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Recent progress in understanding the cellular and genetic basis of plant responses to low oxygen holds promise for developing flood-resilient crops

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Abstract

With recent progress in active research on flooding and hypoxia/anoxia tolerance in native and agricultural crop plants, vast knowledge has been gained on both individual tolerance mechanisms and the general mechanisms of flooding tolerance in plants. Research on carbohydrate consumption, ethanolic and lactic acid fermentation, and their regulation under stress conditions has been accompanied by investigations on aerenchyma development and the emergence of the radial oxygen loss barrier in some plant species under flooded conditions. The discovery of the oxygen-sensing mechanism in plants and unravelling the intricacies of this mechanism have boosted this very international research effort. Recent studies have highlighted the importance of oxygen availability as a signalling component during plant development. The latest developments in determining actual oxygen concentrations using minute probes and molecular sensors in tissues and even within cells have provided new insights into the intracellular effects of flooding. The information amassed during recent years has been used in the breeding of new flood-tolerant crop cultivars. With the wealth of metabolic, anatomical, and genetic information, novel holistic approaches can be used to enhance crop species and their productivity under increasing stress conditions due to climate change and the subsequent changes in the environment.

Keywords: Anoxia, core hypoxia-inducible genes, crop breeding, environmental stress, hypoxia, oxygen sensing, regulation of metabolism, rice, submergence, waterlogging.

Introduction

Water is vitally important for normal functioning of plants; however, it can be harmful when it partially or totally covers plants as a result of a flooding event. In this review, flooding tolerance is defined as a means of survival of conditions that
are fatal to most plants coupled with the capacity to recover sufficiently to produce biomass. This is largely due to a lower availability of oxygen (O₂), carbon dioxide (CO₂), and light in water that hampers respiration and photosynthesis. Adverse conditions caused by flooding or complete submergence cause widespread yield losses to crop plants (Food and Agriculture Organization of the United Nations, 2017, 2021; Wing et al., 2022). In the USA, inundation modelling under the RCP4.5 scenario estimates a 26.4% increase in flood risk by 2050 (Wing et al., 2022). Floods during the growing season have particularly magnified the severity of crop losses, and at the same time there have been large carbon emissions to the atmosphere from the flooded fields (Perkins, 2020). The massive crop losses have led to an increased interest in finding ways to increase flooding tolerance of rice in particular. This was highlighted in a recent review that focused not only on maize, barley, and soybean, but also on other crops that are less commonly studied such as wheat and rapeseed, and which are all relatively flooding intolerant (Mustroph, 2018). Showing the importance of this research field, several other reviews have been recently published. Zahra et al. (2020) reviewed biochemical and molecular approaches aimed at better understanding the complex mechanisms behind tolerance to hypoxic or anoxic conditions. Xie et al. (2021) highlighted that lipids and their metabolites act as important intracellular signalling agents in plants under low-oxygen stress. Many aspects of flooding tolerance and sensitivity have been reviewed before, such as growth, survival, and catabolism (Gibbs and Greenway, 2003), energy requirements (Greenway and Gibbs, 2003), acclimation and genetic diversity (Bailey-Serres and Voessenek, 2008; Fagerstedt et al., 2011), and flooding tolerance in halophytes (Colmer and Flowers, 2008).

In this review we focus on the latest developments in research on plant tolerance to hypoxic conditions (i.e. oxygen concentrations lower than atmospheric 21%; Sasidharan et al., 2017) and, more importantly, we speculate on the future directions of research within the field of hypoxia tolerance. We re-examine some of the most important developments in the field such as the exploration of the core hypoxia response genes (Mustroph et al., 2009) which has led to a wealth of new research results. Another finding is the N-end rule pathway (N-degron pathway) for oxygen level sensing in plants (Gibbs et al., 2011; Licausi et al., 2011) which has led to many studies on the intricacies of the sensing system (Paul et al., 2016; Kunkowska et al., 2023). Research also includes the SUB1A allele effects in improving flooding tolerance in rice (Fukao et al., 2006; Xu et al., 2006; Takeshi et al., 2011), the role of nitric oxide (NO) in the regulation of metabolic events under hypoxia (Gupta et al., 2022), the detection of oxygen levels within tissues or even within cells (Iacopino et al., 2019; Wagner et al., 2019; Pedersen et al., 2021), and the regulation of aerenchyma development (Yamauchi et al., 2019).

One important factor in terms of boosting research on hypoxia in plants and in creating a community of like-minded researchers is the International Society for Plant Anaerobiosis (ISPA), which was founded in 1975 and has since had a large influence on the understanding of plant behaviour under flooded, hypoxic or anoxic conditions (Vartapetian et al., 2011). At its 14th international conference, ISPA was renamed ISPLORE, the International Society for Plant Low Oxygen Research.

Research within ISPA/ISPLORE has developed dramatically over recent years, from descriptive projects on gas exchange, anaerobic metabolism, and anatomical and morphological features, to detailed and intricate research efforts on hypoxia tolerance mechanisms and their regulation at the gene level, and more recently holistic views on flooding tolerance and its regulation in crop plants. Much of the resulting knowledge has been put into practice in crop breeding and used to identify interesting genetic resources especially but not only, in rice by ISPLORE members (Singh et al., 2009; Bailey-Serres et al., 2010; Nghï et al., 2019; Gómez-Álvarez et al., 2023).

Plant diversity in adaptation and acclimation to flooding/hypoxia/anoxia

Plant species have evolved and adapted to flooding in a huge variety of ways. In a study of 91 plant species categorized as wetland, non-wetland, and intermediate, root porosity was found to correlate positively with flooding tolerance (Justin and Armstrong, 1987). This latter study also indicated that many other root morphological and anatomical features affect plant behaviour in flooded soils. This information was exploited in a study on waterlogging stress in okra and maize that were given exogenous ethylene priming which improved root porosity and emergence of adventitious roots (Vwioko et al., 2017). It was also used in experiments published in an article on plant strategies in waterlogged environments (Pan et al., 2022). The results indicated that trait decoupling (i.e. separate regulation of different traits) enables plants to overcome the stresses of a complex flooded environment.

Clearly, there is no general strategy that perfectly explains the adaptation of plants to hypoxia. Instead, flood tolerance involves complex strategies, which is highlighted by a combination of traits regarding induction under flooded conditions. This also does not exclude leaf economics and size-related traits, which all contribute to plant survival in flooding events across a wide range of habitats (Pan et al., 2022). Within crop plant species there is large variation in flooding tolerance, especially in rice cultivars.

Acclimation to oxygen shortage in rice

Rice is traditionally cultivated in flooded conditions thanks to acclimative responses including morphological, physiological, and molecular traits that limit the oxygen shortage. Rice cultivation can be classified according to three ecological situations: deepwater rice that grows in a water depth of >50 cm,
lowland rice that is suitable for irrigated or rain-fed fields of up to 50 cm depth, and upland rice cultivated in non-flooded conditions (Sauter, 2000). However, fields are frequently submerged completely during rainy seasons, and some rice varieties cannot survive prolonged complete submergence (Minami et al., 2018).

The occurrence of different water regimes in cultivated and natural environments has supported the development of ecological adaptations to extreme conditions. Adult plants have two opposing strategies of response to water submergence: a low oxygen quiescence strategy (LOQS) and a low oxygen escape strategy (LOES) (Bailey-Serres and Voesenek, 2008). These are represented by the capacity to escape the water depth through fast and extreme stem/leaf elongation, thus reaching the water surface and continuing aerobic respiration (LOES) and, in contrast, to enter into a state of quiescence underwater. This latter strategy is aimed at reusing energy resources during the subsequent recovery period (Bailey-Serres and Voesenek, 2008).

LOES is found in deepwater rice where an exceptional internode elongation (20–25 cm d⁻¹) reaching several metres in length is manifested during the increase in water depth under months-long deep flooding (Nagai et al., 2010). The quantitative trait locus (QTL) detected on chromosome 12 of the cv C9285 from Bangladesh is responsible for this adaptation, with the group VII-ERF (ethylene response factor) SNORKEL1 (SK1) transcription factor and SK2 being the key components (Hattori et al., 2007, 2008, 2009). The entrapment of ethylene underwater leads to SK1 and SK2 transcription which, through stimulating the production of gibberellic acid (GA), promotes the internode growth of the stem, and thus both cell proliferation and elongation. Subsequently, a genome-wide association study (GWAS) conducted on a panel of deepwater and Asian rice varieties identified a haplotype of the SEMIDWARF gene (SD1), which encodes GA200X2, a gibberellin biosynthetic enzyme that encodes GA200X2, a gibberellin biosynthetic enzyme that activates the development of two types of adventitious roots during partial submergence (Minami et al., 2018).

The presence of the SUB1A-1 allele in some rice accessions mediates flooding tolerance and is regulated by an increase in ethylene under submergence (Xu et al., 2006). Through a feedback mechanism, SUB1A-1 activation reduces ethylene perception, thus suppressing the GA-mediated activation of cell elongation and carbohydrate metabolism-related genes, inducing quiescence. In fact, SUB1A-1 is associated with the accumulation of the DELLA protein SLENDER RICE 1 (SLR19) and the non-DELLA protein SLR like 1 (SLRL1) which dampen GA, thus repressing the elongation of the shoot (Fukao and Bailey-Serres, 2008).

SUB1A-1 is also involved in tolerance to drought stress occurring under the post-submergence recovery period, where it enhances the abscisic acid (ABA) responsiveness and activates genes that are stress related (Fukao et al., 2011). The transfer of the SUB1 QTL through marker-assisted backcrossing into highly productive varieties has led to the production of SUB1 megavarieties which are able to survive flooding and have high yields (Singh et al., 2009; Bailey-Serres et al., 2010). The ERF-VII SUB1A gene is not a substrate of the N-end pathway for proteolysis, suggesting other mechanisms of regulation probably related to enhanced tolerance (Gibbs et al., 2011). Indeed, structural and functional interaction analysis suggests that in SUB1A-1, the C-terminus interacts with the N-terminus, in this way protecting from N-degron-mediated degradation and explaining the enhanced stability (Lin et al., 2019). In the same work, other ERF-VII genes, ERF66 and ERF67, have been found to be substrates of the N-degron and to be transcriptionally up-regulated by SUB1A-1, probably contributing to promote submergence survival in rice accessions harbouring SUB1A-1 (Lin et al., 2019). Finally, MITOGEN ACTIVATED PROTEIN KINASE 3 (MPK3) is involved in a positive loop of regulation with SUB1A-1, where it is activated and subsequently interacts and phosphorylates SUB1A, contributing to acclimation (Singh and Sinha, 2016).

Among cereals, rice has a notable ability to germinate under submergence (Perata et al., 1992; Guglielmini et al., 1995) and elongate the coleoptile with a snorlke-like function (Narsai et al., 2015; Shinohara et al., 2022). Being able to degrade starch under oxygen shortage is critical in order to fuel the organs and their growth (Loret et al., 2003a; Lasanthi-Kudahettige et al., 2007). Rice harbours α-amylase genes that hydrolyse starch and are sensitive to oxygen shortage and...
starvation of sugars which rapidly follow under submergence (Yu et al., 2015). This pathway works independently of the air-dependent activation by GA (Loreti et al., 2003b) and is regulated by hypoxia-dependent calcium signal and sugar starvation (Lee et al., 2009). In this pathway, a calcineurin B-like protein (CBL)-interacting protein kinase CIPK15 is the central regulator contributing to decoding the calcium signal. CIPK15 is a positive regulator of the sucrose-non-fermenting-1-related protein kinase 1A (SnRK1A) and a transcriptional activator of MYBS1 (Lu et al., 2002, 2007; Lee et al., 2009). MYBS1 binds directly to α-amylase subfamily 3 promoters, thus positively regulating starch degradation.

The trehalose-6-phosphate phosphatase 7 gene (TPP7), involved in trehalose-6-phosphate conversion to trehalose, is crucial for the enhanced anaerobic germination capacity (Kretzschmar et al., 2015). The availability of TPP7 in some rice genotypes modifies the T6P/sucrose balance in the seed, thus increasing starch mobilization by α-amylase and the establishment of seedlings under submergence. The TPP7 gene contributes to coleoptile length, as demonstrated by the experiments conducted with the NIL AGI1, containing the TPP7 gene (Kretzschmar et al., 2015). A long coleoptile in japonica rice accessions has been related to the capacity to translocate auxin through the enhanced regulation of AUX1 (Nighi et al., 2019, 2021), in order to favour the extension of the cell during elongation. In parallel, the auxin-dependent signalling is activated in rice through the submergence-dependent dampening of miR399 expression which reduces the degradation of transport inhibitor response 1 (TIR1), the regulator of auxin-responsive gene expression (Guo et al., 2016).

**Acclimation to flooding at the metabolic level**

When a plant experiences a shortage of oxygen, metabolic acclimation occurs in order to produce energy for survival, even without the final oxidative phosphorylation at the mitochondria (Braun, 2020). Pyruvate availability links mitochondrial respiration to the preceding glycolysis in the cytosol. After carbohydrate metabolism through glycolysis, pyruvate is transported to mitochondria and oxidized along the tricarboxylic acid cycle (TCA) to organic acids with the production of NADH, finally leading to ATP production (Braun, 2020).

Under oxygen shortage, energy production from carbohydrates through an acclimation process that includes a shift toward fermentative metabolism. This leads to energy production but can possibly result in an energy crisis, given that the amount of ATP resulting from glycolysis coupled to fermentation is much lower than that obtained through mitochondrial respiration. When respiration is hampered by oxygen shortage, energy production is limited to glycolysis, which leads to the production of some ATP and pyruvate. The availability of substrates and the regeneration of oxidized NAD are vital for the maintenance of glycolysis (Licaisi and Perata, 2009). To maintain the glycolytic flux, the fermentation pathway is activated, consisting of a brief period of lactic acid production and prolonged ethanolic fermentation, which leads to the conversion of NADH back to NAD, allowing glycolysis to continue (Perata and Alpi, 1993).

The end-product of glycolysis, pyruvate, is either converted by lactate dehydrogenase (LDH) to lactate with the regeneration of NADH or it is decarboxylated into acetaldehyde and then ethanol is produced by pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) with regeneration of NAD⁺, respectively. In fact, lactic accumulation can be toxic due to its dissociation which contributes to lowering the cell pH, while membranes are permeable to ethanol which rarely reaches dangerous concentrations (Davies et al., 1974; Davies, 1980; Hanhijärvi and Fagerstedt, 1995). Acetaldehyde accumulation can also be toxic and can also be converted to acetate by ADH (Perata and Alpi, 1991). This reaction, however, depletes the NAD⁺ pool (Kürsteiner et al., 2003). PDC and ADH gene transcripts are induced under low oxygen and are targets of the ERF-VII transcription factors involved in oxygen sensing (Gibbs et al., 2011; Licausi et al., 2011). In this context, recent results reported that the energy sensor TARGET OF RAPAMYCIN (TOR) contributes to the transcriptional activity of RELATED TO AP 2.12 (RAP2.12) through phosphorylation, demonstrating the convergence of oxygen and energy sensing (Kunkowska et al., 2023).

Acidification induced by lactate adjusts the pH to an optimal value for PDC activity with the activation of the ethanolic pathway and subsequent regeneration of NAD⁺ which is required for glycolysis (Davies, 1980). LDH activity stimulates ethanolic fermentation, suggesting the need for lactic fermentation in order for ethanolic fermentation to take place (Dolferus et al., 2008). Lactic fermentation is a prerequisite for plant tolerance to oxygen shortage since ldh1 Arabidopsis mutants show reduced survival under hypoxia (Dolferus et al., 2008).

A further hypothesis explaining the favouring of ethanolic fermentation under oxygen shortage accounts for the activity of PDC in comparison with pyruvate dehydrogenase (PDH) which converts pyruvate to acetyl-CoA. This suggests that an accumulation of pyruvate leads preferentially to the activation of ethanolic fermentation (Tadege et al., 1999). In fact, PDC is a crucial enzyme during fermentation. Arabidopsis PDC1- and PDC2-overexpressing plants have shown increased tolerance to low oxygen and increased ethanol production (Ismoind et al., 2003), while the pdc1 mutant is highly sensitive to anoxia (Kürsteiner et al., 2003). Arabidopsis ADH-overexpressing plants confer anoxia tolerance, but no effect is visible on flooding survival, while adh1 mutants show an enhanced sensitivity to low oxygen, suggesting the crucial role of ADH in plant tolerance (Ismoind et al., 2003).

The rice reduced adh activity (rad) mutant shows a reduced coleoptile length under submergence (Saika et al., 2006), while the maize adh1 mutant is very sensitive to anoxia (Johnson et al.,
Arabidopsis mutants lacking functional PDC (pdc1pdc2 double mutant) and ADH (adhl mutant) have impaired growth in air, suggesting the importance of the fermentative metabolism in plant growth. These results indicate the importance of the ATP supply by fermentative pathways in hypoxic niches irrespective of the surrounding aerobic environment (Ventura et al., 2020).

Activation of the glycolytic flux followed by fermentation enables ATP production to be maintained for a limited length of time. During fermentation, the amount of energy produced is 2–4 mol of ATP per mol of hexose, in comparison with 30–36 mol of ATP produced under normoxia (Greenway and Gibbs, 2003). Early works reported an increase in glycolytic flux under complete anoxia, defined as the ‘Pasteur effect’, in order to produce a higher amount of ATP (Neal and Girton, 1955; Vartapetian, 1982; Gibbs et al., 2000; Bailey-Serres et al., 2012; Banti et al., 2013; Jethva et al., 2022). Subsequent results reported an adaptation of respiration to hypoxia in Pisum sativum when the oxygen availability decreases in order to save oxygen, thus suggesting the existence of a regulatory mechanism that works in a concentration-dependent manner (Zabalza et al., 2009).

Ethanolic fermentation has been analysed in land plant species (Bui et al., 2019), and the results suggest that distant phyla maintain the ability to produce ethanol under hypoxia, while the transcriptional regulation of anaerobic genes is not conserved (Bui et al., 2019). In addition, Arabidopsis adhl mutants maintain the capacity to produce ethanol under hypoxia, suggesting that additional, as yet unidentified, enzymatic pathways are possibly involved (Bui et al., 2019).

The process of glycolysis and thus the survival of the plant require a sugar supply from either sucrose degradation or starch breakdown. Sucrose provides hexose-6-phosphates through two distinct pathways. The sucrose synthase (SUS) pathway firstly produces fructose and UDP-glucose, and is bi-directional, but the catabolic direction is favoured. UDP-glucose is then converted into glucose-1-phosphate (G1P), through the activity of UDP-glucose pyrophosphorylase (UGPPase) which is PPi dependent, and it is subsequently converted into G6P by phosphoglucomutase (PGM). Fructose is then converted into fructose-6-phosphate (F6P) through the activity of fructokinase (FK), which is ATP dependent (Huber and Akazawa, 1986; Stitt, 1998).

The invertase (INV) pathway is unidirectional and catalyses sucrose hydrolysis into fructose and glucose, which are subsequently phosphorylated to F6P and G6P by HK and FK, respectively, using ATP or UDP as energy donors (Renz and Stitt, 1993). Since sucrose degradation to phosphorylated exoses via the INV route requires two molecules of ATP, it is generally assumed that the SUS route is preferred during an oxygen shortage, given that it produces F6P and UDP-Glc, the latter converted to G1P. However, Arabidopsis plants defective in SUS1 and SUS4, which are highly induced by oxygen depletion, have been shown to be as tolerant to low oxygen as wild-type plants (Santaniello et al., 2014). Only when the plants were under sugar starvation was the requirement of SUS for the production of ethanol evident, although compensated partially by the activity of INV. This thus suggests that both the SUS and INV route contribute to sucrose metabolism (Santaniello et al., 2014).

The availability of soluble carbohydrate can be limited under hypoxia when it is prolonged. Leaf transitory starch can instead be a reliable source at night (Smith et al., 2005) or when plants experience hypoxia in darkness (Loreti et al., 2018). In fact, starchless mutants pgm (mutated into the plastidial form of PGM), which are unable to produce starch during the day, and sel (defective in glucan water dikinase), which is unable to degrade starch at night, are highly sensitive to low oxygen conditions, suggesting that survival requires transitory starch (Loreti et al., 2018).

Other products involved in anaerobic metabolism are Ala and γ-aminobutyric acid (GABA). Ala is produced by transferring an amino group from glutamate to pyruvate through Alaaminotransferase (AlaAT) activity with the reversible generation of 2-oxoglutarate (Streeter and Thompson, 1972; Reggiani et al., 1988). Accumulation is likely to be involved in reducing the pyruvate level to prevent the inhibition of glycolysis (Rocha et al., 2010). GABA has also been found to increase under low oxygen as the further conversion of glutamate through glutamate decarboxylase (GAD) (Reggiani, 1999). Both Ala and GABA production may prevent acidification of the cell and reduce carbon/nitrogen loss that can instead be used during the recovery period (Mustroph et al., 2014). There is thus a rapid reconversion of Ala to pyruvate during the recovery period after oxygen shortage in Arabidopsis plants (Miyashita et al., 2007).

Hypoxia is constitutive in some plant tissues where it plays a role in regulation. Hypoxia has been detected in bulky organs such as potato tubers (Geigeberger et al., 2000), fruits (Ho et al., 2010a, b) and seeds (Borisjuk and Rolletschek, 2009), and a low oxygen state has been detected in the phloem (Van Dongen et al., 2003). A constitutive hypoxia state was also recently shown in roots of Arabidopsis despite the potting medium being well aerated, and proved by the high accumulation of PDC and ADH proteins (Ventura et al., 2020). Hypoxia regulates development in the shoot apical meristem (SAM) (Weits et al., 2019) and in the lateral root primordia (Shukla et al., 2019) where fermentation-related transcripts of ADH and PDC are highly expressed. This thus suggests that the energy demand of these hypoxic niches is met by glycolysis plus fermentation.

**The fascinating oxygen-sensing story**

As described in the previous section on rice tolerance to submergence, the discovery of SUB1A in rice was a breakthrough in our understanding of how rice plants tolerate submergence.
In 1996, a group of researchers led by David Mackill at the International Rice Research Institute (IRRI) identified a major QTL on chromosome 9 that was associated with submergence tolerance in rice, which they named QTL SUB1 (SUBmergence 1). It took 10 years for the molecular identity of SUB1 to be revealed. SUB1 is a cluster of three genes that encode ERFs, more specifically those belonging to group VII.

The discovery of SUB1A as the rice submergence tolerance gene boosted research on the members of ERF–VII in Arabidopsis. Papdi et al. (2008) screened for the transcriptional activation of the ADH1–luciferase (LUC) reporter gene. They identified the ERF–VII transcription factor RAP2.12 as being able to sustain a high level of ADH1–LUC bioluminescence as well as an enhanced ADH1 transcription rate (Papdi et al., 2008).

In 2010, RAP2.2, HRE1, and HRE2 (HRE stands for Hypoxia Inducible ERF–VII), three of the five members of the ERF–VII family in Arabidopsis, were identified as being key for the plant's ability to withstand hypoxia (Hinz et al., 2010; Licausi et al., 2010). RAP2.2 is expressed constitutively at high levels in the roots and is induced in shoots by ethylene. Overexpression of RAP2.2 improved plant survival to hypoxia, whereas mutant lines of the gene had poorer survival rates than the wild type (Hinz et al., 2010). HRE1 and HRE2 are also involved in hypoxia acclimation, given that they exert a role in low oxygen signalling and improve the tolerance of the plant to low oxygen by enhancing anaerobic gene expression and ethanolic fermentation (Licausi et al., 2011).

The turning point for research on hypoxia in plants occurred the following year, in 2011. Two research groups independently demonstrated the existence of an oxygen-sensing mechanism in plants that was based on the oxygen-dependent destabilization of the ERF–VII belonging to the subgroup that is constitutively expressed in Arabidopsis (Gibbs et al., 2011; Licausi et al., 2011). The mechanism of oxygen sensing, as illustrated in Fig. 1, is based on the N-end rule pathway (now known as the N-degron pathway), which leads to the degradation of proteins characterized by a specific N-terminal amino acid, the type of amino acid determining whether a protein will be degraded by a proteasome (reviewed in Sasidharan and Mustroph, 2011).

Interestingly, proteins with a Cys residue in the second position, such as the ERF–VII proteins, can be processed by the N-degron pathway in mammals and plants and degraded. Such proteins can become accessible to the protein degradation pathway after oxidation of the N-terminal Cys via a mechanism that was unknown in 2011 (Sasidharan and Mustroph, 2011).

After the identification of group VII ERFs as oxygen-labile proteins due to their N-terminal Cys (after removal of the terminal Met residue), the question that arose was: is the mechanism of Cys oxidation occurring spontaneously in the presence of molecular oxygen or is it enzymatically driven? In 2009, while reviewing the various hypotheses regarding the possible existence of an oxygen-sensing mechanism in plants, Licausi and Perata (2009) revealed, through several microarray analyses of the hypoxic transcriptome, the existence of enzymes requiring oxygen for their activity that are up-regulated in the absence of oxygen. Of these, proteins with an unknown function in plants but with a high similarity to cysteamine oxidases (CDOs) in animals were indeed highly induced by hypoxia (Licausi and Perata, 2009). In Arabidopsis, these CDO-like proteins are At5g39890 and At5g15120 (Licausi and Perata, 2009). Interestingly, At5g39890 was identified as a gene whose transcription is very rapidly induced by hypoxia (within 20 min after exposure to hypoxia) (Loreti et al., 2005).

In 2014, Weits et al. discovered that At5g39890 and At5g15120 are indeed enzymes that use oxygen to oxidize the N-terminal Cys in ERF–VII proteins, leading to their destabilization under aerobic conditions. At5g39890 and At5g15120 were then named PLANT CYSTEINE OXIDASE1 and PLANT CYSTEINE OXIDASE2 (PCO1 and PCO2; Weits et al., 2014). Conversely, under hypoxia, ERF–VII proteins are stable because of PCO inactivity, and are able to activate the transcription of hypoxia–responsive genes (HRGs) by binding to the hypoxia–responsive promoter element (HRPE) present in most HRGs. The HRPE was identified as an evolutionarily conserved promoter element that is necessary and sufficient for hypoxic induction of genes through the ERF–VII2 (VRN2) is regulated by the O2– and NO-sensitive branch of the Arg/N-end rule pathway, thus keeping VRN2 low outside the meristem in the absence of cold or hypoxia (Gibbs et al., 2018; Weits et al., 2019).

Very importantly, it has been shown that VERNALIZATION 2 (VRN2) is regulated by the O2– and NO-sensitive branch of the Arg/N-end rule pathway, thus keeping VRN2 low outside the meristem in the absence of cold or hypoxia (Gibbs et al., 2018).

Notably, ERF–VII proteins also act as NO sensors (Gibbs et al., 2014), through a PCO-independent mechanism (White et al., 2017). Ethylene is a crucial gaseous hormone which, when it is entrapped between the submerged plant and the surrounding water, provides the plant with a specific submergence status signal and primes the plants for hypoxia responses. This occurs because ethylene depletes NO by increasing the NO-scavenger PHYTOGLOBIN1 (Hartman et al., 2019). This leads to enhanced ERF–VII stability prior to hypoxia, which pre-adapts plants for survival under subsequent hypoxia.

Oxygen sensing has recently been revealed to be integrated with several other signalling pathways, including energy sensing via TOR (Kunkowska et al., 2023) and calcium signalling (Fan et al., 2023). Both mechanisms act by activating ERF–VII through phosphorylation, adding a further layer of complexity to oxygen sensing and signalling in plants.

Are differences in oxygen sensing affecting a plant's tolerance to submergence? While it appears that the N-degron-based
Fig. 1. Oxygen sensing in plants. Under normoxia, the presence of oxygen activates PCOs, which utilize it to oxidize the N-terminal Cys residue of ERF-VII proteins (or that of other proteins with a Cys as the N-terminal residue, obviously after removal of the MET residue). Not all proteins showing a Cys residue at the N-terminal are substrates for this pathway. At present, besides ERF-VII, the proteins VRN2 and ZPR2 appear to be substrates for this pathway (see main text for references). ERF-VIIs with oxidized Cys are substrates for the N-degron pathway. Nitric oxide is also an important player in the pathway, although the exact site of action is yet to be identified. Under submergence, the lack of oxygen strongly reduces the activity of PCOs or even makes it impossible. As a consequence, ERF-VIIs are stable, migrate to the cell nucleus, where they bind to genes showing the HRPE element (see main text for the details and references), thus activating their transcription. The activation of hypoxia-responsive genes leads to the production of proteins and enzymes which contribute to adapting the plant’s metabolism to hypoxia and eventually leading to the plant’s tolerance to submergence.
mechanism for oxygen sensing based on PCO/ERF-VII is widespread in the plant kingdom, some evidence suggests that genetic differences in the genes involved in oxygen sensing might define the degree of tolerance to submergence. A recent article reported that in Arabidopsis thaliana, allelic changes in the cis-regulatory elements, in the promoter of the ERF-VII gene, RAP2.12, are responsible for differentially regulating tolerance to drought and flooding (Lou et al., 2022). This implies that there is room for crop improvement by exploring the genetic differences in the genes involved in oxygen sensing, although this might require the discovery of a possibly still unknown component of the pathway.

Core hypoxia gene discovery in Arabidopsis

The considerable progress that has been made in dissecting the molecular mechanisms behind the state of environmental hypoxia in plants, and described in the previous section, took advantage of previous studies of microarray datasets. An initial survey was carried out on Arabidopsis root culture at various time points after the transfer to low oxygen (Klock et al., 2002) which identified a group of differentially expressed genes. This included proteins that were previously suggested to be involved in anaerobic metabolism, together with transcription factors, signal transduction components, and new genes previously not known to be involved. Genes with an analogous expression profile were found to have similar sequence motifs. Accordingly, subsequent work on transcription profiling of the Arabidopsis response to hypoxia led to the identification of over-represented up-regulated genes with promoters of an AtMYB2-binding motif, a G-box-related sequence, and a sugar response element-like motif (Liu et al., 2005).

Subsequently, a transcriptome study on Arabidopsis plants in response to dark anoxia was published in 2005 (Loreti et al., 2005), and the anaerobic transcriptome was analysed both with and without the presence of external sugar. The results showed that, together with a cluster of anaerobic genes involved in sucrose metabolism and alcoholic fermentation, a further group of genes involved in various pathways were positively regulated (Loreti et al., 2005). The application of exogenous sugars showed a lower induction of SUSs and an up-regulation of heat shock protein (HSP) genes, which were subsequently hypothesized as being the result of the early activation of a reactive oxygen species (ROS)-related pathway (Banti et al., 2010; Pucciariello et al., 2012a, b). The reduction in SUS suggested the presence of a sugar starvation signal under anoxia. Anoxia was shown to induce HSPs with an overlap with heat shock and with a heat shock transcription factor, which helps promote anoxia tolerance (Banti et al., 2010).

In addition, the modulation of total RNA in parallel to RNA in large polysome complexes was analysed during anoxia (Branco-Price et al., 2005), revealing different translation responses to anoxia with a significant decrease that suggested possible energy conservation. This prompted a comprehensive analysis of Arabidopsis root responses to a time-course of different concentrations of oxygen (van Dongen et al., 2009), checking transcripts and conducting metabolite profiling. The results revealed that down-regulated genes were related to proteins involved in processes that are energy consuming, most probably in order to save ATP.

The responses of specific cell populations to anoxia were investigated using Arabidopsis lines and revealing the transcriptome (Mustroph et al., 2009). This led to the identification of a core of 49 mRNAs, including genes coding for enzymes involved in anaerobic metabolism but also in signalling (and some uncharacterized proteins) (Mustroph et al., 2009). Subsequently, a cross-kingdom comparison of transcriptomic adjustment under oxygen shortage was conducted considering results from different plants and other organisms (Mustroph et al., 2010) for a total of four kingdoms (Plantae, Animalia, Fungi, and Bacteria). The response was broadly conserved for orthologous genes in anaerobic metabolism, but also in metabolic transport, ROS amelioration, chaperone activity, and ribosome biogenesis. In contrast, a group of hypoxia-responsive unknown protein (HUP) genes were found to be plant specific. Subsequently, several HUPs have been found to be co-regulated by submergence and hypoxia, and mutants of these genes seem to be altered in response to submergence (Lee et al., 2011).

ROS play an important role in both oxidative damage and signalling during hypoxic stress (Blokhina and Fagerstedt, 2010). A bioinformatics approach at the whole-genome level for the analysis of large sets of global expression data revealed the up-regulation of some ROS-producing components and antioxidative defence components in Arabidopsis under anoxia and hypoxia (Blokhina et al., 2014). In addition, the physiological role of induced phytohaemoglobin has been investigated in a transcriptomic assay of Arabidopsis shoots and roots under hypoxia (raw data stored at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/projects/geo/, GSE72168). In brief, the work showed that co-induction of a few genes led to the incorporation of hypoxic metabolites pyruvate, Ala, and GABA shunt constituents glutamate and 2-oxoglutarate, in the TCA cycle under hypoxia.

Determining O₂ in flooded habitats and inside tissues

Due to the gas exchange constraints imposed by flooding, many studies have examined tissue O₂ status in partially or completely submerged plant tissues. A recent meta-analysis of >500 studies and encompassing 112 species showed that O₂ status ranged from effectively 0 kPa in roots to >50 kPa in submerged, photosynthesizing shoots (Herzog et al., 2023). When flooded, tissue O₂ status was particularly low in rhizomes,
potato tubers, and root nodules. Interestingly, light was significantly associated with flooding, showing a submergence-induced 44% increase in tissue \( \text{O}_2 \) in the light, compared with a 42% decline when growing in the dark. The latter demonstrates that submergence can be used as a proxy for hypoxia treatment only in complete darkness; when submerged in light, even terrestrial plants can attain hyperoxic conditions in the shoots (e.g. Pedersen et al., 2006; Rich et al., 2013).

At the onset of soil flooding, radial \( \text{O}_2 \) supply from the soil environment to root tissues ceases within days as the soil soon turns anoxic (Ponnamperuma, 1972). Nevertheless, the growing root tip requires molecular \( \text{O}_2 \) to support mitotic cell division of the apical root meristem (Armstrong and Webb, 1985). Instead, \( \text{O}_2 \) is supplied internally via longitudinal molecular diffusion via porous cortical tissues (Pedersen et al., 2021), and \( \text{O}_2 \) supply to the root tip is further facilitated by preventing \( \text{O}_2 \) loss to the soil via the outer apoplastic barriers (Peralta Ogorek et al., 2023).

Surprisingly, root \( \text{O}_2 \) status is not influenced by light or darkness as long as the root is exposed to air due to the effective \( \text{O}_2 \) exchange between the shoots and the atmosphere (Herzog et al., 2023). However, measurements using miniaturized Clark-type \( \text{O}_2 \) sensors (Revbsch, 1989) show that the stele operates at a significantly lower \( \text{O}_2 \) tension than the cortex, even in roots acclimated to soil flooding (Herzog et al., 2023). The difference in tissue \( \text{O}_2 \) status is due to (i) the higher \( \text{O}_2 \) consumption per tissue mass of the stele (Aguilar et al., 2003), and (ii) the lower diffusivity of the stelar tissues (Armstrong, 1979), both resulting in a lower \( \text{O}_2 \) status of the stele.

Partial or complete shoot submergence produces additional stress on terrestrial plants (Colmer and Voesenek, 2009), whereas aquatic plants possess a range of traits conferring flood tolerance, such as reduced or absent leaf cuticle and/or filamentous leaves both facilitating \( \text{O}_2 \) exchange with the surrounding water (Sculthorpe, 1967). However, superhydrophobic leaf cuticles of terrestrial plants retain a thin gas film upon submergence, and the leaf gas film greatly enhances \( \text{O}_2 \) exchange with the floodwater (Winkel et al., 2011, 2013).

In darkness and during submergence, the only source of \( \text{O}_2 \) is dissolved \( \text{O}_2 \) in the floodwater, and the leaf gas films facilitate the inward flux of \( \text{O}_2 \) via open stomata (Verbolen et al., 2014). \( \text{O}_2 \) is then subsequently distributed via porous tissues to the below-ground organs (Pedersen et al., 2009). In light, however, underwater photosynthesis is the main source of molecular \( \text{O}_2 \) supplied to below-ground tissues via the porous tissues (Pedersen et al., 2013).

In the above studies, molecular \( \text{O}_2 \) was determined using miniaturized Clark-type \( \text{O}_2 \) sensors made from glass capillaries or with \( \text{O}_2 \) optodes consisting of a glass fibre with fluorophores coated onto the tip (Pedersen et al., 2020) (Fig. 2). Even the smallest Clark-type \( \text{O}_2 \) sensor with a tip diameter of only 3 \( \mu \text{m} \) is not suitable for obtaining measurements of cellular \( \text{O}_2 \), but it is an excellent tool for obtaining the \( \text{O}_2 \) status of the SAM of Arabidopsis seedlings of only a few days old (Weits et al., 2019). It is often claimed that Clark-type sensors should be avoided due to their \( \text{O}_2 \) consumption; however, the consumption is very low and it would take 36 years to consume the amount of \( \text{O}_2 \) dissolved in 1 ml of seawater at 20 °C (Gundersen et al., 1998). Nevertheless, glass fibre-based \( \text{O}_2 \) optodes do not consume \( \text{O}_2 \) at all, but the tiny tapered glass fibre needed to construct a sensing tip of <10 \( \mu \text{m} \) is extremely flexible and therefore not suitable for penetrating plant tissues (Pedersen et al., 2020).

There are now sensors that can be used for cellular \( \text{O}_2 \) measurements. These sensors consist of indolequinone, and, depending on the different \( \text{pKa} \) of the dye (DDAO, resorufin, or Me-Tokyo green) and substitution on the methylene group of the indolequinone compounds, the sensors are activated at \( \text{O}_2 \) concentrations ranging from ≤6, ≤4, ≤2, to ≤1 kPa (Wallabregue et al., 2023). Although the indolequinone molecule is relatively large, it is readily taken up by liver cells, and hypoxia has been observed using confocal microscopy. Unfortunately, the reaction is irreversible and once the molecule has been reduced, it does not revert back to its oxidized state even if \( \text{O}_2 \) is re-introduced. However, the technique is a significant step towards subcellular \( \text{O}_2 \) measurements, and genetically encoded oxygen biosensors can be a very useful tool for measurements in plant tissues. The technique has also been optimized with a hypoxia signalling reporter, the five-times repeated HRPE driving the expression of different reporter proteins (Panicucci et al., 2020). Similarly, fluorescent protein-based systems have been used in Arabidopsis to simultaneously monitor many intracellular parameters in leaves under hypoxia (Wagner et al., 2019).

**Morphological and anatomical means of escaping low oxygen conditions**

Various morphological and anatomical modifications offer plants an escape from hypoxic or anoxic environments, which is referred to as the LOES (Bailey-Serres and Voesenek, 2008). It is well known that aerenchyma greatly enhances the diffusion of gases to rhizomes and roots, but to what distances? Diffusion alone cannot supply enough oxygen for root cell respiration at any great distance; for example, in peas only 7–8 cm of the roots can be supplied based on diffusion (Armstrong et al., 1983). In larger plants, a convective flow can take place through humidity-induced pressurization (Armstrong et al., 1996a), thermal osmosis (Grosse, 1996), and Venturi-induced convection (Armstrong et al., 1996b). The Venturi-induced convection is explained in stems by the reduction in gas pressure and increased speed that results when gas flows through a constricted section, such as capillaries in aerenchyma.

To assess the variety of changes in root anatomical features across wetland, non-wetland, and intermediate plants, a total of 91 plant species were studied by Justin and Armstrong (1987).
in control and flooded experimental setups. The variation was large, but the general conclusion was that aerenchyma development greatly enhanced fractional root porosity and enabled root penetration into the flooded soil.

Aerenchyma development can be either constitutive or induced (Visser et al., 1997; Yamauchi et al., 2019). Its development is vital for O₂ and CO₂ gas exchange under waterlogged conditions. The diffusion of O₂ in gases (2.1 × 10⁻⁵ cm² s⁻¹) is 10 000 times faster than diffusion in water (0.2 cm² s⁻¹) (Weast, 1989), which permits a reasonably straight diffusion pathway, and prevents the loss of O₂ along the way, especially if the radial oxygen loss barrier (ROL) is formed (Colmer et al., 1998; Colmer, 2003). As oxygen demand of the root tip quiescent centre cells is low, they are presumably the last cells to die under oxygen deprivation.

Aerenchyma can develop in three ways: lysigenously, where cells die through programmed cell death and large cavities remain in their place; schizogenously, where intercellular spaces enlarge through the splitting of the cell wall middle lamellae; or expansigenously—through cell division and enlargement (Seago et al., 2005). Increases in pectinase and xylanase activity (Branco-Price et al., 2005) as well as in xyloglucan endo-transglucosylase (XET) (Saab and Sacks, 1996) take place under oxygen deprivation, and these may participate in the formation of schizogenous aerenchyma.

Although there are data on the regulation and formation of lysigenous aerenchyma where ethylene, ROS signalling, protein kinases and phosphatases, G-proteins, Ca²⁺, and inositol phospholipids play an important role (He et al., 1996; Steffens et al., 2013; Yamauchi et al., 2016, 2018; Mustroph, 2018),
regulation of the constitutive aerenchyma formation is still not fully understood. Auxin and auxin response factors (ARFs) seem to play an important role in constitutive aerenchyma formation (Yamauchi et al., 2019). The latter authors found that in rice, iaa13 mutant constitutive aerenchyma formation was lower than in the wild type. This was confirmed in indole-3-acetic acid (IAA) and auxin transport inhibitor experiments.

A study on the IAA13 interactor showed that ARF19 had the highest signal activity and, furthermore, a similar transcript accumulation pattern in the tissues to IAA13. To identify genes regulated by AUX/IAA-mediated signalling, a further study by the same authors revealed that two LBD (LATERAL ORGAN BOUNDARIES) genes LBD1-8 and LBD5-3 are transcriptionally regulated through IAA13- and ARF19-dependent signalling in the cortex, while LBD5-3 also plays a role in the formation of lateral roots (Yamauchi et al., 2019). Yamauchi et al. (2017) also showed that ROS-producing RBOH1 activation is stimulated by kinases and is vital for the formation of aerenchyma in rice roots under hypoxia. With maize, information on QTLs for constitutive aerenchyma formation in Zea mianagumensis has been used in producing introgression lines in cultivated maize and this has led to increased root tolerance to low oxygen conditions (Gong et al., 2019).

Certain morphological features, in addition to anatomical means such as aerenchyma, can help plants to obtain sufficient oxygen. Hydrophobic hairs in the leaf sheaths of rice plants seem to contribute to the oxygen supply (Kurokawa et al., 2018). Surprisingly, it was found that a single gene, Leaf Gas Film 1 (LGF1), is required for determining leaf gas films. LGF1 regulates C30 primary alcohol synthesis, which is vital for the formation of epicuticular wax platelets, resulting in leaf hydrophobicity and gas films on submerged leaves.

Interestingly, related plant species can have very different strategies for survival under flooded conditions, depending on the characteristics of their ecological niches. Certain Rumex species, R. palustris and R. acetosa, use different strategies for survival. While in R. acetosa petiole growth is suppressed under flooding, in R. palustris petiole growth is greatly enhanced and leaves reach out into the air (van Veen et al., 2013). At the same time, both species reconfigure their primary metabolic pathways to overcome the stress. Regulation of the petiole elongation involves ethylene and ABA (Benschop et al., 2005), GA (Benschop et al., 2006), expansins, and xyloglucan endotransglucosidases and hydrolases (Vriezen et al., 2000).

Under compound stresses, such as those in salt marshes, plants adapt by exploiting a large fine root biomass (Redelstein et al., 2018), and even in these harsh conditions root competition promotes plant species distribution (Levine et al., 1998).

**Future research and practical applications**

Due to climate change, plant life is suffering from drought and elevated temperatures, and the increased variations in precipitation will have a large impact in the near future (Wing et al., 2022). Plants may be subjected to severe flooding in some seasons, followed by long droughts. As proposed in an ISPLOR conference in Bamberg, Germany (2022), research should focus on the impact on agricultural crops of increased flooding or drought tolerance not only separately, but simultaneously. In the following section we thus focus on the simultaneous breeding of many stress tolerance mechanisms which stem from large-scale or holistic information on genetics and genomics (Fig. 3).

**Examples of large-scale changes achieved in plant functions through a holistic understanding of developmental events and their regulation**

As genetic and genomic knowledge increases, there is increasing information on the systemic tolerance of stress on all levels: genetic, metabolic, and anatomical/morphological. How to solve the regulation of whole metabolic pathways and the development of anatomical features has long been an issue, and now we have increasing information on the regulation of the development of plant structures. One good example is the work by Niko Geldner in Switzerland on the development and functioning of the Casparian strip in the endodermis of roots (Andersen et al., 2015; Doblas et al., 2017). The root endodermis is the innermost cortical cell layer surrounding the vasculature. The endodermal cell walls contain Casparian strips, which are ring-like hydrophobic cell wall impregnations (lignin and suberin) that are involved in the control of transport from the cortex into the xylem elements. In a series of publications, the authors described the endodermal differentiation and molecular markers for the membrane domains vital for the development of the Casparian strip (Alasimone et al., 2010; Roppolo et al., 2014). This work is an excellent example of where a holistic view led to the discovery of an intricate system with a functionally important anatomical structure. The development of the endodermis and Casparian strip may provide clues as to how the ROL barrier is formed in the root hypodermis. The development of the ROL has been described as an adaptive mechanism in rice roots under stagnant flooded conditions (Peralta Ogorek et al., 2023).

Another example of recent advances is in the study of the regulation of cambial activity and the differentiation between xylem and phloem elements in roots by Ari Pekka Mähönen and his group in Finland (Smetana et al., 2019; Zhang et al., 2019; Wang et al., 2020; Ye et al., 2021). With the ingenious use of inducible fluorescent probes, these authors disentangled the hormonal regulation of cambial divisions and the subsequent differentiation between phloem and xylem elements in the roots. These methods could be used to reveal the regulation of the development of aerenchyma in roots and rhizomes, and thus enable us to be one step ahead in the breeding of flooding-tolerant crop plant species (Fig. 3).

The third example is the large international effort in the engineering of nitrogen-fixing nodules in non-nodulating plants...
(for a review, see Pankievicz et al., 2019). These authors are unravelling the complex interactions between nitrogen-fixing bacteria and the plant root, and are exploiting this information in transgenic attempts to create functional root nodules in non-nodulating crop plant species. A similar approach would be beneficial in the study of aerenchyma development.

Our fourth example is Donald Ort’s work in Illinois on the regulation of photorespiration in C₃ plants (South et al., 2018; Cavanagh et al., 2021). With the transformation of only a few genes encoding chloroplast enzymes, they managed to create C₃ plants with greatly diminished photorespiration and with dramatic increases in biomass production.

These kinds of studies show that through directed breeding programmes and/or genetic transformation, in the future we will be able to regulate the carbohydrate consumption in root and rhizome tissue so that reserves can be optimized under flooded conditions, and exhaustion can be restricted.

Another desirable feature would be the regulation of aerenchyma formation in agricultural crop plant species in the case of flooding, and, on the other hand, the development of water-saving features in sudden periods of drought. A holistic approach is suggested for improving root anatomy in a review by Yamauchi et al. (2021). They have used all the available information on root anatomical traits for modelling and automated analyses to be used to improve crop tolerance to soil flooding. The precise engineering of root features requires detailed knowledge, and now we have all the tools for altering and/or adding regulatory mechanisms for metabolic/anatomical/morphological development in many agricultural crop species. Such work requires a panomics approach to improve waterlogging tolerance in plants, as advocated in a review by Tyagi et al., 2023. As the ISPLORE community has researchers in basic research in molecular as well as ecological sciences and crop breeders, using the various methods stated above including modelling (Giraud et al., 2023), and combining the results with crop breeding activity, we are in an advantageous position in creating better performing crop cultivars.

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**Conflict of interest**

The authors have no conflicts to declare.
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