Review

Making sense of low oxygen sensing

Julia Bailey-Serres[1], Takeshi Fukao[1], Daniel J. Gibbs[2], Michael J. Holdsworth[2], Seung Cho Lee[1], Francesco Licausi[3,4], Pierdomenico Perata[4], Laurentius A.C.J. Voesenek[5,6] and Joost T. van Dongen[3]

[1] Center for Plant Cell Biology, Department of Botany and Plant Sciences, University of California, Riverside, CA 92521-0124, USA
[2] Division of Plant and Crop Sciences, School of Biosciences, University of Nottingham, Loughborough LE12 5RD, UK
[3] Max Planck Institute of Molecular Plant Physiology, Am Muehlenberg 1, 14476 Potsdam-Golm, Germany
[4] PlantLab, Institute of Life Sciences, Scuola Superiore Sant'Anna, Piazza Martiri della Libertà 33, 56127 Pisa, Italy
[5] Plant Ecophysiology, Institute of Environmental Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, the Netherlands
[6] Center for Biosystems Genomics, 6708 PB Wageningen, the Netherlands

Plant-specific group VII Ethylene Response Factor (ERF) transcription factors have emerged as pivotal regulators of flooding and low oxygen responses. In rice (*Oryza sativa*), these proteins regulate contrasting strategies of flooding survival. Recent studies on *Arabidopsis thaliana* group VII ERFs show they are stabilized under hypoxia but destabilized under oxygen-replete conditions via the N-end rule pathway of targeted proteolysis. Oxygen-dependent sequestration at the plasma membrane maintains at least one of these proteins, RAP2.12, under normoxia. Remarkably, SUB1A, the rice group VII ERF that enables prolonged submergence tolerance, appears to evade oxygen-regulated N-end rule degradation. We propose that the turnover of group VII ERFs is of ecological relevance in wetland species and might be manipulated to improve flood tolerance of crops.

Improved crop survival of floods is needed

Based on conservative expectations of human population growth, the maintenance of international food security will require a doubling of agricultural productivity in the next two decades [1]. This challenge is exacerbated by severe weather events associated with climate change such as floods, which have occurred with increasing frequency across the globe over the past six decades (Figure 1). However, improvement of crop resilience to water extremes can be accomplished by harnessing natural genetic diversity in breeding programs. An example of this is the use of the rice *SUBMERGENCE 1A* (*SUB1A*) gene, which confers prolonged tolerance to submergence (see Glossary) [2]. The effective *SUB1A-1* allele was isolated from an eastern Indian landrace and has been returned to farmers in high-yielding varieties [3]. This new 'Sub1 rice' promises to help stabilize harvests in rainfed floodplains, which represent 33% of rice acreage worldwide [4]. The task remains to improve flooding tolerance of other crops. Recent comparative studies within and between species have greatly enhanced our understanding of mechanisms that facilitate survival of distinct flooding regimes (Table 1). With new insights into low oxygen sensing and response mechanisms we are optimistic that effective means to lessen crop devastation by flooding can be extended beyond rice paddy fields.

Corresponding author: Bailey-Serres, J. (serres@ucr.edu)

Oxygen deprivation is a frequent component of flooding stress

A key feature of flooding events is the change in levels of three gases, O_2, CO_2 and ethylene, due to a near 10^4 reduction in their diffusion in water relative to air [5–8]. The flooding of root systems – a condition termed waterlogging – has little or no impact in semi-aquatic species such as rice that constitutively form gas conduits (i.e. aerenchyma) between submerged and aerial organs. However, if plants lack gas conduits or lose oxygen from roots, waterlogging rapidly reduces the oxygen concentration within cells [9–11]. The presence of aerobic microbes in the soil can further exacerbate the stress. When both the root and aerial portions of a plant are whelmed by water – a condition termed submergence – cellular oxygen levels can also decline from normoxia. The degree of oxygen deficiency (hypoxia/anoxia) depends on multiple factors including replenishment of oxygen through photosynthesis, inward diffusion from the water layer and cellular consumption of oxygen through metabolic activity. Severe oxygen deficiency compromises mitochondrial respiration [10,12] and leads to an insufficiency in ATP for energy demanding processes [6,13–15]. However, plants can adjust to this energy crisis through increased substrate level ATP production (Figure 2). This is accomplished by catabolism of soluble sugars and in some species or cell types starch [16]. Typically, the increase in glycolytic flux is coupled with regeneration of NAD^+ by fermentation of pyruvate to

Glossary

Anoxia: absence of oxygen.
Direct oxygen sensing: sensing of oxygen via its molecular interaction with a ligand (i.e. enzyme, protein, chemical compound) that results in an effect of cellular consequence.
Hypoxia: oxygen levels below normoxia; the term 'hypoxic' is often used to describe a situation where molecular oxygen is still present, but its level has significantly decreased below 20.6%; cellular oxygen status may be hypoxic or anoxic dependent upon duration, location and metabolic activity.
Hypoxia-responsive genes: genes with transcripts differentially regulated in response to conditions with a low oxygen component.
Indirect oxygen sensing: sensing of change in homeostasis that is a consequence of oxygen deprivation (i.e. change in ATP, ADP, AMP, other metabolite, Ca^{2+}, ROS, pH) that results in an effect of cellular consequence.
Normoxia: typically 20.6% oxygen at 1 atm and 20 °C.
Submergence: waterlogging and partial to complete immersion of aerial system.
Waterlogging: flooding of root system.

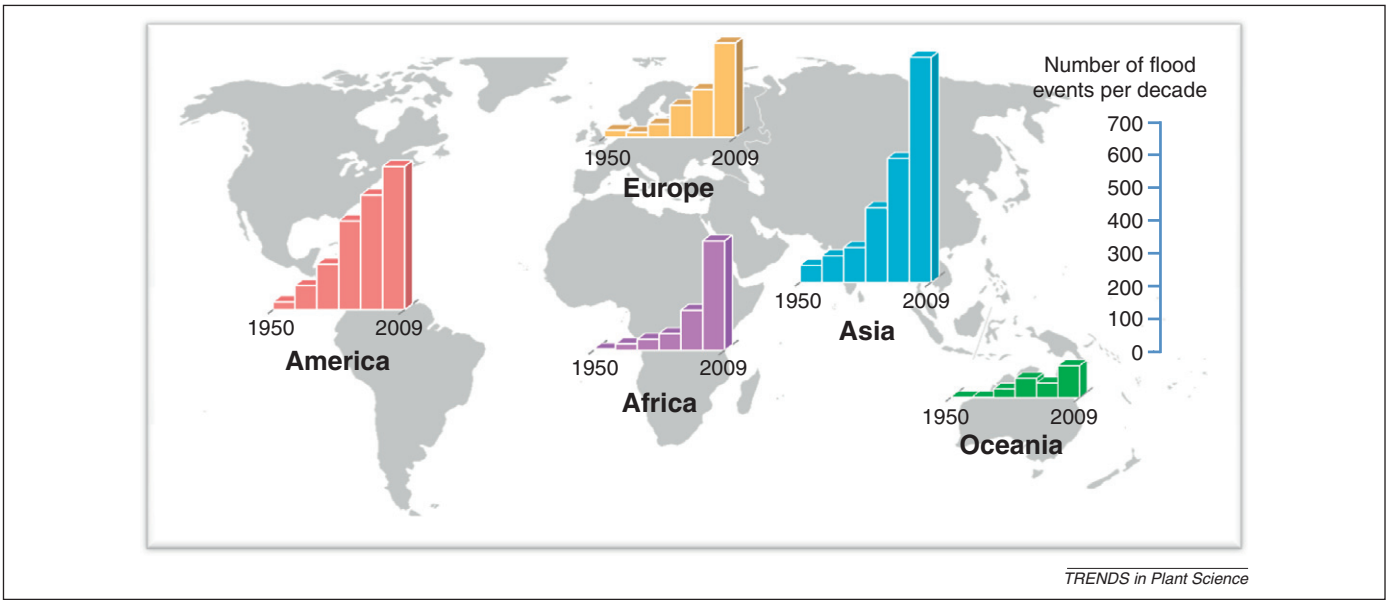

Figure 1. Numbers of floods have increased in each of the past six decades across the globe. Graphs show the number of floods classified as a disaster in the International Disaster Database of the University of Louvain, Belgium for the period from 1950 through 2009 by geographical region [93]. Events include river or coastal floods, rapid snow melts, heavy rainfall and other occurrences that caused significant social or economic hardship. Adapted from a Millennium Ecosystem Assessment map (http://maps.grida.no/go/graphic/number-of-flood-events-by-continent-and-decade-since-1950).

ethanol via pyruvate decarboxylase and alcohol dehydrogenase (ADH). Because ethanol diffuses out of cells into the external milieu, its production depletes the plant's carbon reserves. Therefore, metabolism of pyruvate to alanine provides an alternative, non-detrimental end product of anaerobic metabolism that is observed in a number of species [17,18]. This includes the generation of 2-oxoglutarate as a coproduct, which can be further metabolized to succinate, via the TCA cycle enzyme succinate CoA ligase (SCS), thereby providing additional ATP per molecule of sucrose metabolized. To keep these reactions running, the oxidation of NADH in the mitochondrial matrix is guaranteed by reduction of oxaloacetate via the reversed TCA cycle reaction catalyzed by malate dehydrogenase [19,20]. The malate produced is probably further converted to fumarate and succinate [21], the latter of which could be exported from hypoxic tissue to the aerated parts of the plant. At least in tubers of potato (*Solanum tuberosum*), hypoxia stimulates a rearrangement of the mitochondrial respiratory supercomplexes that enhances regeneration of NAD^+ by the alternative NAD(P)H dehydrogenases [22].

Even though the efficiency of hypoxic ATP production is low compared to aerobic oxidative phosphorylation, it allows cells to survive as long as carbohydrate substrate remains available. Cell death only becomes inevitable when there is insufficient energy for exclusion of protons to the apoplast to prevent membrane depolarization and to maintain a near neutral cytosolic pH [6,23,24]. Avoidance of the severe energy crisis associated with low oxygen stress requires economization of ATP consumption. Means to this end include energy efficient sucrose catabolism through sucrose synthase [25], the preferential use of PPi-dependent enzymes [26], constrained catabolism of storage compounds such as starch, lipid and protein [13], metabolic compartmentalization [27], reduced protein synthesis [28], increased production of heat shock proteins as molecular chaperones [29] and adoption of the K^+-gradient to energize membrane transport [30]. Plant survival of waterlogging or submergence also depends on their ability to limit or endure oxidative stress, which occurs during the transition from normoxia to anoxia as well as upon de-submergence [31–33].

Ethylene initiates submergence survival strategies in rice and wetland species

Recent work has exposed mechanisms of response to submergence that center on growth management. Notable are two antithetical survival strategies displayed by both wild and domesticated species. For example, deepwater rice, cultivated to cope with slowly advancing floods, expends energy reserves in the elongation of internodal regions that are underwater to maintain photosynthetic tissue above the air–water interface [34,35]. Similarly, the wetland dicot *Rumex palustris*, which is well adapted to shallow but prolonged floods, reorients and extends petioles to elevate leaves above the surface of floodwaters [6,7]. However, this 'submergence escape' strategy is unsuccessful if energy reserves are exhausted before escape of the deluge. In wetland species capable of surviving transient floods (e.g. *Rumex acetosa*) [36] and submergence tolerant Sub1 rice [37], a 'quiescence strategy' minimizes energy expenditures for growth until de-submergence.

The genetic determinants and hormonal signaling pathways that underlie the two flooding survival strategies have been identified. In rice, both strategies utilize the phytohormone ethylene and ethylene response factor (ERF) transcription factors. Combined physiological and molecular dissection of submergence responses in rice and *R. palustris* has yielded a model in which a buildup of ethylene in submerged organs initiates a hormonal signaling cascade that reduces the antagonism between abscisic

Table 1. Factors that contribute to survival of flooding or oxygen deprivation

Condition	Species	Comparison	Acclimation or survival response	Causal factors	Refs
Submergence (partial)	Rice (*Oryza sativa* ssp. *indica*)	Deepwater to non-deepwater cultivars; *SK1* and *SK2* transgenics	Rapid underwater internode elongation; escape strategy	*SK1*, *SK2*, ethylene, GA, ABA	[34]
Submergence (complete)	Rice (ssp. *indica, aus* and *japonica*)	Near isogenic lines; *SUB1A-1* transgenics	Growth restriction; quiescence strategy	*SUB1A-1*, ethylene, reduced GA responsiveness	[2,37,73]
Submergence (seed)	Rice (ssp. *indica*)	Anaerobic germination of tolerant to non-tolerant cultivars; *cipk15* mutant	Enhanced coleoptile and shoot elongation	Quantitative trait loci; enhanced starch degradation; CIPK15	[74,75]
Submergence (under anoxia)	Rice (ssp. *indica*)	To wheat	Seed germination and coleoptile elongation	Adjustment of metabolism; reduced oxygen consumption	[76]
	Wheat (*Triticum aestivum*)	To rice	No germination and coleoptile elongation inhibited	Limited adjustment of metabolism and oxygen consumption	[76]
Submergence	Marsh dock (*Rumex palustris*)	Ecotypes with fast and slow underwater elongation responses	Petiole elongation	Fast elongation associated with lower endogenous ABA	[38]
	Common sorrel (*Rumex acetosa*)	To *Rumex palustris*	Limited petiole elongation	Maintenance of ABA under submergence	[36]
	Arabidopsis thaliana	86 accessions	Varied survivability	Unknown	[77]
	Meionectes brownii	Variation in light	Photosynthetic aquatic adventitious roots	Reduced need for shoot photosynthate	[78]
Anoxia	Rice	To wheat	Sucrose or glucose-fed wheat seeds survive longer	α-Amylase produced under anoxia in rice but not in wheat seedlings	[79]
	Chlamydomonas reinhardtii	Wild type to mutant	Transcriptomic and metabolic adjustments	Versatile metabolic adjustments such as H_2 production	[80–82]
	Pondweed (*Potamogeton distinctus)*	To pea (*Pisum sativum*)	Turion elongation and survival	Enhanced H^+ extrusion and stabilization of cytosolic pH	[83]
	Grape (*Vitis* sp.)	Anoxia tolerant (*Vitis riparia*) to intolerant (*Vitis rupestris*)	Improved survival of hypoxia pretreated roots	Fermentation and maintenance of ion homeostasis (e.g. K^+)	[84]
Hypoxia to anoxia	*Arabidopsis thaliana*	Wild type to loss-of-function or other insertion mutants and overexpression transgenics	Low oxygen and/or submergence survival	*HRE1*, *HRE2*, *RAP2.2*, *RAP2.12*; *VERNALIZATION INSENSITIVE 3*; *EXORDIUM 1*; *HEAT SHOCK FACTOR 2a*; *HYPOXIA-RESPONSIVE UNKNOWN PROTEIN* (*HUP*) genes	[29,41, 47,62,63,65, 66,72,85,86]
		Wild type to loss-of-function *prt6* and *ate1ate2* mutants	Low oxygen and/or submergence survival; seed germination under hypoxia	N-end rule pathway components PRT6, ATE1, ATE2	[66,72]
		Wild type to loss-of-function mutants and overexpression transgenics	Seed germination under 0.1% oxygen	NAC transcription factor ANAC102	[87]
Waterlogging	*Lotus japonicus*	Wild type to N-deficient nodular *leghemoglobin* RNAi transgenics	Alanine and succinate accumulation	Modified TCA flux mode	[19]
	Poplar (*Populus* × *canescens*)	Root to shoot	Transcriptomic and metabolic adjustments; limited shoot response	Unknown	[88]
	Cotton (*Gossypium hirsutum*)	Root to shoot	Transcriptomic and metabolic adjustments; shoot growth inhibition	Unknown	[89]
	Maize (*Zea mays*)	Root cell type mRNAs	Aerenchyma formation	Ethylene, Ca^{2+}, ROS; cortex mRNAs	[90]
	Rice	Response to compounds	Adventitious root development	Epidermal cell death mediated by ethylene and ROS	[91]
	Tomato (*Solanum lycopersicum*)	Response to hormone biosynthesis inhibitors	Adventitious root development	Ethylene and auxin	[92]

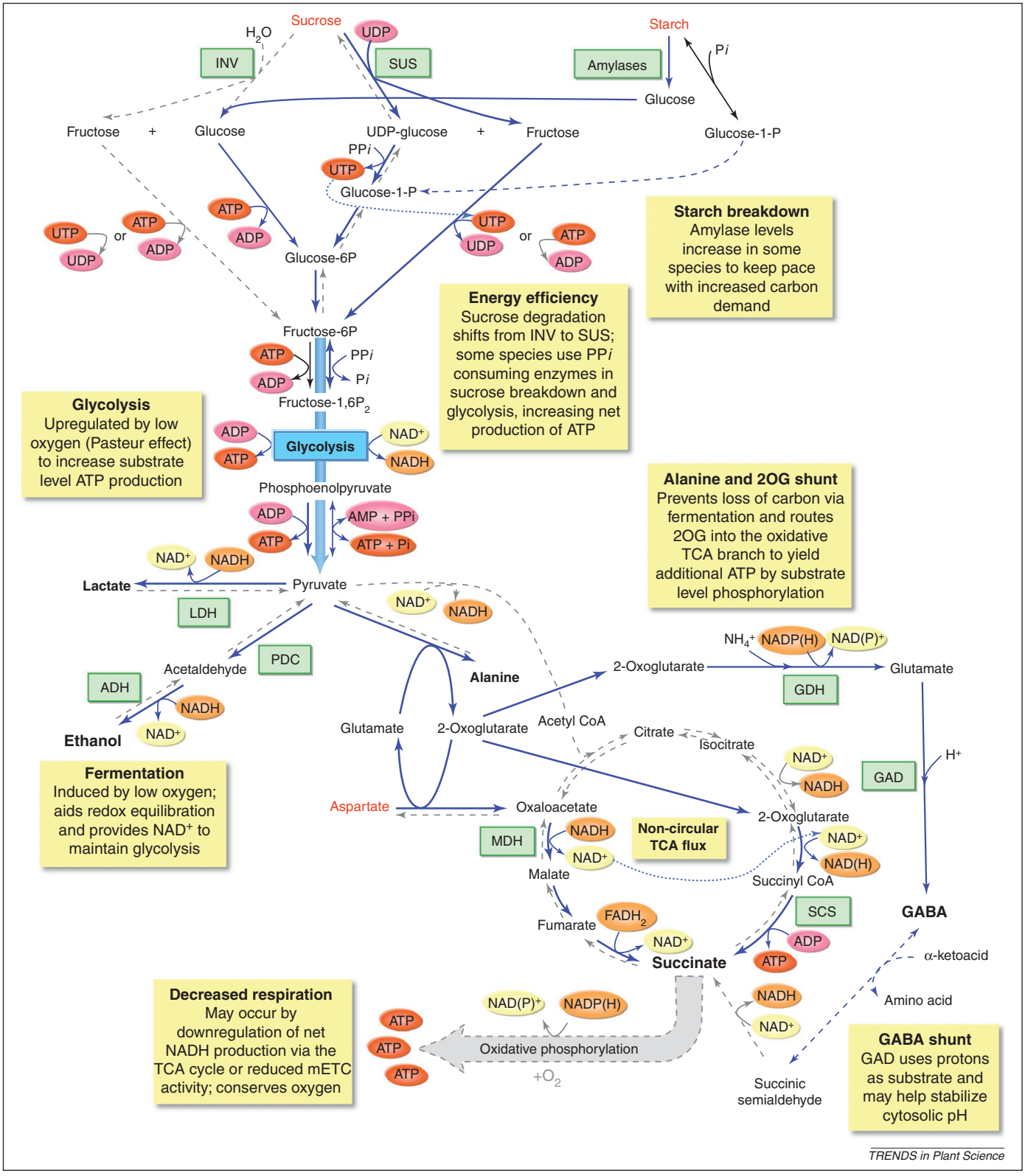

Figure 2. Metabolic reconfiguration under low oxygen stress. Reduced oxygen availability alters metabolism to maximize substrate level ATP production. The model depicts the major known changes that include enhanced sucrose–starch metabolism, glycolysis, fermentation, a modified tricarboxylic acid (TCA) flow, an alanine and 2-oxoglutarate (2OG) shunt and a γ-aminobutyric acid (GABA) shunt. The hypothesis that oxygen is conserved is under further investigation. Yellow boxes summarize notable metabolic adjustments. Blue lines indicate pathways enhanced during the stress, blue dashed lines indicate pathways proposed to be active during the stress and gray dashed lines indicate reactions that are inhibited during the stress. Metabolites that increase during the stress are shown in enlarged black font; metabolites that decrease are shown in red font. Abbreviations are as follows: 2OG, 2-oxoglutarate; ADH, alcohol dehydrogenase; GAD, glutamic acid decarboxylase; GDH, glutamate dehydrogenase, INV, invertase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; PDC, pyruvate decarboxylase; SCS, succinyl CoA ligase; SUS, sucrose synthase.

acid (ABA) and gibberellins (GA), which normally limits cell elongation. In rice, natural variation in the presence and absence of the underwater escape [*SNORKEL (SK) 1* and *2*] and submergence tolerance (*SUB1A*) group VII ERF determinants underlies differential regulation of the hormonal cascade and hormone sensitivities that control underwater growth [8] (Box 1). Two *R. palustris* populations distinguished by fast and slow underwater petiole elongation were differentiated by the maintenance of higher levels of ABA and reduced GA responsiveness in the slow elongating variety during submergence [38]. Consistently, the limited underwater petiole growth and prolonged submergence survival of *R. acetosa* was linked to maintenance of ABA biosynthesis that translated into lower GA responsiveness during submergence [36].

Photosynthesis can continue in submerged leaves and is aided by the gas film that often clings to their surface [39,40]. It follows that the degree of oxygen deprivation in photosynthetic tissue may be less extreme than tissues distant from an oxygen source. Nevertheless, the ethylene-driven underwater elongation of shoot tissue can deplete carbohydrates and lead to an energy crisis.

Similarities in transcriptome response to flooding and oxygen deprivation

Numerous investigations have assessed changes in transcriptomes in response to low oxygen stress or flooding in plants including *Arabidopsis*, rice, poplar (*Populus* × *canescens*) and cotton (*Gossypium hirsutum*) [41,42]. Studies performed on seedlings of the *Arabidopsis* Col-0 ecotype include evaluation of the effects of different severity [43] and duration [28,44] of oxygen depletion, as well as the impact of heat stress prior to anoxia [29]. Because the majority of cellular mRNAs are poorly translated during oxygen deprivation [45], changes in polyribosome-associated mRNAs were used to evaluate dynamics in the stress and recovery responses [28]. In 21 cell types or regions of roots and shoots, polyribosomes were captured by immunopurification to identify transcripts regulated by short-term oxygen deprivation [46]. This approach identified 49 core hypoxia-responsive genes that were strongly induced by the stress across all samples evaluated. Also distinguished were cohorts of mRNAs that were hypoxia-responsive at the organ or cell-specific level, although their modulation was less pronounced than the core

Box 1. Contrasting submergence survival strategies of rice

Most accessions respond to submergence through rapid shoot elongation, which allows emergence from a shallow flood [6]. A limited number of accessions display the ability to survive a slow progressive flood (escape response) or a deep transient flash flood (quiescence response) (Figure I). **(a)** By amplifying the elongation of stem internodes, deepwater rice can outgrow a progressive flood and survive partial inundation for months. This deepwater escape strategy is controlled by the *SNORKEL* (*SK*) locus, which encodes two group VII ERFs, *SK1* and *SK2* [34]. *SKs* are absent from lowland varieties. **(b)** The molecular genetic analysis of the submergence-tolerant accession FR13A revealed that the *SUBMERGENCE 1* (*SUB1*) locus, encoding two or three group VII ERFs, regulates the quiescence response. *SUB1B* and *SUB1C* are invariably encoded at *SUB1* in lowland accessions, whereas *SUB1A* is limited to some *indica* and *aus* landraces [2,60]. The *SUB1A-1* allele is sufficient to confer survival of 2 weeks or longer of complete submergence. **(c)** Model of the core submergence response network that is influenced by *SKs* and *SUB1A*. Genotypes possess either *SK1/SK2*, *SUB1A* or neither. Both *SK1/SK2* and *SUB1A-1* mRNA are ethylene induced. In deepwater rice, *SK1/SK2* and two minor QTLs augment accumulation of bioactive GA in stem internodes during submergence. In submergence tolerant rice varieties, the presence of *SUB1A-1* influences submergence and post-submergence responses in aerial tissue. **(1)** *SUB1A-1* mRNA is ethylene-induced but ultimately limits ethylene biosynthesis [37]. **(2)** *SUB1A-1* promotes accumulation of two negative regulators of GA responses [SLENDER RICE 1 (SLR1) and SLENDER RICE-LIKE 1 (SLRL1)] [73]. **(3)** *SUB1A-1* does not perturb the submergence-induced decline in ABA content but heightens sensitivity to ABA [32]. **(4)** *SUB1A-1* limits induction of genes associated with starch breakdown [37,41,94]. **(5)** *SUB1A-1* enhances upregulation of genes associated with reactive oxygen species (ROS) amelioration and survival of dehydration, thereby improving re-establishment following de-submergence [32]. **(6)** SUB1A-1 interacts with a complex network of proteins [95]. **(7)** *SUB1A-1* transiently restricts the progression to flowering during submergence [96]. In summary, *SUB1A* is remarkably positioned to suspend growth and maintain cell viability during submergence and restore homeostasis during a subsequent recovery period.

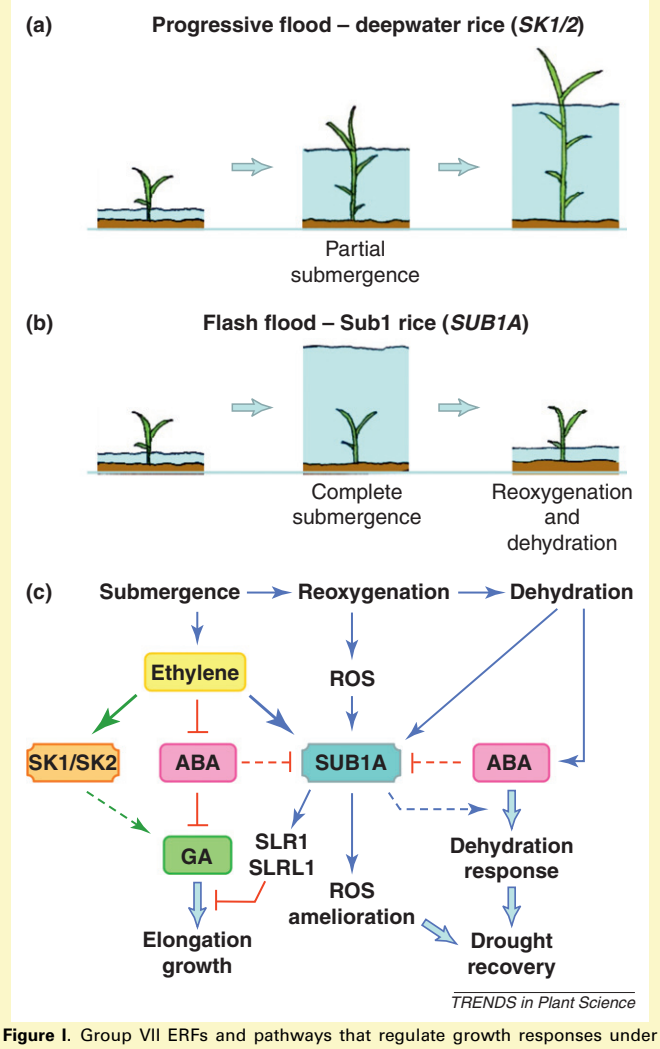

Figure I. Group VII ERFs and pathways that regulate growth responses under distinct flooding regimes.

hypoxia-responsive genes. Of the 49 core hypoxia-responsive genes, 24 were also differentially regulated in roots and rosette leaves during submergence in complete darkness [47]. Finally, meta-analyses that compared transcriptomic adjustments to low oxygen or flooding stress identified conservation in the core network of genes associated with signaling, transcription and efficient anaerobic ATP production that is modulated by oxygen deprivation in a range of plants [41,42].

How do plant cells sense low oxygen stress?

Based on mechanisms in other eukaryotes, both indirect and direct sensing of cellular oxygen status could be responsible for acclimation responses that prolong survival of oxygen deprivation in plants [48]. Indirect sensing mechanisms might include perception of altered energy status through changes in levels of adenylates (ATP, ADP and/or AMP), consumable carbohydrates, pyruvate, cytosolic pH, cytosolic Ca^{2+} or localized production of reactive oxygen species (ROS) and nitric oxide (NO).

Animal and yeast cells sense and adjust energy homeostasis through Sucrose Non-Fermenting 1 (SNF1)/AMP-activated protein kinases [49,50]. The plant energy sensors fall within one clade of SNF1 relatives, the SnRK1s, some of which have been implicated in low oxygen responses. For example, *Arabidopsis* KIN10 and KIN11 are necessary to limit energy consumption during hypoxia [51]. Whereas in rice seeds germinated under oxygen starvation, the depletion of sucrose activates the SnRK1A energy sensor through the activity of a Calcineurin B-like interacting binding kinase 15 (CIPK15) [52]. This signal transduction upregulates transcription of genes encoding α-amylases, which drive catabolism of starch in the seed needed to fuel underwater shoot growth. Logically, a reduction of energy consumption is beneficial when ATP levels decline. A means of energy conservation during low oxygen stress in plants is selective translation and sequestration of mRNAs during hypoxia [28]. Based on evidence from other eukaryotes, the sequestration of a subset of cellular mRNAs, such as the abundant cohort that encodes ribosomal proteins and translation factors, could be regulated through SnRK1s and the Target of Rapamycin kinase [53]. Mitochondria are also thought to contribute to oxygen sensing and signaling in plants, through production of NO and/or release of ROS and Ca^{2+} during the transition from normoxia to hypoxia [54,55], as confirmed in animals [56,57].

In animals, direct oxygen sensing regulates the accumulation of the α subunit of the hypoxia inducible factor (HIF) 1α/β transcription factor [58]. HIF1α is constitutively synthesized but fails to accumulate under normoxia because of oxygen-dependent hydroxylation of specific proline residues that trigger its ubiquitination and 26S proteasome-mediated degradation. As oxygen declines, the prolyl hydroxylases that modify HIF1α are less active. Consequentially, HIF1α accumulates and is trafficked to the nucleus where HIF1α/β can function in transcriptional activation. There is no corollary direct oxygen sensing mechanism in plants, because although they possess prolyl hydroxylases they lack HIF1α [41].

Group VII ERFs regulate low-oxygen acclimation responses

The plant-specific ERF transcription factor family includes over 100 members in rice and *Arabidopsis*, all of which share an APETALA2 (AP2) DNA binding domain [59]. The ERFs have been phylogenetically parsed into ten clades, with the group VII ERFs characterized by a conserved N terminal motif (NH_2-MCGGAI/L) [59] (Figure 3a). Fifteen rice (*japonica* cv. Nipponbare) ERFs were designated group VIIa (*Os*ERF59-72) and VIIb (*Os*ERF73), based on the presence or absence of the conserved N terminal motif, respectively. The single group VIIb ERF corresponds to *SUB1C*, which is found in all rice varieties surveyed [60] and acts downstream of *SUB1A* [37]. Intriguingly, the group VII ERFs encoded by *SUB1A*, *SK1* and *SK2* possess variant N termini relative to the rice group VIIa ERFs. *Arabidopsis* encodes five group VII ERFs (*At*ERF71–75), two of which are hypoxia-responsive genes {*HYPOXIA RESPONSIVE ERF1* and *2* [*HRE1* (*AtERF73*; At1g72360) and *HRE2* (*AtERF71*; At2g47520)]}. As observed for *SUB1A* and the *SKs*, *HRE1* mRNA accumulation is promoted by ethylene, which synergistically enhances its elevation during hypoxia [61,62] (Figure 3b).

Several recent reports indicate that *Arabidopsis* group VII ERFs redundantly regulate hypoxia-responsive gene expression and survival of low oxygen stress. For example, seedling survival of anoxia was more severely compromised in *hre1hre2* double mutant seedlings than in either single mutant or the wild type [61,63]. By contrast, low oxygen sensitivity was lessened in seedlings that constitutively overexpress either *HRE1* or *HRE2* mRNA. The ectopic expression of these ERFs was sufficient to heighten induction of the core hypoxia-responsive gene *ADH1* or ADH enzyme activity during the stress [61,63]. However, because *hre1hre2* seedlings were able to elevate ADH enzyme activity and ethanol production during hypoxia [63], genetic redundancy is likely to extend to the other group VII ERFs [*RAP2.12* (*AtERF75*; At1g53910), *RAP2.2* (*AtERF74*; At3g14230) and *RAP2.3* (*AtEBP*/*AtERF72*; At3g16770)]. Indeed, conditional upregulation of *RAP2.12* was sufficient to elevate expression of a *pADH1:-LUCIFERASE* transgene [64] and *RAP2.2* overexpression improved survival of hypoxia in seedlings [65], whereas the inhibition of either *RAP2.2* or *RAP2.12* expression via miRNA production limited the induction of *ADH1* and several other hypoxia-responsive genes [66]. The impact of ectopic expression of these genes was condition specific, as *HRE1*, *HRE2*, *RAP2.2* and *RAP2.12* overexpression significantly increased levels of *ADH1* mRNA or ADH activity under low oxygen stress but not under normoxia [61,65,66]. Nonetheless, *RAP2.2*, *RAP2.3* and *RAP2.12* mRNAs accumulate under normoxia in association with polyribosomes [46,67], suggesting they are constitutively synthesized. Together, these findings hint that post-translational regulation limits the function of group VII ERFs to periods of low oxygen stress.

Arabidopsis group VII ERFs are degraded via the N-end rule pathway

The conserved N-end rule pathway of targeted proteolysis regulates the half-life of certain cellular proteins based on

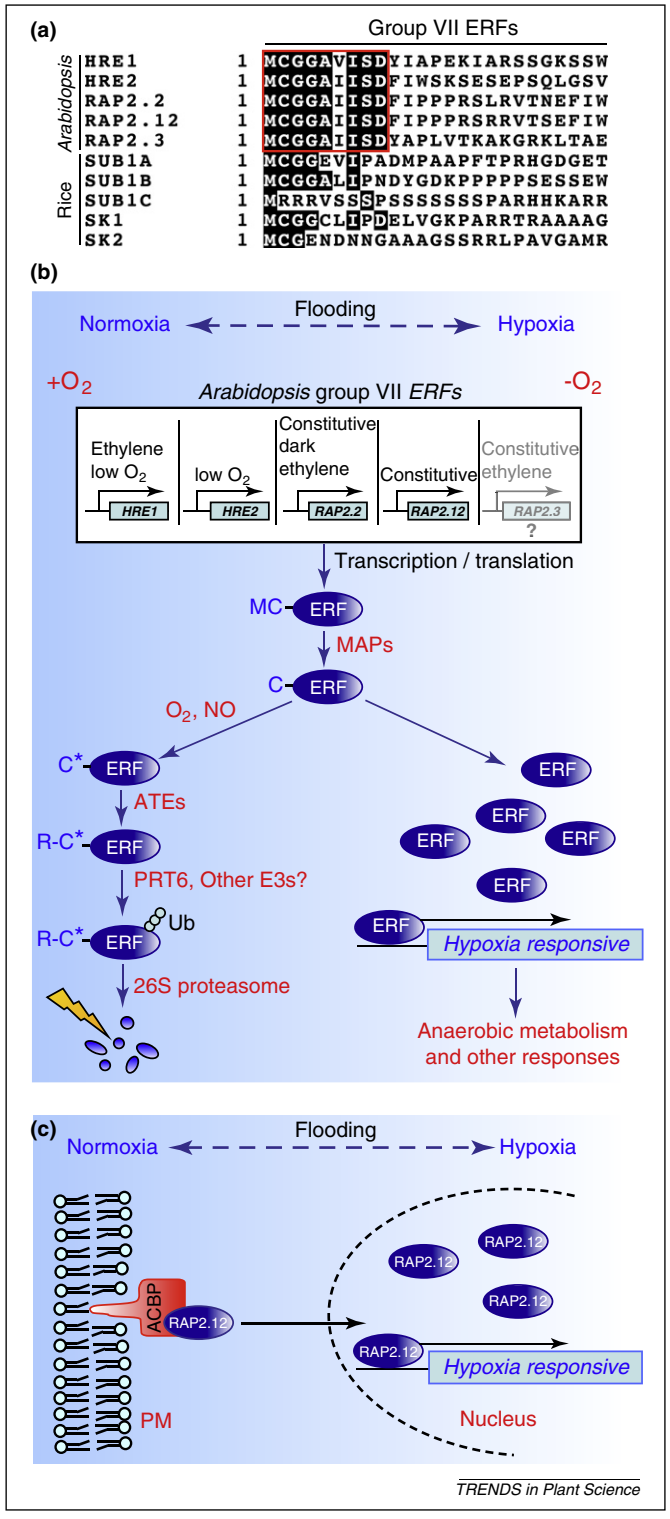

Figure 3. Oxygen sensing via N-end rule pathway-targeted turnover of group VII ERFs. **(a)** N terminal alignment of *Arabidopsis* group VII ERFs. With the exception of SUB1C, all begin with the amino acids 'Met-Cys' (MC). The highly conserved *Arabidopsis* N terminal motif is boxed in red and is less conserved in the proteins at loci associated with submergence responses in rice. **(b)** Homeostatic response to hypoxia is regulated by the N-end rule-mediated proteolysis of group VII ERFs in *Arabidopsis*. Group VII ERF transcription factors are either constitutively expressed and/or differentially transcriptionally regulated in response to variable signals, including low O_2, ethylene and darkness. Four of the five ERFs (HRE1, HRE2, RAP2.2 and RAP2.12) have been implicated in the regulation of hypoxia-responsive genes. Under oxygen-replete conditions (normoxia), ERFs are degraded via the N-end rule pathway of proteolysis. This involves the following steps: (i) the N terminal Met (M) is constitutively cleaved by a methionine aminopeptidase (MAP); (ii) the exposed Cys (C) is converted to an oxidized (C*) form (e.g. Cys-sulfonic acid) by O_2, NO or possibly ROS; (iii) an Arg (R) residue is added to the oxidized Cys by an arginyl tRNA transferase (ATE); and (iv) the argininylated protein is recognized by PROTEOLYSIS 6 (PRT6) or other E3 ligases, which polyubiquitinate the protein, targeting it for proteasomal degradation (26S proteasome). The outcome is prevention of transcription of hypoxia-responsive genes under normoxia. When oxygen becomes limiting (hypoxia), degradation of the ERFs by the N-end rule pathway is inhibited due to a lack of oxygen-mediated Cys_2 oxidation. Stabilized ERFs can then drive the transcription of genes that enhance anaerobic metabolism and other survival responses. Upon return to aerobic conditions, the ERFs are once again destabilized, providing a feedback mechanism that allows the plant to return to aerobic metabolism. **(c)** AtRAP2.12 localization dynamics. At least one group VII ERF, RAP2.12, associates with the plasma membrane (PM) via interaction with ACBP, limiting its turnover under normoxia. During hypoxia RAP2.12 is relocated to the nucleus and activates gene expression. Upon reoxygenation, RAP2.12 is destabilized, presumably as a consequence of Cys_2 oxidation and N-end rule-mediated degradation.

recognition of N terminal residues by specific N-recognin E3 ligases [68]. In plants, 11 amino acids function as destabilizing residues when located at the N terminus of a protein, which coupled with an optimally positioned downstream lysine can act as a degradation signal (N-degron) [69]. In plants and animals but not yeast, a cysteine (Cys) residue at the N terminus can undergo two steps of modification that lead to protein recognition and degradation (Figure 3b). Based on mechanistic studies in mammals, newly synthesized proteins with a Cys as the second residue (i.e. NH_2–Met_1–Cys_2) are constitutively cleaved by a Met amino peptidase (MAP) to yield NH_2–Cys_2. In *Arabidopsis*, a small family of functionally redundant MAPs catalyzes this reaction [70]. The exposed Cys_2 can be spontaneously or enzymatically oxidized in an O_2- or NO-dependent manner to Cys-sulfinate or further to Cys-sulfonate [68]. As a result of oxidation, an arginine residue is added to the NH_2–Cys_2 by an arginyl tRNA transferase (ATE), targeting the protein for recognition by an N-recognin E3 ligase, leading to ubiquitination and 26S proteasome-mediated degradation. In *Arabidopsis*, the genes *ATE1* and *ATE2* encode the Arg transferases [71] and at least one E3 ligase, encoded by *PROTEOLYSIS 6* (*PRT6*), acts as an N-recognin of NH_2–Arg_1–$Cys_2^{oxidised}$ polypeptides [69].

The distinct conservation of the N terminus of group VII ERFs and the serendipitous observation that an *Arabidopsis prt6* mutant constitutively accumulates *ADH1* and other hypoxia-responsive mRNAs in seeds led to the confirmation that group VII ERFs are *bona fide* substrates of the N-end rule pathway in plants [66,72] (Figure 3b). Additional support of this conclusion was obtained through *in vitro* and *in planta* analyses.

An *in vitro* system derived from rabbit reticulocytes [72] was used to confirm that all five *Arabidopsis* group VII ERFs are N-end rule substrates. It was also shown that their instability required Cys_2, as mutation of Cys_2 to the stabilizing residue Ala_2 (NH_2–Met_1–Ala_2) eliminated susceptibility to N-end rule turnover. It was further demonstrated *in planta* that low oxygen stress increased the accumulation of group VII ERFs synthesized with a native N-terminus (NH_2–Met_1–Cys_2), whereas those synthesized with an NH_2–Met_1–Ala_2 N terminus were stable under both normoxia and hypoxia [66,72]. Based on this evidence, the stabilization of group VII ERFs under hypoxia is most probably related to an inhibition of the Cys_2 oxidation that is required before the protein can be arginylated and degraded.

Either the modification of the N terminus of a group VII ERF or disruption of an N-end rule pathway step can affect survival of low oxygen stress or submergence in *Arabidopsis* [66]. For example, stabilization of HRE1 and HRE2 by modification of the N terminus to NH_2–Met_1–Ala_2 was sufficient to improve seed germination and seedling survival under hypoxia [72]. In addition, *ate1ate2* and *prt6* seedlings were less sensitive to hypoxia when grown on sucrose-supplemented medium [72]. The same mutants grown to the rosette stage were more sensitive to submergence in complete darkness [66]. This discrepancy in phenotype might be explained by distinctions in the available carbohydrates in the two survival assays. In the low oxygen experiments, anaerobic metabolism was fueled by sucrose in the medium, whereas in the submergence experiments it was limited to endogenous energy reserves of the plant. Therefore, the absence of PRT6 or ATE activity may enhance anaerobic metabolism to prolong survival in sucrose-fed seedlings but may cause a more rapid onset of energy deficiency in submerged plants. These findings are reminiscent of the earlier proposal that a balance between energy consumption and conservation is crucial to survival of low oxygen stress and submergence [5,37].

It was also observed that the onset of the transcription of hypoxia-responsive genes occurs concomitantly with relocalization of RAP2.12 to the nucleus under hypoxia (Figure 3c) [66]. During normoxia, a GFP-tagged version of RAP2.12 was protected against protein degradation by the N-end rule pathway of proteolysis and excluded from the nucleus via interaction with a plasma membrane (PM)-associated Acyl-CoA binding protein (ACBP1 or ACBP2). RAP2.12 migrated to the nucleus in response to hypoxia and disappeared from the nucleus after reoxygenation. Moreover, transient expression of RAP2.12-GFP in leaves of *ate1ate2* and *prt6* mutants resulted in greater GFP signal intensity in the nucleus under normoxia and following reoxygenation.

In summary, the N-end rule pathway of proteolysis regulates the accumulation of group VII ERFs and consequentially the accumulation of gene transcripts associated with low oxygen responses in *Arabidopsis*. It is proposed that constitutively synthesized group VII ERFs are either degraded or sequestered under normoxia, as confirmed for *RAP2.12*. As oxygen levels fall their degradation becomes limited, PM sequestration is reversed and the ERF is transported to the nucleus and becomes active in gene regulation. Upon reoxygenation, both constitutively expressed and hypoxia-induced group VII ERFs are destabilized. Thus, the N-end rule pathway (i) prevents the excessive accumulation of constitutively expressed ERFs under normoxia; (ii) allows for stabilization of both constitutive and induced ERFs during hypoxia; and (iii) facilitates rapid reversal of ERF-regulated transcription upon reoxygenation. Constitutively expressed group VII ERFs are proposed to encode oxygen sensors that conditionally activate transcription of hypoxia-responsive genes, including other group VII ERFs [66]. The increased synthesis of N-end rule regulated group VII ERFs by ethylene or darkness could further prime cells for acclimation to oxygen deprivation.

Manipulation of N-end rule regulation of group VII ERFs and other proteins

The verification that the N-end rule pathway modulates group VII ERF accumulation in the nucleus in an oxygen-dependent manner exposes the first examples of N-end rule substrates and a homeostatic low oxygen sensor mechanism in plants. Based on available gene sequence data, group VII ERFs with the conserved N-terminus are broadly found in vascular plant species [66]. We propose that future improvement of flooding tolerance could be achieved by manipulation of synthesis and turnover of these proteins (e.g. by overexpression, regulated expression and/or mutation of NH_2–Met_1–Cys_2 to NH_2–Met_1–Ala_2).

Given the crucial importance of modulation of energy reserves during flooding, it is not surprising that variation of group VII ERF susceptibility to oxygen-dependent N-end rule turnover exists in nature. The rice Nipponbare genome encodes 15 group VII ERFs with the conserved N terminus that is consistent with oxygen-regulated N-end rule-targeted proteolysis in *Arabidopsis*. However, neither SUB1A nor SUB1C are N-end rule substrates based on *in vitro* data [72] and the N termini of SK1 and SK2 also deviate from the consensus associated with N-end rule-mediated turnover. This leads us to propose that the escape of SUB1A from N-end rule pathway turnover could allow the ethylene-mediated regulation of *SUB1A-1* to trigger the sequence of events that promotes the energy management associated with submergence tolerance well before oxygen levels reach a critical nadir.

Concluding remarks: direct oxygen sensing via the N-end rule regulates transcription

Alterations in gene expression associated with increased catabolism and substrate level ATP production are a hallmark of reduced oxygen availability and flooding in plants. Group VII ERFs play a prominent role in this process. The identification of an oxygen-dependent protein turnover mechanism that controls the abundance of some but not all group VII ERFs raises several pertinent questions (Box 2). We anticipate that genetic manipulation of the targets of oxygen-regulated N-end rule pathway turnover can provide a means to improve survival under a variety of flooding conditions.

Box 2. Key questions for future experimentation

- Can O_2, NO or ROS-dependent Cys_2 oxidation, arginylation and ubiquitination of group VII ERFs be experimentally confirmed? If so, is the oxidation spontaneous or catalyzed?
- What are the kinetics of oxygen-regulated group VII ERF turnover? Does ERF stabilization occur before oxygen deficiency impairs cytochrome *c* oxidase activity?
- Does the oxygen level affect the interaction between ACBPs and RAP2.12? Does docking of RAP2.12 to ACBP impair Cys_2 oxidation, modification by ATE, or interaction with an E3 ligase? Are other group VII ERFs similarly sequestered?
- What genes and networks are controlled by individual group VII ERFs?
- Is the activity or turnover of SUB1A, which apparently escapes oxygen-mediated N-end rule degradation, controlled upon de-submergence?
- Can manipulation of group VII ERF accumulation and turnover provide an effective strategy to modulate survival of flooding in crops?

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