Waterlogging tolerance: nonsymbiotic haemoglobin–nitric oxide homeostasis and antioxidants

R. K. Sairam[1,*], D. Kumutha[2] and K. Ezhilmathi[1]

[1]Division of Plant Physiology, Indian Agricultural Research Institute, New Delhi 110 012, India
[2]Present address: National Institute for Interdisciplinary Science and Technology, Thiruvananthapuram 695 019, India

Waterlogging is a serious problem which affects crop growth and yield in low-lying, rainfed areas. Most of the rainy-season crops, especially legumes, and to a lesser extent maize and rice are affected by flooding leading to hypoxic or even anoxic conditions. Lack of oxygen shifts the energy metabolism from aerobic mode to anaerobic mode, which in turn adversely affects nutrient and water uptake; so the plants show wilting even when surrounded by excess water. Gaseous plant hormone ethylene plays an important role in modifying the plant's response to oxygen deficiency. Plants normally adapted to growing under waterlogged conditions have mechanisms to cope with this stress in the form of aerenchyma formation, increased availability of soluble sugars, greater activity of glycolytic pathway and fermentation enzymes. Both alcoholic and lactic acid fermentation help in maintaining lower redox potential (low NADH/NAD ratio), and thereby play an important role in anaerobic stress tolerance. Other important biomolecules which are induced under waterlogging, are nonsymbiotic haemoglobins and nitric oxide. Interaction of nonsymbiotic haemoglobins and nitric oxide has been suggested as an alternative to the fermentation pathway, which also rules out the production of toxic alcohol and lactic acid, the latter being the major cause of cytoplasmic acidosis. Waterlogging stress also results in the production of reactive oxygen species, and induction of antioxidant defence enzymes. Waterlogging-induced production of ethylene, H_2O_2 and NO is also involved in signalling and induction of various defence-related genes leading to synthesis of proteins/enzymes imparting hypoxia tolerance.

Keywords: Anaerobiosis, nitric oxide, non-symbiotic-haemoglobin, oxidative stress, waterlogging.

SOIL waterlogging and submergence are abiotic stresses that influence species composition and productivity in numerous plant communities worldwide. Hydrological patterns determine the ecological distribution of species the world over. In rice farming, flooding regimes are manipulated or are accommodated by genotype selection (e.g. deep water and standing water rice) to secure much of the world production of this staple crop[1]. There have also been recent advances toward developing cultivars for lowland areas prone to short-duration flash flooding. For most other crops, excess water is a major constraint to productivity in many regions and situations[2], thus adversely affecting grain yields and growth of pasture species[3].

A major constraint resulting from excess water, at least for poorly adapted species, is an inadequate supply of oxygen to submerged tissues. Diffusion of oxygen through water is 10^4-fold slower than in air. In addition to the threat of oxygen deficiency, excess water also leads to other changes in the soil that influence plant levels of the phytohormone ethylene[4], and the accumulation of products of anaerobic metabolism by soil microorganisms (e.g. H_2S, S^{2-}, carboxylic acids, etc.)[5]. Moreover, when flooding results in complete submergence, availability of carbon dioxide, light and oxygen to the shoots typically diminishes[6].

Growth and development of the vast majority of plant species is impeded by soil flooding, and particularly by complete submergence, both of which can result in death. A variety of morphological and anatomical alterations develop in the root system. In water-saturated soils roots grow only in a small region near the surface and do not exploit a large soil volume as they would under aerated conditions. Reduction of the root respiration rate has been reported in flooding-tolerant and intolerant species. Root systems starved of oxygen are also poor providers of mineral nutrients for both themselves and the shoot systems. Stomatal closure and non-stomatal metabolic alterations are responsible for the reduction of leaf CO_2 incorporation.

Plants invariably wilt within few hours to 2–4 days of imposing a flooding stress[7]. This is a consequence of higher resistance to mass flow of water through the root. Wilting is caused by the inhibition of respiration and loss of ATP synthesis in the roots, which blocks the ion transport systems that normally create the gradient in water potential across the root endodermis. Lack of oxygen thus effectively blocks ATP synthesis in the mitochondria[8]. In the absence of an electron acceptor, NADH oxidation is blocked. Once the mitochondrial respiration stops, the

*For correspondence. (e-mail: rks_ppl@yahoo.co.uk)

adenylate energy charge of the cell (ratio of ATP/ATP + ADP + AMP) declines. In the absence of an adaptive response, the flooded root cell rapidly depletes its available supply of ATP. Plants react to an absence of oxygen by switching from an oxidative to a solely substrate-level phosphorylation of ADP to ATP; the latter reactions predominantly involve glycolysis and fermentation. Plants show induction and up-regulation of genes/enzymes of the glycolytic and fermentation pathways[9–13]. Since glycolysis yields only two ATPs per mole of glucose oxidized as against 38 ATPs by glycolysis–Krebs cycle–electron transfer chain, plants rapidly lose stored root carbohydrates. Under these circumstances carbohydrate starvation is one of the reasons of hypoxia-induced injury and possible death under prolonged waterlogging. Consequently, a species or its genotype with sufficient root sugar levels has better chances of survival[14]. Another important adaptive response is the formation of aerenchyma, specialized tissues in roots, which allow diffusion of gases like O_2 from aerobic shoots to hypoxic/anoxic roots. However, numerous wetland species are highly productive in flood-prone areas. This is achieved by means of a combination of life-history traits such as physical escape from submerged environment[15], avoidance of oxygen deficiency through effective internal aeration[16], anoxia tolerance[17] and a capacity to prevent or repair oxidative damage during re-aeration[18].

These aspects of a plant's response to waterlogging and tolerance mechanisms have been covered earlier[10,19–21]. In this review we present some of the recent work on the role of nitric oxide (NO), nonsymbiotic haemoglobin, reactive oxygen species (ROS) and antioxidant enzymes in waterlogging tolerance and signalling.

Nonsymbiotic haemoglobins

It is now known that there are several classes of haemoglobins (Hbs) in plants. Symbiotic haemoglobins found only in nodules of plants were discovered 62 years ago[22]. Plant haemoglobins, with properties distinct from symbiotic haemoglobins were discovered 18 years ago and are now believed to exist throughout the plant kingdom. They are expressed in different organs and tissues of both dicot and monocot plants[23]. They are induced by hypoxic stress and by oversupply of certain nutrients. The most recently discovered truncated haemoglobin also appears to be ubiquitous and shares some characteristics with nonsymbiotic haemoglobins[24]. There are two classes of nonsymbiotic haemoglobins. Class 2 has similar oxygen-binding properties to symbiotic haemoglobins, but class 1 has dramatically different oxygen-binding properties. Class-1 haemoglobins are induced by hypoxic stress and oversupply of nutrients, and are referred as stress-induced haemoglobins[25]. It is believed that plant and animal haemoglobins originated from the same ancestral globin gene about 1500 million years ago and have been shaped by vertical evolution[26]. Some of the species known to express stress-induced haemoglobins are listed in Table 1.

Function of haemoglobin under hypoxia

One of the major problems faced by plants/tissues experiencing hypoxia/anoxia is an increase in NAD(P)H/NAD(P) ratio, which adversely affects the glycolytic pathway, the only pathway which provides energy under anaerobiosis. To ameliorate this situation, plants use the fermentative pathway employing lactic dehydrogenase (LDH) and alcohol dehydrogenase (ADH) for recycling NAD(P)H to NAD(P). However, accumulation of end-products of these pathways, viz. CO_2, ethanol and lactic acid is toxic for the cell. Another important metabolic process which may recycle NAD(P)H is hypoxia-induced nitrate reductase leading to formation of NO. In this context, the role of nonsymbiotic haemoglobins has also been highlighted in many plant species. Stress-induced haemoglobins have been shown to affect plant metabolism and growth under low oxygen tension. Constitutive expression of barley haemoglobin in wild type and transformed maize cell lines maintained the cell adenine nucleotide levels and energy charge under hypoxic conditions, whereas cells in which haemoglobin expression was suppressed had lower adenine nucleotide levels and energy charge[27]. In similarly transformed alfalfa root cultures, lines constitutively expressing barley haemoglobin maintained root growth during hypoxic treatment, whereas wild-type and lines with suppressed stress-induced haemoglobin expression had slower root growth[28]. It was suggested that nonsymbiotic haemoglobins act in plants to maintain energy status of cells in low oxygen environments and that they accomplish this effect by promoting glycolytic flux through NADH oxidation, resulting in increased substrate-level phosphorylation. In this way, they are used in sequestering oxygen in low oxygen environments, providing a source of oxygen to oxidize NADH to provide ATP for cell growth and development. It was also identified that the expression of RNA of this haemoglobin under

Table 1. Species that express stress-induced haemoglobin

Species	Reference
Parasponia	Landsmann *et al.*[83]
Trema	Bogusz *et al.*[84]
Casuarina	Christensen *et al.*[85]
Hordeum, *Triticum*, *Secale*	Taylor *et al.*[29]
Oryza	Sasaki *et al.*[86]
Chlamydomonas (*chloroplast*)	Couture *et al.*[87]
Glycine, *Pisum*, *Medicago*, *Trifolium*	Andersson *et al.*[88]
Medicago	Seregelyes *et al.*[89]
Arabidopsis	Watts *et al.*[24]
Zea	Guy and Hill[90]
Beta, *Brassica*, *Citrus*	Hunt *et al.*[91]

anoxic conditions is similar to that of a known anaerobic response gene, *ADH* and the expression is an integral part of the normal anaerobic response in barley roots[29].

Nitric oxide

NO is a highly diffusible gas and a ubiquitous bioactive molecule. Chemical properties of NO make it a versatile signal molecule that functions through interactions with cellular targets via either redox or additive chemistry. In plants many environmental and hormonal stimuli are transmitted either directly or indirectly by NO signalling cascades. The ability of NO to act simultaneously on several unrelated biochemical nodes and its redox homeostatic properties suggest that it might be a synchronizing molecule in plants. Because of its diverse role in many metabolic processes and signal transduction cascades, 'NO was declared as molecule of the year' in 1992.

NO is a bioactive molecule able to scavenge ROS and counteracts cytotoxic processes mediated by ROS in plant tissues[30]. NO is also an attractive candidate for involvement in aerenchyma formation in maize roots[31]. The effect may be either direct, through cell necrosis or through regulatory pathways and it may also be selective in relation to the cells that do respond. A similar type of reaction could be responsible for selected cell death during aerenchyma formation in roots exposed to waterlogging[32]. There is an abundance of literature on NO and programmed cell death in many mammalian tissues[33]. Similarly, NO has been implicated in programmed cell death of *Arabidopsis* cell-suspension cultures through its action on signal transduction pathways involving guanylate cyclase[34].

NO synthesis in roots may involve nitrate reductase (NR) and nitric oxide synthase (NOS) or nitrite–nitric oxide reductase (Ni–NOR). In roots, two distinct types of NR are present, one located in the cytosol (cNR) and the other attached to the plasma membrane and facing the apoplast (PM-NR)[35,36]. The potential maximum activity of activated NR, although lower than alcohol dehydrogenase, exceeds the rate of hypoxic ethanol formation by more than three-fold[37]. In *Arabidopsis* root cultures, two NR genes were induced under low oxygen (5%) pressure. The *NR1* gene showed moderate induction after 0.5–4 h and strong induction after 20 h of hypoxia. The *NR2* gene was strongly activated in 2–4 h and even more[38] after 20 h.

There is a 2.5-fold activation of cNR during exposure of plant roots to hypoxia[32], with nitrite reduction being suppressed at the NR step[39]. The limitation of nitrite reduction is connected both with cellular acidification and with increased flux through NR[37,39]. Yamasaki *et al.*[40], using purified cNR from maize showed that a side reaction of cNR is the reduction of nitrite to NO with NADH as an electron donor, probably catalysed by the same molybdenum cofactor-containing domain as in nitrate reduction. NO formation by cNR requires high nitrite concentration[41], as the *Km* of cNR for nitrite is about 300 mM. Though under natural conditions accumulation of nitrite to high concentration within the cell seems unlikely, since nitrite as well as its acid form, nitrous acid, are highly toxic[42] and nitrite is rapidly reduced by NiR, however, under anaerobic condition nitrite does accumulate *in vivo*[39]. Thus under unfavourable anaerobic conditions NO can be formed by cNR. Yamamoto-Katou *et al.*[43] reported that NR is directly involved in elicitin-induced NO production in *Nicotiana benthamiana* under pathogen stress, and availability of substrate NO_2^- may be the rate-limiting step of NO production by NR.

Plasma membrane nitrate reductase (PM-NR) activity was initially demonstrated by Ward *et al.*[44]. It is present only in root tissue where it exceeds the activity of cNR, particularly during the night[36]. It can use both NADH and succinate for reduction of nitrate to nitrite[45]. Regeneration of succinate from fumarate is facilitated by succinate dehydrogenase[46], using reduced ubiquinone produced at complex I. There is thus the possibility that the plasma membrane may have an important role in nitrate reduction during hypoxic conditions. Plasma membrane-bound Ni–NOR is the likely enzyme that converts nitrite to NO, rather than PM-NR. Ni–NOR faces the apoplast and has an activity sufficient to convert all of the nitrite formed by PM-NR to NO[47]. Ni–NOR uses reduced cytochrome *c* for nitrite conversion to NO[48]. Since participation of cytochrome *c* at the plasma membrane is unlikely, it is possible that the physiological electron donor for this reaction could be either another cytochrome or Hb, induced under hypoxic condition (Figure 1). A haem-protein oxidized during this reaction can be reduced by a protein possessing cytochrome reductase activity. The pH optimum of Ni–NOR is favourable for hypoxic conditions (pH 6.1), and it can utilize even low amounts of nitrite (V_{max} is reached at a nitrite concentration of 100 μM)[48].

Cueto *et al.*[49] characterized the NOS activity in roots and nodules of *Lupinus albus*. Fang-Qing *et al.*[50] identified an *Arabidopsis* mutant (Atnos1) that had impaired NO production. Expression of AtNOS1 in Atnos1 mutant plants resulted in overproduction of NO. Thus the results suggested that AtNOSI encodes a distinct NOS which is involved in NO production that regulates growth and hormonal signalling in plants. Available evidences indicate that there are other potential enzymatic sources of NO production in plants, including xanthine oxido-reductase, peroxidase, cytochrome P450 and some haemeproteins. Thus, in plants the enzymatic production of the signal molecule NO, either constitutive or induced by different biotic/abiotic stresses, may be a much more common event than was initially thought.

Haemoglobin and nitric oxide interaction

While hypoxic stress-induced a haemoglobins are widespread in the plant kingdom, their function has not been well elucidated. Dordas *et al.*[51] proposed that at least one

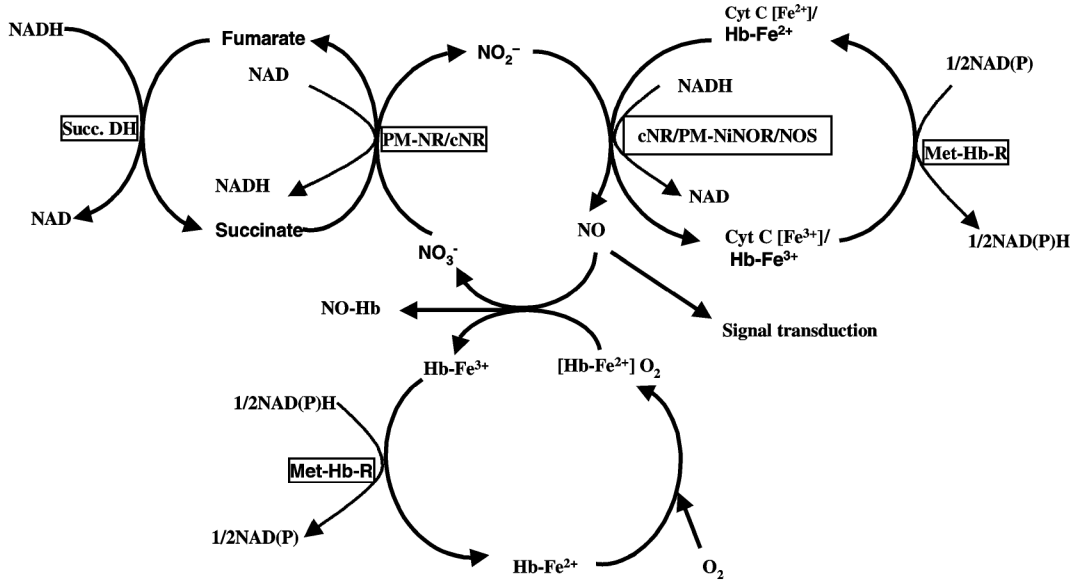

Figure 1. Nitric oxide–nonsymbiotic haemoglobin homeostasis as an alternative to fermentation and possible role in adaptation to hypoxic stress.

of the functions of hypoxic stress-induced haemoglobins is to modulate NO levels in the cell. Dordas *et al.*[51] demonstrated the presence of NO-haem (nitroso-haemoglobin) complexes in both hypoxic maize cell cultures and alfalfa root cultures using electron paramagnetic resonance spectroscopy. Similarly, Perazzolli *et al.*[52] identified that *Arabidopsis thaliana* nonsymbiotic haemoglobin AHb1, scavenges NO through production of S-nitroso-haemoglobin and reduces NO emission under hypoxic stress, indicating its role in NO detoxification. The characteristic signal for the complex is not evident in aerobic systems. Furthermore, using NO traps, it was shown that significant amounts of NO are formed in hypoxic maize cells during the first 24 h of hypoxic treatment[51]. Transformed lines with reduced stress-induced haemoglobin expression produced greater amounts of NO than wild type or overexpressing haemoglobin lines, suggesting that haemoglobin may be involved in the turnover of NO. There is also the possibility that NO may be activating guanylate cyclase, as it is known to do in defence gene induction[53]. The induction of stress-induced haemoglobin in *Arabidopsis* in the presence of elevated nitrate may also relate to a requirement to modulate NO levels[54]. Stress-induced haemoglobins have been implicated in the regeneration of NAD^+ during hypoxia[23], based on the observations that alcohol dehydrogenase activity and CO_2 production are reduced under hypoxia in maize cells constitutively expressing barley haemoglobin[27].

It has been suggested that NO might be involved in a series of reactions to assist in the regeneration of NAD^+ to maintain glycolysis under hypoxic conditions, as an alternative to the use of ADH. Nitrate ion has been shown as a terminal electron acceptor in the denitrification process in flooded soils and has been suggested to play a similar role in plants[55]. NO reacts rapidly with oxyhaemoglobin forming nitrate and methaemoglobin [Hb(Fe^{+3})]. This route for metabolism of NO, with nitrate being recycled, would be advantageous to the hypoxic plant cells exposed to conditions of prolonged soil waterlogging, where nitrates would be severely depleted. Haemoglobins (Fe^{+3}) can be reduced to Hb(Fe^{2+}) via NADH-dependent reductases[56]. This reaction would provide an additional NAD^+ for glycolysis. Thus reduction of nitrate by NR to produce NO and its subsequent oxidation by oxyhaemoglobin have been suggested as mechanisms to maintain plant cell energetics during hypoxia/anoxia[57,58]. Hypoxia-induced (class 1) haemoglobins remain in the oxyhaemoglobin form even at oxygen tension two orders of magnitude lower than that necessary to saturate cytochrome C oxidase. They act, probably in conjunction with a flavoprotein, as NO dioxygenases converting NO back to nitrate, consuming NAD(P)H in the process. The overall system oxidizes 2.5 moles of NADH per one mole of nitrate recycled during the reaction, leading to the maintenance of redox and energy status during hypoxia and resulting in the production of reduced amount of ethanol and lactic acid. Thus they play a major role as an alternative to classical fermentation pathways.

Plant roots that express sufficient haemoglobin soon after exposure to hypoxic stress may modulate levels of NO, produced as a result of the stress, either through reaction of NO with oxyhaemoglobin or through formation of nitrosyl-haemoglobin. This would prevent the accumulation of toxic ethanol and lactic acid, maintenance of ATP levels and energy charge, and delay the onset of cell death[38]. In primary roots, this may provide sufficient time for the plant to develop adventitious roots, needed for prolonged survival under hypoxia. A suggested pathway

of interaction of NO and nonsymbiotic haemoglobins in maintaining redox homeostasis in hypoxic cells is presented in Figure 1.

Reactive oxygen species production and antioxidants

Excessive generation of ROS or oxidative stress is an integral part of many stress situations, including hypoxia. Hypoxic tissues exhibit enhanced ROS generation via xanthine oxidase-mediated acetaldehyde dependent superoxide ($O_2^{\cdot-}$) radical formation. Anaerobic tissue has a high redox potential and the soil environment surrounding the roots contains highly reduced forms of metal ions such as Fe^{2+}, which can readily reduce atmospheric oxygen to superoxide. Therefore, in the interim between return to high oxygen partial pressures and reactivation of the mitochondrial electron transport system, conditions are ideal for the activation of oxygen. Lipoxygenase action on membrane lipids, followed by lipolytic acyl hydrolase-catalysed liberation of FFA underpin a burst in lipid peroxidation on return to normoxia. The main cellular components susceptible to damage by free radicals are lipids (peroxidation of unsaturated fatty acids in membranes), proteins (denaturation), carbohydrates and nucleic acids. Consequences of hypoxia-induced oxidative stress depend on tissue and/or species, membrane properties, endogenous antioxidant content and the ability to induce response in the antioxidant system.

It was observed in a study of soybean roots that a short anoxic stress (1–2 h) increased the potential for superoxide production[59]. Incubation in the presence of exogenous ascorbic acid alleviated post-anoxic injury in these roots. Hydrogen peroxide accumulation under hypoxic conditions has been shown in the roots and leaves of *Hordeum vulgare*[60] and in wheat roots[61]. The presence of H_2O_2 in the apoplast and in association with the plasma membrane has been visualized by transmission electron microscopy under hypoxic conditions in four plant species[62]. Indirect evidence of ROS formation (i.e. lipid peroxidation products) under low oxygen has also been reported[63]. Using diphenylene iodonium chloride, a specific inhibitor of membrane-bound NADPH oxidase, and gene expression studies, it has been shown that there is an increase in NADPH oxidase activity and increased NADPH oxidase–mRNA expression and accumulation of $O_2^{\cdot-}$ and H_2O_2 under hypoxic condition in pigeon pea genotypes[64]. These studies suggest that there is ROS production even during hypoxia, which probably is due to the induction of membrane-bound NADPH oxidase.

There are only a few reports on investigations of the changes in the activity of components of the antioxidative system in response to anoxic or hypoxic conditions. Monk *et al.*[65] reported an increase in superoxide dismutase (SOD) activity when the rhizomes of *Iris pseudacorus* were flooded. Van Toai and Bolles[59] suggested that it probably has a critical role in the survival of the plant when oxygen levels increase as the flooding stress abates. Induction of enzymes involved in the Haliwell–Asada pathway has been shown for anaerobically germinated rice seedlings after transfer to air[66]. Roots of wheat (*Triticum aestivum*) seedlings could cope with the deleterious effects of oxygen radical generation due to post-hypoxia aeration by increasing the glutathione reductase (GR) activity and the glutathione content[67].

Investigations involving 11 species with contrasting tolerance to anoxia have revealed an increase in MDHAR and DHAR in the anoxia-tolerant plants after several days of anoxic treatment. In the intolerant plants activities were low or without any changes. Reduced glutathione (GSH) decreased significantly during the post-anoxic period, while ascorbic acid (AA) showed increased values in the tolerant species[68]. In case of pigeon pea, six-days of waterlogging caused a continuous increase in the activity of SOD, ascorbate peroxidase (APX), GR and catalase (CAT) in tolerant genotypes, while in susceptible genotypes the activities of all the enzymes started declining from the second day onwards[69]. Waterlogging-induced decline in the activity of antioxidant enzymes has also been reported. Biemelt *et al.*[70] reported a slight decrease in the activities of MDHAR, DHAR and GR, or no change in the roots of wheat seedlings under hypoxia, while anoxia caused significant inhibition of enzyme activities and a significant increase in the reduced forms of ascorbate and glutathione. Nevertheless, a rapid decrease in the redox state of both antioxidants was observed during reaeration. Inhibition of GR, APX, CAT and SOD activities has been reported in corn leaves under prolonged flooding, while a short-term treatment led to an increase in the activities[71].

Hypoxic signalling and gene regulation

Hypoxia/anoxia is one of the most important abiotic stresses encountered by most of the higher organisms. Genetic analysis indicates that tolerance is a fairly simple, dominant trait. A recessive factor that increases anaerobic tolerance in plants that are null for ADH activity has been reported in maize[72,73]. Subbaiah and Sachs[74] have also demonstrated how a simple post-translational modification of sucrose synthase by the addition/removal of phosphate can lead to potent changes in the tolerance of seedlings to anoxia.

The coordinate expression of the ANP is accomplished by a common trans-acting factor that interacts with an anaerobic responsive element (ARE) in the promoter region of each gene[75]. Oxygen deficiency changes its conformation, or the nature of its binding to ARE, and thereby promotes transcription. Aerobic mRNA is not translated under anaerobic condition in maize roots, whereas those for ANP are translated, presumably reflecting the recognition of a specific anaerobic signal on the mRNA.

Finally, the anaerobic mRNA is much more stable and has a longer half-life under oxygen deficiency[20].

The anaerobic stress-response of maize offers an opportunity to characterize the regulatory components of a family of 20 genes that are coordinately expressed. The anaerobically induced proteins appear to be encoded by a set of genes, whose expression is stimulated by a deprivation of oxygen. Regulation of protein synthesis under anaerobiosis appears to occur at multiple levels. Subbaiah and Sachs[74] have characterized several genes involved in the anaerobic response, and have demonstrated that Ca^{2+} acts as a key transducer of changes in O_2 availability. An additional aim should be to characterize the promoter elements of the anaerobically induced genes as well as the signalling components downstream to calcium that trigger gene induction.

Dennis *et al.*[21] reported the first results with the hypoxia-inducible AtMYB2 transcription factor. They further reported that transgenic plants may clarify the physiological role of the fermentation pathways, and their contribution to flooding tolerance[21]. Lee *et al.*[76] investigated the transcriptional expression *in vitro* for low oxygen treatment. They reported dramatic increase in the transcript of *TaMyb1* (*Triticum aestivum* Myb transcription factor 1) gene under hypoxia. The transcriptional expression of *TaMyb1* was enhanced by light under hypoxia. *TaMyb1* expression was high in the epidermis, endodermis and the cortex adjacent to the endodermis under hypoxia. *TaMyb1* transcription levels in roots also gradually increased as the result of treatment with ABA, PEG and NaCl. They suggested that the expression of *TaMyb1* in roots could be strongly related to the oxygen concentration in root environment.

In plants, the involvement of Rop signalling in response to hypoxia was revealed by a screen of *Arabidopsis* seedlings that possess a Ds-GUS transposon gene-trap element for genes induced by low oxygen levels[77]. The screen yielded ropgap4-1, a loss-of-function mutant generated by a Ds-GUS insertion in the first exon of ROPGAP4. The ropgap4-1 mutant seedlings induced significantly higher levels of ADH mRNA and specific activity than wild-type seedlings, hinting that ROPGAP4 negatively regulates ADH induction. Rop activation is a prerequisite for hypoxic induction of ADH, because a line overexpressing a dominant negative mutant of ROP2 (35S:DN-rop2) that stably binds GDP, showed no detectable increase in ADH mRNA and enzymatic activity in response to the stress. By contrast, a line producing a mutant ROP2 that constitutively binds GTP (35S:CA-rop2), had elevated ADH activity under control growth conditions[78]. Consistent with this hypothesis, Rop–GTP levels increased dramatically within 1.5 h of hypoxia and then decreased between 12 and 24 h of oxygen deprivation in wild-type seedlings.

The ropgap4-1 seedlings had a brownish, water-soaked appearance after oxygen deprivation, similar to that caused by oxidative stress. This led to the finding that hypoxia promoted an increase in H_2O_2. Significantly higher levels of H_2O_2 were measured in extracts from ropgap4-1 mutants, whereas no rise in H_2O_2 was detected in 35S:DN-rop2 seedlings. In wild-type, ropgap4-1 and 35S:CA-rop2 seedlings, increases in H_2O_2 and ADH activity were inhibited by diphenylene iodonium chloride, an inhibitor of flavin-binding NADPH oxidase. By contrast, ADH activity was induced under 'aerobic' conditions when H_2O_2 was enzymatically generated on the surface of seedlings[77]. These observations support the conclusion that ROS production is a component of the pathway that induces ADH expression under low oxygen conditions[79]. The role of ROS signalling in the induction of transcription factors associated with genes of antioxidant enzymes has also been reported[80,81]. It is thus possible that hypoxia-generated ROS also induce the genes of antioxidant enzymes and *ADH* gene, resulting in the observed increase in the activity of various antioxidative enzymes and ADH.

NO has been reported as a signalling molecule in a wide range of responses in animals and plants. The accumulated evidence suggests that a metabolic pathway involving NO and Hb provides an alternative type of respiration to mitochondrial electron transport under limited oxygen. Hb in hypoxic plants acts as part of a soluble terminal NO dioxygenase system, yielding nitrate ion from the reaction of oxyHb with NO. NO is mainly formed due to anaerobic accumulation of nitrite. The overall reaction sequence, referred to as the Hb/NO cycle, consumes NADH and maintains ATP levels by promoting glycolysis[57]. Hb gene expression appears to influence signal transduction pathways, possibly through its effect on NO, as evidenced by phenotypic changes in normoxic Hb-varying transgenic plants. Ethylene levels are elevated when Hb gene expression is suppressed, which could be a factor leading to root aerenchyma formation during hypoxic stress. Interestingly, both NO and H_2O_2 have recently been found to function as localized and long-range root-derived signals capable of rapidly communicating the redox status and indirectly activating MAP kinase-like activity in the shoots of *A. thaliana*[82]. Figure 2 summarizes various signalling components, genes/enzymes and responses associated with hypoxia tolerance.

Conclusion and perspectives

Examination of regulatory mechanisms and signalling events responsible for triggering responses to oxygen-deficient condition in plants is an interesting area of research. Advances in genome biology, genetic resources and high throughput technologies provide excellent resources for the exploration of oxygen-sensing mechanisms in plant cells. It is imperative to identify sensors and dissect the signalling pathways that occur at the

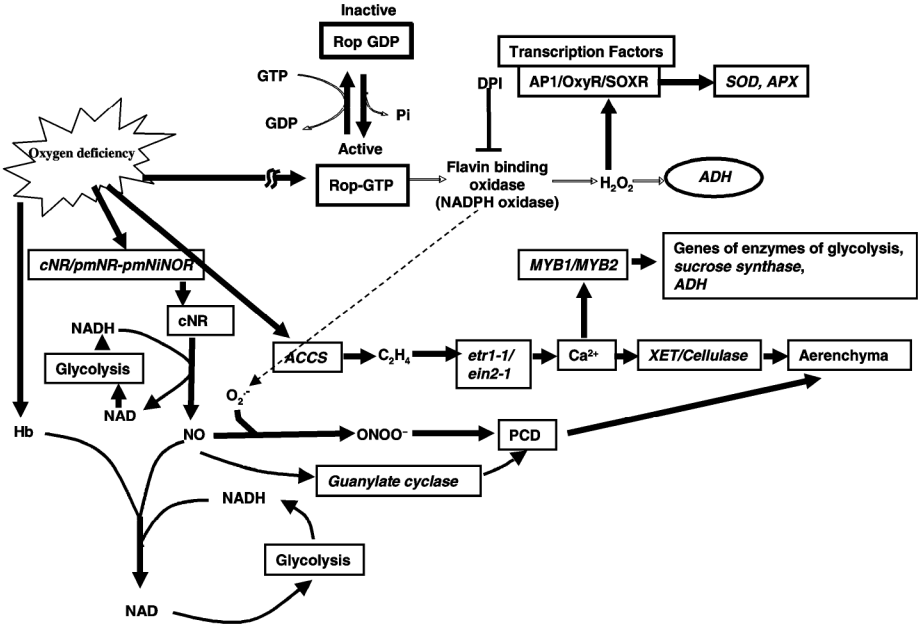

Figure 2. Hypoxia signalling and induction of various genes and associated responses.

cellular, tissue, organ and whole-plant level. Again, the paradoxical ROS may prove to be the second messenger in the response mechanism. Therefore, it is important to investigate the participation of an ROS-sensing mechanism involving PM-NADPH oxidase in different plants species. Further, it will be interesting to determine whether observed increase in NO evolution under flooding condition from roots or soils can positively contribute message in the root-to-shoot communication. Analyses of near-isogenic genotypes that differ in the adaptive response to oxygen deprivation are likely to yield critical information on regulatory mechanisms. Alterations in cytosolic pH and calcium may also have a role in the signalling processes. The importance of changes in adenylate charge, redox status and carbohydrate levels must also be considered. Many questions remain to be answered about the response of individual cells. What could be the basis of differential response between stress-tolerant and intolerant organs and species? Do these differ in cellular signalling and response mechanisms? The involvement of growth regulators such as ethylene, auxin, gibberellins and ABA in hypoxic regulation is also an interesting possibility. The manner in which the energetic needs of meristematic cells are safeguarded and how programmed cell death is promoted or avoided, also need examination. How do cells in the roots and aerial organs communicate over a long distance when there is an oxygen crisis in the roots? Understanding the cell-to-cell and long-distance signalling mechanisms that determine the organ and whole-plant response to oxygen deprivation, viz. regulation of leaf and internode elongation, petiole curvature, aerenchyma formation and adventitious root growth is another inviting area for research. So far we only know a part of the unfolding story, with many more questions still unanswered. Answering these questions will be of relevance to agriculture and will provide knowledge about the fundamental nature of anaerobic plant life.

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