterious to growth and yield (Akter and Islam, 2017). Increased temperatures induce protein unfolding, slow protein production, and escalate oxidation as reactive oxygen species (ROS) are produced (Hassan et al., 2020). Compounds containing highly oxidative oxygen molecules, ROS are devastating in a cell when unchecked since they change oxidation states and can affect function for a variety of cellular processes (Mittler et al., 2022). Under ideal conditions, ROS are highly regulated, with production by NADPH-oxidases and removal controlled via a variety of antioxidants including metabolites such as pigments and active enzymes like superoxide dismutase (SOD) and ascorbate peroxidase (APX) (Mittler et al., 2022). Heat stress, however, proliferates ROS at a much higher rate; therefore, managing ROS under heat stress is essential to continued cellular viability (Lal et al., 2022).

The past twenty years have seen a flood of understanding into the role brassinosteroids (BR) play in various stress responses in plants, including for heat stress in wheat (Krishna, 2003;

Basit et al., 2021). As a short distance signaling hormone, BR has primary roles in plant development but also improves the tolerance to a variety of stressors in a diverse range of crops (Nolan et al., 2017). Several studies have reported an increase in antioxidant activity triggered by exogenous BR application, particularly in antioxidant enzymes like SOD and APX (Cao & Zhao 2008; Kumari and Hemantaranjan, 2018). Photosynthetic capability and resiliency also increase with BR treatment providing energy assimilation and sustained growth when plants undergo heat stress (Zhao et al., 2017). Yet, the longevity of these effects, the specificity of BR effects on various tissues, and differences in plant cultivars or species remain unclear.

Our preliminary research revealed a positive effect of germinating wheat seeds in BR to mild heat stress during the reproductive development, booting stage. During heat stress, the tissue specific yield components of spike length and spikelet number did not have as much loss compared to non-stressed plants; they were protected from heat stress as much as four weeks after BR treatment. Our gene expression analysis of wheat's initial response to heat stress revealed many genes involved in oxidation control and photosynthesis being differentially regulated between control and BR-germinated plants (Chapter 2). We hypothesized that improvements in antioxidant activity and/or photosynthesis prompted by BR treatment may persist from germination to reproduction and wanted to understand: is BR-induced yield component protection from heat stress dependent on antioxidant activity and/or photosynthetic efficiency promoted by BR treatment?

The elicitation of increased antioxidant activity by BR is dependent on an initial ROS-burst following BR application in other crop species (Yan et al., 2015). We limited the BR induction of an ROS burst with diphenyleneiodonium (DPI), an NADPH-oxidase inhibitor, to

test the mechanisms of potential BR effects on antioxidant activity in wheat. We further tested the effects of DPI alone and in combination with the BR synthesis inhibitor propiconazole (PPZ). We then measured antioxidant enzyme activity of SOD and APX, as well lipid peroxidation levels via the accumulation of malondialdehyde (MDA), weekly from germination to heading for the wheat variety ‘Vida’ (PI 642366) (Lanning et al., 2006). We further measured agronomic traits, photosynthetic yields, and yield components with each of these germination treatments before and following a control temperature or heat stress during the booting stage.

We posited that halting an ROS-burst would prevent BR inducement of antioxidant activity and we would see a subsequent return to typical heat loss phenotypes observed in control plants across several yield components. We anticipated that the co-germination of plants in BR and DPI could further alter photosynthetic capability, and we could potentially include this pathway as one for which BR response is dependent on an initial ROS burst. Distinguishing the effect of BR regulation on wheat antioxidant activity during heat stress could be beneficial as oxidation control is important in many different stress responses. Pinpointing existing mechanisms for spring wheat stress tolerance and understanding the role of BR in stress response phenotypes can aid breeding programs to select for more heat tolerant, high yielding wheat in a changing climate.

Methods

Plant material and experimental design

Wheat cultivar ‘Vida’ (PI 642366) seeds were placed in 0.4mL water per seed with either a mock, 1 μ M 2,4-epibrassinolide (EBR), or 5 μ M propiconazole (PPZ) (Lanning et al., 2006). Additionally, each of these treatments had a second seed soak treatment of either 2 μ M

diphenyleneiodonium chloride (DPI) or control which was added at the same time (Appendix B, Supplementary 1). Since each of the treatment chemicals were solubilized in DMSO, a consistent 0.00006% of DMSO was added to all six treatments. The combinations of the germination solutions resulted in six total seed treatments (Figure 1). Seed germination was performed in a sterile petri dish in the dark, covered in aluminum foil to prevent de-etiolation. After 48 hours, the seeds were planted approximately 1" deep in Sunshine mix soil in a 7" pot with four seeds per pot evenly spaced. Pots were placed in growth chambers with a 16-hour photoperiod with fluorescent and LED bulbs at 27,000 Lux light intensity, kept at 22°C at all times. Plants were watered daily depending on soil moisture needs and watered with fertilizer once a week with 100 mg/L 20-20-20 Professional Peters fertilizer per pot. Pot placement within the chamber was in a randomized block design. For pots which did not have 100% seedling emergence, extra seedlings which had undergone the same germination treatment were transplanted around the 12th day to ensure each pot had a minimum of three plants per pot to avoid effects of competition.

At the booting stage, half of the plants from each germination treatment were moved to a growth chamber under the same lighting but kept at 29°C to simulate mild heat stress. The other half of each treatment was kept at 22°C for control comparison. Pots were removed from the 29°C heat stress and returned to the 22°C chamber once half of the plants in the pot had anthers dehisced. Plants were then grown to senescence for agronomic traits and photosynthetic data or destructively sampled each week until heading for enzyme activity data.

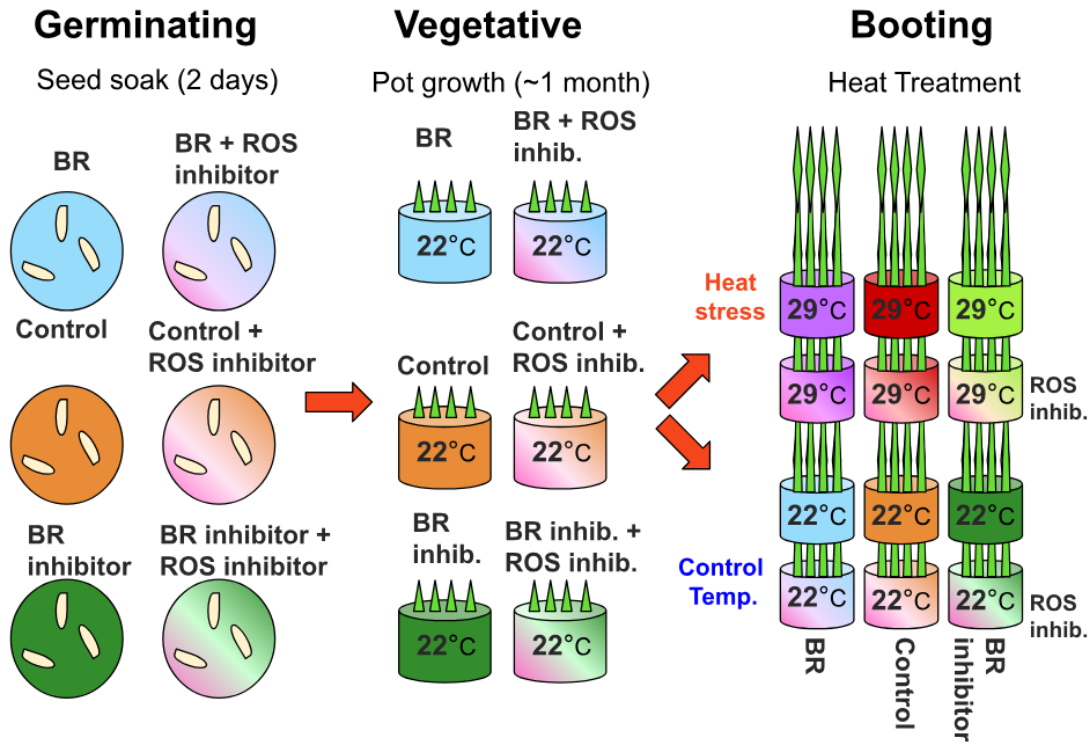


Figure 1: Experimental design. The BR-inhibitor is PPZ and the ROS-inhibitor is DPI, plants were grown with four biological reps, or four plants, per pot.

Agronomic trait measurement

All plants were assessed daily for seedling emergence, tiller emergence, booting time, and heading time. Plant phenotypic abnormalities were assessed 16 days after planting, maximum tiller number was counted between the 22nd and 27th day after planting, and productive tiller number counted following senescence. Tiller height was measured from fully senesced spikes and the heads collected for spike length, spikelet number, seed number, and seed weight assessments. Mature heads were threshed by hand and seed count performed via an automatic seed counter.

Photosynthetic measurements

Once weekly after planting, photosynthetic measurements were taken from two pots, for a total of eight plants per treatment via a Pulse Amplitude Modulator (PAM). The pots were randomly selected for each treatment from different blocks after planting, and these same pots were used throughout the experiment. Measurements were taken on dark-adapted plants four hours prior to dawn, with four plants per pot measured clockwise in the same sequence for every pot and the sequence of which pots to measure randomized but consistent weekly. The primary or flag leaf of each plant was measured approximately one inch below the leaf tip using actinic + yield settings of the PAM to allow measurement of $Y(II)$, NPQ, and $Y(NO)$. Time between initial light pulse and the subsequent measurement was 30 seconds. During the booting heat stress stage, the heat-treated pots were moved to the room temperature chamber 30 minutes before the usual measurement start time and allowed to re-adapt to the dark. The heat-treated plants remained in the control temperature for the duration of the photosynthesis measurements, about two hours, before being returned to the heat chamber. These were plants included in agronomic trait collection but there was no evident effect on final plant development from the brief heat stress removal. Measurements continued weekly until nine weeks after planting, when leaves began to senesce and photochemical measurements approached unmeasurable levels. Two experimental replications were performed with each treatment therefore represented by 32 plants for weeks one to four before heat stress, and 16 plants representing each treatment from weeks five and six during the heat stress.

We measured effective photochemical yield $Y(II)$ which captures the efficiency of the photosystems; efficiency values decrease as systems are damaged by stress (Zahra et al., 2023). We additionally measured non-photochemical quenching $Y(NPQ)$, showing how much excess

energy can be managed, and non-regulated energy loss $Y(NO)$ which is a measure of how much energy is being released into the cells unchecked and therefore could be a generator of additional ROS or reactive compounds (Govindjee, 2014; Li et al., 2021).

MDA – lipid peroxidation quantification

MDA is an end-product of the oxidation of lipids within a cell and therefore increased MDA signals an increase in the oxidation state of plant cells (Ayala et al, 2014). MDA generation as a detector of lipid peroxidation has been well established since the protocol by Heath and Packer (1968). The specific procedure from Sairam and Srivastava (2001) was followed, with slight modification. The plants sampled for MDA quantification were simultaneously sampled for SOD and APX activity, with four plants per replication having tissue divided between the MDA preparation and the enzyme activity preparation which occurred concurrently. Fresh primary or flag leaves from four plants were torn in half, combined to 0.25g, and homogenized in 5mL of 0.1% cold trichloroacetic acid (TCA) with an electric homogenizer between four to nine minutes. The samples were centrifuged at 15,000g for 10 minutes at 4°C and 1mL of the supernatant added to 2mL of 20% TCA and 0.5% of thiobarbituric acid (TBA). The mixture was incubated at $95^{\circ}\text{C} \pm 5^{\circ}\text{C}$ in a hot water bath for 30 minutes and then cooled in an ice bath. Centrifugation at 10,000g for 10 minutes at 4°C was then performed and 200uL of the supernatant transferred to a 96 well microplate. Samples were kept covered in aluminum foil and in ice throughout excepting the time in the hot water bath. Absorbance at 532 and 600 nm was measured and the difference between these values divided by the molar extinction coefficient ($1.56 \text{ M}^{-1} \text{ mL}^{-1}$) used for total quantification. Each treatment was represented by four replications, for a total of 16 plants sampled; sampling order was performed in a randomized

block design with each replication acting as a block. Plants were measured once a week for six weeks and final concentration is reported as nmol/gFW.

Enzyme activity

Fresh primary or flag leaves from four plants per treatment were torn in half, combined with some secondary leaves, and flash frozen in liquid nitrogen. The leaves were ground with mortar and pestle in a thin layer of liquid nitrogen and added to a cold extraction buffer on a tared scale, with $0.5\text{g} \pm 0.02\text{g}$ of ground tissue added to 5mL of buffer. Extraction buffer was prepared with 0.1M KPO_4 and 0.5mM EDTA at pH 7.5. Samples were then filtered through sterile cheesecloth and centrifuged at 15,000g for 15 minutes at 4°C. Supernatant was used for specific SOD and APX activities. Extracts were kept covered with aluminum foil and on ice throughout sample preparation.

For SOD activity, SOD activity buffer was prepared the day before the experiment with 50mM potassium phosphate, 13mM methionine, 50mM sodium bicarbonate, and 0.1mM EDTA then adjusted to 7.8pH with potassium hydroxide. After enzyme extraction and just before beginning the SOD activity assay, 50 μM of nitroblue tetrazolium was added to the SOD activity buffer. Activity of SOD was measured with three technical replicates per treatment replication and a no-light control, with 33 μL of enzyme extract added to 967 μL of buffer. For each technical replicate, 10 μL of riboflavin was added to all samples and placed on ice. The samples on ice were then placed under a fluorescent lamp and the reaction was allowed to take place for 7-15 minutes. Concentration of riboflavin and duration under the light was adjusted weekly to account for differences in enzyme strength, as too much riboflavin or time overwhelmed samples in earlier weeks and complete coloration of every sample occurred, leading to an ability to measure

differences between samples. Each week was compared to respective controls which had the same amount of riboflavin and time under the light as the treated samples. Controls had only 990 μ L of SOD activity buffer and 10 μ L of the riboflavin and were duplicated for each of the technical replications and no light controls each week. After the samples were removed from the light, 200 μ L was aliquoted onto a 96-well microplate and absorption at 560nm recorded.

Quantification was calculated with each technical replicate compared to the corresponding control. Modeling of the activity accounted for all technical replicates without prior averaging.

Activity buffer for APX, with 50mM of potassium phosphate at pH 7, was prepared prior to the experiment and 0.5mM of L-ascorbate added just after enzyme extraction was complete. To a 96-well microplate, 185 μ L of APX activity buffer was added and then 10 μ L of enzyme extract from each enzyme extract was added and change in absorption at 290 nm was measured every 30 seconds for four minutes. Final change in absorption after four minutes was used as a control for enzyme oxidation within the buffer. Then, 5 μ L of 0.2mM hydrogen peroxide (H₂O₂) was added to each sample in the plate and absorption changes measured the same way again. Enzyme extract and H₂O₂ was added and treatment groups measured two or four at a time, for 8-16 samples measured during the same four-minute time period. Placement on the plate was randomized for each treatment each week to avoid biases based on pipetting for the time of H₂O₂ addition to when the absorption measurements actually began. The final change in absorption was calculated following the formula below. The same H₂O₂ stock was used each week so composition of this stock was confirmed weekly by absorption of a dilution series at 290 nm.

$$\frac{(\Delta \text{ sample Abs.} - \Delta \text{ buffer Abs.})}{2.8 \frac{1}{M * cm} * 4 \text{ minutes}}$$

Formula 1: Calculation of APX activity over 4 minutes of measurement

Statistical analysis

Linear modeling was performed for each phenotypic trait reported in this paper and model fit confirmed by visual examination of residual Q-Q plots. Models for agronomic and photosynthetic traits used experimental replicate as a random effect with additional random factors including block, block during heat stress, pot in which the plant was grown, and plant position within the pot. Similarly, for enzyme activity, SOD activity was modeled with experimental replication as a fixed effect, but for both APX and SOD values technical replication was used as a random effect and removed if it did not account for variation. For MDA lipid peroxidation, there was a very strong effect of technical replication, i.e. time sampled, on total MDA, so this was included as a fixed effect in each weekly model. Differences between treatments were determined using ANOVA in R with the car package `anova()` and `emmeans()` pairwise comparison T-tests with multiple testing correction using the sidak method.

Results

Germination in BR Mitigates Yield Component Loss in Response to Heat Stress, but Interrupting an ROS Burst Reduces This Protection

In our preliminary study we found germination in BR protected wheat spike length and spikelet number during reproductive heat stress. We next assessed whether these patterns of BR rescue were dependent on the ROS burst. While most treatments lost spikelet numbers due to early senescence following heat stress, BR only-treated plants had similar spikelet numbers regardless of heat stress, maintaining more spikelets than the control and PPZ only-treated plants (CI [12.94, 15.68], CI[13.47, 16.19], ANOVA, $p=0.04$, Figure 2a). Control and PPZ-treated

plants lost two and one spikelets to heat stress respectively while BR-treated plants lost only half of a spikelet on average (T-test, $p<0.001$, $p=0.07$, $p=0.41$ respectively, Figure 2a).

Germination in PPZ had an effect on total spike length relative to control regardless of reproductive heat stress with PPZ-treated plants having significantly shorter spikes than control plants (ANOVA, $p=0.001$, T-test, $p<0.01$, Figure 2b). With heat stress, germination in BR tended to lessen spike length reduction to heat stress compared to control plants, with BR-treated spikes having more similar lengths regardless of booting temperature (ANOVA, $p=0.07$, CI [5.60, 6.67], CI [4.63, 5.70], Figure 2b, 2d). However, the spike lengths of PPZ-treated plants were also more similar with or without heat stress and there was also less spike length loss to heat for PPZ-treated plants compared to control plants (ANOVA, $p=0.07$, CI [5.24, 6.30], CI [4.35, 5.40], Figure 2b, 2e).

Treatment with PPZ also reduced total seed number production prior to heat stress compared to control plants, but seed number loss from heat stress was not different based on germination treatment (T-test, $p=0.02$, ANOVA, $p=0.57$, Figure 2c). Additionally, seed number was not different prior to or following heat stress comparing control and BR-treated plants (T-test, $p=0.15$, $p=0.7$ respectively, Figure 2c). There was no effect of the DPI treatment on any of the spikelet counts, spike lengths, or seed numbers with or without heat stress (ANOVA, $p=0.5$, $p=0.7$, $p=0.4$ respectively).

For seed size, represented by thousand grain weight (TGW), there was a further effect of germination treatment with or without heat stress (ANOVA, $p=0.03$). Germination in PPZ tended to increase the effects of heat stress on seed size: PPZ only-treated plants lost about 20.8g during heat stress compared to non-stressed counterparts and had significantly smaller seeds compared

to both control and BR-treated plants following heat stress (T-test, $p=0.001$, $p<0.001$, $p=0.01$ respectively, Figure 2f, 2d). Germination in DPI did affect plant response to heat stress for TGW, but only based upon the BR- or PPZ-treatment, with no difference in TGW between the heat stressed control and DPI only-treated plants (ANOVA, $p=0.03$, T-test $p=0.4$ Figure 2f) Co-treatment with DPI affected BR-treated and PPZ-treated plants which underwent heat stress, but did not affect the TGW of control plants alone (T-test, $p=0.03$, $p=0.005$, $p=0.4$ respectively, Figure 2f). Plants treated with both BR and DPI lost about five additional grams in final TGW from heat stress compared to BR only-treated plants, while PPZ-DPI-treated plants gained about five grams compared to PPZ only-treated plants following the heat treatment (T-test $p=0.03$, $p=0.005$, Figure 2f).

Some early plant traits have a downstream effect on yield components, with certain developmental metrics contributing to final yield (Mathan et al., 2016). We measured many developmental phenotypes and found a significant effect of germination treatments on seed and tiller emergence times as well as booting and heading dates (ANOVA, $p<0.001$, Appendix B, Supplementary 2). This developmental delay is in alignment with our own observational data that BR- and PPZ-treated plants developed slightly slower than control plants, regardless of DPI co-treatment. We further observed significant leaf structure abnormalities around the second week of plant growth in the BR-treated and BR-inhibited plants typical of phenotypes from affecting BR levels (Chi-Squared distribution test, $p<0.001$, Appendix B, Supplementary 3). There was no effect of any germination treatment on tiller counts, either maximal or final. For tiller height, which is known to have specific effects on yield, plants treated with only DPI or only

PPZ germination treatments had reduced height compared to control following heat stress (Mathan et al., 2016, T-test, $p=0.001$, $p=0.008$ respectively, Appendix B, Supplementary 4).

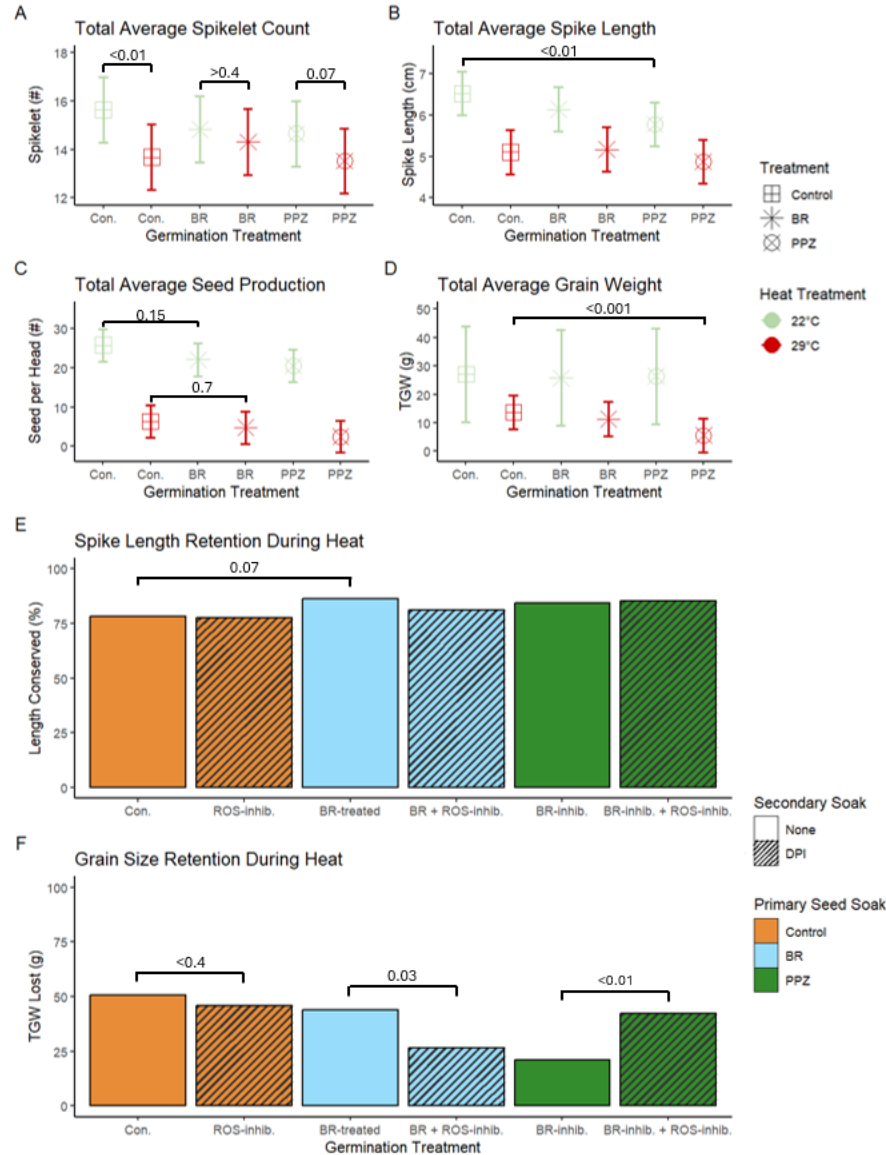


Figure 2: Final agronomic yield components were affected by seed germination treatments. For plots A-D, a single point is the estimated mean of 36 plants with error bars representing confidence intervals, and differences between treatments identified by emmeans pairwise comparisons. For plots E and F, bars represent the total difference of estimated means for each germination treatment between non-stress and stress conditions, with differences identified by ANOVA and emmeans pairwise comparisons. A) Average spikelet number for BR affecting

treatments only, with or without heat stress. B) Average spike length for BR affecting treatments only, with or without heat stress. C) Average seed production for BR affecting treatments only, with or without heat stress. D) Average thousand grain weight for BR affecting treatments only, with or without heat stress. E) Average spike length retention during heat for all treatment combinations. F) Average TGW retention during heat for all treatment combinations.

Germination in BR Changes Plants' Antioxidant Activity During Reproductive Heat Stress

During the reproductive stage, lipid peroxidation increased during the 5th week of development in response to heat stress for all germination treatment groups (Figure 3a-c). Plants co-germinated in BR and DPI which underwent heat stress had increased oxidation when compared to BR only-germinated, PPZ-DPI-treated, and DPI only-treated heat stressed plants (T-test, $p=0.04$, $p=0.01$, $p=0.009$ respectively, Figure 3a-c). However, during the 6th week of development with heat stress, there was reduced lipid peroxidation levels in most plants compared to the non-stress conditions which may indicate a stabilization of oxidation levels within the heat stressed plants (Figure 3a-c). Yet, for PPZ-treated plants, regardless of DPI treatment, there was higher MDA accumulation than the control and BR-treated plants following two weeks of heat stress (T-test, $p=0.005$, $p=0.003$ respectively, Figure 3a-c).

For APX during the fifth week of growth under no stress conditions, there was higher activity in BR-DPI co-germinated plants compared to BR only-treated plants; however, this difference did not persist once heat stress was applied (T-test, $p<0.001$, Figure 3d). With heat stress during the fifth week, only control and BR-DPI-treated plants were different in APX activity compared to the no stress condition, with control plants increasing APX activity in response to heat and BR-DPI-treated plants reducing this (T-test, $p=0.01$, $p<0.001$ respectively, Figure 3d, 3e). Yet, by the second week of heat stress, there was much higher APX activity across all treatments as compared to control temperature conditions (Figure 3d-f). Most

treatments had very similar APX activities following two weeks of heat stress, although co-germination in PPZ and DPI slightly reduced this activity compared to PPZ only-treated plants (T-test, $p=0.05$, Figure 3f).

Unlike APX, SOD activity was affected consistently during the booting stage by germination treatment. Five weeks after planting, without heat stress, there was lower SOD activity overall for BR-germinated plants when compared to the control plants (T-test, $p=0.02$, Figure 3g, 3h). Yet, during this first week of booting, comparing heat stress to the no stress condition, control and PPZ only-treated plants decreased their SOD activity whereas BR-germinated plants maintained SOD activity levels (T-test, $p=0.01$, $p=0.005$, $p=0.99$ respectively, Figure 3g-i). By the sixth week of development, there were similar levels of SOD activity for control plants with or without heat stress, while heat stressed BR- and PPZ-treated plants, regardless of DPI treatment, increased their SOD activity compared to their non-stressed counterparts (T-test, $p=0.3$, $p=0.04$, $p<0.001$ respectively, Figure 3g-i). Although, in accordance with the increase in lipid peroxidation, there was lower SOD activity for PPZ-treated plants overall after two weeks of heat stress when compared to control or BR-treated plants (T-test, $p<0.0001$, $p=0.009$, Figure 3g-i). Treatment with DPI did not have a strong effect on SOD activity during heat stress (ANOVA, $p=0.5$).

During vegetative development, there were differences between the PPZ-DPI co-germinated plants and the plants treated with DPI alone, with higher PPZ-DPI plant APX activity (T-test, $p=0.02$, $p=0.05$) and lower lipid peroxidation during weeks one and two (T-test, $p=0.03$, $p=0.05$, Appendix B, Supplementary 5a, 5b). By week four, there was a drop in APX activity for plants germinated in PPZ alone compared to BR-treated plants, while PPZ-DPI-treated plants

maintained a relatively elevated APX activity (T-test, $p=0.009$, $p=0.007$ respectively, Appendix B, Supplementary 5b). Moreover, just prior to booting during week four, BR-DPI-treated plants tended to have the highest APX activity, with higher activity than plants germinated in DPI alone, which continued during the fifth week without heat stress (T-test, $p=0.03$, Appendix B, Supplementary 5b).

Trends in APX activity and lipid peroxidation appeared inverse over time, with global decreases in APX activity aligning temporally with increases in lipid peroxidation, as notable at weeks two and four (Appendix B, Supplementary 5). Specifically, the increased lipid peroxidation around week four in plants treated only with PPZ matched the lower APX activity observed for this treatment group in comparison to other treatments at this time point. Further, with heat stress, general increases in both enzymes' activities corresponded to a decrease in lipid peroxidation over the same time period, during week six.

For SOD activity, there was no overall consistent effect of germination treatment across the first four weeks of development. During the first two weeks of development as seedlings, BR-treated plants tended to have reduced SOD activity compared to control plants (T-test, $p=0.002$, $p=0.04$ respectively, Appendix B, Supplementary 5c). By the second and third weeks of plant life, PPZ-treated plants began to display reduced activity compared to control, while activity levels of BR-treated plants at the third week began to be more consistent with the control plants (T-test, $p=0.005$, $p=0.02$, $p=0.9$ respectively, Appendix B, Supplementary 5c). Around week four, the plants appeared most similar to one another, though DPI treatment had a specific effect based on the BR additive or inhibitory treatment applied, as SOD activity was lower in

BR-DPI and PPZ-DPI co-germinated plants in comparison to DPI only-treated plants (T-test, $p=0.01$, $p=0.04$ respectively, Appendix B, Supplementary 5c).

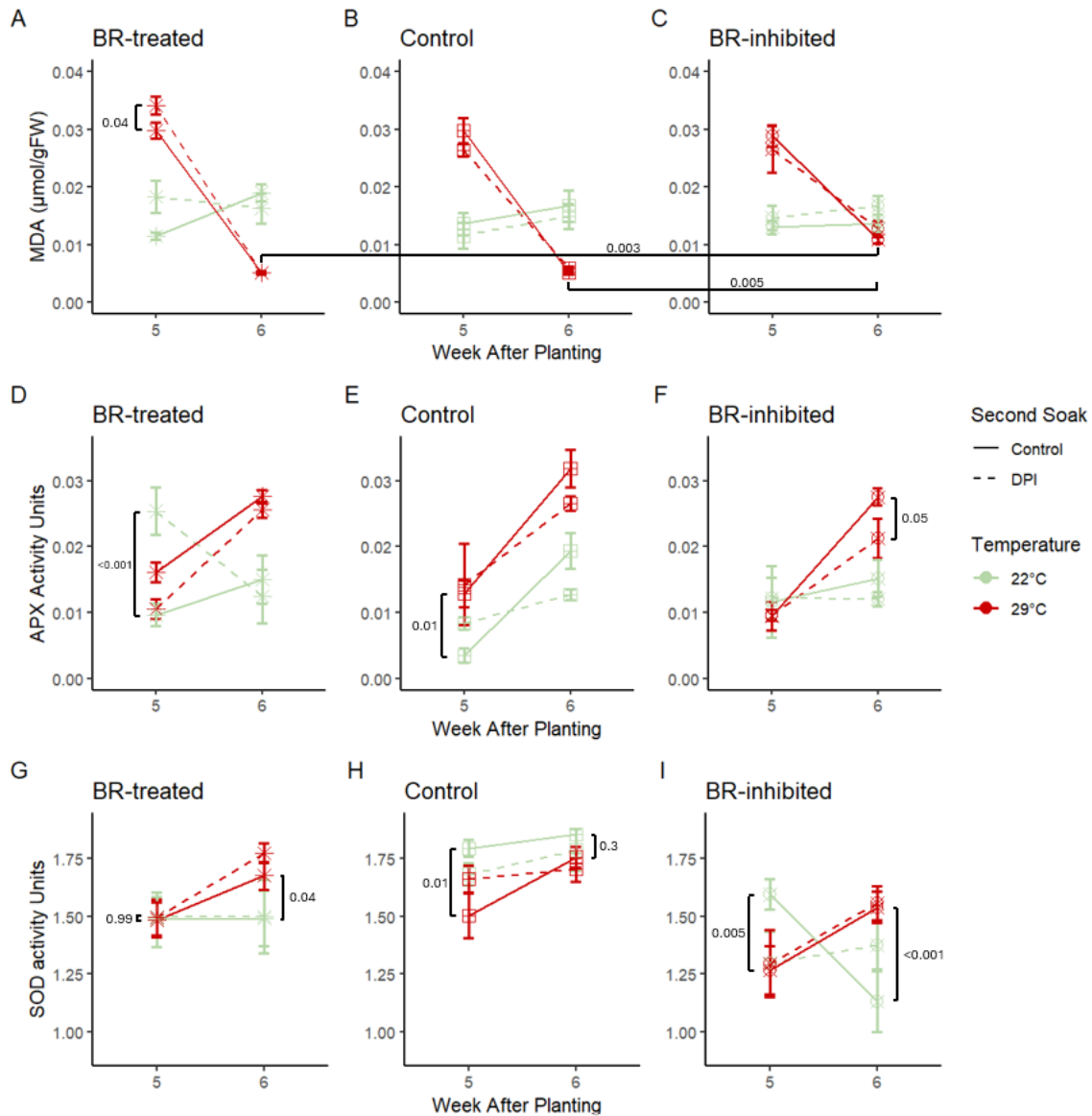


Figure 3: Changes in lipid peroxidation and enzyme activities during heat treatment. Week five after planting is one week into the booting stage with about one week of heat stress, and week six the second week of booting and about two weeks of heat stress. Each data point is represented by four technical replications and four biological samples within each replication. Error bars are standard error. Differences between treatments were identified by emmeans pairwise

comparisons. A-C) Lipid peroxidation, as measured by MDA accumulation for BR-treated, control, and BR-inhibited plants respectively. D-F) APX enzyme activity for BR-treated, control, and BR-inhibited plants respectively. G-I) SOD enzyme activity for BR-treated, control, and BR-inhibited plants respectively.

Photosynthetic Capability During Reproduction is Affected by BR Levels and ROS Proliferation at Germination

Under heat stress conditions, BR treatment improves several photosynthetic aspects including photosynthetic rate in plants including wheat seedlings (Yu et al., 2004; Hairat & Khurana, 2015; Thussagunpanit et al., 2015; Zhao et al., 2017; Pereira et al., 2019). Heat stress predictably revealed differences in photochemistry: across all treatments during the fifth and sixth weeks of development, Y(NO) was higher while Y(II) and Y(NPQ) were lower compared to the non-stress temperature (ANOVA, $p < 0.001$, Figure 4).

Treatment with BR alone did not increase Y(II) and Y(NPQ) during non-stress or heat stress conditions, nor was Y(NO) decreased after heat stress, with similar values in response to initial heat stress for BR-treated and control plants (T-test, $p > 0.05$ for all comparisons, Figure 4). In fact, there was improved Y(NPQ) and lowered Y(NO) for the heat stressed PPZ-treated plants compared to control plants (T-test, $p = 0.01$, $p = 0.02$ respectively, Figure 4e-f, 4h-i). However, plants co-germinated in BR and DPI, compared to BR only-treated plants, tended to increase Y(NPQ) and decrease Y(II) during initial heat stress, indicating improved energy control (T-test, $p = 0.03$, $p = 0.005$ respectively, Figure 4a, 4d). Additionally, there was reduced Y(II) from BR and DPI co-germination when compared to BR only-, PPZ-DPI-, and DPI only-treated plants during heat stress (T-test, $p = 0.04$, $p < 0.001$, $p = 0.02$ respectively, Figure 4a-c).

During the sixth week of development, the germination treatments had more similar photosynthetic qualities which differed primarily based on temperature (Figure 4). Although,

DPI treatment alone negatively affected $Y(II)$ and increased $Y(NO)$ compared to control under heat stress, which was not observed in the BR-DPI or PPZ-DPI heat stressed plants (T-test, $p=0.007$, $p=0.006$ respectively, Figure 4b, 4e).

Once the plants had headed and been moved back to 22°C, around the seventh week of development, there were striking differences in recovery based on germination treatment. Comparing non-stressed plants to their counter parts just removed from heat, plants co-germinated in BR and DPI recovered $Y(II)$ while all other treatments continued to have reduced $Y(II)$ from heat stress effects (T-test, $p>0.05$, $p<0.001$ respectively, Figure 4a-c). Further, PPZ-DPI-treated plants had lower $Y(II)$ and higher $Y(NO)$ from heat stress compared to the PPZ only-treated plants (T-test, $p=0.01$, $p=0.01$ respectively, Figure 4c, 4i). Indeed PPZ-DPI co-treatment appears to curtail photosynthetic efficiency during the heading period even without heat stress, with lower $Y(II)$ compared to both non-stressed PPZ only- and BR-DPI-germinated plants (T-test, $p=0.02$, $p=0.04$ respectively, Figure 4a, 4c).

For the first four weeks of plant development, prior to heat stress, we found no consistent differences between any of the germination treatments in the metrics that we investigated. Though PPZ-germinated plants tended to have higher $Y(NO)$ and lower $Y(II)$ and $Y(NPQ)$ during the first two weeks after planting, this may be confounded by the aforementioned slowed development of these plants compared to the other treatments (Appendix B, Supplementary 6).

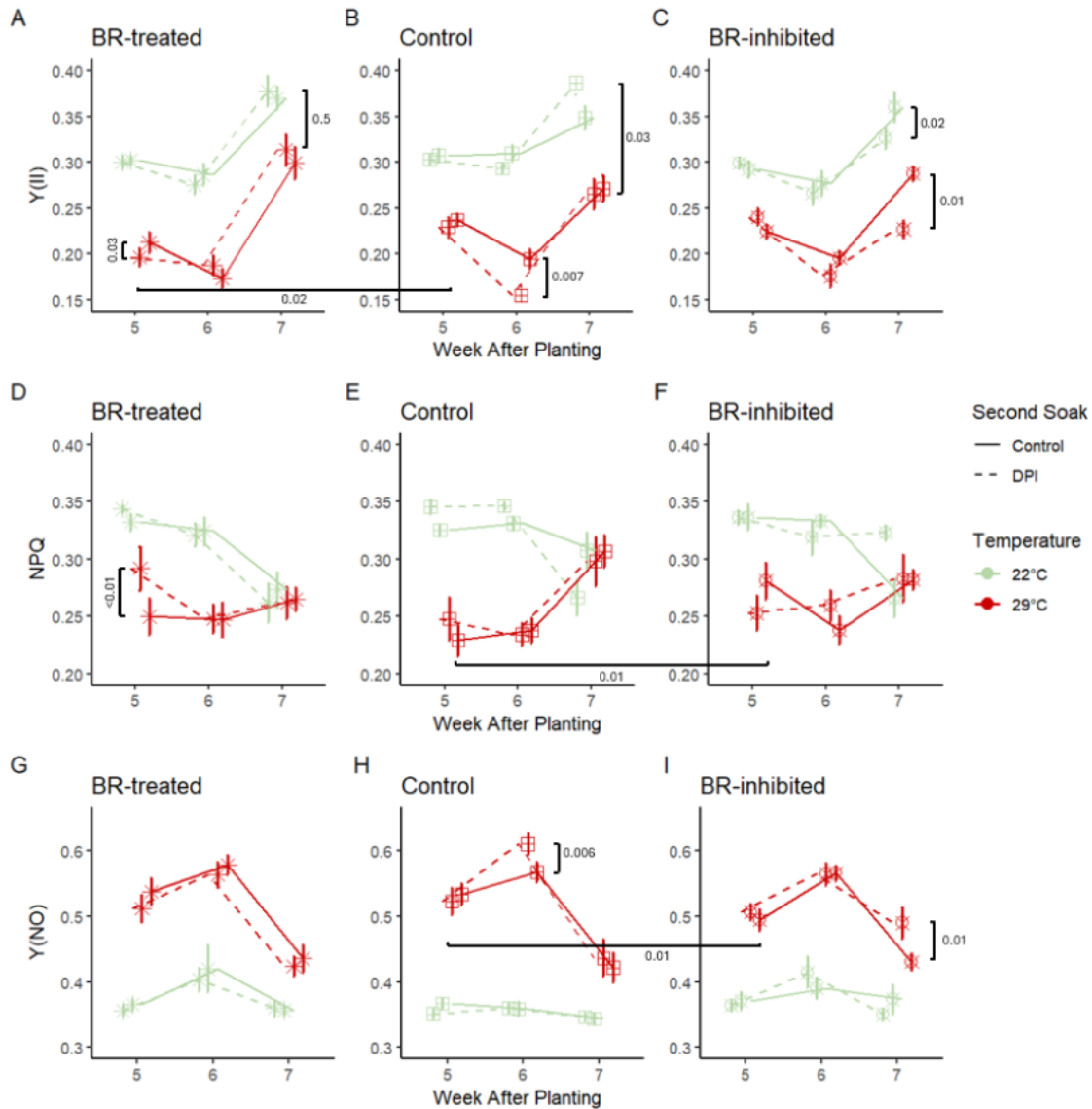


Figure 4: Photochemical changes of flag leaves in response to heat stress, during the booting stage around weeks five or six after planting, and during the heading heat stress recovery stage, around week seven after planting. Each point is representing an average across 16 plants from two experimental replications, and error bars are the standard error. A-C) Effective photochemical yield. D-F) Non-photochemical quenching. G-I) Non-regulated energy loss.

Discussion

Spikelet loss due to heat stress at the booting stage is mitigated by seed germination in BR (Figure 2a). Inhibition of BR synthesis during germination reduced wheat head length,

spikelet numbers, and exacerbated heat stress losses for TGW, indicating that an optimum level of BR is necessary for growth and heat stress tolerance of the wheat head (Figure 2). For TGW, germination with ROS-inhibition disrupted the heat protective or damaging effects of BR and BR-inhibition respectively but had no evident effect when applied alone (Figure 2f). This is similar to research in other crop species where treatment with BR alone reduced plant stress but co-treatment with BR and a ROS inhibitor comparatively increased this stress (Xia et al., 2009; Xia et al., 2011). Specific mechanisms affected by the BR-ROS signal complex may be disrupted by ROS-inhibition leading to higher stress and reduced growth in relevant metrics for the BR-treated and ROS inhibited plants (Nie et al., 2013).

Considering the extensive literature detailing BR improvement of photochemical processes in a variety of plants, we expected to see differences in photochemical traits even during the reproductive stage, based on germination in BR or BR-inhibitor (Janeczko et al., 2011; Hairat and Khurana, 2015). However, we found evidence to suggest that BR reduces photochemical rates in some instances, with a tendency towards lower $Y(II)$ in response to heat compared to control plants (Figure 4a). The co-germination of plants in BR and a ROS synthesis inhibitor exaggerated this impact: these plants had even lower $Y(II)$ during initial heat stress compared to BR treatment alone (Figure 4a). Yet, BR and ROS inhibition co-treated plants had higher $Y(NPQ)$ during initial heat stress and also increased $Y(II)$ much more quickly once removed from heat stress (Figure 4a). Interestingly, plants co-germinated with both BR inhibitor and ROS synthesis inhibitor showed opposite trends across the photosynthesis metrics, with relatively higher $Y(II)$ during the first week of heat stress combined with a reduced $Y(II)$ once removed from heat stress (Figure 4a-c). These findings suggest that there is an effect of BR

which modulates photosynthetic responses to heat stress, and the known initial ROS burst induced by BR can adjust the strength of this effect. Modulation by ROS of this BR effect is evident by the exaggerated differences in these metrics seen in the BR-treated and ROS-blocked plants and reduced performance of these metrics in the BR- and ROS-inhibited plants, as compared to BR-treated or BR-inhibited only counterparts.

Plants with high Y(II) correlate to increased growth; however, processing more energy, when heat stress additionally causes an excess of energy, can result in additional ROS production (Thussagunpanit et al., 2015; Ali et al., 2022; Khorobrykh et al., 2020). Therefore, it may be beneficial to slow the electron transport chain (ETC) function to protect the photosynthetic machinery from oxidation. Alternatively, lower ETR in the BR-treated and ROS-inhibited plants could be reflective of excessive damage to the ETR which is recoverable following heat stress, but not sufficient to rescue seed development deficits. Further, reductions in the ETC results in less energy available for photo assimilation and therefore diminished growth potential. Reduced ETC function during booting may be specifically detrimental as pre-flowering accumulation of carbohydrate reserves in the wheat stem contributes to grain fill during favorable conditions and becomes even more important for grain fill during stress conditions (Bidinger et al., 1977; Austin et al., 1980, Vaezi et al., 2024). Thus, lower ETC function could lead to poor reserve building during booting which may account for BR-treated and ROS-inhibited plants having more TGW loss during heat as compared to the BR only-treated and control plants. Concordantly, plants which had both BR and ROS inhibited during germination had increased ETC function during heat stress and subsequently larger seeds compared to plants which only had BR inhibited. In any case, the ETR is highly adaptable to the temperature wheat plants are grown under and we can

see in this study that there is influence of BR-treatment and ROS signaling on this adaptation. (Yamasaki et al., 2002).

We additionally investigated whether BR treatment had a long-term effect on oxidation states in spring wheat throughout the lifespan of the plant. There have been numerous studies indicating that treatment with exogenous BR can increase antioxidant activity, although the majority of these papers tested activity 1-2 days after BR foliar spray application (Xia et al., 2009; Surgun et al., 2015; Zhao et al., 2017). We found that BR-treated plants actually had a consistently lower activity of SOD the first two weeks after germination treatment and no difference in APX activity or lipid peroxidation levels compared to control plants during vegetative growth (Appendix B, Supplementary 5). Therefore, differences in heat response during the booting stage, induced by BR germination, are not the result of long-term upregulation in activity for these two enzymes, or changes in lipid oxidation states, which could occur prior to heat stress.

The activity of APX was not positively affected by BR treatment or inhibition during germination, as there were no consistent differences in APX activity between control and BR-treated or inhibited plants during the reproductive stage with heat stress. The similarity in control and BR-treated APX activities indicates that APX may not be directly involved in spike growth regulation contributing to the BR spike phenotype (Figure 3d-f). Of note, blocking ROS synthesis during germination did not consistently affect APX or SOD activity compared to counterparts with normal ROS germination signaling. The lack of effects from ROS-inhibition may point to a non-reliance in wheat on an ROS burst during germination for antioxidant regulation during the booting stage, regardless of BR interaction. This is contrary to many

studies which have previously found ROS signaling at the time of BR application to be essential for the BR-response of antioxidant enzymes (Xia et al., 2009; Xia et al., 2011).

For SOD, plants germinated in BR were able to maintain and even increase activity under heat stress while control plants had consistently lower activity during heat as compared to the non-stressed condition (Figure 3g, 3h). Yet, total SOD activity levels were higher in control plants between all stress conditions and reproductive time points compared to both BR-inhibited and BR-treated plants. Therefore, it may not be total SOD activity contributing to the BR favorable phenotype, but rather fine-tuned regulation of SOD in general. Indeed, in this study, high SOD activity is correlated to longer wheat spikes under non-stressed conditions, but differences in SOD activity regulation are correlated to spikelet number protection during heat stress.

Few studies have thus far examined the lasting impact of BR manipulation on antioxidants: rice primed with BR exhibited increased SOD activity compared to controls several weeks later during chromium stress, and similar results were found for long-term response to heat stress in BR-primed wheat (Basit et al., 2021; Kumari and Hemantaranjan, 2018). We detected no increase in SOD activity of heat stressed, BR-treated plants compared to controls, but we did observe reduced activity of SOD for BR-inhibited plants. The difference in findings suggests that the antioxidant response may be sensitive to environmental conditions and genotype. This research adds depth of understanding into how wheat regulates antioxidant activity during the booting stage, under heat stress, and as it specifically relates to yield components.

In this study, we investigated the effects and longevity of exogenous BR on plant enzymatic activity and how this may relate to yield components that we have shown to improve with BR under heat stress. We posited that BR may induce a sustained increase in antioxidant activity even during vegetative growth which primes plants to respond to stress; however, enzyme activity was highly variable across plant development stage and BR treatment even reduced enzyme activity at some time points. By comparing the activities of heat stressed and non-stressed plants, we discovered that, during the booting stage, BR-treated plants were regulating SOD activity differently from control plants to respond to heat stress. We suggest that BR-treated plants, rather than constitutively changing SOD activity from time of germination, may have a fine-tuned response of SOD to heat stress which, when disrupted, leads to intensified spike length losses. The changes in SOD activity regulation observed in BR-treated wheat correlated with improved protection of spikelet number during heat stress.

We also found that germination of wheat seeds in BR, in conjunction with effective ROS production, contributes to photosynthetic regulation in response to heat stress as much as six weeks later. Preventing ROS production and treating with BR during germination decreased photosynthetic activity during heat stress but blocking both increased this. Treatment with BR further increased activity of photosynthesis following heat stress and while dual BR and ROS inhibition decreased it. These disruptions in photosynthetic metrics at the time of booting correlated to changes in seed size which we hypothesize may relate to differences in stem reserve filling.

As increases in crop yield reach a bottleneck, a deeper understanding of plant mechanisms and intricacies is required to continue developing high yielding cultivars. This

research furthers the understanding of wheat response to heat stress and highlights the potent and cascading effects BR can have on this. Pinpointing the processes by which BR improves phenotype despite heat stress will help us identify genes which can be selected for to produce more heat tolerant wheat varieties.

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CHAPTER FOUR

CONCLUSION

Throughout this thesis, I have investigated the nature of heat stress response in spring wheat and how it is affected by brassinosteroid (BR) hormone. Natural plant hormones have substantial control of plant development, and BR in particular improves tolerance to many abiotic and biotic stressors. I found that the application of this hormone as an exogenous seed soak improved the heat tolerance of spring wheat heads for specific yield components. In order to breed more tolerant wheat varieties, I wanted to understand the molecular underpinnings contributing to this phenotype.

Main Findings

In our previous studies, our lab observed that germination in BR allows spring wheat heads to preserve some length and spikelet number after enduring a mild heat stress during the booting stage. It was proposed that since head length is most likely tied to the growth of the structural, rachis tissue, there may be tissue specific mechanisms in the BR-treated wheat contributing to this length. Therefore, I examined gene expression at the booting stage in two specific tissues, the structural rachis and the fertile florets, both with and without heat (Chapter 2). Gene expression was different between BR-treated and control wheat at the booting stage, with or without heat, and it was additionally different based on which tissue was tested. The response of these tissues to heat stress also differed within the BR-treated plants which supported our expectation that BR treatment was affecting the two tissue types differently. More specifically, the gene expression pattern induced by germination in BR persisted separately

within each tissue despite heat stress, pushing each tissue along a different developmental pathway. I focused on expression of redox-related genes since there is extensive literature that purports the benefits of BR on antioxidant activity (Xia et al., 2009; Zhao et al., 2017; Kumari and Hemantaranjan, 2018). I was curious whether this understood effect of BR in wheat was due to increased expression of antioxidants at the time of heat stress. While the BR-treated plants maintained their antioxidant gene expression, I also found that their expression overall was lower than that of the control plants in response to heat stress. This decreased but consistent expression indicates that any potential increase in antioxidant activity from BR treatment in spring wheat is unlikely to be caused by gene regulation changes. Instead, it was more likely that BR treatment affected already present antioxidants: by increasing their activity levels which could occur prior to or during heat stress, and/or by increasing the number of enzymatic antioxidants within the plant prior to heat stress. I set out to test three hypotheses with measurements of antioxidant activity and overall oxidation within the cells. For this, I tested the activity of two key enzymatic antioxidants and overall oxidation levels of these plants from the time of germination until the heading stage, when the heat stress would end according to our experimental design (Chapter 3).

Germination in BR changed the function of several plant processes, impacting the regulation of antioxidant activity in response to heat stress. Interestingly, I could observe differences in ROS-homeostasis, as represented by hydrogen peroxide and lipid peroxidation cellular concentrations, directly after BR seed soak at the 3-day stage of the wheat seedlings. However, other aspects of antioxidant activity were not often affected by the BR germination treatment during the first four weeks of plant development; the enduring effect of BR germination was most impressive only once stress was applied as much as five weeks later. Our

findings failed to support the hypothesis that antioxidant activity was increased in the BR-treated plants. One antioxidant, ascorbate peroxidase (APX), had no difference in activity based on germination treatment, and the other, superoxide dismutase (SOD), had reduced activity with BR treatment compared to control plants during heat stress. Yet overall cellular oxidation levels were worse with our negative BR treatment (PPZ, BR synthesis blocker) and the BR-treated plants had different regulation of SOD activity during stress compared to the control plants. Therefore, we may conclude that BR-induced heat tolerance of the spike length or spikelet number is unlikely to be because of increased antioxidant activity during heat stress. Rather, our enzymatic studies appeared to corroborate the genetic studies – BR treatment diminished expression and activity of specific antioxidants overall, but BR did affect the regulation of these genes/proteins.

In addition to testing the general effect of BR on antioxidant activity, I wanted to explore how BR might be inducing these changes. It is understood that BR application in several plant species activates ROS production, and this ROS signaling is partially responsible for the BR response (Xia et al., 2009; Yan et al., 2015). According to many studies on different crops, blocking ROS production during the time of BR application reverts any antioxidant gains back to untreated levels (Zhang et al., 2010; Nie et al., 2013; Zhou et al., 2014; Xia et al., 2014). I intended to replicate these findings and add wheat to the list of plants for which the BR response, particularly increased antioxidant activity, can be tied back to ROS signaling. However, I did not find evidence to support my hypothesis – indeed, not only did blocking ROS have no effect on any possible BR-induced antioxidant activity, it also did not evidently affect how BR treatment changed antioxidant activity regulation. Differences in environmental and genetic conditions

following BR application could account for deviation from common literature given the known impact experimental differences can have on BR response (Kothari and Lachowiec, 2021).

A more exciting discovery, prompted by what I thought at the time to be secondary findings in the RNAseq study, was the larger effect BR had on photosynthesis activity. Briefly, expression of several photochemical related genes was enriched between BR-treated and control plants at several time points following heat stress. This led me to measure photosynthetic activity of BR-treated or inhibited plants weekly from the time of germination until senescence (Chapter 3). While I, again, did not see differences based on germination treatment prior to heat stress, the response to heat stress was strikingly different based on both BR treatment and on ROS-burst blocking. Treatment with BR appeared to be reducing effective photochemical yields and bolstering excessive energy control during heat stress, and once plants were removed from heat, BR-treated plants more quickly increased photosynthesis rates. This may indicate increased protection of the chloroplast from extensive heat damage and possibly better recovery due to a reduced need for damage repair. Alternatively, we could consider these changes indicative of increased heat sensitivity which necessitates increased photosynthesis during heat recovery to make up for reduced energy assimilation sustained during the heat stress. Fascinatingly, plants which had been treated with BR but had ROS production blocked at the same time had the most pronounced differences from control plants, indicating a role of ROS in attenuating the BR effect on photosynthetic regulation. Plants germinated in both a BR blocker and a ROS blocker had complementary findings to the BR-treated and ROS-blocked plants, increasing photosynthesis rates during heat and reducing them following heat. This evidence supports the necessity of BR

and ROS at the time of germination for key photosynthesis regulations throughout the development of the plant.

These molecular findings, different antioxidant regulation and reduced photosynthetic rates during heat stress, were correlated with specific phenotypic traits relating to the wheat head length that I have found BR to have a specific effect on. Firstly, changes in total SOD activity during booting stage heat stress, as induced by germination in BR or BR inhibitor were correlated to the total length of the finished head and final seed size. Secondly, BR induced SOD activity increases during heat stress compared to the non-stress condition was complementary to spikelet number retention. Thirdly, the BR treatment and ROS inhibition prompted photosynthetic reductions were tied to decreased seed sizes following stress. Taken together, maintenance of antioxidant activity, specifically from SOD, appears to be supported by BR specific pathways, without ROS modulation at the time of application (Figure 1a). This antioxidant activity may protect the wheat head intercalary meristem to allow continued elongation and protect the inflorescence meristem to bolster floral development even during high ROS conditions brought on by heat stress. Additionally, I suggest that treatment with BR causes changes in photosynthetic response to heat stress which is dependent on ROS signaling at the time of germination (Figure 1b). This BR-ROS interaction and modification of photosynthesis allows continued and efficient energy assimilation during heat stress which promotes grain filling once plants are recovering from heat stress.

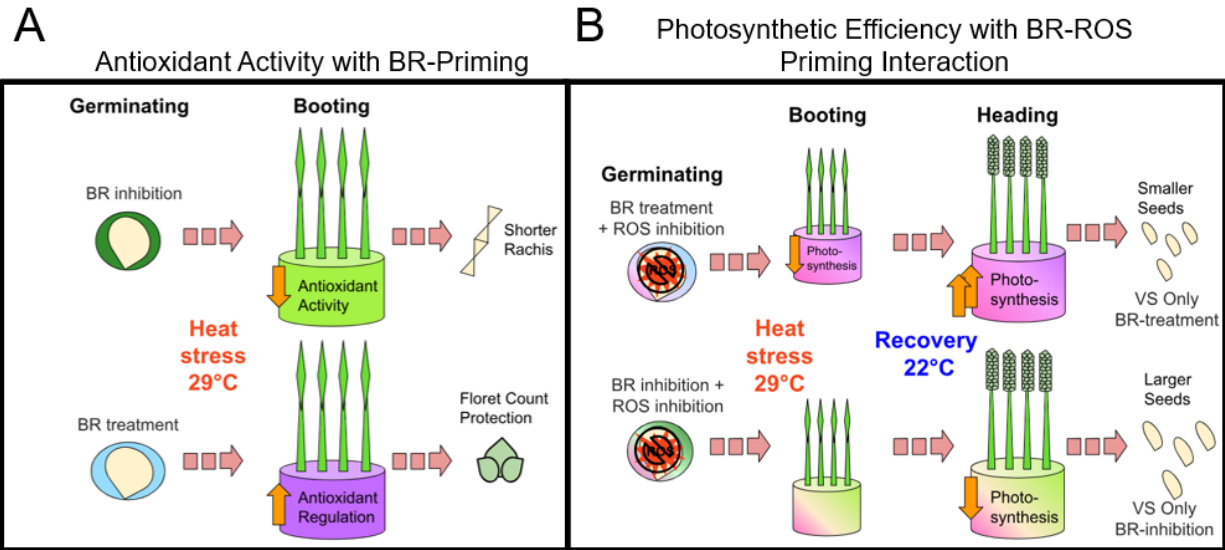


Figure 1: Proposed model of effects of BR-priming on enzymatic activity and photosynthetic efficiency in wheat. A) Inhibition of BR during germination leads to decreased total antioxidant enzymatic activity during the booting stage which results in shorter wheat heads. Treatment with BR during germination leads to improved antioxidant enzymatic regulation during heat stress at the booting stage which results in floret count protection. B) In comparison to only treatment with BR, inhibition of ROS during BR treatment reduces booting stage photosynthetic efficiency during heat stress. This necessitates an increase in photosynthetic efficiency during heading when the heat stress is removed and results in smaller seed fill. Inhibition of both BR and ROS during germination results in normal photosynthetic efficiency during booting and reduced photosynthetic efficiency during heading as it is not required for seed fill, and seeds are subsequently larger compared to BR inhibition alone.

Unique Aspects

Research into BR-induced stress tolerance is ongoing and prevalent in the plant science field. Here, I aimed to delve deeper into the specific mechanisms affecting BR treatment response, how BR response changes over time, how it can vary by tissue, and how this can all be of real-world benefit to crop production.

Investigating tissue specific gene expression is becoming standard in genetic research, with single cell sequencing and the understanding that key differences in tissue developmental features can greatly affect and bury findings during whole tissue sequencing. Many prior genetic

studies of BR response used organs comprised of multiple tissues or only investigated a single tissue type. I intentionally wanted to compare structural and fertile tissues in the wheat head because the effect of BR-induced heat tolerance was so specific to different yield component phenotypes. Treatment with BR did not improve seed number or final yield parameters under heat stress; I hoped that by comparing the fertile tissue to the structural tissue, I could find genetic differences between them which could be responsible for the positive phenotypes.

Another unique aspect of this study is the exceptional aspect of change over time. A majority of the literature investigating BR response do so over short time periods, 0-72 hours after application, or, if they examine phenotypes later in plant development, they only do so at one or two time points. By investigating potential changes throughout the development of the plant, we can improve understanding of when BR regulations have the most effect on plant growth and response. Only two other studies to this author's knowledge have examined the effect of BR germination on spring wheat quite long after application, one looking at chromium stress and the other at field heat conditions (Basit et al., 2021; Kumari and Hemantaranjan, 2018). In each case, these researchers focused on antioxidant activity changes in response to stress for the BR-germinated plants, without further exploration into how BR treatment is inducing these changes.

Lack of understanding in the mechanisms contributing to BR response is a high barrier to accurate knowledge as to how to leverage BR regulation for effectual crop improvements. It is somewhat of a universal in the literature that BR application does increase antioxidant activity and photochemical yield, yet we see this is not the case of 'Vida' (PI 642366) wheat (Lanning et al., 2006). It is further often posited that ROS signaling is contributing to the global effects of BR

response, with the measured disruption of ROS correlating to disruption of antioxidant activity being held as a representation for all BR-induced phenotypes. However, I see in my study that ROS disruption at the time of BR application had pathway and environmental specific effects, not affecting antioxidant activity but affecting photosynthetic response to heat stress.

Variation in BR response studies has been long observed and often attributed to differences in experimental designs. Indeed, given plants' sensitive responses to BR concentrations, the structural diversity in natural BR chemical, and a lack of understanding about the longevity of plant response, this is understandable. Yet, one possibly overlooked mechanistic wrench to a universal BR response is the difference between genetic varieties within crop species. The study I mentioned above, which also measured the effect of BR germination on a later heat stress response, found almost antithetical antioxidant activity to what is reported here. However, crucially, these researchers used different wheat varieties, C-306 and HUW-468 (Kumari and Hemantaranjan, 2018). In our preliminary research, out of the four wheat varieties we tested -- using the same concentration, form, and duration of BR treatment -- only the Vida variety responded positively to BR with improved head length and spikelet numbers following heat stress. Therefore, small changes in genetic regions, gene expression regulation via chromatin folding, or histone modifications which differ between varieties of the same species may all contribute to the response, and course of response, of the plant to BR application.

Further Studies

There is much more research and investigation which could further our understanding of BR regulation in heat stress response. Our studies were limited in the number and type of antioxidants tested -- several researchers have seen an effect of BR on other antioxidant enzymes

which may be accounting for some phenotypic differences I was unable to account for. Further, I tested the activity SOD generally, but there are several forms of SOD within plant cells which localize to different organelles. It would be very interesting to test these SOD separately and narrow which regions of the cell are most affected in SOD activity by BR treatment.

Additionally, testing antioxidant activity in the rachis and floret wheat head tissues in particular would be particularly exciting. Given the tissue specific gene expression I saw, there is reason to believe that enzymatic activity would also be tissue specific. Taking additional time to test the causal tissues, instead of only leaf tissue, could pinpoint differences more correlated to beneficial phenotypes. A further step could be to test photosynthetic potential based on observed developmental stage. I opted to measure all treatments on the same day each week, however there were some differences in developmental rate based on BR treatment. Pinpointing exact developmental stages, rather than temporal plant age, to the changes in photochemical efficiencies could help us rule out growth rate as a confounding factor in our findings.

Research Impacts

This research adds to the deep interest in understanding how BR can affect crops and improve their stress tolerance. Treating spring wheat with exogenous BR and blocking endogenous BR production, we are able to speculate on the typical role of BR by pushing it to its extremes. Molecular understanding of plant processes is essential in the efforts to improve plant phenotype to benefit human needs. Crops like wheat are indispensable to human consumption and are increasingly threatened by environmental stressors. I hope this research will contribute to the fine tuning of heat stress response in spring wheat and will further the efforts to develop wheat varieties suited for production, despite heat stress.

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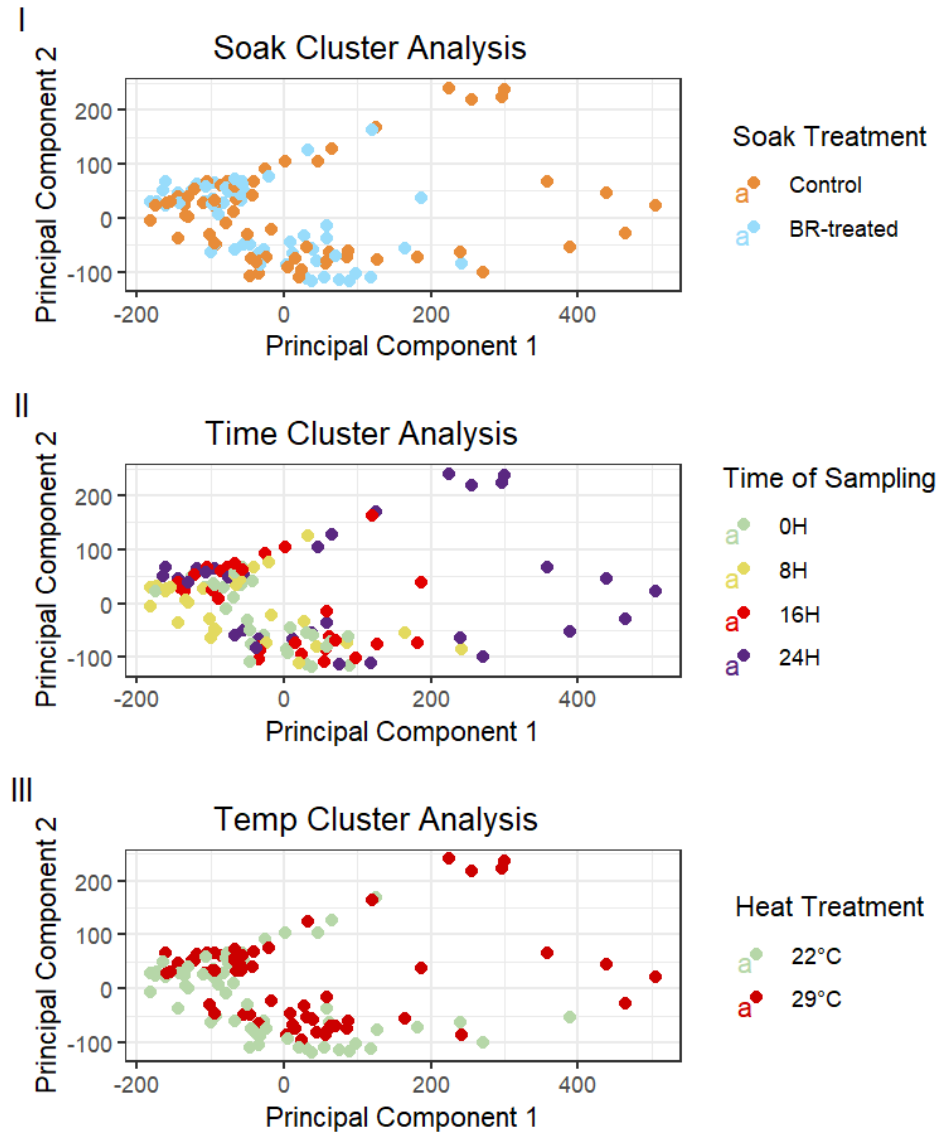
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APPENDICES

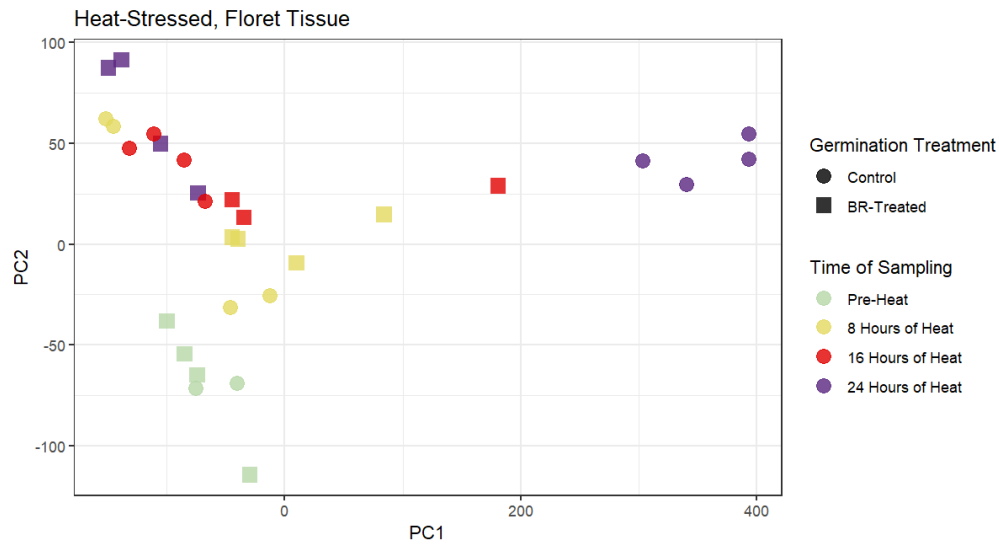
APPENDIX A

APPENDIX A: SUPPLEMENTARY FOR CHAPTER TWO

1. Supplementary 1: Principal Component Analysis.



Supplementary 1a: PCAs of all RNAseq samples colored by various treatment groups, plotted along PC one and PC two. I. Coloration by seed soak treatment. II. Coloration by time of sampling. III. Coloration by temperature treatment. There was some minimal segregation by heat treatment revealed by principal component four only.



Supplementary 1b: Floret tissue PCA of heat-stressed samples revealing segregation of 24H, heat-stressed control samples.

2. Supplementary 2: Gene names and GO terms used to create the expression heatmap containing all heat-annotated genes within dataset

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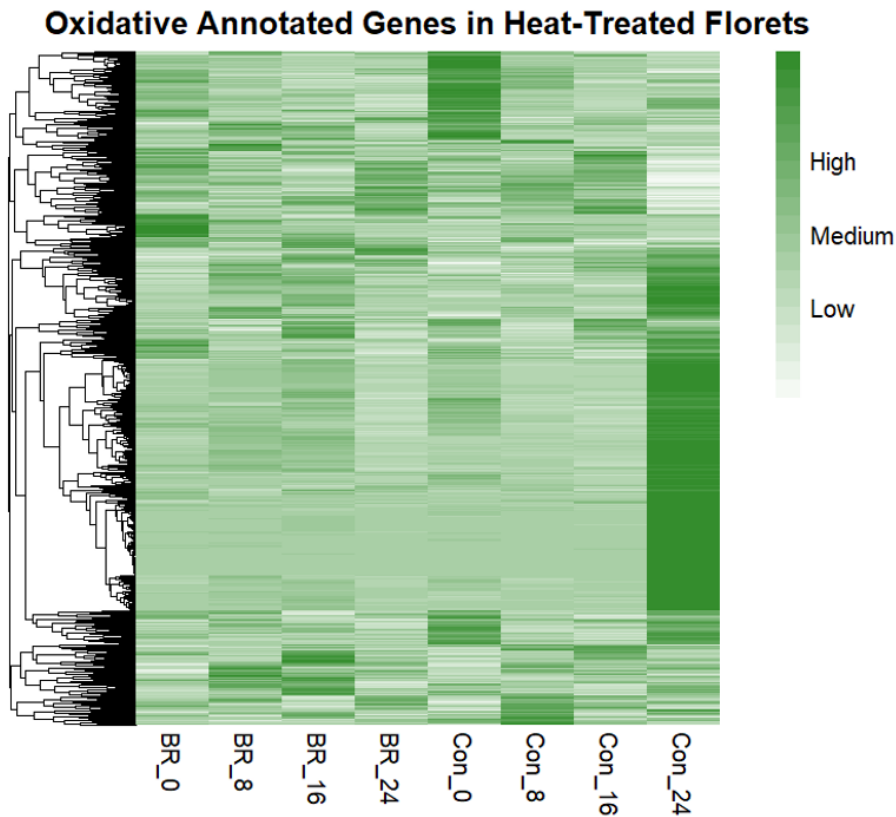
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- b) GO terms used for heat annotation subsetting: "GO:0051082" – unfolded protein binding, "GO:0009408" – response to heat, "GO:0009651" – response to salt stress, "GO:0034605" – cellular response to heat, "GO:0006301" – postreplication repair, "GO:0051085" – chaperone cofactor-dependent protein refolding, "GO:0051787" – misfolded protein binding, "GO:0042026" – protein refolding, "GO:0030544" – Hsp70 protein binding, "GO:0051879" – Hsp90 protein binding, "GO:0031072" – heat shock protein binding, "GO:0006281" – DNA repair, "GO:0006974" – DNA damage response, "GO:1900034" – regulation of cellular response to heat, "GO:1900036" – positive regulation of cellular response to heat, "GO:1900035" – negative regulation of cellular response to heat, "GO:0010286" - heat acclimation, "GO:0070370" – cellular heat acclimation

3. Supplementary 3: Select GO categories among the top 50 enriched for downregulation after 24 hours of heat stress in control plants

- a. Rachis control tissue: translation (GO:0006412), ribonucleoprotein complex (GO:1990904), RNA binding (GO:0003723), cytoplasmic translation (GO:0002181),

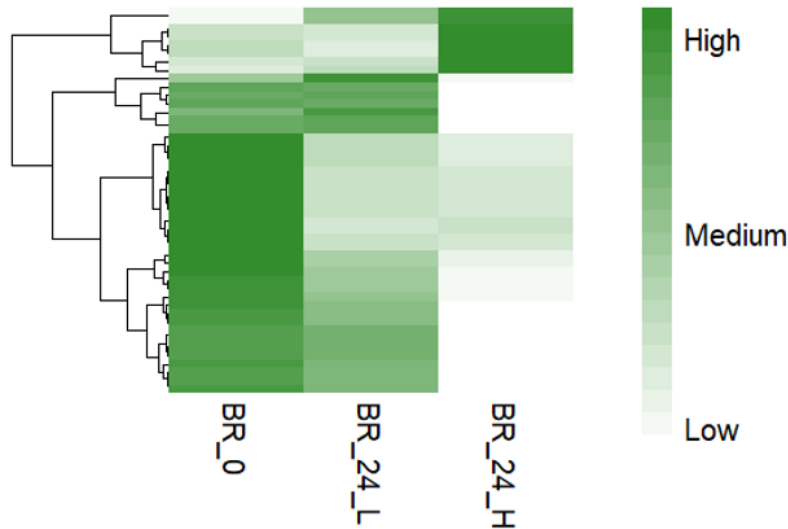
- mRNA binding (GO:0003729), rRNA processing (GO:0006364), mRNA splicing, via spliceosome (GO:0000398), mRNA processing (GO:0006397), tRNA processing (GO:0008033), RNA splicing (GO:0008380), proteasome complex (GO:0000502)
- b. Floret control tissue: rRNA processing (GO:0006364), mRNA splicing, via spliceosome (GO:0000398), ribonucleoprotein complex (GO:1990904), RNA binding (GO:0003723), ribosome (GO:0005840), translation (GO:0006412), mRNA binding (GO:0003729), mRNA processing (GO:0006397), RNA splicing (GO:0008380)
4. Supplementary 4: GO categories related to heat for genes upregulated at zero hours compared to 24 hours after heat stress for BR-treated tissues.
- a. BR-treated rachis: response to heat (GO:0009408), unfolded protein binding (GO:0051082), response to salt stress (GO:0034605), postreplication repair (GO:0006301), chaperone cofactor-dependent protein refolding (GO:0051085), Hsp90 protein binding (GO:0051879), heat shock protein binding (GO:0031072), cellular response to heat (GO:0034605), Hsp70 protein binding (GO:0030544), protein refolding (GO:0042026), misfolded protein binding (GO:0051787)
- b. BR-treated floret: unfolded protein binding (GO:0051082), response to heat (GO:0009408), response to salt stress (GO:0034605), cellular response to heat (GO:0034605)



5.

Supplementary 5: Oxidative Annotated Genes in Heat-treated Rachis. Heatmap of all redox annotated genes sequenced and mapped for floret tissues; each row represents a unique gene and the expression coloration is normalized within each gene or row, with increasing darkness of color relating to increasing expression. Sample names are written as Seed Treatment_Time tested.

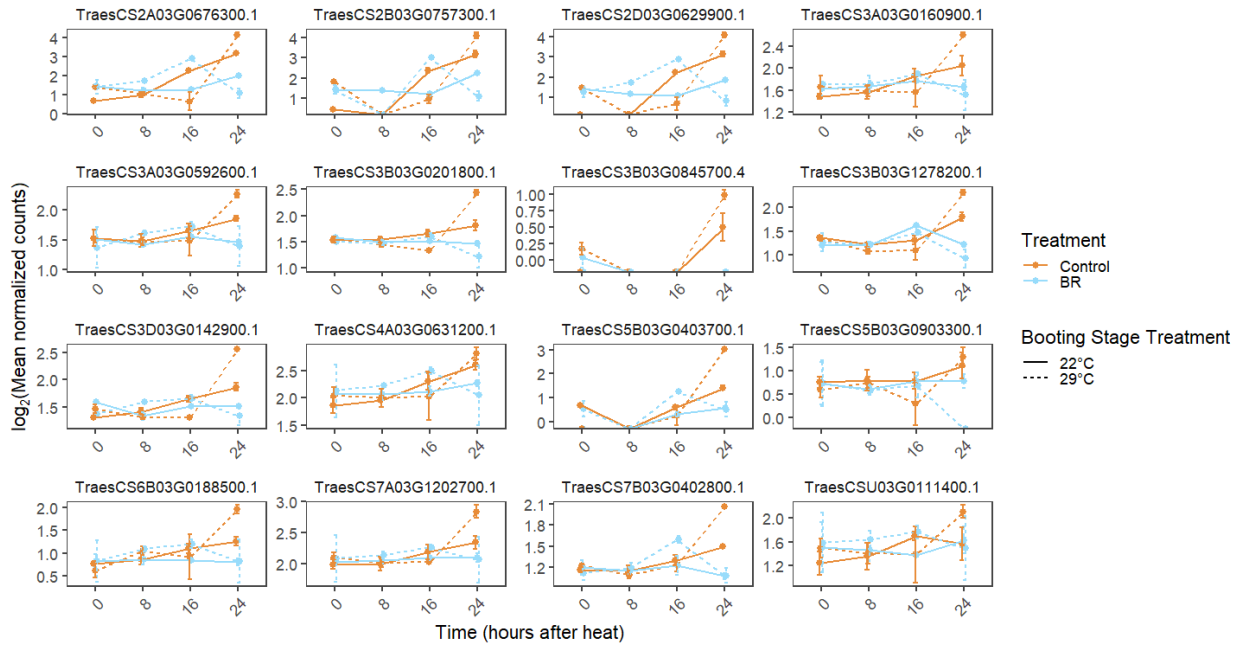
Oxidative Annotated DEGs, 0 to 24h in BR-treated Rachis



6.

Supplementary 6: Oxidative Annotated DEGs, zero to 24 hours in BR-treated Rachis. Expression heatmap of differentially expressed genes between zero and 24 hours of heat stress in BR-treated rachis, specifically DEGs with annotation related to ROS-homeostasis. Columns represent averaged gene expression from zero hours, 24 hours without heat, and 24 hours with heat of control plants from left to right. Expression is normalized within each row, with increasing darkness of color relating to increasing expression.

7. Supplementary 7: GO oxidative categories enriched for DEGs between BR-treated and control floret, upregulated in control tissues at 24 hours of heat: cellular oxidant detoxification (GO:0098869), cellular response to oxidative stress (GO:0034599), response to reactive oxygen species (GO:0004657).
8. Supplementary 8: Comparing control plants at 24 hours with or without heat stress revealed redox categories enriched in the floret tissues such as "Response to oxidative stress", "Cellular oxidant detoxification", "Peroxidase activity", "Hydrogen peroxide catabolic process" (GO:0042744), and "Lactoperoxidase activity" (GO:0140825). Similar categories were not enriched when control rachis with or without heat stress at 24 hours. Finally, we performed additional LRT analysis in DESeq2 to control for the changes over 24 hours in the unstressed control plants and reveal heat specific effects. Several redox categories were still enriched in the rachis including "Peroxisome" (GO:0005777), "Cellular response to oxidative stress" (GO:0034599), "Phenylpropanoid biosynthetic process" (GO:0009699), "L-phenylalanine biosynthetic process" (GO:0009094).



Supplementary 9: Redox-related DEGs expression over time. Time course of expression for redox related genes with the 16 highest LFCs from zero to 24 hours in control plant floret tissue. Plotted read count for BR-treated and control florets over each time point as averaged between each sample in the treatment group and error bars representing the standard deviation.

10. Supplementary 10: Gene names and GO terms used to create the expression heatmap containing all redox-annotated genes within dataset

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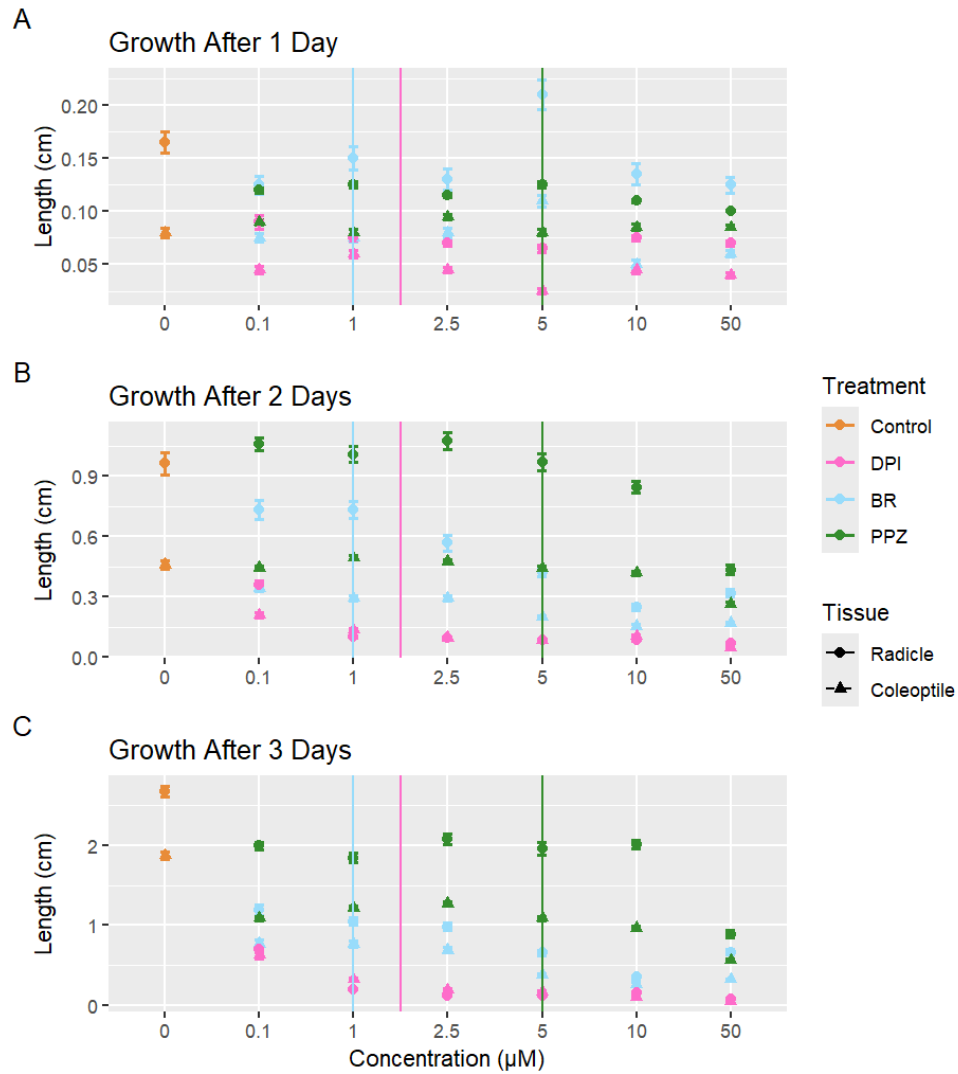
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 TraesCSU03G0111400.1, TraesCSU03G0289800.1

- b) GO terms used for redox annotation subsetting: "GO:0098869" – cellular oxidant detoxification, "GO:0006979" – response to oxidative stress, "GO:0034599" - cellular response to oxidative stress, "GO:0042542" – response to hydrogen peroxide, "GO:0042744" – hydrogen peroxide catabolic process, "GO:0004601" – peroxidase activity, "GO:0005777" – peroxisome, "GO:0009698" – phenylpropanoid, "GO:0009698" – phenylpropanoid metabolic process, "GO:0006950" – response to stress, "GO:0004602" – glutathione peroxidase activity, "GO:0016688" – ascorbate peroxidase activity, "GO:0008379" - thioredoxin peroxidase activity, "GO:0000302" – response to reactive oxygen species, "GO:0004657" – proline dehydrogenase activity, "GO:0006560" – proline metabolic process, "GO:0006562" – proline catabolic process, "GO:0009699" – phenylpropanoid biosynthetic process, "GO:0140825" – lactoperoxidase activity

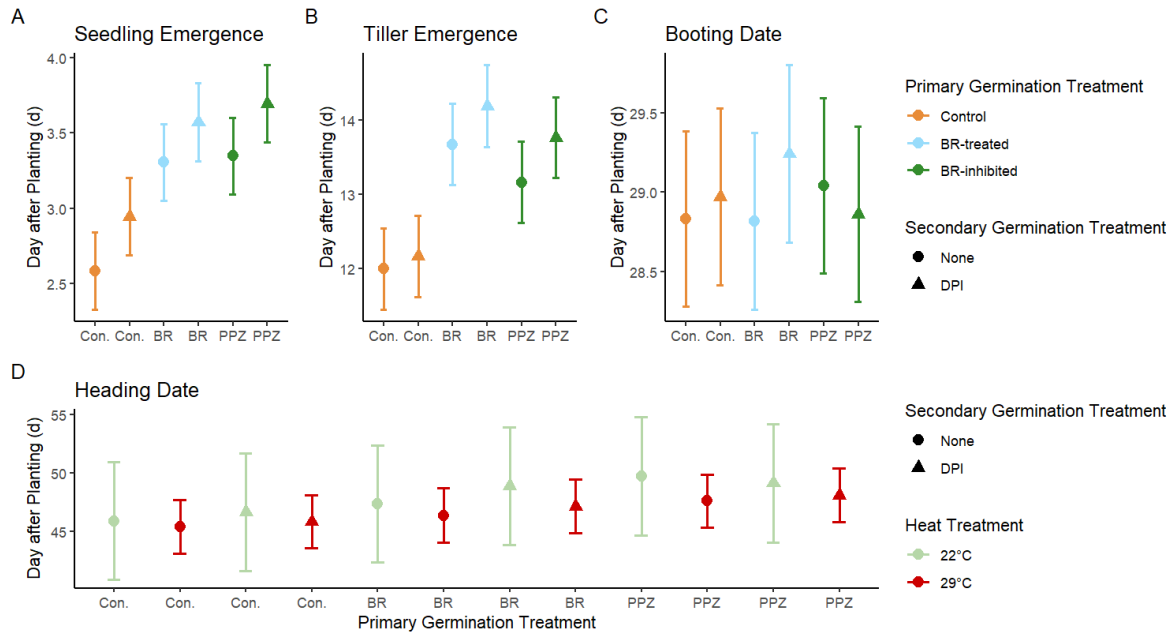
APPENDIX B

APPENDIX B: SUPPLEMENTARY FOR CHAPTER THREE



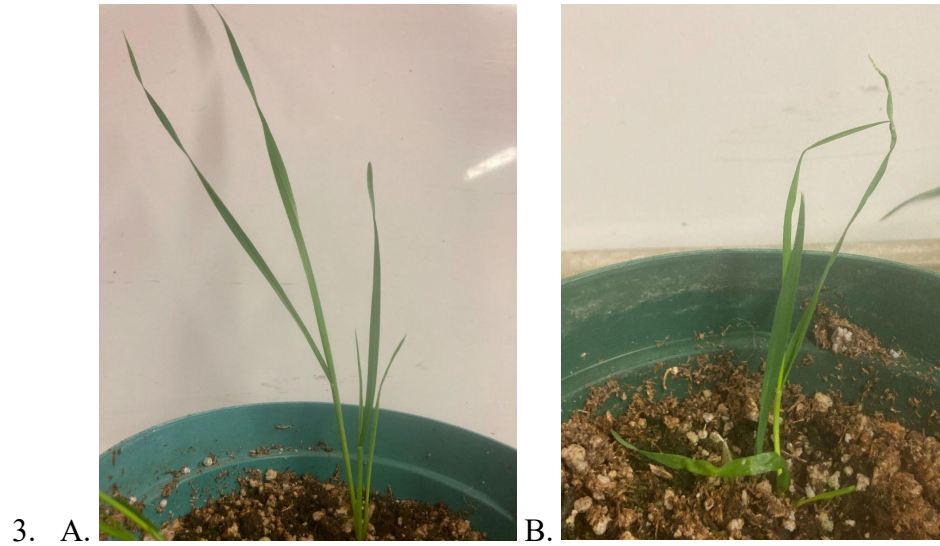
1.

Supplementary 1: Dose response of germination rates to BR, PPZ, and DPI treatments. Ten seedlings per treatment hypocotyl and radicle lengths were measured daily for three days with three experimental replications. Points are the average across all replications with error bars representing the standard error. Colored lines represent the concentration chosen for the given compound in this study, based on evident effect on seedling development and common concentrations used in literature (Kothari and Lachowiec, 2021).

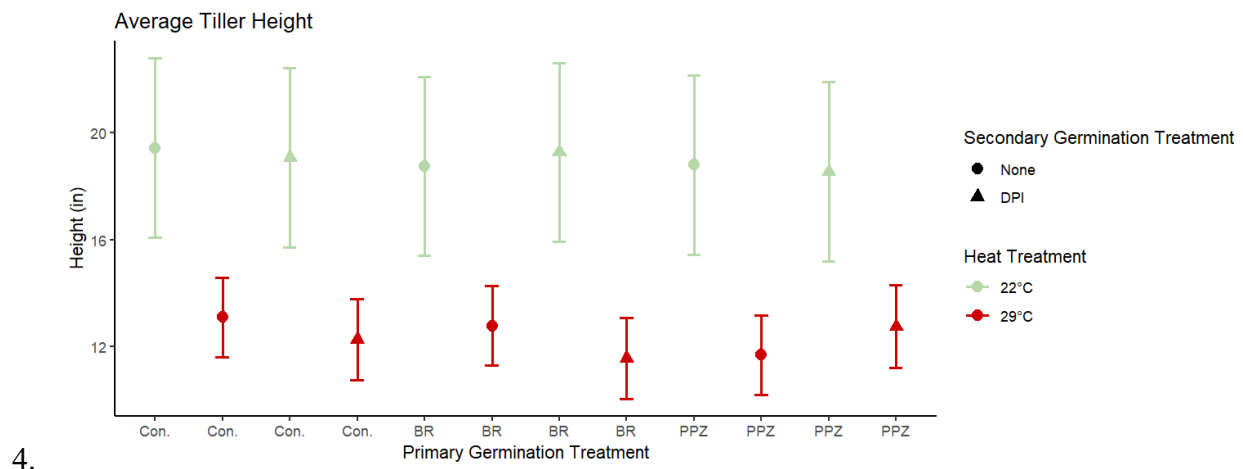


2.

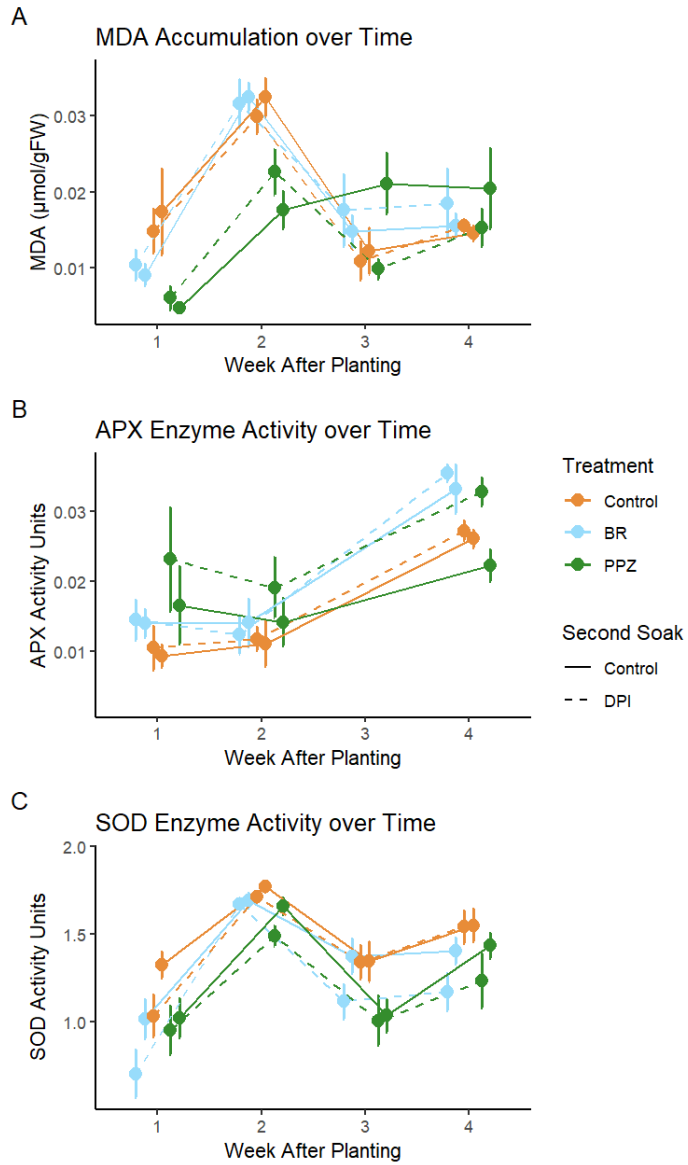
Supplementary 2: Number of days to each growth metric after seeds were planted in soil following seed germination treatment. Each point is the estimated marginal means represented by 36 plants, error bars are the confidence intervals. A) Days to seedling emergence B) Days to tiller emergence. C) Days to booting (at least two of four plants in each pot) D) Days to heading (at least two of four plants in each pot).



Supplementary 3: Leaf structure images, ratings one and two, with one being typical and two being abnormal. Plants were rated for phenotypic abnormalities on the 16th day after seeds were planted in soil, as observable morphological differences tended to disappear around the 25th day. A) Normal leaf structure measured as a one. B) Abnormal leaf structure, with twisting leaves and leaves growing towards the soil measured as a two.

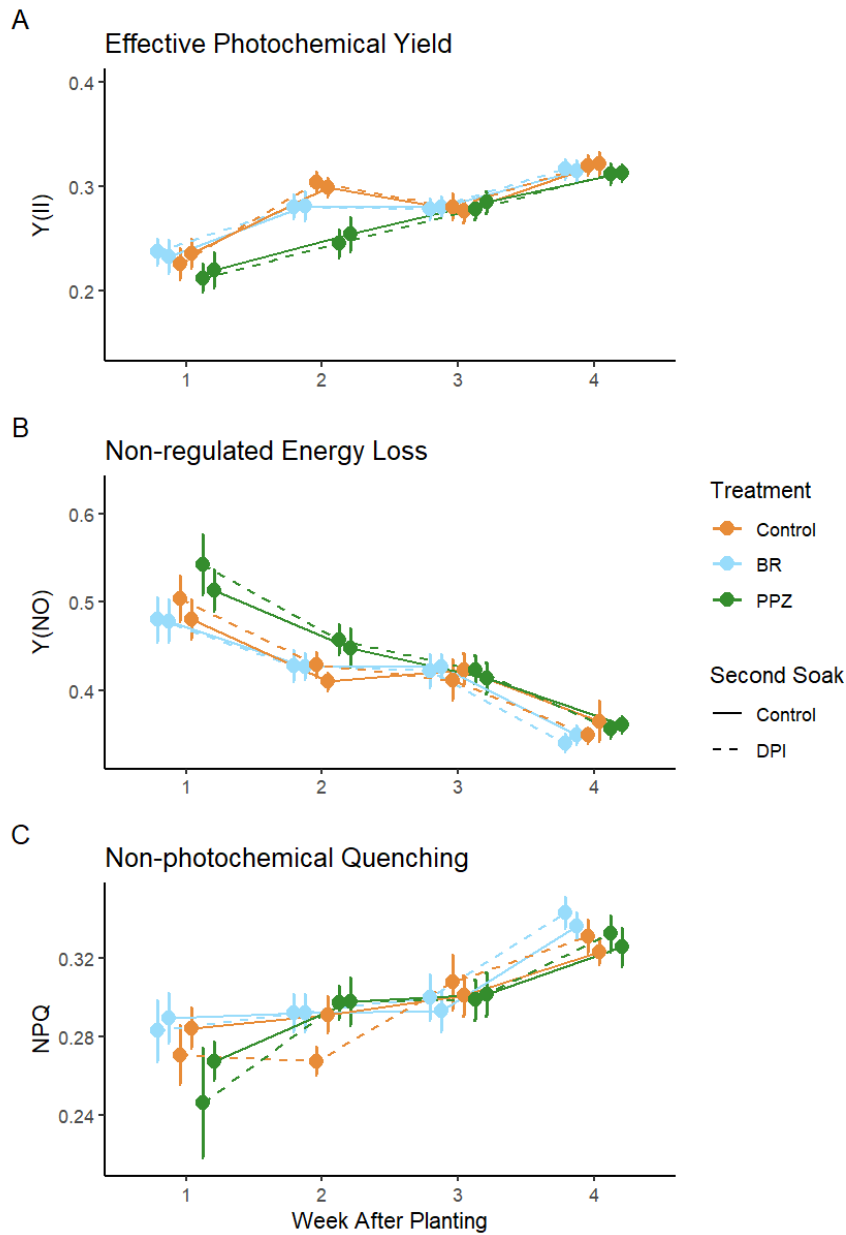


Supplementary 4: Final estimated marginal mean of tiller height, error bars represent confidence interval. Treatments are represented by 36 plants.



5.

Supplementary 5: Changes in peroxidation and antioxidant activity during the first four weeks of plant development. Each data point is represented by four technical replications and four biological samples within each replication. Error bars are standard error. A) MDA accumulation. B) APX activity, week three is omitted due to collection error. C) SOD activity.



6.

Supplementary 6: Changes in photochemical metrics during the first four weeks of plant development. Each point is represented by at least 30 plants with error bars representing the standard error. A) Effective photochemical yield. B) Electron transport rate. C) Non-regulated energy loss. D) Non-photochemical quenching.