

Plant responses to flooding stress

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Most plant species cannot survive prolonged submergence or soil waterlogging. Crops are particularly intolerant to the lack of oxygen arising from submergence. Rice can instead germinate and grow even if submerged. The molecular basis for rice tolerance was recently unveiled and will contribute to the development of better rice varieties, well adapted to flooding. The oxygen sensing mechanism was also recently discovered. This system likely operates in all plant species and relies on the oxygen-dependent destabilization of the group VII ethylene response factors (ERFVII), a cluster of ethylene responsive transcription factors. An homeostatic mechanism that controls gene expression in plants subjected to hypoxia prevents excessive activation of the anaerobic metabolism that could be detrimental to surviving the stress.

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Introduction

Although plants produce oxygen through photosynthesis, the lack of an efficient system to transport oxygen to non-photosynthetic organs implies that these organs can be deprived of oxygen if their anatomy limits oxygen diffusion from outside [1,2]. Additionally, complete submergence of the plant by flooding events may also lead to low-oxygen availability in the aboveground organs, especially when water turbidity limits photosynthesis [3]. When oxygen becomes limiting for respiration plants experience hypoxia, whilst the complete absence of oxygen (anoxia) is even more detrimental to plant survival. Both hypoxia and anoxia trigger extensive reprogramming of gene expression, with induction of the fermentative metabolism, allowing the plant to use glycolysis for ATP production [1]. Climate changes will lead to extremes in water availability that will cause severe drought in some areas, while

flooding due to extreme rainfall events will affect other geographical areas [4]. Unless new crop varieties able to withstand abiotic stresses are developed, productivity will be gravely affected. Until a decade ago little was known about the genes that confer tolerance to submergence, and it is only during recent years that light has been shed on the molecular mechanisms behind oxygen sensing and signalling in plants [2]. In this review we will highlight the most recent findings in the field of plant anaerobiosis, from ecophysiology of plants growing in wetlands to the translation of discoveries made in *Arabidopsis* to crops.

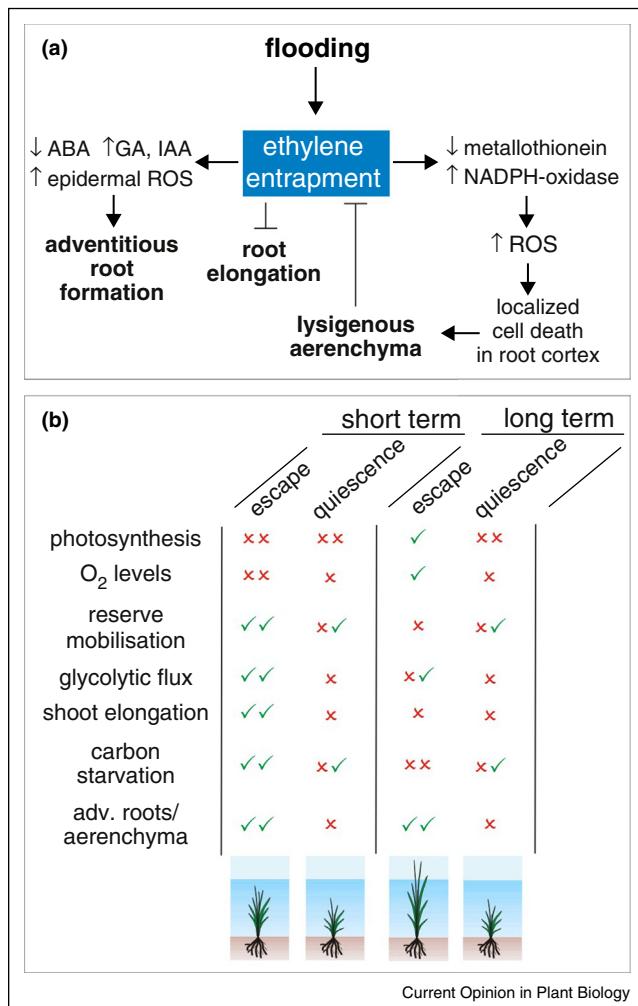
Flooding in the wild

Flooding is a natural occurrence in many ecosystems and therefore many wild species are superbly adapted to watery conditions. Here improved gas exchange with the environment is essential to avoid hypoxia within the plant. To this end, plants can induce and/or constitutively develop aerenchyma, longitudinal connected gas spaces, which provide a rapid means of aerial gas exchange over long distances within the plant [5]. This is usually combined with a change in root architecture to minimize the distance (and therefore diffusive resistance) between the aerial surface and the flooded root tips [6], for instance via adventitious roots, which can create a collection of air conducting snorkels originating from the hypocotyl or stem into the anaerobic substrate. Often aerenchyma are combined with a barrier that prevents oxygen leakage into the surrounding anaerobic soil, which drastically improves flooding tolerance [7].

An extensive aerenchyma system is extremely effective under waterlogged conditions where the shoot remains in aerial contact and can thus funnel air down to the root. During complete submergence, however, the shoot does not make aerial contact oxygen, their effectiveness in funnelling air towards the roots is greatly compromised. In such cases, some wetland plant species, in an attempt to regain aerial shoot contact, display rapid vertical elongation of leaves, internodes or petioles to snorkel for air. This escape strategy is observed in some rice varieties (see below), as well as in several other plant species [8]. In an alternative strategy the plant aims to enter a state of inactivity (quiescence), to be revived once the flood recedes [9,10]. This is also a difficult tactic as energy and carbon utilisation should be kept to a minimum to make reserves last a long time, whilst they should simultaneously be sufficient to maintain cellular integrity (Figure 1a).

The submerged plant: low oxygen and high ethylene

Because of its gaseous nature ethylene hardly leaves the plant under flooded conditions and thus rapidly

Figure 1

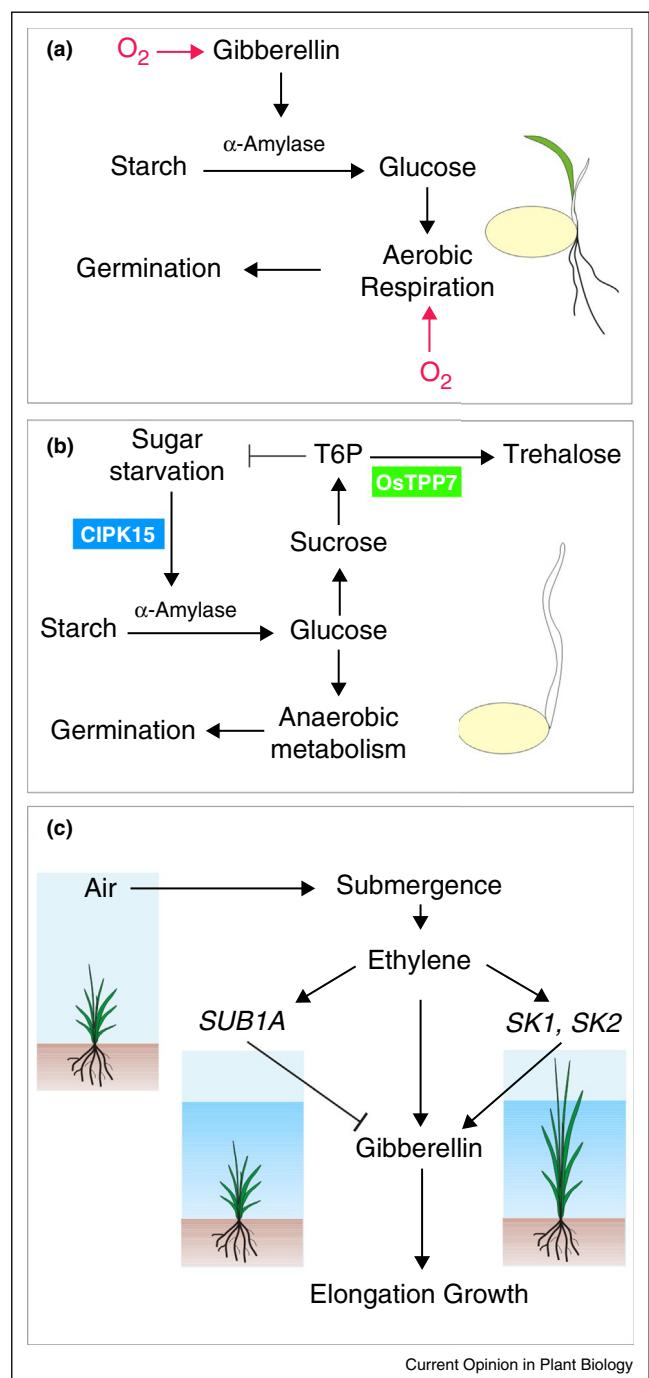
Ethylene is a pivotal regulator of growth survival strategies (a) and root development (b) during submergence and waterlogging. During complete submergence, ethylene induced growth strategies are paramount to survival (a), but both have different short term and long term effects on plant performance, especially since under long term submergence escaping plants will have regained aerial contact. Naturally, photosynthesis is severely reduced by flooding, but through an escape strategy some photosynthesis can be recovered through the re-establishment of aerial contact. This subsequently reduces the need for reserve mobilisation and limits oxygen shortage via aerenchyma. Initially, escaping plants will have low internal O₂ levels, due to their high metabolic activity which is fuelled by a strong glycolytic flux and reserve mobilisation. These high demands, generally mean escaping plants suffer strongly from carbon starvation. Though all these effects are ameliorated once aerial contact is made. Because quiescent plants have low activity, their requirements on reserves and energy are limited. Subsequently, internal O₂ levels would be at a higher steady state and carbon shortage would be considerably lower. However, quiescent plants still rely on reserve mobilisation to sustain cellular functions, both during short term and long term flooding. Root development is also essential to survive flooded conditions. The aqueous environment prevents ethylene to readily leave the plant tissues through gas diffusion. This ethylene entrapment starts a cascade that leads a change in root architecture (b), through for instance the formation of adventitious roots. This includes a hormonal cascade and ROS induced epidermal cell death.

accumulates inside the plant. It is therefore a highly reliable and rapid cue for plants to detect their predicament [11]. Another signal is the oxygen availability. The internal level of these gases is a balance between consumption, production and diffusive resistance. Therefore active, heterotrophic or compact tissue, such as meristems and roots, will rapidly experience low oxygen upon flooding. In photosynthetic tissue the consumption and production of oxygen is dependent on light conditions, and thus also the oxygen availability.

Ethylene is the primary signal for most adaptations to flooding. Ethylene modulates a hormonal cascade of ABA, GA and ultimately auxin to induce adventitious rooting in tomato, *Solanum dulcamarum*, and rice, and [12–14]. However, root emergence also requires ethylene to induce ROS formation in the epidermal cells, leading to their cell death to allow root penetration [15]. Similarly, lysogenous aerenchyma formation, which is formed by apoptosis of specific cells in the cortex, involves an ethylene dependent drop in antioxidant activity. The subsequent increase in ROS leads to the required cell death [16–18]. Interestingly, the important suberin based oxygen barrier is not affected by ethylene, but likely causal genes involved in its formation have been identified [19]. The escape strategy to reach the water surface is also ethylene driven. However, downstream signalling is considered divergent in the plant kingdom, as it was found to act via group VII ERFs in rice (see below), but via genes typical of low light induced elongation in *Rumex palustris* [20–22]. Remarkably, ethylene pre-treatment induced anoxia tolerance of *Rumex palustris* was associated with enhanced hypoxia related gene expression. A behaviour that was absent in *Rumex acetosa*, a species that experiences fewer flooding events and employs a quiescence instead of an escape strategy [20]. This highlights the importance of a link between ethylene and hypoxic signalling pathways.

The high levels of ethylene associated with flooding inhibit root elongation, but through the formation of aerenchyma the excessive ethylene is easily removed. However, species that are ineffective in producing aerenchyma therefore experience strong root growth reduction under flooded conditions [23]. The strong dose dependency of ethylene signalling [24] might play an important role in its contrasting developmental roles during flooding (Figure 2). To avoid detrimental effects associated with high levels of ethylene, some of the species that continuously occupy aquatic or flood-prone environments have

Simultaneously, high levels of ethylene in submerged roots, inhibit root elongation. However, ethylene also leads to a drop in the antioxidant metallothionein and an increase NADPH oxidase, which together leads to an accumulation of ROS. ROS acts as a signal for programmed cell death of specific cortex cells, eventually leading to the formation of lysogenous aerenchyma. As a result, the improved gas diffusion can remove high ethylene levels and thus releases the inhibition on root elongation.

Figure 2

Rice germination and growth under aerial (a) and submerged (b) conditions is regulated at different levels, depending on the genotype as well as the growth-stage. Rice germination under anoxia is very peculiar, with rapid coleoptile elongation (a, b). Only once the water surface is reached and the coleoptile can act as a snorkel do the root and primary leaf develop. Germination under anoxia is extremely challenging because ATP can only be produced through the activity of glycolysis coupled with ethanolic fermentation, which yields only a fraction of the ATP produced by mitochondrial respiration, ready access to starch reserves is thus essential. Under anoxia or hypoxia starch degradation through the gibberellin-induced α -amylase pathway

lost or reduced their capacity to either produce, sense or respond to ethylene [24,25].

Darkness, a typical component of flooding in murky water, is responsible for a large portion of the transcriptomic changes observed during complete submergence in the dark in *Arabidopsis* [26]. This indicates that acclimation to flooding, at least in dark conditions, predominately occurs via sugar and energy signalling, as also was shown in rice [27]. Where the contribution of hypoxia in regulating gene expression in dark submergence acclimation can be minor, hypoxia regulated gene expression is correlated, either positively or negatively, to flooding tolerance in natural variation of *Arabidopsis*, *Rumex* and *Rorippa*, which makes it an important area of study [20,26,28].

Flooding in the field: rice

Rice is remarkably well adapted to submergence (Figure 2) and can even germinate in the complete absence of oxygen [29]. This anaerobic germination (AG) includes a lengthening of the coleoptile, that, analogous to the escape strategy, aims to make aerial contact but considerable variation exists among rice genotypes in coleoptile extension during anoxia [29]. Differently from other cereal seeds that fail to induce the α -amylase enzymes required for starch degradation under anoxia, rice caryopses produce this enzyme, which allows starch degradation coupled to the fermentative metabolism and subsequent germination [30–32]. The rapid depletion of soluble carbohydrates occurring during the first hours of germination under anoxia, together with a possible low-oxygen dependent change in calcium levels, leads to a signalling cascade that finally leads to α -amylase

cannot occur because oxygen is required for gibberellin synthesis (a) and also because rice fails to respond to gibberellins under low-oxygen conditions. In anaerobically germinating rice varieties the low-oxygen conditions (b) require starch degradation through the action of α -amylases, some of which are induced by sugar starvation, rather than gibberellin, and in a feed-back manner is repressed by increased availability of sugars. This feedback loop between sugar starvation and α -amylase acts via a pathway requiring CIPK15. Viable anaerobic germination requires OsTPP7 to reduce the perception of sugars so that sugar induced inhibition α -amylase is prevented, resulting in a strong flux sugars released via starch degradation. This allows rice to feed the anaerobic metabolism with sugars and obtain enough ATP to support germination. In adult rice plants (c) different strategies are observed that allow the rice plant to survive submergence.

Submergence results in ethylene accumulation, that induces SUB1A in genotypes possessing this gene. SUB1A represses growth of submerged plants, thus allowing the plant to preserve carbon reserves, which in turn will allow re-growth of the plant when the water recedes. Instead in deepwater rice varieties ethylene induces the SK genes, which induce fast stem elongation. This results in an 'escape' strategy that allows the plant to keep its leaves above the water surface, thus allowing oxygen to be transported to the submerged parts of the plant through the aerenchyma. Rice varieties that do not possess either SUB1A or SK genes display an intermediate phenotype, with slow stem elongation that depletes the plant from carbon resources without allowing to gain aerial contact.

production. This process begins with the activation of a Calcineurin B-like (CBL), which targets the protein kinase CIPK15, which in turn triggers the SnRK1A pathway that induces the MYBS1 transcription factor which activates the starvation-inducible α -amylase gene *RAmy3D* [27]. There is considerable variation amongst rice varieties in their ability to successfully germinate and establish when submerged in the field, though most activate RAmy3D during this anaerobic germination. AG of rice allows direct sowing instead of transplanting, which is of great importance as it makes rice cultivation more economically sustainable [33^{••}]. A QTL analysis identified *OsTPP7* as the locus responsible for efficient AG. *OsTPP7* encodes a trehalose-6-P-phosphate (T6P) phosphatase, which is non-functional in rice varieties that are unable to establish under submerged conditions [33^{••}]. The presence of the *OsTPP7* in rice accessions was correlated with increased sink strength of elongating coleoptiles, resulting in prolonged tolerance to complete submergence. High sucrose results in high T6P levels and consequently in repression of SnRK1 and downregulation of α -amylases. During anaerobic germination *OsTPP7* misleads the seedling about its sugar status by converting T6P into trehalose. Subsequently the rice seedling can maintain a relative high sugar availability but low T6P levels, which, if high, would repress α -amylases (Figure 2b). The subsequent intense flux of glucose from starch degradation is essential for fuelling glycolysis and lengthening of the coleoptile. Rice germination under anoxia is therefore the consequence of clever sugar management, that allows adept access to starch reserves [34]. To this aim the fine tuning of sugar sensing by keeping lower T6P levels for a given sucrose concentration by the low-oxygen inducible *OsTPP7* appears to be essential [33^{••}].

In some areas of Asia submergence occurs very rapidly and lasts for months, here rice varieties named ‘deep-water rice’ are grown. The adult plant continues to snorkel for air and keeps up with the increasing water level. This trait relies on two group VII ERF genes: *SNORKEL1* and *SNORKEL2* (SK1, SK2) [22]. Only present in deep water rice varieties, they activate a gibberellin-dependent internode elongation, up to 25 cm per day, sufficient to maintain an aerial contact with some of the leaves which allow air transfer to the submerged parts of the plant via aerenchyma (Figure 2c).

Clearly the success of rice in flooded habitats is due to its ability to rapidly regain aerial contact [35]. Interestingly, only a few rice varieties can survive complete submergence for an extended period of time, a phenomena that regularly occurs in so-called flash-floods. These varieties survive thanks to the group VII ERF gene *SUB1A* [36], whose product positively regulates the fermentation capacity, but represses plant growth by restricting gibberellin-signalling [37,38]. Therefore, rice varieties that survive complete submergence

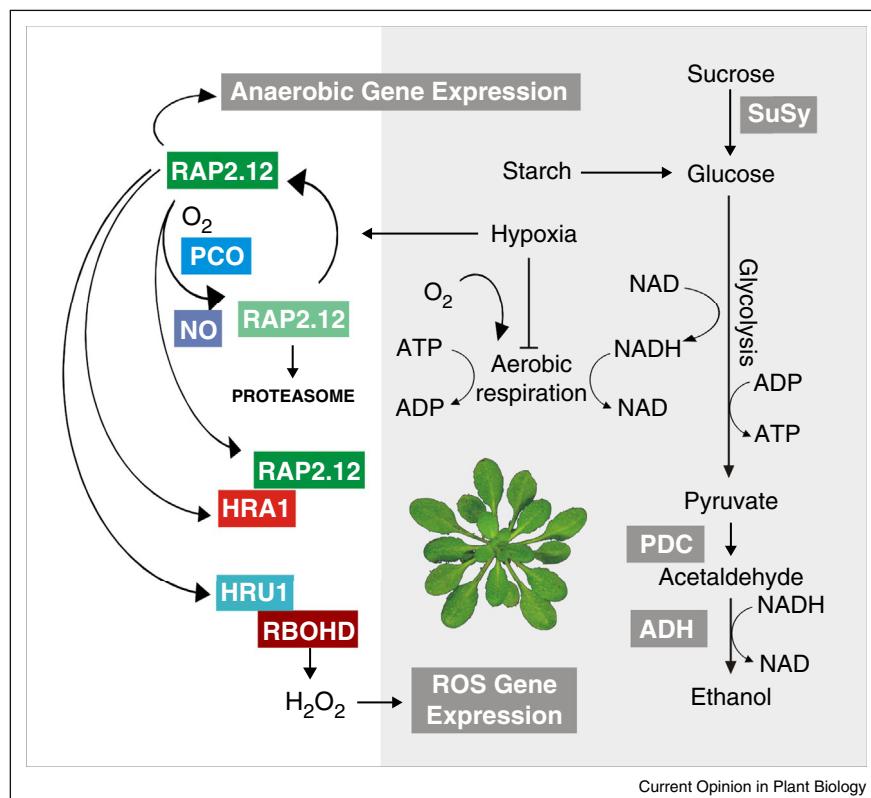
activate, through *SUB1A*, a quiescence strategy that allows them to reduce carbohydrate use to the minimum required for keeping the plant alive, while it waits for water to recede, to continue aerial growth [39] (Figure 2c).

Flooding in the lab: *Arabidopsis* and the N-end rule pathway for oxygen sensing

Arabidopsis is not highly tolerant to submergence [40], nevertheless it made the discovery of oxygen sensing and signalling mechanisms possible [41]. Besides the classical anaerobic genes, several *HYPoxIA-RESPONSIVE UNKNOWN PROTEIN* (*HUP*) genes were identified [42], representing possibly interesting elements in the anaerobic response pathway. Furthermore, an atlas of hypoxic-dependent gene expression in specific cell types was produced and revealed a set of approximately 50 genes that were activated regardless of their cellular identity [43]. This provides an enormous amount of information that could be exploited to elucidate the signalling pathway behind the response of plants to low oxygen. The role of group VII ERFs in rice prompted research on this gene-family in *Arabidopsis*, in which the group VII ERFs are five [44], with the initial identification of two HYP-OXIA-RESPONSIVE ERFs (HRE1 and HRE2) which contribute to hypoxia tolerance and signalling [45]. RAP2.12, another group VII ERF, is not induced by hypoxia, but nevertheless activates ADH [46]. RAP2.12 is regulated by oxygen at the protein level, with oxygen provoking its degradation [47,48]. Only under low oxygen are RAP2.12 and the other two constitutively expressed group VII ERFs, RAP2.2 and RAP2.3, stable and redundantly activate the core anaerobic response [49,50]. This oxygen sensing mechanism relies on the oxygen-dependent oxidation of the group VII ERF N-terminal cysteine (Cys), mediated by the PLANT CYSTEINE OXIDASE (PCO) enzymes [51^{••}]. The oxidised Cys targets RAP2.12 to the proteasome through an N-end-rule pathway of ubiquitin mediated proteolysis (Figure 3). Interestingly, also nitric oxide (NO) is able to induce group VII ERF degradation, indicating that this pathway might also be involved in other processes including seed germination, stomatal closure, and hypocotyl elongation [52^{••}]. Remarkably, oxygen sensing through group VII ERFs was shown to coordinate photomorphogenesis during seedling development [53]. It is presently unknown whether and how group VII ERF cysteine oxidation requires both PCOs and NO.

The RAP2.12 dependent activation of the downstream genes is essential to survive submergence, but also needs to be finely tuned. The HYPOXIA-RESPONSE ATTENUATOR1 (*HRA1*) is a trihelix transcription factor that represses the action of RAP2.12. *HRA1* gene expression is itself activated by RAP2.12 stabilization under hypoxia, indicating the existence of an homeostatic mechanism for regulating the anaerobic response, such

Figure 3



Anaerobic signalling (left) and metabolism (right) in *Arabidopsis*. Under conditions of hypoxia or anoxia respiration in the mitochondria is severely impaired. NADH regeneration to NAD, required to allow glycolysis to proceed, thus occurs through the activation of PDC and ADH. Starch metabolism and sucrose metabolism through sucrose synthase (SuSy) provide the carbon units required for glycolysis. SuSy, PDC, ADH are examples of enzymes encoded by anaerobic genes, whose activation is triggered by hypoxia. Oxygen sensing occurs through ERF-VII genes such as RAP2.12 and RAP2.2 (the latter not shown in figure) that are unstable under aerobic conditions, because PCO enzymes oxidise the N-terminal Cys residue, resulting in degradation of RAP2.12 by the proteasome. Nitric oxide (NO) also induces degradation of ERF-VII proteins. RAP2.12 induces the expression of anaerobic genes, among which is also HRU1, which controls hydrogen peroxide production by RBOHD. The interaction of RAP2.12 with HRA1 dampens the action of RAP2.12.

that it does not harmfully exceed the needs of the plant [54••]. Interestingly, also hydrogen peroxide production under anoxia occurs during the early phases of the stress [55]. Recently, a protein interconnecting the oxygen-sensing machinery with ROS production was identified. HYPOXIA-RESPONSIVE UNIVERSAL STRESS PROTEIN 1 (HRU1) is induced by the oxygen-responsive N-end-rule pathway and affects ROS production, possibly through an interaction with a membrane-localized NADPH-oxidase (RBOHD) and its regulator ROP2 [56•]. Overall these recent findings suggest that hypoxia-dependent signalling is tightly controlled via various signals and proteins in a highly connected network. It is tempting to speculate that excessive activation of the fermentative pathway by RAP2.12 may deplete sugars to a level that induces severe starvation, hampering long term survival and recovery from hypoxia. A highly coordinated network, including HRA1 and HRU1, could prevent such a detrimental scenario.

Translating lab research into better crops

The identification of SUB1A as the determinant for submergence tolerance in rice allowed the breeding of flood-tolerant rice varieties, often called ‘scuba rice’ [4,57,58]. These varieties showed the same yield and quality traits as their non-Sub1 counterparts when grown under non-flooded conditions, but displayed yield advantages of 1 to more than 3 t ha⁻¹ after complete submergence for various durations [59]. This is a great example of rapid translation of a scientific discovery into agricultural improvements in less than ten years since the discovery of SUB1A in 2006 [35]. Experimental evidence showing that SUB1A also contributes to drought tolerance in rice suggests that this trait will contribute to the development of rice varieties better adapted to climate changes [60]. Incorporating flooding tolerance into crops other than rice will be very challenging, given the lack of accessions with flooding tolerance traits. However, the discovery of the oxygen sensing mechanism in *Arabidopsis* could show

great promise for crop improvements. However, both strong and weak hypoxic signalling, that is very large versus moderate induction of group VII ERF targets, has been connected to flooding tolerance [20,26,28,47,48,61]. Nevertheless, barley with reduced expression of the N-end-rule pathway E3 ligase PROTEOLYSIS6 (PRT6) shows increased tolerance to waterlogging [62**].

Incorporating traits from the superbly adapted wetland species will invariably be challenging, but could provide big leaps in flooding tolerance. Aerenchyma formation is a developmentally complex trait and so far we have been unable to import this trait into a species that did not possess it already. Moreover, maize develops aerenchyma upon waterlogging, but despite this ability it still suffers strongly from soil flooding. Other changes in root development, such as enhanced adventitious rooting, might be more promising and pliable to our crops, as these traits are often already present to some extent.

Tolerance to submergence includes the delicate balance between the induction of the fermentative mechanism, that represents a requirement for basal tolerance, and other mechanisms preventing carbon starvation and oxidative stress. Only after we have a complete picture of the many tolerance traits will the development of crop varieties tolerant to waterlogging or submergence be feasible.

Acknowledgement

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