The role of strigolactones and the fungal microbiome in rice during drought adaptation

Beatriz Andreo Jimenez

PROPOSITIONS

1. Drought not only affects rice metabolism but also its root fungal microbiome.
   (this thesis)

2. Through its effect on both strigolactones and abscisic acid, rice *DWARF27* is a promising target for rice drought tolerance breeding programs.
   (this thesis)

3. Vegetation density is increasing in response to climate change (Forzieri et al. 2017; Science. Vol. 356, Issue 6343, pp. 1180-1184), but soil nutrient limitation could jeopardize this process.

4. The recent development of the most powerful antibiotic against superbugs to date (Okano et al. 2017. PNAS vol. 114 no. 26 E5052–E5061), is a good example on how human technology is part of a coevolution process.

5. Let's ignore the impact factor and instead impact society.

6. Symbiosis is the key to success in the natural as well as the social environment.

7. The best way to improve is to compete with ourselves.

Propositions belonging to this thesis entitled

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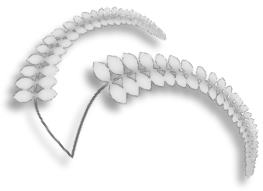
Friday 20 October 2017



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Beatriz Andreo Jimenez

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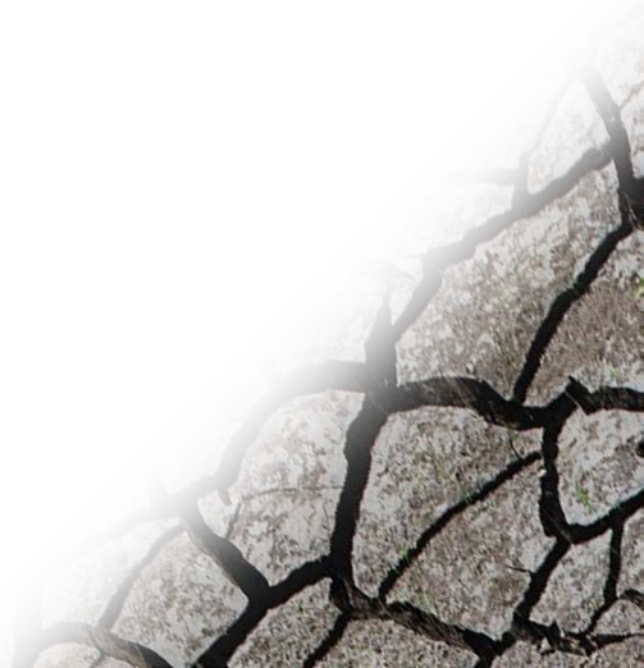
Chapter 1

General Introduction



Rice and its importance as a crop

Rice (*Oryza sativa* L.) is the most produced and consumed crop worldwide, feeding more than 3,000 million people around the globe (FAO). It is the primary food for more than half of the world population, especially in developing countries where water scarcity and drought are threats to food security (Molden, 2009). Rice is a monocot plant that needs well-watered conditions to yield high levels of grain. The majority of rice is cultured in a water layer of about 5-10 cm deep. This flooded condition helps to supress the growth of weeds, to store water during the monsoon season and it allows the growth of the fern *Azolla* sp. that fixes nitrogen through its symbiosis with a bacterium. Flooded conditions also facilitate the plant to dissolve and take up immobile nutrients like phosphorous. The origin of domesticated rice traces back to between 8,200 and 13,500 years ago, and there is evidence supporting a single origin from the wild variety *Oryza rufipogon* Griff. (Molina *et al.*, 2011). This wild ancestor is a semi-aquatic species that grows in diverse environments, from flooded patches to dry upland fields, and even though its yield is low compared with the domesticated varieties, it is well adapted to survive under drought conditions for long periods (Mohapatra *et al.*, 2011). Domesticated rice, including the subspecies 'indica' and 'japonica', has been bred under water logged conditions during the last five thousand years of crop production, which has resulted in a relatively high sensitivity to drought (Xia *et al.*, 2014). Nowadays, 75% of the world rice production comes from irrigated lowland rice, while the other 25% coming from rainfed lowland and upland rice (IRRI database; Fig. 1). Water deficit is by far the main limitation for rice production in rainfed ecosystems, generating a yield gap between rainfed and irrigated rice of approx. 1 to 10 t ha^{-1}.

Water deficit: a problem on the rise in agriculture

The availability of water is declining dramatically and the consumptive water use (CWU) of rice is relatively high (27%) while its water productivity (CWP) is low (0.60 kg / m^3) when compared with other crops like maize (2.19 kg / m^3) (Liu *et al.*, 2009). This makes rice a very inefficient crop in terms of water use. Drought stress (water deficit) is by far the most important factor limiting crop productivity in the world, and improving yield under drought conditions is a major goal in plant breeding nowadays. Plants have evolved many forms of adaptations to water stress, and drought resistance mechanisms can be divided into several types. Drought tolerance is the ability to function when partially dehydrated, usually losing between 1-20% of relative water content, but there are species that are able to survive even when they lose 95%

of relative water content (resurrection plants). Desiccation postponement is the ability to maintain tissue hydration even when conditions are extremely dry, for example in succulent

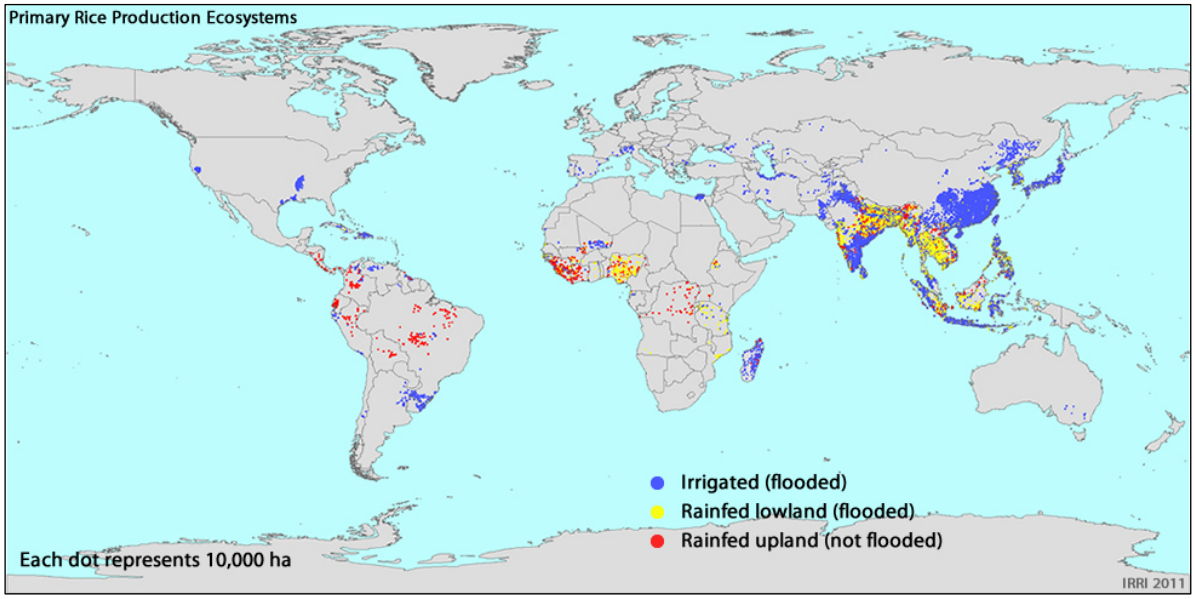


Figure 1. Global production of the three primary rice ecosystems (from IRRI Rice Knowledge Bank: http://www.knowledgebank.irri.org/submergedsoils/index.php/rice-growing-environments/lesson-1).

plants like those in the family Cactaceae. Drought escape is the strategy in which plants complete the lifecycle during the wet season before drought comes in (Moore *et al.*, 2008; Singh, 2011). In rice, water deficit conditions provoke a decrease in overall plant growth and trigger leaf rolling, a mechanism that reduces the transpiration area and leaf dehydration. A limited transpiration rate is an important water-saving mechanism that is also reduced by stomata closure due to the accumulation of abscisic acid (ABA) in leaves. As a result of these responses, nutrient translocation is impaired and grain filling and size are reduced (Pandey & Shukla, 2015). Drought conditions also affect the architecture of the rice root, however the type of these changes and root architecture strategies vary among different cultivars (Kadam *et al.*, 2015). Several cultivars of crop species like maize, wheat and some rice wild varieties have evolved mechanisms leading to improved osmotic adjustment and water use efficiency that allow some degree of drought tolerance in their vegetative tissues. For instance in upland rice, which has some resistance to drought conditions, levels of the aquaporin protein RWC3 are relatively high during drought stress. The accumulation of this aquaporin results in improved root osmotic hydraulic conductivity and leaf water potential (Lian *et al.*, 2004).

Nowadays, cultivation of rice in paddy fields is the dominant practice. This farming practice requires a lot of labour and the waterlogged conditions create a large emission of

greenhouse gases. Furthermore, various water-saving management practices have been developed for rice, such as the use of more drought-tolerant varieties or coupling the growth cycle with low evaporation periods (Bindraban *et al.*, 2006). However, yield penalty seems unavoidable, which makes farmers reluctant to adopt these growing practices. A higher water use efficiency rate, defined as the amount of dry matter or harvestable yield produced per unit of water, is an important characteristic of plants that adapt well to drought. The generation of plants with a more stable water content that maintain yield under drought stress conditions is one of the aims of the current rice breeding programs.

ABA and SLs: signals in the plant adaptation to drought stress

Plant hormones (phytohormones) play an important role in the response and adaptation of plants to stresses that threaten their growth and survival. The stress-related hormone 'par excellence' is ABA because of its role in the adaptation to drought, salinity, cold, pathogen attack and UV damage. In addition to its role in stress responses, it is also involved in developmental processes such as seed maturation, seed and bud dormancy, cell division and floral induction (Nambara & Marion-Poll, 2005; Finkelstein, 2013). ABA acts as a negative regulator of shoot elongation as well. In paddy rice, under submergence, ABA levels decrease rapidly by 75%, resulting in fast shoot elongation through the action of gibberellin and ethylene, hence allowing the rice plant to emerge from the water (Choi, 2011). ABA also plays a key role in rice drought adaptation, as elevated shoot ABA content provokes stomata closure, thus reducing transpiration and water loss, decreasing shoot growth and promoting primary root length (Hong *et al.*, 2013). ABA is a carotenoid-derived molecule generated through the 2-C-methyl-d-erythritol-4-phosphate (MEP) pathway by the cleavage of C_{40} carotenoids (Cutler & Krochko, 1999). One of these C_{40} carotenoids, all-trans-lycopene, is cyclized generating β-carotene (Nambara & Marion-Poll, 2005). The β-carotene is not only an intermediate of the ABA biosynthetic pathway, but also a precursor for the hormone strigolactone (Fig. 2).

ABA, being the active form of the hormone, can be catabolized into a hydroxylated or conjugated form. The product resulting from the first hydroxylation step (hydroxyl-ABA) is still biologically active, however the next catabolic steps, resulting in the generation of phaseic acid (PA), dihydrophaseic acid (DPA) and neo phaseic acid (neo PA) lead to the inactivation of ABA (Nambara & Marion-Poll, 2005). The conjugated form of ABA, ABA-glucose ester

(ABA-GE) is inactive and has a very low permeability for cell membranes, making ABA-GE suited for long-distance translocation and storage (Ye *et al.*, 2012).

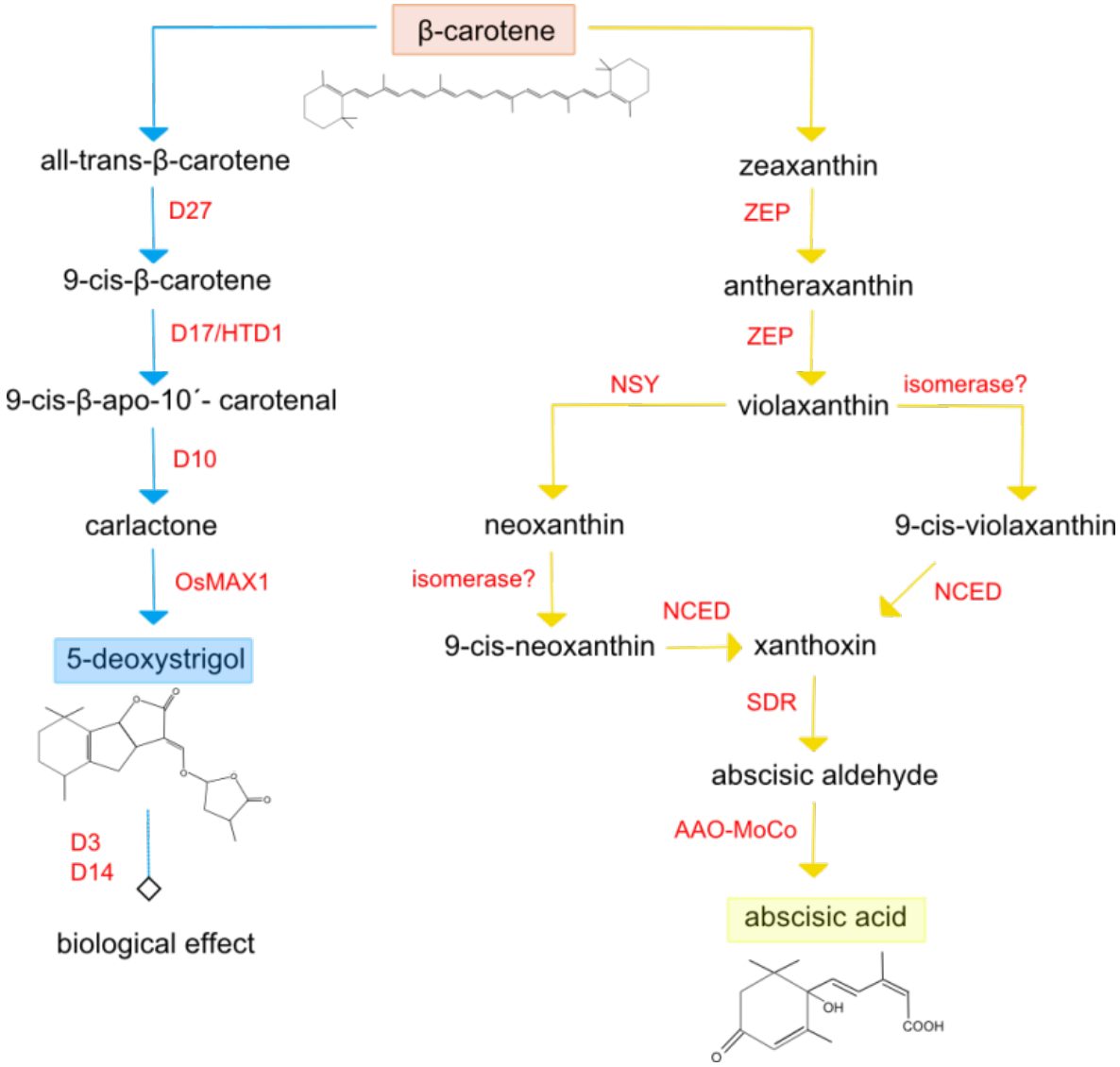


Figure 2. Strigolactones and abscisic acid biosynthetic pathways. Enzymes name for rice are represented.

Strigolactones (SLs) are among the diverse molecules exuded by roots into the surrounding soil. They act as a signal to stimulate hyphal branching of arburscular mycorrhizal fungi (AM fungi) but also induce the germination of root parasitic plant seeds (Besserer *et al.*, 2006). It is important to highlight that they are active in extremely low concentrations. In rice, the concentration of SLs in the exudate varies strongly between genotypes (Jamil *et al.*, 2011).

Their biosynthesis is regulated by the carotenoid isomerase D27, which uses all-trans-β-carotene as substrate (Fig. 2), and two carotenoid cleavage dioxygenases (CCDs) (CCD7 and CCD8). In rice, the CCD8 product, carlactone, is further oxidized to 4-deoxyorobanchol (4DO) which is later hydroxylated to orobanchol, one of the SLs found in some rice ecotypes. These last two steps are regulated by a cytochrome P450 (MAX1) (Zhang *et al.*, 2014). Further downstream the SL biosynthetic pathway, a hydrolase (D14) and an F-box protein (D3) play key roles in the recognition of and response to SLs.

A recent study found a link between the SLs and ABA levels in the plant. ABA biosynthesis tomato mutants had a lower SLs concentration in their root exudates compared with their wild type (López-Ráez *et al.*, 2010). The relation between both hormones under drought stress conditions is an ongoing research topic. SLs seem to have a role in drought adaptation in *Arabidopsis thaliana* (L.) Heynh, as SLs mutants are more sensitive to water and salt stress (Bu *et al.*, 2014; Ha *et al.*, 2014). *Lotus japonicus* L. and tomato SL-depleted plants also show a higher sensitivity to water stress (Liu *et al.*, 2015; Visentin *et al.*, 2016). However, in rice there is currently no experimental evidence reported supporting a potential interaction between ABA and SLs. The discovery of new factors involved in the regulation of the biosynthesis and signalling of carotenoids, including ABA and SLs, under drought could provide us with new insights into their putative roles during the plant adaptation process to water deficit.

SL and ABA biosynthesis already occurs in green algae (Chlorophyta)(Delaux *et al.*, 2012). When plants started to colonise terrestrial habitats, the water content of embryophytes was fluctuating and it has been hypothesised a possible induction of strigolactone biosynthesis and a higher production of ABA (Hartung, 2010; Delaux *et al.*, 2012). Plant symbiosis already started 400 million years ago, between Devonian land plants and AM fungi (Remy *et al.*, 1994; Parniske, 2008). It is known that ABA is needed to make plants susceptible to a symbiosis with AM fungi and that ABA plays a role in the development of arbuscules inside the root (Herrera-Medina *et al.*, 2007). Recently it has been shown that the F-box protein D3, which is involved in SLs perception in rice, plays an important role in the establishment of the symbiosis with AM fungi (Yoshida *et al.*, 2012), whereas the α/β hydrolase D14 does not. In a study with lettuce it was shown that salinity stressed plants have a higher SL production with increasing stress levels, as a “call for help”, and that SLs levels were lower in inoculated plants compared with non-inoculated ones. At the same time ABA levels were lower in inoculated plants, yet the ABA increased with increasing levels of stress in both inoculated and no-inoculated plants

(Aroca *et al.*, 2013). The lower ABA levels in AMF inoculated plants might be caused by an improved water uptake mediated by the fungus. When mild drought was applied to tomato plants inoculated with AM fungi, similar results were obtained for ABA and SLs (Ruiz-Lozano *et al.*, 2015). In rice, however, there is still a need to study how water stress and mycorrhizal fungi interact and ultimately affect SLs and ABA production.

Soil microorganisms and their interaction with plants

The soil ecosystem plays an essential role in nutrient cycling and plant productivity (van der Heijden, 2008) and one of the key components of this environment are the microbial communities. AM fungi are part of these communities, representing obligate symbionts that colonise the roots and their rhizosphere simultaneously. They are associated with 70-90% of land plant species (Parniske, 2008) including rice, are geographically ubiquitous and improve plant growth as well as nutrient uptake - especially phosphorous and nitrogen - and water uptake among other benefits (Robinson-Boyer *et al.*, 2009; Denison & Kiers, 2011). Their filamentous hyphal networks allow the plant to gain access to a greater quantity of water and soil minerals (Singh, 2011). For example, it has been demonstrated that mycorrhizal plants have a higher water use efficiency (WUE) and a higher root turgor (Ruiz-Lozano *et al.*, 1995; Augé, 2001; Asrar *et al.*, 2012; Wu *et al.*, 2013), and are therefore better able to maintain tissue hydration. Even though there are studies which have already shown the role of AM fungi in water uptake by plants under stress conditions, the molecular mechanisms behind this effect are still unknown. Monocots and dicots both form symbioses with AM fungi in which the fungus shows a similar morphology of root colonisation, in spite of the different root system organization characteristic of each plant group. There are several studies exploring the effects of AM fungi on rice under stress conditions such as phosphate starvation, but not so many in relation to drought (Solaiman & Hirata, 1995).

Even though AM fungi are the most studied group among the rhizosphere microbiota, there are also other microorganisms that establish symbioses with plants and help them to cope with different types of environmental stresses. Bacterial endophytes for instance, are present in almost every plant species around the globe, colonizing different tissues, from root to shoot (Santoyo *et al.*, 2016). Some bacteria can boost plant growth, and are thus called plant growth promoting bacteria (PGPB). They are found either around the roots or inside roots (endophytic bacteria). It has been shown that they provide plants with nutrients (especially nitrogen), assist

them in the defense against pathogens and help plants to cope with abiotic stresses (Hardoim *et al.*, 2015; Bacon & White, 2016). Besides AM fungi, there is another class of fungi that has been studied thoroughly during the last years especially because of their role in plant adaptation to biotic and abiotic stress. They are referred as to Class II and Class IV (or dark septate fungal endophytes) fungal endophytes (Rodriguez *et al.*, 2009; Hardoim *et al.*, 2015). The occurrence of Class IV fungal endophytes is restricted to plant roots, while Class II endophytes colonise different plant tissues, from root to shoot. Both groups of fungal endophytes have a wide host range, and their capacity to protect plants against salt and drought stress has been demonstrated in grasses such as rice and wheat (Redman *et al.*, 2011; Hubbard *et al.*, 2014). So far, not much is known about the molecular mechanisms behind these fungal endophyte-root relationships.

Microbial communities and their role in plant fitness

Despite their potential importance for plant fitness, the microbiota present on plants are still understudied both in terms of diversity as well as function. There is a trend, however, to view plants as holobionts, considering the plant and its associated microbiota as the same entity, because the microbiome gives the host additional functions to cope with environmental changes (Friesen *et al.*, 2011; Vandenkoornhuyse *et al.*, 2015). During the last decade there has been a surge in the research on the microbiome composition in plants, especially in roots (Edwards *et al.*, 2015; Bulgarelli *et al.*, 2015; Bai *et al.*, 2015; Angel *et al.*, 2016; Glynou *et al.*, 2016). Inside these microbial communities, species interact with each other in different manners, from synergistic to parasitic, and as a consequence these microbe-microbe interactions affect and determine the host plant fitness (Philippot *et al.*, 2013; van der Heijden & Hartmann, 2016). It has been suggested that plants can recruit specific microbial species when facing a constraint, so they can change their microbiome composition depending on their developmental stage or the environmental conditions (Bonito *et al.*, 2014; Bazghaleh *et al.*, 2015). This process, in which the host-plant controls microorganism recruitment must be under genetic control, since multiple studies demonstrate that host genotype is one of the main factors determining the composition and the structure of the plant microbiome (Fonseca-García *et al.*, 2016; Wagner *et al.*, 2016).

Genome wide association mapping to unravel the basis of the plant response to stress

Another important aspect of how plants adapt to drought conditions is the understanding of the genetic basis of these responses to this type of stress. In rice, substantial genetic variation in response to drought exists. Exploiting such variation in conventional breeding programs has already resulted in drought tolerant varieties. The existing variation in drought tolerance can also be used to explore the genomic regions involved in drought responses in order to unravel how these physiological processes are regulated at the molecular level. Linkage mapping analysis (QTL mapping studies) for rice root traits conferring drought tolerance have been conducted using progenies derived from crosses between modern and traditional varieties (Price *et al.*, 1997; Bernier *et al.*, 2009; Sandhu *et al.*, 2013). Drought-resistance QTLs have been identified for several rice biparental populations and inbred lines (RILs) (Price *et al.*, 1997; Khowaja & Price, 2008; Bernier *et al.*, 2009; Sandhu *et al.*, 2013). However, the use of QTL studies to further unravel the genetic factors involved in drought tolerance is a difficult task. Drought tolerance is a complex trait influenced by many environmental factors such as rainfall rate, soil nutrient availability or plant pathogens. Different rice cultivars are equipped with a repertoire of different drought adaptation mechanisms, each controlled by multiple genes (Price *et al.*, 2002). For these reasons, the detected drought resistance related QTLs by linkage mapping largely depends on the lines selected for the study and generally have a relatively low mapping resolution. Hence further fine mapping is required to narrow down the precise genetic position allowing the final cloning of the underlying gene.

An alternative approach for studying the genetics of natural variation is the use of Genome Wide Association Studies (GWAS). Because these studies take advantage of the presence of the large number of historical recombination in populations of unrelated individuals, mapping resolution is improved. In a typical GWA study, a large number of single nucleotide polymorphisms (SNPs), spread throughout the entire genome, is used to screen for associations between the SNP alleles and the trait of interest (Bush & Moore, 2012). A number of GWAS mapping studies have been performed in rice, but still there is a lot to do regarding drought-tolerance traits. Although it has been shown that GWAS in *O. sativa* is a good approach to detect QTLs responsible for a certain trait, it has not yet been shown to be accurate enough to unravel the genetics that shape natural variation in this species. However, it is expected that

with the use of larger rice populations and denser SNPs map this problem can be solved (Zhao *et al.*, 2011).

Recent studies have shown that there is a link between the host plant and its microbiome composition. This association is observed for both the phyllosphere and the rhizosphere (Peiffer *et al.*, 2013; Bodenhausen *et al.*, 2014; Bonito *et al.*, 2014; Wagner *et al.*, 2016). However, how this link is established or what host factors are responsible for the specific recruitment of certain microorganisms remains to be explored. Only recently, a GWA study using an *A. thaliana* population resulted in the identification of some QTLs responsible for the variation in bacterial and fungal communities in the phyllosphere (Horton *et al.*, 2014). However, no further studies of this kind have been performed for the rhizosphere. Moreover, it is necessary to understand if there is a correlation between the plant associated microbiome and plant fitness, particularly under stress conditions. This kind of information would be relevant to harness specific microorganism assemblies or new genetic mechanisms involved in the host-microbiome relationship that may have an impact on plant fitness. Furthermore this knowledge could potentially be applied for a more sustainable agriculture, especially in a fast changing environment with frequently occurring abiotic stress conditions.

Thesis outline

Given the fact that abiotic stress is the primary cause of crop yield losses worldwide and that food security is challenged by the declining availability of water, especially for rice, exploring how rice adapts to water deficit is a first step for future applications in rice breeding programs. In this thesis I focus on the rice root and rhizosphere. They play an important role in drought adaptation as they are the first plant interfaces to encounter the signs of drought, and signal it to the rest of the plant. In addition, they are important in mitigating or preventing the effect of drought. The main aims of my work are to unravel the mechanisms that regulate the hormonal balance between SLs and ABA in rice, especially under drought, and how the root associated fungal microbiome interacts with the rice plant ultimately affecting plant fitness (Fig. 3).

In **Chapter 2** I discuss the current knowledge on the different roles of strigolactones in the rhizosphere, specifically in regulating root architecture and the establishment of mutualistic symbioses with plant microorganisms, as well as their involvement in how plants respond to and avoid abiotic stresses such as drought and salinity.

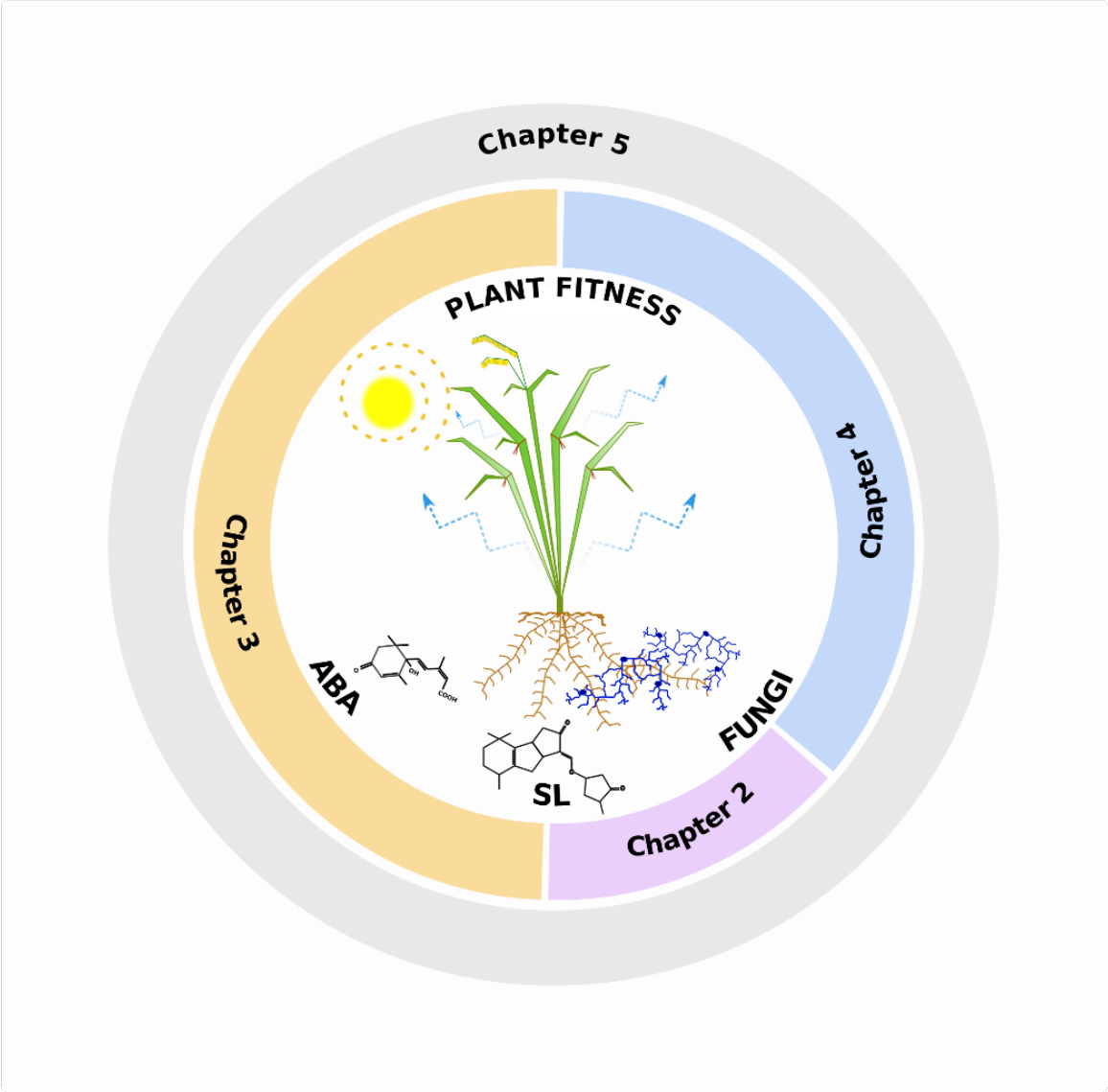


Figure 3. Diagram representing the outline of this thesis. The main targets of my work are to review the current knowledge of strigolactones' role in the rhizosphere, regulating root architecture and the establishment of a mutualistic symbiosis (Chapter 2), to unravel the mechanisms that regulate the hormonal balance between strigolactones (SLs) and abscisic acid (ABA) in rice under drought (Chapter 3) and how the root associated fungal microbiome interacts with the rice plant ultimately affecting plant fitness (Chapter 4 and Chapter 5).

Considering the common origin between strigolactones and abscisic acid and their role in plant development and fitness, in **Chapter 3** I explore the relationship between both phytohormones, using strigolactone mutants, and their impact on rice drought adaptation.

In **Chapter 4** I describe the rice root associated fungal community and how it changes under drought conditions. I study if the fungal microbiome affects grain yield under drought and if these changes are at least in part controlled by the plant genotype.

As mentioned above, strigolactones have a role in the establishment of some root associated microorganisms which help plants to cope with drought stress. In **Chapter 5** I explore within the frame of a Genome Wide Association Study the possible rice loci involved in the symbiosis with mutualistic fungal symbionts and study if these loci are also involved in already known drought adaption mechanisms.

Finally, in **Chapter 6** I discuss the main highlights of my thesis and give an outlook on the possible use of the knowledge generated with my work to improve drought tolerance in rice and the sustainability of the crop in view of its current large water use.

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Chapter 2

Ecological relevance of strigolactones in nutrient uptake and other abiotic stresses, and in plant-microbe interactions below-ground

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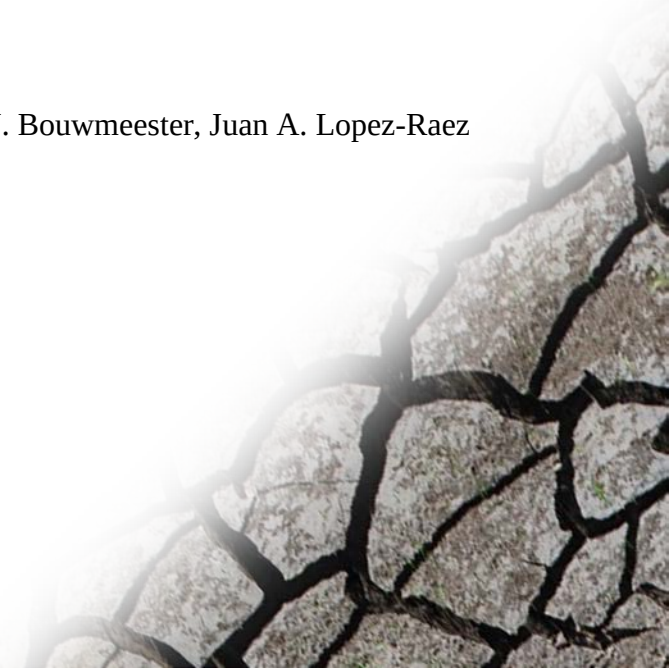

Abstract

Background

Plants are exposed to ever changing and often unfavourable environmental conditions, which cause both abiotic and biotic stresses. They have evolved sophisticated mechanisms to flexibly adapt themselves to these stress conditions. To achieve such adaptation, they need to control and coordinate physiological, developmental and defence responses. These responses are regulated through a complex network of interconnected signalling pathways, in which plant hormones play a key role. Strigolactones (SLs) are multifunctional molecules recently classified as a new class of phytohormones, playing a key role as modulators of the coordinated plant development in response to nutrient deficient conditions, especially phosphorus shortage. Belowground, besides regulating root architecture, they also act as molecular cues that help plants to communicate with their environment.

Scope

This review discusses current knowledge on the different roles of SLs below-ground, paying special attention to their involvement in phosphorus uptake by the plant by regulating root architecture and the establishment of mutualistic symbiosis with arbuscular mycorrhizal fungi. Their involvement in plant responses to other abiotic stresses such as drought and salinity, as well as in other plant-(micro)organisms interactions such as nodulation and root parasitic plants are also highlighted. Finally, the agronomical implications of SLs below-ground and their potential use in sustainable agriculture are addressed.

Conclusions

Experimental evidence illustrates the biological and ecological importance of SLs in the rhizosphere. Their multifunctional nature opens up a wide range of possibilities for potential applications in agriculture. However, a more in-depth understanding on the SL functioning/signalling mechanisms is required to allow us to exploit their full potential.

Introduction

The most important assignment of modern agriculture is to provide global food security in a sustainable manner. Fifty years ago, the challenge to feed the growing world population was solved by the development of new high-yielding crop varieties and high-intensity agricultural management (Gianinazzi et al. 2010). However, optimal production of these improved

varieties/strategies could not be achieved with the natural reserves of nutrients available in most soils. Thus, chemical fertilizers containing nitrogen, phosphorus and potassium (NPK) became an indispensable source of the nutrients required for proper crop growth and food production. However, the cheap source of one of these nutrients, rock phosphate, will be exhausted in a few decades (Cordell et al. 2009). Therefore, there is a need to develop new agronomical strategies to optimize phosphorus (P) usage. Plants can only assimilate P in its inorganic mineral phosphate form, which is usually present in only low concentrations and is rather immobile in the soil, which results in P deficiency (Péret et al. 2011; Schachtman et al. 1998). To cope with P deficiency, plants have evolved a wide array of adaptive responses in plant growth, development, metabolism and interaction with soil microorganisms (Péret et al. 2011; Rouached et al. 2010; Smith and Read 2008).

Strigolactones (SLs) are multifunctional molecules classified as a new class of phytohormones that controls several different processes in plants. They play a pivotal role as modulators of the coordinated development of roots and shoots in response to nutrient deficient conditions, especially phosphorus shortage. Accordingly, SLs regulate above- and belowground plant architecture, adventitious root formation, secondary growth, reproductive development and leaf senescence (Agusti et al. 2011; Gomez-Roldan et al. 2008; Kapulnik et al. 2011a; Kohlen et al. 2012; Rasmussen et al. 2012; Ruyter-Spira et al. 2011; Umehara et al. 2008; Yamada et al. 2014). However, novel roles for SLs are emerging, for example, recently they were also shown to play a role in defence responses (Torres-Vera et al. 2014). Despite their importance as plant hormones, they were initially identified as signalling molecules in the rhizosphere. Here, SLs act as host detection cues for root parasitic plants of the Orobanchaceae and symbiotic arbuscular mycorrhizal (AM) fungi from the phylum Glomeromycota (Fig. 1) (Akiyama et al. 2005; Bouwmeester et al. 2007; López-Ráez et al. 2011b). More recently, a role for SLs in another important plant-symbiotic microorganism interaction in the rhizosphere, nodulation, was described (Fig. 1) (Foo and Davies 2011; Soto et al. 2010).

SL biosynthesis and signalling

SLs are mainly produced in the roots and secreted into the rhizosphere, but biosynthesis also has been suggested to occur throughout the plant, although at low or even undetectable levels (Dun et al. 2009; Xie et al. 2010). They are produced at extremely low levels, being active at pico- and nanomolar concentrations, and are unstable in the soil, which hampers their isolation

and characterization (Xie et al. 2010). To date 19 different SLs have been characterized, but it has been estimated that the total number of natural SLs might be over 1000 (Akiyama et al. 2010; Ćavar et al. 2014; Zwanenburg and Pospíšil 2013). They have been detected in a wide range of monocotyledonous and dicotyledonous plant species, and each plant is producing a blend of different SLs depending on the species (Ruyter-Spira et al. 2013; Xie et al. 2010). All natural SLs isolated and characterized so far have a similar chemical structure, with a structural core consisting of a tricyclic lactone (the ABC-rings) connected via an enol ether bridge to a butyrolactone group (the D-ring) (Fig. 1) (Ćavar et al. 2014; Xie et al. 2010). The bridge between the C- and D-rings can be rapidly cleaved in aqueous and/or alkaline environments, resulting in their short-lived character, which supports their role as signalling molecules (Akiyama et al. 2010; Xie et al. 2010; Zwanenburg and Pospíšil 2013). SLs have recently been classified into two groups of diastereoisomers, the strigol-type and the orobanchol-type, depending on their C-ring orientation (Fig. 1) (Xie et al. 2013; Zwanenburg and Pospíšil 2013). The AB-rings are less conserved than the CD-rings and can be decorated or modified by for example methylation, hydroxylation, acetylation, etc, giving rise to the different SLs known today (Akiyama et al. 2010; Zwanenburg and Pospíšil 2013). The stereochemistry and structural features of the different SLs are important for their biological activity. For example, the CD part is essential for the parasitic weed seed germination inducing activity, but modifications in the A-ring have little effect on this activity (Akiyama et al. 2010; Xie et al. 2010; Zwanenburg and Pospíšil 2013). For their hyphal branching inducing activity in AM fungi the D-ring is also essential, but the bridge between the CD-rings does not necessarily have to be an enol ether (Akiyama et al. 2010; Zwanenburg and Pospíšil 2013). Akiyama and co-workers also showed that the hyphal branching activity depended on the modifications on the AB-ring (Akiyama et al. 2010; Zwanenburg and Pospíšil 2013). The presence of the D-ring is also necessary for hormonal activity of SLs (Boyer et al. 2012). In addition, Boyer and co-workers showed that lipophilicity is an important factor for this activity, with the SLs having a hydroxyl group on the AB-rings being more active (Boyer et al. 2012).

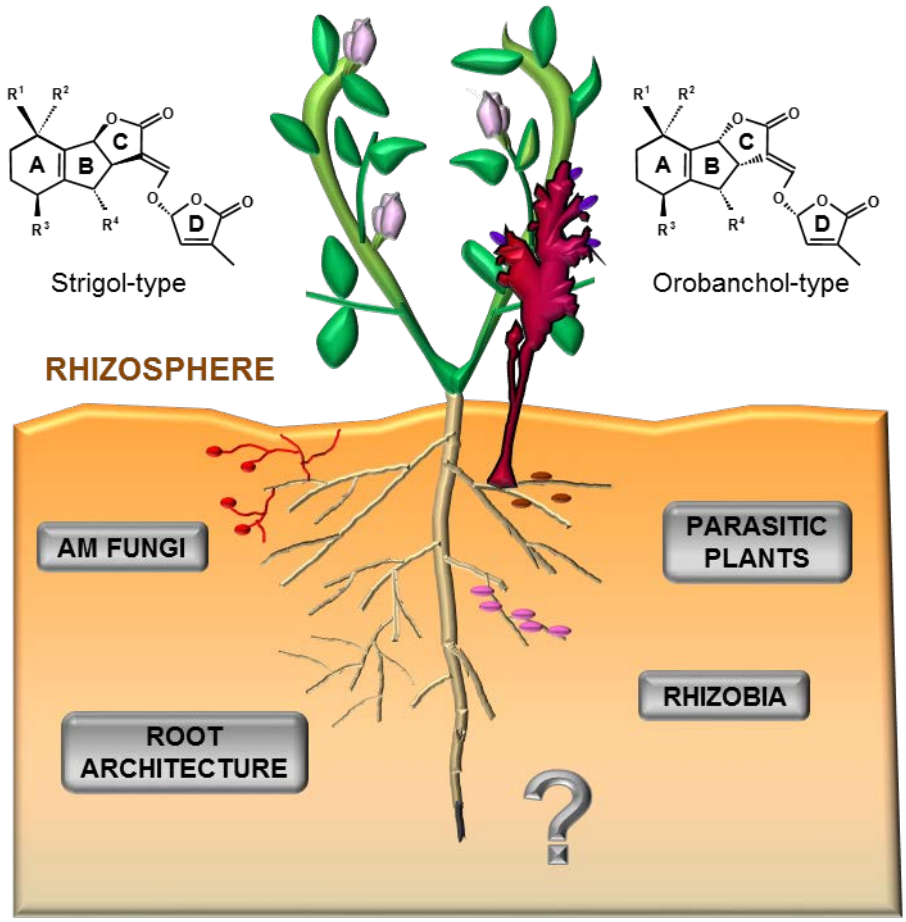

Figure 1. Chemical structures of strigolactones and roles they play belowground. Strigolactones (SLs) are multifunctional molecules playing several different roles in plants. As plant hormones, they modulate root system architecture. In the rhizosphere, they favour the establishment of beneficial associations with arbuscular mycorrhizal fungi (AM fungi) and rhizobia. SLs also promote the germination of root parasitic plants, allowing a parasitic interaction. Novel rhizosphere roles for SLs may emerge as indicated by ?.

SLs biosynthetically derive from the carotenoids (López-Ráez et al. 2008a; Matusova et al. 2005) through the conversion of all-*trans*-β-carotene to 9-*cis*-β-carotene mediated by a β-carotene isomerase (D27) (Alder et al. 2012). 9-*Cis*-β-carotene is transformed into carlactone by sequential oxidative cleavage by two carotenoid cleavage dioxygenases (CCD7 and CCD8) (Alder et al. 2012), and thus SLs belong to the apocarotenoids, as the phytohormone abscisic acid (ABA) (Ohmiya 2009; Walter and Strack 2011). In rice, carlactone is then converted into the strigolactone *ent*-2'-*epi*-5-deoxystrigol by a cytochrome P450, Os900, that is homologous to Arabidopsis MAX1 (Zhang et al. 2014). Another rice MAX1 homolog, Os1400, then converts *ent*-2'-*epi*-5-deoxystrigol into orobanchol (Zhang et al. 2014). Rice has five MAX1 orthologs, of which four - *Os900*, *Os1400*, *Os5100* and *Os1900* - were shown to rescue the

Arabidopsis *max1* mutant phenotype (Challis et al. 2013; Cardoso et al. 2014). Although upon expression in *Nicotiana benthamiana Os5100* and *Os1900* catalysed the conversion of carlactone into *ent*-2'-*epi*-5-deoxystrigol (and minute amounts of 5-deoxystrigol), this occurred with very low efficiency, just as for Arabidopsis *MAX1* (Zhang et al. 2014). The application of labelled carlactone to Arabidopsis resulted in the formation of a product called SL-LIKE1 and not *ent*-2'-*epi*-5-deoxystrigol (Seto et al. 2014), although the level of the latter compound may have been beyond the detection level. SL-LIKE1 was recently identified as methyl carlactonate and showed that it is biologically active in inhibiting shoot branching in Arabidopsis (Abe et al. 2014). Therefore, it seems that in Arabidopsis the thus far reported canonical strigolactones (Goldwasser et al. 2008; Kohlen et al. 2011) are minor side products or artefacts. That could imply that MAX1 and the rice MAX1 orthologs Os5100 and Os1900 have a different enzymatic activity than rice MAX1 orthologs Os900 and Os1400. Interestingly, although Os1400 is absent in the rice cultivar Bala, this line still produces orobanchol. Therefore, there must be an as yet unidentified cytochrome P450 present in the rice genome that has a similar activity as this MAX1 orthologue (Zhang et al 2014, Cardoso et al 2014). Since Arabidopsis MAX1 also lacks the capacity to convert *ent*-2'-*epi*-5-deoxystrigol to orobanchol, the minute amounts of orobanchol observed in Arabidopsis root exudates are also likely to result from a similar mechanism (Zhang et al 2014).

SL perception and signalling require an F-box leucine-rich repeat protein (MAX2) and an α/β-hydrolase (D14) (Gomez-Roldan et al. 2008; Hamiaux et al. 2012; Umehara et al. 2008). Binding of SLs by D14 enables their interaction with MAX2 and this complex facilitates the degradation of the target protein D53 and the transcriptional effector BES1 via the ubiquitin-proteasome system (Jiang et al. 2013; Wang et al. 2013; Zhou et al. 2013), a similar mechanism as for gibberellin perception and signalling. D53 is a class I Clp ATPase protein which acts a repressor of SL signalling, and its degradation prevents axillary-bud outgrowth in rice (Jiang et al. 2013; Zhou et al. 2013). Interestingly, it has been suggested that SLs promote proteasome-mediated degradation of D14 in Arabidopsis, thus limiting their own signalling by a negative feedback loop (Chevalier et al. 2014).

In the present work, we review the current knowledge on the different roles of SLs in the rhizosphere, paying special attention to their involvement in phosphorus uptake by the plant. We focus on their ability to regulate root system architecture and to favour symbiosis establishment with beneficial microorganisms such as AM fungi and rhizobia. Finally, because

of their multifunctional character, the potential use of SLs to develop new more sustainable agricultural strategies will be discussed.

SLs and root system architecture

One of the functions of SLs below-ground is to regulate root development in response to phosphorus shortage (De Cuyper et al. 2015; Kapulnik et al. 2011a; Koltai 2011; Ruyter-Spira et al. 2011). Interestingly, SL biosynthesis is promoted by P-limiting conditions (Table 1) (Foo et al. 2013b; López-Ráez et al. 2008a; Yoneyama et al. 2012; Yoneyama et al. 2007), and it has been suggested that they play a pivotal role as modulators of the coordinated development of roots and shoots under these unfavourable conditions. On the one hand, increased SL production suppresses the outgrowth of axillary branches/tillers (Kohlen et al. 2011; Umehara et al. 2010), while at the same time they affect various aspects of root growth all aimed to improve phosphate foraging (Mayzlish-Gati et al. 2012; Ruyter-Spira et al. 2011; Sun et al. 2014).

Table 1. Effect of different abiotic stresses on SL production and/or SL biosynthetic gene expression and AMF colonisation in different plant species. Stresses include: phosphorus starvation (-P), nitrogen starvation (-N), drought, salinity, low temperature (Low T), high temperature (High T), cadmium (Cd), copper (Cu) and aluminium (Al). The levels are compared with control plants (non-stressed), and are higher (+), lower (-) or not different (=). ND, not determined.

Stress	Plant	Effect on SLs	Effect on AMF colonisation	AM fungus	Reference
-P	*M. truncatula*	+	+	*R. irregularis*	Bonneau et al. 2013
-P	*M. truncatula*	+	ND	ND	Yoneyama et al. 2012
-P	*P. sativum*	+	+	*R. irregularis*	Foo et al. 2013
-P	*O. sativa*	+	ND	ND	Jamil et al. 2011
-P	*O. sativa*	+	ND	ND	Umehara et al. 2010
-P	*S. lycopersicum*	+	ND	ND	López-Ráez 2008
-P	*S. lycopersicum*	+	ND	ND	Yoneyama et al. 2012
-P	*S. bicolor*	+	ND	ND	Yoneyama et al. 2007
-P	*T. aestivum*	+	ND	ND	Yoneyama et al. 2012
-P	*L. sativa*	+	ND	ND	Yoneyama et al. 2012
-P	*A. sinicus*	+	ND	ND	Yoneyama et al. 2012
-P	*A. thaliana*	+	ND	ND	Kohlen et al. 2011

-P	*T. pratense*	+	ND	ND	Yoneyama et al. 2012
-P	*C. officinalis*	+	ND	ND	Yoneyama et al. 2012
-P	*L. japonicus*	+	ND	ND	Liu et al. 2015
-N	*M. truncatula*	=	ND	ND	Yoneyama et al. 2012
-N	*P. sativum*	+	ND	ND	Foo et al. 2013
-N	*O. sativa*	+	ND	ND	Jamil et al. 2011
-N	*S. lycopersicum*	=	ND	ND	Yoneyama et al. 2012
-N	*S. bicolor*	+	ND	ND	Yoneyama et al. 2007
-N	*S. bicolor*	+	ND	ND	Yoneyama et al. 2013
-N	*T. aestivum*	+	ND	ND	Yoneyama et al. 2012
-N	*L. sativa*	+	ND	ND	Yoneyama et al. 2012
-N	*A. sinicus*	+	ND	ND	Yoneyama et al. 2012
-N	*C. officinalis*	+	ND	ND	Yoneyama et al. 2012
Drought	*S. lycopersicum*	ND	=	*R. irregularis*	Aroca et al. 2008
Drought	*T. aestivum*	ND	-	*G. etunicatum*	Al-Karaki et al. 2004
Drought	*T. aestivum*	ND	+	*F. mosseae*	Al-Karaki et al. 2004
Drought	*T. aestivum*	ND	=	*G. etunicatum*	Al-Karaki et al. 2004
Drought	*T. aestivum*	ND	=	*F. mosseae*	Al-Karaki et al. 2004
Drought	*C. lanatus*	ND	+	*R. irregularis*	Omirou et al. 2013
Drought	*C. lanatus*	ND	+	*F. mosseae*	Omirou et al. 2013
Drought	*Z. mays*	ND	-	*G. etunicatum*	Zhu et al. 2012
Drought	*A. majus*	ND	-	*G. deserticola*	Asrar et al. 2012
Salinity	*L. sativa*	-/+	+	*R. irregularis*	Aroca et al. 2013
Osmotic	*L. japonicus*	-	ND	ND	Liu et al. 2015
Low T	*S. bicolor*	ND	-	*R. irregularis*	Augé et al. 2004
Low T	*O. sativa*	=	=	*R. irregularis*	Liu et al. 2013
High T	*M. truncatula*	ND	+	*R. irregularis*	Hu et al. 2014
Cd	*T. aestivum*	ND	-	*F. mosseae*	Shahabivand et al. 2012
Cu	*M. truncatula*	ND	-	*R. irregularis*	Hagerberg et al. 2011
Al	*A. virginicus*	ND	-	*A. morrowiae*	Kelly et al. 2005
Al	*A. virginicus*	ND	+	*G. clarum*	Kelly et al. 2005

Changes in root development during P starvation have been most intensively studied in Arabidopsis. Here, it was shown to stimulate lateral root and root hair formation, as well as their subsequent development, and to inhibit primary root growth (Fig. 2) (reviewed by Niu et al. 2013). In maize and rice, P starvation inhibits lateral root formation, while it promotes primary root growth (Li et al. 2012; Sun et al. 2014). Different responses to low P between these plant species might be due to the fact that Arabidopsis is a non-mycorrhizal plant.

However, we should be careful with generalizing root architectural changes when only studying one specific ecotype or variety for each species. For instance, various Arabidopsis ecotypes displayed a different root architectural response to low P conditions, suggesting that there is natural variation for this response and that it is genetically determined (Chevalier et al. 2003). In Arabidopsis, in the presence of sufficient P, SLs have a suppressive effect on lateral root formation (Fig. 2). Accordingly, SL-deficient mutants have a higher lateral root density (Kapulnik et al. 2011a). They also have a shorter primary root, not only in Arabidopsis, but also in rice and maize (Arite et al. 2012; Guan et al. 2012; Ruyter-Spira et al. 2011). These phenotypes could only be rescued by the application of the synthetic SL analogue GR24 to the SL biosynthesis mutants, but not in those affected in signalling, indicating that SLs regulate root architecture in a MAX2-dependent manner (Kapulnik et al. 2011a; Koltai et al. 2010; Mayzlish-Gati et al. 2012; Ruyter-Spira et al. 2011). Kapulnik and co-workers also showed that the application of GR24 (1 and 3 µM) to Arabidopsis seedlings led to a MAX2- dependent increase in root hair length (Fig. 2) (Kapulnik et al. 2011a; Kapulnik et al. 2011b).

The effect of SLs on the regulation of root system architecture (RSA) was shown to depend on the plant's P status (Kapulnik et al. 2011b; Ruyter-Spira et al. 2011). In contrast to the observed response in the presence of sufficient P, under P limitation SLs promoted lateral root development in Arabidopsis to improve P uptake (Fig. 2) (Ruyter-Spira et al. 2011). The involvement of SLs in the regulation of root architecture occurs through its cross-talk with the phytohormones auxin and ethylene (Kapulnik et al. 2011b; Koltai 2011; Ruyter-Spira et al. 2011). In Arabidopsis, the expression of the auxin receptor TRANSPORT INHIBITOR RESPONSE1 (TIR1) was increased by low P levels. Interestingly, this increase only occurred in wild-type plants but not in the SL signalling mutant (Mayzlish-Gati et al. 2012). Therefore, SLs may regulate RSA by affecting auxin sensitivity. Lateral root development and primary root growth depend on auxin influx from the polar auxin transport stream, which is mainly fed by auxin produced in the apex and young leaves (Aloni 2013; Dubrovsky et al. 2011). In Arabidopsis, GR24 application reduced the auxin level in young developing rosette leaves, resulting in a decreased leaf area (Ruyter-Spira et al. 2011). A logical explanation for this effect could be that because GR24 has an inhibitory effect on the auxin transport capacity of the polar auxin transport stream in the stem (Crawford et al. 2010), auxin levels initially accumulate, which negatively feeds back on auxin biosynthesis. Interestingly, both GR24 application and low P conditions reduced auxin transport and the activity of the auxin reporter DR5::GUS in rice root tips, suggesting that, like in Arabidopsis, SL-mediated root development is regulated

via a reduction of auxin transport from shoot to root (Sun et al. 2014). Indeed, GR24 has been shown to reduce the expression of the gene encoding the auxin efflux protein PIN1 in the stem (Crawford et al. 2010). Moreover, GR24 was found to rapidly (within 10 minutes) induce the depletion of PIN1 from the plasma membrane of stem xylem parenchyma cells (Shinohara et al. 2013). Although GR24 application also caused a reduction of PIN1 protein levels in the provascular region of root tips (Ruyter-Spira et al. 2011), this was only observed after six days when seedlings were grown in the continuous presence of GR24, and is therefore likely a secondary effect due to reduced auxin import from upper parts of the plant. Still, a direct effect on auxin transport capacity in certain regions of the root tip cannot be excluded. Recently, it was indeed observed that GR24 stimulates polar localization of PIN2 in the plasma membrane of root epidermal cells (Pandya-Kumar et al. 2014). Thus, SLs seem to regulate RSA by acting as modulators of the auxin flux hereby altering auxin levels according to the environmental conditions. With respect to the interaction with ethylene, it was proposed that SLs promote its biosynthesis, which in turn induces auxin biosynthesis, transport and signalling in the roots (Stepanova and Alonso 2009). This SL-ethylene-auxin cross-talk has only been proposed for the regulation of root hair elongation (Kapulnik et al. 2011b), although it is very likely that it may also be involved in the regulation of lateral root development, as well as in other SL-mediated processes.

Although we have some ideas about how SLs act in regulating root architecture, we are still far from understanding the exact mechanism and its regulation by environmental conditions. In addition, other phytohormones such as auxin, ethylene, ABA, gibberellins and cytokinins have been shown to be involved in RSA regulation and should be included in this complex signalling network.

Alternative strategies for P uptake: arbuscular mycorrhizas

The soil ecosystem is one of the main factors involved in nutrient cycling and plant productivity, which is intimately related to the associated microbiota (van der Heijden et al. 2008). Root architecture is not only of great importance for the uptake of nutrients and water, it is also vital for the anchorage in the soil and the interaction with symbiotic organisms (Den Herder et al. 2010).

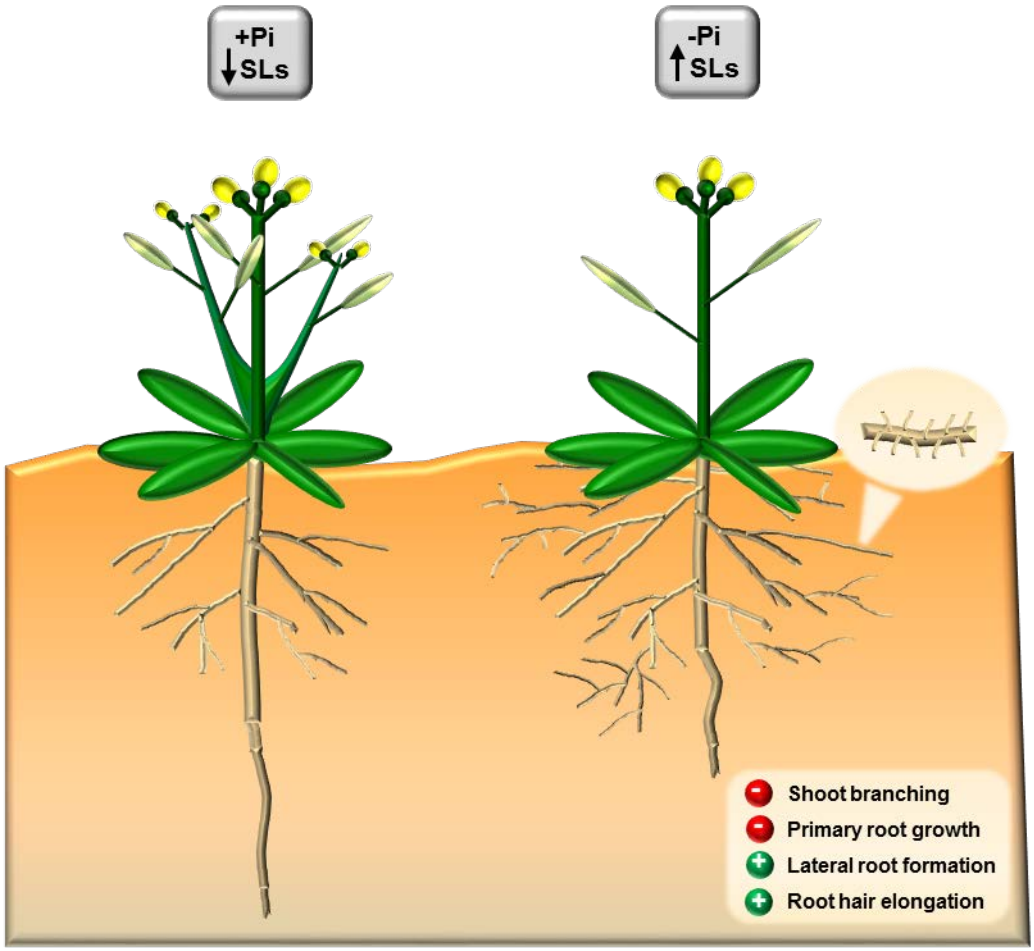

Figure 2. Impact of phosphorus status on strigolactone production and plant development in *Arabidopsis thaliana* (ecotype Columbia). Phosphate (P) deficiency promotes strigolactone (SL) production in the roots, affecting plant architecture. Under these conditions, SLs are involved in reducing primary root growth, inducing lateral root density and development, and stimulating root hair elongation and density. These modifications allow the plant to increase the exploratory capacity of the soil. SLs are also transported to the shoot, where they inhibit shoot branching, hence increasing the root-to-shoot ratio.

Alternatively to the 'direct pathway' of obtaining P by root hairs and lateral roots, another plant strategy to improve P acquisition is by establishing symbiosis with certain soil microorganisms such as AM fungi, the so-called 'AM pathway' (Smith and Read 2008; Smith and Smith 2011). AM symbiosis is one of the most widespread plant associations with beneficial microorganisms. About 80% of land plants, including most agricultural and horticultural crop species, are able to establish this type of symbiosis with fungi from the phylum Glomeromycota (Barea et al. 2005; Smith and Read 2008). It is older than 450 million years and is considered a key step in the evolution of terrestrial plants (Smith and Read 2008). By this mutualistic beneficial association, the fungus obtains photoassimilates from the plant to

complete its lifecycle. In turn, it helps the plant in the acquisition of water and mineral nutrients, mainly P and nitrogen. AM fungi are obligate biotrophs that colonize the root cortex of the host plant, forming specialized and highly branched tree-like structures called arbuscules in the cells of the host, where the nutrient exchange between the two partners takes place (Genre et al. 2013; Gutjahr and Parniske 2013). The hyphae of the fungus grow into the soil far beyond the root rhizosphere and develop an extensive hyphal network that takes up P via fungal high-affinity transporters (Harrison 2005; Smith and Smith 2011), thus acting as 'helper roots' that can search for P beyond the P depletion zone. Accordingly, symbiosis establishment is promoted under P deficiency conditions (Table 1) (Fusconi 2014; Harrison 2005; Smith and Read 2008). A stimulatory effect of nitrogen deficiency has also been reported (Table 1), although its effect seems to be generally weaker than that observed for P (Correa et al. 2014; Nouri et al. 2014). The levels of other essential mineral nutrients such as iron, potassium and calcium do not appear to exert any effect on mycorrhizal colonisation (Fusconi 2014; Nouri et al. 2014).

Mycorrhizal plants can be colonized by several different species of AM fungi, suggesting that there is little host-specificity. However, there are differences in the symbiotic efficiency of one AM species on different plant species and different AM species display different capacity of colonisation on one plant species (Smith and Read 2008). In general, AM symbiosis positively affects plant development and plant fitness, especially under unfavourable conditions. However, neutral or even negative effects on plant growth, attributed to P deprivation and an excessive carbon use by the AM fungus, have also been described (Grace et al. 2009; Li et al. 2008; Smith and Smith 2012). The negative plant response to AM colonisation has been proposed to be associated with the reduced P absorption capacity by the 'direct pathway' induced by the symbiosis and to a lower P uptake capacity by the AM fungus through the 'AM pathway' (Smith and Smith 2012). Therefore, searching for the optimal 'dance partner' is crucial for a mutualistic beneficial association.

It is well known that phytohormone homeostasis is altered during AM symbiosis establishment and functioning (Bucher et al. 2014; Foo et al. 2013a; Gutjahr 2014; Pozo et al. 2015). Some phytohormones control the early steps of the interaction regulating root morphology and preparing the plant to accommodate the fungus, others are involved in later stages controlling the extension of colonisation and/or the lifespan of the arbuscules and some hormones can be involved at the different stages of the symbiosis. Despite their regulatory functions as plant hormones, SLs were initially identified as signalling molecules in the

rhizosphere, where they were shown to act as hyphal branching factors of AM fungi of the Gigasporaceae and germination stimulants in a number of AM fungi of the Glomeraceae (Akiyama et al. 2005; Besserer et al. 2006). It is proposed that plants themselves are able to actively influence the level of mycorrhizal colonisation by controlling the production of SLs depending on the P status (Table 1) (Foo et al. 2013b; López-Ráez et al. 2008a; Yoneyama et al. 2012; Yoneyama et al. 2007). However, the existence of additional molecular signals during the early stages of the interaction has been also suggested (Balzergue et al. 2011). SL perception by a so far uncharacterized receptor in the AM fungus induces profuse hyphal growth and branching - the so-called pre-symbiotic stage -, increasing the chance of encountering the roots of the host plant and facilitating symbiosis establishment (Akiyama et al. 2005; Besserer et al. 2006). Upon recognition of the fungal partner, the plant actively accommodates the fungus within the roots (Bonfante and Genre 2010; Gutjahr and Parniske 2013), but also controls its proliferation and arbuscule development (Reinhardt 2007; Walter 2013). While the importance of SLs in the initial stages of AM fungal colonisation is well accepted, it is not clear whether they also play a role in subsequent steps of the symbiosis.

In addition to SL signalling by the plant, and also before symbiosis establishment, AM fungi produce and release diffusible compounds - Myc factors and short chitin oligomers - into the rhizosphere that act as molecular cues indicating the presence of the fungus in the vicinity of the host root and inducing the plant responses required for a successful colonisation (Bucher et al. 2014; Genre et al. 2013; Maillet et al. 2011). Myc factors consist of a mixture of sulphated and non-sulphated simple lipochito-oligosaccharides that have structural similarities with the rhizobial Nod factors (Maillet et al. 2011). Maillet and co-workers showed that these compounds are not only symbiotic cues that stimulate AM establishment, but also act as plant growth regulators affecting the formation of lateral roots, the AM fungal entry sites. Interestingly, it has been demonstrated that the addition of GR24 elicits the production of short chitin oligomers in the AM fungus *Rhizophagus irregularis* (formerly known as *Glomus intraradices*) (Genre et al. 2013). Therefore, it seems that both partners mutually sense each other and that they respond accordingly. Indeed, using a split-root system with tomato plants, we have recently observed that SL production was higher in roots inoculated with *R. irregularis* compared with non-inoculated roots during the early stages of interaction/colonisation (López-Ráez et al. 2015). This observation suggests that the plant is really sensing the presence of the fungus and that it actively reacts to favour fungal development and symbiosis establishment by promoting SL production. SLs also promote lateral root formation (Ruyter-Spira et al 2011),

therefore, this initial fungal-mediated induction of SLs may serve to increase the number of colonisation sites.

The characterization and a better knowledge on the specificity of these pre-symbiotic signals should pave the way for the development of new environmentally-friendly agricultural strategies based on AM symbiosis.

Effect of other abiotic stresses on SL production and AM symbiosis

In nature, plants are generally exposed to combinations of unfavourable environmental conditions. Besides a better nutrient supply, AM symbiosis provides also increased tolerance against other abiotic stresses such as heavy metals, drought and salinity (Aroca et al. 2013; Evelin and Kapoor 2014; Li et al. 2014; Ruiz-Lozano et al. 2012; Singh et al. 2011). So far, there are, however, no indications that these stresses also have an (positive) effect on symbiosis establishment, in contrast to P shortage.

Water-related stresses

In recent years, harmful effects of water-related stresses such as drought and salinity are rising dangerously, having a major impact on plant growth and development, and being the most important factors limiting crop productivity (Albacete et al. 2014; Sunil Kumar and Garampalli 2013). Moreover, global change is contributing to spread these problems worldwide (Chaves and Oliveira 2004). Therefore, improving the yield under these stress conditions is a major goal nowadays. A concept associated to the adaptation to water related stresses is the water use efficiency (WUE), defined as the amount of dry matter or harvestable yield produced per unit of water. AM symbiosis has the capacity to alter root hydraulic properties, thus helping the plant in the uptake of water under unfavourable conditions. As a consequence, mycorrhizal plants show a higher WUE and root turgor, alleviating the negative effects of water shortage on plant physiology (Al-Karaki et al. 2004; Augé et al. 2015; Bárzana et al. 2014; Li et al. 2014; Wu and Xia 2006). This effect has been associated to an improved nutrient uptake in mycorrhizal plants, which promotes the photosynthetic capacity and growth (Li et al. 2014; Smith et al. 2010). However, the extent of the benefits depends on both the host plant and AM fungal species (Augé et al. 2015). On the other hand, the expression of genes encoding aquaporins is altered in mycorrhizal plants which may play a role in the improved water status in AM plants, although their regulation depends on the type and severity of the stress (Aroca et al. 2007; Bárzana et al. 2014; Uehlein et al. 2007).

Even though it is evident that under drought or salinity AM plants perform better than non-mycorrhizal ones, the effects of water-related stresses in AM symbiosis establishment is not clear and sometimes contradictory (Table 1). Interestingly, an increased SL production under salt stress in the presence of the AM fungus *R. irregularis* was shown in lettuce (Table 1) (Aroca et al. 2013), which might indicate the active promotion of symbiosis establishment. Similarly, the promotion of SL production in mycorrhizal plants has also been observed in lettuce and tomato under drought stress (López-Ráez, Aroca, Ruiz-Lozano et al., unpublished data). In both cases, the induction of SLs occurred in a dose-dependent manner, with the greatest increase under the strongest stress. A different behaviour was observed in the absence of mycorrhization under salinity or drought, where the stress reduced SL production also in a dose-dependent manner (Table 1) (Aroca et al. 2013; López-Ráez, Aroca, Ruiz-Lozano et al., unpublished data). A negative effect on SL production in the absence of mycorrhizal colonization has also been observed in *Lotus japonicus* plants subjected to osmotic stress (Table1) (Liu et al 2015). These results might suggest that plants sense the presence of the AM fungus and that they respond by producing SLs under unfavourable conditions to improve colonization. A relationship between drought and salinity with SLs has also been proposed in the non-mycorrhizal plant Arabidopsis (Ha et al. 2014). Here, a positive effect of SLs on the tolerance to these stresses was observed. Ha and co-workers showed that SL-deficient mutants were hypersensitive to drought and salt stress, and that this phenotype was rescued by exogenous GR24 application. The authors also showed that wild-type plants treated with GR24 were more tolerant to these stresses than untreated plants (Ha et al. 2014). The results from lettuce, tomato and Arabidopsis suggest a different behaviour between mycorrhizal and non-mycorrhizal plants in response to water-related stresses. However, more knowledge is required to decipher how SL regulation is involved in these stress responses and how this regulation is affected by and/or affects AM symbiosis.

As in previous cases, the alteration in the phytohormone homeostasis in mycorrhizal plants has been implicated in the enhanced tolerance against these stresses and here, ABA signalling is the most studied pathway (Calvo-Polanco et al. 2013; Ruiz-Lozano et al. 2012). ABA is considered as the 'stress hormone', as it accumulates rapidly in response to drought and salinity (Hong et al. 2013). Interestingly, a reduction in ABA content has been reported in mycorrhizal roots (Aroca et al. 2008; Aroca et al. 2013; Duan et al. 1996; Estrada-Luna and Davies Jr 2003; Fernández et al. 2014), suggesting that AM plants are less stressed than non-mycorrhizal ones. In contrast, when stressed, an increase in ABA content is generally observed

in mycorrhizal plants (Aroca et al. 2013; Calvo-Polanco et al. 2013), which has been associated with priming for increased stress tolerance. ABA is also necessary for a proper establishment and functioning of the AM symbiosis. It positively regulates arbuscule development and functionality (Herrera-Medina et al. 2007; Martín-Rodríguez et al. 2011). Thus, the increased ABA levels in stressed plants would serve to promote tolerance against stresses, but also to enhance and maintain the symbiosis. Interestingly, there also seems to be a relationship between ABA and SLs. It was shown that the tomato ABA-deficient mutants *notabilis*, *sitiens* and *flacca*, blocked at different steps of the ABA biosynthetic pathway, and wild-type plants treated with specific ABA inhibitors produced less SLs (López-Ráez et al. 2010b). Moreover, a correlation between ABA and SL levels was reported in mycorrhizal lettuce plants subjected to salt stress (Aroca et al. 2013). It seems, thus, that SLs play a dual role under stress conditions. On the one hand, they act as signalling molecules in the rhizosphere favouring AM symbiosis. On the other hand, they form part of the integrative plant hormonal response to unfavourable conditions, interacting with ABA and probably with other stress-related phytohormones to maintain the symbiosis at an optimal level.

Other stresses

Studies on the influence of other abiotic stresses on AM symbiosis are scarce and usually contradictory. A negative effect of low temperature was reported in wheat and sorghum, while no effect was observed in rice (Table 1) (Augé et al. 2004; Hetrick et al. 1984; Liu et al. 2013). Conversely, a positive effect of high temperature on the symbiosis has recently been reported in *Medicago truncatula* (Table 1) (Hu et al 2014). In relation to heavy metals, an inhibitory influence of cadmium on the AM fungus *Funneliformis mosseae* (formerly *Glomus mosseae*) was detected in wheat (Table 1), although mycorrhizal plants were more tolerant than non-mycorrhizal (Shahabivand et al. 2012). A negative effect on AM colonisation was also observed for copper in maize (Table 1) (Hagerberg et al. 2011). Aluminium affected different species of AM fungi in broomsedge (*Andropogon virginicus*), ranging from a negative to a positive effect, depending on the concentration (Kelly et al. 2005). As far as we know, no data about the influence of these abiotic stresses on SL biosynthesis have been reported so far. In any case, it seems that, unlike for the nutritional stress, the effects of other abiotic stresses on SLs and AM symbiosis differ between different species of host plants and AM fungi and probably depend on the severity of the stress. Further research is required to ascertain whether this is the case, but also to understand whether and how these stresses regulate SL production and AM symbiosis, and vice versa.

SLs in other plant rhizosphere interactions

Plant-microbe interactions

The rhizosphere is the narrow soil zone surrounding plant roots and constitutes a very dynamic environment. In addition to AM fungi, it harbours many different organisms and is highly influenced by plant root exudates (Badri et al. 2009; Bais et al. 2006; Barea et al. 2005). Recently, a role for SLs in another important beneficial plant-microorganism association in the rhizosphere - nodulation - was described (Fig. 1) (De Cuyper et al. 2015; Foo and Davies 2011; Soto et al. 2010). Nodulation is established between legumes and certain rhizobacteria collectively known as rhizobia, and dates back about 60 million years (Garg and Geetanjali 2007). This symbiosis is characterized by the development of nodules on the plant roots, where rhizobia fix atmospheric nitrogen, thus improving plant nutrition. Nodules provide the proper micro-environment for nitrogen fixation and nutrient exchange with the host plant in return for photoassimilates (Garg and Geetanjali 2007; Oldroyd and Downie 2008). Accordingly, an increase in SL production under nitrogen deficiency has been shown to occur in pea (Table 1) (Foo et al. 2013b), but also in some non-legume plant species such as rice, sorghum, wheat and lettuce (Table 1) (Jamil et al. 2011a; Yoneyama et al. 2012; Yoneyama et al. 2007). Just as for AM symbiosis, nodulation requires a high degree of coordination between the two partners based on a coordinated molecular communication (Murray 2011; Oldroyd and Downie 2008). However, here SLs do not seem to act as host detection signals (Soto et al. 2010). The chemical dialogue is initiated with the production and exudation of specific flavonoids by the host plant (Badri et al. 2009; Hassan and Mathesius 2012). These flavonoids act as attractants for rhizobial bacteria and inducers of Nod factor biosynthesis, which are structurally similar to the AM fungal Myc factors (see above) (Maillet et al. 2011). Although SLs do not seem to be involved in the pre-symbiotic stage, it has been shown that they are required for optimal nodule number formation (Foo and Davies 2011). Foo and Davies observed that the pea SL-deficient mutant *rms1* (mutated in CCD8) established about 40% less nodules than the corresponding wild-type, and that the phenotype was partially rescued by exogenous GR24 application. Moreover, they showed that GR24 increased the nodule number in wild-type plants (Foo and Davies 2011). More recently, in *Medicago truncatula* it was shown that the effect of GR24 on nodule number is dose-dependent (De Cuyper et al. 2015). De Cuyper and co-workers showed that low concentrations (0.1 μM) of GR24 had a positive effect, while higher concentrations negatively affected the number of nodules. Therefore, SLs play an important role, albeit different, in two

of the most important beneficial interactions in the rhizosphere, further confirming their biological and ecological relevance.

The implication of SLs in other plant-microbe interactions below-ground is not clear. Steinkellner and co-workers showed no response after GR24 application in other beneficial fungal species such as ectomycorrhizal fungi, *Trichoderma* and *Piriformospora indica* (Steinkellner et al. 2007). Regarding fungal pathogens, contradictory data have been reported. On the one hand, no direct effect was observed in fungal pathogens such as *Rhizoctonia solani*, *Fusarium oxysporum* f. sp. *licopersici*, *Verticillium dahliae* or *Botrytis cinerea* at low GR24 concentrations (Steinkellner et al. 2007; Torres-Vera et al. 2014). On the other hand, a negative effect on growth was detected for fungi such as *F. oxysporum* f. sp. *melonis*, *F. oxysporum* f. sp. *mango*, *Sclerotinia sclerotiorum* or *B. cinerea* at higher GR24 concentrations (Dor et al. 2011a). Dor and co-workers also observed increased hyphal branching activity in *F. oxysporum* f. sp. *melonis* and *S. sclerotiorum* (Dor et al. 2011a). Thus, it seems that the effect of SLs on microbes depends on the fungal species and SL concentration.

Root parasitic plants

Long before the discovery of their function as phytohormones and signalling cues for symbiotic plant-microorganism interactions in the rhizosphere, SLs were discovered to be germination stimulants of root parasitic plants of the Orobanchaceae, including the genera *Striga* (witchweeds), *Orobanche* and *Phelipanche* (broomrapes) (Fig. 1) (Bouwmeester et al. 2003; Cook et al. 1966). These obligate parasitic weeds are some of the most damaging agricultural pests, affecting important crops such as rice, maize, sorghum, legumes, tobacco, sunflower and tomato worldwide. They can cause up to 70% losses in crop yields (Gressel et al. 2004; Joel et al. 2007; Parker 2009). Broomrapes are generally found in more temperate regions such as southern Europe, the Mediterranean area, Central Asia and the Americas, and witchweeds appear in warmer areas, mainly in Africa (Parker 2009). Although these parasites affect different hosts in different parts of the world, their lifecycles are broadly similar, starting with seed germination in response to SLs (López-Ráez et al. 2009; Xie et al. 2010). Upon germination, they attach to the roots of the host plant through a specialized organ called haustorium, and acquire all the nutrients and water they need to complete their lifecycle (Bouwmeester et al. 2003; Estabrook and Yoder 1998). After emergence, they produce a large amount of seeds, increasing the seed bank in the soil, which is one of the major problems in the control of these parasites (López-Ráez et al. 2009; Xie et al. 2010). In addition, most of their life cycle occurs below-ground, making diagnosis difficult such that the parasites usually have

already inflicted irreversible damage. As a consequence, these parasitic weeds are difficult to control. Cultural measures such as hand weeding, improvement of soil fertility, crop rotation, sanitation, fumigation or solarisation are being used, but without the desirable success (Joel et al. 2007; Rispail et al. 2007; Scholes and Press 2008). Therefore, new strategies and/or a combination of different methods for a more effective control against these agricultural pests are needed.

Agronomical implications of SL signalling

AM symbiosis as biofertilizer and biocontrol agent

The 'Green Revolution' that took place after the Second World War, was accompanied by over-exploitation of the soil and an excessive use and abuse of agrochemicals such as fertilizers, pesticides and herbicides. Nowadays, due to the public concern about the side effects of these chemicals, there is increasing interest in finding alternatives for more environmentally friendly agriculture. AM symbiosis generally improves the growth of its host plant by facilitating water and mineral nutrient uptake, particularly under stress conditions, although negative effects have also been described, especially in cereals (Grace et al. 2009; Li et al. 2008). Moreover, AM fungi are widely distributed and can colonise most agricultural and horticultural crop species. Indeed, AM fungi are occasionally being used as biofertilisers for enhancing plant growth and biomass production, although much less than conventional fertilisers (Barea et al. 2005; Duhamel and Vandenkoornhuyse 2013; Gianinazzi et al. 2010). Considering the fact that AM symbiosis does also impact the plant's ability to overcome abiotic and biotic stresses, they may not only serve to improve plant nutrition, but also as a biocontrol strategy against different environmental stresses.

SLs are important for AM symbiosis establishment (Akiyama et al. 2005; Besserer et al. 2006; Foo et al. 2013b; Gomez-Roldan et al. 2008; Kohlen et al. 2012). Therefore, breeding for cultivars with high SL production potentially is a strategy to improve mycorrhizal colonisation under agronomical conditions. Alternatively, this could be achieved by the exogenous application of natural SLs or synthetic analogues. On the other hand, we have described above that stress conditions such as nutrient deficiency, drought or salinity influence SL biosynthesis. Thus, another way of promoting AM symbiosis might be by applying controlled stress conditions that do not negatively affect the plant too much. However, when applying these approaches we should keep in mind that SLs are also germination stimulants of root parasitic

plants and that they are involved in multiple physiological functions within the plant. In addition, each plant species is producing a different blend of SLs, which may also depend on the developmental stage and environmental conditions (Ćavar et al. 2014; Xie et al. 2010), although very little is known about their specificity. Therefore, a better understanding of their structure-activity relationship and biology is essential prior to its application. Some progress has already been made, and the effect of structural differences between SLs on AM fungal branching activity, parasitic weed seed germination and shoot branching have been demonstrated (Akiyama et al. 2010; Boyer et al. 2012; Boyer et al. 2014; Yoneyama et al. 2009). Interestingly, SL specificity in transport *in* and *ex planta* has also been reported (Kohlen et al. 2012). Kohlen and co-workers showed that certain SLs are mainly exuded into the rhizosphere, while others are preferentially loaded into the xylem and transported to the shoot. Elucidation of SLs potentially specific for host plant-AM fungus interaction will definitively contribute to a better implementation of AM symbiosis in agro-ecosystems.

Management strategies against root parasitic plants based on SLs

As mentioned above, root parasitic plants are difficult to control because most of their life cycle occurs below-ground. Since these parasites exert the greatest damage prior to their emergence, such strategies should mainly focus on the initial steps of infection, particularly seed germination triggered by SLs and attachment (Fernández-Aparicio et al. 2011; López-Ráez et al. 2009; Yoder and Scholes 2010). Breeding for cultivars with reduced SL production and/or exudation could be a suitable strategy to combat these pests. Indeed, it was shown that the low SL producing tomato mutants *Sl-ORT1* and *high pigment-2* (*hp-2*^{dg}) are more resistant to infection by different *Orobanche* and *Phelipanche* species than the corresponding wild-types (Dor et al. 2011b; López-Ráez et al. 2008b). Genetic variation for low SL production has also been described in other important crops such as sorghum, rice and faba bean (Dor et al. 2011b; Fernández-Aparicio et al. 2014; Jamil et al. 2011b; López-Ráez et al. 2008b; Satish et al. 2012). In sorghum, this genetic variation was used to breed for Striga resistant varieties for use in Africa (Ejeta 2007). In rice, cultivars with lower SL production also displayed reduced infection by *Striga hermonthica* (Jamil et al. 2011b). Similarly, root exudates from faba bean lines resistant against *Orobanche* and *Phelipanche* spp. showed low levels of SLs (Fernández-Aparicio et al. 2014). An alternative approach to obtain resistant plants by reducing SLs is through biotechnology, targeting biosynthesis genes. Indeed, *ccd7* and *ccd8* mutants from different plant species showed a reduced production of SLs (Drummond et al. 2009; Gomez-Roldan et al. 2008; Kohlen et al. 2012; Ledger et al. 2010; Umehara et al. 2008; Vogel et al.

2010). Genetic engineering using RNAi technology on the tomato *CCD7* and *CCD8* genes resulted in a significant reduction in SLs, which correlated with a lower germination of *P. ramosa* seeds (Kohlen et al. 2012; Vogel et al. 2010) and decreased *P. ramosa* infection of the transgenic tomato lines in pot experiments (Kohlen et al. 2012).

AM symbiosis to control root parasitic plants

The fact that SLs play a dual role in the rhizosphere as host detection cues for these parasites and for AM fungi also opens up another possibility to develop new control strategies. It was shown that AM symbiosis in cultivars of maize and sorghum led to a reduction in *S. hermonthica* infection in the field (Lendzemo et al. 2005). Lendzemo and co-workers proposed that this reduced infection was caused, at least in part, by a reduction in the production of SLs in mycorrhizal plants. Similarly, exudates from AM-colonized lettuce, pea, and tomato plants induced less germination of *Orobanche* and *Phelipanche* spp. seeds compared with non-colonized plants (Aroca et al. 2013; Fernández-Aparicio et al. 2010; López-Ráez et al. 2011a). In the case of tomato, it was shown that this reduced germination was caused by a decrease in the production of SLs and that this depends on a fully established symbiosis (López-Ráez et al. 2011a). This down-regulation of SL production likely represents a mechanism to prevent excessive colonisation that could be metabolically costly for the plant, a mechanism known as autoregulation (Staehelin et al. 2011). The results from maize, sorghum, pea, tomato, sunflower and lettuce suggest that the AM-associated decrease in SLs is conserved across the plant kingdom. Since AM fungi colonize roots of most agricultural and horticultural species, AM symbiosis could be used as an environmentally-friendly biocontrol strategy against these root parasites. Interestingly, these crops would also take advantage of all the other well-known benefits of the symbiosis.

All these examples indicate that the development of new strategies to improve crop production and reduce pest infestation by targeting SLs is feasible. However, since SLs are also phytohormones regulating different processes within the plant and affect other beneficial associations in the rhizosphere, the effect of altering SL production should be carefully evaluated before application in agro-ecosystems to avoid possible undesired side-effects.

Future perspectives in SL research and their use in agriculture

Experimental evidence illustrates the biological and ecological importance of SLs in the rhizosphere. Unravelling new roles and functions for the different SLs *in* and *ex planta* is

therefore exciting and promising. Their multifunctional nature opens up a wide range of possibilities for potential applications in agriculture. A number of studies points to differences in biological specificity of individual SLs, although we are still far from a full understanding of how this mechanistically works. Further research on the requirements for specific SLs in the different biological processes should expand our understanding about the biological processes occurring below-ground (Box 1). Moreover, an in-depth knowledge of the mechanisms that regulate SL production and release, and about how they are affected by different environmental conditions is required (Box 1) in order to allow us to exploit the full potential for these signalling molecules in agriculture.

Box1. Outstanding research questions

- Which enzymes catalyse the decoration of the SLs, and where are they expressed?
- How is the production of SLs affected by environmental factors?
- What are the structural requirements of SLs for their different biological functions?
- What are the receptors for SLs in AM fungi?
- What is the lifespan of SLs in the rhizosphere?
- What is the exact mechanism by which SLs regulate root architecture and how are other plant hormones involved?

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Chapter 3

The interaction of strigolactones with abscisic acid during the drought response in rice

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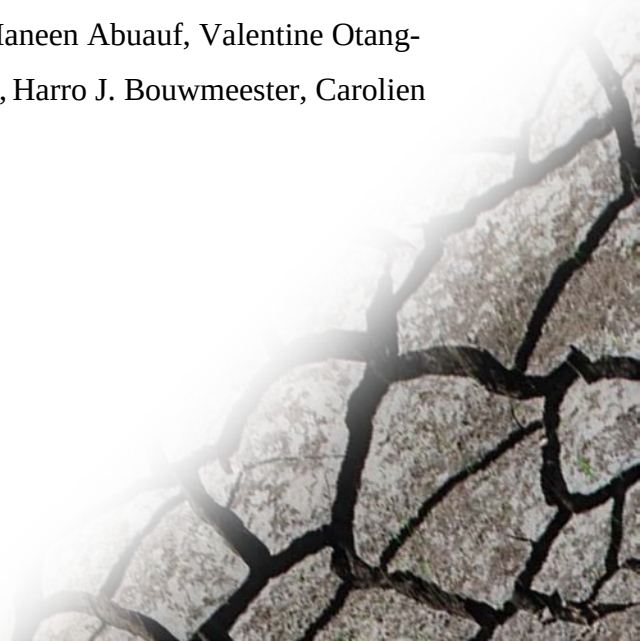

Summary

Both strigolactones (SLs) and abscisic acid (ABA) biosynthetically originate from carotenoids. Considering their common origin, interference of these two hormones at the biosynthetic and/or regulatory level may be anticipated. Here, we show in rice that mild drought simultaneously induces SL production in the root, and ABA production and the expression of SL biosynthetic genes in the shoot. Under control conditions, the ABA concentration was higher in shoots of the SL biosynthetic rice mutants *d10* and *d17* when compared with their wild type, while a similar trend was observed for the SL-perception mutant *d3*. These differences were enhanced under drought. However, during this condition it became apparent that the *d27* mutant failed to increase its ABA level to the same extent as was observed for the other SL mutants and their wild type. Accordingly, *d10*, *d17* and *d3* lines were more drought tolerant than wild-type plants, whereas *d27* displayed increased sensitivity. Over-expression of *OsD27* in rice resulted in increased levels of ABA when compared with wild-type plants. We conclude that the SL and ABA biosynthetic pathways interfere with each other during the drought response in rice, and suggest that this is at least partially mediated by the SL biosynthetic enzyme OsD27.

Introduction

Due to their sessile nature, plants must adjust their growth and development to continuously changing environmental conditions (Wolters and Jürgens 2009). The phytohormones such as abscisic acid (ABA), auxin, cytokinin (CK), gibberellin (GA), ethylene, brassinosteroids (BRs), jasmonic acid (JA), salicylic acid (SA) and strigolactones (SLs) play a central role in regulating these adaptive responses (Santner and Estelle 2009). However, these phytohormones do not act alone but are interconnected and modulate each other at the level of biosynthesis, degradation and signalling by cross-talk mechanisms (Zhang et al. 2013).

ABA is a well-studied phytohormone that not only regulates several developmental processes such as seed maturation and dormancy, but is also intricately involved in the adaptation of plants to abiotic stresses (i.e. drought, salinity, etc.) (Xiong and Zhu 2003, Nambara and Marion-Poll 2005, Chinnusamy et al. 2008). Under drought, ABA levels increase, resulting in stomatal closure, which limits water loss but also results in the accumulation of reactive oxygen species (ROS) (Carvalho et al. 2008). In addition, ABA stimulates the formation of dehydrins and late embryogenesis abundant (LEA) proteins that are involved in osmotic adjustment and other protection mechanisms (Shinozaki and Yamaguchi-Shinozaki

2007). In plants, ABA is derived from C_{40}-carotenoid precursors synthesised in the plastids (Fraser and Bramley 2004, Moise et al. 2014). The first committed step in ABA biosynthesis is the oxidative cleavage of the epoxy-carotenoids 9-*cis*-violaxanthin or 9'-*cis*-neoxanthin, leading to xanthoxin (Schwartz et al. 1997). Both xanthophylls are formed from their corresponding all-*trans*-isomers by (an) unidentified *cis*/*trans*-isomerase(s). All-*trans*-neoxanthin is derived from all-*trans*-violaxanthin, which is synthesized from all-*trans*-zeaxanthin via two epoxidation reactions catalysed by zeaxanthin epoxidase (ZEP). Zeaxanthin itself is the product of the C3-hydroxylation of the ionone rings in all-*trans*-β-carotene, a reaction catalysed by β-carotene hydroxylase (BCH) (for review, see Moise et al. 2014). The oxidative cleavage of 9-*cis*-violaxanthin and/or 9'-*cis*-neoxanthin to xanthoxin is catalysed by 9-*cis*-epoxycarotenoid dioxygenases (NCEDs), which is considered the rate-limiting step in ABA biosynthesis (Tan et al. 1997, Burbidge et al. 1999). The cleavage product xanthoxin is converted, in the cytosol, by a short-chain dehydrogenase/reductase (SDR) to abscisic aldehyde that is oxidised to form ABA by abscisic aldehyde oxidase (AAO3) (Nambara and Marion-Poll 2005) (Supplementary Fig. S5).

SLs were initially identified in root exudates as seed germination stimulants of root parasitic plants of the genus *Striga* (*Striga, Phelipanche* and *Orobanche* spp.) (Cook et al. 1966, Bouwmeester et al. 2003, Xie et al. 2010, Yoneyama et al. 2010) and later identified as hyphal branching stimulants of arbuscular mycorrhizal (AM) fungi (Akiyama et al. 2005, Bouwmeester et al. 2007). By now, SLs are recognised as hormones involved in the inhibition of shoot branching (Gomez-Roldan et al. 2008, Umehara et al. 2008) and a range of other biological processes, including root development, hypocotyl growth, responses to environmental conditions, photo-morphogenesis and secondary growth in vascular plants, as well as protonema branching and colony extension in the moss *Physcomitrella patens* (reviewed in: Ruyter-Spira et al. 2013, Zhang et al. 2013, Kapulnik and Koltai 2014, Waldie et al. 2014, Al-Babili and Bouwmeester 2015). When growing under low phosphate levels, plants display increased biosynthesis and exudation of SLs, which stimulate the hyphal branching of symbiotic arbuscular mycorrhizal (AM) fungi that acquire phosphate from the soil and provide it to the host in return for photoassimilates (Bouwmeester et al. 2007, Yoneyama et al. 2007, López-Ráez et al. 2008).

Based on studies with mutants, so far, eight genes have been identified in several plant species, with roles in SL biosynthesis and signalling. Among them, four have been shown to be required for SL biosynthesis. In rice and *Arabidopsis*, *DWARF27* (*D27*) (Lin et al. 2009, Waters

et al. 2012a) encodes a β-carotene isomerase converting all-*trans*-β-carotene into 9-*cis*-β-carotene (Alder et al. 2012). *MORE AXILLARY GROWTH 3* (*MAX3*)/*DWARF17* (*D17*) (Booker et al. 2004, Zou et al. 2006) and *MORE AXILLARY GROWTH 4* (*MAX4*)/*DWARF10* (*D10*) (Sorefan et al. 2003, Arite et al. 2007), encode carotenoid cleavage dioxygenases (CCDs), CCD7 and CCD8, respectively. *MORE AXILLARY GROWTH 1* (*MAX1*) encodes a cytochrome P450 monooxygenase (Booker et al. 2005, Challis et al. 2013). *In vitro* studies with D27, CCD7 and CCD8 showed that these enzymes are sufficient to convert all-*trans*-β-carotene into carlactone (CL), an intermediate with obvious structural similarities to canonical SLs (Alder et al. 2012, Seto et al. 2014). The formation of CL starts with the D27-catalysed isomerisation of all-*trans*-β-carotene (Bruno and Al-Babili 2016), followed by the CCD7-catalysed stereo-specific cleavage (Bruno et al. 2014) that leads to the intermediate 9-*cis*-β-apo-10'-carotenal that is thereon converted by CCD8 into CL (Alder et al. 2012). Recently, we demonstrated that a rice MAX1 homolog (carlactone oxidase) catalyses the conversion of CL into the SL-parent molecule, 4-deoxyorobanchol (*ent*-2′-*epi*-5-deoxystrigol), by forming the B-C lactone ring, and that a second rice MAX1-homolog (orobanchol synthase) forms orobanchol by introducing a hydroxyl group into 4-deoxyorobanchol (Zhang et al. 2014, Al-Babili and Bouwmeester 2015). *Arabidopsis* MAX1 has been shown to oxidise CL to a different SL-like molecule, carlactonic acid, which is methylated into methyl-carlactonoate (MeCLA) - that can bind to the receptor D14 (see below) - by a yet unidentified enzyme (Abe et al. 2014). More recently, Brewer et al. (2016) revealed that *LATERAL BRANCHING OXIDOREDUCTASE (LBO) converts MeCLA to* an unidentified SL-like compound in *Arabidopsis*, which encodes an oxidoreductase-like enzyme of the 2-oxoglutarate and Fe(II)-dependent dioxygenase (2OGD) superfamily. Moreover, *Arabidopsis lbo* mutant shows a reduced response to CL and MeCLA but not to synthetic SL analogue, GR24 (Brewer et al. 2016). SL mutants have a high-tillering/branching phenotype, which in the case of biosynthetic mutants can be rescued by exogenous application of GR24 (Gomez-Roldan et al. 2008, Umehara et al. 2008, Wang and Li 2011), but not in the SL perception and downstream signalling mutants (Umehara et al. 2008). Up to now, there are three genes known to be involved in the latter process: *MORE AXILLARY GROWTH 2* (*MAX2*)/*DWARF3* (*D3*) encodes an F-box protein (Stirnberg et al. 2002, 2007), *DWARF14* (*D14*), an α/β-fold hydrolase (Arite et al. 2009, Hamiaux et al. 2012, Waters et al. 2012b, de Saint Germain et al. 2013, Kagiyama et al. 2013, Nakamura et al. 2013, Zhao et al. 2013, Chevalier et al. 2014, Toh et al. 2015, Yao et al. 2016, de Saint Germain et al. 2016), and *SUPPRESSOR OF MORE AXILLARY GROWTH2-LIKE 6,7,8* (*SMXL6,7,8*)/*DWARF53* (*D53*) that encodes a target for the SCF^{D3} ubiquitination complex and acts as a repressor of SL

signaling (Jiang et al. 2013, Zhou et al. 2013, Wang et al. 2015). D3 interacts with D14 in a SL-dependent manner and this perception of SL by D14 and the SCF^{D3} complex leads to ubiquitination of SMXL6,7,8/D53 for 26S proteasome-mediated degradation, which enables further growth responses (Morffy et al. 2016).

Considering their common biosynthetic origin (Matusova et al. 2005), a relationship between ABA and SL production may be anticipated. Indeed, ABA-deficient tomato mutants impaired in different steps of ABA biosynthesis exhibit reduced SL levels (López-Ráez et al. 2008, 2010), suggesting that ABA affects SL accumulation. *Vice versa*, Torres-Vera et al. (2014) reported that SL-deficient tomato mutant *slccd8* had reduced levels of ABA. Similarly, a SL deficient *CCD7*RNAi lotus line showed reduced ABA levels when exposed to a combination of phosphate deficiency and osmotic stress, compared with the corresponding wild-type (Liu et al. 2015).

Because in the above mentioned studies SL deficiency was shown to reduce ABA levels, it can be expected that SL deficient plants are compromised in their response to drought. Interestingly, two independent studies investigated drought tolerance in *Arabidopsis* SL mutants (Bu et al. 2014, Van Ha et al. 2014). Van Ha et al. (2014) reported that SLs act as a positive regulator in the plant's response to drought and salt stress. The exogenous application of GR24 rescued the drought-sensitive phenotype of SL-deficient mutants (*max3* and *max4*) but not of the insensitive mutant (*max2*). In addition to this, the *max* mutants showed reduced sensitivity to exogenous ABA compared with wild-type plants. In the study by Bu et al. (2014) the drought sensitivity phenotype was confined to *max2*. However, ABA levels in wild-type and *max2* were similar under control and drought conditions.

To get a better understanding of the relationship between SLs and ABA during drought in rice, in the present study, we first studied whether SLs are affected by drought. Hereto, we quantified SL content and analysed the expression level of SL biosynthesis genes under mild drought conditions. Furthermore, we investigated the impact of SL biosynthesis and signalling on ABA content in rice SL biosynthesis and signalling mutants and *D27* over-expression lines. Our results show that SL biosynthesis/perception interferes with ABA formation as observed in *d10*, *d17* and *d3* mutants, and suggest that the SL biosynthetic enzyme D27 is unique in its capability to regulate the levels of both plant hormones.

Results

SL production is induced under drought

To study the effect of drought on SL production, five-leaf stage rice plants were grown for nine days under a continuous, mild water deficit. This resulted in a reduction in plant biomass, particularly of the shoot (Supplementary Fig. S1a), showing that this level of water deficit is indeed limiting plant growth. As expected, ABA increased in the shoot of stressed plants, but not in the roots (Supplementary Fig. S1b). SL levels strongly increased in the roots when plants were exposed to drought (Fig. 1). Roots of plants grown under drought contained a higher level of 4-deoxyorobanchol and the sum of the three putative methoxy-5-deoxystrigol isomers 1 to 3, identified before in rice (Cardoso et al. 2014; Jamil et al. 2011) when compared with plants grown under control conditions (Fig. 1). These results were supported by the increased expression of some of the SL biosynthetic genes (Fig. 2). In the shoot under drought, expression of *D27*, *Os01g0700900*, *Os01g0701400*, *Os02g0221900* and *Os06g0565100* was upregulated. In the root, expression of *D10* and *Os01g0700900* was upregulated. Of the genes tested, only *D27* was expressed higher in the shoot than in the root.

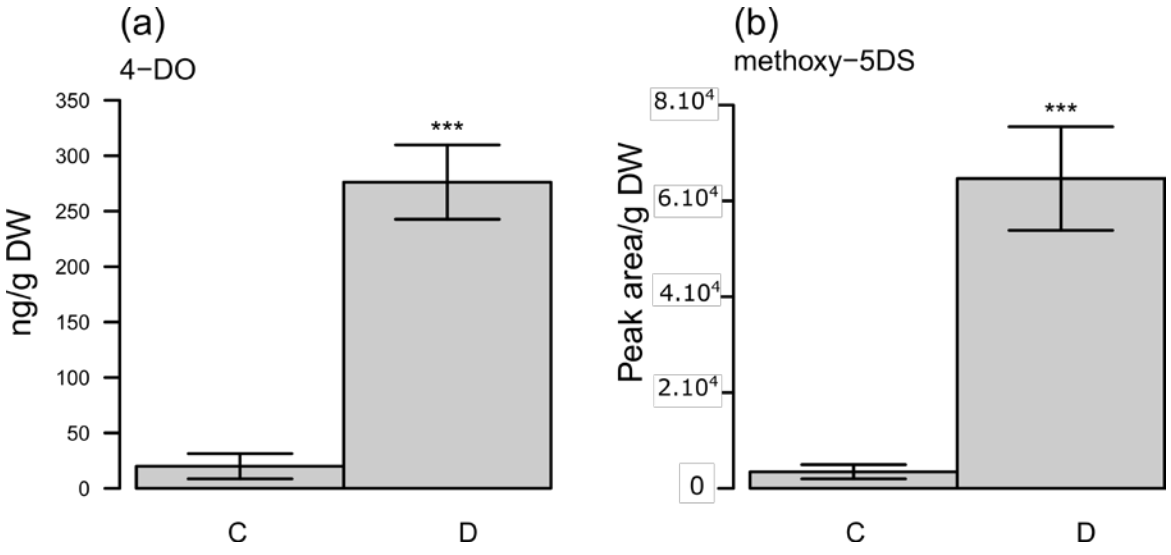

Figure 1. Effect of mild drought on SLs content in rice root extracts. Levels of 4-DO: 4-deoxyorobanchol (a) and methoxy-5DS (as determined by the sum of three methoxy-5-deoxystrigol isomers) (b) was measured in root extracts of rice plants. Mild drought (35-45 % FC) was applied during 9 d. Bars represent the average of five biological replicates (where each replicate consists of a pool of four plants) ± standard error. Significant differences between plants grown in control (C) and drought (D) conditions, as determined by Student's *t*-test, are indicated by asterisks (***$P \leq 0.001$).

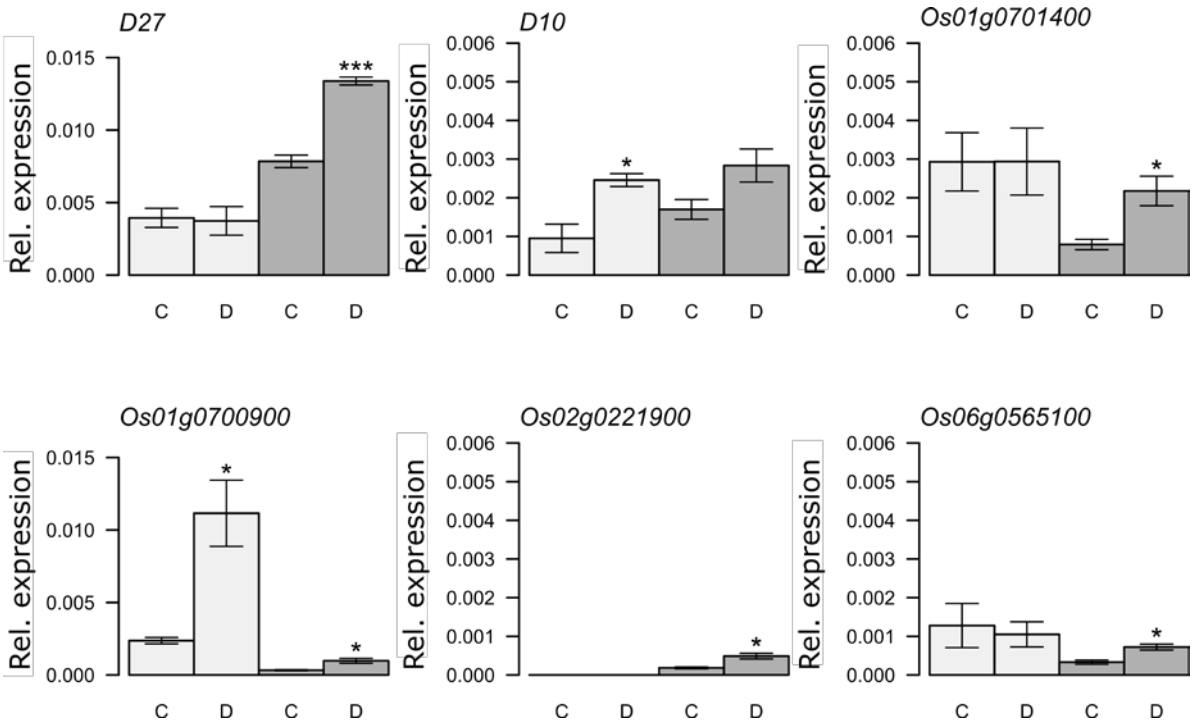

Figure 2. The effect of mild drought on the expression level of SL biosynthetic genes in rice. The expression level of rice strigolactone biosynthetic pathway genes was measured in root (light grey) and shoot tissue (dark grey). Mild drought (35-45 % FC) was applied during 9 d. The *D17* gene was not expressed at detectable levels. Significant differences between control (C) and drought (D) treatments in the different tissues, as determined by Student's *t*-test, are indicated by asterisks (***$P \leq 0.001$, *$P \leq 0.05$). Bars represent the mean of five biological replicates (each replicate consisting of a pool of four plants) ± standard error.

Effect of drought on the survival rate of rice SL mutants

The induction of SL biosynthesis under mild drought could imply that SLs are involved in the adaptation to drought. To explore their contribution to drought tolerance, we analysed how drought affects rice SL mutants. For this purpose, we performed a drought survival assay. The SL deficient *d10* and *d17* and SL insensitive *d3* showed significantly higher survival rates (approximately 85%, 75% and 45%, respectively), compared with wild-type (20%). In contrast, none of the *d27* plants survived (Fig. 3a, b).

As water loss by transpiration is one of the most important parameters contributing to drought sensitivity, we measured water loss rates of detached wild-type and SL mutant leaves. The detached leaves of *d10*, *d17* and *d3* lost water more slowly than wild-type leaves, but *d27* leaves evaporated water significantly faster (Fig. 3c).

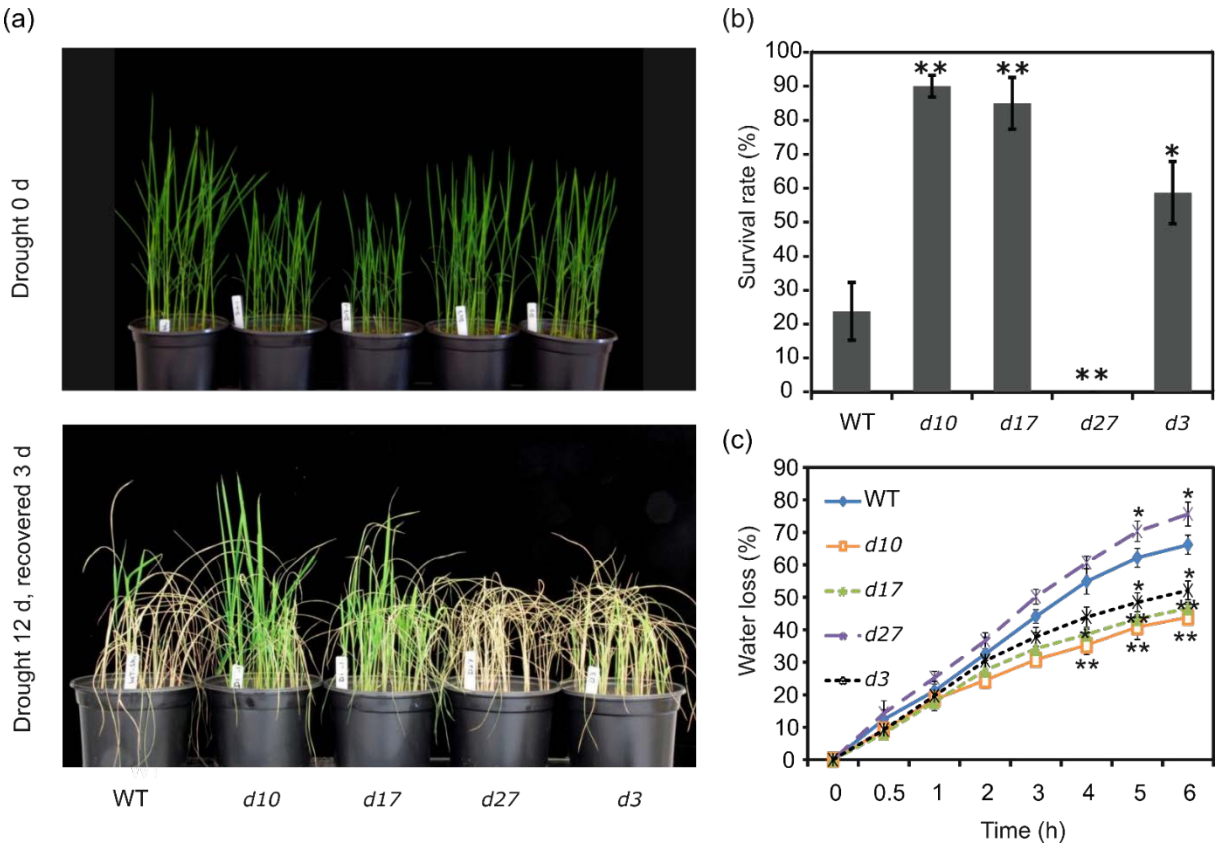

Figure 3. Plant survival – and detached leaf water loss rates of SL-deficient and -insensitive rice mutants and their wild type. (a) Phenotype of five-leaf stage plants before stress and after 12 d of drought followed by re-watering and 3 d of recovery (b) Survival rate after recovery (five independent biological replicates each pot containing 16 plants). (c) Water loss rate of detached flag-leaves (n=9). Bars represent mean ± standard error. Significant differences between mutants and wild-type plants as determined by Student's t-test are indicated by asterisks (*$P \leq 0.05$, **$P \leq 0.01$).

To rule out the possibility that the difference in drought tolerance is due to differences in plant architecture and/or biomass between the mutants, which may cause differences in pot water availability, *d27* and *d10* were analysed in a pooled experiment, in which these two genotypes were grown in the same container. Also under these conditions, the *d27* mutant is more drought sensitive than *d10* (Supplementary Fig. S2).

ABA content in rice SL mutants

To study if the above described differences in drought survival rate between the rice genotypes are linked with ABA levels in the leaf, we quantified ABA in rice SL mutants and wild-type under control conditions and during several time points after water withholding to create different levels of drought (ranging from mild until severe).

Under control conditions, ABA content was significantly higher in *d10* and *d17* when compared with wild-type plants, but did not differ between *d27* and wild-type (Fig. 4a). A tendency for increased ABA levels was observed for *d3*. ABA content was also measured in leaf material that was harvested 2, 6 and 10 days after water withholding. After two days, we observed a higher increase in ABA levels of *d10*, *d17* and *d3* than in wild-type. After six days of water withholding, ABA content was not significantly different between the lines but did show a similar trend when compared with 2 days of water withholding. After 10 days of drought, ABA content was again higher in *d10*, *d17* and *d3*, however, in *d27* ABA levels were significantly lower, when compared with wild-type (Fig. 4b). These results suggest that SL deficiency in rice stimulates ABA production in the shoot, and that the D27 enzyme is needed for this, especially under drought conditions.

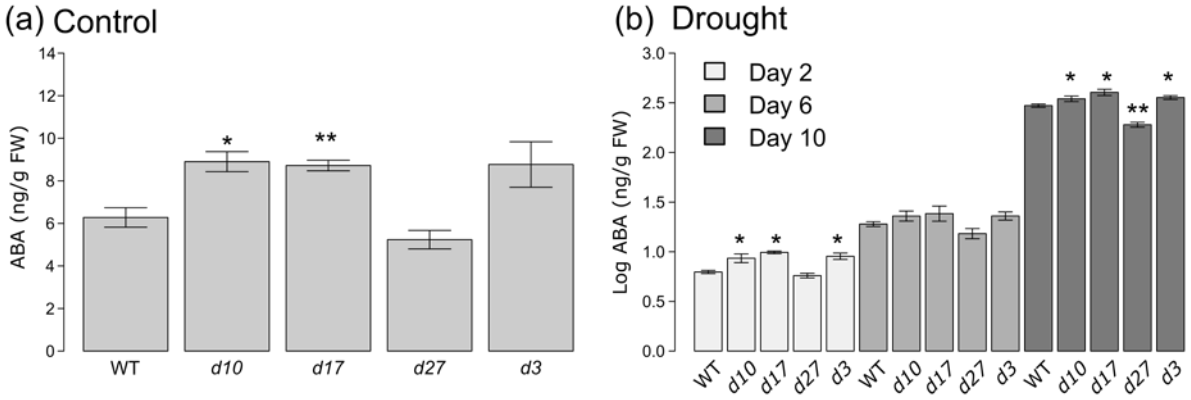

Figure 4. ABA content in shoots of SL deficient and - insensitive rice mutants and their wild type. ABA content was measured in Shiokari wild type (WT), *d10*, *d17*, *d27* and *d3* mutants by UPLC-MS/MS. (a) Shoot ABA in five-leaf stage rice plants grown under non-stressed conditions (normal). (b) From the fifth leaf stage onwards, plants were not watered anymore and shoot ABA was measured after 2, 6 and 10 d. Bars represent mean ± standard error (n=3). Significant differences between mutants and wild type plants, as determined by a pair wise comparison Student's t-test, are indicated by asterisks (*$P \leq 0.05$, **$P \leq 0.01$).

Constitutive over-expression of OsD27 in rice results in increased levels of ABA

Because the lower ABA level in *d27* is in contrast with the higher ABA level observed in the other SL biosynthetic/signalling mutants, we addressed the possible function of *OsD27* in ABA formation. For this purpose we analysed shoot ABA levels in two independent *OsD27* over-expression lines (*D27-OE1* and *D27-OE2*), both under control and drought conditions. The lines did not show morphological changes when compared with their wild-type (Supplementary

Fig. S3a). *D27* over-expression in these lines was confirmed by qRT-PCR and was between 1000- and 2300-fold higher than the corresponding wildtype (Supplementary Fig. S3b).

Under control conditions, shoot ABA content was significantly increased in both *OsD27* over-expression lines *D27-OE1* and *D27-OE2* (approximately 96% and 32%, respectively) compared with wild-type plants (Fig. 5a) while ABA content in *d27* did not differ from that of wild-type. After 10 days of water withholding, only one of the *OsD27* over-expression lines had a higher ABA level than the wild-type, whereas the ABA level in *d27* was significantly lower (Fig. 5b). We also analysed *OsD27* expression in *d10*, *d17* and *d3* mutants, which contain elevated ABA levels under control conditions, and observed a significantly higher expression level of this gene in these SL mutants (Fig. 5c). These results suggest that under control conditions SL deficiency induces a positive feedback on *OsD27* expression in the SL mutants, which may contribute to their increased ABA levels. This would also allow a more rapid increase in the expression of *OsD27* in the SL mutants after drought initiation, which may, through its effect on ABA, contribute to their increased drought tolerance.

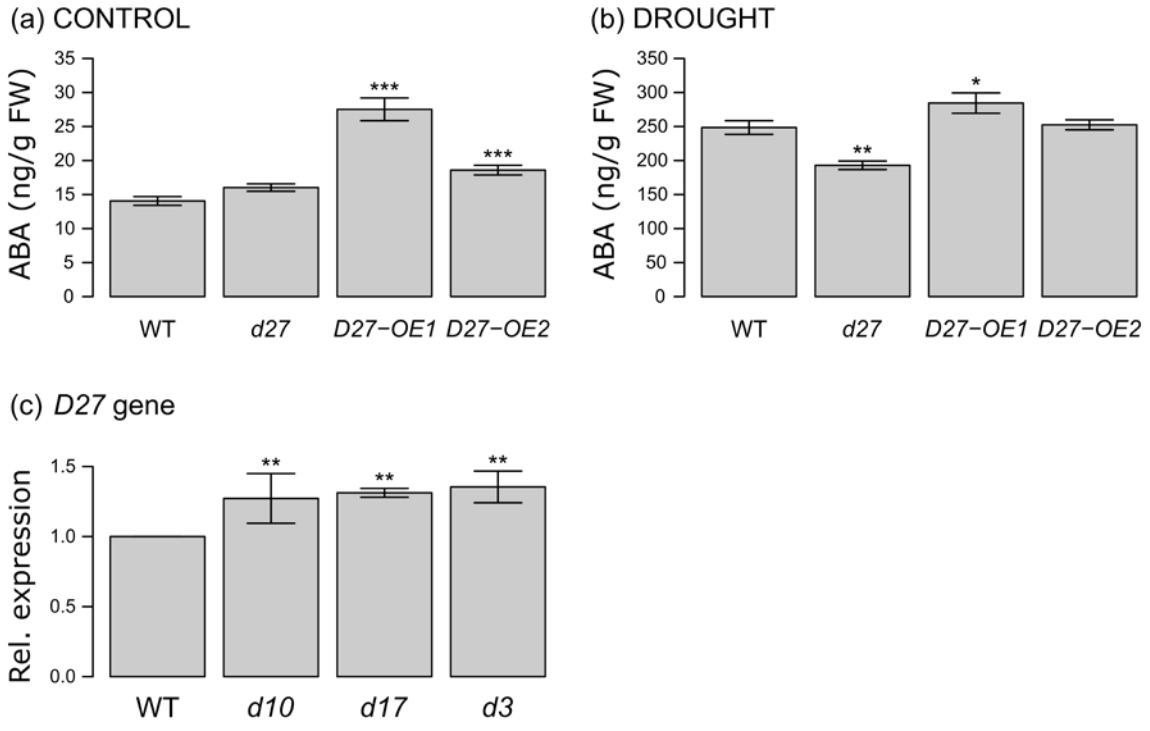

Figure 5. ABA - and *D27* expression levels in shoots of *d27* mutant and D27 over-expressing plants, and their wild type. (a-b) ABA content was measured in shoots of 28-day-old wild-type (WT), *d27* and *D27* over-expression lines (*D27-OE1* and *D27-OE2*) grown under (a) normal conditions and (b) after 10 d of drought. (c) *OsD27* expression in shoot material from wild-type (WT) and *d10*, *d17* and *d3* mutants grown under control conditions. Bars represent mean ± standard errors from six (a, b) and three

(c) biological replicates. Significant differences between mutants, over-expression lines and wild type, as determined by a pair wise comparison Student's *t*-test, are indicated by asterisks (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

Carotenoid content in the d27 mutant and OsD27 over-expression lines

In a further attempt to elucidate how D27 affects ABA biosynthesis, we examined the carotenoid pattern and content in two weeks old wild-type plants, the *d27* mutant and the two *OsD27* over-expression lines, grown under normal conditions. Although all investigated carotenoids (all-*trans*- and 9-*cis*-β-carotene, neoxanthin/violaxanthin and lutein) showed the tendency to be decreased in the *d27* mutant compared to its wild-type, this decrease was only significant for neo-/violaxanthin, lutein and *trans*-beta carotene. Interestingly, the carotenoid levels were increased (two-fold; Fig. 6) in the *D27-OE2* over-expressing line that showed only a minor increase in ABA content (Fig. 5a).

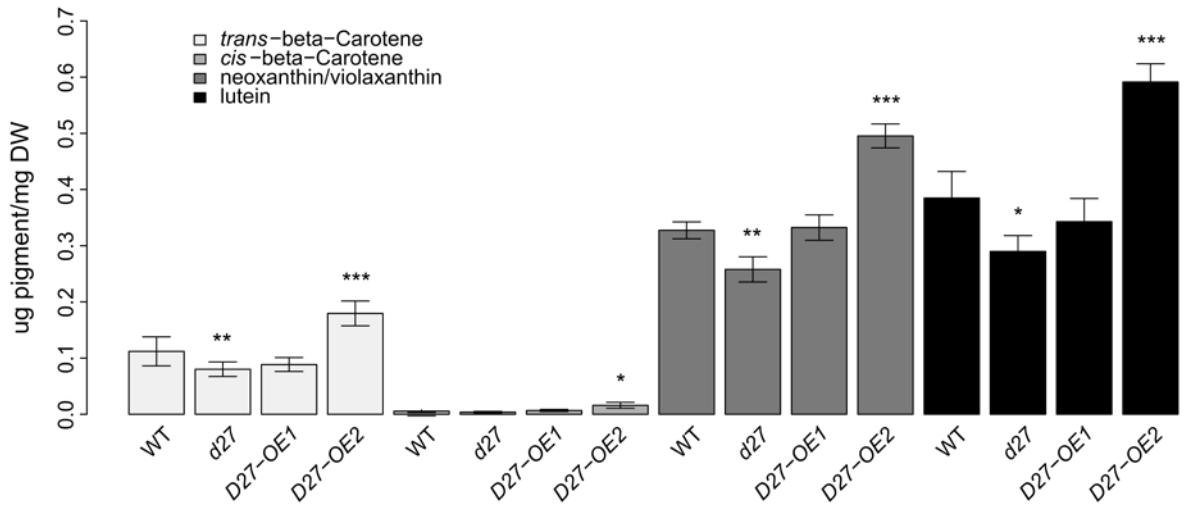

Figure 6. Carotenoid content in *d27* mutant and *D27* over-expressing plants (T2 generation seeds), and their wild-type. Carotenoids content in two-week old rice seedlings were quantified by HPLC. DW: dry weight. Asterisks represent a significant difference with the wild-type after a pairwise Student's *t*-test (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$). Values are means ±SE (n=8).

Discussion

Rice is known to be a large water consumer. Water scarcity is the major limitation for high yield in rain-fed rice and even under mild drought conditions rice yield is severely compromised (Kumar et al. 2008). Exploring ways to produce more rice with less water is essential for food security. This starts with understanding how rice adapts to drought. In the present study the involvement of SLs in the drought response was assessed in *japonica* rice cv. Shiokari, together with its interaction with the abiotic stress hormone ABA.

The results showed that exposure to drought increases expression of some of the SL biosynthetic genes in roots and that this results in increased SL production. Our observations are in contrast with the results in tomato, where drought resulted in decreased SL levels when compared with control conditions (Ruiz-Lozano et al. 2015, Visentin et al. 2016). Also in Lotus, it was shown that water deficit caused by osmotic stress, resulting from a treatment with polyethylene glycol (PEG), inhibited SL production (Liu et al. 2015). The discrepancy between these results and the results obtained during the present study with rice may be inherent to the different levels of drought that the plants were exposed to and the duration of the stress. Different types of drought provoke a different repertoire of plant responses inherent to the different strategies that plants use for their survival during these conditions (Pinheiro and Chaves 2011).

During longer periods of mild drought, plants have the possibility to adapt their architecture. For rice it has been reported that upon drought, plants reduce their leaf production and leaf area, and suppress tillering (Bouman and Tuong 2001). The latter response is a typical response attributed to the SLs and is in line with our observation of increased root SL levels upon exposure to mild drought. Besides this, SLs may also be involved in root architectural changes that occur during drought. At the same time, drought also induced the expression of SL biosynthetic genes, particularly *D27*, in the shoot (Fig. 2), suggesting that SL production is also increased in above ground tissues. However, it is unclear which role shoot based SLs play under these conditions.

It is striking though, that under control conditions, ABA levels in the shoot of SL mutants *d10 and d17* are higher than in wild-type plants. Drought further amplified this difference. Under this condition, all SL mutants under investigation, with the exception of *d27*, showed higher ABA levels than their wild-type control (Fig. 4). This observation once more suggests a

certain level of crosstalk between SLs and ABA, and may point at a regulatory role of SLs in balancing ABA levels. Interestingly, ABA levels in *d27* did not follow the same pattern as in the other SL mutants, and were even lower than in the wild-type.

The effect that SL deficiency/insensitivity had on ABA levels in the SL mutants was reflected in their drought survival rates in dry down assays, where the higher ABA producing lines *d10*, *d17* and *d3* were more drought tolerant and the low ABA producing line *d27* was more drought susceptible when compared with wild-type plants (Fig. 3). These differences correlated with their water loss rates as observed in detached leaf assays. Also other studies showed that water loss is regulated by stomatal aperture through ABA levels (Franks and Farquhar 2001, Hwang et al. 2010). In contrast to our observations, drought tolerance in the SL biosynthetic and signalling mutants *max4*, *max3* and *max2* in *Arabidopsis*, was reported to be decreased (Bu et al. 2014, Van Ha et al. 2014). Interestingly, this increased drought sensitivity in these *Arabidopsis* mutants was explained by a reduced sensitivity to ABA compared with wild-type plants. In the study by Bu et al. (2014), where the drought sensitivity phenotype was confined to *max2,* ABA levels in wild-type and *max2* were similar under control and drought conditions. Also in tomato and lotus, SL deficiency had a different effect on ABA levels than in rice. ABA levels in the SL deficient tomato mutant *slccd8* were reduced (Torres-Vera et al. 2014), and Liu and colleagues showed that in the *CCD7*RNAi lotus line a combination of phosphate starvation and PEG treatment also resulted in lower ABA levels than in the wild-type. Currently, we do not understand the underlying mechanism responsible for the observed differences in ABA levels in SL mutants in rice on the one hand and *Arabidopsis*, tomato and lotus on the other. A genome wide expression profiling approach using these plant species may reveal the explanation for this difference.

The reduced ABA level observed in the rice *d27* mutant, when compared with the other *d*-mutants, suggests that D27 may be involved in the regulation of ABA biosynthesis. ABA levels in the shoot of our two independent constitutive *OsD27* over-expression lines were indeed higher than in the wild-type. If D27 indeed regulates ABA levels, the increased levels of *D27* expression in *d10*, *d17* and *d3*, possibly resulting from SL feedback up-regulation (Arite et al. 2007), could explain the higher ABA levels. The even more pronounced increase in the content of ABA in these mutants under drought is likely caused by the simultaneous high expression of *D27* together with the other well described ABA biosynthesis genes that are specifically induced during this condition (Nambara and Marion-Poll 2005). However, we cannot exclude that SL deficiency also influences other genes in the carotenoid/ABA pathway.

Still, in *Arabidopsis*, *D27* expression is also elevated in *max4* and *max2* mutants, while ABA levels in *max2* were similar to those observed in wild type (Waters et al. 2012a, Bu et al. 2014). Therefore, it would be of interest to also study *D27* over-expression in other plants, to explore whether the effect that it has on ABA levels in rice is occurring throughout the plant kingdom or that it is specific for this plant species.

The underlying mechanism by which D27 controls ABA levels in rice is currently not known. However *in vitro* experiments demonstrated that D27 is not directly involved in the formation of the ABA precursors 9-*cis* violaxanthin or 9'-*cis*-neoxanthin (Bruno and Al-Babili 2016). Therefore a more complex mode of action must be assumed for D27 that ultimately influences the ABA levels in response to drought. Interestingly, phylogenetic analysis of SL biosynthesis and signaling genes shows that *D27* is highly conserved across the entire green lineage, whereas *CCD7*, *CCD8* and SL signaling genes are far less conserved, or not even detected in the Chlorophytes (Delaux et al. 2012; Ruyter-Spira and Bouwmeester 2012). Considering that ABA is already produced in the Chlorophytes (Hartung 2010), and SLs seem to be a later addition to the repertoire of plant hormones, it can be hypothesized that *D27* has a more ancient role, perhaps in regulating ABA biosynthesis.

Although ABA was increased in both *D27* over-expression lines, ABA levels in line *D27-OE1* were higher than in *D27-OE2*. This correlated with a higher expression level of *NCED3* in *D27-OE1* (Supplementary Fig. S4). However, in its turn, line *D27-OE2* contained higher carotenoid levels (viola-/neoxanthin) than *D27-OE1* (Fig. 6). The latter was also observed when ABA biosynthesis gene β-carotene hydroxylase (*DSM2* or *BCH*) was over-expressed in rice (Du et al. 2010), showing that overexpression of ABA biosynthesis genes not always lead to increased levels of ABA but may also lead to accumulation of pathway intermediates. Lutein was also found to be increased in line *D27-OE2* when compared with wild-type. Interestingly, the concentration of lutein and viola-/neoxanthin was found to be decreased in leaves of *d27*. Considering that lutein is the end product of the β,ε branch of carotenoids and ABA the end product of the β,β branch, it is striking that besides β-carotene, also α-carotene has recently been identified as substrate for D27 (Bruno and Al-Babili 2016). These results may imply that D27 is not directly involved in ABA biosynthesis, but that different expression levels of this gene may indirectly affect ABA levels and ABA intermediates by controlling pathway fluxes instead.

Our findings show that SLs play an active role in drought physiology. Moreover, our results suggest a connection between the SL and ABA pathways and a crucial role of *D27* in determining ABA and SL content in rice, pointing out that this gene may be a promising target for drought improvement in crops. Further studies on how D27 fine-tunes the level of ABA and SLs in different stress responses as well as in developmental processes, will expand our understanding of the function of D27.

Materials and Methods

Plant materials, growth conditions and drought treatments

Rice (*Oryza sativa* L. cv. Shiokari) was used in this study. Rice seeds were first surface sterilised with 70% ethanol for 30s and 2% sodium hypochlorite (v/v) for 30 min. The seeds were then rinsed five times in sterile double-distilled water and immersed in water in the dark for two days at 28°C to induce germination. Finally, germinated seeds were transferred to the climate room in pots filled with a mixture of sand and soil (1:1) (for the drought tolerance assay) or silver sand (for others). The conditions in the climate room were: temperature, 28°C day/25°C night; photoperiod, 10-h-light/14-h-dark; 75% relative humidity, and light intensity of 570 μM m^{-2} s^{-1}. The rice plants were watered twice a week using modified half-strength Hoagland nutrient solution (López-Ráez et al. 2008).

To study the effect of long-term mild drought conditions in rice, four seedlings of *japonica* variety Shiokari were grown together in three litre (L) volume pots. The pots were filled with silver sand equally (same soil volume in all the pots) and watered at saturated levels until the five-leaf stage. After this stage drought was imposed by water withholding until a field capacity (FC) of 45% was reached. This condition was maintained during nine days. The drought was maintained by weighing the pots every day and watering with nutrient solution until the desired water level. After the drought period, plant material was collected for further biomass and hormone quantification.

To test the drought tolerance of rice SL mutants (*d10*, *d17*, *d17* and *d3*), seeds of mutants and wild-type were germinated on half-strength Murashige and Skoog (MS) medium. The rice seedlings (16 plants per pot and five pots for each line) were grown in a climate room in three L pots (diameter 19cm, depth 14.5cm) filled with a mixture of sand and soil (1:1). To minimise experimental errors, each pot was filled with the same weight of soil and supplied with the same volume of water. The drought treatment and water-loss rate experiment under dehydration

conditions were performed according to Zhang et al. (2012) with minor modifications. When plants reached the five-leaf stage, watering was withheld for 12 days. Watering was then resumed for three days to allow plants to recover, after which the survival rates were calculated. To rule out the possible effect of differences in plant architecture between the rice lines studied for the drought resistant/sensitive phenotypes, a pooled survival experiment was also performed. In this experiment, eight plants from the *d27* line (drought sensitive) and eight plants from the *d10* mutant line (drought resistant) were sown in the same pot with a total of eight replicates. The rest of the procedure was similar to the drought survival assay performed for the individual lines.

To detect the water loss rate under dehydration conditions, flag leaves were detached from plants (n=9) and weighed at 0, 0.5, 1, 2, 3, 4, 5, and 6 h after their removal from the plant (at 24°C). Water loss rates were calculated as the percentage of initial fresh weight.

Generation of OsD27 over-expression lines in rice

Full-length cDNA of *OsD27* was PCR-amplified using primers *D27-OE-F* and *D27-OE-R* (Supplementary Table S1), digested by *BamHI* and *SpeI*, and then inserted into the corresponding sites downstream of the ubiquitin promoter in the binary vector pTCK303 (Wang et al. 2004). The resulting plasmid was designated as *pUbi::D27* and introduced into rice variety Shiokari using *Agrobacterium tumefaciens* strain *EHA105* as previously described (Hiei et al. 1994).

Strigolactone and abscisic acid analysis

For SL analysis in rice, plants were grown, and exudates were collected and extracted according to Jamil et al. (2011). ABA was analyzed as previously described (López-Ráez et al. 2010) with minor modifications. For SL quantification from root extracts, 0.5 g fresh root tissue was manually ground in liquid nitrogen. Samples were taken in 10 ml cold glass tubes and 2 ml ethyl acetate containing GR24 (0.01 nmol/ml EtOAC-solution of GR24 as internal standard (IS)) was added and mixed by vortexing. After 15 minutes sonication (in a Branson 3510 ultrasonic bath), each sample was centrifuged for 10 min at 2000 rpm. The organic phase was transferred to 4 ml glass vials. The samples were re-extracted with another 2 ml of ethyl acetate (without IS) and combined in the same 4 ml glass vials. The collected samples were dried in a speed vacuum (Thermo Scientific SPD121P SpeedVac). The residue was dissolved in 50 µl of ethyl acetate and diluted with 4 ml of 100 % hexane. This solution was loaded in a pre-conditioned Silica gel GracePure SPE (200mg/3ml) column. After washing with 2 ml of 100 %

hexane, SL was eluted by 2 mL solvent mixtures of hexane: ethyl acetate (10:90). ABA was extracted using 0.2 g of leaves material in a 4 ml glass vial containing 1 ml of ethyl acetate and 0.01 D6-ABA (IS). After 15 minutes of sonication, each sample was centrifuged for 10 min at 2000 rpm. The organic phase was transferred to 4 ml glass vials. The samples were re-extracted with another 1 ml of ethyl acetate (without IS) and combined in the same 4 ml glass vials. The collected samples were dried in a speed vacuum and the residue was dissolved in 50 µl of methanol and diluted with 3 ml of water. This solution was loaded in a pre-conditioned C18 GracePure column (100mg/1ml). ABA was eluted by 1 mL 100% acetone. The solvent mixtures were evaporated under speed vacuum and the residue was dissolved in 200 µl of acetonitrile: water (25:75, v/v). The samples were filtered through Minisart SRP4 0.45 µm filters (Sartorius, Germany) for ultra-performance liquid chromatography-tandem spectrometry (UPLC-MS/MS), as previously described (López-Ráez et al. 2010, Jamil et al. 2011). Data acquisition and analysis were performed using MassLynx 4.1 (TargetLynx) software (Waters).

Analysis of transcript levels

Total RNA was extracted from rice roots and shoots using a combination of three protocols: TRIzol reagent (Invitrogent), the RNeasy Mini Kit (Qiagen), and the DNase-I Kit (Qiagen), as previously described (http://www.untergasser.de/lab/protocols/rna_prep_comb_trizol_v1_0). RNA concentration, quality and integrity were checked using a NanoDrop ND-1000 UV-Vis spectrophotometer and standard gel electrophoresis. Reverse transcription reaction was performed with the Bio-Rad iScript cDNA Synthesis Kit using 1 µg of total extracted RNA following the manufacturer's instructions. Primers used for real-time quantitative RT-PCR (qRT-PCR) analysis are listed in (Supplementary Table S1). qRT-PCR was performed with the Bio-Rad iQ5 instrument using SYBR Green Supermix Bio-RAD to monitor double-stranded DNA (dsDNA) synthesis following the manufacturer's instructions. Three independent biological replicates were used and each PCR reaction was done in triplicate. Relative expression levels of genes were determined using a comparative C_t method as previously described (Livak and Schmittgen 2001) and rice *Ubiquitin* (*ubi*) gene (Supplementary Table S1) was used as the internal control to normalise target gene expression (Lin et al. 2009).

Carotenoid extraction from rice seedlings for quantitative HPLC analysis

The extraction was performed under dim light by adding 2 ml of acetone to 10 mg finely grounded powder of plant material, followed by sonication (duty-cycle 30, output 2) and centrifugation (5 min. at 3000 x g). This procedure was repeated three times, after which 2 ml of PE:DE (2:1 v/v) was added to the collected supernatants. Water was added up to 14 ml

followed by centrifugation for 5 min. at 3000 x g. The organic phase was collected and the extraction was repeated once more. The combined organic phases were evaporated to dryness and dissolved in exactly 100µl of $CHCl_3$ - from which 10µl was injected for HPLC analysis.

Substrates were quantified spectrophotometrically at their individual λ_{max} using molar absorption coefficients given by literature (Britton et al. 1995). Protein concentration was determined using the Bio-Rad protein assay kit. For HPLC analysis, a Waters system equipped with a photodiode array detector (model 996) was used. Separation was performed using YMC-Pack C_{30} reversed phase columns from YMC Europe (150mm length x 3 mm internal diameter; 5 µm particles system 1 and 250mm length x 4.6 mm internal diameter; 5 µm particles, system 2). Column temperature was set at 35°C. Solvent system consisted out of solvent A, methanol/tert-butylmethyl ether (1:1 v/v), and B, methanol/tert-butylmethyl ether/water (5:1:1 v/v). In plant tissue analysis, column was developed at a flow rate of 1 ml/ min with 100% B followed by a gradient to 18% B within 65 min. Then, the flow was subsequently increased to 1,5ml/ min to 0% B within 2 min and maintained for another 11 min. Finally, the column was re-equilibrated to starting conditions for 10 min.

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We thank prof. J. Li and prof. Y. Wang for kindly providing the rice *OsD27* over-expression lines. We thank Francel Verstappen for his technical support at WUR in the Netherlands.

Supplementary material

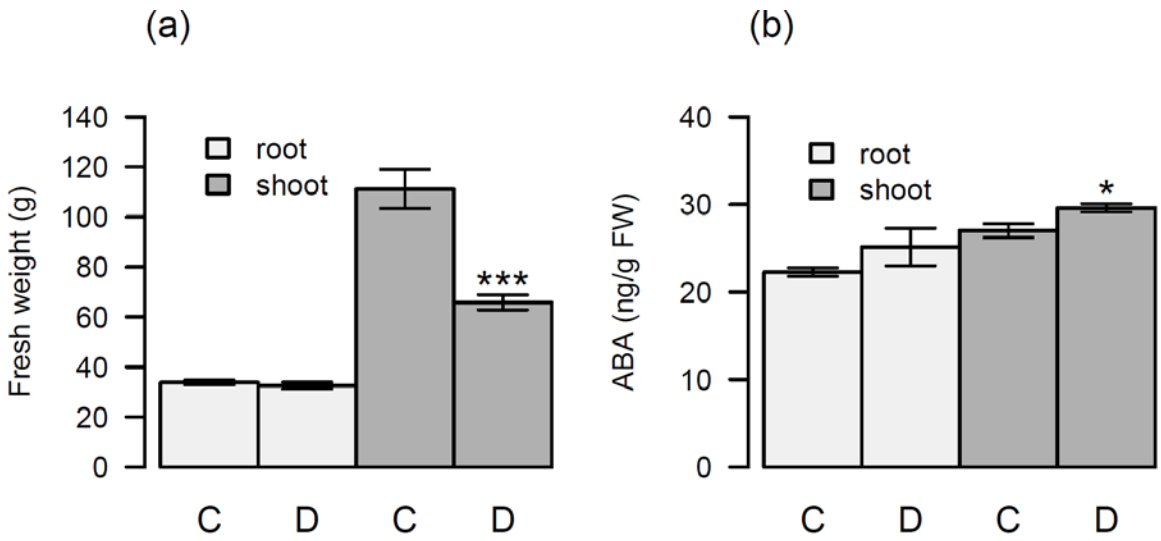

Figure S1. Biomass (a) and ABA content (b) from rice plants under drought and control conditions. Mild drought (35-45 % FC) was applied during 9 d. Data is derived from five biological replicates (and each replicate is represented by a pool of four plants). There are significant differences between treatments, control (C) and drought (D) as revealed by Student's *t*-test (***$P \leq 0.001$, *$P < 0.05$). Bars represent mean ± standard error.

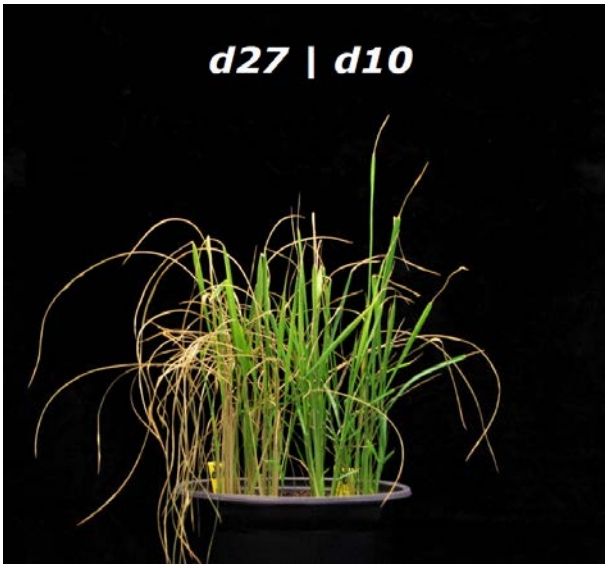

Figure S2. Pooled drought survival assay of the *d27* and *d10* SL biosynthetic mutant lines. Phenotype of five-leaf stage *d27* and *d10* mutant plants after 12 d of drought and 3 d of re-water recovery in a pooled pot experiment.

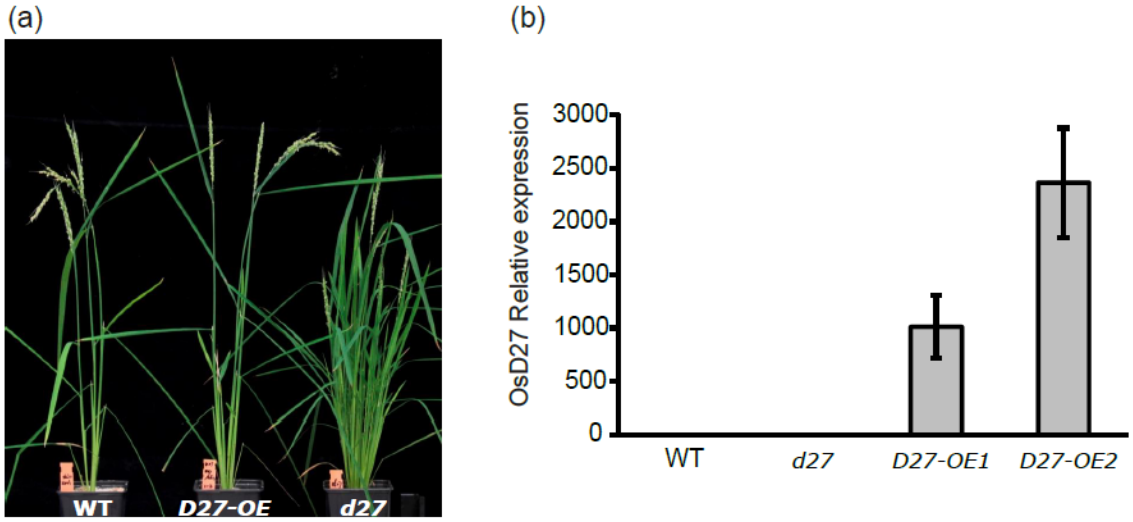

Figure S3. Phenotypic and transcriptional comparison of *D27* over-expressor - and *d27* mutant plants, together with their wild type. (a) Morphology of Shiokari wild-type (WT), *D27* over-expression (*D27-OE*), and *d27* rice plants at post-anthesis stage. (b) The relative expression level of *OsD27* in 19-days-old plants of the two independent over-expression lines (*D27-OE1* and *D27-OE2*) as compared with WT. Values are normalized with the rice *ubiquitin* gene. Bars represent mean ± standard error.

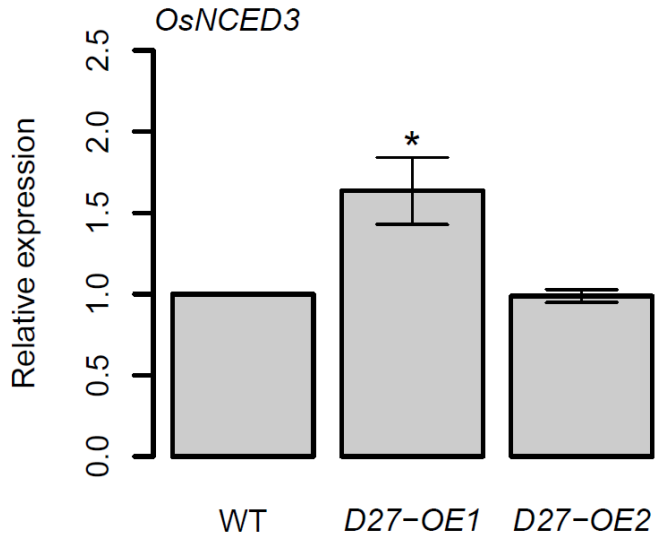

Figure S4. *OsNCED3* expression levels in shoots of *D27* over-expressing plants and their wild-type. Values are normalized with the rice ubiquitin gene. Bars represent mean ± standard error. Asterisk represents a significant difference with the wild-type after a pairwise Student's *t*-test (*, $P < 0.05$).

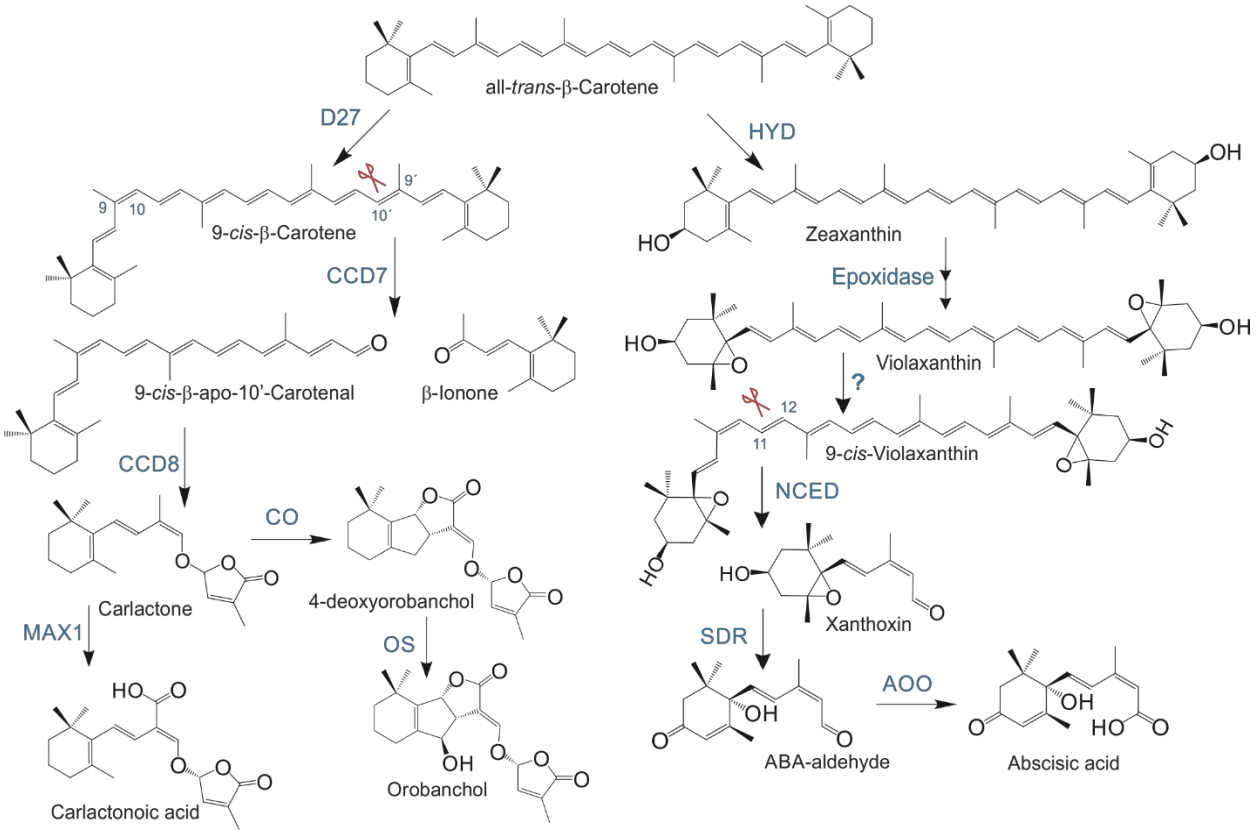

Figure S5. Shared biosynthesis pathway of strigolactone (SL) and abscisic acid (ABA). SL biosynthesis: The conversion of all-trans-β-carotene to carlactone is catalysed by D27, CCD7 and CCD8, respectively (Alder et al. 2012). Rice MAX1 homolog, CO catalyses the conversion of carlactone into the SL-parent molecule, 4-deoxyorobanchol, which is further cleaved by second rice MAX1-homolog, OS to form orobanchol (Zhang et al. 2014, Al-Babili and Bouwmeester 2015). Arabidopsis MAX1 has also been shown to convert carlactone into carlactonoic acid (Abe et al. 2014). ABA biosynthesis: The conversion of all-trans-β-carotene to xanthoxin is catalysed by HYD, epoxidase and NCED, respectively. All-trans-neoxanthin is derived from all-trans-violaxanthin. The conversion of all-trans-violaxanthin and/or -neoxanthin into the corresponding 9-cis-isomer is unidentified. The xanthoxin is further cleaved by SDR and AAO to form ABA (Nambara and Marion-Poll 2005). Abbreviations: HYD (β-carotene hydroxylase), NCED (9-cis-epoxycarotenoid dioxygenases), SDR (short-chain dehydrogenase/reductase), AAO (abscisic aldehyde oxidase), D27 (DWARF27), CCDs (carotenoid cleavage dioxygenases), MAX1 (MORE AXILLARY GROWTH 1), CO (carlactone oxidase), OS (orobanchol synthase).

Table S1. Primer sequences used for the gene expression analysis and over-expression lines.

Primer name	**Sequence**
D27-Q-F	5'-TCTGGGCTAAAGAATGAAAAGGA-3'
D27-Q-R	5'-AGAGCTTGGGTCACAATCTCG-3'
D27-OE-F	5'-AAGGATCCATGGAGACCACCACGCTTGT-3'
D27-OE-R	5'-AAACTAGTTCAGATGGAGCAATTCACAC-3'
OsNCED3-Q-F	5'-CCCCTCCCAAACCATCCAAACCGA-3'
OsNCED3-Q-R	5'-TGTGAGCATATCCTGGCGTCGTGA-3'
Ubi-F	5'-GCCCAAGAAGAAGATCAAGAAC-3'
Ubi-R	5'-AGATAACAACGGAAGCATAAAAGTC-3'
D10-Q-F	5’-CGTGGCGATATCGATGGT-3’
D10-Q-R	5’-CGACCTCCTCGAACGTCTT-3’
D17-Q- F	5’-TCCACAGGATGTTTGGTTACA-3’
D17-Q-R	5’-GTAGCTTGGGTTTATCGCCG-3’
Os01g0701400-Q- F	5’-TGCAGACCAAGTTCCCCTAC-3’
Os01g0701400-Q-R	5’-CACCCATGTTCCCTTTGG-3’
Os01g0700900-Q-F	5’-GAGTTGTGCAAGGAAGTGGG-3’
Os01g0700900-Q-R	5’-GTCCACCTCGAACCACTTGT-3’
Os06g0565100-Q- F	5’-CTCTCCACCAGAAGGGCCTC-3’
Os06g0565100-Q- R	5’-GAGATGATCGTGTTCCTCATCG-3’
Os02g0221900-Q- F	5’-AGGTCATCAAGGAGGCCATG-3’
Os02g0221900-Q- R	5’-CACACGTACGTCCCCTTTG-3’

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Chapter 4

Plant host and drought shape the root associated fungal microbiome in rice

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Summary

Water is an increasingly scarce resource while some crops, such as paddy rice, require large amounts of water to maintain grain production. A better understanding of rice drought adaptation mechanisms could help to reduce this problem. There is increasing evidence for a possible role of root-associated fungi in drought adaptation. In this study we therefore analysed the root fungal microbial community in rice and its relationship with drought tolerance. Hereto, fifteen rice cultivars (*Oryza sativa* ssp. indica) were grown in the field, under well-watered conditions or exposed to a drought period during flowering. The effect of this treatment on the root-associated fungi was analysed by 18S ribosomal DNA sequencing. Grain yield and plant biomass of the cultivars were determined after plant maturation. There was a host genotype effect on the fungal community composition and the drought treatment changed the root-associated fungal community and increased the fungal biodiversity. The majority of OTUs belonged to the *Pezizomycotina* Subphylum. This study shows that drought changes the root-associated fungal community in rice, that the host genotype affects the fungal community structure, and that some fungi may be associated with improved drought tolerance.

Introduction

Global warming is one of the main driving forces that is changing the environment. The higher temperatures caused by global warming further reinforce the effect of drought (Trenberth *et al.*, 2014). Intense drought periods are one of the main causes of grain yield losses in crops worldwide, especially in drought sensitive crops such as rice (*Oryza sativa*), the second most produced and consumed crop in the world. To ensure high productivity, rice requires well-watered conditions and almost 50 % of the fresh water used for crop production worldwide is consumed by rice (Barker *et al.*, 2000). Improving yield under drought is therefore a major goal in rice breeding. The root system is in direct contact with the soil, from which the plants absorb water, and hence root traits are among the critical factors that could ensure good yields under drought stress, including the plant root system and microbiota (van der Heijden, 2008; Comas *et al.*, 2013). Soil microbiota such as arbuscular mycorrhizal fungi (AMF) are strict biotrophs that colonize the plant root and develop a hyphal network to explore the soil. These AMF are associated with about 80 % of land plants (Smith & Read, 2008) and are geographically ubiquitous. They stimulate plant growth by supplying them with mineral nutrients and water that they take up from the soil (Augé, 2001; Singh, 2011).

Another class of root inhabiting fungal micro-organisms, which receive increasing attention, are the so-called Class II fungal endophytes. They have been repeatedly reported to have a role in plant resistance to stresses (Selosse *et al.*, 2004; Rodriguez *et al.*, 2009). This group of fungi belongs mainly to the Ascomycota (mainly Pezizomycotina) and partially to the Basidiomycota. They have a broad host range and colonize the shoots, roots and rhizomes of their hosts (Rodriguez *et al.*, 2009). They can increase plant biomass (Ernst *et al.*, 2003; Redman *et al.*, 2011; Jogawat *et al.*, 2013) and improve tolerance to biotic (Mejía *et al.*, 2008; Maciá-Vicente *et al.*, 2008; Chadha *et al.*, 2015) and abiotic stresses (Hubbard *et al.*, 2014; Yang *et al.*, 2014; Azad & Kaminskyj, 2015).

The root fungal microbiota composition is not static. It can be influenced by environmental factors. Pesticide application, for example, increases the richness of the AM fungal community composition in roots (Vandenkoornhuyse *et al.*, 2003). On the other hand, farming practices such as tillage and ploughing, clearly modify composition and decrease species richness of AM fungi in agricultural soils (e.g. Verbruggen & Kiers, 2010). It has also been shown that monocropping and conventional paddy cultivation reduce the AMF diversity and colonization in rice, and favour the presence of fungal pathogens (Lumini *et al.*, 2010; Esmaeili Taheri *et al.*, 2016). In traditionally flooded rice fields, a lower number of root associated fungal species were found than in upland fields (Pili *et al.*, 2015).

Despite its reported role in plant fitness, the importance of plant colonizing fungal microbiota is underestimated, both in terms of diversity and functionality (Lê Van *et al.*, 2017). However, plants cannot be regarded as standalone entities but rather are holobionts (i.e. the plant and its associated microbiota) in which the microbial community provides additional functions to the host helping to cope with environmental changes and stresses (Vandenkoornhuyse *et al.*, 2015). In this framework of conceptual understanding of the plant as a holobiont, the active recruitment of microbial components to face a constraint could explain the microbiota heterogeneity through space and time. If indeed the host-plant is able to control the recruitment of microorganisms, it is likely that genetic diversity for this trait exists. Indeed, the phyllosphere bacterial community in *Arabidopsis thaliana* (Horton *et al.*, 2014) and wild mustard (Wagner *et al.*, 2016) but also the barley root bacterial microbiota (Bulgarelli *et al.*, 2015) are to some extent host genotype dependent suggesting that plants do not randomly recruit among the available microorganisms. We thus hypothesized herein that changes that occur within the fungal microbiota community composition when plants experience an environmental constraint, are not random, but are at least in part controlled by the plant itself.

To address this hypothesis, we analysed the effect that drought has on the root associated fungal microbiota in a number of different rice genotypes and whether this impacts on rice yield.

Materials and Methods

Plant Materials

Fifteen rice cultivars (*Oryza sativa* ssp. indica) from the International Rice Research Institute (IRRI, Los Baños, Philippines) were used in our study. Ten out of the 15 cultivars were selected to maximise the genetic variation using the SNP information available from a published study (Zhao *et al.*, 2011). The five additional cultivars were selected based on their drought tolerance phenotype, and their information is available in IRGCIS database: http://www.irgcis.irri.org:81/grc/SearchData.htm (Table S3).

Field site and growing conditions

All rice plants were grown at IRRI facilities from December 2012 to March 2013. The upland field (used to grow rice under non-flooded conditions) was localized at 14°08'50.4"N 121°15'52.1"E. There were 15 field blocks (one per cultivar) (0.8 x 2.5 metres) and each block included 48 plants. The soil was a mix of clay (36 %), sand (22 %) and silt (41 %). The plot design was randomized through the field site and there were three replicates per cultivar for both treatments. Plants were grown in waterlogged conditions until 50 % of the plants reached the flowering stage. Then a drought treatment was imposed on half of the replicates by withholding irrigation. After 12 days of drought, the stressed plots reached -46 KPa of soil water potential, while the control plot was saturated with water (100 % of soil field capacity). Then, soil cores (10 x 70 cm) were collected from the plots of the selected 15 cultivars and stored in non-sterile bags at 4°C until further use. Roots were isolated from the soil cores by carefully washing with tap water and a sieve, under non-sterile conditions, to remove all the possible propagules from the root surface, and then frozen in liquid N_2 and stored at -80 °C until further analysis.

DNA isolation and sequencing

Each root sample was grinded to powder with a mortar and pestle using liquid nitrogen, and DNA was extracted from 60–80 mg of plant material with the DNeasy Plant Mini Kit (Qiagen) following the manufacturers protocol. From the extracted DNA, we amplified a fragment of the 18S SSU rRNA gene using general fungal primers (NS22: 5'-

AATTAAGCAGACAAATCACT-3' and SSU0817: 5'-TTAGCATGGAATAATRRAATAGGA-3') (Borneman & Hartin, 2000) and the following thermocycler conditions: 94 ºC for 3 min; 35 cycles of 94 ºC for 45 s, 59 ºC for 45 s (–0.1 ºC / cycle), 72 ºC for 1 min; and 72 ºC for 10 min. Primers were modified to allow the amplicon multiplexing for the sequence production process. For this purpose, a collection of 96 modified SSU0817 primers each containing a specific tag consisting of 10 nucleotides was used. The amplicons were purified with AMPure XP beads (Beckman Coulter). The amplicon size was checked with the Agilent High Sensitivity DNA kit (Agilent Technologies), and the concentration measured with an ultrasensitive fluorescent stain (Quant-IT^{TM}PicoGreen$^{®}$dsDNA Assay kit, Invitrogen). Finally, the purified 560 bp amplicons were diluted to similar concentration, pooled and sequenced (454 GS FLX+ version Titanium, Roche), following the manufacturer's guidelines. All the PCRs were performed twice and sequenced separately. These true replicates were used within our stringent trimming strategy.

Sequence data trimming and clustering:

After demultiplexing, raw reads were filtered to remove sequences containing homopolymers longer than 6 nucleotides, undetermined nucleotides, anomalous length, difference (one or more) in the primer. Reads were also filtered based on quality score. The sequences which have passed all the filters were clustered using DNACLUST (Ghodsi *et al.*, 2011). OTUs are formed of at least two 100 % identical sequences necessarily appeared independently in different replicates. After these steps, a filtration of chimeric sequences was performed using the 'chimeric.uchime' tool within Mothur (v1.31.0, Schloss *et al.*, 2009). The stringent trimming and clustering pipeline used was similar as the one that was used in previous studies (e.g. Ben Maamar *et al.*, 2015; Lê Van et al., 2017). The affiliation statistics to identify OTUs were run using the PHYMYCO-DB database (Mahé *et al.*, 2012). A contingency table was produced to make all the diversity and statistical analyses. We concluded from rarefaction curves that the sequencing depth was high enough to describe the fungal community associated to rice.

Pot experiment with Arthrinium phaeospermum

The endophytic fungus *Arthrinium phaeospermum* was tested in a pot experiment to study if it has effect on rice performance. As host, the cultivar IR36 (indica rice) was selected, based on the higher *A. phaeospermum* presence in roots from the field experiment. The seed coat was removed and seeds were sterilised with 2 % sodium hypochlorite (v / v) and rinsed several times in sterile distilled water. Later seeds were directly sown in small 0.3 litre (L) pots filled with sterilised sand. Plants were watered regularly with modified half-Hoagland nutrient solution

and grown during 7 days in a climate cell. The conditions in the climate cell were: temperature, 28 °C : 25 °C, day : night; photoperiod, 12 h : 12 h, light : dark; 75 % relative humidity, and light intensity of 570 μmoles m^{-2} s^{-1}. As the original *A. phaeospermum* strain from the field could not be isolated at the time that the experiment was done, eight strains that were available from the CBS-KNAW Fungal Biodiversity Centre (Utrecht, The Netherlands) were tested (Table S2), together with *Piriformospora indica*, which was used as a positive control for being a well-known beneficial fungal endophyte (Varma *et al.*, 1999; Sherameti *et al.*, 2008). The fungi were grown in Potato Dextrose Agar (PDA) with rifampicin (50 μg / ml). After the fifth day, plants were inoculated with a 10 millimetre diameter agar disc with mycelia and grown for another three days when the drought treatment was started. When plants were eight days-old, half of the replicates underwent water withholding for six days. In order to avoid plants to wilt and die too soon, plants received a fixed amount of water every day, to keep the stress high but not to lose completely all soil water. After the drought period, all plants were collected and fresh and dried biomass were quantified.

Statistical analysis

All the statistical analyses were performed using R (R core team, 2013). From the normalized contingency matrix, OTU richness, abundance, evenness and diversity index estimators were calculated using *CAR* package (Kindt & Coe, 2005; Fox & Weisberg, 2011). Statistical differences in these measures were analysed using two-way ANOVA. To test for a field position effect on the microbial community results, a Mantel Test was performed using the *VEGAN* package (Oksanen *et al.*, 2015). Each root sample was assigned a field position value (based on two coordinates) and the geographical Euclidean distances were calculated. These distances were subsequently compared with the ecological distances (*Bray-Curtis* method) calculated for the fungal community to analyse if there is a correlation between the field position and the fungal community composition.

Fungal community differences between the different treatments were studied using non-metric multidimensional scaling (NMDS) analysis, after removing rare OTUs (OTUs with abundance < 10 sequences) applying the *Kulczynski* ordination method (Kulczynski, 1928). To test whether significant differences exist between fungal communities from control and drought treatments a permutational multivariate analysis of variance (PERMANOVA) was run with the *adonis* function using the *Bray-Curtis* distance matrix. The analysis was run with the VEGAN package.

To study the correlation between plant performance and the associated fungal community, a Variation Partitioning Analysis (VPA) was performed with the VEGAN package. The VPA model allows to include factors as variables to study if any of them can explain the fungal community composition. In the model OTUs abundance data (without the rare OTUs) were included and as factors 'yield' (described by the yield per square metre) and the rice 'host' (described by the Kinship values from the rice genomic map). As a way to calculate the relative response between treatments, the 'yield robustness' was calculated by the phenotypic plasticity index (PI) (Valladares *et al.*, 2006) defined as yield Mean $_{control}$ – yield Mean $_{drought}$ / yield Mean $_{control}$ (calculated for each cultivar). This index was included as a variable together with the 'host' factor in a new VPA model to study how the plant relative response is correlated with the community. We also run a Pearson correlation analysis with the *rcorr* function in HMISC package, between the independent OTUs and yield under control and drought treatments; the OTUs positively correlated with plant yield with an $R > 0.10$ and $P < 0.05$ were selected.

When exploring changes in community or OTU patterns when plants are exposed to stress conditions, it is advisable to also use qualitative or discrete quantification methods, to avoid that pattern changes are masked by OTU abundance differences (Lozupone & Knight, 2008; Amend *et al.*, 2010; Magurran, 2013). Hence we also estimated the OTU occurrence between treatments for the OTUs positively correlated with yield.

To study if yield is linked to phylogenetic relatedness of the root-fungal microbiota, the phylogenetic signal was calculated using the Blomberg's K statistic (Blomberg *et al.*, 2003) with the *PICANTE* package (Kembel *et al.*, 2010). The OTU abundance matrix was used as trait, where the mean and standard error was calculated for each OTU. The original Ascomycota tree generated by Maximum Likelyhood Estimation was pruned by the yield correlated OTUs. The pruned tree together with the abundance data was used to calculate the phylogenetic signal, with all data together and by treatment (control and drought separately).

Pruned trees where OTUs with less than 10 sequences had been removed, were separately calculated for the main phyla, Ascomycota and Basidiomycota. Sequences were aligned using MAFFT v.7.123b (Katoh & Standley, 2013) and then trimmed with Gblocks v.0.91b (Castresana, 2000). Phylogenetic trees were generated by Maximum Likelihood (ML) using RaxML v.8.00 (Stamatakis, 2014), with the GTRGAMMA model and 1000 boostrap replicates. For a subset of OTUs correlated with yield, a Neighbor Joining (NJ) tree was generated from a pairwise distances matrix of sequences using the SEQINR (Charif & Lobry, 2007) and APE

(Paradis *et al.*, 2004) R packages. All trees were edited using iTOL (http://itol.embl.de, Letunic & Bork, 2011).

To further explore the effect of *Arthrinium phaeospermum* in plant fitness, a linear model analysis was performed using the *STATS* package. The response (plant biomass, water content, root : shoot ratio) and the predictors (treatment 'fungus' and treatment 'drought') were included in a fitted linear model previously to run an ANOVA analysis to see if the categorical variables `fungus´ and `drought´ interact with each other and if they can explain the changes in plant response.

Results

Root-associated fungal community in rice

As the samples were selected from a large field experiment, we performed a Mantel Test to check for the presence of field position effects. This analysis showed that there is no field position effect on the fungal community composition for both treatments (Fig. S1). We analysed a total of 444,757 fungal sequences of 560 bp forming 902 different OTUs. The sequencing depth was high enough to describe the root fungal microbiota (Fig. S2). Given the fragment length and phylogenetic information it contains, the level of resolution of taxonomic identification of OTUs was at the species level within the fungal phyla with the exception of the Ascomycota with a resolution at the species or genus levels. Among this γ-diversity of 902 OTUs detected, only two belonged to the Glomeromycota (i.e. AM fungi). The biggest OTU richness by far was observed for the Ascomycota phylum (784 OTUs), followed by the Basidiomycota (32 OTUs) (Fig. 1). The rest of the OTUs belonged to Chytridiomycota (9 OTUs), Zygomycota (3 OTUs) and an unclassified phylum with 72 OTUs. After filtering out the rare OTUs (OTUs with less than 10 sequences in all analysed samples), we continued our analyses with the remaining 862 OTUs. The γ-diversity in the different treatments was similar, and the majority of OTUs are present under control and drought (Fig. 2). The fungal γ-diversity measure S = 862 and Shannon diversity index H' = 3.5.

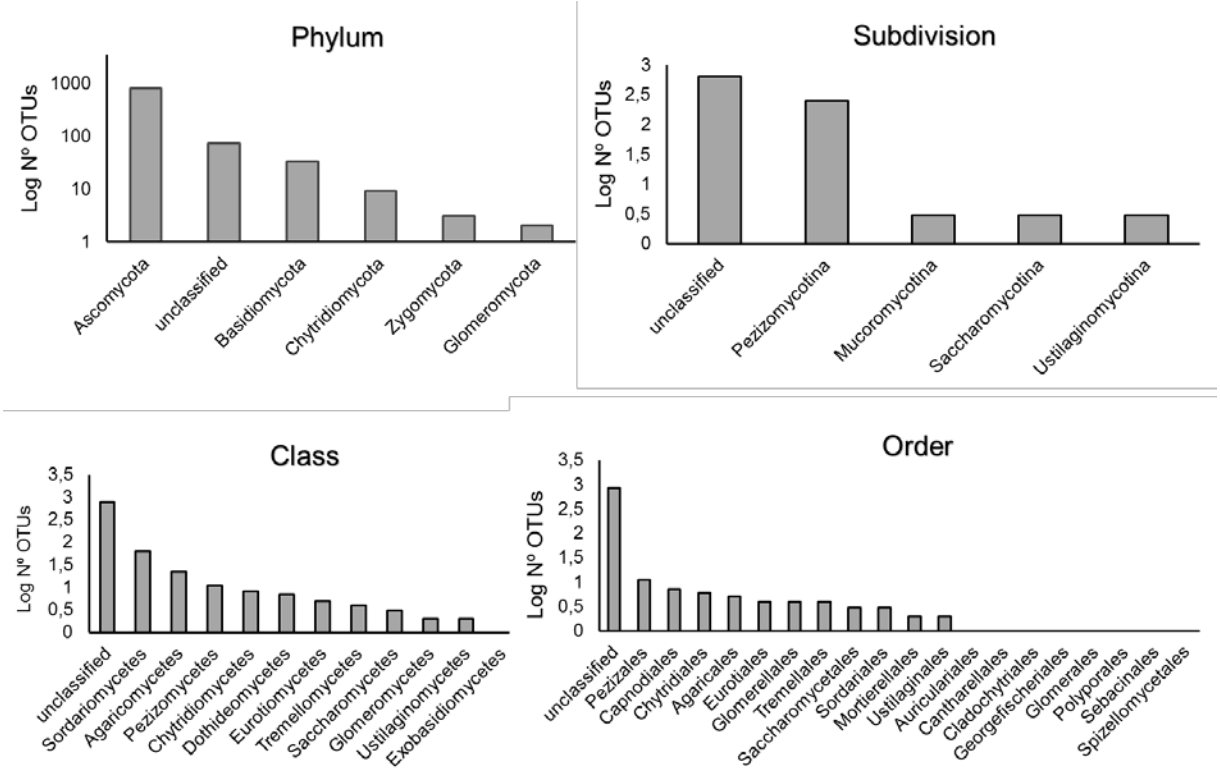

Figure 1. Description of the fungal community in the present study (e.g. γ-diversity). A total of 447,757 sequences was analysed that belonged to 902 OTUs. Bars represent the logarithmic value of the number of OTUs per taxonomic group. The OTU richness per phylum, subphylum, class and order are shown.

The richness and diversity per taxonomic group, however, vary between the control and drought treatment (Fig. 2). The diversity and species richness for the main groups (Ascomycota and Basidiomycota) are higher under drought, while the unclassified phylum shows the opposite pattern. Also for the α-diversity there were small differences in fungal microbiota species richness under control and drought, both with non-normalized as well as with normalized data: $S_{control} = 124$, $S_{drought} = 132$. An uneven distribution of OTUs in the rice fungal microbiota community structure was observed ($J_{eveness}$ index ~ 0.5). These observations correlated what the Shannon diversity index (H') which was higher under drought conditions for all the rice cultivars (Fig. 3), due to an increased OTU richness and the presence of less dominant species. This was confirmed by two-way ANOVA analysis ($P < 0.001$). Interestingly, the change in the diversity index between control and drought was rice cultivar-dependent (Fig. 3) suggesting an effect of the host-plant on fungal biodiversity and changes therein. The effect of drought was also clearly highlighted using Non-metric Multidimensional Scaling (NMDS)

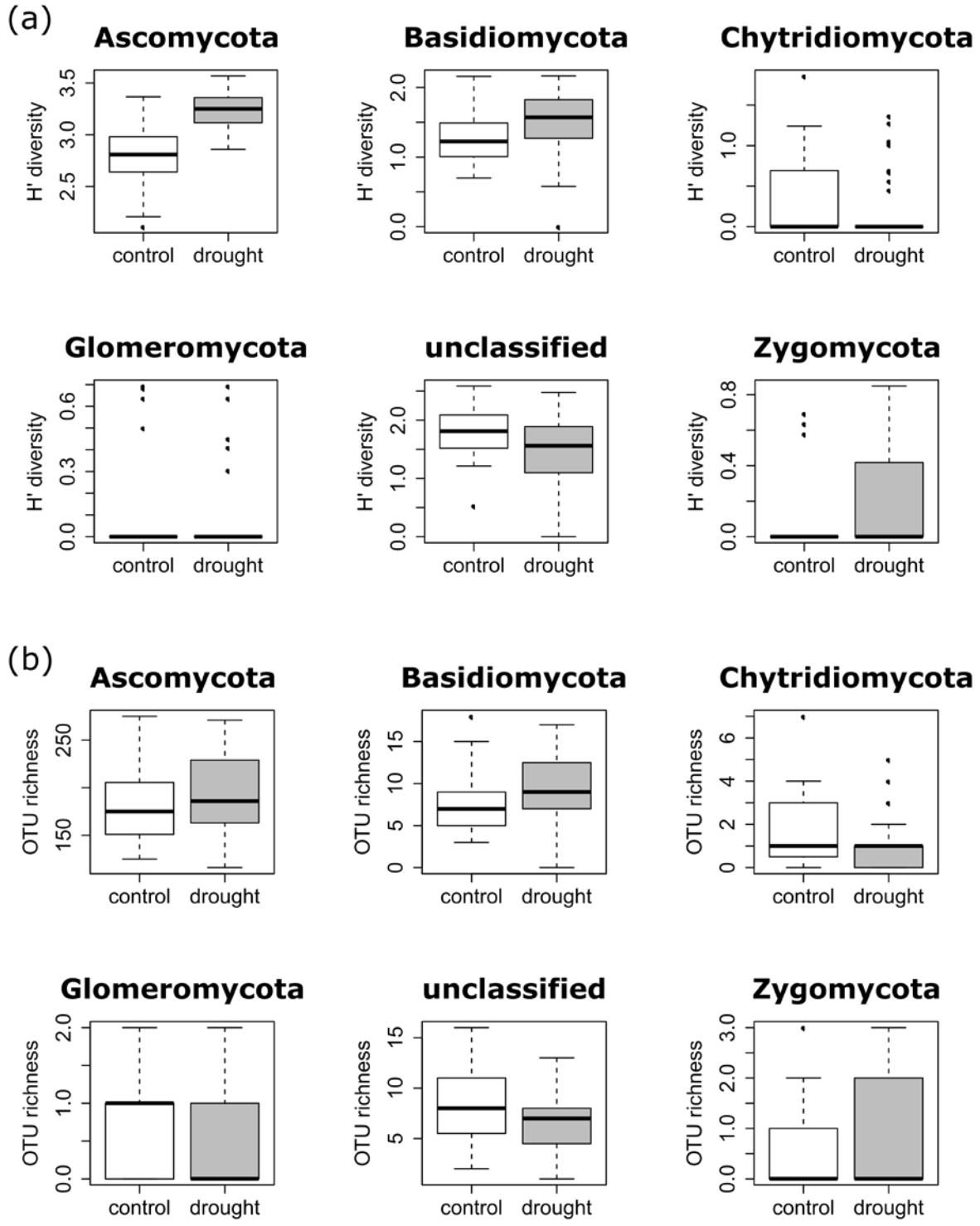

Figure 2. Diversity Shannon index (a) and richness (b) for the different phyla, under control (white) and drought (grey) conditions (i.e. α-diversity). Results show that OTU richness do not differ much between treatments for all the taxa. On the other hand, diversity is higher under drought for Ascomycota and Basidiomycota, while the unclassified group shows the opposite trend.

ordination with a strong differentiation of fungal microbiota communities (i.e. dots in Fig. 4) according to treatment (i.e. drought *versus* control in Fig. 4). A phylogenetic analysis of all frequent OTUs (without the rare OTUs, with less than 10 sequences) was performed for the main phyla: Ascomycota and Basidiomycota (Fig. S4). OTUs within the Sordariomycetes class (from the Pezizomycotina subphylum) and an unclassified group (closely related to Sordariomycetes) dominated (Fig. S4).

In order to test the statistical significance of the NMDS analysis, a PERMANOVA analysis was performed on the NMDS scores. The analysis supports that there is a strong effect of the treatment (control *vs*. drought) ($R^2 = 0.37$; $P = 0.001$). We can conclude from these data that drought has a qualitative and quantitative impact on the fungal community in rice roots.

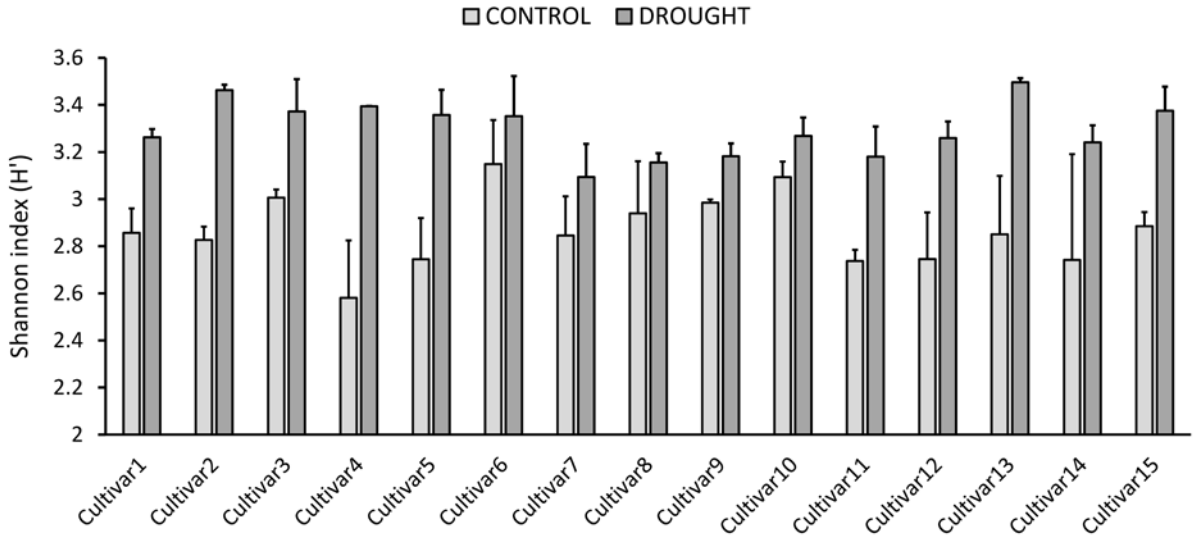

Figure 3. Shannon diversity index for the rice cultivars analysed, under control (light grey bars) and drought (dark grey bars) (i.e. α-diversity). Error bars represent SE. The fungal microbiota Shannon index strongly differs between the treatments (i.e. two-way ANOVA analysis, $P < 0.001$).

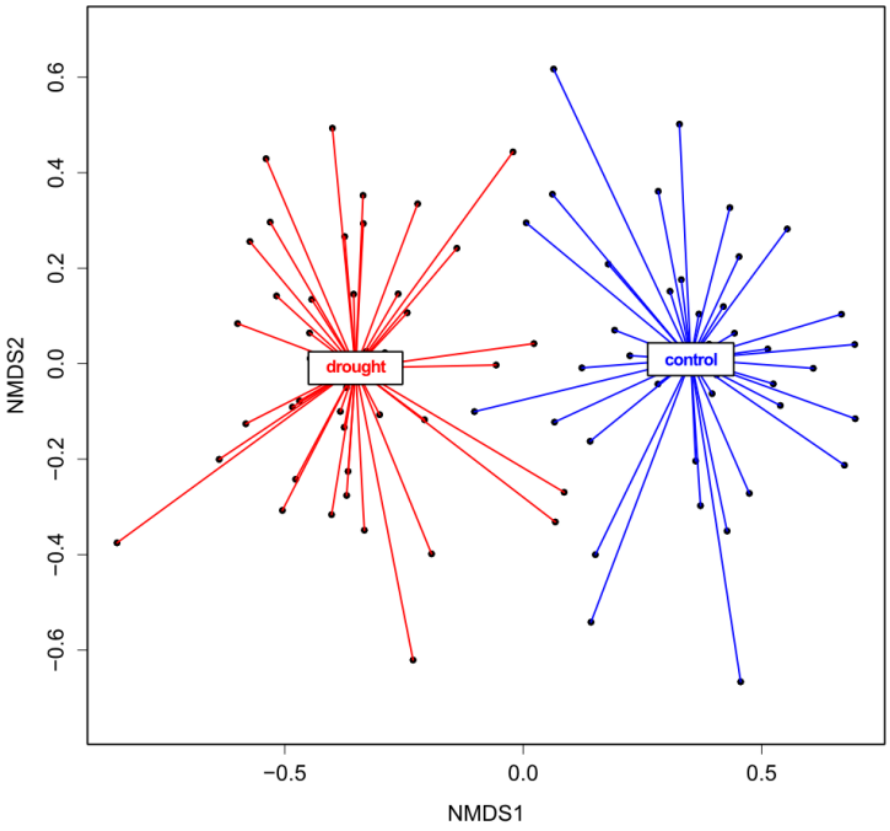

Figure 4. NMDS representing rice root fungal community structure for the two treatments, control and drought. A Bray-Curtis dissimilarity distance (i.e. β-diversity) and a Kulczynski ordination method were used. The statistical analysis (PERMANOVA) showed that the treatments significantly differed in the fungal microbiota composition ($R^2 = 0.37$, $P = 0.001$).

Host and treatment effect on root-associated fungi

To further underpin the effect of drought on the fungal community composition we used Variation Partitioning Analysis (VPA). This analysis compares the root associated microbial community with factors or a group of factors and tests if any of them is correlated with the microbial community structure. In a first VPA model the factors 'treatment', 'host' (genotype Kinship values) and 'yield' were included. Both the 'treatment' effect and the combination 'yield' and 'treatment' significantly explain the variation in fungal community composition (i.e. response matrix) ($P < 0.001$; coefficient of determination, R^2, of 0.22 and 0.38, respectively) (Fig. S5a), that confirms the PERMANOVA results obtained before for the treatment effect. The 'host' effect was very small in this case ($R^2 = 0.01$), also confirmed by the PERMANOVA analysis, which means: no host or block effect on the data. Within a second VPA analysis, we included the 'yield robustness' along with the factor 'host' and the mean abundance of the

OTUs for the different treatments (control and drought) and demonstrated a significant 'host' effect on the fungal community under drought (P = 0.002; coefficient of determination R^2 = 0.13) (Fig S5b).

Effect of fungal endophytes on rice fitness

To address the link between the fungal community and plant fitness under drought, each independent OTU was correlated with seed yield robustness as a proxy for drought tolerance. We found 37 OTUs that were positively correlated with yield (R > 0.10; P < 0.05), of which 13 were occurring more under control and 22 more under drought conditions, while of two the presence did not change between the treatments (Fig. 5). Thirteen out of the 37 OTUs were assigned to Pezizomycotina and the other 24 OTUs are unclassified, but closely related to the Pezizomycotina sub-phylum.

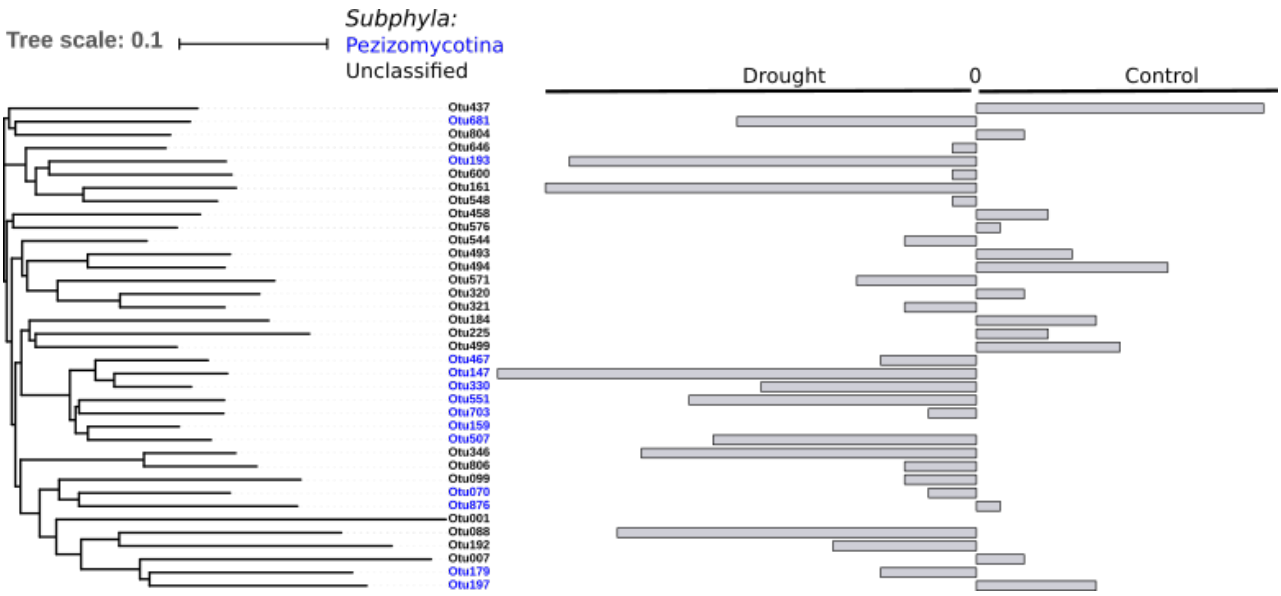

Figure 5. Phylogenetic tree representing the 37 OTUs positively correlated with yield under control and drought conditions. The represented OTUs present a correlation value of R > 0.10 with a P < 0.05. The grey bars provide the OTU occurrence ratio between treatments: OTU occurrence $_{control}$ – OTU occurrence $_{drought}$. The occurrence of only 2 of the 37 OTUs remained unchanged between treatments while 22 of the 37 OTUs increased under drought. There is a strong phylogenetic signal between all yield correlated OTUs (K = 6.6; P = 0.01), indicating that yield correlated OTUs are related.

The effect of specific taxa groups on rice yield was calculated from the phylogenetic signal for yield in comparison with the OTU abundance showing that there was phylogenetic conservation for yield (K = 6.6, P = 0.01) implying that phylogenetically related OTUs are

more associated with similar yields than random OTUs. This relatedness is solely due to the abundance data under drought (K = 8.7; *P* = 0.03).

One of the few OTUs described at specie level and with availability in the fungal stocks centres, *Arthrinium phaeospermum*, was positiviley correlated with yield (Spearman, $R^2 = 0.21$) but not significant (P>0.05). However, this OTU was among the ones contributing to plant yield significantly in the VPA analysis. Because of these reasons plus the fact that it belongs to the Pezizomycotina sub-phylum and the specie has been described in the literature to promote yield, we decided to test it for further experiments. . Therefore, this endophyte was tested in a pot experiment to study its direct effect on rice performance. The results show that the *A. phaeospermum* strains tested did not have a significant possitive effect on the plant shoot biomass under control nor drought conditions (Fig. 6). Moreover, some strains even had a negative effect, just as the control strain *P. indica* (Fig. 6). There was an interaction between the factors 'fungus' and 'drought' for the majority of variables measured (Table S1). Indeed, the majority of fungal strains reduced root biomass under drought (Fig. 6), affecting the root to shoot ratio significantly in the case of strains 2, 4, 7, 8 and *P. indica* (Table S2).

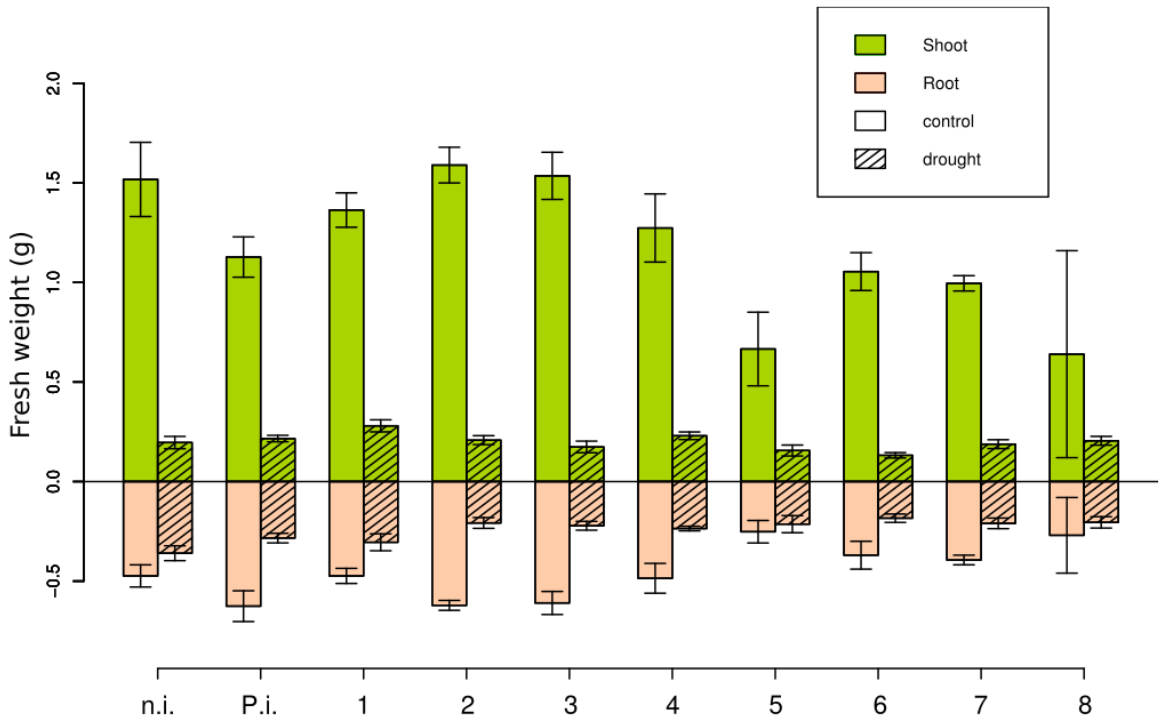

Figure 6. *Arthrinium phaeospermum* effect on plant biomass. There is no effect of inoculation on plant shoot biomass with any of the fungal strains under control nor drought conditions.

Discussion

Endospheric fungal microbiota detection and molecular strategy

There is an increased understanding of the complexity of the root-associated fungal microbiota which is not solely limited to Glomeromycota forming AM association, but also includes other fungi belonging to Zygomycota, Ascomycota and Basiodiomycota (e.g. Vandenkoornhuyse *et al.*, 2002; Lê Van et al., 2017). In the present study the largest group was the Ascomycota phylum (784 OTUs), followed by the Basidiomycota (32 OTUs) (Fig. S4). The Ascomycota and Basidiomycota are also dominant in the roots of other plant species like for example maize (Kuramae *et al.*, 2013), wheat (Vujanovic *et al.*, 2012), poplar (Shakya *et al.*, 2013) and *Agrostis stolonifera* (Lê Van *et al.*, 2017), and they are known to include the so called "dark septate endophytes" (DSEs) which are facultative plant symbionts (Rodriguez *et al.*, 2009). To analyse the entire diversity of the fungal community that is associated with roots, ITS is difficult to use for Chytridiomycota, Zygomycota and Glomeromycota which contain highly divergent ITS copies even within a single spore (e. g. Sanders *et al.*, 1996). Alternatively, the use of the SSU rRNA gene has been proven to be succesful even for unknown fungal species or groups (e.g. Vandenkoornhuyse *et al.*, 2002) across all known fungal phyla, despite the fact that the limited genetic variation contained in the SSU rRNA gene limits the resolution level of identification especially within the Ascomycota. However, to make direct analyses and comparisons of the entire fungal microbiome among plants, to detect a phylogenetic signal (e.g. Lê Van et al., 2017) and to assess the relative abundances of phyla, classes or genera forming the fungal community, the use of a single primer set to generate amplicons is required. The primer set used herein that amplifies the variable regions V4 (partial) and V5 was chosen among other candidate primers from *in silico* amplifications showing that 94 % of the available fungal sequences within the SILVA database (Quast *et al.*, 2013) and only 1.3 % of *Viridiplantae* could be amplified (Lê Van et al., 2017). Amplicon mass sequencing thus allows the description of the fungal community in great detail.

In our study, fungal γ-diversity was S = 862 and Shannon diversity index H' = 3.5. They are of the same order of magnitude as in other crops; lower than in chickpea: H' ~ 4.7; S ~ 800 (Bazghaleh *et al.*, 2015) but higher than in arctic plants: H' ~ 2.8; S ~ 60 (Zhang & Yao, 2015) and other monocots such as wheat: H' ~ 1.8; S ~ 18, and maize: H' ~ 0.9; S ~ 9 (Bokati *et al.*, 2016). These observations suggest that the studied rice cultivars contain a high number of fungal species, which represents a pool for pathogen and mutualistic species.

Drought affects the endophytic fungal microbiome

It has been repeatedly reported that the soil fungal community composition is important and variable with notably a decreased α-diversity under drought conditions (Hawkes *et al.*, 2011; Cregger *et al.*, 2012; Seema B. Sharma & Thivakaran A. Gobi, 2016; Zhang *et al.*, 2016). However, we have to be aware that the stress response in the root associated microbiome might be different from the one in soil. In the present study we now clearly demonstrated that the rice endospheric fungal microbiota changed under drought stress (Fig. 4) with an enrichment of fungal OTUs for all the 15 rice cultivars tested (Fig. 3). This OTU enrichment within the fungal microbiota under drought conditions could be interpreted as an active recruitment of additional fungi by the rice root to face the environmental stress. A high diversity is a better pool for subordinate species (less abundant ones), which have a large influence on the ecosystem where they belong and can improve plant productivity under drought conditions (Mariotte *et al.*, 2015). Herein the increase on fungal species can also be regarded as the enrichment in additional functions mediated by the plant microbiota enabling to mitigate the consequences of drought on host-plants.

In sorghum it has been shown that when water levels are extreme (drought or flooding), roots were colonized by less AM fungal species, however at the same time the abundance of these species was found to be increased. In those experiments, plant biomass was not affected by the water regime, but phosphate intake was increased as a result of a change in the root colonization of plants under non-flooded conditions (Deepika & Kothamasi, 2015).

Reciprocally to this study, Glomeromycota species richness and abundance increased under drought within a diverse panel of plants including wild and cultivated species (Tchabi *et al.*, 2008). Strikingly, in the present study, we only observed two OTUs representing Glomeromycota within the fungal microbial community which were not affected by the different treatments. Although we know that the fungal microbiome is not only composed of Glomeromycota (e.g. Vandenkoornhuyse *et al.*, 2002), rice is unexpectedly poor in AM fungal colonizers in comparison to other Poaceae (e.g. Lê Van et al., 2017) using the same methodological approach. The root endophytic fungal community of rice was dominated by Ascomycota which accounted for about 90 % of the OTUs. We clearly found an increase in Ascomycota OTU richness under drought treatment, as if rice recruited more different fungi. The same holds for the Basidiomycota.

The majority of the OTUs that increased in frequency under drought in our study belonged to the Pezizomycotina subphylum, the most abundant subphylum in the Class II

fungal endophytes. They are well-known for their role in plant performance, boosting plant growth and buffering the effect of environmental stresses and protecting their host-plant against pathogens (Maciá-Vicente *et al.*, 2009; Jogawat *et al.*, 2013; Azad & Kaminskyj, 2015). Other studies also explored how drought influences plant-microbe interactions, showing that fungi have an important effect on plant fitness under drought conditions (Lau & Lennon, 2012; Kaisermann *et al.*, 2015; Classen *et al.*, 2015).

Host genotype affects the fungal microbiome response to drought

We showed with the VPA analysis that the host (i.e. cultivar) determines at least partially the abundance of the root associated fungal community in response to drought (R^2=0.13; P-value=0.01) (Fig. S5). Other studies, using *Arabidopsis thaliana* and barley, also show a host-genotype effect on the root associated microbiome (Lundberg *et al.*, 2012, while in maize and *Microthlaspi*, the root endophyte community composition did not to depend on the host genotype, but was largely determined by the geographical distribution where these cultivars grew (Peiffer *et al.*, 2013; Glynou *et al.*, 2016). Using a GWAS approach for the phyllosphere microbiome composition of *Arabidopsis*, Horton *et al.* (2014) showed that the fungal and bacterial community on leafs is determined at least in part by plant loci responsible for defense and cell wall integrity (Horton *et al.*, 2014).

The results presented herein clearly support the hypothesis that changes occurred within the fungal microbiota community composition when plants experienced an environmental constraint (Fig. 4). The environmental constraint, drought, can be regarded as a strong environmental driver of the endospheric fungal microbiota. Because the fungal microbiota colonizing rice roots do not change stochastically but are modified similarly across plant-cultivars experiencing drought (Fig. 4), we discuss there must be active recruitment of fungal species (in addition to passive recruitment) to enrich the plant-microbiome. This observation is in line with the idea that plants can recruit microorganisms to buffer an environmental stress (Vandenkoornhuyse *et al.*, 2015). In addition to this, in the present study we also demonstrated that the plant cultivar has an effect on the fungal microbiota community composition, which can be interpreted as a genotype-based phenomenon. A so called host-plant preference has also been shown in a study analysing AM fungal communities in co-occurring plant species (poaceae) (e.g. Vandenkoornhuyse *et al.*, 2003). This observation was later explained by a carbon embargo toward less beneficial AM fungi (Kiers *et al.*, 2011), a filtration process allowing a plant to select AM fungal colonizers on the basis of their level of cooperation (Duhamel & Vandenkoornhuyse, 2013).

Root fungal microbiota and rice grain yield

OTUs that are closely related to each other showed similar correlation values for rice grain yield (phylogenetic conservation). Intriguingly their occurrence is higher under drought conditions (Fig. 5), suggesting that they may play a critical role in the rapid adaptation of plants to environmental changes. In a previous study, inoculation of rice with fungal Type II endophytes such as *Fusarium culmorum* and *Curvularia protuberata* resulted in a higher growth rate and yield and a reduced water consumption. Moreover, the rice plants grown under drought stress were more intensively colonized by these fungi in comparison to control plants (Redman *et al.*, 2011). The present study resulted in the identification of 37 different OTUs that belong, or are closely related to the Pezizomycotina which were all positively correlated with yield in plants that were exposed to drought (Fig. 5). Our data suggest that more different fungi than previously believed play a role in rice drought resistance and grain yield. This work opens up new avenues for research for validation and understanding of this induced rice drought resistance by Pezizomycotina and Pezizomycotina-related fungi. As a first step, isolation and culturing of the specific strains that occur on rice and are correlated with the improved yield under drought will be needed.

Arthrinium phaeospermum rice inoculation

Our study provided a new understanding of the complexity of the endospheric fungal microbiota and showed a positive correlation between the presence of particular fungi and crop yield of plants that experienced drought. Among these fungi, one OTU, *Arthrinium phaeospermum,* that was found to be correlated with plant yield and could be identified at the species level. The presence of *Arthrinium* species is often associated with plants from the Poaceae family, suggesting a certain level of host specificity (Yuan *et al.*, 2011). To confirm the role of *A. phaeospermum* in rice drought resistance, different strains of this species were used to inoculate rice plants in a pot experiment. Under control conditions no significant effect (or even a negative effect) of the inoculation was observed on plant shoot biomass, while root biomass was decreased by some of the strains (Fig. 6), also observed under drought. We also found that root biomass investment (root to shoot ratio) under drought was lower for plants inoculated with some of the strains (strain 2, 4, 7, 8 and *P. indica*) compared with the non-inoculated plants ($P < 0.05$). These results seem counter-intuitive as in the community analysis, *A. phaeospermum* was positively correlated with yield, especially under drought. The most likely explanation for this is that we did not use the *A. phaeospermum* strain that caused the effect in the field but rather used strains that were publicly available. Furthermore, the VPA

gave a correlation of this specie with yield, but this analysis cannot discriminate if the correlation is positive or negative, therefore the possible negative yield effect by the original strain from the field can be discussed. To further unravel this, it will be necessary to isolate the corresponding strains from the field and/or plant material analysed. Other explanations for this discrepancy may be that drought induced resistance may be the result of a synergistic/antagonistic effect between several fungal species (Larimer *et al.*, 2010; Aguilar-Trigueros & Rillig, 2016) such that we do not see an effect of a single species. Likewise, a perturbation of the fungal community induced by the inoculation could have blurred any positive effects.

A higher root:shoot ratio, and a longer root length are often characteristics for rice cultivars that are more drought tolerant, as it is a good indicator for a higher water uptake capacity (Comas *et al.*, 2013; Paez-Garcia, 2015). The used rice cultivar for the pot experiment, IR36, is a natural gibberellin (GA) deficient mutant, hence it presents a short primary root length. On the other hand, *A. phaeospermum* is reported to produce GAs (Khan *et al.*, 2008). We did not record the root length in the pot experiment, so it could be that some of the fungal strains may have had an impact on root length rather than on root biomass. Furthermore, the effect of drought on the root to shoot ratio depends on the plant growth stage, and is most evident in older plants (Silva *et al.*, 2012). Therefore, we may have missed the effect that the fungi may have had on root architectural changes in the relatively young plants that were used in the present study. These possibilities should be taken into account for future studies with the same research questions.

Conclusions

Our study is the first one to show that the root associated fungal community changes under drought conditions in rice, towards a higher species diversity. It also shows the existence of specific OTUs (belonging to the Pezizomycotina) of which the presence correlates with yield and of which the abundance increases under drought. Finally, we show that in rice under drought, the host genotype has an effect on the fungal community composition.

Roots can be a potential pool to search for beneficial-plant growth promoting fungi (Fonseca-García *et al.*, 2016; Angel *et al.*, 2016). We could potentially use ‘functional OTU clusters’, specifically tailored for a crop plant species, that we know may have a positive impact on plant performance, and apply them in the field to boost plant productivity under periods of stress. On the other hand, only a maximum of 1.0 % of soil microorganisms can be cultured, so

studying their roles in biological and ecological soil processes is a challenge (Rehman *et al.*, 2016), let alone their possible application in agriculture. Nonetheless, metagenomics can gather very valuable information that could help us to exploit microbial communities and further investigate how microbial 'clusters' are working together to improve plant fitness under stressful environments.

Acknowledgments

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Supplementary material

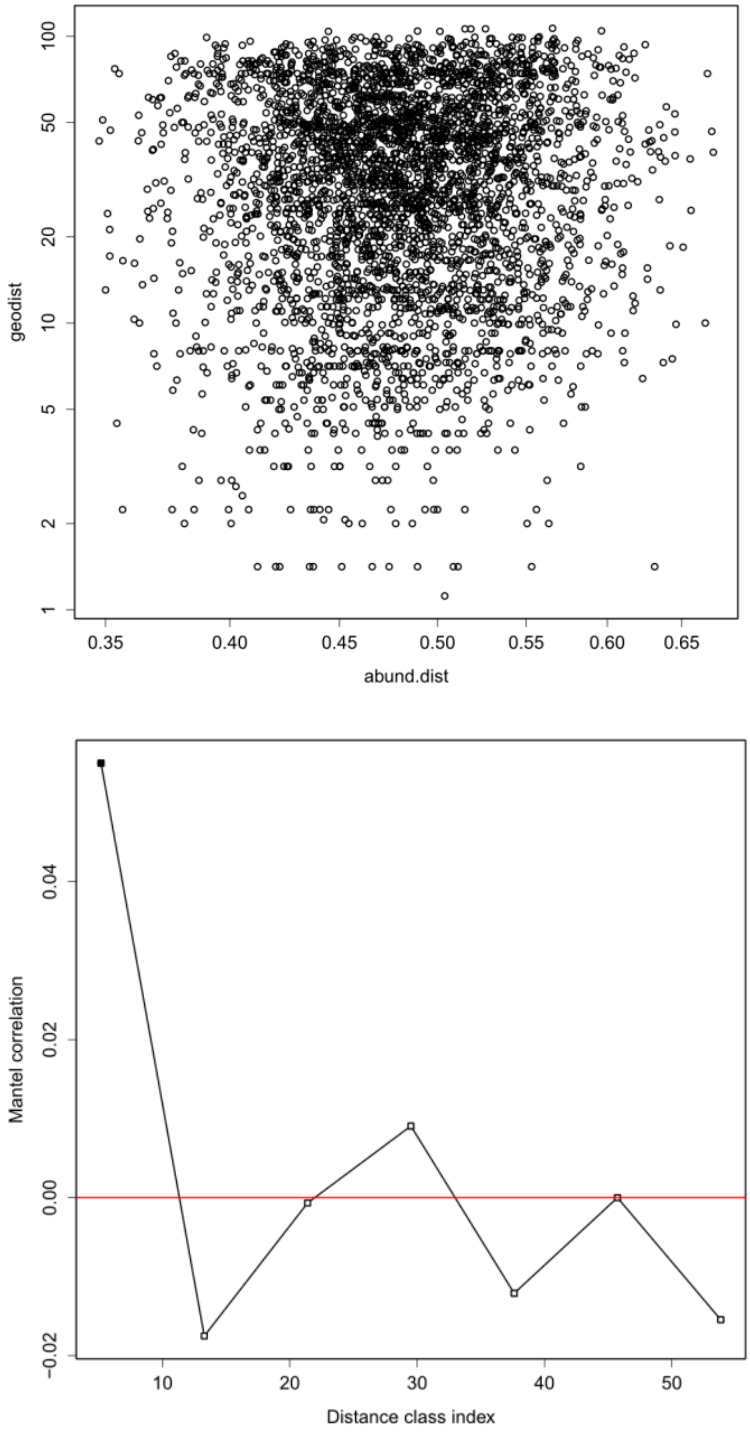


Figure S1. Mantel test to study the field position effect on the fungal community results. Euclidean distances between samples (top) and correlogram (bottom) for all samples. There is no correlation between field position and fungal community, hence no field effect was found.

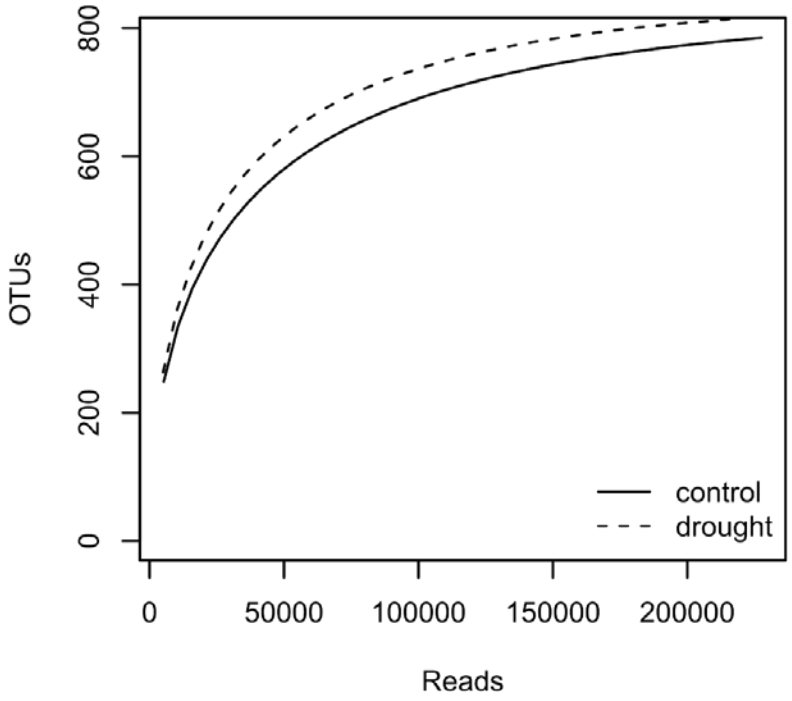


Figure S2. Mean rarefaction curves of the number of fungal OTUs found for the two treatments, control and drought.

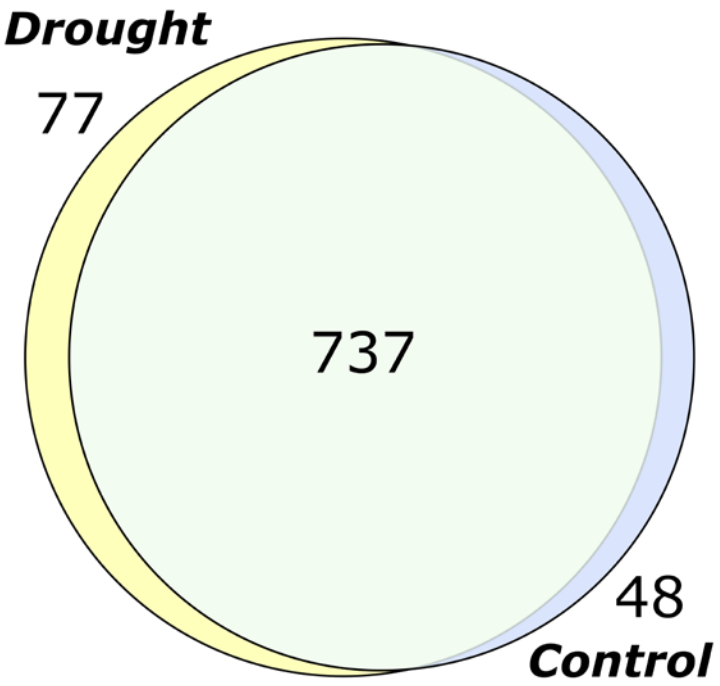


Figure S3. Venn diagram showing the number of OTUs found in control treatment only, in drought treatment only and in both (i.e. γ-diversity). The 40 rarest OTUs were removed from this analysis.

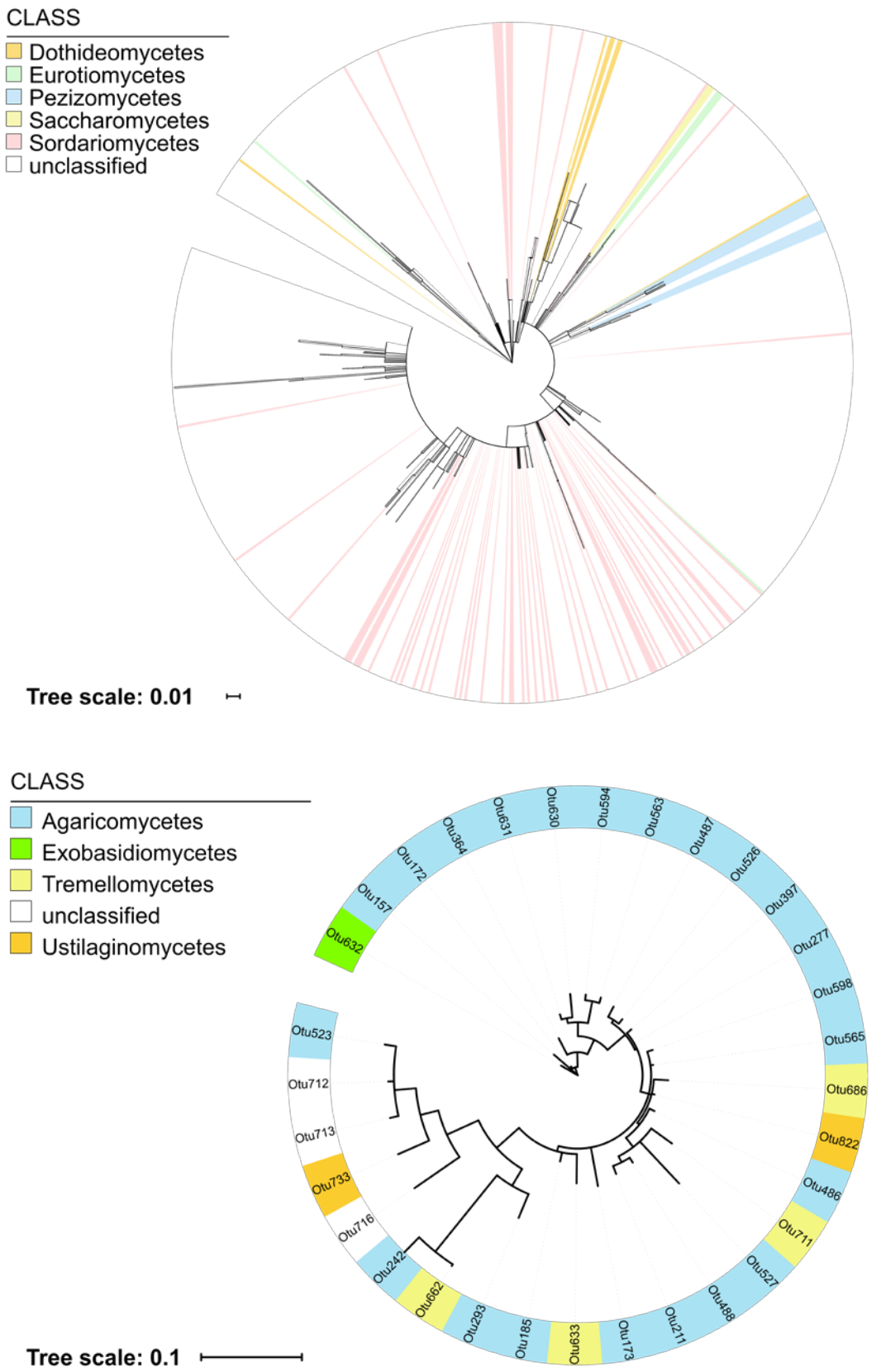
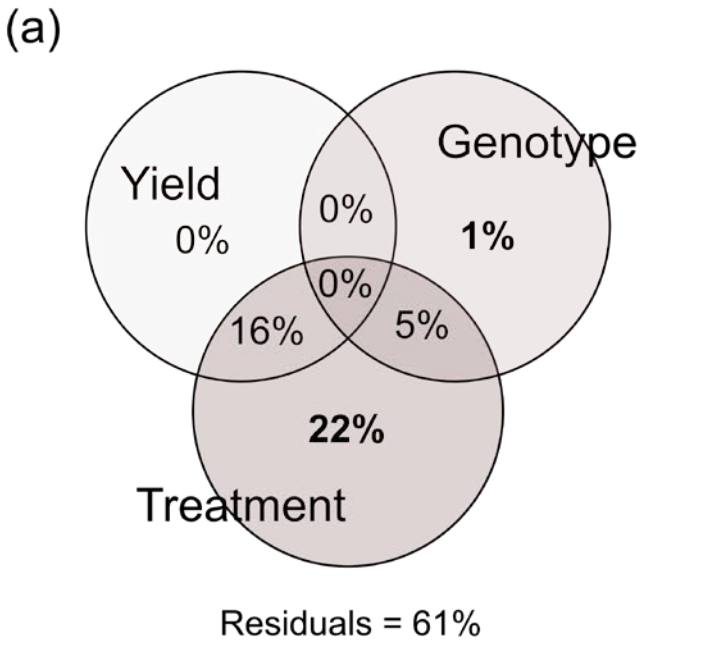


Figure S4. Phylogenetic trees for the main phyla: Ascomycota (top) and Basidiomycota (bottom). For each tree the different classes are represented.



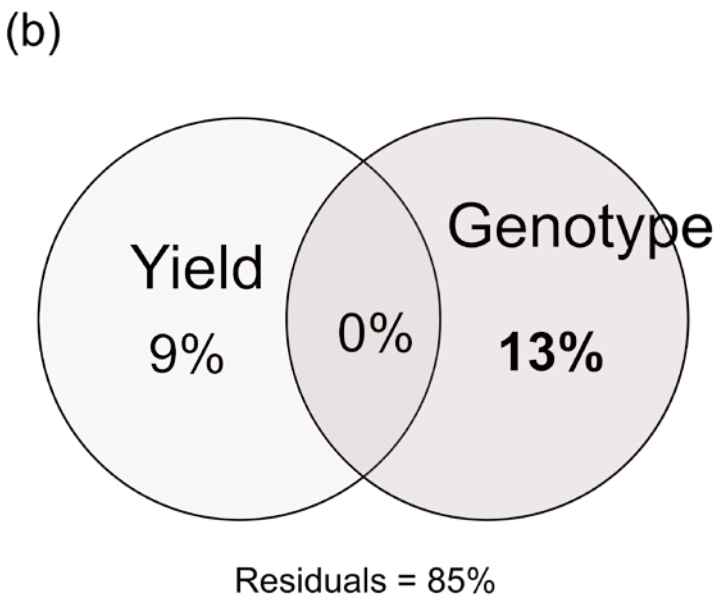


Figure S5. Variation partitioning analysis (VPA) illustrated using Venn diagrams. Each partition represents the variation due to one environmental factor affecting the fungal community abundance. In bold are the adjusted R^2 values for independent factors that contribute significantly. (a) Treatment explains 22% of the variation in community structure ($P < 0.01$). Combined, yield and treatment can explain 16% of the variation in community structure ($P < 0.01$). (b) VPA with the 'robustness' data for yield and the OTUs abundance under drought shows that 13% of the community variation is due to a genotype effect ($P < 0.01$). Data with yield 'robustness' and OTU abundance under control shows a significant 5 % of explanation ($P < 0.05$) (not shown).

Table S1. Summary of the statistics for the linear model fitted for the interaction between treatment 'fungus' (*A. phaeospermum* inoculated *vs.* non-inoculated) and 'treatment' (control *vs.* drought) for a number of different plant traits.

Shoot FW	DF	Sum sq	Mean sq	*F*-value	*P*-value
Fungus	9	3.3087	0.3676	7.6568	**<.0001**
Treatment	1	23.4098	23.4098	487.5605	**<.0001**
Fungus : treatment	9	2.1427	0.2381	4.9585	**<.0001**
Residuals	73	3.5050	0.0480		
Shoot DW	DF	Sum sq	Mean sq	*F*-value	*P*-value
Fungus	9	0.1535	0.0170	4.5058	**<.0001**
Treatment	1	0.8526	0.8526	225.1402	**<.0001**
Fungus : treatment	9	0.0924	0.0102	2.7121	**0.008**
Residuals	73	0.2764	0.0037		
Root FW	DF	Sum sq	Mean sq	*F*-value	*P*-value
Fungus	9	0.5866	0.0651	6.3817	**<.0001**
Treatment	1	1.1844	1.1844	115.972	**<.0001**
Fungus : treatment	9	0.3509	0.0389	3.8177	**0.0005**
Residuals	73	0.7455	0.0102		
Root DW	DF	Sum sq	Mean sq	*F*-value	*P*-value
Fungus	9	0.0654	0.0072	3.7445	**0.0006**
Treatment	1	0.0738	0.0738	37.9897	**<.0001**
Fungus : treatment	9	0.023	0.0025	1.3264	0.2384
Residuals	73	0.1418	0.0019		
Shoot Water	DF	Sum sq	Mean sq	*F*-value	*P*-value
Fungus	9	2.0961	0.2329	8.2902	**<.0001**
Treatment	1	15.3271	15.3271	545.5900	**<.0001**
Fungus : treatment	9	1.3612	0.1512	5.3836	**<.0001**
Residuals	73	2.0508	0.0281		
Root : Shoot FW	DF	Sum sq	Mean sq	*F*-value	*P*-value
Fungus	9	2.4403	0.2711	0.9951	0.4518
Treatment	1	21.5029	21.5029	78.9168	**<.0001**
Fungus : treatment	9	2.9660	0.3296	1.2095	0.3025
Residuals	73	19.8907	0.2725		
Root : Shoot DW	DF	Sum sq	Mean sq	*F*-value	*P*-value
Fungus	9	11.4380	1.2710	0.7615	0.6518
Treatment	1	55.9060	55.9060	33.5010	**<.0001**
Fungus : treatment	9	11.9270	1.3250	0.7941	0.6226
Residuals	73	121.8210	1.6690		

Significant *P*-values are indicated in bold. FW: fresh weight; DW: dry weight; DF: degrees of freedom; Sum sq: sum of squares; Mean sq: mean of squares.

Table S2. Summary of the ANOVA statistics in the fitted linear model for the different *A. phaeospermum* strains effect on fresh root:shoot ratio.

Control	Estimate	Std. error	*t*-value	*P* (> |t|)
(Intercept)	0.3392	0.2334	1.4530	0.1505
Strain 1	0.0177	0.3301	0.0540	0.9573
Strain 2	0.0585	0.3301	0.1770	0.8598
Strain 3	0.0618	0.3301	0.1870	0.8518
Strain 4	0.0453	0.3301	0.1370	0.8912
Strain 5	0.0739	0.3301	0.2240	0.8233
Strain 6	0.0083	0.4367	0.0190	0.9848
Strain 7	0.0570	0.3301	0.1730	0.8632
Strain 8	0.1924	0.4367	0.4410	0.6608
P. indica	0.2588	0.3301	0.7840	0.4356
Drought	Estimate	Std. error	*t*-value	*P* (> |t|)
(Intercept)	1.6698	0.3301	5.0580	**<.0001**
Strain 1	–0.3612	0.4668	–0.7740	0.4415
Strain 2	–1.0452	0.4668	–2.2390	**0.0282**
Strain 3	–0.7106	0.4668	–1.5220	0.1323
Strain 4	–1.0125	0.4668	–2.1690	**0.0334**
Strain 5	–0.3775	0.4668	–0.8090	0.4214
Strain 6	–0.5702	0.5474	–1.0420	0.3011
Strain 7	–0.9423	0.4668	–2.0180	**0.0472**
Strain 8	–1.2077	0.5597	–2.1580	**0.0343**
P. indica	–0.9342	0.4668	–2.0010	**0.0491**

Data under control and drought treatments are shown. Significant *P*-values are indicated in bold text. Std. error: standard error.

Table S3. List of rice cultivars used in this study.

IRGC Nº	Cultivar name	Cultivar Nº
IRGC121705	APO::C1	Cultivar1
IRGC117659	BINULAWAN	Cultivar2
IRGC117623	BR24	Cultivar3
IRGC117684	CHIEM CHANH	Cultivar4
IRGC117691	CO 18	Cultivar5
IRGC117757	IR 36	Cultivar6
IRGC117268	IR 64-21	Cultivar7
IRGC122112	IR 74371-54-1-1::C1	Cultivar8
IRGC120987	IR 77298-14-1-2::IRGC 117374-1	Cultivar9
IRGC117758	IR 8	Cultivar10
IRGC117829	ORYZICA LLANOS 5	Cultivar11
IRGC117841	PAPPAKU	Cultivar12
IRGC117880	SERATOES HARI	Cultivar13
IRGC117915	TKM 6	Cultivar14
IRGC122272	UPLRI-7	Cultivar15

Table S4. List of *Arthrinium phaeospermum* strains used in the pot experiment.

Strain Nº	CBS Nº	Taxon name	Country	Origin
1	CBS 142.55	*Arthrinium phaeospermum*	Japan	soil
2	CBS 463.83	*Arthrinium phaeospermum*[1]	Netherlands	*Phragmites australis*, dead culms
3	CBS 114314	*Arthrinium phaeospermum*	Iran	*Hordeum vulgare*
4	CBS 114315	*Arthrinium phaeospermum*	Iran	*Hordeum vulgare*
5	CBS 114317	*Arthrinium phaeospermum*	Iran	*Hordeum vulgare*
6	CBS 114318	*Arthrinium phaeospermum*	Iran	*Hordeum vulgare*
7	CBS 115473	*Arthrinium phaeospermum*	Hong Kong	wood
8	CBS 134073	*Arthrinium phaeospermum*	USA	*Miscanthus giganteus*
P.i.	CBS 125645	*Piriformospora indica*[2]	India	*Prosopis juliflora* and *Zizyphus nummularia*

[1] Formerly known as *Arthrinium saccharicola*; [2] *P. indica* was included as positive control.

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Chapter 5

Genome Wide Association Mapping of the root fungal microbiome in rice

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Summary

Rice is the second most produced crop worldwide but is also the crop that is most affected by drought. Micro-organisms have been shown to be able to alleviate the effects of drought but how these microbes are recruited by their host is unclear. The aim of the present study was to unravel the genetic factors involved in the selection of fungal micro-organisms by rice, and whether these play a role in the rice adaption process to drought. To accomplish this, the composition of the root associated fungal microbiome in a set of 296 rice cultivars (*Oryza sativa* L. subsp. *indica*), cultivated in the field under water sufficient (control) and - limiting conditions (drought), was characterized. The resulting data were used in a Genome Wide Association Mapping study to screen for associations between rice genomic loci and root associated fungi. The results show that drought induces a change in the composition of the root-associated fungal community. Interestingly, Hypocreales appeared not only to be the most dominant fungal taxon during drought, but were also positively associated with rice productivity under drought. On the other hand, Sordariales were dominant under control conditions. 184 SNPs were found to be associated with 44 root associated fungi (with –log (*P*) >6). Among the *a priori* candidate genes for these plant-microbe interactions are genes that are known to be involved in pathogen defense mechanisms, abiotic stress responses and cell-wall remodeling processes.

Introduction

Rice (*Oryza sativa* L.) is the most important staple food crop worldwide, with a production of around 700 million tons per year (FAOSTAT database: http://www.fao.org/faostat/en/#home). It is a highly water-demanding crop that is mainly grown in paddy fields under water-logged conditions. This environment assures high rice productivity while preventing the growth of weeds, but also results in a low water use efficiency (0.60 kg / m^3) if compared with other major cereal crops, such as maize (2.19 kg / m^3) (Liu et al., 2009). During the last decade climate change has increased the intensity and duration of drought periods (Trenberth et al., 2014), thus yields from high water-demanding crops like rice are in jeopardy. As a response to drought conditions, plants develop different physiological and developmental responses to deal with the stress, e.g. closing stomata to avoid excess evaporation, developing a deeper root system, accumulation of stress-related proteins (Cattivelli, 2008) or establishing mutualistic symbioses with micro-organisms, collectively called the microbiome (Vandenkoornhuyse et al., 2015). Plant microbiomes occupy all plant tissues, from root to shoot, and they are composed of a large

diversity of bacteria and fungi (Hardoim et al., 2015). Fungal root endophytes, such as arburscular mycorrhizal fungi (AM fungi) and Class II and IV fungi (Rodriguez et al., 2009) are important components of the plant microbiome because of their positive effect on plant tolerance to biotic (Chadha et al., 2015; Maciá-Vicente et al., 2008; Mejía et al., 2008) and abiotic stresses such as drought (Azad and Kaminskyj, 2015; Redman et al., 2011; Worchel et al., 2012). They mainly belong to the phyla Glomeromycota (AM fungi) and Ascomycota, and to a lesser extent to the Basidiomycota and the Zygomycota (Rodriguez et al., 2009). AM fungi colonize the roots of about 80 % of land plant species, including embryophytes, and have a very close relation with their hosts in which they entirely depend on plant-provided photosynthates to grow (Smith and Read, 2008), while providing the host with nutrients and water in return (Parniske, 2008). Other non-mycorrhizal root fungal endophytes are also ubiquitous, colonizing all land plants. In rice, drought tolerance has been demonstrated to be promoted by two root fungal endophytes Class II, *Fusarium* sp. (Hypocreales) and *Curvularia* sp. (Pleosporales) (Redman et al., 2011), as inoculated plants showed a higher biomass and yield than non-inoculated plants under drought or salinity stress. Similarly, AM fungi have also shown to improve photosynthetic efficiency and to boost the accumulation of antioxidants in rice plants under drought (Ruiz-Sánchez et al., 2010). In contrast to AM fungi, the majority of non-mycorrhizal fungal endophytes do not have a tight relation with their host and their effect in plants is environment-dependent. How (and if) plants recruit these fungi, and the molecular mechanisms behind these relationships, are not yet understood, especially in the case of non-mycorrhizal fungal endophytes.

It has been demonstrated that the host plant genotype influences the structure and composition of the microbial communities in their leaves (Horton et al., 2014; Wagner et al., 2016) and roots (Bulgarelli et al., 2015) but little is known of the mechanisms that drive this. Plant-microbe associations are complex systems, and therefore holistic study approaches are necessary to describe them, taking into account both environmental and genetic variables that drive these relationships (Salvioli and Bonfante, 2013).

Drought adaptation and yield are complex plant traits that are not easy to study using reverse genetics techniques. However, forward genetics techniques such as whole genome studies have been taking over during the last decade (Han and Huang, 2013; Zargar et al., 2015). In order to find genetic loci that are involved in the regulation of plant-microbe interactions that are relevant during environmental stresses like drought, exploring natural variation from different plant cultivars whole genome studies appear as a promising approach. Genome Wide

Association Analysis (GWA) studies use single nucleotide polymorphism (SNP) maps that allow fine mapping of traits (Brachi et al., 2011). Thanks to the rapid evolution of new generation high throughput sequencing techniques, dense marker maps have become available for several model crops, including rice (McCouch et al., 2016; Rebolledo et al., 2016). Several GWA studies have been performed in rice for grain size, starch quality, aluminum tolerance, panicle morphology and root architecture among others (Famoso et al., 2011; Huang et al., 2010; Kadam et al., 2017; Rebolledo et al., 2016). The use of GWA studies to analyze plant traits that are related to the interaction with microorganisms is new and has just been started to be explored. A recent study with the model plant *Arabidopsis thaliana* found loci for the phyllosphere bacterial and fungal endophyte abundance (Horton et al., 2014). These loci were mainly involved in cell wall related processes, such as xyloglucan biosynthesis and trichome development. So far, however, GWA mapping of the microbial community has not been performed in crops, let alone of the root associated microbiota.

In the present study we aimed to study the presence of genetic loci in rice that are associated with the recruitment of root associated fungal micro-organisms in rice. Moreover, we wanted to study the effect that drought has on both this association as well as on the composition of the root associated microbiome.

Materials and Methods

Plant material and field experiment

As plant material we used 296 cultivars of *Oryza sativa* (L.) subsp. *indica*. This population represents the diversity within the tropical *indica* sub-species, including both traditional and improved landraces (Rebolledo et al., 2016). The experiment was conducted at the International Rice Research Institute (IRRI) facilities during the dry season, from December 2012 to March 2013. The field was localized in the upland site (14°30'N, 121°15'E) and soil was a mix of clay (36 %), sand (22 %) and silt (41 %). Plants were sown in 2.5 x 0.8 m blocks, each one containing 48 plants from the same cultivar. Blocks were laid out with a serpentine design in order to avoid any field effect, with a total of three replicates per block/treatment, and the field was split into two parts, containing the control and drought treatments. To manage the differences in flowering time of the different cultivars, they were sown in different batches to synchronize flowering. Plants were grown in flooded conditions until 50% of the plants reached the flowering stage. Then watering was stopped in half of the field (drought treatment) while the

other half was continuously flooded as control. Drought conditions were maintained during 12 days, until the soil reached a water potential of -46 KPa. Then soil cores (10 x 70 cm) were collected from the inner part of the blocks, and stored in non-sterile plastic bags at 4ºC until further processing. All the 1,776 root samples were cleaned carefully with tap water and a sieve, all under non-sterile conditions, and stored at -80ºC until further analysis.

DNA extraction and sequencing

Roots from the same cultivar and treatment (biological replicates) were pooled together and they were ground to powder with a mortar and pestle using liquid nitrogen. DNA was extracted from 60–80 mg of plant material with the DNeasy 96 Plant Kit (Qiagen) following the manufacturers protocol and finally DNA was diluted ten times. Subsequently a fragment of the 18S SSU rRNA gene was amplified using general fungal primers (NS22: 5'-AATTAAGCAGACAAATCACT-3' and SSU0817: 5'-TTAGCATGGAATAATRRAATAGGA-3') (Borneman and Hartin, 2000), with the following conditions: 95 ºC for 4 min; 40 cycles of 95 ºC for 30 s, 54 ºC for 30 s, 72 ºC for 1 min; and 72 ºC for 7 min. Primers were modified to allow amplicon multiplexing for the sequence production process: a collection of 96 modified SSU0817 primers was generated each containing a specific tag consisting of 10 nucleotides. The PCR products were purified with AMPure XP beads (Beckman Coulter) using a genomic sample preparation platform (BRAVO, Agilent technologies). Amplicons concentration was measured with an ultrasensitive fluorescent stain (Quant-ITTMPicoGreen®dsDNA Assay kit, Invitrogen), and the size was checked with the Agilent High Sensitivity DNA kit (Agilent Technologies). The purified 560 bp amplicons were diluted to similar concentration and pooled using a multiplexing robot. Finally all the fragments (250 bp size) were sequenced twice, in forward (R1) and reverse (R2) directions using a Next Generation Sequencing Platform (Illumina Miseq, Roche), following the manufacturer's guidelines.

Sequences trimming and affiliation

The 5' and 3' primers (on R1 and R2 reads) and the unknown bases (n) were removed from the sequences using the Phyton package 'Cutadapt' (Martin, 2011). The R1 and R2 reads were analyzed separately following the guidelines of the FROGS pipeline (Escudie et al., 2015) and using the galaxy workbench (X.SIGENAE: http://www.sigenae.org/). FROGS pre-process was performed with a custom protocol (Kozich et al., 2013). The clustering step was performed with 'swarm' method (Mahé et al., 2014). Following the pipeline designer's advices, a de-noising step was performed with a maximum distance of aggregation of 1 followed by a second step

with a maximum distance of aggregation of 3. Chimera products were filtered with the FROGS 'remove chimera' tool. A filter was applied to keep OTUs with sequences in at least 5 samples in order to avoid the presence of artifact OTUs. Only R2 results were presented based on affiliation statistics that indicates a better quality of affiliation. OTUs affiliation was performed with 'Blast+' (Camacho et al., 2009) against Silva 128 18S. OTUs were then filtered based on the quality of the affiliations with a threshold of 95% blast coverage and 95% blast identity. After all these steps all multi-affiliation were verified at the Phylum scale and OTUs affiliated to a non-fungal taxon were removed.

Microbial ecology analyses

All the statistical analyses were performed using R (R core team, 2013). From the normalized contingency matrix, OTU richness, abundance, evenness and diversity index estimators were calculated using the package car (Kindt & Coe, 2005; Fox & Weisberg, 2011). Statistical differences in these measures were analyzed using two-way ANOVA. Fungal community differences between the different treatments were studied using non-metric multidimensional scaling (NMDS) analysis, after removing rare OTUs (OTUs with abundance < 10 sequences) applying the Kulczynski ordination method (Kulczynski, 1928). To test whether significant differences exist between fungal communities from control and drought treatments a permutational multivariate analysis of variance (PERMANOVA) was run with the *adonis* function using the Bray-Curtis distance matrix. The analysis was run with the vegan package. We also run a Spearman correlation analysis with the *rcorr* function in Hmisc package, between the independent OTUs and yield under control and drought treatments, and with the yield plasticity, described as the yield relative change in drought compared to control conditions: yield $_{DROUGHT}$ — yield $_{CONTROL}$ / yield $_{CONTROL}$ (Sandhu et al., 2016; Valladares et al., 2006). The OTUs positively correlated with plant yield with an $R > 0.10$ and $P < 0.05$ were selected. Phylogenetic trees were generated by Maximum Likelihood (ML) using RaxML v.8.00 (Stamatakis, 2014), with the GTRGAMMA model and 1000 bootstrap replicates, and plotted and edited using iTOL (http://itol.embl.de, Letunic and Bork, 2011). Heatmap graphs with the correlation values were plotted with corrplot R package (Wei and Simko, 2017).

Population genotyping

We worked with a subset of 296 *indica* cultivar, being the majority of them included in a bigger panel that was genotyped at Cornell University, USA (Kadam et al., 2017; Rebolledo et al., 2016). Only 274 cultivars out of the 296 ones and 90,000 SNPs were used in our study were included in the genotyped population.

Genome Wide Association Analysis

Marker-based estimates of narrow-sense heritability were computed based on all cultivars studied in the present study (Kruijer et al., 2015). GWAs analysis was performed for all OTUs with a frequency above 5%, leaving 82,858 SNPs. Univariate GWAS was performed for each OTU in each of the two conditions separately, following the approach of (Kang et al., 2010), using a relatedness matrix with elements,

$$K_{i,j} = \sum_{l=1}^{N} \frac{(x_{il} - f_l)(x_{jl} - f_l)}{2f_l(1 - f_l)},$$

being N the total number of SNPs, x_{il} the SNP score of accession *i* for SNP *l* and f_l the frequency of that SNP. Two separate univariate analyses were performed, one including OTUs abundance and another one including OTUs occurrence data (OTU presence/absence).

For each OTU, bivariate GWAS was performed on the drought and control conditions simultaneously, using the multi-trait mixed model (MTMM) proposed by Korte et al., 2012 . This is a straightforward extension of the univariate GWAS, and allows to distinguish associations that are common to the two treatments and those that are specific, while accounting for correlation of the genetic background. As the drought/control measurements were clearly made on different plants, residuals were assumed uncorrelated across treatments. To avoid convergence problems we only analyzed OTUs with abundance of at least 5% in both conditions, and used the EM algorithms proposed by (Zhou and Stephens, 2014).

Linkage Disequilibrium analysis and candidate genes selection

For all analyzed traits, candidate genes were selected using LD windows around SNPs with $-\log_{10}(p)$ value larger than 6. As the LD decay rate in rice is ~500 Kb (Mather et al., 2007), we defined windows of 500 Kb upstream and 500 Kb downstream the SNP of interest. We used the function *findLDblocks* with *getLD* (when the number of SNPs included in the block is < 500) and *getLDlarge* (when the number of SNPs included in the block is ≥ 500) from the 'trio' R package (Schwender et al., 2014).

Genes located in the LD blocks were obtained from the MSU Rice Genome Annotation Project database (http://rice.plantbiology.msu.edu), using the version 7.0 (updated on October 2011). A Gene Ontology (GO) enrichment analysis was performed on the set of *a priori* candidate genes using the web based tool AgriGO v2.0 (http://bioinfo.cau.edu.cn/agriGO/index.php) (Du et al., 2010). For some gene candidates *in*

silico gene expression analyses were performed using the RiceXPro (http://ricexpro.dna.affrc.go.jp/) DNA and protein sequence alignments were performed using the EMBL-EBI web based tools MAFFT (http://www.ebi.ac.uk/Tools/msa/mafft/) and Needle (http://www.ebi.ac.uk/Tools/psa/emboss_needle/).

Results

Root associated fungal community description

The raw sequencing data resulted in a total of 15,271,794 sequences belonging to 2,687 different OTUs. The rarefaction curve showed that the sequencing depth was adequate to describe the root associated fungal microbiome in the present study (Fig. S1). 2,347 OTUs were found in both treatments, while 61 (drought) respectively 278 (control) were treatment-specific. The total number of sequences (abundance) and number of OTUs (richness) were lower under drought, while the diversity (γ-diversity) and species balance (J-evenness) were increased (Fig. 1a).

The total fungal community observed in the entire experiment belongs to 12 different phyla, with the Ascomycota as the dominant group, followed by the Basidiomycota and Blastocladiomycota (Fig. S2a). At lower taxonomic levels, the main classes found are Sordariomycetes and a multi-affiliation group (Fig. S2b). Under drought, the diversity (Shannon index) was higher in all phyla (Table S1).

Based on the rank abundance curve (Fig. S3), rare OTUs (with an abundance of < 10 sequences in all samples) were filtered out, resulting in a total of 724 OTUs for further analyses.

Drought effect on the fungal microbiome and correlation with yield

The drought treatment resulted in a significantly different fungal microbiome compared with the control plants (Fig. 1b) (PERMANOVA analysis control vs. drought; $R^2 = 0.70$; $P = 0.001$). As the treatment had a strong effect on the fungal microbiome composition, in later analyses both groups of samples were analyzed separately.

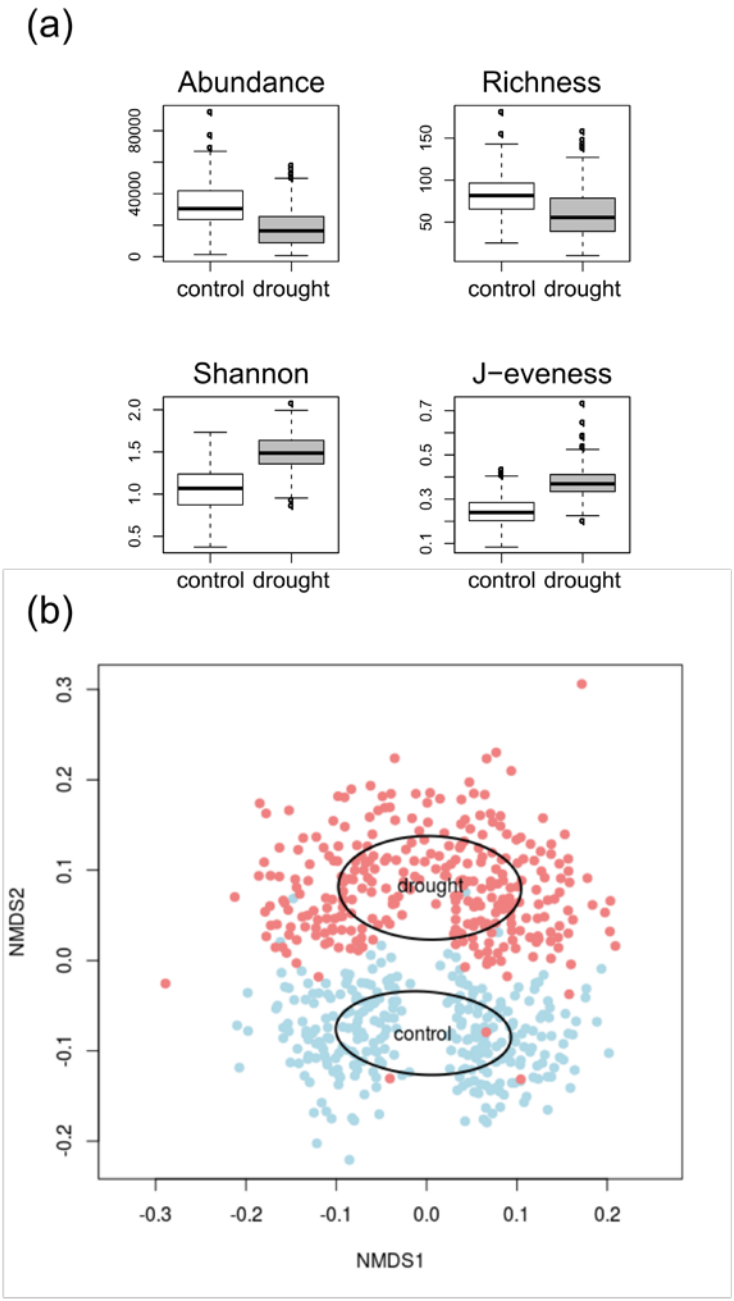


Figure 1. a) Root associated fungal community ecological parameters. Under drought conditions, fungal individuals number (abundance) and OTU species number (richness) are lower when compared to the control, while fungal diversity (Shannon) and OTU species equilibrium (J-eveness) are significantly higher (ANOVA test, P <0.001). b) A Non-Metric Multidimensional scaling analysis (NMDS) shows the drought effect on the root associated fungal community. Drought treatment samples (red dots) present a significantly different qualitative and quantitative fungal microbiome when compared with control samples (blue dots) (PERMANOVA test, P <0.001).

A correlation analysis between the OTUs' relative abundance and yield and yield plasticity (defined as the yield change under drought compared with control conditions) was performed. The correlation analysis resulted in 51 and 46 OTUs that were positively correlated with yield (PYC OTUs) under control and drought conditions, respectively ($P < 0.05$; $R > 0.10$). The number of negatively correlated OTUs (NYC OTUs) was lower; 21 and 18 in control and drought respectively ($P < 0.05$; $R < -0.10$). With regard to the correlations with yield plasticity, the trend is the same: more positively correlated OTUs (25 and 15, in control and drought, respectively) than negatively correlated ones (15 and 17, in control and drought, respectively). Some OTUs were correlated with both yield and yield plasticity, either positively (8 OTUs) or negatively (6 OTUs). Among the yield correlated OTUs, the Sordariomycetes were the most dominant class being more abundant among the positively correlated OTUs (42 % and 52 % from the total OTUs number in control and drought, respectively) than among the negatively correlated ones (38 % and 34 % OTUs in control and drought, respectively). The second most abundant classes are the Chytridiomycetes and Dothideomycetes (being the majority of them from the Order Pleosporales) for negatively and positively correlated OTUs respectively. At the lower taxonomic level the main order, Sordariales, is more abundant among the NYC OTUs (20 %) than the in PYC OTUs (8 %) under control conditions. On the other hand, under drought the Sordariales are not present anymore among the NYC OTUs, and Hypocreales become the majority among the PYC OTUs (11 %).

Univariate Genome Wide Association (GWA) Analysis

With the available SNP information for the population under investigation, we performed a univariate GWA analysis to identify loci that are associated with the occurrence and abundance of each individual OTU as well as the Shannon diversity index. The marker-based narrow-sense heritability analysis gave quite low values for the abundance of the majority of OTUs, with 7% of the OTUs showing heritability (h^2) values between 0.5-0.2, 12% between 0.2-0.1 and the rest below 0.1. For the occurrence data the results are similar, however a bit lower. The heritability for the Shannon diversity data were higher under control (h^2=0.45) than under drought (h^2=0.06).

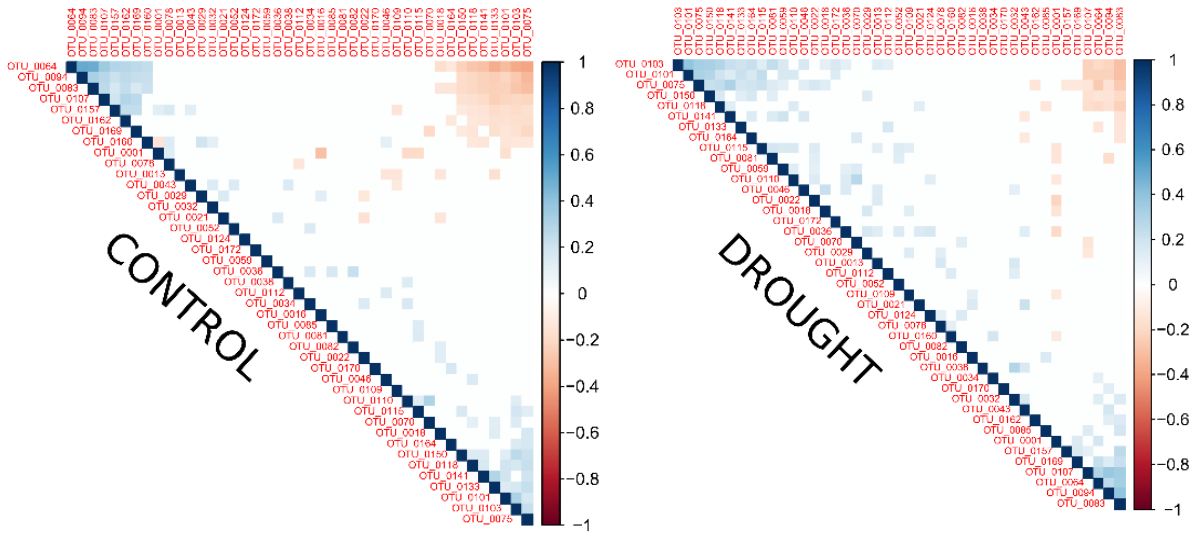
For 44 OTUs, SNPs were identified that were significantly ($- \log_{10}(P) > 6$) associated with their abundance and/or occurrence under control and/or drought conditions (Table S2). These OTUs were associated with a total of 184 SNPs (Table S3). Interestingly, the majority of SNPs were found to be localized on chromosomes 10, 11 and 12 (Fig. S4a). Only a small group of OTUs, 44 out of the 240 studied OTUs, was linked to the majority of SNPs (Fig. S4b).

Furthermore, a correlation analysis between the relative abundances of these 44 OTUs separated the OTUs into two clusters for which the included OTUs abundances were positively correlated with each other. This was observed for both treatments (i.e. blue squares on Fig. 2a). In contrast, OTUs coming from different clusters were found to be negatively correlated with each other (i.e. orange squares in Fig. 2a). OTUs from one cluster might be taxonomically related or have similar biological function in the plant. Indeed, a phylogenetic analysis with the large subunit ribosomal DNA (LSU rDNA) sequences from these OTUs showed that correlated OTUs also cluster together based on their sequence, meaning that they are more related to each other than OTUs that are not correlated (Fig. 2b). Interestingly, among these 44 OTUs, the ones that correlated with yield, turned out to be more or less distributed in two different clusters. One cluster contained PYC OTUs while the other cluster included NYC OTUs (Fig. 2b).

The majority of the 44 OTUs for which the abundance and/or occurrence was found to be associated with a SNP belong to the Ascomycota phylum (80 %) (Table S2). This is not surprising, as it is the dominant phylum in the rice root associated fungal microbiome in this study. The Sordariomycetes were the most represented class, representing 50 % of these 44 OTUs (Table S2). The majority of the 184 observed significant associations were detected in plants grown under control conditions (137 SNPs) (Table S3) and OTU abundance had more associated SNPs (109) than OTU occurrence. 21 SNPs were found in both the OTU abundance as well as the OTU occurrence GWA analyses. Interestingly, some OTUs were associated to several SNPs in different chromosomes (9 OTUs), suggesting that multiple loci control the recruitment of these OTUs. The rest of the OTUs presented either several SNPs in the same genomic LD block (the majority) or just one SNP.

To narrow down the search for *a priori* candidate genes in the present study, we only focused on the traits (OTUs) with associated SNPs that were correlated with plant yield resulting in 13 OTUs (Fig. 2b). 53 % of the correlated SNPs were found on chromosome 11 and were associated with the traits OTU_0001 and OTU_0107, which are both PYC OTUs, and OTU_0018, that is a NYC OTU (Fig. 3; Table S4).

(a)


CONTROL
DROUGHT
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0.6
0.4
0.2
0
−0.2
−0.4
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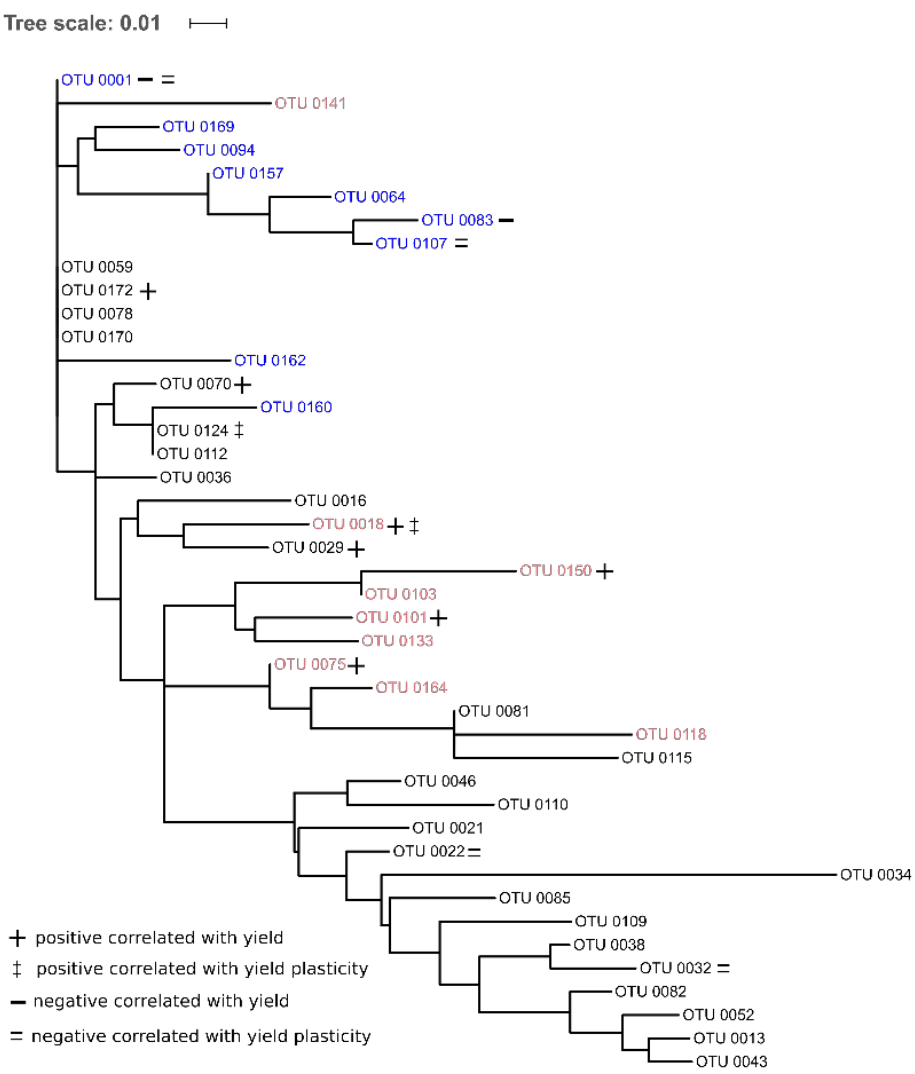
−0.8
−1

(b)


Tree scale: 0.01
OTU 0001 −
OTU 0141
OTU 0169
OTU 0094
OTU 0157
OTU 0064
OTU 0083 −
OTU 0107 =
OTU 0059
OTU 0172 +
OTU 0078
OTU 0170
OTU 0162
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OTU 0160
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OTU 0034
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OTU 0109
OTU 0038
OTU 0032 =
OTU 0082
OTU 0052
OTU 0013
OTU 0043
+ positive correlated with yield
‡ positive correlated with yield plasticity
− negative correlated with yield
= negative correlated with yield plasticity

Figure 2. OTUs associated with SNPs in the present study. (a) Spearman correlation heatmap between the OTUs' relative abundance under control (top) and drought (bottom) conditions. OTUs are ordered by the first principal component (FPC) and only significant correlations are represented ($P < 0.05$). There are two clear OTU clusters that include co-occurring OTUs, and these clusters are negatively correlated with each other. (b) Maximum Likelihood (ML) phylogenetic tree based on OTU large subunit (LSU) rDNA sequences. OTU code color is based on the groups observed in the control correlation heatmap in (a). Heatmap groups cluster in the tree as well. Furthermore, OTUs positively or negatively correlated with plant yield, marked in the tree as + or -, respectively, belong to the same clusters.

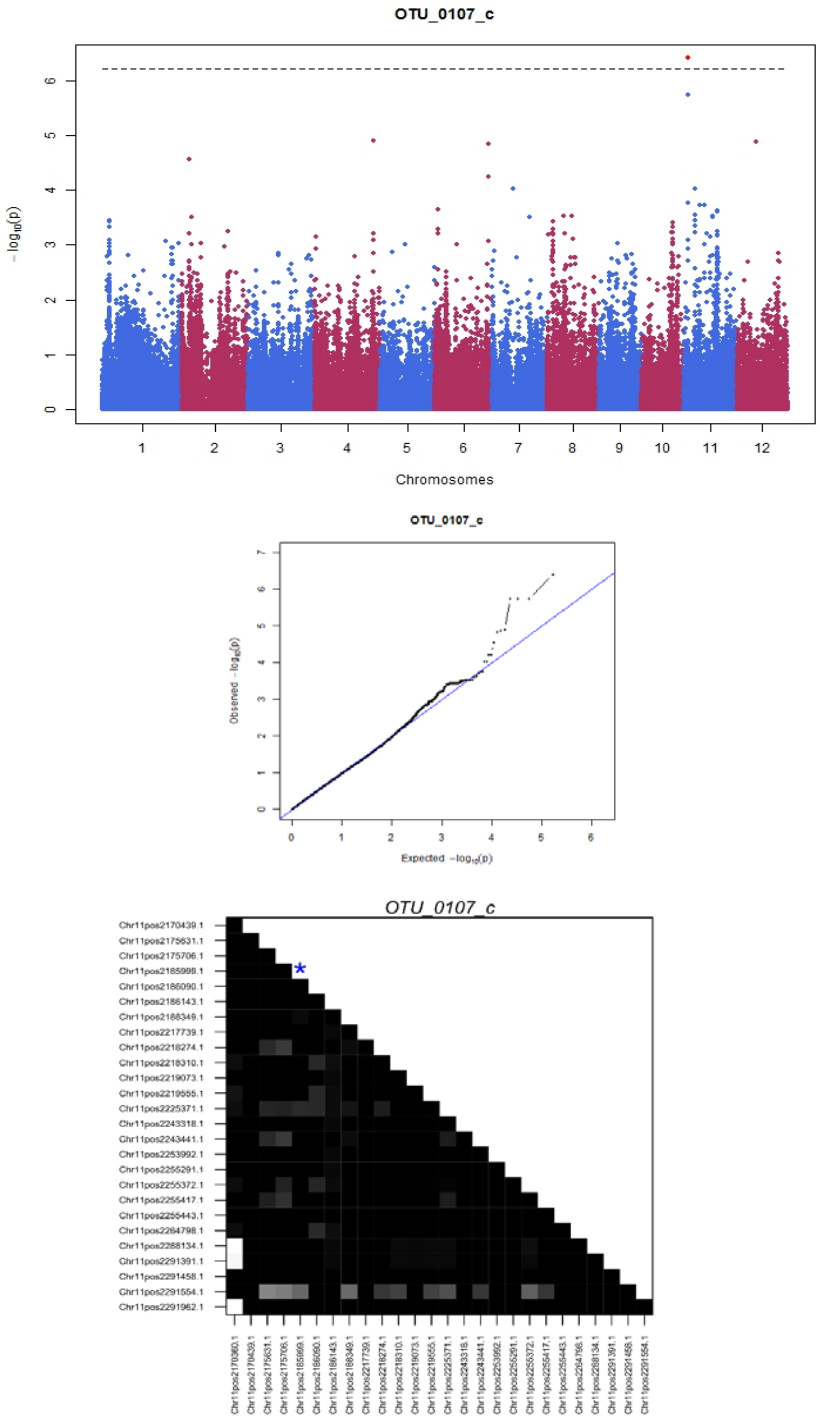
Gene candidates

Before looking into all *a priori* candidate genes in the LD blocks of the SNPs associated with yield correlated OTUs, we retrieved information from all 708 candidate genes for the abundance of the 44 OTUs. A list with their (predicted) functions was retrieved from the MSU Rice database (Table S5). A Gene Ontology (GO) enrichment analysis using the full list of *a priori* candidate genes showed that the candidate genes are enriched in terms involved in *responses to external stimulus*, and *abiotic* (e.g. cupin domain protein, MADS box family protein) and *biotic stress responses* (e.g. PDR ABC transporter, SRPK4). Other biological functions represented are *macromolecule metabolic process* and *secondary metabolic process* (e.g. cytochrome P450). When looking at the top 15 SNPs having the highest LOD score in this study, we found a group of candidate genes related to plant-microorganism communication, such as a gene encoding the SHR5 receptor like kinase, the LTPL1 lipid transfer protein and a tyrosine kinase-like IRAK protein (Table S5).

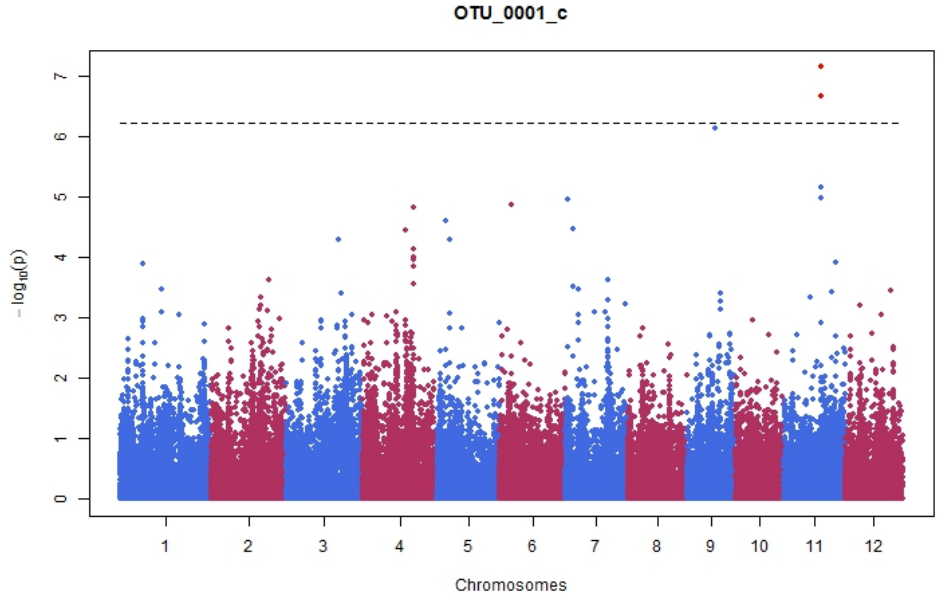
Considering their potential relevance to plant growth and yield, we subsequently focused on the genes included in LD blocks with SNPs associated to yield correlated OTUs (Table 1). Furthermore we selected three OTUs (one PYC and two NYC OTUs) whose associated SNPs are localized on the same chromosome (Chromosome 11). Localization of these SNPs and their LD blocks are shown in Figure 3 (Table S4). From all *a priori* candidate genes on chromosome 11 that are associated with NYC OTUs, the genes encoding the NBS-LRR disease resistance protein (linked to OTU_0001), the powdery mildew resistant protein 5 (linked to OTU_0107) and PHD family proteins such as PHF5 (linked to OTU_0001 and OTU_0107) are all involved in plant pathogen responses (McHale et al., 2006; Müller et al., 2015) which could be relevant for microbiome recruitment as discussed below (Table 1). Among the *a priori* candidate genes for PYC OTUs, we consider the genes encoding WRKY89, chalcone synthase and glycosyl

transferase group 1 protein as interesting, since they are mainly involved in plant resistance processes against biotic stresses and cell-wall remodeling (Dao et al., 2011; Wang et al., 2015). Finally, SNARE proteins, one found as a candidate for one of the PYC OTUs, can be considered as relevant because of their role in symbiotic processes and pathogen responses (Lipka et al., 2007). All these *a priori* candidate genes may be related with OTUs associated with SNPs under control conditions, while just one of them, PYC OTU_0150, was associated under drought conditions.

(a)



(b)


OTU_0001_c
$-\log_{10}(p)$
Chromosomes
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2
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4
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8
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11
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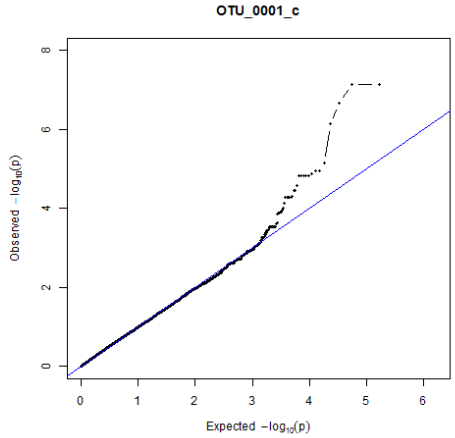
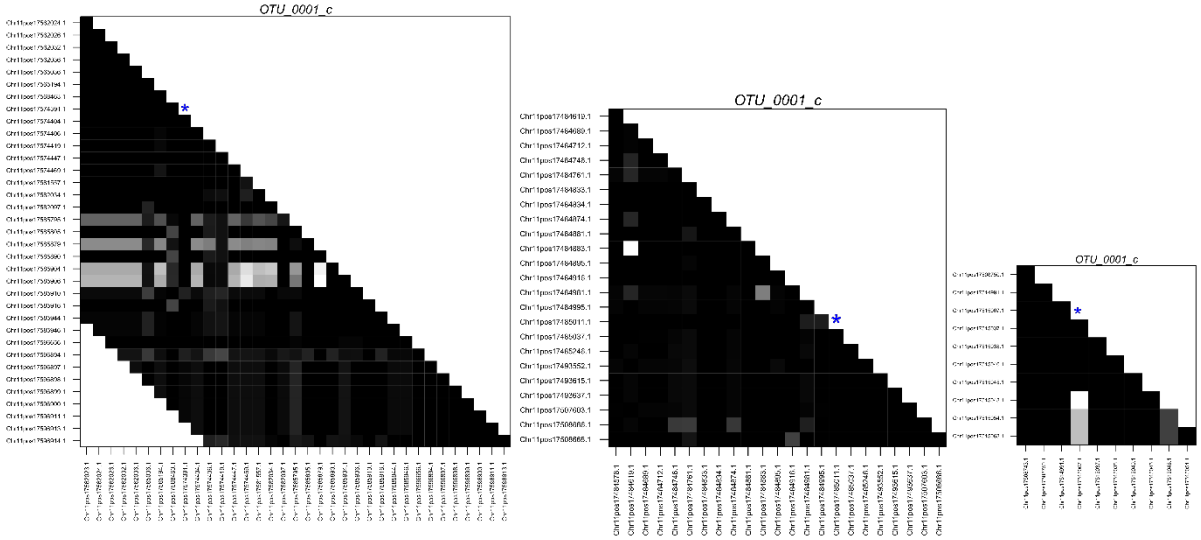
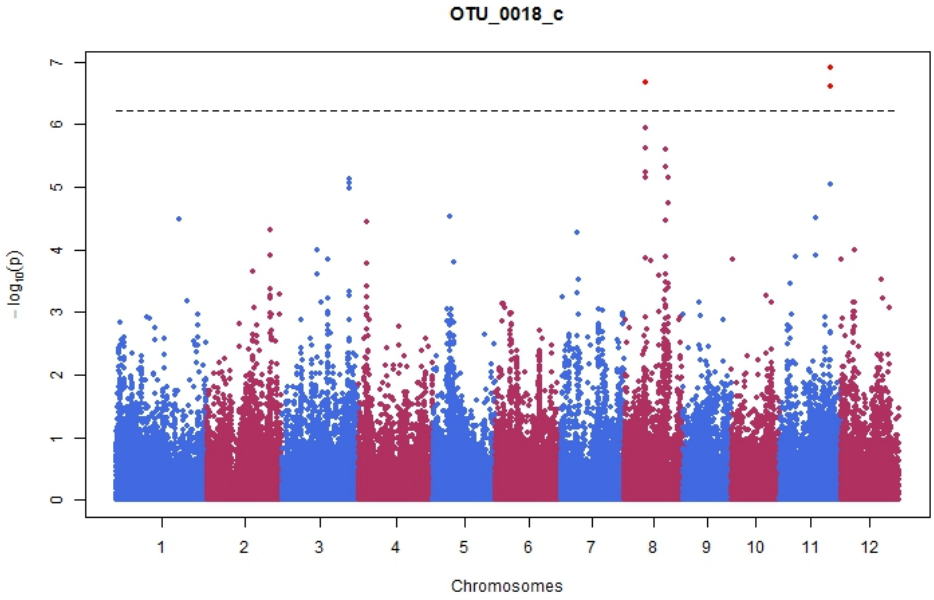

OTU_0001_c
Observed $-\log_{10}(p)$
Expected $-\log_{10}(p)$


OTU_0001_c
OTU_0001_c
OTU_0001_c

(c)


OTU_0018_c
−log10(p)
Chromosomes
1
2
3
4
5
6
7
8
9
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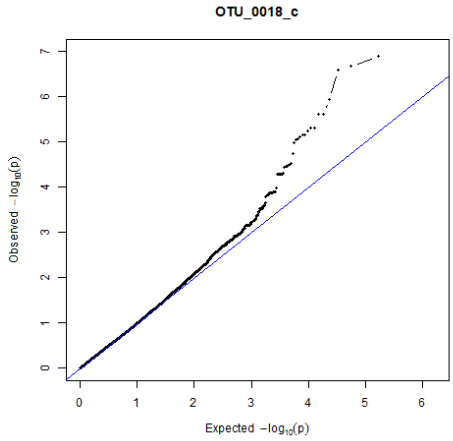
11
12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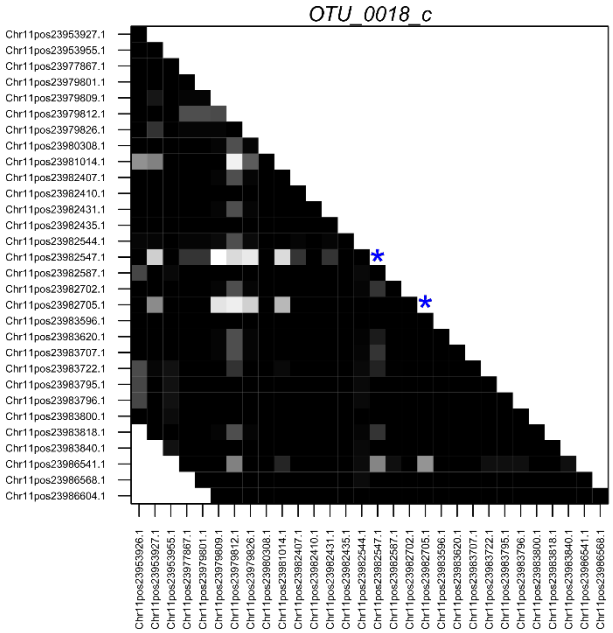
Expected −log10(p)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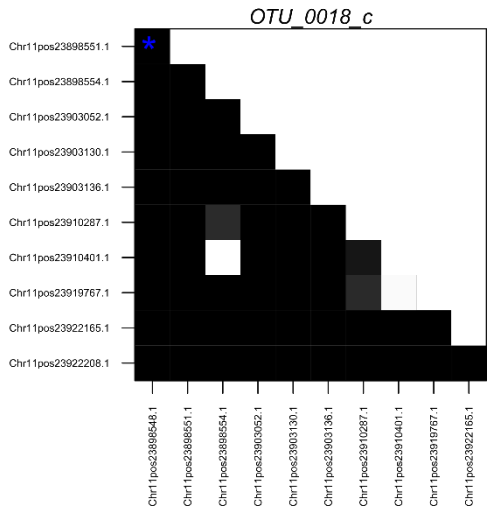
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Chr11pos23910401.1
Chr11pos23919767.1
Chr11pos23922165.1
Chr11pos23922208.1

Figure 3. Manhattan plot, Q-Q plot and LD blocks with SNPs for the OTUs 0107 (a), 0001 (b) and 0018 (c). These OTUs are yield correlated and represented in the different tree clusters in Figure 2. They are also included in the top 40 list with the highest LOD score for all SNPs found in the study. OTU_0001 and OTU_0107 are both negatively correlated with yield and SNPs are localized in chromosome 11. OTU_0018 is positively correlated with yield and is associated with SNPs in chromosomes 11 and 8. LD block plots represent the Dprime (D') values for each SNP pair, with black indicating the highest correlation (D'=1). The associated SNPs are marked with blue asterisks.

Table 1. Description and putative functions of the *a priori* candidate genes located in LD blocks from SNPs that are associated with OTUs correlating with yield and/or yield plasticity. LOD scores of the SNPs, and the name of the associated OTUs are shown. Data were retrieved from the MSU Rice database.

OTUs negatively correlated with yield			
Locus	Description	LOD	OTU
LOC_Os11g30290	PHF5-like_protein_domain_containing_protein_expressed	7.136776	OTU_0001
LOC_Os11g30260	transposon_protein_putative_CACTA_En_Spm_sub-class_expressed	7.136776	OTU_0001
LOC_Os11g30280	transposon_protein_putative_unclassified	7.136776	OTU_0001
LOC_Os11g30100	hypothetical_protein	6.66645	OTU_0001
LOC_Os11g30060	NBS-LRR_type_disease_resistance_protein_Hom-B_putative_expressed	6.66645	OTU_0001
LOC_Os11g30090	transposon_protein_putative_CACTA_En_Spm_sub-class_expressed	6.66645	OTU_0001
LOC_Os05g10730	ABC_transporter_ATP-binding_protein_putative_expressed	6.491966	OTU_0083
LOC_Os05g10780	aminotransferase_classes_I_and_II_domain_containing_protein_expressed	6.491966	OTU_0083
LOC_Os05g10740	pollen-specific_protein_SF21_putative_expressed	6.491966	OTU_0083
LOC_Os05g10710	retrotransposon_protein_putative_unclassified_expressed	6.491966	OTU_0083
LOC_Os05g10770	transcription_factor_jumonji_putative_expressed	6.491966	OTU_0083
LOC_Os11g05160	DNA_binding_protein_putative_expressed	6.399743	OTU_0107
LOC_Os11g05150	hydroxyproline-rich_glycoprotein_family_protein_putative_expressed	6.399743	OTU_0107
LOC_Os11g05100	nucleolar_GTPase_putative_expressed	6.399743	OTU_0107
LOC_Os11g05090	peptidyl-prolyl_isomerase_putative_expressed	6.399743	OTU_0107
LOC_Os11g05130	PHD-finger_family_protein_expressed	6.399743	OTU_0107
LOC_Os11g05080	powdery_mildew_resistant_protein_5_putative_expressed	6.399743	OTU_0107
LOC_Os11g05110	pyruvate_kinase_putative_expressed	6.399743	OTU_0107
LOC_Os11g05070	sodium_calcium_exchanger_protein_putative_expressed	6.399743	OTU_0107
LOC_Os11g05050	stem-specific_protein_TSJT1_putative_expressed	6.399743	OTU_0107
LOC_Os11g05040	transposon_protein_putative_unclassified	6.399743	OTU_0107

OTUs positively correlated with yield			
locus	description	LOD	OTU
LOC_Os11g40090	A49-like_RNA_polymerase_I_associated_factor_family_protein_expressed	6.898341	OTU_0018
LOC_Os08g17410	BRASSINOSTEROID_INSENSITIVE_1_precursor_putative_expressed	6.680293	OTU_0018
LOC_Os08g17500	cinnamoyl-CoA_reductase_putative_expressed	6.680293	OTU_0018
LOC_Os08g17520	flavonol_sulfotransferase-like_putative_expressed	6.680293	OTU_0018

LOC_Os11g40100	GRF-interacting_factor_2_putative_expressed	6.898341	OTU_0018
LOC_Os11g40080	lipin_N-terminal_conserved_region_family_protein_expressed	6.898341	OTU_0018
LOC_Os08g17650	LYR_motif_containing_protein_putative_expressed	6.680293	OTU_0018
LOC_Os08g17700	retrotransposon_protein_putative_Ty1-copia_subclass_expressed	6.680293	OTU_0018
LOC_Os08g17480	retrotransposon_protein_putative_Ty3-gypsy_subclass_expressed	6.680293	OTU_0018
LOC_Os08g17460	retrotransposon_protein_putative_unclassified	6.680293	OTU_0018
LOC_Os08g17380	retrotransposon_protein_putative_unclassified_expressed	6.680293	OTU_0018
LOC_Os08g17600	SNARE_domain_containing_protein_putative_expressed	6.680293	OTU_0018
LOC_Os08g17680	stromal_cell-derived_factor_2-like_protein_precursor_putative_expressed	6.680293	OTU_0018
LOC_Os08g17510	sulfotransferase_domain_containing_protein_expressed	6.680293	OTU_0018
LOC_Os08g17370	transmembrane_9_superfamily_member_putative_expressed	6.680293	OTU_0018
LOC_Os08g17720	transposon_protein_putative_CACTA_En_Spm_sub-class_expressed	6.680293	OTU_0018
LOC_Os08g17400	WRKY89_expressed	6.680293	OTU_0018
LOC_Os08g17640	ZOS8-02_-_C2H2_zinc_finger_protein_expressed	6.680293	OTU_0018
LOC_Os07g17010	chalcone_synthase_putative_expressed	6.745608	OTU_0101
LOC_Os07g16960	glycosyl_transferase_group_1_domain_containing_protein_expressed	6.745608	OTU_0101
LOC_Os07g17120	late_embryogenesis_abundant_protein_putative_expressed	6.745608	OTU_0101
LOC_Os07g16950	Mak16_protein_domain_containing_protein_expressed	6.745608	OTU_0101
LOC_Os07g16970	rab_GDP_dissociation_inhibitor_alpha_putative_expressed	6.745608	OTU_0101
LOC_Os07g16990	retrotransposon_protein_putative_Ty1-copia_subclass_expressed	6.745608	OTU_0101
LOC_Os07g17100	retrotransposon_protein_putative_Ty3-gypsy_subclass_expressed	6.745608	OTU_0101
LOC_Os07g17050	retrotransposon_protein_putative_unclassified_expressed	6.745608	OTU_0101
LOC_Os09g04670	DAG_protein_chloroplast_precursor_putative_expressed	6.960749	OTU_0150
LOC_Os10g38000	dehydration-responsive_element-binding_protein_putative_expressed	7.35287	OTU_0150
LOC_Os09g04624	GDSL-like_lipase_acylhydrolase_putative_expressed	6.960749	OTU_0150
LOC_Os09g04680	photosystem_II_P680_chlorophyll_A_apoprotein_putative_expressed	6.960749	OTU_0150
LOC_Os10g37980	prephenate_dehydratase_domain_containing_protein_expressed	7.35287	OTU_0150
LOC_Os09g04610	retrotransposon_protein_putative_Ty3-gypsy_subclass_expressed	6.960749	OTU_0150
LOC_Os09g04660	retrotransposon_protein_putative_unclassified_expressed	6.960749	OTU_0150
LOC_Os09g04720	SWIB_MDM2_domain_containing_protein_expressed	6.960749	OTU_0150

*Expressed protein and hypothetical protein categories not shown

Discussion

Increasing our knowledge of plant-endophyte interactions with the final aim to improve agricultural plant growth is a rapidly expanding research field. Little is known how environmental changes such as global warming and drought will affect root based microbial populations. In the present study, the combination of a high throughput method for sequencing microbial marker genes and a bioinformatics pipeline resulted in the characterization of the composition of the rice-root associated fungal microbiome. The use of a rice Indica population consisting of 296 accessions that have previously been genotyped (Kadam et al., 2017; Rebolledo et al., 2016), resulting in 82,858 SNPs, allowed us to screen for rice-root-microbiome diversity and to study to which extent this diversity is genetically determined.

A total of 184 different SNPs were found to be associated with the occurrence and/or abundance of 44 independent OTUs. We found several candidate genes involved in plant-microbe interactions that may play important roles in determining OTU abundance in rice roots.

Drought affects the fungal microbiome: the selection of beneficial fungi

Determining the root associated fungal microbiome in a large number of rice cultivars during water sufficient and - limiting conditions, allowed us to study environment specific changes in the microbial community that is present in rice roots. In the present study, sequencing depth was enough to describe the fungal community present in the studied population (Fig. S1).

The results confirmed the drought specific effect on the root associated fungal community, that was described recently in a similar study using 15 rice cultivars that were also present in the current study (Chapter 4)(Fig. 1b). However, in the present study twenty times more rice genotypes were used and the microbiome was analyzed using a slightly different technique. Furthermore, drought caused an increase in species diversity and evenness but at the same time a lower species richness when compared with water-sufficient conditions (Fig. 1a). The increase in fungal diversity in the roots under drought can be considered as an enrichment equipping the host with additional functions, which might assist the plant to obtain additional resources to mitigate the consequences of drought. The positive effect of drought on the species' diversity that we observed is similar to the results in a study in artichoke in which it was shown that the bacterial diversity in the rhizosphere increased under salt stress (Yang et al., 2016). On the other hand, in case of a long term stress, diversity might decrease again as shown to happen for ectomycorrhizal fungi in trees. In the latter study it was shown that after a long period of drought stress, the community shifted to a lower diversity with a few beneficial species as dominant ones (Gehring et al., 2014).

In our study, species richness under drought conditions turned out to be lower, however as evenness was higher, all species were better represented, hence increasing diversity as well. The fact that under control conditions there is a higher species number if compared with a stressful environment is not new. It has been shown in coastal plant population that high salinity conditions lower AM fungi species richness in roots (Guo and Gong, 2014).

In the present study the most represented Subphylum is the Pezizomycotina, confirming what was described in Chapter 4. The most represented class were the Sordariomycetes, which includes the orders Sordariales and Hypocreales, followed by the Dothydiomycetes that includes the order Pleosporales. Sordariales are more present among the NYC OTUs than PYC

OTUs in the control treatment. On the other hand, under drought conditions Sordariales are not present anymore among any of the groups, and Hypocreales take over in the PYC OTUs (11 %). Sordariales are saprobic, plant pathogenic and wood inhabiting fungi. For instance Colletotrichum spp. are an important genus of plant pathogens that affect a large variety of plants (Cannon et al., 2012). On the other hand Hypocreales are the largest order that includes symbiotic non-mycorrhizal plant fungi that are applied as a source of biocontrol agents (Kepler et al., 2017). The fact that Sordariales (mainly pathogenic and saprophytes) are the mayor group among the NYC while Hypocreales (mainly mutualistic) are the main group among PYC OTUs may explain the respective positive and negative effects on yield. In terms of yield plasticity vs. yield stability, OTUs correlated with less yield plasticity, hence higher yield stability, would be a desirable trait in plants to adapt them better to drought conditions.

Pleosporales are only found among the PYC OTUs, and they are similarly represented in both treatments. This order is known to be ubiquitous and to have multiple functions, such as plant pathogens, saprophytes and plant endophytes (Zhang et al., 2009). The Chytridiomycetes, the second main Class among the NYC OTUs and so-called 'primitive' fungi or 'chytrids', live in aquatic environments including capillary water networks around soil particles. The majority of this class behaves as parasites or saprophytes (James et al., 2006).

The interactions between microorganisms and their effect on plant adaptation to the environment is context-dependent (Chamberlain et al., 2014; Laitinen et al., 2016). For instance drought can enhance the growth promoting effect of plant growth promoting bacteria in plants like grapevine (Rolli et al., 2015). In our study, the positive correlation of OTUs are also environment-dependent, being just a small group of OTUs positively correlated with plant yield in both conditions.

Rice loci involved in rice root-fungus interactions

When zooming in at the level of individual candidate genes that may be involved in determining the abundance of any of the 44 OTUs, the SHR5 receptor like kinase caught our attention. This candidate gene (LOC_Os05g16430) is encoding a protein with 64% similarity to the receptor-like kinase SHR5 from the sugarcane. The gene encoding this protein is described to be down-regulated when the plant is associated with endophytic nitrogen-fixing bacteria (Vinagre et al., 2006). The expression of its putative homologue in rice is induced upon infection with the pathogenic fungus Magnaporthe oryzae (data from RiceXPro database). Similarly, two other candidates, the genes encoding the LTPL1 lipid transfer protein and tyrosine kinase-like IRAK

(interleukin-1 receptor-associated kinase), are related with development, stress, symbiosis, as well as innate immunity plant responses and are also up-regulated during disease resistance responses against fungal pathogens such as Magnaporthe oryzae in rice (Park and Ronald, 2012; Wang et al., 2015).

Two other interesting candidate genes encode genes that belong to the family of PHD finger proteins. Generally, PHD finger type genes are up-regulated during bacterial pathogen responses (Müller et al., 2015). Another candidate gene, is a gene encoding a synaptosome-associated protein receptor (SNARE). SNARE domain proteins have a wide range of functions, and have been reported to be involved in cytokinesis, symbiosis/pathogen defense responses and abiotic stress responses (Lipka et al., 2007). Mutations in these genes and their homologues in barley reduce host disease resistance against a pathogenic fungus (Collins et al., 2003; Zhang et al., 2007). Moreover, in Medicago, a mutation in the SNARE protein SYP132A inhibits AM fungi colonization (Pan et al., 2016). Another SNARE protein, SYP71, is involved in nitrogen fixation by symbiotic nodules in Lotus (Hakoyama et al., 2012).

Another group of a priori candidate genes encode cytochrome P450 enzymes and F-box proteins. Two cytochrome P450 candidates (LOC_Os12g04480 and LOC_Os05g29750) show a six and three fold up-regulation, respectively, upon a root infection with Magnaporthe oryzae during which ABA levels were reduced (data from RiceXPro database). The expression levels of all F-box candidate genes identified in the present study were found to be affected (up – or down regulated) by an infection with Magnaporthe oryzae. Both types of proteins are known for their activation in plant responses to symbiotic microorganisms and pathogens through hormonal homeostasis (Fu and Wang, 2011; Piisilä et al., 2015; Young et al., 2005). Another candidate gene, encodes a protein belonging to the glycosyl transferase group 1 proteins. This group is described to be involved in responses to biotic and abiotic stresses such as lignification, cell wall remodeling and hormone activation. Furthermore, one of our candidate glycosyl transferases (LOC_Os03g18890) is three fold down-regulated upon M. oryzae infection and up-regulated by ABA (in silico analysis with RiceXPro database).

The above described available information for these specific candidate genes suggests that they may play a role in the establishment of a symbiosis with endophytes in rice roots. It could be that their expression level depends on the type of microbe interaction (mutualistic or pathogenic) finally affecting the abundance of the respective fungus.

It is important to point out that receptors related to host defense and symbiosis are structurally similar, as they are part of the plant immunity system (Akamatsu et al., 2016; Hacquard et al., 2017) what makes the described candidates very interesting for further investigation.

Rice-fungal interaction loci with a role in plant fitness

Out of all OTUs for which a SNP association was found, thirteen were also correlated with plant yield (Fig. 2). These OTUs are a good target to study loci not only involved in plant-microbe symbioses but also in the combined response of the plant-fungal microbiome and its impact on plant fitness under stressful conditions such as drought. Interestingly, these OTUs grouped in different OTU co-occurrence clusters depending on their correlation with yield (Fig. 2b). Although their taxonomic description is not well defined at lower levels, most OTUs in both co-occurrence clusters are assigned to Sordariomycetes, with one of the NYC OTUs being from the Sordariales and a PYC OTU from the Hypocreales.

Interesting candidate genes for influencing the abundance of OTU0018 and OTU0101 are the genes encoding the WRKY89 transcription factor and chalcone synthase (from the flavonoid biosynthetic pathway). Both proteins play a role in microbial pathogen resistance in rice (Dao et al., 2011; Wang et al., 2007). Furthermore, the two disease resistance genes (NBS-LRR type and the powdery mildew resistance protein 5) that are in the LD-regions of OTUs 0001 and 0107 respectively, are also highly interesting. NBS-LRR type disease resistance genes form a major family in plants (McHale et al., 2006), and their structural and genetic diversity in rice is high (Zhou et al., 2004). As we commented on before, receptors related to host defense and symbiosis are structurally similar, making these resistance genes interesting candidates. Since our results suggest that these genes may also play a role in the establishment of a symbiosis in rice, it probably depends on the kind of plant-microbe relationship whether these genes will positively or negatively affect the abundance of the respective fungus.

Conclusions

Our study provides new candidate genes in rice that might be involved in rice root-fungal interactions, including both pathogens and mutualistic fungi. Furthermore, many of the candidate genes inside these loci have been described to be related to abiotic stress responses, especially among OTUs that are correlated with higher yield. The genes responsible for the abundance/presence of independent OTUs might indirectly contribute to the plant phenotype in

response to water deficit conditions. However, because of the low heritability values, the results should be interpreted carefully and further tested in independent experiments to see how robust they are linked with the trait of interest. An alternative for future approaches would be to include taxonomic groups or correlated clusters as traits instead of individual OTUs in the GWA analysis. Finally, functional studies should be performed to elucidate what type of response these candidate genes regulate in plants. This information would be highly valuable for rice breeding programs and agricultural practices, in order to modulate plants, allowing them to select the most beneficial group of fungi that will finally contribute to a desired phenotype.

Acknowledgements

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Supplementary material

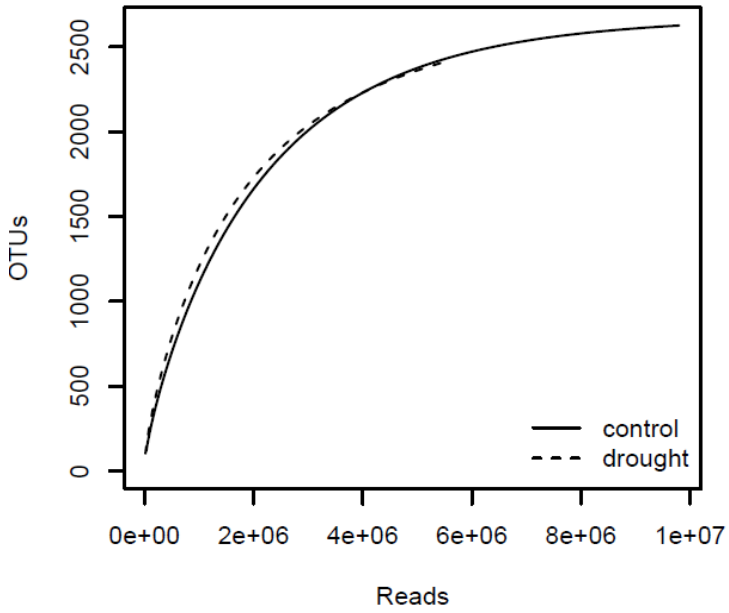
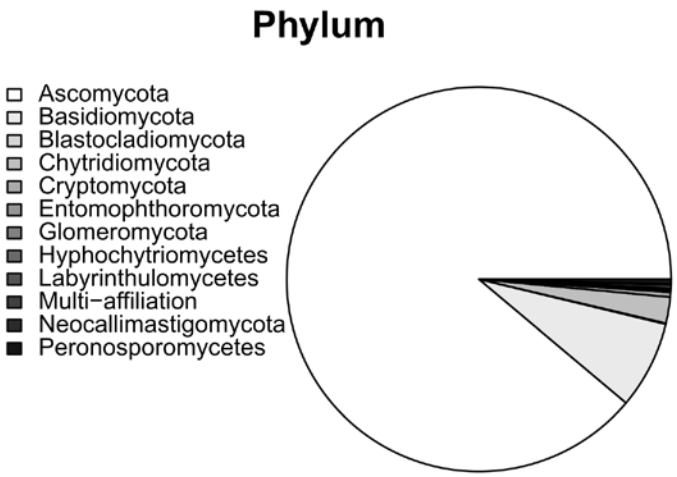


Figure S1. Rarefaction curve relating the sequencing effort to the number of OTUs.

(a)



(b)

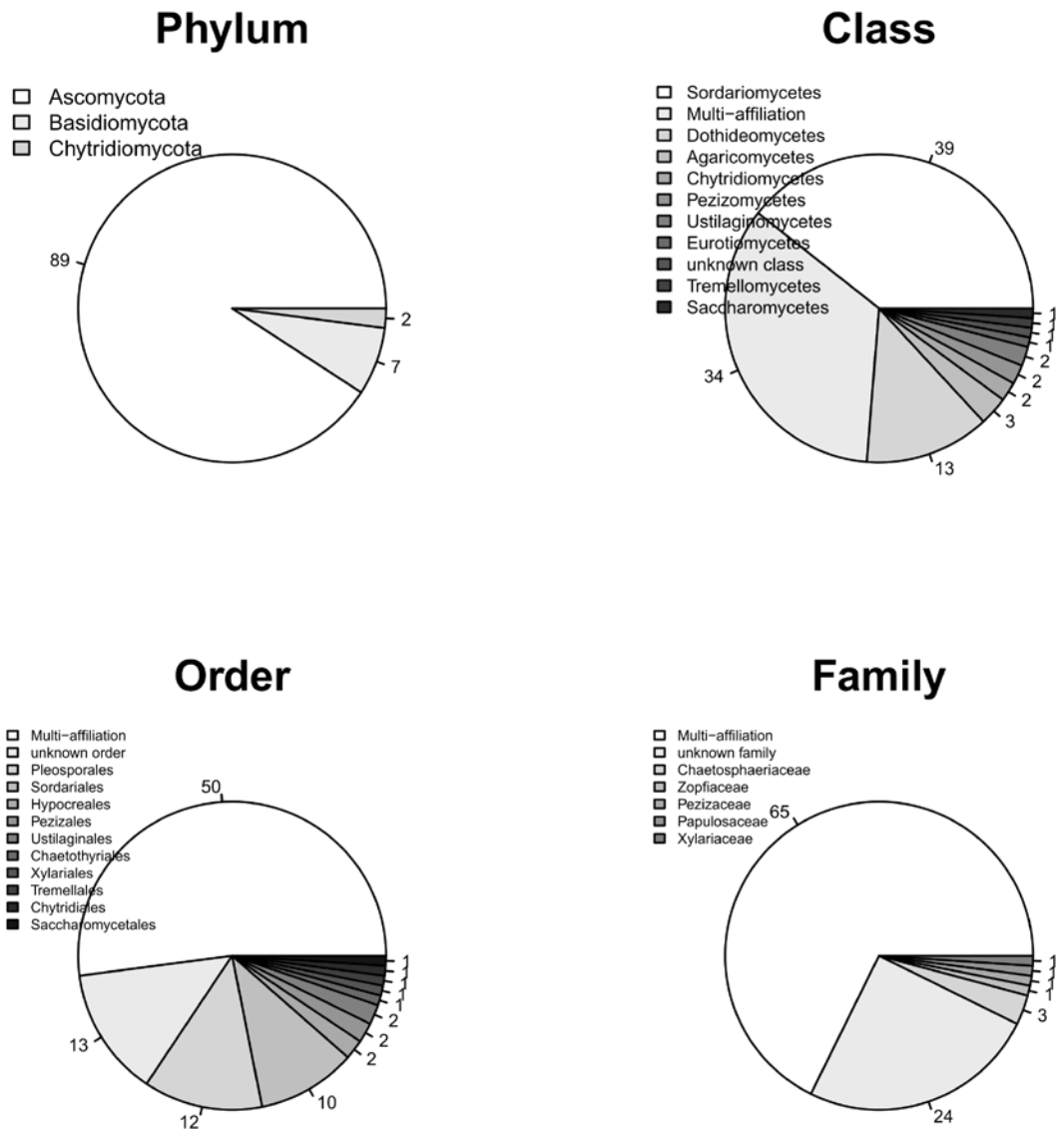


Figure S2. a) Number of OTUs for all phyla that were detected in this study. b) Quantification of OTUs for the main taxonomic levels. Values represent the percentage of OTU number with respect to the total number of OTUs. Only the taxa represented by >1% or the total OTUs are shown.

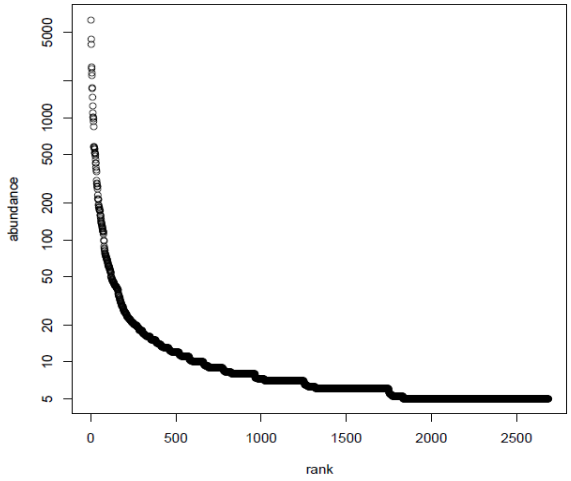


Figure S3. Rank abundance curve representing the abundance data from all OTUs found in the present study (2,687). We considered 'rare' OTUs as the ones represented by less than 10 sequences.

(a) (b)

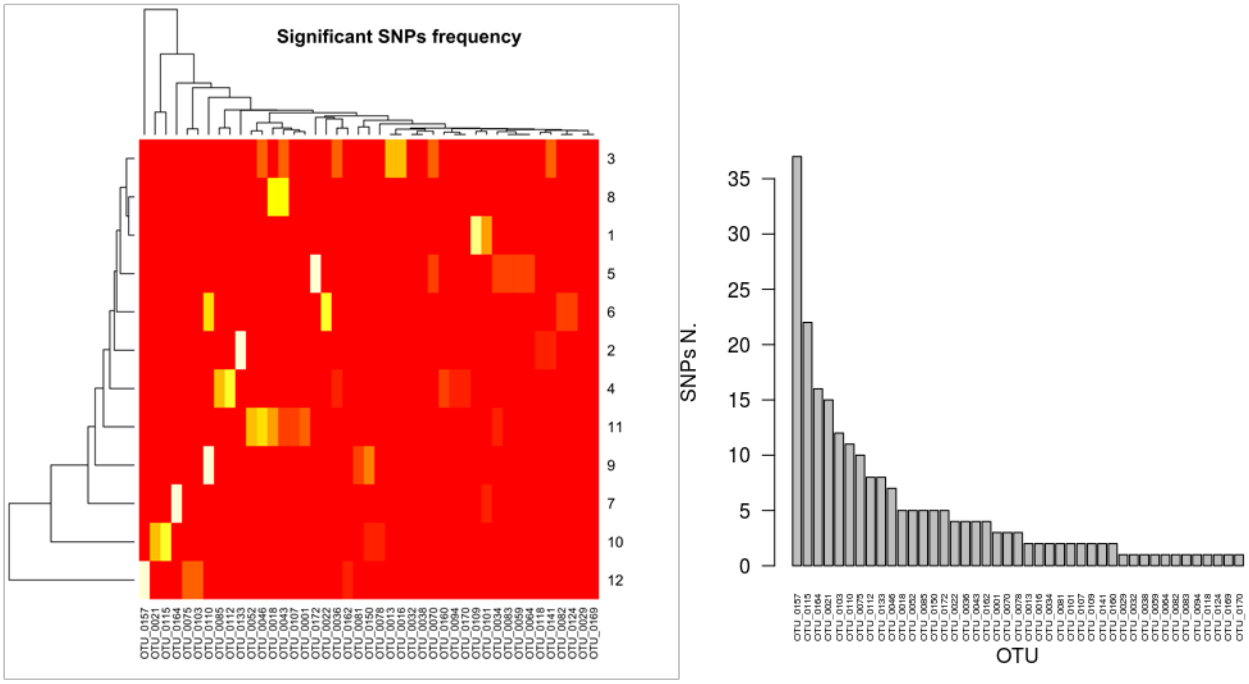


Figure S4. (a) Estimation of SNP frequency. Heatmap with the number of correlated SNPs (-log (p) > 6) in each OTU and chromosome. The frequency gradient colors goes from yellow (high frequency) to red (low frequency). The SNPs are found in several chromosomes. The number of SNPs was corrected by the size of the chromosome. (b) Number of SNPs found to be associated per OTU. There is a small group of OTUs associated with >10 SNPs

Table S1. Ecological parameters in all Phyla found in the study. Total abundance, richness, Jevenness and Shannon index data for both treatments, control and drought, separately.

		CONTROL				DROUGHT			
Phyla	N. OTUs	abundance	richness	Shannon	Jevenness	abundance	richness	Shannon	Jevenness
Ascomycota	2388	9456943	2342	0.96	0.12	5184964	2127	1.40	0.18
Basidiomycota	198	227691	192	1.00	0.20	209235	190	0.91	0.17
Chytridiomycota	59	74257	52	0.87	0.22	68434	55	1.50	0.37
Cryptomycota	11	1186	11	2.10	0.88	5226	11	1.30	0.54
Peronosporomycetes	11	29297	10	0.11	0.05	1440	7	0.45	0.22
Labyrinthulomycetes	7	2061	7	0.94	0.48	556	7	1.30	0.66
Glomeromycota	4	5151	4	0.51	0.37	1654	4	0.51	0.37
Entomophthoromycota	3	48	2	0.23	0.34	1039	3	0.91	0.83
Blastocladiomycota	2	720	2	0.11	0.16	332	2	0.51	0.73
Hyphochytriomycetes	2	536	2	0.13	0.18	989	1	0.00	na
Multi-affiliation	1	17	1	0.00	na	2	1	0.00	na
Neocallimastigomycota	1	12	1	0.00	na	0	0	0.00	0.00

Table S2. Taxonomy description of OTUs correlated with SNPs in the study.

OTU	Phylum	Class	Order	Family	Genus	Species
OTU_0001	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	Chaetomium	Multi-affiliation
OTU_0013	Peronosporomycetes	unknown class	unknown order	unknown family	unknown genus	unknown species
OTU_0016	Ascomycota	Sordariomycetes	Microascales	Multi-affiliation	Multi-affiliation	Multi-affiliation
OTU_0018	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Septofusidium	Septofusidium herbarum
OTU_0021	Ascomycota	Eurotiomycetes	Multi-affiliation	Multi-affiliation	Multi-affiliation	Multi-affiliation
OTU_0022	Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae	Multi-affiliation	Multi-affiliation
OTU_0029	Ascomycota	Sordariomycetes	Lulworthiales	Lulworthiaceae	Lulworthia	Lulwoidea lignoarenaria
OTU_0032	Cryptomycota	unknown class	unknown order	unknown family	unknown genus	unknown species
OTU_0034	Basidiomycota	Microbotryomycetes	Sporidiobolales	Multi-affiliation	Multi-affiliation	Multi-affiliation
OTU_0036	Ascomycota	Sordariomycetes	Sordariales	unknown family	unknown genus	unknown species
OTU_0038	Cryptomycota	unknown class	unknown order	unknown family	unknown genus	unknown species
OTU_0043	Hyphochytriomycetes	unknown class	Hyphochytriales	unknown family	unknown genus	unknown species
OTU_0046	Ascomycota	Orbiliomycetes	Orbiliales	Orbiliaceae	Multi-affiliation	Multi-affiliation
OTU_0052	Peronosporomycetes	unknown class	unknown order	unknown family	unknown genus	unknown species
OTU_0059	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	Chaetomium	Multi-affiliation
OTU_0064	Ascomycota	Sordariomycetes	Sordariales	unknown family	unknown genus	unknown species
OTU_0070	Ascomycota	Sordariomycetes	Sordariales	Multi-affiliation	Multi-affiliation	Multi-affiliation
OTU_0075	Ascomycota	Sordariomycetes	Hypocreales	unknown family	unknown genus	unknown species
OTU_0078	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	Chaetomium	Multi-affiliation
OTU_0081	Ascomycota	Dothideomycetes	Pleosporales	Multi-affiliation	Multi-affiliation	Multi-affiliation
OTU_0082	Labyrinthulomycetes	unknown class	unknown order	Thraustochytriaceae	Thraustochytrium	Thraustochytriidae sp.
OTU_0082	Labyrinthulomycetes	unknown class	unknown order	Thraustochytriaceae	Thraustochytrium	Thraustochytriidae sp.
OTU_0083	Ascomycota	Sordariomycetes	Multi-affiliation	Multi-affiliation	Multi-affiliation	Multi-affiliation
OTU_0085	Ascomycota	Saccharomycetes	Saccharomycetales	unknown family	unknown genus	Metschnikowia sp.
OTU_0094	Ascomycota	Sordariomycetes	Sordariales	Multi-affiliation	Multi-affiliation	Multi-affiliation
OTU_0101	Ascomycota	Sordariomycetes	Multi-affiliation	Multi-affiliation	Multi-affiliation	Multi-affiliation
OTU_0103	Ascomycota	Sordariomycetes	Xylariales	Xylariaceae	Multi-affiliation	Multi-affiliation
OTU_0107	Ascomycota	Sordariomycetes	Multi-affiliation	Multi-affiliation	Multi-affiliation	Multi-affiliation
OTU_0109	Basidiomycota	Agaricomycetes	Multi-affiliation	Multi-affiliation	Multi-affiliation	Multi-affiliation
OTU_0110	Ascomycota	Sordariomycetes	Multi-affiliation	Multi-affiliation	Multi-affiliation	Multi-affiliation
OTU_0112	Ascomycota	Sordariomycetes	Sordariales	Chaetosphaeriaceae	Chaetosphaeria	Chaetosphaeria curvispora
OTU_0115	Ascomycota	Dothideomycetes	Pleosporales	Multi-affiliation	Multi-affiliation	Multi-affiliation
OTU_0118	Ascomycota	Dothideomycetes	Pleosporales	Multi-affiliation	Multi-affiliation	Multi-affiliation
OTU_0124	Ascomycota	Sordariomycetes	Sordariales	Chaetosphaeriaceae	Chaetosphaeria	Chaetosphaeria curvispora
OTU_0133	Ascomycota	Sordariomycetes	unknown order	Papulosaceae	Papulosa	Papulosa amerospora
OTU_0141	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	Multi-affiliation	Multi-affiliation
OTU_0150	Ascomycota	Sordariomycetes	unknown order	unknown family	Ceratosphaeria	Ceratosphaeria lampadophora
OTU_0157	Ascomycota	Sordariomycetes	Sordariales	Multi-affiliation	Multi-affiliation	Multi-affiliation
OTU_0160	Ascomycota	Multi-affiliation	Multi-affiliation	Multi-affiliation	Multi-affiliation	Multi-affiliation
OTU_0162	Ascomycota	Multi-affiliation	Multi-affiliation	Multi-affiliation	Multi-affiliation	Multi-affiliation
OTU_0164	Ascomycota	Multi-affiliation	Multi-affiliation	Multi-affiliation	Multi-affiliation	Multi-affiliation
OTU_0169	Ascomycota	Multi-affiliation	Multi-affiliation	Multi-affiliation	Multi-affiliation	Multi-affiliation
OTU_0170	Ascomycota	Multi-affiliation	Multi-affiliation	Multi-affiliation	Multi-affiliation	Multi-affiliation
OTU_0172	Ascomycota	Multi-affiliation	Multi-affiliation	Multi-affiliation	Multi-affiliation	Multi-affiliation

Table S3. Overview of all SNPs associated with a trait (OTU) resulting from the univariate Genome Wide Association analysis with OTU abundance and occurrence data.

SNP	Chr	LOD	OTU	treatment	GWAs	Nº genes
Chr11pos17485011.1	11	6.666450217	OTU_0001	control	univ	5
Chr11pos17515007.1	11	7.136775549	OTU_0001	control	univ	1
Chr11pos17574391.1	11	7.136775549	OTU_0001	control	univ	8
Chr3pos10676708.1	3	6.934903192	OTU_0013	control	univ	26
Chr3pos10676712.1	3	6.934903192	OTU_0013	control	univ	26
Chr3pos13832700.1	3	7.142413112	OTU_0016	control	ocu	2
Chr3pos13832701.1	3	7.142413112	OTU_0016	control	ocu	2
Chr11pos23898551.1	11	6.898340724	OTU_0018	control	univ	3
Chr11pos23982547.1	11	5.040431725	OTU_0018	control	univ	2
Chr11pos23982705.1	11	6.598680072	OTU_0018	control	univ	2
Chr8pos10626886.1	8	6.680292531	OTU_0018	control	univ	41
Chr10pos19370651.1	10	7.102775792	OTU_0021	control	univ	15
Chr10pos19463063.1	10	6.605058183	OTU_0021	control	univ	15
Chr10pos19463082.1	10	6.605058183	OTU_0021	control	univ	15
Chr10pos19463253.1	10	6.220526238	OTU_0021	control	univ	15
Chr10pos19475539.1	10	7.014817504	OTU_0021	control	univ	15
Chr10pos19540162.1	10	6.839743534	OTU_0021	control	univ	15
Chr10pos19613263.1	10	6.566776699	OTU_0021	control	univ	8
Chr10pos19614941.1	10	6.566776699	OTU_0021	control	univ	8
Chr10pos19650831.1	10	6.659983558	OTU_0021	control	univ	8
Chr10pos19651211.1	10	6.266213671	OTU_0021	control	univ	8
Chr10pos19665687.1	10	6.659983558	OTU_0021	control	univ	8
Chr10pos19668952.1	10	6.659983558	OTU_0021	control	univ	18
Chr10pos19677329.1	10	6.659983558	OTU_0021	control	univ	18
Chr10pos19696466.1	10	6.659983558	OTU_0021	control	univ	18
Chr10pos19720111.1	10	6.659983558	OTU_0021	control	univ	18
Chr6pos14036916.1	6	6.29383718	OTU_0022	control	univ	0
Chr12pos7802338.1	12	6.583071479	OTU_0029	control	univ	12
Chr10pos7126116.1	10	6.381894764	OTU_0032	drought	ocu	0
Chr11pos6537165.1	11	6.429294287	OTU_0034	drought	univ	0
Chr5pos9486096.1	5	7.226471542	OTU_0034	drought	univ	44
Chr12pos12129167.1	12	7.655847896	OTU_0036	drought	univ	0

Chr12pos12129196.1	12	5.311813698	OTU_0036	drought	univ	68
Chr3pos18084680.1	3	6.710248179	OTU_0036	drought	ocu	0
Chr4pos853062.1	4	6.333506572	OTU_0036	drought	univ	4
Chr10pos12448100.1	10	6.451873986	OTU_0038	control	univ	0
Chr11pos21887057.1	11	6.937241653	OTU_0043	control	univ	25
Chr11pos21887062.1	11	6.937241653	OTU_0043	control	univ	25
Chr3pos1810046.1	3	7.026485213	OTU_0043	control	ocu	0
Chr8pos5147568.1	8	6.49430679	OTU_0043	drought	univ	20
Chr11pos565489.1	11	6.348470013	OTU_0046	control	ocu	9
Chr11pos565655.1	11	5.469621147	OTU_0046	control	ocu	9
Chr11pos566626.1	11	5.879077732	OTU_0046	control	ocu	9
Chr11pos568420.1	11	5.879077732	OTU_0046	control	ocu	9
Chr11pos716131.1	11	6.283746548	OTU_0046	control	both	2
Chr3pos30781157.1	3	6.251290219	OTU_0046	control	univ	28
Chr11pos23793418.1	11	5.449555407	OTU_0052	control	both	5
Chr11pos23793446.1	11	6.381503395	OTU_0052	control	both	5
Chr11pos23867206.1	11	6.457837842	OTU_0052	control	univ	0
Chr5pos3027325.1	5	6.327171618	OTU_0059	drought	ocu	0
Chr5pos18146110.1	5	6.604470184	OTU_0064	drought	univ	0
Chr10pos4423993.1	10	6.276679241	OTU_0070	control	univ	0
Chr3pos16889187.1	3	6.297716842	OTU_0070	control	univ	5
Chr5pos15238843.1	5	6.951708769	OTU_0070	drought	univ	0
Chr12pos7239881.1	12	6.494411469	OTU_0075	control	univ	8
Chr12pos7239887.1	12	6.494411469	OTU_0075	control	univ	8
Chr12pos7273712.1	12	6.494411469	OTU_0075	control	univ	8
Chr12pos7302300.1	12	6.494411469	OTU_0075	control	univ	5
Chr12pos7307709.1	12	6.494411469	OTU_0075	control	univ	5
Chr12pos7307710.1	12	6.177055116	OTU_0075	control	univ	5
Chr12pos7307955.1	12	6.494411469	OTU_0075	control	univ	5
Chr12pos7324655.1	12	6.494411469	OTU_0075	control	univ	5
Chr12pos7344618.1	12	7.364486448	OTU_0075	control	univ	5
Chr12pos7344673.1	12	7.364486448	OTU_0075	control	univ	5
Chr10pos11003430.1	10	6.357442032	OTU_0078	drought	univ	7
Chr10pos11008219.1	10	4.198674318	OTU_0078	drought	univ	7
Chr10pos7134016.1	10	6.294446072	OTU_0078	drought	ocu	0
Chr9pos8672174.1	9	7.204770626	OTU_0081	control	both	3

Chr6pos18317559.1	6	6.548290305	OTU_0082	control	univ	3
Chr5pos5890522.1	5	6.491965742	OTU_0083	drought	univ	9
Chr4pos1256419.1	4	5.784883401	OTU_0085	drought	univ	0
Chr4pos1258951.1	4	6.269805032	OTU_0085	drought	univ	0
Chr4pos1258958.1	4	6.269805032	OTU_0085	drought	univ	0
Chr4pos1258961.1	4	6.269805032	OTU_0085	drought	univ	0
Chr4pos1259039.1	4	6.269805032	OTU_0085	drought	univ	0
Chr4pos34656915.1	4	6.28763188	OTU_0094	drought	univ	0
Chr1pos42833365.1	1	6.794021651	OTU_0101	control	univ	0
Chr7pos10052147.1	7	6.745607612	OTU_0101	control	ocu	17
Chr12pos6913711.1	12	5.333353118	OTU_0103	control	univ	6
Chr12pos6916223.1	12	5.923535803	OTU_0103	control	univ	6
Chr12pos6916225.1	12	5.923535803	OTU_0103	control	univ	6
Chr12pos6916237.1	12	6.417827333	OTU_0103	control	univ	6
Chr12pos6932455.1	12	6.411398229	OTU_0103	control	univ	6
Chr12pos6932512.1	12	5.923535803	OTU_0103	control	univ	6
Chr12pos6932526.1	12	5.48289376	OTU_0103	control	univ	6
Chr12pos6932532.1	12	5.48289376	OTU_0103	control	univ	6
Chr12pos6936735.1	12	5.48289376	OTU_0103	control	univ	6
Chr12pos6936795.1	12	5.48289376	OTU_0103	control	univ	6
Chr12pos6936811.1	12	5.48289376	OTU_0103	control	univ	6
Chr12pos6936867.1	12	5.48289376	OTU_0103	control	univ	6
Chr11pos2185999.1	11	6.399743097	OTU_0107	control	univ	17
Chr1pos19422039.1	1	7.251218646	OTU_0109	control	both	0
Chr6pos15086799.1	6	6.23085525	OTU_0110	control	univ	0
Chr6pos15086800.1	6	6.23085525	OTU_0110	control	univ	0
Chr6pos15086801.1	6	6.23085525	OTU_0110	control	univ	0
Chr9pos7644684.1	9	6.428597388	OTU_0110	control	ocu	1
Chr9pos7644691.1	9	6.428597388	OTU_0110	control	ocu	1
Chr9pos7644695.1	9	6.428597388	OTU_0110	control	ocu	1
Chr9pos7644709.1	9	6.428597388	OTU_0110	control	ocu	1
Chr9pos7645588.1	9	2.527966886	OTU_0110	control	ocu	0
Chr9pos7645600.1	9	2.527966886	OTU_0110	control	ocu	0
Chr4pos6775632.1	4	6.235420727	OTU_0112	control	univ	0
Chr4pos6775644.1	4	6.877003572	OTU_0112	control	univ	0
Chr4pos6775650.1	4	6.235420727	OTU_0112	control	univ	0

Chr4pos6775659.1	4	6.235420727	OTU_0112	control	univ	0
Chr4pos6775660.1	4	8.472234528	OTU_0112	control	univ	0
Chr4pos6775665.1	4	6.235420727	OTU_0112	control	univ	0
Chr4pos6775694.1	4	4.792477376	OTU_0112	control	univ	0
Chr10pos16905926.1	10	6.875838733	OTU_0115	drought	univ	20
Chr10pos16963675.1	10	6.908157951	OTU_0115	drought	univ	13
Chr10pos17048354.1	10	6.316693286	OTU_0115	drought	univ	9
Chr10pos17072201.1	10	6.316693286	OTU_0115	drought	univ	9
Chr10pos17089683.1	10	6.875838733	OTU_0115	drought	univ	9
Chr10pos17095109.1	10	6.695264912	OTU_0115	drought	univ	9
Chr10pos17121515.1	10	6.875838733	OTU_0115	drought	univ	4
Chr10pos17121540.1	10	6.875838733	OTU_0115	drought	univ	21
Chr10pos17132637.1	10	6.695264912	OTU_0115	drought	univ	21
Chr10pos17137599.1	10	6.875838733	OTU_0115	drought	univ	21
Chr10pos17141013.1	10	6.695264912	OTU_0115	drought	univ	21
Chr10pos17141015.1	10	6.695264912	OTU_0115	drought	univ	21
Chr10pos17141432.1	10	6.316693286	OTU_0115	drought	univ	21
Chr10pos17159689.1	10	6.875838733	OTU_0115	drought	univ	21
Chr10pos17179336.1	10	6.875838733	OTU_0115	drought	univ	21
Chr10pos17179405.1	10	6.875838733	OTU_0115	drought	univ	21
Chr10pos17193330.1	10	6.316742744	OTU_0115	drought	univ	21
Chr10pos17210385.1	10	6.316742744	OTU_0115	drought	univ	21
Chr10pos17378568.1	10	7.753052167	OTU_0115	drought	univ	0
Chr10pos2147104.1	10	6.727731185	OTU_0115	drought	univ	7
Chr10pos2199166.1	10	6.727731185	OTU_0115	drought	univ	3
Chr10pos2303014.1	10	6.727731185	OTU_0115	drought	univ	18
Chr2pos33555175.1	2	6.258642304	OTU_0118	control	univ	0
Chr6pos30694661.1	6	6.63711074	OTU_0124	control	univ	0
Chr2pos25447359.1	2	6.61867039	OTU_0133	control	ocu	9
Chr2pos25447365.1	2	6.61867039	OTU_0133	control	ocu	9
Chr2pos25447368.1	2	6.61867039	OTU_0133	control	ocu	9
Chr2pos25454511.1	2	6.61867039	OTU_0133	control	ocu	9
Chr2pos25481197.1	2	6.61867039	OTU_0133	control	ocu	9
Chr2pos25511787.1	2	6.61867039	OTU_0133	control	ocu	20
Chr2pos25526770.1	2	6.456629188	OTU_0133	control	ocu	20
Chr2pos25537464.1	2	6.61867039	OTU_0133	control	ocu	20

Chr2pos1248205.1	2	6.531371169	OTU_0141	control	univ	25
Chr3pos26078859.1	3	6.885186027	OTU_0141	control	univ	5
Chr10pos20360888.1	10	7.352869846	OTU_0150	drought	both	3
Chr9pos1920904.1	9	5.678383279	OTU_0150	drought	univ	3
Chr9pos1920905.1	9	6.81871144	OTU_0150	drought	univ	3
Chr9pos2482889.1	9	6.960749432	OTU_0150	drought	univ	10
Chr12pos1753092.1	12	8.486558823	OTU_0157	control	both	14
Chr12pos1797636.1	12	6.872394018	OTU_0157	control	both	14
Chr12pos1870375.1	12	6.385154324	OTU_0157	control	ocu	8
Chr12pos1872241.1	12	4.414196226	OTU_0157	control	ocu	8
Chr12pos1872394.1	12	4.513025749	OTU_0157	control	ocu	8
Chr12pos1881367.1	12	4.266880412	OTU_0157	control	ocu	8
Chr12pos1883341.1	12	6.385154324	OTU_0157	control	ocu	8
Chr12pos1886495.1	12	4.513025749	OTU_0157	control	ocu	8
Chr12pos1886677.1	12	6.385154324	OTU_0157	control	ocu	8
Chr12pos1901368.1	12	7.669098049	OTU_0157	control	both	56
Chr12pos1901483.1	12	6.323782771	OTU_0157	control	both	56
Chr12pos1930064.1	12	4.547192702	OTU_0157	control	both	56
Chr12pos1933706.1	12	6.311204079	OTU_0157	control	both	56
Chr12pos1933712.1	12	6.311204079	OTU_0157	control	both	56
Chr12pos1933713.1	12	6.311204079	OTU_0157	control	both	56
Chr12pos1937568.1	12	6.66908358	OTU_0157	control	both	56
Chr12pos1938734.1	12	5.98301416	OTU_0157	control	both	56
Chr12pos1940192.1	12	4.547192702	OTU_0157	control	both	56
Chr12pos1997708.1	12	5.121494651	OTU_0157	control	ocu	56
Chr12pos1997755.1	12	6.290554581	OTU_0157	control	ocu	56
Chr12pos2001798.1	12	5.121494651	OTU_0157	control	ocu	56
Chr12pos2001943.1	12	6.290554581	OTU_0157	control	ocu	56
Chr12pos2007906.1	12	6.290554581	OTU_0157	control	ocu	56
Chr12pos2023080.1	12	6.290554581	OTU_0157	control	ocu	56
Chr12pos2023096.1	12	6.290554581	OTU_0157	control	ocu	56
Chr12pos2023121.1	12	6.290554581	OTU_0157	control	ocu	56
Chr4pos761084.1	4	6.635099957	OTU_0160	control	ocu	0
Chr12pos19381275.1	12	6.64240433	OTU_0162	control	both	0
Chr7pos20931944.1	7	6.916370722	OTU_0164	control	both	4
Chr7pos20936382.1	7	2.500114161	OTU_0164	control	both	1

Chr7pos20936387.1	7	2.500114161	OTU_0164	control	both	1
Chr12pos18899159.1	12	6.221072863	OTU_0169	control	univ	11
Chr4pos9834024.1	4	6.377331768	OTU_0170	control	univ	0
Chr5pos17207739.1	5	4.955969898	OTU_0172	control	ocu	2
Chr5pos17207759.1	5	4.955969898	OTU_0172	control	ocu	2
Chr5pos17207782.1	5	4.955969898	OTU_0172	control	ocu	2
Chr5pos17208922.1	5	5.685437176	OTU_0172	control	ocu	2
Chr5pos17208953.1	5	6.305488292	OTU_0172	control	ocu	2

Chr: chromosome; uni: SNP comes from univariate analysis with abundance; ocu: SNP comes from univariate analysis with occurrence; both: SNP comes from both analyses; treatment: treatment in which SNP association was found.

Table S4. SNPs that were found to be associated with OTUs that are correlated with yield. Highlighted in bold are the SNPs included in the top 40 SNPs with highest LOD scores in the entire study (LOD > 6.7), coming from OTU_0001, OTU_0018, OTU_101 and OTU_150.

SNP	Chr	LOD	OTU	treatment	GWAs	Nº genes	correlation
Chr5pos5890522.1	5	6.491966	OTU_0083	drought	univ	9	negative
Chr11pos17485011.1	11	6.66645	OTU_0001	control	univ	5	negative
Chr11pos17515007.1	**11**	**7.136776**	**OTU_0001**	**control**	**univ**	**1**	**negative**
Chr11pos17574391.1	**11**	**7.136776**	**OTU_0001**	**control**	**univ**	**8**	**negative**
Chr11pos2185999.1	11	6.399743	OTU_0107	control	univ	17	negative
Chr1pos42833365.1	**1**	**6.794022**	**OTU_0101**	**control**	**univ**	**0**	**positive**
Chr7pos10052147.1	**7**	**6.745608**	**OTU_0101**	**control**	**ocu**	**17**	**positive**
Chr8pos10626886.1	8	6.680293	OTU_0018	control	univ	41	positive
Chr9pos1920904.1	9	5.678383	OTU_0150	drought	univ	3	positive
Chr9pos1920905.1	**9**	**6.818711**	**OTU_0150**	**drought**	**univ**	**3**	**positive**
Chr9pos2482889.1	**9**	**6.960749**	**OTU_0150**	**drought**	**univ**	**10**	**positive**
Chr10pos20360888.1	**10**	**7.35287**	**OTU_0150**	**drought**	**both**	**3**	**positive**
Chr11pos23898551.1	**11**	**6.898341**	**OTU_0018**	**control**	**univ**	**3**	**positive**
Chr11pos23982547.1	11	5.040432	OTU_0018	control	univ	2	positive
Chr11pos23982705.1	11	6.59868	OTU_0018	control	univ	2	positive

Chr: chromosome; uni: SNP comes from univariate analysis with abundance; ocu: SNP comes from univariate analysis with occurrence; both: SNP comes from both analyses; treatment: treatment where SNP association was found; correlation: type of OTU correlation with yield.

Table S5. Description and putative functions of gene candidates found in this study. Information coming from the univariate Genome Wide Association analysis with OTU abundance and OTU occurrence data. Data have been retrieved from the MSU rice database.

Gene functions
3-isopropylmalate_dehydratase_large_subunit_2_putative_expressed
40S_ribosomal_protein_S3a_putative_expressed
60S_ribosomal_protein_L21-2_putative_expressed
A49-like_RNA_polymerase_I_associated_factor_family_protein_expressed
ABC_transporter_ATP-binding_protein_putative_expressed
ABC-2_type_transporter_putative_expressed
actin_putative_expressed
acyl-CoA_synthetase_protein_putative_expressed
aldose_1-epimerase_putative_expressed
allergen-related_putative_expressed
alpha_beta_hydrolase_fold_putative_expressed
AMBP1_-_Antimicrobial_peptide_MBP-1_family_protein_precursor_expressed
aminotransferase_classes_I_and_II_domain_containing_protein_expressed
ammonium_transporter_2_putative_expressed
anaphase-promoting_complex_subunit_11_putative_expressed
ankyrin_repeat_domain_containing_protein_expressed
AP2_domain_containing_protein_expressed
aquaporin_protein_putative_expressed
ASH-like_SPRY_domain_containing_protein_putative_expressed
aspartic_proteinase_nepenthesin_precursor_putative
aspartic_proteinase_nepenthesin-1_precursor_putative_expressed
astaxanthin_synthase_KC28_putative_expressed
AT_hook_motif_domain_containing_protein_expressed
ATP-dependent_RNA_helicase_DHX36_putative_expressed
ATXR_putative_expressed
AWPM-19-like_membrane_family_protein_putative_expressed
beta-amylase_putative_expressed
bolA-like_family_protein_putative_expressed
BRASSINOSTEROID_INSENSITIVE_1_precursor_putative_expressed
BTB8_-_Bric-a-Brac_Tramtrack_Broad_Complex_BTB_domain_expressed
BTBA6_-_Bric-a-BracTramtrack_Broad_Complex_BTB_domain_with_Ankyrin_repeat_region_expressed
caffeic_acid_3-O-methyltransferase_putative_expressed
calcium-transporting_ATPase_plasma_membrane-type_putative_expressed
calmodulin_binding_protein_putative_expressed
calmodulin-like_protein_1_putative_expressed
CAMK_CAMK_like.40_-_CAMK_includes_calcium_calmodulin_depedent_protein_kinases_expressed

carotenoid_synthesis_regulator_CarF_putative_expressed
CCT_motif_family_protein_expressed
CDP-alcohol_phosphatidyltransferase_putative_expressed
chalcone_synthase_putative_expressed
cinnamoyl-CoA_reductase_putative_expressed
COBRA-like_protein_7_precursor_putative_expressed
conserved_hypothetical_protein
CPuORF40_-_conserved_peptide_uORF-containing_transcript_expressed
CRP3_-_Cysteine-rich_family_protein_precursor_expressed
CRS1_YhbY_domain_containing_protein_expressed
CRS2-associated_factor_2_mitochondrial_precursor_putative_expressed
cupin_domain_containing_protein_expressed
CXE_carboxylesterase_putative_expressed
cyclin-dependent_kinase_A-2_putative_expressed
cysteine_synthase_putative_expressed
cytochrome_c-type_biogenesis_protein_dbsD-like_putative_expressed
cytochrome_P450_71E1_putative_expressed
cytochrome_P450_94A1_putative_expressed
cytochrome_P450_putative_expressed
DAG_protein_chloroplast_precursor_putative_expressed
DEAD-box_ATP-dependent_RNA_helicase_putative_expressed
dehydration_response_related_protein_putative_expressed
dehydration-responsive_element-binding_protein_putative_expressed
dehydrogenase_putative_expressed
dimethyladenosine_transferase_putative_expressed
dirigent_putative_expressed
disease_resistance_protein_RPM1_putative_expressed
disease_resistance_RPP13-like_protein_1_putative_expressed
DNA_binding_protein_putative_expressed
DNA_repair_protein_Rad51_putative_expressed
DNA-directed_RNA_polymerases_I_II_and_III_subunit_RPABC1_putative_expressed
domain_of_unknown_function_DUF250_domain_containing_protein_expressed
DTW_domain_containing_protein_putative_expressed
DUF630_DUF632_domains_containing_protein_putative_expressed
DUF677_domain_containing_protein_putative_expressed
EARLY_flowering_protein_putative_expressed
EF_hand_family_protein_putative
embryogenesis_transmembrane_protein_putative_expressed
endoglucanase_putative_expressed
endonuclease_exonuclease_phosphatase_family_domain_containing_protein_expressed
enzyme_of_the_cupin_superfamily_protein_putative_expressed
exonuclease_putative_expressed
exostosin_family_domain_containing_protein_expressed
expressed_protein

FAD-binding_and_arabino-
lactone_oxidase_domains_containing_protein_putative_expressed
F-box_LRR-repeat_protein_2_putative_expressed
ferredoxin-thioredoxin_reductase_variable_chain_putative_expressed
flavonol_sulfotransferase-like_putative_expressed
flavoprotein_wrbA_putative_expressed
GATA_zinc_finger_domain_containing_protein_expressed
GCRP7_-_Glycine_and_cysteine_rich_family_protein_precursor_expressed
GDSL-like_lipase_acylhydrolase_putative_expressed
GEM_putative_expressed
GIL1_putative_expressed
GIY-YIG_catalytic_domain_containing_protein_putative_expressed
glycerol_3-phosphate_permease_putative_expressed
glycosyl_transferase_8_domain_containing_protein_putative_expressed
glycosyl_transferase_group_1_domain_containing_protein_expressed
GRAS_family_transcription_factor_containing_protein_expressed
GRF_zinc_finger_family_protein
GRF-interacting_factor_2_putative_expressed
h_ACA_ribonucleoprotein_complex_subunit_1-like_protein_1_putative_expressed
haloacid_dehalogenase-like_hydrolase_domain-containing_protein_1A_putative_expressed
HEAT_repeat_family_protein_putative_expressed
heat_shock_protein_DnaJ_putative_expressed
heavy-metal-associated_domain-containing_protein_putative_expressed
helix-loop-helix_DNA-binding_domain_containing_protein_expressed
HTH_DNA-binding_protein_putative_expressed
hydroxyproline-rich_glycoprotein_family_protein_putative_expressed
hypersensitive-induced_response_protein_putative_expressed
hypothetical_protein
inhibitor_I_family_protein_putative_expressed
initiation_factor_2_subunit_family_domain_containing_protein_expressed
invertase_pectin_methylesterase_inhibitor_family_protein_putative_expressed
jasmonate-induced_protein_putative_expressed
kinase_pfkB_family_putative_expressed
kinesin_motor_domain_containing_protein_expressed
kinesin_motor_domain_containing_protein_putative_expressed
L11_domain_containing_ribosomal_protein_putative_expressed
late_embryogenesis_abundant_protein_D-34_putative_expressed
late_embryogenesis_abundant_protein_putative_expressed
Leucine_Rich_Repeat_family_protein_expressed
lipin_N-terminal_conserved_region_family_protein_expressed
long_cell-linked_locus_protein_putative_expressed
LTPL1_-_Protease_inhibitor_seed_storage_LTP_family_protein_precursor_expressed
LTPL6_-_Protease_inhibitor_seed_storage_LTP_family_protein_precursor_expressed
LTPL68_-_Protease_inhibitor_seed_storage_LTP_family_protein_precursor_expressed

LTPL79_-
_Protease_inhibitor_seed_storage_LTP_family_protein_precursor_putative_expressed
LTPL94_-_Protease_inhibitor_seed_storage_LTP_family_protein_precursor_expressed
LYR_motif_containing_protein_putative_expressed
Mak16_protein_domain_containing_protein_expressed
membrane_related_protein_CP5_putative_expressed
mitochondrial_glycoprotein_putative_expressed
mitochondrial_transcription_termination_factor-related_putative_expressed
monocopper_oxidase_putative_expressed
MSP_domain_containing_protein_putative_expressed
MTN26L5_-_MtN26_family_protein_precursor_expressed
mttA_Hcf106_family_protein_putative_expressed
mutS_domain_V_family_protein_expressed
MYB_family_transcription_factor_putative_expressed
Myosin_head_domain_containing_protein_expressed
NADP-dependent_oxidoreductase_putative_expressed
NBS-LRR_type_disease_resistance_protein_Hom-B_putative_expressed
nitrilase_putative_expressed
nnrU_putative_expressed
no_apical_meristem_protein_putative_expressed
nuclear_prelamin_A_recognition_factor_putative_expressed
nucleolar_GTPase_putative_expressed
nucleolar_protein_5_putative_expressed
O-methyltransferase_putative_expressed
OsClp3_-_Putative_Clp_protease_homologue_expressed
OsDegp13_-_Putative_Deg_protease_homologue_expressed
OsFBDUF53_-_F-box_and_DUF_domain_containing_protein_expressed
OsFBX358_-_F-box_domain_containing_protein_expressed
OsFBX359_-_F-box_domain_containing_protein_expressed
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OsFBX362_-_F-box_domain_containing_protein_expressed
OsFBX363_-_F-box_domain_containing_protein_expressed
OsFBX390_-_F-box_domain_containing_protein_expressed
OsFBX426_-_F-box_domain_containing_protein_expressed
osFTL7__FT-
Like7_homologous_to_Flowering_Locus_T__gene%3B_contains_Pfam_profile_PF01161
%3A_Phosphatidylethanolamine-binding_protein_expressed
OsMADS73_-_MADS-box_family_gene_with_M-alpha_type-box_expressed
OsMADS74_-_MADS-box_family_gene_with_M-alpha_type-box_expressed
OsPOP20_-_Putative_Prolyl_Oligopeptidase_homologue_expressed
OsProCP3_-_Putative_Lysosomal_Pro-x_Carboxypeptidase_homologue_expressed
OsProCP4_-_Putative_Lysosomal_Pro-x_Carboxypeptidase_homologue_expressed
OsSCP8_-_Putative_Serine_Carboxypeptidase_homologue_expressed

oxidoreductase_aldo_keto_reductase_family_protein_putative_expressed
oxidoreductase_short_chain_dehydrogenase_reductase_family_domain_containing_protein
_expressed
patatin_putative_expressed
peflin_putative_expressed
pentatricopeptide_putative_expressed
pentatricopeptide_repeat_domain_containing_protein_putative_expressed
peptidase_M24_family_protein_putative_expressed
peptidase_T1_family_putative_expressed
peptidyl-prolyl_isomerase_putative_expressed
periplasmic_beta-glucosidase_precursor_putative_expressed
peroxidase_precursor_putative_expressed
peroxisomal_biogenesis_factor_11_putative_expressed
PHD_finger_family_protein_putative_expressed
PHD-finger_domain_containing_protein_putative_expressed
PHD-finger_family_protein_expressed
PHF5-like_protein_domain_containing_protein_expressed
phosphate_phosphate_translocator_putative_expressed
phosphatidylinositol-4-phosphate_5-kinase_putative_expressed
photosystem_II_P680_chlorophyll_A_apoprotein_putative_expressed
Plant_PDR_ABC_transporter_associated_domain_containing_protein_expressed
plastid_division_regulator_MinE_putative_expressed
pleiotropic_drug_resistance_protein_2_putative_expressed
plus-3_domain_containing_protein_putative_expressed
pollen-specific_protein_SF21_putative_expressed
POT_family_protein_expressed
powdery_mildew_resistant_protein_5_putative_expressed
PPR_repeat_containing_protein_expressed
prephenate_dehydratase_domain_containing_protein_expressed
protease_inhibitor_seed_storage_LTP_family_putative_expressed
protein_binding_protein_putative_expressed
protein_phosphotase_protein_putative_expressed
PsbP_putative_expressed
PTEN_putative_expressed
pumilio-family_RNA_binding_repeat_domain_containing_protein_expressed
pyruvate_kinase_putative_expressed
rab_GDP_dissociation_inhibitor_alpha_putative_expressed
RALFL25_-
_Rapid_ALkalinization_Factor_RALF_family_protein_precursor_putative_expressed
response_regulator_receiver_domain_containing_protein_expressed
retrotransposon_protein_putative_Ty1-copia_subclass
retrotransposon_protein_putative_Ty1-copia_subclass_expressed
retrotransposon_protein_putative_Ty3-gypsy_subclass
retrotransposon_protein_putative_Ty3-gypsy_subclass_expressed

retrotransposon_protein_putative_unclassified
retrotransposon_protein_putative_unclassified_expressed
retrotransposon_putative_centromere-specific
retrotransposon_putative_centromere-specific_expressed
ribosomal_protein_L24_putative_expressed
ribosomal_protein_putative_expressed
RING_zinc_finger_ankyrin_protein_putative_expressed
RIPER1_-_Ripening-related_family_protein_precursor_expressed
RNA_recognition_motif_containing_protein_expressed
RNA_recognition_motif_containing_protein_putative_expressed
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saposin-like_type_B_region_1_family_protein_putative_expressed
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SEY1_putative_expressed
SHR5-receptor-like_kinase_putative_expressed
SNARE_domain_containing_protein_putative_expressed
sodium_calcium_exchanger_protein_putative_expressed
splicing_factor_putative_expressed
src_homology-3_domain_protein_3_putative_expressed
SRPK4_putative_expressed
SSE1_putative_expressed
SSXT_protein_putative_expressed
stem-specific_protein_TSJT1_putative_expressed
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sulfotransferase_domain_containing_protein_expressed
SWIB_MDM2_domain_containing_protein_expressed
T-complex_protein_putative_expressed
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thaumatin_putative_expressed
thioredoxin_domain-containing_protein_9_putative_expressed
TKL_IRAK_DUF26-la.5_-
_DUF26_kinases_have_homology_to_DUF26_containing_loci_expressed
transcription_factor_jumonji_putative_expressed
transcription_factor_putative_expressed
transcription_initiation_factor_putative_expressed
translation_initiation_factor_IF-2_putative_expressed
transmembrane_9_superfamily_member_putative_expressed
transmembrane_and_coiled-coil_domain-containing_protein_4_putative_expressed
transmembrane_BAX_inhibitor_motif-containing_protein_putative_expressed
transporter_major_facilitator_superfamily_domain_containing_protein_expressed
transposon_protein_putative_CACTA_En_Spm_sub-class
transposon_protein_putative_CACTA_En_Spm_sub-class_expressed
transposon_protein_putative_Pong_sub-class_expressed
transposon_protein_putative_unclassified

transposon_protein_putative_unclassified_expressed
tRNA_synthetase_class_I_family_protein_putative_expressed
type_II_intron_maturase_protein_putative_expressed
ubiquitin_carboxyl-terminal_hydrolase_family_1_putative_expressed
ubiquitin-conjugating_enzyme_putative_expressed
ubiquitin-like_protein_5_putative_expressed
ulp1_protease_family_protein_putative_expressed
universal_stress_protein_domain_containing_protein_putative_expressed
unknown expressed protein
unknown transcription factor
UP-9A_putative_expressed
vacuolar_protein-sorting_protein_bro1_putative_expressed
WD_domain_G-beta_repeat_domain_containing_protein_expressed
WD_repeat-containing_protein_putative_expressed
white-brown_complex_homolog_protein_11_putative_expressed
WRKY89_expressed
WW_domain_containing_protein_expressed
xyloglucan_galactosyltransferase_KATAMARI1_putative_expressed
YT521-B-like_family_domain_containing_protein_expressed
zinc_finger_C3HC4_type_domain_containing_protein_expressed
zinc_finger_C-x8-C-x5-C-x3-H_type_family_protein_expressed
zinc_finger_family_protein_putative_expressed
ZOS12-03_-_C2H2_zinc_finger_protein_expressed
ZOS8-02_-_C2H2_zinc_finger_protein_expressed

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Chapter 6

General Discussion

Drought affects rice growth and yield. It was already known that in rice ABA plays an important role in the physiological response to drought stress. In Chapter 3 I showed that mild drought also induces SL production in rice roots, and that ABA levels and drought survival were higher in the SL-deficient rice mutants *d10*, *d17* and *d3*, while the *d27* mutant showed the opposite trend. These results suggest a possible link between the SL and ABA biosynthetic pathways during the drought response in rice, which is at least partially mediated by the enzyme OsD27. SLs and ABA also seem to play a role in the rice drought adaptation through microbial symbioses such as those with AM fungi, which I review in Chapter 2. In Chapter 4 I studied also other potentially beneficial microorganisms using an environmental amplicon sequencing approach. I showed that the fungal community associated with rice roots changes and that the fungal species diversity increases under drought conditions. Furthermore, the abundance of one fungal group, the Subphylum Pezizomycotina, was positively correlated with grain yield production under drought, suggesting that it may confer certain tolerance to drought in rice. The results in this Chapter also showed that the host plant genotype has an effect on the composition of the root fungal microbiome. I further investigated this host genotype effect with a Genome Wide Association Study (GWAS) in Chapter 5, which resulted in the identification of 184 different Single Nucleotide Polymorphisms (SNPs) that were found to be significantly associated with the abundance/occurrence of 44 different root fungal operational taxonomic units (OTUs). Some of the associated SNPs were found to be located in genome regions containing interesting candidate genes with putative functions in the plant immune response, as well as in responses to biotic and abiotic stress. Here in Chapter 6 I will discuss the main highlights of this thesis and how these data improve our understanding of drought tolerance in rice, as well as possible follow-up research and potential agricultural applications.

Rice response under water deficit conditions

As mentioned above, the formation of ABA is one of the responses of plants when exposed to drought. However, water deficit conditions trigger a cascade of responses that change the source-sink balance to improve survival and reduce yield penalty (Albacete et al., 2014). In rice, drought provokes a decrease in overall plant growth and causes leaf rolling. The latter serves as a mechanism to reduce the leaf area exposed to wind and UV light, in order to reduce transpiration and subsequent leaf dehydration. The reduction of transpiration is an important water-saving mechanism that is mainly achieved by stomata closure. The stomata close as a result of the production of ABA in leaves. As a result of these responses, nutrient translocation

is impaired and grain filling and size are reduced (Pandey and Shukla, 2015). ABA also interacts with other phytohormones to induce architectural changes in response to water deficit (Davies et al., 2005; Du et al., 2013). During the rice response to drought, ABA interacts with cytokinins (CKs), jasmonic acid (JA) and ethylene in leaves (Kazan, 2015) and auxins (IAA) in roots (Hong et al., 2013). Recent studies have shown that the SLs also play a role in drought adaptation in tomato, *Lotus* and *Arabidopsis* (Bu et al., 2014; Ha et al., 2014; Visentin et al., 2016). ABA levels also seem to be linked with SL levels in tomato through an as yet unknown mechanism (López-Ráez et al., 2010). In this thesis, I also showed that SL levels are affected by drought and that ABA levels differ among SLs-deficient mutants. This suggests that in rice there also is a link between ABA and SLs (Chapter 3). Furthermore, SL-deficient mutants showed different drought survival rates when compared with wild–type plants, which is likely to be mediated through their different levels of ABA.

In Chapter 3 I found that during mild drought, SLs levels in rice roots increase. This is similar to what happens during other abiotic stresses such as phosphate and nitrate starvation, where SLs have been shown to play a role in root architectural changes through an interaction with auxin (Ruyter-Spira et al., 2011; Sun et al., 2014). In rice ecotype Shiokari, low phosphate-induced SL biosynthesis was shown to be involved in the induction of primary root length, and at the same time contributed to a reduction in the length and number of lateral roots (Sun et al., 2014). Drought also affects rice root architecture, and some ecotypes show an increase in primary root length (Gowda et al., 2011; Kadam et al., 2015). A change to longer primary and secondary roots, for example, will result in improved water uptake as the plant now can reach deeper water patches (Gowda et al., 2011).

In *Arabidopsis thaliana* the effect of drought on primary root length is not SL-dependent (Ha et al., 2014). However, different water regimes can result in different responses, and during the latter study only severe water deficiency was tested. Under mild drought, shoot growth is reduced while roots can keep growing (Parent et al., 2010) whereas under severe drought both shoot and root growth are severely affected (Moumeni et al., 2011). It is my hypothesis that while increased SLs biosynthesis during mild drought stimulates root growth, severe drought will shut down SL biosynthesis, ultimately contributing to an arrest in root growth.

The genes the expression of which changes the most in response to drought in rice include aquaporins, transcription factors (TFs) such as AP2/EREBP, bZIP, NAC and MYB types, Late Embryogenesis Abundant (LEA) proteins, osmoprotectant-synthesizing enzymes, protein

kinases such as MAPK, CDPK and SnRK, metallothionein and genes encoding cytochrome P450 family proteins (Hadiarto and Tran, 2011). Indeed, during drought, the ABA increase not only induces stomata closure but also the accumulation of osmoprotectant proteins like LEAs (Hadiarto and Tran, 2011). The ABA-dependent pathway of drought responses in rice involves specifically the TFs OsDREB2, OsNAC10, OsZIP23 and OsAREB1 that induce expression of OsLEA3-1 and several cytochrome P450 genes and F-box proteins (Borah et al., 2017; Degenkolbe et al., 2009; Hadiarto and Tran, 2011). It will interesting to study whether SLs also affect the expression level of genes belonging to these ABA-dependent signalling pathways during different levels of drought severity.

The symbiont-host catch-22

Above, I mentioned that rice plants adapt their responses when experiencing a water deficit and discussed the potential role of SLs on root architecture during drought. As explained in Chapter 2, SLs also have another role in the rhizosphere, where they promote AM fungal hyphal branching and, consequently, colonisation of the roots by these fungi. SLs could potentially act as signals, possibly together with flavonoids and other signalling molecules, also for other mutualistic (and perhaps also pathogenic) microorganisms (Steinkellner et al., 2007). However, such a role for SLs in plant interactions with non-mycorrhizal root-colonising fungi is as yet unknown.

In Chapters 4 and 5 I have shown that drought modifies the root-associated microbiome by enhancing fungal diversity. How plants recruit microorganisms to conform their root microbiome is an emerging research topic. The root-associated microbiome is composed of different groups of microorganisms, including viruses, oomycetes, bacteria and fungi, with the latter two being the most intensively studied (Lareen et al., 2016). There are several factors involved in the establishment of the symbiosis with bacteria and fungi, including hormones and signalling (Akamatsu et al., 2016; Hardoim et al., 2015). In Chapter 5 I performed a GWA study aimed at the identification of rice genomic loci associated with fungal species in the root microbiome in two different treatments, well-watered and drought conditions. I found a group of *a priori* candidate genes that might be involved in regulating the abundance and/or occurrence of specific root fungi. Some of these candidate genes encode disease resistance proteins that are involved in fungal recognition and plant innate immunity responses, such as nucleotide binding site leucine-rich repeat (NBS-LRR) proteins (e.g. SHR5), tyrosine kinase-

like (TKL) IRAK (interleukin-1 receptor-associated kinase) proteins and synaptosome-associated protein receptor (SNARE) proteins (Akamatsu et al., 2016; Lipka et al., 2007; Wang et al., 2014). The majority of plant disease resistance genes that have been characterized to date encode nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins. They have an ancient origin and form one of the biggest gene families in rice, with around 400 genes (McHale et al., 2006). The fact that they are encoded in all land plants together with their ancient origin may lead to the hypothesis that they play a role not only on pathogenic interactions, but also on mutualist associations. Furthermore, some of the molecular strategies used by pathogens and mutualistic microorganisms to colonize roots are similar, only their effect on the plant immune system differs. Microorganisms emit signals called microbe-associated molecular patterns (MAMPs) that can be recognized by plant receptors (PRRs), such as NBS-LRR-like receptors. This can subsequently trigger the plant immune system but whether that happens depends on many factors, a.o. on the fact of whether the micro-organism is pathogenic or mutualistic. For example, *A. thaliana* phosphate-starved roots activate defence responses upon pathogen infection, whereas root transport and cell growth responses are active during association with mutualistic microbes (Hacquard et al., 2016). This supports the idea of plants not activating their immune responses in during the initial stages of the interaction with beneficial root-associated microorganisms to facilitate colonisation.

Among the candidate genes that are present in the LD-regions of SNPs associated to the abundance of fungal OTUs, we also found genes encoding dehydration responsive element binding (DREB) proteins, F-box proteins and cytochrome P450 enzymes. There are studies showing an up-regulation in gene expression for cytochrome P450 enzymes and DREB proteins after pathogen infection in rice (Jain et al., 2017). Two of our candidate cytochrome P450 genes (LOC_Os12g04480 and LOC_Os05g29750) were also up-regulated upon infection with a fungal pathogen of rice, *Magnaporthe oryzae*, and down-regulated upon ABA treatment in root and shoot tissues (data from RiceXpro database: http://ricexpro.dna.affrc.go.jp/). Probably this gene candidates are involved in the plant response to symbiotic fungal OTUs associated with SNPs in certain rice genotypes, by modulating the degree of root colonization by these fungi.

Because the above mentioned *a priori* candidate genes for the regulation of the abundance of specific OTUs (DREB, cytochrome P450s and F-box) are related with the ABA-dependent response to drought, and we observed that ABA and SL levels influence each other, it could be that SLs are also involved in this process. However, no SL related candidate genes were found

in the genomic loci associated with OTU occurrence or abundance, which precludes to conclude on the direct involvement of SLs in the plant's symbiosis with endophytic fungi.

Although we did not find candidate genes for SL biosynthesis or signalling, it is well known that SLs play an important role in the recruitment of AM fungi by roots. This leads to the intriguing question of whether SLs might also be involved in the plant interaction with other fungal symbionts. Experiments performed so far showed that the level of SL exudation into the rhizosphere depends on the plant species and environmental conditions (Chapter 2), and this could hence affect possible interactions with other microorganisms. It has been shown that the transcription factors NSP1 and NSP2, both involved in rhizobium nodulation, affect SL biosynthesis through DWARF27 in rice (Liu et al., 2011). This links SLs to a non-mycorrhizal symbiotic relationship such as bacterial nodulation. Moreover, SLs have been shown to contribute to the resistance against pathogenic fungi (Decker et al., 2017; Steinkellner et al., 2007). Altogether, these data suggest a possible role for SLs in the interaction of plant roots with microorganisms other than non-mycorrhizal fungi. New research, for example involving assessments of microbiomes of SL-deficient mutants versus wild type plants, could help resolve the implication of SLs in determining the structure and composition of root microbiomes.

Using available *in silico* data from gene expression studies in rice, I confirmed that the expression levels of the candidate genes correlated with the abundance of OTU_0021, OTU_0043, OTU_0115, OTU_0150, OTU_0157 and OTU_0172, which encode for DREB -, F-box- and cytochrome P450s proteins, are correlated with one another (Figure 1). Some of these OTUs were linked to SNPs only under control or drought conditions. The correlation in gene expression among the candidate genes linked to the different OTUs suggests a possible similar role during the plant-fungus interaction.

The DREB, P450 and F-box genes, which show the strongest correlation among one another (i.e. thicker edges in the network analysis from Fig. 1), also have their expression changed in response to infection by fungal pathogens, which triggers up-regulation of DREB and P450 genes and down-regulation of the F-box gene (data from RiceXpro database: http://ricexpro.dna.affrc.go.jp/). These transcriptional changes upon fungal infection may suggest a possible role in other fungal root colonisation processes as well. For instance, in *A. thaliana* and rice it has been described that a gene encoding an F-box protein (DIF1) was up-regulated by drought in an ABA-dependent manner. The overexpression of this F-box gene resulted in increased drought sensitivity in both plants (Gao et al., 2017; Yan et al., 2011).

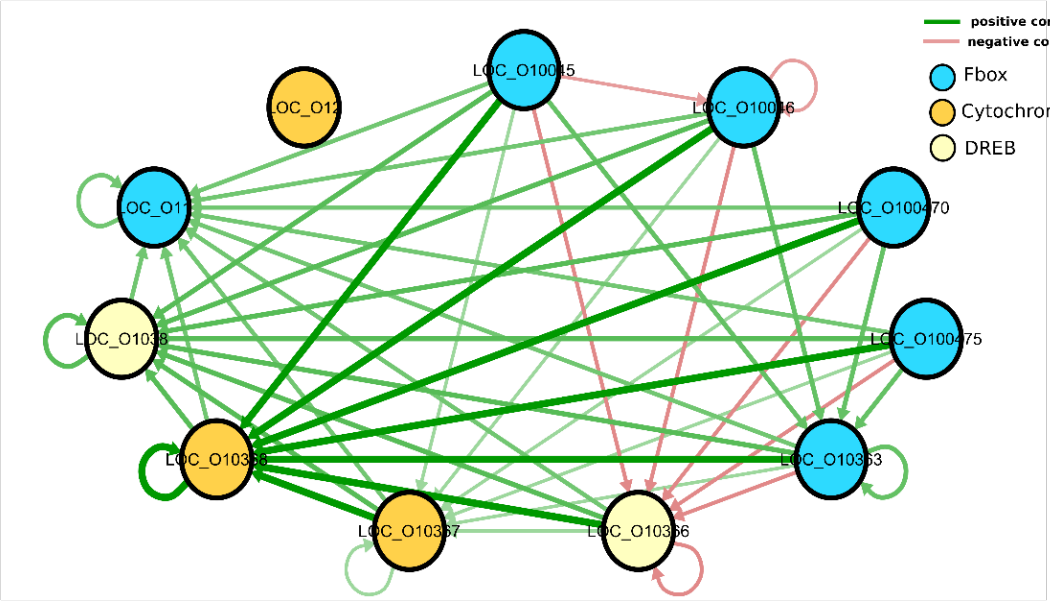

Figure 1. Gene expression correlation network between all cytochrome P450, DREB and F-box genes, that reside in LD blocks of SNPs that were genetically linked with the abundance of specific OTUs in the GWA study (Chapter 5). Correlation intensity is represented by the width of the network edges. Data were retrieved from the PLANEX co-expression database (http://planex.plantbioinformatics.org/).

The *in silico* analyses, together with the results from other studies, support that it would be interesting to study our candidate genes further to explore their role in the fungal-mediated stress responses in rice. Albeit the majority of the SNPs associated with OTUs from our study were found under control conditions, some of them not only correlated with plant yield obtained from plants grown in water sufficient conditions, but also with yield plasticity (difference in yield between control and drought treatments). Possibly, it is important to engage a relationship with a beneficial microbiome already before an environmental stress appears. The connection between root associated fungi, plant environment and plant fitness raises an intriguing question: who is ultimately responsible for improved plant fitness during water deficient conditions? The host or its microbiome? If we see the plant as a holobiont, the answer for this catch-22 would be that both of them are responsible. As commented above, drought triggers plant metabolic and phenotypic changes, including changes of the plant's root microbiome. Likewise, some microbial clusters impact plant fitness in an environment-dependent way, as results from Chapters 4 and 5 showed. There, I found groups of OTUs that positively correlated with plant yield, but these associations were specific for the drought treatment—the effect was not found in control treatments. This has also been shown to occur for the bacterial microbiomes in

grapevine under drought (Rolli et al., 2015). Furthermore, when plants are growing under optimal conditions, the host plant triggers immune responses towards all root fungal endophytes. When growing under less favorable conditions causing stress, the plant's defense is decreased, and the symbiotic response toward mutualistic microbes is activated (Hacquard et al., 2017). These microbial clusters act jointly hereby triggering a response in the host, which will finally lead to the adaptation to the environmental stress (Figure 2).

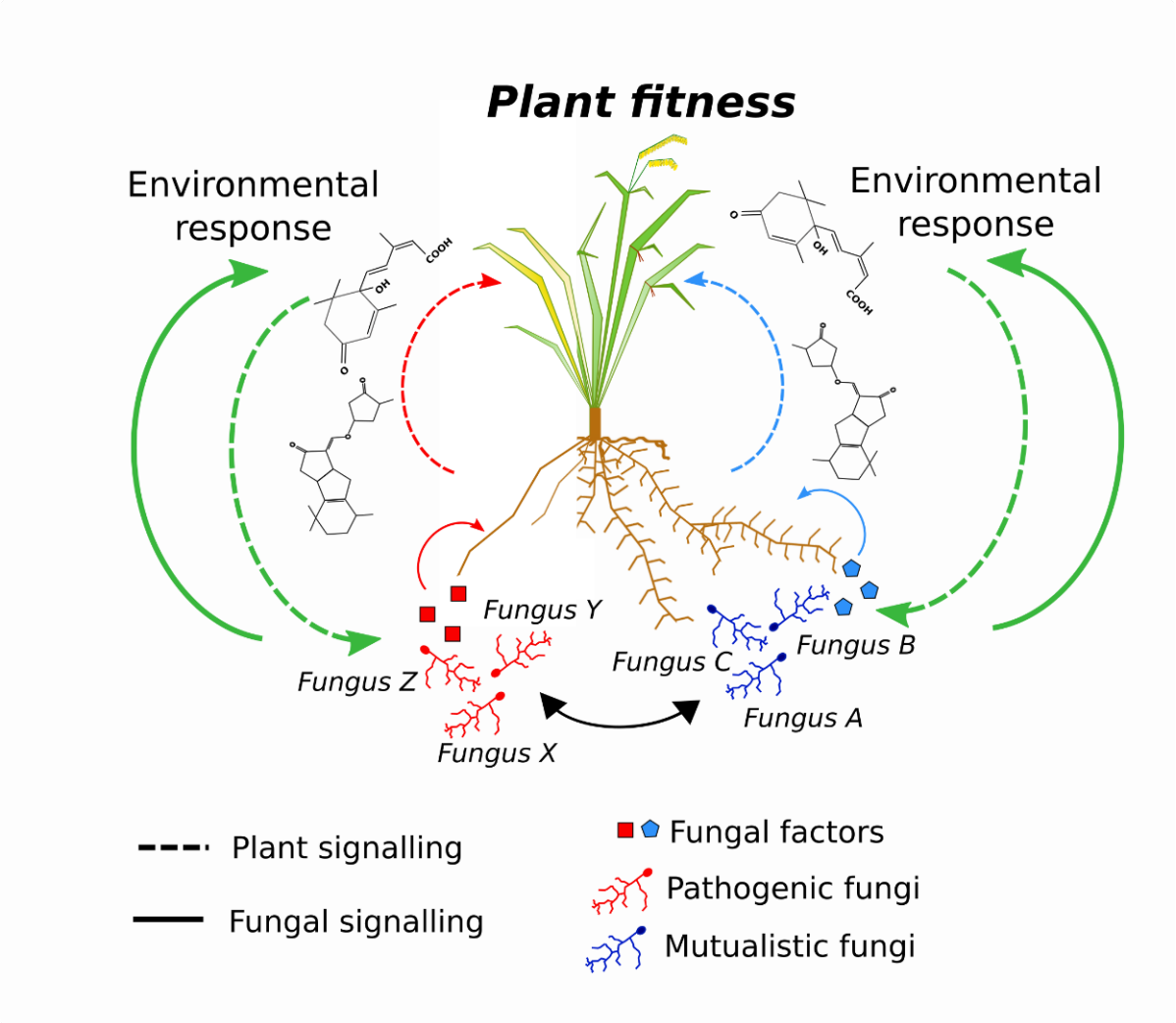

Figure 2. Scheme explaining the hypothetical feedback between a host plant and its root associated fungal microbiome. The host plant recruits a microbiome that is formed by different functional clusters or hubs (e.g. pathogenic/non-pathogenic). Simultaneously, microbial clusters communicate with the plant trough microbial factors. These factors initiate a cascade of biochemical responses in the plant resulting in a specific phenotype. If there is an environmental stress, plants and their microbiome communicate with each other and the host starts a cascade of responses to establish an ultimate adapted/non adapted phenotype. Furthermore, different microbial hubs communicate and influence each other.

Future perspectives: breeding for plant microbiomes?

Currently, the study of microbiomes and their application in agriculture is a hot topic in plant research (Parnell et al., 2016). It has been shown that the host partially influences the recruitment of the microbiome (Bulgarelli et al., 2015; Horton et al., 2014; Wagner et al., 2016) and this also applies to rice (Chapter 4). Furthermore, in *A. thaliana* it has been shown that plants can be influenced to select for beneficial microbial partners that can be transferred to the soil and simultaneously influence the soil microbiota (Panke-Buisse et al., 2015) and its own leaf and root microbiomes (Bai et al., 2015). Associated microbiomes can be considered as plant traits as they are intricately intertwined with plant biology. Moreover, plant associated microorganisms can respond to environmental inputs in favour of the host. Certain microorganisms can be transmitted vertically to new generations and horizontally to other plants in the surrounding environment (Rosenberg and Zilber-Rosenberg, 2016; Vandenkoornhuyse et al., 2015). Additionally, plant associated microbes communicate with each other and form groups (hubs) that can impact plant host fitness (Agler et al., 2016; van der Heijden and Hartmann, 2016), as I also showed in rice (Chapter 4 and 5). For instance, we found two groups of correlated OTUs for which the relation seems stable in both treatments, control and drought. A cluster of correlated OTUs may serve as a toolbox with new genes to be used by the plant in a fast changing environment. Different OTUs from the same cluster could be specifically selected for depending on the environmental condition.

Recent reviews suggest ‘breeding for microbiomes’ (Wei and Jousset, 2017). As part of the plant, its associated microbiome is a trait suitable to be selected to improve plant fitness. There is a possibility to generate artificial microbial communities tailored for particular needs that can be transferred to gnobiotic plants (Beattie, 2015). Indeed, we could select for beneficial microorganisms against a specific stress and then inoculate them to a microorganism-free plant, for example generated *in vitro*. However, this approach relies on gnobiotic systems, meaning that the impact on plant fitness would depend just on the microbial species we would inoculate it with. Thus inoculated plants would be tailored to specific environmental conditions, which would make them unable to respond to other environmental stresses, as they would lack their natural plant microbial diversity. A different approach called ‘holobiont breeding’ is based on the vertical transmission of microorganisms from flowers to seeds. This approach might help to contribute to a desired plant phenotype, with the additional benefit that the microorganisms might spread from plant to soil and be transmitted horizontally (Wei and Jousset, 2017). Still,

with these approaches we are limited to the microbes that can be cultivated, which are a minority, especially among bacteria. However, if we gather more knowledge about how microbial hubs act, and how these microbes influence each other, perhaps applying specific microorganisms could attract or stimulate others from the same hub in the roots or soil. Moreover, we need more information on how interactions in the endophytes-plant-environment network are regulated, what mechanisms stimulate or hinder the survival of microbes in plant tissues and how their transmission to future generations is mediated. One of the drawbacks from the presented candidate genes found to be correlated with the OTUs abundance in the roots is their narrow-sense heritability values. These values were generally very low, being just acceptable for 7% of the OTUs showing positive SNPs. This may hamper the chances of a potential application, as it could not be applied to all rice cultivars. An alternative would be to find loci related to the abundance of taxonomically related OTUs (e.g. Orders) or functionally related OTUs (e.g. positive correlated OTUs). Furthermore, the output of this analysis might have a more realistic potential application, as it would be easier to make plants to select for specific taxa rather than for independent OTUs/species.

My thesis sheds some light on the role of SLs and the host-fungal root microbiome in drought tolerance in an important crop like rice. I found interesting candidate genes, including *SNARE*, *NBS-LRR*, *DREB* and a cytochrome P450 which may be related to plant symbiotic (pathogenic/mutualistic) responses and which heritability values are reasonable (h^2 = 0.15–0.30) in order to be considered for possible applications. This information, as well as the intriguing role of DWARF27 in both ABA and SL biosynthesis, will hopefully provide the opportunity to further explore SLs and microbial traits for future rice breeding programs towards more drought tolerant plants with acceptable yields in a water limiting environment.

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Summary

Rice is the most important human food crop in the world, feeding over half the world's population. However, rice water use efficiency (WUE), as defined by units of yield produced per unit of available water, is the lowest of all grain crops. Therefore, most rice cultivars need water-logged conditions to achieve maximum grain yields. Since global warming and water scarcity pose a threat to crop production and are reinforcing the effect of drought on plant yields, there needs to be a better understanding of rice drought adaptation mechanisms. For agricultural practices it is of interest to be able to select favourable rice traits for drought adaptation without yield penalty.

In the past, many studies have led to the identification of genetic loci that are involved in drought adaptation mechanisms in rice. However, due to the complexity of the traits that contribute to drought tolerance, there is still the need to further zoom into these loci in order to unravel how these mechanisms work at the molecular level during different types of drought and in different cultivars. Furthermore, including plant yield as a target in drought adaptation studies makes it more challenging because yield itself is also a complex trait defined by several other factors. A new approach to address processes involved in rice drought adaptation would be to study the plant as a holobiont. Hence it would be useful to study the presence and diversity of rice associated microbiota and their role in plant fitness under water deficit conditions.

The aim of this thesis is to study novel drought related physiological aspects in rice. First of all, the essence of the plant hormone strigolactone during water deficient conditions, and its interaction with the drought-related hormone abscisic acid, were adressed. Secondly, the composition of the root associated fungal microbiome was characterized in a large collection of rice ecotypes grown in the field, with the aim to explore how this microbiome interacts with the rice plant ultimately affecting plant yield, and to what extent the plant itself is able to control the selection of specific beneficial fungi.

In **Chapter 2**, I review the biological and ecological importance of strigolactones in the rhizosphere. Strigolactones are involved in plant responses to abiotic stresses such as phosphate shortage, drought and salinity. During these conditions, they are involved in stimulating adaptive changes in root architecture and in establishing an interaction between plants and microorganisms such as arburscular mycorrhizal (AM) fungi, rhizobia and root parasitic plants. Due to these characteristics, the agronomical implications of strigolactones and their potential use in sustainable agriculture were addressed.

Considering the common biosynthetic origin of the strigolactones and abscisic acid, and their role in plant development and fitness, the relationship between both phytohormones is explored in rice (**Chapter 3**). It is shown that mild drought simultaneously induced strigolactone production in rice roots, and abscisic acid production and the expression of strigolactone biosynthetic genes in the shoot. Furthermore, abscisic acid levels in the shoot were higher in the strigolactone deficient rice mutants *d10*, *d17* and *d3* when compared with their wild type. These differences were more evident under drought. Interestingly, the drought survival rates of these strigolactone deficient lines were higher as well. However, one of the strigolactone deficient lines, *d27*, failed to increase levels of abscisic acid under drought conditions and showed a more drought sensitive phenotype when compared with the other strigolactone deficient lines. Furthermore, over-expression of the OsD27 gene in rice resulted in increased ABA levels when compared with wild type plants. These results suggest a possible link between the strigolactone and abscisic acid biosynthetic pathways during the drought response in rice, and that this is at least partially mediated by the strigolactone biosynthetic enzyme OsD27.

In **Chapter 4** I describe that the rice root associated fungal community changed under drought conditions. Furthermore, the root associated fungal community diversity (H') was found to be increased as a result of the drought treatment. Interestingly, one group of fungi, all belonging to the Subphylum Pezizomycotina, was positively correlated with plant yield under drought. Finally, the result of this study indicated that the host plant partly determined the composition of the root fungal microbiome.

The latter result triggered me to take this study to the next level, during which I investigated the presence and localization of specific regions in the rice genome that (partly) determine the level of symbioses with mutualistic fungi during water sufficient and limiting conditions (**Chapter 5**). For this purpose, we performed a Genome Wide Association Study (GWAS), using a population of 296 rice cultivars for which the root endophyte microbiome was characterized using a high throughput sequencing pipeline. This led to the identification of 184 different Single Nucleotide Polymorphisms (SNPs) that were found to be significantly associated with the abundance of 44 different root fungal species (OTUs). The majority of the associations were observed under control conditions. Some of the associated SNPs were found to be located in genome regions containing interesting candidate genes with same functions as other genes previously described to be involved in biotic and abiotic stresses.

In **Chapter 6** I discuss the main highlights of the present thesis, and how the data generated during the course of this thesis improve our understanding of drought tolerance in rice. Finally, I comment on possible directions for follow-up research and potential agricultural applications.

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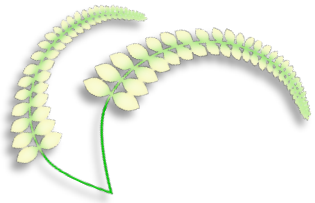

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