Molecular regulation of drought tolerance in rice

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Dedicated to my whole family … !!!

Contents

Chapter *1*

General introduction

Rice plant and water

Rice belongs to the Gramineae, which includes two cultivated and twenty-one wild species of the genus *Oryza*. Cultivated species are *Oryza sativa* (Asian rice) and *Oryza glaberrima* (African rice), of which the first is grown worldwide, while the latter is only grown on a limited scale in West Africa. Cultivated rice (*Oryza sativa*) evolved from a semi-aquatic, perennial ancestor, wild rice (*Oryza rufipogon*), and differentiated into two varietal groups: *indica* and *japonica* (Khush 1997). Like wheat and barley, rice is a C3 species, while sorghum and maize are C4 plants. Rice has different morphological and physiological characteristics than other cereals. One of the most striking differences is the presence of aerenchyma in stems and roots to allow oxygen flow from the leaves to the roots under anaerobic soil conditions. Rice leaves are generally quite thin and the number of stomata is ten-fold higher than in other plants species but are smaller in size. The leaf cuticle of rice is thin, with cuticular resistance for water loss comparatively lower than in other crops. Generally, rice has a lower photosynthetic rate and lower water use efficiency (WUE) than other cereals (Bouman et al. 2007) and as a consequence rice consumes up to two to three times more water than other cereals such as wheat or maize (Bouman 2009). Despite the different morpho-physiological features, rice is an important model species for other cereals due to its small genome size (430 Mb for *japonica* rice). The rice genome is only one sixth the size of the maize genome and 40 times smaller than the wheat genome (IRGSP 2005). Despite large differences in genome size and ploidy level, the genomes of rice and other monocot cereals are highly conserved.

Rice is grown under many different climatic conditions in at least 95 countries. It is used as a staple food for more than half of the world's population, particularly in the Asia-pacific region where 90% of the world's rice is produced and consumed (Khush 2005). Rice production in the world in total consumes about 1,600 km^3 of water, which is about 30% of the fresh water used for agriculture worldwide (Bouman 2009). The global concern about food shortage, however, is increasing with the growing world population and therefore it is crucial to increase rice production in a sustainable manner. Therefore, the world annual milled rice production of 470 million tons (IRRI 2013) should be increased to 850 million tons (so by more than 40%) by 2025 to meet the growing demand. The main constraints for such increase are abiotic stresses which are the primary cause of crop failure worldwide, reducing average yields by more than 50% for most major crop species (Bray et al. 2000). Among the various forms of abiotic stress, drought or water stress is the most limiting factor for rice productivity. Drought affects about 20% of the total rice area in

Asia (Pandey and Bhandari 2008). Therefore, feeding the world's population in the 21st century will require a second revolution in agriculture, to produce more rice with less water implying the efficient use of resources for sustainable crop production (Keating et al. 2010; FAO 2012). Water-saving rice systems, such as the system of rice intensification, ground-cover rice production, alternate wetting-drying and aerobic rice can considerably cut down the unproductive water loss and hence increase WUE. However, these water management practices almost always result in a yield penalty, which has generally been regarded to be unavoidable (Farooq et al. 2009). Although traditional breeding to improve crops for drought tolerance has met some success, it is a slow process. Therefore, the use of a molecular approach to develop new rice cultivars with enhanced drought tolerance provides an alternative, and hopefully fast, avenue to increase total rice production while at the same time improving WUE.

Mechanisms of drought resistance

Plants use various mechanisms to cope with drought stress and can adopt either a *drought escape* or a *drought resistance* strategy. In physiological terms, *drought resistance* can be further classified into *drought avoidance* and *drought tolerance* (Levitt 1980; Price et al. 2002). An overview of the key physiological traits associated with drought resistance in plants is shown in Figure 1.

Drought escape allows the plant to complete its life cycle before the arrival of drought. This escape mechanism usually implies a short life cycle (early flowering and maturity), developmental plasticity and remobilisation of pre-anthesis stored assimilates to the grain. *Drought avoidance* implies maintenance of a relatively high tissue water potential during periods of drought stress. Drought avoidance is usually associated with physiological changes such as reduced epidermal (stomatal and cuticular) conductance for water to reduce water loss, development of a deep and extensive root system for improved water uptake, reduced evaporation surface and reduced absorption of radiation (reduced leaf area and leaf rolling). *Drought tolerance* is the ability of plants to maintain their normal function even with low tissue water potential. This is generally associated with cellular and metabolic changes such as turgor maintenance (by accumulation of compatible solutes) and protoplasmic resistance.

Improving drought resistance is complex and difficult to achieve and this complexity mainly comes from the diversity of the drought resistance mechanisms adopted by plants. In addition, the drought resistance mechanisms have some disadvantages as well. In case of drought escape, a short life cycle generally results in reduced grain yield. Drought avoidance strategies usually result

in reduced carbon assimilation and ultimately yield. Finally, in the case of drought tolerance mechanisms, increased solute concentration for osmotic adjustment may have a negative impact on plant growth. Therefore, adaptation of crops to drought stress must maintain a balance between the drought resistance mechanisms introduced to guarantee sustainable productivity (Mitra 2001).

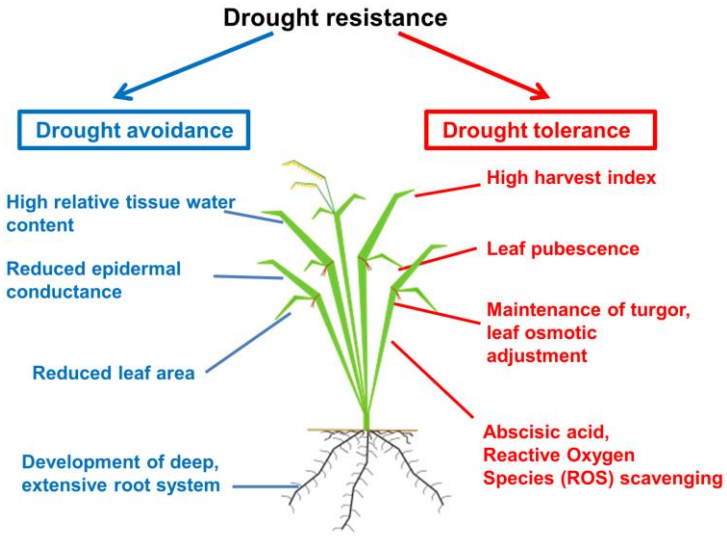

Figure 1. Key traits associated with drought resistance in plants.

In the past decades our understanding of molecular and cellular mechanisms involved in drought stress resistance has strongly increased, enabling us to integrate drought avoidance and tolerance strategies into single improved cultivars. All the research has also shown that many of molecular and physiological responses induced by drought do not match completely with the strict avoidance and tolerance division suggested by Levitt (1980) and others. For example, abscisic acid (ABA) is involved in stomatal conductance and root growth (Schroeder et al. 2001; Liu et al. 2005) (drought avoidance mechanisms), while at the same time, ABA can cause synthesis of dehydrins which function as chaperon-like protective molecules (Xiao and Nassuth 2006) (drought tolerance mechanism). Likewise, accumulated solutes like osmolytes may allow the plants to uptake more water, which is drought avoidance but solutes such as sugars and amino acids may also have a

protective role against membrane and protein damage, which in fact is a drought tolerance mechanism.

Molecular regulation of drought-inducible genes in rice

In response to drought stress, plants activate a wide array of biochemical and molecular processes in order to survive (Bartels and Sunkar 2005; Yamaguchi-Shinozaki and Shinozaki 2006). Any change in the normal growth of plants is perceived as stress and transduced in the form of signals such as protein phosphorylation/de-phosphorylation, calcium signalling, reactive oxygen species and ABA. Overall, the stress response will lead to the induction of gene expression that regulates the suit of biochemical and molecular processes involved in the response to drought mentioned above.

Recently, hundreds of drought-inducible genes in rice have been identified using microarrays, expressed sequence tags and qRT-PCR (Rabbani et al. 2003; Zhou et al. 2007; Gorantla et al. 2007; Degenkolbe et al. 2009; Todaka et al. 2012). Based on these, drought-inducible genes can be classified into two groups: Functional proteins and regulatory proteins (Shinozaki and Yamaguchi-Shinozaki 2007) (Figure 2). The first group (functional proteins), is involved in the protection and repair of plants cells from stress damage such as enzymes for osmolyte biosynthesis, chaperonins, late embryogenesis abundant (LEA) proteins, anti-oxidation or de-toxification proteins and various proteases. The second group (regulatory proteins) is involved in regulation of signal transduction and stress-responsive gene expression such as transcription factors (TFs), protein kinases, protein phosphatases, enzymes involved in phospholipid metabolism and biosynthesis of signalling molecules. The expression of stress-induced genes is largely regulated by specific TFs (Zhu 2002). It has been estimated that the rice genome contains 1,611 TF genes that belong to 37 gene families (Xiong et al. 2005). Stress-related TF families exhibit distinctive binding domains such as dehydration-responsive element binding (DREB) / C-repeat-binding factor (CBF), ethylene-responsive factor (ERF), basic-leucine zipper (b-Zip), ABA-responsive element binding (AREB)/ABRE binding factor (ABF), NAM (no apical meristem), ATAF (*Arabidopsis* transcription activation factor), and CUC (cup-shaped cotyledon) (NAC) conserved domain and homeodomain-leucine zipper (HD-Zip).

TF can bind DNA in a sequence specific manner which in turn can result in activation or repression of transcription. They bind to *cis*-regulatory elements, which are usually present in the proximal promoter region of many stress-inducible genes, including drought. The most common *cis*-regulatory element in ABA regulated genes is the ABA-responsive element (ABRE) which is

recognised by the bZip family. Dehydration-responsive element (DRE)/ C-repeat (CRT) element which is recognised by the DREB/ERF family regulates function in ABA-independent gene expression (Nakashima et al. 2009). One particular large group of TFs involved in stress responses in several plant species is that of the HD-Zip genes (Mukherjee et al. 2009) which is a subgroup of the homeobox genes (described in more details below).

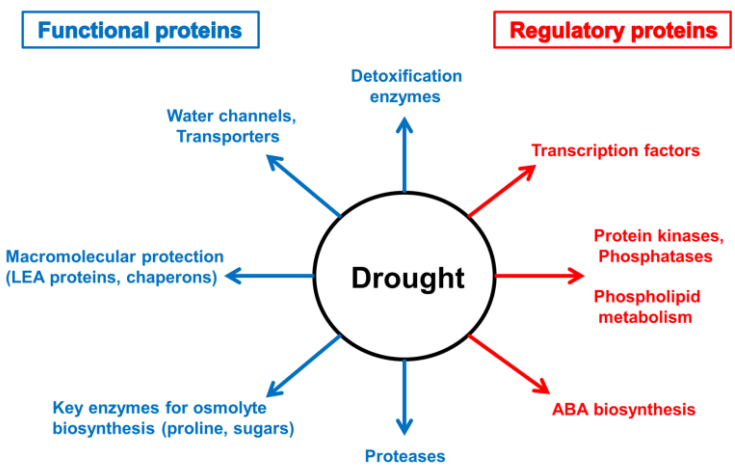

Figure 2. Classification of functions of drought stress-inducible genes. Gene products are classified into two groups. The first group includes proteins generally involved in stress tolerance (functional proteins) and the second group includes proteins involved in the regulation of signal transduction and gene expression (regulatory proteins) (adapted from Shinozaki and Yamaguchi-Shinozaki 2007).

HD-Zip family transcription factors

The homeobox (HB) is a 180 bp consensus DNA sequence which was first identified in the study of homeotic mutants in *Drosophila melanogaster* genes and thereafter in many other eukaryotes. The HB encodes a conserved 60 amino acid DNA-binding domain coined the homeodomain (HD) and its presence defines a whole class of TFs (Gehring 1994). In plants, the HD TFs can be classified into six super-families defined by their gene structures, HD sequence and association with other domains (Ariel et al. 2007). These super-families are the homeodomain-leucine zipper (HD-Zip), homeodomain with a finger domain (PHD finger), a distinctive BELL-type homeodomain (Bell-HD), Zinc finger with a homeodomain (ZF-HD), KNOX related homeobox (KNOX) and Wuschel related homeobox (WOX) (Bharathan et al. 1997; Chan et al. 1998). Among these, HD-

Zip is the largest-family, comprising 48 members out of 89 HD proteins in the *Arabidopsis* genome (Henriksson et al. 2005; Agalou et al. 2008; Mukherjee et al. 2009).

The HD-Zip families are characterised by the presence of a leucine zipper motif (Zip) directly after the HD (Ruberti et al. 1991). The HD is responsible for specific DNA-binding and the Zip domain acts as a dimerization domain (Sessa et al. 1997; Meijer et al. 1997, 2000; Himmelbach et al. 2002). The leucine zipper domain is also present in other transcription factors, like the basic leucine zippers (bZip), but its association with the HD is unique to plants. The HD-Zip super-family has been further divided into four families (I-IV) based on sequence homology, intron/exon pattern and additional conserved domains (Agalou et al. 2008). HD-Zip TFs have been associated with many diverse biological functions including vascular development, embryogenesis, leaf polarity as well as meristem regulation and developmental responses to environmental conditions (Scarpella et al. 2000; Ariel et al. 2007). In my work, I focused on functions of the HD-Zip family I genes, as explained below.

Functions of HD-Zip family I genes

HD-Zip family I TFs from various plant species are mainly involved in the regulation of development in response to changes in environmental conditions (Harris et al. 2011). For example, *Arabidopsis* (*Arabidopsis thaliana*) *Athb-5*, *-6*, *-7*, and *-12* (Johannesson et al. 2003; Olsson et al. 2004; Henriksson et al. 2005), sunflower (*Helianthus annuus*) *Hahb4* (Dezar et al. 2005), medicago (*Medicago truncatula*) *Mthb1* (Ariel et al. 2010) and *Nicotiana attenuata Nahd20* (Ré et al. 2011), are all induced by water deficit and ABA. Furthermore, in rice *Oshox6*, *-22* and *-24* (homologs of *Athb7* and *-12*), in the resurrection plant *Craterostigma plantagineum* (an extremely drought-resistant species) *CpHB-4*, *-5*, *-6* and *-7* (Deng et al. 2002) and in maize (*Zea mays*) 17 *Zmhdz I* genes, are also drought-responsive, either up- or down regulated (Zhao et al. 2011). However, there are just few reports about the role of the HD-Zip family I genes in biological processes. In tomato (*Solanum lycopersicum*), *LeHB1*, is highly expressed in flowers and developing fruits and its over-expression altered the morphology of the floral organs (Lin et al. 2008). In barley **(***Hordeum vulgare*), spikelets are arranged in triplets and in wild barley only the central spikelet is fertile (called two-rowed). *VRS1* encodes a HD-Zip family I transcription factor, of which the loss-of-function mutation, *vrs1*, induces the transition of two-rowed to six-rowed barley, having three fertile spikelets (Komatsuda et al. 2007). Despite its seemingly vital role in the response to drought, the function of the rice HD-Zip family I is poorly understood and so far only 14% (2 out of 14) of the genes belonging to this family have been characterised in rice.

Phytohormones and drought stress response

Plants are sessile organisms and must adjust their growth and development to continuously changing environmental conditions (Wolters and Jürgens 2009), including drought. Plant hormones such as auxin, ABA, cytokinin (CK), gibberellin (GA), ethylene, brassinosteroids (BRs), jasmonic acid (JA), salicylic acid (SA), nitric acid (NO) and strigolactones (Santner and Estelle 2009) play a central role in regulating plant growth and development in response to these environmental changes. However, these plant hormones do not act alone but are interconnected and modulate each other at the level of biosynthesis, degradation and signalling by cross-talk.

ABA is a well-studied phytohormone that is intricately involved in the response of plants to abiotic stresses, especially drought. Under drought, plants show increased levels of ABA, due to induction of ABA biosynthesis, which causes stomatal closure and reduced water loss through transpiration, eventually also limiting cellular growth (Yamaguchi-Shinozaki and Shinozaki 2006). Numerous genes associated with ABA biosynthesis have been well characterised in *Arabidopsis* and this knowledge is highly applicable to other plant species because ABA biosynthesis is highly conserved in angiosperms (Cutler et al. 2010). Exogenous application of ABA induces the expression of several drought-responsive genes (Zhu 2002; Shinozaki et al. 2003). However, several studies report that many drought-related genes do not respond to ABA, suggesting the existence of ABA-dependent and ABA-independent signal transduction pathways in drought stress response (Nakashima et al. 2009).

The roles of other phytohormones have also been reported in regards to drought stress responses directly or via interplay with ABA (Fujita et al. 2006). The earliest response of plants to drought stress is the stomatal closure. This rapid reaction is primarily regulated by the major player ABA (Daszkowska-Golec and Szarejko 2013). However, several studies propose that other hormones such as BRs, SA, JA and NO act synergistically with ABA to regulate stomatal closure, whereas CK and IAA act antagonistically with ABA (Acharya and Assmann 2009; Peleg and Blumwald 2011). Indeed, many drought-related genes are annotated as responsive to other phytohormones such as CK, auxin, JA, ethylene, BRs, and GA (Huang et al. 2008).

Unlike ABA, exposure of plants to drought stress results in decreased levels of CK (zeatin and zeatin riboside) in the xylem. In the same study, however the level of the CK 6-benzylaminopurine (BAP) was increased (Alvarez et al. 2008). Furthermore, in tobacco and rice, expression of *ISOPENTENYL TRANSFERASE* (*IPT*), encoding a rate-limiting enzyme in CK biosynthesis, with a drought/maturation inducible promoter resulted in enhanced drought tolerance (Rivero et al. 2007;

Peleg et al. 2011). These results suggest that increased CK may promote survival under drought stress conditions.

Recently, a role for auxin in abiotic stress response was also postulated. In rice, numerous auxin-responsive genes were differentially affected by various abiotic stresses, including drought (Jain and Khurana 2009). The Gretchen Hagen3 (GH3) gene family is responsible for maintenance of auxin homeostasis through the conjugation of excess indole-3-acetic acid (IAA) to amino acids (Chapman and Estelle 2009). Members of this gene family are also regulated by phytohomones and abiotic stress, including ABA and drought (Du et al. 2012). *TLD1,* a rice *OsGH3.13*, encoding an IAA-amido synthetase, was cloned from a gain-of-function mutation *tld1*-D (an increased number of tillers, enlarged leaf angles, and dwarfism in rice). This gene was exclusively induced by drought stress and over-expression of *TLD1*/*OsGH3.13* in the rice *tld1-D* mutant resulted in IAA deficiency and enhanced drought tolerance, which was due to the accumulation of *LEA* (*late embryogenesis abundant*) mRNA (Zhang et al. 2009). Moreover, a rice carotenoid-deficient mutant, with non-functional PHYTOENE SYNTHASE (*phs3-1)* and a transgenic knock-down of *PHYTOENE DESATURASE* through *PDS*-RNAi were impaired in the biosynthesis of ABA as well as IAA. Under drought stress conditions, the *phs3-1* and *PDS*-knock-down rice showed decreased drought tolerance (Du et al. 2013).

ABA is also a regulator of strigolactone (SL) biosynthesis, as shown using a set of ABA-deficient tomato mutants impaired at various points in the ABA biosynthetic pathway and specific inhibitors for different carotenoid cleaving enzymes (López-Ráez et al. 2008, 2010). As mentioned above, ABA is known to be involved in drought stress tolerance. Therefore, the interaction between ABA and SLs may imply that strigolactones also play a role during drought stress. SLs are rhizosphere communication molecules – that mediate the interaction between plants and parasitic weeds and between plants and arbuscular mycorrhizal (AM) fungi - as well as a relatively new class of plant hormones (as reviewed by Ruyter-Spira et al. 2013; Zhang et al. 2013). The biosynthesis of SLs is highly elevated by abiotic stresses such as nutrient deficiency, particularly phosphate as shown in several plant species, including rice (Jamil et al. 2011). AM fungi improve water and phosphate availability to plants (Ruiz-lozano 2003). In this way, AM plants are more tolerant to abiotic stresses such as drought and nutrient starvation. Considering the role of AM fungi in phosphate uptake, the up-regulation of strigolactone production (which will activate the AM fungi) under phosphate deficiency is not surprising. So far there is no information concerning the regulation of strigolactone biosynthesis by drought stress. The fact that ABA and SLs share a common

biosynthetic origin makes it even more intriguing to explore the mechanism by which they influence each other's levels. All these observations further highlight the importance of phytohormones' cross-talk in the response of plants to drought stress.

Genetic engineering for drought tolerance in rice

Genetic engineering has been used as a powerful tool in the development of drought tolerance in rice. Over-expression of the TFs that regulate the transcription of a large array of down-stream drought-responsive genes seems to be a more attractive and promising approach for improving drought tolerance when compared to the expression of functional protein encoding genes (Hadiarto and Tran 2011). Many TFs, including DREBs, ERF/AP2, Zn-finger, b-Zip, NAC and HD-Zip, can be induced by drought and genetic engineering of these TFs was shown to improve drought tolerance in plants (Shinozaki and Yamaguchi-Shinozaki 2007) (Table 1). For example, over-expression of *AtDREB1A* in rice resulted in improved tolerance to drought and salt stresses (Oh et al. 2005). Similarly, over-expression of *STRESS-RESPONSIVE NAC* (*SNAC1*) induced drought tolerance in rice under field conditions (Hu et al. 2006). Also, over-expression of rice *AP37* enhanced drought tolerance and increased grain yield under field conditions (Oh et al. 2009). Most recently, Yu et al. (2013) showed that over-expression of *ARABIDOPSIS ENHANCED DROUGHT TOLERANCE1* (*ATEDT1*)/*HOMEODOMAIN GLABROUS11* (*HDG11*) gene in rice increases drought tolerance without yield penalty.

As discussed above other regulatory factors like ABA also play an important role in the improvement of drought tolerance in rice. Some of the ABA pathway genes have been manipulated in rice to try to change drought tolerance. *DSM2* encodes a β-carotene hydroxylase (BCH) that catalyses the conversion of β-carotene to zeaxanthin and constitutive over-expression of *OsDSM2* enhanced drought tolerance by increasing the xanthophylls and ABA synthesis in rice (Du et al. 2010). Furthermore, *ABA3*/*LOS5* encodes a Mo-cofactor sulfurase (MCSU), which converts abscisic aldehyde to ABA (last step of ABA biosynthesis). Over-expression of *ABA3*/*LOS5* under the control of constitutive or drought-inducible promoters led to a significant increase in rice grain yield under drought field conditions (Xiao et al. 2009).

Table 1. List of TFs employed to try to enhance drought tolerance in rice

Gene name	Gene type	Source organism	Tolerance in OX (mutant)	Reference
Homeodomain leucine zipper transcription factor				
AtEDT1/HDG11	Homeodomain leucine zipper family IV	*A. thaliana*	Drought ↑	(Yu et al. 2013)
Oshox22	Homeodomain leucine zipper family I	*O. sativa*	Drought ↓	(Zhang et al. 2012)
Oshox4	Homeodomain leucine zipper family I	*O. sativa*	Not reported	(Agalou et al. 2008)
Basic leucine zipper transcription factor				
OsbZIP71	Basic leucine zipper 71	*O. sativa*	Drought ↑	(Liu et al. 2013)
OsbZIP46CA1	Basic leucine zipper 46	*O. sativa*	Drought ↑	(Tang et al. 2012)
OsbZIP16	Basic leucine zipper 16	*O. sativa*	Drought ↑	(Chen et al. 2012)
OsbZIP72	Basic leucine zipper 72	*O. sativa*	Drought ↑	(Lu et al. 2009)
OsbZIP23	Basic leucine zipper 23	*O. sativa*	Drought ↑	(Xiang et al. 2008)
Ethylene-responsive factors				
AtDREB1A	DREB class1 protein	*A. thaliana*	Drought ↑	(Datta et al. 2012)
OsWR1	Rice wax synthesis regulatory gene	*O. sativa*	Drought ↑	(Wang et al. 2012)
OsDREB2A	DREB class2 protein	*O. sativa*	Drought ↑	(Cui et al. 2011)
SUB1A	ERF transcription factor	*O. sativa*	Drought ↑	(Fukao et al. 2011)
DERF1	Drought and ethylene-responsive factor	*O. sativa*	Drought ↓	(Wan et al. 2011)
ARAG1	ABA-responsive DREB gene	*O. sativa*	Drought ↑	(Zhao et al. 2010)
JERF3	Ethylene responsive factor	*L. esculentum*	Drought ↑	(Zhang et al. 2010)
TSRF1	Tomato ethylene response factor	*O. sativa*	Drought ↑	(Quan et al. 2010)
AP37	AP2/ERF domain transcription factor	*O. sativa*	Drought ↑	(Oh et al. 2009)
TERF1	Tomato ethylene response factor	*L. esculentum*	Drought ↑	(Gao et al. 2008)
OsDREB1F	DREB class1 protein	*O. sativa*	Drought ↑	(Wang et al. 2008)
HARDY	AP2/ERF-like transcription factor	*A. thaliana*	Drought ↑	(Karaba et al. 2007)
CBF3/DREB1A	C-repeat/DRE element binding factors	*A. thaliana*	Drought ↑	(Oh et al. 2005)
MYB-Type transcription factor				
OsMYB2	MYB transcription factor	*O. sativa*	Drought ↑	(Yang et al. 2012)
Basic/Helix-loop-Helix transcription factor				
OsbHLH148	Basic/Helix-loop-Helix transcription factor	*O. sativa*	Drought ↑	(Seo et al. 2011)
Zinc-finger transcription factor				
DST	C2H2 Zinc-finger protein	*O. sativa*	Drought ↓	(Huang et al. 2009)
ZFP245	Zinc-finger protein 245	*O. sativa*	Drought ↑	(Huang et al. 2009)
ZFP252	TFIII-type Zinc-finger protein 252	*O. sativa*	Drought ↑	(Xu et al. 2008)
NAC family genes				
NAC10	NAC10 transcription factor	*O. sativa*	Drought ↑	(Jeong et al. 2010)
NAC045	NAC045 transcription factor	*O. sativa*	Drought ↑	(Zheng et al. 2009)
SNAC1	Stress responsive NAC1 transcription factor	*O. sativa*	Drought ↑	(Hu et al. 2006)

Scope of the thesis

The objective of the research presented in this thesis was to get more detailed insight in the molecular regulation of drought tolerance in rice, particularly focussing on transcription factors and plant hormones, as introduced in **Chapter 1**. To achieve that goal I studied the role of abscisic acid and strigolactones in drought stress tolerance in rice and functionally characterised a number of drought-responsive HD-Zip family I transcription factors.

Chapter 2 describes the isolation and characterisation of the rice *Oshox22* gene, which belongs to the plant-specific homeodomain-leucine zipper (HD-Zip) family I of transcription factors. Down-regulation of *Oshox22* expression by T-DNA insertion led to plants with reduced levels of abscisic acid (ABA) and enhanced tolerance towards drought and salt stresses, while over-expression of the gene increased ABA content and decreased drought and salt tolerances. We conclude that Oshox22 affects ABA biosynthesis and regulates drought and salt responses through ABA-mediated signal transduction pathways.

As discussed in Chapter 1, TFs of the homeodomain-leucine zipper (HD-Zip) family I may also affect plant development. Indeed in **Chapter 3**, I report on the role of *Oshox22* in controlling grain length (GL) in rice. An implication of our findings could be that allelic variation in the *Oshox22* promoter is exploited in breeding programmes to modify GL using molecular marker-assisted selection.

The strigolactones are a relatively new class of plant hormones and a possible role in drought tolerance is unknown. **Chapter 4** of this thesis reviews the various roles that strigolactones (SLs) play both in the rhizosphere and as endogenous plant hormone. In addition, I present the current knowledge on the SL biosynthetic and downstream signalling pathways and the interactions of SLs with other phytohormones.

It has been reported that there seems to be a functional link between ABA and strigolactones but the mechanism of that link remained unknown. **Chapter 5** describes the intimate relationship between ABA and SL biosynthesis by a further characterisation of β-carotene isomerase D27 in rice. Our results indicate that *OsD27* plays a role in the regulation of SL as well as ABA biosynthesis, especially in response to environmental constraints such as drought and phosphate deficiency.

Chapter 6 finally discusses the main findings of this thesis and the future perspectives of how the knowledge generated in this thesis can contribute to the improvement of drought tolerance.

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Chapter 2

Function of the HD-Zip I gene *Oshox22* in ABA-mediated drought and salt tolerances in rice

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Abstract

Oshox22 belongs to the homeodomain-leucine zipper (HD-Zip) family I of transcription factors, most of which have unknown functions. Here we show that the expression of *Oshox22* is strongly induced by salt stress, abscisic acid (ABA), and polyethylene glycol (PEG) treatment, and weakly by cold stress. Trans-activation assays in yeast and transient expression analyses in rice protoplasts demonstrated that Oshox22 is able to bind to the CAAT(G/C)ATTG element and acts as a transcriptional activator that requires both the HD and Zip domains. Rice plants homozygous for a T-DNA insertion in the promoter region of *Oshox22* showed reduced *Oshox22* expression and ABA content, decreased sensitivity to ABA, and enhanced tolerance to drought and salt stresses at the seedling stage. In contrast, transgenic rice over-expressing *Oshox22* showed increased sensitivity to ABA, increased ABA content, and decreased drought and salt tolerances. Based on these results, we conclude that Oshox22 affects ABA biosynthesis and regulates drought and salt responses through ABA-mediated signal transduction pathways.

Keywords: Rice, Transcription factor, HD-Zip, Drought stress, Regulation, Abiotic stress.

Introduction

Drought and salt are major abiotic stresses that cause tremendous yield losses in crops all over the world. Due to water shortage and less predictable rainfall patterns resulting from global atmospheric changes, the improvement of stress resistance in crops is now of utmost importance. Consequently, the genetic basis of drought and salt resistance is an intensively studied topic. The plant hormone ABA plays a central role in drought and salt responses through regulating developmental and physiological processes including stomata closure (Leung and Giraudat 1998; Umezawa et al. 2009; Melcher et al. 2010; Huang et al. 2012). Using microarrays, many genes with expression responding to ABA, drought and salt treatments have been identified (Seki et al. 2001, 2002; Rabbani et al. 2003; Bray 2004; Yamaguchi-Shinozaki et al. 2006). Additional characterisation identified key components mediating gene-expression changes in drought responses that include the transcription factors *DREB1A* (Kasuga et al. 2004), *DREB2A* (Sakuma et al. 2006), *SNAC1* (Hu et al. 2006), *OsbZIP23* (Xiang et al. 2008), and *DST* (Huang et al. 2009). Furthermore, a number of transcription factors encoded by homeodomain-leucine zipper (HD-Zip) genes in Arabidopsis, rice and other plants have been implicated in regulating drought tolerance through either ABA-dependent or ABA-independent pathways (e.g. Söderman et al. 1996, 1999; Gago et al. 2002; Himmelbach et al. 2002; Deng et al. 2006; Agalou et al. 2008; Shan et al. 2011). The HD-Zip genes, however, are an abundant group of transcription factors that are exclusively found in plants (Ruberti et al. 1991; Schena and Davis 1992; Aso et al. 1999; Sakakibara et al. 2001; Derelle et al. 2007). HD-Zip proteins, characterised by a DNA-binding HD and a protein-protein interaction Zip domain, have been classified into four families (I to IV) according to their sequence similarities (Ruberti et al. 1991; Morelli and Ruberti 2002). Different members of the HD-

Zip families I and II have been implicated in auxin signalling and transport (Morelli and Ruberti 2002; Sawa et al. 2002), vascular development (Scarpella et al. 2000), and light responses including shade avoidance (Steindler et al. 1999; Wang et al. 2003). Several other members, such as *Athb-6*, *Athb-7* and *Athb-12* from Arabidopsis (Söderman et al. 1996, 1999; Lee et al. 2001; Hjellström et al. 2003; Olsson et al. 2004), *Hahb-4* from sunflower (Gago et al. 2002; Dezar et al. 2005a, 2005b; Manavella et al. 2006), and *CpHB2*, *CpHB6* and *CpHB7* from *Craterostigma plantagineum* (Frank et al. 1998; Deng et al. 2002) are induced by drought and ABA, suggesting a function in ABA-mediated adaptation to drought stress. In agreement, inductions of *Athb-6*, *Athb-7* and *Athb-12* are abolished in the ABA-insensitive mutants *abi1* and *abi2* (Himmelbach et al. 2002; Olsson et al. 2004).

Like in dicots, a subset of HD-Zip family I and II genes is regulated by drought in rice (Agalou et al. 2008). Phylogenetic analysis places *Oshox22*, *Oshox24*, *Athb-7* and *Athb-12* in the same subgroup (γ-clade, Henriksson et al. 2005) of the HD-Zip family I, and *Oshox22* is very likely related to *Oshox24* via an ancient chromosomal duplication (Agalou et al. 2008). Our previous work showed that *Oshox22* is strongly induced by drought which spurred our interest for further studies (Agalou et al. 2008). To gain insight into the function of *Oshox22* in drought and salt tolerances, we performed genetic and physiological studies through mutation and over-expression analyses in rice. Our data showed that *Oshox22* regulates drought and salt stress susceptibility through an ABA-mediated signalling pathway.

Materials and methods

Plant materials and stress treatments

Drought-tolerant cultivar IRAT 112 (upland tropical *japonica*, also named Gajah Mungkur) and drought-sensitive cultivar Nipponbare (lowland *japonica*) were used for most studies described in this paper. Zhonghua 11 (lowland *japonica*) was used for transgenic analyses. The T-DNA insertion mutant *oshox22-1* in Dongjin (lowland *japonica*) background was obtained from the Postech collection in South Korea.

For hormone treatments, 12 day-old Nipponbare seedlings were sprayed with 100 μM ABA, followed by sampling at 0, 1, 3, and 6 h. Alternatively, seedlings at the same stage were irrigated with 10% PEG 6,000 or 200 mM NaCl followed by sampling at 0, 1, 3, and 6 h. For the cold treatment, seedlings were transferred to 4°C and sampled after 0, 1, 3, 6, 12, and 24 h. One whole plant was sampled as one replicate and in total four replicates were used in each RNA extraction.

For drought treatments, about 40 plants of the *oshox22-1* mutant and *Oshox22* over-expression lines were grown in square pots (L26 X W12 X H10 cm) filled with a mixture of sand and soil (1:1) together with wild type, respectively. We stopped watering when seedlings were 12 days old, and watering was resumed for three days when seedlings were 24 days old, after which the survival rates were calculated. For salt treatments, the same amount of 12 day-old seedlings were irrigated with 150 mM NaCl for nine days, after which green leaf rates (green leaf area > 30%) and survival rates were determined. To measure the water-loss rate under dehydration conditions, the second leaves from the top of the plant at the tillering stage (plants were 55 days old) were cut and exposed to air at room temperature (approximately 25°C), and the weight was determined every 30 minutes. Every treatment was done in triplicate.

Subcellular localisation assay

A full-length cDNA of *Oshox22* was amplified from IRAT 112 by RT-PCR using primers Oshox22cdsFW and Oshox22cdsRW (Table S1) based on a full-length cDNA sequence (GenBank accession AY224440) found in a seed-derived cDNA expression library (Cooper et al. 2003). The PCR product was cloned into *pCR2.1-TOPO* (Invitrogen) and sequenced. To create a GFP-tagged construct, the full-length *Oshox22* cDNA was excised by *Eco*RI and ligated into *Eco*RI-digested *pTH2-BN* vector. The cDNA fragment was inserted to the C-terminus of *GFP* and expressed using the Cauliflower mosaic virus (CaMV) 35S promoter. The construct was transformed into rice protoplasts as previously described (Chen et al. 2006; Osnato et al. 2010). Localisation of the GFP-tagged Oshox22 protein was monitored in protoplasts by confocal laser scanning microscopy (Leica SP5) at 24 h after transformation.

Yeast one-hybrid screens

To assay the activation property, the full-length *Oshox22* cDNA PCR product was excised by *Eco*RI and *Bam*HI, then ligated into *Eco*RI/*Bam*HI digested *pAS2-1* (Clontech, GenBank accession U30497), resulting into *pAS2-1-Oshox22.* Partial Oshox22 ORFs were also cloned into *pAS2-1* vector, producing different translational fusions between *Oshox22* and the GAL4 BD. Constructs were sequence-verified before transfer to yeast strain *PJ69-4A* (Table S2) for activation screens. All yeast handling and reporter assays were performed as described before (Meijer et al. 1998, 2000a; Ouwerkerk and Meijer 2001, 2011).

For the DNA binding assay, a full-length cDNA of *Oshox22* was excised from *pAS2-1-Oshox22* with *Eco*RI and *Sal*I and then ligated into *pRED-ATGa* which is replicated via ARS-CEN and maintained via *URA3* selection (unpublished results, P.B.F. Ouwerkerk and A.H. Meijer). The

resulting plasmid *pRED-ATG-Oshox22* was assayed in yeast strains *YM4271-4AH1-HIS3* and *YM4271-4AH2-HIS3* which were made using *pINT1* as the integrative vector system (Meijer et al. 1998) which contains the *HIS3* reporter gene preceded by AH1 or AH2 tetramer-binding sites for HD-Zip proteins (Meijer et al. 2000b).

Binary vector constructions and plant transformation

The *Oshox22* over-expression construct was made in the binary vector *pC1300intB-35SnosEX* (Genbank accession AY560325) as following: the full-length cDNA of *Oshox22* was excised from *pAS2-1-Oshox22* and ligated into binary vector *pC1300intB-35SnosEX*, allowing the gene to be expressed under the control of the CaMV 35S promoter. We transformed rice (Zhonghua 11) as previously described (Scarpella et al. 2000) except that the *A. tumefaciens* strain used was *LBA4404*. Calli used for transformation were obtained from germinating seeds according to Rueb et al. (1994). Plantlets were maintained in culture on half-strength Murashige Skoog (MS) medium with 10 g/l sucrose until transfer to a greenhouse (28°C under a 16 h photoperiod and 85% humidity).

Rice protoplast isolation and transient expression assays

Assays to test DNA binding of Oshox22 involved transient transformations of protoplasts with effector and reporter plasmids. The effector plasmid contained the full-length cDNA of *Oshox22* expressed under the control of the CaMV 35S promoter. The reporter plasmids contained the putative *Oshox22* binding sequences AH1 or AH2 as tetramers upstream of a truncated -90 CaMV 35S promoter directing *GUS* gene expression (Meijer et al. 1997). For protoplast isolation, one hundred rice seeds (Nipponbare) were grown in 10 cm diameter pots (28°C, 85% humidity) in the dark for 12~14 days. Stems and leaves were cut into ~0.5 mm pieces and digested with 25 ml

enzyme solution containing 1.5% w/v cellulase (Sigma) and 0.3% w/v Macerozyme (Sigma) in 50 ml centrifuge tubes. Further preparation of protoplasts and transfection with effector/reporter constructs were as described earlier (Chen et al. 2006; Osnato et al. 2010). Proteins extraction and detection of GUS activity were based on Jefferson et al. (1987). Fluorescence was measured by a Cytofluor 2350 fluorimeter (Millipore).

Northern blot hybridisation

Electrophoresis and northern blotting of RNAs were performed as described by Memelink et al. (1994). Baked blots were pre-hybridised in 1 M NaCl, 1% SDS, 10% dextrane sulphate and 50 µg/ml denatured herring sperm DNA at 65°C, washed with 0.1XSSPE, 0.5% SDS at 42°C and then autoradiographed. Probes were labeled by random priming with ^{32}P-dCTP. Equal loading of RNA samples was verified on the basis of ethidium bromide staining of ribosomal RNA bands.

Real-time qPCR analysis

Total RNAs from different tissues were pre-treated with RNase-free DNase I (Takara), according to the manufacturer's instruction. Reverse transcription reaction was performed with SuperScript™ III reverse transcriptase (Invitrogen) following the manufacturer's instruction. Primers used for real-time PCR analyses of *Oshox22* were QPCR-22FW and QPCR-22RW (Table S1). qPCR was performed with Rotor-Gene 3000 Real-time PCR System using SYBR1 Green to monitor dsDNA synthesis. Relative expression levels of reporter and target genes were determined by the Two Standard Curves Relative Quantification Method using *ACTIN1* (Table S1) as the internal control.

Determination of ABA content

Five seeds each of the homozygous T-DNA insertion mutant *oshox22-1* and over-expression lines together with their corresponding wild type cultivars (Dongjin and Zhonghua 11 respectively) were

surface-sterilised and germinated in half-strength MS salt solution without sucrose and grown at 28°C with a 16/8 h light/dark photoperiod. To determine the ABA level in response to drought stress, 12 day-old *oshox22-1* and over-expression seedlings were stopped watering and sampled after ten days dehydration treatment. Quantification of ABA levels was performed using UPLC-MS/MS with five biological replicates (each 0.2 g of fresh shoot tissue) as described elsewhere, (López-Ráez et al. 2008, 2010). Statistical analyses were performed using Student's t-test.

Stomata density test

Stomata numbers were counted on the upper epidermis of the leaf blades of mutant and over-expression plants grown under normal conditions. Leaf blades were from flag leaves at the stage that the plants just started to flower. For this method, oval surfaces spanning 1 x 2 cm were painted with clear fingernail polish, while avoiding ribbed veins. After the polish had dried it was peeled off by adding a tape on the polish and transferred to a microscopy slide. Finally, the stomata number was counted using DIC microscopy. Five replicates of every leaf were counted and from every plant line seven plants were used and all numbers were converted to number of stomata per square mm to account for variation in microscopes.

Results

Isolation and sequence analysis of *Oshox22*

Previous studies showed that the expression of *Oshox22* in rice is strongly induced by drought, and that the induction is higher in three drought tolerant upland cultivars compared with three lowland cultivars (Agalou et al. 2008). To elucidate the function of *Oshox22*, the full-length cDNA of *Oshox22* was amplified from the rice cultivar IRAT 112 by RT-PCR. The sequence of the amplified cDNA fragment was identical to that of the cDNA from Nipponbare over the whole length. *Oshox22* (Os04g45810) is located on chromosome 4 and encodes a protein of 262 amino acids (AA) including a 61-AA HD domain for DNA binding and a 43-AA Zip domain for protein-protein interactions.

Expression of *Oshox22* under abiotic stress conditions

First, RNA samples from different rice tissues at several developmental stages were analysed by quantitative RT-PCR (qRT-PCR) in Nipponbare. The results showed that *Oshox22* is ubiquitously expressed, with a lower level in stems and higher in panicles and seeds (Figure 1A), which is consistent with earlier observations (Agalou et al. 2008). We then monitored the *Oshox22* expression under different abiotic stress conditions. As shown in Figure 1B, *Oshox22* was rapidly and strongly induced by NaCl, PEG and ABA, and weakly induced by low temperature. These results are in agreement with available microarray data (http://red.dna.affrc.go.jp/RED/).

Subcellular localisation and transcriptional activation function of Oshox22

To confirm that Oshox22 is a nuclear protein, a GFP-tagged *Oshox22* construct under the control of the CaMV 35S promoter was made, with GFP fused at the C-terminus of the full-length Oshox22 protein (construct *35S::GFP-Oshox22*). This construct was introduced into rice

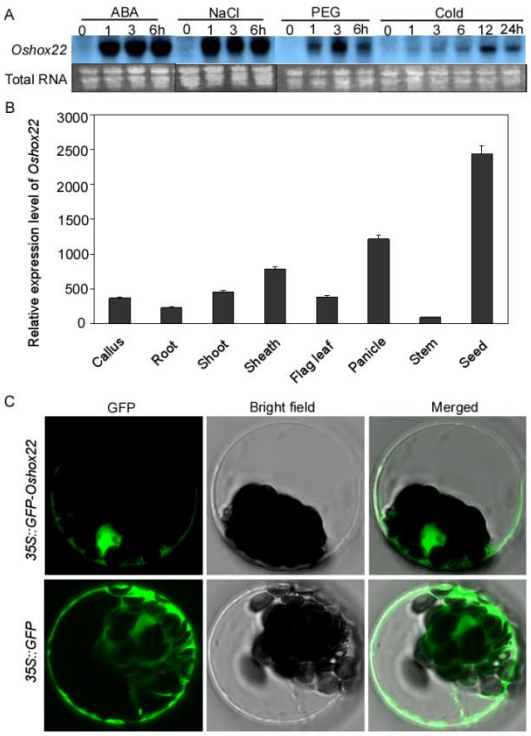

Figure 1. Expression profiling and subcellular localisation of *Oshox22*. **A,** qRT-PCR analyses of *Oshox22* expression in scutellum-derived calli, root, shoot, leaf sheath, flag leave, panicle, stem and mature grain from rice. **B,** Northern blot analysis of *Oshox22* expressions in response to ABA (100 μM), salt (200 mM NaCl), 10% PEG 6,000 and low-temperature (4°C) treatments. **C,** Nuclear localisation of GFP-tagged Oshox22. The GFP-Oshox22 fusion protein, driven by the CaMV 35S promoter, was transiently expressed in rice protoplasts and visualised by confocal laser scanning microscopy. Construct *pTH2* (Chiu et al. 2006) carrying a *GFP* gene driven by CaMV 35S promoter was used as the negative control.

protoplasts by transient transformation and the fusion protein's subcellular localisation was analysed using confocal laser scanning microscopy, where the *35S::GFP* construct served as control. As shown in Figure 1C, the GFP signal was detected specifically in the nuclei of

35S::GFP-Oshox22 transformed protoplasts, while in the control the GFP signal was located primarily in the cytoplasm. These data suggest that Oshox22 is a nuclear-localised protein. In agreement with this observation, a putative nuclear localisation signal sequence (RKRR at the AA 59) was found near its N-terminus, as analysed by WoLF PSORT (http://wolfpsort.seq.cbrc.jp/).

To test whether Oshox22 has any transcription activation property, Oshox22 was fused to the Gal4p binding domain (*GAL4 BD*) and assayed for the ability to induce expression of a *HIS3* gene preceded by Gal4p binding sites. The result showed that the transformed yeast cells were able to grow on medium lacking histidine, with up to 10 mM 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of His3p enzyme activity (Figure 2A). In contrast, no growth was observed of yeast transformed with a control construct without Oshox22 (*pAS2-1*), indicating that Oshox22 has transcriptional activation activity in yeast. To dissect the activation domain(s), a series of seven truncated Oshox22 constructs with deletions from either the N- and C-termini were tested. No activation activity was observed when either the HD or the Zip domains were deleted (Figure 2A), suggesting both domains are required for transcriptional activation in yeast.

Interactions of Oshox22 in yeast and rice with the CAAT(C/G)ATTG sequence

Previous results showed that HD-Zip family I and II proteins are able to interact with pseudopalindromic AH1 (CAAT(A/T)ATTG) and AH2 (CAAT(C/G)ATTG) sequences, respectively (Sessa et al. 1993; Meijer et al. 1997, 1998, 2000b; Palena et al. 1999; Johannesson et al. 2001). To test whether Oshox22 interacts with either the AH1 or AH2 sequences or both, yeast strains containing a chromosomally integrated *HIS3* reporter gene with upstream AH1 or AH2 tetramers (named *4AH1-HIS3* and *4AH2-HIS3* respectively, Meijer et al. 1998, 2000b) were used. The *Oshox22* ORF was cloned into *pRED-ATGa* (named *pRED-ATGa-Oshox22),* allowing for

constitutive expression of full length Oshox22 protein in yeast without fusion to an exogenous activation domain. Construct *pRED-ATGa-Oshox22* was transformed into yeast strains containing constructs *4AH1-HIS3* or *4AH2-HIS3*. The results showed that yeast cells with *4AH2-HIS3* transformed with *pRED-ATGa-Oshox22* grew well on a medium lacking histidine but containing up to 10 mM 3-AT (Figure 2B), whereas no growth was observed in yeast strains with *4AH1-HIS3* or with empty *pRED-ATGa* vector. Thus, in yeast, Oshox22 is able to bind to AH2, but not to AH1, and can activate reporter gene expression by an intrinsic activation domain.

To confirm binding of Oshox22 protein to the AH2 sequence transient expression assays were carried out with effector and reporter plasmids in rice protoplasts. Two reporter plasmids, *4AH1-90-GUS* and *4AH2-90-GUS* were used, in which the AH1 and AH2 tetramers were fused to a CaMV -90 35S minimal promoter. Construct *Pro35S-Oshox22* with *Oshox22* expressed under control of the CaMV 35S promoter was used as an effector.

As shown in Figure 2C, *GUS* expression in protoplasts co-transformed with *Pro35S-Oshox22* and *4AH2-90-GUS* was 3.07 times higher than those co-transformed with *Pro35S-Oshox22* and *4AH1-90-GUS*, and 6.11 times higher than those co-transformed with the empty vector. These data indicate that Oshox22 is capable to activate transcription of the reporter gene when upstream HD-Zip binding sites AH1 or AH2 are present (Figure 2C). The interaction is less effective at the AH1 site than that at AH2, which is in contrast to earlier observations where HD-Zip I proteins mainly interacted with AH1 and HD-Zip II proteins with AH2 (Sessa et al. 1993; Meijer et al. 1997, 2000b; Palena et al. 1999).

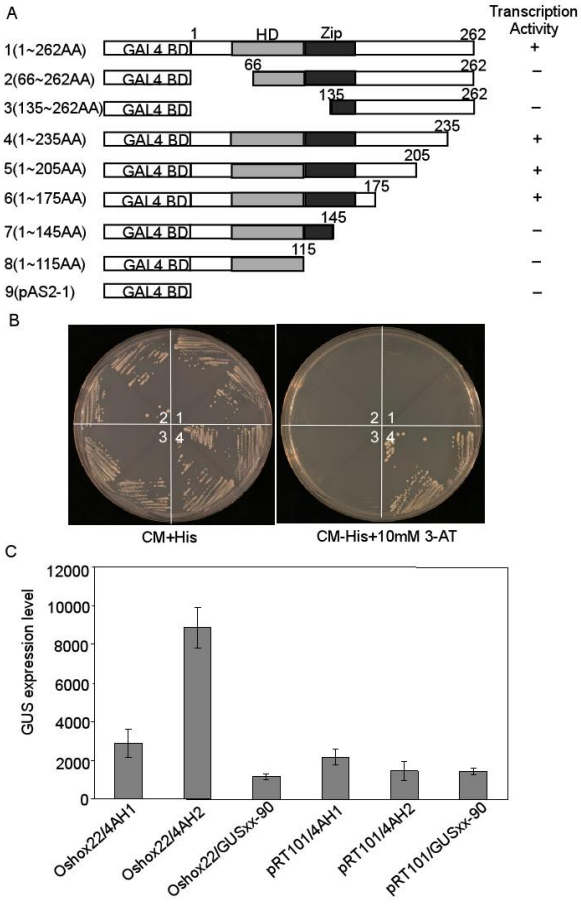

Figure 2. Trans-activation and DNA-binding specificity of Oshox22. **A,** Trans-activation assay in yeast with truncated Oshox22 protein. Fusion proteins of the GAL4 DNA-binding domain and different fragments of Oshox22 were examined for their trans-activation activities in yeast *PJ69-4A*. HD, homeodomain; Zip, leucine zipper. **B,** Trans-activation assay of Oshox22 in yeast. Sections 1 and 2, negative control; sections 3 and 4, Oshox22 fused to GAL4-BD in the vector *pRED-ATGa* was transformed into yeast strains *YM4271-4AH1-HIS3* and *YM4271-4AH2-HIS3*, respectively. **C,** Interactions of Oshox22 with the HD-Zip binding site AH2 (CAAT(C/G)ATTG) and activation of reporter gene expression in a transient expression system using rice protoplasts. Transient expression of *Oshox22* was driven by the CaMV 35S promoter. The Oshox22-OX construct was co-transformed with the reporter constructs *GUSXX-4AH1* or *GUSXX-4AH2*. The empty vectors pRT101 and GUSXX-90 were used as negative controls.

Decreased ABA sensitivity of the *oshox22-1* mutant

We searched the publicly available mutant collections and obtained a putative T-DNA insertion mutant for *Oshox22* in rice (POSTECH_C054121, B-11507) (Jeong et al. 2006) (Figure 3A). The genomic locus of *Oshox22* in this mutant is shown schematically in Figure 3B. Alignment of the flanking sequence tag and the genomic sequence showed that the T-DNA insertion is located at 978 bp before the translational start codon of *Oshox22*. To investigate the function of *Oshox22*, an homozygous T-DNA insertion line (named *oshox22-1*) was identified, and Southern blot analysis showed that only one copy of T-DNA was present in *oshox22-1* (Figure S1).

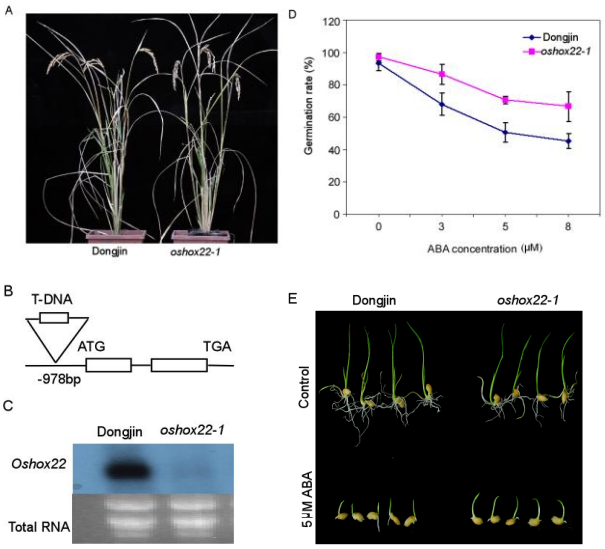

Figure 3. The phenotype and ABA sensitivity of *oshox22-1*. **A,** Morphology of *oshox22-1* at post-anthesis stage. **B,** Schematic representation of the exon-intron structure of *Oshox22* gene and the position of the T-DNA insert in *oshox22-1*. **C,** *Oshox22* expression was down-regulated in *oshox22-1*. **D,** Germination rate of *oshox22-1* on MS medium with 0, 3, 5, and 8 μM ABA for 3 days. **E,** Relative growth of *oshox22-1* on medium with 5 μM ABA at the post-germination stage. Dongjin represent wild-type plants segregated from a heterozygous *oshox22-1* parent.

Northern blot analysis showed that in *oshox22-1* the *Oshox22* transcript was below detection level (Figure 3C). Phenotypic studies showed that *oshox22-1* plants exhibited no obvious morphological difference compared to wild type Dongjin (Figure 3A). The panicle shape and the grain number remained unchanged. When *oshox22-1* was backcrossed with Dongjin, all F_1 and F_2 plants showed the same plant stature in the greenhouse. In the F_2 population, the T-DNA insert segregated in a 3:1 ratio (n=326), suggesting no embryo or gamete lethality was involved.

We further analysed the sensitivity of *oshox22-1* to ABA in germination assays. On MS media with 3 μM and 8 μM ABA, seeds from *oshox22-1* showed significantly higher germination rates (72% and 32%, respectively) than those from wild-type plants segregated from *oshox22-1* backcrosses (40% and 8% on the medium with 3 μM and 8 μM ABA, respectively; Figure 3D). On control media without ABA, the germination rates of *oshox22-1* and wild type seeds showed no significant difference. These results suggest that *Oshox22* may mediate ABA sensitivity. Next, *oshox22-1* seedlings were tested for ABA sensitivity at the post-germination stage using media with different concentrations of ABA. The results showed that shoot development was less inhibited by exogenous ABA in *oshox22-1,* as compared to wild type (Figure 3E). In summary, down-regulation of *Oshox22* expression in the T-DNA insertion line led to compromised ABA sensitivity at germination as well as post-germination stages.

Increased ABA sensitivity of transgenic plants over-expressing *Oshox22*

For over-expression analysis we expressed *Oshox22* under the control of the CaMV 35S promoter (construct Oshox22-OX). Northern blot analysis confirmed that *Oshox22* gene expression levels were increased in the transgenics (Figure S2). Compared to wild type control plants, the Oshox22-OX plants exhibited fewer tillers and decreased height (Figure 4A).

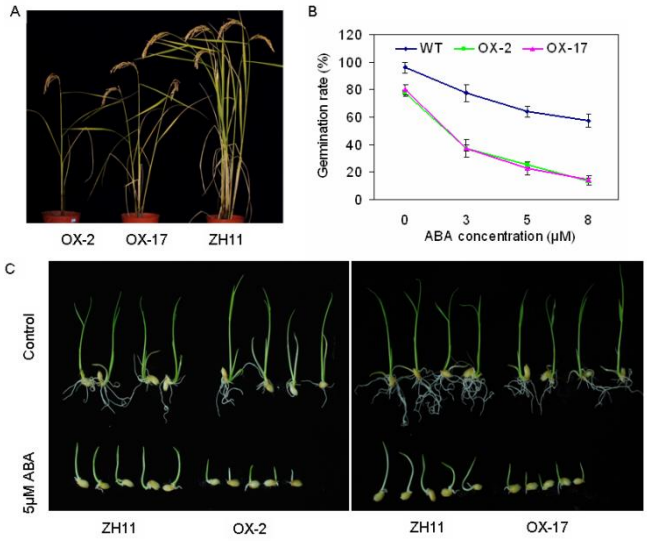

Figure 4. Phenotype and ABA sensitivity of Oshox22-OX plants. **A,** Phenotype of two independent Oshox22-OX lines at mature stage. **B,** Germination rate of seeds from Oshox22-OX plants on medium with 0, 3, 5, and 8 μM ABA for 3 days. **C,** Shoot and root growth of Oshox22-OX plants on MS medium with 5 μM ABA at post-germination stage. ZH11 (Zhonghua 11), wild type segregated from the T_1 line.

We chose two transgenic lines (Oshox22-OX-2 and Oshox22-OX-17) for further analyses. The seed germination rates were reduced 17% in these two lines (Figure 4B). To analyse the sensitivity of the Oshox22-OX plants to ABA, seeds were germinated on solid MS media containing either 0, 3, 5 or 8 μM ABA. As shown in Figure 4B, germination of seeds from both Oshox22-OX lines was severely inhibited by all concentrations of ABA used and the inhibition was much stronger than that observed for wild type seeds. For instance, the germination rates of the two Oshox22-OX lines plated on medium with 3 μM ABA were 40%, as compared to 80% on medium without ABA, whereas in the wild type only a slight reduction (from 98% to 80%) was observed. The relatively low germination rates observed in Oshox22-OX seeds could be due to an increased ABA

sensitivity. We further tested Oshox22-OX seedlings on media containing 0, 3, 5 or 8 μM ABA and found that the growth of Oshox22-OX seedlings was inhibited to a greater extent by 5 μM ABA (Figure 4C). We therefore concluded that over-expression of *Oshox22* led to increased ABA sensitivity at the germination as well as post-germination stages.

Function of *Oshox22* in regulating ABA biosynthesis

Next, endogenous ABA levels were measured of the Oshox22 mis-expression plants grown under control and drought-stress conditions (Figure 5).

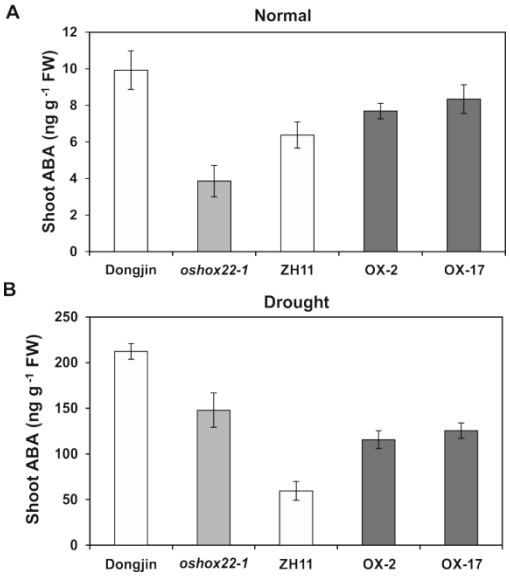

Figure 5. Effects of *Oshox22* mis-expression on ABA levels. ABA levels in *oshox22-1* and Oshox22-OX plants and wild type cultivars Dongjin and Zhonghua 11. **A,** ABA levels under normal condition. **B,** ABA levels after drought treatment. After drought treatment, the ABA levels increased with a factor 21.39, 38.10, 9.34, 15.04 and 15.04 for *oshox22-1*, Dongjin, Zhonghua 11, Oshox22-OX-2 and Oshox22-OX-17, respectively.

Under normal irrigated conditions, the ABA content of wild type Dongjin seedlings was 9.92±1.06

ng.g^{-1} FW, while in *oshox22-1* it was reduced to 3.86±0.86 ng.g^{-1} FW, an average reduction of 60% (Figure 5A). In wild type Zhonghua 11, the ABA content was 6.38±0.72 ng.g^{-1} FW, while in over-expression lines Oshox22-OX-2 and Oshox22-OX-17, the ABA contents were increased to 7.69±0.42 ng.g^{-1} FW and 8.35±0.78 ng.g^{-1} FW, respectively (Figure 5A). Thus, down-regulation of *Oshox22* led to a reduced level of ABA and over-expression led to increased ABA levels, therefore we conclude that *Oshox22* functions in regulating biosynthesis or degradation of ABA in rice. The differences in ABA levels are likely causing changes in sensitivity of germinating and developing seedlings towards exogenous applied ABA. Furthermore, we also tested the ABA contents under drought stress conditions. We found that ABA contents were increased in all plants after drought treatment (Figure 5B), however, there were differences in the levels of induction ranging from 9.34 in wild type Zhonghua 11 to 38.10 in *oshox22-1*. Although the absolute ABA level was lower in the mutant, the level of ABA accumulated 38.31 fold whereas this was 21.39 fold in wild type Dongjin. In the two *Oshox22* over-expression lines the induction level was 15.04 whereas in control Zhonghua 11 plants this was only 9.34, thus not only the absolute ABA levels were higher but also the induced levels were higher.

Drought and salt responses in *oshox22-1* and Oshox22-OX plants

To address the function of *Oshox22* in drought and salt responses, we analysed a homozygous *oshox22-1* line and two Oshox22-OX lines for drought and salt tolerances. When the seedlings were 12 days old (five leaf stage) we stopped watering for 12 days and then resumed watering for three days to measure the survival rates. Drought treatment strongly decreased the survival rate of Oshox22-OX plants, while in *oshox22-1* mutants the survival rate was increased, as compared to their corresponding wild type control groups (Figures 6A and B).

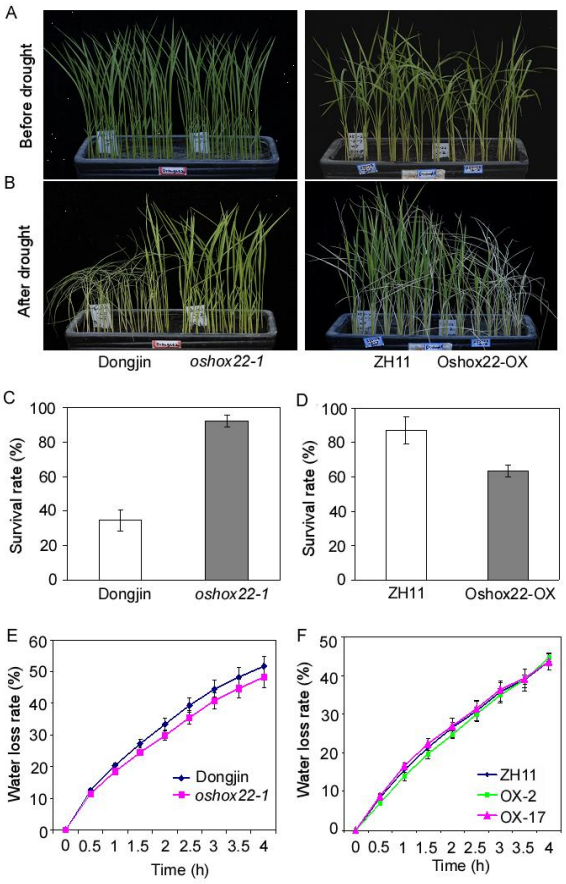

Figure 6. Drought tolerance of *oshox22-1* and Oshox22-OX plants. **A,** Seedlings of *oshox22-1* and Oshox22-OX before applying the drought-stress. **B,** After drought stress treatment. **C,** and **D,** Survival rates of *oshox22-1* and Oshox22-OX plants after application of drought stresses. **E,** Water-loss rates in leaves from *oshox22-1* and corresponding wild type plants. **F,** Water-loss rate in leaves from Oshox22-OX and corresponding wild-type plants.

As shown in Figures 6C and D, 92% of the *oshox22-1* seedlings survived, as compared to 34.7% in the control group. In contrast, 63% of the Oshox22-OX seedlings survived, as compared to 87%

in their control group. From these data we conclude that mutation of *Oshox22* led to increased drought tolerance, while over-expression of *Oshox22* led to decreased drought tolerance.

Furthermore, we measured water-loss rates in leaves from *oshox22-1* and Oshox22-OX plants during the dehydration process. Leaves from *oshox22-1* had significantly lower rates ($P < 0.05$) of water-loss than control plants (Figure 6E). In contrast, Oshox22-OX plants showed no significant difference in water-loss rate as compared to the control (Figure 6F).

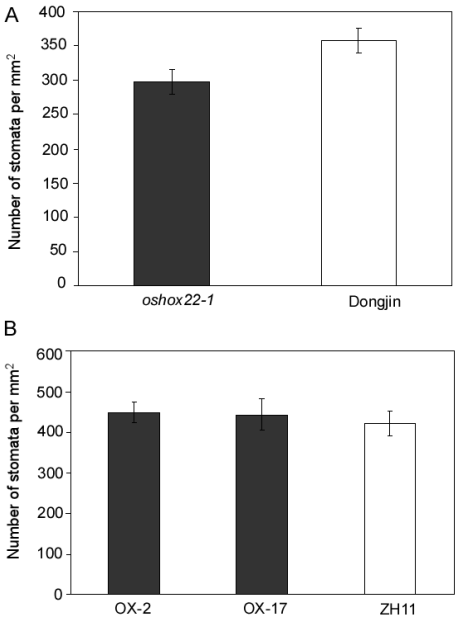

Figure 7. Stomata counting in leaves from *oshox22-1* and Oshox22-OX plants. **A,** The number of stomata on *oshox22-1* leaves compared to wild type ($P < 0.01$, n=10). **B,** The number of stomata on two independent Oshox22-OX lines compared to wild type ($P > 0.05$, n=10). Dongjin in this context is the wild type segregated from heterozygous *oshox22-1*/*Oshox22* line; ZH11 (Zhonghua 11), wild type segregated from an Oshox22-OX T_1 line.

Because stomata density is a critical factor in drought stress, we determined the stomata density in leaf blades of *oshox22-1* plants at flowering stage, Oshox22-OX, and their corresponding wild type control plants. The *oshox22-1* leaves showed 17.8% reduction in stomata density, but stomata density remained unchanged in Oshox22-OX, as compared to leaves from wild type plants (Figure 7). Therefore, the enhanced drought tolerance in *oshox22-1* may partly result from decreased stomata density in leaves.

To evaluate salt tolerance in *oshox22-1* and Oshox22-OX plants, 12-day-old seedlings grown in hydroponic culture were transferred to a 150 mM NaCl solution for nine days and then the green leaf area was measured. The *oshox22-1* plants had significantly more green leaf area (80%) than control plants (36%; Figure 8). In contrast, Oshox22-OX plants had a reduced green leaf area (65%) compared with the control (86%; Figure 8). These results suggest that in rice, down-regulation of *Oshox22* improved salt tolerance, while over-expression of *Oshox22* led to reduced salt tolerance. These data further suggest that *Oshox22* functions as a negative regulator in drought and salt tolerance in rice.

Discussion

In rice and Arabidopsis, about 50% of all homeobox genes (Chan et al. 1998; Jain et al. 2008; Mukherjee et al. 2009) belong to the HD-Zip family. Although some members have been implicated in the regulation of development and stress responses, the functions of most HD-Zip genes are still unknown. Based on mutant and over-expression analyses, we here propose a function for the rice HD-Zip family I gene *Oshox22* in regulating ABA biosynthesis and ABA-mediated drought and salt tolerances in rice.

In this study, we used a GFP-tagged fusion construct to demonstrate that Oshox22 is a nuclear-

localised protein, which is consistent with a function as transcription factor. The DNA-binding and activation properties were analysed in yeast one-hybrid experiments, showing specific binding to a known HD-Zip target sequence and activation of reporter gene expression without the requirement of exogenous activation domains. Further functional analysis in yeast showed that both HD and Zip domains are required for the trans-activation.

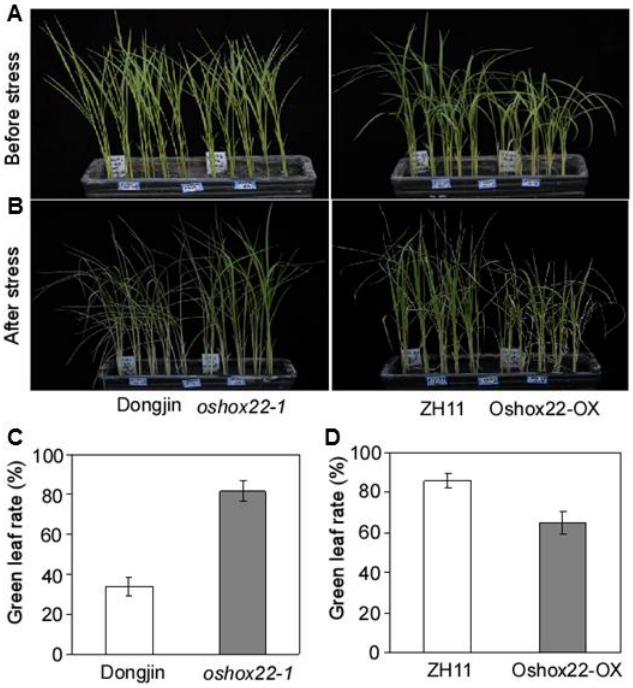

Figure 8. Examinations of salt tolerance of *oshox22-1* and Oshox22-OX plants. **A,** Seedlings of *oshox22-1* and Oshox22-OX before salt treatments. **B,** Plants treated with 150 mM NaCl for nine days. **C and D,** green leaf rates for *oshox22-1* **C**, Oshox22-OX **D,** and their wild type plants after salt treatments.

The function of Oshox22 as a transcriptional activator was further confirmed with transient assays

in rice protoplasts using a *GUS* reporter gene. Sessa et al. (1993, 1997) propose that AH1 (CAAT(A/T)ATTG) and AH2 (CAAT(C/G)ATTG) act as consensus binding sites for HD-Zip I and HD-Zip II proteins, respectively. Apparently, Oshox1 to Oshox7 follow these rules (Meijer et al. 1997, 1998, 2000b). Athb-7 and Athb-12, however, do not seem to bind to either of these consensus sequences and may have totally different binding preferences (Himmelbach et al. 2002; Deng et al. 2006). Our data show that Oshox22 is able to activate gene expression via both AH1 and AH2, but more effectively via AH2 than AH1. Taken together, our data from the yeast and protoplast experiments support the function of Oshox22 as a transcriptional activator, which is typical of HD-Zip I family transcription factors (Ohgishi et al. 2001; Meijer et al. 2000b).

Similar to closely related HD-Zip factors such as *Athb-6*, *Athb-7* and *Athb-12*, our previous (Agalou et al. 2008) and current work shows that expression of *Oshox22* is responsive to drought, salinity and ABA treatments, suggesting its role in regulating stress tolerance. In Arabidopsis, the HD-Zip I protein Athb-6 has been shown to interact with ABI1, a protein phosphatase 2C (Himmelbach et al. 2002). ABI1 is involved in various responses towards ABA including stomata closure, seed dormancy and vegetative growth and thus represents a key component in ABA signalling (Leung et al. 1997; Leung and Giraudat 1998). For *Athb-7* and *Athb-12*, it has been found that their expression is down-regulated in *abi1* mutants (Olsson et al. 2004), which further supports the interaction between ABA signalling and these HD-Zip genes. To perform functional analyses, we generated *Oshox22* over-expression lines and obtained an *oshox22* mutant that contains a T-DNA insertion in the 5' upstream sequence of *Oshox22* and displays strongly down-regulated expression of this gene. Under normal greenhouse conditions, *oshox22-1* plants did not show any visible difference from wild type. However, the endogenous ABA levels in *oshox22-1* seedlings were 60% lower than those of the wild-type Dongjin plants. When grown on medium with ABA,

oshox22-1 seeds showed a higher germination rate than the wild type. On the other hand, rice plants over-expressing *Oshox22* showed a decreased germination rate on medium supplied with ABA suggesting *Oshox22* is involved in an ABA-regulated seed germination process. It should be noted that the over-expression and mutation phenotypes were analysed in different genetic backgrounds. Zhonghua 11 is an important lowland rice cultivar in Chinese agriculture which is easy to transform using *A. tumefaciens* and therefore this is the preferred model for transgenesis in our laboratory. As a mutant allele of *Oshox22* was not present in the Zhonghua 11 RMD T-DNA collection (Zhang et al. 2006), we analysed a mutant allele of *Oshox22* in Dongjin, which is also a lowland *Japonica* rice cultivar. Since ABA sensitivity was decreased upon *Oshox22* mutation in Dongjin background and increased upon over-expression in Zhonghua 11 background, our data suggest that the function of *Oshox22* between these cultivars is conserved.

These results of *Oshox22* effects on ABA sensitivity are only partly consistent with those obtained in Arabidopsis with *Athb-7* and *Athb-12,* in spite of the fact that the latter are in the same HD-Zip I subgroup (γ-clade) as *Oshox22* (Olsson et al. 2004; Henriksson et al. 2005; Agalou et al. 2008). Furthermore, over-expression of *CpHB-7* isolated from *C. plantagineum* in Arabidopsis resulted in increased germination on ABA and thus reduced sensitivity towards ABA (Deng et al. 2006). Plants over-expressing *Athb-6* are also less sensitive to ABA on germination (Himmelbach et al. 2002). Thus, different HD-Zip proteins may be involved in different ABA-mediated signalling pathways and differences of closely related genes in the different species suggest that the signalling pathway may have evolved rapidly.

Although *Oshox22* is induced by ABA, constitutive over-expression of *Oshox22* in Zhonghua 11*,* led to an increased ABA level but compromised drought tolerance. In addition to decreasing ABA sensitivity, however, the *Oshox22* mutation in Dongjin resulted in an increased tolerance towards

drought, which correlated with reduced water-loss efficiencies of mutant seedlings. As numbers of stomata were decreased in *oshox22-1* mutants, regulation of stomata density by *Oshox22* is a possible mechanism underlying enhanced drought tolerance in these mutants. However, a change in stomata density was not found in the *Oshox22-OX* lines although these lines were less drought tolerant. Therefore, most likely stomata density is not the only factor determining drought tolerance under our experimental conditions. We conclude that *Oshox22* negatively regulates drought tolerance in rice. In wheat a similar situation is described where the level of ABA is inversely correlated to the level of drought tolerance. In drought-sensitive cultivars, drought treatment leads to enhanced expression of ABA biosynthesis genes in anthers and ABA accumulation in spikes, while in drought-tolerant wheat the treatment leads to accumulation of lower levels of ABA, which correlates with lower expression of ABA biosynthesis genes and higher level of expression of ABA catabolic genes (e.g. ABA 8'-hydroxylase). Furthermore, wheat *TaABA8' OH1* deletion lines that accumulate higher levels of ABA in spikes are drought sensitive (Ji et al. 2011). We analysed ABA levels in *oshox22-1* mutants and the two over-expression lines as well as their respective wild type backgrounds. We found that mis-expression of *Oshox22* affected the absolute levels of ABA. However, despite difference in induction levels, in all plants ABA levels increased strongly in response to drought, which is in line with the well-known function of this hormone in responses to stress signals including drought and salinity. Higher levels of ABA are due to induction of ABA biosynthesis genes and in turn ABA reprograms plant cells to withstand and survive adverse environmental conditions (reviewed by Leung and Giraudat 1998; Umezawa et al. 2009; Melcher et al. 2010; Huang et al. 2012). We hypothesise that *Oshox22* may plays a role in ABA biosynthesis or degradation and that a lower endogenous ABA level in *oshox22-1* plants and higher level of ABA, respectively correlate inversely with their drought tolerance. Being a

transcription factor, Oshox22 may affect ABA level by directly regulating genes involved in biosynthesis or degradation of ABA.

We propose that *Oshox22* functions as a negative regulator in drought and salt tolerance similar to *OsABI5*, which is a bZIP transcription factor, and is inducible by ABA and high salinity (Zou et al. 2008). Transgenic rice plants over-expressing *OsABI5* were sensitive to ABA and to high-salinity stress as well as to PEG treatment. Similar as with the *oshox22-1* mutant, down-regulation of *OsABI5* in plants using an RNAi approach exhibited increased tolerance to salt as well as to PEG treatment which is a stress condition we did not check for *Oshox22* (Zou et al. 2007, 2008). It seems that several regulators exist that are able to control both drought and salt tolerances. Other examples are the transcription factors *OsbZIP23* (Xiang et al. 2008) and *DST* (Huang et al. 2009). However, there are also differences with *Oshox22*. Like with *Oshox22*, both factors are nuclear-localised transcriptional activators and down-regulation of *OsbZIP23* expression results in decreased ABA sensitivity but tolerance towards salt and drought stress is decreased and over-expression results in an increase of tolerance which is opposite to the results with *Oshox22*. Microarray experiments with *OsbZIP23* mis-expression plants identified sets of genes regulated by *OsbZIP23* amongst which many genes have known functions in stress tolerance (Xiang et al. 2008). Interestingly, the microarray dataset shows that *Oshox22* as well as *Oshox24* which is on a duplicated chromosome segment (Agalou et al. 2008), are higher expressed in the *OsbZIP23* over-expressor indicating that this gene probably acts upstream of the two HD-Zip I genes. We expect that in turn *Oshox22* and Oshox24 also have similar functions in controlling other sets of stress tolerance genes. Future experiments can involve similar experiments for downstream target genes in order to explain how *Oshox22* regulates drought and salt tolerance and ABA biosynthesis.

Taken together, down-regulation of *Oshox22* expression by T-DNA insertion led to plants with reduced levels of ABA and enhanced tolerance towards drought and salt stresses, while over-expression of the gene increased ABA content and decreased drought and salt tolerances. These results support the conclusion that *Oshox22* acts as a negative regulator in stress responses. Since reporter gene studies in yeast and rice cells suggested that *Oshox22* acts as a transcriptional activator, its function as a negative regulator in stress responses might be explained via activation of other repressors. The fact that *oshox22-1* plants showed no significant reduction in yield makes it a potential candidate for improving stress tolerance in rice. Further research is needed to identify allelic variation related to altered levels of *Oshox22* expression for rice breeding.

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Supplemental data

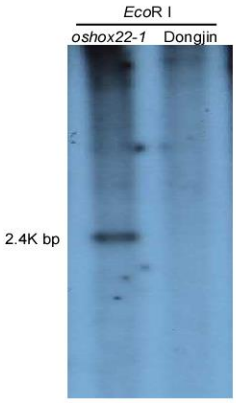

Figure S1. The copy number of the T-DNA insertion in *oshox22-1*. Genomic DNA was digested by *Eco*RI and the *HPT* cDNA fragment was used as probe in the Southern blot experiment.

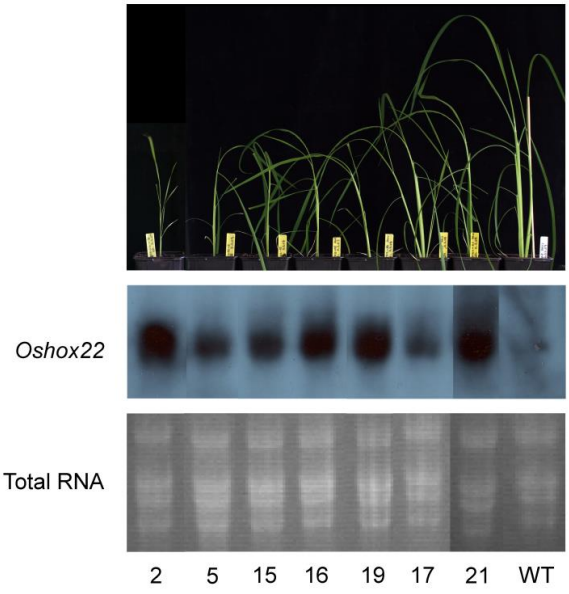

Figure S2. Northern blot analysis of *Oshox22* expression in transgenic *Oshox22* over-expression plants (middle panel). Equal loading of the RNAs was verified by ethidium bromide staining (lower panel). Upper panel: left: phenotypes of the Oshox22-OX plants; right: wild type Zhonghua 11 (ZH11) plant.

Table S1. Primers used in this study

Primer code	Sequence
OsHox22cdsFW	5'- GCTAGGATCGCCATGGATCGG-3'
OsHox22cdsRW	5'- GCTACATGTACACACAAATTAACC-3'
1(1~263aa)-F	5'-CCGGAATTCCGGATGGATCGGGGTGACCACCAC-3'
1(1~263aa)-R	5'-CGGGATCCCGTCAAGCCACCGCATTCCACTCG-3';
2(66~263aa)-F	5'-CCGGAATTCCGGGGAGCAGATACGGTCGCTGGAG-3'
2(66~263aa)-R	5'-CGGGATCCCGTCAAGCCACCGCATTCCACTCG-3';
3(135~263aa)-F	5'-CCGGAATTCCGGCGAGTCCCTCAAGCAAGAGAAG-3'
3(135~263aa)-R	5'-CGGGATCCCGTCAAGCCACCGCATTCCACTCG-3'
4(1~235aa)-F	5'-CCGGAATTCCGGATGGATCGGGGTGACCACCAC-3'
4(1~235aa)-F	5'-CGGGATCCCGTCGTAGTCAAGCTGGTCGTCG-3'
5(1~205aa)-F	5'-CCGGAATTCCGGATGGATCGGGGTGACCACCAC-3'
5(1~205aa)-R	5'-CGGGATCCCGGCGGCTCGAGGTCCAGGCACCC-3'
6(1~175aa)-F	5'-CCGGAATTCCGGATGGATCGGGGTGACCACCAC-3'
6(1~175aa)-R	5'-CGGGATCCCGCTGCTTGCTGCCGTCGTGGCG-3'
7(1~145aa)-F	5'-CCGGAATTCCGGATGGATCGGGGTGACCACCAC-3'
7(1~145aa)-R	5'-CGGGATCCCGAGAGCGAGCTTCTCTTGCTTGAG-3'
8(1~115aa)-F	5'-CCGGAATTCCGGATGGATCGGGGTGACCACCAC-3'
8(1~115aa)-R	5'-CGGGATCCCGTTGGAGCGGAGTGCGGCGTAGT-3'
QPCR-22FW	5'- CCGGCGGACGTGTCGGTGGAGTC-3'
QPCR-22RW	5'- AGCCACCGCATTCCACTCGACGAGC-3'
ACT1-FW	5'-CTTCATAGGAATGGAAGCTGCGGGTA-3'
ACT1-RW	5'-CGACCACCTTGATCTTCATGCTGCTA-3'

Table S2. *S. cerevisiae* strains used in this study

Strain	Genotype
PJ69-4A	*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80ΔMet2::GAL7-lacZ LYS2::GAL1-HIS3 GAL2-ADE2*
YM4271	*MATa ura3-52 his3-200 ade2-101 lys2-801 leu2-3,112 trp1-901 tyr1-501 gal4-Δ512 gal80-Δ538 ade5::hisG*

Chapter 3

Function of the homeobox gene *Oshox22* in controlling grain length in rice

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Abstract

Grain size is a key component of grain quality and yield in rice. Here we report the genetic and molecular characterisation of homeobox gene *Oshox22*, which plays an important role in controlling grain length (GL) in rice. *Oshox22* encodes a homeodomain-leucine zipper (HD-Zip) class I transcription factors on chromosome 4. We found a stable quantitative trait locus (QTL) for GL on this position in four mapping populations. Sequence analysis across the *Oshox22* gene from cultivars Bala and Azucena revealed an extra A base in the promoter of Azucena which has a long grain type. Using a PCR-based insertion/deletion (InDel) CAPS marker assay, we found an association between the A InDel in the *Oshox22* promoter with GL in a core collection of Italian *japonica* rice. Furthermore, expression of *Oshox22* correlates with presence or absence of the A InDel. In addition, expression of *Oshox22* under the control of its native promoter from IRAT112 cultivar (with the A InDel) showed a significant increase in GL compared to the transformation background Zhonghua 11 (without the A InDel). Scanning electron microscopy revealed that enhanced GL was caused by increased cell length in the inner epidermal cells of lemma. Our findings suggest that allelic variation in the *Oshox22* promoter can be exploited in breeding programmes to modify GL using molecular marker-assisted selection.

Keywords: Grain length, Homeobox, QTL mapping, InDel, Rice.

Introduction

Rice is used as a staple food for nearly half of the global population (Khush 2005). Global concern of food shortage, however, is increasing with the population increase and therefore it is crucial to increase rice yield. Yield in rice is composed mainly of three key aspects which are the number of panicles per plant, number of grains per panicle and grain size (or weight (GW)). Grain size is a major agronomic trait that is affected by its length, width, filling and thickness (Xing and Zhang 2010). Recently, several major genes underlying important QTLs for rice grain size were detected and characterised using map-based cloning approach (Bai et al. 2012; Huang et al. 2012; Zhang et al. 2012). Among these are *GS3* and *DEP1*, both genes being negative regulators for grain length. *GS3* encodes a putative transmembrane protein (Fan et al. 2006) and *DEP1* encodes a phosphatidylethanolamine-binding protein-like domain protein (Huang et al. 2009). *TGW6* encodes an indole-3-acetic acid (IAA)-glucose hydrolase and loss-of-function of the gene enhances GW (Ishimaru et al. 2013). *GIF1* encodes a cell-wall invertase required for carbon partitioning during early grain-filling and it functions as a negative factor for grain filling (Wang et al. 2008). *GW2* encodes a RING-type E3 ubiquitin ligase (Song et al. 2007) and *qSW5*/*GW5* encodes a nuclear protein (Shomura et al. 2008; Weng et al. 2008) and both have been reported as negative regulators for grain width. *GS5* encodes a putative serine carboxypeptidase (Li et al. 2011) and *qGW8*/*OsSPL16* encodes a SQUAMOSA promoter-binding protein-like 16 domain protein (Wang et al. 2012), and both are positive regulators for grain width.

In addition, several genes for grain size have recently been identified in other cereals such as maize (*ZmGS3* and *ZmGW2*, orthologues of the rice genes *GW2* and *GS3*, respectively) (Li et al. 2010a, 2010b) and wheat (*TaGW2,* orthologue of the rice gene *GW2*) (Bednarek et al. 2012). Several genes associated with grain size and weight, have also been reported in *Arabidopsis*. Among these are *AGG3,* which has homology with rice *GS3* and *DEP1* genes and is member of a family of heterotrimeric G protein γ-subunits (Li et al. 2012a, 2012b), *APETALA2* (*AP2*) which encodes a transcription factor (Jofuku et al. 2005; Ohto et al. 2005), *DA1* which encodes a ubiquitin receptor (Li et al. 2008), *MINISEED3* (*MINI3*) which encodes a WRKY family gene and *HAIKU2* (*IKU2*) which encodes a leucine-rich repeat (LRR) KINASE gene (Luo et al. 2005).

Grain length (GL) as well as other grain traits in rice were a main target during cereal domestication, it is still important in breeding (Sweeney and McCouch 2007) and has a major impact on global rice grain markets because of the consumer's preference. Generally, a long-slender rice grain is preferred by consumers especially in South and Southeast Asian countries,

Southern China and USA (Unnevehr et al. 1992; Juliano and Villareal 1993; Huang et al. 2012). As GL is a complex quantitative trait that is difficult to address via conventional plant breeding approaches, utilisation of molecular markers for GL can tremendously facilitate exploration and breeding for this trait. An example is *GS3* which is a major QTL for GL in rice on chromosome 3. Fan et al. (2009) designed a marker based on the causal C-A mutation in the second exon of *GS3* and observed a common C-A single nucleotide polymorphism (SNP) between short and long grain rice cultivars. Hence, *GS3* was validated as a functional marker for the selection of long-grain rice lines in breeding programme (Takano-Kai et al. 2009).

In this study, we present genetic and molecular data showing that allelic variation in the promoter of the homeobox gene *Oshox22* controls GL. We found this gene in QTLs for GL in four mapping populations and found an association with GL in an Italian core collection. Using a transgenic approach we confirmed a function of *Oshox22* in controlling GL by regulating cell length of the lemma.

Materials and methods

Plant materials and growth conditions

Rice cultivars used for QTL analysis and rice transformation are listed in Table S1. Seeds were surface sterilised and immersed in water for two days in the darkness at 28°C, and then sowed into soil in a climate chamber (at 28°C, 85% relative humidity and a 12-h light/dark photoperiod 450 µM m^{-2} s^{-1}).

Linkage and QTL analysis

A mapping population of 205 F_6 RILs was produced from a cross of the rice cultivars Bala (*indica* x *aus*) and Azucena (upland *japonica*) by single seed decent (Price et al. 2000). In this study seed of 157 randomly chosen RILs were used to identify QTLs for grain length. This was assessed by placing ten seeds (including the husk) in line and measuring the length. Analysis was conducted as described in (Price et al. 2002). The molecular map covers 1832 cM and has 164 markers whose approximate physical position on the rice genome sequence is given in (Khowaja et al. 2009). The identification of QTLs was performed by composite interval mapping using the programme QTLCartographer version 1.15 (by C.J. Basten, B.S. Weir, and Z.B. Zeng, Department of Statistics, North Carolina State University). Background markers for composite interval mapping were limited to ten and were selected by forward stepwise regression with backward elimination

using the default setting. The default window size was set at 10 cM. Permutation testing indicated that a LOD score of 3.2 was suitable as the genome-wide 5% significance threshold. Bala and Azucena genomes have been analysed by next generation sequencing at 65-fold and 25-fold coverage, respectively. Fastq data has been deposited in the Short Read Archive at NCBI as Acc_ID SRA050654.1 and have been aligned to the Nipponbare reference genome as described in Cardoso 2013 (Pers. Comm. A. Price).

Sequence analysis of *Oshox22*

A 2.9 Kb fragment containing the *Oshox22* ORF together with an intron and 3'-UTR was PCR-amplified from genomic DNA of eight rice cultivars (Nipponare, Bala, T309, 9311, Cabacu, Azucena, IRAT112, IRAT109 (Figure S1) using primers G-NB-22F and G-NB-22R (Table S2). This region contained a 1.2 Kb promoter fragment upstream from the start codon and a 392-bp downstream from the stop codon. The PCR products were subcloned into pCR2.1 Topo (Invitrogen) for sequence analysis (Baseclear, the Netherlands). The sequences were further aligned using ClustalW (Thompson et al. 1994) and displayed with GeneDoc (http://www.cris.com/~ketchup/genedoc.html.) (Figure S1).

Illumina genotyping

The 67 accessions of the European Rice Germplasm Collection (ERGC) that were evaluated in this study are listed in Table S3 and are mainly Italian cultivars. According to a phylogenetic analysis using single sequence repeat (SSR) markers they belong to subgroup IIf and were classified based on their grain type (Faivre-Rampant et al. 2011). The phenotypic data were collected from the available EURIGEN database (http://eurigendb.cirad.fr). The DNA was extracted from one plant per accession. The *Oshox22* SNP was genotyped using an Illumina Veracode assay (http://www.illumina.com/technology/veracode_goldengate_assay.ilmn). Further genotyping and data analyses were conducted accordingly (Courtois et al. 2012).

SNP genotyping using the *Nde*I CAPS marker

Genomic DNA was extracted from the leaves of the rice core collection listed in Table S4 using the high-throughput method (Xu et al. 2005). A 1.2 kb promoter fragment was PCR-amplified from genomic DNA using primers Pro-22F and Pro-22R (Table S2). Conditions of PCR amplification were adjusted according to primer sets as follow: 98°C for 5 min, then 35 cycles each at 98°C for 30 s, 66.1°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 10 min. PCR-amplified products were digested using restriction endonuclease *Nde*I in a total volume of 30 µl

according to the manufacturer's instructions. The digests were separated by gel electrophoresis with 1.0 % agarose gel in 1.0 X TBE buffer and further visualised with UV light (Bio-Rad) after staining with ethidium bromide.

Measurement of grain length

Measurement of GL and GW of fully filled and matured grains was according to (Fan et al. 2006). Harvested paddy rice was air-dried and stored at room temperature for at least three months before testing. Ten randomly chosen grains from each plant were lined up length-wise along a Vernier caliper to measure length, and then arranged by width to measure width. The values were averaged.

Binary vector construction and rice transformation

The construct in which *Oshox22* was expressed from its own promoter with the InDel was made in binary vector pC1300intC (Genbank Accession AF294978) by first cloning two different sub-fragments as following: A 2.3 kb promoter fragment upstream of the translation start of *Oshox22* (corresponding to a region between nucleotide 27121783-27124166 (MSU Osa1 Release 7) with the A InDel in the promoter was PCR-amplified from *O. sativa* L. cv. IRAT112 using primers Pro-IR-22F and Pro-IR-22R (Table S2) and subcloned into pCR2.1 Topo (Invitrogen) for sequence analysis (Baseclear, the Netherlands). A 2.9 kb fragment containing the *Oshox22* ORF together with an intron and 3'-UTR (corresponding to a region between nucleotide 27123201-27126182 (MSU Osa1 Release 7, LOC_Os04g45810) was PCR-amplified from genomic DNA from *O. sativa* L. cv. Nipponbare using primers G-NB-22F and G-NB-22R (Table S2) and cloned into pCR2.1 Topo (Invitrogen) and sequenced. Finally, an *Eco*RI/*Kpn*I promoter fragment and the *Oshox22* ORF-containing *Kpn*I/*Spe*I fragment were combined into binary vector pC1300intC cut with *Eco*RI/*Xba*I. Transformation of *japonica* rice (*O. sativa* L. cv. Zhonghua 11) was performed as previously described by (Scarpella et al. 2000), except that *A. tumefaciens* strain *LBA 4404* was used instead of LBA 1119. Plantlets were maintained in culture on half-strength Murashige-Skoog (MS) medium with 10 g/l sucrose until transfer to a greenhouse (28°C under a 16 h photoperiod and 85 % humidity).

Histological study using cryo-SEM imaging

The inner epidermal cells of the lemma before heading were observed by cryo-scanning electron microscopy. The samples were glued on a brass Leica sample holder by carbon glue (Leit-C, Neubauer Chemicalien, Germany), directly frozen in liquid nitrogen and simultaneously fitted in the

cryo-sample loading system (VCT 100) and frozen in liquid nitrogen. The Leica sample holder was transferred to a non-dedicated cryo-preparation system (MED 020/VCT 100, Leica, Vienna, Austria) onto a sample stage at -93˚ C. In this cryo-preparation chamber the samples were freeze dried for 3 minutes at -93°C at 1.3 x 10^{-6} mBar to remove water vapour from the surface of the sample. The samples were sputter coated with a layer of 10 nm Tungsten at the same temperature. The samples were transferred into the field emission scanning microscope (Magellan 400, FEI, Eindhoven, the Netherlands) on the sample stage at -122°C at 4.0 x 10^{-7} mBar. The analysis was performed with SE at 1 kV, 13 pA. All images were recorded digitally. Cell length in each sample was measured using ImageJ software.

Gene expression analysis

For RNA extraction, tissue from young panicles (2.5 cm and 5 cm in length, before heading) of cultivars as described in Table S3 was harvested and total RNA was obtained using a combination of the protocols for TriPure reagent (Roche), the RNAeasy Mini Kit (Qiagen) and the DNase-I Kit (Qiagen), as previously described (http://www.untergasser.de/lab/protocols/rna_prep_comb_trizol_v1_0.htm). Reverse transcription reaction was performed with an iScript cDNA Synthesis Kit Bio-Rad. Primers used for quantitative RT-PCR (qRT-PCR) analyses of *Oshox22* were QPCR-22F and QPCR-22R (Table S2). qRT-PCR was performed with a Bio-Rad iQ5 analyser using SYBR Green Supermix Bio-RAD to monitor dsDNA synthesis. Three independent biological replicates were used and each PCR reaction was done in triplicate. Relative expression levels of reporter and target genes were determined by the Two Standard Curves Relative Quantification Method using an *ACTIN1* amplicon (Table S2) as internal control.

Results

QTL analysis for grain length

The grain length (GL) of the Bala x Azucena population ranged from 5.35 to 9.15 mm. Their QTL analyses revealed a total of seven QTLs for all of which the Azucena allele increases GL (Table 1). The largest QTL explaining 17.6% of the variation is likely to be related to *GS3* (LOC_Os03g29380) at 16.7 Mb on chromosome 3 (Fan et al. 2006). Of interest in this paper is the QTL on chromosome 4 explaining 10.9% of the variation and localised at 25.6 Mbp. This QTL is not located near the *GIF1* locus (LOC_Os04g33740) that has been shown to affect seed length

(Wang et al. 2008), which is at 20.4 Mbp on the same chromosome, some 5 Mbp or 25 cM away from the QTL peak. Rather the identified QTL showed a tight co-positional relationship with the candidate gene *Oshox22* (LOC_Os04g45810) which is located at 26.9 Mbp (Agalou et al. 2008). The alignment of the sequences from cultivars Bala and Azucena to Nipponbare reveals no polymorphism within the gene, however there is a single insertion of an A base in the promoter of Azucena which was not present in Bala. The A InDel is present at 583 bp upstream of the translation start of *Oshox22* (corresponding to a region between nucleotide 27123582-27123584 in MSU Osa1 Release 7). The presence of the extra A introduced an *Nde*I restriction site that enabled development of a CAPS marker analysis. We further checked by sequence and database analysis and the *Nde*I CAPS marker the variation at this position and found it to be absent in the lowland *japonica* cultivars Nipponbare, Taipei 309, Taichung 65, Tainung 67, but present in the upland tropical *japonica* cultivars IRAT109, IRAT112 and Cabacu (IRAT177) and absent again in the *indica*'s IR36, IR58 and IR64.

Table 1. Summary of main effect QTLs for grain length in rice

Chromosome	Position (cM)	Approx. position on genome (Mbp)	QTL position above or below nearest marker (cM)	LOD	Additive effect (mm)	R^2 (%)
1	211.0	39.0	C949 -6	3.5	0.22	7.6
1	242.4	41.8	R117 -5	6.1	0.31	17.5
3	57.7	10.7	C643-3	3.7	0.22	8.0
3	91.4	16.2	G144 +8	8.4	0.34	17.6
4	75.3	25.6	RM252 +4	5.5	0.25	10.9
8	93.8	20.3	G1073	4.3	0.19	6.7
9	0.0	0.4	P0414D03	3.9	0.19	6.3

We also studied the distribution of the A InDel in Italian *japonica* cultivars. The A InDel was also found in Augusto, Thaibonnet and Baldo but was absent again in Balilla, Carnarolli and Gladio. Together, these initial results show that the A InDel in the *Oshox22* promoter is widespread in *japonica* rice.

Searching the Gramene QTL database and literature identified three more QTLs for GL on chromosome 4 in the vicinity of *Oshox22*. In an Asimori x IR24 population, four QTLs for GL were identified of which the one on chromosome 4 (ID AQGB023) is between markers XNpb331 and C335 (Wan et al. 2005). Marker C335 is at 24.69 Mbp which is rather near to *Oshox22.* Redoña and Mackill (1998) used a Labelle x Black Gora population and identified seven QTLs for GL of which one is near marker RG476 which is at 31 Mbp on chromosome 4 and thus close to *Oshox22*. In a Reiho x Yamada-nishiki population, three QTLs for GL were found of which one is at the lower end of chromosome 4 which peaks at marker RM255 at 30.8 Mb, but at lower significance it seems to extend towards RM241 which is at 26.9 Mb where *Oshox22* is located (Yoshida et al. 2002).

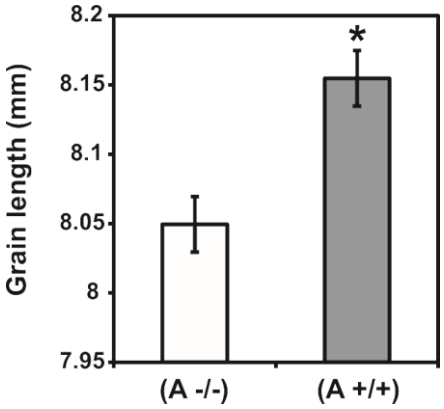

Figure 1. Comparison of GL in F_2 population by crossing IRAT109 (tropical *japonica*, with the A InDel in the *Oshox22* promoter) with Zhonghua 11 (lowland *japonica*, without the A InDel). (A-/-), without the A InDel and (A+/+), with the A InDel. Data are means ±SD ($P < 0.05$, n=336).

In order to verify if the A InDel in the promoter of *Oshox22* identified in Azucena as well as in several other upland type cultivars has any association to GL, we created another F_2 population by crossing the tropical *japonica* IRAT109 (with the A InDel in the *Oshox22* promoter) with lowland

japonica Zhonghua 11 (without the A InDel). The resulting population of 336 F_2 plants was analysed using the *Nde*I CAPS assay. It was found that there are 80 plants with the A InDel in the *Oshox22* promoter (named A+/+), 176 plants are heterozygous (A +/-) and 80 plants without A (A -/-) showing that there is a Mendelian segregation of the variation at the *Oshox22* locus. Next, we measured GL of all 336 plants and found that the A InDel in the *Oshox22* promoter is positively associated to GL (r=0.158*, $P < 0.05$) and increased from an average of 8.049 to 8.154 mm, attributing 1.89% of the variation in GL to the *Oshox22* locus (Figure 1).

The InDel in the *Oshox22* promoter is associated with grain length in rice

The *Nde*I CAPS marker has been used to test for the presence of the A InDel in two different rice diversity collections. Analysis of the Rice Diversity Panel (Zhao et al. 2011) showed that the A InDel was absent in all 12 aromatic rice, absent in all 54 aus, only present in one amongst 68 *indica* cultivars, present in seven out of 85 temperate *japonica*s and 73 out of 81 tropical *japonica* cultivars indicating the gene is mainly present in *japonica* rice with a strong preference for tropical *japonica* but is underrepresented in other rice subpopulations. In this panel, the A InDel does not seem to be associated with GL (results not shown). Although in this particular panel, the A InDel is relatively rare in temperate *japonica*s, it is much more widespread amongst Italian *japonica* rice since the A InDel was found in 43 cultivars out of 95.

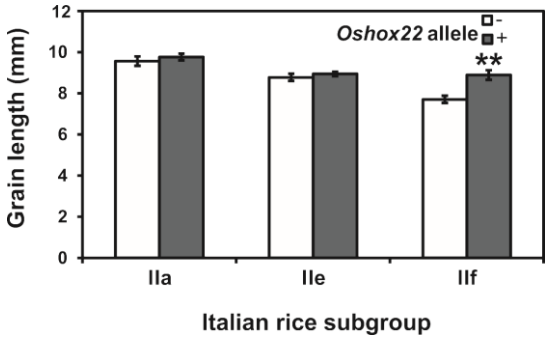

Figure 2. Effect of *Oshox22* alleles on GL in three different groups of Italian rice cultivars. The white bar represents cultivars without the A InDel versus the black bar with the A InDel.

Analysis of genetic diversity of Italian rice grouped them into seven groups named group I, IIa, IIb, IIc, IId, IIe and IIf (Faivre-Rampant et al. 2011). The frequency of the A insertion was larger than 5% in three of these groups. In group IIa, nine out of 13 have the InDel, in IIe 21 out of 35 have it

and in IIf five out of 26 have the InDel. A two-way ANOVA of GL against the presence of the InDel in the three groups (II a, e or f) revealed highly significant effects of both (group F=15.4, p=0.001; insertion F=8.27, *P*=0.005) and their interaction (F=3.82, *P*=0.027). Thus we can conclude that in Italian cultivars the insertion is associated with GL but this seems to be most obvious in group IIf. Figure 2 shows the effect of the allele on GL in this group, where the five cultivars with the insertion have an average grain length of 1.2 mm longer than those without it (*P*=0.005).

Expression pattern of *Oshox22* in young panicles

Earlier work showed that *Oshox22* is ubiquitously expressed, with a lower level in stems and higher in developing panicles and grains (Agalou et al. 2008) and that the gene is involved in tolerances towards drought and salt (Zhang S et al. 2012) suggesting that it has a function in these tissues. This panicle and grain-related function is also confirmed by the present genetics studies that indicate an additional role in determining GL, especially in *japonica* rice. To study this observation in more detail, we analysed the expression pattern of *Oshox22* in young panicles from cultivars with or without the A InDel.

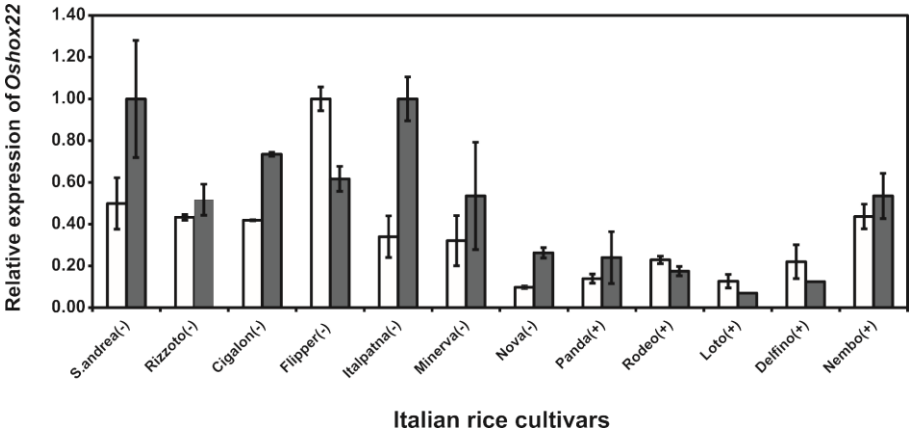

Figure 3. Expression profile of *Oshox22* in young panicles revealed by qRT-PCR. (-) denote, Italian rice cultivars without the A InDel and (+) denote, Italian rice cultivars with the A InDel. White bars indicate, young panicle 2.5 cm in length before heading and black bars represent, young panicle 5 cm in length before heading. All data are based on three biological replications. Data are means ±SD.

As shown in Figure 3, quantitative RT-PCR analysis detected a statistically significant difference in expression between a panel of Italian cultivars from group IIf with and without InDel. The data show that there is a significant association between the InDel and the expression level in 5 cm

long grains (*P*=0.010), with higher expression in lines without the A InDel and lower expression in lines with the A InDel (Figure 3). In addition, Figure S2 shows the same set of expression data in scatter plots versus GL. Although GL is not statistically related with the expression level (*P*=0.132), there is a tendency that the group with longer GL tends to have lower expression of *Oshox22*.

***Oshox22* mis-expression increases grain length**

To further study the effect of the InDel in the *Oshox22* promoter and to confirm the data from the genetics analyses, a construct (ProOshox22-Oshox22) was made with the *Oshox22* gene driven by its own promoter having the A InDel which was from the upland *japonica* cultivar IRAT112.

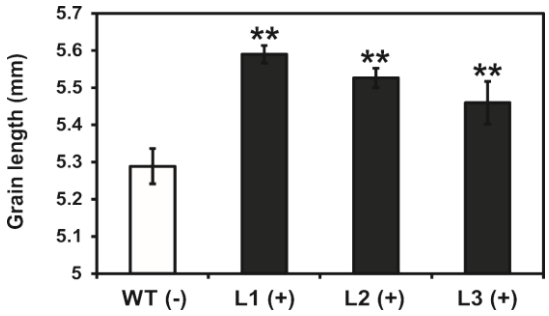

Figure 4. Comparison of GL in wild type Zhonghua 11 (ZH11) and ProOshox22-Oshox22 lines. L1-3 (+), represent three transgenic lines of ProOshox22-Oshox22; WT (-), wild type Zhonghua 11, lowland *japonica* cultivar without the A InDel; ProOshox22-Oshox22, expression of *Oshox22* under the control of its native promoter from IRAT112 with the A InDel. Data are means ±SD (**significant difference at $P < 0.01$, Student's *t* test, n=10).

Transformants were made in a lowland *japonica* type (Zhonghua 11) in which the A InDel is absent. The resulting lines did not show any obvious morphological differences with respect to wild type Zhonghua 11. A total of three lines was selected for further analysis of GL and it was found that in the transgenics, GL increased by 7% from an average of 5.288 to 5.525 mm ($P < 0.01$, n=10) (Figure 4), thereby confirming the results of QTL and the association genetics approaches.

The genetics and transgenic approaches clearly showed an association between the A InDel in the *Oshox22* promoter and an increase in GL. We speculated that this is due to an increase in cell length. To confirm this, the length of cells in the inner epidermal tissues of the lemma were analysed by cryo-SEM (Figures 5A and B). The average cell length in the ProOshox22-Oshox22 and wild type Zhonghua 11 seeds were found to be 129.9±21.8 and 104.6±27.8 µm, respectively.

Thus, the averaged cell length in the lemma in ProOshox22-Oshox22 grains is increased by 32% compared to the wild type ($P < 0.001$) (Figure 5C).

Discussion

In this study, we further characterised a homeobox gene *Oshox22* on chromosome 4, which is positioned on QTLs controlling GL. QTLs for GL on the approximate 25 Mbp position were found in four mapping populations across multiple environments highlighting the QTL stability. By sequence alignments, we did not find any polymorphism within the ORF of *Oshox22*, whereas we identified an extra A base in the corresponding promoter from Azucena (*Japonica*, longer grain length) when compared to Bala (*Indica*, shorter grain length) and the reference genome of Nipponbare, which is present at 583 bp upstream of the translation start of *Oshox22*. Based on this InDel marker assays were developed to further characterise rice populations and collections. In an F_2 population between IRAT109 (upland tropical *japonica*, with the A InDel) and Zhonghua 11 (lowland *japonica*, without the A InDel), we found an association between the presence of the A InDel in the *Oshox22* promoter with GL. Furthermore, in a subgroup IIf of an Italian core collection (Faivre-Rampant et al. 2011), the A InDel is also associated with GL and also determines the expression level of *Oshox22*. Using an Italian core collection we also showed that *Oshox22* is highly expressed in developing panicles and grains (Agalou et al. 2008; Zhang S et al. 2012). In young panicles, we found a significant association between the InDel and GL only in Italian *japonica* rice, subgroup IIf. Our quantitative RT-PCR data showed a significant association between the presence or absence of the InDel and *Oshox22* expression level, with higher expression in Italian lines without the InDel and lower expression in lines with the insertion which suggests that expression level of *Oshox22* is reduced by the presence of the extra A nucleotide in the promoter and therefore we speculate that the extra A is part of a yet unknown transcription factor binding site. Although we don't have statistical support for an association between expression level and GL, the data show that there is a tendency for lower expression when GL increases which would suggest that Oshox22 protein functions as repressor of GL.

Using a transgenic approach with a ProOshox22-Oshox22 construct we could further verify the association between the InDel in the *Oshox22* promoter, the expression polymorphism and GL. Expression of *Oshox22* under the control of its native promoter from cultivar IRAT112 (upland tropical *japonica*, with the A InDel) showed a significant increase in GL compared to the transformation background Zhonghua 11 (lowland *japonica*, without the A InDel).

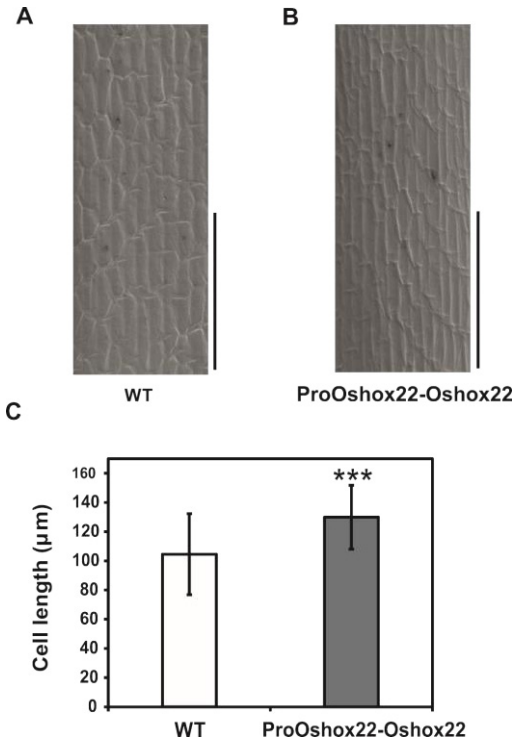

Figure 5. Comparison of cell length between the wild-type Zhonghua 11 (ZH11) and a ProOshox22-Oshox22 line. **A and B,** Inner epidermal cells of lemma of the wild type (ZH11) and ProOshox22-Oshox22 observed by cryo-SEM. Bars = 300 µm. **C,** Quantitative measurement of the cell length of the wild type (ZH11) and ProOshox22-Oshox22 in a 1-mm portion of the central area of the lemma before heading. (mean ± SD, n = 50). ***significant difference at $P < 0.001$, Student's *t* test.

In rice, the palea and lemma (floral organs analogous to sepals) (Yoshida and Nagato 2011) are important organs of the rice floret and partially determine grain shape, GL as well as grain yield (Xing and Zhang 2010). Our data show that the inner epidermal tissues of the lemma from ProOshox22-Oshox22 lines increase in cell length compared to wild type Zhonghua 11. These results suggest that A InDel in the *Oshox22* promoter positively regulates GL by increasing cell size of the lemma. This is supported by earlier studies reporting that loss-of-function mutations in *SRS3* and *SRS5* caused a small and round seed phenotype due to a reduction in cell length in the inner epidermal tissues of the lemma (Kitagawa et al. 2010; Segami et al. 2012).

Cultivated rice (*Oryza sativa*) was originally domesticated from a wild rice progenitor (*O. rufipogon*) and differentiated into two varietal groups; *indica* and *japonica* (Khush 1997). These groups were

further subdivided into the *indica* varietal group (*indica* and *aus*) and the *japonica* varietal group (temperate *japonica*, tropical *japonica*, and aromatic) (Garris et al. 2005; Kovach et al. 2007). Among these, the two types of *japonica* (temperate *japonica* and tropical *japonica*) have a very close genetic relationship and have a lower genetic diversity than *indica* (Glaszmann 1987; Zhang et al. 1992; Ni et al. 2002; Garris et al. 2005). Khush (1997) hypothesised that temperate *japonica* cultivars were derived from tropical *japonica* cultivars. In the rice diversity collections that we studied, the A InDel in the *Oshox22* promoter was detected mainly in tropical *japonica* and only in relatively few temperate *japonica* cultivars, thus apparently this allele was lost during domestication and selection and became underrepresented in lowland *japonica*.

Oshox22 belongs to the so-called homeodomain-leucinezipper (HD-Zip) class of plant-specific transcription factors (TFs) (Ruberti et al. 1991; Schena and Davis 1992; Agalou et al. 2008) which consist about half of the plant homeobox genes (Mukherjee et al. 2009). HD-Zip TFs are characterised by the presence of a DNA binding domain (HD) encoded by a homeobox followed by an adjacent protein dimerisation motif (LZ) and are divided into four subfamilies (I-IV) based on sequence homology (Ruberti et al. 1991; Meijer et al. 1997, 2000; Agalou et al. 2008). HD-Zip TFs have been associated with several diverse biological functions including vascular development, embryogenesis, leaf polarity as well as meristem regulation and developmental responses to environmental conditions (reviewed by Ariel et al. 2007; Harris et al. 2011). Especially, from the HD-Zip I TFs family, many members from several plant species seem to be involved in regulation of development in response to changes in environmental conditions (Harris et al. 2011). For example, *Arabidopsis Athb-5*, *-6*, *-7*, and *-12* (Johannesson et al. 2003; Olsson et al. 2004; Henriksson et al. 2005), sunflower *Hahb4* (Dezar et al. 2005), medicago *Mthb1* (Ariel et al. 2010) and tobacco *Nahd20* (Ré et al. 2011), are induced by water deficit, salt and abscisic acid (ABA). Furthermore, in rice *Oshox6*, *-22* and *-24*, closest homologs of *Athb-7* and *-12*, are also upregulated by water deficit (Zhang S et al. 2012) while *Oshox4* is down-regulated under drought conditions (Agalou et al. 2008) and also plays a role in GA signalling (Dai et al. 2008). Several reports show functions of HD-Zip I genes in developmental processes. In tomato, *LeHB1*, is highly expressed in flowers and developing fruits and its over-expression altered the morphology of floral organs (Lin et al. 2008). In barley, the *Vrs1* gene is encoded by an HD-Zip I gene which is close to *Oshox14*. The loss-of-function mutation of *Vrs1* allowed the transition of two-rowed to six-rowed barley (Komatsuda et al. 2007). Earlier, it was shown that expression of *Oshox22* can be regulated by drought, ABA and salt and that it seems to regulate drought and salt susceptibility through an

ABA-mediated signalling pathway (Zhang S et al. 2012). In addition, in this report we show that *Oshox22* also has a function in controlling GL via determining cell length in the lemma. Currently we do not yet know if this is via the same ABA pathway or via a different pathway.

Taken together, these findings suggest that natural variation in the *Oshox22* promoter can be exploited in breeding programmes to modify GL using molecular marker-assisted selection. However, the exact mechanism of regulation of GL by *Oshox22* is still not clear. Since *Oshox22* is a homeobox gene, it will exert its function via regulation of downstream target genes which we do not know yet. Therefore, more research is needed to elucidate the genetic and biochemical pathways to understand the molecular mechanisms underlying rice GL development and to determine if there are interactions with other known regulators of GL.

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Supplemental data

Table S1. Cultivars used for QTL analysis and rice transformation

Cultivar name	Country of origin	Subspecies
Nipponbare	Japan	*Japonica*
Zhonghua 11	China	*Japonica*
Bala	India	*Indica*
Azucena	Phillippines	*Japonica*

Table S2. Primers used in this study

Primer code	Sequence	Purpose
QPCR-22F	5'-CCGGCGGACGTGTCGGTGGAGTC-3'	Expression analysis
QPCR-22R	5'-AGCCACCGCATTCCACTCGACGAGC-3'	Expression analysis
ACT1-F	5'-CTTCATAGGAATGGAAGCTGCGGGTA-3'	Expression analysis
ACT1-R	5'-CGACCACCTTGATCTTCATGCTGCTA-3'	Expression analysis
Pro-IR-22F	5'-AAGAATTCATTTAGAAACTTGGAAAACGTG-3'	Vector construction
Pro-IR-22R	5'-TAGGATCCTGTGTTCGACCATTCATCCC-3'	Vector construction
G-NB-22F	5'-CGGTACAGAGATCGACGATAATG-3'	Vector construction
G-NB-22R	5'-GAGAAGATTGGAAAGATACGC-3'	Vector construction
Pro-22F	5'-CGGTACAGAGATCGACGATAATG-3'	SNP genotyping
Pro-22R	5'-GGTGGCATCAAGAACTGGTG -3'	SNP genotyping

Table S3. List of the 67 ERGC (European Rice Germplasm Collection) accessions that were genotyped

Sample ID	Accession name and year of release	County of origin	Subspecies	InDel †	Spikelet grain length (mm)	Spikelet grain width (mm)	Grain type ‡
2	Agostano, 1933	Italy	*Japonica*	N	5.53	3.02	Medium
4	Airone, *	Italy	*Japonica*	N	9.22	3.55	Long A
7	Allorio, 1915	Italy	*Japonica*	N	6.33	2.56	Long A
10	Ambra, 1999	Italy	*Japonica*	N	5.33	2.93	Round
11	Americano 1600, 1921	Italy	*Japonica*	N	5.00	5.97	Round
17	Ardizzone, 1925	Italy	*Japonica*	N	5.68	2.70	Medium
19	Ariete, *	Italy	*Japonica*	N	8.83	3.05	Long A
20	Arpa, 2005	Italy	*Japonica*	N	7.30	3.71	Round
25	Balilla, 1924	Italy	*Japonica*	N	7.00	3.58	Round
26	Balilla GG, 1954,	Italy	*Japonica*	N	4.79	3.01	Round
27	Balocco, 1957	Italy	*Japonica*	N	5.40	3.03	Medium
28	Balzaretti, 1932	Italy	*Japonica*	N	5.95	3.03	Medium
31	Bellardone,1938	Italy	*Japonica*	N	5.13	3.09	Round
32	Benito, *	Italy	*Japonica*	N	5.18	2.92	Round
38	Castello, 1978	Italy	*Japonica*	N	5.10	3.00	Medium
39	Castelmochi, 1996	Italy	*Japonica*	N	6.92	3.47	Round
40	Centauro, 2003	Italy	*Japonica*	N	7.77	4.03	Round
43	Creso, 2004	Italy	*Japonica*	N	9.02	3.38	Long A
44	Cripto, 1978	Italy	*Japonica*	N	7.86	3.61	Medium
47	Elio, 1985	Italy	*Japonica*	N	5.32	3.25	Round
53	Feronio, *	Italy	*Japonica*	N	5.22	2.91	Round
54	Ferrarsi, 1957	Italy	*Japonica*	N	5.48	3.13	Medium
55	Flipper, 1997 ⧗	Italy	*Japonica*	N	8.25	3.39	Medium
65	Greggio, 1935	Italy	*Japonica*	N	5.89	2.94	Medium
70	Italico, *	Italy	*Japonica*	N	5.42	3.09	Medium
73	Italpatna, 1960 ⧗	Italy	*Japonica*	N	9.56	3.05	Long A
78	Lencino, 1857	Italy	*Japonica*	N	4.89	2.91	Round
82	Lomello, 1954	Italy	*Japonica*	N	6.63	3.01	Long A
84	Lucero, *	Italy	*Japonica*	N	7.13	3.61	Round
86	Maratelli, 1919	Italy	*Japonica*	N	7.67	3.80	Medium
87	Marte, 2000	Italy	*Japonica*	N	7.00	3.56	Round
91	Molo, *	Italy	*Japonica*	N	8.77	3.11	Long A
92	Monticelli, 1962	Italy	*Japonica*	N	5.56	3.14	Medium
93	Navile, 1970	Italy	*Japonica*	N	5.63	2.73	Medium
99	Nova, 1965 ⧗	Italy	*Japonica*	N	5.80	3.03	Medium
100	Novara, 1933	Italy	*Japonica*	N	5.51	2.24	Medium
105	Originario, 1904	Italy	*Japonica*	N	5.02	3.04	Round
107	Ostiglia, 1850	Italy	*Japonica*	N	5.22	2.98	Round
108	P6, 1915	Italy	*Japonica*	N	5.89	2.95	Medium
109	Padano, 1968	Italy	*Japonica*	N	8.42	3.74	Medium
111	Perla, 1998	Italy	*Japonica*	N	7.04	3.26	Round
116	Precoce Monticelli,1962	Italy	*Japonica*	N	5.51	3.20	Medium

119	Raffaello, 1963	Italy	*Japonica*	N	5.26	2.71	Medium
120	Ranghino, 1887	Italy	*Japonica*	N	4.98	3.03	Round
121	Razza77, 1938	Italy	*Japonica*	N	9.42	3.94	Long A
129	Rizzotto 1950 ⧗	Italy	*Japonica*	N	9.23	3.76	Long A
134	Romanico, 1970	Italy	*Japonica*	N	6.47	2.82	Long A
141	S. Andrea, 1968 ⧗	Italy	*Japonica*	N	9.38	3.92	Long A
150	Selenio, 1987	Italy	*Japonica*	N	6.95	3.45	Round
152	Sesilla, 1968	Italy	*Japonica*	N	5.56	2.86	Medium
162	Trionfo Fassone, 1955	Italy	*Japonica*	N	5.83	2.68	Medium
176	Akitakomachi, *	Japan	*Japonica*	N	7.31	3.31	Round
180	Bomba, *	Spain	*Japonica*	N	7.90	3.49	Round
181	Calmochi-101, 1985	USA	*Japonica*	N	7.52	3.59	Round
182	Cigalon, * ⧗	France	*Japonica*	N	7.50	3.73	Round
190	Giza, *	Egypt	*Japonica*	N	7.48	3.40	Medium
209	Nipponbare, *	Japan	*Japonica*	N	7.46	3.21	Round
214	S101, 1988	USA	*Japonica*	N	7.40	3.42	Round
215	S102, 1996	USA	*Japonica*	N	8.54	3.45	Round
45	Delfino, 2001 ⧗	Italy	*Japonica*	Y	8.22	3.37	Long A
83	Loto, 1988 ⧗	Italy	*Japonica*	Y	8.87	3.37	Long A
89	Minerva, 2003 ⧗	Italy	*Japonica*	N	5.96	2.51	Medium
94	Nembo, 1999 ⧗	Italy	*Japonica*	Y	9.58	3.79	Long A
110	Panda, 1988 ⧗	Italy	*Japonica*	Y	9.87	2.53	Long B
131	Rodeo, 2003 ⧗	Italy	*Japonica*	Y	8.94	3.26	Long A
142	S. Petronio, 1970	Italy	*Japonica*	Y	8.00	3.20	Medium
170	Vitro, 1968	Italy	*Japonica*	Y	6.01	2.61	Long A

* Unknown year

⧗ Cultivars used for gene expression analysis (Figure 3)

† N= cultivars without the A InDel and Y= cultivars with the A InDel

‡ Grain types are Long A= length/width ratio of 2.0–3.0 and length >6.0 mm, Long B= length/width ratio above 3.0 and length >7.0 mm, Medium= length/width ration below 3.0 and length of between 5.2 and 6.0 mm and Round= length/width ratio below 2.0 and length smaller than 5.2 mm (Faivre-Rampant et al. 2011)

Table S4. List of the rice core collection

Cultivar name	InDel†	Spikelet grain length (mm)	Spikelet grain width (mm)	Grain type‡	Subspecies
Santerno	Y	9.87	2.48	Long B	*Japonica*
Ribe (Euribe)	Y	9.27	3.34	Long A	*Japonica*
Piemonte	Y	8.32	3.81	Medium	*Japonica*
Bertone	N	9.08	3.47	Long A	*Japonica*
R.B.Rinaldo Bersani	Y	9.49	3.51	Long A	*Japonica*
Roma	Y	9.29	3.64	Long A	*Japonica*
Saturno	Y	10.04	3.41	Long B	*Japonica*
Redi	Y	9.17	3.91	Long A	*Japonica*
Zena	N	9.11	2.53	Long B	*Japonica*
Tejo	N	8.57	2.89	Long A	*Japonica*
Iido	N	8.28	3.07	Medium	*Japonica*
Lamone	Y	9.29	2.62	Long A	*Japonica*
L 201	Y	9.38	2.5	Long B	*Japonica*
Greppi	Y	7.55	3.02	Round	*Japonica*
Gladio	N	9.23	2.42	Long B	*Japonica*
Giano	Y	10.27	2.51	Long B	*Japonica*
Gange	N	10.15	2.52	Long B	*Japonica*
Fulgente -Iro	Y	8.79	3.31	Medium	*Japonica*
fragrance	N	9.7	2.7	Long B	*Japonica*
Gigante Vercelli	Y	8.82	3.95	Long A	*Japonica*
Lady Wright	Y	7.92	3.1	Medium	*Japonica*
Argo	N	8	3.92	Medium	*Japonica*
Koral	N	9.07	3.09	Long A	*Japonica*
Vialone nano	N	8.17	4.03	Medium	*Japonica*
Volano	Y	9.24	4.06	Long A	*Japonica*
Orione- I	Y	8.11	3.41	Medium	*Japonica*
Alpe	N	8.37	3.2	Long A	*Japonica*
Arborio	Y	9.42	4.04	Long A	*Japonica*
Apollo	Y	10.16	2.56	Long B	*Japonica*
Alice	Y	9.07	3.17	Long A	*Japonica*
Aiace	N	9.16	2.82	Long A	*Japonica*
Asia	Y	10.55	2.46	Long B	*Japonica*
Adelaide Chiappelli	N	9.82	3.9	Long A	*Japonica*
Europa	Y	8.67	3.22	Long A	*Japonica*
Eurosis	N	8.92	2.78	Long A	*Japonica*
Carnaroli	N	9.19	3.8	Long A	*Japonica*
Drago	Y	8.88	3.16	Long A	*Japonica*
Bianca	Y	9.65	3.47	Long A	*Japonica*
Baraggia	N	7.96	3.8	Round	*Japonica*
Baldo	Y	9.46	3.63	Long A	*Japonica*
Augusto	Y	9.08	3.07	Long A	*Japonica*
Ringo	Y	8.99	3.33	Long A	*Japonica*

Jefferson	Y	9.11	2.77	Long B	*Japonica*
Vialone 190- I	N	8.72	4.08	Medium	*Japonica*
Titano	Y	8.59	3.39	Long A	*Japonica*
Smeraldo	N	8.88	3.14	Long A	*Japonica*
SiS- R 215	Y	9.48	2.86	Long A	*Japonica*
Venere	N	7.94	3.08	Medium	*Japonica*
Thaibonnet	Y	9.57	2.44	Long B	*Japonica*
Karnak	N	9.43	3.83	Long A	*Japonica*
Loto	Y	8.87	3.37	Long A	*Japonica*

† N= cultivars without the A InDel and Y= cultivars with the A InDel
‡ Grain types are Long A= length/width ratio of 2.0–3.0 and length >6.0 mm, Long B= length/width ratio above 3.0 and length >7.0 mm, Medium= length/width ration below 3.0 and length of between 5.2 and 6.0 mm and Round= length/width ratio below 2.0 and length smaller than 5.2 mm (Faivre-Rampant et al. 2011)

Figure S1. Alignment of the 1.2 Kb *Oshox22* promoter sequences from the eight rice cultivars. Rectangular shape represents position of InDel. Cultivars without the A InDel; Nipponbare, Bala, T309, 9311 and cultivars with the A InDel; Cabacu, Azucena, IRAT112, IRAT109.

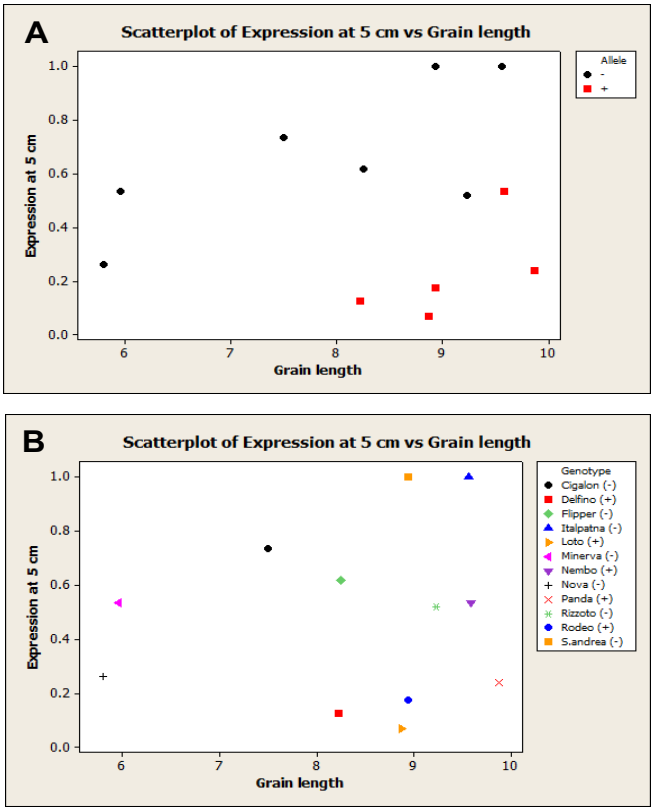

Figure S2. Scatter plot of *Oshox22* expression versus GL in a panel (IIf) of Italian rice cultivars young panicle 5 cm in length (*Y* axis) to its grain length (mm) (*x* axis). **A,** Distribution of the *Oshox22* allele with the A InDel (red squares) and without the A InDel (black dots) versus *Oshox22* expression. **B,** Distribution of Italian rice cultivars with the A InDel (+) and without the A InDel (-) versus *Oshox22* expression. The scatter plot is the same as in **A** but now every cultivar is indicated with its name.

Chapter 4

Strigolactone biosynthesis and biology

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Abstract

Strigolactones belong to a newly identified class of plant hormones that are involved in the inhibition of shoot branching. Prior to this finding, strigolactones were proven to be root rhizosphere signalling molecules that mediate plant-parasitic plant, and the symbiotic plant-AM fungi interactions. More recently, strigolactones were shown to have other biological functions as endogenous plant hormones in shoot development, root architecture, seed germination (also in non-parasitic plants) and to regulate plant developmental processes in interaction with other signalling pathways (i.e. light and senescence signalling) or hormones. Gene discovery in the strigolactone biosynthesis and signal perception pathways is a key step in elucidating the mechanism and mode of action of the existing roles and discovering potential additional roles of strigolactones. Furthermore, the insights in the strigolactone and strigolactone-associated pathways will provide more knowledge for the control of parasitic weeds and improvement of crop yield. In this chapter, we will outline different aspects of the roles that strigolactones play both in the rhizosphere and during plant development. Gene characterisation in strigolactone pathways and strigolactone-related hormone cross-talk will also be addressed.

Keywords: Strigolactone, Seed germination, Biosynthesis, Signal transduction, Biological function, Hormone cross-talk.

Introduction

The rhizosphere is the complex environment surrounding the roots of plants in which a diverse range of organisms interact with each other. Examples are plant-microbe (bacteria, fungi, oomycetes, and viruses), plant-insect, plant-nematode and plant-plant interactions. The fundamental insights in this interplay between plants and the rhizosphere have been extensively reviewed (Estabrook and Yoder 1998; Buee et al. 2009; Lugtenberg and Kamilova 2009; Kawaguchi and Minamisawa 2010). Plants can benefit from some interactions, such as the symbiosis between plants and rhizobia or arbuscular mycorrhizal (AM) fungi, while other interactions, such as with parasitic plants or rhizosphere pathogens, are a source of biotic stress.

Plants or plant roots produce and release into the rhizosphere a multitude of metabolites including sugars, polysaccharides, amino acids, aliphatic acids, aromatic acids, fatty acids, sterols, enzymes, vitamins, phenolics and other secondary metabolites (Bertin et al. 2003; Steinkellner et al. 2007). The volatile plant hormones, ethylene, methyl jasmonate and methyl salicylate, play key roles in mediating plant communication as airborne signals aboveground (Arimura et al. 2002; Engelberth et al. 2004; Heil and Karban 2010). Similarly, in the rhizosphere, it is likely that many root-derived compounds play roles in plant-plant, plant-microbe, and plant-insect chemical communication. For example, flavonoids have been shown to stimulate or inhibit rhizobial nod gene expression, cause chemo-attraction of rhizobia towards the root, inhibit root pathogens, stimulate mycorrhizal spore germination and hyphal branching, and mediate allelopathic interactions between plants (Cooper 2004; Hassan and Mathesius 2012).

Still, there are likely many more plant-derived molecules that await identification as signalling molecules and more biological functions of known signalling compounds to be discovered. The newly identified phytohormone strigolactone is one of the best examples of the discovery of new biological functions of known signalling compounds. Strigolactones are present in the root exudates of many plant species and were identified as seed germination stimulants for root parasitic plants of the Orobanchaceae such as *Striga, Orobanche, Alectra and Phelipanche* spp. decades ago (Cook et al. 1966). Only much later they were also shown to stimulate the symbiosis of plants with arbuscular mycorrhizal (AM) fungi by acting as hyphal branching factors (Akiyama et al. 2005). So far, a variety of different strigolactones have been isolated from a range of plant species and in most cases, one plant species produces more than one strigolactone (Xie et al. 2010).

In this chapter we will review the various roles that strigolactones play both in the rhizosphere and

as endogenous plant hormone. In addition, we will present current knowledge on the strigolactone biosynthetic and downstream signalling pathways and the interactions of strigolactones with other phytohormones.

Strigolactone performance in the rhizosphere

Parasitic seed germination stimulants

Root parasitic plants are integral participants in the strigolactone story. Excellent reviews have focused on the biology, economic importance, and plant resistance approaches of the main root parasitic plant genera *Striga, Orobanche* and *Phelipanche* (Musselman 1980; Rispail et al. 2007; Parker 2009). Several species from these genera belong to the most damaging parasitic weed species worldwide. *Striga* spp. are a major pest in crops such as maize, sorghum, upland rice and millet throughout semi-arid Africa and parts of Asia, while *Orobanche* and *Phelipanche* spp. are problematic in legumes, tomato, tobacco, rapeseed, sunflower, etc. in Southern and Eastern Europe, the Middle East and North Africa (Rubiales 2003). The nature of these parasites is the main reason why they cause significant economic losses in agriculture. They form a connection organ with the host plants' root system, called haustorium, through which they consume water, carbon and nutrients. This inevitably leads to a reduction in the host plant's growth, affects its assimilate partitioning and reproduction, and even disrupts the competitive balance between host and non-host species, leading to changes in community structure (Press and Phoenix 2005).

The persistence of the parasites lies in their tiny seeds which can remain dormant and viable in the soil for over 10 years. They will only germinate upon the perception of specific seed germination stimulants that are released by the host roots (Bouwmeester et al. 2003; Hearne 2009). This ensures that the parasite only germinates when a host plant is within reach. Extensive research work has been performed on the characterisation of these seed germination stimulants, which are of economical and scientific importance for the biology and management of parasitic weeds. In the 1960s and 1970s, strigol and strigyl acetate were discovered to be present in the root exudates of cotton, a non-host of *Striga* (Cook et al. 1966, 1972). More recent publications show that strigol also occurs in root exudates of several host species of *Striga* like maize, sorghum and proso millet, in addition to a closely related but even more active seed germination stimulating compound called sorgolactone (Hauck et al. 1992; Siame et al. 1993). Almost 30 years after the initial discovery of strigol, Butler (1995) coined the name "strigolactones" for these strigol-related compounds. Besides strigol, strigyl acetate and sorgolactone, many other strigolactones have

since then been isolated from the root exudates of a large variety of plants, all functioning – with varying activity - as *Striga* or *Orobanche*/*Phelipanche* seed germination stimulants. Alectrol, which recently was proven to be (+)-orobanchyl acetate (Xie et al. 2008), was originally discovered in cowpea root exudates (Müller et al. 1992). Orobanchol, the first strigolactone isolated from a host of Orobanche (red clover) induces *Orobanche minor* seed germination (Yokota et al. 1998; Mori et al. 1999). Entering the 21st century, with the development of highly advanced quantitative and qualitative analytical methods, more novel natural strigolactones have been identified in the root exudates of many different plant species (Akiyama et al. 2005; Awad et al. 2006; Xie et al. 2007; Matsuura et al. 2008; Xie et al. 2008; Xie et al. 2009a; Xie et al. 2009b; Yoneyama et al. 2010; Kohlen et al. 2011; Ueno et al. 2011b; Jamil et al. 2012) (Table 1). Although in one plant species one or more major known strigolactones can be detected, evidence suggests that usually plant root exudates contain strigolactone mixtures including so far unidentified ones (Cardoso et al. 2011).

In addition to natural strigolactones, several strigolactone analogs have been synthesised and used in seed germination studies (Zwanenburg and Thuring 1997), such as GR24, desmethyl sorgolactone, GR7 and Nijmegen1. Among them, GR24 is the most widely used in strigolactone studies.

Through bioassays with natural strigolactones and the synthesis of strigolactone analogs it has been confirmed that the D ring of the strigolactones is essential for parasitic plant seed germination stimulatory activity (Mangnus et al. 1992; Mangnus and Zwanenburg 1992; Yoneyama et al. 2009b; Ueno et al. 2011a). Interestingly, natural strigolactones exhibit differential activity on different parasitic plant species (Yoneyama et al. 2009a; Kim et al. 2010). Usually, acetates are less active than the corresponding hydroxy-strigolactones (Yoneyama et al. 2009b). Orobanchyl acetate and strigyl acetate were 10 to 100-fold less active as germination stimulant in *O. minor* and *Orobanche ramosa* than orobanchol and strigol (Sato et al. 2005; Xie et al. 2008). However, 7-oxoorobanchyl acetate was more active than 7-oxoorobanchol in *O. minor* as well as *O. ramosa* seed germination (Xie et al. 2009b). As parasitic weed seed germination stimulants, strigolactones seem to play a negative role in the rhizosphere. The fact that strigolactones are still being produced by plants suggests there must be a positive function for strigolactone as well, resulting in selection pressure leading to their persistence in nature.

Table 1. Strigolactones present in different plant species (after year 2000)

Strigolactones	Plant species	Publications
5'-Deoxystrigol and its isomers	*Lotus japonicus*	Akiyama et al. 2005
	Arabidopsis thaliana	Kohlen et al. 2011
	Oryza sativa	Jamil et al. 2011
Ent-2'-*epi*-orobanchol and its acetate	*Vigna unguiculata*	Ueno et al. 2011a, 2011b
	Trifolium pratense L	Xie et al. 2007
	Nicotiana tabacum L	
(+)-4-*O*-acetylorobanchol	*Vigna unguiculata*	Matsuura et al. 2008
(+)-Orobanchyl acetate	*Trifolium pratense L*	Xie et al. 2008
Solanacol	*Nicotiana tabacum L*	Xie et al. 2007
Sorgomol	*Sorghum bicolor*	Awad et al. 2006
		Yoneyama et al. 2009a
Fabacyl acetate	*Pisum sativum*	Xie et al. 2009a
7-Oxoorobanchol and its acetate	*Linum usitatissimum L*	Xie et al. 2009b
7-Hydroxyorobanchol and its acetate	*Linum usitatissimum L*	Xie et al. 2009b

Branching factors in AM symbiosis- a positive role in the rhizosphere

Arbuscular mycorrhizal (AM) fungi are symbiotic organisms in the plant rhizosphere and are playing an important role in agriculture. They can form a beneficial symbiosis with the roots of around 80% of vascular plants and have multiple positive functions in the rhizosphere. They can enhance plant uptake of inorganic phosphate (Pi) and other mineral nutrients from the soil, stimulate plant growth, increase tolerance to drought and protect plant roots against pathogens (Smith and Gianinazzi-pearson 1988; Ruizlozano et al. 1995; Alan 2000; Veresoglou and Rillig 2011). AM fungi can not complete their life cycle without a host, and they have to colonise plant roots after spore germination to obtain carbon for their survival (Smith and Gianinazzi-pearson 1988). This bidirectional benificial relationship between the two organisms consists of two phases, the pre-symbiotic and the symbiotic phase. Exchange of carbon and nutrients occurs through the extensively branched haustoria, known as arbuscules. However, the signalling events for the two phases are not yet fully understood. In the pre-symbotic phase AM fungi exist in the soil as spores which are capable of spontaneous germination without host root contact. The germ tube grows for

one to three weeks but will cease if a host root is not present in the soil. Over the years, experimental evidence has accumulated showing that host roots can release chemical compounds that serve as signals for directional growth and hyphal branching of AM fungi (Koske 1982; Becard and Piche 1989; Giovannetti et al. 1993; Harrison 2005). This is a prerequisite for further root colonisation by the fungus. The signal molecules released by the host are called "branching factors" (Buee et al. 2000). Signal molecules produced by AM fungi, called "Myc factors", are also required for successful colonisation (Kosuta et al. 2003; Maillet et al. 2011).

Many groups have tried to characterise these branching factors. Preliminary evidence has suggested that this factor is a compound of <500D (Giovannetti et al. 1996). Buee et al. isolated a semi-purified fraction from exudates of eight mycotrophic plant species containing active AM fungi branching factors (Buee et al. 2000). Nevertheless, successful isolation and structural characterisation were not achieved until 2005, with the discovery of 5-deoxystrigol as the AM fungal hyphal branching factor from *Lotus japonicus* (Akiyama et al. 2005). This breakthrough for the first time provided evidence that strigolactones, which were only believed to have negative effects, can also be of benefit to plants, making them janus-faced molecules in the plant rhizosphere.

Later Besserer et al. proved that one of the branching factors from the monocot sorghum is sorgolactone. The same study also showed that GR24 stimulated hyphal branching of the AM fungus *Gigantea rosea* by provoking a rapid and strong cellular response which is associated with mitochondrial biogenesis (Besserer et al. 2006). Further study showed that GR24 stimulates the mitosis and growth of the AM fungi by boosting its energy metabolism. Treatment of *G. rosea* with GR24 caused a rapid increase in the NADH concentration, the NADH dehydrogenase activity, and the ATP content of the fungal cell. This powerful action of GR24 on *G. rosea* cells suggests that strigolactones are important plant signals involved in switching AM fungi towards full germination and a presymbiotic state (Besserer et al. 2008). Based on this study, it was also proven that hyphal branching was completely inhibited with the suppression of mitochondrial biogenesis, confirming that AM fungi mitochondria can amplify the strigolactone response (Besserer et al. 2009).

The major advantage of the AM symbiosis for plants is that AM fungi provide a very effective Pi uptake pathway by which Pi is scavenged from the soil and directly delivered to the cortical cells in the roots, reducing Pi depletion in the rhizosphere and improving the plants Pi nutrition (Smith et al. 2011). Pi avaibility can alter phytohormone production in plants. Cytokinin production and the

receptor gene *CRE1* expression are repressed by Pi-limiting conditions in sunflower and *Arabidopsis* (Salama and Wareing 1979; Franco-Zorrilla et al. 2002), but also symbiotic AM fungi are able to induce changes in endogenous plant hormones like cytokinin and auxin (Danneberg et al. 1993; Kaldorf and Ludwig-Muller 2000). Similarly, strigolactone production is also responsive to Pi deficiency. Reduced supply of phosphorus but not of other elements (N, K, Ca, Mg) to red clover significantly promotes the secretion of the strigolactone orobanchol (Yoneyama et al. 2007). Moreover, it has been proposed that increased AM fungal hyphal branching induced by exudates from Pi starved tomato plants is mediated by increased strigolactone production (López-Ráez et al. 2008). A more recent study has demonstrated that the correlation between AM fungal colonisation and strigolactone production is linked to shoot Pi rather than to the external Pi availability or local Pi concentrations present in the root (Balzergue et al. 2011). High Pi conditions, under which strigolactone are hardly produced, did not result in AM symbiosis. Curiously, exogenous GR24 application failed to restore AM symbiosis in these high phosphate-grown plants. Consistently, strigolactone-deficient mutants showed slightly reduced AM fungi colonisation (Gomez-Roldan et al. 2008). Taken together, although it is clear that strigolactones play an important role in AM symbiosis, it is likely there are also other factors involved in the regulation of this process (Nagahashi and Douds 2007; Balzergue et al. 2011).

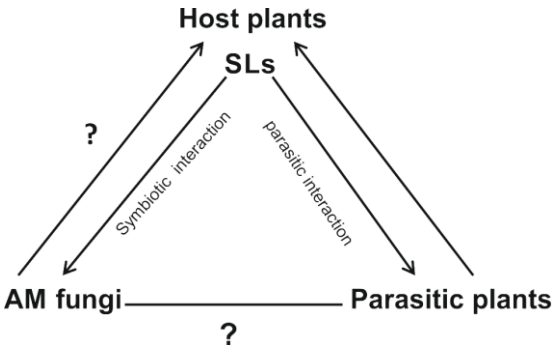

Figure 1. The trilateral relationship of host plants, arbuscular mycorrhizal (AM) fungus and parasitic plants. The host plant facilitates the symbiotic and parasitic interactions with AM fungi and parasitic plants through the exudation of strigolactones. "?" indicates that the mechanism is still unclear.

Interestingly, AM fungal colonisation leads to a decrease in seed germination of the parasitic plant *Striga* and subsequent attachment and emergence (Lendzemo et al. 2007; Sun et al. 2008), and strigolactone production was shown to significantly decrease upon AM symbiosis in tomato (Lopez-Raez et al. 2011). This suggests that the reduced parasitic seed germination upon colonisation by AM fungi is, at least partially, due to decreased strigolactone levels. Possibly, besides the well-documented strigolactone action in plant-parasitic plant and plant-AM fungi symbiotic interactions (Bouwmeester et al. 2007), more complex trilateral relationships exist among host plants, parasites and symbionts mediated by strigolactones or other factors (Figure 1). With respect to the structural requirements of strigolactones to induce hyphal branching in AM fungi, few studies have been performed to date. Truncation of A-and AB-rings in the strigolactone structure causes a dramatic decrease in hyphal branching. The D-ring has been shown to be essential for the branching, while the enol ether bridge in the C-D part is not necessary, leading to the conclusion that the structural requirements for AM fungi hyphal branching are very similar but not identical to those observed in root parasitic weeds seed germination (Yoneyama et al. 2009b; Akiyama et al. 2010).

Strigolactone biosynthetic pathway

In a study aimed at the elucidation of the biosynthetic origin of the strigolactones, Matusova et al. treated maize, sorghum and cowpea plants with inhibitors of various pathways. Screening the plant root exudates for germination stimulatory activity of *Striga* seeds revealed that treatment of the plants with fluridone, an inhibitor of the carotenoid pathway, caused a significant decrease in the germination stimulatory activity of their root exudates (Matusova et al. 2005). Based on this finding, a hypothetical strigolactone biosynthetic pathway was constructed, starting with a carotenoid substrate, such as β-carotene, that would be cleaved by a carotenoid cleavage dioxygenase. Further hydroxylation, decarboxylation and oxidation would then lead to the formation of the A, B and C rings. The D ring (with unknown origin) was hypothesised to be enzymatically coupled to the ABC part as a last step (Figure 2A), leading to the formation of 5-deoxystrigol, which is believed to be the common precursor of other natural strigolactones (Rani et al. 2008; Xie et al. 2010).

As hypothesised in the above described study, in 2008 it was indeed discovered that two carotenoid cleavage dioxygeneases (CCDs) are required for strigolactone biosynthesis (Gomez-Roldan et al. 2008; Umehara et al. 2008). Prior to these findings, these two carotenoid cleavage dioxygeneaes (called *CCD7*/*MAX3* and *CCD8*/*MAX4* in *Arabidopsis*), were reported to be required

for the synthesis of a novel plant signalling molecule controling shoot branching or tillering in *Arabidopsis*, pea (*RMS5* and *RMS1*), petunia (*DAD3* and *DAD1*) and rice (*HTD1*/*D17*/*D10*) (Morris et al. 2001; Sorefan et al. 2003; Booker et al. 2004; Snowden et al. 2005; Zou et al. 2006; Arite et al. 2007; Simons et al. 2007). Plants carrying mutations in these genes show an excessive bushy or branched phenotype. The conclusion from these studies is that strigolactones, or close derivatives, are involved in the regulation of shoot branching. Both *CCD7* and *CCD8* enzymes were shown to be localised in plastids (Auldridge et al. 2006), where also the carotenoid substrates are produced.

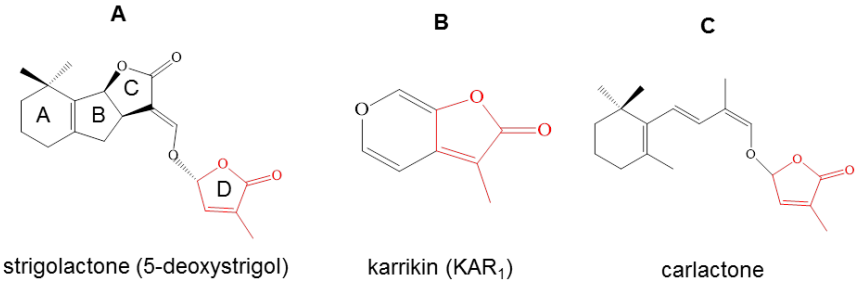

Figure 2. Chemical structures of strigolactones, karrikin and carlactone. **A,** Structure of the strigolactone 5-deoxystrigol; **B,** Structure of karrikin; **C,** Structure of carlactone. Note the the butenolide moiety shared by these three compounds.

D27, an iron-containing protein also suggested to be involved in the strigolactone biosynthetic pathway in rice, shares the same plastidial localisation with the CCDs (Lin et al. 2009). Recently, it has been shown that *D27* is an all-*trans* to 9-*cis*-β-carotene isomerase converting all-*trans*-β-carotene into the CCD7 substrate 9-*cis*-β-carotene (Figure 3). Subsequent incubation of the CCD7 product 9-*cis*-β-apo-10'-carotenal with CCD8 led to a compound called carlactone (Figure 2C). Carlactone restored the tillering phenotype of the strigolactone-deficient rice mutants *d27*, *htd1* (*ccd7*) and *d10* (*ccd8*) but not the signalling mutant *d3* (Alder et al. 2012). The presence of the D-ring in carlactone is highly surprising, makes this compound already strigolactone-like and greatly simplifies the strigolactone biosynthetic pathway. Carlactone clearly is an intermediate in strigolactone biosynthesis already quite close to 5-deoxystrigol (Figure 3). *MAX1* in *Arabidopsis*, encoding a cytochrome P450 *CYP711A1* gene, is involved in the production of the carotenoid-derived branching hormone, and has been suggested to act on a mobile substrate downstream of

CCD7 and *CCD8* (Stirnberg et al. 2002; Booker et al. 2005; Figure 3). Additional experiments have shown that *MAX1* is expressed in all vascular-associated tissues throughout the plant, while the highest *CCD7/CCD8* activity is found mainly in the root (Sorefan et al. 2003; Booker et al. 2004; Booker et al. 2005). Because strigolactone levels are relatively low in *Arabidopsis*, it took some time before analytical evidence for *MAX1* activity in strigolactone production was provided . Kohlen et al. proved that less orobanchol was detected in *Arabidopsis max1* mutants root extracts and root exudates compared with the Col-0 wild-type under Pi-limiting conditions (Kohlen et al. 2011).

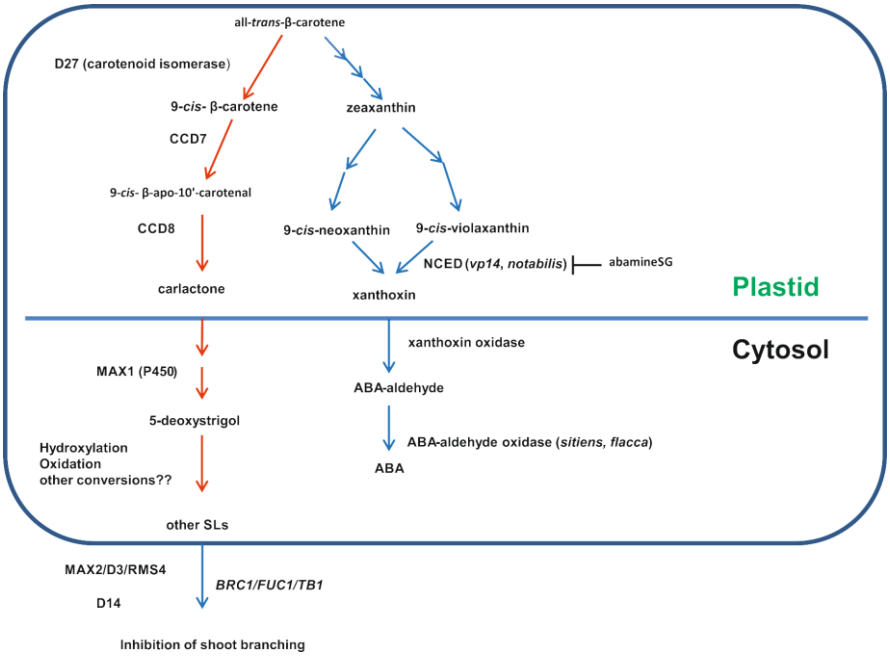

Figure 3. Schematic overview of strigolactone (SL) and abscisic acid (ABA) biosynthesis and downstream pathway.

It is likely that in other plant species, gene redundancy masks the mutational effect of a single CYP450 family member. In rice, there are five putative orthologues of *MAX1* (Booker et al. 2005; Umehara et al. 2010). Interestingly, using a genetic approach in rice, it has been shown that two of these rice P450s are co-localised with a major QTL for strigolactone production on chromosome 1. Both orthologues were capable to rescue the branching phenotype of the *Arabidopsis max1* mutant (Cardoso et al. 2013). Still, the exact enzymatic activity of these MAX1 orthologues (nor of

MAX1 itself) has not been characterised. Since the strigolactone composition in the parents and offsprings of the mapping population was not affected, just the total level, it is likely that these cytochrome P450s are not acting in strigolactone modification but upstream in the common pathway. *MAX1* is a good candidate for the unidentified conversion of carlactone to 5-deoxystrigol (Alder et al. 2012 (Figure 3). Curiously, the moss *Physcomitrella patens* can produce several kinds of strigolactones, but in the moss genome so far no *MAX1* homolog has been reported. This may imply that *MAX1* functions only in the very late steps of strigolactone biosynthesis that lead to shoot branching inhibitors in seed plants or that its function in moss is taken over by other P450s (Proust et al. 2011).

Two *GRAS*-type transcription factors, *NODULATION SIGNALING PATHWAY1* (*NSP1*) and *NSP2*, have been postulated to affect the strigolactone biosynthetic pathway. Liu et al. showed that strigolactone production was disturbed in the *NSP1* and *NSP2* mutants both in the model legume *Medicago truncatula* and in rice, suggesting that these two transcription factors are required for strigolactone biosynthesis. Both in rice and in *Medicago*, *D27* is down-regulated in the *nsp1* and *nsp2* mutants. In addition, in *M. truncatula NSP2* is essential for conversion of orobanchol into didehydro-orobanchol (Liu et al. 2011).

Strigolactone signalling

Strigolactone signal perception and downstream signalling transduction pathway

Strigolactones are essential signalling molecules in establishing plant- parastic plant and plant-AM fungi symbiotic interactions. However, how parasitic plant seeds and AM fungi sense strigolactones secreted from the host roots is still a mystery, awaiting the identification of receptor genes. The sequencing projects of parasitic plants and symbiotic AM fungi are crucial to achieve this and will assist in the elucidation of these mechanisms (http://ppgp.huck.psu.edu/; http://striga.psc.riken.jp/) (Bonfante and Genre 2008; Martin et al. 2008). For the parasitic plant interaction, studies of the seeds before and during germination could be one way to get a better understanding of putative recepter localisation. Joel et al. suggested that the small perisperm cells likely are the location of the germination stimulant receptors in *Phelipanche aegyptiaca* seeds (Joel et al. 2012). Localisation of putrative receptors is one thing but understanding the molecular mechanisms of strigolactone signal perception is still far way.

Identification of genetic mutants in non-parasitic plants has resulted in a lot of progress in the search for putative strigolactone receptors. *Arabidopsis* mutants *max2-1* and *max2-2* showing a

bushy phenotype were isolated from independent M_2 bulks in a screening of M_2 plants for altered shoot branching, indicating that *MORE AXILLARY BRANCHES 2* (*MAX2*) is involved in the regulation of shoot branching (Stirnberg et al. 2002). Later on, grafting experiments showed that wild-type roots did not restore the *max2* bushy phenotype confirming that MAX2 acts in the shoot branching inhibition, while *max2* roots could restore the bushy *max3* phenotype, indicating that MAX2 is not required for the synthesis of the branching signal molecule (Booker et al. 2005). GR24 can rescue the branching phenotype of the strigolactone biosynthetic mutants *max1, max3* and *max4* but not of *max2* (Umehara et al. 2008; Crawford et al. 2010), while in rice the *MAX2* orthologue gene *D3* mutant line (*d3*) is producing even more strigolactone than the wild-type (Yan et al. 2007; Umehara et al. 2008). *MAX2*, encoding an F-box protein with a leucine-rich repeat domain, has been shown to be expressed throughout the plant and is required in the green tissue at each individual node for repression of its associated axillary bud, acting either in the bud itself or close to it. Through a loss of-function test by deleting the F-box domain, it has been proven that this domain is required for *MAX2* function in shoot branching, indicating that *MAX2* is a component of the putative SCF^{MAX2} complex (Stirnberg et al. 2007). Further evidence for this is provided by Stirnberg *et al* who proved that in the SCF^{MAX2} complex MAX2 interacts with the SCF subunits ASK1 and AtCUL1 *in vivo* (Stirnberg et al. 2007). Similarly, the auxin receptor TIR1 and jasmonic acid receptor COI1 are also active in SCF complexes (Tan et al. 2007; Thines et al. 2007; Katsir et al. 2008). In addition to its role in the regulation of shoot branching, *MAX2* was also described to be involved in light signalling. *MAX2*, then named *PLEIOTROPIC PHOTOSIGNALING* (*PPS*), was shown to be essential in both red (R) and far-red (FR) light-induced seed germination and the *pps* mutant seedling was hyposensitive to red and far-red light in a *PHY*-dependent manner (Shen et al. 2007). In parallel, Tsuchiya et al. demonstrated that *max2* seeds had reduced germination compared with wild-type seeds under far-red followed by red light pulses and this germination phenotype could not be rescued by GR24 application. The same study indicated that in a short term (1 hour) situation, strigolactone signals, acting through *LONG HYPOCOTYl 5* (*HY5*) which is a light signalling trancription factor in *Arabidopsis*, can positively regulate *Arabidopsis* seedling growth in a *MAX2* dependent manner. In a long term condition (over 24 hours), strigolactone accumulation may inhibit the *CONSTITUTIVE PHOTOMORPHOGENIC1*(*COP1*) function (Tsuchiya et al. 2010). In the dark, COP1 localises to the nucleus as a repressor of light signalling by degrading the transcription factor *HY5*, while *COP1* will be inactivated by responding to light to move into the cytoplasm (Osterlund et al. 2000). Strigolactone inhibition of *COP1* function is

similar to that of light, which may explain why strigolactones and light can enhance each others function (Tsuchiya et al. 2010).

Interestingly, *MAX2* was also found to be required for the signal transduction of another group of germination stimulating molecules - the karrikins which are smoke derived butenolide compounds (Flematti et al. 2004; Nelson et al. 2011; Figure 2B). Karrikins as well as synthetic strigolactone GR24 can enhance the germination of primary dormant *Arabidopsis* seed in a MAX2 dependent manner (Kipreos and Pagano 2000; Nelson et al. 2011). In addition to their germination stimulatory activity, both karrikins and strigolactones were shown to be involved in the regulation of hypocotyl elongation mediated by *MAX2* (Nelson et al. 2011). Curiously, in contrast to strigolactones, karrikins can not suppress shoot branching (Nelson et al. 2011). In conclusion, *MAX2* is both required for germination and photomorphogenic responses to karrikins and strigolactone, and for strigolactone mediated shoot branching inhibition, indicating that the F-box protein *MAX2* probably has dual roles in both strigolactone and karrikin signalling pathways and that the plants can make a distinction between these two compounds (Nelson et al. 2011). Therefore, it is likely that *MAX2* targets different repressors involved in different signalling pathway during different physiological processes (shoot branching, seed germination and photomorphogenesis) when mediating karrikin and strigolactone signalling.

In addition to *MAX2*, in rice the *HIGH TILLERING 14* (*D14*) was also shown to act in the strigolactone downstream signalling transduction pathway. *D14* was proven to encode an α/β-hydrolase family protein (Arite et al. 2009). Two other rice genes, *HTD2* and *D88,* turned out to be allelic with *D14* (Gao et al. 2009; Liu et al. 2009). In *Arabidopsis*, the *D14* orthologue gene *AtD14* and another *D14-like* α/β-hydrolase family protein (*KAR2*) were also characterised (Waters et al. 2012). Interestingly, the gibberellin (GA) receptor GID1 and a salicylic acid-binding protein *SABP2* also belong to this α/β-hydrolase superfamily (Forouhar et al. 2005; Ueguchi-Tanaka et al. 2005). Based on the GA signalling pathway, a working model has been hypothesised in which *MAX2* could target two types of repressors for proteasomal degradation upon its specific association with At*D14* or *KAR2* in strigolactone and karrikin signalling pathways, respectively (Nelson et al. 2011; Waters et al. 2012). Moreover, it has also been proven that *KAR2* is only responsible for strigolactone and karrikin signalling in the early developmental stages (germination and photomorphogenesis), while *AtD14* is the modulator of *MAX2* required for the regulation of shoot branching, since *kar2* did not display the branching phenotype typical for the strigolactone mutants (Waters et al. 2012). Moreover, additional experimental data showed that *D14* was highly

expressed in the axillary buds where *MAX2::GUS* activity was also detected (Stirnberg et al. 2007; Arite et al. 2009). This spatio-temporal consistency is in line with the proposed model. Although it is still not clear how *MAX2* is interacting with *D14* and *KAR2* in the strigolactone and karrikin signalling pathway, it is clear that *D14* and *KAR2* are the mediators that allow plants to discriminate between strigolactones and karrikins in different developmental stages (Waters et al. 2012).

In addition to putative receptor genes in the strigolactone perception pathway, several studies have focused on the strigolactone downstream transcription factors. More than two decades ago, *TEOSINTE BRANCHED 1* (*TB1*) in maize was already proven to be a major contributor to the change in maize apical dominance during domestication, and the *tb1* mutant showed a branched phenotype (Doebley et al. 1995; Doebley et al. 1997). Later on, this gene was characterised to belong to the *TCP* gene family of putative basic helix-loop-helix transcription factors that are involved in the regulation of organ growth (Cubas et al. 1999). Also in rice, the mutant *fine culm1* (*fc1*), with a loss-of-function mutation in *OsTB1*, displayed a hight-tillering phenotype (Takeda et al. 2003; Choi et al. 2012). Furthermore, the high-tillering phenotype of *fc1* was shown not to be rescued by GR24 application, while over-expression of *FC1* was able to rescue the tillered phenotype of the strigolactone-insensitive mutant *d3-2*, suggesting that *FC1* and its orthologues act downstream of the strigolactone pathway. Interestingly, *FC1* transcription levels were not affected by GR24 application, however, they are downregulated by cytokinin. This may imply that *FC1* is the common converging point through which both strigolactone and cytokinins interact (Minakuchi et al. 2010; Dun et al. 2012).

Strigolactone transport

As a plant hormone and rhizosphere signalling molecule, strigolactone transport is expected to play a key role both in plant development and the rhizosphere, but just few studies exist on this important aspect. The early grafting experiments proved that the long-distance signals, later known as strigolactones, are produced in the roots and transported to the shoot in *Arabidopsis* (Turnbull et al. 2002). Consistently, the strigolactone biosynthetic genes *CCD7* and *CCD8* are expressed highest in the plant root (Sorefan et al. 2003; Booker et al. 2004). Recently, analytical evidence has shown that strigolactones are indeed transported through the xylem from root to shoot. MRM-LC-MS/MS analysis confirmed that the strigolactone orobanchol was present in xylem sap of *Arabidopsis Col-0* and tomato (Kohlen et al. 2011). However, transport is not a prerequisite for shoot branching inhibition, since wild-type shoots grafted to the strigolactone-deficient mutant

max1 and *max3* roots did not show excessive branching (Turnbull et al. 2002). This may imply that local production of strigolactones in the shoot is able to suppress bud activation (Domagalska and Leyser 2011b).

ATP binding cassette (ABC) transporters are well known to be involved in the translocation of the phytohormones abscisic acid (ABA) and auxin (Petrasek and Friml 2009; Kuromori et al. 2010). Recently, a strigolactone transporter, *PDR1,* belonging to the same protein family was identified in *Petunia hybrida* (Kretzschmar et al. 2012). *PDR1* was demonstrated to be expressed in the lateral roots in individual subepidermal cells, which are probably the hypodermal passage cells which serve as entry points for AM hyphae. *Pdr* mutants showed reduced AM fungal colonisation compared with the wilde-type. Consistently, *pdr* mutant root exudates contained reduced strigolactone levels, however, the strigolactone levels in the root extracts were not affected. *PDR1* over-expression lines showed a higher tolerance to the root growth inhibitory effect of high GR24 concentrations (10 and 25μM) also indicating that *PDR1* is indeed functioning as a strigolactone export carrier (Kretzschmar et al. 2012).

Strigolactones as endogenous plant hormone

Strigolactone regulated shoot branching responses to environmental cues

Phytohormones coordinate plant development by modulating growth in response to intrinsic and environmental cues. Light is one of the important environmental signals regulating shoot architecture. A low ratio of red light to far-red light (R/FR) perceived by phytochrome *B* (*PHYB*) reduces shoot branching which is part of the shade avoidance response (Franklin and Whitelam 2005; Franklin 2008). It was proven that *BRC1*, *MAX2* and *MAX4* are required for the phytochrome mediated regulation of shoot branching in *Arabidopsis* (Finlayson et al. 2010). In sorghum, it was also shown that *SbTB1*, an orthologue of *BRC1*, is expressed higher in the buds of *phyb-1* than in the wild-type, suggesting that *PHYB* mediates the regulation of axillary bud outgrowth in response to light signals by suppressing the expression of *SbTB1* (Kebrom et al. 2006). Taken together, these data suggest that strigolactones play a role in the response of shoot branching to light.

Prior to the identification of the role of strigolactones in shoot branching, it was already observed that rice plants growing under Pi deficiency show reduced tillering (Luquet et al. 2005). Low Pi and nitrogen conditions increased strigolactone levels in the root exudates and extracts of various plant species (Yoneyama et al. 2007; López-Ráez et al. 2008). In rice it was also found that the expression levels of the strigolactone biosynthetic genes *D10*, *D17*, *D27* and two putative *MAX1*

orthologues decreased after transferring rice plants from Pi-limiting to Pi-sufficient medium. Consistently, both in *Arabidopsis* and rice it was demonstrated that Pi deficiency led to an inhibition of bud outgrowth in the wild-type but not in strigolactone biosynthetic mutants (Umehara et al. 2010). Hence, enhanced strigolactone production during Pi limiting conditions offers the plant an evolutionary advantage by stimulating nutrient allocation not only within the plant (allocation of nutrients from shoot to root) but also from outside the plant by stimulating its mycorrizhal symbiosis (Umehara 2011).

Root development

Besides regulating shoot architecture as a phytohormone, strigolactones have also been proven to affect plant root development and architecture. In *Arabidopsis*, primary root length of strigolactone-deficient and -insensitive mutants were shorter than those of wild-type plants, and showed a reduction in meristem cell number, which could be rescued by application of GR24 in all mutants except in the strigolactone-insensitive mutant *max2* (Ruyter-Spira et al. 2011). In rice, strigolactones positively regulate the length of crown roots. GR24 application complemented the crown root defect in strigolactone-deficient mutants but not in the insensitive mutant. Also here, the meristematic zone was shorter in strigolactone mutants than in wild-type plants, suggesting that strigolactones may exert their effect on roots via the control of cell division. Under phosphate starvation, the crown roots of wild type, but not of strigolactone mutants, were longer, implying that the increase in strigolactone biosynthesis under low Pi conditions can promote crown root elongation (Arite et al. 2012).

In *Arabidopsis*, strigolactone-biosynthetic and signalling mutants showed a higher lateral root density than wild-type Col-0 (Koltai 2011; Ruyter-Spira et al. 2011). GR24 application negatively affected the formation and subsequent development of lateral roots in wild-type and strigolactone biosynthetic mutants, but not in the *max2* mutant (Ruyter-Spira et al. 2011). Furthermore, strigolactones positively affect root hair (RH) elongation in a *MAX2*-dependent manner. These authors showed that GR24 (10^{-6}M) increases root-hair (RH) length in WT, *max3-11* and *max4-1* of *Arabidopsis*, but not in the strigolactone-insensitive mutant *max2* (Kapulnik et al. 2011a). However, higher concentrations (27 μM) of GR24 led to asymmetric root growth and inhibition of root-hair elongation in tomato (Koltai et al. 2010).

Strigolactones were also proven to regulate adventitious root formation in *Arabidopsis* and pea (Rasmussen et al. 2012). The *Arabidopsis* strigolactone-mutants *max1, max2, max3* and *max4* all had an increased number of adventitious roots, which could be restored by GR24 treatment in all

genotypes except *max2*. These authors showed that strigolactones suppressed the adventitious root formation at or even before the first divisions of the founder cells. Similarly, strigolactones reduced the size of the adventitious rooting zone in pea and GR24 inhibited the numbers of adventitious in a dose-dependent manner.

Seed germination

Besides investigating the germination of parasitic plant seeds triggered by exogenous strigolactone application, strigolactones are now also being studied as endogenous seed germination stimulating hormones. To understand the roles of strigolactones in seed germination, recent studies used *Arabidopsis* as a model system. GR24 stimulated germination of primary dormant (PD) *Arabidopsis Ler* seeds but an approximately 100-fold higher concentration was required than for the least active karrikin. Curiously, the most active karrikin (KAR1) was unable to trigger germination of *O. minor*. These findings suggest that strigolactones and karrikins are acting in a distinct species-specific manner (Nelson et al. 2009). It is likely that the discovery of karrikins, which are structurally related to the strigolactones by sharing a common butenolide moiety (Figure 2), drew the attention of scientists to study the roles of strigolactones in seed dormancy and germination, which are likely mediated by the F-box protein *MAX2* (Waters et al. 2011). Moreover, Toh et al (2012) demonstrated that GR24 rescued the observed reduced germination percentage of thermo-inhibited strigolactone biosynthetic mutant seeds (*max1* and *max3*) but not the signalling mutant *max2*.

Other functions

Besides the roles of strigolactone in seed germination, AM fungal symbiosis, shoot and root development, even more roles for strigolactones are being discovered at a quick pace. Similarly to inhibiting hypocotyl growth in *Arabidopsis* (Tsuchiya et al. 2010), strigolactones negatively regulate mesocotyl elongation in rice in darkness (Hu et al. 2010). In addition, strigolactones were shown to stimulate cambial activity in *Arabidopsis* secondary growth in the stem (Agusti et al. 2011). Moreover, strigolactones were found to regulate the nodule number in pea (Foo and Davies 2011), and can regulate colony growth in moss (Proust et al. 2011). The latter could suggest that strigolactones in the rhizosphere may also act as a quorum sensing-like molecule.

It has also been reported that petunia *CCD8* is involved in the regulation of leaf senescence and flower development (Snowden et al. 2005). Similarly, in Kiwifruit the reduction of *CCD8* expression in RNAi lines resulted in a delay in leaf senecence (Ledger et al. 2010). Consistently, in *Arabidopsis, MAX2* (in this study named *ORE9*) was shown to be a positive regulator acting

upstream in the regulatory cascade of the senescence pathway (Woo et al. 2001; Kim et al. 2011). Taken together, *MAX2* may be the coordinator or mediator in other, yet to be discovered, strigolactone signalling pathways during plant development. Studying its exact localisation throughout the plant may shed more light on these novel aspects.

Mechanisms of strigolactone interaction with other plant hormones

Plants are sessile organisms and must adjust their growth and development to continuously changing environmental conditions (Wolters and Jürgens 2009). Plant hormones such as auxin, ABA, cytokinin (CK), gibberellin (GA), ethylene, brassinosteroids (BRs), jasmonic acid (JA), salicylic acid (SA), nitric acid (NO) and strigolactones (Santner and Estelle 2009) play a central role in regulating plant growth and development in response to these environmental changes. However, these plant hormones do not act alone but are interconnected and modulate each other at the level of biosynthesis, degradation and signalling by cross-talk.

In this part, we summarise the recent findings describing the cross-talk of strigolactones with other plant hormones. Special emphasis will be on the interaction of strigolactones with ABA, auxin, CK and ethylene.

Strigolactones and Abscisic acid

In their study aimed at the elucidation of the origin of strigolactones, Matusova et al. showed that the ABA-deficient mutant *viviparous14* (*vp14*) of maize induced less germination of *S. hermonthica* seeds (Matusova et al. 2005). Later, a similar result was obtained with the tomato mutant *notabilis (not)*, carrying a mutation in a homolog of *vp14* (López-Ráez et al. 2008) (Figure 3). Indeed just as in maize *vp14*, in tomato *notabilis* strigolactone levels were decreased by about 40%. *Vp14* and *notabilis* encode a 9-*cis*-epoxycarotenoid dioxygenase (NCED) catalysing the cleavage of 9-*cis*-epoxycartenoids to xanthoxin, the first step in ABA biosynthesis (Bouwmeester et al. 2007) (Figure 3). The fact that the low ABA levels in these mutants correlate with reduced strigolactone production suggests a role for ABA in the regulation of strigolactone biosynthesis and/or vice versa. Because the possibility that *NCED* itself is involved in the strigolactone biosynthetic pathway, could initially not be excluded, two additional ABA-deficient mutants in tomato - *flacca* and *sitiens* - involved in the last step of ABA biosynthesis were also analysed (Lopez-Raez et al. 2010) (Figure 3). Since also these mutants showed a strong reduction in strigolactone levels, it is more likely that ABA affects strigolactone production rather than that these enzymes are involved

in both pathways. However, the mechanism of how ABA affects strigolactone biosynthesis is still unknown.

ABA plays a vital role in regulating seed development, dormancy and stress tolerance (Davies et al. 2005; Jiang and Hartung 2008). In Arabidopsis, Toh et al. have shown that strigolactones positively regulate seed germination, by counteracting the inhibitory effect of ABA during thermo-inhibition. Under high temperatures (32ºC), application of GR24 decreased ABA levels in *max1-1* but not in *max2-1* seeds. In accordance with this, the expression of the ABA biosynthetic gene *NCED9* was suppressed by GR24. These results suggest that strigolactones decrease the ABA response via the MAX2 F-box protein. Also in the parasitic plant *Striga hermonthica*, application of GR24 during germination reduced ABA compared to the control treatment (Toh et al. 2012) (Figure 4D). Hence the evidence is accumulating that strigolactones regulate seed dormancy/germination by interacting with other plant hormones.

Strigolactones and Auxin

At the physiological level, several studies have provided evidence for the cross-talk between auxin and strigolactones. For instance, in the regulation of shoot branching, it is suggested that strigolactones act synergistically with auxin. Hayward et al. showed that auxin up-regulates strigolactone biosynthetic genes *MAX3* and *MAX4*, whereas in *N*-1-naphthylphthalamic acid (NPA)-treated or decapitated plants the expression of these genes is down-regulated. The regulation by auxin of strigolactone biosynthetic genes is *AXR1* dependent and acts via the AXR1-TIR1 signalling pathway (Hayward et al. 2009; Leyser 2009). Still, the exact mechanism of how strigolactones inhibit the outgrowth of axillary buds has not been resolved. It is hypothesised that strigolactones act as a secondary messenger of auxin inside the buds to repress its outgrowth (Brewer et al. 2009; Dun et al. 2009; Ferguson and Beveridge 2009 (Figure 4B). Other studies suggested that strigolactones act primarily to reduce the capacity of the polar auxin transport (PAT) from the shoot apical meristem. This would inhibit or limit auxin export from the bud into the main PAT stream resulting in inhibition of bud outgrowth (Bennett et al. 2006; Mouchel and Leyser 2007; Ongaro and Leyser 2008; Leyser 2009; Domagalska and Leyser 2011a) (Figure 4A).

Auxin and strigolactones affect each other's levels by feedback mechanisms (Hayward et al. 2009). GR24 application to *Arabidopsis* seedlings resulted in reduced levels of free auxin in rosette leaves (Ruyter-Spira et al. 2011). The strigolactone biosynthetic mutant *max1-1* displayed a higher auxin transport capacity and increase in signal intensity of the auxin reporter *DR5::GUS* in the lower stem and *MAX3* and *MAX4* expression were higher in all max mutants (Hayward et al.

2009). The latter is likely mediated by increased auxin levels caused by the higher auxin transport capacity.

Like in shoot branching, strigolactones also act synergistically with auxin to stimulate secondary growth in plants. It was previously shown that vascular cambium-mediated secondary growth depends on auxin transport (Snow 1935). Interestingly, Agusti et al. showed that although there is an auxin dependent (through *AXR1*) component involved in the strigolactone mediated induction of cambial growth, strigolactones can also directly influence cambium activity independently or downstream of auxin (Agusti et al. 2011). With this new knowledge, it is not unexpected that strigolactone deficient mutants displayed a reduction in cambial activity, and that exogenous application of GR24 could complement this phenotype.

Several studies show that strigolactones interact with auxin to regulate primary root length (Koltai et al. 2010; Ruyter-Spira et al. 2011) (Figure 4C). In *Arabidopsis*, auxin treatment resulted in decreased primary root length accompanied by reduced cell elongation (Rahman et al. 2007). However, higher concentrations of GR24 were able to eliminate the suppressive effect of exogenous auxin application on primary root length in tomato (*Solanum lycopersicon*), accompanied by increased cell length and reduced cell division (Koltai et al. 2010). In addition, exogenous application of GR24 to *Arabidopsis* roots, led to a decrease in PIN1/3/7::GFP intensities in the provascular tissue of primary root tip (Ruyter-Spira et al. 2011). However, since after simultaneous application of NAA and GR24 no reduction in PIN protein levels could be observed, the effect of GR24 is likely not direct but is mediated through reduced local IAA levels instead. Indeed, as previously mentioned, GR24 treated plants were shown to have decreased levels of free IAA in their rosette leafs, implying that strigolactones negatively feedback on auxin levels throughout the entire plant. Consistent with this, *Arabidopsis* strigolactone mutants had shorter primary roots, which were accompanied by higher DR5::GUS signals in the primary root tips.

As described above, lateral root initiation and their subsequent outgrowth are both suppressed by GR24 application (Ruyter-Spira et al. 2011). Interestingly, application of GR24 in the presence of NAA counteracted the inhibitory effect of NAA and stimulated a more rapid outgrowth of lateral root primordia instead. It was postulated that the effect of strigolactones on lateral root formation was mediated through the modulation of local auxin levels (Ruyter-Spira et al. 2011) (Figure 4C). Analogous to this finding, lateral root development during sufficient Pi conditions was faster in strigolactone deficient plants than in the wild-type, while endogenous strigolactones in wild-type

plants were found to stimulate lateral root outgrowth during limiting Pi conditions instead (Ruyter-Spira et al. 2011). It has been hypothesised that the low Pi conditions increase the auxin sensitivity in the roots to such an extent that auxin becomes inhibitory to lateral root outgrowth. Strigolactones will reduce auxin concentration and hence stimulate lateral root outgrowth.

Strigolactones and Ethylene

Ethylene is a gaseous plant hormone that acts synergistically with auxin to regulate root hair elongation (Pitts et al. 1998). In addition to auxin, strigolactones also show a cross-talk with ethylene to regulate root hair elongation (Kapulnik et al. 2011b). Using root hair elongation as an assay, it was found that the effect of GR24 was reduced in the ethylene signalling mutants *ein2* and *etr1* and eliminated when ethylene biosynthesis was blocked. Moreover, root treatment with GR24, resulted in up-regulation of the expression of the ethylene biosynthesis gene *AtACS2*, suggesting that strigolactones and ethylene positively regulate root hair elongation via the same pathway (Kapulnik et al. 2011b). Therefore, the effect of strigolactones on root hair elongation might either be directly via modulation of the auxin flux or indirectly through modulation of ethylene synthesis (Koltai 2011) (Figure 4C).

Strigolactones and Cytokinin

Cytokinins (CKs) are known to act as a promoter of bud outgrowth (Dun et al. 2009; Leyser 2009; Beveridge and Kyozuka 2010). Physiological studies suggest that strigolactones act antagonistically with CK to regulate bud outgrowth (Brewer et al. 2009; Crawford et al. 2010; Liang et al. 2010). Recent work in pea sheds more light on the underlying mechanism (Dun et al. 2012). It was found that axillary buds in strigolactone deficient mutant plants (*rms1*) showed an increased response to 6-benzylaminopurine (BA, synthetic CK), when compared with WT buds. In addition, exogenous application of GR24 in combination with BA, led to a reduction in bud outgrowth in *rms1* but not in the *rms4* strigolactone insensitive mutant. This suggests that strigolactones decrease the CK response via *MAX2*. Furthermore, molecular data show that strigolactones and CK act antagonistically via the common target of *PsBRC1* expression in the bud. Exogenous application of GR24 led to an increase in expression of *PsBRC1*, whereas in decapitated or BA treated pea plants, decreased *PsBRC1* expression was observed (Dun et al. 2012).

Strigolactones and other hormones

There are a few studies indicating the cross-talk between strigolactones and other hormones. Recent work by Toh et al. has shown that strigolactones act synergistically with GA to regulate

seed germination in *Arabidopsis* (Toh et al. 2012) (Figure 4D). Particularly when exposing *Arabidopsis* seeds to high temperature (32°C), GR24 application increased endogenous GA_4 levels in *max1-1* but not in *max2-1* seeds. This suggests that strigolactones increase the GA response via MAX2. However, GR24 application did not increase the transcription of gibberellin-3-oxidase 2, a key enzyme in GA biosynthesis. Therefore, this suggests that the effect of strigolactones on GA is via the regulation of other steps in the GA biosynthetic pathway or through its catabolism or both.

Future perspectives

As an important signal compound in the rhizosphere and a novel phytohormone, strigolactone may have other biological functions besides the above described functions. According to the description above, it is likely that the function and/or activity of the natural strigolactones differs due to structural diversity. To date, over twenty natural strigolactons have been identified, some of which show different capacity in inducing parasitic seed germination and AM fungi branching. Characterisation of strigolactone functional diversification and specifity is highly relevant for agricultural practices. For instance, screening of crop cultivars producing strigolactones which are less potent to trigger parasitic plant seed germination but stimulate more AM fungi colonisation, can be a breeding approach to control parasitic weeds (Cardoso et al. 2011). Hence further unravelling of the strigolactone biosynthesic pathway, including the strigolactone diversification steps, is an important research question. Cytochrome P450s, and O-methyl and acetyl transferases are good candidates for the decoration of strigolactone structures starting from 5-deoxystrigol. In order to discover these genes, a genetic approach, using parental lines that produce different strigolactone profiles, would provide an excellent tool to resolve these questions and to discover enzymes that create the natural variation of strigolactones among plant species. Moreover, from the view of parasitic weed control, elucidation of the strigolactone biosynthesic pathway will also offer posibilities for the development of chemical inhibitors of strigolactone production. With respect to the carotenoid pathway origin, inhibitors of this pathway have been examined in several studies. Fluridone specifically inhibits the second dedicated enzyme in the carotenoid pathway, phytoene desaturase (Li et al. 1996). Treating maize, sorghum and cowpea with fluridone, Matusova et al showed that the root exudates from the treated plants were less active in triggering parasitic seed germination (Matusova et al. 2005). Similarly, the *NCED* specific competative inhibitor abamineSG (Figure 3) and the *CCD* specific inhibitor D2 also reduced strigolactone-production (Lopez-Raez et al. 2010).

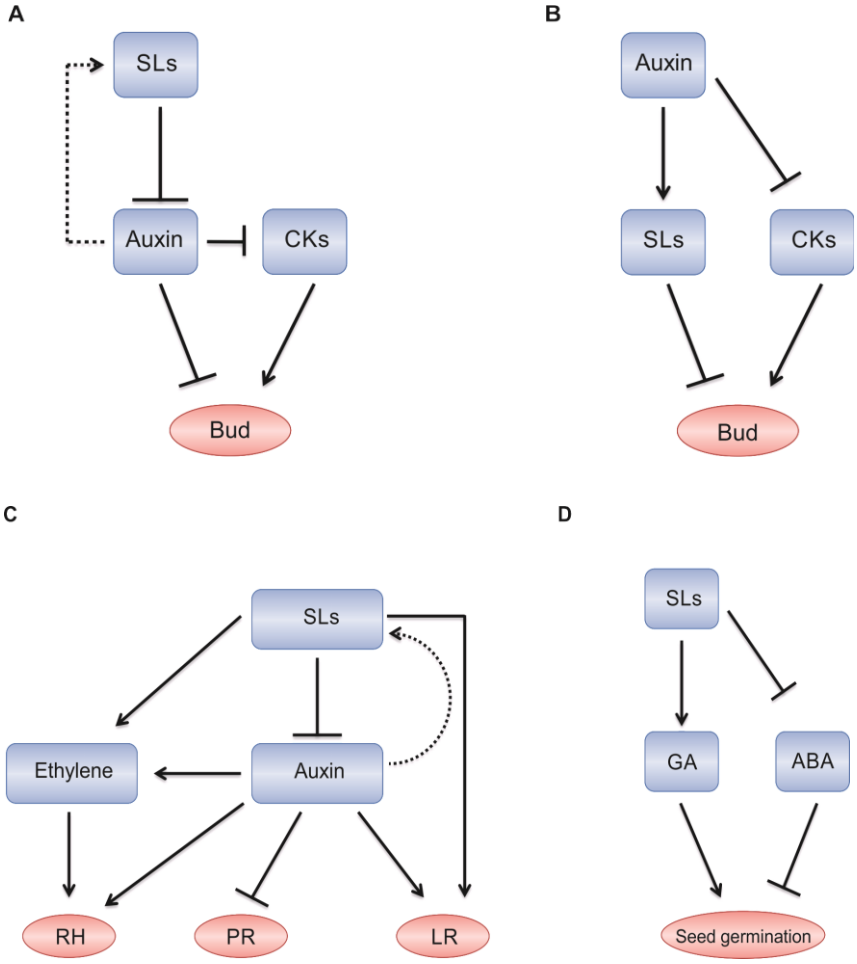

Figure 4. Schematic representation of strigolactones interacting with other plant hormones. **A and B,** For shoot branching; **A,** Canalization hypothesis for bud activation; **B,** Secondary messenger hypothesis for bud activation. **C,** For root architecture. **D,** For seed germination. Arrows represent positive regulation, flat ended lines represent negative regulation, and broken arrows represent feedback. Abbreviations: SLs, strigolactones; CKs, cytokinins; GA, gibberellin; ABA, abscisic acid; RH, root hair elongation; PR, primary root length and LR, lateral root formation.

Besides the above mentioned breeding approach and the use of chemical inhibitors of the strigolactone biosynthetic pathway, synthetic strigolactone analogues may also be used to induce parasitic weeds suicidal germination. Therefore, synthesis of cheap strigolactone analogs having high germination stimulatory activity remains a hot topic (Zwanenburg and Mwakaboko 2011). Furthermore, the control of soil fertility (Pi and N) and AM fungal colonisation are also useful methods to reduce the parasitic weed problem in agricultural practices (Lopez-Raez et al. 2009).

With regard to strigolactones as a phytohormone, it will be of interest to further explore the hormonal cross-talk with other plant hormones. Currently, most progress has been made at the physiological level, while at the molecular and biochemical level, our knowledge is still fragmented. It is not unlikely that strigolactones are involved in even more biological processes than we already know in different development stages, environmental conditions and different tissues. For instance, ABA is a well-known stress hormone known to be involved in drought stress tolerance. The interaction between ABA and strigolactones may imply that strigolactones also play a role during drought stress. However, the mechanisms underlying the ABA-strigolactone cross-talk are still unknown. The fact that they share a common biosynthetic origin (Figure 3) makes it even more intriguing to find out the mechanism by which they influence each other's levels.

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Chapter 5

β-carotene isomerase, DWARF27, links abscisic acid to the strigolactones

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Abstract

Strigolactones (SLs) are carotenoid-derived plant hormones that influence various aspects of plant growth and development in response to specific environmental conditions such as phosphate limitation. SLs and the well-known plant stress hormone abscisic acid (ABA) are both derived from the carotenoid pathway. This common biosynthetic origin may be the basis for a putative coordinated regulatory mechanism between SL and ABA biosynthesis. We have previously demonstrated that SL production was reduced in ABA-deficient tomato plants. Vice versa, in the present study, we investigated the ABA content in SL-deficient and -insensitive *dwarf* (*d*) rice mutants, and link ABA and SL biosynthesis through β-carotene isomerase DWARF27 (D27), which, interestingly, was found to be a common enzyme involved in the biosynthesis of both hormones.

Our results show that the ABA content was increased in *d10*, *d17* and *d3* mutant plants when compared with wild type, while it was reduced in *d27*. In addition, this difference was significantly enhanced by exposure to drought. Interestingly, as a consequence of their enhanced ABA levels, *d10*, *d17* and *d3* plants displayed an increased tolerance to drought compared with wild type plants, while the ABA deficient *d27* plants were more drought sensitive. Transient over-expression of *OsD27* in *Nicotiana benthamiana* enhanced both ABA and SL production. However, constitutive over-expression of *OsD27* in rice plants showed no significant changes in ABA and SL levels under normal conditions. Still, *OsD27* over-expression did result in higher SL levels when compared with wild-type plants during phosphate starvation. This suggests that, likewise, *OsD27* over-expression may only result in increased ABA levels during drought stress conditions.

We conclude that the *OsD27* gene is both involved in SL and ABA biosynthesis, and that, depending on the environmental conditions, the expression of the more downstream SL and ABA specific biosynthetic genes finally determines which and how much of each hormone is being produced. Considering the fact that D27 acts as a beta carotene isomerase, the existence of a so-called *cis* pathway leading to ABA production is being discussed.

Keywords: Abscisic acid, Strigolactones, DWARF27, Drought stress, Rice, Mutants.

Introduction

Carotenoids are ubiquitous lipophilic isoprenoid pigments present in green, photosynthetic plants as well as in some non-photosynthetic micro-organisms (Walter and Strack 2011). Carotenoids play a vital role in photosynthetic light harvesting, stabilisation of lipid membranes, and prevention of photo-oxidative damage (Havaux 1998; Ledford and Niyogi 2005). In flowers and fruits, carotenoids function as colouring agents and attractants for pollinators (Cazzonelli 2011). In addition to the vital role in photoprotection, carotenoids are also precursors of important plant hormones such as abscisic acid (ABA) and the strigolactones (SLs) (Matusova et al. 2005; Nambara and Marion-Poll 2005).

ABA plays a pivotal role in the regulation of seed maturation, desiccation tolerance, and dormancy, as well as 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 in plants increase, which results in stomatal closure to limit water loss and the accumulation of reactive oxygen species (ROS), dehydrins, and late embryogenesis abundant (LEA) proteins for osmotic adjustment (Shinozaki and Yamaguchi-Shinozaki 2007).

In higher plants, ABA is derived from a C_{40}-carotenoid precursor synthesised in the plastids. The first step committed in ABA biosynthesis is the oxidative cleavage of 9-*cis*-violaxanthin or 9-*cis*-neoxanthin, which results in the formation of xanthoxin. Both xanthophylls are supposed to be formed from the corresponding all-*trans*-isomers by an as yet-unidentified *cis*/*trans*-isomerase. All-*trans*-neoxanthin is derived from all-*trans*-violaxanthin, which is synthesised from all-*trans* configured zeaxanthin via two epoxidation reactions catalysed by zeaxanthin epoxidase (ZEP). Zeaxanthin itself is the product of the hydroxylation of the ionone rings in all-*trans*-β-carotene catalysed by β-carotene hydroxylase (BCH) (Davison et al. 2002). The oxidative cleavage of 9-*cis*-violaxanthin and/or 9-*cis*-neoxanthin to xanthoxin is catalysed by 9-*cis*-epoxycarotenoid dioxygenase (NCED) and is considered to be the rate-limiting step in ABA biosynthesis (Tan et al. 1997; Burbidge et al. 1999). Further steps in ABA biosynthesis take place in the cytosol where xanthoxin is converted to abscisic aldehyde catalysed by a short-chain dehydrogenase/reductase (SDR) and then finally ABA is synthesised by the oxidation of abscisic aldehyde catalysed by abscisic aldehyde oxidase (AAO3) (Schwartz et al. 2003).

SLs are carotenoid-derived signalling molecules (Matusova et al. 2005; Rani et al. 2008) initially known as germination stimulants of root parasitic plant seeds (*Striga, Phelipanche* and *Orobanche* spp.) (Cook et al. 1966; Bouwmeester et al. 2003, 2007; Xie et al. 2010; Yoneyama et al. 2010)

and later, as stimulants of arbuscular mycorrhizal (AM) fungi hyphal branching (Akiyama et al. 2005). Recently, SLs have also been recognised as hormones involved in the inhibition of shoot branching (Gomez-Roldan et al. 2008; Umehara et al. 2008). Besides shoot branching, so far, SLs have been shown to control a wide range of plant growth and developmental characteristics including root architecture, hypocotyl growth, photomorphogenesis and secondary growth in vascular plants, and protonema branching and colony extension in the moss *Physcomitrella patens* (as reviewed by Ruyter-Spira et al. 2013; Zhang et al. 2013). Under low phosphate, plants display increased biosynthesis and exudation of SLs, which stimulate the hyphal branching of the symbiotic AM fungi to acquire phosphate from the soil (Bouwmeester et al. 2007; Yoneyama et al. 2007; Jamil et al. 2011; López-Ráez et al. 2008; Kohlen et al. 2011).

Based on studies with mutants, so far, six 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). Mutations in these SL biosynthetic genes result in reduced SL production and exudation and a high-tillering/branching phenotype. The exogenous application of synthetic SL (GR24) rescues the branching phenotype of all these mutants, supporting that all these genes are involved in SL biosynthesis (Gomez-Roldan et al. 2008; Umehara et al. 2008; Wang and Li 2011). The remaining two genes in the pathway are related to SL perception and downstream signalling. *MORE AXILLARY GROWTH 2* (*MAX2*)/*DWARF3* (*D3*) encodes an F-box protein (Stirnberg et al. 2002; Stirnberg et al. 2007) and *DWARF14* (*D14*), encodes an α/β-fold hydrolase (Arite et al. 2009; Hamiaux et al. 2012; Waters et al. 2012b; Kagiyama et al. 2013; Zhao et al. 2013). Mutations in SL signalling genes also exhibit a high-tillering/branching phenotype. However, this phenotype cannot be rescued by the application of GR24. These mutants thus are insensitive to SLs (Umehara et al. 2008).

Considering their common biosynthetic origin (from carotenoids), a relationship between ABA and SL production may be anticipated. Indeed, ABA-deficient tomato mutants impaired at various points in the ABA biosynthetic pathway had reduced levels of SLs (López-Ráez et al. 2008, 2010).

Since in this study three different ABA biosynthetic enzymes were mutated, which all showed reduced SL levels, it is likely that ABA itself, rather than the enzymes, had an effect on SL biosynthesis. Furthermore, it has recently been reported that GR24 application to *Arabidopsis* seeds exposed to a high temperature (32°C) during germination, resulted in a decrease in ABA levels in *max1-1* but not in *max2-1* seeds. This was accompanied by down-regulation of ABA biosynthetic gene *NCED9*. This suggests that SLs decrease the ABA levels via MAX2 through the regulation of the expression of ABA biosynthetic genes (Toh et al. 2012). Also it has been demonstrated that GR24 application to preconditioned parasitic plant, *Phelipanche ramosa*, seeds, triggered the dormancy release which was accompanied by strong and rapid up-regulation of ABA catabolic gene *ABA 8'-HYDROXYLASE* and reduced ABA levels (Lechat et al. 2012). However, the mechanism underlying the ABA-SL cross-talk has not been elucidated yet. To get a better understanding of the relationship between SL and ABA, in the present study, we quantified ABA in SL-deficient and -insensitive rice mutants. We performed genetic and physiological analyses and found that ABA content was significantly increased in *d10*, *d17* and *d3* mutant plants, while it was reduced in *d27*. As a result, *d10*, *d17* and *d3* plants showed increased tolerance to drought, while *d27* plants displayed drought hypersensitivity. In addition, transient over-expression of *OsD27* in *Nicotiana benthamiana* enhanced both ABA and SL production. Our results show that *OsD27* is involved in regulation of SL as well as ABA biosynthesis. D27 activity is required for the formation of both hormones but D27 alone is not sufficient. Rather, it seems that the expression of the more downstream, hormone-specific, biosynthetic genes that is regulated by environmental conditions determines how much of each hormone is produced.

Materials and methods

Plant materials, growth conditions and drought treatments

Rice (*Oryza sativa* L. cv. Shiokari) and *Nicotiana benthamiana* were 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 drought) and silver sand (for others). The conditions in the climate room were as follows: temperature, 28°C day/25°C night; photoperiod, 10-h-light/14-h-dark; 70% relative humidity, and light intensity of 450 μM m^{-2} s^{-1}. The rice plants were watered twice a week using modified half-strength Hoaglands nutrient solution

(López-Ráez et al. 2008). *N. benthamiana* plants were grown in a climate room (temperature, 24°C day/22°C night; photoperiod, 16-h-light/8-h-dark). About four week-old *N. benthamiana* plants were used for the agro-infiltration experiment.

To test the drought tolerance of strigolactone-deficient rice mutants (*d10*, *d17*, *d17* and *d3*), seeds of mutants and the wild-type rice cultivar Shiokari 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 the experimental error, 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 were at the five-leaf stage, watering was withheld for 12 days. Watering was then resumed for 3 days to allow plants to recover, after which the survival rates were calculated.

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

Generation of *OsD27* over-expression lines in rice

To generate over-expression of *D27* plasmid, full-length cDNA of *OsD27* was PCR-amplified using primers D27-OX-F and D27-OX-R (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*. Transformation of *japonica* rice (*O. sativa* L. cv. Shiokari) was performed as previously described (Hiei et al. 1994).

Plasmid construction for transient expression in *Nicotiana benthamiana*

To generate the plasmid for transient expression in *Nicotiana benthamiana*, full-length cDNA of *OsD27* was sub-cloned from plasmid *p35S:OsD27:PJTK13*, kindly provided by Prof. Yonghong Wang (Institute of Genetics, China), while full-length cDNAs of *OsD27likeI* (*Os08g0114100*) and *OsD27likeII* (*Os05g0131100*) (http://rapdb.dna.affrc.go.jp/), were PCR-amplified from *O. sativa* L. cv. Nipponbare. All the PCR products were performed with Phusion polymerase (New England Biolabs) by using primers with the restriction sites of the multi-cloning sites in the entry vector ImpactVectorpIV1A_2.1 containing a CaMV 35S promoter

(www.pri.wur.nl/UK/products/ImpactVector/). All the PCR products were transformed into the cloning vector pJET1.2 (Fermentas), for sequencing confirmation followed by digestion and ligation to linearised entry vector pIV1A_2.1. Primers with restriction sites used are listed in Table S1. After the confirmation of pIV1A_2.1 entry clones by sequencing, LR reactions were performed to transfer the gene fragments into the pBinPlus binary vector (van Engelen et al. 1995), generating *p35S:PBIN*. The binary vector was introduced into *Agrobacterium tumefaciens* strains *AGL0* by electro-transformation. Positive *Agrobacterium* colonies were selected for further infiltration.

Agro-infiltration for transient expression in leaves of *Nicotiana benthamiana* was performed as previously described (van Herpen et al. 2010). Seven days after agro-infiltration, leaves were harvested and 0.5 g of fresh tissue was manually ground in liquid nitrogen. Further, SL and ABA analysis was carried out as described (Jamil et al. 2011; López-Ráez et al. 2010), see below for details.

Strigolactone and abscisic acid analysis

For SL analysis in rice, growing of plants and collection of exudates and SL extraction from exudates were performed as previously described (Jamil et al. 2011). SL and ABA extraction from tissues were performed, as previously reported (Jamil et al. 2011; López-Ráez et al. 2010) with minor modifications. 0.5 g root and 0.2 g shoot fresh tissue was manually ground in liquid nitrogen. Samples were taken in 10 ml cold glass tubes and 2 ml ethyl acetate containing D6-2'-*epi*-5-deoxystrigol and D6-ABA (0.025nmol/ml EtOAC-solution of D6-2'-*epi*-5-deoxystrigol and D6-ABA as internal standards (IS)) was added and mixed by vortexing. After 15 minutes sonification (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 (60:40) gradient. ABA was subsequently eluted by 2 mL solvent mixtures of methanol: ethyl acetate (10:90) gradient. The solvent mixtures were evaporated under speed vacuum and the residue was dissolved in 200 µl of acetonitrile: water: formic acid (25:75:0.1, v/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). SL and ABA analysis was performed using UPLC-MS/MS, as previously described (Jamil et al. 2011; López-Ráez et al. 2010). Data acquisition and analysis were performed using MassLynx 4.1 (TargetLynx) software (Waters).

Analysis of transcript levels

Total RNA was extracted from rice roots, leaves and shoots with 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.htm). 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 quantitative RT-PCR (qRT-PCR) analysis are listed in (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 (Table S1) was used as the internal control to normalise target gene expression (Lin et al. 2009).

Generation of expression plasmids

For the generation of recombinant thioredoxin and maltose-binding protein (MBP) fusion proteins, the cDNA sequence of *OsD27* was inserted into pBAD/THIO-TOPO®TA (Invitrogen) and pMAL-c4x (New England Biolabs)-vectors.

Protein expression and purification

The plasmid *pMAL-c4x-OsD27* was transformed into BL21 Rosetta *E. coli* cells. 3ml of overnight cultures grown in LB (Luria-Bertani) medium were inoculated into 50 ml of 2YT medium [1.6% (w/v) tryptone/1% (w/v) yeast extract/0.5% (w/v) NaCl/ 0.2% (w/v) glucose]. Cultures were induced at OD_{600} of 0.5 with 0.3mM IPTG (isopropyl-β-D-thiogalactoside) and grown for 5h at 28°C. Cells were then harvested and the pMAL fusion protein was purified using Amylose resin (New England Biolabs) according to the manufacturer`s instructions.

Enzymatic assays

Substrates were purified using thin-layer silica gel plates (Merck). Plates were developed in light petroleum/ diethyl ether/ acetone (4:1:1 by vol.). Synthetic cryptoxanthin was provided by BASF and purified by reverse or normal phase thin-layer silica gel plates (Merck). All-*trans*-β-carotene standard was obtained from Roth and 9-*cis*-violaxanthin and 9-*cis*-neoxanthin standards were obtained from CaroteNature (Switzerland).

In *vitro assays* were performed in a total volume of 200 µl. Substrates with 40 µM concentration were mixed with 20 µl of ethanolic 2 % Triton X-100 (Sigma) final concentration 0,2%, dried using a vacuum centrifuge and resuspended in 100 µl 2x incubation buffer containing 100 mM MOPS pH 6.4, 1mg/ml catalase (Sigma), 0,2mM $FeSO_4$. Purified OsD27 was then added to a final concentration of 200ng/ µl. The assays were incubated for 2h at 28°C shaking at 200 rpm in darkness. Extraction was performed by adding two volumes of acetone and three volumes of light petroleum (PE)/ diethyl ether (DE) (1:4, v/v). After centrifugation, the organic phase was collected, dried using a vacuum centrifuge and re-dissolved in 40 µl $CHCl_3$ for HPLC analysis, of which 5µl were injected.

Analytical methods

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 to 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 system 1 (*in vitro* assays D27) the column was developed at a flow rate of 0.75 ml / min. with a gradient from 100% B to 0% B within 20 min. , maintaining final conditions for 4 min. , after that column was re-equilibrated for 6 min.

Results

ABA levels are different in rice strigolactone-biosynthesis and strigolactone-signalling mutants

In earlier work we showed that ABA-deficient tomato mutants have lower SL levels than wild type tomato (López-Ráez et al. 2008, 2010). To determine whether the reverse is also true, we analysed ABA levels in rice SL-mutants. However, under non-stressed environmental conditions, ABA content was significantly higher in *d10* and *d3* compared with wild type, but it was lower in *d27* (Figure 1A). Also in the shoot the ABA content was higher in *d10* and *d17* and lower (though not significantly) in *d27* (Figure 1B). Under prolonged drought stress, ABA content strongly increased in wild type plants, particularly after 10 days of drought. The ABA content after 10 days of drought was significantly higher in *d10*, *d17* and *d3* compared with wild type and was significantly lower in *d27* (Figure 1C).

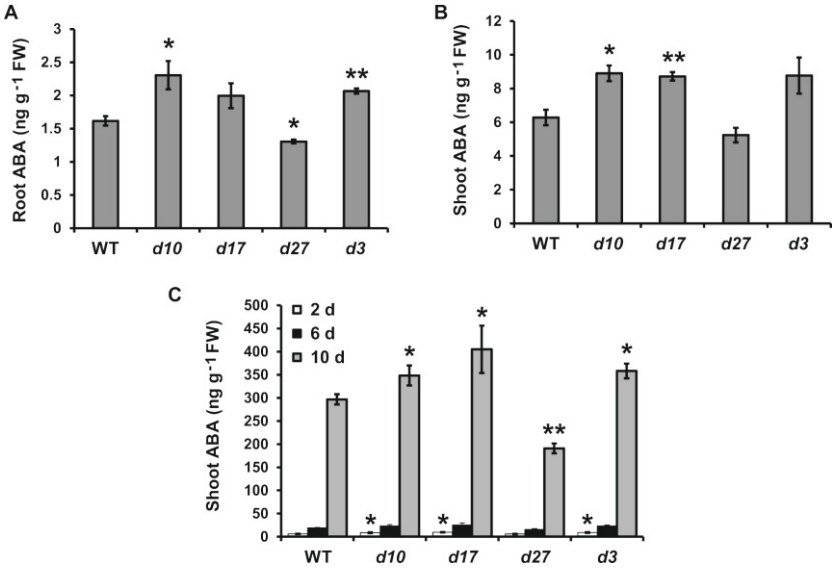

Figure 1. ABA content in rice SL-deficient mutants. ABA content was measured in Shiokari wild type (WT) and *d10*, *d17*, *d27* and *d3* mutants by UPLC-MS/MS. **A,** Root ABA in six-week old rice seedlings under normal conditions. **B,** Shoot ABA in five-leaf stage rice seedlings grown under non-stressed conditions. **C,** From the fifth leaf stage onwards, plants were not watered anymore for a period of 12 d and shoot ABA was measured at 2, 6 and 10 d after drought treatment. Bars represent mean ± SE (n=3). Asterisks indicate significance difference from wild type at * $P < 0.05$ and ** $P < 0.01$ (*t*-test).

D27 is likely involved in ABA biosynthesis

D27 has been shown to catalyse the first dedicated step in SL biosynthesis in rice (Alder et al. 2012). Our results show that *d27* has reduced ABA levels, while the other SL mutants show increased SL levels (Figure 1). This suggests that D27 may also be involved in ABA biosynthesis. Using *A. tumefaciens* mediated transient expression (agro-infiltration) of *D27* in *N. benthamiana* we tried to further elucidate the catalytic functions of D27. Infiltration of *N. benthamiana* with an empty vector alone resulted in a minor, non-significant, increase in ABA compared with non-infiltrated leaf material, but over-expression of *OsD27* led to an even higher increase in ABA levels, significantly higher than the empty vector control (Figure 2A). Also SL production was upregulated in leaves infiltrated with *OsD27* (Figure 2B). Although over-expression of *OsCCD8* did result in enhanced SL levels, this did not affect ABA (Figure 2B). This indicates that the increase in ABA observed with *D27* agroinfiltration are a direct consequence of *D27* over-expression and not of the elevated SL level. Combined, these results suggest that D27 is involved in SL as well as ABA biosynthesis.

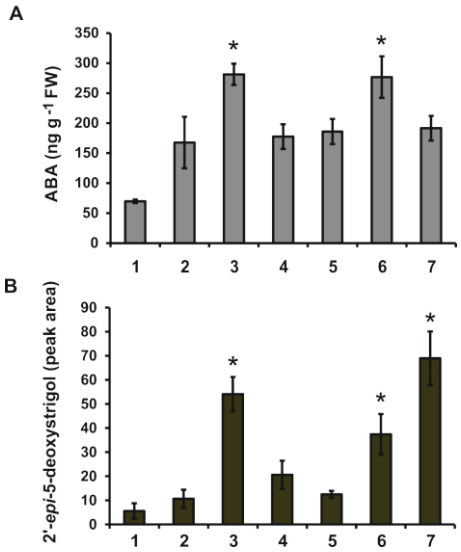

Figure 2. Transient expression of *OsD27* in *N. benthamiana*. ABA and SL quantification in leaves of *N. benthamiana* agro-infiltrated with: **1,** control; no infiltration; **2,** pBin-empty vector; **3,** *OsD27*; **4,** *OsD27likeI*; **5,** *OsD27likeII*; **6,** *OsD27*+ *OsD27likeI*+ *OsD27likeII*; **7,** *OsCCD8*. ABA and SL were analysed using UPLC-MS/MS. Bars represent mean ± SE (n= 5). Asterisks denote a significant difference from plants infiltrated with pBin-empty vector (* $P < 0.05$, ** $P < 0.01$, *t*-test).

Nevertheless, *d27* still produces ABA (Figure 1). In addition, in *Arabidopsis*, grafting *d27* mutant roots to *max4* mutant shoots rescues their branching phenotype, indicating it also still produces SLs (Waters et al. 2012a). In addition, both in rice and *Arabidopsis*, *d27* plants have only a weak tillering/branching phenotype compared with *d17* and *d10*. In *Arabidopsis* two genes, *AtD27likeI* and *AtD27likeII* with relatively high homology with *AtD27,* have been described (Waters et al. 2012a). To study whether these *D27like* genes in rice are redundant with *D27*, we used the amino acid sequence of rice D27 in a BLASTP query to search for D27 like proteins in the *O. sativa* (*japonica* cultivar-group) RefSeq protein database. Just like in *Arabidopsis* we found two *OsD27* like genes in the rice genome, *Os08g0114100* on chromosome 8 and *Os05g0131100* on chromosome 5 (annotated in RAP-DB). These two genes display 41 % and 32 % identity at the amino acid level with rice D27. We named these two genes, *OsD27likeI* and *OsD27likeII*, respectively. We over-expressed *OsD27likeI*, *OsD27likeII* and a combination of *OsD27*+*OsD27likeI*+*OsD27likeII* in *N. benthamiana* leaves. Infiltration with *OsD27likeI* and *OsD27likeII* separately did not result in an increase in ABA nor SL levels compared with wild type (Figure 2), nor did a combination of these two genes with *D27* result in a further increase in ABA compared with *OsD27* alone. Therefore, we can conclude that *OsD27likeI* and *OsD27likeII* are not redundant with *OsD27* in ABA biosynthesis.

Constitutive over-expression of *OsD27* in rice

To further address the biological function of *OsD27*, we generated stable transgenic rice plants over-expressing *D27*. The full-length *OsD27* under the control of the maize ubiquitin promoter was transformed into *japonica* rice Shiokari. Several independent transgenic plants were obtained, and the over-expression of *OsD27* was confirmed by qRT-PCR (Figure S1B). In contrast with *d27*, the *D27* over-expressors did not show morphological changes when compared with the wild type (Figure S1A). Two independent transgenic lines (D27-OX1 and D27-OX2), with high expression were selected for further analyses (Figure S1B).

As previously described, the rice *d27* mutant has reduced SL production and - exudation (Lin et al. 2009). To determine whether over-expression of *OsD27* results in higher SL biosynthesis and/or exudation, root extracts and exudates of *OsD27* over-expression lines were analysed by UPLC-MS/MS. Remarkably, when plants were grown under normal, non-stressed, conditions, the SL level (2'-*epi*-5-deoxystrigol) in root extracts and exudates in the *OsD27* over-expressing lines was significantly lower than in the wild type (Figures 3A and B). Because it is well documented that the amount of SL in rice root exudates and extracts particularly increases upon phosphate starvation

(Jamil et al. 2011; Umehara et al. 2008), we also measured SLs under phosphate starvation. Interestingly, in contrast to the non-stressed situation, now the 2'-*epi*-5-deoxystrigol in exudate and extract was significantly higher in the *OsD27* over-expressing lines compared with wild-type plants (Figures 3C and D). Next, we analysed the ABA content in the same root material, under normal as well as phosphate-starved conditions. There was no difference in the ABA level between the *OsD27* over-expressing lines and the wild type under non-stressed conditions, but under phosphate starvation the ABA level was significantly higher in the *OsD27* over-expressing lines (Figure 3E).

Drought tolerance of strigolactone-deficient and –insensitive rice mutants

ABA is a key signalling molecule for the plant's response to drought stress. It is known that over-expression of *ZEP* and *NCED* (key genes in ABA biosynthesis) in plants result in increased ABA accumulation and enhances drought tolerance (Nambara and Marion-Poll 2005). We therefore evaluated the drought tolerance of the SL-mutants. To test the drought-sensitivity phenotype, *d10*, *d17*, *d27* and *d3* were grown in a mixture of sand: soil (1:1) along with wild type Shiokari. From the fifth leaf stage onwards, plants were not watered anymore for a period of 12 days, after which watering was resumed for 3 d and the survival rates determined. All the SL-deficient and SL-insensitive mutants- *d10*, *d17* and *d3* - had significantly higher survival rates than the wild type (approximately 85%, 75% and 45%, respectively), with the exception of *d27* that did not survive at all, while the wild-type had a survival rate of 20% (Figures 4A and 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 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 (Figure 4C).

Regulation of *OsD27* expression

Previous work showed that *OsD27* is particularly expressed in axillary buds and young panicles, and to a lower extent in roots, sheaths, and leaves (Lin et al. 2009). Considering its role in SL biosynthesis it is not surprising that *D27* expression is induced by phosphate starvation, an environmental condition known to induce SL biosynthesis (Umehara et al. 2010; Liu et al. 2011). Because our results show that D27 is also involved in ABA biosynthesis, we analysed whether drought also regulates the expression of *D27*. Although over-expression of the *D27like* genes in *N. benthamiana* leaves showed that these genes are not redundant with *D27* in ABA biosynthesis, we decided to monitor the expression level of *OsD27*, *OsD27likeI* and *OsD27likeII* in leaves at

different time points during drought stress. We found a slight but significant increase (1.5-fold) in the expression of *OsD27* after 10 days of drought exposure compared with wild-type plants (Figure 5A), supporting a possible regulatory role in ABA production during water limiting conditions. In contrast, expression of *OsD27likeI* and *OsD27likeII* decreased upon drought stress (Figures 5B and C). Under phosphate-starvation, *OsD27* displayed a strong (4-fold) upregulation (Figure S2), consistent with previous reports (Umehara et al. 2010; Liu et al. 2011).

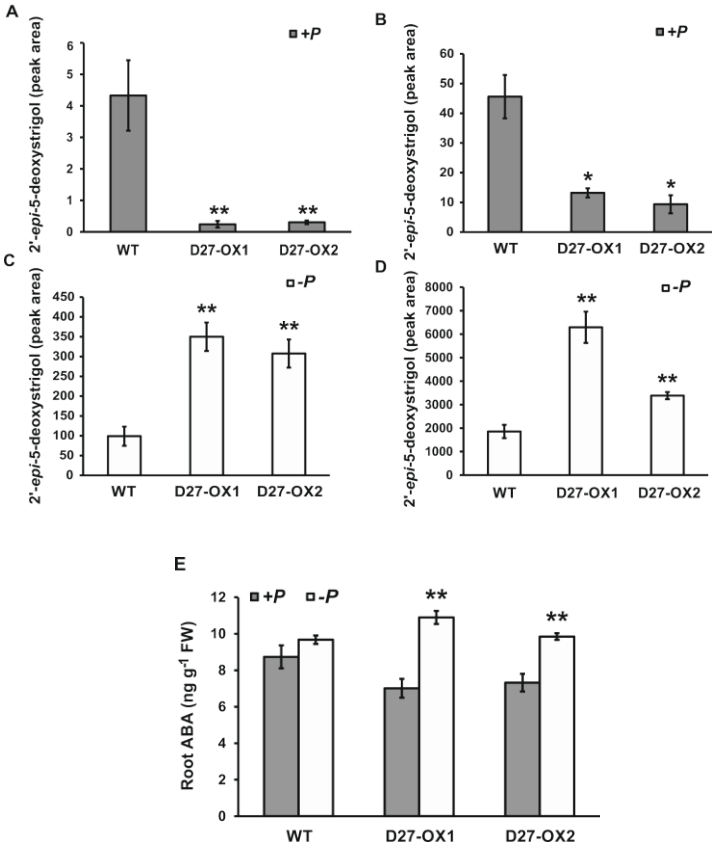

Figure 3. Constitutive over-expression of *OsD27* in rice. **A-D,** Quantification of 2'-*epi*-5-deoxystrigol (according to the peak area) in wild type (WT) and *OsD27* over-expression lines (D27-OX1 and D27-OX2) under sufficient (+*P*) and phosphate-starved (-*P*) conditions; **A and C,** root extracts; **B and D,** root exudates, Asterisks indicate significant difference at * $P < 0.05$ and ** $P < 0.01$ (*t*-test) from wild-type plants. **E,** ABA in roots of wild type (WT) and *OsD27* over-expression lines (D27-OX1 and D27-OX2) under sufficient (+*P*) and phosphate-starved (-*P*) conditions. Asterisks denote a significant difference from –*P* plants (** $P < 0.01$, *t*-test). Bars represent mean ± SE (n=3).

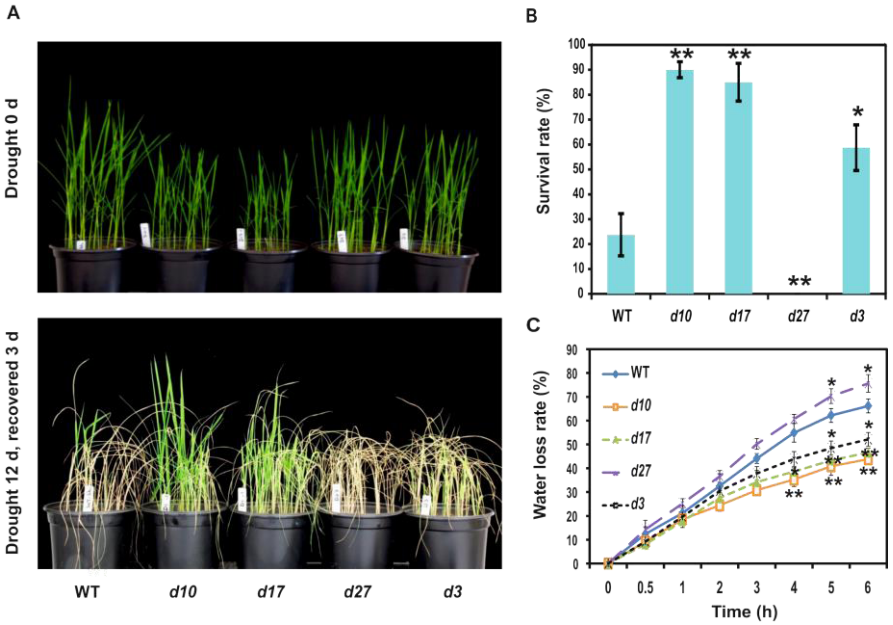

Figure 4. Drought tolerance of rice SL-deficient mutants. **A,** Phenotype of five-leaf stage plants before drought treatment and drought for 12 d then recovery for 3 d. **B,** Survival rate after recovery (five independent biological replicates, n=16). **C,** Water loss rate of detached flag-leaves (n=9). Bars represent mean ± SE. Asterisks indicate significant difference from wild-type plants at * $P < 0.05$ and ** $P < 0.01$ (*t*-test).

A possible explanation for the observed increased ABA levels in the *dwarf* mutants (other than *d27*), could be that the absence of SLs positively feeds back on the expression of SL biosynthetic pathway genes, including *D27* (Arite et al. 2007; Hayward et al. 2009; Waters et al. 2012a). Therefore, we checked the *D27* expression in *d10*, *d17* and *d3* both under non-stressed and drought stress conditions. Although, under non-stressed conditions there was a tendency for higher *OsD27* expression in the SL mutants than in wild type plants, this difference was not significant (Figure 5D). However, drought exposure led to a significant increase in *OsD27* expression in *d17* and *d3*. These results suggest that a higher expression level of *OsD27* in the SL mutants may have contributed to their increased tolerance to drought.

Position of D27 in the ABA biosynthesis pathway

It was previously demonstrated that D27 catalyses the isomerisation of all-*trans*-β-carotene into its 9-*cis*-isomer (Figure 6) and it is therefore the first known 9-*cis*/*trans*-β-carotene isomerase (Alder

et al. 2012). It was also proven that the enzyme catalyses the reaction in both directions (*trans* to *cis* and *cis* to *trans*), demonstrating that the reaction is reversible. Because of the growing evidence that *cis* configured carotenoids play an important role in signalling (Kachanovsky et al. 2012), and according to our results, not only act as the entry point for the SL, but also for the ABA biosynthetic pathway, we investigated other potential substrates for D27.

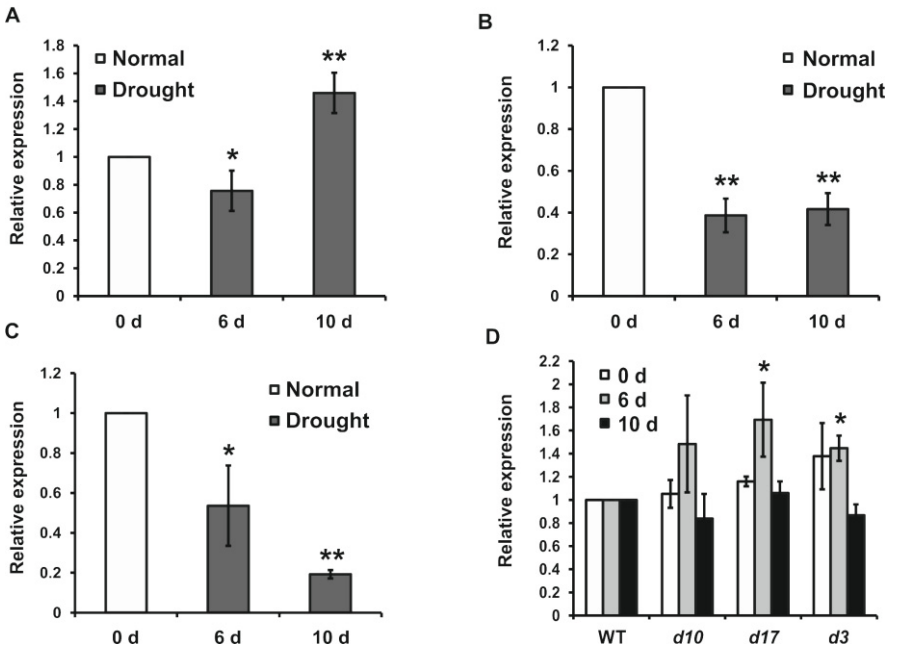

Figure 5. Relative mRNA expression analysis of genes. Expression level of genes under normal and drought conditions in shoots (time course 0, 6 and 10 d), determined by qRT-PCR. **A,** *OsD27*. **B,** *OsD27likeI*. **C,** *OsD27likeII*. **D,** *OsD27* expression in Shiokari wild type (WT) and *d10*, *d17* and *d3* mutants. Bars represent standard errors of three independent replicates. Asterisks indicate significance at * $P < 0.05$ and ** $P < 0.01$ (*t*-test).

Of particular interest in this respect is whether D27 can catalyse the *trans-cis* isomerisation of violaxanthin and neoxanthin for which no enzymes have been identified so far. To address the issue if D27 can directly produce the ABA precursor's 9-*cis*-violaxanthin and 9'-*cis*-neoxanthin, we performed *in vitro* analysis with the all-*trans* substrates and D27. The two substrates were not converted into the corresponding 9-*cis* isomers (Figure 6 VI, VII). This result demonstrated clearly that D27 is not involved in ABA biosynthesis by directly producing the 9-*cis* substrates for the

NCEDs. To investigate other putative substrates for D27, which may lead to other entry points for the ABA biosynthetic pathway, assays were performed with cryptoxanthin (mono-hydroxylated C_{40} xanthophyll) and zeaxanthin (double-hydroxylated) (Figure 6). D27 catalysed the conversion of all-*trans*-cryptoxanthin into the 9-*cis* isomer, but not of zeaxanthin (Figure 6).

Discussion

In earlier work we have demonstrated that ABA-deficient tomato mutants have reduced levels of SLs (López-Ráez et al. 2008, 2010). To get a better understanding of the relationship between SLs and ABA we now quantified ABA levels in SL-deficient (*d10, d17 and d27*) and SL-insensitive (*d3*) rice mutants. Interestingly, we found that *d10*, *d17* and *d3* have increased ABA levels, while *d27* has reduced ABA levels, compared with the wild type. These results spurred our interest to further study the role of D27 in ABA biosynthesis. D27 was recently identified as the first dedicated step in SL biosynthesis (Alder et al. 2012). To gain insight in how D27 influences ABA levels, we used transient expression in *N. benthamiana* and showed that over-expression of *OsD27* (but not of *OsD27likeI* or *OsD27likeII*) was sufficient to induce both SL and ABA biosynthesis. Surprisingly, ABA levels were also increased, although to a lower extent, in leaves infiltrated with the empty vector. Gene expression analysis of empty-vector agro-infiltrated *N. benthamiana* leaves shows a two-fold up-regulation of ABA biosynthetic genes encoding BCH, ZEP and AAO3 (Van der Krol et al., unpublished data). Transient expression of *OsCCD8* in *N. benthamiana* resulted in SL production but this did not result in induction of ABA compared with the empty vector control (Figure 2). These results strongly suggest that D27 has a direct role in ABA biosynthesis and is not affecting ABA through changes in the level of SLs. Interestingly, phylogenetic analysis of SL biosynthesis and signalling genes in the plant kingdom show that *D27* is highly conserved, whereas *CCD7*, *CCD8* and SL signalling genes are less conserved in the Chlorophytes (Delaux et al. 2012; Ruyter Spira and Bouwmeester 2012). In addition, Hartung (2010) reported that ABA is present throughout the green lineage. Combined, these results also suggest an alternative role for D27, possibly in ABA biosynthesis.

Besides lower ABA levels in *d27*, we found relatively high ABA levels in other SL-deficient and SL-insensitive mutants, (*d10*, *d17* and *d3)* compared with the wild type and this difference was more pronounced under drought stress conditions. It has been revealed before that low SL levels induce the expression of SL biosynthesis genes, *CCD7* and *CCD8* through a *MAX2* dependent positive feedback mechanism (Arite et al. 2007; Hayward et al. 2009; Waters et al. 2012a). Similarly, in our

study we show that the expression of *D27* tended to be higher in *d10*, *d17* and *d3* compared with wild-type, especially during drought. These results indeed suggest that the increased ABA level in these mutants, and the resulting tolerance to drought, are caused by positive feedback of the low SL levels on the expression of SL biosynthetic pathway genes including *D27*. It is interesting though, that SL-mutants *d10* and *d3* have a higher root ABA level under non-stressed conditions, while over-expression of *OsD27* did not result in higher ABA levels in roots. Possibly, in SL-mutants the more downstream ABA biosynthetic genes are also upregulated, perhaps as a consequence of increased water losses that occur in these highly tillered plants.

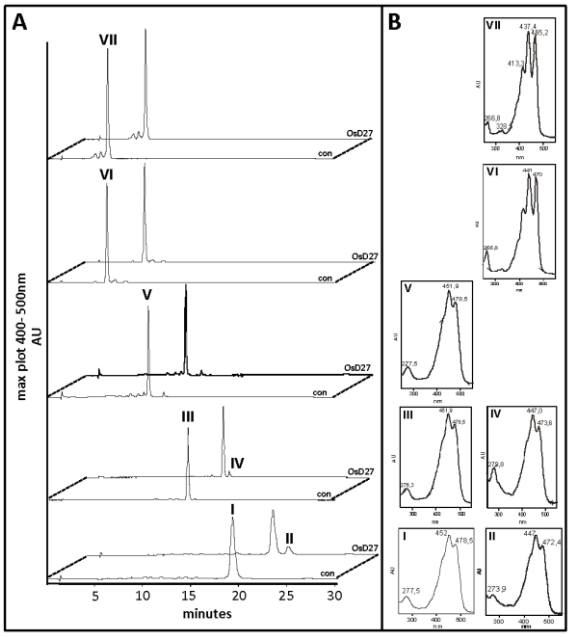

Figure 6. HPLC analysis of D27 *in vitro* incubations for 2h with different substrates. **A,** all-*trans*-β-carotene **(I)** and the emerging product 9-*cis*-β-carotene **(II)**. Incubation with cryptoxanthin **(III)** led to the formation of 9-*cis*-cryptoxanthin **(IV)**, while incubation with zeaxanthin **(V)**, violaxanthin **(VI)** or neoxanthin **(VII)** did not result in product formation. **B,** corresponding UV/vis-spectra.

Similar to ABA levels, under non-stressed conditions, constitutive over-expression of *OsD27* in rice did also not increase SL levels. Only when plants were subjected to phosphate starvation, increased SL levels were observed compared with wild type. This shows that upregulation of the expression of downstream SL biosynthetic genes, such as *CCD7* and *CCD8*, as a result of

phosphate starvation are first required before the higher expression of *D27* can result in higher SL production. These results all suggest that D27 is not the rate-limiting enzyme for ABA and SL biosynthesis and that increased expression levels of genes downstream of *D27* are also required for (increased) biosynthesis of ABA and SLs. Therefore, we can speculate that drought will likely result in increased ABA levels in *OsD27* over-expressing plants as well. Indeed, stress signals like drought are responsible for the higher ABA accumulation in the SL-deficient mutants. We conclude that hormonal feedback mechanisms and environmental conditions - through the regulation of downstream biosynthetic genes – together determine the activation of either the SL or the ABA biosynthetic pathway, or both. Indeed, low phosphate conditions were found to simultaneously increase ABA and SL levels in roots of *D27* over-expressing rice (Figures 3C, D and E). Although in wild type plants only a mild (non-significant) increase in ABA level was detected (Figure 3E), the combination of these results suggests that under low phosphate ABA production in roots is enhanced. It is likely that this effect is easily overlooked which is probably the reason why only few studies, like the one in Castor bean (*Ricinus communis* L.), report similar observations (Jeschke et al. 1997).

It will be of interest to explore the possibility that the reduced SL levels in ABA mutants (López-Ráez et al. 2010) are also the consequence of a hormonal feedback mechanism, in this case a negative feedback down-regulating the expression level of ABA biosynthetic genes, including *D27*. Indeed negative feedback regulation in ABA biosynthesis has been reported. In *Arabidopsis*, ABA deficient mutants displayed reduced expression of all the inducible ABA biosynthetic genes compared with wild-type plants (as reviewed by Xiong and Zhu 2003). This would fit the hypothesis that ABA mutants would have reduced levels of *D27* expression which would also result in reduced SL biosynthesis.

The next question we tried to answer was where in the ABA biosynthetic pathway D27 fits in. D27 is a β-carotene isomerase and it was proven that this enzyme catalyses the isomerisation of all-*trans*-β-carotene to 9-*cis*-β-carotene. However, the latter has not been described to be an ABA intermediate. In this study we show that D27 is not capable to isomerise zeaxanthin nor the downstream biosynthetic products neoxanthin and violaxanthin. The latter two have been hypothesised to be the pathway intermediates *en route* to ABA that are isomerised, however, the responsible enzyme(s) has(ve) not been identified yet. Because D27 was also not able to catalyse this step, D27 cannot directly upregulate ABA biosynthesis by simply producing more 9-*cis*-violaxanthin/9-*cis*-neoxanthin which in turn are converted into ABA. These findings suggest, that in

addition to the *trans*-*cis* isomerisation of epoxidised carotenoids resulting from the activity of the elusive isomerase(s), D27 is channelling 9-*cis*-β-carotene into an alternative "*cis*-pathway", also leading to ABA, possibly even making the postulated *trans*-*cis* conversion of neo- and violaxanthin superfluous (Figure 7). Our results that show that D27 not only acts on all-*trans*-beta-carotene, but also on all-*trans*-beta-cryptoxanthin (the intermediate between beta-carotene and zeaxanthin which only differs from beta-carotene by one hydroxyl-group), also opens up the possibility that this putative "*cis*-pathway" may have multiple starting points.

Using *in vitro* assays, D27 also catalyses the reverse isomerisation of *cis*-β-carotene (Alder et al. 2012). It is likely that these isomers are also actively balanced *in vivo*. By altering this balance, the amounts of SL and ABA, derived from this *trans* and *cis* pathways, can be regulated. This is supposed to be further influenced by the upregulation of downstream acting biosynthesis genes such as *CCD7* for SL production or *ZEP* in ABA biosynthesis (Nambara and Marion-Poll 2005; Umehara et al. 2008). Future studies are needed to shed more light on the relative contributions, ratio and regulation of the "*trans*" and "*cis*" pathways involved in ABA biosynthesis.

Abiotic stresses are the primary cause of crop failure worldwide, reducing average yields by more than 50% for most major crop plants (Bray et al. 2000). Among the abiotic stresses, drought is the most limiting factor, especially in low water use efficiency crops like rice. Drought affects about 20% of the total rice area in Asia (Pandey and Bhandari 2008). Plants have evolved a wide variety of molecular and biochemical mechanisms to survive under adverse growing conditions (Fujita et al. 2006; Shinozaki and Yamaguchi-Shinozaki 2007). One of them is the well-known upregulation of ABA that also seems to mediate the enhanced drought tolerance in the rice SL mutants (except *d27*) in the present study. However, this is not the only mechanism leading to drought tolerance (Yang et al. 2010).

Growing evidence reveals that carotenoids are also exploited in other plant responses to stress (Thompson et al. 2007; Jayaraj and Punja 2008). Over-expression of β-carotene hydroxylase (BCH*)*, which is involved in the conversion of β-carotene to zeaxanthin via β-cryptoxanthin, enhances photo-oxidative tolerance in *Arabidopsis* and drought tolerance in tobacco, by increasing the xanthophyll pigments (Davison et al. 2002; Zhao et al. 2013). A homolog of the *BCH* gene has also been identified and characterised in rice. Du et al. (2010) reported that constitutive over-expression of rice *BCH*, resulted in enhanced drought and oxidative stress tolerance by increasing the xanthophylls and ABA biosynthesis in rice. Therefore, it will be of further interest to explore the

carotenoid levels in all SL biosynthetic mutants and the D27 over-expressor, especially under stressful conditions like drought.

Our findings show that D27 has a central position in the biosynthetic cross-talk between ABA and SL in response to environmental stresses. Despite the complicated regulation, *D27* is a promising candidate for drought improvement in crops. Further studies to explore D27 over-expression in rice during drought stress will expand our understanding of the function of D27.

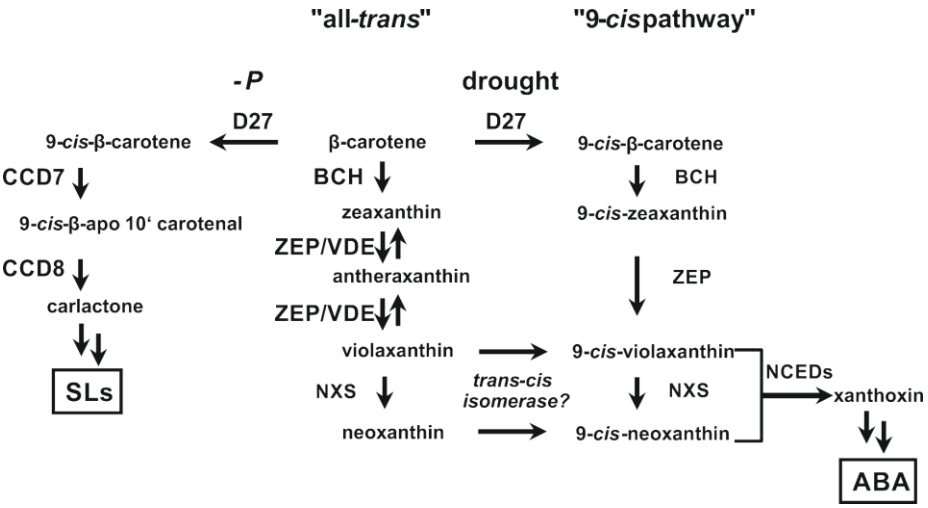

Figure 7. Proposed 9-*cis* pathway for fine tuning/regulation of ABA biosynthesis via D27. Under non-stressed conditions, most of the all-*trans*-β-carotene is channeled into the carotenoid biosynthetic pathway. Under phosphate starvation (-*P*), D27 is upregulated and channeling 9-*cis*-β-carotene towards strigolactone biosynthesis via carlactone. Under drought, D27 channels all-*trans*-β-carotene into a parallel 9-*cis*-pathway which is used to fine-tune ABA biosynthesis in parallel to the (putative) all-*trans* pathway. Abbreviations: BCH (β-carotene hydroxylase), ZEP (zeaxanthin-epoxidase), VDE (violaxanthin-de-epoxidase), NXS (neoxanthin-synthase), NCEDs (9-*cis*-epoxycarotenoid dioxygenases), ABA (abscisic acid), SLs (strigolactones), D27 (DWARF27, β-carotene isomerase).

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Supplemental data

Table S1. Primers sequences used in this study

Experiment	Primer name	Sequence
Overexpression in rice	*D27-OX-F*	5'-AAGGATCCATGGAGACCACCACGCTTGT-3'
	D27-OX-R	5'-AAACTAGTTCAGATGGAGCAATTCACAC-3'
Transient expression in *N. benthamiana*	*D27-N-F*	5'-CATGCCATGGCAATGGAGACCACCACGCTTG-3'
	D27-N-R	5'-ATTTGCGGCCGCTCAGATGGAGCAATTCACACC-3'
	D27I-N-F	5'-GGATCCTATGGCGGCGGCGGCGTTGCTGTTG-3'
	D27I-N-R	5'-TATAGCGGCCGCCTAAGTCTGAAGTTTGGGACAAAT-3'
	D27II-N-F	5'-CTCGAGATGGCCATGGCGCCGCCGTCTCCTC-3'
	D27II-N-R	5'-GCGGCCGCTCAAACTTGGGGGCATTGCAAACCATC-3'
qRT-PCR	*D27-Q-F*	5'-TCTGGGCTAAAGAATGAAAAGGA-3'
	D27-Q-R	5'-AGAGCTTGGGTCACAATCTCG-3'
	D27I-Q-F	5'-CCGAGCAGTTCAAGAAGCTC-3'
	D27I-Q-R	5'-TCAGCACTCCACTCTTCTGC-3'
	D27II-Q-F	5'-GGAAGAGAGCAAATGCCTTG-3'
	D27II-Q-R	5'-TTTAGGGCTTTGTCGGTGTC-3'
	Ubi-F	5'-GCCCAAGAAGAAGATCAAGAAC-3'
	Ubi-R	5'-AGATAACAACGGAAGCATAAAAGTC-3'

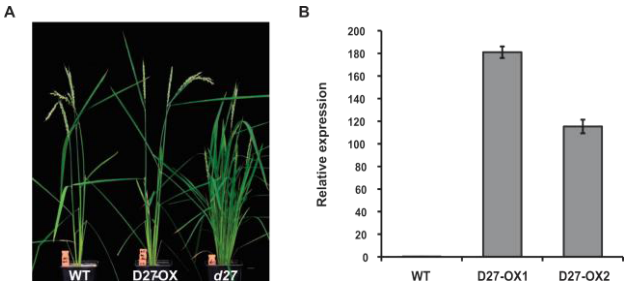

Figure S1. A, Morphology of Shiokari wild type (WT), *D27* over-expression, and *d27* rice plants at post-anthesis stage. **B,** The relative expression level of *OsD27* in roots of the two independent over-expression lines (D27-OX1 and D27-OX2) as compared with the wild type (WT).

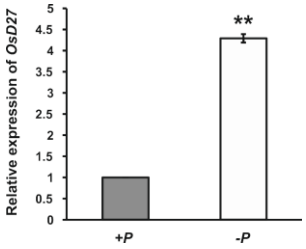

Figure S2. *OsD27* expression in roots of six-week old rice plants grown under sufficient (*+P*) and phosphate-starved (*-P*) conditions determined by qRT-PCR. Statistical significance is indicated by ** $P < 0.01$ (*t*-test).

Chapter 6

General discussion

The overall objective of the research work presented in this thesis was to get more detailed insight in the molecular regulation of drought tolerance in rice, with a particular focus on the role of transcription factors and two groups of plant hormones, ABA and strigolactones. This chapter discusses the main findings of this thesis and future perspectives of engineering for drought tolerance.

An effective strategy, molecular breeding

Rice is used as a staple food for more than half the human population. In order to increase rice production, two major breakthroughs have been achieved in the past 50 years. First, development of semi-dwarf cultivars with high harvest index and yield potential in the 1960s and second, development of rice hybrids in the 1970s. However, in the absence of new silver-bullet technologies, rice production in the 21st century has not increased much and it turns out to be difficult to break the present yield ceiling, while rice production is still facing enormous challenges. The rapid population growth causes a steady increase in the demand for rice while the fast urbanisation and industrialisation put pressure on the available land. In addition, the yield potential has not been fully exploited in many rice growing areas because of the increasing incidence of biotic and abiotic stresses, in particular drought. In addition, the extensive use of chemical fertilisers and pesticides also leads to ecological disruption and environmental pollution. Moreover, the consumer's preference with regards to rice grain quality will continue to play, a major role in global rice market. Rice grain quality is about traits such as appearance (shape and chalk), aroma, texture, stickiness and nutritional properties (reviewed by Fitzgerald et al. 2009; Chen et al. 2012).
In summary, the ideal rice cultivar (novel plant type) has a high grain yield (potential) and improved quality, nutrient-use efficiency, insect and disease resistance and drought tolerance (Zhang et al. 2007). As discussed above, traditional breeding has played a vital role in cultivar evolution over the past 50 years, but now progress is slowing down due to several limitations such as time-consuming selection procedures and complications in suitable genotype selection because of the genetically-complex quantitative nature of most traits. This has resulted in an increasing interest to apply molecular approaches to rice breeding. Marker-assisted selection (MAS), quantitative trait locus (QTL) analysis and transgenic approaches are indeed valuable tools that could speed up breeding in rice to obtain the ideal novel plant types introduced above.

Transcription factors in molecular breeding

Genetic engineering of rice is an efficient and time-saving approach to improve agronomic traits. For example, the gene encoding a protease inhibitor, *COWPEA TRYPSIN INHIBITOR*, *CpTI*, was

introduced into Minghui 86 (an elite rice cultivar in China which is parent line for super-hybrid rice), as a result of which transgenic plants showed an increased resistance to the rice stem borer under field conditions (Huang et al. 2005). Moreover, transgenic rice expressing the *Bacillus thuringiensis*, Bt protein has become a vital component of integrated pest management (Tu et al. 2000). However, the genetically complex quantitative nature of most agronomic traits, including drought, and the potentially harmful side effects, make the transgenic approach also difficult. Drought tolerance is usually controlled by complex gene networks and engineering of a single gene is unlikely to improve this trait. However, Transcription Factors (TFs) have been shown to activate the expression of multiple genes in a coordinated manner and they are therefore attractive and promising targets for application in molecular breeding (Century et al. 2008; Hadiarto and Tran 2011; Hussain et al. 2011). In addition, studies on TFs will improve our understanding of the physiological and molecular mechanisms of drought tolerance.

Although ectopic expression of TFs could activate the expression of multiple downstream stress-responsive genes, it may also trigger additional genes that adversely affect the growth of the plant under normal conditions and/or are not needed for the tolerance mechanism. Common negative effects observed with ectopic over-expression (or knock-down) of stress-tolerance related TFs are the growth retardation and/or delayed phenological development under normal growth conditions (Nakashima et al. 2009). Perhaps an alternative stratgey should use promoters which are expressed in specific tissues or under specific conditions.

The role of the TF *Oshox22* in drought tolerance and ABA homeostasis

In this thesis, I describe the use of *Oshox22*, which belongs to the HD-Zip family I of TFs (Chapter 2). I showed that down-regulation of *Oshox22* expression by T-DNA insertion provides enhanced tolerance towards drought and salt stress in rice without growth retardation or yield penalties both under normal and stress conditions (Zhang et al. 2012). Several families of TFs such as DREB/CBF, ERF, AREB/ABF, NAC and HD-Zip class I and II, are involved in the regulation of the drought response in rice. The drought-responsive TFs can be classified into ABA-dependent and ABA-independent categories, suggesting that drought tolerance is controlled by an extremely complex gene regulatory network (Yang et al. 2010; Shinozaki and Yamaguchi-Shinozaki 2007). Based on mutant and over-expression analyses, we propose that *Oshox22* regulates the drought and salt stress response through ABA-dependent signal transduction pathways (Chapter 2, published in Zhang et al. 2012). The expression of *Oshox22* is induced by drought, salinity and ABA, suggesting its role in regulating stress tolerance. *Oshox22*, is closely related to *Arabidopsis,*

Athb-6, *-7*, and *-12* which are also ABA and drought-responsive. Athb-6 protein has been shown to interact with ABA-INSESNSITIVE1 (ABI1), which is a protein phosphatase 2C (Himmelbach et al. 2002) which is a key component in ABA signalling (Leung et al. 1997; Leung and Giraudat 1998). For *Athb-7* and *Athb-12*, it has been found that their expression is down-regulated in *abi1* mutants (Olsson et al. 2004), which further supports the interaction between ABA signalling and these HD-Zip genes.

Since the discovery of ABA in the 1960s, this hormone has been shown to control a wide range of plant growth and development processes including seed development, seed and bud dormancy and germination. In addition to the vital role in developmental pathways, ABA is also involved in stress adaptation responses, such as stomata closure, and activation of genes required for osmotic adjustment. The major role of ABA seems to be the regulation of the plant water balance and osmotic stress tolerance (Zhu et al. 2002). Several ABA-deficient mutants such as *aba1*, *aba2* and *aba3* have been reported for *Arabidopsis* and rice (Koornneef et al. 1998; Nambara and Marion-Poll 2005; Ye et al. 2012). Under normal conditions, the growth of these mutants is similar as for the wild-type plants. However, under drought, ABA-deficient mutants readily wilt and die if the drought persists.

The endogenous ABA levels are determined by the precise balance between rates of ABA biosynthesis, conjugation and catabolism. However, these rates in a cell cannot entirely explain the endogenous ABA concentration at the site of action when considering the mobile nature of ABA (Seo et al. 2011). There is a common perception that a higher ABA level under drought may confer increased drought tolerance. However, it should be kept in mind that ABA biosynthesis as well as ABA catabolism genes are regulated by drought. Thus, drought susceptible plants may in fact experience a higher degree of stress, which may lead to increased ABA production (Xiong and Zhu 2003; Nambara and Marion-Poll 2005). Nevertheless, several studies showed that drought tolerant cultivars have higher endogenous ABA levels than susceptible cultivars (Iuchi et al. 2001; Perales et al. 2005; Thameur et al. 2011). However, recently, Ji et al (2011) revealed that the level of ABA – in the reproductive organs - is inversely correlated to the level of drought tolerance in wheat. In drought-sensitive cultivars, drought treatment leads to enhanced expression of ABA biosynthesis genes in anthers and ABA accumulation in spikes, while in drought-tolerant wheat the ABA levels remain lower, which correlates with lower expression of ABA biosynthesis genes and a higher level of expression of ABA catabolic genes (e.g. *ABA 8'-HYDROXYLASE*, *ABA8'-OH1*). Furthermore, wheat *TaABA8'-OH1* deletion lines that accumulate higher levels of ABA in the

spikes are drought sensitive. In conclusion, the relationship between endogenous ABA levels and drought tolerance/ sensitivity is complex. In Chapter 2, I describe that the T-DNA *oshox22-1* mutant showed decreased sensitivity to a high concentration of ABA and significantly improved tolerance to drought and high-salinity stresses. However, transgenic rice over-expressing *Oshox22* increased sensitivity to ABA and reduced tolerance to drought and high-salinity stresses. In addition, endogenous ABA levels in *oshox22-1* seedlings in shoot were 60% lower than those of the wild-type plants. Although *Oshox22* is induced by ABA, constitutive over-expression of *Oshox22* in Zhonghua 11, led to an increased ABA level but reduced drought tolerance. In addition to decreasing ABA sensitivity, however, the *oshox22-1* mutant resulted in an increased tolerance towards drought, which correlated with reduced water-loss rate. As numbers of stomata were decreased in *oshox22-1* mutants, regulation of stomata density by *Oshox22* is a possible mechanism underlying enhanced drought tolerance in these mutants. However, a change in stomata density was not found in the *Oshox22-OX* lines although these lines were less drought tolerant. Therefore, most likely stomata density is not the only factor determining drought tolerance under our experimental conditions. We conclude that the *Oshox22* TF negatively regulates drought and salt tolerance in rice by affecting ABA levels through the regulation of genes involved in biosynthesis or degradation of ABA as illustrated in Figure 1.

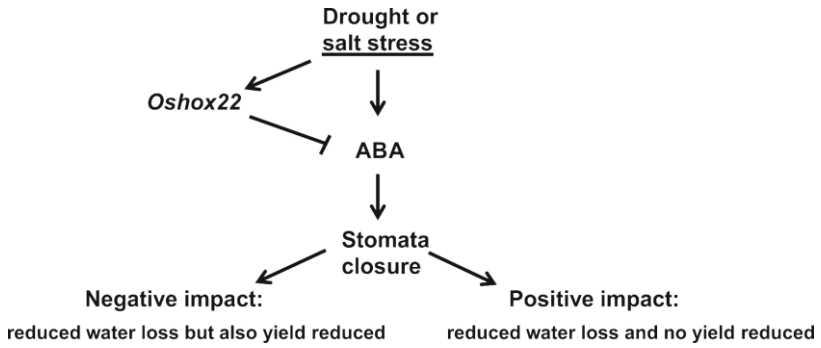

Figure 1. Proposed working model for *Oshox22* function in plant response to drought and salt stresses.

Interaction with other abiotic stresses

Drought or water stress is not only caused by a simple lack of water but also by other abiotic stresses such as salinity and low temperature. Thus, it is likely that the response to all these

abiotic stresses involves many shared molecular components. Indeed, in rice 40% of the genes that are induced by drought or salinity are also induced by cold stress (Shinozaki and Yamaguchi-Shinozaki 2007).

Some of the TFs involved in conferring drought tolerance also convey cross-tolerance to other abiotic stresses, suggesting the activation of genes common to the response pathways of multiple stresses. Most of the ethylene-responsive factors including AP37, HARD and JERF3 have been shown to confer tolerance to multiple abiotic stresses, such as oxidative stress, cold and salinity and drought (Century et al. 2008; Hadiarto and Tran 2011). In Chapter 2, I describe that *Oshox22* also functions as a negative regulator of salt tolerance. Likewise, *OsABI5* - a bZIP TF that is induced by ABA and salinity – upon over-expressing induces sensitivity to ABA and high-salinity stress as well as to a PEG treatment. Similar as with the *oshox22-1* mutant, down-regulation of *OsABI5* induced increased tolerance to salt as well as to PEG treatment (Zou et al. 2008). Hence, it seems that there are several regulators able to control both drought and salt tolerance. Another example is the TF *OsbZIP23* (Xiang et al. 2008). Like with *Oshox22*, *OsbZIP23* is a nuclear-localised transcriptional activator and down-regulation of *OsbZIP23* expression results in decreased ABA sensitivity and decreased tolerance towards salt and drought stress, while over-expression results in increased tolerance, which is opposite to the results with *Oshox22*.

Pleiotropic effects of *Oshox22* mis-expression

Pleiotropy implies that a single gene affects multiple phenotypic traits. For example, in humans the *ARX* gene encodes an Aristales-related homeobox protein and mutations in this gene cause very different phenotypic changes: ambiguous genitalia and lissencephaly (parts of the surface of the brain are more smooth than normal) (Wagner et al. 2011). In plants, two genes, *Ghd7* encoding a CCT domain-containing protein (Xue et al. 2008) and *Ghd8* encoding a HAP3 subunit (Yan et al. 2010), both have been reported as the major QTLs with pleiotropic effects on grain productivity, heading date and plant height in rice. *TMAC2* (*ABA-RESPONSIVE ELEMENTS-CONTAINING GENE 2*) is a negative regulator of ABA and salt stress responses in *Arabidopsis* but also plays a key role in controlling root elongation, floral initiation and starch degradation (Huang et al. 2007). *Oshox22* is a negative regulator of drought and salt tolerance in rice (Chapter 2). Also, I describe that *Oshox22* is playing an important role in controlling the grain length (GL) in rice. I showed that there is a tendency for lower expression of *Oshox22* when GL increases which would suggest that Oshox22 protein functions as repressor of GL (Chapter 3). Thus, *Oshox22* has pleiotropic functions in stress tolerance and GL in rice. Another example of pleiotropy is the *DROUGHT SALT*

TOLERANCE (*DST*) gene, which encodes a zinc-finger TF. Like *Oshox22*, *DST* negatively regulates the drought and salt tolerance in rice (Huang et al. 2009). Recently, it is reported that *DST* also enhances grain yield production in rice through controlling *Gn1a*/*OsCKX2* (*Grain number 1a*/*Cytokinin oxidase 2*) gene expression (Li et al. 2013).

Function of *Oshox22* in determining grain length

Grain size is a major agronomic trait that is affected by its length, width, filling and thickness (Xing and Zhang 2010). Grain length (GL) as well as other grain traits in rice were a main target during cereal domestication, it is still important in breeding (Sweeney and McCouch 2007). These traits have a major impact on the global rice market because of the consumer's preference. Generally, a long-slender rice grain is preferred by consumers especially in South- and South East-Asian countries, Southern China and the USA (Huang et al. 2012). As GL is a complex quantitative trait that is difficult to improve through conventional breeding approaches. The use of molecular markers for GL can substantially improve the breeding process for this trait. An example is *GRAIN SIZE3* (*GS3*) which is a major QTL for GL in rice on chromosome 3. Fan et al (2009) designed a marker, based on the causal C-A mutation in the second exon of *GS3* and observed a common C-A single nucleotide polymorphism (SNP) between short and long grain rice cultivars. Hence, *GS3* was validated as a functional marker for the selection of long-grain rice lines in breeding programmes (Takano-Kai et al. 2009). In Chapter 3, we followed a similar approach and developed a PCR-based insertion/deletion (InDel) CAPS marker for GL based on the allelic variation in the promoter of *Oshox22*. Sequence alignment did not show any polymorphism within the ORF of *Oshox22*, but we identified an extra A base in the *Oshox22* promoter in rice cultivar Azucena (*Japonica*, longer grain length) when compared to Bala (*Indica*, shorter grain length) and the reference genome of Nipponbare. This polymorphism is located at 583 bp upstream of the translation start codon of *Oshox22*. Based on this, InDel marker assays were developed to characterise core collections of rice cultivars and genotype a mapping population. In an F_2 population derived from IRAT109 (upland tropical *japonica*, with the A InDel) and Zhonghua 11 (lowland *japonica*, without the A InDel), we found an association between the A InDel in the *Oshox22* promoter with GL. Furthermore, in an Italian core collection (Faivre-Rampant et al. 2011), the A InDel was also associated with GL. Using a transgenic approach with a *ProOshox22-Oshox22* construct, we could further validate the association between the InDel in the *Oshox22* promoter, the expression polymorphism and GL. Expression of *Oshox22* under the control of its native promoter from cultivar IRAT112 (with the A InDel) showed a significant increase in GL

compared to the transformation background Zhonghua 11 (without the A InDel). To summarise, the polymorphism we identified was confirmed to be associated to GL and in principle this natural allelic variation can be exploited in breeding programmes to modify GL using molecular marker-assisted selection.

In rice, the palea and lemma (floral organs analogous to sepals, Yoshida and Nagato 2011) are important organs of the rice floret, and partially determine grain shape, GL as well as grain yield (Xing and Zhang 2010). We show that the inner epidermal tissues of the lemma in *ProOshox22-Oshox22* lines increase in cell length compared to wild type Zhonghua 11. These results suggest that the A InDel in the *Oshox22* promoter positively regulates GL, by increasing cell size of the lemma. This is supported by earlier studies reporting that loss-of-function mutations in *SMALL ROUND SEED3* and *-5* (*SRS3* and *SRS5*) caused a small and round seed phenotype, due to a reduction in cell length of seeds in the inner epidermal tissues of the lemma (Kitagawa et al. 2010; Segami et al. 2012). Furthermore, we describe that *Oshox22* on chromosome 4 is positioned at the same position as QTLs controlling GL, found in four mapping populations across multiple environments indicating that it is a stable QTL. In conclusion, in Chapter 2, I describe that expression of *Oshox22* is regulated by drought, ABA and salt and that it seems to regulate drought and salt susceptibility through an ABA-mediated signalling pathway. In Chapter 3, we show that *Oshox22* also has a function in controlling GL through changes in the cell length in the lemma. We do not yet know if this is via the same ABA pathway or via a different pathway. As discussed above, ABA also regulates plant growth and development, including seed development. It has been reported that the ABA content in large-size rice grains was higher than in small-size rice grains in the grain filling stage (Kato et al. 1993). The ABA content in shoots of *oshox22-1* mutant seedlings is reduced compared with wild-type plants in the same stage and the *oshox22-1* mutant has a shorter grain length. It would be of interest to further study the role of ABA in determining grain length/size in rice.

The role of abscisic acid in the regulation of strigolactone biosynthesis

As mentioned earlier (Chapter 4), strigolactones (SLs) are apocarotenoids (Matusova et al. 2005; Rani et al. 2008) that were initially known as stimulants of seed germination in root parasitic plants (*Striga, Phelipance* and *Orobanche* spp) (Cook et al. 1966; Bouwmeester et al. 2003, 2007; Xie et al. 2010; Yoneyama et al. 2010) and later, as stimulants of arbuscular mycorrhizal (AM) fungi hyphal branching (Akiyama et al. 2005). Recently, SLs have also been recognised as a plant hormone that inhibits shoot branching (Gomez-Roldan et al. 2008; Umehara et al. 2008). Besides

shoot branching, so far, SLs have now been shown to control a wide range of plant growth and developmental characteristics including root architecture, hypocotyl growth, photomorphogenesis, and secondary growth in vascular plants, and protonema branching and colony extension in the moss *Physcomitrella patens* (Ruyter-Spira et al. 2013; Zhang et al. 2013). ABA and SLs biosynthetically share common precursors, as both are carotenoid derived (Matusova et al. 2005). It was already known that a set of ABA-deficient tomato mutants impaired at various points in the ABA biosynthetic pathway showed reduced levels of SLs (López-Ráez et al. 2008, 2010). Since in the latter study three different ABA biosynthetic enzymes were mutated, which all showed reduced SL levels, it is likely that ABA itself, rather than the enzymes, have an effect on SL biosynthesis. Furthermore, it has been recently reported that application of the synthetic strigolactone GR24 to *Arabidopsis* seeds exposed to high temperature (32^{o}C) during germination, led to a decrease in ABA levels in SL-synthesis mutant, *more axillary growth1* (*max1-1*) but not in SL-signalling mutant, *more axillary growth2* (*max2-1*) seeds. This was accompanied by down-regulation of the key ABA biosynthetic gene, *9-CIS-EPOXYCAROTENOID DIOXYGENASE9* (*NCED9*). This suggests that SLs decrease the ABA levels via F-box protein MAX2 (Toh et al. 2012).

In Chapter 5, I describe that all the known SL-deficient/-insensitive rice mutants, *dwarf10* (*d10)*, *dwarf17* (*d17*) and *dwarf3* (*d3*) have higher ABA levels than the wild type background, except *dwarf 27* (*d27),* which has a lower ABA content. In addition, this difference was more pronounced under drought stress conditions. Interestingly, as a consequence of their enhanced ABA levels, *d10*, *d17* and *d3* plants displayed an increased tolerance to drought when compared with wild-type plants, while the ABA-deficient *d27* was more drought sensitive. It has been revealed earlier that low SL levels induce the expression of SL biosynthesis genes, *CAROTENOID CLEAVAGE DIOXYGENASE 7* and *CAROTENOID CLEAVAGE DIOXYGENASE 8* (*CCD7* and *CCD8*) through a MAX2 dependent feedback mechanism (Arite et al. 2007; Hayward et al. 2009; Waters et al. 2012a). Similarly, here I show that the expression of *DWARF27* (*D27*) tended to be higher in *d10*, *d17* and *d3* mutant backgrounds when compared with wild-type plants, especially during drought. These results indeed suggest that increased ABA levels in these mutants, and the resulting tolerance to drought, is likely caused through a SL feedback mechanism that increases the expression level of SL biosynthetic pathway genes including *D27*.

D27 encodes a novel iron-containing protein (Lin et al. 2009) and is involved in conversion of all-*trans*-β-carotene into 9-*cis*-β-carotene, the first dedicated step in SL biosynthesis (Alder et al. 2012) (Figure 2). However, our results showing that the *d27* mutant has reduced ABA levels, while

the other SL mutants have increased SL levels suggest that D27 may also be involved in ABA biosynthesis. I showed that over-expression of *OsD27* in *Nicotiana benthamiana* leaves alone was sufficient to induce ABA biosynthesis, while we did not find a significant induction of ABA levels in leaves infiltrated with *OsCCD8* compared with the empty vector control. These results strongly suggest that D27 is directly involved in ABA biosynthesis and that ABA levels are not affected by an increase in the level of SLs. Phylogenetic analysis of SL biosynthesis and signalling genes throughout the green lineage showed that *D27* is highly conserved in the plant kingdom, whereas *CCD*7, *CCD8* and downstream signalling genes are less conserved (Delaux et al. 2012; Ruyter-Spira and Bouwmeester 2012). In addition, Hartung (2010) reported that ABA is present throughout the green lineage. This hints at an alternative role for D27, possibly in ABA biosynthesis.

Constitutive over-expression of *OsD27* in rice plants induced no significant changes in ABA and SL levels. However, under phosphate starvation, *OsD27* over-expression did result in higher SL levels when compared with wild type plants. This suggests that likewise, *OsD27* over-expression may only result in increased ABA levels during drought stress conditions (Figure 2) and work to proof this is ongoing. In conclusion, we suggest that *D27* is involved in SL as well as ABA biosynthesis, and that, depending on the environmental conditions, the expression of the more downstream SL and ABA specific biosynthetic genes determine which and how much of each hormone will be produced (Figure 2). In addition, we have also discussed the putative "*cis* pathway" involved in ABA biosynthesis (Chapter 5). Despite this complicated relation between SL and ABA, my results make *D27* a promising candidate for drought improvement in crops. Further studies to assess the effect of *D27* over-expression in rice on drought stress tolerance are ongoing in our group and should further improve our understanding of the function of *D27*.

Future perspectives

Drought tolerance improvement is one of the challenging tasks of this era due to its complex and erratic nature. In order to attain a full understanding about mechanisms of drought tolerance in rice, there is a need to combine the data derived from different studies. Integrative and comparative analyses of multiple transcriptomics, proteomics, phenotyping and metabolomics datasets should provide insights into the key regulators of drought tolerance.

As far as engineering drought tolerant genes is concerned, transgenic approaches will have a wide range of implications in the future. However, this approach can be used to its full potential only when the transgenic work so far mostly done in greenhouses, is further verified under field

conditions, for example *Oshox22* and *D27*. This will allow us to assess the influence of the introduced or silenced genes under diverse stress conditions. Furthermore, there is significant room for improvement of current transgenic techniques. For example, over-expression of drought responsive genes often hampers plant growth under normal conditions. This negative effect could be reduced by using drought stress-inducible promoters (like the *DEHYDRATION-RESPONSIVE29A* (*RD29A*) and tissue or cell specific promoters (e.g. expressed only in guard cells) (Yang et al. 2010). Keeping in mind the genetic diversity of many crop plants and the complex nature of drought tolerance mechanisms, it remains crucial to use yield as the key trait in the breeding for drought tolerance.

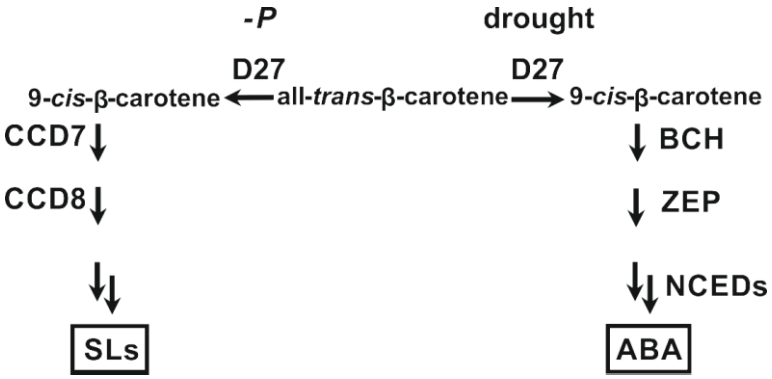

Figure 2. Schematic representation of the intimate relationship between ABA and SLs via D27. Under phosphate starvation (*-P*), D27 is upregulated and channels 9-*cis*-β-carotene towards strigolactone biosynthesis. Under drought conditions, D27 is hypothesised to channel all-*trans*-β-carotene into a parallel 9-*cis*-pathway which then in turn is used to fine-tune ABA content in addition to the all-*trans* pathway. Abbreviations: D27 (DWARF27, β-carotene isomerase), BCH (β-carotene hydroxylase), ZEP (zeaxanthin-epoxidase), NCEDs (9-*cis*-epoxycarotenoid dioxygenases), CCD (carotenoid cleavage dioxygenase), ABA (abscisic acid), SLs (strigolactones).

In this thesis, several functions of *Oshox22* in development and developmental adaptation to stress were described but it is not yet known which downstream target genes are regulated. Therefore, more research is needed to elucidate the genetic and biochemical pathways to understand the molecular mechanisms underlying rice drought tolerance as influenced by *Oshox22*.

In the past decades, the understanding about molecular mechanisms of plant hormones has improved substantially. Receptors, regulators, transcription factors, as well as downstream

responsive genes and proteins have been already identified. However, there are still many aspects to be discovered as is exemplified by the recent discovery of a completely new class of plant hormones, the strigolactones, which are slowly uncovered to play a role in many plant processes, including drought tolerance (this thesis). There are also other major challenges concerning interactions or cross-talk between different plant hormones and fine tuning of gene expression, particularly in crops, under various environmental conditions. In addition, it is also crucial to reveal how plant hormones signalling and changes in gene expression are integrated into phenotype and specific traits.

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Zhang Y, Haider I, Ruyter-Spira C, Bouwmeester H (2013) Strigolactone Biosynthesis and Biology. In: Bruijn FJd (ed) Molecular Microbial Ecology of the Rhizosphere. Vol. 1, John Wiley & Sons, Inc. Hoboken, NJ, U. S. A. pp 355-371

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Summary

Abiotic stresses are the primary cause of crop failure worldwide, reducing average yields by more than 50%. Among the various forms of abiotic stress, drought is the most limiting factor for rice productivity. Drought affects about 20% of the total rice cultivation area in Asia. Understanding the various aspects of drought stress, the response and resistance mechanisms in relation to plant growth is therefore of fundamental importance to improve sustainable agriculture.

Drought tolerance is usually controlled by complex gene networks and engineering of a single gene is unlikely to improve this trait. However, altering the expression of Transcription Factors (TFs) may be a tool for improvement of drought tolerance since they have been shown to activate the expression of multiple genes in a coordinated manner and they are therefore attractive and promising targets for application in molecular breeding or genetic engineering. In addition, studies on TFs will improve our understanding of the physiological and molecular mechanisms of drought tolerance. The overall objective of the work presented in this thesis was to get more detailed insight in the molecular regulation of drought tolerance in rice, with a particular focus on the role of TFs of the homeobox class and two groups of plant hormones, abscisic acid and strigolactones.

In Chapter 2, I described the isolation and characterisation of the rice *Oshox22* gene which is an homeobox gene of the HD-Zip I family. I showed that the expression of *Oshox22* is strongly induced by salt stress, abscisic acid (ABA) and polyethylene glycol (PEG) treatment, and weakly by cold stress. Trans-activation assays in yeast and transient expression analyses in rice protoplasts demonstrated that Oshox22 is able to bind to the CAAT(G/C)ATTG element and acts as a transcriptional activator that requires both the HD and Zip domains. Rice plants homozygous for a T-DNA insertion in the promoter region of *Oshox22* showed reduced *Oshox22* expression and ABA content, decreased sensitivity to ABA, and enhanced tolerance to drought and salt stress in the seedling stage. In contrast, transgenic rice over-expressing *Oshox22* showed increased sensitivity to ABA, increased ABA content, and decreased drought and salt tolerances. These results support the conclusion that *Oshox22* acts as a negative regulator in stress response. Since reporter gene studies in yeast and rice cells suggested that *Oshox22* acts as a transcriptional activator, its function as a negative regulator in stress responses might be explained via activation of other repressors.

As *Oshox22* is highly expressed in developing panicles and grains, in Chapter 4 I investigated the role of *Oshox22* in controlling grain length (GL) in rice. We found a stable quantitative trait locus (QTL) for GL on this position in four mapping populations. Sequence analysis of *Oshox22* in rice

cultivars Bala, Azucena and Nipponbare revealed an extra A base in the Azucena promoter, which is a long grain type rice. Using a PCR-based insertion/deletion (InDel) CAPS maker assay in rice populations and collections, I found an association between the A InDel in the *Oshox22* promoter with GL. Furthermore, expression of *Oshox22* under the control of a promoter with the A InDel in Zhonghua 11 (which does not have the A InDel) resulted in a significant increase in GL in Zhongua 11. Scanning electron microscopy revealed that the enhanced GL was caused by an increased cell length in the inner epidermal cells of the lemma. In addition, the data show that there is a tendency for lower expression of *Oshox22* when GL increases which would suggest that Oshox22 functions as a repressor of GL. These findings suggest that natural variation in the *Oshox22* promoter can be exploited in breeding programmes to modify GL using molecular marker-assisted selection. However, the exact mechanism of regulation of GL by *Oshox22* is still not clear. Since *Oshox22* is a homeobox gene, it will exert its function via regulation of downstream target genes which we do not know yet. Therefore, more research is needed to elucidate the genetic and biochemical pathways to understand the molecular mechanisms underlying rice GL development and to determine if there are interactions with other known regulators of GL.

The strigolactones are a relatively new class of plant hormones and a possible role in drought tolerance is unknown. In Chapter 4 of this thesis, I reviewed the various roles that strigolactones (SLs) play both in the rhizosphere and as endogenous plant hormone. In addition, the current knowledge on the SL biosynthetic and downstream signalling pathways and the interactions of SLs with other plant hormones, such as ABA, is described.

It has been reported that there seems to be a functional link between ABA and SLs but the mechanism of that link remained unknown. In Chapter 5, I studied the intimate relationship between ABA and SL biosynthesis through the further characterisation of β-carotene isomerase D27 in rice. The results show that the ABA content was increased in SL-deficient and -insensitive *dwarf* (*d*) rice mutants, *d10*, *d17* and *d3* compared with wild type, while it was reduced in *d27*. In addition, this difference was significantly enhanced by exposure to drought. Interestingly, as a consequence of their enhanced ABA levels, *d10*, *d17* and *d3* plants displayed an increased tolerance to drought compared with wild-type plants, while the ABA deficient *d27* plants were more drought sensitive. Transient over-expression of *OsD27* in *Nicotiana benthamiana* enhanced both ABA and SL production. However, constitutive over-expression of *OsD27* in rice plants showed no significant changes in ABA and SL levels under normal conditions. Still, *OsD27* over-expression did result in higher SL levels, compared with wild-type plants, under phosphate starvation. This

suggests that likewise, *OsD27* over-expression may only result in increased ABA levels during drought stress conditions. I concluded that the *OsD27* gene is involved in SL as well as ABA biosynthesis, and that, depending on the environmental conditions, the expression of the more downstream SL and ABA specific biosynthetic genes determines which of the two and how much is being produced.

In Chapter 6, I discussed the main findings of this thesis and presented the future perspective of how the knowledge generated in this thesis can contribute to the improvement of drought tolerance and GL in rice.

Samenvatting

Abiotische stress factoren zijn de voornaamste oorzaken voor het wereldwijd tegenvallen of mislukken van oogsten van landbouwgewassen, met opbrengst verliezen oplopend tot meer dan 50%. Onder de verschillende vormen van abiotische stress is droogte de meest beperkende factor voor de productiviteit van rijst. Droogte treft ongeveer 20% van de totale rijstteelt gebieden in Azië. Inzicht in de verschillende aspecten van droogtestress, de respons en resistentie mechanismen met betrekking tot de groei van planten, is daarom van fundamenteel belang om duurzaamheid van de landbouw te verbeteren. Droogtetolerantie wordt door complexe genetische netwerken gereguleerd en het is niet waarschijnlijk dat engineering van een enkel gen deze eigenschap kan verbeteren. Echter, het veranderen van de expressie van transcriptiefactoren (TFs) zou een hulpmiddel voor het verbeteren van droogte tolerantie kunnen zijn, aangezien het is aangetoond dat deze de expressie van meerdere genen op gecoördineerde wijze kunnen activeren. Door deze eigenschappen zijn TFs veelbelovend om in moleculaire veredeling of genetische modificatie toegepast te worden. Bovendien zullen studies over TFs ons begrip van de fysiologische en moleculaire mechanismen van droogte tolerantie verbeteren. De algemene doelstelling van het werk dat in dit proefschrift beschreven wordt, was om meer gedetailleerd inzicht te krijgen in de moleculaire regulatie van droogte tolerantie in rijst, met een bijzondere aandacht voor de rol van TFs van de homeobox klasse en twee groepen van plantenhormonen, abscisinezuur en strigolactonen.

In hoofdstuk 2, beschrijf ik de isolatie en karakterisering van het rijst Oshox22 gen dat een homeoboxgen van de HD-Zip I familie is. Ik toonde aan dat de expressie van Oshox22 sterk geïnduceerd wordt door zoutstress, abscisinezuur (ABA) en polyethyleenglycol (PEG) behandeling, en in mindere mate door koudestress. Trans-activatie assays in gist en transiënte expressie-analyse in rijstprotoplasten toonden aan dat Oshox22 kan binden aan het CAAT (G / C) ATTG promoter element en dat het werkt als een transcriptionele activator die zowel HD en Zip domeinen vereist. Rijstplanten die homozygoot zijn voor een T-DNA insertie in het promotorgebied van Oshox22, vertoonden verminderde Oshox22 expressie, ABA concentraties en ABA gevoeligheid en verbeterde tolerantie voor droogte-en zoutstress in het zaailingstadium. In tegenstelling hiermee, transgene rijst waarin Oshox22 tot overexpressie werd gebracht, vertoonde een verhoogde gevoeligheid voor ABA, verhoogde ABA concentratie, en verminderde tolerantie voor droogte en zout. Deze resultaten ondersteunen de conclusie dat Oshox22 fungeert als een negatieve regulator in reactie op stress. Aangezien reportergen studies in gist en rijst cellen suggereerden dat Oshox22 fungeert als een transcriptionele activator, kan de functie als een

negatieve regulator van stressresponsen wellicht via de activatie van andere repressoren verklaard worden.

Omdat de expressie van Oshox22 tijdens de ontwikkeling van rijst pluimen en granen hoog is, heb ik in hoofdstuk 4 de rol van Oshox22 in het bepalen van graan lengte (GL) onderzocht. We vonden een significant quantitative trait locus (QTL) voor GL op de positie van Oshox22 in vier verschillende mapping populaties. Sequentie-analyse van Oshox22 in rijst cultivars Bala, Azucena en Nipponbare onthulde een extra Adenine in de Oshox22 promotor van Azucena, een cultivar dat gekenmerkt wordt door relatief lange rijst korrels. Met behulp van een PCR-gebaseerde insertie / deletie (INDEL) CAPS marker test die in rijst populaties en collecties wordt gebruikt, vond ik een associatie tussen de Adenine INDEL in de Oshox22 promotor en GL. Bovendien resulteerde expressie van Oshox22 onder de controle van een promoter met de Adenine Indel, in Zhonghua 11 (die zelf niet beschikt over de Adenine Indel) in een significante toename van GL. Scanning elektronenmicroscopie toonde aan dat de verbeterde GL werd veroorzaakt door een toegenomen cel lengte in de binnenste epidermale cellen van het lemma. Bovendien laten de gegevens zien dat er een tendens is voor een lagere expressie van Oshox22 bij toenemende GL, wat erop wijst dat Oshox22 functioneert als een repressor van GL. Deze bevindingen suggereren dat de natuurlijke variatie in de Oshox22 promotor in veredelings programma's kan worden benut om met behulp van moleculaire merker gestuurde selectie GL te wijzigen. Echter, het exacte mechanisme van de regulering van GL door Oshox22 is nog niet duidelijk. Aangezien Oshox22 een homeoboxgen is, zal deze zijn functie uitoefenen via de regulering van andere, verderop gelegen genen in de signaal transductie keten, die we nog niet kennen. Om de moleculaire mechanismen die ten grondslag liggen aan rijst GL ontwikkeling te begrijpen, is daarom meer onderzoek nodig naar de genetische en biochemische routes en mogelijke interacties van Oshox22 met andere bekende regulatoren van GL.

De strigolactonen zijn een relatief nieuwe klasse van plantenhormonen. Het is tot op heden niet bekend of strigolactonen een rol in droogte tolerantie spelen. In hoofdstuk 4 van dit proefschrift, heb ik de verschillende aspecten van strigolactonen die tot op heden bekend zijn, zowel op het gebied als signaal stoffen in de rhizosfeer als endogeen plantenhormoon, samengevat. Bovendien heb ik de huidige kennis over de SL biosynthese en signaal transductie keten, en interacties van SL met andere plantenhormonen zoals ABA beschreven.

Het is beschreven dat er een functioneel verband tussen ABA en SL lijkt te zijn, maar het mechanisme van dit verband is tot op heden onbekend. In hoofdstuk 5, heb ik de relatie tussen ABA en SL biosynthese, door de verdere karakterisering van het β-caroteen isomerase D27 gen, in rijst bestudeerd. De resultaten tonen aan dat, vergeleken met het wildtype, het ABA gehalte in de SL-deficiënte en-ongevoelige *dwerg* (*d*) rijst mutanten, *d10*, *d17* en *d3* is verhoogd, terwijl dit in de *d27* mutant is verlaagd. Bovendien werd dit verschil significant vergroot tijdens blootstelling aan droogte. Interessant genoeg, als gevolg van hun verhoogde ABA niveau, lieten *d10*, *d17* en *d3* planten een verhoogde tolerantie voor droogtestress in vergelijking met wild-type planten zien, terwijl de ABA deficiënte *d27* planten meer droogte gevoelig waren. Transiënte overexpressie van OsD27 in Nicotiana benthamiana verhoogde zowel ABA als SL productie. Constitutieve overexpressie van OsD27 in rijst planten vertoonde geen significante veranderingen in ABA en SL niveaus onder normale omstandigheden. Echter, wanneer planten onder fosfaat tekort werden opgegroeid resulteerde OsD27 overexpressie, in vergelijking met wild-type planten, wel in hogere SL niveaus. Dit suggereert ook dat OsD27 overexpressie alleen tijdens droogte stress in verhoogde ABA niveaus zal resulteren. Aan de hand van deze resultaten heb ik geconcludeerd dat het OsD27 gen zowel betrokken is bij SL als bij ABA biosynthese en dat, afhankelijk van de omgevingsomstandigheden, de expressie van de meer stroomafwaarts gelegen SL en ABA specifieke biosynthetische genen bepaalt welke en hoeveel van de twee hormomen wordt geproduceerd.

In hoofdstuk 6, bespreek ik de belangrijkste bevindingen van dit proefschrift en presenteer ik de toekomst perspectieven van hoe de kennis die in dit proefschrift is verkregen kan bijdragen aan de verbetering van droogte tolerantie en GL in rijst.

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Curriculum vitae

Imran Haider was born in Layyah, Pakistan on 1st July, 1983. After completing higher secondary school education in 2002, he started to study at the University of Agriculture Faisalabad (UAF) of Pakistan, where he obtained his four-year B.Sc. (Hons.) agriculture degree in 2006 with specialisation in Plant Breeding and Genetics. He continued his study in UAF for his M.Sc. (Hons.) agriculture degree and completed his course work. In early 2007, he was awarded an "Overseas MS leading to PhD scholarship" by the Higher Education Commission (HEC) of Pakistan in collaboration with the Netherlands Organisation for International Cooperation in Higher Education (NUFFIC). A few months later, he came to the Netherlands where he joined an MS equivalent educational and research programme at Leiden University. He started his first year with an internship as a Master student in the department of Molecular and Developmental Genetics at the Institute of Biology of Leiden University. He did a research internship entitled "Functional analysis of HD-Zip genes in rice (*Oryza sativa*)" under the supervision of Dr. P.B.F. Ouwerkerk. In late 2009, he relocated to the Laboratory of Plant Physiology of Wageningen University to start his PhD. He continued his MS research topic and worked as a PhD student under the supervision of Prof. Harro J. Bouwmeester, Dr. Carolien P. Ruyter-Spira and Dr. Pieter B.F. Ouwerkerk. The main objective of his research was to get more detailed insight in the molecular regulation of drought tolerance in rice, with a particular focus on the role of drought-responsive HD-Zip family I transcription factors and two groups of plant hormones, abscisic acid and strigolactones. This PhD research work resulted into this thesis.

List of publications

Haider I, Zhang S, Greco R, Casella L, Piffanelli P, Lupotto E, Valè G, Liu C-M, Bouwmeester H, Price AH, Ouwerkerk PBF (2014) Functions of the homeobox gene Oshox22 in controlling grain length in rice (*in preparation for submission*)

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Zhang S, **Haider I**, Kohlen W, Jiang L, Bouwmeester H, Meijer AH, Schluepmann H, Liu C-M, Ouwerkerk PBF (2012) Function of the HD-Zip I gene Oshox22 in ABA-mediated drought and salt tolerances in rice. Plant. Mol. Biol. 80: 571-585

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Education Statement of the Graduate School Experimental Plant Sciences

Issued to: **Imran Haider**
Date: **23 January 2014**
Group: **Plant Physiology, Wageningen University & Research Centre**

1) Start-up phase	*date*
► **First presentation of your project**	
Functional analysis of HD-Zip genes in rice	Jun 19, 2008
► **Writing or rewriting a project proposal**	
Elucidation of the putative role of the rice NCED genes in the production of strigolactones	Apr 02, 2012
► **Writing a review or book chapter**	
Strigolactone biosynthesis and biology, in Molecular Microbial Ecology of the Rhizosphere, Volume 1 (ed F.J. de Bruijn): 355-371	Mar 18, 2013
► **MSc courses**	
Gene Regulation in eukaryotes, Leiden University	Oct 10-Dec 19, 2007
Basic Molecular Biology Techniques, Leiden University	Dec 10-21, 2007
► **Laboratory use of isotopes**	
Safe handling of radioactive isotopes, level 5B, Leiden University	Jan 17-21, 2007
Subtotal Start-up Phase	*15.0 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day, Leiden University	Feb 26, 2009
EPS PhD student day, Utrecht University	Jun 01, 2010
EPS PhD student day, Wageningen University	May 20, 2011
EPS event: ExPectationS career day, Wageningen	Nov 18, 2011
European PhD retreat, John Innes Centre, Norwich, UK	Aug 14-17, 2012
► **EPS theme symposia**	
EPS theme 3 'Metabolism and Adaptation', Amsterdam University	Feb 18, 2009
EPS theme 2 'Interactions between plants and biotic agents', Utrecht University	Jan 15, 2010
EPS theme 1 'Developmental Biology of plants', Wageningen University	Jan 19, 2012
EPS theme 3 'Metabolism and Adaptation', Utrecht University	Apr 26, 2012
► **NWO Lunteren days and other National Platforms**	
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 07-08, 2008
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 06-07, 2009
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 04-05, 2011
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 02-03, 2012
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 22, 2013
► **Seminars (series), workshops and symposia**	
IBL Symposium 2008, Leiden University	Nov 2008
IBL Symposium 2009, Leiden University	Nov 2009
Spotlight scientific biology meetings, Leiden University (10 x per year, 1-2 talks per event)	2009- Mar 2010
Invited seminar: Prof. Dr. Koichi Yoneyama	Jun 25, 2010
Invited seminar: Prof. Dr. Adam Price	Sep 17, 2010

Invited seminar: Dr. Salim Al-Babili	2011
Invited seminar: Dr. Jochem Evers and Dr. Sander van der krol	Oct 11, 2011
Invited seminar: Prof. Dr. Steffen Abel	Mar 20, 2012
Invited seminar: Dr. Inez Hortenze Slamet-Loedin	Jun 29, 2012
Invited seminar: Prof. Dr. Yukihiro Sugimoto	Oct 16, 2012
Invited seminar: Prof. Dr. Ruth Finkelstein	Nov 14, 2012
PP group meeting with Dr. Salim Al-Babili, Freiburg University, Germany	Nov 16, 2012
Invited seminar: Dr. Bas Bouman	2013
► **Seminar plus**	
► **International symposia and congresses**	
American Society of Plant Biologist in Austin, USA	Jul 20-24, 2012
► **Presentations**	
Poster: Summer School Rhizosphere Signalling, Wageningen University	Aug 23-25, 2010
Poster: American Society of Plant Biologist in Austin, USA	Jul 20-24, 2012
► **IAB interview**	Feb 18, 2011
► **Excursions**	
Subtotal Scientific Exposure	*13.6 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
PhD Summer School 'Rhizosphere Signalling', Wageningen University	Aug 23-25, 2010
Bioinformatics: A Users Approach (a practical course), Wageningen University	Aug 30-Sep 03, 2010
► **Journal club**	
Literature discussion: Plant Physiology group, Wageningen University	Apr 2010-May 2013
► **Individual research training**	
Subtotal In-Depth Studies	*5.4 credits**

4) Personal development	*date*
► **Skill training courses**	
Why and how scientists sell science, Wageningen	Oct 28 2010
Techniques for writing and presenting a scientific paper, Wageningen	Dec 14-17, 2010
Workshop: Digital Art, Austin, USA	Jul 21, 2012
Career Perspectives, Wageningen	Sep 20-Oct 25, 2012
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3.2 credits**

TOTAL NUMBER OF CREDIT POINTS*	**37.2**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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Colophon

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