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Shoot architecture from genome to field

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Rice in a different light:
Shoot architecture from genome to field

Rijst in een ander licht:
plantenarchitectuur van genoom tot veld

(met een samenvatting in het Nederlands)

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Contents

“ Modern technology
owes ecology an apology. ”

Alan M. Eddison

Summary

How does a rice plant in the field fight against the weeds growing next to it? Since rice feeds more than half of the world's human population as a staple food, and weeds are a major constraint in modern rice farming, tackling this issue is of great relevance. Driven by climate change, there are shortages of water and the traditional rice farming of transplanting rice into flooded paddy fields needs to adapt. As a consequence, rice farming is transitioning to direct-seeding on non-flooded fields. Besides the advantages of water saving and less labour requirement for transplanting, the major constraint of this system is the weeds. Currently the weeds are suppressed with heavy herbicide usage, but there is an urgent need for more sustainable weed control. This thesis investigated if and to what extent rice shoot architecture could be optimized for improved weed-competitiveness.

We first explored the plethora of natural diversity in shoot architecture by phenotyping a rice diversity panel for relevant weed-competitive traits related to shading and early plant vigour (**Chapter 3**). Based on this, the core traits contributing to increased shading were defined as shoot area, number of leaves, culm height and solidity (the compactness of the shoot). To indicate the shading capacity of a plant, a Shading Rank metric was established, combining these traits in a weighed manner. The investigated shoot architectural traits were then related to their genetic variation using genome-wide association studies (GWAS). This unravelled several novel loci involved in different aspects of plant architecture. The effect of different alleles of these loci on the phenotype were characterised by means of a haplotype analysis, delivering favourable alleles for rice breeding programs.

Since genetic loci related to shoot architecture were studied on individual plants, it was important to validate these findings in field conditions and ultimately verify whether selected varieties would suppress weeds in the field. Therefore, a field assay was undertaken, with selected varieties together with weed and at different planting densities (**Chapter 4**). The field study shows that rice can suppress weeds from 40 up to 70%. Increased rice planting density does suppress weeds stronger, however it also has a negative impact on crop performance, which could outweigh the benefits.

Plants are experiencing inter- and intra-specific competition when growing in close proximity, in an agronomic context either due to high planting densities in monocultures or weeds interfering with crops, and typically display shade avoidance responses to elongate their stems and leaves in order to reach the light and prevent being shaded by neighbours. A reduced ratio between red and far-red light follows from selective absorption of red and blue light for photosynthesis, and is perceived already prior to actual competition, as an early signal for neighbour proximity. Typical shade avoidance responses include increased height growth at the expense of branching in addition to elongation and upward orientation of photosynthetically active foliage (**Chapter 2**).

Rice, as one of the five major global crops, is a particularly interesting study object to elucidate fundamental processes of growth at high densities in monocots. Unlike for the dicot model species *Arabidopsis thaliana*, it is largely unknown how rice senses and responds to high vegetational density. Following this, here we explored the effects of far-red light in rice, to verify to what extent the observed weed-competitive phenotypes are stable, or if they are plastically changing with conditions such as the far-red – a signal for planting density. We performed a range of experiments in the greenhouse (**Chapter 5**), where rice plants were grown under light conditions either or not supplemented with far-red light, leading to a low red to far-red light ratio. It appeared that rice is not showing any strong shade avoidance responses phenotypically, or at the level of gene expression. However, we did observe unprecedented growth enhancement in supplemental far-red light. In order to gain insight into possible effects of far-red photons on photosynthetic activity, gas-exchange measurements on plants under supplemental far-red light were performed (**Chapter 6**). We discovered that, despite the general assumption of far-red light mostly acting as a signal rather than a photosynthetic resource, far-red light strongly enhances carbon fixation in a number of tested rice varieties. At the same time there is only a marginal acclimation effect observed in terms of stomatal dimensions, stomatal conductance and chlorophyll content.

The finding that rice very strongly benefits from supplemented far-red with increased growth, could indicate that at an early stage of proximity shade, far-red would actually increase photosynthesis and promote plant growth. Far-red is always present under natural conditions and in a canopy of dense vegetation it is even enriched by reflection. In the top layers of a canopy where PAR is still high it thus would even lead to increased photosynthetic rates. It is therefore becoming apparent that far-red photons can both be an energy source for

photosynthesis and an early warning signal for proximity shade. It is unclear, how plants balance between these two roles, and further studies are required to elucidate interactions between far-red and PAR for photosynthesis and proximity sensing.

These observations on far-red light effects in rice can give useful insights to make information-based decisions in farming practices such as planting density and planting patterns. Insights from the field assay, can give directions in the choice of rice variety and field management, such as weed and water management. Ultimately, the identification of target genes regulating the shade-casting traits of competitive phenotypes can be integrated in future breeding programmes. This will help to reduce the amount of herbicide usage and enable a more sustainable and climate-change resilient rice-farming.

Samenvatting

Hoe concurreert een rijstplant in het veld tegen het onkruid dat ernaast groeit? Aangezien rijst als basisvoedsel meer dan de helft van de wereldbevolking voedt, en onkruid een groot probleem vormt in de moderne rijstteelt, is het van groot belang dit probleem aan te pakken. Onder invloed van klimaatverandering is er een tekort aan water en moet de traditionele rijstteelt, waarbij rijst wordt geplant in ondergelopen rijstvelden, worden aangepast. Om deze reden is er een tendens in rijstteelt om over te gaan op direct inzaaien op niet-overstroomde velden. Naast de voordelen van waterbesparing en het feit dat er minder arbeidskrachten nodig zijn voor het overplanten, is de grootste beperking van dit systeem de groei van onkruiden. Op dit moment worden onkruiden bestreden door middel van intensief gebruik van herbiciden. Er is echter een dringende behoefte aan een duurzamere onkruidbestrijding. In dit proefschrift werd onderzocht of en in welke mate de architectuur van rijstscheuten kan worden geoptimaliseerd om de onkruiden te onderdrukken.

Als eerste stap werd de natuurlijke diversiteit in plantenarchitectuur onderzocht, door een grote variatie van rijst variëteiten te fenotyperen voor relevante onkruidconcurrerende eigenschappen, in het bijzonder beschaduwing en vroege groeikracht (**Hoofdstuk 3**). Op basis hiervan werden de kernkenmerken gedefinieerd die bijdragen aan de hoeveelheid schaduw: bladoppervlakte, aantal bladeren, halmhoogte en compactheid (van bladeren). Om de beschaduwing capaciteit van een plant aan te geven, is een beschaduwing rangschikking geformuleerd, waarbij deze kenmerken op een gewogen manier werden gecombineerd. De onderzochte kenmerken van de plantenarchitectuur werden vervolgens in verband gebracht met hun genetische variatie door genoom brede associatiestudies (GWAS). Dit bracht verschillende nieuwe genetische loci aan het licht die betrokken zijn bij verschillende aspecten van plantarchitectuur. Het effect van verschillende allelen van deze loci op het fenotype werd gekarakteriseerd via een haplotype analyse, wat gunstige allelen voorspelt voor rijstveredelingsprogramma's.

Aangezien genetische loci gerelateerd aan plantenarchitectuur bestudeerd werden op basis van individuele planten, was het belangrijk om deze bevindingen te valideren in het veld en uiteindelijk na te gaan of geselecteerde variëteiten onkruid zouden onderdrukken. Daarom werd een veldproef uitgevoerd, met geselecteerde variëteiten samen met onkruid en bij verschillende plantdichtheden (**Hoofdstuk 4**). De veldstudie toont aan dat rijst 40 tot 70% van de onkruidgroei

kan onderdrukken in vergelijking met onkruiden die zonder rijst groeien. Een hogere plantdichtheid van rijst onderdrukt het onkruid sterker, maar heeft ook een negatief effect op de rijstgroei zelf, wat de voordelen zou kunnen overschaduwen.

Planten ondervinden inter- en intraspecifieke concurrentie wanneer ze dicht op elkaar groeien. In een agronomische context gebeurt dit door hoge plantdichtheden in monoculturen dan wel door onkruid dat de gewassen verstoort. De planten reageren met schaduw vermijdende reacties door hun stengels en bladeren te verlengen om het licht te bereiken en te voorkomen dat ze door de buren worden overschaduwd. Een verminderde verhouding tussen rood en ver-rood licht is het gevolg van de selectieve absorptie van rood en blauw licht voor de fotosynthese. Dit wordt reeds vóór de eigenlijke concurrentie waargenomen als een vroeg signaal voor de nabijheid van buren. Typische reacties om schaduw te vermijden zijn onder meer een grotere lengtegroei ten koste van vertakking, verlenging en opwaartse oriëntatie van bladeren (**Hoofdstuk 2**).

Rijst is, als een van de vijf belangrijkste gewassen ter wereld, een bijzonder interessant studieobject om de fundamentele processen van groei bij hoge dichtheden in monocotyle planten bloot te leggen. In tegenstelling tot de dicotyle modelplant *Arabidopsis thaliana* is het grotendeels onbekend hoe rijst een hoge vegetatiedichtheid waarneemt en erop reageert. Daarom zijn hier de effecten van ver-rood licht in rijst onderzocht om na te gaan in hoeverre de waargenomen onkruidconcurrerende variëteiten stabiel zijn, of dat ze plastisch veranderen met de omstandigheden, zoals ver-rood licht - een signaal voor plantdichtheid. Om die reden zijn experimenten in de kas uitgevoerd (**Hoofdstuk 5**) waarbij rijstplanten werden gekweekt onder natuurlijk daglicht en daglicht verrijkt met ver-rood licht, wat leidde tot een lage verhouding van rood tot ver-rood licht. Het bleek dat rijst geen sterke schaduw vermijdende reacties vertoont noch fenotypisch, noch op het niveau van genexpressie. De rijstplanten vertoonden daarentegen wel een ongekende toename van groei door extra ver-rood licht. Om inzicht te krijgen in de mogelijke effecten van ver-rood licht op de fotosynthese activiteit werd de gasuitwisseling van CO_2 gemeten onder de verschillende lichtomstandigheden (**Hoofdstuk 6**). Ondanks de algemene aanname dat ver-rood licht vooral als signaal werkt en niet zozeer als fotosynthesebron, bleek dat ver-rood licht de koolstoffixatie in een aantal geteste rijstvariëteiten sterk verhoogt. Tegelijkertijd is er een marginaal acclimatiseringseffect gemeten qua afmetingen van de huidmondjes, de geleiding van huidmondjes voor water en CO_2 en het chlorofylgehalte.

Het resultaat, dat rijst zeer sterk profiteert van ver-rood licht, zou erop kunnen wijzen dat in een vroeg stadium van aanwezigheid van buurplanten waar het licht verrijkt is met gereflecteerd ver-rood licht, dit de fotosynthese zou verhogen en de groei van de plant bevordert. Ver-rood is altijd aanwezig in natuurlijke omstandigheden en onder een dicht bladerdek wordt het zelfs verrijkt door reflectie. In de bovenste lagen van een bladerdek, waar de PAR nog hoog is, leidt het wellicht zelfs tot verhoogde fotosyntheseactiviteit. Het wordt dus duidelijk dat deze fotonen zowel een energiebron voor de fotosynthese kunnen zijn als een vroegtijdig waarschuwingssignaal voor nabijheid van schaduw. Tot op heden is het niet duidelijk hoe planten een evenwicht vinden tussen deze twee eigenschappen van ver-rood licht en verdere studies zijn nodig om dit te verhelderen.

Deze effecten van ver-rood licht in rijst kunnen nuttige inzichten opleveren voor het nemen van data gedreven beslissingen in de landbouw, zoals keuzes voor plantdichtheid en plantpatronen. Inzichten uit de veldexperimenten kunnen helpen bij de keuze van rijstvariëteiten en veldbeheer, zoals onkruid- en waterbeheer. Uiteindelijk kan de identificatie van genen die de schaduwvormende eigenschappen van concurrerende variëteiten reguleren, in toekomstige veredelingsprogramma's worden geïntegreerd. Dit zal helpen om het gebruik van herbiciden te verminderen en een meer duurzame en klimaatveranderingsbestendige rijstteelt mogelijk te maken.

Chapter 1

General introduction

The increasingly pressing climate change affects especially the agricultural sector. Farmers in developing countries suffer the most already by shifting seasons, unpredictable rainfalls, droughts and storms. Especially vulnerable to this are the rice plants grown in small, flooded paddy fields. Therefore, the traditional rice farming system needs to shift to a practice, where rice seeds are sown on dry fields. However, an arising problem under such conditions are the weeds, which were formerly suppressed by the flooded field conditions. Proliferating weeds are now suppressed with increased use of herbicides: an undesirable situation that needs an urgent and sustainable solution. We are addressing this by exploring ways for weed-competitive rice.

Problem and urgency

Climate change

We find ourselves in a world where the effects of climate change are no longer just a theoretical concept, but becoming very realistic: affecting ecosystems, plant, animal and human populations globally and not at least farming systems. Especially farmers, working in close contact with nature, witness how their crop is affected by extreme weather conditions (IPCC, 2022; Pörtner et al., 2022). Destroyed harvests and diminished yields are affecting farmers with reduced income and in the end affect all of us – who eat our daily bread, or bowl of rice. Particularly South-East (SE) Asia will be affected more than other regions in the world from rising sea levels and particularly hit by extreme weather conditions like storms and typhoons, droughts, as well as floods (Pörtner et al., 2022).

Climate impact on crop production and increasing demand

The global population is expected to reach about 10 billion people by 2050 (UN, 2019) and predictions point to highest population growth in SE-Asia and central Africa (Figure 1.1 A). At the same time, climate change is projected to reduce crop yields (Figure 1.1 B) particularly in the regions where food demand is estimated to increase most (Tirado von der Pahlen et al., 2021; Wheeler & von Braun, 2013) and a large part of global cereal crops are grown (Figure 1.1 C).

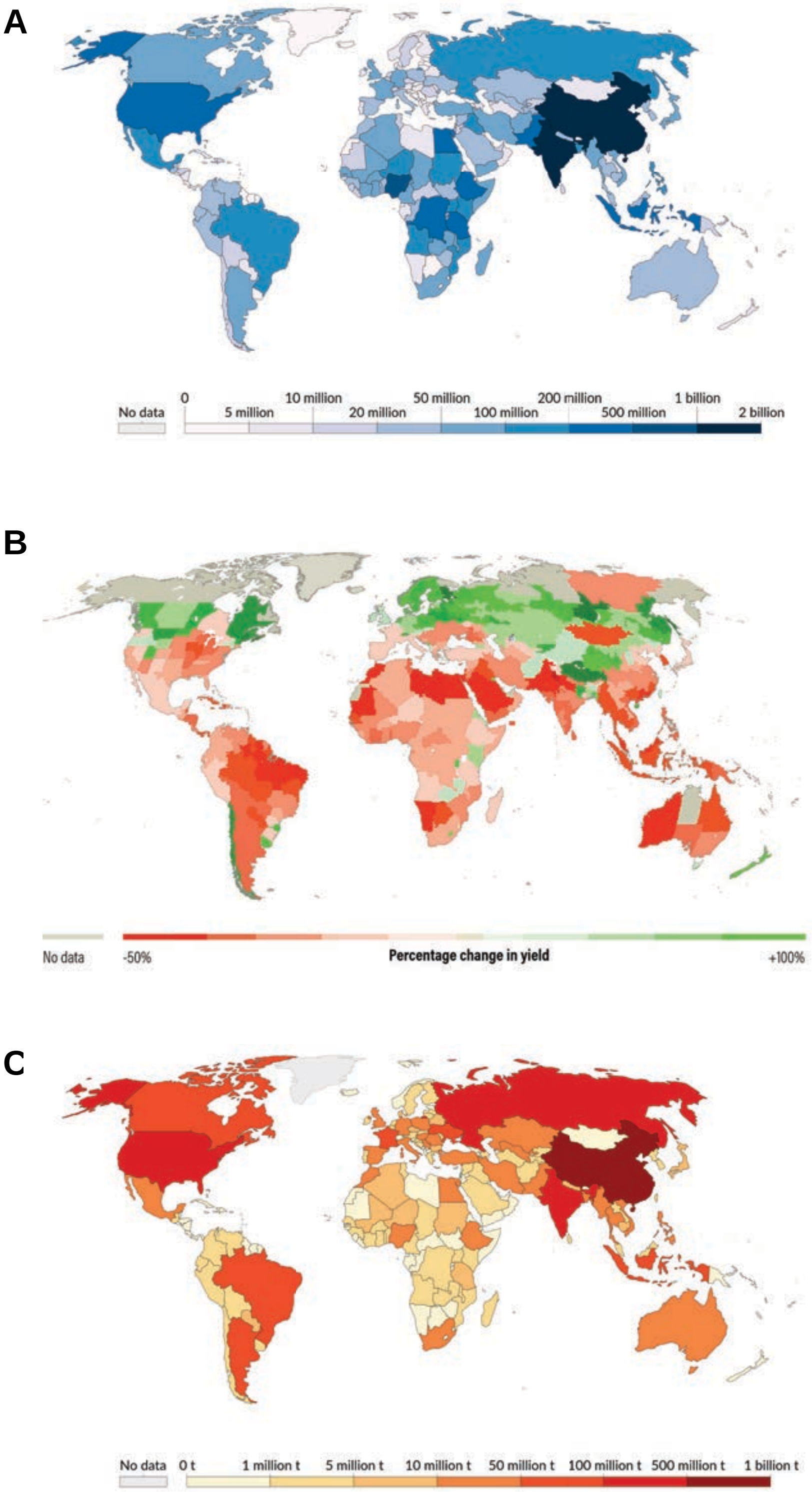

Figure 1.1. Distribution of population growth and climate impact on crop production shows biggest gap lying in the global south. A. Projected human population in 2100 based on data published by United Nations Population Division (2019); visualization created by Edouard Mathieu published online at OurWorldInData.org. Retrieved from https://ourworldindata.org/grapher/population-past-future. **B.** Climate change impact on crop yields in 2015 shown as percentage change in yield between present and 2050; source: WRI 2013. **C.** Global cereal production given in tons in the year 2018. Based on data from UN Food and Agriculture Organization (FAO) (2020): visualization created by Hannah Ritchie and Max Roser published online at OurWorldInData.org. Retrieved from https://ourworldindata.org/agricultural-production#cereals.

Climate change impacts food security globally, but particularly the global south will be hit hardest with destroyed crops (Gornall et al., 2010; Mani et al., 2018), which are the economically weakest regions. Therefore, especially in the global areas of strong population increase and low income, there is a need for affordable staple crops. Together this illustrates a scenario, where life for human beings will become most critical in areas of the global south, where people are already economically disadvantaged and where there is only a small margin to create resilience. Efforts towards climate adapted farming and future resilient crops are indispensable (Gornall et al., 2010; Wheeler & von Braun, 2013) and especially the demand for rice will increase (Bouman & Tuong, 2001; Wing et al., 2018).

Rice as a crop

Rice is a staple crop for at least two-third of the world's population (Meena et al., 2019; Wing et al., 2018) and together with maize and wheat providing two third of the global human caloric intake (FAO, 2010; Wing et al., 2018). For Asia in particular, rice is of immense importance, since 90 % of the global production and consumption is located in Asia. However, increasing rice cultivation with the current methods will be challenged in the future for several reasons, as explained in the following.

Domestication of rice

Similar to other cereal crops, during human settlement Asian rice (*Oryza sativa*) has been domesticated from its grassy ancestor Oryza rufipogon, about 9.000 years ago (Gutaker et al., 2020; Huang et al., 2012; Molina et al., 2011). Much later independently, in Africa rice was domesticated from its wild ancestor *Oryza barthii* and now cultivated to a very small extent as *Oryza glaberrima* (Wing et al., 2018). Further human and natural selection gave rise to a diversion of the *Oryza sativa* species into different subpopulations: the varietal group of the indicas (indica and aus) and the varietal group of the japonicas (temperate japonica, tropical japonica and aromatic) (Gutaker et al., 2020; Wang et al., 2018; Wing et al., 2018; Yang et al., 2018). Indica varieties are commonly grown in tropical regions. Japonica varieties are more tolerant to cold temperatures, but less tolerant to drought, insects and disease (Kennedy & Burlingame, 2003; Wing et al., 2018; Zhao et al., 2011). At present there are, depending on definition and the source, up to 500.000 cultivated *Oryza sativa* varieties (Hamilton, 2016), comprising 98 % of all globally cultivated varieties (Wing et al., 2018).

In the process of domestication of cereals, plant architecture was one of the aspects of strongest selection (Teichmann & Muhr, 2015). One ground breaking event in cereal breeding took place, first starting with wheat in the 1950s and followed by rice in the 1960s, a period referred to as the "Green Revolution", where in a vastly accelerated breeding process modern high-yielding varieties were released (Evenson & Gollin, 2003; Kush & Khush, 2001; Sinclair & Sheehy, 1999; Wing et al., 2018). For rice, the release of the variety IR8 in 1966, developed at the International Rice Research Institute (IRRI), marked the beginning of a new era not only for rice farmers, but for rice producing and consuming countries as a whole (Kush & Khush, 2001; Peng et al., 1999; Sasaki et al., 2002; Wang et al., 2018). Rice production for example increased by 132 % in the period between 1966 and 2000 (Evenson & Gollin, 2003; Kush & Khush, 2001). This was possible with a combination of the newly invented artificial fertilizers, the establishment of irrigation systems and new high yielding crop varieties. The most prominent outcomes of the green revolution were varieties of broad disease resistance, erect, compact and extremely short stature (semi-dwarf varieties), in combination with strong tillering and accelerated life cycle, enabling earlier and bigger harvest, than ever before. This shows, the importance and potential of manipulation of shoot architectural traits in the context of agriculture.

Current state of rice farming

High light intensities, high temperatures and sufficient water availability are the most important factors for optimal rice growth. Situated in the tropical and subtropical regions, Asia and especially SE-Asia (27%) is offering these conditions and delivering more than 90 % of the global rice production (Chauhan et al., 2017). The top ten rice producing countries are all situated in Asia, with the exception of Brazil. China and India together are providing roughly two thirds of the global production (FAO, 2020).

Rice ecosystems and farming practices

Rice production can be classified according to the ecosystem it is grown in and the applied farming practice (Chauhan et al., 2017). With 75% of the annual global rice production, irrigated lowland rice is by far the most important practice, occupying 79 million ha (Chauhan et al., 2017). Here, rice is grown in bounded paddy fields and irrigation water ensures a continuous flooding level keeping anaerobic soil conditions (Chauhan et al., 2017; Kaur & Singh, 2017). After land preparation, different establishment methods can be applied, which are either transplanting or direct seeding (Chauhan et al., 2017; IRRI, 2007). The traditional practice is to transplant four to six weeks old rice seedlings that have been established in nursery fields into the flooded paddy fields (Chauhan et al., 2017). In contrast to transplanting is the practice of direct seeding (DS), where seeds are broadcasted into in wet or dry soil (Chauhan et al., 2017). Dry-DS is the most commonly used method, which is based on sowing dry seeds on fields with unsaturated soil water conditions (Chauhan et al., 2017). In wet-DS pre-germinated seeds are sown on soil that may be anaerobic or aerobic (Weerakoon et al., 2011) and is mostly adopted in irrigated areas. In water-DS seeds are sown into standing water and germination would rely on seed traits for anaerobic germination (Chauhan et al., 2017).

Shortcomings of traditional farming and shift to direct-seeded rice

On the one hand, very high productivity makes transplanted paddy rice an attractive farming practice, with high yields and up to three harvests a year (Chauhan et al., 2017). On the other hand, major constraints exist in terms of the high water requirement (for 1 kg of rice, at least 2.000 litres water are used (Bouman & Tuong, 2001). In addition, transplanting is very labour intensive and an increase of the mechanisation level is difficult. Furthermore, the anaerobic soil conditions lead to relatively high methane emissions (Kumar & Ladha, 2011; Lahue et al., 2016; Lin & Fukushima, 2016)

Threatened by climate change, leading to unpredictable rainfall and typhoons, freshwater is becoming more and more scarce and with a shortage of labour, rice farming needs to adapt (Lin & Fukushima, 2016). Currently a transition from the traditional transplanting system to direct-seeded rice (DSR) is occurring. The DSR system gives the farmer the advantage to be more independent from natural rainfall and water scarcity (Farooq et al., 2011; Kumar & Ladha, 2011; Ray et al., 2013) Besides all the advantages, weeds are now arising as the main biological constraint in this system. Flooded paddy fields haven been a very successful and environmentally friendly way to suppress weeds: plants can typically not germinate in anaerobic conditions under water, so weeds would be unable to establish in these paddy fields, whereas rice was germinated and pre-grown in nurseries and then transplanted. With the transplanting method, rice gets two major advantages over weeds: A size head start because of the pre-growth phase and the flood water suppressing weeds from emerging (Chauhan & Johnson, 2010).

Successful weed control is indispensable, because uncontrolled weed infestation can lead to rice yield losses from 60 % up to complete loss, depending on the conditions (Abdullah Al Mamun, 2014; Dass et al., 2017; Farooq et al., 2011; Raj and Syriac, 2017). Now, farmers tackle this problem by increasing the dosage of herbicide application, which in turn, leads to evolving herbicide-resistance traits in weeds as well as a substantial, undesirable environmental impact. There is, therefore, an urgent need for a more sustainable weed control (Dass et al., 2017; Farooq et al., 2011).

Rice as a scientific model system

Rice is both a very important crop but also an interesting model species, which offers a richness of genetic variation, with high quality genome information available (Wang et al., 2018). Rice has a genome size of 389 Mb, packed into 12 chromosomes, encoding for an estimated 30,000–50,000 genes. Compared to other major cereal crops, rice has with the smallest genome, making functional genomics much more straightforward than for example for wheat, which is tetraploid (Eckardt, 2000; Matsumoto et al., 2005).

Rice scientific resources

Rice was the first crop, and the third largest public genome project, after human and mouse, for which in a community effort the whole genome was sequenced and made publicly available (Eckardt, 2000). The International Rice Genome Sequencing Project (IRGSP) was established in 1998. Several sequencing groups from ten nations worked together, towards the sequencing of the complete rice genome of *Oryza sativa L. ssp. Japonica cv.* Nipponbare (Chen et al., 2019; Matsumoto et al., 2005; Wang et al., 2018). The genomic information is continuously being improved and updated with new sequencing and mapping data (Clark, 2010; Huang et al., 2010; Wei et al., 2021; Yano et al., 2016; Zhao et al., 2011). To date, two major genome annotation projects have been undertaken, both based on *O. sativa* Nipponbare. the first one is the Rice Genome Annotation Project (RGAP) hosted at Michigan State University, with the latest version of release 7 (Oct 31, 2011) available at www.rice.uga.edu (Ouyang et al., 2007). The second one is the Rice Annotation Project Database (RAP-DB), conceptualized in 2004 at the National Institute of Agrobiological Sciences, Tsukuba, Japan, which provides an accurate annotation, based on the IRGSP sequence available at www.apdb.dna.affrc.go.jp, released in its latest version on March 11, 2011 (Kawahara et al., 2013; Sakai et al., 2013). Moreover, an easy conversion scheme between the two differently derived annotations created by RGAP and RAP-DB has been supplied.

A recent genome reannotation was published in December 2018, based on integration of large-scale RNA-seq data as IC4R-2.0 (available at http://ic4r.org) (Sang et al., 2020). In addition, a wide range of databases and analysis tools are available online (McCouch et al., 2016; Wang et al., 2020; Zhao et al., 2015). This offers a plethora of analysis tools, such as: ‘GALAXY’ - a rice genome browser and ‘SNP seek’ (http://snp-seek.irri.org/) provided by IRRI; ‘Gramene’ - a genetic diversity module (http://www.gramene.org/), ‘ricefriend’ (http://ricefrend.dna.affrc.go.jp/), ‘EnsemblPlants’ (https://plants.ensembl.org/Oryza_sativa/), Rice Haplotype Map Project database (http://www.ncgr.ac.cn/RiceHapMap/); tools dedicated to gene expression and network analysis: ‘RiceTOGO’ (https://salad.dna.affrc.go.jp/salad/en/), ‘knetminer’ (https://knetminer.rothamsted.ac.uk/Oryza_sativa/), ‘RiceXPro’ (https://ricexpro.dna.affrc.go.jp/), for protein interaction https://string-db.org/ and pathway analysis https://plantreactome.gramene.org/PathwayBrowser/.

With the development of tools for making targeted genetic adjustments, this has also reached applications in rice. A showcase example of in rice is the successful development of beta-carotene containing rice grains, coined “golden rice” (https://goldenrice.org/). Rice was engineered genetically to produce beta-carotene, with the aim to alleviate blindness and weakened immune system caused by vitamin A deficiency in malnourished population (Paine et al., 2005; Ye et al., 2000). In a great community effort this highly ambitious goal was achieved and after a long way it found its public approval. In 2021 it has been released for farmers to be planted in the Philippines, underway in other countries to be followed (IRRI, 2021; Potrykus, 2001). This is not the only example, where high ambitions and cutting-edge science are brought together in an enormous community effort, to fight major societal challenges. Scientists and institutions across the globe work together with the goal of integrating the C4 photosynthetic pathway in rice and the C4 rice project was kicked off in 2008 (http://c4rice.com/). It is predicted, that C4 rice could have a 50 % increased photosynthetic efficiency and doubled water use efficiency, which would catapult rice yields to satisfy the growing world hunger (Ermakova et al., 2020; Lin et al., 2020; Zhao et al., 2018).

Rice research has benefited strongly from these above-mentioned technological developments and although *Arabidopsis thaliana* is still the plant knowledge system with the largest infrastructure, rice could be the second-best equipped model system, and certainly the best for monocots, for molecular biology research. Although many important plant science discoveries, such as the identification of hormone receptors, are often made initially in Arabidopsis, the gibberellin receptor GID1 was for example first discovered in rice (Hartweck & Olszewski, 2006; Hedden & Sponsel, 2015; Yano et al., 2015) before its identification in Arabidopsis. Relative to Arabidopsis, the life cycle of rice is much slower and genetic transformation procedures are not as straightforward as in Arabidopsis. Nevertheless, it is important to consider that the crop that feeds the largest number of people on our planet, has become a genetically highly tractable model system as well.

Diversity and phenotypic plasticity

The phenotype of a plant is to a certain extent predetermined by its genetic code. Besides the fixed genetic code, the phenotype of an individual plant is further shaped by the genotype x environment interactions depending on the interaction of an individual plant with external factors, of biotic or abiotic nature (Teichmann & Muhr, 2015).

Rice, with the focus in this work on the *Oryza sativa* species, is immensely diverse at the genomic level. As mentioned earlier, due to human an natural selection events it branched into different subpopulations and thousands of subspecies (Huang & Han, 2014; Wang et al., 2018b; Zhao et al., 2018). Subpopulations originate from different climates and agricultural practices (Kennedy & Burlingame, 2003; Wing et al., 2018). Genetic relatedness by and large determines the heritable component of the phenotypic variation (Eizenga et al., 2014; Liakat Ali et al., 2011; McCouch et al., 2016; Zhao et al., 2011). Varieties of the *indicas* are characterized by high tillering and rather shorter shoot with more erect leaves, in contrast to the *japonicas* that have comparatively less tillering, taller stature and droopier and larger leaves (Dingkuhn et al., 2001; Han et al., 2016; Liakat Ali et al., 2011). In addition, varieties vary greatly for other phenotypic traits such as growth vigour and yield related traits (for example Han et al., 2016; Namuco et al., 2009; Zhao et al., 2006a). Varieties belonging to the *Oryza sativa* species offer a vast haplotype diversity, differing in single-nucleotide polymorphisms (SNPs), insertions and deletions (indels) and other structural variants (Matsumoto et al., 2005; Wang et al., 2018b). Different diversity panels and RIL populations have been developed and germplasm is available at the gene-bank IRGC hosted at IRRI. The Rice Diversity Panels 1 and 2 (RDP1 and2) are a collection of ~1500 diverse rice varieties of five subpopulations. These panels have been genotyped using a genome-wide high-density rice array (HDRA) approach and the set of 700k SNPs is available (McCouch et al., 2016). The RDP1 was used for the studies in this thesis. Another widely used diversity panel is the collection of the 3000 Rice Genomes (3KRG), which was also sequenced in the 3000 rice genomes project (3, 2014). The imputed-HDRA set, combines and improved the HDRA and 3KRG data sets (Wang et al., 2018a) and has been further expanded and improved (Morales Id et al., 2020).

This richness in genetic and phenotypic diversity offers a valuable basis and can be taken advantage of for in-depth genome-phenome studies. The release of the sequenced genome gave rise to innumerable QTL and GWAS studies on a variety of traits related to abiotic stresses, such as drought and salinity, and biotic stress resistance and to a large extent on yield related traits; for example (Chen et al., 2019; Guo et al., 2019; Huang et al., 2010; Huang & Han, 2014; Kadam et al., 2018; McCarthy et al., 2008; Subedi et al., 2019; Yano et al., 2016; Zhao et al., 2010, 2011).

Architecture and life cycle

Rice is a monocot and a grass and thus shows the typical anatomy of grasses (*Poaceae*) (Figure 1.2) (Teichmann & Muhr, 2015). The first tissues emerging from the germinating seed are the coleoptile and the radicle. Rice undergoes three major growth phases, from vegetative, over reproductive to ripening phase. The vegetative tissue is formed by the leaves (leaf sheath and leaf blade). The bundle of the leaf sheaths forms the stem like structure, referred to as culm. The point where the leaf blade bends off is the node and the segment in between two leaves is called the internode. At a later stage, side branches are formed out of the basal node, which are called tillers (Figure 1.2), with the process of branch formation and outgrowth, termed tillering.

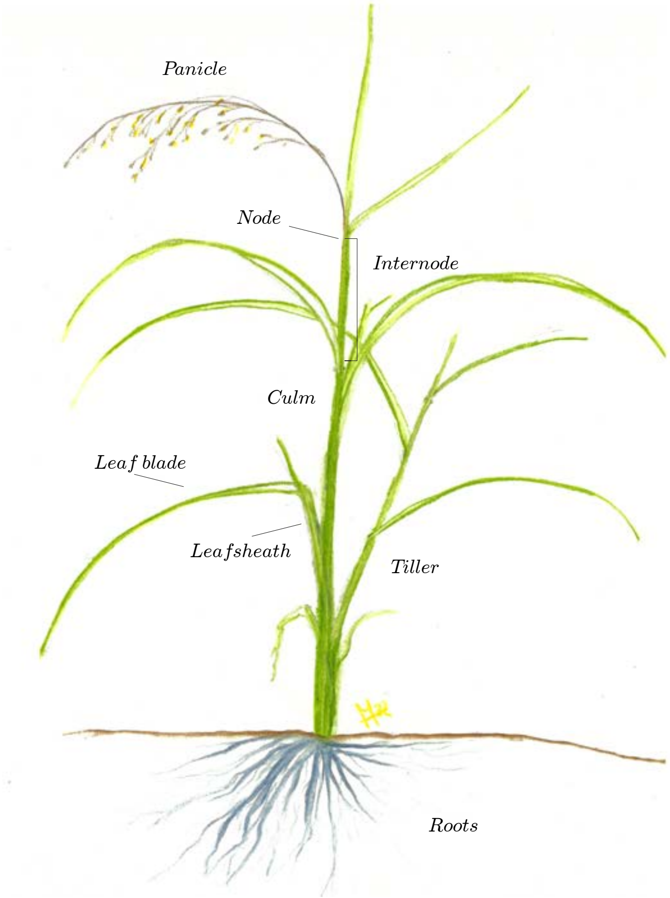

Figure 1.2. Anatomy of *Oryza sativa* at reproductive stage.

After seedling establishment rice starts tillering in the late vegetative phase. After approximately six weeks maximum tillering is reached and formation of new tillers is stopped by transition to the reproductive phase. Stem elongation stops at the time of panicle initiation, the panicle being the whole of flowers (or seeds) and their branched-out stalks. Rice is a short-day plant, indicating that flowering is stimulated by short day length (Brambilla et al., 2017; Izawa et al., 2000). Initiation of panicle formation marks the beginning of the reproductive phase. Panicles are formed at the uppermost internodes of the main culm and the tillers. The emerged panicle (after a process called heading) splits into tiny branches, each end carrying a spikelet. After successful wind pollination, each spikelet is filled and at ripening stage the enclosed rice grains can be harvested. Completing the cycle from seed to seed for a rice plant of a modern cultivated variety takes around three to six months, depending on the genotype. Current rice varieties only differ in the length of their vegetative phase (from 35 to 95 days), whereas the reproductive and ripening phases are relatively fixed (Bardenas & Chang, 1965; Global Rice Science Partnership, 2013; IRRI, 2007).

Plant competition and weed-competitive rice

When several plants grow next to each other, they typically compete for resources essential to their growth, such as light, water and nutrients. In natural environments, competition commonly occurs and even more so in densely planted monocultures of modern agricultural systems. Competition arises above as well as below ground (Mahajan & Chauhan, 2013) and neighbours are detected via different ways, for example via volatile organic compounds (Kegge et al., 2013; Pierik et al., 2003), chemical compounds released from roots (Kegge et al., 2013; Worthington & Reberg-Horton, 2013) or physical contact (Pantazopoulou et al., 2017, 2021; De Wit et al., 2012). Before any of these cues occur, the first neighbour detection cue to be observed is a change in the light quality, where red light is depleted and far-red light is enriched (for example Ballaré & Casal, 2000; Ballaré & Pierik, 2017; Casal, 2012; Franklin, 2008; Roig-Villanova & Martínez-García, 2016). With respect to light, different strategies have evolved to cope with unfavourable light conditions, which can be classified as tolerance, defence and attack strategies. Shade tolerant plants can usually be found in habitats with continuous low light intensities, such as a forest understory, to which plants have adapted with a generally energy conserving metabolism and low growth rates (Casal, 2012; Gommers et al., 2013; Niinemets, 2010; Roig-Villanova & Martínez-García, 2016). Shade avoidance on the other hand, is a way for plants to actively grow away from shade and even or relocating photosynthetic tissue into sunny patches or escape with an accelerated life cycle (more detailed description in the following chapter 2). Although not specifically a response to light, plants can also follow an attack strategy, by negatively affecting their neighbours with releasing compounds deteriorative to their neighbour’s growth – referred to as allelopathy (Guo et al., 2018; Seavers & Wright, 1999; Worthington & Reberg-Horton, 2013), or simply occupying the space faster than competing neighbours by increased growth vigour and extended ground cover. Precisely these traits, found to conferring competitive advantage of wild plants against neighbouring plants, are traits that might be favourable for weed competitive crops. It has been described already that successful competition for light is a highly important factor influencing weed proliferation in an agronomic context, with negative correlation to weed biomass and positive correlation to crop yield (Mahajan & Chauhan, 2013; Namuco et al., 2009). That this would be applicable also for cereals might not seem straightforward, considering the relatively low shading phenotype of grasses, especially in early growth phases. However, that increased shading by cereal crops can be an effective manner to control weeds, has been shown in an approach on more and less uniform planting patterns in wheat (Lu et al., 2020; Marín & Weiner, 2014; Weiner et al., 2010). In addition, there is evidence that certain shoot architectural traits of cereals are aiding to outcompete neighbours through light competition (Chauhan, 2012; Chauhan & Johnson, 2010). In the case of rice, shoot architecture traits that have been linked to weed-competitiveness include increased leaf area and canopy ground cover, droopy leaves and increased tillering capacity, as well as increased early vigour (Cairns et al., 2009; Caton et al., 2003; Chauhan, 2012; Dingkuhn et al., 2001; Haefele et al., 2004; Mahajan & Chauhan, 2013; Namuco et al., 2009; Rao et al., 2007; Worthington & Reberg-Horton, 2013; Zhao et al., 2006). The critical period of weed competition in rice is from the moment of sowing up to 40 – 60 days after sowing in a DSR system (Abdullah Al Mamun, 2014; Azmi et al., 2007; Chauhan & Johnson, 2011; Mennan et al., 2012; Raj & Syriac, 2017). Especially in the context of DSR, shading by the crop canopy would have to occur early in the season and especially seedling vigour would substantially reduce weed growth (Mahajan & Chauhan, 2013; Subedi et al., 2019; D. L. Zhao et al., 2006a).

It has been proposed that even if expressing a certain trait would not confer a fitness advantage for the individual – and not deliver increased yield of the individual plant, it might have advantages for a monoculture plant community (Hedden, 2003; Kush & Khush, 2001; Pingali, 2012; Looking in the Wrong Direction for Higher-Yielding Crop Genotypes, 2019). Taking advantage of this discrepancy between individual plant fitness and performance of the monoculture group, is at the heart of a concept framed as Darwinian Agriculture or Evolutionary Agroecology (Denison, 2012; Weiner et al., 2010; Weiner & Freckleton, 2010; Wu et al., 2021)(for more detail see Chapter 2).

Aim of this thesis and approach

The aim of this thesis is to investigate how rice shoot architecture is regulated, with its functional application in rice cultivation. This thesis investigates i) genetic control of rice shoot architecture, ii) its impact on light availability and weed proliferation and iii) rice architecture and photosynthetic responses to light cues of high planting density. These studies involve detailed investigation of rice on many levels: From the canopy perspective in the field, to the shoot of an individual plant, to distinct shoot traits, to physiological and biochemical traits, over the transcriptome, down to the genome.

Thesis outline

Providing the necessary background information, in **Chapter 2**, a comprehensive review presents a broader view on characteristics of shoot architecture in plant canopies and the behaviour of plants in communities, with a focus on plants in monocultures. In this review also the concept of shade avoidance is introduced with the current knowledge of the field and its meaning for crops.

The experimental work in this thesis focuses on rice and starts with an exploration of natural diversity in shoot architecture at early growth in **Chapter 3**. These shoot architectural traits were related to their genetic loci using genome-wide association studies. The effect of different alleles of the encoding loci on the phenotype were characterised by means of a haplotype analysis.

Upon studying genetic loci related to shoot architecture of individual plants, it is also important to bring clarity into the behaviour of a canopy in a plant community. **Chapter 4** presents the insights gained from a field study, which included rice planted at different densities, in addition to independently planted weeds. This field assay enabled us to not only get a more holistic picture of rice shoot plasticity influenced by density and weed-competition., but also answers the bigger question whether selected varieties would suppress weeds in the field.

Chapter 5 investigates the architectural plasticity further into detail, using far-red light as a specific cue for plant proximity. In addition to detailed description of plasticity of the phenotype, this chapter also describes changes of rice transcriptomes when experiencing proximity shade.

As a continuation of the far-red light studies in chapter, in **Chapter 6** it is investigated how far-red light photons contribute to photosynthesis. Gas-exchange measurements showed that, despite the general assumption that far-red light is mostly a signal rather than a photosynthetic resource, far-red light strongly enhances carbon fixation in a number of rice varieties.

Chapter 7 brings the experimental data together in a general discussion with recommendations for rice farming and photobiology research.

Chapter 2

Light signalling shapes plant-plant interactions in dense communities

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Abstract

Plants growing at high densities interact via a multitude of pathways. Here, we provide an overview of mechanisms and functional consequences of plant architectural responses initiated by light cues that occur in dense vegetation. We will review the current state of knowledge about shade avoidance, as well as its possible applications. On an individual level, plants perceive neighbour-associated changes in light quality and quantity mainly with phytochromes for red and far-red light and cryptochromes and phototropins for blue light. Downstream of these photoreceptors, elaborate signalling and integration takes place with the PHYTOCHROME INTERACTING FACTORS, several hormones and other regulators. This signalling leads to the shade avoidance responses, consisting of hyponasty, stem and petiole elongation, apical dominance and life cycle adjustments. Architectural changes of the individual plant have consequences for the plant community, affecting canopy structure, species composition and population fitness. In this context, we highlight the ecological, evolutionary and agricultural importance of shade avoidance.

Introduction

Plants growing at high densities compete for light, as well as other primary resources such as water and nutrients. In such a crowded environment, shade-intolerant plants are able to adjust their development and physiology to optimize resource acquisition in order to escape from these unfavourable conditions. But how does a plant even detect that there are competing neighbours around? Several environmental cues provide information about the presence of competitors: volatile organic compounds that carry information about neighbouring plants through the air (Pierik, Visser, De Kroon, & Voesenek, 2003), below ground root exudates and volatile organic compounds (Guo et al., 2018; Worthington & Reberg-Horton, 2013) and mechanical interaction via physical touching of neighbour leaves (Wit et al., 2012), all provide information about proximate vegetation. However, the dominant cues for neighbour detection at high planting density are associated with light quality and quantity (Ballaré, 1999; Ballaré & Pierik, 2017; Casal, 2012; Pierik & Wit, 2014; Roig-Villanova & Martínez-García, 2016). Clearly, the presence, intensity and reliability of neighbour cues depends on their proximity: At high planting densities, neighbours are very nearby and cues, be it chemical or visual, will be both strong and reliable. At low densities, or very early stages of stand development, most cues will not be sufficiently strong to elicit major responses in receiving plants. Nevertheless, depending on the specific light cues, a plant can detect whether it is truly shaded, for example, by an overhead canopy (foliage shade) or surrounded by neighbours of similar height (changes in light quality) (Wit et al., 2016).

In this review, we will focus primarily on the changes in light quality that serve as early cues for impeding competition and cause responses in neighbours. These trigger a suite of responses that change plant development and architecture, collectively referred to as the shade-avoidance syndrome (SAS), sometimes even before actual shading occurs (Ballaré, 1999; Ballaré, Scopel, & Sánchez, 1990; Pierik & Wit, 2014; Schmitt, Dudley, & Pigliucci, 1999). In this review, we treat the term ‘canopy’ in its most general sense (Table 2.1), for which we will discuss changes in traits related to canopy architecture. Primarily, we will be focussing on herbaceous plants; responses of perennial, woody plants are mostly beyond the scope of this review.

First, we will provide a detailed description of light cues in canopies, including how these are perceived and processed by plants to plastically regulate development. This will be based largely on knowledge available from the plant model species Arabidopsis thaliana. We then scale up from changes of a single plant to plant communities. We further address the functional consequences of the shade responses and the questions of plasticity and adaptation in this context (Table 2.1).

Table 2.1. Definitions and abbreviations

Adaptation	**Adaptation** refers to heritable, genotypic traits - in contrast to acclimatization - that change a plant's phenotype and physiology and make the organism more fit for a specific environment (Novoplansky 2002). An **adaptive** trait denotes a trait that confers a fitness advantage (Schmitt et al. 1999) and has evolved through natural selection over several generations.
Canopy	The **canopy** is the aboveground portion of a plant community, formed by the collection of individual plant crowns (Campbell & Norman 1990). In general, traits describing canopy architecture include the number, size, shape, distribution and orientation of their leaves (Niinemets 2010; Duursma et al. 2012; Rahman, Duursma, Muktadir, Roberts & Atwell 2018) • LA = leaf area • SLA (specific leaf area) = leaf area / leaf dry weight • Leaf inclination angle or petiole angle • Light interception = amount of light captured
Competition	Competition describes the negative effects on growth of resource restrictions due to neighbouring organisms (Aphalo et al. 1999). **Intra-specific competition** refers to competition between individuals of the same species, e.g. in crop monocultures, whereas **inter-specific competition** refers to competition between different species, e.g. crop-weed competition or naturally mixed-species vegetations.
Phenotypic plasticity	**Phenotypic plasticity** is the capacity of an individual plant to express different phenotypes in response to environmental variation (Smith & Whitelam 1997; Aphalo et al. 1999; Schmitt et al. 1999).
Shade-avoidance syndrome (SAS)	The **shade-avoidance syndrome** refers to the multiple responses of a plant to shade and changes in light quality caused by neighbouring plants (Smith & Whitelam 1997; Roig-Villanova & Martínez-García 2016; Ballaré & Pierik 2017): • Hyponasty (upward movement of leaf or petiole) • Accelerated hypocotyl and internode elongation • Increased apical dominance (reduced branching and tillering) • Accelerated flowering

Detecting light spectral changes in dense stands

Plants have specific spectral absorption and reflection properties, strongly determined by the absorption properties of chlorophyll. When growing in close vicinity, they therefore collectively alter the light composition inside the vegetation. The earliest light cue reflected from neighbouring plants is a change in the red (R) to far-red (FR) ratio (R:FR) (Ballaré et al., 1990).

Plants absorb blue (400–500 nm) and red (635–700 nm) wavelengths through chlorophyll to fuel photosynthesis while reflecting FR wavelengths (700–780 nm). Sunlight has a R:FR of approximately 1.2, but neighbouring plants can reduce this ratio to as low as 0.1 in deep canopy shade (i.e., canopy closure). Interestingly, R:FR can drop already before true shading occurs (Ballaré et al., 1990), due to reflection of FR light by neighbouring plants that are not yet overlapping (Figure 2.1). The initial drop in R:FR is, therefore, an early warning cue of upcoming competition for light and is followed by a decrease of total light intensity and depletion of blue light when the canopy further develops and true shading occurs (reviewed in Pierik & Wit, 2014). In the next paragraphs, the changed light quality due to the (closing) canopy will be discussed (summarized in Figure 2.2). In addition to red, FR, blue and total light intensity, also other factors weigh in, such as the change in light spectrum during the day, the weather, sun flecks and sun zenith angle (Kotilainen et al., 2020). Since these light changes are not caused by neighbouring plants, we do not discuss them in depth in this review and direct the reader to an excellent recent update on the matter by Kotilainen et al. (2020). UV-B light is a potent antagonist of plant responses to FR light enrichment and blue light depletion and will also be decreased inside vegetation due to absorption. When a shade-avoiding plant perceives UV-B through the UVR8 photoreceptor, shade avoidance is inhibited through UVR8 interaction with COP1, resulting in HY5 accumulation (Favory et al., 2009) and through inhibition of PIFs (Hayes, Velanis, Jenkins, & Franklin, 2014; Mazza & Ballaré, 2015). Most mechanistic knowledge discussed here comes from studies on the model species *Arabidopsis thaliana* (Arabidopsis), although some aspects have also been investigated in other species.

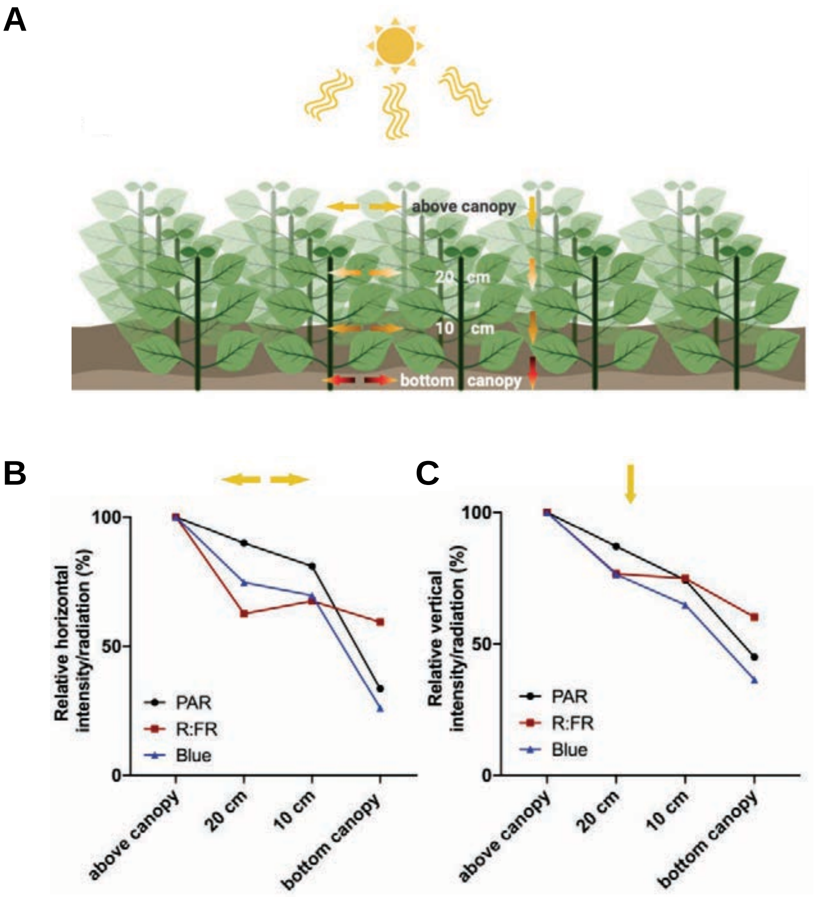

Figure 2.1. Changes in light quality and quantity in different canopy strata. A. The cartoon illustrates a basil (*Ocimum basilicum*) canopy in which PAR (photosynthetic active radiation), blue (λ = 400–499) and R:FR (red to far-red ratio; [R (λ = 650–670): FR (λ = 720–740)]) were measured at different canopy heights. Arrows illustrate the directions of the light measurements at the different heights. Quantifications of **B.** horizontally and **C.** vertically measured PAR (black line), blue (blue line) and R:FR (red line) light at the different canopy heights (above canopy, 20 cm, 10 cm and bottom canopy), expressed as percentage of the values measured above the canopy. The basil canopy consisted of 20 plants that were transplanted 6 days after germination, in a chequerboard pattern with 15 cm distance from each other. The canopy height was 30 cm from soil level. Graphs show light measurements made with a LI-COR LI-180 spectrometer, using a cosine corrected sensor, in a 37-day-old canopy (n = 3). The experiment was performed in the greenhouse facilities of Utrecht University. Created with BioRender.com.

Decreased red to far-red ratio

Plants perceive R and FR light with the phytochrome class of photoreceptors. The dicot species Arabidopsis has five phytochrome genes (PHYA-E), while monocots typically have three (PHYA-C) (Mathews & Sharrock, 1996; Sharrock & Clack, 2002). The phyB receptor is the dominant player in the shade-avoidance response. Phytochromes are photoreversible proteins that are activated by R light (converting phyB into the active Pfr form), rapidly inactivated by FR light and gradually inactivated in the dark (to the inactive Pr form) (Hillman, 1967; Wang &

Wang, 2015). Phytochromes are also sensitive to temperature, and elevated temperature-mediated conversion of Pfr to Pr is one of the mechanisms through which plants sense temperature (Legris, Ince, & Fankhauser, 2019). PhyA can also stay active in FR light and constitutes a negative feedback that can attenuate shade-avoidance responses (Yang et al., 2018).

Depletion of total light intensity and blue light

The reduction in R:FR starts when growing plants in a community are approaching each other. However, when the foliage of plants starts overlapping, this in addition entails a decrease in blue light (low blue light), following from light absorption by the overlaying leaves. The overall quantity of light, the photosynthetically active radiation, also decreases inside vegetation during the growth season. This decrease is registered primarily by the cryptochrome blue light receptors but also through the reduced rates of photosynthesis in low light (Millenaar et al., 2009; Mullen, Weinig, & Hangarter, 2006). The photoreceptors sensitive to blue light are, besides the cryptochromes, phototropins and members of the ZEITLUPE family (Pudasaini & Zoltowski, 2013). Phototropins regulate, amongst others, the phototropic response of seedlings and adult plants towards light-enriched spaces (Briggs & Christie, 2002), whereas the cryptochromes (CRY1 and CRY2) play an active role in elongation responses (Keller et al., 2011; Keuskamp et al., 2011; Pedmale et al., 2016). It is important to note that investigating how plants perceive and respond to the absence of blue light helps to understand the molecular mechanisms of this pathway, but the depletion of blue light alone in a white light spectrum is not a naturally occurring situation. Low blue light in natural conditions will always be accompanied by a decrease in R:FR when indicating canopy shade. Indeed, low R:FR and low blue light pathways converge and are integrated, leading to strong shade avoidance (Wit, Keuskamp, et al., 2016) and regulation of phototropic bending in light-grown plants (Goyal et al., 2016). In addition to convergent action with phyB-sensed R:FR cues, cryptochromes themselves also show sensitivity to another wavelength that is abundant inside canopies, green light, in regulating hypocotyl elongation in Arabidopsis (Sellaro et al., 2010).

Processing canopy light cues

The perception of canopy-associated light cues initiates a signalling cascade that differs between early neighbour detection and canopy shade (Hersch et al., 2014). The combination of different shade cues can either lead to signal intensification and enhance a certain pathway or trigger distinct pathways (Wit, Keuskamp, et al., 2016). In the next paragraphs, we will briefly mention the most important components of shade signalling (summarized in Figure 2.2), resulting in elongation responses of hypocotyls, stems and leaves.

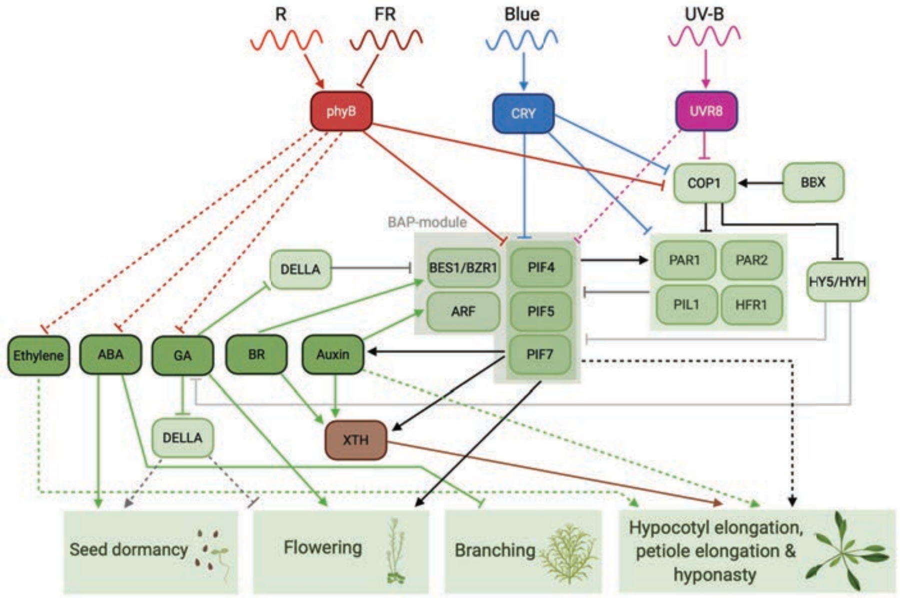

Figure 2.2. Simplified schematic overview of the signal transduction pathway of shade-induced seed dormancy, early flowering, reduced branching, hyponasty and accelerated hypocotyl and petiole elongation. Interactions between important proteins (light green) and hormones (dark green) are shown and result in the different SAS responses. See main text for details. Arrows - positive effect, inhibitors - negative effect, solid lines - confirmed interactions/known signalling pathways, dotted lines - exact mechanisms to be elucidated. Created with BioRender.com

Phytochrome interacting factors

A class of basic helix–loop–helix (bHLH) transcription factors called the PHYTOCHROME INTERACTING FACTORS (PIFs) interact with both phytochromes and cryptochromes (Leivar & Quail, 2011). Binding of the activated phytochromes or cryptochromes to PIFs inactivates them and in many instances even leads to PIF degradation (Huang et al., 2018; Pedmale et al., 2016). The Arabidopsis genome contains eight different PIF genes (PIF1-8), of which PIF4, PIF5 and PIF7 play a major role in the shade-avoidance responses (Hornitschek, Lorrain, Zoete, Michielin, & Fankhauser, 2009). Upon binding of active phytochrome, PIFs are phosphorylated and PIF4 and PIF5 are subsequently degraded (Lorrain, Allen, Duek, Whitelam, & Fankhauser, 2008). Although PIF7 is not rapidly degraded, it still gets inactivated upon phosphorylation (Li et al., 2012). Although a role for PIF8 in shade avoidance has not been tested yet, it was recently shown to repress phyA-dependent light responses (Oh, Park, Song, Bae, & Choi, 2020). PIFs can bind directly to DNA, thereby activating SAS-related genes such as genes encoding cell-wall-modifying enzymes, as well as several growth-promoting hormones, especially auxin (Hornitschek et al., 2012; Li et al., 2012; Pedmale et al., 2016). These molecular processes allow plants to activate the SAS phenotypes, helping them to reposition their photosynthetic organs towards the light.

Hormones

As mentioned above, PIFs interact with hormone pathways that are involved in shade-avoidance control. The best studied and core regulatory hormone is auxin (Iglesias, Sellaro, Zurbriggen, & Casal, 2018; Yang & Li, 2017). Although we give a brief coverage here, we point the readers to Küpers, Oskam, and Pierik (2020) for a much more detailed overview of auxin control in shade-avoidance responses. PIFs directly activate transcription of genes encoding YUCCA enzymes involved in auxin biosynthesis but also of genes encoding auxin transport-associated proteins and proteins relevant in auxin response, such as AUX/IAAs (Wit, Galvão, & Fankhauser, 2016). Auxin is important in growth and development of almost all plant organs. It has been shown that treatment of Arabidopsis plants with additional FR light, thus creating a low R:FR ratio, leads to increased auxin levels in the shoot (Keuskamp, Pollmann, Voesenek, Peeters, & Pierik, 2010; Li et al., 2012; Procko, Crenshaw, Ljung, Noel, & Chory, 2014; Tao et al., 2008). Applying auxin to seedlings or certain organs often mimics the responses associated with SAS (Chapman et al., 2012; Keuskamp et al., 2011; Xu et al., 2018). Auxin synthesis occurs in different parts of the plant, and auxin transport is needed for the shade-avoidance responses to occur, both in hypocotyl elongation (Keuskamp, Pollmann, et al., 2010) and hyponasty (Michaud, Fiorucci, Xenarios, & Fankhauser, 2017; Pantazopoulou et al., 2017). Plant organs can also show contrasting growth responses due to differential auxin responsiveness such as between the leaf lamina and petiole (Wit, Ljung, & Fankhauser, 2015). In rice seedlings, auxin-related genes induced by shade are upregulated in the first leaves, even though the coleoptiles are responding with elongation (Liu, Yang, & Li, 2016). It was shown in *Brassica rapa* seedlings that supplemental FR triggers auxin synthesis in the cotyledons, and auxin is subsequently transported to the hypocotyl to promote elongation (Procko et al., 2014). Shade-induced auxin synthesis is regulated by PIFs, and PIF action in addition can be further promoted by auxin response itself via auxin response factors (ARFs, transcription factors) that increase PIF-dependent gene expression (Oh et al., 2014). However, further studies are necessary to elucidate if such positive feedback also regulates shade avoidance. Auxin and PIFs both lead to the upregulation of a group of cell-wall-modifying enzymes, xyloglucan endotransglucosylase/hydrolases (XTHs) that allow cell-wall modifications needed for the changes in cell growth needed for shade avoidance (Keuskamp et al., 2011; Sasidharan et al., 2010). Cell-wall-modifying proteins are active at low apoplastic pH, and shade exposure is accompanied by acidification of the Arabidopsis petiole apoplast (Sasidharan et al., 2010). This may very well also be auxin-dependent, probably via SMALL AUXIN UPREGULATED proteins that activate plasma membrane ATPases (Fendrych, Leung, & Friml, 2016).

In addition to auxin, also gibberellin synthesis is promoted in low R:FR. Gibberellin promotes SAS by causing degradation of the growth inhibiting DELLA proteins (Feng et al., 2008). In non-shaded conditions, DELLA proteins bind PIFs and thereby inhibit their function (de Lucas et al., 2008; Feng et al., 2008). This is suppressed by low R:FR-mediated, DELLA degradation via gibberellin (Djakovic-Petrovic, de Wit, Voesenek, & Pierik, 2007). Another important hormone regulator of SAS is brassinosteroid (Hayes et al., 2019; Keuskamp et al., 2011; Kozuka et al., 2010; Wit, Galvão, & Fankhauser, 2016). Despite the clear evidence for brassinosteroid involvement, the precise mechanisms are still unknown. Different from gibberellin and auxin, brassinosteroid levels do not seem to increase in shade, and there are even reports of reduced brassinosteroid levels in shade compared to control light (Bou-Torrent et al., 2014; Gommers et al., 2018). At least part of the brassinosteroid involvement occurs via its regulation of BES1/BZR1 transcription factors that interact positively with PIFs to promote target gene expression (e.g., Hayes et al., 2019; Oh et al., 2014). A synergistic relationship between auxin and brassinosteroid has also been proven since both are needed to achieve full hypocotyl elongation under low blue light conditions (Keuskamp et al., 2011). The BZR1-PIF-ARF, BAP module is inhibited by DELLA proteins (Oh et al., 2014).

Two other hormones can be involved in shade-avoidance control, but the molecular mechanisms are less well characterized: ethylene and abscisic acid. Ethylene can promote shoot elongation in a species- and conditions-dependent manner (reviewed in Pierik, Tholen, Poorter, Visser, & Voesenek, 2006). Ethylene is volatile, and its emission is promoted by low R:FR. The hormone can even accumulate in the still air inside a canopy and ethylene-insensitive transgenic tobacco plants had reduced shade-avoidance properties (Pierik, Cuppens, Voesenek, & Visser, 2004; Pierik, Whitelam, Voesenek, de Kroon, & Visser, 2004). In water-adapted terrestrial plants, ethylene has been shown to promote submergence-induced shoot elongation through downregulation of abscisic acid (Benschop et al., 2005; Hoffmann-Benning & Kende, 1992).

However, the role of abscisic acid in shade avoidance so far has been mostly described for its inhibition of branching under low R:FR (Reddy, Holalu, Casal, & Finlayson, 2013). Although abscisic acid can be a strong inhibitor of low R:FR responses, such as accelerated hypocotyl elongation in Arabidopsis (Hayes et al., 2019), it remains to be investigated if this hormone is part of the intrinsic phyB-regulated elongation pathways.

Other regulators

Besides PIFs and hormones, there are other positive and negative regulators important in the shade-avoidance response. ELONGATED HYPOCOTYL5 (HY5) and its homolog HYH are photoreceptor-regulated via CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) E3-ligase that targets HY5 and HYH (Pacín, Semmoloni, Legris, Finlayson, & Casal, 2016). The HY5 and HYH proteins inhibit hypocotyl and petiole elongation in Arabidopsis (Nozue et al., 2015). hy5 mutants show constitutively enhanced hypocotyl elongation, while over-expression of HY5 leads to inhibited elongation (van Gelderen et al., 2018). COP1 also interacts with double B-BOX (BBX) zinc finger transcription factors, of which BBX21 and BBX22 are both involved in early- and long-term SAS responses (Crocco, Holm, Yanovsky, & Botto, 2010). BBX25 interacts with HY5 and enhances COP1 function, lifting the inhibition of the hypocotyl elongation (Gangappa et al., 2013). LONG HYPOCOTYL IN FAR-RED 1 (HFR1), PHYTOCHROME RAPIDLY REGULATED 1 (PAR1) and PAR2 are negative regulators of the shade-avoidance responses (Buti, Hayes, & Pierik, 2020). Plants over-expressing these genes show diminished shade-avoidance responses, whereas reducing the transcript levels of these genes leads to enhanced shade-avoidance responses (Hornitschek et al., 2009; Li et al., 2014; Roig-Villanova et al., 2007; Steindler et al., 1999). It was found that HFR1, PAR1 and PAR2 can interact with PIFs, thereby preventing PIFs from binding to target sequences on the DNA. Indeed, when plants in low R:FR are simultaneously exposed to low blue light, this enhances elongation by suppressing low R:FR-induced HFR1 protein and HFR1 gene expression (Wit, Keuskamp, et al., 2016). The most recent insights into this complex network is that another non–DNA-binding HLH protein, KIDARI (KDR)/PACLOBUTRAZOL-RESISTANCE6 (PRE6) can physically interact with PAR1 and PAR2, as well as several other growth-inhibitory proteins, thereby preventing KDR's targets from inhibiting PIF activity (Buti et al., 2020; Buti, Hayes, & Pierik, 2020). Molecular regulators in species other than Arabidopsis have been thoroughly discussed in reviews by Kebrom and Brutnell (2007) and Carriedo, Maloof, and Brady (2016).

Developmental plasticity in response to light cues

Shade-avoidance responses help plants to grow away from shaded zones in the canopy, into the more light-exposed areas, enabling photosynthesis and consequently growth. Since resources are limiting in dense communities, growth trade-offs between different organs become inevitable. In this section, we will discuss different plant traits underlying SAS that are also summarized in Figure 2.2.

Hypocotyl, petiole and stem elongation

In early canopies, seedlings can already detect neighbours and change their growth forms accordingly. This mostly shows by enhanced elongation of the hypocotyl and reduced growth of the cotyledons. Exposure to the combination of supplemental FR and blue light depletion (low blue) causes an enhanced hypocotyl elongation compared to their separate treatments (Wit, Keuskamp, et al., 2016). In adult Arabidopsis rosette plants, supplemental FR causes petiole and stem elongation (Gommers et al., 2017; Sasidharan et al., 2010). Besides Arabidopsis, supplemental FR elicits internode and stem elongation in stem-forming plants, such as tobacco, sunflower, soybean, spring wheat, maize, tomato, alfalfa and Powell amaranth (Brainard, Bellinder, & DiTommaso, 2005; Caton, Cope, & Mortimer, 2003; Chitwood et al., 2015; Evers, Andrieu, & Struik, 2006; Green-Tracewicz, Page, & Swanton, 2011; Lorenzo et al., 2019; Page, Tollenaar, Lee, Lukens, & Swanton, 2009; Wille, Pipper, Rosenqvist, Andersen, & Weiner, 2017). Exposure to low blue light alone does not necessarily cause a change in petiole elongation in Arabidopsis compared to white light, suggesting that petiole elongation is regulated via different pathways or in a different manner in this species as compared to low R:FR-driven elongation (Pierik, Djakovic-Petrovic, Keuskamp, de Wit, & Voesenek, 2009), but see Keller et al. (2011). Low blue light alone does stimulate internode elongation in various other species, including Stellaria longipes (Sasidharan, Chinnappa, Voesenek, & Pierik, 2008), tobacco (Pierik, Whitelam, et al., 2004), Sinapis alba L. and Datura ferox L., and the strongest elongation in D. ferox L. occurred under combined low R:FR and low blue light levels (Ballaré, Scopel, & Sanchez, 1991).

Hyponasty

Another phenotypic characteristic of shade avoidance is the upward movement of leaves (hyponasty) that typically occurs in rosette plants, such as Arabidopsis. Shade-induced hyponasty leads to a higher leaf lamina position in a canopy, thus preventing chances of being shaded by neighbouring leaves. Hyponasty is typically induced by low R:FR and exposure of just the leaf tip to supplemental FR is already enough to initiate hyponasty through auxin synthesis in the leaf tip and subsequent transport to the petiole (Michaud et al., 2017; Pantazopoulou et al., 2017). Nevertheless, other light cues such as low photosynthetic active radiation can also induce strong upward leaf movement in an auxin-dependent manner (Millenaar et al., 2009). Interestingly, prior to the plant sensing the changed light situation with its photoreceptors, the physical touching of adjacent leaf tips can also trigger hyponasty in dense Arabidopsis monocultures (Wit et al., 2012). The touch-induced hyponasty does not seem to occur though the canonical low R:FR-dependent regulators, but the molecular mechanisms underpinning this response are still to be resolved.

Apical dominance

Plants that have multiple shoot branches, or tillers in grasses, show inhibition of branching or tillering under shaded conditions (Casal, Sanchez, & Deregibus, 1986; Caton et al., 2003; Green-Tracewicz et al., 2011; Wang et al., 2013). The Tb1 gene in maize and orthologs in other species regulates this apical dominance (Doebley, Stec, & Gustus, 1995; Takeda et al., 2003). Mutants in these genes cause plants to tiller in both control and shaded conditions (Kebrom, Burson, & Finlayson, 2006). PhyB mutation in sorghum causes reduced tillering as well, showing a direct link between light perception and changes in tillering (Kebrom et al., 2006).

Life cycle adjustments

Although many species display strong phenotypic plasticity to shade cues, others may not necessarily change their architecture but avoid competition through life cycle tactics such as early flowering or delayed germination. Shade can prolong seed dormancy to ensure germination in favourable light conditions (Casal, Sanchez, Benedetto, & Miguel, 1991; Cumming, 1963; Poppe & Schäfer, 1997; Vazquez-Yanes & Smith, 1982). Exposing seeds to supplemental FR light for example prevents germination while treating seeds with a period of red light lifts the dormancy (Debeaujon & Koornneef, 2000; Lee & Lopez-Molina, 2012; Piskurewicz et al., 2008). Germination of these species with light-sensitive germination depends on a stable pool of phyB, and to achieve this, a period of R light is required. Downstream of phyB, ABA and GA controls seed dormancy and seed germination, respectively (Devlin et al., 1999; Lee et al., 2012; Lee & Lopez-Molina, 2012; Piskurewicz et al., 2008). Shade also causes changes in the later life stages of plants. For annual plants such as Arabidopsis, early flowering is an established SAS trait (Cerdán & Chory, 2003). This early flowering is regulated through PIF4, PIF5 and PIF7 downstream of PhyB (Galvão et al., 2019). It is also at least in part regulated through GA, since silencing GA biosynthesis genes causes late flowering in both control and supplemental FR conditions (Hisamatsu & King, 2008). Interestingly, the perennial species alfalfa (*Medicago sativa*) exhibits delayed flowering upon low R:FR treatment, indicating an uncoupling of the shade-avoidance responses and early flowering (Lorenzo et al., 2019), tentatively associated with different life cycle durations.

Shade avoidance from a plant community perspective

Vegetation is formed by multiple individuals, often from different species, with different sets of response abilities that together shape the 3D structure of a canopy. Here, we will integrate the mechanistic knowledge of SAS from the individual plant to the plant community level (Figure 2.3). We will mostly focus on homogeneous annual plant canopies but will also briefly discuss more complex canopies of mixed species and height stratification. The canopy architecture of a plant community is very dynamic, since it is built by different individuals that may display different plasticities to neighbour proximity. As a consequence, the canopy architecture is highly dynamic.

How does SAS affect canopy architecture?

SAS responses are triggered by shade cues described earlier and lead to modifications of the canopy architecture. Once the canopy architecture changes, this in turn affects the light quality distribution throughout the canopy, thus changing the very light cues that set-in motion the density-induced changes in the canopy architecture, in turn adjusting the individual responses (Figure 2.3).

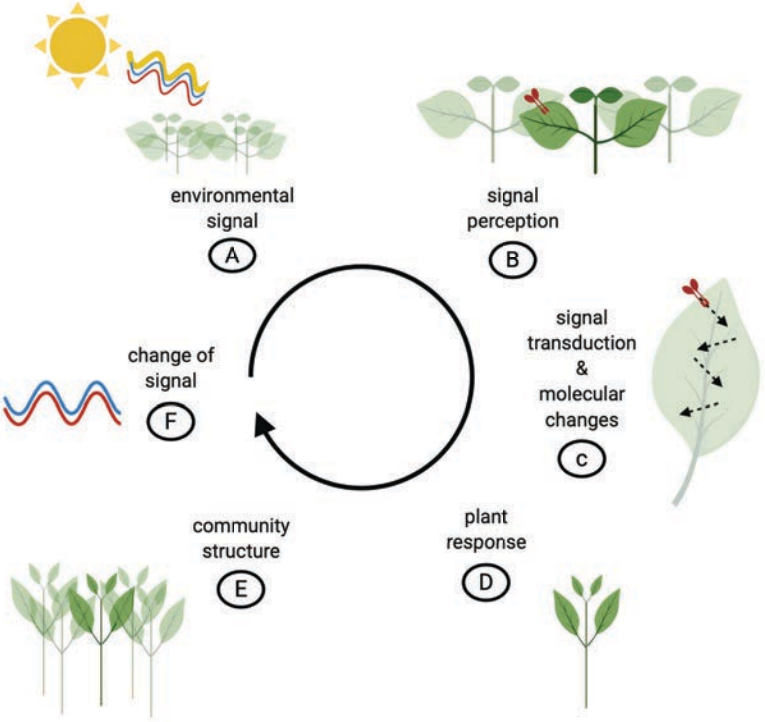

Figure 2.3. Dynamic between signals and architecture. (A) Environmental light signals are (B) perceived by the plant. (C) Signal transduction pathways evoke specific molecular changes and lead to (D) phenotypic responses. (E) This entails architectural changes in the canopy of a plant community, (F) in turn changing the light quality and quantity in a canopy. This loops back in an ongoing feedback of changes in signals and architecture. Created with BioRender.com.

Shade-avoidance responses are a way for plants to forage for light and avoid shaded patches. Phototropism directs plant organs through bending towards light patches in the canopy (Ballaré, 1999; Ballaré, Scopel, Roush, & Radosevich, 1995; López Pereira, Sadras, Batista, Casal, & Hall, 2017). Vertical growth is promoted via hypocotyl, petiole, internode and stem elongation and allows access to higher canopy layers (Schmitt, 1997; Schmitt et al., 1999). To further increase light interception, plants optimize leaf positioning, form new leaves on the upper part of the plant and senesce old leaves on the lower parts (Boonman et al., 2006; Boonman, Prinsen, Voesenek, & Pons, 2009; Maddonni, Otegui, Andrieu, Chelle, & Casal, 2002; Pantazopoulou et al., 2017; Pantazopoulou, Bongers, & Pierik, 2020). A more open canopy structure is generated by consequences of apical dominance with reduced branching and less tiller and leaf formation. Furthermore, leaf angle adjustments from relatively horizontal to a more vertical orientation in response to shade cues further opens the canopy allowing more light penetration down to the lowest regions (Pantazopoulou et al., 2020). The enhanced shoot elongation and senescence come with a trade-off of less photosynthetic active tissue and a lower leaf to stem biomass ratio.

In addition, investment in petiole elongation often leads to a reduction in leaf area (Bongers et al., 2019; Wit et al., 2015). A more open canopy facilitates light penetration deeper into the canopy, allowing better photosynthesis in the lower leaves. This can stimulate whole-plant photosynthesis, but it can of course also foster growth of competing plants at the bottom of the canopy (Box 2.1).

Box 2.1. Agricultural implications

Generally, SAS responses are viewed as undesirable traits in agriculture for their negative effects on yield. This is mainly due to the changes in biomass allocation (Carriedo et al., 2016; Kebrom & Brutnell, 2007; Liu et al., 2016; Roig-Villanova & Martínez-García, 2016; Wille et al., 2017; Wit et al., 2018). Redirecting resources to shade-responsive tissues such as internodes and stems go at the expense of roots, flowers, fruits and seeds. The investment in non-harvestable organs leads to a decrease in crop yield (Boccalandro et al., 2003; Morgan, Finlayson, Childs, Mullet, & Rooney, 2002; Page, Tollenaar, Lee, Lukens, & Swanton, 2010; Robson, McCormac, Irvine, & Smith, 1996). This is the case for some of the most economically important crops such as cereals (Garg et al., 2006; Page et al., 2009) and many vegetables such as tomato or soybean (Wu et al., 2017).

Other negative impacts of SAS in agriculture are increased lodging (Schmitt, McCormac, & Smith, 1995), reduced tuberization, for example, in potatoes (Boccalandro et al., 2003; Jackson & Prat, 2008) and early flowering in crops from the Brassicaceae family, such as cabbage and kale and Asteraceae family like lettuce (Meng, Kelly, & Runkle, 2019). Therefore, suppressing the SAS-induced elongation responses seems an obvious solution in crop monocultures to enhance the harvest index, since more resources would be allocated to harvestable organs (Liu et al., 2016; Robson et al., 1996; Smith, 1995; Wit et al., 2018; Yang, Seaton, Krahmer, & Halliday, 2016).

Furthermore, the previously mentioned SAS responses create a more open canopy structure, that is, a canopy structure that allows more light to penetrate to the leaves in the lowest regions, thereby also facilitating weed growth in the lower zones. One way to counteract this would be to increase sowing density and sowing uniformity (Lu et al., 2020; Weiner et al., 2010) which will lead to a more rapidly closing canopy and stronger light extinction. Alternatively, planting of weed-competitive phenotypes that, for example inhibits weeds from growing and even preventing them from germinating, would also inhibit weed proliferation (Andrew, Storkey, & Sparkes, 2015; Brainard et al., 2005; Mahajan & Chauhan, 2013; Pickett et al., 2014; Raj & Syriac, 2017; Seavers & Wright, 1999; Worthington & Reberg-Horton, 2013). Such weed-competitive phenotypes might consist of horizontal leaves that cast intense shade and high levels of branching/tillering: the opposite of shade avoidance (Pantazopoulou et al., 2020).

Suppression of hyponastic leaf movement might effectively reduce light penetration inside the canopy and at the same time maximize the leaf surface of canopy plants for better photosynthesis, that is, increase their biomass. Indeed, a recent study confirmed that dense stands of non-hyponastic pif7 Arabidopsis mutants had improved rates of canopy closure and suppression of invading competitors as compared to wildtype stands at the same density (Pantazopoulou et al., 2020). Crop orthologs of the Arabidopsis PIF7 gene may thus constitute interesting targets for leaf angle manipulations in crops to improve growth and weed suppression.

Modifications in the structure or the number of tillers in cereals would be another way to enhance canopy closure. Upon shade, inactivation of phyB in cereals leads to accumulation of TEOSINTE BRANCHED 1 (TB1) which in turn activates GRASSY TILLERS 1 (GT1), a class I HD-ZIP transcriptional regulator that suppresses tillering (Carriedo et al., 2016; Kebrom, Brutnell, & Finlayson, 2010; Whipple et al., 2011). Tillering control under high-density shade cues would be another interesting target for cereal breeding towards weeds suppression and crop yield optimization.

It is important to mention that severely suppressing SAS could entail undesired side effects. First, completely inhibiting SAS would also mean impeding the capacity for balancing size inequalities (Pantazopoulou et al., 2020; Weiner et al., 2010; Weiner & Freckleton, 2010) such as phyb mutant in wheat which showed severely reduced stem elongation (Pearce, Kippes, Chen, Debernardi, & Dubcovsky, 2016). Second, whether the elongation response goes at the expense of yield or not depends on what the harvested organ of the crop is. For example, in biofuel crops, such as Miscanthus giganteus, where an increase in shoot biomass is key, it is less relevant which organs have relatively increased or decreased (Danalatos, Archontoulis, & Mitsios, 2007; Warnasooriya & Brutnell, 2014). Each crop has a different canopy architecture, so SAS reduction has to be in agreement and respect of SAS-phenotypic characteristics of each crop plant, in order to increase crop yield and potentially suppress weeds more effectively.

Thus, plant responses to density change the canopy architecture, allowing for better light penetration and escape from shaded patches. Along with these changes, the canopy light cues are highly dynamic too (Figure 2.1). The higher in a canopy, the less red and blue light have been absorbed by neighbouring plants, resulting in a higher R:FR and higher fluence rate of blue and total photosynthetic active radiation. If a plant reaches the top of the canopy, leaves will receive nearly full sunlight but still perceive some FR enrichment from horizontal reflection by proximate neighbours that have grown to similar height. This means that tissues from the same plant are experiencing different light cues at different strata of the canopy. Based on the integration of this information, a plant can fine-tune its responses (Ballaré & Pierik, 2017; Küpers, van Gelderen, & Pierik, 2018).

Shade avoidance and size-asymmetric competition at high planting density

In a plant community, size inequalities will always occur, and smaller plants suffer relatively more shading than larger individuals who may be able to reach direct light. Since light typically comes from above, competition for light is size-asymmetric: a slightly taller individual will absorb a larger fraction of the incoming light as compared to a slightly shorter individual (Weiner, 1985; Weiner & Freckleton, 2010). Thus, the benefits of height growth are disproportional to the height difference between individuals. Shade-avoidance responses, however, tend to work against the development of size inequalities since the shorter individuals that are in the lower canopy layers experience the strongest shade cues (Figure 2.1) (Aphalo et al., 1999; Ballaré, 1999; Ballaré & Scopel, 1997; Ballaré, Scopel, & Sánchez, 1997) (Figure 2.4). Stronger cues tend to elicit stronger responses (Wit, Keuskamp, et al., 2016) and thus especially the suppressed plants show the most pronounced elongation responses, therefore improving their position for light capture. This was illustrated in an elegant study on tobacco plants over-expressing PHYA that show a reduced morphological responsivity to supplemental FR light or neighbours (Ballaré, Scopel, Jordan, & Vierstra, 1994) because phyA represses SAS responses (Ballaré et al., 1994). Size inequalities in a neighbour-insensitive PHYA-overexpressing monoculture increased much more steeply with density than in wildtype monocultures at the same densities, indicating that SAS helps especially the suppressed plants to improve their competitive position (Ballaré et al., 1994). The SAS morphology improves individual plant performance under high density since it facilitates escape from the shade cast by neighbouring plants. This is advantageous for an individual plant, enhancing its Darwinian fitness (Schmitt, Stinchcombe, Heschel, & Huber, 2003; Weiner, 2019; Weiner, Andersen, Wille, Griepentrog, & Olsen, 2010; Weiner & Freckleton, 2010) (Figure 2.4).

If the plant community is composed of only one species, either naturally or by human determination, what advantages does it give to display SAS responses if all neighbouring plants do the same? Resources would then be invested in shade avoidance, but they do not return a benefit to the individual plants since their neighbours achieved the very same (Weiner, 2019; Weiner et al., 2010; Weiner & Freckleton, 2010). At the community level, this is a waste of resources into non-harvestable or non-productive organs (Weiner et al., 2010) (Box 2.1). At the same time, not all individuals will be entirely identical in height, and the slightly shorter individuals will be suppressed disproportionately because of the size-asymmetric nature of competition for light. When densities are very high, density-induced mortality of suppressed seedlings can occur. Under such conditions, the resources available are used less efficiently for the plant community as a whole, since some of the acquired resources are lost again upon mortality (Lu, Jiang, & Weiner, 2020; Weiner et al., 2010). Since size-inequalities and mortality are partly counteracted by SAS responses, the community productivity might still benefit from the expression of some degree of SAS responses by the suppressed individuals (Aphalo et al., 1999).

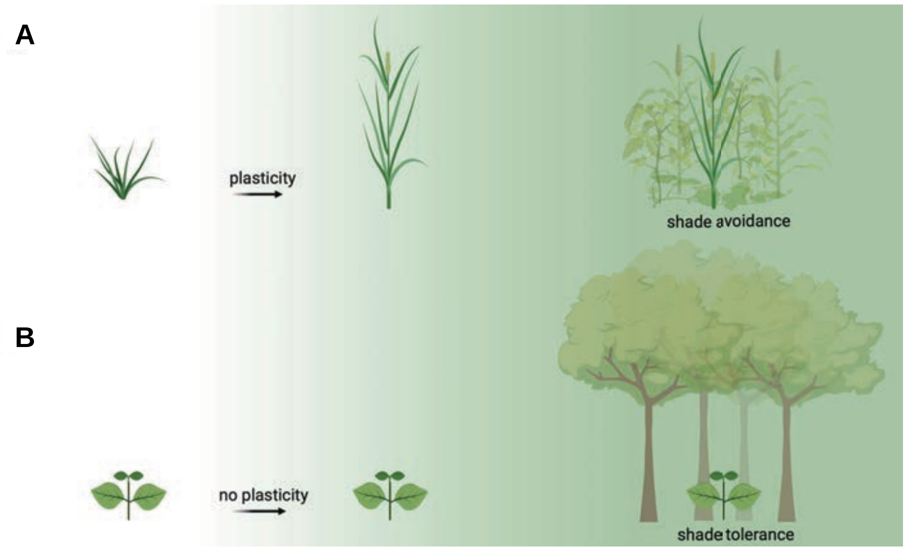

Figure 2.4. Phenotypic plasticity for density-associated shade cues. A. Plants able to respond to increasing neighbour density show plastic SAS responses (plant 1) in contrast to plants that are not responding to density signals and therefore not showing plastic changes in their phenotype (plant 2). **B.** If SAS responses give a fitness advantage, they become an adaptive trait. However, in certain conditions, such as an herbaceous plant growing in a forest understory, suppressing SAS responses yields higher fitness than a plastic response and shade-tolerance then becomes the adaptive trait. Created with BioRender.com.

The adaptive value of shade avoidance

Since environmental conditions are constantly changing, it is essential for plant survival to be able to respond to these changes through phenotypic plasticity (Schmitt, 1997) (Table 2.1), and shade avoidance is a classic example of this. But why would SAS have to be plastic (Figure 2.4), would it not be better to always grow maximally tall? The morphological changes involved in shade avoidance enhance a plant's performance at high density when it is growing in a field together with other plants. It can then escape from the shade created by other plants, ensuring photosynthesis. However, when densities are lower and competition for light is weak or even absent, a shade-avoidance phenotype would confer a fitness disadvantage: a constitutively shade-avoiding plant would be thin and elongated, thereby lodging easily. It would also not form the branches it needs at low densities to grow vigorously (Ballaré et al., 1995; Schmitt, Mccormac & Smith, 1995). This argument also explains why SAS as a plastic response is important in open fields (Bongers et al., 2019), where competition intensity varies strongly with seasons; early in the year, there is hardly any competition, and SAS would be disadvantageous, whereas later on a plant will experience increasing numbers and sizes of competitors and expressing SAS becomes advantageous. In addition, since SAS comes with trade-offs, such as reduced lamina size, plasticity allows the investments to be made when necessary, but costs prevented when not needed (Figure 2.4a). In the earlier sections, we focussed mostly on canopies of relatively similarly sized individuals and mostly took plant density as the dominant variable. In the following sections, we will discuss how SAS expression may vary between different types of canopies and relative plant positions therein, as well as with different additional variables affecting plants, in order to be adaptive.

Why is SAS adaptive?

In order for a plastic response to be adaptive, the phenotype displayed in a certain environment must lead to a fitness advantage in that environment relative to alternative phenotypes (Schmitt, 1997), thus SAS must also result in a fitness advantage to be an adaptive trait (Figure 2.4). This has indeed been confirmed in various studies (Dudley & Schmitt, 1996; Schmitt et al., 1999; Schmitt, Mccormac, & Smith, 1995), and it was shown that too strong or too weak phenotypic changes in response to neighbour presence reduce fitness in dense stands (Dudley & Schmitt, 1995; Keuskamp, Sasidharan, & Pierik, 2010; Pierik et al., 2003; Weijschedé, Martínková, De Kroon, & Huber, 2006; Weinig, 2000a). Naturally, vegetations differ in the intensity of competition for light, and so do species in their ability to respond to this. For example, variation in low R:FR-induced stem elongation rate has been documented between (Gilbert, Jarvis, & Smith, 2001; Gommers et al., 2017; Molina-Contreras et al., 2019; Morgan & Smith, 1978) and even within (Filiault & Maloof, 2012; Sasidharan et al., 2008) species. These observations suggest that natural selection can favour specific degrees of shade-avoidance potential in different habitats. The ability to respond to shade in a plastic way, and to modulate the intensity of this response depending on the precise environmental conditions, allows plants to grow in a relatively wide range of habitats. Indeed, it has been proposed that plasticity acts against the evolution of ecological specialists (Weinig, 2000a). Studies with mutants impaired in R:FR perception show that they are less efficient than the corresponding wild types at foraging for light in heterogeneous light environments, providing direct evidence for the adaptive value of phytochrome-mediated shade avoidance (Ballaré et al., 1995; Schmitt et al., 1995, 1999). It has also been shown that an elongated phenotype due to SAS responses increases fitness over non-elongated plants when growing at high density but reduces fitness at low densities (Aphalo et al., 1999; Donohue, Messiqua, Pyle, Shane, & Schmitt, 2000; Dudley & Schmitt, 1996). In conclusion, although in a dense vegetation, it is the exact position in the canopy that determines a plant's light interception and thus fitness, the ability to be plastic for traits that determine leaf positioning in a canopy allows for optimal light foraging and promotes fitness (Bongers et al., 2019).

Is SAS always adaptive?

Although the fitness advantage of SAS is clear, this does probably depend on such factors as size relative to neighbouring plants, phylogenetic background, ontogenetic stage and the current physiological state. In addition, many other aspects of the environment determine the range of response: population density, availability of resources other than light, time of the year and the type of habitat. For example, a pioneer vegetation of rapidly cycling species that is establishing on bare soil may rely less on phenotypic plasticity, then later successional stages (Lundgren & Sultan, 2005; Weijschedé et al., 2006; Weinig, 2000a, 2000b).

There are also naturally occurring scenarios, where shade avoidance is non-adaptive for specific species. Under some of these conditions, specialization is favoured over plasticity (Weinig, 2000a). In strongly light-deprived habitats, for example, in a forest understory as an herbaceous plant or even for small weeds in a crop field (Weinig, 2000b), the situation is different than in an open grassland (Figure 2.4b). Here, following the SAS escape strategy is unlikely to improve light interception, since outgrowing the neighbouring plants is impossible. Plants adapted to such a forest understory habitat evolved mechanisms to suppress SAS responses and developed ways to be shade-tolerant (Gommers, Visser, St Onge, Voesenek, & Pierik, 2013; Valladares & Niinemets, 2008). Although shade-tolerant plants are typically considered to have very low plasticity (Valladares & Niinemets, 2008), they do show some shade responses, such as an increased specific leaf area and a decreased chlorophyll a/b ratio to optimize light harvesting with minimal carbon investments (Evans & Poorter, 2001; Gommers et al., 2013). It appears that shade-tolerant plants can still sense shade cues but have evolved mechanisms to suppress SAS. Although the molecular mechanisms regulating alternative shade responses are largely unknown, a few recent studies have started to unravel the molecular pathways towards shade-avoidance suppression. In a comparative study on two Geranium species with antithetical shade responses, the shade-tolerant plant G. robertianum was found to be able to respond to low R:FR, but within a few hours, reverse its response and suppresses elongation growth in low R:FR. A candidate regulator of this response flexibility is the atypical HLH protein KIDARI (KDR) that seems to promote shade avoidance in shade-intolerant plants (Gommers et al., 2017), by interacting with other HLH proteins that suppress PIF activity (Buti, Hayes, & Pierik, 2020). Another plant that does not elongate its hypocotyls in response to low R:FR is Cardamine hirsuta, and this is associated with a hyperactive phyA photoreceptor that typically antagonizes phyB-mediated shade-avoidance responses (Molina-Contreras et al., 2019). A phyA (sis1) mutant in this species completely restored hypocotyl elongation in response to low R:FR (Molina-Contreras et al., 2019). These studies indicate that both in C. hirsuta and in G. robertianum, the shade-avoidance machinery is preserved but mechanisms have evolved to suppress it.

Extreme habitats like alpine vegetations, wetlands or saline soils impose strong additional environmental stresses on plant growth, which might overrule the SAS responses (Keuskamp, Sasidharan, & Pierik, 2010). A naturally occurring example of a genotype not expressing SAS in response to low R:FR is the alpine ecotype of Stellaria longipes (Sasidharan et al., 2008). This genotype was collected from alpine sites in the Rocky Mountains where vegetation is extremely sparse and no competition for light occurs. In such environments, plants are typically very short and compact to protect them from the extreme weather conditions. It turns out that this ecotype has lost the ability to elongate in response to low R:FR, whereas a prairie ecotype growing nearby in the lower altitude grasslands is highly responsive to this shade cue (Kurepin, Pharis, Neil Emery, Reid, & Chinnappa, 2015; Sasidharan et al., 2008). Consistently, while the prairie ecotype upregulates cell-wall loosening through expansins in response to low R:FR, this does not occur in the alpine ecotype. Severe low light treatments still elicited internode elongation in both the ecotypes, accompanied by strong induction of several EXPANSIN genes (Sasidharan et al., 2008). Since PIF proteins are known to regulate EXP gene expression, the observed variations between the two Stellaria ecotypes might suggest differences in PIF activity between the ecotypes, but this remains to be investigated.

Next to loss of shade avoidance through evolutionary adaptation, shade-avoidance responses can also be suppressed by local environmental conditions occurring within the lifetime of an individual. A recent example is on abiotic stress, where it was found that exposure to very mildly elevated salt concentrations in the soil inhibits low R:FR-induced hypocotyl elongation. This occurs via an ABA-dependent inhibition of the brassinosteroid-dependent transcription factor BES1 (Hayes et al., 2019). Tentatively, suppressing shade avoidance is important for abiotic stress tolerance by maintaining a relatively small shoot. It will be interesting to study if this is a common feature of other abiotic stresses interacting with plant–plant signalling. At least one other factor, UV-B light, has been shown to also suppresses low R:FR response (Hayes et al., 2014; Mazza & Ballaré, 2015) although this mostly indicates intricate light information integration for optimal light foraging, rather than stress interaction with low R:FR response.

Future perspectives

In this review, we explored the tremendous ecological and agricultural importance of SAS and revealed the complex regulation of the molecular pathways associated to it.

An important aspect for future studies is related to light cue heterogeneity at the (sub)organ level, especially for stem-forming plants receiving different light information from leaves at different heights. It would be important to unravel how this information is integrated at a whole-plant level, if and how self-shading can be distinguished from neighbour plants (Pantazopoulou et al., 2017; Zhang et al., 2020), and how local responses are integrated with systemic responses. Therefore, a major current challenge in shade-avoidance research is to study the molecular mechanisms underpinning the multiple interactions between different light-responsive pathways, for example, R:FR versus blue, and the multiple spatial scales within a plant that senses different light environments. Finally, these already complicated interactions have a very strong temporal component since the canopy develops, causing strong temporal fluctuations of neighbour cues. Studies are needed to understand and predict the reliability of cues that are heterogeneous in time and space.

Despite these open questions, the existing knowledge of SAS from the model plant Arabidopsis should already be translated to crops (Box 2.1). Such translational studies could explore if similar mechanisms are valid for other species and how to adjust them. To create an optimally performing crop plant, rather than entirely suppressing SAS via manipulating the photoreceptors, more subtle approaches might be more promising. Some studies suggest that by targeting downstream effectors of photoreceptors, SAS responses could for instance be limited to a certain developmental stage (Carriedo et al., 2016; Roig-Villanova & Martínez-García, 2016), only affecting specific architectural traits (Devlin, Yanovsky, & Kay, 2003; Wei, Zhao, Xie, & Wang, 2018). It would be very interesting to match concepts from Evolutionary Agroecology/Darwinian Agriculture (Denison, 2012; Weiner et al., 2010), where inhibition of SAS in crop monocultures is proposed to inhibit weed proliferation through enhanced closure of the crop canopy, with the molecular–genetic knowledge and tools for shade-avoidance modulation in Arabidopsis. It might then be possible to target specific genetic loci to select cooperative crops with enhanced communal weed suppression properties.

In vegetable horticulture, the detailed knowledge about SAS pathways can be used not only to target the crop but also the greenhouse light conditions, using LED light spectra (e.g., Demotes-Mainard et al., 2016) to steer architecture and yield.

Chapter 3

Towards increased shading capacity: a combined phenotypic and genetic analysis of rice shoot architecture

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Abstract

Rice feeds more than half of the world’s human population. Major constraints in modern rice farming are weed proliferation and the ecological impact of herbicide application. Increased weed competitiveness of commercial rice varieties requires enhanced shade-casting to limit growth of shade-sensitive weeds and thereby limit the need for herbicides. We aimed to identify traits that increase rice shading capacity based on canopy architecture and associate their expression with the underlying genetic components. We performed a phenotypic screen of a rice diversity panel comprised of 344 varieties, examining 13 canopy architecture traits linked with shading capacity in 4-week-old plants. The analysis revealed a vast range of phenotypic variation across the diversity panel. We first used trait correlation and clustering to identify the core traits that define shading capacity to be shoot area, number of leaves, culm and solidity (the compactness of the shoot). To simplify the complex canopy architecture, these traits were then combined into a Shading Rank metric that is indicative of a plant’s capacity to cast shade. Genome wide association study (GWAS) revealed genetic loci underlying variation in canopy architecture traits, out of which five loci were substantially contributing to shading capacity. Subsequent haplotype analysis further explored allelic variation and identified seven haplotypes associated with increased shading. Identification of traits contributing to shading capacity and underlying allelic variation presented in this study will serve future genomic assisted breeding programmes. The investigated diversity panel, including improved as well as traditional varieties, shows major potential and genetic resources for improvement of elite breeding lines. Implementing increased shading in rice breeding will make its farming less dependent on herbicides and contribute towards more environmentally sustainable agriculture.

Introduction

Rice feeds more than half of the world’s population as a staple food (Kennedy & Burlingame, 2003; Wing et al., 2018). In traditional rice farming, seedlings are transplanted into flooded paddy fields. This works as a natural way to prevent weed infestation, since it gives rice seedlings a size advantage in addition to flood-suppressed germination and growth of weeds. This practice is increasingly problematic, both because of the high manual labour input (Kumar and Ladha 2011; Chakraborty et al. 2017) and because global climate change is reducing the availability of fresh water not only for rice farmers but for the global agricultural sector (FAO, 2019; Oliver et al., 2019). Traditional rice farming system is transitioning towards direct-seeded rice, where rice seeds are directly sown into the fields. This practice drastically reduces the water requirement and labour input (Chauhan et al., 2017; Farooq et al., 2011; Kumar and Ladha, 2011). Besides all of its advantages, the major constraint for direct-seeded rice is abundant proliferation of weeds (Rao et al. 2017; Xu et al. 2019). In direct-seeded rice practice, rice seedlings are directly competing with weeds as they lose their seedling size advantage. Waterlogging cannot be applied to suppress emerging weeds, as most modern rice cultivars do not germinate under water (Chauhan, 2012; Ghosal et al., 2019; Kretzschmar et al., 2015). Currently, weeds are suppressed with herbicides, leading to evolution of herbicide-resistant weeds and ground water pollution. This creates a pressing need for deployment of sustainable weed management options (Chauhan, 2012a; Chauhan & Yadav, 2013; Mennan et al., 2012; Zhao et al., 2006a). One possible solution to this problem is to increase weed-competitiveness of the rice seedling (Rao et al., 2007; Sakamoto et al., 2006; Zhao et al., 2007).

Just like their wild ancestors, shade casting crop varieties compete with invading weeds by reducing the weed’s access to full sunlight, thereby impeding their growth. However, the traits contributing to shading potential were neglected or even selected against in breeding efforts, since tall plants and droopy leaves are generally considered as undesired, because it makes harvesting more difficult. Here we propose to develop weed-competitive rice varieties, by selecting for an ideotype with faster growth and high shade-casting potential on proximate weeds. A big projected shoot area and therefore big ground cover is associated with weed-competitiveness (Caton et al., 2003; Dingkuhn et al., 2001; Haefele et al., 2004; Mennan et al., 2012; Namuco et al., 2009; Rao et al., 2007; Zhao et al., 2006b, 2007). In addition, high number of leaves and tillering capacity as well as plant biomass and early vigour are advantageous for competition against weeds (Haefele et al., 2004; Mahajan & Chauhan, 2013; Namuco et al., 2009; Zhao et al., 2006a), but these are not specific architecture traits.

Shoot architecture traits that help plants to gain advantage over their neighbours through light competition include: increased leaf area, increased planar angle of leaves and tillers and leaf droopiness (Andrew et al., 2015; Brainard et al., 2005; Mahajan & Chauhan, 2013; Seavers and Wright, 1999; Worthington & Reberg-Horton, 2013). Accelerated vertical growth might provide an additional advantage for outcompeting neighbours, yet plant height has been strongly selected against during green revolution of most cereals, including rice. Indeed, there exists great potential for weed suppression in cereal canopies, as has been shown for wheat, where a rapidly closing wheat crop canopy achieved through higher planting density, depleted weeds from access to light (Weiner et al., 2010).

Building on the idea to increase shading for improved weed competitiveness, here (1) we phenotyped a rice diversity panel of 344 globally distributed varieties where we recorded 13 quantitative traits. Based on these, (2) we determined key architectural characteristics of shading potential in early growth phase. (3) We combined these core traits into one parameter to develop the Shading Rank, where the rice varieties were ranked for their shading potential. (4) Genome-wide association study (GWAS) revealed association with eight genetic loci for traits contributing to shade potential. The results of this study form a primer to identification of alleles contributing to increased shading and early plant vigour.

Results

Shoot architectural variation between rice varieties

In order to evaluate the variation in shading potential within the rice diversity panel (Supplemental Table 3.1) we measured 13 traits on 4-week-old seedlings in the screenhouse (Figure 3.1, Table 3.1, Supplemental Table 3.2).

Substantial variation was observed for all measured traits among the varieties belonging to different subpopulations (Figure 3.1; Supplemental Table 3.2). The *indica* subpopulation showed highest dry weight, number of leaves, and number of tillers followed by *aus* subpopulation and *aromatic*, *tropical* and *temperate japonica* ranked lowest for these parameters (Supplemental Table 3.2). Shoot and hull area were also observed to be higher in *indica* and *aus* subpopulations, intermediate in *aromatic* subpopulation and lowest in *japonicas* and *admixture* subpopulations. *Indica* and *aus* on average develop the most compact shoots (highest solidity), contrasting with the low solidity of *japonicas* and *admixed*. In culm height, *indica* and *aus* were shortest and *temperate japonica* and *admixed* subpopulation were tallest. When taking the entire diversity panel of 344 varieties, five traits (shoot area, hull area, solidity, plant height and dry weight) already showed a significant difference between the individual varieties at four weeks after sowing (Supplemental Table 3.2). When grouped together in subpopulations, all traits showed significant differences between subpopulations (Supplemental Table 3.2). Overall, it appears that relatively large variation between subpopulations was observed for traits related to area and branchiness, whereas traits related to height showed only little variation between subpopulations. These differences are clearly determined by differences in genetic background since the growth conditions were constant. The high variation observed for traits related to shading potential suggests that the investigated rice diversity panel offers the genetic variation needed to inspire improvement of shading potential in elite-breeding varieties.

Table 3.1: Description of 13 investigated shoot traits.

Trait	**Unit**	**Description**
Number of leaves		Number of all visible green leaf blades
Number of tillers		Number of side branches classified as tillers as soon as it splits off the culm, having two leaves
Total plant height	cm	Height from soil to the straightened topmost leaf tip
Culm height	cm	Mother stem - from soil to highest node, where youngest leaf blade bends off
Leaf length	cm	Length of longest leaf blade
Projected shoot area	cm²	All green leaf area projected from top view
Convex hull area	cm²	Smallest area enclosing outermost leaf tips
Shoot perimeter	cm	Outline of the projected shoot area
Leaf initiation angle	°	Angle between culm and leaf blade initiation measured for second and third leaf
Tiller angle	°	Angle between the culm and tillers, measured for the left and right outermost tillers
Leaf droopiness	°	Interception angle of two tangents aligned to initiation and tip of leaf blade measured for second and third leaf
Dry weight shoot	g	Dry matter of shoot biomass after drying in oven at 70 C for 48 h
Solidity		Ratio of projected shoot area divided by convex hull area

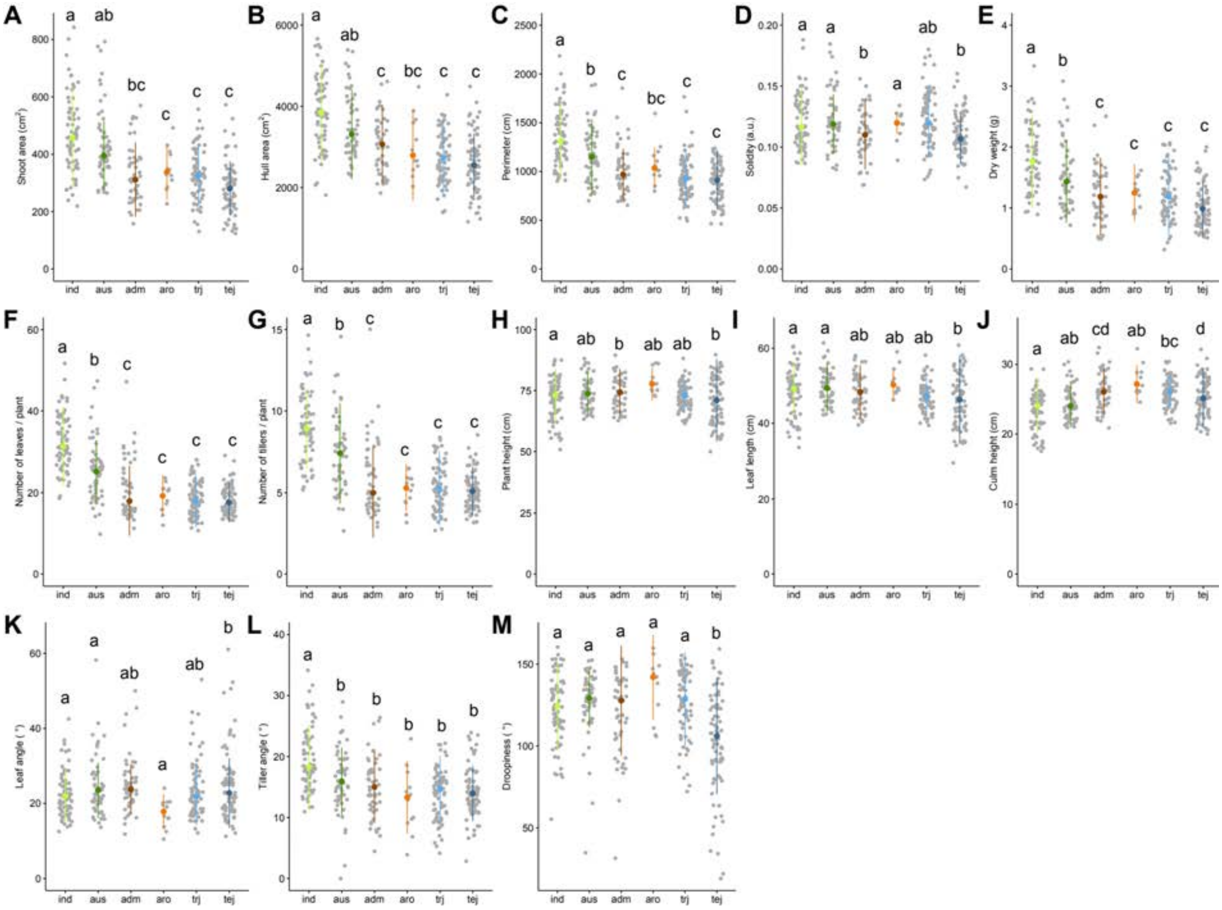


Figure 3.1. Shoot traits in rice differ between subpopulations. Distribution of investigated shoot traits in the screened diversity panel. The plots represent the trait value (y-axis) observed for varieties grouped according to different subpopulations on x-axis. **A.** Shoot area [cm-2], **B.** Hull area [cm-2], **C.** Perimeter [cm], **D.** Solidity, **E.** Dry weight [g], **F.** Number of leaves / plant, **G.** Number of tillers / plant, **H.** Plant height [cm], **I.** Leaf length [cm], J**.** Culm height [cm], **K.** Leaf angle [°], **L.** Tiller angle [°] and **M.** Droopiness [°]. Each data point represents the mean out of 8 replicates for each of the 344 varieties. The colours represent different groups of subpopulations, ind – *indica*, aus, adm – *admixed*, aro -*aromatic*, trj – *tropical japonica* and tej – *temperate japonica*. Letters in the graphs represent the significantly different groups, determined with Tukey's HSD with p-value < 0.05. Mean values for all 13 traits and the sum of the normalized traits including results for Tukey's pairwise post hoc test can be found in Supplemental Table 3.2.

Correlation of shoot architectural traits

To explore the relationship between individually measured traits, and determine which traits are independent of each other, we performed a Pearson correlation analysis (Figure 3.2 A, Supplemental Figure 3.1). Shoot area and hull area showed strong positive correlation with shoot dry weight. Leaf and tiller number were highly correlated with shoot area and dry weight. Height-associated traits, such as plant height, culm height and leaf length, were positively correlated with each other. On the other hand, a negative correlation was found between culm height and number of leaves and tillers. Solidity, leaf angle, tiller angle and droopiness did not display strong correlation with other measured traits.

To examine the types of canopy architecture exhibited within the rice diversity panel, we performed hierarchical clustering (Figure 3.2 B), resulting in seven trait clusters. The clustering shows how traits are grouped together according to the patterns observed across all rice varieties. Taking the correlation and clustering analyses together, we can classify core groups of traits: area-related (shoot area, hull area, perimeter), branchiness (number of leaves and tillers and dry weight), height-related (plant and culm height and leaf length), solidity, leaf angle, tiller angle and droopiness (Table 3.2).

Table 3.2: Core groups of shoot traits. For core groups with multiple traits, we have selected a representative trait as the core trait, shown in bold.

Core groups	**Measured shoot architectural traits**
Area	**Projected shoot area**, convex hull area, perimeter
Branchiness	**Number of leaves**, number of tillers, dry weight
Height	**Culm height**, leaf length, plant height
Solidity	Solidity
Leaf angle	Leaf angle
Tiller angle	Tiller angle
Droopiness	Droopiness

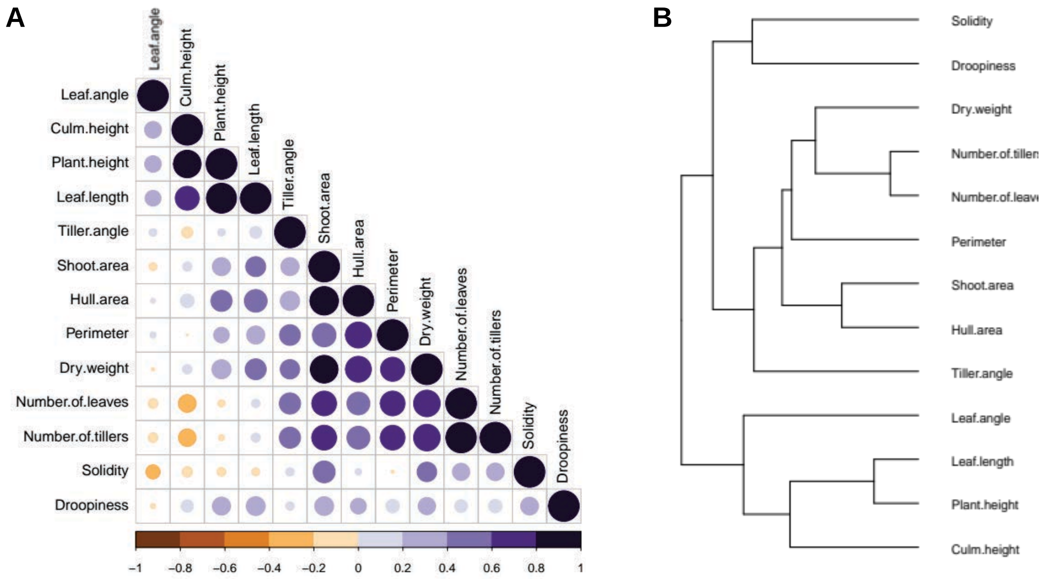


Figure 3.2. Correlation and clustering of 13 shoot traits defines core groups of traits. A. Pearson Correlation coefficients between traits. The colour and size of the circles reflect the strength of the correlation. **B.** Hierarchical Cluster Analysis. Traits are clustered using ward.D2 method. Rows represent 13 studied shoot traits. The values of individual samples are normalized per trait using z-Fisher transformation scaled prior to clustering. Based on a cut off at seven clusters and together with the correlation coefficients, we grouped together the traits into defined core groups.

Defining 'shading potential' from shoot architecture traits

The shading potential of a plant expresses the effectiveness with which it can cover ground area. To quantify shading potential, we ranked varieties for the sum of the core traits contributing to shading potential (projected shoot area, number of leaves, solidity, culm height, leaf angle, tiller angle and leaf droopiness, bold in Table 3.2). To account for the differences in measured units and unit ranges for each trait, the values were rescaled to a range from 0 to 100, whilst keeping the relative differences of trait-values between different varieties unchanged and these relative differences are also reflected in the sum of the normalized trait values. Varieties then were ranked according to their sum of normalized trait values, from 344 (highest) to 1 (lowest), resulting in the Shading Rank (for detailed information see Methods section - Data processing and statistical analysis). The resulting Shading Ranks, within this diversity panel are shown in Supplemental Table 3.3. Since the diversity panel was evaluated 28 days after sowing, a large shoot size of high-ranking varieties also indicates rapid growth and seedling vigour. From the 25 highest ranking varieties, 14 belong to the *indica* subpopulation and eight to *aus*. Low ranking varieties in terms of shading potential include improved varieties such as IR 64 and Nipponbare, ranking 74th and 73rd respectively (Table 3.3). This suggests that some of the current elite rice varieties could have a rather poor shading potential, and through breeding with varieties from *indica* and *aus* subpopulations, the shading potential and weed-competitiveness can possibly be increased.

The distribution of the different varieties with respect to the core trait groups area, branchiness, height and solidity are shown in Figure 3.3, together with top images of representative varieties. None of the top-ranking varieties showed the highest values for all core shading traits (Figure 3.3), hinting at trade-offs between shading traits. For example, Sze Guen Zim ranks highest for shoot area and number of leaves, but is one of the lower-ranking varieties for culm height. The variety with the highest Shading Rank (344), Shim Balte has a very high number of leaves and solidity, but has a close to average culm height. Mudgo reaches a rank of 340, despite its relatively low number of leaves and solidity. At the other end of the spectrum, Della ranks at 49 and is low for all traits except for culm height, whereas Luk Takhar (rank 1) shows low values for all core traits.

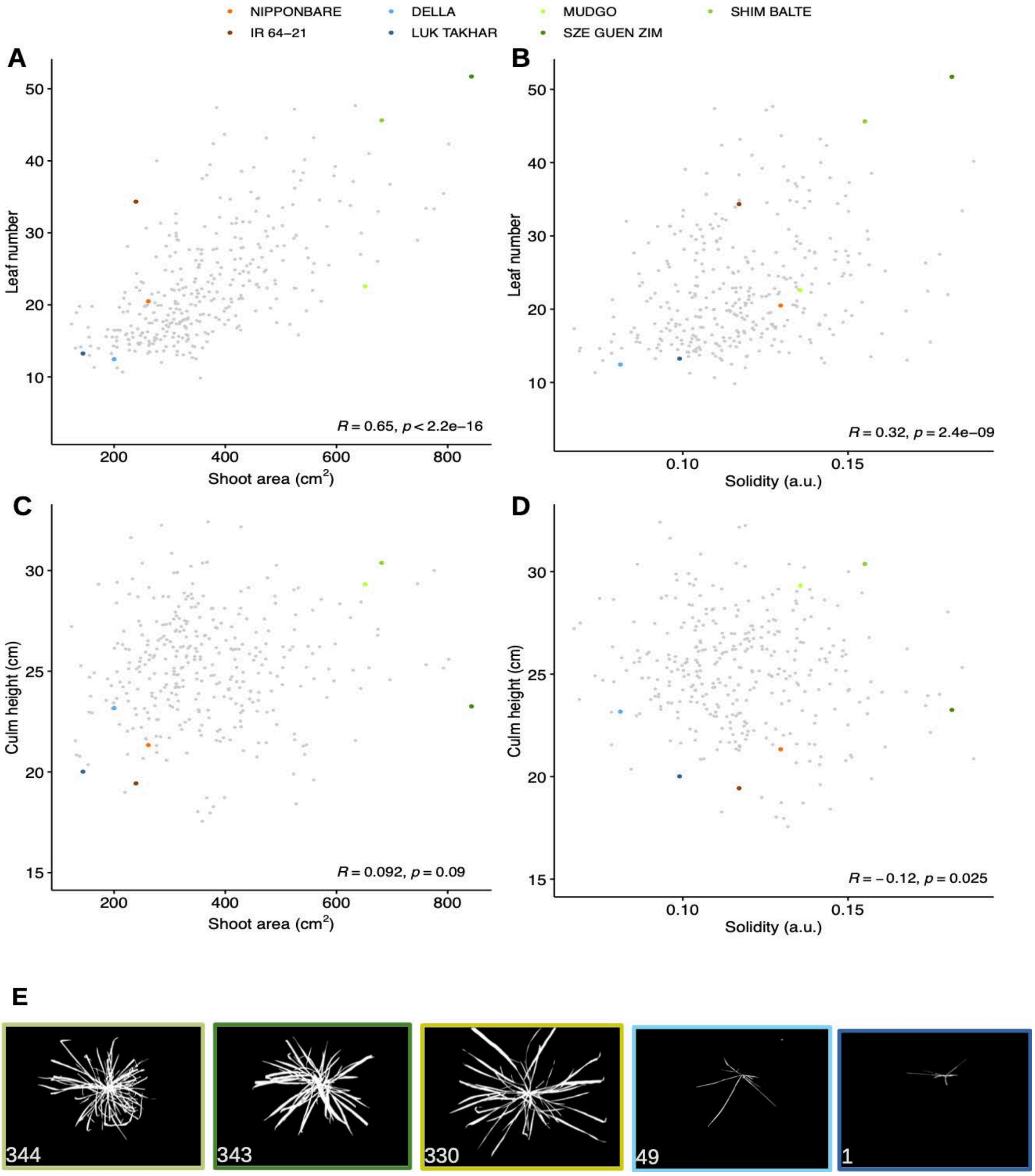
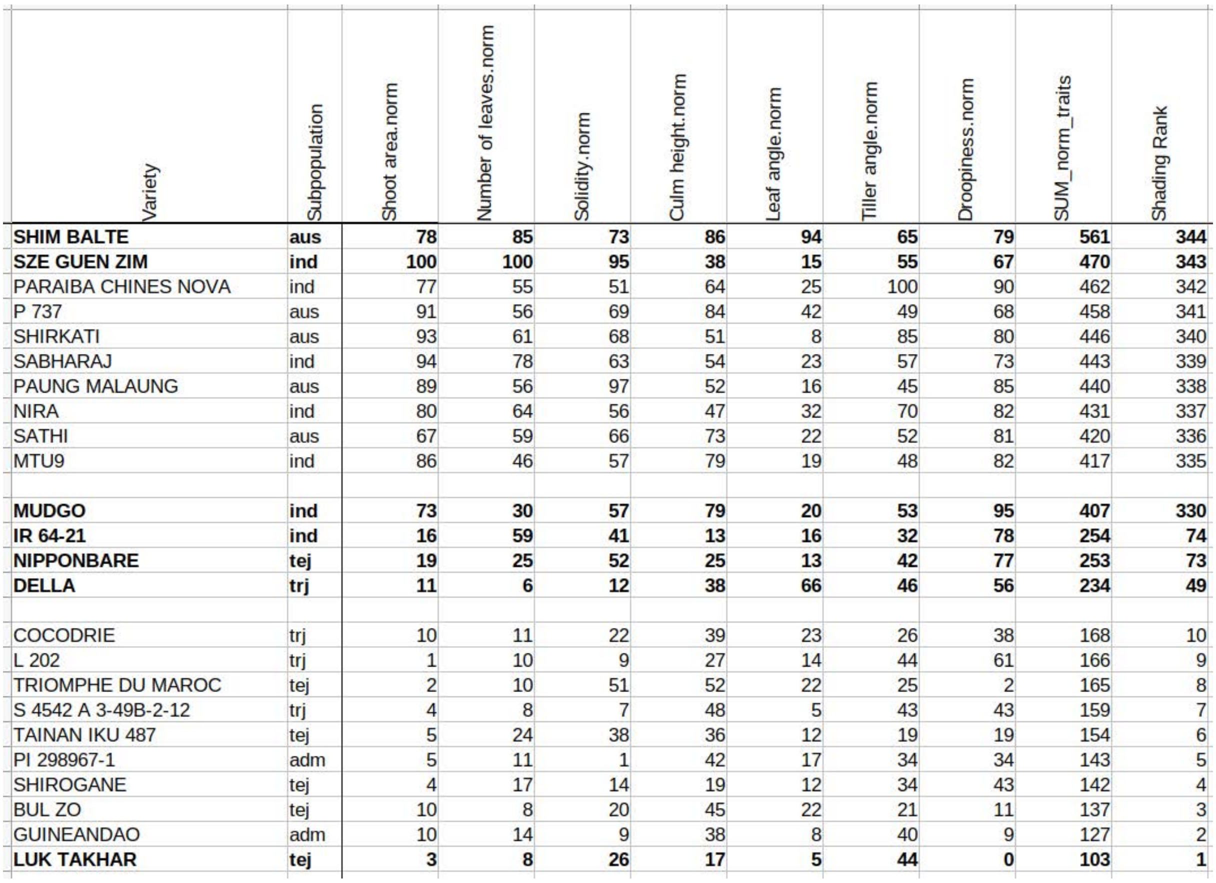


Figure 3.3. Visualization of shading capacity in the investigated rice diversity panel based on core traits for the Shading Rank. A.-D. Scatter plots showing the distribution of 344 rice varieties in pair-wise combination of four core traits, shoot area, number of leaves, solidity and culm height. Representative high (344, 343 and 330) and low (49 and 1) ranking varieties together with Nipponbare (73) and IR 64-21 (74) are highlighted in colours. **B.** Top view images of representative varieties, with colour coded frames. Numbers are respective Shading Ranks as found in Table 3.3.

Table 3.3: Shading Rank for ten highest and ten lowest ranking varieties, and for varieties of special interest (Mudgo, IR 64-21, Nipponbare and Della) with normalized core trait values (between 0 as lowest and 100 highest) compared to the min and max values within the screened panel and the sum of the core traits. Varieties in bold are visualized in Figure 3.3. The Shading Rank ranges from 344 as the highest and 1 as the lowest shading. The list of Shading Ranks for the entire panel can be found in Supplemental Table 3.3.



Variety	Subpopulation	Shoot area.norm	Number of leaves.norm	Solidity.norm	Culm height.norm	Leaf angle.norm	Tiller angle.norm	Droopiness.norm	SUM_norm_traits	Shading Rank
SHIM BALTE	**aus**	**78**	**85**	**73**	**86**	**94**	**65**	**79**	**561**	**344**
SZE GUEN ZIM	**ind**	**100**	**100**	**95**	**38**	**15**	**55**	**67**	**470**	**343**
PARAIBA CHINES NOVA	ind	77	55	51	64	25	100	90	462	342
P 737	aus	91	56	69	84	42	49	68	458	341
SHIRKATI	aus	93	61	68	51	8	85	80	446	340
SABHARAJ	ind	94	78	63	54	23	57	73	443	339
PAUNG MALAUNG	aus	89	56	97	52	16	45	85	440	338
NIRA	ind	80	64	56	47	32	70	82	431	337
SATHI	aus	67	59	66	73	22	52	81	420	336
MTU9	ind	86	46	57	79	19	48	82	417	335
MUDGO	**ind**	**73**	**30**	**57**	**79**	**20**	**53**	**95**	**407**	**330**
IR 64-21	**ind**	**16**	**59**	**41**	**13**	**16**	**32**	**78**	**254**	**74**
NIPPONBARE	**tej**	**19**	**25**	**52**	**25**	**13**	**42**	**77**	**253**	**73**
DELLA	**trj**	**11**	**6**	**12**	**38**	**66**	**46**	**56**	**234**	**49**
COCODRIE	trj	10	11	22	39	23	26	38	168	10
L 202	trj	1	10	9	27	14	44	61	166	9
TRIOMPHE DU MAROC	tej	2	10	51	52	22	25	2	165	8
S 4542 A 3-49B-2-12	trj	4	8	7	48	5	43	43	159	7
TAINAN IKU 487	tej	5	24	38	36	12	19	19	154	6
PI 298967-1	adm	5	11	1	42	17	34	34	143	5
SHIROGANE	tej	4	17	14	19	12	34	43	142	4
BUL ZO	tej	10	8	20	45	22	21	11	137	3
GUINEANDAO	adm	10	14	9	38	8	40	9	127	2
LUK TAKHAR	**tej**	**3**	**8**	**26**	**17**	**5**	**44**	**0**	**103**	**1**

SNPs associated with seedling establishment and shoot architectural traits

The high phenotypic variability found in the studied diversity panel (Supplemental Table 3.4), together with the high genetic variation (Wang et al., 2018b) provides a strong basis for a GWAS. We observed high narrow-sense heritability for all measured traits (Supplemental Table 3.5). We investigated the genomic trait associations on two different SNP sets, both with two different software packages (lme4QTL (Ziyatdinov et al., 2018) and Genomic Association and Prediction Integrated Tool (GAPIT) (Tang et al., 2016; Wang et al., 2018c), see methods for detailed description). The total list of p-values for SNPs association across all measured traits can be found in Supplemental Data 3.3.

Despite solidity being a very complex and likely a poly-genic trait, the analysis revealed a strong association with 14 SNPs in the locus on chromosome 3 (Figure 3.4). Three genomic regions were associated with plant height located on chromosome 3, 5 and 6 (Figure 3.4). The peak on chromosome 3 was also detected for other height related traits: culm height and leaf length (Supplemental Data 3.4). Overall, the associations with culm height showed lower LOD scores (Supplemental Data 3.4), and thus we followed up the loci in plant height. The results for droopiness reveal strong associations with SNPs on chromosomes 1 and 10, sharing the association on chromosome 1 with tiller angle (Figure 3.4, Supplemental Data 3.4). Leaf angle could be associated with a highly significant peak of SNPs on chromosome 12. The associations between leaf or tiller number, found for SNPs on chromosomes 11 and 12, were shared between these two traits (Supplemental Data 3.4). These two loci were also found for dry weight. This suggests that the genetic components underlying formation of new leaves and tillers might have a common genetic constituent, consistent with high correlation in their phenotypes (Figure 3.2). The analysis for dry weight revealed significant associations on chromosomes 3, 7 and 12, overlapping with the associations found for shoot area (Figure 3.4). The strong accumulation of significantly associated SNPs on chromosome 1 were found to be also associated with solidity, shoot area and dry weight, representing three of the core traits. When taking together shading potential as the sum of all core traits, a GWAS on this composite trait yielded a rather random pattern of SNP associations (Supplement Figure 3.4). This further highlights our earlier findings (Table 3.3), that shading can be achieved through various strategies and shading potential, as such, is genetically a highly complex trait. Therefore, genetic mapping of shoot architecture components that contribute to shading capacity is much more effective approach in identifying genetic components that contribute to shading and potential weed competitiveness.

Figure 3.4. GWAS identifies the genetic regions underlying shoot architectural traits and seedling vigour in 4-week-old rice seedlings, reflecting early vegetative growth stage. We used single-trait genome-wide association studies (GWAS) with a mixed linear model (MLM) for Solidity, Plant height, Shoot area, Dry weight, Droopiness and Leaf angle. The Manhattan plots depict single nucleotide polymorphisms (SNPs) with minor allele frequencies (MAF) > 0.05. Negative logarithmic p-values on the y-axis, for 1.7 M SNPs across the 12 rice chromosomes on the x-axis. Genomic regions highlighted in green are loci of interest (L1.1 - L12).

▶

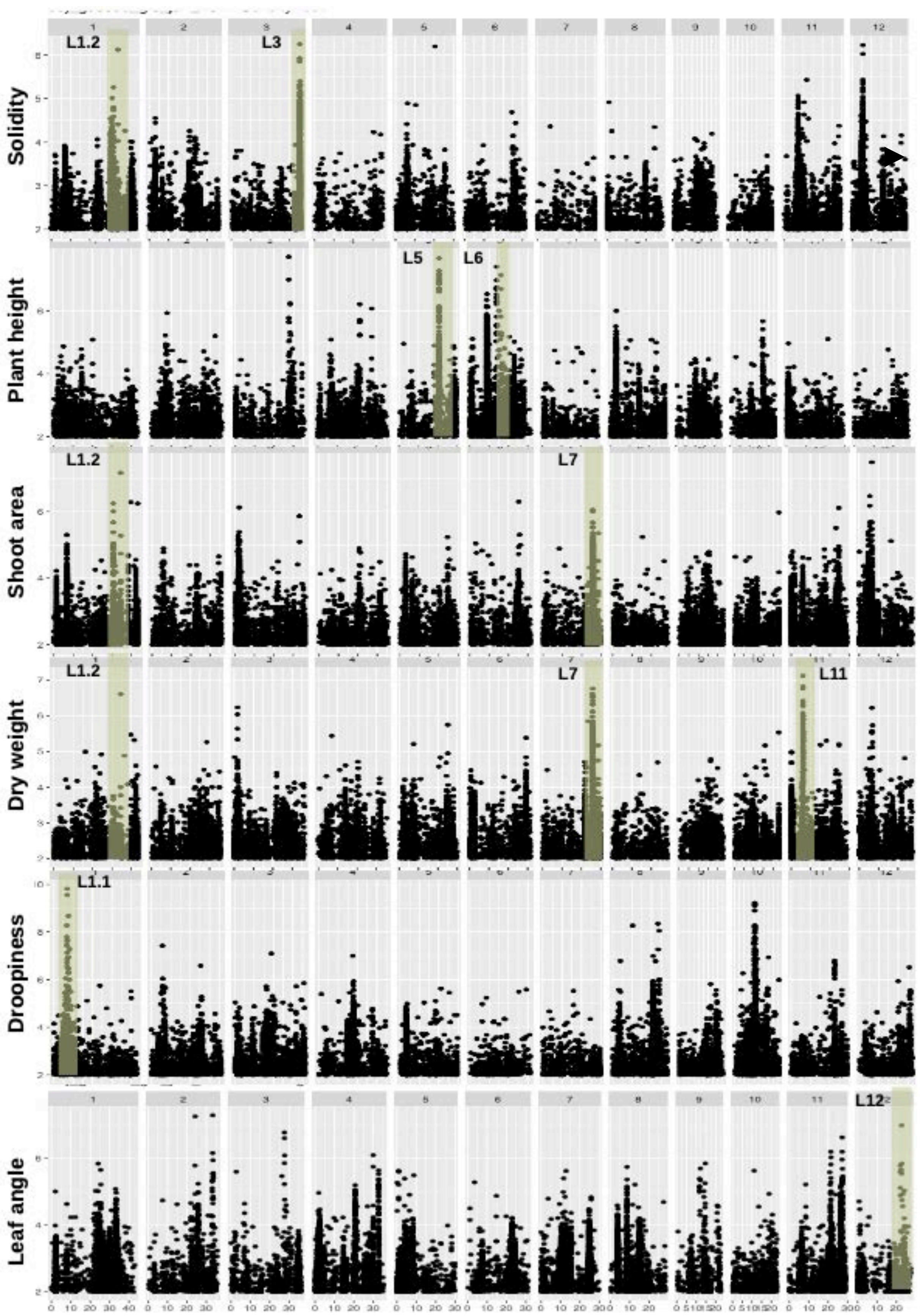
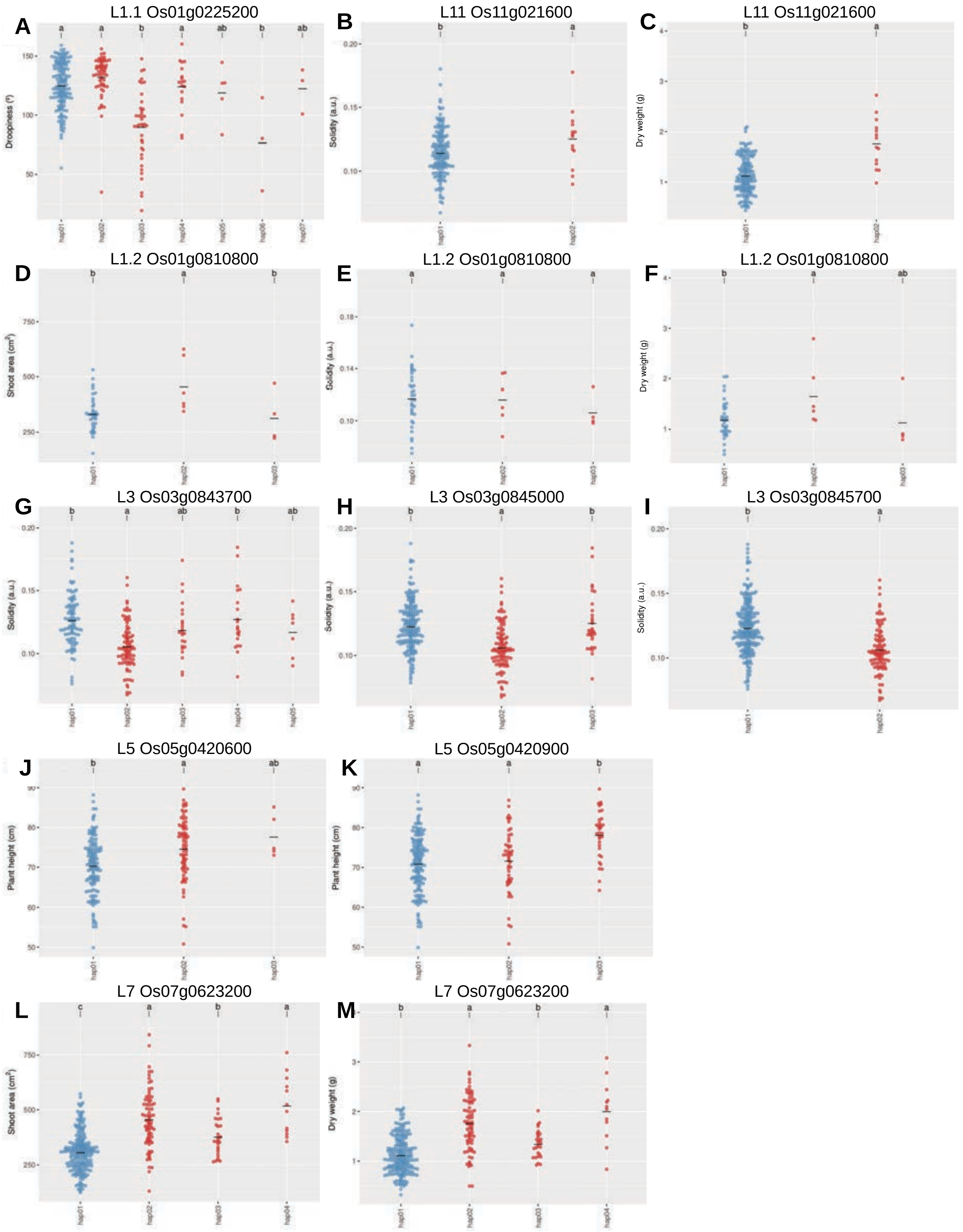

Solidity
Plant height
Shoot area
Dry weight
Droopiness
Leaf angle
1
2
3
4
5
6
7
8
9
10
11
12
L1.2
L3
L5
L6
L1.2
L7
L1.2
L7
L11
L1.1
L12
0 10 20 30 40

Table 3.4: Summary of determined loci and genes of interest of core groups for shading potential with index SNPs (significant SNPs with LOD > 5) and locus span in kb (clumped SNPs with LOD > 4 in local LD up- and downstream). with the Locus ID and gene annotation. Genes represented in Figure 3.5 are highlighted in bold. Full list of SNP positions in loci of interest with gene annotation and gene ontology categories can be found in Supplemental Table 3.6 .

Trait	Locus	Chr	Index SNP_ID	Locus span [kb]	Locus_ID	Gene annotation
Droopiness	**L-1.1**	1	1.01395336	49	**Os01g0225200**	**Predicted protein; BP: GTP binding**
Shoot area Dry weight Solidity	**L-1.2**	1	1.07664139	6	**Os01g0810800**	**Hypothetical conserved gene; BP: protein phosphorylation**
Solidity	**L-3**	3	3.35500735	404	Os03g0841800	GSK3/SHAGGY-like kinase
					Os03g0841850	Hypothetical protein.
					Os03g0843700	FAR1 domain containing protein.
					Os03g0845000	**Similar to Pirin-like protein.**
					Os03g0845700	**Similar to RPB17 (Fragment).**
					Os03g0845800	Conserved hypothetical protein.
					Os03g0848700	Coiled-coil, nucleotide-binding, and leucine-rich repeat protein
Plant height	**L-5**	5	5.20612311	59	Os05g0420500	Conserved hypothetical protein.
					Os05g0420600	**Cytochrome c.**
					Os05g0420900	**Conserved hypothetical protein.**
Plant height	**L6**	6	6.13994152	240	Os06g0269300	TolB-like domain containing protein.
					Os06g0346300	acyl-CoA oxidase/ oxidoreductase
Shoot area Dry weight	**L-7**	7	7.25787749	146	**Os07g0623200**	**Heavy metal transporter protein; ATPase, P-type.**
		7			Os07g0623501	Hypothetical gene.
		7			Os07g0623600	Similar to mRNA, clone: RTFL01-43-H20.
Dry weight	**L-11**	11	11.6059294	23	**Os11g0216000**	**Pyruvate kinase family protein.**
Leaf angle	**L-12**	12	12.085063092	1	Os12g0557800	Pentatricopeptide repeat domain containing protein.

Figure 3.5. Haplotypes for genes of interest linked to core traits for shading capacity. L-1.1 was detected for **A.** Droopiness with haplotypes in the coding sequence of a GTP binding protein (Os01g0225200). L11 found for **B.** Solidity and **C.** Dry weight with haplotypes of a pyruvate kinase family protein (Os11g0216000). L1.2 detected for **D.** Shoot area, **E.** Solidity and **F.** Dry weight with haplotypes for a protein for protein phosphorylation (Os01g0810800). L3 was associated with Solidity, encoding **G.** a FAR1 domain containing protein (Os03g0843700), **H.** a Pirin-like protein (Os03g0845000) and **I.** a RPB17 fragment (Os03g0845700). L5 found for Plant height, encoding **J.** Cytochrome C (Os05g0420600) and **K.** a conserved protein (Os05g0420900). L7 was associated with **L.** Shoot area and **M.** Dry weight encoding only one gene for a heavy metal transporter protein Os07g0623200. Letters indicate significantly different groups, determined with Tukey's HSD, p-value< 0.05. Y-axis trait value, x-axis groups of haplotypes, most abundant haplotype is highlighted in blue.

▶

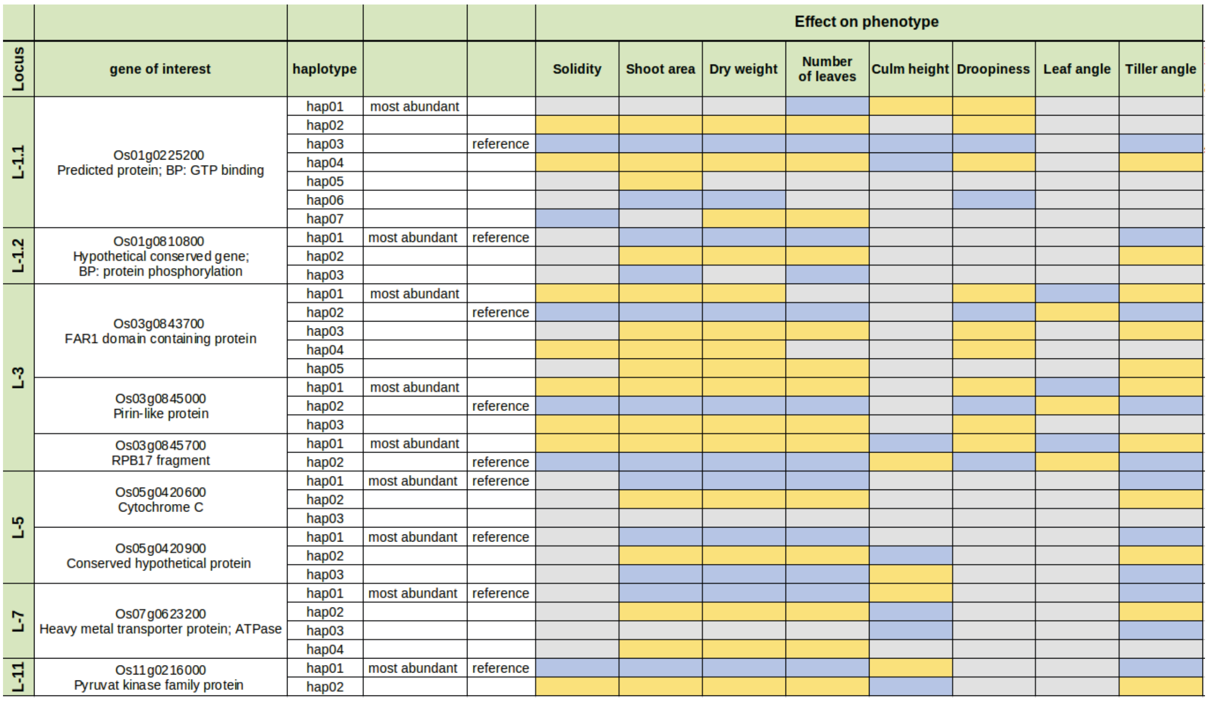

A
L1.1 Os01g0225200
Droopiness (°)
a a b a ab b ab
hap01 hap02 hap03 hap04 hap05 hap06 hap07
B
L11 Os11g021600
Solidity (a.u.)
b a
hap01 hap02
C
L11 Os11g021600
Dry weight (g)
b a
hap01 hap02
D
L1.2 Os01g0810800
Shoot area (cm²)
b a b
hap01 hap02 hap03
E
L1.2 Os01g0810800
Solidity (a.u.)
a a a
hap01 hap02 hap03
F
L1.2 Os01g0810800
Dry weight (g)
b a ab
hap01 hap02 hap03
G
L3 Os03g0843700
Solidity (a.u.)
b a ab b ab
hap01 hap02 hap03 hap04 hap05
H
L3 Os03g0845000
Solidity (a.u.)
b a b
hap01 hap02 hap03
I
L3 Os03g0845700
Solidity (a.u.)
b a
hap01 hap02
J
L5 Os05g0420600
Plant height (cm)
b a ab
hap01 hap02 hap03
K
L5 Os05g0420900
Plant height (cm)
a a b
hap01 hap02 hap03
L
L7 Os07g0623200
Shoot area (cm²)
c a b a
hap01 hap02 hap03 hap04
M
L7 Os07g0623200
Dry weight (g)
b a b a
hap01 hap02 hap03 hap04

Identification of alleles associated with increased shading potential

The genomic regions that consisted of multiple SNPs above the Bonferroni threshold within the calculated local average LD (Table 3.4) were investigated in more detail. Since the traits related to canopy shading potential are the primary focus of this work, we prioritized the loci associated with culm height, shoot area, solidity and dry weight (Figure 3.4). In total we determined six loci to be followed up with a haplotype analysis to identify specific alleles which could contribute to traits determining shading potential. By grouping varieties according to SNPs within one coding region, and examining the phenotypic differences between identified haplotypes, we identified allelic variation associated with high shading potential (Figure 3.5). On chromosome 1 we found two loci, the first one for droopiness (Figure 3.5 A) in the coding sequence for a GTP binding protein (Os01g0225200), where one haplotype (hap03) had a significantly lower droopiness compared to all others. The second locus on chromosome 1, was found to be overlapping between shoot area, solidity and dry weight (Figure 3.5 D-F) in a sequence encoding a protein with protein phosphorylation function (Os01g0810800). The haplotypes of three coding regions in locus 3 (Figure 3.5 G-I), associated with solidity, were observed to have significantly lower solidity than the most abundant haplotype. These are annotated as a FAR1 domain containing protein (Os03g0843700), Pirin-like protein (Os03g0845000) and a RPB17 fragment (Os03g0845700). Locus 5 (Figure 3.5 J-K), associated with plant height, includes Cytochrome C (Os05g0420600) and a conserved hypothetical protein (Os05g0420900), where for both, the most abundant haplotype was linked to the shortest plants. In locus 7, associated with shoot area and dry weight, we found that only one gene (Os07g0623200, annotated as ATPase and heavy metal transporter protein) showed clear separation across the haplotypes, where all the non-reference haplotypes showed higher shading potential, indicated by higher shoot area and dry weight (Figure 3.5 L-M). Within Locus 11, associated with solidity and dry weight (Figure 3.5 B-C), is only one gene located, encoding a pyruvate kinase family protein (Os11g0216000). We found that the second most abundant haplotype was associated with increased shading due to higher dry weight of varieties that were sharing this specific combination of SNPs.

We then summarized these main haplotypes into a table where we express their contribution (positive / neutral / negative) to the expression of the core traits that we used to compile the shading rank. This facilitates a direct impression of how specific alleles, detected as haplotypes as mentioned above, affect all the core traits that together determine the Shading Rank (Figure

3.6). As such, this would facilitate decisions on which alleles would be expected to be a potential source to improve shading potential in elite varieties. From this integrative table it becomes clear that a number of haplotypes are highly desirable. Hap2 from locus L-1.1 (Os01g0225200), hap2 from locus L-1.2 (Os01g0810800), hap3, hap4 and hap5 from locus L-3 (Os03g0843700) and hap3 from another gene in locus L-3 (Os03g0845000) are all alleles that positively affect values of multiple core traits that contribute to shading potential, without negatively affecting any of the other core traits. Since these alleles are not highly abundant in the studied population (Figure 3.6), it is highly likely that they are presently not represented in the current elite cultivars. From this table it also becomes clear that allelic effects on trait expression are quite consistent between shoot area, dry weight and leaf number. Alleles that are positive for these traits, however, are often negative or neutral for culm height. Furthermore, all alleles that seem to stimulate culm height reduce expression of (multiple) other core traits, indicating that within this population there is little potential for improvement of plant height without undesirable trade-offs to other traits. Although only few of the detected alleles affect leaf angles, the ones that do so have the opposite effect on tiller angles.



					Effect on phenotype							
Locus	gene of interest	haplotype			Solidity	Shoot area	Dry weight	Number of leaves	Culm height	Droopiness	Leaf angle	Tiller angle
L-1.1	Os01g0225200 Predicted protein; BP: GTP binding	hap01	most abundant		⬜	⬜	⬜	🟦	🟨	🟨	⬜	⬜
		hap02			🟨	🟨	🟨	🟨	⬜	🟨	⬜	⬜
		hap03		reference	🟦	🟦	🟦	🟦	🟦	🟦	⬜	🟦
		hap04			🟨	🟨	🟨	🟨	🟦	🟨	⬜	🟨
		hap05			⬜	🟨	⬜	⬜	⬜	⬜	⬜	⬜
		hap06			⬜	🟦	🟦	⬜	⬜	🟦	⬜	⬜
		hap07			🟦	⬜	🟨	🟨	⬜	⬜	⬜	⬜
L-1.2	Os01g0810800 Hypothetical conserved gene; BP: protein phosphorylation	hap01	most abundant	reference	⬜	🟦	🟦	🟦	⬜	⬜	⬜	🟦
		hap02			⬜	🟨	🟨	🟨	⬜	⬜	⬜	🟨
		hap03			⬜	🟦	⬜	🟦	⬜	⬜	⬜	⬜
L-3	Os03g0843700 FAR1 domain containing protein	hap01	most abundant		🟨	🟨	🟨	⬜	⬜	🟨	🟦	🟨
		hap02		reference	🟦	🟦	🟦	🟦	⬜	🟦	🟨	🟦
		hap03			⬜	🟨	🟨	🟨	⬜	🟨	⬜	🟨
		hap04			🟨	🟨	🟨	⬜	⬜	🟨	⬜	⬜
		hap05			⬜	🟨	🟨	🟨	⬜	⬜	⬜	🟨
	Os03g0845000 Pirin-like protein	hap01	most abundant		🟨	🟨	🟨	🟨	⬜	🟨	🟦	🟨
		hap02		reference	🟦	🟦	🟦	🟦	⬜	🟦	🟨	🟦
		hap03			🟨	🟨	🟨	🟨	⬜	🟨	⬜	⬜
	Os03g0845700 RPB17 fragment	hap01	most abundant		🟨	🟨	🟨	🟨	🟦	🟨	🟦	🟨
		hap02		reference	🟦	🟦	🟦	🟦	🟨	🟦	🟨	🟦
L-5	Os05g0420600 Cytochrome C	hap01	most abundant	reference	⬜	🟦	🟦	🟦	⬜	⬜	⬜	🟦
		hap02			⬜	🟨	🟨	🟨	⬜	⬜	⬜	🟨
		hap03			⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜
	Os05g0420900 Conserved hypothetical protein	hap01	most abundant	reference	⬜	🟦	🟦	🟦	⬜	⬜	⬜	🟦
		hap02			⬜	🟨	🟨	🟨	🟦	⬜	⬜	🟨
		hap03			⬜	🟦	🟦	🟦	🟨	⬜	⬜	🟦
L-7	Os07g0623200 Heavy metal transporter protein; ATPase	hap01	most abundant	reference	⬜	🟦	🟦	🟦	🟨	⬜	⬜	🟦
		hap02			⬜	🟨	🟨	🟨	🟦	⬜	⬜	🟨
		hap03			⬜	⬜	⬜	⬜	🟦	⬜	⬜	🟦
		hap04			⬜	🟨	🟨	🟨	⬜	⬜	⬜	⬜
L-11	Os11g0216000 Pyruvat kinase family protein	hap01	most abundant	reference	🟦	🟦	🟦	🟦	🟨	⬜	⬜	🟦
		hap02			🟨	🟨	🟨	🟨	🟦	⬜	⬜	🟨

Figure 3.6. Haplotype effect for genes of interest linked to increased values for core traits contributing to shading capacity. The heatmap shows for each for each specific haplotype within each gene of interest, an increased trait value in yellow, no significant change in grey and a decreased value in blue. The most abundant and the haplotype comprising the reference genome are indicated. Additional information about the detected genes in loci of interest can be found in Table 3.4.

Discussion

We studied phenotypic and genetic variation in rice shoot architecture to identify traits and their underlying genetic loci that contribute to canopy shading. We investigated variability across a natural rice diversity panel in shoot architecture at the early vegetative stage. The traits investigated here encompass both early vigour and shade casting through shoot architecture, which are linked to weed suppression in rice fields (Andrew et al., 2015; Brainard et al., 2005; Mahajan & Chauhan, 2013; Seavers and Wright, 1999; Worthington & Reberg-Horton, 2013). Traits related to shoot architecture, such as leaf angle or droopiness, are of special interest as they do not require substantial resource investment while creating more optimal 3D canopy distribution of the shoot biomass for an increased shading potential. Other traits, such as leaf area, number of leaves or shoot biomass, likely require considerable resource investments and are typically associated with growth vigour i.e. rapid seedling establishment.

Shoot architecture traits for shading

Shading potential can be defined in two-dimensional measures, such as ground cover or projected shoot area, or including a third dimension, where plant height is considered as space resource utilization (Zhang et al., 2019). We identified how all measured traits are related to one another and identified core traits that capture the observed variance (Figure 3.2). We identified groups of traits related to branchiness (number of leaves and tillers) and height (plant height, culm height and leaf length), and added solidity, leaf and tiller angles and droopiness to calculate a Shading Rank. The varieties with highest Shading Rank belong to the *indica* and *aus* subpopulation, which have also been found in earlier studies to have higher yield and less weed biomass in weedy fields compared to *japonicas* (Zhao et al., 2006b). We found *admixed, tropical japonica* and *aus* subpopulations to typically range between *temperate japonica* and *indica*. This pattern could be found in the majority of the measured traits and is in line with the phylogenetic relatedness of the different subpopulations (Eizenga et al., 2014; Liakat Ali et al., 2011; McCouch et al., 2016; Zhao et al., 2011). Early seedling vigour is particularly important for weed-competition during the critical period of weed control and some of the high-ranking varieties, such as Shim Balte, Paung Malaung and Sabharaj are also known by breeders for their early vigour. Increased shading ability is intrinsic to early vigour since it follows to some extent from large size. However, the Shading Rank proposed here is more comprehensive to additional traits such as solidity and plant architecture that may involve less resource investment than vigour traits. With this improved way of ranking a plant’s shading capacity, our study exemplifies a new method of selection for high-shading varieties and genetic loci associated with traits that contribute high-shade canopy architecture. It also helps to narrow down any selection to a modest number of core traits, making phenotyping more efficient.

The correlations between traits encapsulated within each of the trait groups that were used for the Shading Rank often underlines natural growth patterns; the more tillers a plant has, the more leaves it bears since each tiller has potential to develop a certain number of leaves. Strong correlation was previously observed between tiller formation and relative growth rate (Dingkuhn et al., 2001). Likewise, in our study number of leaves and leaf area were positively correlated with shoot dry weight (Figure 3.2, Supplemental Figure 3.1). This well-established relationship (Caton et al., 2003; Dingkuhn et al., 2001; Poorter et al., 2012) probably follows from a larger shoot area providing higher capacity for photosynthesis and thereby leading to higher overall growth rate (Caton et al., 2003). However, not all traits showed expected correlations; while solidity is the ratio of shoot area and hull area, it is only weakly correlated with shoot area (Figure 3.2, Supplemental Figure 3.1). This suggests that shoot solidity is independent of how large its total shoot area, leaf number or angles are. Since solidity indicates the uniformity of the plant’s ability to shade its circumference, it is a valuable trait for shading capacity analysis; a large projected shoot area with low solidity would still leave many open spaces within a single plant’s sphere for light penetration where weeds can proliferate. Inverse correlations were found between branchiness (number of leaves and tillers) and height traits. This trade-off between height and branching is well-documented as apical dominance where height growth of the main shoot is promoted at the expense of branching (Roig-Villanova & Martínez-García, 2016; Teichmann & Muhr, 2015). Summarizing, the trends observed within this study are in line with earlier observations, whereas we identify new, informative trait groups that allow interpretations at the canopy level and that contribute independently to the shading potential of rice plants.

Elucidating the genetic components of shading potential

We screened a large diversity panel representing different subpopulations, which adds new information to several available studies on specific subpopulations or recombinant inbred lines (Cordero-Lara et al., 2016; Hoang et al., 2019; To et al., 2019; Wang et al., 2011).

Architecture

The SNP dataset from the rice diversity panel (Eizenga et al., 2014) was combined with the observed phenotypic variation to identify putative genetic loci underlying high shading potential. This variation (Figure 3.1, Supplemental Table 3.4) together with a high trait heritability (Supplemental Table 3.5) provides a strong basis for GWAS. Plant height and leaf length were associated with loci on chromosomes 5 and 6. The locus on chromosome 5 harbours two genes encoding Cytochrome C and a conserved hypothetical protein. The haplotype analysis revealed one allele for both genes that was associated with a highly significant increase in plant height. (Figure 3.5). The locus on chromosome 6 encodes the *Heading Date* (*Hd1*) locus that was also previously associated with plant height in vegetative rice plants (Zhang et al., 2012; Yang et al., 2014). Subedi et al. (2019) performed a GWAS on plant height at plant maturity and found peaks on chromosome 1 and 11. This discrepancy could indicate that at different developmental stages plant height is determined by different genomic regions, but since Subedi et al (2019) used a specifically constructed genetic population stemming from six parents, the genetic starting material was also fundamentally different from the population used here. Interestingly, haplotypes associated with high culm height exhibit low plant height and vice versa (Supplemental Data 3.7). Haplotypes associated with high plant height are typically showing longer leaf length (Supplemental Data 3.7). While all the height related traits were highly correlated at the phenotypic level (Figure 3.2), the lack of common loci for all the traits (Supplemental Data 3.4), and opposite trends within the haplotype groups (Supplemental Data 3.7) suggest that the three components of plant height are regulated independently at the genetic level.

Although we consider solidity a composite trait, we revealed only one strong locus, with several significant SNP associations, on chromosome 3 (Figure 3.4). When we grouped varieties into haplotype groups for two coding regions (Os03g0845000 and Os03g0845700, Figure 3.5 A-B), encoding a Pirin-like protein and a RPB17 fragment within this locus, the phenotype of the haplotype groups appeared to differ not just in solidity, but also shoot area, dry weight and leaf number (Figure 3.5 H-I, Supplemental Data 3.7). This indicates that genetic regulation of solidity could still be associated with traits of plant vigour.

Vigour

Vigour-related traits (i.e., dry weight, shoot area, number of leaves) are all strongly correlated and share associated loci on chromosome 1, 7, 11 and 12 (Figure 3.4, Supplemental Data 3.4).

The locus on chromosome 11 was also reported by (Yang et al., 2014) for dry weight and fresh weight at the late tillering stage, which is comparable to the developmental stage studied here. A closer look into this locus revealed that only one gene is located within the linkage disequilibrium of associated SNPs. Interestingly, the haplotype analysis for SNPs within this gene, encoding a Pyruvate kinase family protein, revealed a significant difference in dry weight between the two haplotype groups (Figure 3.5 G). The significant differences were also observed for shoot area and number of leaves and tillers for the same two haplotype groups. As only one gene was located within this locus and one specific haplotype was related with high biomass, this locus is a promising candidate for follow-up studies and breeding programmes. The locus on chromosome 7 associated with shoot area and dry weight (Figure 3.5 E, F), harbours two genes, where we found that the haplotypes were associated with an increased shoot area and dry weight but also increased number of leaves and tillers. These loci for plant vigour complement those found in a QTL study for height at 7 and 14 days after sowing and fresh weight, using exclusively temperate japonica genotypes (Cordero-Lara et al., 2016), thus having an intrinsically different pool of biological variation that can provide different genomic leads.

Improving shading potential and weed suppression

The large phenotypic variation, high abundance of haplotypes that do not positively contribute to shading potential and the low Shading Rank of several commercially important cultivars in this studied diversity panel together indicate a strong potential for improvement of shading capacity in such varieties. For example, IR 64 and Nipponbare, which gave rise to many of the current widely-grown rice varieties, have a very low Shading Rank, ranking in the lowest quartile of our population (Supplemental Table 3.3). We identified a suite of alleles of the Os01g0225200, Os01g0810800, Os03g0843700 and Os03g0845000 genes that contribute positively to shading potential (Figure 3.6). The IR 64 variety is typically not carrying the favourable alleles for these genes, and this is true for Nipponbare as well. Remarkably, the most abundant haplotype, tends to be the most inferior one for the target traits of high shade casting, in the diversity panel screened here. Based on the insights from this study, we can now guide improvements for shading potential in these varieties through conventional breeding, where we provide information for optimal alleles. Future studies should then resolve if such improved varieties would indeed have superior weed-suppressive properties, as predicted from our analyses. Such tests are especially relevant because rice is a highly plastic species. We have performed our experiments under stable conditions in a controlled environment and it will, therefore, be relevant to perform field trials when testing improved varieties. One obvious factor affecting architecture would be planting density and the associated changes in light composition and availability. Another aspect of weed-competitiveness, which was not covered in our study, would be the root systems, for which the rapidly evolving high throughput phenotyping methods are a major opportunity to resolve comparable questions as done here for shoot architecture. We conclude that breeding for specific vigour traits will likely have additional beneficial effects, as indicated by the haplotype studies. Vigour from root growth can then be an added layer at a later step towards field-grown, weed-competitive varieties that can be farmed in a sustainable manner. Having worked from a broad diversity panel, rather than a focused or limited population, and including traits such as angles, droopiness and solidity, has enabled us to identify alleles in existing varieties that can now be used in rice improvement programs for sustainable weed competitiveness.

Acknowledgements

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Materials and methods

Plant material

344 Asian rice (*O. sativa)* cultivars were used out of an established rice diversity panel (Rice diversity panel 1; RDP1 (Eizenga et al., 2014). In addition, one African rice variety (*O. glaberrima*) TOG7192 was also included. The RDP1 is a collection of purified, homozygous rice varieties spread over 82 countries all over the world. The panel includes landraces and improved rice cultivars from five subpopulations: *indica* and *aus* belonging to the Indica varietal group and *tropical japonica*, *temperate japonica* and *aromatic* which comprise the Japonica varietal group, in addition to the *admixture* group, (Liakat Ali et al., 2011; Zhao et al., 2011). The full panel and detailed information (accession name, accession ID, subpopulation and country of origin) can be found in the Supplemental Table 3.1.

Growth conditions

Rice plants were grown in the screen-house facilities of the International Rice Research Institute (IRRI) in The Philippines, from October 2017 to April 2018. Temperatures ranged from 37 °C during the day to 27 °C at night, with a relative humidity of 75 % and 80 %, respectively and a photoperiod of 11 to 12 hours. Four temporally separated replications were carried out, with three plants per variety within each replicate experiment. Plants were grown in a randomized block design in single pots at a 30 cm x 30 cm distance. In the first experiment, seeds received from the IRRI gene bank were exposed to 40 °C for 5 days, to break dormancy, followed by 24 h at 21 °C. For germination, seeds were put in Petri dishes on wet filter paper and incubated at 32 °C for 24 h. Seeds were planted directly on the soil: 4 seeds were placed per pot (diameter of 16 cm and 13 cm high, without drainage holes) filled with sterilized clay-loam field soil mixed with NPK fertilizer (with 46 / 18 / 60 g per kg soil) and covered with a thin layer of soil. From planting onwards, soil was kept moist. At 7 days after sowing (DAS), surplus seedlings were removed, retaining one seedling per pot. At 14 DAS, fertilizer with 50 % of N of initial concentration was added of first application. From 15 DAS onwards layer of water was maintained for water-logged conditions.

Phenotyping

Plants were measured by hand at 28 das for the following traits: number of leaves, number of tillers, total plant height, culm height, and length of longest leaf. Plants were photographed from the top and side using 2 digital cameras in a fixed imaging set-up at 21 and 28 das. At the last time point, a scan of the blade of the longest leaf was taken and the whole shoot was harvested for analysis of dry weight upon 48 h of drying at 70 °C (IRRI, 2013; Caton et al., 2003). In Table 3.1, each trait, their abbreviations and evaluation methods are described. The raw data for each replicate can be accessed in Supplemental Data 3.1.

Data processing and statistical analysis

In order to extract traits from RGB images, an automatised image analysis pipeline was established using the open source, python based PlantCV software (PlantCV version 3.7) (Fahlgren et al., 2015; Gehan et al., 2017). We made optimisations to the script for detection of monocots, to enable the extraction of values for shoot area, hull area and perimeter. The python script describing the developed pipeline can be accessed at https://plantcv.readthedocs.io/en/stable/ and the adapted Jupiter notebook used for processing all the images at https://doi.org/10.5281/zenodo.4730232 (Supplemental Data 3.2). The measurements of tiller angle, leaf angle and leaf erectness, were done using the free ImageJ software (https://imagej.nih.gov/ij/). Tiller angles were taken between the two outermost tillers and the culm, respectively. The leaf angles were taken between the second and third youngest leaf and the culm, respectively. The leaf droopiness was measured on the same leaves as the interception angle of two tangents aligned to the initiation and the tip of the leaf blade.

The values of the first replicate were excluded for 62 varieties as their position within the greenhouse was more shaded. These positions were excluded from further experimental replication, to ensure equal light conditions for all studied plants. Prior to statistical analysis, the raw data was curated for outliers (using 1.5*IQR away from the mean) and mean was calculated out of the four replicates, with two biological replicates each. Statistical analysis such as Anova, Pearson Correlation and Hierarchical Clustering were performed using R (R Version: 3.6.1-1bionic; R Core Team, 2020) and the online tool MVapp https://mvapp.kaust.edu.sa (Julkowska et al., 2019). The Pearson Correlation coefficients between traits were calculated using raw data. For Hierarchical Clustering traits and individual samples were clustered using ward.D2 method. The values of individual traits were normalized per trait using z-Fisher transformation and scaled prior to clustering. Based on the correlation and clustering analysis, a subset of phenotypic traits, was defined as the core traits. The core traits were shoot area, leaf number, solidity, culm height, leaf angle, tiller angle and leaf droopiness. Then we calculated the Shading Rank as follows.

First, we normalized the trait values $t^{n}_{variety}$ $t^{n}_{variety}=\frac{t_{variety}-min(t_{variety})}{max(t_{variety})-min(t_{variety})}x\,100$ where $t_{variety}$ is the value of a certain trait measured for a certain plant in the investigated population and min and max are the minimum and maximum values of the measured trait in the whole population, with the normalized values ranging from 0 to 100.

Next, we calculated the Shading Score for each variety $SS_{variety}$ $SS_{variety}=\sum_{coretraits} t^{n}_{variety}$ where the sum $\sum$ is calculated as the sum only from the normalized values of the core traits. From this, we get the Shading Rank (SR), which is the rank given to each variety according to its SS, ordering the varieties from 1 (lowest) to 344 (highest). The list of 344 varieties with their normalized core trait values, the sum of normalized core trait values and their Shading Rank can be found in Supplemental Table 3.3.

Phenotype data for GWAS

For the GWAS analyses, the mean values of all phenotypes were included, only *O. glaberrima* TOG7192 was excluded since it does not belong to the *O. sativa* species. We tested for the normal distribution across the recorded traits prior to running the GWAS. The list for all 344 varieties with 13 shoot trait values (as the mean value out of eight replicates, for raw data see Supplemental Data 3.1) which were used as input for GWAS can be found in Supplemental Table 3.4.

Genotype data

For the genotype data we have used two data sets publicly available at http://ricediversity.org/data/index.cfm tools/. As a second dataset, we used the newer version of genomic data imputed HDRA with 4.8 M SNPs, from 3,010 *O. sativa* varieties assembling the established Rice Reference Panel by merging the high-density rice array with 700 K SNPs from in total 1,568 *O. sativa* varieties including RDP1 (rice diversity panel 1), RDP2 and NIAS (national institute of agrobiological sciences) from (McCouch et al., 2016) and 3000 Rice Genomes data sets (Wang et al., 2018). The data was curated by filtering for unique SNPs, 90% call rate (90% minimum count) and minor allele frequency ≥ 5 %. We used the SNP data that adhere to the filtering criteria for 344 varieties that were included in the phenotypic screen, which resulted in total of 1.7 M SNPs remained as an input for the GWAS. As an average genome-wide linkage disequilibrium (LD) decay in rice we used previously calculated values

(Zhao et al. 2011; Huang et al. 2010). LD is calculated by measuring the pairwise SNP LD among the common SNPs (with MAF > 0.05) using r^2, the correlation in frequency among pairs of alleles across a pair of markers, using the software PLINK (http://zzz.bwh.harvard.edu/plink/).

Genome wide association study (GWAS)

We used two different software packages to perform the GWAS. The first is an R package (R version 3.6.1) of Genomic Association and Prediction Integrated Tool (GAPIT) (Tang et al., 2016; Wang et al., 2018c). We employed a mixed linear model (MLM) (Yu et al., 2006) with the optimal number of Principal Components based on the calculated Bayesian information criterion (BIC) for each trait, including as coefficients a kinship matrix (K-matrix), based on clustering analysis to account for genetic relationship between individuals, together with the population structure (Q-matrix). The Manhattan plots for GWAS using the GAPIT can be found in Supplemental Data 3.5, for shoot area, hull area, perimeter, plant height, culm height, leaf length, solidity, number of leaves, number of tillers, dry weight, droopiness, leaf angle, tiller angle and the Sum of normalized traits. Shown are SNPs with MAF > 0.05, with the negative logarithmic p-values on the y-axis, for 1.7 M SNPs across the 12 rice chromosomes along the x-axis. The second software package is lme4QTL (Ziyatdinov et al., 2018). We performed GWAS as described in the paper, taking population structure into account by using a kinship matrix. This kinship matrix was calculated using the cov() function in R 3.6 (Supplemental Figure 3.2). The decomposition matrix to correct for population structure was made by following the lme4QTL protocol. It uses the relmatLmer(), varcov() and decompose_varcov() functions in order. The obtained decomposition matrix, together with the traits and binary SNP matrix is then used in the matlm() function to calculate the significance and effect per SNP. The full list of detected significant SNP associations is presented in Supplemental Data 3.3. As a confirmation for the reliability of SNP trait associations, we correlated the results of the two methods applied here (GAPIT and lme4QTL). We do not expect an exact overlap, as there is a small difference in how the kinship matrix is calculated and GAPIT uses MLM, whereas lme4QTL does not. The narrow sense heritability (h^2) of the analysed traits was calculated with GAPIT (Supplemental Table 3.5). To set the significance threshold the rather conservative Bonferroni correction was applied, calculated by the $-\log^{10}$(p-value of 0.05/Σ SNPs), which corresponds to -log10(0.05/1.700.000) = -7.53 for the imputed HDRA data set. To examine the

GWAS model performance and estimate possible model overfitting, QQ plots were generated (Supplemental Data 3.6).

Post-GWAS analysis

For all follow-up analysis the output of the GWAS on the raw, untransformed phenotype data was used.. **Locus definition:** We determined loci to be of interest, if there are several significantly associated SNPs found in close proximity. Single SNPs passing the threshold were neglected, because whole-genome sequencing data provides enough markers in each linkage disequilibrium block. Since rice has a low rate of LD decay, this makes it more difficult to identify causal genes (Wang et al., 2020). Therefore, the local LD analysis was used to define LD clumps surrounding the index SNPs, using LD clumping in PLINK, where the local LD between SNPs is considered. A strong LD between SNPs is one of the three criteria that must be simultaneously satisfied. The other two criteria are p-value threshold set to 0.01 and physical distance set to 250 kb, given with the R^2 value. We considered SNPs with $–\log^{10}$(p-value) > 5 as index SNPs to perform the analysis and clump SNPs with p-value > 4. For the determination of loci of interest for weed-competitiveness, we focused on the core traits culm height, shoot area, solidity and number of leaves. For culm height and number of leaves single significant SNPs were not found to be surrounded by other significant SNPs within LD and therefore did not meet our selection criteria. Since, dry weight is highly correlated with the traits of branchiness, we included the peaks found for dry weight as a representative locus for branchiness and similarly the loci for plant height as a representative of height related traits.

Gene models: Genetic regions covered by significant SNPs were searched for candidate genes using two different gene annotation models, which were then merged: the Michigan State University (MSU; 31 Oct. 2011 - Release 7; http://rice.plantbiology.msu.edu/) and the Rice Annotation Project Database (RAP-DB; 24 March 2020; https://rapdb.dna.affrc.go.jp/). Other data resources used, were the gene ID converter (https://rapdb.dna.affrc.go.jp/tools/converter), GALAXY – rice genome browser (http://13.250.174.27:8080/?tool_id= getgenes&version=1.0.0) and SNP seek (http://snp-seek.irri.org/).

Haplotype analysis

In order to facilitate the identification of candidate genes within the found loci related to the canopy architecture, we performed haplotype analysis spanning the coding sequence regions of the genes within each locus. For each locus, we used the combined gene model annotation (MSU and RAP-DB) to identify the coding sequences belonging to individual genes (Supplemental Table 3.6). We subsequently compiled all SNPs that were within the coding sequence region into one haplotype and grouped all studied varieties based on their haplotype sequence. The haplotypes represented by two or less varieties were excluded from the analysis, due to low representation. If significant SNPs are too far to be within LD, then they are not defined as a locus and are not included in further haplotype analysis. Since haplotype analysis can only be done for described and annotated genes, SNPs that are not in a coding sequence, cannot be included in haplotype analysis. Based on the haplotype grouping for each coding sequence, we performed an Anova followed by a post-Hoc test for significant differences between the haplotypes within for each measured trait. The individual haplotypes are represented by A/T, where A stands for reference accession sequence, and T for any alternative variant. Supplemental Data 3.8 contains the full list of coding sequences of genes within the defined loci of interest.

All custom R scripts and supplementary data are available upon request to the author.

Supplements

Supplemental Table 3.1. List of rice varieties of screened rice diversity panel (RDP1) and description of origin.

Supplemental Table 3.2. Results for Anova (considered significant with $p < 0.05$) and post-hoc based on Tukey's pairwise comparison of shoot traits between different rice varieties and between different subpopulations, mean out of eight replicates of 344 varieties, the sum of normalized core trait values and their Shading Rank. Raw data can be found in Supplemental Data 3.1.

Supplemental Table 3.3. The list of 344 varieties with their normalized core trait values, the sum of normalized core trait values and their Shading Rank.

Supplemental Table 3.4. List of 344 varieties with 13 shoot trait values (as the mean value out of eight replicates (for raw data see Supplemental Data 3.1.) which were used as input for genome-wide association studies, their normalized trait values, the sum of normalized core trait values and their Shading Rank.

Supplemental Table 3.5. Narrow sense heritability of all analysed traits in genome-wide association studies, calculated in GAPIT.

Supplemental Table 3.6. Full list of SNP positions in loci of interest with gene annotation and gene ontology categories from Rice Annotation Project Database.

Supplemental Data 3.1. List of 344 varieties with raw data of 13 shoot traits from eight replicates.

Supplemental Data 3.2. Python script based on PlantCV used for image analysis.

Supplemental Data 3.3. Association results for GWAS with Lme4QTL using a mixed linear model (MLM) based on the lme4QTL protocol, for shoot area, hull area, perimeter, plant height, culm height, leaf length, solidity, number of leaves, number of tillers, dry weight, droopiness, leaf angle, tiller angle and the Sum of normalized traits of 4-week-old rice seedlings.

Supplemental Data 3.4. Genetic regions underlying shoot architectural traits and seedling vigour in 4-week-old rice seedlings. Single-trait genome-wide association studies (GWAS) using a mixed linear model (MLM) based on the lme4QTL protocol, for droopiness, leaf angle, tiller angle, Sum of normalized traits, number of leaves, number of tillers, culm height, leaf length hull area and perimeter. The Manhattan plots depict the single nucleotide polymorphisms (SNPs) with minor allele frequencies (MAF) > 0.05. Negative logarithmic P-values on the y-axis, for 1.7 M SNPs across the 12 rice chromosomes along the x-axis. P-values of association results for all traits can be found in Supplemental Data 3.3.

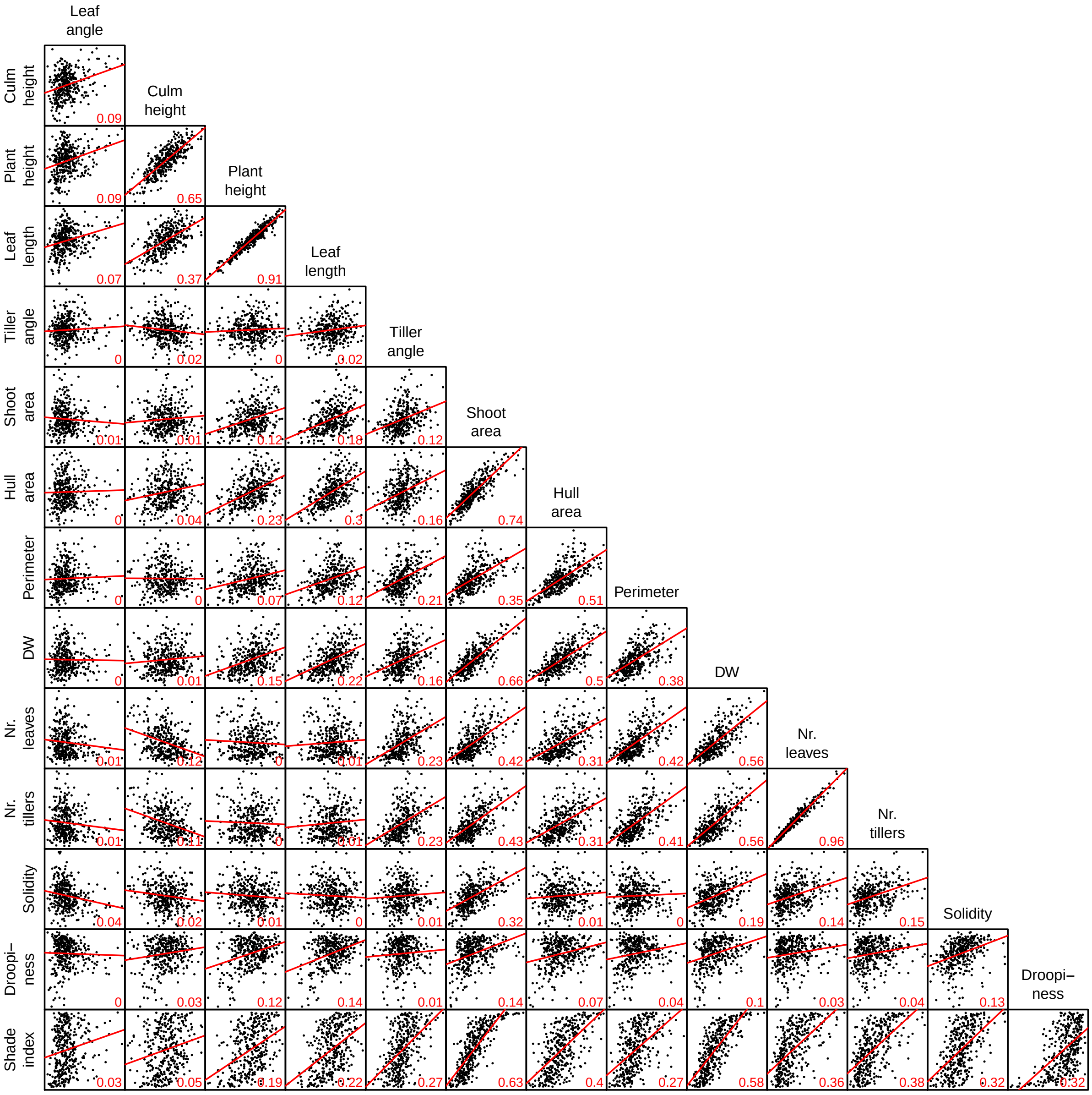
Supplemental Data 3.5. Genetic regions underlying shoot architectural traits and seedling vigour in 4-week-old rice seedlings. Single-trait GWAS using a mixed linear model (MLM) with the GAPIT package in R, for shoot area, hull area, perimeter, plant height, culm height, leaf length, solidity, number of leaves, number of tillers, dry weight, droopiness, leaf angle, tiller angle and the Sum of normalized traits. The Manhattan plots depict the single nucleotide polymorphisms (SNPs) with minor allele frequencies (MAF) > 0.05. Negative logarithmic P values on the y-axis, for 1.7 M SNPs across the 12 rice chromosomes along the x-axis.

Supplemental Data 3.6. QQ-plots with negative logarithmic P values for observed on the y-axis and expected SNP - trait associations on the x-axis.

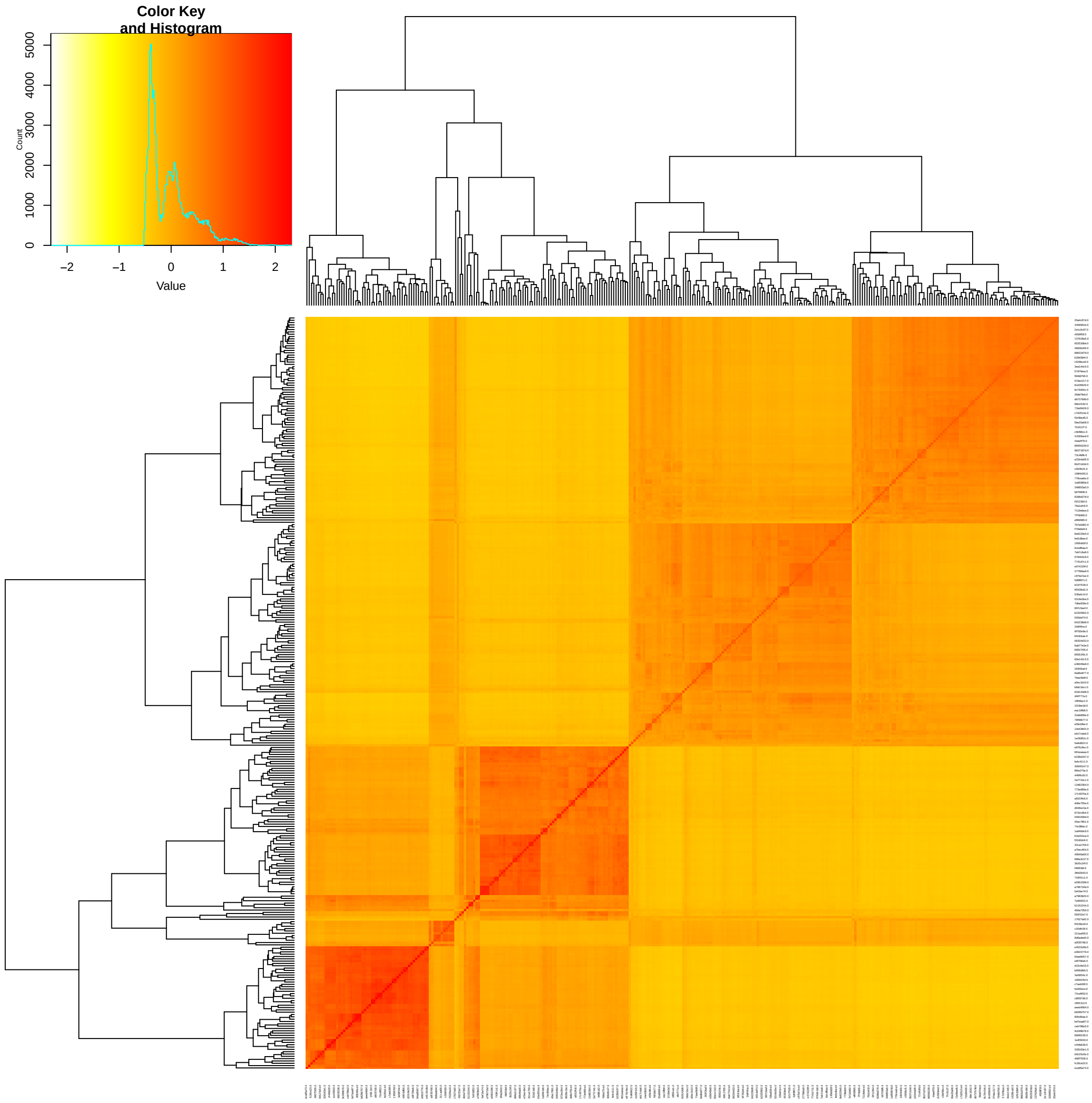
Supplemental Data 3.7. Haplotype groups for all determined loci of interest with their phenotype effect for 13 investigated shoot traits in the order of L1-1_Os01g0225200, L1-

2_Os01g0810800, L3_Os03g0843700, L3_Os03g0845000, L3_Os03g0845700, L5_Os05g0420600, L5_Os05g0420900, L7_Os07g0623200, L11_Os11g216000 and L12_Os12g0557800. In order to provide a complete overview, also graphs for haplotypes shown in main Figure 3.5 are included here, to enable comparison.

Supplemental Data 3.8. List of sequences of genes for loci of interest, with haplotypes for screened varieties.



Supplemental Figure 3.1. Scatter plots and R² values for pair-wise correlation analysis for individual traits.



Supplemental Figure 3.2. Kinship matrix of screened rice diversity panel (RDP1).

Chapter 4

Rice canopy development and architecture can control weed growth

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Abstract

The traditional rice farming system is facing a major transition from transplanting rice seedlings into paddy fields, to direct seeding onto the field. This is driven partly by climate change-related unpredictability of available water for paddy fields, as well as by labour shortage. However, with direct seeding, the weeds infesting rice fields pose a major threat, since there is no field flooding that would suppress weeds like it does in flooded paddy fields. This situation calls for a new sustainable weed-management method. Here, we explored the potential of fighting weeds by depleting them from access to light, on the one hand via high shade casting rice varieties and on the other hand via increased planting density.

The results from our field experiments show, that varieties that we previously selected for having high expected shading abilities reach a larger percent ground cover in the field, together with earlier tillering and larger biomass as compared to varieties with poorer shading ability. The strong rice performance was negatively correlated with weed biomass and weed tiller formation. Canopy height, especially early in the season, also correlated negatively with weed growth.

In addition to high shading potential, an increased planting density also helped suppressing weeds and this beneficial effect of rice density on weed suppression was most obvious in the rice varieties that had a rather modest shading ability at regular planting density. However, the positive effects of increased rice density on weed suppression comes with a penalty for the rice; where individual rice plants had reduced biomass and more investment in height growth at the cost of tillering. In addition, at increased density the ground cover does develop faster, but in the end does not necessarily lead to an earlier complete closure of the canopy.

We see that timing is crucial for the impact on weeds, where shading them early in the season, together with early canopy height shows the strongest negative correlation with end weed biomass. Thus, there is great potential with choosing the right rice variety with strong shade casting and early vigour, to substantially diminish weed growth in a sustainable manner and secure rice yield.

Abbreviations

Treatment groups

rice [R]	rice in monoculture; rice-only
rice [RW]	rice in mixed plots; mixed rice-weed
weed [W]	weed in monoculture; weed-only
weed [RW]	weed in mixed plots; mixed rice-weed
rice [R-HD]	rice in monoculture at high planting density
weed [RW-HD]	weed in mixed plots at high planting density

Timepoints

das	days after sowing
dat	days after transplanting
25 dat	early season
40 dat	maximum tillering stage
53 dat	panicle initiation = end vegetative phase

Traits and Indices

reHT	relative canopy Height; based on RGB camera imagery
NDVI	Normalized Difference Vegetation Index; based on multispectral reflectance imagery
TGI	Triangular Green Index; based on RGB camera imagery
PC	Plot canopy Cover ; based on plot level NDVI and TGI
CC	time to Canopy Closure; derived from PC
% cover	percent ground cover of rice or weed in the mixed plots; based on manual measurements

Introduction

Weeds are one of the major factors resulting in up to 80 % yield loss in rice farming (Bajwa et al., 2015; Caton et al., 2003; Chauhan & Johnson, 2009; Dingkuhn et al., 1999; Johnson et al., 1998; Kraehmer et al., 2016; Mahajan & Chauhan, 2013; Ottis & Talbert, 2007). The fact that most plants cannot germinate under water was successfully exploited to fight weeds in the traditional farming system, where rice seedlings are transplanted into flooded paddy fields.

However, pressing climate change makes this natural way increasingly difficult, with water becoming increasingly scarce and unpredictable rainfall not always making a sustained water level in paddy fields possible anymore. The situation therefore requires a shift towards direct-seeding (Chauhan et al., 2015, 2017). Now weeds also infest rice fields, that are either managed by increased herbicide usage or by hand weeding. In this situation *Echinochloa curs-galli*, emerged as one of the most troublesome weeds (Chauhan & Johnson, 2009; Ottis & Talbert, 2007). It developed herbicide resistance (Heap, 2014) and flood tolerance and at early stages is visually almost indistinguishable from rice plants (Barrett, 1983), making it hard to eradicate even with manual weeding. This situation creates a strong need for a revised weed-management approach. Most weeds are highly sensitive to shade, which delays their development (Chauhan, 2012a; Chauhan & Johnson, 2010). Taking advantage of this, rice plants with increased shading capacity (Caton et al., 2003; Chauhan, 2013; Chauhan & Abugho, 2013a; Dingkuhn et al., 1999; Koarai & Morita, 2003; Mahajan & Chauhan, 2013; Zhao et al., 2006a) could be used as a sustainable solution for weed control. Together with other known weed-competitive traits of rice, such as early growth vigour (Dimaano et al., 2017; Haefele et al., 2004; Mennan et al., 2012; Namuco et al., 2009; Worthington & Reberg-Horton, 2013; Zhao et al., 2006a, 2007), we take this as a possible method, where weed-competitive rice can be incorporated in a combined weed-management protocol.

In Chapter 3 (Huber et al., 2021), we phenotyped a diversity panel of rice for a range of shoot architecture traits that contribute to shading potential of the different varieties. Based on this exercise, we calculated a Shading Rank for each of these varieties, and performed a Genome-Wide Association Study (GWAS) in order to identify genetic loci that could increase shading capacity. Here, we selected a subset of varieties from the screened diversity panel with a very high predicted shading potential, as well as several varieties with an average predicted shading potential. The main aim was to explore how increased shading of rice would work to effectively control weed growth. For this, we first confirmed in the greenhouse that weeds are suppressed via shading and next tested in the field, to confirm if predicted high shade-casting phenotypes of selected varieties, still show the same phenotype, with casting substantial shade early enough. We then investigated how rice suppresses weeds and if this is stronger in rice plants with a predicted high shading potential as compared to varieties that have lower predicted shading potential. In addition to involving variation in phenotypic traits for shading capacity, we also investigated the effectiveness of rice planting density for weed suppression, as well as their interaction.

Results

Is rice able to affect weed growth only by shading?

To validate our previously determined Shading Rank (Chapter 3) and assess functional shading capacity, we performed a greenhouse experiment with rice varieties that have varying Shading Rank and evaluated them for canopy shading. We selected two of the predicted high shading (Shim Balte and Mudgo with a Shading Rank of 344 and 340, respectively) and two predicted low shading rice varieties (Luk Takhar ranking 1 and Della ranking 49) (Table 1). The measurement of the light quantity under the canopies of selected varieties over time revealed a significant reduction under the canopy already after 18 days after sowing (das) of rice (Figure 4.1 A). Indeed, we observed a stronger shading by varieties with a high Shading Rank than by low ranking varieties from 21 das onwards (Figure 4.1 B). This result validates our Shading Rank, at least for the varieties tested and the selection of shoot architecture traits to effectively predict shade-casting.

By carrying out an experiment in pots, with weeds being separated from rice, we were able to study the exclusive effect of rice shoots on growth of a well-known rice weed (*Echinochloa crus-galli*), preventing any belowground interactions via the roots. With the set of two high shading (Mudgo and Shim Balte) and two low shading varieties (Luk Takhar and Della), allowed us to study the isolated effect of canopy shading and how their varying density would be reflected in weed performance of weeds growing below these different canopies. At four weeks of weed growth we saw a significant reduction of number of leaves and tillers, as well as root and shoot biomass of weed plants growing under shade-casting varieties (Figure 4.1 C-F). Although the Della variety was still quite weed-suppressive, the lowest ranking variety Luk Takhar, was not even able to affect weed growth in a significant manner compared to when weed was growing alone. Strongest shading, reflected as strongest reduction in PAR, corresponds to strongest impact on weeds (variety Mudgo) and vice versa, with Luk Thakar showing the lowest PAR reduction and lowest impact on weed growth traits. Although Shim Balte showed a similar PAR reduction as Mudgo did, the impact on weed performance was less strong, indicating that the PAR reduction and effect on weeds can also be partially different. These observations indicate that indeed rice canopies can suppress weed growth significantly and that variation exists between rice cultivars in their ability to do so.

Rice casts substantial shade already in early growth phase

Differences in shoot traits of rice varieties with varying shading capacity

A broader set of rice varieties with different Shading Ranks, ranging from very high to medium (Table 4.1) were grown in the field stations of the International Rice Research Institute (IRRI) in the Philippines. They were planted in field plots and monitored both with manual measurements on site, and with drones equipped with multiple cameras for imaging (Supplemental Figure 4.1).

Plant height (Figure 4.2 A-C) was less variable between the investigated varieties than tiller numbers (Figure 4.2 D-F). One variety, Var_J, was substantially shorter than all others, while having very strong tillering. Figure 4.2 D shows, that the later in the season the bigger the differences in tiller number between varieties become. Tiller development was already significantly different between varieties at 25 dat. Var_J had the most tillers and Var_H and Var_D the fewest (Figure 4.2 E). The overall pattern stayed the same at 40 dat, which was close to panicle initiation for most of the varieties and this was the maximum tillering stage. Here Var_J together with Var_A have the most tillers and Var_H and Var_D the fewest, ranging from 24.5 with the most and 10.2 tillers per plant as the fewest. Interestingly, at the same time-point of 25 dat, Var_H was actually one of the varieties with the highest dry weight. The ranking of varieties observed for tillering is not the same for shoot dry weight (Figure 4.2 G-H). At the end of vegetative stage (53 dat) the shoot dry weight ranges from 11.3 g to 21.3 g per plant (Var_D and Var_B, respectively). Var_B increased its biomass disproportionally more than the other varieties compared to the earlier time point.

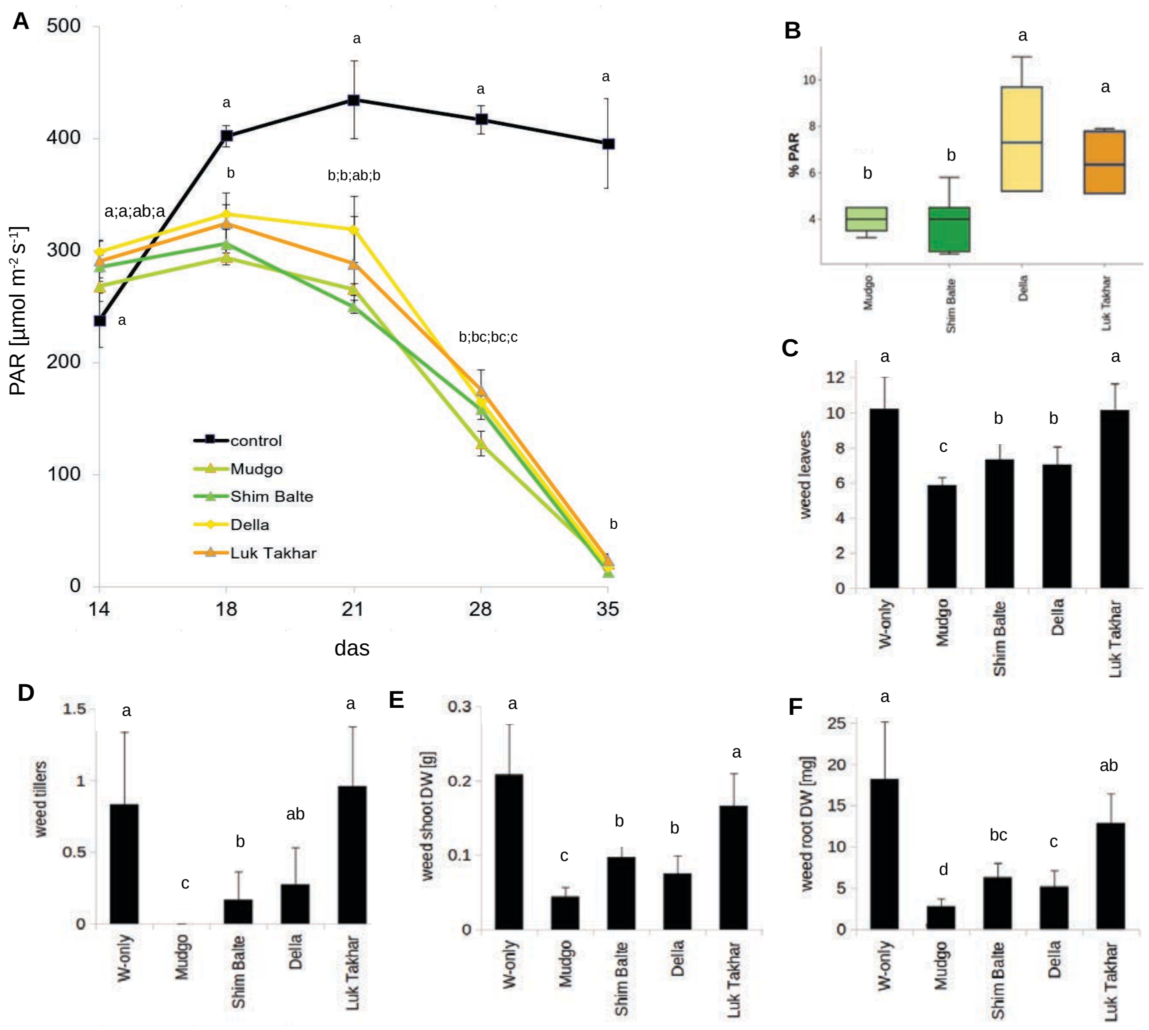


Figure 4.1. Shading Rank predicts canopy shading capacity of high- and low-ranking rice varieties and the effect of variable shading capacity on weed development. A. Absolute PAR (photosynthetic active radiation of 400-700 nm waveband as µmol m-2 s-1) measured over time at the ground level below developing canopies of four different rice varieties in rice-only plots. PAR measurements were recorded 14 days after sowing (das) of rice, which corresponds to the sowing time of the weed, for control (above canopies) n = 4, for below canopy of each rice variety n = 6, per timepoint. **B.** Significant difference in shading capacity between canopies of different rice varieties at 35 das of rice, shown as the reduction of light intensity (% PAR) at the ground level compared to above the canopy, for different rice varieties, where Della and Luk Takhar were classified as low shade-casting (orange) and Mudgo and Shim Balte as high shade-casting (green). Boxes indicate IQ-range with error bars of 2.5 – 97.5 percentile. **C. - F.** The effect of different rice varieties varying in shading capacity on weed C. number of leaves and D. tillers, E. shoot dry weight (DW) as [g] and F. root DW as [mg] at 42 das of rice, which is 28 das of weed. Rice and weed plants were grown in separate pots. Values are means of n = 24 plants with error bars ± SE. Letters indicate significant difference with $p < 0.05$ from Tukey post-hoc test following one-way ANOVA.

Table 4.1. Names and Shading Ranks of studied rice varieties, with their corresponding codes referred to in the results and their shading capacity, categorized by the Shading Rank determined previously (Chapter 3; Huber et al., 2021). The higher the value for the Shading Rank, the higher the predicted shading capacity, with 344 being the highest. Katihan 2 is an elite-breeding line, which was included in this field experiment, with undetermined Shading Rank. A set of four varieties with highest and lowest ranks were grown in the greenhouse experiment, for the field experiment varieties with very high and middle ranging ranks were included, and additionally one released breeding line with undetermined Shading Rank.

Variety	Code	Shading Rank	Greenhouse experiment	Field experiment
SHIM BALTE	Var_A	344	x	x
SABHARAJ	Var_B	339		x
SATHI	Var_C	336		x
MUDGO	Var_D	330	x	x
DM 56	Var_E	300		x
CRIOLLO LA FRIA	Var_F	267		x
BLACK GORA	Var_G	261		x
DJ 123	Var_H	144		x
WAB 501-11-5-1	Var_I	123		x
IR 64-21	Var_J	74		x
DELLA		49	x	
LUK TAKHAR		1	x	
KATIHAN 2	Var_K	na		x

Table 4.2. Description and abbreviation of different treatment groups in the field.

Abbreviation	Description	
[R]	rice in monoculture	Rice-only
[RW]	rice in mixed plots	mixed rice+weed
[W]	weed in monoculture	Weed-only
[R-HD]	rice in monoculture at high planting density	Rice-only at high density
[RW-HD]	mixed plots at high planting density	mixed rice-weed at high density

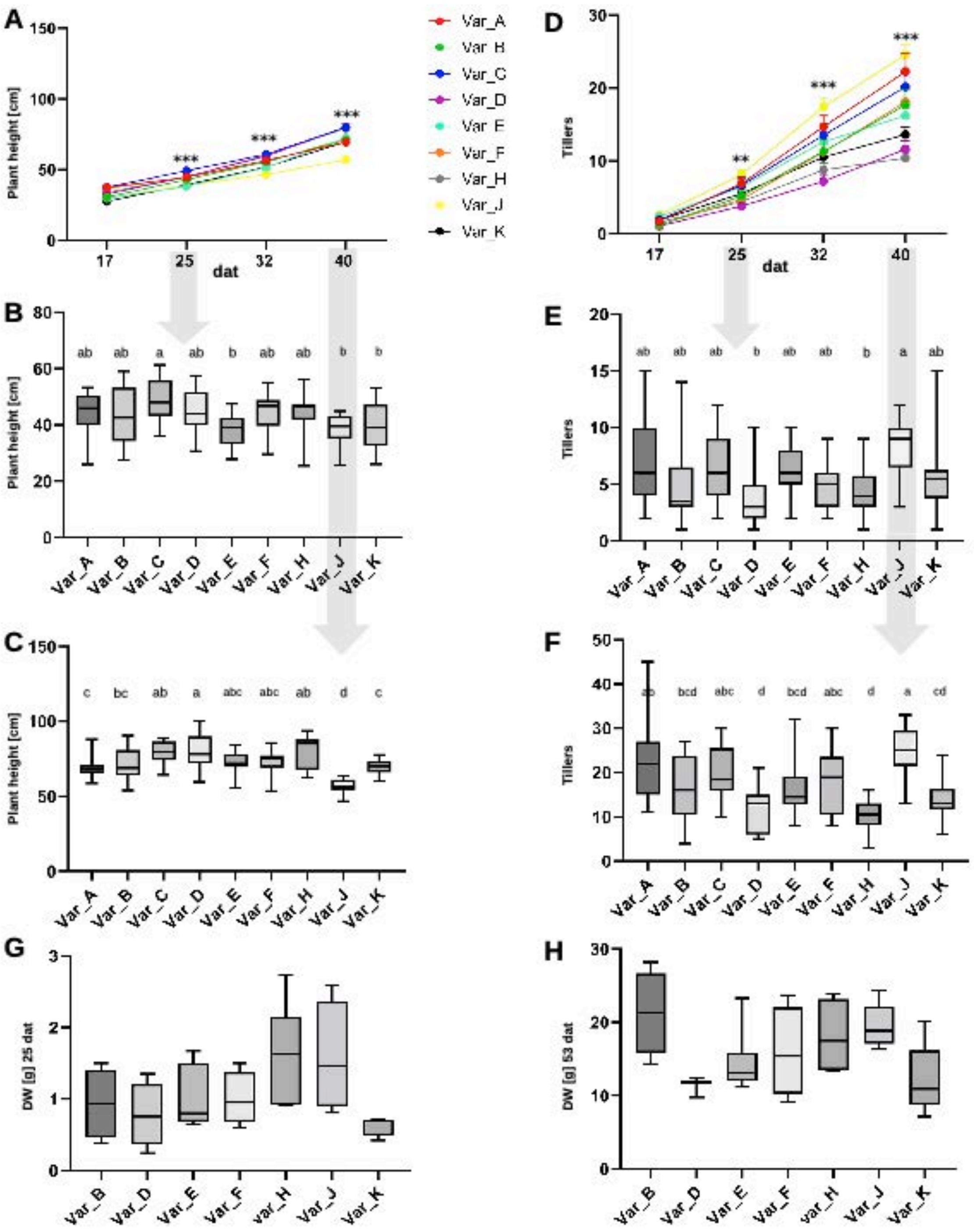


Figure 4.2. Phenotypic differences between rice varieties with varying shading capacity at normal planting density (20 x 20 cm, 25 plants m-2). **A - C.** Rice plant height [cm] A. followed over vegetative growth phase, with detailed differences between varieties at B. 25 and C. 40 dat. **D. - F.** Number of tillers per rice plant D. followed over the time of vegetative growth phase, with detailed differences between varieties at E. 25 and F. 40 dat. For tiller number and plant height, Var_G and Var_I were excluded, because of poor seedling establishment. **G. - H.** Rice shoot dry weight (DW) [g] for different varieties at G. 25 and H. 53 dat. For shoot DW Var_A, Var_C, Var_G and Var_I were excluded. In A. and D. significant differences are indicated with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; for others: letters indicate significant differences with $p < 0.05$ from Tukey post-hoc test following one-way ANOVA. N = 18 plants (3 plots with 2 sampling locations with 3 plants each), min 5 sampling locations. Values are means, boxes indicate IQ-range with error bars of 2.5 – 97.5 percentile. Var – different rice varieties.

Canopy traits and time to reach canopy closure

Time to canopy closure, was inferred from the data for plot canopy cover (Figure 4.3 A-B). For the time to reach 50 % canopy closure the fastest variety (Var_C) took 25.2 days after transplanting (dat) and the slowest (Var_E and Var_K) 30.5 dat. To reach full canopy closure (99 % in our analysis) the range from fastest (Var_D) to slowest (Var_K) variety is from 41.5 to 47.7 dat. Although days to canopy closure of 50 and 99 % correlate, (r = 0.6*, Supplemental Figure 4.3) there are some changes in ranking of the varieties. This indicates that the variety reaching 50 % closure very rapidly is not necessarily also fastest in completely closing the canopy. An example for this is Var_C, which is the fastest reaching 50%, but then takes relatively long to close the canopy fully. Interestingly, the released breeding line Var_K takes the longest to close the canopy. The predicted high shading varieties (Var_A – Var_D) are also among the fastest to reach canopy closure, with Var_D and Var_B being the fastest. However, there was considerable variation in terms of Shading Rank and rate of canopy closure, namely Var_F closing fast but mid-ranking and Var_C ranking high, but taking longer for canopy closure. The triangular green index (TGI), which is based on RGB imagery, together with NDVI using multispectral reflectance imagery, record chlorophyll reflectance, together combine information about canopy cover by photosynthetically active tissue. Thus, although varieties with a very high Shading Rank also have a fast canopy canopy closure, the varieties with an intermediate Shading Rank are much more variable for their respective canopy closure. For the TGI (Supplemental Figure 4.3 A), Var_E clearly seems to be performing the worst, and also Var_K is lower than the rest. These two are also the ones that took the longest for closing the canopy. Var_A and Var_B show the highest values for TGI and are the predicted highest ranking varieties for shading potential.

The average canopy height of a plot (relative plot height) (Figure 4.3 C) measured by the drone, clearly showed Var_H, Var_G and Var_D reaching the highest and Var_J by far the shortest height. This could also be seen for individual plant height (Figure 4.2 D). Generally, tiller number and dry weight are positively correlated (Figure 4.4), and only Var_H presents an exception, as seen in Figure 4.1 B and G. Plant height is strongly negatively correlated with tiller number and shoot biomass, suggesting a trade-off between resource allocation towards branching or height growth. Having established these general descriptions of rice monocultures with different architectures, we next set out to experimentally determine if these architectures are also truly associated with differential weed performance in such rice plots.

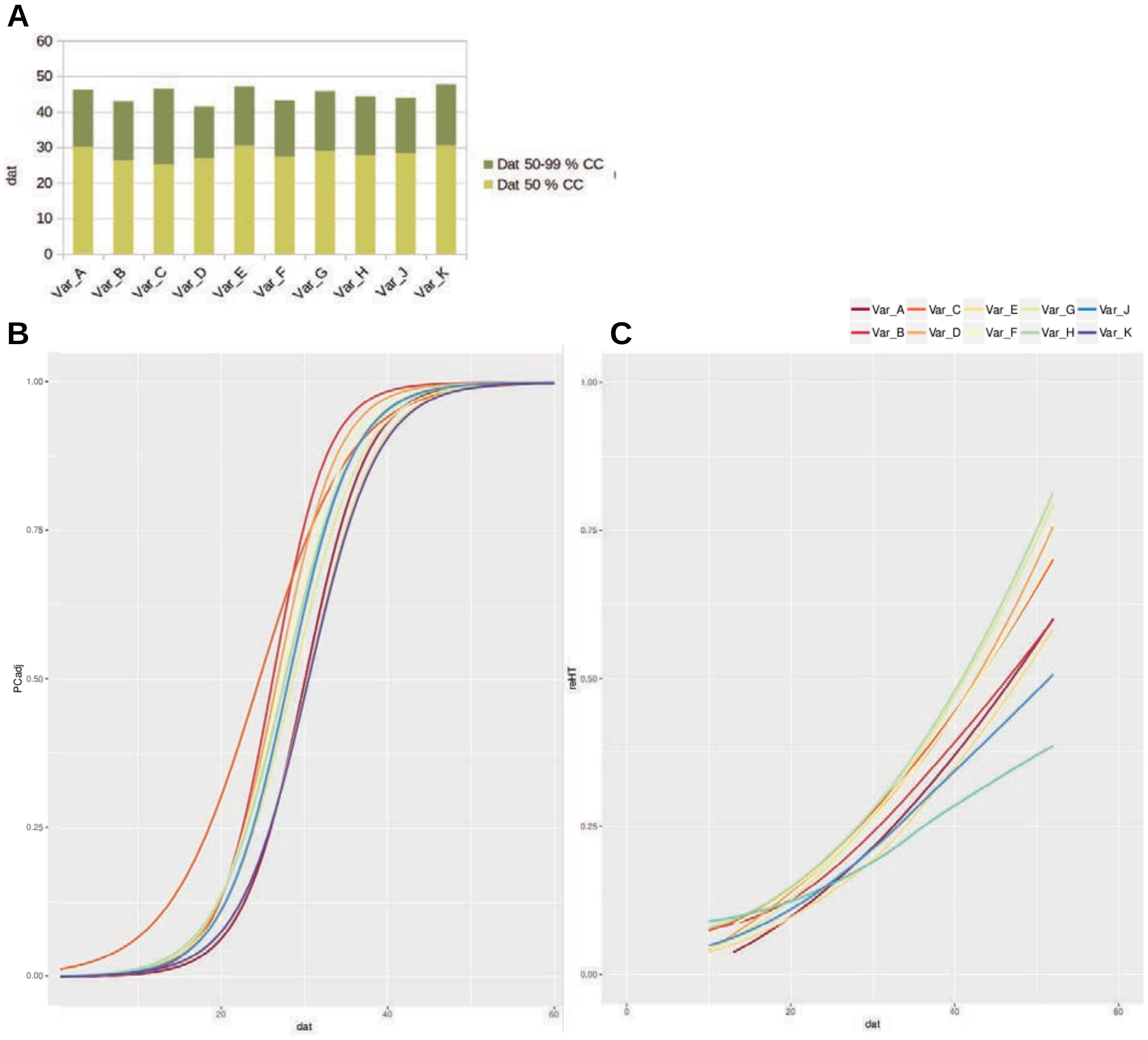
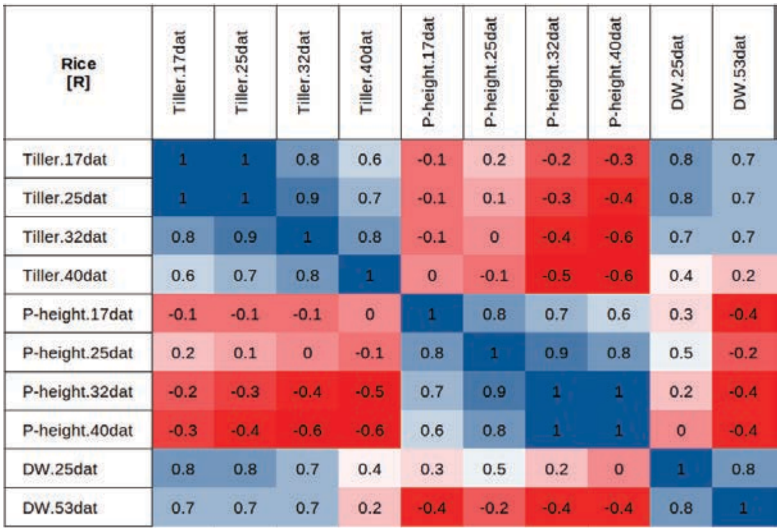


Figure 4.3. Canopy traits and time to reach canopy closure for different rice varieties planted at normal density (20 x 20 cm, 25 plants m-²), monitored with a drone from day of transplanting until 53 days after transplanting (dat). **A.** Time to reach 50% and 99 % canopy closure based on a general linear model fitted to PCadj. **B.** Plot canopy cover adjusted to maximum cover of a plot (PCadj) and **C.** relative plot canopy height (reHT). Var_I was excluded due to poor seedling establishment. Canopy height is based on RGB colour imagery and Plot canopy cover is calculated using indices based on RGB and multispectral camera imagery. Var – different rice varieties.



Rice [R]	Tiller.17dat	Tiller.25dat	Tiller.32dat	Tiller.40dat	P-height.17dat	P-height.25dat	P-height.32dat	P-height.40dat	DW.25dat	DW.53dat
Tiller.17dat	1	1	0.8	0.6	-0.1	0.2	-0.2	-0.3	0.8	0.7
Tiller.25dat	1	1	0.9	0.7	-0.1	0.1	-0.3	-0.4	0.8	0.7
Tiller.32dat	0.8	0.9	1	0.8	-0.1	0	-0.4	-0.6	0.7	0.7
Tiller.40dat	0.6	0.7	0.8	1	0	-0.1	-0.5	-0.6	0.4	0.2
P-height.17dat	-0.1	-0.1	-0.1	0	1	0.8	0.7	0.6	0.3	-0.4
P-height.25dat	0.2	0.1	0	-0.1	0.8	1	0.9	0.8	0.5	-0.2
P-height.32dat	-0.2	-0.3	-0.4	-0.5	0.7	0.9	1	1	0.2	-0.4
P-height.40dat	-0.3	-0.4	-0.6	-0.6	0.6	0.8	1	1	0	-0.4
DW.25dat	0.8	0.8	0.7	0.4	0.3	0.5	0.2	0	1	0.8
DW.53dat	0.7	0.7	0.7	0.2	-0.4	-0.2	-0.4	-0.4	0.8	1

Figure 4.4. Correlation of different shoot traits in rice monoculture [R] grown at normal density (20 x 20 cm, 25 plants m-²). Tiller number per plant (tiller) and plant height (p-height) were manually recorded at 17, 25, 32 and 40 days after transplanting (dat). Shoot biomass was recorded as dry weight (DW) at 25 and 53 dat.

Rice impacts weed growth

To investigate the effect that rice has on weed (*Echinochloa crus-galli*), we compared the weed growing alone in its monoculture without rice, to when it was growing together with different rice varieties.

Using the earlier mentioned drone imaging, we followed the development of rice-only, weed-only and mixed rice-weed plots over time and determined the canopy cover (Figure 4.5 A). These data show that mostly, the combined rice-weed plots close faster than either of the monocultures, which was expected given that the number of plants in the mixture is higher than in either of the monocultures. Early on in plot development, the weed-only plots are always behind, following from these being planted later than the rice. For Var_B the mixed rice-weed plot followed the trajectory of the rice-only, but a bit faster, so the performance of the mixture is mostly being determined by the rice. Var_G is an example where the rice loses against the weed I n the mixed plots, inferring from the weed-monoculture growing very similarly to the mixed plots. We also determined the average canopy height of the different plots from the drone-made images (Figure 4.5 B). Overall, we observed that plot canopy height of the rice-weed mixtures closely followed that of the rice monocultures. This pattern existed even in rice-weed combinations where the rice was relatively short and weeds in the weed monocultures could attain a much greater height themselves. For example, rice Var_J monocultures remain much shorter than weed monocultures. Nevertheless, the rice Var_J – weed mixture never reaches the height that we observe in weed monocultures, but stay at the height of rice. This could indicate that the weeds are suppressed and cannot reach their full potential.

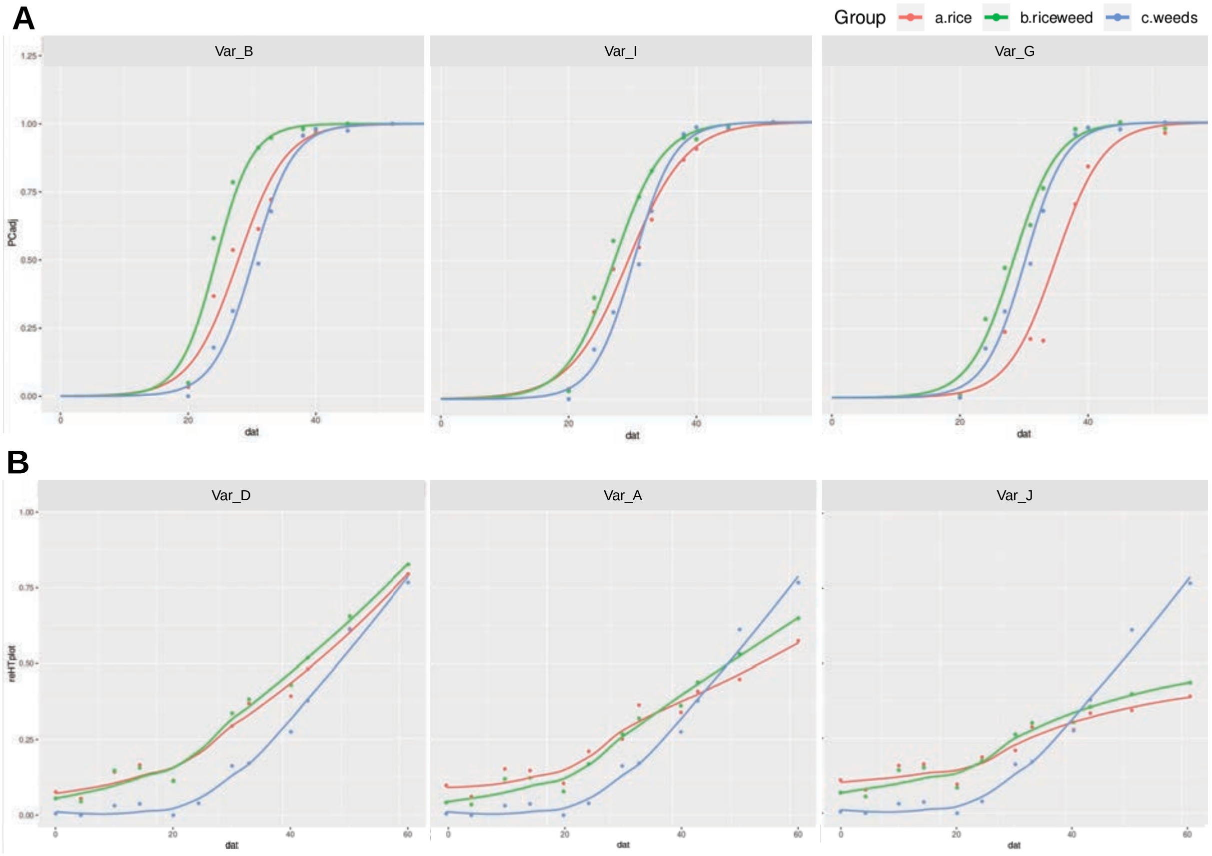


Figure 4.5. Canopy development of different rice varieties in monoculture, compared to weed monoculture and mixed plots grown at normal density (20 x 20 cm, 25 plants m-²), monitored with a drone from day of transplanting until 53 days after transplanting (dat). **A.** Plot canopy cover adjusted to maximum cover of a plot (PCadj) for representative varieties with fast (Var_B), medium (Var_I) and slow (Var_G) canopy cover in rice monoculture and the effect of the trajectory of mixed plots with rice and weeds. **B.** Canopy height (relative plot height, reHT) for representative varieties with tall (Var_D), medium (Var_A) and short (Var_J) canopies in rice monoculture and the effect of the trajectory of mixed plots with rice and weeds. Canopy height is based on RGB colour imagery and Plot canopy cover is calculated using indices based on RGB and multispectral camera imagery. Red – rice monoculture, green – mixed rice+weed, blue – weed monoculture, Var – different rice varieties.

To get more detailed insights into the precise compositions and plant performance in the different plots, we also did manual measurements and harvests. To quantitatively evaluate the impact of rice on weed performance, we assessed the impact on weed tiller number, %-weed cover and weed biomass (Figure 4.6 A-C). Generally, the weed tiller numbers were significantly reduced at the last timepoint of 53 dat, by the presence of rice (Figure 4.6 A). There were also significant differences between rice varieties in the extent to which they suppressed the weed.

The tiller number in weed growing alone was 9.3, when growing together with rice it was reduced significantly, ranging from 5 in Var_F to 3.5 in Var_C. Using the beaded string method, the percentage of ground cover was established (Figure 4.6 B). This method involves detecting at every 20 cm in the canopy from one side to the other if there is a weed and or rice leaf on that position. This is a measure of how much rice compared to weed there is in the total covered area. We observed that in the mixed plots, weed contribution ranged from 32 % in Var_K to 18.5 in Var_D, confirming a major effect of rice on weed proliferation, as observed for weed tillers.

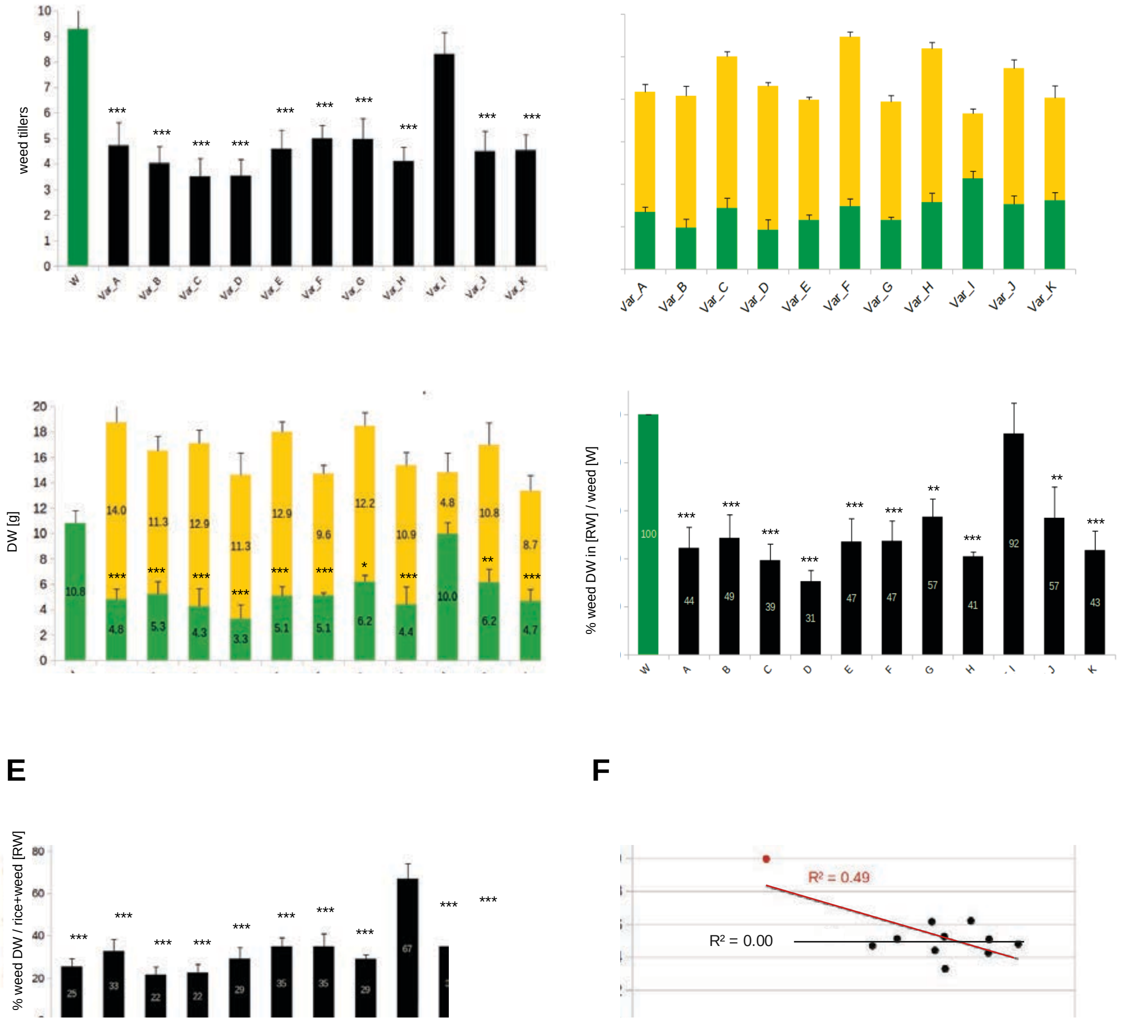


Figure 4.6. Rice impact on weed development evaluated on weeds grown with rice at normal density (20 x 20 cm, 25 plants m-²). **A.** Number of tillers per weed plant at 49 dat of rice. **B.** % cover of weed (green) and rice (yellow) at 45 dat. **C.** Shoot dry weight (DW) per plant of weed (green) and rice (yellow) in same plot [RW] at 53 dat. **D.** % Weed dry weight in mixed plots compared to weed monoculture as % weed DW in [RW] / in [W] and **E.** % Weed dry weight of total plot biomass in mixed plots as % weed DW in [RW] / (rice+weed) in [RW] with weed monoculture in green and weed in mixed plots in black. **F.** ▷

The quantitative weed suppression was determined by measuring weed dry weight (Figure 4.6 C). Growing alone, the weed accumulated a biomass of 10.8 g. In the mixed plots with rice, it was reduced from 6.2 g in Var_G to only 3.1 g in the presence of Var_D. Var_D, Var_C and Var_H gave the strongest suppression of weed growth in terms of weed biomass compared to weed biomass in its monoculture without rice (Figure 4.6 D). A different pattern emerges for Var_I: the weed-only plots grow nearly as well as the weeds in the mixed plots, indicating that the weeds are hardly affected by rice Var_I. When considering the percentage of biomass of the weed compared to the total biomass of mixed plots (Figure 4.6 E), weeds perform worst in the presence of rice Var_C and Var_D (22 % each) and best in the presence of rice Var_I (67 %). When overall comparing rice to weed biomass in the mixed plots (Figure 4.6 F), the correlation is strongly influenced by one variety (Var_I); if this variety is excluded, the correlation is lost. This is consistent with the idea that other rice traits than just biomass determine weed suppression.

Weed suppression is not only explained by shading from rice

Rice canopy cover suppresses weed

To see what contributes most to weed suppression, we correlated rice traits to weed traits (Figure 4.7 A). Final biomass of rice correlated strongly with final %-rice cover ($r = 0.7$) and final weed biomass correlated with %-weed cover ($r = 0.6$). The final weed biomass as well as the fraction weed biomass compared to the total plot biomass are strongly negatively correlated with %-rice cover, and this correlation is strongest early in the season (from $r = -0.8^{***}$ at 17 dat to $r = -0.6^{**}$ at 32 dat, box Figure 4.7 A). Rice varieties showing stronger cover specifically at early dates, corresponding to more weed suppression (Figure 4.7 B-C). Interestingly, the time to 50 % and complete canopy closure of rice monocultures does not correlate with weed dry weight in mixed plots (Figure 4.7 D-E). Correlations between early, mid and late %-rice ground cover and % weed dry weight as % compared to total DW of mixed plots are shown in Supplemental Figure 4.4 A - B and late weed DW in mixed plots in Supplemental Figure 4.4 C-D.

Correlation of weed and rice DW at 53 dat in mixed plots, with regression for all varieties in red and Var_I excluded in black. N = 18 plants (3 plots with 2 sampling locations with 3 plants each), min 5 sampling locations. Values are means with error bars ± SE. Significant differences are indicated with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ from Tukey post-hoc test following one-way ANOVA. [RW] - mixed rice-weed plots, [W] - weed monoculture, W - weed monoculture, Var – weed in plots of different rice varieties.

In Supplemental Figure 4.4 E, predicted shading capacity, based on the Shading Rank of the screened diversity panel, is shown in relation to the effect on weed growth for the varieties included in this field study. As a comparison to the monitored ground cover and canopy cover in the field, also individual shoot area at early vegetative stage determined in an earlier screening on individually grown plants (Chapter 3) is shown in relation to weed biomass (Supplemental Figure 4.4 F).

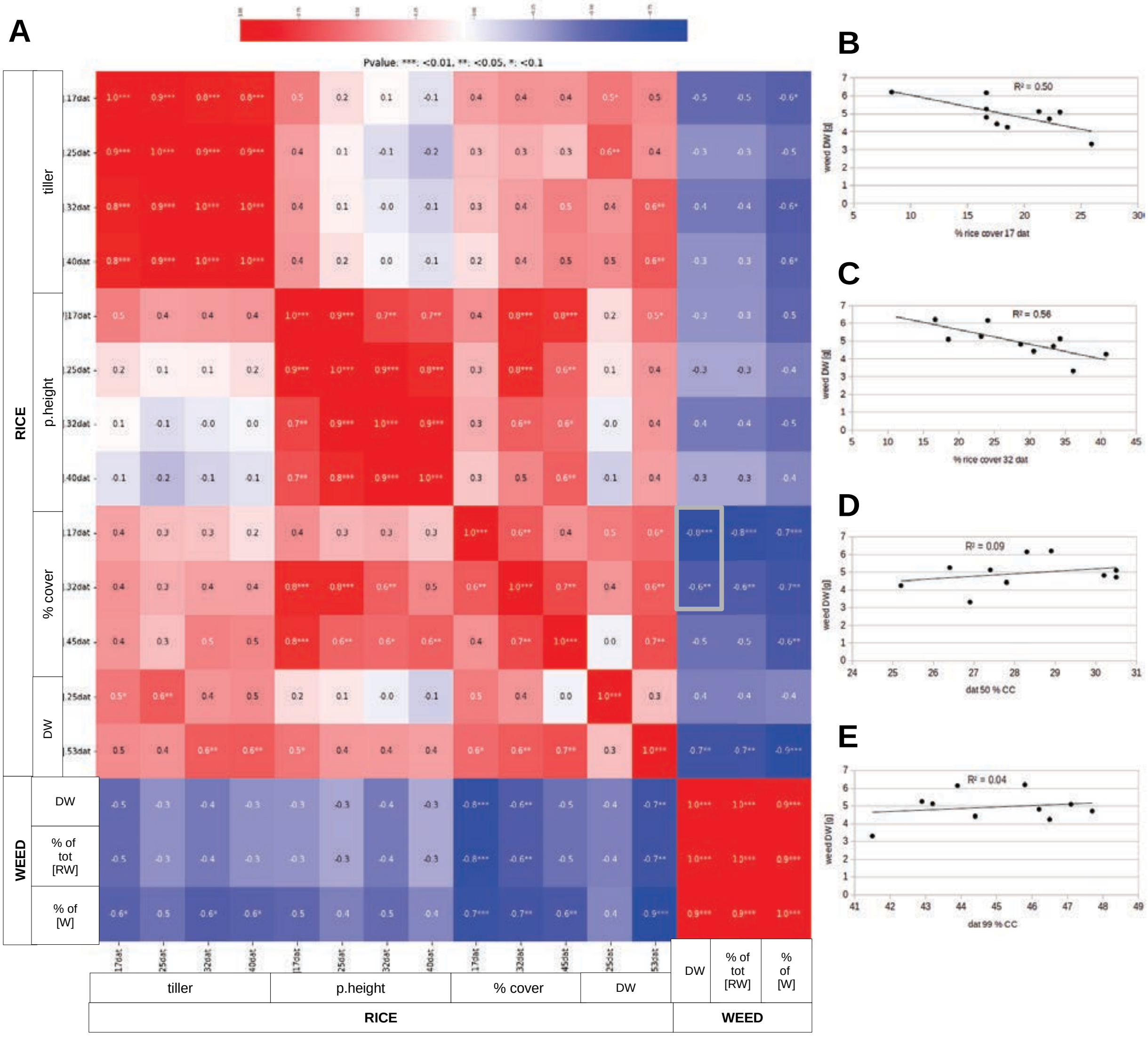
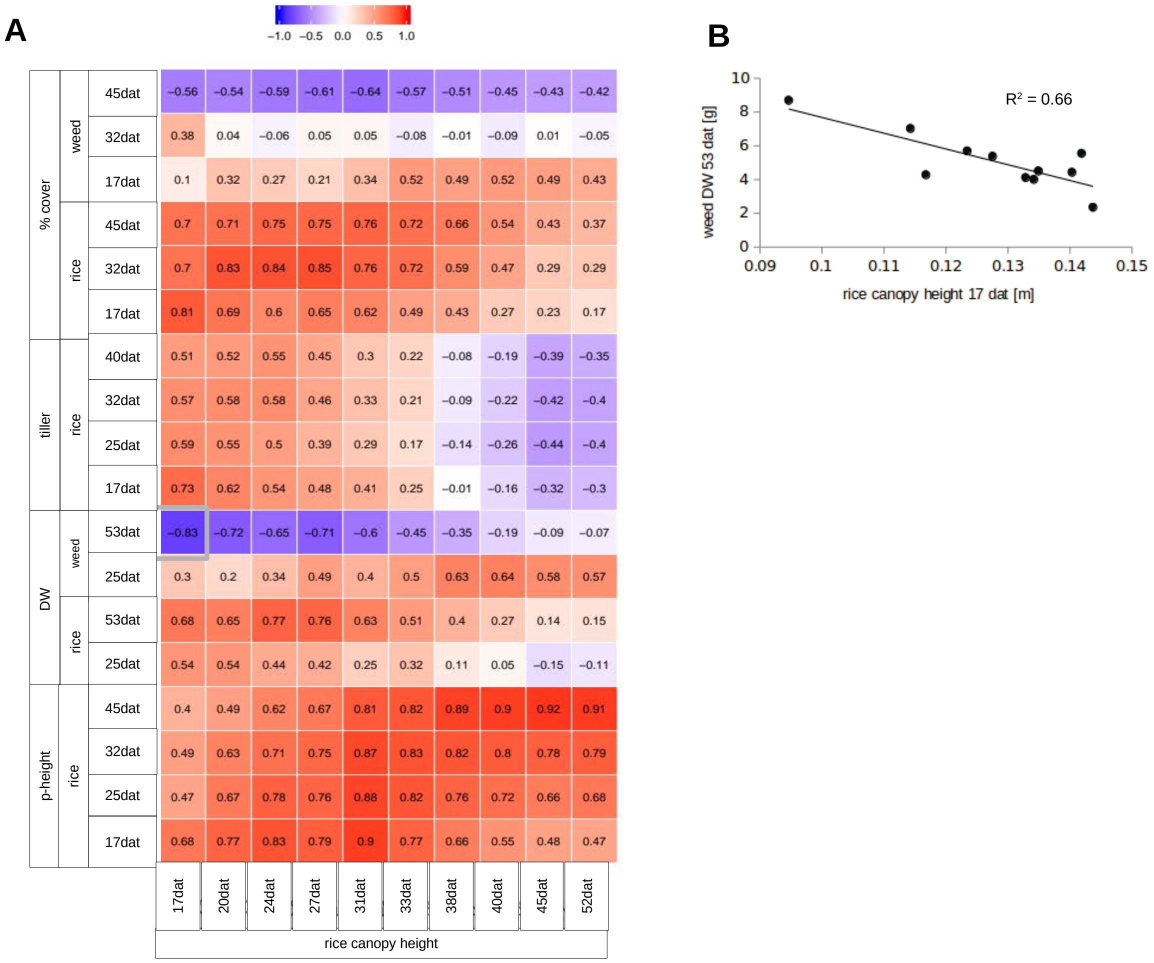


Figure 4.7. Impact of rice shading and other shoot traits on weed growth. A. Correlation matrix of rice traits: number of tillers and plant height at 17, 25, 32, 40 dat and %-rice cover at 17, 32 and 45 dat and rice DW at 25 and 53 dat in mixed rice+weed plots [RW] correlated with weed DW at 53 dat, % weed dry weight of total plot biomass in mixed plots as % of tot [RW] and % weed DW of weed monoculture as % of [W] at 53 dat. **B. - E.** Early shading contributes to stronger weed suppression. Scatter plots of B. early (17 dat) and C. mid (25 dat) %-rice cover with late (53 dat) weed DW, highlighted with box in A. and based on same data. Scatter plots with time to D. 50 % and E. 99 % canopy closure of rice monoculture [R] with late (53 dat) weed DW in [RW]. For B – E, Var_I was excluded.

Impact of rice shoot traits on weed performance

In addition to shading capacity quantified in terms of % canopy cover, rice tiller number is strongly negatively correlated with end weed biomass (r = -0.5 to -0.3 from early to later time points) (Figure 4.7 A). Rice dry weight is negatively correlated with end biomass of weed and even stronger with weed fraction (Figure 4.7 A). The data for development of canopy height (Figure 4.8 A) show, that differences in early season canopy height of different plots (reHT) are strongly correlated with %-rice cover, especially early in the season. Canopy height most strongly negatively correlates with late weed %-cover, and negatively correlated with late season weed biomass (53 dat) (Figure 4.8 B). The negative correlation with final weed biomass, was also seen for individual plant height (Figure 4.7). Together, this indicates that early rice canopy height negatively impacts weed performance later in the season.



			17dat	20dat	24dat	27dat	31dat	33dat	38dat	40dat	45dat	52dat
% cover	weed	45dat	−0.56	−0.54	−0.59	−0.61	−0.64	−0.57	−0.51	−0.45	−0.43	−0.42
		32dat	0.38	0.04	−0.06	0.05	0.05	−0.08	−0.01	−0.09	0.01	−0.05
		17dat	0.1	0.32	0.27	0.21	0.34	0.52	0.49	0.52	0.49	0.43
	rice	45dat	0.7	0.71	0.75	0.75	0.76	0.72	0.66	0.54	0.43	0.37
		32dat	0.7	0.83	0.84	0.85	0.76	0.72	0.59	0.47	0.29	0.29
		17dat	0.81	0.69	0.6	0.65	0.62	0.49	0.43	0.27	0.23	0.17
tiller	rice	40dat	0.51	0.52	0.55	0.45	0.3	0.22	−0.08	−0.19	−0.39	−0.35
		32dat	0.57	0.58	0.58	0.46	0.33	0.21	−0.09	−0.22	−0.42	−0.4
		25dat	0.59	0.55	0.5	0.39	0.29	0.17	−0.14	−0.26	−0.44	−0.4
		17dat	0.73	0.62	0.54	0.48	0.41	0.25	−0.01	−0.16	−0.32	−0.3
DW	weed	53dat	−0.83	−0.72	−0.65	−0.71	−0.6	−0.45	−0.35	−0.19	−0.09	−0.07
		25dat	0.3	0.2	0.34	0.49	0.4	0.5	0.63	0.64	0.58	0.57
	rice	53dat	0.68	0.65	0.77	0.76	0.63	0.51	0.4	0.27	0.14	0.15
		25dat	0.54	0.54	0.44	0.42	0.25	0.32	0.11	0.05	−0.15	−0.11
p-height	rice	45dat	0.4	0.49	0.62	0.67	0.81	0.82	0.89	0.9	0.92	0.91
		32dat	0.49	0.63	0.71	0.75	0.87	0.83	0.82	0.8	0.78	0.79
		25dat	0.47	0.67	0.78	0.76	0.88	0.82	0.76	0.72	0.66	0.68
		17dat	0.68	0.77	0.83	0.79	0.9	0.77	0.66	0.55	0.48	0.47

rice canopy height

Figure 4.8. Rice canopy height is important for weed suppression. A. Correlation matrix of rice canopy height in rice monoculture [R] with traits of rice and weed in mixed plots [RW] (%-weed and %-rice cover at 17, 32 and 45 dat, number of rice tillers at 17, 25, 32 and 40 dat weed and rice DW at 25 and 53 dat and rice plant height at 17, 25, 32 and 40 dat. **B.** Scatter plot showing correlation between early canopy height and late weed biomass, highlighted with rectangle in correlation matrix and based on same data.

Impact of increased planting density on weed performance

With increased planting density of rice, also weed performance is suppressed significantly, evaluated by tiller numbers and shoot dry weight (Figure 4.9 A-B). At normal density weed biomass reduction ranged from 10 to 3.3 g weed shoot biomass, due to different rice varieties (Figure 4.6 C). At higher density the range was only 5.6 to 1.7 g weed biomass (Figure 4.9 B). When looking at weed biomass under rice competition relative to weed in monocultures, the suppressive effect of rice becomes even more apparent (Figure 4.9 C). However, when taking the weed biomass relative to the rice biomass in the same plots, then the effect does not become stronger (Figure 4.9 D), which is different at low density (Figure 4.6 E). This indicates, that rice itself is affected by increased density and does not accumulate that much more biomass.

To quantify whether the suppressive effect is mainly determined by rice variety or by planting density or the interaction of both, we performed a two-way Anova on % weed dry weight with rice variety and planting density as factors. The results show, that the main effects of density and variety are significant in their effect on weed growth, with variety even more significant. Importantly, there is no interaction effect between these two, meaning that high density suppresses weed, for all varieties and vice versa, that the differences in varieties are significant for weed reduction independent of planting density (Figure 4.9 D - box).

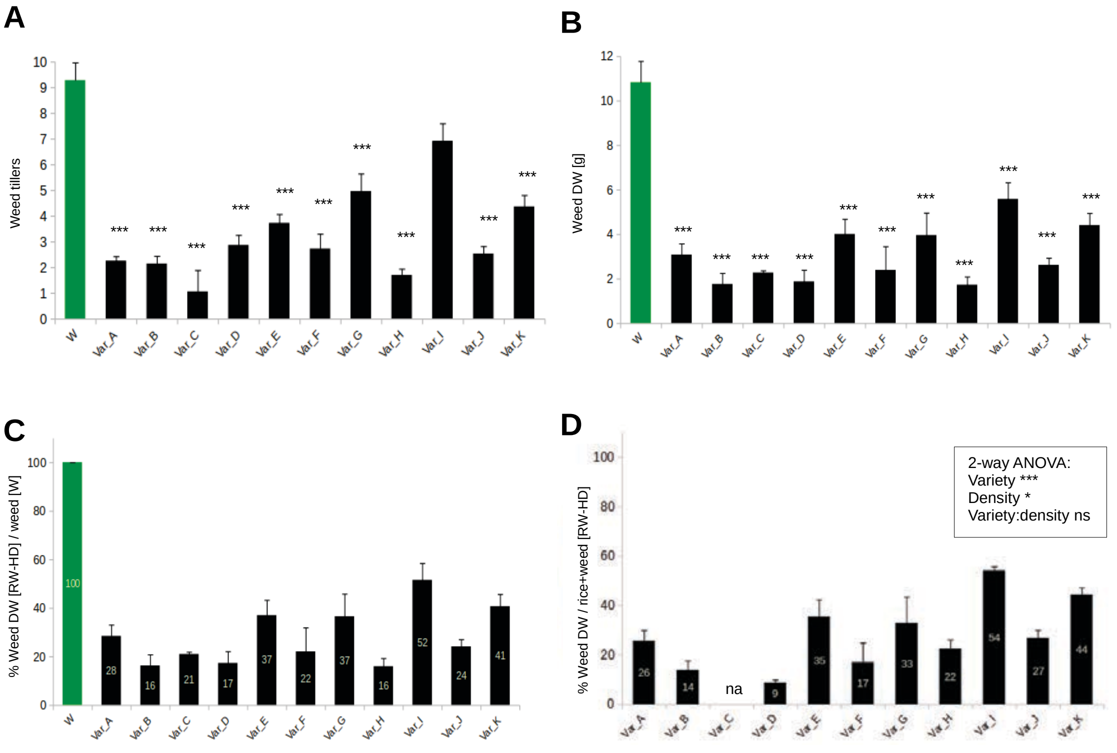


Figure 4.9. Increased rice planting density suppresses weed, evaluated on weed traits grown with rice at high density (HD, 10 x 20 cm, 50 plants m-²). **A.** Number of tillers per weed plant at 49 dat of rice planted at high density. **B.** Weed shoot dry weight (DW) per plant in mixed plots at high density [RW-HD]. **C.** % Weed DW in mixed plots compared to weed monoculture as % weed DW in [RW-HD] / weed in [W]. **D.** % Weed DW of total plot biomass in mixed plots as % weed DW / rice+weed in [RW-HD] with weed in monoculture in green and weed in mixed plots in black; box: main and interaction effect of rice variety and increased planting density compared to normal density (shown in Figure 4.6 C) on weed DW at 53 dat of rice. N = 36 plants (3 plots with 2 sampling locations with 6 plants each), min 5 sampling locations. Values are means with error bars ± SE. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ from Tukey post-hoc test following one-way ANOVA. [RW-HD] - mixed rice-weed plots at high density, [W] - weed monoculture; W - weed monoculture, Var – different rice varieties, na – not available.

Increased planting density negatively affects rice growth

Plant height on average over all rice varieties is increased at higher rice planting density already at 32 dat as compared to normal density (Supplemental Figure 4.5 A-C), even though this effect is only significant for some varieties (Figure 4.10 A), and there indeed is a significant interaction between variety and density (at 40 dat). At 40 dat all varieties had less tillers, for most of them significantly less as compared to normal density. Var_A and Var_J show the largest decrease in tillers, whereas tillering in Var_H and Var_D was less affected (Figure 4.10 B). Starting from 32 dat, on average plants produce significantly fewer tillers (Supplemental Figure 4.5 D-F) when grown in high density.

In the very early stage (at 25 dat) shoot biomass was not affected by increased planting density (Supplemental Figure 4.5 G-H). However, four weeks later, at 53 dat, each of the investigated varieties showed significantly less shoot biomass at high density compared to normal density (Figure 4.10 C) with Var_B and Var_J being most severely affected. At increased planting density, rice plants showed lower tillering and lower biomass but were taller, thus becoming spindlier. Collectively, these data show that at double planting density, the rice plants indeed are suppressed in their individual performance. However, the extent of the negative effect of increased planting density, depends on the variety but not significantly different between varieties (Figure 4.10 C, interaction effect of two-way Anova).

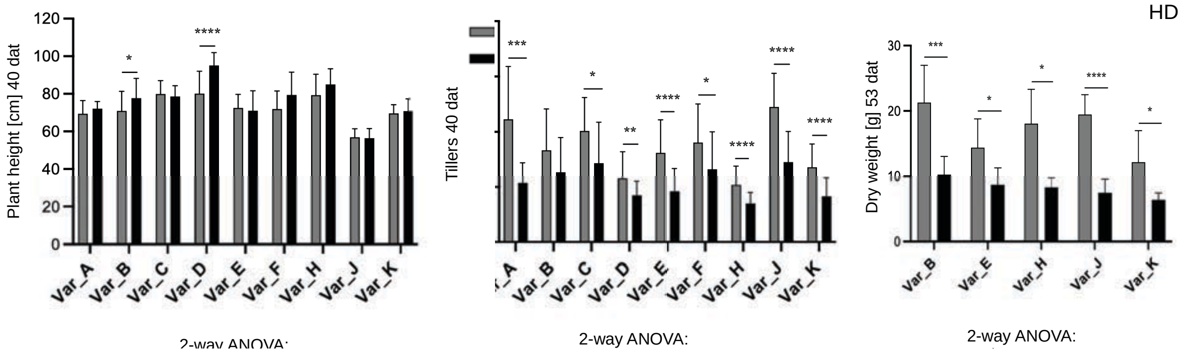


Figure 4.10. Impact of increased planting density on rice performance. Phenotypic differences between different rice varieties planted at high density (HD, 10 x 20 cm, 50 plants m-²) compared to normal density (ND, 20 x 20 cm, 25 plants m-²). **A.** Rice plant height [cm] at 40 days after transplanting (dat) at ND and HD. **B.** Number of tillers per rice plant at 40 dat at ND and HD. For plant height and tiller number Var_G and Var_I were excluded, because of too few replicates. **C.** Rice shoot dry weight [g] at 53 dat at ND and HD. For shoot dry weight Var_A, Var_C, Var_D, Var_F, Var_G and Var_I were excluded, ▶

Plot canopy cover (PCadj from drone) at low density shows that there is a relatively large variance between varieties to reach 50 % but then they converge towards full canopy closure (99 %) (Figure 4.11 A). This means that at a lower planting density, choosing the right variety has a bigger impact on time to reach canopy closure especially in the crucial early vegetative phase (up to four weeks after transplanting) than at higher density. Indeed, at high density, 50 % canopy closure is reached a few days earlier than at normal density (Figure 4.11 B), but interestingly the varieties then vary more in their time it takes to reach the 99 %. This larger variation between varieties in later stage canopy development at high density might indicate that plants are already, differentially, affected by their closer neighbouring plants. Additionally, the TGI shows, that plants at normal density seem to have more photosynthetically active tissue, hinting at healthier plants than at increased planting density (Supplemental Figure 4.2 B).

When planted at higher density, the faster development of the rice canopy follows from double the number of plants in a plot (Figure 4.11 C). This is true for all varieties to reach 50 % canopy closure, however the time to reach complete canopy closure is actually even slowed down for some varieties in higher density. Interestingly, time to reach 50 % canopy closure (light green bars) is proportionally much more reduced with higher planting density, than the time to reach complete canopy cover. For all varieties, the time to 50 % CC is reduced, but then the days needed to reach complete canopy closure is actually increased for all varieties except Var_F, Var_J and Var_K, which are all rather low shading varieties (Figure 4.11 D). Figure 4.11 E shows the reduction in days for total time to canopy closure in high density, which is reduced for all varieties except for Var_B.

because of too few replicates. Main and interaction effects of rice variety and increased planting density on rice are indicated by two-way ANOVA, results below each graph, for each trait respectively. N = 36 plants (3 plots with 2 sampling locations with 6 plants each), min 5 sampling locations. Values are means with error bars ± SE. Significant differences indicated with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ from Tukey post-hoc test following one- or two-way ANOVA. Var – different rice varieties.

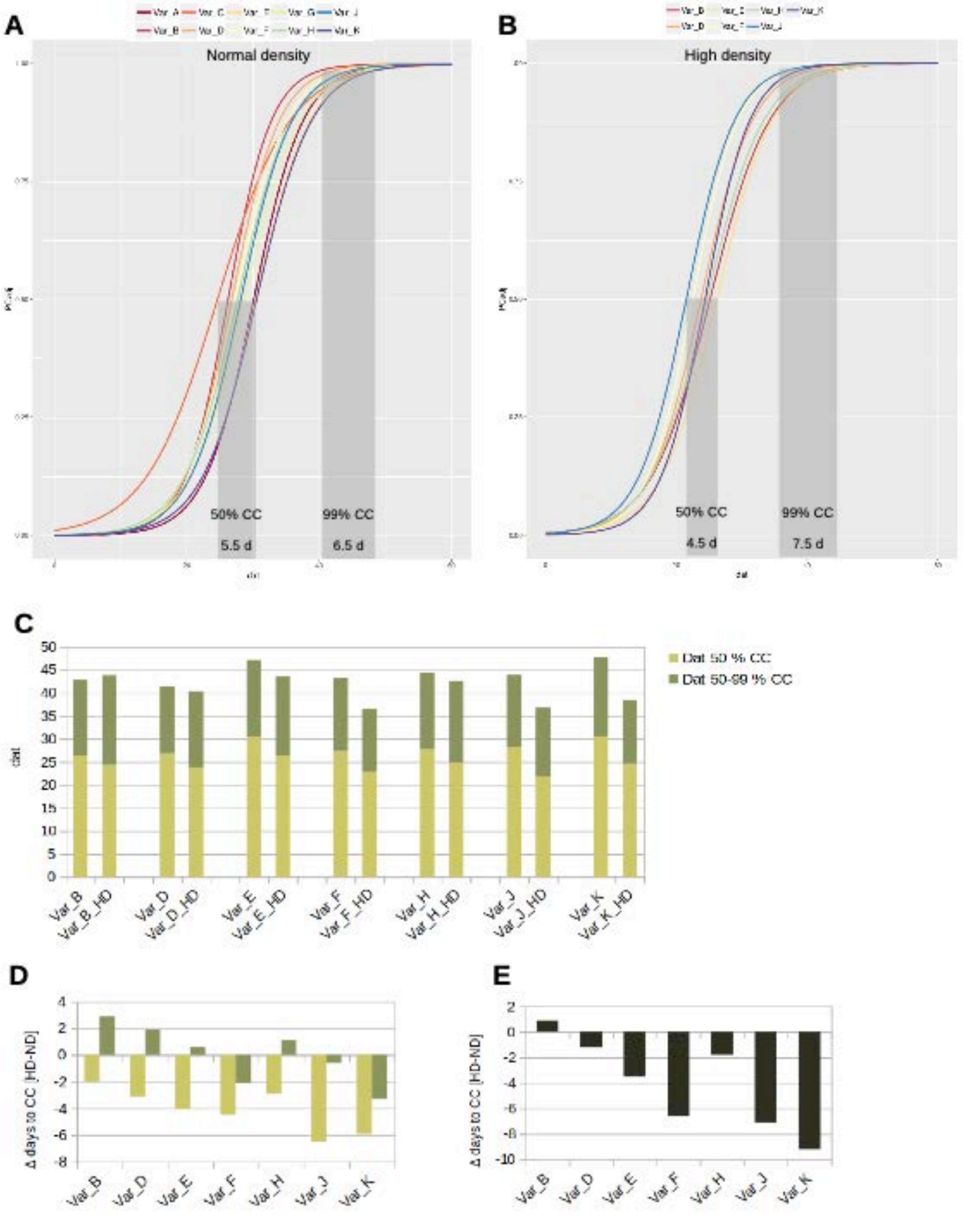


Figure 4.11. Density effect on canopy cover and time to reach canopy closure on different rice varieties planted at high density (HD, 10 x 20 cm, 50 plants m-²) compared to normal density (ND, 20 x 20 cm, 25 plants m-²), monitored with a drone from day of transplanting until 53 days after transplanting (dat). **A - B.** Plot canopy cover adjusted to maximum cover of a plot (PCadj) at normal (A) and high density (B). Lines are based on a sigmoid curve fit. **C. - E.** Time to reach 50 % and 99 % canopy closure for different rice varieties based on a general linear model fitted to PCadj, with C. number of days, D. difference of high to normal density separated for days to reach 50 % and 99 % canopy closure and E. the reduction of days to reach total canopy closure at HD compared to ND. Var_A, Var_C, Var_G and Var_I were excluded due to poor seedling establishment at high density. Var - different rice varieties.

Discussion

The aim of this study was to assess the potential of rice canopy shading to be effectively used to suppress weed growth. For this, we first performed a greenhouse study to identify whether different shading intensities by rice alone would affect weed growth and confirmed that this indeed can happen differentially between rice varieties. These findings then inspired a large-scale field experiment to establish how rice varieties with different shading potential (Chapter 3) grow their canopy and affect proliferation of the weed *Echinochloa crus-galli*.

Predicted high shading rice varieties caste more canopy shade

Besides testing the variable capacity of weed-competitiveness of different varieties, we verified whether the predicted Shading Rank, which is a measure of the shading capacity of a certain variety (Chapter 3), would also be a measure for how well high shade-casting varieties suppress weeds in the field. Var_A, Var_B, Var_C and Var_D were the predicted high shade-casting candidates. With the varieties Var_B, Var_C and Var_D being the fastest to close the canopy in monoculture, this could indeed be confirmed in the field. Var_C was very fast to reach 50 % cover, but a bit slower to close it fully. We observed that time to complete canopy closure in rice monocultures was not strongly correlated with end weed biomass in the rice-weed mixtures. This could indicate that rice canopies in monocultures are not identical to canopies of rice plants competing with weeds. The correlation of variance in Shading Rank to impact on weed end biomass is stronger, than only looking at shoot area of the same varieties. This is indicating that weed suppression is not only caused via shading, measured as canopy closure, but that more factors are playing a role (Supplemental Figure 4.4 E-F). Yet, the rate of canopy development in rice monocultures, is in fact strongly negatively correlated to weed performance in the rice-weed plots (r = -0.7** to -0.8*** from 24 dat to 52 dat, Supplemental Figure 4.3). Similarly, the rate of %-cover is of rice in the mixed plots correlates strongly over different timepoints with canopy development rate in rice monoculture (r = 0.4 to 0.7**, Supplemental Figure 4.3).

Var_B, C and D were the highest for tillering, plant height and accumulated biomass. These varieties, together with Var_H, are also achieving the largest percent ground cover in the mixed plots. This indicates that the relative performance of varieties with a high Shading Rank is consistent when taken to field trials.

High shade-casting varieties suppress weed stronger

Next, we verified whether the high shade-casting ability of these varieties would also translate into strong weed suppression. Indeed, Var_C and Var_D were the varieties with the strongest negative impact on weed biomass. The poorest weed-suppressors, were Var_I with low Shading Rank and Var_K, an elite-breeding line. However, Var_A was predicted to be a very strong shader, but it showed a poor seedling establishment in our field trial, resulting in only a mid-ranging weed suppression. Var_H, a mid-range variety for Shading Rank, was actually also showing a strong effect on weeds. Interestingly, even though Mudgo (Var_D) is one of the lowest tillering varieties, it is the strongest weed suppressing variety, when looking at weed biomass and weed tillers. Therefore, we can indeed validate that plants with high shading potential, based on their various shoot architecture traits, have a stronger suppressive effect on weeds (Supplemental Figure 4.4). Also other field studies showed a direct relation between early light extinction and later weed biomass (Chauhan, 2013; Koarai & Morita, 2003; Pérez de Vida et al., 2006) together with our results strengthening that indeed shading does have a strong impact on weed performance. However, we also confirm that under field conditions additional factors play into weed suppression, such as allelopathy, nutrient deprivation or root interaction (Chauhan et al., 2017; Chauhan & Johnson, 2010; Kraehmer et al., 2016).

Other factors contribute to weed suppression

Different rice varieties show different canopy growth development and therefore a different impact on the weed. Data from our field experiment show that early season plot height is negatively correlated with later weed biomass. In addition, maybe more than shading itself is nutrient deprivation by rice – early tillering and biomass accumulation indirectly lead to weed suppression.

Rice plant characteristics often reported to be associated with weed competitiveness, i.e. positively correlated for yield under weedy conditions and negatively correlated with weed biomass, are at early crop stage: shading related traits, such as leaf area, canopy ground cover (Caton et al., 2003; Dingkuhn et al., 1999; Mahajan & Chauhan, 2013; Namuco et al., 2009; Zhao et al., 2006b) The competitive ability of rice is often associated with light interception-related traits (Rao et al., 2007); plant height (Chakraborty et al., 2017; Chauhan, 2012b, 2013; Garrity et al., 1992; Haefele et al., 2004; Mennan et al., 2012); architectural traits including droopy leaves, leaf angle and canopy structure (Chauhan, 2013; Rao et al., 2007; Worthington

& Reberg-Horton, 2013; Zhao et al., 2006); seedling vigour, together with tillering capacity and early biomass (Caton et al., 2003; Dingkuhn et al., 2001; Fischer et al., 1997; Haefele et al., 2004; Mahajan & Chauhan, 2013; Mennan et al., 2012; Namuco et al., 2009; Ni et al., 2004; Pérez de Vida et al., 2006; Rao et al., 2007; Worthington & Reberg-Horton, 2013; Zhao et al., 2006).

Rice performance in monoculture does not predict rice competitiveness

Time to reach complete canopy closure of rice in monoculture does not correlate with weed suppression in mixed plots. However, progression of plot canopy cover in rice monoculture does negatively correlate with weed biomass in mixed plots. This could be a consequence of how time to canopy closure was estimated, since the data close to approaching full canopy closure shows a long tail to reach 100 % and therefore increases errors. Irrespective of these technical complications, it is clear that all rice varieties affect the weed strongly. However, a study of Zhao et al., 2006a describes that early measurements of crop vigour, canopy ground cover, height, tillers, vegetative crop biomass and plant erectness in monoculture were all positively correlated with yield related traits under weed competition and negatively with weed biomass; also Caton et al., 2003 found least vigorous rice cultivars in monoculture, scored as height gain rate, also being least productive in weed competition. Correlations between canopy characteristics under monoculture and competition indicated that leaf area index, specific leaf area and tillering ability were predictive of competitiveness (Dingkuhn et al., 1999). Namuco et al., 2009 claim that early leaf area and dry weight of rice seedlings are traits that can be used for mass screening of rice for competitiveness against weeds, however our study suggests, that this might be not so straightforward and more confounding factors come in, evoking a plastic phenotypic response in rice.

Timing is crucial

We showed, that the timing of shading by rice is crucial in order to be effective against weeds. Varieties that are slower in the progression of development of canopy cover, are less effective at weed suppression. This also appears from the observation that the late and mid-season rice data are not so strongly correlated with weed growth as early season, meaning that at the point when the canopy is closed the impact on the weed has already occurred. This is in line with the known importance of early season effective weed management, which largely determines how much weeds will be impacted later in the season (Evers & Bastiaans, 2016; Koarai & Morita, 2003). In a direct-seeding system, the critical period of weed competition has been reported to be in the range of 12 - 60 days after sowing of rice (Azmi et al., 2007; Chauhan & Johnson, 2011), where effective control of weeds at initial stages of rice growth, ensures rice yields to be unaffected (Raj & Syriac, 2017) The earlier the rice occupies the space and the faster the canopy closes, the less it will be affected by weed competition (Dass et al., 2017).

Increased rice planting density to improve weed suppression

More straightforward than getting the right varieties for farmers would be simply to increase the rice planting density to achieve the same suppressive effect on weeds. Indeed, weed suppression is increased with higher planting density, which was especially the case for varieties that tend to have a rather weak shading phenotype under standard density (Var_F, Var_I and Var_J). The data indicate the weed is more effectively suppressed at this increased density as compared to the standard density, which was also described in other studies (Chauhan et al., 2011; Chauhan & Abugho, 2013b; Dass et al., 2017; Heap, 2014; Ottis & Talbert, 2007). However, genetic diversity presents a large variance in suppression, indicating that choosing the right variety might have a stronger effect than increasing planting density. Additionally, with increased density, rice plants compete amongst themselves (intraspecific competition) and shade each other. Rice plants respond to this with increased height growth at the expense of tillering, a typical shade avoidance response (Chapter 2). At high density the poorer shading varieties benefit from it with faster canopy closure. However, although strong shading varieties also reach 50 % canopy closure faster, they already start competing with each other and do not reach full canopy closure faster than at normal density (see Var_B and Var_D marginally close the canopy faster, compared to Var_E and Var_K). Rice is known for its astonishing high plasticity and for filling up empty spaces with tillers, which is also the reason why plants grown at lower density show more tillers and more vigorous growth and more yield per plant (Bahuguna et al., 2021) The higher number of plants at increased density might not even make up for the loss of yield of each individual plant, compared to growing them at lower density, where they would show optimal performance (Heap, 2014; Zhao et al., 2007). However, if potential yield per area of land would be similar between the two densities, the somewhat improved reduction of weed performance at higher rice density might still present an advantage (Heap, 2014). Testing these ideas would clearly involve similar field trials that would have to be continued until the rice yield stage in order to validate the suitability in terms of yield.

High shading rice varieties as a sustainable weed management

Generally, predicted high shade-casting varieties are also the fastest to close the canopy in monocultures and amongst the strongest in affecting the weeds in the rice-weed mixtures. Low ranking varieties for shading potential on the other hand, together with the released breeding line of undetermined shading potential, showed slower canopy closure and less weed suppression. However, it should be kept in mind that this works only when comparing rice varieties with the highest Shading Ranks against the rest, a simple correlation analysis between Shading Rank and weed suppression does not yield a clear pattern. The effect of rice canopies, was not only manifested in the reduction of weed biomass, but also in number of weed tillers. The number of tillers formed by the weed, correlates strongly with seed production (Chauhan & Abugho, 2013). Reducing weed tillering would thus result in reduced weed seed production, and thereby weed infestation over the years, by reducing the weed seed bank. The classic weed management in rice occurs by flooding of the rice fields, which suppresses weeds (Heap, 2014; Zhao et al., 2007). However, in many locations, water shortage is increasing and using scarce fresh water for field flooding is therefore not very attractive anymore. An effective integrative approach might be to keep the rice fields flooded very early in the season only, and then further suppressing the weeds by the shading ability of the rice while the fields are drained (Toulotte, 2022). This would combine two sustainable modes of weed suppression, that would save precious irrigation water together with securing yields (Chauhan, 2012b, 2013; Chauhan et al., 2011; Mahajan et al., 2015, 2017; Ottis & Talbert, 2007).

Acknowledgements

We thank Jerico Bigornia, Bjorn Manuel and Leodegario Dela Rosa who were invaluable with their assistance and practical support in preparing, executing and maintaining the field experiment. and Caesar Arloo Centeno for assistance with drone imaging at the International Rice Research Institute. We thank Alba Schielen, Armin Geluk and Yorrit van de Kaa for their help with phenotyping in the greenhouse experiment at Utrecht University.

Materials and methods

Greenhouse experiment: Effect of rice shade on early *E. crus-galli* development

Seed origins

Four rice (*O. sativa)* varieties were selected for their high (Shim Balte and Mudgo) and low (Della and Luk Takhar) shade-casting potential based on Shading Ranks of 344, 330, 49 and 1, respectively (Huber *et al.*, 2021). Seeds were obtained from plants grown in the greenhouse at the International Rice Research Institute (IRRI), Los Baños, the Philippines, in wet season of 2018, stored at 6°C in the dark. The weed seeds of *Echinochloa crus-galli* were originally collected by the Weed Science team at IRRI, Los Baños, The Philippines, in a lowland field (IRRI – M5), within the period Sept-Nov 2016. Seeds were then further multiplied under the natural light and temperature conditions of The Philippines (12 h dark 23-27°C / 12 h light 30-40°C), in non-flooded pots in an IRRI screenhouse. Seeds were received in September 2018 and were kept in a dark and dry place.

Germination and growth

Rice seeds were exposed to 40 °C for 24 h to break dormancy, followed by 24 h at 21 °C. For germination, seeds were put in Petri dishes on wet filter paper and incubated at 32 °C for 24 h. Pre-germinated seeds were directly planted on soil, with five seeds for each variety, per pot (10 x 10 x 11 cm) in a substrate mix of black soil, agra-vermiculite 0-1.5 mm and sand in a ratio of 5 : 3 : 2 together with 6 g Osmocote NPK-Mg 15-4-9 (+1) (2.4 g/L of soil) and 20% Yoshida nutrient solution (Yoshida, 1976) with a double iron dose (Sequestreen = Fe-EDTA) and pH 6.5 (1l per kg substrate). One week after seeding, only four plants per pot were retained. For each weed pot, six dehulled *E. crus-galli* seeds were put to germinate 14 days after rice sowing (das) to simulate transplanting conditions in the field where two weeks old rice seedlings are transplanted. The same pot size and soil mixture were used as for the rice, with thinning after one week, to keep four weed plants per pot.

Plants were grown in the greenhouse facilities of the Botanical Gardens, Utrecht University, in The Netherlands, in February 2021. Temperatures were set to 29°C during the day and 25°C during the night and a 12 h photoperiod from 8 am to 8 pm, with a minimal light intensity of 400 µmol m^{-2} s^{-1} and artificial light (Valoya, Model Rx400 500mA 5730, Spectrum AP673L) switching on if sunlight flux rate dropped below 400 ɥmol m^{-2} s^{-1}. Automatic watering kept soil in pots saturated. Pots of rice and weed were arranged in a chessboard-like layout with the weed pots in between rice pots. The experiment units (the eight weed plants that were measured per plot) were surrounded by bordering plants to avoid border effects on the experimental units (see experimental design Supplemental Figure 4.6).

Experimental design

Pots were arranged at a distance of 10 cm in mixed plots, where each plot included the three treatment groups of rice only, weed only and rice and weed mixed, in three repeated blocks. The experiment units (the eight plants that were measured) were surrounded by bordering plants to avoid border effects on the experimental units (Supplemental Figure 4.6).

Measurements

Light intensity (photosynthetic active radiation (PAR) of 400-700 nm waveband) was measured every week at the ground level between plants at two locations for each of the three repeated blocks. For reference, PAR was measured four times above the plant canopy at the same time, to calculate light extinction.

Weed plants were harvested for shoot and root biomass measurements at 28 days after sowing (42 days after rice sowing). Plant material was dried at 80°C for three days and weighed per plant. In addition, the number of leaves and tillers per plant were counted.

Field experiment: Effect of rice shade and planting density on *E. crus-galli*

Seed origins, germination, growth

The field experiment took place in a field at the IRRI, Los Baños, the Philippines from January to March 2020. Eleven different rice varieties were selected based on their high (Shim Balte, Sabharaj, Sathi and Mudgo), intermediate (DM 65, Criollo La Fria,Black Gora and Wab 501-11-5-1) or low (IR 64-21 and Var_DJ 123) shade-casting capacity described in (Huber *et al.*, 2021), including an elite-breeding line with no data about its shading capacity (Katihan 2) (Table 4.1). Rice seeds were provided from the gene-bank IRGC, IRRI. *E. crus-galli* seeds were collected in a lowland field and multiplied in control conditions by the Weed Science team at the IRRI.

Germination protocol was followed as described above. Rice seedlings were raised in trays for 14 days, after which the seedlings were manually transplanted into the field. On the day of transplanting rice seedlings, *E. crus-galli* seeds were sown in trays and raised for 10 days before transplanting into the field. The seed to seed age difference between rice and *E. crus-galli* seedlings was 14 days to simulate field conditions, with rice having a two weeks age advantage.

Treatment details and experimental design

A total of eleven rice varieties was grown at standard planting density at a spacing of 20 cm x 20 cm (25 plants m^{-2}) and high planting density at a spacing of 10 cm x 20 cm (50 plants m^{-2}). Additionally, weeds of *E. crus-galli* were planted into rice plots at both densities. As a control, *E. crus-galli* was grown alone without competition from rice, giving in total five different treatment groups (Table 2).

In mixed rice-weed plots, a density of 50 *E. crus-galli* plants m^{-2} was maintained by manually transplanting 10-days old seedlings of *E. crus-galli* between rice rows at a spacing of 10 cm plant-to-plant. Similarly, in weed-only plots, a density of 50 *E. crus-galli* plants m^{-2} was established.

A factorial randomized complete block design was followed with three replications, with rice variety (11 rice varieties), planting density (normal and high density) and weed competition (rice-only and mixed rice+weed). The experimental layout of the field is provided in Supplemental Figure 4.7. The planting pattern in both rice-only and mixed rice+weed plots is given in Supplemental Figure 4.8 A-B. Plots size was 2.6 m x 2.4 m = 6.24 m^2.

Soil preparation, herbicide and fertilization

A stale seedbed approach was practised for 20 days prior to rice transplanting to minimize the background soil weed seed bank by stimulating emergence with tillage and irrigation and then killing them with glyphosate application. Prior to transplanting, field was puddled and then levelled. One day after rice transplanting, an application of pre-emergence herbicide (Sofit) and shallow flooding was maintained until weed seedlings transplanting to achieve good weed control of background weeds in the plots. After weed transplanting, soil was kept saturated. With manual weeding, emerging weeds other than *E. crus-galli* were removed throughout the experiment. A standard fertilizer and pest management was followed uniformly in all plots. DAP (Di-ammonium Phosphate) with phosphorus (P; 0.7 kg/ha) and nitrogen (N; 0.4 kg/ha) and MOP (Muriate of Potash) with potassium (K; 0.3 kg/ha) were applied as basal with puddling operation in the form of complete fertilizer N-P-K (18-46-60) and nitrogen (N; 1.1 kg/ha) was applied at early (0-7 days after transplanting with 0.4 kg/ha) and at active tillering with 0.7 kg/ha.

Measurements

Several traits to follow rice and weed development were recorded manually (Supplemental Figure 4.8 C). Rice and weed maximum shoot height (measured with stretched leaves) and tiller number were recorded at 17, 25 and 40 days after transplanting (dat) of rice. Shoot biomass of rice and weed was harvested at 25 and 53 dat, dried several days at 80°C and weighed. All biomass data referred to in this study, only comprises biomass of shoot samples. Rice and weed yield data could not be gathered due to COVID-19 related lock-down leaving the experiment abandoned at 53 dat.

To assess the percent ground/canopy cover, two methods were used. First, ground cover was assessed manually, referred to as percent ground cover of weed and rice, in the mixed plots at two week intervals following the beaded string method (Sarrantonio, 1991). A 3.6 m string with beads at 20 cm distance (with a total of 18 beads) was placed diagonally over the plot and the number of beads hitting either rice or weed leaves were recorded. Measurements were done two times per plot, giving 36 beads in total. Additionally the canopy development was recorded with a drone, two times per week equipped with a near infrared and red edge camera. Dates of manual and drone measurements were carried out from 3 to 53 days after transplanting, exact dates for each of the measurements and number of replicates are given in Supplemental Figure 4.9. With data provided by drone images, we could derive plot canopy cover (PC), which is based on a combination of NDVI (Normalized Difference Vegetation Index) and TGI (triangular green index). Values for each index were calculated for each plot and timepoint. Drone imaging also provided data for relative plot canopy height (reHT).

Data analysis

In this chapter, data of rice [R], rice [RW], weed [RW] at normal density and weed [W] and rice [R-HD] was analysed. For drone data a full plot size was of about 5.3 m^2. For analysis and generated graphs, only plots with a minimum of 4 m^2 (plots in red in Supplemental Figure 4.7 A) were included.

To quantify the effect on weed suppression, we determined the relation of weed biomass in mixed plots compared to weed monoculture as

% weed in [RW] = DW in [RW] x 100 / DW in [W]

and the relation of weed biomass to total plot biomass in mixed plots as

% weed in [RW] = DW in [RW] / (rice+weed) DW in [RW],

where [RW] refers to mixed rice-weed plots and [W] to weed monoculture.

From drone images, pixel values are extracted of each plot, using the median value of the pixels per m2 for each plot; were only the green area of a plot was included. The following indices were calculated. Normalized difference Index, where NDVI = (NIR-Red) / (NIR +Red) which is based on multispectral reflectance; and the Triangular green index, where TGI = ((660-450) * (R-G) - (660-520) * (R-B)) * (-0.5), which is based on RGB values.

To estimate what percent of the plot is covered in vegetation, information of both TGI and NDVI were combined, using the following

Plot cover (PC) = (PCRGBplot +PCMSplot) / 2

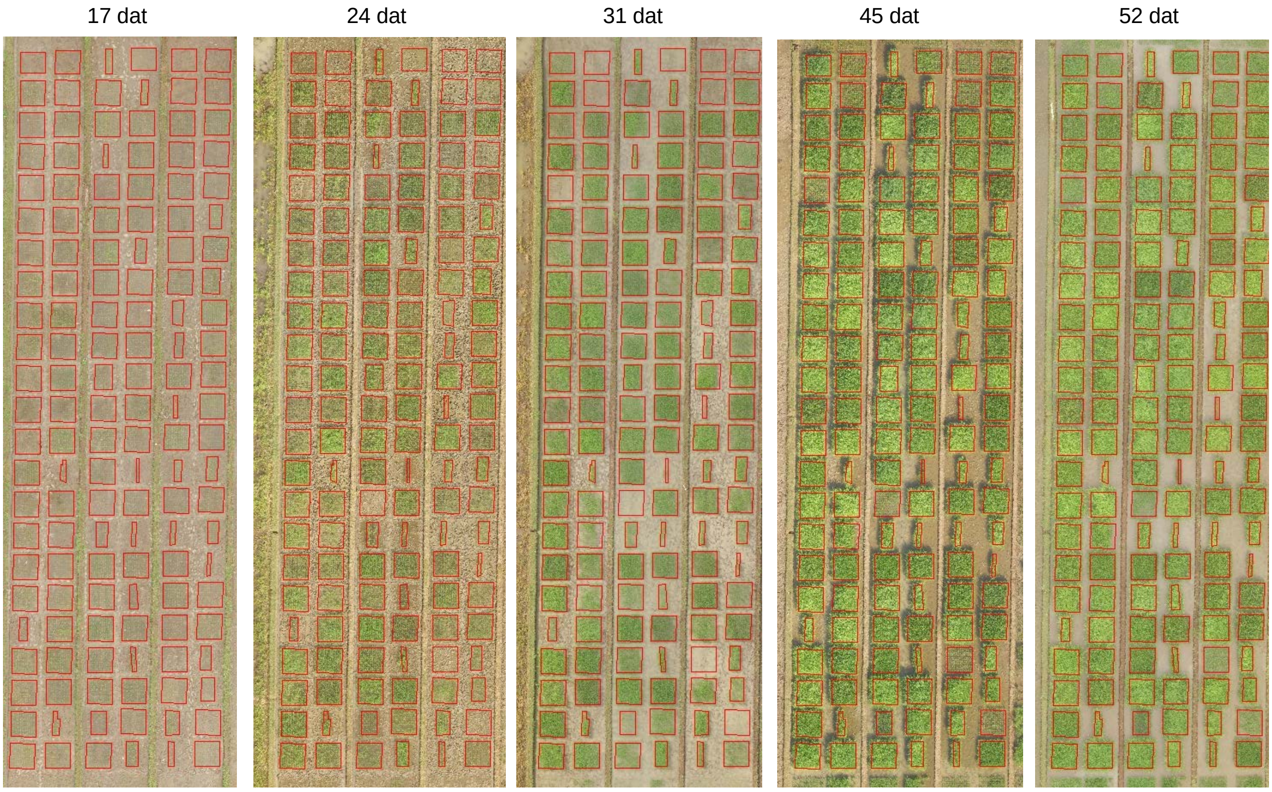
The values of PC were then adjusted for each variety between 0 and 1, by taking the maximum (maxPC) for each variety, assuming that all varieties reached at this point the stage of closed canopy; from which adjPC, was calculated as

adjPC = PC / maxPC

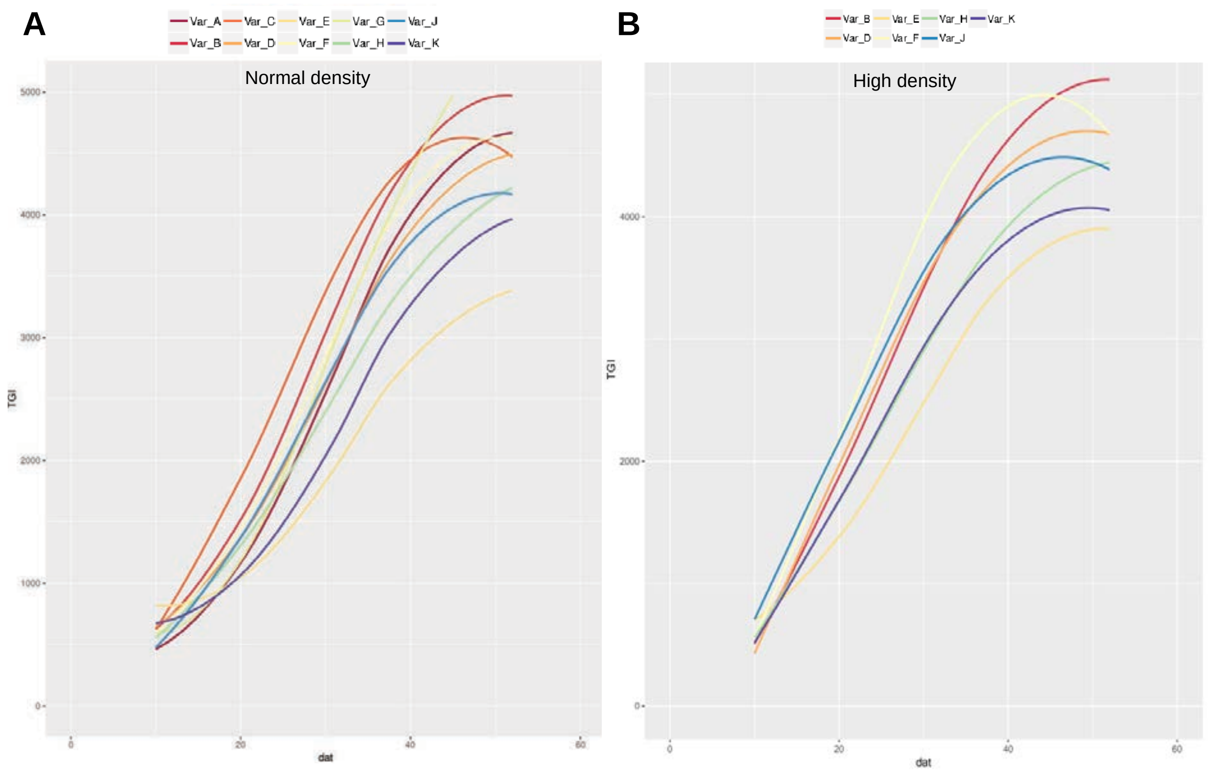
for a given plot of a given variety. We estimated the time (days) to 50 % and 99 % canopy cover for each variety using sigmoid curve fitted to adjPC values based on a generalized linear model (GLM) with bionomial function. Practically, 99 % of canopy cover for a plot corresponds to canopy closure.

Correlation tables show r values, based on the least square means (lsm) for each variety-treatment-density combination. The lsm values were calculated using the raw data files for manual and drone data, excluding plots which either had poor coverage or missing reps (tag=1), or were small (area < 4.0). Scatter plots show r^2 values.

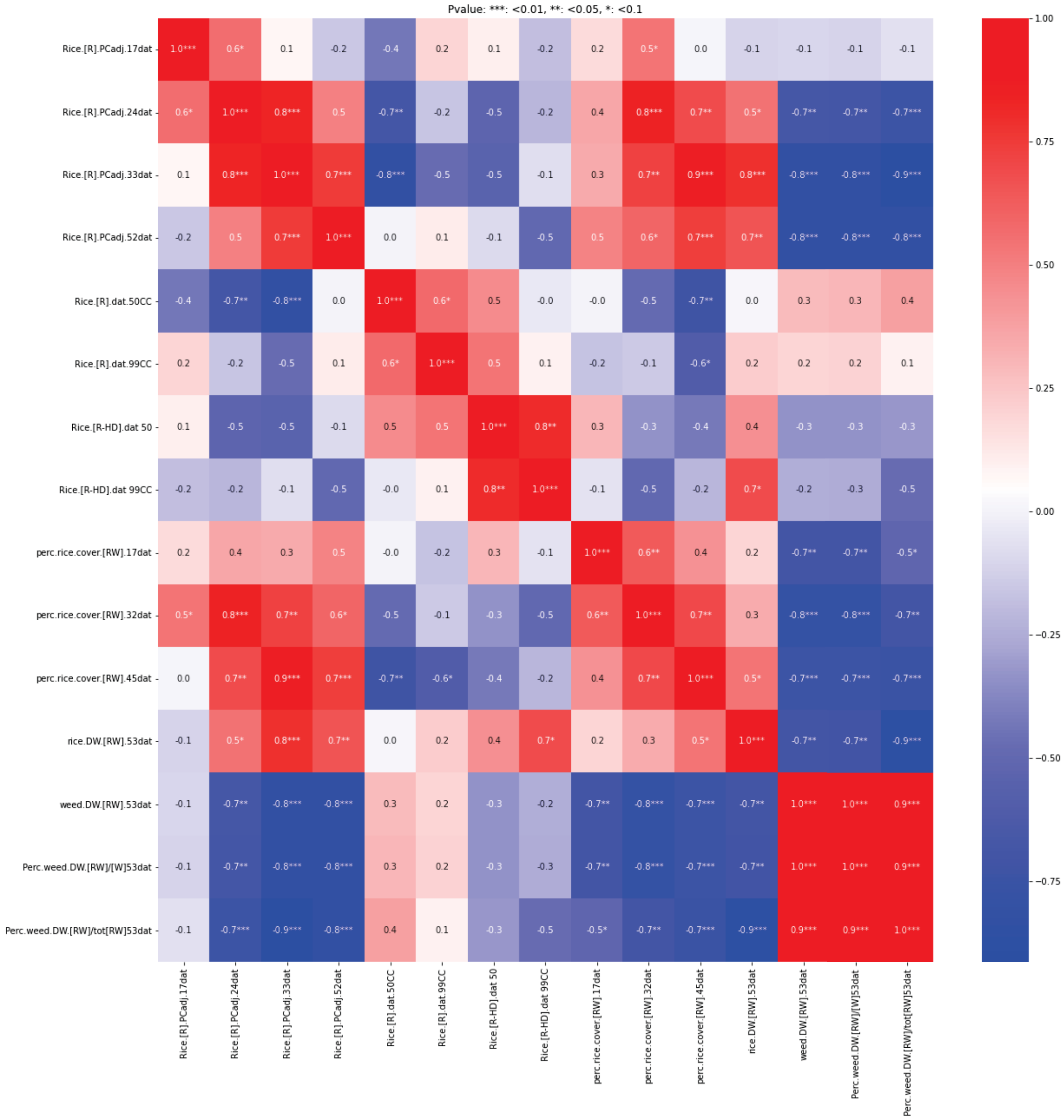
Supplements



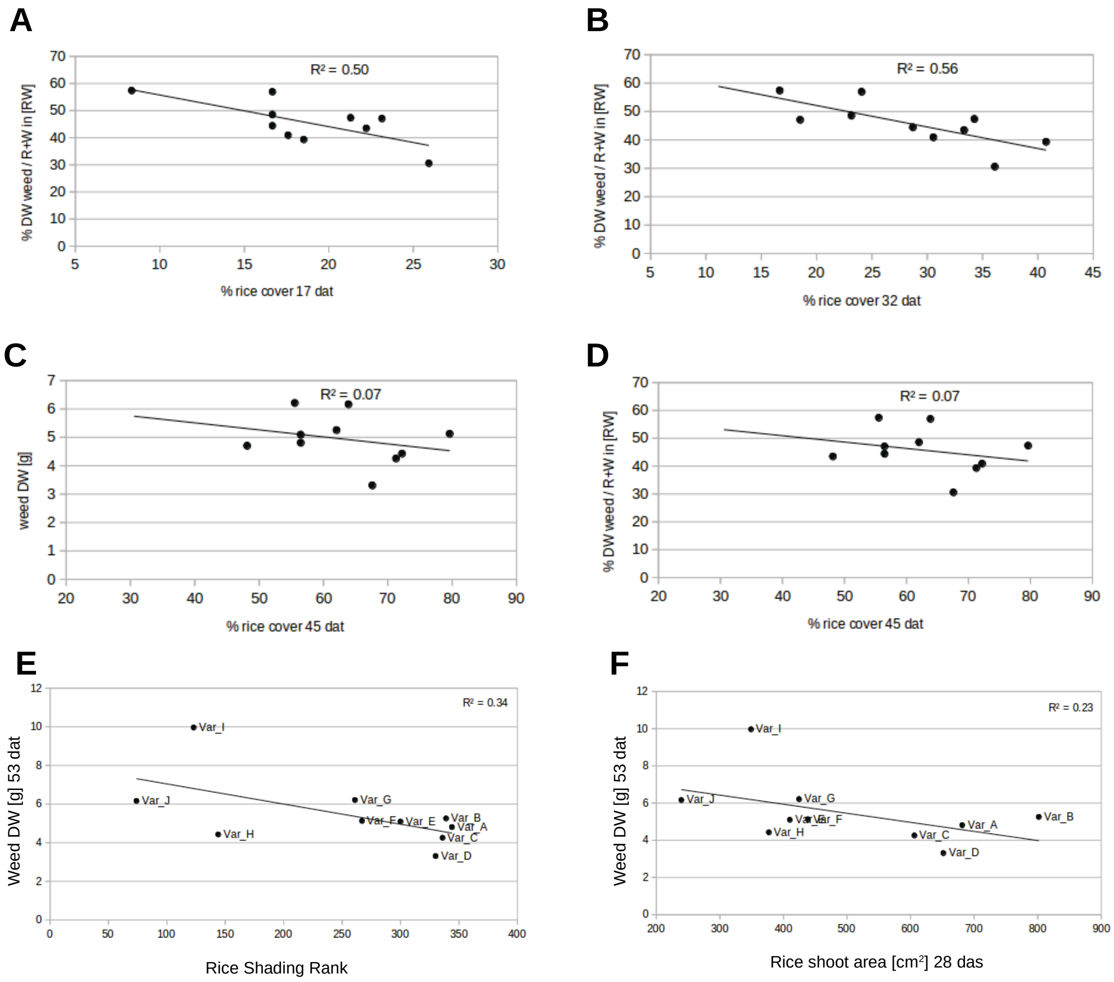
Supplemental Figure 4.1. Aerial drone imaging of the field showing the canopy development of single plots followed from 17 - 52 days after transplanting (dat) of rice, weeds were transplanted at 10 dat.



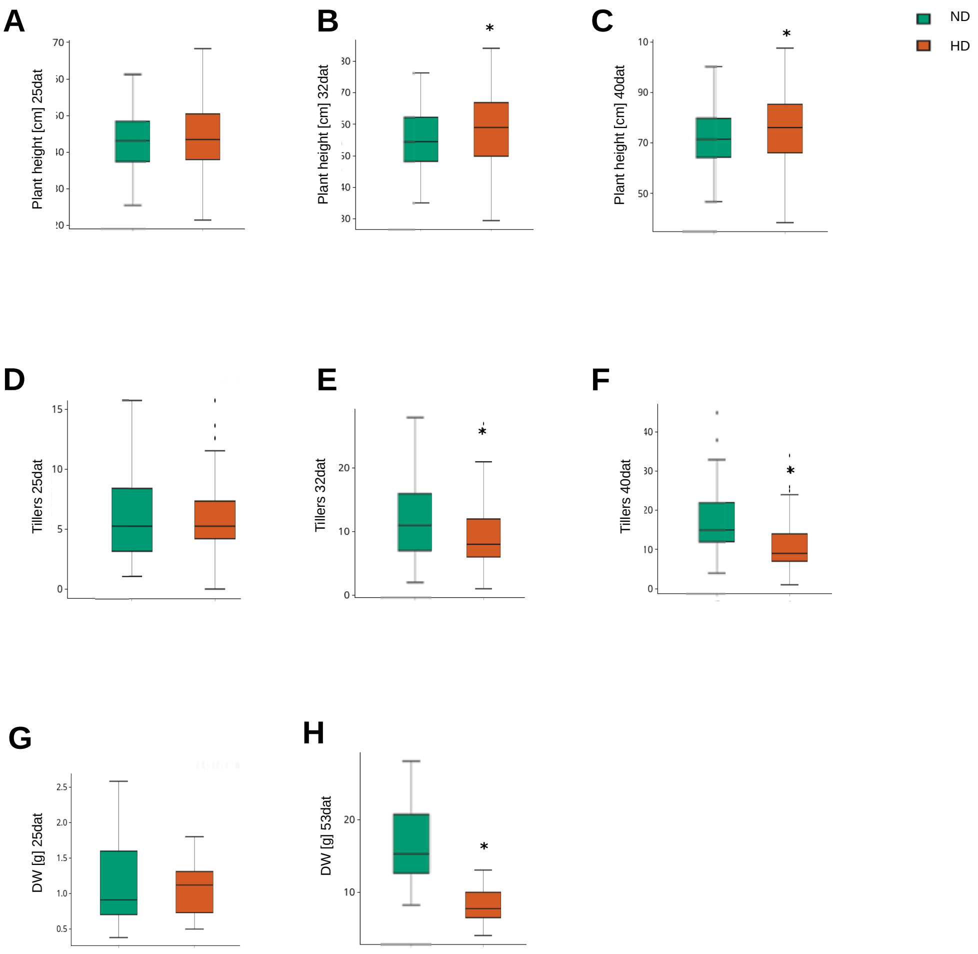
Supplemental Figure 4.2. Density effect on rice development shown as triangular green index (TGI) on different rice varieties planted at **A.** normal density (ND, 20 x 20 cm, 25 plants m-^{2}) compared to **B.** high density (HD, 10 x 20 cm, 50 plants m-^{2}), monitored with a drone from day of transplanting until 53 days after transplanting (dat). Lines are based on a quadratic curve fit. TGI is based on RGB camera, capturing chlorophyll content , indicative for photosynthetically active tissue. Var - different rice varieties.



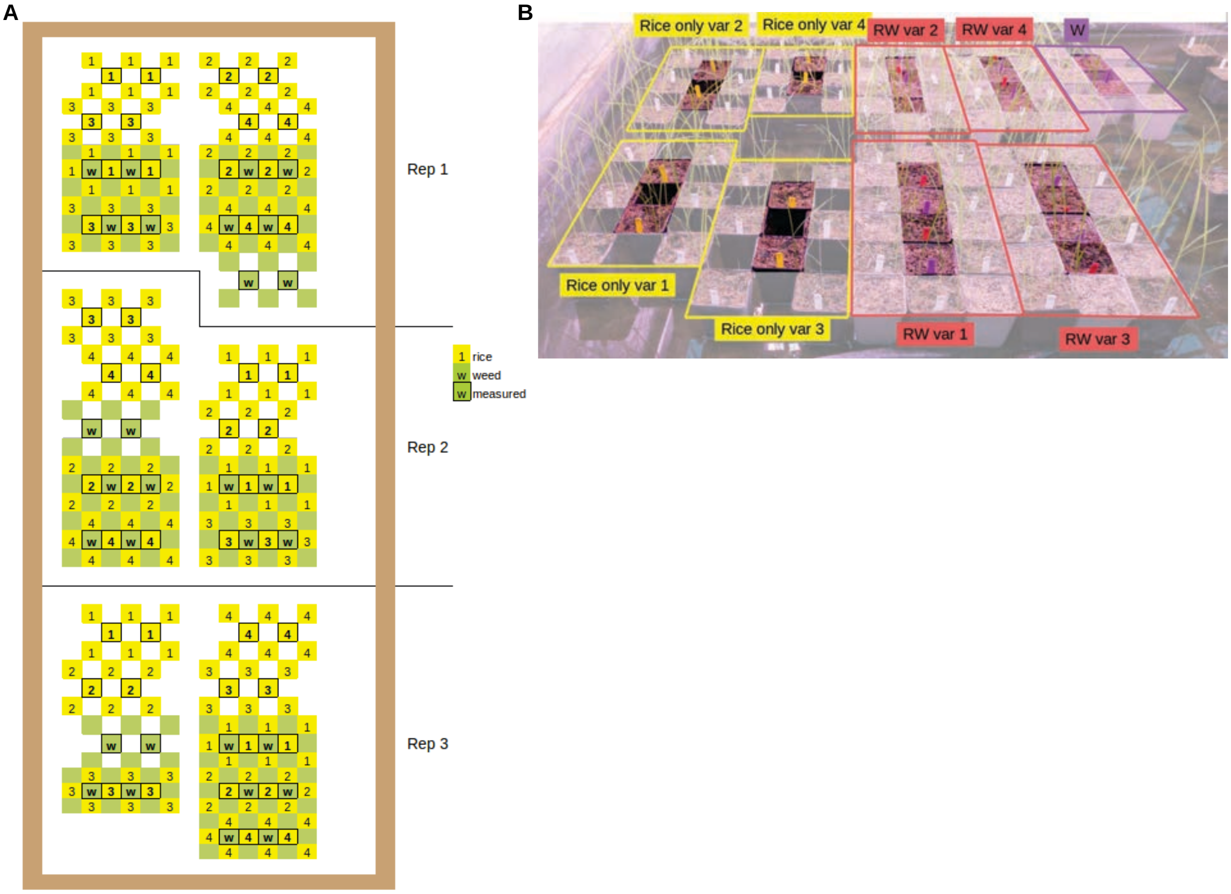
Supplemental Figure 4.3. Trait values of rice grown in monoculture are not correlated with rice grown mixed plots or at high density and impact on weed biomass in mixed plots. Rice: plot canopy cover at 17, 24, 33, 45, 52 dat, time to 50 % and 99 % canopy closure in rice monoculture at normal density [R] and high density [R-HD], %-rice cover at 17, 32 and 45 dat [RW] and rice DW. Weed: %-weed cover 45 dat, weed DW, % DW compared to total plot biomass in [RW], % DW compared to weed monoculture [W].



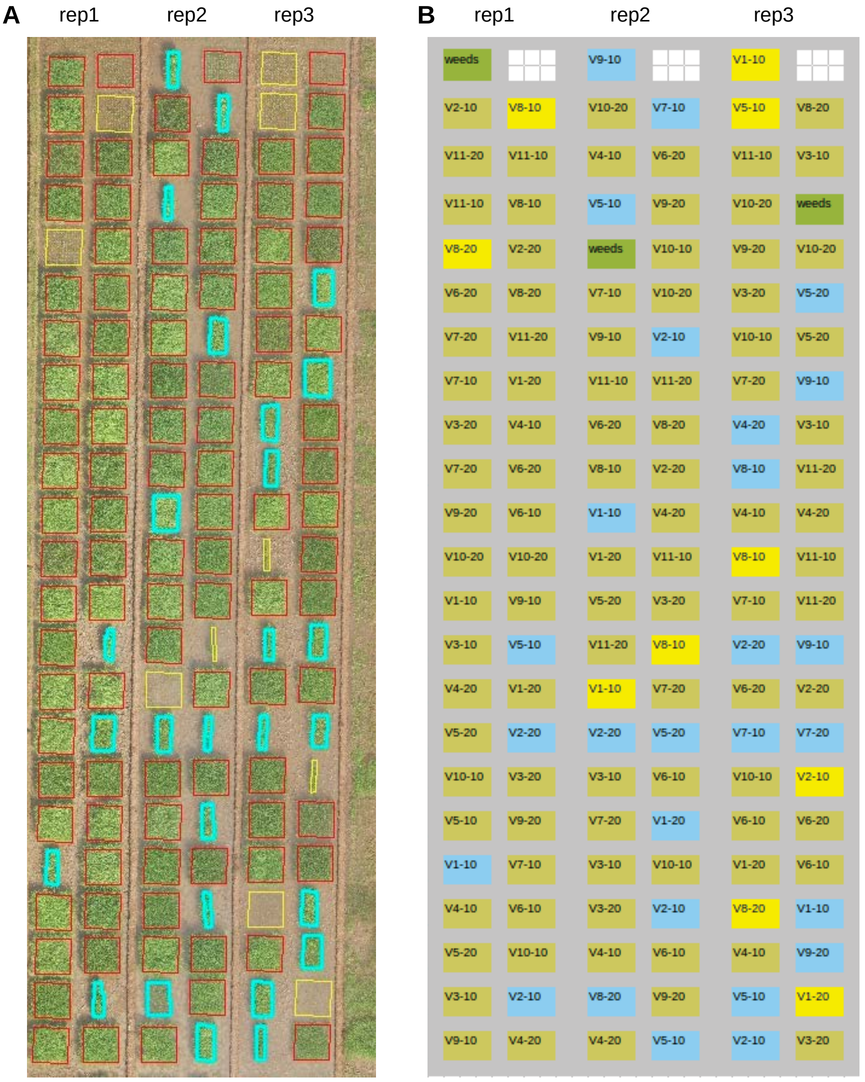
Supplemental Figure 4.4. Impact of variance in early shading on later season weed suppression. A. - B. Correlation scatter plots of %-rice ground cover at A. 17 dat and B. 32 dat with % weed dry weight of total plot biomass in mixed plots as % weed DW in [RW] / (rice+weed) in [RW]. **C. - D.** Correlation scatter plots of %-rice ground cover 45 dat with C. weed dry weight at 53 dat and D. % weed dry weight of total plot biomass in mixed plots as % weed DW in [RW] / (rice+weed) in [RW]. R^2 values of regression Var_I excluded. **Correlation of Shading Rank and shoot area of rice with weed biomass. E.** Shading capacity of rice as the Shading Rank (1 as lowest and 344 as highest shading) on x-axis and **F.** shoot area at 28 days after sowing (das) of plants grown in pots on x-axis in relation to and weed shoot dry weight at 53 dat on y-axis. Data for Shading Rank and shoot area is based on a screen of a rice diversity panel in an earlier study (Chapter 3).



Supplemental Figure 4.5. Increased planting density affects rice growth. Phenotypic differences between different rice varieties planted at high density (HD, 10 x 20 cm, 50 plants m^{-2}) compared to normal density (ND, 20 x 20 cm, 25 plants m^{-2}). **A. - C.** Rice plant height [cm] at 25, 32 and 40 days after transplanting (dat). **D. - F.** Number of tillers per rice plant at 25, 32 and 40 dat. For plant height and tiller number Var_G and Var_I were excluded, because of too few replicates. **G. - H.** Rice shoot dry weight [g] per rice plant at 25 and 53 dat. For shoot dry weight Var_A, Var_C, Var_D, Var_F, Var_G and Var_I were excluded, because of too few replicates. N = 18 plants for ND and 36 plants for HD (3 plots with 2 sampling locations with 3 plants or 6 each), min 5 sampling locations, if lower then excluded. Values are means, box indicating +/- IQ-range, whiskers indicating 2.5 – 97.5 percentile, with significant difference based on two-sample t-test with * $p < 0.05$.



Supplemental Figure 4.6. Experimental design of rice-weed competition in greenhouse experiment. A. Schematic of the experimental design of the rice-weed competition experiment in the greenhouse as randomised mixed plot design with three replicates (Rep 1 - 3). W - weed pot, 1 to 4 - rice pots of four different varieties. The plants used for measurements are indicated with borders. **B.** Image of experimental set up of Rep 1 with “Rice-only” plots in yellow, “Weed-only” plots in purple and “Rice-Weed” plots in red. The highlighted pots in the picture indicate the pots per replication block with measured plants.

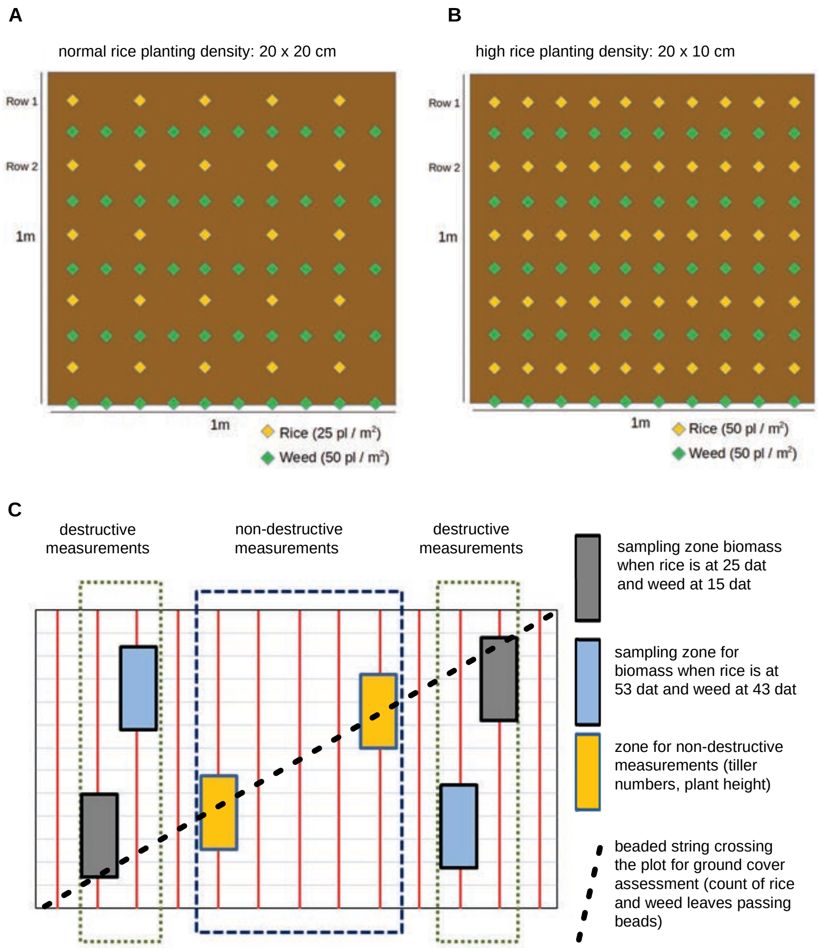


rep1		rep2		rep3	
weeds		V9-10		V1-10	
V2-10	V8-10	V10-20	V7-10	V5-10	V8-20
V11-20	V11-10	V4-10	V6-20	V11-10	V3-10
V11-10	V8-10	V5-10	V9-20	V10-20	weeds
V8-20	V2-20	weeds	V10-10	V9-20	V10-20
V6-20	V8-20	V7-10	V10-20	V3-20	V5-20
V7-20	V11-20	V9-10	V2-10	V10-10	V5-20
V7-10	V1-20	V11-10	V11-20	V7-20	V9-10
V3-20	V4-10	V6-20	V8-20	V4-20	V3-10
V7-20	V6-20	V8-10	V2-20	V8-10	V11-20
V9-20	V6-10	V1-10	V4-20	V4-10	V4-20
V10-20	V10-20	V1-20	V11-10	V8-10	V11-10
V1-10	V9-10	V5-20	V3-20	V7-10	V11-20
V3-10	V5-10	V11-20	V8-10	V2-20	V9-10
V4-20	V1-20	V1-10	V7-20	V6-20	V2-20
V5-20	V2-20	V2-20	V5-20	V7-10	V7-20
V10-10	V3-20	V3-10	V6-10	V10-10	V2-10
V5-10	V9-20	V7-20	V1-20	V6-10	V6-20
V1-10	V7-10	V3-10	V10-10	V1-20	V6-10
V4-10	V6-10	V3-20	V2-10	V8-20	V1-10
V5-20	V10-10	V4-10	V6-10	V4-10	V9-20
V3-10	V2-10	V8-20	V9-20	V5-10	V1-20
V9-10	V4-20	V4-20	V5-10	V2-10	V3-20

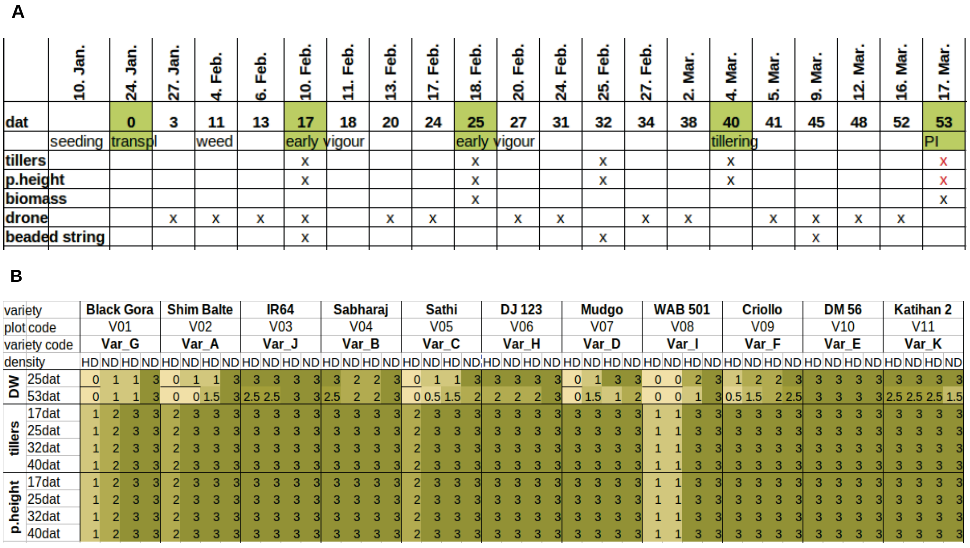
C

	Plot Code	**Variety Code**
Black Gora	V01	**Var_G**
Shim Balte	V02	**Var_A**
IR64	V03	**Var_J**
Sabharaj	V04	**Var_B**
Sathi	V05	**Var_C**
DJ 123	V06	**Var_H**
Mudgo	V07	**Var_D**
WAB 501	V08	**Var_I**
Criollo	V09	**Var_F**
DM 56	V10	**Var_E**
Katihan 2	V11	**Var_K**

Supplemental Figure 4.7. Experimental design of rice-weed competition in the field. A. Aerial view of field layout of the rice-weed competition experiment. For analysis of drone data, the evaluated plot area was adjusted to fit the area covered by plants within a plot > 4 m-2. **B.** Schematic depicting the mixed plot design of the rice-weed competition field experiment. **C**. Names of rice varieties with their plot codes and variety codes. Weeds – weed monoculture, R – rice monoculture, RW – mixed rice + weed, V1 to V11 – code of rice varieties, 20 – normal density (20 x 20 cm), 10 - high density (10 x 20 cm). Plots indicated in red are the full size plots that were analysed. Plots in blue (reduced row number) and yellow (poor seedling establishment) were not included in the analyses.



Supplemental Figure 4.8. Planting scheme for rice and weed in the field plots of 2.6 m x 2.4 m = 6.24 m-2 for **A.** normal rice density of 20 x 20 cm (25 plants m-2) and **B.** high density of 20 cm x 10 cm (50 plants m-2). Weed density remains the same in both cases: 50 plants m-2. C. Schematic depicting the zones for sampling and measurements.



A

	10. Jan.	24. Jan.	27. Jan.	4. Feb.	6. Feb.	10. Feb.	11. Feb.	13. Feb.	17. Feb.	18. Feb.	20. Feb.	24. Feb.	25. Feb.	27. Feb.	2. Mar.	4. Mar.	5. Mar.	9. Mar.	12. Mar.	16. Mar.	17. Mar.
dat		**0**	**3**	**11**	**13**	**17**	**18**	**20**	**24**	**25**	**27**	**31**	**32**	**34**	**38**	**40**	**41**	**45**	**48**	**52**	**53**
	seeding	transpl		weed		early vigour				early vigour						tillering					PI
tillers						x				x			x			x					x
p.height						x				x			x			x					x
biomass										x											x
drone			x	x	x	x		x	x		x	x		x	x		x	x	x	x	
beaded string						x							x					x			

B

variety		**Black Gora**				**Shim Balte**				**IR64**				**Sabharaj**			
plot code		V01				V02				V03				V04			
variety code		**Var_G**				**Var_A**				**Var_J**				**Var_B**			
density		HD	ND	HD	ND	HD	ND	HD	ND	HD	ND	HD	ND	HD	ND	HD	ND
DW	25dat	0	1	1	3	0	1	1	3	3	3	3	3	3	2	2	3
	53dat	0	1	1	3	0	0	1.5	3	2.5	2.5	3	3	2.5	2	2	3
tillers	17dat	1	2	3	3	2	3	3	3	3	3	3	3	3	3	3	3
	25dat	1	2	3	3	2	3	3	3	3	3	3	3	3	3	3	3
	32dat	1	2	3	3	2	3	3	3	3	3	3	3	3	3	3	3
	40dat	1	2	3	3	2	3	3	3	3	3	3	3	3	3	3	3
p.height	17dat	1	2	3	3	2	3	3	3	3	3	3	3	3	3	3	3
	25dat	1	2	3	3	2	3	3	3	3	3	3	3	3	3	3	3
	32dat	1	2	3	3	2	3	3	3	3	3	3	3	3	3	3	3
	40dat	1	2	3	3	2	3	3	3	3	3	3	3	3	3	3	3

variety		**Sathi**				**DJ 123**				**Mudgo**				**WAB 501**			
plot code		V05				V06				V07				V08			
variety code		**Var_C**				**Var_H**				**Var_D**				**Var_I**			
density		HD	ND	HD	ND	HD	ND	HD	ND	HD	ND	HD	ND	HD	ND	HD	ND
DW	25dat	0	1	1	3	3	3	3	3	0	1	3	3	0	0	2	3
	53dat	0	0.5	1.5	2	2	2	2	3	0	1.5	1	2	0	0	1	3
tillers	17dat	2	3	3	3	3	3	3	3	3	3	3	3	1	1	3	3
	25dat	2	3	3	3	3	3	3	3	3	3	3	3	1	1	3	3
	32dat	2	3	3	3	3	3	3	3	3	3	3	3	1	1	3	3
	40dat	2	3	3	3	3	3	3	3	3	3	3	3	1	1	3	3
p.height	17dat	2	3	3	3	3	3	3	3	3	3	3	3	1	1	3	3
	25dat	2	3	3	3	3	3	3	3	3	3	3	3	1	1	3	3
	32dat	2	3	3	3	3	3	3	3	3	3	3	3	1	1	3	3
	40dat	2	3	3	3	3	3	3	3	3	3	3	3	1	1	3	3

variety		**Criollo**				**DM 56**				**Katihan 2**			
plot code		V09				V10				V11			
variety code		**Var_F**				**Var_E**				**Var_K**			
density		HD	ND	HD	ND	HD	ND	HD	ND	HD	ND	HD	ND
DW	25dat	1	2	2	3	3	3	3	3	3	3	3	3
	53dat	0.5	1.5	2	2.5	3	3	3	3	2.5	2.5	2.5	1.5
tillers	17dat	3	3	3	3	3	3	3	3	3	3	3	3
	25dat	3	3	3	3	3	3	3	3	3	3	3	3
	32dat	3	3	3	3	3	3	3	3	3	3	3	3
	40dat	3	3	3	3	3	3	3	3	3	3	3	3
p.height	17dat	3	3	3	3	3	3	3	3	3	3	3	3
	25dat	3	3	3	3	3	3	3	3	3	3	3	3
	32dat	3	3	3	3	3	3	3	3	3	3	3	3
	40dat	3	3	3	3	3	3	3	3	3	3	3	3

Supplemental Figure 4.9. Timepoints of measurements and number of replicates of field experiment. A. Timepoints of sampling and different measurements and **B.** number of samples and measurements per variety and density for each timepoint. Dat – days after transplanting of rice, p.height – plant height, PI – panicle initiation, DW – dry weight, HD – height density, ND – normal density

Chapter 5

Phenotypic and transcriptomic response of rice to low R:FR

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Abstract

Plants are equipped with the phenotypic plasticity to respond to early signals for approaching vegetational shade, before they are actually being shaded. These phenotypic changes are tuned to increase light interception and collectively referred to the shade avoidance syndrome (SAS). Resource allocation to vegetative structures rather than seeds, being the harvestable organs of the majority of the globally grown staple crops, makes it unfavourable in agriculture. Since rice is cultivated in dense monocultures, the investigation of SAS is of special interest. To this end, knowledge in the field of SAS is scarce, even more so for rice. To shed light on the very unknown territory of shade avoidance in monocots and rice in particular, we exposed seedlings of different rice varieties to low R:FR by adding FR light to the treated group. The surprising results after four weeks of treatment showed very mild phenotypic results, changes that we recorded were opposed to what is commonly described as SAS. We noted increased leaf and tiller formation and very marginal height response. In addition, response varied greatly between varieties. RNAseq analysis of shoot tissue of the same seven different varieties, revealed a remarkably low number of differentially expressed genes and only a fraction of them being reported as SAS induced genes. To explore this further, we applied the same low R:FR treatment, but at lowered growth temperatures, since an interplay of SAS and thermomorphogenesis is known. Phenotypes of the treated group, showed the same results; similarly, for tested wild rice varieties. To our knowledge, this is the very first study exploring response of rice to low R:FR, revealing a completely lacking SAS phenotype.

Abbreviations and definitions

SAS	Shade Avoidance Syndrome
PAR	Photosynthetic Active Radiation; number of photosynthetically active photons that fall on a given surface each second. It is defined as the photons in the range of 400 to 700 nm measured as μmol photons m^{-2} s^{-1}.
R:FR	red to far-red light ratio
WL	white light
WL+FR	white light with supplemented far-red light
das	days after sowing
CPMs	Counts Per Million
FC	Fold Change
logFC	logarithmic of the Fold Change
DEG	Differentially Expressed Gene; the difference of treated to control group
FDR	False Discovery Rate; also called q-value; it is the corrected p-value
GO	Gene Ontology

Introduction

Light is essential for plant life. Plants have evolved to thrive on our planted by harvesting light energy to assimilate CO_2 and together with water form organic compounds via the process of photosynthesis. In this spectacular process, especially photons of red and blue light are absorbed, whereas light outside the waveband of 400 to 700 nm defined as photosynthetic active radiation (PAR) is mostly reflected or transmitted. Even though, far-red light (FR) in the wavelength from 700 to 800 nm lies outside of this range, it carries important information for plants about their environment. Since neighbouring plants reflect FR light, but absorb red, the ratio of red to far-red (R:FR) decreases with increasing vegetation density. The R:FR of sunlight is about 1.2 on a clear day and can decreases to 0.3 - 0.4 in dense vegetation and drop to 0.1 under deep canopy shade (Li, 2019; Roig-Villanova & Martínez-García, 2016). FR light was reported to be the crucial early warning signal for approaching vegetational shade. In this context, it is important to distinguish between proximity shade and canopy shade (Ballaré & Casal, 2000; Casal, 2013; Roig-Villanova & Martínez-García, 2016). The first occurs when plants are growing in close proximity, without direct shading, but with a reduction of the R:FR to about 0.5 – 0.3 resulting from FR reflection. The latter refers to actual shade caused by overgrowing vegetation, which leads to a reduction in PAR and a R:FR well below 0.3.

When low R:FR is perceived by a shade sensitive plant, it triggers a response referred to as the shade avoidance syndrome (SAS). SAS describes the phenomenon of changes in plant architecture, with the main characteristics of rapid elongation and height growth at the cost of leaf formation and branching (in addition to other attributes such as reorientation of leaves, described in more detail in chapter 2) (Keuskamp et al., 2010; Pierik & De Wit, 2014). These architectural changes take place before the plant is actually shaded and enable the plant to reach more light, when vegetation grows dense. Therefore, expressing SAS gives plants a fitness advantage, which makes SAS an adaptive trait (Franklin, 2008).

Rice is very sensitive to shade, be it vegetational shade or simply reduced light intensity, and density. As it was described in the previous chapter, rice is impacted by closely growing neighbours in the field, either by weeds or other rice plants, but also by increased planting density. Sensitivity of rice to high density and shade is well documented. Several studies have reported elongation of internodes, reduced tillering and reduced biomass in response to density and shade (Evers et al., 2006; Finlayson et al., 2007; Kebrom et al., 2006; Takano et al., 2001, 2005; Warnasooriya & Brutnell, 2014). This was also observed in our own experiments, where all tested varieties in general had reduced growth. Leaf and tiller formation in addition to culm height and leaf length were reduced in low light conditions. Solidity was lower, tiller angles were smaller and leaves were more erect (unpublished data; BSc thesis Linda Nooren).

Several aspects of light perception and signalling have been studied in rice with mutants and overexpression of phytochromes (phyA, phyB, phyC), phytochrome interacting factor like proteins (PILs) (Garg et al., 2006; Gu et al., 2011; Hu et al., 2020; Iwamoto et al., 2011; Izawa et al., 2000; Liu et al., 2016; Takano et al., 2009). However, a mechanistic understanding of the response to proximity shade induced by low R:FR, is to date vastly unexplored in rice.

Rice together with maize and wheat are the most important crops worldwide, yet, knowledge on SAS in cereals, to date is very limited. A common phenotype of SAS is out-growing neighbours while de-prioritizing investments into harvestable organs, which would compromise the yield of crops (Carriedo et al., 2016; Franklin & Whitelam, 2005; Kebrom & Brutnell, 2007; Liu et al., 2016; Wille et al., 2017). Therefore, it is of particular interest to study the response of rice to low R:FR, as a representative of monocots in general and a cultivated cereal in particular. Here, we are exploring in detail how SAS is manifested in rice from phenotype down to gene regulation.

Results

Supplemental FR effects on rice shoot architecture

To investigate the shade avoidance responses in rice, we grew rice seedlings from different varieties belonging to different subpopulations in the greenhouse. The light environment was a mix of sunlight and artificial white light (WL) with a ratio of red to far-red light (R:FR) of approximately 2.0. The treatment group was exposed to supplemental FR light added to the white light background (WL+FR) lowering the R:FR to 0.2 (detailed description can be found in Materials and Methods, Supplemental Figure 5.1). In this study, we are referring to the treatment as supplemental FR treatment. The investigated varieties were chosen based on their differences in phenotypes in a broad screen of a rice diversity panel described in Chapter 3. Indeed, the selected varieties were already different under the WL conditions, and this was also observed under supplemental FR treatment already at 14 days after sowing (das) (Supplemental Figure 5.2) and was followed up to 28 das (Figure 5.1).

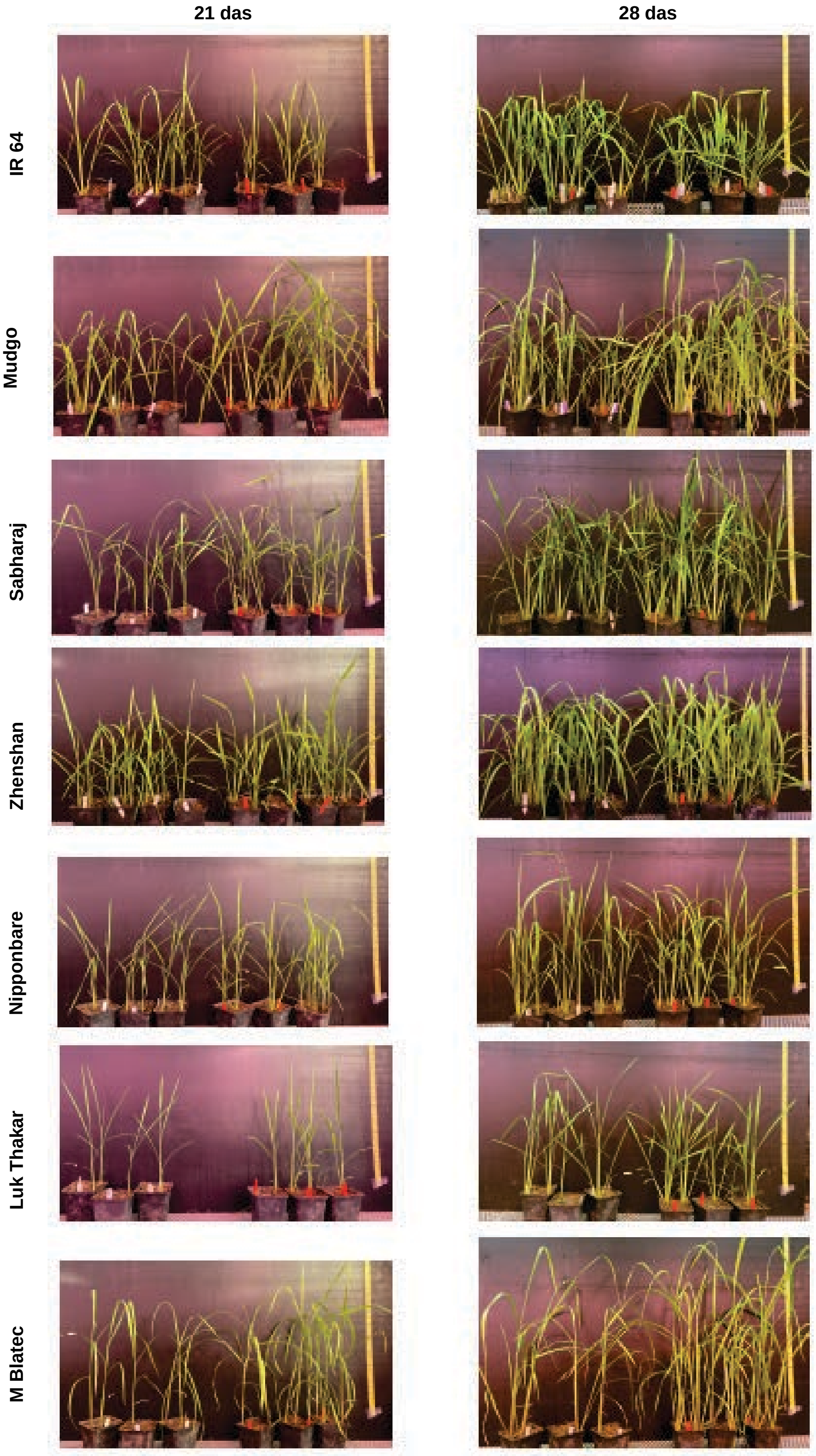


Figure 5.1. Phenotype of rice seedlings exposed to supplemental far-red (FR) at 21 and 28 days after sowing (das) of the varieties of the subpopulations *indica* (IR 64, Mudgo, Sabharaj, Zhenshan), *temperate japonica* (Nipponbare, Luk Thakar) and *admixed* (M Blatec), visualizing their differences in phenotypes under white light (white labels, left) and under FR treatment (red labels, right).

Phenotypic plasticity in response to low R:FR in cultivated rice

In order to probe branching traits, we recorded leaf and tiller number (Figure 5.2). Seedlings of 21-days produced more leaves and more tillers, when exposed to +FR. Nipponbare, Mudgo and Luk Thakar showed the strongest increase. One week later, leaf number and tillering were significantly increased in all varieties, except for IR64 (Supplemental Figure 5.3 A-B). Since many plant species experiencing low R:FR are known to respond with stem or petiole elongation, we additionally measured height and length of different plant organs (Figure 5.3). Culm height was longer in four of the seven varieties at 21 das (Figure 5.3 A), however the pattern was changed one week later (Supplemental Figure 5.3 C), where IR64, Zhenshan and Luk Thakar grow longer in WL. Low R:FR-exposed plants on average had more internodes than WL-grown plants (Figure 5.3 B). Elongation of the internodes, differed between varieties and between the internodes (second and third youngest) that were measured (Figure 5.3 C-D). There was no significant response measurable at the older internode (Figure 5.3 C), in the developmentally younger internode some varieties do show a strong difference (Figure 5.3 D), where some varieties show an elongation response (Mudgo, Sabharaj, M Blatec) but others also show shorter internodes (IR64). Interestingly, M Blatec has shorter older internodes but longer younger internodes, compared to the WL-grown plants. The same applies for leaf length, where the difference between control and treated group are stronger in the younger leaves (Figure 5.3 E-F).

Comparing height growth to branching is an indication for apical dominance, and we observed indeed that under low R:FR conditions, the ratio culm height : number of tillers increased as compared to WL in all varieties except IR64, indicating enhanced apical dominance in low R:FR (Figure 5.4). We also followed changes of leaf inclination angle and leaf erectness upon +FR treatment (Figure 5.5). The leaf inclination angle to the vertical is higher in most of the varieties in low R:FR at 7 das, but this effect almost disappears at a later stage of 21 das (Figure 5.5 A-B). In 7-days old seedlings, most of the varieties had less erect leaves +FR, here as well the effect got milder later on, at 21 das, four out of the tested eight varieties have significant changes, where one variety has more erect leaves (Della) and the others droopier leaves in +FR (Figure 5.5 C-D).

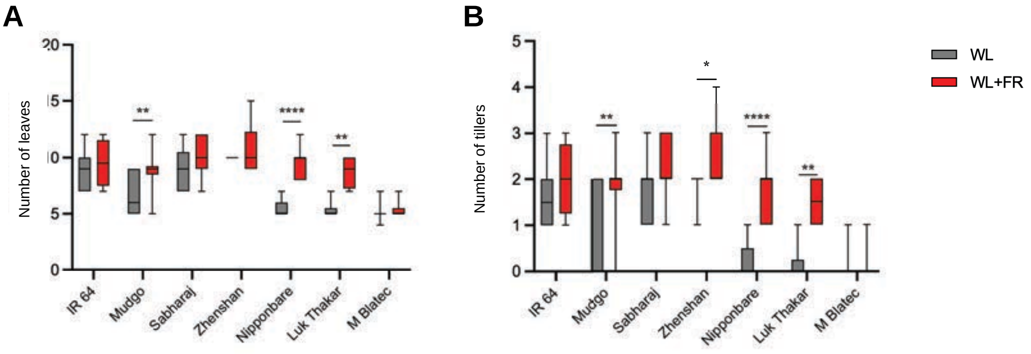
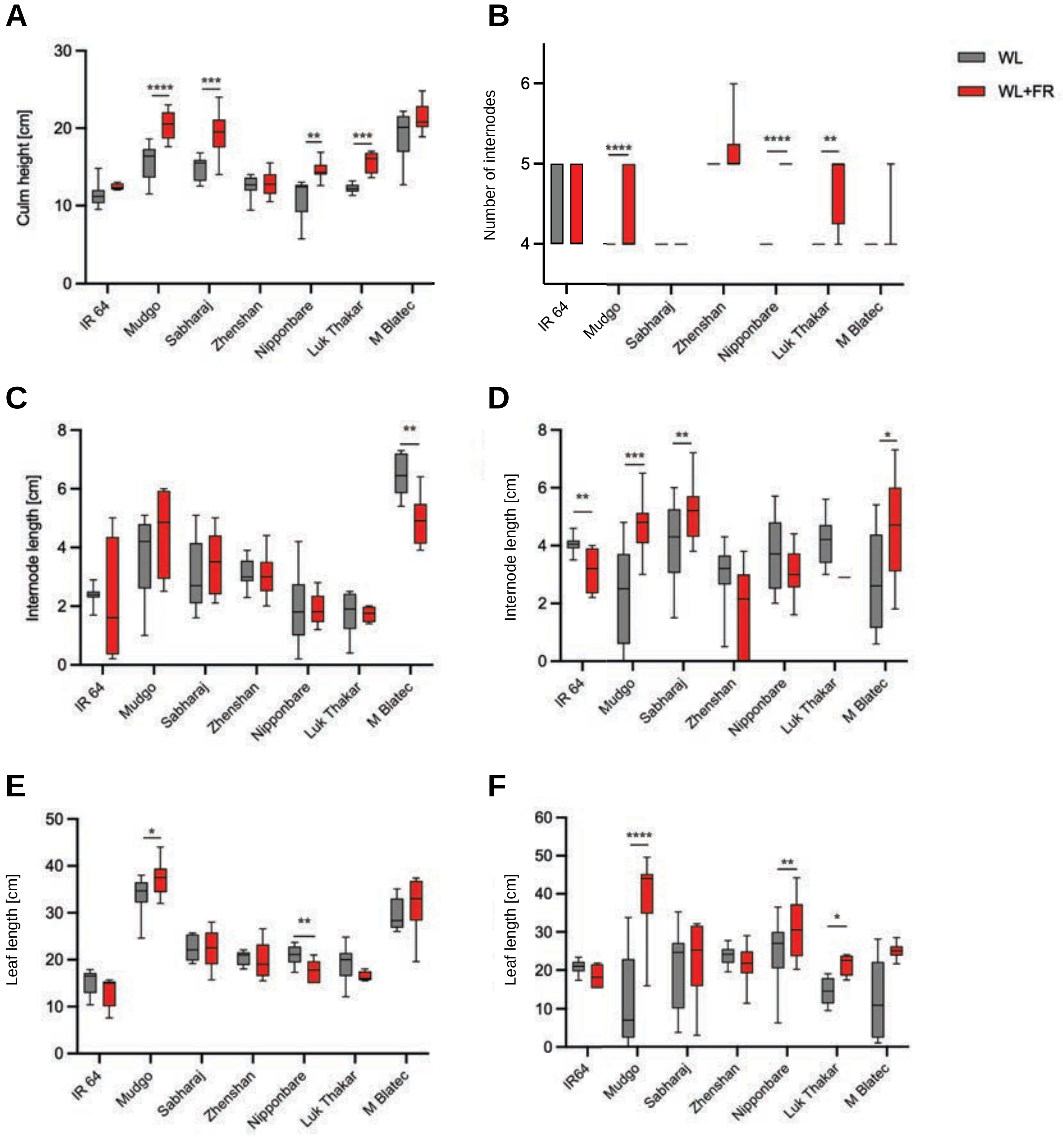


Figure 5.2. Branching traits in response to supplemental FR treatment in different rice varieties at 21 days after sowing (das). Comparison of **A.** leaf number and **B**. tillering of seven different rice varieties: IR 64, Mudgo, Sabharaj, Zhenshan, Nipponbare, Luk Thakar, M Blatec grown in control (WL) and supplemental FR (WL+FR). Values are means 6 - 14 plants; boxes indicate IQ-range with error bars of 2.5 – 97.5 percentile; significant differences are indicated with p-value < 0.05 *, < 0.01 **, < 0.001 *** .



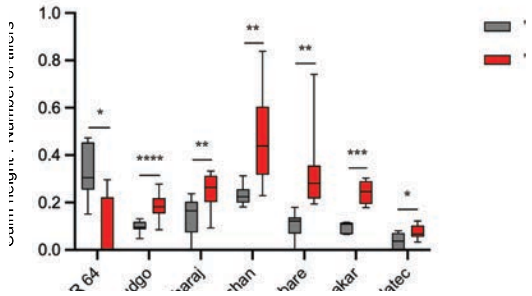


Figure 5.3. Elongation traits in response to supplemental FR treatment in different rice varieties at 21 days after sowing (das). Comparison of **A.** culm height B. number of internodes, length of **C.** third and **D.** second youngest internode and length of **E.** third and **F.** second youngest leaf of plants grown in control (WL) and supplemental FR (WL+FR). Values are means 6 - 14 plants; boxes indicate IQ-range with error bars of 2.5 – 97.5 percentile; significant differences are indicated with p-value < 0.05 *, < 0.01 **, < 0.001 ***.

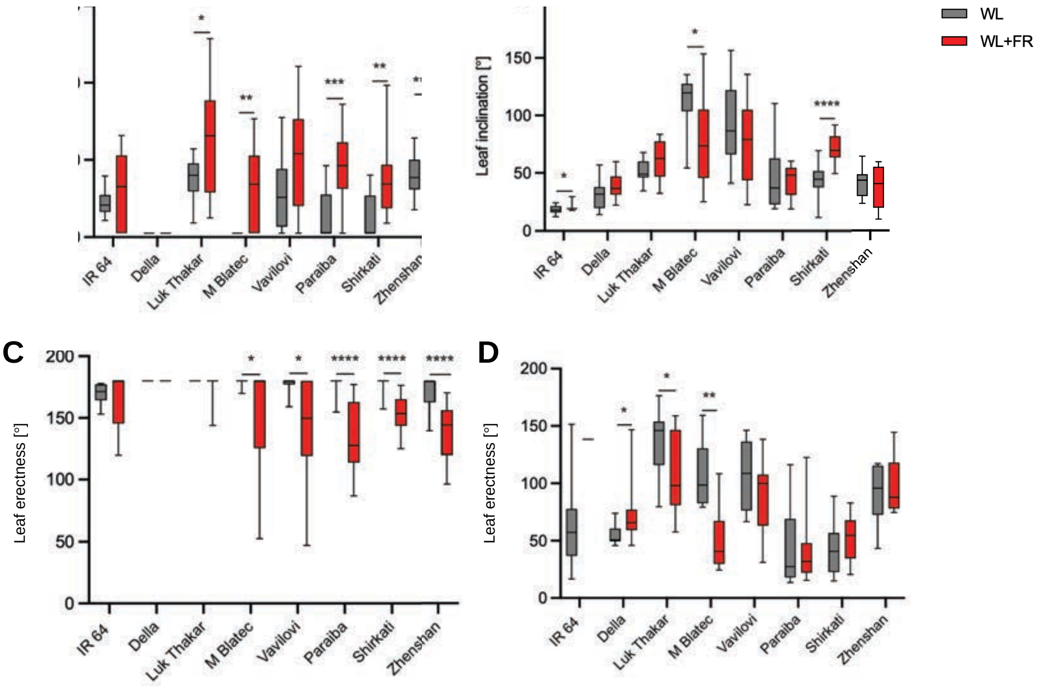


Figure 5.4. Apical dominance of plants grown in control (WL) and supplemental FR (WL+FR), as the ratio of culm height to number of tillers at 28 das. Values are means 6 - 14 plants; boxes indicate IQ-range with error bars of 2.5 – 97.5 percentile; significant differences are indicated with p-value < 0.05 *, < 0.01 **, < 0.001 ***.

◁ **Figure 5.5. Leaf inclination angle (A-B) and leaf erectness (C-D)** of plants grown in control (WL) and supplemental FR (WL+FR) at 7 days after sowing (das) and at 21 das, respectively. Values are means 10 - 14 plants; boxes indicate IQ-range with error bars of 2.5 – 97.5 percentile; significant differences are indicated with p-value < 0.05 *, < 0.01 **, < 0.001 ***. Bigger value for inclination angle = more open; 90° = horizontal; bigger value in erectness = less droopy; 180° = erect.

FR response in other species and wild rice

Given the observation that the cultivated rice responses to low R:FR were of very modest magnitude, even when exposing them for several weeks, we verified if in this greenhouse set-up we could still observe the expected stronger responses in other species. Therefore, we tested plants of other species, that were known to show strong SAS. We selected tomato (*Solanum lycopersicum)* and maize (*Zea mays*) (Supplemental Figure 5.4 A, C). Seedlings of both species were strongly elongated after 21 days of +FR treatment (Supplemental Figure 5.4 B). Since the set-up clearly was suitable for SAS induction in other species, we verified if the mild responses of rice were specific for cultivated rice, or also a feature of wild rice species. We, therefore, included wild ancestors of our tested cultivated *Oryza sativa* varieties: two *Oryza nivara* and two *Oryza rufipogon* varieties (Figure 5.6 A-B). As seen before in the tested *O. sativa* species, all wild rice varieties responded with increased tillering in +FR (Figure 5.6 C). Interestingly, the two *O. nivara* varieties showed opposing effects on culm height, one with longer and one with shorter culms under +FR treatment and the same applies in a milder version, for *O. rufipogon* varieties (Figure 5.6 D). Importantly, the responses of the ancestral species were of similar magnitude as those observed in the cultivated varieties.

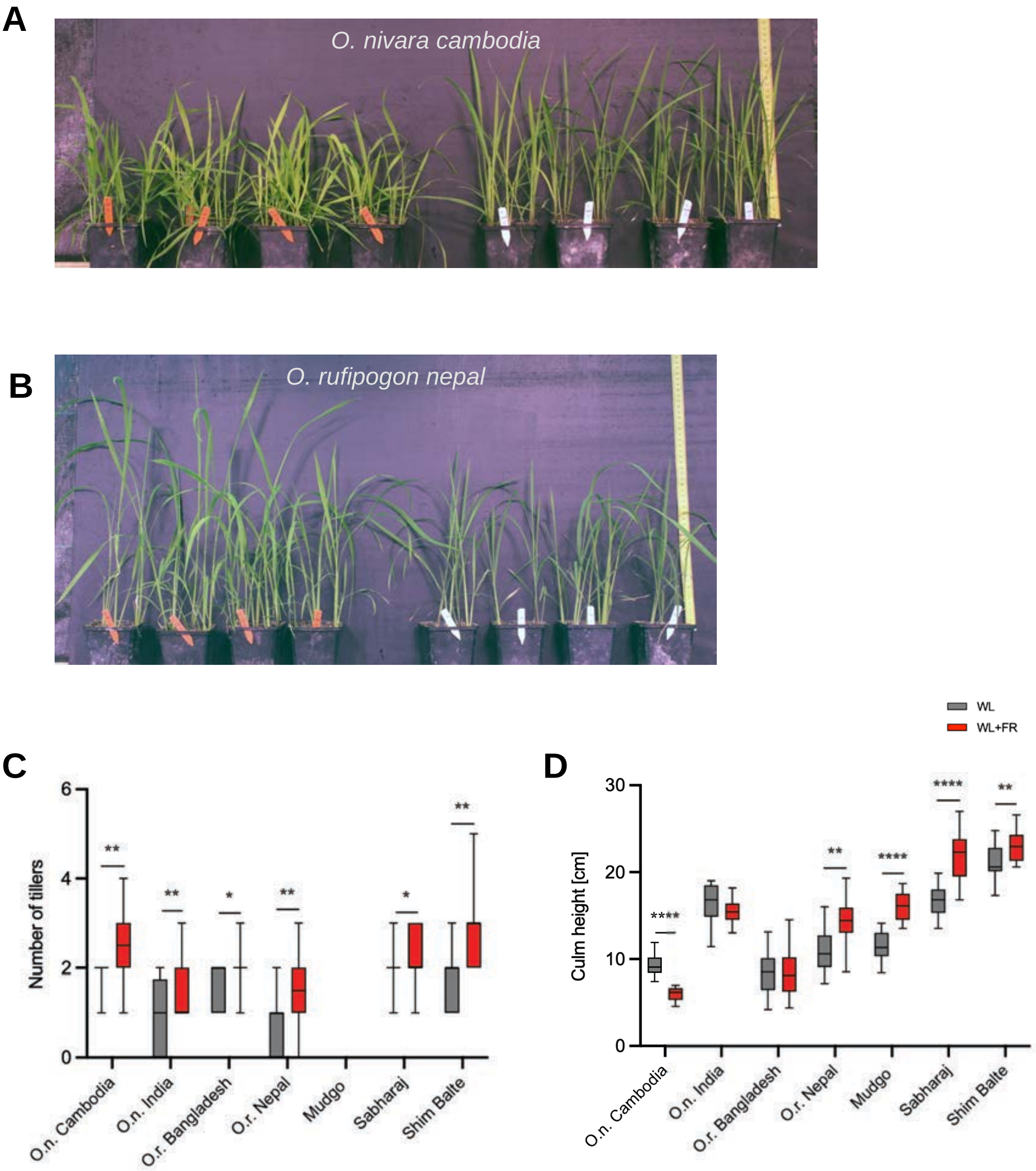


Figure 5.6. Wild rice shows similar response to +FR as cultivated rice. Three *Oryza sativa* varieties (Mudgo, Sabharaj, Shim Balte) were compared to four wild rice varieties, with two of the *Oryza nivara* (*india* and *cambodia* – **A.** representative image) and two *Oryza rufipogon* varieties (*bangladesh* and *nepal* – **B**. representative image) in their response to +FR for **C.** tillering and **D.** culm height. Values are means 12 - 24 plants; boxes indicate IQ-range with error bars of 2.5 – 97.5 percentile; significant differences are indicated with p-value < 0.05 *, < 0.01 **, < 0.001 ***, < 0.0001 ****.

FR response at lowered temperature

It was found previously that elevated temperature triggers a phenotype that is reminiscent of shade avoidance in Arabidopsis, and this acts through the phyB-PIF pathway, similar to low R:FR responses (Casal and Balasubramanian, 2019; Romero-Montepaone *et al.*, 2020, (Legris *et al.*, 2016). We therefore hypothesized that under the high temperatures that we used to grow rice, potentially there could be constitutive activation of the pathway already, which would explain the very weak shade avoidance responses to low R:FR. We, therefore, also performed a low R:FR experiment in a growth chamber where we reduced the temperatures by 5 °C from a 30 °C / 25 °C to a 25°C / 20 °C day-night regime (Figure 5.7).

Leaf number and tiller number were marginally affected, but generally increased under supplemental FR treatment (Figure 5.7 A-B). Culm height was not increased in response to +FR, except for M Blatec, and contrasting response with decreased culm height occurred in IR64 and Zhenshan (Figure 5.7 C). The observation of contrasting responses in height was similar to the results in the greenhouse (Supplemental Figure 5.3 C). Leaf length was decreased in +FR for some varieties (Figure 5.7 D). Length of second and third youngest internode (Figure 5.7 E-F), was consistent with observations in the greenhouse, where the younger tissue shows stronger response, with consistently shorter internodes in +FR. Importantly, all effects again were very modest, indicating that the ambient temperature is not likely explaining the modest rice response to low R:FR.

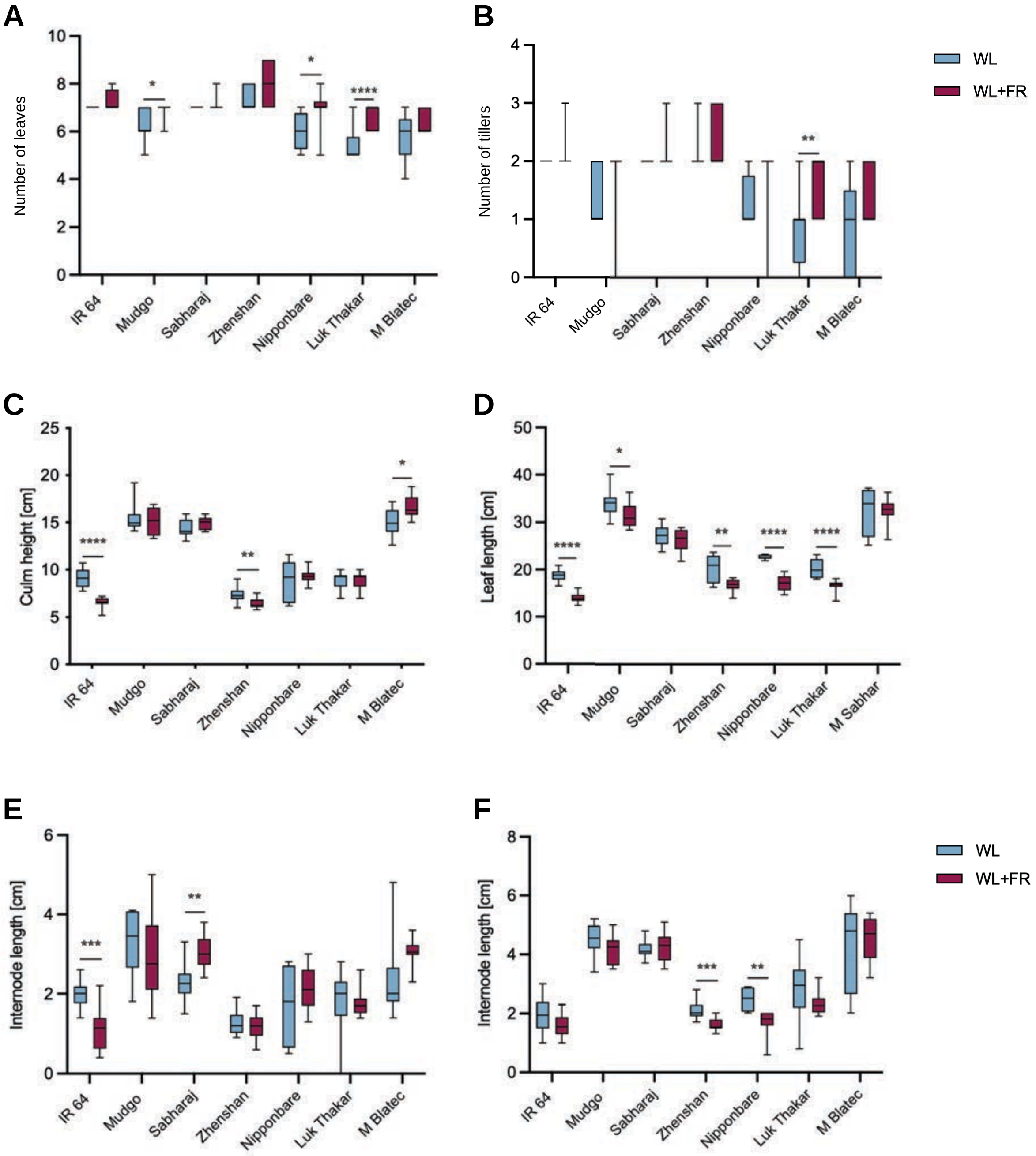


Figure 5.7. Effect of lowered temperature on phenotypic response to supplemental FR in different rice varieties at 21 days after sowing (das). Comparison of **A.** leaf number, **B.** tillering, **C.** culm height, **D.** length of second youngest leaf and **E.** second and **F.** third youngest internode. Rice plants were grown in control (WL) and supplemental FR (WL+FR) at 25 °C day and 23 °C night temperature. Values are means 10 - 14 plants; boxes indicate IQ-range with error bars of 2.5 – 97.5 percentile; significant differences are indicated with p-value < 0.05 *, < 0.01 **, < 0.001 ***.

Genome-wide transcriptomic responses to low R:FR

Differential gene expression upon +FR in rice seedlings of different varieties

To get insight into the transcriptomic regulation under low R:FR, we carried out an RNAseq analysis on 5-day old rice seedlings of six different varieties exposed for 24 hours to supplemental FR or kept under control conditions. Total RNA was extracted from the whole shoot material of four biological replicates and sequenced as described in Materials and Methods. To get an overall impression of the transcriptomic data, we performed a PCA (principle component analysis) on the normalized and filtered CPMs per sample (six varieties, two treatments, four replicates) (Figure 5.8). This unbiased approach shows that samples mostly cluster for variety, and that treatment is not really determining a segregation (Figure 5.8 A-B), neither are replicates explaining the variation (Supplemental Figure 5.6.C). When zooming in on the top 500 highest expressed genes the patterns stay the same, with a clear clustering for varieties but not for treatment. This is the case for all varieties, except for IR64 (Figure 5.8 C). We then zoomed in on the genes that differ in their abundance between control and treatment to investigate transcriptomic changes in response to +FR treatment. After this selection, treatment was mostly explaining PC2 of the PCA analysis (with 18 % of variation), which is still a low degree determining segregation, but more pronounced than in the unbiased approach (Supplemental Figure 5.6 A, B). The variation of samples, only for CPMs for 434 DEGs of unique response, is still mostly determined by variety, and treatment only as a secondary effect (Figure 5.9 A). PC1 is explained by variety (36 %) and PC2 is by treatment (18 %) (Figure 5.9 B).

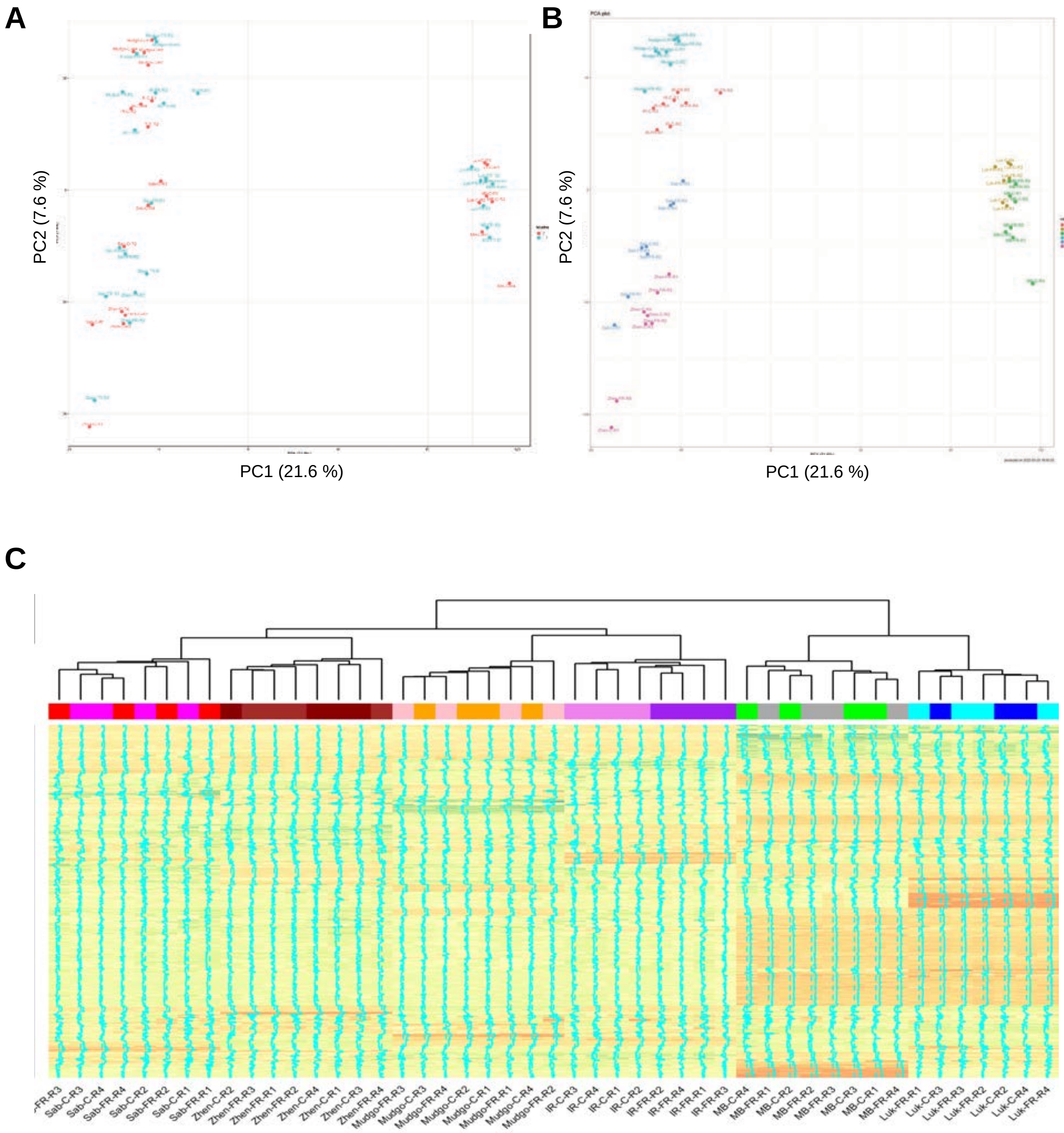


Figure 5.8. CPMs of total reads after normalizing and filtering. Principle Component Analysis (PCA) showing contribution to sample variation with colour code for **A.** treatment and **B.** varieties; PC1 on x-axis and PC2 on y-axis. **C.** Hierarchical clustering of samples based on top 500 expressed genes. Abbreviation of varieties: Luk – Luk Thakar, MB – M Blatec, IR – IR64, Sab – Sabharaj, Zhen – Zhenshan. Treatment groups with white light grown control (C) and supplemental FR light (FR) grown plants; biological replicates: R1 – 4.

We then focussed on the unique DEGs per variety and for the two groups of subpopulations: Luk Takhar (2) and M Blatec (9) are *japonicas* and Mudgo (27), IR64 (29), Sabharaj (35) and Zhenshan (31) are *indicas*. The DEGs of *indicas* and *japonicas* are not just the sum of their belonging varieties, when calculated separately for the subpopulations, with FR treatment as main effect, this results in a higher number of DEGs, with 319 and 21 DEGs, respectively (Figure 5.9 C-D). It is striking, how little the two *japonica* varieties respond in their gene regulation to the +FR treatment; with only two DEGs Luk Thakar is basically not responsive. The number of DEGs for the *indica* varieties is overall higher than for the *japonicas*. We found four DEGs unique to M Blatec and IR64, and three unique for Zhenshan. Only one gene is in common for all the varieties (LOC_Os09g27750, encoding an ethylene-forming enzyme) and three more genes are shared among five out of the six tested varieties (LOC_Os04g58200, for PROTOCHLOROPHYLLIDE OXIDOREDUCTASE A; LOC_Os04g41130, protein of phosphatidyl-ethanolamine-binding protein family; LOC_Os03g37450, na) (Figure 5.9 C). The bar plot in Figure 5.9 D visualizes the proportion of up and down regulated DEGs per variety, subpopulation and for the 379 DEGs as a general response to +FR treatment.

To get a better understanding of how a general response to +FR treatment is regulated in rice seedlings, we further investigated the relative abundance of transcripts (shown as $\log_2$ of the CPMs in Figure 5.10) and the strong influence of genetic background on the gene regulation. The hierarchical clustering analysis revealed a separation of control versus +FR samples in all varieties (indicated with grey and dark red on the horizontal axis), with *japonicas* (Luk Thakar and M Blatec) splitting from *indicas* (Figure 5.10 A). This separation of subpopulations is consistent for both, control and +FR treated plants. The clustering analysis of the abundance of transcripts resulted in seven main clusters (indicated by grey boxes on the vertical axis). The *indica* group (IR64, Mudgo, Sabharaj, Zhenshan) showing separated clusters in +FR with a distinct group of transcripts highly abundant (box A, cluster 3) and a distinct group of low abundance (box B, cluster 7-8) in +FR compared to control. Also, the transcriptional change in response to +FR compared to control, i.e. how much more or less a certain gene is expressed upon treatment (shown in Supplemental Figure 5.7 as $\log_2$ of the fold changes) shows the same pattern, where the *japonicas* are clustering from *indicas*. Dynamics of specific up and downregulation of the indicas and japonicas (Figure 5.10. B, C, respectively) were analysed separately.

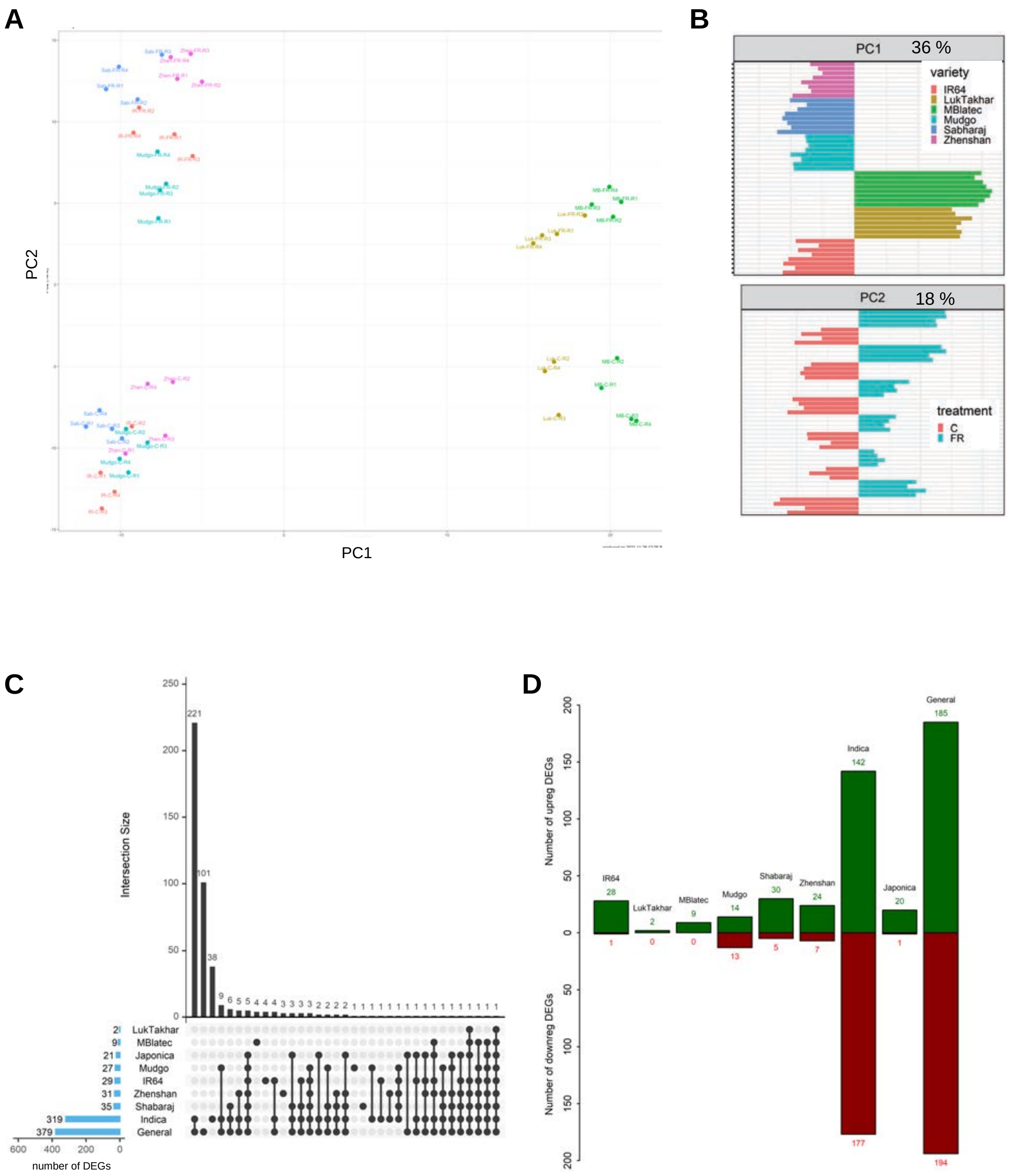


Figure 5.9. Differential gene expression upon supplemental FR exposure in rice seedlings. A. PCA of CPMs of 434 DEGs for unique FR response with FDR < 0.05 with colour code for variety, PC1 on x-axis and PC2 on y-axis with **B.** detailed contribution per **PC**. Treatment groups with white light grown control plants (C) and supplemental FR light (FR) grown plants; biological replicates: R1 – 4. **C.** Shared DEGs for general FR response with FDR < 0.05 and **D.** number of up and down regulated genes between different groups of subpopulations and varieties.

Identities of DEGs found for +FR response

In order to probe the identities of the individual DEGs, we looked into the strongest up and down regulated genes among the 434 DEGs that were found for unique response to +FR in young rice seedlings (DEGs sorted for min and max $\log_2$FC of CPMs with FDR < 0.05) (Figure 5.11 A). The strongest up regulated genes are described to encode FLOWERING PROMOTING FACTOR 1 (FLP1) and FLP1-LIKE PROTEIN (LOC_Os02g26210 and LOC_Os07g47450, respectively). Among the 15 most up regulated DEGs were genes encoding AMINOPHOSPHOLIPID ATPase 1 (LOC_Os08g30380), RAC-LIKE GTP-BINDING PROTEIN 2 (LOC_Os01g35850), HISTONE H2A 12 homologue (LOC_Os04g13530), a member of the PHOSPHATIDYL-ETHANOLAMINE-BINDING protein family (LOC_Os04g41130) and a FAR1-related protein (LOC_Os03g42970), DEGs also strongly up regulated with a higher significance are PHOTOSYSTEM II subunit R and an ethylene-forming enzyme.

The most strongly down regulated gene was LOC_Os01g04510, which is not yet described. The second most down regulated gene is LOC_Os10g26940, encoding the beta subunit of POLYGALACTOURINASE 1, involved in abiotic stress response. Interestingly, among the most down regulated genes we found LOC_Os07g08150, a homologue of the Arabidopsis Chlorophyll A-B binding protein family, and LOC_Os03g12660, CYTOCHROME P450 90B2, described as BRASSINOSTEROID C-22 HYDROXYLASE, involved in Brassinosteroids biosynthesis. Other strongly down regulated genes are those encoding, ALDEHYDE OXIDASE 1 homologue (LOC_Os03g57720), a Peroxidase superfamily protein (LOC_Os07g48020) and an alpha/beta-Hydrolases superfamily protein (LOC_Os04g09540). Many of the detected DEGs have not yet been characterized and are only hypothetical putative proteins and/or not found to have Arabidopsis homologues.

Finally, we performed a gene ontology (GO) enrichment analysis where the detected DEGs are grouped together into categories according to their biological processes they are contributing to. The defined GO groups are sorted for their significance (smallest FDR) (Figure 5.11 B). Surprisingly, the five most significantly enriched GOs are all associated with photosynthesis, whereas GO enrichments with slightly less significant enrichment are related to SAS, with processes involved in Auxin signalling and response pathways. Additionally, the GO localization analysis shows that virtually all gene products of the significant GO categories are localized in the Chloroplast (Figure 5.11 C).

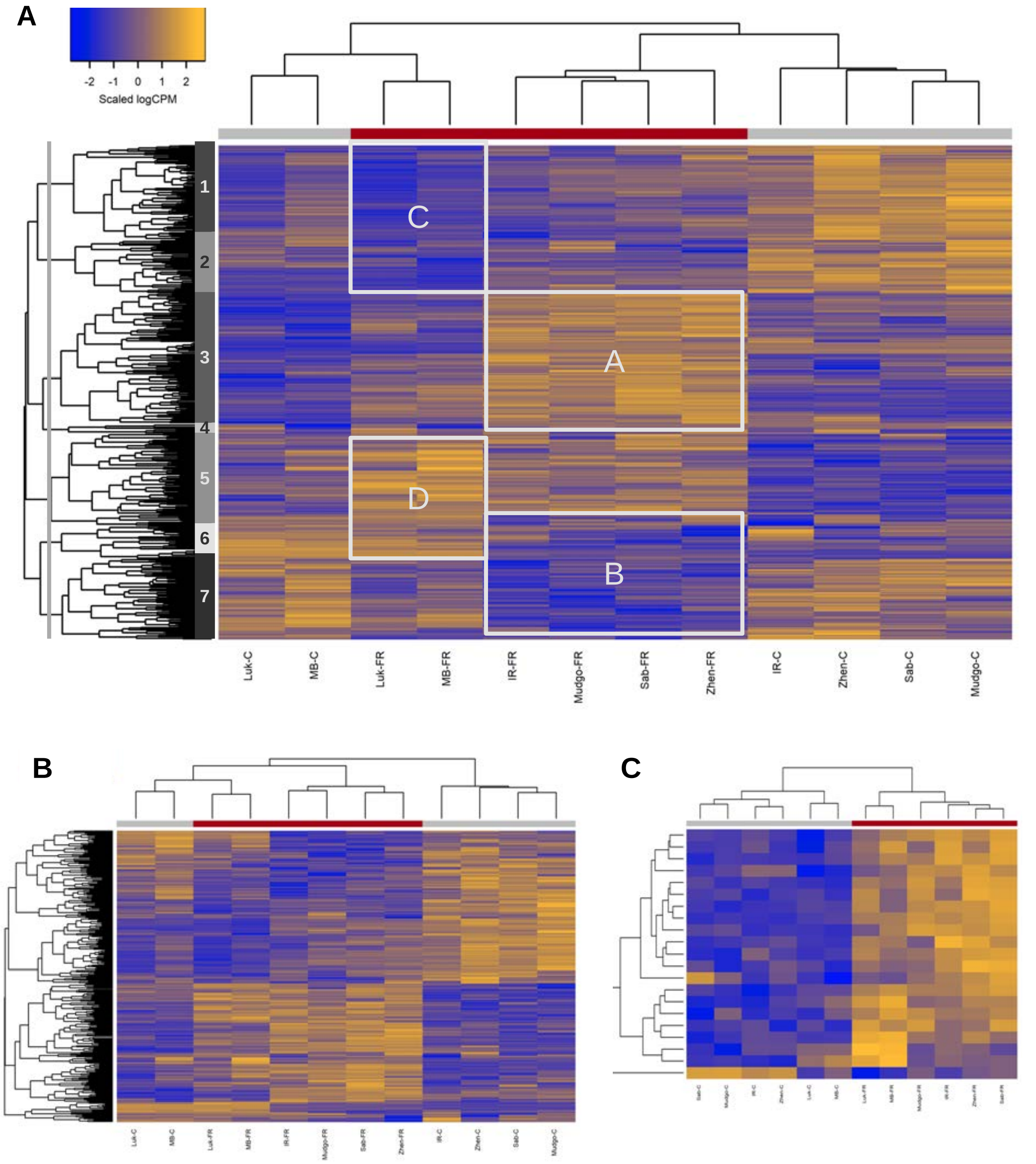


Figure 5.10. Heatmap and clustering for CPMs of differentially expressed genes (DEGs) upon supplemental FR treatment, of A. all DEGs found for unique response (434 DEGs); **B.** indica response, with 319 specific DEGs and **C.** japonica response, with 21 specific DEGs; with an FDR < 0.05 and no logFC threshold. Colour gradient for log2CPMs from blue to yellow for low to high abundance; grey and red indicating control and +FR treatment in bar on top of heatmap. Abbreviation of varieties: Luk – Luk Thakar, MB – M Blatec, IR – IR64, Sab – Sabharaj, Zhen – Zhenshan. Treatment groups with white light grown control (C) and supplemental FR light (FR) grown plants.

Gene_ID	logFC	fdr	Gene.name	Gene.description	Best.arabi.name	arabi.symbol	arabi.defline
LOC_Os07g47450	5.79	0.00	-	-	AT4G31380.1	FLP1	FPF1-like protein 1
LOC_Os11g19270	5.76	0.00	-	-	-	-	-
LOC_Os07g14460	5.26	0.00	-	-	-	-	-
LOC_Os10g01420	5.26	0.00	-	-	-	-	-
LOC_Os03g37450	4.32	0.00	-	-	-	-	-
LOC_Os08g30380	4.16	0.00	-	-	AT5G04930.1	ALA1	aminophospholipid ATPase 1
LOC_Os05g32770	3.79	0.00	-	-	-	-	-
LOC_Os02g26210	3.70	0.00	-	-	AT5G24860.1	ATFPF1,FPF1	flowering promoting factor 1
LOC_Os01g35850	3.68	0.01	-	Rac-like GTP-binding protein 2	AT4G28950.1	ARAC7,ATRAC7,ATROP9, RAC7,ROP9	RHO-related protein from plants 9
LOC_Os08g10870	2.98	0.00	-	-	-	-	-
LOC_Os04g13530	2.84	0.00	-	Similar to Histone H2A	AT5G02560.1	HTA12	histone H2A 12
LOC_Os03g17200	2.58	0.00	-	Similar to plant-specific domain TIGR01589 family protein	AT3G55240.1	AT3G55240.1	Plant protein 1589 of unknown function
LOC_Os04g21350	2.45	0.00	-	-	AT5G10625.1	AT5G10625.1	-
LOC_Os04g41130	2.42	0.00	-	-	AT1G65480.1	FT	PEBP (phosphatidylethanolamine-binding protein) family
LOC_Os03g42970	2.41	0.02	-	-	AT4G38180.1	FRS5	FAR1-related sequence 5
LOC_Os03g12660	-1.10	0.01	OsDWF4	Cytochrome P450 90B2, Brassinosteroid C-22 hydroxylase, Brassinosteroids biosynthesis	AT3G50660.1	CLM,CYP90B1, DWF4,PSC1,SAV1,SNP2	Cytochrome P450 superfamily protein
LOC_Os04g09540	-1.17	0.01	-	Similar to Acyl-protein thioesterase 2	AT1G52700.1	AT1G52700.1	alpha/beta-Hydrolases superfamily protein
LOC_Os07g48020	-1.17	0.01	-	-	AT5G05340.1	AT5G05340.1	Peroxidase superfamily protein
LOC_Os03g57720	-1.30	0.03	-	-	AT5G20960.1	AAO1,AO1,Aoalpha, AT-AO1,ATAO,AtAO1	aldehyde oxidase 1
LOC_Os11g35540	-1.31	0.00	-	-	-	-	-
LOC_Os07g08150	-1.36	0.01	-	Similar to Low molecular mass early light-inducible protein HV90, Chloroplast precursor (ELIP)	AT3G22840.1	ELIP,ELIP1	Chlorophyll A-B binding family protein
LOC_Os02g46500	-1.46	0.00	-	-	AT2G35930.1	PUB23	plant U-box 23
LOC_Os05g03960	-1.53	0.02	-	-	-	-	-
LOC_Os10g26940	-2.06	0.00	RD22	beta subunit of polygalacturonase 1, Abiotic stress respons	AT1G70370.1	PG2	polygalacturonase 2
LOC_Os01g04510	-2.12	0.04	-	-	-	-	-

ID	Term ID	Term Name	p_{adj} (query_1)
1	GO:0015979	photosynthesis	4.156×10^{-7}
2	GO:0015995	chlorophyll biosynthetic process	9.242×10^{-5}
3	GO:0046148	pigment biosynthetic process	6.024×10^{-4}
4	GO:0015994	chlorophyll metabolic process	9.219×10^{-4}
5	GO:0042440	pigment metabolic process	2.415×10^{-3}
6	GO:0033014	tetrapyrrole biosynthetic process	2.638×10^{-3}
7	GO:0009734	auxin-activated signaling pathway	6.128×10^{-3}
8	GO:0071365	cellular response to auxin stimulus	6.584×10^{-3}
9	GO:0006779	porphyrin-containing compound biosynthetic pr...	6.644×10^{-3}

Term ID	Term name	$-\log_{10}(p_{adj})$
GO:0009507	chloroplast	
GO:0009536	plastid	
GO:0009570	chloroplast stroma	
GO:0009526	plastid envelope	
GO:0009532	plastid stroma	
GO:0009523	photosystem II	
GO:0042170	plastid membrane	
GO:0042651	thylakoid membrane	
GO:0009521	photosystem	
GO:0034357	photosynthetic membrane	
GO:0043231	intracellular membrane-bounded organelle	
GO:0043227	membrane-bounded organelle	
GO:0010287	plastoglobule	

Figure 5.11. Investigation of biological processes and function of specific DEGs. A. 15 strongest up and 10 strongest down regulated genes (with highest and lowest logFC). **B.** Gene ontology categories of biological processes for DEGs in +FR response and **C.** localization in cell compartments of GOs; based on 434 DEGs found for unique response with FDR < 0.05.

Discussion

The main aim of this study was a first exploratory investigation of how shade avoidance in response to low R:FR is manifested in rice, not only on a phenotypic but also on a transcriptomic level. For this, we exposed rice seedlings to supplemental FR light from right after germination until four weeks of growth and recorded several shoot related traits. Samples for transcriptomic analysis were taken from 5-day old seedlings undergoing a 24 hour +FR treatment.

Modest shade avoidance phenotypes in rice seedlings

A typical characteristic of SAS is enhanced elongation (height) growth at the cost of branching. What we observed, however, in rice seedlings was the opposite, with more leaves and tillers and no significant elongation response. Hence, also the ratio of height to branching was lower in +FR treated plants compared to WL-grown plants, which is contrary to the known SAS manifestation. Measurements on different tissues, revealed that it was mostly the tissue that was just formed in low R:FR and is developmentally younger which was responding stronger, where in the same tissue at a later stage the differences to control grown plants disappear or are even reversed.

Based on our results, where we investigated a selection of phenotypically different rice varieties of different subpopulations, we could not see a clear common response to supplemental FR for the traits we measured. The response is very much dependent on the variety, i.e. the phenotype is mainly determined by genetic background rather than FR treatment. Depending on the variety there is an increase or decrease in culm height, length of youngest internode and length of youngest leaf in response to FR compared to control; or no response at all. The increased number of internodes could partly explain longer culms in +FR, rather than an elongation of internodes (Figure 5.3 A-D). It appears that the low R:FR-response phenotypes cannot directly be predicted from the phenotype under control light conditions (Figure 5.1). However, what is consistently found to be in common, is an increase in leaf number and tillering under +FR conditions (Figure 5.2). Also, there is a trend towards less erect leaves and wider leaf inclination angles, however only at the very early seedling stage (Figure 5.5).

The responses strongly vary between varieties, where some are responding in opposing ways to the typical SAS and some do not show a response. This indicates a dynamic in the manifestation of +FR treatment, which is determined by genetic background specific to a variety.

Transcriptome responses to supplemental FR in rice varieties

Generally, in our RNAseq study we observed very mild responses to the +FR treatment, and much stronger signatures between varieties (Figure 5.7). Differential gene expression is mainly determined by variety not by treatment, and other possible factors are only marginally explaining the clustering of samples, such as biological replicate, natural habitat or phenotypic response (Figure 5.8. A-B, Supplemental Figure 5.6 C).

The Mudgo variety, when compared to the other tested varieties, has the strongest response in phenotype with increased elongation growth, and the generally observed increased leaf and tiller number. Mudgo was also among the varieties with stronger changes in gene expression response to +FR. Sabharaj is phenotypically and transcriptionally very similar to Mudgo, with a slightly stronger transcriptomic response (with the highest number of DEGs), but less explicit phenotypical responses (Figure 5.1, 5.3 and 5.8). The varieties Zhenshan and M Blatec differ strongly in their phenotype in control, where Zhenshan with a compact and short statured shoot and M Blatec having very long and droopy leaves. This contrast is maintained in their response to +FR, where Zhenshan decreases culm height and M Blatec increases elongation of culm and internode (Figure 5.3). This contrast is also manifested in the transcriptomic signature (Figure 5.10), with opposite regulation of the same genes in addition to regulation of different genes. IR64 and Zhenshan are most similar in their phenotypes, and in their gene expression. M Blatec and Luk Thakar show a very similar response in their phenotype with decreased trait values for elongation and height and only mild increase in leaf number and tillering. Luk Thakar overall shows a significant response with shorter culms and leaf length. In fact, Luk Thakar and also IR64, show a phenotype in +FR, which is in the opposite direction to what would be expected for SAS, with decrease in elongation and increase in branching. Interestingly, these two varieties are very different in their transcriptomic signature, in their constitutive gene expression under control conditions and as well as in terms of differential gene expression upon +FR (clustering in Figure 5.10 A), and also in terms of number of DEGs (Figure 5.9 C) with Luk Thakar basically not responding at all.

Investigating DEGs

In this dataset, we found the genes for FLP1 and FLP1–LIKE protein among the strongest up-regulated genes. In addition to its role in initiation of flowering, in rice it is also involved in internode elongation during the reproductive phase in rice. Strongly induced was also the transcription of the genes encoding members for PHOTOSYSTEM II subunit R. This is interesting, because FR photons can only be absorbed by Photosystem I (Zhen et al., 2021; Zhen & van Iersel, 2017), where it leads to excitation of electrons, which are then replenished by electrons from Photosystem II (Yamazaki, 2010). It has been found in other species that with enriched FR conditions the ratio of Photosystem II / I is increased (Tan et al., 2022). Among the strongest up-regulated genes, we found also are two genes described in rice to be involved in ethylene biosynthesis (*ACC OXIDASE 1* and *2*). It has been found that Ethylene plays a role in SAS (Kegge et al., 2015; Pierik et al., 2003, 2006). The strongest down regulated gene was *POLYGALACTURONASE 2*, whose protein product is involved in cell wall loosening. This is surprising, since increased growth via cell elongation would typically involve cell wall loosening (Sasidharan et al., 2008). Similarly surprising, was to find genes involved in photosynthetic activity in the most up, as well as in the most down-regulated genes, such as Chlorophyll A-B binding family protein and photosystem II light harvesting complex gene *B1B2*. *CYTOCHROME P450* was also among the most down-regulated genes, which is described to have a Brassinosteroid C-22 hydroxylase function in rice.

However, there are some of the known SAS involved players, that are slightly up-regulated. Among the highest are genes involved in auxin signalling and biosynthesis, such as *YUCCA6* (a homologue of Arabidopsis *YUCCA9*) and *OsSAUR30*, which are described to have a similar function in rice as in Arabidopsis. A very prominent candidate is *PHYTOCHROME INTERACTING FACTOR 3* homologue, where a similar function in rice as in Arabidopsis is not yet confirmed.

Different from the five phytochromes identified in Arabidopsis, grasses only have three phyA, phyB and phyC (Sawers et al., 2005). A triple mutant, where all three phytochromes are non-functional, showed the same phenotype as wild type plants grown in darkness, because they cannot perceive light (Takano et al., 2009). PhyA is essential for perception of FR light, but not perceiving red light (Takano et al., 2001). PhyA is involved in regulation of plant height and internode elongation as well as branching. *PHYA* overexpression led to reduced growth and a reduction in plant height as well as branching (Garg et al., 2006; Kong et al., 2004; Yun-jia & Liesche, 2017).

Also in rice, it has been found, that phyB plays a major role in suppressing SAS. PhyB mutants exhibit a constitutive SAS phenotype also under control light conditions, with increased plant height and enhanced apical dominance (Kebrom et al., 2006). In addition, phyB regulates the stomatal density and stomatal length in rice (Gu et al., 2011). However, in contrast to these studies, it was also found that phyB mutants in rice showed decreased internode elongation (Takano et al., 2009) and decreased plant height under control light (Sun et al., 2017), which would suggest that phyB might have a different role in SAS in rice, compared to other species. PhyB is the receptor for R:FR, phyA only responds when R:FR is lower than 0.3 and together with low PAR, so it could be that phyB does not trigger the SAS pathway in rice but only phyA.

Why does rice show such a mild SAS response?

We consistently documented a very minimal response, both on phenotypic and on transcriptomic level of rice exposed to +FR. Based on follow up studies with wild rice and +FR treatment under growth conditions with lowered temperature, we need to reject the hypotheses of rice having lost the ability to respond to low R:FR during domestication, nor does temperature seem to play an inhibitory role. This is at least true for our experimental conditions and the limited number of varieties tested.

It is intriguing, that rice is a species that is strongly responding to low light intensity and planting density, but not to low R:FR. This raises the question to what it is responding, i.e. which environmental cue is perceived and translated into a phenotypic response. It could for example be, that low blue light intensity is more relevant in rice, or that a response is only triggered, when actual physical contact to neighbouring plants occurs; both are described as cues involved in neighbour detection (De Wit et al., 2012; Keller et al., 2011; Keuskamp et al., 2011; Pantazopoulou et al., 2017, 2021). The strong regulation of processes related to photosynthesis and most of the transcriptomic products being localized in the chloroplast, are hinting at other processes strongly interacting with commonly described SAS pathways (Figure 10 B-C).

Comparison of rice with other monocots and cereals

It would be plausible to assume, that grasses in general are not responsive to proximity shade. However, reduced bud outgrowth, a highly conserved characteristic trait of SAS, is observed in many plants, including grasses (Evers et al., 2006; Kebrom & Brutnell, 2007; Tan et al., 2022; Whipple et al., 2011). Proximity shade, mimicked by low R:FR, results as suppressed tillering in different grass species (Casal et al., 1986; Dubois & Brutnell, 2009; Ugarte et al., 2010). Several crop cereals have been studied in their response to low R:FR treatments. Sorghum (*Sorghum bicolor*) showed inhibited bud outgrowth (Kebrom et al., 2006) in addition to leaf sheath elongation (Finlayson et al., 2007). Studies in barley (*Hordeum vulgare)* reported reduced tillering and elongation of leaf blade (Skinner & Simmons, 1993) under low R:FR. Similarly, cessation of tillering was documented in wheat (*Triticum aestivum*), however results of elongation response were not found consistently (Evers et al., 2006; Ugarte et al., 2010; Wille et al., 2017). Based on to dates findings, also maize (*Zea mays*) is responsive to R:FR, where stem elongation, less leaf formation and smaller leaf inclination was repeatedly observed (Maddonni et al., 2002; Sawers et al., 2005; Whipple et al., 2011). In contrast to rice and other cereals, during domestication maize was selected for apical dominance, i.e. it was bred against tillering (Doebley et al., 1997; Whipple et al., 2011). Also, domestication of millet (foxtail millet, *Setaria italica*) favoured selection against tillering (Whipple et al., 2011).

Different studies with low R:FR treatments on wild relatives of current crop cereals, reported reduced tillering in teosinte (tillering ancestor of maize) (Tian et al., 2019; Whipple et al., 2011), reduced tillering and increased leaf sheath length in Italian ryegrass *(Lolium multiflorum)* (Casal et al., 1990) and weedy millet (*Setaria viridis*) exhibiting culm elongation and reduced tillering (Warnasooriya & Brutnell, 2014). Combined these findings suggest that absence of SAS is not a general feature to cereals.

Conclusion

We discovered a species that is not responding to low R:FR, in a way which was so far considered to be widely universal for plants. This study reveals that rice, at least under the tested conditions, is not exhibiting typical SAS phenotypic nor transcriptomic changes. It has been documented that cereals are in principle respond with SAS triggered by low R:FR. Why in rice this is not the case, remains an open question. This exemplifies how little we know about the complexity of interacting environmental and genetic factors as well as other circumstances of an individual plant, which is awaiting to be discovered.

A better mechanistic understanding of interactions in plant canopies would have great potential for implementation in an agronomic context, where plants are grown in dense monocultures (Morgan et al., 2002; Perico et al., 2022; Sawers et al., 2005)

Acknowledgements

We want to thank Yorrit van de Kaa for his support in greenhouse work and assistance with phenotyping and Basten Snoek for fruitful discussions and input on the statistical analysis for the RNAseq evaluation.

Materials and methods

Plant material and growth conditions

Seed material of different *Oryza sativa* varieties (IR64, Nipponbare, Luk Takhar, M Blatec, Mudgo, Sabharaj and Zhenshan) was harvested from plants grown in the greenhouse at the International Rice Research Institute (IRRI), Los Baños, the Philippines, in wet season of 2018, stored at 6°C in the dark. Seeds of wild rice varieties (*Oryza nivara*, HK87 originating from India and SRANGE from Cambodia) and *Oryza rufipogon* (NABO from Nepal and from Bangladesh) were provided by the International Rice Genebank Center (IRGC) at IRRI.

Prior to sowing, the seeds were pre-germinated, with 37 °C for 24 h, followed by 24 h at 21 °C and after transferring into Petri-dishes with wet filer paper, incubated at 32 °C for 24 h. Seeds were directly planted into soil, with five seeds per pot (10 x 10 x 11 cm). The substrate was a mix of black soil, agra-vermiculite 0-1.5 mm and sand in a ratio of 5 : 3 : 2 together with 6 g Osmocote NPK-Mg 15-4-9 (+1) (2.4 g/L of soil) and 20% Yoshida nutrient solution (Yoshida et al. 1976) with a double iron dose (sequestreen = Fe-EDTA) and pH 6.5 (1l per kg substrate). Two weeks after seeding, only four plants per pot were retained. Maize (*Zea mays*, variety EFF) and tomato (*Solanum lycopersicum*, variety Moneymaker) seeds were incubated for 24 h at room temperature on wet filter paper prior to seeding. Independent experiments were performed for: analysis of phenotypic response of cultivated rice (*Oryza sativa)*, wild rice varieties (sample number was, maize and tomato screening and investigation for leaf and tiller angle measurements on *Oryza sativa* varieties were performed as independent experiments, respectively.

Light treatment

For experiments with light treatment, plants were grown in the greenhouse facilities of the Botanical Gardens, Utrecht University, in The Netherlands, in summer and autumn of 2021 (Supplemental Figure 5.1 A). Temperatures were set to 30 °C during the day and 25 °C during the night and a 12 h photoperiod from 8 am to 8 pm, with automatic watering keeping the soil saturated. Pots were arranged at 10 cm distance.

For control group the light intensity was min 400 μmol m^{-2} s^{-1} of sunlight and artificial light (Valoya, Model Rx400 500mA 5730, Spectrum AP673L) switching on if sunlight flux rate dropped below 400 ɥmol m^{-2} s^{-1}. The treatment group received in addition to the WL background, FR from LEDs. Light intensity in PAR range was the same between the control and treatment group, where approximately 500 ɥmol m^{-2} s^{-1} FR light was added (Supplemental Figure 5.1 B).

Low temperature treatment

Experiments for light treatments under lowered temperature, plants were grown in a climate chamber at Utrecht University, in The Netherlands (Supplemental Figure 5.1 C). With controlled conditions of a 12 h photoperiod (8 am – 8 pm) at 70 % relative humidity. Temperatures were set to 25 °C day and 20 °C night. Soil was kept saturated with pots in trays with a constant level of water. Similar to the treatment in the greenhouse, light for control group was set to min 400 µmol m^{-2} s^{-1}, for treatment group FR was supplemented. Ratios of R:FR were 2.0 for control and 0.21 for FR treatment.

Phenotypic measurements and analysis

Leaves and internodes were recorded with numbering from the bottom up. Internode 1 is the oldest and the youngest is the newest from on the top, same for leaves. Definitions of internodes and leaf number were followed from (Izawa et al., 2000; Liu et al., 2016). If a tiller was formed, then the leaf sheath of the first leaf on the tiller was recorded as the internode. The height of the highest node, was noted as the culm height (Supplemental Figure 5.1 D).

For statistical analysis, tissue of same developmental stage was compared. If there was a tissue not (yet) formed under one of the treatment groups, the value 0 was considered. Angle were determined in ImageJ using digital images taken from the side. For leaf erectness a smaller value refers to more droopy leaves and 180 ° is a completely erect leaf. For leaf inclination a smaller value shows a more vertical leaf and with 90 ° the leaf bends off the culm horizontally. Statistical analysis was performed in R and data visualization with GraphPad Prism.

Transcriptome analysis

Experimental design and plant material

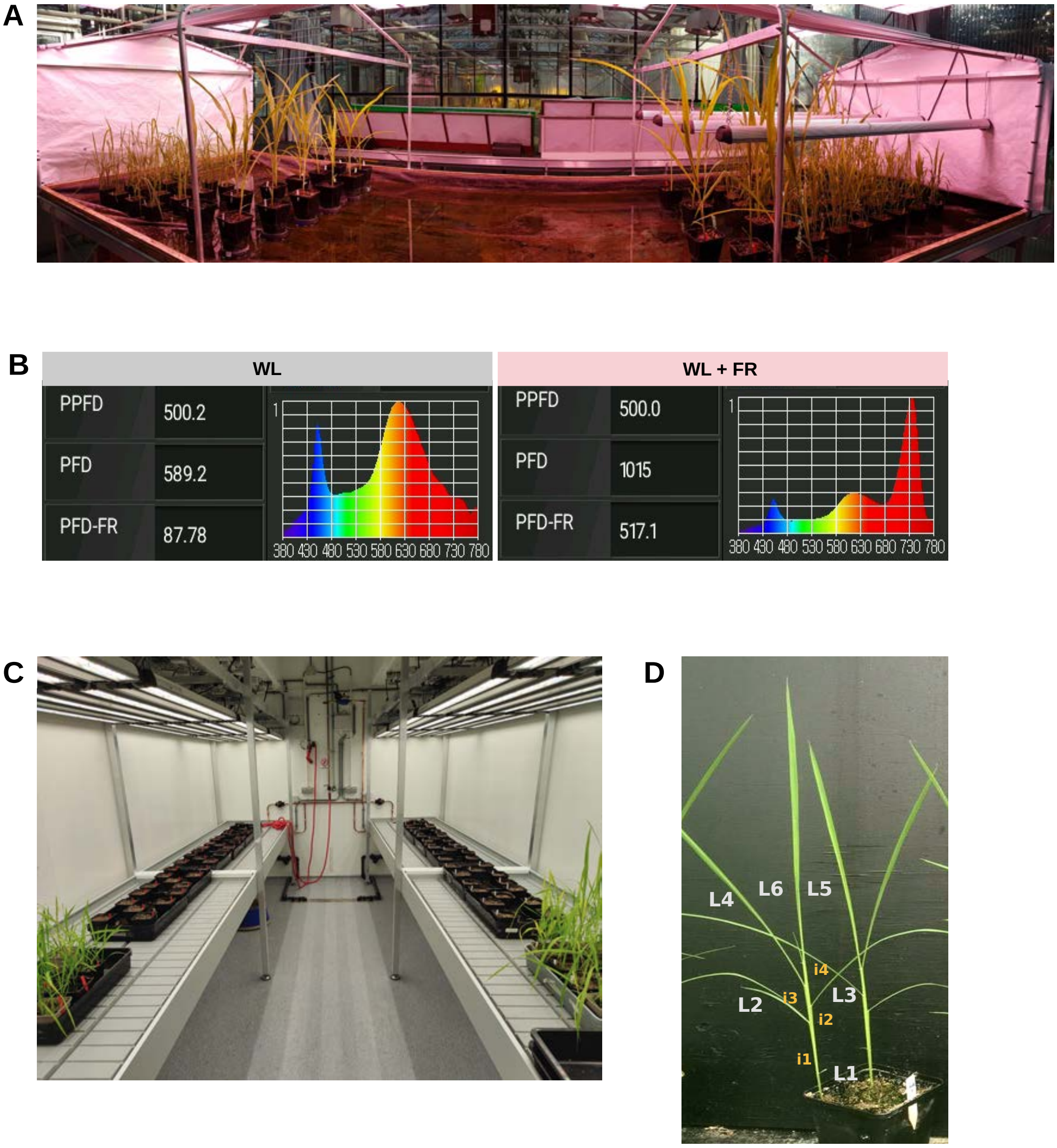
Plants for transcriptome analysis were grown, following the same procedure as for phenotyping. After five days in WL, so that seedlings were big enough to have enough tissue, the treatment group was exposed to supplemented FR light for 24 hours. In an independent experiment, it was shown, that seedlings after having grown for five days in WL and then receiving FR light, show the same phenotype at four weeks as the group receiving FR light starting from germination. The whole shoot of six varieties was sampled, with four plants pooled in one sample. The experiment was repeated four times, resulting in 48 samples (6 varieties x 2 treatments x 4 replicates). AT harvesting, tissue was snap frozen in liquid nitrogen and stored at -80 °C until grinding with Retsch grinder. From tissue powder, RNA was isolated using the Qiagen RNeasy kit with on-column DNAse treatment and quality was checked with the Bioanalyzer, before sequencing.

RNA sequencing and data analysis

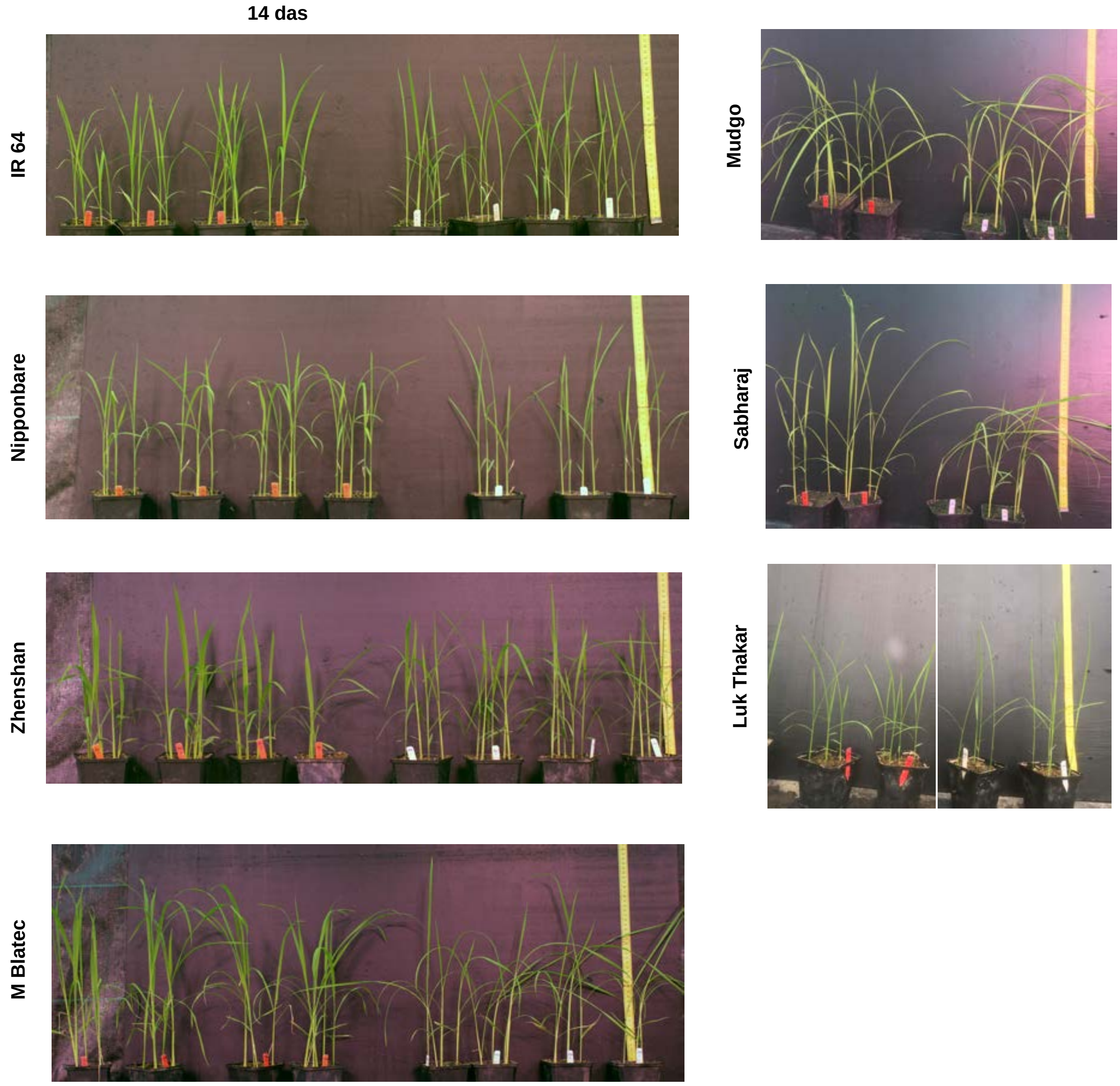
Libraries were sequenced on an Illumina NextSeq 2000 platform at 1 x 50 bp read length yielding around 20 – 30 million reads per sample, performed at USEQ, Utrecht, the Netherlands. The total number of raw reads grouped per variety are shown in Supplemental Figure 5.5 A. To optimize mapping with Kallisto (Bray et al. 2016), the reads were aligned to two available reference transcriptomes (*indica* and *japonica*; 'Osativa_323_v7.0.annotation_info.txt; based on MSUv7 (Feb. 7, 2012, retrieved from http://rice.plantbiology.msu.edu/) using a range of Kmer lengths for the reference index (Supplemental Figure 5.5 C). The mapping was equally high for all varieties, and yielded the highest alignment rates with the Japonica transcriptome with a Kmer index of 17 (~90 % mapped reads). Transcript abundances of the mapped reads were then quantified with Kallisto.

Counts per million (CPMs) were then obtained with the cpm() function of edgeR v3.36.0 (Robinson et al., 2010; Lun et al., 2016). Genes with more than 1 cpm in at least 3 samples were considered expressed and included in the analysis. This resulted in 25.925 out of 42.189 genes (61.45 %) considered for further downstream analysis. Prior to determining Fold Changes and significance the counts were normalized (TMM, trimmed mean of M-values) by correcting for differences in library sizes and compositional biases (Supplemental Figure 5.5 B). Fold changes were subsequently determined with the Bioconductor R package edgeR v3.36.0. Differentially expressed genes (DEGs) were estimated based on the response to treatment by each variety. Resulting P values were adjusted for multiple comparisons with the Benjamini-Hochberg method yielding a false discovery rate (FDR) criterion. Genes with FDR values lower than 0.05 were considered differentially expressed. Detailed information about the statistics for each graph can be found in the respective figure legends. All custom R scripts are available upon request to the author.

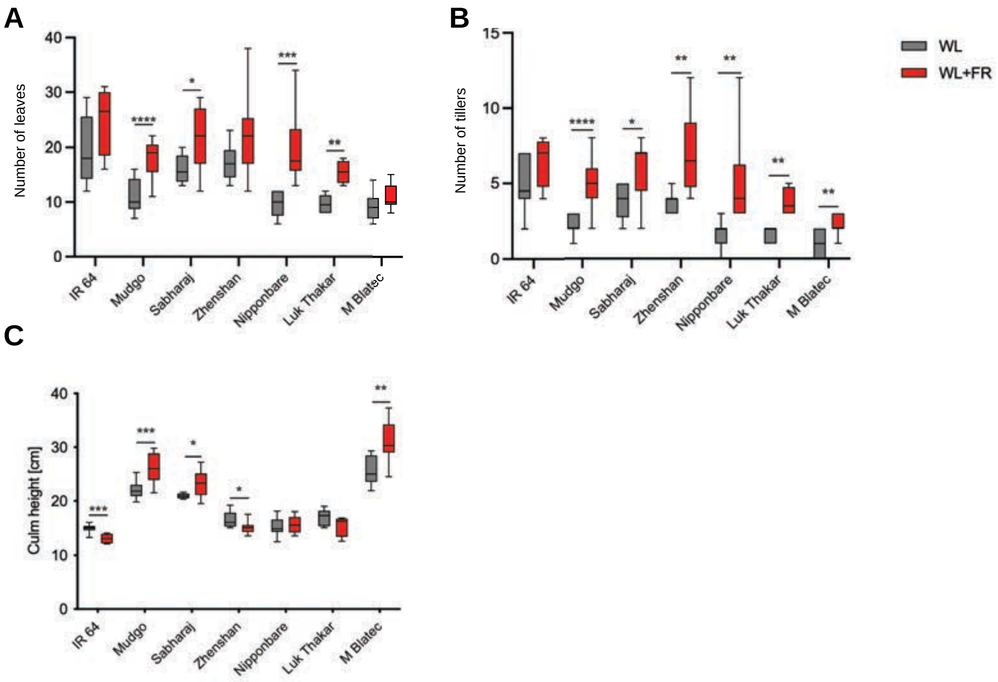
Supplements



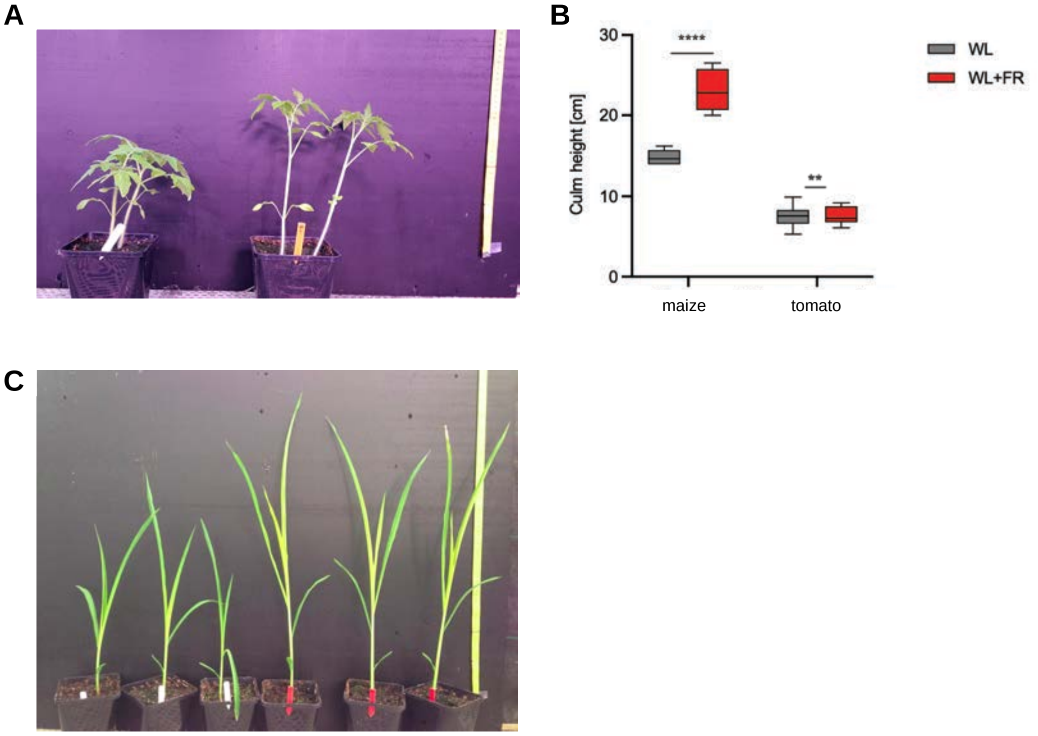
Supplemental Figure 5.1. Experimental set up for far-red light treatment in the greenhouse and growth chambers. A. Greenhouse compartment with **B.** light spectra of control (WL) and WL+FR (FR) light treatments and the respective values for total photon flux density (PFD), far-red (PFD-FR) and photosynthetic photon flux density (PPFD); values are in µmol photons m-2 s-1. **C.** Climate chamber for low temperature and FR light treatments. **D.** Classification for phenotyping of leaf number (L1 – L6) and internodes (i1 – i4), here of a representative 3-weeks-old rice plant.



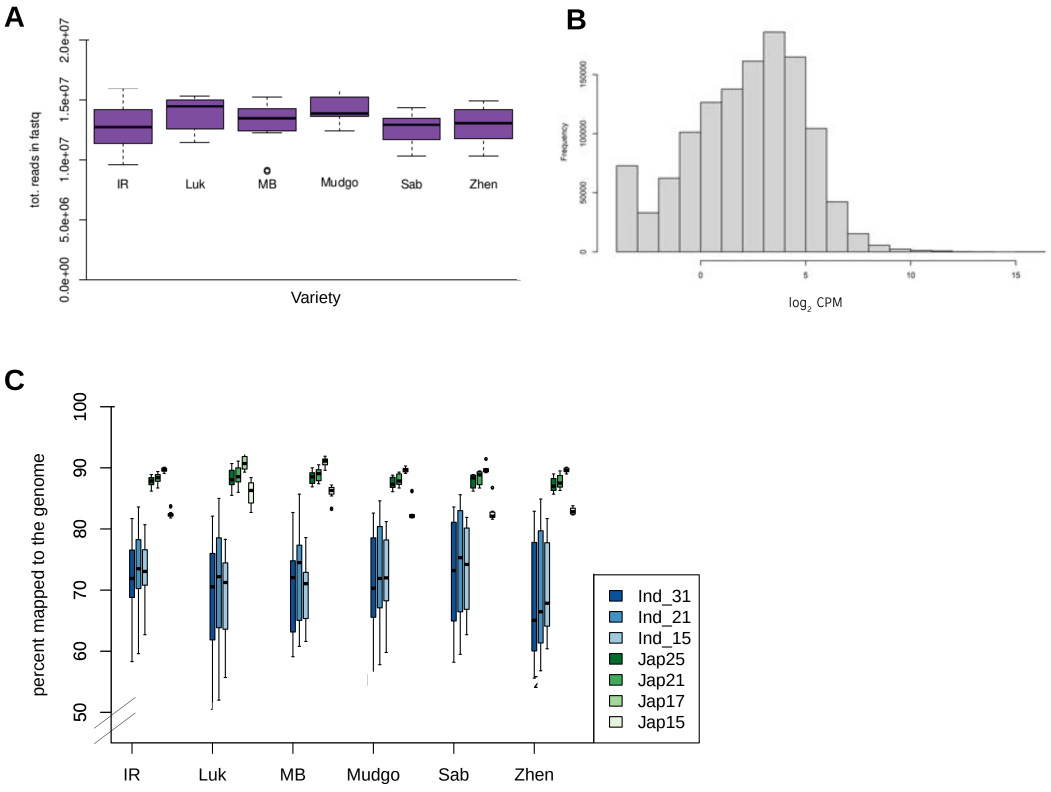
Supplemental Figure 5.2. Phenotype of rice seedlings exposed to supplemental far-red at 14 days after sowing (das) of different rice varieties.



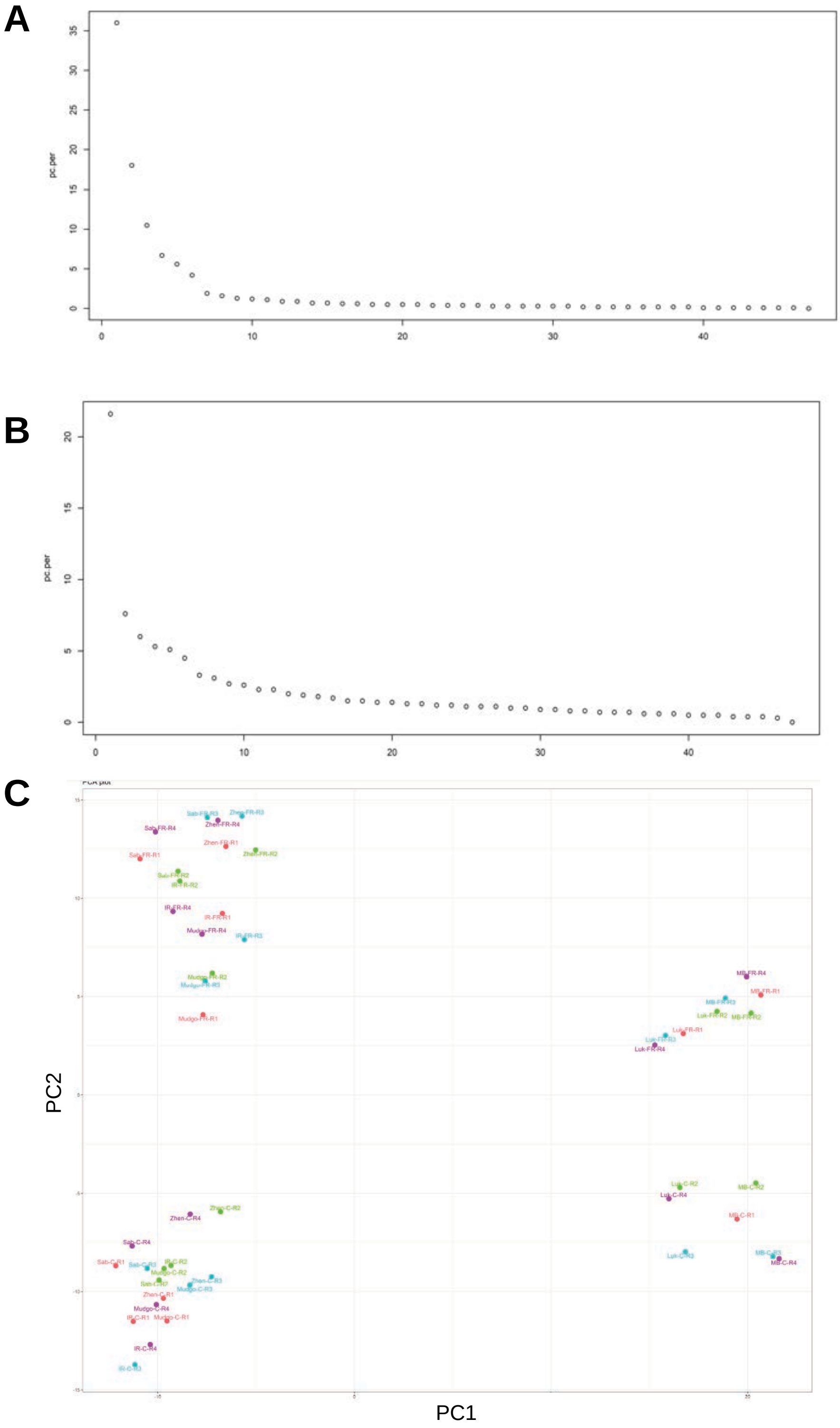
Supplemental Figure 5.3. Comparison of different traits in response to supplemental FR treatment in different rice varieties at 28 days after sowing (das). **A** leaf number, **B.** tillering and **C.** culm height of plants grown in control (WL) and supplemental FR (WL+FR)**.** Values are means 6 - 14 plants; boxes indicate IQ-range with error bars of 2.5 – 97.5 percentile; significant differences are indicated with p-value < 0.05 *, < 0.01 **, < 0.001 *** .



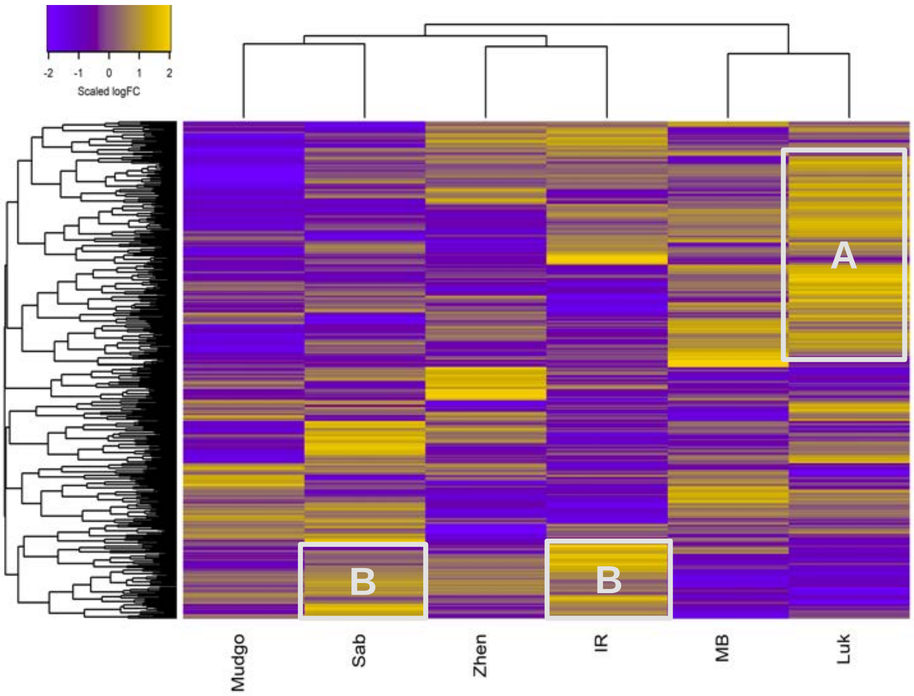
Supplemental Figure 5.4. A. Tomato (*Solanum lycopersicum*) and C. maize (*Zea mays*) exhibit **B.** elongation of culm and stem under +FR treatment at 21 days after sowing (das). Values are means 6 - 8 plants; boxes indicate IQ-range with error bars of 2.5 – 97.5 percentile; significant differences are indicated with p-value < 0.01 **, < 0.0001 **** .



Supplemental Figure 5.5. Quality of reads and mapping. A. Number of raw reads with samples grouped per variety. **B.** After filtering and normalizing 25925 counts per million (CPMs) remained, shown with frequency (y-axis) of CPMs (as $\log_2$ on x-axis). **C.** Based on percentage of reads mapped, we choose jap17 (japonica reference with length of 17 nucleotides; indicated number refers to length of nucleotide mapping. Jap – japonica, Ind – indica and varieties: Luk – Luk Thakar, MB – M Blatec, IR – IR64, Sab – Sabharaj, Zhen – Zhenshan.



Supplemental Figure 5.6. Eigenvalues of Principal Components (PCs) for CPMs of +FR response. A. Eigenvalues per PC for unique DEGs (434 DEGs, FDR < 0.05); cumulative variation of PC1 – PC3 is 63 % of variance. **B.** Eigenvalues per PC of total CPMs; cumulative variation of PC1 – PC6 is 48 % of variance; Percent per PC on y- axis, index of PC on x-axis; **C.** PCA on total CPMs with samples coloured by replicate, PC1 on x-axis and PC2 on y-axis. Abbreviation of varieties: Luk – Luk Thakar, MB – M Blatec, IR – IR64, Sab – Sabharaj, Zhen – Zhenshan. Treatment groups with white light grown control (C) and supplemental FR light (FR) grown plants; biological replicates: R1 – 4.



Supplement Figure 5.7. Heatmap and clustering for logFC of differential gene expression upon supplemental FR treatment. Unique response genes calculated as the difference between white light grown and supplemental FR light (FR) grown plants (434 DEGs; with FDR < 0.05). Colour gradient for log2FCs from blue to yellow for strongest down to strongest up regulation; grey and red indicating control and +FR treatment in bar on top of heatmap. Abbreviation of varieties: Luk – Luk Thakar, MB – M Blatec, IR – IR64, Sab – Sabharaj, Zhen – Zhenshan.

Chapter 6

A dual role for far-red photons: signal and resource

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Abstract

Light is essential for plant growth as it drives photosynthesis. However, only photons within 400-700 nm waveband are considered photosynthetically active radiation (PAR). Outside of this range are UV-light (300–400 nm) and far-red (FR, 700 – 800 nm) light and although these are not considered photosynthetically active, they have important environmental signalling functions, especially in close vegetation. When shade-intolerant plants perceive a FR-enriched environment, they show phenotypic changes described as the shade avoidance syndrome (SAS). Rice varieties show rather modest and variable SAS responses, but surprisingly strongly increased rates of development and biomass accumulation under supplemental FR light conditions. Here we investigate if FR enriched light can promote photosynthesis to explain the enhanced biomass accumulation under these conditions. To explore the hypothesis that FR exposure would promote photosynthesis, we first investigated morphological and biochemical acclimation responses of stomata and chlorophyll content of rice plants. We recorded an unchanged number, but slightly smaller stomata and decreased values for chlorophyll content in plants exposed to supplemental FR. We then investigated the instantaneous effect of FR photons on rates of photosynthesis, by performing gas-exchange measurements. These measurements revealed that under FR-enriched conditions, CO_2 fixation nearly doubled as compared to the regular conditions with identical PAR. These observations are consistent with the observed FR-induced increase of biomass of these plants. The increased photosynthesis under FR-enrichment is not accompanied by changes in stomatal conductance, indicating that the increased rates of photosynthesis are a direct effect of FR absorption. We conclude that FR photons can enhance photosynthesis when added to a PAR background. This observation can have consequences for how to consider FR photons in plant science: in addition to being an environmental signal, they are also a source of harvestable energy.

Abbreviations and definitions

SAS	Shade Avoidance Syndrome
PPFD	Photosynthetic Photon Flux Density = Photosynthetic active radiation (PAR); PPFD is the amount of light within the PAR region or the number of photosynthetically active photons that fall on a given surface each second. It is defined as the photons in the range of 400 to 700 nm measured as μmol photons m^{-2} s^{-1}.
PFD	Photon Flux Density; PFD is the number of photons that fall on a given surface each second in the range of 380 to 780 nm, which includes part of UV and FR, in addition to PAR, measured as μmol photons m^{-2} s^{-1}.
R:FR	red to far-red light ratio
WL	white light
WL+FR	white light with supplemented far-red light
das	days after sowing

Measurement groups

- control plants grown in WL (WL) and treated plants grown with supplemental FR (FR pre-treated)
- under two FR light settings: off and on

Parameters of gas-exchange measurements
(de Lobo *et al.*, 2013; Evans et al., 2014; Wohlfahrt & Gu, 2015)

g_s	stomatal conductance [mol H_2O m^{-2} s^{-1}]
P	Photosynthesis [μmol CO_2 m^{-2} s^{-1}]
P_g	gross photosynthetic rate; gross photosynthesis is true photosynthesis (carboxylation);
P_{gmax}	maximum gross photosynthetic rate
P_N	net photosynthetic rate; = net Assimilation rate of CO_2, net photosynthesis is gross minus photo respiration and dark respiration
P_{Nmax}	maximum net photosynthetic rate
$P_N(I_{max})$	maximum net photosynthetic rate obtained at $I = I_{max}$
I	intensity of photosynthetic photon flux density (PPFD)
I_{comp}	light compensation point
I_{sat}	light saturation point
RD	dark respiration
ϕ	quantum yield
$\phi(I_{comp} - I_{200})$	quantum yield between I_{comp} and $I = 200$ μmol photons m^{-2} s^{-1}

Introduction

Light is an essential resource for a plant to thrive as it is the plant’s sole source of energy. Therefore, it is not surprising that reductions in light lead to reduced energy in the form of photons that can drive photosynthesis. Less energy availability on its term leads to reduced potential for plant growth, accompanied by many phenotypic and physiological consequences (Poorter *et al.*, 2019).

However, other than quantity, also light quality, as determined by the composition of the spectrum, plays fundamental roles for plants, providing cues about the environment they are in (Chapter 2, Huber *et al.*, 2020). In the previous chapter, we investigated light quality as a cue for phenotypic adjustments, and focussed on a reduced red to far-red ratio (R:FR) as an early neighbour detection cue that elicits responses of the shade avoidance syndrome (SAS) (Chapter 2). The vast majority of the studies on this topic, were done with the dicot model plant *Arabidopsis thaliana*, but here we study responses in rice (*Oryza sativa*). Typically R:FR ratios are adjusted by FR supplementation to a stable PAR background. This is based on the convention that FR photons are not considered part of photosynthetic active radiation (PAR) and hence do not contribute to photosynthetic activity.

Surprisingly, we observed only marginal SAS responses and transcriptomic changes under FR-enriched light in several rice varieties (Chapter 4). However, what we did observe for all rice cultivars tested in response to FR enrichment, was an increase in number of leaves and tillers. Recent studies on individual species (Li & Kubota, 2009; Stutte, Edney & Skerritt, 2009; Park & Runkle, 2017; Kalaitzoglou *et al.*, 2019) or a large variety of different species (Zhen & Bugbee, 2020a; Zhen & Bugbee, 2020b) show increased biomass formation under supplemental FR treatment. These studies, together with the results we showed in the previous chapter, suggest that supplemental FR promotes biomass accumulation. This would imply that FR photons would add to photosynthesis. Indeed, in the light reactions of photosynthesis, Photosystem I can absorb FR photons (Emerson, 1958; Zhen, van Iersel & Bugbee, 2021), even though these are not considered part of the photosynthetically active radiation.

Based on our observations in Chapter 4, combined with above-mentioned insights from literature, we investigate here at different physiological and morphological levels if supplemental FR light promotes growth in rice, and if additional growth is associated with photosynthesis. To this end, we combined morphological measurements on plant growth, development and stomatal density and size, with measurements of photosynthetic gas exchange under high and low R:FR light conditions in four rice varieties. For the photosynthesis measurements, in addition to measuring the instantaneous effect of FR on CO_2 fixation, we also compared FR-acclimated plants against a control group.

Our data indicate that FR photons add substantially to the rate of photosynthesis, presumably by direct absorption fuelling the light reactions, whereas acclimation to supplemental FR had a significant, yet small contribution.

Results

FR promotes growth and rate of development

Phenotypic changes in supplemental FR (+FR) treated rice plants occur during tissue formation, where plants in +FR form certain organs earlier than in the control group grown under WL. We observed accelerated leaf formation in +FR treated plants compared to their controls, consistently for all varieties (Figure 6.1, Supplemental Figure 6.1 A-C), leading to significantly more leaves at 28 days after sowing (das). Similarly, tillering is accelerated in +FR treated plants (Figure 6.2). Although we showed previously that culm height was not differing between treatment groups for all varieties (Chapter 4), when considering the formation of internodes, this is still a bit faster under +FR treatment (Figure 6.3, Supplemental Figure 6.1 D-F). Consistent with these rates of organ development, we see higher biomass in +FR, for shoots as well as roots, clarifying that the stronger vigour in shoot growth, it is not due to a shift in resource allocation, at the expense of the root system (Figure 6.4, Supplemental Figure 6.1 G-H).

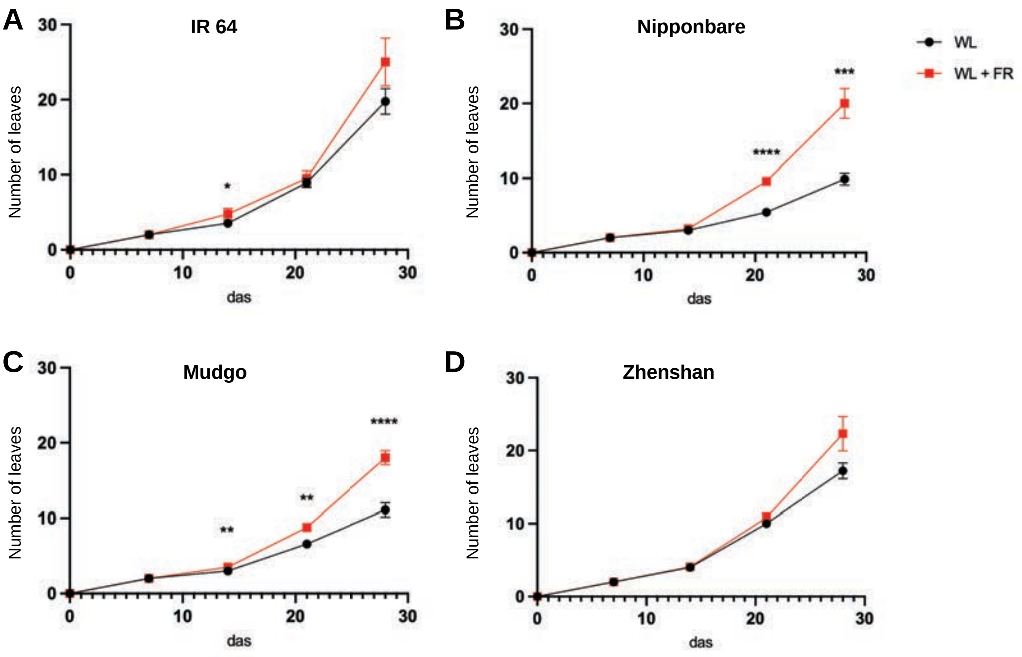


Figure 6.1. Increased leaf formation rate of rice seedlings exposed to supplemental far-red (WL+FR) recorded weekly from day of sowing until 28 days after sowing (das) in the varieties **A**. IR 64, **B.** Nipponbare, **C**. Mudgo and **D**. Zhenshan. Values are means 4 - 10 plants; significant differences following two-sample t-test are indicated with p-value < 0.05 *, < 0.01 **, < 0.001 ***, < 0.0001 ****; error bars are ± SE.

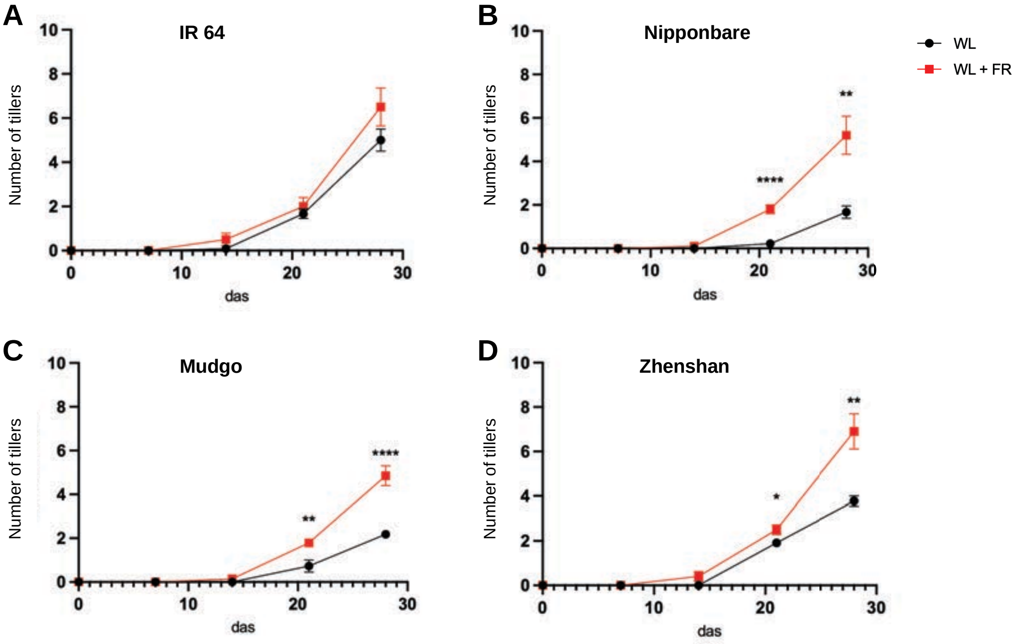


Figure 6.2. Increased tillering rate of rice seedlings exposed to supplemental far-red (WL+FR) recorded weekly from day of sowing until 28 days after sowing (das) in the varieties **A**. IR 64, **B.** Nipponbare, **C**. Mudgo and **D**. Zhenshan. Values are means 10 - 14 plants, 4 for IR64; significant differences following two-sample t-test are indicated with p-value < 0.05 *, < 0.01 **, < 0.001 ***, < 0.0001 ****; error bars are ± SE.

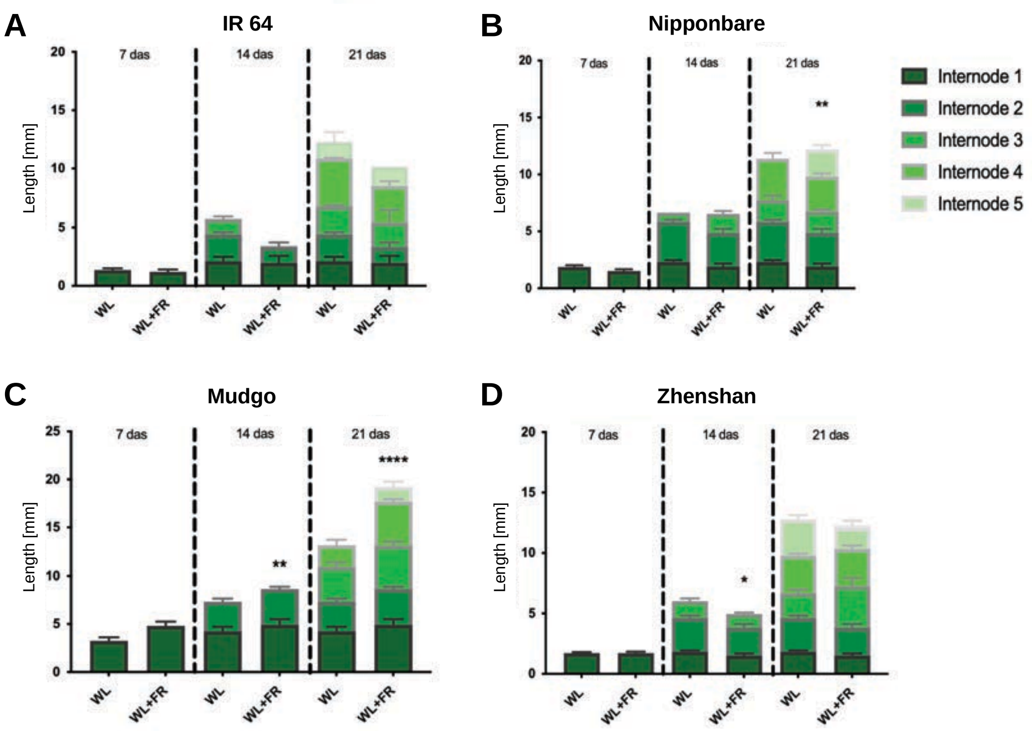


Figure 6.3. Increased rate of internode formation in rice seedlings exposed to supplemental far-red (WL+FR). Length and number of internodes was recorded weekly from day of sowing until 21 days after sowing (das) in the varieties **A**. IR 64, **B.** Nipponbare, **C**. Mudgo and **D**. Zhenshan. Values are means 10 - 14 plants, 4 for IR64; significant differences following two-sample t-test are indicated with p-value < 0.05 *, < 0.01 **, < 0.001 ***, < 0.0001 ****; error bars are ± SE.

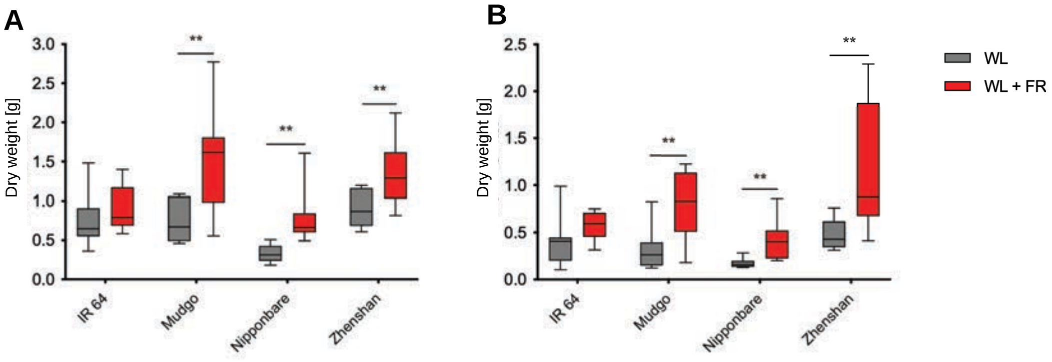


Figure 6.4. Increased biomass of rice seedlings of different varieties exposed to supplemental far-red (WL+FR) at 28 days after sowing (das). **A.** Shoot and **B.** root biomass recorded as dry weight in g of the varieties IR 64, Mudgo, Nipponbare and Zhenshan. Values are means of 5 - 12 plants, significant differences following two-sample t-test are indicated with p-value < 0.05 *, < 0.01 **; boxes indicate IQ-range with error bars of 2.5 – 97.5 percentile.

FR has only minor effects on stomata and chlorophyll content

In order to understand how plants in +FR can gain more biomass, even though the PAR (400-700 nm) light is not different from the control group, we first investigated if the number and size of stomata in the leaves of +FR grown plants differed from those grown in WL. In monocots, stomata are arranged parallel along the veins of a leaf (Figure 6.5 A, Supplemental Figure 6.2 A). Stomatal density, as the number of stomata per length of vein (i.e. within one inter-vein segment), was not different between treatment groups, consistently in all varieties (Figure 6.5 B). However, when looking at the number of stomata in 1 mm long leaf sections, the total number of stomata in WL is higher than in +FR due to the trend of a higher number of segments in WL-grown plants (Supplemental Figure 6.2 B-D). Rice shows a morphology of stomata characteristic for grasses (Figure 6.5 C) and these were longer for plants grown in WL than for plants in +FR, whereas the width is only marginally increased (Figure 6.5 D-E). The chlorophyll content of plants grown in WL is higher than in +FR (Figure 6.6 A), but the chlorophyll A/B ratio is not affected by presence of +FR (Figure 6.6 B).

Summarising, the measurements on stomata and chlorophyll, all indicate that in +FR light there is certainly not a higher predicted potential for carbon gain as chlorophyll levels are on average reduced in +FR compared to control light, stomatal number is not affected by FR and stomatal size is even slightly reduced in +FR.

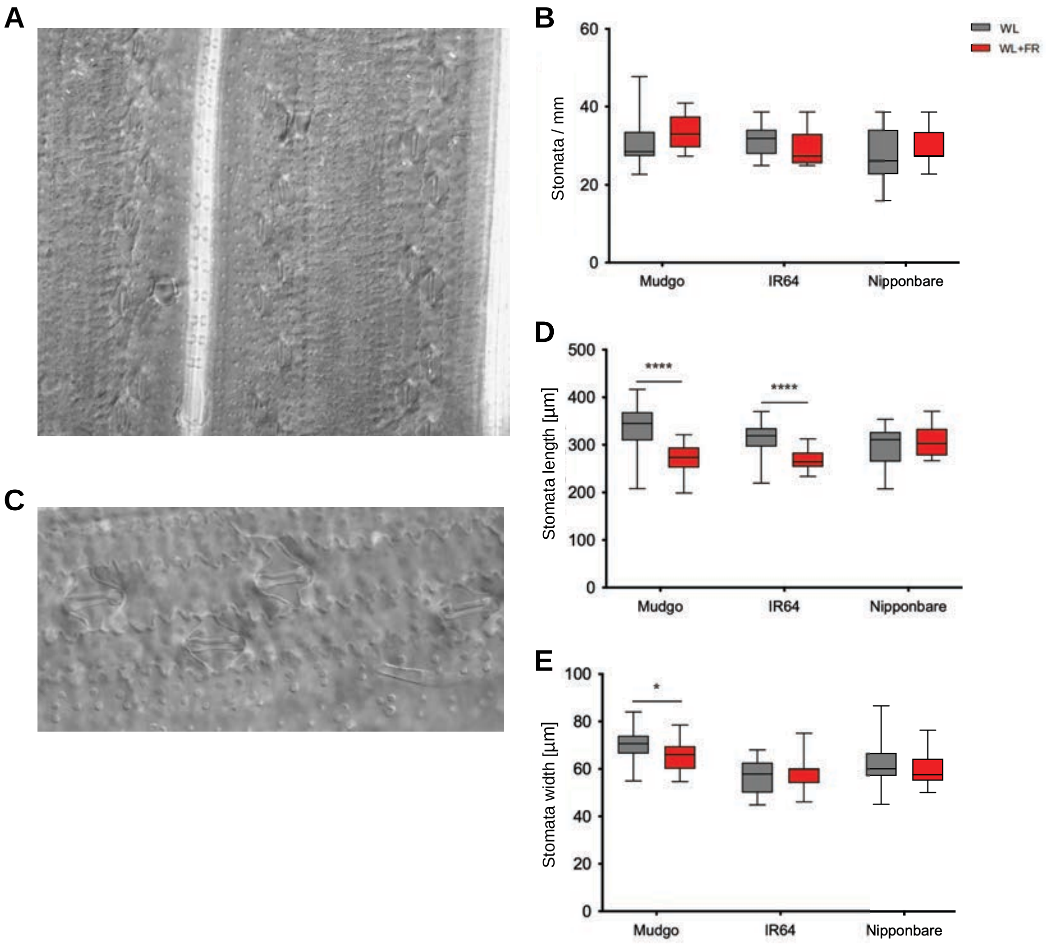


Figure 6.5. Stomatal density and morphology of 28 days old rice plants exposed to supplemented FR (WL+FR) and control group grown at WL. **A.** Apical surface of leaf blade at 200-fold magnification of representative area used for quantification of **B.** stomatal density as number of stomata per mm of vein length. **C.** Representative area investigated for stomatal morphology at 400-fold magnification with dimensions of **D.** stomatal length and **E.** width in µm. Measurements are taken on third youngest leaf, for stomatal density n = min 12 counts in 3 biological replicates, for stomatal dimensions n = min 16 counts in 3 biological replicates. Significant differences following two-sample t-test are indicated with p-value < 0.05 *; boxes indicate IQ-range with error bars of 2.5 – 97.5 percentile.

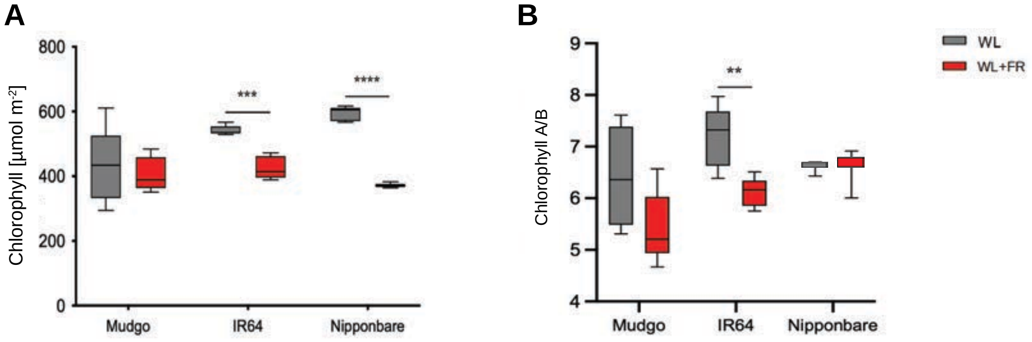


Figure 6.6. Chlorophyll content of 28 days old rice plants exposed to supplemented FR (WL+FR) and control group grown at WL. **A.** Total Chlorophyll content determined with a chlorophyll content meter as µmol m-2 and the **B.** ratio of Chlorophyll A/B determined with leaf disc extraction. Measurements are taken on third youngest leaf, for both methods n = 5 biological replicates. Significant differences following two-sample t-test are indicated with p < 0.05 *, < 0.01 **, < 0.001 ***, < 0.0001 ****; boxes indicate IQ-range with error bars of 2.5 – 97.5 percentile.

FR light promotes photosynthesis

After investigating possible morphological and physiological aspects of the observed increased growth under supplemental FR, we next analysed what the actual photosynthetic activity of plants is under our light treatment conditions (Figure 6.7). For measurements on instantaneous effects of FR on photosynthesis, plants from both, the WL and +FR treatments, were placed under WL or WL with supplemental FR light, similar to the growth conditions of the +FR treatment. We measured gas-exchange with a LI-COR 6400 using a leaf chamber that has a transparent top, allowing measurements under ambient light conditions (Supplemental Figure 6.3 A). Settings of the IRGA (infra-red gas analyser) were set the same for both groups (Supplemental Figure 6.3 B). Light reaching the plants in WL and +FR was different only for total photon flux density (PFD), but not in PAR (Supplemental Figure 6.3 C-E), neither did the leaf temperature inside the cuvette differ between FR light on and off (Supplemental Figure 6.3 F). Measurements of the WL treatment started with gas-exchange with FR switched off and then on, whereas +FR treated plants were first measured with FR on and then off. The combined measurements were conducted in a time span of several minutes to minimize potential changes in stomatal conductance to non-native light conditions.

We observed a very strong instantaneous effect on photosynthesis when FR lamps were switched on, measured as CO_2 exchange. In +FR there was nearly a doubled rate of carbon fixation as compared to plants in WL. This strong effect of FR on CO_2 fixation was consistent between varieties, and present both in plants that were pre-grown in WL and in plants grown in +FR (Figure 6.7 A). Although there were no significant effects of FR acclimation on photosynthesis, there was a significant interaction effect of pre-treatment and FR-light presence during measurement (box Figure 6.7 A). When looking at the difference in rate of photosynthesis between FR light on and off for acclimated and non-acclimated plants separately, we see an asymmetric response to FR supplementation (Figure 6.7 B). A two-way Anova was performed to analyse the effect of pre-treatment and variety on the difference in photosynthetic rate when FR lamps are switched on. The main effect of pre-treatment was highly significant as well as the effect of variety, but there is no interaction effect, i.e. it does not depend on the variety to what extent photosynthetic rate increased when FR lamps are switched on. Consistently, the response is larger in the FR pre-treated group. This is even stronger when only comparing FR-grown plants with FR on and WL-grown plants with FR off, where the basal photosynthetic rate of plants in the native environment they are acclimated to, is vastly increased by FR (Figure 6.7 C). Nevertheless, although the acclimation has a significant effect on the responsiveness to instantaneous FR photons, this effect is small as compared to the overall large instantaneous effect of FR for driving photosynthesis, which is independent of variety and pre-treatment.

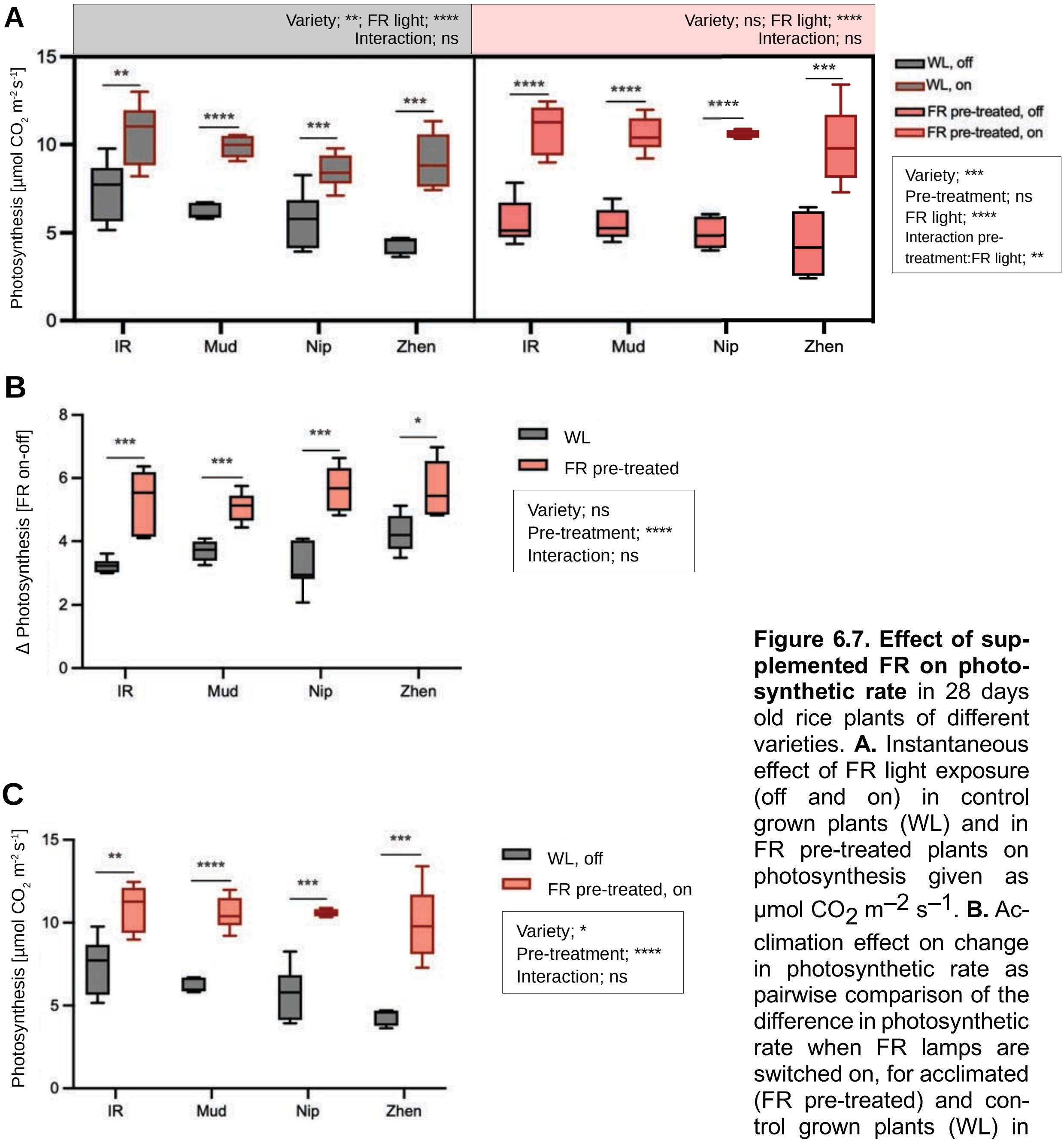


Figure 6.7. Effect of supplemented FR on photosynthetic rate in 28 days old rice plants of different varieties. **A.** Instantaneous effect of FR light exposure (off and on) in control grown plants (WL) and in FR pre-treated plants on photosynthesis given as µmol CO_2 m^{-2} s^{-1}. **B.** Acclimation effect on change in photosynthetic rate as pairwise comparison of the difference in photosynthetic rate when FR lamps are switched on, for acclimated (FR pre-treated) and control grown plants (WL) in different varieties. **C.** Acclimation effect on photosynthetic rate between native groups (WL-off and FR-on). Measurement groups consist of two treatments: control grown in WL and treated plants grown with supplemental FR (FR pre-treated) under two light settings: off and on. Values are means of 6 plants of each measurement group. Box-plots indicate IQ-range with error bars of 2.5 – 97.5 percentile. Significant differences from pairwise (in A.) and two-sample t-test (in B. and C.) are indicated with p-value < 0.05 *, < 0.01 **, < 0.001 ***, <

0.0001 ****; boxes show results for three- and two-way ANOVA. Abbreviations for varieties: IR - IR 64, Mud - Mudgo, Nip - Nipponbare, Zhen - Zhenshan.

Stomatal conductance is not affected by FR

Although we observed no strong differences in number and size of stomata, the opening of the stomata could still be different between +FR and WL plants. We therefore also determined stomatal conductance in our gas exchange measurements. We observed no clear acclimation effect for stomatal conductance towards +FR (Figure 6.8 A), since plants grown in WL versus those grown in +FR in their respective native light environment, had similar stomatal conductance. Two-way Anova results show that differences in conductance were independent of treatment and only determined by variety (box Figure 6.8 A). We then performed a dedicated experiment to verify whether plants would change their stomatal opening due to instantaneous switching on of FR lights. For this, we shifted a group of plants of two varieties, to the respective different light environment four hours prior to measurements, giving the stomata enough time to adjust. Under FR exposure, FR-acclimated IR64 plants (native plants) had a slightly higher conductance than WL grown plants (shifted plants), whereas Nipponbare plants did not show this effect (Figure 6.8 B), indicating that the stomatal opening is probably not strongly adjusted to changed FR light conditions.

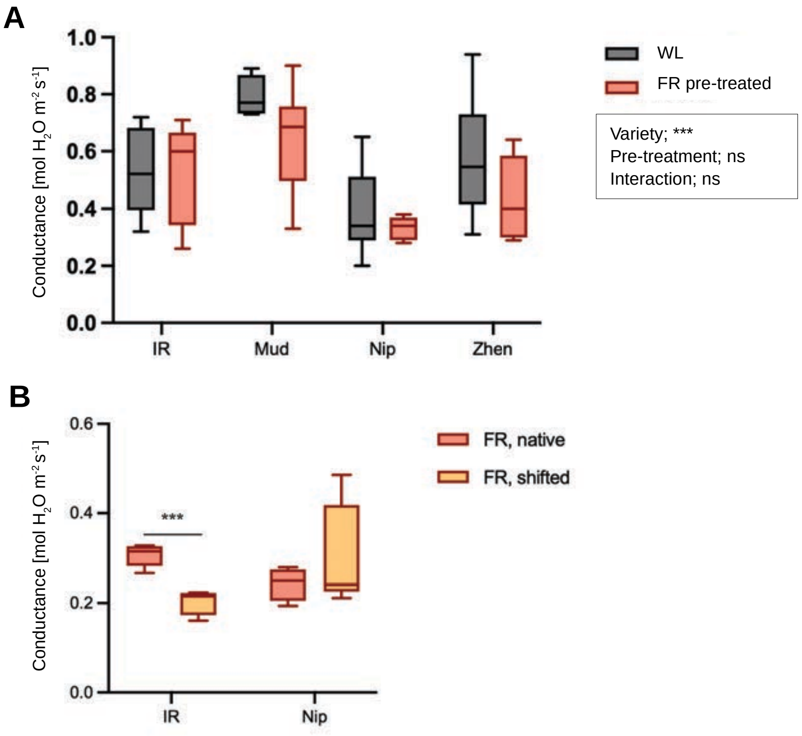
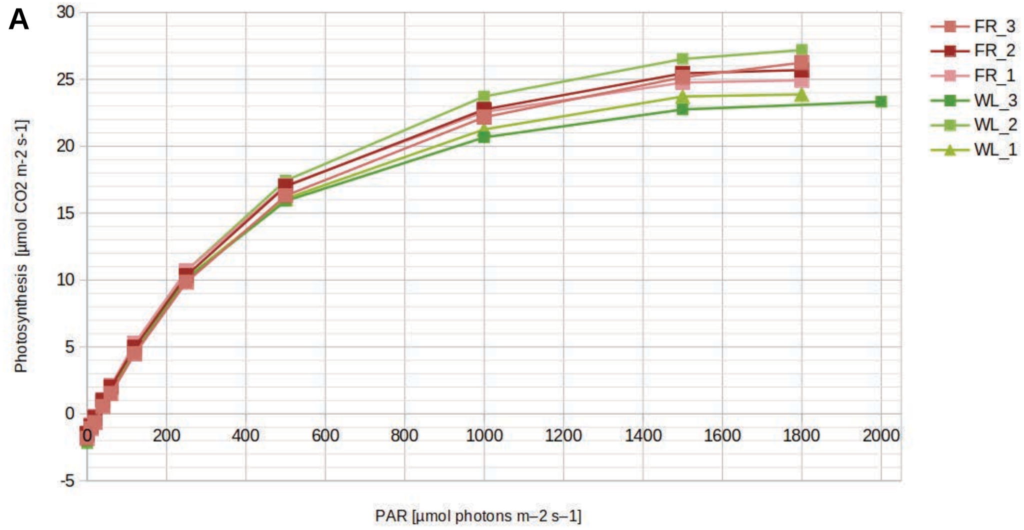


Figure 6.8. Effect of FR on stomatal conductance in 28 days old rice plants of different varieties. **A.** Acclimation effect, between native groups (C off and FR on) and **B.** adjustment effect, under one light condition (FR on) between native and shifted group (C shifted and FR native). Measurement groups are of two treatments: C (control) and FR pre-treated under two light settings: off and on. Values are means of 6 plants of each measurement group. Box-plots indicate IQ-range with error bars of 2.5 – 97.5 percentile. Significant differences from two-sample t-tests are indicated with p-value < 0.05 *, < 0.01 **, < 0.001 ***, < 0.0001 ****; boxes show results for two-way ANOVA. Abbreviations for varieties: IR - IR 64, Mud - Mudgo, Nip - Nipponbare, Zhen - Zhenshan.

Comparing the efficacy of PAR and FR photons for photosynthesis

To investigate long term acclimation effects of plants exposed to supplemental FR, we performed light response curve measurements on the variety Nipponbare (Figure 6.9). Here we used a closed cuvette with internal LEDs as a light source to manipulate precisely the light intensity given in the PAR range. The light response curves describe how the rate of photosynthesis varies as a function of light, providing information on relevant parameters of photosynthetic activity, including maximum photosynthetic capacity, quantum yield, light compensation and light saturation points as well as respiration rate (Herrmann, Schwartz & Johnson, 2020; Du et al., 2020). Generally, the light response curves follow the typical trajectory of rice plants (Xiang-Sheng *et al.*, 2006; Ye, 2007) and are similar for the two treatment groups (Figure 6.9 A). Already apparent from the curves, none of the derived parameters showed a significant difference due to pre-treatment of plants (Figure 6.9 B).

These light response curves (Figure 6.9 A) enable us to compare CO_2 fixation at any given PAR, with the measurements we performed with open top cuvettes, where FR was reaching the leaves during the measurements. Such comparison might help answer the question if FR photons are effectively driving photosynthesis as photons within the PAR range do. Plants were grown at approximately 380 µmol PFD photons m^{-2} s^{-1} in control and approximately 900 PFD µmol photons m^{-2} s^{-1} with supplemented FR (= 380 µmol PAR photons m^{-2} s^{-1} + 400 µmol FR photons m^{-2} s^{-1}), which are the light environments they were acclimated to (Supplemental Figure 6.3 C). When performing the gas exchange measurements, the actual light reaching leaves in the cuvette was only approximately 170 µmol PAR photons m^{-2} s^{-1}. FR could not be determined by the internal light sensor, but inferring from a 55% reduction of PAR measured outside (Supplemental Figure 6.3 E), a similar 55% reduction of FR would still translate into 400 PFD µmol photons m^{-2} s^{-1} (PAR + FR) at leaf level in the cuvette. At 170 µmol PAR photons m^{-2} s^{-1} in the light-response curve we see approximately 5 µmol CO_2 fixation m^{-2} s^{-1} (Figure 6.9 A), which is roughly the same as observed for Nipponbare control plants with FR lights off (Figure 6.7 A). Similarly, when reading the CO_2 fixation at 380 µmol PAR photons m^{-2} s^{-1} in the light response curve, this equals to 12-14 µmol CO_2 fixation m^{-2} s^{-1} . In the FR-on situation, FR-acclimated plants fixed 10.6 µmol CO_2 m^{-2} s^{-1} . This comparison indicates that FR photons are not entirely as effective as PAR photons to drive CO_2 fixation, but are surprisingly close.



B

[µmol CO2 m-2 s-1]	**WL**	± SE	**+FR**	± SE	**significance** P < 0.05
Gross photosynthesis (I_{max})	**26.7**	1.2	**27.7**	0.8	ns
Net photosynthesis (I_{max})	**23.7**	0.3	**22.72**	1.3	ns
Light compensation point	**27.1**	2.5	**26.1**	2.2	ns
Light saturation point	**1,101**	51	**1,155**	55	ns
Dark respiration	**1.80**	0.20	**1.68**	0.12	ns
Quantum yield	**0.048**	0.001	**0.048**	0.001	ns

Figure 6.9. Disentangling the effect of light quality from light quantity in control grown and FR pre-treated in 28 days old rice plants of the variety Nipponbare. **A.** Light response curve showing the photosynthetic activity [µmol CO_2 m-2 s-1] on the y-axis, at a given light intensity as µmol PAR photons m-2 s-1 on the x-axis. **B.** Parameters from fitted model to light response curve: maximum gross and net photosynthetic capacity, light compensation and light saturation point, quantum yield and dark respiration, with the results of two-sample t-test between treatment groups. N = 3 plants, with 4 technical replicates of measurements.

Discussion

We simulated the red:far-red light ratio of vegetation shade by supplementing FR light to a standard PAR light background. Our results show that rice plants grown in supplemental FR light conditions have an accelerated rate of development as well as enhanced biomass accumulation. This has been observed as well in other studies, in different plant species, including vegetables and ornamentals, where the increase in dry weight was attributed to increased leaf area, providing higher light interception (Li & Kubota, 2009; Stutte et al., 2009; (Stutte, Edney & Skerritt, 2009; Park & Runkle, 2017; Kalaitzoglou *et al.*, 2019; Zhen & Bugbee, 2020b, 2020a). We show here, that in rice a major effect of FR light occurs through a direct promotion of photosynthesis, independent of leaf area.

Photosynthesis is enhanced in supplemented FR

We observed there were two factors contributing to enhanced CO_2 fixation in +FR light conditions: strong instantaneous photosynthesis by FR photons and a modest long-term acclimation effect. The finding that +FR grown plants showed a stronger CO_2 fixation response to +FR in the photosynthesis measurement could not be explained at the level of gas exchange itself: There were no differences in stomatal density and some varieties grown in +FR even had slightly smaller stomata. Furthermore, there were no significant differences in stomatal conductance between WL and +FR-grown plants. In addition, chlorophyll levels were slightly reduced in +FR-grown plants as compared to control plants, as were the chlorophyll a/b ratio, both indicative of a mild shade acclimation in these plants (Li & Kubota, 2009; Kalaitzoglou *et al.*, 2019). The weak patterns of stomatal conductance and chlorophyll under FR-enriched growth conditions would rather reduce than improve photosynthesis. We, therefore, conclude that any acclimation responses that we measured do not likely explain the enhanced biomass accumulation in +FR as compared to control light. Other acclimation effects, as derived from light-response curves for CO_2 fixation, also showed no major adjustments, further corroborating that the enhanced growth under +FR growth conditions in the different rice varieties does not follow from strong photosynthetic acclimation effects. The acclimation effect on carbon assimilation in gas exchange measurements is small and in none of the relevant parameters describing the photosynthetic rate, derived from the light response curve, we see a significant difference. Similar to the morphology data, actual stomatal conductance was not changed between treatment groups. Interestingly, Kalaitzoglou *et al.*, 2019 have described the possibility of a higher net photosynthesis at a lower stomatal conductance in supplemental FR than in control light. Similarly, Zhen & Bugbee, 2020 recorded a higher canopy photon capture and increased daily carbon gain (net photosynthesis minus respiration at night) with supplemental far-red photons. Inspired by the observations, we determined CO_2 fixation rates in the presence or absence of supplemental FR photons. The vastly augmented rate of CO_2 fixation in +FR clearly indicates that FR photons can indeed be used to drive photosynthesis directly. This accelerated rate of photosynthesis could potentially explain the observed accelerated rates of development and enhanced biomass accumulation in +FR.

Regulation of photosynthesis and stomatal conductance under +FR

Our observation that stomatal conductance was not enhanced with the increased photosynthetic activity and change in light environment, indicates that conductance is regulated independently of the rate of photosynthesis under FR-enriched conditions. This is very different from the common regulation of stomata where with increasing PAR levels stomata follow with increased opening position (Assmann & Jegla, 2016; Inoue & Kinoshita, 2017). This adjustment is normally fast (stomata adjust within approximately 20 minutes) and happens even on the same plant at the level of individual leaves, depending on how shaded or sun-lit they are. In our FR-enrichment conditions, however, we observe that at the same rate of gas exchange, following from stomatal conductance, rice plants can fix more CO_2 in the presence of supplemental FR photons. The lack of enhanced stomatal opening in +FR indicates that, although FR can power photosynthesis, stomata do not respond to FR as a cue for enhanced photosynthesis, unlike their responsiveness to blue and red light intensity (Chen 2012; Matthews 2020).

Strong increase in photosynthesis but lack of acclimation

Although the differences between FR-acclimated and non-acclimated plants in their photosynthetic responsiveness to instantaneous FR was small, the FR-acclimated plants did benefit significantly more from FR photons than did the non-acclimated plants. Accordingly, in Chapter 4 we did observe a significant gene ontology signature, where photosynthesis-related processes, in addition to shade avoidance signatures, were prominent. Follow-up studies would be required to pinpoint exactly what this modest acclimation effect is really comprised of and if this is associated with the light reactions, the Calvin cycle or even anatomical parameters such as leaf morphology and/or anatomy.

These findings have two important consequences: i) Assuming CO_2 is not limited, the increase of photosynthesis while stomatal opening remains unchanged, would allow plants to perform more CO_2 fixation without a penalty of losing water via evaporation and ii) powering photosynthesis with FR photons could be used as a way to save water, via reduced stomatal opening, while driving photosynthesis. However, the draw-back of the latter would inevitably be that the rate of photorespiration would increase, since little CO_2 can enter and therefore oxygenation reactions by Rubisco would be relatively prominent. Indeed, we observed that at 400 μmol PAR photons m^{-2} s^{-1} the nett CO_2 fixation rates are higher than at the equivalent PFD based on 170 PAR + 230 FR μmol photons m^{-2} s^{-1}, which could result from enhanced photorespiration but further studies are needed to establish this. Some pioneering studies show that FR photons can be used as effectively to drive photosynthesis as has been commonly described for PAR photons. Park & Runkle, 2017 saw, when substituting red with FR, a similar shoot dry weight compared to plants receiving the same number of photons within the PAR range. Similar observations were made by Zhen & Bugbee, 2020a, who found this effect to be constant for 14 investigated plant species, including C3 and C4 species. FR can be absorbed only in Photosystem I (Zhen & van Iersel, 2017; Zhen, van Iersel & Bugbee, 2021), whereas PAR can power both Photosystem I and II. Thus, a strongly skewed balance between the number of photons of FR and PAR would have major consequences for the balance between Photosystem I and II activation and thus for coordinated electron transport. To some extent, FR photons can even increase the efficiency of the use of photons in the PAR range (Zhen & Bugbee, 2020a), possibly because if there is too much light of shorter wavelength, FR can balance out an over-excitement of Photosystem II, by draining electrons via exciting Photosystem I (Yamazaki, 2010).

Future perspectives

We have shown here that FR photons, in addition to their established function as a signal for neighbour proximity, are also actively powering CO_2 fixation in the process of photosynthesis. The different rice varieties used here, all have rather poor shade avoidance-like responses to +FR but very strong growth and photosynthetic responses to +FR. It remains to be studied if the signalling and resource functions of FR go hand-in-hand or if plants with strong shade avoidance responses show little photosynthetic responses to +FR and vice-versa. Also, further studies are needed to elucidate the extent to which positive effects of FR on photosynthesis depend on the PAR levels.

Acknowledgements

We thank Jannes van der Meijden and Jeroen Koch for their substantial work in carrying out phenotyping experiments in the greenhouse and growth chambers, data processing and visualization, as well as Yorrit van de Kaa for his manual support. We thank Astrid Odé and Jan A. Lankhorst[b] for their help in conduction gas exchange measurements and data processing.

Materials and methods

Plant material and growth conditions

Oryza sativa seeds of the varieties IR-64, Nipponbare, Mudgo and Zhenshan were obtained from plants grown in the greenhouse at the International Rice Research Institute (IRRI), Los Baños, the Philippines, in wet season of 2018, stored at 6 °C in the dark.

As a pre-germination treatment, seeds were kept at 37 °C for 24 h, followed by 24 h at 21 °C. For germination, seeds were put in Petri dishes on wet filter paper and incubated at 32 °C for 24 h, which were planted with a tweezer 0.5 mm deep into the soil, with five seeds for each variety, per pot (10 x 10 x 11 cm) in a substrate mix of black soil, agra-vermiculite 0-1.5 mm and sand in a ratio of 5 : 3 : 2 together with 6 g Osmocote NPK-Mg 15-4-9 (+1) (2.4 g/L of soil) and 20% Yoshida nutrient solution (Yoshida, 1976) with a double iron dose (Sequestreen = Fe-EDTA) and pH 6.5 (1 l per kg substrate). Two weeks after seeding, only four plants per pot were retained.

Plants were grown in the greenhouse facilities of the Botanical Gardens, Utrecht University, in The Netherlands, in summer and autumn of 2021. Temperatures were set to 29 °C during the day and 25 °C during the night and a 12 h photoperiod from 8 am to 8 pm, with a minimal light intensity of 400 μmol m^{-2} s^{-1} and artificial light (Valoya, Model Rx400 500mA 5730, Spectrum AP673L) switching on if sunlight PAR dropped below 400 μmol m^{-2} s^{-1}. Automatic watering kept soil in pots saturated, with flooding up to a level of 5 cm and draining four times a day.

Light treatment conditions

The treatment group received in addition to the WL background, FR from LEDs (Valoya FR). Light intensity in PAR range was the same between the control and treatment group. PFD was 380 μmol photons m^{-2} s^{-1} in control (FR 43 + PPFD 338) and 906 μmol photons m^{-2} s^{-1} (FR 570 + PPFD 340) in +FR. Detailed information about the light spectra can be found in Supplemental Figure 6.3 C-D.

Measurements of stomatal morphology

For measurements on stomata of leaf samples of 28 days old plants were fixed following the protocol of (Sharma, 2017) with a 24 h incubation in 95% ethanol : acetic acid (7:1), followed by a 2x washing with 70% ethanol and incubation in 1 N potassium chloride. Samples of the 3rd youngest leaf were observed at the abaxial side using a light microscope (Zeiss Fluorescence Stereo Macroscope) with 200 – fold magnification (10 x 20) for counting number of stomata and a 400-fold magnification (40 x 10) for measuring stomatal dimensions (stomatal length and stomatal width, as described in (Boer *et al.*, 2016)) see (Wu *et al.*, 2020) Stomatal density was expressed as the number per vein length i.e. in one leaf segment; the stomata in one leaf segment do not differ, this segment is based on the veins surrounding the leaf segment, and is 1 mm long. However, the size and number of segments was highly variable between varieties. The minimal stomatal density in 1 mm leaf section is the number of stomata in one segment multiplied by the total number of segments in the leaf (Supplemental Figure 6.2 B-D).

Chlorophyll content

Samples of the third youngest leaf were analysed for their chlorophyll content, with a minimum of five biological replicates, using two different methods. One is non-destructive, using a Chlorophyll Content Meter (CCM) (Gitelson, Buschmann & Lichtenthaler, 1999; Buschmann, 2007). The second method is destructive, but allowing to quantify not only total but also Chlorophyll a and b content, via extraction of leaf discs following the protocol described in (Sharma, 2017).

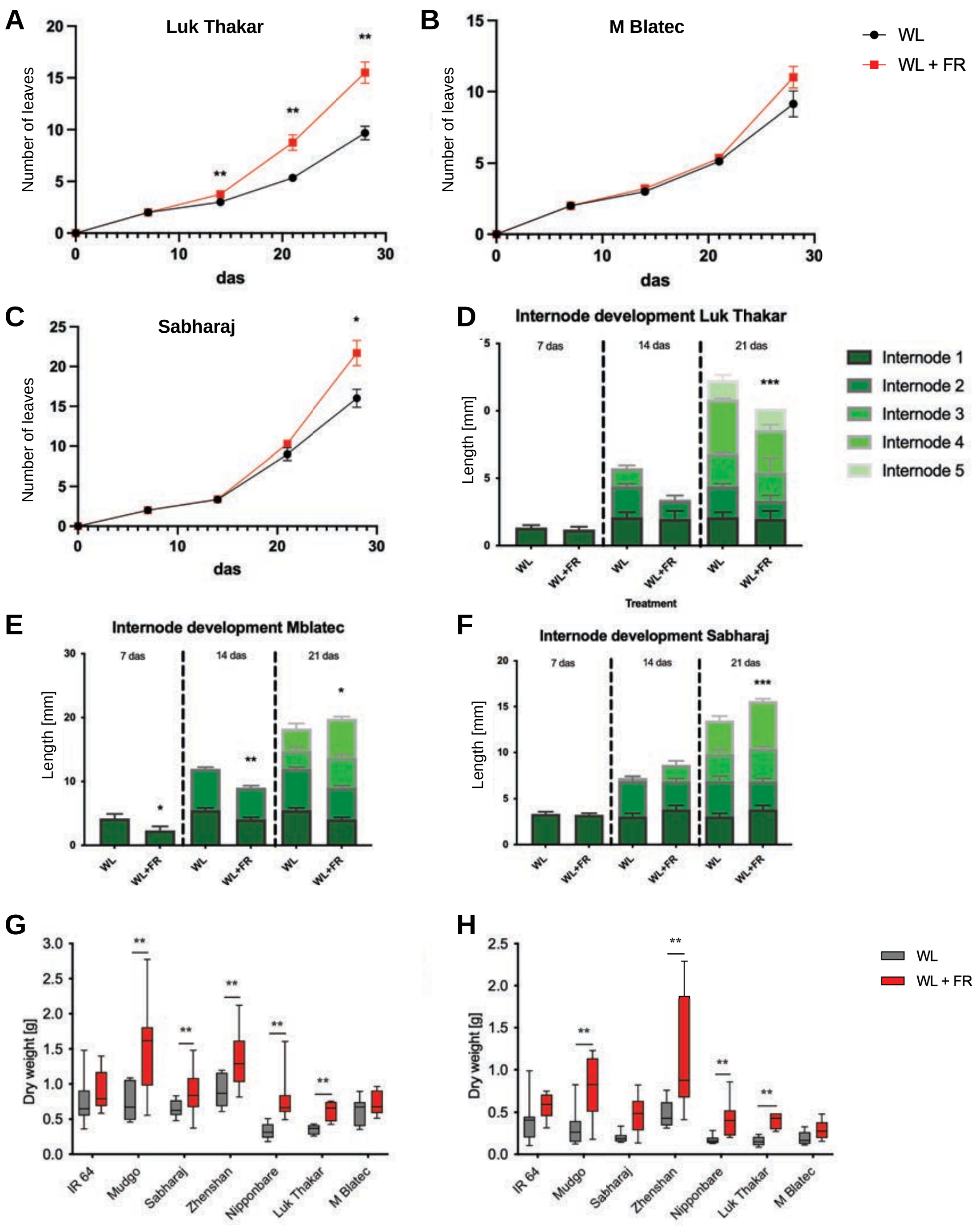
Gas exchange measurements

Gas exchange was measured using a LI-COR 6400XT with a 2x3 cm measuring cuvette (LiCor Inc., Lincoln, NE, USA) with a transparent top, allowing outside light to penetrate the leaves (Supplemental Figure 6.3 A). The parameters of the infra-red gas analyser were set to a flowrate of 500 µmol s^{-1}, the CO_2 flow of the sample to 400 µml m^{-2} s^{-1} and the block temperature to 30 °C (Supplemental Figure 6.3 B). Relative humidity of the sample was approximately 70%. The sequence of measurements was for each treatment group first under their “native” light environment and then "changed”. This means that for control grown plants first with FR lamps were off and then switched on and for FR-acclimated plants vice versa, leading to four measurement groups: C-off, C-on, FR-on, FR-off. The variables we have investigated for statistical analysis were photosynthetic rate and stomatal conductance. We also compared leaf temperature, CO_2 of the sample and internal PAR inside the cuvette throughout the measurements (Supplemental Figure 6.3 E-F), which give insight in potential confounding factors.

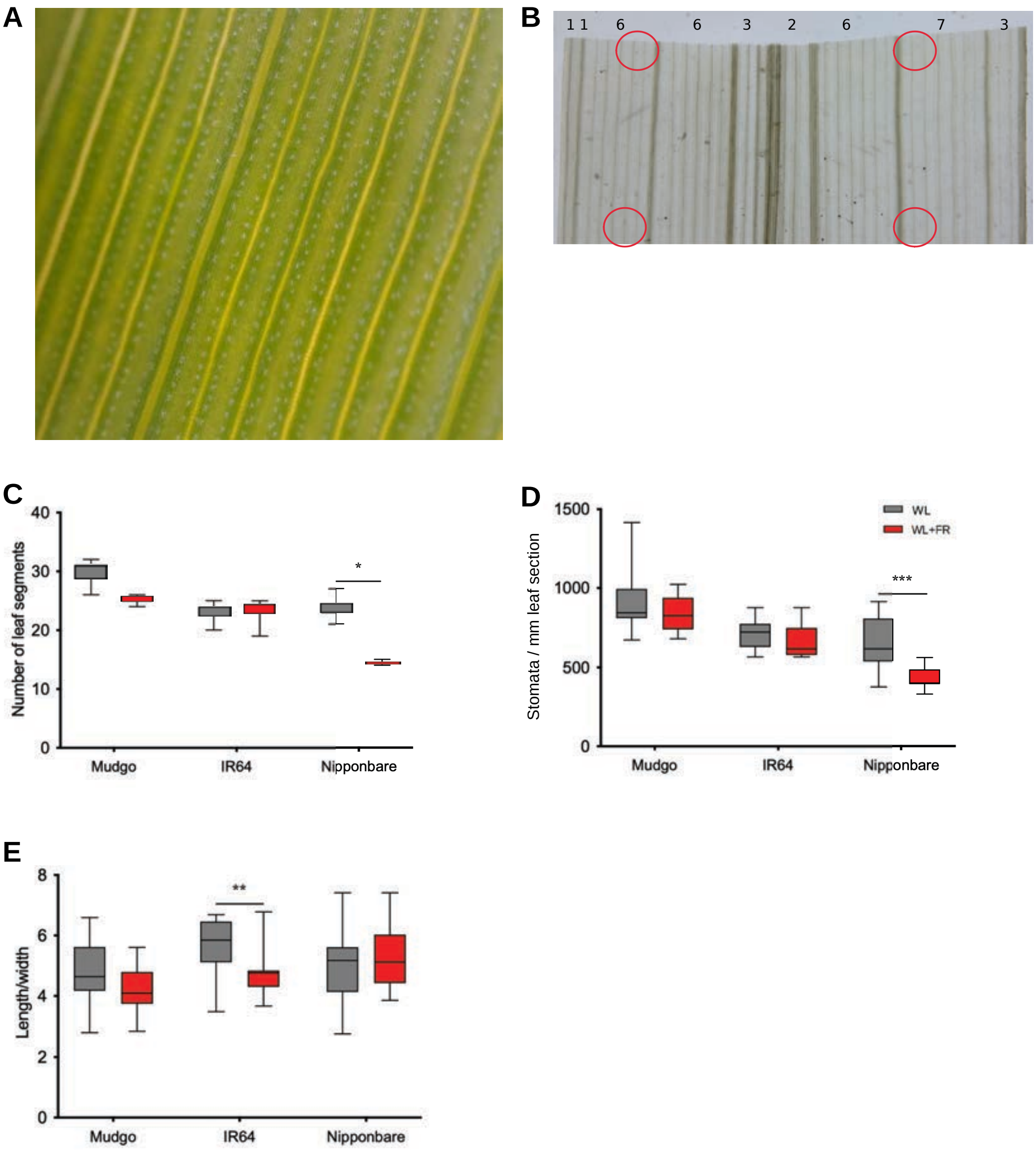
Light response curves were measured using gas exchange using LI-COR 6400XT with a closed measuring cuvette equipped with LED light source (2x3 cm with red and blue LEDs). Following the protocol in (Evans *et al.*, 2014) with minor adjustments using a flow rate of 400 µmol s^{-1}. Measurements for light response curves were taken with plants of the variety Nipponbare of the two treatment groups grown under control and FR supplemented conditions.

Measurements for light response curves were performed under ambient CO_2 concentration with set PAR intensities starting at high going to low light intensities: 1800 (first replicate was started at 2000), 1500, 1000, 500, 250, 120, 60, 40, 20, 10, 0 µmol photons m^{-2} s^{-1}. By following the analysis protocol described in (de Lobo *et al.*, 2013), we fitted equation 11 to determine the following parameters: light compensation point (I_{comp}), light saturation point (I_{sat}), maximum gross photosynthetic rate (P_{gmax}), maximum net photosynthetic rate obtained at Imax ($P_N(I_{max}$), dark respiration (RD) and quantum yield at the range between I_{comp} and I = 200 µmol photons m^{-2} s^{-1} ($\phi(I_{comp} - I_{200}$).

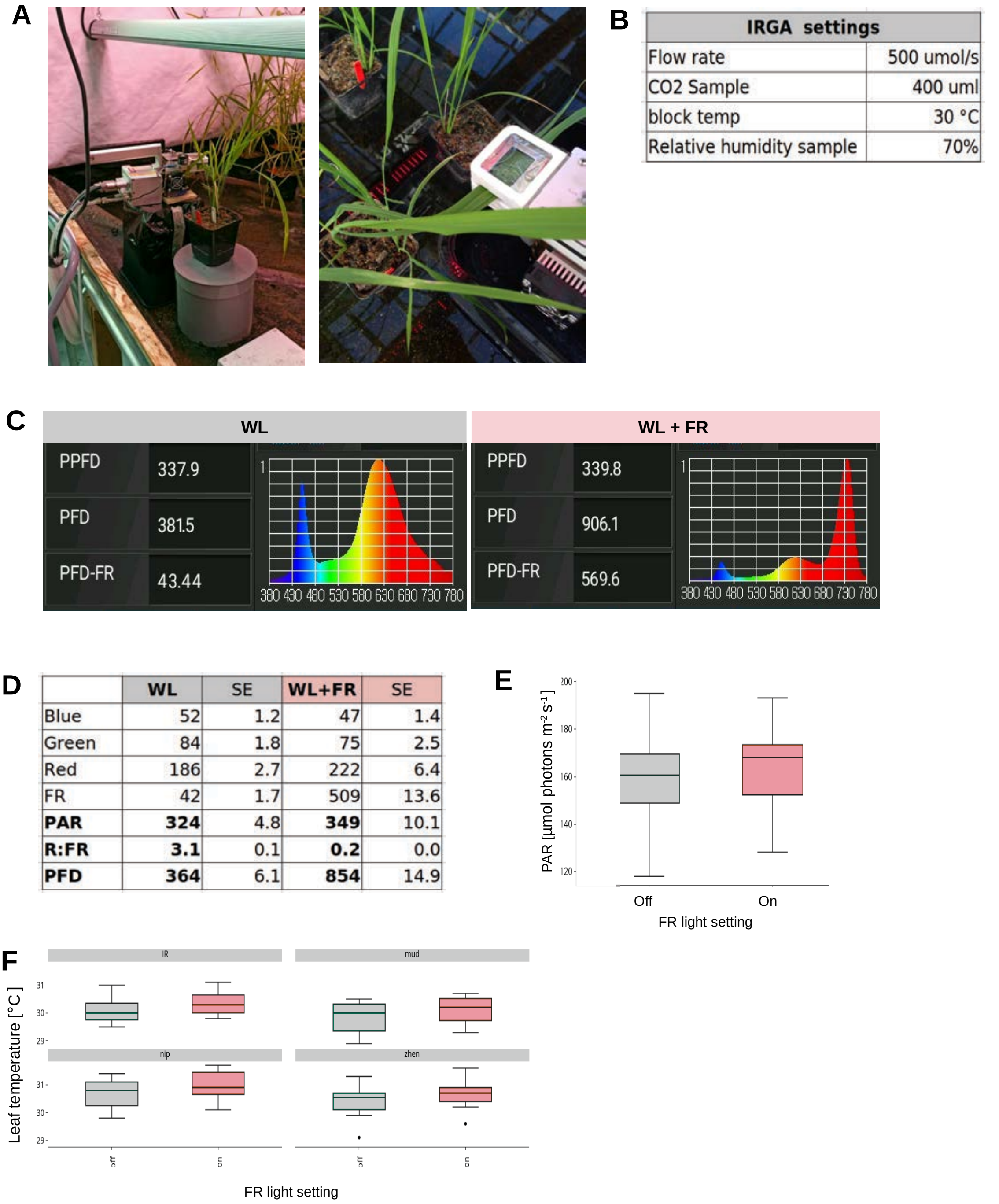
Supplements



Supplemental Figure 6.1. Increased leaf and internode formation rate and biomass of rice seedlings exposed to supplemental far-red (FR) recorded weekly from day of sowing until 28 days after sowing (das) in Luk Thakar, M Blatec and Sabharaj for **(A-C)** leaf number and **(D-F)** internode number. Comparison between varieties of **G.** shoot and **H.** root biomass at 28 das recorded as dry weight. Values are means of min four up to ten plants, significant differences are indicated with p-value < 0.05 *, < 0.01 **, < 0.001 ***; error bars are ± SE.



Supplemental Figure 6.2. Stomatal morphology and density of 28 days old rice seedlings exposed to supplemental FR. **A.** Adaxial side of leaf blade of plant under control conditions at 30x magnification. **B**. Example of distribution of leaf segments with circles are areas used for quantification of stomatal density. **C.** Number of leaf segments in WL and WL+FR grown plants and **D.** stomatal density as the minimal density based on segments in 1 mm leaf section, which is the number of stomata in one segment multiplied by the total number of segments in the leaf. **F.** Ratio of stomatal length to width. Measurements are taken on third youngest leaf, for stomatal density n = min 12 counts in 3 biological replicates, for stomatal dimensions n = min 16 counts in 3 biological replicates. Significant differences are indicated with p-value < 0.05 *, < 0.01 **, < 0.001 *** ; error bars are ± SE.



IRGA settings	
Flow rate	500 umol/s
CO2 Sample	400 uml
block temp	30 °C
Relative humidity sample	70%

	WL	WL + FR
PPFD	337.9	339.8
PFD	381.5	906.1
PFD-FR	43.44	569.6

	WL	SE	WL+FR	SE
Blue	52	1.2	47	1.4
Green	84	1.8	75	2.5
Red	186	2.7	222	6.4
FR	42	1.7	509	13.6
PAR	**324**	4.8	**349**	10.1
R:FR	**3.1**	0.1	**0.2**	0.0
PFD	**364**	6.1	**854**	14.9

Supplemental Figure 6.3. PAR is constant between groups, but PFD is strongly increased in +FR treatment. A. Set up of gas exchange measurement using the of the LI6400XT with **B.** IRGA settings for measurements. **C.** Light spectra of control group in white light (WL) and far-red treated group (WL+FR), with **D.** values for blue, green, red, far-red, total photosynthetic active radiation (PAR), red to FR ratio (R:FR) and total photon flux density (PFD); values are in µmol photons m-2 s-1. **E.** PAR measured inside the measuring cuvette of the open top leaf chamber with FR lights on and off and **F.** differences of leaf temperature between FR light on and off were not significant.

Chapter 7

General discussion

Main findings

When a plant responds to its environment, there are many signalling steps from signal perception to the observable changes in the phenotype (summarized in Buccitelli & Selbach, 2020). This thesis explores several dimensions of rice shoot architecture and regulation of response to environment: in space, in time and in magnitude (from whole canopy down to single gene level). One goal of this thesis was to explore the potential for improved weed suppression by rice plants, through optimized shoot architecture. In addition, plasticity of rice shoot architecture and growth in the presence of far-red light, the dominant planting density cue, was explored.

As a starting point, a large rice diversity panel was screened for phenotypic variation in shoot architecture (Chapter 3) with a focus on traits that are related to early growth vigour, as well as on additional traits such as solidity, leaf angle, tiller angle and leaf droopiness. A genome-wide association study on the key traits, pinpointed underlying genetic loci and facilitated the characterization of the alleles in these loci which are most optimal for a high shade casting rice plant with increased weed- competitiveness.

This study was followed up with experiments to test how stable these phenotypes of selected varieties are under field conditions and under neighbour competition (Chapter 4). The predicted competitive varieties identified in Chapter 3 were indeed high-shading in the field. The suppressive effect of rice on weeds, was strongest for varieties with a predicted high shade-casting ability, based on their high Shading Rank. Weed tillering was strongly suppressed and weed biomass was reduced to 30 % by competitive rice canopies as compared to their performance in the absence of rice. In addition, increased rice planting density also effectively suppresses weed growth, but has negative effects on rice performance itself, as seen from reduced tillering and biomass.

After exploring phenotypic plasticity in the field, Chapter 5 further investigated this under controlled conditions with planting density-associated light quality manipulations. Using far-red (FR) light as the known cue for proximity shade, responses to light quality could be studied independently from possible other interactions in the field. Surprisingly, rice seedlings exposed to supplemental FR showed increased tillering and biomass and only some varieties showed increased height. This is in contrast with classic shade avoidance responses. A transcriptomic analysis revealed very weak changes in gene expression in response to FR. Indeed, the transcriptomic signature rather revealed induced gene expression related to photosynthetic activity, more than the expected shade avoidance signatures.

Inspired by the consistent observations of increased biomass and faster developmental in +FR treated plants, it was then investigated if there were effects of FR photons on photosynthesis (Chapter 6). Gas-exchange measurements revealed an almost doubled CO_2 assimilation rate under FR-enriched conditions. These findings indicate that FR photons, unlike the conventional definition of photosynthetically active radiation being restricted to the 400-700 nm waveband, can substantially power CO_2 fixation in rice.

Contribution to improved rice farming

Weeds compete directly with rice plants and reduce rice yield. The crucial period for crops susceptible to weed damage is in the early vegetative growth phase of the crop, where weeds cause most yield loss when competing with rice plants in the first 40 – 60 days after rice establishment (Chauhan & Johnson, 2011; Mennan et al., 2012; Raj & Syriac, 2017). Therefore, in the studies of this thesis, the focus was on the early growth stages of rice. Based on the findings in the conducted field assay, we can confirm that rice indeed is able to substantially suppress weed proliferation. Weed biomass could be reduced by 40 % up to 70 % compared to when weed was growing alone. These data also show that the capacity of weed-suppression immensely depends on the rice variety, which underlines the fact, that choosing the right variety, can be a strong component of integrative weed management. We observed, that increased rice planting density similarly led to weed reduction, however came along with negative impacts on rice growth and is therefore not advisable in terms of weed-management.

Leads towards improving weed-competitive varieties

The here presented approach is different from what has been done so far, where selection has mainly focused on increase of shoot biomass, with the aim of increased yield. The aim was not to learn how to increase growth rate, but primarily to investigate how shoot architecture can be optimized, without major increased resource investments. The use of a large diversity panel, including representatives of all the six main subpopulations of cultivated rice (*indica*, *aus*, *aromatic*, *admixed*, *tropical* and *temperate japonica*), for a phenotypic screen and the genome wide analysis provide a highly diverse genetic pool, which is essential for these types of analysis and adds value for follow up application. In this work, genetic loci were associated with important shoot traits, that are crucial for increased shading and can therefore be valuable for breeding weed-competitive varieties. Especially shoot solidity could be of great relevance for breeding optimal cops that maximise shoot biomass within a given land area. This trait has not received significant attention in published studies on rice improvements so far. Architectural traits such as leaf angles, tiller angles and leaf erectness can also supply resources for manipulating plant architecture with other aims than weed-competitiveness, such as maximising light interception and decrease of lodging. The genetic loci associated with these traits are not only important for optimal shoot architecture, but in addition these traits are intrinsic to early growth vigour, which in turn are key traits for seedling establishment, especially valuable in direct seeded systems.

Without any doubt, the green revolution was a blessing for farmers as well as consumers, however the almost exclusive focus in breeding on increased yield, brought along shortcomings (Pingali, 2012). Not only that the modern varieties are highly dependent on fertilization and optimized irrigation, but also pesticides and herbicides. In respect to weed-competitiveness, some aspects of shoot architecture that were selected for during the green revolution, such as strong tillering, are also of advantage. However, the very strong selection against height, shows its drawback in weed-competitiveness, as this study here exemplifies. Well-known improved varieties, such as IR64 and Nipponbare, opposed to most of the other lines in the RDP which are traditional varieties, were close to the worst in their ranking for shading capacity (Chapter 3). These varieties were bred to be short and to invest a maximal amount of their assimilates into their reproductive organs – the harvestable grains (Hedden, 2003; Teichmann & Muhr, 2015; Wing et al., 2018). For successful weed-competition of rice, more than one shoot trait is necessary for increased shading capacity (as manifested in the Shading Rank in Chapter 3). It is rather a sum of architecture as well as vigour traits, and as part of this, also height and tiller angle should be considered in future breeding.

Improved (rice) farming practices

Weeds can be well suppressed by flooding the rice fields, but this water table on the fields is difficult to maintain and leads to undesirable methane emissions. We propose alternative ways to tackle the weeds in dry seeded rice systems. It was shown in the Chapters 3 and 4 that, by choosing the right variety and perhaps also adjusting planting densities, an improved weed suppression can be achieved. If the genetic loci of interest are introgressed in the highest yielding varieties, and these measures are then combined with other cultural and mechanical practices such as optimized field preparation, using crop residue for mulching and uniformed seeding patterns preparation, this forms a valuable part of a sustainable integrated weed management (IWM) (Chauhan, 2012; Mahajan et al., 2014; Raj & Syriac, 2017).

This thesis shows that there exists strong potential for weed suppression by both optimising the rice farming practice as well as the rice varieties themselves. Although this study focused on rice, weed infestation is not a problem specific to rice farming. This study can be taken as a representative of the principle of suppressing weeds via shading of the crop. Crops can do more than they have been selected for, in terms of disease and abiotic stress resistance, but also about weed competitiveness (Lu et al., 2020; Park et al., 2003; Teichmann & Muhr, 2015; Westwood et al., 2018). Increased shading potential of the crop has a clear potential for improvement towards sustainable weed suppression, which can be applied also in breeding programmes of other mayor cereal crops (Marín & Weiner, 2014; Park et al., 2003; Peerzada et al., 2017; Seavers & Wright, 1999; Wolfe et al., 2008; Wu et al., 2021). The overall principle is that crops could be engineered to optimize productivity at the crop canopy level, rather than at the individual plant level (Warnasooriya & Brutnell, 2014; Weiner et al., 2010).

Contributions to photobiology research

Shade avoidance responses

In the plant photobiology research domain, the term ‘shade’ is not always clearly defined. In most of the studies simulated shade is used, either by applying green filters that absorb red and blue light, or by manipulating the intensity of FR light with FR-emitting lamps. There are two main types of shade to be distinguished (Ballaré & Casal, 2000; Casal, 2013; Fiorucci & Fankhauser, 2017; Roig-Villanova & Martínez-García, 2016):

- proximity shade is without direct shading, but mimics nearby neighbouring vegetation; treatments with moderate or strong reduction in the R:FR;
- canopy shade or vegetational shade, is when vegetation is leading to actual shading, where both, PAR and R:FR are reduced.

As outlined in Chapter 2, low R:FR signals to plants that there is nearby neighbouring vegetation, and this typically elicits growth responses that help plants to avoid becoming shaded. This is achieved with elongation and height growth accompanied by a trade off in branching and leaf formation. In addition, photosynthetically active tissue is concentrated in the higher parts of the plants where they are maximally exposed to light. All these characteristics together are defined as the Shade Avoidance Syndrome (SAS). It is important to keep in mind, that FR light is the crucial trigger for the phenotypic responses occurring under proximity shade. These plastic responses are adaptive, giving the plant a fitness advantage. Under very low light intensities of deep vegetation shade, these shade avoidance traits are also displayed, but they are accompanied by overall slow growth due to reduced photosynthesis.

It is well studied that rice is strongly adversely affected by low light intensities, resulting in diminished growth in general and impaired tillering in particular, as well as reduced yield (Kikuchi et al., 2017; Takano et al., 2001, 2005; Warnasooriya & Brutnell, 2014; Wu et al., 1998). In the field experiment presented in Chapter 3, where the effect of higher planting density was investigated, there was no difference in terms of rice performance between the two planting densities early in the season, where a reduction in R:FR but no change in PAR can be expected. Later in the season, when growing canopies also lead to a reduction in light intensity, a reduction in tillering and biomass under high compared to normal density was recorded, indicating that rice plants do respond to planting density.

However, how responses of rice to proximity shade are manifested was unknown. This was investigated by subjecting rice to low R:FR treatment. After four weeks of treatment, there was no reduction in branching, but the opposite; leaf and tiller formation were increased. Plant height and internode elongation were only marginally increased, and only in some of the tested varieties. Leaves did also not become more upward oriented, but rather became droopier (less erect) and leaf inclination angles became more horizontal. Consistent with the lack of pronounced shade avoidance responses, an RNAseq analysis revealed very weak transcriptional responses to supplemental FR. Based on literature evidence, rice does carry the necessary molecular sensory and signalling components for neighbour proximity signalling and shade avoidance, i.e. phytochromes (Gu et al., 2011; Hu et al., 2020; Kebrom et al., 2006; Shin & Park, 2014; Sun et al., 2017; Takano et al., 2001, 2009)and phytochrome interacting factors (Nkamura et al., 2007; Yun-jia & Liesche, 2017; Zhou et al., 2014). So, why does rice not respond with strong universal shade avoidance responses? In later experiments, described in

Chapter 6, a vast increase in photosynthetic carbon fixation was unravelled when rice was given extra FR light. This might be part of the explanation to the question why there is such a weak SAS response to treatment with supplemental FR. However, the question remains, why low R:FR is not triggering a SAS response.

Possible reasons for marginal response to supplemental FR in rice

Low R:FR as a signal for SAS in rice

It is intriguing, that rice is responsive to low light intensity and planting density with respect to shoot architecture, but not to low R:FR. This raises the question, to what cue it is responding, i.e. which environmental cue is perceived and translated into a phenotypic response at high planting density. Low R:FR is one of the main and earliest cues for early warning of approaching vegetational shade, but it is not the only cue (Huber et al., 2020; Pierik & De Wit, 2014). It is possible, that for example low blue light intensity is more relevant in rice or when low R:FR occurs in combination with low PAR (De Wit et al., 2016). An option could also be, that a response is only triggered, when actual physical contact to neighbouring plants occurs; both are described as cues involved in neighbour detection (Keller et al. 2011; Keuskamp et al. 2011; Pantazopoulou et al. 2017, 2021; Sasidharan et al. 2008; De Wit et al. 2012). The regulation of processes related to photosynthesis and most of the transcriptomic products being localized in the chloroplast, are hinting at other processes strongly interacting with commonly described SAS pathways, consistent with the photosynthetic responses, rather than shade avoidance (Chapter 6).

Alternative neighbour proximity response strategy

When plants are growing in unfavourable light conditions, they follow different coping mechanisms, as described in the introduction. One of them is the avoidance strategy, which is the most studied and best understood, described as the SAS (Chapter 2). In the here investigated response of rice, none of the characteristics of SAS could be recorded, neither on the phenotype nor on the transcriptomic level. Even if there was slight induction of some of the typical SAS genes, this was a marginal number compared to what is recorded for strong shade avoiding species, such as Arabidopsis or in a shade sensitive Geranium species, where the number of induced DEGs is about ten times higher (e.g. Gommers et al., 2017; Küpers, 2022). There are also species that acclimate to low light intensities. Various species are known to be shade tolerant, for example because they grow in a forest understory (Kurepin 2015; Pons 2014;

Matsumoto 2005). These species would typically not show pronounced SAS responses to supplemental FR. However, these species do show a strong transcriptomic response, either to be able to make metabolic adjustments for limiting resource consumption and/or suppressing SAS induced pathways. This was shown in a *Geranium robertianum, a* species found in forest understories, which showed up-regulation of SAS genes but at the same time induced genes that were counteracting the activity of these genes and therefore did not exhibit a SAS phenotype (Gommers et al., 2017). Similar observations were made for *Cardamine hirsuta* (Molina-Contreras et al., 2019). However, unlike rice, shade tolerant species grow in chronically low light habitats where shade avoidance responses would be maladaptive since surrounding trees cannot be outgrown.

Following from the above, rice does not classify as a shade avoider nor as a shade tolerant plant. It is possible, that rice would be following a more offensive coping mechanism. In supplemental FR leaves and tillers become more horizontal. Combined with the observed stimulated tillering, this might help rice to outgrow potential upcoming neighbours and occupy the space before competitors do. Only when the light intensity drops some elongation is induced at the cost of branching.

Phylogenetic differences

Shade avoidance is an adaptive response since it promoted individual plant fitness in many species. If in the course of evolution plants with the potential to express SAS before being shaded had higher fitness than those who did not, why would this not be the case in rice? First of all, much of our knowledge about these phenotypic and transcriptomic changes is based on studies in Arabidopsis. The anatomy, physiology and even more so the genetic equipment of Arabidopsis is fundamentally different from a grass, such as rice. Certainly, there are signalling pathways in common, and many gene homologues between rice and Arabidopsis have been identified. It can, however, not be assumed that gene or protein function is always conserved between these two distinct species (Kebrom & Brutnell, 2007; Sawers et al., 2005). Even within the group of cereals there are differences in gene functions due to deletions, different copy number or expression patterns (Bennetzen et al., 2007). So, it may not be surprising that an SAS response would look different in rice than in Arabidopsis.

What the anatomy of a monocot would allow as a response to low R:FR might look different from a rosette plant (Sawers et al., 2005). Based on recent evolutionary studies, for example the hyponastic petiole angle in a rosette plant would be comparable to decreased tiller angle in grass and the hypocotyl or stem elongation would be comparable to the leaf sheath, i.e. internode elongation in the early vegetative stage of a grass (Richardson et al., 2021). This would still mean that one could expect increased height growth and reduced branching, which was not recorded here. It is., therefore, unlikely that the lack of shade avoidance in rice follows from the grass anatomy and architecture. Indeed other grasses, such as maize, can show pronounced elongation responses to FR light (Dubois et al., 2010).

Natural habitat

Shade avoidance, and especially its fitness benefit, depend on the habitat that the perceiving plant is adapted to. Both, Arabidopsis and rice, are adapted to open fields, Arabidopsis as a pioneering plant, and rice as a grass and most of the species in these open habitats, are known to express the SAS when shaded (Gommers et al., 2013). The natural region of rice to grow in is the tropics and subtropics, which is a very high light environment. Rice requires a minimum light intensity, which is far higher than for many plants growing in northern latitudes, which would experience this as high light stress. So, one explanation for the absence of a typical SAS phenotype, despite the very low R:FR, is, because even though when a rice plant is growing close to other plants, it will still be ensured of having enough light and can “afford” to ignore the approaching shade signal.

Domestication

Human cultivation selected rice to be adapted to grow at high densities in monocultures, where neighbouring plants barely grow taller and in addition the chances are high that the neighbouring plants are of their own kind. Darwinian fitness of a single plant would probably not be beneficial for the plant population, in this case a crop monoculture, since whatever investment a plant makes, this investment will also be made similarly by its identical neighbours (Pantazopoulou et al., 2021; Weiner, 2019; Weiner & Freckleton, 2010; Weiner et al., 2010). During the green revolution, one of the main objectives was to select plants with reduced height. Since some of the genes hitherto selected for during the green revolution are also involved in the SAS pathway, this might have entailed some selection against SAS plasticity. An example for this is the growth hormone Gibberellin (GA) and players in its downstream pathway. It was discovered that green revolution dwarf rice varieties, were defective in a metabolic enzyme for GA synthesis, leading to lower GA levels (Monna et al., 2002; Sasaki et al., 2002; Spielmeyer et al., 2002). In addition, a mutation in the *SLR1* gene, encoding a DELLA protein, resulted in enhanced DELLA stability in the presence of GA, thus preventing GA-induced height growth (Chandler & Harding, 2013; Hartweck & Olszewski, 2006; Hedden, 2003).

Maize, rice and wheat have been selected for erect canopies with genetic selection for small leaf angles and optimal leaf orientation (Warnasooriya & Brutnell, 2014). Although traditional breeding programs have selected for increased performance under high planting density, at least for maize and wheat, it was hypothesized that this could indicate increased tolerance for limited resources, rather than selection against shade avoidance and indeed modern cultivars even respond to low R:FR with stronger elongation than old varieties (Sawers et al., 2005; Wille et al., 2017). Given the strong selective pressure imposed by breeders who strive for increasing yields at increasing plant densities (Duvick, 1997), some studies showed that certain shade responses in crop plants may, in fact, be enhanced relative to their weedy ancestors (Kebrom & Brutnell, 2007). Also, the results shown here for wild rice species indicate that their response is in the same order of magnitude as compared to cultivated varieties (in terms of trait values, but also in terms of treatment duration until a response resulted in a significant difference). Thus, domestication does not per se select against shade avoidance, and wild rice varieties are also not shade avoiding, indicating that the lack of classic R:FR-induced shade avoidance responses in rice are not likely related to the domestication process.

What distinguishes rice from other cereal crops?

All cereal crops are grasses (*Poacea*), which belong to the clade of monocots and originated 55-70 million years ago (Kellogg, 2001). Cereals of tropical origin and adapted to short day lengths include rice, maize, and sorghum, whereas wheat and barley were originally domesticated in the Fertile Crescent and are considered long day species (Brambilla et al., 2017). Later they diverged into semi-tropical cereals, including rice, maize and sorghum, and into temperate species, that include wheat, barley and rye (oat), flowering under long day conditions (Sawers et al., 2005). Some studies show that wheat and barley do respond to supplemental FR with reduced tillering, but also minor elongation (Wille 2017; Evers 2006; Ugarte 2010; Skinner 1993;). A study on low R:FR on barley showed biomass reallocation to the shoot and increased leaf area (Kegge et al., 2015). This group of cereals is adapted to being mostly cultivated in northern latitudes, where days are long in summer and short in winter and a generally lower light intensity. As discussed in Chapter 5, other cereal crops, such as maize and sorghum, do exhibit elongation of internodes and leaf sheaths under shade (Evers et al., 2006; Finlayson et al., 2007; Kebrom et al., 2006; Sawers et al., 2005; Takano et al., 2001, 2005; Warnasooriya & Brutnell, 2014).

Cereals such as sorghum, maize and millet, a close relative of maize, diverged earlier, and like rice, their main areas of cultivation are the tropics and subtropics (Figure 7.1). This region is characterised by very high light intensities and close to constant day length in addition to high temperatures, which these cereals are well adapted to. However, what distinguishes all of them from rice, is that they are adapted to dry areas and that they are all C4 species. It would be interesting to further research if there is an interaction between drought adaptation, C4 metabolism and expression of SAS that could explain the specific status of rice on this spectrum of species.

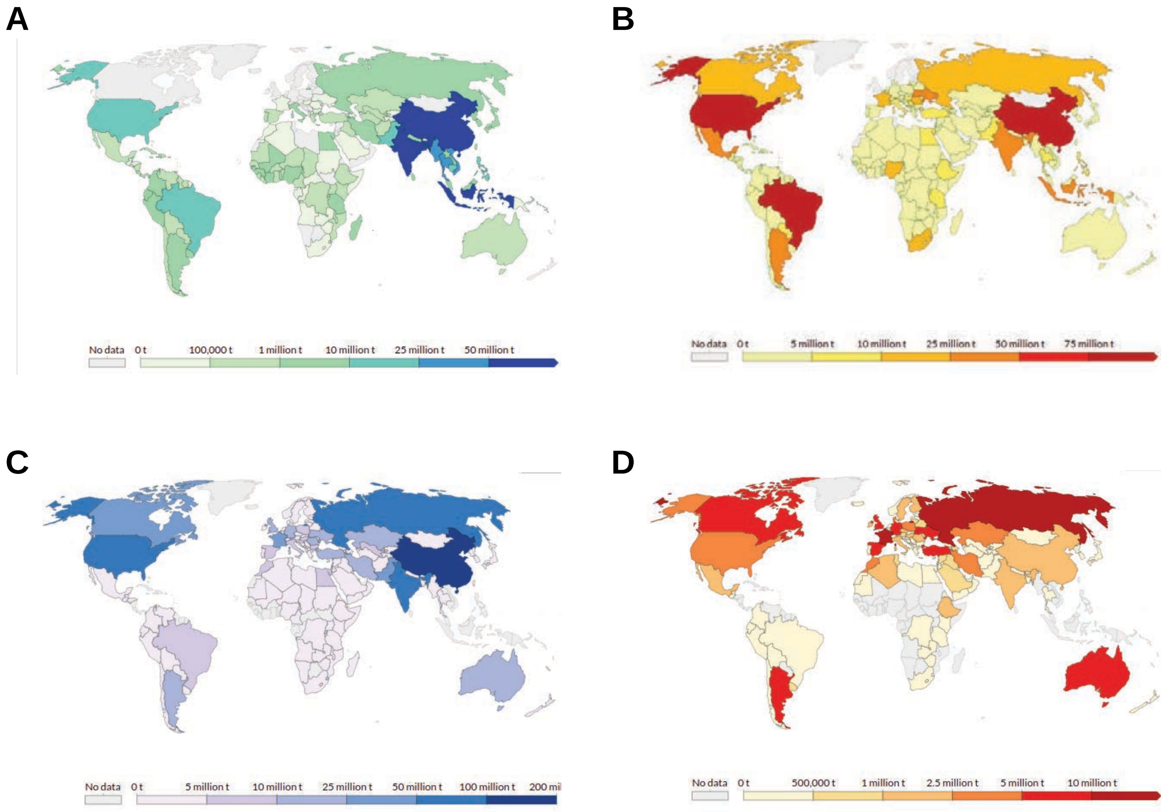


Figure 7.1. Global production areas of mayor cereal crops. A. Paddy rice, **B.** corn, **C.** wheat and **D.** barley, given in tons in the year 2018. Based on data from UN Food and Agriculture Organization (FAO) (2020): visualization created by Hannah Ritchie and Max Roser published online at OurWorldInData.org. Retrieved from https://ourworldindata.org/agricultural-production#cereals.

FR is more than a signal

A highly unexpected finding in Chapter 6 was that FR photons are not just a signal for proximity shade triggering SAS, but also an energy source for driving photosynthesis (FR photons exciting photosystem I). Whether FR is perceived as a signal for shade and elucidating the SAS or substantially fuelling photosynthesis and leading to more assimilation, might not be a black and white difference between plants, but possibly a combination of both. It cannot be fully excluded that rice does respond to low R:FR as a signal for neighbour proximity, but that any subsequent shade avoidance response is masked by the strong promotive effect of FR on growth and development. For example, if low R:FR signalling through phytochromes would reduce tillering as a form of apical dominance, the strong promotive effect of FR on CO_2 fixation might simultaneously strongly promote tillering, with the net result being a promotion, rather than inhibition of tillering.

At this point it is not clear, how FR and PAR depend on each other. In a plant canopy, PAR goes down but it is possible that up to a certain level FR could compensate the effect on photosynthesis, since FR photons will be enriched where PAR is depleted. Studies with supplemented FR light added to a higher background PAR did find increased biomass in tomato, lettuce and soybean (Tan et al., 2022). These observations might indicate that FR photons can drive photosynthesis, and promote growth, only in relatively high light environments. FR only excites Photosystem I, which drains electrons from Photosystem II to Photosystem I, from which it follows that there must be enough WL present providing a balance of available electrons that can be provided from Photosystem II (Yamazaki, 2010; Zhen et al., 2021). It will be important to investigate the interdependencies of PAR and FR fluence rates for photosynthesis in great detail, in order to understand where in the canopies FR can and cannot compensate losses in PAR. This is scientifically important, but can also have important opportunities in cropping systems with full control over light quality and quantity, such as vertical farming solutions and greenhouses.

Conclusion

Our findings, that rice very strongly benefits from supplemented FR with increased growth, could mean that at an early stage of proximity shade, FR would actually increase photosynthesis and plant growth. FR is always present under natural conditions and in a canopy of dense vegetation it is even enriched by reflection. A canopy consists of many layers, with different light conditions, where in the top part increased FR reflection of surrounding vegetation might also lead to increased photosynthetic rates. To this end it is unclear, how specific this phenomenon is for rice. Further studies on responses in young rice canopies and the role of FR light can give useful insights to make information-based decisions in farming practices such as planting density, planting patterns, choice of variety and field management, such as weed and water management.

Future directions

Recommendations for rice breeding and farming practices

Engineering future-ready rice would benefit from including shoot architectural traits using the necessary genomic information supplied here. Introgressing favourable alleles of relevant genes into high yielding varieties would give rise to new varieties delivering secured yields under weedy conditions.

In addition to the genetic insights for weed-competitiveness, we also gained more understanding of improvement in rice farming practices. Increased density might be an option for better weed-suppression, but is counterproductive for crop performance. Getting a better understanding of a combination of different practices for IWM will be necessary to give advice to farmers for optimized sustainable farming practices. The method of alternate-wetting and drying (AWD) as a field water management would be a promising option, where in the early crop season water would suppress weeds, before rice casts substantial shade. As a first step, high weed suppressive varieties in combination with AWD could be explored in field trials, to validate new farming practices.

Recommendations for photobiology

In addition to using the genetic loci identified in this thesis to breed for more weed-competitive rice, the presented work on FR responses points towards an interesting area for plant photobiology research. The discovery that FR photons can power CO_2 fixation suggests that we might need to reconsider how to define photosynthetic active radiation. These discoveries present a great opportunity to explore further, how much the FR contribution to photosynthesis is a general feature of most plant species if they are growing in high light environment and whether there is a trade-off between increased photosynthesis and SAS under FR enrichment. To what extent the two effects of FR light as energy and signal are exclusive, either affecting plant architecture or photosynthesis, needs further research. The uncoupling of stomatal opening from photosynthetic rate under FR enrichment, as well as the weak acclimation to FR also need further investigation. Opposing results were found in other studies, where under high FR light an increased number of stomata was observed in chrysanthemum and *Rotala hippuris* leaves but a reduction was seen in leaves of tobacco and cucumber (Tan et al., 2022).

Disentangling the dual role of FR photons

To study the interplay of FR as an energy source for photosynthesis and as an early warning signal for proximity shade, several approaches can be followed. FR can probably only be used for photosynthesis when also WL is present. Whether the amount of PAR compared to FR occurs in absolute terms (i.e. a minimum amount needed) or a relative amount (i.e. PAR needs to increase with increased FR) for FR to be effective for photosynthesis requires detailed follow-up studies. This could be investigated by testing different quantities of PAR and FR in combination and measuring chlorophyll fluorescence characteristics, such as the Fv/Fm of the photosystems (Zhen & van Iersel, 2017). The initial question, of how the SAS phenotype is manifested in rice, still remains. To shed light on the SAS response in rice, the dual effect of FR needs to be disentangled, which could be done for example by end of day FR treatment, where FR is only applied, when there is no WL present and therefore not being able to drive photosynthesis. Rice is highly plastic and strongly impacted by reduced light intensity and planting density. Investigating further to what signal it is responding, if it is not low R:FR, and how this is mechanistically regulated, is a field of further research.

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About the author

Martina was born on the 2nd of March 1990 in a forgotten corner of the Austrian countryside, surrounded by mountains and forests. She had the privilege to grow up in close relation to nature, at the ridge of the Hohe Tauern National Park.

There she spent her childhood and received her primary education. She then moved a few valleys further, where she completed her higher education with honours in 2009. Her studies were focused on logistics and hotel industry.

To extend her horizon, she left the boundaries of her home country, to first work on the Canary Islands and then cross the ocean to explore the rainforest and rural areas of Central America. There she developed a fascination for biology and decided to pursue her studies in Biology at the University of Salzburg. She obtained a master's degree with honours in 2015, after having completed internships in forestry and in laboratory analysis, at a hospital and a dairy factory. She spent long periods abroad in Finland and in Colombia, and she finally reached the Netherlands, where she joined the Plant-Environment Signaling group at Utrecht University for an internship. This brought her in contact with plant research and opened up the opportunity of pursuing a PhD.

She embarked on this new adventure in the search for weed-competitive rice for sustainable farming, under the guidance of Prof. dr. Ronald Pierik and co-supervision of Dr. Kaisa Kajala. Throughout the project, she has worked together with Justine Toulotte, whose work has focused on flooding tolerance in the major rice weed *Echinochloa crus-galli*. The project brought Martina all the way to the Philippines, where she worked in the rice fields at the International Rice Research Institute in Los Baños, in collaboration with Dr. Virender Kumar.

This thesis is the result of her scientific work throughout this journey.

Along the way, she has been part of the Experimental Plant Sciences PhD council, founding member of Scientist4Future NL, and executive board member of the KlimaatHelpdesk. Moreover, she has engaged in several public outreach activities, including a fruitful art-science project together with two photographers. In her future career, she will explore these activities further, as a passionate biologist motivated to contribute to a more sustainable and fair world.

Publications and preprints

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Rice in a different light: Shoot architecture from genome to field Martina Huber