

Evolutionary insights into the regulation of hypoxia adaptation across plant lineages



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Chapter 1 – Introduction to low-oxygen stress and signaling in plants

Plant physiological response to hypoxia

Molecular oxygen (O_2) is required for energy production via oxidative phosphorylation in aerobic organisms, including plants. Yet, plant tissues frequently encounter O_2 limitations due to restricted gas diffusion, O_2 metabolic consumption, or its sequestration by oxygen-binding proteins. When intracellular O_2 levels drop below the affinity of cytochrome c oxidase, aerobic respiration is impaired, leading to hypoxia and severe metabolic disruptions. Flooding events, especially complete submergence, are major causes of hypoxia, since O_2 diffusion in water is ~10,000 times slower than in air (**Figure 1**) (Armstrong et al., 2000). The increasing frequency and severity of flooding events threaten ecosystems and agriculture, causing global crop losses and damages of ~US\$21 billion (FAO, 2021). This trend highlights the urgent need to better understand how plants sense and adapt to flooding (Hirabayashi et al., 2013).

Based on the amount of water involved and the organs affected, water excess can be classified as waterlogging, which predominantly affects the root system, or submergence, when the water covers, entirely or partially, the aerial tissues (Sasidharan et al., 2017). Alongside the water level, stress severity is further shaped by the plant developmental stage, the perturbed soil microbiome, the accumulation of toxic reduced compounds, and the persistence of the condition (Striker, 2012). To cope with flooding, most angiosperms have evolved two contrasting adaptive strategies: low-oxygen escape syndrome (LOES) and low-oxygen quiescent syndrome (LOQS) (Bailey-Serres and Voesenek, 2008; Bailey-Serres et al., 2010). LOES strategy promotes elongation, growth, reduces leaf thickness and alters cell structure to facilitate gas exchange and improve aerobic metabolism. As these traits are energy-consuming, the LOES strategy is mainly exploited in flood-prone environments where the benefits to plant fitness outweigh the costs. On the other hand, LOQS strategy involves temporary reduction of metabolism and growth during oxygen-deprived conditions, preserving energy and resources until physiological conditions are restored.

Interestingly, hypoxia is not restricted to environmental stress; it also occurs physiologically in seeds, storage organs, meristematic tissues and active callus cells, suggesting that oxygen acts as a diffusible signal coordinating development (**Figure 1**) (Van Dongen et al., 2003; Rolletschek et al., 2007; Licausi et al., 2011a; Shukla et al., 2019; Weits et al., 2019, p. 201; Koo et al., 2024). Although hypoxic niches have been identified in a range of tissues, their spatial extent, dynamics, and physiological relevance remain difficult to resolve due to the limited availability of non-invasive tools capable of mapping oxygen distribution in living plant tissues

with high resolution. This technical limitation has constrained our ability to directly link oxygen availability with local developmental and stress responses.

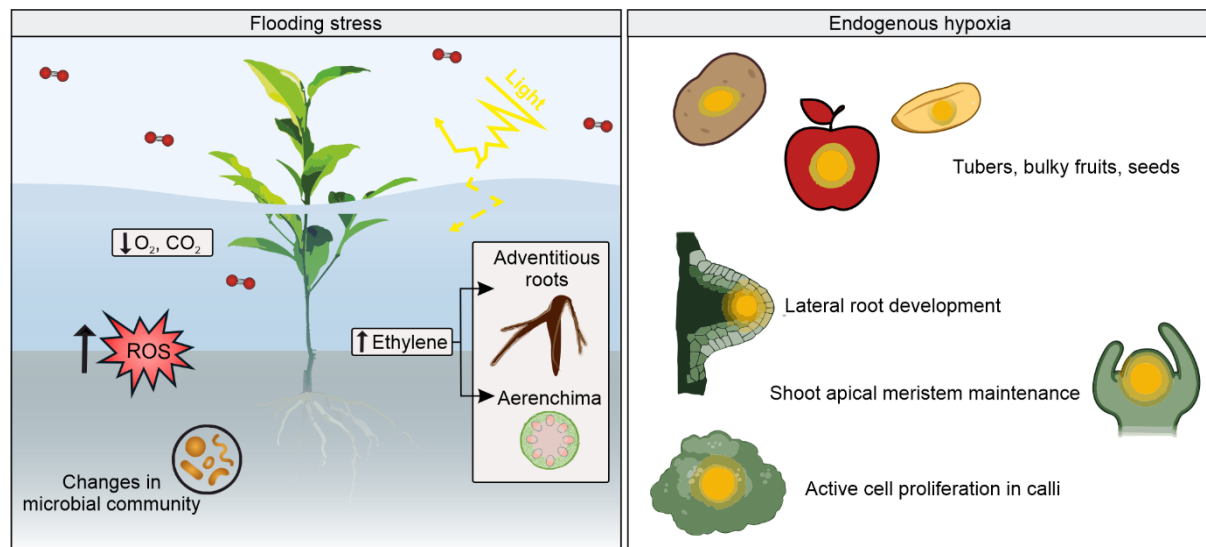


Figure 1 – Hypoxic conditions in plants. Plant hypoxia can arise from environmental stress or from endogenous developmental processes. (Left) Under flooding stress, oxygen (O_2) and carbon dioxide (CO_2) diffusion is restricted and light penetration is reduced. This leads to the accumulation of reactive oxygen species (ROS) and the phytohormone ethylene, which trigger molecular signaling and adaptive responses such as the production of adventitious roots and aerenchyma. The strongly reducing conditions established in the flooded soil also induce shifts in the microbial communities that surround the plant. (Right) Under physiological conditions, localized low-oxygen niches occur in various organs and developmental stages. These endogenous hypoxic zones contribute to accommodating developmental processes, including tuber, fruit, and seed formation, lateral root development, shoot apical meristem maintenance and active cell proliferation in calli.

Oxygen limitation severely impacts plant metabolism. Because oxygen serves as the terminal acceptor in the mitochondrial electron chain, aerobic respiration is impaired under hypoxia, lowering ATP production and adenylate charge, and restricting NADH reoxidation, which can in turn inhibit glycolysis (Waters et al., 1991, p. 1; Perata and Alpi, 1993). To compensate, hypoxic plants enhance carbohydrate catabolism by promoting starch and sucrose mobilization to fuel glycolysis. In addition, plants also employ PPI-dependent phosphofructokinases to maintain glycolytic flux under low ATP availability, while lactate and ethanol fermentation regenerate NAD^+ to allow continued ATP production (Bui et al., 2019; Ventura et al., 2020).

Concurrently, hypoxia-induced reductions in gas exchange lead to ethylene accumulation, partly through root-to-shoot translocation of its precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) (Jackson, 2002; Sasidharan et al., 2018). Although the range of ethylene may fluctuate across species, its accumulation is considered a main indicator of flooding stress, triggering anatomical adaptation traits, such as aerenchyma and adventitious roots, that facilitate root aeration (**Figure 1**) (Colmer, 2003; Loreti and Striker, 2020).

Reactive oxygen species (ROS) also play a central role in hypoxia signaling. Indeed, periods of oxygen deprivation during submergence are frequently followed by rapid reoxygenation upon water recession. Both hypoxia and reoxygenation provoke transient ROS accumulation from mitochondrial dysfunction and NADPH oxidases (e.g., RBOHD), generating a ROS burst (Baxter-Burrell et al., 2002; Chen et al., 2012; Barreto et al., 2022; Peláez-Vico et al., 2023). Reoxygenation-associated ROS production can be particularly damaging, as the sudden restoration of oxygen coincides with disrupted redox homeostasis and metabolic imbalance (Fukao et al., 2019). However, ROS act as both stressors and signals, activating Ca^{2+} influx and Ca^{2+} -dependent kinases such as CPK16, which further activate RBOHD. This ROS- Ca^{2+} feedback loop amplifies systemic signaling and fine-tunes hypoxia responses (Banti et al., 2010, p. 20; Wu et al., 2020; Bakshi and Gilroy, 2025). This cross-talk between ROS and low-oxygen response extends to transcriptional regulation, as ROS-responsive genes are modulated by hypoxia-inducible transcription factors and hypoxia-responsive proteins, such as hypoxia-responsive universal stress protein 1 (HRU1), are upregulated by ROS-activated heat-shock factors (Banti et al., 2010, Gonzali et al., 2015, p. 201; Giuntoli et al., 2017b; Yao et al., 2017). As a result, plant responses to flooding cannot be fully understood by considering hypoxia alone, but require integration of oxygen deprivation and subsequent reoxygenation.

Finally, flooding alters soil chemistry and microbiomes, leading to phytotoxic ion accumulation (Fe^{2+} , Mn^{2+} , S^{2-}), elevated CO_2 , and reduced nutrient availability (Mg^{2+} , Ca^{2+}), further compromising plant fitness (Colmer and Voesenek, 2009; Unger et al., 2009; Compant et al., 2019; Francioli et al., 2021). Shifts in microbial communities can also affect plant health, emphasizing the interplay of abiotic and biotic factors in flood stress.

Together, these observations highlight the complexity of low-oxygen stress, integrating metabolic, hormonal, and signaling pathways, emphasizing the importance of understanding hypoxia sensing both under environmental stress and in physiological contexts. Despite significant progress in identifying adaptive traits and regulatory components, flooding remains a major constraint on plant performance, and improvements in flooding tolerance are often accompanied by trade-offs with growth and development. A deeper mechanistic understanding of oxygen sensing and stress integration is therefore required to identify new leverage points for enhancing flooding resilience without compromising plant fitness.

Low-oxygen sensing in the green lineage

The molecular components of low-oxygen sensing in flowering plants have been widely studied in the last decade, revealing the pivotal role of Group-VII Ethylene Response Factors (ERFVII) and their post-translational regulation by Plant Cysteine Oxidases (PCOs) (Licausi et al., 2011b; Weits et al., 2014). This pathway extends beyond hypoxia signaling, targeting additional substrates such as LITTLE ZIPPER2 and the Polycomb Repressive Complex 2 (PRC2) subunit VERNALIZATION2 (VRN2), extending oxygen-dependent control to developmental regulation and epigenetic memory (Gibbs et al., 2018; Weits et al., 2019; Labandera et al., 2021; Osborne et al., 2025).

ERFVII proteins belong to the APETALA2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF) superfamily, characterised by a conserved 60 aa AP2 DNA-binding domain (Xie et al., 2019). Members of the AP2/ERF superfamily have been demonstrated to play several roles in regulating plant growth, developmental programs, phytohormones response, drought stress and plant-pathogens interactions (Licausi et al., 2013; Papdi et al., 2015; Vicente et al., 2017). In *Arabidopsis thaliana*, the ERFVII group consists of five members: RELATED TO APETALA2 2, 3 and 12 (RAP2.2, RAP2.3, RAP2.12), that are ubiquitously and constitutively expressed and post-translationally regulated under hypoxia, and HYPOXIA RESPONSIVE 1 and 2 (HRE1 and HRE2), transcriptionally induced by hypoxic conditions (Gasch et al., 2016).

Among the ERF family, members of the group VII are frequently characterised by a penultimate cysteine residue in the N-terminal CMVII-1 domain (MCGGAI) (Nakano et al., 2006), which is fundamental for ERFVII's role as hypoxia transducers. Under aerobic conditions, the stability of ERFVII proteins is regulated by the N-degron pathway (Gibbs et al., 2011; Varshavsky, 2011). According to this pathway (**Figure 2**), the CMVII-1 domain is co-translationally recognised by methionine aminopeptidases (MetAP) to remove the initial methionine. The exposed cysteine is then subjected to oxidation by PCOs, using O₂ as co-substrate, resulting in the formation of a cysteine-sulfinic (SOOH) or cysteine-sulfonic (SOOOH) acid adduct. Consequently, the exposed oxidised cysteine undergoes arginylation by the tRNA-arginyl-transferase (ATE). The presence of an exposed N-terminal arginine triggers the ubiquitination by the E3 Ubiquitin-ligase PROTEOLYSIS (PRT6) in concerted action with BIG/Dark Overexpression of CAB1/Transport Inhibitor Response 3 (BIG), driving the target to proteasomal degradation (Graciet and Wellmer, 2010; Zhang et al., 2024). Notably, this process is also sensitive to treatments that inhibit nitric oxide (NO) accumulation, although the exact step that requires NO has yet to be identified (Gibbs et al., 2014). Under hypoxia, PCO activity is suppressed, stabilizing ERFVII, which accumulate in the nucleus to activate hypoxia-responsive genes.

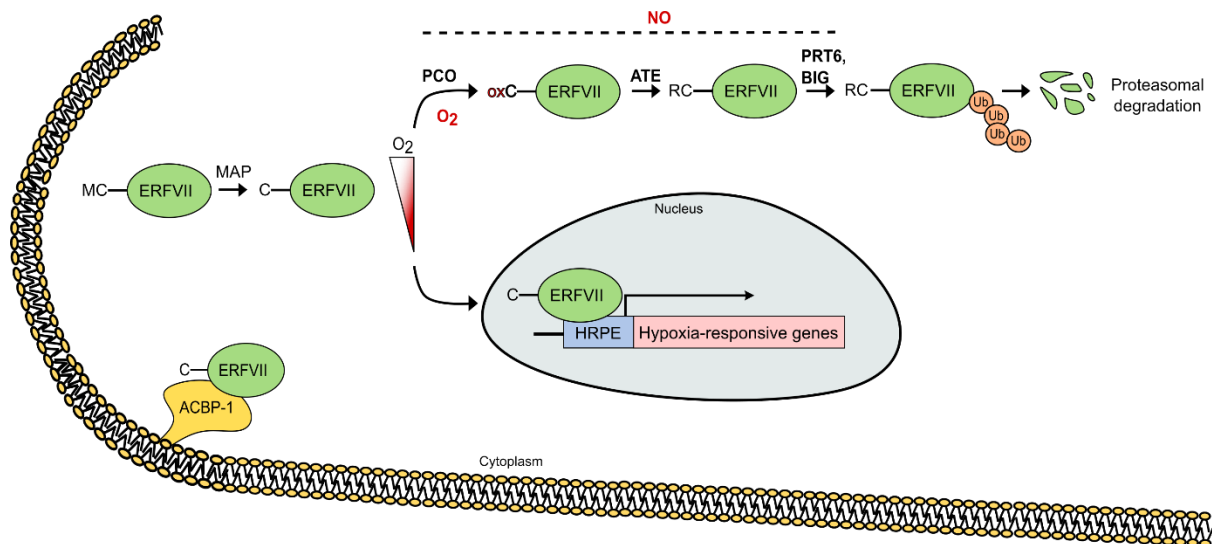


Figure 2 – O₂-dependent destabilization of ERFVII regulates hypoxia response in angiosperms. The N-terminal MCGGAI motif undergoes MetAP-mediated methionine removal, leaving an exposed cysteine which, in presence of O₂ is targeted for oxidation by PCO. The resulting Cys-sulfinic or Cys-sulfonic acid is sequentially modified by ATE triggering proteasomal degradation after ubiquitination by PRT6 and BIG, in a NO-dependent manner (Gibbs et al., 2014; Van Dongen and Licausi, 2015; Zhang et al., 2024). Hypoxia inhibits PCO activity, stabilizing ERFVIIs, which translocate to the nucleus and bind hypoxia-responsive promoter element (HRPE) to induce gene expression (Gasch et al., 2016). RAP2.12 stability is further regulated by sequestration at the plasma membrane via ACBPs and its release under hypoxia (Schmidt et al., 2018a). Additional abbreviations: M, methionine; C, cysteine; oxC, oxidized cysteine; R, arginine; Ub, ubiquitin.

ERFVII activity is also modulated by subcellular localization. Under aerobic conditions, ERFVIIs can evade degradation by associating with plasma membrane-localized acyl-CoA-binding proteins (ACBP1/2) through a RAYD motif, despite lacking hydrophobic transmembrane domains (Licausi et al., 2011b; Schmidt et al., 2018b). Hypoxia-mediated energy crisis shifts the CoA pool, releasing RAP2.12 from the membrane, allowing nuclear translocation and activation of the hypoxic response (Zhou et al., 2020). Once in the nucleus, ERFVIIs mediate transcriptional regulation by binding to a specific promoter element, a repeated 12-bp consensus sequence (AAAACCA[G/C][G/C]GC), known as the hypoxia-responsive promoter element (HRPE) (Gasch et al., 2016). Although the mechanism of ERFVII-HRPE interaction has yet to be described, a core transcriptional response of forty-nine genes containing the consensus sequence has been demonstrated (Mustroph et al., 2009). ERFVII-induced anaerobic responses include the transcriptional activation of pyruvate decarboxylase (*PDC*), alcohol dehydrogenase (*ADH*) and several transcription factors and,

remarkably, PCO itself. Notably, constitutive RAP2.12 expression is detrimental to plant fitness, suggesting that a tight regulation is crucial for cell homeostasis (Licausi et al., 2011b). One negative feedback mechanism involves HYPOXIA RESPONSE ATTENUATOR1 (HRA1), a trihelix transcription factor induced by RAP2.12 that represses anaerobic gene expression via direct interaction with RAP2.12 (Giuntoli et al., 2017a, 2014)

Additional regulatory layers integrate metabolic status and energy signaling cascades. The Target of Rapamycin (TOR) kinase phosphorylates the C-terminal activation domain of RAP2.12 in response to sugars, enhancing its transcriptional activity (Kunkowska et al., 2023). Mitogen-activated protein kinases (MPK3/6) and Ca²⁺-dependent protein kinase CPK12 also phosphorylate RAPs, promoting their stability, nuclear localization, and transcriptional output (Chang et al., 2012; Fan et al., 2023). Under hypoxia, the interaction of CPK12 and RAP2.12 is additionally facilitated by the IQ DOMAIN-containing protein 22 (IQD22), amplifying the transcriptional activation of hypoxia-responsive genes (Zhao et al., 2025). Thus, ERFVII activity is orchestrated through the interplay of oxygen-dependent degradation, subcellular dynamics, phosphorylation, and feedback loops, ensuring a balanced hypoxia response.

Evolution of oxygen sensing across kingdoms

Oxygen availability is a fundamental determinant of growth and development in multicellular organisms. To survive hypoxic conditions, diverse homeostatic mechanisms have evolved across kingdoms. Striking parallels exist between plant and animal oxygen-sensing systems, reflecting convergent evolution in response to the universal challenge of low-oxygen.

In animals, oxygen sensing was first characterised through the hypoxia-inducible factor (HIF) pathway. HIF transcription factors regulate angiogenesis, erythropoiesis, and glycolysis by binding hypoxia response elements (HREs) in target genes (Epstein et al., 2001; Kaelin and Ratcliffe, 2008). Under normoxia, HIF-1 α is hydroxylated by Fe(II)- and 2-oxoglutarate-dependent prolyl hydroxylases (PHDs), which target it for von Hippel-Lindau (pVHL)-mediated degradation. Additional oxygen-sensitive mechanisms include Jumonji C domain lysine demethylases (KDMs), which regulate chromatin methylation in an oxygen-dependent manner (Shmakova et al., 2014; Chakraborty et al., 2019).

More recently, research on PCOs guided the identification of their functional homolog in humans, cysteamine dioxygenase (ADO) (Masson et al., 2019). Like PCOs, ADO catalyses oxygen-dependent oxidation of N-terminal cysteine residues, thereby regulating substrate stability via the N-degron pathway. Experimentally confirmed substrates include RGS4, RGS5, IL-32, and ACAD10, which are implicated in cardiovascular development, immune regulation, tumor progression and autophagy (**Figure 3a**) (Lee et al., 2005; Shim et al.,

2022). Remarkably, the first three of these proteins are also transcriptional targets of HIF, giving them dual oxygen-dependent regulation: transcriptional induction by HIF and post-translational turnover by ADO. This layered control enables tight regulation of hypoxia-responsive pathways. Importantly, ADO can functionally substitute for plant PCOs in *Arabidopsis*, revealing a convergent function among eukaryotes (Masson et al., 2019).

Thiol dioxygenases (TDOs), including PCOs and ADOs, form therefore a unifying enzymatic family central to oxygen sensing across life. TDOs are a widespread family of non-heme Fe(II)-dependent enzymes that catalyse the oxidation of thiol substrates (R-SH) to sulfinic acids (R-SO₂H) using molecular oxygen as a co-substrate. The first member identified, cysteine dioxygenase (CDO), was discovered in rat liver in 1966, where it catalyses the conversion of cysteine to cysteine sulfinic acid (CSA) (Ewetz and Sörbo, 1966). Since then, TDOs have been described in bacteria, fungi, plants, and animals, showing a patchy yet evolutionarily significant distribution (Dominy et al., 2006; Licausi et al., 2011b; Masson et al., 2019; Flückiger et al., 2020; Weits et al., 2023).

Despite low primary sequence conservation, TDOs share a characteristic cupin β -barrel fold that coordinates an Fe(II) ion at the active site (Perri and Licausi, 2024). The geometry of this metal centre, together with flexible loop regions, confers substrate specificity and catalytic versatility. Some TDOs, such as mammalian CDO, act on free cysteine to regulate sulfur metabolism. Others, such as plant cysteine oxidases (PCO) and animal cysteamine dioxygenase (ADO), preferentially oxidize N-terminal cysteine residues of proteins, thereby coupling oxygen availability to proteostasis (**Figure 3a**). Phylogenetic analyses suggest that PCO/ADO enzymes diverged early from ancestral CDO-like proteins, coinciding with the emergence of complex life forms requiring oxygen-sensitive regulation of developmental and signaling pathways. Across plant lineages and kingdoms, thiol dioxygenases emerge as a conserved enzymatic framework linking oxygen availability to protein regulation.

In plants, however, oxygen sensing via PCO has been studied most extensively in angiosperms. Phylogenetic analyses have shown that while the enzymatic machinery of the N-degron pathway is broadly conserved across the green lineage, PCO-encoding genes display notable variability (**Figure 3b**) (Weits et al., 2023). In *Arabidopsis*, two clades of PCOs have been identified: A-type PCOs, which are constitutively expressed, and B-type PCOs, which are transcriptionally-induced under hypoxia via ERFVII-mediated recognition of HRPE motifs. While both clades are present in spermatophytes, bryophytes retain only A-type isoforms, suggesting this represents the ancestral state. Notably, PCO-like sequences are also present in green algae, where they display features of both clades. Despite conservation of the enzymatic components, canonical N-degron substrates such as ERFVIIIs, VRN2, and ZPR2 are absent in non-vascular plants (**Figure 3b**).

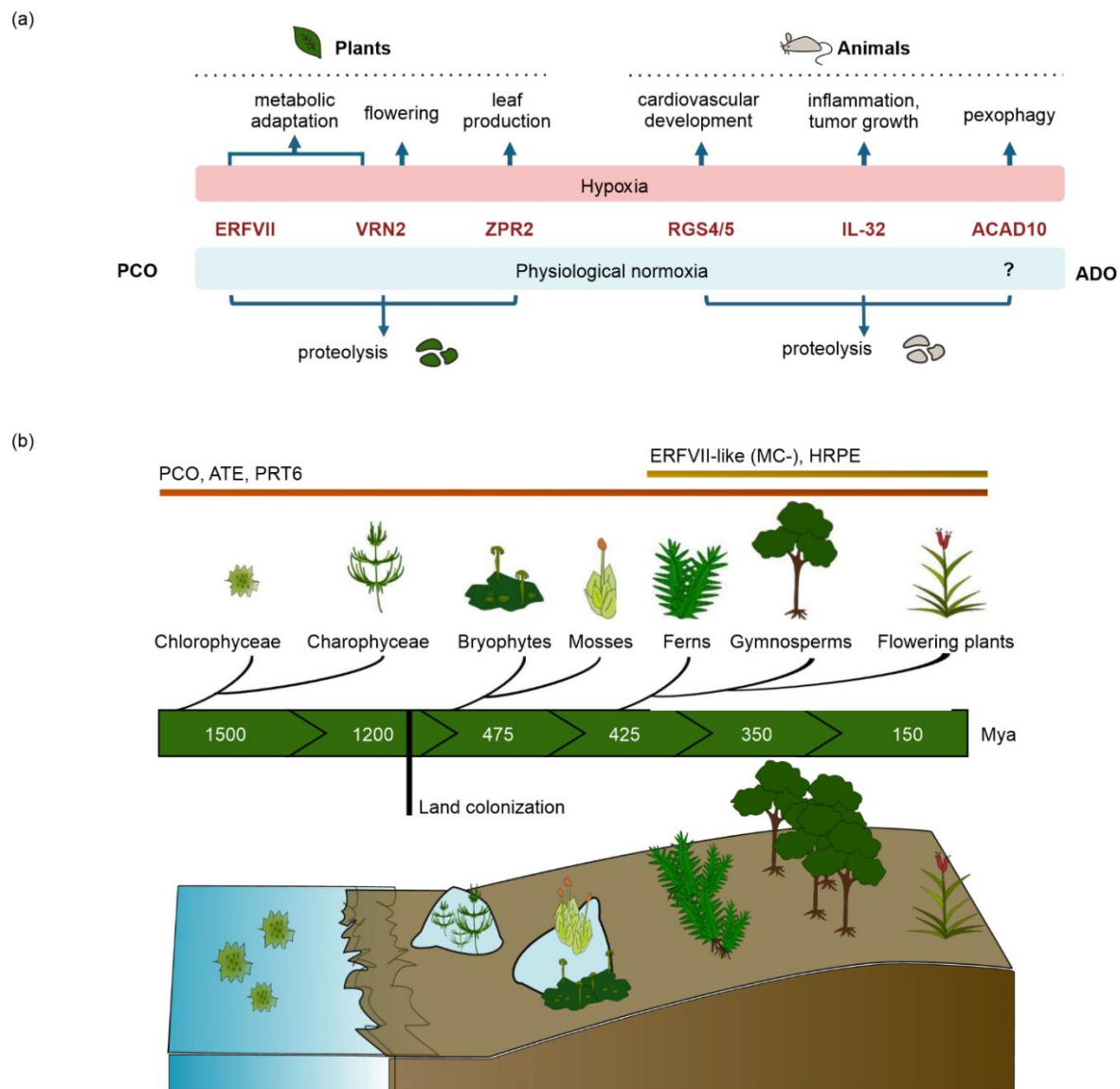


Figure 3 – N-degron oxygen sensing across eukaryotes. (a) Evolution of N-terminal cysteinyl proteins and their oxidation by PCO and ADO, respectively, in plants and animals, establishes a convergent oxygen sensing mechanism to control the adaptation to low-oxygen and stress conditions. Adapted from (Perri and Licausi, 2024). (b) N-degron enzymatic components (ATE, PRT6, PCO) are broadly conserved in the green lineage, but canonical ERFVII substrates and HRPE emerged with vascular plants, enabling transcriptional plasticity in hypoxia responses (Weits et al., 2023; Dalle Carbonare et al., 2025).

A recent study from our group demonstrated that, while a core-response set of metabolic genes is ubiquitously induced by hypoxia in all land plants, ERFVII-dependent regulation of hypoxia-responsive genes is a relatively recent innovation of vascular plants (Dalle Carbonare et al., 2025). Recruitment of ERFVII, together with the HRPE regulatory element, established

a dedicated oxygen-sensing module in this clade of land plants that expanded hypoxia responses beyond basal metabolic adjustments. This innovation likely coincided with the evolution of roots and vascular tissues, supporting plant adaptation to fluctuating oxygen levels in soils and the colonization of terrestrial habitats. This evolutionary framework raises the question of how plants sensed and responded to low-oxygen before the emergence of ERFVII-dependent transcriptional control. Addressing this question requires investigation of divergent land plant lineages prior to vascular innovations. The liverwort *Marchantia polymorpha*, which lacks canonical ERFVII-mediated oxygen sensing yet retains conserved metabolic responses to hypoxia (Dalle Carbonare et al., 2025), represents a suitable system to examine ancestral low-oxygen adaptations and to assess the conservation of enzymatic oxygen-sensing components.

Together, these considerations underscore that oxygen sensing in plants cannot be understood as a single pathway or response, but instead emerges from the interplay of factors. Spatially restricted oxygen gradients arise both during development and under flooding, shaping cellular environments that must be interpreted through oxygen-dependent enzymatic and regulatory processes. These processes operate within dynamic stress scenarios, where periods of hypoxia are frequently followed by reoxygenation and associated redox perturbations, necessitating flexible integration with metabolic and signaling networks. Viewed across plant evolution, core oxygen-dependent metabolic responses appear deeply conserved, while regulatory layers that confer transcriptional and developmental plasticity have been progressively elaborated in specific lineages. Understanding how these layers intersect is essential for defining the principles of plant oxygen sensing and forms the conceptual foundation for the work presented in this thesis.

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AIM OF THE WORK

Plants frequently experience low-oxygen conditions during environmental stresses such as flooding, as well as during normal developmental processes that generate localized hypoxic niches. Although major components of plant hypoxia responses have been identified, the spatial organization of oxygen availability within tissues, the integration of oxygen sensing with other stress signals, and the evolutionary diversification of these mechanisms across land plants remain incompletely understood.

The aim of this thesis was to elucidate how plants sense and adapt to low-oxygen conditions by integrating physiological, molecular, and evolutionary approaches. Addressing these gaps is essential both for advancing fundamental plant biology and informing strategies aimed at improving plant resilience to flooding stress.

RESEARCH QUESTIONS

To achieve this aim, this thesis addresses the following research questions, each targeting a distinct and unresolved aspect of plant low-oxygen sensing:

1. How spatially heterogeneous is oxygen availability within living plant tissues, and can hypoxic microenvironments be directly visualised *in planta* using non-invasive tools?
2. Which molecular mechanisms integrate hypoxia and oxidative stress signaling during flooding and subsequent reoxygenation?
3. How does functional variation among N-terminal cysteine dioxygenases influence low-oxygen tolerance, and what trade-offs exist between stress resilience and growth?
4. To what extent are oxygen-sensing mechanisms and physiological responses conserved across land plants, and which components represent lineage-specific innovations?

Together, these research questions define distinct knowledge gaps and provide a coherent framework for the experimental and comparative approaches employed throughout this thesis.

OUTLINE OF THE THESIS

This thesis begins by examining the physiological and molecular foundations of low-oxygen stress in plants, with a particular focus on flooding tolerance and hypoxia perception.

Chapter 2 presents the application of two novel hypoxia-activated fluorescent probes to visualise oxygen distribution in living tissues. Applied to *Arabidopsis*, these probes revealed both experimentally-induced and naturally occurring hypoxic niches, providing a powerful platform for dissecting oxygen dynamics *in planta*.

Chapter 3 investigates the cross-talk between hypoxia and oxidative stress during flooding and reoxygenation using biochemical and physiological approaches. It demonstrates that ERFVII transcription is repressed upon reoxygenation by ROS-mediated inhibition of PCOs, redirecting transcriptional responses toward oxidative stress and uncovering a mechanism that integrates oxygen and ROS signals to optimize plant performance under fluctuating conditions.

Chapter 4 explores the functional diversity of N-terminal cysteine dioxygenases by substituting endogenous *Arabidopsis* PCOs with homologues from species with divergent sequences, structures, and kinetics. This comparative approach illustrates how N-Cys dioxygenation shapes gene expression under control and hypoxic conditions and highlights trade-offs between stress resilience and plant growth.

Finally, **Chapter 5** situates these findings in an evolutionary context by examining the low-oxygen response in the liverwort *Marchantia polymorpha*, a sister clade to vascular plants. Transcriptomic and metabolomic analyses under hypoxia and submergence identify both conserved and lineage-specific adaptations relative to angiosperms, providing insights into the origins and diversification of oxygen-sensing pathways.

Collectively, these chapters integrate physiological, molecular, and evolutionary perspectives to provide a cohesive understanding of oxygen sensing and adaptation in plants. This approach aims to improve our understanding of plant adaptation and lays the groundwork for strategies to enhance crop resilience under oxygen-limited conditions.

Chapter 2 – Hypoxia-activated fluorescent probes as markers of oxygen levels in plant cells and tissues

Commentary

Recent years have witnessed major progress in understanding the formation of hypoxic niches in plant tissues and their role in shaping specific physiological responses. A key driver of this progress has been the development of tools that enable the study of oxygen levels and plant responses with unprecedented spatial and temporal resolution. Chapter 2 highlights a critical technological advancement that directly supports the overarching goal of this thesis: to deepen our understanding of how plants sense and adapt to low-oxygen conditions.

In this chapter, novel hypoxia-activated fluorescent probes are applied in *Arabidopsis thaliana*, introducing a novel, non-invasive approach to visualise and quantify oxygen distribution within plant cells and tissues. This capability overcomes a longstanding limitation in plant hypoxia research, where accurate, spatially resolved oxygen measurements were constrained by invasive techniques or dependence on genetic biosensors.

The selective fluorescence of 4NB-Resorufin and MeIQ-Resorufin under hypoxic conditions allows the detection of both experimentally induced low-oxygen environments and naturally occurring hypoxic niches, such as lateral root primordia. These probes provide a powerful tool to map the dynamic oxygen landscape *in planta*, laying the foundation for mechanistic studies of oxygen sensing and signaling explored in later chapters. Notably, the ability of MeIQ-Resorufin to detect mild hypoxia enhances the resolution of oxygen gradients, enabling a finer understanding of how localized oxygen availability intersects with developmental cues and stress responses.

This technological innovation complements the molecular investigations presented later in the thesis. Moreover, by offering a versatile, chemical-based approach applicable across diverse tissues and potentially multiple species, these probes have significant applied value, including the development of strategies to improve plant tolerance to hypoxia-related stresses.

Overall, Chapter 2 bridges mechanistic insights with technological innovation, advancing the toolkit available to plant biologists and reinforcing the thesis' integrated approach to unravelling oxygen sensing and adaptation in plants.

Hypoxia-activated fluorescent probes as markers of oxygen levels in plant cells and tissues

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Summary

- Low-oxygen signaling in plants is important in development and stress responses. Measurement of oxygen levels in plant cells and tissues is hampered by a lack of chemical tools with which to reliably detect and quantify endogenous oxygen availability. We have exploited hypoxia-activated fluorescent probes to visualise low-oxygen (hypoxia) in plant cells and tissues.
- We applied 4-nitrobenzyl (4NB-) resorufin and methyl-indolequinone (MeIQ-) resorufin to *Arabidopsis thaliana* whole cells and seedlings exposed to hypoxia (1 % O₂) and normoxia (21 % O₂). Confocal microscopy and fluorescence intensity measurements were used to visualise regions of resorufin fluorescence.
- Both probes enter *A. thaliana* whole cells and are activated to fluoresce selectively in hypoxic conditions. Similarly, incubation with *A. thaliana* seedlings resulted in hypoxia-dependent activation of both probes and observation of fluorescence in hypoxic roots and leaf tissue. MeIQ-Resorufin was used to visualise endogenous hypoxia in lateral root primordia of normoxic *A. thaliana* seedlings.
- Oxygen measurement in plants until now has relied on invasive probes or genetic manipulation. Use of these chemical probes to detect applied and endogenous hypoxia has the potential to facilitate a greater understanding of oxygen concentrations in plant cells and tissues, allowing correlation of oxygen availability with acclimative and developmental responses to hypoxia.

Keywords: Bioreduction, Fluorogenic probes, Hypoxia, Oxygen-sensing, Resorufin

Introduction

Plants require oxygen for the production of ATP and other important biomolecules and rely on photosynthesis and diffusion or convection to deliver oxygen to cells and tissues. Regions of low-oxygen and oxygen gradients are common in plants (Weits *et al.*, 2021) due to high metabolic oxygen consumption, as well as physical barriers to diffusion. The latter arise from hydrophobic and waxy layers present at the surfaces of some tissues which prevent gas diffusion, including in legume root nodules where anaerobic conditions are critical for effective nitrogen fixation (Venado *et al.*, 2022), the cuticle and suberized cell layers in seeds in which low-oxygen is an important developmental cue (Borisjuk & Rolletschek, 2009; Langer *et al.*, 2023) and the deposition of suberin at the surfaces of adventitious roots formed to prevent radial oxygen loss (Peralta Ogorek *et al.*, 2024). For example, oxygen concentrations of <1 % are reported in seeds (Borisjuk & Rolletschek, 2009). Shoot apical meristems and lateral root primordia have metabolically-driven low O₂ tensions of ~3 % and <5 % O₂, respectively (Shukla *et al.*, 2019; Weits *et al.*, 2019) and O₂ gradients across *Ricinus communis* (castor bean) stems can range from 21 % at the surface to 7 % in the vascular tissue (van Dongen *et al.*, 2003). In addition to endogenous variations in O₂ tension, plants can be exposed to hypoxia (where oxygen is at a lower concentration than physiologically typical) through submergence (Bailey-Serres *et al.*, 2012) and pathogen infection; *Botrytis cinerea*-infected *Arabidopsis* leaves and crown gall tumours arising from infection of wounded tissue by *Agrobacterium tumefaciens* result in O₂ tensions of 2-5 % (Kerpen *et al.*, 2019; Valeri *et al.*, 2021).

Molecular pathways which drive adaptive responses to hypoxia or molecular outcomes of low-oxygen niches are well understood (Weits *et al.*, 2021), however, the intracellular oxygen concentrations which trigger these responses are not accurately known. To date it has been challenging to accurately quantify oxygen levels in plant cells and tissues non-invasively (Akter *et al.*, 2021): the most widely used method of oxygen measurement is the Clark-type electrode which measures oxygen concentrations at distinct locations (Geigenberger *et al.*, 2000; Colmer *et al.*, 2020). Mini-electrodes enable resolution to 10 µm, and can reveal cross-sections of O₂ gradients across tissues (Weits *et al.*, 2019), but the electrodes are invasive (thus creating physical damage) and also consume O₂ locally. Luminescence quenching-based probes have also been used but are similarly invasive, and can be impacted by other quenching agents (Papkovsky, 1993; Pedersen *et al.*, 2016; Mori *et al.*, 2019; Akter *et al.*, 2021). A number of biosensor cassettes have been applied to measure oxygen levels *in vivo* without this invasive treatment (reviewed in (Weits *et al.*, 2021)). These have exploited a heterologous mammalian O₂-sensing system to drive luciferase activity (Iacopino *et al.*, 2019) and used a hypoxia-responsive promoter element to drive expression of dual fluorescence proteins (Panicucci *et*

al., 2020). Although effective, these strategies require significant engineering and are not easily applicable to a range of plant tissues or species. There are no chemical probes reported to date applicable in plants for O₂ sensing. Since they do not require genetic manipulation or invasive procedures, chemical probes represent a promising system to investigate oxygen sensing in plant cells and shed light on physiological responses to these conditions. An ideal oxygen-sensing probe should have high sensitivity to hypoxia, potentially finely discriminating among oxygen levels, should be non-toxic, permeable to plant cells, detectable in plant systems, and potentially reversible to accurately reflect real-time oxygen dynamics. Considering that the structure of an elaborate cell wall strictly limits the uptake of large molecules in plants (Tepfer & Taylor, 1981), efficient tissue permeability needs to be tested for chemical probes. In the case of fluorescence-based chemical probes, avoiding fluorescence spectral overlap between probe and chlorophyll is also a prerequisite consideration for such O₂-sensing in plants.

Hypoxia-activated imaging agents have been used extensively in mammalian cell and tissue biology to visualise intracellular hypoxia *via* fluorescence or immunostaining (Nordsmark *et al.*, 2003; Koch, 2008; Elmes, 2016; Close & Johnston, 2022). These chemical tools exploit the oxygen-sensitive activity of reductase enzymes: in low-oxygen, these enzymes can catalyse bioreduction of redox sensitive functional groups to generate products that can be visualised. One such example is 4-nitrobenzene-resorufin (4NB-Resorufin); hypoxia-dependent bioreduction of 4NB-Resorufin results in cleavage of the 4NB group to release the fluorescent resorufin (**Figure 1**). The proposed mechanism for the bioactivation of resorufin-based probes in mammalian and bacterial systems relies on the fact that the attachment of 4-nitrobenzyl group to the phenol of resorufin results in a non-fluorescent compound. In normoxia, nitroreductase catalyses one electron reduction of the nitro group but the compound is rapidly back oxidized and the resorufin remains uncleaved; however, further reduction of the group occurs under hypoxic conditions leading to the release of the resorufin fluorophore (Collins *et al.*, 2018). Bioreductive moieties such as the nitroaryl groups, quinones, and *N*-oxides have been used in hypoxia activated prodrugs (HAPs) in mammalian systems (McKeown *et al.*, 1995; Sharma *et al.*, 2019; Skwarska *et al.*, 2021; Tosun *et al.*, 2023). Additionally, it was shown that isopropyl β-D-1-thiogalactopyranoside (IPTG) in conjugation with the 4-nitrobenzene moiety is inactive, but hypoxia-dependent activation of the molecule releases the IPTG that induces the expression of GFP protein in bacteria (Collins *et al.*, 2018).

We hypothesised that hypoxia-activated fluorescent probes could be applied to plant cells and tissues as a way to visualise low-oxygen conditions and thus contribute to a clearer understanding of oxygen signaling processes in plants. We therefore investigated two resorufin-based fluorogenic probes, 4NB-Resorufin and a recently reported methyl-

indolequinone-conjugated resorufin probe (MeIQ-Resorufin, which has been used in human cancer cell lines (Wallabregue *et al.*, 2023) to determine whether they could permeate plant cells and tissues to report on low-oxygen conditions. 4NB-Resorufin has been reported to permeate bacterial cells (Collins *et al.*, 2018), therefore we tested whether this probe might also be able to cross plant cell walls. MeIQ-Resorufin has recently been reported to be activated at higher oxygen concentrations than 4NB-Resorufin (Wallabregue *et al.*, 2023) therefore we also tested the possibility that this probe could be applied to plants, and in combination, the probes could differentiate oxygen concentrations in *Arabidopsis thaliana* cell and tissue cultures. Our results reveal that these hypoxia-activated probes are effective at visualising low-oxygen conditions (herein termed hypoxia) in this model plant, thus paving the way for an expanded toolkit in detecting and measuring hypoxic conditions in plants.

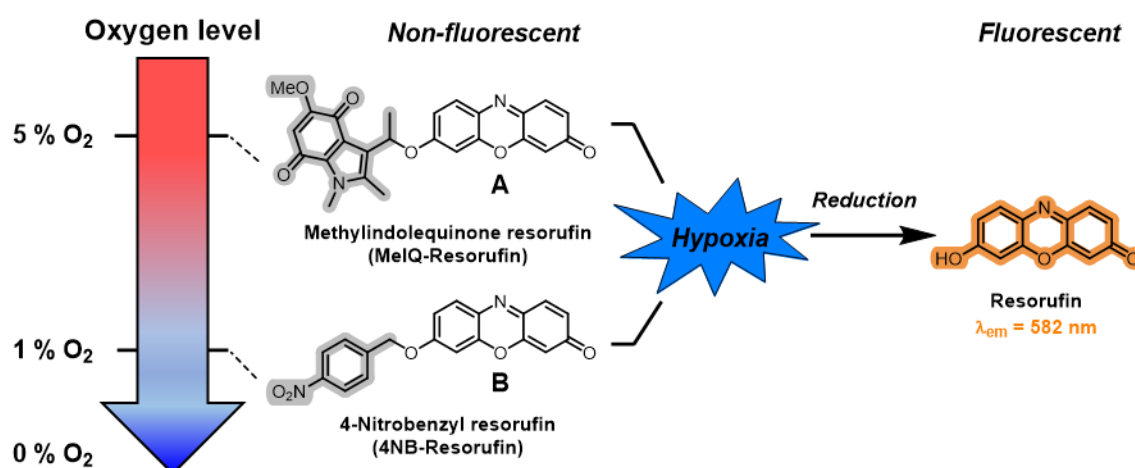


Figure 1 – Schematic representation of bioreductive hypoxia-activated chemical probes. Methylindolequinone resorufin (MeIQ-Resorufin, **A**) and 4-nitrobenzyl resorufin (4NB-Resorufin, **B**): reduction under hypoxic conditions liberates fluorogenic resorufin.

Results

4NB-Resorufin and MeIQ-Resorufin can permeate *Arabidopsis thaliana* whole cells and show hypoxia-dependent activation

We first set out to determine whether the 4NB- and MeIQ-Resorufin probes could permeate *Arabidopsis thaliana* cells. Four-day old whole autotrophic suspension-cultured cells were incubated with either 10 μ M probe or 1 % DMSO loading control prior to shaking in the dark for 2 hours under normoxia (21 % O₂) or hypoxia (1 % O₂). Cells were visualised using confocal microscopy to detect fluorescence arising from resorufin ($\lambda_{\text{ex/em}}$ 561/570-590 nm), chlorophyll ($\lambda_{\text{ex/em}}$ 561/650-750 nm) and propidium iodide (PI) ($\lambda_{\text{ex/em}}$ 488/604-622 nm). Propidium iodide (PI) staining was exploited for live/dead cell imaging, since this dye is not cell-permeable in intact membranes (Riccardi & Nicoletti, 2006).

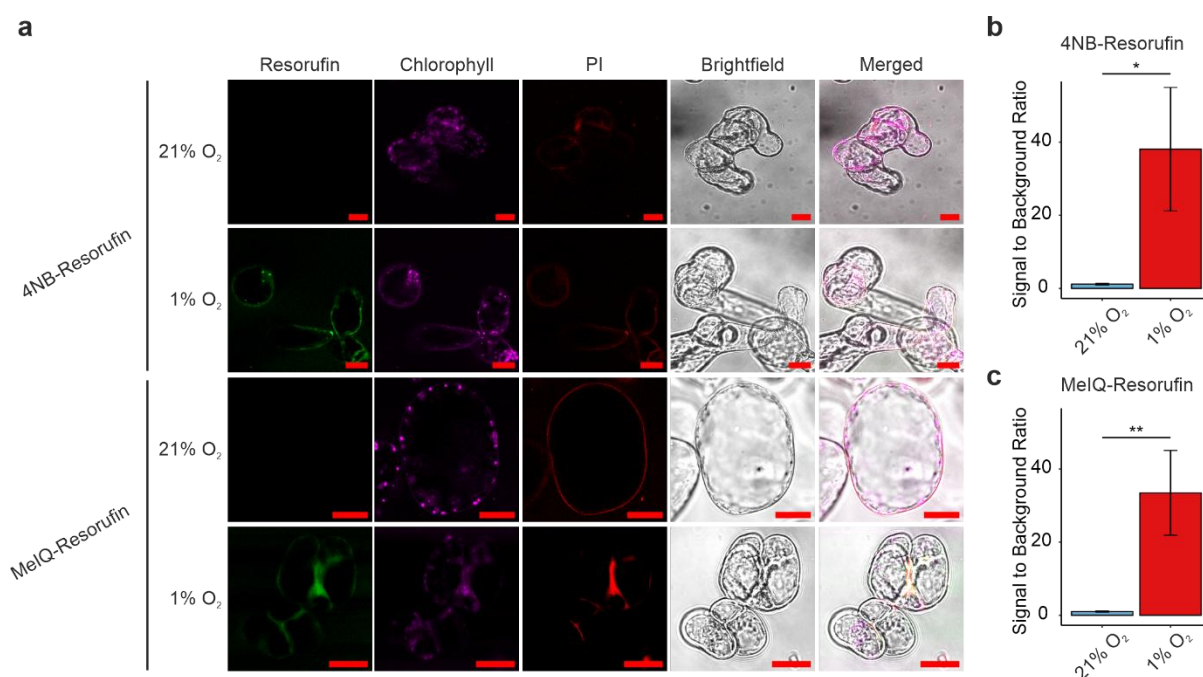


Figure 2 – Hypoxic incubation of *Arabidopsis thaliana* cells with 4NB-Resorufin and MeIQ-Resorufin results in fluorescence. (a) 1 mL *Arabidopsis thaliana* whole cells were incubated with 10 μ M 4-NB-resorufin or MeIQ-Resorufin at 21 % or 1 % O₂ for 2 hours. Cells were examined for fluorescence of resorufin (green, 570-590 nm), chlorophyll (purple, 650-750 nm) and propidium iodide (PI, red, 604-622 nm). Images show representative cells from ≥ 3 x wells containing 1 mL cells; scale bars: 20 μ m. (b, c) Quantification of resorufin fluorescence intensity in cells treated with 4NB- or MeIQ-Resorufin upon hypoxia or normoxia represented by the ratio of the signal intensity to the background ($n \geq 3$). Statistical significance was determined using two-sided Student's t-test (* p-value < 0.05, ** p-value < 0.01).

The arising images (**Figure 2a**, **Figure S1**) and signal quantification (**Figure 2b**) show that when cells were incubated with either 4NB-Resorufin or MeIQ-Resorufin in hypoxic conditions, significant increase in fluorescence in the 570-590 nm range was observed in cytosolic regions of the cells, consistent with resorufin fluorescence (**Figure 2b, c**). No fluorescence was observed at this wavelength range when cells were incubated with either 4NB-Resorufin or MeIQ-Resorufin in normoxia (**Figure 2a**). Similarly, no fluorescence was observed if cells were incubated in hypoxia and treated with 1 % DMSO only, nor if either probe was incubated in MS media, in hypoxia and in the absence of cells (**Figure S2**). Chlorophyll autofluorescence was also observed in the presence of both 4NB-Resorufin and MeIQ-Resorufin and when cells had been incubated in both normoxia and hypoxia. PI fluorescence was used to demonstrate that the probes were entering cells and that the cells remained viable. PI fluorescence emission was only observed at cell surfaces, indicating the probes were not toxic to the cell under the conditions tested. PI staining was then used to determine the proportion of dead or damaged cells as a function of increasing concentrations of each probe (**Figure S3a, b**). Across the different concentrations tested, there was no significant increase in PI staining with increasing concentration of probe, relative to DMSO control. There was some increase in PI staining in hypoxic control cells compared to normoxic control cells, although this was not significant. Overall, the results indicate that, under the conditions used, the presence of the probes does not cause significant damage to the cell membranes and is no more damaging to cells than hypoxia itself.

The results therefore indicate that both probes are able to permeate Arabidopsis whole cells and enter the cytoplasm. The observed fluorescence in hypoxia but not in normoxia indicates that the probes have been bioreductively cleaved to form resorufin in a manner that is dependent on a sustained decrease in oxygen availability. This is consistent with the bioreductive properties of these probes in bacterial and mammalian systems (Collins *et al.*, 2018; Wallabregue *et al.*, 2023). Although the reductase enzyme(s) responsible for bioreduction of the probes have not been identified in plants, the same probes are reduced by nitroreductase and CYP450 enzymes in mammalian and bacterial cells (O'Connor *et al.*, 2016; O'Connor *et al.*, 2017; Collins *et al.*, 2018; Wallabregue *et al.*, 2023), suggesting that similar enzymes may be enabling probe activation in plant cells. These probes are therefore able to detect whether whole plant cells grown in culture are hypoxic.

Quantitative fluorescence detection of hypoxia-dependent bioreduction of 4NB-Resorufin and MeIQ-Resorufin in a plate-based format

We next wanted to determine whether the probes could be exploited to detect cellular hypoxia in a higher-throughput plate-based format by measuring fluorescence intensity, and whether

such an assay can be used to quantify fluorescence levels as a function of O_2 concentration. By exposing cells to different concentrations of O_2 we also wanted to determine whether the bioreduction and probe fluorescence were the same for both probes. 1 mL of autotrophic *A. thaliana* four-day old cell cultures were transferred to 12-well plates and incubated for 2 hours in a plate reader where the atmospheric environment was controlled at concentrations of O_2 ranging from 0.2 % to normoxia (21 % O_2). Plates were kept in dark conditions throughout the experiment to avoid O_2 production through photosynthesis, therefore glucose was added to the culture medium to maintain cells in a heterotrophic state. Fluorescence intensity was measured over time for each condition (**Figure 3a, b**) and average intensity (from 15-120 minutes) was used to determine overall fluorescence levels (**Figure 3c, d**) at each O_2 concentration.

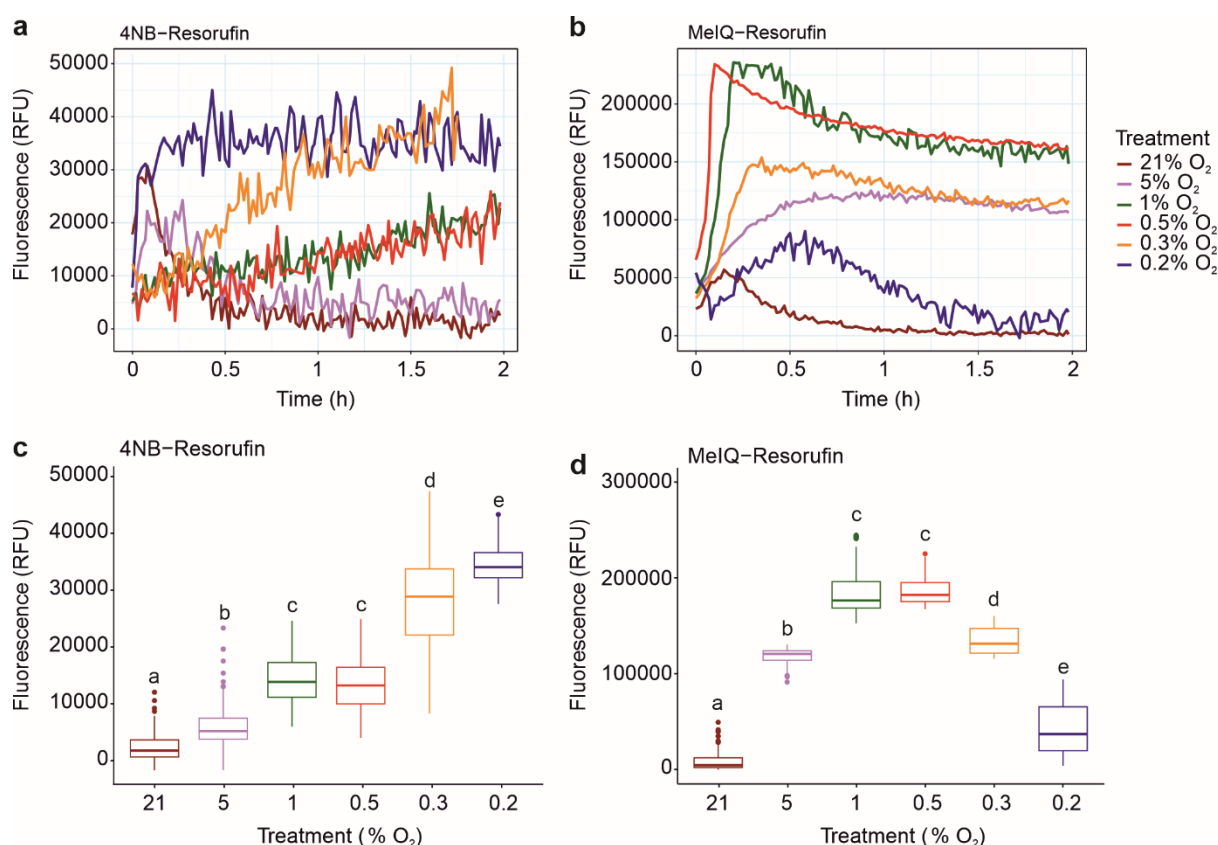


Figure 3 – Fluorescence intensity of bioreduced probes varies with O_2 concentration. Hypoxic incubation of *Arabidopsis thaliana* cells with 4NB-Resorufin (**a**) or MeIQ-Resorufin (**b**) shows variation in O_2 -dependent activation of fluorescence with time. Averaged fluorescence intensity (across the duration of 15-120 minutes) shows a difference in the O_2 concentration at which the most intense signal is achieved for 4NB-Resorufin (**c**) and MeIQ-Resorufin (**d**). In all experiments, 1 mL *Arabidopsis thaliana* whole cells were incubated with 10 μ M of the probes at varying O_2 concentrations. Data in boxplots (**c**) and (**d**)

were taken from ≥ 3 independent experiments; one-way ANOVA analysis followed by Tukey HSD *post-hoc* test revealed that fluorescence intensity was significantly different from that measured in normoxia in all cases (letters indicate statistical differences between groups, p -value < 0.001).

As detected using microscopy, resorufin fluorescence was observed when cells were equilibrated with both probes in hypoxia compared to cells exposed to normoxia. Cells incubated with 4NB-Resorufin at O_2 concentrations of 1 % and below showed a sustained increase in fluorescence over the course of 2 hours; when the O_2 concentration was 0.2 %, a maximum and sustained level of fluorescence was observed after approximately 15 minutes (**Figure 3a**). There was an overall increase in fluorescence intensity as O_2 concentration reduced from 1 % to 0.2 % (**Figure 3c**). When cells were incubated with MeIQ-Resorufin, a more rapid increase in fluorescence was observed at all O_2 concentrations measured except 21 % (**Figure 3b**). The absolute levels of fluorescence attained appeared to be dependent on O_2 concentration, with highest levels of fluorescence observed for 1 % and 0.5 % O_2 (**Figure 3d**). This fluorescence was not sustained, however, and over the course of 2 hours, fluorescence levels decreased, most notably in the presence of 0.2 % O_2 .

MeIQ-Resorufin derived fluorescence appeared to be detected at higher O_2 concentrations compared to fluorescence observed in the presence of 4NB-Resorufin at equivalent O_2 levels (**Figure 3b, d**), consistent with comparison of these probes in mammalian cells (Wallabregue *et al.*, 2023), suggesting MeIQ-Resorufin may be capable of detecting milder hypoxia than 4NB-Resorufin. However, absolute levels of fluorescence were approximately 5-fold higher in the presence of MeIQ-Resorufin compared to 4NB-Resorufin, which may reflect greater uptake of this probe into the cell, faster intracellular diffusion or a greater propensity for MeIQ-Resorufin bioreduction in the *A. thaliana* cell environment. In line with this, the observed decline in fluorescence signal with time for MeIQ-Resorufin suggests it is sequestered more rapidly: resorufin fluorescence is quenched by acidic conditions (pH 6.5 and below) (**Figure S4**) suggesting the decline in fluorescence could be a result of vacuolar sequestration of high levels of resorufin derived from the MeIQ-conjugated probe. Alternatively, resorufin fluorescence quenching could derive from reduced intracellular pH associated with lactate fermentation under hypoxia (Wagner *et al.*, 2019). Overall, the results indicate that the probes are capable of detecting intracellular hypoxia in a semi-quantitative manner in a plate-based assay, thus extending their utility as hypoxia-sensing probes.

4NB-Resorufin and MeIQ-Resorufin can detect hypoxic conditions in *Arabidopsis thaliana* seedling tissues

To confirm the biological relevance of 4NB- and MeIQ-Resorufin as hypoxia probes, we next sought to determine whether cell permeability and bioreduction of the probes could be observed in *A. thaliana* whole seedlings under hypoxic conditions. We anticipated that additional incubation time would be required for diffusion and uptake of probe in seedlings compared to cells; given we also observed a decrease in resorufin fluorescence in whole cells after prolonged incubation with MeIQ-Resorufin at low O₂ concentrations, we first determined whether a stable fluorescence signal could be observed in seedlings incubated with 4NB- and MeIQ-Resorufin at severe hypoxia (0.1 % O₂) over the course of 6 hours. The fluorescent signal intensity was not strong in cultured cells (**Figure 2**) therefore we increased the probe concentration in these experiments to 100 µM to ensure adequate signal intensity in a system with more complex tissue structure (including potential barriers to uptake). The arising data (**Figure S5**) revealed a time-dependent increase in resorufin fluorescence intensity over 6 hours, suggesting that the probes were sufficiently stable to elicit a detectable signal in the whole-seedling environment.

We therefore proceeded to examine seedling tissues exposed to normoxia (21 % O₂) or hypoxia (1 % O₂) by confocal microscopy following incubation with both probes (Fig. 4). When seedlings were incubated with 4NB-Resorufin (**Figure 4(I)**), hypoxia-dependent fluorescence was observed in root tissue (**Figure 4(I)a, b**) and leaves (**Figure 4(I)c, d**). In the root tissue, fluorescence was observed in the cortex and vascular tissue; in the leaf tissue, fluorescence was observed, albeit not homogeneously. Overall, these images indicate that 4NB-Resorufin was able to permeate the seedling tissue and translocate through the plant, likely *via* the vasculature. MeIQ-Resorufin was similarly able to permeate seedling tissue and fluoresce in a hypoxia-dependent manner. MeIQ-Resorufin derived fluorescence was observed in vascular regions of the root and near-homogeneously in the leaf tissue (**Figure 4(II) a-d**). These data suggest translocation of the probe throughout the plant tissue including to the leaf mesophyll layer. As observed in cells, a significant increase in fluorescence was only observed in the presence of either probe (and hypoxia) (**Figure 4e, f**); seedlings treated with equivalent volumes of DMSO did not show fluorescence at 570-590 nm in either hypoxia or normoxia, while chlorophyll autofluorescence was observed in these seedlings in leaf tissue (**Figure S6**).

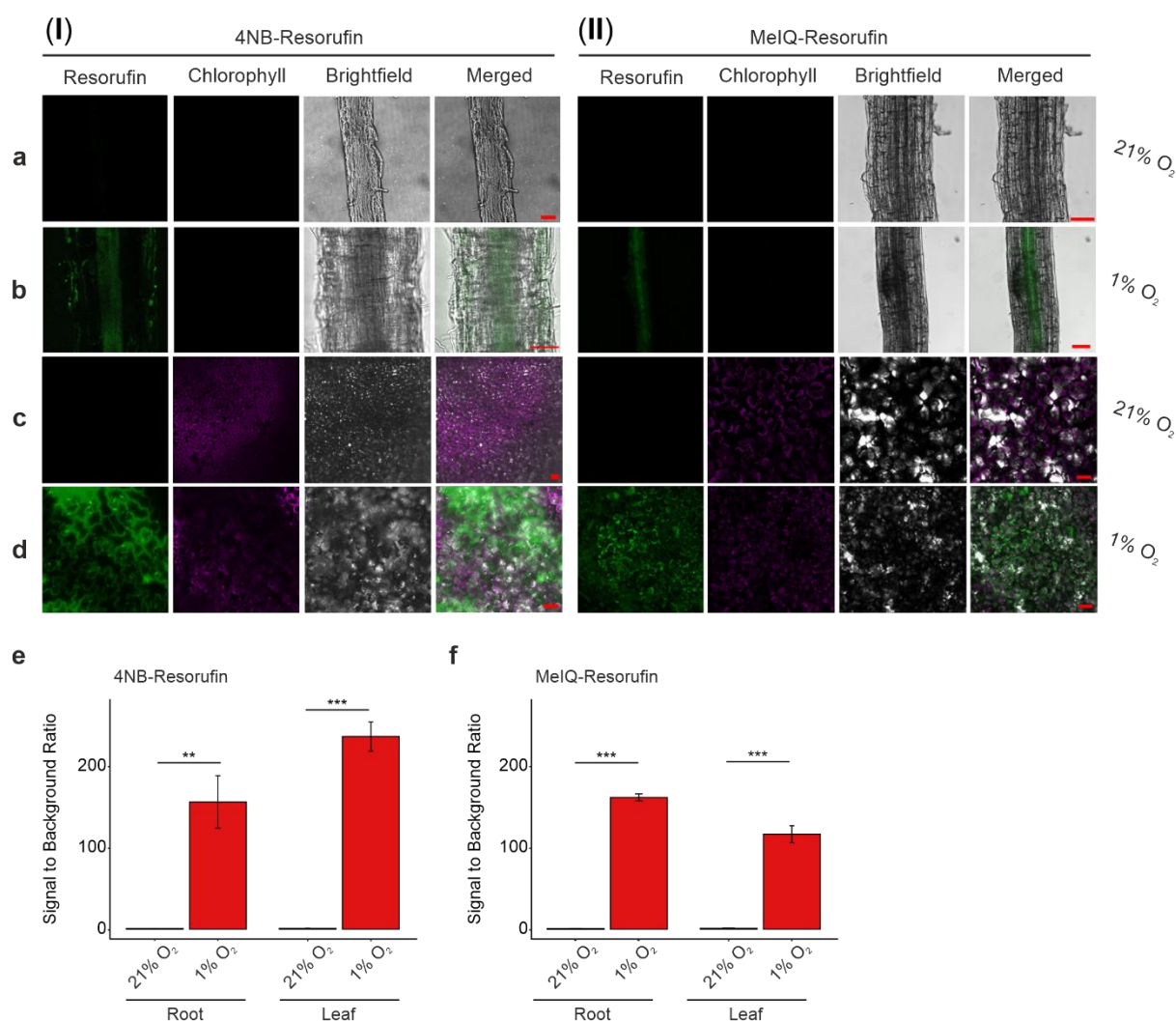


Figure 4 – Biochemical hypoxia-responsive chemical probes show fluorescence in hypoxic tissue of Arabidopsis. Two-week old *Arabidopsis* seedlings were incubated with **(I)** 4NB-Resorufin or **(II)** MeIQ-Resorufin for 6 hours in normoxic (21 % O₂) or hypoxic (1 % O₂) conditions. The live root (**a**, **b**) and leaf (**c**, **d**) of the seedlings were imaged using a confocal (Zeiss LSM880/LSM780) microscope using a 561 nm laser to excite and fluorescence of the resorufin measured at 570 nm to 600 nm (green) as well as chlorophyll autofluorescence measured at 650 nm to 750 nm (purple). The leaf tissue experiments in panel **(I)** were performed with 50 μ M 4NB-Resorufin, otherwise 100 μ M probes used. Scale bars: 50 μ m. (**e**, **f**) Quantification of resorufin fluorescence intensity in seedlings treated with 4NB- or MeIQ-Resorufin upon hypoxia or normoxia represented by the ratio of the signal intensity to the background ($n = 3$). Statistical significance was determined using two-sided Student's t-test (** p-value < 0.01, *** p-value < 0.001).

To confirm that seedling intracellular O₂ concentrations were hypoxic under the experimental hypoxia settings, we employed an *A. thaliana* line containing a luciferase reporter cassette

under the control of the hypoxia-responsive promoter element (HRPE). This HRPE is known to promote ERFVII-mediated gene upregulation under hypoxic conditions (Gasch *et al.*, 2016; Akter *et al.*, 2024). When seedlings were exposed to normoxia or hypoxia under the experimental conditions used for probe incubation, we detected a significant difference in luciferase activity, commensurate with elevated luciferase expression in hypoxia (**Figure S7**), confirming that the seedlings were experiencing a hypoxic environment.

We next checked whether incubation of seedlings with the probes could affect plant growth. Seven-day old *Arabidopsis* seedlings were treated with 100 μ M 4NB-Resorufin, MeIQ-Resorufin or resorufin (**Figure S8**). No treatment, or treatment with 10 % DMSO, were used as negative controls. Physiological measurements were taken after four days of the treatment, during which seedlings were kept in 16:8 photoperiod conditions, in air. Consistent with our results for cell culture, we did not observe any apparent phenotypic differences between seedlings treated with the probes compared to the DMSO negative controls (**Figure S8a**). No statistically significant differences were measured in primary root length, growth rate and fresh weight compared to DMSO-treated seedlings, albeit the comparison with untreated seedlings indicates a mild negative effect due to DMSO (**Figure S8b, c, d**). These results suggest, therefore, that 4NB-resorufin, MeIQ-Resorufin and their reduced form do not affect biomass or root growth in *Arabidopsis*, in the conditions used in this study. Collectively, these results demonstrate that the probes are capable of acting as fluorescent markers of hypoxia in live plant tissue as well as in cells.

MeIQ-Resorufin can detect endogenous hypoxia in *Arabidopsis thaliana* root lateral primordia

Finally, we sought to determine whether these probes could be used to detect physiologically relevant hypoxia in an otherwise normoxic environment. *Arabidopsis* root lateral primordia represent a physiological hypoxic niche, arising due to rapid cell division and high metabolism and demonstrated by localised ERF-VII stabilisation and hypoxic-response gene upregulation (Shukla *et al.*, 2019). We tested whether MeIQ-Resorufin could detect hypoxia in root lateral primordia by incubating seven-day old *Arabidopsis* seedlings with 100 μ M probe for 2 hours in either normoxic or hypoxic conditions. We selected to use MeIQ-Resorufin for this experiment given the higher levels of fluorescence observed for this probe in the whole cell cultures. Under normoxic conditions, MeIQ-Resorufin derived fluorescence was not typically observed in primary root tissue, as expected (**Figure 4**), while fluorescence was consistently observed in root lateral primordia regions at all stages of development (**Figure 5, Figure S9**). When these tissues were incubated in hypoxia, fluorescence was usually observed in all regions of the root, including the lateral primordia (**Figure 5, Figure S9**), consistent with previous

experiments. These results confirm that the MeIQ-Resorufin probe can be used to visualise regions of hypoxia in otherwise normoxic *Arabidopsis* tissues.

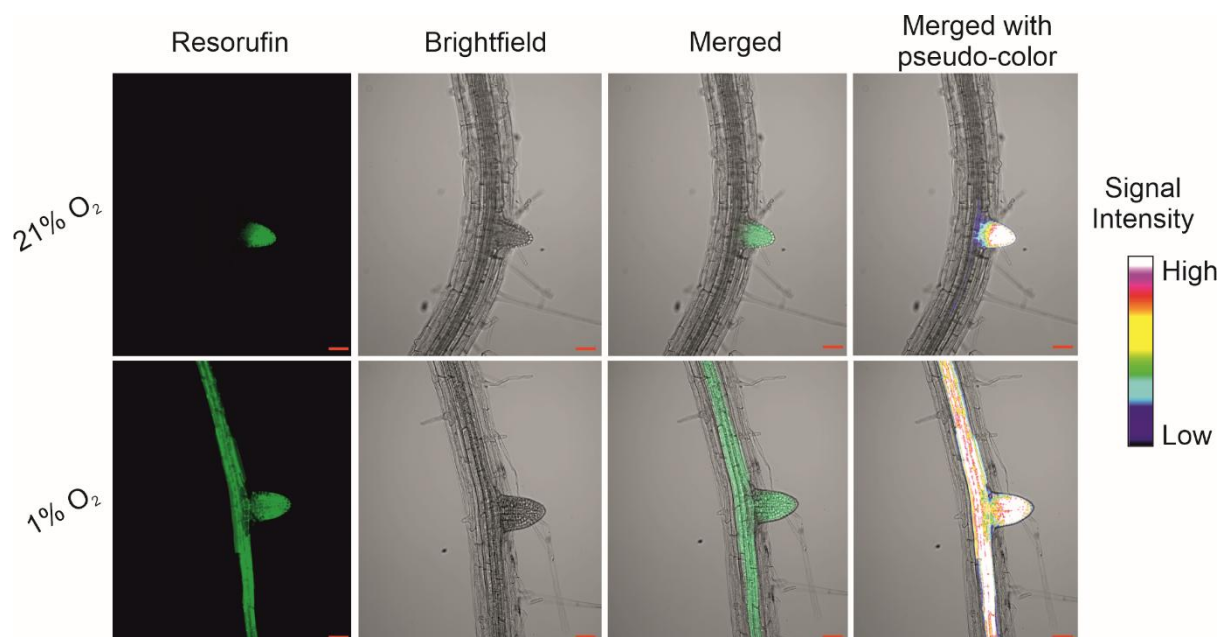


Figure 5 – Bioreductive hypoxia-responsive chemical probes show fluorescence in hypoxic tissue of *Arabidopsis*. Seven-day old seedlings were incubated with 100 μM MeIQ-Resorufin for 2 hours in normoxic (21 % O_2 v/v) or hypoxic (1 % O_2 v/v) environments. MeIQ-Resorufin fluorescence was imaged using a Zeiss LSM800 Airyscan laser scanning confocal microscope with 561 nm excitation laser light and 565-616 nm emission detection. Scale bars 50 μm ; signal intensity bar refers to pseudocolour.

Discussion

The hypoxia-sensing fluorescent probes 4NB-Resorufin and MeIQ-Resorufin are capable of being reduced under hypoxic conditions to release fluorescent resorufin, both *in vitro* and in bacterial and mammalian cells (Collins *et al.*, 2018; Wallabregue *et al.*, 2023). In this work, we have shown that these probes are also capable of being used to detect hypoxic conditions in plant cells, specifically the model plant *Arabidopsis thaliana*. Moreover, we were able to demonstrate that the probes can also be applied to *Arabidopsis thaliana* seedlings and fluoresce exclusively in hypoxia, both when it is exogenously applied (1 % O_2) as well as endogenous hypoxia which exists in rapidly metabolising lateral root primordia. Normoxic tissues remained non-fluorescent. This work demonstrates that hypoxia-sensitive bioreductive enzymes must be present and active in the plant cells and tissues examined, similar to those in other kingdoms (Elmes, 2016; Cenas *et al.*, 2021) in order to result in release of fluorescent resorufin. We cannot rule out that transport dynamics, diffusion barriers, metabolism or other factors affect the homogeneity of probe distribution in seedlings, and

would therefore recommend interpreting relative intensities of fluorescence in seedling tissues with caution. Indeed, distribution of each probe may differ due to their differing structures. This may explain why MeIQ-Resorufin derived fluorescence is observed only in root vascular tissue (known to be most susceptible to hypoxic conditions (Armstrong *et al.*, 1994; Zabalza *et al.*, 2008; Colmer *et al.*, 2020)), while 4NB-Resorufin derived fluorescence can also be observed in cortical cells. Further studies are necessary to provide detailed information that correlates probe properties with distribution characteristics, particularly in tissues with internal barriers to diffusion.

We were also able to measure probe-derived fluorescence intensity in a plate-based format. Here, the use of whole cell cultures reduces issues of homogeneous probe distribution and also enables higher-throughput analyses that correlate fluorescence intensity with O₂ availability. However, this was dependent on which probe was used: MeIQ-Resorufin derived fluorescence was significant at 5 % O₂, in contrast to fluorescence derived from 4NB-Resorufin at the same O₂ concentration. This indicates that MeIQ-Resorufin is able to detect ‘mild’ hypoxia (5 % O₂ to 0.5 % O₂) in contrast to 4NB-Resorufin which gave significant fluorescence values at 1 % O₂ and below. The mild hypoxia-dependent response of MeIQ-Resorufin is consistent with the hypoxia-sensitivity results observed for this probe in mammalian A549 lung cancer cells and HCT116 colorectal cancer cells spheroids (Wallabregue *et al.*, 2023). In these systems MeIQ-Resorufin begins producing significant fluorescence at 4 % O₂, where a 5-fold increase compared to normoxic controls was observed after 2 hours incubation. Fluorescence increase further intensifies at lower oxygen concentrations with a maximum of 30-fold increase at <0.1 % O₂ compared to normoxic controls. In this study, the overall fluorescence levels derived from MeIQ-resorufin were approximately 5-fold higher than those from 4NB-resorufin. Time-course experiments conducted in *Arabidopsis thaliana* whole plants and cell lysates at 0.1 % O₂ further highlight these differences (**Figure S5** and **Figure S10**): MeIQ-Resorufin exhibited a significant hypoxia-induced fluorescence increase, with 134-fold and 157-fold enhancements compared to the DMSO control in plant cell lysates with and without protease inhibitor cocktails, respectively, after 100 minutes. Similarly, a 196-fold increase was observed in whole plants after 360 minutes. In contrast, 4NB-Resorufin showed only modest fluorescence increases under the same conditions, with 1.6-fold and 1.7-fold increases in cell lysates with and without protease inhibitor cocktails, respectively, and a 2-fold increase in whole plants compared to the DMSO control. Notably, the fluorescence increases between the two probes differed significantly, with 117-fold and 59-fold higher activation for MeIQ-Resorufin in plant cell lysates without protease inhibitor cocktail and whole plants, respectively. These findings clearly demonstrate that the differences in activation are predominantly due to the inherently higher bioreductive reactivity of the indolequinone group

compared to the 4NB-benzyl group, though a minor contribution from probe uptake cannot be excluded.

In hypoxic mammalian cells, bioreduction of MeIQ-resorufin is rapid (Wallabregue *et al.*, 2023) which aligns with the observed higher fluorescence derived from MeIQ-resorufin than 4NB-Resorufin in hypoxic plant cells. Notably in cell cultures, the high levels of fluorescence observed with MeIQ-resorufin were not stable over time, with decreases in fluorescence being observed at low O₂ (< 0.5 % O₂) concentrations. This may be due to acid-quenching of resorufin fluorescence (**Figure S4**), either in mildly acidic conditions associated with hypoxia in cytosol (Felle, 2005), or to vacuolar xenobiotic compartmentalisation (Coleman *et al.*, 1997), where pH levels will likely lead to a significant decrease in fluorescence. Despite the observation of this effect in whole cells, MeIQ-derived resorufin fluorescence showed prolonged stability in tissues which may reflect improved tolerance of hypoxia-induced metabolic acidosis in tissues compared to isolated whole cells (Felle, 2005). Importantly, the ability of MeIQ-resorufin to detect mild hypoxia with relatively high signal intensity enabled its use for the detection of endogenous hypoxia in lateral root primordia and may make this the most practical of the two probes for future use *in planta*.

The probes themselves are not significantly bioreduced in the presence of reactive oxygen species (**Figure S11** and **Figure S12**), which are known to be elevated in prolonged hypoxia (Jethva *et al.*, 2023). However, they are susceptible to prolonged photobleaching and high concentrations of NaCl (**Figure S13** and **Figure S14**), so care should be taken in choosing conditions for their application. It is also important to note that bioreduction of these probes is irreversible, so they are not able to indicate real-time oxygen dynamics; development and application of reversible oxygen probes in plants would be of significant interest (Takahashi *et al.*, 2012; Zhang *et al.*, 2021). Nevertheless, the bioreductive ability of these chemical probes to detect and stain for hypoxic conditions in plant cells and tissue will be a valuable and timely addition to the suite of techniques available to measure O₂ availability in the field of plant hypoxia research, both with respect to acute hypoxia (as an environmental stress) and chronic hypoxia (as a developmental cue). They can be applied without the requirement for genetic engineering of biosensors or the challenging aspects of probe measurement (including tissue damage and handling challenges) (Weits, 2021). A summary of the properties we observed for each probe in *A. thaliana* cells and seedlings can be found in Table **S1**; determination of probe permeability and hypoxia-activation in crop plants will expand their utility. Furthermore, the inference that bioreductive enzymes are present and active in Arabidopsis cells indicates that the same principle of bioreduction could be used to trigger hypoxic activation of other molecules, a principle which has been exploited in mammalian hypoxia biology (Collins *et al.*, 2018) and which may enable intervention in a range of situations where hypoxia is relevant.

Materials and Methods

MeIQ-Resorufin and 4NB-Resorufin synthesis

MeIQ-Resorufin and 4NB-Resorufin were synthesised as described previously (Wallabregue *et al.*, 2023). To yield MeIQ-Resorufin, diisopropyl azodicarboxylate was added dropwise to a solution of 3-(1-hydroxyethyl)-5-methoxy-1,2-dimethyl-1*H*-indole-4,7-dione, resorufin and triphenylphosphine in anhydrous tetrahydrofuran at room temperature under argon. The reaction mixture was stirred vigorously for 24 hours at 50 °C, then concentrated under reduced pressure. The resulting residue was dissolved in ethyl acetate, washed with aqueous sodium hydrogen carbonate, water, aqueous hydrochloric acid (1 M solution), brine, dried (sodium sulfate), filtered, and concentrated under reduced pressure. The crude material was purified using silica gel flash column chromatography. The residue was dissolved in ethyl acetate and precipitated by adding hexane. The precipitation procedure was repeated twice. The precipitate was then dried under reduced pressure to give the title compound as an orange solid. The timescale for this synthesis is approximately three weeks.

To prepare 4NB-Resorufin 4-nitrobenzylbromide in *N,N*-dimethylformamide was added to a solution of resorufin and potassium carbonate, stirred at 40 °C for 3 hours, then cooled to room temperature. The solution was diluted with ethyl acetate and washed with brine. The aqueous layer was extracted with ethyl acetate. The organic components were combined, dried (sodium sulfate), filtered and concentrated under reduced pressure. The crude residue was recrystallized from dichloromethane, followed by trituration with diethyl ether to yield the title compound as a red solid. Further details are provided in the Supplementary Information. All the probes were dissolved in DMSO to reach a stock concentration of 1 mM and stored at -20 °C, in black Eppendorf tubes to avoid light penetration. The timescale for this synthesis is approximately 1-2 days.

Plant materials and growth conditions

A. thaliana Columbia-0 (Col-0) ecotype was used for seedlings. For *in vitro* propagation, sterilised seeds were cultivated on half-strength Murashige & Skoog (MS) medium (Murashige & Skoog, 1962) supplemented with 1 % (*w/v*) sucrose and 0.8 % (*w/v*) agar, and grown vertically at 22°C, 16:8 day:night photoperiod 100 $\mu\text{mol photons m}^{-2}.\text{s}^{-1}$. Two-week old seedlings were used for the experiments. *A. thaliana* Landsberg erecta ecotype was used for whole cell cultures; these were propagated in full-strength MS medium, supplemented with 3 % (*w/v*) glucose, 2.5 $\mu\text{g/mL}$ 1-naphthaleneacetic acid (NAA), 0.25 $\mu\text{g/mL}$ kinetin adjusted to pH 5.8, and renewed weekly. Experiments were performed with cells from cultures which had been growing for four days following sub-culture.

Probe incubation

For incubation with cell cultures, 1 mL of four-day old cell culture was incubated with a final concentration of 10 μ M of probe, for two hours (based on studies with similar probes in mammalian cells (Wallabregue *et al.*, 2023) as well as time course studies of fluorescence intensity in the presence of each probe, Fig. 3). 1 mL of cell cultures incubated with 1 % DMSO and 1 mL of MS incubated with either 1 % DMSO or 10 μ M of probe were used as controls. For seedlings treatment, two-week old *A. thaliana* seedlings were incubated with 1 mL of liquid MS medium supplemented with 100 μ M of probe unless otherwise mentioned, for six hours, to allow time for tissue entry and diffusion. Seedlings incubated with 10 % DMSO, MS incubated with the same amount of probe or DMSO, were used as negative controls. All the experiments were performed using 12-well plates, with three biological replicates per plate, unless otherwise stated. Each biological replicate is represented by 1 mL of Arabidopsis cell suspension or one two-week old seedling for the cell culture and seedling experiments, respectively.

Hypoxia treatment

All the hypoxic treatments were conducted using a CLARIOstar® plate reader (BMG LabTech) supplemented with an Atmospheric Control Unit (ACU) to regulate O₂ gas level. Probe sensitivity to hypoxia was evaluated at 5, 1, 0.5, 0.3 and 0.2 % O₂, using the ACU, flushed with N₂ (g), for two hours (cell culture treatment) or six hours (seedlings), in the dark; the ACU was turned off for controls in normoxic conditions. Plates were shaken at 100 rpm every 30 seconds before fluorescence detection. For each cycle, 20 readings per well were taken and the mean was calculated. Resorufin was detected upon excitation at 550 nm and emission at 585 nm, using a dichroic filter of 567.5 nm. Two-week old *A. thaliana* Col-0 seedlings expressing a HRPE:nLUC construct (Akter *et al.*, 2024) were used for *in vivo* luciferase detection upon treatment under normoxic or hypoxic conditions (1 % O₂ *v/v* O₂/N₂), for one hour in the dark, using the CLARIOstar® plate reader (BMG LabTech). Four biological replicates were employed in the experiment and wild-type genotype was used as control. Luminescence was detected using the Nano-Glo® Vivazine™ live cell substrate (Promega), following the manufacturer's instructions. Measurements were collected for 20 minutes, 7 measurements per minute were taken and the mean was calculated.

Confocal imaging

Four-day old *A. thaliana* cell cultures and two-weeks old seedlings were used for probe detection after hypoxic treatment (1 % O₂ *v/v* O₂/N₂) for two hours (cell culture) or six hours (seedlings). For propidium iodide (PI) staining, cell cultures were incubated with 10 μ g/mL of PI solution for 5 minutes, then mounted on slides. Imaging was performed using either ZEISS

LSM 780 (Micron, Department of Biochemistry, University of Oxford) or ZEISS LSM880 Airyscan microscope (Department of Biology, University of Oxford), equipped with 25 x objective lens, upon laser excitation at 561 nm and collection at 570-590 nm for Resorufin imaging, excitation at 488 nm and collection at 604-622 nm for PI, excitation at 561 nm and collection at 650-750 nm for chlorophyll. For lateral root primordia, *A. thaliana* seedlings were grown on half-strength MS supplemented with 0.5 % sucrose for seven days, then incubated with 100 μ M MeIQ-Resorufin for 2 hours in 6-well plates in normoxic (21 % O₂) or hypoxic (1 % O₂) conditions. After 10 minutes washing in distilled water, seedlings were imaged using a Zeiss LSM800 Airyscan laser scanning confocal microscope (University of Utrecht) with 561 nm excitation laser light and 565-616 nm emission detection. Fluorescence intensity was calculated using ImageJ software (Schindelin *et al.*, 2012). Six measurements were taken for each image, divided between background and signal (three measurements per category). The signal intensity was quantified as the ratio of the mean signal measurement to the mean of the background measurements for each image. This process was repeated for three images per probe, under each experimental condition.

Root Length and Fresh Weight Measurements

To measure plant tolerance to the probe, *A. thaliana* seedlings were grown vertically on squared plates for seven days and then treated with 100 μ M 4NB-Resorufin, MeIQ-Resorufin or resorufin, or 10 % DMSO as negative control, under air and 16:8 photoperiod conditions. After four days of treatment, plates were scanned using EPSON Perfection V750 PRO scanner with a resolution of 720 dots per inch. Primary root length was assessed using ImageJ (Schindelin *et al.*, 2012). 21-28 biological replicates were used for root length measurements. Growth rate per day was assessed as the increase in length of the primary root after four days of treatment compared to beginning of treatment. Fresh weight was measured after four days of treatment using four biological replicates, each consisting of seven whole seedlings.

Spectroscopy

Fluorescence spectra were recorded using a HORIBA Jobin Yvon FluoroLog3 fluorimeter (Hamamatsu R928 detector and a double-grating emission monochromator). The standard conditions for acquiring emission and excitation spectra are room temperature and steady-state mode unless otherwise stated. Fluorescence spectra were obtained by using GraphPad Prism software.

UV-visible spectra were recorded on a V-770 UV-Visible/NIR Spectrophotometer equipped with Peltier temperature controller and stirrer using disposable polystyrene cuvettes of 1 cm path length. Experiments were conducted at 25 °C unless otherwise stated. UV-visible spectra were plotted using GraphPad software.

pH measurements of solutions were taken using an Oakton pH meter.

Reactive oxygen species preparation and assays

General procedure for hydrogen peroxide (H₂O₂) assay:

To a quartz cuvette on ice (0–4 °C) containing Milli-Q water and H₂O₂ solution, a probe solution was added. The cuvette was sealed and left at room temperature for 5 minutes. At this point, the final concentrations of H₂O₂ and the hypoxia-imaging probe were 100 μM and 1 μM, respectively. The fluorescence spectra were then measured using the specified parameters at the given time point. Further details are provided in the Supplementary Information.

General procedure for hydroxyl radical (HO•) assay:

Preparation

The hydroxyl radical (HO•) was generated using the Fenton reaction. Ferrous chloride (1 eq.) was added to a solution of H₂O₂ (10 eq.). Upon mixing, the solution turned orange and was used immediately for analysis.

Assay

To a quartz cuvette on ice (0 – 4 °C) containing Milli-Q water and generated HO• solution, probe solution was added. At this stage, the final concentrations of HO• and the hypoxia-imaging probe were 100 μM and 1 μM, respectively. The solution was homogenized by pipetting for 10 seconds, then the cuvette was sealed and left at room temperature for 5 minutes. Fluorescence spectra were measured under the specified parameters. Further details are provided in the Supplementary Information.

General procedure for light stability assay:

To a vial containing DMSO, a probe solution was added. An aliquot was taken from the reaction solution and transferred to a quartz cuvette, followed by the addition of Milli-Q water. The solution was homogenized by pipetting, then the fluorescence spectra were recorded using the specified parameters, serving as the initial time point (T = 0, where T represents time). The vial was then sealed and placed in an aluminum box, positioned 20 cm away from a white LED and illuminated for 4 hours. After illumination, another aliquot was collected, transferred to a quartz cuvette, and diluted with Milli-Q water. The fluorescence spectrum was subsequently recorded under the specified parameters. Further details are provided in the Supplementary Information.

General procedure for the assay with different pH or ionic strength buffers:

To a quartz cuvette containing buffer with the desired pH or NaCl concentration, a probe solution was added. The solution was homogenized by pipetting for 10 seconds, then the

cuvette was sealed and left at room temperature for 10 minutes (NaCl), 5 or 60 minutes (pH buffers). The fluorescence spectra were then recorded using the specified parameters. Further details are provided in the Supplementary Information.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 10.3.0 or R Statistical Software (version 4.1.3, Foundation for Statistical Computing, Vienna, Austria). Normal distribution of data was evaluated through the Shapiro-Wilk test. Based on the outcome of the normality test, the appropriate tests were performed to evaluate differences. Additional details are provided in the legend of the corresponding figure.

Resource availability

Data acquired in this study are available in the Supplementary Information or on request from the corresponding author. The time required to synthetically prepare each probe is given in the Methodology section. Requests for resources (including 4NB-Resorufin and MeIQ-Resorufin) can be made to the corresponding author.

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Author contributions

MP, MSK, ALDW, VV and AMR conducted experiments and analysed data, assisted by ENS and HB. EF, SJC and EMH conceived the research. EF and DW designed and supervised the research. SJC supervised the chemistry aspects of the research. MP, MSK, ALDW, and EF wrote the manuscript with assistance from all co-authors. MP, MSK and ALDW contributed equally to this work.

Completing interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Supplementary Information

The following Supporting Information is available for this article:

Figure S1 – Additional images of *A. thaliana* cells incubated with 4NB-Resorufin and MeIQ-Resorufin.

Figure S2 – Fluorescence at 570-590 nm is resorufin-dependent and conditional on cellular uptake.

Figure S3 – 4NB-Resorufin and MeIQ-Resorufin are not toxic to *Arabidopsis thaliana* cells and seedlings under the conditions used.

Figure S4 – Resorufin, MeIQ-Resorufin, and 4NB-Resorufin fluorescence is quenched at mildly acidic pH.

Figure S5 – MeIQ-Resorufin and 4NB-Resorufin bioreduction in *A. thaliana* seedlings at 0.1 % O₂.

Figure S6 – Hypoxic fluorescence in *A. thaliana* tissues is a result of incubation with 4NB-/MeIQ-Resorufin.

Figure S7 – *A. thaliana* seedlings elicit a typical hypoxic response after 1 hour incubation in hypoxia (1 % O₂).

Figure S8 – 4NB-Resorufin, MeIQ-Resorufin and their reduced form resorufin do not affect growth in *Arabidopsis* seedlings.

Figure S9 – Additional images of MeIQ-Resorufin-derived fluorescence in *A. thaliana* lateral root primordia, including at different stages of development.

Figure S10 – MeIQ-Resorufin and 4NB-Resorufin bioreduction in *A. thaliana* cell lysates under hypoxia.

Figure S11 – Stability of 4NB-Resorufin under treatment with hydrogen peroxide or hydroxyl radical.

Figure S12 – Stability of MeIQ-Resorufin under treatment with hydrogen peroxide or hydroxyl radical.

Figure S13 – Resorufin, MeIQ-Resorufin, and 4NB-Resorufin fluorescence is quenched after light irradiation.

Figure S14 – Resorufin, MeIQ-Resorufin, and 4NB-Resorufin fluorescence is quenched in buffer with high salt concentrations.

Table S1 – Comparison of chemical probe properties

Chemistry Experimental Details General experimental techniques plus different *in vitro* assays, synthetic procedures and HPLC chromatograms for preparation of 4NB-Resorufin and MeIQ-Resorufin.

References

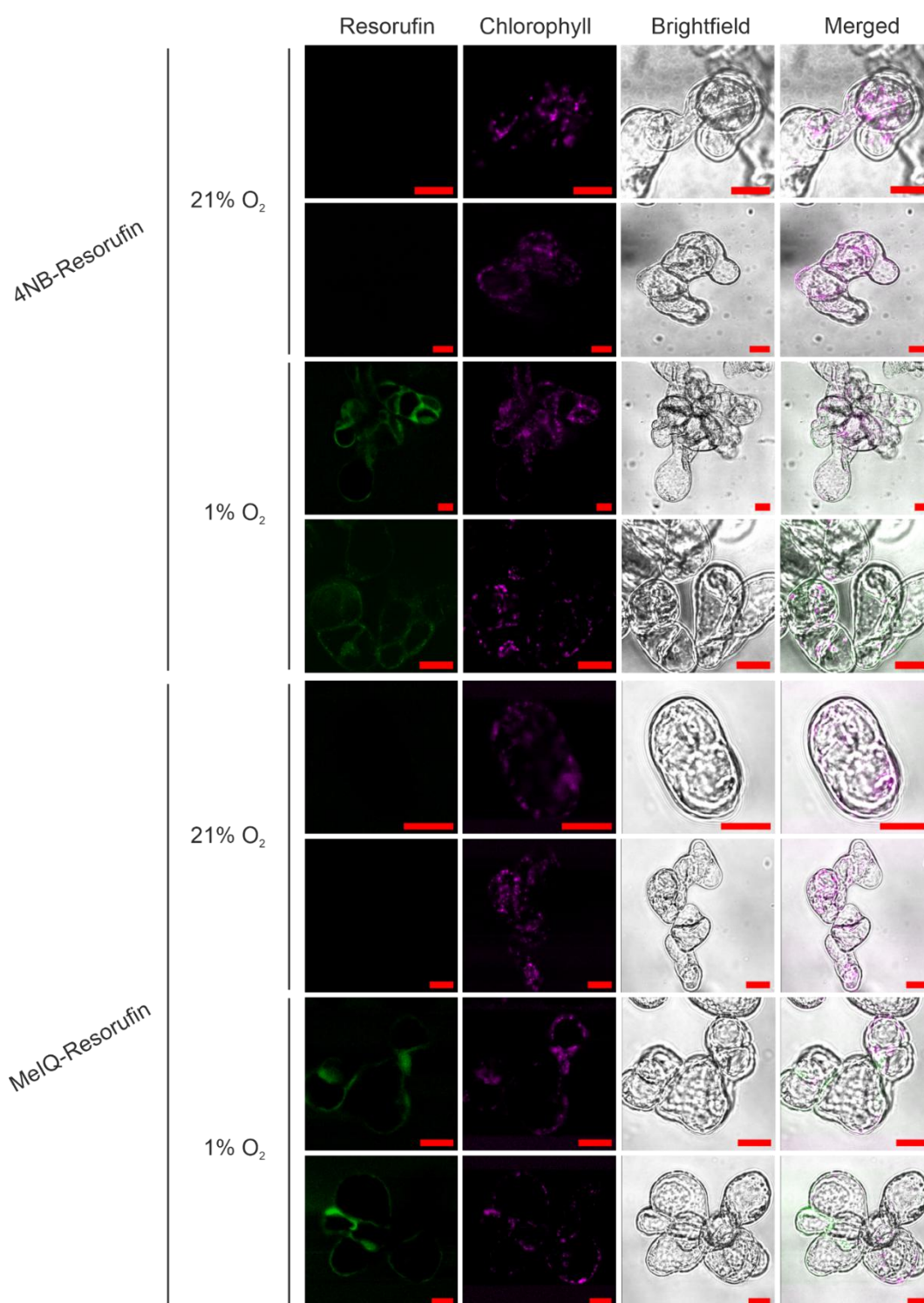


Figure S1 – Additional images of *A. thaliana* cells incubated with 4NB-Resorufin and MeIQ-Resorufin. *A. thaliana* cell cultures were incubated with 10 μ M of either 4NB-Resorufin or MeIQ-Resorufin under normoxic (21 % O₂) or hypoxic (1 % O₂) conditions for 2 hours. Fluorescence signals were collected for resorufin (green, 570-590 nm) and chlorophyll autofluorescence (purple, 650-750 nm). Scale bars: 20 μ m.

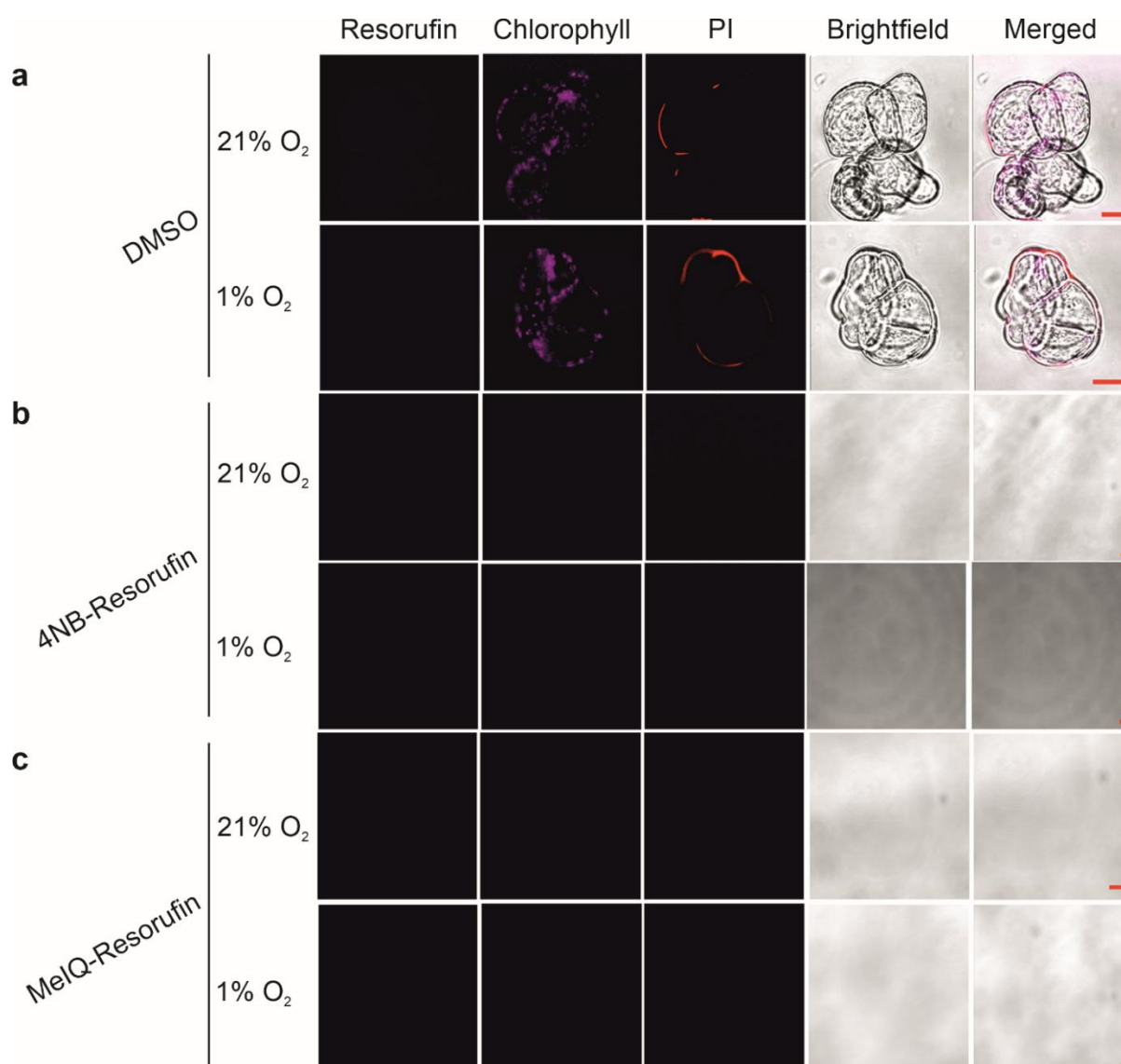


Figure S2 – Fluorescence at 570-590 nm is resorufin-dependent and conditional on cellular uptake. Confocal images demonstrating that fluorescence observed in hypoxic *A. thaliana* cells is dependent on the presence of 4NB- or MeIQ-Resorufin and its ability to penetrate cells. Four-day old *A. thaliana* whole cell cultures were incubated for 2 hours with 1 % DMSO (**a**) under normoxic (21 % O₂) or hypoxic conditions (1 % O₂). MS media (in the absence of cells) was incubated with 10 μM of either 4NB-Resorufin (**b**) or MeIQ-resorufin (**c**) in normoxia or hypoxia, respectively. Scale bars: 20 μm.

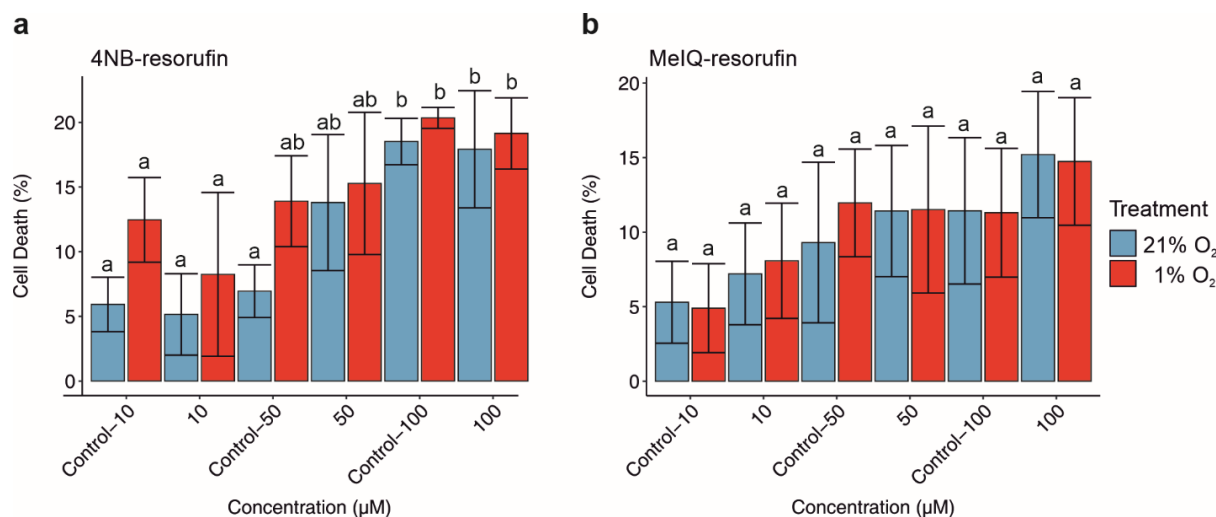


Figure S3 – 4NB-resorufin and MeIQ-resorufin are not toxic to *Arabidopsis thaliana* cells under the conditions used. *Arabidopsis* cells (four-day old) in MS media (1 mL) were treated with varying concentrations (10, 50 and 100 μM) of 4NB-Resorufin (**a**) or MeIQ-Resorufin (**b**) in normoxia (21 % O₂) or hypoxia (1 % O₂) for 2 hours followed by propidium iodide (PI) staining, followed by confocal imaging of the cells. DMSO was used as a control for each concentration. Cells stained internally with PI dye were considered dead cells. Dead cells were scored from triplicate slide images within a fixed area (0.83 μm x 0.83 μm) taken at 20x magnification using Zeiss LSM780. Letters indicate statistical differences between conditions analysed using two-way ANOVA, followed by Tukey HSD *post-hoc* test (p-value < 0.05).

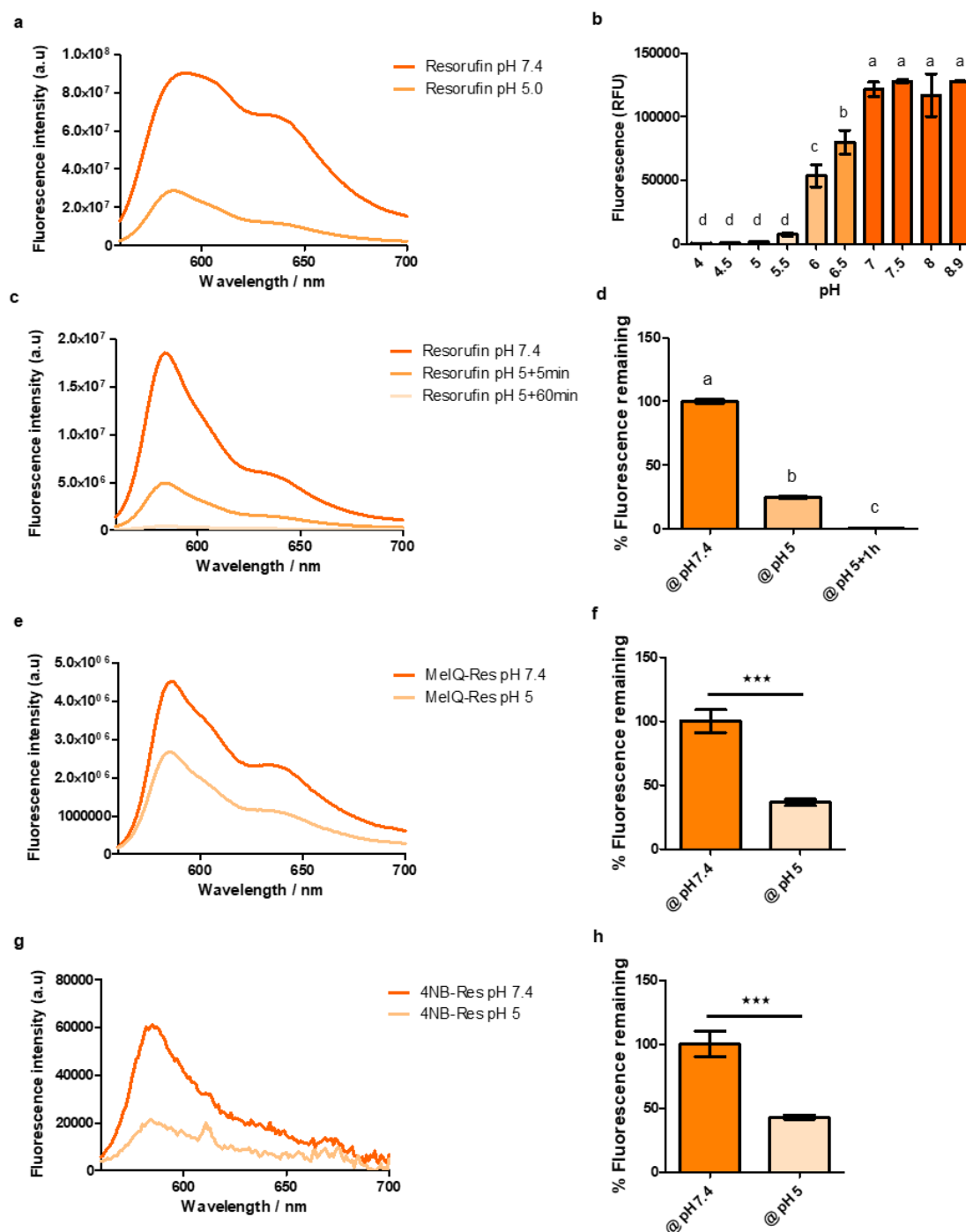


Figure S4 – Resorufin fluorescence is quenched at mildly acidic pH. (a) 3000 μL of potassium phosphate buffer (500 mM) with adjusted pH (5 or 7.4) was mixed with resorufin (10 μM) in a quartz cuvette. The fluorescence emission was measured from 560 to 700 nm (excitation 545 nm, slits 3, 3 nm) under ambient conditions. (b) 200 μL of sodium phosphate buffer (200 mM) with adjusted pH (4 - 8.9) was mixed with resorufin (10 μM). The plate was agitated orbitally for 30 s at 100 rpm for homogeneous mixing before measuring the fluorescence emission at 580 nm (excitation, 550 nm). Data represent mean of triplicate measurements ($n = 3$), and the vertical bar represents standard deviations of the

measurements. Letters indicate statistical differences between conditions analysed using one-way ANOVA, followed by Tukey HSD *post-hoc* test (p-value < 0.05). **(c)** Resorufin (1 μ M) in Milli-Q water adjusted to the indicated pH, following the general procedure. **(d)** Quantification of the average fluorescence intensity from three replicates shows a decrease in resorufin fluorescence at pH 5, where the fluorescence intensity at pH 7.4 is normalized to 100 %. Statistical significance was assessed using one-way ANOVA, followed by Tukey HSD *post-hoc* test (p-value < 0.05), where letters indicate statistical differences between groups. **(e)** MeIQ-Resorufin (1 μ M) in Milli-Q water adjusted to the indicated pH, following the general procedure. **(f)** Quantification of the average fluorescence intensity from three replicates shows a decrease in MeIQ-Resorufin fluorescence at pH 5, with the intensity at pH 7.4 normalized to 100%. Statistical significance was assessed using two-sided Student's t-test (***p-value < 0.001). **(g)** 4NB-Resorufin (1 μ M) in Milli-Q water adjusted to the indicated pH, following the general procedure. Fluorescence intensity data represent the mean of three replicates, collected with excitation at 545 nm and slit widths set to 3 nm for both excitation and emission. **(h)** Quantification of the mean fluorescence intensity from three replicates shows a decrease in 4NB-Resorufin fluorescence at pH 5, where the fluorescence intensity at pH 7.4 is normalized to 100%. Statistical significance was assessed using two-sided Student's t-test (***p-value < 0.001).

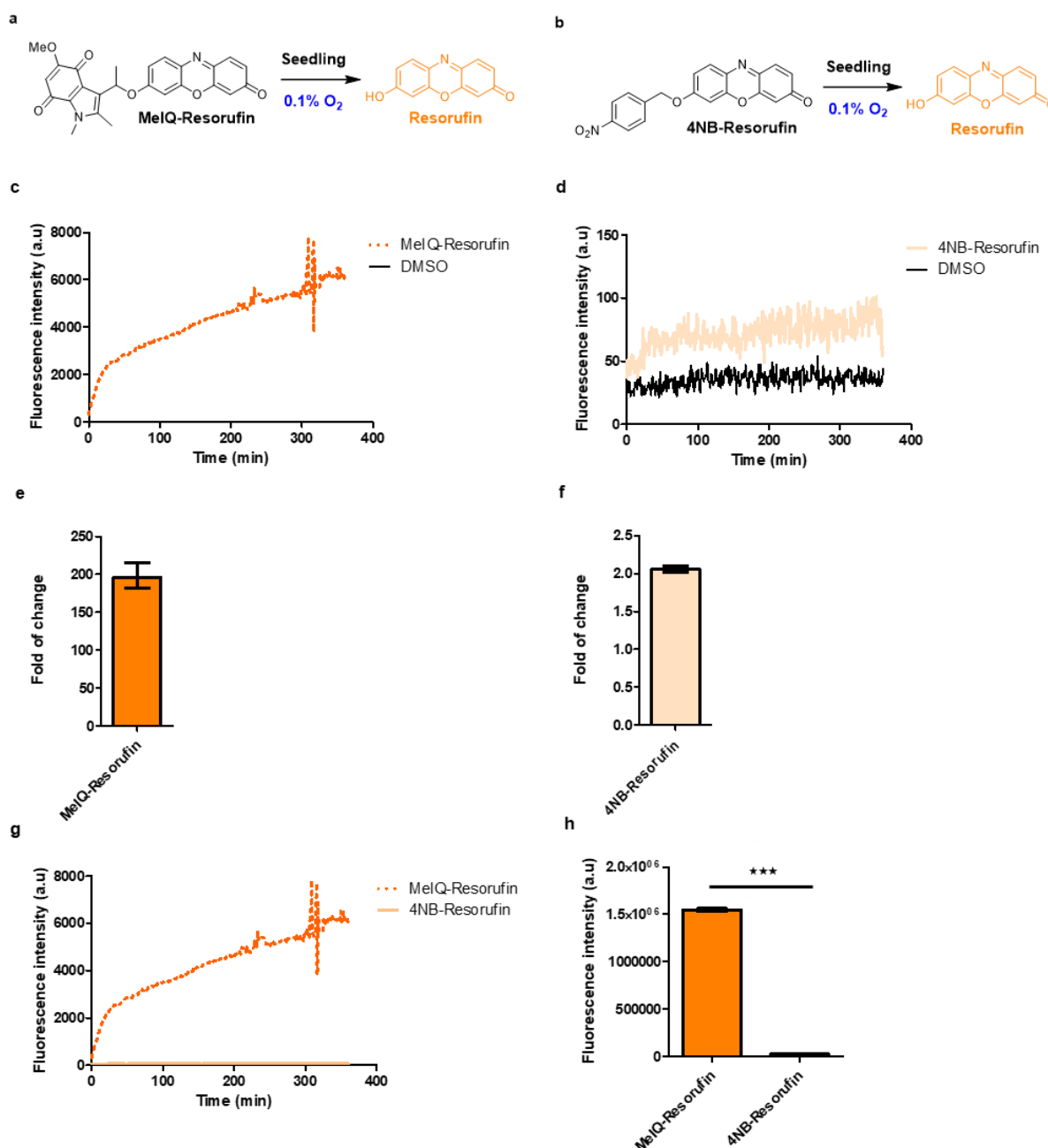


Figure S5 – MeIQ-Resorufin and 4NB-Resorufin bio-reduction in *Arabidopsis thaliana* seedlings at 0.1 % O₂. (a, b) Proposed reactions of MeIQ-Resorufin and 4NB-Resorufin with *Arabidopsis* seedlings leading to the formation of resorufin. (c, d) Hypoxic incubation of whole *Arabidopsis* seedlings (10-day old) in 500 µL of Milli-Q water containing 100 µM of MeIQ-Resorufin (c) or 4NB-Resorufin (d) was performed for 360 minutes at 25 °C. The mean fluorescence intensity of three biological replicates measured after 360 minutes, DMSO was used as a control for each concentration. (e) Quantification of the fluorescence increase for MeIQ-Resorufin observed in panel c (where the fluorescence is normalized to the DMSO control). (f) Quantification of the fluorescence increase for 4NB-Resorufin observed in panel d (where the fluorescence is normalized to the DMSO control). (g) Mean fluorescence

intensity of three biological replicates measured after 360 minutes reveals a difference in the rate at which maximum intensity is achieved for MeIQ-Resorufin (dashed line) compared to 4NB-Resorufin (solid line). **(h)** Quantification of the fluorescence increase for MeIQ-Resorufin and 4NB-Resorufin observed in panel g. Data are representative of three biological replicates; statistical significance was assessed using two-sided Student's t-test (**p-value < 0.001).

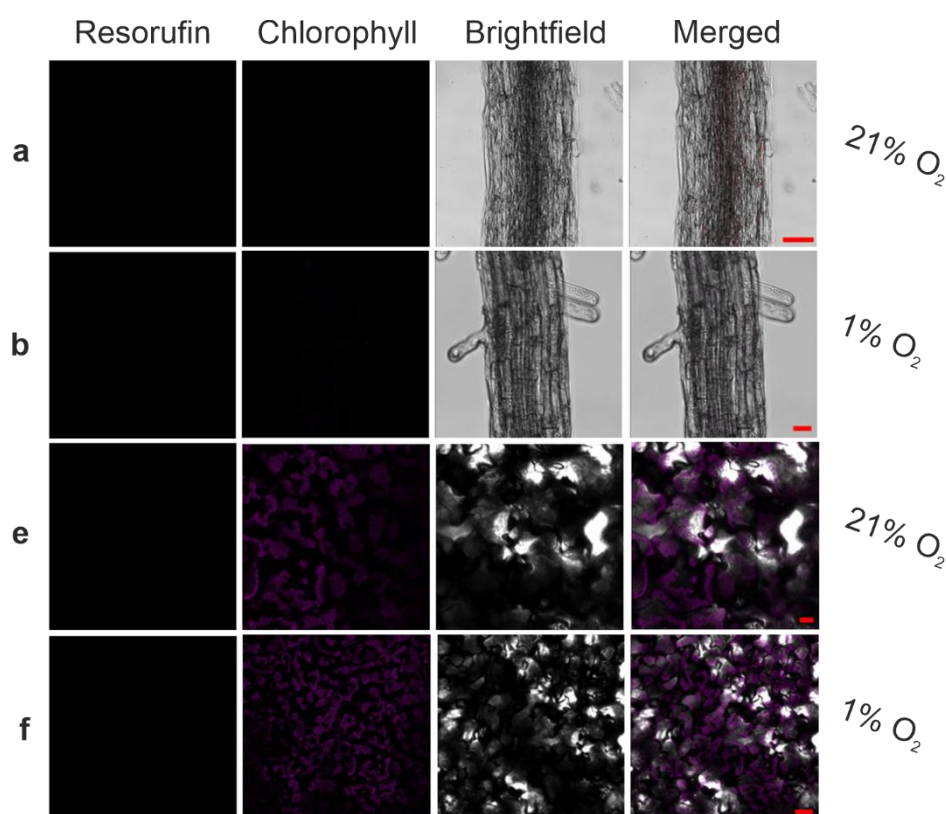


Figure S6 – Hypoxic fluorescence in *Arabidopsis thaliana* tissues is a result of incubation with 4NB-/MeIQ-Resorufin. *A. thaliana* seedlings were floated in media containing an equivalent volume of DMSO to that used in treatment with 4NB-/MeIQ-Resorufin (50 μ L/100 μ L, respectively). No fluorescence was observed under either normoxia or hypoxia in roots (**a**, **b**) or leaf tissue (**c**, **d**). Scale bars: 50 μ m.

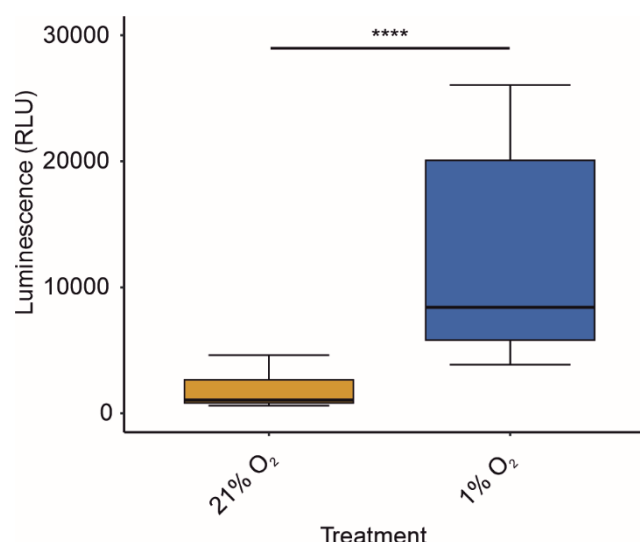
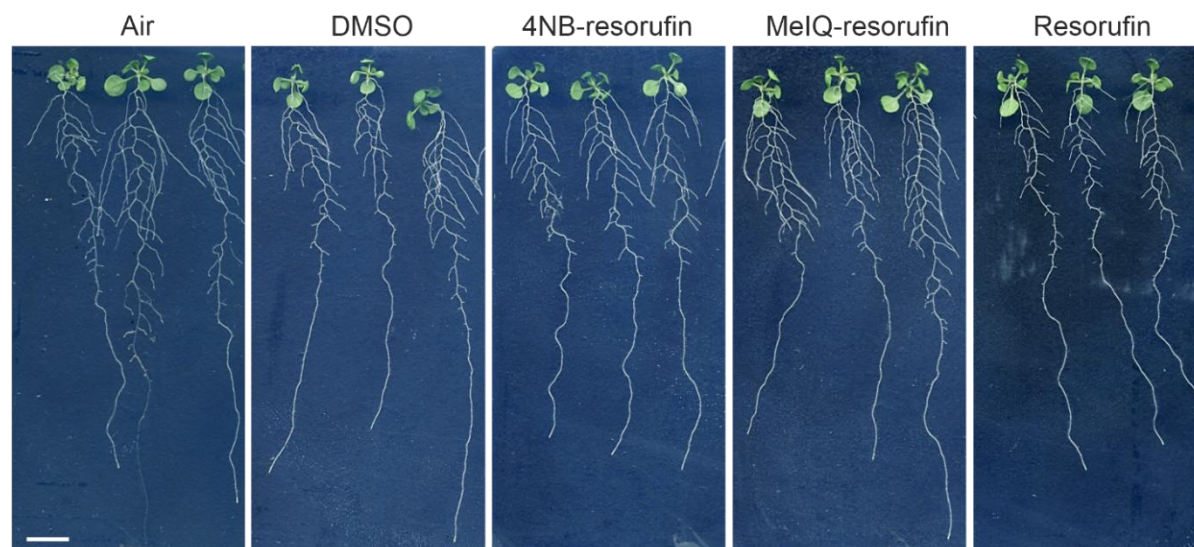
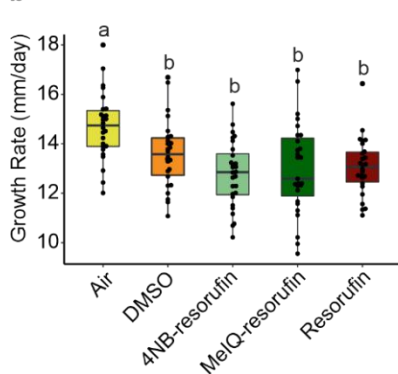


Figure S7 – *Arabidopsis thaliana* seedlings elicit a typical hypoxic response after 1 hour incubation in hypoxia (1 % O₂). *A. thaliana* wild-type seedlings expressing a hypoxia-responsive cassette comprising hypoxia-response element (HRPE) and the UTR-ADH promoter upstream of a gene encoding firefly luciferase enzyme. When a plant experiences hypoxia, hypoxia-responsive transcription factors recognize the HRPE motif and upregulate downstream genes (Gasch *et al.*, 2016). Luciferase expression therefore indicates the seedlings are experiencing hypoxia. Two-weeks old seedlings were incubated in 1 mL MS media for 1 hour in the Clariostar plate reader coupled to the atmospheric control unit, either in hypoxia (1 % O₂) or normoxia (21 % O₂). A vivazine™-based live assay system was employed to determine luminescence arising from luciferase activity. Data are representative of four biological replicates, statistical significance was assessed using Kruskal-Wallis, followed by Dunn's test. Seedlings exposed to hypoxia showed significantly higher levels of luciferase activity (*** p-value < 0.001).

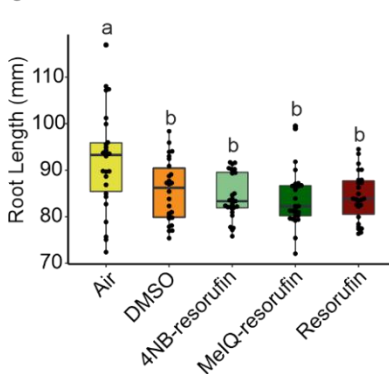
a



b



c



d

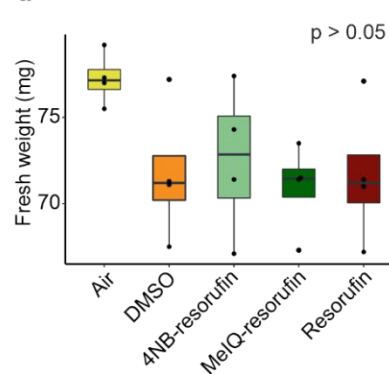


Figure S8 – 4NB-Resorufin, MeIQ-Resorufin and their reduced form resorufin do not affect growth in Arabidopsis seedlings. (a) Phenotypes of seven-day old Arabidopsis seedlings, after 4 days of treatment with mock (Air), 10 % DMSO, 100 μ M 4NB-resorufin, MeIQ-resorufin or resorufin. Scale bar: 1 cm. (b, c, d) Measurements of growth rate per day, primary root length ($n \geq 21$) and fresh weight of the entire seedling ($n = 4$) at the end of the treatment. Letters indicate statistical differences between conditions analysed using one-way ANOVA, followed by Tukey HSD *post-hoc* test.

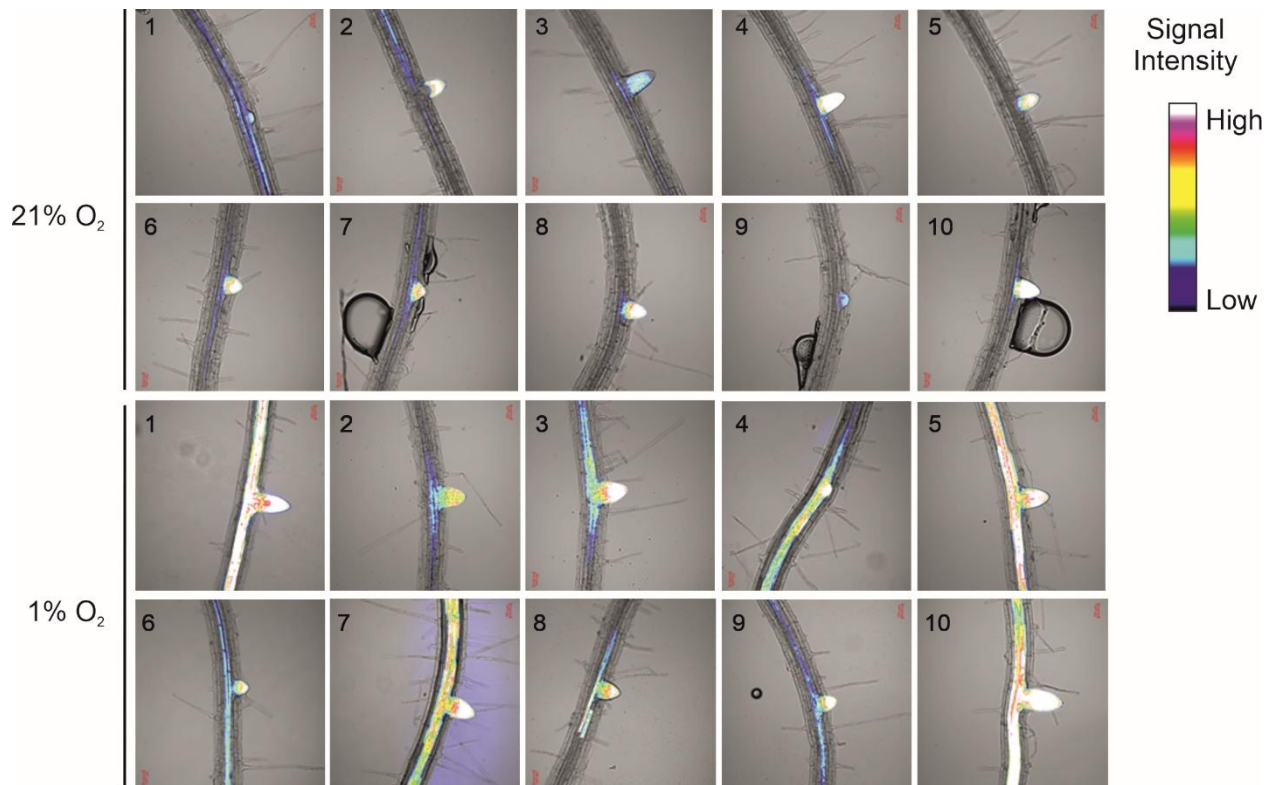


Figure S9 – Additional images of MeIQ-Resorufin-derived fluorescence in *Arabidopsis thaliana* lateral root primordia, including at different stages of development. In three separate experiments at 21 % O₂, 14 lateral roots from a total of four plants were imaged (10 shown below). The hypoxic environment in the lateral root primordia (Shukla *et al.*, 2019) is shown by intensity of fluorescence (indicated by pseudo-colour); lateral root primordia at a range of stages of development were shown to be hypoxic. In 2/14 cases (for example #1) a fluorescent signal was also seen in the root, however in these cases signal intensity was lower in the main root than in the lateral root primordia. In two separate experiments at 1 % O₂, 16 lateral roots from a total of four plants were imaged (10 shown below). In most cases, fluorescent signal was observed in both lateral roots and the main root, however in 2/16 cases (for example #2), fluorescence was only observed in the lateral root. Scale bars = 50 μ m.

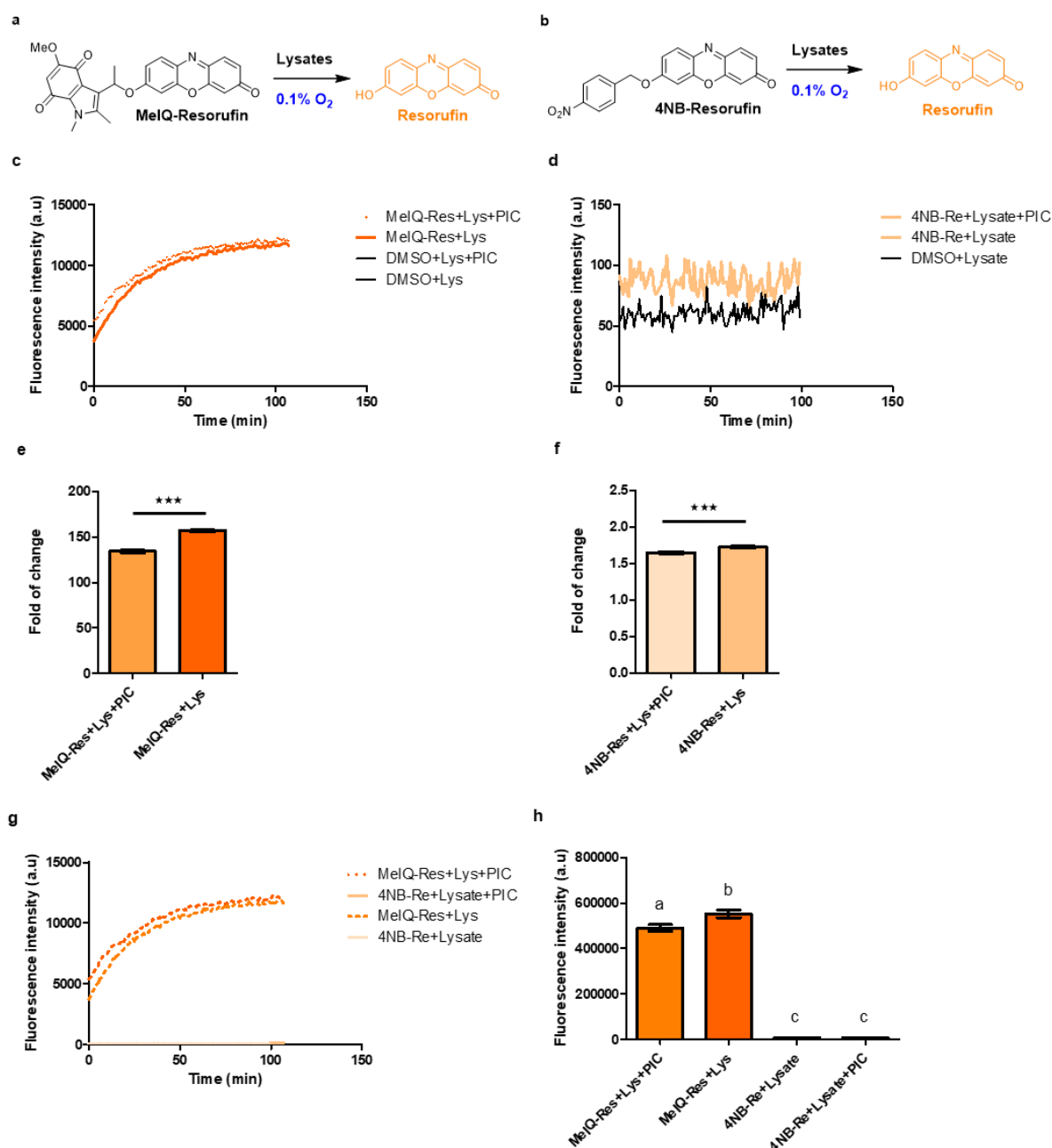


Figure S10 – MeIQ-Resorufin gets bio-reduced faster than 4NB-Resorufin at 0.1 % O₂ concentration in *Arabidopsis thaliana* lysates. (a, b) Proposed reactions of MeIQ-Resorufin and 4NB-Resorufin with *Arabidopsis* lysates leading to the formation of resorufin. **(c, d)** Hypoxic incubation of *Arabidopsis* lysates (10 days old, 147 μL) with or without protease inhibitor cocktail (PIC), NADH solution (1.5 μL of a 10 mM stock solution), and 10 μM of either MeIQ-Resorufin **(c)** or 4NB-Resorufin **(d)** was conducted for 100 minutes at 25 °C. Average fluorescence intensity of three biological replicates measured after 100 minutes, DMSO was used as a control for each concentration. **(e)** Quantification of the fluorescence increase for MeIQ-Resorufin observed in panel c (where the fluorescence is normalized to the DMSO control). **(f)** Quantification of the fluorescence increase for 4NB-

Resorufin observed in panel d (where the fluorescence is normalized to the DMSO control). (g) Average fluorescence intensity of three biological replicates measured after 100 minutes reveals a difference in the rate at which maximum intensity is achieved for MeIQ-Resorufin (dashed line) compared to 4NB-Resorufin (solid line). Statistical significance was assessed using two-sided Student's t-test (**p-value < 0.001). (h) Quantification of the fluorescence increase for MeIQ-Resorufin and 4NB-Resorufin observed in panel g. Data are representative of three biological replicates; statistical significance was assessed using one-way ANOVA, followed by Tukey HSD *post-hoc* test, where letters indicate statistical differences between groups (p-value < 0.05).

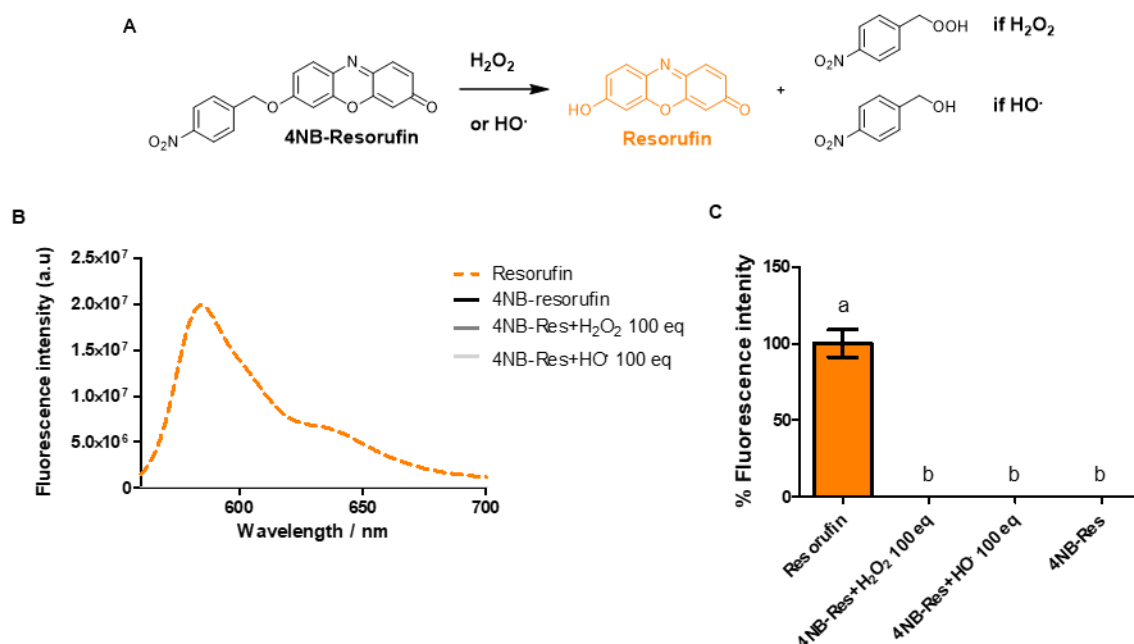


Figure S11 – Treatment of 4NB-Resorufin with hydrogen peroxide or hydroxyl radical does not result in the release of resorufin. (a) The proposed reaction mechanism of 4NB-Resorufin with hydrogen peroxide or hydroxyl radicals predicts the formation of resorufin and corresponding nitrobenzyl derivatives. However, no evidence of resorufin or nitrobenzyl derivatives was observed. (b) 4NB-Resorufin (1 μM) in Milli-Q water (pH 7) was treated with hydrogen peroxide (100 eq.) or hydroxyl radicals (100 eq.) for 10 minutes, following the general procedure. Fluorescence intensity data are the mean fluorescence intensity of three biological replicates and were collected with excitation at 545 nm and slit widths set to 3 nm for both excitation and emission. (c) Quantification of the mean fluorescence intensity of three biological replicates shows no increase after 10 minutes of treatment with hydrogen peroxide or hydroxyl radical, indicating the stability of 4NB-Resorufin (where the fluorescence of untreated resorufin is normalized to 100 %). Data are representative of three biological replicates; statistical significance was assessed using one-way ANOVA, followed by Tukey HSD *post-hoc* test, where letters indicate statistical differences between groups (p-value < 0.05).

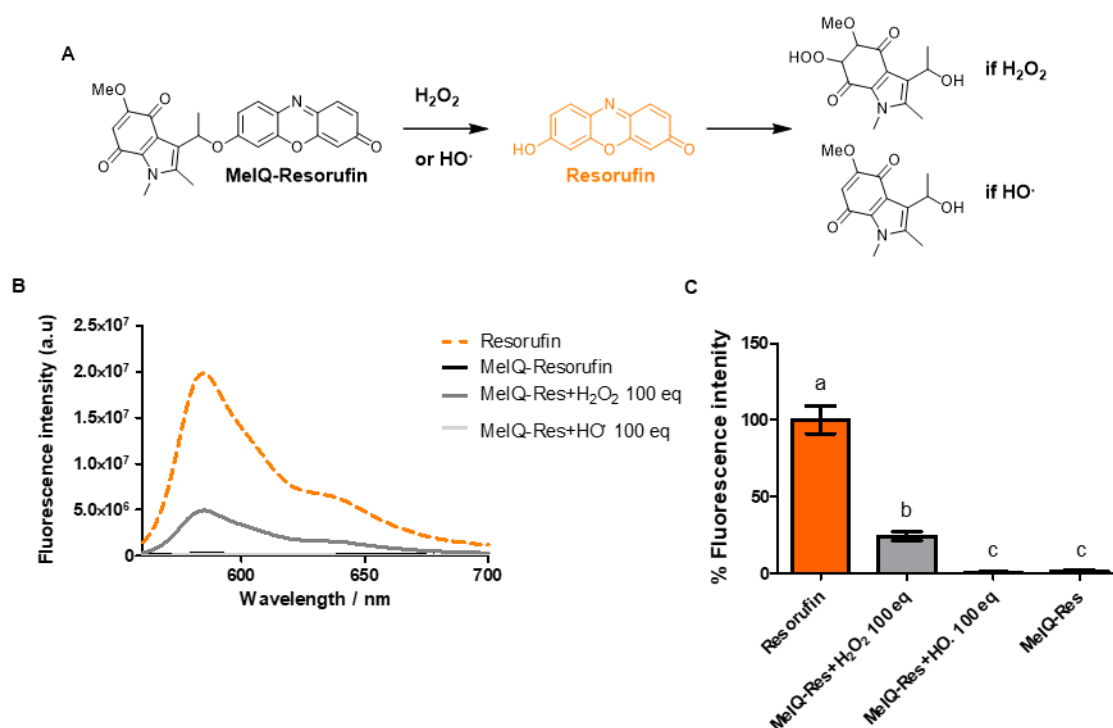


Figure S12 – Treatment of MeIQ-Resorufin with hydrogen peroxide or hydroxyl radical results in only a modest release of resorufin in the former case. (a) The proposed reaction of MeIQ-Resorufin with hydrogen peroxide or hydroxyl radicals predicts the formation of resorufin and corresponding nitrobenzyl derivatives. (b) MeIQ-Resorufin (1 μM) in Milli-Q water (pH 7) was treated with hydrogen peroxide (100 eq.) or hydroxyl radicals (100 eq.) for 10 minutes, following the general procedure. Fluorescence intensity data are the mean fluorescence intensity of three biological replicates and were collected with excitation at 545 nm and slit widths set to 3 nm for both excitation and emission. (c) Quantification of the mean fluorescence intensity of three biological replicates shows a 24 % increase after 10 minutes of treatment with hydrogen peroxide, indicating modest release of resorufin (where the fluorescence of untreated resorufin is normalized to 100 %). Data are representative of three biological replicates; statistical significance was assessed using one-way ANOVA, followed by Tukey HSD *post-hoc* test, where letters indicate statistical differences between groups (p-value < 0.05).

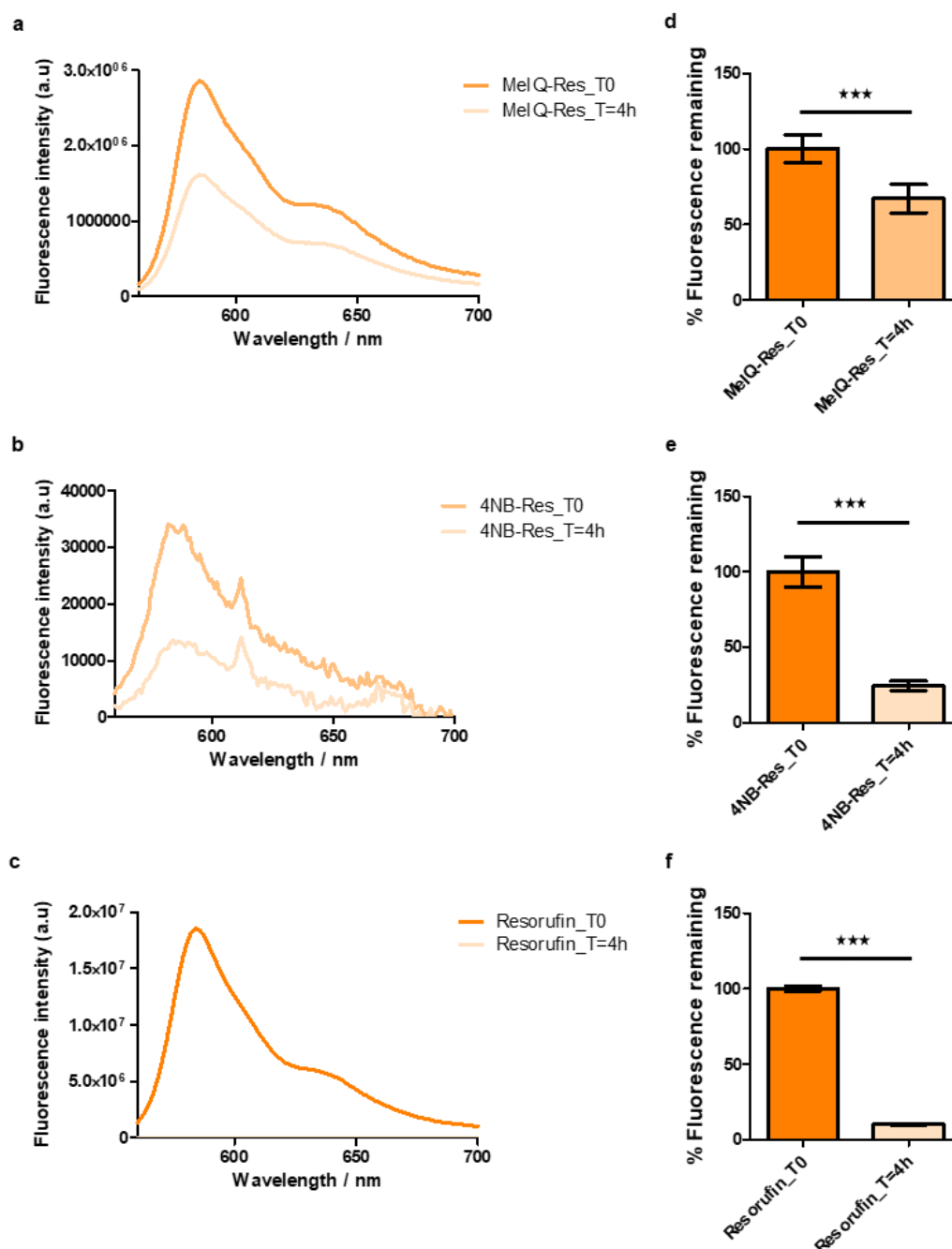


Figure S13 – Fluorescence of MeIQ-Resorufin, 4NB-Resorufin, and Resorufin is quenched after illumination with white LED (photobleaching). (a) MeIQ-Resorufin (1 μ M) in Milli-Q water (pH 7) and exposed to light as described in the general procedure. (b) 4NB-Resorufin (1 μ M) in Milli-Q water (pH 7) and exposed to light as described in the general procedure. (c) Resorufin (1 μ M) in Milli-Q water (pH 7) and exposed to light as described in the general procedure. Fluorescence intensity data represent the mean of three replicates and were collected at the time points specified in the general procedure with excitation at 545 nm and slit widths set to 3 nm for both excitation and emission. (d) Quantification of the mean fluorescence intensity from three replicates shows a decrease in MeIQ-Resorufin fluorescence

after 4 hours ($T = 4$ hours), normalized to the fluorescence of untreated MeIQ-Resorufin at the initial time point ($T = 0$, set as 100 %). (e) Quantification of the mean fluorescence intensity from three replicates shows a decrease in 4NB-Resorufin fluorescence after 4 hours ($T = 4$ hours), normalized to the fluorescence of untreated 4NB-Resorufin at the initial time point ($T = 0$, set as 100 %). (f) Quantification of the mean fluorescence intensity from three replicates shows a decrease in Resorufin fluorescence after 4 hours ($T = 4h$), normalized to the fluorescence of untreated Resorufin at the initial time point ($T = 0$, set as 100%). Statistical significance was assessed using two-sided Student's t-test (**p-value < 0.001).

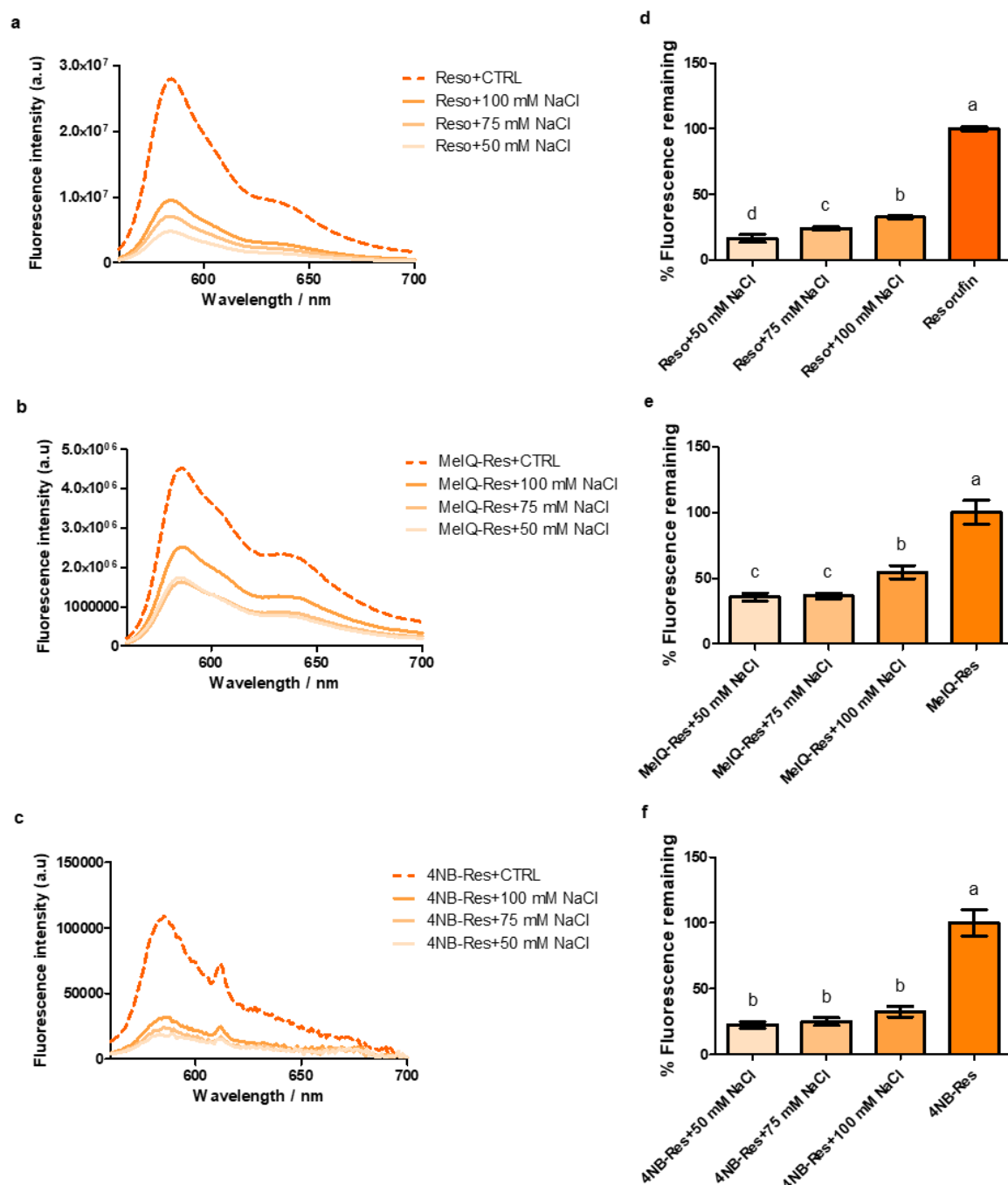


Figure S14 – Fluorescence of Resorufin, MeIQ-Resorufin, and 4NB-Resorufin is quenched in buffer with varying concentrations of NaCl. (a) Resorufin (1 μ M) in aqueous buffer containing different concentrations of NaCl, as described in the general procedure. (b) MeIQ-Resorufin (1 μ M) in aqueous buffer containing different concentrations of NaCl, as described in the general procedure. (c) 4NB-Resorufin (1 μ M) in aqueous buffer containing different concentrations of NaCl, as described in the general procedure. Fluorescence intensity data represent the average of three replicates, collected with excitation at 545 nm and slit widths set to 3 nm for both excitation and emission. (d) Quantification of the mean fluorescence intensity from three replicates shows a decrease in Resorufin

fluorescence at different salt concentrations, normalized to the fluorescence of untreated Resorufin (set as 100 %). **(e)** Quantification of the mean fluorescence intensity from three replicates shows a decrease in MeIQ-Resorufin fluorescence at different salt concentrations, normalized to the fluorescence of untreated MeIQ-Resorufin (set as 100 %). **(f)** Quantification of the mean fluorescence intensity from three replicates shows a decrease in 4NB-Resorufin fluorescence at different salt concentrations, normalized to the fluorescence of untreated 4NB-Resorufin (set as 100 %). Data are representative of three biological replicates; statistical significance was assessed using one-way ANOVA, followed by Tukey HSD *post-hoc* test, where letters indicate statistical differences between groups (p -value < 0.05).

Table S1 – Comparison of properties of 4NB-Resorufin and MeIQ-Resorufin. ^a 10 μ M probe in whole cells at 1 % O₂ for 2 hours; ^b 100 μ M probe in seedlings at 1 % O₂ for 6 hours; ^c 100 μ M probe in seedlings at 0.1 % O₂; ^d incubation in aqueous solution at pH 5; ^e 100 μ M probe in 10 % DMSO for 4 days; ^f after 4 h illumination with white LED; ^g incubation with 50 mM NaCl.

	4NB-Resorufin	MeIQ-Resorufin
Signal Intensity ^a	Low	High
Spatial distribution ^b (note probe structure may impact distribution)	Roots: vascular regions & cortex (near-homogenous) Leaf: non-homogenous	Roots: vascular regions (homogenous) Leaf: near homogenous
Response time in seedlings ^c	Slow	Very fast (within minutes)
Quenching in mild acidic pH ^d	57 %	62 %
Toxicity in DMSO ^e	Not toxic (negligible impact on response)	Not toxic (negligible impact on response)
Photobleaching in DMSO ^f	58 %	48 %
Quenching with NaCl ^g	78 %	65 %

Chemistry experimental section

Chemicals were purchased from Sigma Aldrich UK, Alfa Aesar UK, and Fluorochem, and were used as supplied unless stated. Brine refers to a saturated aqueous solution of sodium chloride. Anhydrous solvents were obtained from an MBRAUN Solvent Purification System 5 and stored under an argon atmosphere over 3 Å molecular sieves. Petroleum ether refers to the fraction of light petroleum ether boiling in the range 40–60 °C. *In vacuo* refers to the removal of solvent using a Buchi® rotary evaporator under reduced pressure in a water bath at 40 °C.

¹H NMR spectra were recorded on Bruker AVC500 (500 MHz) or Bruker AVH400 (400 MHz) spectrometers using deuteriochloroform (unless indicated otherwise) as a reference for the internal deuterium lock. The chemical shift data for each signal are given as δ_{H} in units of parts per million (ppm) relative to tetramethylsilane (TMS) where δ_{H} (TMS) = 0.00 ppm. The multiplicity of each signal is indicated by s (singlet); d (doublet); dd (doublet of doublets); or m (multiplet). The number of protons (n) for a given resonance signal is indicated by nH. Coupling constants (*J*) are quoted in Hz and are recorded to the nearest 0.1 Hz. Identical proton coupling constants (*J*) are averaged in each spectrum and reported to the nearest 0.1 Hz. The coupling constants are determined by analysis using MestreNova software. Bruker Topspin was used to plot the spectra. Spectra were assigned using COSY, NOESY, HSQC, and HMBC experiments as necessary.

Mass spectra were acquired on a VG platform spectrometer and an Agilent 6120 spectrometer (low resolution). Electrospray ionization spectra were obtained on Micromass LCT Premier and Bruker MicroTOF spectrometers, operating in a positive or negative mode, as indicated, from solutions of MeOH or MeCN. *m/z* values are reported in Daltons and followed by their percentage abundance in parentheses. Electron ionization/field ionization (EI/FI) was carried out on a Waters GCT with a temperature-programmed solids probe inlet. Samples were introduced in glass tips directly into the source where they were vaporized and analysed. In EI the ionization is by electron impact, electrons being provided by a filament. In FI the ionization is in an intense electric field which causes quantum electron tunnelling of a valence electron.

Melting points were determined using a Griffin capillary tube melting point apparatus and are uncorrected.

Analytical thin-layer chromatography (TLC) was carried out on normal phase Merck silica gel 60 F254 aluminum-supported chromatography sheets. Visualisation was done by absorption of UV light (λ_{max} 254 and 365 nm) or thermal development after staining in an

aqueous solution of potassium permanganate. UV light was provided by a LF – 206.LS 230V – 50 Hz from UVIttec Limited.

Flash column chromatography was performed manually using Geduran® silica gel 60 (40-63 μm) eluting with solvents as supplied, under a positive pressure of air or nitrogen on a Biotage® Selekt Flash Pure Purification System using Biotage® KP-Sil SNAP or Biotage® Sfär Silica cartridges.

General procedures for reactive oxygen species (ROS) preparation and assays (Zhang et al., 2015)

General procedure for hydrogen peroxide (H_2O_2) assay:

Preparation

The concentration of H_2O_2 (stock solution) was determined by measuring its absorption at 240 nm, using an extinction coefficient of $43.6 \text{ cm}^{-1}\text{M}^{-1}$. To prepare the assay solution, 15 μL of H_2O_2 (30 wt. % stock solution) was added to 5 mL of Milli-Q water in a 15 mL Falcon tube. Using the aforementioned method, the concentration of the resulting solution was determined to be 28.8 mM.

Assay

To a 3 mL quartz cuvette on ice (0–4 °C) containing Milli-Q water (pH 7.4, 2986.5 μL) and H_2O_2 solution (10.5 μL of a 28.8 mM stock solution in Milli-Q water), a probe solution (3 μL of a 1 mM stock solution in DMSO) was added. The cuvette was sealed and left at room temperature for 5 minutes. At this point, the final concentrations of H_2O_2 and the hypoxia-imaging probe were 100 μM and 1 μM , respectively. The fluorescence spectra were then measured using the specified parameters at the given time point.

General procedure for hydroxyl radical ($\text{HO}\cdot$) assay:

Preparation

The hydroxyl radical ($\text{HO}\cdot$) was generated using the Fenton reaction. To prepare the assay solution, 15 μL of H_2O_2 (30 wt. % stock solution) was added to 5 mL of Milli-Q water in a 15 mL Falcon tube, resulting in a 28.8 mM solution. From this, 1 mL of the H_2O_2 solution (28.8 mM, 10 eq.) was transferred to another 15 mL Falcon tube, and ferrous chloride (2.9 mM, 1 eq.) was added. The solution turned orange and was used immediately for the assay.

Assay

To a 3 mL quartz cuvette on ice (0–4 °C) containing Milli-Q water (pH 7.4, 2986.5 μL) and $\text{HO}\cdot$ solution (10.5 μL of a 28.8 mM stock solution in Milli-Q water), a probe solution (3 μL of a 1 mM stock solution in DMSO) was added. At this stage, the final concentrations of $\text{HO}\cdot$ and

the hypoxia-imaging probe were 100 μM and 1 μM , respectively. The solution was homogenized by pipetting for 10 s, then the cuvette was sealed and left at room temperature for 5 minutes. The fluorescence spectra were measured under the specified parameters.

General procedure for light stability assay:

To a 1 mL transparent vial containing DMSO (45 μL), a probe solution (5 μL of a 10 mM stock solution in DMSO) was added. An aliquot (3 μL) was taken from the reaction solution and transferred to a 3 mL quartz cuvette, followed by the addition of Milli-Q water (pH 7, 2997 μL). The solution was homogenized by pipetting for 10 s, then the fluorescence spectra were recorded using the specified parameters, serving as the initial time point ($T = 0$, where T represents time). The vial was then sealed and placed in an aluminum box, positioned 20 cm away from a white LED (OSRAM, 11 W, 52 mA, 1521 lm), and illuminated for 4 hours. After illumination, another aliquot (3 μL) was collected, transferred to a 3 mL quartz cuvette, and diluted with Milli-Q water (pH 7, 2997 μL). The fluorescence spectrum was subsequently recorded under the specified parameters.

General procedure for the assay with high ionic strength buffers:

To a 3 mL quartz cuvette containing buffer with the desired NaCl concentration (2997 μL), a probe solution (3 μL of a 1 mM stock solution in DMSO) was added. The solution was homogenized by pipetting for 10 s, then the cuvette was sealed and left at room temperature for 10 minutes. The fluorescence spectra were then recorded using the specified parameters.

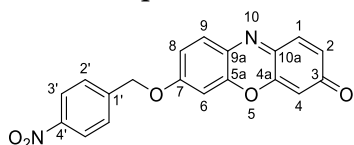
General procedure for the assay with different pH buffers:

To a 3 mL quartz cuvette containing buffer at the desired pH (2997 μL), a probe solution (3 μL of a 1 mM stock solution in DMSO) was added. The solution was homogenized by pipetting for 10 s, then the cuvette was sealed and incubated at room temperature for 5 or 60 minutes. The fluorescence spectra were then recorded using the specified parameters.

Synthetic procedures

7-[(4-Nitrobenzyl)oxy]-3H-phenoxazin-3-one (4-NB Resorufin)

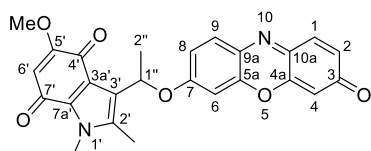
4-nitrobenzylbromide (89.1 mg, 0.412 mmol, 1.0 eq) in N,N-Dimethylformamide (1.5 mL) was added dropwise to a solution of resorufin (97.0 mg, 0.412 mmol, 1.0 eq) and potassium carbonate (85.5 mg, 0.618 mmol, 1.5 eq) in N,N-Dimethylformamide (3.5 mL). The solution was stirred at 40 $^{\circ}\text{C}$ for 3 hours. The solution was diluted with ethyl acetate (100 mL) and washed with brine (3×50 mL). The aqueous layer was extracted with ethyl acetate (3×30 mL). The organic components were combined, dried (sodium sulfate), filtered and concentrated in vacuo. The crude residue was recrystallized from dichloromethane, followed



by trituration with diethyl ether (3×10 mL) to yield the title compound (112 mg, 65%) as a red solid; R_f = 0.44 (50 % ethyl acetate in petroleum ether), mp: decomposes >240 °C (from diethyl ether), [Lit. (Collins *et al.*, 2018) 289–292 °C]; ^1H NMR (400 MHz, CDCl_3) δ 8.22 (2H, d, J = 8.6, H -3'), 7.68 (1H, d, J = 8.9, H -9), 7.56 (2H, d, J = 8.6, H -2'), 7.35 (1H, d, J = 9.7, H -1), 6.95 (1H, dd, J = 8.9, J = 2.7, H -8), 6.80 (1H, d, J = 2.7, H -6), 6.77 (1H, dd, J = 9.7, J = 2.0, H -2), 6.25 (1H, d, J = 2.0, H -4), 5.22 (2H, s, $\text{C1}'\text{CH}_2\text{O}$).; LRMS m/z (ES+) 349.0 ($[\text{M}+\text{H}]^+$, 4.27%). Analytical HPLC (Dionex Acclaim™ 120 C18 column [$5\ \mu\text{m}$, $120\ \text{\AA}$, $4.6 \times 150\ \text{mm}$]; 95:5 H_2O : MeCN $\rightarrow$ 5:95 H_2O : MeCN: H_2O with 0.1 % TFA modifier, 10 min; 5 min hold; $1.5\ \text{mL min}^{-1}$] Ret. Time = 10.6 min. Purity: 98.4%. These data are in accordance with literature (Collins *et al.*, 2018).

5'-Methoxy-1',2'-dimethyl-3-(1-((3-oxo-3H-phenoxazin-7-yl)oxy)ethyl)-1H-indole-4',7'-dione (MeIQ Resorufin)

Diisopropyl azodicarboxylate (0.120 mL, 0.602 mmol, 3.0 eq.) was added dropwise to a solution of methyl indolequinone (Wallabregue *et al.*, 2023) (50.0 mg, 0.201 mmol, 1.0 eq.), resorufin (107 mg, 0.502 mmol, 2.5 eq.) and triphenylphosphine (132 mg, 0.502 mmol, 2.5 eq.) in anhydrous tetrahydrofuran (3 mL) at room temperature under argon. The reaction mixture was stirred vigorously for 24 hours at 50 °C, then concentrated *in vacuo*. The residue was then dissolved in ethyl acetate (50 mL), washed with aqueous sodium hydrogen carbonate (3×20 mL), water (3×20 mL), aqueous hydrochloric acid (50 mL, 1 M solution), brine (2×20 mL), dried (sodium sulfate), and concentrated *in vacuo*. The crude material was purified using silica gel column chromatography eluting with petroleum ether:ethyl acetate (gradient 10 to 100 % ethyl acetate). The residue was dissolved in ethyl acetate (2.5 mL) and precipitated by adding hexane (50 mL). The orange precipitate was filtered, washed with hexane (50 mL), and the precipitation procedure was repeated twice. The precipitate was then dried *in vacuo* to give the title compound as an orange solid (36 mg, 40%): R_f 0.22 (petroleum ether:ethyl acetate, 1:1); m.p. 77–79 °C (ethyl acetate); [Lit. (Wallabregue *et al.*, 2023) 78 °C]; ^1H NMR (600 MHz, CDCl_3) δ 7.62 (d, J = 8.9 Hz, 1H, H -9), 7.38 (d, J = 9.8 Hz, 1H, H -1), 6.92 (dd, J = 8.9, 2.6 Hz, 1H, H -8), 6.80 (dd, J = 9.8, 2.0 Hz, 1H, H -2), 6.78 (d, J = 2.6 Hz, 1H, H -6), 6.36 (q, J = 6.5 Hz, 1H, H -1'), 6.29 (d, J = 2.0 Hz, 1H, H -4), 5.66 (s, 1H, H -6'), 3.86 (s, 3H, OCH_3), 3.82 (s, 3H, NCH_3), 2.30 (s, 3H, $(\text{C}-2')\text{CH}_3$), 1.68 (d, J = 6.5 Hz, 3H, H -2''); LRMS m/z (ESI+) 445 ($[\text{M}+\text{H}]^+$, 90%); Analytical HPLC (Dionex Acclaim™ 120 C18 column [$5\ \mu\text{m}$, $120\ \text{\AA}$, $4.6 \times 150\ \text{mm}$]; 95:5 H_2O : MeCN $\rightarrow$ 5:95 H_2O : MeCN: H_2O with 0.1 % TFA modifier, 10 min; 5 min hold; $1.5\ \text{mL min}^{-1}$] Ret. Time = 10.6 min. Purity: 93.5%. The spectroscopic data are in good agreement with literature values (Wallabregue *et al.*, 2023).

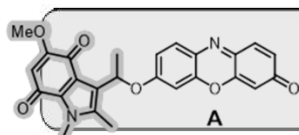


HPLC Traces

75'-Methoxy-1',2'-dimethyl-3-(1-((3-oxo-3H-phenoxazin-7-yl)oxy)ethyl)-1H-indole-4',7'-dione (MeIQ Resorufin) @254 nm

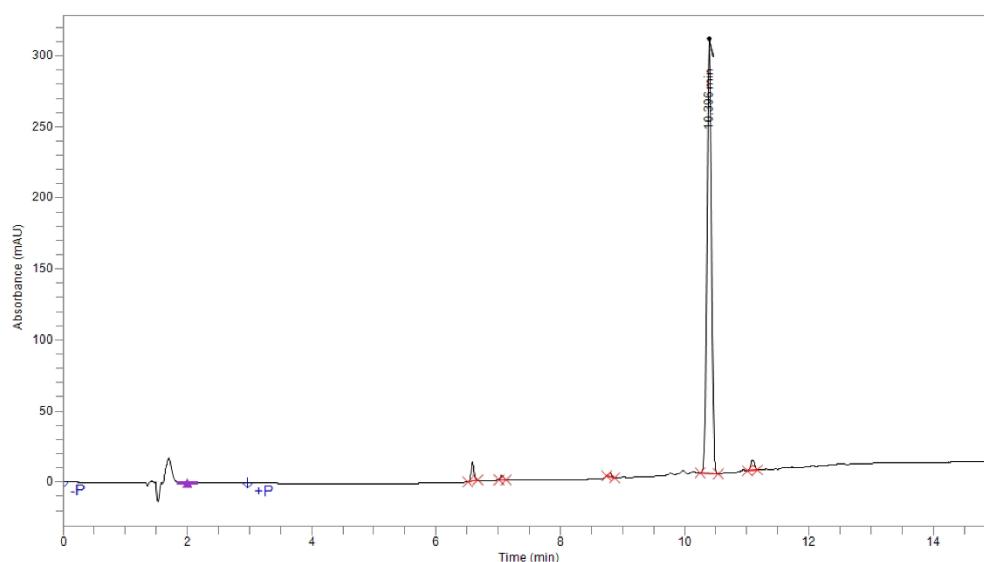
OXAW51-2

10/2/2021



Acquisition Method Purity short run @254 nm
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 Injection Volume 40
 Sample Name OXAW51-2
 Sample Description
 Batch Description

OXAW51-2 : Injection 1

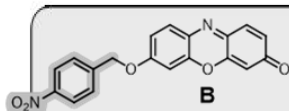


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8.798	2,791.6	11,067.8	0.73
10.396	305,851.7	1,417,074.4	93.53
11.093	7,270.8	30,670.2	2.02
Total		1,515,123.0	100.00

7-[(4-Nitrobenzyl)oxy]-3H-phenoxazin-3-one (4-NB Resorufin) @254 nm

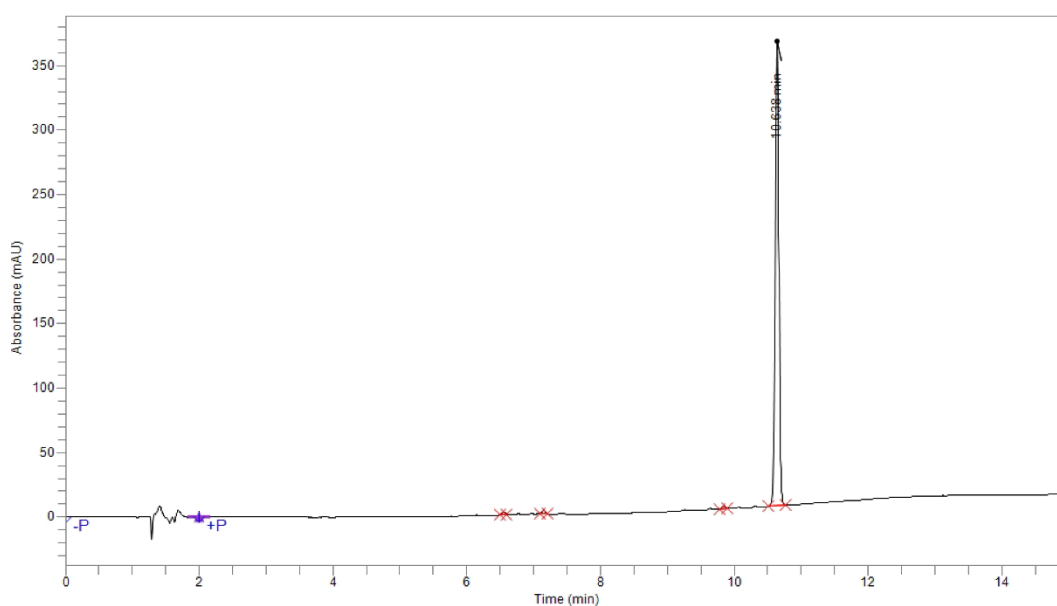
OX-AW91@254

6/4/2021



Acquisition Method Purity short run @254 nm
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 Sample Name OX-AW91@254
 Sample Description
 Batch Description

OX-AW91@254 : Injection 1



Time	Height	Area	Area %
6.550	1,914.1	6,323.4	0.47
7.148	3,003.3	8,985.0	0.67
9.844	2,022.7	6,576.5	0.49
10.638	360,908.8	1,318,842.1	98.37
Total		1,340,727.0	100.00

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Statement of authorship

Statement of Authorship for joint/multi-authored papers for PGR thesis

To appear at the end of each thesis chapter submitted as an article/paper

The statement shall describe the candidate's and co-authors' independent research contributions in the thesis publications. For each publication there should exist a complete statement that is to be filled out and signed by the candidate and supervisor (**only required where there isn't already a statement of contribution within the paper itself**).

Title of Paper	Hypoxia-activated fluorescent probes as markers of oxygen levels in plant cells and tissues
Publication Status	☒ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and unsubmitted work written in a manuscript style
Publication Details	Perri, M.*, Khan, M.S.*, Wallabregue, A.L.D.*, Voloboeva, V., Ridgway, A.M., Smith, E.N., Bolland, H., Hammond, E.M., Conway, S.J., Weits, D.A., Flashman, E., 2025. Hypoxia-activated fluorescent probes as markers of oxygen levels in plant cells and tissues. <i>New Phytologist</i> . https://doi.org/10.1111/nph.70202

Student Confirmation

Student Name:	Monica Perri
Contribution to the Paper	Conducted and designed experiments: cell and seedlings incubation with probes, confocal imaging, root length and fresh weight measurements (Figures 2-4, Supplementary

	Figures 1-3, 6-8); analysed data; contributed to writing the manuscript.
Signature 	Date 12/08/2025

Supervisor Confirmation

By signing the Statement of Authorship, you are certifying that the candidate made a substantial contribution to the publication, and that the description described above is accurate.

Supervisor name and title: Professor Francesco Licausi		
Supervisor comments I hereby confirm that the above Declaration of Authorship and Contributions provided by Ms Monica Perri is accurate and truthful. The statement correctly reflects her own scientific and scholarly contributions to the work presented in her thesis, as well as the contributions made by collaborators, which are appropriately acknowledged. I endorse this declaration as a fair and transparent account of authorship and contributions.		
Signature 	Date	10/09/2025

This completed form should be included in the thesis, at the end of the relevant chapter.

Chapter 3 – H₂O₂ repurposes the plant oxygen-sensing machinery to control the transcriptional response to oxidative stress

Commentary

Chapter 3 expands our understanding of hypoxia signaling by investigating the sophisticated crosstalk between hypoxia and oxidative stress during flooding and subsequent recovery phase in plants. This chapter identifies a critical regulatory mechanism in Arabidopsis, where group VII Ethylene Response Factors (ERFVII) remain stabilised upon reoxygenation, contrary to expectations, as a result of reactive oxygen species (ROS)-mediated inhibition of Plant Cysteine Oxidases (PCOs). This finding underscores how plants integrate oxygen and ROS signals to finely tune transcriptional responses during the challenging transition from hypoxia to normoxia.

The sustained stabilization of ERFVII after reoxygenation is particularly striking, revealing a dual role: these transcription factors repress classical hypoxia-responsive genes but simultaneously activate genes involved in ROS homeostasis and oxidative stress protection. By demonstrating that PCOs function not only as oxygen sensors but also as redox sensors, this chapter extends the canonical beyond oxygen sensing into a broader redox regulatory framework. It reveals a nuanced mechanism whereby the plant oxygen-sensing machinery integrates both oxygen availability and oxidative stress signals to modulate gene expression, allowing plants to balance metabolic adaptation with protection against oxidative damage.

In summary, the findings of Chapter 3 advance the thesis's aim by elucidating how plants integrate intersecting signals from fluctuating oxygen levels and oxidative stress, an essential process for survival under natural and agricultural flooding conditions. These discoveries deepen our understanding of plant stress resilience and open new avenues for engineering crops capable of withstanding the combined challenges of flooding and oxidative stress.

H₂O₂ repurposes the plant oxygen-sensing machinery to control the transcriptional response to oxidative stress

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Summary

Understanding plant responses to flooding is crucial for strategies to increase resilience. Plants respond to submergence-induced low-oxygen (hypoxia) through decreased Plant Cysteine Oxidase (PCO) activity which stabilises group VII Ethylene Response Factors (ERFVII), master regulators of adaptive metabolic and anatomic responses. Plants must also survive desubmergence stress, which includes a burst of reactive oxygen species (ROS) and rapid metabolic re-acclimation to normoxia. Restoration of PCO function and ERFVII degradation may be expected on reoxygenation. In contrast, we report here that ERFVII are important in post-submergence recovery, remaining stable upon reoxygenation through ROS-mediated inactivation of PCOs. Stabilised ERFVII are retained at hypoxia-responsive promoters, becoming repressors of typical hypoxia marker genes but upregulators of genes involved in ROS homeostasis and oxidative stress protection. Our findings suggest that PCOs respond to both oxygen and ROS to coordinate ERFVII stability and regulate timely responses to damaging fluctuations in oxygen availability.

Keywords

Reactive oxygen species, peroxide, oxidative stress, hypoxia, reoxygenation, desubmergence, plant cysteine oxidases, ethylene response factors.

Introduction

All aerobic organisms require oxygen for efficient energy generation; they therefore have mechanisms capable of sensing decreased oxygen availability (hypoxia) to coordinate an adaptive response. Plants experience hypoxia as both an acute stress, such as that caused by submergence, or as a developmental cue in rapidly metabolizing tissues ¹⁻³. In both scenarios, the molecular response to the hypoxic conditions has been well characterised and involves decreased activity of oxygen-sensing Plant Cysteine Oxidase enzymes (PCOs) ⁴⁻⁶. PCOs create a direct molecular link between environmental O₂ and stabilization of transcriptional regulators including ERFVII ⁷. Using molecular O₂, PCOs catalyse the oxidation of the N-terminal cysteine residue of their substrates, forming cysteine sulfinic acid (SO₂H) ⁸. This initiates degradation of the substrate via the Cys/Arg branch of the PRT6 N-degron pathway in normoxic plants ⁹. Alternately, low O₂ (hypoxia) limits PCO activity resulting in substrate stabilisation ⁸. In the case of ERFVII, the stabilised transcription factors bind to hypoxia-responsive promoter elements (HRPE) resulting in the elevated expression of a number of core hypoxia-responsive genes (HRGs) required to switch from aerobic respiration to fermentative respiration ¹⁰⁻¹². This adaptive response helps plants survive short periods of hypoxia arising from decreased oxygen diffusion when plants are submerged during a flood event.

While the acute hypoxia experienced by submerged plants is itself stressful, the reoxygenation that occurs on desubmergence is also challenging. Plants must readapt to aerobic respiration and photosynthesis, as well as cope with photoinhibition and desiccation while enduring sugar and energy shortage ¹³. One outcome of hypoxia followed by rapid reoxygenation in both plants and mammals is a burst of elevated reactive oxygen species (ROS), in particular the superoxide anion radical (O₂•⁻) and hydrogen peroxide (H₂O₂) ¹⁴⁻¹⁹. These ROS are specific molecular derivatives of oxygen produced during normal aerobic metabolism, primarily in chloroplasts and mitochondria with high electron flow and metabolic rates ²⁰. Under hypoxia, O₂•⁻ and H₂O₂ production occurs through inefficient O₂ reduction at electron transport chains, as well as by NADPH oxidases such as RBOHD ^{21,22}. Upon reoxygenation, the reactivation of mitochondrial and photosynthetic activities, involving proteins which may have been damaged during hypoxia, causes electron leakage in the electron transport chains and membrane-associated processes ²³, which subsequently further increases ROS formation, culminating in a ROS burst ²⁴. There is evidence linking this response to hypoxia/reoxygenation in submerged plants: submergence stress in *Arabidopsis thaliana* (hereafter *Arabidopsis*) is reported to induce systemic ROS wave responses ²⁵ and in *Arabidopsis* rosettes, desubmergence is reported to

induce expression of plasma membrane-localized NADPH oxidase/respiratory burst oxidase that produces superoxide in the apoplastic space ¹⁸.

Given that hypoxia stress entails ROS production at its onset and after reoxygenation, there is likely to be cross-talk between cellular responses to both signals. Indeed, in *Arabidopsis* the hypoxia-responsive universal stress protein 1 (HRU1) is reported to be upregulated by the ROS-related transcription factor heat-shock factor 2A ^{26,27} and ROS-responsive genes are activated by hypoxia-responsive ERFVII in *Arabidopsis* ^{28,29} and SUB1A in rice ³⁰. However, it is not known whether there is a direct interaction between ROS and the plant oxygen-sensing machinery. ERFVII have been demonstrated to play a crucial role in modulating the response to multiple stresses ^{31–33}, but might be expected to be susceptible to PCO-initiated degradation upon reintroduction of oxygen ¹³. Here, we report that *Arabidopsis thaliana* *erfVII* knock-out mutants showed impaired recovery following reoxygenation compared to wild-type plants, as well as elevated ROS in response to prolonged hypoxia and reoxygenation. We therefore investigated the contribution of ROS (specifically H₂O₂) ²¹ to PCO-ERFVII mediated responses to hypoxia and reoxygenation. We found that upon both reoxygenation and direct H₂O₂ treatment, ERFVII in fact remain stable and localised in plant nuclei and that this is due to ROS-mediated PCO-inactivation. Interestingly, we find that ERFVII repress HRG expression under these conditions while instead activating expression of genes associated with oxidative stress. These data indicate that the PCO-ERFVII signaling pathway can sense both oxygen depletion and a corresponding ROS surge, allowing them to distinguish between hypoxia and oxidative stress and coordinate the appropriate cellular response.

Results

ERFVII are required for post-hypoxia recovery in Arabidopsis seedlings

Given that plant recovery from a submergence event requires survival from the desubmergence-associated ROS burst, we first investigated whether ERFVII stabilisation during hypoxia contributes to tolerance of this ROS burst and the likely resulting oxidative stress by comparing recovery from reoxygenation in Arabidopsis wild-type and *erfVII* mutant plants. We subjected seven-day old seedlings to severe hypoxia (0.1 % O₂, no light) or normoxia (21 % O₂, no light) for 24 h; their growth phenotype was analysed immediately after the treatment and after 4 days reoxygenation, during which plants were maintained in 16:8 photoperiod under ambient atmospheric conditions (**Figure 1A, B**). While we did not observe differences between the wild type and the mutant immediately at the end of the hypoxic treatment, after 4 days of reoxygenation *erfVII* seedlings that had been exposed to hypoxia demonstrated strongly decreased survival in comparison to the wild type (**Figure 1B, C**). Root growth was impaired after reoxygenation in the *erfVII* seedlings (**Figure 1D**), which resulted in lower biomass accumulation compared to wild type (**Figure 1E**). Although we cannot rule out from this experiment that the differences observed are due to inability of the *erfVII* seedlings to mount an adaptive response to hypoxia, they nevertheless indicated the possibility that ERFVII contribute to the plant's ability to cope with reoxygenation stress.

Based on previous studies where constitutive RAP2.2, RAP2.3 and RAP2.12 protein expression caused increased levels of ROS-related genes in Arabidopsis seedlings¹¹, we speculated that ERFVII may function to limit ROS accumulation following reoxygenation. To examine this, we first used 3,3'-diaminobenzidine (DAB) staining as an indicator of H₂O₂ levels. Seven-day old Arabidopsis seedlings were treated with 0.1 % O₂ for 24 h then stained with DAB 0 h, 1 h, and 6 h after reoxygenation, in darkness. Control plants were kept under aerobic conditions for 24 h in the dark, followed by staining for the same time periods. When compared after reoxygenation, increased DAB staining was observed in *erfVII* seedlings compared to wild type after 1 h, indicating higher production of H₂O₂ in *erfVII* than in wild-type seedlings upon reoxygenation (**Figure 1F, G**). To validate these findings and to investigate the live dynamics of H₂O₂, we then used the roGFP2-Orp1 fluorescent biosensor³⁴ in both *erfVII* mutant and wild-type plants during 6 h hypoxia of severe (0.1 % O₂) and mild (1 % O₂) hypoxia followed by reoxygenation (21 % O₂) (**Figure 1H, Figure S1A**). In both seven-day old seedlings and leaf discs from five-week old plants, we observed an increase in sensor oxidation upon reoxygenation which was significantly greater in *erfVII* mutant than Col-0 plants (**Figure 1J, Figure S1C**). Already over the course of the 6 h hypoxic period, there was also a gradual increase in sensor oxidation, however this increase was only

significantly greater in *erfVII* than Col-o in all plants in the later stages of severe hypoxia (0.1 % O₂) (**Figure 1I, Figure S1B**).

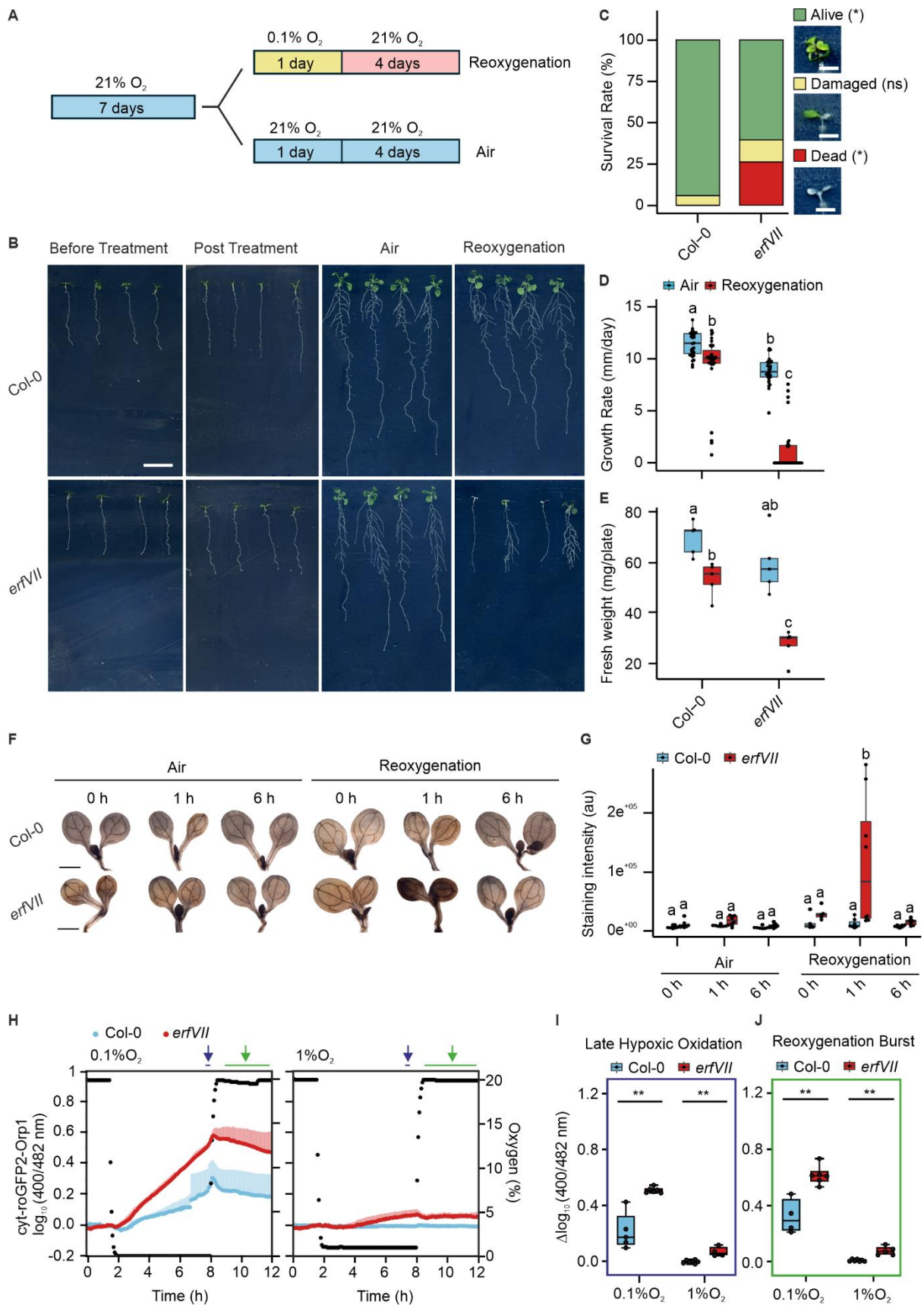


Figure 1 – ERFVIIIs mediate plant tolerance upon reoxygenation. (A) Schematic diagram of experimental design; seven-day old *Arabidopsis* seedlings were exposed to severe hypoxia (0.1 % O₂) or air (21 % O₂), in darkness, for 24 h and subsequently returned to aerobic conditions (16:8 photoperiod) for 4 days to allow for reoxygenation. (B) Phenotype of Col-o and *erfVII* seedlings before and after hypoxia treatment (or air control) and after 4 days reoxygenation (scale bar, 1 cm). (C) Percentage of live, damaged, or dead seedlings after 4 days of post-hypoxia reoxygenation ($n = 4$, each replicate representing ~7 seedlings). (D) Growth rate of primary roots after 4 days of reoxygenation ($n \geq 25$). (E) Fresh weight after 4 days of reoxygenation ($n = 4$, each replicate representing ~7 seedlings). (F) DAB staining of Col-o and *erfVII* seedlings following exposure to 0.1 % or 21 % O₂ for 24 h in darkness, and subsequently returned to aerobic conditions (at dark) for 0, 1 or 6 h (scale bar, 0.5 cm). (G) Quantification of DAB staining intensity represented in arbitrary units (au) ($n \geq 5$). (H-J) Multi-well fluorimetry of cytosolic oxidative stress using seven-days old *Arabidopsis* seedlings expressing the biosensor roGFP2-Orp1 in Col-o and *erfVII* background over time, each normalized to the baseline oxidative state before the start of hypoxic treatment ($n \geq 4$, each replicate consists of ~6 seedlings). (I-J) Amplitudes of late hypoxic roGFP2-Orp1 oxidation before reoxygenation (blue arrow, I) and maximum oxidative burst during reoxygenation (green arrow, J), each normalized to the baseline oxidative state before the start of hypoxic treatment. Statistical analyses were conducted using: (C) the χ^2 test followed by a post-hoc test for survival analysis where asterisks indicate statistical differences between Col-o and *erfVII* ($p < 0.05$); (D-G) two-way ANOVA followed by Tukey's HSD test, $p < 0.05$ where different letters indicate statistically different groups ($p < 0.05$); (H) two-sided Student's t-test ($p < 0.001$). (I, J) Statistical significance of differences determined using Mann-Whitney test ($p < 0.05$).

These results are consistent with the finding of relatively lower levels of H₂O₂ upon desubmergence in the aerial tissue of rice expressing *SUB1A*, an N-degron pathway resistant ERFVII, compared with plants lacking *SUB1A*³⁰. Together, the results suggest that ERFVII contribute to recovery post-submergence by reducing accumulation of ROS upon reoxygenation.

ERFVIIIs are stabilised in *Arabidopsis* seedlings by both post-hypoxia reoxygenation and oxidative stress

During hypoxic conditions, ERFVIIIs are known to be stabilised and accumulate in the nucleus^{35,36} (**Figure 2A**). Since our results indicate that ERFVIIIs also contribute to growth recovery after reoxygenation, we investigated the abundance and localization of RAP2.12 in *Arabidopsis*

cells in conditions that mimic post-submergence reoxygenation, as well as upon the addition of exogenous ROS in the form of tert-butyl hydroperoxide (TBHP) ^{37,38}. We treated transgenic *Arabidopsis* seedlings constitutively expressing RAP2.12 fused with green fluorescent protein (GFP) with or without TBHP (1 mM) in normoxia, hypoxia, and after reoxygenation (3 h post-hypoxia, consistent with the timing of oxidative damage of lipids reported in *Arabidopsis* after desubmergence ¹⁸). We then looked for RAP2.12-associated GFP signal in *Arabidopsis* root cells using confocal imaging. As expected, we observed elevated RAP2.12-GFP localization in the nuclei of hypoxic plants compared to normoxic plants (**Figure 2B**). Upon reoxygenation, ERFVII may be presumed to destabilise with the resumption of PCO enzyme function in aerobic conditions, however we found that the RAP2.12-GFP signal persisted in the nucleus after 3 h reoxygenation treatment, both in the presence and absence of TBHP. Interestingly, we observed that TBHP treatment resulted in an increase in RAP2.12-GFP signal in the nuclei of plants exposed to both air and hypoxia (**Figure 2B**). We confirmed these observations using a second transgenic *Arabidopsis* line expressing RAP2.3-GFP (**Figure S2A**). A time-course observation of TBHP-treated, normoxic plants showed stabilisation of RAP2.3 within less than 2 h (**Figure S2B**). Collectively, these data revealed that ERFVIIs remain stable in the nucleus upon reoxygenation, when plants are exposed to ROS as well as O₂.

To probe ERFVII stability in a more quantitative manner, we generated an *Arabidopsis* line constitutively expressing the full RAP2.3-coding sequence fused in frame with the nanoluciferase enzyme (RAP2.3-nLuc). Seven-day old RAP2.3-nLuc seedlings were treated with or without TBHP (1 mM) in normoxia, hypoxia and after reoxygenation, as in the subcellular localisation experiment. As expected, in the absence of TBHP luciferase activity was higher in hypoxic seedlings than in normoxic ones, consistent with N-degron pathway-mediated degradation of RAP2.3 in normoxia (**Figure 3A**). TBHP-treated seedlings in normoxia exhibited significantly higher luciferase activity compared to controls, consistent with ERFVII stabilisation under aerobic conditions when cells are exposed to H₂O₂ (**Figures 2B, 3A**). We confirmed that this phenomenon is dose- and time-dependent (**Figure 3B, C**) with RAP2.3 stabilization reaching a plateau at 2 h of treatment (**Figure 3C**). Furthermore, during reoxygenation, and in the absence of exogenous TBHP, luciferase activity did not decrease from hypoxic levels, remaining significantly higher than in normoxic seedlings (**Figure 3A**). Taken together, these measurements quantitatively confirm the stability of ERFVII upon both oxidative stress and during the reoxygenation period. A second ERFVII reporter line expressing a 1-28 amino acid fragment of RAP2.12 fused with the firefly luciferase enzyme (RAP2.12₁₋₂₈-FLuc)⁴ exhibited similar behaviour (**Figure S3A**), with elevated FLuc activity in TBHP-treated aerobic and reoxygenated seedlings, further confirming our findings.

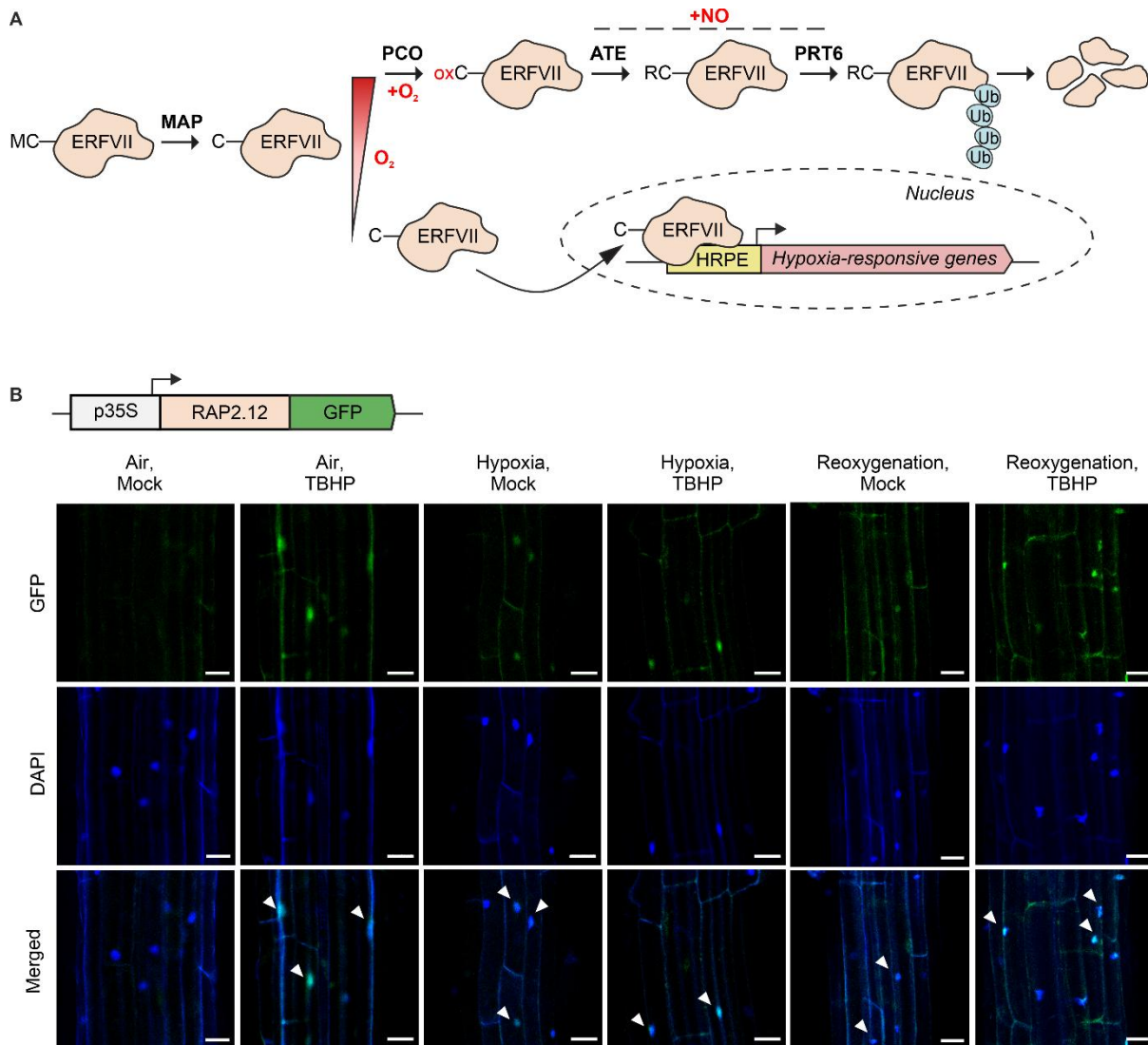


Figure 2 – RAP2.12 localizes to the nucleus upon oxidative stress as well as during reoxygenation. (A) Schematic representation of N-degron pathway-controlled destabilisation of ERFVII. Methionine (M) in the N-terminal position is target of the methionine aminopeptidase enzyme (MAP), leaving an exposed cysteine which, in the presence of oxygen (O_2) is targeted for oxidation by plant cysteine oxidase (PCO). The resulting Cys-sulfinic acid is sequentially modified by arginyl aminotransferases 1/2 (ATE) triggering proteasomal degradation after ubiquitination by proteolysis E3 ligase (PRT6), in presence of nitric oxide (NO). Upon hypoxic conditions, ERFVII are stabilised and relocate to the nucleus where they bind the hypoxia-responsive promoter element (HRPE) triggering the hypoxic response. Additional abbreviations: M, methionine; C, cysteine; oxC, oxidized cysteine; R, arginine; Ub, ubiquitin. (B) Localization of 35S:RAP2.12-GFP (green) in seven-day old Arabidopsis seedlings upon 1 mM TBHP treatment in normoxia (21 % O_2 v/v O_2/N_2), hypoxia (1 % O_2 v/v O_2/N_2) or after 3 h of reoxygenation. Nuclear localization is confirmed using DAPI staining (blue). White arrows indicate nuclear colocalization (scale bar, 50 μ m).

ROS-induced ERFVII stabilisation is conferred via the N-terminus

The observations obtained with the RAP2.12₁₋₂₈-FLuc line indicated that the prevention of the predicted O₂-dependent degradation exclusively required the N-terminal region of ERFVIIIs, known to contain the conserved N-degron MCGGAI 35. We therefore sought to determine whether H₂O₂-mediated ERFVII stabilisation is conferred via modification of its Nt-Cys (the catalytic target of the PCOs) rather than other ERFVII residues. We used a transgenic RAP2.3-nanoluciferase fusion in which the N-terminal cysteine of the transgenic RAP2.3 is mutated to alanine (C2A-RAP2.3-nLuc), thereby preventing PCO-initiated proteolysis of RAP2.3. We examined the luciferase signal in C2ARAP2.3-nLUC seedlings exposed to air or hypoxia and treated with or without TBHP. In contrast to Cys2-RAP2.3-nLuc (**Figure 3A-C**), the luciferase signal of C2A-RAP2.3-nLUC did not vary significantly upon hypoxia or TBHP treatment (**Figure 3D**). This suggested that prevention of ERFVII degradation is specifically due to the inability of the N-degron pathway to process protein N-termini and that TBHP-induced oxidative stress cannot increase ERFVII stability when ERFVIIIs are no longer N-degron substrates.

The comparative observations of luciferase activity in RAP2.3-, RAP2.12₁₋₂₈- and C2A-RAP2.3-linked reporter lines suggested that ERFVII stabilization upon TBHP treatment or upon post-hypoxia reoxygenation is linked to the protection of N-terminal cysteine from the N-degron pathway. To dissect the step at which N-degron processing is inhibited under these conditions, we next used a heterologous DLOR (Double Luciferase Oxygen Reporter) reporter assay developed in the budding yeast *Saccharomyces cerevisiae* 39. Here, the relative luciferase output (FLuc/RLuc) of the DLOR reporter is strictly dependent on transgenic PCO enzymes (in this case AtPCO4) expressed in yeast, which otherwise lack N-terminal cysteine oxidation activity. TBHP treatment of exponentially grown yeast caused a fast and dose-dependent increase of DLOR output, thus demonstrating TBHP-induced ERFVII stabilization, similar to our observations in plants (**Figures 3E, S3B, C**). The optical density (OD) of the TBHP-treated cultures was slightly lower in TBHP-treated cells compared to control, but this was dose-independent in contrast to the dose-dependent effect on FLuc activity, indicating a specific effect of TBHP on the DLOR system (**Figure S3D**). Having confirmed the stabilisation of ERFVII in PCO-expressing yeast, we then used this system to independently probe each step of the N-degron pathway for susceptibility to TBHP. The Arg/N-degron pathway in yeast is mediated by ATE1-dependent arginylation of Nt-Glu-, Nt-Asp- or Nt-Cys-SOO(O)H-initiating proteins and UBR1-catalyzed ubiquitination of Nt-Arg- proteins 9,40. We used two alternative versions of DLOR, D-DLOR and R-DLOR, in which the Nt-Cys (C) of the RAP2.12₂₋₂₈ domain is mutated to aspartate (D) or arginine (R), respectively 41. C-DLOR, D-DLOR and R-DLOR were all treated with 0.5 mM TBHP for 30 min and the relative FLuc

activity was measured (**Figure 3E**). The signal was increased by TBHP treatment only in C-DLOR yeast, whereas it did not change significantly in D-DLOR and R-DLOR strains (**Figure 3E**). These results indicate that TBHP does not interfere with ATE1 and UBR1 function, thereby suggesting that ROS-induced ERFVII stabilization in yeast occurs through inhibition of the PCO-catalysed step, suggesting that this is also the case in plants.

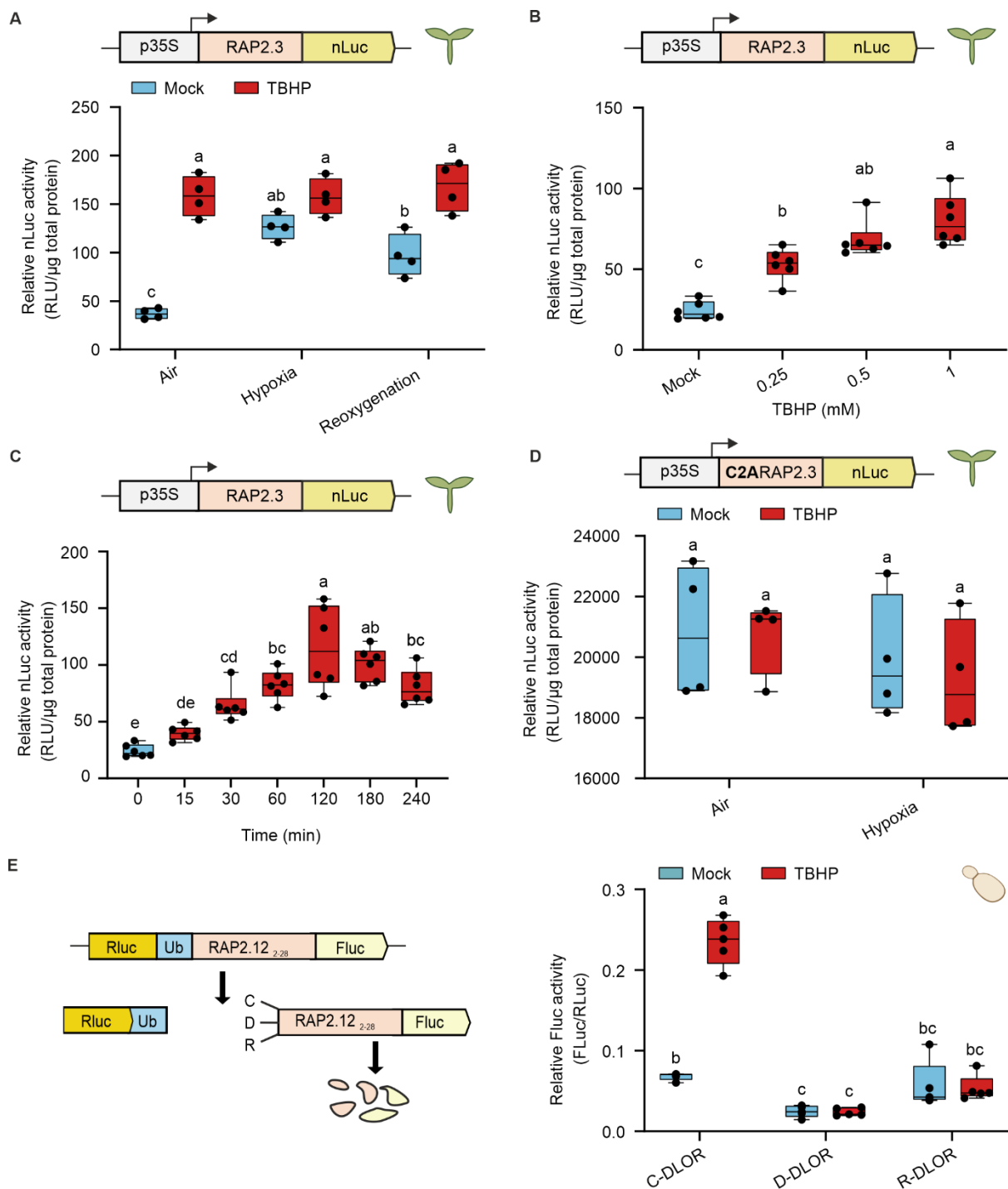


Figure 3 – Oxidative stress increases ERFVII stability in a N-degron pathway-dependent manner. (A) Relative nLuc activity of 35S:RAP2.3-nLuc seedlings treated under hypoxic (1 % O₂ v/v O₂/N₂) or aerobic (21 % O₂ v/v O₂/N₂) conditions for 6 h, followed by 3 h

reoxygenation, 1 mM TBHP or mock treatment ($n = 4$). **(B)** Relative nLuc activity of 35S:RAP2.3-nLuc seedlings treated with different TBHP concentrations upon aerobic conditions over 4 h ($n = 6$). **(C)** Relative nLuc activity of 35S:RAP2.3-nLuc seedlings treated with 1 mM TBHP upon aerobic conditions measured over 6 h ($n = 6$). **(D)** Relative nLuc activity of 35S:C2ARAP2.3-nLuc variant upon 1 mM TBHP or mock treatment, under hypoxic or (1 % O_2 v/v O_2/N_2) or aerobic (21 % O_2 v/v O_2/N_2) conditions ($n = 4$). **(E)** Relative FLuc activity in yeast cultures expressing AtPCO4 together with either C-DLOR, R-DLOR, or D-DLOR and exposed to 0.5 mM TBHP or mock treatment for 30 min. Statistical differences were evaluated using one-way ANOVA **(B)** or two-way ANOVA **(A, D, E)** ($p < 0.05$) followed by Tukey HSD test ($p < 0.05$).

H₂O₂ inhibits recombinant PCO enzymes

We set out to determine the biochemical mechanism that leads to inhibition of Nt-Cys sulfinylation in plant cells. We first examined whether TBHP or H₂O₂ treatment could inhibit PCO activity. We treated 10 μ M recombinant AtPCO4 with 50 μ M TBHP or H₂O₂ and then tested its ability to catalyse oxidation of a 14-mer peptide corresponding to the RAP2.12 N-terminus (hereafter RAP₂₋₁₅) using liquid chromatography-mass spectrometry (LC-MS) as described previously⁴¹. Both treatments led to a significant reduction in AtPCO4-catalysed RAP₂₋₁₅ oxidation compared to untreated enzyme (**Figure 4A**). In a dose-dependence assay, we found 3 to 5 μ M H₂O₂ to be sufficient to inhibit 50 % activity of 10 μ M AtPCO4 (**Figure 4B**). We also assessed the sensitivity of all five AtPCOs to H₂O₂ (**Figure 4C**) and found that all of these enzymes showed significantly decreased activity in response to H₂O₂ exposure, with strongest inhibition for AtPCO4 and AtPCO5.

Given that Cys residues are susceptible to non-enzymatic oxidation, we also tested whether H₂O₂ could directly oxidise RAP₂₋₁₅ under the same conditions and thus generate potential N-degrons. We found that high concentrations (1 mM) H₂O₂ treatment resulted in a small amount of spontaneous RAP₂₋₁₅ Nt-Cys oxidation (**Figure 4A**), comprising sulfenic acid (+16 Da), sulfinic acid (+32 Da), or sulfonic acid (+48 Da) (**Figure S4**), however the levels of RAP₂₋₁₅ oxidation were low (5.3 %) compared to AtPCO4-catalysed oxidation. Overall, therefore, the results indicate that the primary effect of H₂O₂ and TBHP on N-degron pathway mediated ERFVII stability is inhibition of PCO activity.

To explore the mechanism of H₂O₂-mediated PCO enzyme inhibition, we first tested whether H₂O₂ causes oxidative modification of the AtPCO4 enzyme. Whole protein mass spectrometry of H₂O₂-treated enzyme revealed a +209 Da mass increase (**Figure S5A**) which we considered likely due to non-specific oxidation of susceptible residues. Cys residues are particularly prone

to oxidative modification to Cys-sulfinic acid, therefore we used a sulfinic acid-specific DiaAlk probe ⁴² which confirmed that Cys residues of *AtPCO4* undergo sulfinic acid modification upon H_2O_2 treatment (**Figure S5B**). Further interrogation using LC-MS/MS based proteomic analysis of H_2O_2 treated and non-treated *AtPCO4* showed that Cys165, Cys172 and Cys190 of *AtPCO4* form sulfinic (+31.98 Da) and sulfonic acids (+47.97), all of which are close to the *AtPCO4* active site (**Figure D**), with the highest proportion of oxidation occurring on Cys165 upon H_2O_2 treatment (**Figure S5C-F**). Oxidation of any of these Cys residues could therefore potentially negatively impact the ability of *AtPCO4* to bind substrate or catalyse oxidation. The PCOs are Fe(II)-dependent thiol dioxygenases ⁸ (**Figure 4D**), therefore as well as protein modification, ROS-mediated oxidation of PCO active site Fe(II) could result in their inhibition. Continuous wave electron paramagnetic resonance (CW-EPR) spectroscopy revealed that H_2O_2 treatment resulted in an increase in rhombic Fe(III) signal at the *AtPCO4* active site, similar to that seen upon treatment of isolated $Fe(II)_{\text{aqueous}}$ with H_2O_2 (**Figure 4E**). This confirmed that Fe(II) oxidation is indeed another possible mechanism for PCO inhibition.

Finally, we tested whether the H_2O_2 mediated inhibition of *AtPCO4* could be restored using known cellular reductants glutathione and ascorbate ^{43,44} as well as a synthetic thiol reductant dithiothreitol ⁴⁵. None of the reductants were able to recover the activity of H_2O_2 -treated enzyme (**Figure 4F**), consistent with the duration of ERFVII stabilisation seen *in vivo*. Collectively, the results indicate non-specific impacts of H_2O_2 treatment on PCO function, likely combining both catalytic inactivation and impacts on protein structure, which together result in the ERFVII stabilisation observed *in vivo*.

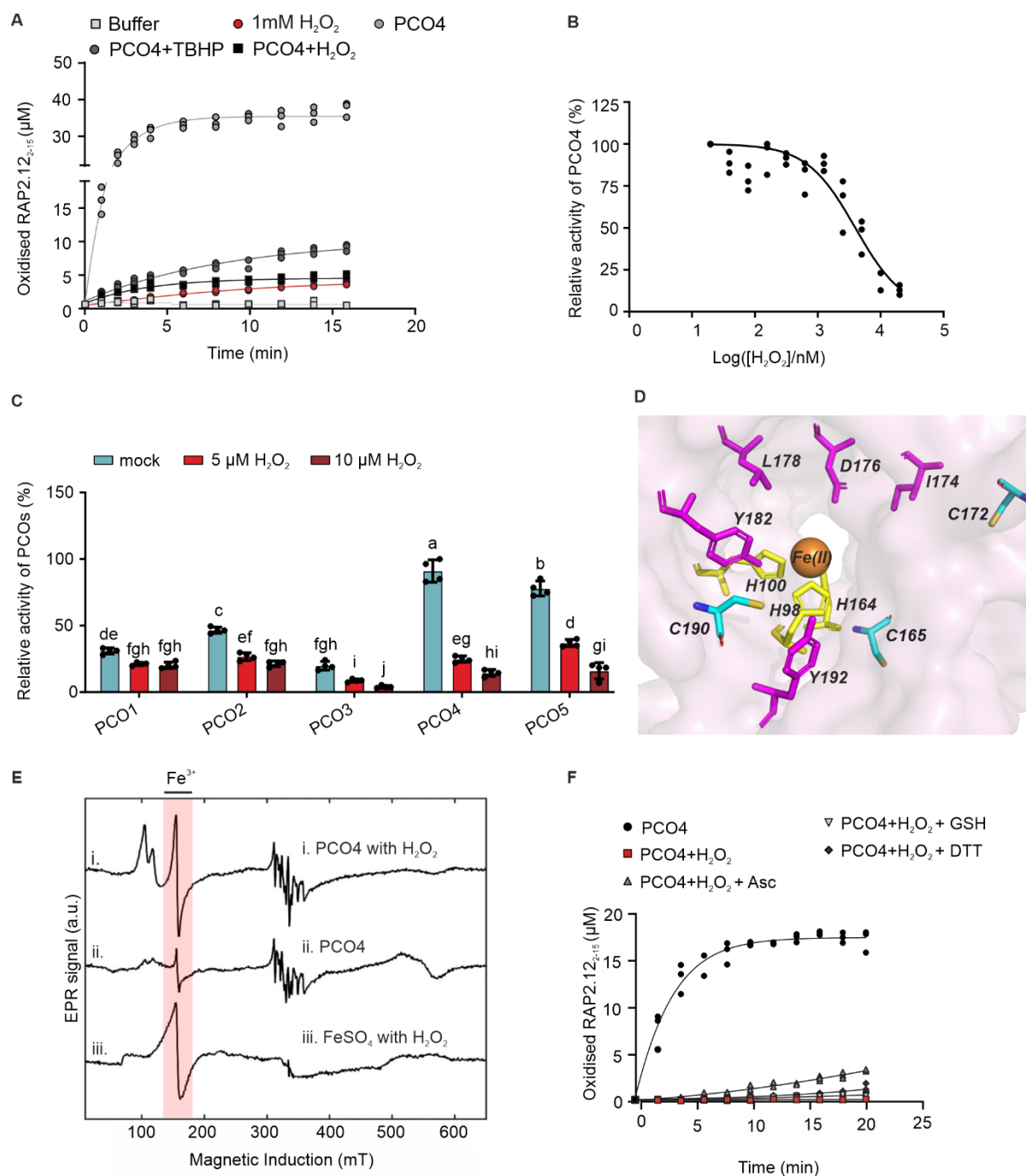


Figure 4 – H₂O₂ inhibits recombinant AtPCO enzymes. (A) The activity of 50 μM H₂O₂- and 50 μM TBHP-treated or non-treated recombinant PCO4 enzyme-catalysed oxidation of RAP2₂₋₁₅. Non-enzymatic RAP2₂₋₁₅ oxidation by 1 mM H₂O₂ was included to compare with the oxidation of RAP2₂₋₁₅ by PCO4 ($n = 3$). (B) H₂O₂ dose-dependent effect on PCO4 enzyme activity ($n = 3$). (C) H₂O₂-mediated inhibition of PCOs1-5; statistical differences were evaluated using two-way ANOVA followed by Tukey HSD test ($p < 0.05$). Error bars display standard deviation (S.D.) ($n = 3$). (D) Active site view of crystal structure of AtPCO4 (PDB: 6S7E); Fe²⁺ cofactor (orange sphere) is bound by a triad of His residues His98, His100,

and His164 (highlighted in yellow), Cys residues found to be oxidised by H_2O_2 shown in cyan. (E) X-band CW-EPR spectra of (i) AtPCO₄ with H_2O_2 , (ii) AtPCO₄ only and (iii) FeSO_4 (aqueous) with H_2O_2 . Spectrum in (i) shows Fe(III) signal intensity at 150 mT similar to that seen in (iii), but with signal splitting at $g_{\text{eff}} = 4.29$, $g'_{\text{eff}}=6.4$ and $g''_{\text{eff}}=5.7$, first as typical rhombic coordination of Fe(III) with two axially-symmetric species. (F) Activity restoration test of H_2O_2 -treated PCO₄ enzyme, using known cellular reductants glutathione (GSH), ascorbate (Asc) and dithiothreitol (DTT) ($n = 3$).

ROS-triggered oxidative stress disrupts hypoxia-responsive gene expression

Our results indicate that elevated ROS, as occurs in reoxygenation, causes inhibition of PCO activity, leading to stabilization of ERFVII. This unexpected result contrasts with previous reports that show rapid repression of hypoxia-inducible genes upon reoxygenation^{46,47} as well as lack of activation of the same set of genes under oxidative stress conditions⁴⁸. To address this conundrum, we set out to investigate whether ROS signaling affects the hypoxia response in Arabidopsis by measuring the expression of hypoxia-responsive genes (HRGs) upon hypoxia, reoxygenation and direct treatment with TBHP. We exposed seven-day old seedlings to 1 mM TBHP or mock control under normoxia, hypoxia and reoxygenation. We monitored the expression of six hypoxia marker genes: three involved in fermentative metabolism (*Alcohol Dehydrogenase 1 (ADH1)*, *Pyruvate Decarboxylase 1 (PDC1)* and *sucrose synthase 1 (SUS1)*), the *non-symbiotic hemoglobin 1 (Hb1)* and two transcription factors (*HYPONIC RESPONSE ATTENUATOR1 (HRA1)*, and *LATERAL ORGAN BOUNDARY DOMAIN 41 (LBD41)*)¹², using real-time qPCR. This analysis confirmed upregulation of all six genes in hypoxia and their downregulation under reoxygenation conditions (**Figure 5A**, **Figure S6A**), consistent with findings from previous studies⁴⁶. Interestingly, TBHP-treated plants showed significantly lower expression levels of all hypoxia-responsive genes compared to untreated plants under hypoxia (**Figure 5A**, **Figure S6A**), with expression levels similar to aerobic conditions. These genes also showed low expression levels in reoxygenated plants treated with TBHP. These results are surprising given that we observed ERFVII stabilisation on TBHP treatment and/or reoxygenation and suggested that ROS interfere with the plant's hypoxia responses, even while plants are experiencing hypoxia stress. Consistent with these observations, expression of the core hypoxia-responsive genes in the *RAP2.3-nLUC* transgenic Arabidopsis lines was also elevated in hypoxia, but significantly decreased upon TBHP-treatment under hypoxia and/or under reoxygenation (**Figure S7A**). To determine whether TBHP-treatment promoted expression of oxidative stress responsive genes, we investigated expression of *Cysteine-Rich Receptor-Like Kinase (CRK36)*, *GLUTATHIONE S-TRANSFERASE (CLASS TAU) 24 (GSTU24)* and *zinc-finger protein 12 (ZAT12)* in

Arabidopsis wild-type seven-day old seedlings under similar treatments (**Figure 5A, Figure S6B**). These genes are reported to be triggered by ERFVII transcription factors ²⁸. We found that all three genes were upregulated upon TBHP treatment (**Figure 5A, Figure S6B**). These results confirmed that the plants did indeed experience oxidative stress as a result of TBHP treatment, and demonstrate that TBHP treatment does not inhibit their transcriptional machinery, but selectively acts to prevent expression of HRGs.

HRGs are controlled by ERFVIIIs through their interaction with the Hypoxia Responsive Promoter Element (HRPE) ^{11,49,50}. To understand how TBHP treatment resulted in lowering of HRG expression, we investigated whether oxidative stress decreases the binding of ERFVIIIs to HRPEs. To do this, we used a transgenic Arabidopsis line with a synthetic promoter harbouring a five-time repeat of HRPE driving the expression of a gene encoding a nanoluciferase enzyme (*nLuc*). We measured the expression level of *nanoluciferase* as well as the expression of the same six core hypoxic genes described above, in air, hypoxia and reoxygenation with or without TBHP-mediated oxidative stress (**Figure 5B, Figure S7B**). We found that *nLuc* expression was upregulated under hypoxic conditions, but downregulated upon oxidative stress, similar to the HRGs (**Figure 5B, Figure S7B**). These results indicate that TBHP treatment interferes with the transactivation of genes whose promoter includes at least one HRPE, and not through a different DNA motif.

We next raised the question of whether oxidative stress hinders the ability of ERFVII to physically interact with the HRPE. We performed a chromatin immunoprecipitation (ChIP) experiment using a GFP-tagged truncated version of RAP2.12 lacking 13 amino acid residues at the N-terminus (Δ 13RAP2.12-GFP ^{28,35}). Due to the absence of the Cys- residue at the N-terminus, RAP2.12 is constitutively stable and therefore able to transactivate the hypoxia response. Seven-day old seedlings were treated with TBHP upon normoxic conditions, followed by evaluation of the enrichment of the promoter regions of four hypoxia-responsive genes, *ADH1*, *HB1*, *HRA1* and *LBD41*, together with three ERFVII-dependent oxidative stress-responsive genes *ZAT12*, *ATH8* and *GSTU24* (**Figure 5C**). *UBQ10* and *EIF4A1* promoters, which do not respond to ERFVII, were used as negative controls. ChIP-qPCR revealed an enrichment for all genes upon normoxic conditions, when compared to the negative controls, confirming that Δ 13RAP2.12-GFP binds to the promoter regions of these genes. A significant reduction in genomic enrichment was only observed for the *LBD41* promoter upon TBHP treatment, while no statistical difference was measured for any other promoters. These results suggest that oxidative stress only mildly affects RAP2.12 binding to selected genomic regions. Additional regulation is therefore likely required to reach the complete suppression of the transcriptional hypoxic response observed in this study (**Figure 5A, 5B, Figure S6A**).

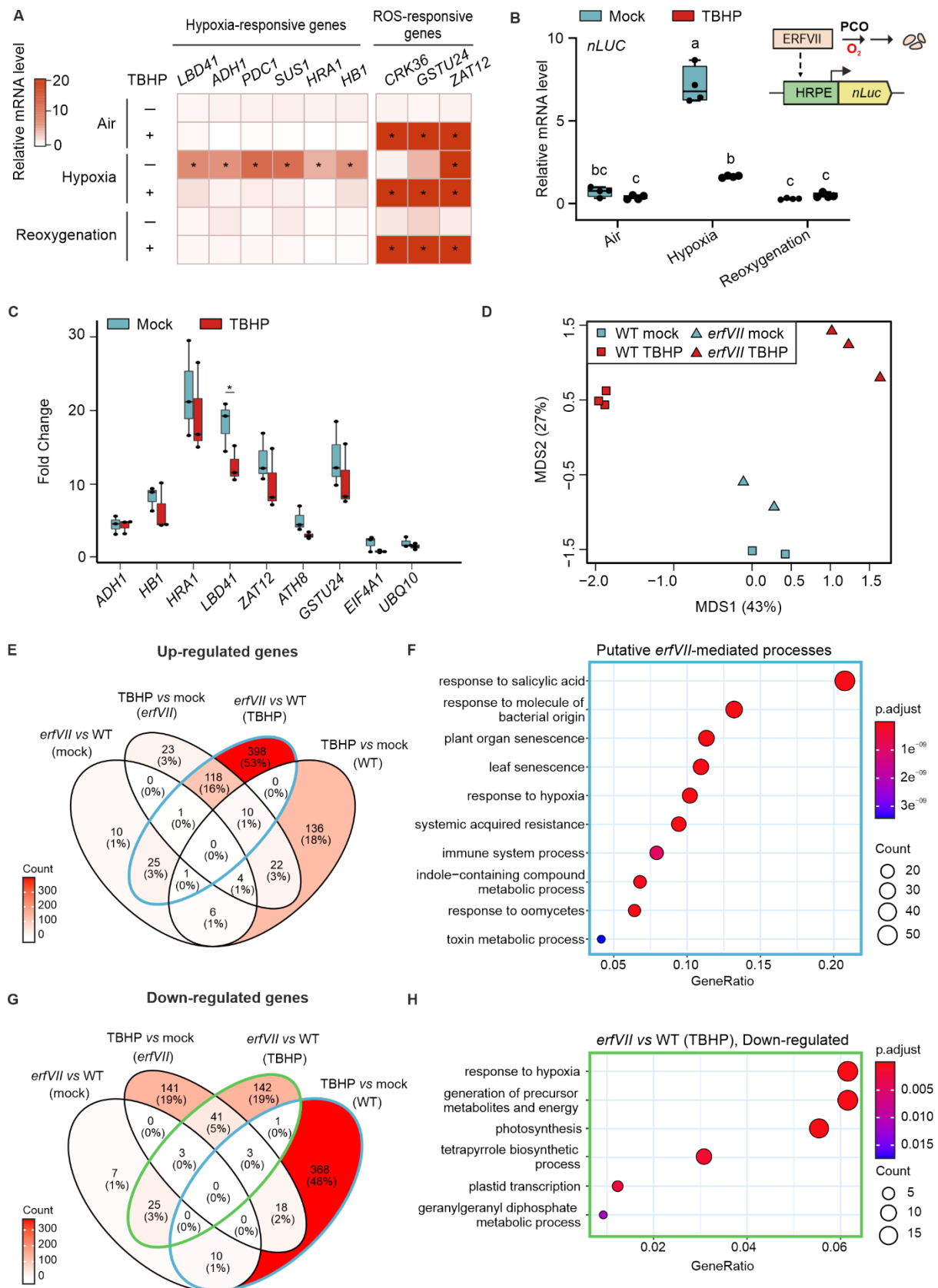


Figure 5 – Oxidative stress induces an ERFVII-dependent transcriptional change, without affecting the ERFVII DNA binding affinity. (A) Relative expression of hypoxia-responsive and ROS-responsive genes in air, hypoxia or reoxygenation, upon 1 mM

TBHP or mock in Col-o seedlings ($n = 4$). Full dataset is shown in Figure S5. **(B)** Relative expression of *nLuc* in HRPE:*nLuc* seedlings treated with 1 mM TBHP or mock under air, hypoxia or reoxygenation ($n = 4$). Statistical analyses were conducted using two-way ANOVA followed by Tukey HSD test ($p < 0.05$). Different letters indicate statistically different groups. **(C)** ChIP-qPCR analysis of $\Delta 13\text{RAP2.12-GFP}$ for hypoxia-responsive genes (*ADH1*, *HB1*, *HRA1*, *LBD41*) and oxidative stress genes (*ZAT12*, *ATH8*, *GSTU24*) promoters, compared to negative controls (*EIF4A1*, *UBQ10*). Asterisks indicate statistically significant differences between the two mean values for each gene (two-sided Student's t-test, $p < 0.05$, $n = 3$). **(D)** Multidimensional scaling plot (MDS) conducted on the normalized gene expression values of *erfVII* and Col-o seedlings treated with TBHP or mock. Horizontal and vertical coordinates show MDS1 and MDS2, respectively, with the amount of variance contained in each component (43 % and 27 %). Each point in the plot represents a biological replicate. Differences in symbols and colour indicate difference in genotype and treatment, respectively. **(E)** Venn Diagram indicating the overlap of up-regulated genes for *erfVII* or Col-o treated upon TBHP or mock conditions. **(F)** GO enrichment results for biological process of genes that are up-regulated genes in *erfVII* seedlings compared to wild type, upon TBHP treatment, and down-regulated in the wild type upon TBHP treatment compared to mock. **(G)** Venn diagram indicating the overlap of down-regulated genes for *erfVII* or Col-o treated upon TBHP or mock conditions. **(H)** GO enrichment results for biological process of the down-regulated genes in TBHP-treated *erfVII* seedlings compared to wild type. Circle size indicates the gene count per GO term, with colour maps indicating the False Discovery Rate (FDR) value ($p.\text{adjust}$).

ERFVIIIs control transcriptional reprogramming during oxidative stress

Having shown that ERFVII stability upon oxidative stress does not induce the canonical hypoxia response, we set out to investigate more broadly the contribution of ERFVII transcription factors to the transcriptional response to oxidative stress. To this end, we sequenced the poly(A)-enriched transcriptome of seven-day old wild-type and *erfVII* seedlings treated with 1 mM TBHP. We included untreated seedlings of both genotypes as controls. Multidimensional scaling (MDS) analysis (**Figure 5D**) confirmed a moderate effect of ERFVII inactivation under control conditions⁵¹, where only 92 genes were differentially expressed ($|\text{FC}| > 1.5$, $p < 0.05$) between the mutant and the wild type (**Fig. 5E**). The group of significantly downregulated genes included four of the 49 core hypoxia-responsive genes, likely expressed in cells physiologically exposed to low-oxygen tension² (**Table S6**). The *erfVII* mutant seemed to modulate gene expression as if experiencing a mild oxidative stress under control conditions, as demonstrated by the separation of samples in the dimension primarily affected by TBHP (**Figure 5D**). Moreover, exposure to TBHP exerted a clearly

distinct effect on the two genotypes. Indeed, pairwise comparisons revealed that the *erfVII* mutant induced a higher number of genes than the wild type (**Figure 5E**). We found 282 genes that were both up-regulated in the *erfVII* mutant upon TBHP treatment compared to the wild type and down-regulated in the wild type upon TBHP treatment compared to mock conditions. This set of genes included those involved in salicylates-mediated response, senescence, hypoxia, and oxidative stress responses (**Figure 5F**). This indicates that TBHP-stabilised ERFVIIs repress these transcriptional responses. In contrast, 136 genes (18 %) were not up-regulated by TBHP treatment in the *erfVII* mutant, opposite to what we observed in the wild type, indicating that TBHP-stabilised ERFVIIs also act as activators of expression of this subset of genes (**Figure 5E**). Additionally, the 142 genes which were downregulated upon TBHP treatment in *erfVII* seedlings, but not in the wild type, were enriched in GO terms related to plastids, chlorophyll biosynthesis and photosynthesis (**Figure 5G, H**). Overall, our results expand the molecular role of ERFVIIs as both activators and repressors of transcription (in a gene-selective manner) in response to hypoxia and oxidative stress, providing a potential rationale for their implication in responses to both reoxygenation and a broader range of biotic and abiotic stresses.

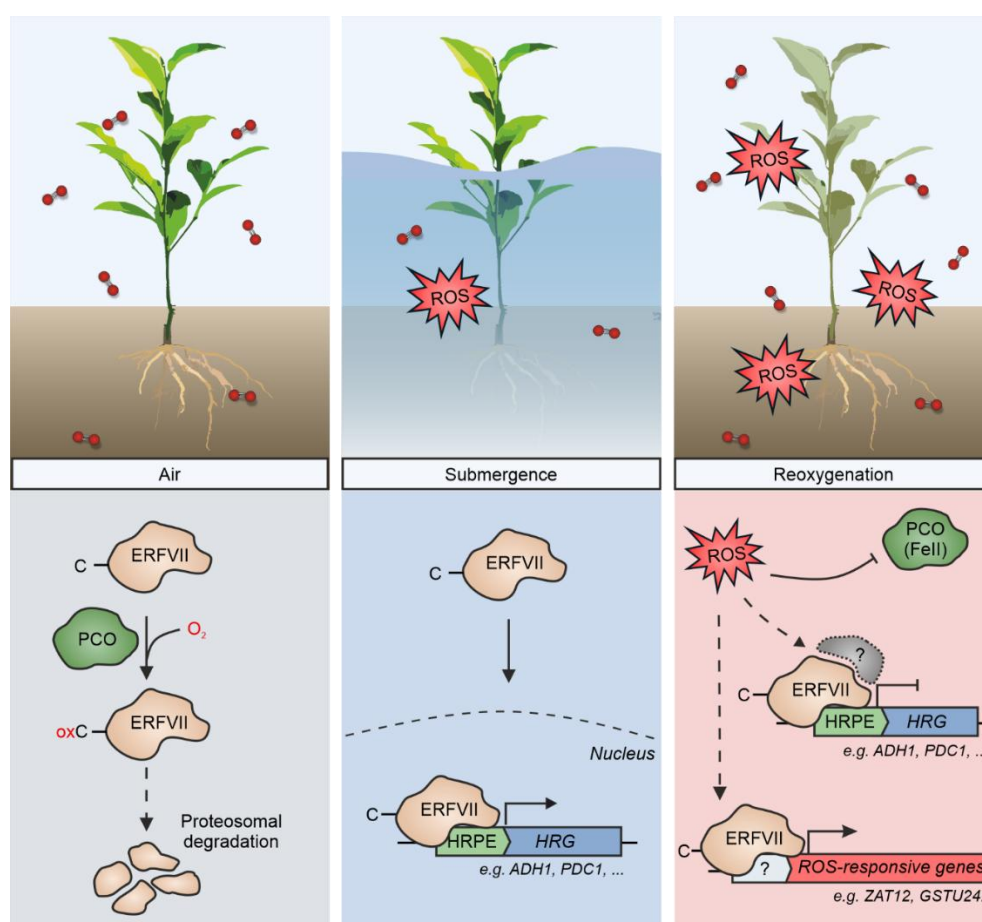


Figure 6 – PCO-ERFVII signaling as dual sensor for low-oxygen and oxidative stress. ERFVIIs transcription factors are constitutively expressed under aerobic conditions

and continuously degraded via the N-degron pathway. Upon low-oxygen conditions, such as submergence, lack of oxygen reduces PCO activity, leading to ERFVIIs stabilization and relocalization into the nucleus, where they activate anaerobic genes expression. Upon reoxygenation, a ROS burst occurs which inhibits PCO activity, therefore stabilizing ERFVIIs. Additionally, ROS redirects the transcriptional machinery inducing the expression of oxidative stress genes and repressing the canonical hypoxic response.

Discussion

Plant flood survival requires tolerance to both submergence-induced hypoxia and the reoxygenation stress associated with desubmergence. The molecular response to submergence-induced hypoxia is well-characterised, comprising PCO/O₂-dependent regulation of ERFVII stability and the consequent upregulation of genes enabling adaptive metabolic reconfiguration^{4,10,35,36}. What is less well characterised is the impact on this oxygen-sensing machinery of the ROS burst known to be associated with reoxygenation.^{13–19} We therefore set out to determine whether there is any cross-talk between reoxygenation, ROS, and PCO-ERFVII function. We first confirmed that ERFVIIs have a role in facilitating both tolerance to, and recovery from, hypoxia-reoxygenation stress (**Figure 1**). We then confirmed that ERFVIIs persist in the nucleus after reoxygenation (**Figures 2, 3**) and collected evidence that this is caused by H₂O₂-mediated inactivation of the plant Nt-Cys-degron pathway at the PCO level, leading to ERFVII stabilisation (**Figure 4**). Surprisingly, we found that ROS-stabilised ERFVIIs do not promote, but rather repress, the expression of hypoxia-responsive genes (**Figure 5**). Finally, we uncovered that ERFVIIs modulate transcription of a different set of genes in response to ROS than they do in response to hypoxia (**Figure 5**) suggesting the PCO-ERFVII signaling pathway can be redirected in a stress-specific manner (**Figure 6**). ERFVII transcription factors have previously been proposed to mediate responses to multiple stresses, including high salinity, heat stress and high temperature^{30–32}. These stresses are proposed to impact the degradation of ERFVIIs via the N-degron pathway, for example salt stress is reported to decrease NO production^{32, 52} and is speculated to impair ATE or PRT6 function³⁹. Our data, showing that exogenous supply or endogenous production of H₂O₂ results in PCO inhibition and ERFVII stabilisation, suggests that other conditions that involve enhanced H₂O₂ accumulation, such as cold stress⁵ or pathogen attack^{53,54}, may also invoke ERFVII stabilisation via this method.

PCOs are able to respond sensitively to O₂ availability through the kinetic effect on ERFVII oxidation. The response of PCOs to the presence of H₂O₂ however is less finely tuned; inhibition of PCO function by H₂O₂ appeared to occur in a less specific manner, likely via a combination of both oxidation of the active site Fe(II) to Fe(III), as detected by EPR, and via

oxidation of Cys residues in or near the active site, as detected by Cys-sulfinic acid-detecting probes and tryptic digest-mass spectrometry. It is also possible that other oxidative modifications took place that we were unable to detect. These modifications are all capable of impacting substrate binding and catalysis to decrease or ablate the activity of the enzyme. Notably, there is a precedent for ROS-mediated inhibition of oxygen-sensing enzymes in mammalian systems, where H₂O₂-mediated inhibition of Factor Inhibiting HIF, an Fe(II) and 2-oxoglutarate-dependent oxygenase, has been reported⁵⁵. We were not able to observe repair of H₂O₂-mediated PCO inhibition with cellular antioxidants *in vitro*, however it would be of interest to examine whether modulation of redox status in cells could impact ERFVII stabilisation, or indeed whether enzymes that can reverse Cys oxidation may have a role in protecting PCOs from ROS-mediated damage.

Our intriguing findings that upon ROS treatment, despite ERFVII stabilisation and retention at HRPEs, HRGs are ‘turned off’ while responses to oxidative stress are ‘turned on’, provides an explanation for the role of ERFVII as a major hub to control adaptation to different adverse environmental conditions³² (**Figure 6**). The versatility of ERFVII in producing stress-tailored responses is surprising and can be explained by their interaction with distinct proteins and DNA motifs. Our results indicate that oxidative stress causes only mild dislocations of ERFVII from hypoxia-responsive genes and more likely turns ERFVII from positive to negative regulators of gene transcription. This could explain the limited overlap between the transcriptional changes observed between hypoxia and other stresses that involve ERFVII stabilisation, despite the crucial role these TFs play in enabling tolerance. A systematic survey of their protein and DNA partnerships under different conditions will reveal the complex regulatory networks orchestrated by these TFs. The trihelix TF HRA1, previously reported to associate with RAP2.12 and decrease its trans-activation capacity, is a promising candidate for such a role. The different assortment of conserved motifs identified in ERFVII in vascular plants suggests a plethora of different interactions depending on the group member. For example, the Hypoxia Responsive ERFVII HRE1 and HRE2 seem to play a minor role in the early activation of hypoxic genes, although it is possible they are majorly involved in other transcriptional responses³⁵. The generation of ERFVII variants devoid of specific domains will support the hunt and the characterisation of these interactions.

The ability of ERFVII to regulate different subsets of genes in response to different stimuli raises questions about the evolution of these TFs. Was the last common ancestor of the ERFVIIs, which likely appeared in the last common ancestor of land plants⁵¹, a generic regulator of stress tolerance required to cope with an environment of increasing ROS? Or rather did the ERFVIIs evolve first as signal transducers for hypoxia, and only later expand their role to accommodate the response to other stresses? In today’s environment, however, it

appears that the PCO-ERFVII sensing and signaling nexus is more nuanced than a simple response to fluctuations in O₂ availability. Its sensitivity to oxidative stress, including that which occurs upon reoxygenation, allows dynamic control in response to a range of cues. The overall impact of this ability to switch between subsets of genes explains the key role played by ERFVIIIs in plant survival of both submergence and desubmergence.

Acknowledgements

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Author Contributions

S.A., M.P., B.G., M.S., E.F. and F.L. conceived and designed the study. S.A., M.P., M.L-P., B.F., S.L., L.D.C., V.S., Y.T., D.Z., W.M., D.M.G. and P.B. conducted experiments. All authors analysed data and prepared figures. S.A., M.P., E.F. and F.L. wrote the manuscript with input from all authors.

Declaration of Interests

The authors declare no competing interests.

Supplemental information

Document S1, containing Figures S1-S8 and Tables S1-S5.

Document S2, excel file containing fold change data of RNA sequencing.

STAR Methods

Experimental model and subject details

Plant materials and growth conditions

A. thaliana Columbia-o (Col-o) was used as wild-type ecotype. The genotypes RAP2.12₁₋₂₈-FLuc, RAP2.12-GFP and Δ 13RAP2.12-GFP have been previously described^{4,35}. Seeds were sown in a soil:vermiculite 3:1 ratio mixture, stratified at 4 °C in the dark for 3 days, then germinated at 22 °C/20 °C with a 16h:8h, light:dark photoperiod and 100 μ mol photons m⁻² s⁻¹ intensity. For *in vitro* propagation, seeds were sterilised using 70 % ethanol for 1 min, incubated in 10 % sodium hypochlorite (NaClO) for 10 min, followed by six washes in 1 mL sterile distilled water. Growth in liquid medium was performed by inoculating 100 μ L of seeds suspension, corresponding to 20-40 seeds, in 1 mL of sterile half-strength MS medium (basal salt mixture 2.15 g L⁻¹, pH 5.7) supplemented with 1 % sucrose in each well of 6-well plates. For growth in solid media, seeds were incubated in the dark at 4 °C for 2 days and subsequently on half-strength MS⁵⁶ medium, supplemented with 1 % (w/v) sucrose and 0.8 % (w/v) agar, and grown at 22°C, 16:8 day:night photoperiod 100 μ mol photons m⁻² s⁻¹ intensity. All experiments were conducted on seven-day old seedlings.

Yeast strains and culture

A haploid parental strain BY4742 (Mat α ; his3- Δ 1; leu2- Δ 0; lys2- Δ 0; ura3- Δ 0) was co-transformed following the LiAc/SS carrier DNA/PEG method⁵⁷ with PCO4-pAG415GPD and different versions of the DLOR-pAG413GPD (C-, D- or R-DLOR). All plasmids used for yeast expression were produced in previous works⁵⁸. Prior to transformation, cells were grown at 30°C on YPDA (20 g L⁻¹ peptone, 10 g L⁻¹ yeast extract, 20 g L⁻¹ of glucose (Duchefa) and 20 mg L⁻¹ adenine hemisulfate (Sigma-Aldrich), supplied with 20 g L⁻¹ agar (Duchefa) when necessary). Transformants were selected on SD medium, containing 6.7 g L⁻¹ Yeast Nitrogen Base (DIFCO), 1.37 g L⁻¹ Yeast Dropout Medium (Sigma-Aldrich) and 20 g L⁻¹ glucose, plus supplements (0.16 M uracil, 0.8 M histidine- HCl, 0.8 M leucine and 0.32 M tryptophan (Sigma-Aldrich) when complete), with 20 g L⁻¹ agar when solid.

Bacterial strains

Bacterial strain *Escherichia coli* BL21 (DE3) was used for expression of recombinant Plant Cysteine Oxidases (PCOs). Bacteria were cultured in 2YT medium at 37°C until OD₆₀₀ reached 0.6, protein expression was induced by addition of 0.8 mM Isopropyl- β -D-thiogalactoside (IPTG, Sigma-Aldrich) at 18°C for 16 h with 170 rpm in shaking incubator (Eppendorf, UK).

Method Details

Construct generation

For the generation of 35S:RAP2.3-*nLuc* construct, an 806 nt long synthetic string containing Arabidopsis codon-optimised *nLuc* sequence including RAP2.2 intron (**Table S1**) was synthesised in the pMK-RQ backbone by Geneart (Thermo Fisher Scientific). The destination vector pK7GWnL2 was generated as ligation between pK7GW2⁵⁹ and GWnLuc-intron after restriction using XbaI and MluI (Thermo Fisher Scientific). Arabidopsis RAP2.3 CDS was amplified from Col-o cDNA without stop codon (RAP2.3Δstop) introducing overlapping *AttB* sites using PCR using GoTaq® DNA polymerase (Promega). Entry clone vector was then generated as a BP reaction between RAP2.3 CDS PCR product and pENTR/D-TOPO (Life Technologies). The resulting entry vector was finally recombined into the hereby generated pK7GWnL2 destination vector using the LR clonase mix II (Thermo Fisher Scientific). Primers for RAP2.3Δstop cloning and screening are listed in **Table S2**. For the generation of 35S:RAP2.3-GFP construct, entry vector containing RAP2.3 CDS was recombined with pK7GW2F⁵⁹ destination using the LR clonase mix II (Thermo Fisher Scientific).

For HRPE-*nLuc* construct design, DNA containing the SacI-attR1-ccdB-attR2-NanoLuc-HindIII sequence (**Table S3**) was *de novo* synthesised by GeneArt service (Thermo-Fisher Scientific, Waltham, MA, USA) and cloned into the pBGWL7 Gateway destination vector⁶⁰. The entry vector containing the HRPE:5'-UTR ADH1 sequence⁵⁰ was recombined into the destination vector by Gateway cloning.

For recombinant protein production, the five *pco* genes from Arabidopsis had previously been cloned into the NdeI and XhoI sites of pET28a (Novagen), transformed into *E. coli* NEB5α competent cells (New England Biolabs) and sequences validated by Sanger sequencing (Source Biosciences) as previously described⁶¹.

Arabidopsis transformation

Agrobacterium-mediated transformation was performed to obtain RAP2.3-nLUC, RAP2.3-GFP and HRPE:nLuc stable transgenic lines using the floral dip method as previously described⁶². T₀ seeds were selected for resistance on agarized half-strength MS medium supplemented with the corresponding antibiotic and subsequently transferred in soil. The presence of the transgene was detected by PCR using GoTaq® DNA polymerase (Promega). T₃ generation plants were used for the experiments.

Chemical treatment

Oxidative stress in plants was induced by 1 mM TBHP treatment diluted in MilliQ water for 6h in normoxia and 6h in hypoxia. After 3 h, the media was replenished with TBHP.

Low-oxygen and reoxygenation treatments

For hypoxia treatments, seedlings were grown in six-well plates in liquid media and subjected to anaerobic conditions inside the Hypoxic Workstations (Whitley) continuously flushed with an artificial humidified atmosphere containing a mixture of oxygen (1%) and nitrogen gases (99 %) at 22°C for 6 h. For severe hypoxic treatment, seedlings were grown vertically in square plates and treated with 0.1 % O₂ v/v O₂/N₂ for 24 h. During the hypoxic and anoxic treatments, the seedlings were maintained in the dark to avoid oxygen release by photosynthesis. Seedlings used for control samples were maintained in aerobic conditions (21 % O₂ v/v O₂/N₂) at dark for an equal amount of time. After low-oxygen, plants were transferred to the aerobic growth conditions for reoxygenation treatment.

Yeast treatments

For TBHP treatments, colonies were inoculated in 5mL of -his -leu SD medium, grown overnight, diluted to half in fresh media and further diluted to OD₆₀₀ = 0.1. Cultures were grown for 5 h prior to the treatment. TBHP was then supplied for up to 30 min at different concentrations (0, 0.25, 0.5 and 0.75 mM). 50 µl of culture were harvested for luciferase assays, centrifuged at 15,000 rpm for 5 min, frozen and extracted in 50 mL of PLB. Luciferase was measured using the Dual-Luciferase® Reporter (DLR™) Assay System (Promega) as described before ³⁹. 1 mL of culture was used for OD₆₀₀ spectrometric measurements.

Root length and survival rate measurements

To assess reoxygenation tolerance, seven-day old seedlings were treated as previously mentioned. Primary root length was measured both before and after 4 days of recovery, fresh weight and survival rate were assessed following 4 days of recovery. Transparent squared plates containing Arabidopsis seedlings were scanned using the EPSON Perfection V750 PRO scanner with a 720 dots per inch resolution. Growth rate was measured as increase in length of the primary root divided by days of recovery. Primary root length was assessed using ImageJ ⁶³.

Histochemical H₂O₂ staining and quantification

Reactive oxygen species were visualised using 3,3'-diaminobenzidine (DAB, Fluorochem Ltd) staining to detect H₂O₂ using methods described previously ⁶⁴ with minor modifications. Seedlings were incubated with either 1mg/mL DAB, vacuum infiltrated for 5 min and incubated for 4-5 h at dark shaking. After staining, seedlings were washed with distilled water and bleached in several washes of 70 % ethanol. 5-8 seedlings were analysed per condition using the Leica M165C stereo microscope with 2.5x magnification, followed by quantification of pixel intensity using ImageJ ⁶³.

Fluorescent biosensing of oxidative stress in Arabidopsis

Arabidopsis wild-type plant lines stably expressing the protein sensor roGFP2-Orp1 with cytosolic and nuclear localization have been described before ³⁴. Sensor lines in *erfVII* background ⁶⁵ were generated by Agrobacterium-mediated transformation via floral dip with a pH2GW7:cyt-roGFP2-Orp1 expression construct. Positive transformants were selected on selection medium with resistance marker Hygromycin B and based on fluorescence. Measurements were performed using two independent sensor lines to control for any potential effects of sensor insertion loci. Replicates from the two lines were then combined for each genotype.

Leaf discs of five-week old plants and seven-days old seedlings were submerged in wells of a 96-well plate filled with 200 μ L standard assay medium (10 mM MES (pH 5.8 with KOH), 10 mM MgCl₂, 10 mM CaCl₂, 5 mM KCl). A single leaf disc was placed in each well with the abaxial side up for the leaf disc experiment whereas 5 to 6 seedlings were placed per well in case of seedlings experiment. Ratiometric readout of the biosensor was performed using a multiwell fluorimeter (ClarioStar Plus, BMG Labtech) in top optics mode, using 30 excitation flashes distributed in a 3 mm orbital averaging diameter (leaf discs) and in a 3 mm spiral averaging diameter (seedlings). Samples of both genotypes were measured side by side using the same gain for the fluorophore channels (Ex1: F:400-10, Ex2: F482-16; Dichroic Mirror F:LP504; Em: F:520-10) for all samples for maximum comparability. Wild-type plants without sensor expression were included for background correction. Emissions of each sample were collected every 189 seconds (leaf discs) and 243 seconds (seedlings).

Oxygen gradients were performed by targeted influx of N₂ into the reader system using an atmospheric control unit. For leaf disc experiments, different O₂ concentrations were tested on consecutive days using material from the same plants and maintaining consistent measurement parameters. For seedlings experiments, different batches of plants of the same age were used for each O₂ concentration. *In vivo* responsiveness of the roGFP2-Orp1 protein sensor was routinely validated after the experiments using the same tissue by subsequent treatments with 20 mM DTT (1,4 Dithiothreitol) to drive the sensor to a fully reduced state, followed by two washes with standard assay medium before addition of 20 mM DPS (2,2'-Dithiodipyridine) to oxidize the sensor. For data analysis, wild type autofluorescence was subtracted from biosensor intensities before calculation of 400 nm / 482 nm ratios for each timepoint. Ratio data were log₁₀-transformed to increase symmetry.

Confocal imaging

Seven-day old seedlings were used for GFP detection after treatment. For nuclear localization, seedlings were stained in PBS containing 1 μ g/mL 4',6-diamidino-2-phenylindole (DAPI,

Thermo Fisher Scientific) and washed three times in PBS. Imaging was performed using ZEISS LSM 880 Airyscan microscope (Department of Biology, University of Oxford), equipped with a 25x objective lens, upon laser excitation at 405 nm and collection at 410-495 nm, for DAPI imaging, and excitation at 488 nm and collection at 498-560 nm, for GFP imaging.

Luciferase assay

Total proteins were extracted in passive lysis buffer (Promega). Firefly Luciferase activities were measured using the ONE-Glo Luciferase Assay kit (Promega) and the Nano-Glo® Luciferase Assay System (Promega) was used to measure the activity of NanoLuc® luciferase enzyme. Luciferase signal was normalized based on total protein concentration using Bradford assay ⁶⁶.

Recombinant protein production

Expression and His6-tag affinity purification of *At*PCOs were as previously described ⁶¹. Following affinity purification, the His6-tag was cleaved using TEV protease and the cleaved tag removed using a HisTrap HP column (GE Healthcare). Proteins were then purified with a HiLoad 26/600 Superdex 75 prep grade size exclusion column (GE Healthcare) equilibrated with 50 mM Tris (pH 7.5) and 0.4 M NaCl. Protein purity was assessed with SDS-PAGE.

In vitro H₂O₂ oxidation assay of PCOs

10 μM of recombinant PCOs enzymes (PCO 1 to 5) were incubated with H₂O₂ or equal volume of H₂O in 50 mM HEPES buffer at pH 7.4 (herein termed reaction buffer) at 4°C for 30 minutes. Excess H₂O₂ was removed using a Micro Bio-Spin P-6 chromatography column (Bio-Rad), equilibrated with reaction buffer. 200 μM RAP₂₋₁₅ peptide (CGGAIISDFIPPPR, purchased from GL Biochem (China)) was reacted with 1 μM PCO (H₂O₂ treated or non-treated) at 25 °C for the required time before 5 μL samples were quenched in 45 μL of 5 % formic acid to stop the enzymatic reaction. Peptide masses were subsequently analysed using an Agilent RapidFire RF360 sampling robot connected to an Agilent 6530 Accurate-Mass Q-ToF mass spectrometer operated in positive electrospray mode. Product distributions were assigned via the relative integrated areas of the peaks corresponding to products of interest. Spectra were visualised on Qualitative Analysis (Version B.07.00) and Agilent RapidFire Integrator (Version 4.3.0.17235) was used to calculate integrated peak areas.

H₂O₂ oxidation assay of RAP₂₋₁₅ peptide

200 μM RAP₂₋₁₅ stock solutions were prepared in reaction buffer and treated with 400 μM freshly prepared dithiothreitol (DTT) for 45 minutes at room temperature (25 °C) to ensure peptides were monomeric (in all experiments unless otherwise mentioned). Peptides were then treated with H₂O₂ under conditions required by the experiment. Time-course

experiments were conducted in 2 mL deep well plates and analysed in real time using RapidFire mass spectrometry as described above.

Peptide fragmentation by LC-MS/MS

20 μ M of DTT reduced RAP₂₋₁₅ was treated with 1 mM H₂O₂ at room temperature for 1h. 5 μ L sample was diluted with 45 μ L 5 % formic acid for measurement. LC-MS/MS was carried out using an Acquity UPLC system coupled to a Xevo G2-XS Q-ToF mass spectrometer on a Chromolith Performance RP-18e 100-2 mm HPLC column (Merck) at 40 °C as above. Ions with a m/z ratio of 1474.7 (+32 Da of AtRAP₂₋₁₅) were selected for sequential fragmentation under a collision energy of 80V. Fragment assignment was made via comparison of the obtained spectrum with computationally predicted fragment patterns, calculated using the University of California, San Francisco webpage tool Protein Prospector (Version 6.3.1).

Protein analysis

Protein mass measurement: 100 μ M of recombinant AtPCO4 enzyme was incubated with 1 mM H₂O₂ or equal volume of H₂O in reaction buffer for 1 h at 25 °C. Excess H₂O₂ was removed using a Micro Bio-Spin P-6 chromatography column (Bio-Rad) equilibrated with reaction buffer. Total mass of the H₂O₂ treated or nontreated AtPCO4 was measured using RapidFire mass spectrometry as described above.

Cysteine oxidation detection

After passing the column, H₂O₂ treated or nontreated AtPCO4 were incubated with 1 mM BioDiaAlk in dark for 1 h at 25 °C, followed by 10 mM DTT reduction for 1 h at 25 °C. Equal amount proteins were separated on SDS-PAGE, transferred into polyvinylidene difluoride (PVDF) membrane followed by streptavidin-HRP blot at 1:1000 dilution or anti-his-HRP blot at 1:10,000 dilution, the protein signal was visualised by chemiluminescence (ECL Plus, Pierce).

LC-MS/MS data acquisition

15 μ g recombinant AtPCO4 enzyme was incubated with H₂O₂ or equal volume of H₂O in reaction buffer for 1 h at 25 °C. After removing excess H₂O₂, the enzyme was reduced with 85 mM DTT in 50 mM ammonium bicarbonate (Ambic) for 40 min at 56 °C, followed by 55 mM Iodoacetamide (IAA) incubation in 50 mM Ambic for 30 min in the dark at RT. To eliminate excess IAA, samples were reduced again with 85 mM DTT in 50 mM Ambic for 10 min in the dark at RT. In-solution trypsin digestion was performed by adding trypsin in a 1:50 (W/W) ratio overnight at 37 °C, followed by desalting using C18 ZipTip. The resulting tryptic peptides were resuspended in 40 μ L of Milli-Q water with 2 % acetonitrile and 0.1 % formic acid, and 2 μ L were analysed on a NanoAcquity-UPLC system (Waters) connected to an Orbitrap Elite

mass spectrometer (Thermo Fischer Scientific) possessing an EASY-Spray nano-electrospray ion source (Thermo Fischer Scientific). The peptides were trapped on an in-house packed guard column (75 μm i.d. $\times$ 20 mm, Acclaim PepMap C18, 3 μm , 100 Å) using solvent A (0.1 % Formic Acid in water) at a pressure of 140 bar. The peptides were separated on an EASY-spray Acclaim PepMap® analytical column (75 μm i.d. $\times$ 50 mm, RSLC C18, 3 μm , 100 Å) using a linear gradient (length: 100 minutes, 3 % to 60 % solvent B (0.1 % formic acid in acetonitrile), flow rate: 300 nL/min). The separated peptides were electrospray directly into the mass spectrometer operating in a data-dependent mode using a CID-based method. Full scan MS spectra (scan range 350-1500 m/z, resolution 120000, AGC target 1e6, maximum injection time 250 ms) and subsequent CID MS/MS spectra (AGC target 5e4, maximum injection time 100 ms) of 10 most intense peaks were acquired in the Ion Trap. CID fragmentation was performed at 35 % of normalized collision energy, and the signal intensity threshold was kept at 500 counts. The CID method used performs beam-type CID fragmentation of the peptides.

Processing data

The analysis was performed with Peaks 8.5. The raw MS file was searched against the TAIR database. Trypsin with a maximum of 3 missed cleavages and one unspecific end was selected as the protease. Carbamidomethylation (Cysteine) was set as a fixed modification, and oxidation (Methionine) and deamination (Asparagine, Glutamine) were set as variable modifications. Precursor mass tolerance was set as 15 ppm. Fragment mass tolerances for CID were set to 0.8 Da, respectively. All spectra were manually validated. All peptides present at $-\log P > 20$, and spectra were manually checked, validated, or disqualified.

Electron paramagnetic resonance (EPR) of AtPCO4

EPR spectroscopy was performed on a Bruker EMXmicro spectrometer with a Premium bridge connected to an ER4122SHQE-W1 cavity fitted to an Oxford Instruments ESR900 cryostat. The microwave source operated at 9.3891(17) GHz. The spectra were recorded at 10 K with liquid helium cryogen. Protein (200 μM) and control solutions were frozen in liquid N_2 . Spectra were obtained as two 5 min scans from 10 to 650 mT using time constant 20.48 ms, a microwave power of 200 uW modulation amplitude of 1 mT, and a modulation frequency of 100 kHz.

RNA extraction and real-time qPCR analyses

Total RNA was extracted from 60-80 mg of plant material using the phenol-chloroform extraction method as described previously ⁴. RNA concentration was quantified using a NanoDrop ND-1000 (Thermo Scientific) and RNA integrity was tested on 1 % agarose gel. One microgram of total RNA was subjected to DNase Treatment (Thermo Scientific™) and retro-transcribed using qPCRBIO cDNA Synthesis Kit (PCR Biosystems). Real-time quantitative

PCR was performed with QuantStudio 5™ Real-Time PCR System (Applied Biosystems) using a Power SYBR™ Master Mix (Thermo Fisher Scientific™). *Ubiquitin10* (*AT4G05320*) was used as housekeeping gene for Arabidopsis analysis. Four biological replicates were extracted for each condition, each represented by two technical replicates and the average expression was calculated. The primer pairs used in the real-time qRT-PCR are listed in **Table S4**. Relative expression of each individual gene was calculated using the 2^{-ddCt} method ⁶⁷.

Chromatin Immunoprecipitation Assay

ChIP was performed using a modified version of ⁶⁸ Chromatin was extracted from 2 g of seven-day-old $\Delta 13\text{RAP2.12-GFP}$ seedlings grown in sterile liquid half-strength MS medium, supplemented with 1 % w/v sucrose, under controlled conditions (16h:8h light:dark photoperiod, at 22 °C). Seedlings were treated with 1 mM TBHP, or DMSO as control, in 1 mL of fresh liquid 1 % w/v sucrose half-strength MS medium for six hours in the dark. After chromatin sonication, a volume corresponding to 2 % of the total lysate volume was set aside to be used as ‘input’ sample. 5 μg of GFP antibody (Roche, cat. No. 11814460001) were used for immunoprecipitation and the antibody was pulled down from the nuclear lysate after sonication using Dynabeads™ Protein G magnetic beads (Thermo Scientific). At the end of the reverse cross-link step, DNA was purified using QIAquick PCR Purification Kit (Qiagen) and eluted in a final volume of 30 μL . Enrichment of genes in the chromatin immunoprecipitate was detected through real-time qPCR (CFX384 Touch Real-Time PCR Detection System, BioRad) with a triple technical replicate for each of the four biological replicates, applying the percent input method. To calculate the ratio between immunoprecipitated (ip) DNA and input DNA, Log_{250} was subtracted from the raw Ct values of the input, before the $\text{Ct}_{\text{ip-input}}$ value was obtained. The final enrichment was calculated as 2^{-ddCt} . The primer sequences used are listed in **Table S5**.

RNA sequencing

Col-o and *erfVII* seedlings were grown for 7 days in a six-well plate in liquid media, followed by 6 h treatment with 1 mM TBHP, at dark, or mock. At the end of the treatment, samples were harvested and frozen in liquid nitrogen. RNA was isolated using GeneJET RNA Purification Kit (Thermo Scientific™) as per manufacturer’s instruction. RNA sequencing was exploited in paired-end mode using the Illumina Sequencing PE150 on the NovaSeq 6000 platform (Novogene). Transcriptomic analyses were conducted in R software (version 4.3.1). After quality check using FastQC, reads were aligned on *Arabidopsis thaliana* full genome (TAIR 10) using *Rsubread* ⁶⁹ version 2.16.1) and counted using *featureCounts* software ⁷⁰ multi-dimensional scaling (MDS) plot was used to assess similarities and differences between samples based on their gene expression profiles. Differentially expressed genes were identified using *edgeR* ⁷¹ version 3.42.4). Differentially expressed genes with $\geq |1.5|$ expression fold

change and FDR <0.05 (**Table S6**) were selected for subsequent analysis. GO term enrichment analysis of the differentially expressed genes was conducted using *clusterProfiler*⁷²(version 4.10.1).

Statistical analyses

Statistical analyses were performed using GraphPad Prism 10.2.3(403) and R Statistical Software (version 4.3.1, Foundation for Statistical Computing, Vienna, Austria). Normal distribution and homogeneity of variances of data were evaluated through Shapiro-Wilk test and Levene's test, respectively. Student's t-test, ANOVA or Kruskal-Wallis test followed by Tukey's HSD post hoc test ($p < 0.05$) were performed to establish the statistical significance of differences. Additional information is provided in figure legends.

Additional Software

Graphs were made and annotated using GraphPad Prism 10.2.3(403) and R Statistical Software (version 4.3.1, Foundation for Statistical Computing, Vienna, Austria).

Data availability

RNA sequencing raw data generated for this study have been deposited in the Sequence Read Archive (SRA) at the National Centre for Biotechnology Information under BioProject ID PRJNA1171625. Any additional information required to reanalyse the data reported in this paper is available from the lead contacts upon request.

Materials availability

All unique/stable reagents generated in this study are available from the lead contacts without restriction.

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Supplementary information

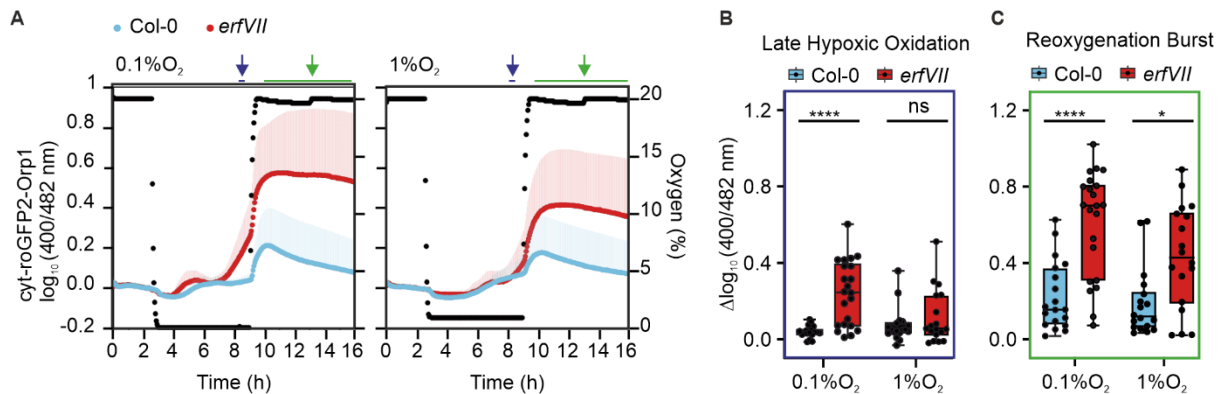


Figure S1 – Ratiometric readout of roGFP2-Orp1 biosensor in Col-o wildtype and *erfVII* leaf discs over time. (A) Multi-well fluorimetry of cytosolic oxidative stress using submerged leaf discs of five-week old Arabidopsis plant lines stably expressing the biosensor roGFP2-Orp1 in Col-0 wild type and *erfVII* background, each normalized to the baseline oxidative state before the start of the hypoxic treatment ($n \geq 18$). (B-C) Amplitudes of late hypoxic roGFP2-Orp1 oxidation before induction of reoxygenation (blue arrow, B) and maximum oxidative burst during reoxygenation (green arrow, C) (significance of differences determined using Mann-Whitney U test, $p < 0.05$, $n \geq 18$).

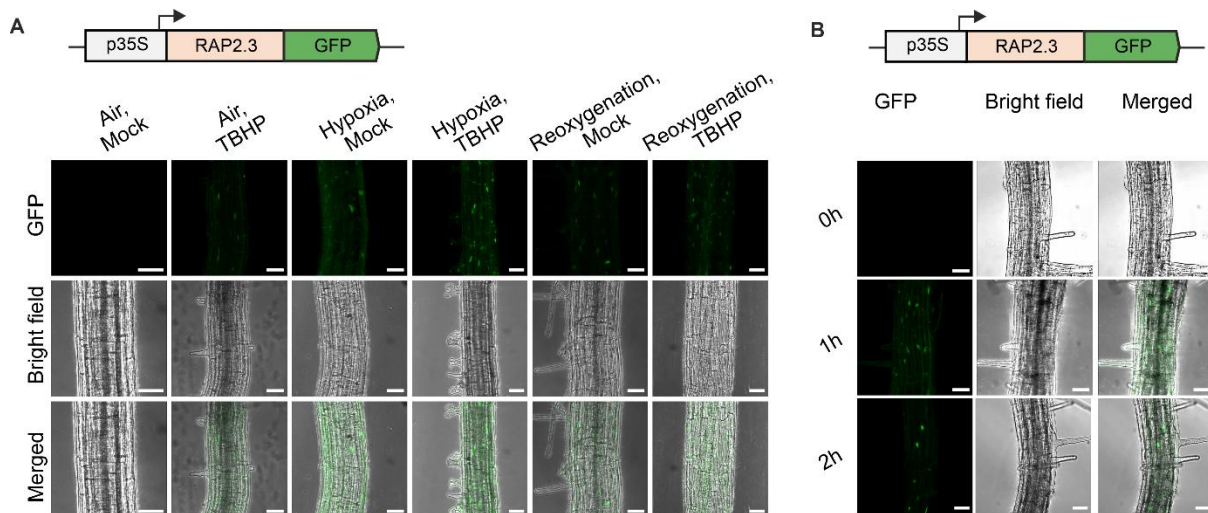


Figure S2 – RAP2.3 localizes to the nucleus upon oxidative stress and reoxygenation in a time-dependent manner. (A) Stabilization of 35S:RAP2.3-GFP (green) in seven-day old Arabidopsis seedlings upon 1 mM TBHP or mock treatment in normoxia (21 % O₂), hypoxia (1 % O₂) or after 3 h of reoxygenation (scale bar, 50 µm). (B) Time-dependent stabilization of 35S:RAP2.3-GFP (green) in seven-day old seedlings over 2 h of 1 mM TBHP treatment in air (scale bar, 50 µm).

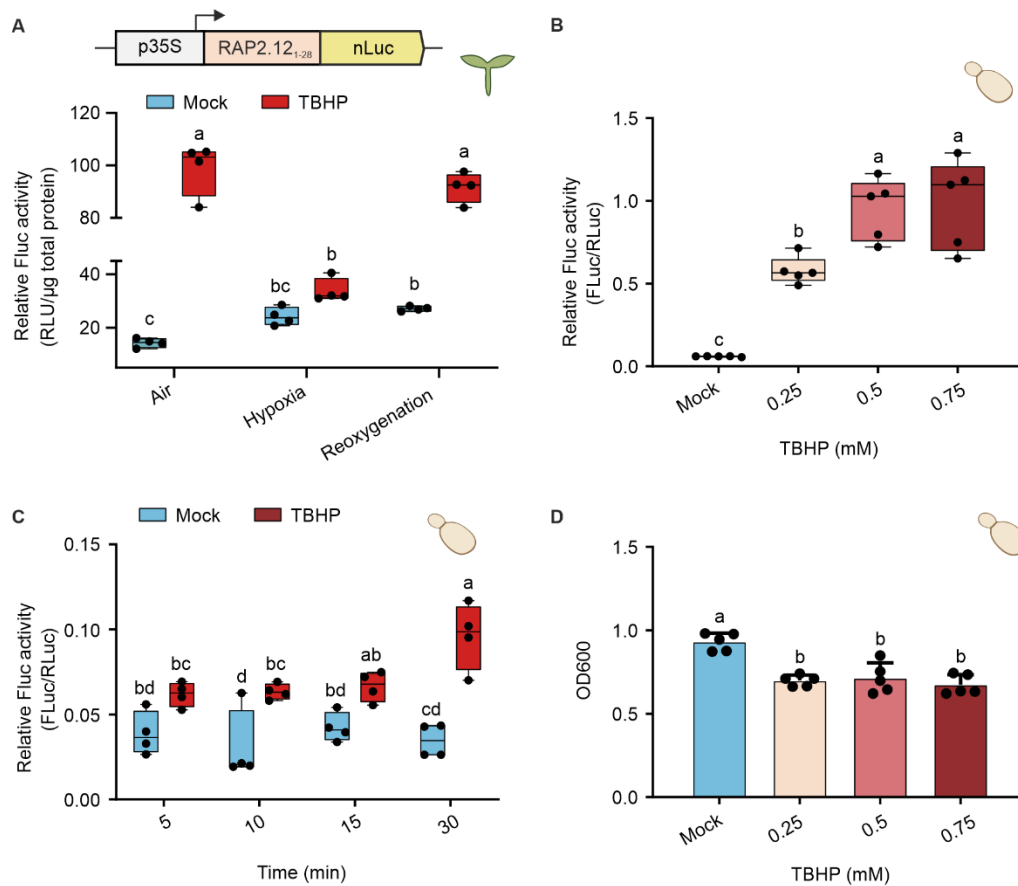


Figure S3 – Measurement of ERFVII stability upon oxidative stress in plant and yeast-based reporter systems. (A) Relative FLuc activity of 35S:RAP2.12₂₋₂₈-FLuc seedlings in air, hypoxia and reoxygenation upon 1 mM TBHP or mock treatment ($n = 4$). (B) Relative FLuc activity of yeast expressing C-DLOR and PCO4 over time. (C) Effect of TBHP doses on C-DLOR activity ($n = 5$). (D) Measurement of optical density at 600 nm (OD600) of yeast culture subjected to different doses of TBHP ($n = 5$). Two-way (A, C) or one-way (B, D) ANOVA followed by Tukey's HSD test ($p < 0.05$), where different letters indicate statistically different groups.

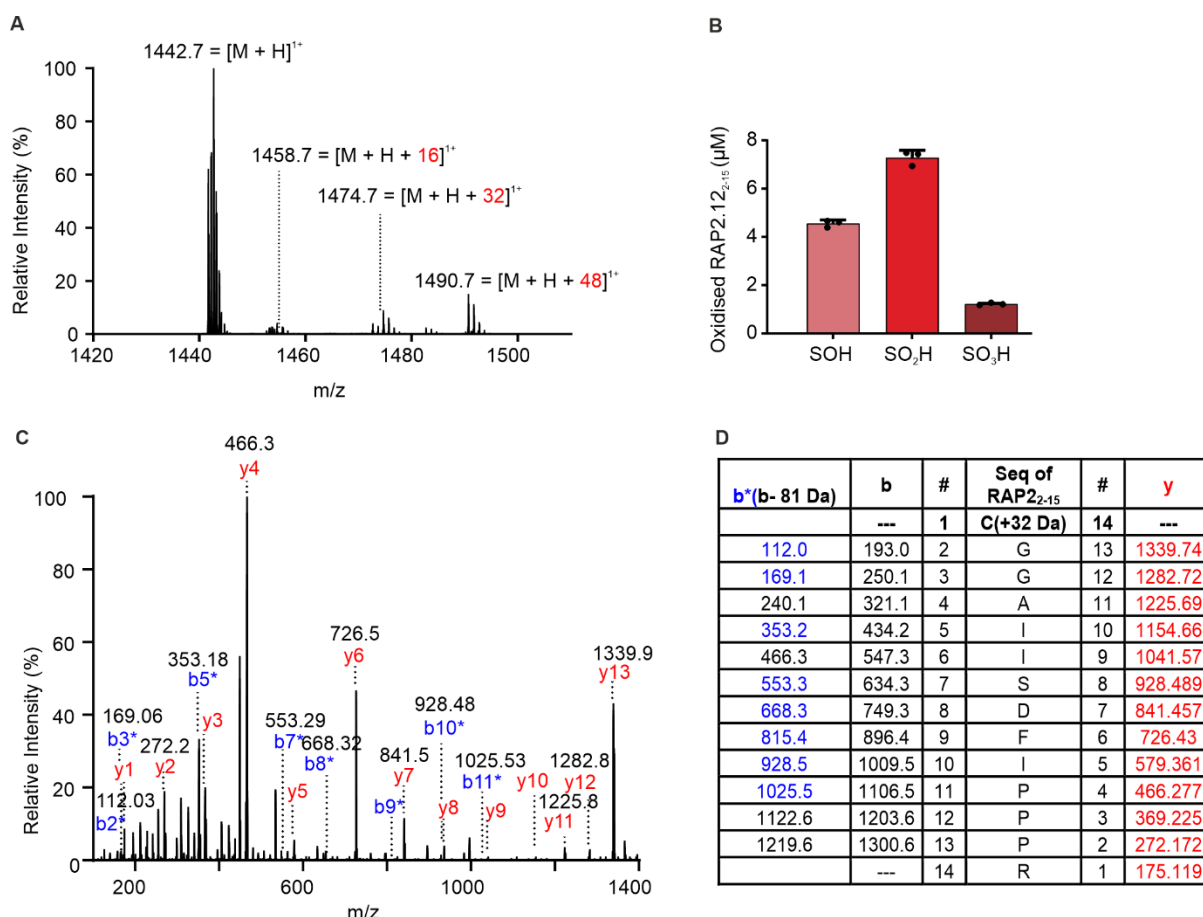


Figure S4 – Direct H₂O₂ oxidation of RAP₂₋₁₅ peptide N-terminal cysteine. (A) UPLC-Mass spectrometry spectra showing mass increases of RAP₂₋₁₅ after 1 mM H₂O₂ treatment for 1 h⁻¹. (B) H₂O₂ causes sulfenic (SOH), sulfinic (SO₂H) and sulfonic acid (SO₃H) modifications on the N-terminal cysteine residue of RAP₂₋₁₅ after 1 mM H₂O₂ treatment for 1 h (*n* = 3). (C) Tandem mass spectrometry confirms H₂O₂-mediated oxidative modification on Nt-Cys of RAP₂₋₁₅; Spectrum shows b ions and y ions of fragmented 1474.7 Da peptide (Nt-Cys-SO₂H) following H₂O₂ treatment RAP₂₋₁₅ (collision energy 80 V). Expected y ions were observed, however b ions were predominantly observed with a consistent mass loss of 81 Da, termed b* ions. These ions likely correspond to loss of SO₂ and NH₃ upon fragmentation, as it has been observed previously for an oxidative modification on N-terminal cysteine² (D) Table showing the matched mass of the predicted fragments (b, b* and y) with the observed mass of RAP₂₋₁₅ (1474.7 Da) under H₂O₂ treatment.

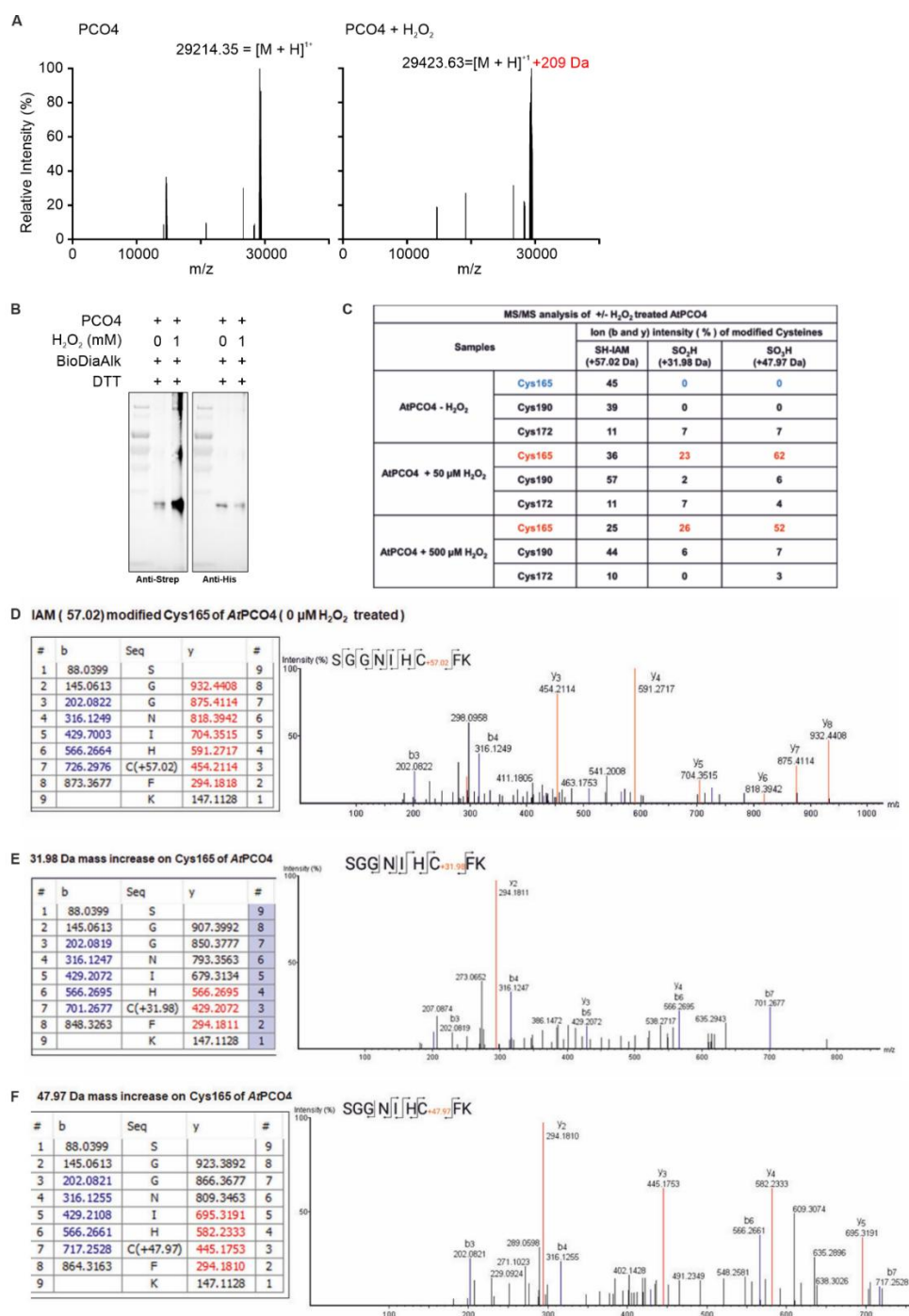


Figure S5 – H₂O₂ inhibits recombinant PCO enzymes. (A) Intact protein mass spectra of 100 μM H₂O₂ treated and non-treated 10 μM AtPCO4 measured by RapidFire mass spectrometry. (B) Streptavidin blot of BioDiaAlk labeling of sulfinic acids in PCO4. (C) Table showing percentage of ion (b and y) intensity of H₂O₂-treated and non-treated PCO4. (D) IAM (+57.02) modifications on b ion and y ion fragments of the peptide containing Cys165 of PCO4. (E) Sulfinic acid (+31.98) modifications on b ion and y ion fragments of the peptide containing Cys165 of PCO4. (F) Sulfonic acid (+47.97) modifications on b ion and y ion fragments of the peptide containing Cys165 of PCO4.

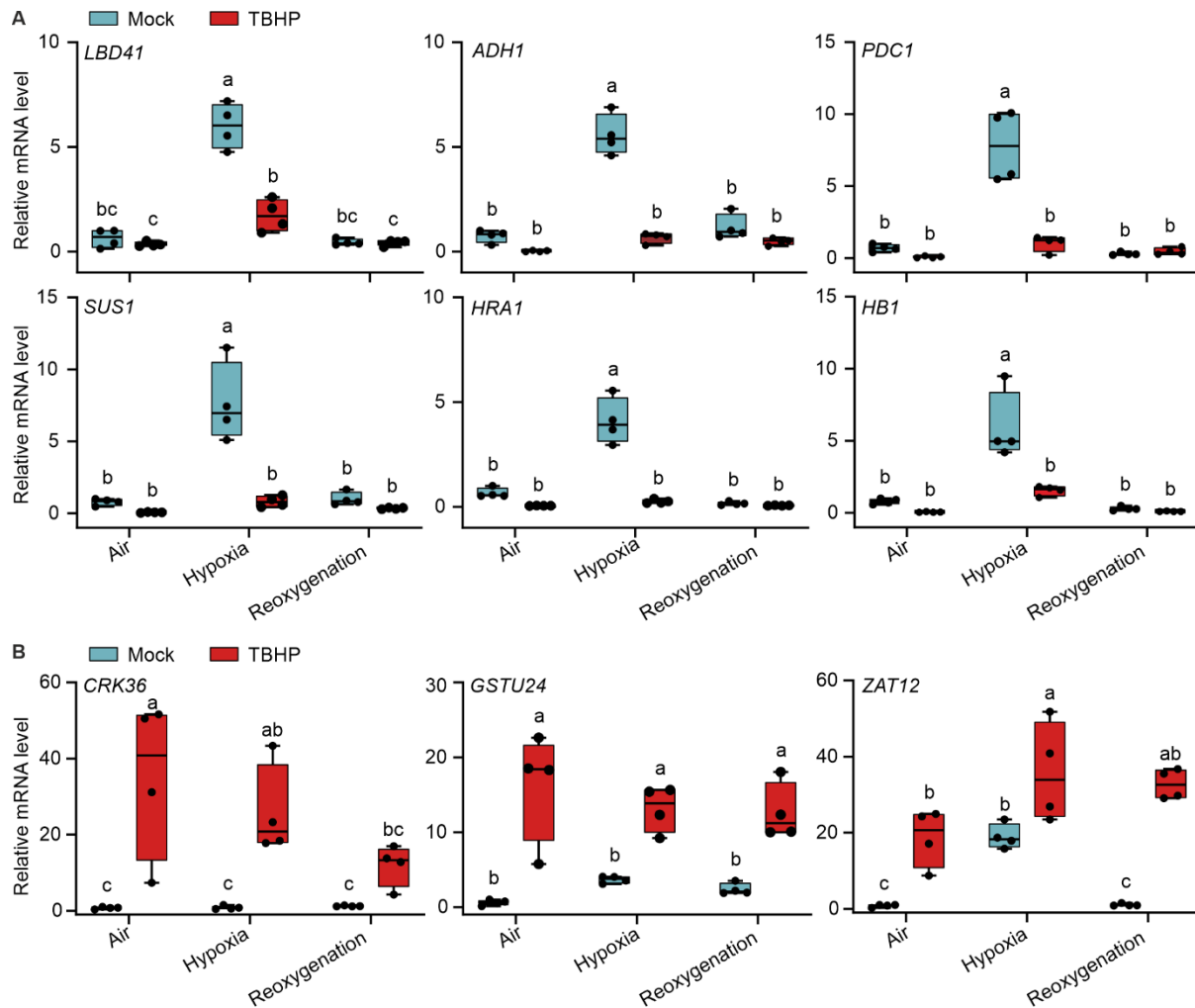


Figure S6 – ROS-triggered oxidative stress disrupts hypoxia-responsive gene expression, without damaging the transcriptional machinery. (A) Relative expression level of six hypoxia-responsive genes *LBD41*, *ADH1*, *PDC1*, *SUS1*, *HRA1*, *HB1* in air, hypoxia or reoxygenation, upon 1 mM TBHP or mock treatment in seven-day old Col-o seedlings ($n = 4$). (B) Relative expression level of ROS-responsive genes *CRK36*, *GSTU24* and *ZAT12* in air, hypoxia or reoxygenation, upon 1 mM TBHP or mock treatment ($n = 4$). Statistical analyses were conducted using two-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Different letters indicate statistically different groups.

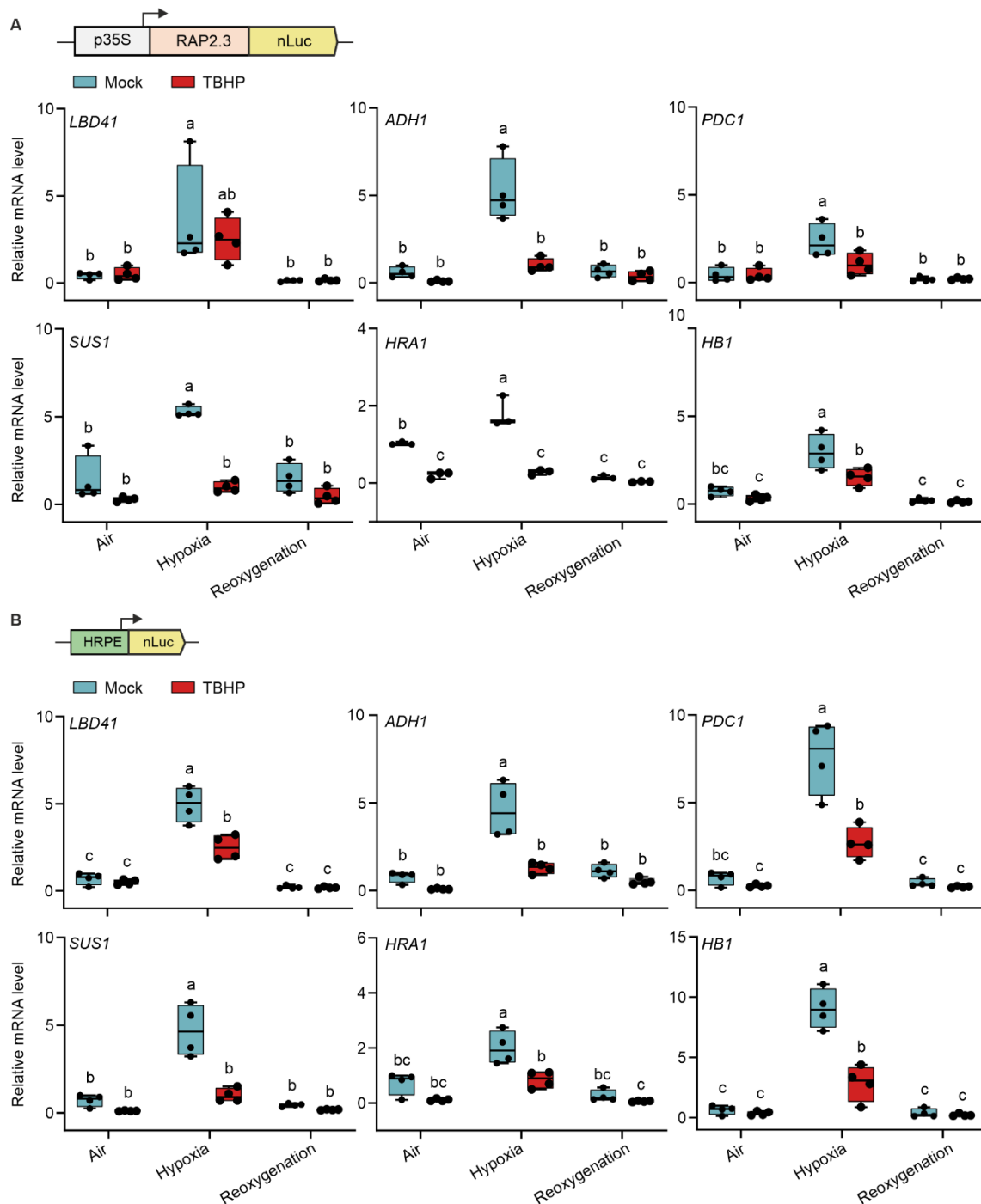


Figure S7 – Hypoxic response in 35S:RAP2.3-nLuc and in HRPE:nLuc seedlings. Relative expression of six hypoxic responsive genes (*LBD41*, *ADH1*, *PDC1*, *SUS1*, *HRA1* and *HB1*) in 35S:RAP2.3-nLuc (**A**) and HRPE:nLuc (**B**) seven-day old seedlings subjected to air, hypoxia or reoxygenation upon TBHP or mock treatment ($n = 4$). Statistical analyses were conducted using two-way ANOVA followed by Tukey HSD test ($p < 0.05$). Different letters indicate statistically different groups.

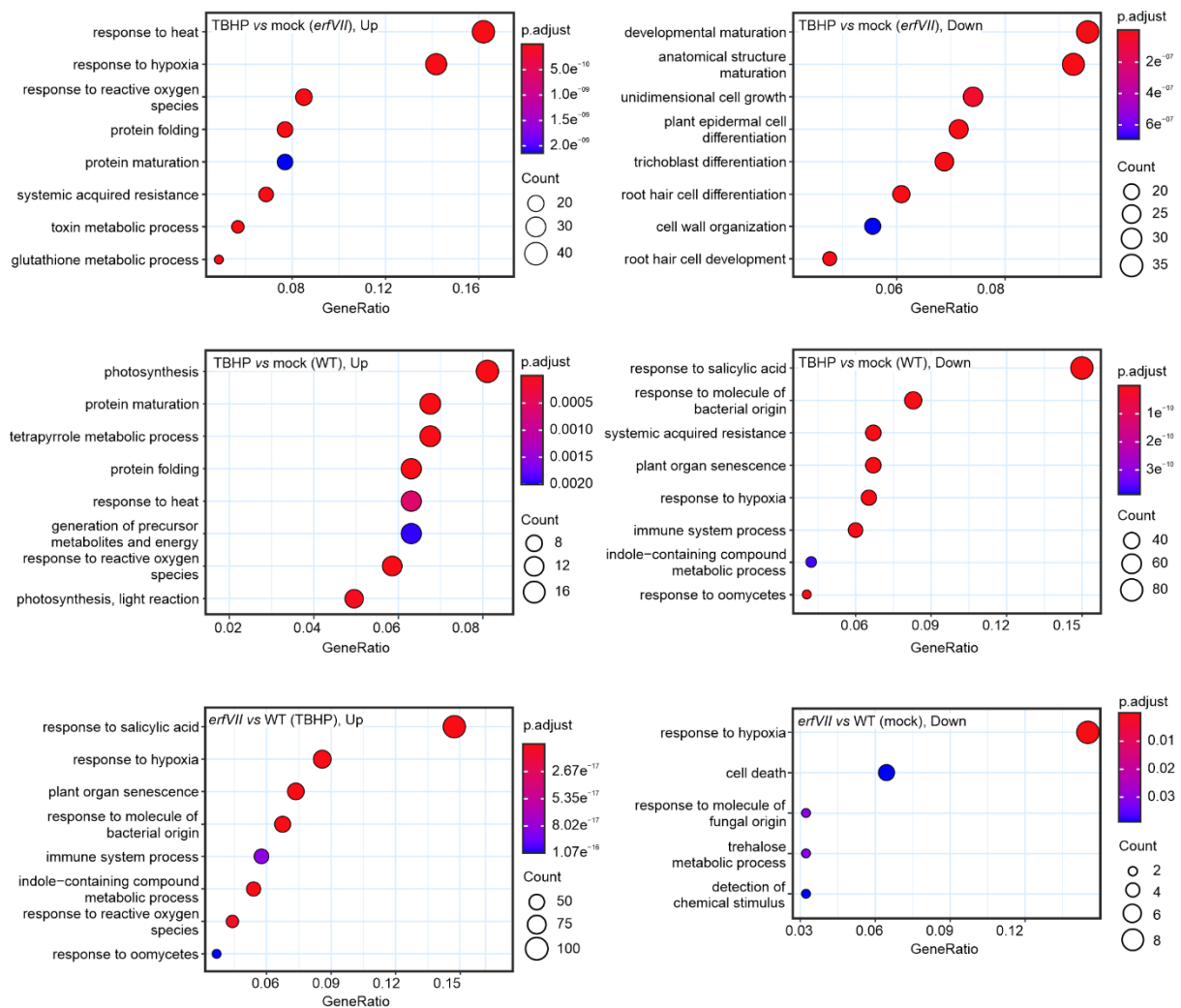


Table S1 – Sequence of Arabidopsis codon-optimised *nLuc*-intron synthetic sequence.

ATGGTTTTACCCCTTGAGGACTTCGTTGGAGATTGGAGACAGACCGCTGGATACAACCTTG
 ATCAGGTGTTGGAGCAAGGTGGGGTGTTCATCTTTGTTCCAGAACCTCGGAGTTAGCGTGAC
 CCCTATCCAGAGAATCGTTCTCTCTGGTGAGAACGGGCTCAAGATCGATATCCACGTGATC
 ATCCCTTACGAGGGACTTAGCGGAGATCAGATGGGACAGATCGAGAAGATTTTCAAGGTG
 GTGTACCCTGTGGACGACCACCACTTCAAGGTTATCCTCCATTACGGtaaataatttagggattgactta
 gtttgagcgattgaattggttagaaattaagtttcgattctatttagctctgttcatagatacctttatctgcttacgattttgattgtgtata
 aagcatgaggtttgatttgtgtgatatttatatgcatatgaggttgagtatgagacttgatttgtgtgatgagatttgattagagattgtt
 atgagttatttatagaagaactcttttgtttgtttgtttcactactagGAACCCTCGTGATCGATGGTGTGACCCCA
 AACATGATCGACTACTTCGGTAGACCGTACGAGGGAATCGCTGTGTTTCGATGGAAAGAAGA
 TTACCGTCACTGGGACCCTCTGGAACGGGAACAAGATTATCGATGAGAGGCTCATCAACCC
 GGACGGCTCACTTTTGTTCAGAGTGACTATCAACGGTGTGACCGGTTGGAGACTTTGCGAG
 AGAATTTTGGCTGGAAGCAGCGGAGCTATCGCCATGGAATGA

Table S2 – List of primers for generation and screening of RAP2.3-nLuc and RAP2.3-GFP transgenic line.

Primer Name	Primer
RAP2.3 Δ stop Fw	AAAAAAGCAGGCTCCATGTGTGGCGGTGCTATTAT
RAP2.3 Δ stop Rv	CAAGAAAGCTGGGTGCTCATACGACGCAATGACAT
AttB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCC
AttB2	CACCCAGCTTTCTTGTACAAAGTGGTCCCC
nLuc Rv	AGTCCCTCGTAAGGGATGAT
GFP Rv	ACAACTCCAGTGAAAAGTTC

Table S3 –Sequence of Arabidopsis codon-optimised HRPE-nLuc synthetic construct.

ctcccatatggctgacctgcaggcggccgactagtgatatcacaagttgtacaaaaagctgaacgagaaacgtaaaatgatataa
 atatcaatatattaaattagattttgcataaaaaacagactacataatactgtaaaacacaacatatccagtcactatggcggccgcatt
 aggcaccccaggctttacattttatgcttcggctcgataatgtgtggattttgagtaggatccggcgagattttcaggagctaaggaa
 gctaaaaatggagaaaaaaatcactggatataccaccgttgatataatcccaatggcatcgtaaagaacattttgaggcatttcagtcagtt
 gctcaatgtacctataaccagaccgttcagctggatattacggcctttttaagaccgtaaagaaaaataagcacaagttttatccggcc
 ttatttcacattcttgcggcctgatgaatgctcatccggaattccgtatggcaatgaaagacggtgagctgggataggatagtggtc
 acccttggtacacgcttttccatgagcaaaactgaaacgcttttcacgctctggagtgaaataaccacgacgatttcggcagtttctacacata
 tattcgcaagatgtggcgtgttacggtgaaaacctggcctatttccctaaagggtttattgagaatatgttttcgtctcagccaatccctg
 ggtgagtttcaccagttttgatttaaactggccaatatggacaacttcttcccccgtttccacatgggcaaatattatacgcaaggc
 gacaagggtgctgatccgctggcgattcaggttcatcatgccgtctgtgatggcttccatgtcggcagaatgcttaataaatacaacagt
 actgcgatgagtggcaggcgggcggtgtaaacgcgtggatccggcttactaaaagccagataacagtatgcgtatttgcgcgctgatttt
 tgcggtataagaatatatactgatgtatatacccggaagtatgtcaaaaagaggtgtgctatgaagcagcgattacagtgacagttgac
 agcgacagctatcagttgctcaaggcatatatgatgtcaatatctccggtctggtaagcacaacctatgcagaatgaagcccgtcgtctgc
 gtgccgaacgctggaaagcgggaaatcaggaagggtggctgaggtcgcccggtttattgaaatgaacggctcttttctgacgagaa
 cagggactggtgaaatgcagtttaaggtttacacctataaaagagagagccgttatcgtctgtttgtggatgtacagagtgatattattga
 cacgccccggcgacggatggtgatccccctggccagtgacgctctgtgtcagataaaagtctcccgtaactttaccgggtggtgcatat
 cgggggatgaaagctggcgcatgatgaccaccgatatggccagtggtccggtctccgttatcgggggaagaagtggctgatctcagccac
 cgcgaaaatgacatcaaaaacgccattaacctgatgttctggggaatataaatgtcaggctcccttatacacagccagctctgcaggtcg
 accatagtactggatattgtgttttacagtattatgtagtctgtttttatgcaaaatctaatttaatatattgatatttatatcattttacg
 tttctcgttcagctttctgtacaaagtgtgataaaaaatggttttcacccttaggacttcgttgagattggagacagaccgctggat
 acaaccttgatcaggtgttgagcaaggtggggtgtcatctttgtccagaacctcggagtttagcgtgaccctatccagagaatcgttct
 ctctggtgagaacgggctcaagatcgatatccacgtgatcatcccttacgagggacttagcggagatcagatgggacagatcgagaag
 attttcaagggtggttacctgtggacgaccaccaacttaagggttatctccattacggaacctcgtgatcgatggtgtgaccccaaca
 tgategactacttcggtagaccgtacgaggaatcgctgtgttcgatggaaagaagattaccgtcactgggacctctggaacgggaac
 aagattatcgatgagaggtcatcaaccggacggctcactttgttcagagtactatcaacggtgtgaccggttgagactttgcgag
 agaattttggctggaagcagcggagctatcgccatggaatgaccgcgccatgctagagtcgcaaaaatcaccagtctctctacaa
 atctatctctctatattttctccagaataatgtgtgagtagtcccagataagggaattagggttcttatagggttcgctcatgtgttgagc
 atataagaaaccttagtatgtattgtattgtaaaatacttctatcaataaaatttctaattcctaaaacaaaatccagtgacctgcag
 gcatgcgacgtcgggccc

Table S4 – List of primers for qRT-PCR.

Gene	AGI Code	Forward Primer	Reverse Primer
<i>UBQ10</i>	AT4G05320	GGCCTTGATAATCCCTGATGAATAAG	AAAGAGATAACAGGAACGGAAAC ATAGT
<i>LBD41</i>	AT3G02550	TGAAGCGCAAGCTAACGCA	ATCCCAGGACGAAGGTGATTG
<i>ADH1</i>	AT1G77120	TATTCGATGCAAAGCTGCTGTG	CGAACTTCGTGTTTCTGCGGT
<i>PDC1</i>	AT4G33070	CACAGAATCTTCAATGTTCTTACC	CCATGATAAAAGCGTACATGGAA
<i>SUS1</i>	AT5G20830	ACGCTGAACGTATGATAACGCG	AACCCTGGAAAGCAAGGCAAG
<i>HRA1</i>	AT3G10040	ACAACCACCGCAACAGAATCC	TCTCCGCAATTCTCGCCAT
<i>HB1</i>	AT2G16060	AATGATTTATAACTGCAGGTGGC	TCATAAGCCTGACCCCAAGC
<i>CRK36</i>	AT4G04490	CCGGATGCGGAGGAGGATTT	TACCGCTATCTCTTGCCCGC
<i>GSTU24</i>	AT1G17170	GAGACTTGCCCCGACAATAA	CTCGCCGTAACATTACCTT
<i>ZAT12</i>	AT5G59820	CATCACAACACTACTATCACACCAAATC	ATCCACCGTCGACTTGATCT
<i>nLuc</i>	NA	CCAGAACCTCGGAGTTAGCG	CTGTCCCATCTGATCTCCGC

Table S5– List of primers for ChIP-qPCR.

Gene	AGI Code	Forward Primer	Reverse Primer
<i>UBQ10</i>	AT4G05320	TCCCTCCCTTTAAGCACCAG	TCCGGTCCTAGATCATCAGTTCA
<i>EIF4AI</i>	AT3G13920	TGTTTTGCTTCGTTTCAAGGA	GCATTTTCCCGATTACAAC
<i>ADH1</i>	AT1G77120	GCAAAACCAAATACGCCCC	TAATCTGTCCGGTGGTAGAC
<i>LBD41</i>	AT3G02550	GAGAGAGTCACAAAGATCCGCCC	GAAGAACTGGGGCCCACACTTAG
<i>HB1</i>	AT2G16060	CCATGTGCTCTGTACTGGTAATGGA	TTATACCACTTGGTGTGGTTGGC
<i>HRA1</i>	AT3G10040	GCAGTGGTTTTGGGAGCCGT	TTTGCCAAAACCAGCCCCTTG
<i>ZAT12</i>	AT5G59820	TACGCGGTGTCGCAAATCGT	TGGGTAAGGAAGTGGCAGCG
<i>ATH8</i>	AT1G69880	AGGTTTACATGCAACTTTCCGCT	CTGGTGGACCAAGTAGCCGT
<i>GSTU24</i>	AT1G17170	TCAAGTGCGCCAAAAGGAAAGA	AGGGGTTTTGAATCGCATTTTGCT

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1. White, M. D., Kamps, J. J. A. G., East, S., Kearney, L. J. T. & Flashman, E. The plant cysteine oxidases from *Arabidopsis thaliana* are kinetically tailored to act as oxygen sensors. (2018) doi:10.1074/jbc.RA118.003496.
2. Masson, N. et al. Conserved N-terminal cysteine dioxygenases transduce responses to hypoxia in animals and plants. *Science* (80-.). 364, 65–69 (2019).

Statement of authorship

Statement of Authorship for joint/multi-authored papers for PGR thesis

To appear at the end of each thesis chapter submitted as an article/paper

The statement shall describe the candidate's and co-authors' independent research contributions in the thesis publications. For each publication there should exist a complete statement that is to be filled out and signed by the candidate and supervisor (**only required where there isn't already a statement of contribution within the paper itself**).

Title of Paper	H ₂ O ₂ repurposes the plant oxygen-sensing machinery to control the transcriptional response to oxidative stress
Publication Status	☐ Published ☐ Accepted for Publication ☒ Submitted for Publication ☐ Unpublished and unsubmitted work written in a manuscript style
Publication Details	Akter, S.*, Perri, M.*, Lavilla-Puerta, M., Ferretti, B., Carbonare, L.D., Shukla, V., Telara, Y., Zhang, D., Gunawardana, D.M., Myers, W.K., Giuntoli, B., Flashman, E., Licausi, F., 2024. H ₂ O ₂ repurposes the plant oxygen-sensing machinery to control the transcriptional response to oxidative stress. https://doi.org/10.1101/2024.10.21.619351

Student Confirmation

Student Name:	Monica Perri
Contribution to the Paper	Conducted and designed experiments: generation of cloning construct and transgenic lines, tolerance assays on seedlings, DAB staining and quantification, confocal imaging, luciferase assays, hypoxic and reoxygenation treatments, RNA

	extraction, qPCR and RNA-seq (processing and analysis) (Figures 1-3, 5-6 ; Supplementary Figures 1-2, 5-7); analysed data; contributed to writing the manuscript.	
Signature 	Date	12/08/2025

Supervisor Confirmation

By signing the Statement of Authorship, you are certifying that the candidate made a substantial contribution to the publication, and that the description described above is accurate.

Supervisor name and title: Professor Francesco Licausi		
Supervisor comments I hereby confirm that the above Declaration of Authorship and Contributions provided by Ms Monica Perri is accurate and truthful. The statement correctly reflects her own scientific and scholarly contributions to the work presented in her thesis, as well as the contributions made by collaborators, which are appropriately acknowledged. I endorse this declaration as a fair and transparent account of authorship and contributions.		
Signature 	Date	10/09/2025

This completed form should be included in the thesis, at the end of the relevant chapter.

Chapter 4 – Manipulating plant oxygen sensing through NCO substitution reveals trade-offs between growth and flooding tolerance

Commentary

Chapter 4 builds directly on the previous chapter's efforts to enhance plant resilience to low-oxygen stress by adopting an evolutionary and comparative approach. While the prior chapter identified novel molecular mechanisms that could improve hypoxia tolerance within *Arabidopsis*, this chapter expands the scope by exploiting natural variation in N-terminal cysteine oxidases (NCOs) from highly divergent eukaryotic organisms to investigate how structural and kinetic differences influence ERFVII stability, hypoxia-responsive gene expression, and stress tolerance.

Through structural analyses, biochemical characterisation and complementation assays in the *Arabidopsis* *apco* mutant, this chapter demonstrates that NCO enzymes from divergent eukaryotes vary significantly in their ability to oxidize ERFVII peptides and regulate hypoxia signaling. Notably, plants expressing less efficient NCOs exhibited enhanced survival under flooding due to partial stabilization of ERFVIIs and a primed hypoxic response. However, this benefit came with a trade-off of reduced growth and delayed development under physiological oxygen conditions.

By offering a novel strategy to increase low-oxygen resilience through enzyme substitution, this chapter builds on previous findings and highlights the evolutionary diversity of NCOs as a valuable resource for fine-tuning oxygen sensing and optimizing plant resilience.

Manipulating plant oxygen sensing through NCO substitution reveals trade-offs between growth and flooding tolerance

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Author Contributions: M.P. designed and performed the research, analysed the data and wrote the manuscript. Y.H., X.C., M.L.-P. performed the research and analysed the data. R.L. analysed the data. T.B. contributed new reagents and analysed the data. A.P. designed the research. E.F. designed the research and analysed the data. F.L. designed the research, analysed the data and wrote the manuscript.

Competing Interest Statement: The authors declare no competing interest.

Classification: Biological sciences/Plant Biology

Keywords: cysteine dioxygenase, hypoxia, flooding, oxygen sensing, mathematical modelling

Abstract

The oxygen-dependent degradation of Ethylene Response Factors VII (ERFVIIIs) through the N-degron pathway is central to regulating the transcriptional responses to hypoxia in vascular plants. Plant Cysteine Oxidases (PCOs) control this step by catalysing the oxidation of an N-terminal Cys residue exposed by ERFVIIIs. In the present study, we investigated the functional impact of replacing Arabidopsis PCOs with diverse N-terminal cysteine oxidases (NCOs) from across the three eukaryotic kingdoms, hypothesizing that structural and kinetic differences may influence gene regulation of ERFVII targets under hypoxia and thus impact stress tolerance. Combining structural analyses, *in vitro* biochemical characterisation and *in planta* complementation assays we observed that not all tested NCOs are functionally equivalent to the endogenous PCOs. In fact, despite the remarkable conservation of catalytic motifs, we identified key differences in enzyme architecture that appear to affect the enzyme's capacity to regulate hypoxia responses in plants. Notably, NCO efficiency in oxidising ERFVII peptides inversely correlated with hypoxic gene expression under aerobic conditions and enhanced submergence survival, suggesting that partial ERFVII stabilization primes plants to cope with hypoxia. However, enhanced basal expression of hypoxia-responsive genes in turn correlated negatively with development and biomass accumulation, pointing to a trade-off between growth and stress resilience. Our findings demonstrate that tuning NCO activity can reshape the transcriptional and physiological hypoxia response, suggesting it is possible to enhance plant resilience under fluctuating oxygen conditions through enzyme engineering and precision breeding.

Significance statement

Control of low-oxygen responses to improve crop flooding tolerance is a long-sought objective of molecular plant breeders. The oxygen-dependent oxidation of N-terminal cysteines in transcription factors is thought to be a key step in modulating the transcriptional response to hypoxia. In this study, we tested this hypothesis by substituting endogenous N-terminal cysteine dioxygenases with homologues from different species characterised by highly divergent sequences, structures, and kinetic properties. We show that indeed these variations effectively and predictably influence gene transcription in plants exposed to hypoxia, thereby affecting their tolerance to submergence. However, we also demonstrate that, unexpectedly, these substitutions impact plant growth and development under aerobic conditions, revealing a trade-off between flooding stress resilience and biomass accumulation or yield.

Introduction

Low-O₂ responses in vascular plants rely on proteolytic control of Cys-initiating (N-Cys) members of the group VII of the ethylene response factors (ERFVIIs) (1). In angiosperms, an additional contribution to accommodate chronic, acute and fluctuating hypoxia is provided by the Polycomb Repressive Complex (PRC) 2 subunit Vernalization 2 (VRN2) and the LITTLE ZIPPER 2 (ZPR2) (2–4).

These N-Cys proteins undergo O₂-dependent destabilisation via post-translational regulation through the N-degron pathway (5–7). This involves Plant Cysteine Oxidases (PCOs), non-heme iron (Fe(II))-dependent dioxygenases, which act as main O₂ sensors by catalysing oxidation of the N-terminal cysteine (8, 9). The resulting sulfinylated cysteine is recognised for arginylation by arginyltransferase (ATE), which leads to ubiquitination by the E3 Ubiquitin-ligase PROTEOLYSIS 6 (PRT6) and BIG, thereby targeting the protein to the proteasome for degradation (10, 11). Notably, N-Cys degron-mediated proteolysis also requires nitric oxide (NO) (12). Oxygen depletion under hypoxic conditions prevents PCO activity, stabilising N-Cys substrates, which then accumulate in the nucleus to trigger the activation of hypoxic response genes, including those coding for anaerobic metabolism (**Figure 1a**) (13, 14).

The identification of PCOs in vascular plants led to the discovery that the human homolog, 2-aminoethanethiol dioxygenase (ADO), also acts as an enzymatic O₂ sensor, alongside the Prolyl Hydroxylases (PHDs) and histone demethylases (15–17). ADO catalyses the oxidation of N-terminal cysteine residue of N-Cys proteins involved in cell signaling such as the regulator of G-protein signaling 4 and 5 (RGS4/5), interleukin-32 (IL-32) and the member 10 of the acyl-CoA dehydrogenase family (ACAD10), thus modulating angiogenesis, inflammation and autophagy (17–19). Sequence alignments revealed conserved catalytic site features between PCO and ADO, despite the absence of shared targets (20, 21). Moreover, experiments *in planta* demonstrated the ability of *Homo sapiens* ADO (HsADO) to complement the homozygous quadruple *pco1/pco2/pco4/pco5* (*4pco*) mutant (17). A recent study using an *in vitro* reporter assay also confirmed that HsADO can turn over ERFVII peptides, alongside those corresponding to the endogenous substrates Regulator of G-protein signaling RGS4 and RGS5 (22). Finally, a cross-kingdom survey revealed that N-terminal cysteinyl-dioxygenases (NCOs) are ubiquitously distributed across plants and animals, with PCO-like proteins also found in the chytrid lineage of fungi (21).

The most common cause of acute hypoxia for plants is flooding, due to four orders of magnitude decrease in the gas-exchange rate in water compared to air (23). Limitation in O₂ availability severely impacts cell metabolism, including aerobic respiration, leading to reduced ATP production (24, 25). Upon hypoxia detection, plant cells reconfigure their metabolism to

ensure basal energy production even when aerobic respiration is impossible. This includes the activation of fermentation and the promotion of starch and sucrose catabolism (26–28). While anaerobic metabolic adaptation is conserved across land plants, recent evidence suggests that ERFVII as N-degron substrates emerged relatively late, with the evolution of vascular plants (1). O_2 concentrations below aerobic levels have also been reported to occur physiologically in plant tissues such as seeds, ripe fruits, and bulky storage organs (29–31). The physiological relevance of oxygen sensing is also highlighted by the developmental defects observed in the homozygous *4pco* mutant, including delayed germination and growth, increased leaf serration, and flower sterility (17, 20, 21). Recent studies also demonstrated the establishment of endogenous hypoxic niches in shoot and root meristems, thus designating the role of O_2 as a diffusible signaling molecule (21, 32). Additionally, fluctuations in O_2 levels between day and night have been recently reported to impact shoot growth (33).

Modulation of the O_2 sensing pathway to anticipate, enhance or redirect regulation of adaptive genes is a highly sought goal of plant hypoxia research (34). This can be achieved by interfering with the N-degron substrates, such as the ERFVIIs, or the O_2 -dependent effectors, such as the NCOs (35). Previous studies have shown that increasing the levels or stability of ERFVII transcription factors enhances submergence and hypoxia tolerance in plants: for example, in *Arabidopsis*, overexpression of RAP2.2, RAP2.12, HRE1, and HRE2 improved tolerance to low- O_2 , though constitutively stable RAP2.12 impaired growth and development (5, 6, 36).

Since constitutive ERFVII stabilisation can be detrimental, precise tuning is necessary to ensure a beneficial effect. Acting on the molecular switch that regulates ERFVII in hypoxia, such as N-Cys oxidation, could be the key to modulate intensity and dynamics of the molecular response to hypoxia. PCOs are regarded as the ideal target to manipulate, due to their more specialised activity, and thus likely reduced number of substrates, compared to ATE and PRT6/BIG. Moreover, the identification of key residues within PCO enzymes, along with examination of reported and predicted structures of NCO enzymes in which some of these amino acids are not conserved, provides an opportunity to understand how to modulate the activity of these enzymes and to predict the consequences of their molecular engineering (20, 37–39).

Results

Predicting hypoxia response dynamics in case of alteration of PCO properties

We first assessed the theoretical impact of varying PCO activity or levels on the expression dynamics of a generic *Hypoxia-Responsive Gene* (*HRG*) by applying a previously established model based on ordinary differential equation (ODE) (40). For this prediction, we assumed a

simplified cell environment with a single ERFVII (RAP2.12) and a single PCO (PCO4). We calculated the effects of varying *PCO4* expression levels, its Michaelis-Menten constants (K_M) for O_2 and RAP2.12, and its catalytic rate constant (k_{cat}) (**Figure 1b**). Our ODE-based simulations predicted constitutively high *HRG* expression in the case of reduced *PCO4* levels or activity. Alteration of substrate affinity (K_M) showed high potential to enhance the magnitude of *HRG* induction under hypoxia (1 % O_2). Taken together, these predictions suggest that reducing *PCO* expression or modifying the enzyme to decrease its catalytic rate are effective strategies for priming plants against hypoxia. In contrast, altering substrate affinity is predicted to be an effective strategy to modulate the intensity of the response during hypoxia. We therefore set out to test experimentally whether NCOs with drastically different kinetic parameters would produce the effects predicted by our ODE model, and whether this would actually help plants to cope with low- O_2 stress.

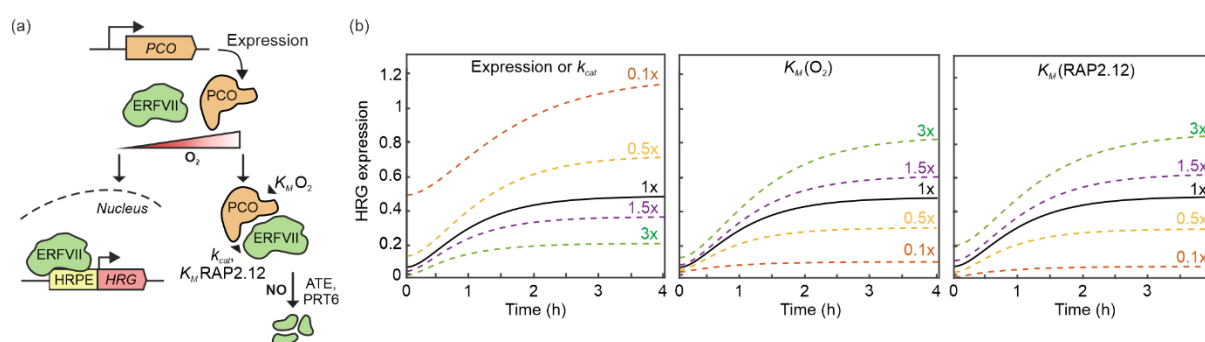


Figure 1 – Hypoxic gene induction as a function of PCO features. (a) Oxygen-dependent regulation of ERFVIIs in flowering plants. ERFVII substrates are characterised by an N-terminal cysteine which, in presence of oxygen (O_2) is targeted for oxidation by Plant Cysteine Oxidases (PCOs). The resulting Cys-sulfinic or Cys-sulfonic acid triggers proteasomal degradation of ERFVIIs. The activity of PCOs is influenced by their expression levels as well as their kinetic parameters (K_M and k_{cat}) for substrates such as RAP2.12 and O_2 . In the absence of O_2 , PCOs are unable to oxidize the N-terminal cysteine, leading to stabilization of the ERFVIIs, which in turn can enter the nuclei and activate *HRG* by binding the hypoxia-responsive promoter element (HRPE). (b) Prediction dynamics of a generic *HRG* at 1 % O_2 (v/v) depending on PCO4 expression levels or catalytic activity (k_{cat}), O_2 affinity ($K_M(O_2)$) and RAP2.12 affinity ($K_M(RAP2.12)$) over time. Dotted lines of different colours indicate fold variation of the parameter level (black line) described in Shukla, *et al.*, 2024 (40).

Sequence and structure divergence among eukaryotic NCOs

We next explored the divergence of NCO sequences within the eukaryotic domain. We selected 50 NCO sequences from the animal, plant and fungal kingdoms and reconstructed their phylogeny based on amino acid sequence comparison (**Supplementary Figure 1a**). This analysis confirmed the taxonomic clustering across the three kingdoms and guided our selection of NCOs for subsequent biochemical characterisation. Specifically, we chose *Homo sapiens* ADO (HsADO) and *Amphimedon queenslandica* ADO (AqADO) from the animal kingdom, *Spizellomyces punctatus* ADO (SpADO) from fungi, and *Marchantia polymorpha* PCO (MpPCO), *Klebsormidium nitens* PCO (KnPCO), and *Arabidopsis thaliana* PCO4 (AtPCO4) from plants. HsADO was included due to its previously demonstrated ability to complement the *Arabidopsis 4pco* mutant (17). AqADO was chosen as a representative of early metazoan lineages (41). SpADO belonging to the chytrid clade, represented fungal NCOs (21, 42). Finally, MpPCO and KnPCO, from bryophytes and algae, respectively, were selected to explore PCO diversity among non-plant clades that do not have ERFVIIIs as Cys-degron substrates. AtPCO4 served as a control throughout the study.

We looked into structural differences among the selected NCOs. We relied on the structures which have been solved by X-ray crystallography for AtPCO4 and HsADO (PDB: 6S7E and 7REI, respectively) whereas we used the AlphaFold2-predicted structures (43) for the remaining NCOs. The core active site, comprised of two β -barrels, is conserved across all six enzymes (**Figure 2a(I)**). Analysis of the internal substrate-binding cavity revealed varying volumes: HsADO and SpADO possessed smaller pockets (276.8 Å³ and 316.8 Å³, respectively) compared to AtPCO4 (568.4 Å³). In contrast, AqADO, MpPCO, and KnPCO exhibited cavity volumes comparable to AtPCO4 (681.2 Å³, 680.6 Å³ and 587.3 Å³, respectively), suggesting a shared potential to accommodate protein N-termini (**Supplementary Figure 2**). Moreover, analysis of the electrostatic potential surrounding the active site revealed notable differences, implying variations in the interaction with substrates (**Figure 2a(II)**). For example, HsADO and AqADO exhibited a similar predicted three-dimensional structure and overall size to AtPCO4 but displayed more negative electrostatic charge around their active sites. This electrostatic difference could explain the previously demonstrated substrate preference of HsADO for positively charged peptides (22). Outside the active site, SpADO featured an additional N-terminal α -helix and an internal loop (residues 250-260) and was more positively charged than AtPCO4 at the C-terminal region. MpPCO and KnPCO showed conservation at the core active site but contained additional internal loops and a less positively charged external surface (**Figure 2a**).

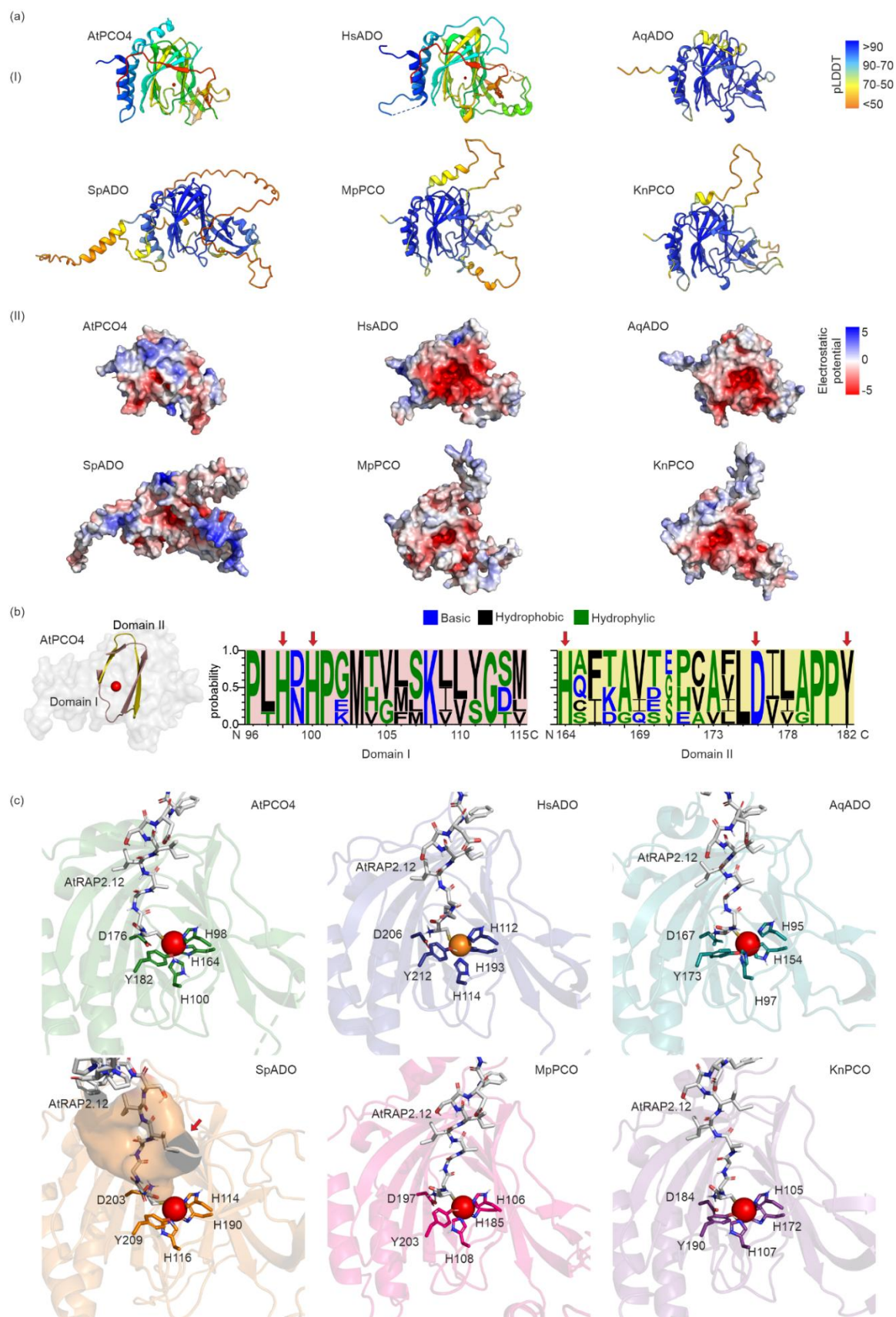


Figure 2 – Structural comparison of selected NCOs. (a, I) 3D structures of AtPCO4 and HsADO (PDB: 6S7E and 7REI, respectively) and AlphaFold2 prediction of AqADO,

SpADO, MpPCO and KnPCO shown in cartoon representation. AlphaFold2 predictions are coloured according to the predicted local distance difference test (pLDDT) values (red: low accuracy in prediction, blue: high accuracy in prediction). **(a, II)** Surface representation of NCO enzymes using electrostatic colour map. **(b)** Multiple sequence alignment of amino acid residues in Domain I and II of AtPCO4 active site. Amino acids are coloured by hydrophobicity. Red arrows mark ubiquitously conserved residues putatively involved in iron coordination (H98, H100, H164 in AtPCO4) and substrate binding (D176 and Y182 in AtPCO4). **(c)** Molecular docking of the first 14 amino acids of RAP2.12 (grey) inside the NCOs showed in **a**. Coloured sticks mark amino acids involved in iron coordination (His) or substrate binding (Asp and Tyr). The orange cloud in SpADO indicates the volume occupied by an extra loop which potentially interferes with substrate access.

When zooming in on the two β -barrel domains (Domain I: residues 96-115, Domain II: residues 164-182 in AtPCO4) that coordinate the iron centre in AtPCO4 (**Figure 2b**), alignment of these domains revealed high sequence similarity, including conserved histidine residues (H98, H100, H164), which are critical for iron coordination, aspartate (D176) and tyrosine (Y182) proposed to be necessary for substrate binding in AtPCO4 (20, 39). A high level of sequence similarity for these residues was also found more generally across all the analysed NCO sequences (**Supplementary Figure 1b**).

To assess the potential for these NCOs to be functionally relevant *in vivo*, we first performed an *in silico* analysis of their interaction with the PCO-substrate RAP2.12₂₋₁₅ peptide using molecular docking (**Figure 2c**). HsADO, AqADO, MpPCO and KnPCO exhibited similar RAP2.12₂₋₁₅ molecular trajectories to AtPCO4. Instead, SpADO showed altered positioning, with an additional internal loop for SpADO (residues 254-259, indicated by the orange surface shading) hindering peptide access to the active site.

NCOs vary in the catalysis of ERFVII oxidation

To test the ability and efficacy of the six selected NCOs to regulate ERFVII in plants, we first compared their kinetic parameters for Arabidopsis RAP2.12 and oxygen. Notably, even among Arabidopsis PCOs, different isoforms exhibit different kinetic activities, with AtPCO4 having the highest catalytic turnover among the five PCOs, potentially influencing substrate preferences and different roles of PCOs under stress or physiological conditions (44). Based on this, we hypothesized that NCOs with greater sequence divergence might display even more distinct kinetic properties. While kinetic data for AtPCO4, MpPCO, and KnPCO were already available from previous studies, which demonstrated their oxygen-dependent activity on

RAP2.12 (44, 45), we extended the analysis to AqADO, HsADO, and SpADO. Therefore, we expressed these three NCO proteins in *Escherichia coli* and purified them as described previously (44). We determined the kinetic parameters of AqADO and HsADO for a peptide corresponding to the first 14 amino acids of RAP2.12 (RAP2.12₂₋₁₅). Substrate turnover using varying RAP2.12₂₋₁₅ concentrations was used to estimate catalytic parameters via Michaelis-Menten fitting (**Figure 3a, Supplementary Table 1**). Subsequently, we determined the K_M (O_2) values of AqADO and HsADO with respect to RAP2.12₂₋₁₅ oxidation, in order to assess their oxygen-sensing potential (**Figure 3b, Supplementary Table 2**).

It is worth noting that we used 200 μM RAP2.12 peptide in this study to determine oxygen affinity, which is lower than that used to determine kinetic parameters of AtPCO4, MpPCO and KnPCO in previous studies (44, 45).

Full kinetic data including standard errors are provided in **Supplementary Table 1, 2**. These data confirmed that AqADO and HsADO can function as cysteine dioxygenases on the RAP2.12₂₋₁₅ peptide in an oxygen-dependent manner, similar to AtPCO4. In contrast, SpADO showed no substrate turnover over time (**Figure 3c**), indicating its inability to oxidize RAP2.12 *in vitro* (at least under the conditions used in this study) consistent with the *in silico* predictions.

Comparative analysis of K_M values for RAP2.12₂₋₁₅ revealed the highest peptide affinity (11 μM) for MpPCO, even higher than AtPCO4 (690 μM) or any other AtPCOs (44, 45), followed by AqADO and HsADO (206 μM and 392 μM , respectively), with KnPCO showing the lowest (914 μM) (**Figure 3d**). The high affinity of MpPCO for the RAP2.12₂₋₁₅ peptide is particularly remarkable considering that even the most affine PCO in Arabidopsis, AtPCO2, has a K_M almost 8 times higher than MpPCO (90 μM) (44).

When considering O_2 affinity, MpPCO again showed the lowest K_M (3.2 %) (45), even lower than that reported for any other AtPCOs (44). Additionally, all the other NCOs tested in this assay displayed higher K_M (O_2) compared to the value of 17.3 % reported for AtPCO4 in the literature, with HsADO exhibiting the lowest affinity (81.5 %). The high affinity of MpPCO for both oxygen and RAP2.12₂₋₁₅ determined the highest catalytic efficiency (k_{cat} / K_M) (0.39 $s^{-1} \mu M^{-1}$ for O_2 and 0.75 $s^{-1} \mu M^{-1}$ for RAP2.12₂₋₁₅) among all tested NCOs, despite its low catalytic constant (k_{cat}) (15.5 s^{-1} for O_2 and 8.2 s^{-1} for RAP2.12₂₋₁₅). Notably, MpPCO and KnPCO exhibited the highest and similar levels of catalytic efficiency towards O_2 (0.39 $s^{-1} \mu M^{-1}$ and 0.17 $s^{-1} \mu M^{-1}$, respectively) (45). This suggests that MpPCO and KnPCO could be catalytically active in plant cells, and thus repress ERFVII accumulation, even at low- O_2 concentrations.

In contrast, AqADO showed kinetic values for O_2 similar to those of AtPCO4 but had a higher RAP2.12₂₋₁₅ peptide affinity. This indicates that AqADO would respond similarly to AtPCO4

under decreasing O₂ concentrations; however its strong peptide affinity suggests a stronger interaction with ERFVII substrates, which could enhance the substrate recognition and therefore NCO activity. Finally, HsADO showed comparable catalytic efficiency towards RAP2.12₂₋₁₅ compared to AtPCO4, but reduced affinity for O₂ (44). Although this may reflect the conditions under which this parameter was determined, it nevertheless suggests that HsADO may be unable to effectively target ERFVII in mild hypoxic conditions.

We next predicted the impact of these kinetic parameters on gene expression under hypoxia. We integrated the kinetic values of each NCO into the above-described ODE-based model to simulate generic hypoxia-responsive gene (*HRG*) expression under hypoxic conditions (1 % O₂ v/v) (**Figure 3f**). As expected, the model predicted no *HRG* induction in hypoxia with MpPCO, whereas control of RAP2.12 by AqADO, HsADO and KnPCO increased *HRG* levels compared to the levels obtained with AtPCO4.

To validate the predictions on cysteine dioxygenase activity of the selected NCOs, we utilized yeast as a heterologous expression system (**Figure 3g**). Yeast, possessing the Arg/N-degron pathway components (ATE1 and UBR1) but lacking endogenous NCOs and ERFVII, provides a suitable environment for assessing NCO activity without interference. We co-transformed yeast cells with each of the NCO enzymes and the Dual Luciferase Orthogonal Reporter (DLOR) system (46), enabling *in vivo* evaluation of NCO catalytic activity. Co-transformed yeast cells were subjected to hypoxia (1 % O₂ v/v) or normoxia (21 % O₂ v/v) for 6 hours. As expected, the ERFVII-based, Cys2-substrate was stabilised under hypoxia in yeast expressing AtPCO4. Significant substrate stabilization was also observed in yeast expressing AqADO, HsADO, and MpPCO. Conversely, KnPCO expression did not result in RAP2.12 accumulation under hypoxia. In fact, the substrate was already stable under normoxic conditions (**Supplementary Figure 4**). The lack of substrate stabilization in the presence of KnPCO contradicts the *in vitro* data, overall suggesting that KnPCO is unable to effectively target RAP2.12 for degradation *in vivo*.

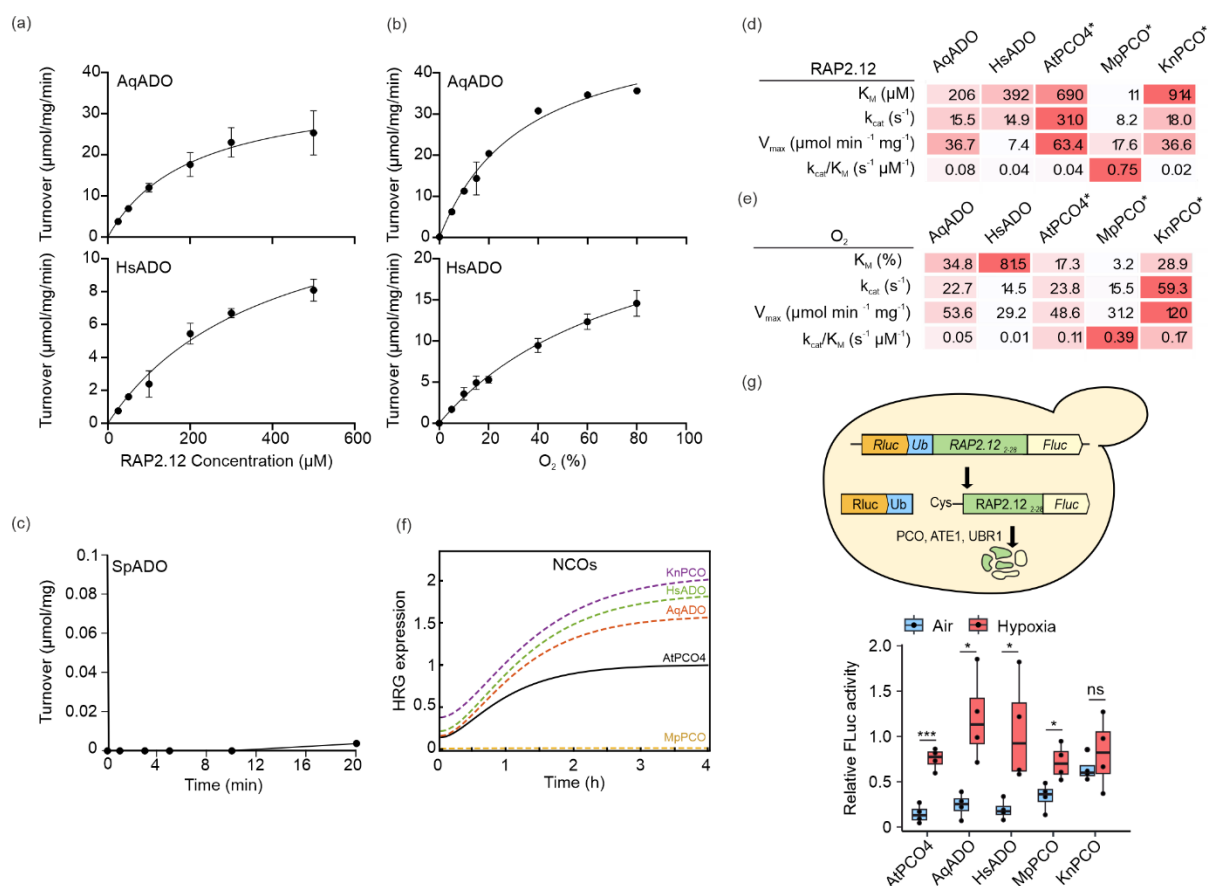


Figure 3 – Characterisation of NCO activity. Michaelis–Menten kinetic plots showing the Cys dioxygenation activity of AqADO and HsADO in relation to RAP2.12₂₋₁₅ peptide (a) and O₂ (b) concentrations. (c) Cys oxidation rate for SpADO using 200 μM of RAP2.12₂₋₁₅ peptide. (d) Kinetic values of selected NCO towards the RAP2.12₂₋₁₅ peptide. Colour formatting to identify highest values with strongest red hue is applied by row. (e) Kinetic values of selected NCO towards O₂. Colour formatting in (d, e) to identify highest values with strongest red hue is applied by row. Asterisks in (d, e) indicate data presented in previous publications (44, 45). (f) ODE model prediction of HRG expression by RAP2.12 when this is regulated by different NCOs. (g) On top, a scheme depicting the DLOR assay in *S. cerevisiae* (46). On the bottom, relative FLuc activity in yeast cultures expressing NCO together with the DLOR substrate in hypoxia (1 % O₂ v/v) and normoxia (21 % O₂ v/v) for 6 hours. Asterisks indicate statistical difference between air and hypoxia as evaluated using a two-sided Student's t-test ($n = 4, p < 0.05$).

Substitution of endogenous PCO with different NCOs affects growth and development in Arabidopsis

To assess NCO activity *in planta* and its impact on growth and development, we first tested whether the six selected NCOs localize where known substrates are expected to be found: the

cytosol and the nucleus. To this end, we transiently transformed Arabidopsis wild-type leaves with constructs bearing codon-optimized *NCO* genes, fused to GFP and placed under the control of the 35S constitutive promoter (CaMV). All selected NCOs localised to the cytosol and nucleus as expected (**Figure 4a**, **Supplementary Figure 5**).

To test functional complementation capacity of the six selected NCOs, we took advantage of the quadruple *4pco* mutant, which displays severe developmental and growth defects. We stably introduced the *NCO*-coding sequences in this genetic background. We placed the six *NCO*-coding genes under the control of the *AtPCO4* promoter so that they could be expressed at levels comparable with endogenous *PCOs* and subjected to the same transcriptional and post-transcriptional regulation. We selected two independent lines per NCO construct, with comparable transgene expression levels. We also verified that the insertion of the NCO-bearing T-DNA in these lines did not inactivate genes related to growth, development or O₂ sensing (**Supplementary Figure 6d-k**).

As for *PCO4* complementation, two lines with slightly different expression levels were included (**Figure 4b**) to assess the effect of expression level on plant physiology. As expected, plants expressing *AtPCO4* complemented the mutant phenotype. However, the different gene expression levels led to phenotypic differences. The lower-expressing line (#2) exhibited reduced rosette fresh weight, seed size and different germination rate compared to #1 (**Figure 4d, e, f**). Such broad phenotypic consequences related to relatively small differences in *PCO4* expression, reveal the important contribution of *PCOs* to plant growth and fitness.

Plants expressing *SpADO* resembled the *4pco* mutant, including stunted growth, pale serrated leaves, and flower sterility (**Figure 4b**) (17, 21). Similarly, expression of *KnPCO* could not fully prevent growth defects, including pale leaves and reduced growth, although these were less severe than in *4pco* and in *SpADO*-complemented lines. Nonetheless, the plants could not produce seeds (**Figure 4b**). The *4pco* mutant expresses *HRG* at high levels under normoxic conditions, as a consequence of its inability to repress *ERFVII* activity (21). To confirm whether these lines displayed a *4pco*-like phenotype also at the transcriptional level, we assessed the expression of two *HRGs*, *ADH1* and *LBD41*, under normoxic conditions. Both *SpADO*- and *KnPCO*-expressing lines showed increased hypoxic response upon normoxia similar to *4pco* plants, indicating a constitutive hypoxic response (**Figure 4c**). These results, consistent with *in silico* and *in vitro* data (**Figure 2**, **Figure 3**), indicate that *SpADO* and *KnPCO* cannot functionally complement the *4pco* mutant.

In contrast, expression of *HsADO*, *AqADO*, and *MpPCO* rescued the *4pco* phenotype, similarly to *AtPCO4*, although we could detect differences at the level of plant vegetative growth and reproductive development among these lines (**Figure 4b**). We therefore decided to exclude non-complementing genotypes and focus our analyses on investigating the effect of

complementing NCOs on plant physiology. AqADO-complemented plants showed increased fresh biomass accumulation compared to AtPCO4 #1 (**Figure 4d**). MpPCO-complemented plants exhibited decreased fresh weight, increased leaf number, delayed flowering, decreased seed area, and delayed germination, both with and without stratification, similarly to AtPCO4 #2 (**Figure 4d, e, f, Supplementary Figure 7a-d**). One of the two MpPCO-expressors, line #2, presented an additional pale seed phenotype (**Supplementary Figure 7d**). HsADO-complemented plants showed similar fresh weight and leaf number as line AtPCO4#1, although they also exhibited delayed flowering and decreased seed area (**Figure 4d, e, f, Supplementary Figure 7a-d**).

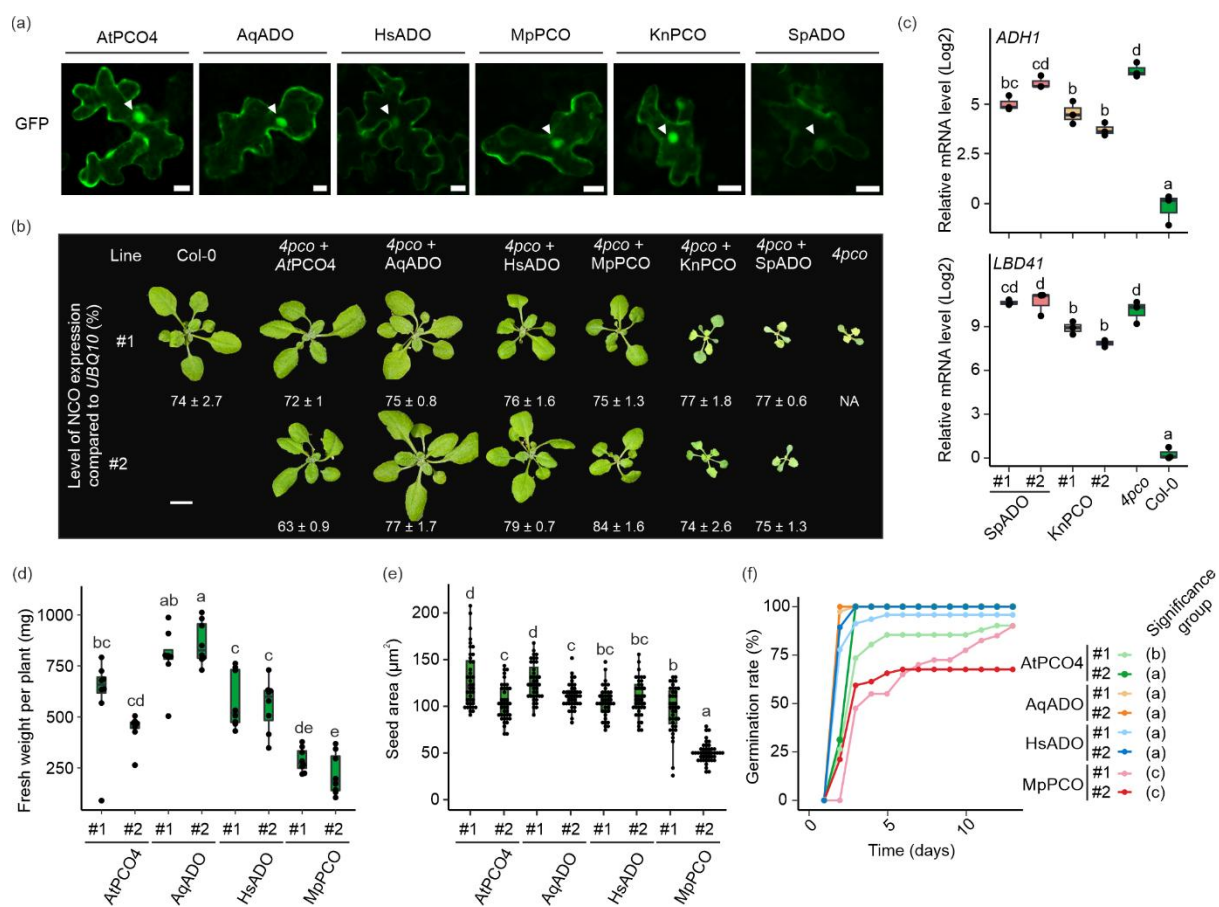


Figure 4 – Complementation of Arabidopsis 4pco mutant with selected NCOs. (a) Confocal images of NCO:GFP fusion proteins in *Agrobacterium*-infiltrated Arabidopsis leaves. Scale bar: 10 μm . (b) Phenotypic differences of 17-day old, NCO-expressing plants compared to the Col-o wild type. Two independent lines are shown for each selected NCO. Relative Expression Level (REL) of each NCO to the expression of the *UBQ10* gene in the respective NCO-expressing line is shown under each plant. Scale bar: 1 cm. (c) Relative mRNA level (log2) of two hypoxia-responsive genes (*ADH1*, *LBD41*) in non-complementing lines expressing SpADO and KnPCO compared to Col-o and the 4pco mutant ($n = 3$). (d) Fresh

weight measurement of 17-day old NCO-complemented plants ($n \geq 7$). (e) Measurement of seed area of NCO-complemented lines compared to Col-o. Statistical differences were evaluated using one-way ANOVA, followed by Tukey HSD post-hoc test ($n = 40$, $p < 0.05$) (c, d, e). (f) Germination rate of NCO-complemented seeds after three days of stratification. Statistical difference was evaluated using the Kaplan-Meier survival analysis ($n \geq 49$, $p < 0.05$).

Transcript profiling reveals a negative correlation between *HRG* expression and growth

Next, we analysed transcriptional changes in NCO-expressing plants to investigate the molecular basis of the observed phenotypic differences. To this end, we sequenced the poly(A)-enriched transcriptome of 7-day-old NCO-expressing seedlings grown under atmospheric (normoxic) conditions. Multidimensional scaling analysis (MDS) revealed only moderate transcriptional differences between *AtPCO4*- and *AqADO*-expressing plants, while *HsADO*- and *MpPCO*-expressors exhibited more divergent transcriptomic profiles (**Supplementary Figure 8a**). Differential gene expression analysis was performed to identify genes significantly altered ($|FC| > 1$, $p\text{-adj} < 0.05$) when compared with PCO4-complemented plants, followed by Gene Ontology (GO) enrichment analysis to assess functional differences. *MpPCO*-expressing plants showed the most up- and down-regulated genes (DEGs), with 175 upregulated and 62 downregulated genes (**Figure 5a**). *AqADO*-expressors exhibited mostly upregulated genes compared with *AtPCO4* plants (55 up- and 13 down-regulated genes) while *HsADO* expression caused the least changes (25 up- and 19 down-regulated genes) (**Figure 5a**). Remarkably, the overlap between differentially expressed genes was extremely limited among NCO-expressor lines (**Figure 5b**). GO enrichment analysis revealed that expression of all three NCOs led to the up-regulation of low-oxygen inducible genes, although only *MpPCO*-expressors exhibited higher levels of core hypoxia-response genes, especially those that require ERFVII for their up-regulation, under low-oxygen (47, 48) (**Figure 5c-d**, **Supplementary Figure 8c**). A similar, though statistically non-significant, trend was observed in *HsADO*-expressing plants (**Figure 5d**). Additionally, *MpPCO*-expressing plants showed enrichment of GO terms associated with immune response and stress-related hormones (**Supplementary Figure 8c**). While it is difficult to explain the enhanced growth and biomass accumulation observed for *AqADO*-expressing plants based on the DEGs list, on the other hand the reduced size of *MpPCO*-expressors is potentially associated with the activation of the hypoxic response under aerobic conditions (**Figure 5c-d**), as observed in case of constitutive ERFVII stabilisation (49). These observations suggest that reduced *in planta* activity of these NCOs toward PCO substrates may lead to incomplete repression of the hypoxia response under normoxic conditions. However, the little overlap in DEGs suggests

that, while HsADO and AqADO, differently from MpPCO, can effectively repress ERFVII activity, other substrates could evade the cysteine dioxygenation catalysed by them and trigger signaling events that result in alteration of gene expression.

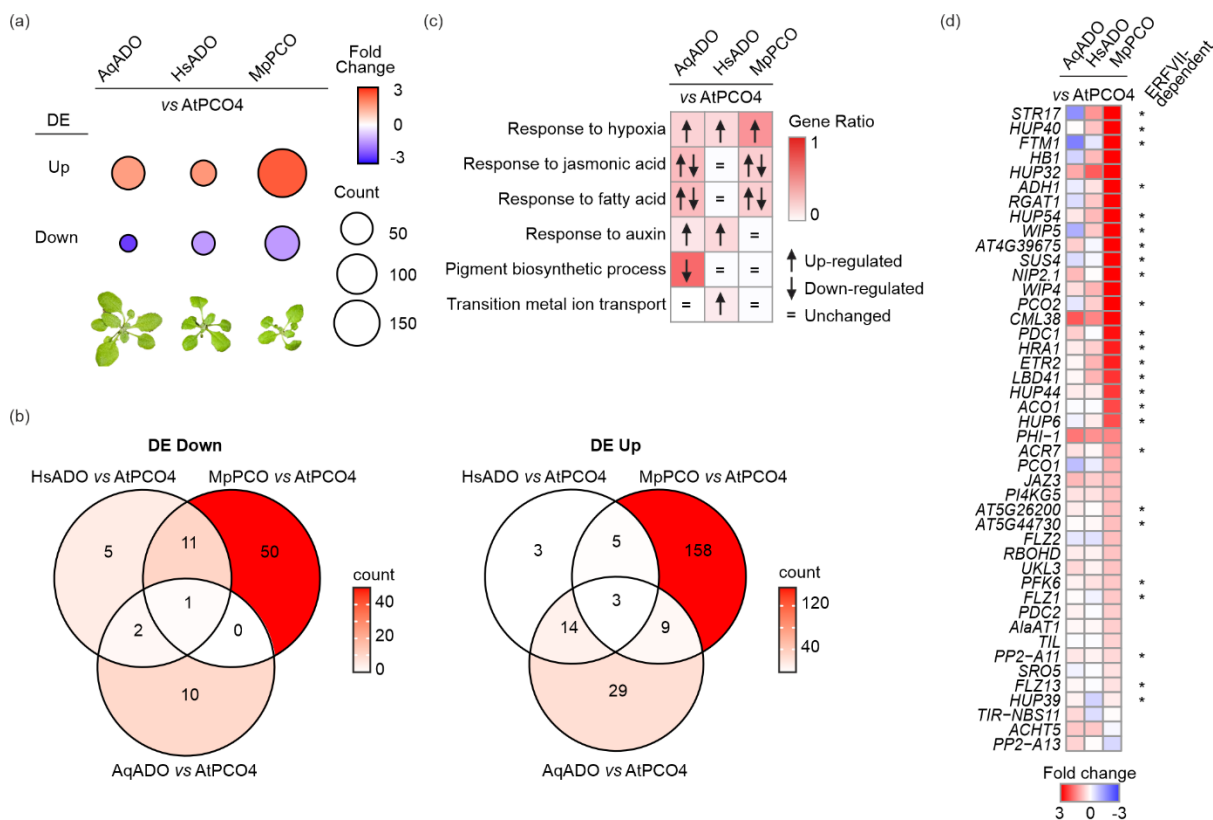


Figure 5 – Transcriptional changes in *NCO*-expressing seedlings grown under aerobic conditions. (a) Differentially expressed genes in AqADO-, HsADO- and MpPCO-complemented lines compared to AtPCO4-complemented ones. (b) Venn diagrams showing the limited overlap of differentially upregulated and downregulated genes among the three contrasts displayed in (a). (c) Comparison of changes in main gene ontology categories among NCOs. (d) Differential expression of hypoxia-core genes in *NCO*-expressing lines compared to *AtPCO4*-expressing lines.

NCOs with reduced activity on ERFVII increased tolerance to submergence

Finally, we investigated the effect of different NCO enzymes on the plant's low- O_2 response. First, we measured the expression of three *HRGs* (*LBD41*, *ADH1*, and *PDC1*) in plants subjected to 24 hours of normoxia (21 % O_2 v/v) or hypoxia (1 % O_2 v/v), followed by 1 hour of reoxygenation (21 % O_2 v/v) under dark conditions (**Figure 6a**). Statistical analysis revealed a significant increase in the expression of all three hypoxia-responsive genes in MpPCO #1 plants under normoxic conditions (**Figure 6a, Supplementary Figure 9a**). Also, MpPCO #2 exhibited a tendency towards elevated *HRG* expression in air, although this increase was

not statistically significant. As expected, hypoxia induced expression of these genes in all genotypes. Notably, both HsADO-complemented lines and MpPCO #1 plants showed significantly higher expression levels compared to all other genotypes, including the AtPCO4 #1 control. Upon reoxygenation, *HRGs* expression decreased, though MpPCO #1 and, for *ADH1*, MpPCO #2, maintained higher expression relative to other genotypes. These findings suggest that the specific activity of each NCO enzyme can differentially modulate the hypoxic response of plants, with MpPCO leading to constitutively higher expression of hypoxia-responsive genes, which persisted during reoxygenation.

Next, we assessed the impact of NCO enzyme complementation on tolerance to low-O₂ stress. 17-day-old soil-grown plants were subjected to full submergence in the dark for 2.5, 3, or 3.5 days. Control plants were maintained in the dark for 3.5 days. Following desubmergence and a 4-day recovery period under standard photoperiod conditions, survival rates were determined. Across all time points, *MpPCO*-expressing lines and *AtPCO4* #2 demonstrated enhanced survival (**Figure 6b, c**). HsADO-complemented lines also showed a trend of increased survival compared to *AtPCO4* #1 control (**Figure 6b, c**), though to a lesser extent than MpPCO-complemented and *AtPCO4* #2 lines. Already under dark conditions, these genotypes displayed slower growth and, for *MpPCO*-expressing lines, reduced biomass accumulation compared to the *AtPCO4* #1 control (**Figure 6d**), consistent with the previous fresh weight and seed area measurements (**Figure 4d**). To further investigate their tolerance, we assessed tissue damage by categorizing plants as 'dead', 'alive', or 'damaged' (**Supplementary Figure 9b**). This analysis revealed that, despite improved survival, MpPCO- and HsADO-complemented lines still exhibited significant levels of damage compared to control conditions in the dark.

Collectively, these results indicate that plants with a more active NCO enzyme exhibited higher biomass (e.g., AqADO-complemented lines and *AtPCO4* #1) and lower survival rates compared to plants with reduced NCO activity that exhibited an initial reduced fresh weight (MpPCO-complemented lines and a trend in HsADO-complemented lines), but despite experiencing damage, exhibited greater resilience to submergence.

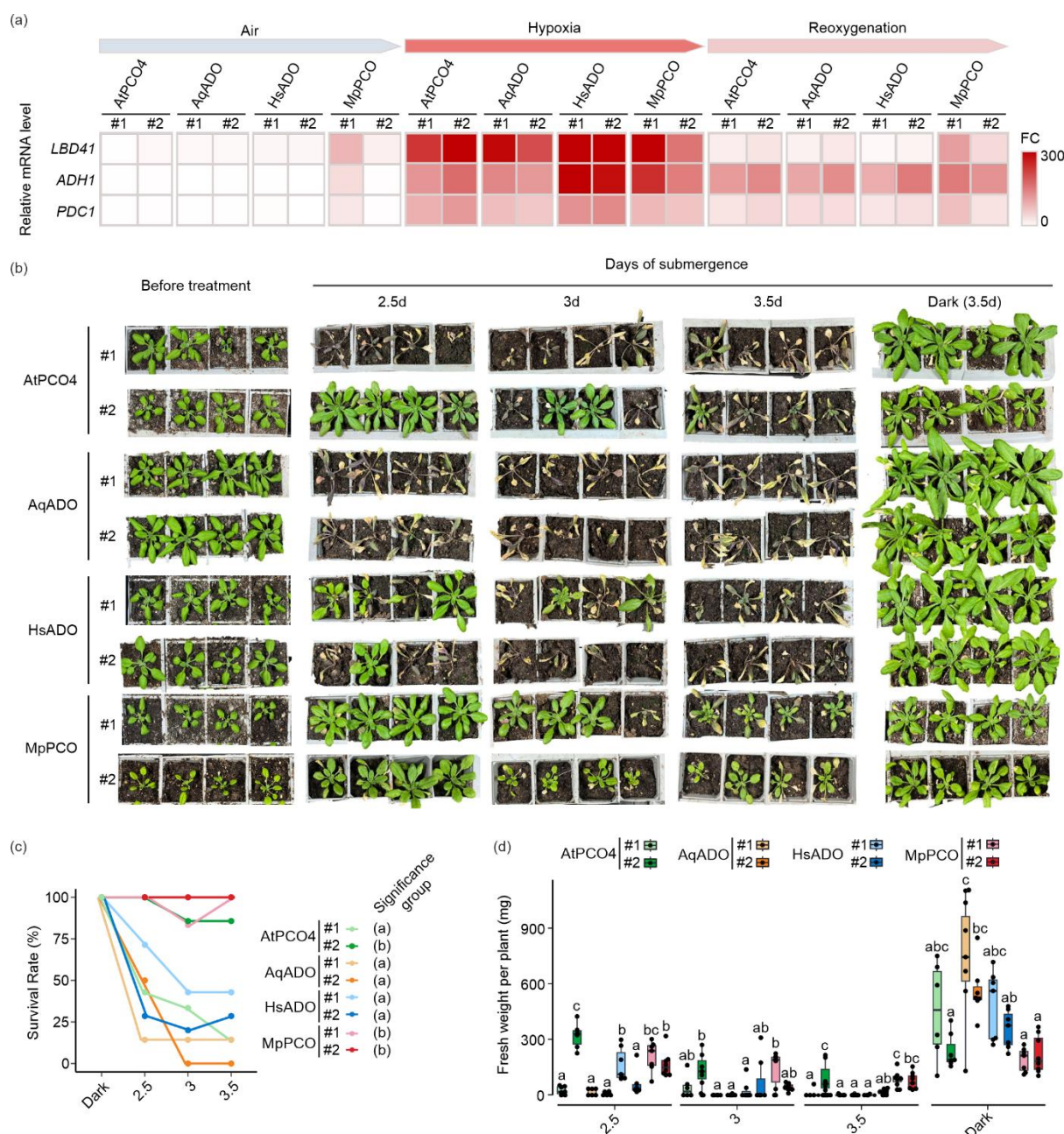


Figure 6 – Effect of NCO-complementation under low-oxygen conditions. (a) Heatmap representing the relative mRNA level of three hypoxia-core responsive genes (*ADH1*, *LBD41*, *PDC1*) upon 24 hours of normoxia (21 % O_2 v/v), hypoxia (1 % O_2 v/v) or hypoxia followed by 1 hour of reoxygenation (21 % O_2 v/v). All treatments and controls were kept in the dark. Statistical significance was evaluated using one-way ANOVA among treatments, followed by Tukey HSD post-hoc test ($n = 3$, $p < 0.05$). (b) Phenotype of 17-day old NCO-complemented plants submerged for 2.5 to 3.5 days in the dark. Photos for each plant were taken before the treatment and four days after the end of the submergence treatment. Control plants were kept in dark for 3.5 days ($n = 8$). (c) Survival rate of submerged plants analysed four days after the end of the submergence treatment. Statistical analysis of survival rate was conducted using the Kaplan-Meier survival analysis method. Letters indicate significant

differences among groups. **(d)** Fresh weight of plants four days after desubmergence. Statistical differences were evaluated using one-way ANOVA, followed by Tukey HSD post-hoc test ($n \geq 6$, $p < 0.05$).

Discussion

In the present study, we tested the consequences of substituting endogenous *Arabidopsis* PCOs with homologs from different eukaryotic lineages. The motivation to undertake such a broadly cross-species approach relies on the opportunity offered by natural variation in the three eukaryotic kingdoms to explore the evolutionary space available for N-terminal cysteine oxidases. Our results confirm that variations in NCO sequences, structures and kinetic properties do impact Cys2-substrate activity, and show how this affects plant growth and development. This transgenic substitution alters the basal expression levels of genes involved in adaptations to low-oxygen availability and their dynamics when cells experience fluctuations in oxygen availability, ultimately affecting the plant's capacity to cope with submergence conditions.

Although NCOs are ubiquitously found in plants and animals, the number of intra-specific variants varies between kingdoms. While only one or two isoform variants are found in metazoans, in flowering plants, where they constitute the main cellular oxygen sensor, their number expanded remarkably, even when considering genome duplication events (17, 21). The observation that this is not the case for the other enzymes that participate to the N-cys-degron, such as ATE and PRT6/UBR, suggests the need, in flowering plants, for finely-tuned control of N-Cys substrate stability, and the opportunity to achieve this through a cell-specific suite of PCO isoforms. For example, thorough characterisation of *Arabidopsis* PCOs has revealed that isoforms with limited sequence diversity exhibit rather distinct kinetic activities, potentially influencing substrate preferences and different roles of PCOs under stress or physiological conditions (44). Therefore, we speculated that NCOs with higher sequence divergence might have even more diverse kinetics.

Indeed, a comparison of six NCOs from across the eukaryotic domain revealed even greater differences in affinity for the canonical ERFVII peptide and for O₂, and activity (**Figure 3a-e**). This is most likely due to differences in sequence and structure among the enzymes, which impact, besides catalysis, substrate interaction and selectivity. Phylogenetic and structural analyses confirmed that NCOs are conserved across eukaryotic lineages, not only in their primary amino acid sequences but also in core structural features, such as the β -barrel catalytic pocket and residues essential for the iron coordination and substrate interaction (**Supplementary Figure 1, Figure 2**). These conserved features support a shared

enzymatic mechanism and a potentially ancestral functional role. Nevertheless, we observed key structural variations among homologs, particularly in electrostatic surface charge distribution and substrate pocket volume. Such divergence likely reflects lineage-specific substrate specialization, consistent with the kingdom-specificity of known NCO targets such as plant ERFVIs and animal RGS proteins. Our docking simulations further support this hypothesis, as the fungal SpADO displayed divergent docking trajectories for the Arabidopsis RAP2.12 peptide, suggesting substrate incompatibility. These findings also highlight the usefulness of *in silico* modelling in predicting substrate interaction, particularly for peptide-like substrates.

It is plausible that some of the tested NCOs, including ADO, may also turn over small thiol-containing molecules rather than peptide-based substrates (50). In such cases, structural features like differences in the binding pocket volume may be indicative of this substrate preference, although further biochemical validation is needed. Importantly, while structural and docking data can be informative, they do not always correlate with *in vivo* function. For instance, SpADO exhibited a markedly altered three-dimensional structure, including an additional loop potentially occluding the active site, and failed to catalyse RAP2.12 oxidation *in vitro* or complement the *apco* mutant phenotype. Conversely, KnPCO, despite showing minimal structural deviation, was similarly inactive, suggesting potential adaptation to non-angiosperm-specific substrates or limitations in our docking approach, which focused on peptide segments rather than full proteins.

Variation in sequence and structure is also likely to impact the interaction with other proteins, including potential post-translational regulators of PCO activity. Y2H and co-immunoprecipitation surveys indicate only a few potential interactions of Arabidopsis PCOs, PCO2 with the SEC5A subunit of the exocyst complex, PCO3 with the sulfurtransferase SUFE1 and PCO5 with the subunit 5A COP9 signalosome (51, 52). While this study was not designed to explore this aspect of PCO regulation, it can explain the discrepancy between the predicted and measured effects of NCOs and HRGs (**Figure 3f-g, Figure 6a**). The DLOR assay in yeast, where NCO expression levels are substantially comparable across cells, exhibited a trend of enhanced substrate stability in hypoxia for *HsADO*- and *AqADO*- expressing cells. However, the model indeed overestimated KnPCO and MpPCO capacity to induce Cys2-substrate degradation. This could raise doubts about the capacity of our ODE-based model to effectively predict the effect NCO substitutions on HRG expression. As stated above, protein-protein interactions are likely to impact this. Additional complications are determined by cell heterogeneity in plant tissues and the impact of NCO and ERFs transcription and translation towards the determination of *HRG* expression. Because of well-known feedback mechanisms

such as *ERFVII*s and *PCOs* gene induction by hypoxia (21, 53), *HRG* expression predictions are especially challenging in plants.

Despite the afore-described limitations, our approach defines an effective pipeline for improving the control of hypoxia responses that can be conveniently conjugated with the rational-design or directed evolution of NCO. When applied through precise gene editing (54), this has the potential to affect the plant's ability to respond to hypoxia exactly in the desired trajectory, as the impact on gene expression in this case would be negligible. This is crucial, when looking at the phenotypes observed for plants grown under aerobic conditions in this study. Substitution of *PCO4* with *HsADO*, *AqADO*, and *MpPCO* affected rosette biomass accumulation, flowering time, and seed size (**Figure 4, Supplementary Figure 7**). This phenotypic variability suggests that differences in kinetic efficiency or substrate interaction dynamics markedly influence growth and developmental outcomes. For example, *AtPCO4* #2 line exhibited only mild *HRG* induction and yet showed marked developmental delays, consistent with computational predictions that reduced *PCO* expression drives *HRG* upregulation. Similarly, *HsADO*, characterised by a smaller pocket volume and reduced catalytic efficiency toward O_2 , also led to altered phenotypes in planta, including trends toward increased *HRG* expression, albeit without statistically significant differences. These results suggest that phenotypic differences likely reflect organ or tissue-specific roles for NCOs, associated with the need to regulate different substrates. The N-Cys-degron substrates identified so far participate to various developmental processes, including root and leaf development and shoot apical meristem activity (3, 4, 32, 55–57). The comparison of transcript profiles of *AqADO*- and *MpPCO*-expressing plants compared to *PCO4*-complemented ones suggests the existence of additional substrates. There are more than 200 MC-proteins in Arabidopsis, and more Cys2-proteins can be created by endoproteolysis (6, 58).

When evaluating the effect of flooding, plants complemented with *MpPCO* and *HsADO*, which exhibited delayed seed germination, slow vegetative development, and late flowering, displayed enhanced submergence survival. These findings suggest that a trade-off may exist between growth and stress tolerance. One possibility is that enhanced hypoxic response in low-efficiency NCO-complementing lines directly influences developmental and growth regulators. However, it is also plausible that the altered phenotype is due to the regulation of other N-degron targets, such as *VRN2* or *ZPR2*, which are known to play roles in flowering and developmental transitions. Alternatively, the link may be indirect: delayed development could allocate fewer resources to growth and more to survival mechanisms when plants experience stress. This aligns with prior studies on strategies evolved by plants to reduce growth to improve flooding tolerance due to enhanced carbon storage or metabolic flexibility

(59, 60). We consider this approach particularly valuable given the expanding roles of ERFVIIIs in responding to drought, heat, salt, oxidative, and osmotic stresses, often mediated through ABA and ROS signaling pathways (61).

The elevated basal hypoxic response in MpPCO lines suggests partial stabilization of ERFVIIIs under normoxia. This is supported by prior mutagenesis studies in *AtPCO4*, where disruption of substrate-binding residues (e.g., H164 and D176) resulted in increased *HRG* expression (20). While this constitutive activation is not necessarily advantageous under normal conditions, it raises the question as to whether constitutive or elevated *HRG* expression could enhance hypoxia tolerance by priming the response system. A similar concept has been observed in ethylene-treated plants, where *PHYTOGLOBIN1* induction enhances flooding tolerance (62). Similarly, pre-exposure to waterlogging has been shown to improve stress resilience via redox, transcriptional, and metabolic reprogramming (63–66). Finally, N-degron signaling has been previously linked to chromatin remodelling, via the VRN2 substrate (2, 4, 28), both of which could contribute to long-term priming to low-oxygen stress. It remains an open question whether a moderate, constitutive hypoxic signature, as observed in the *MpPCO*-expressing lines, is broadly beneficial or if it risks detrimental trade-offs under non-stress conditions. These observations highlight the broader role of ERFVII signaling beyond hypoxia. NCO-mediated differential RAP2.12 stability likely affects redox homeostasis, with implications for both hypoxic stress and the subsequent reoxygenation. Modulation of ERFVIIIs has been shown to impact H₂O₂ signaling and activate specific transcriptional responses during reoxygenation (67, 68). The phenotypic variability observed across our complemented lines likely reflects differences in energy use, redox homeostasis, and developmental state. Given this, modulating ERFVII stability via NCO activity has far-reaching implications for plant stress resilience more generally.

In summary, our findings reveal that, while the core NCO structure is conserved across eukaryotes, variations in kinetic properties, substrate pocket size and electrostatic surface potential significantly alter downstream hypoxic responses *in planta*, development, and stress tolerance. This is evidenced by the differential complementation phenotypes in the *Arabidopsis apco* mutant and the enhanced submergence tolerance observed in lines expressing less efficient NCO variants. These differences reflect a complex interplay between enzyme expression and kinetics, developmental regulation and ability to promptly respond to an environmental cue. By integrating structural, biochemical, and physiological analyses, this data highlights the potential for tuning the hypoxic response through targeted engineering of PCO and ultimately improve plant survival under low-oxygen stress.

Material and Methods

Phylogenetic analysis

Phylogenetic analyses were performed using MEGA X (69). NCO sequences were aligned using the MUSCLE algorithm (70). Phylogenetic trees were obtained using the maximum-likelihood algorithm (71) and a bootstrapping method based on 1000 repeats. Multiple sequence alignment of 50 NCO sequences and selected ones are provided in **Supplementary Tables 9, 10**.

3D structure prediction and molecular docking

AtPCO4 and HsADO crystal structures were retrieved from the Protein Data Bank (PDB: 6S7E and 7REI, respectively). Predicted structures for AqADO, SpADO, MpPCO and KnPCO were generated using AlphaFold2 (43). Electrostatic surfaces were measured using the APBS Tool2.1 plugin in PyMOL (v3.1.3) (72). Pocket volumes were measured by the Fpocket webserver 1.0.1 (73). All structures were visualised using Pymol (74).

An AlphaFold predicted structural model of RAP2.12 (structure ID: AF-AoA1S3YXC8-F1-v4) was used to generate substrate peptide files for docking. The RAP2.12 protein was truncated to display only the first 14 amino acids (reflective of experimental conditions). The N-terminal was modified using PyMOL (v3.1.3) (74). For RAP2.12₂₋₁₅, the N-terminal cysteine was modified to remove the hydrogen from the thiol group thereby improving metal binding (modified amino acid labelled: CYF) and the amino group was protonated. Iron-containing structures for AqADO, KnPCO, MpPCO and SpADO were obtained by alignment with AtPCO4 and subsequently complementation with Fe at the active site. Prior to docking analyses, all water molecules were removed. A 2+ charge was applied to the iron (heteroatom, HETATM) by editing the .pdb file. The protein and RAP2.12₂₋₁₅ files were uploaded to the HADDOCK webserver (75, 76) in .pdb format. Two active residues, along with Fe which is key for coordination of the N-terminal Cys, were selected for each NCO: AtPCO4 (D164, Y182), HsADO (D206, Y212), AqADO (D167, Y173), SpADO (D203, Y209), MpPCO (D197, Y203), KnPCO (D184, Y190). HADDOCK was executed using default input parameters. Three stages of docking were applied: (1) randomisation of orientations and rigid-body minimisation (ito) (2) semi-flexible simulated annealing in torsion angle space (3) refinement in Cartesian space with explicit solvent (water), to generate 1000 models, best 200 of which were refined in stages 2 and 3. The final docked models were clustered based on similarities (RMSD) and assigned a z-score indicating how many standard deviations from the average the cluster is located. .pdb files of docked results were visualised in PyMOL (v 3.1.3) (74).

Modelling HRG expression as a function of NCO parameters

To describe the expression dynamics of Hypoxia-Responsive Genes (*HRGs*) in the N-degron pathway-based O_2 sensing system, we employed a previously developed mathematical model which consists of two coupled Ordinary Differential Equations (ODEs) (40).

For simplification, we assumed that all reactions occur in a homogeneous environment, disregarding compartmentalization or microenvironments within the cell. Additionally, we assumed that all reaction species – including RAP2.12, AtPCO4, O_2 , and mRNA – are uniformly distributed throughout the system. To ensure comparability, we set the initial concentration of AtPCO4 at steady state under normoxic conditions, representing the baseline (0 timepoint) in our simulations. Furthermore, we assumed that AtPCO4 protein levels remain constant following exposure to hypoxia (1 % O_2 v/v). This assumption is reasonable within a short timeframe, as protein synthesis is significantly reduced under hypoxic conditions (77). By keeping AtPCO4 levels unchanged, we can analyse the dynamic hypoxic responses based solely on its catalytic activity and affinities for O_2 and RAP2.12.

For our simulations, we used previously fitted rate constants – k_3 , K_{M1} , and K_{M2} – representing AtPCO4 catalytic activity and its affinities for O_2 and RAP2.12 in the AtPCO4-mediated degradation of RAP2.12 (40). To model hypoxic responses, we obtained the initial concentrations of RAP2.12 and HRG mRNA from steady-state simulations performed under normoxic conditions (21 % O_2 v/v) for 6 hours. Subsequently, we conducted dynamic simulations under 1 % O_2 v/v conditions for 4 hours. To explore how the N-degron pathway responds when regulated by enzymes with different kinetic properties than AtPCO4, we varied k_3 , K_{M1} , and K_{M2} across a range of 10 % to 300 %, mimicking different catalytic activities and affinities for O_2 and RAP2.12. This approach allowed us to assess how the dynamics of hypoxic responses vary when regulated by NCO from different species through the N-degron pathway.

For additional *HRG* simulations based on different NCOs, the parameters were obtained by using MATLAB's 'Talwar', 'Fair' and 'Huber' weight functions within the *nlinfit* function. The rate constants k_6 , K_{M4} , and K_{M5} as well as k_9 , K_{M10} , and K_{M11} were fitted for the AqADO- and HsADO-mediated degradation of RAP2.12, respectively. Meanwhile, k_7 , K_{M6} , and K_{M7} , and k_8 , K_{M8} , and K_{M9} were re-fitted for the MpPCO- and KnPCO-mediated degradation of RAP2.12 (45). This three-dimensional nonlinear fitting is depicted in **Supplementary Figure 3**. All parameters and equations are shown in **Supplementary Tables 3 - 5**.

Plant Material and Growth Condition

A. thaliana Columbia-0 (Col-0) was used as wild-type ecotype. Quadruple mutant *4pco* seeds were obtained as previously described (17). Seeds were sown in a soil:vermiculite 3:1 ratio mixture, stratified at 4°C in the dark for three days, then germinated at 22°C/20°C with a

16h:8h, light:dark photoperiod and 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ intensity. For *in vitro* propagation, sterilised seeds were cultivated on half-strength MS (78) (Duchefa) medium, supplemented with 1 % (w/v) sucrose and 0.8 % (w/v) agar, and grown at 22°C/20°C, 16:8, day:night photoperiod and 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ intensity, after stratification at 4°C for three days in the dark. For leaf agroinfiltration, Col-o plants were grown at 22°C, 12:12 day:night photoperiod for three weeks and subsequently used for *Agrobacterium* infiltration as previously described (79).

Construct design and plant transformation

For PCO substitution, cDNAs codon-optimized sequences for Homo sapiens ADO (HsADO), Amphimedon queenslandica ADO (AqADO), Spizellomyces punctatus ADO (SpADO), Marchantia polymorpha PCO (MpPCO), Klebsormidium nitens PCO (KnPCO), Arabidopsis thaliana PCO4 (AtPCO4) were synthesized (GeneArt, Thermo Fisher Scientific) and cloned into pENTR™/D-TOPO™ (Thermo Fisher Scientific™). pENTR-HsADO, pENTR-MpPCO, pENTR-KnPCO, pENTR-AqADO and pENTR-SpADO were recombined in pH7pPCO4GW (20) using the Gateway™ LR Clonase™ II Enzyme mix (Thermo Fisher Scientific™).

The recombined vectors were used to obtain stable transgenic lines in Col-o using *Agrobacterium*-mediated transformation using the floral dip medium as previously described (80). Seeds were harvested and surface sterilised with 70 % ethanol and 10 % commercial bleach, followed by six washes in sterile water. T₀ seeds were screened for hygromycin resistance and subsequently transferred in soil. The presence of the transgene and the T-DNA inside the sequence of the PCO5 gene, to verify its homozygous/heterozygous state, was detected by PCR using PCRBIO Taq Mix Red polymerase (PCR Biosystems) (**Supplementary Figure 6a-c**). The sequences of primers used for screening the transgene in **Supplementary Table 6**. T₃ transgenic plants were used for experimental procedures.

For *Agrobacterium*-mediated transient expression in Col-o leaves, NCO entry vectors were recombined in pK7WGF2 gateway.

TAIL-seq based identification of T-DNA insertions

Thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) was used to identify the DNA flanking regions to the NCO transgene insertion. High quality DNA was extracted from NCO-complemented lines using the Monarch® Genomic DNA Purification Kit (New England Biolabs). Rounds of TAIL-PCR were conducted as previously described (81) with insertion-specific primers listed in **Supplementary Table 7**. TAIL-PCR products were purified and sequenced using the Nanopore platform (Source Bioscience Ltd). Raw reads were aligned to the *A. thaliana* TAIR10 genome with *minimap2* (v2.24) (82) using -ax map-ont -A2 -B4 -O6,24 -E2,1 -k9 -w2, and insertion sites were identified with *SAMtools* (v1.16) (83).

Measurement of physiological traits

To analyse phenotypical differences, 17-day old plants grown in soil were used to measure fresh weight and leaf number, using at least five biological replicates.

Confocal imaging

Agroinfiltrated Arabidopsis leaf discs were used for GFP localization. For nuclear localization, leaf discs were stained with 1 $\mu\text{g mL}^{-1}$ DAPI (Invitrogen™) for 20 minutes, followed by three washes in PBS before visualisation. Imaging was performed using ZEISS LSM 880 Airyscan microscope, equipped with a 20x objective lens, upon laser excitation at 405 nm and collection at 410-492 nm, for DAPI excitation, or at 488 nm and collection at 495-560 nm, for GFP imaging.

Seed area and germination rate

For determining seed area, 40 dried seeds were imaged using ZEISS Stemi 508 stereo microscope and then measured using the ImageJ software (84).

For germination assays, eight seeds per genotype were sowed in the same plate of MS medium supplemented with 1 % sucrose with or without 3 days of stratification at 4°C. Five biological replicates were analysed. The germination rates were recorded every 24 hours for 13 days. Germination was considered complete at the first protrusion of the radicle.

RNA extraction and RT-qPCR analysis

Total RNA was extracted from 60-80 mg of plant material using the phenol-chloroform extraction method as described previously (8). One microgram of total RNA was subjected to DNase Treatment (Thermo Scientific™) and retro-transcribed using Maxima Reverse Transcriptase (Thermo Scientific™). Real-time quantitative PCR was performed with QuantStudio 5™ Real-Time PCR System (Applied Biosystems) using a SYBR® Green PCR Master Mix (Thermo Fisher Scientific™). *Ubiquitin10* (AT4G05320) was used as housekeeping gene. Target genes relative expression levels were analysed using the comparative $2^{-\Delta\Delta C_t}$ method (85). Three biological replicates were extracted for each condition, each represented by two technical replicates and the average expression was calculated. The sequences of primers used for cDNA amplification are listed in **Supplementary Table 8**.

RNA-sequencing

NCO-expressing seeds were stratified for one week and subsequently grown vertically on 1/2 MS medium supplemented with 1 % sucrose under a 16:8 light/dark photoperiod for 7 days. Samples were harvested at the emergence of the first true leaves and immediately frozen in liquid nitrogen. Total RNA was extracted using the GeneJET RNA Purification Kit (Thermo Scientific™), following the manufacturer's instructions. RNA sequencing was performed in

paired-end mode (PE150) on the NovaSeq 6000 platform (Illumina) by Novogene. Transcriptomic analyses were conducted using R software (v4.3.1). After quality assessment with FastQC, reads were aligned to the *Arabidopsis thaliana* reference genome (TAIR10) using the *Rsubread* package (v2.16.1) (86) and quantified using *featureCounts* package (87). A Multi-Dimensional Scaling (MDS) plot was generated to evaluate similarities and differences in gene expression profiles across samples. Differentially expressed genes (DEGs) were identified using the *edgeR* package (v3.42.4) (88). Fold change data and TPM are provided in **Supplementary Tables 11, 12**. Genes with an absolute log₂ fold change ≥ 1 and a false discovery rate (FDR) < 0.05 (**Supplementary Table 11**) were selected for further analysis. Gene Ontology (GO) term enrichment analysis of the DEGs was performed using the *clusterProfiler* package (v4.10.1) (89). Complete GO results are provided in **Supplementary Tables 13-15**.

Low-O₂ treatment

NCO-complemented seedlings were grown in vertical plates for one week and subsequently treated with normoxic (21 % O₂ v/v) or hypoxic (1 % O₂ v/v) conditions for 24 hours using the H35 Hypoxystation (Don Whitley Scientific). During both treatments, plates were kept at dark to avoid O₂ release from photosynthesis. For reoxygenation, after the hypoxic treatment plates were then exposed to normoxic condition for 1 hour, at dark. For each condition, 3 biological replicates were employed, each consisting of 10 seedlings.

Submergence treatment

For submergence treatment, seeds were sown in a Sinclair Modular Seed soil:John Innes compost No. 2:vermiculite 2:2:1 ratio mixture, stratified at 4°C in the dark for three days, then germinated at 22°C/20°C with a 16h:8h, light:dark photoperiod and 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ intensity. After 17 days, plants were submerged with approximately 23 cm of tap water in black tanks for 2.5 to 3.5 days, with control plants maintained at dark only for the longest duration of the treatment. After the treatment, plants were moved to aerobic photoperiodic growth conditions. Survival was measured four days after desubmergence, and plants were scored as 'alive' for plants that produced new leaves, 'damaged' for plants with at least one dead leaf, and 'dead' otherwise. Fresh weight was measured four days after the end of the treatment. Survival analysis was conducted using the Kaplan-Meyer test.

Luciferase measurements

Total proteins were extracted in passive lysis buffer (Promega). Firefly Luciferase activities were measured using the ONE-Glo Luciferase Assay kit (Promega) and the Nano-Glo® Luciferase Assay System (Promega) was used to measure the activity of NanoLuc® luciferase

enzyme. Luciferase signal was normalized based on total protein concentration using Bradford assay (90).

Heterologous NCO proteins expression and purification

The coding sequences of AqADO and SpADO were cloned into the NdeI and XhoI restriction sites of pET28a (Novagen) as described in White et al. 2018 (44) before transformation into *E. coli* BL21 (DE3) (New England Biolabs) for protein production. Expression of the heterologous protein and purification were also carried out as described in White et al. 2018 (44).

Enzyme kinetic characterisation of AqADO, HsADO and SpADO

The kinetic assays were performed according to the protocol with slight modifications (91). To determine the affinities of enzymes for RAP2.12, the previously synthesised 14-amino-acid RAP2.12₂₋₁₅ peptide (GL Biochem) (44) was prepared in reaction buffer at pH 8.0 (50 mM bis-tris propane [1,3-bis(tris(hydroxymethyl)methylamino)propane] and 50 mM NaCl dissolved in LC/MS-grade water). To maintain a reductive environment, 5 mM TCEP, 20 μ M FeSO₄, and 1 mM ascorbate were added to the samples. Aliquots (100 μ L) of RAP2.12₂₋₁₅ at different concentrations (25, 50, 100, 200, 300, and 500 μ M) were transferred into silicone-sealed MS vials and equilibrated with an 80:20 nitrogen-to-oxygen ratio using a mass flow controller (Brooks Instruments) to mimic atmospheric conditions. The samples were then pre-incubated in a benchtop thermocycler (Eppendorf) at 25°C for 10 min. Reactions were initiated by injecting 1 μ L of enzyme at a final concentration of 0.1 μ M using a gas-tight syringe (Hamilton) and were allowed to proceed for 2 min (within the linear range of activity). Reactions were terminated by injecting 100 μ L of 10 % formic acid, and if necessary, samples were further diluted in 5 % formic acid to prevent detector saturation. The same method was applied to determine enzyme affinities for O₂, except that 200 μ M aliquots of RAP2.12₂₋₁₅ were equilibrated with different nitrogen-to-oxygen ratios to mimic O₂ levels of 0%, 5%, 10%, 15%, 20%, 40%, 60%, and 80%. Oxidation was monitored using ultrahigh-performance liquid chromatography-mass spectrometry (UPLC-MS), and turnover was quantified by comparing the areas under the product and substrate ion peaks extracted from the total ion current chromatogram. UPLC-MS measurements were obtained as previously described (44). All kinetic figures and parameters were generated using Prism (GraphPad).

*DLOR assay using *S. cerevisiae**

A haploid parental strain BY4742 (Mat α ; his3- Δ 1; leu2- Δ 0; lys2- Δ 0; ura3- Δ 0) was co-transformed following the LiAc/SS carrier DNA/PEG method (92) with the DLOR-pAG413GPD (35) and each pENTR-NCO recombined into pAG415GPD (Addgene plasmid

#14146) via LR. Transformants were selected on yeast synthetic drop-out medium lacking leucine and histidine; 6.7 g L-1 Yeast Nitrogen Base (DIFCO), 1.37 g L-1 Yeast Dropout Medium (Sigma-Aldrich) and 20 g L-1 glucose, plus 0.16 M uracil and 0.32 M tryptophan. Positive colonies were grown in a 96-well plate, as described previously (35). For hypoxic treatments, cells were incubated in the H35 Hypoxystation (Don Whitley Scientific), at 1 % O₂ (v/v), 30°C for 6 hours. Luciferase activity was measured using the Dual-Luciferase® Reporter (DLR™) Assay System (Promega).

Statistical Analyses

All statistical analyses were performed using R Statistical Software (v4.4.2, Foundation for Statistical Computing, Vienna, Austria). Additional details are provided in the corresponding sections.

Additional Software

Graphs were made using and R Statistical Software (v4.4.2, Foundation for Statistical Computing, Vienna, Austria).

Data availability

RNA sequencing raw data generated for this study were deposited in the Sequence Read Archive (SRA) at the National Centre for Biotechnology Information under BioProject ID PRJNA1292127.

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Author Contributions

M.P. and F.L. conceived and designed the research. M.P., Y. H., X.C, M. L-P., R. L. and T.B. conducted experiments and analysed the data. Y.H. designed and applied mathematical modelling under the supervision of A.P., M.P. and F.L. wrote the manuscript with input from all authors.

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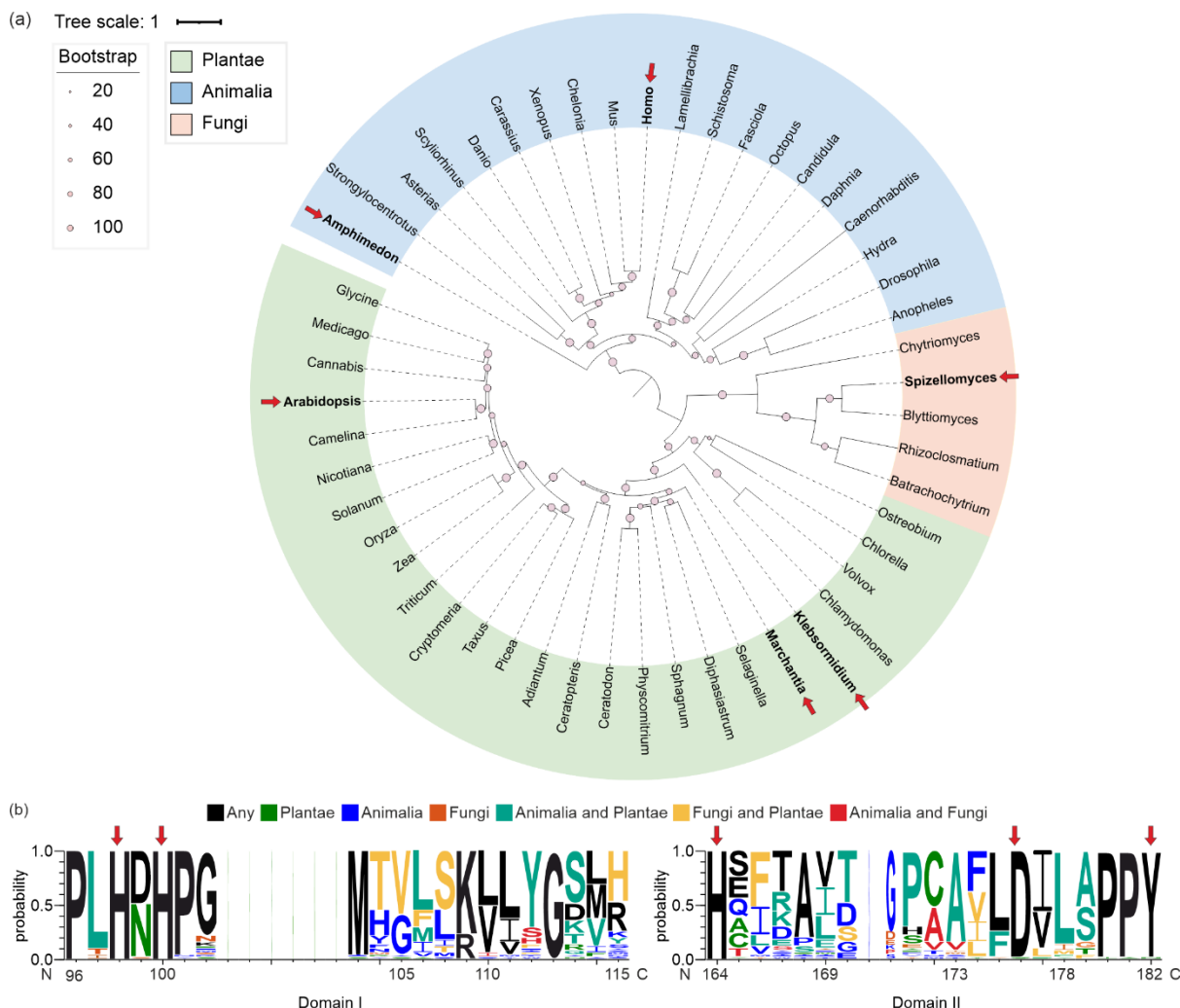
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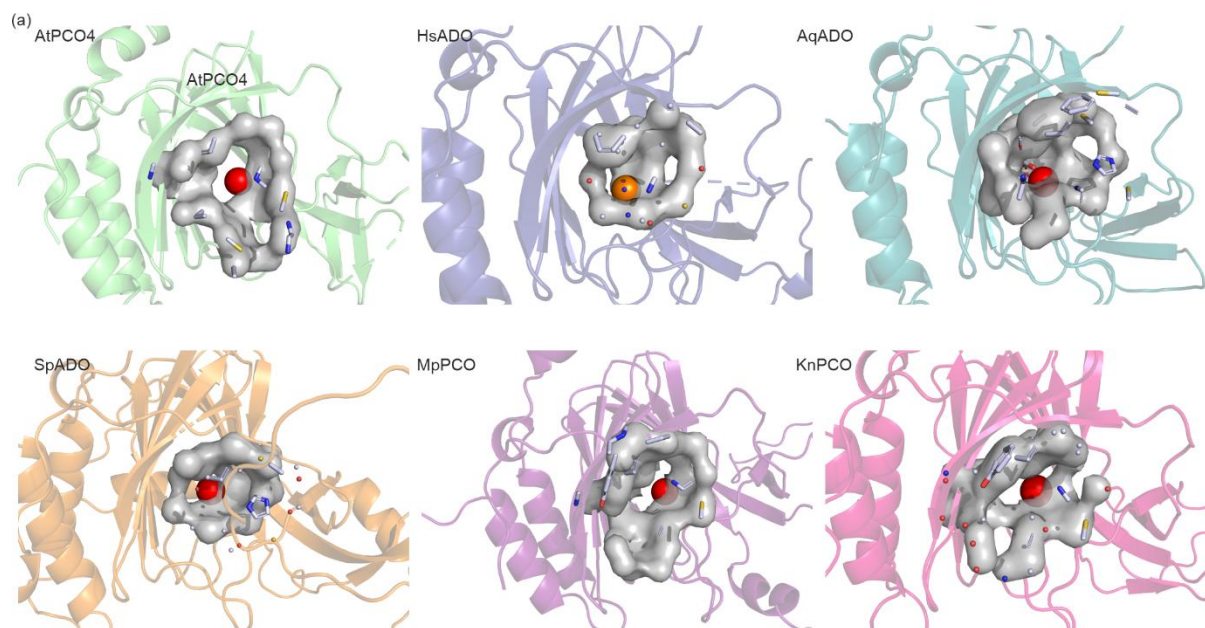
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Supplementary Information



Supplementary Figure 1 – Phylogenetic analysis of NCO sequences. (a) Evolutionary conservation of 50 NCO sequences across the three eukaryotic kingdoms (plants in green, fungi in orange, animals in blue), inferred using the Maximum Likelihood method and JTT matrix-based model. The size of the circles on branches indicates the bootstrap values (1000 bootstrap replicates). (b) Conservation of amino acid residues constituting the AtPCO4 active site in 50 NCO sequences. Amino acids are coloured by kingdom affiliation and their position in AtPCO4 is shown at the bottom.

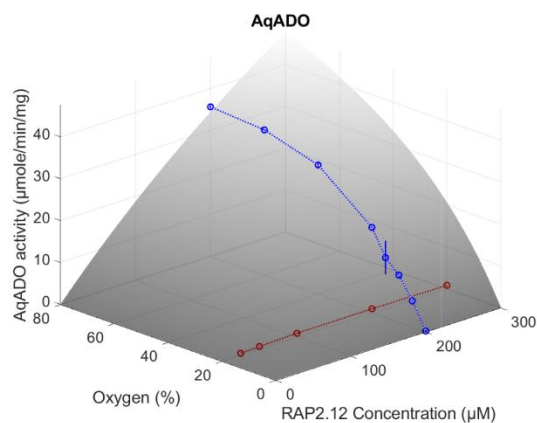


(b)

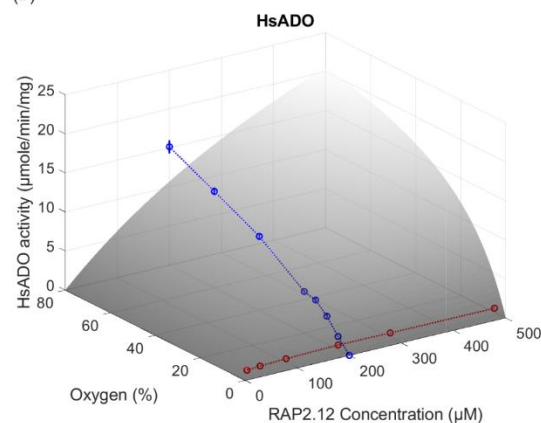
	AtPCO4	HsADO	AqADO	SpADO	MpPCO	KnPCO
Pocket Volume ($\text{\AA}^3$)	568.4	276.8	681.2	316.8	680.6	587.3
Atoms involved	Y73, S83, G85, F87, I95, H98, N99, H199, M103, D176, L188, P181, Y182, S183, H189, C190, Y191, F227, I228	F101, I109, H112, H114, H193, I195, F204, D206, L208, Y212, C220, Y222, F256, C258	F70, N72, E75, N80, A82, F84, M92, H95, H97, M104, H154, I156, F165, D166, L169, Y173, C180, Y183, F209, C211	F70, N72, E75, N80, A82, F84, M92, H95, H97, M104, H154, I156, F165, D167, L169, Y173, C181, Y183, F209, C211	Y81, E86, S91, G93, F95, I103, H106, H108, F187, D197, L199, P202, Y203, C211, Y213, F252, V254, Q255, R256	Y80, E85, S90, G92, F94, I102, H105, H107, F174, D184, V186, Y190, C198, Y200, I238

Supplementary Figure 2 – Predicted internal pockets in selected NCOs. (a) Structural differences among putative substrate-binding pockets (shown in surface representation) in 3D structures of AtPCO4 and HsADO (PDB: 6S7E and 7REI, respectively) and AlphaFold2 prediction for AqADO, SpADO, MpPCO and KnPCO (shown in cartoon representation). (b) Binding pocket volume ($\text{\AA}^3$) and residues surrounding the pocket region. Substrate pockets were identified using the FPocket web server (73).

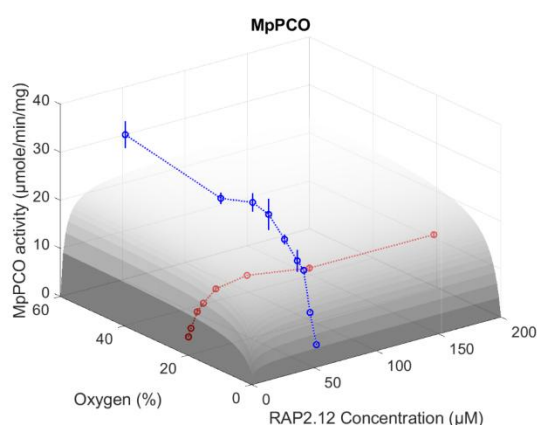
(a)



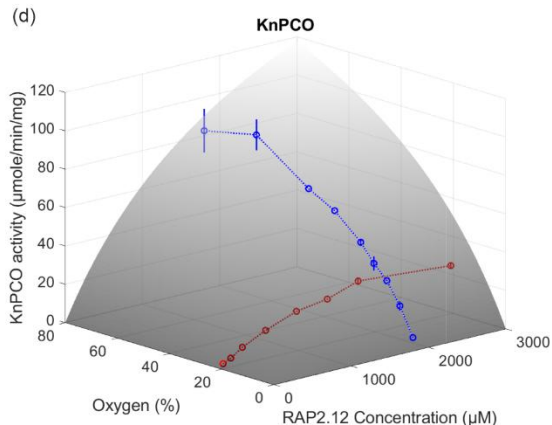
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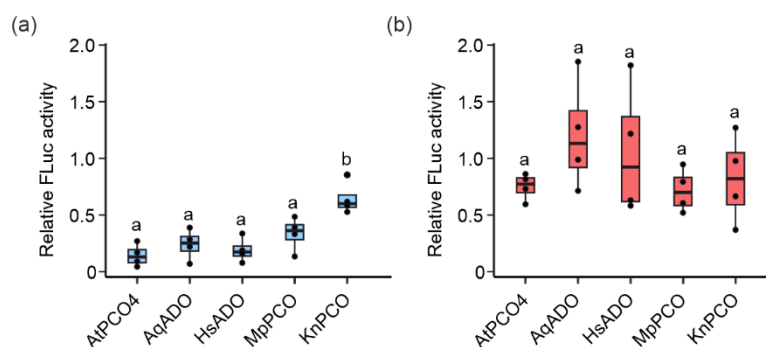
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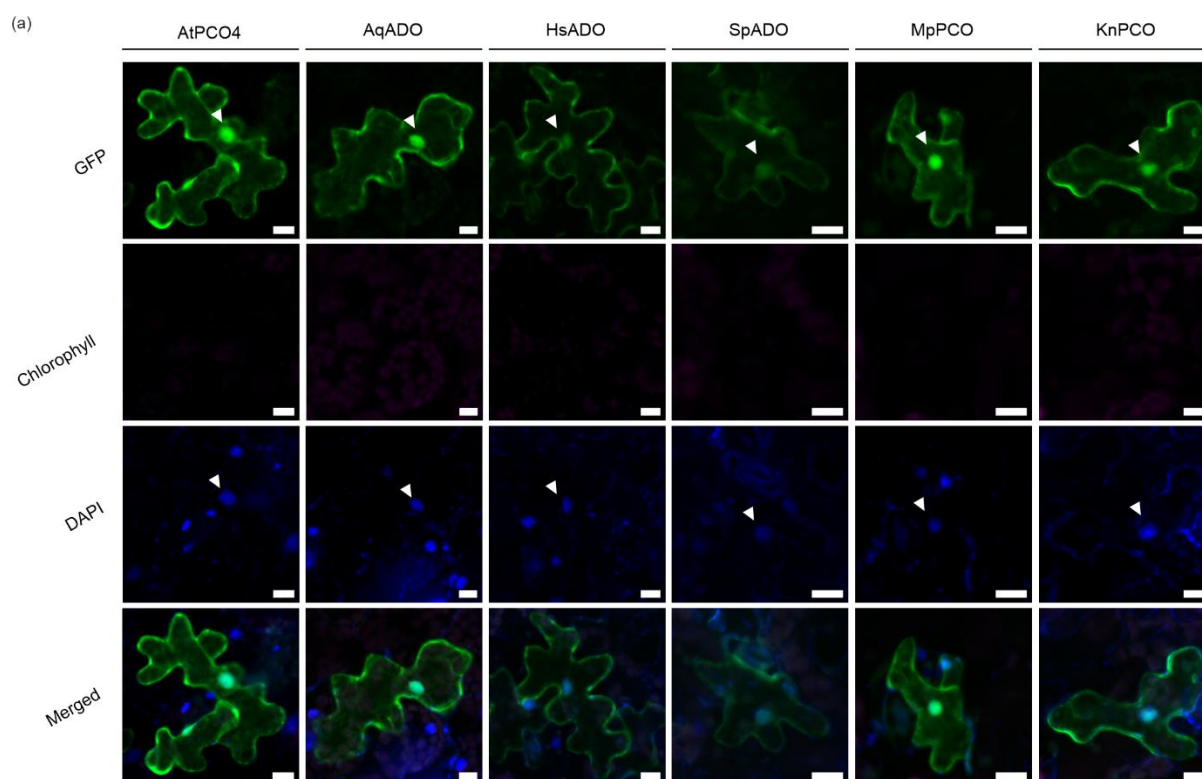
(d)



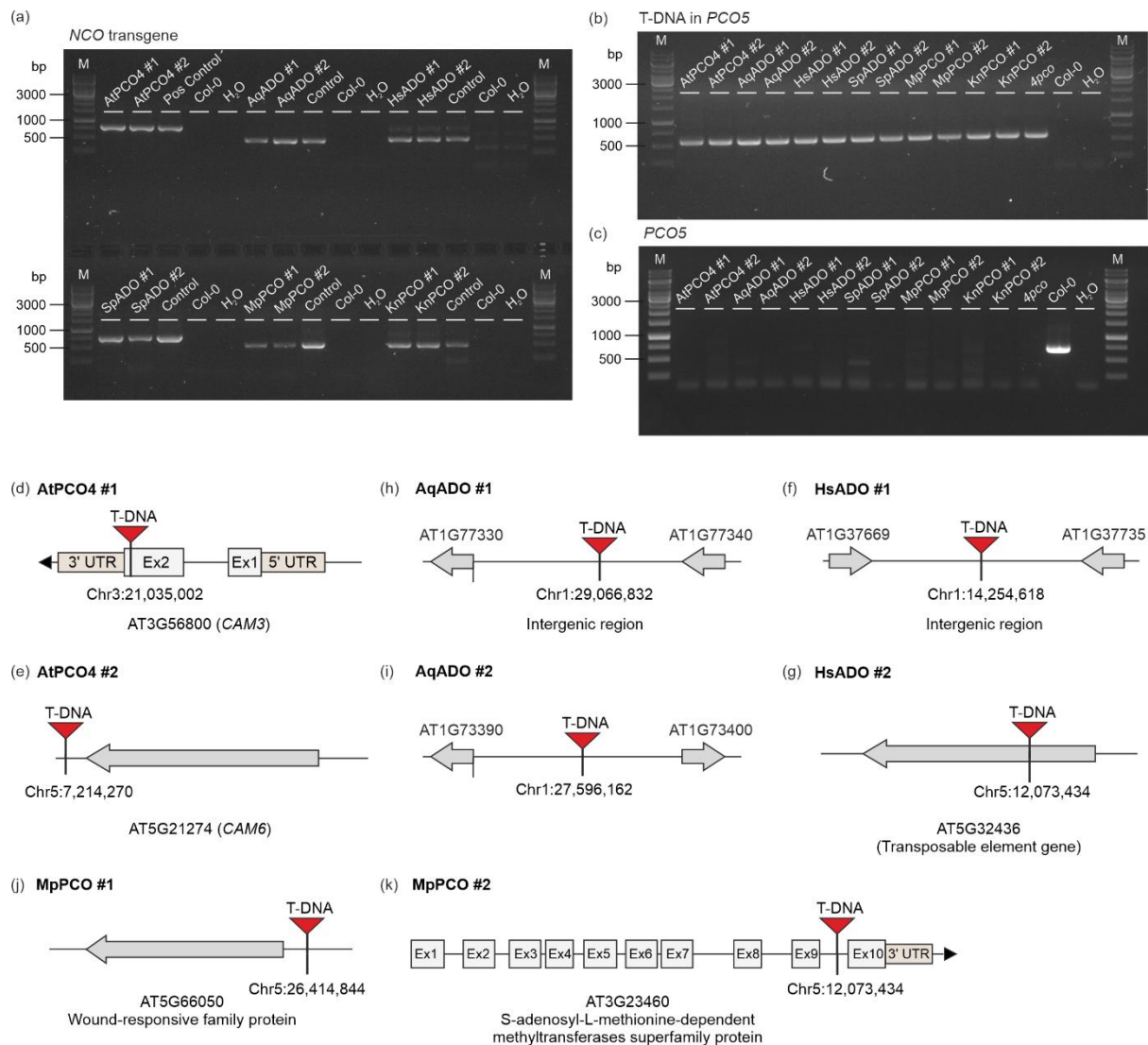
Supplementary Figure 3 – 3D Visualisation and non-linear kinetic fitting of RAP2.12/NCO systems. The 3D surface plot (grey) illustrates the predicted activity of NCOs including AqADO (a), HsADO (b), MpPCO (c) and KnPCO (d), as a function of both oxygen and RAP2.12 concentrations. Red curves represent enzyme kinetics across varying substrate concentrations, while blue curves depict kinetics across different oxygen levels. Experimental kinetic data for KnPCO and MpPCO were extracted from Taylor-Kearney, *et al.*, 2022 (45).



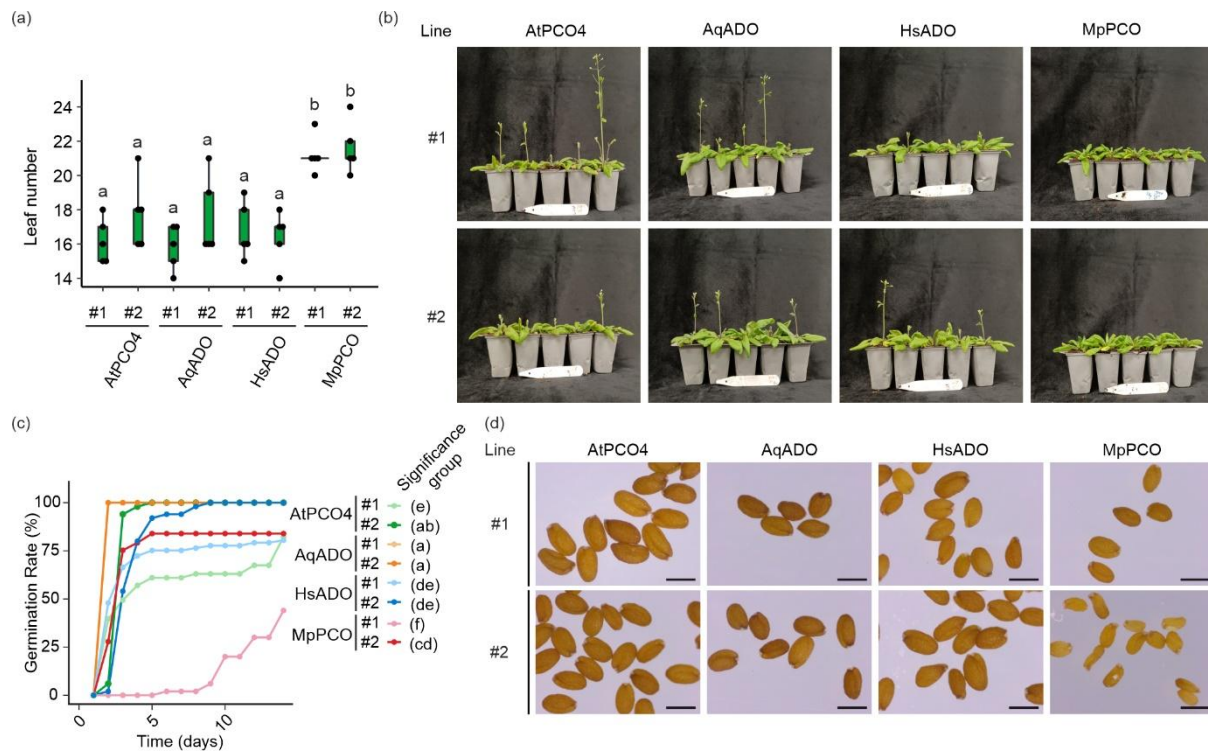
Supplementary Figure 4 – Differential DLOR substrate stabilization in NCO-expressing yeast. Relative FLuc activity in yeast cultures expressing NCO together with the DLOR substrate in normoxia (21 % O₂ v/v) **(a)** or hypoxia (1 % O₂ v/v) **(b)** for 6 hours. Letters indicate statistical differences between groups as evaluated using one-way ANOVA, followed by Tukey's HSD post hoc test (n = 4, p < 0.05).



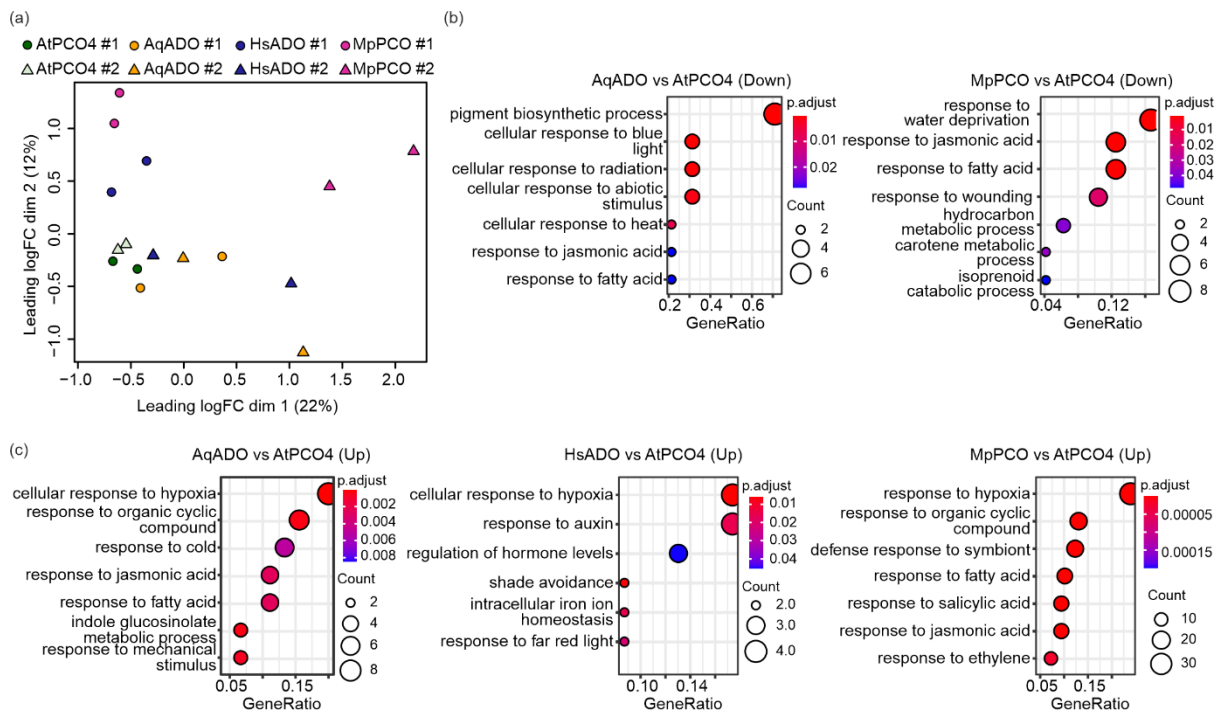
Supplementary Figure 5 – Nuclear localization of NCO proteins. Confocal images of NCO:GFP fusion proteins in *Agrobacterium*-infiltrated leaves. Localization in the nuclei was confirmed by DAPI staining. From top to bottom: GFP, chlorophyll, DAPI and overlay channels. White arrow indicates the nucleus. Scale bar: 10 μ m.



Supplementary Figure 6 – Genotyping of NCO-complemented plants. PCR detection of NCO-complemented plants in *4pco* homozygous background. **(a)** PCR detection of NCO-specific transgene in each independent line. The vector used to transform *Agrobacterium* was used as a positive control; Col-o DNA and H₂O were used as negative controls. **(b)** PCR detection of T-DNA in *PCO5*. DNA from *4pco* homozygous plants was used as a positive control; Col-o DNA and H₂O were used as negative controls. **(c)** PCR detection of *PCO5*. DNA from Col-o plants was used as a positive control, DNA from *4pco* homozygous plants and H₂O were used as negative controls. M: Marker. **(d-k)** Genomic locations of NCO transgene insertion in NCO-complementing lines identified via TAIL-PCR. Red arrows indicate the position of the T-DNA insertion.

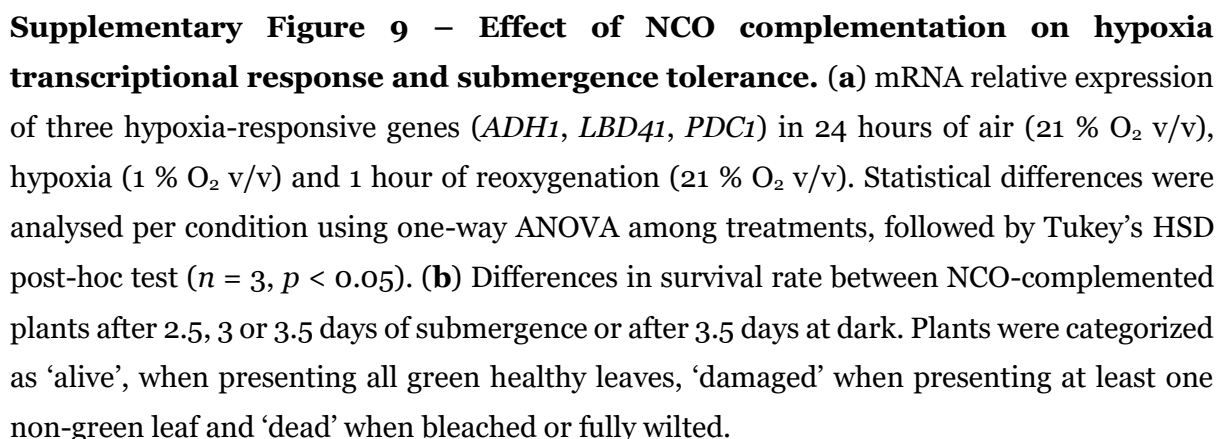


Supplementary Figure 7 – Phenotypic effect of complementation of Arabidopsis *4pco* mutant with selected NCOs. (a) Measurement of leaf number in 17-day old NCO-complemented seedlings. Statistical differences were evaluated using one-way ANOVA, followed by Tukey's HSD post-hoc test ($n = 5$, $p < 0.05$). (b) Differences in flowering time among selected 25-day old NCO-complemented plants ($n = 5$). (c) Germination rate of NCO-complemented seeds without prior stratification. Statistical difference was evaluated using the Kaplan-Meier survival analysis ($n \geq 49$, $p < 0.05$). (d) Phenotype of seeds in different NCO-complemented plants compared to the Col-o wild type. Scale bar: 0.2 mm.



Supplementary Figure 8 – Transcriptional changes in *NCO*-expressing seedlings.

(a) Multidimensional scaling plot (MDS) conducted on the normalized gene expression values of 7-day old *NCO*-expressing seedlings. Horizontal and vertical coordinates show MDS1 and MDS2, respectively, with the amount of variance contained in each component (22 % and 21 %). Each point in the plot represents a biological replicate. Two independent lines with two biological replicates each were sequenced per *NCO*-complementation. Differences in colour and symbol indicate different *NCO*-complementation and independent lines, respectively. (b-c) Pairwise analysis of GO terms associated with transcripts differentially downregulated (b) and upregulated (c) in *AqADO*-, *HsADO*- and *MpPCO*-expressing lines compared to *AtPCO4*. Circle size indicates the gene ratio per GO term, with colour maps indicating the False Discovery Rate (FDR) value ($p.adjust$).



Supplementary Table 1 - Steady-state kinetic parameters derived for NCO and activity toward RAP₂₋₁₅ at atmospheric O₂.

	<i>AqADO</i>	<i>HsADO</i>	<i>AtPCO4</i>	<i>MpPCO</i>	<i>KnPCO</i>
K_M RAP _{2.12-15} (μM)	205.7 ± 53.56	392 ± 91.61	690 ± 110	11 ± 1.1	914 ± 144
k_{cat} RAP _{2.12-15} (s^{-1})	15.51 ± 1.79	14.91 ± 1.99	31 ± 3.49	8.2 ± 0.5	18.0 ± 1.2
V_{max} RAP _{2.12-15} ($\mu mol\ min^{-1}\ mg^{-1}$)	36.66 ± 4.23	7.39 ± 0.99	63.4 ± 7.13	17.6 ± 1.1	36.6 ± 2.4
k_{cat}/K_M RAP _{2.12-15} ($s^{-1}/\mu M$)	0.08 ± 0.02	0.04 ± 0.01	0.04 ± 0.01	0.75 ± 0.09	0.02 ± 0.01

Supplementary Table 2 - Steady-state kinetic parameters derived for NCO and activity toward O₂.

	<i>AqADO</i>	<i>HsADO</i>	<i>AtPCO4</i>	<i>MpPCO</i>	<i>KnPCO</i>
$K_M \text{ O}_2$ (%)	34.84 ± 3.86	81.5 ± 14.39	17.3 ± 1.69	3.2 ± 0.6	28.9 ± 4.1
$k_{cat} \text{ O}_2$ (s ⁻¹)	22.67 ± 1.14	14.48 ± 1.55	23.8 ± 0.9	15.5 ± 0.7	59.3 ± 3.7
$V_{max} \text{ O}_2$ (μmol min ⁻¹ mg ⁻¹)	53.6 ± 2.69	29.2 ± 3.12	48.6 ± 1.83	31.2 ± 1.4	120.2 ± 7.6
$k_{cat}/K_M \text{ O}_2$ (s ⁻¹ /μM)	0.05 ± 0.01	0.01 ± 0.01	0.11 ± 0.01	0.39 ± 0.08	0.17 ± 0.03

Supplementary Table 3 – Reactions and reaction rates of mathematical model.

	Reactions	Reaction Rates	Description
v_1	$\rightarrow \text{RAP2.12}$	k_1	RAP2.12 synthesis
v_2	$\text{RAP2.12} \rightarrow \text{null}$	$k_2 \cdot [\text{RAP2.12}]$	RAP2.12 degradation
v_3	$\text{RAP2.12} + \text{O}_2 + \text{AtPCO4} \rightarrow \text{AtPCO4}$	$k_3 \cdot [\text{AtPCO4}] \cdot \frac{[\text{O}_2]}{K_{M1} + [\text{O}_2]} \cdot \frac{[\text{RAP2.12}]}{K_{M2} + [\text{RAP2.12}]}$	RAP2.12 degradation through oxidation by AtPCO4
v_4	$\text{RAP2.12} \rightarrow \text{mRNA} + \text{RAP2.12}$	$k_4 \cdot \frac{[\text{RAP2.12}]}{K_{M3} + [\text{RAP2.12}]}$	HRG mRNA production by RAP2.12
v_5	$\text{mRNA} \rightarrow \text{null}$	$k_5 \cdot [\text{mRNA}]$	HRG mRNA decay
v_6	$\text{RAP2.12} + \text{O}_2 + \text{AqADO} \rightarrow \text{AqADO}$	$k_6 \cdot [\text{AqADO}] \cdot \frac{[\text{O}_2]}{K_{M4} + [\text{O}_2]} \cdot \frac{[\text{RAP2.12}]}{K_{M5} + [\text{RAP2.12}]}$	RAP2.12 degradation through oxidation by AqADO
v_7	$\text{RAP2.12} + \text{O}_2 + \text{HsADO} \rightarrow \text{HsADO}$	$k_9 \cdot [\text{HsADO}] \cdot \frac{[\text{O}_2]}{K_{M10} + [\text{O}_2]} \cdot \frac{[\text{RAP2.12}]}{K_{M11} + [\text{RAP2.12}]}$	RAP2.12 degradation through oxidation by HsADO
v_8	$\text{RAP2.12} + \text{O}_2 + \text{KnPCO} \rightarrow \text{KnPCO}$	$k_8 \cdot [\text{KnPCO}] \cdot \frac{[\text{O}_2]}{K_{M8} + [\text{O}_2]} \cdot \frac{[\text{RAP2.12}]}{K_{M9} + [\text{RAP2.12}]}$	RAP2.12 degradation through oxidation by KnPCO
v_9	$\text{RAP2.12} + \text{O}_2 + \text{MpPCO} \rightarrow \text{MpPCO}$	$k_7 \cdot [\text{MpPCO}] \cdot \frac{[\text{O}_2]}{K_{M6} + [\text{O}_2]} \cdot \frac{[\text{RAP2.12}]}{K_{M7} + [\text{RAP2.12}]}$	RAP2.12 degradation through oxidation by MpPCO

Supplementary Table 4 – Ordinary differential equations of the kinetic model.
The reaction rates v_1 – v_9 are given in Supplementary Table 3.

Model	Left-hand Sides	Right-hand Sides	Simulated Concentrations (μM)	Initial
RAP2.12/AtPCO4	$d[\text{RAP2.12}]/dt$	$v_1-v_2-v_3$	0.00090	
	$d[\text{RNA}]/dt$	v_4-v_5	0.07230	
RAP2.12/AqADO	$d[\text{RAP2.12}]/dt$	$v_1-v_2-v_6$	0.00990	
	$d[\text{RNA}]/dt$	v_4-v_5	0.08140	
RAP2.12/HsADO	$d[\text{RAP2.12}]/dt$	$v_1-v_2-v_7$	0.00150	
	$d[\text{RNA}]/dt$	v_4-v_5	0.12210	
RAP2.12/KnPCO	$d[\text{RAP2.12}]/dt$	$v_1-v_2-v_8$	0.00230	
	$d[\text{RNA}]/dt$	v_4-v_5	0.18710	
RAP2.12/MpPCO	$d[\text{RAP2.12}]/dt$	$v_1-v_2-v_9$	0.00005	
	$d[\text{RNA}]/dt$	v_4-v_5	0.00390	

Supplementary Table 5 – Parameter values used in the kinetic model.

Concentrations and the Michaelis-Menten constants (Kms) are given in μM , except Kms for O_2 are in %. Rate constants (ks) are expressed in s^{-1} , except k_1 is nM s^{-1} .

Parameters	Description	Values	References
k_1	RAP2.12 protein synthesis rate	0.005	(93, 94)
k_2	RAP2.12 degradation rate	0.0002	(93)
k_3	Catalytic rate constant for AtPCO ₄ -mediated oxidation of RAP2.12	26.5878	(40)
K_{M1}	Michaelis-Menten constant for O_2 as a substrate of AtPCO ₄	16.4217	(40)
K_{M2}	Michaelis-Menten constant for RAP2.12 as a substrate of AtPCO ₄	270.2505	(40)
NCOs	Protein concentration of AtPCO ₄ , AqADO, HsADO, KnPCO and MpPCO	0.1	(95)
k_4	Activation rate constant for HRG expression by RAP2.12	0.0016	(95)
K_{M3}	Michaelis-Menten constant for HRG expression	0.05	(40)
k_5	HRG mRNA decay rate	0.00038	(96)
k_6	Catalytic rate constant for AqADO-mediated oxidation of RAP2.12	153.4026	Fitted
K_{M4}	Michaelis-Menten constant for O_2 as a substrate of AqADO	262.7200	Fitted
K_{M5}	Michaelis-Menten constant for RAP2.12 as a substrate of AqADO	233.0448	Fitted
k_7	Catalytic rate constant for MpPCO-mediated oxidation of RAP2.12	12.1853	Re-fitted (45)
K_{M6}	Michaelis-Menten constant for O_2 as a substrate of MpPCO	0.9715	Re-fitted (45)
K_{M7}	Michaelis-Menten constant for RAP2.12 as a substrate of MpPCO	10.8438	Re-fitted (45)
k_8	Catalytic rate constant for KnPCO-mediated oxidation of RAP2.12	185.3389	Re-fitted (45)

K_{M8}	Michaelis-Menten constant for O_2 as a substrate of KnPCO	63.5650	Re-fitted (45)
K_{M9}	Michaelis-Menten constant for RAP2.12 as a substrate of KnPCO	2360.5	Re-fitted (45)
K_9	Catalytic rate constant for HsADO -mediated oxidation of RAP2.12	33.9905	Fitted
K_{M10}	Michaelis-Menten constant for O_2 as a substrate of HsADO	123.8549	Fitted
K_{M11}	Michaelis-Menten constant for RAP2.12 as a substrate of HsADO	156.6104	Fitted

Supplementary Table 6 – List of primers for screening of transgenic lines.

Primer Name	Sequence
attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCC
attB2	CACCCAGCTTTCTTGTACAAAGTGGTCCCC
pPCO4 Fw	ATGTGCGATTCATTTCCCAA
PCO4 Rv	GGGCTTTGAGCCGTCATCTCAGTATCCTTC
AqADO Rv	ATGATTTTCTTGAGCTTTTC
HsADO Rv	CGCCTCCGGGCCAGAAGCCG
SpADO Rv	GTGTTCGAAGACCTCACAGG
MpPCO Rv	GCCAGTCACAGCAGAATATGGA
KnPCO Rv	CGAGTCCAACATCAGCGCAA
LBb1	GCGTGGACCGCTTGCTGCAACT
PCO5 Fw	GCCCATTTAGGTAGCTGCAGTG
PCO5 Rv	AGCTTCCTGTTTCGAGACCAA

Supplementary Table 7 – List of primers used for TAIL PCR.

Primer Name	Primer
LAD1-1	ACGATGGACTCCAGAGCGGCCGCVNVNNNGGAA
LAD1-2	ACGATGGACTCCAGAGCGGCCGCBNNNNNGGTT
LAD1-3	ACGATGGACTCCAGAGCGGCCGCVNVNNNCCAA
LAD1-4	ACGATGGACTCCAGAGCGGCCGCBDBNNNNCGGT
tail_RB0	GGTGAAAAGGGCGAATTCTGCA
tail_RB1	GGCGAATTCTGCAGATAAACTATCAGTGTTTG
tail_LB0	GTAATGCATGACGTTATTTATGAGATGGGTT
tail_LB1	TATCGCGCGCGGTGTCATCTATG
tail_AC1-A	AACACATCACGATGGACTCCAGAG
tail_AC1-B	AGAGTGCGACGATGGACTCCAGAG
tail_AC1-C	CCGTATATACGATGGACTCCAGAG
tail_AC1-D	CTTGTTTGACGATGGACTCCAGAG
tail_AC1-E	GAGATAACACGATGGACTCCAGAG
tail_AC1-F	GTACAGGAACGATGGACTCCAGAG
tail_AC1-G	TCTCGCCTACGATGGACTCCAGAG
tail_AC1-H	TGCTCCGAACGATGGACTCCAGAG

Supplementary Table 8 – List of primers used for qRT-PCR.

Gene	AGI Code	Forward Primer	Reverse Primer
<i>UBQ10</i>	AT4G05320	GGCCTTGTATAATCCCTGATGAATAA G	AAAGAGATAACAGGAACGGAA ACATAGT
<i>LBD41</i>	AT3G02550	TGAAGCGCAAGCTAACGCA	ATCCCAGGACGAAGGTGATTG
<i>ADH1</i>	AT1G77120	TATTCGATGCAAAGCTGCTGTG	CGAACTTCGTGTTTCTGCGGT
<i>PDC1</i>	AT4G33070	CACAGAATCTTCAATGTTCTTACC	CCATGATAAAGCGTACATGGA A
<i>PCO4</i>	AT2G42670	CGAACCAGAGGATCCATCACAAGA	GCCGTCATCTCAGTATCCTTC ACC
<i>AqADO</i>	NA	AGCCAGATCACCCGAAGGA	AAGCGGCAGGAGCATCAGTC
<i>HsADO</i>	NA	CGGTGGTGGACAAAGGCCTA	AGCAGGTCCTTCAACAGCGT
<i>MpPCO</i>	NA	CACACACCAATGGTTGCCAG	GGGGTTGACCCAGTCATACG
<i>KnPCO</i>	NA	GACCTCAGCACCTCCTAGA	AGGAGCAACAACATCGAGCA

Supplementary Table 9 – Multiple sequence alignment of NCO sequences in the eukaryotic kingdom using CLUSTAL Omega.

Chytriomycetes	-----MVMIAALLAQRLHDILYGNSTG	23
Batrachochytrium	-----MPLLQAIGLFSERLFAQ	17
Rhizoclosmatium	-----MLLTAIGLVAHELFTN	16
Blyttiomycetes	-----MSLLHTIGLLAHLRFSR	17
Spizellomyces	-----MSPPCINESDSSPTTCCFPFCTEMENRRTQSLMDMSHLQRIQVLAQVFAA	51
Chlamydomonas	-----MQQNGTFASYNQTSSSGGSSGSSSTLQAFFSSAKAAVLR	39
Volvox	-----MHSQSAVAGS-QT--ANSQQTSGSRLQDFFTAARRAVLH	36
Ostreobium	-----AEEQGHGVGCETRTPLTAQRERRPCSAPAVR	31
Chlorella	-----MPHRSPVRLQFLMDVAQATFNQ	23
Picea	-----MTAVQRLYDICKASFTS	17
Cryptomeria	-----M-LEEQWFCTMTAVQRLYEICKASFTS	26
Taxus	-----NIVFQH-CLELWFCTMTTVQRLYDSCASFTS	31
Triticum	-----MAAIQKLYEVCKASLSE	17
Oryza	-----MPIASRRPLNGSSRRGWICLFV-FSRTSKCKMPKIKSLSNACKVSFSP	49
Zea	-----MPKIKSLSNACRVSFSP	17
Arabidopsis	-----MPYFAQRLYNTCKASFSS	18
Camelina	-----MPYFAQRLYNTCKSSFSS	18
Nicotiana	-----MPAVQKLYNACKASLSP	17
Solanum	-----MPAVQKLYNACKASLSP	17
Cannabis	-----MPYYIQRLFNTCKASFSP	18
Glycine	-----MPYYIQRLYRLCNASFSP	18
Medicago	-----MPYCVQRLYRLCKASFSP	18
Klebsormidium	-----MASKVQKLYDACRAAFGA	18
Adiantum	-----MCLEERLWPEPAE--DGSEQDPRPPIQRLYEFMTVVFEM	38
Selaginella	-----MTAMTAVQKLYEVCKASFSC	20
Ceratopteris	-----MREESQSAPAVDESSVMPEHAMAVQKLYESCRATFTR	38
Ceratodon	-----MTMSSAPAIQRLYDVCKKVFTS	22
Physcomitrium	-----MAMTSTPAVQKLYDVCKKVFTP	22
Diphasiastrum	-----MSAIQKLHDVCKASFSL	17
Marchantia	-----MMTAVQRLHDVCKATFTM	18
Sphagnum	-----MGAMSTTAVQKLYDVCKATFSA	22
Caenorhabditis	-----MDSPARHLQMAIRKIALLSRSF	22
Anopheles	-----MSALFARVFRQARQTFEY	18
Drosophila	-----MTTHFSNVLQAFKTFDR	18
Candidula	-----MAAPILKLAQLSTKIFGVLSF	21
Octopus	-----MALLIQEVSKAARRAFSG	18
Fasciola	-----MTTKIATVANLALHAFQQHSV	21
Schistosoma	-----MTTKIATVANLAFHAFQQHSA	21
Hydra	-----MASLIQRIVRQAATTFPN	18
Daphnia	-----MGSRIENVIRTAVQTFAT	18
Amphimedon	-----MGVVK-MSGVLVHQLVHQAARTFSR	23
Lamellibrachia	-----KCSMNGTKIQLAAQAFQTFSK	22

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Strongylocentrotus	-----MAALQKVTRQAWDIFRL	17
Asterias	-----MANLQPIQKVAKLALETFKY	20
Scyliorhinus	-----MPGDKMASSLIQTIARQARTTFKL	24
Xenopus	-----MPRDD-MSSLIQKVARQARQTFRS	23
Carassius	-----MMPRDS-MTSTVQKIARQALATFRN	24
Danio	-----MTSTVQKIAKQALATFRN	18
Chelonia	MQGAVRAGAEGRALPRSTAESRGRAGSAERDYLGGTMPRDN-MASLIQIRVAKQARITFRG	59
Homo	-----MPRDN-MASLIQRIARQACLTFRG	23
Mus	-----MPRDN-MASLIQRIARQACLTFRG	23
Chytriomycetes	ADEGS-----P-AGK	32
Batrachochytrium	PTPTH-----PPPSA	27
Rhizoclostridium	PSERA-----	21
Blyttiomycetes	FPEPH-----PQR	25
Spizellomyces	FPGRH-----PEA	59
Chlamydomonas	GGLNE-----TNT	47
Volvox	GTVNE-----SNP	44
Ostreobium	TPATGTDAFP-----AGRLRNLGAGPPC	55
Chlorella	PGGP-----TR	29
Picea	SGPR-----SP	23
Cryptomeria	NGPR-----SP	32
Taxus	SGPR-----SL	37
Triticum	KGPS-----SP	23
Oryza	DGPI-----SD	55
Zea	EGPI-----SE	23
Arabidopsis	DGPI-----TE	24
Camelina	DGPI-----SD	24
Nicotiana	NGPV-----SE	23
Solanum	NGPV-----SE	23
Cannabis	NGPV-----SE	24
Glycine	NGPA-----SE	24
Medicago	DGPV-----SE	24
Klebsormidium	KGPA-----SK	24
Adiantum	LIVVGKG-----IDMFEQ	52
Selaginella	VQGV-----AP	27
Ceratopteris	NAGSF-----PA	45
Ceratodon	T-SVP-----SE	28
Physcomitrium	A-SVP-----LE	28
Diphasiastrum	LGAVP-----SP	24
Marchantia	S-APP-----SA	24
Sphagnum	SSGVP-----SA	29
Caenorhabditis	D-----GSA	26
Anopheles	TTVS-----DPKFA	27
Drosophila	AN-----HASFN	25
Candidula	E-----KITK	26

Octopus	-----SLKEPLFQ	26
Fasciola	HVTLASTAALTIGTSAGSTPGSGSLNAQTSSHSPTDGSGAVPDSPNHAPRSASCPNGLP	81
Schistosoma	RA-----ALSPASANINNGMGDSHPCTNGNSPTHSDKPVSPNLPVKHKQLS	67
Hydra	SL-----TVS	23
Daphnia	KPNGS-----F-----VNANVS	30
Amphimedon	EK-----TLP	28
Lamellibrachia	YR-----RTDAFS	30
Strongylocentrotus	FFKD-----RNANI	26
Asterias	VNKG-----DKHVFL	30
Scyliorhinus	LPLA-----ETGRFQ	34
Xenopus	GG-----GGPSA	30
Carassius	PSAVG-----E-----HGKVFL	36
Danio	PSVIG-----E-----HNKVFL	30
Chelonia	PA-----PGPGFG	67
Homo	SGGGR-----GASDRDAASGPEAPMQPGFP	48
Mus	SSTGS-----E-----GPAPGFP	36
Chytriomycetes	GRGDGATAMATPTPD-TDSLVRANPANTDTVANVLRLLLLKIALP-----	76
Batrachochytrium	HTTARLLQSLTKLL---FKLKLESLA-----	50
Rhizoclostridium	ADLEEALTLLELRLT-PAMLKL-----	42
Blyttiomycetes	EVIAEKLGLVKLVLT-TEQLNLQHVK-----	50
Spizellomyces	DAILDRLCSLIRDVT-LGHLKVKP-----	82
Chlamydomonas	ETVNSLL-RLMSHIS-LEELGLDPMVQDPFLGALKLH-----	83
Volvox	DTVAQLA-NMMGAIR-LEELGLDSDMVADPFLGALKLH-----	80
Ostreobium	VAKDQAPAIPEPAM-DDGQERRPRLQELYDVGWRTFGRGGGAPSREEVLAVLGALREV	114
Chlorella	QQVEALQ-RTLSLVP-LEELGLSPQQREQQHEVHSSSSSRSCSPGLGDGAHMLHDELER	87
Picea	DVLQNVN-DILDTIK-PSDVGLEEEALV-RGWSGPAFG-----	58
Cryptomeria	EILQNVH-DVLDTIK-PSDVGLEEAALVAHRWSGSSHG-----	68
Taxus	EFLQNVN-DVLDTIK-ASDVGLEEGELVARGWSGSPYG-----	73
Triticum	EAVENVN-AVLDMIT-PSDVGLECEAQAVRVWRRPRVL-----	59
Oryza	EALERVN-ALLDEIR-PIDVGLDNEAQIARNWNNSTRQ-----	91
Zea	EALERVN-ALLDMIR-PLDVGLENEAQIARNWSSSTRP-----	59
Arabidopsis	DALEKVR-NVLEKIK-PSDVGIEQDAQLARSRSGLN-----	59
Camelina	EALEKVR-NVLEKIK-PSDVGIEQEAQLARTRSGPLN-----	59
Nicotiana	DALEKVR-SLLDKIK-PSDVGLEQEAQLVRSWNGTLH-----	58
Solanum	DALEKVR-SLLDKIK-PSDVGLEQEAQLVRSWNSTLH-----	58
Cannabis	ESLEKVR-AMLEKIK-PSDVGLEQEAQLVRNWPGLTP-----	59
Glycine	EAIEKVR-EKLERIK-PSDVGLEQEAQVVRNWSGSM-----	59
Medicago	EVVKVR-EKLDRIK-PSDVGLEQEAQVVRNMSRTVL-----	59
Klebsormidium	ENLQLLQ-EALDSVT-CADVGLDPVTDG-ETRGFGFFG-----	59
Adiantum	RHRECKESLMADNIK-PIDVGLDESMPHNDRGFGFFG-----M	90
Selaginella	EAVARVN-AVLDDIK-CSDLGLQEDNCQRNGRST-----	59
Ceratopteris	QGLQVR-SILDSIK-PSDVGLEDAPIQQNSRTNLLG-----R	82
Ceratodon	AQVSNNV-AVLDTIK-PQDLGLTIQSDQQNR---GDRG-----F	62
Physcomitrium	AQVAKVR-AVLDSIK-PQDLGLGTIDQHN-----RG-----L	58

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Diphasiastrum	PAIQTVR-NALDNIE-CADVGLDWKEAQDDDGFGYYG-----L	61
Marchantia	QALQVR-TILDSIQ-ASDVLDEVDAMSQERGFGFFG-----	60
Sphagnum	AALERV-LALENVK-ALDVGLEEEQ--ERGFGFFG-----	63
Caenorhabditis	ESINIIQ-SIVNNFDSRIFQRLIPGRISRVIQ-----	58
Anopheles	TNLTALR-RLVDQLT-LADIGLDGSLVATDTFQ-----	58
Drosophila	ANLQHLR-QLTDELT-YRDLHLREELFRNVG-----	54
Candidula	DHLSPLI-RAMNNIS-SEDVYFDPKEIEERDAEL-----	58
Octopus	EKLNELV-KLMNQVR-GRDLNFPALTERSKNN-----	57
Fasciola	VALQRL-LISVHNLK-PKDIGFDTSWVSDSQVY-----	112
Schistosoma	ESLSRLL-ACVRSLT-PKDVGFDIRWISDSNIS-----	98
Hydra	SNLENLI-ALVEGLT-ANDLGLKADAKDALA-----	52
Daphnia	HNMNLR-QLLSEICPADDLGLTRYSGQPRFPL-----	62
Amphimedon	EEMEKLK-KIMSQIT-PKDFNLDSTAIKPDWDY-----	59
Lamellibrachia	EQLTNLR-DLMNHIT-KVEVNFNSQLVKNKEVY-----	61
Strongylocentrotus	DGLKALQ-ETMSKIQ-ASDLNIDASRAMIEQQRLPPNPA-----	63
Asterias	DKLESLL-ASVNRIR-AADVGVDLTANALP-----	58
Scyliorhinus	DNLQQLN-SLLEKIQ-VDDLNLGPRRNQG-----	61
Xenopus	ESLGQLR-KLVSVQR-AEDVGLGVSRS-----	56
Carassius	ENLSKLK-SLVAEVR-AADLRIAPRTSASAPV-----	66
Danio	ENQSKLK-SLLAEVR-AADLKIAARTPESAPV-----	60
Chelonia	ENLHRLQ-QLLNEVQ-AEDLHLAPRGFAAVPG-----	97
Homo	ENLSKLK-SLLTQLR-AEDLNIAPRKATLQ-----	76
Mus	ENLSLLK-SLLTQVR-AEDLNIAPRKALPQ-----	64
Chytriomycetes	-----MLSVQIPSVNSIEYYHIWGSE-SMTMCLFVLKKGSTLPIHSHPN-----M	120
Batrachochytrium	-----ILSPQHPIEFYPLYETK-DFAICIFVLKKGTTMPTHDHPG-----M	90
Rhizoclostridium	-----EKPPSSPIEYSNIYDCP-AFNIAAFVIRKGETMPIHDHPN-----M	82
Blyttiomycetes	-----VIDPVEYYNIYESD-VFTLCVFALKGGTTMPTHDHPQ-----M	87
Spizellomyces	-----AQYPVEYYPIYESS-VYTMCFVLKRGTTMPTHDHPK-----M	119
Chlamydomonas	-----LRDSRIKYMRIYEDHT-LTFGLFCFPAGATIPLHNHPG-----M	121
Volvox	-----LRDSRIKYMRIYEDPS-LTFGLFCFPAGTVIPLHNHPDGHSANPS	124
Ostreobium	PIEEVGLPGLGRRGRSARSITYLPVKEDRR-LHLGVFCMPPRATIPLHDHPG-----M	167
Chlorella	ERGRYPCTESEA-LDPRPPITYLLIYEDAR-VSLGIFCLPARAKIPLHNHPG-----M	139
Picea	INGRRH-----RYMPPITYLHIHECER-FSIGIFCMPSSIIPLHNHPG-----M	102
Cryptomeria	VNGRRGRNGSP---RYFSPITYLHIHECEN-FSIGIFCMPVSSVIPLHNHPG-----M	117
Taxus	INGRKRNGGL---RYTSPITYLHIHECEK-FSIGIFCMPASSTIPLHNHPG-----M	122
Triticum	----NRKTVL----HSSPAIRYRHIYECKS-FSIGIYCIPASSIIPLHNHPG-----M	103
Oryza	PNGRRGRNGAN---QFTSPIKYLHIHESES-FSMGIFCMPSSVIPLHNHPG-----M	140
Zea	SNRRGRNGAN---QFAAPIKYLHIHECES-FSMGIFCMPSSVIPLHNHPG-----M	108
Arabidopsis	----ERNGSN----QSPPAIKYLHLHECDS-FSIGIFCMPSSMIPLHNHPG-----M	103
Camelina	----ERNAL----QSPPAIKYLHLHECDS-FSIGIFCMPSSMIPLHNHPG-----M	103
Nicotiana	----DRNGGI---RSPPIKYLHIHECDS-FSMGIFCMPSSIIPLHNHPG-----M	102
Solanum	----ERNEL---QPPPIKYLHLHECDS-FSMGIFCMPSSIIPLHNHPG-----M	102
Cannabis	----ERNGRH---QSLPPIKYLHLHEDDS-FSIGIFCMPSSIIPLHNHPG-----M	103
Glycine	----EHNGSH---QSLPPIKYLHLHECDS-FSIGIFCMPSSIIPLHNHPG-----M	103

Medicago	-----EQNGSH----HSLPAIKYLHLHECDS-FSIGIFCMPSSIIPLHNHPS-----M	103
Klebsormidium	RQGPWGRHSAAP--PRWAPPISYQHIIYEDQH-ISIGIFCLPTCASIIPLHNHPG-----M	110
Adiantum	FNGQKGRHSSMV--SRWAPPISYKHIYECQE-FSMGIFCLPTSASIIPLHNHPG-----M	141
Selaginella	-----DQPP--ESAAPAITYLHLYECNK-FSMGIFCLPQSAALPLHNHPG-----M	102
Ceratopteris	INGRRGK-----VSPAISYLHLYECNY-FSMGIFCLPKSASIIPLHNHPG-----M	126
Ceratodon	VDDVQGNWRSIL--PRYAPPISYLHLYECDL-FSMGIFVLPTSASIIPLHDHPG-----M	113
Physcomitrium	GDDGKEKCRPIL--SRYVPVISYLHLYECDL-FSMGVFVLPASASIIPLHNHPG-----M	109
Diphasiastrum	VKGCRGRLSAMV--PRWAQPIITYLHLYEKENFFSMGIFCLPTSAAIPLHNHPG-----M	113
Marchantia	TNGRRGRHTPMV--ARWAPPISYLHLYECED-FSMGIFCLPTSAAIPLHNHPG-----M	111
Sphagnum	SNGETGRHSSMV--PRWSPPISYLHLYECEQ-FSMGIFCLPTSAAIPLHNHPG-----M	114
Caenorhabditis	-----L---RKETSGIRYADVYQDDYCHVNTFGLLKSGLKIPLHDHPN-----Q	99
Anopheles	-----RPTKAPCTYGVGFEND-CFAMSVFVLRENYTMPLHDHPR-----M	97
Drosophila	-----SHRAPCSYMHIFEDD-RFSMSLFIVRGASTIPLHDHPM-----M	92
Candidula	-----QK---LGEVAPVTYMHIIHQDH-NFTMAVFVVKAGSVLPLHDHPK-----M	99
Octopus	-----NAPVTYIIHIFEDE-VYSMGIFVLKENSRIPLHDHPG-----M	93
Fasciola	-----AAPVAYVHITENE-VFSMGIFILRSGSRIPLHDHPG-----M	148
Schistosoma	-----SAPVAYVHIMENE-VFSMGIFILKPGSRIPLHDHPG-----M	134
Hydra	-----IYPQAPVTHVSIYEGK-NFTMGVFILHPGMAIPLHDHPG-----M	91
Daphnia	-----NLF---SSKAAPVSYMEIFENQ-TVSIIGIFVLKDGASIIPLHDHVG-----M	104
Amphimedon	-----PP---FVTDAPAAFMNVYECs-EFNVAIFMLKANKEMPLHDHPE-----M	100
Lamellibrachia	-----QAE---SGGKPPVTYVHLWEDD-VFSMGIFVLNRNGAKIPLHDHPG-----M	103
Strongylocentrotus	--AFEAAAGGD---PSQRAVPGYMHIFEDG-VMTMGVFIIREGSRIPLHNHPG-----M	111
Asterias	-----P---VPATAPVGYMFIAENS-AVSMGVFVVERGCRLPLHDHNG-----M	98
Scyliorhinus	-----PRHGPPVTYMHICETE-YFSMGVFLQGGACIPLHDHPG-----M	100
Xenopus	-----GGKGPVVTYMHICETS-CFSMGVFLLRPGACIPLHDHPG-----M	95
Carassius	-----PS---QRVAPPVTYMHIIYETD-TFSMGVFLKKTGASIIPLHDHPG-----M	107
Danio	-----PH---QRIAPPVTYMHICETD-SFSMGVFLKKTGASIIPLHDHPG-----M	101
Chelonia	--AGCSRAGAG---AGAVPPVSYMHICETE-SFSMGVFLLRSGACIPLHDHPG-----M	145
Homo	-----PL---PPNLPVVTYMHIIYETD-GFSLGVFLKSGTSIPLHDHPG-----M	117
Mus	-----PL---PRNLPVVTYMHIIYETE-GFSLGVFLKSGTCIPLHDHPG-----M	105

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Chytriomycetes	TVFSRVIHGDHLVKTYYHLEGDSHANTSSNSRSSLPTASASEGVTPASSPRLFQTTTQPS	180
Batrachochytrium	TVFTKLISGDMHVKTFFELINDSNSILKNAKCQL-----	123
Rhizoclostridium	TVFTKLIHGDHLVKTFFELMDETGPVRRARVITDKICTASTTTPSNSFTSSSNQHSPPSAH	142
Blyttiomycetes	MVFSKIVIRGDLRVRTYQFAEDESHPHT-VV-DPESGELVKVRSASAS-----	132
Spizellomyces	TVFSKIVSGDLHVRTYEFAKGSTAQAHAVV-DPRTGDLVQARPTKAG-----	165
Chlamydomonas	TVFSRLLFGRLRVSAFDWVVPPEPIT--EG-----	150
Volvox	RSATRLLFGQLRVSAFDWVVPAPIT--SG-----	153
Ostreobium	TVLSRVLYGRVIRITSYDWAEGAGGGAG-----	194
Chlorella	TVLSRVLYGDMHVCSDYDWAEPGDADA---VE-----	167
Picea	TVLSKLLYGSMHAKAFDWINPSEAAD---TS-----	130
Cryptomeria	TVLSKLLYGSVHAKAYDWIDPSEVSD---SS-----	145
Taxus	TVLSKLLYGSMHAKAYDWNPDSEVAD---SS-----	150
Triticum	TVLSKLLYGKVHVHAKAYDWIDIEEPGN---LS-----	131
Oryza	TVLSKLLYGTLHAESYDWIDVTDPTDQLQEL-----	171

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Zea	TVLSKLLYGRHLHAESYDWVDIPDHPI--DQL-----	137
Arabidopsis	TVLSKLVYGSMDHVKSYSYDWLEPQLTEPE-DPS-----	133
Camelina	TVLSKLVYGSMDHVKSYSYDWLEPELTEPD-DQL-----	133
Nicotiana	TVLSKLVYGSFHVKAYDWIDIPGPSV--LTE-----	131
Solanum	TVLSKLVYGSFHVKAYDWINIPGPLV--PLE-----	131
Cannabis	TVLSKLIYGSLYVNSYDWLDLPRPDD--P-F-----	131
Glycine	TVLSKLLYGSMDVKSYSYDWIDAPGSND--P-S-----	131
Medicago	TVLSKLIYGSLYVRSYDWIDVPGFTY--S-S-----	131
Klebsormidium	VVMSKLLYGDMDHVRYSYDWAQEAAPPVGPQHP-----	141
Adiantum	TVFSKLLYGSMDHVKAYDWVDLN-----KVL-----	166
Selaginella	TVLSKLIYGSMDHVKSYSYDWVPSDHTA---SS-----	130
Ceratopteris	TVMSKLLYGSMDHVKAYDWVDHN-----DVS-----	151
Ceratodon	TVLSRLLYGNMDHVRAYDWVEPHDGRNLRNPA-----	144
Physcomitrium	TVLSRLLYGSMDHVRAYDWVDPHDEKLNLRNPS-----	140
Diphasiastrum	TVFSRLLYGSMDHVRYSYDWNPFSELSG-DAS-----	143
Marchantia	TVLSKLLYGSMDHVKAYDWNPCDEKLNSDPS-----	142
Sphagnum	TVLSRLLYGSVHVVRAYDWNPFDEKLNADPS-----	145
Caenorhabditis	HAVMKVYQGSVKIRSFISIICDESDDDEGGAASSSSA---SPVRV-----	143
Anopheles	HGLLRVVSAGVQICSYSSELARRDADELLVGV-----GK-----	130
Drosophila	FGLLRCIWQQLMVDSFSHQLGPDEPLTYDP-----	122
Candidula	HGLLKVLYGKVRISSTYEDVQKPLPENIARLQVSQS-----QKRS-----	139
Octopus	FGIIKVIHGTLSLESFDEVPVSSEQPTSSASTTATSFNN---EFKKF-----	137
Fasciola	YGILRVMYGSRLCRSFTPLQNLKPSHPAYPRSSLTEAFP---GSG-W-----	191
Schistosoma	YGILKVLTVGSVRCRSTRLKNVKSTTDLGNYKPSLSVFD---DSK-W-----	177
Hydra	NGICKVLYGSIKLTSEFGLQSRNFMKGTS-----	121
Daphnia	YGILKVLYGSLNVQSYSSIDLPGQTNSIVH-----	135
Amphimedon	HGLMKILSGSMCVTSYNRPEKRGNSY-----	127
Lamellibrachia	HGLIKVLEGKAKLNYSYDLVNEI PVE-----VLP---QLHPW-----	138
Strongylocentrotus	HGLLKVLYGDISVRTFNTITEDWTKFPISKF-EGFPEN-----EPP-----	151
Asterias	HGVIKVLSGTIRMHSYDLTTEDEVNSIKIPD-SLAHPNS---ESDEE-----	141
Scyliorhinus	HGLLKVLYGSVAVKCFDKEETEPGAGF---A-AGVQFNP---PLLQC-----	140
Xenopus	HGLLKVLYGKLRIISGFDRLEASEPP-----EALAFSP---PLLPY-----	132
Carassius	HGMLKVIYGKVRISCFDRDLKPRD-----GA-SGAHFSP---PLVPF-----	145
Danio	YGMLKVIYGKVRISCFDRDLKPRD-----GA-SGVQFNP---PLMPF-----	139
Chelonia	NGMLKVLYGSLRIACLDPLPPAAPGA-----	172
Homo	HGMLKVLYGTVRISCMDKLDAGGGQRPRALP-PEQQFEP---PLQPR-----	160
Mus	HGMLKVLYGTVRISCMDKLDTGAGHRR--PP-PEQQFEP---PLQPL-----	146
: *		
Chytriomycetes	SHAQHPNLPNARLHRDE---IISH-----TSPGLTSILEINSNTPNLHTFTAVS-DHV	230
Batrachochytrium	-----	123
Rhizoclostridium	PPSPALTH-----ASE-----PYSTETIFKIFPSDGNLHYSYSAVS-DYA	181
Blyttiomycetes	-----RTR-----TIP-----STSPDSSLVIRPDGGPNMHSFTAAS-PEV	166
Spizellomyces	-----LNR-----IIS-----ASDPESLLLIRPEGGNMHSFTAAS-EEV	199
Chlamydomonas	-----SGHRAQLVYDA---TVEAS-----SPPLVLFPPASGGNLHEFTAET--PC	189
Volvox	-----SA-RARLVCA---VIGPA-----TPPLVLFPPSSGGNLHEFTAET--PC	191

Ostreobium	-----GGAARRVADE---VLSGP-----TGTSLLTPVHANVHSLEALT--AC	231
Chlorella	-----LPRAARRVDS---TLTAA-----DQPTVLFDPAGGNIHEFTALT--DC	206
Picea	-----KAKLAKLVKDC---EMSAP-----CDTTILYPTSGGNLHSFRALT--PC	169
Cryptomeria	-----KAKLAKLVKDC---EMSAP-----CDTTILYPTYGGNIHSFRALS--PC	184
Taxus	-----KAKLAKLVKDC---EMSAP-----CDTTILYPTRGGNIHSFRAMS--PC	189
Triticum	-----KVRPAKLVVRDG---EISAP-----CAAMVLRPTGGGNVHALKAIT--PC	170
Oryza	-----SVRPARLVDR---EMSAP-----E-TTILYPNRGGNIHTFRAIT--PC	209
Zea	-----QTRPAKLVKDC---EMTAP-----E-TTILYPNAGGNIHTFRAIT--PC	175
Arabidopsis	-----QARPAKLVKDT---EMTAQ-----SPVTTLYPKSGGNIHCFKAIT--HC	172
Camelina	-----QARPAKLVKDK---EMTAP-----TPATTLYPKSGGNIHCFKAIT--HC	172
Nicotiana	-----GTRPAKLVKDC---DMTAP-----CGTTVLYPTSGGNIHCFKAIS--PC	170
Solanum	-----GARPAKLVKDC---DMTAP-----CGTTALYPTSGGNIHCFKAIT--PC	170
Cannabis	-----EARPAKLVDRD---EMIAP-----CPATILYPTSGGNIHCFRALT--PC	170
Glycine	-----EARPAKLVKDT---EMTAP-----SPTTVLYPTSGGNIHCFRAIT--PC	170
Medicago	-----GARPAKLVKDT---EMTAP-----SPTTILYPTNGGNIHCLRAIT--PC	170
Klebsormidium	-----PRRNAVLVCDE---VLSAP-----CDALVLYPTSGGNIHAFTGV--PC	180
Adiantum	-----QPRLAKLVVDR---TMSAP-----CEPAVLYPTSGGNMHAFTAVT--SC	205
Selaginella	-----TPRLAMPVKDH---VLVAP-----CDTAVLYPTSGGNIHSFTAVT--SC	169
Ceratopteris	-----HPRLAKLVVDR---TLTAP-----CESSILYPKSGGNMHVFTAIT--PC	190
Ceratodon	-----IARQAKLVVDH---VLVTGPNETEPLRAPATEVLYPTSGGNIHSFTALT--SC	192
Physcomitrium	-----IARQAKLVVDH---VLVTGPNETEPLRAPATEVLYPTSGGNIHAFTALT--SC	188
Diphasiastrum	-----TPRLAKLVADH---VLTAP-----CESAVLFPSGGNIHAFTALT--PC	182
Marchantia	-----VPRSAKLVVNR---VLTAP-----CETATLHPTSGGNIHAFTAVT--PC	181
Sphagnum	-----RPRLAKLVVDH---VLTAP-----CETAVLYPTTGGNIHAFTALT--PC	184
Caenorhabditis	---RYE-GEIVLS-----SGSEQ-----QHSAVLGPRNGNIHEVTSLE--PH	179
Anopheles	---GQR-HVLVAEEPEKI---ISSSP-----GDCALLTPTERNFHEITAIG-GPA	172
Drosophila	---HQ-TVVKVNVEEPK---LVTPA-----SPCATLTPRKRNYHQIAQIGSGVA	164
Candidula	---NSC-IIKTVYRHNDI---VLSET-----DECCVLSPSVGNFHEIQPET-KFA	181
Octopus	---LRD-YDRTPKPFLEVPKKASVN-----DDCCVLSPKGNLHEVRPDG-GPA	182
Fasciola	---QFR-DLIVARPHQDV---VLNVE-----SCAQLLTPTEGNLHEVTPVD-GPA	233
Schistosoma	---QLT-DLIARPHQDT---VLSPD-----HQPCLLFPPIEGNLHEITPVD-GPA	219
Hydra	-----KYVQVKRIPEK---ILTAD-----TKSQFFLPPIREIYHSMKATD-GPA	160
Daphnia	-----QPQYLKARRFPIS---CINEK-----DSPAVLSPSEHNLTHTWTVG-GPA	176
Amphimedon	-----IARFFRSI---LATPA-----TEPCHFTPEEGNIHQIKAQD-SHV	163
Lamellibrachia	---QIQ-MTRVVSKCKEE---EVNAD-----DVPCLLTPDEGNVHEVCAVD-GPM	180
Strongylocentrotus	---KKHLLAPTRLGIDQ---HFTAS-----SEAVLLTPREGNYHSLESVG-GPA	193
Asterias	---LMRNLIPIVRKKAEAK---IITPE-----SKPCVLSPMESNVHSLESID-GPA	184
Scyliorhinus	---QQA-TLRRSLQSSTK---VLDQA-----SGPCILTPHNNMHQIDALD-GPA	182
Xenopus	---QYN-CVCRAALRSAG---DFGDN-----SPPCLLAPHRENHQAISAE-GPA	174
Carassius	---QSS-SLRPAVLRSVG---EYTEE-----NGPCVLSPQKDNIHQIDAVID-GPT	187
Danio	---QRG-SLRPSVLKSVG---EFTED-----SSPCVLSPQQDNIHQIDAVID-GPT	181
Chelonia	-----RRRALLRSRQ---LYTPA-----SPPCLLSPHTDNLHQIDAVID-GPA	210
Homo	---ERE-AVRPGVLSRA---EYTEA-----SGPCILTPHRDNLHQIDAVID-GPA	202
Mus	---ERE-AVRPGVLSRA---EYTEA-----SGPCVLTPHRDNLHQIDAVID-GPA	188

Chytriomycetes	VVFDLIFPPYDE-----DARMCSYYQERHDSANISG----	261
Batrachochytrium	-----	123
Rhizoclostratium	IIVDIMGPPYAV-----GEREITYFKEVPLSNNNT--TEF	214
Blyttiomycetes	MILDVIGPPYNL-----TDRPCTYYRELARRAGTGGRR	201
Spizellomyces	VLLDIIGPPYNS-----EDRPCTYFREVSWDEGC-----K	229
Chlamydomonas	AVLDLLTPPYEP-----P--TRNCTYYRLQRPPMVWVVAR--	222
Volvox	AVLDLLTPPYEP-----P--ERNCTYYRVQRQAASWPVGL--	224
Ostreobium	AVLDVLAPPYAP-----S-KGRDCTYFRECPGGDA-----	260
Chlorella	AVLDLMSPPYST-----E-DGRDCTYYSIVSEEQQ-----	235
Picea	ALLDVLAPPYST-----D-NGRHCSYYRKLPKR-IPSG----	200
Cryptomeria	ALLDVLAPPYST-----E-EGRHCTYYRKSFRK-VPSG----	215
Taxus	ALLDVLAPPYST-----D-EGRHCTYYRKSRLK-VPPG----	220
Triticum	AILDILSPPYSS-----K-DGRHCSYFRRRQKS-HPTG----	201
Oryza	ALFDVLSPPYSA-----E-KGRDCSYFRKSSVRETPPV----	241
Zea	ALFDVLSPPYSA-----E-DGRHCSYFRKSQMN-QPPV----	206
Arabidopsis	AILDILAPPYSS-----E-HDRHCTYFRKSRRDLPG-----	204
Camelina	AILDILAPPYSS-----E-HDRHCTYFRKSRRDLPG-----	204
Nicotiana	AIFDILSPPYSS-----E-DGRHCTYFRRSPIGDLPG-----	202
Solanum	AIFDILSPPYSS-----E-DGRHCTYFRRSVPADLPG-----	202
Cannabis	AIFDILSPPYSS-----E-NGRHCTYFRTSPRKDLPGS----	202
Glycine	AIFDILSPPYSS-----D-HGRHCTYFRRSQRKDLPVN----	202
Medicago	AVFDILSPPYSS-----E-DGRHCTYFRQSQRKDLPVN----	202
Klebsormidium	AVLDVVAPPYDP-----E-SGRHCTYYRETPVPAMVGK----	212
Adiantum	AVLDILAPPYSA-----Q-DGRHCTYYRDFPYSSFAS-----	236
Selaginella	AVLDVLAPPYNT-----T-AGRPCVYYRLAAATSP-----	198
Ceratopteris	AVLDVLAPPYSA-----E-DGRHCTYFRALPYASRTG-----	221
Ceratodon	AVLDVLAPPYSP-----G-TGRHCTYYRATSNDGV-----	221
Physcomitrium	AVLDVLAPPYAP-----A-TGRHCTYYRITSHEGE-----	217
Diphasiastrum	AVLDVLTTPYSP-----I-DARHCIYYRESKFCGYPH-----	213
Marchantia	AVLDVLAPPYSA-----V-TGRHCTYYREAHVSGSN-----	211
Sphagnum	ALLDVLAPPYSP-----A-TGRHCTYYHNI PRAVFSGSD----	217
Caenorhabditis	TYFCDFFIANS-----NCNYYRVDESSEPL-----	205
Anopheles	AFFDILSPPYNTASQPQYYFYRKVPVPRHLAELEPI-GSPDKPSPLHD-----	219
Drosophila	AFFDILSPPYDAD-----MPTY-GPRQCRFYRAT-----	192
Candidula	AFLDVLAPPYEG-----D-CDSGCHYYKDLNPG-----	208
Octopus	AFLDILAPPYDT-----D-SDRRCNYYEITKGHSPP-----	212
Fasciola	VFLDILAPPYDH-----DLGTRECRFYKEVILLGNHVPTRPV	270
Schistosoma	VFLDILAPPYDH-----DLGTRECRFYKEVII P-QMNSNALL	255
Hydra	AFFDILAPPYRT-----KD-YKTDCHYFRELTVSEHPEIDLEK	197
Daphnia	SFLDILAPPYDPSGT-----LNGG-EVRDCYYSVDVAGEDGMP-----	213
Amphimedon	AFLDILAPPYAP-----E-EGRDCTYYFKDTKQSN-----	192
Lamellibrachia	AFLDILSPPYDN-----VRKCHYYKTLSPVSTDNRTT--	212
Strongylocentrotus	AFLDILSPPYDP-----V-IGRDCQYFKELKSLQPS-----	224
Asterias	AFLDVLSPYDP-----D-TGRDCVYYTETPFSSSNLKL---	217
Scyliorhinus	AFLDILAPPYDP-----E-XGRDCHYFKVLQTVSEGPCKA--	216
Xenopus	AFLDILAPPYDP-----A-DGRDCHYYQLIHPAAPPGETD--	208

Carassius	AFLDILSPPYDP-----D-EGRDCHYYKVLHAHSEASDR---	220
Danio	AFLDILAPPYDP-----D-EGRDCHYYKVLQAHSEAADK---	214
Chelonia	AFLDILAPPYDP-----E-NGRDCHYYRLLEAPAG--PA---	241
Homo	AFLDILAPPYDP-----D-DGRDCHYYRVLEPVRPKEAS---	235
Mus	AFLDILAPPYDP-----E-DGRDCHYYRVVEPIRPKEAS---	221
Chytridiomycetes	-----DVVTTVATELSRMESSALIGKEQNAELHASISRLSVHEDGY--SSESRED	309
Batrachochytrium	-----	123
Rhizoclostridium	VVENGSTPSSAALFDSFSSSSSDSCRIPTTGSVKRKRSETGLSDDAADQVVAK-KQTSS--	271
Blyttidiomycetes	RASGGVVRAGPPQVAGLASVSALGEAAPVASREDTAAGSAPATVPTRRAHPI-PPVSAAA	260
Spizellomyces	LVAMGVEATAPG----KKSNNKKKKRRRAVGPERKTDAMPIQENGSA NPC-PPPPST	284
Chlamydomonas	-----SAGGRAPVGPPLPALGEVVELEV---FE-PPDSFEV	254
Volvox	-----PGSRRVVA-QKMRVGDEVELEV---FD-PPGSFEV	255
Ostreobium	-----GQGDVLLLEP---CE-PPADFEV	278
Chlorella	-----QNSVEPPLVLLSC---FE-PPLDFVI	257
Picea	-----LQLNGIDVASYQLAWLED---YQ-PPDDFV	227
Cryptomeria	-----LQVEGINIPGHQLAWLEE---YQ-PPEDFV	242
Taxus	-----LQVNETNVPSHLFAWLEE---YQ-PPEDFV	247
Triticum	-----ILWDRTR--ESEFVWLEE---YQ-PRDNFVI	226
Oryza	-----VLPGEIN--SAEVIWLEEELEDHQ-PPEGFV	269
Zea	-----VLPAGEID--SSQVWLEEELEDHQ-PPEGFV	234
Arabidopsis	-----LEV DGEV--VTDVTWLEE---FQ-PPDDFVI	229
Camelina	-----IEVDGEV--VTDVTWLEE---FQ-PPDDFVI	229
Nicotiana	-----LEV DGV--FSDVTWLEE---VQ-PPDDFV	227
Solanum	-----LEV DGV--FSDVTWLEGE---FQ-LPDDFMI	227
Cannabis	-----LELDGV--IPEVTWLEE---HQ-PPDNFVI	227
Glycine	-----VQLNGVT--VSEVTWLEE---FQ-PPDNFVI	227
Medicago	-----LELDGV--VSEVTWLEE---FQ-PPDDFVI	227
Klebsormidium	-----RDHKSSAVTDESIWLEE---FS-PPDDFVI	238
Adiantum	-----SIEDESTDGDIDLAWLEE---CQ-PSDDFIV	262
Selaginella	-----EATDSAEHPVHLQE---FR-PSSEFV	222
Ceratopteris	-----RSLTEEQRKELSLHDLVWLEE---SR-PPDDFV	251
Ceratodon	-----AEPGLVGWLEE---FH-PPDDFV	241
Physcomitrium	-----VEASDQPGSIEWLEE---FH-PPDDFV	241
Diphasiastrum	-----DSTESEQELDLQEPYVWLEE---SQPSSDDFVI	244
Marchantia	-----PGGIIANGHEHREESQVETMLEE---YL-PPDDFV	242
Sphagnum	-----GSDGLQENGDNAAENPNYEWLEG---FQ-PPEDFV	249
Caenorhabditis	-----VAGKSAILNR---IR-CPSDFYC	224
Anopheles	-----DERPRYVLET---IP-NPDHYC	238
Drosophila	-----AEGSQVQLHC---IP-SPDITYC	211
Candidula	-----KTSIGGTTWLKE---IP-QPSFYWC	229
Octopus	-----RPLSQPEESSQKISWLQ---IQ-QPPDFWC	239
Fasciola	DLSSLTEAGSDVKC---ASNHQHSDSDVQRVNGLSGSLIYMVE---TN-QPKDYWC	322
Schistosoma	SQNNLHNNNNNEEL---SSS---ADSSDPDLCSNQSEKDSVVYLVE---TN-QPSDYWC	304
Hydra	LK-----EYNEMLNLEEKLNLEDLTWLTE---VP-TPLDYC	230

Daphnia	-----SNSEIRLLKK---TS-CPPSFWC	232
Amphimedon	-----QDENLIWLIS---GQ-NPSWFSC	211
Lamellibrachia	-----QGASNLEGIVCLIE---TA-QPHDFYC	235
Strongylocentrotus	-----SESDPHWLMC---IS-QPHDFWC	243
Asterias	-----KETPVDLRWLLR---IP-QPSSFWC	238
Scyliorhinus	-----S-----PAPSPVQEAVWLLE---VP-QPSDFWC	240
Xenopus	-----GPAASDNCTGGAAQKEMWLLE---IP-QPDDFWC	238
Carassius	-----KSEAQQGDLWLME---IP-QPSEFWC	243
Danio	-----KSEVQDQGDVWLME---IP-QPSEFWC	237
Chelonia	-----ADPHALPREVWLLE---TP-QAADFWC	264
Homo	-----SSACDLPREVWLLE---TP-QADDFWC	258
Mus	-----GSACDLPREVWLLE---TP-QADDFWC	244
Chytriomycetes	TDGDSKSPKVPAPFSKGESHKTGALKNHSSSLFEEDHEVNSEGLQMRVLVELDYGDEIESVA	369
Batrachochytrium	-----	123
Rhizoclostridium	----SQNSNSSTVVSTFNQHYCRKALVSPNTSAKSFDMDCSAAKCIHESNGGSNASFCQ	327
Blyttiomycetes	PRMSSSLPSLSEDDDDADEEADSDDSGSPRGTSAPAFDPSLDALDLSACEDDADSDLVW	320
Spizellomyces	PTLDLTPPPVRSSN-----TTISNSSSEAS-----LDLLNTSGTDEMCW	323
Chlamydomonas	VSGLYPGDPVS-----	265
Volvox	VSGCYPGDPVV-----	266
Ostreobium	QRGEYRGVVIEG-----	290
Chlorella	NQGRYRGVTPRCKLLPHSGSMGVSAGGQSPPTTSVHTAGGQEGHREPLLQQPSSPLSSPS	317
Picea	QRGLYRGPKVVP-----	239
Cryptomeria	QRGLYKGQKVVL-----	254
Taxus	QRG-----	250
Triticum	RRDLYTGPTLEL-----	238
Oryza	ARGLYKGPIVIR-----	281
Zea	ARGLYKGPIVIR-----	246
Arabidopsis	RRIPYRGPIVIRT-----	241
Camelina	RRVPYRGPIVIRT-----	241
Nicotiana	RRGQYRGRIKTI-----	239
Solanum	RRGQYKGRVIKT-----	239
Cannabis	RRGLYKGPIVIRT-----	239
Glycine	RRGLYRGPIVIRT-----	239
Medicago	RRGLYRGPIVIRT-----	239
Klebsormidium	ERGLYRGPKVQGV-----	252
Adiantum	DGAPYSGPRIIP-----	274
Selaginella	HRGNYRGPKVVPSI-----	236
Ceratopteris	TPAPYTGPTIIP-----	263
Ceratodon	QRGLYRGPIRISSYN-----	256
Physcomitrium	QRGIYRGPIRIPSRN-----	256
Diphasiastrum	QEGFYKGPKIKP-----	256
Marchantia	QRGEYRGPRVGP-----	254
Sphagnum	QRGIYRGPKVVPSMSGSNMVTTPALWNKSLRRRSTMLASHMRTRYELKLSKAVLGSEDNA	309
Caenorhabditis	DIMDFPSFEKFD-----	236

Anopheles	DTVHYTPPGFMDYEEGTAPPPPPYESTTDRPV-----	270
Drosophila	DVVDTPESVMQAAFQCADEVYADNASGTQ-----	240
Candidula	DTIKYKGPLLTLCL-----	242
Octopus	DESDYNGPEIPSH-----	252
Fasciola	ESAEYVGPSVV-----	333
Schistosoma	ESAEYNGPSIV-----	315
Hydra	NTLEYTGPTFSVKS-----	244
Daphnia	DSLTYCGPDIKHLQHELN-----	250
Amphimedon	IPQPYNGPKVD-----	222
Lamellibrachia	DQHTYEGPEIQPVWFENDSTDSS-----	258
Strongylocentrotus	DEVHYPGPEISIPS-----	257
Asterias	HQQSYLGPPVNLGQL-----	253
Scyliorhinus	GGEPYPGPKVVPDREAGQME-----	260
Xenopus	GGEAYTGPKVSV-----	250
Carassius	GGEPYPGPEVSL-----	255
Danio	GGEPYPGPKVTL-----	249
Chelonia	GGEPYPGPKVSP-----	276
Homo	EGEPYPGPKVFP-----	270
Mus	EGEPYPGPKVLP-----	256
Chytriomycetes	YVGQQVKRDQLERGSLLTDAELVQLGGRVAMMVD RMSASTQPGQGNPSGK-----	419
Batrachochytrium	-----	123
Rhizoclostridium	LQVDPSGEMEVTKEYQGLPVRDFLKLLEIAHLNGKDRVETLTKICDRIGKTFDRLVASL	387
Blyttiomycetes	LAEAPDVDYDCVERAYMGPRI--FARDMIVDASDEEIIYVLADALLAGQILGASGPAGGGE	378
Spizellomyces	LVEDPDVDYRCVERLYRGEV--AKVE-----RYANVELGII-----	358
Chlamydomonas	-----	265
Volvox	-----	266
Ostreobium	-----	290
Chlorella	SAATTHSGRSGGGAQLERSNLIEAPSS-----	344
Picea	-----	239
Cryptomeria	-----	254
Taxus	-----	250
Triticum	-----	238
Oryza	-----	281
Zea	-----	246
Arabidopsis	-----	241
Camelina	-----	241
Nicotiana	-----	239
Solanum	-----	239
Cannabis	-----	239
Glycine	-----	239
Medicago	-----	239
Klebsormidium	-----	252
Adiantum	-----	274
Selaginella	-----	236

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Ceratopteris	-----	263
Ceratodon	-----	256
Physcomitrium	-----	256
Diphasiastrum	-----	256
Marchantia	-----	254
Sphagnum	AFHHAGKTNLVTTNFSVNGST-----	330
Caenorhabditis	-----	236
Anopheles	-----	270
Drosophila	-----	240
Candidula	-----	242
Octopus	-----	252
Fasciola	-----	333
Schistosoma	-----	315
Hydra	-----	244
Daphnia	-----	250
Amphimedon	-----	222
Lamellibrachia	-----	258
Strongylocentrotus	-----	257
Asterias	-----	253
Scyliorhinus	-----	260
Xenopus	-----	250
Carassius	-----	255
Danio	-----	249
Chelonia	-----	276
Homo	-----	270
Mus	-----	256

Chytriomycetes	-----	419
Batrachomyces	-----	123
Rhizoclostridium	VVAQS	392
Blyttomyces	HQGQR	383
Spizellomyces	-----	358
Chlamydomonas	-----	265
Volvox	-----	266
Ostreobium	-----	290
Chlorella	-----	344
Picea	-----	239
Cryptomeria	-----	254
Taxus	-----	250
Triticum	-----	238
Oryza	-----	281
Zea	-----	246
Arabidopsis	-----	241
Camelina	-----	241
Nicotiana	-----	239

Chapter 4

Solanum	-----	239
Cannabis	-----	239
Glycine	-----	239
Medicago	-----	239
Klebsormidium	-----	252
Adiantum	-----	274
Selaginella	-----	236
Ceratopteris	-----	263
Ceratodon	-----	256
Physcomitrium	-----	256
Diphasiastrum	-----	256
Marchantia	-----	254
Sphagnum	-----	330
Caenorhabditis	-----	236
Anopheles	-----	270
Drosophila	-----	240
Candidula	-----	242
Octopus	-----	252
Fasciola	-----	333
Schistosoma	-----	315
Hydra	-----	244
Daphnia	-----	250
Amphimedon	-----	222
Lamellibrachia	-----	258
Strongylocentrotus	-----	257
Asterias	-----	253
Scyliorhinus	-----	260
Xenopus	-----	250
Carassius	-----	255
Danio	-----	249
Chelonia	-----	276
Homo	-----	270
Mus	-----	256

Supplementary Table 10 – Multiple sequence alignment of selected NCO sequences using CLUSTAL Omega.

Amphimedon	MGVVKMSGLVHQLVHQARRTFSRE-----KTLPEEMEKLKKIMSQ	40
Homo	MPRDNMASLIQRIARQACLTRGSGGGRGASDRDAASGPEAPMQPGFPENLSKLKSLLTQ	60
Spizellomyces	-MSPPCINESDSSPTTCCFPCTEMENRR-----TQSLMDMSHLQRIGVLAYQ	47
Arabidopsis	-----MPYFAQRLYNTCKASFSSDG-----PITEDALEKVRNVLEK	36
Klebsormidium	-----MASKVQKLYDACRAAFGAKG-----PASKENLQLLQEALDS	36
Marchantia	-----MMTAVQRLHDVCKATFTMSA-----PPSAQALQVRVTILDS	36
	. . * . . : . :	
Amphimedon	ITPKDFNLDSTAIDKPWDYPPFV-----TDAPAAFNMVYECSEFNVAIFM	85
Homo	LRAEDLNIAPRKATLQ---PLPP-----NLPPVTYMHIYETDGFSLGVFL	102
Spizellomyces	VFAAFPGRHPEADAILDRLCSLIRDVTGLHLKVKP---AQYPVEYYPIYESSVYTMCVFV	104
Arabidopsis	IKPSDVGIEQDAQLARSRSGPLNERNGSNQ-----SPPAIKYLHLHECDSFSIGIFC	88
Klebsormidium	VTCADVGLDPVTDG-ETRGFGFFGRQGPWGRHSAAPPRWAPPISYQHIYEDQHISIGIFC	95
Marchantia	IQASDVGLDEVDAMSQERGFGGFTNGRRGRHTPMVARWAPPISYLHLYECEDFSMGIFC	96
	: . : : * . . : : *	
Amphimedon	LKANKEMPLHDHPEMHGLMKILSGSMCVTSYNRPEKRGNS-----	126
Homo	LKSGTSIPLHDHPGMHGMLKVLYGTVRISCMDKLDAGGGQRPRALPEQQFEPPLQPRER	162
Spizellomyces	LKRGTTMPTHDHPKMTVFVSKIVSGDLHVRTYEFAGSTAQAHAHVDPRTG-----DLVQA	159
Arabidopsis	MPPSSMIPLHNHPGMTVLVSKLVYGS MHVKS YDWLEPQLTEPE-DPSQ-----	134
Klebsormidium	LPTCASIPLHNHPGMVVMVMSKLLYGDMHVRSYDWANQEAPPVGPQHPP-----	142
Marchantia	LPTS AQIPLHNHPGMTVLVSKLLYGS MHV KAYDWNPCDEKLNSDPSV-----	143
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Amphimedon	--YIARFFRS-ILATPATEPCHFTPEEGNIHQIKAQDSHVAFLDILAPPYAPEEGRDCTY	183
Homo	EAVRPGVLRRAEYTEASGPCILTPHRDNLHQIDAVEGPAAFLDILAPPYDPDDGRDCHY	222
Spizellomyces	RPTKAGLNRIISASDPESLLLIRPEGGPNMHSFTAVSEEVLLDIIGPPYNSE-DRPCTY	218
Arabidopsis	-ARPAKLVKDTEMTAQSPVTTLYPKSGGNIHCFKAIT-HCAILDILAPPYSSEHDRHCTY	192
Klebsormidium	-RRNAVLCDEVLSAPCDALVLYPTSGGNIHAFTGVT-PCAVLDV VAPPYDPESGRHCTY	200
Marchantia	-PRSAKLVVNRVLTAPCETATLHPTSGGNIHAFTAVT-PCAVLDV LAPPYSAVTGRHCTY	201
	. * : * : . . * : : . * * *	
Amphimedon	YFKDTKQSN-----	192
Homo	YRVLEPVRPK-----	232
Spizellomyces	FREVSWEDEGCKLVAMGVEATAPGKKSKNKKKKRRRAVGPERKTDAMPIQENGSA PNCP	278
Arabidopsis	FRKSRRED-L-----	201
Klebsormidium	YRETPVPA-M-----	209
Marchantia	YREAHSVGSN-----	211
	:	
Amphimedon	-----QDENLIWLISGQNPSWFSICIPQ	214
Homo	-----EASSA-----CDLPREVWLETPQADDFWCEGE	261

Chapter 4

Spizellomyces	PPPPSTPTLDLTPPPVRSSNTTISNSSSEASDLLNTSGTDEMCWLVE-DPDVDYRCVER	337
Arabidopsis	-----PGELEVDGEVV-----T---DVTWLEEFQPPDDFVIRRI	232
Klebsormidium	-----VGKRDHKSSAV-----T--DESIWLEEFSPDDFVIERG	241
Marchantia	-----PGGIIANGEHR-----EESQVETMLEEYLPDDDFVQRG	245

* . :

Amphimedon	PYNGPKVD-----	222
Homo	PYPGPKVFP-----	270
Spizellomyces	LYRGEFPAKVERYANVELGII	358
Arabidopsis	PYRGPVIRT-----	241
Klebsormidium	LYRGPKVQGVR-----	252
Marchantia	EYRGPRVGP-----	254

* * :

Statement of authorship

Statement of Authorship for joint/multi-authored papers for PGR thesis

To appear at the end of each thesis chapter submitted as an article/paper

The statement shall describe the candidate's and co-authors' independent research contributions in the thesis publications. For each publication there should exist a complete statement that is to be filled out and signed by the candidate and supervisor (**only required where there isn't already a statement of contribution within the paper itself**).

Title of Paper	Manipulating plant oxygen sensing through NCO substitution reveals trade-offs between growth and flooding tolerance
Publication Status	☐ Published ☐ Accepted for Publication ☒ Submitted for Publication ☐ Unpublished and unsubmitted work written in a manuscript style
Publication Details	Perri, M., He, Y., Chirinos, X., Lavilla-Puerta, M., Latter, R., Bancroft, T., Papachristodoulou, A., Flashman, E., Licausi, F., 2025. Manipulating plant oxygen sensing through NCO substitution reveals trade-offs between growth and flooding tolerance. https://doi.org/10.1101/2025.07.24.665511

Student Confirmation

Student Name:	Monica Perri
Contribution to the Paper	Conducted and designed experiments: phylogenetic analysis, 3D structure prediction, generation of cloning constructs and transgenic lines, TAIL-PCR, measurements of physiological traits (fresh weight, seed area), confocal imaging, RNA extraction, qPCR and RNA-seq (processing and analysis), low-oxygen treatments, submergence tolerance assays (Figures 1-2, 4-6 ;

	Supplementary Figures 1-2, 5-9), data analysis; write-up of the manuscript.	
Signature 	Date	12/08/2025

Supervisor Confirmation

By signing the Statement of Authorship, you are certifying that the candidate made a substantial contribution to the publication, and that the description described above is accurate.

Supervisor name and title: Professor Francesco Licausi		
Supervisor comments I hereby confirm that the above Declaration of Authorship and Contributions provided by Ms Monica Perri is accurate and truthful. The statement correctly reflects her own scientific and scholarly contributions to the work presented in her thesis, as well as the contributions made by collaborators, which are appropriately acknowledged. I endorse this declaration as a fair and transparent account of authorship and contributions.		
Signature 	Date	10/09/2025

This completed form should be included in the thesis, at the end of the relevant chapter.

Chapter 5 – Transcriptomic and metabolic profiling of low-oxygen adaptations in *Marchantia polymorpha*

Commentary

Chapter 5 situates the findings of this thesis within an evolutionary context by examining the low-oxygen response in the liverwort *Marchantia polymorpha* (hereafter *Marchantia*), a sister clade to vascular plants. Oxygen availability in plants is highly variable, shaped by environmental factors such as flooding and the lack of transport systems, often resulting in hypoxic conditions. In response, plants reorganize their metabolism to sustain energy production when aerobic respiration is impaired. While hypoxia sensing and adaptation are well-characterised in angiosperms, the evolutionary origins and conservation of these mechanisms across non-vascular plants remain poorly understood. *Marchantia* provides a critical model to address this gap. Transcriptomic and metabolomic analyses reveal that hypoxia alone induces only modest changes, while submergence triggers extensive reprogramming resembling angiosperm flooding responses. By linking molecular and metabolic responses in bryophytes to those in angiosperms, this work sheds light on the evolution and diversification of low-oxygen responses across the green lineage.

Transcriptomic and metabolic profiling of low-oxygen adaptations in *Marchantia polymorpha*

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Abstract

The availability of molecular oxygen (O₂) in plants fluctuates across organs and developmental stages, shaped both by environmental factors such as flooding and by the lack of a dedicated O₂ transport system. These conditions often lead to hypoxia, which plants mitigate by reorganizing their metabolism to sustain basal energy production when aerobic respiration is impaired. Although major studies have investigated hypoxia sensing in angiosperms, the evolutionary origins and conservation of this response across land plants remain poorly understood. To address this gap, we examined the molecular and physiological responses to hypoxia in the liverwort *Marchantia polymorpha*. Using transcriptional and metabolic analyses, we identified both conserved and lineage-specific features of its low-oxygen response compared with angiosperms. Transcriptome profiling showed that hypoxia alone triggered only a modest response, whereas submergence induced much stronger transcriptional reprogramming. Similarly, metabolomic analyses revealed extensive changes under submergence resembling angiosperm flooding responses, while hypoxia alone produced weaker and directionally distinct metabolic shifts. Together, these findings uncover unique patterns of molecular and metabolic adjustment in *M. polymorpha* while also emphasizing parallels with angiosperm flooding responses.

Introduction

Molecular oxygen (O_2) is indispensable for aerobic organisms, functioning as the terminal electron acceptor in the mitochondrial electron transport chain and enabling efficient ATP production. In plants, however, oxygen availability is often limited because tissues rely primarily on passive diffusion for gas exchange. Several factors contribute to oxygen limitation, including environmental conditions and internal constraints, such as diffusion barriers within bulky organs, localized sequestration, or high respiratory demand. When intracellular O_2 concentrations fall below the affinity of cytochrome c oxidase, aerobic respiration is impaired, resulting in hypoxia. Although hypoxia has traditionally been associated with environmental stressors, particularly flooding, where oxygen diffusion in water is restricted (Armstrong et al., 2000), low-oxygen niches have also been documented in developing seeds, ripening fruits, and storage organs (Licausi et al., 2011; Rolletschek et al., 2007; Van Dongen et al., 2003). More recently, hypoxic niches have been detected in shoot and root meristems, highlighting oxygen's potential role as a diffusible signaling molecule (Shukla et al., 2019; Weits et al., 2019).

Limitation in oxygen availability severely impacts plant metabolism. Oxygen depletion reduces oxidative phosphorylation, causing a decrease in ATP production and adenylate energy charge (Perata and Alpi, 1993). At the same time, reduced oxidative phosphorylation limits NADH reoxidation to NAD^+ , which may ultimately inhibit glycolysis (Narsai et al., 2011). To sustain energy production upon oxygen limitation, plants have evolved multiple adaptive mechanisms. Starch and sucrose catabolism provide hexose substrates for glycolysis, while pyruvate generated by glycolysis is redirected into fermentative pathways, with ethanol fermentation predominating in most plant tissues (Bui et al., 2019; Ventura et al., 2020). This process regenerates NAD^+ , allowing glycolysis to continue by providing a limited supply of ATP (Narsai et al., 2011; Ventura et al., 2020).

In addition to fermentation, low-oxygen conditions also induce reprogramming of amino acid metabolism, promoting alanine accumulation through alanine aminotransferase (AlaAT) and GABA transaminase (GABA-T), both of which use pyruvate as a substrate (Miyashita and Good, 2008; Mustroph et al., 2014, p. 201). This alanine pool has been hypothesized to function as a transient carbon reservoir, which can be reconverted into pyruvate through alanine deamination upon reoxygenation, thus facilitating recovery (Diab and Limami, 2016). Collectively, these strategies sustain short-term survival but also impose significant costs, such as rapid carbon depletion and reduced ATP production, increasing the risk of cell death under prolonged hypoxia.

While these responses have been extensively characterised in angiosperms, much less is known about how non-vascular land plants cope with oxygen deprivation. This represents a

significant gap in our understanding of the evolution of low-oxygen tolerance. To address this gap, we selected the liverwort *Marchantia polymorpha*, hereafter *Marchantia*, as a model system to investigate the response to low-oxygen conditions in non-vascular plants. *Marchantia* has emerged as a powerful experimental system in plant biology due to its relatively simple, low-redundancy genome, which facilitates the study of stress-response pathways (Bowman et al., 2017). Moreover, *Marchantia* has been increasingly used as a model for stress biology, having demonstrated resilience to diverse abiotic stresses, including heat, cold, salinity, osmotic imbalance, and nutrient deficiency, making it particularly suitable for comparative studies of stress acclimation across plant lineages (Tan et al., 2023). In this study, we investigated transcriptional and metabolic adaptations of *Marchantia* to low-oxygen conditions. By comparing its responses with those described in angiosperms, this study aims to identify both conserved and lineage-specific features of low-oxygen adaptation, thereby providing new insights into the evolution of hypoxia response across land plants.

Results

Changes in *Marchantia* transcriptome upon hypoxia and submergence

To characterise the transcriptional responses of *Marchantia* to low-oxygen conditions, thalli were grown under normoxia (21 % O₂ v/v) for two weeks and subsequently subjected to either hypoxia (1 % O₂ v/v) or submergence, for 3 or 12 hours, at dark (**Figure 1a**). Plants incubated at dark under aerobic conditions (21 % O₂) for the same duration were included as controls. Plants appeared healthy with no visible signs of distress immediately after the treatment and after four days of recovery (**Supplementary Figure 1**). Total RNA was extracted, and poly(A)-enriched transcripts were used for transcriptome analysis. Multidimensional scaling (MDS) analysis revealed that the primary source of variance was the treatment itself (57 % along dimension 1), while the treatment duration explained 18 % of the variance (dimension 2) (**Figure 1b**). Both hypoxia and submergence induced a change in transcriptomes in the same direction along dimension 1, consistent with hypoxia being a component of the submergence response.

However, differential expression analysis ($|FC| \geq 1.5$, $p_{adj} < 0.05$) revealed that the magnitude and timing of transcriptome changes differed between treatments (**Figure 1c-d**). Hypoxia resulted in 42 and 226 upregulated genes at 3 h and 12 h, respectively, compared to 680 and 724 upregulated genes under submergence. Fold-change magnitudes also differed, with maximum changes reaching ~ 6-fold under hypoxia and ~ 10-fold under submergence. Longer exposure increased transcriptional changes in both conditions, although their temporal dynamics diverged. In hypoxia, the majority of transcriptomic changes were evident

only after 12 h. In contrast, submergence induced extensive transcriptional changes already at 3 h, with ~ 39 % overlap between upregulated genes at 3 h and 12 h, indicating both a stable core and distinct sets of genes activated over time. The amount of downregulated genes also increased with treatment duration, but was again more pronounced under submergence (67 genes at 12 h under hypoxia and 377 genes at 12 h under submergence).

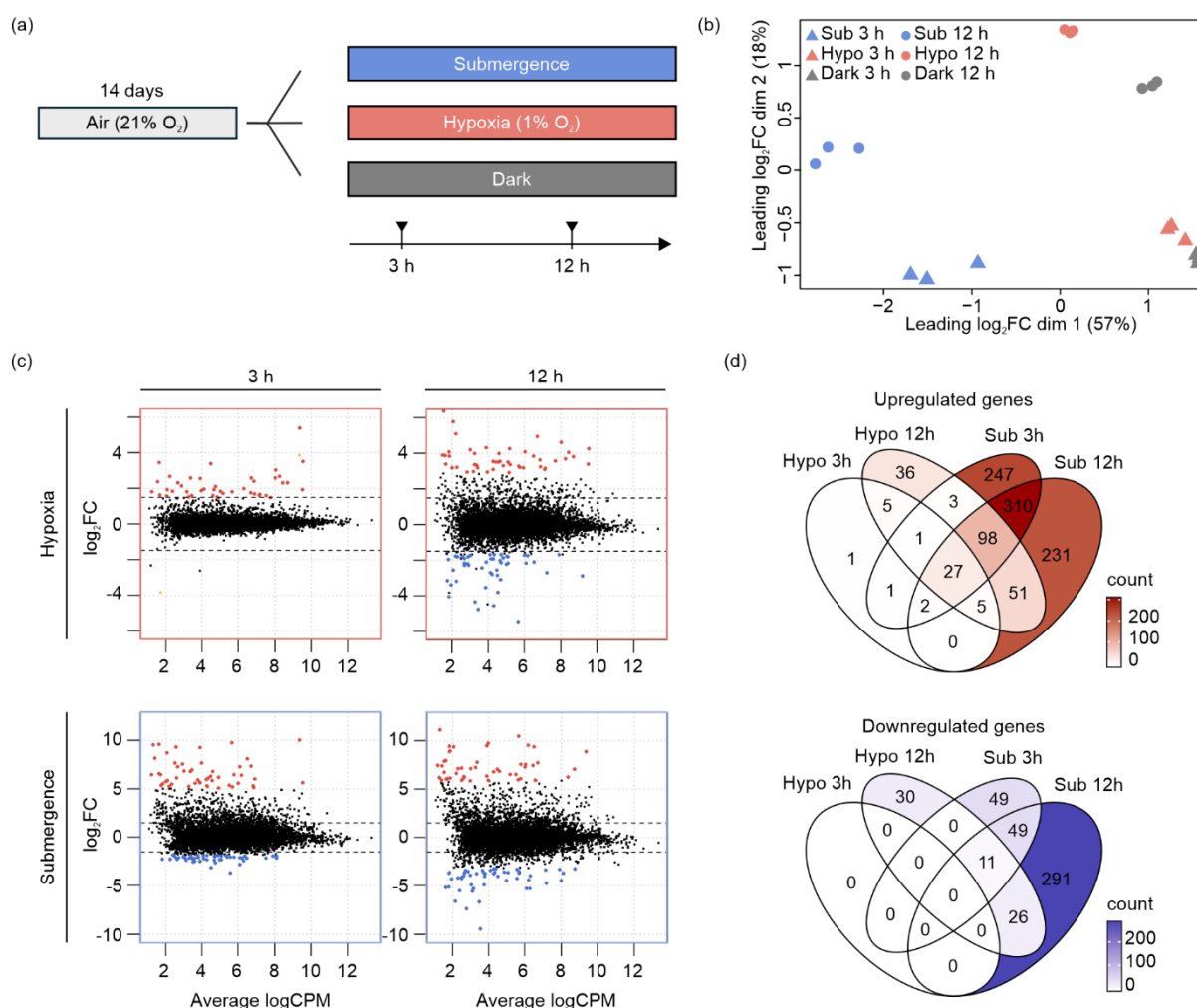


Figure 1 – Transcriptional changes in *Marchantia thalli* under low-oxygen conditions. (a) Experimental design and time points for transcript analyses. *Marchantia* plants were grown aseptically for 14 days in photoperiod conditions, then subjected to hypoxia (1 % O₂ v/v) or submergence for 3 and 12 h, at dark. Control plants were maintained under normoxia (21 % O₂ v/v) at dark for the same duration of time. (b) Multidimensional scaling (MDS) plot of *Marchantia thalli* upon hypoxic, submergence and control conditions at 3 h and 12 h. (c) Funnel plot for differentially expressed genes (log₂FC) in plants exposed to hypoxia or submergence versus dark controls at 3 h and 12 h. Each dot represents a gene; significantly differentially expressed genes (p.adj < 0.05) are highlighted in red or blue, for up-regulated and down-regulated genes, respectively. (d) Venn Diagram indicating the overlap of

upregulated or downregulated genes ($|\text{FC}| \geq 1.5$, $p.\text{adj} < 0.05$) after 3 h or 12 h of hypoxia or submergence compared with dark controls.

Functional enrichment highlighted distinct temporal and condition-specific processes (**Figure 2a**). After 3 h of submergence treatment, genes involved in mechanical stress responses, including response to wounding, and oxidoreductive activity were strongly induced, while genes related to ion binding and transporters were repressed, including H(+)-ATPase (*AHA-like*), cation exchangers (*CAX-like*), nitrate transporter (*NTR-like*), likely reflecting the combined effects of the water column and altered solubility of gases and ions. After 12 h, submergence induced upregulation of genes involved in primary and secondary metabolism: carbohydrate metabolism, amino acid and flavonoid biosynthesis. In contrast, few significant functional enrichments were found after 3 h of hypoxic treatment, including alteration in metal ion binding and transport, while 12 h of hypoxia primarily affected genes related to redox homeostasis, highlighting the more limited impact of oxygen limitation alone compared to submergence.

Despite these differences, a conserved transcriptional core was identified across treatments and time points. Both hypoxia and submergence (particularly at 12 h) induced common gene sets associated with stress responses (late embryogenesis abundant (*LEA*) hydroxyproline-rich glycoprotein), carbon metabolism and fermentation (pyruvate decarboxylase (*PDC*), phosphofructokinase (*PFK*), NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (*GAPC*) and citrate synthase(*CSY*)), redox homeostasis (peroxidases (*PRX*), polyamine oxidase-like (*PAO5-like*)), and calcium signaling, with some of these responses already detectable at 3 h (**Figure 2b**). A total of 98 upregulated genes were shared between hypoxia (12 h) and submergence (3 h and 12 h), associated to GO terms including oxidoreductase activity, glycolysis, carbohydrate metabolism and phenylpropanoid pathway (**Figure 1d**, **Figure 2b**). This overlap indicates a core low-oxygen response that is rapidly activated under submergence but develops more slowly under hypoxia. Collectively, these results demonstrate that while hypoxia alone induces a restricted transcriptional response, submergence triggers a broader, faster response that integrates oxygen limitation with additional metabolic and stress-related adjustments.

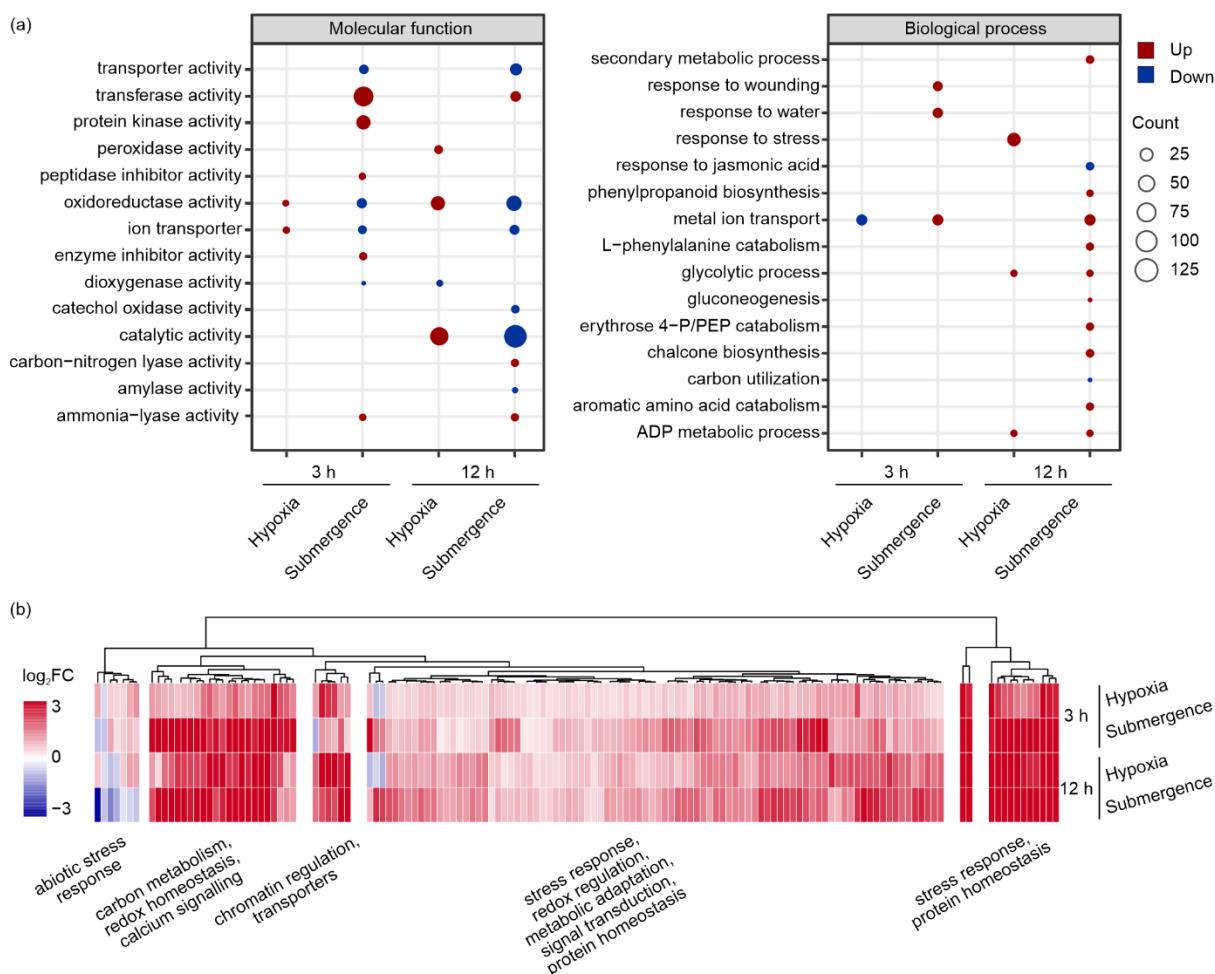


Figure 2 – Functional analysis of transcriptional responses in *Marchantia thalli* upon low-oxygen conditions. (a) Summary of upregulated or downregulated gene ontology (GO) terms (molecular function and biological process) in *Marchantia* plants treated for 3 h and 12 h upon hypoxia or submergence compared to dark control ($|FC| > 1.5$, $p_{adj} < 0.05$). (b) Heatmap showing the clustering of differential expression genes across low-oxygen treatments and time points.

Changes in metabolites

We next investigated changes in primary metabolism under hypoxia and submergence, compared to aerobic conditions. To increase temporal resolution, we applied the same treatments as in the transcriptomic analysis but increased the sampling time points (1.5 h, 3 h, 6 h, 12 h, and 24 h), with controls maintained at dark for the same duration of time (**Figure 3a**). Metabolite profiling by GC-MS and LC-MS revealed changes in sugars, organic acids, and amino acids, providing a comprehensive view of metabolic adjustments to oxygen limitation in *Marchantia*. Principal component analysis (PCA) of metabolite levels revealed distinct temporal trajectories (**Figure 3b**). Hypoxia induced mild changes compared to dark controls,

while submergence caused stronger metabolic shifts, particularly at 3 h, 12 h and 24 h, with the strongest changes observed at 12 h. Interestingly, the metabolic response to submergence was oriented in the opposite direction to that of hypoxia along PC2, already evident at 3 h, indicating fundamentally distinct rearrangements. By 24 h, hypoxia responses largely overlapped with the dark control, likely reflecting starvation, while submergence continued to induce substantial changes. This was further supported by fold-change analysis of significantly changed metabolites relative to the dark control ($|FC| > 1$, $p_{adj} < 0.05$) (**Figure 3c**). Both treatments required at least 3 h to induce significant changes, except for a few metabolites, including a decrease in fructose and glucose under submergence and an increase of glutamine, fructose-6-phosphate (Fru-6P), and pyroglutamic acid. Overall, submergence induced a broader and stronger metabolic reprogramming than hypoxia, in agreement with the transcriptomic patterns (**Figure 1c**).

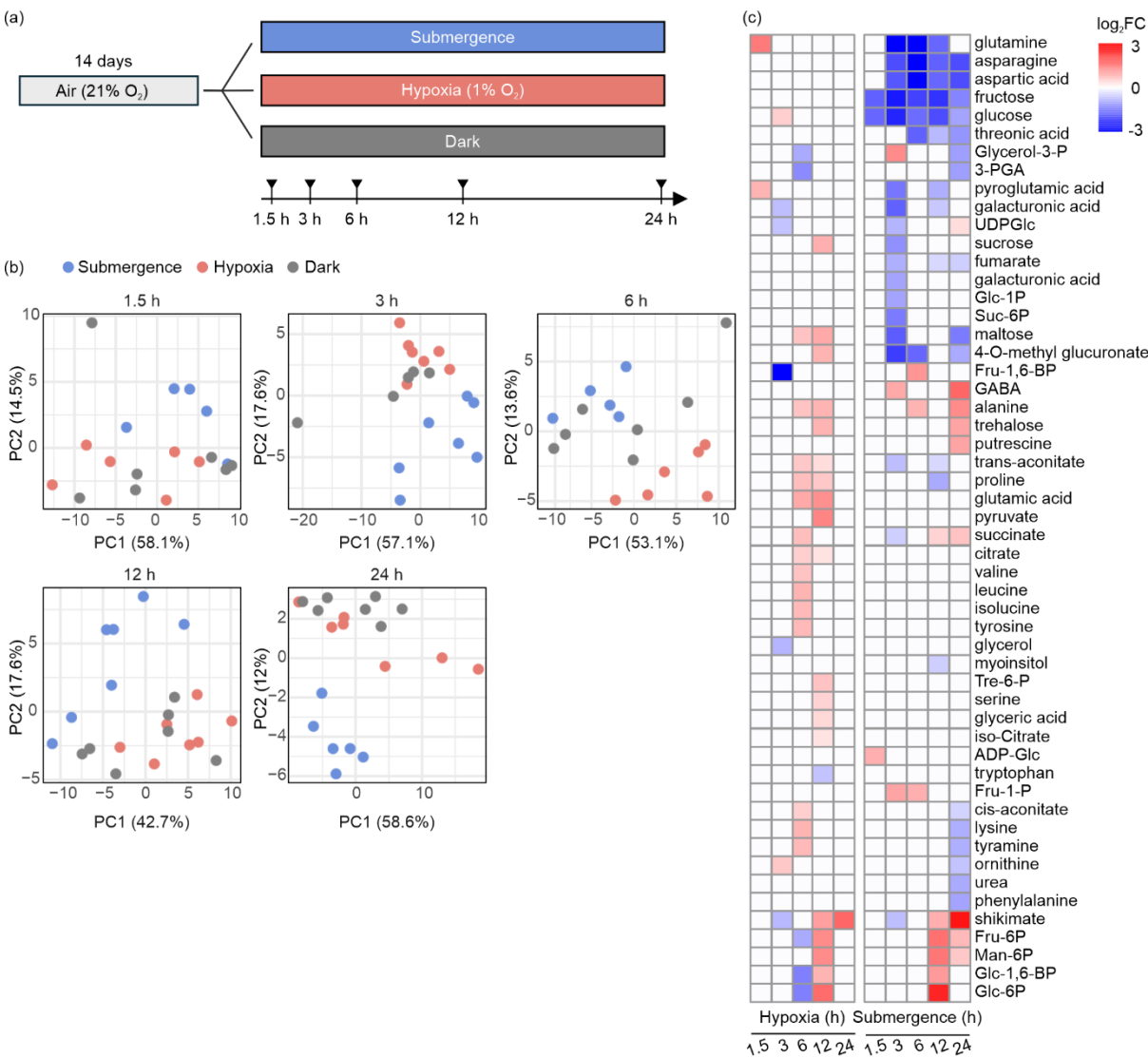


Figure 3 – Metabolic changes in 14-days old *Marchantia thalli* upon low-oxygen conditions. (a) Scheme of experimental design and timepoints of sample collection for

metabolic analyses. *Marchantia* thalli were grown aseptically for 14 days in photoperiod conditions and subsequently treated with hypoxic conditions (1 % O₂ v/v) or submergence for 1.5, 3, 6, 12 and 24 h. Control plants were maintained under normoxic conditions (21 % O₂ v/v) at dark and sampled at the same time points. **(b)** Principal component analysis (PCA) of metabolic changes under hypoxic, submergence or control conditions (dark) over time. **(c)** Heatmap representing significant differences in metabolites. ($|FC| > 1$, $p_{adj} < 0.05$).

To investigate the functional significance of the observed changes, we mapped the affected metabolites to their respective primary pathways and compared their abundance with the expression profiles of the corresponding enzymes identified from the transcriptomic analysis. We focused on three main categories: (i) central carbon and nitrogen metabolism, (ii) sugar and starch metabolism, and (iii) amino acid biosynthesis.

Under hypoxia, the induction of key glycolytic enzymes, including phosphofructokinase (*PFK*), glyceraldehyde-3-phosphate dehydrogenase (*GAPC*) and phosphoglycerate kinase (*PGK*), together with depletion of glycolytic intermediates at 3 and 6 h and the accumulation of pyruvate at 12 h, indicated enhanced glycolytic flux (**Figure 4a**). Although fermentation products such as lactate and ethanol were not detected, transcriptional upregulation of pyruvate decarboxylase (*PDC*) suggested activation of fermentative metabolism to reduce pyruvate accumulation and regenerate NAD⁺. Increased levels of glutamate and alanine at 6 h and 12 h further supported the redirection of pyruvate through the alanine aminotransferase/NADH-dependent glutamate synthase (AlaAT/NADH-GOGAT) pathway, consistent with increased glycolysis. The increase in glutamate was likely responsible for the accumulation of metabolites in the ornithine pathway, such as ornithine itself, which is derived from glutamate by the activity of arginine amidohydrolase (*ARG*), although *ARG* expression remained unchanged. Transient changes were also observed in the TCA and glyoxylate cycles, with accumulation of intermediates at 6 and 12 h and decreased malate synthase (*MLS*) expression after 12 h, indicating adjustments of these pathways in response to oxygen limitation. Succinate accumulated at 6 h, consistent with a temporary block in the TCA cycle, but levels remained unchanged from 12 h onward, suggesting partial recovery of the metabolic flux towards oxidative phosphorylation. In parallel, phosphorylated sugars such as Fru-6P, glucose-6-phosphate (Glu-6P), and mannose-6-phosphate (Man-6P) decreased during the first 6 h, consistent with increased glycolytic demand, but increased again after 12 h (**Figure 4b**). This change may reflect either a reduction in glycolytic activity or the mobilization of sugars from starch degradation, the latter hypothesis supported by the observed increase in glucose and maltose. Alternatively, prolonged incubation in darkness may have introduced starvation effects that contributed to this shift, reducing the glycolytic flux and leading to the

accumulation of sugar phosphate intermediates. Changes in amino acid metabolism were more limited, although shikimate decreased at 3 h and accumulated after 12 h, while several amino acids, including leucine, valine, lysine, isoleucine and tyramine, transiently accumulated at 6 h (**Figure 4c**). These changes likely reflect the diversion of excess pyruvate into amino acid biosynthesis as a strategy to balance carbon flux and temporarily store carbon under hypoxia.

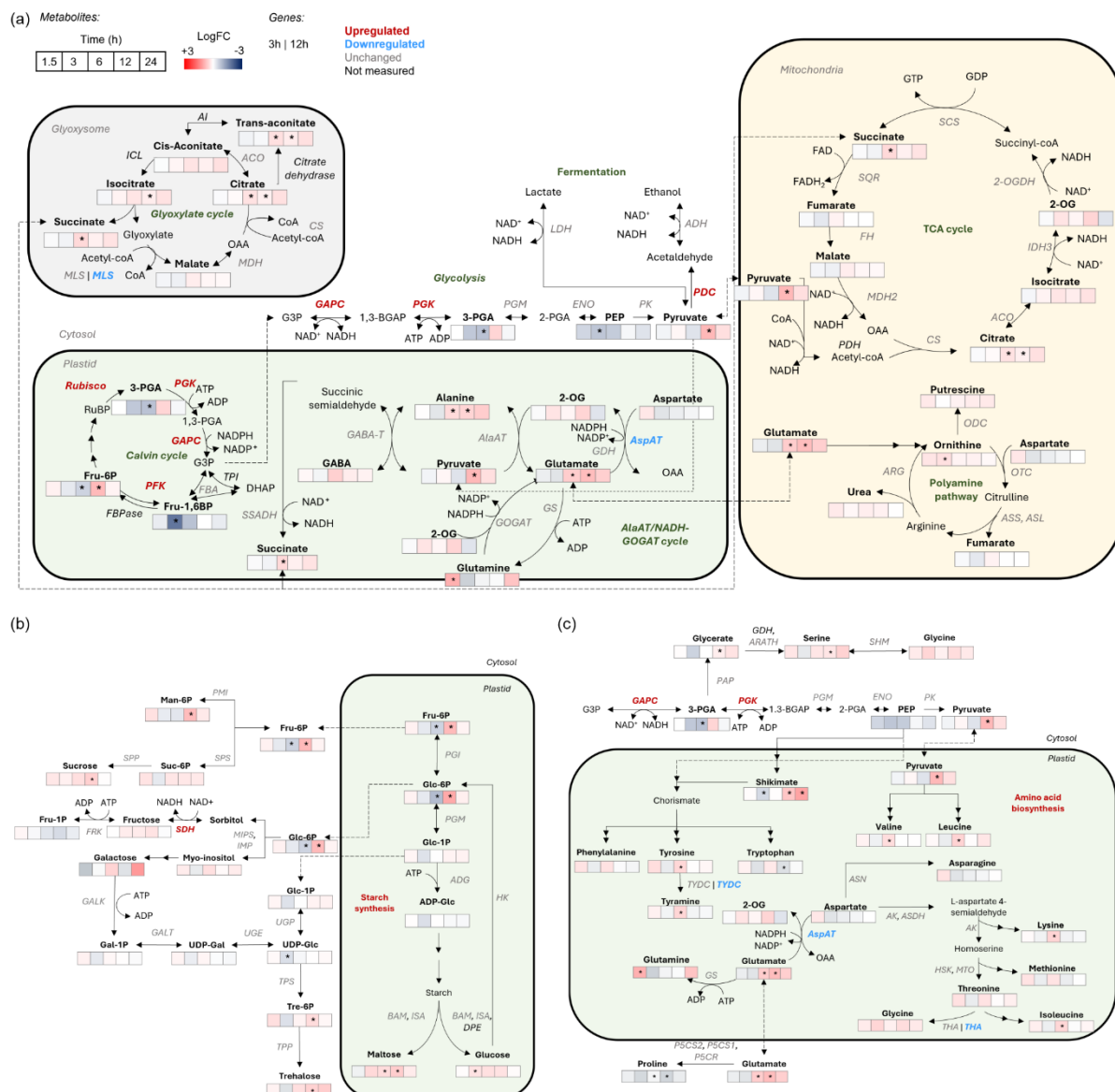


Figure 4 – Metabolic alterations under hypoxia over time. (a) Pathway changes in photorespiration, glycolysis, the TCA cycle, the AlaAT/GOGAT pathway, and the glyoxylate cycle. (b) Changes in sugar and starch metabolism. (c) Shifts in amino acid biosynthesis. Metabolite levels are shown as fold changes (LogFC) relative to dark control conditions at five

time points (1.5 h, 3 h, 6 h, 12 h, and 24 h). Stars (*) indicate statistically significant changes ($p_{\text{adj}} < 0.05$). Corresponding enzyme expression patterns at 3 h and 12 h, derived from RNA-seq analysis, are also displayed. Enzymes with identical expression patterns at both time points are shown once, whereas those with differential expression are displayed twice, separated by “|”, with the order representing 3 h and 12 h, respectively. Abbreviations used: *2-OGDH*, 2-oxoglutarate (2-OG) dehydrogenase; *1,3-BGAP*, 1,3-bisphosphoglycerate; *ACO*, aconitase; *ADG*, ADP-glucose (ADP-Glc) pyrophosphorylase; *AI*, aconitate isomerase; *AK*, aspartate kinase; *AlaAT*, alanine aminotransferase; *ALS*, acetolactate synthase; *ARATH*, cysteine synthase; *ARG*, arginine amidohydrolase; *ASDH*, aspartate-semialdehyde dehydrogenase; *ASL*, argininosuccinate lyase; *ASN*, asparagine synthetase; *AspAT*, aspartate aminotransferase; *ASS*, argininosuccinate synthase; *BAM*, beta-amylase; *CSY*, citrate synthase; *DPE*, disproportionating enzyme; *ENO*, phosphoenolpyruvate enolase; *FBA*, fructose-bisphosphate aldolase; *FH*, fumarase; *FRK*, fructokinase; *GABA-T*, GABA transaminase; *GALK*, galactose kinase; *GALT*, galactose-1-phosphate uridyltransferase; *GAPC*, NAD-dependent glyceraldehyde-3-phosphate (G3P) dehydrogenase; *GDH*, glycerate dehydrogenase; *GOGAT*, glutamate synthase; *GS*, glutamine synthetase; *HK*, hexokinase; *HSK*, homoserine kinase; *ICL*, isocitrate lyase; *IDH3*, isocitrate dehydrogenase 3; *ISA*, isoamylase; *MDH*, malate dehydrogenase; *MIPS*, inositol-3-phosphate synthase; *MLS*, malate synthase; *MTO*, threonine synthase; *ODC*, ornithine decarboxylase; *OTC*, ornithine carbamoyltransferase; *P5CR*, pyrroline-5-carboxylate reductase; *P5CS*, pyrroline-5-carboxylate synthase; *PAP*, 3-phosphoglycerate (3-PGA) phosphatase; *PDC*, pyruvate decarboxylase; *PFK*, phosphofructokinase; *PGI*, phosphoglucosomerase; *PGK*, phosphoglycerate kinase; *PGM*, phosphoglucomutase; *PK*, pyruvate kinase; *PMI*, phosphomannose isomerase; *SCS*, succinyl coenzyme A synthetase; *SDH*, sorbitol dehydrogenase; *SHM*, serine hydroxymethyltransferase; *SPP*, sucrose-phosphate phosphatase; *SPS*, sucrose-phosphate synthase; *SQR*, succinate dehydrogenase; *SSADH*, succinic semialdehyde dehydrogenase; *THA*, threonine aldolase; *TPP*, trehalose-6-phosphate (Tre-6P) phosphatase; *TPS*, trehalose-6-phosphate synthase; *TYDC*, tyrosine decarboxylase; *UGE*, UDP-D-galactose 4-epimerase; *UGP*, UDP-Glucose (UDP-Glc) pyrophosphorylase.

Under submergence, glycolysis was also strongly activated, with upregulation of *PFK*, *GAPC*, *PGK*, and phosphoenolpyruvate enolase (*ENO*) (**Figure 5a**). Unlike hypoxia, however, most glycolytic intermediates remained stable, apart from increased Fru-6P and Fructose-1,6-biphosphate (Fru-1,6-BP) at 6 h and 12 h, suggesting rapid consumption of intermediates fueling into downstream metabolism. *PDC* expression was also elevated, suggesting induction of fermentative metabolism. An early alteration in the TCA and glyoxylate cycles was

detectable within 3 h, characterised by reduced levels of fumarate and succinate and increased expression of citrate synthase (*CSY*) and malate synthase (*MLS*). These changes may reflect either an early blockage of the cycles or a strong activation leading to depletion of intermediates, consistent with the upregulation of key enzymes. After 6 h, however, succinate accumulated while fumarate levels continued to decline, suggesting that the conversion of succinate to fumarate was impaired, potentially due to the limited reducing power resulting from inhibition of the mitochondrial electron transport. The glyoxylate cycle showed fewer changes, although trans-aconitate levels decreased at 3 and 12 h, possibly reflecting limited precursor availability. Similar to hypoxia, *MLS* expression increased at 12 h, although no changes in malate content were detected. In contrast, nitrogen metabolism under submergence showed distinct rearrangements relative to hypoxia. The *AlaAT*/*GOGAT* pathway was activated, reflected by increased alanine accumulation at 6 and 24 h, most likely from pyruvate conversion. Elevated γ -aminobutyric acid (*GABA*) levels at 3 and 24 h further suggested redirection of nitrogen metabolism, although no transcriptional changes were detected in *AlaAT* or gamma-aminobutyric acid-transaminase (*GABA-T*). Together, the increase in alanine and *GABA*, combined with reduced glutamine and aspartate, suggested that glutamate was preferentially consumed by *AlaAT* to generate alanine and 2-oxoglutarate, reducing its availability to produce aspartate, via aspartate aminotransferase (*AspAT*), or glutamine, via glutamine synthetase (*GS*). This is consistent with reduced expression of *GOGAT* and *GS* at 3 h, which later normalized at 12 h. Sugar metabolism showed a contrasting behaviour compared to hypoxia (**Figure 5b**). Phosphorylated sugars (*Fru-6P*, *Glc-6P*, *Man-6P*, *Fru-1P*) increased steadily from 3 to 24 h, while hexoses such as maltose and glucose declined (from 3 h and 1.5 h, respectively). This pattern indicates rapid phosphorylation of available sugars to sustain glycolysis, although no upregulation of hexokinase (*HK*) or phosphoglucosomerase (*PGI*) was detected. Interestingly, beta-amylase (*BAM*) and isoamylase (*ISA*), involved in starch degradation were downregulated, suggesting reduced mobilization of starch reserves under submergence. Shikimate again displayed a biphasic response, declining at 3 h and accumulating after 12 h, similar to hypoxia (**Figure 5c**). Amino acid biosynthesis was less affected overall, though several trends were apparent: asparagine levels decreased, likely reflecting reduced aspartate availability and downregulation of asparagine synthetase (*ASN*); proline decreased at 12 h, and lysine declined at 24 h, consistent with reduced glutamate pools (redirected toward alanine production) and asparagine depletion.

Overall, these results indicate that, while both hypoxia and submergence activate glycolysis and reconfigure nitrogen metabolism, submergence induces broader and more sustained metabolic adjustments. This was particularly evident for sugar metabolism and TCA cycle

(a) Metabolites: Time (h) 1.5 3 6 12 24 LogFC +3 -3 3h | 12h

Upregulated
Downregulated
Unchanged
Not measured

Glyoxysome

Trans-aconitate
Cis-Aconitate
ICL
Isocitrate
Citrate
Citrate dehydratase
Glyoxylate cycle
Glyoxylate
OAA
Acetyl-coA
CoA
Malate
MDH
Succinate
MLs | MLs

Cytosol

3-PGA
1,3-BGAP
PGK
PGM
2-PGA
ENO
PEP
PK
Pyruvate
Lactate
LDH
Ethanol
ADH
Acetaldehyde
PDC
LDH

Plastid

Rubisco
3-PGA
1,3-PGA
GAPC
G3P
Fru-6P
PFK
FBPase
Fru-1,6BP
DHAP
TPI
FBA
SSADH
Succinate
GABA-T
GABA
AlaAT
Alanine
2-OG
GDH
Glutamate
AspAT
Aspartate
OAA
GOGAT
GOGAT
ATP
ADP
GS | GS
Glutamine

Mitochondria

GTP
GDP
SCS
Succinate
Succinyl-CoA
FAD
FADH₂
SQR
Fumarate
FH
Malate
MDH
OAA
CSY
Citrate
2-OG
IDH
Isocitrate
ACO
Putrescine
ODC
Ornithine
Aspartate
OTC
Polyamine pathway
Citrulline
Arginine
Fumarate
Urea
ARG
ASS, ASL

(b)

Cytosol

Man-6P
Fru-6P
Sucrose
Suc-6P
SPP
SPS | SPS
Fru-1P
FRK | FRK
Fructose
SDH
Sorbitol
MIPS, IHP
Glc-6P
Glc-1P
GALC | GALC
Galactose
Myo-inositol
GALC | GALC
Gal-1P
UDP-Gal
UGE
UDP-Glc
TPS
Tre-6P
TPP
Trehalose

Plastid

Fru-6P
PGI
Glc-6P
PGM
Glc-1P
ATP
ADP-Glc
ADG
Starch
BAM, ISA
Maltose
Glucose

(c)

Cytosol

Glycerate
GDH, ARATH
Serine
SHM
Glycine
PAP
GAPC
G3P
NAD⁺ NADH
3-PGA
ATP
ADP
1,3-BGAP
PGM
2-PGA
ENO
PEP
PK
Pyruvate

Plastid

Shikimate
Chorismate
Phenylalanine
Tyrosine
Tryptophan
Tyramine
TYDC | TYDC
2-OG
NADPH NADP⁺
Glutamine
GS | GS
Glutamate
ADP ATP
Aspartate
Asn | Asn
Valine
Leucine
Asparagine
L-aspartate 4-semialdehyde
AK, ASDH
Lysine
Homoserine
HSK, MTO
Methionine
Threonine
Glycine
THA | THA
Isoleucine
PSCS2, PSCS1, PSCR
Proline
Glutamate

Amino acid biosynthesis

215

Discussion

Many studies have focused on metabolic and transcriptomic changes upon hypoxia in model angiosperms such as *Arabidopsis* and rice (*Oryza sativa*). In contrast, the response of non-vascular plants has remained largely unexplored. Here, we characterised the transcriptomic and metabolic responses of the liverwort *Marchantia polymorpha*, revealing both conserved and divergent adaptation strategies when compared with angiosperms.

Transcriptome dynamics

The scale and timing of transcriptomic reprogramming differed between *Marchantia* and angiosperms. In *Arabidopsis*, exposure to 3 % O₂ was sufficient to induce more than 100 transcripts within 1 h and to repress a similar number after 2 h (Liu et al., 2005). By contrast, *Marchantia* exhibited only modest transcriptional changes after 3 h of hypoxia, and even after 12 h, the number of differentially expressed genes (226 upregulated, 67 downregulated) remained considerably lower than in angiosperms (**Figure 2**) (Mustroph et al., 2010; Tamura and Bono, 2022). Despite this limited transcriptional plasticity, activation of redox homeostasis pathways was observed, consistent with conserved low-oxygen responses in higher plants (Mustroph et al., 2010; Van Dongen and Licausi, 2015). A different pattern emerged under submergence. In *Arabidopsis*, submergence for 7 h induced over 300 genes, with a similar number activated after 12 h (Lee et al., 2011), and 4 h of submergence in different *Arabidopsis* ecotypes resulted in the upregulation of up to 500 genes alongside fewer than 100 consistently downregulated genes (van Veen et al., 2016). Given the lower genome redundancy in *Marchantia*, a weaker response might be expected if transcriptional dynamics were comparable to those of angiosperms. However, we observed over 600 genes upregulated and a similar number downregulated following submergence (**Figure 2d**).

This pattern of expression contrasts with angiosperms, where hypoxia alone induces stronger transcriptomic responses than submergence, with responses also being tissue-specific (Mustroph et al., 2009). In *Marchantia*, minimal transcriptional changes occurred under hypoxia, particularly at 3 h, compared with the extensive reprogramming observed under submergence (**Figure 2c**). Tissue-specific differences may also exist in *Marchantia*; for example, transcriptional rearrangements in rhizoids could have been masked in our study by the predominance of thallus tissue in the sampled material. Developmental stage may further influence oxygen sensitivity, as younger tissues in angiosperms show stronger transcriptional responses and greater tolerance than older tissues (Giuntoli et al., 2017). A similar age-dependent effect may also affect transcriptional changes in *Marchantia*.

These observations align with previous studies across land plants, showing that 8 h of hypoxia induces reduced transcriptional changes in non-vascular plants (including *Marchantia*)

compared to angiosperms, suggesting lower sensitivity to low-oxygen alone (Dalle Carbonare et al., 2025). The molecular basis of these differences in *Marchantia* remains to be elucidated. The N-degron pathway, responsible for oxygen-dependent transcriptional regulation in angiosperms, is only partially conserved in *Marchantia*. While the enzymatic components (PCO, ATE, PRT6) are conserved (Weits et al., 2023), the main angiosperm substrate, Cys-initiating ERFVII transcription factor, is absent (Dalle Carbonare et al., 2025). This suggests that alternative transcription factors, potentially other ERFs or MYB proteins, induced by hypoxia, may drive the hypoxic and submergence responses in *Marchantia*.

Reprogramming of central metabolism

In angiosperms, fermentative metabolism is activated under hypoxia to regenerate NAD^+ when oxidative phosphorylation is impaired (Perata and Alpi, 1993). Our data suggest that this is also present in *Marchantia* under both hypoxia and submergence, as indicated by transcriptional activation of *PDC*. However, direct metabolite profiling (ethanol, lactate) would be needed to confirm flux changes. Interestingly, ethanol production has been reported in *Marchantia* (Bui et al., 2019), but the absence of high-homology *ADH*-like gene suggests the existence of an alternative ethanol-producing pathway.

The dynamics of TCA intermediates highlight further divergence from angiosperms. Under hypoxia, we observed accumulation of glutamate and alanine but not GABA, consistent with a delayed activation of the GABA shunt (**Figure 4a**). In contrast, in angiosperms such as soybean roots, 3 h of hypoxia induces increases in alanine, glutamate, and GABA, suggesting faster activation of the AlaAT/GOGAT pathway compared to *Marchantia* (António et al., 2016). Succinate increased only at 6 h and returned to baseline by 12 h, suggesting a partial blockage of the TCA cycle. Under submergence, depletion of fumarate and citrate at 3 h was followed by succinate accumulation at 6 h, consistent with inhibition of succinate dehydrogenase due to restricted mitochondrial electron transport (**Figure 5a**), a pattern also documented in angiosperms, including poplar, rice and *Arabidopsis* already after 2 h of low-oxygen (Narsai et al., 2011).

As in angiosperms, GABA accumulation occurred independently of changes in *GABA-T* transcription, supporting post-translational control of metabolic acclimation (Mustroph et al., 2014). Furthermore, pyruvate was redirected into amino acid metabolism: hypoxia promoted accumulation of alanine and aspartate, whereas submergence led to their depletion and later GABA accumulation (**Figure 6**). This progression mirrors patterns in angiosperms, where amino acids act as nitrogen stores and precursors of stress-protective metabolites (Diab and Limami, 2016; Yuan et al., 2023).

Sugar metabolism also displayed treatment-specific shifts. Hypoxia led to accumulation of soluble sugars, likely via starch degradation, whereas submergence depleted hexoses, suggesting stronger flux into glycolysis (**Figure 6**). Similar context-dependent starch dynamics have been described in potato tubers, where moderate hypoxia sustained starch synthesis while anoxia inhibited it (Geigenberger et al., 2000). Measuring starch reserves and oxygen availability in *Marchantia* under submergence will be essential to clarify these dynamics.

Secondary metabolism and signaling

A striking feature of the submergence response in *Marchantia* was the strong activation of phenylpropanoid and chalcone biosynthesis, potentially associated with amino acid depletion (**Figure 2a**, **Figure 5c**). In angiosperms, flavonoids such as anthocyanins play central roles in ROS scavenging, including during hypoxia (Gong et al., 2022; Gulzar et al., 2024; Pina et al., 2021). Although *Marchantia* does not produce anthocyanins, it synthesises auronidins, red pigments derived from chalcone intermediates (Berland et al., 2019; Bowman et al., 2017; Piatkowski et al., 2020). Previous work has shown that production of auronidins is induced in *Marchantia* under abiotic stresses such as low light and nutrient deprivation (Albert et al., 2018; Jibrán et al., 2024; Rico-Reséndiz et al., 2020). Additionally, flavonoid extracts from *Marchantia* display antioxidant activity (Wang et al., 2016). In our study, induction of *PAL*, *CHS*, and *CHIL*, involved in the production of auronidin precursors, under submergence highlights a potential convergent strategy with angiosperms for ROS mitigation.

Upon submergence, we also observed the induction of calcium-dependent protein kinases (*CPK-like*) already after 3 h of treatment (**Figure 2b**). This suggests potential contribution of Ca^{2+} signaling to the submergence response in *Marchantia*, although changes in cytosolic Ca^{2+} occur early and are unlikely to be primarily regulated at the transcriptional level. Therefore, it is possible that the induction of CPK-like proteins participates in a feedback or feedforward mechanisms. In angiosperms, such proteins regulate the hypoxia responses through phosphorylation of RBOHD and ERFVII factors (Fan et al., 2023; Yu et al., 2024). Calcium signaling has been shown to regulate stress acclimation to other abiotic stresses in *Marchantia*, such as drought (Tansley et al., 2023). While the oxygen-dependent stabilisation of ERFVII is not conserved in bryophytes (Dalle Carbonare et al., 2025; Weits et al., 2023), our data suggest that CPK-mediated signaling may still contribute to submergence acclimation.

By contrast, several ion transporters, including a CAX-like vacuolar $\text{H}^+/\text{Ca}^{2+}$ exchanger, were downregulated under submergence (**Figure 2a**). Similar downregulation of ATP-consuming transporters was observed in *Arabidopsis* under hypoxia (van Dongen et al., 2009). Notably, loss of CAX function has been linked to improved submergence tolerance in rice (Yang et al.,

2022). In *Marchantia*, transporter downregulation may thus represent an energy-conserving strategy that also maintains cytosolic Ca^{2+} for signaling rather than vacuolar sequestration.

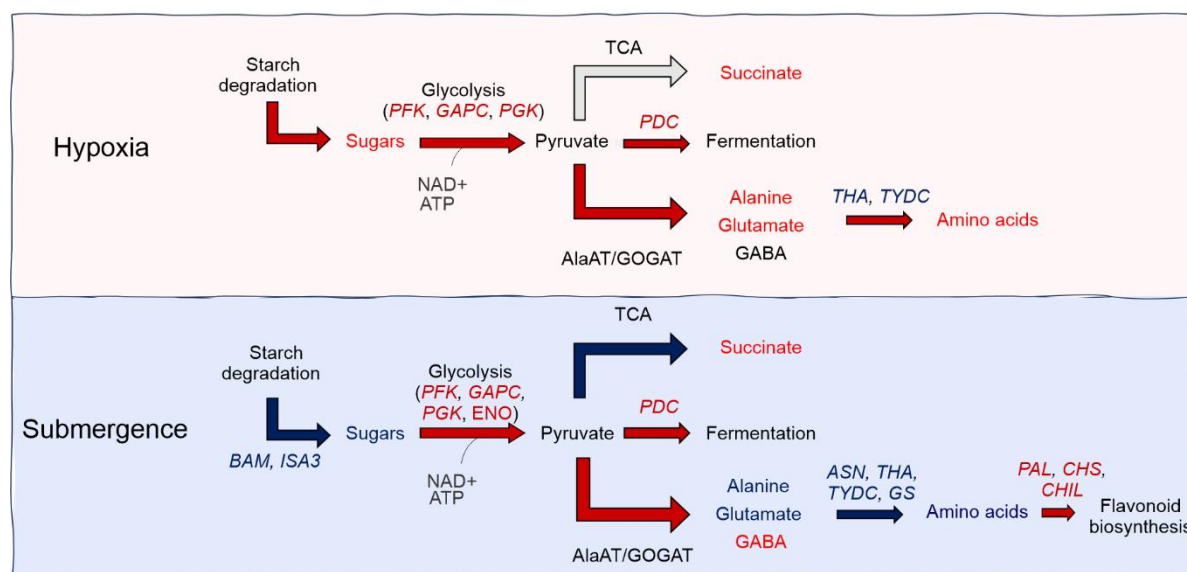


Figure 6 – Summary scheme of pathway affected upon hypoxia and submergence. Hypoxia led to hexose accumulation, enhanced glycolysis, and likely fermentation, together with activation of the AlaAT/GOGAT pathway, resulting in increased amino acid content. In contrast, submergence caused hexose depletion, activation of fermentation and AlaAT/GOGAT metabolism, GABA accumulation, and a decline in amino acids. Submergence also impaired the TCA cycle, as indicated by succinate accumulation.

Hypoxia and submergence produce largely different responses

Submergence triggered broader transcriptomic and metabolic shifts than hypoxia alone, with responses more closely resembling angiosperm submergence adaptations. These effects cannot be attributed to oxygen deprivation alone, suggesting additional effectors. One possibility could be ethylene signaling. Previous studies have shown that *Marchantia* produces ethylene, although through an ACC-independent pathway (Booker and DeLong, 2015; Katayose et al., 2021). Additionally, ethylene production in *Marchantia* has been associated with increased tolerance to heat, salt stress, and nutrient deficiency (Bharadwaj et al., 2022). Since ethylene is a central regulator of submergence survival in angiosperms (Hartman et al., 2021), its involvement in *Marchantia*'s adaptation should be tested. Submergence also restricts diffusion of both O_2 and CO_2 , potentially leading to photosynthetic disruption (Colmer and Voesenek, 2009; Greenway et al., 2006). Finally, nutrient deficiency may increase this stress, as indicated by the downregulation of multiple nutrient transporters, which further require ATP, specifically under submergence. In angiosperms, structural root modifications such as aerenchyma and adventitious roots mitigate nutrient limitation under

flooding (Pedersen et al., 2021). *Marchantia*, lacking roots, relies on rhizoids for anchorage and nutrient absorption (Jones and Dolan, 2012). Rhizoid plasticity has been previously reported under phosphate starvation and drought (Lu et al., 2024; Rico-Reséndiz et al., 2020). Whether similar modifications occur under submergence remains unknown but could help explain the stronger molecular impact compared to hypoxia.

It is important to keep in mind that *Marchantia* naturally inhabits humid environments and is likely adapted to periodic submergence (During, 1979). In contrast, our experimental hypoxia treatments, non-gradual and conducted in the dark, do not occur in nature. This suggests that *Marchantia* may have a limited ability to sense the low-oxygen component of natural submergence, so hypoxia alone could trigger a quiescence strategy that may not be adaptive. Observations from natural habitats indicate that submerged *Marchantia* typically escapes or acclimates, highlighting the need for caution when interpreting experimental hypoxia.

Conclusion

In conclusion, our study revealed that *Marchantia* responds to hypoxia and submergence through a combination of conserved low-oxygen pathways and clade-specific strategies unique to non-vascular plants. While hypoxia alone elicited relatively modest transcriptional and metabolic changes, submergence triggered extensive reprogramming across central metabolism, secondary metabolism, and signaling pathways, suggesting that additional factors beyond oxygen limitation contribute to acclimation. Future studies should investigate the contribution of these additional factors and the molecular mechanisms underlying their regulation. This knowledge not only deepens our understanding of plant adaptation to low-oxygen but also provides valuable insights for developing crop improvement strategies in habitats increasingly threatened by flooding under climate change.

Material and Methods

Plant materials and growth conditions

M. polymorpha accession Cam-2 (female) was grown in Magenta™ vessels on half-strength MS medium (Murashige and Skoog, 1962) (Duchefa), supplemented with 0.5 % (w/v) sucrose and 0.8 % (w/v) agar. Plants were grown at 22°C/20°C, 16:8 h light:dark photoperiod with a light intensity of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Hypoxic and submergence treatment

For hypoxic treatment, two-week-old thalli were exposed to 1 % O₂ (v/v) for 3 and 6 hours in the dark using a Whitley H85 Hypoxystation (Don Whitley Scientific). Three biological replicates were collected for each time point, each consisting of five thalli. For submergence treatments, thalli of the same age were submerged in three volumes of sterile deionized water for 1.5, 3, 6, 12, and 24 hours in the dark. Five to seven biological replicates were collected per condition. Control plants for both hypoxic and submergence treatments were maintained in the dark for the corresponding durations.

Transcriptome analysis

Total RNA was isolated using the Monarch® Total RNA Miniprep Kit (NEB) as per manufacturer's instructions. RNA sequencing was exploited in paired-end mode using the NovaSeq 6000 platform (PE150 mode) (Novogene). Transcriptomic analyses were conducted using R software (version 4.4.2). After quality check using FastQC, reads were aligned to *Marchantia polymorpha* reference genome (v3.1) using *Rsubread* package (v2.16.1) (Liao et al., 2019) and quantified using *featureCounts* software (Liao et al., 2014) in R (v4.4.2, Foundation for Statistical Computing, Vienna, Austria). Multidimensional scaling (MDS) plots were used to evaluate similarities and differences between samples based on gene expression profiles. Differentially expressed genes (DEGs) were identified using edgeR (v3.42.4) (Chen et al., 2025). Differentially expressed genes with a $|\text{Log}_2 \text{fold change}| \geq 1.5$ and a false discovery rate (FDR) < 0.05 were selected for further analysis. Fold change data and TPM are provided in Supplementary Tables 1, 2. Gene Ontology (GO) enrichment analysis of DEGs was performed using gProfiler.

Metabolite analysis

Soluble metabolites were extracted and analysed as described in (Lunn et al., 2006). Tissue homogenization was carried out in liquid nitrogen, followed by metabolite extraction using methanol and water. Polar metabolites were isolated through chloroform purification, after which the polar phase was dried by vacuum centrifugation. Finally, the samples were analysed

using GC-MS and LC-MS in collaboration with Dr John Lunn and Dr Alisdair Fernie at the Max-Planck-Institute (Potsdam-Golm, Germany). Principal component analysis (PCA) was performed in R. For statistical analysis, metabolites showing differential concentrations were selected based on a $|\text{Log}_2 \text{ fold change}| \geq 1$ and an adjusted p-value (p_{adj}) < 0.05 . Full fold change metabolite data is provided in Supplementary Tables 3-4.

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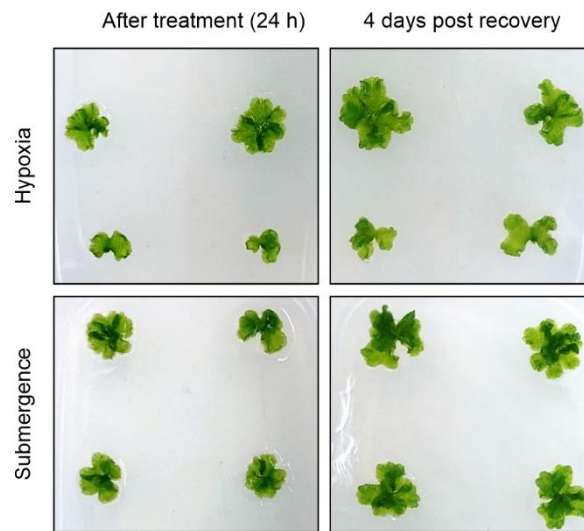
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Supplementary Information



Supplementary Figure 1. Images showing *Marchantia* thalli after 24 hours of hypoxic and submergence treatment. Pictures were taken 4 days after the end of the treatment.

Statement of authorship

Statement of Authorship for joint/multi-authored papers for PGR thesis

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The statement shall describe the candidate's and co-authors' independent research contributions in the thesis publications. For each publication there should exist a complete statement that is to be filled out and signed by the candidate and supervisor (**only required where there isn't already a statement of contribution within the paper itself**).

Title of Paper	Transcriptomic and metabolic profiling of low-oxygen adaptations in <i>Marchantia polymorpha</i>
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☒ Unpublished and unsubmitted work written in a manuscript style
Publication Details	Monica Perri, Saleh Alseekh, Regina Feil, Alisdair R Fernie, John Lunn, Francesco Licausi*

Student Confirmation

Student Name:	Monica Perri
Contribution to the Paper	Conducted and designed experiments: low-oxygen and submergence treatments; RNA extraction and RNA-seq (processing and analysis), data analysis; write-up of the manuscript.

Signature 	Date	12/08/2025
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Supervisor Confirmation

By signing the Statement of Authorship, you are certifying that the candidate made a substantial contribution to the publication, and that the description described above is accurate.

Supervisor name and title: Professor Francesco Licausi		
Supervisor comments I hereby confirm that the above Declaration of Authorship and Contributions provided by Ms Monica Perri is accurate and truthful. The statement correctly reflects her own scientific and scholarly contributions to the work presented in her thesis, as well as the contributions made by collaborators, which are appropriately acknowledged. I endorse this declaration as a fair and transparent account of authorship and contributions.		
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Discussion

Plants frequently encounter oxygen limitation during environmental challenges such as flooding and throughout various developmental processes. Despite this, the mechanisms by which they sense low-oxygen and adjust their responses are only partly understood. Most current understanding of plant hypoxia responses comes from studies in angiosperms, whereas other plant clades have been far less explored (Reynoso et al., 2019; Weits et al., 2023; Dalle Carbonare et al., 2025). Improving our understanding beyond model species is crucial for advancing fundamental plant biology and informing strategies to enhance crop resilience under stress. In this thesis, I investigated how plants sense and respond to low-oxygen by integrating physiological, molecular, and evolutionary approaches. Rather than pointing to a single regulatory pathway, the results indicate that oxygen sensing reflects the combined effects of spatial variation in oxygen availability, signal integration during hypoxia and reoxygenation, and evolutionary diversification of conserved enzymatic components.

An important starting point for this work was the spatial organization of oxygen availability within plant tissues. The development and application of the hypoxia-sensitive fluorescent probes 4NB-Resorufin and MeIQ-Resorufin enabled direct visualisation of hypoxic niches in *Arabidopsis* tissues, revealing both experimentally induced hypoxia and naturally occurring hypoxic domains such as lateral root primordia. Importantly, MeIQ-Resorufin showed greater sensitivity to mild hypoxia and higher overall fluorescence than 4NB-Resorufin, highlighting its potential as a practical tool for non-invasive oxygen mapping *in planta*. By identifying where hypoxia occurs, this work provides spatial context for interpreting the molecular responses examined in later chapters, which necessarily operate within oxygen gradients rather than uniform tissue environments. However, differences in probe distribution across tissue types underscore the influence of plant anatomy and transport dynamics on probe performance. In addition, the irreversible nature of probe activation limits temporal resolution during rapid oxygen fluctuations. Despite these constraints, the probes represent a valuable addition to the hypoxia research toolkit, particularly because they do not require genetic manipulation and can therefore be applied across species. Future work combining such probes with reversible oxygen sensors or redox reporters would allow a more detailed analysis of oxygen dynamics during hypoxia-reoxygenation transitions.

Within this spatially heterogeneous context, plants must integrate oxygen signals with additional cues that arise during flooding stress. A key finding of this thesis is that transitions from hypoxia to reoxygenation represent a critical regulatory phase rather than a simple return to normoxia. Reoxygenation was shown to be associated with reactive oxygen species

accumulation, which in turn modulates the activity of plant cysteine dioxygenases. This work revealed that reactive oxygen species, particularly H_2O_2 , inhibit plant cysteine dioxygenases (PCOs), stabilizing ERFVII transcription factors even after oxygen levels recover. Unexpectedly, stabilised ERFVII under these conditions repressed hypoxia-responsive genes while activating oxidative stress pathways. These findings demonstrate that the PCO-ERFVII module functions as a versatile signaling hub, integrating oxygen and ROS signals to tailor transcriptional responses according to the prevailing stress. Such flexibility may explain why ERFVII are implicated in tolerance to a broad range of stresses, including salinity, temperature, and pathogen attack, beyond hypoxia alone.

While this work establishes that the PCO-ERFVII module integrates hypoxia and reoxygenation signals, an important unresolved question concerns how differential regulation between hypoxia- and oxidative stress-responsive outputs is achieved at the molecular level. One possibility to be addressed in future studies is whether ERFVII undergo stress-specific post-translational modifications or interact with distinct protein partners under hypoxia versus reoxygenation, thereby altering their transcriptional activity.

The functional consequences of modulating oxygen-sensing enzymes were further explored by manipulating of cysteine dioxygenases themselves. Substitution of Arabidopsis PCOs with homologous NCOs from animals, fungi, algae, and non-flowering plants revealed that subtle structural differences, such as substrate pocket volume or electrostatic charge distribution, have profound consequences for enzyme kinetics and downstream transcriptional programs. Lines with low *NCO* expression, reduced catalytic activity or lower substrate efficiency exhibited delayed development yet improved survival under submergence, revealing a trade-off between growth and stress tolerance. Conversely, this trade-off is not reciprocal, as previous studies have shown that overexpression or constitutive stabilization of PCO substrates also impairs plant growth (Gibbs et al., 2011; Hinz et al., 2010; Licausi et al., 2011).

The observation that PCOs are susceptible to inhibition by ROS further raises the possibility that oxygen-sensing enzymes integrate multiple chemical signals beyond oxygen alone. Recent evidence indicates that hydrogen sulphide can also inhibit PCO activity (Telara et al., 2025), suggesting that thiol dioxygenases may act as broader chemical during stress. Whether such inhibitory mechanisms are plant-specific or conserved across NCOs from different lineages remains unknown. Comparative sequence analyses combined with *in vitro* assays across divergent NCOs could clarify whether sensitivity to ROS or other small molecules represents an ancestral feature or a lineage-specific adaptation. It should also be noted that ERFVII stability in the cross-species study was inferred indirectly from transcriptional outputs, and additional regulatory layers may therefore remain undetected.

Additionally, the identification of novel N-degron substrates and clarification of their physiological roles will be critical for informing the selection of optimal NCO candidates to improve stress tolerance. It is also important to consider that the N-degron pathway is not the only mechanism controlling hypoxia responses; for example, recent work from our group has identified an oxygen-sensing mechanism that reprograms chromatin in roots, which may also contribute to submergence tolerance (Zhang et al., 2025). Overall, these results demonstrate that enhanced hypoxia signaling does not universally improve plant performance and that oxygen-sensing pathways must be finely tuned to balance resilience with developmental progression.

The observation that substitution of Arabidopsis PCO with *Marchantia polymorpha* PCO (MpPCO) produced distinct physiological outcomes motivated the extension of this work into an evolutionary context. Accordingly, Chapter 5 examined low-oxygen responses in the non-vascular liverwort *Marchantia polymorpha*. In contrast to angiosperms, hypoxia alone triggered only a modest transcriptional response in *Marchantia*, whereas submergence induced a broad transcriptomic and metabolic reprogramming. This included activation of fermentative metabolism, reconfiguration of amino acid pathways, and induction of auronidin biosynthesis as a putative ROS-scavenging mechanism. The absence of ERFVII transcription factors in *Marchantia* points to alternative regulators of oxygen and stress responses in early land plants, leaving open the possibility that the N-degron pathway may act on as-yet unidentified substrates. Moreover, the pronounced submergence response suggests that additional cues, such as ethylene accumulation or nutrient limitation, play central roles in shaping stress adaptation. These findings place angiosperm oxygen-sensing mechanisms within a broader evolutionary framework, indicating that conserved metabolic responses to low-oxygen predate the emergence of ERFVII-dependent transcriptional regulation.

Future work combining flux-based metabolic analyses with higher temporal resolution transcriptomics would help resolve metabolic pathway dynamics in *Marchantia* and identify regulatory networks. The recurrent involvement of ROS responses further suggests that hypoxia-redox integration may represent a conserved survival strategy, although the underlying mechanisms remain to be elucidated in the absence of ERFVIIs in bryophytes (Dalle Carbonare et al., 2025). Finally, given that hypoxia also occurs physiologically, the hypoxia-activated probes validated in this study provide an opportunity to explore endogenous hypoxic niches in *Marchantia* and other green lineages, particularly when combined with tissue-specific transcriptomic or metabolomic resources.

Conclusion

In this work, I have shown that plants adapt to low-oxygen through a combination of conserved enzymatic mechanisms, flexible transcriptional networks, and lineage-specific innovations. By developing novel chemical probes, investigating crosstalk between combined stresses and extending analysis to evolutionary contexts, this thesis expands both the experimental approaches and conceptual understanding of plant oxygen sensing.

Viewed across all chapters, the results point to a coherent framework in which spatial oxygen landscapes define local physiological contexts, molecular oxygen- and redox-dependent processes interpret these cues during dynamic stress transitions, and evolutionary diversification shapes how these responses are deployed across plant lineages. In this framework, hypoxia is not only a stress condition but also a physiological signal, and successful adaptation depends on regulation that is finely tuned rather than maximally activated.

Looking ahead, elucidating additional regulators of oxygen-dependent pathways, improving spatial and temporal resolution of oxygen and redox measurements, and translating these insights into crops through context-dependent modulation of oxygen sensing will be important steps toward improving resilience to flooding and other oxygen-limiting stresses.

Ultimately, this work contributes to a broader understanding of how plants cope with oxygen limitation and provides a foundation for developing more sustainable and climate-resilient agriculture.

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