Characterizing the effects of growth-limiting drought on Asian rice (*Oryza sativa* L.)

by

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ABSTRACT

Drought stress is one of the main constraints to global food production and productivity. Understanding the physiological and molecular mechanisms underlying responses to growth limiting drought conditions, which is the agriculturally relevant water limiting conditions, is a major goal for plant science research and a prerequisite to enhancing the climate change resilience for improving global food security. Here, I aimed to investigate how rice seedlings respond to constant and nearly stable water-limiting conditions and to determine whether there were qualitative or quantitative differences in their responses to two levels of water-limiting stress. To this end, I developed a growth-limiting drought assay and used it to examine the extent of growth limitation, photosynthetic performance and transcriptome changes in rice seedlings under two levels of mild water limiting conditions. The two growth conditions resulted in intensity-dependent changes in growth and the global transcriptome profile. A stable and consistent water limiting condition was associated with reduced CO_2 and light utilization efficiency in rice seedlings. Global transcript profiling revealed a distinct transcriptional program in terms of the number of differentially expressed genes, but similar functional categories were responsive to the two-levels of growth-limiting drought stress. This research provides valuable insight into potential phenotypic plasticity, and physiological and transcriptional programs that coordinate the response of rice plants to nearly stable drought stress conditions.

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LIST OF ABBREVIATIONS

ABA	abscisic acid
A_{net}	net CO_2 assimilation
CK	cytokinins
CO_2	carbon dioxide
DEG	differentially expressed gene
DREB	dehydration-responsive element-binding
E	transpiration
FAO	Food and Agriculture Organization
GA	gibberellic acid
GLD	growth-limiting drought
GO	gene ontology
g_{sw}	stomatal conductance
IPCC	Intergovernmental Panel on Climate Change
iWUE	intrinsic water use efficiency
JA	jasmonic acid
J_{max}	maximum electron transport rate
LEA	late embryogenesis abundant
PAR	photosynthetically active radiation
PCA	principal component analysis
RH	relative humidity
RNA-Seq	RNA sequencing
ROS	reactive oxygen species
RWC	relative water content
SMP	soil matric potential
SWCg	gravimetric soil water content
V_{cmax}	maximum carboxylation rate

CHAPTER 1: INTRODUCTION

1.1 Introduction and Overview

Drought is a major limiting factor affecting crop production worldwide and is predicted to increase due to the rapidly changing climate (IPCC, 2021). For instance, data from the Food and Agricultural Organization (FAO) showed a downward trend in the amount of water available for agriculture across all the continents of the world between 2010 to 2017 (FAO, 2020). Drought can have a wide-ranging impact on the growth and yield of major crop species, leading to a significant decline in production (Lesk et al., 2016; Zhang et al., 2018). Climate change is exacerbating the problem of drought, and according to predictions from climate change models, the areas of the world that are prone to drought will continue to expand, affecting more agricultural regions (Dai, 2013; FAO, 2020). Therefore, developing crop varieties that can maintain high yields under water-limited conditions is essential. Developing drought-tolerant varieties is particularly important considering the increasing global human population and the need to ensure an adequate food supply (Tilman et al., 2011). However, developing drought-tolerant crops requires a deep understanding of the physiological mechanisms underlying plant responses to drought stress and the molecular genetic factors contributing to drought tolerance. By identifying and targeting these factors, it may be possible to breed or genetically engineer crops with improved tolerance to drought, leading to higher yields and more sustainable agriculture.

Rice is a primary staple food for more than half of the world's population. It is grown in more than 100 countries and on every continent except Antarctica (Fukagawa & Ziska, 2019; Muthayya et al., 2014). Because rice is widely cultivated, it has been the subject of extensive

research, resulting in a large amount of data and a well-annotated genome available for study (Song et al., 2018). Therefore, rice is a popular monocot model for studying molecular physiological responses to various stresses such as flooding, high temperature and water deficit conditions. In addition, the global population is expected to continue to grow, and as it does, the demand for food, including rice, is also likely to increase. Understanding the molecular basis of stress response in rice could help improve rice production and other cereal crops.

Plants, being sessile organisms, have developed complex systems to adapt to changing environmental conditions at the growth, cellular, physiological, and molecular levels (Baerenfaller et al., 2012; Osakabe et al., 2014; Yamaguchi-Shinozaki & Shinozaki, 2006). For example, when plants experience abiotic stress, such as drought, they activate stress-responsive genes and increase the production of phytohormones to modulate growth and respond to stress (Yoshida et al., 2014). In rice, studies in the past have unravelled the molecular component of the response to drought stress by profiling the global gene expression landscape underlying the response to drought (Degenkolbe et al., 2009; Hadiarto & Tran, 2011; Lv et al., 2019; Moumeni et al., 2011; Smita et al., 2013; Wang et al., 2011). A critical component identified was the ABA signalling pathway, which involved the production of ABA that induced the expression of stress-responsive genes leading to cellular and physiological responses (Mundy & Chua, 1988; Rabbani et al., 2003). Upon drought stress, ABA accumulates, leading to stomatal closure to decrease the rate of water loss by reducing the transpiration rate (Wilkinson & Davies, 2002; Yoshida et al., 2014). However, the reduction in stomatal conductance also affects the CO_2 assimilation rate and reduced photosynthate production – a short-term response to the water deficit (Chaves, 1991). Over a longer-term, mild drought leads to shoot growth retardation through changes in photosynthetic organs and accumulation of metabolites to alleviate the stress (Robertson et al.,

2022; Todaka et al., 2017; Verelst et al., 2013). By making these adjustments, plants can optimize their growth and survival in their new growth environments.

To mitigate the impacts of drought on plants, it is essential to evaluate the molecular and physiological underpinnings of tolerance to drought in a reproducible growth condition that recapitulates nearly field environments (Dubois & Inzé, 2020; Lovell et al., 2016). The vast majority of molecular understanding of the response to drought stress in rice is based on hydroponically grown rice responses to osmotic stress or root drying in the laboratory (Lenka et al., 2011; Wilkins et al., 2016; Zhou et al., 2007). Therefore, validating early osmotic stress studies that examined the molecular basis of the drought stress response in a soil-based system is very challenging, limiting its usefulness in targeted stress response studies. It has been demonstrated that the types of growth environment can alter the physiology and gene expression response to water deficit treatment (Lovell et al., 2016; Wilkins et al., 2016). Stress-induced physiological and gene expression patterns become less pronounced, with respect to both the number of affected genes and the magnitude of the changes, as the complexities of the growth environments increase (Lovell et al., 2016). One plausible reason is that the plant grown in the field is exposed to a complex environment with multiple stressors simultaneously (Mittler, 2006; Plessis et al., 2015). Furthermore, drought stress in field crops often manifests as prolonged reduced water availability rather than shock treatment experienced in control environments (Lovell et al., 2016). Therefore, it is essential to consider the growth environment when studying plant stress response, as it may influence the results obtained.

Implementing physiologically relevant drought stress requires painstakingly monitoring the soil water content and the soil matric potential (SMP), which can be used to assess and control the level of drought stress on plants (Dowd et al., 2019). Platforms such as PHENOPSIS (Granier et

al., 2006) and the SMP monitoring system (Todaka et al., 2017) have been developed to monitor and control soil moisture content for plants grown under mild drought conditions. These systems have helped investigate the responses of plants treated with mild drought stress. In this study, we describe the development of a standardized and physiologically relevant drought stress assay for rice seedlings. This method differs from other drought assays in that it allows for plant growth under a nearly stable soil water contents due to highly reduced evapotranspiration. Using this method, we determined the growth, physiology, and gene expression pattern of rice seedlings under two levels of reduced water availability. The general objectives of this study were to determine how rice seedlings respond to consistent and nearly stable water-limiting conditions and whether responses to two levels of water-limiting stress were qualitatively or quantitatively different.

1.2 Statement of thesis objectives

The specific objectives of this study will be in two parts.

Objective 1: Describe the development of a growth-limiting drought assay for juvenile rice seedling.

Objective 2: Investigate the effects of water limitation on growth, physiology and quantify the gene expression changes of rice seedling in two levels of growth-limiting drought conditions.

CHAPTER 2: LITERATURE REVIEW

2.1 Background and Relevance

The global human population is expected to reach approximately 10 billion by 2050 (United Nations, 2019), increasing the need to boost crop production and productivity in order to meet the projected demand. This requires expanding the area under cultivation worldwide to increase food production (Tilman et al., 2011). Climate change factors are exacerbating this problem by shrinking the production area around the world (Tilman et al., 2011; Zhang & Cai, 2011). The effects of global warming are expected to last longer in agricultural areas, resulting in high temperatures, changing precipitation patterns, and elevated CO_2 levels. These factors can further limit agriculture production by increasing the frequency of droughts, flooding, and soil salinity, which limit agricultural land and water use. Climate change is also projected to increase the risk of crop failure and decrease agriculture productivity, potentially reducing the yield of staple foods like rice and wheat by up to 30% (Jain et al., 2015). One strategy to combat this problem is to enhance productivity of crops grown on currently available arable land through the use stress-tolerant crops that can grow without a yield penalty under stressful environmental conditions (Cattivelli et al., 2008). Therefore, understanding the molecular mechanisms that make crops resistant to climate change stressors, such as drought, is a crucial step in producing drought-tolerant crops.

2.2 Rice is an important staple food and a model monocot

Rice (*Oryza sativa* L.) is a globally important cereal that provides more than 80% of food energy intake for half of the world's population, especially in Asia (Fukagawa & Ziska, 2019; Seck et al., 2012). Rice production is significantly affected by abiotic stressors such as drought, high

temperature, and flooding (Kumar et al., 2022; Pathak et al., 2021). Upland rice is grown on about 23 million hectares globally (Bekis, 2019); these crops are rain-fed and are particularly susceptible to drought caused by reduced rainfall. Therefore, it is essential to develop drought-resistant crop varieties that will remain productive in water-limiting environments. This is critical to ensure food security of the growing world population and food demand (Dhankher & Foyer, 2018). In addition, rice is a widely used model organism in plant biology research. One of the main reasons for this is its relatively small genome size (approximately 389 Mb) (International Rice Genome Sequencing Project & Sasaki, 2005), with about 50,000 genes (Goff et al., 2002; Yu et al., 2002), which is about one-tenth the size of the human genome and about one-fifth the size of the maize genome. The small genome size makes it easier for scientists to map and identify the location of genes and study their functions (Delseny et al., 2001). Rice is a low-cost model because it has a short generation time and can be grown in large quantities (Itoh et al., 2005). Its popularity as a model organism is also due to the ease of genetic manipulation because it is amenable to a variety of molecular biology techniques, such as genetic transformation and genome editing (Bajaj & Mohanty, 2005; Delseny et al., 2001), which allows scientists to introduce new traits or study the function of specific genes.

Rice is a member of the Poaceae family, the Ehrhartoideae subfamily, and the Oryzeae tribe (Kellogg, 2009). Monocots represent one of the two major clades of flowering plants (Soltis & Soltis, 2004), which include important crops like maize, wheat, and sugarcane. The evolutionary relationships of monocots, including rice, have been extensively studied (Chase, 2004; Givnish et al., 2018; Molina et al., 2011). Furthermore, rice is a close evolutionary relative of other important cereal crops like wheat, barley, and maize, making it a useful model for understanding the genetic and molecular mechanisms underlying cereal crop improvement (Gale et al., 2007;

Sasaki & Antonio, 2005). Research into rice genetics and genomics have the potential to improve rice yield and nutritional quality, which is critical for food security in many parts of the world.

2.3 Defining drought and water limitation

As a preface to the literature review relating to water limitation mechanisms in plants, the distinction between "drought" and "water limitation" requires clarification. For many disciplines working on "drought", it is a period of below-average precipitation (Mapedza & McLeman, 2019). Here, I refer to "drought" in terms of plant-water relationships, which I used interchangeably with "water limitation". In the molecular physiology context, drought responses are plants' responses to altered water status (Verslues et al., 2006). Drought occurs when the soil water become less available to the plant because it has a lower free energy state than well-watered conditions (Juenger & Verslues, 2022). Drought is a serious abiotic stress that affects crop production worldwide (Fahad et al., 2017), and the frequency and severity of drought have increased due to climate change. Climate events causing increased drought occurrence can lead to food insecurity and economic losses for farmers and rural communities (Naumann et al., 2021). For example, studies of drought impact on yield have shown that a slight decrease in precipitation can lead to a significant decrease in crop yields (Fishman, 2016); this problem becomes severe in areas dependent on irrigation. In addition, water limitations can significantly impact developing countries due to their higher dependence on agriculture and limited access to irrigation and other water management technologies (Hyland & Russ, 2019). Developed countries are also affected by drought, with examples of drought events in the US, Europe and Australia causing significant economic losses (Naumann et al., 2021; Ziolkowska, 2016).

2.4 Plant response to water limitation

Plant responses to water limitation seem to consist of two major mechanisms: stress avoidance and tolerance (Claeys & Inzé, 2013; Verslues et al., 2006). While the latter is targeted at protecting against cellular damage, the former is a strategy used by plants to minimize water loss and maintain adequate water availability by optimizing water uptake. Primary mechanisms of drought avoidance in plants take place through the regulation of stomatal closure. In response to water-limiting conditions, plants regulate stomatal closure to reduce water loss and maintain water balance, which ensure that tissue water potential is maintained close to well-watered conditions (Verslues et al., 2006). Additionally, plants can also avoid drought stress by actively reducing their leaf surface area (Clauw et al., 2015, 2016), which helps plants to minimize water loss through reduced transpiration and maintenance of cellular water balance. In rice, drought susceptible genotypes showed smaller flag leaves under drought stress, which negatively affect their yield (Kumar et al., 2021). These strategies are a short-term response to declining soil water availability orchestrated by stomatal control. Under long term drought conditions, plants develop deeper root systems as an important drought avoidance mechanism. Although, root growth at the expense of the shoot leads to an imbalanced root/shoot ratio, this mechanism is essential to access water from deeper soil layers and maintain water availability during drought conditions (Gowda et al., 2011). In addition to these mechanisms, other components of drought avoidance mechanism including elasticity in leaf rolling, high cuticular resistance and increased water permeability (Blum et al., 1989; Gowda et al., 2011; Verslues et al., 2006), which are all of potential importance for developing drought tolerant crops.

In cases where water limitation becomes too severe or under long term drought exposure, stress avoidance mechanisms are insufficient to maintain cellular homeostasis (Bandurska, 2022;

Claeys & Inzé, 2013). The tolerance mechanisms are required to prevent cellular damage due to the dehydrating effect of limited water availability (Bandurska, 2022; Cruz de Carvalho, 2008; Verslues et al., 2006). This mechanism takes place primarily through osmotic adjustment, which include detoxification of reactive oxygen species (ROS), accumulation of protective proteins and osmo-protectants (Azhar & Rehman, 2018; Claeys & Inzé, 2013). Osmotic adjustment ensures continue plant growth by facilitating balanced cell osmotic potential, the gradient for water influx and the maintenance of plant turgor pressure (Azhar & Rehman, 2018). Also, the accumulation of solutes, such as sugars, amino acids, and organic acids in plant cells helps to maintain cell turgor pressure and prevent dehydration (Takahashi et al., 2020). For example, under mild drought conditions, sugar and starch were found to accumulate at higher quantities in rice (Todaka et al., 2017) and Arabidopsis leaves (Skirycz et al., 2009). Drought-induced accumulation of metabolites have been hypothesized to be backup sources for compatible solutes to reduce cell water potential in severe drought conditions (Takahashi et al., 2020; Todaka et al., 2017). Another important mechanism of drought tolerance is the production of stress-responsive proteins (Verslues et al., 2006). These protective proteins help plants to cope with drought stress by protecting cellular components from damage, increasing water uptake and retention, and improving water transport within the plant (Riyazuddin et al., 2022). Some examples of stress-responsive proteins include dehydrins, LEA (LATE EMBRYOGENESIS ABUNDANT) proteins, and aquaporins. While the role of these protective proteins are still subject of extensive research, at least in part, they act as chaperones that protect cellular protein and membrane structure under low osmotic potential; they also act as a compatible solute and control of reactive oxygen species thereby contributing to drought tolerance in plants (Riyazuddin et al., 2022; Verslues et al., 2006).

It is important to note that the mechanisms of drought avoidance and tolerance in plants are dynamic and can involve different strategies, which may not occur in a linear progression over time or severity of stress (Verslues et al., 2006). Research has shown that different plant species may have unique drought tolerance mechanisms, and these mechanisms can vary depending on the severity and duration of the drought condition. To help plants adapt to drought stress, a number of factors and mechanisms work separately or in concert.

2.5 Effect of water limitation on plant growth and development

Numerous studies have shown that plants experience reduced growth under drought conditions, due to a variety of physiological and morphological changes (Dubois et al., 2013; Harb et al., 2010; Skirycz, Vandenbroucke, et al., 2011; Todaka et al., 2017; Verelst et al., 2013). Therefore, growth regulation is a key component of how plants respond to drought, which is a mechanism primarily aimed to reduce the evaporation surface (Claeys & Inzé, 2013). The first morphological manifestation of reduced water availability is to actively decrease leaf area (Skirycz, Vandenbroucke, et al., 2011). This reduction in leaf growth by water limitation occur through the inhibition of cell division and expansion (Claeys & Inzé, 2013). Recent studies suggest that water limitation can impact nearly all aspects of the cell cycle machinery and cell wall, which is an indication of active growth restriction mechanisms (Claeys et al., 2012; Claeys & Inzé, 2013). Another pathway that explains this growth regulation is that water limitation altered levels of hormones, such as the ethylene and cytokinin, which are crucial regulators of cell division and differentiation (Dubois et al., 2013). Above mentioned evidence suggests that the final leaf area decreased significantly in limited water conditions due to the reduction in both number of cells and size as reported in Arabidopsis (Aguirrezabal et al., 2006), and rice (Todaka et al., 2017). More interestingly, growth compensation have also been reported in which the

drought stressed plant extend their growth duration to compensate for the slow growth under water limiting conditions. It is important to also add that the timing, duration, and severity of the stress and the developmental stage all affect how plants respond to drought and how much it can tolerate (Panda et al., 2021). While the effects of water deficit are usually measured in terms of survival, mild non-lethal drought significantly affect plant growth, development and yield. For example, mild drought conditions during the critical flowering stage can lead to a significant reduction in rice yield, with studies reporting decreases of up to 50% or more compared to well-watered conditions (Zhang et al., 2018). Therefore, painstakingly measuring the changes in plant growth over time can serve as a monitoring system to understanding mechanisms of plant growth and development under mild drought stress and avenues for developing drought tolerant crops.

While the effect of water limitation on aboveground organs could be detrimental to biomass accumulation and yield, it important to note that plant roots are an important component of drought avoidance and tolerance at the forefront of sensing and responding to drought stress (Gowda et al., 2011; Janiak et al., 2016). Water limitation can also significantly impact root growth by altering its structure and functions including nutrient and water uptake (Gowda et al., 2011). In rice, studies have shown that root systems can lengthen and produce more biomass when there is a slight reduction in soil water availability (Fonta et al., 2022). Lower soil water potential was associated with increased length, diameter and volume of first-order lateral roots in maize, although this observation was genotype specific (Dowd et al., 2019). The ability of plants to establish a deeper and more intricate root system is one of the most crucial adaptations that they made to continue growing in water-scarce environments (Gowda et al., 2011; Wasson et al., 2012). This phenomenon is highly prevalent in a mild drought condition, where plants may allocate more resources toward root growth in order to explore deeper soil layers for water, while

simultaneously reducing the shoot growth to improve plant water balance. However, increased investment in root growth can come at the expense of aboveground growth, leading to an imbalanced root – shoot ratio.

2.6 Physiological responses of plant to water limitation

Water limitation is a common stress that plants encounter in their natural environments, and various physiological responses have been developed to tolerate the stress while maintaining growth (Gupta et al., 2020). Under mild water limitation, plants can adjust their photosynthetic rate to conserve water and maintain their carbon balance (Flexas, Bota, et al., 2006). This process may involve downregulation of photosynthesis to reduce water loss or the enhancement of water use efficiency through the optimization of photosynthetic parameters. Among various physiological and biochemical changes that occur in plants under water stress, alterations of chlorophyll content have been extensively studied (Sanchez et al., 1983; Todaka et al., 2017; Zhuang et al., 2020). Chlorophyll is the most abundant pigment in plants, responsible for capturing light energy during photosynthesis. Reduction in chlorophyll content under water stress can have a significant impact on plant growth, yield, and survival (Christ et al., 2014; Emerson, 1929; Katz et al., 1978). The mechanism behind the reduction in chlorophyll content under water stress is not fully understood. However, it is believed that water stress can cause oxidative stress in plants, leading to the degradation of chlorophyll molecules (Aarti et al., 2006).

While there has been a number of published reports on drought constraints on chlorophyll content and photosystem apparatus, there is still much debate about what constitutes the main physiological target responsible for photosynthetic limitation (Chaves et al., 2009; Pinheiro & Chaves, 2011). Photosynthesis is one of the primary biological processes to be affected by water

availability, and it has a significant influence on yield. Drought constraints on photosynthesis could occur directly by limiting CO_2 diffusion through the stomatal and mesophyll or indirectly through the alteration of carbon metabolism (Flexas et al., 2004). Mesophyll limitation is a phenomenon in which the rate of photosynthesis is limited by the diffusion of CO_2 from the intercellular spaces to the sites of carboxylation in the chloroplasts due to a thickened mesophyll layer. However, the extent of mesophyll limitation is still subject to debate because of variations in the methods used to estimate the mesophyll conductance (Chaves et al., 2009). Nevertheless, limited soil water availability results in decreased CO_2 diffusion through the leaf mesophyll, which constitutes one of the main non-stomatal limitations to photosynthesis (Flexas et al., 2012). For instance, Flexas et al., 2006 demonstrated that mesophyll conductance responds rapidly to desiccation and that exogenous application of abscisic acid (ABA) can induce a reduction in mesophyll conductance even in well-watered plants.

Stomatal limitation constitutes the main factor contributing to decreased net CO_2 assimilation rates by the leaves under water limiting conditions (Chaves, 2002). Therefore, stomatal regulation is an important aspect of water conservation mechanisms for adaptation to water limiting environments. Under mild stress, a small decline in stomatal conductance can be beneficial for plants as it allows for water saving and improves water-use efficiency (Medrano, 2002). The regulation of stomatal conductance and its response to water limitation stress are mediated by the hormone abscisic acid (ABA), which is synthesized in the leaves or transported from the roots (Pinheiro & Chaves, 2011). This stomatal response to ABA is modulated by various external and internal factors, including plant hormones, age, developmental stage and intracellular K^+ composition (Andrés et al., 2014; Merilo et al., 2015), as well as other

environmental factors such as light, temperature, in addition to soil water content (Araújo et al., 2011).

Apart from the diffusion limitations by the mesophyll and stomata, there is biochemical limitation to photosynthesis. This component is estimated to be smaller than the diffusion limitation in a mild water limiting condition. Since changes in plant biochemistry may be required for downregulation of photosynthetic metabolism, this component is likely to be orchestrated by transcriptional reprogramming (Chaves et al., 2009; Pinheiro & Chaves, 2011). A recent example in cotton showed that low intercellular CO_2 levels in leaves was associated with decreased soil water content which led to a deactivation of Rubisco enzyme (Carmo-Silva et al., 2012).

2.7 Role of plant hormones in adaptation to growth limiting drought stress

Plant hormones play an essential role in regulating plant growth and development under water limiting conditions. Abscisic acid (ABA) is a well-known hormone that affects plant drought response. In response to water deficit, ABA is synthesized and triggers stomatal closure, which lowers transpiration and water loss. ABA also stimulates the buildup of osmo-protectants, like proline and sugars, which help plants cope with reduced water availability. For example, a number of studies have shown that ABA-deficient mutants are more sensitive to drought stress than wild-type plants (Verslues & Bray, 2006; Zhao et al., 2016). Transcriptome wide profiling in Arabidopsis have also revealed the important role of ethylene and GA in plant adaptation to mild water limitation (Huang et al., 2008; Skirycz, Claeys, et al., 2011; Skirycz et al., 2009). For example, ethylene production in *OsDERF1* rice mutants positively increases drought tolerance by regulating osmolyte accumulation and ethylene biosynthesis (Wan et al., 2011). Important

role of GA biosynthesis transcripts in regulation of leaf growth has been shown in *Brachypodium distachyon* (Verelst et al., 2013), in addition to GA's role in enhancing plant tolerance to drought stress by promoting root growth and increasing water use efficiency (Colebrook et al., 2014). Other plant hormones appear to be involved either directly or indirectly as well; cytokinins, auxins, jasmonate, and brassinosteroids have also been implicated in plant adaptation to drought stress. Cytokinins have been shown to promote root growth, increase water uptake, facilitate carbon and nitrogen metabolisms leading to enhance tolerance to limited water availability (Reguera et al., 2013). Understanding the molecular mechanisms underlying these hormone-mediated drought responses will be essential for developing strategies to improve plant drought tolerance and enhance crop yield under water-limited conditions.

2. 8 Molecular mechanisms of the response to water limitation

Plant response to water limitation consists of morphological, biochemical, physiological and molecular responses coordinated by complex gene regulatory networks (Yamaguchi-Shinozaki & Shinozaki, 2006). Many recent studies have shown that drought responses are coordinated by a large number of regulators of small effect, which is in contrast with the relatively small number of major effect loci for submergence tolerance (Septiningsih et al., 2012; Zhang et al., 2017) and high-temperature response (Kilasi et al., 2018; Lafarge et al., 2017). This view is supported by genome wide association studies (GWAS) (Bhandari et al., 2020; Li et al., 2017) and quantitative trait locus (QTL) analyses (Fleury et al., 2010; Lanceras et al., 2004; Sabar et al., 2019) which are used to identify genomic variants that are associated with drought tolerance. These studies have identified a large number of genetic loci of small effect on variance in drought stress response. Moreover, plants produce various drought-responsive regulatory mechanisms such as avoidance and tolerance, depending on the stress context because different plant tissues,

developmental stages and varieties respond differently to drought stress (Demirevska et al., 2009; Fahad et al., 2017; Skirycz et al., 2009). Furthermore, the regulation of drought stress is a function of stress duration, which triggers early or later responses depending on the nature of the stress (Harb et al., 2010). For example, a time-series study of the global gene expression pattern of Arabidopsis and maize revealed temporal responses to water deficit and demonstrated that different functional gene sets were regulated at early, middle, and late drought stages (Bechtold et al., 2016; He et al., 2020).

The phytohormone abscisic acid (ABA) is produced in response to drought stress and enables plants to cope with water deficit by coordinating complex gene regulatory networks (Lata et al., 2011). ABA triggers stomata closure and the induction of other stress-related genes (Shinozaki & Yamaguchi-Shinozaki, 2006; Todaka et al., 2015). Canonical drought responses include the ABA-dependent and independent signalling pathways (Mizoi et al., 2012; Shinozaki & Yamaguchi-Shinozaki, 2006; Yamaguchi-Shinozaki & Shinozaki, 2005). The ABA-dependent pathway plays a vital role in drought response and it is orchestrated by genes possessing ABA-responsive elements (ABRE) in their promoter regions (Yamaguchi-Shinozaki & Shinozaki, 2006). ABRE-binding factors (AREB/ABFs), including members of the basic leucine zipper (bZIP) and APETALA2/ethylene-responsive element binding protein (AP2/EREBP) families, bind to ABRE motifs and initiate the transcription of downstream target genes involved in drought tolerance mechanisms (Fujita et al., 2013; Yoshida et al., 2014). In contrast, the ABA-independent response is activated by genes possessing a dehydration response element (DRE) controlled by the expression of DREB and NAC transcription factor (TF) families (Lata et al., 2011; Nakashima et al., 2014; Shinozaki & Yamaguchi-Shinozaki, 2006). Expression of transcription factors ensures osmotic balance, cellular damage repairs, and protection through the

production of various enzymes and proteins such as LATE EMBRYOGENESIS ABUNDANT (LEA) proteins, antioxidants and transporters, and proteases (Hirayama & Shinozaki, 2010; Joshi et al., 2016).

Transcriptome analyses using microarray and RNA-seq have identified several drought inducible genes involved in coordinating response to water limitation in plants (Lovell et al., 2016; Lv et al., 2019; Moumeni et al., 2011; Skirycz et al., 2009; Smita et al., 2013; Todaka et al., 2017; D. Wang et al., 2011). Several of these genes are involved in producing essential metabolic proteins, while a host of others are involved in regulating stress signal perception and transduction (Yamaguchi-Shinozaki & Shinozaki, 2006). Extensive investigation from gene regulatory network predictions showed several transcriptional regulators to be involved in controlling drought response in rice (Groen et al., 2020, 2022; Wilkins et al., 2016). Functional experimental studies involving knockout and overexpression of these TFs in rice (Ahn et al., 2017; Huang et al., 2018) has only led to incremental changes in drought stress tolerance.

The observation that experimental manipulating dehydration responsive TFs has resulted in only slight enhancements in drought tolerance highlights the complexity of plant responses to drought (Harb et al., 2010). This observation may be attributed to the fact that survival in severe drought conditions and tolerance under mild drought stress may involve distinct mechanisms (Skirycz, et al., 2011). In light of this, future research directions could be directed towards elucidating the intricate gene regulatory networks underlying growth-limiting drought conditions. This could involve exploring molecular understanding of the interplay between different growth-limiting drought tolerance mechanisms. By focusing on these aspects, we can aim to enhance crop resilience to water limitation in a way that specifically addresses the challenges posed by growth-limiting drought conditions.

CHAPTER 3: MATERIALS AND METHODS

3.1 Establishment of a consistent potting mix-based growth-limiting drought assay

Sunshine mix #4 (Sun Gro Horticulture, Agawam, Massachusetts) was used to establish a growth-limiting drought experiment due to its high porosity. The potting mix was dried at 55 ° C for 48 hours in a soil drying oven until the soil reached constant weight and all the water evaporated (Dowd et al., 2019). The gravimetric soil water content was then measured following the method described by Dowd et al. 2019. Briefly, the dried potting mix was mixed with a proportional weight of water to achieve desired soil-to-water ratio (SWCg, gram of water per gram of potting media). For the initial assessment of plant growth in this potting media, 120g of soil was weighed into a zip lock bag, and then 120, 150, 180, 300, 360 and 600 grams of water were added to generate 1, 1.25, 1.5, 2.5, 3 and 5 g/g gravimetric soil water contents.

3.2 Plant growth and water-limiting conditions

Rice seeds (Nipponbare, GSOR #100) used in this study were obtained from Dale Bumpers National Rice Research Centre, Stuttgart, Arkansas, USA. Seeds were first germinated on moist Whatman filter paper at 30°C for 72 hours before planting. Seven seeds showing signs of germination and radicle emergence were planted about 2cm below the surface of each shuttle pot (11×11×9.53 cm) containing 120 g of soil and varying soil water content depending on SWCg as previously described above. To study the effects of limited soil water availability on growth, the SWCg of the control was set at 3.0g/g, while the water-limiting drought conditions were set at 1.5 g/g and 1.25 g/g. This growth experiment was carried out with three to four biological replicates for each treatment. After planting, pots were placed in trays, with a cheesecloth underneath each pot, to reduce the water loss through evaporation (Dowd et al., 2019). Plants

were grown in a Conviron growth chamber, with a 12-hour day/night cycle at 400 μmol photosynthetically active radiation (PAR) at canopy height, 30°C/28°C day/night temperature, and 90% relative humidity. To ensure the plants were growing in consistent soil water availability, each pot was weighed daily and watered back to its target weight using a spray bottle to wet the potting mix. Additionally, cheesecloth was dampened every day to slow evaporation from the soil, as previously described by Dowd et al. 2019. Around five hours after the growth chamber light was on, the pots were weighed and rewatered to the target each day.

3.3 Measurement of growth rate and morphological changes of rice seedlings under water-limiting conditions

In order to capture the growth rate under the three consistent soil water levels, plant morphological traits were measured. Plant emergence was recorded daily for each soil water content until all the plants in a pot had emerged. The plant height was measured from soil level to the zenith of the longest leaves between days five to ten after planting. Length of the third leaf, which is the distance from the soil level to the tip of the leaf, was measured and documented from the day of its emergence until it reached full expansion. The leaf elongation rate was then estimated from the daily change in leaf length (n = 20 – 30 leaves). For the root length, all plants from three pots for each of the three soil water conditions were selected, and soil was carefully washed from the root system, the bare roots were laid on a flat surface, and the length was measured from the shoot base to the longest root tip day ten after planting. Root length measurements were taken from three biological replicates of at least three individual plants.

3.4 Chlorophyll pigment extraction

Chlorophyll pigment extraction was performed on a fully expanded third leaf from water-limiting conditions (1.5 g/g) and well-watered control (3.0g/g). This experiment was planted and grown in Sunshine Mix #1 (Sun Gro Horticulture, Agawam, Massachusetts) as described above. The third fully expanded leaf tissue was cut into seven longitudinal sections, and a ~2cm middle segment was used for the chlorophyll pigments extraction. Leaf tissue was weighed to determine the starting fresh weight and immediately flash frozen in liquid nitrogen and stored frozen in a -80°C freezer. For the chlorophyll pigment extraction, the tissues were homogenized in 600 µl of ice-cold 80% acetone, filtered through miracloth to remove debris, and then the filtrate was centrifuged at 5000g for five minutes to remove fine debris. The absorbance of the supernatant was measured using a spectrophotometer plate reader (SYNERGY microplate reader, BioTek) at 663 nm (Chlorophyll a), 645 nm (Chlorophyll b) and 750nm (non-specific absorption). The Chlorophyll contents were calculated per fresh weight using the formula below, as described by (Pocock et al., 2004).

$$\textit{Chlorophyll a (mg/g)} = \textit{(12.7 * A663) – (2.69 * A645)/ fresh weight *extraction volume}$$

$$\textit{Chlorophyll b (mg/g)} = \textit{(22.9 * A645) – (4.68 * A663)/ fresh weight *extraction volume}$$

$$\textit{Total chlorophyll (mg/g)} = \textit{Chlorophyll a} + \textit{Chlorophyll b}$$

3.5 Chlorophyll fluorescence measurements

The maximum photosystem II efficiency (*Fv/Fm*) was measured on the dark-adapted leaves using LI-COR 6800, 1 hour before the light was on in the growth chamber. The leaves were placed in the cuvette for 20 minutes under constant CO_2 480 ppm supply, 0 µmol m^{-2} s^{-1} light,

flow rate 100 μmol m^{-2} s^{-1}, RH 65%, leaf temperature 30ºC and fan speed 5000 rpm. Although, the CO_2 concentration used was higher than current atmospheric CO_2 concentration, this provides a more realistic representation of indoor growth chamber and future atmospheric conditions due to global warming. A rectangular flash 10,000 μmol m^{-2} s^{-1} of light flash was used, and the *Fv/Fm* was recorded. All measurements were conducted in four to five replicated for well-watered control (3.0 g/g) and water-limiting treatment (1.5 g/g).

3.6 Physiological and gas exchange measurement

Rice seedlings used for all the gas exchange and photosynthesis experiments were grown in Sunshine Mix #1 following the same procedure described for the growth assays. For gas exchange parameters, the measurements were conducted on the third fully expanded rice leaves using LI-COR 6800 mounted with a 01A fluorometer (LI-COR Biosciences, Lincoln, Nebraska, USA). Because the leaves did not cover the cuvette area, the middle portions of the four leaves were arranged in parallel in the cuvette, such that they did not overlap. The leaves covered approximately 75% of the cuvette area, and all gas exchange measurements accounted for this. Point measurements of net CO_2 assimilation (A_{net}), transpiration (E), stomatal conductance (g_{sw}), and intrinsic water use efficiency ($iWUE$) were made at the midpoint of the day (six hours after the light was on) by placing leaves inside the cuvette, allowing the leaves to acclimate to the cuvette conditions, and then taking measurements every 30 seconds for five minutes. The cuvette conditions were set to match those in the chamber with respect to light intensity (400 μmol m^{-2} s^{-1}), temperature (30ºC), and humidity (RH 65%). While CO_2 supplied concentration was set to 480 ppm, the flow rate and the fan speed was set to 100 μmol m^{-2} s^{-1}, and 5000 rpm, respectively. The gas exchange parameters investigated are described in Table 3.1.

Table 3.1: Description and symbol of gas exchange and fluorescence parameters studied.

Description	Label	Units
Net photosynthetic rate	A_{Net}	μmol m^{-2} s^{-1}
Transpiration rate	E	mol m^{-2} s^{-1}
Stomatal conductance	g_{sw}	mol m^{-2} s^{-1}
Intercellular CO_2	C_i	μmol mol^{-1}
Maximal chlorophyll fluorescence in a dark-adapted leaf	F_m	
Maximal chlorophyll fluorescence in a dark-adapted leaf	F_m'	
Minimal chlorophyll fluorescence in a dark-adapted leaf	F_0	
Minimal chlorophyll fluorescence in a dark-adapted leaf	*F0'*	
Steady-state fluorescence	F_s	
Variable chlorophyll fluorescence	F_v	
PSII maximum efficiency in dark-adapted leaves	F_v/F_m	
Non-photochemical quenching	*NPQ*	
PSII operating efficiency	*PhiPSII*	
Electron transport rate	*ETR*	
Intrinsic water use efficiency	*iWUE*	μmol CO_2 mol^{-1} H_2O
The quantum yield of the carboxylation rate	*PhiCO2*	μmol CO_2 $mmol^{-1}$ photons

3.7 Response curves

Light responses were measured six hours after the light was on. The cuvette conditions were set to match those in the chamber with respect to temperature (30ºC), and humidity (RH 65%). While the CO_2 was set to (480 ppm), the flow rate was set to 100 µmol m^{-2} $s^{-1,}$ and the fan speed was set to 5000 rpm. Light intensity was increased to 2000 µmol m^{-2} $s^{-1,}$ and leaves were allowed to acclimate for 15 minutes before the first measurement. Subsequently, the light was reduced stepwise to 1800, 1500, 1200, and 1000 µmol m^{-2} s^{-1}, where measurements were taken after six minutes at each step, and then to 800, 700, 600, 400, 300, 200, 150, 25, 0 µmol m^{-2} s^{-1} with measurements taken after three minutes at each step. This experiment was repeated on five biological replicates, each of which included four plants, for each of the soil water conditions.

The CO_2 response curves (*A/Ci*) were carried out under non-limiting light conditions (1200 µmol m^{-2} s^{-1}). The cuvette conditions were set to match those in the chamber with temperature (30ºC) and humidity (RH 65%); the flow rate was set to 100 µmol m^{-2} $s^{-1,}$ and the fan speed was set to 5000 rpm. The plants were supplied with CO_2 concentrations in the cuvette of 800, 600, 500, 485, 405, 300, 200, 100, 75, 50, 25 and 0 mmol m^{-2} s^{-1} CO_2. For the *A/Ci* curve, the plants were allowed 2.5–5 min to stabilize for each reference CO_2 concentration before the data points were logged. Similarly, the CO_2 response curve measurement was repeated on five biological replicates, each of which included four plants, for each of the soil water conditions.

3.8 Modelling gas exchange parameters

Photosynthetic measurements from light response curves were fitted using the R package "*photosynthesis*" (Stinziano et al., 2021) to obtain the maximum photosynthetic rate at saturated CO_2 and the light compensation point. Next, photosynthetic parameters from the CO_2 response

curve were modelled to estimate the maximum velocity of Rubisco carboxylation rate (V_{cmax}) and maximum electron transport rate (J_{max}) using the R package "*plantcophys*" (Duursma, 2015). All response curve analyses and model parametrization were done using R 4.1.1. Statistically significant differences in the model parameters between the control and water limiting conditions were calculated by Student's t-test *($P < 0.05$).*

3.9 RNA extraction and sequencing library preparation

For RNA isolation, shoot tissues were collected six hours after the light was on, flash frozen in liquid nitrogen, and stored at -80 ºC freezer until use. Three replicates of tissue samples from each SWCg were collected by cutting just below the first leaf. Each replicate consisted of three representative plants grown in the same pot. The tissue was ground to fine powder by bead mill homogenization. Total RNA was extracted from plant tissues using the Qiagen RNeasy plant (Qiagen, Toronto, ON, Canada) mini kit following the manufacturer's instructions. RNA quality and quantity were checked using spectrophotometry (Table 3.2) and agarose gel electrophoresis (Figure 3.1). High-quality RNA from samples of three water statuses with three replications per treatment were sent to Genome Québec for cDNA synthesis, library construction and RNA sequencing. The RNA sequencing libraries were generated from the nine samples and sequenced for paired-end 100 base pair reads on the Nova-seq Illumina platform following the NEBNext-mRNA stranded library protocol (New England Biolabs, Whitby, ON, Canada).

Table 3.2: RNA concentrations from tissue samples used for RNA sequencing.

#ID	Sample	Conc (ng/μl)	A260/A280	A260/A230	RIN
1	Os.Nip.W.3.pil.1	858.6	2.15	2.24	7.8
2	Os.Nip.W.3.pil.2	1891.4	2.19	2.26	10
3	Os.Nip.W.3.pil.3	2241.9	2.19	2.17	7.2
4	Os.Nip.W.1.5.pil.1	1510.6	2.17	2.12	7.5
5	Os.Nip.W.1.5.pil.2	2728.9	2.18	2.35	7.6
6	Os.Nip.W.1.5.pil.3	1030.4	2.14	2.10	6.8
7	Os.Nip.W.1.25.pil.1	1454.6	2.17	2.32	7.6
8	Os.Nip.W.1.25.pil.2	1173.2	2.15	2.24	7.4
9	Os.Nip.W.1.25.pil.3	1447.8	2.17	2.12	7.4

RIN – RNA integrity number was obtained from Genome Québec QC reports.

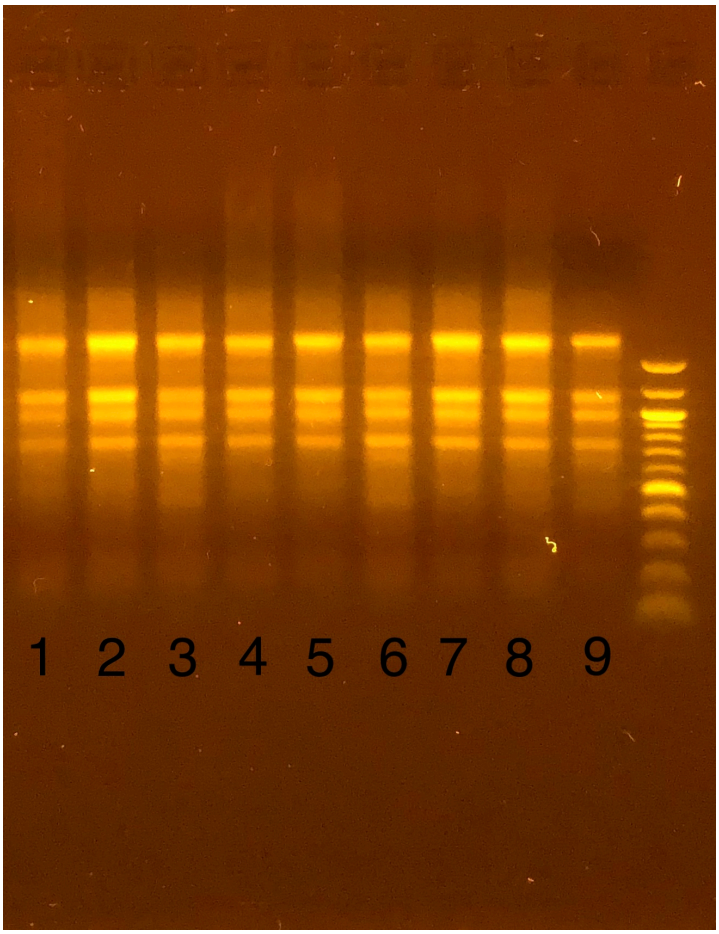

Figure 3.1: High quality RNA extracted from rice leaves were checked using agarose gel electrophoresis. Sample numbers indicated below the bands in black matched the ID number in Table 3.2 above.

3.10 RNA sequencing data processing and analysis

All bioinformatic analyses were performed on the Compute Canada cluster managed by the Digital Research Alliance of Canada (https://alliancecan.ca/en). Sequencing reads in the *fastq* formats were first passed through FastQC (Andrews, 2010) and trimmed using Trimmomatic (Bolger et al., 2014). The trimming parameters include removing adaptor sequences and the reads with quality scores less than 25%. The resulting high-quality reads were aligned to the rice Nipponbare reference genome (Kawahara et al., 2013) using HISAT2 (Kim et al., 2019), and the SAM files converted to BAM using SAMtools (H. Li et al., 2009). Transcript abundance was quantified using featureCount (Liao et al., 2014). All subsequent downstream analyses were performed in R 4.11(R Core Team, 2022). Differential gene expression analysis was carried out using EdgeR (Robinson et al., 2010) between the two water-limiting conditions and the well-watered control. Differentially expressed genes were identified using absolute $\log_2$FC greater than zero and a false discovery rate (FDR) of less than 0.05 by the Benjamini–Hochberg method (Benjamini & Hochberg, 1995).

3.11 Data exploration, clustering and GO term enrichment analysis

To explore the primary source of variation and the relationship between the samples, principal component analysis was performed with the modified plotPCA function using the DESeq2 package (Love et al., 2014). UpSet plots were generated using UpsetR (Conway et al., 2017) and SuperExactTest (Wang et al., 2015) scripts adapted and modified from Bjornson et al., 2021. Differentially expressed genes were hierarchically clustered to identify similar expression modules using the cutreeDynamic function (Langfelder et al., 2008) of the WGCNA package (Langfelder & Horvath, 2008). The 12 clusters identified were manually assigned to five broad

expression groups based on similarity in their expression pattern. Gene ontology enrichment analysis was performed using the topGo (Alexa & Rahnenführer, 2022) package for the gene sets that showed similar expression patterns as identified by cutreeDynamic. For the GO term enrichment analysis, Fisher's exact test was used, and biological processes with an adjusted p-value of less than 0.01 were considered significant. All code used for analysis in this thesis is available in the supplemental material.

CHAPTER 4: RESULTS

4.1 Examination of phenotypic effects and growth changes of two consistent water-limiting conditions reveals the extent of growth reduction in rice

A system was developed for studying the effects of growth limiting drought on rice seedlings that could be sustained for a period of at least two weeks. The growth conditions we developed were physiologically relevant to juvenile rice plants and provided near-constant soil water content to the plants throughout the study. These growth conditions enabled us to control the variations in soil water contents for the duration of the experiment. Using this system, we established a range of different gravimetric soil water contents to determine when the soil water becomes limiting to rice growth. Rice plants were grown in varying soil water contents (1.0 - 5.0 grams of water per gram soil) over a period of ten days and found that both extreme water deficit and excess water inhibit plant growth (Figure 4.1A – B). Based on this, we selected two growth limiting soil water conditions (1.25 g/g and 1.5 g/g), which were between the plant permanent wilting point (< 1.0 g/g), and well-watered control conditions (3.0 g/g) to further characterize the effect of drought on the growth, physiology, and transcriptome of rice seedlings. Rice plants grown in 1.25 g/g and 1.5 g/g SWCg were smaller than plants grown in the 3 g/g conditions (Figure 4.2A), with a 26% and 16% reduction in height, respectively (Figure 4.2B). The lengths of the third seedling leave decreased by 23% in 1.5 g/g and 26% in 1.25 g/g water-limiting conditions (Figure 4.2C). Moreover, the root length increased by about 35% in the two water-limiting conditions at ten days after planting (Figure 4.2D).

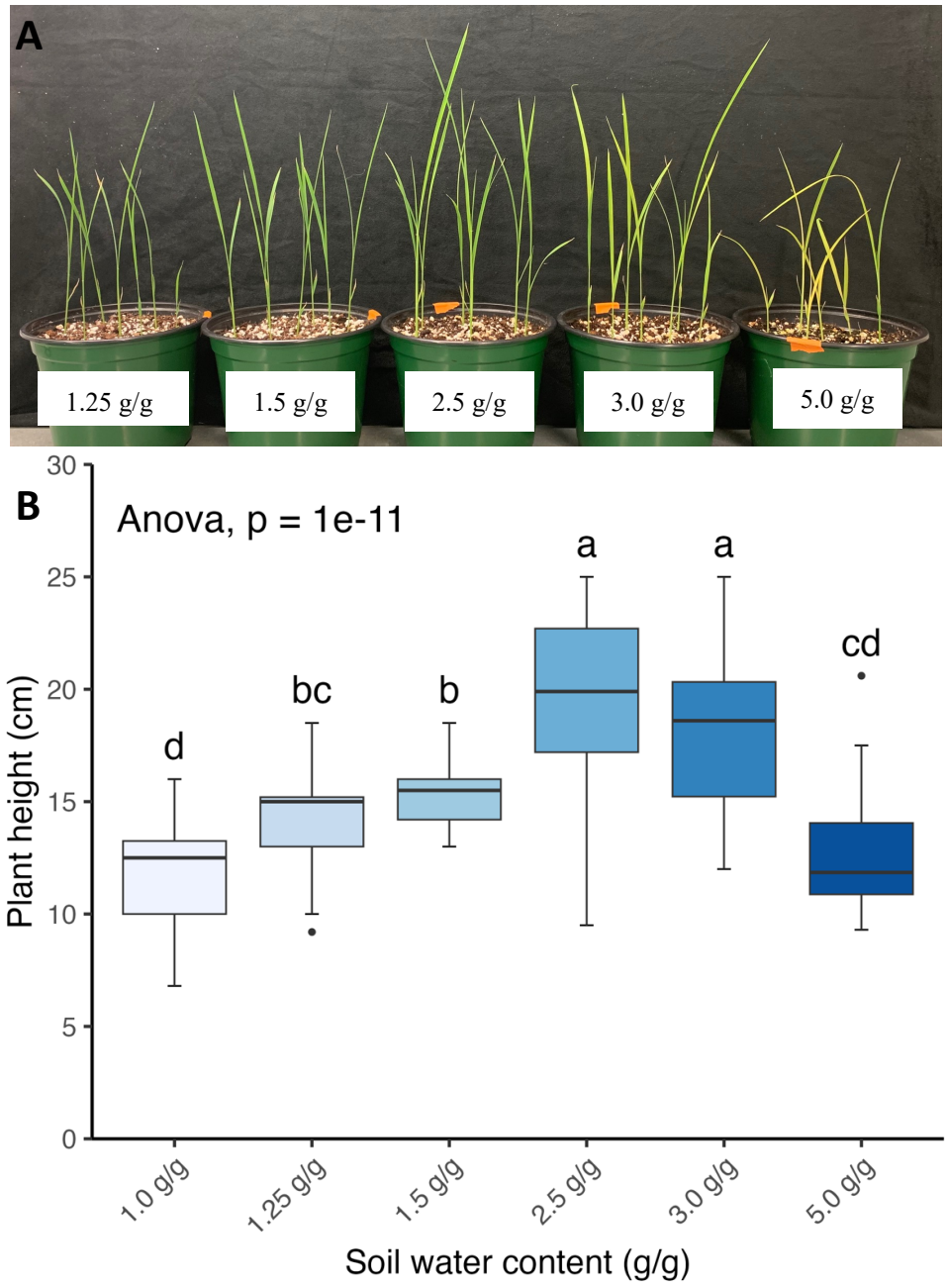

Figure 4.1: Morphological analysis of rice plants under consistent soil water conditions. (A) Photograph of representative rice plants grown in five gravimetric soil water contents (5.0 g/g, 3.0 g/g, 2.5 g/g, 1.5 g/g, and 1.25 g/g). (B) The plant height of rice seedlings at ten days after growing in six consistent soil water contents (n = 21 - 28). Boxplot shows the median and interquartile range of the measurement visualized per treatment. Letters indicate statistically different means from other SWC by analysis of variance ($P < 0.05$).

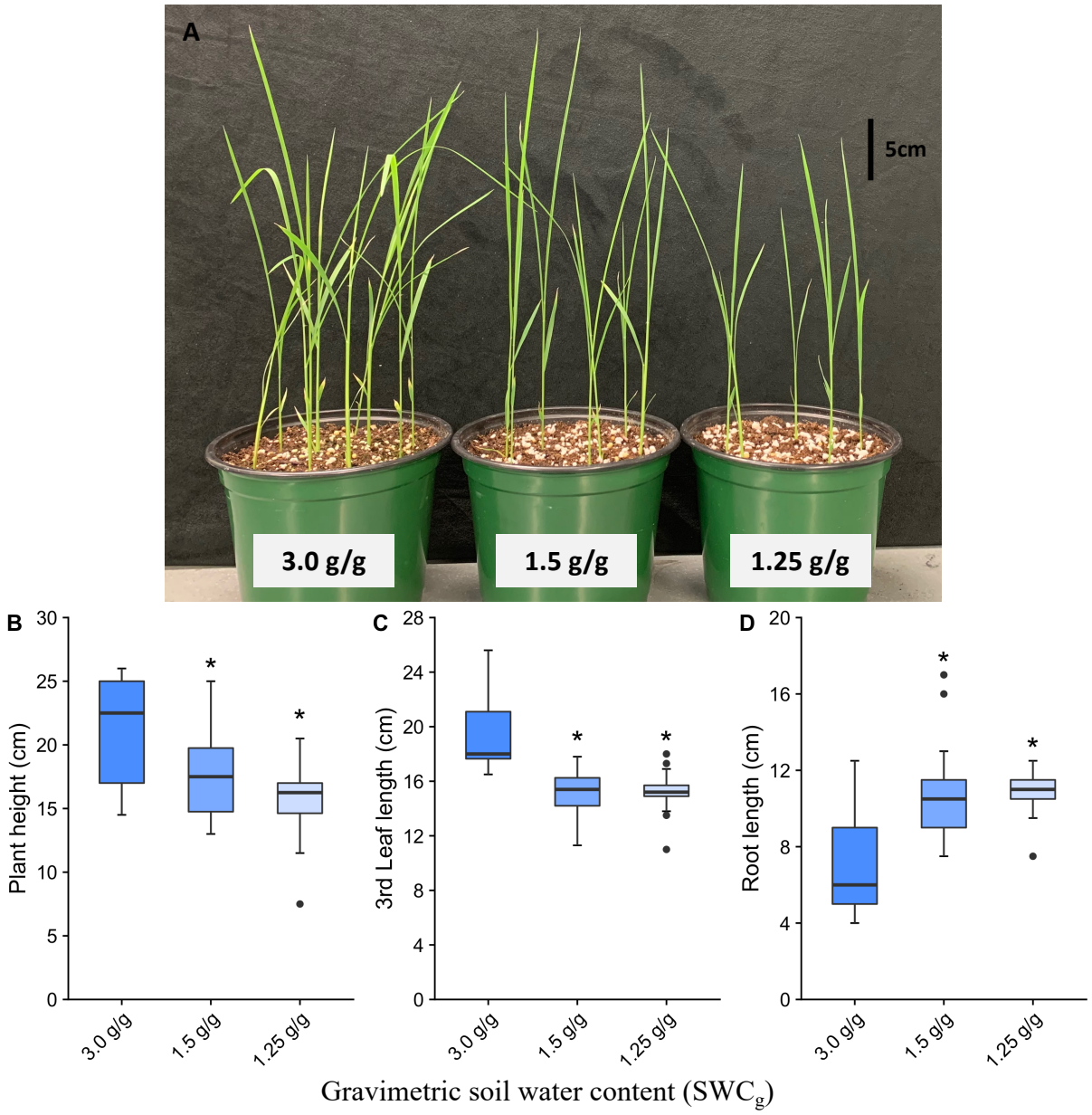

Figure 4.2: Morphological characterization of rice plants grown in three SWCg conditions. (A) Representative rice plants grown in three gravimetric soil water contents (Left to right: 3.0 g/g, 1.5 g/g, and 1.25 g/g). Photo was taken at the full expansion of the third leaves and the plants were at different ages, where 3.0 g/g, 1.5 g/g and 1.25 g/g were at 14, 15 and 16 days after planting, respectively) (B). The plant height (n = 21 - 22), (C) leaf lengths of third leaves (n = 21 - 22), and (D) root lengths (n = 16 - 17) of rice plants grown in each of three SWCg. Boxplots show the median and interquartile range of the measurements per treatment. Asterisk (*) indicates a statistically significant difference by pairwise t-test between the treatment conditions and well-watered control ($P < 0.05$).

We next examined the growth of rice plants over the period of ten days in constant soil water contents (Figure 4.3A) to gain further insights into the shoot growth reduction. The control plants had significantly higher plant height throughout the experiment than the two drought conditions (Figure 4.3B). Five days after planting, the seedling height in the well-watered control group was 19% taller than the 1.25 g/g (lowest) soil water content. By ten days after planting, the height difference had increased, and the plants in the well-watered control were 22% taller than the 1.25 g/g soil water content. It is worth noting that the plant height was measured five to ten days after planting, while the elongation rates were monitored from the point of emergence until the leaves became fully expanded. Owing to the plant growth inhibition in the limited soil water conditions, we monitored the length of the third leaf to capture the leaf expansion rate and the duration of growth. At full expansion, the third leaves in the control group were 12 % longer than those in the 1.25 g/g water-limiting condition (Figure 4.3C). Notably, the third leaves in well-watered controls reached full expansion earlier compared to the growth-limiting drought conditions, with a rapid elongation rate for the first four days, after which the elongation rate slowed considerably (Figure 4.3D).

In contrast, the leaf expansion rate in the two reduced water availability groups was slower, and the leaves could not reach full expansion until six to seven days after emergence (Figure 4.3C - D). While the leaves in well-watered control had higher elongation rate, the leaves in the drought conditions exhibited a prolonged growth phase (Figure 4.3D), although the drought conditions did not exceed the control in terms of final length (Figure 4.3C). These results indicate that consistent water-limiting conditions reduced the leaf growth rate and prolonged the time to reach full expansion.

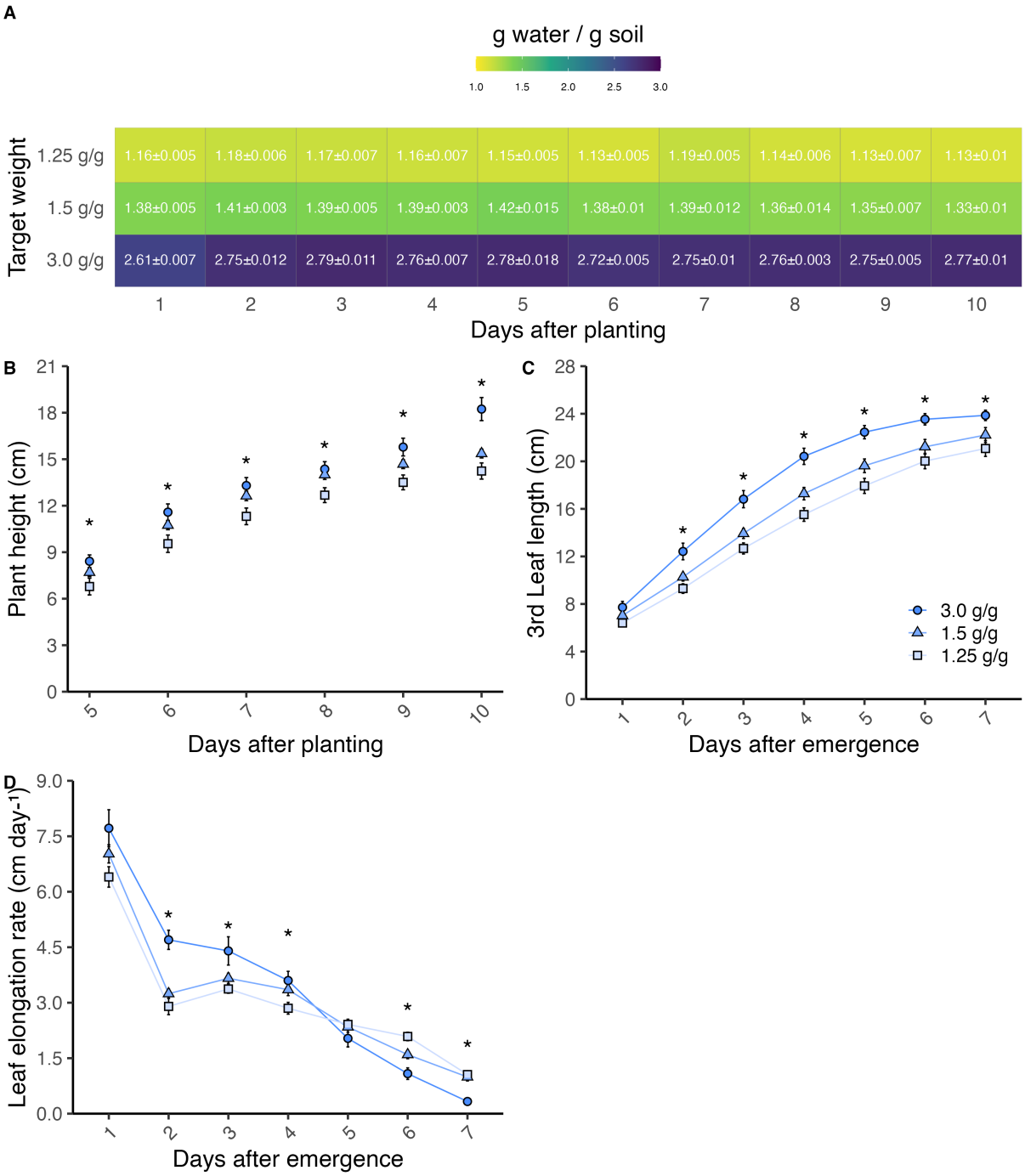

Figure 4.3: The effect of limited soil water availability on the growth of rice plants. (A) Gravimetric soil water contents in the pots under 3.0 g/g, 1.5 g/g, and 1.25 g/g treatments before daily rewatering. Values in the heatmap represent mean ± standard error of SWCg from four pots per treatment. (B) Overall plant height from day five to ten after planting. (C) The leaf length measured daily until the full expansion. (D) The leaf elongation rate from the day of emergence until the full expansion. Each data point represents measurements from 20 – 30 plants for the leaf length, plant height and elongation rate. Asterisk (*) indicates a statistically significant difference by analysis of variance *($P < 0.05$)* at each timepoint between the three gravimetric water contents.

4.2 Growth-limiting drought stress reduces PSII operating efficiency but not chlorophyll content

Due to challenges in maintaining healthy plants in Sunshine Mix #4 under well-watered control conditions, we posited that the problems were from the potting mix used. We repeatedly observed stunted growth and yellowing leaves in rice seedlings grown in Sunshine Mix #4, even when they were well-watered and received appropriate nutrients. Therefore, chlorophyll pigments and gas exchange experiments were performed on plants grown in Sunshine Mix #1 with 1.5 g/g and 3.0 g/g SWCg conditions only. We measured the chlorophyll pigments (chlorophyll 'a' and 'b') and fluorescence efficiency in the mid-section of the third fully expanded leaves (about 14 – 16 days after planting). Although there was a slight reduction in chlorophyll 'a' (Figure 4.4A) and chlorophyll 'b' (Figure 4.4B) pigments in the drought condition, these differences were not statistically significant at $P < 0.05$. We also found no significant difference in the total chlorophyll pigments of the control and the water-limiting condition (Figure 4.4C).

Next, we measured the Fv/Fm, which is the maximum chlorophyll fluorescence, in rice leaves dark adapted for 20 minutes. Fv/Fm is the ratio of the variable fluorescence (Fv) to the maximum fluorescence (Fm) the plant can achieve under optimal conditions. The data showed no significant difference in the maximum efficiency of photosystem II (PSII) between the water-limiting and control leaves (Figure 4.4D). However, when we measured the photosystem II operating efficiency (PSII) in light-adapted leaves, we found that the chlorophyll fluorescence (PhiPSII) was significantly reduced by 5% under water-limiting conditions compared to the well-watered control (Figure 4.4E).

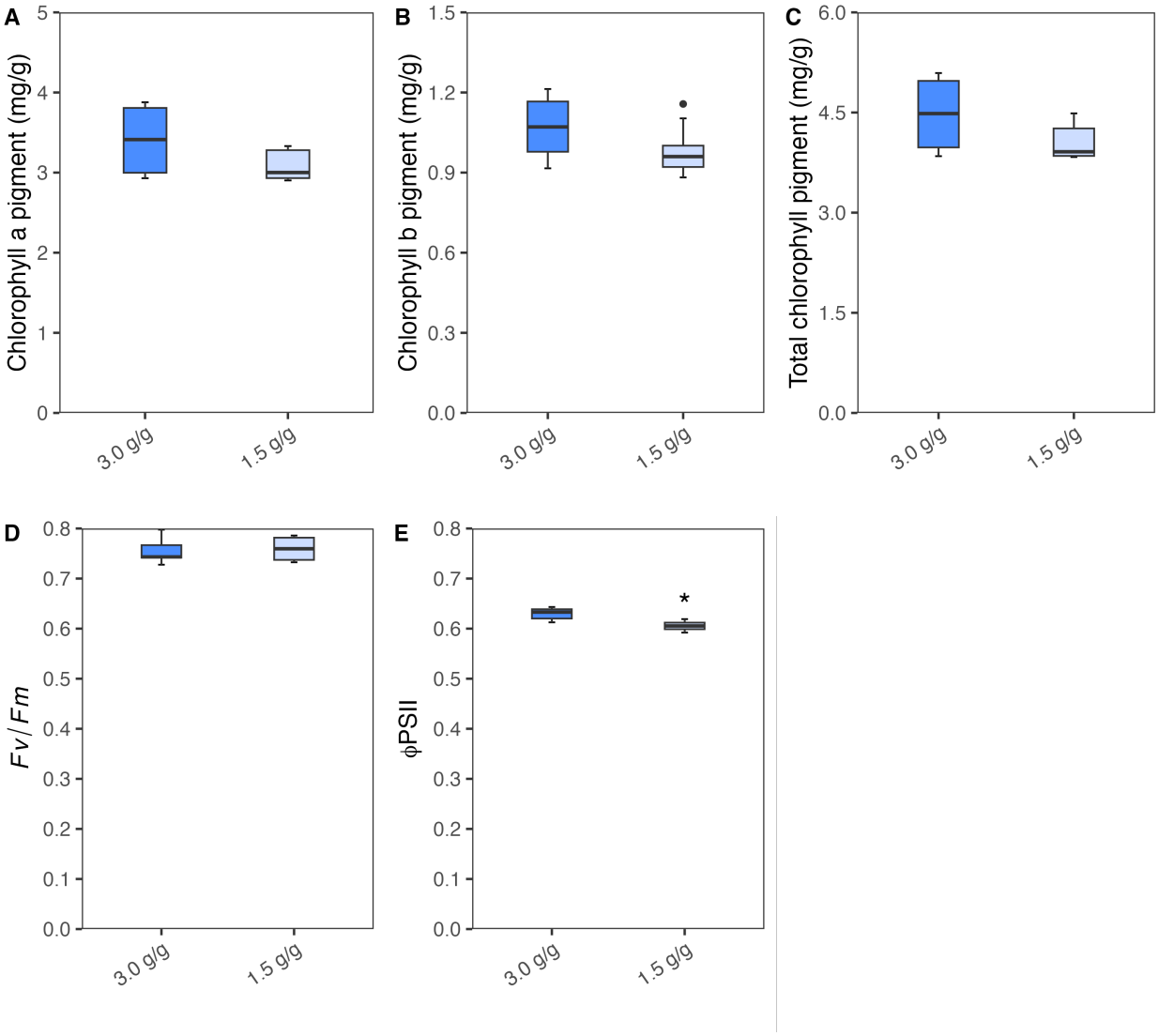

Figure 4.4: Chlorophyll pigment content and chlorophyll fluorescence efficiency of rice leaves under growth limiting drought stress. (A) Chlorophyll "a", (B) Chlorophyll "b", and (C) total chlorophyll pigment content of mid-section of rice leaves in two soil water contents (3.0 g/g and 1.5 g/g). For chlorophyll analysis, each replicate is made of five – seven leaf segments. (D) Maximum PSII light efficiency under dark adaptation (*Fv/Fm*), and (E) operating PSII light efficiency under the light adaptation of rice leaves in two soil water contents (3.0 g/g and 1.5 g/g). Asterisks (*) indicate statistically significant differences by Student's t-test ($P < 0.05$). Boxplot represents three to five biological replicates.

4.3 Growth-limiting drought reduces the photosynthetic capacity of rice leaves

To investigate whether decreased shoot growth rate observed under drought conditions was due to the reduced photosynthetic capacity, we measured the CO_2 assimilation, stomatal conductance, transpiration rates and water use efficiency. We observed a 35% reduction in the steady-state net CO_2 assimilation rate (A_{net}) of rice leaves grown in 1.5 SWCg (Figure 4.5A), a 69 % reduction in stomatal conductance (g_{sw}) (Figure 4.5B), and a 60% reduction in transpiration rate (E) (Figure 4.5C), and the intrinsic water use efficiency ($iWUE$) increased by 48% under drought conditions (Figure 4.5D). This suggests that the rice leaves in growth-limiting conditions take less CO_2 and reduce the rate of water loss by transpiration, thereby increasing their water use efficiency.

Since the maximum chlorophyll fluorescence efficiency was not significantly affected, but the CO_2 assimilation rate was drastically reduced, we checked the PhiCO_2 rate (the quantum yield of carboxylation rate) to gain insight into the ratio of light absorption per CO_2 assimilation rate. The water-limiting condition had a 29% reduction in the quantum yield of the carboxylation rate (Figure 4.5E).

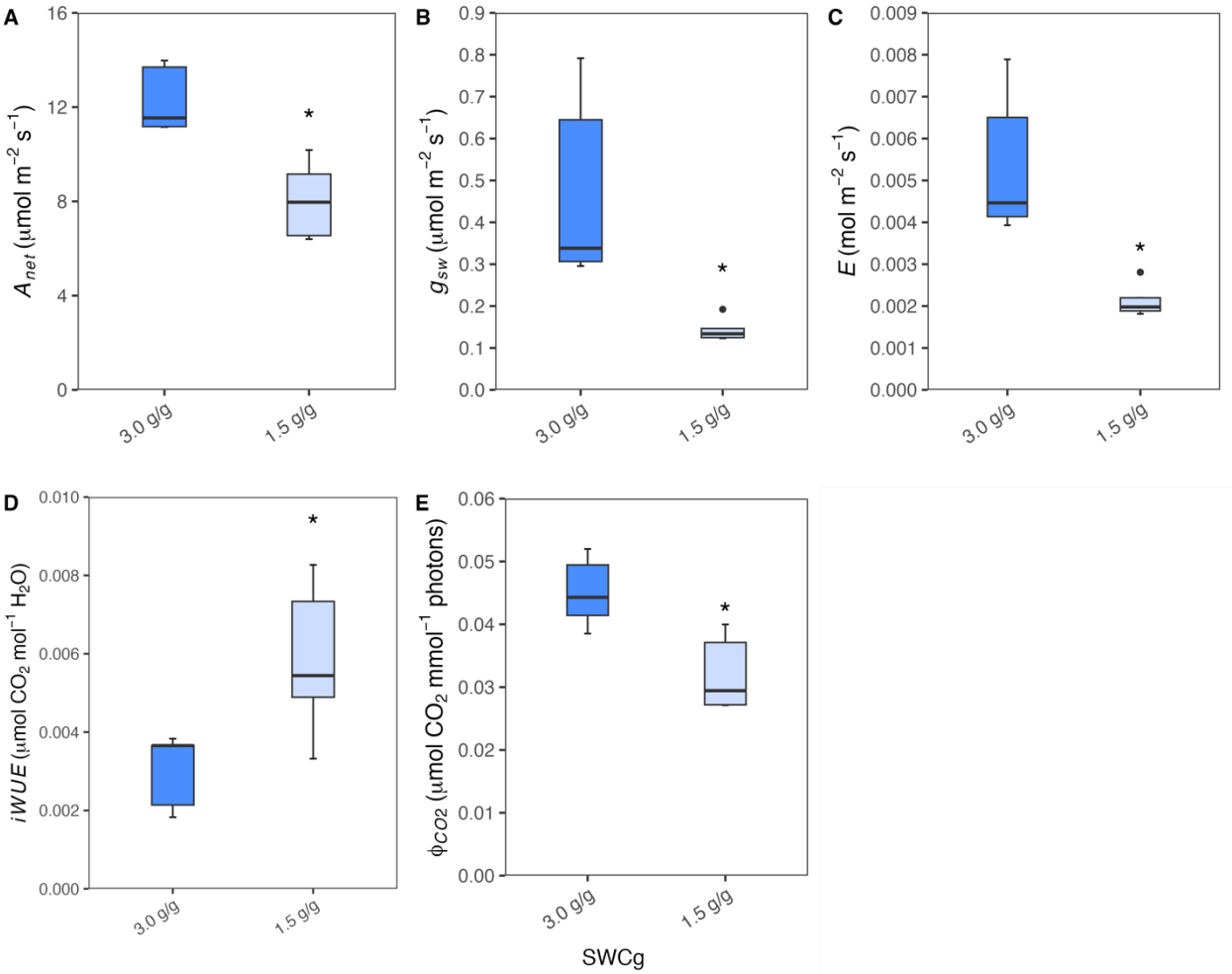

Figure 4.5: Gas exchange measurements of rice leaves in growth-limiting and well-watered conditions. (A) Net photosynthetic rate, (B) stomatal conductance, (C) transpiration rate, (D) intrinsic water use efficiency (iWUE), and (E) phi CO_2 rate of rice leaf grown under water-limiting and well-watered conditions measured at steady light and CO_2 conditions. Boxplot represents four to five biological replicates showing the median and interquartile range values for each set of measurements. Asterisks (*) indicate statistically significant differences by Student's t-test *($P < 0.05$)* at each timepoint between the water-limiting and well-watered control.

We next measured photosynthetic responses to a range of light intensities (0 - 2000 μmol m^{-2} s^{-1}) to understand when light becomes limiting for photosynthesis in rice leaves in well-watered and water limiting growth conditions. The net CO_2 assimilation rates were significantly reduced in water-limiting condition compared to control across the range of light intensity used (Figure 4.6). For instance, the net photosynthetic rate increased with increasing light availability up to 800 - 1000 μmol m^{-2} s^{-1}, with leaves in 1. 5 g/g (water-limiting condition) reaching maximum A_{sat} of 11.96 μmol m^{-2} s^{-1} at 800 μmol m^{-2} s^{-1} of light intensity, no further increases in A_{net} could be achieved through the provision of additional light intensity. In contrast, the leaves in 3.0g/g (well-watered control) reached A_{sat} of 22.15 μmol m^{-2} s^{-1} at around 1000 μmol m^{-2} s^{-1} of light intensity.

The initial increase in the net assimilation rate at high light intensity was supported by a rapid increase in the stomatal conductance (Figure 4.7A) and transpiration rate (Figure 4.7B) also stabilized at a similar light intensity as the net assimilation rate. The photosystem II operating efficiency (Figure 4.7C) decreased with increased light intensity leading to elevated non-photochemical quenching (NPQ) (Figure 4.7D) in rice leaves. However, the NPQ was significantly higher in leaves treated with water-limiting compared to the control (Figure 4.7D). Furthermore, because the step increase in light intensity also led to an initial increase in net assimilation that was greater than the magnitude of increase in stomatal conductance in the growth-limiting condition, this response led to increased iWUE compared with control (Figure 4.7F).

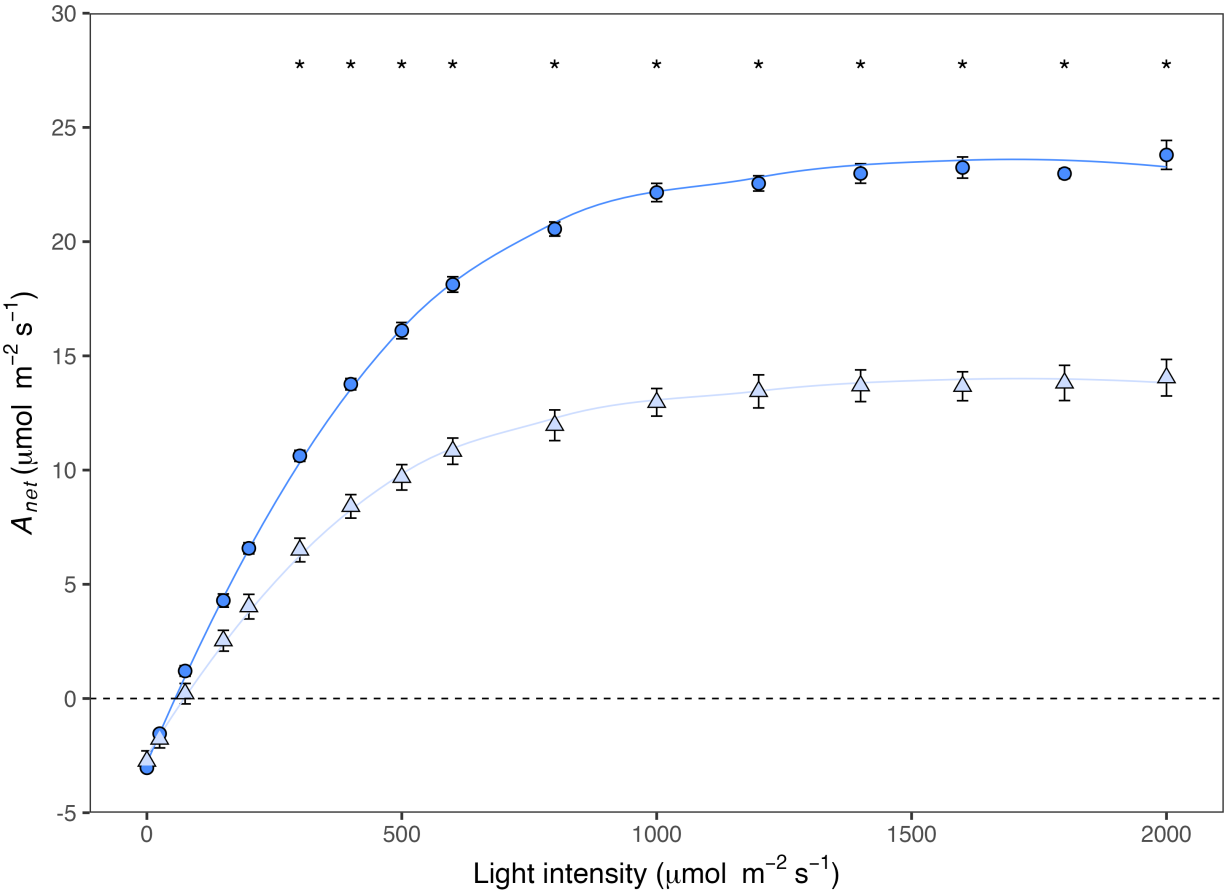

Figure 4.6: The net photosynthetic rate during the light response curve. Each point represents four to five biological replicates showing the mean and standard error of measurement taken. Asterisks (*) indicate statistically significant differences by Student's t-test *(P < 0.05)* at each timepoint between the water-limiting (triangles) and well-watered control (circles).

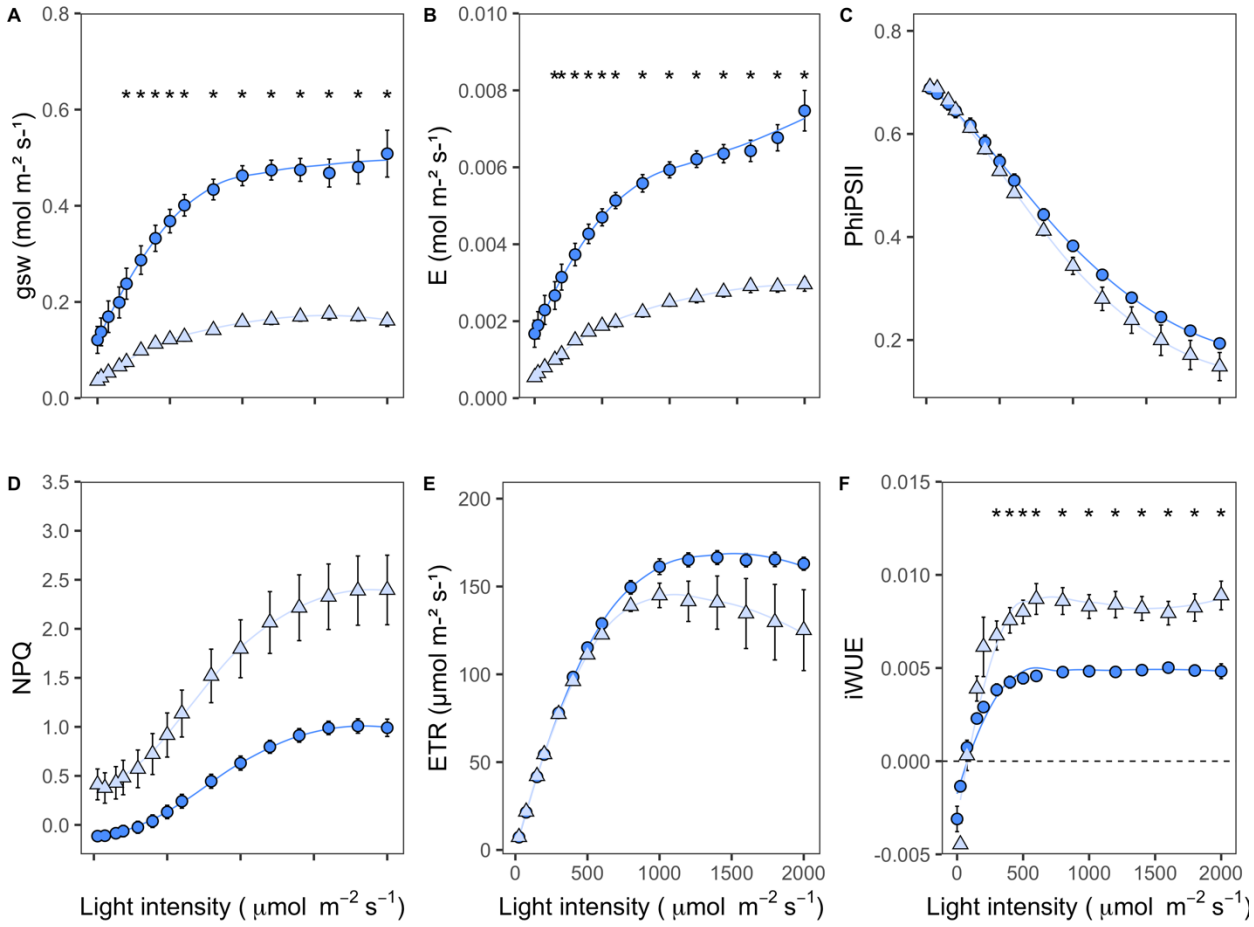

Figure 4.7: Gas exchange parameters during the light response curve. (A) Stomatal conductance, (B) transpiration rate, (C) photosystem II operating efficiency, (D) non-photochemical quenching, (E) electron transport rate, and (F) intrinsic water use efficiency. Each point represents four to five biological replicates showing the mean and standard error of measurement taken. Asterisks (*) indicate statistically significant differences by Student's t-test *($P < 0.05$)* at each timepoint between the water-limiting (triangles) and well-watered control (circles).

We performed a CO_2 response (A/Ci) curve with varying CO_2 concentrations to further investigate the photochemistry underpinning growth limitation during reduced water availability. A/Ci curves are used to estimate the *in vivo* biochemical limitation to photosynthesis. The net photosynthetic rates were significantly lower in water-limiting conditions, and this difference became apparent at higher CO_2 concentrations. For example, the intercellular CO_2 contents in the drought condition were reduced and highly variable compared to the control despite having the same leaf CO_2 concentration exposure (Figure 4.8). Stomatal conductance (Figure 4.9A), transpiration rate (Figure 4.9B), PhiPSII (Figure 4.9C), and electron transport rate (Figure 4.9D) were lower in the water-limiting condition across all the CO_2 concentrations used during the CO_2 response curve. However, the NPQ (Figure 4.9E) and iWUE (Figure 4.9F) increased with an increase in the leaf intercellular CO_2 concentration, and the magnitude of the increase in iWUE was two-fold higher in the growth-limiting condition compared with the control.

When we modelled the light response (A/Qin) curve, we found that the rice leaves in water-limiting conditions had a 35% reduction in the maximum photosynthetic rate at saturating CO_2 (Figure 4.10A), as well as a 46% decrease in dark respiration rate compared to well-watered control (Figure 4.10B). Additionally, rice leaves in water-limiting required 28% more light to reach a point where photosynthesis and respiration are in equilibrium (Figure 4.10C). We also modelled the A/Ci curve to understand the mechanisms by which the reduction in CO_2 assimilation takes place and found a 31% reduction in the maximum rate of Rubisco carboxylation (V_{cmax}) (Figure 4.10D), as well as a 31% reduction in electron transport (J_{max}) under drought conditions (Figure 4.10E). Taken together, our results suggest that rice in water-limiting conditions had reduced photosynthetic performance due to the reduced ability to effectively utilize available light and CO_2 during photosynthesis.

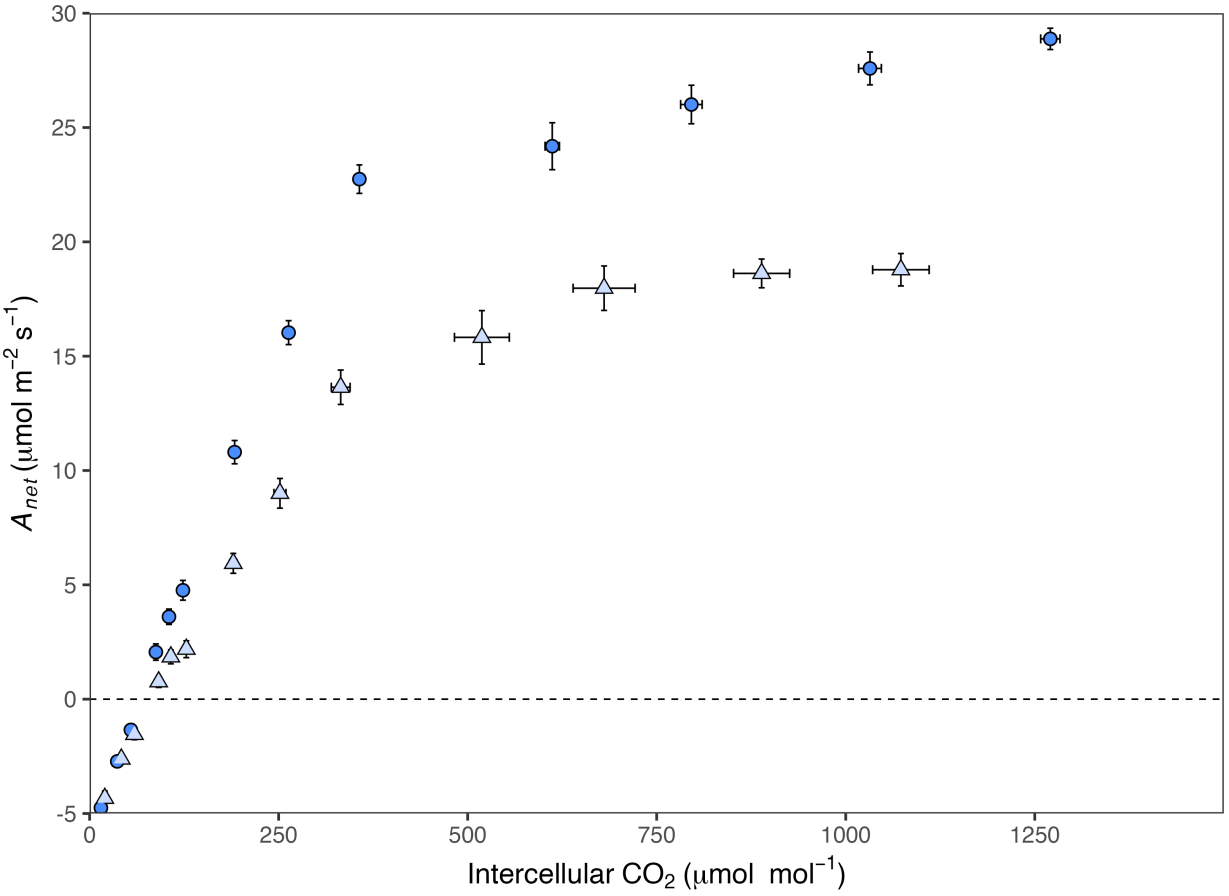

Figure 4.8: The net photosynthetic rate during the CO_2 response curve. Each point represents four to five biological replicates showing the mean and standard error of measurement taken.

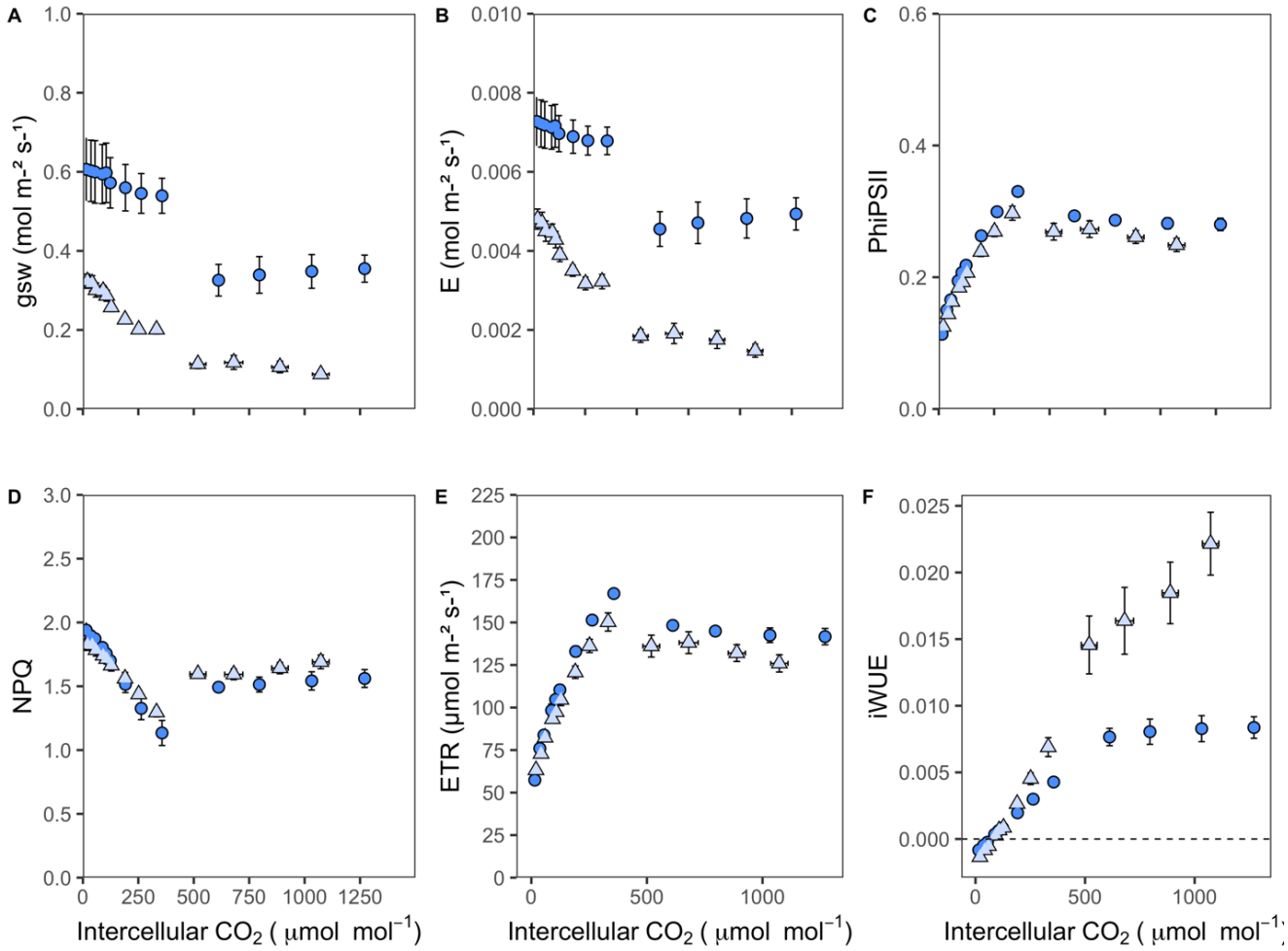

Figure 4.9: Gas exchange parameters during the CO_2 response curve. (A) Stomatal conductance, (B) transpiration rate, (C) photosystem II operating efficiency, (D) non-photochemical quenching, (E) electron transport rate, and (F) intrinsic water use efficiency. Each point represents four to five biological replicates showing the mean and standard error of measurement taken.

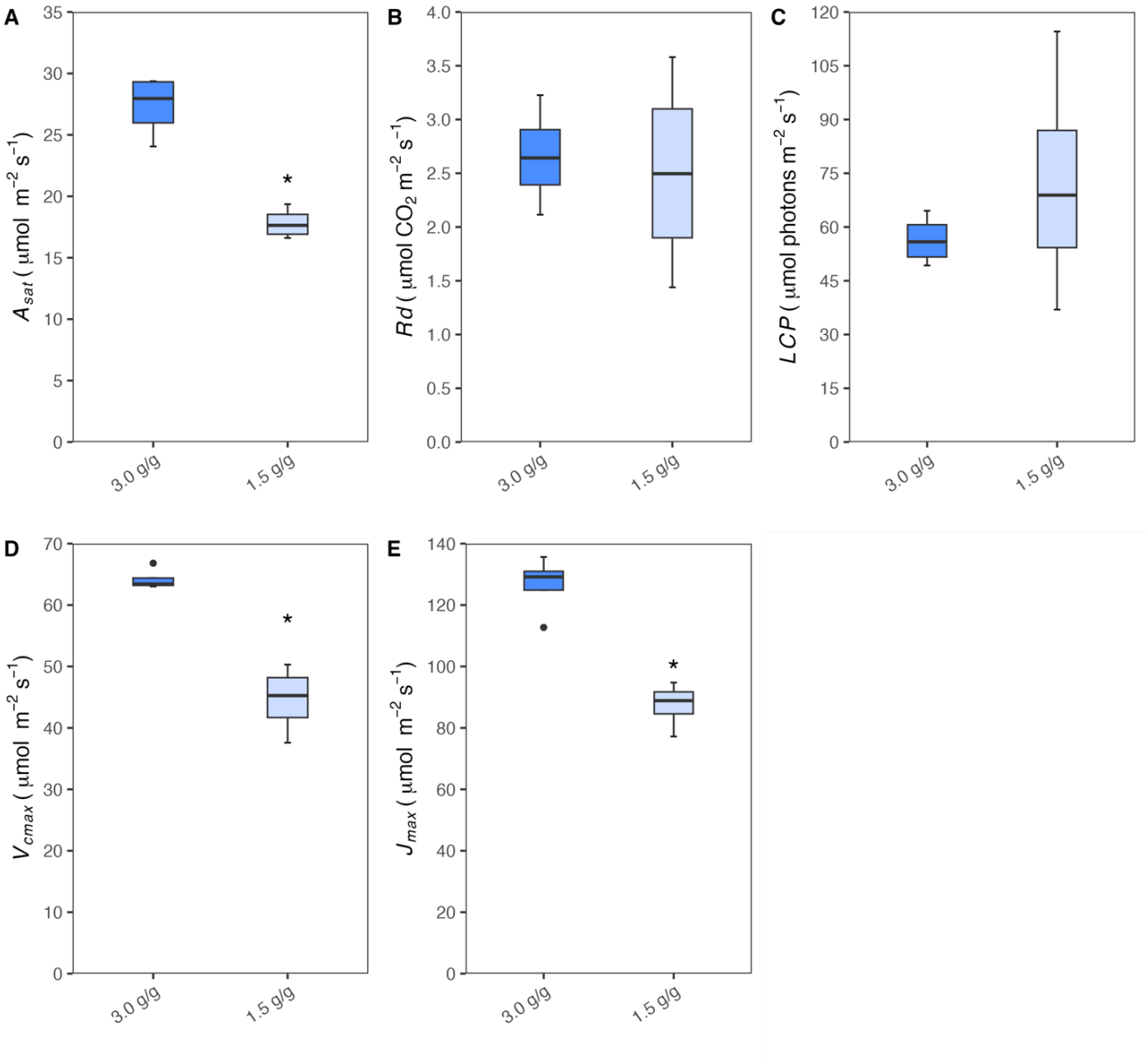

Figure 4.10: Parameters from modelling the light and CO_2 response curves. (A) Maximum assimilation rate at saturating light, (B) light compensation point, (C) dark respiration rate, (D) the maximum rate of Rubisco carboxylation (Vc_{max}), and (E) potential rate of electron transport (J_{max}) of rice leaves in growth-limiting drought and well water control. Boxplot represents four to five biological replicates showing the median and interquartile range values for each set of measurements. Asterisks (*) indicate statistically significant differences by Student's t-test ($P < 0.05$).

4.4 Global transcriptomic analysis reveals DEGs underlying the response to growth-limiting drought in rice

To further explore the transcriptome changes associated with growth-limiting drought (GLD), we conducted RNA-seq to measure the global gene expression profile of rice leaves in two growth-limiting conditions (1.25 g/g and 1.5 g/g) and a well-watered control (3.0 g/g). We analyzed 330 million high-quality sequencing reads, of which about 99% passed the quality control trimming uniquely mapped to the *Oryza sativa* Nipponbare reference genome (Kawahara et al., 2013) (Table 4.1).

First, we performed a principal component analysis on normalized read counts to understand the global transcriptomic response to reduced soil water availability. The first principal component explained 62% of the variation observed in the dataset and the samples were partially separated into well-watered and water-limiting conditions along the PC1 axis. The second component explained 21% of the variance in the dataset (Figure 4.11A). Cumulatively, we identified 2494 genes that were differentially expressed in the two drought conditions. In all, 222 of upregulated DEGs commonly responded to water-limiting conditions, while 126 DEGs in 1.25 g/g and 35 DEGs in 1.5 g/g were uniquely upregulated to these drought treatment conditions (Figure 4.11B). On the other hand, of the 1493 downregulated genes in our drought conditions, 350 and 747 genes were unique to 1.25 g/g and 1.5 g/g, respectively, while 396 downregulated genes were common to the two treatment conditions (Figure 4.11C).

Table 4.1: Summary of reads generated from RNA sequencing and the processing steps. Surviving and dropped reads are from Trimmomatic. Overall alignment rate is from HISAT2, and overall gene assignment rate is from featureCounts.

Sample ID	Raw reads	Surviving reads (%)	Overall alignment rate %	Overall gene assignment rate %
W-3.0g/g-pil-1	37425297	36616005 (97.84)	98.90	78.30
W-3.0g/g-pil-2	41890723	40861859 (97.54)	99.04	77.70
W-3.0g/g-pil-3	37425430	36346355 (97.12)	98.76	76.10
W-1.5g/g-pil-1	30190446	29525380 (97.80)	99.00	80.20
W-1.5g/g-pil-2	41496793	40554374 (97.73)	99.02	80.40
W-1.5g/g-pil-3	39321430	38312945 (97.44)	98.61	69.30
W-1.25g/g-pil-1	32173620	31398010 (97.59)	98.99	80.80
W-1.25g/g-pil-2	37025916	36045341 (97.35)	98.80	77.20
W-1.25g/g-pil-3	34549420	33592206 (97.23)	98.78	75.70
Total	331499075	323252475 (97.52)	98.88	77.30

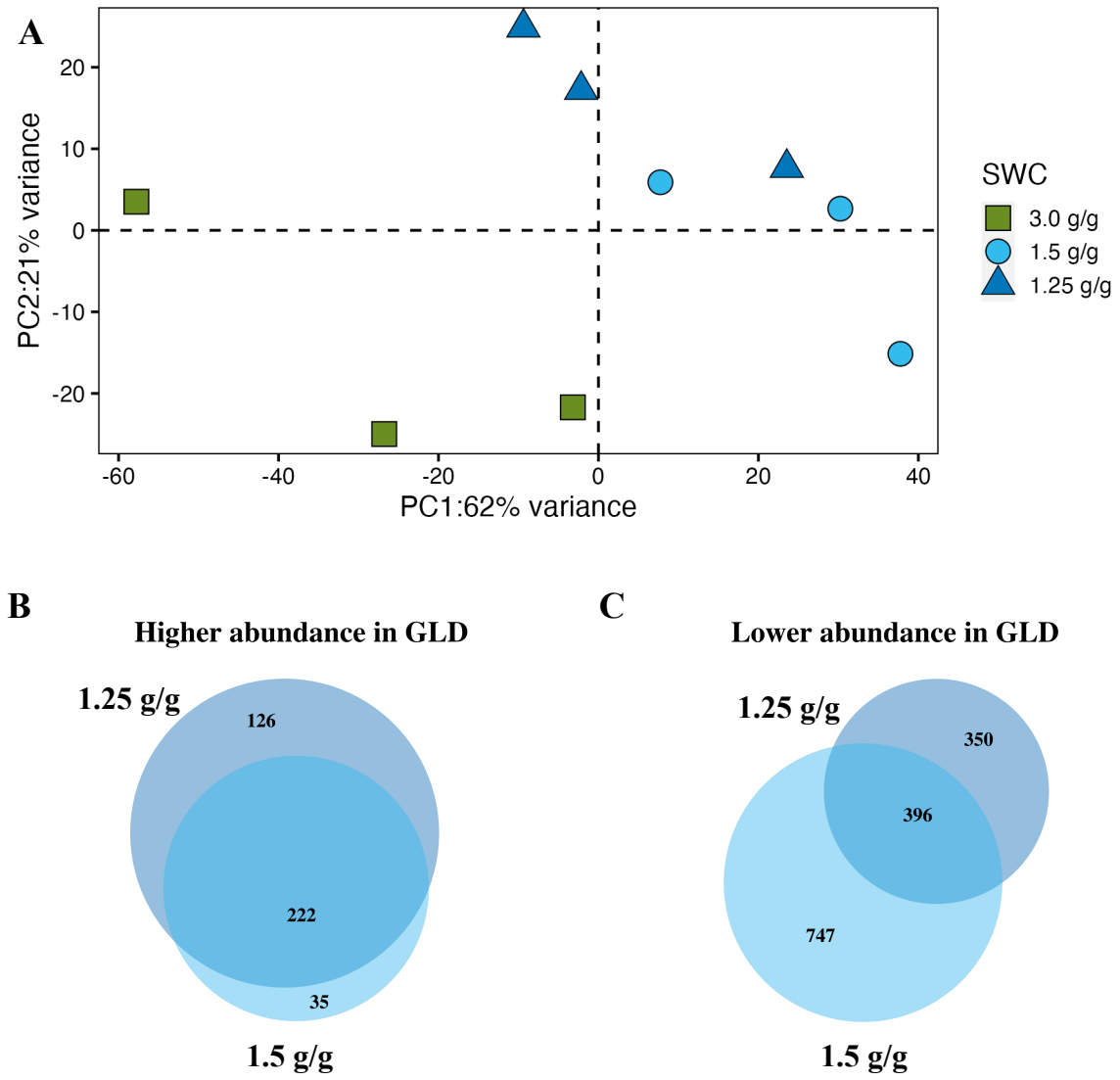

Figure 4.11: Transcriptome profile of rice in response to water-limiting conditions. (A) Principal component analysis (PCA) of all expressed genes (normalized counts). Each point represents a single replicate of pooled tissue from three rice plants. (B) Venn diagram of upregulated differentially expressed genes (DEGs) in response to two levels of growth-limiting drought (GLD). (C) Venn diagram of the downregulated DEGs in response to two levels of water-limiting conditions.

4.5 Different gene sets but similar functional categories are involved in regulating growth at two levels of reduced water availability

To understand the functional significance of genes that responded to water-limiting conditions, we performed a co-expression analysis and hierarchical clustering of all the significantly up and downregulated genes, resulting in 1701 differentially expressed genes (DEGs) assigned to clusters based on their expression profiles using the complete linkage method (Figure 4.12A). Through this analysis, we identified 13 clusters of genes with varying expression patterns (Figure 4.12B), further categorized into five dominant expression modules (Figure 4.13A-B). These modules emphasized the quantitative differences in gene expression between the two drought stress treatments.

To identify the biological processes associated with these clusters, we analyzed the genes in each cluster using gene ontology (GO) enrichment analysis. The enriched GO terms revealed various stress-responsive terms overrepresented in these clusters (Figure 4.13C). Cluster I, which included 227 genes whose expression was higher in both growth-limiting conditions, was enriched for GO terms associated with abiotic stress responses, including response to stress ($P = 2.1 \times 10^{-4}$), response to heat ($P = 1.09 \times 10^{-3}$), and cellular response to nitric acid ($P = 6.19 \times 10^{-3}$) and hydrogen peroxide catabolic processes ($P = 6.19 \times 10^{-3}$). Cluster II, which includes 81 genes whose abundance increased progressively with the severity of drought stress, was enriched for GO terms associated with cellular ion homeostasis ($P = 4.3 \times 10^{-6}$), response to hydrogen peroxide ($P = 9.8 \times 10^{-9}$), response to water deprivation ($P = 3.8 \times 10^{-4}$), and response to abscisic acid ($P = 4.56 \times 10^{-3}$).

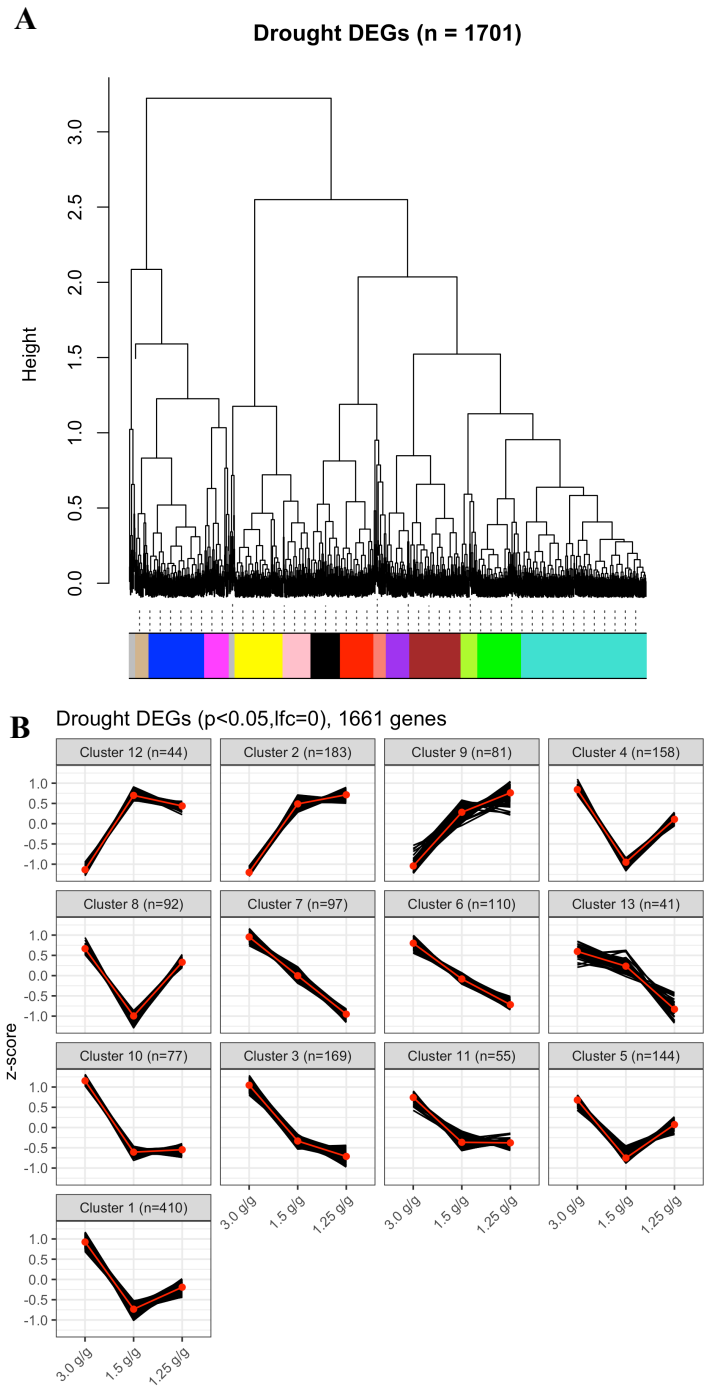

Figure 4.12: Hierarchical clustering analysis of all genes differentially expressed in the two growth-limiting conditions. (A) Dendrogram clustering of all differentially expressed genes based on the expression pattern in the three gravimetric soil water contents. (B) Expression profile of 13 clusters identified by cutTreeDynamics. Clusters were arranged based on similarity of their expression patterns.

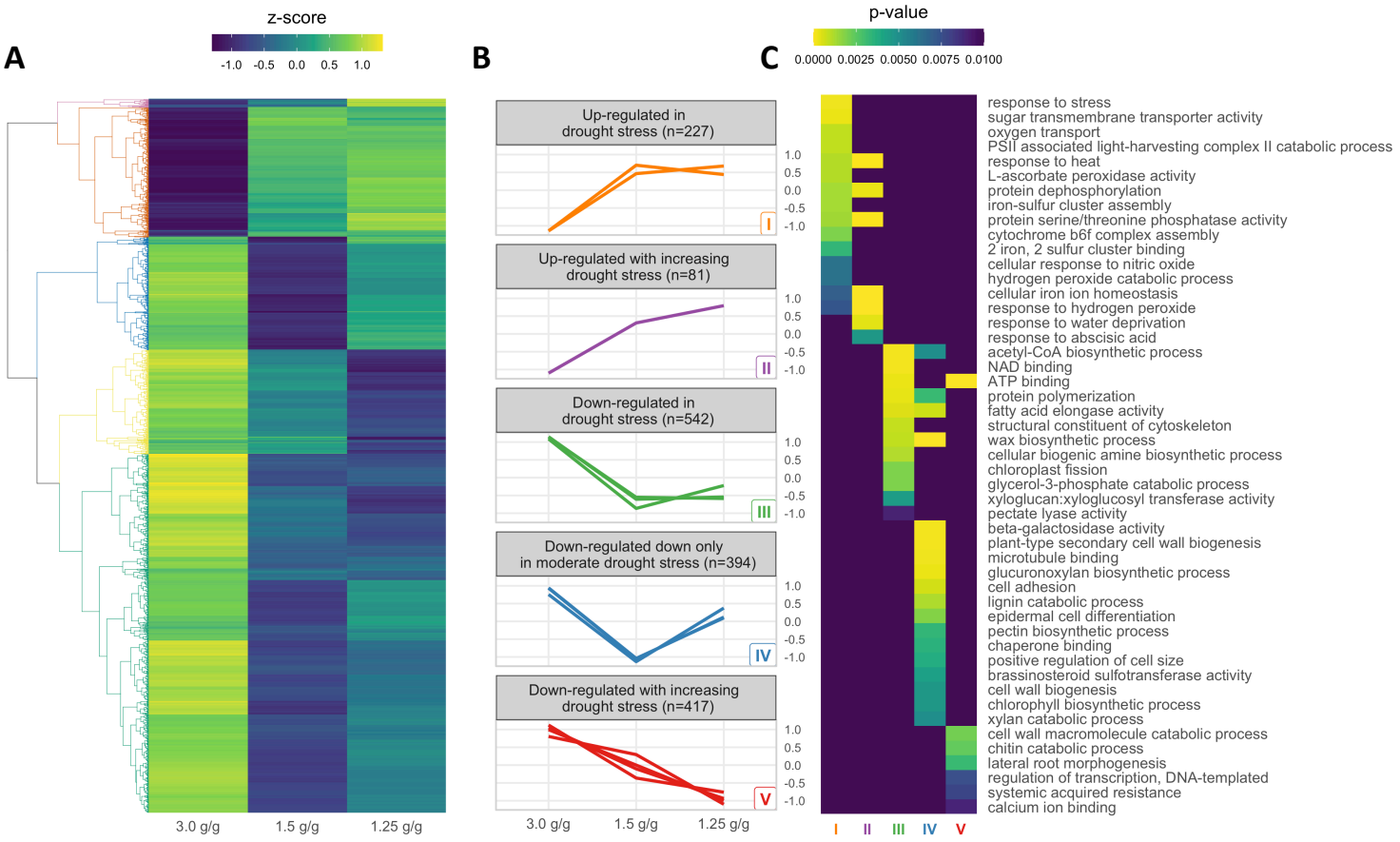

Figure 4.13: Expression profile of DEGs and functional GO categories of expression cluster. (A) Heatmap of DEGs expression profile in water-limiting conditions. Visualized values are the scaled expression levels of all the DEGs hierarchically clustered to identify genes with similar expression patterns. (B) Average scaled expression profile of clusters. (C) Heatmap of GO term enrichment analysis of the expression clusters.

Cluster III, which consists of 542 genes whose transcripts were downregulated at both levels of water-limiting treatments, showed enrichment of GO terms associated with ATP binding ($P = 2.6 \times 10^{-4}$), protein polymerization ($P = 3.2 \times 10^{-4}$), wax biosynthesis ($P = 8.8 \times 10^{-4}$), chloroplast fission ($P = 1.89 \times 10^{-3}$), and xyloglucosyl transferase activity ($P = 4.52 \times 10^{-3}$). Cluster IV, which consists of 394 genes whose transcripts were downregulated in moderate water-limiting treatment, showed a strong association with GO terms related to the cell wall, including secondary cell wall biogenesis ($P = 1.7 \times 10^{-4}$), cell adhesion ($P = 6.6 \times 10^{-4}$), lignin catabolic process ($P = 1.17 \times 10^{-3}$), pectin biosynthesis process ($P = 3.27 \times 10^{-3}$), and chlorophyll biosynthesis process ($P = 4.57 \times 10^{-3}$). Lastly, cluster V, which consists of 417 genes whose transcripts were decreasing with increasing drought stress, was enriched for GO terms involved in the regulation of transcription ($P = 7.55 \times 10^{-3}$) and calcium ion binding ($P = 8.96 \times 10^{-3}$).

4.6 Photosynthesis and cell wall-associated gene expression changes in rice seedlings during limited water availability

Next, we investigated the expression of some known drought-responsive, photosynthesis-related, cell wall-related, and starch biosynthesis-related genes in the transcriptome dataset (Table 4.2). The expression of *9-CIS-EPOXYCAROTENOID DIOXYGENASE 3* (*OsNCED3*), a gene involved in ABA biosynthesis pathways (Huang et al., 2018), had a 0.5- and 2-fold increase in the plants grown at 1.5g/g and 1.25 g/g SWCg, respectively, indicating a marked increase in transcript accumulation with increased water limitation. The *ABA STRESS RIPENING 6* (*OsASR6*) transcription factor (Jiang et al., 2022), induced by ABA, was upregulated about 1.5-fold in the two water-limiting conditions (Table 4.2). The expression of genes related to photosynthesis, 1*0 KDA PHOTOSYSTEM II POLYPEPTIDE* (*OsPsbR1*) (Li et al., 2017) and chloroplast development, *NUCLEOSIDE DIPHOSPHATE KINASE 1* (*OsNDPK1*) (Ye et al.,

2016) were decreased by about 2 to 3-fold under the two water-limiting conditions. Additionally, the small *EPIDERMAL PATTERNING FACTOR-LIKE 2* (*OsEPFL*) gene, required for stomatal development in rice (Mohammed et al., 2019), was downregulated by about 1.5-fold after ten days in water-limiting conditions. Meanwhile, the *PHOSPHATASE 2Cs* (*OsPP2C50*) gene, a stomatal closure/opening signalling component (Min et al., 2019), was upregulated by 1.5-fold in water-limiting conditions. The *SOLUBLE STARCH SYNTHASE I* (*OsSSI*) gene (N. Fujita et al., 2006; Hirose & Terao, 2004) was upregulated in our two water-limiting conditions. The expression of genes related to the cell wall and cell wall components was also examined. The *RECEPTOR KINASE 11* (*OsWAK11*) and *MONOSACCHARIDE TRANSPORTERS* (*OsMST3*) genes, which are involved in cell wall synthesis (Toyofuku et al., 2000), were decreased by 1 to 1.5-fold in our reduced soil water availability treatments. The *OsWSL1* gene, well-known for its role in synthesizing cuticular waxes in rice leaves (Xia et al., 2015), was slightly decreased under growth-limiting conditions. The *LIPID TRANSFERASE* (*OsLTPG18*) (Edstam et al., 2013) and *TREHALOSE BIOSYNTHESIS* (*OsTPP3*) (Jiang et al., 2019) genes were also accumulated at lower abundance in the water-limiting conditions compared to the control. These expression patterns were consistent with the reduction in shoot growth and photosynthesis of rice leaves in water-limiting conditions.

Table 4.2: Log_2 fold expression of drought-responsive, photosynthesis and cell wall-related, genes in the two growth-limiting conditions.

Gene ID	Log_2 fold change	
	1.25 g/g	**1.5 g/g**
AP2;EREBP129 (LOC_Os01g04800)	-4.06	-2.96
OsSBDCP1 (LOC_Os01g63810)	0.98	1.09
OsEPFL9 (LOC_Os01g68598)	-2.90	-4.53
OsASR6 (LOC_Os01g73250)	1.48	1.42
OsWAK11 (LOC_Os02g02120)	-3.12	-1.02
OsNCED3 (LOC_Os03g44380)	2.36	0.53
OsLEA21 (LOC_Os05g28210)	-12.38	-12.42
OsPP2C50 (LOC_Os05g46040)	0.99	0.73
OsSSI (LOC_Os06g06560)	2.56	2.38
OsWSL1 (LOC_Os06g39750)	-2.98	-3.00
OsMST3 (LOC_Os07g01560)	-2.70	-1.53
OsPsbR1 (LOC_Os07g05360)	-2.12	-3.35
OsLTPG18 (LOC_Os07g07930)	-2.34	-2.00
OsTPP3 (LOC_Os10g40550)	-2.31	-2.00
OsNDPK1 (LOC_Os12g36194)	-0.94	-1.16

4.7 Phytohormones-associated responses in rice seedlings following reduced water availability for a prolonged period

Since the drought stress implemented in this study did not cause leaf wilting and growth was still maintained, we studied the expression levels of some genes associated with phytohormones, including cytokinin (CK), jasmonic acid (JA), gibberellic acid (GA), ethylene, and indole-3-acetic acid (IAA), which are involved in regulating plant growth (Table 4.3). Notably, the expression of *CYTOKININ OXIDASE/DEHYDROGENASE* (*OsCKX2*), an enzyme that degrades cytokinin (Yan et al., 2021), was significantly downregulated under drought conditions. In addition, the expression of *CYTOKININ-O-GLUCOSYLTRANSFERASES* (*LOC_Os07g30330*) (Li et al., 2019) also decreased under water-limiting conditions. GA has been shown to play a crucial role in leaf growth and expansion in rice (Jathar et al., 2022), and the expression of GA synthesis-related genes such as *GIBBERELLIN 20 OXIDASE 2* (*Os20ox2*) (Qin et al., 2013), *LOC_Os02g01332* (*gibberellin-regulated 60S ribosomal protein L9*), and *OsCPS1* (encoding an enzyme in the GA biosynthesis pathway) (Ma et al., 2022) were downregulated under water-limiting conditions. However, another GA-related gene, *OsSWEET3a*, involved in the C13-hydroxylation pathway of GA biosynthesis (Morii et al., 2020), showed a 3-4-fold increase in transcript levels under these conditions. A number of ethylene-related genes, including *OsACO2*, *OsACO5*, *OsACO7*, *OsERF65*, and *OsERF66*, were downregulated after water-limiting treatments. On the other hand, *OsEREBP-5* and *OsEIL*, which are involved in ethylene-mediated drought and cold responses, were upregulated under these conditions. The expression of the JA gene *OsAOS2* was also decreased by 3-fold under water-limiting conditions. Similarly, two IAA-related genes (*OsIAA2* and *OsIAA7*) had lower transcript abundance in the two water-limiting

stress conditions. These results indicate that water-limiting conditions affected the expression of genes relating to various phytohormones involved in plant growth and development.

Table 4.3: Transcript abundance of phytohormone-related genes in two levels of growth-limiting drought conditions

Gene ID	Associated hormone	Log_2 fold change	
		1.25 g/g	1.5 g/g
OsCKX2 (LOC_Os01g10110)	Cytokinin	-3.61	-2.70
LOC_Os07g30330	Cytokinin	-2.12	-1.34
OsERF66 (LOC_Os03g22170)	Ethylene	-4.99	-2.81
OsACO7 (LOC_Os01g39860)	Ethylene	-2.67	-1.74
OsACO2 (LOC_Os09g27820)	Ethylene	-2.71	-1.54
OsACO5 (LOC_Os05g05680)	Ethylene	-1.66	-1.24
OsERF65 (LOC_Os07g42510)	Ethylene	-1.69	-1.39
OsEIL (LOC_Os02g36510)	Ethylene	2.51	2.51
OsEREBP-5 (LOC_Os09g11480)	Ethylene	1.34	1.34
Os20ox2 (LOC_Os01g66100)	GA	-1.71	-1.23
LOC_Os02g01332	GA	-1.12	-1.06
OsCPS1 (LOC_Os02g17780)	GA	-1.14	-0.36
OsSWEET3a (LOC_Os05g12320)	GA	3.56	3.08
LOC_Os08g15149	GA	1.71	1.51
OsIAA2 (LOC_Os01g09450)	IAA	-1.21	-0.85
OsIAA7 (LOC_Os02g13520)	IAA	-1.97	-0.18
OsAOS2 (LOC_Os03g12500)	JA	-3.78	-3.06

CHAPTER 5: DISCUSSION

Understanding the physiological and molecular mechanisms underlying growth limitation under mild drought stress or reduced water availability is critical for identifying avenues to improve plant resilience to agriculturally relevant drought conditions (Yang et al., 2010). In this study, we developed a growth method to study the physiological and transcriptomic changes underlying shoot growth reduction under two levels of reduced soil water availability in rice seedlings. Our data showed that the reduction in growth under low soil water availability was associated with reduced capacity for light and CO_2 utilization efficiency in rice leaves. Furthermore, we identified a suite of differentially expressed genes in response to the two levels of drought stress imposed.

5.1 Nearly stable reduced water availability inhibits shoot growth and leaf elongation

Plants have a capacity for phenotypic plasticity that allows for adaptive allocation of their resources in response to changing environmental conditions (Bradshaw, 1965). Reduced leaf size is an active way plants respond to environmental stressors, including limited water availability (Clauw et al., 2015, 2016) triggered by cell-signalling due to reduced turgor pressure (Zhang et al., 2020). In this study, we studied the impact of steady, growth-limiting drought on rice leaves. Growth was reduced in rice seedlings as the gravimetric soil water content decreased from 3.0 g/g to 1.25 g/g. Although the 1.25 g/g SWCg treatment had only 10% less water than the 1.5 g/g condition, there was a noticeable difference in growth performance between these conditions. The dramatic difference in growth observed within the narrow SWCg may be attributed to the fact that the soil water potential decreases with decreasing gravimetric soil water until a point at which the reduction in SWCg leads to an exponential decrease in soil water potential (Dowd et

al., 2019; Juenger & Verslues, 2022). Although water availability was controlled by monitoring the soil water content in this study, soil water potential provides a better picture of the level of stress experienced by measuring the free energy of soil water and its driving force for movement within the plant (Or et al., 2022). It is possible that 1.25 g/g SWCg caused a more significant decrease in soil water potential than 1.5 g/g condition, even though the difference in soil water content between the two conditions was relatively small. The observed difference in growth performance could be explained by the fact that the rice seedlings in the 1.25 g/g SWCg condition may have been subjected to more severe water stress than those in 1.5 g/g SWCg condition. This highlights the need for plant biologists to employ a consistent metric system to determine the level of stress applied in order to facilitate reproducibility across experimental setups.

We also found that by five days after planting, stressed leaves showed a slower expansion rate compared to control leaves, indicating that the plants were experiencing limited water availability. During this growth period, the elongation rate of control leaves remained much higher than that of stressed leaves. Only six to seven days after the third leaf emerged did the expansion of the stressed leaves surpass that of the control leaves. However, more was needed to make up for the final size difference, as the elongation rate had already significantly declined. In the monocot model species *Brachypodium distachyon*, low leaf expansion rates under mild drought stress were compensated by a prolonged duration of growth (Verelst et al., 2013). Similarly, we observed that water limitation impacted both the leaf growth, size and the duration of the leaves to reach full length. These similarities in growth inhibition strategy under drought stress in both *Brachypodium* and rice may be attributed to the inability of plants to coordinate growth at the cellular level because water is required to maintain cell turgor and growth (Zhang

et al., 2020). While this current study does not examine histological details of reduced leaf size under water limitation, the reduced leaf growth may be due to reduced cell proliferation, expansion, and cell number per leaf or by a combination of these factors in any or all tissue and cell layers. For example, studies have shown that water limitation affects cell expansion and proliferation, resulting in stunted growth. Consistent water limitation reduces the average size of leaf cells by up to 40% (of parenchymatous cell) in rice (Todaka et al., 2017), 23% in *Brachypodium* and 40% in Arabidopsis, while a smaller number of cells per leaf was reported in Arabidopsis (Aguirrezabal et al., 2006; Skirycz et al., 2009). Therefore, growth alterations observed in water-limiting conditions suggest an active response mechanism of plants to their new environment. Since reduced growth and increased leaf development duration may be a viable way to increase plant biomass productivity under stressful conditions, particularly as improvements to biomass yield become increasingly important due to the growing energy demand for biofuel (Alalwan et al., 2019). Nevertheless, biomass accumulation during the vegetative stage is still required to maximize seed yield at harvest (Barik et al., 2019; Prince et al., 2015). While this study investigated some aspects of how plants respond to constant water limitations at developmental and growth levels, questions remain how the growth and developmental shifts to water limitation are coordinated at spatial-temporal scale and cellular resolution. For example, what are the intrinsic developmental programs that are integrated to control leaf growth economy under stressful conditions, and can some of these trade-offs be overcome while still maintaining drought tolerance? These questions, among others, were recently raised by a group of plant scientists that need to be addressed in the face of global climate change and food security challenges (Verslues et al., 2022).

5.2 Growth inhibition under a stable water deficit is concomitant with a reduced photosynthetic rate

While chlorophyll contents and activities play a key role in determining light absorption and energy transfer during photosynthesis in plants (Emerson, 1929; Katz et al., 1978), there exists the possibility that changes in chlorophyll content may not lead to a significant effect on photosynthetic performance (Wang et al., 2012). Here, we found no significant decrease in chlorophyll contents of rice leaves in water-limiting conditions, which may be attributed to resistance of the photosystem apparatus to mild drought. Further examination of chlorophyll fluorescence by measuring Fv/Fm showed no significant decrease in maximum photosystem II efficiency in growth-limiting drought conditions compared to controls. It is noteworthy that we only examined the chlorophyll pigment accumulation and fluorescence efficiency in 1.5 g/g SWCg, which had 50% water less than the well-watered control condition, indicating that the treatment severity may be insufficient to cause a significant reduction to PSII maximum efficiencies and chlorophyll content. In Arabidopsis, lower Fv/Fm ratios were associated with decreasing relative water content (RWC) (Woo et al., 2008). A significant reduction in the Fv/Fm ratio only occurred in severe drought conditions when the RWC of the leaves had reached approximately 40 – 50% in rice (Todaka et al., 2017). While our data showed that the observed decrease in growth under water-limiting conditions was not associated with reduced efficiency of the photosystem II apparatus, it would be overly ambitious to state that the photosynthetic apparatus was completely unaffected by the mild drought conditions. There are many factors that can contribute to the functioning of PSII (Kalaji et al., 2016; Nixon et al., 2010), and not all of them were examined in this thesis. For instance, previous research on algae has demonstrated that examination of electron transfer at the donor and acceptor sides of PSII can reveal the extent

of damage caused by salt stress by determining changes in the characteristics of flash-induced chlorophyll fluorescence kinetics (Gong et al., 2008). Future studies that use a range of soil water contents, from mild to severe drought conditions, could help elucidate the effects of growth-limiting drought stress on the photosynthetic apparatus.

One hypothesis about reduced shoot growth under mild drought stress is that plants may reduce their growth in order to conserve resources, such as photosynthates, in anticipation of severe droughts (Claeys & Inzé, 2013; A. Gupta et al., 2020; Skirycz & Inzé, 2010). Here, the carbon assimilation rate and stomatal conductance were inhibited with limited water availability and were associated with reduced plant growth. The decrease photosynthetic rate observed under lower SWCg conditions could be due to partial stomatal closure, leading to a reduced CO_2 assimilation rate during photosynthesis. While I did not measure stomatal closure, closing the stomata helps plants conserve available water, thus ensuring that the plant can maintain a higher leaf water potential in anticipation of severe drought conditions (Caine et al., 2019; A. Gupta et al., 2020). It is important to note that gas exchange experiments reported in this study were conducted at a slightly elevated CO_2 concentration of 480 ppm supplied to the leaves. While it has been shown that elevated CO_2 promotes stomatal closure (Chater et al., 2015), I believe that the difference observed between the growth-limiting conditions and well-watered controls in this study holds for the current ambient atmospheric CO_2 condition. Additionally, the global CO_2 concentration has been increasing, with an average of 2 ppm per year (Maginn, 2010), thus, the elevated CO_2 concentration used here may be prevalent in future climate conditions.

Interestingly, rice seedlings under reduced water availability conditions showed a higher intrinsic water use efficiency (*iWUE*) than control plants, although this did not result in better growth performance. These results suggest that plants under lower water availability had more robust

control of the instantaneous rates of photosynthesis and transpiration, which helped the plants adjust to the changing environmental conditions. Improved water economy highlights the strong control of the stomatal aperture in plants, which prioritizes water conservation at the cost of potential carbon gain. This emphasis on water conservation is due to the inherent need to maintain high leaf water potential (Luo, 2010; Turner & Thomas, 1998).

Step changes in light intensity along with saturated CO_2 assimilation rate are widely used to provide insight into the kinetic properties of enzymes that play roles in photosynthesis (Sharkey et al., 2007). Here, we found that water limitation inhibited the photosynthetic rate as irradiation levels changed from low to high compared to control plants, indicating a reduced capacity to utilize the photosynthetically active radiation available to the leaves. The decreased CO_2 assimilation likely resulted from increased non-photochemical quenching at a high light intensity (Anderson et al., 2021), which could not be countered by leaves growing under water limitation. The other possibility is that water limitation treatment probably influenced the photosynthetic rate through the regulation of the Rubisco carboxylation rate, as evidenced by decreased V_{cmax} and J_{max} in drought-treated rice leaves. Rubisco is an enzyme that plays a crucial role in regulating CO_2 fixation and has been implicated as a potential link between the plant response to environmental stresses and photosynthesis (Jensen, 2000; Perdomo et al., 2017). While the rate of carbon gain is dependent on Rubisco activity which is influenced by water availability (Bota et al., 2004), and other factors (Taylor et al., 2022), *in vivo* measurements only provide an estimation of potential activity. Thus, direct biochemical measurements could provide a better understanding of how Rubisco activity is impacted by water availability and could be an opportunity to fine-tune Rubisco bioengineering for the improvement of drought tolerance.

5.3 Degree of water limitation defines discrete patterns of gene expression and functional responses in rice

My initial hypothesis was that the two levels of water limitation would elicit different qualitative and quantitative transcriptome-wide responses. As expected, global RNA sequencing revealed changes in gene expression that are unique to the two levels of growth-limiting drought conditions. Drought-induced transcriptional responses are an observation well supported by a number of studies in Arabidopsis (Harb et al., 2010), switchgrass (Lovell et al., 2016), rice (Todaka et al., 2017), poplar (Robertson et al., 2022), and tomato (Nicolas et al., 2022). In part, the difference in the set of genes in the two water-limiting conditions may be explained by obvious physiological and growth differences or stress severity. Nevertheless, molecular mechanisms underlying why plants exhibit these stress intensity-dependent responses need to be further examined. For example, future studies could examine if the response was mediated by chromatin accessibility to facilitate transcriptional machinery binding and other regulatory proteins to increase the transcriptional landscape of the response. In addition to the significant gene expression shifts in response to water-limiting conditions, clustering of DEGs showed varying expression profiles, with the enrichment of classical drought-responsive GO terms among the upregulated clusters such as response to stress, cellular ion homeostasis, and water deprivation similar to other drought studies (Harb et al., 2010; Huang et al., 2008). Notably, maintaining cell ion homeostasis appears to be a drought tolerance mechanism deployed by plants to counter the effect of drought (Mulet et al., 2020; Nieves-Cordones et al., 2019). For example, plasma membrane H^+ -ATPase, which is a member of the ATPases subfamily, was associated with improved photosynthetic efficiency in rice leaves by facilitating stomatal opening in response to light induction (Zhang et al., 2021). Additionally, the over-expression of

potassium channel gene *OsTPKb* in rice confers drought tolerance through the enhancement of the cytoplasm/vacuole K^+ ratio in the roots and shoots (Ahmad et al., 2016). Together, their findings and our gene expression data highlight the complexity of the regulation of water limitation response in plants.

While ABA is often considered the central player in signalling transduction pathways during the abiotic stress response (Yamaguchi-Shinozaki & Shinozaki, 2006; Yoshida et al., 2014), there is increasing evidence that other phytohormones may play important roles in regulating plant growth under drought conditions (Nishiyama et al., 2011). Here, in addition to the drought-induced expression of *9-CIS-EPOXYCAROTENOID DIOXYGENASE 3* (NCED3) , a characteristic gene in drought stress ABA biosynthesis and signalling pathways (Huang et al., 2018), water limitation also affects biological processes related to cell wall modification and cytokinin related gene expression changes. Previous studies of mild drought stress response also found that expression of cytokinin oxidase was differentially affected in the expansion and maturation zones of the leaf due to their role in promoting cell wall loosening under drought stress (Verelst et al., 2013). Todaka et al., 2017 observed a decrease in the endogenous accumulation of cytokinin molecules in the basal region and leaves of rice under drought conditions. Their study suggests that differential control of cytokinin activity may be a key mechanism through which plant control growth under water limitations. GA is another major plant growth hormone integral to the signalling and regulation of leaf growth. Examination of expression levels of *GIBBERELLIN 20 OXIDASE 2* (*Os20ox2*) and *ENT-COPALYL DIPHOSPHATE* (*OsCPS1*) involved in the biosynthetic pathways of GA reveals downregulation in response to water-limiting conditions. This decrease in the expression of GA-related genes in growth-limiting drought conditions suggests a plausible explanation for reduced leaf growth

under water-limiting conditions. GA has been suggested to play a primary role in regulating rice leaf elongation via the regulation of cell division. For instance, a knockout mutant of the GA biosynthesis enzyme *OsGA20OX2* gene caused reduced leaf length in both cultivated and wild rice through the control of cell division rate size of the division zone (Jathar et al., 2022).

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

Drought stress in food crops is one of the major causes of food insecurity around the world. Efforts have been put forward to improve drought tolerance in food crops due to climate change and the increasing need for water to support crop production. While conventional and biotechnological approaches have been used to improve crop performance under reduced water availability, the gains achieved so far have been only incremental. Despite these advances, drought events may not always threaten plant survival in agricultural environments but rather affect plant growth and development – characteristics that may also limit yield. Earlier studies that form much of stress physiology and molecular understandings of drought stress response focused on dehydration treatments that caused severe osmotic stress for the plant. Going forward I believe that laboratory efforts should be focused on investigating drought response that are physiologically relevant by precisely monitoring soil water content and imposing mild non-lethal water limitation similar to agricultural field.

Through this study, I developed a standardized growth-limiting drought screening assay for juvenile rice plants. The peat-based growth media method provides a reliable system for monitoring the stress level by controlling the amount of soil water which may be relevant for studying drought stress response in other important staple crops such as wheat, barley and oat. In using growth-limiting drought assays, I was able to study plant growth, development, physiology and transcriptomic responses to reduced water availability in rice seedlings. Furthermore, I gained a better understanding of physiological and transcriptomic responses underlying growth limitation in rice as a result of reduced water availability. One key advantage of this method is that it allows for stress initiation at the time of seedling emergence without relying on the conventional approach of drying down the soil to induce drought stress. While this method is

promising for studying the basis of growth limitation by drought, further optimization of these protocols are likely required for other monocot plant species due to differences in growth rate, development, and resource requirements. Additionally, it is possible to automate the system in order to accurately and efficiently monitor plant growth and development under different environmental conditions that will facilitate improvement of drought tolerance in food crops.

My next objective was to investigate the phenotypic plasticity, physiological adaptation, and transcriptomic responses of rice of seedlings to consistent reduced soil water contents. One main finding of this study was that growth limitation was associated with reduced CO_2 and light utilization efficiencies in rice leaves grown in water limiting conditions. These data provide the groundwork for exploration of the transcriptomic landscape of the rice seedling under control conditions and two water limiting conditions. Through the transcriptome analysis, I identified groups of differentially expressed genes that showed unique and common responses to increasing water limitation intensity. In addition, transcriptome data revealed various stress tolerance mechanisms elicited in response to water limiting conditions including the expression of genes related to cell ion homeostasis which could be involved in maintaining normal osmotic state despite the limited water for growth. Together, these experiments provide insight to phenotypic flexibility and transcriptome changes that accompany plant response to water limitation, even when the soil water availability was only slightly changed.

While the data presented here provided preliminary insight to growth, physiological and transcriptomic responses to growth-limiting drought condition, further investigations will be required to answer additional biological questions relating to molecular coordination of plant response to growth-limiting drought condition. Future research is required to investigate the molecular mechanisms underpinning growth-limiting drought in both space and time to further

resolve the complex response. Given the limitation of bulk RNA-analysis to capture the tissue heterogeneity present in leaf tissues, thus, an important next line of action is to use spatially resolved transcriptomic analyses to further tease apart the complexity of responses to water limiting condition. For example, single-cell sequencing technology which is currently being optimized in the lab of Dr. Olivia Wilkins at the University of Manitoba offers an opportunity to answer further questions such as: what are the leaf spatial transcriptional program underlying response to drought stress?; and what are the cell-type-specific transcriptional programs modulating drought response? Insights gained from studying the cell-type-specific transcriptional programs during drought stress using a single-cell sequencing platform could have significant implications for improving stress responses in plants. For example, identifying key genes and regulatory networks involved in drought responses at the cellular level could provide a comprehensive understanding of the mechanisms governing stress tolerance. This knowledge can be harnessed to develop targeted strategies for enhancing stress responses in crops, such as genetic engineering or breeding programs that aim to modulate the expression of specific genes or regulatory elements in drought-responsive cell types.

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