Plant Biotechnology Journal
Open Access

Plant Biotechnology Journal (2025) **23**, pp. 395–411 doi: 10.1111/pbi.14504

Mitochondrial AOX1a and an H_2O_2 feed-forward signalling loop regulate flooding tolerance in rice

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Received 28 May 2024;
revised 17 September 2024;
accepted 19 October 2024.
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Keywords: rice, seed germination, flooding, alternative oxidase, hydrogen peroxide, root development.

Summary

Flooding is a widespread natural disaster that causes tremendous yield losses of global food production. Rice is the only cereal capable of growing in aquatic environments. Direct seeding by which seedlings grow underwater is an important cultivation method for reducing rice production cost. Hypoxic germination tolerance and root growth in waterlogged soil are key traits for rice adaptability to flooded environments. Alternative oxidase (AOX) is a non-ATP-producing terminal oxidase in the plant mitochondrial electron transport chain, but its role in hypoxia tolerance had been unclear. We have discovered that *AOX1a* is necessary and sufficient to promote germination/coleoptile elongation and root development in rice under flooding/hypoxia. Hypoxia enhances endogenous H_2O_2 accumulation, and H_2O_2 in turn activates an ensemble of regulatory genes including *AOX1a* to facilitate the conversion of deleterious reactive oxygen species to H_2O_2 in rice under hypoxia. We show that *AOX1a* and H_2O_2 act interdependently to coordinate three key downstream events, that is, glycolysis/fermentation for minimal ATP production, root aerenchyma development and lateral root emergence under hypoxia. Moreover, we reveal that ectopic *AOX1a* expression promotes vigorous root and plant growth, and increases grain yield under regular irrigation conditions. Our discoveries provide new insights into a unique sensor–second messenger pair in which *AOX1a* acts as the sensor perceiving low oxygen tension, while H_2O_2 accumulation serves as the second messenger triggering downstream root development in rice against hypoxia stress. This work also reveals *AOX1a* genetic manipulation and H_2O_2 pretreatment as potential targets for improving flooding tolerance in rice and other crops.

Introduction

Climate change is shifting weather patterns to greater extremes, and the frequency of flooding events is rising. Flooding is one of the most devastating natural disasters, causing significant crop yield losses worldwide. Flooding can be classified into two general categories: partial submergence, when water covers roots and some of the aerial tissue; and complete submergence, when aerial tissues are all underwater. Flooding restricts oxygen (O_2) diffusion, so submerged plant tissues suffer O_2 deficiency, resulting in impaired sugar metabolism, oxidative respiration, energy generation and, ultimately, growth retardation or even death. Hypoxia triggers cessation of the Krebs/tricarboxylic acid (TCA) cycle and oxidative phosphorylation, shifting ATP production from the mitochondrial electron transport chain (mETC) to ethanol fermentation (Taiz *et al.*, 2015), which supports glycolysis by re-oxidizing NADH to NAD^+ and thereby enables the minimal ATP production required for plant cell survival under hypoxia.

Rice is the only cereal capable of germination and growth under aquatic conditions, and it does so by employing various anatomical, metabolic or developmental mechanisms to adapt to periods of hypoxia or anoxia (Colmer, 2003; Das and Uchimiya, 2002; Fukao and Xiong, 2013; Lee *et al.*, 2009). Under hypoxia, tolerant rice genotypes exhibit speedy germination and coleoptile elongation, and they can hydrolyse starch into metabolizable sugars to fuel the anaerobic metabolism necessary for the root and shoot development that enables faster emergence above the water surface (Lee *et al.*, 2014; Yu *et al.*, 2021). Direct seeding, an important cultivation practice for rice, is increasingly being adopted by farmers in rain-fed and irrigated fields in Asia due to advantages of reduced labour, energy, water and herbicide costs, as well as ease of mechanization (Kumar and Ladha, 2011). Rapid and uniform germination and seedling establishment underwater promote seedling vigour, nutrient uptake and grain yield in direct seeding systems. However, most rice varieties are extremely sensitive to hypoxia during germination and early seedling growth stages.

The mETC is the site of oxidative phosphorylation and ATP generation in eukaryotes. Plant mitochondria are a source of reactive oxygen species (ROS), including superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radical ($OH^{\bullet}$), which are toxic byproducts of aerobic metabolism but also serve as signalling molecules regulating plant growth and stress responses (Mittler, 2017). H_2O_2, converted from superoxide anions by superoxide dismutases (SODs), is considered an important redox signalling molecule due to its relatively stable lifetime and rapid and reversible oxidation of target proteins (Garcia-Santamarina *et al*., 2014). H_2O_2 can oxidize thiol groups of cysteines in target proteins, leading to alterations of protein structures and functions (Garcia-Santamarina *et al*., 2014). The signalling roles of H_2O_2 in stress acclimation and pathogen defence are drawing increasing attention, yet the mechanism of H_2O_2-mediated signal transduction remains largely unclear (Smirnoff and Arnaud, 2019).

In plants, alternative oxidase (AOX) localized on the inner mitochondrial membrane is a non-energy-producing terminal oxidase in the mETC that can relax the highly coupled electron transport process in mitochondria, and thus maintains metabolic homeostasis by directly reducing O_2 to water (Vanlerberghe, 2013). Under stress, the mETC generates more ROS, which activates AOX expression. Consequently, AOX sequesters electrons from the ubiquinone pool, preventing ROS overproduction and alleviating the detrimental effects of stress on mitochondrial respiration (Vanlerberghe, 2013). AOX also regulates the synthesis of $O_2^{\bullet-}$, H_2O_2 and NO (Alber *et al*., 2017) that can serve as important signalling molecules in response to various stresses, so AOX-mediated metabolic activities and signalling are important for plant biotic and abiotic stress responses (Del-Saz *et al*., 2018; Vanlerberghe, 2013).

The role of AOX in hypoxia tolerance is controversial. During flooding, root tissues become hypoxic and their metabolism switches to fermentation-based processes with limited respiratory activity. In rice, *AOX1a* mRNA levels decline under hypoxia, so *AOX1a* has been regarded as unnecessary for hypoxic conditions (Tsuji *et al*., 2000). However, Cyt C oxidase displays nitrite reductase activity under hypoxia, generating NO from nitrite, with NO then inhibiting Cyt C oxidase activity (Poyton *et al*., 2009). In contrast, AOX is NO-resistant, continuing to function effectively as an oxidase in the presence of NO under hypoxia (Gupta *et al*., 2012). Hence, at low O_2 levels, it has been suggested that consumption of O_2 shifts from Cyt C oxidase toward AOX, despite the lower affinity for O_2 of AOX compared to Cyt C oxidase (Affourtit *et al*., 2001).

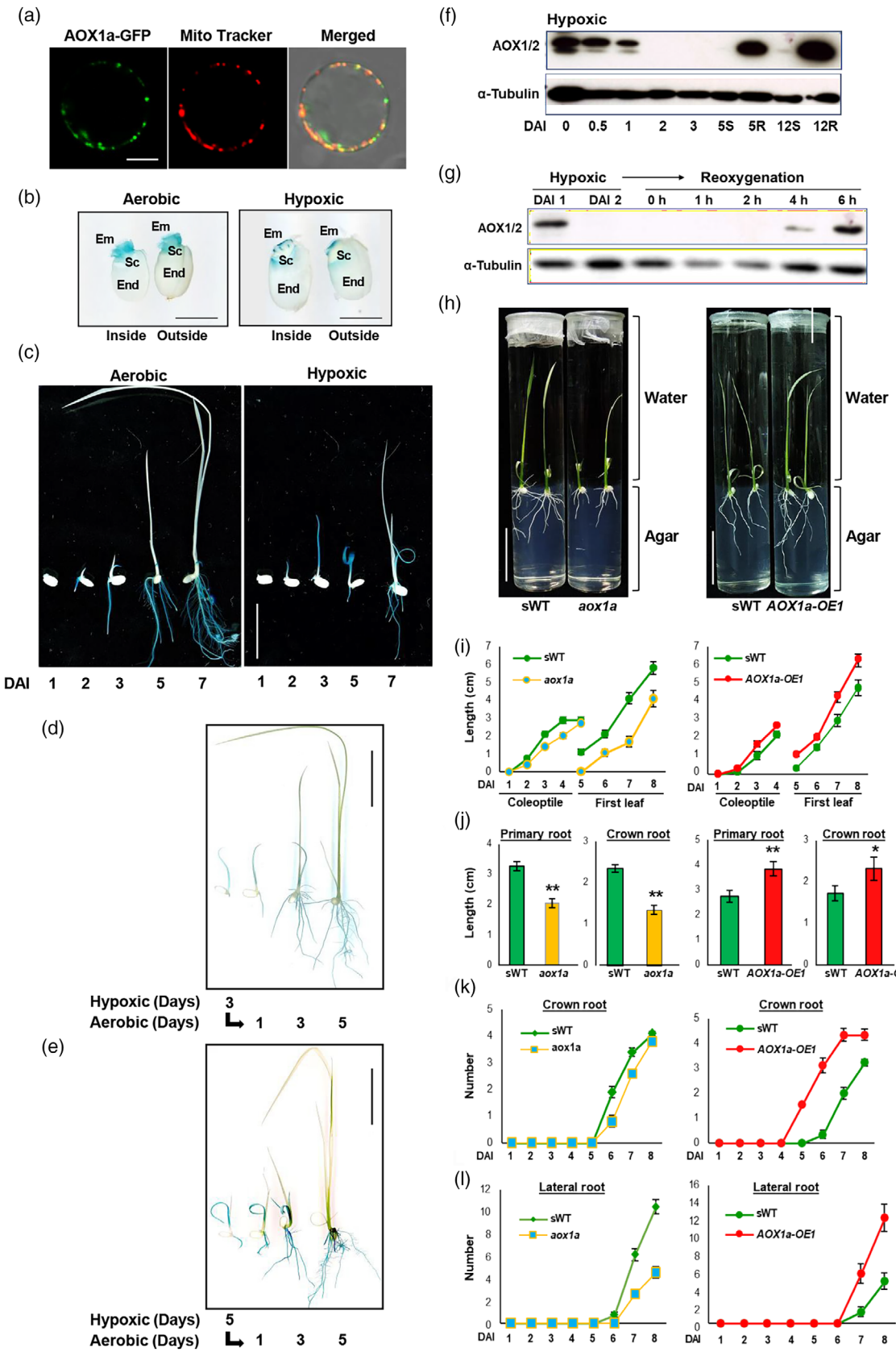
Despite the relative tolerance of rice to flooding and hypoxia, root development under water is restricted in most rice varieties (Miro and Ismail, 2013), which affects anchorage and nutrient uptake in soil. Development of an aerated and vigorously growing root system during seedling establishment and entire plant growth under flooding is a fundamental requirement for the flooding tolerance displayed by plants. Understanding the mechanism by which rice tolerates flooding and devising strategies to improve it are not only fundamental scientific endeavours in their own right, but they are also vital to agricultural production and food security. In the present study, we discover an *AOX1a*-H_2O_2 feed-forward regulatory loop that constitutes an intriguing sensor-second messenger pair for rice responses to hypoxia, with H_2O_2 functioning as the second messenger in the hypoxia signalling pathway, amplifying the low O_2 signal perceived by AOX1a. This work also reveals *AOX1a* genetic manipulations, such as transgenic engineering or genome editing, and H_2O_2 pretreatment as potential targets for improving flooding tolerance in rice and other crops.

Results

AOX1a is specifically expressed in embryos and roots and is necessary and sufficient for promoting seedling growth under hypoxia

We revealed that the coding sequence of AOX1a fused to GFP (AOX1a-GFP), is localized in mitochondria (Figure 1a, Figure S1). We generated transgenic rice expressing the *AOX1a* promoter fused to *GUS* (*AOX1a:GUS*), and found that the *AOX1a* promoter was active in embryos, roots and coleoptiles, but not in leaves, under both aerobic and hypoxic conditions (Figure 1b, c). The *AOX1a* promoter was weaker in embryos under hypoxia than under aerobic conditions. Seeds germinated and seedlings grown under hypoxia for 3 or 5 days were then recovered under aerobic conditions for up to 5 days, and the *AOX1a* promoter activity increased with time (Figure 1d,e). Notably, we also found that levels of AOX1a protein were high in dry seeds (0 DAI), and remained high in roots up to 5 DAI under aerobic conditions (Figure S2). In contrast, AOX1a protein accumulated up to 1 DAI but became undetectable at 2–3 DAI, and increased again at 5

Figure 1 *AOX1a* is localized in mitochondria, specifically expressed in embryos and roots, and is necessary and sufficient to promote rice seedling growth under hypoxia. (a) Rice protoplasts were transfected with plasmid *Ubi:AOX1a-GFP*, incubated in MS medium for 20 h, and stained with the mitochondrial marker MitoTracker® Red CMXRos for 10 min before being examined under confocal microscopy. Scale bar = 10 μm. Additional images are shown in Figure S1. (b) Seeds of an *AOX1a:GUS* line were germinated under aerobic or submerged conditions for 1 day and stained 16 h for GUS activity. Em, embryo; End, endosperm; Sc, scutellum. Scale bar = 5 mm. (c) Seeds of an *AOX1a:GUS* line were germinated and the resulting seedlings were grown under aerobic or submerged conditions for 7 days. DAI, day after imbibition. All seedlings were stained for GUS activity for 2 h. (d, e) Seeds of the *AOX1a:GUS* line were germinated under water in glass jars for (d) 3 days or (e) 5 days, and water was receded. Glass jars were uncapped and seedlings inside were recovered in air for 1, 3 and 5 days. All seedlings were stained for GUS activity for 1 h. Scale bar = 3 cm. (f, g) WT seeds were germinated, seedlings were grown under different conditions for various times, and proteins were extracted and subjected to Western blot analysis using anti-AOX and anti-tubulin antibodies. (f) After growing under hypoxia, entire seedlings were collected before 3 DAI and then shoots and roots were harvested separately at 5 and 12 DAI. S, shoot; R, root. (g) Seedlings were grown under hypoxia for 2 days, then water was removed to allow seedlings to grow in air for up to 6 h. (h–l) Seeds of the *aox1a* (left panel) and *AOX1a-OE1* (right panel) lines were germinated and the resulting seedlings were grown under hypoxia for 8 days. (H) Phenotype of 8-day-old seedlings under hypoxia. Additional seedlings are shown in Figure S6B,C. Scale bar = 3 cm for (c) and (h). (i) Length of coleoptiles and first leaves. (j) Length of primary and crown roots. (k) Number of crown roots. (l) Number of lateral roots. Error bars represent SD of 12 replicates.


(a)
AOX1a-GFP
Mito Tracker
Merged
(b)
Aerobic
Hypoxic
Em
Sc
End
Inside
Outside
(c)
Aerobic
Hypoxic
DAI 1 2 3 5 7 1 2 3 5 7
(d)
Hypoxic (Days) 3
Aerobic (Days) 1 3 5
(e)
Hypoxic (Days) 5
Aerobic (Days) 1 3 5
(f)
Hypoxic
AOX1/2
α-Tubulin
DAI 0 0.5 1 2 3 5S 5R 12S 12R
(g)
Hypoxic
Reoxygenation
DAI 1 DAI 2 0 h 1 h 2 h 4 h 6 h
AOX1/2
α-Tubulin
(h)
Water
Agar
sWT aox1a
sWT AOX1a-OE1
(i)
sWT
aox1a
AOX1a-OE1
Length (cm)
DAI
Coleoptile
First leaf
(j)
Primary root
Crown root
Length (cm)
sWT aox1a sWT AOX1a-OE1
(k)
Crown root
Number
DAI
(l)
Lateral root
Number
DAI

DAI in roots under hypoxia (Figure 1f, Figure S2). AOX1a protein accumulated at 1 DAI, disappeared at 2 DAI under hypoxia, but started to accumulate again 4 h after reoxygenation (Figure 1g). These results indicate that the expression of *AOX1a* is developmentally regulated, and suppressed by hypoxia and induced by aerobiosis.

We generated *AOX1a* knockout mutants via CRISPR-Cas9-mediated genome editing (Figure S3A), and obtained two homozygous knockout mutant lines: *aox1a-7-9* with a 36-basepair (bp) deletion and *aox1a-18-7* with a 1-bp insertion, respectively, in the coding region of *AOX1a* (Figure S3B,C). We also generated many transgenic lines carrying *Ubi:AOX1a*, and overexpression (OE) lines *AOX1a-OE1* and *AOX1a-OE2* were used in subsequent experiments (Figure S4). Seeds from the *aox1a* and *AOX1a-OE* lines were germinated in test tubes either filled with water (hypoxic) or without excess water (aerobic). The O_2 levels in water in which seedlings were submerged ranging from 4.7 to 5.6 mg/L (148.3–176.8 μM) at 0–8 days after imbibition (DAI) (Figure S5), which is within the range of those (3.0–5.3 mg/L) in floodwater of a rice paddy (Senapati *et al.*, 2019) (see also Appendix S1: Results and Discussion).

We found that root growth of *AOX1a-OE* lines slightly increased compared to WT and *aox1a* lines under the aerobic condition (Figure S6A). Seedlings of *aox1a* mutant grew more slowly than segregated wild type (sWT) under hypoxia, whereas those of *AOX1a-OE* lines grew more rapidly under the same condition (Figure S6B,C). Two additional *aox1a* knockout mutants displayed similar hypoxia-intolerant phenotypes (Figure S7), indicating that the phenotypes displayed by the gene-edited knockout lines were not due to off-target editing. Quantitative analyses revealed that the length of coleoptiles, first leaves, and primary and crown roots was shorter in *aox1a* but longer in *AOX1a-OE* seedlings compared to sWT, up to 8 DAI under hypoxia (Figure 1h–j). Moreover, the number of crown and lateral roots (LRs) was lower in *aox1a* but higher in *AOX1a-OE* seedlings compared to sWT under hypoxia (Figure 1k,l).

AOX1a-H_2O_2 self-perpetuating feed-forward regulation controls seed germination and seedling development under hypoxia

Although the germination rate of all lines was 100%, we found that coleoptile elongation was slower in *aox1a* but faster in *AOX1a-OE1* relative to sWT under hypoxia (Figure 2a). We have demonstrated previously that *CIPK15* and *αAmy* are necessary for hydrolysis of starch to sugar in germinating embryos under hypoxia (Lee *et al.*, 2009, 2014). In the current study, we detected that mRNA levels of *CIPK15* and *αAmy3* in germinating embryos were reduced in *aox1a* but increased in *AOX1a-OE1* relative to sWT at 1 DAI (Figure 2b). We also observed that the concentration of endogenous H_2O_2 was significantly lower in *aox1a* but higher in the *AOX1a-OE1* line compared to sWT at 1 DAI under hypoxia (Figure 2c).

Next, we pretreated sWT seeds overnight with H_2O_2 and let seedlings grow under aerobic or hypoxic conditions for 2 more days, which revealed that coleoptile and root development was faster in seedlings from H_2O_2-pretreated seeds than those from seeds not subjected to that pretreatment, under both aerobic and hypoxic conditions (Figure 2d). We determined that seed H_2O_2 pretreatment induced accumulations of *AOX1a*, *AOX1b*, *CIPK15*, *SnRK1A* and *MYBS1* mRNAs in these seedlings (Figure 2e). We pretreated *aox1a* seeds with H_2O_2 overnight, and then grew the seedlings under hypoxia, which revealed that root growth of *aox1a* could be partially restored relative to sWT (Figure 2f). These results indicate that the H_2O_2 treatment could induce the expression of *AOX1a* and genes essential for germination as well as promote root development.

Ascorbic acid (AsA) serves as an antioxidant that scavenges superoxide anions and H_2O_2 (Asada, 1992). Exogenously applied AsA was previously found to lower electrolyte leakage, lipid peroxidation and H_2O_2 accumulation upon chilling stress in tomato (Elkelish *et al.*, 2020). However, we found that primary root length was significantly reduced, crown root growth was abolished, and H_2O_2 accumulation was reduced, under hypoxia upon treating sWT and *AOX1a-OE1* roots with AsA (Figure 2g, Figure S8, Appendix S1: Results and Discussion), indicating that reduced levels of endogenous H_2O_2 impairs root growth under hypoxia.

Next, we performed RNA sequencing (RNAseq) on 3-day-old seedlings (seeds pretreated with or without H_2O_2 overnight and let seedlings grow under aerobic or hypoxic conditions for 2 days). We found that H_2O_2 upregulated or downregulated (fold-change ±2) 1557 and 1988 genes, respectively (Dataset S1). Four *AOX1* genes were upregulated (Figure 3). Moreover, genes encoding H_2O_2-generating enzymes MnSOD and Cu/ZnSOD were also highly upregulated, whereas a number of H_2O_2 scavenging enzymes (APX1, GR2, GPX1, GPX3 and UCP1) were downregulated (Figure 3, Table S1). Genes encoding CIPK15, SnRK1a and MYBS1, which induce expression of αAmy and other enzymes involved in sugar production, glycolysis and fermentation (Lee *et al.*, 2014; Yu *et al.*, 2015), were all upregulated (Figure 3). The expression of *αAmy3* peaks within 0.5–1 DAI (Figure 2b) and declines in germinated embryos after 2 DAI normally, therefore, its expression has decreased whereas other *αAmy* genes, for example, *αAmy7*, *αAmy8* and *αAmy10*, were increased in the RNAseq analysis (Figure 3), which is consistent to our previous studies (Lee *et al.*, 2009; Yu *et al.*, 1996).

The RNAseq data shows that genes for ABA and GA biosynthesis were suppressed, and for GA inactivation (the GA2ox family) were induced (Figure 3), indicating that the levels of ABA and active forms GAs (GA_1 and GA_4) were reduced. In the meantime, genes for auxin biosynthesis, transport, signalling and positive regulation were significantly induced (Figure 3). These results indicate that the regulation of post-germination seedling development shifted from the conventional GA and ABA antagonistic pathways to the AOX1a-H_2O_2-regulated auxin-dependent pathway.

AOX1a and H_2O_2 promote lateral root emergence by enhancing the expression of cell wall remodelling enzymes/proteins under hypoxia

We have shown that LR number was lower than sWT in *aox1a* but greater in *AOX1a-OE* (Figure 1l). Accordingly, we assessed if *AOX1a* and H_2O_2 promotes LR initiation and/or elongation. Compared to sWT, we detected no difference in numbers of lateral root primordia (LRP) in the region 0–1 cm from the root tip of *aox1a* and *AOX1a-OE* seedlings. However, we observed notably fewer LRP and LR in *aox1a* but significantly more LR in *AOX1a-OE* relative to sWT in regions 1–2 cm and 2–4 cm from the root tip (Figure 4a,b). LR development in H_2O_2-pretreated sWT roots was promoted to a similar extent to those of the *AOX1a-OE* line compared to non-treated sWT roots (Figure 4a,b).

We observed that GUS was highly expressed in the root vascular bundles, epidermal cell (EPC) layers and emerging LR of

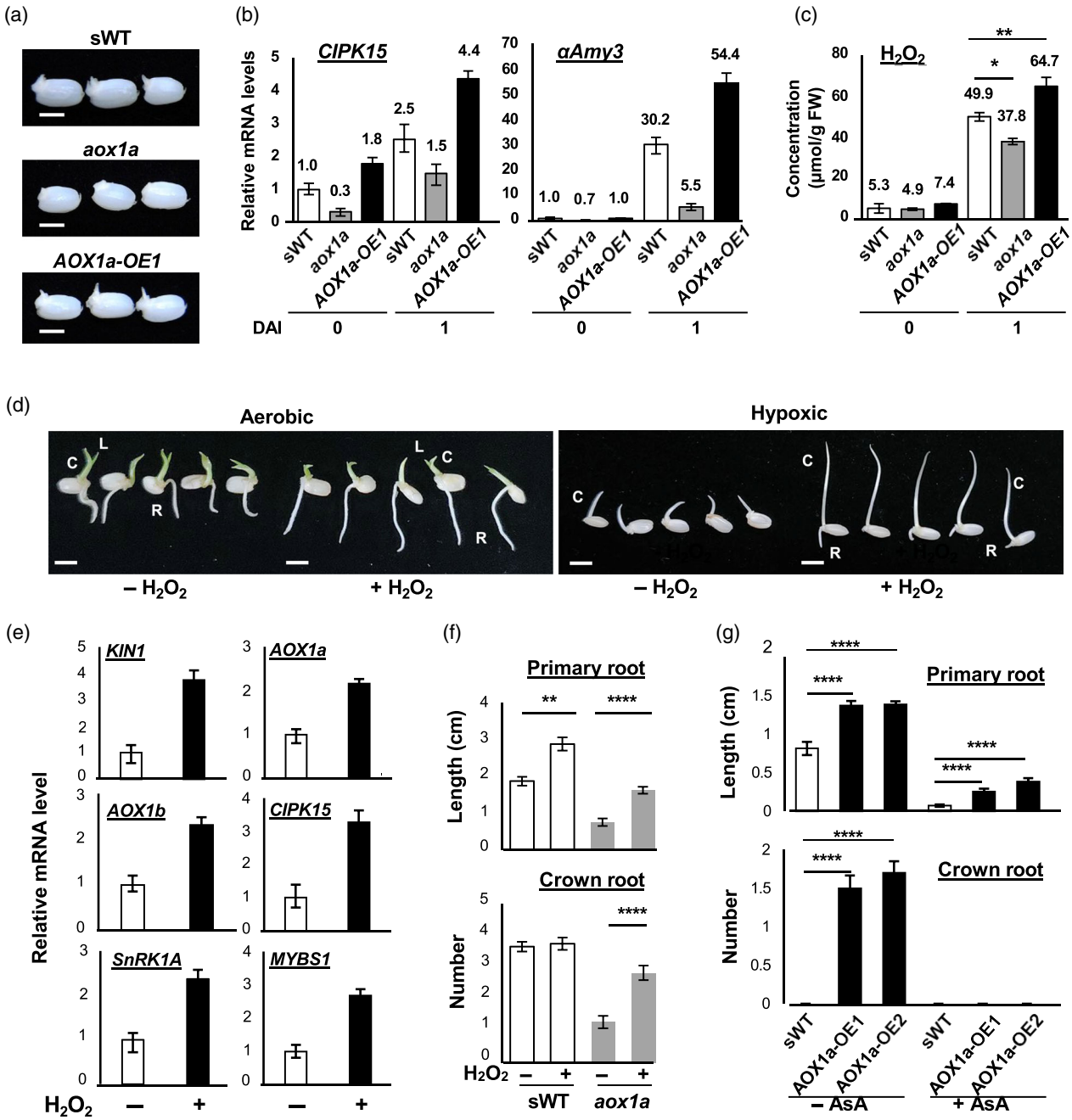


Figure 2 *AOX1a* and H_2O_2 mutual regulation controls germination and seedling development under hypoxia. Seeds of WT, *aox1a* and *AOX1a-OE1* lines were germinated under submergence. (a) Germinated seeds at 1 DAI. Scale bar = 3 mm. (b, c) Embryos at 0 and 1 DAI were collected for (b) qRT-PCR analysis of *αAmy3* and *CIPK15* mRNAs and (c) Measurement of H_2O_2 concentration. (d–f) Seeds were pretreated with water or H_2O_2, germinated, and the seedlings were then grown under aerobic or hypoxic (submerged in water) conditions. (d) Morphology of WT seedlings 2 days after H_2O_2 pretreatment. C, coleoptile; L, first leave; R, root. Scale bar = 0.5 cm. (e) mRNAs were extracted from seedlings in (d) and subjected to qRT-PCR analysis. (f) Length and number of *aox1a* roots 8 days after H_2O_2 pretreatment. Error bars represent SD of three independent experiments. (g) Seeds were germinated, and then seedlings were submerged in water with or without 0.1 mM AsA for 5 days.

the *AOX1a:GUS* line under hypoxia (Figure 4c,d). Upon being oxidized by H_2O_2, 3,3′-Diaminobenzidine (DAB) turns brown that can be used to detect H_2O_2 accumulation in plant tissues *in situ*. DAB staining of *AOX1a:GUS* roots revealed that the tissues displaying H_2O_2 accumulation were similar to those that expressed GUS under hypoxia (Figure 4c,d). During LR emergence, the *AOX1a* promoter was highly active in root EPC layers overlaying the emerging LR, as well as in emerged LR, where H_2O_2 had also strongly accumulated (Figure 4e). Importantly, pretreating the seeds with AsA reduced H_2O_2 and GUS accumulation in these tissues under hypoxia (Figure 4f).

LRP develop into LR as they extend through many cell layers, ultimately breaking through epidermal cell junctions toward the rhizosphere, with LR progression being facilitated by

enzyme-weakened cell walls (Vilches-Barro and Maizel, 2015). Several genes encoding cell wall remodelling enzymes/proteins were downregulated in *aox1a* and upregulated in *AOX1a-OE* under hypoxia, including the genes encoding α-expansins (*EXPA32* and *EXPB4*), laccase (*LAC24*) and pectin methylesterases (*PME12* and *PME34*) (Figure 4g), all of which have been reported

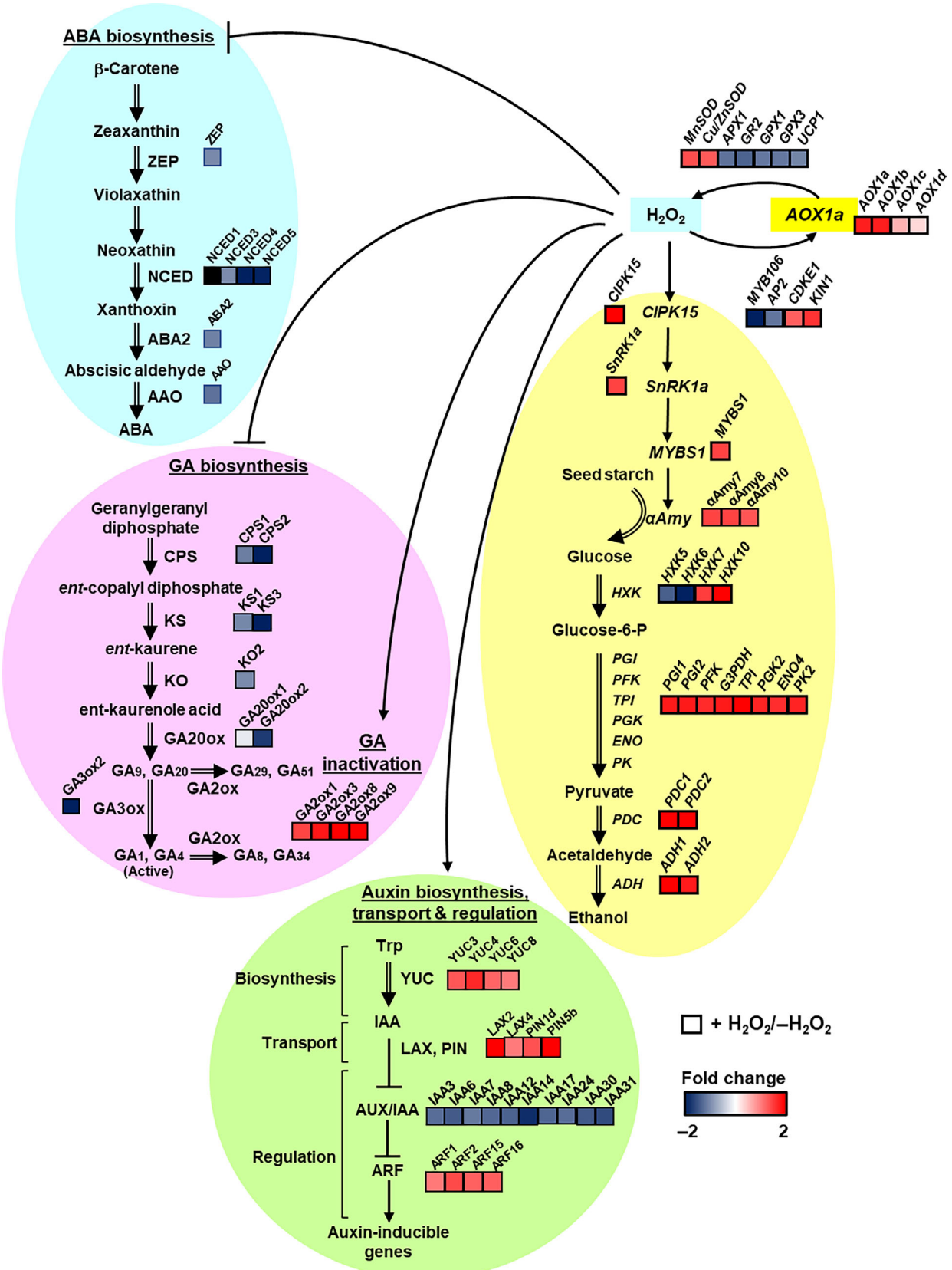


Figure 3 *AOX1a*-H_2O_2 self-perpetuating feed-forward regulation of genes involved in H_2O_2 production, sugar metabolism, ABA and GA biosynthesis, and auxin biosynthesis, transport and regulation under hypoxia. Seeds were pretreated with or without H_2O_2 overnight and germinated, and then the seedlings were grown under water for 2 days. Total RNAs were extracted from seedlings and subjected to RNAseq analysis. The fold-change colour scale ranges from −2 to 2, with red and blue representing higher and lower transcript levels respectively. The details of all genes and fold change of mRNA levels are provided in Table S1.

previously as being impacted or upregulated by auxin or as being associated with epidermis cell wall loosening during LR emergence in Arabidopsis (Vilches-Barro and Maizel, 2015). Pretreating *aox1a* seeds with H_2O_2 significantly enhanced and recovered the expression of these genes in the sWT and *aox1a* lines, respectively, under hypoxia (Figure 4h). In contrast, AsA treatment impaired the expression of these genes in the sWT and *AOX1a-OE* lines under hypoxia (Figure 4i). These results indicate that *AOX1a* and H_2O_2 upregulate the expression of these cell wall remodelling enzymes/proteins essential for LR emergence under hypoxia.

AOX1a promotes the conversion of ROS to H_2O_2 under hypoxia, and both *AOX1a* and H_2O_2 promote aerenchyma and root development via the ethylene-dependent pathway

We have already shown herein that H_2O_2 upregulates the expression of genes encoding ROS-generating enzymes (*MnSOD* and *Cu/ZnSOD*) and downregulates genes encoding ROS-scavenging enzymes (*APX1* and *GR2*) in 3-day-old seedlings (Figure 3). We also found reduced expression of *MnSOD* and *Cu/ZnSOD* in roots of the 7-day-old *aox1a* line, whereas respective expression was increased in the *AOX1a-OE* line (Figure 5a). In contrast, expression of *APX1* and *GR2* was increased in *aox1a* but reduced in *AOX1a-OE* (Figure 5a). Histochemical analysis is commonly used to detect accumulations of superoxide anions and H_2O_2 in plants (Smirnoff and Arnaud, 2019). We stained seedlings with nitroblue tetrazolium (NBT) and found that superoxides mainly accumulated in the coleoptile and roots, with levels being higher in *aox1a* but lower in *AOX1a-OE* relative to sWT (Figure 5b,c, Figure S9A). In contrast, though DAB staining of seedlings revealed that H_2O_2 also primarily accumulated in the coleoptile and roots, the levels were lower in *aox1a* but higher in *AOX1a-OE* compared to sWT (Figure 5d,e, Figure S9B). These experiments demonstrate that *AOX1a* induces the expression of enzymes that convert superoxides to H_2O_2 and suppresses the expression of enzymes that scavenge H_2O_2. Consequently, roots maintain relatively high levels of H_2O_2 to promote root growth under hypoxia.

Aerenchyma development facilitates vertical O_2 transmission, enhances hypoxia tolerance, and is an indicator of active root growth (Yamauchi *et al.*, 2018). H_2O_2 has been shown previously to promote aerenchyma development in stems and roots of rice (Steffens *et al.*, 2011). Rice root aerenchyma is constitutively formed in well-drained soils and it is further induced upon soil becoming waterlogged, in a process mediated by an ethylene-ROS-dependent programmed cell death (PCD) pathway (Drew *et al.*, 2000; Yamauchi *et al.*, 2018). We observed that aerenchyma development in seminal and crown roots was impaired in the *aox1a* line relative to sWT but enhanced in the *AOX1a-OE1* line with or without H_2O_2 pretreatment (Figure 5f,g). H_2O_2 pretreatment of the *aox1a* line recovered aerenchyma development in seminal roots by 19% and promoted aerenchyma growth in crown roots from zero to 56% under hypoxia (Figure 5g). Endogenous H_2O_2 concentrations in the roots were lower in *aox1a* but higher in *AOX1a-OE* relative to sWT (Figure 5h), which was correlated with reduced and enhanced *ACC SYNTHASE 1* (*ACS1*) and *ACC OXIDASE 5* (*ACO5*) mRNA levels in *aox1a* and *AOX1a-OE1* line respectively (Figure 5i). We also observed that exogenous H_2O_2 induced the expression of *ACS1* and *ACO5* (Figure 5j).

AOX1a promotes root growth under flooding and grain yield in rice paddy

We wanted to determine how *AOX1a* affects root growth in flooded pot soil under greenhouse conditions. We observed that plant height and root length were shorter in *aox1a* but longer in *AOX1a-OE* relative to sWT, and that shoot and root biomass were significantly reduced in *aox1a* but increased in *AOX1a-OE* compared to sWT, after complete flooding (Figure 6a–c) or partial flooding (Figure 6d–f) for 2 weeks.

Moreover, we found that leaves were greener for the *AOX1a-OE* line compared to sWT (Figure S10A,C), with levels of chlorophyll a, chlorophyll b and total chlorophyll being correspondingly reduced for *aox1a* but increased for *AOX1a-OE* compared to sWT for 1-month-old plants grown in the greenhouse (Figure 6g). Furthermore, we detected that *AOX1a-OE* leaves were wider than those of sWT (Figure 6h, Figure S10B, D). Notably, the photosynthesis rate of the *aox1a* plants was lower than that of sWT, whereas it was enhanced for *AOX1a-OE* plants (Figure 6i). We also found that 1000-grain weight, grain size and panicle number per tiller were increased in *AOX1a-OE* lines and reduced in the *aox1a* mutant compared to sWT (Figure 6j,k, Figure S11). Accordingly, we evaluated grain yield in rice paddies with regular irrigation. Importantly, grain yield decreased for *aox1a* (Figure S12), but increased by 14–49% in the *AOX1a-OE* lines compared to sWT (Figure 6l).

KIN1 is a key regulator of the *AOX1a*-H_2O_2 feed-forward loop that controls gene expression and promotes rice germination and seedling growth under hypoxia

In Arabidopsis, the protein kinase KIN10, a sucrose non-fermenting 1 (SNF1)-related protein kinase 1 (SnRK1), integrates stress, darkness, and sugar signalling with growth, and its overexpression enhances sugar starvation tolerance (Baena-Gonzalez *et al.*, 2007). Arabidopsis cyclin-dependent Kinase E1 (CDKE1) has also been shown previously to interact with KIN10 in the nucleus and to regulate the *AOX1a* promoter in response to H_2O_2 (Ng *et al.*, 2013). In rice, *KIN1* is the homologue of Arabidopsis *KIN10* (Table S2). Our RNAseq analysis indicated that both *KIN1* and *CDKE1* mRNAs were increased by seed H_2O_2 pretreatment under hypoxia (Figures 2e and 3).

We obtained a T-DNA activation-tagged rice *KIN1* mutant (*KIN1-Act*) that its roots grew more vigorously than sWT under hypoxia (Figure 7a,c). *KIN1* mRNA accumulation was significantly increased (by 6-fold) in the *KIN1-Act* line under aerobic conditions (Figure S13). We also obtained a T-DNA knockout-tagged rice *KIN1* mutant (*kin1*), which displayed impaired root growth

relative to sWT under hypoxia (Figure 7b,d). Expression of *AOX1a* and genes essential for H_2O_2 generation, cell wall-remodelling, sugar metabolism, and ethylene biosynthesis was all upregulated, whereas that of genes for H_2O_2 scavenge was downregulated, in the *KIN1-Act* line under hypoxia (Figure 7e). In contrast, the reverse was true for the *kin1* line under the same conditions

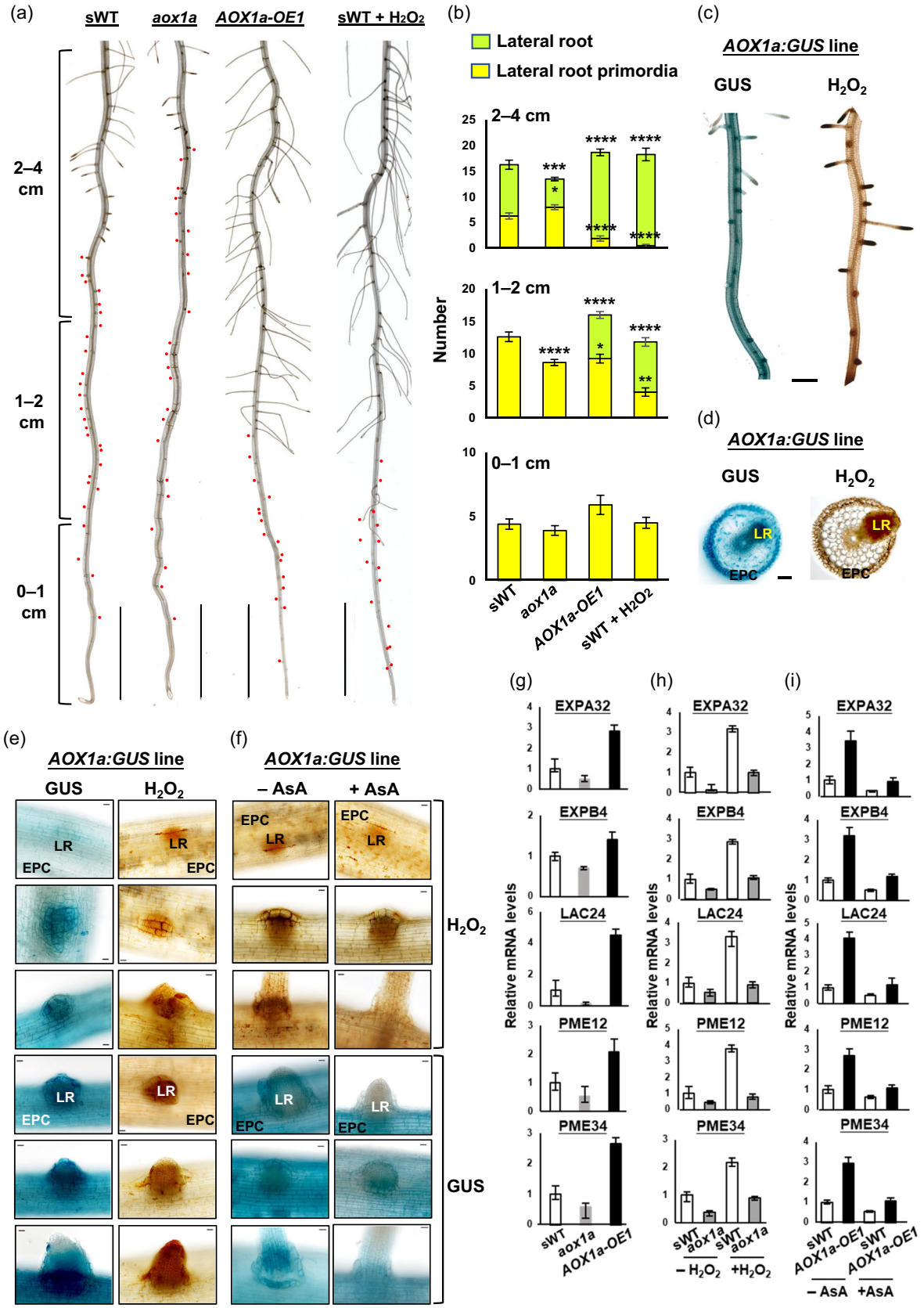


Figure 4 *AOX1a* and H_2O_2 both promote lateral root emergence by enhancing the expression of cell wall remodelling enzymes/proteins under hypoxia. (a, b) Seeds were pretreated with or without H_2O_2 and germinated overnight, and seedlings were grown under hypoxic conditions for 11 days. (a) Morphology of crown roots. Lateral root primordia (LRP) and lateral roots (LR) in three root segments (0–1, 1–2 and 2–4 cm from the root tip) were examined. Scale bar = 5 mm. Red dots mark the positions of LRP. (b) Numbers of LRP (yellow bar) and LR (green bar) in (a) were quantified. Error bars represent SD of four replicates. (c–f) *AOX1a:GUS* seeds were germinated and the seedlings were submerged in water for 11 days. (c) Segments of primary roots. (d) Cross-sections of primary roots. (e, f) Detection of LR emergence. (e) Roots were stained for GUS and H_2O_2. (f) Roots were treated with or without AsA. Scale bar = 20 μm. EPC, epidermal cell; LR, lateral root. (g–i) mRNA was extracted from roots 11-day-old seedlings and subjected to qRT-PCR analysis. (g) Roots without any pretreatment. (h) sWT and *aox1a* roots developed from seeds with or without H_2O_2 pretreatment. (i) Roots of sWT and *AOX1a-OE* seedlings submerged in water with or without 0.1 mM AsA.

(Figure 7f). Coleoptile elongation was slower in *kin1* but faster in *KIN1-Act* relative to sWT under hypoxia (Figure 7g), but the germination rate of all lines was 100%. The accumulation of *AOX1a* mRNA increased earlier and to higher levels in seedlings of the *KIN1-Act* line compared to sWT at the post-germination stage (Figure 7h). These results indicate that *KIN1* is an upstream positive regulator for the *AOX1a*-H_2O_2 feed-forward loop regulating root development under hypoxia (Figure 7i).

Discussion

The *AOX1a*-H_2O_2 feed-forward regulatory network coordinates three key downstream events necessary for germination and root development under hypoxia

Rice AOX is encoded by four genes, with expression of *AOX1a* and *AOX1b* being similarly induced by low temperature, drought and high salt in the leaves, roots and shoots of rice seedlings (Ohtsu *et al*., 2002; Saika *et al*., 2002). In the present study, we observed that both *AOX1a* and *AOX1b* were highly induced under aerobic conditions in 3-day-old seedlings, but suppressed under hypoxic conditions (Figure 7j,k). The *AOX1a* and *AOX1b* mRNA levels were increased by approximately two- to three-fold (Figures 2e and 7k), however, the relative mRNA level of *AOX1a* was higher than that of *AOX1b* under hypoxia compared to aerobic conditions either with or without H_2O_2 pretreatment (Figure 7k). *AOX1a* and *AOX1b*, but not *AOX1c*, are induced by drought, cold, salinity and heat (Li *et al*., 2013), and *AOX1c* was reported not expressed in rice seedling roots (Saika *et al*., 2002). Additionally, we found that *AOX1c* and *AOX1d* were only slightly induced by H_2O_2 (Table S1). Therefore, we explored *AOX1a* function using gain- and loss-of-function genetic analyses, and revealed that *AOX1a* plays an important role in the hypoxia tolerance of rice.

We show that *AOX1a* and H_2O_2 are interdependent, with both being sufficient and necessary to promote germination under hypoxia via the *CIPK15-SnRK1a-MYBS1*-dependent pathway by activating genes involved in sugar production and metabolism, glycolysis and alcoholic fermentation (Figure 3). Our RNAseq analysis of 3-day-old seedlings developed from H_2O_2-pretreated seeds under hypoxia confirmed the existence of an H_2O_2-*AOX1a* self-perpetuating feed-forward loop. Exogenous H_2O_2 induces the expression of four *AOX1* genes under hypoxia. Moreover, overexpression of *AOX1a* induces the accumulation of endogenous H_2O_2 and the expression of antioxidants such as the H_2O_2-generating enzymes MnSOD and Cu/ZnSOD, as well as suppresses H_2O_2-scavenging enzymes such as APX1 and GR2, at the early stage of seedling development (Figure 5a).

Our RNAseq analysis suggests that the traditional antagonism between GA and ABA in regulating seed germination and seedling growth has been shifted to the auxin-mediated root development in response to H_2O_2 (Figure 3). High ABA concentrations inhibit germination and root growth (Chen *et al*., 2015), and low GA concentrations promote root growth (Lo *et al*., 2008). Despite suppression of GA biosynthesis and enhancement of GA inactivation, no effect on shoot growth was detected in the *AOX1a-OE* lines or in seedlings from H_2O_2-pretreated seeds. Auxin regulates many aspects of root development: LRP initiation, LR elongation, and LR emergence (Jung and McCouch, 2013). The LRP develops into an LR as it transits through multiple cell layers, ultimately piercing parental root epidermal cell junctions toward the rhizosphere in a process that is facilitated by enzymatic weakening of cell walls (Vilches-Barro and Maizel, 2015). In Arabidopsis, H_2O_2 promotes LR elongation and emergence by promoting cell wall remodelling of the parental epidermal cells overlaying LR (Orman-Ligeza *et al*., 2016). In rice, H_2O_2 influences auxin distribution and induces root growth under cadmium-stressed conditions (Zhao *et al*., 2012). We showed that both *AOX1a* and H_2O_2 induce the same sets of cell wall remodelling enzymes/proteins (Figure 4g,h) known to be upregulated by auxin and associated with epidermis cell wall separation during LR emergence in Arabidopsis (Vilches-Barro and Maizel, 2015).

In rice, aerenchyma provides a longitudinal channel for distributing O_2 from the shoots to root tips, contributing to plant tolerance of waterlogged soil (Yamauchi *et al*., 2018). The apoplastic superoxide production in rice roots that is mediated by the ethylene-induced NADPH oxidase, Respiratory Burst Oxidase Homologue that converts O_2 to ROS, enhances H_2O_2 levels in the apoplast and cytosol, induces PCD, and subsequently promotes aerenchyma development in the cortex (Yamauchi *et al*., 2018). We have shown herein that both *AOX1a* and H_2O_2 induce the expression of genes encoding the ethylene biosynthesis enzymes ACS1 and ACO5 and they promote aerenchyma development under hypoxia (Figure 5f–j). *AOX1a*-induced development of an aerated root system in waterlogged soil is a fundamental requirement for plant flooding tolerance, as evidenced by the weaker root system displayed by the *aox1a* line but the vigorous root growth of *AOX1a-OE* under both partial and complete flooding conditions (Figure 6a–f).

The *AOX1a*-H_2O_2 regulatory loop prevents the accumulation of deleterious ROS by generating H_2O_2 that promotes plant growth under hypoxia

Mitochondrial redox homeostasis plays key roles in seed viability and gametophyte development. In rice, *AOX1a* is important for biomass accumulation in seeds (Ji *et al*., 2023), and in soybean, *AOX2b* is important for pollen development and fertility (Chai *et al*., 2010). We also found that grain size and weight, and panicle number per tiller, were reduced in the *aox1a* mutant but increased in the *AOX1a-OE* lines compared to sWT

(Figure 6j,k, Figure S11), indicating that *AOX1a* is necessary and sufficient for seed setting and development. These outcomes explain the pre-existence of AOX proteins in dry seeds (0 DAI), and the expression of *AOX1a* appears to be developmentally regulated under hypoxia (Figure 1f). AOX protein accumulation disappeared under hypoxia but

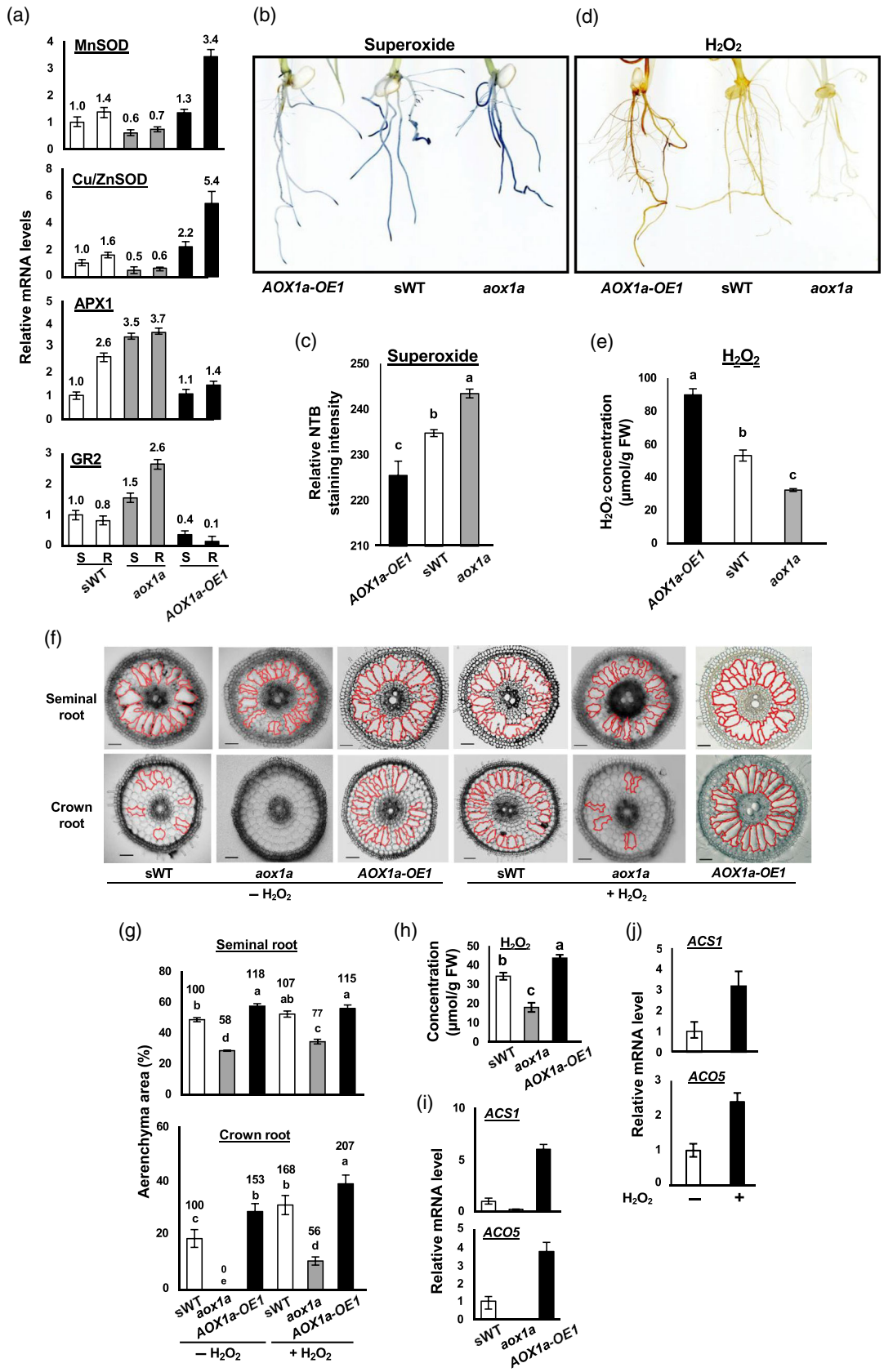


Figure 5 *AOX1a* promotes the conversion of ROS to H_2O_2 under hypoxia, and both *AOX1a* and H_2O_2 promote aerenchyma and root development via the ethylene-dependent pathway. (a–e) Seeds were germinated on ½ MS agar medium and the seedlings were submerged in water for (a) 5 days and (b–e) 7 days. (a) mRNAs were extracted and subjected to qRT-PCR analysis using gene-specific primers. (b) Seedlings were stained for superoxides using NBT reagent, with root portions shown here. Images of whole seedlings are shown in Figure S9A. (c) Images in (b) were quantified using ImageJ software. Error bars are SD for 12 replicates. (d) Seedlings were stained for H_2O_2 using DAB reagent, with root portions shown here. Images of whole seedlings are shown in Figure S9B. (e) Roots in (d) were used to measure H_2O_2 concentration. (f–j) Seeds were pretreated with or without H_2O_2, germinated, and the seedlings were submerged in water for 11 days. (f) Cross sections were made at 2 and 4 cm behind the tips of crown roots and seminal roots respectively. The area of aerenchyma is outlined in red. Scale bars = 50 μm. (g) Aerenchyma area as a percentage of total cortex cell area in roots prepared for (f). Error bars are SD for 12 replicates (12 root sections from 12 plants). (h) H_2O_2 concentration in the seedlings from (g). (i) Total RNAs were extracted from seedlings in (g) for qRT-PCR analysis using gene-specific primers. (j) sWT seedlings were grown from seeds with or without H_2O_2 pretreatment, and total RNAs were extracted for qRT-PCR analysis using gene-specific primers.

reappeared after reoxygenation (Figure 1g), also indicates that the expression of *AOX1a* is regulated by O_2.

AOX expression has been shown previously to enhance photosynthesis in various plant species under different stress conditions (Del-Saz *et al.*, 2018). In tobacco plants, *AOX* overexpression results in increased plant biomass relative to WT after prolonged moderate water deficit, with the beneficial effect on plant growth being associated with the ability of AOX to maintain a higher level of mitochondrial respiration and improved chloroplast energy balance and photosynthesis (Dahal and Vanlerberghe, 2018). We have shown herein that expression of *AOX1a* under the control of the *Ubi* promoter in transgenic rice results in overexpression of both *MnSOD* and *Cu/ZnSOD* in shoots and roots (Figure 5a). Moreover, we found that chlorophyll content, leaf width and photosynthesis rates were all increased in the *AOX1a-OE* line that, combined with the vigorous root growth observed under flooding, may account for the ultimate increase in grain yield in rice paddy (Figure 6, Appendix S1: Results and Discussion).

The *AOX1a*-H_2O_2 feed-forward regulatory loop constitutes a sensor-second messenger pair for rice responses to hypoxia/flooding stress

We found that *KIN1* regulates *AOX1a* (Figure 7e,f), and both *KIN1* and *AOX1a* are needed for germination and coleoptile elongation under hypoxia (Figures 2a and 7g), confirming *KIN1* is a key upstream regulator of *AOX1a*. The mRNA levels of regulatory genes – *CDKE1* and *KIN1* – are activated by H_2O_2, whereas *MYB106* and *AP2* are suppressed by it (Figure 3). Homologues of these genes have also been shown to regulate similarly the *AOX1a* promoter in Arabidopsis in response to mitochondrial or chloroplast stress and/or H_2O_2 (Table S2). The activated *AOX1a* then induces *MnSOD* and *Cu/ZnSOD* expression, leading to an increase in H_2O_2 accumulation. Based on our findings, we propose a functional model illustrating the signalling pathway that regulates germination, root development and plant growth in rice in response to hypoxia/flooding (Figure 7i). Since the expression of *AOX1b* is also induced by H_2O_2, although to a lesser extent than *AOX1a* under hypoxia (Figure 7k), and the expression of *AOX1b* was unaltered by *AOX1a* knockout (Figure S14), we cannot completely rule out the possibility that *AOX1b* can still play a role in the hypoxia response process.

The AOX1a-H_2O_2 regulatory loop constitutes an intriguing sensor-second messenger pair for rice responses to hypoxia/-flooding stress. We have demonstrated that *AOX1a* is necessary and sufficient for seed germination, coleoptile elongation and root growth under hypoxic conditions. Although AOX1a could exert other functions in a normal aerobic environment, it becomes the primary mitochondrial oxidase when O_2 tensions are lowered during hypoxia (Affourtit *et al.*, 2001). The ROS species generated by AOX1a are converted to H_2O_2 due to SODs, expression of which is also enhanced by *AOX1a* overexpression. Perhaps the most novel observation in the current work is the induction of *AOX1a* expression by H_2O_2, forming a self-perpetuating feed-forward loop, leading to the accumulation of high levels of H_2O_2 (Figure 7h).

In summary, our discoveries provide new insights into a unique sensor-second messenger pair in which *AOX1a* acts as the senor perceiving low O_2 tension, and H_2O_2 accumulation serves as the second messenger triggering downstream root development against hypoxia stress in rice. We also highlight *AOX1a* as a potential target for genetic modifications, such as transgenic engineering or genome editing, to enhance flooding tolerance in rice and other crops. Moreover, since monocots and dicots share the conserved H_2O_2-dependent regulatory pathways that elicit *AOX1a* expression and flooding tolerance displayed by rice, our study lays the foundation for further explorations of how the mechanism has diverged, leading to differences in how flooding tolerance is manifested in plant species. Such analyses would not only answer fundamental scientific questions, but would also prove to be of vital importance to agricultural production and food security.

Materials and methods

Plant materials

The *japonica* rice line *Oryza sativa* cv Tainung 67 was used in this study. Plasmids were introduced into *Agrobacterium tumefaciens* strain EHA105, and rice transformation was performed as described previously (Chen *et al.*, 2002). The gene-knockout mutants *aox1a* (M0030882) and *kin1* (M0037269), and the gene-activation mutant *KIN1-Act* (M0101986) were obtained from the Taiwan Rice Insertional Mutant (TRIM) population (http://trim.sinica.edu.tw/) (Hsing *et al.*, 2007; Lo *et al.*, 2016). All plant materials were freshly harvested and stored at −80 °C prior to experiments. Sterilized seeds were placed on ½ MS agar medium and pressed down slightly into agar, before being germinated in glass tubes in a growth chamber at 25 °C, with a day/night cycle of 16 h/8 h. For hypoxic treatment, autoclaved water was carefully poured to the top of test tubes to fill up the space, and then the tubes were sealed with parafilm. The condition under which shoots were exposed to air was designated as 'aerobic', and for shoots submerged in water it was designated as 'hypoxic'.

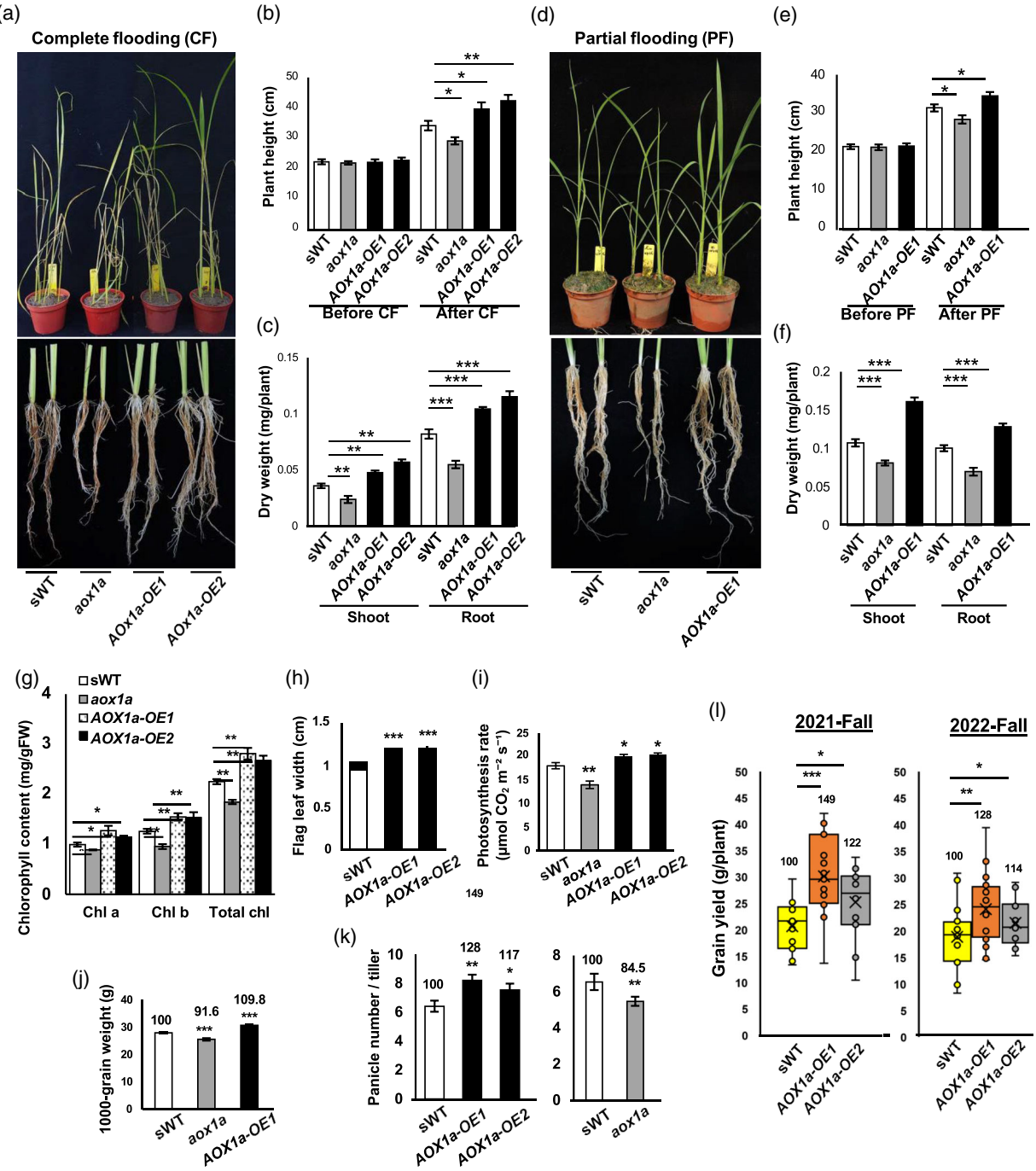


Figure 6 *AOX1a* promotes root growth under flooding and grain yields in rice paddy. (a–c) Seeds were germinated in soil and the seedlings were then grown under complete flooding (CF, water depth of 15 cm above the leaf tips at the beginning of experiment) for 14 days. (a) Plant and root morphology under CF. (b) Plant height under CF. (c) Shoot and root dry weights under CF. (d–f) Seeds were germinated in soil and then the seedlings were grown under partial flooding (PF, water depth of 5 cm above the soil surface throughout the experiment) for 14 days. (d) Plant and root morphology under PF. (e) Plant height under PF. (f) Shoot and root dry weights under PF. Error bars are SD for 12 replicates. (g) Chlorophyll content of 30-day-old plants. (h) Flag leaf width of 120-day-old plants. $n = 9$. (i) Photosynthesis rate of 30-day-old plants. $n = 6$. (j) One thousand-grain weight. $n = 6$. The numbers above bars are % relative to the value in WT. (k, l) Rice lines were cultivated in rice paddy in Fall season of 2021 and 2022. (k) Panicle number per tiller of lines grown in 2021. $n = 10$. (l) Total grain yields of individual plants. The box plot was constructed using the Microsoft Excel program, and statistical analysis was carried out with the Student's *t*-test. The value of total grain weight per plant in the sWT was set to 100%, and all other values were calculated relative to this value. Error bars represent SE of 10 replicates.

Plasmid construction

For the *AOX1a*-overexpressing construct, the full-length coding sequence of *AOX1a* (999 base pairs, bp) was amplified by polymerase chain reaction (PCR) from rice (*Oryza sativa* L. ssp. japonica cv Tainung67) cDNA using a Phusion high fidelity DNA polymerase (Thermo Scientific, Waltham, MA, USA) and cloned into pCR-4-TOPO vector (Invitrogen, Waltham, MA, USA).

Following sequencing verification, the *AOX1a*-TOPO clone was sub-cloned into Gateway entry vector pSMY between the *Ubi* promoter and *GFP-Nos*-terminator using a Multi-Round LR recombinase-mediated Gateway™ system (Invitrogen, Waltham, MA, USA), generating p*Ubi:AOX1a-GFP*. For the *AOX1a* promoter-driven *GUS* construct, we amplified a 2.0-kilobase promoter region of *AOX1a* from the rice genome and inserted it into plasmid pCAMBIA1305, generating p*AOX1a:GUS*. To generate the genome-editing construct, we designed a guide RNA complementary to the second exon of *AOX1a*. This guide RNA was cloned into destination Cas9 expression vector pRGEB31 (Xie and Yang, 2013), generating an AOX1a-CRISPR-Cas9 construct. All plasmids were introduced into *Agrobacterium tumefaciens* strain EHA105, and rice transformation was performed as described previously (Chen *et al.*, 2002).

Subcellular localization analysis

Protoplasts were isolated from rice leaf sheath and transfected with plasmids as described previously (Muzaffar *et al.*, 2024). Fluorescence images were collected using a Zeiss LSM 780 confocal microscope. GFP fluorescence images were detected with the laser line of excitation/emission at 488/505 nm. Mitochondria were stained using MitoTracker® Red CMXRos (red), a derivative of X-rosamine, for 10 min according to the manufacturer's instructions prior to examination under confocal microscopy. Red fluorescence images were detected with the laser line of 579–599 nm.

GUS activity assays and GUS staining

GUS activity in shoots and roots of the *AOX1a:GUS* and *CIPK15:GUS* lines was assayed as described previously (Lin *et al.*, 2014). GUS staining of seeds and seedlings of the *AOX1a:GUS* line was conducted as described previously (Chen *et al.*, 2015).

Generation and selection of genomic *AOX1a* knockout lines

Twenty T_0 independent transgenic *AOX1a-CRSPR-Cas9* lines were selected for DNA sequencing. Genomic *AOX1a*-edited T_0 lines were cultivated for propagation of T_1 seeds, and then T_1 plants were self-pollinated to propagate T_2 seeds. T_2 plants were DNA sequenced to select *aox1a* knockout homozygous and T-DNA-free lines. Two *AOX1a* knockout lines, *aox1a*-7 and *aox1a*-18, harbouring a 12-bp deletion and a 1-bp insertion, respectively, at the DNA editing target site were generated. The *aox1a*-18 knockout line has a stop codon at the 33rd amino acid (counting from the first ATG), resulting in premature termination of the *AOX1a* peptide.

O_2 concentration measurement

A high-resolution Oxygraph-2k respirometer (O2k, Oroboros Instruments, Innsbruck, Austria) was used to measure O_2 concentration in water. Measurements were carried out at 37 °C. The standard O_2 level in water with stirring at 750 rpm was set as 100%, and the level in water containing Na_2SO_3 without stirring was set as 0%. Four ml of water was quickly collected from test tubes containing rice seedlings at various time points and measured for O_2 concentration.

H_2O_2 quantification

H_2O_2 concentration in tissues was determined as described previously (Loreto and Velikova, 2001). Entire seedlings (0.1 g) were homogenized with 1.5 mL of 1% (w/v) TCA. The homogenate was centrifuged at 13 000 rpm and 4 °C for 10 min, and then 0.5 mL of the supernatant was added to 0.5 mL of 10 mM potassium-phosphate buffer (pH 7.0) and 1 mL of 1 M KI. The absorbance of the supernatant was measured at 390 nm. A standard calibration curve of H_2O_2 was plotted in a range from 1 to 1000 μmoL/mL. The H_2O_2 concentration in samples was calculated by comparison to the standard curve and values are expressed as μmol/g fresh weight.

Seed H_2O_2 pretreatment

Sterilized seeds were pretreated with 50 mM H_2O_2 overnight, germinated on ½ agar MS medium, and the resulting seedlings were grown under aerobic or hypoxic conditions.

Examination of germination and seedling development

For rice seeds germinated under aerobic/normoxic conditions at 28 °C, the coleoptile and radicle emerge out of the embryo simultaneously at 1 DAI. Under hypoxic conditions, the coleoptile emerges out of the embryo at 1 DAI, whereas the radicle emerges at 3–4 DAI. With H_2O_2 pretreatment and germination under hypoxic conditions, the coleoptile emerges out of the embryo at 1 DAI and the radicle emerges at 2–3 DAI (including the date of H_2O_2 pretreatment), with subsequent seedling development being faster with H_2O_2 pretreatment than without it.

Quantitative real-time qRT-PCR

Total RNA was extracted from rice tissues using Trizol reagent (Invitrogen) and 1–5 μg of total RNA was used for cDNA synthesis using SuperScript™ III Reverse Transcriptase (Invitrogen). The cDNA was diluted 10-fold for storage. Quantitative RT-PCR was performed as described previously (Chen *et al.*, 2019), using *UBQ5* as the internal reference for RNA quantification.

Western blot analysis

Western blot analysis of proteins was conducted as described previously (Chen *et al.*, 2015). The anti-plant alternative oxidase 1 and 2 (AOX) (AS04054) and anti-α-tubulin (AS10680**)** antibodies were purchased from Agrisera (Vännäs, Sweden). The anti-AOX antibodies were generated with a conjugated synthetic peptide derived from a fully conserved C-terminal consensus motif from plant AOX isoforms including *Arabidopsis thaliana* AOX1A, AOX1B, AOX1C and AOX2 and *Solanum lycopersicum* and *Oryza sativa AOX1D. The anti-α-tubulin antibodies were generated with a conjugated* peptide derived from tubulin alpha chain sequences including *Arabidopsis thaliana* tubulin α-1, α-2/α-4, α-5 and α-6 chains.

RNAseq and bioinformatics analyses

Total mRNA was extracted from 3-day-old seedlings, which were developed from H_2O_2-pretreated seeds and grew under water for 2 days, using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). RNA quality control and quantification were carried out using a Bioanalyzer 2100 system (Aligent, San Jose, CA, USA). cDNA libraries were prepared with a TruSeq standard mRNA kit (Illumina, San Jose, CA, USA) according to the manufacturer's instructions. cDNA sequencing was performed using a 500-high output v2 sequencing kit and an Illumina Nextseq500 instrument. Bioinformatics analysis was conducted in CLC Genomics Workbench software (v11.0.1, Qiagen, Hilden, Germany).

Detection of H_2O_2 by DAB staining

Roots of 7-day-old seedlings were collected. Then, DAB was dissolved in low pH (1–2) water overnight. The solution was

filtered and neutralized with 200 mM Na_2HPO_4 to pH 7 for use as the staining solution. Roots of 7-day-old seedlings were placed in 50-mL Falcon tube containing staining solution and vacuum-processed three times (each time for 5 min) to ensure complete infiltration. The tube was then covered with aluminium foil and placed on a shaker at a speed of 60 rpm for 4–5 h. Then the DAB staining solution was removed with a bleaching solution (ethanol:acetic acid:glycerol = 3:1:1). The tube was then carefully placed in a boiling water bath for 15 ± 5 min, before replacing the boiled bleaching solution with fresh bleaching solution and allowed to stand for 30 min. Roots were kept in 70% ethanol at 4 °C and photographed using an optical microscope (Z1; ZEISS Microscopy Co. Ltd., Oberkochen, Germany) with a CCD camera (DP70; Olympus Optical Co. Ltd., Shinjuku, Tokyo, Japan).

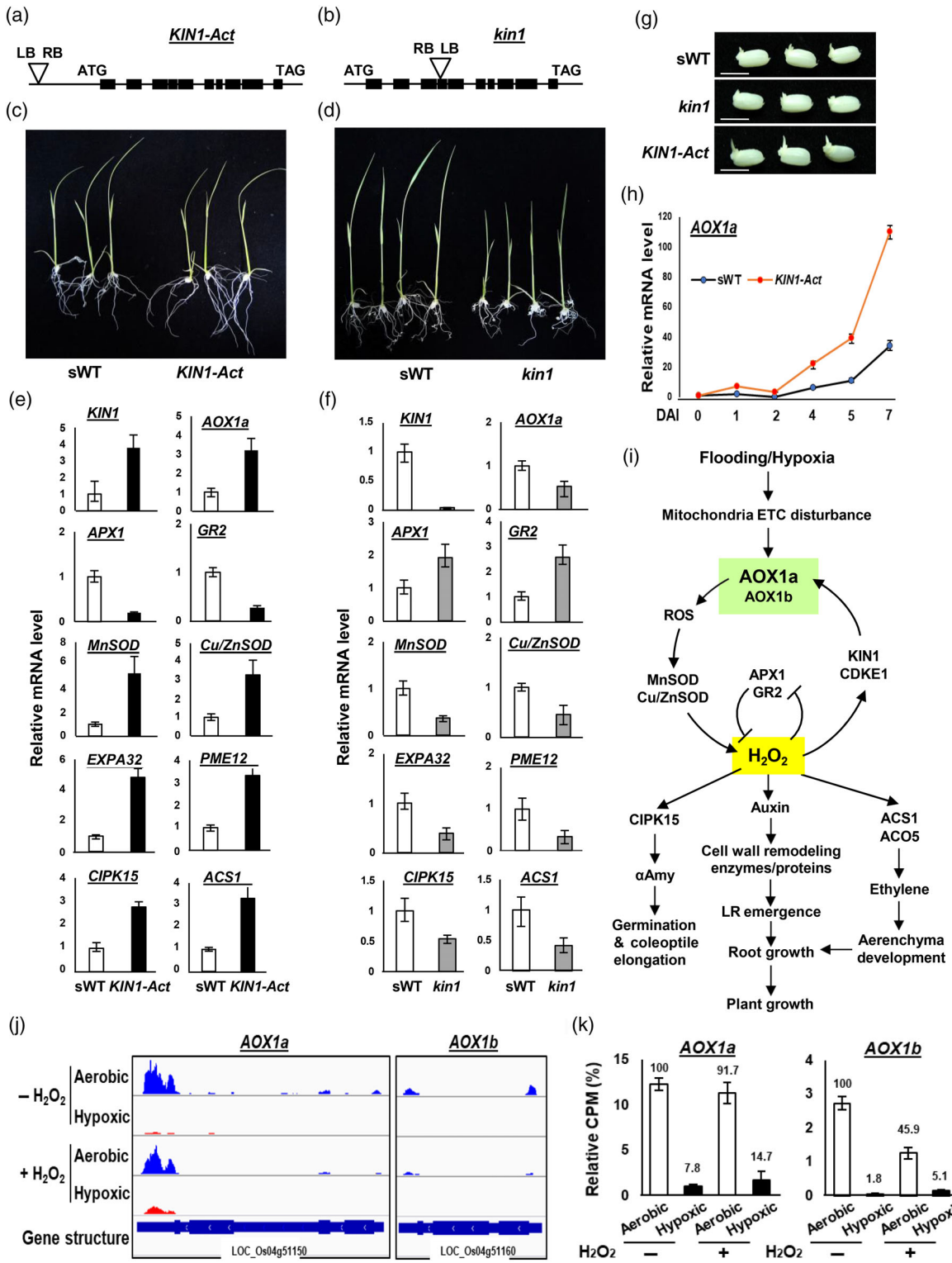


Figure 7 *KIN1* is a key regulator of the *AOX1a*-H_2O_2 feed-forward loop that controls gene expression and promotes rice germination and seedling growth under hypoxia. (a, b) The T-DNA inserted 4 kb upstream of ATG in *KIN1* coding region causes *KIN1* activation (*KIN1-Act*), and inserted in the fourth exon it causes *KIN1* knockout (*kin1*). (c–f) *KIN1-Act* and *kin1* mutant lines were germinated on ½ MS agar medium and the seedlings were then submerged in water for 11 days. (c, d) Morphology of seedlings. (e, f) mRNA was extracted from roots and subjected to qRT-PCR analysis using gene-specific primers. (g) Seeds of WT, *kin1* and *KIN1-Act* lines were germinated under submergence for 1 day. (h) Seeds of sWT and the *KIN1-Act* line were germinated on ½ MS agar medium and then seedlings were grown under hypoxia for 7 days. mRNA was extracted from entire seedlings and subjected to qRT-PCR analysis for *AOX1a*. (i) The *AOX1a*-H_2O_2 self-perpetuating feed-forward regulatory loop controls *AOX1a* expression and H_2O_2 accumulation and promotes germination and seedling growth under hypoxia. Details of the model are described in the text. (j) The RNAseq data shown in Figure 3 were annotated with the IGV software (https://software.broadinstitute.org/software/igv/). (k) Quantification of *AOX1a* and *AOX1b* mRNA levels in (j), presented as values of count per million (CPM). The CPM value under aerobic condition without H_2O_2 treatment was set as 100%, and the other values relative to this value were calculated.

Detection of superoxides by NBT staining

O_2^- levels were detected as previously described (Song *et al.*, 2021) with minor modifications. Five-day-old seedlings were placed in a solution containing 1 mg/mL NBT in 50 mM sodium phosphate buffer (pH 7.5). Seedlings were then vacuumed and incubated with gentle shaking for 2 h in the dark. The staining solution was replaced with absolute ethanol in a boiling water bath for 30 min to remove chlorophyll. Seedlings were then transferred to plates with water for imaging.

ROS imaging

Stained samples were scanned using an EPSON Perfection 4990 PHOTO scanner. In common with all other assays based on insoluble products, DAB staining does not follow the Beer–Lambert Law. Consequently, to provide insights into the extent of staining, we processed the DAB-stained samples in ImageJ (Ver. 1.43u; US National Institutes of Health, Bethesda, MD, USA). Thus, to visualize colour intensity, each image was imported into ImageJ and a target area was selected for editing using the rectangle tool. This target area was 'inverted' to create a reversed image for export. Quantification of NBT staining was based on the pixel intensity of equal squared areas (regions of interest, ROI) of roots by ImageJ.

Root anatomy and microscopy

Root cross-sections were prepared from 4 mm-long segments excised from rice roots. To examine aerenchyma, crown roots were sectioned at 20 mm and 40 mm from the tip of the crown and seminal roots, respectively, by hand-sectioning with a razor blade. Sections were photographed using an optical microscope (Z1; ZEISS Microscopy Co. Ltd., Oberkochen, Germany) with a CCD camera (DP70; Olympus Optical Co. Ltd.). Aerenchyma is formed by fusion of parenchyma cells that have undergone PCD and exhibit an irregular cavity. The area of aerenchyma was determined using ImageJ software (Ver. 1.43u; US National Institutes of Health, Bethesda, MD, USA). Aerenchyma area was calculated (in %) by dividing total aerenchyma area by total root area from cross-sections. To analyse LR numbers, 20 to 30 cross-sections were prepared from 4 mm root segments.

Chlorophyll measurement

The chlorophyll contents were determined as described previously (Ni *et al.*, 2009).

Photosynthesis rate measurement

Leaf photosynthetic rate was measured on the uppermost fully expanded leaves of 1-month-old rice plants grown in the greenhouse using a portable gas-exchange system (LI-6800; LI-COR Inc., Lincoln, NE) equipped with a fluorometer leaf chamber (6800-01A) with the following settings: fluorescent light source of 1200 μmol photons m^{-2} s^{-1} (90% red/10% blue); flow rate of 500 μmol s^{-1}; CO_2 of 450 μmol mol^{-1} in a fixed chamber with size of 1 cm × 3 cm. Six biological replicates of each genotype were measured. Student's *t*-test was performed to show statistically significant differences.

Field trial

Field trials were carried out at the Agricultural Experimental Station of National Chung Hsing University at Wufeng, Taichung, Taiwan. The irrigated field (100% watering) was flooded with 1–5 cm of water until the end of the active tillering stage (30–40 days after transplanting). Water was then drained for 10–15 days at the late tillering stage, and flooded again with 3–10 cm of water until the milky stage. To evaluate grain weight per plant in the paddy, 25-day-old seedlings were transplanted into soil, with 25 × 25 cm spacing between each plant. A total of 10 plants of each line were grown in the paddy. Seeds were harvested after ripening, dried, and their weight per plant was determined as described previously (Chen *et al.*, 2015).

Statistical analyses

Error bars indicate the SE or SD from three independent experiments. Significance levels from Student's *t*-test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and $****P < 0.0001$. n.s. indicates no significant difference. For bar plots displaying letters above the bars, statistical analysis was performed by one-way ANOVA with a post-hoc Tukey HSD. Significant levels are labelled at $P < 0.05$.

Primers

Primers used for plasmid constructions and qRT-PCR analyses are listed in Table S3.

Acknowledgements

We thank Dr. Sue Lin-Chao for scientific advice, Dr. John O'Brien for critical review of this manuscript, Ms. Min-Wei Tsai for rice transformation, Ms. Ku-Ting Chen for fieldwork, Ms. Yi-Ju Lee for O_2 measurement, Dr. Chen-Hsin Yu at the Genomics Core, Bioinformatics-Biology Service Core and Imaging Core at the Institute of Molecular Biology, Academia Sinica, for bioinformatics and imaging analyses. We also thank Dr. Chih-Yu Lin and Gong-Min Lin at the Metabolomics Core Facility of Agricultural Biotechnology Research Center, Academia Sinica, for conducting the LC-QTOF analysis and data processing. This work was supported by grants from the Innovative Translational Agricultural Research (ITAR) Program of Academia Sinica (AS-108-ITAR-TD08,

AS-109-ITAR-TD08, AS-110-ITAR-TD06, and AS-KPQ-111-ITAR-11108), Ministry of Science and Technology (MOST 109-2326-B-001-004, MOST 110-2326-B-001-011, and NSTC 112-2311-B-005-007), and in part by the Advanced Plant Biotechnology Center from the Featured Areas Research Center Program within the framework of the Higher Education Sprout Project by the Ministry of Education (MOE) in Taiwan.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting information

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

Figure S1 Rice AOX1a is localized in mitochondria.
Figure S2 AOX pre-exists in dry seeds and its accumulation at early stage of seedling development is suppressed under hypoxia.
Figure S3 Generation of *AOX1a* knockout lines.
Figure S4 Generation of *AOX1a* overexpression lines.
Figure S5 O_2 concentration for hypoxic treatment of rice.
Figure S6 *AOX1a* is necessary and sufficient to promote rice seedling growth under hypoxia.
Figure S7 *AOX1a* is necessary for rice seedling growth under hypoxia.
Figure S8 AsA-impaired root growth is not attributable to low pH.
Figure S9 AOX1a reduces superoxide accumulations under hypoxia.
Figure S10 *AOx1a* enhances leaf width.
Figure S11 *AOX1a* increased rice seed size.
Figure S12 *AOX1a* is important for rice grain yield.
Figure S13 *KIN1* is an upstream regulator of AOX1a under hypoxia.
Figure S14 The accumulation of *AOX1b* mRNA is unaltered in the *aox1a* under hypoxia.
Table S1 Fold-change in transcript levels of signaling and regulatory genes by seed H_2O_2 pretreatment in 3-day-old seedlings under hypoxia as analyzed by RNAseq.
Table S2 Rice and Arabidopsis homologous genes regulating *AOX1a* promoters.
Table S3 List of primers.