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A quantitative trait locus conferring flood tolerance to deepwater rice regulates the formation of two distinct types of aquatic adventitious roots

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Summary

• A key trait conferring flood tolerance is the ability to grow adventitious roots as a response to submergence. The genetic traits of deepwater rice determining the development and characteristics of aquatic adventitious roots (AAR) had not been evaluated.
• We used near-isogenic lines introgressed to test the hypothesis that the impressive shoot elongation ability of deepwater rice linked to quantitative trait loci 1 and 12 also promote the development of AAR.
• The deepwater rice genotype NIL-12 possessed expanded regions at the stem nodes where numerous AAR developed as a response to submergence. Two types (AR1 and AR2) of roots with distinct timing of emergence and large differences in morphological and anatomical traits formed within 3 (AR1) to 7 (AR2) d of submergence. The mechanical impedance provided by the leaf sheath caused AR2 to emerge later promoting thicker roots, higher elongation capacity and higher desiccation tolerance. Upregulation of key genes suggests a joint contribution in activating the meristem in AAR enhancing the development of these in response to submergence.
• The morphological and anatomical traits suggested that AR2 is better adapted to long-term flooding than AR1. We therefore propose that AR2 in deepwater rice functions as an evolutionary defence strategy to tackle periodic submergence.

Introduction

Floods affect natural wetlands as well as arable land, and frequencies and intensities have increased over the past decades as a result of ongoing climate change (Pedersen et al., 2017). Rice, as a crop, is also affected by flooding in rain-fed and irrigated lowlands where plants in some areas face high risk of submergence early in their life cycle (Bailey-Serres et al., 2012). However, a special type of rice, deepwater rice, is cultivated in natural ecosystems where long-lasting floods of several months and alternating water levels are a natural feature requiring a range of traits to cope with cycles of submergence and desubmergence (Catling, 1992). A well-studied trait of deepwater rice is its remarkable snorkelling capacity, where internode elongation of up to 30 cm d−1 enables the plant to keep track of rising waters. The snorkelling helps acquiring O2 from the atmosphere so that internal aeration is sustained (Mori et al., 2019). Moreover, formation of numerous aquatic adventitious roots growing from the nodes of the inundated part of the stem occurs soon after the onset of partial submergence (Lorbiecke & Sauter, 1999). In the present study, we report on the formation of two types of aquatic adventitious roots differing significantly in the timing of emergence and in key morphological and anatomical traits. We found that the leaf sheath delayed temporal and spatial expression of genes related to root development upon submergence, determining the timing of root emergence and even enhancing the root thickness and their elongation capacity at late stages of submergence.

Formation of adventitious roots emerging from stem nodes as a response to partial submergence is known to occur in many species of wetland plants (Pedersen et al., 2006; Ayi et al., 2016; Zhang et al., 2017b). These roots often do not reach the soil surface but remain floating in the floodwater and are thus referred to as aquatic adventitious roots (Rich et al., 2011). The function of aquatic adventitious roots in deepwater rice is related to nutrient acquisition from the floodwater of nitrogen (Khan et al., 1982) and of phosphorous (Rother & Whitton, 1988). Interestingly, deepwater rice was reported to form two types of aquatic adventitious roots (Inouye & Mochizuki, 1980). Within a few days of partial submergence, type 1 roots emerge from the node just below the point of attachment of the leaf sheath whereas type 2 roots emerge several days later from a position just above, thereby
penetrating the leaf sheath. These two types of roots have also been reported in paddy rice (Kawata et al., 1963).

In deepwater rice, adventitious root primordia develop at each newly formed node as part of the developmental programme (Lorbiecke & Sauter, 1999; Itoh et al., 2005). When plants become partially submerged, growth of adventitious root primordia is triggered. Like for other gasses, molecular diffusion of ethylene is severely impeded by inundation since the diffusion coefficient is 10 000-fold lower in water than in air (Armstrong, 1979). Therefore, radial diffusion of endogenously produced ethylene is restricted from tissues surrounded by water (Colmer & Voesenek, 2009). The accumulated ethylene acts as a submergence signal and promotes the emergence of adventitious roots in deepwater rice (Suge, 1985; Lorbiecke & Sauter, 1999, Rumex palustris (Peeters et al., 2002), tomato (Phatak et al., 2001) and mung bean (Robbins et al., 1985). The involvement of ethylene as signalling molecule is further demonstrated by the fact that pharmacological or genetic inhibition of ethylene perception impairs adventitious root emergence (Clark et al., 1999; McDonald & Visser, 2003; Lin & Sauter, 2018; Qi et al., 2019).

Quantitative trait locus (QTL) analysis has been successfully employed in rice to identify genetic loci conferring tolerance to complete submergence in paddy rice (Xu et al., 2006) or partial submergence in deepwater rice (Hattori et al., 2009). Using this approach, members of the ethylene response factor (ERF) transcription factor family have been identified as key regulators of submergence acclimation (Voesenek & Bailey-Serres, 2015). The ERF transcription factors SNORKEL1 and SNORKEL2 (SK1/SK2) in deepwater rice (Hattori et al., 2009) and SUBMERGENCE IA (SUB1A) in paddy rice (Xu et al., 2006) provide tolerance to flooding by inducing contrasting survival strategies. In deepwater rice, SK1 and SK2 are induced by ethylene to promote rapid stem elongation as an escape response, whereas in paddy rice, SUB1A confers tolerance to submergence by restricting stem growth as a quiescence response with both strategies relying on the contrasting modulation of gibberellic acid signalling. Growth arrest is a successful strategy to save carbohydrates during short-term flooding of 1–2 wk (Xu et al., 2006) since photosynthesis under water is greatly restricted due to slow diffusion of CO₂ and low light (Winkel et al., 2013). By contrast, the escape response of deepwater rice invests in continuous stem growth that ensures contact of the shoot to atmospheric O₂, CO₂ and light and hence ensures energy supply when plants are partially submerged for several weeks or months (Hattori et al., 2009).

Submergence is a compound stress, and therefore, tolerance is conferred by a range of traits. Since stem elongation and formation of aquatic adventitious roots occur soon after inundation, we hypothesized that regulation of stem growth and adventitious roots is genetically linked. We therefore tested the hypothesis that QTLs promoting stem growth in response to submergence in deepwater rice also promote the growth of adventitious roots. Three QTLs on chromosomes 1, 3 and 12, respectively, of deepwater rice have been shown to promote accelerated stem growth when introgressed into paddy rice (Hattori et al., 2009). We employed the respective near-isogenic lines NIL-1, NIL-3, NIL-12 and NIL-1 + 3 + 12 to test our hypothesis. We report that the submergence tolerance locus QT12L, which harbour 17 genes including the ERF transcription factor genes SK1 and SK2, promotes the development of an aquatic adventitious root system with a high degree of developmental plasticity, which is highly adjusted to short-term, long-term and recurrent flood events. We further show that the two types of aquatic adventitious roots, which were reported previously (Inouye & Mochizuki, 1980) but never characterized, have distinct anatomical features, emerge with different lag phases and have different growth potentials, features that all contribute to a high degree of adaptability of the aquatic adventitious root system to diverse flooding regimes.

Materials and Methods

The experimental procedures are conceptually described in Fig. 1 and in the text below; the extended version of all methods is found in Supporting Information Methods S1.

Plant material and treatments

Seeds of the near-isogenic rice lines 1, 3, 12 and 1 + 3 + 12 (NIL-1, NIL-3, NIL-12 and NIL-1 + 3 + 12 cv Taichung 65 (T65), Oryza sativa L. japonica subgroup) in which the quantitative deepwater rice trait loci 1, 3 and/or 12 (QT1L, 3 and 12) of the deepwater rice cultivar C9285 (O. sativa L. indica subgroup) were introgressed into lowland (paddy) rice T65 (Hattori et al., 2009) were obtained from Dr Motoyuki Ashikari (Nagoya University, Japan). Plants were grown for 14 wk in a climate chamber and partially (~2/3 of the shoot) submerged in tap water (Lorbiecke & Sauter, 1999) to induce the growth of aquatic adventitious roots (Fig. 1).

Following partial submergence, T65 produced only one type of root while NIL-12 produced two types of aquatic adventitious roots at node 3 (Fig. 3, see later). Type 1 roots emerged from nodes early during submergence whereas type 2 roots emerged several days later at the nodal region above type 1 roots; these two types of roots are subsequently referred to as adventitious root 1 (AR1) and adventitious root 2 (AR2), respectively. After the indicated duration of partial submergence (Fig. 1), numbers of aquatic adventitious roots were counted and root length determined. For recurrent submergence/de-submergence, T65 and NIL-12 plants were partially submerged for 3 d, de-submerged for 3 d and partially submerged for another 6 d with numbers of AR1 and AR2 counted every 3 d. For a time-course analysis of root growth, plants were partially submerged for 60 d and lengths of AR1 and AR2 were measured at nodes 3 and 5 every 2 (until day 40) or 5 d (after day 40). The influence of the leaf sheath on AR2 emergence (NIL-12 plants with or without the leaf sheath removed at node 4; numbering of nodes follows Lorbiecke & Sauter (1999)) was partially submerged for 28 d. Finally, to test the effect of mechanical impedance on AR1 emergence and anatomy, the region where AR1 emerge from at node 4 was wrapped with five layers of Parafilm® before submergence for 7 d.
Histological analyses and SEM

Adventitious roots (ARs) of NIL-12 were cross-sectioned at positions 5, 25, 45 and 65 mm behind the apex and analysed using IMAGEJ (v.1.43u; US National Institutes of Health). Percent of aerenchyma was calculated by dividing the aerenchymal area by the total cortical area (Lin et al., 2021). Visualization of suberin and lignin deposition was obtained using the approach of Abiko et al. (2012), and the permeability of the apoplast was assessed following Soukup et al. (2007). Nodal segments with emerging roots were imaged using scanning electron microscopy using the approach of Steffens & Sauter (2009). See Methods S1 for further details.

Radial O₂ profiles

Radial O₂ profiles of aquatic adventitious roots attached to stem segments were measured using O₂ microsensors following the procedure in Lin et al. (2021). Stems possessing internodes with several 8–10-cm-long AR1 or AR2 were cut from the main stem; AR1 had some laterals in this position, and these remained in place during the measurements. The stem piece with one target root (additional roots were trimmed off) was fixed on a metal mesh, positioned in a small aquarium and submerged into DI water at air equilibrium. Using an O₂ microsensor (OX-10; Unisense A/S, Aarhus, Denmark), radial measurements were taken in steps of 10–25 μm starting 400–
500 µm from the root surface inside the DBL. See Methods S1 for further details.

Respiration of root tissues

Oxygen consumption by root segments was measured using the approach of Colmer et al. (2019). Eight–ten-cm-long AR1 or AR2 was cut into 20-mm-long segments corresponding to different positions of the root: 20 mm behind the root tip (10–30 mm), 40 mm behind the root tip (30–50 mm) and 60 mm behind the root tip (50–70 mm). At these positions, AR1 had formed several lateral roots while AR2 had no laterals. Therefore, the respiration rates of AR1 were carried out leaving or removing the lateral roots. The 20-mm-long segments were placed into 1-ml glass vials containing tap water at air equilibrium. An O₂ optode was inserted into each vial to measure O₂ concentration. The respiration rates were calculated by Rate (SensorTrace Suite v.3.2) and divided by fresh mass (FM, final unit, nmol O₂ g⁻¹ FM s⁻¹). See Methods S1 for further details.

Radial water loss

Radial water loss (RWL) from AR1 and AR2 was measured using gravimetric measurements following the approach of Peralta Ogorek et al. (2021). Adventitious roots of 8–10 cm length had the most apical 3 cm removed and shortened into 5-cm-long segments. The diameter of 10 root segments of AR1 and five root segments of AR2 was measured using a digital calliper. The decline in root mass as water evaporated was recorded automatically every 30–60 s for 1 h using 4-digit analytical balance (Analytical Balance ME54; Metler Toledo). The roots were further dried at 50°C for 3 d enabling calculation of the total water content and cumulated water loss. Rates of RWL were obtained based on the cumulated water loss and surface area of the root segments (final unit, µmol H₂O m⁻² s⁻¹). Finally, to compare the two root types, the RWL rates at the time point where 50% of the total water content was lost were extracted. See Methods S1 for further details.

Transcriptomic analysis

Plants with or without leaf sheath were submerged for 9 h, and RNA samples were collected at the region where AR2 would emerge with nonsubmerged plants as control. RNA extraction was performed using RNeasy Plant Mini Kit (Qiagen) following the manufacturer’s protocol. Raw reads of FASTQ format were processed to ensure the quality and further mapped to the reference genome (https://rapdb.dna.afrc.go.jp/). Differential expression analysis between plants with or without leaf sheath (three biological replicates per treatment) was performed using DESeq2 R package (Dai et al., 2021). The resulting P values were adjusted using the Benjamini & Hochberg’s (1995) approach for controlling the false discovery rate. Gene Ontology (GO) was used to annotate genes to biological processes, molecular functions and cellular components, and Kyoto Encyclopaedia of Genes and Genomes (KEGG) was used to annotate genes to pathway. GO enrichment analysis of differentially expressed genes was implemented by the CLUSTERPROFILER R package, in which gene length bias was corrected, and this package was also used to test the statistical enrichment of differential expression genes in KEGG pathways (http://www.genome.jp/kegg/). See Methods S1 for further details.

Statistical analyses

Statistical analyses were performed using Minitab 17 (Minitab Ltd, Coventry, UK). Comparison of means or variance was performed using Student’s t-test, one-way ANOVA or two-way ANOVA depending on experimental design. For these parametric tests, the assumptions were tested using Shapiro–Wilk for normality and Brown–Forsythe for equal variance. In cases where analysis of variance showed significant effects of treatment, a Tukey post hoc test was applied. The number of replicates and tests used are denoted in the figure captions and in Fig. 1.

Results

Deepwater rice QTL1 and QTL12 promote the growth of aquatic adventitious roots

Hattori et al. (2009) identified several QTLs in deepwater rice contributing to rapid stem elongation as an escape response to submergence. We tested the hypothesis that regulation of stem growth may be genetically linked to the development of aquatic adventitious roots as another important trait conferring tolerance to partial submergence. We employed four near-isogenic lines (NIL-1, NIL-3, NIL-12 and NIL-1+3+12) harbouring a single QTL, or three QTLs, introgressed into paddy rice. Phenotypic (Figs 2a,b, S1) and quantitative analysis (Fig. 2c) revealed that deepwater rice C211, NIL-1, NIL-12 and NIL-1+3+12, but not NIL-3, plants had more and longer adventitious roots after 7 d of partial submergence as compared to the paddy rice T65. NIL-12 and NIL-1+3+12 grew as many adventitious roots as deepwater rice (Fig. 2c), and root elongation was enhanced in NIL-12 and NIL-1+3+12 compared with paddy rice but less than in deepwater rice (Fig. 2d). The number of nodes was higher in all NILs than in paddy rice but was less than in deepwater rice (Fig. 2e) as also reported by Hattori et al. (2009). In paddy rice, adventitious roots had emerged from the lower part of the node below the insertion site of the leaf sheath, whereas in NIL-1, NIL-12, NIL-1+3+12 and deepwater rice, adventitious roots emerged both at the lower part of the node and also at the upper part of the node covered by the leaf sheath (Figs S1, 3a–c). Formation of additional adventitious roots in partially submerged deepwater rice plants was hence achieved by expanding the nodal region where adventitious roots emerge and is partly genetically encoded by QTL1 and QTL12.

We characterized the adventitious root system further using NIL-12 as a deepwater rice model. Notably, the two types of adventitious roots, AR1 and AR2, showed distinct differences in diameter, AR2 being thicker than AR1 (Fig. 3d–f), whereas AR1 had grown longer than AR2 after 7 d of submergence (Fig. 3g).
Since root structure influences root function (Lynch, 2019), we next quantified the anatomical differences between AR1 and AR2 (Figs 3e–l, S2). AR2 had more cortex cell layers, a larger stele and larger ratios of cortex-to-stele and xylem-to-stele, traits that have been functionally linked to drought response (Lynch et al., 2021; Yamauchi et al., 2021).

AR1 and AR2 differ in anatomy and respiration rate

To further investigate the differences between the two types of roots, we compared the respiration rate of AR1 and AR2 (Fig. 4a, b). Respiration rates were determined at 20, 40 and 60 mm from the root tip in 8–10-cm-long roots. At this length, AR1 developed lateral roots whereas AR2 did not, revealing another difference between the two types of adventitious roots. AR1 with its lateral roots had significantly higher rates of O2 consumption than AR2 (Fig. 4b). However, when lateral roots were pruned, respiration rates of AR1 declined to levels resembling those of AR2 at equivalent positions. Since respiration requires O2, we compared aerenchyma formation in AR1 and AR2 (Fig. S3). While the total cross-sectional area of aerenchyma was larger in AR2, the percentage of aerenchyma per cortex area was similar in AR1 and AR2 from the root tip to the mature zone (Fig. S3) in accordance with the observation that respiration rates did not differ.

We proposed that the formation of AR2 might be an adaptation to extended flooding periods. As previously shown,
emergence and elongation of AR2 at the onset of submergence were delayed compared with AR1 (Inouye & Mochizuki, 1980), yet the growth ability of both root types under long-term flooding remained unexplored. To fill this knowledge gap, we performed a time-course analysis of elongation of AR1 and AR2 (Fig. 4c). As described previously, AR1 emerged faster and elongated rapidly within the first 10 d. Thereafter, growth rates declined, and growth arrested after 3 wk. By contrast, AR2 emergence was delayed by 5 d. Subsequently, AR2 elongated for 6 wk reaching nearly twice the length of AR1 (Fig. 4c).

Aside from the root type-specific difference in growth behaviour, we also noted a dependence on developmental stage of the node. AR1 and AR2 that emerged at node 5 emerged sooner than at the younger node 3 (Fig. 4c) in accordance with previous observations that adventitious root primordia within each node grow larger with age (Lorbiecke & Sauter, 1999). Taken together, the

**Fig. 3** NIL-12 plants of *Oryza sativa* generate two distinct AR types at their nodes. (a) NIL-12 stem node indicating the two positions at which AR develop by a dashed purple and red line. Bar, 50 mm. (b) Nodal cross sections at the positions indicated in (a). ARs that develop at the lower position are termed AR1, and ARs that develop at the upper part of the node that is covered by the leaf sheath are termed AR2. Bars, 1 mm. (c) Scanning electron micrograph depicting AR1 and AR2. Bar, 300 μm. (d) Representative elongated AR1 and AR2. Bar, 5 mm. (e) Cross sections of AR1 and AR2 from the fourth youngest node of NIL-12 were cut 5 mm behind the root tip and stained with toluidine blue. Bar, 0.2 mm. (f) Tracheary elements (TE), central cylinder (CC), cortex (Co) and outer cell layers (OCL) are shown for AR2. The cross sections were taken 5 mm behind the root tip. Bar, 0.2 mm. (g–l) Mean lengths, cross-sectional area, cortex area, stele area or xylem to stele ratio of AR1 and AR2 were determined at the fourth youngest node of NIL-12 plants that were submerged for 7 d (±SE, n = 18–24, **, P < 0.01; Student’s t-test). AR, adventitious root; NIL, near-isogenic line. For the box plots, the horizontal line indicates the median, the box indicates the 50% quartiles, the whiskers indicate the range, and the circles indicate individual data points.
gradient of adventitious root development along the stem and the different growth kinetics of AR1 and AR2 at each node are well suited to acclimate to rising water levels and to maintain a functional system of aquatic adventitious roots over a long period of flooding.

Although there was no difference in aerenchyma:cortex ratio between AR1 and AR2 (Fig. S3), we tested whether radial oxygen supply from the floodwater might differ (Lin et al., 2021). Oxygen profiles were taken at fixed position behind the root tip (Fig. 4d–h) in 8–10-cm-long roots representing the exponential growth phase of AR1 and AR2 (Fig. 4c). At the surface of AR1, but not AR2, O₂ levels steadily declined, indicating that oxygen consumption occurred through the surface of AR1 but not AR2 (Fig. 4e,f). O₂ levels were lower in the stele than in the cortex as described previously (Lin et al., 2021) with no differences between AR1 and AR2, indicating that the gas diffusion barrier in AR2 that prevented oxygen uptake from the floodwater did not alter O₂ supply to the root tissue under the conditions tested (Fig. 4g,h). Suberin and lignin are cell wall components that can restrict radial O₂ diffusion. Staining for suberin and lignin at 20, 40 and 60 mm behind the tip of AR1 and AR2 showed increasing deposition of both components from tip to base (Fig. 5a,b). Yet, diffusion of periodic acid revealed that AR1 did not form an apoplastic barrier even in the mature root zone, whereas AR2 had restricted radial diffusion at 40 and 60 mm from the root tip (Fig. 5c), demonstrating that AR1 and AR2 differ in their ability to develop a diffusion barrier.

Developmentally and functionally distinct aquatic adventitious roots may provide desiccation resistance during de-submergence

It is conceivable that adventitious roots of deepwater rice become transiently exposed to air when water levels fluctuate supporting the hypothesis that the ability to restrict gas, including water vapour, diffusion may protect AR2 from water loss under such conditions. Therefore, we measured RWL, and both types of roots visibly shrank in diameter within 1 h (Fig. 6a), but AR1 lost water at a higher rate and desiccated faster than AR2 (Fig. 6b–d), suggesting that AR2 is partially protected from drying out by the gas diffusion barrier. In addition, the lower surface area to volume ratio of the thicker AR2 roots would also slow down desiccation.

The possible advantage of the two types of roots in transient flood events was tested by exposing paddy rice and NIL-12 plants to a submergence/de-submergence regime (Fig. 6e–g). After 3-d submergence, AR1 but not AR2 emerged from paddy and deepwater rice. Following de-submergence, all AR1 desiccated and only a few AR1 emerged from remaining root primordia during a second submergence period (Figs 6i, S4). By contrast, AR2 emerged from those nodes during resubmergence in both paddy and NIL-12 plants, with more and longer AR2 observed in NIL-12 rice revealing high plasticity in establishing an aquatic adventitious root system during short-term (few days), long-term (weeks to months) and transient flooding. Under the current experimental conditions, Evan’s blue staining of the roots showed that AR1 and AR2 both died after 3 d of de-submergence (Fig. S4).

The leaf sheath determines AR2 key anatomical features

Adventitious root 1 were thinner than AR2 and emerged faster, and their maximum length was shorter. AR2 developed at a position covered by the leaf sheath providing a mechanical barrier and possibly also influencing AR2 anatomy. We therefore removed the leaf sheath before submergence and analysed root length and diameter after 7 d of submergence (Fig. 7a). In the absence of a leaf sheath, AR2 grew longer and thinner than AR2 having to penetrate the leaf sheath (Fig. 7b–e). We compared AR2 emergence with or without the leaf sheath present in a time-course experiment (Fig. 7f,g). In the absence of the leaf sheath, AR2 emerged faster and ceased growing after 3 wk reminiscent of the growth behaviour of AR1 (Fig. 4c), indicating that the leaf sheath determines key root characteristics. Interestingly, AR1 emerging from nodes that were wrapped with Parafilm® mimicking mechanical impedance grew shorter but did not increase in diameter (Figs S5, S6). The difference in root diameter of AR2 that emerged in the presence or absence of the leaf sheath was due to fewer files of cortex cells and a smaller stele (Figs 7d,e, S6). Notably, the cortex-to-stele ratio was reduced in AR2 with the leaf sheath removed, being intermediary of AR2 and AR1 (Fig. S6). We therefore conclude that the nodal position at which AR2 develop determines their key anatomical features.

The leaf sheath determines molecular responses to submergence

To gain insight into the molecular responses triggered by the leaf sheath in the node from which the AR2 develop, we performed a RNA-seq analysis of the node in three conditions: (1) partially submerged with the leaf sheath present; (2) partially submerged without the leaf sheath; and (3) with the leaf sheath present but not submerged (controls). In the absence of the leaf sheath, nearly four times more genes were differentially expressed during submergence compared with plants with the leaf sheath present (Fig. S7). Of the 1578 upregulated genes, 892 were also differentially expressed when the leaf sheath was removed, and of the 1142 downregulated genes, 504 were regulated in the same direction with the leaf sheath removed indicating common responses (Fig. S7A). We compared genes that were previously described as submergence-upregulated in rice such as hypoxia core genes, PDC and ADH (Reynoso et al., 2019; Fig. S7). As a response to submergence, we found increased expression of nearly all submergence signature genes in plants where the leaf sheath had been removed. By contrast, with the leaf sheath present, these signature genes were not upregulated to the same extent, showing that the leaf sheath acts as ‘shield’ protecting the nodal tissue from hypoxia responses. A similar pattern of regulation was observed for group VII ERF transcription factor genes (Fig. S7). Analysis of ABA biosynthesis genes (Fig. S8) revealed downregulation in the AR2 area with leaf sheath that was enhanced when the leaf sheath was removed (Fig. S8) in accordance with the finding that downregulation of ABA levels is an early response to

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submergence (Hoffmann-Benning & Kende, 1992; Minami et al., 2018).

Genes that exclusively relate to adventitious root development have not been described. However, adventitious roots and crown roots have in common their origin from aerial nodes, suggesting that they may be regulated by common or overlapping gene networks (Ge & Wang, 2012; Wu & Cheng, 2014; Meng et al., 2019). We therefore analysed the expression of genes that were previously shown to be involved in crown root initiation and emergence in rice (Fig. 7h). The auxin receptor OsTIR1 and the stem cell regulator OsWOX11 were significantly induced in the absence of the leaf sheath, whereas the cytokinin response...
**Fig. 4** Comparison of oxygen supply and respiration rate of AR1 and AR2 in *Oryza sativa* L. (a) AR1, but not AR2, developed lateral roots. Bars, 1 mm. (b) Respiration rates of AR1 without (lateral removed) or with lateral roots (original condition) and AR2 (not forming laterals at these positions at this time point) were determined at different distances from the root tip. Root segments 20 mm (10–30 mm), 40 mm (30–50 mm) and 60 mm from the root tip (50–70 mm) were collected from 8 to 10-cm-long roots of submerged NIL-12 plants. A comparison of respiration rates between root types and positions was conducted on log-transformed data with one-way ANOVA followed by a Tukey test where different lowercase letters indicate significant differences, \( P < 0.01, n = 5 \) for AR1 at all positions, \( n = 6 \) for AR2 at positions 20 and 40 mm and \( n = 5 \) at 60 mm from the tip. (c) Average lengths (±SD indicated by shading, \( n = 27 \)) of AR1 and AR2 at the 3rd and 5th youngest nodes (nodes 3 and 5) were continuously determined during 60 d of submergence. AR1 and AR2 grown at node 5 of NIL-12 after 60 d of submergence. Bar, 10 cm. (d) An AR fixed immersed in water to measure oxygen with a microsensor at 60 mm from the tip (red star). The cut ends of the stem to which the AR was connected were sealed with silicone tubing and small glass beads. To enable diffusional supply of \( O_2 \) through the stem, the silicone tubes were punctured and left in contact with the air above the tank while preventing flooding of the pit cavity. The bulk water was gently purged with air to keep the oxygen tension near atmospheric equilibrium and to maintain a stable diffusive boundary layer. (e, f) Radial oxygen profiles taken at 60 mm from the tip of AR1 (e) or AR2 (f). The red arrow indicates \( O_2 \) diffusion from the water into the root. The red asterisk indicates the central stele. (g, h) Median cortex (g) or stele (h) \( pO_2 \), calculated from \( n = 5 \) radial \( O_2 \) profiles per root type, are depicted as box-whisker plot showing mean (+), median (horizontal line), 50% quartiles (box), minimum and maximum (bars), and the circles indicate individual data points. Statistical comparisons between root types and positions were conducted using a two-way ANOVA; for cortex (g), \( P = 0.0111 \) for Type, \( P = \text{ns} \) for Position and \( P = \text{ns} \) for Type \( \times \) Position; for stele (h), \( P < 0.0001 \) for Type, \( P = 0.0137 \) for Position and \( P = \text{ns} \) for Type \( \times \) Position. A post hoc Tukey test showed significantly different stele \( O_2 \) between AR1 and AR2 at 40 and 60 mm from the tip (*, \( P < 0.05 \)). AR, adventitious root; NIL, near-isogenic line.

**Fig. 5** In *Oryza sativa*, a gas diffusion barrier is constitutively formed in AR2 but not AR1. (a) Suberin staining in AR1 or AR2 shown at the distances from the root tip indicated. Suberin lamellae are visualized by the yellow-green colour indicated by yellow arrowheads. Blue arrowheads point to cell wall auto-fluorescence. Bars, 20 \( \mu m \). (b) Lignin staining in AR1 or AR2 is visible in pink and indicated by red arrowheads. Bars, 20 \( \mu m \). (c) Periodic acid diffusion occurs through the apoplast in AR1 but not in AR2. Purple arrowheads indicate the cortex cell layers where periodic acid is detected. Bars, 50 \( \mu m \). AR, adventitious root.
regulator gene OsRR1 was induced only with the leaf sheath present. Genes supporting cell division and elongation in crown roots were exclusively upregulated in the absence of the leaf sheath (Fig. 7i), whereas genes involved in lateral root development were not upregulated or downregulated (Fig. 7j), indicating that removal of the leaf sheath led to regulation of genes at the node which are specifically involved in crown root/adventitious root development. Since ethylene promotes the growth of adventitious roots in rice (Lorbiecke & Sauter, 1999), we extracted genes encoding proteins involved in ethylene biosynthesis or signalling.

ACS6, ACO3 and ACO5 were upregulated whereas ACO2 was downregulated when the leaf sheath was removed (Fig. 8a,b). Ethylene signalling genes were in general more strongly upregulated by submergence when the leaf sheath was present. (b) Cumulated water loss (% of total water content) of 8-cm-long AR1 or AR2 roots exposed to dry air under constant temperature (23°C) and relative humidity of 30%. Each data point shows mean ± SE. (c) Values of cumulated water loss were converted to radial water loss (RWL) rates based on total surface area. Each data point shows mean ± SE. (d) RWL rates at the time point where 50% of the total water content were lost were used to contrast the two root types applying a two-tailed t-test on log-transformed data (**, P < 0.01) illustrated as a box-whisker plot showing mean (+), median (horizontal line), 50% quartiles (box), minimum and maximum (bars), and circles showing individual data points. Number of replicates was four for AR1 roots and five for AR2 roots. (e) Representative plant images showing ARs at node 3 of T65 and NIL-12 exposed to the submergence/de-submergence regime indicated. Bar, 20 mm. (f, g) Mean number of viable AR1 (f) or AR2 (g) counted at node 3 of T65 and NIL-12 plants exposed to the submergence/de-submergence (Sub/De-sub) regime indicated (±SE, n = 21, P < 0.05; two-way ANOVA with Tukey test, where different lowercase letters indicate significant differences). (h) Mean number of viable AR1 or AR2 submerged only once in NIL-12 plants (±SE, n = 9, P < 0.05; two-way ANOVA with Tukey test, where different lowercase letters indicate significant differences). (i) Mean number of viable AR1 or AR2 after two times of submergence interrupted by de-submergence in NIL-12 plants (±SE, n = 9, P < 0.05; two-way ANOVA with Tukey test, where different lowercase letters indicate significant differences). AR, adventitious root; NIL, near-isogenic line.
ETR2 and CTR2 were both upregulated whereas related genes encoding signalling isoforms were downregulated (Fig. 8b). Expression analysis revealed stronger upregulation of ethylene-related genes in the absence of the leaf sheath but did not reveal whether responsiveness to ethylene was altered by the leaf sheath. We therefore excised stem sections and treated them with the ethylene-releasing compound ethephon through the lower cut surface and scored AR2 growth after 7 d (Fig. 8c–e). With this treatment, more AR2 emerged without leaf sheath and roots grew longer, showing that the leaf sheath does not impair ethylene responsiveness, but may rather act as a physical barrier that promotes radial growth and delays emergence.

Discussion

Deepwater rice evolved along rivers and lake banks (Catling, 1992), and to survive recurrent floods, it developed a series of adaptive traits to avoid drowning (Voosenk & Bailey-Serres, 2015). Among them is the stunning ability to elongate its internodes enabling deepwater rice to remain in contact with...
atmospheric O₂, CO₂ and light, thereby preventing the adverse impact of complete submergence. In other species, some adaptive traits such as inducible aerenchyma in soil roots and adventitious root development occur simultaneously as a response to flooding (Pedersen et al., 2021), and oftentimes they share an overlapping gene network (Sasidharan et al., 2018). We therefore investigated if the previously described deepwater rice QTLs involved in internode elongation are also involved in the formation of aquatic adventitious roots. Indeed, responses to partial submergence and the following emergence of aquatic adventitious roots in deepwater rice are under strong genetic control. QTL1 and QTL12 not only enhanced the internode elongation but also significantly increased the number of adventitious roots and their growth rate. Importantly, NIL-12 formed additional ectopic thick roots as compared to paddy rice. Below, we discuss these findings in the context of function during episodic flood events and the genetic network involved in responses to partial submergence.

Is the delayed emergence of the AR2 roots an evolutionary defence against multiple flood events?

Rice possesses a variety of root traits such as aerenchyma and a barrier to radial O₂ loss enabling growth in flooded soils (Kuroha & Ashikari, 2020). However, deeper floods causing partial or complete submergence of the shoot exert additional stress to the soil roots because the distance from the atmosphere to the root tips exceeds the diffusive transport capacity of O₂ resulting in a dysfunctional soil root system. The growth of a secondary set of roots from the stem therefore alleviates the functional loss of soil roots (Lorbiecke & Sauter, 1999; Sauter, 2013). In wild wetland plants, the aquatic adventitious roots assist plants taking up water and nutrients (Cumbus & Robinson, 1977; Zhang et al., 2017b) demonstrating the capacity of aquatic adventitious roots to replace the function of soil roots. Moreover, aquatic adventitious roots floating in water can also act as gills facilitating diffusion of O₂ from O₂-rich floodwater into the submerged part of the stem (Ayi et al., 2016; Lin et al., 2021), and in other situations, aquatic adventitious roots develop functional chloroplasts enabling O₂ and carbohydrate production (Rich et al., 2011, 2013).

Recurrence of submergence followed by de-submergence expose the aquatic adventitious roots to desiccation during times when the floodwater recedes. After a few days of exposure to the air, the roots perish, and although AR1 lose tissue water significantly faster than AR2 (Fig. 6a–d), we consider the observed difference in desiccation tolerance of minor functional importance since both types of roots desiccate within an hour or two when exposed to dry air. Plants exposed to recurrent submergence therefore rely on the ability to continue forming adventitious roots, an ability lacking in most other rice cultivars (Steffens & Rasmussen, 2016; Zhang et al., 2017a). We show that AR1 emerges several days before AR2, and the latter is only formed during prolonged submergence (Fig. 6e–g). After recurrent changes in water level, AR1 only had little initiation, while AR2 continued to develop (Fig. 6e–g). We therefore propose that the delayed emergence of AR2 is an effective defence strategy developed by deepwater rice to sustain recurrent flood events. We further suggest that AR2 is particularly important for deepwater rice to cope with prolonged flooding as these AR2 keep growing and remain functional for longer times compared with AR1.

Functional aspects of the contrasting key morphological and anatomical traits of AR1 and AR2

Key morphological and anatomical traits differ in the two types of aquatic adventitious roots. AR2 are significantly thicker than AR1, and thick roots are considered to have higher biomechanical force enabling penetration into compacted soils (Bengough et al., 2011; Chimungu et al., 2015), but such strength would be of little importance in floodwater. Thick roots would, however, restrict radial O₂ loss into the floodwater during periods of hypoxia. The surface area to volume ratio (SA : V) is lower in a thick root, that is, the relative surface in contact with hypoxic floodwater per volume of root tissue is smaller reducing the diffusive O₂ flux into the floodwater in the same way that thick roots lose less O₂ to anoxic soils (Pedersen et al., 2021). The significantly larger diameter of AR2 is mainly due to extra files of cortical cells, whereas the tissue porosity of the AR1 and AR2 is similar (Figs 3h,i, S2). Therefore, the cross-sectional area of aerenchyma is substantially higher in AR2 leading to greater capacity of O₂ diffusion towards the tip (Colmer, 2003) during times of low O₂ content in the floodwater (Setter et al., 1988).

In 18 species of wild Poaceae, the cortex-to-stele ratio (CSR) and xylem-to-stele ratio (XSR) were both found to increase with increasing soil water content (Yamauchi et al., 2021). Both of these traits differ significantly in AR1 and AR2 roots and exceed the values characteristic for waterlogged Poaceae. The CSR value of c. 15 for Poaceae in wet soils is similar to the mean CSR of AR1 (14.6) with AR2 having 1.8-fold higher CSR (Fig. 3k) indicating acclimation to high soil water content in both roots, but more so in AR2. XSR shows a similar pattern with numerical values (0.26) of AR2 exceeding those observed for the wild Poaceae (c. 0.14) and AR1 being similar (0.14) (Fig. 3i). Since AR1 and AR2 are both formed in floodwater, the similarity with roots from high soil water content is expected and demonstrates that these two traits are also strongly reliant on ‘soil water content’ in deepwater rice. However, the functional importance of both traits is lacking experimental evidence.

Moreover, AR2 forms a partial barrier to radial O₂ in its basal parts whereas the barrier is lacking in AR1. A root barrier to radial O₂ loss would have little if any function in floodwater with high O₂ since O₂ to sustain aerobic metabolism would be supported by influx of O₂ from the floodwater. In severely hypoxic floodwater, however, the barrier to radial O₂ loss could serve a similar role as the one that has been evidenced in waterlogged, anoxic soils (Colmer, 2003) since O₂ supply would be based on diffusion from the submerged parts of the stem (Lin et al., 2021). With AR2 obtaining a maximum length of 35 cm compared with only 19 cm in AR1, the O₂ supply to the root tip would become critical during low environmental O₂. In such a situation, even a partial barrier to radial O₂ loss in combination with the much higher diffusional transport capacity of the porous cortex of AR2...
(as mentioned previously) could become crucial to sustaining root growth.

Contrasting anatomy and timing of root emergence are controlled by mechanical impedance exerted by the leaf sheath

We found that mechanical impedance exerted by the leaf sheath significantly influenced the timing of emergence and key morphological traits of the aquatic adventitious roots. The influence of mechanical impedance on root anatomy and morphology is well described for soil roots where mechanical stress is exerted by soil compaction determining morphological changes of roots (Lynch, 1995; Correa et al., 2019). High soil compaction reduces root growth in rice (Iijima et al., 1991; Grzesiak, 2009) and increases root thickness of those roots that do grow (Potocka & Szymanowska-Pułka, 2018). Therefore, we suggest that the mechanisms regulating the timing of emergence and the root anatomical traits are similar to those involved in response to soil compaction. In fact, radial expansion of the root tip has been observed as a response to soil compaction (Correa et al., 2019), but the underlying mechanism remains unclear. Interestingly, restriction of root growth in compacted soils is not only controlled by mechanical stress but also mediated by ethylene signalling suggesting an involvement of molecular control on the root growth (Pandey et al., 2021). However, whether ethylene signalling is also involved in regulating root thickness remains unknown.
After removal of the mechanical barrier as presented by the leaf sheath, AR2 became longer during short-term submergence and the root diameter decreased significantly (Fig. 7c–e). Functionally, the aquatic adventitious roots of deepwater rice (Khan et al., 1982) and other aquatic plants (Cumbus & Robinson, 1977; Zhang et al., 2017b) are important sites of nutrient uptake. Considering that AR1 emerges immediately below the anchor point of the leaf sheath, there is no natural mechanical barrier hindering root emergence. Nevertheless, we also investigated the influence of mechanical impedance on AR1 emergence and found that AR1 grew shorter with imposed mechanical stress exerted by Parafilm®. Unlike AR2, the diameter of AR1 remained unchanged. We therefore conclude that the root diameter is not only determined by the mechanical stress, but also linked to their specific initiation site. The presence of the leaf sheath clearly delays the emergence of AR2 at the early growth stages in our study, but once emerged, AR2 grows longer than AR1.

QTL1 and QTL12 likely contain key genes involved in AR growth upon submergence

Three QTLs have previously been shown to regulate stem elongation in deepwater rice upon submergence (Hattori et al., 2009). Here, we revealed that QTL1 and QTL12 promote the growth of aquatic adventitious roots during submergence, indicating that stem elongation and formation of adventitious roots might be genetically coupled when deepwater rice responds to submergence. For example, QTL1 harbours a gibberellin biosynthesis gene, SEMIDWARF1 (SD1) responsible for the internode elongation under submergence (Kuroha et al., 2018). Similarly, QTL12 contains two ERFVII transcription factors, SK1 and SK2, which are essential for the submergence response. Whether QTL12-promoted growth of adventitious roots relies on SKs remains unknown, given that 17 genes are included within the introgressed QTL region (Hattori et al., 2009). Interestingly, internode elongation is linked to the absorption of ions and water by upregulated proton pumps and aquaporins causing swelling of the vacuoles (Muto et al., 2011). Similar to the study by Muto et al. (2011), we also found significant upregulation of vacuolar H⁺-pyrophosphatase, tonoplast intrinsic protein and also plasma membrane intrinsic protein (Fig. S9), indicating a possible involvement of these genes in AR growth.

Compared with paddy rice, NIL-12 grew more AR2, and this prompted us to investigate the genetic regulation of AR2 development upon submergence through transcriptome analysis. Reduction in mechanical stress by removal of the leaf sheath revealed a gene network involved in crown root/adventitious root development. The WUNSCHEL-related homeobox gene WOX11 is seen as a key crown root growth regulator, regulated by auxin or cytokinin (Zhao et al., 2015), and WOX11 is expressed in crown roots activating the meristem (Zhao et al., 2009). In our study, expression of WOX11 was strongly induced by submergence, indicating that WOX11 is also required for initiation and growth of adventitious roots in response to submergence. Surprisingly, genes such as the crown rootless genes (CRLs) or other genes related to crown root initiation and emergence did not respond to submergence in AR2; CRLs have been reported to be crucial for crown root development in rice (Inukai et al., 2005; Kitomi et al., 2011). It is assumed that these genes are key regulators for crown root de novo formation in rice, rather than the main molecular signal for adventitious root development during flood events. By contrast, the expression of the genes associated with root cell division and expansion was substantially upregulated, even though these genes perform distinct molecular work in plants. GH3.6 and GH3.8 are involved in the auxin signalling pathway (Mao et al., 2020), whereas OsDGL1 participates in the sugar metabolism (Qin et al., 2013). Recently, OsGLU3 has been described to be responsible for the cell wall loosening (Zhang et al., 2012). Consequently, upregulation of these genes suggests a joint contribution in activating the meristem in ARs enhancing the development of these roots in response to submergence. In conclusion, adventitious root formation as a response to submergence might follow its own pattern albeit with some overlapping gene network shared between crown root and adventitious root formation.

Conclusion and outlook

We propose that AR2 in deepwater rice could function as an evolutionary defence strategy to tackle periodic and long-term submergence. The morphological and anatomical traits suggested that AR2 is better adapted to flooding than AR1. The leaf sheath was the key factor influencing growth rate, elongation capacity and root diameter of AR2. As compared to paddy rice (T65), NIL-1 and NIL-12 produced more AR2 during flooding, indicating that key genes controlling AR2 development are included in QTL1 and QTL12. We suggest to further investigate the genes in these two QTLs regulating AR2 growth. Moreover, the functions of AR2 with respect to water and nutrient uptake during partial submergence have to be unravelled to justify introducing aquatic adventitious roots in modern cultivars for areas experiencing long-term shallow flooding.

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Competing interests
None declared.

Author contributions
CL, OP and MS designed the project and the experiments; CL, LLPO, DL and OP performed and analysed experiments; CL, OP and MS wrote the manuscript.

References


Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Aquatic adventitious root growth differs in paddy rice T65, deepwater rice C9285 and NIL-1, NIL-3, NIL-12 and NIL-1 + 3 + 12 after 7-d submergence.

Fig. S2 Adventitious root 1 and adventitious root 2 differ in several anatomical aspects.
**Fig. S3** Aerenchyma formation in adventitious root 1 and adventitious root 2.

**Fig. S4** Phenotypes of submerged, de-submerged and resubmerged adventitious roots.

**Fig. S5** Mechanical impedance does not promote radial growth of adventitious root 1.

**Fig. S6** Anatomical traits of adventitious root 1 and adventitious root 2 emerging with or without mechanical impedance.

**Fig. S7** Removal of the leaf sheath enhances the expression of submergence-related genes at the node region where adventitious root 2 emerge.

**Fig. S8** Leaf sheath delays repression of genes associated with ABA biosynthesis and signalling in response to submergence.

**Fig. S9** Key genes involved in internode elongation of deepwater rice at the adventitious root 2 region of node 4 in NIL-12.

**Methods S1** Extended version of Materials and Methods.

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