

Utrecht University

Oxygen sensing mechanisms in plants

Valerie Hemelaers

Supervisor: Rashmi Sasidharan

Utrecht University

Biology department

April 2012



Preface:

The goal of this thesis is to reflect on the knowledge that I have obtained during my master environmental biology at the Utrecht University. I would like to thank to all the staff from the biology department at the Utrecht University and in particular my mentor Rashmi Sasidharan, who guided and supported me through the writing process.

Summary:

Oxygen sensing mechanisms are important for organisms to adapt their survival strategy when faced with low oxygen conditions. These survival strategies are morphological, physiological and biochemical adaptations to the lack or reduction in oxygen. Oxygen is a major electron acceptor that is needed for the aerobic metabolism. For plants during floods, oxygen diffuses slower in water than in air, creating an oxygen deficit. This results in the plant's biochemical adaptation from an aerobic to an anaerobic respiration. This is achieved through the glycolysis and the fermentation pathway. During flooding, ethylene also accumulates and serves as a signal to activate hypoxic responsive-genes. Other organisms such as bacteria, fungi and animals have oxygen sensing mechanisms. In these mechanisms, the heme protein plays a central role as an oxygen sensor. For plants, the group VII of ethylene response factor (ERF) transcription factors seems to have an important role as a mediator for oxygen sensing. The group VII of ERF transcription factors are regulated through the N-end rule proteolysis pathway. The N-end rule pathway has an oxygen dependent sequence which would be responsible for oxygen sensing. The conserved sequence of the N-end rule pathway is responsible for the degradation of the ERF under aerobic conditions. When there is no more oxygen available, the oxidation of cysteine in the N-end rule pathway cannot occur. This leads to the inhibition of the degradation pathway. The research around direct oxygen sensing mechanisms for plants still needs to be completed, but I believe that recent studies are close to find the answer behind the 'unknown' factor that is behind the activation and regulation group VII of ERF transcription factors and/or N-end rule proteolysis pathway.

INTRODUCTION:

In this thesis, I investigate the oxygen sensing mechanisms in plants. In the first chapter a general description is given of the adaptive responses that plants undergo during oxygen deprivation. This chapter gives an understanding of all the pathways that are activated and repressed during hypoxia in plants. In the second chapter, the sensing mechanisms in other organisms (fungi, bacteria and animals) are evoked. There are many similarities between organisms for oxygen sensing mechanisms. Understanding similarities and differences between other organisms gives insights to further insight that I will explore in the last chapter. In chapter 4, I will go into detail to the current knowledge of oxygen sensing mechanism in plants and I will present my own view on the gaps in the current knowledge in the last chapter.

Content

Preface:	1
Summary:	2
INTRODUCTION:	3
Chapter1: WHEN ARE PLANTS DEPRIVED OF OXYGEN AND WHAT ARE THE ADAPTIVE RESPONSES TO COMPENSATE FOR THE ENERGY CRISIS CAUSED BY OXYGEN DEPRIVATION?	5
Introduction:.....	5
WHEN ARE PLANTS DEPRIVED OF OXYGEN?	5
WHAT ARE THE ADAPTIVE RESPONSES?	5
Biochemical changes:	5
Morphological changes:	7
Summary:	8
Conclusion:	9
Chapter 2: WHAT ARE THE OXYGEN SENSING MECHANISMS IN OTHER ORGANISMS?	10
Introduction:.....	10
OXYGEN SENSING MECHANISMS IN BACTERIA	10
OXYGEN SENSING MECHANISMS IN ANIMALS	11
OXYGEN SENSING MECHANISMS IN FUNGI	13
Summary:	14
Conclusion:	15
Chapter 3: THE INDIRECT AND DIRECT OXYGEN SENSING MECHANISMS IN PLANTS	16
Introduction:.....	16
The indirect and direct oxygen sensing mechanism:	16
N-end rule degradation pathway:.....	17
Summary:	17
Conclusion:	18
Chapter 4: CONCLUSIONS AND FURTHER RESEARCH	19
Introduction:.....	19
Main Conclusions:.....	19
Future research possibilities:.....	20
List of abbreviations:.....	22
References:.....	26

Chapter1: WHEN ARE PLANTS DEPRIVED OF OXYGEN AND WHAT ARE THE ADAPTIVE RESPONSES TO COMPENSATE FOR THE ENERGY CRISIS CAUSED BY OXYGEN DEPRIVATION?

Introduction:

This chapter will address the conditions from when plants are deprived of oxygen and continue with the adaptive responses as a result from oxygen deprivation. These responses lead to biochemical and morphological changes in a plant. The oxygen sensing mechanisms are most likely linked to the adaptive responses that is why it is important to have a full understanding of this subject.

WHEN ARE PLANTS DEPRIVED OF OXYGEN?

Oxygen is a major electron acceptor, which is used in the oxidative phosphorylation pathway for the production of ATP. A plant has an oxygen deficit, when oxygen levels drop below 21% (Geigenberger P. 2003). Plants can be deprived of oxygen due to a change in external environment like submergence, waterlogging, flooding and a higher microbial activity. Also during times and in dense tissues when the metabolic rate of a plant is very high, a lack of oxygen might occur. During waterlogging, submergence and flooding, the root system is flooded and diffusion rate of O₂, CO₂ and ethylene is strongly reduced. These conditions trigger adaptive responses in the plant.

WHAT ARE THE ADAPTIVE RESPONSES?

It has been observed that a plant demonstrates adaptive responses to low oxygen levels. These adaptive responses are biochemical and morphological changes.

Biochemical changes:

The biochemical changes reduce the plants energy consumption by halting the synthesis of sucrose, amino acids, proteins and lipids and by giving a decrease in wound response (Geigenberger P. 2003). This conserved energy is redirected for the transcription and translation of stress proteins that are needed for the plants survival. These stress proteins are called anaerobic polypeptides (ANP) (Drew M.C. 1997). For example in seedlings of maize, there are many ANPs produced during oxygen deprivation in their roots. These ANPs (sucrose synthetase, glucose phosphate isomerase, fructose 1,6 biphosphate aldolase, alcohol dehydrogenase 1 and 2 (ADH), pyruvate decarboxylase (PDC), lactate dehydrogenase (LDH)) are all part of the glycolysis and the fermentation pathway (see Figure 1).

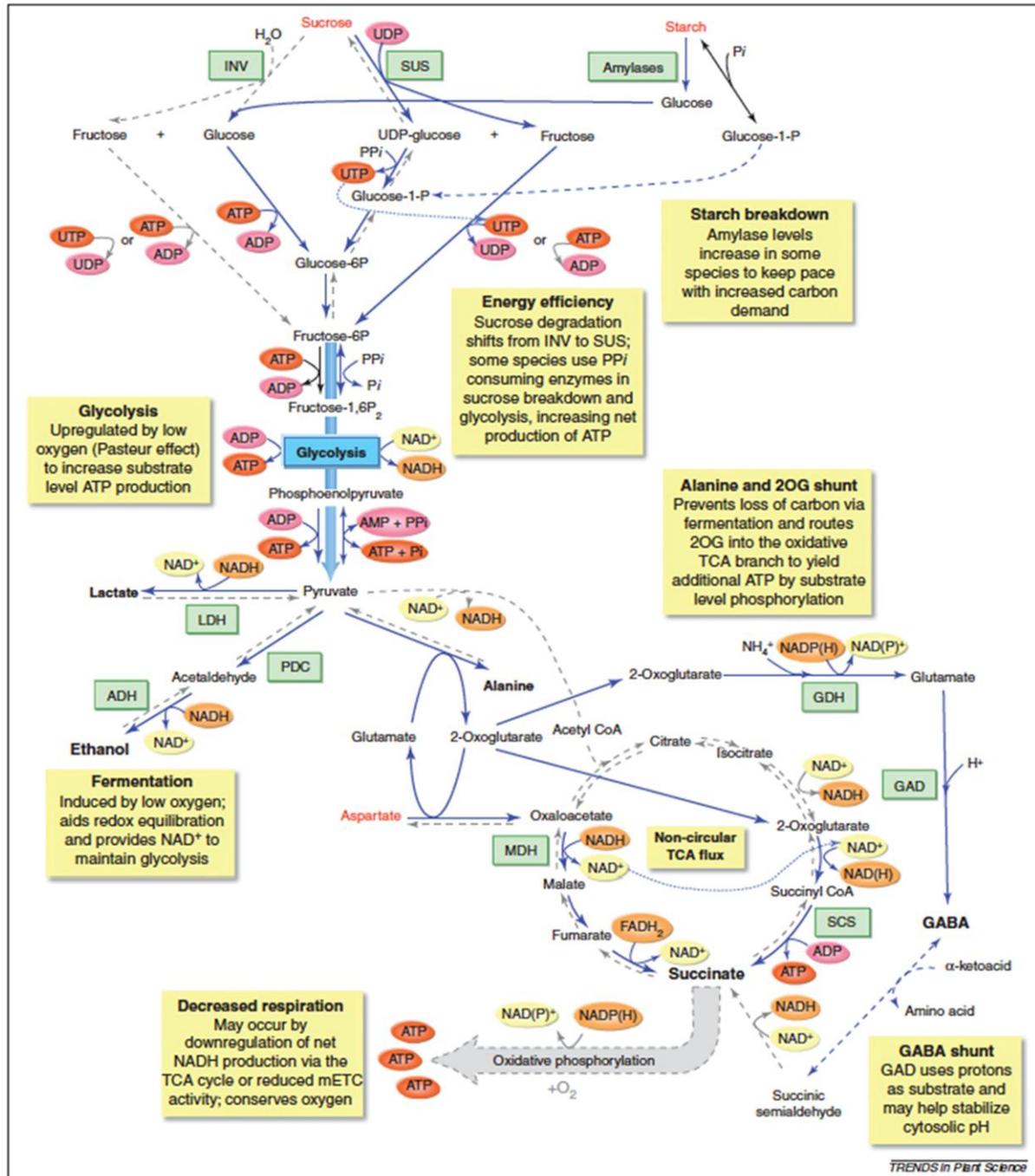


Figure 1. Representation of the energy conserving pathways within a plant during oxygen deficiency. When oxygen levels drop below 21%, transcription and translation of anaerobic polypeptides (ANP) begins. These ANPs (sucrose synthetase, glucose phosphate isomerase, fructose 1,6 biphosphate aldolase, alcohol dehydrogenase 1 and 2 (ADH), pyruvate decarboxylase (PDC), lactate dehydrogenase (LDH)) are involved in the glycolysis and fermentation pathway. To prevent carbon loss in the form of ethanol from the fermentation pathway, the 'alanine and 2-oxoglutarate shunt' (2OG) promotes the production of succinate in the tricarboxylic acid (TCA) cycle. These anaerobic pathways will provide less energy than the aerobic pathways and lower the metabolic rate of the plant during low oxygen conditions. (Figure from 'Making sense of oxygen sensing' by Bailey-Serres et al. 2012).

The glycolysis and fermentation pathways are activated during low oxygen conditions. The ANPs from the fermentation pathway produce NAD^+ which is needed for other pathways. However at the end of the fermentation pathway, ethanol is remaining as end-product which diffuses in the external environment, thus forming a carbon loss. If pyruvate is converted to alanine instead of going through the fermentation pathway, then the carbon loss can be avoided. This alternative pathway which prevents carbon loss is called 'the alanine and 2-oxoglutarate shunt'. Here 2-oxoglutarate is formed which leads to the formation of succinate through succinate CoA ligase (SCS) in the tricarboxylic acid (TCA) cycle. These anaerobic pathways makes the pH within the plant more acidic. However, the pH can be regulated through the gamma-aminobutyric acid (GABA) shunt. Here glutamate decarboxylase (GAD), the rate limiting enzyme in GABA synthesis, uses protons as substrate and may help stabilize cytosolic pH. The plant can produce less energy during ATP synthesis because of the low gradient in the electron transfer chain in the mitochondria. Due to the down regulation of the NADH production in the Krebs Cycle or the reduced mitochondrial electron transport chain (mETC) activity leads to a decreased metabolic activity which leads to the conservation of energy (see Figure 1). (Bailey-Serres et al. 2012).

Morphological changes:

Ethylene accumulates during hypoxia and induces a plant's survival strategy embedded in its genome.

There are two types of strategies: avoidance and quiescence strategy.

The quiescence strategy is used during flash flooding in rice plants when they are submerged for a maximum of 14 days. Rice cultivars that are tolerant to flash flooding stop elongation to consume less energy. This energy will be needed for re-growth and recovery from reactive oxygen species (ROS). When flooding is from the deepwater type, tolerant rice plants use the avoidance strategy. This strategy consists of elongation until leaves reach the water surface. This

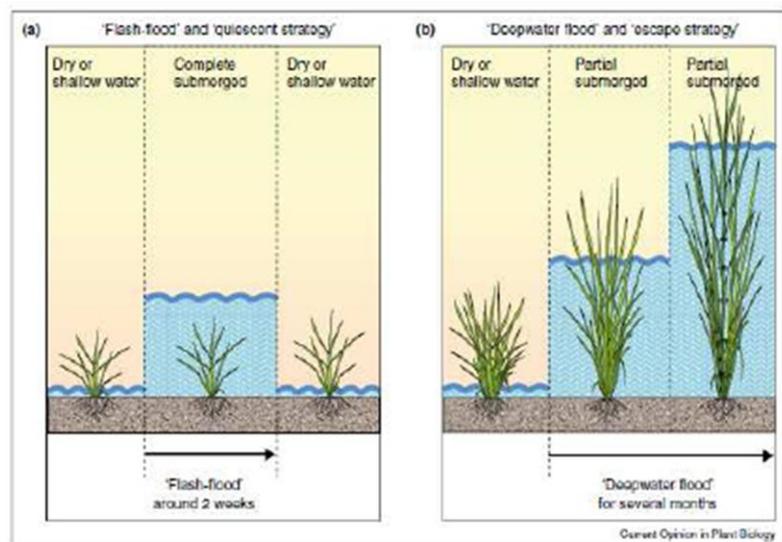


Figure 2. Adaptive responses during flash floods (a) which lasts for 2 weeks and deepwater floods (b) which lasts several months. During flash floods the quiescence strategy is used to conserve energy until the waters recede. For the escape strategy, the plant will elongate to reach the surface and therefore restore aerobic respiration. (Figure from Rice growth adapting to deepwater. Hattori et al 2011).

process consumes a lot of carbohydrates, but if the foliage can reach the surface, respiration and photosynthesis can be resumed (see Figure 2) (Hattori, Nagai, & Ashikari, 2011).

Both the traits of tolerance to flash water and deepwater, are quantitative trait loci in rice (QTL). This means that this phenotypic characteristic is inherited and can be attributed to the interaction of genes with their environment (Hattori, Nagai, & Ashikari, 2011). The QTL of tolerant rice cultivars that are responsible for using the quiescence strategy is located on the Submergence1 (Sub1) locus on chromosome 9. For rice cultivars with the avoidance strategy, the QTL is located on the SNORKEL (SK) genes. In both these strategies, ethylene is accumulated. However only in the avoidance strategy elongation occurs. The elongation is due to activation of the SK genes by ethylene during submergence. These SK genes will regulate downstream genes that induce gibberellic acid (GA) accumulation. This accumulation induces internode elongation. For the quiescence strategy, ethylene accumulation induces the activation of Sub1, which is responsible for the inhibition of shoot elongation and carbon consumption. (Hattori, Nagai, & Ashikari, 2011).

These genetic factors and signaling pathways responsible for the avoidance and quiescence strategy are part of group VII of the ethylene response factor (ERF) transcription factor. These are believed to play an important role in the direct oxygen sensing mechanisms in plants. (see chapter 3)

Summary:

There are several ways a plant can have limited access to oxygen, these are submergence, waterlogging, flooding and a higher microbial activity. Therefore, adaptations have to be made when oxygen as an electron acceptor is no longer available. These adaptations are biochemical and morphological. When the aerobic pathway is no longer available anaerobic polypeptides (ANPs) are transcribed and translated. These are necessary to drive the glycolysis and fermentation pathway. Because ethanol is an end product of the fermentation pathway which leaks into the external environment, the alanine and 2 oxoglutarate shunt is used as an alternative pathway. Here, 2-oxoglutarate is eventually transformed to succinate in the Krebs Cycle. The diminished production of NADP⁺ will serve as signal to the plants to lower its metabolic activity.

Survival strategies are embedded in a plant's genome. During floods, ethylene accumulates and serves as a signal to initiate survival strategies. When a flash flood occurs, plants with the quiescence strategy imbedded on their Sub1 locus, will conserve energy and produce antioxidants against the damage from ROS in the photosynthetic machinery. Once the photosynthesis is protected, the quiescent type can proceed to other metabolic functions like growth. Plants with the avoidance strategy embedded in their SK genes will elongate in attempt to reach the surface

and resume aerobic metabolism. These genes are part of group VII of the ethylene response (ERF) transcription factors. These are believed to play an important role in the oxygen sensing mechanisms. This topic will be further elaborated in chapter 3.

Conclusion:

The adaptive responses of a plant during hypoxia are of great importance to conserve energy. This change of strategy during periods of limited oxygen availability needs to occur swiftly. This entails that a plants could have an oxygen sensing mechanism that activates the transcription and translation of genes responsible for these adaptive responses. However, I could be that plants only activate these adaptive responses when energy levels are low or because the pH becomes more acidic (see chapter 3). But since oxygen sensing mechanisms have been discovered in other organisms (see chapter 2) , it could be assumed that plants also have direct oxygen sensing mechanism.

Chapter 2: WHAT ARE THE OXYGEN SENSING MECHANISMS IN OTHER ORGANISMS?

Introduction:

This chapter will give an understanding of different oxygen sensing mechanisms for bacteria, animals and fungi. Evidence for oxygen sensing mechanisms has been extensively investigated for many organisms. However, for plants there are still some gaps in the knowledge that need to be investigated. These new insights on oxygen sensing mechanisms are crucial to engineer flood resistant crops. These improved crops, would have great importance for populations who rely on agriculture. By comparing oxygen sensing mechanisms between different organisms, there might be possible interesting similarities found with plants.

OXYGEN SENSING MECHANISMS IN BACTERIA

There are two oxygen sensing mechanisms that have been found for the facultative anaerobe *Escherichia coli*. These cytoplasmatic heme containing sensors are: the two-component ArcAB system and the fumarate and nitrate reduction (FNR) transcriptional regulator. These systems react to environmental signals, that is why bacteria can adapt in many habitats. Also these bacterial oxygen sensors usually have an N-terminal histidine kinase sensory domain and a C-terminal cytoplasmic transmitter domain. (Oshima T. et al, 2002)

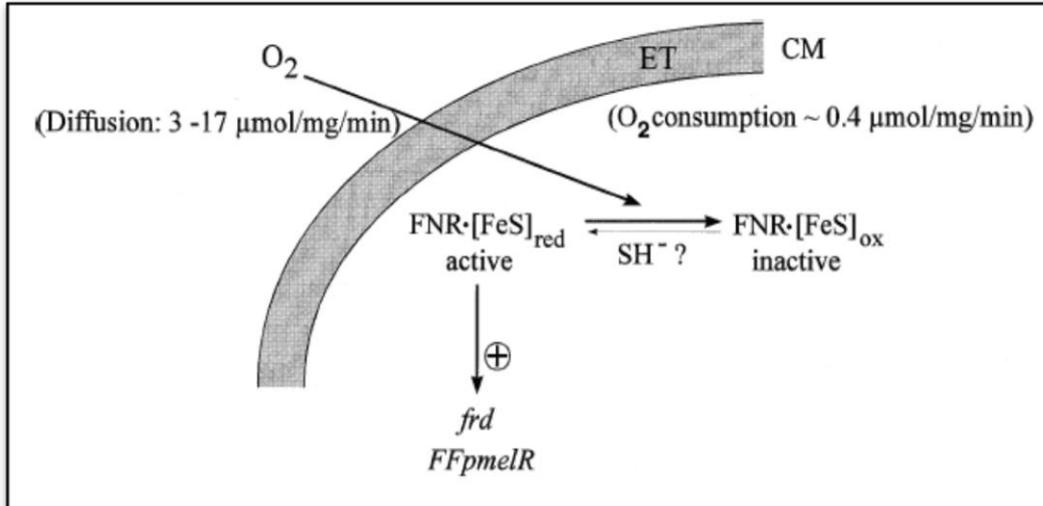


Figure 3. The fumarate and nitrate reduction (FNR) transcriptional regulator. This bacterial oxygen sensing mechanisms constructed in two steps which are reversible. First step: during normoxia, the diffusion rate of oxygen is 3 to 17 $\mu\text{mol/mg/min}$. FNR is inactivated due to the oxidation. During hypoxia, the FeS cluster becomes reduced. FNR takes its homodimeric form, binds to DNA. This leads to the expression of anaerobic respiratory processes. (Figure from Unden G. and Schirawski J., 1997. The oxygen-responsive transcriptional regulator FNR of *Escherichia coli*: the search for signals and reactions).

The fumarate and nitrate reduction (FNR) transcriptional regulator, regulates the genes that need to be expressed during hypoxia and also acts as an oxygen sensing mechanism. When sufficient oxygen levels are present, FNR is oxidized and is inactive and has a heterodimeric form (see Figure 3). FNR contains a $[4\text{Fe}-4\text{S}]^{2+}$ cluster per subunit and has special redox properties. When this cluster is exposed to oxygen it converts to a $[2\text{Fe}-2\text{S}]^{2+}$ cluster. This leads to a conformational change of FNR from a homodimer to a heterodimer form. This conformational change results in the unbinding of FNR to DNA during normoxia. (Kiley P.J. and Beinert H., 2003). These redox properties lead thus to the deactivation of FNR during normoxia and activation of genes that are responsible for expressing anaerobic respiratory processes during hypoxia.

Another oxygen sensing mechanism in bacteria is the two component system ArcAB. The ArcAB system regulates many operons that are involved in the citric acid cycle and energy metabolism (Gunsalus R.P. and Park S.J., 1994). The ArcAB system consists of a membranous sensor ArcB and a response regulator ArcA (see figure 4). The ArcB sensor possesses a C-terminal extension with a receiver domain and a second histidine protein kinase domain that is located in the membrane of the bacteria. During absence of oxygen, ArcB autophosphorylates and activates ArcA. This activation is inhibited during normoxia by quinones which are then oxidized (Georgellis D. et al, 2001). The transphosphorylation of ArcA during hypoxia by ArcB leads to the repression of the genes that are responsible for the aerobic metabolism and activates genes for anaerobic metabolism (Malpica R. et al, 2006; Luo C.C. et al, 2009).

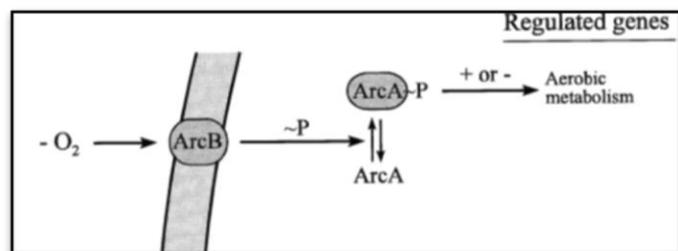


Figure 4. The ArcAB system The oxygen sensor ArcB and the response regulator ArcA. During hypoxia ArcB activates ArcA through transphosphorylation, leading to the activation of genes for the anaerobic metabolism. (Figure from Uden G. and Schirawski J., 1997. The oxygen-responsive transcriptional regulator FNR of *Escherichia coli*: the search for signals and reactions).

OXYGEN SENSING MECHANISMS IN ANIMALS

The oxygen sensing mechanism in animals is regulated by a heme protein (see Figure 5). This heme protein has two subunits (gp91 and p22) and contains an iron molecule and a flavin group (FL). When this heme protein comes in contact with oxygen, it is transformed to peroxide, through the proton gift of NADPH. Then reactive oxygen species (ROS) are formed from oxygen. This last reaction is catalyzed by iron and is called the Fenton reaction. The formation of ROS degrades the hypoxia-inducible factor-1 α (HIF-1 α). This HIF-1 α together with aryl hydrocarbon

receptor nuclear translocator (ARNT or also called HIF-1 β) forms together a hypoxia-inducible factor (HIF). The formation of this heterodimer is thus repressed during normoxia. (Hochachka P.W. Et al. 2001)

During hypoxia, HIF-1 α is not repressed and forms a heterodimer with ARNT. This

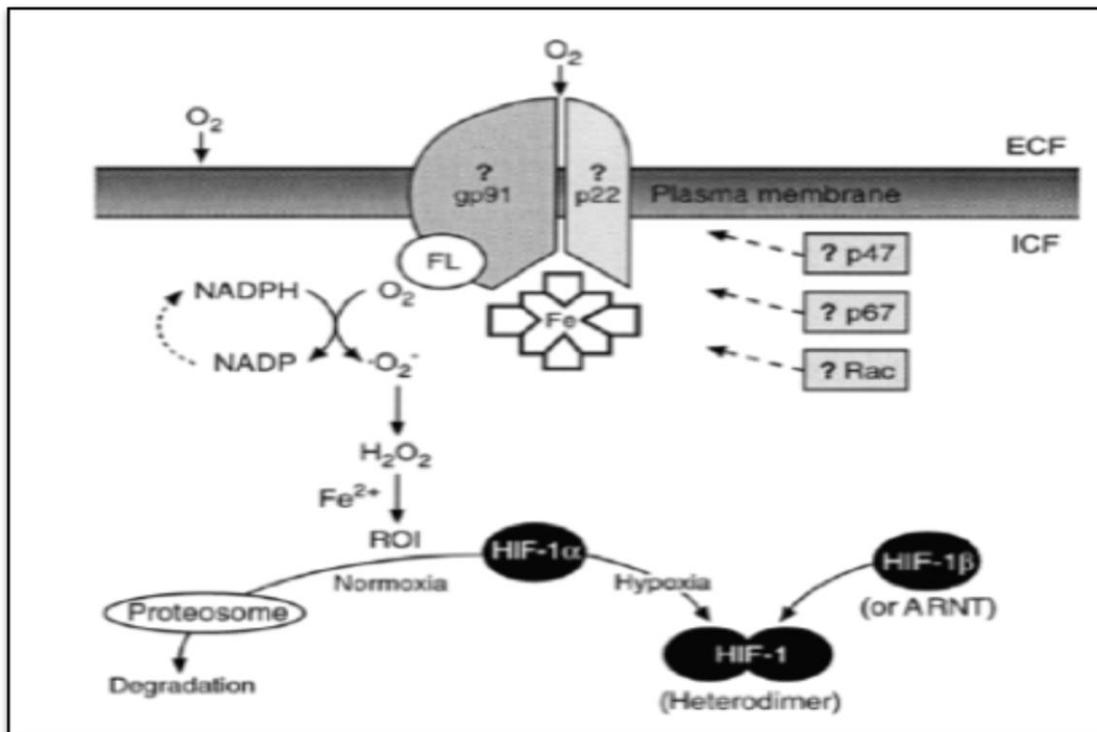


Figure 5. Oxygen sensing mechanism for animals. A heme protein containing iron and a flavin group (FL) represses the formation of HIF-1 α through the formation of reactive oxygen species during normoxia. When oxygen is no longer available, HIF-1 α is not repressed and can bind to aryl hydrocarbon receptor nuclear translocator (ARNT or also called HIF-1 β) and form hypoxia-inducible factor (HIF). (Figure from Hochachka, P.W. and Lutz, P.L. (2001). Mechanism, origin, and evolution of anoxia tolerance in animals.)

heterodimer is transported to the nucleus of the cell. There this heterodimer binds to DNA. For liver cells, for example, this will lead to the production of erythropoietin (EPO), phosphoglycerate kinase (PGK) and lactate dehydrogenase (LDH). These will help an animal acclimatize to the oxygen deficiency. In muscle cells a different pathway is used that does not involve HIF-1 α (see Figure 6). Here during normoxia, Sp3 is used as a repressor and is bound to Sp1. When oxygen is no longer available, Sp3 becomes reduced and detaches from Sp1. Sp1 is transported to the nucleus and binds to DNA. This leads to the formation of PGK and enolase in the myocyte. (Hochachka, P.W. and Lutz, P.L. (2001)

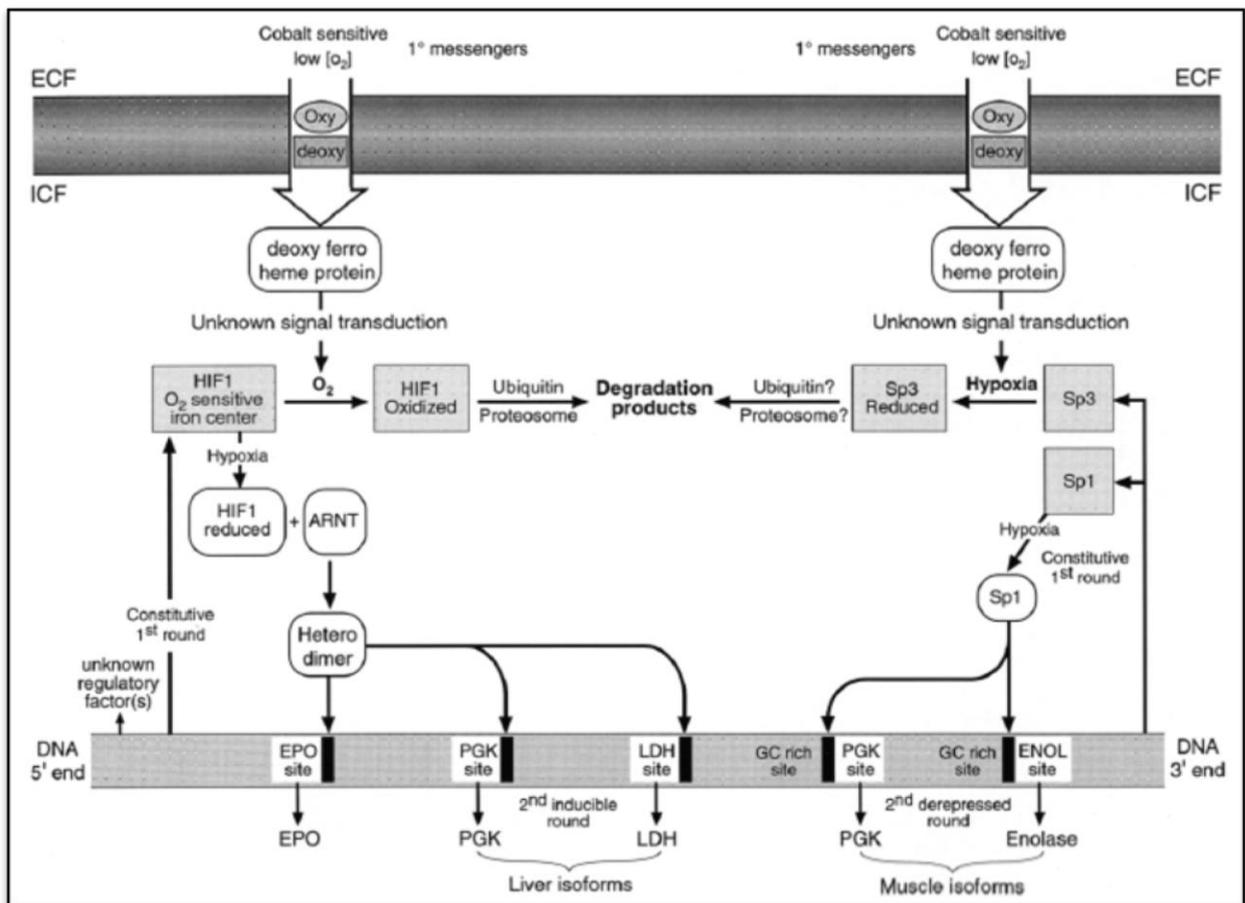


Figure 6. Oxygen sensing mechanism in liver cells and myocytes. On the left, the pathway for liver cells is shown. This pathway uses HIF-1 α during hypoxia, to bind to DNA and to form EPO, PGK and LDH. The pathway on the right uses the sp3, as a repressor in myocytes. During hypoxia, sp3 is detached from sp1. Sp1 is transported to the nucleus and PGK and enolase is formed. (Figure from Hochachka, P.W. and Lutz, P.L. (2001). Mechanism, origin, and evolution of anoxia tolerance in animals.)

OXYGEN SENSING MECHANISMS IN FUNGI

Recent studies found that both sterol and heme levels played an important role in the oxygen sensing mechanisms for *Saccharomyces cerevisiae*. The biosynthesis of heme and sterols depends on the amount of oxygen that is available. Oxygen is required for two steps in heme biosynthesis, the formation of protoporphyrinogen IX by coproporphrinogen oxidase III and the formation of protoporphyrin IX by protoporphyrinogen oxidase IX oxidase (Hon T. et al, 2003). The activation of the transcription factor Hap1p, occurs when sufficient heme levels are present. (see Figure 7). Here heme will bind to the second regulatory domain. (Kwast K.E. et al, 1998). When Upc2p is activated this leads to the expression of ergosterol

biosynthesis (ERG) genes among other hypoxic genes. Rox1p and Mot3p are responsible for the inhibition of the transcription of hypoxic genes under aerobic conditions. However Upc2p activates these hypoxic genes during hypoxia when sterol levels become limited. (Davies B. S. J. and Rine J. (2006)

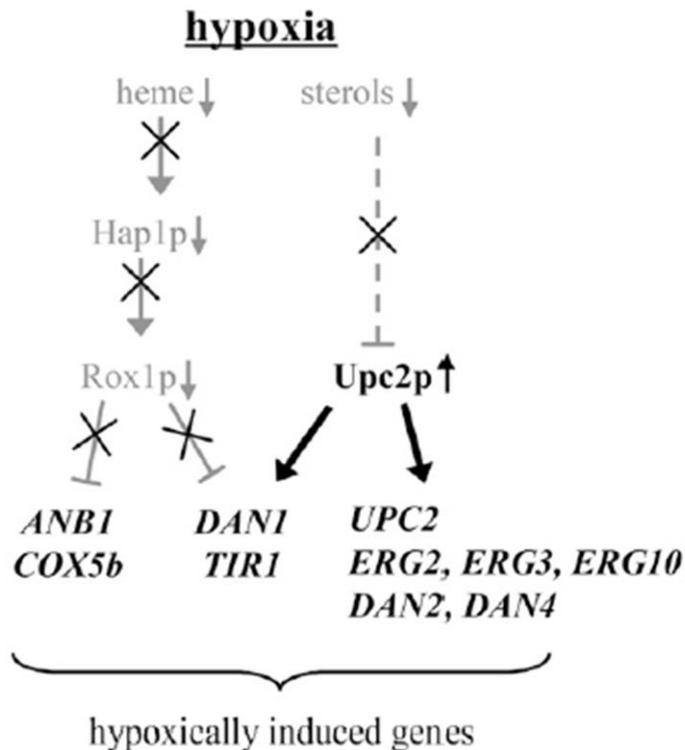


Figure 7. Simplified Model for oxygen sensing mechanism in *Saccharomyces cerevisiae*. (Figure from Davies B. S. J. and Rine J. (2006). A Role for Sterol Levels in Oxygen Sensing in *Saccharomyces cerevisiae*.)

Summary:

It seems that heme plays a central role in oxygen mechanisms for bacteria, animals and yeast. For bacteria there are two oxygen sensing mechanisms: the fumarate and nitrate reduction (FNR) transcriptional regulator and the two component system ArcAB. These regulate downstream the deactivation of the aerobic pathway and the activation of the anaerobic pathway. For animals, a heme protein with two subunits serves as an oxygen sensor that leads to the heterodimerization of HIF1. HIF 1 leads in turn to the transcription of for example erythropoietin (EPO), phosphoglycerate kinase (PGK) and lactate dehydrogenase (LDH). These will help an animal acclimatize to the oxygen deficiency. For yeast, heme and also sterol levels play an important role in oxygen sensing. The biosynthesis of heme depends on oxygen

levels. In the absence of heme, Hap 1p is not activated, which in turn do not activate Rox1p. The absence Rox 1p and Mot 3p then prevent the inhibition of hypoxic genes. Therefore, during hypoxia, the diminished levels of heme and sterols lead to the expression of hypoxic induced genes. All these organisms have heme playing a central role as a homeostatic oxygen sensor. However, for plants it has been suggested that heme cannot play a central role, because the dissociation constant of oxygen to heme is too low to signal a plant during oxygen deprivation (Abbruzzetti Z. et al., 2011).

Conclusion:

Animals, bacteria and fungi all have oxygen sensing mechanisms. These mechanisms are usually two-component systems that have an oxygen reacting protein. These cascade reactions are mostly inhibited during normoxia and activated when the oxygen supply is low. These oxygen sensing mechanisms could have similarities with the oxygen sensing mechanisms for plants. The oxygen sensing mechanisms for plants will be further discussed in chapter 3.

Chapter 3: THE INDIRECT AND DIRECT OXYGEN SENSING MECHANISMS IN PLANTS

Introduction:

In this chapter the oxygen sensing mechanisms will be investigated based on the most recent available literature. There have been many recent discoveries about how plants sense the lack of oxygen, but there are still some questions to be answered.

According to literature, there are two types of oxygen sensing mechanisms. There is an indirect and a direct oxygen sensing mechanism. The indirect oxygen sensing mechanism consists of the plant sensing a change in energy conservation status. When the anaerobic pathways are activated due to the lack of oxygen several changes arise in the plant that the plant would sense. These changes involve pH, cytosolic calcium, ATP levels and the production of ROS and NO. The direct oxygen sensing mechanism would involve group VII ERF (Ethylene Response Factor) transcription factors. These ERF transcription factors were found to be stabilized during hypoxic stress and degraded during normoxia through the N-end rule proteolysis.

The indirect and direct oxygen sensing mechanism:

When oxygen levels are below 20.6%, plants are in hypoxic stress (Bailey-Serres J. et al, 2012). The gene expression is then altered to start many adaptation mechanisms to conserve the plant's energy status (see chapter 1).

These adaptations results in a change in homeostasis in the plant, which the plant would sense. This indirect sensing mechanism would sense the drop in energy levels including ATP and carbohydrates as well as the drop in cytosolic pH as a result from the anaerobic pathways. Also the Ca^{2+} is release from mitochondria which is necessary for the altered gene expression during hypoxia, would be sensed by the plant. And finally the accumulation of NO and ROS during hypoxia would also be sensed by the indirect oxygen sensing mechanism. (Bailey-Serres and Chang, 2005)

There has been indications for *Arabidopsis thaliana* that the group VII of the ERF transcription factors would be involved in the direct oxygen sensing mechanism. There are ten clades of ERF transcription factors. Recent studies have found that for *Arabidopsis thaliana* group VII of the ERF would be involved in the direct sensing mechanisms. Group VII ERF consists of *HRE 1*, *HRE2*, *RAP 2.2*, *RAP 2.12*, *RAP 2.3*, *SUB1A*, *SUB1B*, *SUB1C*, *SK1* and *SK2*. These transcription factors all begin with the amino acids 'Met-Cys' except for *SUB1C* (Bailey-Serres et al., 2012). In experiments where the transcription factors *HRE1*, *HRE2*, *RAP2.2* and *RAP2.12* were overexpressed, the ADH activity was enhanced only during hypoxia but not during normoxia (Hess, N. et al. (2011), Hinz, M. et al. (2010)). Also from experiments with *Arabidopsis thaliana*, It was also discovered that *RAP2.2*, *RAP2.3*

and *RAP2.12* are constitutively synthesized (Mustroph, A. et al. (2009); Mustroph, A. and Bailey-Serres, J. (2010)). Presumably, some of the group VII ERF transcription factors are regulated through transcription in response to hypoxia, ethylene and darkness, other transcription factors are constitutively synthesized. During re-oxygenation the group VII ERF transcription factors are degraded through the N-end rule degradation pathway.

N-end rule degradation pathway:

The conserved terminus which ends with 'NH₂-Met-Cys' of the group VII ERF transcription factors are targeted for proteolysis during oxygen replete conditions. First the 'Met-Cys' terminus is cleaved by the methionine aminopeptidase (MAP). Methionine is detached and cysteine becomes exposed. Cysteine is then oxidized by O₂, NO or ROS. Afterwards, the arginyl tRNA transferase (ATE) adds an arginine residue to the oxidized Cysteine at the N-terminus. The added arginine is recognized by PROTEOLYSIS 6 (PRT6) or other E3 ligases. These polyubiquitinate the N-terminus, which makes it a target for proteasomal degradation (26S proteasome). (Licausi, F. et al. (2011)). This mechanism prevents during normoxia the transcription of the ERF transcription factors which activates of the anaerobic pathway. During hypoxia this mechanism is stopped because there is not enough oxygen available for the oxidation of cysteine.

During normoxia, *RAP2.12* is bound to the plasma membrane through the action of the Acyl-CoA binding protein (ACBP), which prevents it to move to the nucleus. After *RAP2.12* has been moved to the nucleus during hypoxia, it becomes also targeted for degradation during the N-end rule pathway when re-oxygenation occurs. (Licausi, F. et al. (2011)).

However, *SUB1A*, *SUB1C*, *SK1* and *SK2* are not degraded through the N-end rule pathway. It was suggested that that these transcription factors are regulated with the accumulation of ethylene during flooding. This strategy is different compared to the other ERF transcription factors but this response conserves more energy during flooding. The anaerobic pathway would be activated earlier even before the first signs of hypoxic stress would occur. (Gibbs, D.J. et al. (2011))

The N-end pathway for proteolysis has therefore an important function as an homeostatic sensor of severe low oxygen levels through the deactivation of group VII ERF transcription factors during re-oxygenation.

Summary:

There are two categories of oxygen sensing mechanisms. There is an indirect oxygen sensing mechanism, where a plant senses differences in the metabolism (the drop in PH, fluctuations in Ca²⁺ levels, change in ATP and carbohydrate levels, the production of NO and ROS) resulting from the change in energy status. There is also an indirect sensing mechanism that involves the group VII ERF transcription factors.

The accumulation of these transcription factors is regulated through the oxidation of cysteine in the N-end rule pathway of proteolysis. When there is no oxygen available, the oxidation of cysteine cannot occur and the cascade from this degradation pathway is withheld. Thus, the ERF transcription factors are degraded during normoxia and not during hypoxia. The SUB1A, SUB1C, SK1 and SK are exceptions in the group VII ERF transcription factors because they are not degraded through the N-end rule pathway. It was suggested that these respond to an accumulation of ethylene during flooding, which would activate the energy saving pathways before hypoxic stress would occur.

Conclusion:

Here evidence has been found that plants might also possess an oxygen sensing mechanism that activates the transcription and translation of genes that are necessary for the adaptive responses during hypoxia. The answers for the gaps in knowledge for the oxygen mechanisms for plants, are still being investigated. These answers could be found due to similarities with other organisms. This will be further explored in the next chapter.

Chapter 4: CONCLUSIONS AND FURTHER RESEARCH

Introduction:

This chapter gives a general reflection on the different chapters from this thesis and what is still lacking in the current knowledge in oxygen sensing mechanisms. The goal of this thesis was to summarize and comment on the mechanisms for oxygen sensing in plants. This topic has already been extensively researched for other organisms (animals, yeast and bacteria). Because there might exist similarities between these organisms and plants when it comes to oxygen sensing mechanisms, I included these in my literature research. Hemoglobin and hemoglobin-like-molecules play an important role as an oxygen sensor in animals and yeast. But due to the low dissociation constant of these for plants, it was reasoned that it would be part as an oxygen sensor for plants. The evidence found in this thesis suggests that the direct oxygen sensing mechanisms between these other organisms are quite different than these for plants.

Main Conclusions:

Flooding is a worldwide problem, but especially in developing countries. Due to climate change, there will be more precipitation and cyclones. This will have a great impact on farmers in the poorest parts of the world, because their main income comes from agriculture. When a plant is flooded transportation of O₂, CO₂ and ethylene is slower because gasses diffuse slower in water than in air. Research shows that for some rice cultivars during submergence, ethylene accumulates and *SUB 1A* is activated. Presumably this activation would start the adaptive response even before an oxygen deficit would start to be noticed by the plant. This early activation would conserve energy by adapting the metabolism earlier to oxygen deprivation. When oxygen levels drop below 20.6%, the plant undergoes biochemical changes. The production of ATP from the oxidative phosphorylation is lowered, due to the lack oxygen that serves as an electron acceptor. Carbohydrate catabolism starts through the glycolysis and fermentation pathway. Meanwhile the pH becomes more and more acidic. These changes would be indirectly sensed by the plant and would decrease the metabolic rate as a response.

Evidence was found that the group VII of the ERF transcription factors were involved as an homeostatic oxygen sensor at very low levels of oxygen. The group VII ERF factors exist among all plants. The survival traits from a plant depends on the expression of these group VII transcription factors. The regulation of these transcription factors occurs in response to the accumulation of ethylene, a dark period and/or hypoxia. *SUB1A*, *SUB1C*, *SK1* and *SK2* are regulated through ethylene accumulation. Whereas the expression of remainder members of the group VII ERF transcription factors are regulated through the N-end rule proteolysis pathway. These recent studies point out that plants probably possess an oxygen sensing mechanism just as other organisms do. There are still some gaps in the

knowledge. The answers could be found by analysing similarities with other organisms.

The investigation of the sensing mechanisms in other organism (bacteria, yeast and animals) led to the conclusion that heme plays a central role in the oxygen sensing mechanisms. For bacteria there are two heme dependent oxygen sensing mechanisms: the fumarate and nitrate reduction (FNR) transcriptional regulator and the two component system ArcAB. These regulate downstream the deactivation of the aerobic pathway and the activation of the anaerobic pathway. For animals, a heme protein with two subunits serves as an oxygen sensor that leads to the heterodimerization of HIF1 which will eventually lead to the acclimatization to low oxygen levels. For yeast, heme and also sterol levels play an important role in oxygen sensing. The biosynthesis of heme depends on oxygen levels. In the absence of heme, Hap 1p is not activated, which in turn do not activate Rox1p. The absence Rox 1p and Mot 3p then prevent the inhibition of hypoxic genes. Therefore, during hypoxia, the diminished levels of heme and sterols lead to the expression of hypoxic induced genes. All these organisms have heme playing a central role as a homeostatic oxygen sensor. However, for plants it has been suggested that heme cannot play a central role, because the dissociation constant of oxygen to heme is too low to signal a plant during oxygen deprivation (Abbruzzetti Z. et al., 2011).

Future research possibilities:

Recent research demonstrated the importance of group VII ERF transcription factors as mediators for oxygen sensing mechanisms. But there has yet to be discovered which factor that stands in the first line before passing the 'oxygen deprivation signal' to group VII of ERF. The increase in transcription and translation of group VII ERF transcription factors could be triggered to an unknown enzyme, a hormone, a specific decrease percentage of oxygen, ... Also the oxidation of the Cys terminal of the N-terminal of the N-end rule pathway proteolysis might have an unknown precursor that reacts to low oxygen conditions.

Also because hemoglobin was excluded as an oxygen sensor because the dissociation constant was too low, It would be interesting to compare speeds of turnover rate of group VII ERF transcription factors during hypoxia. Additionally the mechanism behind the ACBP complex that keeps RAP2.12 from moving to the nucleus still needs to be elucidated.

In conclusion, I believe that some unknown protein or enzyme acts as an direct oxygen sensing mechanism such as has been found with animals. This 'unknown factor' probably reacts to oxygen and perhaps is part of a two-component system. And finally I presume that this 'unknown factor' increases the transcription and translation of the group VII of ERF transcription factors and/or the N-end rule degradation pathway. This is because group VII of ERF transcription factors exist

among all vegetation and because the N-end rule degradation pathway has a conserved terminal motive.

List of abbreviations:

ACBP: Acyl-CoA binding protein

ADH: alcohol dehydrogenase

ROS: Reactive oxygen species

NO: Nitric Oxide

References:

- Abbruzzetti, S., Faggiano, S., Spyrakis, F., Bruno, S., Mozzarelli, A., Astegno, A., Dominici, P., and Viappiani, C. (2011). Oxygen and nitric oxide rebinding kinetics in nonsymbiotic hemoglobin AHb1 from *Arabidopsis thaliana*. *IUBMB Life* 63: 1094–1100
- Bailey-Serres, J., and Chang, R. (2005). Sensing and signalling in response to oxygen deprivation in plants and other organisms. *96*: 507–518.
- Bailey-Serres J., Fukao T., Gibbs D.J., Holdsworth M.J., Lee S.C., Licausi F., Perata P. Voesenek L.A.C.J., van Dongen J.T. (2012). Making sense of low oxygen sensing. *Trends in Plant Science* 17: 3
- Bailey-Serres, J., and Voesenek, L.A.C.J. (2008). Flooding stress: Acclimations and genetic diversity. *Annu. Rev. Plant Biol.* 59: 313–339.
- Boutilier RG, St-Pierre J. 2000. Surviving hypoxia without really dying. *Comp Biochem Physiol*, 126A: 481-490.
- Davies B. S. J. and Rine J. (2006). A Role for Sterol Levels in Oxygen Sensing in *Saccharomyces cerevisiae*. Department of Molecular and Cellular Biology, Division of Genetics, Genomics, and Development, University of California, Berkeley, California 94701-3202
- Drew MC. 1997. Oxygen deficiency and root metabolism: injury and acclimation under hypoxia and anoxia. *Annual Review of Plant Physiology and Plant Molecular Biology* 48: 223–250
- Geigenberger P. 2003. Response of plant metabolism to too little oxygen. *Current Opinion in Plant Biology* 6: 247–256.
- Georgellis D, Kwon O, Lin EC (2001). Quinones as the redox signal for the Arc two-component system of bacteria. *Science*, 292:2314-2316.
- Gibbs, D.J. et al. (2011) Homeostatic response to hypoxia is regulated by the N-end rule pathway in plants. *Nature* 479, 415–418
- Gunsalus RP and Park SJ (1994). Aerobic-anaerobic gene regulation in *Escherichia coli*: control by the ArcAB and Fnr regulons. *Re Microbiol*, 145:437-450
- Hattori, Y., Nagai, K., & Ashikari, M. (2011). Rice growth adapting to deepwater. *Current opinion in plant biology*, 14(1), 100-5. Elsevier Ltd
- Hess, N. et al. (2011) The hypoxia responsive transcription factor genes ERF71/HRE2 and ERF73/HRE1 of *Arabidopsis* are differentially regulated by ethylene. *Physiol. Plant.* 143, 41–49

Hinz, M. et al. (2010) Arabidopsis RAP2.2: an ethylene response transcription factor that is important for hypoxia survival. *Plant Physiol.* 153, 757–772

Kiley PJ, Beinert H. (2003). The role of Fe–S proteins in sensing and regulation in bacteria. *Current Opinion in Microbiology* 66: 181–185.

Licausi, F. et al. (2011) Oxygen sensing in plants is mediated by an N-end rule pathway for protein destabilisation. *Nature* 479, 419–422

Loui C.C. , Chang A.C. and Lu S. (2009). Role of the ArcAB two-component system in the resistance of *Escherichia coli* to reactive oxygen stress

Malpica R, Sandoval GR, Rodriguez C, Franco B, Georgellis D (2006): Signaling by the arc two-component system provides a link between the redox state of the quinone pool and gene expression. *Antioxid Redox Signal*, 8:781-795.

Mustroph, A. et al. (2009) Profiling translomes of discrete cell populations resolves altered cellular priorities during hypoxia in Arabidopsis. *Proc. Natl. Acad. Sci. U.S.A.* 106, 18843–18848

Mustroph, A. and Bailey-Serres, J. (2010) The Arabidopsis translome cell-specific mRNA atlas: mining suberin and cutin lipid monomer biosynthesis genes as an example for data application. *Plant Signal. Behav.* 5, 320–324

Oshima, T., Aiba, H., Masuda, Y., Kanaya, S., Sugiura, M., Wanner, B. L., Mori, H. and Mizuno, T. (2002). Transcriptome analysis of all two-component regulatory system mutants of *Escherichia coli* K-12. *Molecular Microbiology*, 46: 281–291.

Poyton RO. 1999. Models for oxygen sensing in yeast: implications for oxygen-regulated gene expression in higher eucaryotes. *Respiration Physiology* 115: 119–133.

Uden G and Schirawski J. (1997) . The oxygen-responsive transcriptional regulator FNR of *Escherichia coli*: the search for signals and reactions. *Mol Microbiol*, 25:205-210.

West, A.H., and Stock, A.M. (2001). Histidine kinases and response regulator proteins in two-component signaling systems. *Trends Biochem Sci* 26: 369–376.