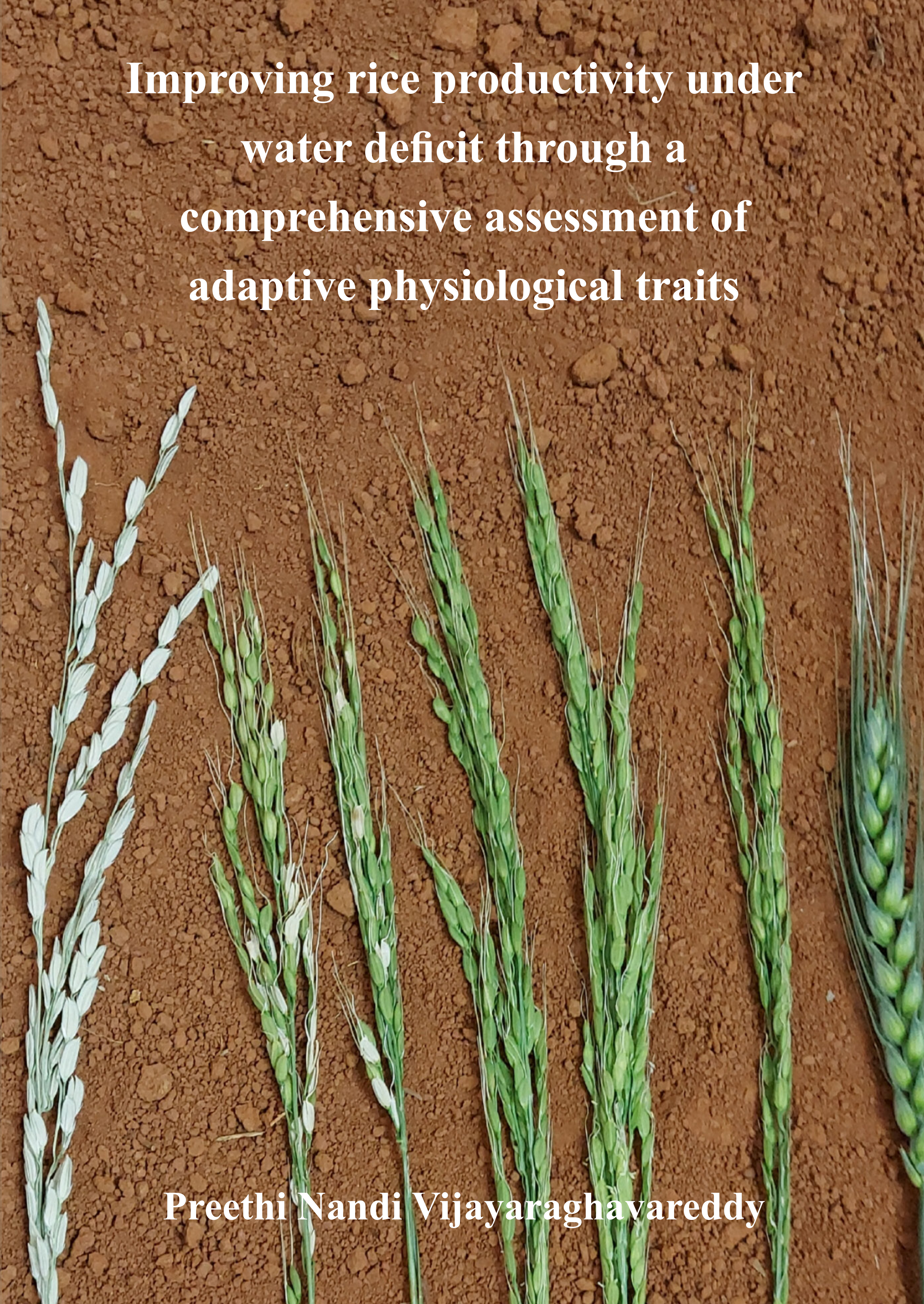
Improving rice productivity under water deficit through a comprehensive assessment of adaptive physiological traits



Preethi Nandi Vijayaraghavareddy

Propositions

1. Inefficient free radical regulation during water deficit is a greater concern in rice than in wheat.
   (this thesis)

2. Developing a reliable stress imposition protocol is more challenging than phenotyping the responsive traits *per se*.
   (this thesis)

3. Significance of correlations between traits is good but increases the risk of losing diversity.

4. Virtual conferences need to be continued beyond the Covid-19 pandemic for the benefit of students.

5. Technophobia is a major threat to the agricultural sector.

6. Simultaneous progress in food security and nutritional security is difficult.

7. Opportunities for PhD candidates need to be created to get funding for a postdoc position by developing a proposal based on their thesis outcome.

Propositions belonging to the thesis, entitled

“Improving rice productivity under water deficit through a comprehensive assessment of adaptive physiological traits”

Preethi Nandi Vijayaraghavareddy

Wageningen, 25th October 2021

Improving rice productivity under water deficit through a comprehensive assessment of adaptive physiological traits

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Improving rice productivity under water deficit through a comprehensive assessment of adaptive physiological traits

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In memory of his inspiration

dedicated to

my teacher

Prof. M. Udayakumar

Abstract

Preethi Vijayaraghavareddy (2021) "*Improving rice productivity under water deficit through a comprehensive assessment of adaptive physiological traits"***.** PhD thesis, Wageningen University & Research, Wageningen, The Netherlands, 170 pp.

Global climate change, especially in the view of severe changes in precipitation pattern, is posing a great threat for the agricultural sector, mainly in crops with large water consumption like rice. Major advances have occurred in rice cultivation system to grow rice with lower water input. But the lower yields compared with traditional puddle cultivation is the major disadvantage. It is estimated that there is a need to produce 50% more rice by 2050. To meet this challenge, we need rice varieties with higher yield potential under limited water supply. Therefore, it is important to identify physiological traits to improve drought tolerance and productivity of rice under water limitation. The main goal of this thesis is to compare the physiological basis of drought adaptation among rice genotypes and between rice and wheat.

In an initial experiment, rice cultivars adapted to puddle, aerobic and upland ecosystems were compared for their response to drought stress imposed at critical growth stages of the crop. All cultivars investigated showed significant reduction in yield under drought and the largest reduction was noticed when drought stress coincided with flowering. Comparatively, the aerobic rice cultivar showed higher tolerance irrespective of stage of drought occurrence than lowland and upland cultivars. Upon analysis of the results, reduction in leaf area, increased spikelet sterility and reduced source capacity were seen as the major reasons for the observed yield loss when drought occurred during vegetative, reproductive and grain filling phases, respectively. At all growth stages, maintenance of photosynthesis contributed to higher yield in the drought tolerant aerobic cultivar.

For a specific understanding of drought tolerance at one particular stage, two contrasting rice genotypes were again compared to capture the drought response during grain filling by using a high-throughput phenomics platform and a metabolomics approach. The outcome revealed the relevance of maintaining a high source and its transport efficiency for a high productivity. The maintenance of transport of assimilates facilitated an enhanced accumulation of carbohydrates and secondary metabolites in the tolerant rice cultivar. Phenylproponoid pathway metabolites which are involved in protective mechanisms against reactive oxygen species (ROS) were the most prominent changes among differentially expressed metabolites.

Besides within rice, comparison was also made between two C_3 cereals, rice and wheat. Wheat is known to be drought adaptive and consumes less water than rice. Rice was more susceptible to drought-induced oxidative damage than rice. Although scavenging capabilities were similar between species, rice was not able to detoxify the ROS sufficiently. In contrast, wheat possessed better activation of a photo-protection mechanism which prevented ROS production itself. The wheat cultivar also exhibited a significant increase in osmotic adjustment, epicuticular wax content, with a lower leaf temperature. These protection mechanisms in wheat led to a higher photosynthetic efficiency and capacity than in rice. In line with this, there was a higher expression of photosynthetic genes in wheat than in rice.

Overall findings in this thesis suggest that although certain mechanisms are valuable in the aerobic rice cultivar, it is not sufficient to withstand drought. The outcome of the comparative analysis between species clearly indicate the need for introgressing a few important physiological traits to grow rice like wheat.

Keywords: Drought, rice, wheat, growth stages, aerobic cultivar, lowland cultivar, metabolome profiling, high-throughput phenomics platform, acquired tolerance traits, reactive oxygen species, scavenging enzymes, gene expression, non-photochemical quenching, photosynthesis.

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CHAPTER 1

General introduction

Cultivating rice

Rice (*Oryza sativa* L.) is the prominent food crop of Asian countries like India. It plays a vital role in the food security of these countries (Molden, 2010). India is a major consumer of rice but it is also the second largest producer in the world. Considering its importance, the United Nations declared 2004 as the International Year of Rice.

The traditional cultivation practice is growing rice in a semi-aquatic flooded condition, known as puddle or lowland system. This lowland system requires large amounts of fresh water. It has been claimed that for the production of 1 kg of rice grain 2500 litres of water is required (Bouman et al., 2009). However, rice shows an incredible adaptability to various soil and climatic conditions (Shresta et al., 2016; Tahir et al., 2018). Nevertheless, growing paddy rice has to compete for water with other sectors of society. The diminishing water resources for cultivating rice, especially prevalent under climate change conditions, led to the development of less water-demanding cropping systems (Bindraban et al., 2006). Among the existing rice ecosystems, lowland (both irrigated and rainfed) and deep water cultivation systems are practices that consume large quantities of water. In contrast, upland and aerobic cultivation are considered as production systems that show much higher water productivity. Upland rice cultivation is growing rice in fields without bunds and with good soil drainage (Atlin et al., 2006). Although this method saves water significantly, it is considered as a low-yielding cultivation practice (Bernier et al., 2008). Aerobic rice is a newly developed method of growing rice with low water input. In this method rice will be cultivated in non-flooded fields with supplementary irrigation when rainfall is inadequate (Bouman and Tuong, 2001). This method not only saves water, but also requires less labour and also lowers the emission of greenhouse gases compared with the puddle system. However, the system requires a different portfolio of cultivars compared with the puddle system: drought tolerant cultivars are desperately needed.

Drought impacts productivity

Global climate change in the form of entailed shifts in rainfall pattern accompanied by increasing temperature has led to more episodic drought and increased risk of food insecurity (Wang et al., 2007; Zhang et al., 2018). Drought is a major climatic constraint to plant productivity in many parts of the world, especially of arid and semi-arid terrestrial ecosystems

(Swemmer et al., 2007). Severe drought in India during 2002-2003 affected major cropping areas (Wassmann et al., 2009). The estimate of global yield loss in major cereals (such as maize, wheat and rice) due to drought was 1820 million Mg (Lesk et al., 2016). Among these cereals, rice, being semi-aquatic, is highly water demanding. It consumes ~50% of the total irrigation fresh water resources (Upadhyay, 2016; Wu et al., 2017). Efforts have been made to develop production systems that perform well in non-puddle conditions, for example by developing drought-tolerant aerobic and upland rice cultivars, or by using the System of Rice Intensification method (Nishimura et al., 2008; Uphoff et al., 2011; Kadiyala et al., 2012). However, low yields compared with lowland production systems remain a major drawback. But improving low water demanding cultivation methods are necessary because of increasing risk of more demand than available water (physical water scarcity) and poor management of available water (economic water scarcity). It has been predicted that by the year 2025, puddled cultivation practices may experience physical water scarcity whereas cultivation practices such as aerobic or upland rice may undergo economic water scarcity in Asia (Tuong and Bouman, 2003). Hence there is an urgent need to develop high-yielding rice cultivars with superior capability to utilize water more efficiently than the present-day genotypes.

The need to compare rice with wheat

In order to increase rice yield under drought many cultivars were developed using conventional, molecular and genetic engineering approaches. Although these cultivars were able to maintain higher yields, these yield increases were not consistent and sufficient enough to meet the target productivity. This could be due to the quantitative and complex nature of drought. It is also very challenging to identify relevant drought tolerance traits and to introgress these traits into high-yielding cultivars (Sheshshayee et al., 2018). The narrow genetic variation in rice for these traits also made it difficult to obtain drought tolerance (Basu et al. 2010; Singh et al. 2016). Hence, it is becoming more important to assess the variability in traits for drought tolerance within and between species. The species that exhibit, higher drought tolerance ability are often targeted to study drought responsive mechanisms.

Rice and wheat are C_3 cereals with significant differences in their water requirement and drought adaptation. Rice requires 2-5 times more irrigation than wheat. Despite lower water requirement the wheat yield is significantly higher (Fischer and Edmeades, 2010). With the diverse adaptability of rice to different environmental conditions, efforts to grow rice like wheat can improve its water productivity without decreasing yield. To achieve this, it is important to expose both rice and wheat to the same level of soil moisture stress to understand the drought tolerance mechanisms (Prabha et al., 2009). Such a comparative analysis will help to identify the traits and mechanisms required to achieve drought tolerance in rice with higher productivity.

Effects of drought on growth during critical phases of rice

With the change in precipitation pattern due to climate change, drought may occur at different growth phases and be of varying intensity and duration; all these factors determine the magnitude of yield loss (Kumar et al., 2014). It is important to study the plant's responses to drought at different growth phases because upregulation of drought tolerance mechanisms will also be distinct, depending on stage of drought occurrence. Hence, improving certain traits useful for a specific phase may not be sufficient to provide tolerance to drought at all critical growth phases. For example, improving seedling vigour that is associated with drought tolerance at seedling establishment phase is not useful when drought occurs at flowering phase (Sallam et al., 2018).

Drought impedes seed germination and seedling establishment during crop establishment (Osakabe et al., 2014). Drought stress during the vegetative phase of rice mainly results in reduction in tiller number, plant height, and leaf expansion leading to delayed and/or reduced canopy cover or overall source for grain filling. This decrease in canopy traits causes a yield loss of up to 20% (Babu et al., 2003). It has also been shown that stress during the vegetative phase results in redirection of carbohydrates to root growth to enhance water uptake. Drought during flowering reduces productivity because of poor panicle exertion, reduced pollen viability, and increased spikelet sterility. The flowering phase is considered as the most susceptible to drought stress with approximately 50% yield reduction in rice (Raju et al., 2014). During grain filling, drought reduces the grain filling rate and duration in rice (Wang et al., 2006; Zhang et al., 2014). Additionally, altered grain composition due to lower source activity and translocation of assimilates will result in poor quality of the grains (Yang and Zhang, 2006; Barnabás et al., 2008). However, varieties respond differently to stress because there is genetic variation in drought tolerance. For example, aerobic cultivars developed to cope with water scarcity will have more tolerance than lowland cultivars adapted to puddle conditions. Hence, comparison of rice genotypes adapted to different ecosystems is useful to explore and exploit mechanisms of drought tolerance at different growth phases.

High-throughput phenotyping approaches to connect phenomics to genomics

A deeper understanding of the biological processes by developing innovative approaches to speed up the improvement of stress resilience and characterizing novel traits is highly essential to incorporating them into breeding programmes (Nolan and Santos, 2012; Rogers et al., 2015). High-throughput phenotyping plays a crucial role in successful breeding programmes by linking the phenomics to genomics. Over the last decade many phenotyping platforms were developed to screen large sets of plants under both controlled greenhouse conditions and naturally varying environments. Additionally, these facilities with controlled irrigation will have the ability to alter timing, frequency and intensity of drought episodes (Niinemets, 2010). Hence the trait values can be measured more accurately by mimicking various natural drying conditions. These automated controlled irrigation platforms are very effective to capture plant stress responses at physiological and molecular level. Moreover, the "-omics" platforms now allow for extensive mining of the drought responsive genes, proteins and metabolites to improve drought tolerance. Hence integrating phenomics and genomics can establish stronger relationships between gene and trait or metabolite and phenotype (Arbona et al., 2013).

Among these omics approaches, metabolomics is widely used to detect a vast array of metabolites, thus helping identification of bio-markers to assist crop improvement (Heyman and Dubery, 2016). Under drought, many metabolites will be altered to avoid or to tolerate stress. Metabolomics profiling will provide a comprehensive view of differential regulation of metabolites among the genotypes representing the physiological state of the plant (Arbona et al., 2013; Turner et al., 2016). With increasing precision in phenotyping and identification of metabolites, there is a growing interest in combing phenomics with genomics data (Rutkoski et al., 2016). Using these multiple data set now it is possible to study the drought tolerance mechanisms more accurately.

Chapter 1

Importance of acquired drought tolerance mechanisms in plants

Drought stress stimulates several drought tolerance mechanisms, such as stomatal closure, leaf rolling, and enhanced root growth (Zlatev and Lidon, 2012).These drought adaptive traits are classified into different categories.Based on the level of organisation and response to environmental clues, drought tolerant traits are mainly divided into “constitutive” and “acquired” tolerance traits (Sheshshayee et al., 2018). Many efforts are being made to improve constitutive traits such as root traits, specific leaf area, stomatal number and size (Raju et al., 2014; Sheshshayee et al., 2018). Additionally plants also require certain acquired tolerance traits, which only express during mild stress conditions and later provide tolerance when the stress becomes severe (Sung et al., 2003; Collins et al., 2008).Under natural conditions, plants experience gradually progressive stress (mild stress to severe stress) and hence will have opportunities to trigger acquired response (Fig. 1). In contrast, exposing plants directly to severe stress conditions will lead to reduction in physiological activities due to very poor activation of acquired traits (Niinemets, 2010). Under stress conditions where soil moisture is gradually depleted, plants tend to alter mechanisms to maintain better metabolism. Initially, plants will upregulate certain mechanisms to maintain tissue turgor. These mechanisms include increasing osmolyte production such as proline, glycine betaine, sugars, and polyamines (Moinuddin et al., 2005; Ruggiero et al., 2017). Osmolytes are compatible solutes that enhance cell potential to maintain leaf turgor. The upregulation of these osmolytes protects cellular structure and function and delays damage due to dehydration (Taiz and Zeiger, 2006). Later, plants will also upregulate photoprotection and scavenging mechanisms to prevent the damaging effects of free radicals known as reactive oxygen species (ROS).

ROS are regarded as bi-products of plant aerobic metabolism and are produced due to partial reduction of atmospheric O_2. In plants there are four forms of cellular ROS, singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^\bullet$) (Mittler et al., 2004). During normal conditions, plants will maintain the minimum level of ROS. Occurrence of abiotic stress will create an imbalance between production and scavenging of these ROS radicals. Hence under drought stress conditions, managing the ROS is very crucial for the survival of the plants. To manage the ROS production and scavenging, many of the protective mechanisms will be induced as an adaptive strategy. These acquired mechanisms include osmotic adjustment, management of ROS production by scavenging enzymes, non-photochemical quenching, and upregulation of many drought responsive genes (Fig. 1). These traits are collectively referred to as acquired traits and the level of their upregulation will determine the tolerance ability of the plant.

Management of free radicals in plants

During drought conditions, plants will adopt a water-saving strategy and provoke stomatal closure to reduce transpiration flux, but this strategy will concomitantly limit the entrance of carbon dioxide (CO_2) and thus photosynthesis. This results in an imbalance between light harvesting and its utilization for CO_2 reduction causing ROS overproduction and ultimately cell death (Mittler, 2002). Furthermore, these accumulated ROS molecules react with proteins, lipids and secondary molecules and generate highly electrophilic compounds, called reactive carbonyl compounds (RCCs), such as malondialdehyde, methyl glyoxoal, and protein carbonyls. These free radicals bring functional change to macromolecules such as changes in charge and solubility (Pamplona, 2011) (Fig. 2). To mitigate free radical accumulation, plants have developed several protective mechanisms including non-photochemical quenching (NPQ), production of antioxidants and enzymes for scavenging. NPQ is a dissipation of excessive excitation energy in the form of heat at the Photosystem II (PSII) antenna complex (Müller et al., 2001). Upregulation of these protective mechanisms during stress will determine the tolerance of plants, if any imbalance in production and scavenging results in over-accumulation of ROS and RCCs that lead to damages to many photosynthetic and Calvin cycle enzymes (Mano, 2012). For example, during drought conditions, higher light intensity associated with reduced CO_2 supply causes photoinhibition and ROS production, and reduces PSII photochemistry which alone lowers the quantum yield of CO_2 assimilation (Filek, 2006).

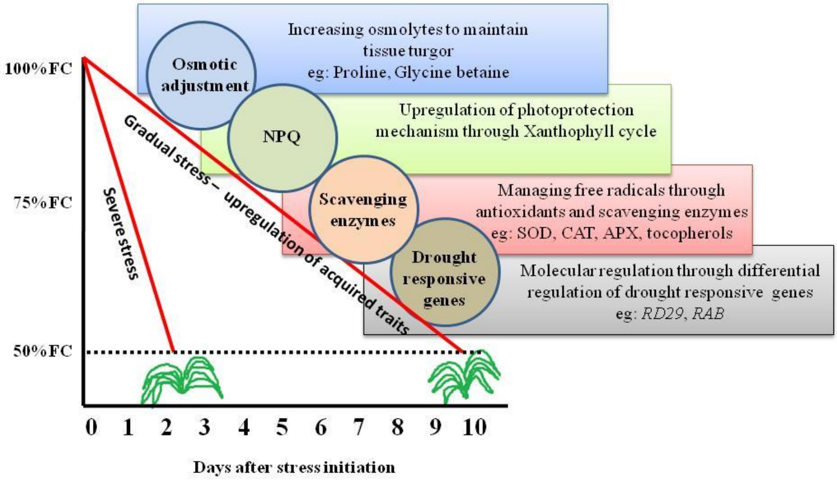

Figure 1. Differential response of plant to severe stress and gradual stress. When the plants are exposed to gradual and rapid (severe stress) reduction in soil moisture from 100% field capacity (FC) to 50%FC (Y-axis) the upregulation of drought responsive traits will be different between stresses. Severe stress conditions is withholding water until it reaches to 50%FC (dotted line). Gradual stress mimics the natural drought condition, where plants will take 10 days to reach 50%FC due to progressive soil drying. Under gradual stress, plants will initially upregulate certain acquired mechanisms such as osmotic adjustment to maintain tissue turgor, non-photochemical quenching (NPQ) for photo-protection. Later plants will also upregulate scavenging activities by increasing antioxidants and enzymes like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidise (APX), tocopherols etc. Molecular regulation will also get activated through upregulation of many drought responsive genes like responsive to desiccation (RD29), responsive to abscisic acid (RAB) etc. These are collectively referred to as acquired mechanisms which protect plants when the stress becomes sever. But when the plants are subjected to sever stress directly, lack of protective mechanisms may lead to death of the plant (Senthil-Kumar et al., 2003; Sung et al., 2003; Sheshshayee et al., 2018).

To overcome this, plants have adopted Zeaxanthin mediated energy dependent quenching as the most prominent NPQ process since it operates on a time scale of seconds to minutes (Niyogi and Truong, 2013) (Fig. 2). Additionally, in order to cope with continuous ROS and RCC production, plants have a suite of enzymatic and nonenzymatic antioxidants, which function as an extremely efficient cooperative system to detoxify ROS and RCC

(Karuppanapandian et al., 2011). Enzymatic antioxidants include catalase, ascorbate peroxidises, glutathione peroxidise, superoxide dismutase, aldo-keto reductase, and alcohol dehydrogenase (Cruz de Carvalho, 2008). Non-enzymatic antioxidants include ascorbic acid, glutathione, tocopherols, carotenoids, flavonoids, and phenolic compounds (Das and Roychoudhury, 2014) (Fig. 2).

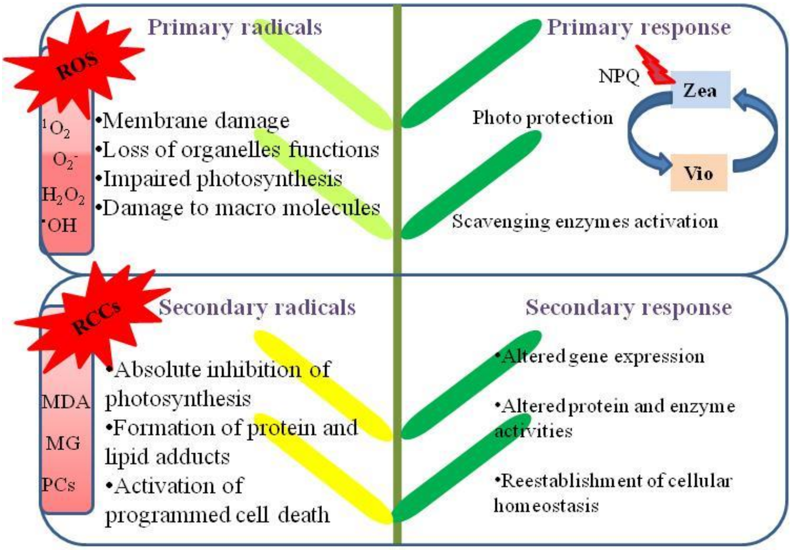

Figure 2. Effect and response of plants to accumulation of primary (reactive oxygen species; ROS) and secondary (reactive carbonyl compounds; RCC) radicals in plants (Vemanna et al., 2020).

Objectives

Drought is one of the most widespread climatic disasters threatening agriculture productivity. Developing rice cultivars with improved abilities to withstand drought is very crucial. Although many efforts are being made to develop cultivars for limited water conditions such as aerobic cultivation practice, concomitant reduction in yield was noticed compared with rice grown under puddle conditions. Therefore, improving rice productivity under water limited conditions to achieve the yield targets needed to ensure food security remains a great challenge. With the increasing significance of physiological breeding for crop improvement, identification of relevant physiological traits by different approaches is a pre-requisite.

In view of the background knowledge as outlined in earlier sections of this general introduction, the main aim of this thesis was to compare the stress response of rice cultivars adapted to different ecosystems and also to compare drought-sensitive rice with drought-adaptive wheat. The key objectives of the study are as follows:

1. To investigate genetic variability in stress response at different phenological phases in rice;
2. To understand the stress induced alteration in metabolome profiling of contrasting rice genotypes during grain filling;
3. To verify the variability in acquired tolerance mechanism in rice and wheat using a high-throughput phenomics facility;
4. To comparatively assess reactive oxygen species management and photosynthetic characteristics at the vegetative phase in rice and wheat.

Achieving these objectives in view of inter- and intra-species comparisons will help identify and understand the key traits that are essential to breed for improved drought tolerance with sustained yield in rice.

Methodology

To achieve the above mentioned objectives, I framed the methodology into two parts: firstly to compare contrasting genotypes within the rice species and secondly to make a comparison between contrasting rice genotypes on the one hand and with a drought-tolerant wheat genotype on the other hand. An initial experiment was conducted to understand the response to drought imposed at different critical growth phases for rice genotypes adapted to lowland, upland and aerobic ecosystems, respectively. From this, I identified two contrasting genotypes that were then characterised to assess the differential regulation of metabolites at grain filling. These two rice genotypes were further compared with wheat to understand the variability in acquired mechanisms using a high-throughput phenotyping facility. The phenotyping facility consisting of an automated irrigation system was used to create a dry-down protocol to mimic natural soil drying conditions. With the help of this facility, a gradual stress imposition was created to induce acquired mechanisms such as managing reactive oxygen species, and a comparison was made again between rice genotypes and between rice and wheat. Furthermore, these two rice genotypes and one wheat genotype were grown in controlled greenhouse conditions to capture the stress response at the vegetative phase. In this experiment, the superior drought tolerant traits of wheat compared with rice were identified by mainly focusing on ROS management and photosynthetic efficiency and capacity.

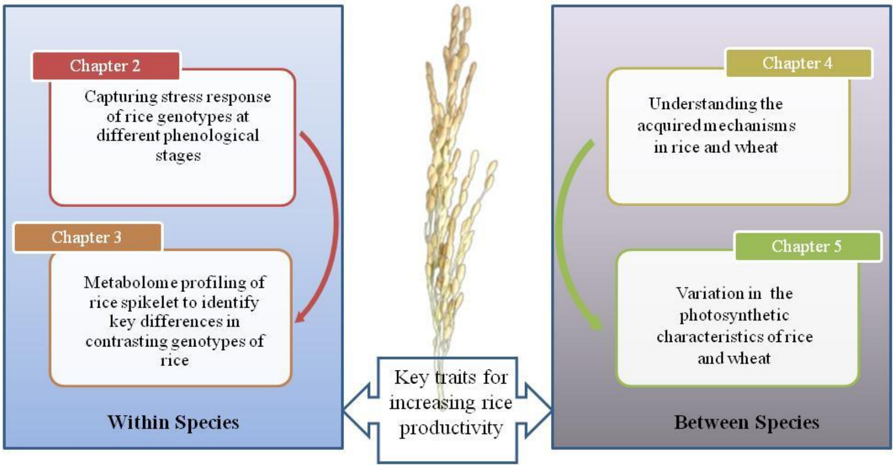

Figure 3. Outline of the thesis

Thesis outline

This thesis has six chapters including this General Introduction (Chapter 1). The main aim of my work is to unravel the drought responsive mechanisms between rice genotypes and also between rice and wheat (Fig. 3).Chapters 2-6 are outlined as follows.

In **Chapter 2**, I report the key outcome from the experiment conducted to assess the stress response of rice genotypes differing in their adaptability at different growth phases. The key traits which determine the drought tolerance at different growth phases among lowland, upland and aerobic rice cultivars will be described.

Considering the importance of source supply for grain filling, in **Chapter 3**, I explore the metabolite profiling of spikelets in two contrasting rice genotypes to reveal whether higher accumulation of carbohydrates and secondary metabolites in tolerant rice can help maintain improved spikelet fertility.

In **Chapter 4**, I compare rice and wheat for their acquired drought tolerance mechanisms using a novel high-throughput phenomics facility. I study the stress response in rice and wheat mainly with regard to reactive oxygen species management to examine whether and why the wheat genotype studied has higher acquired drought tolerance than some contrasting rice genotypes.

In **Chapter 5**, I describe the variation in drought responses of rice and wheat at the vegetative phase grown under controlled environmental conditions. The differential response in photosynthetic capacity and efficiency attributed to drought tolerance will be described.

Finally in **Chapter 6**, I discuss the key conclusions of my thesis and give an outlook on important drought tolerance mechanisms in rice and wheat. I also discuss what traits have to be improved in rice to grow it like wheat.

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CHAPTER 2

Responses of lowland, upland and aerobic rice genotypes to water limitation during different phases

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Chapter 2

Abstract

Rice yield reduction due to water limitation depends on its severity and duration and on the phenological stage of its occurrence. We exposed three contrasting rice genotypes, IR64, UPLRi7 and Apo (adapted to lowland, upland and aerobic conditions, respectively), to three water regimes (puddle,100% and 60% field capacity) in pots during the vegetative (GSI), flowering (GSII) and grain filling (GSIII) stages. Stress at all the three stages significantly reduced yield especially in lowland genotype IR64. Effect of water limitation was more severe at GSII than at the other two stages. Stress at GSI stage reduced both source activity (leaf area and photosynthetic rate) and sink capacity (tiller number or panicle number per pot). When stress was imposed at GSII, spikelet fertility was most affected in all the three genotypes. In both GSII and GSIII, although leaf area was constant in all the three water regimes, estimated relative whole-plant photosynthesis was strongly associated with yield reduction. Reduced photosynthesis due to stress at any given stage was found to have direct impact on yield. Compared to the other genotypes, Apo had deeper roots and maintained a better water relation, thus, higher carbon gain and spikelet viability, and ultimately, higher biomass and productivity under water-limited conditions. Therefore, screening for these stage-dependent adaptive mechanisms is crucial in breeding for sustained rice production under water limitation.

Key words: water limitation; phenology; upland; aerobic; *Oryza sativa*; yield

Introduction

Rice is a major staple food crop, providing 35%-80% of total calories required for populations of Asia (IRRI, 1997; Yang et al., 2009). It is mainly grown in semi-aquatic environments in paddies, and rice production in such a system requires 3000-4000 L of water to produce 1 kg of grains (Bouman, 2009). Most rice production is in irrigated lowland ecosystems in Asia. However, the industrial and household requirements are rapidly withdrawing fresh water away from agriculture in this region. This urgently necessitates efforts towards devising water saving agronomies as well as improving (or at least sustaining) rice productivity under water-limited conditions (Bouman and Tuong, 2001).

Many efforts have been made to modify cultivation practices, like SRI (system of rice intensification) (Uphoff et al., 2010), upland (Nishimura et al., 2008), rainfed lowland (Zeigler and Puckridge, 1995) and aerobic (Kadiyala et al., 2012) systems, to reduce water consumption in rice production. The emphasis in these systems has generally been to decrease the 'E' component of 'ET' (Evapo-transpiration). However, these systems have one thing in common: yields are reduced compared to the irrigated lowland (paddy) ecosystem. Efforts have been made to identify genotypes with good yields in upland and aerobic conditions. Although these genotypes were selected for cultivation in water-limited conditions, yield penalties are still severe. Under water-limited conditions, reduced field capacity (FC) has a major impact on yield depending on crop growth stage that coincides with the stress, its intensity and duration. Understanding the physiological mechanisms that get affected under stress and assessing the variability in these mechanisms among various genotypes is crucial for further crop improvement.

Three growth stages, i.e. vegetative (GSI), flowering (GSII) and grain filling (GSIII) stages, are characterized as distinctive growth periods that are differentially influenced by biotic or abiotic stresses (Boonjung and Fukai, 1996; Sarvestani et al., 2008). At all phenological stages, water limitation potentially affects size and capacity of both source and sink for rice growth. Stress at GSI can impede leaf expansion (thus reducing source size) and inhibit tillering (thus reducing both sink and source). The carbon assimilatory capacity of the canopy remains small due to a reduced photosynthetic rate combined with the reduction in canopy leaf area. These factors can lead to lower final biomass production (Praba et al., 2009). GSII is the most critical stage of yield formation, and water limitation at this stage can adversely affect both carbon source and reproductive development, resulting in reduced yields (Jin et al., 2013). GSIII also represents a critical stage, during which reduction in the grain yield can be caused by reduced leaf photosynthesis as well as lower green leaf area by advanced leaf senescence.

Rice varieties that adapt to different ecosystems with diverse adaptive strategies to grow under water-limited conditions have been developed, with better extraction of water by roots and increased cellular-level tolerance (CLT) under stress (Raju et al., 2014), increased water use efficiency (WUE) (Sheshshayee et al., 2003) and reduced leaf transpiration through wax layers (Zhang et al., 2007). Plants with good root systems can maintain better tissue water potential with cooler canopy (Ramu et al., 2016) and that plants with high CLT have improved spikelet fertility in stress conditions (Raju et al., 2014). Significant progress has also been made in developing suitable cultivars for greater adaptation to diverse rice growing agronomic practices, which leads to the identification of UPLRi7 for upland conditions and Apo for aerobic cultivation (Ouyang et al., 2017). IR64, a mega variety in Southeast Asia (Mackill et al., 2018), is predominantly grown in puddled conditions and is known to be sensitive to water limitation (Dharmappa et al., 2019). The objective of this study was to understand the stress response of these three rice genotypes known to adapt to upland, aerobic and lowland rice ecosystems, respectively, and to assess their yielding ability under stress imposed at GSI, GSII and GSIII stages.

Materials and methods

Plant growth and stress conditions

Three rice genotypes, which were bred for lowland (IR64), upland (UPLRi7) and aerobic (Apo) conditions, were selected because they had similar phenology. For instance, all the three genotypes took 85-90 days to reach 50% flowering (Ouyang et al., 2017). Pot experiments were conducted in the University of Agricultural Sciences, Bengaluru, India. Experiments were done in an open area under a mobile rain-out shelter, which protected plants against rainfall. Whenever required and during nights the shelter was drawn over the experimental area, thus pots were maintained at the specified water regime. Pots (24 L) were filled with equal amounts (20 kg) of red soil with recommended fertilizers added. Direct sowing was followed, and thinning was done at 21 days after sowing (DAS) to maintain one plant per pot. Plants were well watered manually until the stress imposition. During treatments, three water regimes were maintained viz., puddle, 100% FC and 60% FC. Puddle treatment was created with standing water of 5 cm above the soil in the pot. 100% FC and 60% FC treatments were maintained following the gravimetric approach (Udayakumar et al., 1998): the pots were weighed daily using a hanging load cell balance (Essae-Teraoka, Japan), the reduction in weight of the pots as a result of evapo-transpiration was recorded, and on the early morning the pots were replenished with the exact same amount of water evapo-transpired in the preceding day to maintain a given FC. There were five replicates for each treatment and arranged in randomized-block design. Three separate experiments were conducted for each of the three phenological stages.

- GSI experiment was conducted from January to April of 2016, in which the three water regimes were maintained at the time of maximum tillering stage (30 to 50 DAS).
- GSII experiment was conducted from July to October 2016, in which the three water regimes were maintained particularly at the flowering (85 DAS). Water was withheld from flag-leaf stage, such that the required stress level was reached at the time of flowering, and maintained for 10 days.
- GSIII experiment was conducted from July to October of 2017, in which the three water regimes were maintained after flowering (100 DAS) until grain maturity.

Relative water content

To assess the effect of different water regimes on tissue water status, relative water content (RWC) was measured. Ten leaf discs of 1 cm^2 each were punched out of the second fully expanded leaf counted from the apex. Fresh weight was recorded immediately. The leaf discs were immersed in distilled water in a beaker for 6 h, and were gently blotted on a filter paper to remove the water adhering to the surface. Fresh weight of this tissue was taken to represent turgid weight. Then, the samples were dried in an oven (65 ºC-70 ºC) until constant weight, and then dry weight was recorded. RWC (%) was estimated according to Babitha et al. (2015).

Gas exchange

Leaf net-photosynthetic rate (A) and stomatal conductance (gs) were measured with a portable gas-exchange system Li-6400 (LiCOR-Inc, Lincoln, Nebraska, USA). The ambient concentration of CO_2 in the leaf chamber of the Li-6400 was kept at 701.8 mg/m^3, and the photosynthetically active radiation (PPFD) was 1500 µmol $m^{-2}\cdot s^{-1}$). All measurements were made at 5 days after stress imposition on the second fully expanded leaf from the top for the GSI experiment, and on the flag leaf for the GSII and GSIII experiments.

Soil and plant analyzer develotrnent (SPAD) value

SPAD value was calculated by averaging nine readings per leaf using a portable, non-destructive chlorophyll meter (SPAD-502, Minolta, Japan). Mean of the measurements was calculated to arrive at a single value per pot. Measurements were recorded at 5 days after reaching 60% FC.

Morpho-physiological parameters

Plant height and number of tillers were recorded before harvest. Plant height was measured from surface of soil to tip of the longest leaf. Roots were separated from shoot to measure root length before drying. All the plant parts were oven-dried at 70 ºC to record stem weight, leaf weight and root weight, and total biomass per pot (TBM) were calculated. Specific leaf area was calculated by dividing fresh leaf area by its dry biomass. Total leaf area (TLA) per pot was calculated by multiplying final dry leaf biomass with specific leaf area, which was determined from a subsample of leaves.

Yield parameters

Panicles were harvested from individual plants after maturity. Grains were manually separated from panicles. Spikelet fertility was calculated as the number of filled grains divided by the total number of grains. Weight of filled grains was recorded to obtain grain yield per pot.

Statistical analysis

Two-way ANOVA was conducted by using GenStat (15th edn) (http://www.genstat.co.uk/). The generated least significant difference for each parameter was used to check the significance level in all the three experiments. Standard correlation/regression procedures were performed using Microsoft Excel.

Results

Effect of stress at GSI (vegetative stage)

RWC, a simple proxy for water relations, decreased significantly as stress level increased in all the genotypes (Fig. 1A). IR64 showed 10.1% and 17.1% reductions in RWC under 100% FC and 60% FC, respectively, relative to the puddle treatment, while UPLRi7 (10.0% and 13.9%) and Apo (0.5% and 2.8%) showed significantly lower reductions in RWC (Fig. 1A). SPAD value was not affected in any of the genotypes (Supplementary Fig. S1A). Gas exchange parameters like stomatal conductance (*gs*) and net assimilation rate (*A*) decreased with increasing stress in all the genotypes, albeit to different extents. While *A* decreased by 33.0% and 54.8% for IR64 under 100% FC and 60% FC compared to puddle, this reduction was much less for UPLRi7 (11.7% and 34.0%) and Apo (19.4% and 27.5%) (Fig. 1B). UPLRi7 maintained higher stomatal conductance compared to the other two genotypes and it was highest with 0.7 mol/($m^2 \cdot s$) under puddle condition. *gs* decreased significantly in all three genotypes with highest reduction for IR64 and UPLRi7 under 60% FC (Fig. 1C). Apo recorded lower gs under the two stress regimes and a lower decrease in *gs* under 60% FC compared to the other two genotypes (Fig. 1C).

The reduction in soil moisture status resulted in a significant reduction in TLA in all the three genotypes (Table 1). UPLRi7 and IR64 showed a stronger reduction in TLA under 100% FC than Apo. Under 60% FC, all the genotypes showed a very strong reduction with IR64 showing the strongest one (57.7%) (Table 1). Both plant height and tiller number were sensitive to water limitation and IR64 showed a higher reduction than the other two genotypes. Although reduction in tiller number was low in Apo, plant height was significantly reduced compared to puddle conditions (Supplementary Table S1). Whereas, both IR64 and UPLRi7 showed significant reduction in tiller number and plant height under 100% FC and 60% FC. Stem weight decreased more in IR64 and Apo than in UPLRi7 with increased water limitation (Supplementary Table S1).

Reduction in plant height and tiller number closely matched the reduction in total biomass (TBM). Even under 100% FC, an overall reduction in TBM ranging from 16.8% to 25.0% was noticed (Table 1). Under 60% FC, IR64 recorded the strongest reduction in TBM.

This effect of water limitation on physiological responses led to an average yield decrease of 13.2% and 27.6% under 100% FC and 60% FC, respectively (Table 2). IR64 recorded the highest reduction by 35.1% under 60% FC. Similarly, UPLRi7 was also sensitive to vegetative stress with a reduction of 17.7% under 100% FC and 31.7% under 60% FC. Apo showed the least reduction in yield under both 100% FC and 60% FC (6.0% and 16.2%, respectively).

Stress response at GSII (flowering stage)

There was a significant reduction in RWC under both 100% FC and 60% FC over puddle condition in all the genotypes. This reduction was strong in IR64, especially under 60% FC (Fig. 1D). Reduction in chlorophyll content was highly significant under 60% FC for IR64 and UPLRi7 (Supplementary Fig. S1). Water limitation at GSII affected both assimilation rate and stomatal conductance (Fig. 2E and F). IR64 showed more than 45% reduction in *A* and *gs* while both UPLRi7 and Apo being drought adaptive also showed > 24% of reductions in *A* and gs under 60% FC.

As expected, water limitation during the GSII experiment had no considerable influence on plant height and tiller number (Supplementary Table S1). Similarly, TLA showed no significant changes in response to stress (Table 1). Reduction of TBM was marginal in all the genotypes under 60% FC. A mild water limitation (100% FC) under GSII significantly reduced yield. This reduction in yield was stronger when severity of stress increased to 60% FC. An overall yield loss of 22.3% and 44.4% was recorded under 100% FC and 60% FC, respectively. IR64 had 50.7% yield loss and UPLRi7 had 46.1%. Apo showed a significant reduction of 36.3% under 60% FC over the puddle condition (Table 2).

Stress effect at GSIII

RWC was significantly lower for IR64 than for UPLRi7and Apo when imposed to stress. The latter two genotypes showed a marginal reduction (upto 6.5%) in RWC under 60% FC compared with the puddle treatment (Fig. 1G). Under 60% FC, RWC of IR64 decreased by more than 19%. Gas exchange parameters recorded a similar trend. IR64 showed marked reduction in both *A* and *gs* in both the water regimes compared to puddle treatment (Fig. 1H and I).

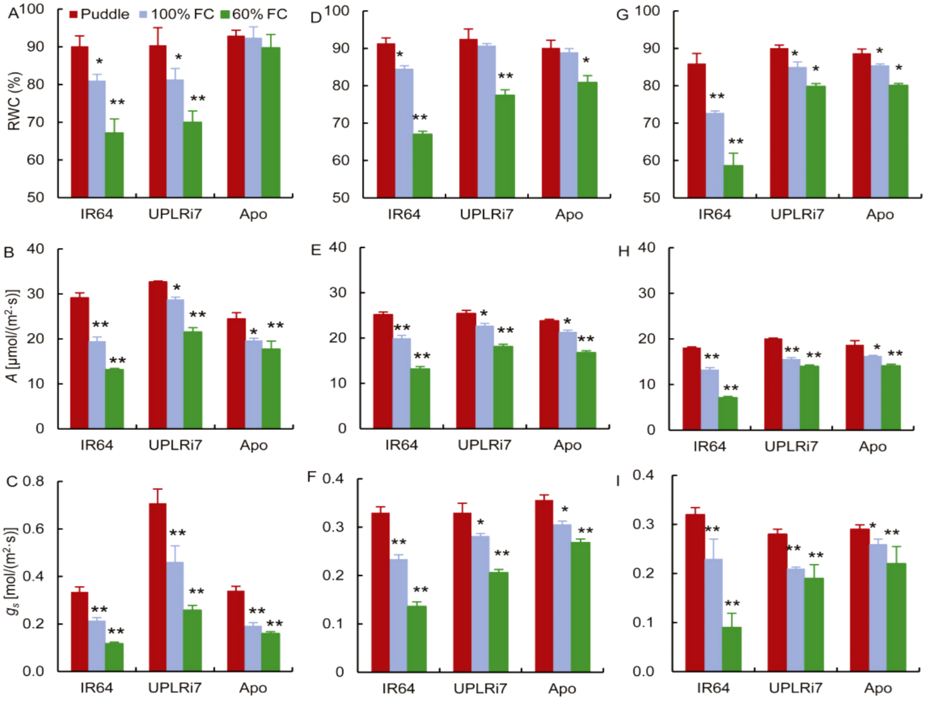

Figure 1: Effects of water limitation on various physiological characteristics of three contrasting genotypes. A, Relative water content (RWC) at the vegetative stage (GSI). B, Photosynthetic rate (*A*) at GSI. C, Stomatal conductance (*gs*) at GSI. D, RWC at the flowering stage (GSII). E, *A* at GSII. F, *gs* at GSII. G, RWC at the grain filling stage (GSIII). H, *A* at GSIII. I, *gs* at GSIII. FC, Field capacity. Data indicate mean ± SE (n = 5). * and ** indicate significant differences from puddle at the 0.05 and 0.01 levels within genotypes, respectively.

SPAD values were generally lower in IR64 than in UPLRi7 and Apo at all the phenological stages. Chlorophyll content of IR64 decreased significantly under both 100% FC and 60% FC (Supplementary Fig. S1). On the other hand, there was not much change in leaf area, plant height and tiller number in all the three genotypes (Table 1 and Supplementary Table S1). Shoot weight and TBM were significantly reduced in IR64 and UPLRi7 especially under 60% FC. An overall yield loss of 12.2% to 27.4% was recorded under 100% FC and 60% FC, respectively (Table 2). Under 60% FC, IR64 and UPLRi7 had the highest reduction

(41.6% and 32.4%, respectively), whereas Apo had a marginal reduction in yield (9.8%).

Responses of spikelet fertility to stress at three stages

When stress was imposed during the GSI stage, a slight reduction in spikelet fertility was noticed (Fig. 2A). A decrease in spikelet fertility was the most dominant yield component accounting for the observed yield reduction at GSII in all the three genotypes. IR64 had the greatest reduction in spikelet fertility followed by UPLRi7 (Fig. 2B). Although Apo showed smaller reduction in spikelet fertility than the other genotypes, a reduction of 36.3% under 60% FC was observed irrespective of its adaptability to water-limited conditions. Stress at GSIII reduced the number of filled grains especially under 60% FC. IR64 had the highest reduction (18.0%) and Apo had the lowest reduction (9.5%) under 60% FC (Fig. 2C).

Aerobic cultivar Apo had higher water extraction ability under stress

Significant effects of stress treatments at different growth stages on root traits were observed for all the three genotypes. In all the three growth stages, Apo and UPLRi7 maintained higher root growth compared to IR64 under stress. At GSI, root length was increased significantly in all the genotypes under 60% FC. Apo showed the highest increase of 40.8% and 42% under 100% FC and 60% FC, respectively (Fig. 2D). UPLRi7 also showed a significant increase of 18.2% and 31.3% under 100% FC and 60% FC, respectively, compared to the puddle condition. Interestingly, cultivar with the highest root length showed the lowest root weight (Supplementary Fig. S2). UPLRi7 and Apo had the highest root length under 60% FC but root weight was significantly lower for these two genotypes than for IR64.

The increasing trend of root length in all the genotypes under stress conditions during GSI was not observed in GSII and GSIII. Root weight was marginally decreased by stress in GSII (Supplementary Fig. S2). At GSIII, IR64 and UPLRi7 showed marginal reduction in root length under 60% FC (Fig. 2F).

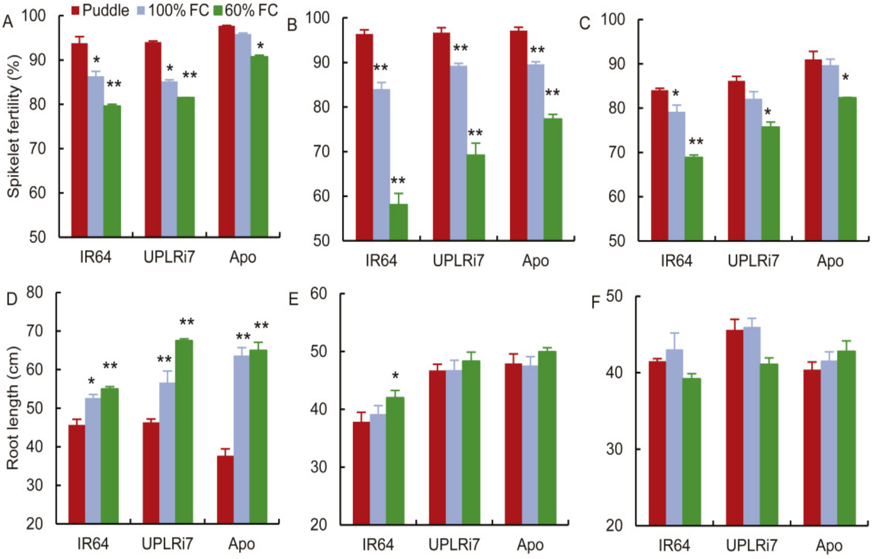

Figure 2. Effects of stress on spikelet fertility and root length among the genotypes. A, Spikelet fertility at vegetative stage. B, Spikelet fertility at flowering stage. C, Spikelet fertility at grain filling stage. D, Root length at vegetative stage. E, Root length at at flowering stage. F, Root length at grain filling stage. FC, Field capacity. Data indicate mean ± SE (n = 5). * and ** indicate significant differences from puddle at the 0.05 and 0.01 levels within genotypes, respectively.

Source to sink imbalance was the major contributor for yield loss at any given stage

At all the three growth stages, significant reduction in photosynthesis under 100% FC and 60% FC over puddle was noticed. Reductions in *A* and yield were significantly related in all the three stages (Fig. 3A). However, a strong linear relationship was shown between reduction in total leaf area and yield only at GSI stage (Fig. 3B). We computed an index as an indication of relative whole-plant photosynthesis by multiplying total leaf area with single leaf photosynthetic rate (the term 'relative' is used because the estimations are not absolute values which would require considering the vertical decrease of *A* with lowering leaf positions). Reduction in this index was strongly related with reduction in yield in all the three phenological stages (Fig. 3C). Reproductive growth (sink) was examined along with photosynthetic rate (source activity) to explain yield loss at all the three stages. Reduction in *A* and spikelet fertility were significantly correlated at GSII and GSIII (Fig. 3D).

Table 1. Effects of water limitation on total leaf area (TLA, cm^2/plant) and total biomass (TBM, g/plant). GSI, Vegetative stage; GSII, Flowering stage; GSIII, Grain filling stage. * and ** indicate significant differences from puddle at the 0.05 and 0.01 levels within genotypes, respectively.

Stage	**TLA in IR64**			**TLA in UPL Ri7**			**TLA in APO**		
	Puddle	**100% FC**	**60% FC**	**Puddle**	**100% FC**	**60% FC**	**Puddle**	**100% FC**	**60% FC**
GSI	2431.7	2110.7**	1026.8**	1839.6	1396.5**	965.5**	1977.4	2019.7	1240.5**
GSII	2221.9	2151.8	2197.6	2155.9	2033.4	2076.6	2067.9	2079.7	1947.5
GSIII	2410.6	2463	2384.3	1779.4	1747.6	1706	1913.4	1957.4	1903.4

Stage	**TBM in IR64**			**TBM in UPL Ri7**			**TBM in APO**		
	Puddle	**100% FC**	**60% FC**	**Puddle**	**100% FC**	**60% FC**	**Puddle**	**100% FC**	**60% FC**
GSI	35.38	29.43**	20.47**	31.25	25.18**	20.05**	32.87	24.63**	19.95**
GSII	39.52	37.43	34.97*	33.19	32.45	30.63	36.68	35.94	34.85
GSIII	34.87	32.89	28.59*	33.83	32.28	29.72*	36.06	35.58	32.6

Table 2. Yield (g/plant) for the three contrasting genotypes at different growth stages and stress treatments. GSI, Vegetative stage; GSII, Flowering stage; GSIII, Grain filling stage; FC, Field capacity. * and ** indicate significant differences from puddle at the 0.05 and 0.01 levels within genotypes, respectively. Values in the parentheses are the reduced percent compared to the puddle condition.

Stage	**IR64**			**UPL Ri7**			**APO**		
	Puddle	**100% FC**	**60% FC**	**Puddle**	**100% FC**	**60% FC**	**Puddle**	**100% FC**	**60% FC**
GSI	29.24	24.54(16.1)*	18.97(35.1)**	20.36	16.76(17.7)*	13.90(31.7)**	28.59	26.86(6.0)	23.94(16.2)*
GSII	27.2	19.24(29.2)**	13.4(50.7)**	19.06	15.18(20.3)**	10.27(46.1)**	26.56	21.95(17.4)*	16.91(36.3)**
GSIII	27.9	22.91(16.6)*	16.03(41.6)**	16.8	14.2(15.4)*	11.36(32.4)**	26.72	25.42(4.8)	24.10(9.8)*

Source to sink imbalance was the major contributor for yield loss at any given stage

At all the three growth stages, significant reduction in photosynthesis under 100% FC and 60% FC over puddle was noticed. Reductions in *A* and yield were significantly related in all the three stages (Fig. 3A). However, a strong linear relationship was shown between reduction in total leaf area and yield only at GSI stage (Fig. 3B). We computed an index as an indication of relative whole-plant photosynthesis by multiplying total leaf area with single leaf photosynthetic rate (the term 'relative' is used because the estimations are not absolute values which would require considering the vertical decrease of *A* with lowering leaf positions). Reduction in this index was strongly related with reduction in yield in all the three phenological stages (Fig. 3C). Reproductive growth (sink) was examined along with photosynthetic rate (source activity) to explain yield loss at all the three stages. Reduction in *A* and spikelet fertility were significantly correlated at GSII and GSIII (Fig. 3D).

Discussion

Crop improvement strategies are aiming at identification of specific traits with precise phenotyping methods (Vijayaraghavareddy et al., 2017; Sheshshayee et al., 2018). But the major limitation is that response of plants is widely different at specific phenological stage. Further, these physiological responses could potentially vary between genotypes with known history of adaptation to specific agro-ecosystem.

Stress at any phenological stage reduces plant productivity

During all the phenological stages, reduced water availability significantly affected plant performance and productivity. Stress at any phenological stage reduced yields by 15.9% (100% FC) to 33.1% (60%FC), compared to puddle conditions (Table 2). For rice being semi-aquatic, even a mild water limitation reduces yield significantly (Yang et al., 2019). IR64 had the greater reduction in yield under 100% FC in all the three phenological stage because of its adaptability to lowland condition, whereas Apo, a genotype adapted to aerobic condition, showed lower reduction under 100% FC over puddle condition in all the three phenological stages. Though this trend was consistent under 60% FC, reduction in yield at GSII was significantly higher even for aerobic genotype Apo. Therefore, among the growth stages, GSII was most sensitive even for mild water limitation (Yang et al., 2019) with an overall yield

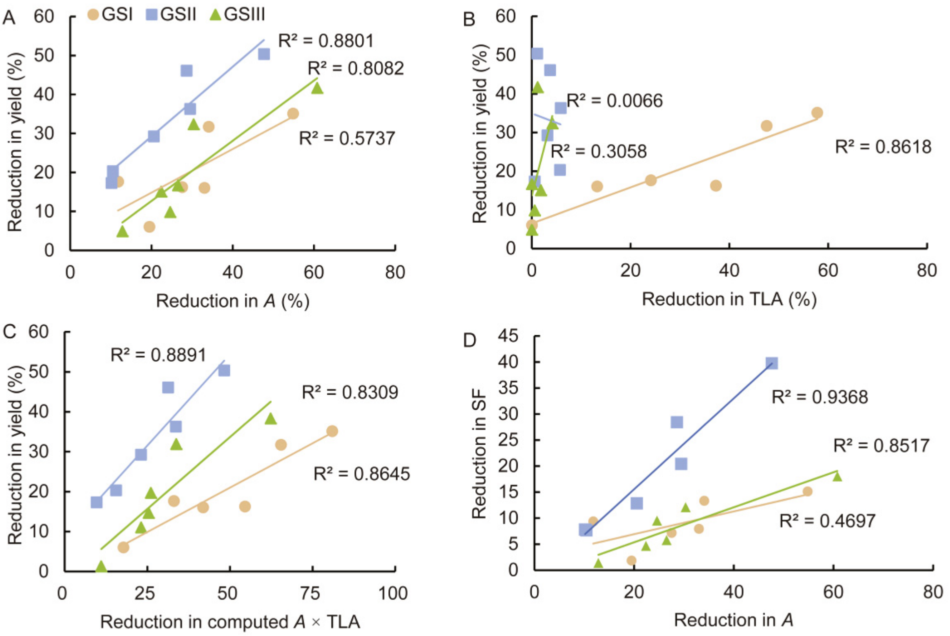

Figure 3. Linear regression among reductions in several traits. A, Linear regression of reductions in assimilation rate (*A*) and yield. B, Linear regression of reductions in total leaf area (TLA) and yield. C, Linear regression of reductions in computed *A* and yield. D, Linear regression of reductions in assimilation rate (*A*) and spikelet fertility (SF). GSI, Vegetative stage; GSII, Flowering stage; GSIII, Grain filling stage. Reduction in yield over puddle was calculated for the means under 100% and 60% field capacity. ** indicates significance at the 0.01 level.

loss of 22.3% and 44.4% under 100% FC and 60% FC, respectively.

Increasing root length assists to maintain tissue turgor

Maintenance of water relations under low soil moisture condition is an important drought adaptive strategy. In aerobic and water limited conditions, root traits are important for water uptake from deeper layers of soils (Raju et al., 2014). Increase in root length was observed at GSI (Fig. 2D). But the influence of water limitation has no effect on root length at GSII and GSIII stages (Fig. 2E and F). The maximum root growth was observed at GSII and GSIII. It is most unlikely that a period of 15 days stress may have a significant influence on physical root length. However, GSI, which represents a period of active growth, showed a maximum increase in root length under water-limited conditions (Fig. 2D). Root growth is known to be stimulated by soil moisture deficit and significant genetic variability has also been demonstrated for this response (Kadam et al., 2015), and this will be more pronounced if carbon assimilatory capacity and available carbon resources for remobilisation are not significant. Genotypes with better root system can maintain higher RWC due to efficient water mining ability (Nguyen et al., 1997; Raju et al., 2014). This could be an adaptive mechanism in upland genotype UPLRi7 and aerobic genotype Apo, hence showing significant increase in root length at GSI stage (Fig. 2D).

Reduced spikelet fertility is the determining factor for reduced yield in all phenological stages

In GSI, the most prominent influence of stress was reduced leaf expansion and gas exchange. Decreases in canopy cover when plants experience stress during vegetative stage are well documented and have been attributed to reduced cell expansion and cell division (Boonjung and Fukai, 1996; Sarvestani et al., 2008; Praba et al., 2009). Reduction in turgor has a direct effect on carbon assimilation rates (Turk et al., 1980; Praba et al., 2009). The combined effect of reduced leaf area, tiller number and photosynthetic rate significantly impedes total carbon assimilatory capacity (source), which may lead to decreased spikelet fertility. Spikelet fertility was significantly lower at GSII stage than at the other stages (Fig. 2B). Spikelet fertility at GSII stage is governed by a number of processes ranging from anther development, anther dehiscence, pollen viability, stigmatic receptivity, pollen efficiency, fertilization and ovule abortion, and all of these processes are extremely sensitive to stress (Shi et al., 2018). Besides, the spikelet water relations could further exacerbate the stress effect (O'Toole et al., 1984; Selote and Khanna-Chopra, 2004). Although a significant reduction in spikelet fertility was also noticed at GSIII (Fig. 2C), factors like lack of availability of photosynthates for grain filling and negative source-sink interactions were the major contributors to reduce spikelet fertility at GSIII. Hence, yield losses arise from reduced source size resulting from a decrease in amount of photosynthates for grain filling, reduced number of spikelets and/or from reduction in efficiency of grain filling (Roitsch, 1999; Madani et al., 2010).

Reduction in source availability will influence yield

Influence of source on growth and productivity can be viewed from two perspectives:

reduction in canopy cover (Cakir, 2004) and reduction in photosynthetic rate per unit leaf area (Serraj et al., 1999). In other words, source influences growth and productivity because of a combination of these two factors collectively responsible for whole-plant photosynthesis. Yield reduction when stress coincides with grain filling (GSIII) could be resulted from lack of carbohydrates for grain filling and/or due to reduced synthesis of current photosynthates and their translocation (Madani et al., 2010). The reduction in leaf area during GSII and GSIII showed no or any association with reduction in yield (Fig. 3B). However, reduction in photosynthetic rate was strongly associated with reduction in yield (Fig. 3A). Further, the reduction in the relative whole-plant photosynthesis showed a much stronger reduction in yield in all the stages. A change in source-sink interaction has been suggested as one of the major factors responsible for yield reduction under stress (Roitsch, 1999). Stress during the later phenological stages is well known to induce leaf senescence which decreases green leaf area and thus reduces canopy photosynthesis (Jagadish et al., 2015). However, we noticed no considerable reduction in green leaf area due to stress during GSII and GSIII stages (Table 1), which is against the normally noticed trend. There could be two possible reasons. Firstly, stress was provided for a period of 15 days, which may not be severe enough to cause changes in leaf area. Secondly, total leaf area was computed by multiplying leaf dry weight with specific leaf area. As we had only taken the green and functional leaves for recording leaf weight, this method can cause little error in determining functional leaf area.

Among the growth stages, moisture stress at GSII significantly affects the yield irrespective of adaptability of a genotype. Reductions in biomass, spikelet fertility and carbon gain seem to be major determining factors of yield under stress at GSI, GSII and GSIII stages, respectively. Reduction in yield was significantly high for lowland cultivar IR64 even at a 100% FC, which is a mild water limitation compared with puddle condition. Although the aerobic genotype Apo maintained much higher yield in water limited conditions (100% FC and 60% FC), there is a need of further improvement to reduce yield loss especially when stress induced at GSII stage. Hence, a genotype which maintained source to sink balance like Apo can be used for further crop improvement programmes. This information would therefore be most useful in improving the performance of rice crops which differ in their adaptability.

Acknowledgements

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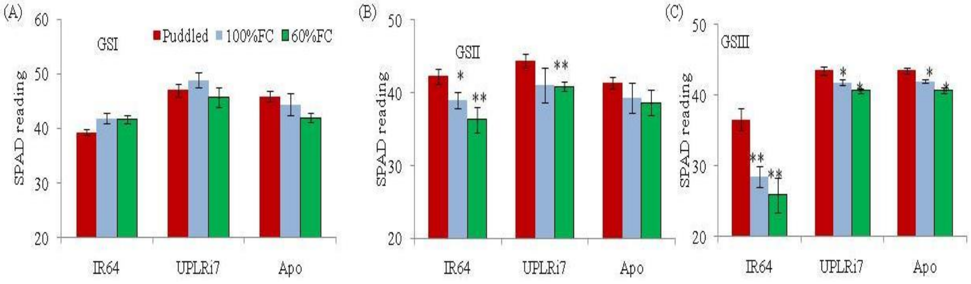
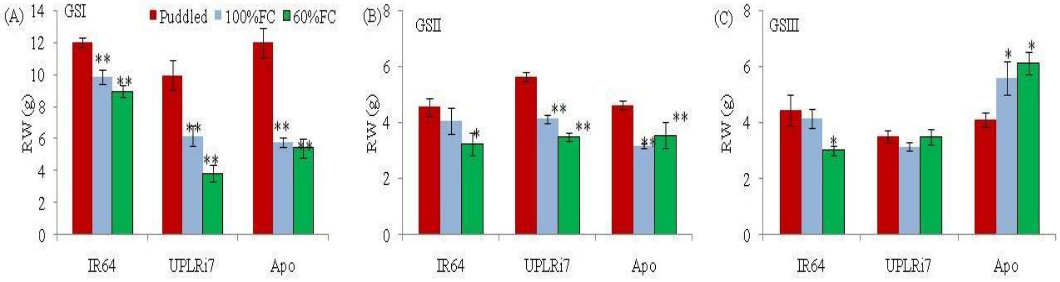
Supplementary information in Chapter 2



Supplementary Figure S1: Effect of water limitation on SPAD value at GSI, GSII and GSIII in rice genotypes. Water limitation effect on SPAD value in lowland IR64, upland UPLRi7 and aerobic Apo genotypes due to stress at (a) GSI, (b) GSII and (c) GSIII stage. Error bars indicates the standard error of mean. *Significantly different from puddle at p≤0.05 and **p≤0.01 level within genotype.



Supplementary Figure S2: Effect water limitation on root weight (RW) at GSI, GSII and GSIII in rice genotypes. Water limitation impact on root weight in lowland IR64, upland UPLRi7 and aerobic Apo genotypes due to stress at (a) GSI, (b) GSII and (c) GSIII stage. Error bars indicates the standard error of mean. *Significantly different from puddle at p≤0.05 and **p≤0.01 level within genotype.

Supplementary Table S1: Effect of water limitation on total plant height, number of tillers and stem weight. Reduction in on total plant height, number of tillers and stem weight was calculated for low land IR64, upland UPLRi7 and aerobic Apo at GSI, GSII and GSIII growth stages over puddle conditions

	% reduction over puddle condition																	
	Plant height (cm)						**Number of tillers**						**Stem weight (g/plant)**					
	IR64		**UPL Ri7**		**Apo**		**IR64**		**UPL Ri7**		**Apo**		**IR64**		**UPL Ri7**		**Apo**	
Stage	**100 % FC**	**60 % FC**	**100 % FC**	**60 % FC**	**100 % FC**	**60 % FC**	**100 % FC**	**60 % FC**	**100 % FC**	**60 % FC**	**100 % FC**	**60 % FC**	**100 % FC**	**60 % FC**	**100 % FC**	**60 % FC**	**100 % FC**	**60 % FC**
GSI	15.9	35.0	10.1	25.4	22.1	29.1	15.5	29.3	12.0	25.3	15.4	12.6	13.8	43.2	10.1	7.8	16.5	32.4
GSII	1.1	4.3	4.7	8.4	7.4	5.2	2.9	2.9	3.8	2.5	1.4	2.9	4.7	4.2	6.8	13.2	-0.8	1.2
GSIII	5.4	6.2	6.0	9.0	1.3	2.2	2.9	5.5	-1.2	5.0	2.9	1.4	15.8	19.4	7.5	13.9	6.8	11.2

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CHAPTER 3

Metabolome profiling reveals impact of water limitation on grain filling in contrasting rice genotypes

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Abstract

Drought significantly decreases crop productivity, especially in high water consuming crops like rice. Grain filling is one of the important critical growth phases in rice and drought during this phase leads to significant reduction in yield. In this study, a comparison was mad between IR64 (drought susceptible) and Apo (drought tolerant) rice genotypes to capture the response to water limitation (50% field capacity (FC)) compared with the control (100% FC) during grain filling. Plants were grown in a high-throughput phenomics facility for précis imposition of moisture stress during grain filling. Apo performed better in water limited conditions with lower reduction of photosynthetic rate and maintenance of lower leaf temperature than IR64. Days from sowing to maturity, spikelet fertility and seed weight were more impeded by water limitation in IR64 than in Apo. Unlike Apo, IR64 did not show any decrease in transpiration rate at 50% FC compared with 100% FC. Metabolomic profiling of spikelets at grain filling showed distinct effects of water limitation on accumulation of metabolites, especially in Apo. Secondary metabolism, mainly the phenylpropanoid pathway involved in scavenging mechanisms, was upregulated in Apo. Accumulation of most amino acids was significantly higher in IR64 than in Apo. Due to higher rates of photosynthesis under stress, most carbohydrates accumulated more in Apo than in IR64 at 50% FC. Sucrose transporters were significantly upregulated in water limited conditions especially in Apo. Overall, thanks to higher source capacity, more source to sink transport and better scavenging, Apo showed a lower reduction in yield than IR64.

Keywords: spikelet metabolomics; water limitation; sucrose transport; phenomics; Source-to-sink ratio

Introduction

The reproductive phase, spanning the duration from pollination and fertilization until the completion of grain filling, is an important growth phase in cereals directly determining productivity. Even a mild stress during the reproductive phase can cause a stronger yield reduction than stress during other growth phases (Vijayaraghavareddy et al., 2020a). In a semi-aquatic plant like rice, drought during the reproductive phase significantly reduces yield (Pantuwan et al., 2002; Kadam et al., 2014, 2018). Exposure of plants to drought during flowering and during early grain filling can lead to reduced spikelet fertility and also reduced grain size and quality (Lyman et al., 2013). Pollen development and its viability are affected by drought at flowering, caused by decreased carbohydrate metabolism, sugar transport and starch accumulation (Saini et al., 1984; Dorion et al., 1996). Increased spikelet temperature associated with reduced soil water status under drought stress has also been shown to affect pollen viability and pollen germination (Jagadish et al., 2010; Prasad et al., 2017; Sharma et al., 2018).

Apart from flowering, the initial phase of grain filling is also crucial in determining grain size and quality. Drought stress during grain filling impairs leaf gas exchange, phloem loading and assimilates translocation (Farooq et al., 2009). Effect of drought on photosynthesis due to reduced leaf turgor leading to stomatal closure significantly reduces source strength. Moreover, reduced turgor in phloem cells inhibits phloem loading and translocation of assimilates towards the sink (Sevanto, 2014). Stress during grain filling causes early senescence, enhances assimilate remobilization and shortens the grain filling period (Farooq et al., 2009). It has also been shown that drought significantly affects grain composition by altering the nitrogen pool, free amino acid levels, C/N ratio and mineral accumulation (Zhang et al., 2014; Ghanbari et al., 2013). These effects on chemical composition of the grains are associated with many transporters and enzymes. Many sucrose transporters (SUTs) are known to play a crucial role in export of sucrose from source to sink (Xu et al., 2018). Stress significantly affects the levels of these transporters depending on species (and genotype within species), stress type, stress level and duration, and growth phase during stress occurrence (Miyazaki et al., 2013; Phan et al., 2013; Xue et al., 2016). Enzymes like starch synthase and starch branching enzymes are also affected by stress (Hurkman et al., 2003).

To understand drought stress responses and to identify drought adaptive mechanisms, it is important to phenotype specific physiological traits such as leaf turgor, water use and water use efficiency. Similarly, to assess the stress response at cellular level, it is crucial to have a precise imposition and maintenance of a specific level of stress (White et al., 2012; Vijayaraghavareddy et al., 2020b). The advent of omics and phenomics approaches provided a means to gain a complete view of plant responses to stress at cellular, tissue or organism level (Vijayaraghavareddy et al., 2020b).

As drought response spans diverse mechanisms, combining omics approaches has great relevance in understanding plant response to stress at molecular levels. Among several omics approaches, metabolomics has shown a diverse utility in understanding plant responses to stress. This approach monitors the end products of the stress response and therefore reflects the direct physiological status of the plant (Casartelli et al., 2019). Findings from metabolomics studies can be used to design strategies for enhanced stress tolerance.

While several studies have reported the mechanistic differences between contrasting genotypes in their response to water limitation, we hypothesize that a metabolomic approach would lead to a more functional characterization of the stress response. Hence, we adopted a metabolomics approach to unravel the response of two contrasting rice genotypes (IR64 and Apo) to water limitation imposed during the reproductive growth phase. The genotypes were grown under precisely maintained water limited conditions using the plant phenomics facility as described by Vijayaraghavareddy et al. (2020b). The differential regulation of secondary metabolism under water limiting conditions would provide subtle protection to cellular functions translating to an overall improvement in drought adaptation at canopy level.

Materials and methods

Plant material and experiment

Two rice (*Oryza sativa* L.) genotypes contrasting in tolerance to water limitation, IR64 (susceptible) and Apo (tolerant), were selected (Vijayaraghavareddy et al., 2020a). As the genotypes differed in the time taken to reach flowering, sowing of Apo was staggered over a period of 3 and 6 days to obtain synchrony of flowering with IR64 so as to impose stress precisely at the same time and at the same phenological phase (early grain filling) of the two genotypes, thereby avoiding the influence of microclimatic differences. To impose water limitation, the plant phenomics facility established at the University of Agricultural Sciences, Bengaluru, India, was used. Plastic containers of 24-L capacity were filled with red soil and farmyard manure in a 3:1 ratio (w/w). The containers were placed on special load cells which determined the weights of each container in real-time. An automated, software driven irrigation system comprising of solenoid valves was used for dispensing precise volumes of water to replenish water lost due to evapo-transpiration. This facility allowed the precise maintenance of a specific soil moisture regime (Vijayaraghavareddy et al., 2020b). This system kept the soil at 100% FC (field capacity). To arrive at field capacity, a known weight of fresh soil sample (200 g) in three replicates was taken and dried at 80°C for three days. After taking the weight the dried soil samples were taken in separate cotton-cloth bag and submerged into water for saturation and hanged from a stand overnight, to drain off excess water. Using the fresh weight, dry weight and saturation weights of soil, the field capacity of soil was calculated as follows

$$\%FC = \frac{Saturated\ soil\ wt\ (g) - Dry\ soil\ wt(g)}{Dry\ soil\ wt\ (g)} \times 100$$

At flowering, a gradually progressing stress regime was initiated by the “dry-down” protocol using the software which ensured a user defined ramping of soil moisture status ensuring required reduction in %FC per day (Vijayaraghavareddy et al., 2020b). Stress imposition was initiated when each of the genotypes were at 50% flowering (85 days after sowing (DAS) for IR64 and 91 DAS for Apo). Soil moisture status was reduced by 5% per day irrespective of the differences in leaf area and/or transpiration rate. For the stress treatment, 50% soil moisture status was reached on the 10th day after stress imposition and this was maintained for 10 days thereafter. For recovery, 50% FC was elevated to 100% FC by immediately adding water required to reach 100% FC through automated irrigation and maintained at 100% FC till harvest. For the control treatment 100% FC was maintained throughout as describe previously. Five replicates were maintained for each genotype in each treatment and pots were arranged in a complete randomised design. Spikelet samples for metabolomics were collected at milky stage (100 DAS for IR64, 106 DAS for Apo). As our major intent of the investigation was to examine the changes in metabolites and physiological responses to stress at milky stage, all sampling and physiological observations, described below, were made five days after soil moisture attained 50% FC. Beyond this stage the spikelets would have passed the milky stage.

Relative water content

The fresh weight of leaves (FW) was measured and leaves were then immersed in water overnight at room temperature. The water saturated leaves were blot dried with filter paper and weighed to determine turgid weight (TW). Dry weight (DW) was determined by drying these leaves in an oven at 80°C for two days. The relative water content (RWC, %) was calculated using the formula (Jones and Turner, 1978):

$$RWC = \frac{FW - DW}{TW \ - DW} \times 100$$

Leaf temperature, spikelet temperature and gas exchange measurements

The leaf temperature and spikelet temperature of plants were measured using the Fluke thermal imaging system (Fluke Technologies Pvt. Ltd., Everette, Washington, USA). Measurements were made during bright sunshine hours between 09:00 to 11:00 h. Fluke smart view IR analysis software was used to analyse the images taken from the same distance. To avoid noise by variation in background temperature, leaves and spikelets were marked using software and mean temperature was exported to Microsoft Excel for further analysis. Net photosynthetic rate and stomatal conductance were measured in the flag leaf and second leaf (top canopy), fourth leaf (middle canopy) and fifth leaf (lower canopy), where leaf numbers were counted from the top. Gas exchange measurements were recorded using a LI-6400XT Portable Photosynthesis System (LI-COR Inc., Lincoln, NE, USA). Photosynthetic photon flux density (PPFD) was maintained at 1500 µmol m^{-2} s^{-1}, a fixed block temperature of 25°C and a chamber CO_2 concentration of 400 µmol mol^{-1}. The CO_2 dosing unit of LI-6400XT was used to fix the reference CO_2 concentration at 400 µmol mol^{-1}. SPAD chlorophyll meter readings of these leaves were also recorded, with six readings per leaf using a portable, non-destructive chlorophyll meter (SPAD-502, Minolta, Japan).

Yield parameters

Days from sowing to maturity was recorded. Plants were harvested at maturity and dissected into root, stem, leaf and grain. Panicle structures were discarded. To arrive at total biomass, leaf, root and stem were oven dried at 65°C for three days and dry weight was assessed. Grains from panicles were threshed and numbers of filled and chaffy (unfilled and half filled) grains were counted to calculate spikelet fertility. Number of spikelets per plant was also counted. All filled grains were weighed to arrive at grain yield per plant. One hundred filled grains were separated and weighed to determine the test weight.

Transpiration and derived parameters from phenomics platform

The phenomics platform is an evapo-transpiration (ET) interfaced automated irrigation system which works based on a gravimetric approach. It measures ET in real-time throughout the growing period of the crop (Vijayaraghavareddy et al., 2020b). The soil surface of all pots was covered with equal quantities of plastic beads to minimize direct soil evaporation. A set of pots without plants were maintained to assess direct soil evaporation. This evaporation measured from pots without plants was subtracted from ET from pots with plants to determine transpired water. The transpired water was summed over the entire experimental period to arrive at cumulative water transpired (CWT). Mean transpiration rate (MTR) per leaf area was calculated for the entire crop period from CWT and leaf area measured at harvest. Water use efficiency was calculated using total dry matter at harvest and CWT. Transpiration rate during stress period was calculated from day of initiation of stress to end of stress period.

RT-PCR analysis for sucrose transport genes

Tissues from second leaf and flag leaf of 100% and 50% FC plants in three replicates were used to extract total RNA using the phenol–chloroform method according to Datta et al. (1989), and cDNA was synthesized by oligo (dT) primers using Moloney murine leukaemia virus reverse transcriptase (MMLV-RT; MBI Fermentas, Hanover, MD). The cDNA pool was used as a template to perform RT-PCR analysis according to the manufacturer's protocol (Opticon 2; MJ research, USA & MJ Bioworks, Inc.). The reaction mixture for the quantitative real-time RT-PCR was prepared following the protocol of SYBR green PCR master mix (TAKARA SYBR Green qPCR Kit). Comparative threshold method was used to arrive at relative expression levels of selected genes. The PCR conditions were 94°C for 3 min, 25 cycles of 94°C for 30 s, 52–58°C for 30 s, 72°C for 40 s and a final extension of 72°C for 5 min. Ubiquitin was used as internal control for normalization.

Non-targeted metabolomic analysis of rice genotypes

Spikelets (at early grain filling phase) from both 100% FC and 50% FC were collected (between 10:00 to 11:00 h) and immediately frozen in liquid nitrogen. Tissues were stored at -80 ◦C, until extraction. Extraction of metabolites was done using the protocol described by Pushpa (2014). Frozen samples were ground using a pre-cooled pestle and mortar. Finely ground tissue (25 mg) was transferred to a microcentrifuge tube and 50% ice cold methanol (400 µL) was added. To the aliquot, internal standard reserpine was added for abundance correction. The mixture was stirred and sonicated at 40 kHz in a water bath for 15 min at room temperature. The mixture was centrifuged at 13,000 rpm for 10 min at room temperature. The obtained supernatant was passed through a 0.22 mm poly (vinylidene difluoride) membrane filter. The filtrate was collected in glass vials and stored at -20°C.

Non-targeted metabolomics analysis was conducted using a Dionex UltiMate 3000 ultraperformance liquid chromatography (UPLC) system coupled with a heated electrospray ionization (HESI) probe with a Q Exactive Orbitrap mass spectrometer (Thermo Scientifics, USA). Metabolites were separated on a Waters Acquity UPLC ethylene bridged hybrid (BEH) C18 column (1.0 × 100 mm, particle size 1.7 µm) kept at 40°C. Mobile phase A consisted of 0.1% formic acid in water and B was 0.1% acetonitrile at a flow rate of 0.1 mL/min using gradient mode. Total run time was 30 min. Extract (3 µL) was injected for the analysis. The data were acquired by MS/MS through data-dependent acquisition (DDA). Profiling experiments were performed in Electro Spray Ionization (ESI)-positive and negative ionization using the polarity-switching mode as described by Gunnaiah and Kushalappa, (2014). Thermo Scientific, Xcalibur RAW files were converted to mzXML files using an MS converter. Raw data was processed with MZmine2 software (Pluskal et al., 2010). Prior to peak detection, baseline for all the peaks were corrected in relation to the total ion chromatograms. Chromatograms (>0.2 min) were built from the peaks with >10000 intensity. Peaks were deconvoluted from the chromatograms with the centWave algorithm, aligned across the samples. Only peaks consistent in two replicates were considered for the analysis. The analysed data was exported to MS-Excel for further statistical analysis.

Statistical analysis

Two-way ANOVA with interaction was conducted by using GenStat (15th edition) (http://www.genstat.co.uk/). Least significant difference (LSD) was used to check the significant difference between genotypes and treatments. For metabolomics analysis, the data on relative intensity of peaks (m/z = mass/charge ratio) were subjected to Student's t-test to check the significance of the difference between genotypes and between treatments and of the genotype × treatment interaction. Only peaks which showed significant difference were used for metabolite annotation using databases (Plantcyc and KEGG). The ANOVA table for detected metabolites is given in a Supplementary Table S1.

Results

Physiological response to water limitation of contrasting rice genotypes

RWC was significantly lower at 50% FC than at 100% FC in both genotypes but did not differ between genotypes at both water regimes (Fig. 1a). Although tissue water status was comparable between IR64 and Apo, the leaf and spikelet temperatures showed significant differences between genotypes at both 100% FC and 50% FC. Increase in leaf temperature at 50% FC over 100% FC was significantly higher in IR64 than in Apo (Fig. 1b). Spikelet temperature increase at 50% FC was also significantly higher in IR64 than in Apo (Fig. 1c). Photosynthetic rate per unit leaf area (A) and stomatal conductance (gs) decreased with a lower leaf position when measured on the same date in both genotypes. Further, these gas exchange parameters were also significantly lower under water-limited conditions than without stress. Apo maintained higher A and gs in all leaf positions compared with IR64 (Fig. 1d). The reduction in photosynthesis and stomatal conductance at 50% FC compared with the control was significantly higher in IR64 than in Apo in all measured leaves (Fig. 1d and e). Similarly, chlorophyll content was also significantly reduced in IR64 in all leaf positions compared with Apo in 50% FC (Supplementary Fig. S1).

Morpho-physiological parameters significantly reduced under water limited conditions

Since the stress imposition was initiated on 90 DAS when the canopy cover had already reached its maximum value, no significant reduction in leaf area of both genotypes was observed at 50% FC compared with 100% FC (Fig. 2a). However, 50% FC significantly reduced total dry matter compared with 100% FC in both genotypes and the difference was markedly higher for IR64 than for Apo (Fig. 2b). Days necessary to maturity was significantly lower at 50% FC compared with 100% FC in IR64 but there was no difference between treatments in Apo (Fig. 2c). The total number of spikelets per plant was significantly higher for IR64 compared with Apo in both treatments with no effect on spikelet number of treatments in both genotypes (Fig. 2d). Apo maintained significantly higher spikelet fertility compared with IR64 and the percent reduction in spikelet fertility in Apo was significantly lower under 50% FC condition than in the control treatment (Fig. 2e). Weight of 100 grains was significantly lower for IR64 than for Apo whereas the 50% FC condition had a significantly lower 100-grain weight than 100% FC in IR64 only (Fig. 2f).

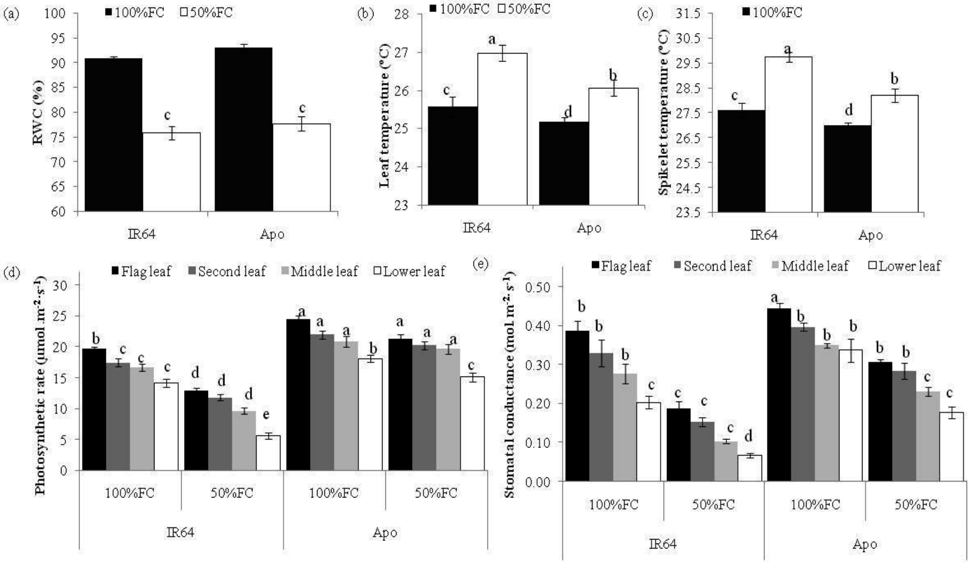


Figure 1. Response of two contrasting rice genotypes to water limitation stress: (a) relative water content (RWC), (b) leaf temperature, (c) spikelet temperature, (d) photosynthetic rate, and (e) stomatal conductance measured in flag leaf and 2nd leaf (top canopy), 4th leaf (middle canopy) and 5th leaf (lower canopy). Different letters indicate significance ($P < 0.05$) from the LSD post-hoc test determined using two-way ANOVA with genotypes × treatments interactions.

Accordingly, grain yield was significantly higher in Apo than in IR64 under 50% FC and this genotype also showed the smallest percent reduction in grain yield caused by water-limiting conditions (Fig. 2g).

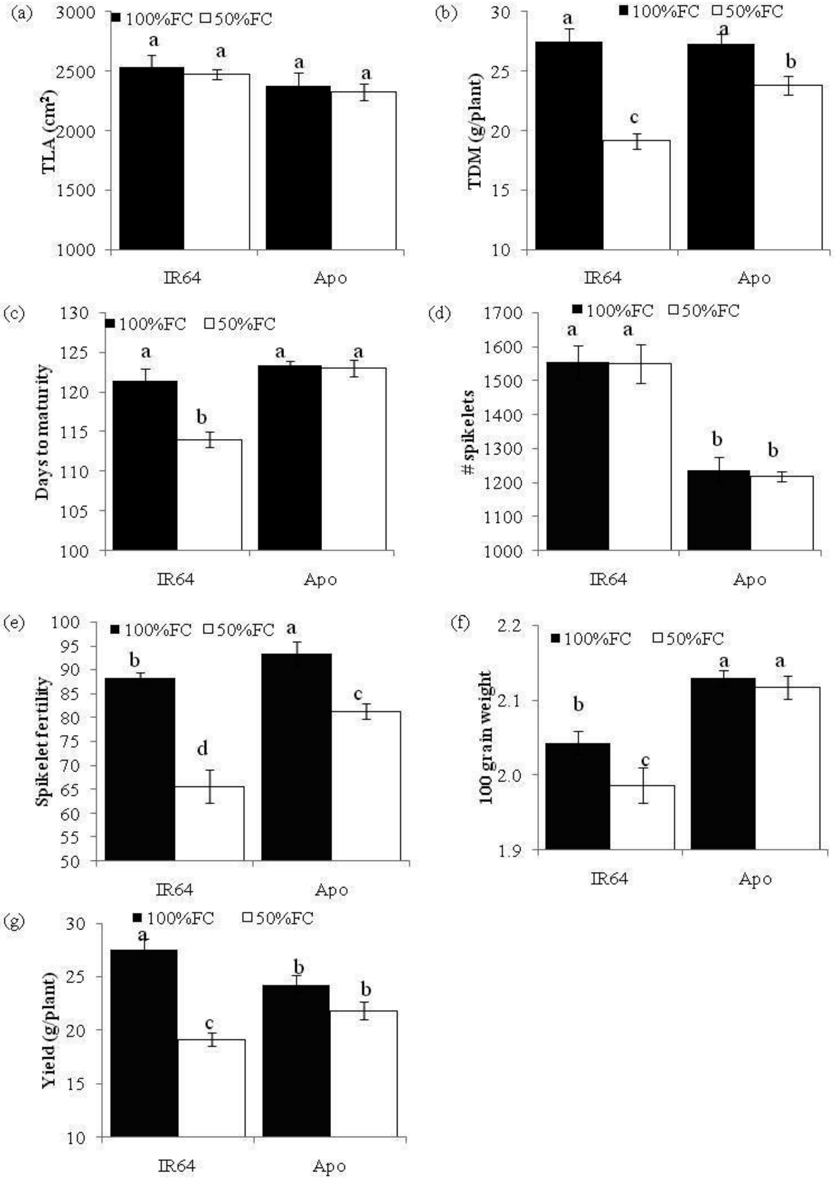


Figure 2. Morpho-physiological response of two contrasting rice genotypes to water limitation stress: (a) total leaf area (TLA), (b) total dry matter (TDM), (c) days to maturity, (d) number of spikelets, (e) spikelet fertility, (f) 100-grain weight, and (g) yield, in IR64 and Apo under 100% FC and 50% FC conditions. Different letters indicate significance ($P < 0.05$) from the LSD post-hoc test determined using two-way ANOVA with genotypes × treatments interactions.

Variation in transpiration

Water transpired during the stress period as well as during the entire crop cycle was measured at the phenomics platform. Total transpiration rate during the entire growth duration was significantly different between the two genotypes. The mean transpiration rate (MTR) of Apo was significantly higher in both 100% and 50% FC conditions compared with IR64. But a stress induced reduction in MTR was only noticed in Apo (Fig. 3a). Transpiration rate during the stress period was lower at 50% FC than at 100% FC in both genotypes but the difference was significant only for Apo (Fig. 3b and Fig. S2). But, in IR64, a marginal reduction in transpiration was noticed only during noon hours (Fig. S2). WUE of IR64 was lower at 50% FC than at 100% FC, while for Apo WUE was significantly higher at 50% FC than at 100% FC (Fig. 3c).

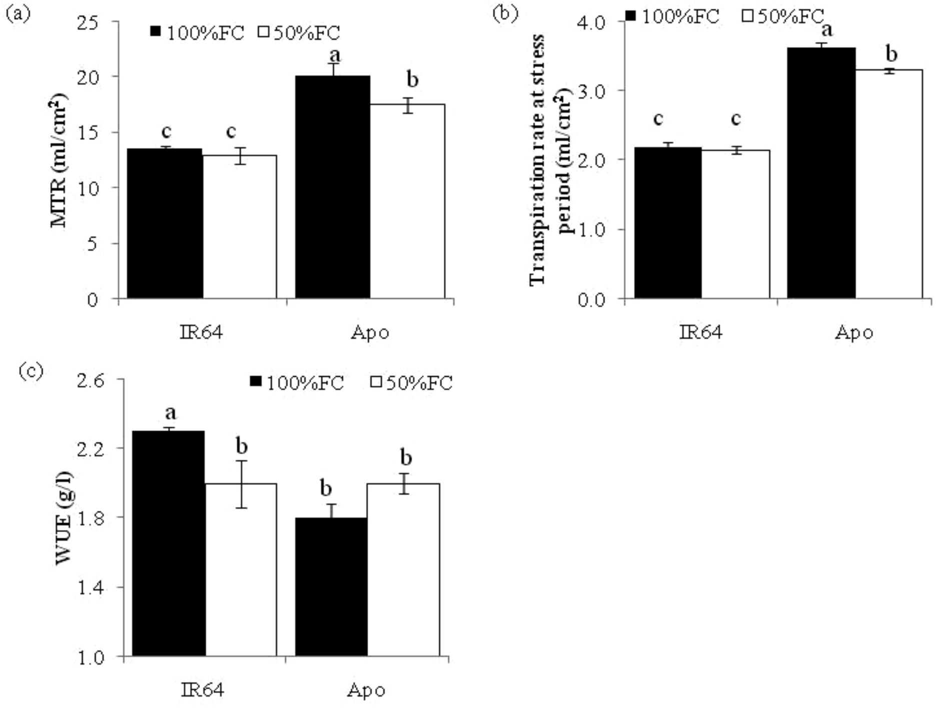


Figure 3. Variations in (a) mean transpiration rate (MTR) for entire crop period, (b) transpiration rates during stress period, and (c) whole-plant water use efficiency (WUE) of the two contrasting rice genotypes in response to different water limitations. Different letters indicate significance ($P < 0.05$) from the LSD post-hoc test determined using two-way ANOVA with genotypes × treatments interactions.

Metabolomics profile from spikelets of contrasting rice genotypes

From spikelet metabolome profiling, 63 metabolites were identified and annotated from the two contrasting rice genotypes grown at two water regimes (100% FC and 50% FC) (Supplementary Table S2). The metabolites were classified into four groups: secondary metabolites (28), amino acids (20), nucleic acids (7) and carbohydrates (8). The metabolite profiles of the two genotypes and also of the two stress treatments were analysed by a heat map. Large variation was observed in metabolite content between genotypes even at 100% FC. The heat map clearly demonstrated that the response to water limitation (50% FC) was significantly different between IR64 and Apo (Fig. 4). Secondary metabolites showed larger increase or decrease in content in both IR64 and Apo than the other groups of chemicals (Fig. 4). Principal component analysis also segregated IR64 and Apo with large variability (PC1, 49.2% and PC2, 28.9% of variance). Apo showed large differences in metabolomics content between 100% FC and 50% FC and hence segregated but this was not observed in IR64 (Fig. 5a).

Variability in metabolome content due to water limitation

To understand the stress response, changes in metabolome content in response to water limitation (50% FC) in comparison with the control (100% FC) were calculated and expressed as fold change for each group of chemical components. Most secondary metabolites were either up- or down-regulated under stress in both IR64 and Apo, whereas most amino acids were up-regulated in IR64 but down-regulated in Apo (Fig. 5b). The metabolites of nucleic acid metabolism revealed interesting trends. Two metabolites, allantoate and xanthosine in IR64 were significantly lower under stress, while only a marginal difference was noticed in Apo. For carbohydrate metabolism, Apo showed larger up- or downregulation of metabolome content than IR64 (Fig. 5b). In order to detect specific responses of these genotypes, metabolites which were down-regulated in one genotype and up-regulated in the other at 50% FC compared to 100% FC were selected from each group of metabolites (Supplementary Table S3). Among the secondary metabolites, 11 metabolites showed differential response in IR64 and Apo. Pelargonidin involved in anthocyanin biosynthesis was significantly up-regulated in IR64 and down-regulated in Apo. Coumaraldehyde, 4-coumaroylshikimate,

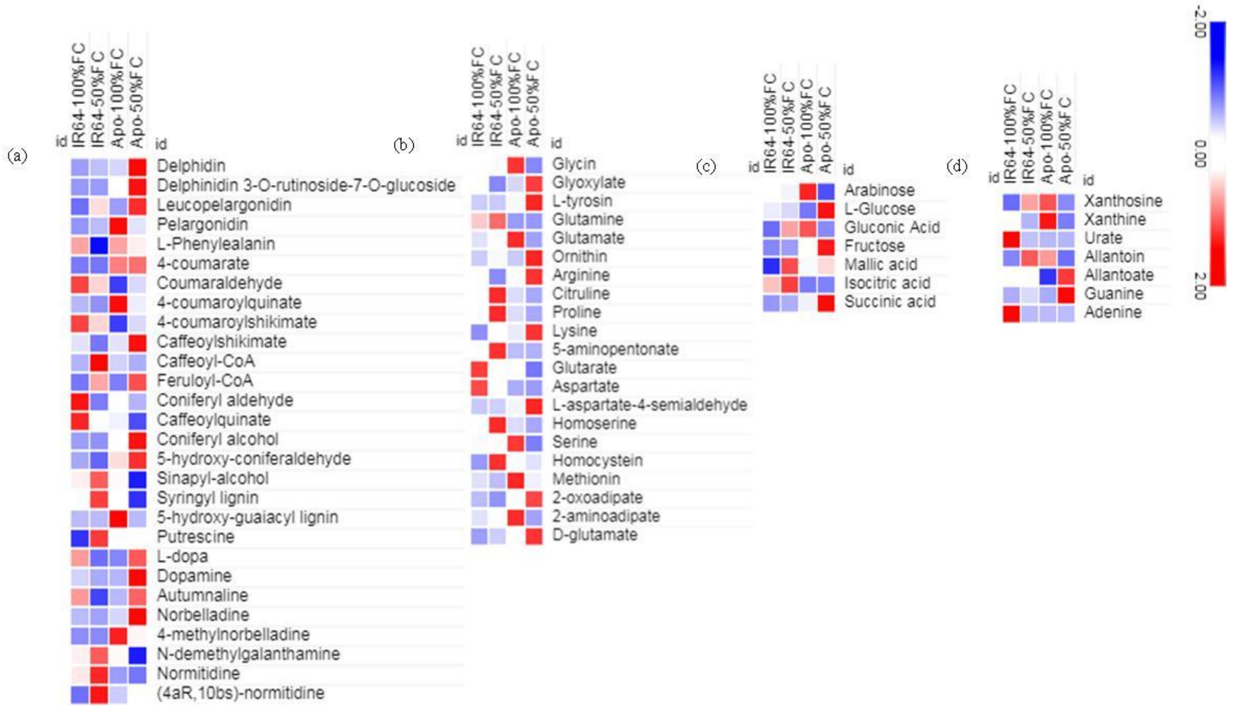


Figure 4. Heat map showing metabolite level in spikelets of two contrasting rice genotypes (IR64 and Apo). Log2 fold difference is indicated by color code of (a) secondary metabolites, (b) amino acids, (c) carbohydrate metabolism, and (d) nucleic acid metabolism. Two-way ANOVA for these metabolites are given in Supplementary Table S1.

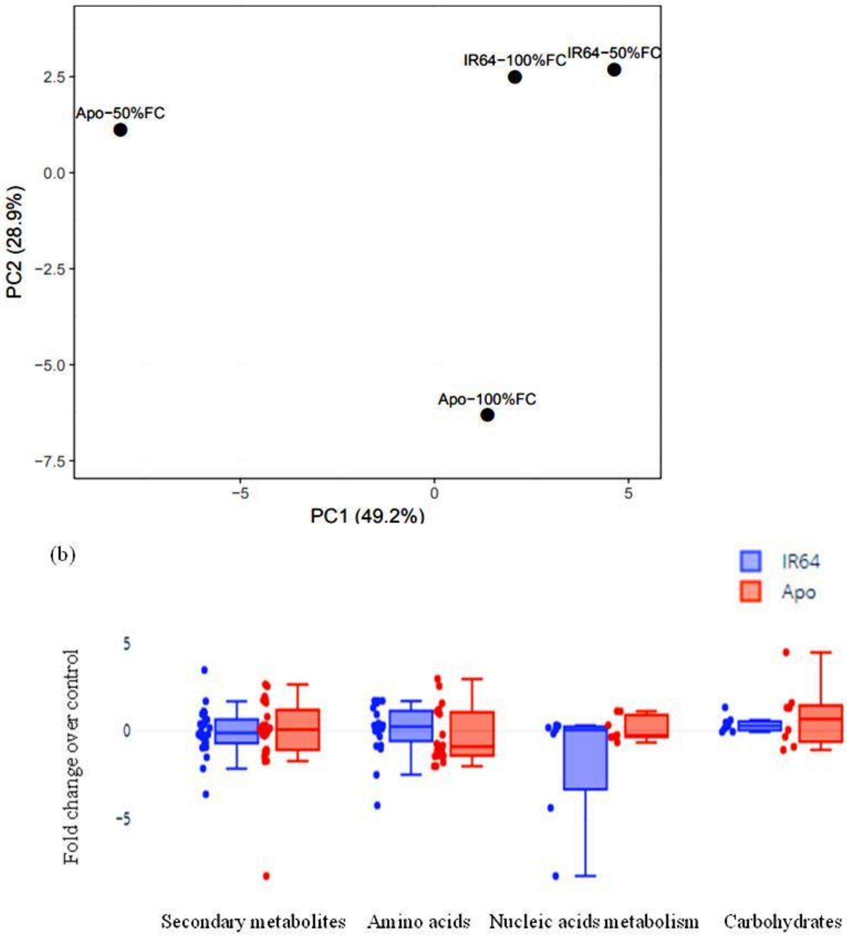
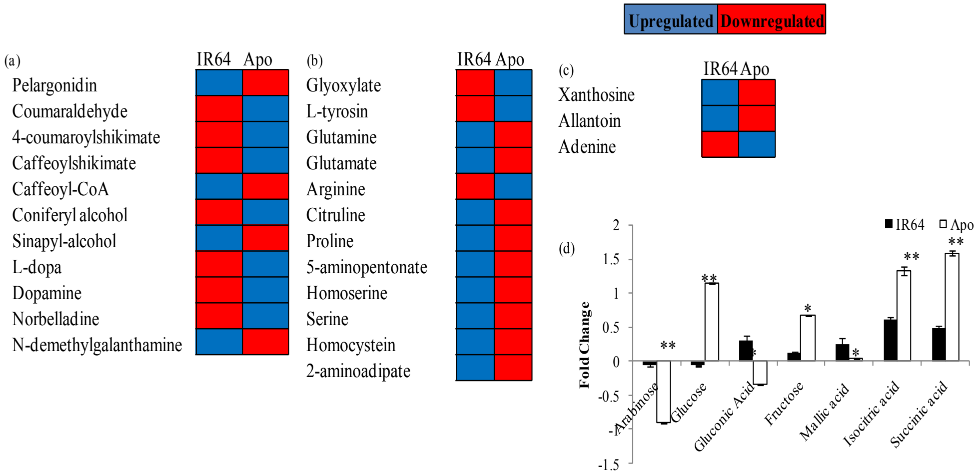


Figure 5. (a) Principal component analysis (PCA) for rice spikelet metabolome of two contrasting genotypes (IR64 and Apo) in two water regimes (100% FC and 50% FC), and (b) box plot showing up or down regulation of metabolites in 50% FC compared to 100% FC in IR64 and Apo.

caffeoylshikimate and coniferyl alcohol which are involved in phenylpropanoid biosynthesis were significantly up-regulated in Apo and down-regulated in IR64 (Fig. 6a). Caffeoyl-CoA and sinapyl-alcohol which are also involved in phenylpropanoid biosynthesis were downregulated in Apo and up-regulated in IR64 (Fig. 6a). Among the 12 amino acids, five metabolites, glyoxylate, L-tyrosin, arginine, xanthosine and allantoin were significantly down-regulated in IR64 and upregulated in Apo (Fig. 6b). Seven other metabolites of the amino acid metabolism showed a decrease in Apo and an increase in IR64 under stress (Fig. 6b). D-glutamate showed significant up-regulation in both the genotypes. From the nucleic acid metabolism, adenine was significantly upregulated in Apo but not in IR64. Xanthosine and allantoin were significantly up-regulated in IR64 and down-regulated in Apo (Fig. 6c).

Variability in carbohydrate content in spikelet in contrasting rice genotypes

Since the tolerant rice genotype Apo maintained significantly higher photosynthetic rate even at 50% FC (Fig. 1d), carbohydrate levels in spikelets were assessed. Glucose content was significantly higher at 50% than at 100% FC in Apo but slightly lower at 50% than at 100% FC in IR64 (Fig. 6d). Although fructose content was significantly higher at 50% than at 100% FC in both genotypes, the difference was significantly higher in Apo than in IR64.



🟦 Upregulated 🟥 Downregulated

(a)

	IR64	Apo
Pelargonidin	🟦	🟥
Coumaraldehyde	🟥	🟦
4-coumaroylshikimate	🟥	🟦
Caffeoylshikimate	🟥	🟦
Caffeoyl-CoA	🟦	🟥
Coniferyl alcohol	🟥	🟦
Sinapyl-alcohol	🟦	🟥
L-dopa	🟥	🟦
Dopamine	🟥	🟦
Norbelladine	🟥	🟦
N-demethylgalanthamine	🟦	🟥

(b)

	IR64	Apo
Glyoxylate	🟥	🟦
L-tyrosin	🟥	🟦
Glutamine	🟦	🟥
Glutamate	🟦	🟥
Arginine	🟥	🟦
Citruline	🟦	🟥
Proline	🟦	🟥
5-aminopentonate	🟦	🟥
Homoserine	🟦	🟥
Serine	🟦	🟥
Homocystein	🟦	🟥
2-aminoadipate	🟦	🟥

(c)

	IR64	Apo
Xanthosine	🟦	🟥
Allantoin	🟦	🟥
Adenine	🟥	🟦

Figure 6. Metabolites which are significantly up-regulated in one genotype and down-regulated in another genotype from (a) secondary metabolites, (b) amino acid metabolism, (c) nucleic acid metabolism (d) Fold change in identified carbohydrate content in 50% FC compared to 100% FC. * Significant difference in fold change between the genotypes (*P < 0.05, **P < 0.01, ns – nonsignificant).

Expression pattern of sucrose transporter genes

Levels of up- or down-regulation of transcript levels on some of the sucrose transport genes were analysed at 50% FC and fold change over 100% FC was calculated (Fig. 7). In Apo, all the sucrose transport genes were significantly upregulated at 50% FC in both flag leaf and second leaf. Among them *OsSUT1*, *OsSUT2* and *OsSUT3* showed significantly higher up-regulation in both flag leaf and second leaf than *OsSUT4* and *OsSUT5*. In IR64, only *OsSUT4* and *OsSUT5* showed significant upregulation in both leaves; *OsSUT1* showed marginal downregulation in flag leaf and up-regulation in second leaf, while *OsSUT3* showed significant downregulation in IR64 in both leaves.

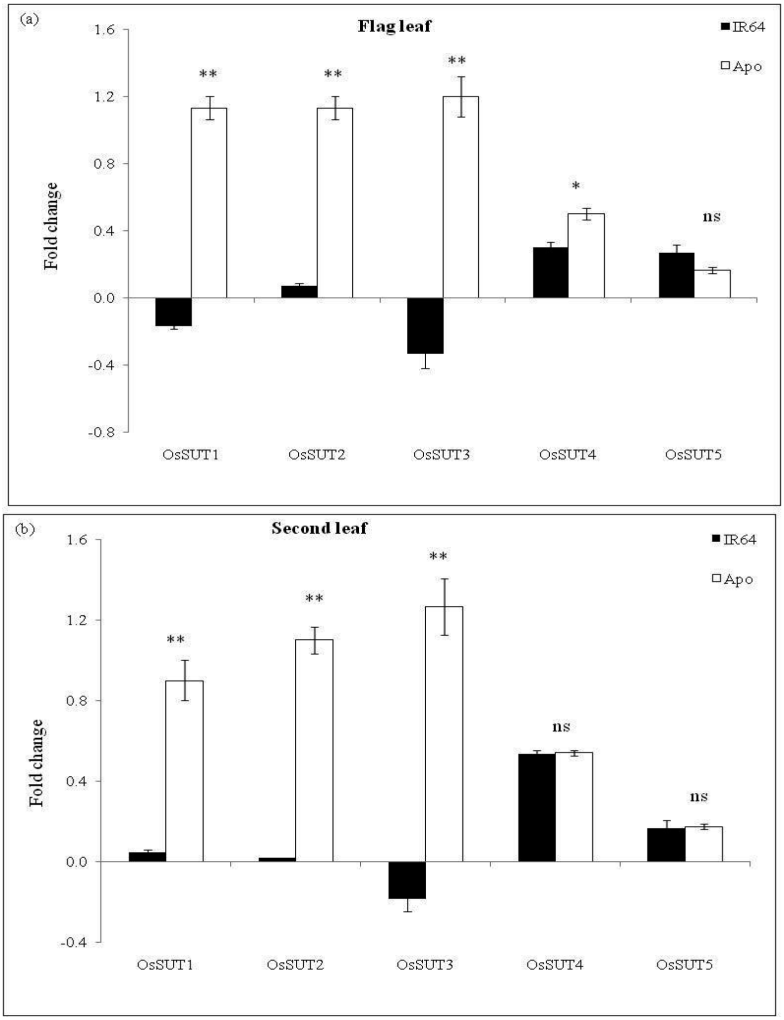


Figure 7. Fold change in sucrose transport genes in IR64 and Apo at 50% FC in (a) flag leaf, and (b) second leaf. * Significant difference in fold change between the genotypes (*P < 0.05, **P < 0.01, ns – nonsignificant).

Discussion

Water limitation during all growth phases (vegetative, flowering, and grain filling periods) of rice significantly reduces productivity (Vijayaraghavareddy et al., 2020a). Among all growth phases, the phenological phase coinciding with flowering and fertilization appears to be the most sensitive to stress. Besides the stress effect on reproductive development, plant photosynthesis and hence the source strength to support grain filling can also be an important constraint under water limitations. This could arise mainly due to reduction in current photosynthesis, phloem loading, and remobilization leading to source to sink imbalance (Sevanto, 2014; Fabre et al., 2020). The major lacuna, however, has been the imposition of a specific level of stress at a specific period exactly coinciding with anthesis and fertilization phase. A drought susceptible genotype (IR64) and a tolerant genotype (Apo) were grown in the high-throughput phenomics facility. Besides determining several parameters associated with tissue water relations and carbon assimilation, spikelets were also collected from both 100% FC and 50% FC to understand differences in stress response metabolome.

Maintenance of source strength during stress is crucial for better grain filling

Source strength is often considered as a trait closely linked to potential yield (Murchie et al., 2009; Zhu et al., 2010). The strength of the source is dependent on the proportion of newly formed highly photosynthesising leaves and the number of older leaves with lower photosynthesising ability (Suriyagoda et al., 2010). Hence any reduction in photosynthesis in the lower leaves, which arises both due to senescence and low light availability, would contribute to significant yield loss. Therefore, we measured photosynthetic rate and stomatal conductance in four leaves from different positions (Fig. 1d, e). The source capacity of the plant is also determined by the total leaf area. Leaf area growth generally is maximised at flowering in rice and hence no significant difference was noticed between treatments (Fig. 2a). But water limitation significantly decreased the leaf turgor in both genotypes, which would affect photosynthesis both due to stomatal closure and due to the stress effect on carbon metabolism directly (Evans and Rawson, 1970; Sheshshayee et al., 2018). Thus, reduction in source strength in IR64 during grain filing had a significant effect on productivity. The most appropriate comparison of stress response on source strength is possible only when the tissue turgor is maintained at a comparable level despite the differences in total transpiration between the genotypes. RWC of the two genotypes was comparable in both water regimes (Fig. 1a). Smaller effects of stress on gas exchange traits in Apo (Fig. 1 d, e) represented the maintenance of a much higher source strength compared to IR64.

Reduction in transpiration with higher carbon gain determines water use efficiency

Differences in water use and carbon assimilation (or biomass production) influences water-use efficiency (WUE) at single leaf level or at the whole plant level (Sheshshayee et al., 2018). Increasing WUE is often considered as a useful mechanism to sustain productivity under water-limited conditions (Richards, 2000). However, selection for higher WUE may not always result in higher growth rates or productivity depending on the controlling physiological traits (Sheshshayee et al., 2003, 2013). In the present experiment also, the drought adaptive genotype Apo, although had lower WUE, was associated with higher biomass accumulation and yield (Figs. 2 and 3c). For attaining higher water productivity, especially under limited water condition, it is important to minimize water loss without reduction in carbon gain. Although biomass was lower under stress than in the control treatment in both genotypes, the percent reduction was much smaller in Apo than in IR64, indicating a better maintenance of carbon assimilation (Fig. 2b). Plants close stomata in water-limited conditions (Peters et al., 2018). Despite a higher reduction in transpiration rate, Apo maintained higher photosynthetic rates compared with IR64 in 50% FC and hence maintained higher biomass (Figs. 1d and 3a). Therefore, WUE of Apo increased significantly at 50% FC compared with IR64 (Fig. 3c).

Metabolome profiling showed different pattern of response to water limitation

Besides the source strength, sink capacity is an equally important factor that helps maintain productivity under stress. Apo maintained higher spikelet fertility under stress and recorded higher total number of filled spikelets compared with IR64. A cooler spikelet temperature in Apo under stress (Fig. 1c) could be a cursory indicator of better spikelet metabolism resulting in higher grain filling. Spikelet metabolomes showed distinct variability in fold changes between genotypes and between treatments (Fig. 4). Principal component analysis completely segregated the two genotypes, reiterating the significant differences in metabolite profiles of the two genotypes under 100% and 50% FC (Fig. 5a). The positive or negative difference in metabolome content between 50% FC and 100% FC was significant in Apo but not in IR64 except for nucleic acid metabolism. This differential accumulation of metabolites could be associated with differences in adaptability of these genotypes to different ecosystems (Vijayaraghavareddy et al., 2020a). Amino acids serve as precursors for several metabolites which in turn are involved in stress protection mechanisms (Sircar and Parekh, 2019). In accordance with this result, secondary metabolites were significantly upregulated in Apo than IR64, indicating that amino acid pool might have been diverted for secondary metabolism (Fig. 5b). Among the secondary metabolism pathways, the phenolics groups comprise a large number of secondary metabolites having antioxidant properties. Hence, they are capable of scavenging free radicals produced because of the exposure to water limitation. Many studies have shown that accumulation of different phenolic compounds from phenylpropanoid pathway resulted in tolerance to water limitation by detoxifying harmful free radicals (Sharma et al., 2019). A tolerant genotype Apo, showed significant up-regulation of phenylpropanoid pathway in water limited conditions, suggesting that increased biosynthesis of phenolics helped maintain a more balanced metabolism in Apo (Fig. 5b).

Source availability and its transport determines metabolome flux in spikelets

The up- or down-regulation of metabolome content in spikelets is mainly regulated by source availability and its transport (Sevanto, 2014). Although Apo showed lower MTR in 50% FC, the decrease in morning transpiration was lower than during afternoon hours (Fig. S2). Despite a reduced water use, a higher photosynthetic activity even under stress indicated a better maintenance of metabolism in Apo. Furthermore, vapour pressure deficit-driven stomatal closure during the afternoon hours resulted in the higher WUE in Apo, especially under stress. On the other hand, although MTR was not lower in IR64 at 50% FC, higher chlorophyll degradation could result in reduced photosynthesis (Fig. S1). The higher source availability in Apo was evident from the higher fold change in metabolite content in spikelets. During stress conditions, water limitation significantly reduces transport and phloem loading which are also the major factors that determine extent of grain filling (Farooq et al., 2009). For Apo, despite its lower MTR under stress, the upregulation of sucrose transport genes clearly explained better source to sink transport (Fig. 7). Hence higher fold change in carbohydrate content and secondary metabolism in Apo was mainly by source and regulation of transport; as a result, a 100-grain weight under 50% FC was similar to that under the control (Fig. 2f). The increase in secondary metabolites in grains is known to protect cells from oxidative burst by acting as effective scavengers (Sircar and Parekh, 2019). The significant increase in secondary metabolites was only observed in the tolerant genotype Apo. This up-regulation of secondary metabolites is evident from higher spikelet fertility in Apo than in IR64 (Fig. 2e). Overall, maintenance of source strength and translocation resulted in higher metabolome flux into spikelets of Apo and was associated with lower reduction in grain yield under stress compared to IR64 (Fig. 2g).

Conclusions

Understanding plant response to stress at a specific growth phase is crucial for crop improvement. Combining many high-throughput approaches can pave a way to assess plant response to stress. Use of a high-throughput plant phenomics facility and a metabolomics approach allowed to assess differences in mechanisms of stress response between tolerant and susceptible rice genotypes. Under stress, with similar leaf turgor, IR64 showed significantly higher reduction in photosynthesis than Apo. A better assimilation and enhanced transport of assimilates to the sink by up-regulation of transporters resulted in higher metabolomics change in Apo at 50% FC compared with IR64. In Apo, the higher fold change of carbohydrate content resulted in no reduction in 100-grain weight. The significant up-regulation of secondary metabolites which are known to protect against stress was only noticed in Apo. The upregulation of the metabolome flux in Apo facilitated the higher grain yield under stress conditions compared with IR64.

Acknowledgements

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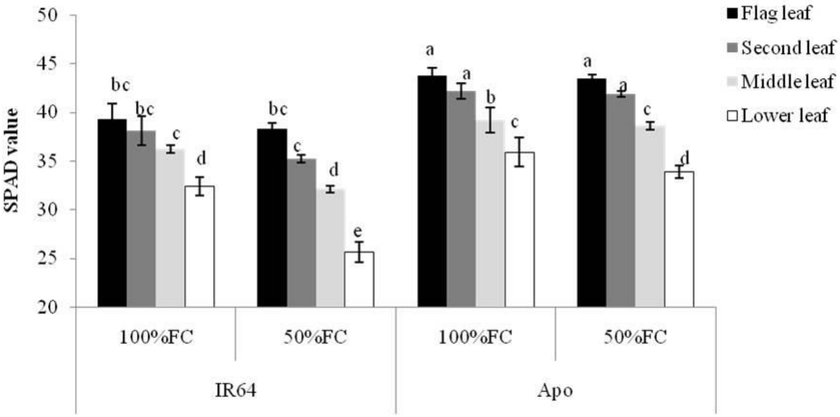
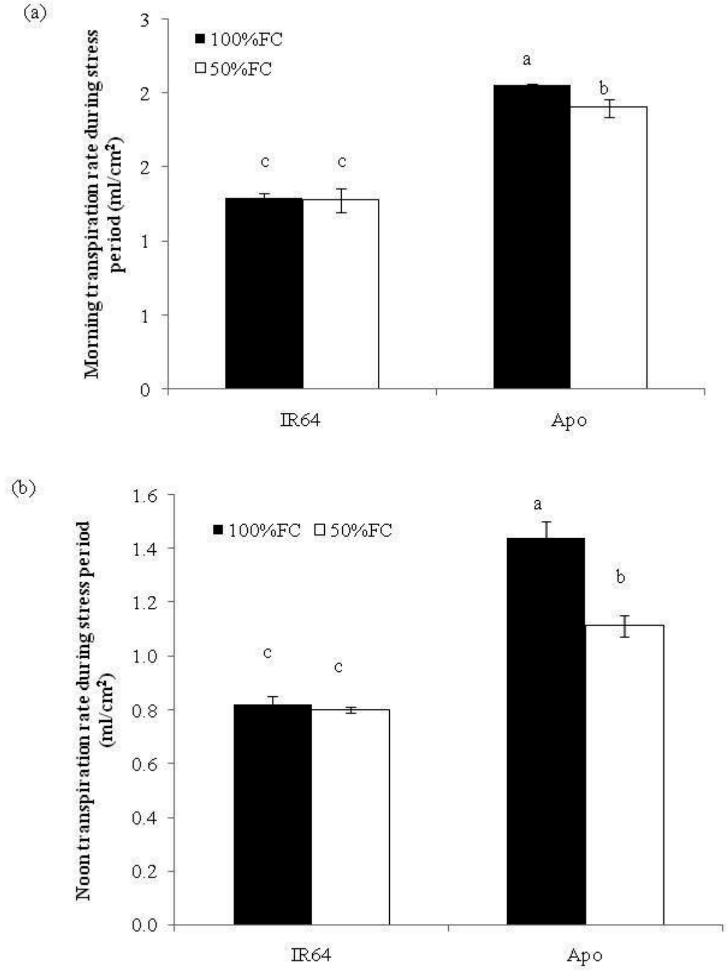
Supplementary information in Chapter 3



Supplementary Figure S1: SPAD value in two rice genotypes under 100 and 50% FC conditions. Measurements were made flag leaf, second leaf, middle leaf and lower leaf. Different letters indicates significance ($P < 0.05$) from the LSD post-hoc test determined using two-way ANOVA with genotypes × treatments interactions.



Supplementary Figure S2: (a) Morning and (b) noon transpiration rates of IR64 and Apo during stress period. Morning transpiration was considered from 6:00 to 12:00 hours and noon period was between 12:00 to 18:00 hours. Different letters indicates significance ($P < 0.05$) from the LSD post-hoc test determined using two-way ANOVA with genotypes × treatments interactions.

Chapter 3

Supplementary Table S1: Results of ANOVA ($P < 0.05$) for detected metabolites

Compound	Genotype (G)	Treatment (T)	G × T
Delphinidin	3.38E-12	5.93E-23	5.21E-12
Delphinidin 3-O-rutinoside-7-O-glucoside	7.77E-07	6.64E-37	4.43E-26
Leucopelargonidin	5.41E-08	1.25E-50	6.19E-08
Pelargonidin	4.42E-06	5.79E-16	1.34E-12
L-Phenylalanin	4.42E-12	1.77E-12	4.00E-19
4-coumarate	6.60E-03	3.40E-05	1.53E-04
Coumaraldehyde	4.54E-12	1.57E-38	0.00053517
4-coumaroylquinate	4.95E-12	2.39E-51	0.000025
4-coumaroylshikimate	3.03E-11	1.11E-23	9.60E-06
Caffeoylshikimate	4.11E-18	9.16E-08	5.50E-06
Caffeoyl-CoA	4.44E-13	6.30E-10	2.50E-08
Feruloyl-CoA	1.74E-15	5.88E-36	7.70E+01
Coniferylaldehyde	1.07E-26	5.12E-33	1.74E-10
Caffeoylquinate	5.80E-10	3.15E-19	1.00E-06
Coniferyl alcohol	1.45E-34	5.61E-20	0.00090627
5-hydroxy-coniferaldehyde	4.40E-24	7.14E-27	1.95E-23
Sinapyl-alcohol	1.71E-51	6.38E-10	0.00017794
syringin	5.09E-18	9.66E-05	6.44E-10
Ferulic acid	1.99E-10	9.45E-15	1.94E-09
Putrescine	8.41E-31	1.90E-23	7.41E-12
L-dopa	8.21E-12	1.34E-05	2.50E-07
Dopamine	2.59E-06	4.75E-11	9.50E-05
Autumnaline	6.59E-10	3.87E-12	8.08E-05
Norbelladine	4.02E-08	4.86E-10	1.66E-10
4'-O, N-dimethylnorbelladine	8.65E-06	3.64E-05	1.84E-09
N-demethylgalanthamine	1.75E-13	3.96E-10	1.23E-15
Normitidine	1.25E-06	1.26E-05	3.47E-10
(10bS, 4aR)-normaritidine	3.66E-07	1.12E-10	6.35E-05
Glycine	1.20E-09	2.10E-06	4.36E-18
Glyoxylate	3.12E-09	1.01E-07	3.01E-44
L-tyrosin	2.47E-07	2.61E-06	2.17E-06

Supplementary Table S1 (Continued)

Glutamine	2.90E-11	8.89E-06	3.87E-19
Glutamate	4.41E-15	8.84E-07	2.72E-19
Ornithin	4.03E-27	8.85E-07	0.1013
Arginine	2.14E-27	9.04E-07	1.04E-10
Citrulline	6.59E-10	4.56E-08	1.37E-33
Proline	4.02E-08	9.71E-07	7.85E-27
Lysine	8.65E-06	7.96E-07	1.83E-23
5-aminopentanoate	1.75E-13	9.76E-08	8.80E-41
Glutarate	8.11E-05	4.14E-14	1.21E-33
Aspartate	8.58E-05	3.65E-31	2.80E-08
L-aspartate-4-semialdehyde	1.32E-07	8.01E-14	2.84E-07
Homoserine	1.47E-09	1.01E-06	2.56E-07
Serine	5.70E-05	4.74E-20	3.90E-08
Homocystein	0.022796	4.42E-09	2.91E-07
Methionin	2.29E-13	1.07E-26	2.30E-07
2-oxoadipate	1.64E-12	1.83E-38	1.96E-08
2-aminoadipate	4.46E-15	1.51E-30	3.15E-07
Xanthosine	6.68E-06	1.25E-14	1.48E-18
Xanthine	8.78E-07	3.08E-11	4.36E-18
Urate	8.35E-35	3.08E-11	3.01E-44
Allantoin	6.00E-16	8.32E-31	2.17E-06
Allantoate	2.62E-17	4.31E-26	3.87E-19
Guanine	9.78E-16	2.69E-16	2.72E-19
Adenine	3.68E-23	1.06E-13	0.1013
D-glutamate	1.96E-07	4.93E-35	1.04E-10
Arabinose	0.076037	1.46E-28	1.37E-33
L-Glucose	4.93E-35	8.66E-14	7.85E-27
Gluconic Acid	6.31E-10	0.00092584	1.83E-23
Fructose	2.03E-11	7.91E-12	1.63E-15
Mallic acid	6.57E-05	0.0010896	5.60E-06
Isocitric acid	2.13E-06	0.0039842	7.40E-04
Succinic acid	5.70E-05	2.91E-12	6.50E-04

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Supplementary Table S2: Identified compounds, its chemical structure, mass and KEGG ID

Group	Compound	Chemical structure	Exact mass (Daltons)	KEGG ID
Secondary metabolites	Delphinidin	$C_{15}H_9O_7$	303.05048	C05908
Secondary metabolites	Delphinidin 3-O-rutinoside-7-O-glucoside	$C_{33}H_{40}O_{21}$	773.21403	
Secondary metabolites	Leucopelargonidin	$C_{15}H_{14}O_6$	290.07904	C03648
Secondary metabolites	Pelargonidin	$C_{15}H_9O_5$	271.06065	C05904
Secondary metabolites	L-Phenylalanin	$C_9H_{11}NO_2$	165.07898	C00079
Secondary metabolites	4-coumarate	$C_9H_7O_3$	164.04734	C00811
Secondary metabolites	Coumaraldehyde	$C_9H_8O_2$	148.05243	C05608
Secondary metabolites	4-coumaroylquinate	$C_{16}H_{17}O_8$	338.10017	C12208
Secondary metabolites	4-coumaroylshikimate	$C_{16}H_{15}O_7$	320.08960	C02947
Secondary metabolites	Caffeoylshikimate	$C_{16}H_{15}O_8$	336.08452	C10434
Secondary metabolites	Caffeoyl-CoA	$C_{30}H_{38}N_7O_{19}P_3S$	929.14690	C00323
Secondary metabolites	Feruloyl-CoA	$C_{31}H_{40}N_7O_{19}P_3S$	943.16255	C00406
Secondary metabolites	Coniferylaldehyde	$C_{10}H_{10}O_3$	178.06299	C02666
Secondary metabolites	Caffeoylquinate	$C_{16}H_{17}O_9$	354.09508	C00852
Secondary metabolites	Coniferyl alcohol	$C_{10}H_{12}O_3$	180.07864	C00590
Secondary metabolites	5-hydroxy-coniferaldehyde	$C_{10}H_{10}O_4$	194.05791	C12204
Secondary metabolites	Sinapyl-alcohol	$C_{11}H_{14}O_4$	210.08921	C02325
Secondary metabolites	syringin	$C_{17}H_{24}O_9$	372.14203	C01533
Secondary metabolites	Ferulic acid	$C_{10}H_9O_4$	194.05791	C01494
Secondary metabolites	Putrescine	$C_4H_{14}N_2$	88.10005	C00134
Secondary metabolites	L-dopa	$C_9H_{11}NO_4$	197.06881	C00355
Secondary metabolites	Dopamine	$C_8H_{12}NO_2$	153.07898	C03758
Secondary metabolites	Autumnaline	$C_{21}H_{27}NO_5$	373.18892	C16707
Secondary metabolites	Norbelladine	$C_{15}H_{17}NO_3$	259.12084	C16701
Secondary metabolites	4'-O, N-dimethylnorbelladine	$C_{17}H_{21}NO_3$	287.15214	
Secondary metabolites	N-demethylgalanthamine	$C_{16}H_{19}NO_3$	273.13649	C12173
Secondary metabolites	Normitidine	$C_{16}H_{19}NO_3$	273.13649	
Secondary metabolites	(10bS, 4aR)-normaritidine	$C_{16}H_{19}NO_3$	273.13649	C21635

Supplementary Table S2 (Continued)

Amino acids	Glycine	$C_2H_5NO_2$	75.03203	C00037
Amino acids	Glyoxylate	C_2HO_3	74.00039	C00048
Amino acids	L-tyrosin	$C_9H_{11}NO_3$	181.07389	C00082
Amino acids	Glutamine	$C_5H_{10}N_2O_3$	146.06914	C00064
Amino acids	Glutamate	$C_5H_8NO_4$	147.05316	C00025
Amino acids	Ornithin	$C_5H_{13}N_2O_2$	132.08988	C00077
Amino acids	Arginine	$C_6H_{15}N_4O_2$	174.11168	C00062
Amino acids	Citrulline	$C_6H_{13}N_3O_3$	175.09569	C00327
Amino acids	Proline	$C_5H_9NO_2$	115.06333	C00148
Amino acids	Lysine	$C_6H_{15}N_2O_2$	146.10553	C00047
Amino acids	5-aminopentanoate	$C_5H_{11}NO_2$	117.07898	C00431
Amino acids	Glutarate	$C_5H_6O_4$	132.04226	C00489
Amino acids	Aspartate	$C_4H_6NO_4$	133.0375077183	C00049
Amino acids	L-aspartate-4-semialdehyde	$C_4H_7NO_3$	117.04259	C00441
Amino acids	Homoserine	$C_4H_9NO_3$	119.05824	C00263
Amino acids	Serine	$C_3H_7NO_3$	105.04259	C00065
Amino acids	Homocystein	$C_4H_9NO_2S$	135.03540	C00155
Amino acids	Methionin	$C_5H_{11}NO_2S$	149.05105	C00073
Amino acids	2-oxoadipate	$C_6H_6O_5$	160.03717	C00322
Amino acids	2-aminoadipate	$C_6H_{10}NO_4$	161.06881	C00956
Amino acids	D-glutamate	$C_5H_8NO_4$	147.05316	C00217

Nucleic acid metabolism	Xanthosine	$C_{10}H_{12}N_4O_6$	284.07568	C01762
Nucleic acid metabolism	Xanthine	$C_5H_4N_4O_2$	152.03343	C00385
Nucleic acid metabolism	Urate	$C_5H_4N_4O_3$	168.02834	C00366
Nucleic acid metabolism	Allantoin	$C_4H_6N_4O_3$	158.04399	C02350
Nucleic acid metabolism	Allantoate	$C_4H_7N_4O_4$	176.05455	C00499
Nucleic acid metabolism	Guanine	$C_5H_5N_5O$	151.04941	C00242
Nucleic acid metabolism	Adenine	$C_5H_5N_5$	135.05450	C00147
Carbohydrates	Arabinose	$C_5H_{10}O_5$	150.05282	C00216
Carbohydrates	L-Glucose	$C_6H_{12}O_6$	180.06339	D09924
Carbohydrates	Gluconic Acid	$C_6H_{11}O_7$	196.05830	C00257
Carbohydrates	Fructose	$C_6H_{12}O_6$	180.06339	C00095
Carbohydrates	Mallic acid	$C_4H_4O_5$	134.02152	
Carbohydrates	Isocitric acid	$C_6H_8O_7$	192.0270026115	C00311
Carbohydrates	Succinic acid	$C_4H_4O_4$	118.02661	C00042

Supplementary Table S3: Fold change in 50% FC over 100% FC for detected metabolites

Group	Compound	IR64	Apo
Secondary metabolites	Delphinidin	0.63	1.95
Secondary metabolites	Delphinidin 3-O-rutinoside-7-O-glucoside	0.92	1.75
Secondary metabolites	Leucopelargonidin	1.69	1.78
Secondary metabolites	Pelargonidin	0.44	-1.46
Secondary metabolites	L-Phenylalanin	-3.62	-0.29
Secondary metabolites	4-coumarate	-0.28	0.07
Secondary metabolites	Coumaraldehyde	-0.21	0.31
Secondary metabolites	4-coumaroylquinate	-0.96	-1.72
Secondary metabolites	4-coumaroylshikimate	-0.21	0.31
Secondary metabolites	Caffeoylshikimate	-2.15	1.71
Secondary metabolites	Caffeoyl-CoA	3.47	-1.12
Secondary metabolites	Feruloyl-CoA	0.66	0.80
Secondary metabolites	Coniferylaldehyde	-0.66	-0.16
Secondary metabolites	Caffeoylquinate	-0.93	-1.74
Secondary metabolites	Coniferyl alcohol	-0.96	1.60
Secondary metabolites	5-hydroxy-coniferaldehyde	-0.04	0.09
Secondary metabolites	Sinapyl-alcohol	0.38	-1.24
Secondary metabolites	syringin	0.17	-0.21
Secondary metabolites	Ferulic acid	0.32	-8.29
Secondary metabolites	Putrescine	1.12	0.06
Secondary metabolites	L-dopa	-0.25	0.28
Secondary metabolites	Dopamine	-0.74	2.54
Secondary metabolites	Autumnaline	-0.38	0.28
Secondary metabolites	Norbelladine	-1.51	2.65
Secondary metabolites	4'-O, N-dimethylnorbelladine	-0.28	-1.08
Secondary metabolites	N-demethylgalanthamine	0.38	-1.24
Secondary metabolites	Normitidine	0.96	0.00
Secondary metabolites	(10bS, 4aR)-normaritidine	1.01	0.15

Supplementary Table S3 (Continued)

Amino acids	Glycine	-0.05	-0.74
Amino acids	Glyoxylate	-0.84	1.03
Amino acids	L-tyrosin	-4.26	2.97
Amino acids	Glutamine	0.43	-0.83
Amino acids	Glutamate	0.30	-2.02
Amino acids	Ornithin	0.18	1.11
Amino acids	Arginine	-2.51	1.57
Amino acids	Citrulline	1.71	-1.42
Amino acids	Proline	1.71	-1.42
Amino acids	Lysine	0.94	1.21
Amino acids	5-aminopentanoate	1.60	-0.96
Amino acids	Glutarate	-1.02	-1.80
Amino acids	Aspartate	-0.87	-1.12
Amino acids	L-aspartate-4-semialdehyde	0.30	2.56
Amino acids	Homoserine	1.71	-1.42
Amino acids	Serine	0.12	-1.09
Amino acids	Homocystein	1.32	-0.22
Amino acids	Methionin	-0.32	-1.46
Amino acids	2-oxoadipate	0.00	0.96
Amino acids	2-aminoadipate	0.30	-2.02
Nucliec acid metabolism	Xanthosine	0.31	-0.34
Nucliec acid metabolism	Xanthine	-0.16	-0.66
Nucliec acid metabolism	Urate	-4.40	-0.36
Nucliec acid metabolism	Allantoin	0.28	-0.25
Nucliec acid metabolism	Allantoate	0.06	1.12
Nucliec acid metabolism	Guanine	0.18	1.11
Nucliec acid metabolism	Adenine	-8.29	0.32
Carbohydrates	D-glutamate	1.34	1.30
Carbohydrates	Arabinose	-0.05	-0.91
Carbohydrates	L-Glucose	-0.06	1.15
Carbohydrates	Gluconic Acid	0.31	-0.34
Carbohydrates	Fructose	0.12	0.67
Carbohydrates	Mallic acid	0.25	0.05
Carbohydrates	Isocitric acid	0.61	1.32
Carbohydrates	Succinic acid	0.48	1.59

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CHAPTER 4

Acquired traits contribute more to drought tolerance in wheat than in rice

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Chapter 4

Abstract

Drought tolerance is governed by constitutive and acquired traits. Combining them has relevance for sustaining crop productivity under drought. Mild levels of stress induce specific mechanisms that protect metabolism when stress becomes severe. Here, we report a comparative assessment of "acquired drought tolerance (ADT)" traits in two rice cultivars, IR64 (drought susceptible) and Apo (tolerant), and a drought-tolerant wheat cultivar, Weebill. Young seedlings were exposed to progressive concentrations of methyl viologen (MV), a stress inducer, before transferring to a severe concentration. "Induced" seedlings showed higher tolerance and recovery growth than seedlings exposed directly to severe stress. A novel phenomic platform with an automated irrigation system was used for precisely imposing soil moisture stress to capture ADT traits during the vegetative stage. Gradual progression of drought was achieved through a software-controlled automated irrigation facility. This facility allowed the maintenance of the same level of soil moisture irrespective of differences in transpiration, and hence, this platform provided the most appropriate method to assess ADT traits. Total biomass decreased more in IR64 than in Apo. The wheat cultivar showed lower levels of damage and higher recovery growth even compared to Apo. Expression of ROS-scavenging enzymes and drought-responsive genes was significantly higher in Apo than in IR64, but differences were only marginal between Apo and Weebill. The wheat cultivar showed significantly higher stomatal conductance, carbon gain, and biomass than the rice cultivars, under drought. These differences in ADT traits between cultivars as well as between species can be utilised for improving drought tolerance in crop plants.

Keywords: acquired drought tolerance traits; induction response; reactive oxygen species; methyl viologen; phenotyping platform

Introduction

Rice (*Oryza sativa* L.), being a semi aquatic species, is generally cultivated under puddle conditions. However, with the impending climate change combined with domestic and industrial demands for fresh water, cultivation of rice in the conventional puddle system is fast becoming less feasible (Elliott et al., 2014). This necessitates development of suitable water-saving agronomic practices to save water. Practices such as semi-irrigated aerobic cultivation ar known to save more than half of the water used and avoid the destruction of soil structure while puddling (Kato et al., 2015). However, a concomitant yield loss by more than 40% when grown under the aerobic system has been observed (Torres and Henry, 2018; Sheshshayee et al., 2018). Therefore, we should attempt to enhance the genetic potential that can produce more biomass and yield with reduced water requirement.

Selection for higher absolute yields under water-limiting conditions was one of the major approaches for improving rice productivity. Although this approach provided initial dividends, further improvement in rice productivity has become hard to achieve (Sheshshayee et al., 2018). To overcome this bottleneck, a focused trait-based breeding approach has been proposed and is being widely adapted (Reynolds and Langridge, 2016). This led to a significant increase in enumerating physiological and morphological traits and establishing their functional relevance in conferring stress tolerance in rice plants (Sheshshayee et al., 2018). Depending on the level of organization and response to external stimuli, drought adaptive traits are broadly classified as “constitutive,” “responsive,” and “acquired” tolerance traits (Sheshshayee et al., 2018). Traits that are always expressed such as root number, stoma number, specific leaf area, and epicuticular waxes are referred to as "constitutive traits or integral traits" (Blum, 1996). Traits that are expressed always but would significantly change in response to stress such as root length and osmotic adjustment are often referred to as “responsive” traits (Sheshshayee et al., 2018). On the other hand, “acquired tolerance traits” are conspicuously absent under well-watered conditions but get upregulated when plants experience gradual induction of a stress (Sung et al., 2003). De novo gene expression has been shown to increase when plants experience mild stress levels. This upregulation of gene expression is known to induce specific mechanisms that provide protection to cellular metabolism when stress gets severe. Acquired tolerance is therefore associated with the upregulation of several diverse processes such as maintenance of redox homeostasis, regulation of gene expression, protein turnover, DNA/protein repair mechanisms, osmotic adjustment, and membrane stability including specific metabolomic changes (Vemanna et al., 2016). Maintenance of cellular metabolism is dependent upon the abilities of the plant to sustain positive tissue turgor and to maintain cell membrane integrity. While turgor maintenance is governed mostly by constitutive traits such as root and leaf surface characters (Raju et al., 2014), maintenance of cellular metabolism even under decreasing tissue turgor is more associated with acquired tolerance traits (Raju et al., 2014).

Maintenance of cell membrane integrity is in turn dependent on the ability to manage oxidative stress (Vemanna et al., 2016). Increased production of reactive oxygen species (ROS) is an inevitable consequence of stress encountered by plants. Naturally, plants have evolved several mechanisms to balance the ROS homeostasis under stress through upscaling several ROS scavenging mechanisms (Sheshshayee et al., 2018). Increased activity of some key ROS scavenging enzymes like ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione peroxidise (GPX), dehydroascorbate reductase (DHAR), and catalase (CAT) has been reported in many species. Wheat genotypes that show increased activity of these enzymes also display considerable stress tolerance (Pour-Benab et al., 2019). Interestingly, these protective mechanisms are generally upregulated when plants experience mild levels of stress and hence develop tolerance to stress when the severity increases. These mechanisms that provide acquired tolerance display large genetic diversity, which also depends on the type of stress and the progression of stress occurrence (Rizhsky et al., 2004; Choudhury et al., 2017).

The premise of this investigation is based on the hypothesis that a combination of constitutive traits with acquired tolerance traits would comprehensively improve drought adaptation. Methods for determining constitutive traits have been developed under both laboratory and field conditions (Sheshshayee et al., 2018; Blum, 1996). Methods for determining acquired tolerance require the induction of young seedlings with a mild level of stress before transferring the seedlings to severe or lethal stress levels. A few laboratory experimental protocols have been developed where, young seedlings are induced with a gradually increasing temperature until it reaches lethal levels. This method, referred to as Temperature Induction Response (TIR), is a convenient assay for assessing acquired tolerance (Senthil-Kumar et al., 2003 and 2004). Similarly, inducing changes in cellular metabolism with the use of specific stress “inducers” such as methyl viologen (MV) is also a simple approach to assess acquired tolerance at the seedling stage (Nareshkumar et al., 2020). Many studies have documented significant genetic variability in acquired tolerance levels using these experimental protocols. Furthermore, several studies reported that crop genotypes with higher acquired tolerance recorded improved growth and performance under drought stress conditions. Rice genotypes with higher acquired tolerance had higher spikelet fertility under stress and hence were associated with a superior yield (Raju et al., 2014).

Realising the importance of acquired tolerance in drought adaptation, several methods to quantify this trait were developed (Kumar et al., 2014; Viajayaraghavareddy et al., 2017). Although these methods provide clues on genetic variations in the propensity to respond to stress, these methods rely on the induction of response through priming young seedlings with temperature and/or stress-inducing molecules. Examining the stress response by providing drought itself as an inducer has been the most important limitation.

To capture these stress-responsive mechanisms, precise imposition and accurate maintenance of a specific stress regime are of paramount importance. Advancements in high-throughput phenotyping approaches led to the establishment of high-end phenomic platforms that are increasingly being used for imposing stress and for capturing genetic variability in stress responses (Vadez et al., 2015). Gravimetric principles have, by and large, been the approach for determining water lost by a potted plant. Automated water dispensing systems have also been developed in these high-end phenomics platforms to maintain a specific soil moisture status. However, these platforms are limited by the number of gravimetric determination of water loss in a day. Thus, mimicking the progression of stress in natural conditions still remains a major challenge. We developed a novel phenomic platform with an automated irrigation system that is interfaced with transpirational water loss. Thus, the system maintains the water content in the soil within 1% of the fixed field capacity and hence represents an excellent system to examine stress response of plants. The most important feature of this facility is its ability to progressively decrease the water added so as to bring the soil to a specified level of soil moisture stress. This transpiration-interfaced automated irrigation system can therefore accurately mimic a field-like drought progression scenario. The other most prominent feature of this facility is its ability to maintain the same level of stress despite any possible differences in transpiration rates and hence represents an excellent system to compare stress responses of genotypes within as well as across species. Therefore, this system provides an opportunity for a comparative assessment of rice and wheat (*Triticum aestivum* L.) for the differences in their acquired tolerance levels.

Rice and wheat are the two most extensively consumed C_3 cereals, with phenomenal differences in water use patterns that render wheat to be better adapted to drought than rice. Therefore, growing rice like wheat would have phenomenal significance in saving water. Comparing the stress response of these two cereals to water limitation would provide scientific insights to prepare rice for limited water resources. Rice and wheat are known to differ in several morpho-physiological traits and hence have reduced water requirement. While large differences in water absorption through root system architecture, metabolic status, etc., are well known to be superior in wheat (Praba et al., 2009; Kadam et al., 2015; Ouyang et al., 2017), no systematic study has ever been done to assess the differences in acquired tolerance between these cereal species. We hypothesise that when acquired tolerance traits are combined with constitutive traits such as roots and water use efficiency, such genotypes would have comprehensively higher adaptability to drought. We examined rice and wheat cultivars for the differences in acquired tolerance besides other traits. The major intent of this study was to examine the relevance of acquired tolerance in imparting drought adaptation, by comparing tolerant and susceptible rice cultivars and also rice and wheat. An initial experiment was conducted to assess the best suitable stress imposition approach (induction and lethal) to study acquired mechanisms of rice and wheat using MV which is a potent inducer of oxidative stress. Further experiments were done using the phenomic facility by following an induction protocol. The results clearly demonstrate the superior drought tolerance in wheat compared to even a known drought-adaptive cultivar of rice.

Materials and Methods

Plant material and approach

Two rice cultivars, IR64 and Apo, and one wheat cultivar, Weebill, were used in the experiments. IR64 is a drought-susceptible, high-yielding, lowland rice cultivar, and Apo is a drought-tolerant, high yielding, and aerobic rice cultivar. The wheat cultivar Weebill is known to be drought tolerant (Ouyang et al., 2017). Two types of a stress imposition method and three independent experiments were conducted at the research facilities of the University of

Agricultural Sciences, Bengaluru, India (12° 58′N, 77° 35′E): the first one used a chemical stress inducer with young seedlings (48 h old), and the second was to examine the drought stress response both at the seedling (15 days after sowing (DAS)) and vegetative (35 DAS) stages. Drought treatment was imposed using the phenomic platform with an automatic irrigation capability (described later).

Experiment 1: Stress imposition using methyl viologen.

Methyl viologen (MV) is an artificial electron donor to both mitochondrial and chloroplast electron transport (Mano et al., 2001; Babitha et al., 2015a; Cui et al., 2018) and hence generates reactive oxygen species (ROS). At high concentrations, MV can be lethal. To assess the effect of MV at the young seedling stage, 48 h-old uniformly germinated seedlings were used. An induction protocol was developed by treating young seedlings with progressively increasing concentrations of MV before transferring the seedlings to a "severe" concentration. To determine this lethal concentration, uniformly germinated rice seeds of cv. IR64 were spread on 13cm diameter Petri plates with two layers of wet filter papers. Each plate had 10 seedlings. Plates with 48 h-old seedlings were exposed to different concentrations of MV to determine the "severe stress" concentration of MV, i.e., the concentration at which about 95% of the seedlings died (Supplementary Fig. S1). A concentration of 10 µM MV was found to be "severe" or lethal. An induction protocol was developed that involved transferring the 48 h-old seedlings to sequentially increasing concentrations of MV, i.e., 2, 4, 6, and 8 µM of MV. Seedlings were allowed to stay for a duration of 3 hours in each of the concentrations before being transferred to the next higher concentration, and subsequently, the "induced" seedlings were transferred to 10 µM concentration of MV (lethal stress). Three plates were maintained for each treatment, and a completely randomized design was adopted for statistical analysis. To every plate, 10 ml of each concentration of MV was added. One set of seedlings was directly transferred to 10 µM MV, to represent the "severe stress" treatment, while a separate set of seedlings was continuously kept in plates wetted with distilled water to represent the "absolute control." Seedlings in all treatments were exposed to 600 µmol m^{-2} s^{-1} of light intensity in a controlled growth chamber with an air temperature maintained at 30°C with 60% RH throughout the experiment period. Shoot and root lengths were recorded between 10:00 and 12:00 hours at the end of the stress period (72 hours from the stress imposition).

Recovery growth was assessed 48 hours after transferring the seedlings from the induction and severe stress treatments to distilled water. Comparison was made between induction and lethal treatments within cultivars. Measurements of membrane damage, reactive oxygen species (ROS), and reactive carbonyl compound (RCC) production, antioxidant activities, etc., were made with the seedlings subjected to induction stress, and the results were compared in relation to that of the seedlings which were directly exposed to sever stress.

Quantification of superoxide (O_2^-) by nitrotetrazolium blue chloride (NBT) staining. Seedlings were transferred to NBT solution to detect superoxide radicals. Seedlings were immersed in 0.2% NBT solution dissolved in 50mM sodium phosphate buffer with a pH of 7.5. NBT reacts with O_2^- to form a dark-blue insoluble formazan compound. The seedlings were transferred to a bleaching solution to remove chlorophyll. Tissues were ground in 0.1% acetic acid (Kumar et al., 2014). The samples were centrifuged at 10,000 rpm for 10 min, and absorbance was read at 560 nm.

Quantification of hydroxyl (·OH) radicals.

The presence of hydroxyl radicals in seedlings was quantified using the method described by (Aruoma, 1994). Seedlings exposed to MV stress as described above were immediately transferred and homogenized in 1.2 ml of 50mM sodium phosphate buffer (pH 7.0) and centrifuged at 12,000 rpm at 4°C for 10 min. The supernatant was collected (0.5 ml) and 0.5 ml of 50mM of sodium phosphate buffer (pH 7.0) and 1 ml of 25mM sodium phosphate buffer containing 2.5mM 2-deoxyribose were added to the supernatant, and this mixture was incubated at 35°C in the dark for 1 h. After incubation, 1 ml of 1% thiobarbituric acid (TBA, Sigma, USA) and 1 ml of glacial acetic acid were added and the mixture was boiled for 10 min and cooled immediately on an ice bath. Absorbance was recorded at 532 nm.

Measurement of malondialdehyde (MDA) content.

Fresh leaf tissue (0.2 g) was homogenized in 0.1% trichloroacetic acid (TCA) and centrifuged at 14,000 rpm for 15 min. 2.5 ml of 0.5% thiobarbituric acid (TBA) in 20% TCA was added to 1.0 ml of the supernatant, and the mixture was incubated at 95°C in a water bath. After 30 min of incubation, it was cooled immediately and centrifuged at 10,000 rpm for 30 min.

Absorbance was determined at 532 and 600 nm, and MDA concentration was estimated by subtracting the OD at 600 nm from the OD at 532 nm as a correction for nonspecific turbidity (Nisarga et al., 2017).

Quantification of methylglyoxal (MG).

Fresh tissue (100 mg) was collected from both control and treated seedlings and ground in a known volume of distilled water. The extract was centrifuged at 11,000 rpm at 4°C for 10 min. 250 µl of 7.2mM 1,2-diaminobenzene and 100 µl of 5M perchloric acid were added to the supernatant (650 µl). The absorbance in the mixture was measured at 336nm using a spectrophotometer (SpectraMax Plus 384, Spinco Biotech Pvt Ltd, Bangalore) (Nisarga et al., 2017).

α,α-Diphenyl-β-picryl-hydrazyl (DPPH) **assay for total scavenging activity.**

The total free radical scavenging activity was measured as described by McCune and Johns (McCune and Johns, 2002). The reaction mixture containing 1 ml of the methanolic leaf extract and 1 ml of DPPH solution (0.3mM) was incubated in the dark for 10 min. Absorbance was read at 517 nm, and percent inhibition was calculated over the control.

Quantification of superoxide dismutase (SOD) activity.

SOD activity was quantified by using the photochemical NBT method as described by Beyer et al., (1987). In this method, assay buffer containing L-methionine (300 mg/10 ml), NBT-2HCl (14.1 mg/10 ml), and Triton X-100 (1%) were added to a glass tube. 20 µl of the sample extracted using phosphate buffer was delivered to this mixture. To initiate the reaction, 10 µl of riboflavin (4.4 mg in 100 ml) was added. The tube was illuminated at a light intensity of 600 µmol m^{-2} s^{-1} along with the control (without the sample). Absorbance at 560 nm was recorded in all the tubes, and a percentage decrease in NBT reduction due to SOD activity was calculated. The percent increase in SOD activity due to the stress effect was calculated over the control.

Quantification of membrane damage by the Evans blue technique.

Seedlings exposed to MV stress were immediately transferred to 5 ml tubes containing 0.25 g of Evans blue dye prepared in 0.1M $CaCl_2$ (pH 5.6). Seedlings were immersed completely in Evans blue dye and incubated for one hour in the dark. After incubation, seedlings were washed thoroughly using distilled water to remove any stain adhered to the surface. Later, seedlings were transferred to 2 ml Eppendorf containing 1 ml of 1% sodium dodecyl sulphate (SDS) and ground using a tissue lyser. Suspension was centrifuged at 10,000 rpm for 10 min. The supernatant was collected, and absorbance was recorded at 600 nm (Viajayaraghavareddy et al., 2017).

Quantification of proline content.

Fresh tissue (0.1 g) was homogenized in 3% sulfosalicylic acid. The homogenate was centrifuged at 6000 g for 20 min. Acids ninhydrin (2 ml) (1.25 g of ninhydrin, 20 ml of 6M orthophosphoric acid, and 30 ml glacial acetic acid) and glacial acetic acid (2 ml) were added to a 2 ml aliquot and the mixture was boiled at 100°C for 1 h, and the reaction was terminated in an ice bath. 4 ml of toluene was added to this mixture to extract chromophores from the aqueous phase, and absorbance was read at 520 nm. The proline content was determined from a standard curve and expressed as μmol g^{-1} fresh weight (Bates, 1973).

Experiment 2: Drought stress imposition using an automated drought simulator phenotyping platform.

Two independent experiments were conducted at the minilysimeter- (MLM-) based drought simulator platform to examine the response of plants to drought stress and to assess acquired tolerance. The first experiment (Experiment 2A) was carried out using 15-day-old seedlings in containers (10 l capacity) filled with 10 kg of a rooting mixture comprising red sandy-loam soil mixed with farm yard manure in a 3 : 1 (W/W) proportion. The second experiment (experiment 2B) was carried out using 35-day-old plants using larger containers (20 l capacity) filled with 20 kg of a rooting mixture.

Acquired tolerance for drought stress can be measured only when the plants are exposed to a gradually progressing stress. The novel phenomic platform established at our centre was utilised to generate a specific level of drought stress in container-grown plants. This facility consists of containers (minilysimeters (MLM)) placed on individual load cell balances and is capable of determining the weights in real time. The load cell balances are capable of determining weights up to 50 kg with an accuracy of ±50 g (Figs. 1a–c). The weight of containers decreases due to evapo-transpiration, and hence, this gravimetric approach represents a very accurate method to determine water used by plants. Custom designed software recognises this weight reduction and commands a solenoid value to dispense accurate volumes of water to replenish the water lost by evapo-transpiration. The irrigation water is dispensed into a 2 cm diameter tube that has a round coil of tube at the middle of the container. The tubes are perforated to facilitate uniform distribution of water in the container. This setup ensures the maintenance of soil moisture within 1% of the set water regime in the container. The most important feature of this software is its capacity to bring down the water level at a rate determined by the user. This transpiration-interfaced irrigation software dispenses a predesignated proportion of water so as to provide an accurate rate of depletion of water. This "drydown" protocol is capable of precisely mimicking the field conditions as well as creating a gradually developing stress condition (Fig. 1d). The software controlling the automated irrigation is also capable of maintaining the soil moisture status at any notified level, and hence, this system is the most appropriate method for assessing acquired tolerance (patent application number is 201941028751). The other major feature of this automated irrigation facility is its ability to precisely maintain a highly comparable tissue water status for cultivars independent of any differences in their transpiration rates (Figs. 1e and 1f). The entire facility is established under naturally varying conditions. The weather parameters that prevailed during the experimental period were obtained from the weather station of the University, located around 300m from the experimental plot (Supplementary Tables S1 and S2).

Experiment 2A: Stress imposition at the seedling stage.

Stress imposition was initiated at 15 days after sowing (DAS) for rice and wheat seedlings by automated irrigation. A set of five containers were maintained at 100% field capacity (FC) while another five containers were subjected to gradual stress. The software was tuned to ensure a 5% reduction in soil water status per day irrespective of the differences in evapo-transpiration. The containers designated as "water stressed" reached 45% FC on the 11th day after stress imposition. Stress level was maintained at 45% FC for an additional 10 days. Afterwards, soil water status was elevated to 100% FC for recovery. To measure relative water content (RWC), leaf discs were made from both control and stress samples and fresh weight was recorded. Leaf discs were floated on distilled water in Petri plates for 5 h, and turgid weight was recorded. Leaf discs were oven dried at 65°C for three days, and dry weight was recorded. The RWC was calculated using the formula: RWC% = (Fresh weight − dry weight)/(turgid weight − dry weight) × 100. Total chlorophyll content was measured using the acetone : DMSO (1 : 1) method. Leaf discs were incubated in 10 ml of acetone : DMSO (1 : 1) overnight in the dark. Absorbance was measured at 663, 645, and 652nm to calculate total chlorophyll content as described by Arnon (1949). MDA and total proline were measured in replicates of three using the protocol described above in the second leaf collected during bright sunshine hours (10:00 to 11:00 hours). Plant height, tiller number, and biomass were measured a week after recovery in three replicates.

To prove that gradual drought stress upregulates drought responsive scavenging enzymes, ascorbate peroxidase (APX), FeSOD, and catalase (CAT), aldo-keto reductase (AKR1) Δ1- pyrroline-5-carboxylate synthase (P5CS) expression levels were measured. Along with scavengers, ER stress-responsive genes, binding protein (BiP1) and calreticulin (CRT1) activity were also measured by expression studies. Leaf tissues from seedlings grown at 100% and 45% FC in replicates of three were used to extract total RNA using the phenol-chloroform method according to Datta et al. (1989), and cDNA was synthesized by oligo (dT) primers using Moloney murine leukaemia virus reverse transcriptase (MMLV-RT; MBI Fermentas, Hanover, MD). The cDNA pool was used as a template to perform RT-PCR analysis according to the manufacturer’s protocol (Opticon 2; MJ Research, USA, and MJ Bioworks Inc.). The quantitative real-time RT-PCR was performed with the fluorescent dye SYBR Green (TAKARA SYBR Green qPCR Kit). The relative expression levels of the selected genes under a given stress condition were calculated using the comparative threshold method. The PCR was performed with SYBR dye, and the PCR conditions were 94°C for 3 min, 25 cycles of 94°C for 30 s, 52–58°C for 30 s, and 72°C for 40 s, and a final extension of 72°C for 5 min. Ubiquitin was used as an internal control for normalization.

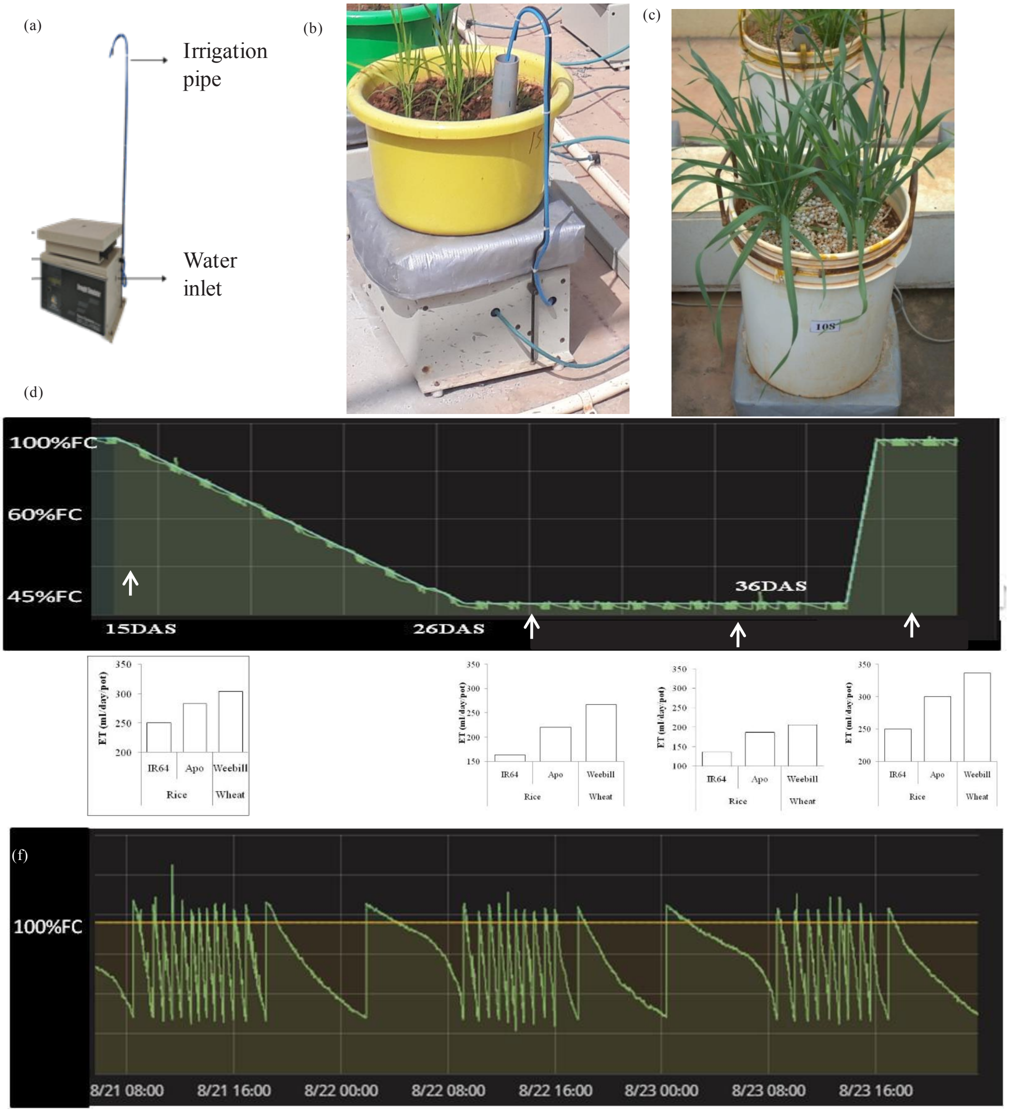

Figure 1: Drought stress imposition protocol for rice and wheat using a plant phenomic platform. (a) Minilysimeter with an irrigation pipe. (b) Pots of 10 l capacity and (c) 20 l capacity were kept on lysimeters with the automated irrigation facility. (d) Gradual moisture stress (drydown) was imposed with a reduction of 5% of FC per day to reach 45% FC which was then elevated to 100% FC after 10 days. (e) Bar graph indicating evapo-transpiration data of IR64, Apo, and the wheat cultivar Weebill for one day. (f) The graph is indicating real-time water transpired and added per day.

Experiment 2B: Stress imposition at the vegetative stage.

In a set of 10 containers, the plant population was progressively thinned to maintain two uniform and healthy plants per container by 25 DAS. Stress imposition was initiated on the 35th DAS, as described above. A set of five containers for each cultivar was designated as "45% FC" while the other five were maintained at 100% FC (well-watered). The automated irrigation system of the drought simulator platform regulates the water added to containers, and plants reached 45% FC on the 11th day after stress imposition. Soil water status was maintained at 45% FC for additional 10 days and later elevated to 100% FC and maintained until maturity. During the stress period, photosynthetic rate and stomatal conductance were measured using a portable photosynthetic system (LICOR 6400, Lincoln, Nebraska, USA). RWC and MDA content were measured in the second leaf collected during bright sunshine hours (10:00 to 11:00 hours) using the protocol described above. The canopy temperature was measured in 100% and 45% FC plants using the Fluke thermal imaging system. Images taken from the canopy using a Fluke camera were analysed using SmartView software. Data was exported to Microsoft Excel to analyse all the data points of the image. To avoid background noise, the canopy was outlined to arrive at mean temperature (Ming-Han et al., 2015). At harvest, from three replicates, shoot, leaves, and roots were separated, oven dried at 65°C for three days, and weighed; values were computed to arrive at total leaf area and total biomass.

Statistical analysis

All the data were analysed to check statistical significance using Genstat (15th edition) (http:// www.genstat.co.uk/). The goal of this investigation was to compare the effect of "induction" stress on plant performance when stress levels become severe. For this comparison, a one-way statistical algorithm was used in experiment 1, where various parameters of induced and severely stressed plants were compared. Further, the comparisons were also made between the two rice cultivars (Apo and IR64) and between the tolerant rice cultivar Apo and wheat cultivar, Weebill in induction treatment only. In experiment 1, methyl viologen, a stress inducer, was used. In experiment 2, the comparison was made at 45%FC between the two cultivars of rice and between the tolerant rice cultivar Apo and wheat cultivar, Weebill. Plants were exposed to progressive drought stress using the automated phenomics platform, and the statistical significance of several parameters was compared by performing a one-way

ANOVA. Comparisons were also made between Apo and Weebill using the same algorithm.

Results

Wheat has higher oxidative stress tolerance than rice.

A significant reduction in growth was observed in MV treated seedlings of the rice cultivars, with a strong reduction in root length, especially in the susceptible cv. IR64 at the end of the stress period (Figs. 2a and b and Supplementary Table S3a). The wheat cultivar, Weebill, on the other hand showed better tolerance with sustained shoot and root growth compared to the tolerant rice cv. Apo (Figs. 2b and c). Neither of the two rice cultivars survived the severe stress treatment, but wheat proved to be more tolerant to the severe stress treatment (Fig. 2a). In the induction treatment, recovery growth of seedlings was significantly superior to that of seedlings directly exposed to the severe stress treatment for both cereals (Figs. 2d and 2e). Rice cv. Apo had a higher recovery when stress was gradually induced than cv. IR64. Wheat had significantly more root and shoot growth than the tolerant rice cultivar Apo when the stress was gradually induced (Figs. 2d and e and Supplementary Table S3b).

Generation of reactive oxygen species (ROS) is an inevitable consequence of the stress response, and the ability to scavenge these ROS is considered a stress-adaptive mechanism. There was a significant increase in O_2^- in all the cultivars of both rice and wheat (Table 1). However, the increase in ROS production was significantly lower in the seedlings receiving induction treatment than in the seedlings directly exposed to severe stress treatment. Similar observations were made for ·OH accumulation. However, between the two rice cultivars, there was a marginally lower accumulation of O_2^- and ·OH in cv. Apo than in cv. IR64 in the induction treatment. Weebill recorded the lowest levels of O_2^-, while ·OH accumulation was not significantly different in the induction treatment than that in tolerant rice cv. Apo (Table 1).

Lipid peroxidation and glycation lead to the production of several cytotoxic compounds called reactive carbonyl compounds (RCCs), which are consequences of oxidative stress. Seedlings exposed directly to severe levels of MV experience an oxidative burst leading to a significant increase in MDA and MG content in the susceptible rice cv. IR64 compared to Apo (Table 1). But when seedlings were induced with mild stress levels of MV, the increase in MDA and MG were significantly lower and it was lowest in Weebill. The MDA and MG

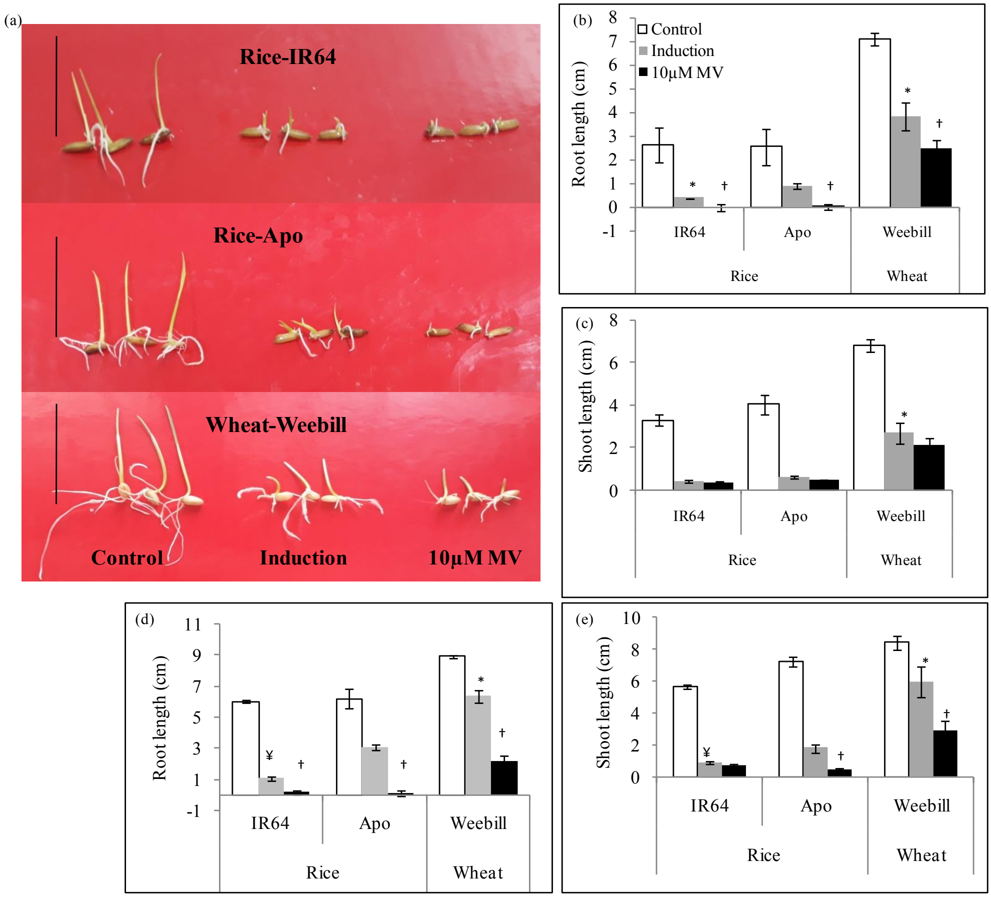

Figure 2: Effect of methyl viologen (MV) on plant growth in induction and severe stress treatments (10 µM) in both rice (IR64 and Apo) cultivars and the wheat (Weebill) cultivar (Experiment 1). Effect of MV on (a) plant growth, (b) root length, and (c) shoot length measured at the end of the stress period (72 hours after stress imposition). Seedlings from all the treatments were subjected to recovery growth for two days, and (d) root length and (e) shoot length were measured after the recovery period. Scale bar = 8 cm. †Significant difference between induction and severe stress treatments within cultivars at $p \leq 0.05$. ¥Significant difference between rice cultivars (IR64 and Apo) in the induction treatment at $p \leq 0.05$. *Significant difference between tolerant rice cv. Apo and wheat cv. Weebill in the induction treatment at $p \leq 0.05$.

levels in the wheat cultivar were lower than those in the tolerant rice cultivar, Apo. These data indicate the enhancement of acclimation response upon induction (Table 1).Lipid peroxidation and glycation processes are known to damage the cell membrane stability. Evans blue staining, a sensitive assay to determine membrane damage, revealed that Apo experienced less membrane damage compared with IR64 indicating a superior induction response in the tolerant cultivar. The membrane damage of the wheat cultivar Weebill was lower than that of Apo indicating an even better protective mechanism operating in wheat compared to rice (Table 1).

As a stress response, ROS-scavenging antioxidants and enzymes increased in both induction and severe stress treatments as compared to the control for both cereals. Proline content was not significantly different between the induction and severe stress treatments in rice (Fig. 3a). But fold change in proline was significantly higher for wheat in severe stress compared to induction treatment (Fig. 3a). To assess the acquired tolerance through their abilities to scavenge ROS, a percent increase in total antioxidant activity and SOD activity was measured (Figs. 3b and c). Both rice cultivars showed significant increases in total antioxidant and SOD activities when induced with gradually increasing stress levels. The levels of induction of SOD activity and total antioxidants in wheat were comparable with those in the tolerant rice cultivar Apo (Fig. 3c). But the SOD activity in the wheat cultivar was significantly higher even in the severe stress treatment which indicates a greater ability to scavenge ROS in wheat compared to rice (Fig. 3c).

Variability in acquired mechanisms at the seedling stage.

Rice and wheat seedlings (15 days old) were subjected to gradual drought stress of 45% FC using the phenotyping platform (Fig. 4a). The gradual stress imposition protocol that was adopted in these experiments ensured that the seedlings were "induced" with milder stress levels before reaching the target stress of 45% FC. At the end of the stress period, cv. IR64 was more wilted indicating poor tolerance to stress (Fig. 4a). Morpho-physiological parameters were measured a week after recovery (Fig. 4b). Although both the rice cultivars had a similar reduction in plant height at 45% FC, the reduction in the tiller number was marginally higher for cv. IR64 than for cv. Apo. But wheat was unaffected by a reduction in soil moisture in terms of both plant height and tiller number (Figs. 4c and d). No significant reduction in root length and weight was recorded for rice cultivars. Interestingly, in wheat, although root biomass decreased under stress, a significant increase in root length was observed (Figs. 4e and f). Wheat plants recovered faster with higher biomass compared to cv. Apo (Fig. 4g). The total biomass recorded after recovery was significantly less in rice cv. IR64 than in rice cv. Apo, while wheat recorded significantly higher total biomass accompanied with the least percent reduction in total biomass even when compared with the tolerant rice cv. Apo at 45% FC (Fig. 4g).

The automated irrigation system precisely maintains a specific soil water status by gravimetrically recognising the water loss from each individual container. Therefore, maintenance of a given moisture status irrespective of differences in water use or root traits is the most prominent feature of the stress imposition system adopted. Accordingly, the relative water content (RWC) did not differ significantly at 45% FC between species (Table 2). Interestingly, no reduction was found in the chlorophyll content relative to 100% FC in both rice and wheat. However, the wheat cultivar Weebill recorded higher chlorophyll content than the rice cultivars in both well-watered and 45% FC conditions (Table 2). As a biomarker that reflects stress, the MDA content increased significantly in both rice cultivars, but more so in IR64 than in Apo. Wheat had much lower MDA levels than the drought-tolerant rice cultivar Apo (Table 2). The highest increase in the proline content was seen in wheat and was marginally higher even under well-watered conditions compared to that in rice. Between rice cultivars, IR64 had a higher increase in proline than tolerant rice cv. Apo in 45% FC (Table 2).

Upregulation of drought-responsive genes was crucial for acquired mechanisms.

The tolerant rice cv. Apo showed significant increases in drought-responsive genes. The ROS scavenging enzymes, ascorbate peroxidase (APX), FeSOD, and catalase (CAT), are known to balance the ROS in the system by detoxifying and were significantly upregulated in cv. Apo compared to cv. IR64 under stress. Further, reactive carbonyl compound scavengers, aldo-keto reductases (AKR1), showed significant upregulation of transcript in cv. Apo than in cv. IR64 (Fig. 5). An endoplasmic reticulum (ER) chaperon-binding protein (BiP1) activity was more enhanced in cv. Apo than in cv. IR64. But another ER stress-responsive gene

Table 1: Effects of methyl viologen on ROS and RCC production in seedlings of two rice cultivars and one wheat cultivar (Experiment 1). Accumulation of superoxide (O_2^-), hydroxyl radical (·OH), alondialdehyde (MDA), and methylglyoxal (MG) contents and extent of membrane damage measured by the Evans blue technique in control, induction, and severe (10 µM) treatments of methyl viologen. †Significant difference between induction and severe stress treatments within cultivars at $p \leq 0.05$. $^{¥}$Significant difference between rice cultivars (IR64 and Apo) in the induction treatment at $p \leq 0.05$. *Significant difference between tolerant rice cv. Apo and wheat cv. Weebill in the induction treatment at $p \leq 0.05$.

Parameters	Treatment	Rice		Wheat
		IR64	Apo	Weebill
O_2^- (Absor × 1000)	**Control**	127 ± 9.6	122 ± 6.0	104 ± 7.2
	Induction	955 ±37.6$^{¥}$	836 ± 18.4	601 ± 19.1*
	10µM MV (Severe)	1668 ± 45.5†	1268 ± 39.4†	916 ± 23.4†
·OH (Absor × 1000)	**Control**	26 ± 2.1	29 ± 2.1	31 ± 5.5
	Induction	141 ± 2.8$^{¥}$	102 ± 2.0	101 ± 5.0
	10µM MV (Severe)	275 ± 7.1†	218 ± 1.4†	173 ± 4.6†
MDA (µg/gFW)	**Control**	12.6 ± 4.5	12.8 ± 1.5	18.6 ± 1.5
	Induction	59.1 ± 0.9	55.2 ± 0.3	45.0 ± 1.1*
	10µM MV (Severe)	62.3 ± 0.9†	58.8 ± 1.0†	53.0 ± 0.4†
MG (µg/gFW)	**Control**	13.3 ± 1.5	18.6 ± 3.2	15.6 ± 3.5
	Induction	52.0 ± 2.6$^{¥}$	45.3 ± 1.5	35.6 ± 3.0*
	10µM MV (Severe)	78.0 ± 1.0†	65.3 ± 4.0†	56.3 ± 4.1†
Membrane damage (ngEvansblue/gFW)	**Control**	0.7 ± 0.01	0.6 ± 0.15	0.6 ± 0.01
	Induction	3.1 ± 0.32$^{¥}$	2.0 ± 0.08	1.8 ± 0.08*
	10µM MV (Severe)	5.1 ± 0.34†	3.9 ± 0.25†	3.7 ± 0.05†

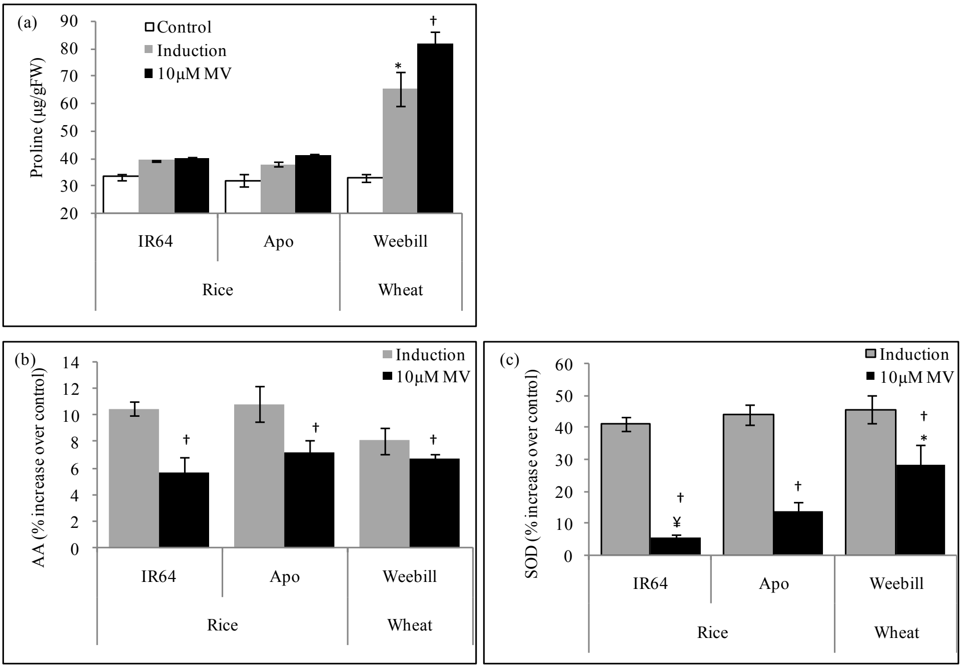

Figure 3: Effect of methyl viologen (MV) on scavenging activity in the induction treatment compared to the severe stress treatment (10 µM MV) in both rice (IR64 and Apo) cultivars and the wheat (Weebill) cultivar (Experiment 1). (a) Proline content. (b) Percent increase in antioxidant activity (AA) over the control measured by the DPPH method. (c) Percent increase in superoxide dismutase (SOD) activity over the control measured at the end of the stress period. †Significant difference between induction and severe stress treatments within cultivars at $p \leq 0.05$. ¥Significant difference between rice cultivars (IR64 and Apo) in the induction treatment at $p \leq 0.05$. *Significant difference between tolerant rice cv. Apo and wheat cv. Weebill in the induction treatment at $p \leq 0.05$.

calreticulin (CRT1) activity did not show any variation between IR64 and Apo (Fig. 5). However, interestingly, the gene involved in proline biosynthesis Δ1-pyrroline-5-carboxylate synthase (P5CS) showed a greater upregulation in the susceptible rice cultivar IR64 (Fig. 5). The transcription response was compared between the tolerant rice cultivar (Apo) and wheat (Weebill). Although ROS-scavenging enzymes FeSOD and CAT showed a marginally greater increase in wheat than in the cultivar Apo, there were no detectable differences in the activity of APX between wheat and the rice cultivar Apo. AKR1 showed significantly higher expression levels in wheat than in cv. Apo. ER stress-responsive genes, CRT1 and BiP1, also showed marginal increases in the transcript level in both tolerant rice (Apo) and wheat (Weebill) cultivars (Fig. 5). Fold change in expression of P5CS was significantly higher in wheat compared to that in tolerant rice cv. Apo (Fig. 5).

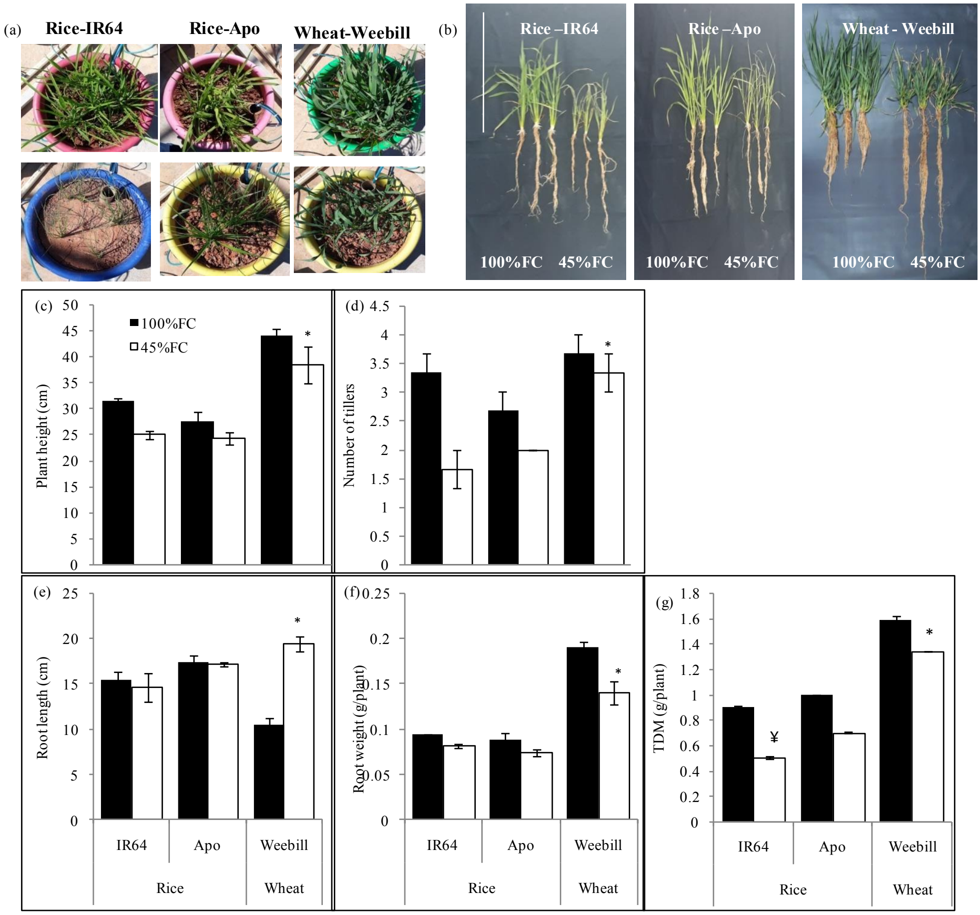

Figure 4: Effect of gradual induction of moisture stress on morphophysiological parameters of IR64 and Apo (rice) and Weebill (wheat) (15 DAS-Experiment 2A). (a) Phenotype of plants in 100% FC and 45% FC at the end of the stress period and (b) 7 days after recovery. Effects of moisture stress on (c) plant height, (d) number of tillers, (e) root length, (f) root weight, and (g) total dry matter (TDM) measured after recovery. Scale bar = 45 cm. ¥Significant difference between rice cultivars (IR64 and Apo) in the induction treatment at $p \leq 0.05$. *Significant difference between tolerant rice cv. Apo and wheat cv. Weebill in the induction treatment at $p \leq 0.05$.

Table 2: Effects of gradual imposition of drought stress on physiological parameters of two rice cultivars (IR64 and Apo) and one wheat cultivar (Weebill) (Experiment 2A). Gradual drought stress was imposed at 15 DAS using an automated irrigation and plant phenotyping platform. Physiological parameters like relative water content (RWC), total chlorophyll, malondialdehyde (MDA), and total proline were measured at 100% FC and 45% FC conditions. $^{¥}$Significant difference between rice cultivars (IR64 and Apo) in the induction treatment at $p \leq 0.05$. *Significant difference between tolerant rice cv. Apo and wheat cv. Weebill in the induction treatment at $p \leq 0.05$.

		Rice		**Wheat**
Parameters	**Treatment**	**IR64**	**Apo**	**Weebill**
RWC (%)	**100%FC**	91.6 ± 2.4	93.4 ± 2.9	88.0 ± 0.9
	45%FC	60.4 ± 5.4	62.5 ± 3.5	62.6 ± 1.2
Total chlorophyll (mg/gFW)	**100%FC**	0.8 ± 0.02	0.7 ± 0.06	1.3 ± 0.07
	45%FC	0.6 ± 0.01	0.6 ± 0.06	1.0 ± 0.12*
MDA (µg/gFW)	**100%FC**	14.4 ± 1.8	63.1 ± 6.5	29.3 ± 3.4
	45%FC	315.4 ± 3.7$^{¥}$	265.7 ± 6.9	247.0 ± 19.7*
Total Proline (µg/gFW)	**100%FC**	75.1 ± 24.1	76.2 ± 6.03	126.1 ± 5.3
	45%FC	396.4 ± 14.8$^{¥}$	259.5 ± 24.4	408.4 ± 33.6*

Higher carbon gain helped wheat to maintain higher growth rates.

At the same soil moisture level of 45% FC, both cereals had lower RWC compared to 100% FC (Fig. 6a). The automated irrigation system maintained the soil moisture status precisely and at the prescribed level irrespective of differences in water mining (roots) and transpiration. Despite similar RWC, cv. Apo maintained a lower canopy temperature at 45% FC compared to cv. IR64. However, in comparison with tolerant rice cv. Apo, wheat had marginally lower canopy temperature (Fig. 6b). Significantly greater assimilation rate and stomatal conductance were maintained in wheat at both 100 and 45% FC than in rice cv. Apo (Figs. 6c and d). Among rice cultivars, susceptible rice cv. IR64 exhibited significantly lower photosynthetic rate and stomatal conductance at 45% FC. As a stress effect, the percent increase in MDA content was significantly lower in wheat than in the drought-tolerant rice cv. Apo (Fig. 6e). Of the rice cultivars, MDA content was significantly higher in cv. IR64 than in Apo at 45% FC. Total biomass was measured on 80 DAS; the percent reduction in total biomass was least in the wheat cultivar Weebill, and it was highest in the rice cultivar IR64. Between the rice cultivars, Apo maintained better carbon gain and hence more biomass accumulation even under 45% FC (Fig. 6f).

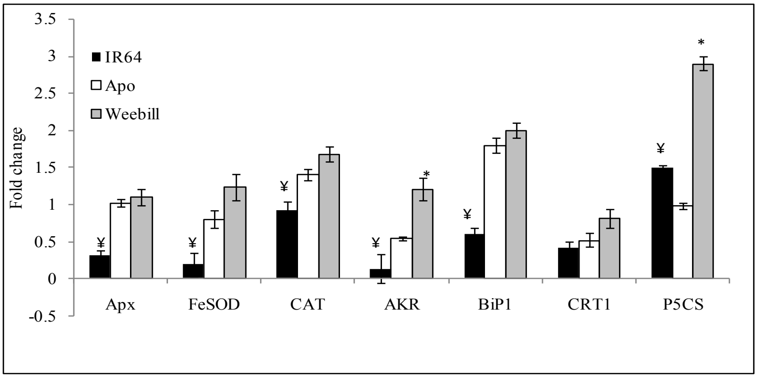

Figure 5: Real-time quantitative RT-PCR analysis for drought responsive genes in cv. IR64 and cv. Apo (rice cultivars) and cv. Weebill (wheat). The second leaf of 100% FC and 45% FC treatments was used from experiment 2A. ¥Significant difference between rice cultivars (IR64 and Apo) in the induction treatment at $p \leq 0.05$. *Significant difference between tolerant rice cv. Apo and wheat cv. Weebill in the induction treatment at $p \leq 0.05$.

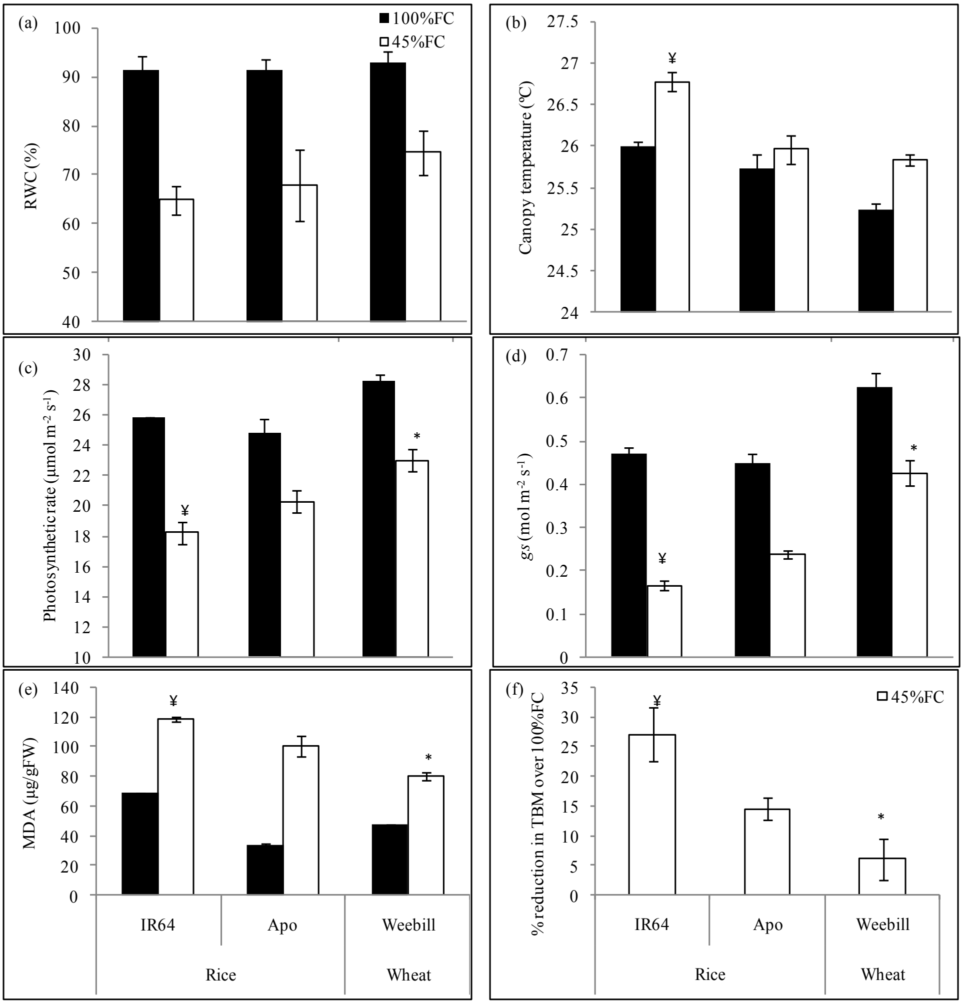

Figure 6: Effect of gradual induction of moisture stress (45% FC) on morphophysiological parameters of IR64 and Apo (rice) and Weebill (wheat) cultivars at the vegetative stage (35 DAS-experiment 2B). (a) Relative water content (RWC). (b) Canopy temperature. (c) Photosynthetic rate. (d) Stomatal conductance (gs). (e) Malondialdehyde (MDA) content was measured during stress. (f) Biomass was measured at 80 DAS and expressed as percent reduction in total biomass (TBM) over 100% FC. $^{¥}$Significant difference between rice cultivars (IR64 and Apo) in the induction treatment at $p \leq 0.05$. *Significant difference between tolerant rice cv. Apo and wheat cv. Weebill in the induction treatment at $p \leq 0.05$.

Discussion

Plants have naturally evolved several mechanisms to survive under stressful conditions. A number of mechanisms have been ascribed to these survival strategies, which range from morphological traits associated with maintenance of tissue turgor to physiological mechanisms that help maintain metabolism. Most of these traits are constitutively present and sometimes may also be responsive to stresses (Sheshshayee et al., 2018). While these constitutive traits have been shown to provide stress adaptation, a set of traits, often referred to as "acquired" tolerance traits, also are known to provide significant levels of tolerance to stresses. These acquired drought tolerance mechanisms or traits are known to be upregulated or expressed only when the plants experience stress, unlike the constitutive traits. In natural conditions, most abiotic stresses like drought and temperature progress gradually and plants would initially experience a mild level of stress and have an opportunity to trigger an acquired response (Mittler, 2006). It is well documented that an array of genes is upregulated when plants experience a progressive stress and mechanisms regulated by these genes subsequently provide tolerance when the stress aggravates and becomes severe (Senthil-Kumar et al., 2003 and 2004). These acquired mechanisms are well documented for temperature tolerance (Senthil-Kumar et al., 2003, Babitha et al., 2015a). Studies have shown that seedlings of the induction treatment had a higher recovery than those exposed directly to the severe stress treatment. In an earlier study with diverse rice genotypes, Raju et al., (2014) reported that the genotypes that had better acquired tolerance (they referred to this trait as cellular level tolerance) maintained higher spikelet fertility. Thus, it can be visualised that a genotype that has a higher propensity to respond to stress at the young seedling stage would also reflect a higher level of tolerance to stress at later phenological stages as well (Raju et al., 2014).

Breeding programs predominantly selected genotypes with higher absolute yields under stress with the premise that high yields under stress would result in an automatic selection of acquired tolerance traits. Although this argument is acceptable, there are significant genotypic and species variations in the mechanisms that govern acquired tolerance. However there was not much research on understanding the component mechanisms leading to drought adaptation through acquired tolerance. The major aim of this investigation was, therefore, to examine the variability in acquired tolerance levels between drought-susceptible (IR64) and drought-tolerant (Apo) rice cultivars and a tolerant wheat cultivar (Weebill). Since wheat can sustain better growth and productivity under water-limited conditions compared to rice, we aimed to understand the differences in the acquired tolerance levels between a wheat cultivar (Weebill) and the two rice cultivars.

Upregulation of protective mechanisms helps in acquiring better tolerance in induction treatment.

Capturing the variability in acquired traits or mechanisms depends on accurate phenotyping for the responses coupled with precise methods to impose induction and severe stress treatments. Many compounds such as methyl viologen (Babitha et al., 2015a), DTT (Babitha et al., 2015b), NaCl (Allakhverdiev et al., 2000), and Rose Bengal (Fischer et al., 2010) have been used to induce stress and capture genetic variability in stress response. Most of these molecules as well as naturally occurring stresses are known to induce an oxidative burst through the generation of reactive oxygen species (ROS). Oxidative stress in turn induces membrane damage and inhibits most metabolic activities, thereby leading to reduced growth rates. Therefore, to maintain the cellular homeostasis, it is essential to either decrease the production of ROS or develop abilities to effectively scavenge and ultimately detoxify the ROS by antioxidants or scavenging enzymes (Sheshshayee et al., 2018; Poljsak et al., 2013). Further, it is also demonstrated that increases in such scavenging enzymes and antioxidants were high only when plants experienced mild induction treatment compared to plants exposed directly to a severe stress treatment. Hence, rice seedlings directly exposed to the severe stress treatment did not show any recovery growth (Figs. 2 and 3). Therefore, it is plausible that variability in production and scavenging mechanisms would determine the susceptibility and tolerance mechanisms of a cultivar. In our study, the susceptible cultivar IR64 showed higher ROS and RCC level damage with lower recovery growth in the induction treatment than the tolerant cv. Apo. Although scavenging mechanisms were comparable between wheat cv. Weebill and tolerant rice cv. Apo, the ROS levels were much lower for wheat, which hence showed better recovery in the induction treatment (Table 1). Recovery hardly occurred after the direct severe stress treatment.

Precise maintenance of soil moisture is essential to capture ADT traits.

Lack of precise stress-imposing methods increased the difficulty in understanding these acquired mechanisms of stress response. The most critical requirement in capturing genetic variability in acquired drought tolerance depends on the ability to maintain the same levels of stress to plants irrespective of differences in evapo-transpiration. To capture the induction response, the current study was done using a phenotyping platform with an automated irrigation facility that dispensed precise volumes of water. The volume of water to be dispensed was determined gravimetrically in real time, and the automated solenoid system dispenses water when the soil moisture status decreased by 1% of the set field capacity. Thus, it was possible to precisely maintain the same level of soil moisture in containers irrespective of differences in transpiration and water mining (Fig. 1). Accordingly, both cereals showed similar leaf tissue water status at 100% FC and at 45% FC irrespective of their differences in leaf area and root traits (Table 2). This task could only be achieved when the containers were irrigated very frequently based on gravimetric determination of water loss. The automated system was also equipped with a software controlled stress imposition protocol that permitted the maintenance of a gradual water loss regime until the containers reached the target of 45% FC from the well-watered status of 100% FC. To achieve this, the software commanded the solenoid valves to dispense only a calculated fraction of the transpired water. This setup provided an excellent option to create gradual induction stress treatment before reaching a severe stress level.

Although tissue water status was similar in both cereals, the reduction in total biomass was significantly different. The rice variety IR64 recorded a higher tiller number in 100% FC, and reduction due to stress was significantly higher. Hence, differences in reduction of biomass were mainly due to the variability in acquired drought-tolerant mechanisms (Fig. 4).

Differences in ROS balance and carbon assimilation determine growth under stress. Imbalance between ROS production and scavenging leads to the generation of a class of secondary radicals referred to as reactive carbonyl compounds (RCCs) through their reactions with lipids and proteins (Niki, 2009; Zimniak, 2011). These RCCs undergo several modifications with proteins by forming Schiff's bases, Maillard products, and Amadori compounds. ROS-scavenging enzymes like SOD and APX provide the first line of defence against oxidative stress. Similarly, RCC-scavenging enzymes like aldo-keto reductases (AKR) catalyze the reduction of aldehydes and ketones to their respective alcohol using the electrons from NADPH (Ramu et al., 2016; Nisarga et al., 2017). Besides these ROS- and RCC-detoxifying strategies, plants have also developed mechanisms to overcome oxidative stress damage through protecting proteins through folding and unfolding mechanisms. A stress-responsive gene, BiP1, is involved in protein folding and protection (Babitha et al., 2015b). The other unfolded protein pathway gene, CRT1, is known to bind to Ca^{2+} in ER and acts as a calcium buffer and helps in the folding of newly synthesized proteins (Babitha et al., 2015b). The rice cv. Apo had higher expression of these scavenging enzymes and unfolded protein response than cv. IR64 (Fig. 5) and hence showed lower ROS level and better recovery growth. Although wheat is more tolerant than rice, a fold increase in expression of these scavenging enzymes and drought-responsive genes was only marginally higher than that in the drought-tolerant rice cultivar Apo (Fig. 5). This could be due to lower levels of ROS production in wheat by reducing excess electron or a better quenching mechanism (Latowski et al., 2013) or by better photochemistry with higher carbon assimilatory capacity. With similar tissue water status, higher photosynthetic rate and higher stomatal conductance might be additional advantages for wheat to maintain higher growth in stress conditions compared with the drought-tolerant rice cv. Apo (Fig. 6).

Conclusion

Expression of acquired tolerance traits is highly dependent on the method of stress imposition. Most reports adopt withholding of water to container-grown plants and record observations when the stress levels reach the target FC. This approach causes a sudden buildup of stress which neither happens in natural field conditions nor gives an opportunity for the plants to stimulate acquired response. Therefore, recovery growth of rice seedlings exposed directly to a severe stress completely retarded compared to induction. Mimicking the stress conditions that occur naturally is, therefore, an important criterion while studying acquired drought responses in plants. In this background, the system used for creating stress is novel and quite precise in creating a gradual drought stress to container-grown plants. This system is also capable of creating a specific and comparable stress regimen independent of differences in transpiration rates between cultivars.

Balancing between ROS production and detoxification is crucial for survival and growth under stress or on its alleviation. Of the two rice cultivars tested, cv. Apo had higher tolerance to stress with higher expression of scavenging enzymes and drought-responsive genes. Wheat seemed to have two strategies: (1) lower production of ROS and RCC hrough the maintenance of tissue turgor and (2) increasing scavenging activity to detoxify free radicals to maintain redox homeostasis. Although cv. Apo seemed to have good scavenging mechanisms, they were not sufficient to detoxify the total ROS produced. Hence, our findings reveal that understanding and further dissecting the acquired drought tolerant mechanism though a precise stress imposition protocol and between-species comparison will help in crop improvement. Improving these acquired mechanisms along with adaptive traits through precise phenotyping for these traits would be very crucial while developing rice varieties suitable for cultivation under the semi-irrigated aerobic conditions.

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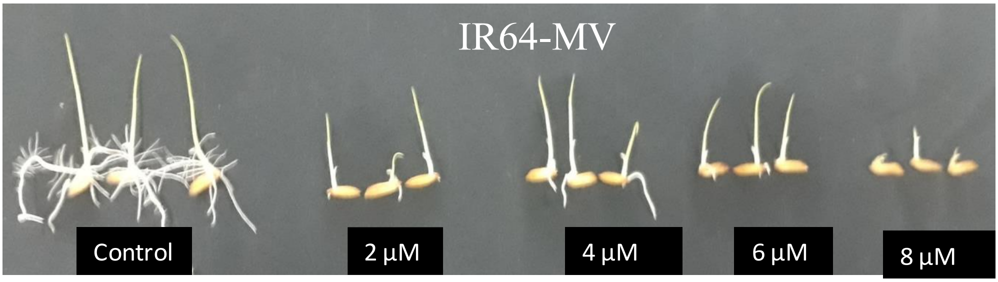
Supplementary information in Chapter 4



Supplementary Figure S1: Standardization of sever dose of MV using rice cultivar IR64. Seedlings were directly transferred to 2, 4, 6 and 8 mM and observations were recorded 48 hours after stress imposition

Supplementary Table S1: Weather parameters during Experiment 2A:

	Air temperature °C		RH (%)		Bright sun shine hours	Rainfall (mm)
	Min	Max	Min	Max		
October 1 to October 7	20.1	27.9	58	90	4.1	47.4
October 8 to October 14	19.4	28.8	55	93	5.1	174.6
October 15 to October 21	18.5	27.2	58	92	5.5	42
October 22 to October 28	18.3	29.1	51	87	9.1	0
October 29 to November 4	18.2	25	69	94	4.6	11.4
November 5 to November 11	16.9	27.2	64	86	4.6	0

Supplementary Table S2: Weather parameters during Experiment 2B:

	Air temperature °C		RH (%)		Bright sun shine hours	Rainfall (mm)
	Min	Max	Min	Max		
January 29 to February 4	11.9	29.1	39	90	10.5	0
February 5 to February 11	13.9	29.9	34	82.9	10.3	0
February 11 to February 18	14.6	29.4	33.6	83.8	10	0
February 19 to February 25	15.8	31.9	35.5	84.8	9.9	0
February 26 to March 4	15.6	30.9	36.3	85.9	9.1	0
March 5 to March 11	20.1	32.3	41	87	8.4	1.4
March 12 to March 18	19.9	32.3	42	84	8.5	0
March 19 to March 25	20.5	33.4	40	78	9.4	0
March 26 toApril 1	20.5	35	35	82	9.3	0
April 2 to April 8	21.5	34.6	38	82	8.6	1.6
April 9 to April 15	22.4	35.7	36	82	8.5	0
April 16 to April 22	21.9	34.6	36	84	7.7	0
April 23 to April 29	22.4	35.4	36	84	8.8	0

Supplementary Table S3: Percent reduction in growth of rice and wheat seedlings due to MV induced stress at (a) end of stress period and (b) after recovery.

(a)

	Cultivars	%reduction in RL over control		%reduction in SL over control	
		Induction	Lethal	Induction	Lethal
Rice	IR64	84.8	100.0	88.9	0.1
	Apo	64.9	98.7	87.6	0.0
Wheat	Weebill	46.0	64.8	68.3	0.3

(b)

	Cultivars	%reduction in RL over control		%reduction in SL over control	
		Induction	Lethal	Induction	Lethal
Rice	IR64	82.8	97.8	84.1	88.2
	Apo	50.5	98.7	75.1	93.5
Wheat	Weebill	28.8	76.0	29.2	65.2

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CHAPTER 5

Production and scavenging of reactive oxygen species confer to different sensitivity of rice and wheat to drought stress

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This chapter is under review

Abstract

Drought poses a serious threat to crop production worldwide. To support crop improvement programmes for developing plants with enhanced tolerance to drought, it is highly desirable to understand drought tolerance differences within and between species. We compared the vegetative growth of two C_3 cereals, rice (IR64, drought susceptible, and Apo, drought tolerant) and wheat (Weebill) plants under well-watered (100% field capacity, 100% FC) and water-limited (60% FC) conditions. The reduction in biomass of wheat was smaller due to a higher rate of photosynthesis associated with maintenance of tissue turgor compared to rice genotypes. Drought caused greater inhibition of Photosystem II quantum efficiency, carboxylation efficiency and regeneration of RuBP in IR64 than in Apo. Transcript levels of photosynthesis-related genes were also significantly more repressed in IR64, whilst the wheat genotype showed smaller reduction than Apo. Despite higher non-photochemical quenching, a smaller increase in scavenging enzymes in IR64 resulted in more accumulation of reactive oxygen species (ROS) in 60% FC than in 100% FC compared to Apo. As a photoprotection mechanism, increased levels of non-photochemical quenching resulted in lower ROS accumulation in wheat despite the similar increase in scavenging enzyme transcript levels as in Apo, signifying the importance of preventing oxidative burst for enhanced drought tolerance. In Apo, upregulation of the 9-*cis-epoxycarotenoid dioxygenase* gene implies the use of xanthophylls pool for the ABA biosynthesis. Our data suggests that regulating photosynthesis and oxidative stress in the wheat genotype enhanced drought tolerance. Improving these traits for rice is crucial to develop drought-tolerant rice genotypes.

Keywords: drought; gene expression; non-photochemical quenching; photosynthesis; reactive oxygen species.

Introduction

Drought stress induces a series of processes leading to the generation of oxidative stress in plants. Limited availability of water causes the closing of stomata, resulting in the excess of available light energy relative to the available intercellular CO_2. Therefore, the quantity of harvested light energy and generated reducing power can easily surpass the rate of its utilization by the Calvin-Benson-Bassham (CBB) cycle, thus causing an accumulation of reactive oxygen species (ROS) (Grieco et al., 2020). Overproduction of ROS in various organelles causes oxidative damage to cell components, and activates programmed cell death pathways. The excessive light absorption of stressed plants exacerbates the inactivation of Photosystem II (PSII) under drought (Valladares and Pearcy, 1997). The combination of drought and high light intensity causes oxidative damage to chloroplasts and results in decreased photosynthesis through enhanced production of ROS (Niyogi, 1999; Davletova et al., 2005).

Plants have evolved several photo-protective mechanisms to manage this excess energy, thereby avoiding or minimizing cellular damage. These mechanisms include thermal dissipation of light energy, increased photorespiration, and enhanced activity of antioxidant systems under drought conditions (Wingler et al., 1999; Müller et al., 2005; Šircelj et al., 2007). Thermal dissipation of excess energy within the light-harvesting complexes (measured as non-photochemical quenching, NPQ) is believed to be an important, quickly activated, regulatory mechanism (Lavaud and Kroth, 2006) involving the xanthophyll cycle (Jahns and Holzwarth, 2012). Increasing NPQ may thus enhance crop performance under stress by reducing cellular damage (Kromdijk et al., 2016; Hubbart et al., 2018). Plants with genetically altered NPQ effectively limit photoinhibition (Krah and Logan, 2010; Hubbart et al., 2018).

Antioxidant enzymes and other enzyme-dependent mechanisms control levels of ROS in plant cells. Over the last two decades, attempts have been made to improve photoprotection through genetic engineering by manipulating antioxidant enzymes (Ishikawa and Shigeoka, 2008; Nisarga et al., 2017). Apart from activating ROS scavenging mechanisms, avoiding ROS production is equally important. A superior photosynthetic efficiency and/or a higher availability of substrate CO_2 would help consume more electrons and hence can significantly reduce ROS production. Therefore, enhancing carbon uptake by maintaining tissue turgor with an improved photosynthetic machinery is one of the best approaches to reduce ROS production.

Rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.) are two C_3 cereals that contribute to over 90% dietary energy requirements of the world. These crops are grown in contrasting environments. While wheat production is predominantly confined to the cool temperature ecosystems, rice is more adapted to tropical climates. These species also differ in their water requirement, with rice requiring much more water than wheat. However, with receding water availability as a result of climate change and competition for water by non-agricultural sectors, there have been serious efforts to explore opportunities of growing rice with less water (Kadam et al., 2015; Ouyang et al., 2017 and 2020; Vijayaraghavareddy et al., 2020a). It is envisaged that rice, if grown like wheat in dry soil, will experience much more photo-oxidative stress than wheat because of its semi-aquatic origin. Hence, comparison between rice and wheat to explore the possible mechanisms to minimize redox burst during drought will provide good leads for crop improvement. In this study, we used two rice genotypes and one wheat genotype to compare their responses to water limitation. Given that managing oxidative stress either through avoiding production of ROS or effectively scavenging them is known for plants to perform well under stress, our major aim was to compare rice and wheat and ascertain how they differ in their drought stress responses in terms of managing oxidative stress.

Materials and Methods

Two rice cultivars, IR64 and Apo, and one wheat cultivar, Weebill, were used in this study. Rice genotypes were classified as drought sensitive (IR64) and tolerant (Apo) on the basis of our previous experiment (Vijayaraghavareddy et al., 2020a). Wheat cultivar Weebill is known to be drought tolerant (Ouyang et al., 2017). The experiment was carried out at University of Agricultural Sciences, Bengaluru, India (12°58’N, 77°35’E) during August to October 2017. Experiments were conducted in controlled greenhouse, set at 22°C/18°C day/night temperature and relative humidity of 60% for wheat. For rice, 29°C/21°C day/night temperature and 60% relative humidity was maintained. Seeds were planted in containers of 20-L capacity filled with sandy loam soil and farm yard manure in the proportion of 3:1 (w/w). Plants were thinned at 15 days after sowing to maintain two plants per pot.

Drought stress imposition and dry matter assessment

A gravimetric method was followed to impose stress. The containers were maintained at 100% field capacity (100% FC) until 30 days after sowing and stress was introduced thereafter. To impose drought stress, irrigation was withheld to a set of containers. Five replicates were maintained for each genotype in each treatment and pots were arranged in a complete randomised design. Both rice genotypes reached 60% FC on the 5th day after stress imposition, and for wheat genotype Weebill it took 7 days to reach 60% FC. All the physiological measurements were made at five days after stress attained to 60% FC. Soil surface was tightly covered with plastic beads to avoid evaporation. Containers were weighed twice a day between 09:00 to 10:00 hours and 15:00 to 16:00 hours. Water lost by transpiration was replenished to maintain target field capacity. The experiment was terminated at booting stage and plants were harvested. Stems, leaves and roots from the plants were separated and oven dried at 60°C for 3 days. Root length was measured and photographs were taken. Total dry matter was computed by summing stem, leaf and root weights.

Osmotic adjustment

The fully expanded leaf was collected and was squeezed using a sterile syringe to extract the sap and osmolality (in mmol L^{-1}) was measured using a vapour pressure osmometer (Vapro, Model 5520; Wescor Inc., Logan, UT, USA). The osmotic potential (MPa) was calculated using:

$$\psi\pi = -cRT\times10^{-9}$$

where c is the osmolality (mmol L^{-1} H_2O), R is the universal gas constant (8314 $L.Pa.K^{-1}.mol^{-1}$) and T is the temperature (37°C, i.e. 310 K). Difference between the turgid osmotic potential of 100% FC and 60% FC was calculated to arrive at osmotic adjustment (Praba et al., 2009).

Total chlorophyll content

Chlorophyll content was determined by incubating a known weight of 10 leaf discs from single plant in 10 mL of acetone (80%): dimethylsulphide (DMSO) (1:1) solution in the dark overnight. Three biological replicates were maintained for each treatment. The absorbance of each extract was measured at 663, 645 and 652 nm using a spectrophotometer. The total chlorophyll content was calculated using the equation given by Arnon (1949) and expressed in mg (g fresh weight)$^{-1}$.

Leaf temperature and epicuticular wax content

The leaf temperature was measured using the Fluke thermal imaging system (Fluke Technologies Pvt. Ltd., Everette, Washington, USA) as described by Vijayaraghavareddy et al., (2020b). Epicuticular wax content was measured by the colorimetric approach based on the colour change generated by the reaction of wax to the acidic $K_2Cr_2O_7$ as described by Mamrutha et al., (2010). The absorbance of samples was measured spectrophotometrically at a wavelength of 590 nm. Carnauba wax (Sigma,St. Louis, Missouri, USA) of different concentrations was used to generate a standard graph (Samdur et al., 2003). The total epicuticular wax was expressed as µg (g fresh weight)$^{-1}$.

Carbon isotope discrimination ($\Delta^{13}C$)

Stable carbon isotope ratio was measured using an Isotope Ratio Mass Spectrometer (Delta Plus, Thermo Fischer scientific, Bremen, Germany), at the National Facility for stable isotopes, Department of Crop Physiology, UAS, Bangalore, India. Dried leaf samples homogenized into a fine powder was used for the estimation of carbon isotope discrimination ($\Delta^{13}C$) (Reddy et al., 2020). The calculation was done as per the notation proposed by Farquhar et al. (1989) considering the carbon isotope composition of air ($\delta^{13}C$) as -8.00‰.

Superoxide accumulation (O_2^-)

Nitrotetrazolium blue chloride (NBT) staining assay in accordance with Vijayaraghavareddy et al., (2020b) was used for the quantification of O_2^- radicals. Leaves excised from the plants were immersed into a 0.2% NBT (prepared in 50mM sodium phosphate buffer, pH = 7.5) and incubated for an hour in darkness. The presence of blue coloured compound, formazan, indicates the presence of superoxide. Later, the chlorophyll was removed using a bleaching solution (acetic acid: glycerol: ethanol (1:1:3, v/v/v) solution). Further, to quantify total O_2^- accumulation, the tissues were ground in 0.1% acetic acid and supernatant obtained at 10,000 rpm for 10 min and absorbance was measured at 560 nm.

Malondialdehyde content

Malondialdehyde (MDA) as the by-product of lipid peroxidation was extracted using 5 mL of 5% trichloro acetic and centrifuged at 12,000 rpm for 15 min. To the extract (1 mL), 2 mL of 0.5% TBA in 20% trichloro acetic was added and incubated at 95°C for 30 min. The reaction was stopped immediately by cooling on an ice bath. Absorbance was recorded at 532 nm and 600 nm and non-specific turbidity was removed by subtracting A600 from A532. Different concentrations of pure MDA (Sigma Aldrich) were used for the standard curve preparation (Nisarga et al., 2017).

Evans Blue staining

The Evans Blue technique, as described by Vijayaraghavareddy et al. (2017), was used for the quantification of membrane damage. Leaf samples from the control and stress treatments were completely immersed in 5 mL of Evans Blue dye solution prepared by dissolving 0.25 g of dye in 0.1 M $CaCl_2$ (pH 5.6). After incubation for 1 hour in darkness, samples were thoroughly rinsed in distilled water to remove unbound dye adhered to the surface. Further, the staining was observed using light microscope (Magnus Analytics, model: MLX).

Gas exchange and chlorophyll fluorescence measurements

Gas exchange and chlorophyll fluorescence parameters were measured using a LICOR-6400XT (Lincoln, NE, USA). Measurements were made on the second fully expanded leaves at five days after stress attained 60% FC. Both the light and CO_2 response curves were measured at 21% oxygen. The CO_2 response curves were measured at a photosynthetic photon flux density (PPFD) of 1500 µmol m^{-2} $s^{-1}$and leaf temperature of 25°C, with ambient CO_2 increased stepwise: 50, 60, 70, 80, 100, 150, 250, 400, 650, 1000, and 1500 µmol mol^{-1}. For light response curves, light intensity was increased stepwise in the order of 30, 50, 70, 100, 200, 500, 1000, 1500, 2000 µmol m^{-2} s^{-1} while keeping the CO_2 concentration constant at 400 µmol mol^{-1}. From these measurements (see Supplementary Figs. S1 and S2), we derived the following photosynthetic parameters:

- CE, the carboxylation efficiency, which was the value of the linear slopes obtained in the initial low part of the CO_2 response curves (i.e. 50, 60, 70, 80 and 100 µmol mol^{-1});
- ϕ_{CO2},quantum efficiency of CO_2 assimilation, which was the value of the linear slopes

of the initial points of the light response curves (i.e. 30, 50, 70, 100 and 200 μmol m^{-2} s^{-1});

- $A_{max(1500)}$, the light saturated (1500 μmol m^{-2} s^{-1}) photosynthetic rate at high CO_2 (1500 μmol mol^{-1});
- $A_{max(400)}$, the light saturated (1500 μmol m^{-2} s^{-1}) photosynthetic rate at ambient CO_2 (400 μmol mol^{-1});
- TPU, the rate of triose phosphate utilization. Our measured photosynthetic rate within high C_i levels of A-C_i curves decreased slightly with increasing C_i in IR64, and increased non-significantly with increasing C_i in both Apo and wheat (see Supplementary Fig. S1), suggesting that TPU limitation most likely occurred. Therefore, we calculated values of TPU, as $(A+R_d)/3$ (Sharkey, 1985), where A is the mean photosynthetic rate at the two highest CO_2 levels (1000 and 1500 μmol mol^{-1}) of A-C_i curves, R_d is day respiration, estimated as the intercept of the linear regression between photosynthetic rate against $PPFD{\cdot}\phi_{PSII}/4$ using the initial points of the light response curves (i.e. 30, 50, 70, 100 and 200 μmol m^{-2} s^{-1}) (Yin et al., 2009, 2011).

For chlorophyll fluorescence measurements, plants were dark adapted for 30 minutes. The Fo and Fm values were measured under low modulated light over a period of 0.8 second and after an application of a 0.8-second pulse of saturating light (~8000 μmol $m^{-2}s^{-1}$), respectively. Actinic light (red light) was applied to measure the steady-state chlorophyll fluorescence (Fs). In the light-adapted state, Fm' was recorded after applying the saturating pulse. Apparent quantum yield of PSII (ϕ_{PSII}) was calculated as: ϕPSII= (Fm'–Fs)/Fm' and non-photochemical dissipation of excitation energy (NPQ) was calculated as: NPQ= (Fm–Fm')/Fm'.

RNA extraction

Total RNA was extracted from leaf tissue harvested from both control and stress plants using the the phenol-chloroform method (Datta et al., 1989). The strand of cDNA was synthesized through reverse transcription mediated by M-MLV reverse transcriptase by an oligo (dT) primer and was used as the template for RT-PCR. The quantitative real-time PCR (qRT-PCR) was performed with the fluorescent dye from TAKARA SYBR Green qPCR Kit. The conditions for the PCR amplification were set as follows: one cycle at 94°C for 3 min, followed by 25 cycles of denaturation at 94°C, 30 s; 52– 58°C, 30 s; 72°C, 40 s and 72°C, 5 min per cycle. All reactions were performed in three replicates for each cDNA sample. The housekeeping ubiquitin gene was used as the reference gene and relative expression of the genes was estimated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Gene-specific primers were designed using Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Ye et al., 2012).

Statistical analysis

Two-way ANOVA with interaction was conducted by using GenStat (15th edition) (http://www.genstat.co.uk/). Least significant difference (LSD) was used to check the significant difference between genotypes and treatments.

Results

Effect of drought stress on growth and biomass

As a result of drought during the vegetative stage, significant reductions in plant height, tiller number and total leaf area were observed in both rice genotypes with stronger reductions in IR64 than in Apo (Fig. 1a). Marginally smaller reductions in wheat genotype Weebill than in rice genotype Apo were observed for these traits (Fig. 1a). Root weight was significantly decreased in IR64 in 60% FC compared with 100% FC. Root length was maintained in both the species and hence no reduction was noticed in 60% FC. Similarly, no significant change in root volume and root weight was found in Apo and Weebill (Fig. 1b). Comparatively, the highest root weight and root length were observed in wheat genotype Weebill in both treatments. Of the rice genotypes, root length and weight were significantly higher in Apo than in IR64 in both treatments (Fig. 1b, c). Stem and leaf weights were significantly reduced in both species with the strongest reduction in rice genotype IR64 (Fig. 1d). In rice genotype Apo and wheat genotype Weebill, reduction in stem weight was ~15% but reduction in leaf weight was significantly higher in Apo than in Weebill. The reduction in total biomass was significantly higher in IR64 than in Apo. Wheat genotype Weebill showed least reduction in biomass (Fig. 1d).

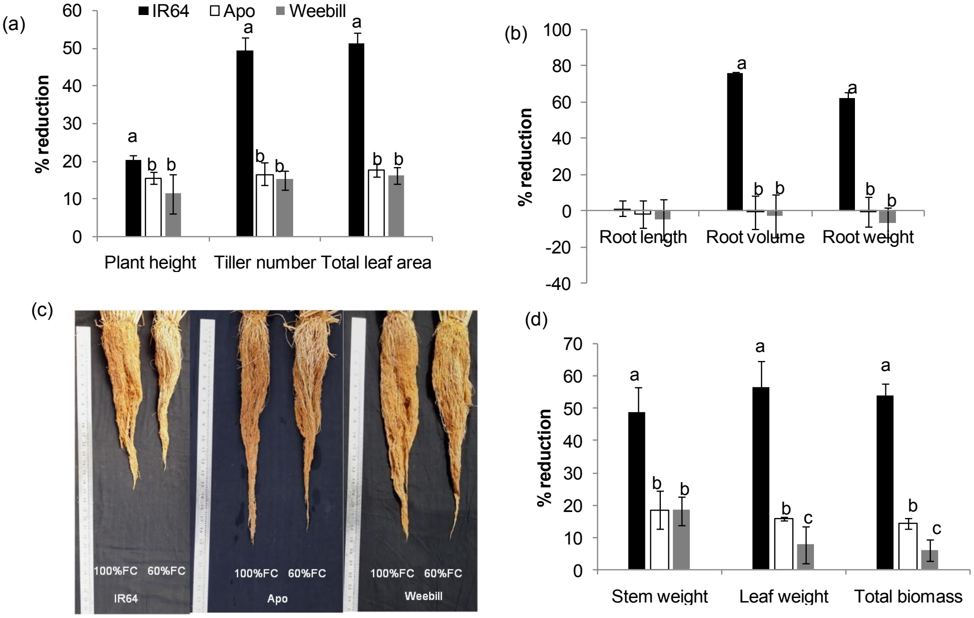

Figure 1: Effect of drought stress on morpho-physiological parameters of two rice genotypes (IR64 and Apo) and one wheat genotype (Weebill). Percent reduction at 60% FC over 100% FC in (a) plant height, tiller number, and total leaf area and in (b) root length, root volume and root weight; (c) photographs of the root systems of the various genotype × treatment combinations; (d) at booting stage, plants were harvested from both 100% and 60% FC to assess the percent reduction at the booting stage in stem weight, leaf weight, and total biomass. Error bars indicate the standard error of the mean (mean ± SE). Different letters indicate significance ($P<0.05$) from the LSD post-hoc test determined using two-way ANOVA with genotype × treatment interactions.

Physiological responses to drought

Wheat genotype Weebill showed stronger osmotic adjustment in 60% FC than the rice genotypes, with IR64 showing stronger osmotic adjustment than Apo (Fig. 2a). Total chlorophyll content was marginally reduced in IR64, whereas Apo and wheat genotype Weebill showed no marked differences between treatments (Fig. 2b). The wax content of wheat genotype Weebill was higher than that of the rice genotypes in both 100% and 60% FC. Among rice genotypes, wax load was significantly increased in Apo, whereas IR64 showed no difference between treatments (Fig. 2c). With higher wax content, Weebill maintained lower leaf temperature in both treatments. Similarly, between rice genotypes, increase in leaf temperature in 60% FC over 100% FC was lower for Apo than for IR64 (Fig. 2d). Carbon isotope discrimination ($\Delta^{13}C$) of the leaf was used as surrogate of water use efficiency (WUE). Wheat showed higher $\Delta^{13}C$ in 100% FC than rice, suggesting lower WUE. In 60% FC, Apo showed significantly higher WUE than IR64 and wheat genotype Weebill. The percent increase in WUE at 60% FC was significantly higher in Weebill than in the rice genotypes (Fig. 2e).

Accumulation of free radicals and their scavenging

Water limitation significantly induced the accumulation of free radicals in both rice and wheat. Of the rice genotypes, IR64 showed higher accumulation of superoxide (O_2^-) and malondialdehyde (MDA) content in 60% FC than Apo. Levels of O_2^- and MDA were significantly lower in wheat genotype Weebill than in rice genotypes (Fig. 3a, b). With more free radicals in IR64, significant membrane damage was observed in 60% FC using Evans Blue staining technique. Compared with the rice genotypes, membrane damage was significantly lower in Weebill at 60% FC (Fig. 3c). The expression of scavenging enzymes, ascorbate peroxidase (APX), iron containing superoxide dismutase (FeSOD), catalase (CAT) and aldo-keto reductase (AKR), was significantly upregulated in 60% FC in both species. Comparatively, fold change in expression at 60% FC over 100% FC was significantly lower in IR64 than in Apo and Weebill. The fold change in expression of these enzymes was not significantly different between rice genotype Apo and wheat genotype Weebill (Fig. 3d).

Effect of drought on photosynthesis parameters

Weebill maintained a higher stomatal conductance than both rice genotypes. Of the rice genotypes, IR64 showed significantly higher reduction in stomatal conductance in 60% FC compared with 100% FC than Apo did (Fig. 4a). The light saturated maximum photosynthetic rate under ambient CO_2 ($A_{max(400)}$) and that under high CO_2 ($A_{max(1500)}$) conditions were significantly higher for wheat than for the rice genotypes. Of the rice genotypes, reduction in $A_{max(400)}$and $A_{max(1500)}$was significantly lower in Apo than in IR64 (Fig. 4b, c). Quantum efficiencies of CO_2 assimilation (ϕ_{CO2}) and carboxylation efficiency (CE) were significantly affected in both species; comparatively a stronger reduction was noticed in rice genotype IR64 than in Apo. But wheat maintained significantly higher ϕ_{CO2} and CE in both 100% FC and 60% FC than the rice genotypes (Fig. 4d, e). Similarly, rate of triose phosphate utilization (TPU) was also significantly higher for wheat in both water regimes than in rice. Between the rice genotypes, the reduction of TPU was significantly higher for IR64 than for Apo (Fig. 4f). Chlorophyll fluorescence measurements were carried out to assess non-photochemical quenching (NPQ) and quantum efficiency of PSII (ϕ_{PSII}). NPQ was significantly higher in both rice genotypes at 100% FC compared with the wheat genotype Weebill. In 60% FC, although no significant difference was observed within and between species, the percent increase in NPQ was significantly higher for wheat genotype Weebill than for the rice genotypes (Fig. 4g). IR64 did not show any significant difference in NPQ values between 100% and 60% FC (Fig. 4g). The ϕ_{PSII} was significantly lower in both species at 60% FC than at 100% FC. Between the species, reduction in ϕ_{PSII} was lower in wheat genotype Weebill and of the rice genotypes, Apo showed lower reduction than IR64 (Fig. 4h).

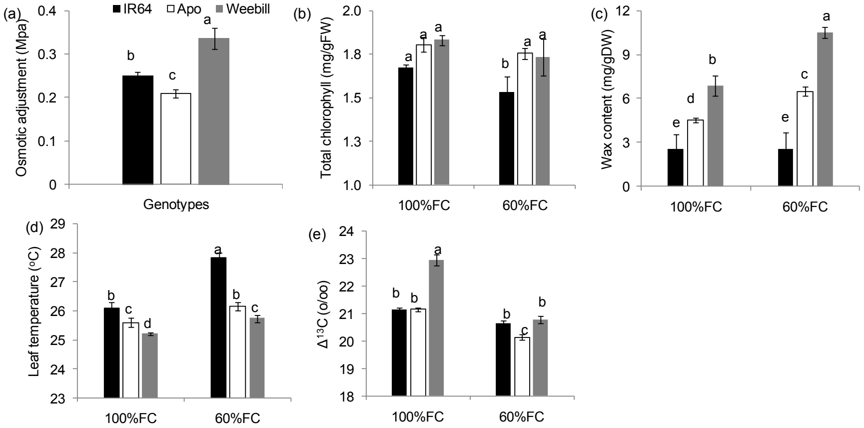

Figure 2: Effect of drought on physiological parameters of two rice genotypes (IR64 and Apo) and one wheat genotype (Weebill) at 100% and 60% FC. (a) osmotic adjustment at 60% FC; (b) total chlorophyll content; (c) epicuticular wax content; (d) leaf temperature; (e) carbon isotope discrimination ($\Delta^{13}C$). Error bars indicate the standard error of the mean (mean ± SE). Different letters indicate significance ($P<0.05$) from the LSD post-hoc test determined using two-way ANOVA with genotype × treatment interactions.

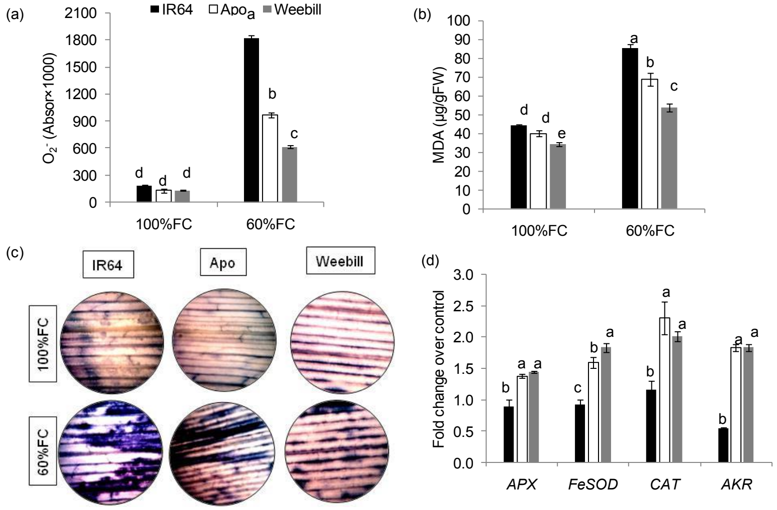

Figure 3: Effect of drought on ROS accumulation and scavenging in two rice genotypes (IR64 and Apo) and one wheat genotype (Weebill): (a) superoxide (O_2^-); (b) malondialdehyde (MDA) content; (c) photographs of Evans blue staining; (d) fold change in expression levels of scavenging enzymes: ascorbate peroxidase (APX), iron containing superoxide dismutase (FeSOD), catalase (CAT) and aldo-keto reductase (AKR). Error bars indicate the standard error of the mean (mean ± SE). Different letters on (a) and (b) indicate significance ($P<0.05$) from the LSD post-hoc test determined using two-way ANOVA with genotype × treatment interactions. One-way ANOVA was carried out to determine significant difference between genotypes for gene expression analysis (d).

Expression of photosynthesis-associated genes

To assess the effect of drought on photosynthesis-associated genes, expression studies were done for selected genes. PSI P700 apoprotein A1 (*psaA*), and Photosystem II protein D1 (*psbA*) expression levels were significantly downregulated in 60% FC compared with 100% FC in rice genotypes with a stronger reduction in IR64 than in Apo. No significant difference between 100% and 60% FC was observed in transcript levels of both *psaA* and *psbA* in the wheat genotype Weebill (Fig. 5a). Similarly, no significant change in expression levels of light-harvesting chlorophyll-protein 2 (*LHCP2*) was observed in Weebill. Of the rice genotypes, Apo maintained higher transcript levels than IR64 in 60% FC compared with 100% FC (Fig. 5a).

Expression levels of RuBisCO small subunit (*rbcS*) and large subunit (*rbcL*) were significantly downregulated in both rice genotypes, and, comparatively, reduction was higher in IR64 than in Apo. The reduction in *rbcS* and *rbcL* expression in 60% FC compared with 100% FC was very marginal in wheat genotype Weebill (Fig. 5b). Transcript levels of triose phosphate isomerase 1 (*TPI1*) were downregulated in both species with higher reduction in rice genotype IR64 than Apo. A similar trend was observed for fructose-1,6-bisphosphatase (*FBPase*) and sedoheptulose-1,7-biphosphatase (*SBPase*) transcript levels. Relative expression values of *TPI1*, *FBPase* and *SBPase* transcripts were similar for rice genotype Apo and wheat genotype Weebill at 60% FC (Fig. 5b).

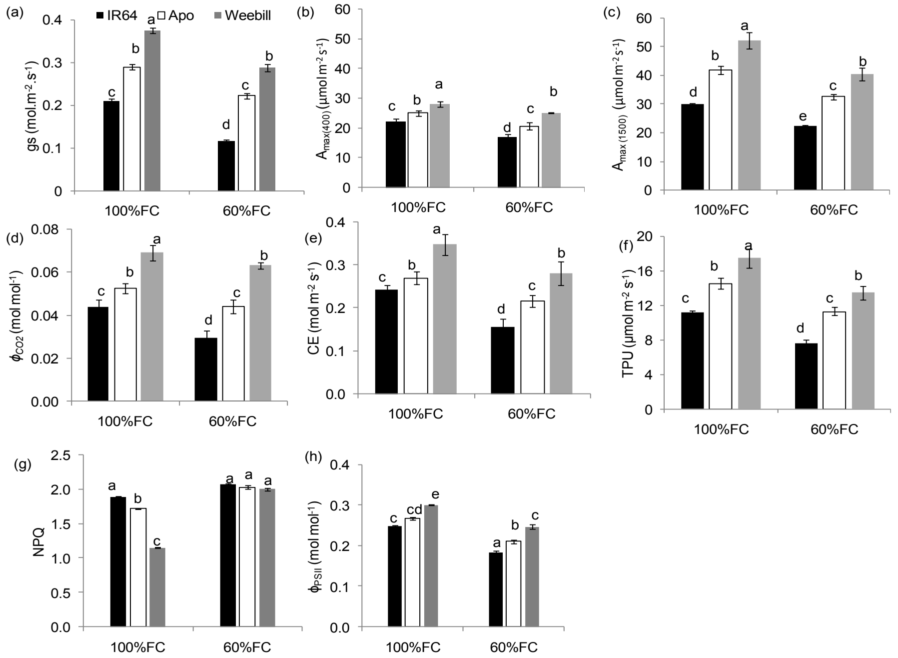

Figure 4: Effect of drought in two rice genotypes (IR64 and Apo) and one wheat genotype (Weebill). (a) stomatal conductance (gs) at 1500 µmol m^{-2} s^{-1} of light intensity and ambient [CO_2]; (b) light-saturated maximum photosynthetic rate under ambient [CO_2] ($A_{max(400)}$); (c) light-saturated maximum photosynthetic rate at high [CO_2] ($A_{max(1500)}$); (d) quantum efficiency on the incident light basis(ϕ_{CO2}); (e) carboxylation efficiency (CE); (f) triose phosphate utilization (TPU); (g) non-photochemical quenching (NPQ) at 1500 µmol $m^{-2}s^{-1}$ of light intensity and ambient [CO_2]; and (h) apparent quantum yield of PSII (ϕ_{PSII}) at 1500 µmol $m^{-2}s^{-1}$ of light intensity and ambient [CO_2]. Error bars indicate the standard error of mean (mean ± SE). Different letters indicate significance (*P*<0.05) from the LSD post-hoc test determined using two-way ANOVA with genotype × treatment interactions.

Expression of xanthophyll cycle and ABA genes

Expression of *zeaxanthin epoxidase* (*ZEP*) and *violaxanthin de-epoxidase* (*VDE*) genes was upregulated in both species in 60% FC compared with 100% FC. The relative expression levels of *ZEP* were significantly higher in rice genotype Apo, whereas expression was similar for IR64 and wheat genotype Weebill in 60% FC (Fig. 6a). *VDE* expression was significantly higher in the wheat genotype compared with both rice genotypes at 60% FC. Relative expression levels of *VDE* for both rice genotypes were similar at 60% FC (Fig. 6b). Since the xanthophyll cycle is also involved in ABA biosynthesis, expression of *9-cis-epoxycarotenoid dioxygenase* (*NCED*) was analysed. A key biosynthetic gene *NCED* expression was significantly upregulated in Apo compared with IR64 (Fig. 6c).

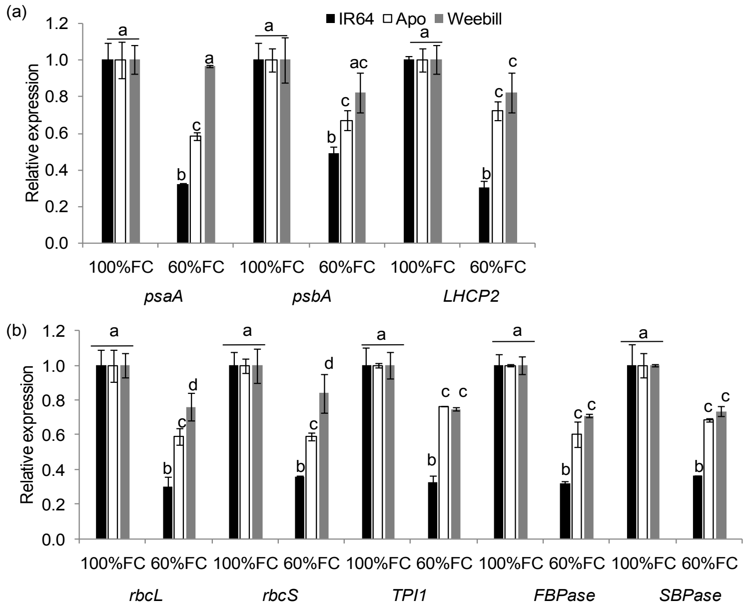

Figure 5: Effect of drought in two rice genotypes (IR64 and Apo) and one wheat genotype (Weebill) on expression of photosynthetic genes. (a) PSI P700 apoprotein A1 (*psaA*), Photosystem II protein D1 (*psbA*), and light-harvesting chlorophyll-protein 2 (*LHCP2*) and expression of genes associated with Calvin-Benson-Bassham (CBB) cycle (b) RuBisCO large subunit (*rbcL*)and small subunit (*rbcS*), triose phosphate isomerase 1 (*TPI1*), fructose-1,6-bisphosphatase (*FBPase*) and sedoheptulose-1,7-biphosphatase (*SBPase*). Error bars indicate the standard error of the mean (mean ± SE). Different letters indicate significance (P<0.05) from the LSD post-hoc test determined using two-way ANOVA with genotype × treatment interactions.

Discussion

Drought stress will negatively influence the plant processes mainly by decreasing the carbon gain due to stomatal closure to minimize water loss (Sheshshayee et al., 2018). The reduction in activity of the CBB cycle combined with a high rate of electron flow due to high light intensities produces ROS (Foyer, 2018). In order to mitigate ROS production, plants have developed protective mechanisms such as quenching of excess electrons, and scavenging them by antioxidants and enzymes (Kasajima et al., 2011). Comparative studies within and between species have great relevance in understanding these drought responsive mechanisms. In this study, two rice genotypes (IR64 and Apo) and one wheat genotype (Weebill) were compared to assess the drought responsive mechanisms by gravimetric approaches during the vegetative phase. The effects of drought on tissue turgor, water use efficiency, photosynthetic capacity, photosynthetic efficiency and regulation of oxidative stress through quenching of excess electrons and/or by scavenging were captured in rice and wheat.

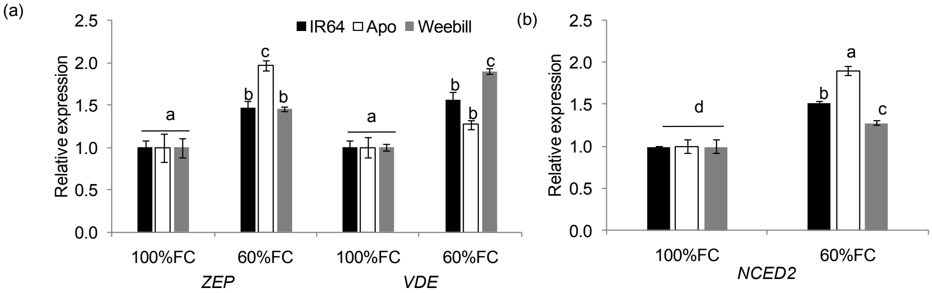

Figure 6: Effect of drought in two rice genotypes (IR64 and Apo) and one wheat genotype (Weebill) on gene expression of (a) zeaxanthin epoxidase (*ZEP*) and violaxanthin de-epoxidase (*VDE*); (b) 9-cis-epoxycarotenoid dioxygenase (*NCED*). Error bars indicate the standard error of the mean (mean ± SE). Different letters indicate significance ($P<0.05$) from the LSD post-hoc test determined using two-way ANOVA with genotype × treatment interactions.

Wheat has higher carbon gain through maintenance of higher leaf turgor and lower leaf temperature than rice varieties.

Maintenance of tissue turgor is crucial to sustain growth and productivity under stress conditions. Osmotic adjustment is one such important adaptive process which can reduce some of the harmful effects of drought through accumulation of solutes to maintain tissue turgor (Hatzig et al., 2014). Additionally, deposition of epicuticular waxes to avoid heat load on leaves by reflecting radiation is proven to impart drought tolerance by decreasing leaf temperature. In this study, wheat with its extensive root system maintained significantly higher wax content and osmotic adjustment and hence maintained cooler leaves. Of the rice genotypes, although IR64 maintained higher osmotic adjustment, less ability to increase wax accumulation resulted in higher leaf temperature and hence no decrease in WUE (represented by $\Delta^{13}C$) was noticed compared to Apo (Fig. 2). IR64 is adapted to the lowland ecosystem and hence even a small decrease in water availability will negatively influence the plant metabolism (Vijayaraghavareddy et al., 2020a). Therefore the increase in osmotic adjustment may not be sufficient to maintain metabolism of plants under water limitation. Lowering leaf temperature in stress condition is a great advantage to minimizing water loss. It is clear from Fig. 2 that the increased wax load attributed to the lower leaf temperature and increased water use efficiency. In wheat genotype Weebill and tolerant rice genotype Apo, a significant increase in wax load resulted in increased WUE compared with IR64. In Apo, although osmotic adjustment was lower, increased wax load might be an adaptive trait helping sustain growth under water limitation (Fig. 2). These results are in agreement with a study by Cheng et al. (2020), who found that enhanced cuticular wax biosynthesis has relevance in increasing drought tolerance by decreasing water use. Hence these traits which maintain tissue turgor have great relevance in imparting drought tolerance due to its close association with photosynthetic rate by influencing stomatal conductance (You and Chan, 2015).

Regulation of photosynthesis contributes to drought tolerance

Photosynthesis is one of the primary processes affected by drought, due not only to stomatal closure but also to alterations in the photosynthetic metabolism (Lawlor and Cornic, 2002) and in antenna size and quantum efficiency of Photosystem II (PSII) (Hao and Papadopoulos, 1999).Water limitation results in downregulation or degradation of RuBisCO and CBB cycle enzymes and hence reduces carbon fixation (Feller et al., 2008). The initial slope of CO_2 response curves represents the carboxylation efficiency (CE) of RuBisCO and the photosynthetic rate at ambient CO_2 concentration ($A_{max(400)}$) directly reflects the capacity of RuBisCO carboxylation (Von Caemmerer and Farquhar 1981; Lawlor and Cornic, 2002). Additionally, TPU is a mechanism by which triose phosphate, the first reduced compound of the CBB cycle, is utilised in starch and sucrose synthesis. In stress conditions, if the starch and sucrose synthesis will not increase with increasing photosynthetic rate at highest substomatal CO_2 concentration, then the Pi needed for ATP synthesis will be decreased (Kiirats et al., 2009; Fabre et al., 2019).TPU limitation in stress conditions triggers the energy dependent quenching and as a consequence, it deactivates RuBisCO (Takizawa et al., 2008). In our study, higher carboxylation efficiency indicates better protection of CBB cycle enzymes in wheat than in rice genotypes from photodamage (Fig. 4e,f).This was evident from the higher transcript levels of RuBisCO subunits (*rbcs* and *rbcl*) for better photosynthesis along with regeneration of RuBP through maintenance of *FBPase* and *SBPase* transcript levels in wheat at 60% FC than in the rice genotypes (Fig. 5b). Similarly the end product synthesis capacity relative to carbon fixation was significantly higher for wheat compared with the rice genotypes which is indicated by higher TPU and TPI1 expression levels. Between rice genotypes, IR64 showed higher sensitivity to drought with significant reduction in carboxylation efficiency and TPU compared with Apo.

As consequence of reduced carbon fixation the utilization of NADPH decreases which results in overloading of electron transport chain due to reduced supply of NADP. This will lead to the formation of ROS via the Mehler reaction (Ramu et al., 2020). The increased ROS production causes photoinhibition of PSII and also suppresses the *de nova* synthesis of PSII repair proteins (Aro et al., 2005). But the degree of photoinhibition is highly dependent on the balance between damage to PSII (mainly the D1 and D2 proteins) and activation of the repair process (Nishiyama et al., 2006; Nelson and Yocum, 2006). As a result, many genes associated with photosynthetic pathways will be highly altered to maintain metabolism (Wong et al., 2006). Ghotbi-Ravandi et al., (2014) reported that a drought-sensitive genotype of barley exhibited a larger decrease in D1 protein content and quantum yield in response to severe drought stress than a tolerant genotype. In our study, higher rates of D1 protein turnover in wheat provided stability to photosynthetic electron transport rate from damaging actions of ROS and hence resulted in higher quantum efficiency compared to rice genotypes. Between the rice genotypes the highest reduction in quantum efficiency was noticed in IR64 indicating the sensitivity of the genotype to drought (Fig. 4d) and hence triggering the activation of photoprotection mechanisms resulting in competition between thermal dissipation and photochemical reaction (Zhu et al., 2004). Besides quantum yield, measurement of maximum photosynthetic rate at non-limiting CO_2 and light conditions explains the non-diffusive limitations on photosynthesis ($A_{max(1500)}$). Between species, $A_{max(1500)}$, which is an indicator of J_{max} (maximum electron transport rate), was significantly higher in wheat than in rice genotypes, indicating superior photosynthetic capacity in wheat compared with rice in both water regimes. Between the rice genotypes, $A_{max(1500)}$was significantly more decreased in IR64 indicating the inhibition effects of drought on photosynthesis in this sensitive rice genotype compared with the tolerant rice genotype Apo (Fig. 4c).

Balancing ROS production and scavenging imparts drought tolerance in wheat

The suppression to carbon fixation and PSII repair exacerbate the ROS generation in drought conditions. The balance between ROS production and their scavenging is most important to sustain growth under stress (Asada, 1999). The published information suggests the existence of significant genetic diversity for this ROS regulation (Lekshmy et al., 2021). Additionally, plants upregulate an extensive spectrum of antioxidants and scavenging enzymes to ensure optimum cellular ROS (Kanzaki et al., 2017). It was always predicted that the level of increase in scavenging mechanisms to detoxify ROS determines the drought tolerance of plants. But in one of our earlier studies, we observed a similar increase in transcript levels of scavenging enzymes in 30-day-old seedlings for wheat genotype Weebill and tolerant rice genotype Apo (Vijayaraghavareddy et al., 2020b). Our present study also confirms that the accumulation of ROS in wheat was significantly lower than in rice genotype Apo despite similar increases in transcript levels of scavenging enzymes (Fig. 3). This could be due to an upregulation of mechanisms to prevent production of ROS itself which could be very high in wheat and hence there is no need to further increase transcript levels of scavenging enzymes.

Increased non-photochemical quenching in wheat prevents the redox burst

Increasing NPQ is often considered as a rapid feedback response in stress conditions by dissipating excess light energy to prevent further ROS formation (Ballottari et al., 2007). Therefore, NPQ is one of the important mechanisms to avoid ROS hyper accumulation during drought. The increase in NPQ values suggests that the photoprotection mechanism may aid in the stress tolerance in wheat plants by restraining the ROS production but not in rice genotype

Apo. The higher ROS accumulation in IR64 may be due to stronger photoinhibition and lower transcript levels of scavenging genes compared with genotype Apo (Fig. 3). The molecular regulation of NPQ depends on two important genes of xanthophylls cycle, zeaxanthin epoxidase (*ZEP*) and violoxanthin de-epoxidase (*VDE*) (Jahns et al., 2009). Suppression of these genes results in lower NPQ levels with higher damage to PSII (Niyogi et al., 1998). In this study, the increases in NPQ were also evident from the expression of *ZEP* and *VDE*, both being upregulated significantly in wheat and the rice genotypes but the level of upregulation was very distinct in these genotypes (Fig. 6a).The xanthophyll precursor pool also plays an important role in ABA biosynthesis (Zhou et al., 2015). In stress conditions, both *ZEP* and 9-*cis-epoxycarotenoid dioxygenase* (*NCED*) transcript levels will increase to reduce water loss through ABA accumulation (Ye et al., 2011). But the higher expression of *VDE* to regulate the xanthophylls cycle has shown to antagonise the ABA biosynthesis (Zhou et al., 2015). Therefore, the levels of *VDE* expression will determine the NPQ and ABA accumulation. In wheat and rice genotype IR64, the higher transcript levels of *VDE* resulted in lower ABA biosynthesis which was evident from lower expression of *NCED2*. Although NPQ and *VDE* were significantly increased in IR64, the higher ROS accumulation could be due to its adaptability to lowland conditions and hence 60% FC will be a more severe stress in IR64 than in the aerobic cultivar Apo. However, Apo showed lower *VDE* levels but higher *NCED2* expression indicating utilization of the xanthophyll pool for ABA biosynthesis (Fig. 6).

Conclusions

Under drought stress conditions, photosynthesis processes are negatively affected but significant diversity exists for the extent of damage. This study using C_3 cereals rice and wheat shows distinct variations in their adaptive mechanisms to drought. The wheat genotype with its stronger adaptive mechanisms to maintain tissue turgor sustained growth under stress by increasing WUE with less reduction in carbon assimilation than in both rice genotypes. Within rice, the tolerant genotype Apo showed higher WUE at 60% FC than IR64 due to lower leaf temperature through accumulation of epicuticular waxes. With the maintenance of tissue turgor, both wheat and tolerant rice genotype Apo maintained higher photosynthetic capacity and efficiency than IR64. Lower decrease in transcript levels of genes associated with photosynthesis also suggests less damage to photosynthetic machinery in wheat and rice genotype Apo than in IR64. Apo was also no match to the wheat genotype Weebill in regulating photosynthesis even in well-watered (100% FC) conditions. Other than scavenging of ROS, the higher NPQ levels and maintenance of photosynthesis in wheat prevented the ROS formation compared with drought tolerant rice genotype Apo. Although IR64 showed higher NPQ, the biomass of IR64 was lower than that of Apo because of more damage to photosynthetic machinery associated with lower increase in scavenging enzymes. Unlike in rice, the maintenance of superior photosynthetic machinery and redox balance in wheat helped sustain growth under stress conditions. Hence, better managing of ROS production need to be addressed for improving rice adaptation to drought.

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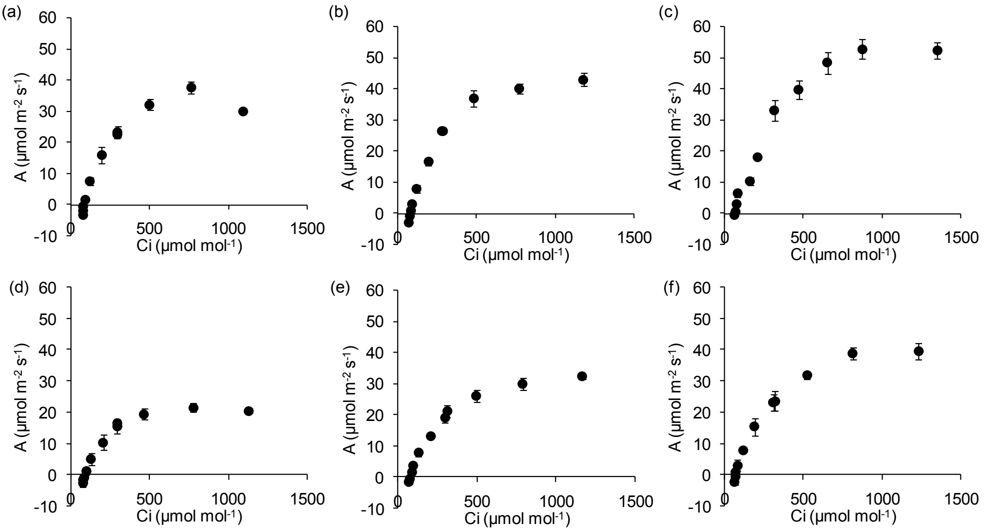
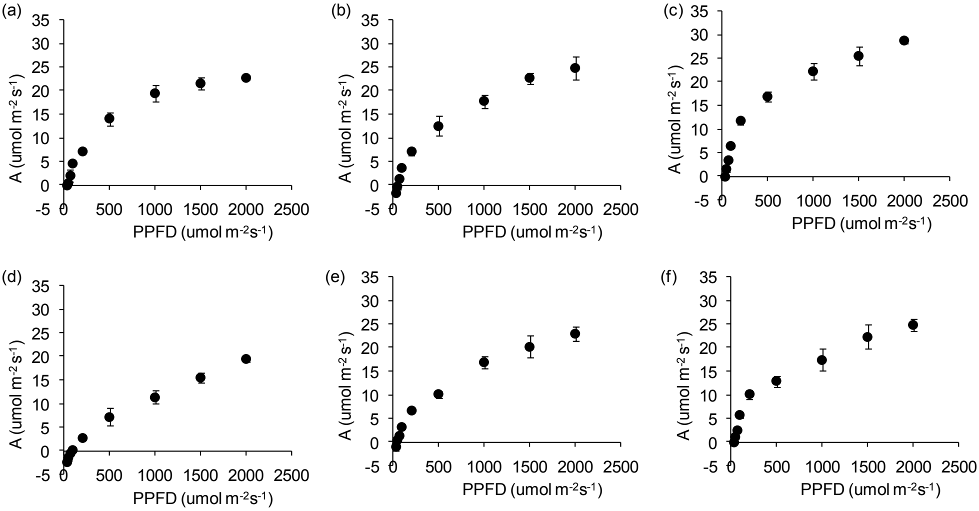
Supplementary information in Chapter 4



Supplementary Figure S1: CO_2 response curves for photosynthetic rate (A) in rice and wheat genotypes grown at two water regimes, 100% FC (a-c) and 60% FC (d-f). Data presented for rice genotypes IR64 (a and d) and Apo (b and e) and for wheat genotype Weebill (c and f). Error bars indicate the standard error of the mean (mean ± SE).



Supplementary Figure S2: Light response curves for photosynthetic rate (A) in rice and wheat genotypes grown at two water regimes, 100% FC (a-c) and 60% FC (d-f). Data presented for rice genotypes IR64 (a and d) and Apo (b and e) and for wheat genotype Weebill (c and f). Error bars indicate the standard error of the mean (mean ± SE).

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CHAPTER 6

General discussion

A constant effort was made towards genetic enhancement to develop rice cultivars that have greater resilience to water-limited conditions as well as in devising appropriate cultivation practices that save water. Genetic enhancement has largely relied on selecting for high absolute yield under water-limiting conditions (Venuprasad et al., 2007). Although this approach showed initial success, this strategy of selection is not expected to provide the required breakthrough in improving yield under drought, because genetic variation in yield among the already improved cultivars is limited and a significant G×E interaction for yield *per se* may further complicate yield improvement (Araus et al., 2008; Reynolds et., 2009; Fischer et al., 2014). Therefore, the concept of physiological breeding is getting more attention to improve yield under drought (Reynolds and Langridge, 2016).

A successful physiological breeding programme requires identification of superior traits for drought tolerance through phenomics and genomics. Significant progress has been made in phenomics by developing high-throughput phenotyping platforms combined with imaging tools to efficiently capture traits of interest (Granier and Vile, 2014). With the availability of genomics and phenomics technologies, comparisons between genotypes can now be made with more precision, allowing to identify and dissect complex drought tolerance traits. In previous chapters of this thesis, I have made an attempt to compare rice genotypes, which are differing in their adaptability to drought, amongst themselves as well as with another C_3 cereal, i.e. wheat. Here in this Chapter, I will discuss, from a broad perspective, the main highlights of the thesis and how these important findings can contribute to improving the drought tolerance in rice.

Stress response of rice genotypes adapted to different ecosystems

Among cereal crops, rice is the major consumer of fresh water, but the decline in irrigation resources is posing a great challenge to realize the expected rice productivity (Asharf, 2010). The diminishing water supply for rice production led to the development of water-saving cultivation practices like the aerobic production system (Chapter 1). The aerobic cultivation, like the upland rice practices, resulted in higher water productivity, but the observed yields were significantly lower than those in the lowland cultivation system. Bouman et al. (2005) reported 32% yield reduction in an aerobic condition compared with the puddle condition. Therefore, reaching high yields under limited water supply requires new rice cultivars with drought tolerance characteristics as well as high yielding potential (Lafitte et al., 2002). To achieve this, rice genotypes adapted to different ecosystems need to be compared and characterized.

The physiological evaluation in this thesis confirmed that the negative effect of drought on productivity was smaller for the aerobic rice cultivar compared with the cultivar adapted to lowland conditions. Although the upland cultivar maintained lower reduction in yield compared with lowland types, the actual yields under well-watered conditions were very low (Chapter 2). Hence a low-yield potential is inhibiting a wide spread of upland cultivars (Lafitte et al., 2002). When comparing the aerobic cultivar and the lowland cultivar, the aerobic cultivar performed superiorly irrespective of stage of occurrence, duration and intensity of stress (Chapters 2-5). This effect of drought on genotypes adapted to different ecosystems that we observed is similar to results in other studies: the aerobic genotypes are highly tolerant to drought stress occurring at different growth stages (Zaman et al., 2018). Genotypes developed for such cultivation practices will be often highly water-use efficient when subjected to drought conditions (Bouman et al., 2006). Similarly, this thesis showed that the water use efficiency (WUE) of the aerobic rice cultivar increased under stress without much decrease in yield, but the increase in WUE was not observed in the lowland cultivar (Chapters 2 and 4). This indicates the differential drought response of these cultivars adapted to different ecosystems.

Many constitutive and drought adaptive traits will contribute to the differences in WUE (Blum, 2009). A deeper root system promotes soil moisture capture thus helping maintaining plant metabolic processes (Sheshshayee et al., 2018). This thesis clearly demonstrates the differential response of root growth between aerobic and lowland cultivars at different water regimes (Chapters 2 and 5). Aerobic cultivars are reported as deeper rooting types both in aerobic and drought stress conditions which is attributed to their ability to adapt to soil conditions with lower availability of moisture. Hence increased root growth helps maintain higher stomatal conductance, lower canopy temperature and more carbon gain due to more uptake of water compared with shallow rooted genotypes (Phule et al., 2019). The increased root length in the aerobic rice cultivar was attributed to lower leaf temperature and maintenance of a higher source activity compared with lowland cultivar. Despite higher yield under drought, a significant reduction in yield was also observed in the aerobic cultivar when stress occurred at critical growth stages. Hence the challenge is to further improve the aerobic cultivar to decrease the yield reduction under drought. This could be possible by identifying and introgressing specific drought adaptive traits into aerobic cultivars.

Role of drought adaptive traits at specific growth stages

As explained above, improving the aerobic cultivars is the best option to increase crop productivity under limited water supply. To achieve this, there is a need to identify specific important drought adaptive traits through comparison between genotypes of a species. Apart from intensity and frequency of drought, the phenological timing at which drought occurs is equally important; the impact of drought on overall performance of the crop differs greatly depending on the growth stage during which the drought occurs (Sarto et al., 2017). The traits that contribute to final grain yield differ at each growth stage, and the extent to which these are impacted by drought determines the tolerance of the genotype. Indeed, severe drought occurrence at flowering results in crop failure, but several previous studies reported significant yield loss also when drought occurs at the vegetative or the grain filling stage (Wang et al., 2010; Kadam et al., 2015). More information on this is required. Understanding all responses to drought during various stages could be achieved by comparing rice genotypes at different growth stages to refine the limiting factors.

To fill this knowledge gap, initial comparison was made within the rice species. I evaluated the drought response of rice genotypes at different growth stages. In my experiments a thorough understanding of the specific effects of drought at different growth stages among rice cultivars allowed demonstrating the relevance of certain traits in determining yield. Drought during the seedling establishment stage and the vegetative stage reduced tiller number, plant height and leaf area. Praba et al. (2009) reported a significant reduction in leaf expansion rate in the tolerant genotype Apo when compared with the sensitive genotype IR64. Consistent with this observation, in my study both at the seedling establishment stage and the vegetative stage, the tolerant rice genotype Apo maintained larger leaf area and higher tiller number than the sensitive cultivar IR64. This could be due to the maintenance of tissue turgor, lower leaf temperature and higher carbon gain associated with deeper root growth for more extraction of water (Chapters 2 and 4). Thus, an optimum leaf development and adequate leaf area are crucial for creating yield (Reddy et al., 2020).

Drought during the vegetative stage will negatively influence the canopy cover due to reduced photosynthesis. In my study, a positive association between reduction in total leaf area and reduction in yield was only found when the stress occurred during the vegetative stage (Chapter 2). This finding suggests that introgressing traits associated with leaf development to aerobic rice cultivars has relevance when stress occurs during the vegetative stage. So, these leaf traits have to be further improved.

At flowering, a mild drought stress even for a short period can cause significant yield loss due to the negative effect of drought on pollen development and viability (Praba et al., 2009). Effect of drought also severely inhibits photosynthesis leading to reduced carbohydrate accumulation and transport for the pollen development (Saini et al., 1984; Dorion et al., 1996). Therefore, reduction in yield due to drought at flowering is proportional to the reduction in rate of photosynthesis. The data in my study also indicates that yield under drought at flowering depends on the supply of assimilates from concurrent photosynthesis and the tolerant genotype maintained higher spikelet fertility by virtue of higher rate of photosynthesis compared with the upland and lowland cultivars (Chapter 2).

Although drought stress during flowering is primarily responsible for a reduction in spikelet fertility (Jagadish et al., 2010; Rang et al., 2011), the impact of drought stress during early grain filling, which affects the source to sink transport, could equally influence yield loss, and also grain quality, due to incomplete grain filling. Some studies have shown that drought-induced premature senescence causes loss of productivity due to decreases in assimilatory duration (Egli, 2004). This was evident from the stay-green hybrids of rice (Cha et al., 2002), maize (Wang et al., 2012) and sorghum (Zhou et al., 2014), which revealed that late onset of senescence contributes to a significant increase in productivity. This thesis also supports the notion that maintenance of photosynthesis during flowering or late onset of senescence helps create higher yield under drought (Chapters 2 and 3). With lower carbon gain, a significant reduction in 1000-seed weight was noticed only in the sensitive genotype, indicating the limitation in supply of assimilates.

Apart from the source strength, carbohydrate transport efficiency and sink activity can help sustain rice yield under drought at the grain filling stage (Lemoine et al., 2013). Tonoplast localised sucrose transporters (SUTs) are known to function as sugar transporters from source leaves to sink organs (Eom et al., 2011). Downregulation of these SUTs has shown to decrease yield due to impairment of long-distance transport (Sun et al., 2019). Decreased source as well as transport results in abnormal packaging of storage compounds leading to chalkiness (Dhatt et al., 2019). To understand the influence of these two processes on grain filling, metabolome profiling of spikelets to know the chemical composition of the grains at that stage is necessary and is often less studied than the effect of drought during flowering (Lawas et al., 2019).

In the experiment described in Chapter 3, rice genotypes with contrasting sensitivity to drought were selected to understand the influence of drought on spikelet metabolism using non-targeted metabolome profiling. The analysis of the data showed a higher carbohydrate and lower amino acids accumulation in the drought-tolerant genotype. This was consistent with the results in studies by Impa et al. (2019) and Dhatt et al. (2019) in wheat and rice, respectively, under high night temperature conditions. Amino acids are the precursors for secondary metabolites (Pott et al., 2019). Therefore, in tolerant genotypes the metabolic flux of amino acids might redirect towards the secondary metabolism such as the phenylpropanoid pathway. In rice, the importance of the phenylpropanoid pathway in the production of flavonoids and coumarins to detoxify reactive oxygen species (ROS) is well studied (Vogt, 2010). The higher upregulation of the phenylpropanoid pathway in the tolerant rice genotype confirms the importance of these secondary metabolites in avoiding ROS production for higher spikelet fertility (Chapter 2). These candidate metabolites identified in contrasting rice genotypes can be a potential target for crop improvement.

Collectively, this study emphasizes the importance of certain drought adaptive traits in specific growth stages and also signifies the maintenance of photosynthesis at all stages will determine the tolerance ability. These specific mechanisms at different growth stages were superior in the aerobic cultivar. Therefore, to further improve the aerobic cultivar, there is a need to compare with another drought tolerant species like wheat. Understanding the constitutive and acquired traits between species in managing reactive oxygen species and maintenance of higher photosynthesis will deliver the possible mechanisms that need to be improved in rice.

Differences in response to drought between rice and wheat

Rice is considered to be highly susceptible to drought stress compared with dry land cereals. Rice and wheat are C_3 cereals which significantly differ in drought adaptation and water use. Wheat requires half of the water used by lowland rice cultivation and is highly drought tolerant. As I discussed above, although aerobic cultivars are drought tolerant and water productive, it is not enough to meet the required food security. Therefore, to study the possible drought tolerance mechanisms, a comparative assessment between species was made in this thesis. A tolerant aerobic rice genotype was compared with a wheat genotype for many constitutive and acquired drought adaptive traits (Chapters 4 and 5). Here the differences in upregulation of these traits between species will be discussed.

Traits associated with maintenance of tissue turgor during drought

Maintenance of tissue turgor under drought has been implicated in maintaining leaf temperature, stomatal conductance, carbon gain and growth. Traits, such as increased root growth, osmotic adjustment, epicuticular waxes, have shown to be positively involved in regulating leaf turgor under drought (Basu et al., 2016; Sheshshayee et al., 2018). It has been reported that drought stress triggered a wide range of anatomical traits to different levels (Kadam et al., 2015). Between species, as a drought effect, increases in root weight density and root thickness were observed in wheat but not in rice (Kadam et al., 2015). In this experiment, both at the seedling stage and the vegetative stage, root length was significantly higher in wheat compared with rice in both stress and non-stress conditions (Chapters 4 and 5). This indicates that wheat has better ability for acquisition of water compared with rice. Additionally, osmotic adjustment and proline contents were significantly higher in wheat than in rice. Increase in osmotic adjustment has shown to help maintain the metabolic activity of a plant (Farooq et al., 2009). Hence in rice breeding improving osmotic adjustment will be important.

In plants, increase in leaf temperature as a result of drought induced stomatal closure is often noticed. Although increase in osmotic adjustment is useful under mild stress, under prolonged severe stress with depleting moisture status, plants will close the stomata to avoid transpiration leading to increased leaf temperature (Sheshshayee et al., 2018). There have been many studies focused on understanding the importance of epiculticular waxes in reflecting radiations to keep leaf temperature lower (Ruggiero et al., 2017). The transgenic plants with increased wax content have been shown as effective in minimizing heat load and increased water use efficiency (Mamrutha et al., 2010). Consistent with this, in wheat the wax load was significantly higher than in rice both under well-watered and drought-stress conditions and hence wheat maintained a higher WUE and a lower leaf temperature than rice. Although an increase in wax content under stress was observed in the aerobic cultivar, this might not have been sufficient to reduce the heat load and to maintain protein and enzyme activities. Screening for genotypes with higher osmotic adjustment and higher wax content and introgressing these traits into elite background will be an effective strategy to produce cultivars of rice that can be grown under minimal water like wheat.

Acquired tolerance mechanisms

Unlike constitutive traits, the acquired drought tolerance traits will be expressed only during stress conditions (Sung et al., 2003; Senthil-kumar et al., 2007). Among several traits, preventing the generation of and scavenging there active oxygen species (ROS) and reactive carbonyl compounds (RCCs) are the most relevant acquired tolerance responses (Chapter 1) (Nisarga et al., 2017; Vemanna et al., 2020). In plants, non-photochemical quenching (NPQ) acts as a photoprotective mechanism which upregulates during stress conditions in order to dissipate excess energy and prevent PSII damage and ROS production (Kromdijk et al., 2016). It has been evident that NPQ deficient mutants of *Arabidopsis thaliana* produced more ROS compared with the wild type (Havaux et al., 2000). Therefore, it appears that, under drought stress, NPQ also protects PSII from ROS by preventing PSII mediated electron transport. In my experiment, a higher increase in ROS levels was observed in rice compared with wheat (Chapter 4). In aerobic rice cultivar increased levels of zeaxanthin might be diverted to ABA biosynthesis rather than for NPQ like in wheat (Chapter 5). This was also evident from the significant reduction in transpiration and increased expression of the ABA biosynthesis gene in aerobic rice during drought (Chapters 3 and 5).

To reduce the detrimental effect of ROS, plants possess a highly efficient and sophisticated antioxidant system that includes both non-enzymatic and enzymatic defence mechanisms (Gill and Tuteja, 2010). Studies have shown that the tolerance ability of plants is associated with antioxidant capacity (Moussa and Abdel-Aziz, 2008). Contradictory to these studies, in my experiments, although wheat had lower ROS levels and better tolerance to drought than rice, a similar increase in scavenging mechanisms was observed in wheat compared with aerobic rice (Chapters 4 and 5). The degree of expression of protective mechanisms depends on severity of stress experienced by the plant (Meena et al., 2017). If NPQ suppresses ROS formation this may lead to plants being more tolerant and hence upregulation of scavenging beyond requirement will not be necessary. This response was observed in wheat, where preventing ROS production through higher NPQ resulted in a surplus upregulation of scavenging activity (Chapter 5). Therefore, it is apparent that, in rice, activation of ROS prevention pathway is the major limitation. Intriguingly, these management strategies have a significant role in preventing ROS-induced damage to the photosynthesis machinery.

Maintenance of photosynthesis

The photosynthetic machinery is highly vulnerable to oxidative damage (Krieger-Liszkay et al., 2008). Chapter 2 demonstrates that maintenance of the photosynthesis is the key for sustained yield under drought at any crop growth stage of rice. Higher ROS accumulation impairs the *denovo* synthesis of the D1 protein and also molecules in the thylakoid system, particularly poly-unsaturated fatty acids and other membrane lipids (Przybyla et al., 2008). Similarly, high ROS levels inactivate the Calvin cycle enzymes by oxidizing their thiol groups (Halliwell, 2006). When the antioxidant capacity is not enough to manage ROS, a decrease in expression of many chloroplast and nuclear genes and also in Rubisco activity has been reported in many plant species (Irihimovitch and Shapira, 2000; Pfannschmidt, 2003). Even under well-watered conditions, the photosynthesis of rice was significantly lower than that of wheat. In drought conditions a significant reduction in relative expression of photosynthetic genes was smaller in rice than in wheat, indicating more damage to the photosynthetic machinery (Chapter 5). Consistent with the molecular analysis, the photosynthetic efficiency and capacity was significantly more decreased under drought in rice than in wheat. Although the upregulation of scavenging mechanisms was significantly increased in rice, as in wheat, continuous production of ROS might result in significant damage to the photosynthetic system (Chapter 5). The comparative analysis between species from this thesis suggests that, although the aerobic cultivar is better adopted to stress, it is no match to wheat even under well-watered conditions. Therefore, for rice to grow like wheat under water limited conditions there is a need to introgress key traits which are superior in wheat to obtain higher yield. My study is a useful source to narrow down the important traits required for physiological breeding.

A technical consideration of high-throughput phenomics platforms for capturing acquired tolerance traits

Regardless of the aspects discussed above, precision phenotyping is crucial for studying the plant response to drought. Given the potential of computation in plant phenotyping, many automated phenomics platforms with specific targets have been developed. Specifically, for drought-stress imposition, several high-throughput phenotyping facilities have been developed with state-of-the-art technologies that can control timing, frequency and intensity of drought episodes (Granier and Vile, 2014; Lekshmy et al., 2020). To create drought stress, withholding of water until the desired field capacity was reached was the common practice. Although this method is widely used, it is not precise as it will not mimic the natural drought conditions due to quick soil drying and will also be influenced by differences in canopy and thus in rate of water withdrawal (Poorter et al., 2012). Development of the gravimetric method with weighing pots daily was well established (Udayakumar et al., 1998; Earl, 2003), but it was difficult to implement in large sets of genotypes or breeding lines due to high labour-hour requirements (Lekshmy et al., 2020). Moreover, maintaining the desired field capacity by weighing pots once in a day and replenishing water is not accurate as transpiration is a continuous process and hence soil moisture depletes progressively throughout the day. Additionally, it is also depending on canopy cover, a genotype with more leaf area will exhaust soil moisture more quickly than types with less leaf area. Hence, genotypes with a large canopy may experience a more severe stress due to a quicker withdrawal of water. Therefore, the gravimetric approach by manual weighing leads to error in phenotyping for drought adaptive traits in a platform of lines differing in their canopy. This calls for the development of phenomics platforms with automated weighing and irrigation facility for capturing the drought adaptive traits.

In my study, an automated phenomics facility was used to create the water regimes. With the help of automated irrigation, soil moisture was maintained within 1% of the set water regime in the container (Chapters 3 and 4). In natural conditions, plants experience progressive drought stress, which means, plants experience mild stress before reaching more severe drought conditions. In such conditions, plants upregulate many acquired mechanisms at mild drought stress which protect plants when stress becomes severe (Chapter 1). When the drought is imposed by withholding water, such protective mechanisms will not upregulate leading to death of plants (Niinemets et al., 2009). Hence mimicking such natural soil drying conditions will be more appropriate to capture acquired drought adaptive traits. The phenomics facility used in this study had a user-defined dry-down protocol which was helpful in creating drought mimicking natural drying conditions. Using this facility, we were able to maintain similar moisture status irrespective of differences in leaf area, root growth and species (Chapters 3 and 4). Hence, this phenotyping platform to create drought stress was more appropriate in making both inter- and intra-specific comparisons for measuring acquired tolerance mechanisms.

Final remarks and future research needs

Among cereals, rice is more susceptible to drought, and hence with increasing scarcity of water, improving its adaptation to water limitation is the major challenge. To achieve this, comparative studies to capture stress response as well as to identify traits that needs further enhancement is necessary. In this thesis, experimental work of comparison between genotypes adapted to different ecosystems has shown that improving aerobic cultivars can help achieving the required productivity under diminishing water availability (Chapter 2). At all growth stages, aerobic genotypes performed better than lowland and upland cultivars. Although the percent reduction in yield under drought was lower for the aerobic cultivar, the potential yield was poorer compared with the lowland cultivar under irrigated conditions. Hence more focus is needed on improving the potential yield of aerobic rice genotypes under well-watered conditions and also on easing the yield reduction due to drought. Furthermore, this thesis allowed to demonstrate that traits contribution to yield differs significantly based on stage of drought occurrence. Canopy cover, spikelet fertility and source maintenance were positively associated with yield at the vegetative, flowering and grain filling phase respectively (Chapter 2). At all these stages photosynthesis showed significant association with yield, thus our data confirms that introgression of specific traits which helps in increasing photosynthesis is the key for achieving higher yield.

In Chapter 3, I focused on understanding the drought response of lowland and aerobic rice cultivars during grain filling. The fast growth of phenomics and genomics is leading us towards precision identification of traits. In this thesis, I used the combined approach of a high-throughput phenomics platform and a metabolomics approach. The study revealed that the higher carbohydrate metabolism and secondary metabolism are important in regulating yield under drought. The upregulation of phenylproponoid pathway protected the cell from ROS induced damage in tolerant genotype. These candidate metabolites identified in this study (Chapter 3) could be used as markers to screen large germplasm or mapping populations. However, some more experiments are needed for validating these metabolites by growing the genotypes in field conditions at different locations.

Chapters 4 and 5 investigate the physiological responses of rice and wheat to drought. Acquired drought tolerance mechanisms, mainly the ROS regulation, were superior in wheat compared with rice. However, upregulation of scavenging mechanisms was similar between aerobic rice cultivar and wheat. But the activation of the photo-protection mechanism was higher in wheat than compared to rice. These mechanisms in wheat helped maintain higher expression of photosynthetic genes leading to higher photosynthetic capacity and efficiency compared with rice. The data suggests that the increase in scavenging mechanism alone might not serve as an efficient drought tolerant trait. Thus, the comparative analysis between rice and wheat presented in this thesis suggests the need of improving several drought adaptive traits in rice. These include: 1) osmotic adjustment, 2) epicuticular waxes, 3) improving photosynthesis, and 4) preventing ROS production (Chapters 4 and 5). This thesis suggests that the comparative analysis between species is an effective strategy and further experiments with other dry land C_3 cereals will help identify important traits to improve rice productivity. Beyond the physiological traits, effective identification of novel mechanisms between species requires the use of phenomics and genomics tools and such approaches would be able to identify certain traits for crop improvement. Screening of and introgression of these traits into aerobic rice cultivars should be done to elucidate the percent improvement in yield under drought. Together, these approaches help narrow down the traits and can guide breeding efforts leading to the development of improved global food security.

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Summary

Rice is the major food crop to half of the world population. Although the production of rice has increased over time in the wake of the Green Revolution, major shortfalls caused by climatic aberrations such as drought have resulted in a stagnation of rice production. Best production of rice comes from puddled cultivation practice that consumes significant amounts of water. With the immense increase in demand for fresh water, saving water and sustaining production under water-limited conditions are the two main challenges to be addressed for attaining food security. These challenges necessitate a genetic enhancement to improve yield potential as well as to sustain productivity under water deficit. Several water-saving agronomic approaches have been evolved to save water from rice cultivation, such as the system of rice intensification (SRI) method, upland methods and semi-irrigated aerobic cultivation. Among these, the semi-irrigated aerobic condition is known to save significant volumes of irrigation water. However the major lacuna of this approach is the yield reduction which is not acceptable under the current situation of reducing food security. Hence it is important to develop strategies to improve rice cultivars for water-limited conditions.

The aim of this thesis was to understand the physiological responses of rice genotypes differing in their adaptability and also to compare these responses between rice and wheat. Initially, these within-species comparison studies form a basis to understand the superior and inferior mechanisms of drought tolerance in cultivars adapted to different ecosystems. Secondly, comparison with drought adapted cereal wheat helped to identify the traits which need to be introgressed into rice genotypes for tolerance and sustained productivity.

In Chapter 2, I compared the rice genotypes adapted to aerobic, lowland and upland methods of cultivation practice at vegetative, flowering and grain filling phase. Among these, the aerobic cultivar performed better at all growth stages. At the vegetative stage, reduction in canopy cover significantly contributed to yield reduction. In flowering and grain filling phases, reduction in spikelet fertility and source availability showed significant association with yield reduction. At all growth stages, source availability and efficiency were found to be crucial for higher productivity under drought. These results suggest the need to study the stress response at a particular stage using advanced phenomics and genomics techniques.

The finding in Chapter 2 shows that, apart from flowering phase, drought during grain filling significantly affected yield. Yet the limited detailed information available in literature on stress effects during grain filling made me focus on this particular stage. In Chapter 3, I used a phenomics platform and a metabolomics approach to study the stress response of two contrasting rice genotypes. During this stage source to sink transport was crucial for complete grain filling and drought inhibited the transport efficiency. In the susceptible cultivar a stronger reduction in photosynthesis and transport was noticed than in the tolerant cultivar. This led to a differential metabolome profiling in developing grains. In order to cope up with drought-induced oxidative damage, plants had certain protective mechanisms and the extent of upregulation of these mechanisms determines the stress tolerance of plants. In the tolerant genotype, an increase in metabolites of the phenylproponoid pathway indicated a unique mechanism of drought adaptation to detoxify free radicals.

In Chapter 4, I compared the two species rice and wheat to study the acquired drought tolerance mechanisms, mainly focusing on reactive oxygen species (ROS) regulation. This study was conducted using a high-throughput phenomics facility with automated irrigation, where soil moisture is maintained precisely irrespective of differences in canopy cover. Wheat had a superior tolerance at the seedling as well as at the vegetative stage. But an interesting outcome was that the upregulation of scavenging mechanisms was similar between the tolerant rice and wheat cultivars.

These results of Chapter 4 made me to execute a further experiment with the same set of genotypes. In this experiment I investigated the differences in ROS production and scavenging abilities and also in photosynthetic mechanisms between rice and wheat. In wheat, photoprotection mechanisms prevented ROS production which led to less ROS accumulation. Hence the activated of scavenging mechanisms were sufficient to detoxify the produced ROS. But in the tolerant rice cultivar the poor activation of the photoprotection mechanism resulted in higher ROS accumulation. Other than these mechanisms, higher osmotic adjustment and epicuticular wax content helped wheat to maintain its metabolic activities better than rice. This was evident from a smaller reduction in photosynthesis in wheat than in rice.

In the General discussion (Chapter 6), I discussed the results and the main outcomes of Chapters 2-5. I also discussed the future prospects on how to improve rice productivity under water-deficit conditions. The importance and need of introgression of specific traits identified from the comparison study between rice and wheat are also discussed.

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Preethi Vijayaraghavareddy

Wageningen, The Netherlands, 2021

List of Publications

Preethi Vijayaraghavareddy, Vanitha Adhinarayanreddy, Ramu S. Vemanna, Sheshshayee Sreeman, and Udayakumar Makarla. "Quantification of membrane damage/cell death using Evan's blue staining technique." Bio-protocol 7, no. 16 (2017): 2519

Sheshshayee Sreeman, **Preethi Vijayaraghavareddy**, Rohini Sreevathsa, Sowmya Rajendrareddy, Smitharani Arakesh, Pooja Bharti, Prathibha Dharmappa, and Raju Soolanayakanahally. "Introgression of physiological traits for a comprehensive improvement of drought adaptation in crop plants." Frontiers in Chemistry 6 (2018): 92.

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PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)

- Identification and importance of physiological traits for improving drought tolerance in rice.

Writing of project proposal (4.5 ECTS)

- Understanding rice drought tolerance mechanisms: the whole-plant physiology and metabolomics of acclimation and adaptation to field stress

Post-graduate courses (5.5 ECTS)

- Molecular approaches for improving physiological traits; University of Agricultural Sciences, Bangalore, India (2016)
- Climate change and crop growth; University of Agricultural Sciences, Bangalore, India (2016)

Deficiency, refresh, brush-up courses (3 ECTS)

- Physiology and molecular aspects of photosynthesis, carbon and nitrogen metabolism; University of Agricultural Sciences, Bangalore, India (2017)
- Physiological and molecular responses of plants to abiotic stresses; University of Agricultural Sciences, Bangalore, India (2018)

Competence strengthening / skills courses (4 ECTS)

- Technical writing and communication skills; University of Agricultural Sciences, Bangalore, India (2017)
- Disaster management; University of Agricultural Sciences, Bangalore, India (2018)

PE&RC Annual meetings, seminars and the PE&RC weekend (0.6 ECTS)

- PE&RC Last years weekend (2021)

Discussion groups / local seminars or scientific meetings (7 ECTS)

- Science club meetings; University of Agricultural Sciences, Bangalore, India (2016-2018)

PE&RC Training and Education Statement

- Department curricular seminar; University of Agricultural Sciences, Bangalore, India (2016-2021)
- Contemporary webinar series; Regional Centre for Biotechnology, Faridabad, India (2020-2021)

International symposia, workshops and conferences (5.5 ECTS)

- International Plant Physiology Congress; New Delhi, India (2015)
- InterDrought-V Conference; Hyderabad, India (2017)
- International Plant Physiology Conference; online, Jammu, India (2020)
- Translating Physiological Tools to Augment Crop Breeding; online, ICAR-Indian Institute of Wheat & Barley Research (2021)

Lecturing / supervision of practicals / tutorials (3 ECTS)

- Principles of plant physiology, metabolic processes and growth regulation (2016)

Supervision of MSc students (9 ECTS)

- Physiological strategies to minimise unproductive water loss in rice (*Oryza sativa*)-analysis using germplasm lines
- On the regulation of water use efficiency by carboxylation among diverse rice germplasm accessions
- Influence of nocturnal and diurnal transpiration on water use efficiency (WUE) of rice (*Oryza sativa*)

Curriculum vitae

Preethi Vijayaraghavareddy was born in Nandi, a village in Karnataka province, India, on 28th May 1989. She obtained her Bachelor of Science (BSc) in 2011, and her Master of Science (MSc) in 2013, in Agriculture with specialisation in Crop Physiology from the University of Agricultural Science, Bangalore, India. Immediately after completion of her MSc study, she joined the Department of Crop Physiology as a Senior Research Fellow. In September 2014, she started as a sandwich PhD student at the Centre for Crop Systems Analysis (CSA) at Wageningen University & Research, in Wageningen, the Netherlands, in a project in close collaboration with the University of Agricultural Sciences, Bangalore, India. This study reports on her six years of research work on improving rice productivity under water deficit through a comprehensive assessment of adaptive physiological traits.

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