

Regulation of adventitious root system architecture in response to flooding in rice (*Oryza sativa* L.)

By

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1. Abbreviations

1-MCP	methylcyclopropene
2,4-D	2,4-dichlorophenoxyacetic acid
ABA	abscisic acid
ABCB	ATP-Binding Cassette family B
AIM1	ABNORMAL INFLORESCENCE MERISTEM1
α -NAA	1-naphthalene acetic acid
ANOVA	analysis of variance
ARF	AUXIN RESPONSE FACTOR
ARs	adventitious roots
B	blue light
CKX	cytokinin oxidase/dehydrogenase
CLSM	confocal laser scanning microscopy
CPT1	Coleoptile Phototropism1
CRL1/ARL1	CROWN ROOTLESS1/ADVENTITIOUS ROOTLESS1
CRL4	CROWN ROOTLESS4
CRL5	CROWN ROOTLESS5
CT	X-ray Computer Tomography
D	darkness
DRO1	DEEPER ROOTING 1
EIL1	EIN3-LIKE 1
EIN3	ETHYLENE INSENSITIVE3
FR	far-red light
GA	gibberellic acid
GSA	gravitropic setpoint angle
GUS	β -glucuronidase
IAA	indole-3-acetic acid
IPA	indole-3-pyruvate
LOB	LATERAL ORGAN BOUNDARIES
MRI	Magnetic Resonance Imaging

ABBREVIATIONS

MT2b	METALLOTHIONEIN 2b
N	Nitrogen
NAL1	NARROW LEAF1
NBT	Nitro Blue Tetrazolium
NIL	nearly isogenic line
NPA	naphthylphtalamic acid
NPH3	NONPHOTOTROPIC HYPOCOTYL3
NRL	NPH3/RPT2-like
P	Phosphate
PAT	polar auxin transport
PCD	programmed cell death
PG56	Pin Gaew 56
PIN	PIN-FORMED
PINE1	PRE MATURE INTERNODE ELONGATION1
QTL	quantitative trait locus
R	red light
RBOH	respiratory burst oxidase homolog
RNAi	RNA interference
ROS	reactive oxygen species
RR	response regulator
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SA	salicylic acid
SD1	SEMIDWARF1
SK1	SNORKEL1
SK2	SNORKEL2
SOR1	Soil-surface Rooting1
T65	Taichung 65
Trp	tryptophan
WL	white light
WL+E	white light with 150 μ M ethephon
WOX11	Wuschel-related homeobox 11

ABBREVIATIONS

TWD1 TWISTED DWARF 1

ZH11 Zhonghua 11

2. Table of Contents

1	Abbreviations	I
2	Table of Contents.....	V
3	Zusammenfassung	1
4	Summary	3
5	Introduction.....	5
	5.1 Control of root system architecture by phytohormones and environmental signals in rice.....	6
6	Chapter 1	18
	6.1 Control of adventitious root architecture in rice by darkness, light and gravity	19
7	Chapter 2	34
	7.1 Polar auxin transport determines adventitious root emergence and growth in rice.....	35
8	Chapter 3	51
	8.1 QTL12 with the ethylene response transcription factor genes <i>SNORKEL1</i> and <i>SNORKEL2</i> promotes adventitious root formation and growth in rice.....	52
9	Discussion and perspectives.....	94
	9.1 The establishment of a secondary root system in plants during submergence	94
	9.1.1 The generation of a secondary root system is beneficial for plants to survive flooding.....	94
	9.1.2 Adaptive traits in response to flooding are genetically linked	95
	9.2 AR initiation and AR emergence are possibly controlled by different phytohormone	96
	9.2.1 Auxin signalling pathway or polar auxin transport probably mediate the de novo genesis of AR primordia	96
	9.2.2 SKs mediate the ethylene-induced AR growth during submergence	98
	9.2.3 SKs possibly promote AR formation through auxin or cytokinin signalling pathways	99
	9.3 The AR system architecture in rice is controlled by genetic factors and environmental cues	100

TABLE OF CONTENTS

9.3.1 Novel technologies are helpful to study and optimize root system architecture	100
9.3.2 Light rather than gravity is the main regulator governing the AR angle in rice	101
9.3.3 Polar auxin transport is crucial for the determination of AR angle	103
9.3.4 Optimization of the root system architecture to support growth in cereals	104
10 References	105
11 Acknowledgements	110
12 Curriculum Vitae.....	111
13 Declaration of author contributions	113

3. Zusammenfassung

Überflutung ist eine starke Umweltbelastung, die die Ernteerträge weltweit beeinträchtigt. Reis (*Oryza sativa* L.) ist im Gegensatz zu den meisten anderen Nutzpflanzen eine semi-aquatische Pflanze mit einer hohen Überflutungstoleranz. Das gasförmige Phytohormon Ethylen reichert sich in überfluteten Reispflanzen an und steuert eine Reihe von Anpassungsreaktionen. In Tiefwasserreis, der mehrere Monate lang stark überflutet wird, induziert Ethylen eine Streckung der Internodien und das Wachstum von Adventivwurzeln (AR). Das Stängelwachstum hilft überfluteten Pflanzen, ihre Blätter über dem Wasser zu halten. In Reis bilden sich konstitutiv AR Primordien an den Nodien aus, die bei Überflutung auswachsen und beschädigte oder tote Wurzeln ersetzen. Das AR-Wachstum, das mit dem programmierten Tod von darüberliegenden Epidermiszellen einhergeht, wird durch Ethylen vermittelt, das sich in überfluteten Pflanzenteilen anreichert. ARs unterstützen oder ersetzen das Hauptwurzelsystem, indem sie Wasser, Nährstoffe und Sauerstoff aus dem Wasser aufnehmen. Der Tiefwasserreis C9285 besitzt drei *quantitative trait loci* (QTLs), die Gene für Überflutungstoleranz enthalten. QTL12 enthält zwei Gruppe VII Ethylen Response Faktor (ERF) Transkriptionsfaktorgene, *Snorkel1* (*SK1*) und *Snorkel2* (*SK2*), von denen gezeigt wurde, dass sie das schnelle Stängelwachstum induzieren. In dieser Arbeit wurde gezeigt, dass QTL12 auch die Bildung und das Wachstum von AR bei Überflutung fördert. Neben den typischen ARs am Knoten entwickeln sich ARs auch an der Basis des Internodiums. Beide AR Typen unterscheiden sich morphologisch, die AR, die sich am Internodium entwickeln sind deutlich dicker und besitzen mehr Zellschichten. *EIN3-LIKE 1* (*OsEIL1*) ist ein Transkriptionsfaktor, der *SKs* reguliert. Die Überexpression von *OsEIL1* in Nicht-Tiefwasserreispflanzen zeigte einen geringen Einfluss auf die AR-Bildung und das Wachstum, was darauf hindeutet, dass *SKs* für die Wurzelentwicklung essentiell sind. Der Spiegel an reaktiven Sauerstoffspezies (ROS) in der nahezu isogene Linie NIL-12 ist im Vergleich zu Wildtyp-Pflanzen signifikant höher, was darauf hinweist, dass *SKs* die AR-Bildung und das Wachstum möglicherweise durch Hochregulieren des ROS-Spiegels vermitteln.

Unter natürlichen Bedingungen wachsen Reispflanzen in einem Tag/Nacht-Zyklus. Hochwasser kann jedoch trüb sein, wodurch die Lichtintensität unter Wasser geringer ist und

die Photosyntheserate unter Wasser verringert ist. Eine reduzierte Photosyntheserate bedeutet, dass unter Wasser weniger Sauerstoff produziert wird, was den Sauerstoffmangel in den Wurzeln verstärkt. Diese Arbeit zeigt, dass Dunkelheit nicht nur den Metabolismus verändert, sondern auch eine tiefgreifende Auswirkung auf die Entwicklung des Wurzelsystems hat. Im Dunkeln wird das Wachstum von ARs induziert, wobei die Wurzeln nach oben wachsen. Einen vergleichbaren Wachstumswinkel weisen Wurzeln auf, die im Boden wachsen, was darauf hindeutet, dass dieses Wachstumsverhalten für die Pflanze vorteilhaft ist. Schwerkraft ist ein Umweltfaktor, der die Wachstumsrichtung von Organen beeinflusst. Ein Klinostat-Experiment zeigte, dass der Wachstumswinkel bei fehlender Schwerkraftrichtung etwas abgeflacht war, was zeigt, dass die Adventivwurzeln antigravitrop wachsen. Hellrotes, dunkelrotes und blaues Licht induzieren ein negativ-phototropes Wurzelwachstum, wobei blaues Licht am effektivsten ist. *CPT1 (Coleoptile Phototropism1)* ist eine Komponente des Phototropinsignalweges. *cpt1* Pflanzen zeigten eine verminderte Empfindlichkeit gegenüber blauem Licht, was darauf hinweist, dass Phototropin das Blaulichtsignal vermittelt. Darüberhinaus wird die Adventivwurzelarchitektur durch die Rotlichtzeptoren Pr und Pfr reguliert. Die Richtung des AR-Wachstums wird somit genetisch und durch die Umweltfaktoren Licht und Schwerkraft gesteuert.

Auxin ist bekannt dafür, dass es die Bildung von Seitenwurzeln fördert. In dieser Arbeit wurde gezeigt, dass der Auxin-Transportinhibitor Naphthylphthalaminsäure (NPA) das Auswachsen von Adventivwurzeln unterdrückt. Die Expression von Genen, die für Auxin-Efflux-Carrier kodieren, wurde mit *OsPIN:β-Glucuronidase*-Reporterlinien untersucht. *OsPIN1b* und *OsPIN1c* werden in der Wurzelhaube und in der Stele exprimiert, was auf eine Rolle bei der Wurzelentwicklung hindeutet. *OsPIN2* wird in der Epidermis über den Wurzelprimordien und in der Wurzelspitze exprimiert. Der Auxinreporter *DR5:VENUS*, nimmt in wachsenden Wurzelprimordien zu und nimmt in Epidermiszellen über den Primordien ab, die einen programmierten Zelltod erleiden, und damit das Auswachsen der Primordien erleichtern. Zusammengefasst deuten die Ergebnisse darauf hin, dass lokale Auxingradienten das Auswachsen, die Wachstumsrate und die Wachstumsrichtung von ARs bestimmen und dass gerichteter Auxintransport die Architektur des AR-Systems wesentlich mitbestimmt.

4. Summary

Flooding is a severe environmental stress that lowers crop production worldwide. Unlike most other crops, rice is a semi-aquatic plant with high flooding tolerance. The gaseous hormone ethylene accumulates in flooded rice plants and controls a number of adaptive responses. In deepwater rice (*Oryza sativa* L.), that get highly flooded for several months, ethylene induces internodal elongation and adventitious root (AR) growth. Stem growth helps flooded plants to keep their leaves above the water. In rice, ARs are constitutively formed and emerge at the nodes of flooded plants to replace damaged roots. AR growth is mediated by ethylene that gets trapped inside submerged plant parts and is coordinated with programmed death of epidermal cells that cover root primordia. ARs support or replace the main root system by taking up water, nutrients and oxygen from flood waters and by providing anchorage. The deepwater rice variety C9285 harbours three main quantitative trait loci (QTLs) that confer flooding tolerance. QTL12 includes two group VII ERF (ethylene response factor) transcription factor genes, *Snorkel1* (*SK1*) and *Snorkel2* (*SK2*) that were shown to induce rapid stem elongation. This study showed that QTL12 also promotes AR formation and growth upon submergence. Aside from the typical ARs that form at the node, a second type of ARs develop at the base of the internode and these two types of roots are morphologically different. *EIN3-LIKE 1* (*OsEIL1*) is a transcription factor, that directly targets the SK genes. The overexpression of *OsEIL1* in nondeepwater rice plants lacking SK genes shows a minor effect on AR formation and growth indicating that SKs are essential for the enhanced formation of ARs. ROS levels were significantly higher in the near-isogenic line NIL-12 compared with wildtype plants suggesting that Snorkels may mediate AR formation and growth by upregulating the ROS level. In brief, the data revealed a major role for QTL12 in AR development.

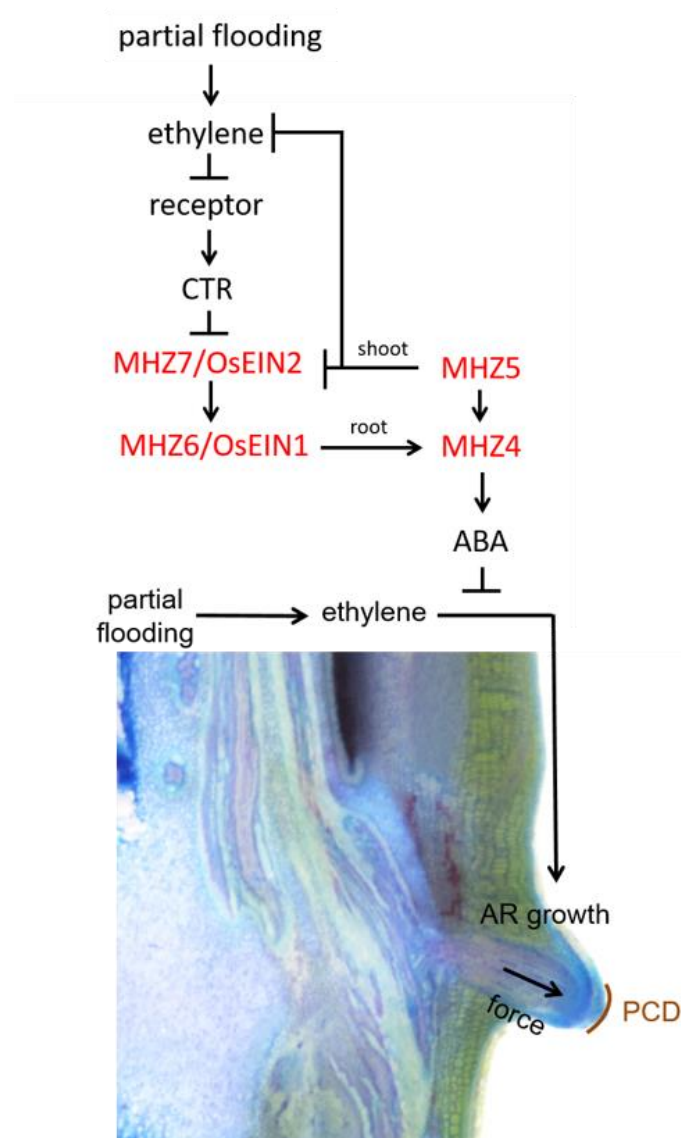
Naturally, rice plants grow in a light/dark cycle. However, flood water can be turbid thereby reducing underwater light intensity and underwater photosynthesis even in the daytime. Reduced photosynthesis means that less oxygen is produced underwater making oxygen shortage of roots more severe in the dark. We describe that darkness not only alters underwater metabolism but has a profound developmental effect on the root system. In the dark, growth of ARs is induced at stem nodes of deepwater rice with roots growing at an

upward angle. A similar AR growth pattern is observed in soil suggesting that this root behaviour is advantageous to the plant. Gravity is one of several environmental factors that are integrated to adjust the angle of root growth which, in turn, determines the soil or water layers that will be explored by these roots. Clinostat experiments revealed that gravity increased the root angle suggestive of an antigravitropic growth component. Red, far-red and blue light induced a negative phototropic response of ARs, with blue light as the most effective light signal. *cpt1* (coleoptile phototropism1), a component of phototropin signaling, showed a decreased sensitivity to blue light indicating that phototropin and phytochrome signalling regulate the root system architecture. The AR growth angle is thus controlled genetically and by the environmental cues light and gravity.

The auxin transport inhibitor naphthylphthalamic acid (NPA) effectively repressed root emergence. The expression of auxin efflux carrier genes was investigated by using *OsPIN::β-glucuronidase* reporter lines. *OsPIN1b* and *OsPIN1c* were active in the root cap and root stele suggestive of a role in root development. *OsPIN2* was specifically expressed in epidermal cells above the root primordia and at a lower level in the root tip indicating a role for polar auxin transport in AR emergence. Auxin activity as detected with the *DR5::VENUS* reporter increased in the root primordia during AR emergence and decreased in epidermal cells above ARs that undergo programmed cell death prior to AR emergence. The results indicate that local auxin gradients determine emergence, growth rate and growth direction of ARs revealing a deep impact of controlled auxin transport on the overall architecture of the AR system.

5. Introduction

Control of root system architecture by phytohormones and environmental signals in rice



Control of root system architecture by phytohormones and environmental signals in rice

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ABSTRACT

Drought and flooding are environmental extremes and major threats to crop production. Water uptake is achieved by plant roots which have to explore new soil spaces to alleviate water deficit during drought or to cope with water excess during flooding. Adaptation of the root system architecture helps plants cope with such extreme conditions and is crucial for plant health and survival. While for dicot plants the well studied model plant *Arabidopsis thaliana* has provided insight into the genetic and molecular regulation of the root system, less information is available for monocot species, which include the agronomically important cereal crops. Rice (*Oryza sativa* L.) is a semi-aquatic monocot plant that develops strong tolerance to flooding. Flooding tolerance of rice is closely linked to its adaptive root system. The functional root system of rice is mainly composed of crown roots and is shifted to nodal adventitious roots during flooding which allows rice to maintain oxygen supply to the roots and to survive longer periods of partial submergence as compared with other crops. Likewise, a number of drought-tolerance traits of rice are the result of an altered root system architecture. Hence, the structure of the root system adapts to, both, flooding and drought. Understanding the regulatory mechanisms that control root system adaptation to extreme environments is a key task for scientists to accelerate the breeding efforts for stress-tolerant crops. This review summarizes recently identified genes and molecular mechanisms that regulate root system architecture in rice in response to drought and flooding.

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Rice; adventitious roots; root system architecture; flooding; drought; crown rootless mutants; mao hu zi mutants

Introduction

The necessity for food production is growing with a worldwide increasing population. The World Bank pointed out that 83 million people in 45 countries were starving in 2017 (Prosekov and Ivanova 2018). A current global problem that urgently needs to be addressed is to satisfy the demand for grain yield in view of climatic changes and in view of less usable water and less cultivable land in the future. Among the approaches that are taken, improvement of crop production under adverse and extreme conditions is thought to be effective and reasonable in the present stage (Mickelbart et al. 2015). Extreme weather conditions, causing flooding and drought, are threatening crop growth, eventually resulting in loss of grain yield at harvest. Rice (*Oryza sativa* L.) is an important cereal crop that provides the main source of food for many people in Asia and Africa. Identifying the crucial traits responsible for stress resistance, and breeding rice

varieties with these traits such that plants thrive under extreme conditions, are major tasks for scientists.

Roots are indispensable for autotrophic plants. The functional root system anchors the plant, facilitates water uptake, acquisition of mineral nutrients, and engages in the formation of plant-microorganism communities in the rhizosphere (Orman-Ligeza et al. 2013). Roots are most prone to flooding conditions, and are faced with the necessity to ensure water and nutrient supply under oxygen-deficient conditions that limit ATP synthesis (Sauter 2013). The root system adapts to low oxygen and other abiotic stresses as well as to pathogenic and beneficial organisms. Root system architecture describes the three-dimensional shape and the spatial arrangement of roots in soil. The root system is made up of the primary root, lateral roots and adventitious roots (ARs). The three-dimensional root system architecture is determined by the position of the roots, root numbers, root lengths and the root

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growth angles (Rogers and Benfey 2015). The root system is regulated by both, genetic factors and environmental signals and is continuously adapted to exploit the soil for water and nutrient resources under given and oftentimes changing environmental conditions.

Substantial progress has been made in understanding the development of lateral roots and root growth regulation in the dicot model plant *Arabidopsis*. In contrast to *Arabidopsis*, the root system of cereal plants is composed mainly of ARs. Even though some physiological and molecular mechanisms that control lateral and AR development are shared, there are also root type-specific or species-specific differences that reflect the phylogenetic plant origin or the adaptation to an ecological niche. As a monocot model plant with the advantage of a relatively small-sized genome, rice is widely used in molecular-genetic studies. Several rice genomes have been sequenced to date (Wang et al. 2018) providing genomic information that help in the characterization of gene functions and in transcriptome studies. Efficient transformation systems, mutant collections and the generation of introgression lines further aid in functional gene analysis. These tools have led to the identification and functional characterization of a number of genes with a role in root development and growth in recent years (Meng et al. 2019). Nonetheless, the molecular network that regulates AR formation, growth and spatial orientation is still largely unknown. An exciting challenge in the future will be to decipher the molecular control of the root system architecture and dissect functional genes for breeders to improve crop tolerance to biotic and abiotic stress. This review focuses on our current understanding of the regulatory mechanisms and molecular players associated with AR system development in rice. In addition, ideotypes are proposed for root system architecture in response to drought and flooding conditions for the aim of further research.

Types of roots in rice system architecture

Root systems consist of embryonic and postembryonic roots (Fig. 1). Unlike many dicot plants that rely on the primary root and branching lateral roots, most cereal plants, including rice, wheat and barley, form a dense fibrous root system made up of crown roots and nodal ARs (Rebouillat et al. 2008; Coudert et al. 2010). Rice seeds germinate with a thick embryonic radicle. During seedling development, four or five crown roots

grow from the coleoptile node (Fig. 1A). These seedling roots play an important role in exploring and monitoring soil conditions (Sauter 2013). At the postseedling stage, AR primordia develop at the stem nodes to eventually form the mature rice root system. ARs are commonly classified into underground and above ground ARs. It depends on the ecotype and on the environment whether ARs are above or under ground. In general, lowland rice produces more underground ARs and few above ground ARs. Deepwater rice is adapted to grow in areas with fluctuating water levels to which they adapt through stem growth and the development of ARs from above ground stem nodes. In addition, ARs form lateral roots to enlarge the area that is explored by the root system (Fig. 1B). Lateral ARs can be categorized into large lateral and small lateral roots, that display different root lengths (Rebouillat et al. 2008). Hence, the different root types contribute to the root system architecture in rice.

Crown and adventitious root formation and growth in rice is controlled by phytohormones

Root formation and growth in rice is mediated by phytohormones. Auxin and cytokinins are essential for the *de novo* genesis of root primordia. But recent reports also describe the involvement of other hormones, such as abscisic acid (ABA) and gibberellic acid (GA), in root formation and growth.

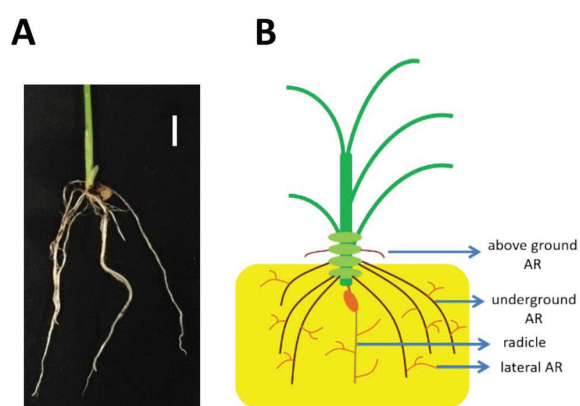


Figure 1. Root types and schematic root system architecture in rice plants. (A) Root system of a 10-day-old rice seedling of the cultured variety Nipponbare (bar = 1 cm). (B) Scheme of a mature rice root system in soil. The root system architecture in rice is mainly composed of the radicle with lateral roots, above ground adventitious roots (AR), underground ARs and lateral ARs.

Auxin

Previous studies have extensively reported on the role of auxin on the *de novo* genesis of ARs. In plants, auxin is synthesized mainly through the highly conserved TAA/YUC pathway (Zhao 2012). Indole-3-pyruvate (IPA) is produced by tryptophan (Trp) aminotransferase of Arabidopsis (TAA) from Trp and converted to natural indole-3-acetic acid (IAA) by YUCCA flavin monooxygenases through oxidative decarboxylation (Mashiguchi et al. 2011; Won et al. 2011). Overexpression of *OsYUCCA1/OsYUC1* in rice plants results in more crown root formation (Yamamoto et al. 2007; Zhang et al. 2018). Similarly, overexpression of *OsYUC3*, *OsYUC5*, *OsYUC6*, *OsYUC7*, *OsYUC8* and *OsYUC11* promotes crown root initiation indicating that the YUCCA gene family universally regulates root formation by controlling the biosynthesis of endogenous auxin. The disruption of *TAA1* resulted in reduced crown root formation, supporting the crucial role of auxin in AR formation in rice (Zhang et al. 2018).

Rice *CROWN ROOTLESS 1/ADVENTITIOUS ROOTLESS 1* (*CRL1/ARL1*), a plant-specific *LATERAL ORGAN BOUNDARIES* (*LOB*) family member, is essential for AR initiation. It is auxin-regulated and acts downstream of auxin response factors (ARFs) (Inukai et al. 2005; Liu et al. 2005). A number of additional crown rootless mutants were identified, some of which have been functionally characterized and shown to be involved in auxin-controlled AR initiation and growth. *CRL4/OsGNOM1* is a guanine nucleotide exchange ADP-ribosylation factor that controls cycling and polar localization of the auxin efflux carrier PIN1 (Kitomi et al. 2008; Liu et al. 2009). *crl4* plants display reduced crown root and lateral root numbers together with impaired gravitropism. *CRL5*, an AP2/ERF transcription factor, is regulated by auxin through ARF transcription factors. It promotes crown root formation through inhibition of cytokinin signaling (Kitomi et al. 2011). *CRL6* encodes a protein from the large chromodomain, helicase/ATPase, and DNA-binding domain (CHD) family. Root initiation and development are under the control by *CRL6* through downregulation of *OsIAAs*, transcriptional repressors of *ARF* genes in the auxin signaling pathway (Wang et al. 2016). Although *CRL2* and *CRL3* mutants were shown to have fewer crown roots, their gene functions have yet to be elucidated (Inukai et al. 2001; Kitomi et al. 2008). The auxin efflux carrier protein *OsPIN1* mediates auxin-dependent AR emergence. In *OsPIN1* RNA interference (RNAi) plants, AR emergence is reduced,

a phenotype that is partly reverted by application of the synthetic auxin α -naphthylacetic acid (α -NAA) (Xu et al. 2005). *OsPIN1:: β -galactosidase* reporter gene analysis revealed expression of *OsPIN1b* and *OsPIN1c* in growing AR primordia and local expression of *OsPIN2* in epidermal cells above AR primordia supporting the idea that polar auxin transport contributes to AR growth and possibly to death of epidermal cells that cover AR primordia which facilitates AR emergence (Steffens and Sauter 2005; Lin and Sauter 2019).

The Ca^{2+} -independent Ser/Thr kinase gene *OsRPK1* functions as a negative regulator of AR development by inhibiting polar auxin transport via PIN proteins. Knockdown of *OsRPK1* promoted AR initiation (Zou et al. 2014). The *Aux/IAA* gene *OsIAA23* is required for the auxin-mediated maintenance of the quiescent center in postembryonic roots. *Osiaa23* mutants have fewer crown and lateral roots compared with the wild-type (Jun et al. 2011). *NARROW LEAF 1* (*NAL1*) encodes a putative trypsin-like serine/cysteine protease. Loss of *NAL1* results in a decreased number of ARs, a phenotype that was rescued by exogenous auxin, suggesting that *NAL1* also controls AR growth through the auxin signaling pathway as was shown for several crown rootless mutants (Cho et al. 2013). Moreover, *OsCAND1*, that is responsible for maintaining the G2/M cell cycle transition, regulates crown root formation through the control of the endogenous auxin level. *Oscand1* shows an abnormal crown root formation that could be partly compensated by exogenous auxin (Wang et al. 2011). Additionally, a point mutation of *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3* (*OsSPL3*) repressed the auxin signaling pathway, resulting in a rootless phenotype further supporting the conclusion that auxin plays a crucial role in AR formation in rice (Shao et al. 2019).

Cytokinins

Growing evidence demonstrates the involvement of cytokinins in AR emergence and growth in rice. Cytokinin receptors activate two types of primary response regulators (RRs), designated type-A and type-B RRs. Type-A RRs act as negative regulators whereas type-B RRs have activating activity. In the presence of cytokinin, type-A RR genes display distinct expression patterns suggestive of specific functions of OsRR proteins in response to cytokinin (Gao et al. 2014). Rice type-A RR6 negatively regulates shoot and root growth. Overexpression of *RR6* results in a severe rootless phenotype (Hirose et al. 2007). *OsCKX4*, a cytokinin oxidase/dehydrogenase

(CKX) family gene, is targeted by auxin response factor OsARF25 and the type-A response regulators OsRR2 and OsRR3. Overexpression of *OsCKX4* in rice promotes crown root formation (Gao et al. 2014). Finally, *CRL5* is a positive regulator of crown root formation that acts by repressing cytokinin signaling (Kitomi et al. 2011).

The *WUSCHEL*-related homeobox gene *WOX11* is expressed in emerging ARs. It maintains cell division in the root apical meristem. In rice, *wox11* knockout plants and *wox11*-RNAi lines show reduced crown root formation, whereas overexpression of *WOX11* results in ectopic AR growth, indicating that *WOX11* is essential for the development of crown root formation in rice (Zhao et al. 2009). The AP2/ERF protein ERF3 interacts with *WOX11* and co-regulates crown root development. Knockdown of *ERF3* in a *wox11* mutant background showed a more severe rootless phenotype whereas overexpression of *ERF3* partially rescued the *wox11* phenotype. Both, *WOX11* and *ERF3* target the type-A *RR2* gene that regulates crown root initiation in response to cytokinin (Zhao et al. 2015). *WOX11* also recruits an ADA2-GCN5 histone acetyltransferase module to regulate root-specific genes involved in cell division and root meristem activity (Zhou et al. 2017) suggesting that AR formation is also regulated at the epigenetic level. Additional evidence suggests that the TAA/YUC pathway functions together with *WOX11*, indicating that auxin and cytokinin signaling pathways merge at the level of *WOX11* (Zhao et al. 2009; Zhang et al. 2018).

Ethylene and abscisic acid

Unlike auxin and cytokinin, that are involved in the *de novo* genesis of rice roots, ethylene represses radicle root growth, and was shown to promote AR emergence and growth in rice. The ethylene signaling pathway of rice remained unexplored for a long time due to the lack of mutants (Rzewuski and Sauter 2008). The transcription factor *ETHYLENE INSENSITIVE 3* (*EIN3*) is a key regulator of ethylene signaling in Arabidopsis. The homologous gene *OsEIL1* of rice, when expressed in the Arabidopsis *ein3* mutant background, partially compensated the short-root phenotype (Mao et al. 2006) suggesting that key signaling elements are conserved between dicot and monocot plants. Nonetheless, specific regulatory mechanisms in ethylene signaling of root development need yet to be elucidated. The ethylene response factor gene *OsERF2* regulates the levels of ethylene and abscisic acid (ABA) and is essential

for embryonic root growth (Xiao et al. 2016). In recent years, a number of rice mutants were identified that develop more ARs (Yin et al. 2018). These mutants were termed *mao hu zi* (*mhz*), which means 'cat whiskers' (Chen et al. 2018). Several of the corresponding *mhz* genes were identified through map-based cloning and their functions were studied (Fig. 4A). *MHZ7* is the rice homolog of Arabidopsis *EIN2* (Merchante et al. 2013; Li et al. 2015). *MHZ7:GUS* reporter analysis revealed expression of *MHZ7* in both the embryonic root and in ARs. Overexpression of *MHZ7* led to a short, twisted radicle in rice seedlings (Ma et al. 2013). *MHZ6* encodes a homolog of Arabidopsis *EIN1*. Loss of *MHZ6* results in roots that are insensitive to ethylene (Yang et al. 2015). *MHZ7* and *MHZ6* are considered central components in the ethylene signaling pathway based on the crucial role of orthologous genes *EIN2* and *EIN1* in Arabidopsis. *MHZ3* is an endoplasmic reticulum-localized membrane protein that is required for the stabilization of *MHZ7* (Ma et al. 2018). *MHZ5* encodes a carotenoid isomerase involved in carotenoid biosynthesis. *MHZ5* gene expression was induced by ethylene and results in ABA accumulation in the root, which, in turn, inhibits root growth. *MHZ4* is homologous to *ABA4* of Arabidopsis, that was previously shown to regulate the interplay between ethylene and ABA. Overexpression of *MHZ4* enhanced the ethylene sensitivity of seedling roots and increased the endogenous ABA level. Disruption of *MHZ4* induced AR formation at the nodes (Ma et al. 2014). ABA promotes expression of *MAIF1* that codes for an F-box domain protein localized at the plasma membrane and in the nucleus. Ectopic overexpression of *MAIF1* enhanced root growth (Yan et al. 2011). Taken together, these findings suggest that *MHZ4* and *MHZ5* mediate the interaction between ethylene and ABA pathways in regulating root growth in rice (Yin et al. 2015). The mutant analysis revealed molecular components of the ethylene-ABA crosstalk that was previously described at the physiological level (Steffens et al. 2006).

Gibberellic acid and salicylic acid

The hormonal network that regulates AR development and growth in rice includes additional hormones but the molecular mechanisms of their activities have not been exhaustively studied. The AP2/ERF transcription factor *OsSHB* targets the gene *KS1*, which encodes the entkaurene synthase of gibberellic acid (GA) biosynthesis

controlling proliferation and elongation of root meristem cells. *Osshb* knockout plants have fewer and shorter roots compared with the wildtype (Li et al. 2015) supporting previous physiological studies that revealed enhanced AR growth in the presence of GA (Steffens et al. 2006). The 3-hydroxyacyl-CoA dehydrogenase *ABNORMAL INFLORESCENCE MERISTEMI 1* (*AIM1*) controls root mitotic activity in rice by promotion of salicylic acid (SA) synthesis and repression of redox-related genes and of genes involved in scavenging of reactive oxygen species (ROS), with elevated ROS levels as a result (Xu et al. 2017).

The growth angle of rice ARs is determined by genetic factors and by the environment

Root system architecture is determined by the number and position of roots, the growth rates and the root growth angles. The functional root system adjusts these parameters to optimize exploitation of the soil and to ensure uptake of nutrients and water in an inhomogeneous soil substrate or when soil parameters change. Analysis of root systems in their natural underground environment is difficult. Non-destructive 3D-imaging techniques such as Magnetic Resonance Imaging (MRI) and X-ray Computer Tomography (CT) elegantly address this issue and allow visualization of entire root systems in soil with a high resolution (Metzner et al. 2015). In general, roots grow toward higher water potential, nutrients and oxygenated areas. By contrast to the soil-borne root system, nodal ARs that emerge upon flooding grow in an aquatic environment where other environmental cues become important.

One such factor is light, which regulates growth of above-ground ARs and of roots that grow at the soil surface. In rice, above-ground ARs grow upward in the dark whereas in the light, ARs grow downward (Lin and Sauter 2018). The AR growth angle is determined by far red, red and blue light revealing negative phototropism controlled by phytochrome (Pr and Pfr) and blue light signaling likely through phototropin (Lin and Sauter 2018). Roots are sensitive to gravity with primary roots displaying positive gravitropism whereas lateral and ARs grow at an angle that is genetically determined, the gravitropic setpoint angle, and also subject to regulation by environmental cues (Baldwin et al. 2013; Rogers and Benfey 2015). Non-vertical root

growth is the result of differential growth rates at opposite sides caused by an asymmetric auxin distribution across the root tip. *DEEPER ROOTING 1* (*DRO1*) is an auxin-regulated gene that promotes a more vertical root growth angle resulting in an overall deeper root system. *DRO1* is expressed in the cell elongation zone at the root tip and is responsive to gravity (Uga et al. 2013).

It is well established that polar auxin transport contributes to asymmetric auxin distribution in the root (Petrasek and Friml 2009; Adamowski and Friml 2015). PIN-formed (PIN) proteins are auxin efflux carriers localized at the plasma membrane that are responsible for auxin efflux from the cell. PIN efflux carriers are key determinants of directed auxin transport in the plant and for generating auxin gradients. In Arabidopsis, PIN1 drives acropetal auxin transport in the central cylinder of roots. PIN3, PIN4 and PIN7 redirect the auxin flux in the root cap to the peripheral cell layers while PIN2 directs the basipetal auxin transport toward the root base. PIN5, PIN6 and PIN8 are located at the endoplasmic reticulum and their functions in roots are still largely unknown (Adamowski and Friml 2015). In rice, at least 12 PIN proteins have been predicted based on homologies to Arabidopsis PINs (Fig. 2A). OsPIN1a-d are the rice homologs of AtPIN1 and OsPIN2 is homologous to AtPIN2. AtPIN5 has 3 homologs in rice, OsPIN5a, OsPIN5b and OsPIN5c. OsPIN10a and OsPIN10b are most related to PIN3, PIN4 and PIN7. So far, the functions of OsPINs in determining the root growth angle is largely unclear. The auxin flux mediated by PIN proteins in the rice root can be predicted based on these homologies and the functional characterization of PINs in Arabidopsis (Fig. 2B). OsPINs show different expression patterns in the radicle and in above-ground ARs (Wang et al. 2009; Lin and Sauter 2019). Despite the fact that AR growth direction is largely determined by light, light did not much alter *PIN* gene expression in ARs. It is conceivable that OsPIN localization and activities are regulated through posttranscriptional mechanisms (Eysholdt-Derzso and Sauter 2017; Lin and Sauter 2019). The *OsPIN2* mutant exhibited decreased sensitivity to gravity and showed an increased root growth angle (Wang et al. 2018) suggesting that the gravitropic setpoint angle is in part determined by polar auxin transport via OsPIN2.

In addition to efflux carriers, the auxin influx carrier gene *OsAUX1* also positively regulates the root growth

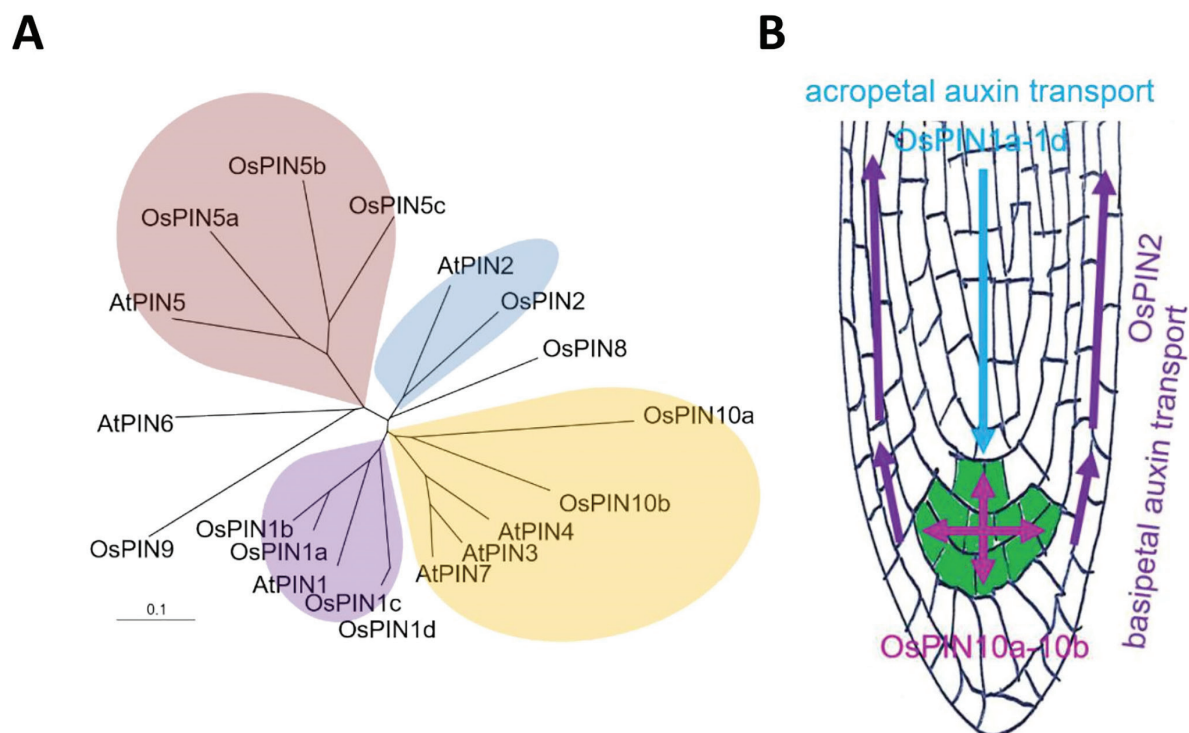


Figure 2. Phylogenetic relationship of PIN proteins in Arabidopsis and rice and predicted polar auxin transport (PAT) in the rice root. (A) Phylogenetic analysis of PIN family members in rice and Arabidopsis. A multiple sequence alignment was carried out with Clustal Omega and a phylogenetic tree was generated using Treeview; bar = 0.1. (B) Predicted schematic PAT through rice PIN proteins.

angle. *Osaux1* plants have a shallower root system than wild type. In addition, *OsAUX1* promotes root hair elongation thereby improving the phosphate acquisition in low phosphate conditions. *OsAUX1* plays an important role in auxin transport from the root apex to the differentiation zone (Giri et al. 2018). In summary, investigations of the molecular mechanisms that control the root growth angle in rice have just begun, which holds true also for other cereal crops. Understanding the regulatory network that controls the root growth angle will be a prime task for future research and will help design cereal root systems adapted to a challenging environment.

Changes in root system architecture in response to drought

Drought causes grain yield loss due to water deficit in the plant (Atkinson and Urwin 2012). Severe or long-lasting drought renders the root system damaged or dysfunctional which limits the uptake of water and

nutrients or makes it impossible (Rogers and Benfey 2015). For the benefit of the plant and with regard to agricultural yield, an adjustable root system that confers drought tolerance is a desirable trait.

A number of transcription factors have been studied that enhance plant survival under drought conditions. Genes of the NAC domain family of rice have received significant interest due to their specific roles in drought adaptation of the root system (Ooka et al. 2003). Plants that express *NAC9* under a root-specific promoter, acquire thicker roots with an enlarged stele and large aerenchyma. Several genes related to root morphogenesis as well as cell elongation and lignin and suberin biosynthesis for walls of the rhizodermis were found to be upregulated in the root of *NAC9* overexpressed transgenic plants through microarray analysis (Redillas et al. 2012). The morphological changes might account for the improved grain yield under drought conditions. Similarly, *OsNAC10* expression driven by a root-specific promoter caused an increase in root volume, length, dry weight, and diameter together with enhanced

grain yield, both, in non-stressed and drought conditions (Jeong et al. 2010). *SNAC1* is a stress-related NAC family member, that is involved in the adaptation to drought and salt stress. When overexpressed in cotton (*disambiguation*), *SNAC1* produced more roots compared with wildtype plants (Liu et al. 2014). Similarly, *NAC045* also confers drought tolerance in rice plants (Zheng et al. 2009), even though the root phenotypes have not been described yet. In summary, NACs confer drought tolerance mainly by increasing the root volume, both, in length and diameter, and by changing the root anatomy. In addition, *RSOsPR10*, a rice root-specific pathogenesis-related protein, also enhances drought resistance in plants by enhancing the root mass (Takeuchi et al. 2016). *WOX11* mediates drought resistance in plants by controlling the number of crown roots, lateral roots and root hairs (Cheng et al., 2016). In contrast, *DEEPER ROOTING 1 (DRO1)* increases the root angle allowing the plant to explore water resources in deeper soil layers (Uga et al. 2013; Fig. 3A).

It is well supported that endogenous ABA levels increase in response to drought (Kuromori et al. 2018). ABA alleviates drought stress mainly through enhanced net photosynthesis, stomatal closure and the resulting decreased transpiration rate (Teng et al. 2014). On the other hand, ABA inhibits root growth and hampers water transport. ABA synthesis is mainly detected in young root tips but not in mature roots, which is thought to be an early response to drought (Schachtman and Goodger 2008). However, growing evidence suggests that a high ABA content in long-lasting droughts restricts the root growth, which is not beneficial for plants (Shi et al. 2015).

In general, a deep and thick root system confers higher resistance to drought. Thick roots have a larger diameter that enhances the penetration ability in soil allowing faster and deeper growth of roots. In addition, thick roots with additional xylem strands can transport more water, which is beneficial for water uptake and will reduce axial water stress (Yambao et al. 1992). It was estimated that xylem with a pit anatomy is less leaky and transports water more efficiently (Pandey et al. 2015).

During plant breeding, a number of advantageous abiotic stress tolerance genes were lost. It is however possible to identify the respective traits through QTL mapping in the wild rice species. Deeper root is a good example providing the potential for improved rice resistance to drought. The challenge ahead is thus to

identify the genes that improve drought tolerance and minimize crop loss.

Adaptative role of rice root system architecture upon flooding

Flooding and soil water-logging are threatening many crops and weather extremes with severe floods are increasing worldwide (Mustroph 2018). When flooded, the root system is prone to oxygen shortage which limits its uptake and transport capacity for nutrients and water. The maintenance of a functional root system is a prerequisite for plants to survive flooding (Sauter 2013). Several plant species including rice (Lorbiecke and Sauter 1999), tomato (*Solanum lycopersicum*) and tamarack (*Larix laricina*) (Calvo-Polanco et al. 2012) developed an adaptive strategy. They replace the damaged roots by ARs through *de novo* formation or by promoting emergence of existing AR primordia. In rice, ARs are formed at each node during nodal development and remain buried until triggered to emerge by flooding. In tamarack, long-term submergence results in the complete loss of the original roots which are gradually replaced by ARs (Calvo-Polanco et al. 2012). Nodal ARs provide the advantage of a reduced distance for O₂ transport from the shoot. The porosity in flooding-induced ARs is higher compared with unflooded roots due to aerenchyma formation (Webb and Jackson 1986; Laan et al. 1989; Drew et al. 2000; Argus et al. 2015). Furthermore, ARs tend to float at or near the water surface which facilitates uptake of atmospheric oxygen (Dawood et al. 2016). In several plants, including *Tecticornia pergranulata*, aquatic ARs develop chloroplasts and are photosynthetically active to partially compensate for the decreased rate of photosynthesis under water with O₂ release as a side effect (Rich et al. 2008). Deepwater rice, adapted to long-lasting floods, develops more nodes than lowland rice which allows deepwater rice plants to form more ARs. When floods recede and deepwater rice stems bend to the ground, ARs can grow into the soil thereby avoiding root desiccation and providing anchorage for the plant (Fig. 3A-B).

In rice, induction of AR emergence is triggered by ethylene that gets trapped in the flooded plant (Fig. 4B). Ethylene promotes cell cycle activity at the nodes (Lorbiecke and Sauter 1999), indicating that AR growth is distinctly regulated compared to primary or lateral root growth that is inhibited by ethylene. Inhibition

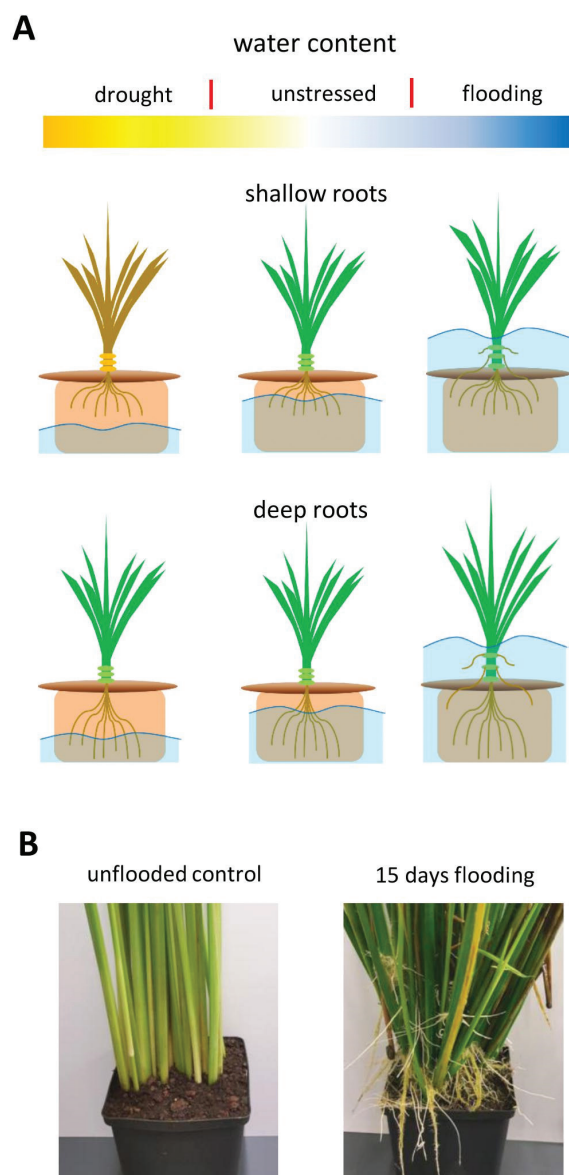


Figure 3. Root systems during drought and flooding. (A) Scheme of a shallow and a deep root system in drought or flooding conditions. (B) Adventitious root growth in 3-month-old Nipponbare plants grown under non-flooded conditions or exposed to partial submergence for 15 days.

of ethylene perception by 1-methylcyclopropene (1-MCP) abolished AR emergence revealing a requirement for ethylene signaling (Lin and Sauter 2018). The AR primordium is covered by the nodal epidermis that acts as a physical barrier (Sauter 2013). Programmed death of epidermal cells above AR primordia occurs prior to AR emergence which weakens the epidermal barrier and protects the AR meristem from injury (Steffens and

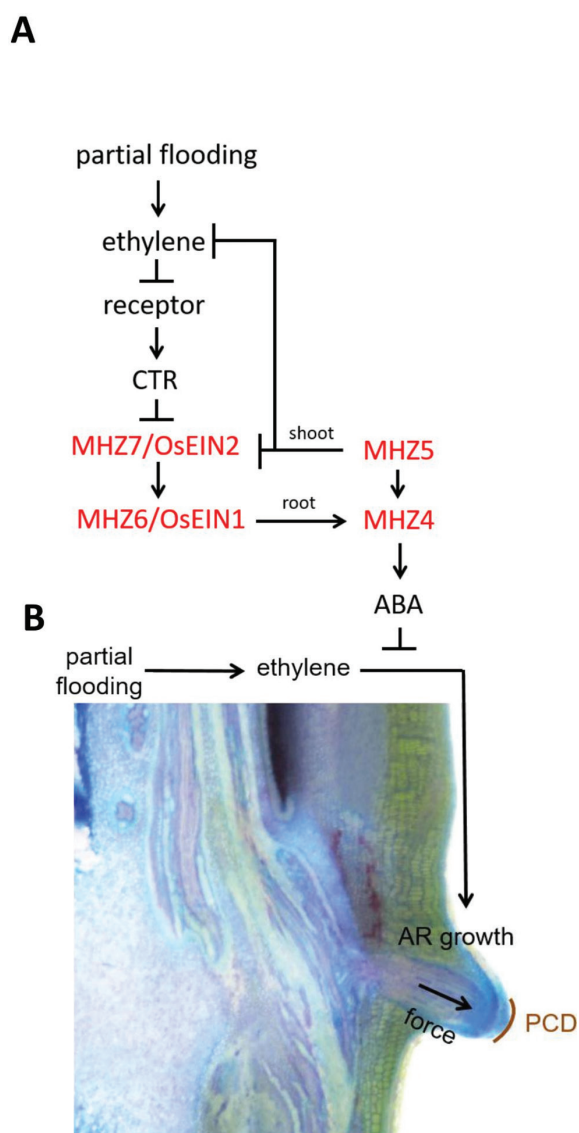


Figure 4. Model of ethylene signaling during submergence. (A) The ethylene signaling pathway of rice based on the analysis of *mao hu zi* (*mhz*) mutants. (B) Ethylene signaling of adventitious root growth and epidermal cell death (PCD) in rice. A nodal cross section was stained with toluidine to visualize the root primordium.

Sauter 2005). Epidermal cell death and AR growth are induced by ethylene, promoted by GA and inhibited by ABA (Steffens et al. 2006) indicating that the interaction and balance of these phytohormones coordinates growth and death in a timely and spatially coordinated manner. Hydrogen peroxide produced by NADPH oxidase at the plasma membrane acts downstream of ethylene in cell death induction. In addition, reduced expression of the ROS scavenger gene *METALLOTHIONEIN*

2B (*MT2b*) enhances the accumulation of reactive oxygen species and promotes cell death (Steffens and Sauter 2009). The spatial control that limits epidermal cell death to sites where ARs emerge is provided by a mechanical signal coming from the growing AR (Steffens et al. 2012). Both, mechanical force and ethylene signaling via reactive oxygen species are required to trigger the death of epidermal cells.

In rice, ethylene is a positive regulator of AR growth. ARs are crucial for plant survival of submergence. While the blueprint for the ethylene signaling pathway in rice has been established (Fig. 4A) the distinct molecular mechanisms that drive AR growth still need to be uncovered. More so, the majority of studies carried out in rice to date focused on seedlings rather than on mature plants where the adaptive root systems may be controlled differently. And finally, different rice cultivars have developed different survival strategies with an escape response in deepwater rice cultivars and a quiescence response in lowland rice (Voesenek and Bailey-Serres 2009). It is conceivable that different strategies exist in the development of the AR system. To test this hypothesis, a systematic comparison of root system architecture in different rice types will have to be done.

Conclusion

Root system architecture in rice is regulated developmentally and by environmental cues. Both regulatory venues rely on phytohormones as key signals. To date, a few genes that are functionally associated with rice root development have been identified and characterized, but the regulatory network that integrates endogenous and exogenous signals still needs to be uncovered. An improved understanding of the molecular mechanisms that control root system development and determine root system architecture will help accelerate breeding of plants with a root system that is optimized to cope with drought and floods to ensure stable crop production.

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6. Chapter 1

Control of adventitious root architecture in rice by darkness, light, and gravity



white light

dark

Control of Adventitious Root Architecture in Rice by Darkness, Light, and Gravity¹[OPEN]

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Rice (*Oryza sativa*) is a semiaquatic plant that is well adapted to partial flooding. Rice stems develop adventitious root (AR) primordia at each node that slowly mature but emerge only when the plant gets flooded, leading to the formation of a whole new secondary root system upon flooding. AR growth is induced by ethylene that accumulates in submerged plant tissues due to its lowered diffusion rate in water. Here, we report that the architecture of the secondary root system in flooded rice plants is controlled not only by altered gas diffusion but also by gravity and light. While ethylene promotes the emergence and growth of ARs, gravity and light determine their gravitropic setpoint angle (i.e. the deviation of growth direction relative to vertical). ARs grow upward at about 120° in the dark and downward at 54° in the light. The upward growth direction is conserved in *indica* and *japonica* rice varieties, suggestive of a conserved trait in rice. Experiments with a klinostat and with inverted stem orientation revealed that gravity promotes upward growth by about 10°. Red, far-red, and blue light lead to negative phototropism in a dose-dependent manner, with blue light being most effective, indicating that phytochrome and blue light signaling control AR system architecture. The *cpt1* (*coleoptile phototropism1*) mutant, which lacks one of the phototropin-interacting CPT proteins, shows reduced sensitivity to blue light. Hence, the gravitropic setpoint angle of rice ARs is controlled by genetic and environmental factors that likely balance the need for oxygen supply (upward growth) with avoidance of root desiccation (downward growth).

Root system architecture is determined by a number of parameters, including root growth rate, the rate at which lateral roots and adventitious roots (ARs) are formed, and the angles at which lateral roots and ARs grow with respect to the gravity vector. Root structure is highly adaptive. It is shaped by environmental signals such as gravity, light, nutrients, and water availability to ensure plant survival and propagation (Atkinson et al., 2014; Bellini et al., 2014). Gravity is an invariant physical signal and, hence, well suited to provide spatial orientation for plants. Gravity is sensed by starch statoliths in specialized root cap cells termed statocytes (Baldwin et al., 2013). Displacement of the statoliths as a result of an altered root orientation with regard to gravity triggers a signal in columella cells that causes an asymmetric distribution of auxin between the upper and lower sides of the lateral root cap and the epidermis of the root elongation zone. The auxin gradient causes differential growth on the upper and lower

root sides and, eventually, root bending (Ottensschläger et al., 2003; Swarup et al., 2005; Sato et al., 2015). The mechanism of gravity sensing by displacement of statoliths is well suited to explain the positive gravitropic growth of primary roots that ensures penetration into soil. By contrast, many lateral roots and ARs grow at a nonvertical angle that has been termed the gravitropic setpoint angle (GSA; Digby and Firn, 1995). The branching of secondary roots ensures radial exploration of the soil. The nonvertical growth of lateral roots is species specific and genetically determined (i.e. independent of gravity; Guyomarc'h et al., 2012). However, the angle at which lateral roots grow also is environmentally controlled, as was shown for low-phosphate conditions in *Arabidopsis* (*Arabidopsis thaliana*) that cause a more vertical growth of lateral roots (Bai et al., 2013; Roychoudhry et al., 2017). However, relatively few studies have focused on the endogenous and environmental control of the GSA so far, with most of the information gained from *Arabidopsis*. In order to learn more about the control of the GSA and to identify general regulatory mechanisms that control secondary root architecture, it is useful to extend the studies to other plant species. Rice (*Oryza sativa*) is a monocot model plant that is well suited to extend our understanding of GSA regulation to an agriculturally important crop species and provide insights that may contribute to crop improvement.

Light is a key environmental cue for plants. It provides orientation for the shoot and root and triggers developmental reprogramming such as deetiolation in

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M.S. and C.L. conceived the project; C.L. and M.S. designed experiments; C.L. performed and analyzed experiments; M.S. wrote the article with contributions from C.L.

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seedlings. Plants have different photoreceptors with characteristic action spectra. They perceive red light via the phytochrome receptor. The inactive phytochrome Pr is converted to its active form Pfr by red light absorbance. This conversion is reversible. Absorbance of far-red light by Pfr converts the active phytochrome to its inactive form Pr (Li et al., 2011). In rice, phytochromes are encoded by three genes, PhyA to PhyC, whereby PhyA is responsible mainly for mediating photomorphogenic responses (Takano et al., 2009; Roy et al., 2013). Some physiological responses, such as shade avoidance, depend on the ratio of Pr to Pfr. In shade avoidance, light gradients that are perceived by leaves induce the growth of petioles or leaf blades toward higher light intensities to optimize photosynthesis (Briggs, 2014). Shade avoidance is triggered by an increased ratio of far-red to red light that naturally occurs in the shade that is perceived by phytochrome (de Wit et al., 2016).

UV-A/blue light is perceived via the blue light receptors phototropin and cryptochrome, whereby phototropin is the main blue light receptor mediating phototropic responses (Fankhauser and Christie, 2015). Positive phototropism also serves to orient the shoot in the direction of light for efficient photosynthesis by means of directed growth (Fankhauser and Christie, 2015). While shoots display positive phototropism, roots most often respond to light by negative phototropic growth, driving them into the dark. In *Arabidopsis*, negative root phototropism was shown to have a beneficial effect under drought conditions with increased plant size at maturity (Galen et al., 2007), indicating that the control of root growth direction by light can help the plant to adapt to adverse environmental conditions. The blue light receptor phototropin is encoded by two genes in flowering plants, *PHOT1* and *PHOT2*, whereby *PHOT1* is responsible mainly for root phototropism (Fankhauser and Christie, 2015). In *Arabidopsis* roots, light was shown to redirect auxin flux by altering the polarized distribution of the auxin-transporting PIN proteins such that one side of the root grows faster while the other side grows slower, resulting in phototropic root bending (Ding et al., 2011; Sassi et al., 2012).

Root system architecture is determined not only by primary and lateral roots but also by shoot-borne roots. The formation of ARs can be developmentally programmed, as is the case for crown and brace roots of cereals, or be induced by environmental signals, such as flooding (Bellini et al., 2014). Submergence poses a number of challenges to plants, the most obvious of which is reduced oxygen availability. In flooding-adapted plants, postsubmergence root architecture differs largely from the primary root system. Flooding promotes the establishment of an AR system that takes on the task to supply the plant with water, nutrients, and anchorage (Ayi et al., 2016). It can support or even replace the soil root system that suffers most from oxygen shortage during flooding (Sauter, 2013). In semiaquatic rice, AR primordia are constitutively

formed at the stem nodes without emerging from them as long as the node is not submerged (Lorbiecke and Sauter, 1999). Upon submergence, the gaseous hormone ethylene accumulates in the submerged plant parts due to its lowered diffusion rate in water as compared with air. Ethylene acts as a hormonal trigger that promotes the growth and emergence of ARs in concert with gibberellic acid and abscisic acid (Steffens et al., 2006).

While deep rooting is a beneficial trait for drought resistance, it is unfavorable during flooding, where shallow rooting helps avoid hypoxic or anoxic soil layers. In contrast to the primary root system, ARs grow in the oxygen-rich surface layers of the flood water, from which they can take up oxygen produced in underwater photosynthesis (Winkel et al., 2013; Ayi et al., 2016). ARs benefit from oxygen dissolved in flood water yet need to avoid desiccation in air. Light may be a helpful signal to guide root orientation under water. In this study, we report on the regulation of AR growth direction in rice by gravity and light signals.

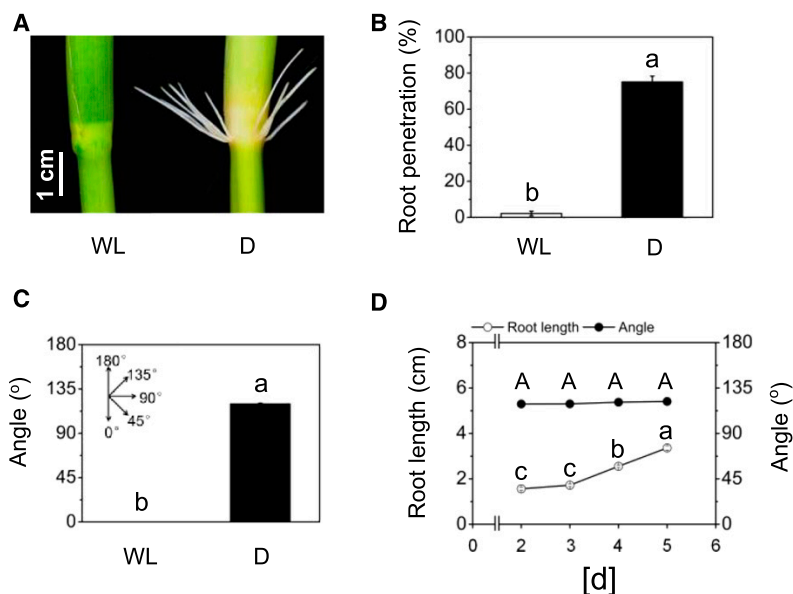
RESULTS

Growth of Adventitious Rice Roots Is Induced in the Dark

Rice plants form AR primordia at their stem nodes that emerge to develop into ARs upon plant submergence. AR growth is promoted by ethylene that accumulates in submerged tissue (Lorbiecke and Sauter, 1999). In this study, we show that exposure of excised deepwater rice stems of the cv PG56 to continuous darkness for 2 d likewise results in AR growth (Fig. 1, A and B). In the dark, 75% of root primordia grew out as compared with only 2.2% when stems were kept in the light. Interestingly, the angle at which ARs grew in the dark was, on average, 119.8° such that roots pointed upward (Fig. 1, A and C). A time-course analysis revealed that the upward angle was maintained while ARs continued to elongate for at least 5 d (Fig. 1D). To test if a similar growth behavior was observed in intact plants under flooding conditions, plants were partially submerged or kept in air in the light or in the dark for 3 d (Fig. 2). Partial submergence in either continuous light or continuous darkness induced AR growth. While the average growth angle in continuous light was 46.8°, it was 119.3° in the dark, confirming that the response observed in excised stem sections was a physiological response that likewise occurs in flooded plants. In the dark, no ARs emerged from intact plants. In rice plants, senescence-induced ethylene formation was measured no earlier than after 6 d of complete darkness (Fukao et al., 2012). When plants are submerged, ethylene that is continuously produced is trapped to physiologically active levels more rapidly.

To test if the upward growth of ARs occurred only in a gaseous or liquid medium or also in solid medium such as soil, we tested AR growth behavior in cv PG56 stems kept in soil for up to 3 d (Fig. 3). Stem nodes were placed 8 cm below the soil surface. The number of

Figure 1. ARs of rice emerge and grow upward in the dark. A, cv PG56 rice stem sections were exposed to white light (WL) or darkness (D) for 2 d. ARs emerged in the dark but not in the light. B, Percentage of penetrated roots. C, Average angle of ARs at the third youngest node. Bars in B and C indicate means \pm SE from four independent experiments, with 30 stem sections analyzed in total per treatment. Different letters indicate significantly different values between treatments ($P < 0.05$, Student's *t* test). D, Time-course analysis of AR elongation and root growth angle in the dark. Values are means \pm SE of three independent experiments, with 18 stems analyzed per time point. Uppercase letters indicate significantly different values of root angle between different time points, and different lowercase letters indicate significantly different values of root length between different time points ($P < 0.05$, ANOVA with Tukey's test).



emerged roots at this node increased from 0% at 0 h to 30.6% after 14 h, to 73.3% after 24 h, and to 96.9% after 2 d (Fig. 3A). To analyze the root growth angle, we carefully dug out stems from the soil after 2 d. The average length of ARs after 2 d was 1.6 cm and the average growth angle was 113.5°, indicating that upward growth of AR in rice was independent of the physical nature of the medium. To visualize AR growth direction in solid medium, we placed the lower 10-cm part of rice stem sections in Styrofoam beads for 3 d and analyzed the AR system in situ with x-ray computer tomography (Fig. 3B). ARs grew at an upward angle in situ as observed in air and in water, supporting the conclusion that the angle of AR growth is not determined by the medium in which roots grow.

Upward Growth of ARs in the Dark Is a Conserved Developmental Trait in Rice

We next investigated AR development by studying different age nodes. ARs form at the youngest node and slowly grow over time without penetrating the epidermis as long as the node is kept in the light and not exposed to ethylene (Lorbiecke and Sauter, 1999). We analyzed ARs at the first (i.e. youngest) to the fourth (i.e. oldest) nodes of cv PG56 after exposure to continuous darkness or to light as a control for 2 d (Fig. 4). The percentage of roots that had penetrated after 2 d in the dark increased with developmental age of the node. None of the root primordia had emerged at the youngest node, while 64.3% of all primordia had emerged at the oldest node (Fig. 4A). Only ARs at the third and fourth nodes were long enough to determine the growth angles (Fig. 4B). ARs grew upward at both nodes, with similar angles of 121.4° at the third node and 117.4° at the fourth node. Taken together, the

rate of emergence of ARs is dependent on their developmental stage, whereas the growth angle is independent of the development age of the node.

The studies described so far were carried out with the deepwater rice variety cv PG56, which raised the question of whether the observed trait was a characteristic of rice that is regularly exposed to long-term (several months) severe flooding (several meters) or whether the upward growth of ARs was generally conserved in rice. To answer this question, we studied five lowland rice cultivars that are adapted to short-term (fewer than 2 weeks), less severe (less than 1 m) flooding events. The deepwater and lowland rice varieties that we studied are not only adapted to different flooding regimes but also belong to different rice subspecies (Table I). The rice cv PG56 belongs to the *indica* subspecies, whereas the lowland rice cultivars belong to the *japonica* subspecies.

Rice stems from lowland rice cv ZH11, T65, Nipponbare, Bomba, and Kinmaze and from the deepwater rice cv PG56 were exposed to continuous darkness for 3 d. Darkness induced AR emergence in all cultivars (Table I). The angles of the lowland rice cultivars varied between 104.6° and 115.5° and, hence, were slightly lower than the 120.7° measured for cv PG56. Nonetheless, roots grew upward in all genotypes even prior to internode elongation (Supplemental Fig. S1, A and B). In conclusion, dark-induced AR growth in an upward direction is conserved in rice, suggesting that it is of benefit to its semiaquatic lifestyle.

Dark-Induced AR Growth Is Mediated by Ethylene Signaling

It has been reported previously that, in rice, an extended dark period results in ethylene formation,

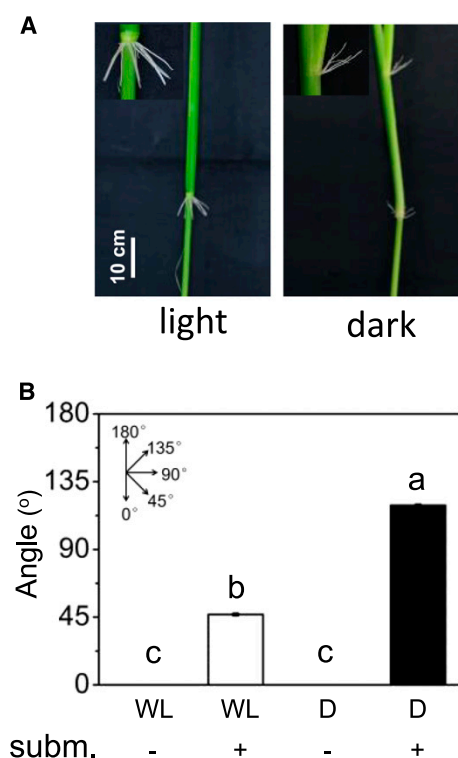


Figure 2. The direction of AR growth in partially flooded plants is determined by light. A, cv PG56 rice plants were partially submerged for 3 d in the light or in the dark. ARs grow downward in the light and upward in the dark when flooded. B, Mean \pm SE angles of ARs that emerged at the third youngest node were determined in three independent experiments from 18 to 24 plant stems exposed to white light (WL) or darkness (D). Different letters indicate statistically significant differences ($P < 0.05$, ANOVA with Tukey's test).

which, in turn, promotes dark-induced senescence (Fukao et al., 2012). To answer the question of whether ethylene was a trigger for AR growth in the dark, we exposed cv PG56 stems kept in air to $5 \mu\text{L L}^{-1}$ 1-methylcyclopropene (1-MCP), an inhibitor of ethylene perception, and kept them either in the light as a control or in the dark (Fig. 5A). In the light, no roots penetrated regardless of the 1-MCP treatment (Fig. 5B). In the dark, AR growth was induced in untreated stems whereas no ARs penetrated in the dark when stems had been pretreated with 1-MCP, indicating that AR growth in the dark is mediated by ethylene signaling.

AR Emergence and the AR Growth Angle Are Controlled by Red and Blue Light Signaling

To better understand which light signaling pathways control AR emergence and the growth angle of ARs, we studied the effects of red, far-red, and blue light. As controls, cv PG56 stems were exposed to continuous

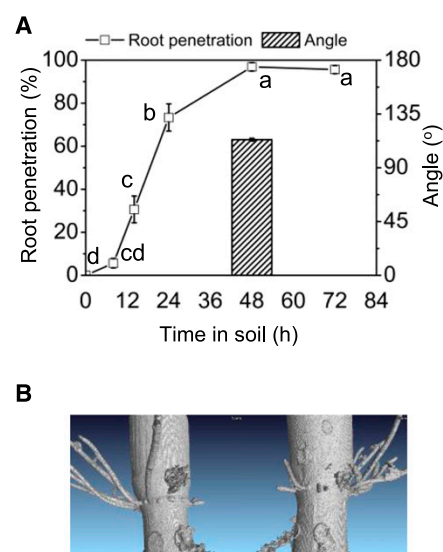


Figure 3. ARs emerge and grow upward in soil. A, AR penetration at the third node of cv PG56 stem sections that were buried in soil was monitored for up to 72 h. The AR growth angle was determined after 48 h. Values are means \pm SE analyzed from 15 stems in three independent experiments. Different letters indicate significant values between each time point ($P < 0.05$, ANOVA with Tukey's test). B, CT image of third nodes with ARs visualized after 3 d in styrofoam beads.

darkness or treated with $150 \mu\text{M}$ of the ethylene-releasing compound ethephon in white light for 3 d to induce AR emergence. Ethephon treatment in white light caused about 80% of all AR primordia that were present at the third node to emerge, while no roots emerged in white light without ethephon (Fig. 6A; Steffens et al., 2006).

Exposure to far-red light reduced root penetration with increasing fluence rate, from 84.8% in the dark to 19.1% at $78 \mu\text{mol m}^{-2} \text{s}^{-1}$, and was 2.2% in $43 \mu\text{mol m}^{-2} \text{s}^{-1}$ white light (Fig. 6B). In red light, we also observed decreasing AR penetration rates with increasing light intensity, a response that leveled out at 18.4% root penetration in stems exposed to $3.3 \mu\text{mol m}^{-2} \text{s}^{-1}$ and higher red light fluences (Fig. 6B). Red light had a stronger effect at low fluence rates than far-red light, while red and far-red light had similar inhibitory effects at higher fluence rates. The growth angle of emerged roots declined from 118.3° in the dark to 76.9° at $78 \mu\text{mol m}^{-2} \text{s}^{-1}$ far-red light (Fig. 6C). The angle of emerged ARs was likewise decreased by red light in a dose-dependent manner, from 104.8° at $0.03 \mu\text{mol m}^{-2} \text{s}^{-1}$ to 91° at $13.2 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 6C), but again, red light appeared to be more efficient than far-red light at low light intensities. Roots that emerged in the presence of ethylene in white light grew at an angle of 54.2° . The results revealed that both Pr (favored by far-red light exposure) and Pfr (favored by red light exposure) regulate AR emergence and growth angle. Taken together, our analysis

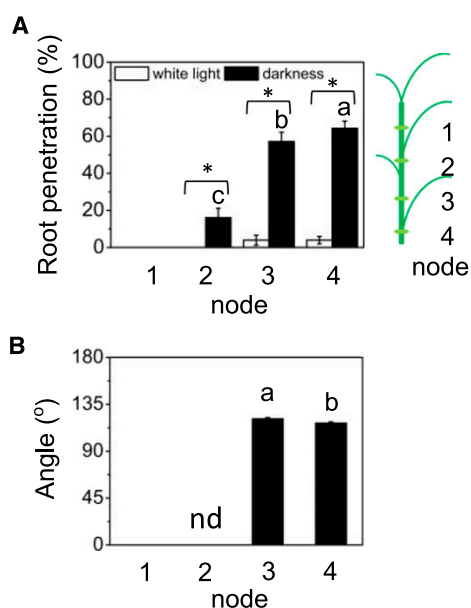


Figure 4. Developmental gradient of ARs in the dark. A, cv PG56 stem sections were excised 2 cm below the first, second, third, or fourth node and kept in white light or darkness for 2 d. Subsequently, root penetration was determined. B, Mean \pm SE root angles at each node were determined from 16 stems analyzed in three independent experiments. Different letters indicate statistically significant differences between nodes in the dark ($n = 16$; $P < 0.05$, ANOVA with Tukey's test). Asterisks indicate significant differences between dark and light treatments at each node ($P < 0.05$, Student's t test; nd, no data).

revealed an inhibitory effect of phytochrome signaling on AR emergence and upward growth. The results further show that inhibition of AR penetration is more sensitive to red light than to far-red light at low fluence rates. However, neither far-red nor red light signaling in the low ($1\text{--}1,000\ \mu\text{mol m}^{-2}$) and very low ($0.1\text{--}1\ \mu\text{mol m}^{-2}$) fluence ranges that were analyzed here (Li et al., 2011) can fully account for light regulation of AR penetration and growth angle, suggesting that another light signaling pathway may be involved.

Since phototropic responses are commonly regulated by the blue light receptor phototropin, we tested the effect of blue light on AR penetration and growth angle (Fig. 7). The cv PG56 stems were exposed to white light, to darkness, or to different intensities of blue light for 3 d. Following these treatments, root penetration rates (Fig. 7A) and the angle of penetrated roots (Fig. 7B) were determined. Root penetration was inhibited in a dose-dependent manner. At $3.8\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ and higher blue light intensities, the percentage of emerged roots was as low as in white light. Similarly, the angle of ARs decreased in a dose-dependent manner and reached 58.9° at $8\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ blue light, which was comparable to the 54.2° observed in $43\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ white light, suggesting that blue light signaling

is a major signal regulating AR emergence and growth direction.

A Role for CPT1 in Blue Light Regulation of the AR Growth Angle

NONPHOTOTROPIC HYPOCOTYL3 (NPH3) has been identified as a signaling component involved in hypocotyl and root phototropism in *Arabidopsis* (Motchoulski and Liscum, 1999). The rice COLEOPTILE PHOTOTROPISM1 (CPT1) protein is an NPH3 ortholog (Haga et al., 2005). Seedlings of the *cpt1* deletion mutant of the rice cv Nihonmasari are unresponsive to blue light with regard to the induction of positive phototropism of the coleoptile and show decreased blue light sensitivity in negative phototropism of the primary root (Haga et al., 2005). We employed the *cpt1* mutant to answer the question of whether AR phototropism involves signaling via CPT1. To verify the CPT1 deletion, DNA was isolated from wild-type cv Nihonmasari, from *cpt1* plants, and from cv PG56 plants. A DNA fragment within the deleted region was amplified with gene-specific primers from cv Nihonmasari and PG56 but not from *cpt1* plants (Fig. 8, A and B), confirming the CPT1 gene deletion. Next, stem sections from wild-type cv Nihonmasari and from the *cpt1* mutant were kept in the dark, exposed to blue light, or exposed to white light with $150\ \mu\text{M}$ ethephon for 7 d (Fig. 8C). Root penetration was high in the dark and decreased in both cv Nihonmasari and *cpt1* in a dose-dependent manner, with no statistically significant difference between genotypes (Fig. 8D). ARs grew upward in the dark with no difference in growth angle between cv Nihonmasari and *cpt1*. The growth angle decreased with increasing blue light intensity in both genotypes (Fig. 8E). However, *cpt1* displayed reduced sensitivity to blue light such that angles were larger in *cpt1* as compared with cv Nihonmasari at any given intermediary blue light intensity. ARs of cv Nihonmasari reached the lowest angle of 81.8° at $1.8\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$, whereas ARs of *cpt1* reached 79.9° at $17.1\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$. These results suggest that CPT1 participates in blue light regulation of AR growth angle but not of AR emergence and, hence, suggest a specific role for CPT1 in negative AR phototropism in rice.

The Growth Direction of ARs Is Reversibly Controlled by Light

We next tested whether regulation of the AR growth angle by light was a reversible process. We exposed stem sections to either continuous white light combined with $150\ \mu\text{M}$ ethephon to promote downward growth of ARs or to continuous darkness to promote upward growth. In addition, we exposed stems to 2 d of darkness followed by 2 d of light plus ethephon treatment, or to 2 d of light plus ethephon followed by 2 d of darkness (Fig. 9). ARs that grew upward in the dark

Table 1. Upward growth of ARs in the dark is a conserved trait in rice

ARs of the six rice genotypes, cv Pin Gaew 56 (PG56), Zhonghua 11 (ZH11), Taichung 65 (T65), Nipponbare, Bomba, and Kinmaze, were analyzed. Rice stem sections including the third youngest node were cut and incubated in white light with 150 μM ethephon (WL+E) or in the dark for 4 d before growth angles were determined. Angle values indicate means \pm SE from 15 to 30 stems analyzed in three independent experiments. Letters a and b indicate significant differences between treatments in a given variety ($P < 0.05$, Student's *t* test).

Cultivar	Subspecies	Ecotype	Angle	
			Dark	WL+E
PG56	<i>indica</i>	Deepwater	120.7 \pm 0.6 a	57.8 \pm 1.2 b
ZH11	<i>japonica</i>	Lowland	104.6 \pm 0.5 a	60.2 \pm 1.9 b
T65	<i>japonica</i>	Lowland	110.8 \pm 0.9 a	59.7 \pm 0.9 b
Nipponbare	<i>japonica</i>	Lowland	113.9 \pm 0.9 a	62.7 \pm 1.7 b
Bomba	<i>japonica</i>	Lowland	115.5 \pm 1.2 a	48.8 \pm 1.3 b
Kinmaze	<i>japonica</i>	Lowland	112.3 \pm 1.0 a	64.6 \pm 1.2 b

changed their growth direction when transferred to the light, and ARs that grew downward in the light changed their growth direction to an upward angle when moved to the dark (Fig. 9A). The changes in growth angle are depicted as cartoon drawings (Fig. 9B).

Gravity Plays a Minor Role in Determining AR Growth Direction

Plants are constantly exposed to gravity. Primary roots and shoots orient their growth direction along the gravity vector either in a positive or negative manner, while the angle of lateral roots or ARs deviates from the gravity vector. To investigate the impact of gravity on the GSA of ARs, we performed a klinostat experiment in which stem sections were vertically rotated at 5 rpm in the dark for 3 d (Fig. 10A). The angle of rotated roots was 106° as compared with 120.4° in nonrotated roots, indicating that gravity induced a negative gravitropic response of 14.4° (Fig. 10B). To further test the effect of gravity, we performed two experiments in which stem sections were oriented in an upright or inverse orientation (Fig. 10, C and E). In one setup, the stems were placed in a beaker in either orientation (Fig. 10C). In a second experiment, water was supplied in a test tube to the lower cut surface of the stem irrespective of its orientation. In stems kept in a beaker (Fig. 10, C and D), the angle changed from 123.4° in the upright orientation with roots exposed to 1 g to an angle of 101.3° in inverse oriented stems with roots exposed to -1 g. Hence, applying gravity from the opposite direction caused a change in growth angle of 22.1°. A similar result was obtained with stems supplied with water from test tubes at the lower cut surface (Fig. 10E). An angle of 116.4° was measured in the upright orientation and one of 96.6° in the inverse orientation, resulting in a difference of 19.8° (Fig. 10, E and F).

Gravity is sensed by starch-containing statoliths in specialized root cap cells called statocytes. We

performed starch staining of ARs to analyze at which developmental stage the statocytes develop in AR primordia. We obtained cross sections from each node that were stained with Lugol's solution to visualize starch as a brownish stain (Fig. 11). We also isolated AR primordia from each node for staining. The results revealed that statoliths were present in the root cap of nonemerged ARs at all nodes, including the youngest node (Fig. 11). These observations suggested that AR primordia can sense gravity at a very early stage. However, the GSA is determined by gravity to only a

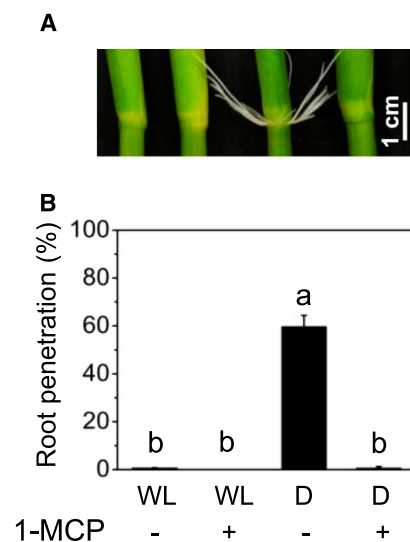


Figure 5. AR growth in darkness is suppressed by 1-MCP. A, Stem sections of rice cv PG56 were kept in white light (WL) or darkness (D) with or without 5 $\mu\text{L L}^{-1}$ 1-MCP for 2 d to inhibit ethylene perception. B, Percentage of penetrated roots. Results are means \pm SE from three independent experiments, with 20 to 22 stems analyzed. Different letters indicate statistically significant differences ($P < 0.05$, ANOVA with Tukey's test).

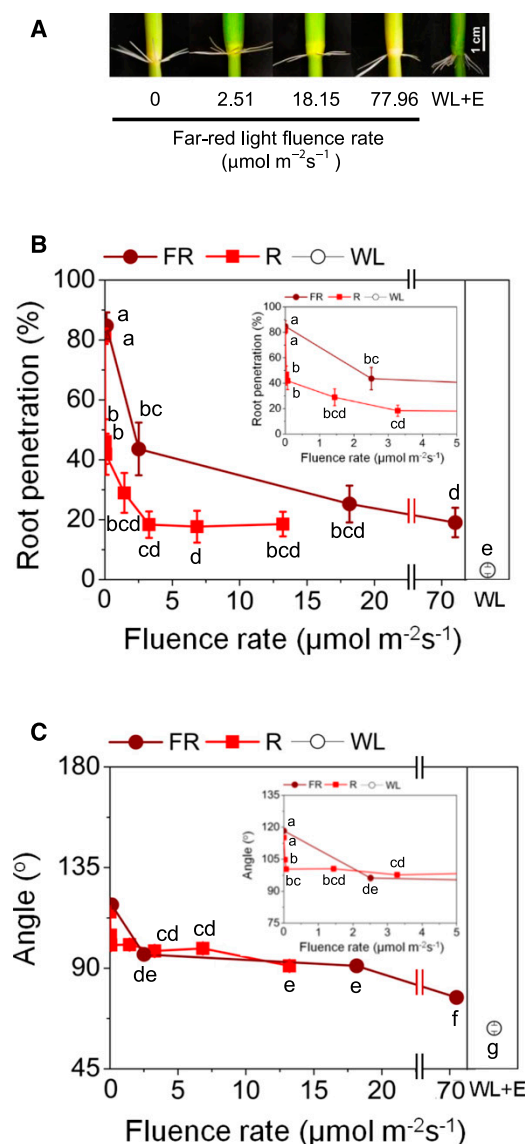


Figure 6. AR emergence and the growth angle of ARs are controlled by red and far-red light signaling. A, Stem sections including the third node show a fluence rate-dependent change in growth angle when exposed to far-red light. For comparison, a stem is shown that was exposed for 3 d to white light in the presence of $150 \mu\text{M}$ ethephon (WL+E). Ethephon releases ethylene that promotes AR growth even in the light. B, Percentage of ARs that penetrated at the third node in darkness (0), red light (R), far-red light (FR), or white light (WL) after 3 d. The inset shows a higher resolution of the low fluence range. In B and C, results are means \pm SE from four independent experiments, with 18 to 30 stems analyzed per treatment. Different letters indicate statistically significant differences ($P < 0.05$, ANOVA with Tukey's test).

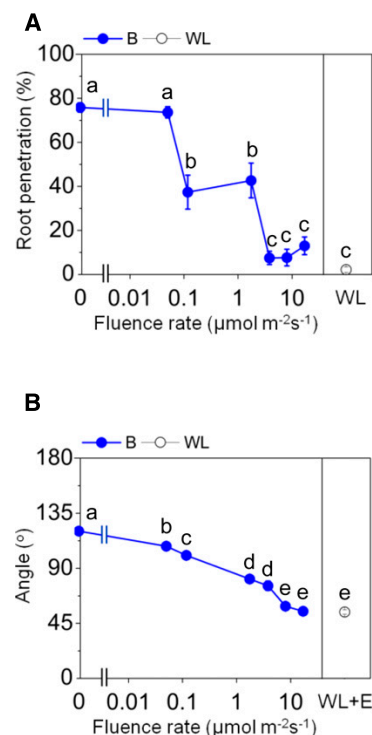


Figure 7. Blue light fully inhibits the emergence and upward growth of ARs. A, Percentage of penetrated roots at the third node of cv PG56 stems exposed to darkness (0), blue light (B), or white light (WL) for 3 d. B, Average root growth angles in increasing blue light fluence rates and in white light in the presence of $150 \mu\text{M}$ ethephon (WL+E). Values are means \pm SE from three independent experiments, with 16 to 20 stems analyzed per treatment. Different letters indicate statistically significant differences ($P < 0.05$, ANOVA with Tukey's test).

minor degree whereby ARs respond to gravity with a negative gravitropic response.

DISCUSSION

Physiological Function of Upward AR Growth

Rice is a semiaquatic plant. With the exception of upland rice, many rice varieties are cultivated in paddy fields. Depending on the ecological niche to which a particular rice variety is adapted, it can, on top, encounter more or less severe flooding events. In waterlogged or flooded soil, gas diffusion is limited and roots compete with microorganisms for oxygen. For roots, a continuous supply of oxygen from the shoot via internal air spaces becomes crucial (Pedersen et al., 2009). When fully submerged, shoots do not have access to atmospheric oxygen; however, submerged shoots can photosynthesize and thereby increase endogenous oxygen levels and enrich the flood waters with oxygen. During flooding, the original root system in the soil is

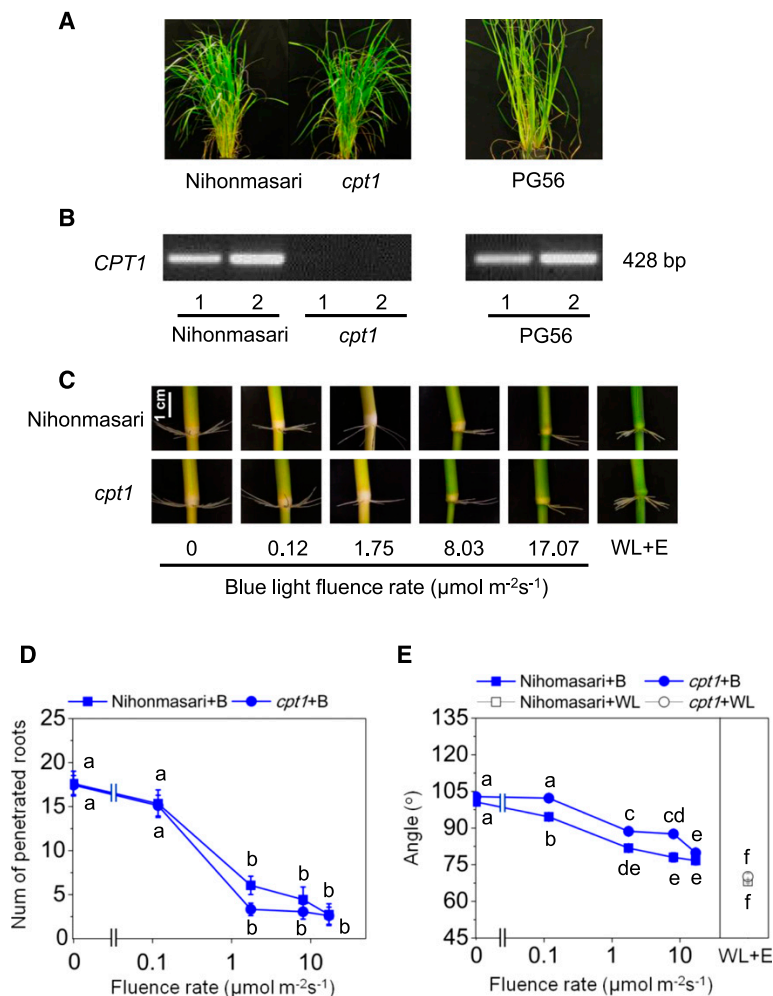


Figure 8. Blue light signaling of AR growth direction is partially impaired in the *cpt1* mutant. A, A *cpt1* plant, its wild-type background cv Nihonmasari, and a cv PG56 rice plant. B, A 428-bp fragment within the *CPT1* gene was amplified from cv PG56 and Nihonmasari but not from *cpt1*. DNA was isolated from two individual plants for each genotype. C, Emerged ARs at the third node of cv Nihonmasari and *cpt1*. *cpt1* is less sensitive to low blue light fluence rates. D, Number of ARs penetrated at the third node of stems that were exposed to increasing blue light (B) intensities for 6 d. E, Average root angles at the third node of stems that were exposed to increasing blue light intensities for 6 d or kept in white light in the presence of 150 μM ethephon (WL+E). Results in D and E are means \pm SE obtained from three independent experiments, with 18 stems analyzed per treatment. Different letters indicate significantly different values ($P < 0.05$, ANOVA with Tukey's test).

supported or replaced by aquatic ARs that emerge from the stem nodes. ARs are more easily supplied with oxygen, as they are closer to the photosynthesizing shoot, and aquatic ARs can take up oxygen from the flood water (Ayi et al., 2016). The survival of *Alternanthera philoxeroides* plants was higher with aquatic ARs as compared with plants with aquatic ARs removed (Ayi et al., 2016). Root oxygen in fully submerged rice plants was shown to be determined by underwater photosynthesis (Winkel et al., 2013). For the wetland grass *Meionectes brownii*, Rich et al. (2013) showed that, irrespective of partial or full submergence, the deeper parts of submerged stems and aquatic roots received their oxygen from underwater photosynthesis during the day and from the oxygen-enriched flood water at night. Hence, light is a determinant of oxygen supply under water.

We observed that rice ARs show an unusual growth behavior in the dark: they grow upward. In the light, the growth direction changes, resulting in downward

growth, a response that indicates negative phototropism. Phototropism is generally seen as a means to optimize plant growth and development. Shoot phototropism serves to optimize the photosynthetic ability of plants. Unlike shoots, roots in general display negative phototropism that directs roots into the soil to avoid desiccation and ensure that roots can take up nutrients and water and provide anchorage. In addition, root growth is directed in a positive gravitropic manner that further drives downward growth. Phototropic and gravitropic responses are mediated by auxin that can be transported polarly by auxin efflux carriers. Directed auxin flow allows for an asymmetric auxin distribution and, hence, for differential growth rates on the upper and lower sides of a root or on the shoot side facing the light versus the shaded side and, hence, to organ bending (Fankhauser and Christie, 2015; Su et al., 2017). In rice, AR growth direction is determined by gravity to only a minor degree. In fact, ARs display negative gravitropism. Loss of directional gravity in a

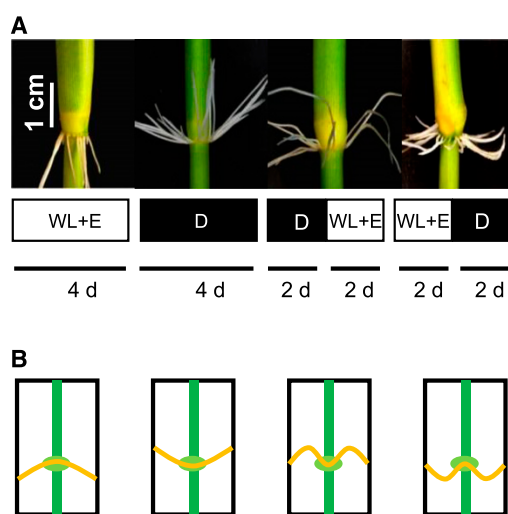


Figure 9. The growth direction of ARs is reversibly regulated by light. A, Rice stem sections were exposed to white light + ethephon (WL+E) and dark (D) regimes as indicated: WL+E or D for 4 d, D for 2 d followed by WL+E for 2 d, or WL+E for 2 d followed by 2 d of D. Ethephon was applied at a final concentration of $150 \mu\text{M}$. The white light intensity was $43 \mu\text{mol m}^{-2} \text{s}^{-1}$. B, Schematic diagrams indicating the changes in root growth directions as shown in A.

klinostat resulted in a reduced upward growth angle in the deepwater rice cv PG56 by 14° , and reversion of the gravity vector with respect to stem orientation reduced upward growth by 20° to 22° . Hence, the gravitational signal enhances upward AR growth direction. Deeper rooting1 (DRO1) is a rice gene that makes roots grow deeper into soil and, thereby, provides drought avoidance ability in upland rice (Uga et al., 2013). IR64 is a paddy field rice variety with a shallow root system. Interestingly, roots of the DRO1-near isogenic IR64 line that was equipped with the DRO1 trait had a root system that reached twice as deep without having a higher root dry mass and with only marginally longer roots, suggesting a function of DRO1 in the determination of the root growth angle and, hence, genetic control of the root system architecture and the function of the root system.

With regard to environmental control of the root system, we found that light had a strong impact. In non-flooded conditions, light fully inhibited AR emergence. In partially submerged plants or in the dark, ARs emerged due to the activity of ethylene that accumulates in these conditions (Steffens et al., 2006; Fukao et al., 2012). The growth angle of emerged ARs changed from about 120° in the dark to 54° in white light, thus reversing the growth direction from upward to downward (Fig. 12). Taking previous and our findings together, we conclude that the growth angle that deviates from the vertical, the GSA, is, in part, genetically programmed (e.g. via DRO1) and environmentally controlled by gravity and light, with light exerting the stronger environmental effect. We

hypothesize that absence of light is an indicator for oxygen-deficient soil that ARs try to avoid through upward growth as an escape response. Light indicates an environment that could be in flood water but also could be in air, which bears the danger of desiccation for the root. Hence, in the light, ARs grow downward.

Regulation of AR Growth by Light

Plants sense light by a number of photoreceptors, including phytochromes, cryptochromes, and phototropins. Plant photoreceptors are specialized to perceive different light qualities (wavelengths) and to measure light intensity (fluence rate). The information gained from light signals helps plants to adapt to their environment. The red light receptor phytochrome exists in two isoforms that are interconvertible, the far-red light receptor Pfr and the red light receptor Pr (Li et al., 2011). Rice has three phytochrome genes, *PHYA*, *PHYB*, and *PHYC*. *PHYA* and *PHYC* interact to mediate red and far-red light responses, likely by stabilization of *PHYC* by *PHYA* (Takano et al., 2005; Xie et al., 2014). *PHYB* modulates red light signaling by regulating the Arabidopsis response regulator ARR4 (Sweere et al., 2001). Cryptochrome and phototropin are blue light receptors with distinct functions (Christie et al., 2015). While cryptochrome regulates photomorphogenesis, phototropic responses are generally controlled by phototropin that is encoded by two genes, *PHOT1* and *PHOT2*, in flowering plants (Fankhauser and Christie, 2015). In Arabidopsis, root phototropism is mediated by *PHOT1* that is active over a broad range of blue light intensities (Christie et al., 2015). In addition, the red light receptor phytochrome also has been implicated in the regulation of phototropism.

In rice, we observed two distinct responses to light, AR emergence and a change in AR growth angle. Root penetration was inhibited from about 80% in the dark to 2.2% in white light and was reduced to 18.4% in red light and to 19.1% in far-red light, clearly supporting a role of both red and far-red light signaling in the control of AR emergence. Root emergence is furthermore controlled by blue light that reduced the number of penetrated roots in a dose-dependent manner, with a significant reduction observed at $0.12 \mu\text{mol m}^{-2} \text{s}^{-1}$ blue light and a full inhibition to 7.5% at $3.8 \mu\text{mol m}^{-2} \text{s}^{-1}$. Hence, light of different quality is able to prevent root penetration, with blue light being more effective than red/far-red light. In rice seedlings, continuous white light irradiation was shown to inhibit the growth of seminal roots (Shimizu et al., 2009). Partial inhibition of seminal root growth was observed with red, far-red, and blue light. Analysis of phytochrome mutants revealed that far-red light was perceived exclusively by *PhyA*, whereas red light was perceived by both *PhyA* and *PhyB*. Little or no role was assigned to *PhyC* in growth inhibition of the seminal roots. Light can penetrate soils to some degree, with red light penetrating deeper (several centimeters) than blue light that is perceived in shallow soil. In flood

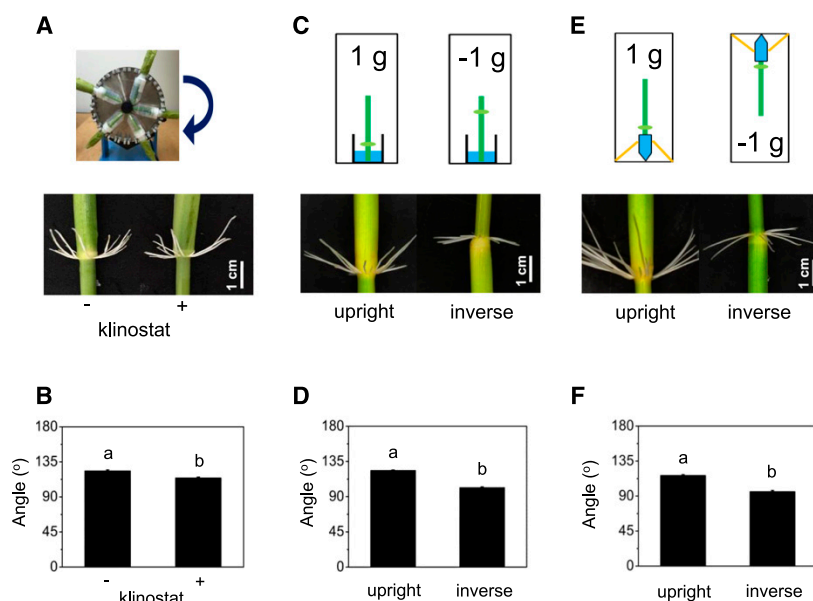


Figure 10. Gravity plays a minor role in determining the setpoint angle of ARs. A, Photograph showing the klinostat setup. B, Angle of ARs growing at the third node of PG56 stems that were rotated at 5 rpm on a klinostat for 3 d in the dark. Means \pm SE were analyzed in three independent experiments. Different letters indicate different values between treatments ($n = 15$; $P < 0.05$, Student's *t* test). C, Schematic showing stems that were placed upright or in an inverse orientation in a beaker. D, Average angle of ARs grown for 3 d in the dark as indicated in C. Means \pm SE were analyzed in three independent experiments. Different letters indicate different values between treatments ($n = 16$; $P < 0.05$, Student's *t* test). E, Schematic indicating stems that were placed upright or in an inverse orientation. Water was supplied through a 10-mL tube attached to the lower cut surface in either setup. F, Average root angle of stems kept for 3 d in the dark as indicated in E. Means \pm SE were analyzed in three independent experiments on a total of 14 stems per treatment. Different letters indicate significant differences between treatments ($P < 0.05$, Student's *t* test).

waters, numerous factors, including turbidity and chlorophyll content, determine the quality and intensity of penetrating light (Dev and Shanmugam, 2014). It may be advantageous for plants to perceive a whole range of light with different photoreceptors to control AR growth in an environment where not only light intensity but also light quality varies.

As long as the rice stem does not get flooded or buried in soil, ARs will not grow. In flooded plants, emergence of root primordia is promoted even in the light by ethylene that gets trapped naturally due to its low gas diffusion rate in water. In prolonged darkness, plants start to senesce, a process that is promoted by ethylene (Johnson and Ecker, 1998; Wang et al., 2002). Ethylene accumulation during an extended dark period has been shown previously for rice and *Arabidopsis* (Fukao et al., 2012; Rasmussen et al., 2017). In this study, we show that inhibition of ethylene signaling abolishes dark-induced AR emergence, supporting the conclusion that AR growth is mediated by ethylene both in the dark and during submergence. Interestingly, the SUB1A-1 locus, which confers submergence tolerance in rice, dampens ethylene formation and delays senescence-associated processes such as chlorophyll breakdown (Fukao et al., 2012). It is conceivable

that rice genotypes that carry the SUB1A-1 locus have delayed AR growth in the dark compared with rice varieties that follow an escape strategy that is driven by ethylene. In rice seedlings, seminal root growth is inhibited by white light. Analysis of phytochrome mutants revealed that seminal roots were inhibited by far-red light perceived by PHYA and by red light through both PHYA and PHYB signaling, whereas PHYC played no role in this response (Shimizu et al., 2009). In addition, blue light partially inhibited the growth of seminal roots, indicating that, in rice, phytochrome and blue light signaling regulate both seminal and AR growth, but to different degrees.

The growth angle of ARs was reversed in light in a gradual, dose-dependent manner, indicating that the AR system can adjust to a wide range of light intensities. Blue light was as effective as white light in redirecting ARs downward, whereas red and far-red light caused a partial redirection of ARs at the light intensities analyzed. Taken together, AR emergence and growth angle are under the control of red and blue light receptors and are inhibited by red and blue light to different degrees.

Blue light signal transduction via phototropin involves NPH3 of the NRL (NPH3/RPT2-like) family (Holland et al., 2009) that interacts directly with PHOT1

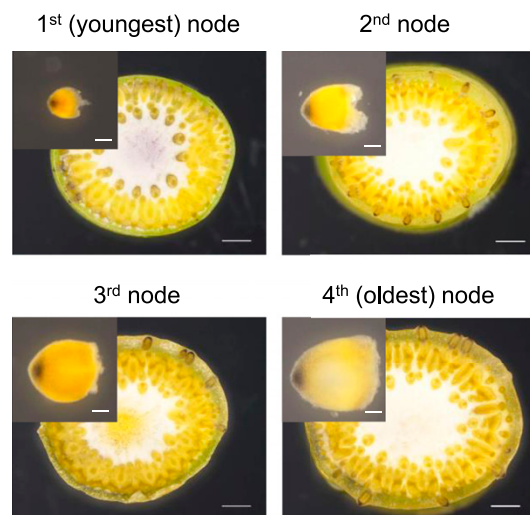


Figure 11. AR primordia possess statoliths. Cross sections of the first, second, third, and fourth nodes of rice cv PG56 were stained with Lugol's solution to visualize starch-containing statoliths in the root caps of AR primordia. Bars = 1 mm. Insets show enlarged images of isolated AR primordia. Bars = 0.1 mm.

(Motchoulski and Liscum, 1999). NPH3 acts as a substrate adapter in a CULLIN3-based E3 ubiquitin ligase (Roberts et al., 2011), with PHOT1 being a substrate in Arabidopsis. Both NPH3 and CUL3 were shown to be required for PHOT1 ubiquitination following blue light irradiation and may serve to internalize or degrade PHOT1 depending on the degree of ubiquitination (Roberts et al., 2011). CPT1 is a rice ortholog of Arabidopsis NPH3 (Haga et al., 2005). The *cpt1* mutant displays reduced negative phototropism of the primary root of rice seedlings in response to unilateral blue light, while the gravitropic response was not changed. Analysis of AR growth angle in the wild-type cv Nihonmasari and in *cpt1* mutant plants revealed no difference in blue light inhibition of AR emergence. However, *cpt1* plants showed a decreased sensitivity toward blue light with respect to the negative phototropic response, indicating that the regulation of the AR growth angle is mediated in part by CPT1. The *cpt1* mutant was still responsive to higher fluence rates of blue light, possibly due to the activity of redundant NRL family members. Haga et al. (2005) predicted 26 CPT1 homologs in rice.

In summary, we have shown that secondary root system architecture in rice is altered substantially during flooding. Ethylene promotes AR growth, and far-red, red, and blue light signaling control AR growth direction (Fig. 12). We propose that the environmental control of the AR system serves to keep ARs close to the waterlogged soil surface and, thus, to escape oxygen shortage, which, in turn, supports plant survival during flooding.

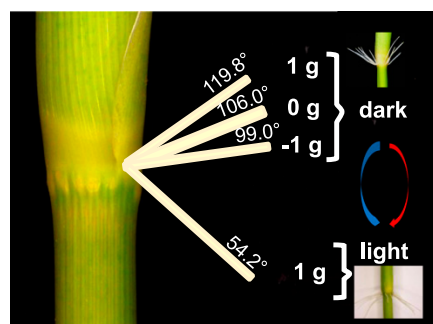


Figure 12. Scheme summarizing the impact of light and gravity on AR growth direction. The growth angle of 119.8° at 1 g in the dark was taken from Figure 1C, that of 106° at 0 g in the dark was taken from Figure 10B, that of 99° at -1 g in the dark was taken from Figure 10, D and F, and that of 54.2° in white light + ethephon was taken from Figure 7B.

CONCLUSION

Rice is a semiaquatic plant that generates AR primordia at each stem node that emerges upon flooding. For rice plants, stem nodes experience extended darkness when they are buried in soil or submerged in very turbid flood waters. In either case, the nodes are likely to become oxygen deficient. In the dark, ARs emerge and grow upward, possibly to escape this low-oxygen environment. The AR growth angle is modulated moderately by gravity, whereas the decision between upward and downward growth is regulated by light signaling through red and blue light receptors (Fig. 12). Hence, light is the most important environmental signal that shapes AR architecture in rice during flooding. We propose that light is an indirect cue for oxygen availability. Lack of this signal in the dark triggers an escape response.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seeds of rice (*Oryza sativa indica*) cv PG56 were originally obtained from the International Rice Research Institute. The *japonica* cv Nihonmasari and the *cpt1* mutant were donated by Moritoshi Iino (Osaka City University). The *japonica* cv Kinmaze was provided by Ko Shimamoto (Plant Molecular Genetics, Nara Institute of Science and Technology). The *japonica* cv ZH11 was obtained from Ping Wu (Zhejiang University). The *japonica* cultivar T65 was obtained from Motoyuki Ashikari (Nagoya University). The *japonica* cv Nipponbare was obtained from the Genome Research Centre. And the *japonica* cv Bomba was obtained from José L. García-Martínez (Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia). Plants were grown for 12 to 20 weeks according to Sauter (1997). Stem sections with a total length of 20 cm were cut 2 cm below the third node and incubated in a 150-mL beaker with 20 mL of tap water and 150 μ M ethephon where indicated. A plastic cylinder covered the beaker to ensure high humidity (Steffens and Sauter, 2005). Stem sections were kept in a growth chamber at 27°C and 70% humidity. To inhibit ethylene perception, stems were treated with 5 μ L L⁻¹ 1-MCP.

For partial submergence of rice plants, a 600-L tank was filled with tap water 2 d before plant treatment to adjust the water temperature to an average of 26.8°C. Experiments were carried out in the same growth chamber as experiments with stem sections at 27°C and 70% relative humidity. During partial

submergence, ~30% of the leaves remained above the water surface (Sasidharan et al., 2017).

Light Treatments

For soil experiments, 20-cm-long stem sections were placed in soil to a depth of 8 cm to monitor ARs at the third node that was 2 cm above the lower cut surface (6 cm below the soil surface). After the treatment, stems were removed from the soil, washed twice with tap water, and ARs were analyzed. Partially submerged plants were exposed to either darkness or light. The white light intensity perceived by leaves that remained in air was at least $362.3 \mu\text{mol m}^{-2} \text{s}^{-1}$ (EYE CERA ARC CMT220/W/BH; IWASAKI).

To incubate stem sections in the dark, the cylinder with which stems were covered was wrapped in aluminum foil. For treatment with white light, stems were exposed to $43 \mu\text{mol m}^{-2} \text{s}^{-1}$ white light (EYE CERA ARC CMT220/W/BH; IWASAKI). For far-red light treatment, a light-emitting diode (LED) lamp (Philips; LED E27 far-red light lamp) with a maximum wavelength of 730 nm was used. To provide blue or red light, an LED light (Thomann; LED Flood Panel 150) was used accordingly. The wavelengths of red light irradiance were 620 to 640 nm, and the wavelengths of blue light were 460 to 480 nm. The LED lamps were fixed 1 m above the cylinder, and light intensity was measured 2 cm below the third node of the stem sections. Five different light intensities were used for red and blue light treatments. Light intensities were measured from 250 to 799 nm with a double monochromator (DMC150; Bentham). Irradiance was measured every 1 nm as skip size and was normalized as $\text{mW m}^{-2} \text{nm}^{-1}$.

Measurement of Root Penetration Rate, Root Angle, and Root Length

The total number of AR primordia and the number of emerged roots were counted at each node, and the percentage of emerged ARs was calculated from that. The numbers given in the graphs are average percentages obtained from all stem sections (i.e. nodes) analyzed. The number of stems analyzed is provided for each experiment in the figure legends. Images of ARs were taken with a digital camera (PowerShot; SX220HS). The root angle was measured using ImageJ software (version 146r; National Institutes of Health). To determine AR lengths, the roots were excised and their lengths were measured with a ruler.

X-Ray Computed Tomography Imaging

Twenty-centimeter-long stem sections were cut such that the third node was 4 cm above the lower cut surface. Stems were placed up to 9 cm in Styrofoam. After 3 d of incubation in the dark, the buried part of the stem was scanned in a 180-kV nanofocus computed tomography system (GE Sensing & Inspection Technologies) and visualized with Volume Graphics VGStudio MAX software (GE Sensing & Inspection Technologies) at a voxel resolution of $120 \mu\text{m}$ or less.

Gravity Experiments and Statolith Staining

For clinostat experiments, each stem section was fixed in a 10-mL tube (Falcon) filled with 10 mL of tap water such that the third node remained outside the tube. Tubes were sealed with Parafilm to prevent water leakage. In order to provide an atmosphere of high humidity, a 50-mL centrifuge tube (Falcon) was placed above this setup. Stem sections were rotated vertically at a speed of 5 rpm in the dark. For inverse experiments, in one setup, stem sections were placed in a beaker in either upright or inverse orientation. In another setup, a stem section was fixed with its lower cut surface in a 10-mL tube (Falcon) filled with 10 mL of tap water and placed upright or inverted in a cylinder to ensure high humidity. In either setup, stems were incubated in the dark for 3 d. Subsequently, AR growth angles were determined.

In order to visualize the starch-containing statocytes in the root cap, cross sections including the AR primordia were cut from the first, second, third, and fourth nodes of cv PG56. In addition, we isolated intact ARs from each node. The samples were stained with Lugol's solution for 1 min and washed once with distilled water for 1 min.

Statistical Analysis

Statistical analyses were performed using Minitab. Cell death rates were calculated with $\arcsine\sqrt{(x/100)}$ formation to obtain distributed data. Comparison of

means was performed for statistical significance with an ANOVA Tukey's test or two-sample Student's *t* test. Constant variance and normal distribution of data were verified before statistical analysis. The *P* value was set to $P < 0.05$.

Accession Number

The accession number for CPT1 is AB186127.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. ARs growing at the first and second nodes of rice stems excised from deepwater rice plants.

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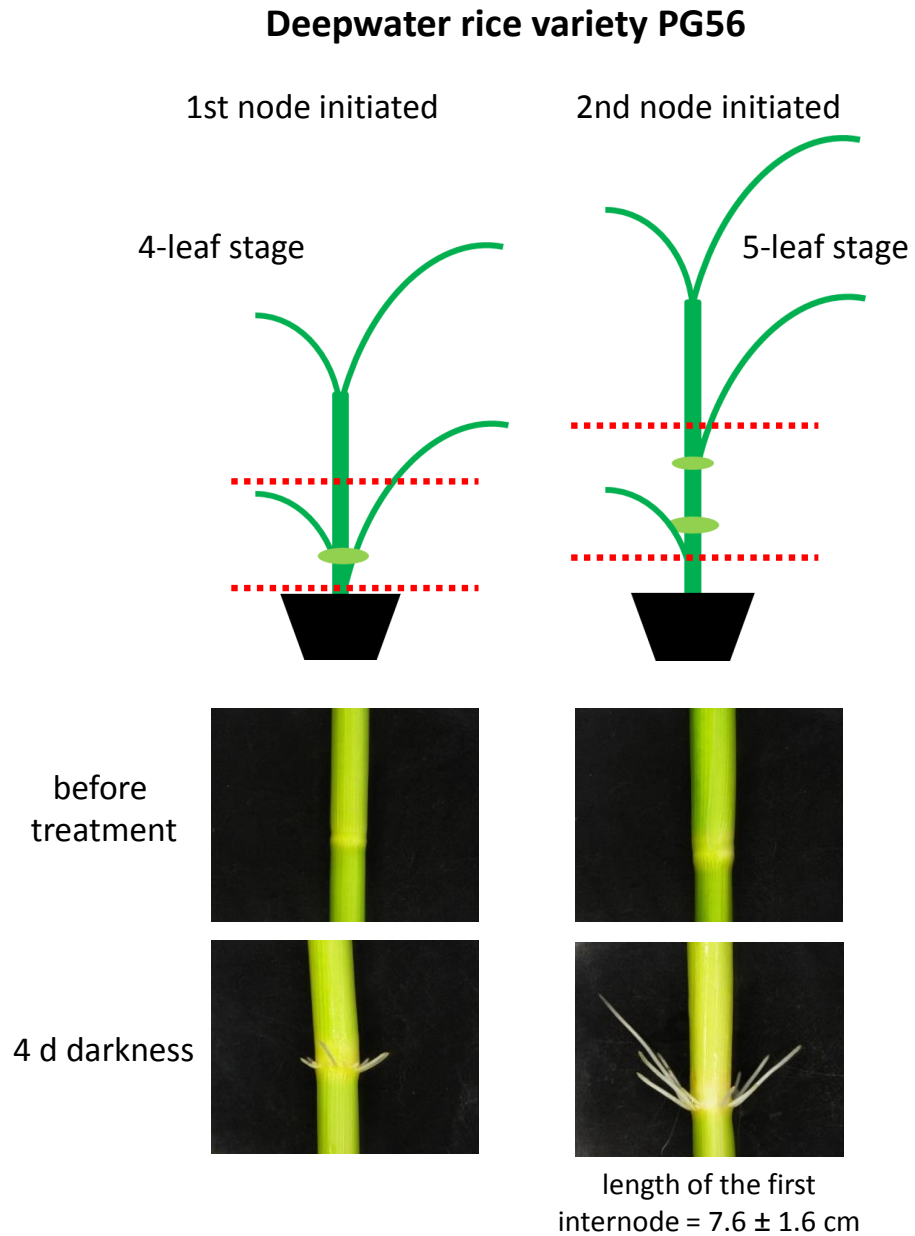
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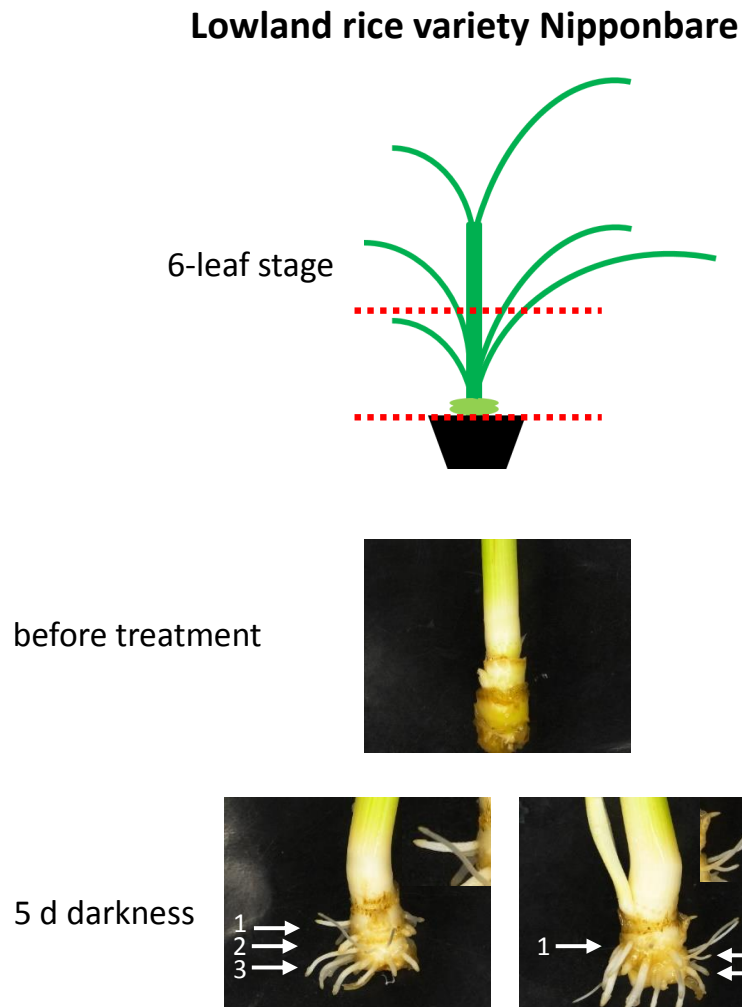
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Supplemental Figure S1A



Supplemental Figure S1A: Rice stems with one or two nodes were excised from deepwater rice plants, cultivar Pin Gaew 56 (PG56), that were in the 4- or 5-leaf stage (schematic), respectively, and exposed to 4 days of darkness. Shown are adventitious roots growing upward at the first (left) and second (right) node.

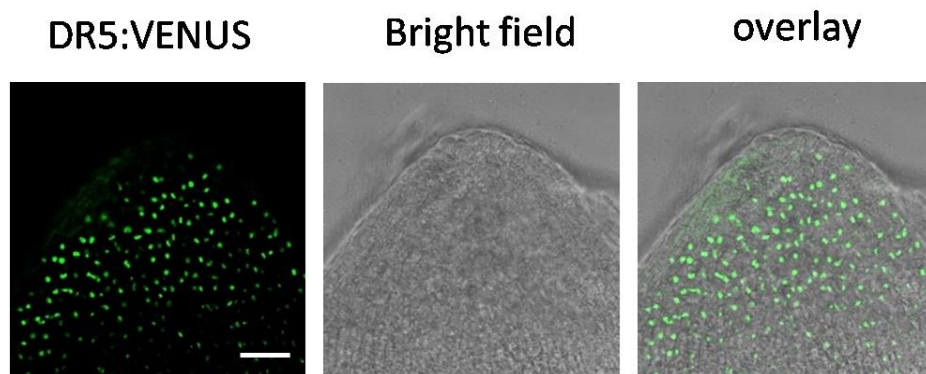
Supplemental Figure S1B



Supplemental Figure S1B: Rice stems were excised from Nipponbare plants in the 6-leaf stage and exposed to 5 days of darkness. Numbers indicate nodes (1-3: from youngest to oldest). Internodal elongation has not occurred. Yet, some of the adventitious roots at nodes 2 and 3 grow upward.

7. Chapter 2

Polar auxin transport determines adventitious root emergence and growth in rice





Polar Auxin Transport Determines Adventitious Root Emergence and Growth in Rice

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Flooding is a severe limitation for crop production worldwide. Unlike other crop plants, rice (*Oryza sativa* L.) is well adapted to partial submergence rendering it a suitable crop plant to understand flooding tolerance. Formation of adventitious roots (ARs), that support or replace the main root system, is a characteristic response to flooding. In rice, AR emergence is induced by ethylene and in the dark where roots grow upward. We used the synthetic auxins 2,4-D and α -NAA, and the auxin transport inhibitor naphthylphthalamic acid (NPA) to study emergence, growth rate and growth angle of ARs. While α -NAA had no effect, NPA and 2,4-D reduced the root elongation rate and the angle with a stronger effect on root angle in the dark than in the light. Furthermore, NPA delayed emergence of AR primordia suggesting that efflux carrier-mediated auxin transport is required for all aspects of directed AR growth. Expression analysis using *OsPIN:GUS* reporter lines revealed that *OsPIN1b* and *OsPIN1c* promoters were active in the stele and root cap in accord with their predicted role in acropetal auxin transport. *OsPIN2* was expressed at the root tip and was reduced in the presence of NPA. Auxin activity, detected with *DR5:VENUS*, increased in primordia following growth induction. By contrast, auxin activity was high in epidermal cells above primordia and declined following growth induction suggesting that auxin levels are antagonistically regulated in AR primordia and in epidermal cells above AR primordia suggesting that auxin signaling contributes to the coordinated processes of epidermal cell death and AR emergence.

Keywords: adventitious roots, polar auxin transport, auxin efflux carrier, root angle, root emergence, rice, flooding

INTRODUCTION

Rice is semi-aquatic showing remarkable tolerance to partial submergence. Adventitious root (AR) primordia form at each node during normal development that emerge during submergence to replace the original dysfunctional root system (Sauter, 2013). AR formation is regarded as a characteristic response to flooding not only in rice but also in other flooding tolerant and flooding-intolerant plants such as tomato (*Lycopersicon esculentum*) (Vidoz et al., 2010) and pecan tree (*Carya illinoensis*) (Smith and Ager, 1988). ARs are located closer to the shoot than the original root system thereby facilitating oxygen supply. Furthermore, ARs take up oxygen from flood waters further improving their oxygen status. Recent work on rice revealed that ARs grow upward in the dark such that roots get closer to the oxygen-rich water surface (Lin and Sauter, 2018). The hormones and molecular mechanisms that control AR growth direction need yet to be elucidated. This study sets out with the mechanistic analysis by studying the role of auxin transport and

signaling in AR growth. In the past decades, the number of flooding events has increased worldwide causing increasing crop losses (Voeseinek and Bailey-Serres, 2015). It is hence rather timely to decipher in more depth the molecular mechanisms that control AR growth in flooded crop plants.

In rice and *Solanum dulcamara*, the gaseous hormone ethylene that accumulates in submerged tissues due to a reduced diffusion rate in water, promotes AR growth (Drew and Sisworo, 1979; Lorbiecke and Sauter, 1999; Dawood et al., 2016). Both, ethylene-induced AR emergence and AR growth rate are enhanced by gibberellic acid and inhibited by abscisic acid (Steffens and Sauter, 2005; Steffens et al., 2006).

Unlike growth of nodal ARs, growth of crown roots in rice seedlings is inhibited by ethylene. Inhibition of root growth by ethylene and auxin has been reported for Arabidopsis where ethylene and auxin pathways interact (Stepanova et al., 2007; Swarup et al., 2007). A recent study in rice seedlings revealed that auxin signaling downstream of ethylene is required for root growth inhibition by ethylene (Chen et al., 2018) suggesting similar hormonal crosstalk in monocots and dicots. Auxin signal transduction through SCF^{TIR1/AFBs}-Aux/IAAs has been well studied (Salehin et al., 2015). The E3 ubiquitin ligase SCF^{TIR1/AFB} recognizes Aux/IAA proteins as a target upon binding of auxin to the F-Box subunit TIR1/AFB resulting in their ubiquitination and proteasome-dependent degradation. Canonical Aux/IAA proteins act as transcriptional repressors of auxin response factors (ARFs). Their degradation allows transcription of ARFs which regulate auxin-responsive genes including most Aux/IAA genes, hence providing a negative feedback loop (Salehin et al., 2015). In rice seedlings, ethylene signaling induces *OsIAA26* expression whereby the *OsIAA26* protein stability is controlled by the auxin-dependent E3 ubiquitin ligase Soil-surface Rooting 1 (SOR1). This study hence revealed a molecular link between ethylene and auxin signaling in rice root development.

A widely used tool to monitor auxin distribution *in planta* is DR5-based auxin-inducible reporters. The DR5 promoter contains several ARF binding sites resulting in reporter gene induction in the presence of auxin (Ulmasov et al., 1997). A more recently developed auxin reporter uses the destabilizing DII-domain of an Aux/IAA protein that is fused to the fluorescent VENUS protein. An increase in auxin results in degradation of DII-VENUS as an immediate response to SCF^{TIR1/AFB} activation through auxin binding (Brunoud et al., 2012). Local auxin accumulation due to synthesis or polar auxin transport can be tracked with these reporters (Brumos et al., 2018) which aids in deciphering the contribution of an auxin gradient to a development process. Chemiosmosis is widely accepted as mechanism of polar auxin transport (Rubery and Sheldrake, 1974; Raven, 1975; Adamowski and Friml, 2015). While auxin diffuses passively into the cell through the membrane or by way of facilitated diffusion through AUX1/LAX influx carriers, the efflux of the IAA anion requires auxin efflux carriers of the PIN-FORMED (PIN) protein family or ATP-Binding Cassette family B (ABCB) transporters. PIN and ABCB activity is a target of the auxin transport inhibitor 1-naphthylphthalamic acid (NPA) and of flavonoids that interfere with the interaction of ABCB and its partner TWISTED DWARF 1 (TWD1) (Bailly et al., 2008).

The direction of auxin flux and the establishment of an auxin gradient are achieved by the polar distribution of PINs in the plasma membrane (Tanaka et al., 2006). For Arabidopsis, seven PIN genes and for rice, twelve PIN genes were reported (Wang et al., 2009). In Arabidopsis roots, auxin moves acropetally in the central cylinder driven by AtPIN1 activity. Auxin is laterally deflected in the root cap mediated by AtPIN3, AtPIN4 and AtPIN7 activity and moves basipetally in the peripheral cell layers due to AtPIN2 localized in the apical part of the plasma membrane (Vanneste and Friml, 2009). The PIN1 clade has diversified into four members in rice, OsPIN1a-d. OsPIN10a and OsPIN10b are most closely related to AtPIN3, AtPIN4 and AtPIN7, whereas the PIN2 clade is represented by a single member in Arabidopsis and rice (**Supplementary Figure S1**).

In rice, little is known about the role of auxin and polar auxin transport in the development of ARs. A number of genes related to crown root formation and growth have been identified (Liu et al., 2005; Zhao et al., 2009, 2015). *Crown-rootless 4 (crl4)* and *OsGNOM1* show reduced crown root initiation and delayed lateral root differentiation (Kitomi et al., 2008; Wang et al., 2011). *OsGNOM1* controls PIN1 trafficking within the cell, and thus alters polar auxin transport that is required for the formation of an auxin gradient which triggers the asymmetrical division of parenchyma cells that develop into crown roots (Richter et al., 2010). Interestingly, an RNAi-knockdown line for the *OsPIN1b* gene displays a reduced AR penetration rate at the seedling stage (Xu et al., 2005).

In this study, we investigated auxin activity and *OsPIN* gene promoter activities with high spatial resolution during AR emergence and growth. Our results indicate that local auxin gradients determine AR penetration, growth rate and growth direction and thereby have a deep impact on the overall architecture of the AR system.

RESULTS

Inhibition of Auxin Transport Inhibits AR Penetration and Growth and Reduces the Root Growth Angle

To study the role of auxin on AR growth in rice, we employed the auxin transport inhibitor naphthylphthalamic acid (NPA) and two auxin analogs, 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthalene acetic acid (α -NAA). Rice stem sections were treated with NPA, 2,4-D or α -NAA in the dark (**Figure 1**). Darkness induced AR emergence with an upward direction of AR growth as described previously (Lin and Sauter, 2018). The root penetration rate, i.e., the number of AR primordia that emerged from the node in relation to the total number of AR primordia, given as percentage, was not significantly altered by 2,4-D or α -NAA at concentrations of 0.3 to 5 μ M whereas inhibition of auxin efflux at 5 μ M NPA significantly reduced AR penetration (**Figure 1B**). The length of penetrated ARs was reduced by NPA and 2,4-D in a dose-dependent manner, with an average of 0.3 cm at 5 μ M 2,4-D compared to 1.9 cm in the absence of 2,4-D. By contrast, α -NAA did not inhibit AR elongation revealing a functional similarity

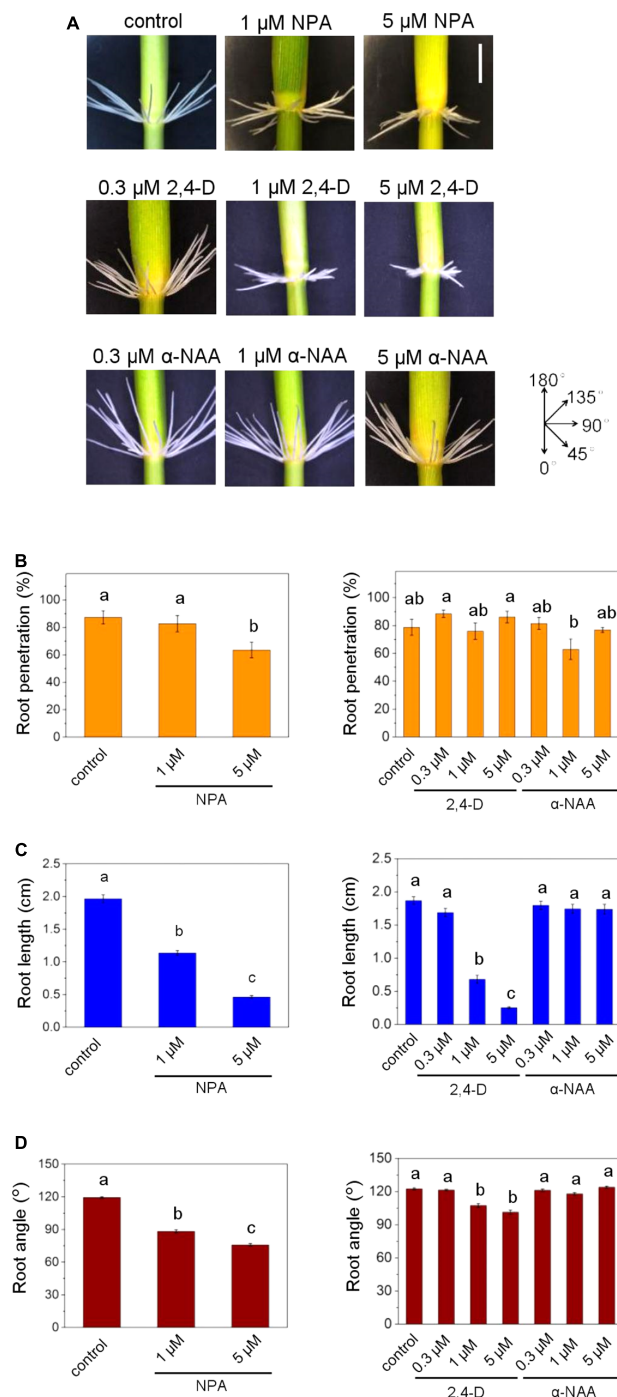


FIGURE 1 | Auxin transport inhibition alters emergence, growth rate and angle of adventitious roots (ARs) in rice. **(A)** ARs were analyzed at the third node using stem sections kept in the dark for 3 days in the presence of NPA, 2,4-D or α -NAA at the concentrations indicated. Phenotypes of ARs exposed to the auxins 2,4-D or α -NAA or to the auxin transporter inhibitor NPA; bar=1 cm. **(B)** Percentage of penetrated ARs. **(C)** Average lengths of penetrated ARs. **(D)** Mean growth angles of ARs. Bars in **B–D** indicate means (\pm SE) of ARs measured from nine stems per treatment in three independent experiments. Different letters indicate statistically significant differences ($P < 0.05$; ANOVA with Tukey test).

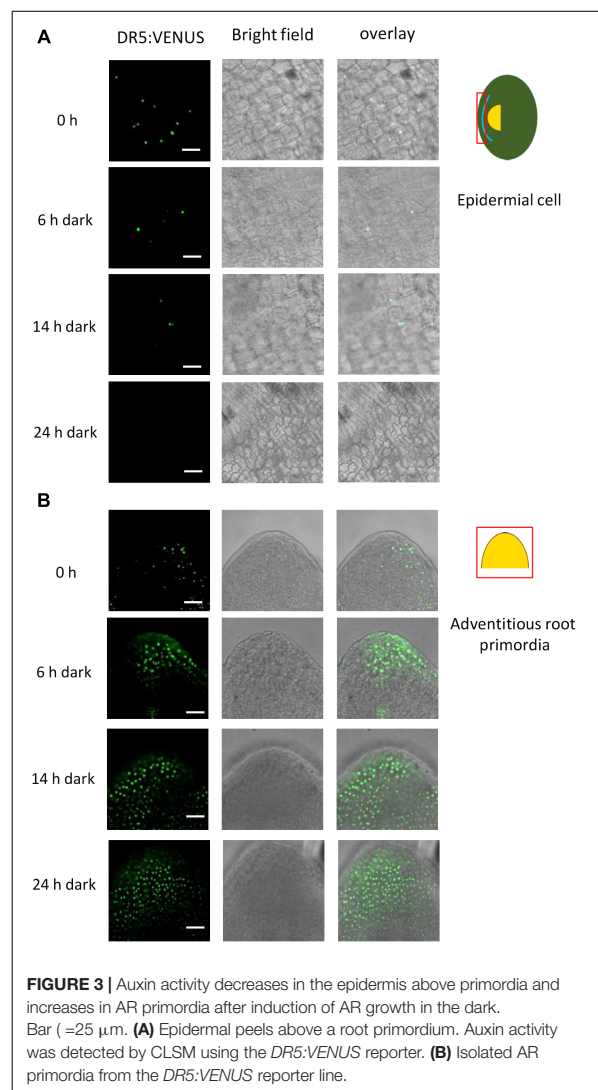
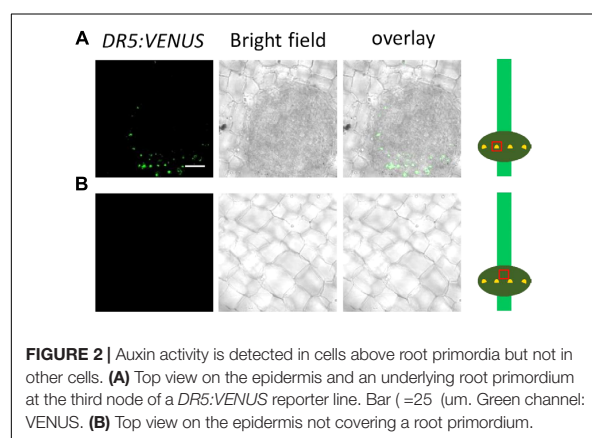
between auxin efflux inhibition by NPA and 2,4-D activity and a functional difference between the two synthetic auxins 2,4-D and α -NAA (Figure 1C). Similarly, the AR angle decreased in the presence of 2,4-D and NPA but not after exposure to α -NAA (Figure 1D). Taken together, our data suggested that increased intracellular auxin levels induced by chemical inhibition of auxin efflux through NPA or by supplying 2,4-D, that is taken up by cells but not actively secreted by auxin efflux carriers, reduced AR elongation and growth angle whereas the auxin analog α -NAA that is actively exported from the cell through efflux carriers (Delbarre et al., 1996; Simon et al., 2013) did not.

Adventitious root emergence is facilitated by programmed death of epidermal cells covering root primordia (Mergemann and Sauter, 2000). To study a possible involvement of auxin in epidermal cell death, we employed a *DR5:VENUS* auxin reporter line (Figures 2A,B). Fluorescence microscopy revealed auxin activity in epidermal cells above root primordia and/or the primordia but not in other epidermal cells indicating a specific function of auxin.

For better spatial resolution, we peeled off the epidermis above a root primordium and isolated root primordia (Figure 3). Interestingly, when AR growth was induced by transfer to the dark, auxin activity appeared to decline within 6 h in epidermal cells covering a primordium (Figure 3A). At the same time, auxin activity increased in AR primordia following growth induction (Figure 3B). A time course analysis of epidermal cell death (Figure 4A) and root penetration (Figure 4B) showed that 5 μ M NPA delayed both and resulted in reduced auxin activity at the AR apex (Figure 4C). These findings suggest that auxin activity coordinates epidermal cell death and AR emergence. The data further indicated that polar auxin transport through efflux carriers controls auxin levels.

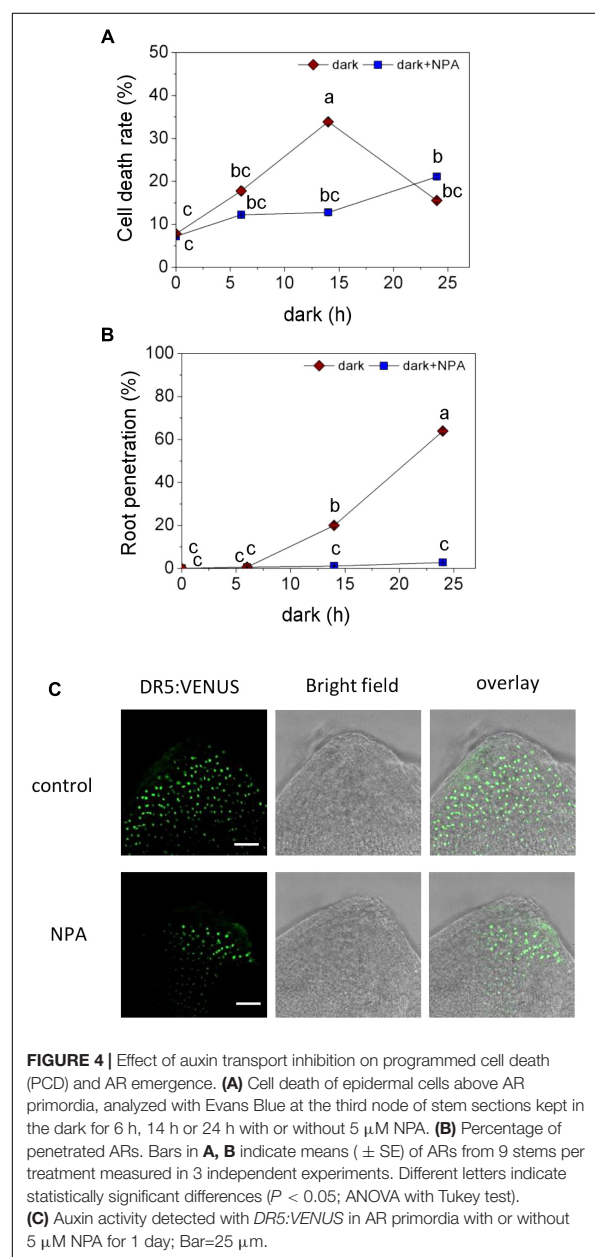
OsPIN Genes Are Differentially Expressed in ARs and in Epidermal Cells Above ARs

PINs are carrier proteins that facilitate auxin efflux from plant cells and are responsible for directed auxin flux in plant organs.

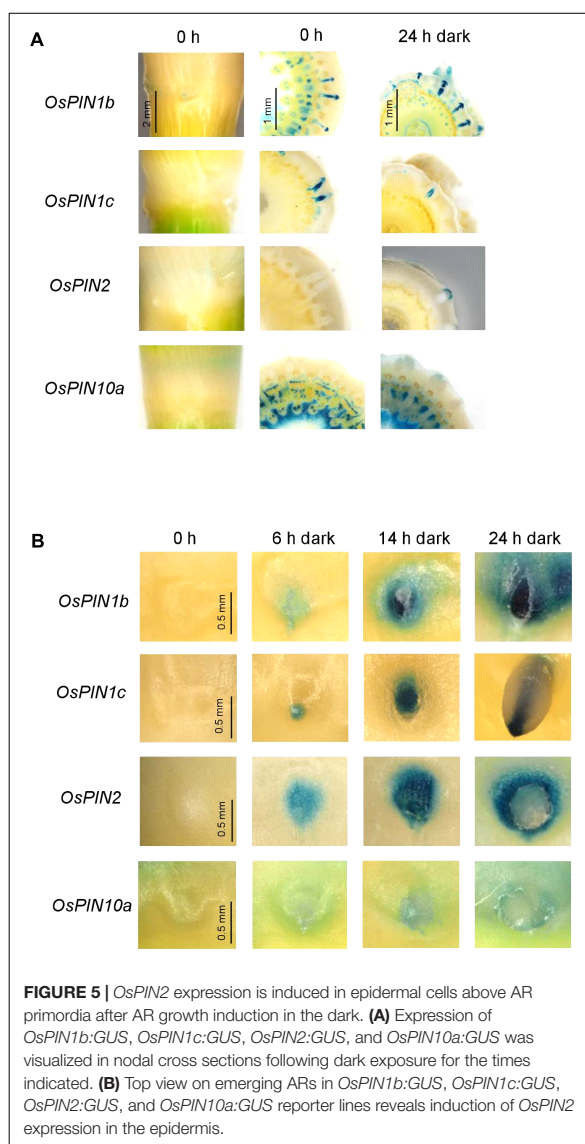


PINs are encoded by gene families with 7 members in Arabidopsis and 12 members in rice (Supplementary Figure S1) and have mostly been studied in Arabidopsis (Křeček et al., 2009). In Arabidopsis roots, PIN1 drives acropetal auxin transport in the central cylinder, PIN3, 4, and 7 redirect the auxin flux in the root tip to the outer cell layers and PIN2 drives basipetal auxin flow in the outer root cell layers. To find out whether homologous transporters in rice might be involved in generating auxin gradients in ARs and at the site of AR emergence, we obtained GUS reporter lines for the Arabidopsis *PIN1* orthologs *OsPIN1b* and *OsPIN1c*, for the *PIN2* ortholog *OsPIN2* and for *OsPIN10a* which is phylogenetically most closely related to PINs 3, 4 and 7 (Supplementary Figure S1).

OsPIN1b:GUS and *OsPIN1c:GUS* was expressed in AR primordia and penetrated ARs (Figure 5A and



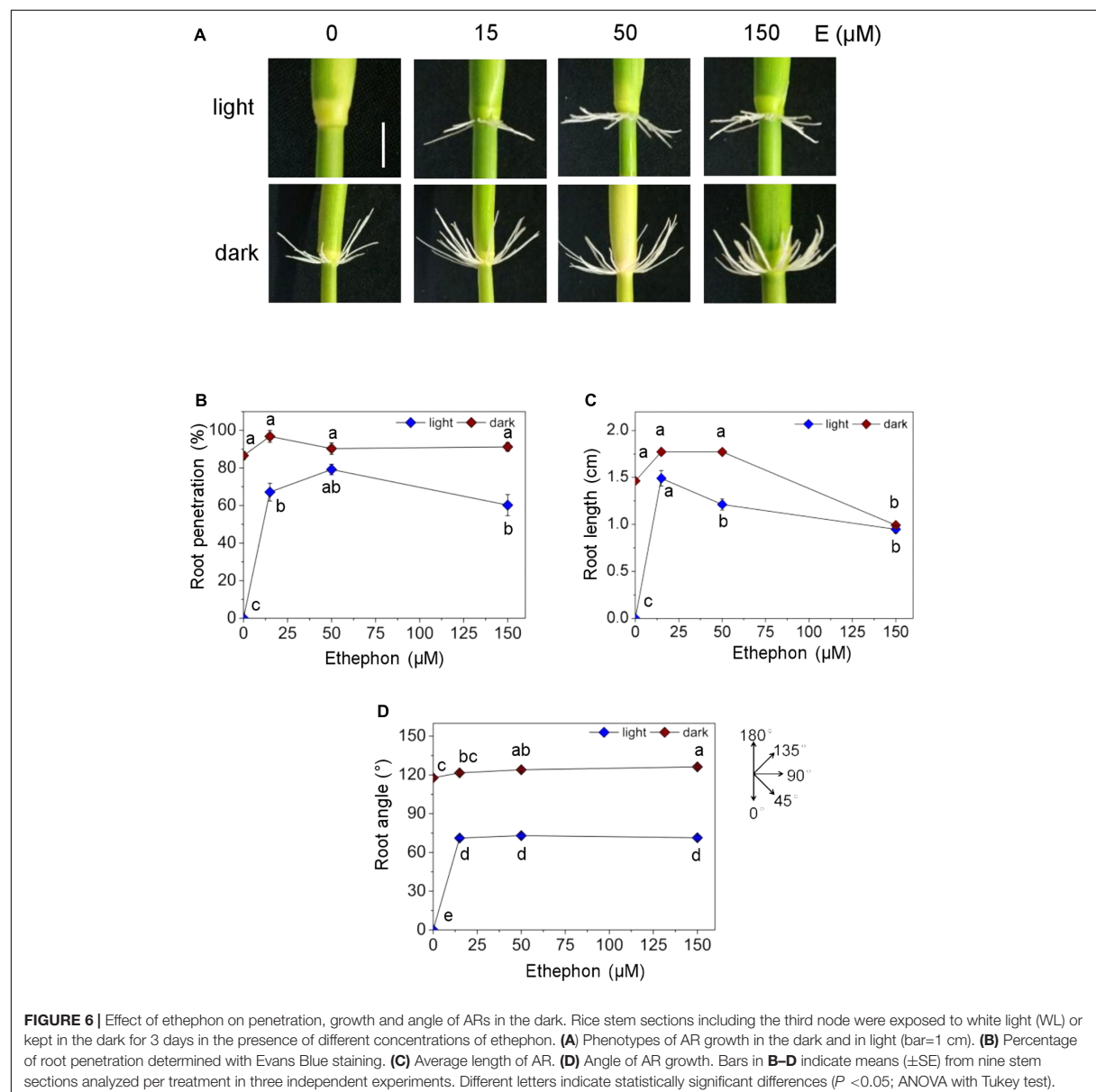
Supplementary Figure S2A). *OsPIN2:GUS* expression was not detected in uninduced nodes and appeared in epidermal cells above AR primordia within 6 h after transferring to the dark to trigger AR emergence (Figures 5A,B). *OsPIN2:GUS* was also active at the apex of emerged ARs in what appeared to be the root cap (Supplementary Figure S2). *OsPIN10a:GUS* expression was observed at AR tips within 24 h after transfer to the dark and was maintained during subsequent AR elongation (Figure 5A and Supplementary Figure S2A). Overall, the results revealed



regulation of *OsPIN* gene expression following growth-inducing dark treatment. Interestingly, *OsPIN2* expression was induced specifically in epidermal cells above AR primordia following growth induction coincident with reduced auxin activity in these cells (Figure 3A). These findings suggest that *OsPIN2*-mediated auxin depletion in the epidermis promotes epidermal cell death and root penetration.

Auxin Transport Is Required for AR Growth Promotion by Ethylene

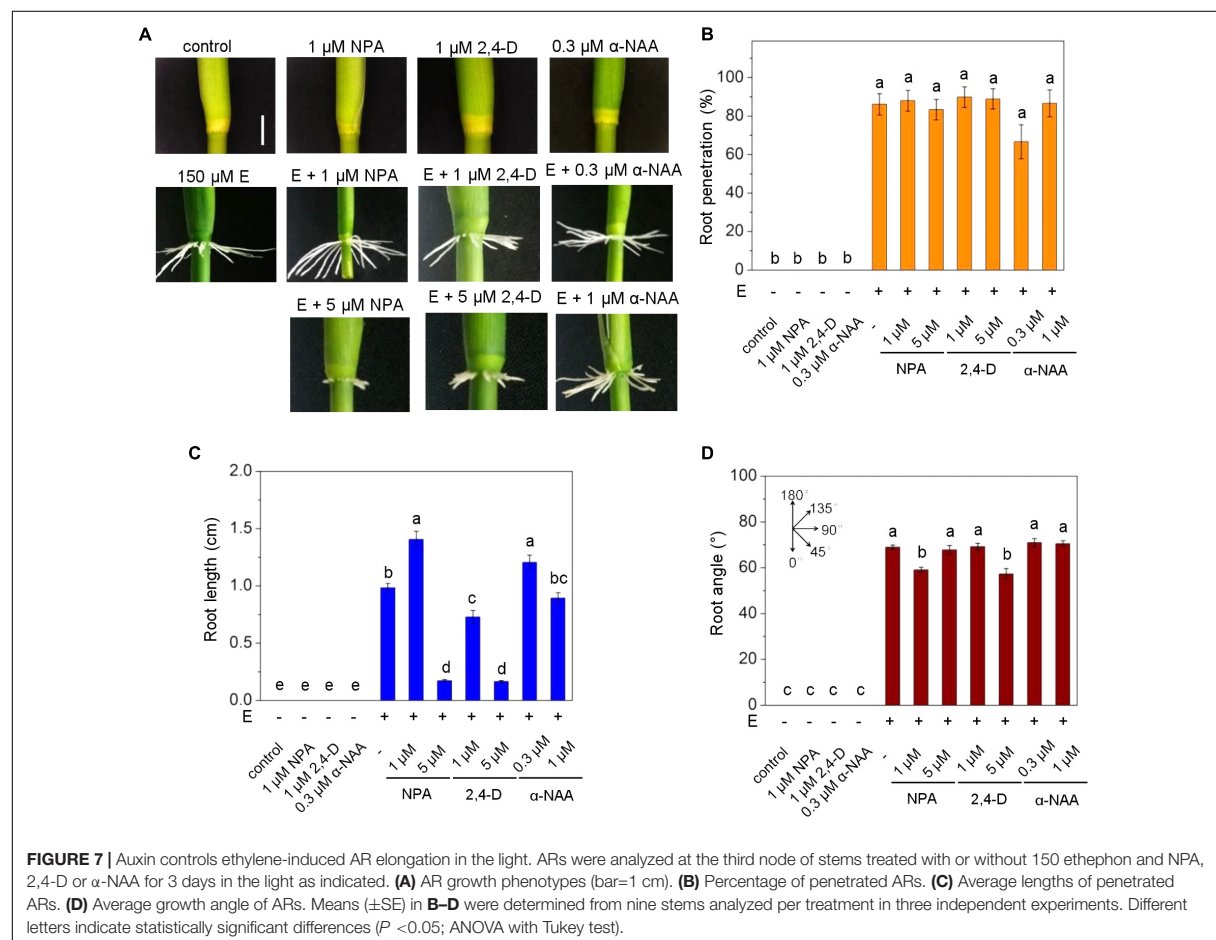
Adventitious roots emerge in the dark but not in the light (Figures 6A,B). Emergence in the dark was related to dark-induced ethylene synthesis (Fukao et al., 2012) which is in



accord with the observation that ethylene promotes emergence and growth of ARs in the light (Figures 6A,B; Mergemann and Sauter, 2000). A dose-response analysis of AR growth revealed that root emergence was elevated to 67.1% at 15 μM ethephon, an ethylene-releasing compound, and remained at this level at higher concentrations while the emergence rate in the dark was close to 100% (Figure 6B). AR elongation was similar in the light and in the dark at 15 and 150 μM ethephon and was lower in the light than in the dark at 50 μM ethephon possibly indicating different responsiveness to ethylene (Figure 6C). ARs grew upward in the dark and downward in the light as described

previously (Figures 6A,D; Lin and Sauter, 2018). While the growth angle in the light was independent of ethephon, the growth angle in the dark increased slightly but significantly from 117.8 to 126.2 $^{\circ}$ with increasing ethephon concentration.

In order to investigate a possible cross-talk between auxin and ethylene in AR growth, we exposed stem sections to ethephon together with NPA, 2,4-D or α -NAA for 3 days (Figure 7). Neither 2,4-D or α -NAA nor auxin transport inhibition by NPA altered AR penetration in the light in the long term (Figure 7B) whereas root length was significantly elevated from 1 to 1.4 cm at 1 μM NPA and reduced to 0.2 cm at 5 μM NPA revealing



a dose-dependent opposite response. Root elongation was also promoted by low ($0.3 \mu\text{M}$) α -NAA and was inhibited by 1 and $5 \mu\text{M}$ 2,4-D in a dose-dependent manner suggesting that the native auxin activity is at a suboptimal level in ethephon-induced ARs (**Figure 7C**). The growth angle was slightly reduced at $1 \mu\text{M}$ NPA and $5 \mu\text{M}$ 2,4-D and was unaffected by α -NAA (**Figure 7D**). These findings support the idea that ethylene overrides the inhibitory effect of NPA on AR emergence in the long run whereas control of AR elongation and, to a lesser degree, growth angle by auxin is not. Since NPA and 2,4-D reduced the growth angle in the dark and, to a minor degree, in the light, we next investigated a possible contribution of auxin in redirecting AR growth.

Time Course Analysis of AR Redirection

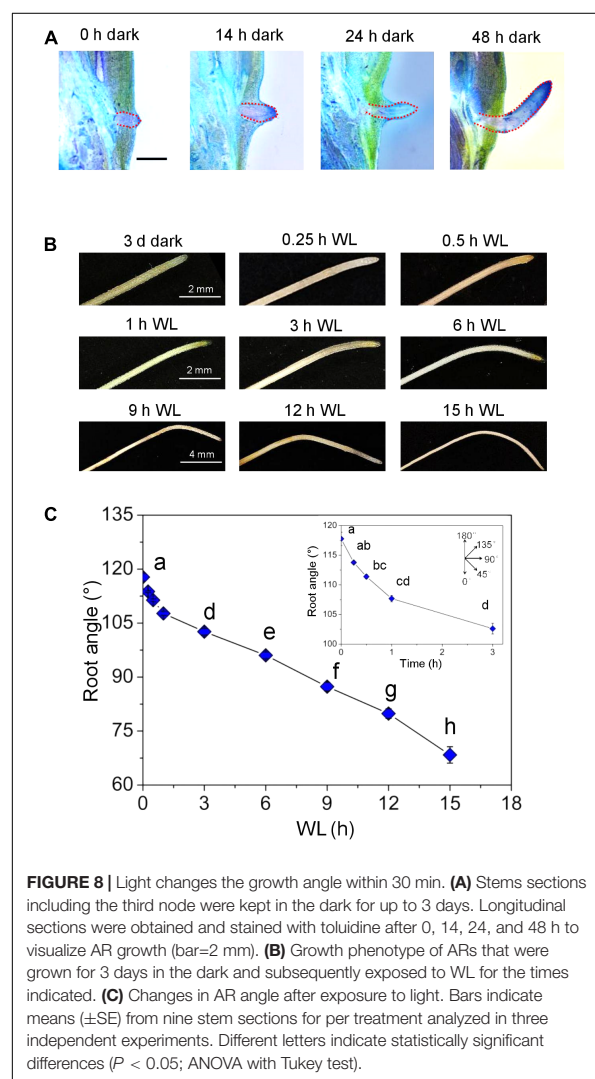
To study the mechanisms underlying redirection of AR growth, we established a time course of AR bending induced by light (**Figure 8**). Stem sections were exposed to darkness for 2 days to induce AR growth at an upward angle as visualized by toluidine-stained cross sections (**Figure 8A**). In the dark, emerged ARs were bending upward after 24 h. The upward angle of growth was

fully established after 48 h. After 3 days in the dark, stems were exposed to white light (WL) and the root angle was monitored. Roots grew at an angle of 117.8° in the dark (**Figures 8B,C**). A significant change in growth direction to 111.4° was measured after 30 min. Within 15 h, the growth angle reached 68.3° which is about the angle at which ARs grow in the light (**Figure 6D**).

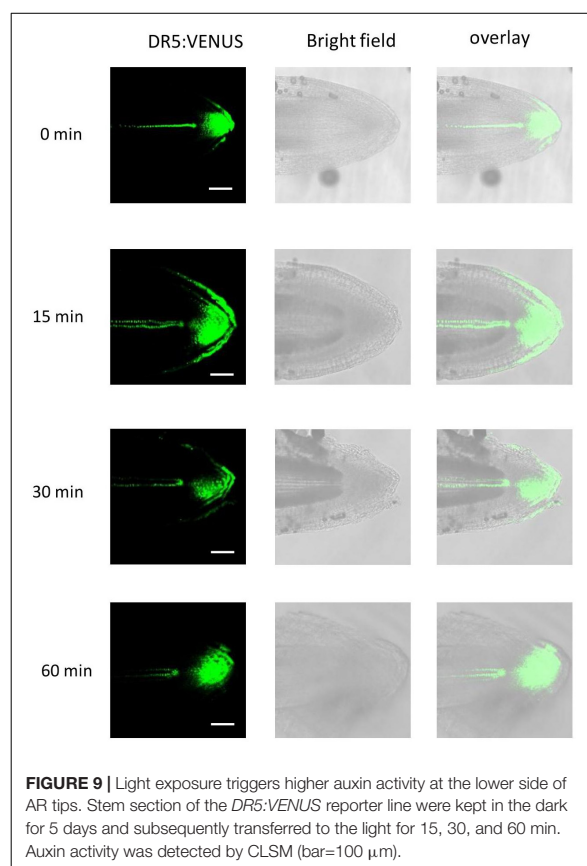
Gravitropic root growth is initiated by starch-containing statoliths in root cap statocytes. To exclude a possible role of statoliths in light-induced redirection of AR growth, statoliths were visualized at the root tip as brown precipitate from Lugol's starch staining (**Supplementary Figure S3**). Neither exposure of stems to darkness in an upright or inverse orientation, nor a shift from dark to light for 1, 3 or 6 h altered the detectable pattern of starch staining.

OsPIN Expression and Auxin Distribution in ARs That Change Their Growth Direction

To test whether a change in growth direction of ARs is accompanied by altered expression of *OsPIN* genes, we



transferred rice stems that were kept in the dark for 3 days to induce AR growth at an upward angle, to the light for 0.5, 1, or 3 h (**Supplementary Figure S2A**). *OsPIN1b:GUS* expression was detected in the stele and in the root cap. Expression in the root cap was intensified within 3 h of exposure to light. A similar expression pattern was observed when ARs were exposed to ethephon in the light indicating that *OsPIN1b* expression is under light control (**Supplementary Figure S2B**). *OsPIN1c:GUS* was active at the root apex, xylem and stele tissues but not in lateral root cap cells. Neither transfer from dark to light (**Supplementary Figure S2A**) nor growth in the light with ethephon changed *OsPIN1c:GUS* expression (**Supplementary Figure S2B**). *OsPIN2:GUS* and *OsPIN10a:GUS* displayed a patchy expression pattern at AR tips in what appeared to be root cap cells that appeared to become stronger within 3 h after transfer to the light and weaker in the presence



of NPA (**Supplementary Figure S2C**). Overall, the expression patterns were in accord with *OsPIN1b* and *OsPIN1c*-driven auxin transport in the stele, redistribution of auxin at the root tip and auxin transport in root cap cells by *OsPIN2* and *OsPIN10a*. Altered expression of *OsPIN1b* and *OsPIN2* suggested an altered auxin distribution in response to the dark-to-light switch.

To test if altered *OsPIN* gene expression affects auxin distribution, we employed the *DR5:VENUS* reporter (**Figure 9**). AR growth was induced in the dark. After 5 days in the dark, the stems were transferred to the light and auxin distribution was visualized at the root tip for up to 1 h. Auxin appeared to accumulate at the lower side within 30 min suggesting that elevated auxin inhibits growth at this side resulting in downward bending of the root (**Figure 8C**). In summary, polar auxin transport likely regulates the angle at which ARs grow indicating that auxin plays a key role in shaping the AR system architecture in rice.

DISCUSSION

Plant root systems mainly consist of secondary roots that develop postembryonically from the primary root as lateral

roots or from the shoot as ARs. Auxin is a key hormone that regulates secondary root formation and growth (Olatunji et al., 2017). In tomato, induction of ARs during flooding is mediated by ethylene that promotes auxin flux toward the submerged stem thereby increasing local auxin activity (Liu et al., 2009; Vidoz et al., 2010). In rice, the Lateral Organ Boundaries Domain (LBD) transcription factor Crown Rootless1/Adventitious Rootless1 (CRL1/ARL1) acts downstream of ARFs to control the formation of ARs (Inukai et al., 2005; Liu et al., 2005). Consequently, *arl1* mutants have fewer ARs at their stem nodes. While auxin is well known to promote formation of secondary roots including formation of ARs, the role of auxin in AR emergence and growth is less well studied. In rice, AR formation is developmentally programmed whereas emergence and growth are induced by environmental cues, rendering rice highly suitable to study these aspects. Flooding promotes AR emergence through ethylene signaling. The AR growth-promoting activity of ethylene is enhanced by gibberellic acid and repressed by abscisic acid (Steffens et al., 2006).

Our current study supported a role for polar auxin transport in shaping the AR system since inhibition of polar auxin transport by NPA reduced AR emergence, AR elongation growth and the angle at which roots grew in the dark. A similar phenotype was observed after treatment with the synthetic auxin 2,4-D. PIN proteins facilitate auxin efflux of the natural auxin IAA and of the synthetic auxin α -NAA but not of 2,4-D supporting the conclusion that high levels of 2,4-D accumulate in plant cells and thereby mimic inhibition of efflux carriers by NPA (Delbarre et al., 1996). By contrast, α -NAA did not similarly, alter AR growth, presumably because local auxin activity did not increase. Interestingly, when ARs were induced by ethylene to grow in the light, the growth rate was strongly repressed by NPA and 2,4-D whereas emergence and growth angle were not or only marginally affected suggesting that responsiveness to auxin differs in the light and in the dark. A previous study on rice showed that repression of the auxin efflux carrier gene *OsPIN1* repressed AR emergence supporting the conclusion that locally altered auxin activity controls AR development (Xu et al., 2005).

Analysis of defined PIN genes revealed differential expression patterns in ARs and epidermal cells above ARs. Expression patterns were in accord with their predicted functions based on protein similarity to Arabidopsis homologs. *PIN1b* and *PIN1c* were predominately expressed in the central cylinder as is *AtPIN1* which facilitates acropetal auxin movement. *PIN1b* and *PIN1c* were further expressed in the root cap where expression was enhanced when roots were exposed to light possibly indicating that *PIN1b* contributes to control of AR growth direction. Expression of *OsPIN1c* in AR primordia agrees with the observation that auxin levels are high at the primordia apex. High auxin activity at the root apex has also been shown for Arabidopsis (Petersson et al., 2009).

An unexpected and previously not reported finding was the accumulation of auxin specifically in epidermal cells covering root primordia but not in other epidermal cells. Programmed

cell death (PCD) of epidermal cells was suggested to facilitate AR emergence (Mergemann and Sauter, 2000). Epidermal cells covering AR primordia were shown to have a unique molecular identity that allows them to undergo PCD in response to a specific trigger (Steffens and Sauter, 2009). Induction of PCD requires the hormonal signal ethylene and in addition a mechanical signal that provides spatial resolution and thereby limits PCD to the site where an AR emerges (Steffens et al., 2012). Ethylene signaling sets the stage in two ways, for one by promoting AR growth and, secondly, by activating the cell death program that, however, is executed only in cells that perceive mechanical force exerted by the growing AR primordium. Auxin that accumulates in epidermal cells could be synthesized locally in the epidermis itself or in the root tip from where it is transported to epidermal cells above it. However, auxin activity was low in AR primordia. Alternatively, efflux carrier-mediated auxin transport in epidermal cells could generate an auxin maximum in these cells. However, none of the efflux carrier genes analyzed was expressed at detectable levels in epidermal cells. Finally, it is possible, that local auxin synthesis is responsible for elevated auxin activity in these cells. The decline in auxin activity in epidermal cells above AR primordia upon growth induction of ARs in the dark occurs in parallel, and possibly as a consequence of, elevated *OsPIN2* expression in these epidermal cells and suggests a role of polar auxin transport in epidermal cells during AR emergence. Inhibition of polar auxin transport by NPA delayed epidermal cell death and reduced the rate of AR emergence. Taken together, our findings indicate that controlled local auxin activities through polar auxin transport contribute to rapid weakening of the epidermis as a physical barrier. At the same time, auxin activity in the root meristem increases likely promoting cell division activity.

The growth angle contributes substantially to AR system architecture. It is controlled by red and blue light signaling (Lin and Sauter, 2018). Upward AR growth in the dark is reverted to a downward angle in the light. The change in growth angle occurs within 30 min. Polar auxin transport is required to maintain an upward growth angle in the dark. It seems reasonable to assume that directed auxin flux is altered to change AR growth direction after exposure to light. Fluorescence auxin reporter data indicate that auxin accumulates at the lower side within 30 min. Auxin is known to inhibit root growth already at low concentrations. It is hence conceivable that growth is slowed down at this side resulting in downward bending of the AR tip. Whether differential expression of auxin carrier genes or regulation at the protein level alters efflux carrier activity remains to be resolved. In Arabidopsis, the growth direction of the primary root changes in response to hypoxic conditions such that the primary root no longer grows downward (Eysholdt-Derzso and Sauter, 2017). While auxin activity was asymmetrically distributed between epidermal cells of either side of the primary root, AtPIN2 protein, responsible for auxin transport in this cell layer, was symmetrically distributed. AtPIN2 distribution in the plasma membrane is regulated via phosphorylation/dephosphorylation (Santner and Watson, 2006; Michniewicz et al., 2007; Adamowski

and Friml, 2015) pointing to multiple levels of auxin transport regulation in plants.

CONCLUSION

Adventitious root emergence is controlled by polar auxin transport likely through activation of the auxin efflux carrier gene *OsPIN2* in epidermal cells above AR primordia and subsequent depletion of auxin in these cells. Polar auxin transport furthermore controls AR elongation and determines growth direction. Taken together, local auxin activities controlled by polar auxin transport through *OsPIN* efflux carriers is a major determinant of AR system architecture in rice.

MATERIALS AND METHODS

Growth Conditions and Treatments

The seeds of the deepwater rice variety PG56 (*Oryza sativa* L., Pin Gaew 56) were originally obtained from the International Rice Research Institute (Philippines). The *OsPIN:GUS* lines in the rice *japonica* cultivar Nipponbare were generously donated by Prof. Chuanzao Mao (Zhejiang University, China). The *DR5:VENUS* line of the *japonica* rice cultivar 9522 was generously provided by Prof. Zheng Yuan (Shanghai Jiao Tong University, China). The *VENUS* protein is directed to the nucleus for better detection and resolution (Yang et al., 2017). Seeds were germinated in a 12.5 × 12.5 cm Petri dish with a surface-moist Whatman filter paper at 27°C in the dark for 4 days. Seedlings were transferred to 1.7-liter pots with a soil mixture consisting of two thirds of soil and one third of volcanic soil. Plants were grown in a growth chamber with 16 h light (362.3 μmol m⁻²s⁻¹) at 27°C and 8 h dark at 19°C with 70% relative humidity for 12–14 weeks (Sauter, 1997). Stems were excised 2 cm below the third youngest node with a total length of 20 cm (Mergemann and Sauter, 2000). Stem sections were incubated in 150 ml beakers with 20 ml aqueous solution with or without different concentrations of ethephon, 2,4-D or α-NAA or NPA. Plastic cylinders covered the beakers to ensure high humidity. To investigate the effect of light on the growth angle of ARs, stem sections were either kept in the dark for 3 days to induce roots to emerge and subsequently exposed to WL (43 μmol m⁻²s⁻¹) for the times indicated or exposed to light in the presence of the ethylene-releasing compound ethephon.

To determine the root angle, each AR was photographed (Canon SX 220 HS) and the angle measured with ImageJ (Fiji). Subsequently, roots were excised to determine the root length with a ruler.

Evans Blue and Lugol Staining

Evans Blue staining was used for epidermal cell death determination and to better visualize AR penetration at the rice stem. Five mm of the node were cut and stained in a 2% (w/v) Evans Blue solution for 3 min followed

by two washing steps in tap water. Root penetration was determined using a light microscope (Olympus SZ61, Japan) and the percentage was calculated based on the total number of AR initials that was taken as 100%. For Lugols staining, 1 cm of the root tip was isolated and stained with Lugols' solution for 1 min. Subsequently, the samples were washed once in distilled water. The starch-containing statoliths were visualized and photographed using a binocular (Nikon H600L, Japan).

Histochemical GUS Analysis

Histochemical β-glucuronidase (GUS) staining was performed according to Blázquez et al. (1997) with minor modifications. Rice stem sections were kept in the dark or exposed to WL for the duration indicated with or without 150 μM ethephon or 5 μM NPA. Isolated ARs or nodes were rapidly transferred to 90% pre-cooled acetone for 20 min and subsequently washed twice with 50 mM sodium-phosphate buffer (pH 7.2) for 5 min on a shaker. After removing the washing solution, 1 ml staining buffer (50 mM sodium phosphate, 0.2% [v/v] Triton X(-100, 2 mM K₄[Fe(CN)₆], 2 mM K₃[Fe(CN)₆], and 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide [Duchefa], pH 7.5) was added to each sample. Samples were kept in a vacuum chamber for 10 min followed by shaking at 37°C in the dark (*OsPIN1b:GUS* and *OsPIN1c:GUS* for 1 h, *OsPIN2:GUS* and *OsPIN10a:GUS* overnight). The staining buffer was removed and samples were incubated in 70% ethanol to remove chlorophyll. GUS staining was visualized using a binocular (Nikon H600L, Japan).

Confocal Laser Scanning Microscopy

Fluorescence images of *DR5:VENUS* samples were taken with a Leica SP5 confocal laser scanning microscope (CLSM). For an overview, a 5 mm segment containing an AR primordium and the surrounding nodal tissue was excised from the node. Root primordia and the epidermis were isolated and prepared on a slide with a drop of 10% (w/v) glycerol for imaging. Imaging of *VENUS* was done at 510–550 nm and bright field was regarded as a control. ARs that were transferred from dark to light were marked on the upper and lower sides to allow for proper orientation of the root in CLSM.

Statistical Analysis

Statistical analysis was performed using Minitab. The *P*-value was set to *P* < 0.05. Comparison of means was analyzed for statistical difference with an analysis of variance (ANOVA) (Tukey test) or a two sample *t*-test.

AUTHOR CONTRIBUTIONS

CL and MS designed the project. CL performed the experiments. MS wrote the manuscript with contributions from CL.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2019.00444/full#supplementary-material>

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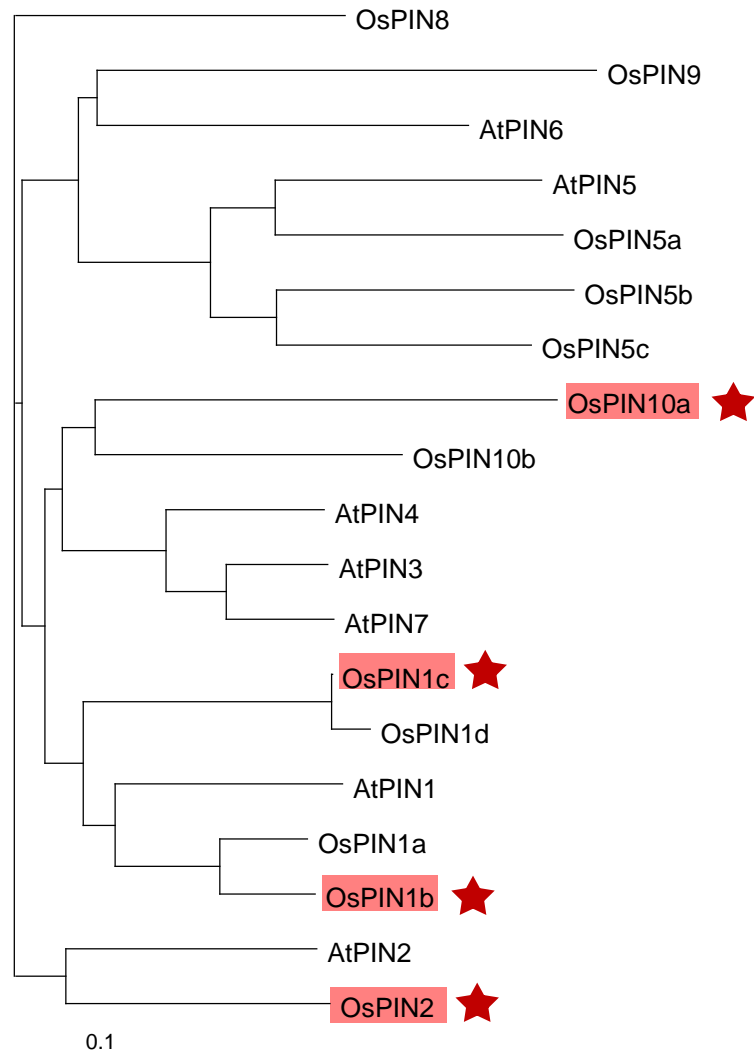
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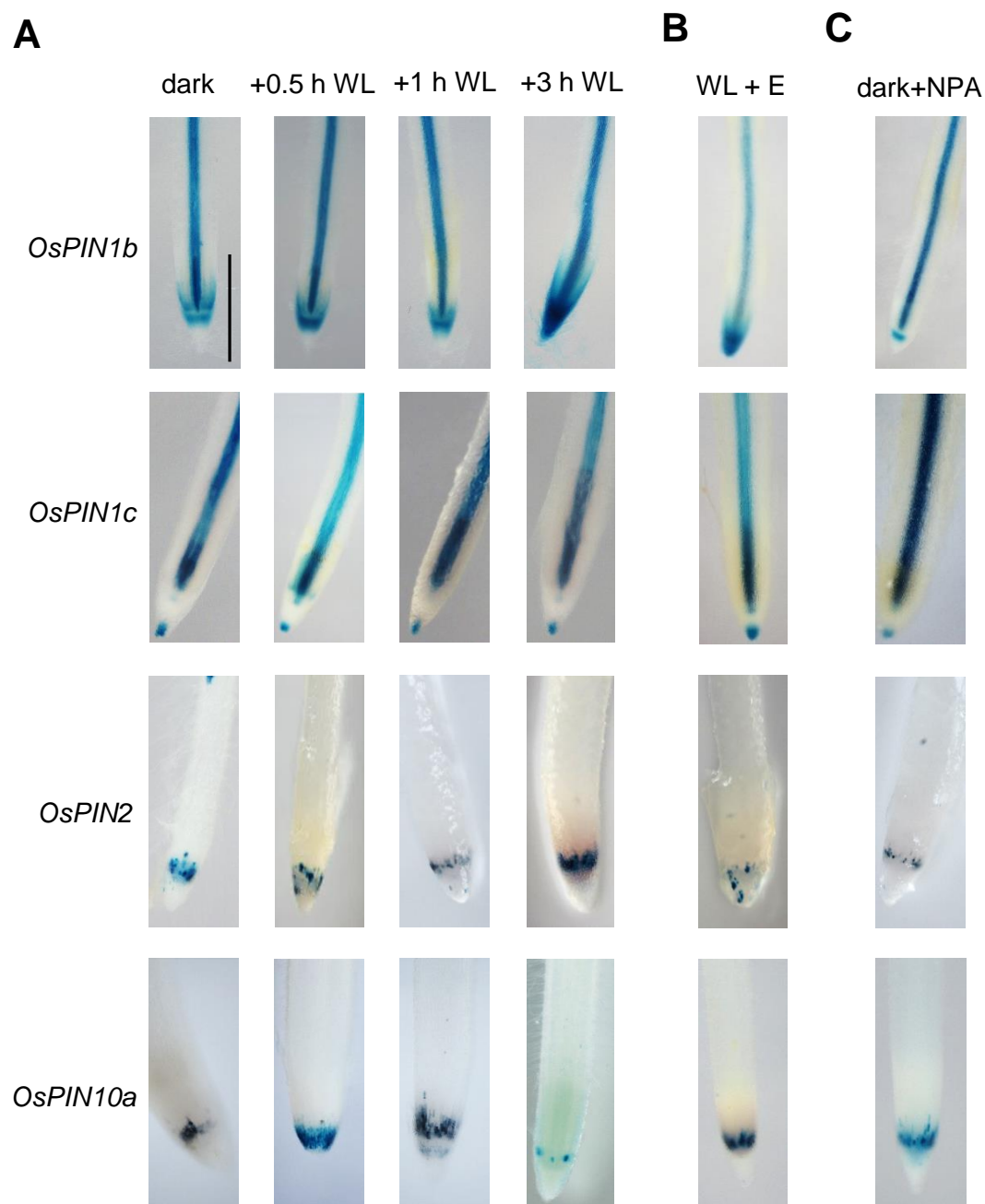
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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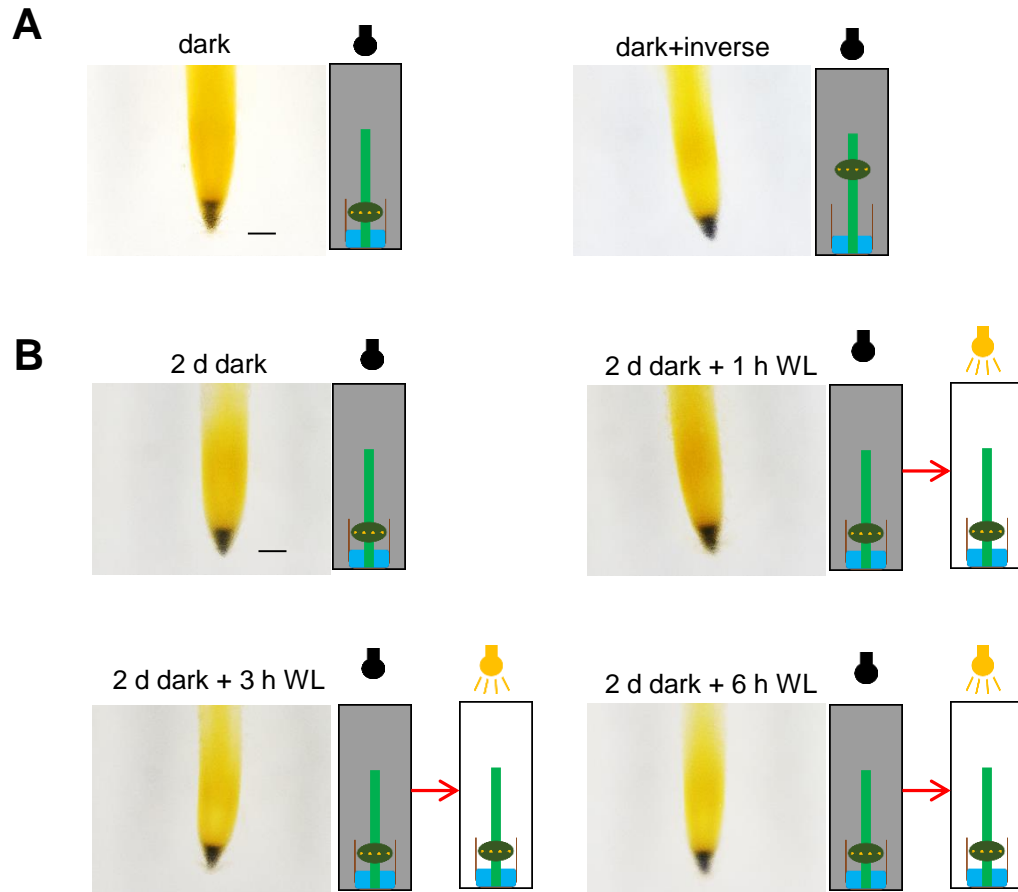
Supplementary Data



Supplementary Figure S1: Phylogenetic analysis of PIN family members in rice and arabidopsis. Multiple sequence alignment was performed with Clustal Omega and a phylogenetic tree was generated by Treeview; bar=0.1. Red stars indicate PIN genes that were analyzed with GUS reporter lines.



Supplementary Figure S2: Histochemical GUS analysis of *OsPIN* expression in rice ARs. Stem sections were kept in the dark for 3 d (dark), 3 d dark and 0.5 h white light (3 d Dark + 0.5 h WL), 3 d dark and 1 h white light (3 d Dark + 1 h WL), 3 d dark and 3 h white light (3 d Dark + 3 h WL), white light and 150 μ M ethephon (WL + E) for 3 d, or in the dark with 5 μ M NPA (dark + NPA). Images were obtained with a binocular (bar=0.5 mm).



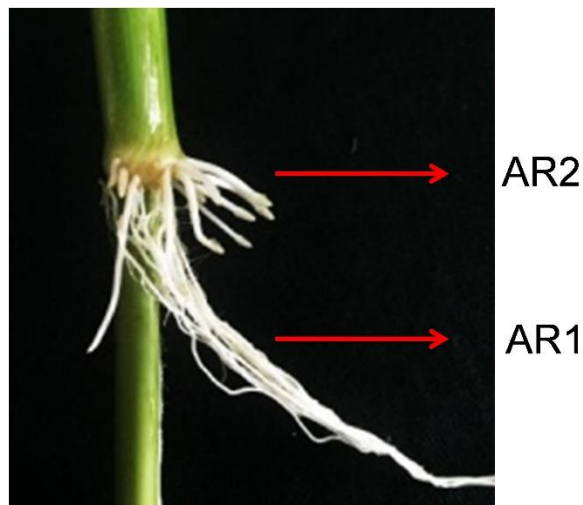
Supplementary Figure S3: Visualization of statoliths in AR caps.

(A) Stems were placed upright or in an inverse orientation in a beaker. After exposure to darkness for 2 d or 2 d darkness followed by 3 h light, ARs were isolated and stained with Lugols solution to visualize statolith-containing root cap cells (bar = 0.2 mm).

(B) Rice stems were kept in the dark for 2 d and subsequently exposed to light for 1 h, 3 h or 6 h. Isolated ARs were stained with Lugol solution as described in (A) (bar=0.2 mm).

8. Chapter 3

**QTL12 with the ethylene response transcription factor genes
SNORKEL1 and *SNORKEL2* promotes adventitious root formation and
growth in rice**



NIL-12, node 4
5 d submergence

Short title: Adventitious root growth in rice

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QTL12 with the ethylene response transcription factor genes
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Abstract

Flooding is one of the most severe environmental stresses that lower crop production worldwide. Unlike most other crops, rice is a semi-aquatic plant with a high flooding tolerance. The gaseous hormone ethylene accumulates in flooded rice plants and controls a number of adaptive responses. In deepwater rice (*Oryza sativa* L.), that get severely flooded for several months, ethylene induces internodal elongation and adventitious root (AR) growth. Stem growth helps flooded plants to keep their leaves above the water. Rice constitutively forms AR primordia that emerge upon submergence in response to ethylene. ARs support or replace the main root system by taking up water, nutrients and oxygen from flood waters and by providing anchorage. Furthermore, additional AR primordia and higher root length were observed in NIL-12 as compared to T65 upon submergence. OsEIL1 activates SKs through targeting the promoter region of the SKs. Overexpression of the *OsEIL1* shows a minor effect on the AR formation and growth during submergence indicating that SKs are essential for the root formation and penetration. In addition, accumulated ROS was visualized in NIL-12 and C9285 but not T65 by NBT staining suggesting that SKs mediate the root growth possibly through upregulating the ROS level. Using the near-isogenic line NIL-12 with QTL12 in the T65 background we reveal a major role for QTL12 in AR formation and development.

Key words: flooding, rice, adventitious root, QTL12, NIL-12, snorkel, ROS

Introduction

An increasing occurrence of flooding events as a result of climate change threaten crop production worldwide (Lesk et al., 2016). The proper strategy to reduce or avoid yield losses during flooding is to improve the resistance and survival ability of crop plants under submerged conditions (Loreti et al., 2016). Rice (*Oryza sativa* L.) is a semi-aquatic plant that has distinct adaptive traits to cope with changing and hostile environments. The study of adaptation to submergence in rice as a monocot model plant could help to establish an understanding of the flooding tolerance in cereal crop plants in general. Roots are prone to flooding due to the resulting reduction in soil O₂ (Pedersen et al., 2017). Under submerged conditions, the original root system can become dysfunctional. In order for the plant to survive, new roots need to develop. In rice, flooding induces growth of adventitious roots (ARs) at the nodes (Sauter, 2013). The floating ARs acquire O₂ from the flood water that is enriched in oxygen by underwater photosynthesis, and from the shoot through aerenchyma that facilitate O₂ oxygen supply to the root. AR primordia are formed at the nodes during plant development and emerge when flooding occurs. Root emergence and elongation growth are mediated by the gaseous phytohormone ethylene, are further promoted by gibberellic acid (GA) and inhibited by abscisic acid (ABA) (Steffens et al., 2006).

Previous work on the initiation and development of ARs in rice focussed mainly on crown root formation in seedlings. A number of genes associated with AR formation and development in rice have been described. *CROWN ROOTLESS1/ADVENTITIOUS ROOTLESS1* (*CRL1/ARL1*) encodes a LATERAL ORGAN BOUNDARIES (LOB) family member, that is required for AR initiation (Inukai et al., 2005; Liu et al., 2005). It is an auxin-regulated transcription factor, that acts downstream of AUXIN RESPONSE FACTORS (ARFs). Lack of *CRL1/ARL1* results in a reduced number of ARs. *CROWN ROOTLESS5* (*CRL5*) is an ERF transcription factor, that mediates the auxin-induced AR formation through the repression of cytokinin signaling. The *cr15* mutant has a severely reduced AR number and a reduced AR length (Kitomi et al., 2011). The *WUSCHEL*-related homeobox gene *WOX11* maintains cell division in the root meristem. The *wox11* mutant also has fewer ARs. *WOX11* interacts with the ERF transcription factor ERF3 that co-regulates a negative primary cytokinin response regulator (RR), the type-A *RR2* gene in response to cytokinin (Zhao et al., 2009; Zhao et al., 2015; Zhou

et al., 2017). In *Arabidopsis*, *ABA4* is an enzyme of the neoxanthin synthesis pathway and is hence crucial for ABA synthesis. Rice *MHZ4* is homologous to *ABA4* and was previously shown to mediate the crosstalk between ethylene and ABA. Overexpression of *MHZ4* enhanced the ethylene sensitivity of seedling roots and increased the endogenous ABA level. Disruption of *MHZ4* induced AR formation at the nodes (Ma et al., 2014). Mutants of the above-described genes provide a framework for the molecular analysis of *de novo* AR formation. In particular, it is still largely unknown how environmental cues and genetic programs regulate these genes to integrate control of AR formation and growth during submergence.

In deepwater rice, internodes elongate upon submergence (Kende et al., 1998). Internodal elongation helps the plants to keep their foliage above the water during severe floods and allows for gas exchange with the atmosphere, thus maintaining oxygen supply. In all rice varieties including deepwater rice, AR primordia develop and grow at each node to support the uptake of the water and nutrients (Sauter, 2013). Both, internode elongation and AR growth are important adaptive traits in response to submergence (Voisenek and Bailey-Serres, 2015). Three main quantitative trait loci (QTLs), QTL1, QTL3 and QTL12, were identified in the deepwater rice variety C9285 that confer flooding tolerance when introduced to the non-deepwater rice variety T65 (Hattori et al., 2009). Among these, QTL12 that includes two ERF transcription factor genes termed *SNORKEL1* and *SNORKEL2* (*SKs*), shows the highest impact on stem elongation in response to submergence. The expression of *SKs* is induced by flooding, specifically in response to ethylene, that gets trapped inside plants upon submergence due to decreased gas diffusion. Interestingly, AR emergence from above ground nodes in response to flooding is mediated by ethylene (Mergemann and Sauter, 2000). To date, it has not been studied whether *SKs* are involved in ethylene-induced AR growth. Here, we investigated the role of QTL12 in submergence-induced AR formation and growth using the near-isogenic line NIL-12 that possesses QTL12 from the deepwater rice variety C9285 in the T65 non-deppwater rice background. Results show that QTL12 promotes AR initiation even under non-submerged conditions and accelerates AR growth after submergence thereby leading to the formation of an extensive secondary root system.

Results

QTL12 promotes node formation and enhances AR growth during submergence

In previous work, positional cloning was performed to identify three QTLs that regulate internode elongation in deepwater rice as an adaptation to flooding (Hattori et al., 2009). The non-deepwater rice variety Taichung65 (T65) shows limited internode elongation under deepwater conditions. In contrast, the deepwater rice C9285 accelerates stem growth to keep the leaves above the water under deepwater conditions. Using the progeny of T65 and C9285, three major QTLs, that are located on chromosomes 1, 3 and 12, were identified that mediate stem elongation in deepwater conditions. The corresponding single near-isogenic lines (NIL) and a pyramiding line of all three QTLs were subsequently generated for further analysis. It was shown that NIL-12 has the strongest effect on stem elongation when partially flooded. In addition, NIL-1 and NIL-3 had an additive effect on stem elongation during submergence (Hattori et al. 2009). To test if QTL1, QTL3 and QTL12 participate in the regulation of AR growth in flooding conditions, T65, NIL-1, NIL-3, NIL-12, NIL-1+3+12 and C9285 were partially submerged for 7 days (Figure 1A and B). NIL-1, NIL-12 and NIL-1+3+12 produced additional above ground nodes compared with T65 as previously reported (Hattori et al., 2009) (Figure 2A). Detailed analysis of stems revealed that NIL-12, in particular, has more penetrated roots at each node compared with NIL-1, NIL-3 and T65 suggesting that QTL12 harboured critical genes that promote root development during flooding. In addition, NIL-1 showed a higher number of ARs compared with T65 indicating that the regulation of AR development was controlled by several different QTLs. Analysis of root lengths revealed that NIL-12 showed a higher average root length compared with T65, NIL-1 or NIL-3, indicating that NIL-12 not only promotes root formation but also root elongation (Figure 2B). However, the observation of a higher average root length in C9285 as compared with NIL-12 suggested the existence of other regulators that mediate root elongation during submergence. Here, we further focussed on the role of QTL12 in the formation and development of ARs.

NIL-12 plants were higher than T65 at 45 d, 60 d, 75 d and 90 d of growth supporting a role of QTL12 in stem growth (Figure 3A and B). In rice, the formation of AR primordia at the node is genetically programmed. The total number of AR primordia per stem is hence partially controlled by the number of nodes (Figure 3C). The numbering of nodes was given from the youngest to the oldest with the youngest node designated as node 1. An average of 3.9 nodes was formed in 45-d-old C9285 plants. Node 1 was initiated in NIL-12 after 60 d of growth and in T65 after 75 d of growth. The number of nodes in NIL-12 was higher than in T65 at all time points analyzed thereafter. At 90 d, the average number of nodes reached 8.1, 4.1 and 1.8 in C9285, NIL-12 and T65 respectively, confirming a role of QTL12 in above-ground node formation.

As outlined above, more ARs were generated in NIL-12 compared with T65 and fewer than in C9285 after 7 d of submergence (Figure 4A and B). To better visualize ARs at the nodes of one stem, the leaves were removed (Figure 4B). Then, ARs were collected from each node from a total of 10 stems for better visualization. NIL-12 clearly had more ARs than T65, whereas C9285 had even more ARs than NIL-12 (Figure 4C). Finally, the numbers of penetrated roots after submergence were determined in the 3 genotypes (Figure 5). C9285 and NIL-12 plants produced several fold more ARs at nodes 2 and 3 than T65 plants. The AR numbers of NIL-12 and C9285 were similar at nodes 2 to 5. However, since C9285 plants produced additional nodes compared with NIL-12, the overall numbers of ARs were higher in C9285 plants (Figure 5A). ARs of NIL-12 and C9285 were on average longer as compared to T65. Root lengths were comparable at nodes 2 to 4 between C9285 and NIL-12, whereas C9285 plants had significantly longer roots at node 5 compared with NIL-12 (Figure 5B). The AR fresh and dry weights of NIL-12 were significantly higher than of T65 supporting the conclusion that QTL12 improved the root production (Figure 5C). In conclusion, QTL12 promotes both penetration and elongation of ARs upon submergence. It was further observed that some ARs penetrated in air-grown NIL-12 but not in T65 plants (Figure S2).

NIL-12 contains the *SNORKEL1* (*SK1*) and *SNORKEL2* (*SK2*) genes that were described to regulate stem elongation during prolonged flooding (Hatorri et al, 2009). Both *SK1* and *SK2* encode ERF transcription factors with similar predicted protein structures (Figure S1). We next investigated the expression of *SK1* and *SK2* at node 2 of unsubmerged plants and of plants that were submerged for 1 day. RT-PCR analysis suggested induction of *SK1* and *SK2* expression in, both, C9285 and NIL-12 but not in T65 after 1 d submergence (Figure 4D).

QTL12 promotes formation of two types of ARs.

NIL-12 plants (Figure 6A) unlike T65 (picture not shown) formed two types of AR primordia, for one the 'classical' AR that form at the node of all rice types which we termed type 1 AR (AR1) and a second type that developed at the base of the internode which we designated as type 2 AR (AR2). AR2 have to penetrate not only the epidermis but also the leaf sheath that wraps around the stem. After 5d of submergence, AR1 were visible in all genotypes, whereas AR2 were present in NIL-12 and C9285 but not in T65.

The average root length of AR1 at node 3 of NIL-12 was 7.4 cm, which was significantly higher compared with an average length of 0.4 cm in T65. To characterize AR1 in more detail, cross sections were obtained in the 3 genotypes and stained with toluidine blue for better contrast (Figure 7A). The roots of NIL-12 had more cortex cell area and slightly more cortex cell layers in comparison with C9285 and T65 (Figure 7B and C), however, no significant differences were observed with regard to the number of tracheary elements between the genotypes (Figure 7D).

In order to compare the anatomical structures of AR1 and AR2, cross sections were obtained from roots at node 4 of NIL-12 and C9285 (Figure 8A, B). AR2 were significantly thicker, had a higher number of cortex cell layers and significantly more tracheary elements compared with AR1, possibly indicating functional characteristics for the two root types (Figure 8D and F). The average root length of AR1 was higher than AR2 in both NIL-12 and C9285 after 7-day submergence, possibly as a result of delayed development of AR2 (Figure 8C).

Lysigenous aerenchyma formation in the root allows for rapid O₂ movement from the shoot to the root tip. Cortex cells undergo programmed cell death and are absorbed resulting in the formation of air spaces from the root base to the tip (Yamauchi et al. 2018). To investigate the formation of lysigenous aerenchyma in AR1 and AR2, root cross sections were cut 5 mm, 25 mm, 45 mm, 65 mm above the root tip (Figure 9A). Extensive aerenchyma were observed in the mature zone, whereas no aerenchyma were present at the root tip in both AR1 and AR2. In AR2, more cortex cell layers contributed to aerenchyma formation than in AR1 (Figure 9B and C) suggesting that O₂ movement may be facilitated in AR2 over AR1. However, further experiments are needed to verify this hypothesis.

QTL12 enhances AR formation

AR1 form at the nodes in all rice genotypes including C9285, NIL-12 and T65. To investigate if QTL12 is involved in the *de novo* genesis of root primordia, cross sections from node 2 were cut to visualize and count AR1 primordia. NIL-12 generated more root primordia than T65 (Figure 10A and B). On average, NIL-12 had 20.1 AR1 primordia, which was significantly more than the 12.4 AR1 primordia counted in T65 indicating that QTL12 promotes the formation of AR1 primordia. C9285 had an average of 20.9 root primordia, which was significantly more than NIL-12 indicating that genes other than those present in QTL12 contribute to AR1 genesis (Figure 10B).

The number of AR2 primordia was not determined due to their scattered distribution at the base of the internode. However, it was interesting to know whether AR2 primordia are formed in T65. The starch-containing statoliths that are present in the root cap can be visualized by Lugols' staining. After removing the leaf sheath from node 3 Lugols' staining was performed to visualize root tips. Brownish spots were detected at the internode bases of T65, NIL-12 and C9285 (Figure 11A) suggesting that the genes required to generate AR2 exist in all three genotypes. However, elongated AR2 were observed only in NIL-12 and C9285 after 7 days of submergence (Figure 9B) indicating that QTL12 promotes AR2 growth.

Overexpression of *OsEIL1* shows a minor effect on the AR formation and growth.

The rice EIN3-like protein OsEIL1 binds to the promoters of *SK1* and *SK2* and activates expression of *SKs* (Hattori et al., 2009). *SKs* are present in deepwater rice but not in lowland rice. It is conceivable that the ethylene-regulated OsEIL1 promotes AR formation through activation of *SKs*. To test if upregulation of *OsEIL1* enhances AR2 growth, two *OsEIL1* overexpression lines were employed and plants exposed to submergence. Overexpression of *OsEIL1* did not significantly increase internode length, plant height or leaf numbers (Figure 12A, B, C and D). To investigate the function of *OsEIL1* in AR formation and growth, Nipponbare wildtype, *OsEIL1ox-1* and *OsEIL1ox-2* plants were submerged for 7 days (Figure 13). The nodal region with roots attached were isolated from each genotype to visualize AR

growth at nodes 1 to 4 (Figure 13B and C). In addition, the root penetration rate and root lengths were determined. Submergence induced AR growth in both Nipponbare and OsEIL1 overexpression lines from node 2 to node 4. However, there were no significant differences between the genotypes (Figure 14A and B). Our data indicated that overexpression of *OsEIL1* did not promote AR growth in air or submergence, suggesting that QTL12 acts independent of OsEIL1 on AR development.

ROS accumulation at sites of AR emergence and in tips of emerged ARs

Emergence of ARs is preceded by local epidermal cell death that requires H_2O_2 which acts downstream of ethylene (Steffens and Sauter, 2009). To test if QTL12 promotes AR growth by promoting ROS accumulation, Nitro Blue Tetrazolium (NBT) was used, that reacts with O_2^{\bullet} to form blue NBT-formazan (Romero-Puertas et al, 2004). Nodes from air-grown T65, NIL-12 and C9285 plants and from plants submerged for 2 d were stained with NBT for 1 h. No staining pattern was observed at the nodes 2 and 3 of T65 in air-grown conditions (Figure 15A). Some staining was observed at nodes 3 and 4 of NIL-12 (Figure 15A) and pronounced staining was detected at nodes 2 to 4 in C9285 indicating that comparably high levels of O_2^{\bullet} were produced in C9285 even at non-submerged conditions. After submergence, still no staining was observed in T65 (Figure 15B), while epidermal cells above root primordia were stained at node 2 in NIL-12. From older nodes of NIL-12 and from all nodes of C9285 ARs had emerged and the AR tips showed intense staining suggestive of ROS accumulation. Taken together, the data suggest that QTL12 accelerates AR development and emergence possibly through enhanced ROS formation.

Discussion

Internode elongation and AR emergence are two closely controlled traits

Deepwater rice has a number of adaptive traits for survival in flooding condition, of which internode elongation, that enables the plant to acquire atmospheric O₂, is one of the most important ones (Kende et al. 1998). Aside from accelerated stem elongation, growth of nodal ARs ensures uptake of water and nutrients when the main root system becomes dysfunctional during flooding (Sauter, 2013). In general, both adaptive responses are induced when the water level rises during flooding, however the hormonal networks that trigger them are seemingly different. Internode elongation is mainly controlled by gibberellic acid (GA), that accelerates the cell cycle in the intercalary meristem in the stem (Lorbiecke and Sauter, 1999). In contrast, AR emergence and growth are mediated by ethylene, and further promoted by GA. GA alone shows a minor effect on AR penetration (Steffens et al., 2006). Increasing evidence suggests crosstalk of ethylene and GA in the co-regulation of plant development (Pierik et al., 2004; Hattori et al., 2009; Iqbal et al., 2017). In rice, the expression of *SKs* is specifically induced in response to ethylene but not GA, however, an increased GA₁ content was detected in C9285 but not in T65 upon submergence (Hattori et al., 2009). In addition, NIL-12 plants which harbour *SK* genes promote internode elongation as a result of increased GA content and enhanced GA signaling. Expression of *SKs* is induced in response to ethylene and promoted growth not only of internodes but also of ARs. AR growth was previously shown to be induced by ethylene and *SKs* appear to be one type of transcription factors that can relay this signal (Lorbiecke and Sauter, 1999). It was furthermore observed that AR growth is enhanced by QTL1 containing the gibberellin biosynthesis gene *SD1* (*SEMIDWARF1*), that increases the content of bioactive GA₄ (Kuroha et al, 2018). It is therefore conceivable that internode elongation and AR growth are two traits that are co-regulated by an ethylene-GA signal network.

Since type 1 ARs are located at the nodes, the total number of AR1 is positively correlated with the number of nodes. AR2 form at the base of the internodes which requires

internodes to elongate to provide space for the development of AR primordia. Therefore, internode elongation is essential for the development of AR2 primordia.

Differences in AR1 and AR2 anatomy may be important for flooding resistance

Rapid AR emergence during submergence is crucial to alleviate stress of water and nutrient uptake caused by flooding (Sauter, 2013). SKs promote emergence of AR2 which are morphologically and anatomically distinct from AR1 suggestive of a functional difference. AR1 emerge prior to AR2 possibly due to a delayed development of AR2 primordia. AR1 primordia develop at the node at an initial stage when the node is formed (Lorbiecke and Sauter, 1999). AR2 primordia develop at the base of the internodes possibly after the internode has elongated, a hypothesis that needs to be tested. Epidermal cell overlying root primordia undergo programmed cell death prior to root emergence (Steffens and Sauter, 2005). AR2 primordia are covered by the epidermis and by the leaf sheath that represents an additional physical barrier that needs to be overcome (Figure 8). Penetration of the leaf sheath by AR2 may require additional time and a higher mechanical force.

The anatomy of roots determines their ability to adapt to flooding, in particular with respect to aerenchyma formation (Coudert et al., 2010). Root aerenchyma is critical for the O₂ supply from the shoot to the root in stagnant conditions (Drew et al. 2000). In rice, lysigenous aerenchyma in roots is constitutively formed, and enhanced when plants are submerged in response to increased ethylene levels in the roots (Evans, 2004; Yamauchi et al. 2017). Root aerenchyma formation results from programmed death and lysis of cortex cells. Interestingly, the cortex cell area and the number of the cortex cell layers in AR2 are significantly higher than in AR1 (Figure 8), providing an enlarged area for aerenchyma formation in AR2 as seen in cross sections of elongated roots (Figure 9). Taken together, it is conceivable that AR2 might enable more efficient movement of O₂ as compared to AR1, which may be of advantage during flooding. However, data of the O₂ status and of respiration rates in AR1 and AR2 are needed to test this hypothesis. It would also be interesting to investigate the benefits of AR2 for flooding resistance in the future.

SKs might be involved in de novo AR formation

QTL12 promotes the formation of nodes, of AR1 and the growth of AR2 during submergence (Figure 16). *SKs* in QTL12 are activated by ethylene signaling and enhances the root penetration, which is consistent with previous findings (Steffens et al., 2006). It is still elusive how *SKs* mediate *de novo* root formation. In plants, auxin positively regulates root genesis, while cytokinins inhibit it (Su et al., 2011). NIL-12 shows an increased number of AR1 primordia (Figure 10), with *SKs* being induced by ethylene (Hattori et al., 2009). It is possible that *SKs* control auxin synthesis or signaling. Recent research described that the stability of Aux/IAA protein is required for the ethylene inhibition on the root growth in rice (Chen et al., 2018). Several genes associated with auxin signaling were shown to promote root initiation in rice. *CROWN ROOTLESS1/ADVENTITIOUS ROOTLESS1 (ARL1/CRL1)* is an auxin-regulated transcription factor required for crown root formation. Disruption of *ARL/CRL1* results in a crown rootless phenotype (Inukai et al., 2005; Liu et al., 2005). *CROWN ROOTLESS5 (CRL5)* encodes a transcription factor, that participates in the root initiation in rice through repressing the cytokinin signaling pathway independent from *ARL1/CRL1* (Kitomi et al., 2011). The *Wuschel*-related homeobox 11 gene (*WOX11*) maintains the cell division in the root apical meristem in response to cytokinins (Zhao et al., 2009; Zhao et al., 2015). Interestingly, overexpression of *WOX11* in plants constitutively formed the abnormal more ARs in the air-grown condition, indicating that *wox11* plays a crucial role in root development. As transcription factors, *SKs* might mediate the root *de novo* genesis through binding to the promoter region and activates the expression of these candidate genes.

SKs are essential for AR formation and emergence

OsEIL1 is essential for the ethylene-regulated plant growth. Overexpression of *OsEIL1* results in a phenotype with a slightly short primary root in rice. In addition, Overexpression of *OsEIL1* rescues the severe rootless phenotype of the *ein3* mutant of Arabidopsis (Mao et al., 2006). Further studies indicated that *OsEIL1* binds to the promoter of *SKs* and activates their expression (Hattori et al., 2009). In cultivar rice, *OsEIL1* is present whereas *SKs* are missing. We test the function of *OsEIL1* on the AR growth. Overexpression of *OsEIL1* in the

Nipponbare background without *SKs* shows a minor impact on the formation of AR number and AR length indicating that *SKs* are required for root genesis and growth. It is also possible that *OsEIL1* activates AR growth in the short term without a detectable difference after 7 days. In addition, AR2 are not generally observed in *OsEIL1ox* plants indicating a limited function of *OsEIL1* in the root genesis, that complies with our hypothesis that ethylene promotes the emergence and growth but not AR formation whereas *SKs* promote AR formation and growth. Taken together, *SKs* play an important role in root formation and growth.

Conclusion

Rice is a semi-aquatic crop plant, that is tolerant to submergence. AR growth in the flooding partially rescues the plant through the uptake of water and nutrients. In flooding, ethylene is responsible for the penetration and growth of ARs. *SKs* encode AP2/ERF transcription factors, that are present in deepwater rice in response to flooding not only through internode elongation but also the AR growth. In addition, *SKs* also mediate the root formation without submergence. We propose that *SKs* are crucial for the root formation and root growth to help plants alleviate the stress from flooding.

Materials and Methods

Plant growth conditions

Seeds of rice (*Oryza sativa* L. ssp japonica) cultivar Taichung65 (T65) and the nearly isogenic lines 1 (NIL-1), 3 (NIL-3), 12 (NIL-12) and the pyramiding line NIL-1+3+12 were obtained from Motoyoki Archikari (Nagoya University, Japan). Seeds of the *Oryza sativa* L. ssp indica cv C9285 were generously donated by the National Bioresource Project Rice (NBRP, Japan). Two independent *OsEIL1* overexpression lines were provided by Ping Wu (Zhejiang University, China). The rice plants were grown for 12 to 14 weeks according to Sauter (1997). For partial submergence of the rice plants, a 600-L plastic tank was filled with tap water and

the temperature was adjusted to 27°C for 3 days prior to partial plant submergence. About 70% of the shoot was submerged for up to 7 days (Sasidharan et al., 2017). In order to clearly visualize the ARs for documentation, the surrounding leaves were removed with a blade.

Bioinformatic analysis and modelling of protein structure

Information of the *SK* gene sequence was obtained from the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/>, NCBI, USA). The protein structure of SK1 and SK2 was predicted using phyre2 (<http://www.sbg.bio.ic.ac.uk/~phyre2>) with Aterf96 in Arabidopsis as a template to model the structure.

Measurement of root primordia number, number of penetrated roots, root length, plant height, internode length, fresh weight and dry weight

The number of root primordia was determined from nodal cross sections obtained with a razor blade. The number of penetrated roots was counted at each node of the genotypes indicated. The root length, plant height and internode length were measured with a ruler. For the determination of the fresh weight, the roots from 10 stems every genotype were collected as one replicate and three independent measurements were performed. The same root samples were heated to 80°C for 8 h in a drying oven and the dry weight was subsequently determined with a fine scale.

Toluidine staining

In order to visualize the root primordia, longitudinal cross sections of the stems including node 3 were obtained using a razor blade, stained with 0.25% (w/v) toluidine blue and subsequently washed twice with distilled water. The root primordia were visualized and photographed with a binocular (Nikon H600L, Japan). Excised roots were embedded in 0.4% agar in a 1.5-ml tube for 3 min and cut with a blade by hand. The root slices were stained

with 0.25% (w/v) toluidine blue for 1 min and washed two times with distilled water. The root cross sections were visualized with a binocular (Nikon H600L, Japan). The number of cortex cell layers and the number of tracheary elements were counted from cross sections of different roots. The root cross section area was determined by ImageJ (Fuji).

Lugols' staining and NBT staining

A 5 mm-long stem section including node 3 was cut and stained with Lugols' solution for 3 min. The samples were subsequently washed by distilled water for two times. The starch statoliths at the root primordia tip were detected as a brownish precipitate and visualized with a binocular (Nikon H600L, Japan). For the detection of O_2^{\bullet} , stem sections with root primordia or elongated roots were stained with 2 mM Nitro Blue Tetrazolium (NBT) dissolved in 50 mM potassium phosphate, pH 7.0 for 10 min and washed with distilled water for two times.

The expression analysis of SKs

The samples were collected from the second youngest node termed node 2 of C9285, NIL-12 and T65 plants that were exposed to partial submergence for 24 h or kept in air as a control. Total RNA was isolated and Reverse Transcriptase PCR (RT-PCR) was performed. The relative expression level of SKs was investigated with transcript abundance of *OsActin1* used as a control.

Statistical analysis

Minitab was applied for statistical analyses. The comparison of means was performed for statistical significance with an ANOVA Tukey's test or two-sample Student's *t* test. Constant variance and normal distribution of data were verified before statistical analysis. The *P* value was set to $P < 0.05$.

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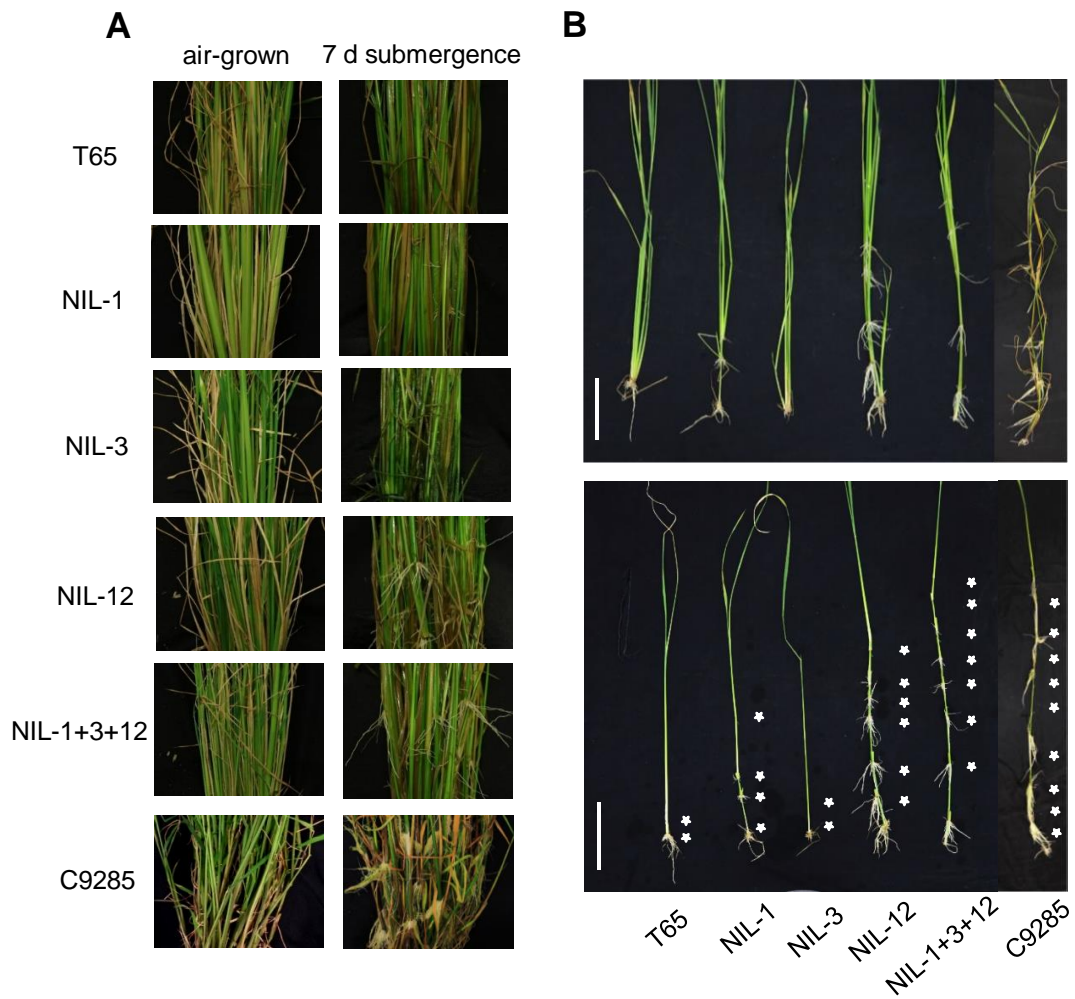


Figure 1: Adventitious roots are induced to grow after flooding.

A, T65, NIL-1, NIL-3, NIL-12, NIL-1,3,12 and C9285 plants were exposed to 7 d of partial submergence or kept in the air as a control.

B, ARs of T65, NIL-1, NIL-3, NIL-12, NIL-1+3+12 and C9285 after 7 d submergence are shown at single stems isolated with leaves attached (top) or with leaves removed (bottom). Asterisks indicate the position of nodes; bar=10 cm.

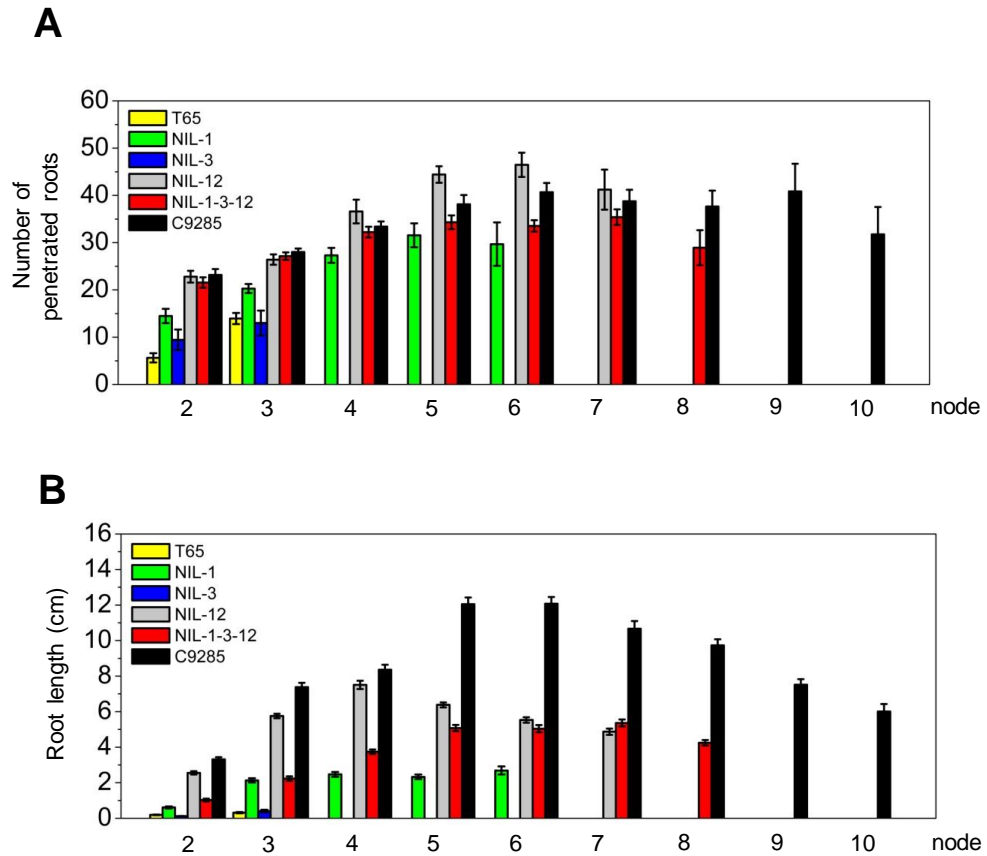


Figure 2: QTL1 and QTL12 but not QTL3 promote AR formation and growth.

A, Three-month-old rice plants were submerged for 7 d and the number of emerged ARs were determined. Results are from 3 independent experiments. Means \pm SE with $n=9-27$, $P<0.05$; Tukey test.

B, Lengths of ARs of 3-month-old rice plants that were submerged for 7 d. Results are from 3 independent experiments. Shown are means \pm SE, $n=9-27$, $P<0.05$; Tukey test.

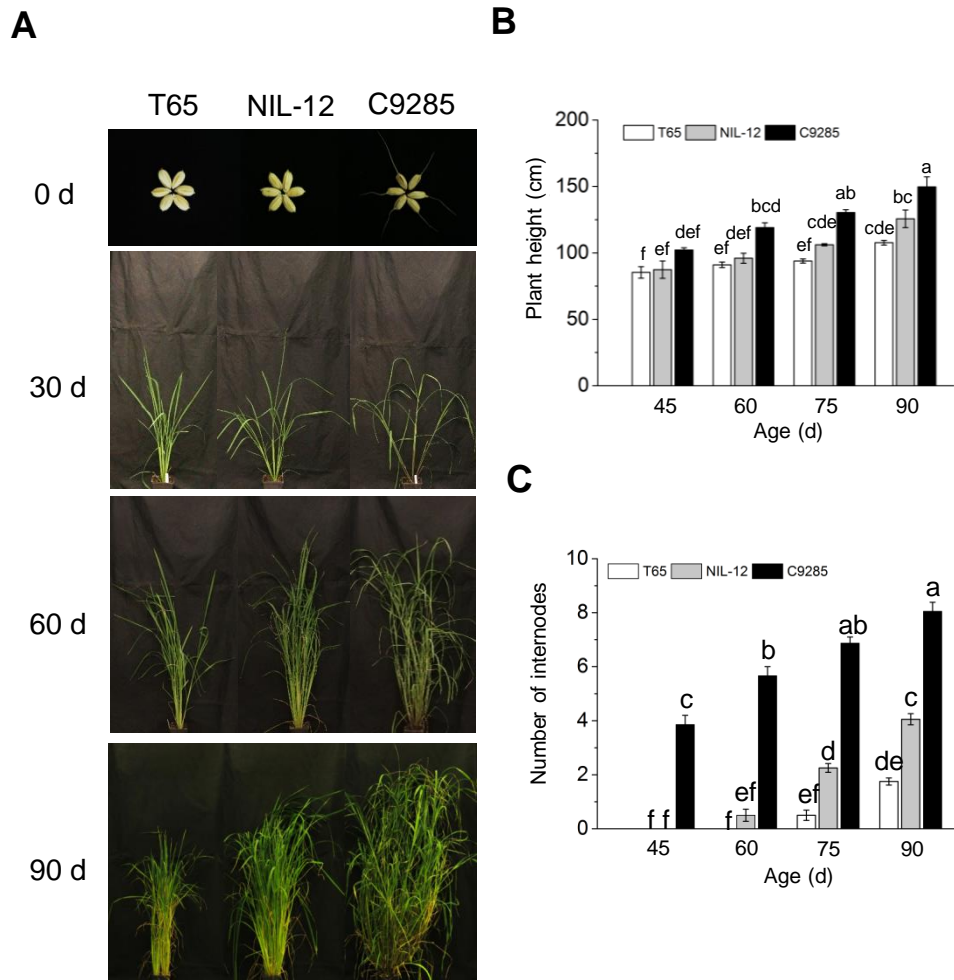


Figure 3: Plant height and internode elongation are controlled by QTL12.

A, T65, NIL-12 and C9285 seeds and plant growth 30 d, 60 d and 90 d after planting.

B, Plant heights of T65, NIL-12 and C9285 were measured after 45 d, 60 d, 75 d and 90 d.

Bars indicate means (\pm SE, $n=9$, $P<0.05$; Tukey test) from 3 independent experiments.

C, Numbers of aboveground nodes in C9285, NIL-12 and T65 at 45 d, 60 d, 75 d and 90 d after planting (means \pm SE, $n=5$, $P<0.05$; Tukey test).

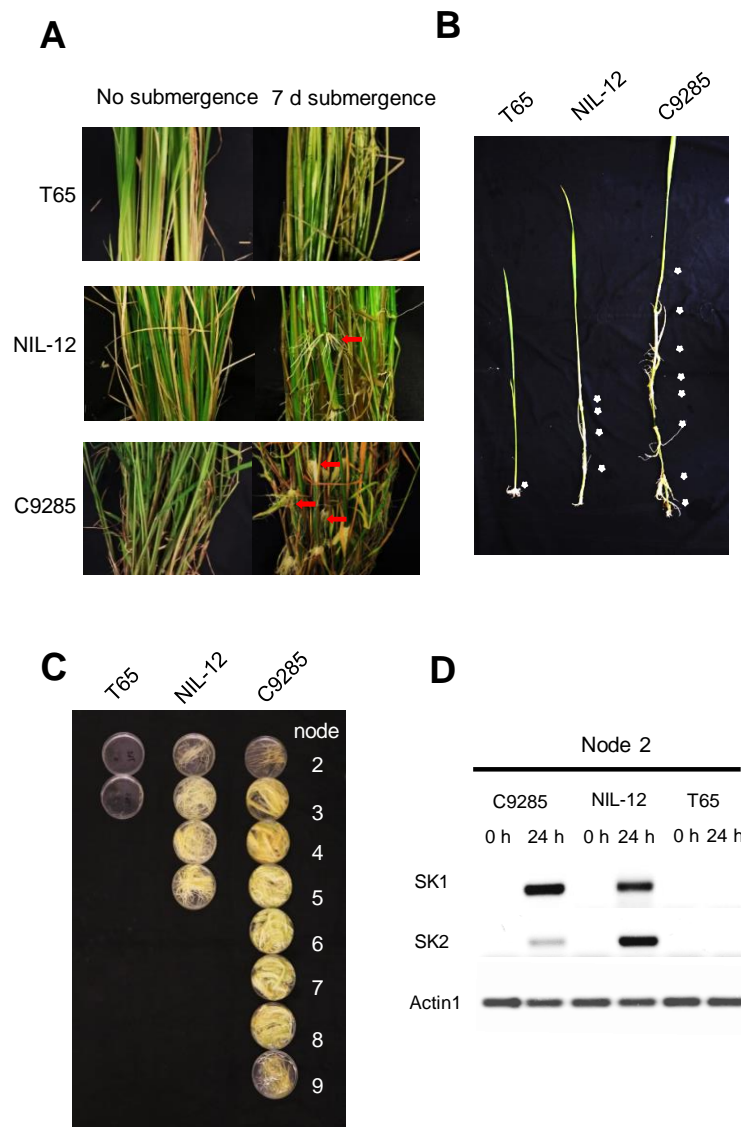


Figure 4: QTL12 promotes adventitious root growth after submergence.

A, Three-month-old rice plants were partially flooded for 7 d to induce ARs (pointed out by red arrows).

B, Single stems were excised and leaves were removed to visualize ARs. Asterisks indicate the position of the nodes in each genotype; bar=10 cm.

C, ARs from each node were collected from 10 stems per genotype.

D, Gene expression of SKs at node 2 before and after 24 h submergence as determined by RT-PCR.

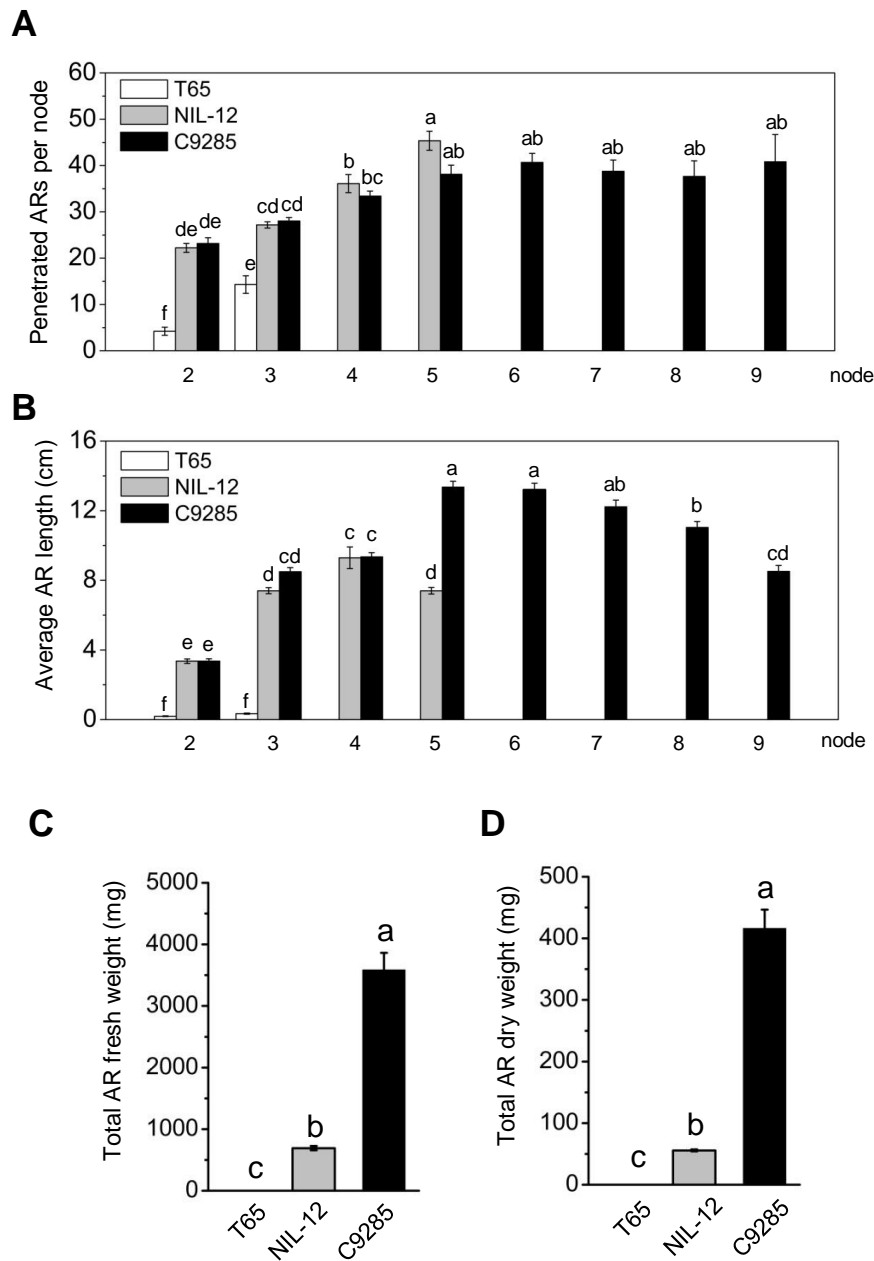


Figure 5: AR penetration, AR length and AR weight in T65, NIL-12 and C9285.

A, T65, NIL-12 and C9285 plants were partially submerged for 7 days and the number of penetrated ARs were determined at each node (means \pm SE, $n=30$, $P<0.05$; Tukey test).

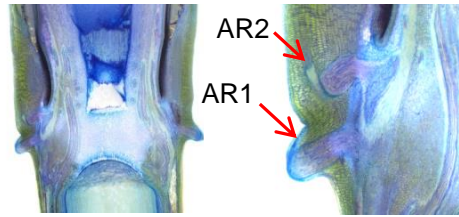
B, Average AR lengths from different nodes in T65, NIL-12 and C9285 (means \pm SE, $n=90-270$, $P<0.05$; Tukey test).

C, The total fresh weight of ARs from 10 stems was determined for each genotype (means \pm SE, $n=30$, $P<0.05$; Tukey test).

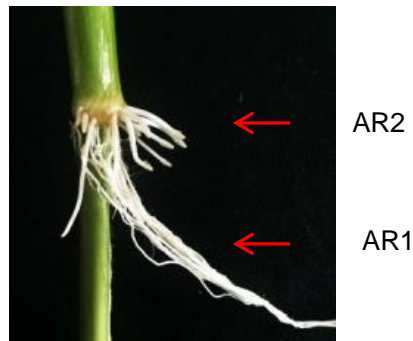
D, The total dry weight of ARs from 10 stems was determined for each genotype (means \pm SE, $n=30$, $P<0.05$; Tukey test).

A

Toluidine staining



B



NIL-12, node 4
5 d submergence

Figure 6: QTL12 promotes formation of two types of adventitious roots.

A, A cross section of the 3rd youngest node of C9285 stained with toluidine revealed two types of ARs: type1 (AR1) at the node and type2 (AR2) above the node. AR1 primordia are covered by the epidermis while AR2 primordia are covered by the epidermis and the leaf sheath. Red arrows to AR1 and AR2 primordia.

B, ARs growing at node 4 of a NIL-12 plant after 5 d of submergence. Red arrows point to the two types of ARs.

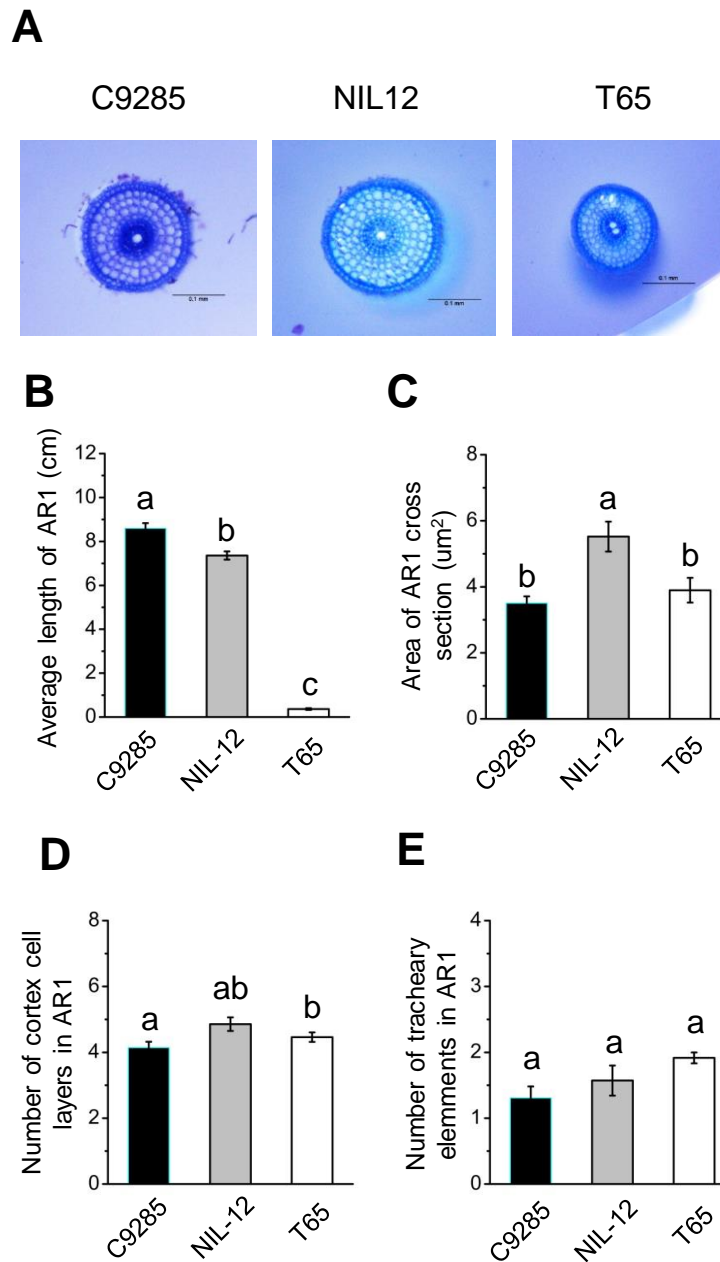


Figure 7: The morphological and anatomical characteristics of AR1 in C9285, NIL-12 and T65.

A, Cross sections of AR1 from node 3 of C9285, NIL-12 and T65 were cut 5 mm from the root tip and stained with toluidine blue.

B, Average lengths of AR1 from node 3 of C9285, NIL-12 and T65 (means \pm SE, $n=18$, $P<0.05$; Tukey test).

C, Cross section areas of AR1 (means \pm SE, $n=18$, $P<0.05$; Tukey test).

D, Number of cortex cell layers in AR1 (means \pm SE, $n=18$, $P<0.05$; Tukey test).

E, Number of tracheary elements in AR1 (means \pm SE, $n=18$, $P<0.05$; Tukey test).

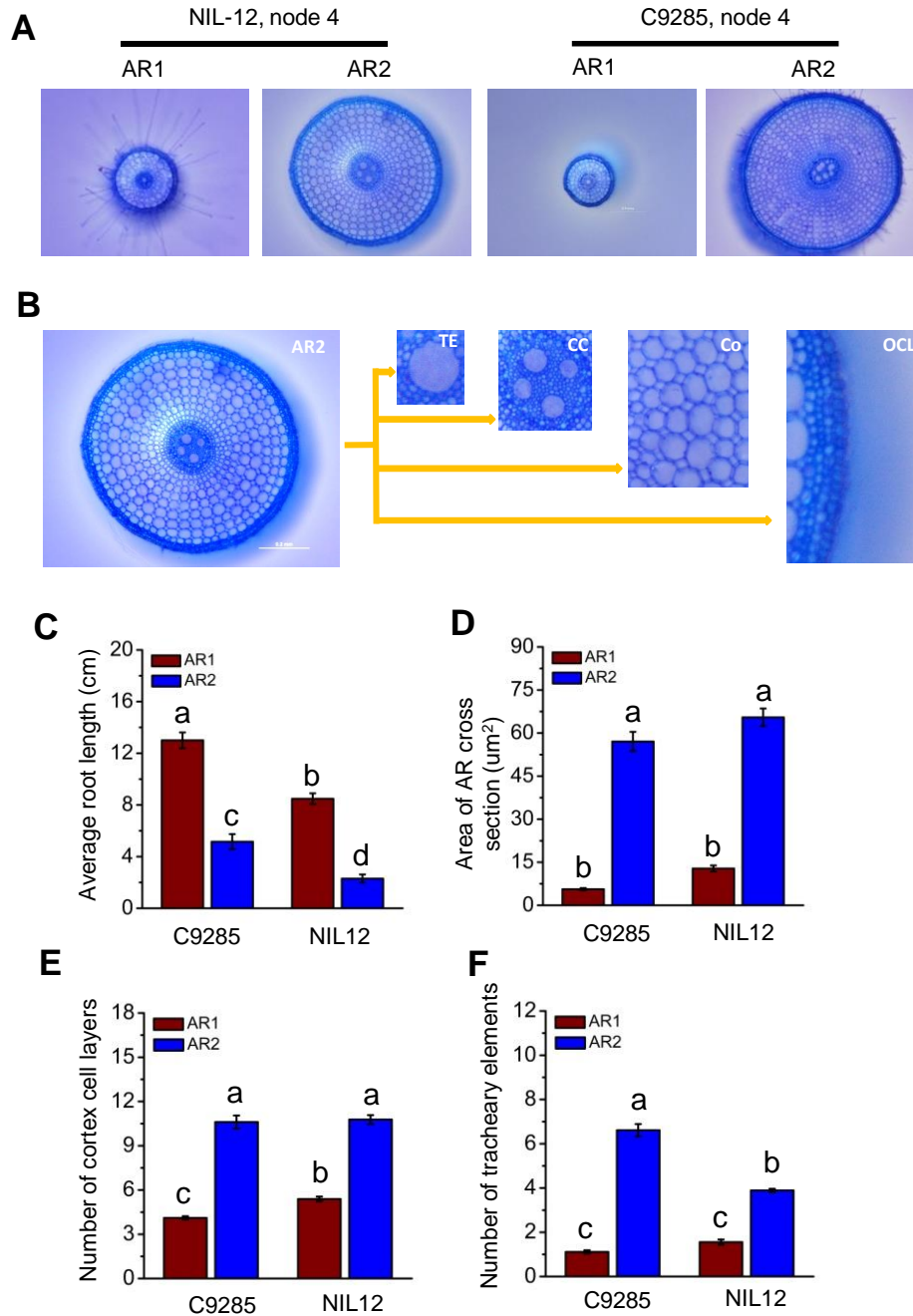


Figure 8: AR1 and AR2 differ in morphology and anatomy.

A, Cross sections of AR1 and AR2 from node 4 of NIL-12 and C9285 were cut 5 mm above the root tip and stained with toluidine blue.

B, Anatomy of tracheary elements (TE), central cylinder (CC), cortex (Co), and outer cell layers (OCL) in AR2 of NIL-12 5 mm from the root tip.

C, Average root lengths of AR1 and AR2 from node 4 of NIL-12 and C9285 plants submerged for 7 days (means \pm SE, $n=5$, $P<0.05$; Tukey test).

D, Average AR cross section area (means \pm SE, $n=18$, $P<0.05$; Tukey test).

E, Number of cortex cell layers (means \pm SE, $n=18$, $P<0.05$; Tukey test).

F, Number of tracheary elements (means \pm SE, $n=18$, $P<0.05$; Tukey test).

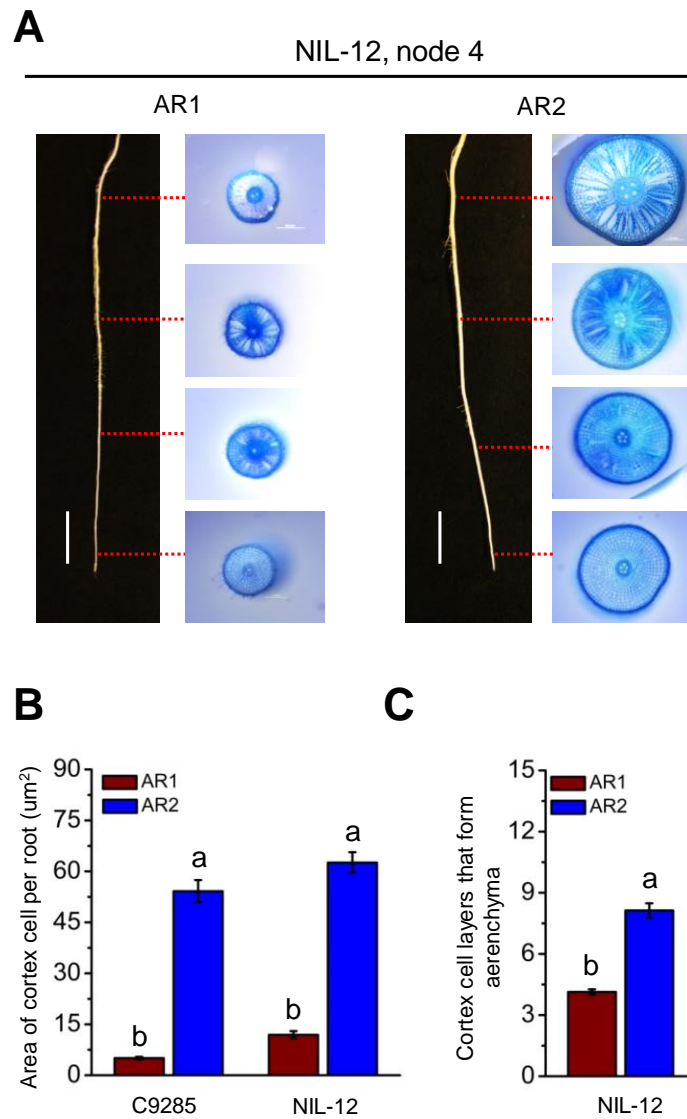


Figure 9: Aerenchyma formation in AR1 and AR2 during flooding.

A, Cross sections were obtained from AR1 and AR2 of node 4 of NIL-12 after 7 d of partial submergence; bar=1 cm. Root cross sections were cut 5 mm, 25 mm, 45 mm, 65 mm from the root tip.

B, The cortex area per root was determined in NIL-12 and C9285 (means \pm SE, $n=18$, $P<0.05$; Tukey test).

C, Number of cell layers that contribute to aerenchyma formation in AR1 and AR2 of NIL-12 (means \pm SE, $n=18$, $P<0.05$; t test).

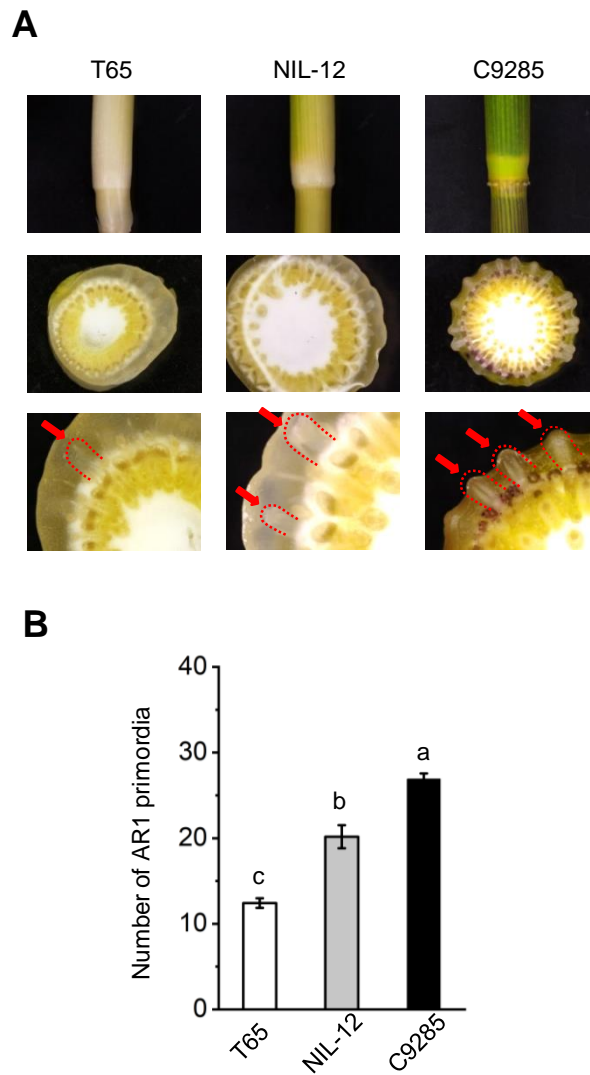


Figure 10: QTL12 promotes AR formation under unflooded conditions.

A, Stems with node 2 and cross sections of node 2 from air-grown T65, NIL-12 and C9285 plants. Red arrows point to AR1 primordia.

B, The number of AR1 primordia was determined at node 2 of air-grown 3-month-old T65, NIL-12 and C9285 plants (means \pm SE, $n=15$, $P<0.05$; Tukey test).

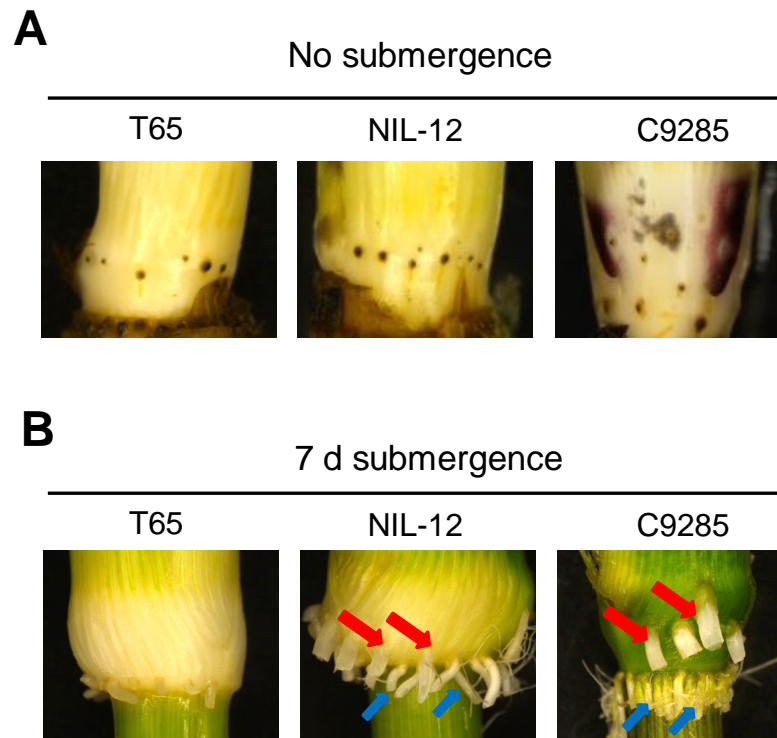


Figure 11: Submergence induces the growth of AR2 beneath the leaf sheath.

A, The leaf sheath at node 3 was removed and the stems were stained with Lugol's solution to visualize root caps. The stained pattern indicates the root primordia.

B, After 7 d of submergence, AR2 (indicated by red arrows) have emerged in NIL-12 and C9285 but not in T65. Note: the roots were cropped for better visualization. The red arrow indicates AR2 and the blue arrow indicates AR1.

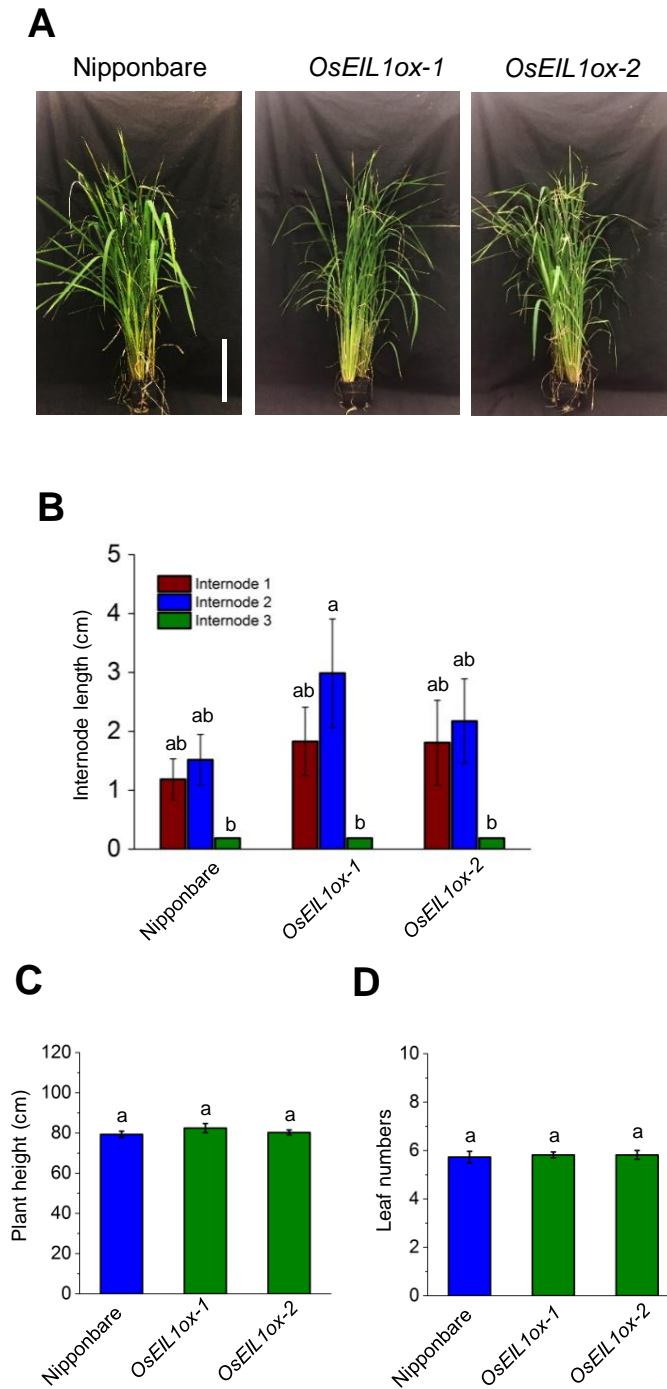


Figure 12: *OsEIL1* does not regulate growth of Nipponbare plants.

A, Nipponbare, *OsEIL1ox-1* and *OsEIL1-ox2* plants were grown for 90 d (bar=20 cm).

B, Internode lengths were measured in 90-d-old Nipponbare and two independent *OsEIL1ox* lines. Results are means \pm SE from 3 independent experiments with n=12-14, $P < 0.05$, Tukey test.

C, Plant heights of 90-d-old Nipponbare and two independent *OsEIL1ox* lines. Results are means (\pm SE) from 3 independent experiments with $n=6$, $P<0.05$, Tukey test.

D, Leaf numbers of 90-d-old Nipponbare and two independent *OsEIL1ox* lines. Results are means (\pm SE) from 3 independent experiments with $n=6$, $P<0.05$, Tukey test.

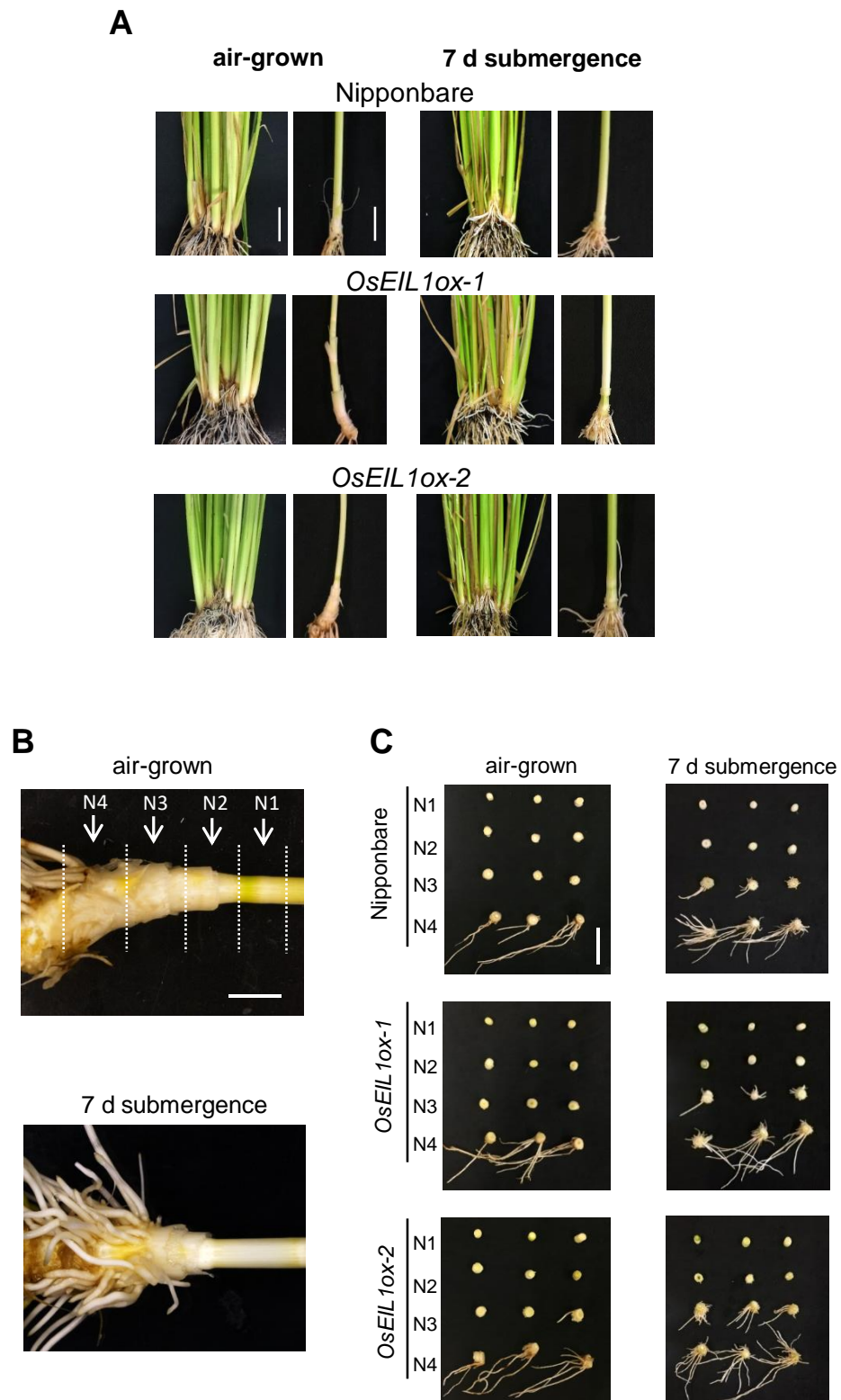


Figure 13: Overexpression of *OsEIL1* does not promote AR growth in Nipponbare.

A, Three-month-old rice plants were partially flooded for 7 d. Stems were excised and leaves were removed to visualize ARs; bar=10 cm.

B, In Nipponbare, nodes develop with minor internodal growth. ARs develop underground at node 4 and in response to submergence at nodes 3 and 4. Arrows indicate the position of nodes; bar=1 cm.

C, Nipponbare, *OsEIL1ox-1* and *OsEIL1ox-2* plants were submerged for 7 d or kept in air as a control. Subsequently, nodes were excised to visualize ARs; bar=1 cm and applies to all pictures.

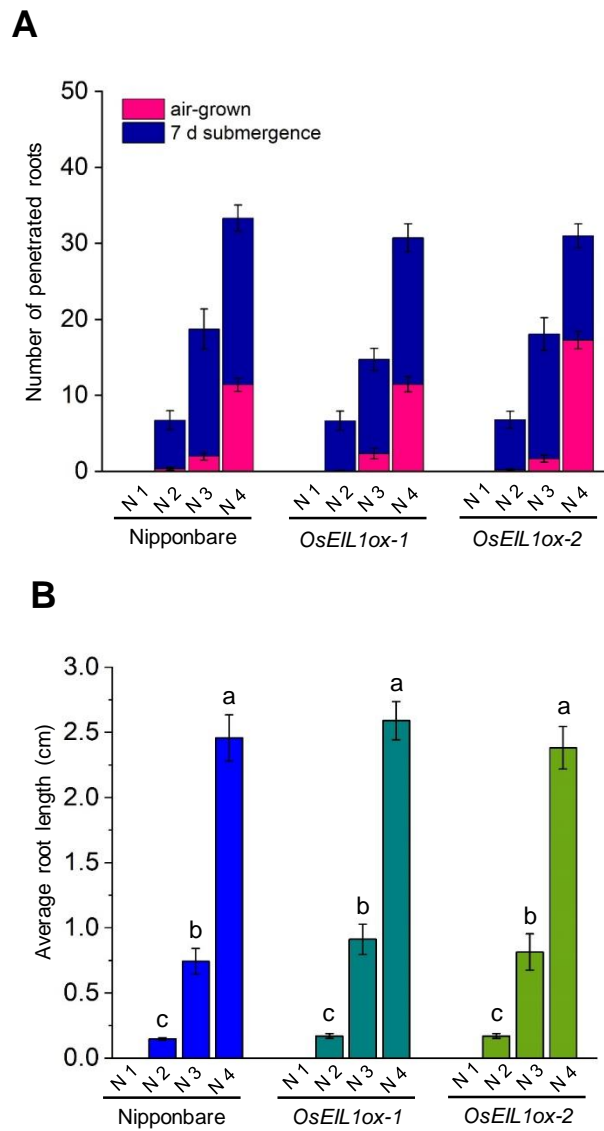


Figure 14: Nipponbare, *OsEIL1ox-1* and *OsEIL1ox-2* have comparable root numbers and root lengths.

A, Number of penetrated roots in air-grown and submerged Nipponbare, *OsEIL1ox-1* and *OsEIL1ox-2* plants (means \pm SE, $n=6$, $P<0.05$; Tukey test).

B, Root lengths at nodes1 to 4 in Nipponbare, *OsEIL1ox-1* and *OsEIL1ox-2* plants (means \pm SE, $n=50-80$, $P<0.05$, Tukey test).

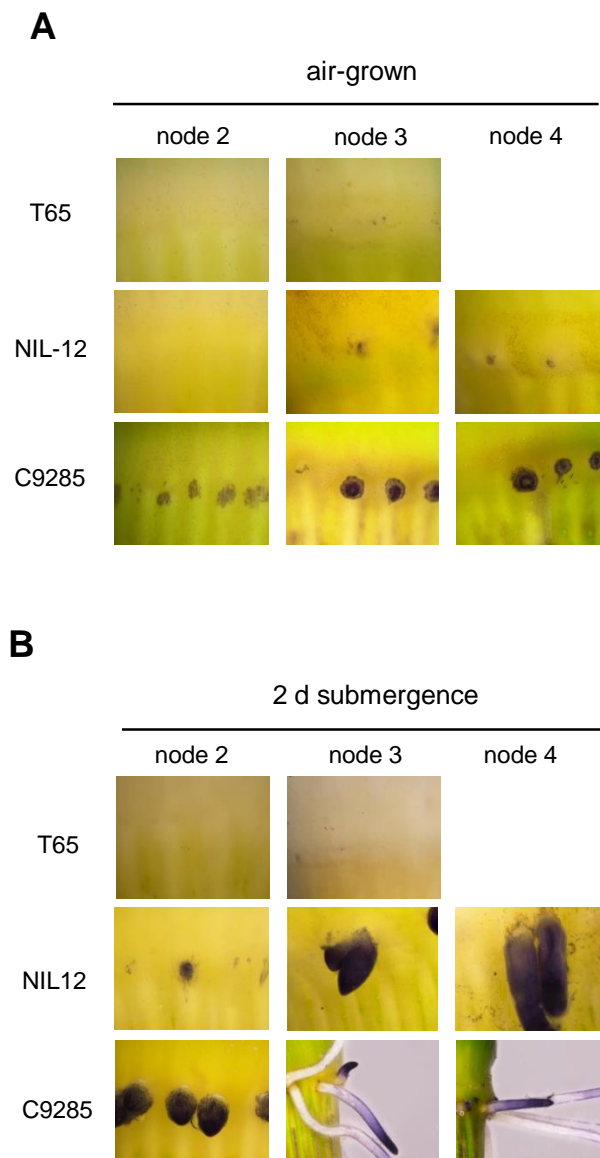


Figure 15: C9285 have elevated ROS levels at sites of AR emergence.

$O_2^{\cdot -}$ levels were determined by NBT staining in nodes 2 to 4 of Nipponbare, NIL-12 and C9285 plants.

A, $O_2^{\cdot -}$ accumulation at nodes of air-grown plants.

B, $O_2^{\cdot -}$ accumulation at nodes of plants that were partially submerged for 2 d.

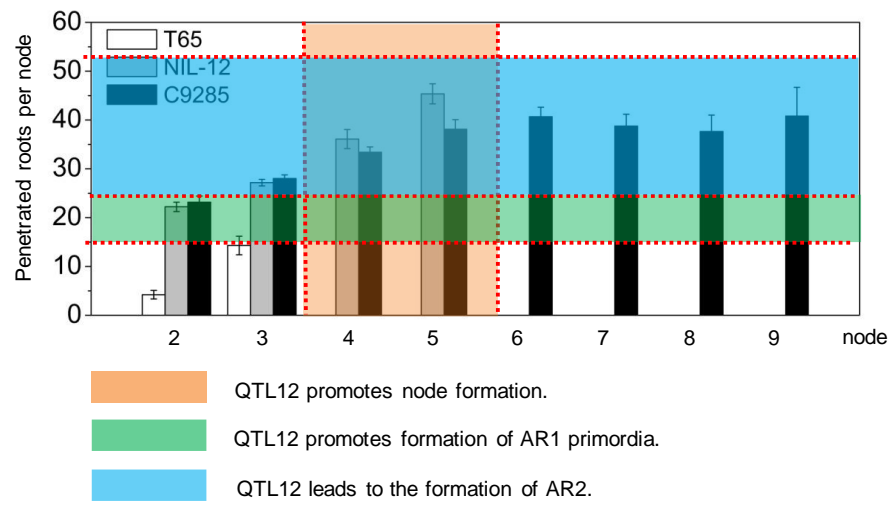


Figure 16: Scheme summary of the effects of QTL12 on the formation of nodes, AR1 and AR2 in comparison to T65 and C9285.

Supplementary Data

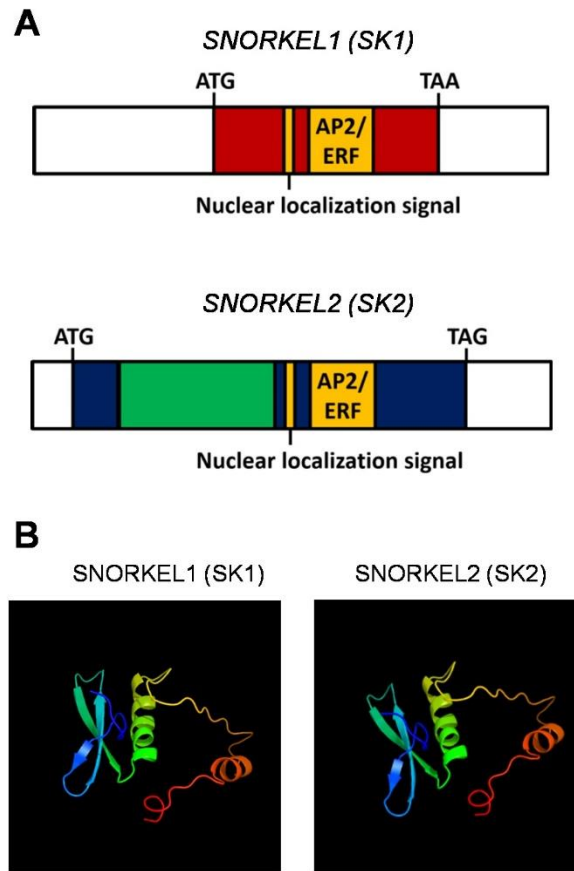


Figure S1: Structure of snorkel genes and their predicted protein structures.

A, Gene structure of *snorkels* (SKs). Red and blue represent exon. Green indicates the intron. White represents the non-coding region.

B, The protein structures of SKs were predicted using Phyre2 (<http://www.sbg.bio.ic.ac.uk/~phyre2>). The predicted protein is based on the Aterf096 with 99.95% confidence. Image colour by rainbow N→C terminus.



Figure S2: QTL12 promotes AR growth in mature plants.

T65, NIL-12 and C9285 plants were grown for 105 d. Several ARs have grown at node 3 as indicated by the red arrows.

Internode	C9285				NIL-12				T65			
	45 d	60 d	75 d	90 d	45 d	60 d	75 d	90 d	45 d	60 d	75 d	90 d
1	6,6±2.4	6,7±2.4	6,6±1.0	1,6±1.1	ND	2,9±1.3	1,0±0.7	1,2±0.7	ND	ND	0,3±0.2	7,1±1.4
2	12,0±1.5	10,2±1.7	9,0±1.1	1,7±1.3	ND	ND	9,7±1.7	9,0±1.9	ND	ND	ND	9,7±1.5
3	13,9±1.7	12,4±1.2	12,2±0.9	9,3±2.8	ND	ND	10,7±2.0	12,6±0.6	ND	ND	ND	ND
4	6,2±1.7	14,1±1.5	11,9±0.7	10,5±1.3	ND	ND	ND	9,7±1.2	ND	ND	ND	ND
5	ND	14,1±2.3	14,3±1.0	11,5±1.0	ND	ND	ND	ND	ND	ND	ND	ND
6	ND	3,9±1.0	13,8±1.4	15,7±0.8	ND	ND	ND	ND	ND	ND	ND	ND
7	ND	ND	6,0±1.2	15,7±0.7	ND	ND	ND	ND	ND	ND	ND	ND
8	ND	ND	ND	20,0±1.4	ND	ND	ND	ND	ND	ND	ND	ND
9	ND	ND	ND	12,0±1.8	ND	ND	ND	ND	ND	ND	ND	ND

Table S1: QTL-12 promotes internodal elongation in air-grown plants.

Internode lengths were measured in T65, NIL-12 and C9285 plants 45 d, 60 d, 75 d and 90 d after planting; ND: not detectable.

Experiments	Name of the primers	Primers 5' - 3' sequence
RT-PCR	SK1-FP	ACGGTATCCCTGAACTACTG
	SK1-RP	TCGTAGCGACAGCCGTACTG
	SK2-FP	CACTGGAGGCAACGAATG
	SK2-RP	TAAAAGGACCAGAGGCAGC
	OsActin1-FP	TCCATCTTGGCATCTCTCAG
	OsActin1-RP	GTACCCTCATCAGGCATCTG

Table S2: The primer sequence that used in this work

9. Discussion and perspectives

9.1 The establishment of a secondary root system in plants during submergence

9.1.1 The generation of a secondary root system is beneficial for plants to survive flooding

Crop yield is severely threatened by increasing rainfall events worldwide in recent years (Bailey-Serres et al., 2012). Continuous flooding is detrimental for plants, due to the decreased O₂ supply in the water for respiration. As compared with aboveground tissues, plant roots are first prone to submergence. In particular, the roots in soil compete with soil microorganisms for O₂ that renders the situation even worse (Jacoby et al., 2017). The main root system often encounters irreversible damage and becomes dysfunctional in long-term flooding. The ion transport, uptake of water and nutrients are impaired, ultimately leading to plant senescence and death (Sauter, 2013).

Rice (*Oryza sativa* L.), belonging to the grass family, together with maize, wheat and barley, is an important cereal crop supporting the global food supply. Cereals as monocot plant have a main root system which primarily consists of the postembryonic roots. Unlike other crop plants, the majority of rice species are semi-aquatic and partially tolerant to flooding. To date, several adaptive traits in rice have been described to ensure their survival ability in water. Ethylene biosynthesis is induced, and trapped inside plants upon submergence, due to the limited gas diffusion in water. AR primordia in rice are constitutively formed at the node and do not emerge until triggered by ethylene accumulation as a result of flooding. Similarly, the AR primordia are genetically formed and induced to grow upon submergence in bittersweet (*Solanum dulcamara*), and *Rumex palustris* (Negi et al., 2010; Dawood et al., 2016). Several other species, such as *Eucalyptus* spp. and tamarack, *de novo* form the ARs when submerged (Steffens and Rasmussen, 2016). The main roots in *Larix laricina* (tamarack) are completely replaced with ARs after six-month submergence (Steffens and Rasmussen, 2016). The *Alternanthera philoxeroides* with ARs exhibit a higher survival ability as compared with plants with ARs removed (Ayi et al., 2016).

Thus the establishment of a secondary root system consisting of ARs is a universal adaptive strategy for plants to survive flooding. In addition, *Tecticornia pergranulata* and *Meionectes brownii* also produces aquatic roots with chloroplasts supplying O₂ for respiration (Rich et al., 2008; Rich et al., 2011). However, most of the underwater roots still require O₂ from the atmosphere or from underwater photosynthesis of shoot and leaves. The additional ARs formed at the node will provide anchorage, sufficient water and nutrients for plants.

9.1.2 Adaptive traits in response to flooding are genetically linked

The emerging ARs elongate for exploration of the accessible nutrients and water. The aerenchyma in the root is genetically formed and further promoted as a result of the programmed death of specific cortex cell at the mature zone of the roots (Yamauchi et al., 2017; Yamauchi et al., 2019). The aerenchyma formation is helpful to enhance the O₂ diffusion in the root. Constitutive aerenchyma is genetically formed and induced by IAA13 and ARF-dependent auxin signalling (Yamauchi et al., 2019). Ethylene, Ca²⁺ signalling and ROS are required for the formation of the induced lysigenous aerenchyma upon flooding (Yamauchi et al., 2017). The aquatic root is generated *de novo* in the water, rendering the category between constitutive aerenchyma and lysigenous aerenchyma difficult. In NIL-12 plants, the aerenchyma was observed in both AR1 and AR2 roots indicating that both aquatic roots are functional to adapt to flooding. However, AR2 exhibit more cortex cell layers which lyse and form aerenchyma after elongation. So far, the functional difference between these two types of roots is still elusive.

Internode elongation at the stem is another adaptive trait, which allows the plant foliage to stay above the water, for the acquisition of O₂ from the atmospheric environment. In mature plants, GA biosynthesis is highly induced in response to submergence. GA activates the cell division in the intercalary meristem leading to the stem elongation (Lorbiecke and Sauter, 1998). Three main and two minor quantitative trait loci (QTL) in deepwater rice have been identified to be involved in the stem elongation in response to submergence (Hattori et al., 2009; Nagai et al., 2012). In addition, the single lines and a pyramiding nearly isogenic line (NIL) were generated. The QTL12 containing two AP2/ERF transcription factors encoded by *Snorkel1* (*SK1*) and *Snorkel2* (*SK2*), show the most

significant influence on the internode elongation. NIL-1 is another main QTL, containing a GA biosynthesis gene, *SD1* (*SEMIDWARF1*). NIL-1 shows a significant increase in the GA₄ level compared with wildtype plants upon submergence (Kuroha et al., 2018). In contrast, *PREMATURE INTERNODE ELONGATION1* (*PINE1*) negatively controls the internode elongation through repressing the GA sensitivity (Gomez-Ariza et al., 2019). In general, the internode elongation and the AR growth both occur in submerged conditions. QTL1 and QTL12 remarkably increase the root number and promote the root growth during submergence indicating that these two adaptive traits are closely related. In addition, the AR2 develop at the base of the internode, which requires stem elongation for space of root initiation.

In summary, the adaptive traits that develop during submergence in deep water aim to either acquire O₂ from the atmosphere or increase O₂ diffusion to ultimately improve respiration. It is conceivable that these adaptive traits are coordinated and function together to help the plants survive in the deepwater condition.

9.2 AR initiation and AR emergence are possibly controlled by different phytohormones

9.2.1 Auxin signalling pathway or polar auxin transport probably mediate the *de novo* genesis of AR primordia

AR primordia are constitutively formed from ground meristem at the young node of rice plants. Auxin signalling and polar auxin transport (PAT) play an important role in the *de novo* genesis of AR primordia in plants (Vanneste and Friml, 2009). PIN proteins are the auxin efflux carriers, which are mainly responsible for PAT in plants (Naramoto, 2017). The application of the auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA) effectively inhibited the root emergence in a dose-dependent manner. In rice, at least 12 predicted PIN proteins have been identified referring to PIN sequence in Arabidopsis (Lin and Sauter, 2019). Four PIN1 homologs (*OsPIN1a–1d*) are highly expressed in the roots compared with other tissues (Wang et al., 2009; Li et al., 2019). *OsPIN1b* and *OsPIN1c* promoters were active at the growing AR tip indicating that PAT is crucial for the AR development. A mutation of

OsPIN1b in rice results in a rootless phenotype (Liu et al., 2005). The *pin1a pin1b* double mutant exhibits an even more severe rootless phenotype (Li et al., 2019). The epidermal cells above the root primordia undergo programmed cell death (PCD) before root penetration (Steffens and Sauter, 2005). Interestingly, the expression of *OsPIN2* was exclusively visualized in epidermal cells above the emerging root primordia. Therefore, it is conceivable that these OsPIN proteins in rice coordinate the process of root emergence (Lin and Sauter, 2019). To verify our hypothesis that OsPIN2 mediates the PCD at the epidermis above the root primordia, the CRISPR/Cas9 technology will be applied to knock out OsPIN2 in wildtype to investigate the PCD above the root primordia in the future study.

The auxin reporter *DR5:VENUS* allows for the timely tracking of auxin activity with a high resolution. We investigated how auxin activity changes during root emergence. An increased auxin activity was observed in the growing AR primordia together with decreased signals at the epidermis above it in the dark indicating that local auxin accumulation in AR primordia is essential for the AR development. In addition, the application of NPA slowed down auxin accumulation, that is consistent with the observation that NPA inhibits root emergence. It remains to be analyzed whether submergence also induces an increase in local auxin activity at the node where root primordia develop similar to that in the dark.

TAA/YUC is a highly conserved auxin synthesis pathway in the plant kingdom (Zhao, 2012). Indole-3-pyruvate (IPA) is synthesized by tryptophan (Trp) aminotransferase of Arabidopsis (TAA) from Trp and further converted to natural indole-3-acetic acid (IAA) by YUCCA flavin monooxygenases through oxidative decarboxylation (Mashiguchi et al., 2011; Won et al., 2011). Growing evidence suggested that YUCs are one of the key genes mediating the biosynthesis of endogenous auxin in plants. Overexpression of *OsYUCCA1/OsYUC1*, *OsYUC3*, *OsYUC5*, *OsYUC6*, *OsYUC7*, *OsYUC8* and *OsYUC11* led to ectopic AR growth in rice suggestive of a universal function of *YUCCA* genes in regulating root formation (Yamamoto et al., 2007; Zhang et al., 2018). Application of exogenous auxin was used to investigate the function of auxin on AR growth. A higher concentration of 2,4-D had an inhibitory impact on AR growth similar to NPA, however, α -NAA did not promote the root growth, possibly because α -NAA did not increase the local auxin concentration in plant cells, of which the innate regulatory mechanism remains unclear. (Delbarre et al., 1996).

9.2.2 SKs mediate the ethylene-induced AR growth during submergence

The roots gradually mature after AR initiation, but do not emerge until triggered by ethylene. The AR penetration in rice is regulated by environmental factors. Flooding induced AR emergence due to increased accumulation of ethylene. In addition, continuous darkness also promoted root penetration (Lin and Sauter, 2018). The application of the ethylene biosynthesis inhibitor 1-methylcyclopropene (1-MCP) efficiently inhibited AR growth (Steffens and Sauter, 2005; Lin and Sauter, 2018) indicating that dark-induced AR growth is mediated by ethylene as well. Both blue light and red light significantly decrease root penetration rate, which is suggestive of a universal light inhibition. Even though only a minor difference in ethylene levels was observed between T65 and NIL-12 in deepwater conditions, SKs that are harboured by NIL-12 are induced by ethylene signalling, rapidly inducing the AR growth. ROS act downstream of ethylene, that is required for the PCD above the root primordia (Steffens and Sauter, 2009). The source of the ROS is possibly from the Ca^{2+} -dependent respiratory burst oxidase homologs (RBOHs) localized in the plasma membrane (Steffens and Sauter, 2009). ROS accumulation was observed at the epidermis above the root primordia and the growing root tip in NIL-12 and C9285 but not T65, indicating that SKs might control AR penetration upon flooding through the regulation of ROS level.

Transcriptome data show that the expression of *OsGA20ox2* is highly induced in C9285, but not T65 (Minami et al., 2018). *OsGA20ox2* is a GA biosynthesis gene, which is also known as *SD1* (*SEMI DWARF1*) located at chromosome1 in deepwater rice. NIL-1 shows a higher internode elongation compared with T65 as a result of more synthesized GA_4 (Kuroha et al., 2018). The additional root formation and more rapid root penetration in NIL-1 compared with T65 was consistent with the previous report that GA promotes the root growth together with ethylene in a synergistic manner (Steffens et al., 2006). *OsSHB* is an AP2/ERF transcription factor who targets the gene *KS1* encoding the ent-kaurene synthase of gibberellic acid (GA) biosynthesis. *OsSHB* controls the proliferation and elongation of root meristem cells. *Osshb* mutant plants have fewer and shorter roots compared with the wildtype (Li et al., 2015) further supporting the role of GA in AR growth.

ABA acts antagonistic to ethylene, repressing root growth (Steffens et al., 2006). *ABA4* mediates the interplay between ethylene and ABA in Arabidopsis (Dall'Osto et al., 2007). *MHZ4* in rice is a homolog of *ABA4*. Overexpression of *MHZ4* enhanced the ethylene sensitivity of seedling roots and increased the endogenous ABA level. Disruption of *MHZ4* induced AR formation at the nodes (Ma et al., 2014). It remains unknown whether ABA levels are altered at defined region where roots emerge to promote AR penetration in NIL-12.

The present evidence together with our data indicated that AR emergence is controlled by different phytohormones. It would be interesting to know whether SKs target *OsSHB* or *MHZ4*, and hence hormone homeostasis to promote AR growth upon submergence.

9.2.3 SKs possibly promote AR formation through auxin or cytokinin signalling pathways

Root formation and emergence are both promoted with the introgression of QTL12, including SK1 and SK2, into T65. SKs are AP2/ERF transcription factors, that specifically respond to ethylene and regulate AR emergence. So far, it remains unknown which genes are regulated by SKs and how SKs activate the downstream genes that promote AR formation. Auxin promotes *de novo* genesis of root primordia and cytokinins act antagonistically to auxin. Hattori (2009) reported that SKs shows no response to exogenous auxin or cytokinins, however, it is reasonable to assume that the additional AR primordia that are generated in NIL-12 are due to an increased local auxin activity or a decreased cytokinin content at the node, where the root primordia initiate. Hence, we hypothesize that auxin and/or cytokinin signalling pathways act downstream of SKs.

OsNAC2 is a NAC transcription factor, negatively controlling the crown root formation in rice (Mao et al., 2019). *OsNAC2* binds to the promoters of the IAA signalling related gene *OsARF25* and the cytokine oxidase gene *OsCKX4*, thereby integrating the auxin and cytokinin signalling pathways. Overexpression of *OsCKX4* in rice results in more crown root formation (Gao et al., 2014). Auxin response factors (ARFs) are transcription factors that mediate auxin signalling. The expression of *OsARF1* and *OsARF8* was significantly higher in C9285 compared

with T65 after submergence (Minami et al., 2018), which lead us to consider whether SKs bind to the promoters of these *ARFs*, controlling AR formation. An increased *DR5:VENUS* fluorescence indicating enhanced auxin activity was visualized at the emerging root primordium (Lin and Sauter, 2019). Hence, hybrid crosses, generating QTL-12/*DR5:VENUS* and T65/*DR5:VENUS* lines, will be useful to compare local differences in auxin activity between T65 and NIL-12 using CLSM. The *TCSn:GUS* reporter is a cytokinin reporter. Its expression is positively regulated by cytokinins and lowered by auxin (Tao et al., 2017). We will also generate QTL-12/*TCSn:GUS* and T65/*TCSn:GUS* lines to compare changes in cytokinin levels related to QTL12 (SKs).

Auxin and cytokinins likely play an important role in *de novo* AR genesis, while ethylene is mainly responsible for the root penetration during submergence. SKs specifically respond to ethylene mediating the AR penetration. It is however conceivable that residual SK activity targets the auxin and/or cytokinin signalling pathway, to promote AR formation. Hence, it is suggested that SKs control both, AR formation and AR emergence.

9.3 The AR system architecture in rice is controlled by genetic factors and environmental cues

9.3.1 Novel technologies are helpful to study and optimize root system architecture

The root system architecture is determined by the root number, the position of the roots, the root lengths and the root growth angle. A functional and effective root system architecture optimizes the uptake of water and nutrients to meet the demand for plant growth. However, specific root system adaptations improve the plant ability to cope with stress conditions. Root elongation, formation of lateral roots and of root hairs enable the root system to have access to a larger soil area to acquire sufficient water and nutrients. Based on the consideration that roots, in general, grow in dark conditions in the soil, specialized technology is needed to comprehensively analyze the root system architecture. In recent decades, non-destructive 3D-imaging techniques such as Magnetic Resonance

Imaging (MRI) and X-ray Computer Tomography (CT) have provided a high spatial resolution for the visualization of root systems (Metzner et al., 2015) which highly improved the study of root system architectures. Previously the generation of mutants rendered the study of specific gene functions in cereals difficult and tedious. In recent years, the CRISPR/Cas9 became a basic and applied technology for genome editing that can be used to generate mutants for functional gene analysis (Yin et al., 2017). The new technologies described here will be fundamentally helpful for root research in plants.

9.3.2 Light rather than gravity is the main regulator governing the AR angle in rice

A majority of work has been focused on lateral and adventitious root initiation and on root growth, whereas the regulation of the root angle is still largely unknown. GSA is genetically determined and influenced by environmental factors. We described that in rice, ARs at aboveground nodes grow upward in the dark, that has not been reported before (Lin and Sauter, 2018). Primary roots tend to grow in a vertical direction as a result of gravitropism (Su et al., 2017). The gravity is sensed through the starch statolith accumulated at the root tip in plants, which plays a critical role in redirecting the root growth. In contrast, lateral roots slowly sense the gravitropism after emergence and the statolith is not detected within 1 day emergence, that indicated an alternative response to gravity (Guyomarc'h et al., 2011). Starch statolith were also observed in initiating root primordia from node 1 to node 4 indicating that ARs perceive gravity at an early developmental stage. Surprisingly, when the rice stems were placed on a clinostat, where plants no longer perceive a gravity signal, the AR angle of inverted stems decreased indicating that ARs negatively respond to gravity. The underlying molecular mechanism of the negative gravitropic response of ARs remains unknown.

When ARs that grew upward in the dark were transferred to the light, the AR growth changed to a downward direction indicative of negative phototropism. These observations indicated that light is a key regulator that reshapes AR growth in rice. To date, several photoreceptors have been described in plants including phytochromes, cryptochrome and phototropin, which regulate plant growth and development in distinct manners. The red

light phytochrome receptors have two interconvertible isoforms, P_{fr} and P_r . In rice, PHYA, PHYB and PHYC are involved in red light and far-red light responses (Li et al., 2011; Takano et al., 2015). Cryptochrome and phototropins are two blue light receptors with different functions. Cryptochrome mainly regulates photomorphogenesis, while phototropins, encoded by *PHOT1* and *PHOT2* in rice, regulate phototropic responses. Blue light showed effective inhibition of the AR growth direction at low fluences. By comparison red and far-red light induced negative phototropism at higher fluence rates indicating that the root growth angle is controlled by blue, red and far-red light with a high sensitivity toward blue light.

In Arabidopsis, NPH3 is a member of the NRL (NPH3/RPT2-like) family and is required for the phototropic response by directly interacting with PHOT1. Rice CPT1 is homologous to NPH3 of Arabidopsis. The *cpt1* mutant that lacks a key gene of the blue light signal transduction pathway (Haga et al., 2005) is not sensitive to unilateral blue light in the coleoptile and less sensitive to unilateral blue light in the primary root as compared with the wildtype cultivar Nihonmasari. In our study, ARs of the *cpt1* mutant also showed a decreased sensitivity to blue light as compared with the wildtype. However, *cpt1* was still responsive to high blue light, presumably because the phototropic response is partially compensated by one or more of the 26 orthologues of this family in rice (Haga et al., 2005).

In general, blue light reaches the soil surface, while red light can penetrate deeper into the soil as compared with blue light. Therefore, the roots at the surface perceive both blue light and red light, whereas the roots in the depth only sense red light (Dev and Shanmugam, 2014). In flooding conditions, light quality and intensity are influenced by the turbidity of the water and by the chlorophyll content (Dev and Shanmugam, 2014). Downward root growth away from the soil surface may help to avoid desiccation while the upward growth of ARs provides access to O_2 in shallow turbid water. In summary, long-term submergence damages the main root system. Therefore the uptake of essential ions and water partially or fully relies on the new-born AR system. The establishment of a secondary root system is a crucial adaptation for improved plant tolerance to submergence.

9.3.3 Polar auxin transport is crucial for the determination of AR angle

Asymmetric auxin distribution in the root tip determines the root growth direction (Band et al., 2012; Tian et al., 2014). Auxin accumulation was observed at the lower side of the AR tip after 30 min of white light irradiation and the AR tip exhibited a phototropic response within 30 min (Lin and Sauter, 2019). Roots are more sensitive to auxin than the shoot and root growth is inhibited at low auxin concentrations (Tomas and Perrot-Rechenmann, 2010). Therefore, the auxin accumulated at the lower side likely led to growth inhibition, thus resulting in downward root bending. In Arabidopsis, AtPIN2 is mainly responsible for the basipetal auxin flux to directionally move auxin from the tip to the shoot. In Arabidopsis, an asymmetric auxin distribution in epidermal cells of the primary root tip was observed in response to hypoxia and resulted in primary root bending (Eysholdt-Derzso and Sauter, 2017). However, AtPIN2 was found to be symmetrically distributed at both sides of the primary root. Similarly, in rice, *OsPIN2* was expressed at the root tip, however, no asymmetrical expression was observed with the *OsPIN2:GUS* analysis. Phosphorylation is required for the regulation of AtPIN2 protein (Adamowski and Friml, 2015) indicating that auxin activity is controlled both at the transcriptional and posttranslational level. Knock out of *OsPIN2* decreased the root sensitivity to gravity, leading to a shallow root growth phenotype (Wang et al., 2018). Introgression of *OsPIN2* in *Atpin2* mutant rescued the root phenotype indicating that PIN2-mediated basipetal auxin transport is conserved in monocots and dicots.

The *ospin1a ospin1b* double mutant shows a decreased gravitropic response, while the single mutant of *OsPIN1a* and *OsPIN1b* did not exhibit different root phenotypes suggesting that *OsPIN1a* and *OsPIN1b* function redundantly. Even though the *OsPIN1c* promoter was active in growing ARs and in lateral roots, the single mutant of *OsPIN1c*, *OsPIN1d* and the double mutant *ospin1c ospin1d* did not significantly alter the root phenotype indicating that *OsPIN1c* and *OsPIN1d* might not be important for root development (Li et al., 2019). These observations render several PIN family members as candidate genes that modulate root system architecture.

9.3.4 Optimization of the root system architecture to support growth in cereals

The root system architecture plays a pivotal role in plant fitness, plant performance and crop yield. The optimization of the root system architecture depends on the nutrient demand of the crop together with local environmental conditions. In soil, gravitropism directs the roots to grow downward to acquire the essential nutrients for growth, on the one hand, and to protect the root from desiccation on the other hand. Root system architecture depends on the ecotype and on environmental cues. In cultivated soil, nitrogen (N) and phosphate (P) are essential and oftentimes limiting for plant growth. A large amount of work focuses on root responses to nitrogen (N) and phosphate (P) use efficiency (Niu et al., 2013; Postma et al., 2014). In low P conditions, the primary root growth is inhibited. Lateral roots and root hairs are largely promoted for uptake of P in a defined space. In addition, roots tend to grow in a vertical direction for soil exploration. By contrast, a shallow root system is preferable for N uptake since nitrogen is in general provided at the soil surface.

However, a specialized root system architecture needs to be considered to cope with environmental extremes. For instance, deeprooting systems enable the root to penetrate vertically to obtain water from deep soil layers for support of the aboveground tissue in drought conditions. During flooding, as we proposed, the rapid generation of a secondary root system is necessary for plants to improve their survival ability.

In summary, rice is a semi-aquatic plant that is tolerant to submergence. AR primordia genetically develop at the stem nodes and emerge when triggered by continuous darkness or flooding as a result of ethylene accumulation. The ERFVII transcription factors *Snorkel1* and *Snorkel2* promote not only the number of root primordia but also the root penetration rate. Light inhibits the dark-induced AR emergence and growth. The AR growth angle is regulated to a minor degree by gravity, and to a large degree by light signalling through blue light and red light receptors. Hence, the root system architecture is significantly influenced by flooding and flooding-associated light conditions to optimize root growth direction for O₂ acquisition.

10. References

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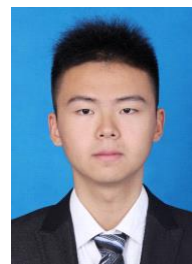
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13. Declaration of author contributions

I hereby declare that the thesis “Regulation of adventitious root system architecture in response to flooding in rice (*Oryza sativa* L.)” was performed and submitted to Christian-Albrechts-Universität zu Kiel. Apart from the supervisor’s guidance, the design of the paper is all my own work. The thesis has not been submitted to any other university. The accepted papers were listed below. The chapter 3 is presented as an unpublished manuscript and has not yet been submitted to any Journals. The thesis has been prepared subject to the Rules of Good Scientific Practice of the German Research Foundation. No academic degree has ever been withdrawn.

Author abbreviations: CL, Chen Lin; MS, Margret Sauter

The introduction was accepted as a review paper in the *Israel Journal of Plant Sciences* cited as follows:

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CL and MS wrote the article with contributions from CL

The work that presents chapter 1 was published in *Plant Physiology* cited as follows:

Lin C, Sauter M (2018) Control of adventitious root architecture in rice by darkness, light, and gravity. *Plant Physiology* 176(2): 1352-1364.

MS and CL conceived the project; CL and MS designed experiments; CL performed and analyzed the data; M.S. wrote the article with contributions from CL

The work that presents chapter 2 was published in *Frontiers in Plant Science* cited as follows:

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MS and CL conceived the project; CL and MS designed experiments; CL performed and analyzed experiments; MS wrote the article with contributions from CL

DECLARATION OF AUTHOR CONTRIBUTIONS

The work in chapter 3 is presented as an unpublished manuscript.

MS and CL conceived the project; CL and MS designed experiments; CL performed and analyzed experiments; CL wrote the article with the supervision of MS.

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Chen Lin