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Flooding tolerance in the major rice weed *Echinochloa crus-galli*

Overstromingstolerantie in het grote rijstonkruid *Echinochloa crus-galli*

(met een samenvatting in het Nederlands)

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Chapter 1
General introduction

Excerpts of this chapter will be published in: Toulotte JM, Pantazopoulou CK, Sanclemente MA, Voesenek LACJ, Sasidharan R (2022), Water stress resilient cereal crops: lessons from wild relatives. *Journal of integrative plant biology*, accepted

Flooding tolerance in plants

Most crops are sensitive to waterlogging (root submergence) and submergence (partial or complete submergence of aerial parts). Just a few days of flooding can seriously damage plants and will result in significant agricultural losses. The primary consequence of flooding is impaired gas diffusion between the plant and its environment. The gas diffusion rate in water is approximately 10,000 times slower than in air, resulting in limited delivery of oxygen and carbon dioxide. This leads to an impairment of cellular respiration and of photosynthesis and consequently, an energy crisis that can ultimately kill the plant (Jackson, 1985; van Dongen and Licausi, 2015; Voesenek and Bailey-Serres, 2015). Light availability can also be limited if plants are submerged in muddy water, further restricting photosynthesis. Impaired gas diffusion also causes accumulation of the gaseous hormone ethylene. While this is an important trigger of various adaptive responses (Sasidharan and Voesenek, 2015), long-term ethylene build-up can also be harmful (Stearns and Glick, 2003). In addition, oxygen deficiency in the soil causes the reduction of oxidized compounds, which can be toxic to plants and alters nutrient availability (Elzenga and van Veen, 2010). Together with limited energy production, these changes cause injury to roots and ultimately to the whole plant, leading to plant death in severe cases (Drew and Lynch, 1980; Kirk et al., 2014). The period following floodwater retreat presents another stressful scenario for flooded plants. The return to aerial conditions is associated with excessive reactive oxygen species (ROS) accumulation leading to oxidative stress and drought like symptoms associated with malfunctioning roots (Yeung et al., 2019).

Tolerant plants have developed different strategies to cope with submerged conditions, including various morphological, metabolic and growth adjustments. These include alterations that facilitate hypoxia escape or when the flooding is too deep, hypoxia endurance. Escape traits typically permit avoidance of oxygen deficiency by improving internal aeration and enhancing underwater gas exchange. These include root traits such as the formation of a barrier against radial oxygen loss (ROL) - in roots. This ROL barrier prevents the diffusion of oxygen to the surrounding anoxic soil, thanks to a deposition of lignin and suberin in the outer root cell layer (Colmer, 2003; Colmer et al., 2019; Pedersen et al., 2020). Such barriers might also prevent the influx of toxic compounds from the soil into the plant. Detoxification of excessive damaging oxygen radicals and of other toxins also helps prevent further injuries during submergence and recovery (Ismail et al., 2009; Ismail et al., 2012; Colmer et al., 2014; Yeung et al., 2019).

Often the primary roots are replaced by shoot-borne adventitious roots that can be rich in aerenchyma (Visser et al., 1996; Yamauchi et al., 2017). Aerenchyma formation can occur in roots, stems and leaves (Alpi and Beevers, 1983; Kawai and Uchimiya, 2000; Abiko et al., 2012). These interconnected gas

filled spaces improve internal aeration by offering a low resistance pathway for the diffusion of air to flooded tissues from plant parts still above the water. Aerenchyma can be formed constitutively or in response to waterlogging. This process is induced directly by the intercellular presence of ROS and of ethylene (Ni et al., 2019). Some species have hydrophobic leaves facilitating the formation of gas films underwater which enhance gas exchange and photosynthesis (Colmer and Pedersen, 2008; Pedersen et al., 2009). An upward movement of leaves (hyponasty) and petiole and/or shoot elongation can facilitate an escape response of plants from floodwaters (Hattori et al., 2008; Van Veen et al., 2013; Ayano et al., 2014; Voesenek and Bailey-Serres, 2015; Yamauchi et al., 2018; Pucciariello, 2020). In contrast, some plant species display a quiescence strategy upon submergence, meaning that shoot elongation is inhibited, and energy expenditure is limited until the water level decreases (Xu et al., 2006; Akman et al., 2012).

In addition to these different morphological and anatomical mechanisms, plants can also adjust their metabolism to oxygen limited conditions. This involves a switch to anaerobic metabolism to continue to produce energy (ATP) or permits the anaerobic mobilisation of starch to germinate underwater (Kretzschmar et al., 2015). This allows many wetland species to germinate anaerobically or to tolerate hypoxia during prolonged submergence. In plants that are tolerant to flooding, genes encoding alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH) are increasingly expressed during submergence (Fukao et al., 2003). These enzymes regenerate NAD+ required to generate energy through glycolysis, by converting pyruvate to acetaldehyde and ethanol.

Flooding tolerance in rice

Rice has traditionally been grown in flooded environments owing to its relative tolerance to waterlogged conditions (Mackill et al., 2012). Indeed, modern rice cultivars derive from aquatic ancestral landraces. However, prolonged and deep flooding can also kill rice plants. Most rice varieties die within 14 days of complete submergence and only a few can withstand longer submergence durations (Xu et al., 2006). Being farmed in low lying flood-prone areas, rice cultivation has always been vulnerable to flooding. But the heightened threat of flooding due to climate change makes the need for the development of flood-tolerant rice cultivars more urgent. In rice farming areas, flooding patterns can vary widely, with each requiring a different survival mechanism. Flooding regimes can vary from long-term partial submergence (stagnant flooding) to complete plant submergence of short (flash floods) or long (deep-water flood) duration.

Semi-aquatic plant species like rice can survive flooding better than most crops because they possess several of the aforementioned adaptive traits. Rice plants have hydrophobic leaf surfaces and form gas films when submerged, they

also form adventitious roots, aerenchyma tissues and ROL barriers in roots (Colmer et al., 2019).

Flash flood-tolerant rice varieties typically show growth retardation when submerged and are highly tolerant of post-submergence stress. An example is the Indian flood-tolerant variety FR13A derived from a traditional landrace (Dhalputtia, an aus variety). The observed high submergence tolerance of FR13A was found to be conferred by the (*Submergence1*) *SUB1* QTL on chromosome 9 (Xu et al., 2006). The *Sub1* locus is composed of a cluster of three ethylene response factors (*ERF*) genes located in tandem, named *Sub1A*, *Sub1B* and *Sub1C*. Tolerant genotypes possess the tolerant *Sub1* haplotype *Sub1A-1/Sub1C-1* (Singh et al., 2010). The dampening of underwater shoot growth imposed by *Sub1A* is mediated by an accumulation of the GA signalling repressors Slender rice (SLR1) and SLR1 Like-1 (SLRL1) (Fukao and Bailey-Serres, 2008). The resulting repression of gibberellic acid (GA) action subsequently results in restricted leaf and internode elongation. The downregulation of energetically expensive growth is accompanied by a reduced expression of genes involved in carbohydrate metabolisms such as alpha-amylases and sucrose synthases. In contrast, the expression of genes encoding fermentation enzymes is activated. Thus, *Sub1A* directs a general energy conservation strategy while prioritizing core hypoxia acclimation responses (Bashar, 2019).

This conservative strategy is especially beneficial for plants following desubmergence. *Sub1A* containing varieties show superior recovery associated with a better energy balance and higher drought and oxidative stress tolerance (Fukao et al., 2006; Fukao et al., 2011; Tamang and Fukao, 2015). The introduction of the *Sub1A* locus into several varieties of high-yielding rice allowed them to tolerate complete submergence for 2 weeks. For example, the introgression of the *Sub1* loci into the Indian variety Swarna (Swarna-Sub1), resulted in high submergence tolerance in field trials without any negative influence on yield, plant height, harvest index and grain quality (Xu et al., 2006). Although *SUB1A-1* derives from the aus sub-group of indica rice (Xu et al., 2006), alleles have been also found in other *Oryza* species such as *Oryza nivara* and *Oryza rufipogon* accessions, also belonging to the A-genome group. Other species such as *Oryza rhizomatis* and *Oryza eichingeri*, belonging to the C-genome group, do not possess the *Sub1A-1* allele but still are tolerant to flooding alluding to *Sub1A*-independent mechanisms. Indeed, submergence tolerance is a common feature of many wild *Oryza* species that grow in wet habitats but do not possess *Sub1A*. Deeper investigation of such species is warranted to uncover novel tolerance mechanisms and loci (Niroula, 2012).

Many delta and river basin regions around the world experience long-lasting floods several meters deep. In such flooding scenarios, deep-water rice varieties thrive. They do so by utilizing an escape strategy where energy and carbohydrates are invested in stem elongation. This allows deep-water rice to emerge above the water surface (over 50 cm to several meters) and continue normal oxygen uptake facilitated by aerenchyma. Deepwater rice varieties, though not high yielding, are an important crop in flood-prone regions of countries like Thailand, Bangladesh and Cambodia. The genetic and molecular mechanisms underlying their spectacular growth responses have been extensively studied (Kuroha et al., 2017; Kuroha et al., 2018). A QTL mapping approach identified three QTLs that explained the submergence-induced elongation in the deep-water rice cv C9285, a japonica varietal group from Bangladesh (Wang et al., 2013). The major QTL on chromosome 12 contained the genes *SNORKEL 1* (*SK1*) and *SNORKEL 2* (*SK2*). Like the *SUB1* genes, the *SK1s* are also ERFs. Ethylene accumulation in submerged internodes upregulates *SK1* and *SK2* gene expression. These transcription factors subsequently trigger downstream responses culminating in GA biosynthesis and stimulation of internodal elongation.

Coleoptile elongation is also seen as one of the major contributors to flooding tolerance in plants (Kato-Noguchi and Morokuma, 2007). This means that flood-tolerant rice varieties that show such an escape strategy will be more tolerant to flooding than tolerant rice varieties that show a quiescence strategy during early development. This elongation, however, costs a lot of energy. To have a sustainable energy trade-off, rice plants first produce the coleoptile before the radicle and will invest less, or nothing at all, in root formation (Fox et al., 1994).

Rice farming and weeds

Weeds can be defined as any plant species growing in human-controlled settings (fields, gardens, lawns, parks, roadsides etc.) that are unwanted for varied reasons including the negative impact on crop yields or the aesthetics of a place. The invasiveness and persistence of weeds in diverse environments are attributed to a suite of morphological and physiological traits that allow them to germinate and settle in new environmental conditions and to generate and efficiently spread seeds. The highly invasive nature of weeds and their ability to adapt to extreme environments also make them good models for studying plant environmental stress adaption (Vigueira et al., 2013; Clements and Jones, 2021; Sharma et al., 2021). Weed infiltration in agricultural fields is particularly worrying and a major threat to crop productivity.

In rice fields as well, weed infestation causes drastic reductions in crop yields. It is estimated that for rice, weeds may result in yield losses ranging from 23

to 100% under aerobic systems (Jabran and Chauhan, 2015). The extent of weed inflicted losses is dependent on several factors including, the weed species and density, the rice ecosystem, the management practice used and the rice cultivar itself. Weeds growing in rice fields have to be adapted to quickly germinate in reduced and hypoxic soils and sometimes continue growth under submerged conditions (Gibson et al., 2002; Chauhan and Johnson, 2009; Chauhan and Johnson, 2010). The negative impact of weeds on rice yields is because of their ability to compete with rice for critical resources such as water, nutrients and light or by changing the pH of the soil (Bastiaans et al., 2008). Wilson et al. (2014) show that high weed infestation can absorb 60-80% of the available nitrogen in the soil at the expense of the crop. Species like *Echinochloa colona* or *E. crus-galli* can also suppress the germination or growth of rice by allelopathic effects (Sitthinoi et al., 2017; Khanh et al., 2018). As a result, these weeds can quickly outcompete rice in their early growth stages compromising the growth and finally grain yield of rice (Manandhar et al., 2007; Shukla et al., 2015). Another trait related to their invasiveness is the ability to form considerable quantities of seeds. Depending on the environmental conditions, *Echinochloa* species can produce several thousands of seeds per plant (Gibson et al., 2002), that can persist in the soil for 8-9 years (Chul and Moody, 1989; Chin, 2001).

Among the several weed control methods in use in rice farming, is water-based weed management which exploits the high flood tolerance of rice (Tuong et al., 2000; Kaya-Altop et al., 2019) and the high sensitivity of certain weeds to early flooding. For rice farmed in irrigated lowland systems in paddies, nursery-grown rice seedlings are transplanted to flooded soils. Prior to the preparation of the field for transplantation includes treatment with general herbicide application to control mixed weed flora. Most nonaquatic weeds cannot germinate or develop underwater, while the transplanted flood-tolerant rice is able to grow in waterlogged conditions. However, in recent times, the emergence of several flood-tolerant weeds has reduced the efficacy of this water-based weed management strategy. Several weed species such as *Ammannia prieriana, Sphenoclea zeylanica and Heteranthera callifolia* (Kent and Johnson, 2001), *Echinochloa oryzoides* (Pearce and Jackson, 1991), *E. phyllopogon* and *E. crus-pavonis* (Fox et al., 1998), *E. crus-galli* (Holm et al., 1977; Fukao et al., 2003; Chauhan and Johnson, 2011), *E. glabrescens* (Opeña et al., 2014) or *Cyperus rotundus* (Peña-Fronteras et al., 2009; Fuentes et al., 2010) are commonly found in rice flooded environments. Flood-tolerant weed species utilize a suite of morphological, physiological and metabolic adaptations to cope with the compound stress inflicted by flooding. Many of these are similar to those present in flood-tolerant rice varieties. These include exodermal suberin in adventitious roots to prevent oxygen loss in *Echinochloa spp.* (Ejiri and Shiono, 2019), cuticle hydrophobicity, permeability and leaf gas film formation for underwater gas exchange in *Glyceria fluitans* (Dennis Konnerup and

Pedersen, 2017), aerenchyma tissue formation in *Cyperus rotundus* stems and roots (Fuentes et al., 2010), a higher content of carbohydrates and a higher amylase activity, as well as the ability to maintain high-soluble sugars during early growth in *C. rotundus* tubers (Fuentes et al., 2010; Peña-Fronteras et al., 2009).

Thesis outline

Considering the huge relevance of rice as a staple food crop, and as a source of livelihood for the world's poorest farmers, investigating ways to reduce weed-related losses in rice fields is both urgent and necessary. The main aim of this thesis was to investigate the extreme flood tolerance of aggressive rice weeds found in the rice flooded agro-environment. The identification of flood-tolerant weeds and an understanding of the underlying resilience mechanisms and tolerance traits can allow the identification of alternative flooding regimes to suppress weed growth but also provide novel insights into the evolution of flooding stress tolerance.

In **Chapter 2**, a selection of rice weeds and their biotypes were surveyed for their resilience to complete submergence at different developmental stages. Based on this screen of these highly flood-tolerant weed species, *Echinochloa crus-galli* was chosen for further in-depth molecular and physiological interrogation.

In **Chapter 3,** the high flood tolerance of *Echinochloa crus-galli* was confirmed by further phenotypic and physiological characterisation. This was followed by a comparative transcriptomics approach to probe the observed high tolerance of *Echinochloa crus-galli* to submergence and post-submergence stress. Using mRNA-sequencing the *Echinochloa crus-galli* molecular response to flooding was compared to that of two other species, including rice (four genotypes in total) representing a relevant spectrum of tolerance strategies. This resulted in the identification of both common and *E. crus-galli* specific responses to flooding stress.

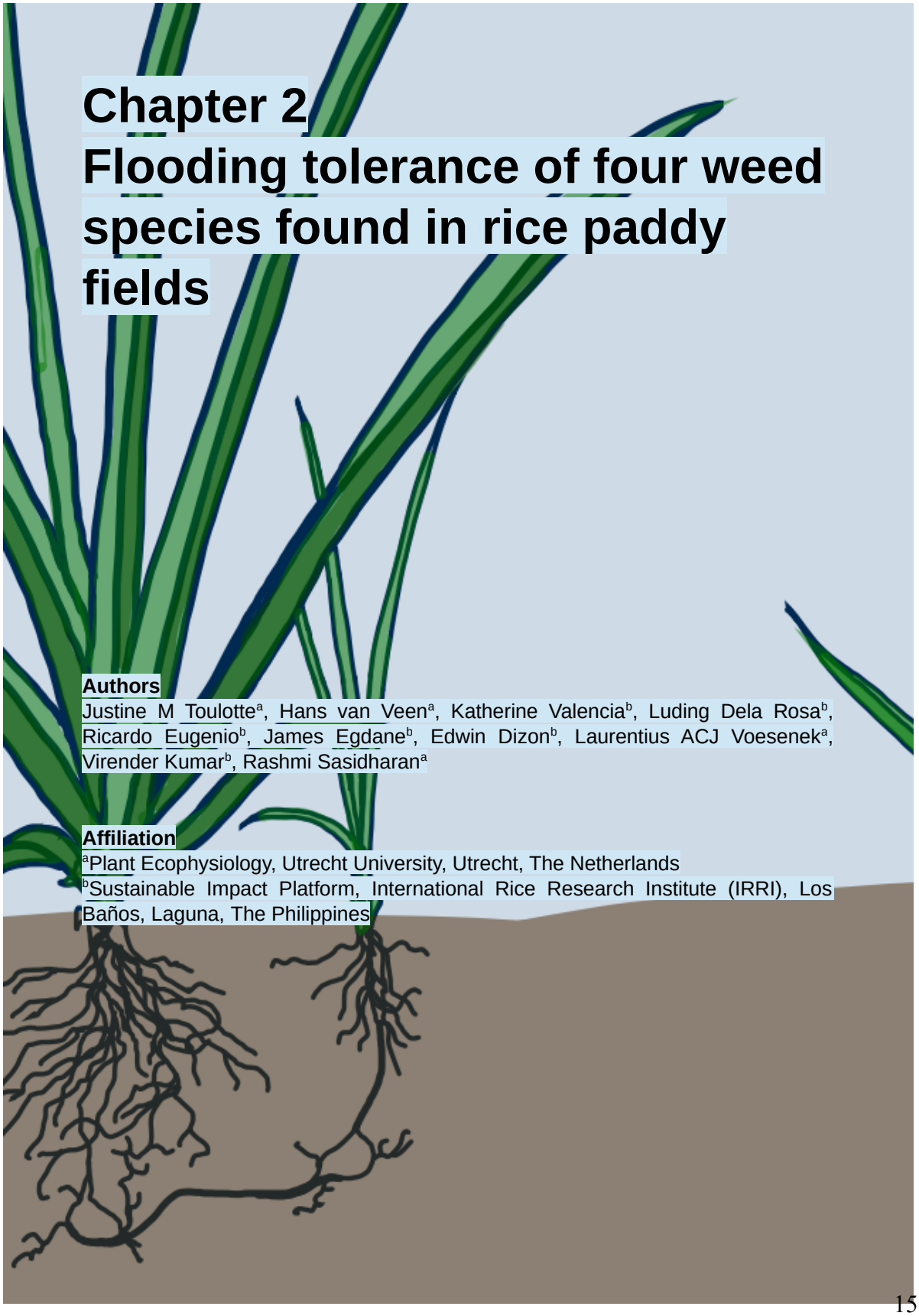
To further probe the molecular basis of *E. crus-galli* high flooding tolerance, a multi-species (18 species) orthology comparison was performed in **Chapter 4**. The orthology analysis coupled with a general linear model approach revealed common and shared responses between species, but also species-specific responses. The aim of this chapter was also to investigate the conservation of flooding responses in Poaceae species and gain insight into the evolution of flooding tolerance in monocot grasses.

Chapter 5 investigates alternative weed management strategies for *E. crus-galli* based on reducing weed development through shade cast by the crop (rice). Both greenhouse and field rice-weed competition experiments revealed the

high sensitivity of the weed to light-limited conditions. We also show that combining complete submergence and shade does not result in additional weed suppression. These results suggest that weed management protocols using early flooding, followed by natural shade from high shade-casting rice cultivars might more efficiently suppress weed growth in rice fields.

In **Chapter 6** we integrate the main findings in this thesis and discuss the evolution of flooding tolerance, what makes *Echinochloa* so resilient to flooding and how better weed subdual can be achieved by the incorporation of weed competitive rice into existing water-based weed management protocols.

Chapter 2
Flooding tolerance of four weed species found in rice paddy fields



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Abstract

Weed infestation dramatically reduces rice yields. In the cultivation of paddy rice, this is overcome by the transplantation of rice seedlings to flooded fields, where the anaerobic conditions prevent the establishment of most weeds. The efficacy of this method, however, is undermined during the last decades by the emergence of several flood-tolerant weed species. It is of interest to understand the tolerance mechanisms in these species from the perspective of developing alternative weed management practices and as interesting models for the evolution of flooding acclimation. Here we investigated flooding tolerance in different biotypes of four major rice weed species: *Echinochloa crus-galli* (common barnyard grass), *Echinochloa colona* (jungle rice), *Leptochloa chinensis* (Chinese sprangletop) and *Cyperus rotundus* (purple nutsedge). Our results revealed considerable tolerance to flooding conditions in all biotypes and species at both the germination and post-germination stages. Within each species, there was no correlation between the collection site (flooded or drained) and the observed tolerance. Even though submergence could successfully repress germination and growth, seeds could germinate, and seedling growth was resumed upon desubmergence. These findings present interesting model systems to further probe the evolution and molecular basis of flooding tolerance. Additionally, these results could have implications for the current water-based weed management protocols in use.

Introduction

Echinochloa spp., *Leptochloa spp.* and *Cyperus rotundus* species are considered among the most important weed species infesting rice (Kraehmer et al., 2016). Water is an important weed control tool in lowland transplanted rice farming. After eradication of the first batch of germinated weeds by chemical methods during the field preparation, fields are flooded and 2-4 week-old rice seedlings are transplanted into these flooded fields. This suppresses or kills most weeds and favours the growth of the rice seedlings. Water depth is generally kept at 5-10 cm through most of the season and water is gradually drained prior to harvest. These waterlogged conditions have been shown to be a very efficient weed control method (Chamara et al., 2018; Kaya-Altop et al., 2019). For most weed species, germination and establishment are impaired underwater, while the rice seedlings get a size advantage and growth head start against the weeds. However, there are several weed species such as *Ammannia prieriana, Sphenoclea zeylanica and Heteranthera callifolia* (Kent and Johnson, 2001), *Echinochloa oryzoides* (Pearce and Jackson, 1991), *E. phyllopogon* and *E. crus-pavonis* (Fox et al., 1995), *E. crus-galli* (Holm et al., 1977; Fukao et al., 2003; Chauhan and Johnson, 2011), *E. glabrescens* (Opeña et al., 2014) or *Cyperus rotundus* (Peña-Fronteras et al., 2009; Fuentes et al., 2010) that have evolved tolerance to flooded conditions, necessitating the needs for integrated weed management protocols that combine other weed control methods with water management.

Seed germination is regulated by several types of phytohormones and various environmental factors including oxygen (Christianson et al., 2009; Park and Hasenstein, 2016). Starch is the main storage material in cereal seeds, that will be converted to soluble sugars during seed germination (Loreti et al., 2003). Under anaerobic conditions, wheat and barley seeds cannot germinate - like most cereal species - although they have a high starch content. This is due to a lack of α-amylase activity (Perata et al., 1992), which is very important for seed germination underwater. On the contrary, rice seeds are able to germinate, grow and survive underwater. This is primarily due to their ability to express α-amylase under low oxygen conditions (hypoxia) (Damaris et al., 2019).

However, not all rice varieties are capable of anaerobic germination (AG). Some rice seeds can germinate and extend their coleoptiles under hypoxic and even anoxic conditions, but fail to develop roots and leaves (Ella and Setter, 1999). In a study published in 2012 by Ismail et al., only 0.23% of more than 8000 rice (*Oryza sativa*) accessions and IRRI breeding lines, survived (over 70% of survival) and were able to form shoots and roots within 3 weeks, after dry sowing followed by flooding at 8-10 cm height. Among the metabolic processes described to be likely associated with the tolerance to AG, was the ability to initiate and to maintain

carbohydrate catabolism to provide the necessary energy to the seed and to the seedling later (Miro and Ismail, 2013).

Several QTLs associated with rice AG-tolerance have been identified (Hsu and Tung, 2015; Kim and Reinke, 2018; Jeong et al., 2019; Ghosal et al., 2019; Yang et al., 2019; Ghosal et al., 2020; Mondal et al., 2020), paving the way for the development of AG-tolerant rice varieties. These varieties could be one of the solutions to the weed infestation problem in Direct-Seeded Rice (DSR) and could be used in association with controlled water management in the fields. The technique could be beneficial to suppress flooding-sensitive weeds economically and in an environmentally friendly manner (Illangakoon et al., 2020). Another avenue for the exploration of AG-tolerance is flood resistant weeds themselves. AG tolerance is reported for several weed species such as *Echinochloa crus-galli*, *Scirpus juneoides* or *Sphenoclea zeylanica* (Kennedy et al., 1980; Pons and Schroder, 1986; Moon et al., 1999; Estioko et al., 2014) and it would be interesting to explore how these weeds evolved to the flooded conditions and if the identified molecular features could be transferred to new rice varieties.

When a seed is able to germinate (or a tuber to sprout) underwater, the just-emerged seedling has to face a multitude of stresses: limited gas exchange, limited nutrient availability and water uptake, phytotoxicity and intermediates from the anaerobic carbon metabolism, changes in soil pH, etc. Several weed species from flood-prone areas have been described as having typical flood-adaptive features.

Although flooding in the early stages of a rice crop may reduce weed density, once lowland weeds have emerged and passed the seedling stage, their growth may be unaffected by flooding. The greater the delay in flooding, the less growth is likely to be affected. As an example, flooding at 15 days after emergence resulted in a 53% reduction in the growth of *Eclipta prostrata* compared with the unflooded control, though flooding at 20 days after emergence did not affect the growth of this weed (Lee and Moody, 1988).

While the emergence of flooding tolerance in weeds is a problem, it also presents an opportunity to investigate and identify the evolution of flooding tolerance and novel tolerance mechanisms. To this end, we investigated flooding resilience in four major rice weed species: *Echinochloa crus-galli, Echinochloa colona, Cyperus rotundus* and *Leptochloa chinensis*. For each species, biotypes were collected from flooded and dry conditions. We hypothesized that biotypes from frequently flooded environments would show a higher resilience to flooding.

The main goals were to:

- Characterize the effect of submergence on germination and early seedling growth in major rice weed species
- Document potential natural variation in flooding tolerance between biotypes of weed species.

Flooding tolerance was observed in all tested weed species at the germination and at the early growth stages. There were no significant effects of submergence depth on tolerance and no variation between the different biotypes within species. Emergence and early development were impaired drastically while the plants were underwater, but germination and seedling growth continued once the water was drained. Early flooding of the fields could therefore be useful to suppress weeds and give the rice a growth head start and competitive advantage in fields.

Materials and methods

Plant material collection

All *Echinochloa crus-galli, Echinochloa colona* and *Leptochloa chinensis* seeds used here were originally collected by the Weed Science team (International Rice Research Institute (IRRI), Los Baños, The Philippines), from different locations (Fig. 2.1) in The Philippines, within the period Sept-Nov 2016. Seeds were then further bulked 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 the IRRI screenhouse. Seeds harvested in 2017 seeds were used in this study, which was performed in Jan-April 2018 at IRRI. For one biotype of *E. crus-galli* used in the germination assay (Ecg13), seeds collected in an upland field (October 2015), were used directly. The conditions in this upland field sampling site are considered similar to the ones used for the seed reproduction of the other biotypes in the IRRI screenhouse. Seeds were kept in a dark and dry place until needed for the experiment.

Cyperus rotundus tubers were collected on IRRI field sites and near Iloilo city (Santa Barbara region) a few days before the experiments (Nov 2018) (Fig. 2.1). The sites chosen were upland (non-flooded) and lowland rice fields (experiencing continuous irrigation). Tubers were isolated from each other by cutting the surrounding rhizomes and washing away the soil. The tubers were then briefly sterilized (1 min) in a bath of bleach:Milli-Q water (1:1) and then washed five times with Milli-Q water. Tubers were then stored in the dark at 6ºC, in a wet paper in sealed plastic bags for 1 or 2 days before using them for experiments.

Experiment 1: Effect of submergence on weed germination / sprouting

Plant growth and treatments

Echinochloa and *Leptochloa chinensis*

Plastic canisters of 7 cm x 7 cm dimensions were filled with 5 cm depth of sterilized soil from IRRI fields. Per submergence depth, four canisters placed in four different cubicles were used. Each canister had 50 seeds that were placed on the soil surface and then covered by a thin layer of soil, watered and placed the next day in the submergence set-up (Fig. s2.1).

Cyperus rotundus tubers

Tubers of 7 to 11 mm width per 18 to 22 mm length and of 0.5 to 1.7 g (depending on the biotype), were stored in the dark at 6ºC and then directly pushed at 2 cm depth into the soil (sterilized soil from IRRI fields) in plastic trays of 42 cm x 72 cm and of 15 cm soil depth. The trays were then placed in the submergence set-up so that the water was 4 cm above the soil surface. For each biotype, 41 to 50 tubers were followed per treatment (control and submerged).

Submergence treatments

Echinochloa and *Leptochloa chinensis*

Submergence experiments were conducted in the Weed Science Team screenhouse at IRRI, Los Baños. The different submergence depths were obtained by placing canisters on a step-ladder placed in a cubicle that could be flooded (Fig. s2.1). The cubicles were flooded with tap water that was continuously circulated during the submergence phase of the experiment. During the recovery phase, the water was drained and the canisters were watered every day manually. Temperature was recorded throughout the experiment duration by several Tinytag data loggers (*www.geminidataloggers.com*), hung on top of the set-up. Temperatures varied between 25-26°C by night and 27-36°C by day, for a light cycle of 12 h / 12 h. Light intensity during the day was of 150-300 $\mu mol.m^{-2}.s^{-1}$. Relative Humidity was 40-60% during the day and 90-95% during the night. The different submergence depths were: Control conditions (normal watering), saturated conditions (level of water= soil surface= 0 cm), 1 cm, 2 cm, 3 cm, 5 cm and 8 cm of water from the soil surface. The submergence duration was 12 days, followed by a recovery phase of 10 days. Different cubicles were used, where all the different biotypes were submerged simultaneously. Four cubicles per species were used, each cubicle being an independent technical repetition.

Cyperus rotundus tubers

The germination assay took place in the submergence greenhouse at IRRI, Los Baños. For the submerged conditions, water depth was 4 cm above the soil surface. Submergence treatment was 13 days, followed by a recovery phase of 17 days. Control condition trays were watered manually every day. Standing tap water was used and was circulated once the algae would appear on the surface. During the recovery phase, trays were watered manually every day. Temperature was recorded continuously during the experiments using several Tinytag data loggers (*www.geminidataloggers.com*), hung on top of the set-up. Temperatures varied between 25-26°C by night and 34-40°C by day, for a light cycle of 12 h / 12 h. Light intensity during the day was of 150-300 $\mu mol.m^{-2}.s^{-1}$. Relative Humidity varied from ~30% during the day to ~70% during the night.

Emergence monitoring

For all species, the soil emergence of the seedlings was counted daily or every two days. Time to 50% emergence was estimated by fitting a combination of two logistic models to the emergence data, where the first was based on emergence data from submergence and the second based on the recovery. To this end, Generalized Linear Models with binomial errors was implemented in R. Time to 50% emergence was subsequently assessed with the dose.p() function from the library “MASS”.

Experiment 2: Effect of submergence on weed germination / sprouting

Plant growth and treatments

Echinochloa and *Leptochloa chinensis*

Echinochloa seeds were first dehulled (seed coats removed) to increase the % germination. Then, seeds were germinated on wet paper in the screenhouse (12 h dark 23-27°C / 12 h light 30-40°C). *Leptochloa chinensis* seeds were directly put to germinate under the same conditions, without pre-treatment. After 3 days (Ech) or 1 day (Lc), 9 (Ech) or 12 (Lc) germinated seeds per canister were transplanted in soil, 3 canisters per timepoint, and installed in the submergence set-up. For each biotype, 27 (Ech) or 36 (Lc) seedlings (3 canisters) were followed per treatment (control and submerged).

Cyperus rotundus tubers

Per each sealed petri dish, six *Cyperus rotundus* tubers were placed between two wet Whatman paper sheets. The petri dishes were then kept in the screenhouse (12 h dark 27°C / 12 h light 42°C) to allow the tubers to sprout. After 2 days, sprouted tubers were transplanted into the trays filled with sterilized soil from IRRI fields, at 2 cm depth (the entire sprouted tuber is below the soil surface).

Trays were submerged to a depth of 4 cm above the soil surface. For each biotype, 6 to 50 tubers per treatment (biotype and control or flooding) were followed.

Submergence treatments

Submergence experiments were conducted in the Weed Science Team screenhouse at IRRI, Los Baños, under the same condition of water, light, temperature and relative humidity described above.

Echinochloa and *Leptochloa chinensis*

The different submergence depths were: Control conditions (normal watering), 10 cm and 20 cm of water above the soil surface for the *Echinochloa* species and 5 cm and 12 cm of water above the soil surface for *L. chinensis*. The submergence duration was 8 days (Ech) or 10 days (Lc), followed by a recovery phase of 10 days for all species. Different cubicles were used, where all the different biotypes were submerged simultaneously. Three cubicles per species were used, each cubicle being an independent technical repetition.

Cyperus rotundus tubers

The early growth assays took place in the submergence greenhouse at IRRI, Los Baños, at the same time as for the germination assay. For the submerged conditions, water depth was 4 cm above the soil surface. Submergence treatment was 13 days, followed by a recovery phase of 14 days.

Measurements of early growth and development

Echinochloa and *Leptochloa chinensis*

Biomass was assessed at the end of the submergence period and at the end of recovery. For all conditions, all plants coming from one canister were harvested, pooled (9-12 plants), shoot and root were separated, dried at 80°C for 3 days and then weighed. Maximum stretched shoot height, as well as the number of true leaves and tillers per plant, were recorded during the recovery phase.

Cyperus rotundus tubers

For all conditions, the number of emerged shoots per tuber, the maximum stretched shoot height, as well as the number of true leaves per tuber (only the main shoot was counted), were recorded at different timepoints of the experiment. Submerged plants were followed till the 14th day of recovery.

Statistical analyses

The statistical tests were performed with the free software jamovi (https://www.jamovi.org). The effect of submergence on pre-germinated seedlings of *Echinochloa* and *Leptochloa chinensis* was analyzed with a 2-way Analysis of Variance (ANOVA). Multiple comparisons were performed with Tukey's HSD

(honestly significant difference) test. The effect of submergence on pre-sprouted tubers of *Cyperus rotundus* was analyzed with a 1-way Analysis of Variance (ANOVA).

Results

Experiment 1: Seedling emergence is delayed by complete submergence but recovers following drainage

The effect of complete submergence on seedling emergence was monitored in various biotypes of three major weed species: *Echinochloa crus-galli, Leptochloa chinensis* and *Cyperus rotundus* (Fig. 2.1). The percentage of seed germination was monitored over time under well-drained (Control) and submerged conditions (Fig. 2.2). To assess if an increase in the submergence depth would impose a more severe stress, several water depths were also compared (Fig. s2.2 and s2.3). The germination of the seeds under control conditions recorded at the end of the recovery period was set at 100%. Here, we compared the relative germination under complete submergence normalized to the corresponding control, for each timepoint, to be able to compare between biotypes.

A

Code	Species	Location collection	Environment
Ecg08	*Echinochloa crus-galli*	K, IRRI, Laguna	Lowland
Ecg09	*Echinochloa crus-galli*	UD2, IRRI, Laguna	Lowland
Ecg10	*Echinochloa crus-galli*	M5, IRRI, Laguna	Lowland
Ecg11	*Echinochloa crus-galli*	200, IRRI, Laguna	Lowland
Ecg13	*Echinochloa crus-galli*	CRF area, IRRI, Laguna	Upland
Ecol05	*Echinochloa colona*	UQ, IRRI, Laguna	Lowland
Ecol07	*Echinochloa colona*	?	Upland
Lc01	*Leptochloa chinensis*	San Matias, Iloilo	Lowland
Lc02	*Leptochloa chinensis*	Sinibaan, Iloilo	Lowland
Lc03	*Leptochloa chinensis*	Cogonan, Nasugbu, Batangas	Lowland
Lc06	*Leptochloa chinensis*	UC, IRRI, Laguna	Upland
IR-Low	*Cyperus rotundus*	300, IRRI, Laguna	Lowland
IR-Up	*Cyperus rotundus*	MN, IRRI, Laguna	Upland
ILO-Low	*Cyperus rotundus*	Santa Barbara, Iloilo	Lowland
ILO-Up	*Cyperus rotundus*	Santa Barbara, Iloilo	Upland

B

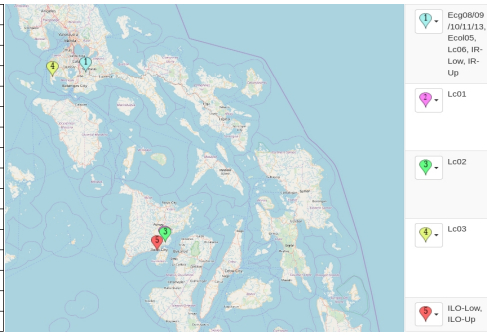


Figure 2.1: Four different weed species from diverse growing environments used in this study.
A. Table listing the different biotypes of *Echinochloa crus-galli* (Ecg), *Echinochloa colona* (Ecol), *Leptochloa chinensis* (Lc) and *Cyperus rotundus* (Cr) used in this study. Numbers indicate different biotypes. Low= lowland (prone to flood); Up= upland (dry fields); ILO= ILOILO; IR= IRRI. Also listed are the original sampling location and growing environment.
B. Map showing the sampling locations in the Philippines. 1= IRRI fields, 2= San Matias, Iloilo, 3= Sinibahan, Iloilo, 4= Cogonan, Nasugbu, Batangas, 5= Santa Barbara, Iloilo

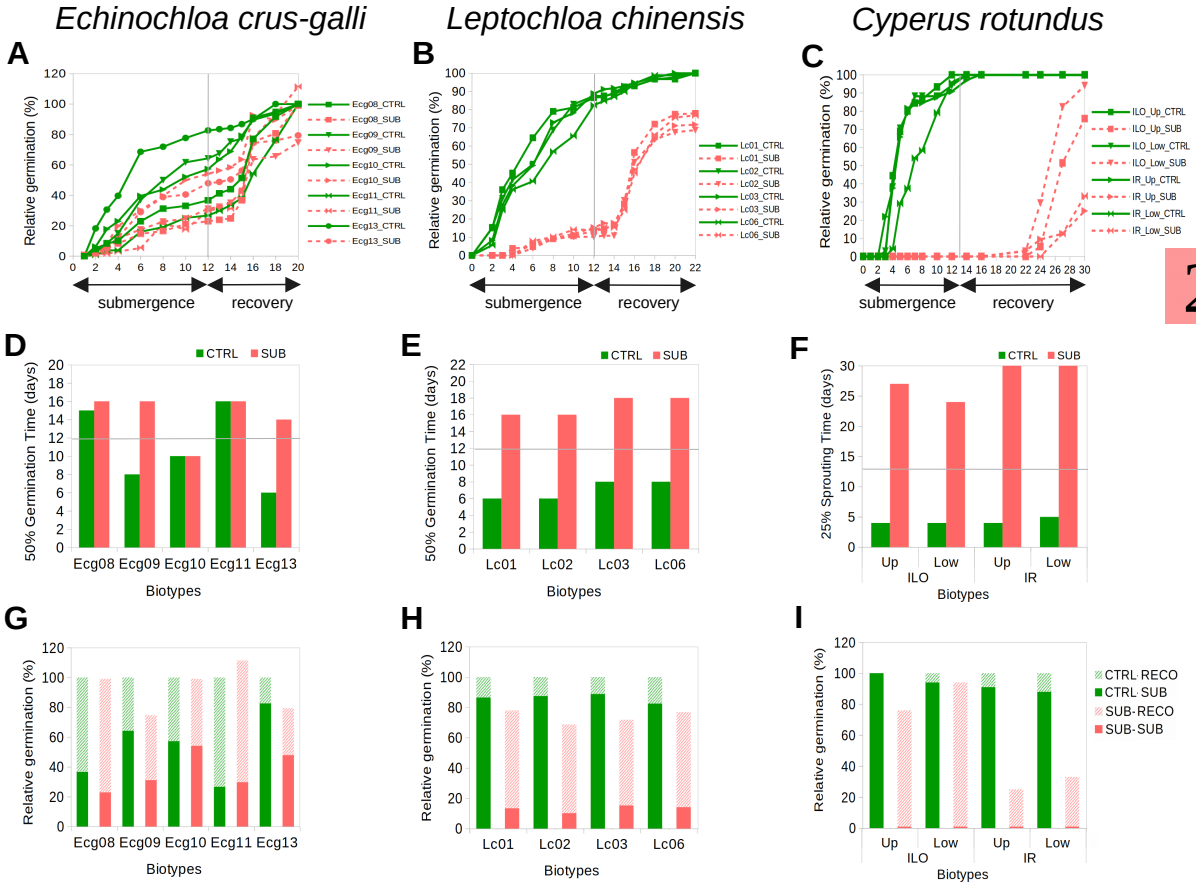


Figure 2.2: Anaerobic germination / sprouting of different weed species and biotypes.
A-C. Relative seed germination percentages for (**A**) *Echinochloa crus-galli* (Ecg) (**B**) *Leptochloa chinensis* (Lc) and relative tuber sprouting percentage for (**C**) *Cyperus rotundus biotypes*. The germination / sprouting under complete submergence (SUB) was normalized to the corresponding control (CTRL) values for each timepoint. For the controls, germination recorded at 20 days (Ecg), 22 days (Lc) or 30 days (Cr) (end of recovery period) was set as 100%.
D-E. Number of days after which 50% germination was achieved (GT50%) for Ecg (**D**) and Lc (**E**) under control (CTRL) and submerged (SUB) conditions.
F. Number of days after which 25% of the tubers had sprouting (ST25%) for Cr (50% sprouting was not reached for all conditions at the end of the experiment).
G-I. Relative germination / sprouting values at the end of the submergence phase and at the end of the recovery phase for Ecg (**G**), Lc (**H**), Cr (**I**).
For (**A-I**) CTRL= Well drained, SUB= Submerged for 5 cm from the soil surface for Ecg and Lc and 4 cm from the soil surface for Cr, Ecg= *Echinochloa crus-galli*, Lc= *Leptochloa chinensis*, Cr= *Cyperus rotundus,* ILO= Iloilo, IR= IRRI, Up= Upland, Low= Lowland. Submergence treatment was 12 days for Ecg and Lc and 13 days for Cr, indicated by the grey dashed line. Recovery after submergence (well drained conditions) was 8 days for Ecg, 10 days for Lc and 17 days for Cr. Green bars= control (CTRL) conditions, red bars= submerged (SUB) conditions. Bars are means± SEM. n(Ecg)= 200, n(Lc)= 200, n(Cr)= 41-50.

E. crus-galli biotypes varied in their ability to germinate underwater (Fig. 2.2A). While submergence clearly delayed germination in some biotypes (Ecg08, Ecg09 and Ecg13), others (Ecg10 and Ecg11) were not affected at all (Fig. 2.2A, 2.2D and 2.2G). The 50% germination time (GT-50) values for Ecg10 and Ecg 11 were similar under control and submerged conditions (10 days and 16 days), while for submerged Ecg 9 and 13 the GT-50 values more than doubled (16 days and 14 days respectively) relative to control (8 days and 6 days respectively) (Fig. 2.2D). In general, an increase in the depth of submergence did not have a significant effect on germination. For all biotypes, the same germination is observed from 1 to 8 cm of water depth (Fig. s2.2). When the water was drained after 12 days of submergence, all biotypes continued to germinate, eventually approaching the maximum germination recorded for the control group (Fig. 2.2A and 2.2G). Even biotypes Ecg09 and Ecg13 where germination was considerably impaired during submergence, showed a remarkable recovery following drainage (Fig. 2.2G).

While submergence clearly delayed the seed germination in *L. chinensis*, there was hardly any variation across biotypes (Fig. 2.2B, 2.2E and 2.2H). The GT-50 values more than doubled for all biotypes when seeds were submerged (Fig. 2.2E). In control conditions, seeds needed between 6 and 8 days to reach 50% germination, whereas the submerged seeds needed between 16 and 18 days to reach it. Like *E. crus-galli*, varying submergence depth had no significant effect on germination in any of the *L. chinensis* biotypes (Fig. s2.3). All biotypes showed a remarkable increase in germination following desubmergence. Depending on the biotype, 10-15% of the seeds could germinate underwater, but most of the submerged seeds (69 to 78%) germinated during the recovery phase (Fig. 2.2H).

In contrast with the two other weed species, complete submergence was effective in completely suppressing tuber sprouting of *C. rotundus* for all the biotypes (Fig. 2.2C, 2.2F and 2.2I). All biotypes sprouted very rapidly in control conditions (Fig. 2.2C and 2.2I). Between 4 and 7 days were necessary to reach 50% emergence and between 12 and 16 days were sufficient to get 100% emergence. However, following water drainage, *C. rotundus* shoots started to emerge from the soil within 5 to 11 days post-submergence (Fig. 2.2C). The two biotypes collected from Iloilo sprouted faster than the 2 biotypes collected at IRRI (Fig. 2.2C and 2.2I).

Experiment 2: Complete submergence negatively affects the development of young seedlings

The effect of complete submergence on the performance of pre-germinated seedlings for *E. crus-galli* and *Echinochloa colona*, *L. chinensis* and *C. rotundus* was assessed. Weed survival and various shoot traits such as shoot biomass and height, number of leaves and tillers were monitored. For *E. crus-galli,*

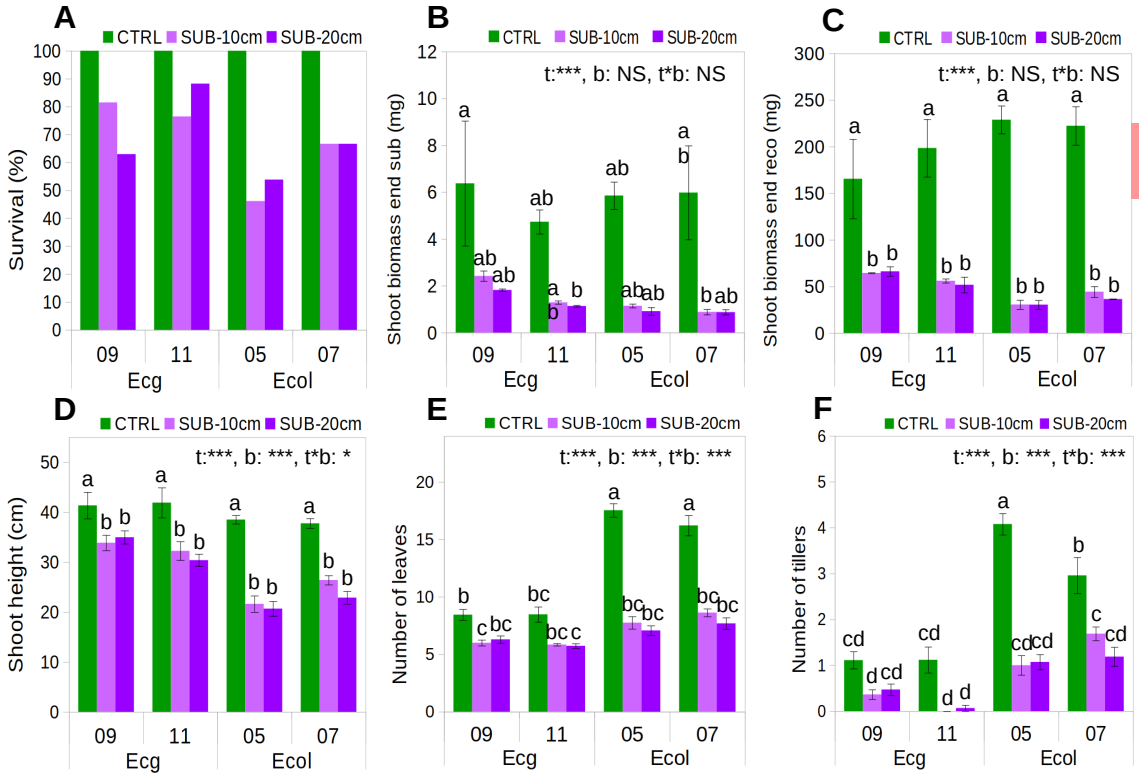


Figure 2.3: The effect of submergence on pre-germinated seedlings of *Echinochloa* species.

A. Percentage plant survival, after 8 days of complete submergence followed by 10 days of recovery.

B-C. (**B**) Shoot biomass after 8 days of complete submergence and (**C**) after 10 days of recovery.

D-F. (**D**) Shoot height, (**E**) Number of leaves per shoot and (**F**) Number of tillers per shoot after 10 days of recovery (following 8 days of submergence). CTRL= Well drained, SUB-10cm and SUB-20cm= Submerged for 10 or 20 cm above canopy, Ecg= *Echinochloa crus-galli*, Ecol= *Echinochloa colona*, mg= milligrams. Bars represent means ± SEM. n= 27. The 2-ways ANOVA test (* P< 0.05, ** P< 0.01 and *** P< 0.001. NS, not significant) is showing the significant differences for biotype (b), treatment (t) and the interaction biotype*treatment (b*t). For **D-F**, « b: *** » indicate the difference between the two species, not between the different biotypes within the same species. Different letters indicate significant differences by a Tukey's HSD test (p< 0.05), within species/biotypes and treatments.

Echinochloa colona and *L. chinensis*, two submergence depths were applied to assess the effect of increasing depth.

All tested biotypes of *Echinochloa crus-galli* and *Echinochloa colona* were significantly affected by the complete submergence period. For all biotypes, submerged seedlings had a lower survival (Fig. 2.3A) and a lower biomass after 8 days of submergence compared to control seedlings (Fig. 2.3B). The trend was similar after 10 days of recovery (Fig. 2.3C). Submerged seedlings also had a significantly lower shoot height (Fig. 2.3D) and formed fewer leaves (Fig. 2.3E) and tillers (Fig. 2.3F) compared to the non-submerged seedlings. For all measured traits, the submergence effects were bigger for the *E. colona* seedlings than for the *E. crus-galli* seedlings. Also, there were no differences between the two submergence depths of 10 or 20 cm (Fig. 2.3A-F). Only one significant submergence effect was observed for the number of leaves for Ecg09 when the seeds were submerged below 10 cm water, compared to the control seeds ($p= 0.02$). For the other submerged conditions, no significant submergence effects were observed for the *E. crus-galli* biotypes for the number of leaves and tillers. In contrast, the differences between the submerged and the control plants were significant for the *E. colona* biotypes (Fig. 2.3E and 2.3F). Within the same species, no differences were observed between the biotypes. Interestingly, there was a significant difference between the two *Echinochloa* species in terms of leaf and tiller number in control conditions (Fig. 2.3E-F). It is also interesting to note that for a similar shoot height and biomass, *E. colona* biotypes formed three to four times more tillers and two times more leaves than *E. crus-galli* biotypes (Fig. 2.3C-F).

For *L. chinensis*, the submergence treatment clearly killed more than 50% of the seedlings for all biotypes, and even 80% for the biotype 02 (Fig. 2.4A). All measured traits were negatively affected by submergence. As for *Echinochloa,* varying the submergence depths (5 or 12 cm of water above the canopy) caused no significant differences (Fig. 2.4A-F). Similar to *E. crus-galli* and in contrast with *E. colona*, submerged and non-submerged *L. chinensis* seedlings did not show any significant differences in terms of number of leaves and tillers (Fig. 2.4E and 2.4F). However, shoot height (Fig. 2.4D) and shoot biomass (Fig. 2.4B and 2.4C) were significantly impacted. The only trait differences noted between the different biotypes were for the shoot biomass in control conditions (Fig. 2.4B), but this difference was lost 10 days later (Fig. 2.4C).

The submerged pre-sprouted *C. rotundus* tubers were assessed after 14 days of recovery following 13 days of submergence (Fig. 2.5A-D). The percentage of tubers with at least one shoot emerging from the soil (Fig. 2.5A), the number of shoots per tuber (Fig. 2.5B), the shoot height of the main shoot per tuber (Fig.

2.5C) and its number of leaves (Fig. 2.5D) were recorded and compared. The Iloilo biotype tubers emerged faster than those of the IRRI biotype after 14 days of recovery. For the upland and lowland Iloilo biotypes, 68% and 62.5% tubers sprou-

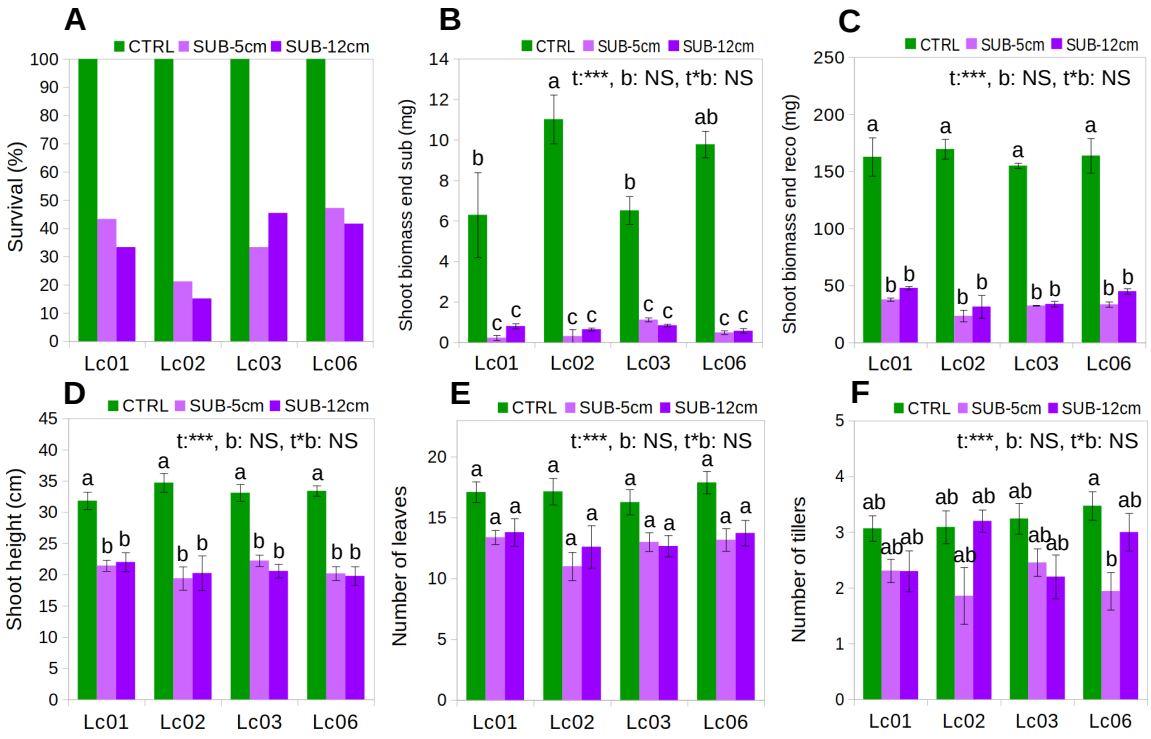


Figure 2.4: The effect of submergence on pre-germinated seedlings of several *Leptochloa chinensis* biotypes.
A. Percentage plant survival after 10 days of complete submergence followed by 10 days of recovery.
B-C. (**B**) Shoot biomass after 10 days of complete submergence and (**C**) after 10 days of recovery following 10 days of submergence).
D-F. (**D**) Shoot height, (**E**) Number of leaves per shoot and (**F**) Number of tillers per shoot after 10 days of recovery (following 10 days of submergence). CTRL= Well drained, SUB-5cm and SUB-12cm= Submerged for 5 or 12 cm above canopy, *Lc*= *Leptochloa chinensis*, mg= milligrams. Bars represent means± SEM. n= 36. The 2-ways ANOVA test (* P< 0.05, ** P< 0.01 and *** P< 0.001. NS, not significant) is showing the significant differences for biotype (b), treatment (t) and the interaction biotype*treatment (b*t). Different letters indicate significant differences by a Tukey's HSD test (p< 0.05), within biotypes and treatments.

ted, whereas 27.5% (11 upland tubers out of 40) and 10% (1 lowland tuber out of 10) of IRRI biotype tubers emerged at that time (thus also explaining the lack of standard error of the mean (SEM) for this biotype (Fig. 2.4). When comparing the remaining three biotypes, despite the difference in sprouting percentage, emerged sprouts showed similar development, with one-two shoots on average per tuber, a shoot height between 10,5 and 13,3 cm and around four leaves.

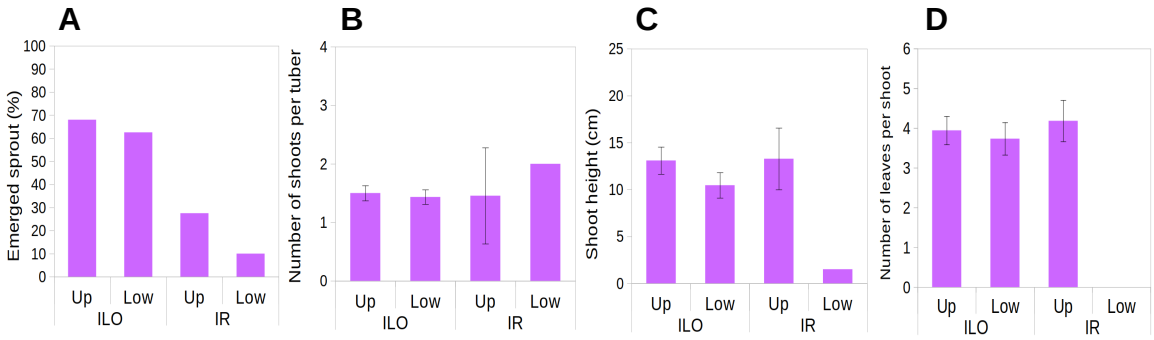


Figure 2.5: The effect of submergence on pre-sprouted tubers of *Cyperus rotundus* biotypes.
(**A**) Percentage of plants emerged from the soil surface after 13 days of submergence followed by 14 days of recovery. (**B**) Number of emerged shoots per tuber, (**C**) maximum shoot height and (**D**) number of leaves per shoot for the main shoot, after 14 days of recovery (following 13 days of submergence). ILO= Iloilo, IR= IRRI, Up= Upland, Low= Lowland. Bars represent mean± SEM. n= 6-50. Submerged IR-Low tubers are emerging slower then the other biotypes. After 14 days of recovery, only one tuber out of six emerged from the soil, explaining why there is no bars for **B**-**D**. The 1-way ANOVA test is showing no significant differences between biotypes for the three tested ones (ILO-Up, ILO-Low and IR-Up) for **B**-**D**.

Discussion

In lowland rice fields, controlled flooding is a frequently used as an effective method for suppressing weeds such as *C. iria, E. colona, E. crus-galli, E. glabrescens*, and *F. miliace*a (Smith and Fox, 1973; Civico and Moody, 1979; Diop and Moody, 1984; Kent and Johnson, 2001; Chauhan and Johnson, 2010). Although such water-based weed management strategies can be important for farmers, the effectiveness can be highly weed species-dependent, and several of them including *Monochoria vaginalis* (Burm. f.) Kunth or *Sphenoclea zeylanica* Gaertn. are well adapted to flooded conditions (Pons, 1982; Kent and Johnson, 2001). Furthermore, some weeds that were previously described as sensitive to flooding are becoming problematic in paddy fields with the evolution of new flood resilient biotypes (Ismail et al., 2012). The emergence of such tolerant weed varieties implies the acquisition of traits that enhance survival in wet soils. Since

this compromises the effectiveness of water-based weed management protocols, understanding flooding responses in these weeds is therefore essential. Besides, the identification of genotypes with contrasting tolerance could be exploited to study the mechanistic basis of enhanced survival in flooded soils and the evolution of flooding tolerance. While previous studies have documented genotypic variation in flooding tolerance (Angaji et al., 2010; van Veen et al., 2016; Yu et al., 2019; Di Bella et al., 2020; Rumanti et al., 2020), insufficient information was provided regarding the original sampling location of the biotypes tested. This makes comparisons between studies and correlation of tolerance to environmental parameters at a location complicated.

Here our goal was to evaluate the effectiveness of flooding in suppressing germination and emergence of four major rice weed species and thereby document their relative tolerance. In addition, we surveyed several biotypes from wet and dry locations with the goal of identifying biotypes with contrasting tolerance.

Anaerobic germination in three major rice weeds

Our results showed that while *E. crus-galli* and *L. chinensis* seeds were to some extent able to germinate underwater, flooding completely suppressed *C. rotundus* tuber emergence from the soil surface. These observations corroborate previous studies (Chauhan and Johnson, 2008; Peña-Fronteras et al., 2009; Estioko et al., 2014).

Despite the complete suppression of tuber emergence in *C. rotundus* during submergence, sprouting recovered following drainage, as observed before, for both upland and lowland biotypes (Peña-Fronteras et al., 2009). During the recovery period, it took more time for *C. rotundus* than for the other species to emerge from the soil, i.e. 9 days after water removal for *C. rotundus* tubers, compared to 2 days for *E. crus-galli* and *L. chinensis* seeds. Within species, no differences were observed between the different biotypes of *L. chinensis* and between the different biotypes of *C. rotundus*. Furthermore, the capacity to emerge did not correlate with the collection site (upland / dry fields or lowland / prone to flood fields) in The Philippines (Fig. 2.1). In our experiment, some variations have been noticed for the different *E. crus-galli* biotypes, but these differences are probably not due to the field location. Indeed, the «recorded as upland» IRRI field where the *E. crus-galli* biotype 13 seeds were collected could actually have been flooded for a period in the year, so the seeds might have experienced submergence before. Moreover, a larger number of biotypes need to be screened to assign differences related to upland and lowland collection sites.

Flooding has also been reported to have a suppressive effect on the emergence and dry matter of *L. chinensis*, and germination was strongly stimulated

by light and warm fluctuating temperatures (Chauhan and Johnson, 2008). *L. chinensis* also has been described to be strongly suppressed by standing water (1,5 cm), and its establishment was found to be prohibited by water depths of 5 cm (Mortimer et al., 2005; Chauhan and Johnson 2008). In our experiments, 5 cm water depths drastically repressed seed germination, but 10 to 15% of the *L. chinensis* submerged seeds still could emerge underwater.

One major difference between *L. chinensis* seeds, *E. crus-galli* seeds and *C. rotundus* tubers is their size and therefore their carbohydrates reserves. Larger seeds with greater carbohydrate reserves can emerge from greater burial depths (Baskin and Baskin, 1998). *L. chinensis* seeds are tiny with 1000 seeds weighing only 67.7 mg (Chauhan and Johnson, 2008). In contrast, the large tubers of *C. rotondus* facilitate their survival for weeks underwater, to sprout once the water is drained. In a study comparing a *C. rotundus* upland and lowland biotype, tolerance was related to tuber size and ability to utilize it (Peña-Fronteras et al., 2009). The tolerant lowland biotype with larger tubers also had more energy for use during flooding due to its ability for anaerobic starch utilization. This was reflected in its superior amylase activity and higher soluble sugar content in the tubers during flooded conditions and correlated with greater sprout emergence during recovery. Fuentes et al. (2010) also observed that the lowland biotype developed even larger tubers with increasing floodwater depth, probably as an adaptive feature for flooded-soil conditions. It also developed thicker stems and larger aerenchyma air spaces than the upland biotype. The lowland tubers also had lower lactate dehydrogenase activity under flooded conditions than the upland types, a feature necessary to avoid lactic acid accumulation and consequent cellular acidosis, associated with cell death under low-oxygen stress (Roberts et al., 1984).

A similar comparison of tolerant and intolerant *E. crus-galli* seeds revealed elevated activities of several anaerobic metabolism enzymes such as aldolase, aldehyde dehydrogenase (ALDH) and pyruvate decarboxylase (PDC) under anoxia in the tolerant subspecies (Fukao et al., 2003; Estioko et al., 2014).

Early seedling growth and vigour under flooded conditions

Although most weeds cannot grow and survive in the waterlogged soil in rice paddies, some have adapted to and become dominant in these habitats (Kraehmer et al., 2016). Factors including the reduced oxygen level, the accumulation of carbon dioxide and toxic gaseous products of anaerobic decomposition, and the presence of reduced forms of chemical radicals and gases (methane, nitrogen, nitrogen oxides, and sulphides), may affect the growth of weeds in submerged soils (Smith and Fox, 1973).

Flooding in the early stages of a rice crop may reduce weed density, but once the lowland weeds have emerged and progressed beyond the seedling stage,

their growth is more likely to be unaffected by flooding, as observed with *E. crus-galli* (Chauhan and Johnson, 2011). Other studies have shown that the greater the delay in flooding, the less weed growth is likely to be affected. When the flooding was delayed to 21 DAS, *C. difformis, C. iria*, and *F. miliacea* had little growth reduction (Chauhan and Johnson, 2009b). When flooding was delayed to 21 DAS for *F. miliacea*, 10 cm flood depth was required to suppress its growth (Begum et al., 2006). Similar observations were made on *E. crus-galli, E. colona*, and *Ludwigia hyssopifolia* (Sahid and Hossain, 1995). How flooding affects weeds at an early stage is therefore very important for field weed management protocols. We therefore investigated the effects of submergence on the four weed species from the 1st day of emergence.

Our results revealed that all tested biotypes of *Echinochloa* and *L. chinensis* were negatively affected by the submergence treatment in terms of shoot biomass, shoot height, number of leaves and tillers. At the end of the submergence period and compared to their respective air controls, *L. chinensis* seedlings survival was the lowest. The shoot biomass was also the most significantly and negatively impacted for the *L. chinensis* seedlings than for *Echinochloa*, especially for the *E. crus-galli* seedlings. *Echinochloa* seeds are bigger (1.9 mg per *E. crus-galli* seed, 1.1 mg per *E. colona* seed) than those of *Leptochloa* (0.07 mg per seed) and so may represent larger energy reserves, which could explain superior growth and survival of *Echinochloa* seedlings during the submergence stress.

Survival of flooded conditions is not restricted to only the submerged phase. Often, when floodwater recedes, despite surviving submergence stress, plants can succumb to the new set of stressors imposed by reoxygenation. Post submergence ROS accumulation can cause injuries including photoinhibition, impaired carbohydrate replenishment, desiccation stress, and senescence (Yeung et al., 2019). Without being able to overcome this desubmergence stress, plants are prone to die. Intermittent flooding can then be an interesting tool to manage submergence-tolerant but desubmergence-susceptible weeds in fields, as they might not stand the successive phases of anoxia-reoxygenation. Therefore, with regard to using flooding as a weed management protocol, assessing the responses of weeds to both submergence and recovery is relevant.

At the end of recovery, several traits including biomass of the submerged *E. colona* seedlings relative to control were more reduced than for *L. chinensis* and *E. crus-galli* seedlings. This suggests that *E. colona* is more susceptible to reoxygenation stress. This might be explained by the fact that this species does experience less lowland conditions compared to the two other species (IRRI, A Handbook for Weed Control in Rice, 1991). We observed that the *L. chinensis* species exhibits better recovery in terms of biomass recorded 10 days post-

submergence. Taking both submergence and reoxygenation into consideration, *E. crus-galli* seedlings performed the best since the submerged seedlings were very comparable to corresponding control seedlings. In this study, we found *E. crus-galli* to be the least morphologically impacted by flooding and therefore the most tolerant weed species. This has been observed in previous studies and could be explained by multiple morphological and metabolic adaptations that facilitate the survival of anaerobic conditions (Mujer et al., 1993; Fox et al., 1998; Khedr et al., 2017).

The effect of flooding depth, duration and timing on germination and seedling development

Several studies (Kent and Johnson, 2001; Begum et al., 2006; Singh et al., 2010; Ismaila et al., 2015; Ghosh et al., 2017; Chamara et al., 2018) have examined the effects of varying flooding time, duration and depth on emergence and growth of different weed species, and showed that these three parameters are important to consider for new water management protocols in fields. Increasing water depth reduces light penetration to the seedlings and to the soil surface, preventing germination of seeds where light is a requirement. Moreover, thermal fluctuation constitutes a known germination stimulant, and these fluctuations are dampened when the water depth increases. Finally, the stress imposed by flooding e.g: impaired gas exchange and its consequences, becomes increasingly severe as the duration increases.

An increase in the duration and depth of flooding significantly reduced seedling emergence of *L. chinensis* (Chauhan and Johnson, 2008). With flooding depths of 2 cm, *L. chinensis* emergence decreased by 26% after 2 days of flooding and further decreased by 72% where the soil had been flooded continuously for 7 days. Similar results have been observed with the weeds *C. difformis, C. iria, F. miliacea*, and *Ludwigia hyssopifolia* (Begum et al., 2006; Chauhan and Johnson, 2009a; Chauhan and Johnson, 2009b). Seedling emergence of *E. crus-galli* was not influenced by the varying depth of 0 to 10 cm in Estioko et al. (2014), nor from 2.5 to 10 cm in Sahid and Hossain (1995) but a flooding depth of 10 cm was enough to completely inhibit seedling emergence according to Benvenuti et al. (2001). In our study, we tested the effect of varying depths for the different biotypes of *E. crus-galli* and *L. chinensis*. However, no effect of flooding depth (1 to 8 cm of water) was observed and there was an equally efficient reduction of weed germination for all biotypes and for both species at all depths.

However, it is possible that obvious effects are observed with increasing depths where light and oxygen levels might be significantly affected. Seedling survival has been reported to decrease considerably even in tolerant genotypes

due to the limitation of oxygen and light transmission underwater due to the presence of algae for example (Ella et al., 2010).

It would be interesting then, to test the germination and seedling growth of these rice weeds when decreasing the light drastically in intensity and/or quality, or to combine the submergence treatment with a dark treatment. Alternating flooding could also be tested as an efficient weed management practice in fields as some of these weeds can be sensitive to subsequent reoxygenation and resubmergence phases.

Flooding tolerance in four rice weeds

Our data revealed high tolerance to flooding among the tested weed species. Although emergence and early development were significantly impaired by long-term flooding, seeds were still able to germinate and seedlings were able to further develop during submergence and following desubmergence. This indicated that prolonged submergence suppresses germination but does not kill the weeds.

Cyperus rotundus tubers were not able to sprout underwater but could emerge post-submergence. As *C. rotundus* is extremely flood-tolerant and has a major impact on rice yields, applying a long-term submergence could be effective in suppressing tuber sprouting and allow the rice to gain a competitive size advantage.

The lack of tolerance variation between biotypes did not support our hypothesis that genotypes from flooded environments would be more tolerant. However, the characterization of flooding tolerance of these major weed species paves the way for further investigation into the molecular mechanisms underlying the observed tolerance.

Supplemental data

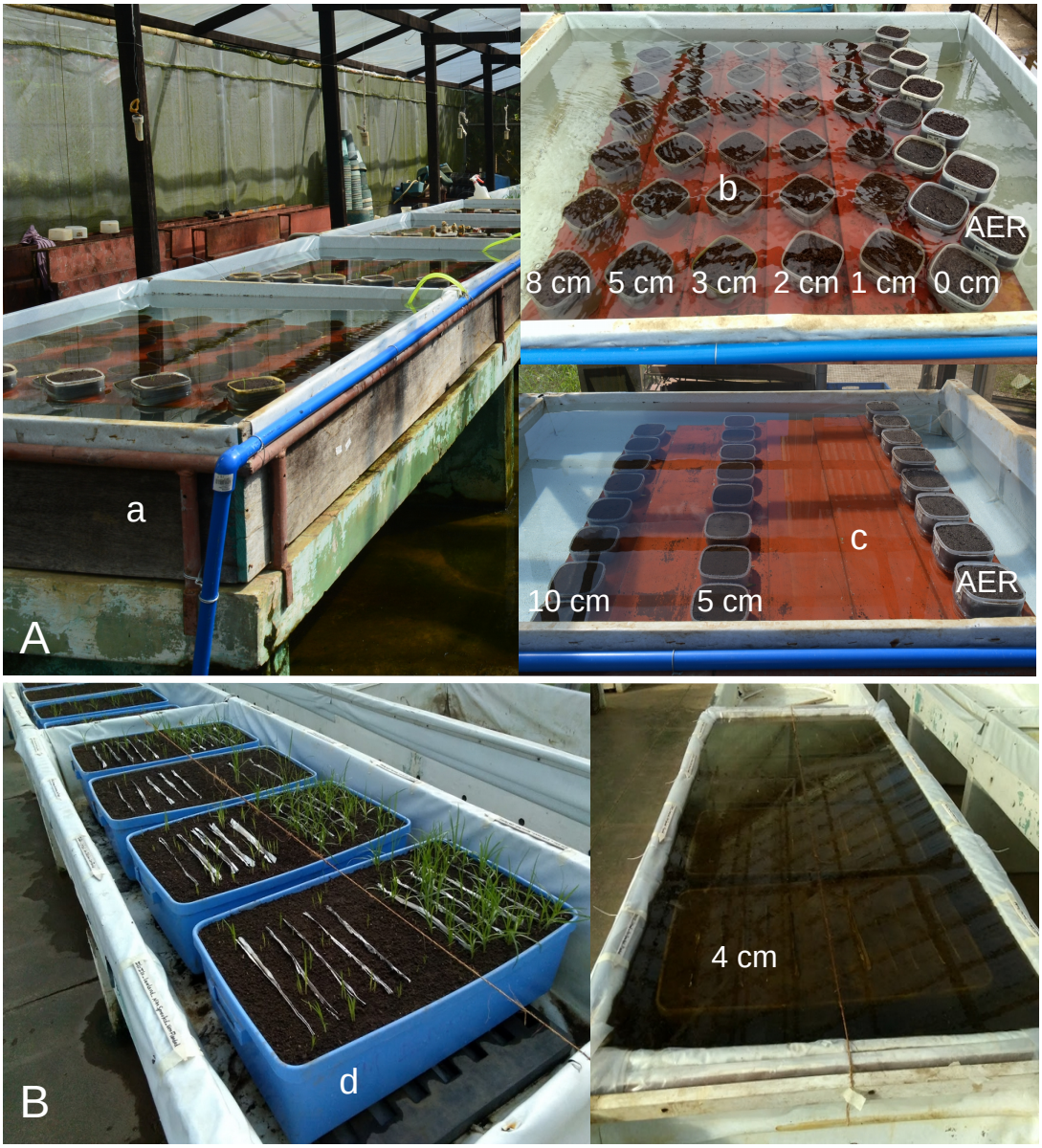


Figure s2.1: Submergence set up where the different biotypes of (A) *Echinochloa* and *Leptochloa chinensis* and (B) *Cyperus rotundus* were submerged.
a= cubicle, b= canister, c= step ladder, d= tray. Indicated cm are the depths of water on top of the soil surface in centimeters.

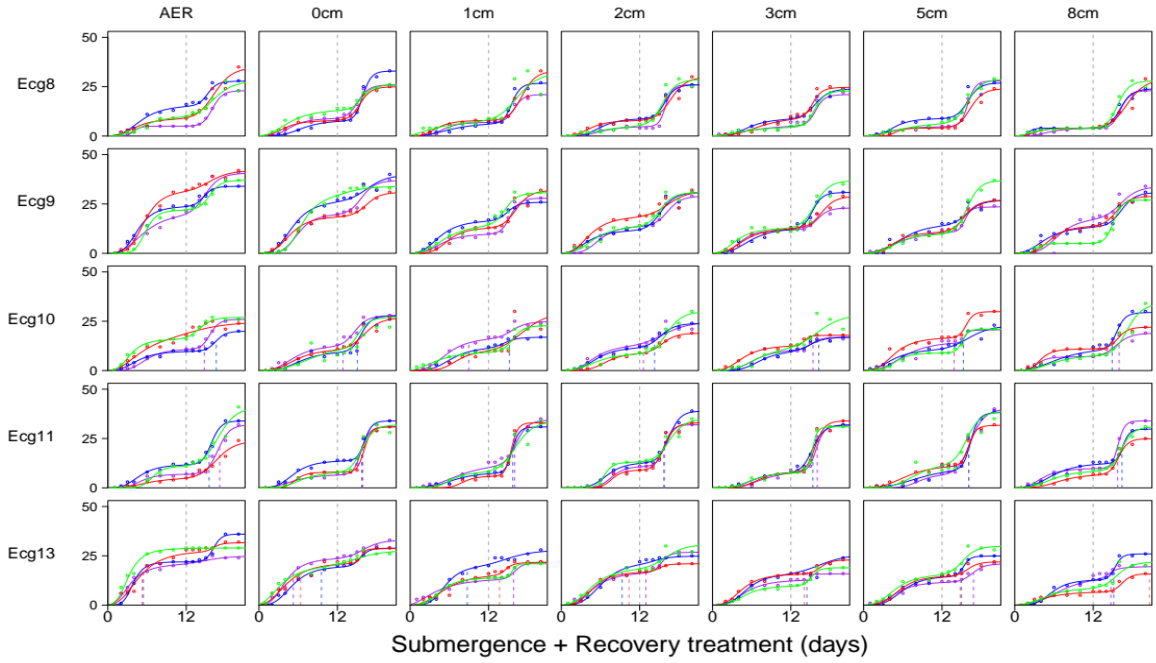


Figure s2.2: Anaerobic germination of different biotypes of *Echinochloa crus-galli* under different submergence depths.
Absolute seed germination on a total of 50 seeds per repetition x 4 repetitions, n= 200. The different biotypes are the different lines of plots, the different levels of submergence treatments are in column. The grey dash line marks the time of desubmergence (12 days) and the start of the recovery period (10 days). The different colours represent the different repetitions, and the corresponding colored dash lines indicate the number of days after which 50% relative germination was achieved (GT50%) in all conditions.

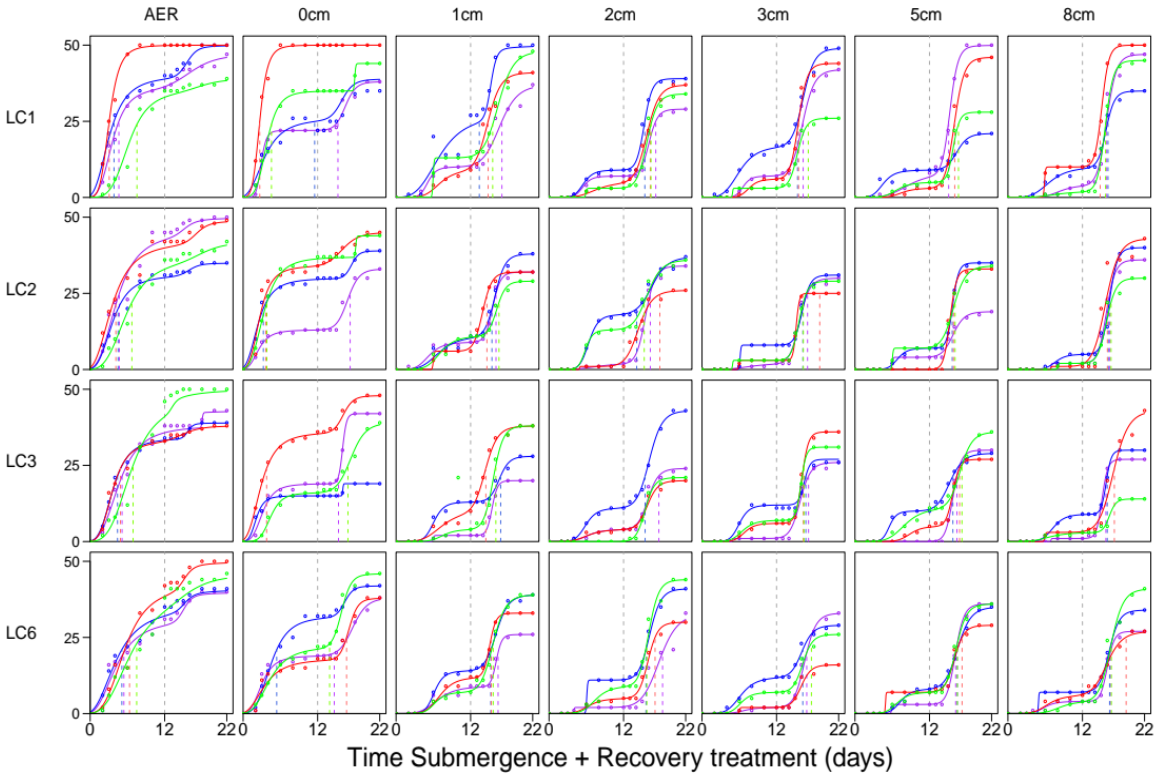
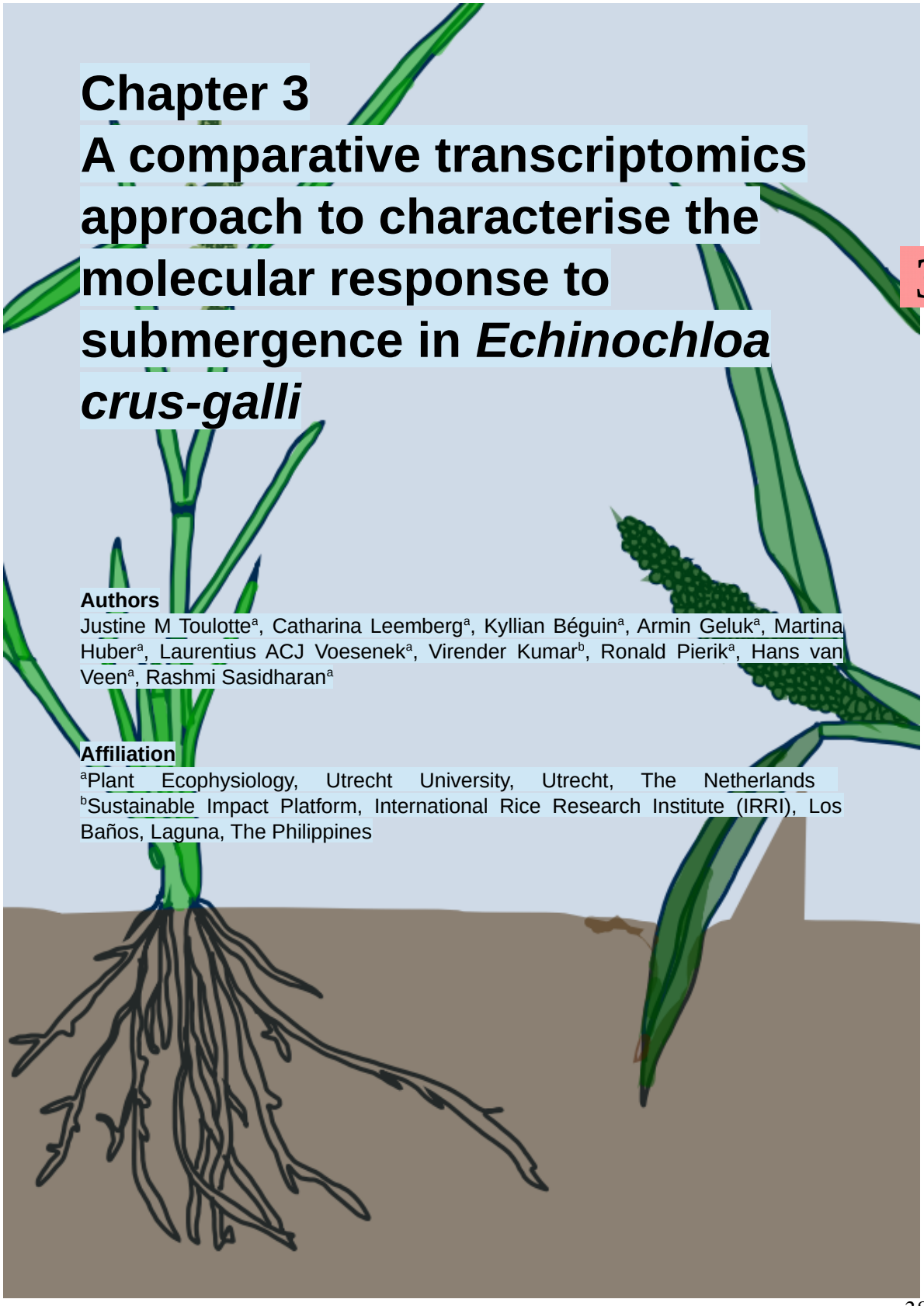


Figure s2.3: Anaerobic germination of different biotypes of *Leptochloa chinensis* under different submergence depths.
Absolute seed germination on a total of 50 seeds per repetition x 4 repetitions, n= 200. The different biotypes are the different lines of plots, the different levels of submergence treatments are in column. The grey dash line marks the time of desubmergence (12 days) and the start of the recovery period (10 days). The different colours represent the different repetitions, and the corresponding colored dash lines indicate the number of days after which 50% relative germination was achieved (GT50%) in all conditions.



Chapter 3
A comparative transcriptomics approach to characterise the molecular response to submergence in *Echinochloa crus-galli*

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Abstract

Echinochloa crus-galli (L.) Beauv (barnyard grass) is a worldwide notorious weed. Its success in flooded environments like paddy fields is detrimental to rice yields but makes it an attractive system to study flooding tolerance mechanisms. Despite some basal characterisation of its flooding resilience, a comprehensive analysis of the molecular responses to flooding has not yet been done for this species. Such analyses could be useful in the identification of unknown tolerance-related genes and processes. Here we used a transcriptomics approach to get a better insight into the response of *E. crus-galli* to long-term submergence. To be able to compare and contrast transcriptome responses in relation to tolerance to flooding, we included the following two other monocot species with varying tolerance levels to submergence: (1) *O. sativa* (genotype FR13A with known tolerance to submergence and genotype IR42 with relatively less tolerance to submergence), and (2) *Z. mays* (highly sensitive to submergence). Furthermore, each of these species possesses different morphological adaptations to submergence. Our results showed that *E. crus-galli* was highly tolerant to submergence and post-submergence stresses. This was associated with a faster and better-targeted regulation of oxidative stress responses and metabolic responses, such as deriving energy from alternative sugar pathways under submergence, and a possible gibberellin-mediated response facilitating its continued growth under submergence and during post-submergence recovery phase. Additionally, *E. crus-galli* shared a group of common responses with *O. sativa*, including a set of recovery genes typically linked to drought-responses stress.

Introduction

Echinochloa crus-galli (L.) Beauv (barnyard grass) is a highly problematic weed worldwide (Randall, 2012; Bajwa et al., 2015; Shabbir et al., 2019). This is attributed partly to its high plasticity to varied environments, including flooded paddy fields where it severely competes with the rice crop and reduces its yield if not controlled effectively (Bajwa et al., 2015). Agricultural weeds such as *Echinochloa* have evolved to survive in specific natural or agricultural ecosystems by developing adaptive mechanisms. Accordingly, the ability of *E. crus-galli* to persist in flooded environments (Maun and Barrett, 1986; Bajwa et al., 2015), makes it an attractive model to study related resilience mechanisms.

While there have been some efforts to describe flooding tolerance in *Echinochloa* spp. (Kennedy et al., 1980; Chauhan and Johnson, 2011; Estioko et al., 2014), very little is known about the molecular mechanisms underlying this tolerance. Submergence imposes a compound stress on affected plants. Reduced gas diffusion in the aqueous environment results in oxygen decline and carbon dioxide limitation. This coupled with a decrease in light quantity, limits photosynthesis and causes an energy crisis (Henry et al., 2020). Flood tolerance in plants is associated with adaptive strategies to facilitate internal aeration such as the formation of a barrier against radial oxygen loss (ROL) in roots, the formation of aerenchyma in roots, stems and leaves, the growth of adventitious roots, the formation of gas films on leaves, as well as the induction of leaf hyponasty and/or elongation of stems and leaves to reach the water surface (reviewed in Voesenek and Bailey-Serres, 2015; Yamauchi et al., 2018; Ejiri et al., 2021). Additionally, some species resort to metabolic adjustments such as the use of anaerobic metabolism to germinate underwater or to tolerate hypoxia at later stages and to cope with the energy crisis (reviewed in Ismail et al., 2012; Miro and Ismail, 2013). Anaerobic germination is facilitated by the ability to mobilize starch under anaerobic conditions and enable fast coleoptile elongation (Loreti et al., 2016). Submergence tolerance is also related to the ability to cope with post-submergence stress. The transition back to terrestrial conditions poses a second stress for plants emerging from prolonged submergence. The ability to overcome associated oxidative and dehydration stress relates to faster recovery of growth (Yeung et al., 2018; Yeung et al., 2019). Several studies have characterised flooding tolerance in *Echinochloa* species. Various *Echinochloa* species are able to germinate and grow in hypoxic soils (Kennedy et al., 1980; Rumpho and Kennedy, 1981; Pearce and Jackson, 1991; Fukao et al., 2003; Chauhan and Johnson, 2011; Estioko et al., 2014; Peralta Ogorek et al., 2019) via the upregulation of aldehyde dehydrogenase (ALDH) that may play a role in the detoxification of acetaldehyde produced through ethanol metabolism (Fukao et al., 2003; Chauhan and Johnson, 2011; Estioko et al., 2014). *Echinochloa* can also form gas films on leaves when submerged, aerenchyma in roots when waterlogged or submerged (Ogasawara et

al., 2000), adventitious roots and a suberin barrier which prevents ROL and facilitates establishment in anaerobic soils (Ejiri and Shiono, 2019).

As described in Chapter 2, our results support these observations. Amongst the weed species screened, *E. crus-galli* demonstrated the highest potential to survive several days of complete submergence when flooded before germination, and at the pre-emerged-from-soil germinated seeds stage. It also had a superior capacity to recover upon reoxygenation. Based on these observations, here we concentrated further investigations on *E. crus-galli*, and specifically on biotype 09 (Ecg09). Ecg09 showed a high percentage of survival after submergence at the pre-emerged seed stage and after post-submergence at the seedling stage. This biotype also germinated more rapidly compared to biotype 11 in control conditions.

The main aim of this chapter was to use an mRNA sequencing approach to understand the molecular response of this species to flooding. The transcriptome response of *E. crus-galli* was compared with two other monocot species: rice (*Oryza sativa* L.) and maize (*Zea mays* L.). Two *O. sativa* varieties were used: (1) FR13A, a highly tolerant landrace that can survive more than two weeks underwater (Xu et al., 2006; Bailey-Serres et al., 2010) and (2) IR42, mainly classified as a variety sensitive to submergence (Das et al., 2005; Winkel et al., 2014; Singh et al., 2020), although sometimes also as «intermediately tolerant» as it can survive several days of submergence but is sensitive to post-submergence stress (Ella et al., 2003). *O. sativa* IR42 displays a shoot elongation escape strategy when submerged (Das et al., 2005). *O. sativa* FR13A in contrast, limits underwater shoot elongation growth via a quiescence strategy (Xu et al., 2006; Bailey-Serres et al., 2010). This energy limiting response is controlled by the ethylene-responsive factor (ERF) SUB1A. *O. sativa* FR13A is also highly tolerant to post-submergence conditions and recovers quickly following desubmergence. To contrast with the long-term submergence tolerance of *O. sativa* and *E. crus-galli*, we also included genotype B73 of *Z. mays*. Maize is very sensitive to flooding (Zaidi et al. 2004; Lone et al., 2009) despite possessing several flood adaptive traits, such as adventitious roots (Mano et al., 2005), lysigenous aerenchyma (Drew et al., 2000; Yamauchi et al., 2011), and leaf gas films underwater (personal observation).

This panel of three species and four genotypes thus provides a relevant spectrum of tolerance strategies that can be compared to determine potential molecular responses associated with submergence tolerance. In this study, we first performed a basic morphological analysis of the responses of these genotypes to prolonged submergence and post-submergence recovery. Per species, the transcriptome was analyzed in the shoots of seedlings at physiologically relevant

moments and reflecting early and late responses to submergence and recovery. This approach allowed a comparison of the common and contrasting responses between species during and after stress removal. Our analyses revealed that:

- Contrasting morphological responses to submergence stress exist between the two tolerant genotypes *E. crus-galli* and *O. sativa* FR13A. *O. sativa* FR13A restricts growth while *E. crus-galli* continued forming new leaves during the submergence stress period.
- *E. crus-galli* is likely able to sustain its growth/development by effective transcriptional activation of alternative carbon usage pathways, unlike *O. sativa*.
- The *GIBBERELLIN 20-OXIDASE* is only induced by submergence in *E. crus-galli* and this might explain the sustained growth of *E. crus-galli.*
- Common recovery genes between *O. sativa* and *E. crus-galli* reflect the importance of coping with dehydration stress during the post-submergence recovery phase.
- The high tolerance of *E. crus-galli* is reflected in its fast and coordinated transcriptome responses to both submergence and recovery, in contrast to the slow response of submergence sensitive *Z. mays*.
- In general, major typical hormonal and core hypoxia responses were not very different between the four genotypes.

Materials and methods

Seed origins and germination

Echinochloa crus-galli biotype 09 (Ecg09) seeds were originally collected by the Weed Science team at the International Rice Research Institute (IRRI), Los Baños, The Philippines, in a lowland field (IRRI – UD2), within the period Sept-Nov 2016. Seeds were then further bulked 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. For germination, dehulled seeds were put to germinate for 4 days in Petri dishes between two wet Whatman™ papers in an incubator (12 h light (120+/-50 µmol.m^{-2}.s^{-1}) 35°C / 12 h dark 25°C, 70% relative humidity).

Oryza sativa FR13A seeds are from IRGC, IRRI (ref: IRGC 6144). Seeds were collected in 2006 in “DS” site. *Oryza sativa* IR42 seeds come from Amelia Henry’s group, IRRI Los Baños (ref: IR42 - Sub 17DS-12 (Sub-Demo#10)). Seeds were received in June 2019 and were stored in the dark at 6°C. For germination, rice seeds were transferred to 37°C in the dark for 3 days, and then at room temperature in the dark for 1 day, and sown in Petri dishes for 3 days in the same incubator conditions as *E. crus-galli* seeds.

Zea mays genotype B73 seeds were provided by Karen Koch's lab (University of Florida) and were received in February 2019. Seeds were kept in the dark at 6°C. Seeds were sterilized with 15% bleach solution for 15 min and rinsed 6 times with Milli-Q water. Seeds were then placed in Petri dishes between two wet Whatman™ papers at room temperature on the lab bench for 3 days.

Plant growth

Per pot, one seedling was transplanted at 3 (*O. sativa* and *Z. mays*) or 4 (*E. crus-galli)* days after sowing. Canopy pots were used (perforated Round Pots 6° Azalea – MXC 5,5 plastic pot of 5 cm diameter top, 3.5 cm diameter bottom, 5.5 cm depth, 78 ml) with a mixture of 50% black soil / 20% sand / 30% agra-vermiculite 0-1.5 mm + 20% Yoshida nutrient solution (Yoshida, 1976) with a double iron dose (sequestreen= Fe-EDTA), pH 6.5 + osmocote NPK-Mg 15-4-9 (+1) (2.4 g/L of soil). Seedlings grew in the greenhouse for 8 (*Z. mays*) to 12 (*O. sativa* and *E. crus-galli*) days, in a 12 h light (200+/-20 µmol.m^{-2}.s^{-1}) 29+°C / 12 h dark 24+°C cycle conditions, with ventilation, in trays that were manually watered.

Submergence procedure

Submergence experiments were conducted in the greenhouse of the Botanical Gardens, The Science Park, Utrecht University. When plants reached the 3-leaf stage, healthy plants with a homogeneous shoot height were selected and completely submerged, all at the same time, in ~240 L tanks (~60 x ~60 x ~64 cm), with a minimum depth of 16 cm above the tallest plant (Fig. 3.2A). Tanks were filled with tap water the day before submerging the plants, for water temperature acclimation. Tanks were all connected together and to a bigger tank providing a constant flowing water of 27°C. A UV pump was connected in between, in order to reduce the algae growth. Light underwater at plant level was 150+/-20 µmol.m^{-2}.s^{-1}. During the recovery phase, plants were removed from the water and placed next to the tanks in trays in the same light, temperature and ventilation conditions as during their growth phase. Watering was done manually.

Long term submergence treatment and measurements

Plants of *O. sativa* and *E. crus-galli* were completely submerged for 15 days and allowed to recover for 14 days. Pictures, number of leaves and shoot height were measured before, at the end of submergence and at the end of recovery. Number of leaves and green index representing the health of the newly formed leaves were recorded at the end of submergence and throughout the recovery period. Survival was scored based on if the plants were still alive (based on visible green tissue). Only plants that were alive were counted for each trait that was followed. For the end of the submergence period, n=10 plants for each species. For the end of the recovery period, n=7, 10 and 9 plants respectively for

E. crus-galli, *O. sativa* FR13A and *O. sativa* IR42. For shoot height, a paired samples T-test was performed with the open software jamovi (https://www.jamovi.org).

Z. mays plants were completely submerged for 2 to 5 days and recovered for 7 days. Pictures, number of leaves and shoot height were measured before, at the end of submergence and at the end of recovery. Chlorophyll content was estimated with a chlorophyll content meter CCM-300 (Optisciences) at the end of recovery, on the 2/3 length from the base of the youngest leaf. Survival was scored based on if the plants were still alive (not rotten). Only plants that were alive were measured (n=1 to 5 plants). For shoot height and chlorophyll content, a paired samples T-test was performed with the open software jamovi.

RNAseq harvest

For the RNAseq experiment, plants were completely submerged for 2 to 5 days, and following desubmergence, put to recover for 1 day. Timepoints were as followed (Fig. 3.2B):

PS = pre-sub → day 0 at 2 pm
ES = early sub → day 0 at 6 pm (4 h sub)
NS = night sub → day 1 at 8 am (18 h sub)
MS = mid sub → day 2 at 2 pm (48 h sub) (= de-sub for maize)
LS = late sub → day 5 at 2 pm (120 h sub) (= de-sub for Ecg09/FR13A/IR42)
ER = early reco → day 2 (maize) / day 5 (Ecg09/FR13A/IR42) at 6 pm (48 h / 120 h sub + 4 h reco)
LR = late reco → day 3 (maize) / day 6 (Ecg09/FR13A/IR42) at 2 pm (48h / 120h sub + 24 h reco)

The light period was between 8 am and 8 pm. For each harvest timepoint, three (*Z. mays*) to five (*O. sativa* and *E. crus-galli*) plants were taken out of the water from 3 different tanks. Entire shoots were harvested and pooled in aluminium envelopes, directly frozen in liquid nitrogen, and stored at -80°C. The experiment was done three times, corresponding to the three time replicates a, b and c on the MDS plots (Fig. s3.3B).

Preparation and sequencing of the RNAseq samples

The frozen shoot samples were ground into a powder with a mortar and pestle. A fraction of about 0.1 ml of this powder was used for total RNA extraction. RNA extraction was performed with the RNeasy Plant Mini Kit protocol (Qiagen, Germany) with an on-column DNAse treatment step (RNase-Free DNase Set, Qiagen, Germany). The RNA was collected in RNAse-free water. The RNA purity, quality and quantity were checked with the Implen NanoPhotometer® and by running the samples on a SyBrGreen gel. A minimum of 1.5 µg of RNA per sample was prepared and sent to Macrogen Europe BV, Amsterdam, The Netherlands, for

the quality check of the samples, preparation of the cDNA library (TruSeq stranded mRNA) and paired-end (2 x 150 bp) Illumina Next-Generation Sequencing (NovaSeq).

RNAseq analyses

RNAseq analyses were performed with the R version 3.6.3 combined with a mix of Bioconductor packages and standalone bioinformatics tools.

1. Read trimming and mapping.

First, low quality reads with low basecall quality scores or adapter contamination were trimmed or removed with 'cutadapt' and the settings -e 0.07, --no-indels, --nextseq-trim=20 -m 30 (Martin, 2011). The trimmed and filtered reads were aligned and counted to the cDNA fasta files from the corresponding reference genomes (Guo et al., 2017; Du et al., 2017; Jiao et al., 2017) with the kallisto program (Bray et al., 2016).

2. Differential expression analysis.

Genes with more than 12 read pairs in at least 3 out of 18 (*Z. mays*) or 21 (*O. sativa* and *E. crus-galli*) samples were kept. The resulting count data was subsequently used for expression analysis with the edgeR and limma package (Robinson and Oshlack, 2010; Ritchie et al., 2015). First, the count data were normalized for size and compositional bias with the TMM method (Trimmed Mean of M-values). Then, fold Change, P values and Counts per Million (CPMs) were calculated using tag-wise dispersion and assuming a negative binomial distribution of the count data. To observe the data in time and between experimental repetition (Fig. s3.3B), an MDS (MultiDimentional Scaling) plot for each species was performed with the inbuilt edgeR function. Here, distances between samples were estimated by the 2000 most variable genes among all samples. Gene description, motif information and *Arabidopsis*- and *O. sativa*-related genes were available for the *O. sativa* indica and *Z. mays* genomes (Du et al., 2017; Jiao et al., 2017). Gene descriptions for *E. crus-galli* were created by blasting to the rice (Oryza sativa v7.0) and maize genomes (Zea mays RefGen_V4).

3. Hierarchical clustering

Scaled CPM values of the DEGs were submitted to a hierarchical clustering. Euclidean distances were calculated between genes and used as input for agglomerative hierarchical clustering employing the ward squared method. To visualise the regulation patterns of the cluster of gene expression, a line plot was drawn with the expression of each gene in time (1 gene= 1 line), as well as the average expression with a thicker line (Fig. s3.7).

4. Gene Ontology enrichment

Gene Ontology (GO) enrichment was performed with the bioconductor packages GO.db to retrieve GO descriptions and GOseq to assess enrichment of the GOterms. The analyses were done assuming a hypergeometric distribution. The most important GO terms were assessed manually (Fig. s3.7, Fig. 3.3).

5. Targeted pathway analysis

For the generation of heatmaps of hormones, sugars and hypoxia-response genes (Fig. s3.4 s3.5 and s3.6), DEGs were selected based on P adj. <0.001. The information about each selected pathway (gene name, description and pathway code) were retrieved manually from various online databases: PlantCyc (https://plantcyc.org), KEGG (Kyoto Encyclopedia of Genes and Genomes; https://www.genome.jp) and TAIR (The Arabidopsis Information Resource; https://www.arabidopsis.org). Heatmaps were generated with R with the function «ComplexHeatmap».

6. Recovery cluster analysis

To explore the shared recovery genes, *E. crus-galli*, *O. sativa* FR13A and *O. sativa* IR42 genes were mined from the selected «recovery» clusters (see Fig. 3.5A). To compare the expression in the three different species, a common gene annotation was created by BLASTing each species to *O. sativa*. A Venn diagram assayed the degree of overlap between all species, which were tested with the Fisher exact test yielding, the odd ratios, for each species pair. To plot the Core Recovery Genes heatmaps, genes of each species, with corresponding *Z. mays* genes were retrieved thanks to the common *O. sativa* annotation available in each species file. Heatmaps in figures 3.5E and s3.8 were generated with the function «ComplexHeatmap».

Sugar measurements

Glucose, sucrose, fructose and starch were measured from samples harvested at the same time as for the RNAseq samples. Measurements were performed on 2 replicates consisting of three to five pooled plant shoots per timepoint (PS, MS, LS, LR). For each sample, 20 to 40 mg of frozen shoot powder was accurately weighed, and metabolites were extracted with $HClO_4$, buffered by HEPES and KOH. After centrifugation, 40 µL of the supernatant was used for the different soluble sugars measurements and the pellet for the starch measurement. Then, glucose, sucrose, fructose and starch were measured with a commercial kit (Megazym Assay Kit, Ireland) following the manufacturer's protocol. All concentrations were compared to a corresponding standard curve. Two-way ANOVAs (timepoint and treatment) were done per species, followed by a Tukey post hoc test with the open software jamovi.

Respiration rate measurement

Seeds were germinated and plants were grown in a cabinet (12 h light (120+/-50 µmol.m^{-2}.s^{-1}) 35°C / 12 h dark 25°C, 70% relative humidity), in similar soil and pots as used in the greenhouse. Submergence was done in the same cabinet under the same environmental conditions, in a ~10 L glass tub (~25 x ~17.5 x ~23.5 cm), pre-filled with tap water 2 to 3 hours before submerging the plants, for water temperature acclimation and oxygen stabilization. Two 3-leaf stage plants were completely submerged for 48 h before measurement, while two plants were directly sampled for respiration rate assessment. For *O. sativa* FR13A, *O. sativa* IR42 and *E. crus-galli*, the respiration rate was followed on the whole third leaf, whereas for *Z. mays*, the respiration rate was followed on a fragment of the middle of the leaf as the whole leaf did not fit in the 4 ml vials. In each vial, a fresh leaf fragment was placed weighing between 8 to 50 mg. The concentration of oxygen over time was measured with an OX-MR 400-600 µm sensor connected to the Microrespiration system from Unisense (https://www.unisense.com/MicroRespiration_System). The slope of the oxygen depletion is dependent on oxygen concentration in time and on the corresponding solution volume in the vial. Thus, vials were pre-filled with the Smart and Barko solution (Smart and Barko, 1985), with the pH adjusted to 6.5 and with a salinity of 6%, and weighed before and after adding the solution and the leaf, to calculate the real volume of the solution where the leaf was submerged and respiring. Vials with their leaf were then placed in a 30°C water bath. Each vial contained a stir bar for constantly moving the solution inside the vial. Two control solutions for calibrating the oxygen sensor before measuring the samples were used: 0% oxygen solution and saturated 100% oxygen solution. Each sample was measured for 1 minute, four times, every 20 minutes to follow the respiration rate in time. The software (Unisense Rate) calculates 2 types of oxygen consumption rates: (1) within measurements based on the decline in oxygen whilst the optode is inserted into the solution and (2) between measurements based on the decline in oxygen in between two successive measurements in the vial. After measurement, leaves were dried in the oven at 80°C and weighed after 2 days to report the respiration rate per mg of dry biomass. The rate of oxygen depletion in the vials was divided by the dry weight (DW) to calculate the respiration rate in nmol of oxygen consumed per mg of dry weight biomass per hour. The whole experiment was done twice. In total, two to six leaves from different plants per treatment were measured. A 2-way ANOVA (timepoint and treatment) per species, followed by a Tukey post hoc test were done with the open software jamovi.

Associated files

3a. Fold changes of *E. crus-galli*, *O. sativa* FR13A, *O. sativa* IR42 and *Z. mays* in response to submergence and recovery stress per gene (Time series, 1 file per species) “Chapter3and4a_FC_FDR_OG_speciesX”

3b. GO enrichment analyses per timepoint “GOUP/DO_timepointX”
3c. GO enrichment analyses of clustering per species “Chapter3c_GOper clust_SpeciesX.txt”

Results and Discussion

Echinochloa crus-galli is highly tolerant to long term complete submergence

Submergence tolerance of vegetative-stage weed *E. crus-galli* biotype 09 (Ecg09) was assessed together with two varieties of *O. sativa* previously reported as submergence tolerant and moderately tolerant, *O. sativa* FR13A and *O. sativa* IR42 respectively (Fukao and Bailey-Serres, 2008; Singh et al., 2020). Three-leaf stage plants of both species were completely submerged for a period of 15 days. Both varieties of *O. sativa* and *E. crus-galli* survived the submergence period (Fig. 3.1A) and were scored healthy based on the visible green and turgid shoot parts (Fig. 3.1B and s3.1). However, a detailed assessment of various plant traits revealed some distinctions between the underwater responses. In line with previous reports (Fukao et al*.,* 2006), *O. sativa* FR13A plants showed a typical quiescent strategy during the submergence period (Fig. 3.1 C). *O. sativa* FR13A possesses the SUB1A allele, which upon induction during submergence, orchestrates the quiescence strategy typified by restricted underwater growth (Fukao and Bailey-Serres, 2008; Bailey-Serres et al., 2010). In contrast, submergence triggered shoot elongation in both *E. crus-galli* and *O. sativa* IR42 (Fig. 3.1C and s3.1). However, *E. crus-galli* was the only species that formed new leaves during the submergence period (Fig. 3.1D and E).

The effect of the post-submergence phase was assessed based on measurements during a 2-week recovery period. At the end of this recovery phase, 100% of *O. sativa* FR13A plants survived. As expected for this tolerant variety (Ella et al., 2003; Collard et al., 2013; Singh et al., 2020), plants remained green (Fig. 3.1B) and all were able to form new leaves following desubmergence (Fig. 3.1D and E). In contrast, both *E. crus-galli* and *O. sativa* IR42 showed signs of post-submergence stress with survival scores of 70% and 90% respectively (Fig. 3.1A). However, *E. crus-galli* has a higher tolerance to post-submergence stress and recovered better, as reflected in the higher percentage of green plants and new leaf formation during recovery (Fig. 3.1B and D). While *O. sativa* IR42 plants formed one new leaf on average in 14 days of recovery, *E. crus-galli* and *O. sativa* FR13A produced an average of two to three new leaves in the same period (Fig. 3.1E). None of the three genotypes grew in height during this period (Fig. 3.1C).

In addition to *E. crus-galli, O. sativa* FR13A and *O. sativa* IR42, we included the flooding sensitive monocot *Z. mays* B73 to broaden the tolerance range in our study. For *Z. mays*, the survival of the plants was assessed at the end

of the different duration of submergence period, and after 7 days post submergence. *Z. mays* plants were able to survive only up to 3 days of submergence, with three out of five plants dying at the end of the 7 days recovery period. After 4 days of complete submergence, all the plants were dead following 7 days of recovery (Fig. s3.2B). This submergence sensitivity was consistent with previous reports (Zaidi et al. 2004; Lone et al., 2009; Campbell et al., 2015). The

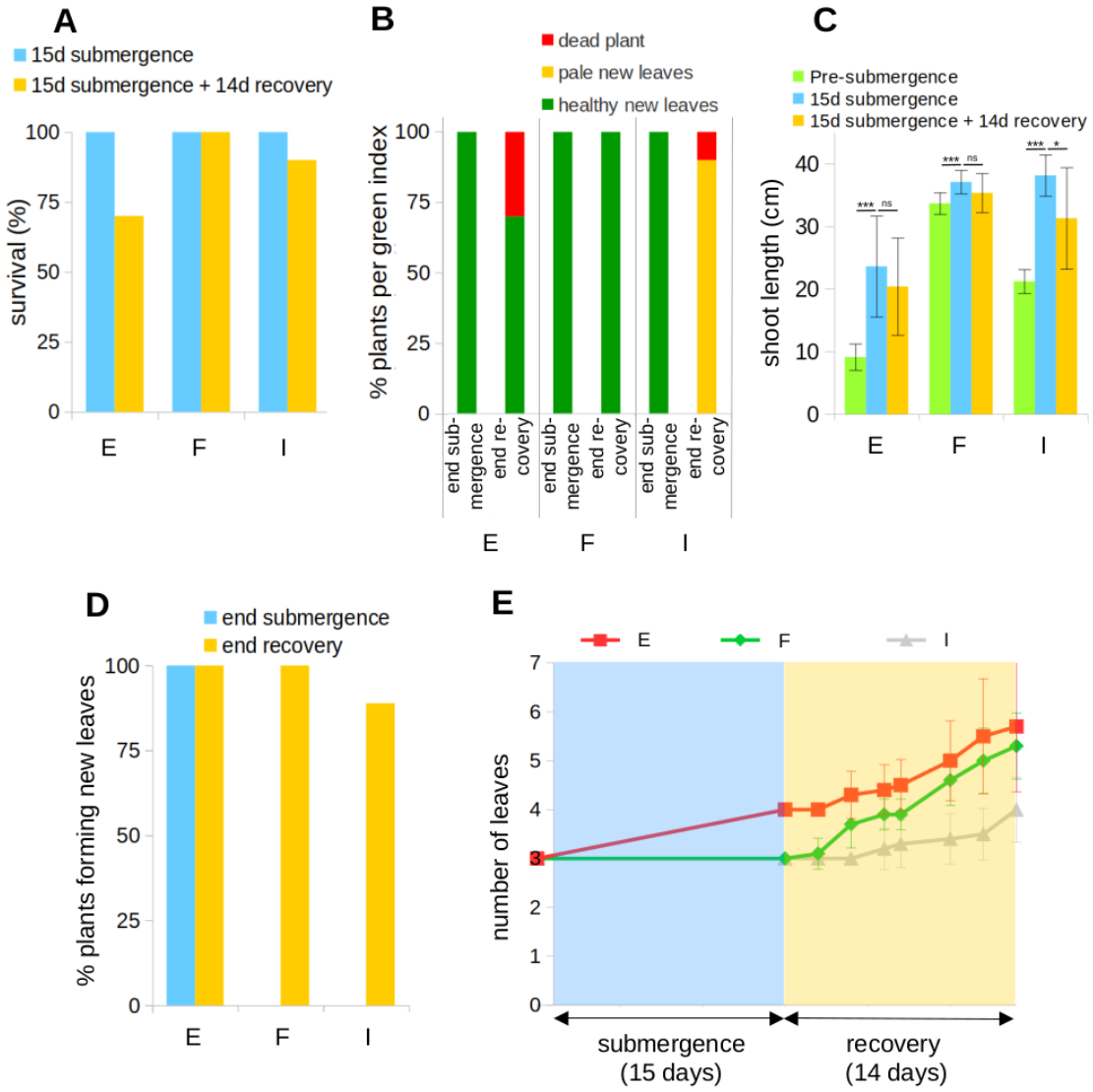


Figure 3.1 *Echinochloa crus-galli* survives prolonged complete submergence and has superior recovery compared to rice.
A. The percentage of plants that survived 15 days of complete submergence (blue bars) and after 15 days of complete submergence followed by 14 days of recovery in control conditions (orange bars). Plants of *Echinochloa crus galli* (E), *Oryza sativa* FR13A (F) and IR42 (I), were submerged at 3 leaf stage. n= 10 plants per species. Survival was scored based on if the plants were still alive (not rotten).
B. Performance of plants at the end of 15 days of submergence and after 14 days of recovery in control conditions based on a 'green index'. The green index categories were dead (red), healthy new leaves growing (green) and only pale new leaves (yellow). Shown are the percentages of plants per species in each category at the end of submergence or recovery. n= 10 plants per species. *Echinochloa crus galli* (E), *Oryza sativa* FR13A (F) and IR42 (I)
C. Shoot lengths of *Echinochloa crus galli* (E), *Oryza sativa* FR13A (F) and IR42 (I) plants before (pre-sub; green bars), after 15 days of complete submergence (15 days sub; blue bars), and after 14 days of recovery following desubmergence (15 days sub + 14 days reco; orange bars). n= 10 plants except for the recovery timepoints where n= 7 for plants *E. crus-galli* and n= 9 plants for *O. sativa* IR42. Values are means +/- SD. ns= non significant, *= p-val< 0.05, ***= p-val< 0.001 from paired samples T-test.
D. The percentage of plants that have formed new leaves after 14 days of complete submergence (end submergence; blue bars) and after two weeks of recovery following desubmergence (end recovery; orange bars). Only plants that were alive were counted. n=10 plants for submergence, n= 7, 10, 9 plants respectively for *Echinochloa crus galli* (E), *Oryza sativa* FR13A (F) and IR42 (I) at the end of the recovery period.
E. Total leaf number per plant during the submergence treatment and in the recovery period following desubmergence. The blue box represents the submergence time (15 days), while the orange box represents the recovery time (14 days). *Echinochloa crus galli* (E), *Oryza sativa* FR13A (F) and IR42 (I).

shoot length of *Z. mays* plants increased underwater, but there was no further growth during recovery (Fig. s3.2C). Although no new leaves were formed during submergence, leaf formation resumed during recovery after 2 and 3 days of submergence (Fig. s3.2D). Another sign of submergence sensitivity was accelerated senescence in the youngest leaf of the submerged *Z. mays* plants, correlating with the severity of the submergence treatment. A significant effect of chlorophyll loss in the leaf was detected from 3 days of submergence (Fig. s3.2E).

Thus, varied responses to submergence culminated in the observed tolerance for both flooding and recovery in *O. sativa* FR13A and *E. crus-galli*, flood tolerance but recovery sensitivity in *O. sativa* IR42 and high sensitivity to both flooding and recovery for *Z. mays*.

Transcriptomic responses to submergence in three monocot species

Following the phenotypic assessment, a transcriptomic approach was performed to characterise and to compare the molecular responses to submergence stress in *Z. mays*, *O. sativa* and *E. crus-galli* in a similar submergence setup. Plants of each of the four genotypes were completely submerged and shoot samples were harvested both during submergence and recovery (Fig. 3.2). The submergence duration was 5 days, except for *Z. mays* plants which were submerged for 2 days as they do not survive more than 3 days in the current setup (Fig. s3.2). Samples were harvested before the submergence treatment (Pre sub; PS); after 4 hours of complete submergence to capture rapid acclimation responses (early submergence response (Early sub; ES)); directly after the first night to observe the effects of darkness and corresponding starvation associated with the night period and an eventual switch in the carbon metabolism to the corresponding lower oxygen availability (Night sub; NS); after 2 days (Mid sub; MS) for all species and 5 days (Late sub; LS) for the two *O. sativa* varieties and *E. crus-*

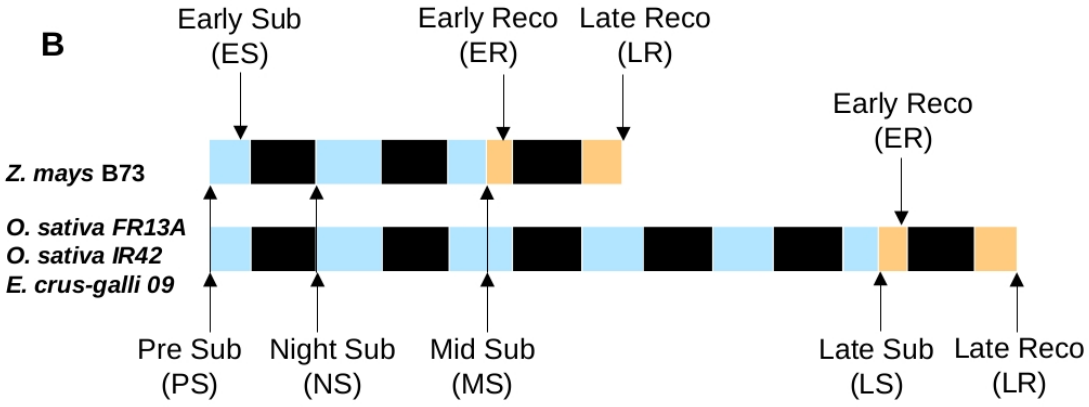
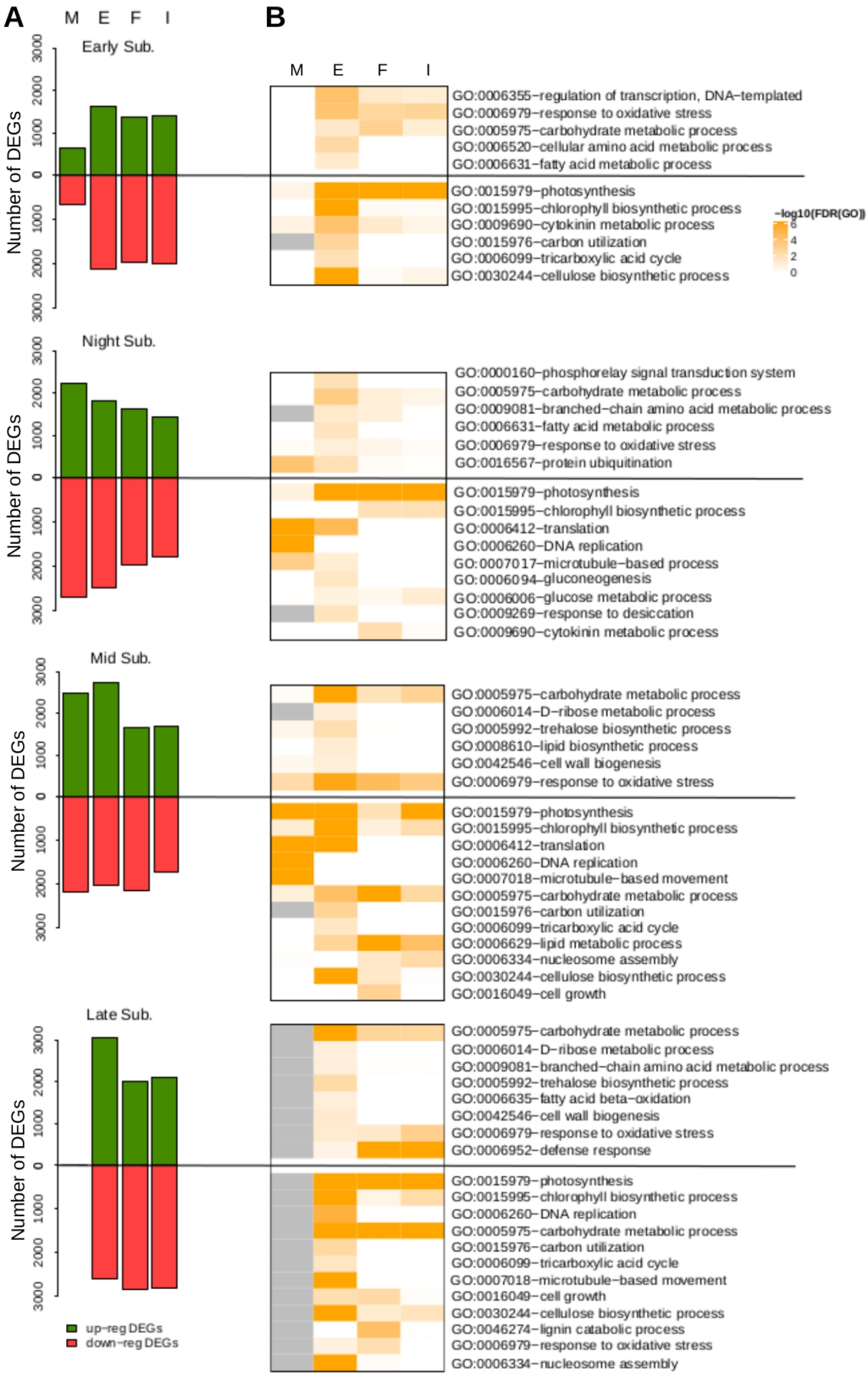


Figure 3.2: Experimental setup and sampling design for the transcriptomic survey of submergence responses in *Echinochloa,* rice and maize.
A. Image showing the set up in the greenhouse used for submerging the plants. Plants have been submerged (white tubs) and let to recover on the side.
B. Schematic depicting the experimental design and harvest timepoints for transcriptome profiling using RNA sequencing. *Z. mays* was completely submerged for 2 days while *E. crus-galli*, *O. sativa* FR13A and *O. sativa* IR42 were completely submerged for 5 days. Blue and orange boxes represent the submergence and recovery periods respectively. Each black box represents one night. Black arrows indicate the sampling timepoints. PS= Pre-Submergence, ES= Early Submergence (4 hours), NS= Night Submergence (18 hours), MS= Mid Submergence (2 days), LS= Late Submergence (5 days), ER= Early Recovery (5 days Sub + 4 hours Reco), LR= Late Recovery (5 days Sub + 1 day Reco). Plants were submerged at 2pm (PS) and harvested at 2pm for the timepoints MS, LS, LR, at 8am for the timepoint NS and at 6pm for the timepoints ES and ER.

galli, to see eventual changes in responses to prolonged submergence. Recovery sampling points included a 4 hours (Early reco; ER) and a 1 day (Late reco; LR) post-submergence harvest to detect any reoxygenation responses, as well as to assess the extent of return to the pre-submerged status.

Following RNA sequencing, reads were mapped to respective reference genomes (Guo et al., 2017; Du et al., 2017; Jiao et al., 2017). Approximately 70% of *E. crus-galli* and 90 % of *O. sativa* and *Z. mays* reads were successfully mapped to their respective genomes leading to total mapped read pairs per sample ranging from 28 to 38 million (Fig. s3.3A). A multidimensional (MDS) scaling plot was plotted per species to assess the clustering of replicates and the degree of similarities between different samples (Fig. s3.3B). The three replicates always cluster per samples for all the species. Submergence caused a clear shift from pre-submergence conditions to the right. A reoxygenation related shift up and left was also obvious, tending to move back towards the pre-submergence status. This shift to return to the pre-submergence state appeared to be the most pronounced for *E. crus-galli*, then for *O. sativa* genotype FR13A and weakest for *Z. mays* genotype B73 and *O. sativa* genotype IR42.

To assess the magnitude and nature of the transcriptomic responses to the early and late submergence, night time, and early and late recovery, we quantified the number of differentially expressed genes (DEGs; $| \log_2 FC | > 1$ and $P_{adj.} < 0.001$) and retrieved enriched Gene Ontology (GO) categories among those DEGs (Fig. 3.3A and 3.3B). The recovery effect on the transcriptome was assessed by calculating differential expression relative to the desubmergence timepoint (2 d for *Z. mays* and 5 d for *O. sativa* and *E. crus-galli*) (Fig. 3.3).


A
M E F I
Early Sub.
Number of DEGs
3000 2000 1000 0 1000 2000 3000
B
M E F I
GO:0006355−regulation of transcription, DNA−templated
GO:0006979−response to oxidative stress
GO:0005975−carbohydrate metabolic process
GO:0006520−cellular amino acid metabolic process
GO:0006631−fatty acid metabolic process
GO:0015979−photosynthesis
GO:0015995−chlorophyll biosynthetic process
GO:0009690−cytokinin metabolic process
GO:0015976−carbon utilization
GO:0006099−tricarboxylic acid cycle
GO:0030244−cellulose biosynthetic process
−log10(FDR(GO))
6
4
2
0
Night Sub.
Number of DEGs
3000 2000 1000 0 1000 2000 3000
GO:0000160−phosphorelay signal transduction system
GO:0005975−carbohydrate metabolic process
GO:0009081−branched−chain amino acid metabolic process
GO:0006631−fatty acid metabolic process
GO:0006979−response to oxidative stress
GO:0016567−protein ubiquitination
GO:0015979−photosynthesis
GO:0015995−chlorophyll biosynthetic process
GO:0006412−translation
GO:0006260−DNA replication
GO:0007017−microtubule−based process
GO:0006094−gluconeogenesis
GO:0006006−glucose metabolic process
GO:0009269−response to desiccation
GO:0009690−cytokinin metabolic process
Mid Sub.
Number of DEGs
3000 2000 1000 0 1000 2000 3000
GO:0005975−carbohydrate metabolic process
GO:0006014−D−ribose metabolic process
GO:0005992−trehalose biosynthetic process
GO:0008610−lipid biosynthetic process
GO:0042546−cell wall biogenesis
GO:0006979−response to oxidative stress
GO:0015979−photosynthesis
GO:0015995−chlorophyll biosynthetic process
GO:0006412−translation
GO:0006260−DNA replication
GO:0007018−microtubule−based movement
GO:0005975−carbohydrate metabolic process
GO:0015976−carbon utilization
GO:0006099−tricarboxylic acid cycle
GO:0006629−lipid metabolic process
GO:0006334−nucleosome assembly
GO:0030244−cellulose biosynthetic process
GO:0016049−cell growth
Late Sub.
Number of DEGs
3000 2000 1000 0 1000 2000 3000
GO:0005975−carbohydrate metabolic process
GO:0006014−D−ribose metabolic process
GO:0009081−branched−chain amino acid metabolic process
GO:0005992−trehalose biosynthetic process
GO:0006635−fatty acid beta−oxidation
GO:0042546−cell wall biogenesis
GO:0006979−response to oxidative stress
GO:0006952−defense response
GO:0015979−photosynthesis
GO:0015995−chlorophyll biosynthetic process
GO:0006260−DNA replication
GO:0005975−carbohydrate metabolic process
GO:0015976−carbon utilization
GO:0006099−tricarboxylic acid cycle
GO:0007018−microtubule−based movement
GO:0016049−cell growth
GO:0030244−cellulose biosynthetic process
GO:0046274−lignin catabolic process
GO:0006979−response to oxidative stress
GO:0006334−nucleosome assembly
up−reg DEGs
down−reg DEGs

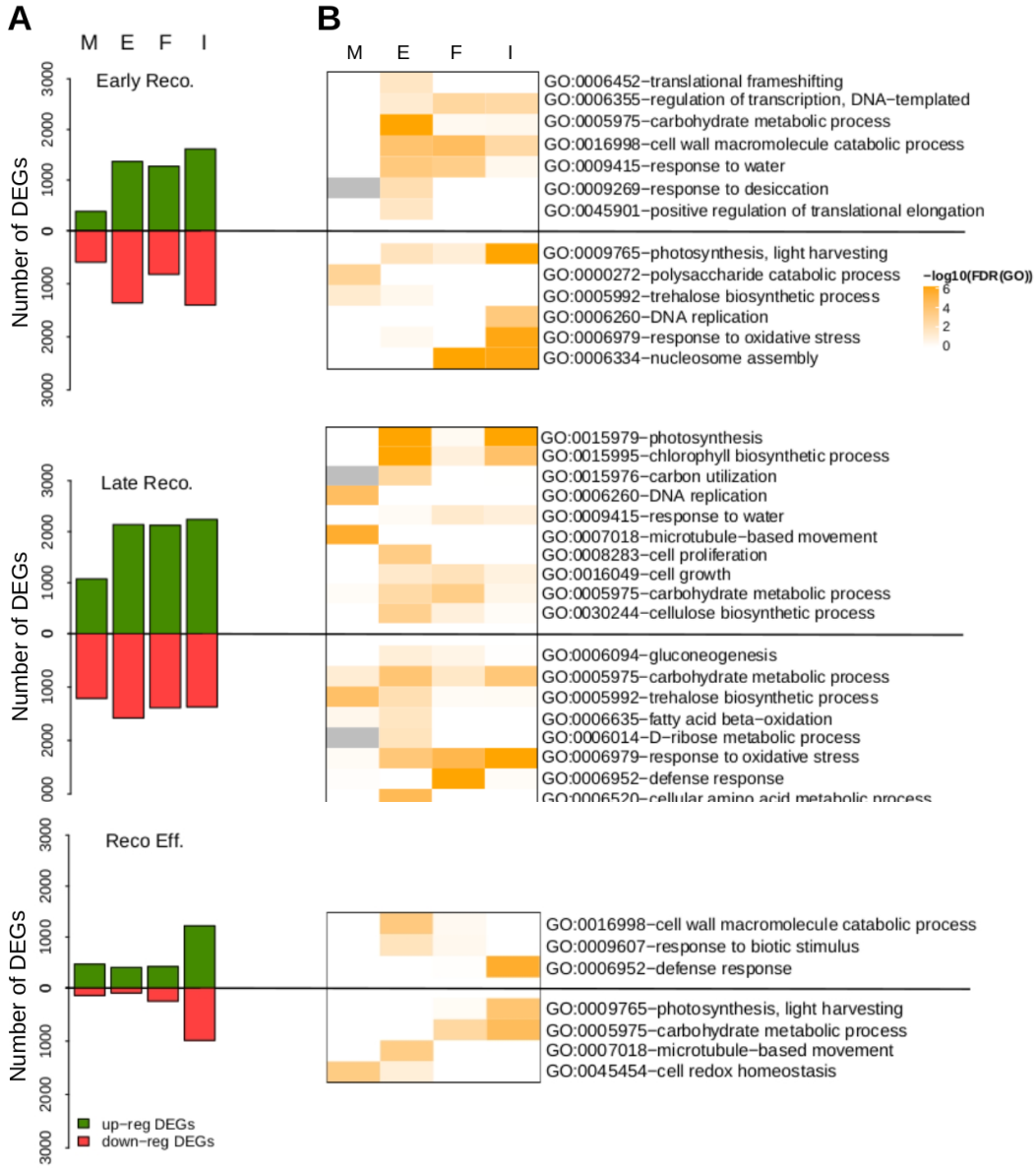


Figure 3.3: Genome-wide transcriptome responses to submergence and recovery in three monocot species

A. The number of Differentially Expressed Genes (DEGs) (|log2FC|> 1 and Padj.< 0.001) per submergence and recovery timepoint for the three species; (upregulated- green bars; downregulated- red bars).

B. A selection of significant Gene Ontology (GO) categories associated with the DEGs plotted in (A) per submergence and recovery timepoint. GO terms are depicted alongside their corresponding up and downregulated DEG cohorts for each timepoint. GO terms are visualized as a heatmap with the strength of the colour indicating the significance of the enrichment of that GO term in the corresponding up or downregulated DEG clusters.

The 4 columns represent the 4 genotypes in this order: M= *Z. mays*, E= *E. crus-galli,* F= *O. sativa* FR13A, O= *O. sativa* IR42. Early Sub= Early Submergence (4 hours), Night Sub= Night Submergence (18 hours), Mid Sub= Mid Submergence (2 days), Late Sub= Late Submergence (5 days), Early Reco= Early Recovery (5 days Sub + 4 hours Reco), Late Reco= Late Recovery (5 days Sub + 1 day Reco), Reco Eff.= Recovery Efficiency is the comparison of the Late Recovery to the Pre-Submergence timepoint.

A few generic trends could be discerned. At the early and the night submergence timepoints, the number of downregulated DEGs exceeds the upregulated DEGs while the mid and late submergence timepoints have a similar number of up and downregulated DEGs (Fig. 3.3A). As submergence progressed, the number of DEGs (up and down) increased. The submergence intolerant *Z. mays* had a delayed response, with about half the number of DEGs compared to the other species at the early submergence timepoint. This is also reflected in the selected GO categories (Fig. 3.3B) where almost no significant enrichment was detected for *Z. mays* categories. In contrast, there were more DEGs in *Z. mays* after the first night and this was maintained at the last 2 days submergence timepoint. In contrast to *Z. mays*, *E. crus-galli* was the most responsive at the early submergence timepoint. It also showed a stronger response to submergence at mid and late submergence than *O. sativa* as reflected in the higher number of DEGs at these timepoints and the many enriched GO terms. Despite the more quiescent nature of *O. sativa* FR13A and the shoot elongation behaviour of *O. sativa* IR42, the responses of the two *O. sativa* genotypes were similar across the timepoints in terms of number of DEGs and enriched GO terms. The slow transcriptomic reprogramming and poor GO term enrichment (except the downregulation in Night Sub. and Mid Sub.) suggest that *Z. mays* lacks a targeted and efficient gene regulatory network responsive to flooding cues, whereas *E. crus-galli* outperformed the *O. sativa* genotypes.

General responses for the four genotypes enriched among downregulated genes were associated with chlorophyll biosynthesis and photosynthesis, whilst many metabolic processes were also enriched among upregulated genes (Fig. 3.3B). For all submergence timepoints, the most GO categories were significantly downregulated for *E. crus-galli* compared to the other species. *E. crus-galli* uniquely showed enrichment of carbon utilization, TCA cycle and chlorophyll and cellulose biosynthetic processes under downregulated genes. A general repression of energetically expensive anabolic processes and selective translation is considered an essential energy conserving response that could prolong survival underwater (Cho et al., 2019; Hwang et al., 2020). In our dataset, translation was downregulated in both *Z. mays* and *E. crus-galli* during submergence. However, this downregulation of translation during submergence was reversed only in *E. crus-galli* during recovery, showing a reactivation of the protein production machinery upon reoxygenation. Both *O. sativa* genotypes showed a highly similar response to submergence, both in DEGs and enriched GO terms. However, cell growth-associated DEGs were downregulated already at 2 days of submergence in *O. sativa* FR13A and from 5 days of submergence in *E. crus-galli*, a response not identified for *O. sativa* IR42. In general, DEGs associated with response to oxidative stress and carbohydrate metabolic process were upregulated in all species. Notably, both *O. sativa* varieties upregulated genes associated with

defense responses after 5 days of submergence. Defense response signatures are often found in flooding studies (van Veen et al., 2013; van Veen et al., 2016; Jung et al. 2010) and could be the result of ethylene accumulation in the plant (Voesenek and Sasidharan, 2013), a hormone also involved in defense response (Aerts et al. 2021).

Transcriptomic responses to post-submergence recovery in three monocot species

The recovery effect on the transcriptome was assessed by calculating differential expression relative to the desubmergence timepoint (2 d for *Z. mays* and 5 d for *O. sativa* and *E. crus-galli*) (Fig. 3.3). During recovery, more DEGs were upregulated than downregulated for all species (Fig. 3.3A). For the early recovery (4 h) timepoint, the number of *Z. mays* DEGs were lower than for the other species as it was the case during early submergence. DEGs associated with carbohydrate metabolic process and utilization and responses to desiccation are upregulated in *E. crus-galli* only (Fig. 3.3B). Common upregulated GO categories in the two *O. sativa* genotypes and *E. crus-galli* were the photosynthesis-related processes, regulation of transcription, response to water, cell growth and cellulose biosynthetic process while the response to oxidative stress was downregulated.

To assess how effectively plants could return to their original air grown conditions, we also defined a Recovery Efficiency (Reco Eff.) comparison, which calculated the difference in responses after 1 day of recovery compared to the control condition. Except for *O. sativa* IR42, all species approached their pre-submerged transcriptome status after 1 day of recovery (Fig. 3.3A). The incomplete recovery of *O. sativa* IR42 was enriched for genes associated with photosynthesis, whilst defense response activated during submergence did not return to a situation prior to flooding. *E. crus-galli* was in general still upregulating more cell wall-related processes whereas *O. sativa* FR13A and *O. sativa* IR42 rebalanced their carbohydrate metabolic processes.

Submergence stress calls for a use of alternative sugar metabolism in all species

The appropriate management of energy and carbohydrate reserves underwater is an important element of surviving prolonged submergence. Different plant species showing different morphological adaptive strategies will manage and utilize their energy stores differently. An underwater escape response would involve more starch and soluble sugar catabolism than a quiescence response. During submergence, the activation of pathways facilitating the use of reserves alternative to starch, such as amino acids and fatty acids might be vital for fuelling starving plant organs by redirecting energy rich metabolites from older leaves to sink meristems and roots. These metabolic adjustments and energy management

strategies triggered during submergence will also determine the speed and quality of recovery following desubmergence. A large portion of the GO terms that were enriched among the DEGs are associated with carbon metabolism. To go beyond the overall impression of GO enrichment we focussed on carbohydrate levels, respiration rate and investigation of specific metabolic routes.

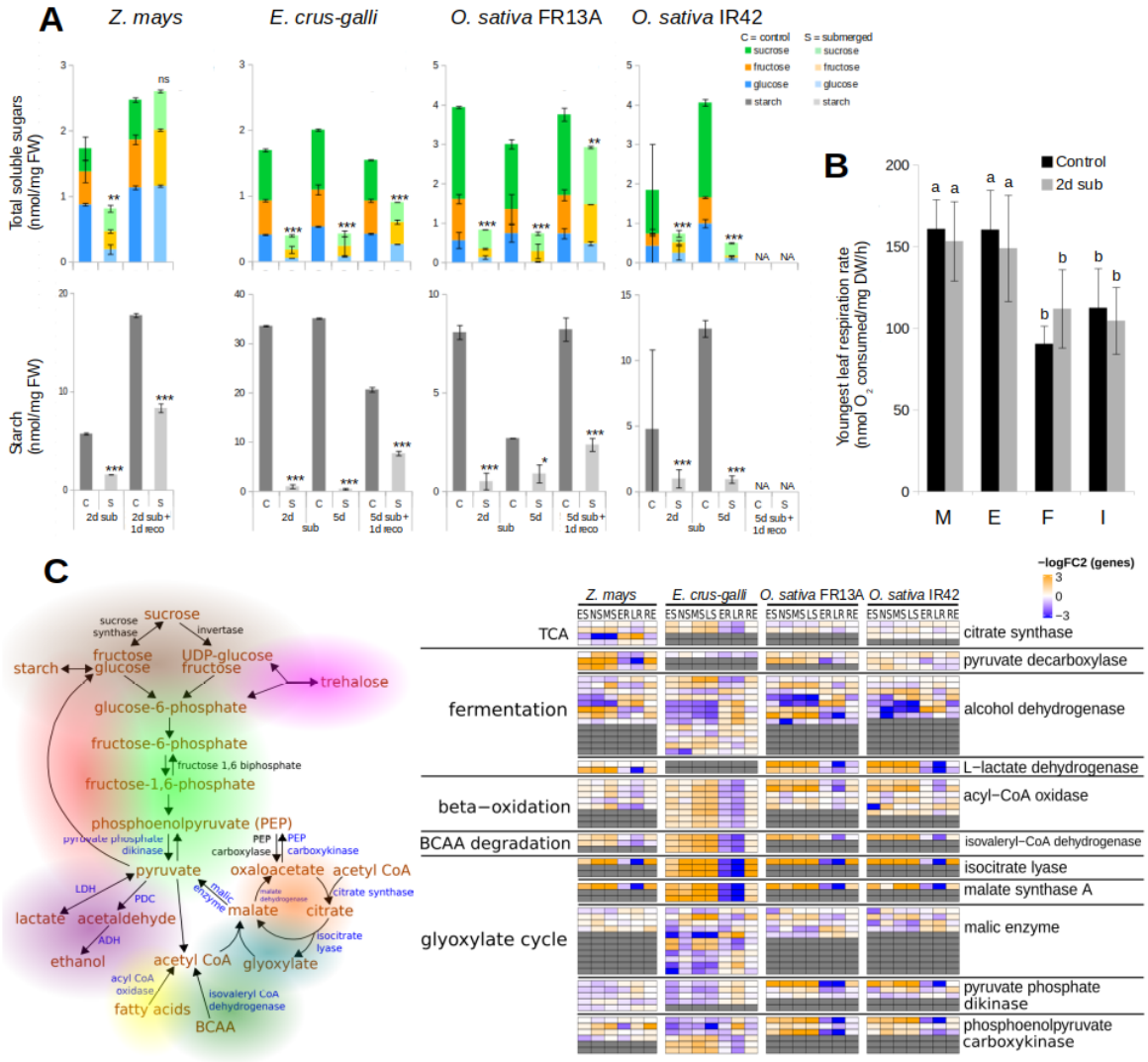


Figure 3.4: Carbohydrate metabolism during submergence and recovery in three monocot species.
A. Total soluble sugar (glucose, fructose, sucrose) and starch content in shoots of *Z. mays*, *E. crus-galli*, *O. sativa* FR13A and *O. sativa* IR42, after 2 or 5 days of complete submergence (sub) and after 1 day post-submergence (1d reco). C= control (air); S= submerged. NA= no data available, ns= non significant, *= p-val< 0.05, **= p-val< 0.01, ***= p-val< 0.001 from Tukey post hoc test after 2-way ANOVA (timepoint and treatment) per species. n= 2 replicates of 3 to 5 pooled plant shoots.
B. Respiration rate of the youngest (3rd) leaf of each species before (control) and after 2 days of complete submergence (2d sub). M= *Z. mays* B73, E= *E. crus-galli* 09, F= *O. sativa* FR13A, I= *O. sativa* IR42. Tukey post hoc test after 2-way ANOVA (species and treatment). Different letters above the bars represent statistically significant differences (p-val< 0.05). Letters represent grouped data. n= 2 to 6 leaves from different plants.
C. Heatmap of a selection of metabolic enzymes that showed strong treatment or species-specific regulation. Gene names and associated pathways are shown next to the heatmap. One gene can have several copies, represented by individual horizontal rows next to a gene name. Orange represents up- and blue downregulation. Grey boxes are the gene copies that do not exist or lack of a significant regulation (FDR< 0.001). Submergence and recovery timepoints are depicted in columns per species. ES= Early Submergence (4 hours), NS= Night Submergence (18 hours), MS= Mid Submergence (2 days), LS= Late Submergence (5 days) are the submergence timepoints compared to Pre-Submergence, and ER= Early Recovery (5 days Sub + 4 hours Reco) and LR= Late Recovery (5 days Sub + 1 day Reco) are the recovery timepoints compared to the end of the Submergence period (MS for maize, LS for the 3 others). RE= Recovery Efficiency is the comparison of the Late Recovery to the Pre-Submergence timepoint. The subpathways to which the genes belong are also indicated on the left of the heatmap in a schematic depicting some carbohydrate metabolic pathways. Selected genes from the heatmaps are highlighted in blue. Subpathways depicted are: starch and sucrose metabolism (brown), glycolysis (light green), TCA (orange), glyoxylate cycle (blue), gluconeogenesis (red), fermentation (purple), threalose metabolism (pink), fatty acid beta-oxidation (yellow), BCAA degradation (dark green).

To investigate the effect of submergence on carbohydrate metabolism, starch and total soluble sugars (sucrose, fructose, glucose) were measured in the three species (Fig. 3.4A). As expected, submergence caused a significant reduction in the content of both starch and soluble sugars in all the species after 2 or 5 days of submergence in comparison to their relative controls. One day following desubmergence, the levels of sugars and starch recovered considerably in all species, but they were still significantly lower than controls. Notably, *O. sativa* had twice the amount of soluble sugars (sucrose, fructose, glucose) than *Z. mays* and the weed, which had respectively two to four times more starch than the two *O. sativa* varieties. Next, underwater respiration rates of leaves from the three species were measured to assess the effect of submergence on oxygen consumption and therefore carbohydrate and energy reserves (Fig. 3.4B). Basal respiration rates were measured in the youngest formed leaf of the four plant

genotypes in control conditions and after 2 days of complete submergence. While submergence had no significant effect on the respiration rate of the same leaf in any of the species, notably, the basal respiration rate of control plants of the C4 species (*Z. mays* and *E. crus-galli*) was one and a half times higher than the one in the two C3 *O. sativa* (Fig. 3.3B).

Metabolite profiling studies have indicated the importance of mobilizing alternative carbon sources such as amino acids and fatty acids during starvation periods (Dietrich et al. 2011, Usadel et al. 2008). Such pathways could be critical, especially during prolonged submergence for sustaining especially the young leaves and meristem as starch levels get depleted. In such instances, activation of senescence in older leaves can redirect much-needed reserves towards these tissues (Law et al., 2018). In our dataset, several GO categories associated with alternative sugar pathways were upregulated during submergence (Fig. 3.3B). Notably, in *E. crus-galli*, these were significantly upregulated from the beginning of submergence and persisted till late submergence timepoints (fatty acid, BCAA, D-ribose metabolic processes, trehalose biosynthetic process). A surge in amino acid levels upon flooding has been observed before (Medina et al., 2009; Ferner et al., 2012) and indicates protein breakdown likely in senescing leaves or the need to synthesize polyamines involved in senescing tissues (Cohen et al., 1979; Mizrahi et al., 1989; Besford et al., 1993; Soudry, 2005; Sobieszczuk-Nowicka, 2016; Sobieszczuk-Nowicka, 2019). Especially consumption of branched-chain amino acids is a common observation triggered by autophagy in starving leaves (Dietrich et al., 2011).

To further decipher these metabolic acclimations to submergence, we looked at the expression of the genes encoding enzymes from the major carbohydrate utilizing pathways across the four species. Figure s3.5 shows the differential expression of these genes for all timepoints (ES to LR), and the differential expression of the genes between 1 day of recovery compared to control condition (RE) with key responding enzymes highlighted in Fig. 3.4C. Interestingly, genes associated with sucrose, fructose, glucose and starch pathways do not seem to be strongly and consistently regulated, despite the changes noted in Fig. 3.4A. Glycolysis did show an overall downregulation during submergence for both *O. sativa* genotypes and very clear downregulation for *E. crus-galli*, whilst *Z. mays* showed many contrasting responses in the glycolytic pathway. The TCA cycle was dampened in all three species. However, citrate synthase, also part of the glyoxylate cycle, did show upregulation in *E. crus-galli*. Lactate dehydrogenase was induced in both *O. sativa* genotypes and *Z. mays*, whereas in *E. crus-galli* no ortholog expression was detected. PDC and ADH showed a variety of responses, which could be due to difficulty for accurately annotating these enzymes. Since respiration remains unchanged in the initial days of submergence, the exact role of

activating the fermentation pathway is unclear. The actual fermentative flux might prove to be minimal since this is highly dependent on sugar availability (Summers et al., 2000; Huang et al., 2005; Santaniello et al., 2014), which was low (Fig. 3.4A) and coincide with transcriptional glycolytic downregulation.

In correspondence with the GO enrichment analysis, the pathways that address alternative carbon sources showed strong regulation. For all species and genotypes, fatty acid breakdown (beta-oxidation) and breakdown of branched-chain amino acids (BCAAs) were activated. The breakdown products of these pathways can fuel the TCA cycle and respiration. The carbon backbones can also preserve the glyoxylate cycle for subsequent gluconeogenesis. The key glyoxylate enzymes ICL (IsoCitrate Lyase) and MLS (MaLate Synthase A), invariably were activated upon flooding. Though the key enzymatic routes channelling towards gluconeogenesis (ME-PPDK/PCK) showed highly variable responses, it might be that one route is preferred over the other and this indicates challenges with enzyme annotation.

Established flood responses: hormones and hypoxia-responsive genes

Hormones are well-established regulators of several submergence-induced adaptive traits. In particular, ethylene, ABA and GA actions are implicated in the induction of traits such as enhanced shoot elongation, adventitious root formation and aerenchyma during submergence. Likewise, genes typically induced upon hypoxia (Mustroph et al., 2009; Mustroph et al., 2010) are often associated with flooding. The enrichment analysis yielded few clues regarding their relevance. Given their importance in the literature we further explored these key pathways.

Interestingly, the hormonal transcriptome signatures were largely similar across the three species (Fig. s3.4). In our dataset, ethylene metabolism (*ACC*; 1-AminoCyclopropane-1-Carboxylic acid) and signalling (*ERF1*; Ethylene-Responsive transcription Factor 1) were upregulated. Their regulation is considered a reporter for ethylene accumulation (Voesenek and Van Der Veen, 1994). ABA-related genes were downregulated during submergence in all species, particularly *NCED* (9-cis-epoxycarotenoid dioxygenase) which is the critical rate-limiting enzyme in the ABA biosynthetic pathway (Fig. s3.4). Both types of *O. sativa* (quiescence and escape) showed a strong decline of ABA when submerged (Hattori et al. 2009; Ram et al. 2002). Our data suggest this might be a common trait among Poaceae.

The upregulation of *SUB1A* (present in *O. sativa* FR13A) is engendered by a submergence-induced accumulation of ethylene. In SUB1A containing varieties, shoot growth is restricted by the increase in the GA response repressors Slender Rice-1 (*SLR1*) and SLR1 Like-1 (*SLRL1*), which reduce GA responsiveness (Fukao

and Bailey-Serres, 2008). Increased GA levels on the other hand are associated with escape growth (Hattori et al., 2009; Raskin and Kende 1984). Varieties that lack SUB1A (like *O. sativa* IR42, Septiningsih, 2008; Winkel et al., 2014; Singh et al., 2020) do not show growth restriction underwater. Of note in relation to the gibberellin pathway is that the biosynthetic enzyme gibberellin 20 oxidase (*GA20ox*) is strongly upregulated only in *E. crus-galli*, which elongates underwater, but not in the other species. *SD1* (*SEMIDWARF1*) encodes a GA20-oxidase for gibberellin biosynthesis, generating a semidwarf phenotype (Kuroha et al., 2018). Indeed, it has been described that *GA20-oxidase* transcription is under negative feedback regulation (Fukazawa et al., 2017) and may be partially diminished in deepwater rice, leading to higher *SD1* transcript accumulation relatively compared to in other rice genotypes, in response to submergence.

The cytokinin patterns seem to be stronger in the two *O. sativa* varieties, with biosynthesis strongly downregulated during submergence and recovery upon reoxygenation, a similar but milder form of regulation was found in *E. crus-galli* but absent in *Z. mays*. The exact role of cytokinin in flooding acclimation remains unclear. On the other hand, jasmonates are increased and aid upon recovery in *Arabidopsis* (Yuan et al., 2017). Also, for our three species, we found a strong jasmonate signalling and biosynthesis responses upon recovery, though *Z. mays* showed a much milder response.

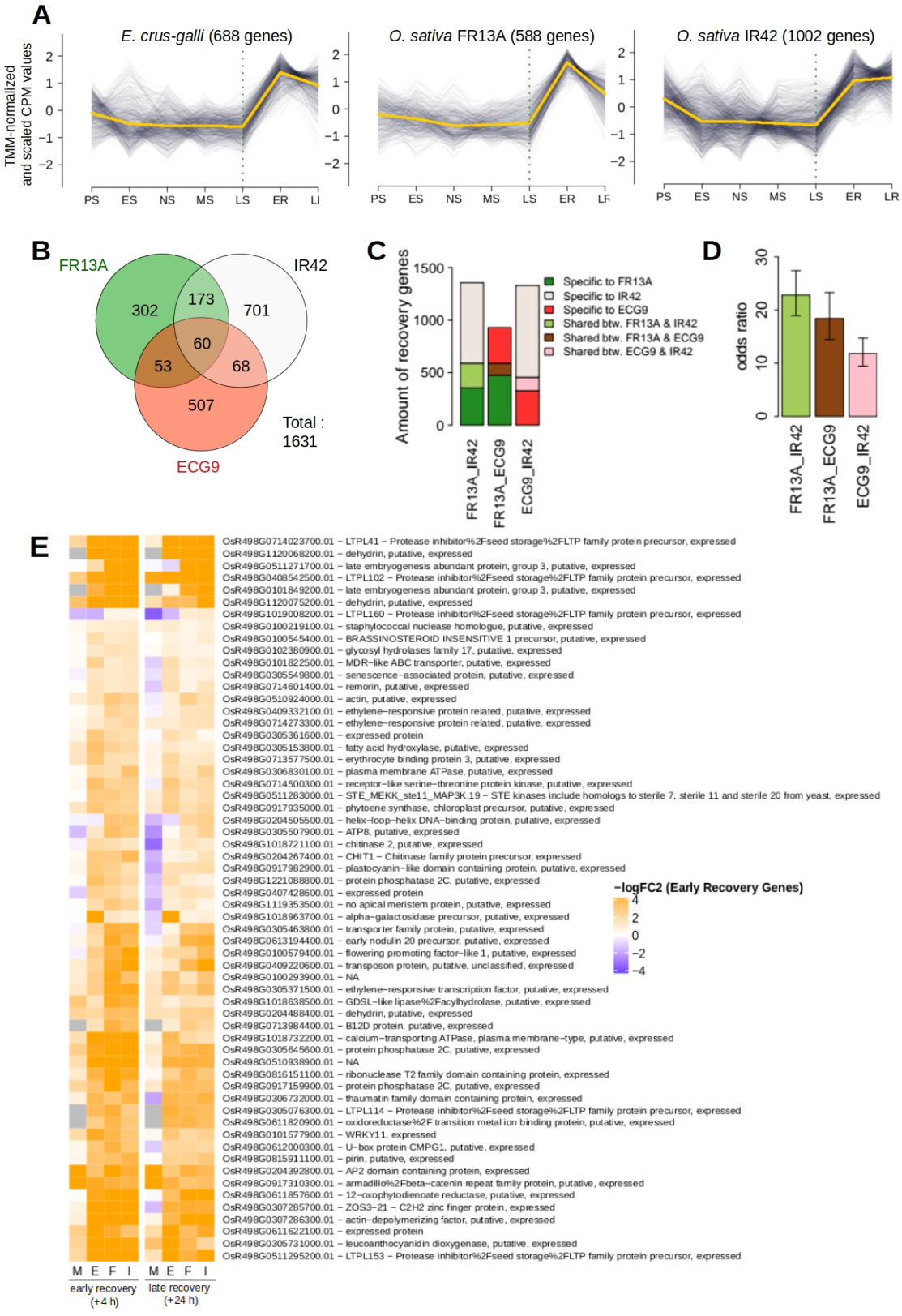
Transcriptomic work in *Arabidopsis* identified a set of genes induced upon hypoxia regardless of cell type (Mustroph et al., 2009), a majority of which are direct targets of the cellular oxygen sensing machinery (Gasch et al., 2016). These genes show decent conservation among the plant kingdom (Mustroph et al., 2010; Reynoso et al., 2019). Also in the three studied species, these genes show a strong upregulation upon flooding (Fig. s3.6). Shoot tissue typically does not reach extremely low levels of oxygen, even under dark respiring conditions. Nonetheless, a clear upregulation of the hypoxic core genes was found. We expected a stronger response for the night timepoint with associated lower oxygen levels, but this is not apparent from the data. It can be that the oxygen and energy levels are still enough for the metabolic activities during the first night of submergence and that a difference would be observed between day and night only after several days of submergence. Another possibility is, on the contrary, that the genes are already induced at the maximum extent from the early submergence timepoint and cannot be more induced at night. Hypoxia genes regulation seemed independent of species. Indeed, only poor relationships exist with the magnitude of transcriptional induction of core hypoxia genes and tolerance to flooding (Loreti et al., 2016).

Core recovery genes shared between *O. sativa* and *E. crus-galli* after desubmergence are not differentially expressed in the intolerant species *Z. mays*

Flooding is a sequential stress of increasing severity of hypoxia and energy shortage, followed by a post-submergence phase that poses its own stresses that again need to be dealt with, all together determining tolerance. We therefore further explored the transcriptome data to identify recovery-specific DEGs. For this, we first performed a hierarchical clustering of all submergence-responsive shoot DEGs over time for the three species (Fig. s3.7) to visualize the dynamics of the transcriptome responses. Seven distinct expression clusters were observed for *O. sativa* FR13A while DEGs of the other genotypes clustered into nine different expression profiles. This representation of the data can help to visualize the dynamics of co-expressed genes across subsequent timepoints and can be thought of as distinct regulatory modules. Several expression profiles were very similar between species. For instance, Cluster 2 in maize and Cluster 1 in the weed and the two *O. sativa* varieties show a clear effect of the night period that can be due to regulation by the circadian clock and other day/night cues. Furthermore, the lack of light and expected reduced oxygen levels would also imply a strong role of starvation signalling in these genes.

To focus on recovery effects, we looked for clusters that show a recovery-specific regulation. These were cluster 5 in *E. crus-galli* and *O. sativa* FR13A and cluster 6 in *O. sativa* IR42. These genes (Fig. 3.5A) barely respond to flooding but are specifically upregulated upon desubmergence and stay highly expressed also after 24 hours of recovery. Interestingly, such a recovery specific cluster does not exist in *Z. mays*, which might be related to the poor capacity of the plant to recover. Next, we identified the genes from these «recovery» clusters that are shared between rice and the weed, and genes that are species-specific (Fig. 3.5B). Most recovery genes were species-specific, especially for *O. sativa* IR42 (701 genes) compared to *O. sativa* FR13A (302 genes) or *E. crus-galli* (507 genes). Nonetheless, we identified a strongly significant overlap between the species and genotypes, suggesting the presence of a conserved set of recovery responsive genes. Indeed, we identified 60 «core recovery genes» common to both rice varieties and the weed (Fig. 3.5E). Clearly, these genes are upregulated in the weed and the rice at both recovery timepoints, while only a few of these genes are upregulated in maize during recovery.

Post-submergence stress is typically associated with leaf dehydration and wilting (Fig. s3.1 and s3.2) and recovery is assisted by the ability to limit this (Yeung et al., 2018; Yeung et al., 2019). Accordingly, the core recovery list included genes such as the «dehydrins» and the «late embryogenesis abundant (LEA) protein» that are particularly protective against dehydration damage (Hanin, 2011;



OsR498G0714023700.01 – LTPL41 – Protease inhibitor%2Fseed storage%2FLTP family protein precursor, expressed
OsR498G1120068200.01 – dehydrin, putative, expressed
OsR498G0511271700.01 – late embryogenesis abundant protein, group 3, putative, expressed
OsR498G0408542500.01 – LTPL102 – Protease inhibitor%2Fseed storage%2FLTP family protein precursor, expressed
OsR498G0101849200.01 – late embryogenesis abundant protein, group 3, putative, expressed
OsR498G1120075200.01 – dehydrin, putative, expressed
OsR498G1019008200.01 – LTPL160 – Protease inhibitor%2Fseed storage%2FLTP family protein precursor, expressed
OsR498G0100219100.01 – staphylococcal nuclease homologue, putative, expressed
OsR498G0100545400.01 – BRASSINOSTEROID INSENSITIVE 1 precursor, putative, expressed
OsR498G0102380900.01 – glycosyl hydrolases family 17, putative, expressed
OsR498G0101822500.01 – MDR−like ABC transporter, putative, expressed
OsR498G0305549800.01 – senescence−associated protein, putative, expressed
OsR498G0714601400.01 – remorin, putative, expressed
OsR498G0510924000.01 – actin, putative, expressed
OsR498G0409332100.01 – ethylene−responsive protein related, putative, expressed
OsR498G0714273300.01 – ethylene−responsive protein related, putative, expressed
OsR498G0305361600.01 – expressed protein
OsR498G0305153800.01 – fatty acid hydroxylase, putative, expressed
OsR498G0713577500.01 – erythrocyte binding protein 3, putative, expressed
OsR498G0306830100.01 – plasma membrane ATPase, putative, expressed
OsR498G0714500300.01 – receptor−like serine−threonine protein kinase, putative, expressed
OsR498G0511283000.01 – STE_MEKK_ste11_MAP3K.19 – STE kinases include homologs to sterile 7, sterile 11 and sterile 20 from yeast, expressed
OsR498G0917935000.01 – phytoene synthase, chloroplast precursor, putative, expressed
OsR498G0204505500.01 – helix−loop−helix DNA−binding protein, putative, expressed
OsR498G0305507900.01 – ATP8, putative, expressed
OsR498G1018721100.01 – chitinase 2, putative, expressed
OsR498G0204267400.01 – CHIT1 – Chitinase family protein precursor, expressed
OsR498G0917982900.01 – plastocyanin−like domain containing protein, putative, expressed
OsR498G1221088800.01 – protein phosphatase 2C, putative, expressed
OsR498G0407428600.01 – expressed protein
OsR498G1119353500.01 – no apical meristem protein, putative, expressed
OsR498G1018963700.01 – alpha−galactosidase precursor, putative, expressed
OsR498G0305463800.01 – transporter family protein, putative, expressed
OsR498G0613194400.01 – early nodulin 20 precursor, putative, expressed
OsR498G0100579400.01 – flowering promoting factor−like 1, putative, expressed
OsR498G0409220600.01 – transposon protein, putative, unclassified, expressed
OsR498G0100293900.01 – NA
OsR498G0305371500.01 – ethylene−responsive transcription factor, putative, expressed
OsR498G1018638500.01 – GDSL−like lipase%2Facylhydrolase, putative, expressed
OsR498G0204488400.01 – dehydrin, putative, expressed
OsR498G0713984400.01 – B12D protein, putative, expressed
OsR498G1018732200.01 – calcium−transporting ATPase, plasma membrane−type, putative, expressed
OsR498G0305645600.01 – protein phosphatase 2C, putative, expressed
OsR498G0510938900.01 – NA
OsR498G0816151100.01 – ribonuclease T2 family domain containing protein, expressed
OsR498G0917159900.01 – protein phosphatase 2C, putative, expressed
OsR498G0306732000.01 – thaumatin family domain containing protein, expressed
OsR498G0305076300.01 – LTPL114 – Protease inhibitor%2Fseed storage%2FLTP family protein precursor, expressed
OsR498G0611820900.01 – oxidoreductase%2F transition metal ion binding protein, putative, expressed
OsR498G0101577900.01 – WRKY11, expressed
OsR498G0612000300.01 – U−box protein CMPG1, putative, expressed
OsR498G0815911100.01 – pirin, putative, expressed
OsR498G0204392800.01 – AP2 domain containing protein, expressed
OsR498G0917310300.01 – armadillo%2Fbeta−catenin repeat family protein, putative, expressed
OsR498G0611857600.01 – 12−oxophytodienoate reductase, putative, expressed
OsR498G0307285700.01 – ZOS3−21 – C2H2 zinc finger protein, expressed
OsR498G0307286300.01 – actin−depolymerizing factor, putative, expressed
OsR498G0611622100.01 – expressed protein
OsR498G0305731000.01 – leucoanthocyanidin dioxygenase, putative, expressed
OsR498G0511295200.01 – LTPL153 – Protease inhibitor%2Fseed storage%2FLTP family protein precursor, expressed

Figure 3.5: Recovery-specific gene expression in shoots of *Echinochloa crus-galli* and *O. sativa.*
A. Selected clusters from Figure s3.5 showing the expression profile where the genes were only expressed during the recovery period in *E. crus-galli*, *O. sativa* FR13A and *O. sativa* IR42. Hierarchical clustering (distance method= "euclidean", hierarchical clustering method= "ward.D2") was made on scaled significant DEGs (|log2FC|> 1 and Padj.< 0.001). Individual gene patterns are plotted in grey. Orange lines indicate the mean expression level for all genes in a cluster. The number of genes per cluster is indicated above each panel. The x-axis represents the different timepoints when the tissue was harvested: PS= Pre-Submergence, ES= Early Submergence (4 hours), NS= Night Submergence (18 hours), MS= Mid Submergence (2 days), LS= Late Submergence (5 days), ER= Early Recovery (5 days Sub + 4 hours Reco), LR= Late Recovery (5 days Sub + 1 day Reco). The dashed green vertical bar represents the end of complete submergence and beginning of recovery.
B. Venn Diagram of the shared and specific recovery genes of each species. The recovery specific genes depicted in 'A' for each species were used as input in a Venn diagram to assess the degree of overlap between each couple of species and between all species.
C. Inter-and intra species pair-wise comparison of shared and unique recovery-specific genes. The x-axis indicates the species being compared. Coloured boxes break down the total number of recovery genes displayed into being unique or shared across the compared species.
D. Odds ratio corresponding to the overlapping genes in (C) for each couple of species, showing the high confidence of the shared genes when the odds ratio is high.
E. Heatmap showing the expression of the 60 common Core Recovery Genes (CGR) identified in 'B' in *E. crus-galli*, *O. sativa* FR13A and *O. sativa* IR42 at 4h (early) and 24h (late) recovery, in *E. crus-galli*, *O. sativa* FR13A, *O. sativa* IR42 and *Z. mays*. Orange represents up- and blue downregulation. The 4 columns represent the species in this order: M= *Z. mays*, E= *E. crus-galli*, F= *O. sativa* FR13A, O= *O. sativa* IR42.

Candat et al., 2014), and genes associated with ABA and drought stress tolerance, such as the «no apical meristem (NAC) protein» (Kim et al., 2014) or the «protein phosphatase 2C (PP2C)» (Zhang and Gan, 2012). This is in accordance with previous studies in *Arabidopsis* and *O. sativa*, where the upregulation of such genes was associated with enhanced recovery (Yeung et al., 2018). In addition to imposing a quiescence strategy, SUB1A also enhances post-submergence recovery (Locke et al., 2017). This is in part associated with an enhanced responsiveness to ABA and the stronger upregulation of genes associated with drought. Notably, ABA signatures were strongly enhanced during recovery in all the genotypes (Fig. S3.4). In addition, the core recovery genes include a few transcription factors such as *AP2*, *WRKY* and *bHLHs*, which could participate in orchestrating the recovery response (Fukao et al., 2006, van Veen et al., 2014).

In *Arabidopsis*, superior recovery is associated with suppressing senescence and retaining water. Ethylene synthesis and ABA accumulation immediately following submergence stimulate senescence and water loss via ORE1 and SAG113 in recovery-sensitive *Arabidopsis* accessions (Zhang and Gan,

2011; Yeung et al. 2018). A clear ABA signature was found in the core recovery genes in our data, which includes species and genotypes with excellent recovery. We could not detect clear differential expression patterns for *SAG113* orthologs, which is essential to utilize ABA signalling to aid senescence and dehydration (Yeung et al., 2018). The lack of *SAG113* regulation suggests that in these monocots, ABA is not associated with accelerated dehydration in contrast to *Arabidopsis* (Yeung et al. 2018).

Another hormone showing a strong transcriptome signature during recovery was jasmonic acid (JA), with a decline during flooding but a strong and rapid increase again during recovery (Fig. s3.4). JA has been linked to the amelioration of ROS-mediated oxidative stress in *Arabidopsis* (Yuan et al., 2017). The excessive accumulation of ROS is a hallmark of reoxygenation and accordingly the ability to limit ROS damage is beneficial for recovery. While no clear ROS associated genes were detected in the core recovery cluster, there was a notable induction of genes associated with the response to oxidative stress in *E. crus-galli* already from the first submergence timepoint. This could represent an enhancement of the antioxidant status and ROS ameliorating capacity in this species, thus making it better prepared for reoxygenation stress. On the contrary, *Z. mays* does not show any JA signature, and this would suggest a poor ROS-antioxidant response and corresponding poor recovery. Post-submergence tolerance of *O. sativa* FR13A harbouring SUB1A is also attributed to a superior ability to limit oxidative damage (Ella et al., 2003; Fukao et al., 2011). This is reflected in restricted lipid peroxidation and chlorophyll damage during recovery and correlates with the green phenotype of *O. sativa* FR13A shoots following desubmergence.

Indeed, it can be speculated that the recovery cluster is associated with an immediate adjustment and response to recovery conditions and signals such as ROS and dehydration. *O. sativa* IR42 is relatively recovery stress sensitive, implying that these core recovery genes are unlikely factors to be crucial for tolerance to reoxygenation. Overall, the vast array of transcriptomic reprogramming occurs already during flooding and recovery-specific genes are highly shared between sensitive and tolerant reoxygenation behaviours. We therefore hypothesize that tolerance to reoxygenation is determined mostly by changes that have occurred during the submergence phase, rather than by gene expression patterns observed during reoxygenation.

Conclusions

Although both *O. sativa* FR13A and *E. crus-galli* are very tolerant to submergence stress, the obvious difference is their behaviour underwater. While *E. crus-galli* continues its growth and even forms a new leaf underwater, *O. sativa* FR13A clearly stays quiescent, as previously recorded (Xu et al., 2006; Bailey-

Serres et al., 2010). Although these two tolerant species evidently used different strategies to cope with submergence, we observed a depletion of soluble sugars and starch in both (Fig. 3.4A). However, the transcriptome survey revealed distinct metabolic readjustments during submergence stress. Unlike *O. sativa* FR13A activating its fermentation pathway, especially the lactate dehydrogenase, *E. crus-galli* seems to use the alternative pathways (BCAA and beta-oxidation pathways) to get energy (Fig. 3.3B, 3.4C and s3.5). The glyoxylate cycle and gluconeogenesis related genes are upregulated in both species, especially those encoding for the isocitrate lyase and malate synthase A enzymes, but *E. crus-galli* does not upregulate genes encoding for the pyruvate phosphate dikinase and the phosphoenolpyruvate carboxylase like *O. sativa*. But unlike *O. sativa*, it does downregulate the phosphoenolpyruvate carboxykinase gene. From a carbon economic point of view, the downregulation of energy consumption does not lead to a reduced maintenance cost (Fig. 3.4B).

E. crus-galli also shows superior recovery like *O. sativa* FR13A. While the economic usage of carbohydrates and energy in *O. sativa* FR13A is associated with its ability to recover from prolonged submergence, *E. crus-galli* elongates during submergence like *O. sativa* IR42 and is even able to make leaves underwater and recover better. Its better recovery is reflected in the rapid reversion of most processes such as chlorophyll biosynthesis and resumption of carbohydrate metabolism and translation following desubmergence (Fig. 3.3). The new leaves formed underwater might also boost photosynthesis recovery in this phase. *E. crus-galli* shows a stronger induction of the *GIBBERELLIN 20-OXIDASE* gene compared to the other genotypes, which might explain the superior recovery of *E. crus-galli*, allowing for a continuation of development underwater and after 2 weeks of being submerged. In addition, the transcriptome survey indicated an early and persistent response to oxidative stress in *E. crus-galli*. These responses might be associated with an enhancement of the antioxidant status that makes it better prepared for the stresses of recovery. A core recovery response has been identified in *E. crus-galli* and in the two *O. sativa* varieties, mainly corresponding to typical drought-stress responses and to transcription factors regulation, that *Z. mays* is not expressing at all, even after 1 day post-submergence (Fig. 3.5E).

The high tolerance of *O. sativa* and *E. crus-galli* was starkly contrasted with the extreme sensitivity of *Z. mays* to submergence (Fig. s3.2) despite possessing some flood-adaptative morphological traits. Despite the contrasting resilience to submergence between *Z. mays* and *E. crus-galli*, these two species are genetically more closely related than with *O. sativa*. This was reflected in some common responses between these species, like the leaf respiration rate (Fig. 3.4B), the high level of starch reserves in their shoots and high consumption during the submergence period (Fig. 3.4A). In the transcriptome dataset, *Z. mays*

responded very differently as compared to the other three genotypes (Fig. s3.4, s3.5 and s3.6). It appears not to perceive and respond to the stress as quickly and strongly as the other species. In contrast, *E. crus-galli* responses were fast, and it could effectively modulate entire pathways, as exemplified by the many enriched GO terms, and metabolic pathways (Fig. s3.3B, 3.3A, and s3.7). No strongly significant GO categories for *Z. mays* were identified (Fig. 3.3B). All these trends were also reflected during recovery, implying overall that *Z. mays* has low conservation of responses to submergence which are not very coordinated. Despite the observed phenotypes (Fig. s3.2), it was remarkable that the level of total soluble sugars and of starch also recovered in *Z. mays* post submergence. It is possible that although *Z. mays* can resume main metabolic pathways and photosynthesis, it is highly intolerant to post-submergence oxidative stress and succumbs to associated injury. Indeed, an ability to maintain ROS homeostasis was associated with submergence tolerance in *Z. mays* and linked to the major submergence tolerance QTL Subtol6 (Campbell et al., 2015).

In this chapter, we described the major responses of four genotypes varying in their tolerance to flooding. The behaviour among the species overall is very similar, and hormones (Fig. s3.4) and typical hypoxia responses (Fig. s3.6) mattered little. Despite many common responses in general, we could note phenotypic and metabolic differences, and recovery genes shared between rice and weed. The large-scale view in this chapter focussed on processes rather than individual genes. This has potentially prevented an insight into the key players orchestrating the response. Moreover, GO categories and BLAST-based annotations tend to clump many genes together. These limitations can be overcome via an investigation of orthologous gene groups, which can reveal common and species-specific genes (evolutionary comparison), and comparison of the different individual gene to gene expression variation in response to flooding rather than comparing general processes. This will help identifying key gene regulators for flooding tolerance and is the goal of Chapter 4.

Supplemental data

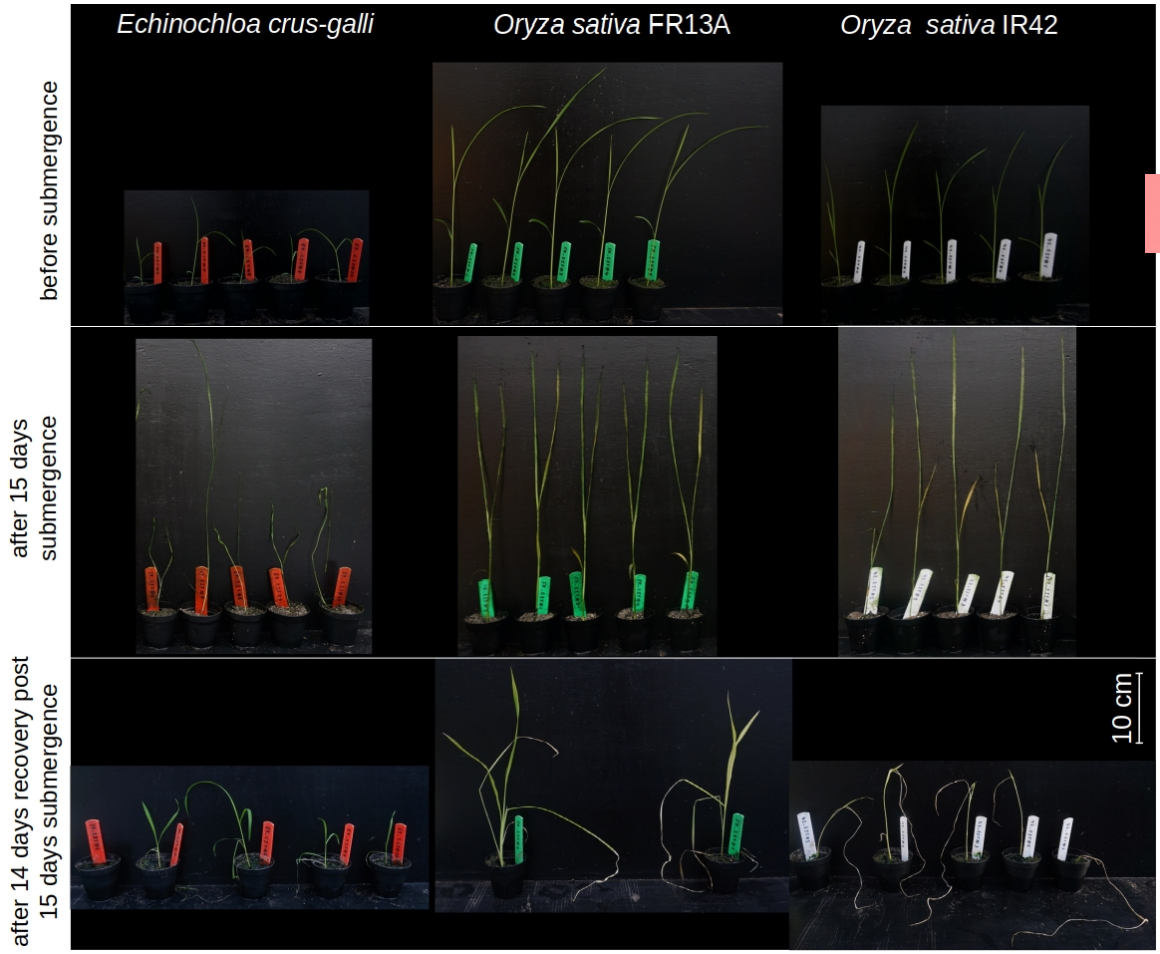


Figure s3.1: The effects of complete submergence on *E. crus-galli* and *O. sativa.* Representative images of *E. crus-galli*, *O. sativa* FR13A and *O. sativa* IR42 plants before and after 15 days of complete submergence and after 14 days of recovery.

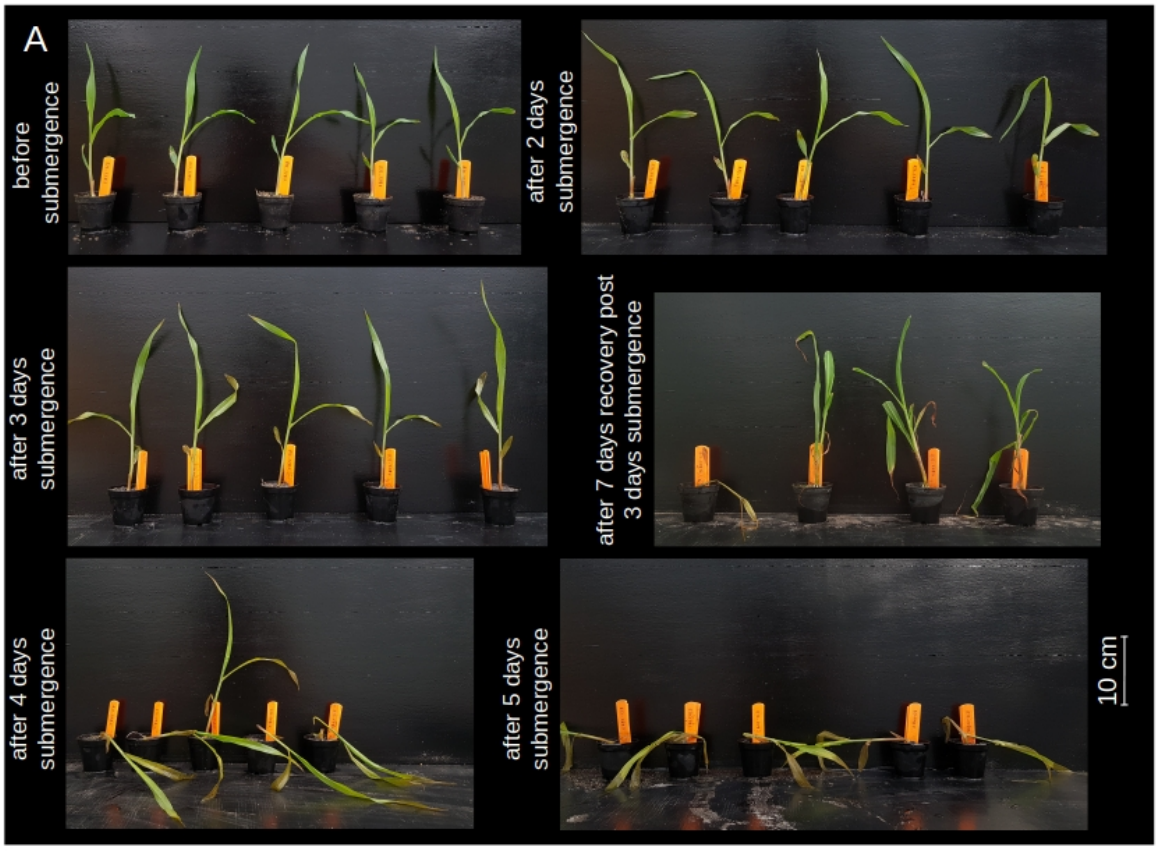

A
before submergence
after 2 days submergence
after 3 days submergence
after 7 days recovery post 3 days submergence
after 4 days submergence
after 5 days submergence
10 cm
B
end submergence
end recovery
survival (%)
100
75
50
25
0
S2+R7
S3+R7
S4+R7
S5+R7
C
pre-submergence
end submergence
end recovery
shoot length (cm)
30
20
10
0
ns
**
***
D
end submergence
end recovery
% plants forming new leaves
100
75
50
25
0
E
pre-submergence
end submergence
end recovery
chlorophyll content of 3rd leaf (mg/m2)
400
300
200
100
0

Figure s3.2: The effects of complete submergence on *Zea mays*.
A. Representative images of *Z. mays* plants before and after 2, 3, 4 and 5 days of complete submergence, and after 7 days of recovery post 3 days submergence.
B. The percentage of plants still alive after 2, 3, 4 and 5 days of complete submergence and after 7 days of recovery. Plants of *Z. mays* were submerged at 3 leaf stage. n=5 plants. Survival was scored based on if the plants were still alive (not rotten).
C. Shoot lengths of *Z. mays* plants before (pre-submergence; green bars), after 2, 3, 4 and 5 days of complete submergence (end submergence; blue bars), and after 7 days of recovery following desubmergence (end recovery; orange bars). Only plants that were alive were measured. n=1 to 5 plants (see B). Values are means +/- SD. ns= non significant, **= p-val< 0.01, ***= p-val< 0.001 from paired samples T-test.
D. The percentage of plants that have formed new leaves after 2, 3, 4 and 5 days of complete submergence (end submergence; blue bars) and after 7 days of recovery following desubmergence (end recovery; orange bars). Only plants that were alive were counted. n=1 to 5 plants (see B).
E. Total chlorophyll content in the third leaf of *Z. mays* plants before (pre-submergence; green bars), after 2, 3, 4 and 5 days of complete submergence (end submergence; blue bars), and after 7 days of recovery following desubmergence (end recovery; orange bars). Measurements were done on the leaf directly with a chlorophyll content meter CCM-300 (Optisciences). Only plants that were alive were measured. n=1 to 5 plants (see B). Values are means +/- SD. ns= non significant, ***= p-val< 0.001 from paired samples T-test.

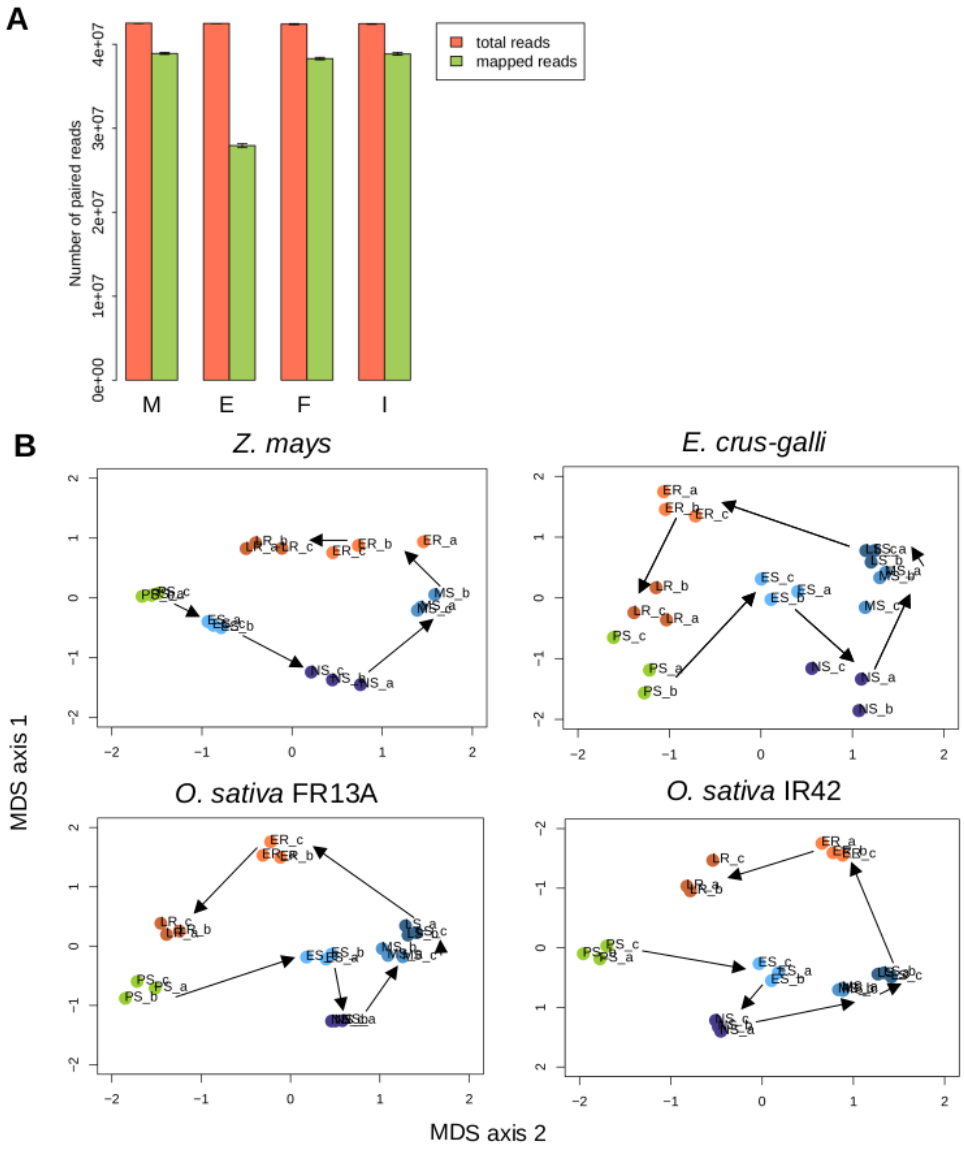


Figure s3.3: RNA sequencing quality assessment.
A. Number of total paired reads (red bars) and corresponding mapped paired reads (green bars) for M= *Z. mays*, E= *E. crus-galli*, F= *O. sativa* FR13A and I= *O. sativa* IR42. Each bar represents the average of the 3 experimental repetitions per 6-7 timepoints.
B. Multi-dimensional scaling (MDS) plots showing the clustering of the 3 biological replicates (a, b, c) per timepoint and species. PS= Pre-Submergence, ES= Early Submergence (4 hours), NS= Night Submergence (18 hours), MS= Mid Submergence (2 days), LS= Late Submergence (5 days), ER= Early Recovery (5 days Sub + 4 hours Reco), LR= Late Recovery (5 days Sub + 1 day Reco). The arrow points to subsequent timepoints starting from the first timepoint and ending with the last recovery timepoint. Green dots represent the Pre-Submergence timepoint, the blue dots represent the timepoints during submergence, and the orange dots represent the timepoints during recovery.

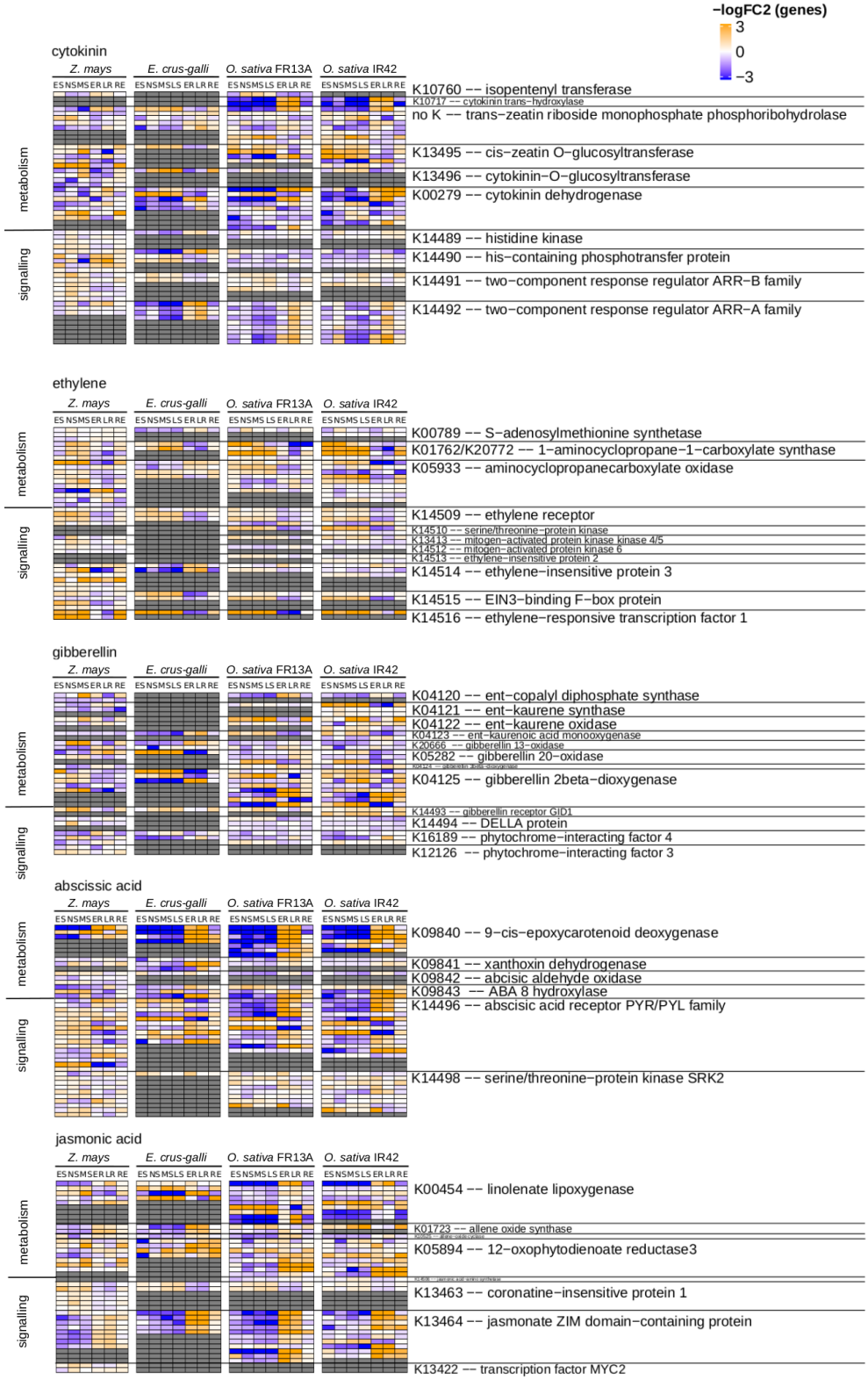
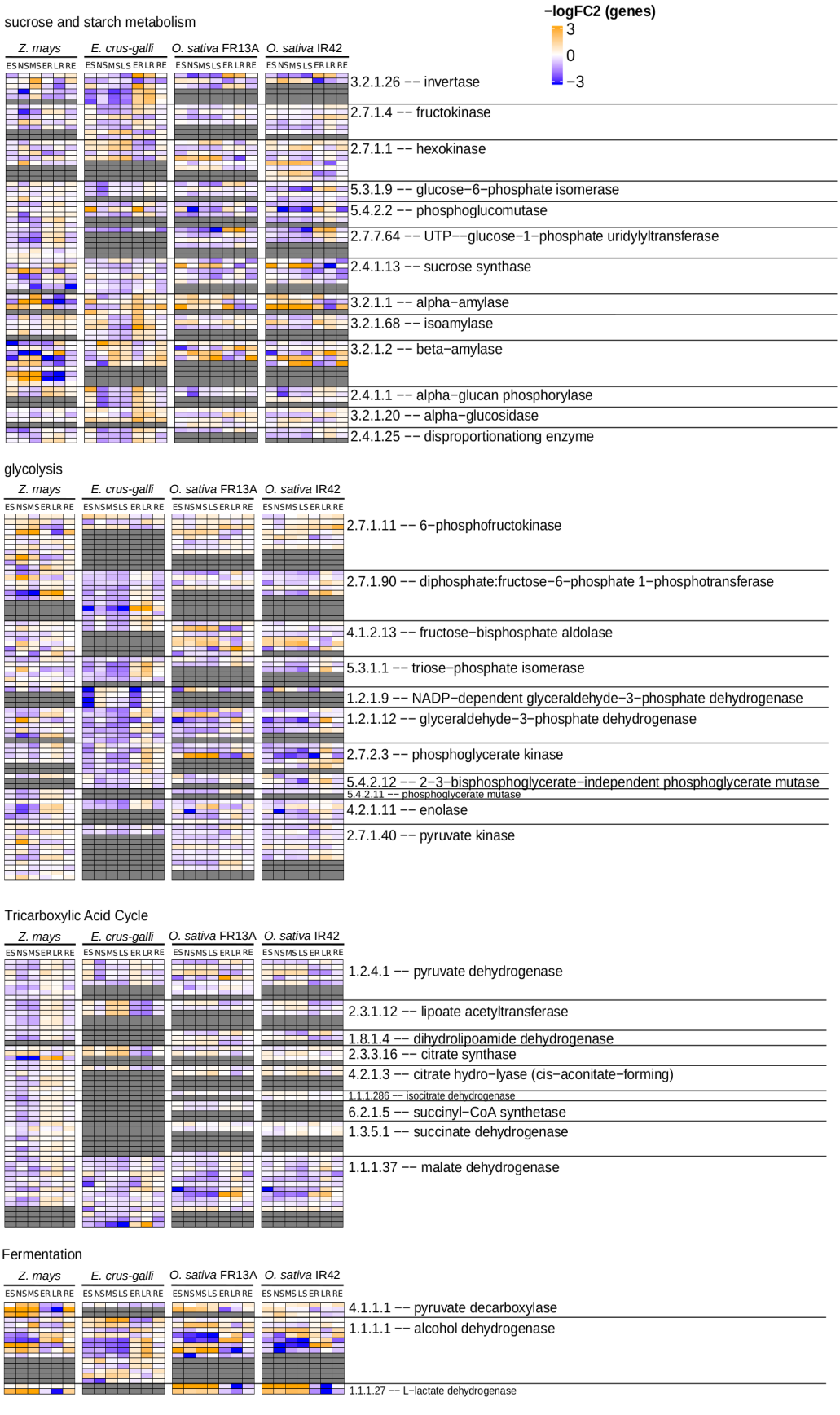

−logFC2 (genes)
3
0
−3
cytokinin
Z. mays
E. crus-galli
O. sativa FR13A
O. sativa IR42
ES NS MS ER LR RE
metabolism
signalling
K10760 −− isopentenyl transferase
K10717 −− cytokinin trans−hydroxylase
no K −− trans−zeatin riboside monophosphate phosphoribohydrolase
K13495 −− cis−zeatin O−glucosyltransferase
K13496 −− cytokinin−O−glucosyltransferase
K00279 −− cytokinin dehydrogenase
K14489 −− histidine kinase
K14490 −− his−containing phosphotransfer protein
K14491 −− two−component response regulator ARR−B family
K14492 −− two−component response regulator ARR−A family
ethylene
K00789 −− S−adenosylmethionine synthetase
K01762/K20772 −− 1−aminocyclopropane−1−carboxylate synthase
K05933 −− aminocyclopropanecarboxylate oxidase
K14509 −− ethylene receptor
K14510 −− serine/threonine−protein kinase
K13413 −− mitogen−activated protein kinase kinase 4/5
K14512 −− mitogen−activated protein kinase 6
K14513 −− ethylene−insensitive protein 2
K14514 −− ethylene−insensitive protein 3
K14515 −− EIN3−binding F−box protein
K14516 −− ethylene−responsive transcription factor 1
gibberellin
K04120 −− ent−copalyl diphosphate synthase
K04121 −− ent−kaurene synthase
K04122 −− ent−kaurene oxidase
K04123 −− ent−kaurenoic acid monooxygenase
K20666 −− gibberellin 13−oxidase
K05282 −− gibberellin 20−oxidase
K04125 −− gibberellin 2beta−dioxygenase
K14493 −− gibberellin receptor GID1
K14494 −− DELLA protein
K16189 −− phytochrome−interacting factor 4
K12126 −− phytochrome−interacting factor 3
abscissic acid
K09840 −− 9−cis−epoxycarotenoid deoxygenase
K09841 −− xanthoxin dehydrogenase
K09842 −− abcisic aldehyde oxidase
K09843 −− ABA 8 hydroxylase
K14496 −− abscisic acid receptor PYR/PYL family
K14498 −− serine/threonine−protein kinase SRK2
jasmonic acid
K00454 −− linolenate lipoxygenase
K01723 −− allene oxide synthase
K05894 −− 12−oxophytodienoate reductase3
K13463 −− coronatine−insensitive protein 1
K13464 −− jasmonate ZIM domain−containing protein
K13422 −− transcription factor MYC2

Figure s3.4: Heatmaps showing hormonal signatures during submergence and recovery in the three species.
Gene names and associated pathways (signalling or metabolism) are shown next to the heatmap. One gene can have several copies, represented by individual horizontal rows next to a gene name. Orange represents up- and blue downregulation. Grey boxes are the gene copies that do not exist or lack of a significant regulation (FDR< 0.001). Submergence and recovery timepoints are depicted in columns per species. ES= Early Submergence (4 hours), NS= Night Submergence (18 hours), MS= Mid Submergence (2 days), LS= Late Submergence (5 days) are the submergence timepoints compared to Pre-Submergence, and ER= Early Recovery (5 days Sub + 4 hours Reco) and LR= Late Recovery (5 days Sub + 1 day Reco) are the recovery timepoints compared to the end of the Submergence period (MS for maize, LS for the 3 others). RE= Recovery Efficiency is the comparison of the LR to the Pre-Submergence timepoint.


sucrose and starch metabolism
−logFC2 (genes)
3
0
−3
Z. mays
E. crus-galli
O. sativa FR13A
O. sativa IR42
ES NS MS ER LR RE
3.2.1.26 −− invertase
2.7.1.4 −− fructokinase
2.7.1.1 −− hexokinase
5.3.1.9 −− glucose−6−phosphate isomerase
5.4.2.2 −− phosphoglucomutase
2.7.7.64 −− UTP−−glucose−1−phosphate uridylyltransferase
2.4.1.13 −− sucrose synthase
3.2.1.1 −− alpha−amylase
3.2.1.68 −− isoamylase
3.2.1.2 −− beta−amylase
2.4.1.1 −− alpha−glucan phosphorylase
3.2.1.20 −− alpha−glucosidase
2.4.1.25 −− disproportionationg enzyme
glycolysis
2.7.1.11 −− 6−phosphofructokinase
2.7.1.90 −− diphosphate:fructose−6−phosphate 1−phosphotransferase
4.1.2.13 −− fructose−bisphosphate aldolase
5.3.1.1 −− triose−phosphate isomerase
1.2.1.9 −− NADP−dependent glyceraldehyde−3−phosphate dehydrogenase
1.2.1.12 −− glyceraldehyde−3−phosphate dehydrogenase
2.7.2.3 −− phosphoglycerate kinase
5.4.2.12 −− 2−3−bisphosphoglycerate−independent phosphoglycerate mutase
5.4.2.11 −− phosphoglycerate mutase
4.2.1.11 −− enolase
2.7.1.40 −− pyruvate kinase
Tricarboxylic Acid Cycle
1.2.4.1 −− pyruvate dehydrogenase
2.3.1.12 −− lipoate acetyltransferase
1.8.1.4 −− dihydrolipoamide dehydrogenase
2.3.3.16 −− citrate synthase
4.2.1.3 −− citrate hydro−lyase (cis−aconitate−forming)
1.1.1.286 −− isocitrate dehydrogenase
6.2.1.5 −− succinyl−CoA synthetase
1.3.5.1 −− succinate dehydrogenase
1.1.1.37 −− malate dehydrogenase
Fermentation
4.1.1.1 −− pyruvate decarboxylase
1.1.1.1 −− alcohol dehydrogenase
1.1.1.27 −− L−lactate dehydrogenase

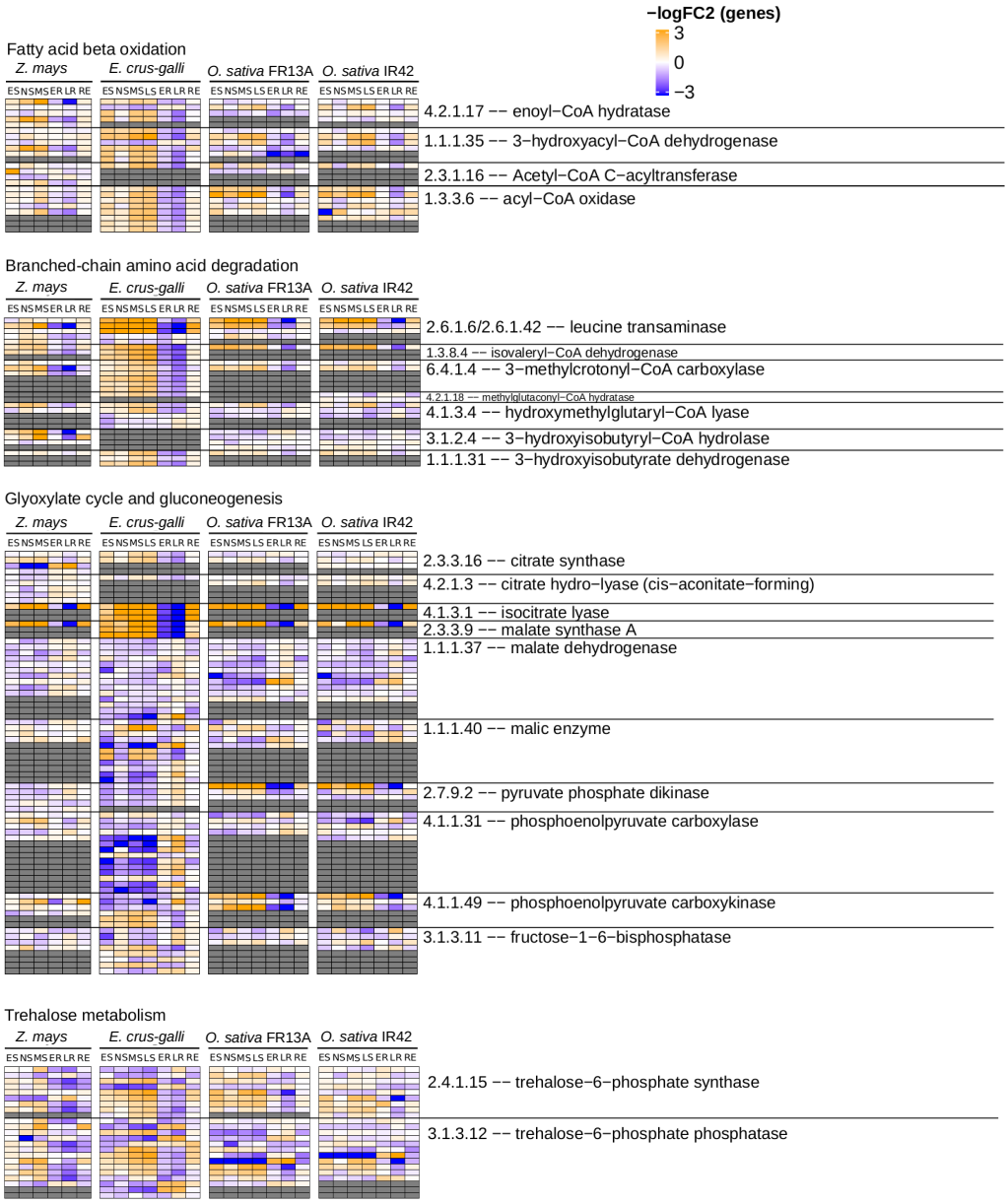


Figure s3.5: Heatmaps showing the regulation over time during submergence and recovery of sugars-related genes per subpathways.
Gene names and associated enzymatic reaction codes are shown next to the heatmap. One gene can have several copies, represented by individual horizontal rows next to a gene name. Orange represents up- and blue downregulation. Grey boxes are the gene copies that do not exist or lack of a significant regulation (FDR<0.001). Submergence and recovery timepoints are depicted in columns per species. ES= Early Submergence (4 hours), NS= Night Submergence (18 hours), MS= Mid Submergence (2 days), LS= Late Submergence (5 days) are the submergence timepoints compared to Pre-Submergence, and ER= Early Recovery (5 days Sub + 4 hours Reco) and LR= Late Recovery (5 days Sub + 1 day Reco) are the recovery timepoints compared to the end of the Submergence period (MS for maize, LS for the 3 others). RE= Recovery Efficiency is the comparison of the Late Recovery to the Pre-Submergence timepoint.

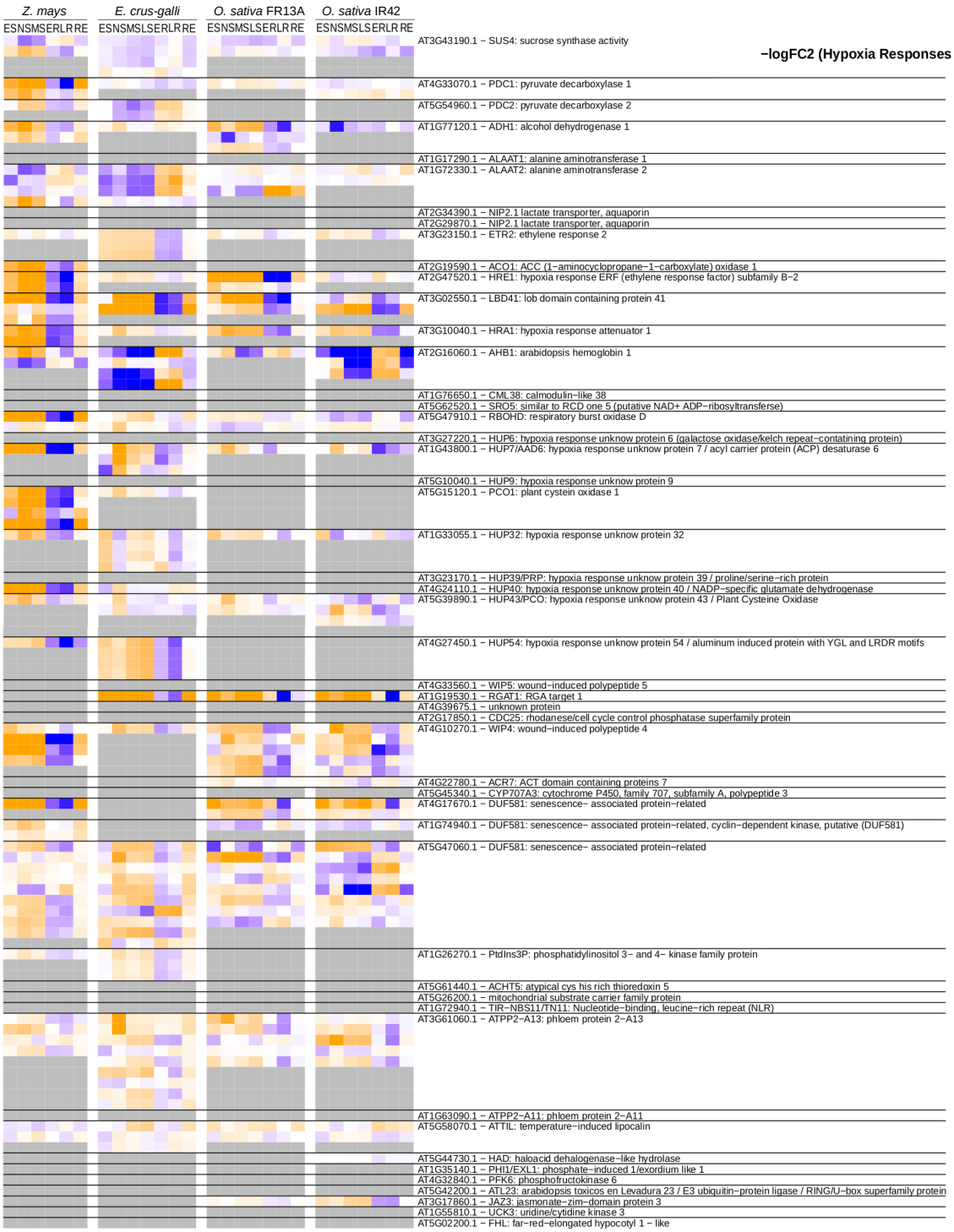
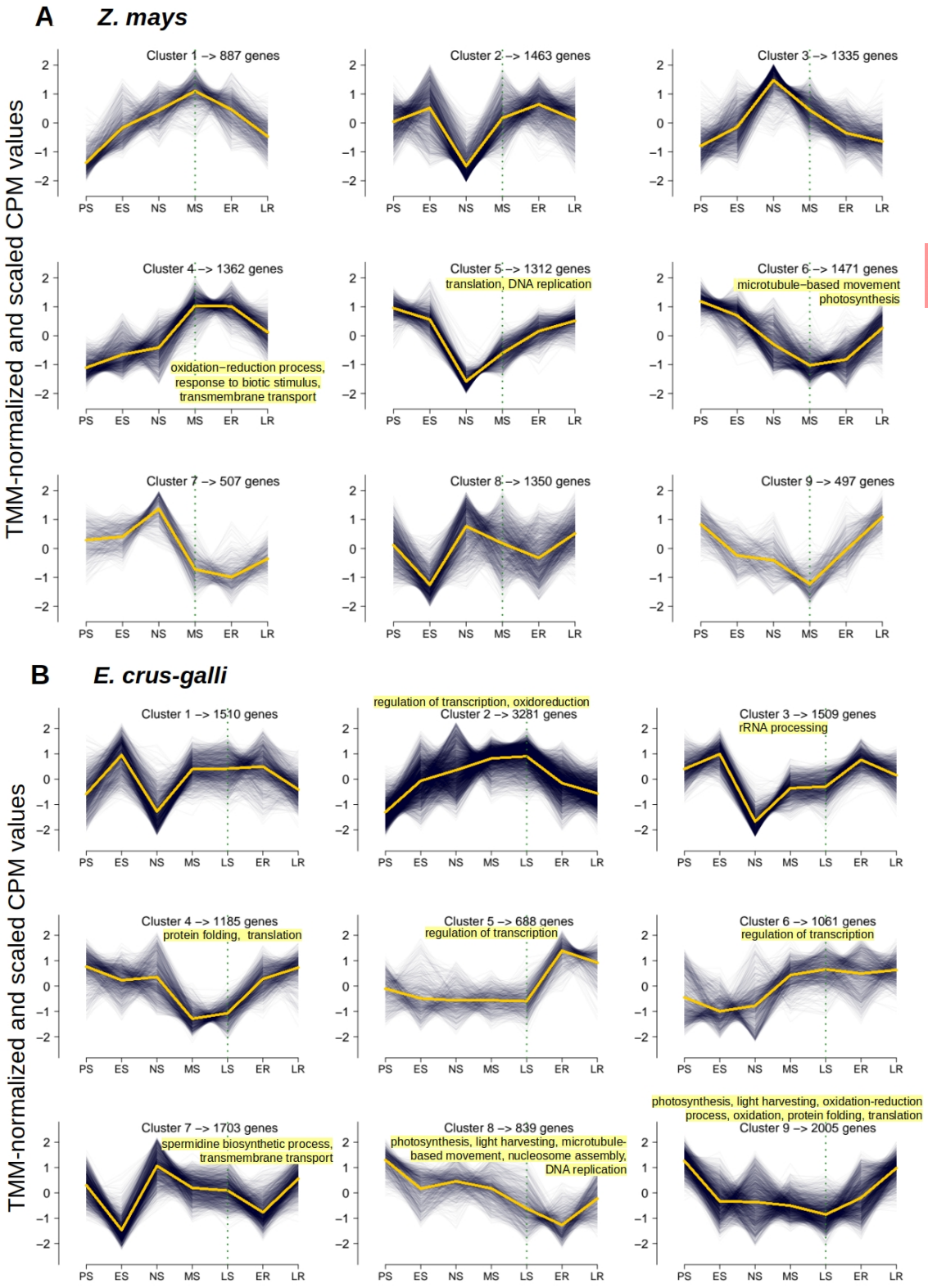

Z. mays
E. crus-galli
O. sativa FR13A
O. sativa IR42
ESNSMSLSERLRRE
−logFC2 (Hypoxia Responses Genes)
4
0
−4
AT3G43190.1 – SUS4: sucrose synthase activity
AT4G33070.1 – PDC1: pyruvate decarboxylase 1
AT5G54960.1 – PDC2: pyruvate decarboxylase 2
AT1G77120.1 – ADH1: alcohol dehydrogenase 1
AT1G17290.1 – ALAAT1: alanine aminotransferase 1
AT1G72330.1 – ALAAT2: alanine aminotransferase 2
AT2G34390.1 – NIP2.1 lactate transporter, aquaporin
AT2G29870.1 – NIP2.1 lactate transporter, aquaporin
AT3G23150.1 – ETR2: ethylene response 2
AT2G19590.1 – ACO1: ACC (1−aminocyclopropane−1−carboxylate) oxidase 1
AT2G47520.1 – HRE1: hypoxia response ERF (ethylene response factor) subfamily B−2
AT3G02550.1 – LBD41: lob domain containing protein 41
AT3G10040.1 – HRA1: hypoxia response attenuator 1
AT2G16060.1 – AHB1: arabidopsis hemoglobin 1
AT1G76650.1 – CML38: calmodulin−like 38
AT5G62520.1 – SRO5: similar to RCD one 5 (putative NAD+ ADP−ribosyltransferse)
AT5G47910.1 – RBOHD: respiratory burst oxidase D
AT3G27220.1 – HUP6: hypoxia response unknow protein 6 (galactose oxidase/kelch repeat−contatining protein)
AT1G43800.1 – HUP7/AAD6: hypoxia response unknow protein 7 / acyl carrier protein (ACP) desaturase 6
AT5G10040.1 – HUP9: hypoxia response unknow protein 9
AT5G15120.1 – PCO1: plant cystein oxidase 1
AT1G33055.1 – HUP32: hypoxia response unknow protein 32
AT3G23170.1 – HUP39/PRP: hypoxia response unknow protein 39 / proline/serine−rich protein
AT4G24110.1 – HUP40: hypoxia response unknow protein 40 / NADP−specific glutamate dehydrogenase
AT5G39890.1 – HUP43/PCO: hypoxia response unknow protein 43 / Plant Cysteine Oxidase
AT4G27450.1 – HUP54: hypoxia response unknow protein 54 / aluminum induced protein with YGL and LRDR motifs
AT4G33560.1 – WIP5: wound−induced polypeptide 5
AT1G19530.1 – RGAT1: RGA target 1
AT4G39675.1 – unknown protein
AT2G17850.1 – CDC25: rhodanese/cell cycle control phosphatase superfamily protein
AT4G10270.1 – WIP4: wound−induced polypeptide 4
AT4G22780.1 – ACR7: ACT domain containing proteins 7
AT5G45340.1 – CYP707A3: cytochrome P450, family 707, subfamily A, polypeptide 3
AT4G17670.1 – DUF581: senescence− associated protein−related
AT1G74940.1 – DUF581: senescence− associated protein−related, cyclin−dependent kinase, putative (DUF581)
AT5G47060.1 – DUF581: senescence− associated protein−related
AT1G26270.1 – PtdIns3P: phosphatidylinositol 3− and 4− kinase family protein
AT5G61440.1 – ACHT5: atypical cys his rich thioredoxin 5
AT5G26200.1 – mitochondrial substrate carrier family protein
AT1G72940.1 – TIR−NBS11/TN11: Nucleotide−binding, leucine−rich repeat (NLR)
AT3G61060.1 – ATPP2−A13: phloem protein 2−A13
AT1G63090.1 – ATPP2−A11: phloem protein 2−A11
AT5G58070.1 – ATTIL: temperature−induced lipocalin
AT5G44730.1 – HAD: haloacid dehalogenase−like hydrolase
AT1G35140.1 – PHI1/EXL1: phosphate−induced 1/exordium like 1
AT4G32840.1 – PFK6: phosphofructokinase 6
AT5G42200.1 – ATL23: arabidopsis toxicos en Levadura 23 / E3 ubiquitin−protein ligase / RING/U−box superfamily protein
AT3G17860.1 – JAZ3: jasmonate−zim−domain protein 3
AT1G55810.1 – UCK3: uridine/cytidine kinase 3
AT5G02200.1 – FHL: far−red−elongated hypocotyl 1 − like

Figure s3.6: Heatmaps showing the regulation over time during submergence and recovery of hypoxia-responsive genes.
Gene names are shown next to the heatmap. One gene can have several copies, represented by individual horizontal rows next to a gene name. Orange represents up and blue downnregulation. Grey boxes are the gene copies that do not exist or lack of a significant regulation (FDR<0.001). Submergence and recovery timepoints are depicted in columns per species. ES= Early Submergence (4 hours), NS= Night Submergence (18 hours), MS= Mid Submergence (2 days), LS= Late Submergence (5 days) are the submergence timepoints compared to Pre-Submergence, and ER= Early Recovery (5 days Sub + 4 hours Reco) and LR= Late Recovery (5 days Sub + 1 day Reco) are the recovery timepoints compared to the end of the Submergence period (MS for maize, LS for the 3 others). RE= Recovery Efficiency is the comparison of the Late Recovery to the Pre-Submergence timepoint.


A
Z. mays
TMM-normalized and scaled CPM values
Cluster 1 –> 887 genes
Cluster 2 –> 1463 genes
Cluster 3 –> 1335 genes
Cluster 4 –> 1362 genes
oxidation−reduction process, response to biotic stimulus, transmembrane transport
Cluster 5 –> 1312 genes
translation, DNA replication
Cluster 6 –> 1471 genes
microtubule−based movement
photosynthesis
Cluster 7 –> 507 genes
Cluster 8 –> 1350 genes
Cluster 9 –> 497 genes
PS ES NS MS ER LR
B
E. crus-galli
TMM-normalized and scaled CPM values
Cluster 1 –> 1510 genes
regulation of transcription, oxidoreduction
Cluster 2 –> 3281 genes
Cluster 3 –> 1509 genes
rRNA processing
Cluster 4 –> 1185 genes
protein folding, translation
Cluster 5 –> 688 genes
regulation of transcription
Cluster 6 –> 1061 genes
regulation of transcription
Cluster 7 –> 1703 genes
spermidine biosynthetic process, transmembrane transport
Cluster 8 –> 839 genes
photosynthesis, light harvesting, microtubule-based movement, nucleosome assembly, DNA replication
photosynthesis, light harvesting, oxidation-reduction process, oxidation, protein folding, translation
Cluster 9 –> 2005 genes
PS ES NS MS LS ER LR

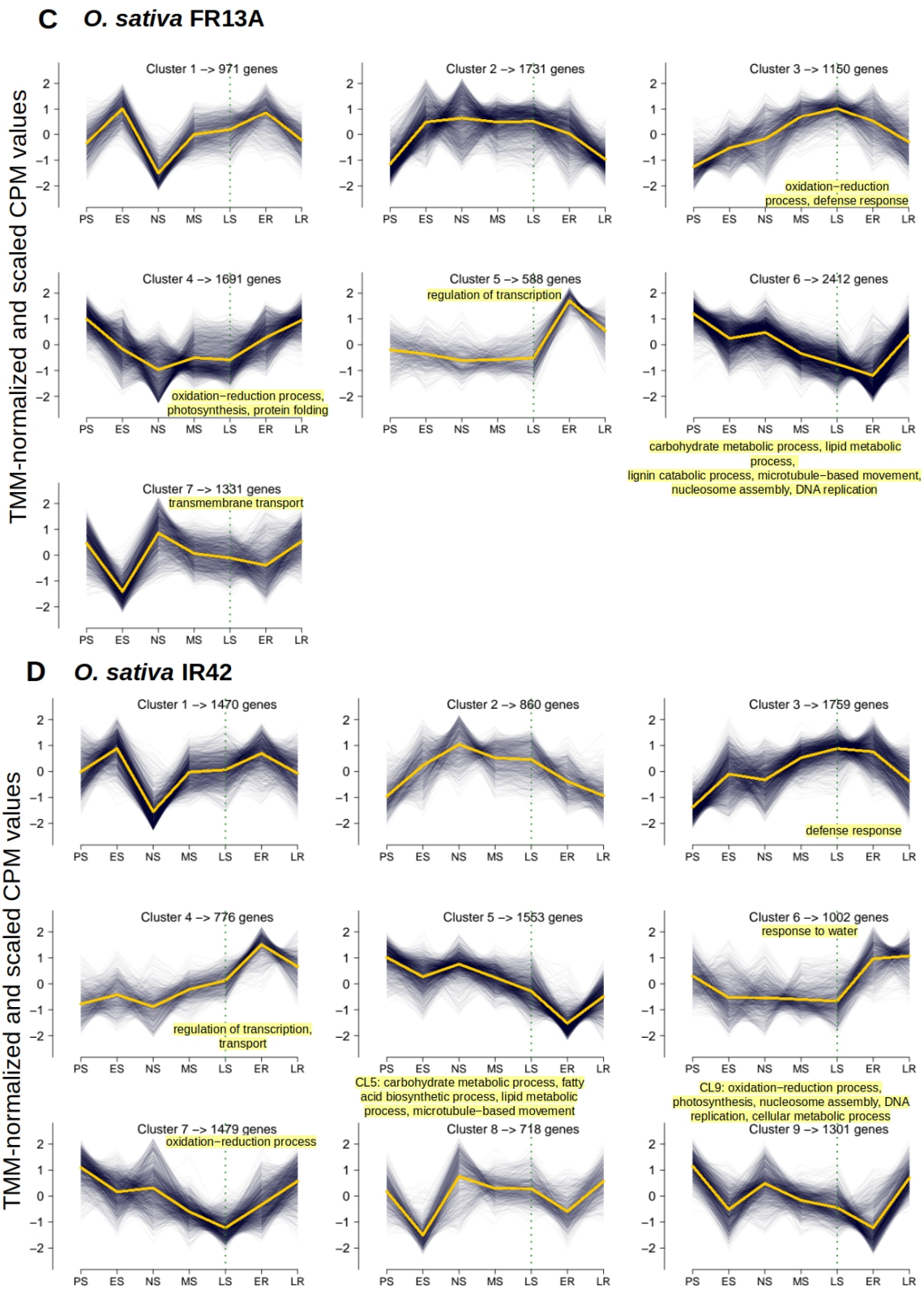

C O. sativa FR13A
TMM-normalized and scaled CPM values
Cluster 1 –> 971 genes
Cluster 2 –> 1731 genes
Cluster 3 –> 1150 genes
oxidation−reduction process, defense response
Cluster 4 –> 1691 genes
oxidation−reduction process, photosynthesis, protein folding
Cluster 5 –> 588 genes
regulation of transcription
Cluster 6 –> 2412 genes
carbohydrate metabolic process, lipid metabolic process, lignin catabolic process, microtubule−based movement, nucleosome assembly, DNA replication
Cluster 7 –> 1331 genes
transmembrane transport
PS ES NS MS LS ER LR
D O. sativa IR42
TMM-normalized and scaled CPM values
Cluster 1 –> 1470 genes
Cluster 2 –> 860 genes
Cluster 3 –> 1759 genes
defense response
Cluster 4 –> 776 genes
regulation of transcription, transport
Cluster 5 –> 1553 genes
CL5: carbohydrate metabolic process, fatty acid biosynthetic process, lipid metabolic process, microtubule−based movement
Cluster 6 –> 1002 genes
response to water
CL9: oxidation−reduction process, photosynthesis, nucleosome assembly, DNA replication, cellular metabolic process
Cluster 7 –> 1479 genes
oxidation−reduction process
Cluster 8 –> 718 genes
Cluster 9 –> 1301 genes
PS ES NS MS LS ER LR

Figure s3.7: Hierarchical clustering of all significant submergence responsive shoot DEGs over time, for (A) *Z. mays*, (B) *E. crus-galli*, (C) *O. sativa* FR13A and (D) *O. sativa* IR42.

Hierarchical clustering (distance method = "euclidean", hierarchical clustering method = "ward.D2"), on scaled significant DEGs (| log2FC | >1 and Padj. <0.001)) was used to visualise the regulation patterns of all DEGs that were significantly regulated in response to submergence. Individual gene patterns are plotted in grey. Orange lines indicate the mean expression level for all genes in a cluster. The number of genes per cluster is indicated above each panel. The x-axis represents the different timepoints when the tissue was harvested: PS= Pre-Submergence, ES= Early Submergence (4 hours), NS= Night Submergence (18 hours), MS= Mid Submergence (2 days), LS= Late Submergence (5 days), ER= Early Recovery (5 days Sub + 4 hours Reco), LR= Late Recovery (5 days Sub + 1 day Reco). The dashed green vertical bar represents the end of complete submergence and beginning of recovery. The GO categories of the most significant clusters (log10(GoCat)) are indicated. If no GO category is indicated, no important or no clear GO category can be associated.

Figure s3.8: Expression of recovery genes shared between *Echinochloa crus-galli* and *O. sativa* FR13A in shoots.
Heatmap showing the expression of the 53 common recovery genes identified in *E. crus-galli* and *O. sativa* FR13A at 4h (early) and 24h (late) recovery, in *E. crus-galli*, *O. sativa* FR13A, *O. sativa* IR42 and *Z. mays* B73. Orange represents up- and blue downregulation. The 4 columns represent the species in this order: M= *Z. mays*, E= *E. crus-galli*, F= *O. sativa* FR13A, O= *O. sativa* IR42.

Chapter 4

Identification of *Echinochloa crus-galli*-specific flooding tolerance genes via an orthogroup analysis

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Abstract

Echinochloa crus-galli is a highly flood-tolerant species displaying higher submergence recovery capacity compared to rice and maize. Such differences in tolerance might be attributed to the genetic composition of the species. However, a direct comparison of transcriptome responses to submergence did not reveal tolerance related differences. *E. crus-galli* has an allohexaploid genome, augmenting the chances for novel flooding tolerant genes to be present and expressed compared to the two diploids rice and maize. Accordingly, here we investigated whether species-specific gene composition and expression might explain the observed variation in flooding tolerance. To this end, we performed a multi-species orthology comparison based on gene sequences from 18 grass species, spread across two clades and five lineages. We described the orthogroup composition of each species, from commonly shared across the phylogeny tree to lineage-specific. Subsequently, submergence transcriptome data was reassessed in relation to the orthology composition of our four genotypes of interest using two different approaches. Both Venn Diagram and GLM revealed complementary information regarding the shared and unique flooding responses despite common orthogroup composition. Notably, the flooding responses are very phylogeny-dependent rather than tolerance dependent. There was minimal overlap in the flooding transcriptome responses between the tolerant *E. crus-galli* and tolerant *O. sativa* FR13A. Thus, the data gives an insight into a possible separated adaptation to flooding stress between *Echinochloa* and Oryzoideae lineages. A closer look at *Echinochloa* lineage-specific responses revealed that flooding tolerance in *E. crus-galli* might derive from a combination of shared responses and largely lineage-specific responses. The latter included several non-annotated genes that deserve further investigation as potential tolerance regulators. Understanding the function and role of these novel candidate flooding tolerance genes exclusively expressed in *E. crus-galli* could reveal new tolerance mechanisms and provide clues to the invasiveness of this weed.

Introduction

There is considerable variation in flooding tolerance in the plant kingdom. However, to date, we only have a limited understanding of what causes some species to be flood-tolerant and others intolerant, especially at a molecular physiological level. A major aim of this thesis is a molecular level characterisation of the notorious invasive weed *E. crus-galli* towards understanding its extreme flood tolerance. Whilst published work (Kennedy et al., 1980; Maun and Barrett, 1986; Moon et al., 1999; Estioko et al., 2014; Bajwa et al., 2015; Kraehmer et al., 2016) and the results presented in Chapter 2, confirmed the high flood resilience of *E. crus-galli,* the underlying molecular mechanisms are poorly understood. To address this knowledge gap, we used an mRNA sequencing approach (Chapter 3) to characterize the submergence-induced transcriptomic responses of *E. crus-galli* and compared them to that of two other Poaceae species (maize, rice).

This panel of genotypes spanned a wide tolerance range and had alternative physiological responses to submergence. The highly intolerant maize died within 3 days underwater, while the two rice varieties (FR13A and IR42) and the weed survived 15 days of complete submergence. Though *E. crus-galli* and *O. sativa* FR13A recovered strongly after submergence, during submergence their shoot development progressed or halted, respectively. This suggested that *O. sativa* FR13A and *E. crus-galli* achieve tolerance in different ways. Developmental suppression and the corresponding tolerance of *O. sativa* FR13A have been ascribed to the SUB1A locus (Xu et al., 2006, Fukao et al., 2006). The time-series expression analyses using mRNAseq identified many commonly regulated processes, but relatively few distinct species-specific responses. No clear *E. crus-galli*-specific tolerance trait was revealed, aside possibly from the catabolism of alternative resources (lipids/amino acids) and the stronger upregulation of *GIBBERELLIN 20-OXIDASE* compared to the other genotypes. *E. crus-galli* also exhibited the best recovery phenotype. Recovery vigour is the combined result of responses during submergence and acclimation following reaeration. We found that while *E. crus-galli* shared common recovery responses with rice, it also had 507 *E. crus-galli*-specific genes, which might play a key role in its higher tolerance to reoxygenation.

In the previous chapter, the analysis of the transcriptome data was focused on characterising the regulation of processes rather than on the regulation of individual genes during flooding (submergence and recovery). This approach is valuable in providing a general overview of transcriptomic behaviour and is a good proxy of physiological flooding and recovery responses. However, for direct comparisons between species, it is prone to miss key species-specific features due to poorly assigned GO categories or multiple viable BLAST hits. A key solution is to directly compare orthogroups (OGs). OGs encompass a set of genes from multiple species that are closely related as they are descended from a single gene in the

most recent common ancestor (Emms and Kelly, 2015). An OG thus only consists of orthologs between species, and in the case of lineage-specific gene duplications or polyploidy, also within species paralogs (Sonnhammer and Koonin, 2002).

OG inference and use in transcriptomic comparisons have been successfully utilized in closely related species pairs to identify genes responsible for environmental induced petiole elongation (van Veen et al., 2013; Gommers et al., 2017), and in the identification of core responses to a fungal pathogen and hypoxia across groups of species scattered among the diverse angiosperm lineages (Reynoso et al., 2019; Sucher et al., 2020). Here relatively few OGs were identified that contained orthologs for all species of interest. Instead, many genes were found in OGs that were specific to only one species. The relevance of those species-specific genes for environmental stress resilience is hard to judge. In our study, such species-specific genes could be several of the 507 *E. crus-galli* specific recovery genes (Chapter 3). Flood tolerance of *E. crus-galli* is not unique within its genus and likewise, relatives of *O. sativa* are considered flood-tolerant. The wide array of recently available grass genomes allows us to include several additional, closely related species to each of the four genotypes used in the mRNAseq here. This additional phylogenetic support will facilitate distinguishing species-specific gene anomalies from novel lineage-specific OGs. The latter could be crucial to indicate the importance of lineage-specific gene composition and behaviour and contrast it to the transcriptomic behaviour of conserved OGs.

Following the comparison of global transcriptomic responses between species in Chapter 3, here we conducted a comparison of flooding responses of conserved and lineage-specific orthologs. This chapter presents the results of the OG comparison between five lineages among the Poaceae, with three of them representing the genotypes studied by mRNAseq in the previous chapter. The aims of this chapter were (1) to determine whether flooding responses are conserved in all Poaceae species, are lineage-specific, or a combination of both and (2) to identify *Echinochloa crus-galli*-specific responses to flooding stress, leading to the description of candidate tolerance genes that could be interesting to study further. Our investigation uncovered that:

- Tolerance to flooding in *E. crus-galli* is a combination of (1) shared and common responses, (2) shared but specific responses (amino acid utilisation and transport, fatty acid degradation, growth and heavy metal transport and detoxification) and of (3) *Echinochloa* lineage-specific responses (more plant growth and development regulation, carbon usage management, aquaporin regulation and several antioxidative and detoxification responses).
- The two rice varieties have near identical transcriptome behaviour including those from lineage-specific OGs. The tolerance signature of FR13A relates to some differential growth processes and to potassium

ion homeostasis, not previously associated with SUB1A mediated tolerance.
- Transcriptomic behaviour of Poaceae-wide conserved orthologs is primarily related to phylogeny and shows few tolerance specific patterns.
- *O. sativa* FR13A and *E. crus-galli* possess distinct sets of orthologs that could point to their high tolerance, indicating independent evolution and mechanisms to survive underwater.
- The GLM approach coupled with an orthology comparison was a satisfying method to address *E. crus-galli* hexaploidy biases and to discover lineage-specific genes in both tolerant genotypes (*O. sativa* FR13A and *E. crus-galli)*.

Overall, our results present a new view on how flooding tolerance is achieved in the plant kingdom. Not only could we describe common responses between the four genotypes or by groups of genotypes in Chapters 3 and 4, but we also identify the importance of lineage-specific responses to flooding. Altogether, we conclude that part of flooding tolerance is shared, but part of it got acquired independently later, through adaptation to recurring submergence events.

Materials and methods

Methods described in Chapter 3

This chapter continues analyses of the dataset generated and described in Chapter 3. Subsequently, the methods regarding seed origin, germination and plant growth are as described in Chapter 3. Likewise for the submergence treatments, the harvest of plant material and the preparation of RNA samples for sequencing. Bioinformatics methods for RNAseq were identical, starting with quality trimming with cutadapt, read alignment with Kallisto and estimation of fold changes and calling differentially expression by gene per species using edgeR.

Transcriptome building of *E. colona* and *E. glabrescens*

A. Seed origins and germination

Echinochloa colona and *Echinochloa glabrescens* seeds were originally collected by the Weed Science team at the International Rice Research Institute (IRRI), Los Baños, The Philippines, in a lowland field (IRRI – UD2), within the period Sept-Nov 2016. Seeds were then further bulked 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 Utrecht in September 2018 and were kept in a dark and dry place. For germination, dehulled seeds were put to germinate for 4 days in Petri dishes between two wet Whatman™ papers in an incubator (12 h light (120+/-50 $\mu molm^{-2}s^{-1}$) 35°C / 12 h dark 25°C, 70% relative humidity).

B. Plant growth and treatments

Per pot, one seedling was transplanted at 4 days after sowing. Canopy pots were used (perforated Round Pots 6° Azalea – MXC 5,5 plastic pot of 5 cm diameter top, 3.5 cm diameter bottom, 5.5 cm depth, 78 ml) with a mixture of 50% black soil / 20% sand / 30% agra-vermiculite 0-1.5 mm + 20% Yoshida nutrient solution (Yoshida, 1976) with a double iron dose (sequestreen= Fe-EDTA), pH 6.5 + osmocote NPK-Mg 15-4-9 (+1) (2.4 g/L of soil). Seedlings grew in the greenhouse in a 12 h light (200+/-20 $\mu molm^{-2}s^{-1}$) 29+°C / 12 h dark 24+°C cycle conditions, with ventilation, in trays that were manually watered. Plants of both *Echinochloa* species were grown and harvested to build a reference transcriptome. In order to have plant material representing the most complete expressed complement of genes, per species, when the plants reached the 3-leaf stage (12 days), subsets were put in varying environmental conditions. These were: high light conditions (1000+/-60 $\mu mol.m^{-2}s^{-1}$ with a Red:Far Red ratio of 2.98) for 5 days; Low light conditions (320+/-10 $\mu mol.m^{-2}s^{-1}$ with a Red:Far Red ratio of 0.58) for 5 days; complete submergence for 4 days; 8 days without watering. A subset of plants was also left under control conditions and harvested after 4 days. Three to eight plants per condition were pooled together (shoot and root separately) in aluminium envelopes, directly frozen in liquid nitrogen, and stored at -80°C until RNA extraction (See methods in Chapter 3). An equal amount of total RNA coming from both shoot and root tissues per treatment were pooled for sequencing the two final samples.

C. Transcriptome building

RNAseq analyses were performed with the R version 3.6.3 combined with a mix of Bioconductor packages and standalone bioinformatics tools. For both *Echinochloa* species, low quality reads with low basecall quality scores or adapter contamination were first trimmed or removed with 'cutadapt' and the settings -e 0.07, --no-indels, --nextseq-trim=20 -m 30 (Martin, 2011). Of the processed reads, 52,220,654 and 52,278,976 reads were assembled with the Trinity program (Grabherr et al., 2011) for *E. colona* and *E. glabrescens* respectively. Kmer length was set to 31 with a minimal Kmer abundance of 1. Reads were mapped onto the assemblies by bowtie2 (Langmead and Salzberg, 2012) and transcript coverage was determined from the resulting BAM files with the concordant alignments only and the samtools coverage function. Expression abundance rank was determined by Kallisto read mapping and counting with default settings, and subsequently based on the calculated FPKM (Fragments per Kilobase transcript per million reads). Transcript length as a function of abundance rank was compared to RNAseq of *E. crus-galli* plants grown and harvested alongside *E. colona* and *E. glabrescens* but mapped to the *E. crus-galli* reference genome (*E. crus-galli* line STB08, collected from rice paddy fields in the lower Yangtze River region of China (30°17′ N, 119°57′ E), published in Guo et al., 2017) with Kallisto (Bray et al.,

2016). The Trinity program defines several isoforms (splice/sequence variants) hypothesized to represent a single gene. Only the longest isoform with a mean coverage of at least 2 was used for orthology analysis. Moreover, only genes with at least 12 reads were included.

Orthogroups comparison, composition analysis and responses

A. Analysis of the orthogroup composition of 18 Poaceae species

Genomes and the corresponding primary transcripts were sourced as described in Table s4.1. To identify Orthologous groups (OG), the Orthofinder2 method was used (Emms and Kelly, 2015; Emms and Kelly, 2019), but it was adapted to transcript sequences, rather than protein sequences to facilitate the inclusion of the *E. colona* and *E. glabrescens de novo* transcriptome assemblies. First, an all-vs-all BLAST was performed for the 18 species using a discontiguous megaBLAST and reporting alignments with Evalue< $1e^{-5}$ (Altschul et al., 1990). Subsequently, the orthofinder program normalized a BLAST score for alignment length, then for each gene kept only the BLAST scores higher than the minimum score of the reciprocally best BLAST hit with the other 17 species and applied the orthoMCL algorithm on the remaining BLAST graph network with an inflation factor of 2 to separate the OGs (Li et al., 2003). Based on the collective set of gene trees for the OGs, Orthofinder2 produced a species tree concordant with the known taxonomy (http://www.timetree.org).

OGs, gene composition and phylogeny were subsequently analyzed with R 3.6.3 and the R packages ape() for “Analyses of Phylogenetics and Evolution” and phytools() for “Phylogenetic Tools for Comparative Biology”. Distinct sets of OGs were determined as follows. For each OG, the number of genes from each species was counted to know the OG composition. OGs were regrouped in different categories:

1. OGs were considered conserved if they belonged to a minimum of eight (out of 18) species and having a minimum of one species represented per lineage (Pooideae, Oryzoideae, Paniceae – flood-tolerant, Paniceae – flood sensitive, Andropogoneae).

2. OGs were lineage-specific if at least one species of each individual sub-lineage is present and contained at least four (BOP), three (Pooideae), three (Oryzoideae), five (PACMAD), four (Paniceae), three (flood-tolerant Paniceae), three (flood sensitive Paniceae) or three (Andropogoneae) species.

3. OGs belong to the lineage lost categories if they are shared by all lineages but one, and are present in at least eight species, except for OGs missing in BOP (at least in five species) and PACMAD (at least in four species).

4. The remaining OGs that were represented by multiple species were considered unplaced.

OG data for the direct comparison between *E. crus-galli, O. sativa* and *Z. mays* was a subset from the conserved OGs of the 18 species analysis where any of these three species was present.

B. Transcriptomic responses of the conserved OGs in *Z. mays, E. crus-galli, O. sativa* FR13A and *O. sativa* IR42

Differentially expressed genes were selected and calculated as described in Chapter 3 (see: RNAseq analyses, 2. Differential expression analysis.). Genes/loci with |logFC|> 1 and FDR< 0.001 were selected and attributed to each OG shared between the three species. A homemade Venn Diagram R function was used to classify the differentially expressed OGs (DEOs) in different categories depending on if the OGs were expressed in only one, two, three or all genotypes and if they were upregulated or downregulated, this for each timepoint. In figure s4.3, a closer look at one category was proposed. The expression in time of the unique DEOs in *E. crus-galli* and *O. sativa* FR13A for at least one timepoint (both up- and downregulated categories) were plotted in a heatmap generated with the function ComplexHeatmap().

To measure the distance between each pair of species, a score for upregulation (1), for downregulation (-1) or for non-regulation (0) was first given. Then, the average score for each timepoint within each species and each OG was determined. Expression scores thus ranged from -1 where all genes in an OG were downregulated, to +1 where all were upregulated. Subsequently, the absolute difference in the regulation score between a species pair was determined for each timepoint within an OG and averaged per OG. Therefore, distances per OG for a species pair ranged from 0 (identical regulation for all timepoints) to 2 (opposite regulation for all timepoints).

C. OG responses in *Z. mays, E. crus-galli, O. sativa* FR13A and *O. sativa* IR42

To determine OG based expression values adhering to a continuous scale and provide an opportunity to be employed in a statistical model to obtain genotype*treatment effects, the read counts of individual OG members were summed. These summed read counts, provided a weighted expression average of all gene members which were determined in the negative binomial model with edgeR, analogous to the approach with single genes. To identify the OGs affected by flooding differently between species, per timepoint a full factorial model was implemented. Here OGs with a significant genotype*treatment interaction effect (FDR< 0.0001) for at least one timepoint were considered. Additionally, to pursue OGs specific to a lineage and genotype, OG expression was determined for all timepoints on a genotype basis.

OGs affected by flooding depending on their genotype were clustered. To this end expression data, where for each genotype the ‘pre-submergence’ values

(logFC) were set to zero, was first scaled across all species. Distances between OGs were based on the Euclidean approach with the dist() R function. Subsequent agglomerative hierarchical clustering used the Ward's minimum variance method; hclust() R function with 'ward.d2'.

Gene Ontology (GO) enrichment was performed with the bioconductor packages GO.db to retrieve GO descriptions and GOseq to assess enrichment of the GOterms. The analyses were done assuming a hypergeometric distribution. The most important GO terms were assessed manually for each cluster.

D. Lineage-specific OG responses to flooding stress

DEOs in the four genotypes were counted for each lineage-specific (PACMAD, Andropogoneae, Paniceae, *Echinochloa*, BOP, Oryzoideae) OGs and for the conserved OGs. Association between the direction of regulation and lineage specificity was assessed by a Fisher's-exact test. In figure 4.6, the red box represents the OGs highlighted in figure 4.7 (*Echinochloa* or flood-tolerant Paniceae lineage) and the green box represents the OGs highlighted in figure 4.8 (Orizoideae lineage). The OGs have been selected and the expression of *E. crus-galli* or of *O. sativa* FR13A and IR42 DEOs was plotted over time in heatmaps generated with the function ComplexHeatmap() after clustering with hclust(). For *E. crus-galli*, DEOs were separated depending if they were annotated (based on *O. sativa* blasting) or not. For the *O. sativa* heatmap, a combination of *O. sativa* Indica and *Arabidopsis* annotation was used.

Associated files

4a. Fold changes of *E. crus-galli*, *O. sativa* FR13A, *O. sativa* IR42 and *Z. mays* in response to submergence and recovery stress per gene with associated OG (Time series, 1 file per species) "Chapter3and4a_FC_FDR_OG_speciesX"
4b. OrthoFInder output file "Orthogroups"
4c. OrthoFInder output file "Unassigned Orthogroups"
4d. Fold changes of *E. crus-galli*, *O. sativa* FR13A, *O. sativa* IR42 and *Z. mays* in response to submergence and recovery stress per OG "Chapter4d_logFCperOG_speciesX"
4e. Clustering and GO enrichment analyses per OG "GOenrich_byClust"

Results and Discussion

Poaceae restricted analysis allows for high discrimination of orthologs

To reliably identify OGs, we selected three to four species per phylogenetic lineage, providing a relatively balanced representation of the grass-like Poaceae (Fig. 4.1A). The Poaceae family, which includes the four genotypes studied here, contains two major clades. Here the BOP (Bambusoideae; Oryzoideae; Pooideae)

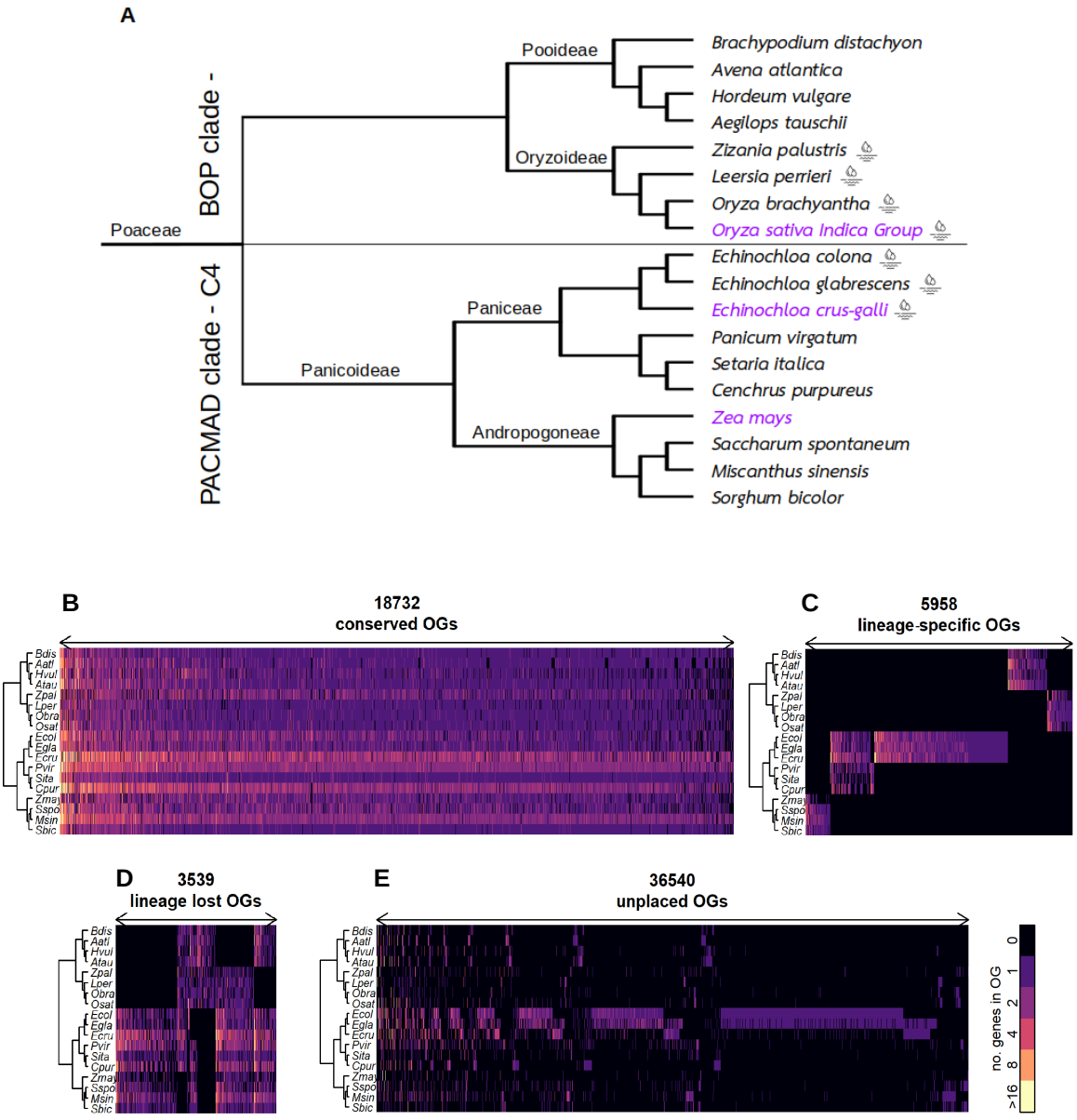
A
BOP clade - Poaceae
PACMAD clade - C4
Pooideae
Oryzoideae
Panicoideae
Paniceae
Andropogoneae
Brachypodium distachyon
Avena atlantica
Hordeum vulgare
Aegilops tauschii
Zizania palustris
Leersia perrieri
Oryza brachyantha
Oryza sativa Indica Group
Echinochloa colona
Echinochloa glabrescens
Echinochloa crus-galli
Panicum virgatum
Setaria italica
Cenchrus purpureus
Zea mays
Saccharum spontaneum
Miscanthus sinensis
Sorghum bicolor
B
18732
conserved OGs
C
5958
lineage-specific OGs
D
3539
lineage lost OGs
E
36540
unplaced OGs
Bdis
Aatl
Hvul
Atau
Zpal
Lper
Obra
Osat
Ecol
Egla
Ecru
Pvir
Sita
Cpur
Zmay
Sspo
Msin
Sbic
no. genes in OG
0
1
2
4
8
>16

Figure 4.1: Evolutionary relationships and gene conservation between 18 species spanning the Poaceae.
A. Phylogenetic tree showing the two clades (PACMAD and BOP) and relatedness between the species of the Poaceae (grasses) family, with the indication of tolerance to submergence ( ) identified in the concerned species. Distances and lengths of the lines are not representative of evolutionary time but do represent the genetic relatedness between species published in the literature. RNAseq-studied species are highlighted in purple.
B. Orthogroups (OGs) shared between the 18 species (conserved OGs). OGs were considered conserved if they belonged to a minimum of eight species and having minimum one species represented per lineage (Pooideae, Oryzoideae, Paniceae – flood tolerant, Paniceae – flood sensitive, Andropogoneae).
C. Orthogroups that are lineage-specific, where at least one species of each individual sub-lineage is present and contain at least four (BOP), three (Pooideae), three (Oryzoideae), five (PACMAD), four (Paniceae), three (flood tolerant Paniceae), three (flood sensitive Paniceae) or three (Andropogoneae) species.
D. Orthogroups that are shared by all lineages but one, where representatives of lineages defined in **B** must be present. The shown OGs are present in at least eight species, except for OGs missing in BOP (at least in five species) and PACMAD (at least in four species).
E. Orthogroups that are neither conserved between species (**B**), nor lineage-specific (**C&D**). Here only OGs are shown that are shared by at least two species. Species specific OG counts are in Supl. Figure s4.2A.
For **B-E**, each vertical line represents one OG, and the cell color indicates the number of genes per OG (black= no genes, to yellow= more than 16 genes in the OG).

clade covers only C3 species and the PACMAD (Panicoideae; Arundinoideae; Chloridoideae; Micrairoideae; Aristidoideae; Danthonioideae) clade only C4 species. Within the BOP, the lineage of Oryzoideae includes the rice *Oryza sativa* Indica Group and three additional Oryzoideae relatives species and is predominately flood-tolerant. The inclusion of four BOP species from the Pooideae tribe provides an opportunity to identify genes specifically unique to the flood-tolerant Oryzoideae lineage. Analogously, in the PACMAD clade, maize is part of the Andropogoneae, surrounded by three other sensitive species, whereas *E. crus-galli* is part of the Paniceae lineage. Most Paniceae species are flooding intolerant, thus, two other species of *Echinochloa* were added to confirm gene presence in the genus members of *E. crus-galli*-specific flooding-responsive genes. Flooding tolerance is found in two distinct lineages of the phylogenetic tree, namely the Paniceae - Tolerant (*Echinochloa*) and the Oryzoideae (Indicated by a flooding sign in Fig. 4.1A). This suggests that this tolerance (or part of it) derives from independent adaptive traits. Monocot grass species are mainly intolerant to flooding. However, rice is able to grow in flooded conditions since its domestication from the aquatic wild rice *O. rufipogon* around 10,000 years ago (Gross and Zhao, 2014; Stein et al., 2018; Qiu et al., 2019). *Echinochloa* belongs to the Paniceae lineage which are typically dry-land grasses. *E. crus-galli* presence has been increasingly reported in paddies and other wetlands around the globe for decades

(Leeson et al., 2005) and could have evolved flooding tolerance in another way than rice did, via new adaptive traits and molecular responses. We will attempt to answer this supposition in this chapter.

An array of recent genome sequencing projects made many grass genomes publicly available, partially guiding our choice of species (Table s4.1). For *Echinochloa spp.*, only the genome of *E. crus-galli* was available. To get a fast estimate of gene content, we therefore assembled the transcriptomes of *E. colona* and *E. glabrescens* from material harvested from an array of tissue (entire four leaf-stage plants) and conditions (complete submergence, drought, high light, low light, and normal conditions of light and watering). Approximately 150,000 transcripts were identified for each species with a mean coverage of at least two (Fig. s4.1A and B). Completeness of the assembly was assessed by comparing the distribution of transcript lengths of expression cohorts to the transcript lengths of the genome of *E. crus-galli* (Fig. s4.1C). This indicates a close to full length assembly for the 50,000 most expressed genes. After the top 90,000 expressed transcripts, assembly length becomes extremely short, and these could reflect artifacts in the assembly. Interestingly, *E. crus-galli* contains 108,771 genes (Guo et al., 2017), close to the 90,000 figure after which assembly quality drops. Transcripts with a mean coverage of two and at least 12 reads per kilobase were included in the OG analysis (Fig. S4.1A).

With the species spanning the distinct lineages of the Poaceae (Fig. 4.1A), we identified OGs with OrthoFinder2 (Emms and Kelly, 2015; Emms and Kelly, 2019). For 70-90% of the genes in each species, an ortholog was found in at least one other species. For 3-20% of the genes, no orthologs in another species were assigned, but paralog(s) were identified within the genome of the species. For 2-13% of genes, neither orthologs nor paralogs were found (Fig. s4.2A). The majority of OGs contained genes from either a very few species or had a representative from the majority of species (Fig. s4.1B). We defined 18,732 OGs that were present in at least eight species and in all lineages (conserved OGs). Most conserved OGs were represented by one gene only. However, some species tend to be represented by two or three genes per OG (Fig. 4.1B). Species having more genes per OG were mainly in the Paniceae group, except for *S. italica*, and were all multiploid species (> diploid) (Table s4.2). The diploid *M. sinensis* also contained many genes per OG, but *M. sinensis* does contain a higher number of chromosomes and has a high, probably duplicated, gene count (Fig. s4.2A, Table s4.2).

Though species-specific genes were highly abundant, a lot were shared across multiple species (Fig. s4.2A), so we could make lineage-specific groups, which counted for an elevated amount of shared OGs (5,958) between phylolineages (Fig. 4.1C). Likewise, we could also determine 3,539 OGs lost in (a)

lineage(s) (Fig. 4.1D). The prevalence of lineage-specific OGs was also apparent from comparing pairwise shared OGs between species. This clearly demonstrated enhanced overlap within the 5 lineages explored here (Fig. S4.2C). Most of the 36,540 unplaced OGs were more abundant in the *Echinochloa* species (Fig. 4.1E) and could indicate an artifact of the assembly. Likewise, *Echinochloa* species also possessed the most abundant lineage-specific OGs (Fig. 4.1C). Those could also be artifacts of the transcriptome assemblies, but could nonetheless contain interesting novel flooding tolerance genes and will be inspected further here.

Overall, our analysis on these Poaceae species identified many small OGs and we observed a good identification of the lineage specificity. This is in contrast with many other comparative studies (van Veen et al., 2013; Gommers et al., 2017; Reynoso et al., 2019; Sucher et al., 2020). This can be attributed to the lack of well-sequenced and well-annotated genomes in the past, as well as the lack of accurate orthology tools which are now being improved. The update of orthology tools to include length biases and to facilitate multiple species comparisons proves helpful for applying OG identification on restricted or custom sets of species (Emms and Kelly, 2019), such as the Poaceae here. Additionally, the increased availability and relative ease of transcriptome assemblies create the possibility to define lineage-specific genes more robustly and accurately.

Conserved OGs show little overlap in flood responses between the four genotypes, rather transcriptome behaviour is associated with phylogenetic lineage

To find an accurate representative transcriptomic response for an OG, fewer paralogs are better. To explore the implication of OG structure and size on transcriptomic comparisons, we highlighted the OG relationships between *Z. mays*, *O. sativa* and *E. crus-galli*. The high amount of conserved OGs is also apparent from a shared set of 16,012 OGs between them (Fig. 4.2A). Notably, most OGs are still restricted to one of the three species (*Z. mays*: 3,798, *O. sativa*: 7,835, *E. crus-galli*: 22,379). Nonetheless, the conserved OGs are of central relevance to directly compare the 4 genotypes. For the vast majority of conserved OGs, rice and maize are represented with a single gene. In contrast, *E. crus-galli* is mostly represented by 3 distinct genes (Fig. 4.2B). *E. crus-galli* is hexaploid and thus contains multiple sets of chromosomes in excess in comparison with maize and rice, which are both diploid species. It means that overall, the comparisons of transcriptomic regulation would compare one *O. sativa* gene to one *Z. mays* gene to three *E. crus-galli* genes. This analysis includes only these three species.

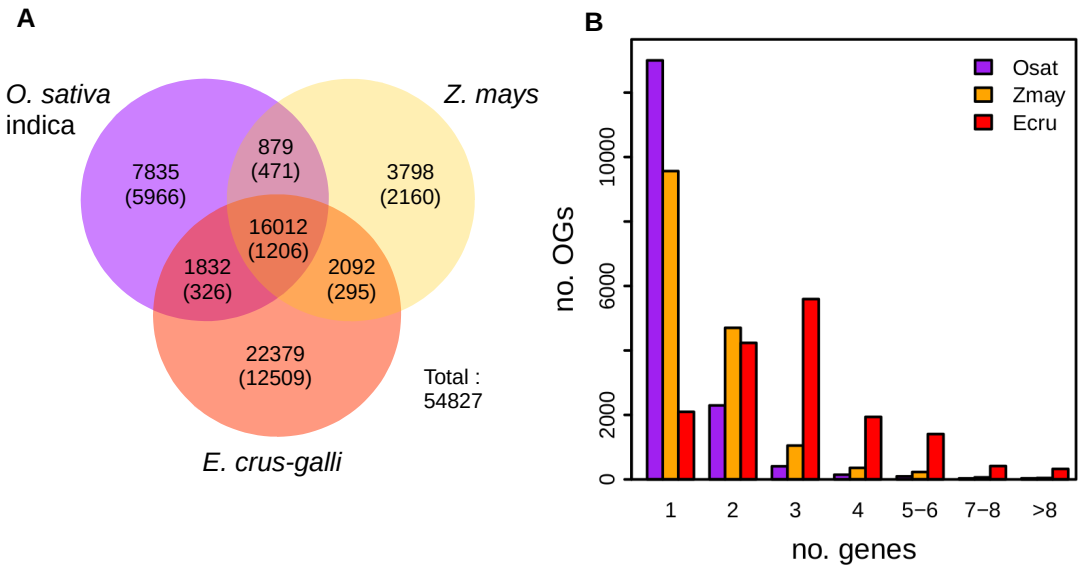

Figure 4.2: Gene and orthogroup composition of *Oryza sativa* (Osat), *Zea mays* (Zmay) and *Echinochloa crus-galli* (Ecru).
A. Venn Diagram highlighting shared orthogroups (OGs) of each species that was used for mRNAseq. The total number of OGs is indicated together with the number of OGs composed of one gene copy per species only (in brackets). All OGs identifed are included here, these are the multispecies, single species and genes placed in an OG by themselves.
B. Histogram of OG size (no. Genes) per species.

The OGs with distinct behaviour in the tolerant genotypes or showing a strong conserved response could provide clues regarding the mechanisms underlying flooding tolerance. We therefore characterized for each genotype and timepoint whether an OG was up- or downregulated (defined as Differentially Expressed OGs; DEOs) given at least one gene in the OG showed a response (| log2FC|> 1, FDR< 0.001). This allowed us to assess per timepoint the overlap in OG flooding responses. We detected more genotype-specific DEOs (Fig. 4.3A-B) than DEOs shared by all species (Fig. 4.3C-H). In general, more downregulation was observed during submergence and more upregulation was observed during recovery. The few conserved responses are the DEOs shared between the 4 genotypes (Fig. 4.3G-H) and could be genes related to hormones, sugar metabolism, hypoxia response and dehydration responses described in Chapter 3. Based on the number of DEOs, *Z. mays* is the least responsive species both at the early submergence and the early recovery timepoints (ES and ER), corresponding

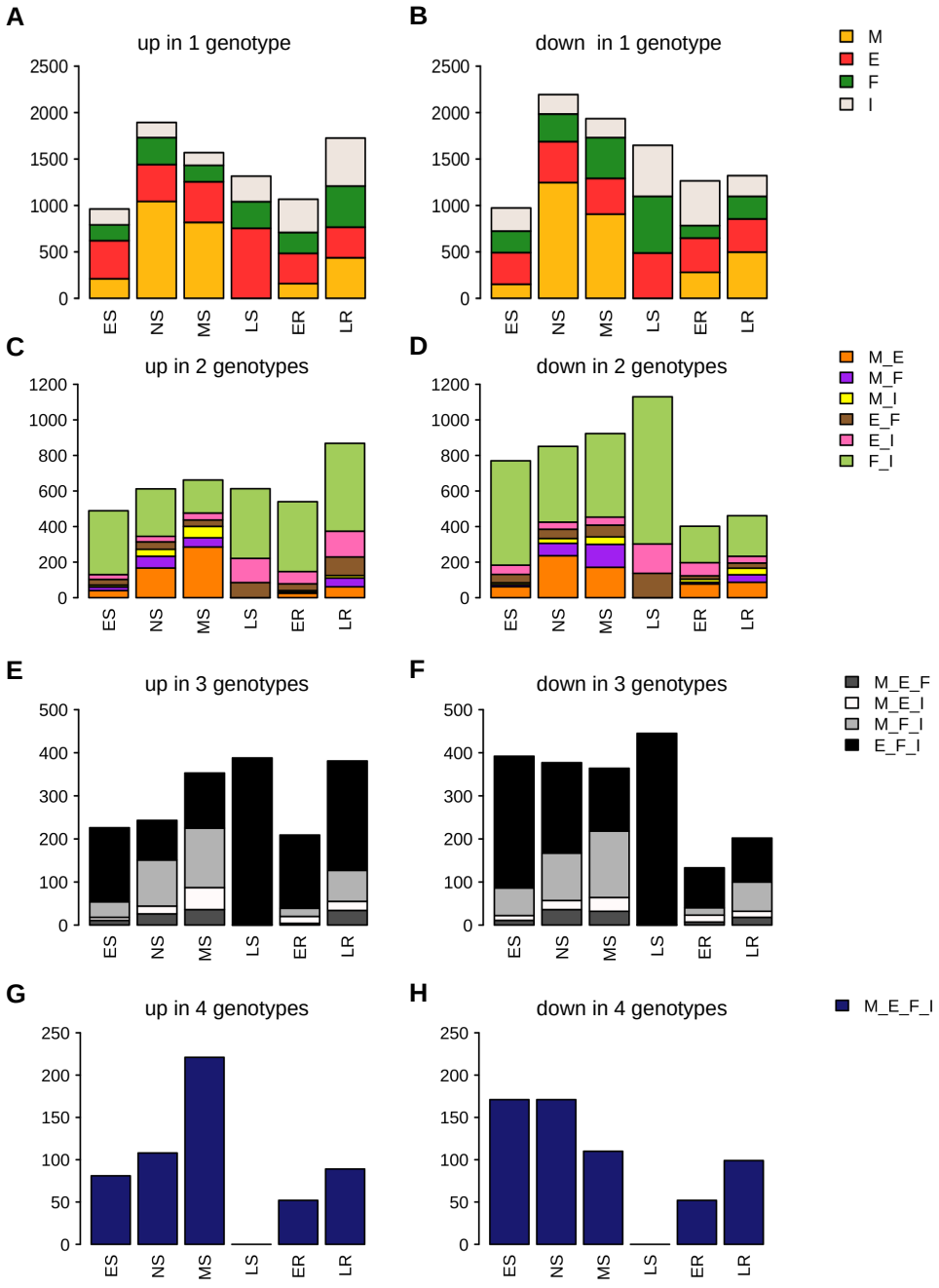
A
up in 1 genotype
B
down in 1 genotype
M
E
F
I
C
up in 2 genotypes
D
down in 2 genotypes
M_E
M_F
M_I
E_F
E_I
F_I
E
up in 3 genotypes
F
down in 3 genotypes
M_E_F
M_E_I
M_F_I
E_F_I
G
up in 4 genotypes
H
down in 4 genotypes
M_E_F_I
ES
NS
MS
LS
ER
LR

Figure 4.3: Specific and shared differentially expressed orthogroups in *Z. mays (M), E. crus-galli (E), O. sativa* FR13A (F) and *O. sativa* IR42 (I).
The number and overlap of Differentially Expressed Orthogroups (DEOs). A DEO contains at least one gene with |logFC|> 1 and FDR< 0.001 for the corresponding submergence or recovery timepoint and genotype. Analysis for upregulation is shown on the left, downregulation is on the right. Submergence and recovery timepoints are depicted in columns. ES= Early Submergence (4 hours), NS= Night Submergence (18 hours), MS= Mid Submergence (2 days), LS= Late Submergence (5 days), ER= Early Recovery (5 days Sub + 4 hours Reco), LR= Late Recovery (5 days Sub + 1 day Reco).
A-B. Orthogroups specific to each genotype that are (**A**) up- or (**B**) downregulated in time. **C-D.** Orthogroups shared between 2 genotypes that are (**C**) up- or (**D**) downregulated in time. **E-F.** Orthogroups shared between 3 genotypes that are (**E**) up- or (**F**) downregulated in time. **G-H.** Orthogroups shared between the 4 genotypes that are (**G**) up- or (**H**) downregulated in time.

to delayed stress perception in these phases, and a delayed response. This also correlates with Chapter 3 observations.

Interestingly, DEOs are mainly clustered in C3/BOP and C4/PACMAD (phylogeny) groups. Nonetheless, the shared DEOs between *E. crus-galli* and *O. sativa* FR13A observed in figure 4.3C-D could represent shared responses associated with flooding tolerance. The behaviour of these shared DEOs across the different timepoints, and in all the four genotypes was visualized in a heatmap (Fig. s4.3) to detect any potentially distinct patterns of flooding responses. Despite the selection based on the E_F cluster, the two rice varieties showed a highly similar pattern of regulation. The relatedness between the two C4 genotypes (*Z. mays, E. crus-galli*) and the two rice varieties was very evident. However, this pattern could be the consequence of OG selection based on a single timepoint. This can be different for each gene, while shared tolerance could be based on gene behaviour at one or several timepoints. Additionally, OGs with many gene representatives are biased to show regulation of their most variable member, leading to biased FC estimates for an OG.

Even if the Venn Diagram approach poorly pulls out key OGs of interest, it excellently shows large scale patterns in the data, such as the low conservation and high species specificity. We indeed observed a significant overlap of OG responses between the two rice varieties, but we wanted to determine the extent of similarity between the genotypes. For this, we calculated the (dis)similarity in transcriptional regulation between pairs of genotypes for each OG (see methods and Fig. 4.4 legend). We found that most distances of the shared OGs between pairs of genotypes are low (score= 0); Fig. 4.4A-D). This confirmed that in general, the flooding responses of the four genotypes are similar, or restricted to few timepoints. However, distances between *Z. mays* and *E. crus-galli* tended to be

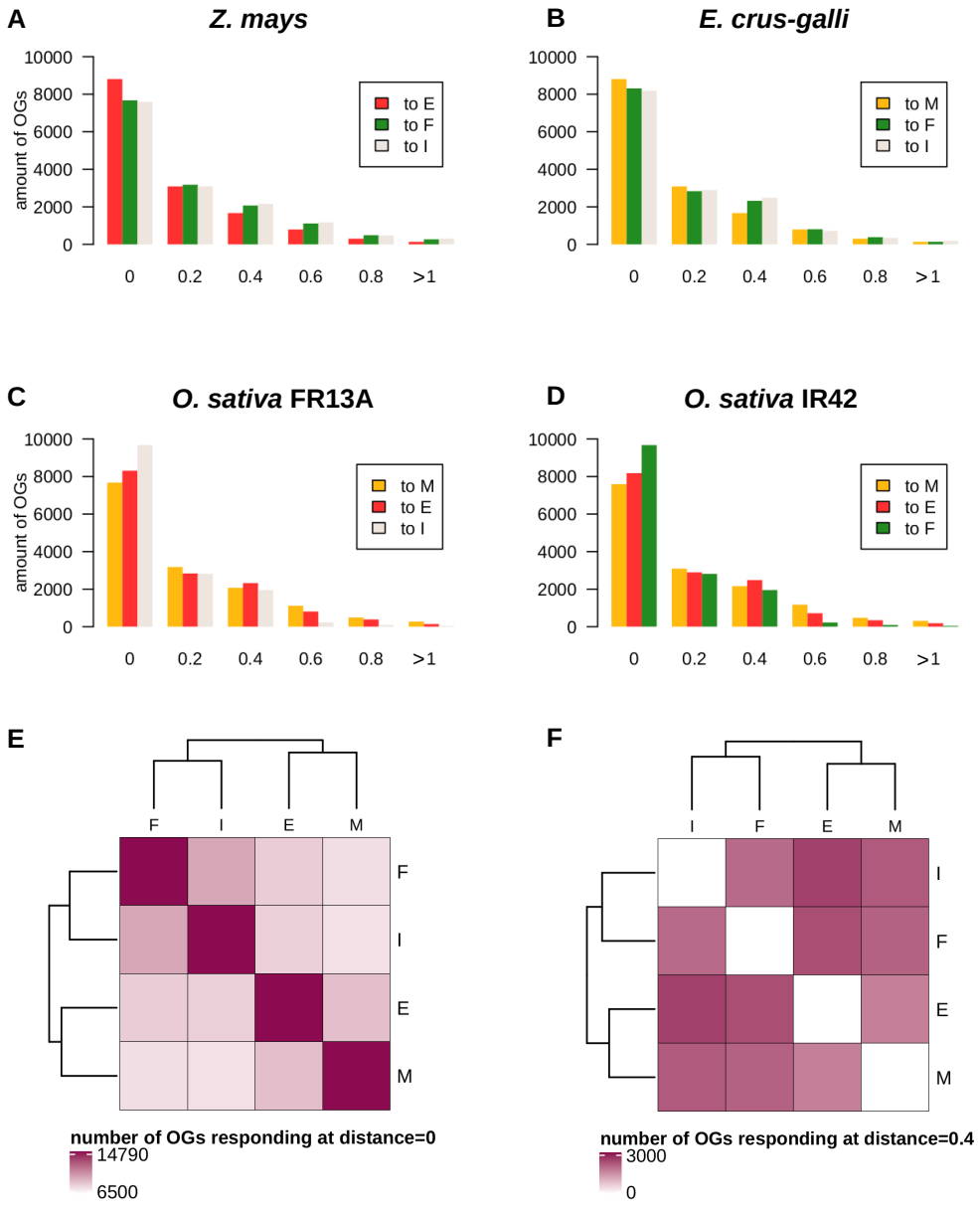

Figure 4.4: Distances and similarities between *Z. mays* (M), *E. crus-galli* (E), *O. sativa* FR13A (F) and *O. sativa* IR42 (I).
A-D. Histograms of the differences in OG regulation between pairs of genotypes, where the amount of OGs is shown as a function of the distance between that species and the species of reference. Because OGs can contain only one or several genes, a score for upregulation (1), for downregulation (-1) or for non-regulation (0) was given for each gene (|logFC|> 1, FDR< 0001), and the average score over the timepoints was given to each OG. Subsequently, the absolute difference in regulation score for each timepoint per OG was determined for each species pair. The resulting average distance thus ranges from 0 (identical regulation for all timepoints) to 2 (opposite regulation for all timepoints).
E-F. Matrices of the amount of OGs responding to flooding among the shared OGs at two given distances between species (**E**: distance of 0; **F**: distance of 0.4).

smaller than compared to *O. sativa*. Vice versa, the *O. sativa* genotypes were more similar and more distant to the PACMAD species (Fig. 4.4A-D). Clustering of similarity (number of OGs with a low distance) or dissimilarity (number of OGs with a high distance) between species pairs reiterated that *Z. mays* and *E. crus-galli* cluster together, and *O. sativa* FR13A clusters with *O. sativa* IR42 (Fig. 4.4 E-F), indicating that the responses to flooding depend on the phylogeny rather than on the tolerance to flooding stress for these genotypes.

Species-specific transcript responses indicate distinct and independent development of tolerance mechanisms in *E. crus-galli* and *O. sativa* FR13A related to alternative resource use and potassium transport respectively

Intersecting DEOs from genotypes and timepoints showed overall patterns in transcriptome behaviour but proved a poor approach to pinpoint key transcripts that could explain tolerance. A key factor here are OGs that just miss the cut-off or meet the cut-off for a few timepoints only. Given that OGs contain genes that are functionally highly similar, we summed the read counts of the individual OG members to produce a weighted mean response of an OG. These count data were fitted to a negative binomial model with a genotype, a treatment, and the interaction effect (Robinson and Oshlack, 2010). This allowed us to effectively retrieve all OGs with a strongly significant treatment*genotype interaction (FDR< 0.0001) for at least one timepoint (9,637 OGs retrieved out of the 16,013 shared OGs), and subsequently cluster and visualize their magnitude of regulation along a continuous expression scale (Fig. 4.5).

The two rice genotypes *O. sativa* FR13A and IR42 showed near identical expression patterns although they are respectively submergence tolerant and intolerant genotypes. *E. crus-galli* and *O. sativa* FR13A, despite being both tolerant genotypes, exhibited a completely different regulation pattern for most clusters, reinforcing the hypothesis that part of their tolerance to flooding evolved separately and is of a different nature. *E. crus-galli* regulation had a closer resemblance to flood sensitive maize than the two rice genotypes, even if only few common responses could be observed between *Z. mays* and *E. crus-galli* and many other clusters behaved differently. In general, the results from this approach echo our previous findings (Fig. 4.3 and 4.4) and illustrate very well that the responses of the shared OGs are mainly phylogenetic-specific responses (*Z. mays, E. crus-galli, O. sativa*).

Surprisingly, *Z. mays* had three downregulated and two upregulated clusters in the night timepoint that *E. crus-galli, O. sativa* FR13A and *O. sativa* IR42 do not regulate. The night timepoint (NS) seems the most separating factor between genotypes raising the question of the importance of night-time regulation for stress tolerance. For that particular timepoint, *Z. mays* started to show signs of

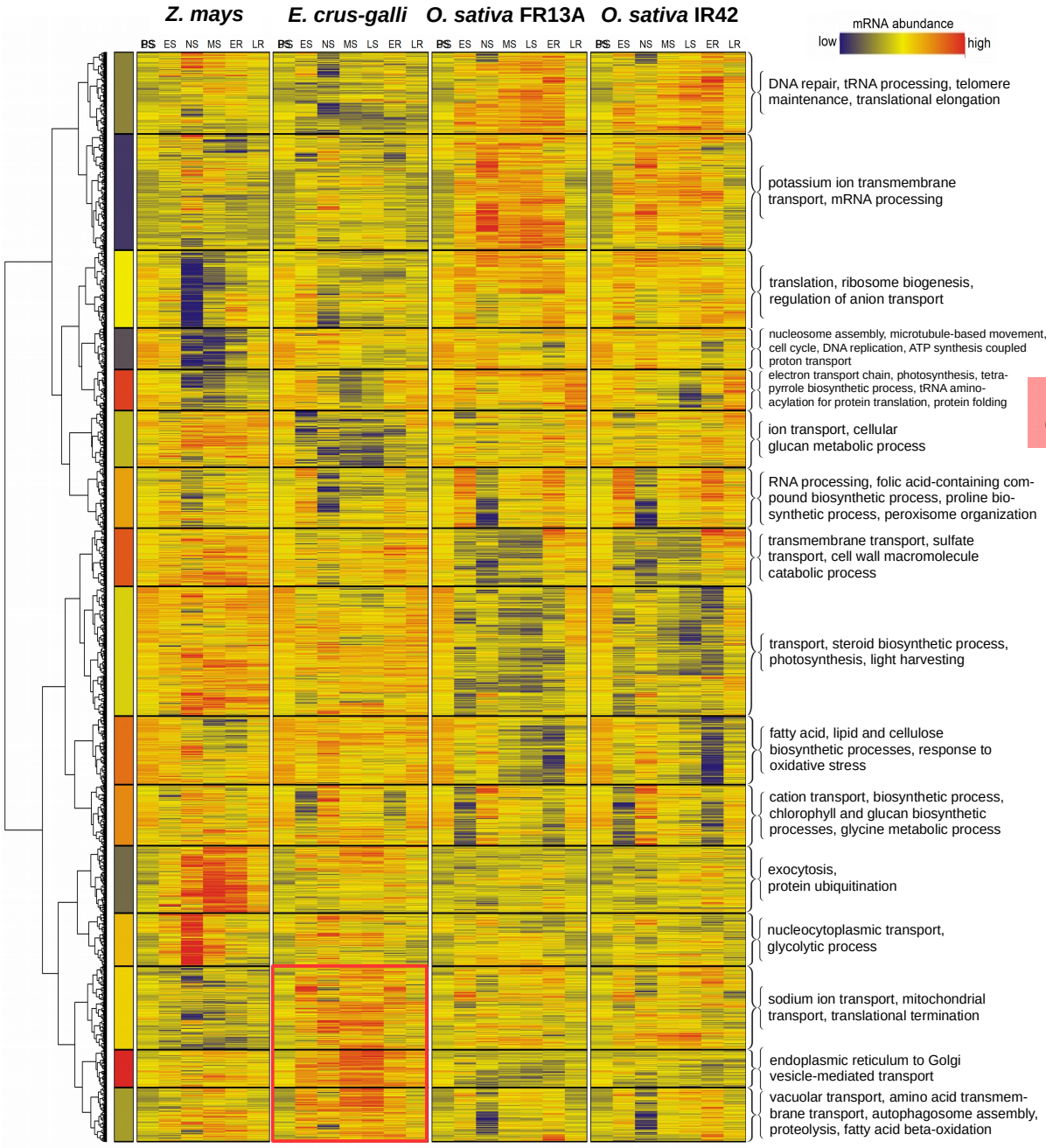

Figure 4.5: Transcriptional response of conserved OGs in *Z. mays, E. crus-galli, O. sativa* FR13A and *O. sativa* IR42, with significant treatment*genotype interactions.
Expression of the shared OGs per submergence and recovery timepoints for the four genotypes. OGs with a strongly significant treatment*genotype interaction (FDR< 0.0001) for at least one timepoint are shown. The data are logFCs scaled across all genotypes. Red represents high transcript abundance and blue low transcript abundance. Submergence and recovery timepoints are depicted in columns. PS= Pre-Submergence, ES= Early Submergence (4 hours), NS= Night Submergence (18 hours), MS= Mid Submergence (2 days), LS= Late Submergence (5 days), ER= Early Recovery (5 days Sub + 4 hours Reco), LR= Late Recovery (5 days Sub + 1 day Reco). Hierarchical clustering (euclidean distance and ward agglomeration method) and cluster division are shown on the left side. GO enrichment for each cluster was analyzed and the main significant categories are indicated on the right side. The specific *E. crus-galli* over-expressed OGs are indicated in the red box.

downregulating protein biosynthesis (“translation”, “protein folding”) and cell division and DNA replication (“microtubule-based movement”, “cell cycle”, “DNA replication”, “nucleosome assembly”). It seems to initiate protein degradation via the upregulation of the GOcat “protein ubiquitination”. Lastly, it also seems to catabolise sugars via the strong upregulation of the “glycolytic process”. This could reflect its slow start to express genes to acclimatize to carbon and energy limiting conditions associated with flooding. Subsequently, when night-time arrives, *Z. mays* finds itself in a severe energy crisis. Another NS cluster that was clearly regulated by *E. crus-galli, O. sativa* FR13A and *O. sativa* IR42, but only mildly in *Z. mays*, could be regulated by the circadian clock and other day/night cues, given the categories: “chlorophyll”, starch (“glucan biosynthetic processes”) and “glycine metabolic process” which are often associated with photorespiration (Timm and Hagemann, 2020).

Despite the high degree of similarity in responses between the two rice varieties, two main differences were noted. First, *O. sativa* FR13A had a higher upregulation of potassium ion transport, which could potentially be associated with its tolerance. Potassium dynamics have been associated with flooding responses, where retaining intracellular potassium levels is generally considered advantageous. Indeed, knocking out K+ efflux channels improved waterlogging tolerance of *Arabidopsis* (Wang et al. 2017). In fact, it is hypothesized that potassium could acts as a metabolic switch where high cellular levels stimulate anabolism and low levels preserve energy (Armengaud et al., 2009; Shabala, 2017; Demidchik, 2014). Potassium transport also plays a crucial role in phloem (un)loading dynamics, where mobile K+ gradients provide an energy source during brief energy shortages and allow for shared energy source between distant cell populations (Gajdanowicz et al., 2011). Though we cannot provide an exact role for K+ in *O. sativa* FR13A, it seems plausible that energy management, which is crucial for flood tolerance, might be a key function of K+ transport. The second difference is the downregulation of photosynthetic metabolism in the late submergence timepoint (LS) in IR42, which could be correlated with the sensitive phenotype of this variety during recovery after two weeks of complete submergence (Chapter 3). In contrast, FR13A did not regulate this cluster underwater but it does regulate it faster and stronger than IR42 in recovery, which provides an explanation for its greener and new formed leaves.

Three clusters showed upregulation only in *E. crus-galli* (red box in Fig. 4.5). This upregulation occurred mainly during submergence rather than recovery, though performance during submergence can be a major factor determining recovery success. Most GO categories indicated DEOs associated with transport of a variety of compounds (sodium ions, amino acids) and in a variety of cellular compartments. Also, a strong induction of beta-oxidation of fatty acids was found, in line with results from Chapter 3. Interesting and annotated DEOs among the

1,552 OGs contained in these three clusters are listed in Table 4.1. A lot of key metabolic enzymes are present, including “invertase”, “phosphoenolpyruvate carboxylase kinase”, “glyoxylase”, “long-chain acyl-CoA ligase” and “Enoyl-CoA hydratase”. Several groups are associated with amino acids “amino acid permease”, “ornithine aminotransferase” and “threonine aldolase”. This confirmed what was described in Chapter 3 regarding *E. crus-galli* being the only genotype favouring the usage of alternative energy resources, but our results here place an additional accent on the importance of amino acid transport.

Table 4.1: Selected conserved orthogroups shared in *Z. mays, E. crus-galli, O. sativa* FR13A and *O. sativa* IR42 but only upregulated in *E. crus-galli*.
List of the selected OGs coming from the overexpressed *E. crus-galli* genes clusters (selected based on genotype*treatment interaction effects), indicated by a red box in Figure 4.7.

OG numbers	OG category
OG0004932 ; OG0008084 ; OG0009339 ; OG0011074 ; OG0014992 ; OG0016406	nudix hydrolase homolog
OG0009692	ornithine aminotransferase
OG0000067 ; OG0002403 ; OG0002721 ; OG0002782 ; OG0004804 ; OG0006057 ; OG0006717 ; OG0007903 ; OG0009467 ; OG0011185 ; OG0012665 ; OG0017060 ; OG0018211	amino acid permease / branched-chain aminotransporter
OG0004103 ; OG0011843	alanine:glyoxylate aminotransferase
OG0000144 ; OG0001976 ; OG0000055 ; OG0001415 ; OG0008927 ; OG0016790	peroxidase
OG0000746	threonine aldolase
OG0000395 ; OG0002673 ; OG0008933 ; OG0009722 ; OG0013385	invertase
OG0002359	long-chain acyl-CoA ligase
OG0004852 ; OG0013887	enoyl-CoA hydratase
OG0011864 ; OG0000226 ; OG0005087 ; OG0011307 ; OG0012057 ; OG0015469 ; OG0017818	heavy metal transport/detoxification
OG0014772 ; OG0002021 ; OG0002638 ; OG0004794 ; OG0010788 ; OG0012313 ; OG0014442	protein phosphatase 2C family protein
OG0010393	gibberellin 20 oxidase
OG0003996 ; OG0009064	vacuolar iron transporter
OG0019305	ammonium transporter
OG0000284 ; OG0006868	senescence-related gene
OG0001130	phosphoenolpyruvate carboxylase kinase
OG0001136	lactoylglutathione lyase / glyoxalase
OG0019940	growth-regulating factor

Some specific stress-coping categories were also upregulated: “nudix hydrolase homolog”, “peroxidase” and “senescence-related gene”. Some growth response associated genes were present too: the gene coding for the enzyme “gibberellin 20 oxidase” involved in GA biosynthesis (Ayano et al., 2014) which was also identified in Chapter 3 and the “growth-regulating factor” DEO that could mediate the observed stem elongation underwater (van der Knaap et al., 2000; Choi et al., 2004;). Notably, many *PP2C* DEOs were upregulated and could

prepare the plant to cope with the loss of water and dehydration during recovery (Zhang and Gan, 2012; Chen et al., 2021). This gene was part of the core recovery gene cluster defined in Chapter 3, that is common between *E. crus-galli* and the two rice varieties. Interestingly, a high number of DEOs associated with heavy metal transport and detoxification were also found to be upregulated. QTLs associated with tolerance of the toxic compounds commonly found in reduced flooded soils have been discovered in *Z. mays* inbred lines and in *Z. nicaraguensis*, a relative of *Zea mays* (Mano et al., 2006; Mano and Omori, 2013; Mano and Nakazono, 2021), and this could potentially be an important feature for *E. crus-galli* tolerance to flooding.

***Echinochloa*-specific flooding responses: tolerance by novel gene evolution**

The three *E. crus-galli*-specific OG clusters upregulated upon complete submergence (Fig. 4.5 and Table 4.1) were conserved OGs (Fig. 4.2). These were OGs represented also by the other genotypes, but they do not upregulate them. Most flooding responses were lineage-specific when regarding the conserved OGs. In addition, the numerous lineage-specific DEOs could also represent a valuable and essential resource of tolerance mechanisms. The near identical response of the two *O. sativa* genotypes further indicates that lineage-specific genes could hold the main clue to explain variation in flooding tolerance in the Poaceae specifically, or the plant kingdom overall. We therefore tested whether there was considerable regulation of lineage-specific DEOs regarding the three species of interest (Fig. 4.1C and Fig. 4.6).

Firstly, we saw around 2,000-2,500 conserved OGs being regulated upon submergence (Fig. 4.6). Again, based on the number of DEOs, *Z. mays* responded slowly and regulated a lower amount of conserved OGs in early submergence (ES) and early recovery (ER). In general, more downregulation is noted during submergence while more upregulation is noted during recovery, which was a trend also observed in Chapter 3. This contrasted somewhat with lineage-specific DEOs. Regarding lineage-specific DEOs, the two rice genotypes showed a balanced response between up- and downregulation for the Oryzoideae-specific OGs (around 30 to 40 each). *Z. mays* and *E. crus-galli* even upregulated more DEOs that are specific to their lineage (respectively Angropogoneae and Paniceae/*Echinochloa*). We verified if the observed upregulation was associated more with lineage-specific DEOs using a Fisher-exact test (Fig. s4.4). As expected, association with lineage-specific DEOs upregulation was found deeper into the phylogenetic lineage with significant association regarding submergence, and reverse association regarding recovery, for the *Echinochloa*, and Oryzoideae.

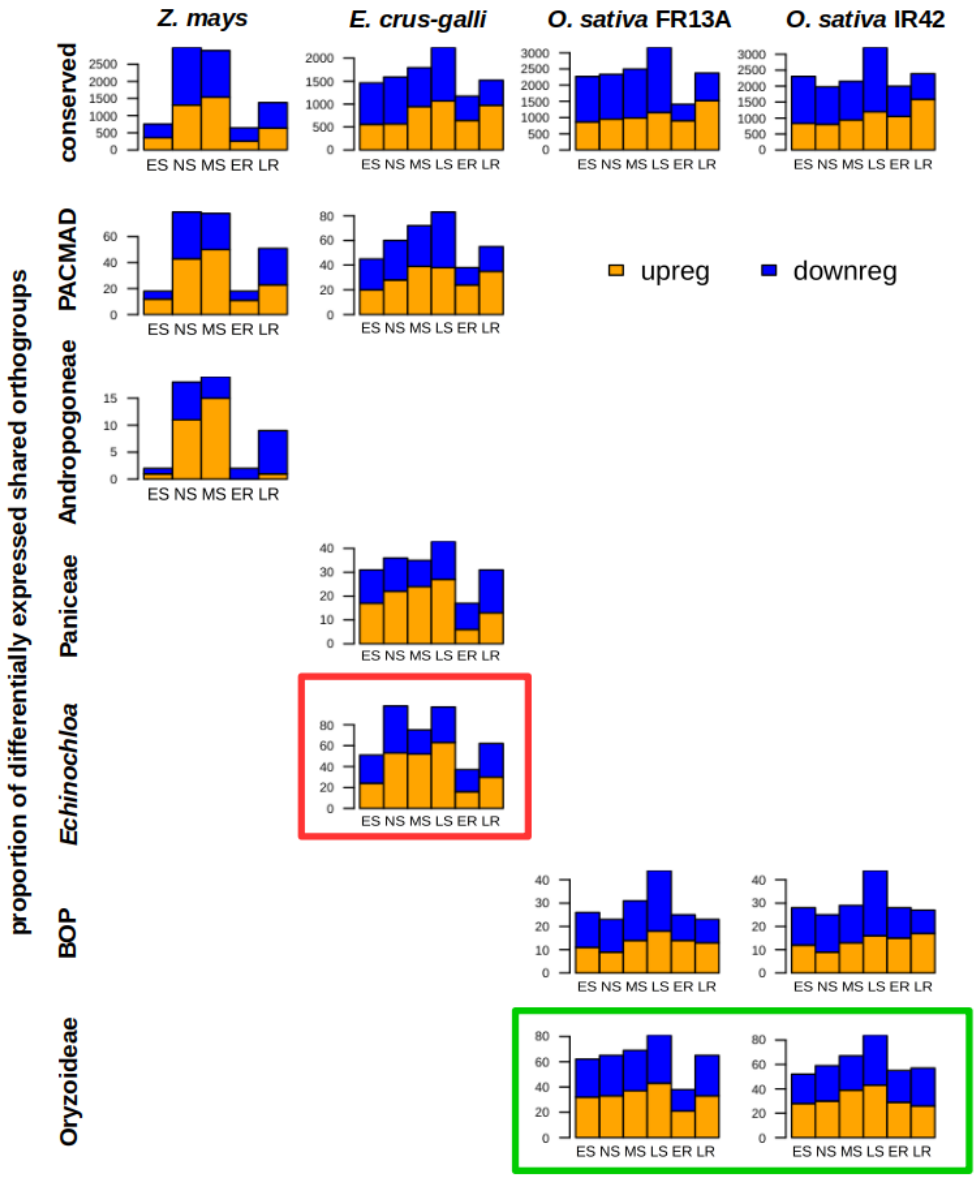

Figure 4.6: Lineage specific orthogroup responses to flooding stress.
Bar plots of the proportion of differentially expressed conserved orthogroups (DEOs) between the 18 species or only belonging to the PACMAD, Andropogoneae, Paniceae, *Echinochloa*, BOP, Oryzeae lineages over time, based on the expression of the *Z. mays*, *E. crus-galli*, *O. sativa* FR13A and *O. sativa* IR42 genes in response to flooding. The number of upregulated DEOs (logFC(gene)> 1 and FDR< 0.001) is indicated in orange whereas the number of downregulated DEOs (logFC(gene)< -1 and FDR< 0.001) is in blue. Submergence and recovery timepoints are depicted in columns. PS= Pre-Submergence, ES= Early Submergence (4 hours), NS= Night Submergence (18 hours), MS= Mid Submergence (2 days), LS= Late Submergence (5 days), ER= Early Recovery (5 days Sub + 4 hours Reco), LR= Late Recovery (5 days Sub + 1 day Reco). The statistical analyses on the ratio of up- and downregulation (Fisher's-exact test) for each pair-lineage comparison is indicated in Figure s4.4. The red box represents the OGs highlighted in Figure 4.7 and the green box represents the OGs highlighted in Figure 4.8.

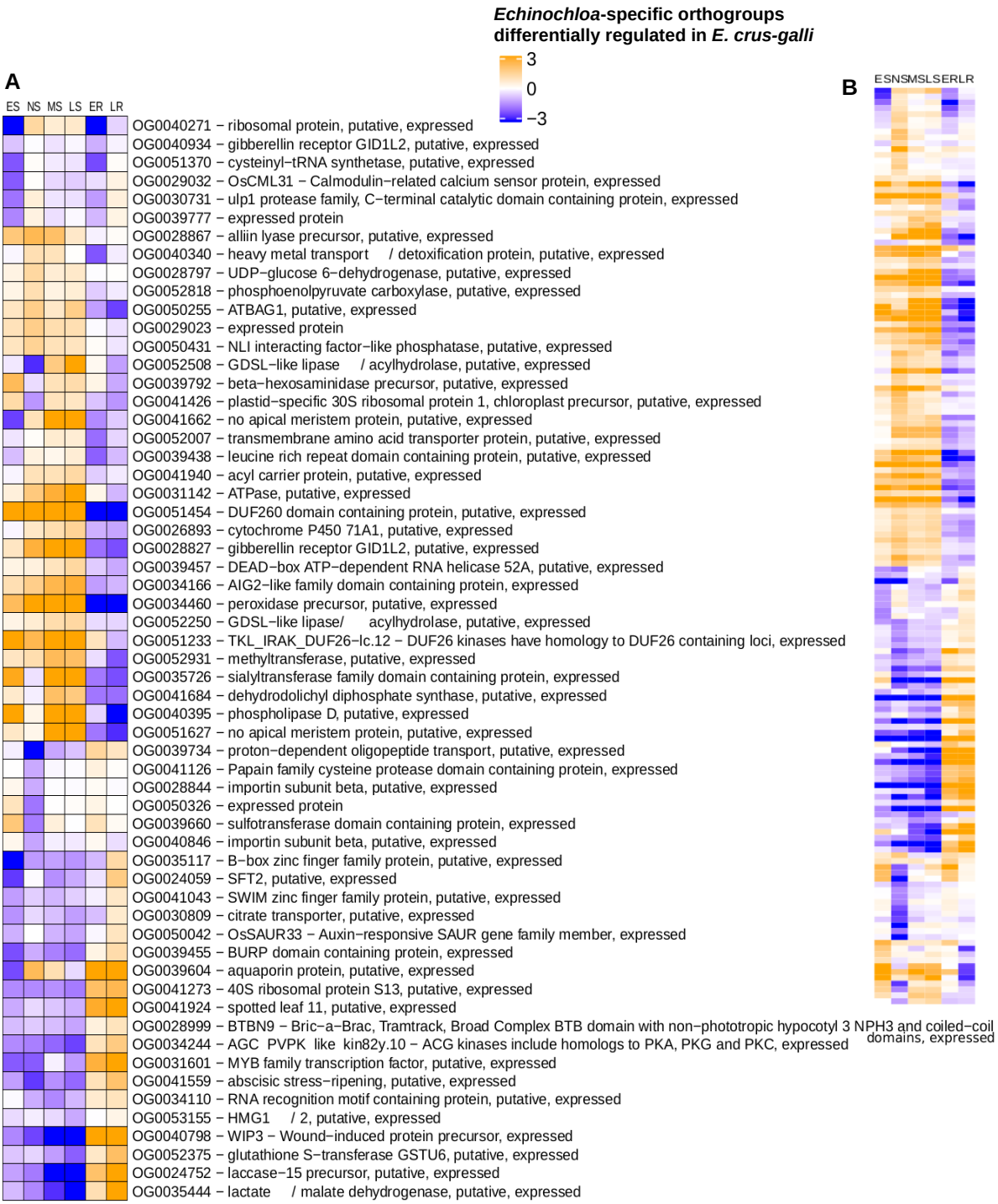

Figure 4.7: Differentially expressed *Echinochloa* spp. specific OGs in *E. crus-galli* upon flooding.

A-B. Heatmaps of the annotated (**A**) or not annotated (**B**) orthogroups that are exclusively present in the *Echinochloa* tribe, which are up- or downregulated in *E. crus-galli* upon submergence and/or during recovery (|log2FC|> 1 and Padj.< 0.001) (red box in figure 4.6). Orange represents up- and blue downregulation. Names and annotations of the orthogroups are shown next to the heatmap. Submergence and recovery timepoints are depicted in columns per species. ES= Early Submergence (4 hours), NS= Night Submergence (18 hours), MS= Mid Submergence (2 days), LS= Late Submergence (5 days), ER= Early Recovery (5 days Sub + 4 hours Reco), LR= Late Recovery (5 days Sub + 1 day Reco).

Having a stronger upregulation of the lineage-specific DEOs compared to the conserved DEOs allows the species to respond to a specific stress they may have had to adapt to. During recovery, more downregulation in the lineage-specific DEOs compared to the conserved DEOs response correlates with the return to normoxia.

Aside from the quantitative response, the question desired to be answered is: Which *Echinochloa*-specific OGs are regulated in *E. crus-galli* during the flooding stress? More *E. crus-galli Echinochloa*-specific DEOs were detected (about 40 to 90 depending on the timepoint) than Paniceae-specific DEOs (about 18 to 42 depending on the timepoint). Even if *Echinochloa*-specific DEOs are more numerous than Paniceae-specific DEOs (Fig. 4.1C), we can hypothesize that *Echinochloa* species have more flooding tolerant-specific genes than the Paniceae sub-family species. By focusing the analysis on the *Echinochloa*-specific DEOs (Fig. 4.6 red box), all DEOs were retrieved and plotted in figure 4.7. Not more than 59 DEOs could be annotated, and still 157 DEOs up- or downregulated upon flooding do not have any annotation available (Fig. 4.7). Orthology analysis clearly placed these genes separate from other Poaceae, based on no similar sequence, or very poor BLAST scores. Annotation of these genes would require a dedicated study on conserved protein domains and phylogeny.

Among the annotated *E. crus-galli* DEOs upregulated during submergence and downregulated during recovery, we found OGs mainly encoded proteins involved in sugar metabolic pathways (“UDP-glucose-6-dehydrogenase”, “phosphoenolpyruvate carboxylase” and “transmembrane amino acid transporter protein”), in growth (“gibberellin receptor GID1L2” - *GIBBERELLIN INSENSITIVE DWARF1)* (Hartweck and Olszewski, 2006), but also involved in the antioxidative/detoxification response (“peroxidase”, ‘heavy metal transport”, “no apical meristem protein”). *DUF260* is also strongly upregulated this way. It encodes a LATERAL ORGAN BOUNDARIES (LOB) DOMAIN (LBD) protein, which are transcription factors with key roles in the regulation of different plant development processes and stress responses (Grimplet et al., 2017). Secondly, a group of OGs downregulated during submergence but upregulated during recovery, regroup genes participating in photosynthesis (“lactate/malate dehydrogenase”) and the TCA cycle (“citrate transporter”), hormonal responses (“auxin-responsive SAUR, abscisic-stress ripening - transcription factor”), and the response to some abiotic stresses (“laccase-15-precursor” Os*LAC15*, “glutathione-S-transferase *GSTU6*” and “wound-induced-protein *WIP3*”). *WIP3* is a gene involved in the response to biotic agents but is regularly found among drought stress responsive genes (Sircar and Parekh, 2015; Liu et al., 2017). Genes encoding “aquaporins”, which are transmembrane water transporters, were also upregulated during the night timepoint (NS) and during recovery (ER, LR). *E. crus-galli* also carried unassigned

Figure 4.8: Differentially expressed OGs common to the Oryzoideae tribe in *O. sativa* FR13A and *O. sativa* IR42 upon flooding.

Oryzoideae orthogroups that are up- or downregulated upon submergence and/or during recovery in *O. sativa* FR13A and in *O. sativa* IR42 (green box in figure 4.6). Names and annotations of the orthogroups are shown next to the heatmap. Orange represents up- and blue downregulation. Grey boxes are the orthogroups that do not exist or lack significant regulation (|log2FC|> 1 and Padj.< 0.001). Submergence and recovery timepoints are depicted in columns. PS= Pre-Submergence, ES= Early Submergence (4 hours), NS= Night Submergence (18 hours), MS= Mid Submergence (2 days), LS= Late Submergence (5 days), ER= Early Recovery (5 days Sub + 4 hours Reco), LR= Late Recovery (5 days Sub + 1 day Reco). The Venn diagram on the side indicates the number of specifically or commonly regulated orthogroups between *O. sativa* FR13A and *O. sativa* IR42. The number of annotated ones are in brackets.

flooding responsive OGs that were not shared with *E. colona* and *E. glabrescens*. These OGs are likely to be composed of non-true genes and likely unrelated to the superior tolerance of the *Echinochloa* genus and therefore of lesser interest.

The two genotypes *O. sativa* FR13A and IR42 showed highly similar transcriptome behaviour (Chapter 3 and Fig. 4.3, s4.3, 4.4C-F, 4.5, 4.7). To seek differences that could explain the higher tolerance of *O. sativa* FR13A we explored the annotated DEOs for *O. sativa* FR13A and IR42 from the Oryzoideae tribe (Fig. 4.8). Within the Oryzoideae, 103 DEOs were commonly regulated in both genotypes, while 25 DEOs (including 19 annotated ones) were present or differentially regulated only in *O. sativa* FR13A. In this latter group, we noticed a few DEOs upregulated during submergence and downregulated upon recovery including the "pseudo-response regulator*"* (*PRR*) which is a family of transcriptional repressors of the circadian clock expression genes (Nakamichi et al., 2010; Toda et al., 2019), the "phytosulfokines precursor" (*PSK*) which are thought to have a role in growth and development and in adaptation to biotic and abiotic stresses (Sauter et al., 2015) and the "*OsFBDUF13*" thought to be involved in the control of shoot branching under low nitrogen cultivation (Kwon et al., 2021). "Enoyl-CoA isomerase" (*ECI*), involved in the beta-oxidation of unsaturated fatty acids was downregulated during submergence. The "carboxylesterase" (*CXE*), encoding an enzyme involved in shoot branching and tillering was also downregulated during submergence and upregulated during recovery (Roesler et al., 2021). In general, several growth processes are downregulated underwater and reactivated again when the water recedes in *O. sativa* FR13A, marking its difference with IR42 despite huge similarities in transcriptome behaviour. The growth and development controlled by the above-mentioned genes could add to / depend on the SUB1A quiescence locus (Jung et al., 2010) which does not appear in this list as it is not contained in the Indica genome.

Conclusions

Altogether, our data suggest that the flooding tolerance in *E. crus-galli* is a combination of shared responses and lineage-specific responses. As the latter encompasses most of *E. crus-galli* tolerance, it emphasizes the importance of favouring an OG comparison in addition to a unique transcriptomics comparison. With the advent of modern genomics, the sequences of many genomes are becoming quickly available, paving the way for trustworthy OG comparative analyses aimed at disentangling plant responses to any biotic and abiotic stresses.

Comparing OGs instead of genes, coupled with a GLM approach to analyze the differential responses in time, appeared to be the best approach in our study. Taking the maximum or the average expression of all genes belonging to each OG and then performing an OG-to-OG comparison permitted the correction of ploidy differences between species. One benefit for a species to contain multiple

copies of the same gene in its genome is to allow for redundancy of function. To respond to a stress, only some genes of the OG can be transcribed, and a manual selection not considering the OG composition could lead to different or incomplete results. In our study, we compared one rice gene to one maize gene to three *E. crus-galli* genes on average, which corresponds to their different ploidy levels. The choice of species was well-balanced to perform the analyses. Moreover, going further away in the phylogeny would make the small differences between close species invisible, for instance among the different rice varieties or the different *Echinochloa* species. Thus, restricting the tree to the Poaceae was an intelligent selection.

In Chapter 3, we identified several common gene regulation patterns and clusters in response to flooding in the four genotypes, including the highly flood sensitive maize. These conserved responses therefore do not correlate with tolerance to the stress. Major differences found with the transcriptomics analysis were found again with the OG analysis performed here. We confirmed that part of maize intolerance resides in its slow response to both submergence and desubmergence, which contrasts with the quick response of *E. crus-galli* in both situations. The data suggest that upon desubmergence, *E. crus-galli* can rapidly reverse its metabolic machineries which would permit a faster recovery of growth and development. This is one of the main differences compared to the rice varieties and could explain the better phenotypic index after two weeks of recovery described in Chapter 3. However, among the conserved OGs, no strong recovery responsive clusters were observed in *E. crus-galli* (Fig. 4.5). This could be because acclimation upon reoxygenation mainly requires *Echinochloa*-specific genes. Additionally, we hypothesize that the ability to cope with post-submergence stress is determined by responses during submergence.

Defining the lineage-specific responses allowed for the identification of *E. crus-galli*-specific responses of OGs that are unique to the *Echinochloa* clade. Interesting upregulated OGs during submergence were linked to shoot growth (growth-regulating factor) and to heavy metal transport and detoxification. The upregulated OGs during recovery were linked to photosynthesis, hormones and sugar metabolism regulation, water transport via aquaporins, antioxidative, abiotic stress (*LAC*, *GSTU6* and *WIP3*) and detoxification responses (Fig. 4.7). We noted many non-annotated genes that could play a crucial role in submergence responses of *E. crus-galli* and are therefore interesting candidate genes to characterize further.

With the Venn Diagram approach, we showed that the two tolerant genotypes *E. crus-galli* and *O. sativa* FR13A do not share a lot of DEOs. The responses show significant separation according to phylogeny rather than tolerance (Fig. 4.3, s4.3, 4.4, 4.5), which indicates that *E. crus-galli* and *O. sativa*

FR13A have distinct molecular responses that contribute to flooding tolerance and perhaps also explaining their contrasting phenotypic behaviour underwater. We compared *E. crus-galli* tolerance responses shared with *O. sativa* FR13A with a GLM (general linear model) approach, but the only common responses found were the downregulation of photosynthesis and starch biosynthesis during the night, which is expected due to the circadian clock (Fig. 4.5). We mainly identified DEOs that are upregulated in *E. crus-galli* but not regulated in *O. sativa* FR13A (Table 4.1). We confirmed the importance of alternative sugar metabolism and GA biosynthesis (*GA20OX)* to mediate shoot elongation. Several genes encoding for growth, transporters, key metabolic enzymes and oxidative stress responses were upregulated, all potentially important for both submergence and recovery phases. In addition, many upregulated DEOs were associated with heavy metal transport and detoxification. This could be an important feature for *E. crus-galli* tolerance to flooding by handling the reduced toxic compounds formed in flooded soils.

By selecting various C3 and C4 species, we also aimed to answer the question: Do photosynthesis-related processes contribute to the flooding tolerance? Indeed, we could hypothesize that *E. crus-galli* photosynthesis machinery, being a C4 species, would be more efficient and/or less damaged underwater and consequently, directly operational when the carbon dioxide (and light) are available again for photosynthesis. Since the corresponding GO categories were not especially regulated in unique *E. crus-galli* OGs or between the different genotypes in general, we cannot conclude on a potential photosynthetic advantage for C4 species on their flooding tolerance. Yet, the global transcriptomics responses cluster by phylogeny and the leaf structure, photosynthesis mechanism and energy storage are different in C3 and C4 species. One way to further explore this hypothesis would be to measure the actual photosynthetic efficiency in addition to the sugar usage under flooding treatment.

Polyploidy, providing a much higher range of genetic diversity than diploidy, augments the chances of the concerned species to differentially regulate particular genes, in order to cope with the given stress(es) and/or to facilitate the invasion of that species in new environments (Pandit et al., 2001; Van de Peer et al., 2017; Rutland et al., 2021). On the other hand, strong and recurrent selective events could favour polyploidy and this can also give an indication about the occurrence of a particular environmental condition for the plants and about their specific adaptation to it (Comai, 2005; Rutland et al., 2021). This is what could have resulted with *E. crus-galli*, which is an allohexaploid species that arose from the hybridization between tetraploid *E. oryzicola* (paternal donor) and an unknown diploid species (maternal donor) (Ye et al., 2020) and that is found in diverse environmental conditions (reviewed in Bajwa et al., 2015). Part of its tolerance might have appeared quite recently, independently to the rice flooding tolerance mechanisms.

Supplemental data

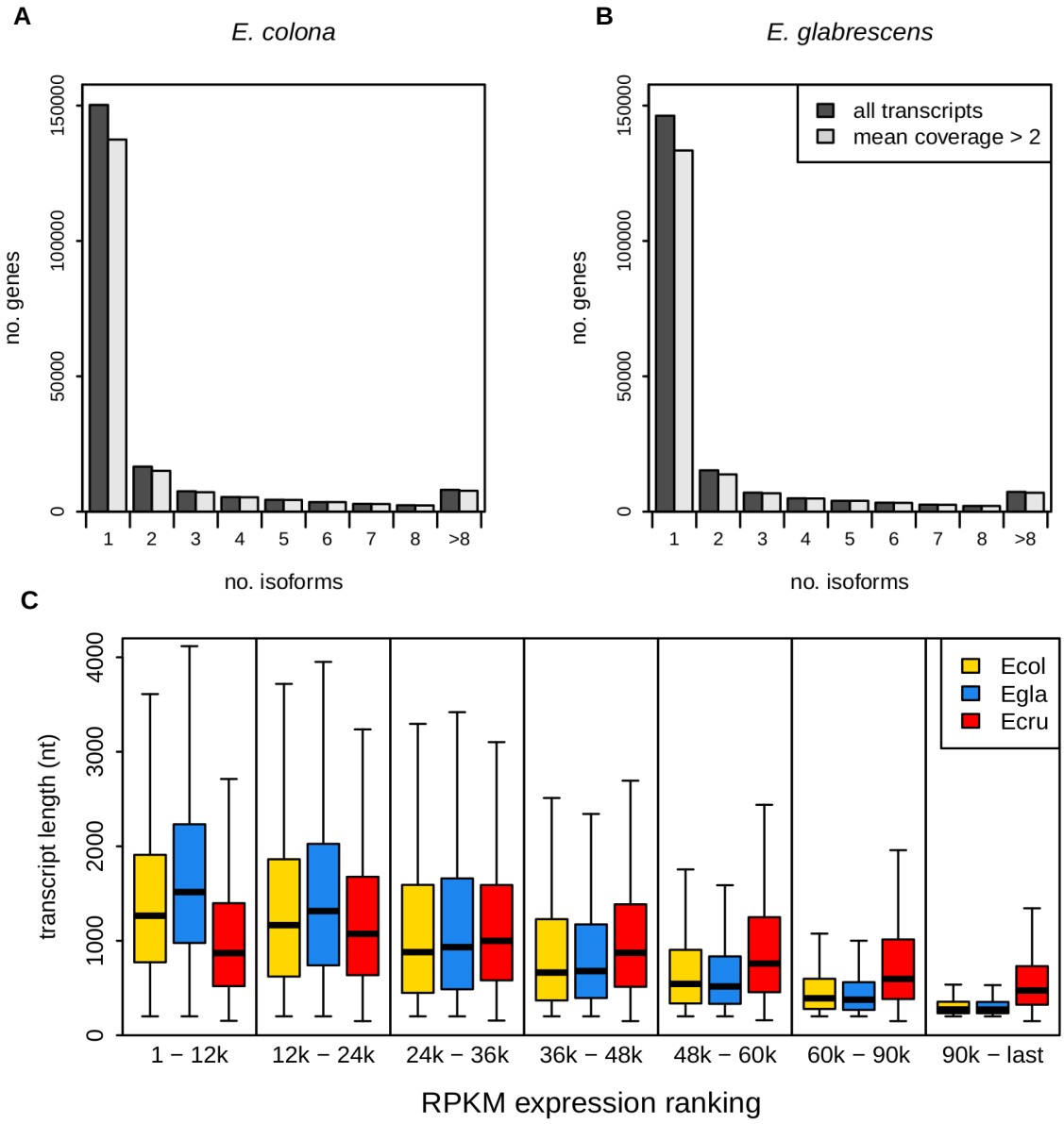

Figure s4.1: Assembly statistics of the transcriptomes of *Echinochloa colona* and of *Echinochloa glabrescens.*

A-B. Number of genes having one or more isoforms (alleles or splice variants) in (**A**) *E. colona* and (**B**) *E. glabrescens*. Dark grey bars depict all assembled transcripts, whereas light grey bars are the transcripts with a mean coverage of at least two.

C. Box plots of transcript lengths of *E. colona* (Ecol), *E. glabrescens* (Egla) and the *E. crus-galli* (Ecru) genome. Separate plots are for high and low abundance genes (Reads Per Kilobase of transcript, per Million mapped reads, RPKM). With the most abundant 12,000 genes on the left, on the lowest on the right.

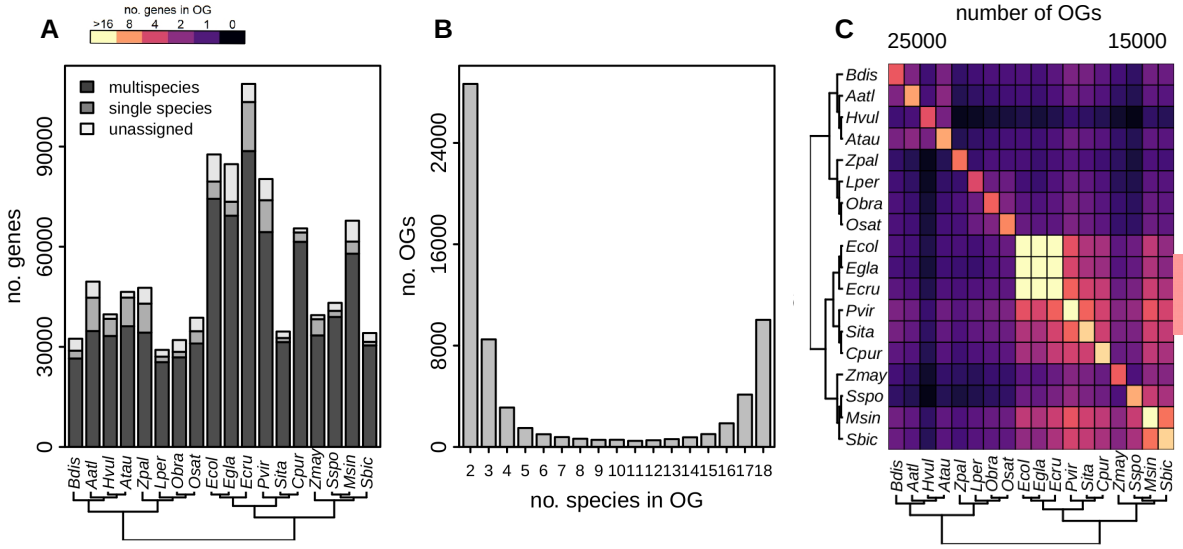

Figure s4.2: Family composition and relationships between the 18 species used in the orthogroup analysis.

A. Barplot of the number of genes assigned to orthogroups (OGs) that are either shared with one or more other species (multispecies), specific to that species with at least two genes per OG (single species), or genes not sharing an OG with any other genes (unassigned).

B. Barplot of the number of OGs with representives from two to 18 species.

C. Matrix indicating the number of OGs shared between a pair of species. High amounts of shared OGs are indicated by a lighter color. The relatedness of the species indicated on the left and at the bottom corresponds to the phylogeny indicated in Figure 1.

Table s4.1: Composition of the sequenced genome of the 18 species used in the orthogroup analysis.

name	species	clade	subfamily	tribe	size sequenced genome	genome composition	source
Bdis	*Brachypodium distachyon*	BOP	Pooideae	Brachypodieae	271.9 Mb	2n= 2x= 10	The International Brachypodium Initiative 2010
Aatl	*Avena atlantica*	BOP	Pooideae	Poeae	3.685 Gb	2n= 2x= 14	Maughan et al., 2019
Hvul	*Hordeum vulgare*	BOP	Pooideae	Triticeae	5.1 Gb	2n= 2x= 14	Beier et al., 2017
Atau	*Aegilops tauschii*	BOP	Pooideae	Triticeae	~4.3 Gb	2n= 2x= 14	Luo et al., 2017
Zpal	*Zizania palustris*	BOP	Oryzoideae	Oryzeae	1.29 Gb	2n= 2x= 30	Haas et al., 2021
Lper	*Leersia perrieri*	BOP	Oryzoideae	Oryzeae	267 Mb	2n= 2x= 24	Stein et al., 2018
Obra	*Oryza brachyantha*	BOP	Oryzoideae	Oryzeae	261 Mb	2n= 2x= 24	Chen et al., 2013
Osat	*Oryza sativa* Indica Group	BOP	Oryzoideae	Oryzeae	390.6 Mb	2n= 2x= 24	Du et al., 2017
Ecol	*Echinochloa colona*	PACMAD	Panicoideae	Paniceae	/	/	Toulotte et al., non published
Egla	*Echinochloa glabrescens*	PACMAD	Panicoideae	Paniceae	/	/	Toulotte et al., non published
Ecru	*Echinochloa crus-galli*	PACMAD	Panicoideae	Paniceae	1.27 Gb	2n= 6x= 54	Guo et al., 2017
Pvir	*Panicum virgatum*	PACMAD	Panicoideae	Paniceae	1.129 Gb	2n= 2x= 18	Lovell et al., 2021
Sita	*Setaria italica*	PACMAD	Panicoideae	Paniceae	~400 Mb	2n= 2x= 18	Bennetzen et al., 2012
Cpur	*Cenchrus purpureus*	PACMAD	Panicoideae	Paniceae	1.97 Gb	2n= 4x= 28	Yan et al., 2021
Zmay	*Zea mays*	PACMAD	Panicoideae	Andropogoneae	2.106 Gb	2n= 2x= 20	Jiao et al., 2017
Sspo	*Saccharum spontaneum*	PACMAD	Panicoideae	Andropogoneae	3.36 Gb	1n= 4x= 32	Zhang et al., 2018
Msin	*Miscanthus sinensis*	PACMAD	Panicoideae	Andropogoneae	1.68 Gb	1n= 1x= 19	Mitros et al., 2020
Sbic	*Sorghum bicolor*	PACMAD	Panicoideae	Andropogoneae	655.2 Mb	2n= 2x= 20	McCormick et al., 2017

Table s4.2: Composition of the real genome of the 18 species used in the orthogroup analysis.

name	number of chromosomes	real genome composition	real ploidy level
Bdis	5	2n= 2x= 10	diploid
Aatl	7	2n= 2x= 14	diploid
Hvul	7	2n= 2x= 14	diploid
Atau	7	2n= 2x= 14	diploid
Zpal	15	2n= 2x= 30	diploid
Lper	12	2n= 2x= 24	diploid
Obra	12	2n= 2x= 24	diploid
Osat	12	2n= 2x= 24	diploid
Ecol	9	2n= 6x= 54	hexaploid
Egla	9 ?	2n= 6x= 54 ?	hexaploid ?
Ecru	9	2n= 6x= 54	hexaploid
Pvir	9	2n= 4x= 36, 2n= 8x= 72	tetra/octa- ploid
Sita	9	2n= 2x= 18	diploid
Cpur	7	2n= 4x= 28	allotetraploid
Zmay	10	2n= 2x= 20	diploid
Sspo	8	2n= 8x= 64	octoploid
Msin	19	2n= 2x= 38	diploid
Sbic	10	2n= 2x= 20	diploid

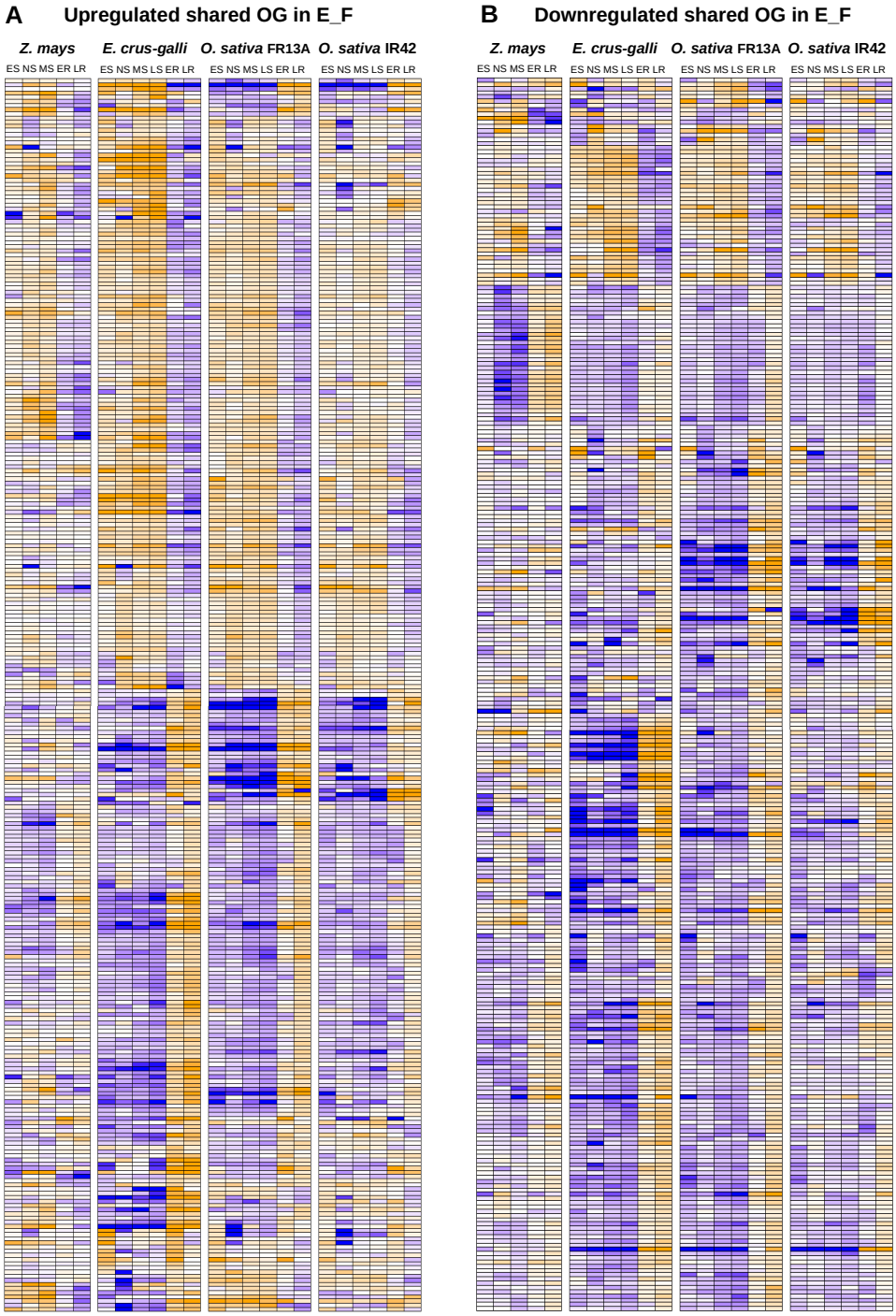

Figure s4.3: Heatmaps of the shared orthogroups upregulated in *E. crus-galli* and *O. sativa* FR13A.

A-B. Top regulated DEOs of the conserved orthogroups in the four genotypes that are (**A**) upregulated (logFC(gene)> 1 and FDR< 0.001) or (**B**) downregulated (logFC(gene)< -1 and FDR< 0.001) in *E. crus-galli* and *O. sativa* FR13A only for at least one timepoint. ES= Early Submergence (4 hours), NS= Night Submergence (18 hours), MS= Mid Submergence (2 days), LS= Late Submergence (5 days), ER= Early Recovery (5 days Sub + 4 hours Reco), LR= Late Recovery (5 days Sub + 1 day Reco). Data corresponding to «E_F» in Figure 4.3C.

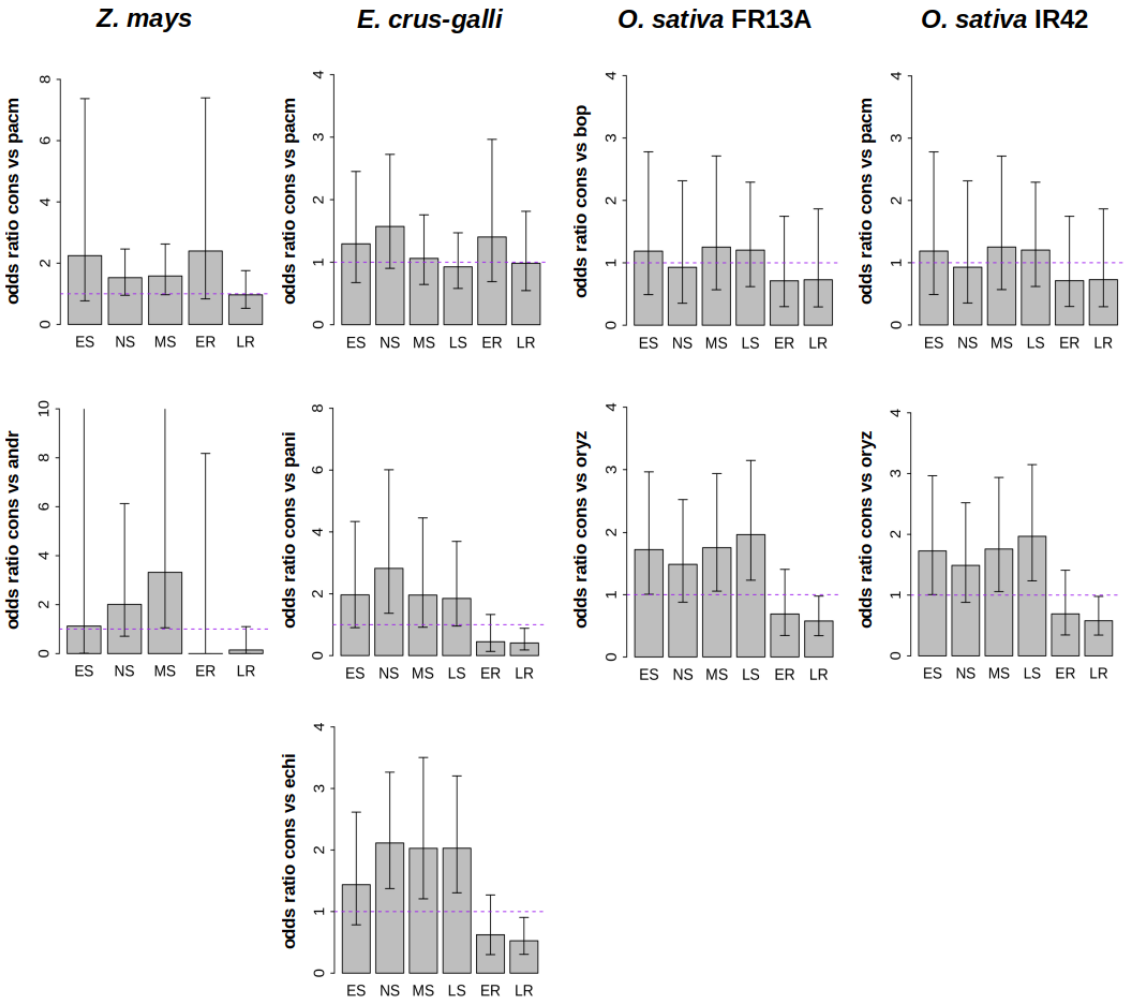

Figure s4.4: Association between upregulation and lineage specificity compared to conserved orthogroups (Odds ratios) for *Z. mays*, *E. crus-galli*, *O. sativa* FR13A, *O. sativa* IR42.

Odds ratios and 95% confidence interval resulting from the Fisher's-exact test on the number of up- and downregulated DEOs between conserved and lineage specific orthogroups. The odds ratio are calculated per timepoint per genotype with data from Figure 4.6. The horizontal line at y= 1 represents the equilibrium between up- and downregulation. Values greater than 1 indicate association of upregulation with lineage specificity. Lineages: cons= conserved orthogroups between the 18 species, pacm= only belonging to the PACMAD, andr= only to Andropogoneae, pani= only to Paniceae, echi= only to *Echinochloa*, bop= only to BOP, oryz= only to Oryzeae. Submergence and recovery timepoints are depicted in columns per genotype. ES= Early Submergence (4 hours), NS= Night Submergence (18 hours), MS= Mid Submergence (2 days), LS= Late Submergence (5 days), ER= Early Recovery (5 days Sub + 4 hours Reco), LR= Late Recovery (5 days Sub + 1 day Reco).

Chapter 5
Management of *Echinochloa crus-galli* through shade and submergence-based practices

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Abstract

Echinochloa crus-galli is a highly competitive and persistent rice weed, responsible for high rice yield losses. Its phenotypic similarity to rice makes manual weeding at the vegetative stage difficult. It has also evolved multiple resistances against commonly used herbicides for its control. These factors warrant the need to identify alternative management strategies which can (1) reduce dependence on herbicides and minimize risks associated with their overuse, and (2) reduce labour use in manual weeding as labor is increasingly scarce and expensive. One promising approach to suppress weeds including *E. crus-galli* is to incorporate shading stress as a complement to the water-based field management. This would also permit reduced water and herbicides usage. Most weeds are sensitive to shade, with light limitation significantly delaying their development. Here we explored the efficacy of shade on the growth and development of *E. crus-galli*. The light was manipulated either artificially or through varying rice planting densities or using rice varieties with variable light-intercepting properties. Our experiments revealed a high sensitivity of *E. crus-galli* to shade. A considerable reduction of different weed growth traits below the canopies of high shading rice varieties was observed. Early shading, either provided by the selected rice variety or a relatively high density rice planting, was crucial for the reduction of *E. crus-galli* growth and biomass. Rice varieties reduced weed biomass to 45-65% at 43 days after transplanting the weed and this increased to 47-82% when combined with high plant density. We also tested whether shade alone or in combination with early complete submergence could provide a more effective suppression of *E. crus-galli*. Our data revealed that the shade treatment was as efficient in reducing weed development as was complete submergence (respectively 82% and 89% of weed biomass reduction after 8 days of treatment). Additionally, simultaneous application of both stresses did not result in synergistic effects. These results suggest that *early* flooding for shorter periods during the first weeks after rice sowing/transplanting, followed by a shade-based management via the selection of high shading rice varieties and manipulating rice planting density can be an effective approach for the management of *E. crus-galli* in rice.

Introduction

Echinochloa crus-galli (L.) Beauv. is classified as the third worst weed in modern agriculture (Michael, 2003; Leeson et al., 2005; Heap, 2014; Kraehmer et al., 2016). It produces a large quantity of seeds that can stay dormant in the soil for 8-9 years (Chul and Moody, 1989). Consequently, its propagation as well as its seed bank builds up in the fields over time (Gibson et al., 2002; Clay et al., 2005). It is a highly competitive weed as reported yield losses caused by this weed in rice ranged from 21 to 79%, depending on the cropping system and weed management (Stauber et al., 1991; Ottis and Talbert, 2007; Wilson et al., 2014; Bajwa et al., 2015).

Managing *Echinochloa* species infestation in paddy fields has become challenging. This is because of several factors including (1) manual weeding – one of the common weed control methods in smallholder farming in Asia and Africa is becoming less economically viable because of rising labour shortages and consequently wage costs (Ahmed et al. 2021; Bajwa et al. 2015). Moreover, *E. crus-galli* is phenotypically similar to rice which makes it indistinguishable from rice at the seedling stage, and hence manual weeding is extremely difficult and less effective (Barrett et al., 1983); (2) *E. crus-galli* has evolved resistance (cross and multiple resistance) to several commonly used herbicides in rice – making chemical weed control challenging for *E. crus-galli* as one of the world's most serious herbicide-resistant weeds (Heap, 2019); and (3) *E. crus-galli* is becoming increasingly tolerant to flooding/water-based management practice (Estioko et al. 2014; Ismail et al., 2012; Peralta et al., 2019; Kaspary et al. 2020). These factors warrant the need to develop alternative non-chemical weed management options for this weed species and combine them with herbicide use as an integrated weed management strategy. As described in Chapter 2, flooding-based protocols applied in the field showed varying *E. crus-galli* reduction depending on the timing and duration of the stress application.

Crop-weed competition experiments have shown that time of crop and weed emergence, weed density and crop planting density significantly affect crop competitiveness and yield losses (Bhowmik and Reddy, 1988; Norris, 1992; Ottis and Talbert, 2007; Chauhan and Johnson, 2011; Chauhan, 2012; Chauhan and Abugho, 2013a; Daas et al., 2017). Another promising way to reduce weed infestation is to exploit the documented sensitivity of these weeds to light-limited conditions. This can be achieved through the use of high shade-casting rice varieties alone or in combination with high planting density. Chauhan (2013) has shown that a reduction of 50-75% light intensity (600 to 300 µmol m^{-2} s^{-1} compared to 1200) could reduce *Echinochloa* plant height, biomass and seed production. Another study that tested the effect of three planting densities of two rice cultivars on *Echinochloa glabrescens* growth found that higher rice density reduced weed development (Chauhan and Abugho, 2013a). This included a reduction in weed

leaf number, leaf area, shoot biomass by 83% and weed seed production by 88% compared with when the weed was not growing with rice. While this reduction was significant, the researchers concluded that rice interference alone was not sufficient as it requires a few initial days to acquire sufficient canopy and cast significant shade on weeds. Therefore, to achieve efficient and effective weed management would require a combination of methods. Flooding is another important weed control strategy in paddy rice fields. Accordingly, the effect of flooding (2 cm water layer) together with rice interference on *E. crus-galli* growing in pots was tested (compared to aerobic conditions) with different nitrogen fertilization rates (Chauhan and Abugho, 2013b). Growth and seed production of *E. crus-galli* declined by 84-86% and 82-87% respectively with the increase in rice density compared with the weed-only plots without rice interference, irrespective of the nitrogen fertilization or water regime. Therefore, a weed management strategy involving shading of weeds via the use of high shade-casting rice varieties or combined with existing water-based management protocols could be effective.

To test this, we investigated the effect of shade, submergence and their combination on *E. crus-galli*. The effect of shade on weed development was tested in the greenhouse and in the field, where the extent of shading was manipulated either by using rice varieties with variable shade-casting capacities or by increasing the rice planting density. We also monitored the extent of shade cast by rice during canopy development to determine the duration needed to reach the threshold level of shading causing weed suppression. A first selection of two high and two low shade-casting rice (*O. sativa*) varieties was used in a pot experiment in the greenhouse to monitor the effects on weed growth. The shading capacity of these rice varieties is achieved through different shoot architectural traits (Huber et al., 2021). The aim was to identify how long we need to manage weeds at an early stage by other methods (e.g. flooding) before crop-induced shading can effectively suppress weeds. Subsequently, a larger selection of 11 different rice varieties with variable shade-casting capacity, also selected from Huber et al. (2021), were evaluated in the field. Different weed traits were followed during the vegetative stage development of the weed and the ground cover for each plot was recorded over time through two distinct methods to monitor the association of canopy shade and weed development. Finally, to understand the impact of flooding and shading alone and in combination on weed suppression, we tested whether a combination of shade and flooding could lead to more effective weed suppression and therefore provide useful insight for future field management of this weed.

Our data revealed that *E. crus-galli* development is significantly affected by shade and that early shading is important for optimal weed suppression. Combining shading and submergence did not further reduce weed growth. The equal efficacy of both stresses thus offers an alternative to the current water-based management. We propose a shortening of the flooding duration followed by a

second step of natural shade-imposition from early and high shade-casting rice cultivars. Such a design would save precious freshwater resources and provide a low chemical weed suppression approach and should be further field tested in both dry and wet fields.

Materials and methods

Experiment 1 (Greenhouse): Effect of rice shade on early *Echinochloa crus-galli* development

Seed origins

Four different rice (*O. sativa)* varieties were selected for their high (Shim Balte and Mudgo) or low (Della and Luk Takhar) shade-casting potential based on Huber et al. (2021). Seeds were provided from International Rice Genebank Center (IRGC), International Rice Research Institute (IRRI).

E. crus-galli (biotype 10) seeds 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, growth and experimental set-up

Plants were grown in the greenhouse facilities of the Botanical Gardens, The Science Park, 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}. 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 Fig. s5.1).

For each rice variety, five seeds were put to germinate per pot (10 x 10 x 11 cm) with a mixture of 50% black soil / 20% sand / 30% agra-vermiculite 0-1.5 mm + 20% Yoshida nutrient solution (Yoshida, 1976) with a double iron dose (sequestreen= Fe-EDTA), pH 6.5 + osmocote NPK-Mg 15-4-9 (+1) (2.4 g/L of soil). Pots were directly placed in the set-up in the greenhouse. A week later, only the pots in which there were 5 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. A

week later, thinning was done to retain four weed plants per pot. The experiment includes three repeated blocks (Fig. s5.1) and was performed once.

Measurements

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

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 3 days and weighed per plant. In addition, the number of leaves and tillers per plant were counted.

Experiment 2 (Field): Rice-Weed competition assay

Seed origins, germination, growth

The field experiment took place in a field at the IRRI, Los Baños, the Philippines from January to March 2020. A selection of 11 different rice varieties was made based on their high (V02, V04, V05 and V07), intermediate (V01, V08, V09, V10) or low (V03 and V06) shading potential based on Huber et al. (2021). An elite-breeding line with no data about its shading capacity was also included (V11) (Table 5.1). Rice seeds were provided from 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, Los Baños, the Philippines. Rice seedlings were raised in trays for 14 days and the 14 days rice seedlings were then manually transplanted into the main field. On the day of transplanting rice seedlings into the main field, *E. crus-galli* seeds were sown in trays and raised for 10 days before transplanting into the main field. The seed to seed age difference between rice and *E. crus-galli* seedlings was 14 days to simulate rice conditions with 2 weeks age advantage to rice.

Treatment details and experimental design

To assess the shade-casting effect of rice varieties and rice planting density on the suppression of *E. crus-galli* growth, a field experiment was conducted. A total of 11 rice varieties were evaluated with standard rice planting density with spacing 20 cm x 20 cm= 25 plants/m^2 and with high planting density with spacing 20 cm x 10 cm= 50 plants/m^2. These rice varieties with both planting densities were grown with and without *E. crus-galli* competition (Rice-only and Rice+Weed). A fully weedy treatment was also included as a check where *E. crus-galli* was grown alone without competition from rice (Weed-only). In R+W plots, a density of 50 *E. crus-galli* plants/m^2 was maintained by carefully manually transplanting 10-days old seedlings of *E. crus-galli* between rice rows at a spacing

of 10 cm plant-to-plant. Similarly, in weedy plots (W-only), a density of 50 *E. crus-galli* plants/m^2 was established. The experiment design was a factorial randomized complete block with three replications. The factors were: variety (11 rice varieties), rice planting density (25 plants/m^2 and 50 plants/m^2) and weed competition (Rice-only and Rice+Weed). The details of the 11 rice varieties with their shading ability are provided in Table 5.1. The layout of the study is given in figure s5.2. The planting pattern (crop geometry) in both rice alone and R+W plots is given in Fig s5.3 (A-B). Plots size was of 2.6 m x 2.4 m, i.e.= 6.24 m^2. In this Chapter, only the R+W plots and W-only plots were analyzed.

Soil preparation, herbicide and fertilization

A stale seedbed approach was practiced for 20 days prior to rice transplanting to minimize the background soil weed seed bank by stimulating germination/emergence with tillage and irrigation and then killing them with glyphosate application. Prior to transplanting, the field was puddled and then leveled. 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, the soil was kept saturated. Manual weeding to remove emerging weeds other than *E. crus-galli* was done throughout the experiment.

A standard fertilizer and pest management practices were 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 the weed development were recorded manually. The weed's maximum shoot height (measured with stretched leaves) and tiller number were recorded at 25 and 39 days after weed transplanting. The weed shoot biomass was harvested at 25 and 43 days after weed transplanting, dried several days at 80°C and weighed.

To assess the percent ground/canopy cover, two methods were used. First, ground cover was assessed manually at two-week intervals until it reached 100% using a beaded string method (Sarrantino, 1991). For the beaded string method, a 2 m string was used and beads were placed at every 20 cm distance with a total of 10 beads. To estimate percent canopy/ground cover, this 2 m string was placed diagonally twice in rice+weed plots. The number of beads hitting either rice canopy or weed canopy was counted to estimate percent ground cover by rice or weed. The second ground cover assessment was performed through image analysis of

top pictures of the field, taken two times per week with a drone with a near infrared and red edge cameras (Fig. s5.4). For the image analysis, a full plot size was about 5.3 m^2. Only plots with rice and weeds (R+W) on a minimum of 4 m^2 (plots in red in Fig. s5.2) were analyzed. The NDVI (Normalized Difference Vegetation Index) data were retrieved for each plot and the PC (Percent Cover) was calculated for each variety. The PC (referred to as relative ground cover) at 24 dat of rice/14 dat of weed and at 52 dat of rice/42 dat of weed were compared with the manually measured ground cover at early, mid and late timepoints (17, 32 and 45 dat of rice) and to see if rather early or late ground cover correlated most with the weed shoot biomass at 52 dat of rice/42 dat of weed (Fig. s5.6 and s5.7). The value for the weed biomass for these plots was calculated following %weed = weed biomass/ total (rice+weed) biomass.

We estimated the time taken (days) to 50% and 99% ground cover for each variety using a sigmoid curve using a generalized linear model (GLM) with binomial function with adjusted Percent Cover (adjPC) (Fig. s5.5 and Table s5.1). Assuming that all the varieties reached a stage of closed canopy at maxPC (maximum Percent Cover), the adjPC equals the PC divided by the maxPC for a given variety. It was calculated for each timepoint. Practically, 99% of ground cover for a plot corresponds to canopy closure.

Weed yield could not be estimated because the trial was abandoned at 53 dat of rice / 43 dat of weed because of COVID-19 related lockdown in the country.

Experiment 3 (Greenhouse): Effect of artificial canopy shade coupled with complete submergence treatment on *E. crus-galli* development.

Seed origins and germination

E. crus-galli (biotype 09) seeds were originally collected by the Weed Science team at the International Rice Research Institute (IRRI), Los Baños, The Philippines, in a lowland field (IRRI – UD2), within the period Sept-Nov 2016. Seeds were then further bulked 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. For germination, dehulled seeds were put to germinate for 4 days in Petri dishes between two wet Whatman™ papers in an incubator (12 h light (120+/-50 µmol m^{-2} s^{-1}) 35°C and 12 h dark 25°C, 70% relative humidity).

Plant growth

Per pot, one seedling of *E. crus-galli* was transplanted at 4 days after sowing. Canopy pots were used (perforated Round Pots 6° Azalea – MXC 5,5 plastic pot of 5 cm diameter top, 3.5 cm diameter bottom, 5.5 cm depth, 78 ml) with a mixture of 50% black soil / 20% sand / 30% agra-vermiculite 0-1.5 mm + 20%

Yoshida nutrient solution (Yoshida, 1976) with a double iron dose (sequestreen= Fe-EDTA), pH 6.5 + osmocote NPK-Mg 15-4-9 (+1) (2.4 g/L of soil). Seedlings grew in the greenhouse for 12 days, in a 12 h light (200+/-20 µmol m^{-2} s^{-1}) 29°C / 12 h dark 24°C cycle conditions, with ventilation, in trays that were manually watered.

Shade and submergence treatment

To assess the effect of shading and submergence alone and in combination on *E. crus-galli* growth, a greenhouse study was conducted. The pot experiments were conducted in the greenhouse of the Botanical Gardens, The Science Park, Utrecht University. A total of four treatments were evaluated: (1) Shading stress only - mimicked by using a single layer of Lee Fern green filter, (2) complete submergence stress only, (3) combination of both treatments 1 and 2, and (4) no shading (transparent filter without impact on the light) and no submergence stress (normal watering) (=control conditions). This experiment was done once. When plants reached the 3-leaf stage, healthy plants with a homogeneous shoot height were selected and placed under the different treatments in ~240 L tanks (~60 x ~60 x ~64 cm) (Fig. s5.8). Six plants per treatment were used, except treatment 3 for which eight plants were used. For submergence, tanks were filled with tap water the day before submerging the plants, for water temperature acclimation. Tanks were all interconnected and connected to a bigger tank providing constant flowing water of 27°C. Water flowed through a UV pump connected in between to reduce algae growth. The temperature in the tanks below the filters was on average 33°C during the day. Light in control conditions (under a single layer of transparent plastic filter) at the plant level was 280+/-30 µmol m^{-2} s^{-1} PAR. Light in shade condition (under a single layer of Lee Fern green filter) was 100+/-10 µmol m^{-2} s^{-1} i.e. a reduction of half to two third of light intensity, which is similar to a reduction measured below rice leaves (410 to 200 µmol m^{-2} s^{-1} PAR). Control plants were also placed in the tanks with a transparent plastic filter on top but were not submerged. The duration of the treatment was continuous for 8 days (Fig. 5.4A).

Measurements

Plant shoot height was measured, and the number of leaves was counted at the start and after 3 and 8 days of treatment. Root length, shoot and root biomass were determined at the start and after 3 and 8 days of treatment. Tissues were weighed after 3 days at 80°C. Pictures were taken after 8 days of treatment.

Experiment 4 (Greenhouse): Effect of sequential or simultaneous combinations of artificial shade and complete submergence treatment on pre-submerged *E. crus-galli* development

Seed origins, germination, plant growth and application of shade and submergence treatment were the same as for experiment 3.

Sequential or simultaneous combinations of artificial shade and complete submergence treatment

The duration of the treatment was different to experiment 3, as the plants were first pre-submerged for 3 days before being subjected to the different combinations of artificial shade and complete submergence treatment for 5 days. All along the experiment, one batch of control plants was neither submerged nor shaded for comparison (Fig. 5.5A).

Measurements

Per treatment, 13 plants were measured. Plant shoot height was measured and the number of leaves was counted at the start and after 3 and 8 days of treatment. Root length, shoot and root biomass were determined at the start and after 8 days of treatment. Tissues were weighed after 3 days at 80°C. Pictures were taken after 8 days of treatment.

5/ Statistical analyses

All analyses were performed with the free software available at https://jamovi.com. For Experiment 1/ and 2/, PAR data, the number of leaves or tillers, shoot height, root and shoot biomass were analyzed with a 1-way ANOVA (variety) followed by a Tukey post hoc test (Fig 5.1, 5.2 and 5.3). Correlation plots with the Pearson correlation values were generated for each interaction between the manually measured early, mid and late ground cover (beaded string count) with the early and late relative ground cover assessed through image analysis (PC) from high and low shade-casting rice varieties with *E. crus-galli* end shoot biomass at 43 dat of weed at normal and high density planting (Fig. s5.6 and s5.7). A 2-way ANOVA (variety*density) test was performed on end weed biomass (43 weed dat) to see the impact of both factors on weed development by these two treatments combined. Data used for the statistical analyses was the average of weed shoot biomass of *E. crus-galli* plants at 53 rice dat and 43 weed dat for all R+W plots or for all R+W plots excluding incomplete plots (V1/V2/V5) and poorly-established variety V8. W-only plots are not included in the analyses (Table 5.2). For experiments 3/ and 4/, the number of leaves, shoot and root lengths and shoot and root biomass were analyzed with a 2-way ANOVA (treatment*timepoint) followed by a Tukey post hoc test (Fig 5.4 and 5.5).

Results

Effect of rice interference on *E. crus-galli* development

A rice-weed competition experiment was performed in the greenhouse to evaluate the suppressive effect of a selection of rice varieties on *E. crus-galli*. Since the goal was to investigate the weed-suppressive effects of shade and determine how early the shade would influence weed development, the rice

varieties chosen varied in their 'shading capacity'. We selected two high shade-casting varieties (Mudgo and Shim Balte) and two low shade-casting varieties (Della and Luk Thakar). This classification is based on the predicted shading capacity of these rice varieties achieved through a combination of different shoot architectural traits as described in Huber et al. (2021). Rice seeds were sown two weeks prior to the weed to mimic a typical field situation where rice gets a competitive advantage over the weed and time to shade it. To confirm the shading classification of the selected rice varieties, the PAR was measured at the soil level of the developing rice canopies (thus reflecting the light intensity and quality that the weed would experience) every week till harvest. In the panel of four rice genotypes used here, PAR reduction was significant compared to control conditions observed from 18 days after rice sowing (das) (Fig 5.1A). At 28 days after rice sowing, PAR was significantly lower under two high shade-casting varieties compared to the two low shade-casting ones, especially under Mudgo as compared to Luk Takhar (Fig 5.1A).

This correlated with a greater weed suppression reflected in a stronger reduction in the number of leaves and tillers and the shoot and root biomass of *E. crus-galli* grown under the canopy of the two high shade-casting varieties versus the low shade-casting ones and compared to the weed-only plots, at 42 das of rice or 28 das of weed (Fig 5.1 B-F). *E. crus-galli* grown under the canopy of the high shade-casting Shim Balte variety showed a shoot biomass reduction of 50% compared to the W-only control plots and of about 55% compared to under the canopy of the low shade-casting Luk Thakar. *E. crus-galli* grown under the canopy of the high shade-casting Mudgo variety showed an even greater reduction with about 75% shoot biomass reduction compared to the W-only control plots and of about 72% compared to under low shade-casting Luk Thakar. For all parameters followed, we observed a greater reduction of weed development (number of tillers and leaves) for Mudgo than for Shim Balte. The Della variety, although qualified as a low shade-casting variety, also caused a reduction of all weed development parameters measured, but its shading capacity (PAR) was greater than for Luk Thakar.

Effect of low and high shade-casting rice varieties on *E. crus-galli* development in the field at normal and high rice planting density

Following the confirmation of the weed suppressive effects of rice associated with shading capacity, we next investigated the effect of crop competition on *E. crus-galli* performance under a field setting. The weed was again grown together with rice, but the selection of rice varieties was expanded. A panel of 11 rice genotypes was used, ranging from high to low shading ability (Table 5.1) (Huber et al., 2021). The shading capacity of these rice varieties was estimated using different shoot architectural traits as described in Huber et al. (2021). In addition, each of the rice varieties was transplanted at a normal density (20 x 20

cm - 25 plants / m^2) and at a high density (20 x 10 cm - 50 plants / m^2) to further exaggerate the shading effect and assess the effect on weed (Fig. s5.2 and s5.3). The density of *E. crus-galli* seedlings (50 plants / m^2) did not vary and they were transplanted to the fields 10 days after rice transplantation.

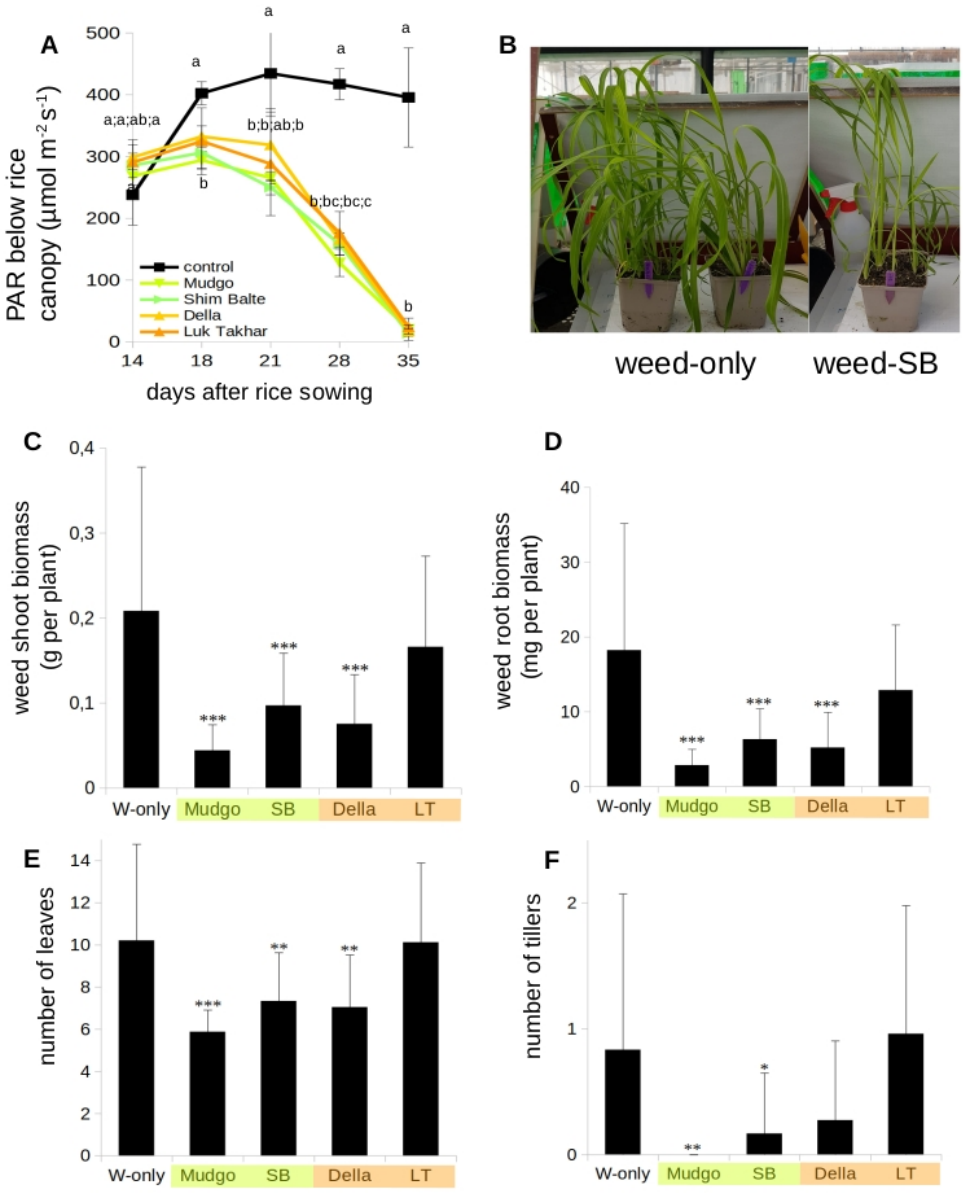


Figure 5.1: Effect of variable rice shading capacity on *E. crus-galli* development.
A. Absolute Photosynthetically Active Radiation (PAR) values measured over time at the soil level below developing canopies of four different rice varieties in rice-only plots. Measurements were repeated four times for control (above canopies) and six times below each rice variety canopy per timepoint, starting at 14 rice days after sowing (das), which corresponds to the sowing time of the weed. Values are means +/- SD. Significance per group for p-val< 0.05 is indicated with letters from Tukey post hoc test after 1-way ANOVA (variety).
B. Representative images of weeds growing in weed-only plots (left) or below the canopy of the high shade-casting variety Shim Balte (right) at 28 das of weed.
C-F. The effect of four rice varieties varying in shading capacity on *E. crus-galli* shoot biomass (**C**), root biomass (**D**), number of leaves (**E**) and number of tillers (**F**) at 42 rice das and 28 weed das. Rice and weed plants were grown in separate pots. High shade-casting varieties are coloured in green and low shade-casting varieties in orange SB= Shim Balte, LT= Luk Thakar. n= 24 plants. Values are means +/- SD. *= p-val< 0.05, **= p-val< 0.01, *** = p-val< 0.001 from Tukey post hoc test after 1-way ANOVA (variety).

At normal rice density (Fig. 5.2), weed shoot height showed a moderate but significant reduction compared to weed-only plots at the later timepoint, although this was observed only for around half of the tested rice varieties (Fig. 5.2A). A more pronounced effect was observed for tiller number (Fig. 5.2B). Compared to the weed-only plots (W), weed tiller number was strongly reduced when growing with rice (except V02 and V06) until about 50% maximum when growing with V11, at the 25 dat of rice (Fig. 5.2B). This trend was maintained at the 49 dat of rice, and growth with rice (except with variety V08) reduced weed tiller number by half (compared to the weed-only plot). At 53 dat of rice, rice also reduced weed shoot biomass by about 45-65% (except variety V08) (Fig. 5.2C). The four high shade-casting rice varieties (V02, V04, V05 and V07) reduced weed development, with V05 and V07 performing the best in suppressing all three measured weed traits. Interestingly, two low shade-casting rice varieties (V03, V06) efficiently reduced weed growth, while the intermediate shade-casting variety V08 was ineffective at weed suppression in the field, attributed to the poor establishment of this variety in the field.

At the high rice density planting, all the measured weed development traits were more strongly affected compared to the normal planting density, at the later timepoint and compared to the W-only plots (Fig. 5.3). A statistical analysis evaluating the effect of the factors “rice density” and “rice variety” for the weed biomass at the later timepoint is presented in Table 5.2. The planting density is indeed a significant influence even when excluding varieties that are incomplete (with less than 3 replicated field plots: V01/V02/V05) or poorly established (V08). Rice planting density can therefore be an important factor for weed suppression, and doubling the rice density seems more effective than differences between the selected varieties at the field level. However, the importance of optimizing the architecture of individual plants remains relevant. As for the low planting density,

the most pronounced effect at both timepoints was observed for weed tiller number except in the R+W V08-weed plot. Weed shoot height and biomass were significantly reduced only at the late timepoint by all rice varieties (except V01, V05 and V08 for height). Stronger reduction in all traits was observed with the four high shade-casting varieties V02, V04, V05 and V07, but also with the varieties V03 and V06, classified as low shade-casting. Compared to normal rice density plantation, weed shoot biomass was on average reduced two times more in high rice density plantation. The reduction in tiller number was twice more than in the low density rice planting and was observed for the high shade-casting varieties V02 and V04, low shade-casting varieties V03 and V06 and the intermediate variety V09. The variety V05 did not show this reduction in tiller number, which is due to a lack of plot repetitions for that variety at high density.

Table 5.1: Rice varieties used in the field assay and their shading ability based on Huber et al. 2021.

Code	Variety	Shading capacity
V01	Black Gora	middle
V02	Shim Balte	high
V03	IR64 21	low
V04	Sabharaj	high
V05	Sathi	high
V06	DJ 123	low
V07	Mudgo	high
V08	Wab 501	middle
V09	Criollo	middle
V10	DM 56	middle
V11	Katihan 2	?

In addition to weed traits, we also measured the time of rice+weed canopy closure in the field plots. A faster canopy closure is strongly correlated with higher shading capacity and improved crop competition over weeds. To assess the development of the rice and weed over time, aerial drone images of the fields (Fig s5.4) were analyzed. A sigmoid curve using a generalized linear model (GLM) with a binomial function was generated to calculate the number of days until 50% and 99% of ground cover. The average number of days to reach 99% of ground cover indicating canopy closure was determined for each rice variety at normal and high density (Fig. s5.5B and Table s5.1). This value helped to describe the speed of

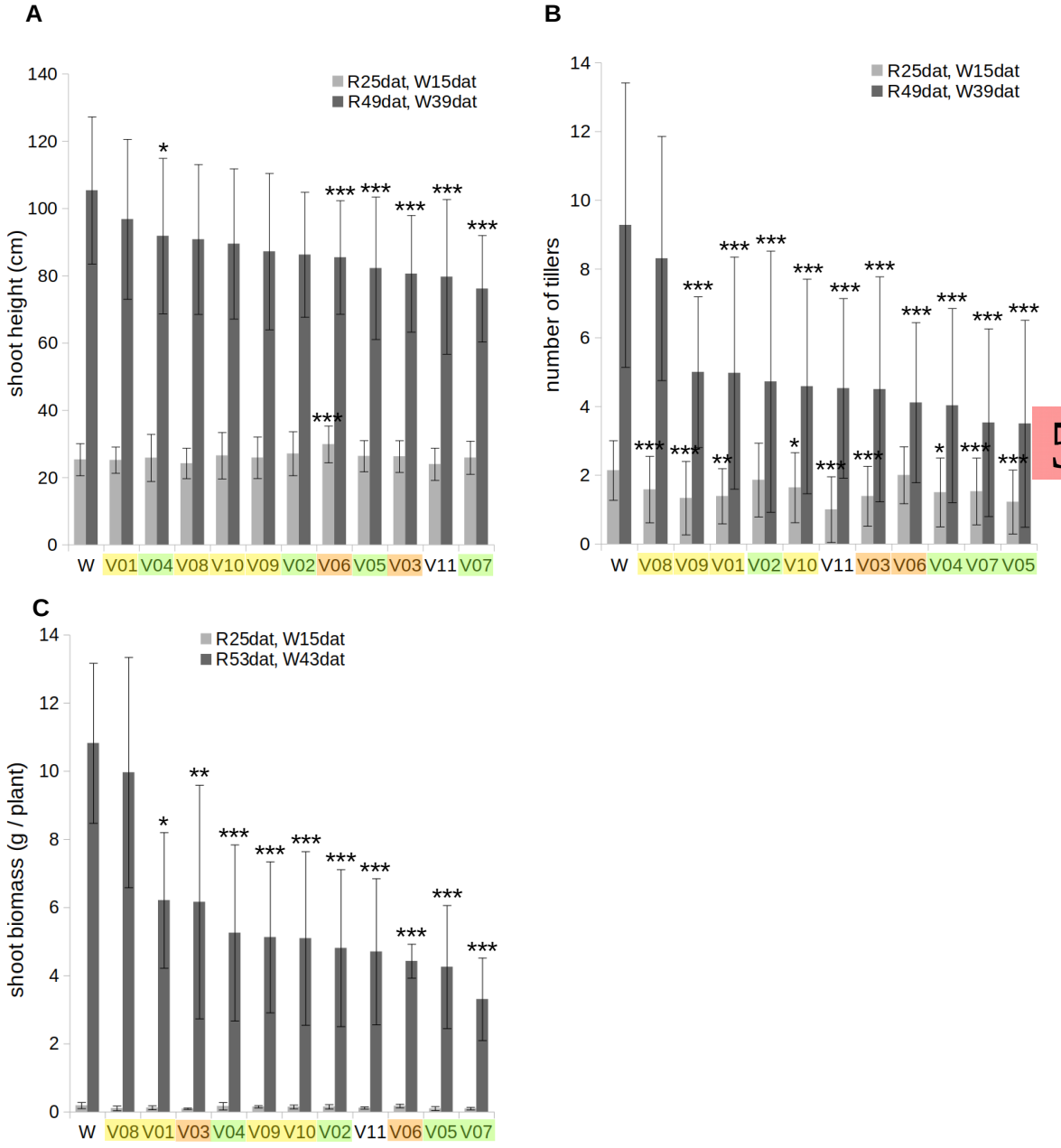


Figure 5.2: Effect of low and high shade-casting rice varieties on *E. crus-galli* development in the field at normal rice density planting.
A-C. Shoot height (**A**), number of tillers (**B**) and shoot biomass (**C**) of *E. crus-galli* plants. The two sampling points shown are when the 'days after transplanting' (dat) to the field for rice and weed was 25 d and 15 d respectively (R25dat, W15dat; light gray bars) and 49/53 d or 39/43 d respectively (R49/R53 dat, W39/43 dat; dark gray bars). Rice was transplanted at normal density (20 x 20 cm). Measurements were done on n= 36 plants. Values are means +/- SD. *= p-val< 0.05, **= p-val< 0.01, ***= p-val< 0.001 from Tukey post hoc test after 1-way ANOVA (variety). The x-axis indicates various rice varieties (V01-V11) as also indicated in Table 5.1. High shade-casting varieties (V02, V04, V05, V07) are coloured in green, low shade-casting varieties (V03, V06) in orange, intermediate shade-casting varieties (V08, V09, V10) in yellow and the unknown shade-casting variety in white (V11). W indicates weed-only plot.

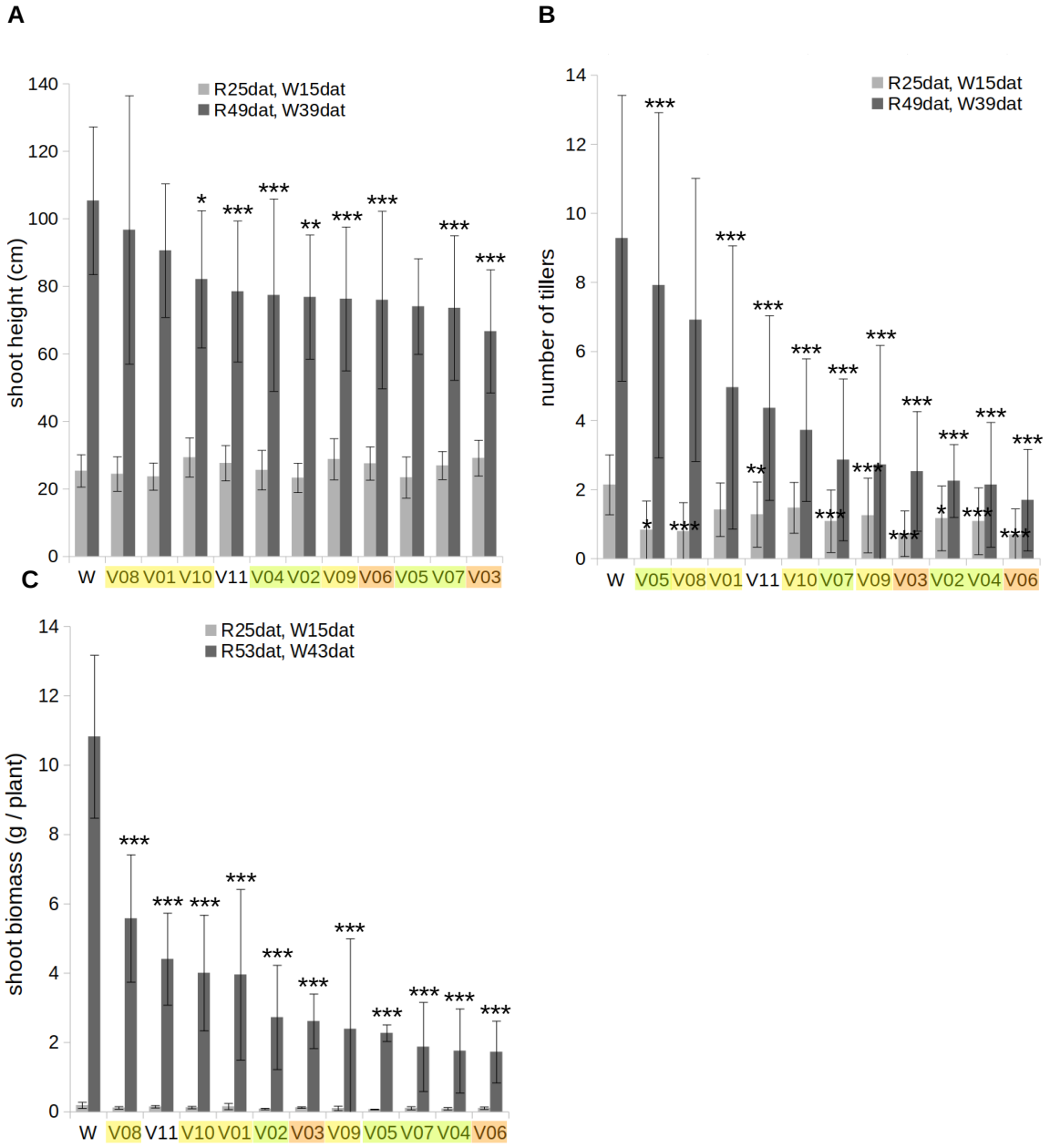


Figure 5.3: Effect of low and high shade-casting rice varieties on *E. crus-galli* development in the field at high rice density planting.
A-C. Shoot height (**A**), number of tillers (**B**) and shoot biomass (**C**) of *E. crus-galli* plants. The two sampling points shown are when the 'days after transplanting' (dat) to the field for rice and weed was 25 d and 15 d respectively (R25dat, W15dat; light gray bars) and 49/53 d or 39/43 d respectively (R49/R53 dat, W39/43 dat; dark gray bars). Rice was transplanted at high density (20 x 10 cm). Measurements were done on n= 36 plants, except for V01 and V08 (24 plants) and for V02 and V05 (12 plants). Values are means +/- SD. *= p-val< 0.05, **= p-val< 0.01, ***= p-val< 0.001 from Tukey post hoc test after 1-way ANOVA (variety). The x-axis indicates various rice varieties (V01-V11) as also indicated in Table 5.1. High shade-casting varieties (V02, V04, V05, V07) are coloured in green, low shade-casting varieties (V03, V06) in orange, intermediate shade-casting varieties (V08, V09, V10) in yellow and the unknown shade-casting variety in white (V11). W indicates weed-only plot.

Table 5.2: Interaction effect of different shade-casting rice varieties and density on *E. crus-galli* shoot biomass in the field.
A-B. Data used for the statistical analyses were the average of weed shoot biomass of *E. crus-galli* plants at 53 rice dat and 43 weed dat for n= 6 plants (with 2 sampling locations of 6 plants for each of the 3 replicated plots). Significant differences for rice variety effect (var), density effect (dens) and the interaction variety*density (var*dens) are shown for all R+W plots (**A**) or for all R+W plots excluding incomplete plots (V1/V2/V5) and the poor-established variety V8 (**B**). W-only plots are not included in the analyses. Significant differences are indicated with *= p-val< 0.05, **= p-val< 0.01, ***= p-val< 0.001, ns= non significant from 2-way ANOVA (variety*density).

A ANOVA - end weed biomass - all plots

	Sum of Squares	df	Mean Square	F	p	
variety	194.0	10	19.40	4.633	< .001	***
density	150.4	1	150.41	35.911	< .001	***
variety ✻ density	39.3	10	3.93	0.937	0.503	ns
Residuals	410.5	98	4.19			

B ANOVA - end weed biomass - complete and well-established plots

	Sum of Squares	df	Mean Square	F	p	
variety	42.6	6	7.09	1.91	0.091	ns
density	100.4	1	100.42	27.03	< .001	***
variety ✻ density	28.4	6	4.74	1.27	0.280	ns
Residuals	260.1	70	3.72			

canopy closure per rice-weed plot and provided an indication of the timeline of shading on the weeds by the crop. At normal density, the top three varieties reaching canopy closure the fastest were the high shade-casting varieties V05 and V04 in 35.2 and 36.6 dat respectively, and the intermediate shade-casting variety V09 in 36.8 dat. At high density, the ranking changed. The top three fastest canopy closure times were recorded for the high shade-casting variety V07 (35.7 dat) and the two low shade-casting varieties V03 and V06 in 35.7 and 36.6 dat respectively (Table s5.1 and Fig. s5.5B). In general, the increase in planting density does not appear to dramatically decrease the time to canopy closure (35.2-42.5 dat in normal density and 35.6-43.8 dat in high density). We also determined the number of dat to reach 50% ground cover (Fig. s5.5A) to see if complete canopy closure correlated with earlier partial canopy shade. While the ranking was similar between normal and high density, it was more distinct when comparing the values at 50% and 99% ground coverage. Notable exceptions at normal density were the high

shade-casting varieties V05 and V04. These were amongst the fastest to reach 50% of ground cover and subsequently also achieved earlier canopy closure.

To assess how early canopy closure affects weed performance, we correlated all ground cover values (measured manually or drone-based) at early, mid, and late timepoints of normal and high rice planting density per rice variety with the end shoot biomass of the weed. At both rice densities, the two methods showed a strong positive correlation. This was +0.64 and +0.66 at early/mid timepoints (24 and 17/32 rice dat) in normal density and +0.33 and +0.63 in high density (Fig. s5.6 and s5.7). Lower correlations between the two methods were noted at later timepoints (52 rice dat and 45 rice dat) in normal density (+0.33) and high density (+0.50). The high shade-casting varieties mainly clustered at the left of the graph (green dots), associated with a stronger reduction of weed biomass at 43 weed dat and with a higher ground cover (all timepoints). At lower planting density, weed biomass at 43 weed dat correlated more strongly and negatively with ground cover at early timepoint (rel.gr.cov.early at 24 rice dat) with image analysis and at mid-timepoint for manual data (%cov.mid at 32 rice dat) (Fig. s5.6). For the mid-timepoint, the negative correlation of ground cover with the weed biomass at 43 weed dat was -0.76 in normal density and -0.66 in high density. Late ground cover (rel.gr.cov.late at 52 rice dat and %cov.late at 45 rice dat) showed a poor correlation with the weed biomass at 43 weed dat in both normal density (-0.15 with rel.gr.cov.late and -0.27 with %cov.late) and high density (-0.43 with rel.gr.cov.late and -0.27 with %cov.late).

Effect of sequential or simultaneous combinations of artificial shade and complete submergence treatment on *E. crus-galli* development

Following confirmation of the effectiveness of shading on suppressing weed development, we therefore tested whether combining a shade treatment with flooding would be a more effective and efficient weed management method than either one alone. We used two combinations of flooding and shade: simultaneous or sequential, to determine the most efficient stress pattern to reduce weed development. Experiments were performed in the greenhouse, and shade was mimicked using a layer of green filter, thus allowing a more controlled manipulation of light conditions without any rice allelopathic interference at the root level and an easier combination of the two stress treatments (Fig. s5.8).

First, we tested the effect of similar durations of shade and submergence (8 days) single stresses and a simultaneous combination of both stresses (Fig. 5.4A). The use of artificial shade with a green filter resulted in a reduction of half to two third of PAR (280 to 100 µmol m^{-2} s^{-1}), which corresponds to the difference above and below the closed rice canopy measured in a different set-up (410 to 200 µmol m^{-2} s^{-1}). The two single stress treatments also allow for a comparison of the relative effect of shade compared to submergence (Fig. 5.4A). After 3 days of

treatment, only root length was significantly reduced for all treatments. After 8 days, a significant reduction of all measured traits (number of leaves, shoot and root length, shoot and root biomass) for the treated plants was seen compared to the control plants. Shade stress and submergence stress did not differ from each other in terms of weed growth suppression in all the measured development traits. Compared to the control plants, plants under shade or submergence stress had one or two leaves less, shoot length was one third shorter and root length was two third shorter and shoot and root biomass were reduced more than 6 to 10 times respectively (Fig. 5.4 and Fig. s5.9A). We observed that either stress alone already led to a severe growth suppression that was not further increases when the two stresses were applied together.

Since submergence and shade were both comparably efficient in suppressing *E. crus-galli* development, and a combination of both treatments did not exaggerate the effects, we investigated the effects of sequential stress combinations. We explored whether a pre-treatment of submergence could be followed by shade treatment to reduce weed growth. This combination would also reduce water usage in fields as shade would increase with the development of the rice canopy and this could replace water treatment for the rest of the crop cycle. To test this in the greenhouse, we first pre-submerged plants for 3 days, and we subsequently applied either single submergence or shade treatments, or the combination of both for 5 days (Fig. 5.5A). After 3 days of submergence pre-treatment, a significant effect was observed only for leaf number. Submergence caused the reduction in leaf number for all treatment groups except for the group that was under control condition following desubmergence (Fig 5.5B). After 8 days, a significant reduction was observed for all measured traits (number of leaves, shoot and root length, shoot and root biomass) in all treatments compared to the control plants. In addition, we noticed a significant reduction in the number of leaves, root length and shoot biomass of plants that got another stress treatment after being 3 days pre-submerged, compare to the control plants that only were pre-submerged for 3 days. In the submergence, shade and combination treatment groups, no difference was noted, except for shoot length as submerged plants were significantly elongated. A 3-day submergence treatment already had a noticeable effect on weed development compared to the control plants: a slight reduction in leaf formation, a reduction in shoot and root length and a reduction of about 75% of shoot biomass and 85% of root biomass (Fig. 5.5B-F and Fig. s5.9B).

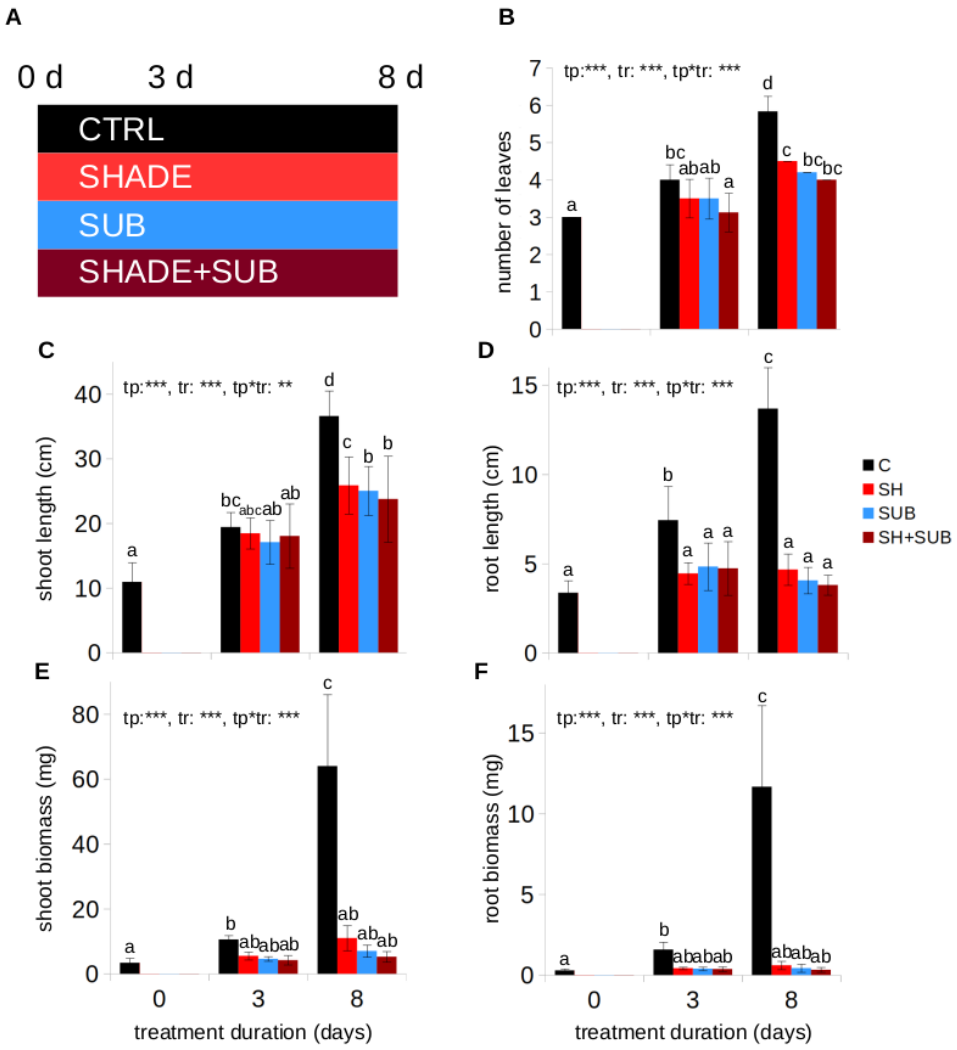


Figure 5.4: Effect of simulated canopy shade coupled with complete submergence treatment on *E. crus-galli* development.

A. Experimental design of the experiment for studying the effect of artificial shade coupled with complete submergence treatment on *E. crus-galli.* sub= submergence. Canopy shade was mimicked by using a single layer of Lee Fern green filter which reduced the PAR to 1/2-1/3. Measurements were done at 0, 3 and 8 days.

B-F. Leaf number (**B**), shoot (**C**) and root (**D**) length, shoot (**E**) and root (**F**) biomass of *E. crus-galli* plants at the start and after 3 or 8 days of single (C= Control; SH = shade; SUB= submergence) or combined (SH + SUB= shade + submergence) treatment. n= 5-8 plants. Values are means +/- SD. Significant differences (*= p-val< 0.05, **= p-val< 0.01, ***= p-val< 0.001) from Tukey post hoc test after 2-way ANOVA (treatment*timepoint) for timepoint (tp), treatment (tr) and the interaction timepoint*treatment (tp*tr) are shown on top of the graphs. Significance per group for p-val< 0.05 is indicated with letters.

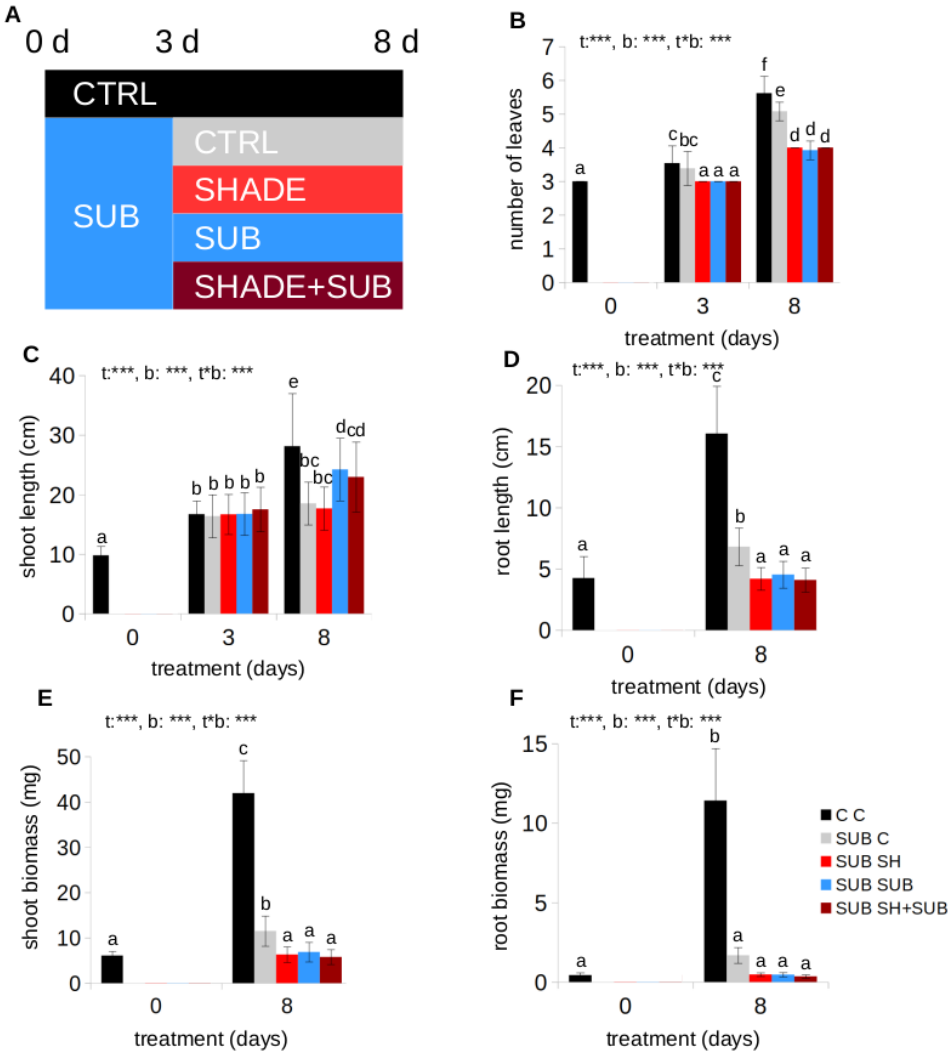


Figure 5.5: Effect of sequential or simultaneous combinations of simulated canopy shade and complete submergence treatment on pre-submerged *E. crus-galli* development.
A. Experimental design of the experiment for studying the effect of artificial shade coupled with complete submergence treatment on *E. crus-galli* plants that were pre-submerged for 3 days. sub= submergence. Canopy shade was mimicked by using a single layer of Lee Fern green filter which reduced the PAR to 1/2-1/3. Measurements were done at 0, 3 and 8 days.
B-F. Number of leaves (**B**), shoot (**C**) and root (**D**) length, shoot (**E**) and root (**F**) biomass of *E. crus-galli* plants at the start of the experiment (0 days), after 3 days of submergence pre-treatment and subsequently a further 5 days of control conditions (SUB C), shade (SUB SH), submergence (SUB SUB) or combined shade and submergence (SUB SH+SUB). n= 13 plants. Values are means +/- SD. Significant differences (*= p-val< 0.05, **= p-val< 0.01, ***= p-val< 0.001) from Tukey post hoc test after 2-way ANOVA (treatment*timepoint) for timepoint (tp), treatment (tr) and the interaction timepoint*treatment (tp*tr) are shown on top of the graphs. Significance per group for p-val< 0.05 is indicated with letters.

Discussion

The documented sensitivity of *Echinochloa spp.* to shade presents a promising avenue for managing its invasiveness in rice fields. It implies that weed suppression could be potentially achieved via the use of rice varieties with superior shade-casting properties. Rice-weed competition experiments performed both in the greenhouse and in field conditions supported this hypothesis. In general, our results demonstrated that shade cast by crop (rice) interference is effective in suppressing the growth of *E. crus-galli.*

Weed competitive rice varieties

The imposition of shade either artificially or by shade-casting varieties alone or in combination with high rice planting density effectively reduced weed development as reflected in a significant reduction in almost all measured weed traits. Amongst the rice varieties tested, V05 (Sathi) and V07 (Mudgo), both previously classified as high shade-casting (Huber et al., 2021), emerged as the best candidates for suppressing weed development in the field through shade, even at a low planting density. These varieties also achieved canopy closure relatively fast (Table s5.1). Mudgo also showed a significant reduction of weed growth in the greenhouse pot assay. These varieties ranked very high for shading ability in Huber et al. (2021) and are characterized as being tall with a large and compact developed shoot area, high droopiness of the leaves and larger tiller angle than other varieties at early vegetative stage. Two other high shading varieties 02 (Shim Balte) and 04 (Sabharaj) also performed well with respect to weed suppression but were delayed in the days needed to reach canopy closure. Shim Balte (SB) canopies did show a good reduction in PAR and correspondingly effectively suppressed weed development in the greenhouse pot assay. While in general all the high shade-casting varieties performed well with respect to restricting weed development, the field data did not indicate a very strong correlation with shading capacity. Notably, the two varieties previously identified as low shade-casting (V03 - IR64 and V06 - DJ123) also performed well in reducing weed development (all measured traits) at both densities. The ranking of the rice varieties in Huber et al., (2021) was based on plants grown in single pots, and not in community plots as it was done here with the field assay. In the field, several other factors might affect the competitive ability of rice, such as potential weed allelopathic effects on rice (Sitthinoi et al., 2017; Majeed et al., 2018; Khanh et al., 2018), proximity with neighbouring rice plants which can modify shoot architecture, or the microbiome that could affect some rice varieties more than others (Edwards et al., 2019; Wang et al., 2020). *E. crus-galli* is documented to affect rice growth by using until 60-80% of the nutrients in soil, thus depriving rice roots of much needed nutrients (Wilson et al., 2014).

Importance of early shade for weed control

The monitoring of the dynamics of canopy closure using both drone-based imaging and manual measurements (beaded string) revealed the importance of early shading and faster canopy closure for weed suppression. Early and mid-season rice ground cover negatively correlated with later season weed biomass in both normal and high-density treatments and appeared to have the strongest effect on reducing weed biomass. This indicates that genetic variation in early ground cover has a strong effect on weed growth. However, at the early timepoint, this correlation was weak for the drone data. This can be explained by the noise from the pixels from the ground at early stages of crop development (water, soil and thin leaves). In the later vegetative stage, complete canopy closure is reached (plateau), so percent cover and weed biomass do not correlate much (-0.15 under normal and -0.43 under high rice planting density). In general, the manual ground cover estimation was more accurate (stronger correlation in both density plots). However, the drone-based imaging analyses was very efficient for retrieval of the data for 50% of ground cover and for 99% (canopy closure). These values were very useful in associating shading capacity of rice cultivars with weed suppression. Thus, an effective weed suppressing variety must not only possess relevant shade-casting traits, but these traits must also be expressed relatively early in the canopy development. Kropff van Laar (1993) demonstrated that only few days of crop advantage could make the difference in the balance between crops and weeds. A greater early rice growth and tillering ability have been repeatedly described as producing higher rice grain yields when in competition with weeds (Wu et al., 1998; Zhao et al., 2006).

Importance of rice planting density for weed control

Growth dynamics are different at high planting densities and in this situation, genetic variation in early cover had little influence on weed cover. Earlier canopy closure can be achieved by choosing high shade-casting varieties but also higher rice planting density. At a doubled density as compared to standard, the high shade-casting varieties V04 and V05 were among the slowest varieties, needing more days to reach canopy closure (43.6 and 38.6 dat) although at normal planting density they were the fastest varieties to reach canopy closure (36.6 and 35.2 dat). Clearly the planting density influences the canopy closure time for some varieties. In this aspect, V05 emerged as a promising variety. It had significant weed development reduction at both densities and achieved earlier canopy closure at normal density (35.2 dat). However, at higher densities, it takes 3 more days (38.6) to close the canopy. This can be explained by the rice elongating, rather than augmenting tillers number and biomass, as the rice plants are closer together (Thesis Martina Huber, 2022).

At later timepoints, the rice variety factor is much more important than the density factor. There was little time difference in canopy closure (99% ground

cover) between normal and high planting density (Fig s5.5A and B). Therefore, it is better to use higher shade-casting rice varieties than a higher density, as at normal density, rice plants are healthier (also observed in Saju et al., 2019), they produce more tillers and so probably more yield per plant, to achieve similar shading of the weed. In addition, increasing the rice planting density in the field would automatically call for more seedlings, with higher labour and energy requirements for their growth in the nursery and transplanting. Moreover, the weed *E. crus-galli* is not taller at high rice densities and does not compete with the rice for light and it is still shaded. At higher densities, the elongation triggered by light competition between the rice seedlings will divert energy usage from grain formation and filling and negatively impact rice yield. However, it must be noted that the weed shoot biomass and tiller number are twice as much reduced in the higher rice density plots as compared to normal planting density plots and it is still an interesting factor to manipulate for weed suppression. A reduction in *E. crus-galli* tillering due to higher rice densities has been previously observed especially at high *E. crus-galli* density (more than 30 plants / m^2) (Mennan et al., 2012). In addition to the link between biomass and seed yield, the number of tillers can also be linked to the panicle density and so the weed seed production and reducing it could therefore diminish field infestation (weed seed bank) (Kawano and Tanaka, 1968; Miller et al., 1991; Wu et al., 1998). It might also delay weed seed production so that rice could be harvested before weed seeds would start shattering and contaminating the rice.

Towards a new water- and shade-based protocol to test in the field for weed suppression

While our study identified candidate rice varieties for effective weed suppression, more sustainable weed management for longer term would require a combination with other methods as part of an integrated weed management protocol. It is especially promising to avoid yield losses due to weeds in areas where farmers do not have a lot of options for weed control or in low input agriculture systems as it can help reducing herbicide use. Even if early weed control is managed by other methods, weed competitive varieties can suppress later emerging weeds. Generally, there is a negative linear relationship between rice grain yield and weed biomass and so the highest reduction in weed biomass we observed (80%) can have a significant impact on rice yield.

In this study, we explored the potential of a combination of shade and flooding as an alternative weed management strategy for *E. crus-galli* in rice cultivation. These experiments revealed a similar effect on weed development upon treatment with equal duration of artificial shade or complete submergence. However, combining the two treatments did not lead to any additive effects. While this does not serve the purpose of more severe weed suppression, it does offer possibilities for adjustments to current water-based weed management strategies.

As our sequential stress experiments also reveal, replacing part of the flooding phase in the field with shade (imposed by high shade-casting varieties or density manipulation) would help to reduce the use of herbicides at later stages, but also minimize water usage. Additionally, this type of protocol will also potentially reduce the methane emissions from stagnant water in the field (Adhya et al., 1994; Wassmann et al., 2000; Nayak et al., 2006).

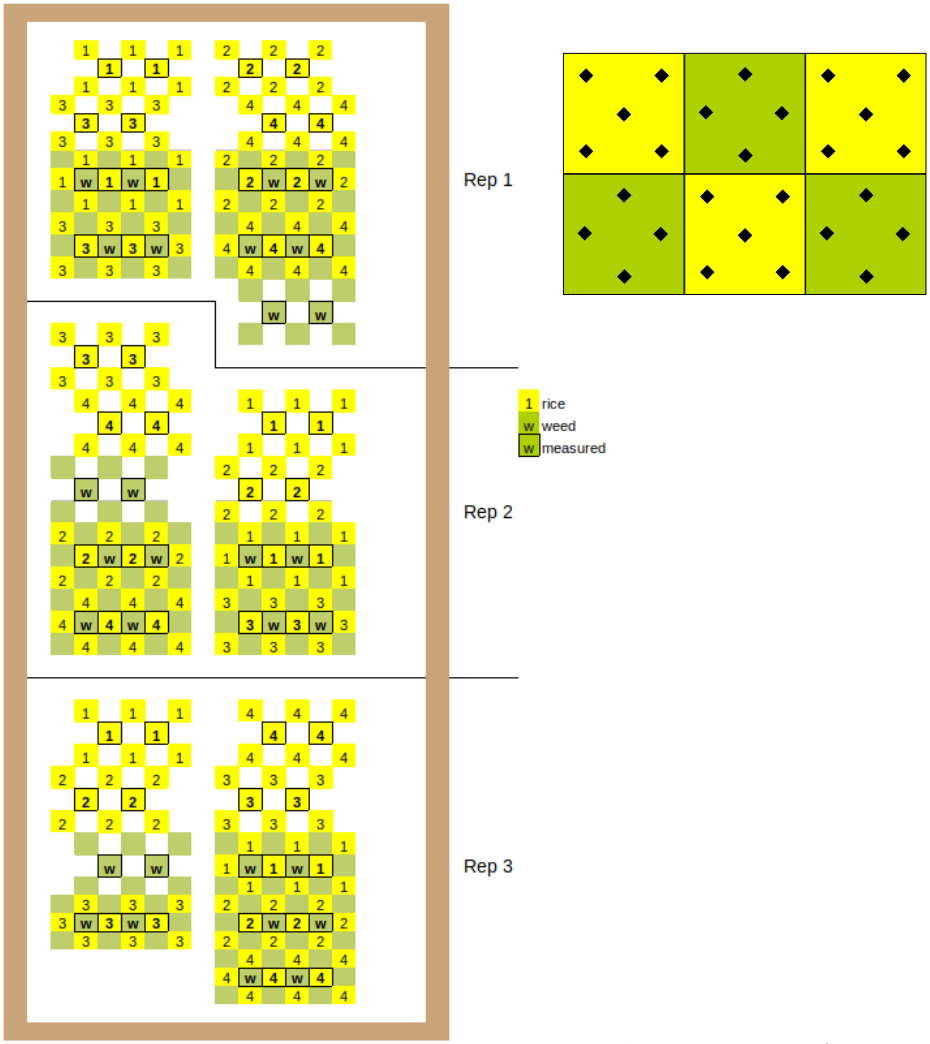
Towards implementation of this research

Most rice-weed competition assays examined traits in the late vegetative, maturation and panicle stages. Not many monitored rice competitiveness from the early stages after crop transplantation and could correlate the early potential of different rice varieties to efficiently reduce weed growth.

While weed yield was not measured in our experiments, the observed negative impact on vegetative traits suggests a likely impact on yield. The high shading rice varieties identified here can help reducing weed infestation in both dry fields and fields using water-based weed management, by their early vigour and providing early canopy closure. These varieties also possess specific traits/genes responsible for weed suppression (Huber et al., 2021). While these are not the highest yielding varieties, these selected cultivars can be a source for high yielding breeding varieties for weed suppression by transferring weed suppressive traits. Finally, the shade and flooding combinations proposed to combat *E. crus-galli* still need field testing, to determine the best time frame for the switch from water-based management to shade-based management. However, our results provide a promising start.

Supplemental data

A



B

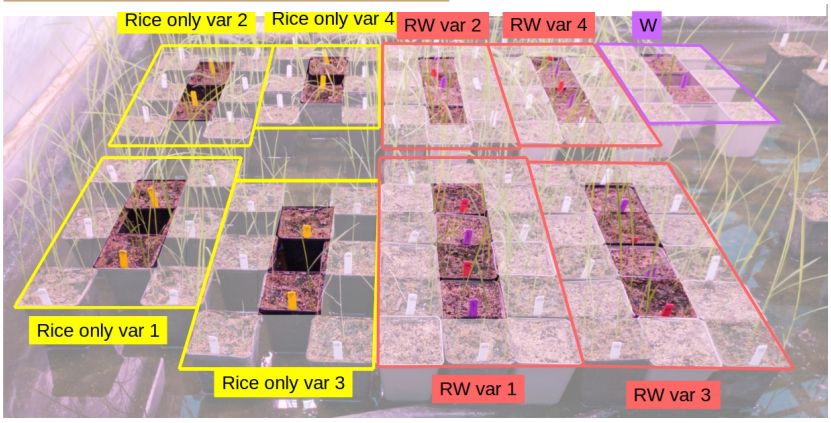
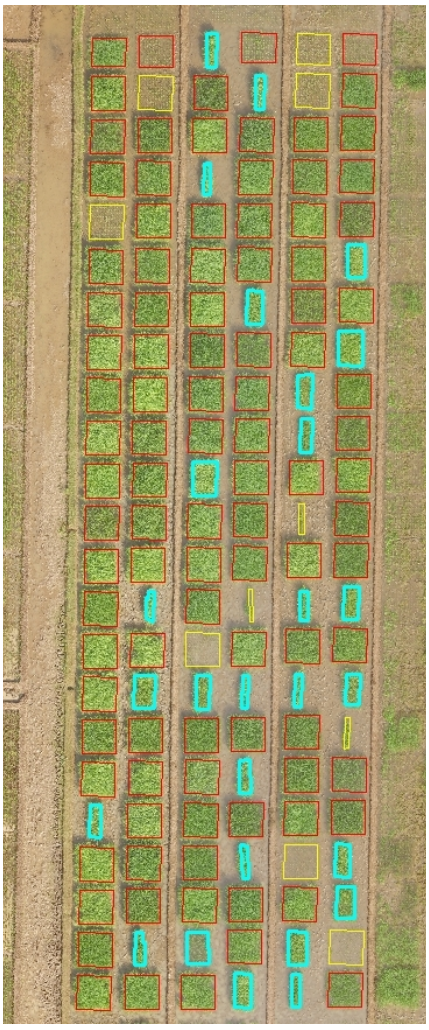


Figure s5.1: Experimental design of the rice-weed competition experiment in the greenhouse.
A. Schematic showing the experimental design. “w” indicates the weed pot and “1” to “4” the rice pots with varieties 1 to 4. The plants measured were in the pots indicated in bold and with borders. The plots are repeated three times in the set up. On the right side are shown a subset of the sowing pattern for rice (yellow pot - 5 plants) and for the weed (green pots - 4 plants).
B. Image of the experimental set up, Rep 1. “Rice-only” pots are indicated in yellow, “Weed-only” pots in purple and “Rice-Weed” pots in red. The highlighted pots in the picture indicate the pots per replication block with measured plants.

A



B

Rep1		Rep 2		Rep 3	
weeds	V8-R-10	V9-R-10	V7-R-10	V1-R-10	V8-RW-20
V2-RW-10	V11-R-10	V10-R-20	V6-R-20	V5-R-10	V3-RW-10
V11-R-20	V8-RW-10	V4-R-10	V9-RW-20	V11-RW-10	weeds
V11-RW-10	V2-RW-20	V5-R-10	V10-RW-10	V10-RW-20	V10-R-20
V8-R-20	V8-RW20-	weeds	V10-RW-20	V9-RW-20	V5-R-20
V6-RW-20	V11-RW-20	V7-RW-10	V2-RW-10	V3-RW-20	V5-RW-20
V7-R-20	V1-RW-20	V9-RW-10	V11-R-20	V10-R-10	V9-RW-10
V7-RW-10	V4-RW-10	V11-R-10	V8-RW-20	V7-RW-20	V3-R-10
V3-RW-20	V6-R-20	V6-RW-20	V2-RW-20	V4-R-20	V11-R-20
V7-RW-20	V6-RW-10	V8-RW-10	V4-RW-20	V8-RW-10	V4-RW-20
V9-RW-20	V10-RW-20	V1-RW-10	V11-RW-10	V4-RW-10	V11-R-10
V10-R-20	V9-RW-10	V1-RW-20	V3-RW-20	V8-R-10	V11-RW-20
V1-RW-10	V5-R-10	V5-RW-20	V8-R-10	V7-RW-10	V9-R-10
V3-RW10-	V1-R-20	V11-RW-20	V7-RW-20	V2-R-20	V2-RW-20
V4-R-20	V2-R-20	V1-R-10	V5-R-20	V6-RW-20	V7-R-20
V5-RW-20	V3-R-20	V2-R-20	V6-R-10	V7-R-10	V2-R-10
V10-RW-10	V9-R-20	V3-R-10	V1-R-20	V10-RW-10	V6-R-20
V5-RW-10	V7-R-10	V7-R-20	V10-R-10	V6-RW-10	V6-R-10
V1-R-10	V6-R-10	V3-RW-10	V2-R-10	V1-RW-20	V1-RW-10
V4-R-10	V10-R-10	V3-R-20	V6-RW-10	V8-R-20	V9-R-20
V5-R-20	V2-R-10	V4-RW-10	V9-R-20	V4-R-10	V1-R-20
V3-R-10	V4-RW-20	V8-R-20	V5-RW-10	V5-RW-10	V3-R-20
V9-R-10		V4-R-20		V2-RW-10	

Figure s5.2: The rice-weed competition field experiment.
A. Aerial view of the rice-weed competition field assay. The plot area was adjusted to fit the green area of plots >4 m2.
B. Schematic depicting the design of the rice-weed competition field experiment. weeds= weed-only plots; R= rice-only plots; RW= rice+weed plots; V1 to V11 = 11 varieties of rice with varying shade casting capacity, 20= normal rice planting density (20 x 20 cm); 10= high rice planting density (20 x 10 cm).
Plots indicated in red are the full size plots that were analyzed. Plots in blue (smaller plots) and yellow (plots with poor establishment) were not included in the analyses. Plots analyzed in this chapter were 'RW-20', 'RW-10' and 'weeds'.

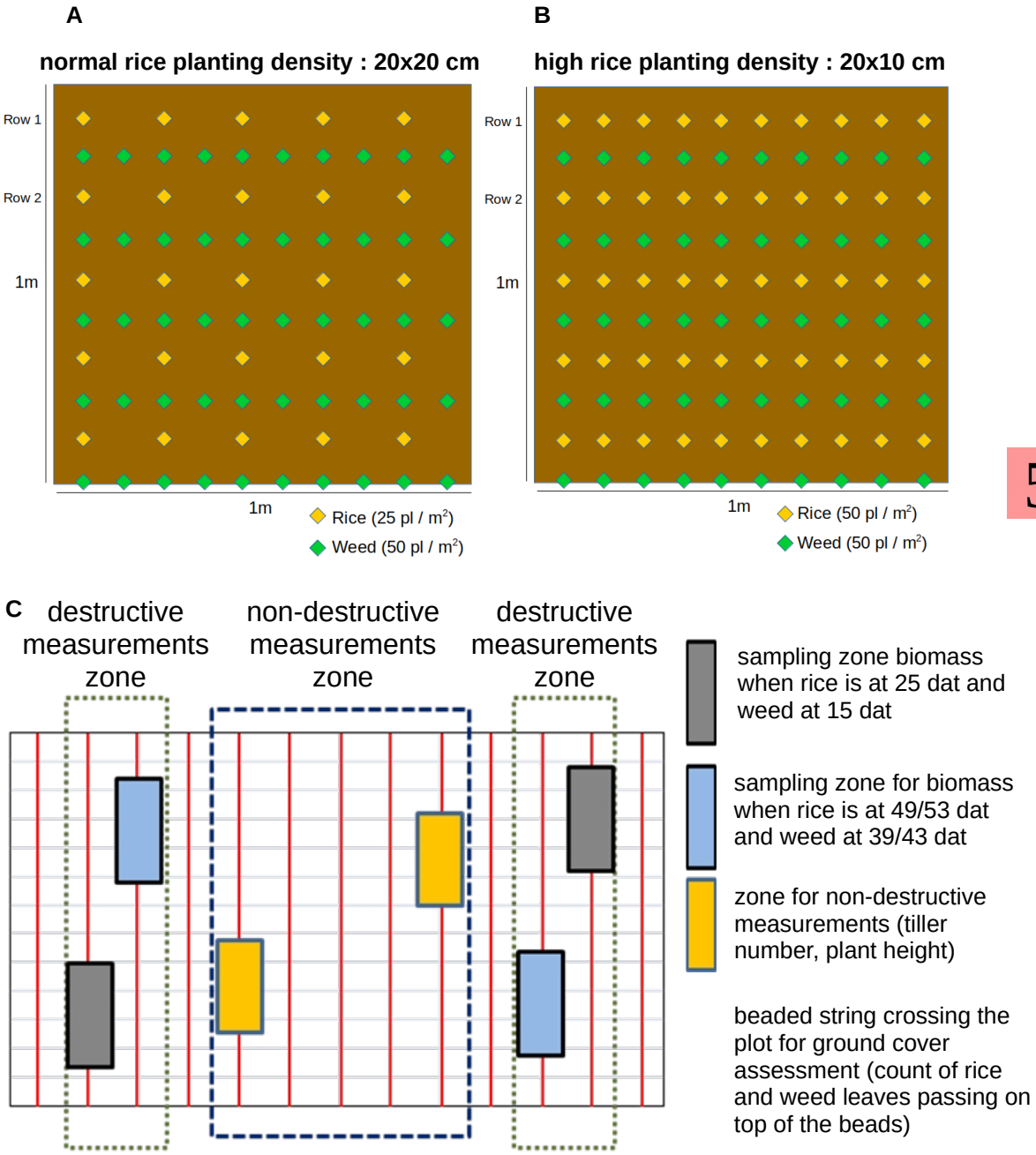


Figure s5.3: The rice-weed transplantation scheme for the field experiment.
A-B. Schematic depicting the design for rice and weed transplantation. Real size of the field plots was of 2.6 m x 2.4 m= 6.24 m2. Normal rice planting density (**A**) is of 20 x 20 cm (25 plants / m2) and high density (**B**) of 20 cm x 10 cm (50 plants / m2). Weed density remains the same in both case: 50 plants / m2. Orange diamonds depict the rice and green diamonds depict the weed.
C. Schematic depicting sampling and measurement areas.

Table s5.1: The average number of days to canopy closure (99% of the ground is covered by the rice+weed canopy) for each of the rice varieties and in the weed-only plot, at normal and high rice densities.
Values are retrieved from a general linear model with binomial option of the % of ground cover through time. High shade-casting varieties are coloured in green, low shade-casting varieties in orange, intermediate-shading varieties in yellow.

	Normal density	High density
V01	41,7	40,9
V02	40,4	38,7
V03	37,5	35,7
V04	36,6	43,8
V05	35,2	38,6
V06	37,7	36,6
V07	38,1	35,6
V08	42,5	40,5
V09	36,8	36,5
V10	41	38
V11	37,9	36,2
W	**44,2**	

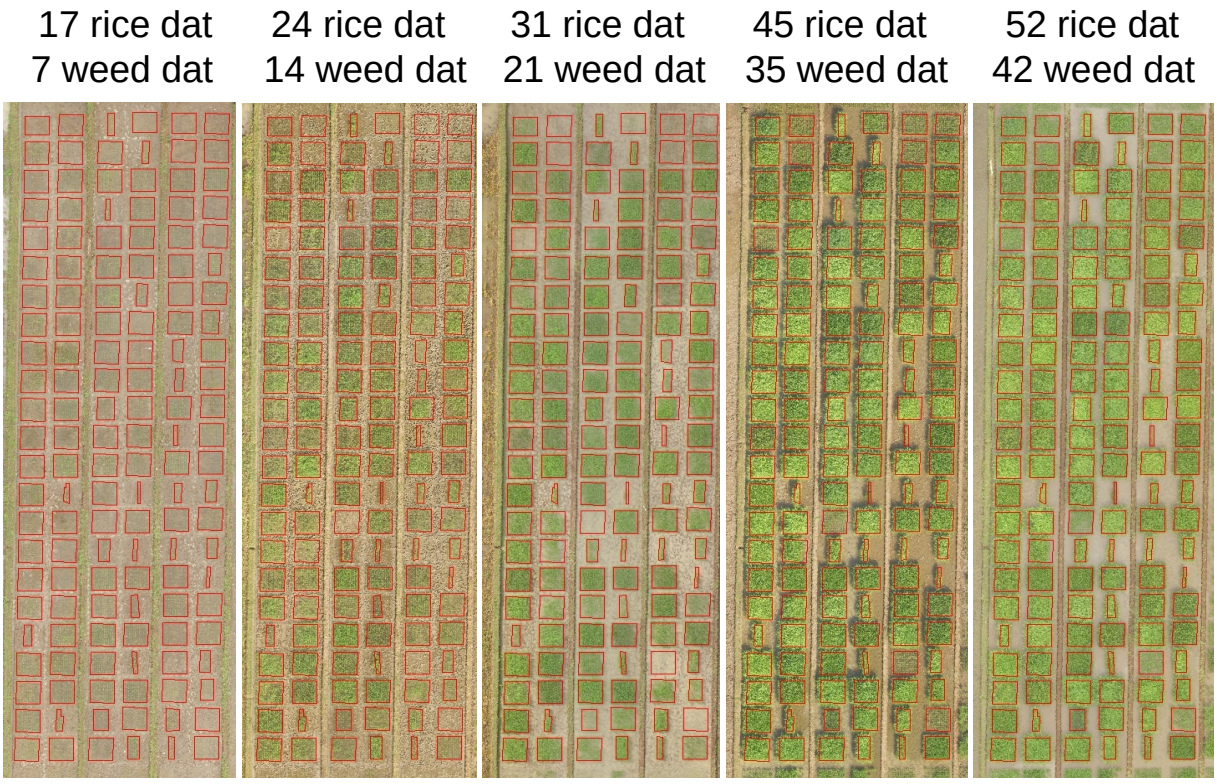


Figure s5.4: Aerial drone imaging of the field assay through time.
dat= days after transplanting.

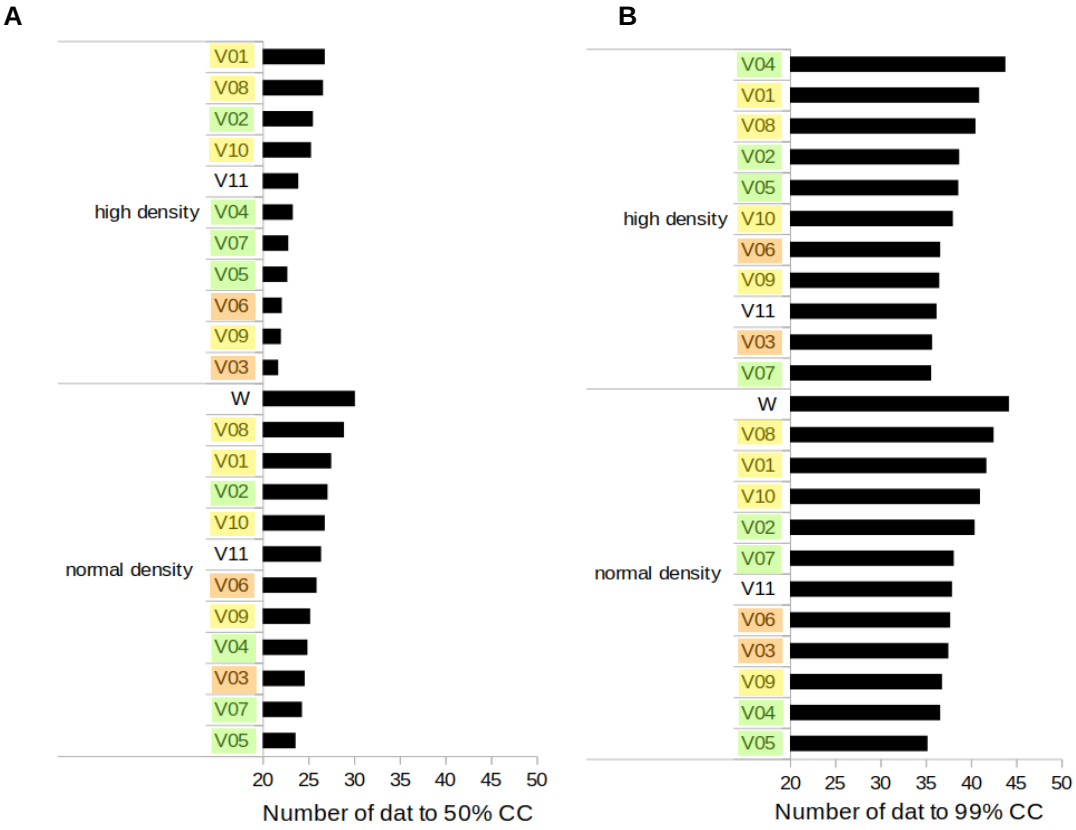


Figure s5.5: Time to reach 50% and 99% of canopy closure.
A-B. Barplots depicting the number of days after rice transplanting needed to reach 50% (**A**) and 99% (**B**) of ground cover for the 11 rice varieties in Rice+Weed plots, in normal (20 x 20 cm) or high (20 x 10 cm) rice planting densities. w= weed-only plot, v1 to v11= 11 varieties of rice. High cast-shading varieties are colored in green, low cast-shading varieties in orange, intermediate-shading varieties in yellow.

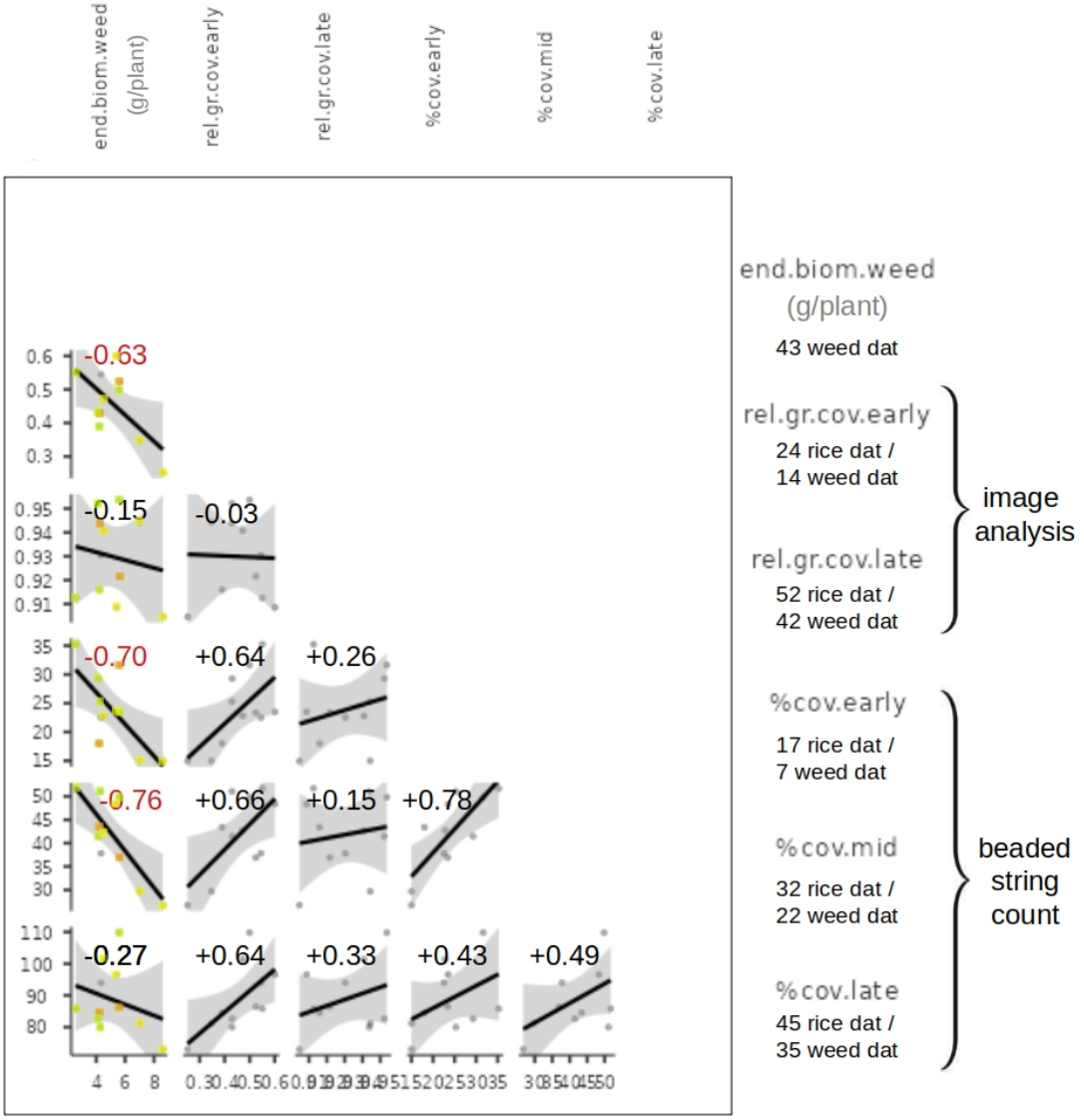
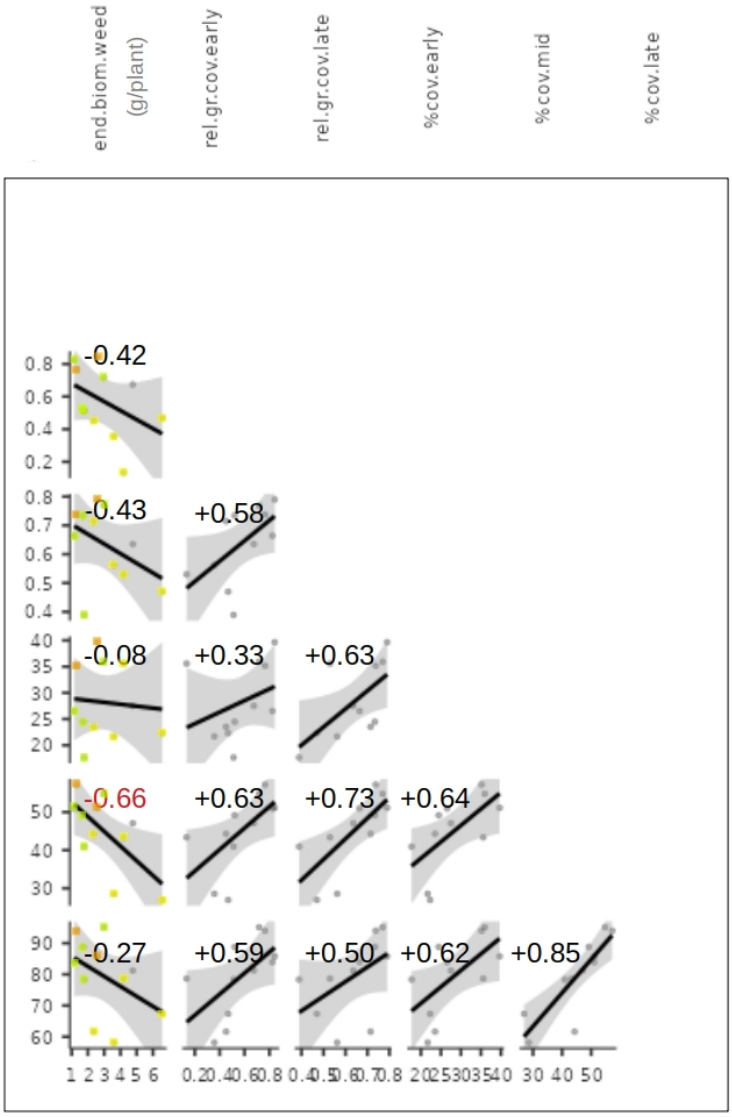


Figure s5.6: Correlation matrix between the ground cover and *E. crus-galli* end shoot biomass at normal rice planting density.
Correlation plots between the manually measured early, mid and late ground cover (beaded string count) with the early and late relative percent ground cover assessed through drone image analysis (PC) from high and low shade-casting rice varieties with *E. crus-galli* end shoot biomass at 43 weed dat, where rice is transplanted at normal density (20 x 20 cm). Manual cover and ground cover is measured in Rice+Weed plots. Plot generated with https://jamovi.com. Pearson correlation for each line plot is indicated with the negative or positive sign of the interaction. Red indicates the strongest negative correlation of ground cover with the end weed shoot biomass. High shade-casting varieties are coloured in green, low cast-shading varieties in orange, intermediate-shading varieties in yellow.



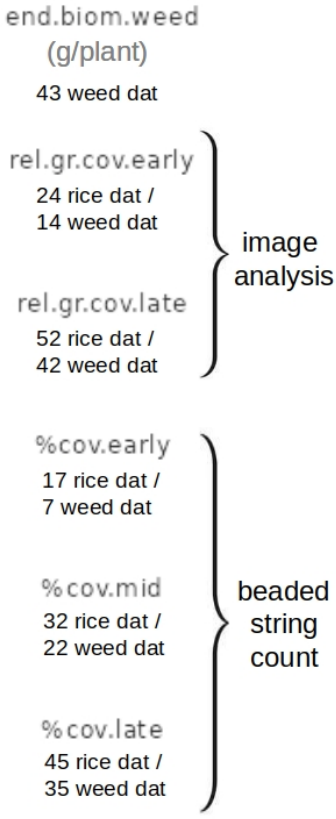


Figure s5.7: Correlation matrix between the ground cover and *E. crus-galli* end shoot biomass at high rice planting density.
Correlation plots between the manually measured early, mid and late ground cover (beaded string count) with the early and late relative percent ground cover assessed through drone image analysis (PC) from high and low shade casting rice varieties with *E. crus-galli* end shoot biomass at 43 weed dat, where rice is transplanted at high density (20 x 10 cm). Manual cover and ground cover is measured in Rice+Weed plots. Plot generated with https://jamovi.com. Pearson correlation for each line plot is indicated with the negative or positive sign of the interaction. Red indicates the strongest negative correlation of ground cover with the end weed shoot biomass. High shade-casting varieties are coloured in green, low cast-shading varieties in orange, intermediate-shading varieties in yellow.

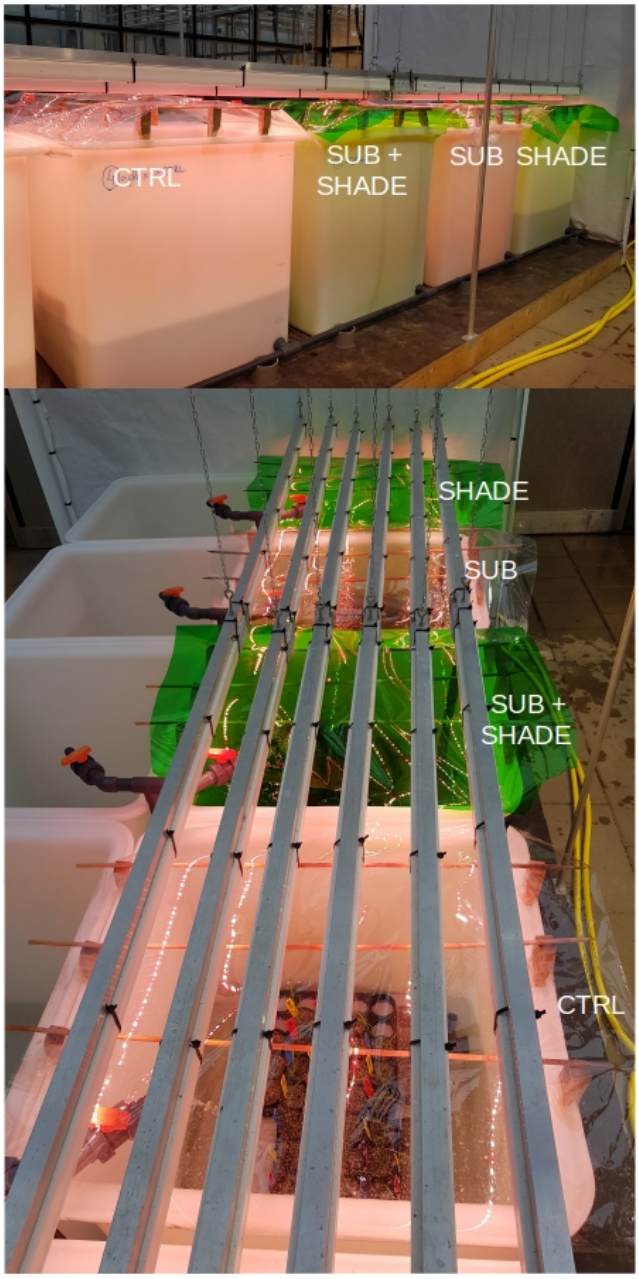


Figure s5.8: Experimental design and set up of the artificial shade and complete submergence experiments in the greenhouse.
Tanks were filled completely with tap water for submergence, and a single layer of Lee Fern green plastic filter was placed on top for the shade treatment. A transparent filter was used for control conditions. CTRL= control, SUB= complete submergence.

Figure s5.9: Representative images of *E. crus-galli* plants placed under artificial shade and complete submergence in the greenhouse.
A. Representative images of *E. crus-galli* plants after 8 days of single (C= Control; SH= shade; SUB= submergence) or combined (SH + SUB= shade + submergence) treatments.
B. Representative images of *E. crus-galli* plants after 5 days of single (C= Control; SH= shade; SUB= submergence) or combined (SH + SUB= shade + submergence) treatment, following 3 days of complete submergence treatment.
sub= submergence. Shade was mimicked by using a single layer of Lee Fern green filter which reduced PAR and R/FR ratio to 1/2-1/3 of that in control conditions.

Chapter 6
Summarizing discussion

Why study weeds?

Weeds are among the greatest source of crop losses, causing about 10% of reduction in crop productivity globally (Oerke, 2006). Their ability to invade and colonise a considerable range of environments indicates substantial environmental adaptability. In general, invasive plant species are better equipped than endemic plants or crops to adapt to new environmental conditions. Invasive weed species can even survive extreme weather like storms, floods and other similar events associated with climate change (Young et al., 2017). As an example, several weeds have been reported to have invaded several coasts in the United States after major flooding events, attributed to their high ability for seed dispersal and colonisation in coastal environments (Rouifed et al., 2011; Colleran and Goodall, 2015; Tougas-Tellier et al., 2015; Charbonneau et al., 2017). This has triggered a recent interest in these species as models for investigating environmental stress resilience mechanisms.

The fact that weeds abound in interesting tolerance traits could be used to address emerging agronomic challenges (Vigueira et al., 2013; Clements and Jones, 2021; Sharma et al., 2021). For example, several studies on *Echinochloa spp.* growing in paddy fields have identified various flood tolerance traits linked to improved anaerobic germination, photosynthesis, and post-submergence survival. These traits could potentially be introgressed in sensitive rice varieties to heighten their flood tolerance (Bouhache and Bayer, 1993; Ismail et al., 2012; Covshoff et al., 2016; Khedr et al., 2017). In a similar vein, wild relatives of crop species also represent a relatively untapped source of resilience traits. The transfer of these traits into domesticated crop varieties offers promising avenues for maintaining productivity or minimizing yield losses in the current climate of unpredictable weather patterns (Toulotte et al., 2022).

In recent times, the generation of new adaptive traits occurring on relatively short timescales caused by rapidly changing environments (higher frequency of stressful events) has gathered traction (Reznick et al., 2019). Indeed, ‘agricultural weeds’ provide good examples of such short-term evolution. Faced with human-inflicted increasingly stronger eradication measures, these species are forced to change rapidly to survive these hostile agro-environments. By virtue of this, they also represent excellent models for the investigation of the evolution of resilience mechanisms and the identification of novel tolerance mechanisms. This in turn delivers crucial knowledge towards revising existing weed management protocols in fields.

How and why is *Echinochloa crus-galli* so tolerant to flooding?

E. crus-galli was continuously reported as tolerant to water-based management practices in the last decades (Estioko et al. 2014; Ismail et al., 2012; Peralta et al., 2019; Kaspary et al., 2020). Its ability to persist in flooded environments (Maun and Barrett, 1986; Bajwa et al., 2015) has been described in several studies (Kennedy et al., 1980; Chauhan and Johnson, 2011; Estioko et al., 2014). However, an in-depth study investigating the underlying mechanisms had not been realized until now. Previous studies documented the morphological and physiological responses correlating with the observed flooding tolerance in various *Echinochloa* species.

These studies have revealed variation in the ability to tolerate flooding in different *Echinochloa* species at various developmental stages. Among the traits linked to this tolerance and of relevance for early establishment in flooded environments is the ability for anaerobic germination. Anaerobic germination was associated with the upregulation of genes such as aldehyde dehydrogenase (ALDH) which can detoxify acetaldehyde generated during anaerobic fermentation. Besides, the activities of alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC) were reduced, which is supposed to contribute to their faster growth compared to rice (Kennedy et al., 1980; Rumpho and Kennedy, 1981; Pearce and Jackson, 1991; Fukao et al., 2003; Chauhan and Johnson, 2011; Estioko et al., 2014; Peralta Ogorek et al., 2019). In addition, some *Echinochloa* can also benefit from morphological features such as the formation of aerenchyma, adventitious roots with radial oxygen loss (ROL) barriers and leaf gas films, all important traits to improve internal aeration and potentially facilitate underwater photosynthesis (Ogasawara et al., 2000; Ejiri and Shiono, 2019). While these studies provided a substantial characterisation of flooding survival of *Echinochloa*, a molecular understanding of these traits was missing. However, in this regard, with the recent sequencing of the *E. crus galli* genome (Guo et al., 2017) a major bottleneck has been removed, providing various opportunities for probing its stress responses, via genomics-based approaches.

In this thesis, the focus on *E. crus galli* was spurred by its high tolerance in our flooding screen, together with its tremendous relevance as a persistent weed in paddies and the availability of a sequenced genome. We characterised the survival of *E. crus-galli* under complete submergence at different stages probing potential variation amongst natural biotypes (Chapter 2). We then documented stress responses at the phenotypic and physiological levels to aid better comprehension of a subsequent transcriptomics investigation (Chapter 3). We also examined its gene composition to gain insights into its supposed recent evolution of flooding tolerance in comparison with other grasses species (Chapter 4).

In a screen for submergence tolerance with other major paddy field weeds, *E. crus-galli* showed the overall highest survival and recovery score. In general, the high resilience to flooding was observed at all developmental stages tested. Complete submergence at early developmental stages revealed that the negative effects of the stress on *E. crus-galli* were highly dependent on stress timing and duration (Chapter 2). Pre-germinated seedlings when submerged, could still develop below 20 cm of water, and recovered well even after being underwater for 8 days. Similarly, germination occurred even below 8 cm of water and *E. crus-galli* exhibited higher seed germination rates underwater, compared to the other tested weeds. Water could delay but not kill the seeds that did not germinate underwater, as evidenced by resumed germination following water removal without any loss in viability. In general, *E. crus-galli* showed the best survival and recovery compared to the other paddy field weeds tested.

Vegetative stage (3-leaf stage) *E. crus-galli* plants could withstand 15 days of complete submergence, even continuing growth underwater as evidenced by an increase in shoot height and formation and extension of new leaves (Chapter 3). It also displayed the highest resilience to reoxygenation stress following desubmergence better even than the well-documented post-submergence resilience and recovery capacity of the tolerant *O. sativa* FR13A variety (Fukao et al., 2006). In addition to a high survival rate, it also generated more and healthier leaves. The analysis of the shoot transcriptomes revealed that this ability to grow and tolerate submergence could be linked to (1) a different way to generate energy, based on the utilization of alternative sugar pathways involving branched-chain amino acids and beta-oxidation, (2) the utilization of the gibberellin pathway to potentially assist underwater growth and (3) a global, faster response to submergence and recovery including a targeted regulation of oxidative stress responses and metabolic responses. Considering that reoxygenation-induced oxidative stress is a major contributor to post-submergence injury, the induction of genes associated with its amelioration might underlie the faster and better post-stress survival and performance of *E. crus-galli*. The identification of a cohort of recovery genes commonly regulated between *E. crus-galli* and *O. sativa*, also revealed the importance of mitigating tissue dehydration following desubmergence.

For further insight into *E. crus-galli* flooding tolerance, we also scrutinized its gene composition in comparison with a panel of 17 other grass species (Chapter 4). Partly due to its polyploidy, this species possesses more lineage-specific genes. These *Echinochloa* lineage-specific responses included regulation of genes associated with plant growth and development, carbon usage management, water transport (aquaporins) and antioxidant and detoxification activity. The lack of annotation of several of these genes however indicates that the exact functional relevance in flood adaptive responses will require further

exploration. These genes presumably were acquired later during repeating independent flooding events. They therefore are of high relevance as they could be important for the introgression of new flooding tolerant traits in sensitive species, and also for understanding the invasiveness of this species.

Results in Chapters 2 and 3 indicated that flooding-based management in the field must be implemented as early as possible for effective weed control. At later developmental stages, water has less impact on weed control and therefore, must be supplemented with other methods. We found that *E. crus-galli* development was significantly affected by shade (Chapter 5). Here too, early shading stress was important for optimal weed control. Notably, while independently both complete submergence and shade could limit weed growth, a combination did not result in additive effects. However, a sequential combination of these two stresses could still be an effective way to regulate *E. crus-galli* in the rice fields. This could involve using early flooding in the first few weeks following rice transplantation. Water would permit weed reduction until the selected rice variety can develop enough to cause significant shading of the weed at which point the water can be drained. That way, valuable freshwater resources could be preserved and less herbicide applications would be needed.

New insights into plant flooding tolerance

Rice is a well-established model for studying flooding stress and provides well-studied examples of distinct flood-adaptive strategies. While rice is cultivated in flooded paddies and is relatively flood-tolerant, deep or long-lasting floods are harmful even for rice and only some varieties are known to withstand these conditions (Xu et al., 2006; Hattori et al., 2011). *O. sativa* FR13A is a highly tolerant landrace that can survive more than two weeks underwater via a quiescent strategy (Xu et al., 2006; Bailey-Serres et al., 2010). 70% of this tolerance can be attributed to the SUB1 locus and the *SUB1A* gene, induced by the flooding-induced accumulation of ethylene. While the SUB1A-mediated quiescence and resulting tolerance are well-described, we also found additional components in FR13A linked to differential growth processes and to potassium ion homeostasis that could also contribute to its observed high resilience.

Varieties that lack SUB1A (like *O. sativa* IR42 investigated here, Septiningsih, 2008; Winkel et al., 2014; Singh et al., 2020) continue growth underwater. An increase in gibberellin is associated with the escape response of shoots in deepwater rice to breach the water surface (escape strategy) (Raskin and Kende 1984; Hattori et al., 2009). *SD1* (*SEMIDWARF1*) encodes for a GA20-oxidase for gibberellin biosynthesis and is responsible for a semidwarf phenotype under submergence (Fukazawa et al., 2017; Kuroha et al., 2018). Of the genotypes studied here, both *O. sativa* IR42 and *E. crus-galli* continued shoot growth

underwater. While the gene coding for the gibberellin 20 oxidase (*GA20ox*) was strongly upregulated in *E. crus-galli,* this was absent in *O. sativa*. The two genotypes might thus use different mechanisms to trigger growth underwater. Despite the higher submergence tolerance and superior recovery of FR13A and *E. crus-galli,* their transcriptomic profiles differed considerably. Rather, the FR13A transcriptome profile was near identical to that of IR42.

The possession of flood-adaptive morphological traits or activation of core flooding associated molecular responses does not always correlate with tolerance. This is evident in *Z. mays* which possesses hydrophobic leaves (personal observation), adventitious roots (Mano et al., 2005) and can form lysigenous aerenchyma (Drew et al., 2000; Mano et al., 2006; Yamauchi et al., 2011), but is highly sensitive to complete submergence (Chapter 3) and here was chosen as a sensitive species to contrast the responses found in the other tolerant genotypes. Despite displaying close physiological and transcriptomics response patterns with tolerant *E. crus-galli,* neither strongly significant GO categories, genes, pathways nor orthogroups for *Z. mays* were identified, in accordance with its sensitivity to flooding. Notably, during submergence, it did retain a high sugar content and displayed conventional flooding induced transcriptomic adjustments shared with tolerant species. However, in general, transcriptomic responses to submergence and recovery were delayed, suggesting a general inability for timely coordination of essential responses. This likely also made it unable to cope with post-submergence oxidative stress and thus succumb to reoxygenation injury.

Surprisingly, major typical hormonal and core hypoxia responses were activated in a similar way in both tolerant and sensitive flooding genotypes, suggesting that hypoxia responses do not play a crucial role in submergence tolerance in the studied species. The decline in tissue oxygen levels is moderate when submergence occurs in sufficiently illuminated conditions. In fact, studies even report hyperoxic conditions in shoot tissues of plants (Mori et al., 2019; Muller et al., 2021). However diurnal fluctuations in oxygen levels - involving a nighttime decline that recovers during the day - are common in submerged shoots (Rijnders et al., 2000; Colmer and Pedersen, 2008; Muller et al., 2021). In our dataset, despite obvious night-responsive transcriptome patterns, there were no genotype-specific differences in this regard that could be associated with tolerance. However, the orthology analyses did reveal *Z. mays* specific clusters not regulated in the other genotypes, linked to the reduction of growth-related metabolism and to the enhancement of protein degradation and sugar catabolism. We speculate that the delayed response of *Z. mays* to submergence signals results in an inability to adjust metabolism causing it to consequently expend valuable energy at night and leading to an energy crisis.

The multi-species orthology comparison proved to be a useful approach for deciphering what determines flooding tolerance of a species. The multi-species transcriptomics analysis provided a global picture of common and genotype-specific molecular responses, whereas the orthology analysis facilitated a more in-depth examination of lineage-specific responses. In both cases, transcriptome responses were more related to phylogeny (BOP/C3 and PACMAD/C4 species) than overall flooding tolerance. There was minimal overlap in the flooding transcriptome responses between the tolerant *E. crus-galli* and tolerant *O. sativa* FR13A. The analysis revealed separated sets of orthologs linked to flooding tolerance and a small number of commonly differentially expressed orthogroups. These results could reflect an independent evolution of mechanisms to cope with submergence in these species, which could also explain their contrasting phenotypic behaviour underwater.

Echinochloa spp. presence in rice fields was described 8000 years ago. *Echinochloa esculenta* (Japanese barnyard millet) was cultivated in drylands in Japan together with rice about 850 B.C. (Gross and Zhao, 2014). *E. crus-galli* growing in the same paddy fields evolved adaptive and competitive traits to evade removal from rice fields (Barrett, 1983), as well as herbicide resistance later (Norsworthy et al., 2014). Therefore, we could hypothesize that *E. crus-galli*, being rapidly adaptive, could also have developed new flooding tolerant mechanisms to sustain themselves in rice paddies. It could have evolved its own set of flooding tolerance mechanisms to utilize in addition to shared tolerance traits (Chapter 4). Given the immense flood tolerance and adaptability of *E. crus-galli* and related weeds, they will prove hard to suppress with traditional paddy field approaches, especially when the weeds reach the air. Our preliminary experiments with shading suggest that low light can be equally effective on *E. crus-galli* development and the use of high shade-casting rice varieties would be a fruitful option to pursue weed control after submergence (Chapter 5).

Concluding remarks and future perspectives

The results of this thesis support the usefulness of weeds to study previously unidentified resilience mechanisms. Further investigation of the *E. crus-galli* genes identified here will potentially unravel novel pathways and functions underlying the high flooding tolerance of this species. It also raises questions about other related species in the *Echinochloa* lineage, also infesting paddy fields like *E. colona* and *E. glabrescens* (Alberto et al., 1996; Rao et al., 2007; Gross and Zhao, 2014; Covshoff et al., 2016). For example, do these species share common tolerance strategies with *E. crus-galli* or do they possess unique adaptation mechanisms? The rapid pace of sequencing and progress towards completion of the *E. colona* genome by the International Weed Genomics Consortium (IWGC;

https://www.weedgenomics.org/) and direct comparisons with the *E. crus-galli* genome will definitely aid answering such questions.

Flooding is a compound stress and the ability to survive inundation is thus attributed to a suite of traits that can be regulated via different mechanisms. The arsenal of traits employed and the underlying mechanisms depend on the species, the severity of the applied stress, developmental stage or even the interaction with other environmental effects. Our analyses indicate that tolerance found in *O. sativa* and *E. crus-galli* evolved separately and is achieved through different routes with distinct molecular profiles. Given that tolerance to flooding likely was achieved multiple times advocates widening the research portfolio into tolerant relatives of our crops. This would provide opportunities to understand paths leading to adaptation and mimic those in our domesticated plants and gain insight into diversification and adaptation in the plant kingdom.

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Layman summary

Whereas most crops cannot survive even a few days of submergence, rice is an exception. Several rice varieties can grow in fields with standing water while providing significant yields. Flooding seriously damages plants, primarily by drastically reducing the necessary gas exchange and the light needed for photosynthesis and energy generation. The flooding tolerance of rice has been exploited for managing weeds in rice fields. By flooding the rice fields, weeds are prevented from germinating or growing underwater, while rice remains unaffected. Yet, decades of this practice have led to the evolution of flooding tolerance in certain endemic weed species, decreasing the effectiveness of this natural weed control method.

In agronomy, weeds are generally defined as unwanted plants because of their negative impact on crop yields. However, their ability to adapt to extreme environments also make them good models for studying plant environmental stress adaptation. Thus, studying the evolved tolerance of rice weeds is important, not only from the perspective of devising alternative weed management strategies but also discovering novel stress resilience mechanisms. Besides, fresh water resources are becoming scarce. Therefore, water usage in rice fields must be reduced and new weed management methods requiring less water, involving submergence combined with other methods, should be explored and tested.

The aim of this thesis was to investigate flooding tolerance in rice weeds to discover new flood tolerant traits. This provides the opportunity to better understand plant adaptation to flooding stress but also to uncover genetic mechanisms towards enhancing crop flood resilience. Another aim of this thesis work was to test and to find more efficient and less-water demanding methods of weed management.

We started our investigation by screening different weed species and several of their ecotypes for submergence tolerance (Chapter 2). Among them was *Echinochloa crus-galli* (barnyard grass), often referred to as one of the worst weeds worldwide and described by scientists and farmers as highly submergence tolerant. In agreement with this, this species also emerged as one of the most submergence tolerant weeds in our tolerance screen. Thus, subsequent work focussed on further characterisation of the morphological, physiological and molecular responses of this weed to submergence and post-submergence conditions. For a comparative assessment, we included in our investigations an intolerant cultivar of maize and two varieties of rice (one tolerant and one intolerant).

Chapter 3 presents the survival assessment to a long period of complete submergence of the tolerant weed, maize and the two rice varieties, confirming their reported flooding tolerance/intolerance. This validated our experimental set-up and revealed that flooding tolerance in the tolerant weed and in the tolerant rice was achieved by different means. Since flooding imposes hypoxia and an energy crisis in affected plants, the ability to conservatively use oxygen and sugar is expected to be linked to tolerance. However, there were no major differences between our three species in sugar and oxygen consumption, indicating similar resource management

underwater. In-depth analyses of the transcribed and sequenced RNA indicated many commonly regulated genes but also revealed crucial mechanisms associated with differential growth and metabolic responses to water stresses, i.e. underwater stress (hypoxia and reduction of light) and post-submergence stress (reoxygenation and re-illumination).

The exploration of the high flooding tolerance of this weed through an orthology comparative analysis of its genome composition to a set of 17 other grass species (Chapter 4) revealed a conservation of flooding responses in the grass family, as well as species-specific flooding tolerance responses in the weed. We hypothesize that these species-specific flooding tolerance responses could have been acquired by selective pressure exerted by the relatively recent exposure to flooding conditions. The exploration of the function and regulation of these weed specific genes is expected to reveal novel insights into flood response and resilience mechanisms.

Considering the high flood tolerance of this weed, in Chapter 5, we explored alternative strategies for suppressing its growth in rice fields. New water-based management protocols were tested alone or in combination with shade, cast artificially or by the crop, i.e. different rice varieties. Experiments in the greenhouse and in field lead to the conclusion that weed management protocols using early flooding followed by natural shade from high shade-casting rice cultivars might be the most efficient way to naturally suppress weed growth in rice fields. This would also be a more water conservative regime to control weeds.

In conclusion, this thesis (1) confirms the high tolerance of *E. crus-galli* to flooding, to both submergence and post-submergence stresses, (2) finds that the weed shares common responses with other grass species, including rice, to cope with flooding stress, (3) but has also acquired other specific flooding tolerance traits that should be studied further. Moreover, (4) we provide new insights regarding the evolution of the flooding tolerance in *E. crus-galli* and (5) how, in general, weeds growing in the paddy fields could be better controlled, by integrating weed-competitive rice, in particular by incorporating high shade-casting varieties, into existing water-based weed management protocols.

key-words: flooding tolerance; rice weeds; *Echinochloa crus-galli*; submergence; post-submergence; maize; rice; RNAseq; gene orthology; field experiments

Samenvatting

De meeste gewassen kunnen slechts enkele dagen overleven tijdens een overstroming, rijst is hierbij een uitzondering. Verschillende rijst variëteiten kunnen groeien in velden met een laagje water en nog steeds een hoge opbrengst leveren. Echter, een overstroming is erg schadelijk voor de plant omdat de diffusie snelheid van gassen drastisch verminderd, en toegang tot licht voor fotosynthese en energy flink wordt verhinderd. De watertolerantie van rijst wordt gebuikt om onkruid tegen te gaan. In een laag water kunnen onkruid slecht kiemen en groeien en krijgt het zo geen kans. Echter een geschiedenis van dit land gebruik heeft ertoe geleid dat sommige onkruid soorten tolerantie voor de overstroming in rijstvelden ontwikkelden, met serieuze gevolgen voor de effectiviteit van deze natuurlijk wijze van onkruid beheers.

Binnen de agronomie kenmerkt een onkruid zich als een ongewenste plant, aangezien deze de gewas opbrengst negatief beïnvloed. Echter, de capaciteit van onkruid om met extreme condities om te gaan, zoals de nieuwe rijst onkruiden, maakt hen juist een zeer geschikt studieobject om stress adaptatie te begrijpen. Het bestuderen van geëvolueerd rijst onkruid is daarom dus niet alleen belangrijk voor beheer strategieën, maar ook om nieuwe mechanismen van stress tolerantie te onderzoeken. Daarnaast worden zoetwaterbronnen steeds schaarser en moeten de rijstvelden met minder water toe. Naast het klassieke rijstveld moeten dus gezocht worden naar alternatieven om onkruid tegen te gaan die minder water kosten.

Het doel van dit proefschrift was om nieuwe eigenschappen van overstromingstolerantie te ontdekken in onkruid van rijstvelden. Dit geeft niet alleen de mogelijkheid om plant adaptatie aan overstromingsstress beter te begrijpen, maar ook om genetische mechanisme ten aanzien van het verbeteren van de bestendigheid van gewassen te vatten. Daarnaast had dit proefschrift als doel meer efficiënte, en minder water vragende onkruid beheer strategie te vinden en te testen.

In Hoofdstuk 2 hebben we allereerst diverse onkruidsoorten en ecotypes getest op hun overstromingstolerantie. Hiertussen zat ook *Echinochloa crus-galli*, door boeren en wetenschappers beschreven als wereldwijd het grootste onkruid probleem en extreem overstromingstolerant. Inderdaad, van alle soorten die we testten bleek deze de beste prestatie bij overstroming. Vervolg werk was om de soort morfologisch, fysiologisch en moleculair verder te onderzoeken. Ter vergelijking gebruikten we mais, een zeer overstromingsgevoelige soort. En daarnaast uit het rijstveld een overstromingstolerante en een overstromingsgevoelige rijst variëteit.

Hoofdstuk 3 presenteert overleving en prestaties van *E. crus-galli*, mais en de twee rijst variëteiten, die hun classificaties als ofwel gevoelig of tolerant onderschrijven. Dit valideerde ons experimenteel systeem en liet zien dat tolerantie

in het onkruid en in rijst op verschillende manieren bereikt werd. Overstroming leidt tot een lage zuurstof beschikbaarheid in de plant en een energiecrisis. Daarom is de capaciteit om minder zuurstof en suikers te gebruiken geassocieerd met tolerantie. Echter, er waren nauwelijks verschillen in zuurstof en suiker gebruik, wat wijst op vergelijkbaar metabolische activiteit. De analyse van miljoenen RNA sequenties tijdens diverse fases van de overstroming suggereert vergelijkbare regulatie van genen in mais, *E. crus-galli* en rijst. Dit identificeerde ook de regulatie van processen als groei, metabolisme en factoren typisch voor de onderwater omgeving zoals laag zuurstof en laag licht, en juist het omgekeerde na afloop van de overstroming.

Om belangrijke genen te achterhalen aan de hand van de vergelijking tussen de soorten is het vereist om verwante genen tussen de soorten aan elkaar te koppelen. Om dit robuust te doen gebruiken we 17 extra grassoorten (Hoofdstuk 4). De daaropvolgende soortvergelijking liet zowel geconserveerde reacties in de gras family zien als soort-specifieke en tolerantie specifieke reacties in *E. crus-galli*. Onze hypothese is dat specifieke reactie van het tolerante onkruid recent zijn ontwikkeld door selectiedruk vanuit de rijstveld condities. Door het verkennen van de functie en regulatie van deze genen verwachten we nieuwe inzichten in overstromingstolerantie mechanismen te verkrijgen.

Gezien de enorme overstromingstolerantie van dit onkruid verkennen we in Hoofdstuk 5 alternatieven strategieën om *E. crus-galli* te onderdrukken. Op water gebaseerde beheer strategieën werden getest met of zonder schaduw, verzorgd door rijst of kunstmatig toegediend. Experimenten in het veld en in de kas leidde tot de conclusie dat onkruid beheer met eerst een overstroming gevolgd door laag licht veroorzaakt door rijst variëteiten met veel schaduw de meest efficiënte wijze van onderdrukking verzorgd in de rijstvelden. Zo een beheerregime zou water-efficiënt onkruid onderdrukken.

In conclusie, dit proefschrift (1) bevestigd de hoge tolerantie van *E. crus-galli* ten aanzien van zowel overstromingsstress en de stress van de daaropvolgende periode, (2) vindt gedeelde responsen van *E. crus-galli* met andere gras soorten, inclusief rijst, om met overstroming om te gaan, (3) maar heeft ook unieke tolerantie specifieke reacties die verder onderzocht dienen te worden. (4) We bieden nieuwe inzichten in met betrekking tot de evolutie van overstromingstolerantie en (5) hoe onkruid in rijstvelden beter onder controle gehouden kan worden door de schaduw van rijstvariëteiten.

Résumé

Alors que la plupart des plantes cultivées ne peuvent pas survivre plus de quelques jours de submersion, le riz est une exception. Plusieurs variétés de riz peuvent pousser en champs avec une eau stagnante, tout en offrant des rendements importants. La submersion endommage gravement les plantes, principalement en réduisant considérablement les échanges gazeux et la lumière, tous deux nécessaires à la photosynthèse et à la production d'énergie. La tolérance du riz à la submersion a été exploitée pour contrôler les adventices dans les rizières. En inondant les rizières, la germination et la croissance des adventices sont considérablement réprimées, tandis que le riz n'est pas affecté. Pourtant, des décennies de cette pratique ont rendu certaines espèces d'adventices endémiques tolérantes aux inondations, diminuant l'efficacité de cette méthode de désherbage naturelle.

En agronomie, les adventices sont généralement définies comme des plantes indésirables en raison de leur impact négatif sur les rendements des cultures. Cependant, leur capacité d'adaptation aux environnements extrêmes en fait également de bons modèles pour étudier l'adaptation des plantes aux stress environnementaux. Ainsi, l'étude de l'évolution de la tolérance des adventices du riz à la submersion est importante, non seulement dans la perspective de concevoir des stratégies alternatives de gestion des adventices, mais aussi celle de découvrir de nouveaux mécanismes de résilience aux stress hydriques. Par ailleurs, les ressources en eau douce se raréfient. Par conséquent, la consommation d'eau dans les rizières doit être réduite et de nouvelles méthodes de gestion des adventices qui nécessitent moins d'eau doivent être explorées et testées, par exemple en combinant une submersion plus efficace avec d'autres méthodes.

L'objectif de cette thèse était d'étudier la tolérance à la submersion chez une adventice du riz, afin de découvrir de nouveaux traits de tolérance des plantes à la submersion. Cela offre l'opportunité de mieux comprendre l'adaptation des plantes au stress provoqué par les inondations, mais aussi de découvrir des mécanismes génétiques permettant d'améliorer la résilience des cultures à celles-ci. Un autre objectif de ce travail de thèse était de tester et de trouver des méthodes de gestion des adventices plus efficaces et moins gourmandes en eau dans les rizières.

Nous avons commencé notre étude en examinant la tolérance de différentes espèces d'adventices et de plusieurs de leurs écotypes à la submersion (Chapitre 2). Parmi elles, se trouvait *Echinochloa crus-galli* (panic pied-de-coq), souvent désignée comme l'une des pires adventices au monde et décrite par les scientifiques et les agriculteurs comme très tolérante à la submersion. Effectivement, cette espèce est également apparue comme l'une des plus tolérantes aux périodes de submersion et de post-submersion dans notre essai de tolérance. Ainsi, nous nous sommes concentré.e.s sur une caractérisation plus

poussée des réponses morphologiques, physiologiques et moléculaires de cette adventice en réponse aux conditions de submersion et de post-submersion. Pour une évaluation comparative, nous avons inclus dans nos investigations un cultivar de maïs intolérant et deux variétés de riz (une tolérante et une intolérante).

Le chapitre 3 présente l'évaluation de la survie d'*E. crus-galli*, du maïs et des deux variétés de riz, à une longue période de submersion et de post-submersion, confirmant ainsi leur respective tolérance/intolérance aux inondations. Les résultats ont validé notre dispositif expérimental et ont révélé que la tolérance aux inondations chez l'adventice tolérante et chez le riz tolérant était permise par des moyens différents. Étant donné que les inondations imposent une hypoxie et une crise énergétique chez les plantes touchées, nous avons émis l'hypothèse que la capacité à conserver ou à réguler différemment l'oxygène et le sucre devrait être liée à la tolérance. Cependant, il n'y avait pas de différences majeures entre nos trois espèces dans la consommation de sucre et d'oxygène, indiquant une gestion similaire de ces ressources sous l'eau. Des analyses approfondies de l'ARN transcrit et séquencé ont indiqué une régulation de nombreux gènes en commun, mais ont également révélé une régulation différentielle des mécanismes cruciaux associés à la croissance et aux réponses métaboliques induites par le stress hydrique, c'est-à-dire le stress de la submersion (hypoxie et réduction de la lumière) et le stress post-submersion (réoxygénation et ré-illumination).

L'exploration de la haute tolérance aux inondations d'*E. crus-galli* a été rendue possible par une analyse orthologique comparative de sa composition génomique à celle de 17 autres espèces de graminées (Chapitre 4). Cette analyse a révélé une conservation des réponses aux inondations dans la famille des graminées, ainsi que des réponses de tolérance spécifiques à l'espèce *Echinochloa*. Nous émettons l'hypothèse que ces réponses de tolérance aux inondations spécifiques à cette espèce pourraient avoir été acquises par la pression sélective exercée par l'exposition relativement récente aux conditions d'inondations. L'exploration de la fonction et de la régulation de ces gènes spécifiques à l'espèce *Echinochloa* devrait révéler de nouvelles informations sur les mécanismes de réponse et de résilience aux inondations.

Compte tenu de la grande tolérance aux inondations de cette adventice, avec le chapitre 5, nous avons exploré des stratégies alternatives pour réprimer sa croissance dans les rizières. De nouveaux protocoles de gestion basés sur la submersion ont été testés seuls ou en combinaison avec l'ombre, apportée artificiellement ou apportée directement par la culture du riz, c'est-à-dire par différentes variétés de riz. Des expériences en serre et sur le terrain ont conduit à la conclusion que les protocoles de gestion des adventices utilisant une inondation des rizières précoce suivie d'un ombrage naturel amené par des cultivars de riz à fort ombrage pourraient être le moyen le plus efficace de réprimer naturellement la croissance des adventices dans les rizières, tout en étant plus économe en eau.

En conclusion, cette thèse (1) confirme la haute tolérance d'*E. crus-galli* aux inondations (à la fois aux stress de submersion et de post-submersion), (2) constate que cette espèce partage des réponses communes avec d'autres espèces de graminées, y compris le riz, pour faire face au stress d'inondation, (3) mais a également acquis d'autres traits spécifiques de tolérance aux inondations qui mériteraient d'être étudiés plus amplement. De plus, (4) nous apportons de nouvelles informations sur l'évolution de la tolérance aux inondations chez *E. crus-galli* et (5) comment, en général, les adventices qui poussent dans les rizières pourraient être mieux contrôlées, en intégrant aux protocoles de submersion existants des variétés de riz plus compétitives avec les adventices, comme par exemple des variétés avec une forte capacité d’ombrage.

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Founder and committee members

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Curriculum Vitae

Justine Martine Toulotte was born on the 4th of February 1991 in Beuvry, France. She obtained her national degree with “Life and Earth Science” and “Spanish” specialities, in France in 2009. She then studied “Biological Engineering Technology, Biochemical and Biological Analysis” in the University Institute of Technology “A” in Lille in 2009-2011. Her first short-term internship took place in Pamplona, Spain, hosted by Dr. Milagro, and supervised by Dr. Usune Etxeberria and Dr. Ana Laura de la Garza. She was testing new plant compounds to treat diabetes in rats. Afterwards, Justine studied one year of Experimental Plant Sciences bachelor in the University of Le Havre and “Hortithèque” Formation Centre of Mont-Saint-Aignan, followed by an internship in Le Ctifl, working among other things in the optimization of nitrogen fertilization in bell pepper crop. Then, she studied her first year of Master’s in Science and Plant Production in University of Rennes 1 and “AgroCampus Ouest” School, followed by a short-term internship in the lab of Dr. Bernard Dumas in LRSV, CNRS-UPS, Plant Immunity and Effectors, Toulouse. She worked in the development of a method of natural protection against the fungus *Alternaria brassicicola* responsible for black spot disease on Brassicaceae. She pursued with another year of Master’s in Molecular Biology and Plant Biotechnology in the University of Strasbourg. That year, she got the opportunity to do a 6-months internship in the lab of Sebastian Schornack (SCLU; Sainsbury Laboratory - University of Cambridge, The United Kingdom), supervised by Dr. Thomas Rey, working on the role of a candidate gene of *Medicago truncatula* during the interaction with *Phytophthora palmivora* and an endomycorrhizal fungus. She stayed working as a junior lab technician in the same lab on different plant-microbes interactions for 3 years. She then joined the Plant Ecophysiology (now called Plant-Environment Signaling) group at Utrecht University, under the supervision of Prof. dr. L.A.C.J. (Rens) Voesenek and Dr. Rashmi Sasidharan, where she studied the flooding tolerance in the major rice weed *Echinochloa crus-galli*. This research has jointly been done with the thesis work of Ms. Martina Huber, supervised by Prof. dr. Ronald Pierik and Dr. Kaisa Kajala. During her PhD research, Justine went to do part of her experiments at IRRI (International Rice Research Institute) in Los Baños, The Philippines, under the supervision of Dr. Virender Kumar. This thesis is the result of her PhD research.

Publications list

Rey, T., Chatterjee, A., Buttay, M., **Toulotte, J. M.** and Schornack, S. (2015). *Medicago truncatula* symbiosis mutants affected in the interaction with a biotrophic root pathogen. *New Phytol*, 206: 497-500. https://doi.org/10.1111/nph.13233

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