Plant oxygen deprivation stress and function of plant mitochondria under anoxia

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Doctoral dissertation

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TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The doctoral thesis is based on the following three articles and one manuscript. In the text they are referred to by their Roman numerals.

I) Blokhina O.B., **Virolainen E.**, Fagerstedt K.V., Hoikkala A., Wähälä K. and Chirkova T.V. (2000) Antioxidant status of anoxia-tolerant and -intolerant plant species under anoxia and reaeration. Physiologia Plantarum 109: 396-403.

II) Virolainen E., Blokhina O., Fagerstedt K. (2002) Ca^{2+}-induced high amplitude swelling and cytochrome *c* release from wheat (*Triticum aestivum* L.) mitochondria under anoxic stress. Annals of Botany 90: 509-516.

III) Blokhina O., **Virolainen E.**, Fagerstedt K.V. (2003) Antioxidants, oxidative damage and oxygen deprivation stress: a review. Annals of Botany 91: 179-194.

IV) Virolainen-Arne E.I., Fagerstedt K.V. (2020) ATP use under anoxia and F_1F_o-ATPase activity of anoxia-intolerant wheat (*Triticum aestivum* L.) and anoxia-tolerant yellow flag iris (*Iris pseudacorus* L.) root mitochondria.A manuscript.

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I) Experimental design with co-author KVF; Experimental work: Vitamin E compound isolation and analysis with HPLC; Interpretation of results with co-authors AH and KW; Writing of the publication with co-author OBB who acted as the author in charge.

II) Experimental design with co-author OBB; Experimental work: equal contribution of all experiments with co-author OBB; Interpretation of results with co-author OBB; Writing of the publication with co-author OBB.

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IV) Experimental design with co-author KVF; Experimental work: Isolation of mitochondria, anoxia incubations of mitochondria, analysis of mitochondrial ATP content, assays of F_1F_o-ATPase and succinate: cytochrome c oxidoreductase enzyme activities, mitochondrial oxygen consumption assay; Interpretation of results; Writing of the manuscript, acted as the author in charge.

ABBREVIATIONS

AA	ascorbic acid
ABA	abscisic acid
ADP	adenosine diphosphate
ANP	anaerobic protein
ANT	adenine nucleotide translocator
AOX	alternative oxidase
APX	ascorbate peroxide
ATP	adenosine triphosphate
Ca^{2+}	calcium ion
CsA	cyclosporine A
CypD	cyclophilin D
DHA	dehydroascorbic acid
DHAR	dehydroascorbate reductase
DTE	dithioerythritol
ETC	electron transport chain
GABA	γ-aminobutyric acid
GR	glutathione reductase
GSH	reduced glutathione
GSSG	glutathione disulfide
HR	hypersensitive response
HRU1	hypoxia responsive universal stress protein 1
IF1	ATPase Inhibitory Factor 1
INV	invertase
JA	jasmonic acid
K_m	Michaelis-Menten constant
LEA	late embryogenesis abundant protein
Mg^{2+}	magnesium ion
MDHA	monodehydroascorbate
MDHAR	monodehydroascorbate reductase
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
NR	nitrate reductase
PAP	3´-phosphoadenosine 5´-phosphate
PCD	programmed cell death
PiC	phosphate carrier
PRX	peroxiredoxin
PT	permeability transition
PTP	permeability transition pore
ROS	reactive oxygen species
SA	salicylic acid
SOD	superoxide dismutase
SUS	sucrose synthase
TCA	tricarboxylic acid cycle
TEM	transmission electron microscopy
VDAC	voltage-dependent anion channel

ABSTRACT

Flooding stress and concomitant anoxia stress of plants has been in the focus of abiotic stress research for several decades. Despite that, there are several poorly studied areas in the field of flooding stress research such as responses of the anoxia-tolerant wild plant species and the function of plant mitochondria under oxygen deprivation stress. Contents and redox states of the small molecular antioxidants, that is ascorbate, glutathione and tocopherols, were determined in roots or rhizomes of four anoxia-tolerant and -intolerant plant species under anoxia and reoxygenation. Results of the study demonstrate that no correlation could be detected between the anoxia-tolerances of individual plant species and in the contents of given antioxidant under anoxia-reoxygenation stress but some correlation was observed between redox states of the glutathione pools and anoxia tolerances of the iris species studied. Permeability transition was induced under high calcium ion concentration in wheat root mitochondria. Properties of the permeability transition in wheat mitochondria were characterized and results of the study demonstrated several similarities with the properties of the mammalian permeability transition. However, several features of the permeability transition observed in wheat root mitochondria diverge from the features found in plant mitochondria of different species or origin of tissue. Anaerobic ATP use of mitochondria isolated from roots of anoxia-sensitive wheat and anoxia-tolerant yellow flag iris was determined. Results of the study demonstrate that wheat mitochondria show high ATP hydrolyzing activity while mitochondria of yellow flag iris hydrolyze ATP molecules at a low rate under anoxia. Results of the study correlate well with the anoxia tolerances of the species referring to divergent survival strategies of the species under oxygen deprivation. While mitochondria of yellow flag iris, a species tolerating long-term anoxia, consume ATP at a sparing rate thus avoiding depletion of the ATP pool under anoxia, wheat mitochondria consume ATP molecules at a fast rate leading to abrupt ATP depletion and possibly to cell death. Investigating the mechanisms underlying plant anoxia-tolerance would benefit breeding of cultivated plants in order to have more flooding tolerant cultivars in future. Several aspects of plant anoxia-tolerance are still unknown and should be investigated such as properties and function of mitochondria in anoxia-tolerant wild plant species and function of the plant F_1F_o-ATP synthase under anoxia.

TIIVISTELMÄ

Kasvien tulvastressiä ja siitä aiheutuvaa hapenpuutestressiä on tutkittu vuosikymmeniä, mutta useat tutkimusalueet kuten hapettomuutta sietävien luonnonkasvien vasteet sekä kasvimitokondrioiden toiminta hapenpuutteessa tunnetaan silti yhä heikosti. Pienimolekyylisten antioksidanttien, askorbiinihapon, glutationin ja tokoferolien, määrät sekä antioksidanttien pelkistyneiden ja hapettuneiden muotojen keskinäiset määräsuhteet hapettomuudessa ja sen jälkeisissä hapellisissa oloissa mitattiin neljän hapettomuutta sietävän ja hapettomuudelle herkän kasvin juurista ja juurakoista. Tulokset osoittivat, että kasvien hapettomuuden sieto ei korreloinut hapettomuudesta ja sen jälkeisistä hapellisista oloista johtuvien antioksidanttien määrien muutosten kanssa, mutta kurjenmiekkojen hapettomuuden siedoilla havaittiin olevan jonkin verran korrelaatiota niiden juurakoista määritetyn glutationin pelkistyneen ja hapettuneen muodon määräsuhteiden muutosten kanssa. Vehnänjuurista eristettyjen mitokondrioiden läpäisevyyden muutoksen ominaisuudet määritettiin. Tulokset osoittivat, että vehnän juurimitokondrioiden läpäisevyyden muutoksella on useita yhteneväisiä ominaisuuksia nisäkäsmitokondrioiden läpäisevyyden muutoksen kanssa, mutta useat havaituista ominaisuuksista eroavat muista kasvilajeista tai eri kasvin osista eristettyjen mitokondrioiden läpäisevyyden muutoksen ominaisuuksista. Hapettomuudelle herkän vehnän ja hapettomuutta hyvin sietävän keltakurjenmiekan juurista eristettyjen mitokondrioiden ATP molekyylien hapettomuuden aikainen kulutus määritettiin. Tulokset osoittivat, että vehnän juurimitokondriot hydrolysoivat ATP molekyylejä hapettomuudessa nopealla tahdilla, kun puolestaan keltakurjenmiekan juurimitokondriot hydrolysoivat ATP molekyylejä hapettomuudessa oleellisesti hitaammalla tahdilla. Saadut tulokset korreloivat hyvin tutkittavien lajien hapettomuuden siedon kanssa tulosten viitatessa lajien selviytymisstrategiaan hapenpuutestressissä. Pitkäkestoista hapettomuutta sietävän keltakurjenmiekan mitokondriot kuluttavat säästeliäästi ATP molekyylejä välttäen solujen ATP varastojen hupenemisen hapettomuudessa, kun vehnän mitokondriot kuluttavat nopeasti ATP molekyylejä hapettomuudessa, jonka seurauksena solujen ATP varastot tyhjenevät nopeasti johtaen solujen kuolemaan. Kasvien hapettomuuden siedon mekanismien selvittäminen hyödyttäisi viljelykasvien tulvansiedon muokkaamista ja siksi hapettomuutta sietävien luonnonkasvien mitokondrioiden sekä kasvimitokondrioiden ATP syntaasin toimintaa hapettomuudessa tulisi tutkia.

1 INTRODUCTION

Plants are obligate aerobic organisms and they need oxygen, above all, for aerobic respiration, but there are also other oxygen-dependent metabolic pathways in cells such as haem, sterol and fatty-acid biosyntheses (Geigenberger 2003). Shortage of oxygen is a common feature in several plant organs and in tissues such as seeds, meristems, phloem, fruits and storage organs (Geigenberger 2003; Bailey-Serres and Voesenek 2008). There are several reason for the oxygen shortage observed in plant tissues such as high metabolic activity, high cell density of tissues or restricted oxygen entry of the tissue or organ. Lowered oxygen concentration of plant tissues and organs have been shown to lead to severe deterioration of cell metabolism such as partial inhibition of glycolysis, respiration, and both sucrose breakdown and uptake, but it has been shown that lack of oxygen can lead to a severe inhibition of amino acid synthesis in growing tissues of potato tubers, inhibition of seed production in several crop plants and to declined sugar retention in the phloem and ion loading into the xylem cells due to lowered oxygen tensions in the tissues in question (Geigenberger *et al.* 2000).

There are also environmental factors such as for example soil flooding, which cause oxygen deprivation stress in plant tissues (**III**; Sasidharan *et al.* 2017). Oxygen deprivation of plant cells starts rapidly especially in nonphotosynthetic cells and in darkness under flood leading to hypoxic stress i.e. lowered oxygen concentration, or anoxic stress i.e. total absence of oxygen in plant cells (Bailey-Serres and Voesenek 2008). After flooding, oxygen returns into previously hypoxic or anoxic plant cells i.e. reoxygenation, which in turn leads to a stress due to increased formation of reactive oxygen species (ROS) in plant cells. Flooding stress of plants has long been in the focus of abiotic stress research but its importance is becoming more significant due to the global climate change which increases flooding events especially in major farming regions (Voesenek *et al.* 2016). Incidences of flooding soils around the world have increased significantly from the 1950s' with a consequence that floods have become one of the major abiotic stresses of cultivated plants causing considerable crop losses in the world (Pedersen *et al.* 2017). As a result of these environmental changes, considerable efforts are needed urgently to produce more flooding-tolerant crop plants

(Voesenek *et al.* 2016). It has been suggested that plant breeding programmes would benefit considerably of the research on truly flooding-tolerant wild plant species, instead of Arabidopsis and rice cultivars, since wild plants have stress tolerance mechanisms so far inadequately investigated (Voesenek *et al.* 2014). In earlier research on the flooding tolerance of wild plant species especially the anoxia-tolerant species sweet flag (*Acorus calamus* L.) has been studied extensively over the years (Crawford and Braendle 1996). The extremely high anoxia tolerance of the rhizomes of the sweet flag is a result of several features such as large reservoir of storage carbohydrates and high stability of membrane lipids under prolonged anoxia and reoxygenation stress. Anoxia tolerance of the wetland plant species, yellow flag iris (*Iris pseudacorus* L.), has been studied extensively during the last decades (Tyler *et al.* 1970; Hetherington *et al.* 1983; Monk *et al.* 1987; Monk *et al.* 1989; Hanhijärvi and Fagerstedt 1994; Hanhijärvi and Fagerstedt 1995). In addition to the ability of the rhizomes to withstand prolonged anoxic stress, there are unique features in the rhizomes of *I. pseudacorus* such as the 13-fold increase in the enzyme activity of superoxide dismutase (SOD) during long-term anoxia incubation and concomitant post-anoxia (Monk *et al.* 1987). There are also more recently published studies on flooding survival strategies of wild plants such as *Rumex palustris* and *R. acetosa* (van Veen *et al.* 2013) and *Rorippa sylvestris* and *R. amphibia* (Sasidharan *et al.* 2013).

Several essential aspects of plant responses to oxygen deprivation stress have been unraveled during the last years such as the mechanism of oxygen sensing (Gibbs *et al.* 2011; Licausi *et al.* 2011) and signaling networks (Gibbs *et al.* 2015) in plants under low oxygen stress. Despite the long-term research on the oxygen deprivation stress of plants, knowledge on the plant mitochondrial functions under oxygen deprivation or anoxia stress is still fragmentary (Shingaki-Wells *et al.* 2014). There are still only a few articles published in which plant mitochondria have been shown to function under ananerobiosis such as the anaerobically functioning mitochondria of *Echinochloa* species (Fox and Kennedy 1991; Fox *et al.* 1994) and nitrite-driven ATP synthesis of rice and barley mitochondria under anoxia (Stoimenova *et al.* 2007). How the F_1Fo-ATPase of plant mitochondria functions under anoxia is still largely unknown.

The aims of my doctoral thesis have been to investigate how long-term anoxic stress affects the contents and the redox states of small molecular antioxidants i.e.

tocopherols, ascorbic acid and glutathione, in the roots of rice (*Oryza sativa*) and wheat (*Triticum aestivum*) and in the rhizomes of two species, the anoxia-tolerant yellow flag iris (*Iris pseudacorus*) and the anoxia-sensitive garden iris (*Iris germanica*). Studying the functional properties of plant mitochondria has been the main focus in my thesis: permeability transition of isolated wheat root mitochondria was characterized (**II**), and ATP consumption of the F_1Fo-ATPase of wheat and yellow flag iris mitochondria under anoxia stress was investigated (**IV**).

1.1 Oxygen deprivation stress and plant metabolism

Plant metabolism is significantly altered under low oxygen stress and extreme metabolic changes has been shown to take place in oxygen deprived plant cells (Bailey-Serres and Voesenek 2008; Banti *et al.* 2013). One of the consequences of oxygen deprivation is inhibition of the oxidative phosphorylation and mitochondrial ATP synthesis since oxygen is needed as the final electron acceptor in the mitochondrial electron transport chain (Geigenberger 2003). Under low oxygen stress, ATP is produced by glycolysis in oxygen depleted tissues which in turn leads to energy crisis of cells due to the low amount of ATP molecules produced by glycolysis in comparison to the aerobic ATP production (Bailey-Serres and Voesenek 2008).

Decreasing oxygen concentration in the environment leads to cell metabolism in which cytosolic glycolysis and fermentation dominate (Bailey-Serres and Voesenek 2008; Banti *et al.* 2013). Starch degradation under oxygen deprivation is a rare feature among plant species but starch degrading enzymes, amylases, are induced in some wetland species such as *A. calamus* and *Potamogeton pectinatus* under low oxygen stress (Bailey-Serres and Voesenek 2008). According to the study by Arpagaus and Brändle (2000), enzyme activity of α-amylase and amount of free sugars increased in the rhizomes of *A. calamus* under strict anoxia. It has been proposed that functional α-amylase enzyme is a prerequisite for maintaining glycolysis and hence long-term survival of *A. calamus* in oxygen depleted soils. Also in rice seeds (*Oryza sativa*) α-amylase enzyme is induced under anoxia enabling the breakdown of the stored starch and germination of the seed in anaerobic environment but the α-amylase enzyme is not

induced in wheat seeds, and hence the seeds cannot germinate under anoxia (Perata *et al.* 1992). Sucrose degradation for the maintainence of glycolysis can be carried out by two independent metabolic pathways, sucrose synthase (SUS) or invertase (INV). Under low oxygen stress SUS pathway has been shown to be induced in a variety of plant species due to the more energy-saving sucrose degrading reactions of the pathway (Bailey-Serres and Voesenek 2008; Banti *et al.* 2013). It is obviously an advantage for the energy deprived cells to direct sucrose catabolism via SUS pathway since the degradation consumes only one pyrophosphate molecule in the pathway while two molecules of ATP are consumed in the INV pathway.

There are several possible end products in the anaerobic carbohydrate metabolism since pyruvate, the end product of glycolysis, can be used for different metabolic pathways such as ethanol and lactate fermentation (Bailey-Serres and Voesenek 2008). While they are the major pathways in the anaerobic metabolism leading to the major end products i.e. ethanol and lactate, there are also pathways leading to formation of minor end products such as alanine, γ-aminobutyric acid (GABA), succinate and occasionally also malate. Accumulated alanine can be used for energy metabolism of cells under reoxygenation of tissues since alanine can be converted to pyruvate. Succinate is an intermediate of the tricarboxylic acid cycle (TCA). Its accumulation in plant tissues under anoxia has been explained to be a consequence of stalling of the TCA cycle at the succinate dehydrogenase step due to lack of oxygen. This in turn leads to succinate accumulation in cells (Shingaki-Wells *et al.* 2011). GABA accumulation under hypoxia involves upregulation of enzymes such as glutamate decarboxylase, but under reoxygenation accumulated GABA can be converted to succinate i.e. via the GABA shunt, in order to fuel the operation of the TCA cycle (Mustroph *et al.* 2014).

Protein metabolism of plants is greatly altered under low oxygen stress (Kennedy *et al.* 1992; Shingaki-Wells *et al.* 2011). Ethanolic fermentation dominates cell metabolism under oxygen deprivation but fermentation produces only low amounts of ATP molecules which leads to decreased rates of protein synthesis in cells (Shingaki-Wells *et al.* 2011). Anoxic treatment induces also synthesis of new proteins i.e. anaerobic proteins (ANPs), which are mainly enzymes of glycolysis and fermentation or enzymes related to sugar metabolism (Sachs *et al.* 1996). Several of the ANPs have also been shown to be induced in rice (Huang *et al.* 2005). In addition to the induced enzymes of

sugar metabolism there are also enzymes involved in scavenging ROS (Hashiguchi *et al.* 2009) and a putative cell wall loosening and degrading enzyme, xyloglucan endotransglycosylase (Sachs *et al.* 1996), which both are induced under anoxia.

In a recent study by Shingaki-Wells and colleagues (2011) amino acid and protein metabolism of wheat and rice coleoptiles under anoxia were studied revealing major differences in the strategy of adaptation between the species. Protein profile of anoxia tolerant rice coleoptiles responded rapidly to anoxia leading to changes in abundances of several dozens of proteins but the protein profile of anoxia-intolerant wheat coleoptiles responded weakly to anoxia leading to almost unaltered proteome. Accordindg to the study, anoxic treatment of rice increased the abundance of sucrose synthase enzymes, and several enzymes of both glycolysis and fermentation in the coleoptiles. In addition, the abundances of several enzymes such as those involved in the biosynthesis of amino acids alanine, serine and glycine, two group 3 late embryogenesis abundant proteins (LEA) and several ROS degrading enzymes were significantly increased under anoxia. The study showed also great differences in the metabolites of rice and wheat coleoptiles under anoxia of which the accumulation of aminoa acids such as alanine, serine and glycine, was significant in anoxic rice coleoptiles.

1.2 Plant metabolism under reoxygenation

In post-anoxia oxygen returns into anoxic tissues leading to several reactions which produce free radicals of oxygen i.e. ROS (Gibbs and Greenway 2003). Several changes take place in plant cells under oxygen deprivation stress such as acidification of the cytoplasm, decreasing production of ATP leading to lowered cellular energy metabolism, saturation of respiratory electron transport chains and accumulation of high amounts of reducing equivalents in the reduced form, which favour formation of ROS under reoxygenation (VanToai and Bolles 1991; Biemelt *et al.* 1998; **III**).

First studies on radical formation in plant tissues after anoxic treatment were performed in the early 1990's revealing the link between birth of radicals and post-anoxic injury

of plant tissues (Crawford *et al.* 1994). According to the study, radical formation was detected by electron paramagnetic resonance spectroscopy in reoxygenated plant tissues following anoxia treatment. The study brought out for the first time the similarities between post-anoxic injury of plant tissues and post-ischemic i.e. ischemia-reperfusion, injury of animal tissues.

Reoxygenation has been shown to cause injuries in cells which are estimated to result mainly from the generation of ROS (VanToai and Bolles 1991; Biemelt *et al.* 1998; **III**). ROS can cause damage in plant tissues by both reacting with proteins and nucleic acids and by oxidizing polyunsaturated fatty acids which leads to lipid peroxidation and in the end injuries to membrane structures (Gibbs and Greenway 2003). It has been demonstrated in several studies that lipid peroxidation increases especially in anoxia-sensitive plant species in post-anoxia (Hetherington *et al.* 1982; Chirkova *et al.* 1998; Blokhina *et al.* 1999; **III**). Involvement of ROS in the lipid peroxidation of anoxic tissues has been, however, reassessed by Rawyler and colleagues (2002). In their experiments with the anoxia-sensitive potato tuber cells it was demonstrated that membrane damage occurs already during the anoxia stress under conditions where energy metabolism of cells is low and ATP production of cells falls below a certain treshold value. This leads to activation of lipolytic acyl hydrolase and lipid hydrolysis. Consequently, membrane damage of anoxia-treated potato tuber cells occurs independently of ROS formation in post-anoxia. According to the study, it has been assessed that lipid peroxidation occurs at a late stage during the course of anoxia-reoxygenation and only in membranes which have been already severely injured due to anoxia-induced lipid hydrolysis.

Antioxidant defence system protects cells against the injuries originating in a burst of reactive oxygen species commonly detected in reoxygenation of tissues (Crawford and Braendle 1996; **III**; Gibbs and Greenway 2003). Activation of the system under anoxia or hypoxia treatment has been shown in several studies (Monk *et al.* 1987; Biemelt *et al.* 1998; Garnczarska 2005) leading to either increased activities of antioxidant enzymes or increased amounts of small molecular antioxidants (Skutnik and Rychter 2009). However, there are also opposing results wherein activities of the antioxidative enzymes or total amounts of small molecular antioxidants have decreased under hypoxia or anoxia incubation (**I**; Garnczarska 2005; Goggin and Colmer 2005). In

addition, there are studies where total amounts of small molecular antioxidants and activities of the enzymatic antioxidants increased under reoxygenation phase subsequent to the anoxia incubation of tissues (Skutnik and Rychter 2009).

1.3 Oxidative stress and plant antioxidantive defence system

The concept of oxidative stress has changed considerably during the last decades (Sies *et al*. 2017). In mid 1980's oxidative stress was described as "a disturbance in the prooxidant-antioxidant balance in favor of the former" but the concept became old-fashioned when multitude of studies on signaling function of ROS emerged. The concept still lacks a definition but there are several key hallmarks of oxidate stress such as enhanced ROS formation and oxidative damage to cellular components leading to cellular damage which in turn causes deterioration in cell functions and eventual cell death (Foyer and Noctor 2011). Since the view of ROS in plant metabolism has considerably widened during the last years it has been suggested that the term "antioxidative systems" should be replaced with a term "ROS processing systems" which describes more precisely the present understanding of ROS-antioxidant interactions (Noctor *et al.* 2017).

Antioxidant defence system of plants

The antioxidative defence system of plant cells consists of both enzymatic and non-enzymatic antioxidants (Foyer *et al.* 1994; Noctor and Foyer 1998; **III**). Non-enzymatic antioxidants are small molecules such as ascorbate (Vitamin C), glutathione, tocopherols (Vitamin E compounds) and carotenoids (Foyer *et al.* 1994), but there are a variety of plant compounds such as for example phenolics which show antioxidantive activities in plant cells (Larson 1988; **III**). In addition, some of the housekeeping compounds like amino acids and sugars have antioxidative properties (Noctor *et al.* 2017). There are also numerous enzymatic antioxidants in plant cells, including superoxide dismutases, several peroxidases, catalase and enzymes which are involved in synthesis and regeneration of small molecular mass antioxidants (Foyer *et al.* 1994;

Noctor and Foyer 1998; **III**), and whose antioxidative action has been described in detail in several reviews (Noctor and Foyer 1998; **III**).

Ascorbate occurs in all subcellular compartments such as chloroplast, mitochondria, peroxisomes, vacuoles and in the apoplastic fluid but highest concentrations of ascorbate have been found in the cytosolic compartment and peroxisomes (Smirnoff 2011). Ascorbate is a powerful antioxidant being able to react directly with singlet oxygen, hydroxyl radicals, superoxide (Noctor and Foyer 1998; **III**) and ozone but ascorbate reacts also with hydrogen peroxide in an ascorbate peroxidase (APX) catalyzed reaction (Smirnoff 2011). Ascorbate has been suggested to be the most important reducing substrate for detoxification of hydrogen peroxide (H_2O_2) in the APX catalyzed reaction which starts a series of reactions called the ascorbate-glutathione cycle (**Fig 1.**) (Noctor and Foyer 1998). In addition to the previous, ascorbate can also scavenge tocopheroxyl and carotenoid radicals thus regenerating tocopherols and carotenoids (Smirnoff 2011). The tripeptide glutathione (γ-glutamylcysteinylglycine) is the principal low-molecular-weight thiol metabolite in plant cells (Noctor *et al.* 2012). Several cell compartments contain glutathione but highest concentrations are found in mitochondria, cytosol and chloroplasts. Reduced glutathione (GSH) has been shown to scavenge singlet oxygen, hydroxyl radicals and it also regenerates ascorbate in the ascorbate-glutathione cycle (**Fig 1.**) (Foyer *et al.* 1994; **III**). According to current understanding ascorbate and glutathione are integral parts of plant redox signaling and core components of redox homeostasis in plant cells (Foyer and Noctor 2011). They differ from other plant ROS scavenging compounds in several ways since ascorbate and glutathione have stable oxidized forms which are regenerated to their reduced forms by NAD(P)H dependent high-capacity enzyme-based systems. Ascorbate and glutathione are also coupled to peroxide metabolism via specific peroxidases such as APX and glutaredoxin-dependent peroxiredoxins (PRX) which show glutathione-dependent peroxidase activity. Overall, ROS scavenging of the ascorbate-glutathione-NAD(P)H cycle leads to transient or sustained adjustments in the components of the cycle which can be sensed leading to signal transduction and finally changes in multiple signaling pathways. In a recent research for example by Caviglia and colleagues (2018) ethylene signaling was altered in ascorbic acid deficient Arabidopsis mutants. Studies with *Arabidopsis* mutants have altogether brought out that both ascorbate and glutathione are metabolites with a multitude of significant

functions in plant cells having important roles in redox homeostasis and cell signaling, but they have also significant roles in development and defense reactions (Foyer and Noctor 2011).

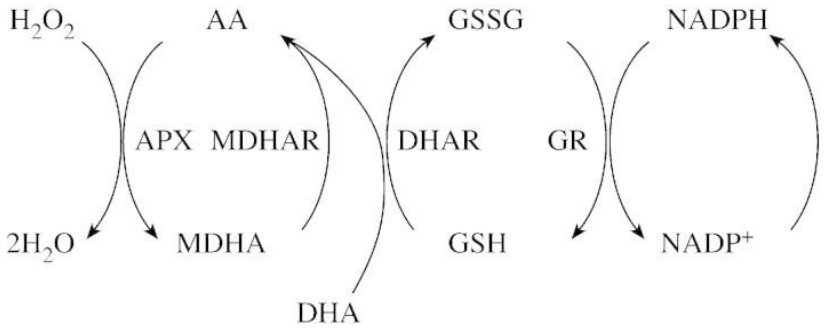

Fig. 1. Ascorbate-glutathione cycle. AA, ascorbic acid; DHA, dehydroascorbic acid; MDHA, monodehydroascorbate; APX, ascorbate peroxidase; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; GSH, reduced glutathione; GSSG, glutathione disulfide; GR, glutathione reductase. From the original publication by May *et al.* (1998).

Both carotenoids and Vitamin E compounds i.e. tocopherols and tocotrienols, are antioxidants localized in plant membranes (Noctor *et al*. 2015). A variety of reactive oxygen species are formed in chloroplasts causing photo-oxidative damage to the photosynthetic apparatus in thylakoid membranes (Jahns and Holzwarth 2012). There are, however, several ways to protect the photosynthetic machinery against the damage. Carotenoids are able to lower the formation of reactive oxygen species but they can also act as antioxidants by reacting with any reactive oxygen species formed in thylakoid membranes (Noctor *et al.* 2015). According to several studies zeaxanthin has been shown to have a central role in the protection of the photosynthetic apparatus against photo-oxidative damage since it is the major player in the deactivation of excited singlet chlorophyll molecules that is called nonphotochemical quenching. Also zeaxanthin has antioxidative functions in thylakoid membranes where it scavenges singlet oxygen (Jahns and Holzwarth 2012). Tocopherols and tocotrienols, collectively called tocochromanols, belong to a poorly studied group of prenyllipids which have been recently reviewd by Szymańska and Kruk (2018). Prenyllipids are lipophilic compounds which have been shown to function as antioxidants but also as electron and proton carriers in electron transport chains of bacteria, plants and animals.

Antioxidative function of tocochromanols in biological membranes is based on either scavenging of reactive oxygen species, especially singlet oxygen, physically deactivating singlet oxygen by a charge transfer mechanism or acting as chain-breaking antioxidants by donating hydrogen ions to peroxyl radicals in the lipid peroxidation cascade of polyunsaturated fatty acids (**III**; Noctor *et al.* 2015). In addition, one of the antioxidative functions of tocochromanols is the ability to react directly with reactive nitrogen species (Mène-Saffrané and DellaPenna 2010). According to animal studies it has been suggested that γ-tocopherol could react with nitric oxide (NO) being a putative regulator of NO levels both in animal and plant cells. There are also several *in vivo* and *in vitro* studies which demonstrate evidence of synergistic function of zeaxanthin and tocopherols in the protection of membrane lipids against peroxidation (Jahns and Holzwarth 2012).

Reactive oxygen species (ROS) of plant cells

Formation of ROS is an unavoidable consequence of aerobic metabolism (Apel and Hirt 2004). ROS are generated principally in two different ways: by transferring excess energy to ground state oxygen ($^3O_2$) which leads to formation of singlet oxygen, or by reduction of the ground state oxygen which first leads to formation of superoxide radical ion and hydrogen peroxide and hydroxyl radical are formed later in the sequence of reactions (**Fig. 2.**). Perhydroxyl radical alias hydroperoxyl radical is instead formed by protonation of superoxide radical ion (**Fig. 2.**) which occurs in aqueous solutions (Vranová *et al.* 2002).

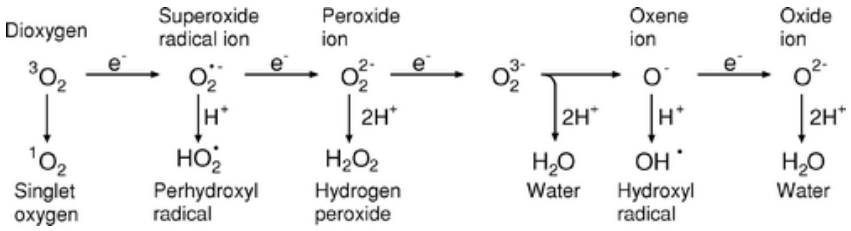

Figure 2. Generation of different reactive oxygen species (ROS) by energy transfer or sequental univalent reduction of ground state triplet oxygen, $^3O_2$. From the original publication by Apel and Hirt (2004).

Detrimental effects of ROS to cell structures have been known for decades (Noctor and Foyer 1998; Vranová *et al.* 2002; **III**, Apel and Hirt 2004). Hydroxyl radical is the most reactive of the active oxygen species being able to react with all biological molecules (Vranová *et al.* 2002). Superoxide radical ion shows only moderate reactivity but nevertheless, it is able to reduce quinones and affect activities of transition metal-containing enzymes. Singlet oxygen is highly reactive and it has been shown to oxidize a multitude of cellular compounds such as fatty acids of membrane lipids, thiols, DNA bases, several amino acid side chains in proteins and secondary metabolites (Triantaphylidès and Havaux 2009). Hydroperoxyl radicals have been shown to subtract hydrogen ions from polyunsaturated lipids and lipid hydroperoxides which in turn initiates lipid auto-oxidation (Vranová *et al.* 2002). Hydrogen peroxide have been shown to for example to inactivate activities of the enzymes of Calvin-Benson cycle and copper/zinc superoxide dismutase by oxidizing their thiol groups. Research during the last decade has, however shown that ROS and redox-signaling have significant functions in several plant processes such as plant metabolism and development, stress responses, and both gene expression and translation (Dietz *et al.* 2016; Noctor *et al.* 2017). According to recent studies, singlet oxygen, which is produced in leaf chloroplasts at the reaction centre of PSII, is suggested to initiate cell signaling pathway in high light leading to for example stimulation of jasmonic acid (JA) biosynthesis (Mullineaux *et al.* 2018). Hydrogen peroxide is relatively stable and less reactive active oxygen species making hydrogen peroxide a suitable signaling molecule in cells (Saxena *et al.* 2016; Mullineaux *et al.* 2018). Hydrogen peroxide has been studied extensively during the last years and it has been shown to be involved in several signaling cascades vital in plant growth and development. Formation of hydrogen peroxide is also upregulated in hypoxia pointing to a role in low oxygen stress signaling (Saxena *et al.* 2016). Recent research show also that hydrogen peroxide has interaction with other signaling molecules such as abscisic acid (ABA), salicylic acid (SA), nitric oxide (NO) and ethylene (Saxena *et al.* 2016). Hydroxyl radicals have a significant role in several plant developmental processes such as seed germination, elongation growth and fruit ripening (Müller *et al.* 2009). Hydroxyl radicals formed in cell walls have also been shown to attack cell wall polysaccharides leading to loosening of cell walls which is essential during the course of several plant developmental processes.

Reactive oxygen species are formed in several cell compartments such as mitochondria, chloroplasts, peroxisomes, plasma membrane and apoplastic compartment (Noctor and Foyer 2016) of which mitochondria, peroxisomes and chloroplasts are considered to be the most significant compartments in ROS metabolism (Noctor and Foyer 2016). In biological systems ROS are produced either enzymatically or nonenzymatically (**III**; Sasidharan *et al.* 2018). There are several enzymes in plant cells which are involved in ROS formation acting in various plant cell compartments such as xanthine oxidase in peroxisomes and NADPH oxidases of plasma membrane. Electron transport chains of mitochondria and chloroplasts produce ROS nonenzymatically since the electron transport chains tend to leak electrons which leads first to formation of superoxide radicals and subsequently other forms of ROS.

Although ROS are formed continuously in several cell organelles it has been shown that during abiotic stress, such as drought, high light or temperature extremes, formation of ROS tends to increase (Apel and Hirt 2004). Increased formation of ROS is also a characteristic response in plants under low oxygen stress (**III**; Pucciariello and Perata 2017; Sasidharan *et al.* 2018). Hydrogen peroxide has been shown to accumulate in plant tissues under low oxygen stress and concomintant reoxygenation in several plant species (Biemelt *et al.* 2000; Blokhina *et al.* 2001; Vergara *et al.* 2012). According to the study by Vergara and colleagues (2012) hydrogen peroxide accumulation in grapevine buds under hypoxia stress was produced by electron transport chain of mitochondria. Controlled ROS formation under oxygen deprivation stress has been shown to be indispensable for ROS signaling and plant adaptive responses during the stress (Sasidharan *et al.* 2018). In an earlier study by Pucciariello and colleagues (2012) on low-oxygen signaling of plants a transient production of H_2O_2 by NADPH oxidase was detected in Arabidopsis at the onset of anoxia. A ROS-dependent signaling of low oxygen stress has been suggested to be an additional mechanism to the oxygen sensing mechanism presented first by Gibbs and colleagues (2011) and Licausi and colleagues (2011) (Pucciariello and Perata 2017). According to later studies a link between the oxygen sensing mechanism and ROS signaling has been described, and hypoxia responsive universal stress protein 1 (HRU1) has been suggested to act as a hub connecting the oxygen sensing mechanism to ROS signaling (Pucciariello and Perata 2017).

1.4 Mitochondria and permeability transition (PT)

A phenomenon called permeability transition (PT) was first described in mammalian mitochondria in late 1970' by Robert A. Haworth and Douglas R. Hunter (Hunter *et al.* 1976; Haworth and Hunter 1979; Hunter and Haworth 1979a; Hunter and Haworth 1979b). Permeability transition of mitochondria can be defined as a sudden permeability increase of the mitochondrial inner membrane to solutes up to 1.5 kDa which results from the opening of a regulated channel called the permeability transition pore (PTP) (Bernardi *et al.* 2015; Biasutto *et al.* 2016). Permeability transition has been studied in several organisms such as in yeast, *Drosophila*, mammals (Bernardi *et al.* 2015) and plants (De Col *et al.* 2018). Though regulation of the mammalian PTP has been studied for several decades (Bernardi *et al.* 2015), structure of the PTP is still under investigation. Several models for the identity of PTP have been proposed over the last decades such as for example the tripartite model which suggests that the PTP consists of cyclophilin D (CypD), adenine nucleotide translocator (ANT), voltage-dependent anion channel (VDAC) and AAA-type protease (Biasutto *et al.* 2016). Recently published studies, however, demonstrate that a high-conductance channel, which have all the properties of the mammalian PTP, is formed from the mitochondrial F-ATP synthase after Ca^{2+}-induced transformation in the enzyme complex (Urbani *et al.* 2019). Structure of the mitochondrial F_1F_O -ATP synthase is highly conserved among diverse organisms such as animals and plants (Bernardi *et al.* 2015) and it has been speculated that the structure of the plant PTP could also be similar to the PTP of mammalian mitochondria (Zancani *et al.* 2015).

According to Bernardi and colleagues (1998) there are over 40 compounds or factors which regulate the mammalian PTP. The regulating compounds or factors have been shown to act as inducers or inhibitors of the open-closed transitions of the PTP in mammalian mitochondria (Bernardi *et al.* 1998; Bernardi 1999; Bernardi *et al.* 2015; Biasutto *et al.* 2016). Studies with isolated mammalian mitochondria have demonstrated that accumulation of Ca^{2+}-ions into mitochondrial matrix is a prerequisite for permeability transition to occur though the ions are not able alone to induce opening of the PTP. There are several factors which, together with matrix accumulated Ca^{2+} ions, induce the open conformation of the PTP such as oxidative stress, increased electron flux within complex I and concomintant formation of ROS, oxidation of the

matrix pyridine nucleotides, glutathione and critical dithiols, depolarization of the inner mitochondrial membrane, matrix inorganic phosphate and CypD. In addition to the previous, there are also several compounds or factors which favour the closed conformation of the PTP in mammalian mitochondria such as matrix divalent cations (Mg^{2+}, Mn^{2+} and Sr^{2+}), adenine nucleotides i.e. ADP and ATP, reduced matrix pyridine nucleotides, glutathione and dithiols, matrix acidification, matrix quinones such as ubiquinone 0 and decylubiquinone, high membrane potential and cyclosporine A (CsA).

Mitochondrial permeability transition has a central role in several cell death pathways in mammalian cells (Lemasters *et al.* 2002). Several factors such as oxidative stress, hypoxia/anoxia and toxic chemicals induce opening of the mitochondrial PTP leading to swelling of the mitochondrial matrix, disruption of the outer membrane and finally, release of intermembrane proapoptotic proteins including cytochrome *c*. Permeability transition of mitochondria has been shown to be involved in several mammalian diseases and disorders (Bernardi *et al.* 2015; Biasutto *et al.* 2016). In ischemia-reperfusion of heart tissue, PTP opens only in reperfusion due to several inducing factors existing in the reoxygenation phase such as increased formation of ROS, high matrix pH and Ca^{2+}-ion transport into the mitochondrial matrix (Biasutto *et al.* 2016). Disorders in the regulation of the PTP have been shown to lead to excessive opening of the PTP which, in turn, give rise to several diseases such as Ullrich congenital muscular dystrophy and several neurodegenerative diseases (Bernardi *et al.* 2015). In contrary of the previous, decreased propensity of the open conformation of the PTP in cancer cells cause resistance to apoptosis leading to avoidance of cancer cell death (Bernardi *et al.* 2015).

There are currently a few studies which demonstrate features of the mammalian permeability transition taking place in isolated plant mitochondria (Vianello *et al.* 1995; Fortes *et al.* 2001; Arpagaus *et al.* 2002; Curtis and Wolpert 2002; Tiwari *et al.* 2002; **II;** De Col *et al.* 2018). The studies show, however, that there are major differencies in the characteristics of the permeability transition in different plant species or cultivars of the same species such as sensitivity of the permeability transition to CsA or involvement of matrix accumulated Ca^{2+}-ions in plant permeability transition (Zancani *et al.* 2015). Physiological roles of the permeability transition in plants are suggested to

be related to developmental processes, such as in formation of perforations in the leaves of the aquatic monocot *Aponogeton madagascariensis* (Lord *et al.* 2013), and also to plant responses under abiotic and biotic stresses such as for example heavy metal toxicity or pathogen induced stress (Zancani *et al.* 2015). Plant PCD and permeability transition have been shown to be connected, and characteristic features of PT such as cytochrome *c* release, collapse of the mitochondrial membrane potential, mitochondrial swelling and inhibition of plant PCD by CsA have been detected in several studies (Zancani *et al.* 2015). However, the link between permeability transition of plant mitochondria and plant PCD is still for the most part unknown (Van Aken and Van Breusegem 2015; Zancani *et al.* 2015).

1.5 Mitochondria and plant stress

Role of mitochondria in plant stress responses has been under investigation for decades (Jones 2000; Van Aken *et al.* 2009; Huang *et al.* 2016; Belt *et al.* 2017; Wagner *et al.* 2018). Research on plant mitochondria has shown that ROS production of mitochondria is the key function which connects mitochondria to several stress-related processes such as PCD, retrograde signaling, redox signaling, plant hormone signaling and pathogen defence such as the hypersensitive response (HR) (Huang *et al.* 2016). Under biotic and abiotic stresses plant mitochondria produce ROS which has been shown to cause massive perturbations in the energy physiology of mitochondria but ROS have also been suggested to lead to the induction of mitochondrial signaling (Wagner *et al.* 2018). Mitochondrial energy signaling, arising from functionally inhibited mitochondria, has been studied with plants under low oxygen stress since oxygen deprivation causes dysfunctions in the mitochondrial electron transport chain which are suggested to induce the signaling. Several molecules and physiological parameters have been proposed to act as signals or second messengers in the mitochondrial energy signaling pathway including high rate of mitochondrially produced ROS, NO, posttranslational modifications of proteins such as succinylation of proteins, changes in adenylate ratios or mitochondrial membrane potential, PAP (3´-phosphoadenosine 5´-phosphate), calcium ion fluxes and plant hormones such as abscisic acid (ABA) and auxin. Contribution of the proposed molecules and physiological parameters in the

mitochondrial signaling is, however, for the most part largely still unknown. Evidence of hydrogen peroxide as a likely candidate for a signaling molecule in plant mitochondria has, however, been accumulated during the last years (Huang *et al.* 2016; Belt *et al.* 2017; Wagner *et al.* 2018). In a recent article by Belt and colleagues (2017) salicylic acid was shown to increase enzyme activity of succinate dehydrogenase, i.e. Complex II of the mitochondrial ETC, and induce succinate-dependent hydrogen peroxide formation in *Arabidopsis* mitochondria.

1.6 Mitochondrial functioning under oxygen deprivation stress

Knowledge of the mitochondrial functioning under anoxia stress especially in anoxia-tolerant species is still very poorly known (Galli *et al.* 2014). According to earlier studies there are several common features shared by anoxia-tolerant animals taking place under low oxygen stress such as reduced metabolism of the whole animal called either hypometabolism (Galli *et. al* 2014) or metabolic depression (St-Pierre *et al.* 2000). Metabolic depression has been observed in several anoxia-tolerant animal tissues such as in frog muscles and turtle brains (West and Boutilier 1998) but similar metabolic adjustments have also been observed in tissues of anoxia-tolerant plant species (Pfister-Sieber and Brändle 1994). Reduced rate of metabolism under anoxia leads to lowered ATP consumption both in anoxia-tolerant animals (West and Boutilier 1998) and plants (Pfister-Sieber and Brändle 1994) which in turn balances with the low level of ATP synthesis produced by glycolysis.

Studies on animals having capacity to withstand long-term anoxia has brought out new information on anoxia-tolerance showing that changes in the properties of mitochondria have fundamental role in the long-term anoxia tolerance (Galli and Richards 2014). Several changes have been observed to take place in mitochondria of anoxia-tolerant animals such as reduction of the respiration capacity, elevation of both nitrite and NO levels, inhibition of the F_1F_o-ATPase activity and suppression of ROS formation. According to a recently published research by Bundgaard and colleagues (2019) cold acclimated heart mitochondria of anoxia-tolerant freshwater turtles (*Trachemys scripta elegans*) reduce respiration rate under prolonged anoxia by reducing substrate oxidation which was estimated to result from concerted downregulation of several enzyme

complex activities. According to earlier studies anoxia-tolerant animal species might also be able to regulate ROS production by suppressing ROS formation at the onset of anoxia and under reoxygenation (Galli and Richards 2014). ROS production was suppressed in heart mitochondria of freshwater turtles (*T. scripta*) after exposure to prolonged anoxia and reoxygenation but the mechanism of ROS suppression is still unknown (Bundgaard *et al.* 2018). The study shows, however, that the suppression of ROS is tightly associated with the overall decrease in the mitochondrial aerobic function. As a consequence of anoxia stress nitrite levels have been shown to increase substantially in tissues of several anoxia-tolerant animal species (Galli and Richards 2014). Both nitrite and its bioactive form NO have been shown to modify mitochondrial function in several ways under ischaemia-reperfusion stress such as by S-nitrosation of the critical thiol groups in the mitochondrial Complex I carried out by NO which in turn, leads to reduced formation of ROS and further in cytoprotection under the stress (Murillo *et al.* 2011). Elevation of the cytosolic and mitochondrial Ca^{2+} levels are common consequences under anoxia stress but especially accumulation of Ca^{2+}-ions in mitochondria can be detrimental since it can lead to PTP formation and cell death (Galli and Richards 2014). There are some studies on Ca^{2+}-ion homeostasis under anoxia observed in anoxia-tolerant turtle brain cells (*T. scripta*) demonstrating that the cytosolic Ca^{2+}-levels are maintained at low level under anoxia through suppression of ion channels and glutamate receptors which both are involved in Ca^{2+} transport. In addition, studies on the characteristics of PTP of some anoxia-tolerant animal species show that PTP of anoxia-tolerant animal species is less sensitive to highered Ca^{2+}-ion concentrations than the mammalian PTP which has been speculated to be a physiological adaptation to low oxygen stress.

Responses of plant mitochondria under low oxygen stress have been under focus during the last decades (Shingaki-Wells *et al.* 2014). Enzyme activities and both transcript and protein levels of the TCA cycle enzymes are overall downregulated under anoxia stress in plants such as rice, poplar and *Arabidopsis* (Shingaki-Wells *et al.* 2014) but the TCA cycle of the anoxia-tolerant wild plant species barnyard grass (*Echinocloa phyllopogon*) is functional under anoxia and the levels and also patterns of the TCA cycle enzyme activities are on the whole almost similar to the activities found in barnyard grass seedlings grown in air (Fox and Kennedy 1991). It has been estimated that the anaerobically functioning TCA cycle of barnyard grass may possibly have a role in the

anaerobic metabolism and thus contributing to the exceptional anoxia-tolerance of the species. Long-term hypoxia stress has been shown to induce modifications in respiratory supercomplex compositions and changes in the activities of respiratory protein complexes in potato tuber mitochondria (Ramírez-Aguilar *et al.* 2011). According to the study, hypoxia stress induced for example a reduction in the activity of the Complex I within a respiratory supercomplex but instead an increasement of the Complex I enzyme activity was detected in the individual Complex I monomer. Complex I was also shown to dissoaciate from the respiratory supercomplex, which was containing also Complex III and IV, under hypoxia, the modification in the supercomplex composition being suggested to be a regulatory mechanism and part of the acclimation of respiration under low oxygen stress.

Nitrite (NO_2^-) and NO metabolism have been shown to have significant functions in plant mitochondria under low oxygen stress (Stoimenova *et al.* 2007; Gupta and Igamberdiev 2016; Gupta *et al.* 2017; Gupta *et al.* 2020). In an earlier study by Stoimenova and colleagues (2007) it was shown that NO_2^- can function as a terminal electron acceptor in isolated rice and barley root mitochondria which leads both to formation of NO and ATP synthesis under anoxia. Gupta and colleagues (2017) showed also that incubation of isolated pea (*Pisum sativum*) root mitochondria in NO_2^- under hypoxia led to increased ATP synthesis. The study show also that incubating pea root mitochondria with NO_2^- under hypoxia resulted in improvement of mitochondrial integrity and energization of the inner mitochondrial membranes but the incubation decreased lipid peroxidation and formation of ROS. Incubation of hypoxic pea root mitochondria in NO_2^- affected also enzyme activities of the electron transport chain complexes since activites of the Complex I and the supercomplex I+III_2 were increased. Overall, NO_2^- has been shown to have an essential role in protecting mitochondria under hypoxia and contributing mitochondrial integrity and function under low oxygen stress (Gupta *et al.* 2017).

Mitochondria play the main role in production of NO under hypoxia since reduction of NO_2^- leading to formation of NO occurs at Complex III, the alternative oxidase (AOX) and also at Complex IV, in which the major part of the NO is produced (Gupta *et al.* 2020). Electron transport chains of root mitochondria in several plant species have been shown to reduce NO_2^- to NO under low oxygen stress (Gupta and Igamberdiev 2016).

NO molecules produced in mitochondrial matrices diffuse through the membranes and are converted to nitrate (NO_3^-) by the cytosolic class 1 phytoglobins. NO_3^- molecules formed in cytosol are in turn reduced to NO_2^- by the cytosolic enzyme nitrate reductase (NR), and the NO_2^- molecules are imported back into the mitochondrial matrices by either putative nitrite transporter or passive diffusion (Gupta and Igamberdiev 2016; Gupta *et al.* 2020). Altogether, phytoglobin-NO cycle recycles both NO_3^- and NO_2^- molecules but the cycle has been suggested to have a significant role in oxidating cytosolic NADPH and NADH molecules under oxygen deprivation since the NADH/NADPH pool is to a great extent reduced under low oxygen environment (Gupta and Igamberdiev 2016). While ATP is produced glycolytically under low oxygen and glycolysis consumes NAD^+ molecules the phytoglobin-NO cycle thus contributes in maintaining the operation of glycolysis under oxygen deprivation stress.

1.7 Mitochondrial F_1F_o-ATPase and low oxygen stress

Inhibition of the Complex V, i.e. F_1F_o-ATP synthase, has been evaluated to be one of the fundamental survival strategies of anoxia-tolerant animal species under anoxia since the reverse function of the F_1F_o-ATP synthase, which is activated in oxygen deprivation, hydrolyses rapidly ATP molecules leading abruptly to energy crisis in cells (Galli and Richards 2014). According to several studies (St-Pierre *et al.* 2000; Duerr and Podrabsky 2010; Galli *et al.* 2013; Pamenter *et al.* 2016; Gomez and Richards 2018) mitochondrial F_1F_o -ATP synthase has been shown to be inhibited in various tissues of anoxia-tolerant animal species under anoxia stress. In an early study by St-Pierre and colleagues (2000) ATP consumption of anoxia-tolerant frog (*Rana temporaria*) skeletal muscle mitochondria under short-term anoxia incubation was measured. Results of the study demonstrated that ATP use of the F_1F_o -ATP synthase was minor under anoxia and it was caused by substantial inhibition in the ATPase activity. Activity of the F_1F_o-ATP synthase has been shown to be inhibited considerably also in tissues of other anoxia-tolerant animals such as in diapausing embryos of annual killifish (*Austrofundulus limnaeus*) (Duerr and Podrabsky 2010) and in heart, brain and liver tissues of freshwater turtle (*Trachemys scripta*) (Galli *et al.* 2013; Pamenter *et al.*

2016; Gomez and Richards 2018). Still today very little is known on the anaerobic function of plant F_1F_o-ATP synthase (Shingaki-Wells *et al.* 2014).

The underlying mechanisms of the anaerobic F_1F_o-ATPase inhibition are still largely unknown. It has been suggested that the F_1F_o-ATPase inhibition might come from the binding of the ATPase inhibitory factor 1 (IF1) to the F_1-subunit, transport limitation at the adenine nucleotide carrier, modification of the K_m of the enzyme for ATP or from the inhibition of the F_1F_o-ATPase by ADP (St-Pierre *et. al.* 2000) but also inhibition through protein S-nitrosylation has been suggested as the inhibitory mechanism of the Complex V (Gomez and Richards 2018). In a recent study by Gomez and Richards (2018) putative mechanisms of the F_1F_o-ATPase inhibition in anoxic tissues of freshwater turtle (*T. scripta*) were investigated. According to the results, long-term anoxia led to a drastic decrease in the activity of F_1F_o-ATPase in tissues of freshwater turtle but S-nitrosylation did not have any effect on the Complex V activity and the K_m of the enzyme for ATP was neither affected. However, proteomics analysis revealed significant anoxia-induced decreases in three peripheral stalk subunits of Complex V which have in mutational analysis been shown to reduce ATPase activity in yeast mitochondria.

2 AIMS OF THE STUDY

In my thesis I have tried to find answers to several questions concerning plant responses to oxygen deprivation stress. The key questions of the research articles in my thesis are:

1) How long term incubation of rhizomes and roots under anoxia affect the quantities of low molecular mass antioxidants and the redox states of the antioxidants in plant species with differing tolerance to anoxia stress? (I, III)

2) Is the opening of the permeability transition pore (PTP) of plant mitochondria similarly regulated as animal PTP? (II)

3) How do the root mitochondria and the mitochondrial F_1F_o-ATPase function in anoxia-tolerant and -sensitive plant species under anoxia stress? (IV)

3 MATERIALS AND METHODS

The materials and methods are described in detail in the research articles I, II, and IV. The list of the methods are given in an alphabetical order in Table 1. The Roman numerals refer to the original articles listed in Page 1.

Table 1. Methods used in the original articles. The brackets refer to a method which has been performed by co-authors in the publication in question.

Method	**Publication**
Anoxia incubations of rhizomes of *Iris* spp.	I
Anoxia incubations of roots of cereals	(I)
Ascorbic acid (AA) assay	(I)
Bradford method	II, IV
Ca^{2+}-ion transport assay with indicator dye Arsenazo III	(II)
Determination of glutathione	(I)
Determination of mitochondrial volume changes	(II)
Determination of ATP content by chemiluminescence reaction	IV
High-Performance Liquid Chromatography (HPLC) of Vitamin E compounds	I
High-Performance Liquid Chromatography (HPLC) of Vitamin E compounds and mass spectrometry (MS)	(I)
Mitochondria isolation from plant roots	II, IV
Oxygen consumption assay of isolated mitochondria	II, IV
Phenol Red assay for enzyme activity assay of F_1F_o-ATPase	IV
Safranine O method for membrane potential determination	(II)
Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)	II
Solvent extraction method of Vitamin E compounds	I
Statistical analysis	(I),I, IV
Succinate:cytochrome *c* oxidoreductase activity assay	II, IV
Transmission electron microscopy (TEM)	(II)
Western blotting	II

4 RESULTS AND DISCUSSION

4.1 Anoxia followed by reaeration leads to a decrease in the content and in the redox state of small molecular antioxidants in both anoxia-tolerant and –intolerant plant species

Measurements of the content of small molecular antioxidants i.e. ascorbate, glutathione and tocopherols, in rhizomes of *Iris germanica* and *Iris pseudacorus* and in the roots of wheat (*Triticum aestivum*) and rice (*Oryza sativa*) show that there are considerable differencies in the initial contents of the antioxidants between the species. The initial amount of glutathione in the rhizomes of *I. germanica* and ascorbate in the rhizomes of *I. pseudacorus* were multifold higher when compared to the amounts detected in the other species studied (**I**). In addition, the initial contents of both tocopherols, i.e. α-and β-tocopherols, were higher in the rhizomes of *I. germanica* in comparison to the amounts measured in the rhizomes of *I. pseudacorus* (**I**). The study revealed also a major difference between the cereals and the *Iris*-species since the predominant form of the ascorbate pool was the oxidised form i.e. dehydroascorbic acid (DHA) in cereals, while the reduced form, ascorbate (AA), dominates the pool in *Iris*-species (I).

Anoxic treatment followed by reoxygenation led to a decrease in the total contents of ascorbate and glutathione in all the species studied resulting especially from the considerable decreases in the reduced forms of ascorbate and glutathione which did not, however, lead to simultaneous increases in the contents of the oxidized forms of the antioxidants in question (**I**). However, long-term anoxia incubations of the rhizomes and roots with subsequent reoxygenation led to a decreased content of both reduced and oxidized forms of ascorbate and glutathione in all the species studied (**I**). Furthermore, declined contents of the reduced forms of both ascorbate and glutathione led to decreased redox states i.e. ratio between the reduced and the oxidized form of a given antioxidant, of the antioxidant pools in question in all the species studied except in the rhizomes of *I. pseudacorus* in which the redox state of the glutathione pool remained at the same level as in the aerated control rhizomes of the species (**I**). Incubation of rhizomes of *I. germanica* and *I. pseudacorus* for a prolonged time under anoxia led also to a decrease in the content of α-and β-tocopherols in both species studied (**I**).

Responses of plant ascorbate and glutathione pools in roots or rhizomes under anoxia/hypoxia stress with or without subsequent reaeration have been under study in several research articles (**I**; Biemelt *et al.* 1998; Lin *et al.* 2004; Garnczarska 2005; Goggin and Colmer 2005; Skutnik and Rychter 2009; Luo *et al.* 2012). In an earlier study by Biemelt and colleagues (1998) total amount of ascorbate increased significantly in roots of wheat seedlings under anoxia treatment which was not the case in roots of barley (Skutnik and Rychter 2009) wherein the total content of ascorbate did not increase significantly until in postanoxia. In reoxygenation experiments of wheat seedlings the amount of total ascorbate stayed at higher level due to the accumulation of ascorbate under anoxia though the redox state of the ascorbate pool decreased in reaeration (Biemelt *et al.* 1998). Instead, the redox state of the ascorbate pool increased in the roots of barley (Skutnik and Rychter 2009) under reaeration. Instead results of the total content of ascorbic acid and the redox state of the ascorbate pool observed in the rhizomes of *Irises* and in the roots of wheat and rice show opposite changes when compared to the results presented above (**I**). Changes in the total content of glutathione observed in cereal roots are more consistent since total amount of glutathione has been shown to decrease significantly both in roots of wheat (**I**; Biemelt *et al.* 1998; Goggin and Colmer 2005) and barley (Skutnik and Rychter 2009) under anoxia. In reoxygenation, however, total content of glutathione has been shown to increase both in wheat (Biemelt *et al.* 1998) and barley roots (Skutnik and Rychter 2009) but the content decreased significantly both in the rhizomes of *I. germanica* and *I. pseudacorus* and in the roots of wheat and rice under reoxygenation phase (**I**). Changes in the redox state of the glutathione pool under reoxygenation vary to a great extent between studies since redox state of the glutathione pool increased significantly in barley roots (Skutnik and Rychter 2009) while the redox state of the glutathione pool remained similar to the aerated controls of the species in rhizomes of *I. pseudacorus* (**I**). In addition, redox state of the glutathione pool decreased significantly in the rhizomes of *I. germanica* and in the roots of both wheat and rice under reaeration (**I**).

There are only few studies on the changes in the tocopherol content of roots or rhizomes under hypoxia or anoxia stress (**I**; Chirkova *et al.*1998; Lin *et al.* 2004). In a study by Chirkova and colleagues (1998) there were divergent responses in the content of tocopherols observed in roots of wheat and rice under anoxia since the tocopherol content of wheat roots multiplied rapidly at the onset of anoxia but declined during the

next two days, while tocopherol content of rice roots increased slightly only at the end of the 7-days incubation both under anoxia and aeration. According to Lin and colleagues (2004), no significant changes were observed in the α-tocopherol content in the roots of several tomato or eggplant cultivars under short-term flooding stress experiments. Instead, incubation of the rhizomes of *I. germanica* and *I. pseudacorus* under anoxia led to significant decreases in both α- and β- tocopherol contents only under prolonged anoxia incubations i.e. 45 and 12 days for the rhizomes of *I. pseudacorus* and *I. germanica*, respectively (**I**).

There is a strong probability that oxidative stress, with a burst of ROS, accounts for the observed decreases in the total amounts of small molecular mass antioxidants in reoxygenation phase detected in the rhizomes of *Iris*-species and in the roots of wheat and rice (**I**). In the reaerated tissues rapid increase of ROS depletes especially the reduced forms of ascorbate and glutathione due to scavenging of ROS, which was observed as a decrease in the redox states of the antioxidants in question in the study (**I**). However, no concurrent increases in the oxidized forms of ascorbate and glutathione were detected in the studied species (**I**) which can be due to increased catabolism of dehydroascorbic acid (DHA) in stress since DHA is easily catabolized to oxalate and tartrate especially if the rereduction to ascorbate is slowing down (Noctor and Foyer 1998). The results could also be due to the antioxidantive capacities of the species studied which might have been exceeded under anoxia-reoxygenation stress. The low amounts of the oxidized forms of ascorbate and glutathione could also arise from declined activities of the antioxidant enzymes responsible for the regeneration of ascorbate and glutathione i.e. the enzymes of the ascorbate-glutathione cycle shown in the **Figure 1.** Altogether, it can be seen in the results (**I**) that there was no correlation between the anoxia tolerance of a given plant species and the total content of a given antioxidant under anoxia or anoxia with subsequent reoxygenation. However, measuring redox states of antioxidants show some correlation with anoxia tolerances of the species studied. Redox state of the glutathione pool i.e. GSH/GSSG, decreased both in the rhizomes of the anoxia-sensitive *I. germanica* and in the roots of wheat and rice under anoxia but no decrease was observed in the redox state of the anoxia-tolerant *I. pseudacorus* despite the long-term anoxic incubations of the species. According to the results the GSH/GSSG ratio of the *I. pseudacorus* stayd at the same level between the experiments of aerated and anoxia-incubated rhizomes (**I**). Consequently, changes

in the redox states of antioxidants under oxidative stress may be a more significant factor than the changes in total amounts of antioxidants when estimating antioxidative capacity of a given plant species under stress.

The predominant tocopherol isomer in rhizomes of both *Iris*-species studied was β-tocopherol (**I**) which is a new finding for the distribution of tocopherol isomers in plants. In a closer analysis in mass spectrometry the assumed β-tocopherol extracted from the rhizomes was estimated to be β-dehydrotocopherol (**I**). In photosynthetic tissues α-tocopherol is in general the predominant isomer but in seeds or seed oils β-, γ- and δ- tocopherols and tocotrienol isomers predominate (Dellapenna and Mène-Saffrané 2011). Tocomonoenols i.e. dehydrotocopherols, were first found in etiolated shoots of maize and barley but later they have been found in seed oils and leaves of several plants species bur they have also been found for example in kiwi fruit and *Arabidopsis* seeds (Szymańska and Kruk 2018). It has been suggested that tocomonoenols may be precursors of tocopherols but on the whole metabolism of tocomonoenols is poorly known.

There are only few studies on the responses of the tocopherol pool under plant oxygen deprivation stress (Chirkova *et al.*1998; **I**; Lin *et al.* 2004; Paradiso *et al.* 2016). Nevertheless, results indicate highly diverse responses in the tocopherol pool of the species studied under low oxygen stress since content of tocopherols has been shown to increase in wheat roots (Chirkova *et al.* 1998) and in *Arabidopsis* suspension cell cultures (Paradiso *et al.* 2016) but a significant decrease in the tocopherol content was observed both in the rhizomes of *I. pseudacorus* and *I. germanica* (**I**) and in shoots of wheat seedlings (Chirkova *et al.* 1998). In addition, no significant changes were detected in the content of tocopherols in roots of tomato and eggplant cultivars under flooding stress experiments (Lin *et al.* 2004) and in roots of rice seedlings under anoxia (Chirkova *et al.* 1998). The diversity in the responses may depend on factors such as plant species or plant organ used in the experiment, but also experimental set-up could affect the results. In most of the studies duration of the anoxia experiments were short, continuing either for some hours (Paradiso *et al.* 2016) or three to seven days (Chirkova *et al.* 1998; Lin *et al.* 2004) while the anoxic incubations of *I. germanica* and *I. pseudacorus* continued up to 12 days and 45 days, respectively (**I**). Only the most long-term anoxic incubations of the *Irises* led to significant decreases in the contents of

tocopherols in *I. pseudacorus* and *I. germanica*, respectively, while no significant changes were detected under shorter incubations (**I**). Studies on the anoxia-tolerances of rhizomes of several plant species have demonstrated that rhizomes of *I. pseudacorus* can survive at least 28 days experimental anoxia while rhizomes of *I. germanica* can survive only 7-10 days under similar conditions (Crawford and Braendle 1996). It can be concluded of the results presented in (**I**) that decreased tocopherol pools detected in the rhizomes of *I. pseudacorus* and *I. germanica* result most probably from increased breakdown of tocopherols and simultaneous inhibition of the tocopherol synthesis under prolonged anoxia. According to Munné-Bosch (2005), α-tocopherol levels respond in general to environmental stresses due to stress-induced changes in tocopherol degradation and recycling but also gene expression of the tocopherol synthesis pathway can be altered under stress.

Research on antioxidants of animal heart tissues in ischemia-reperfusion has brought out a concept of antioxidant networking in which different antioxidants are linked and acting as a part of a network instead of acting individually (Haramaki *et al.* 1998; Marczin *et al.* 2003). According to the study by Haramaki and colleagues (1998) hydrophilic antioxidants, ascorbate and glutathione, were at the forefront of the antioxidative defence system in myocardial cells being depleted in mild oxidative stress while the levels of hydrophobic antioxidants, such as tocopherols, were not affected. Both hydrophilic and hydrophobic antioxidants were, however, depleted in severe oxidative stress. Similar pattern was seen in the contents of small molecular mass antioxidants in response to the length of anoxic incubation in the rhizomes of *Iris*-species (**I**). Duration of the incubations of the rhizomes varied according to the anoxia-tolerance of the species so that the rhizomes of anoxia-tolerant *I. pseudacorus* and anoxia-sensitive *I. germanica* were incubated up to 45 days and 12 days, respectively. According to the results presented in (**I**), only amounts of the hydrophilic antioxidants, ascorbate and glutathione, decreased significantly under short-term anoxic incubations while no significant decreases were observed in the contents of tocopherols. However, significant decreases in the contents of tocopherols were seen only in the long-term anoxia incubations of *Iris*-species with simultaneous decreases of the hydrophilic antioxidants. It could be concluded of the results (**I**) that short-term anoxia induced mild oxidative stress in the rhizomes studied but the long-term incubations under anoxia exerted severe stress which led to changes in the small molecular mass antioxidants

similar to the ones described in research article by Haramaki and colleagues (1998). Thus both ascorbate and glutathione could be at the forefront of the antioxidative defence system under mild oxidative stress also in plant cells leading to a depletion of the hydrophilic antioxidants but simultaneously protecting both hydrophobic antioxidants, such as tocopherols, and other structures, such as proteins, nucleic acids and cell membranes, from destruction (Marczin *et al.* 2003).

4.2 Wheat root mitochondria under high calcium concentration show several features of the mammalian permeability transition

Energized wheat root mitochondria were shown to be able to transport calcium ions (Ca^{2+}) in the presence of inorganic phosphate (**II**). Ca^{2+}-ion uptake of mitochondria led to the accumulation of Ca^{2+}-ions in the mitochondrial matrices which were shown to be released to the surrounding medium either spontaneously or by addition of the Ca-ionophore A23187. Under high Ca^{2+}-ion concentration high amplitude swelling of the mitochondrial matrices was detected both spectrophotometrically and in transmission electron microscopy (TEM) but no swelling of mitochondrial matrices could be detected when high magnesium ion (Mg^{2+}) concentration substituted for the addition of Ca^{2+}-ions. Calcium induced swelling of wheat mitochondria was not inhibited by CsA together with dithioerythritol (DTE) suggesting to a differently regulated PTP when compared to the regulation of the mammalian PTP. High Ca^{2+}-ion concentration in the presence of inorganic phosphate induced also substantial cytochrome *c* release in wheat root mitochondria, but CsA in the reaction medium together with DTE could not inhibit the release. In addition to the previous, neither high Mg^{2+}-ion concentration in the presence of inorganic phosphate nor high Ca^{2+}-ion concentration without inorganic phosphate could induce cytochrome *c* release in isolated wheat root mitochondria.

At the moment features of permeability transition (PT) have been described in studies with plant mitochondria isolated from pea stems (Vianello *et al.* 1995; De Col *et al.* 2018), potato tubers (Fortes *et al.* 2001; Arpagaus *et al.* 2002), wheat roots (**II**), oat leaves (Curtis and Wolpert 2002) and Arabidopsis cell cultures (Tiwari *et al.* 2002). Regardless of the minor amount of studies on plant PT they show major differencies in the features of PT in different plant species or cultivars of the same species. Requirement of matrix accumulated calcium ions to induce plant PT is one of the major

differences in the studies. Though accumulation of calcium ions in the mitochondrial matrices of wheat root mitochondria leading to PTP opening was shown in the study (**II**) there is a contrary result observed by Fortes and colleagues (2001) demonstrating features of PT in potato tuber mitochondria even though the mitochondria were shown not to be able to transport calcium ions. Altogether, accumulation of high matrix calcium concentration, either by spontaneous calcium transport or by the addition of calcium ionophore, was shown to induce PT in some of the studies (Arpagaus *et al.* 2002; Curtis and Wolpert 2002; Tiwari *et al.* 2002; **II**; De Col *et al.* 2018) but high calcium concentrations outside mitochondrial matrices could seemingly also induce PT (Fortes *et al.* 2001).

Accumulation of matrix located calcium ions is an absolute requirement for the induction of the mammalian PT (Bernardi 2018; Carraro and Bernardi 2016; Bernardi *et al.* 2015). According to recent studies on mammalian PTP and its identity, it has been suggested that PTP forms through a mechanism which is strictly dependent on Ca^{2+} ions (Giorgio *et al.* 2018). According to the hypothetical model presented by Giorgio and colleagues (2018), matrix Ca^{2+} ions bind to the catalytic part of the F_1F_o-ATPase thus replacing bound Mg^{2+} ions which, in turn, lead to a conformational change in the enzyme complex and formation of PTP. Plant mitochondrial calcium uniporter which share several features of the mammalian mitochondrial calcium uniporter, was characterized first in 2017 by Teardo and colleagues. There are six genes in *Arabidopsis* genome which are homologs of the mammalian mitochondrial calcium uniporter channel proteins. According to the study by Teardo and colleagues (2017) two of the six *Arabidopsis* homologs localize only in mitochondria showing particularly high expression in *Arabidopsis* roots, while only intermediate or low expression levels were detected in other plant tissues studied. Whether matrix located Ca^{2+} ions have similar function also in the plant mitochondrial PT has not been studied yet but contradictory results with plants indicate that calcium ions may have divergent roles in different plant PT studies. It is also possible that the contradictory results on calcium transport of potato tuber mitochondria (Fortes *et al.* 2001; Arpagaus *et al.* 2002) might refer to two different channels having distinct properties which has been demonstrated to exist in rat liver mitochondria (Kushnareva and Sokolove 2000). In addition, studies on yeast mitochondria show that they do not possess a calcium uniporter but still the mitochondria show typical features of PT such as increased intracellular calcium ion

concentration ([Ca^{2+}]), increased ROS generation, mitochondrial depolarization, ATP depletion, matrix swelling and release of cytochrome *c* and other preapoptotic proteins (Carraro and Bernardi 2016).

In addition to matrix accumulated Ca^{2+} ions, several inducing or inhibiting factors are required to bring about PT in mammalian mitochondria (Bernardi *et al.* 2015). There are several factors which promote onset of mammalian PT such as high matrix pH, matrix located Pi, oxidation of matrix pyridine nucleotides and dithiols while matrix Mg^{2+} ions, low matrix pH, CsA, thiol reductants and adenine nucleotides promote inhibition of PT. Studies on plant PT show that plant mitochondria collectively have at least some shared PT inhibiting or inducing factors with the mammalian ones such as the inhibitory effect of Mg^{2+}-ions on PT (Fortes et al. 2001; Arpagaus *et al.* 2002; Curtis and Wolpert 2002; **II**; De Col *et al.* 2018) and the inducing effect of thiol oxidants such as diamide and phenylarsine oxide on PT (Fortes *et al.* 2001; Arpagaus *et al.* 2002; De Col *et al.* 2018). However, plant mitochondria of different origin show divergent sensitivities to some of the mammalian PT inducing or inhibiting factors, such as CsA and inorganic phosphate (Pi). In mammalian mitochondria inhibition of PT by CsA has been shown to be one of the main characteristic features of the mammalian PT (Carraro and Bernardi 2016). Studies on the mechanism of inhibition has proved that CsA binds to a matrix located enzyme, cyclophilin (CyP) D, leading to the inhibition of its enzyme activity (Bernardi *et al.* 2015). PT of isolated brain mitochondria, however, show PT which is not inhibited by CsA (Brustovetsky and Dubinsky 2000). Results of the plant mitochondria studies show that the effect of CsA is highly diverse among the plant studies since PT was inhibited by CsA both in potato tuber mitochondria (Arpagaus *et al.* 2002) and Arabidopsis cell cultures (Tiwari *et al.* 2002) and the onset of PT was delayed in pea stem mitochondria (Vianello *et al.* 1995; De Col *et al.* 2018) but studies with mitochondria isolated from potato tubers (Fortes *et al.* 2001) and wheat roots (**II**) demonstrate PT which is insensitive to CsA. PT of yeast mitochondria has been studied for several decades and one of the characteristic features of the yeast PT is that it is not inhibited by CsA (Carraro and Bernardi 2016; Jung *et al.* 1997). Studies on plant PT demonstrate also that mitochondria isolated from different plant species show divergent responses to added inorganic phosphate (Pi). Pi has been shown to act as an inducer of PT in potato tuber mitochondria (Arpagaus *et al.* 2002) and wheat root mitochondria (**II**) but instead, PT of pea stem mitochondria was shown to be inhibited in the presence

of added Pi (De Col *et al.* 2018) which is also the case with yeast mitochondria (Jung *et al.* 1997).

In the course of the mammalian permeability transition several events take commonly place in mitochondria such as depolarization of the inner membrane, Ca^{2+}-ion release from the mitochondrial matrices, mitochondrial swelling, rupture of the mitochondrial outer membranes and release of proapoptotic proteins including cytochrome *c* (Giorgio *et al.* 2018). Similar features have also been observed in the plant PT studies but the detected events show contrasting features in plant mitochondria isolated from different plant species or plant tissues. Several of the events observed in mammalian PT were demonstrated in wheat root (**II**) and in potato tuber (Arpagaus *et al.* 2002) mitochondria such as disruption of the membrane potential, Ca^{2+}-induced mitochondrial swelling and release of cytochrome *c*. In addition to the previous, Ca^{2+}-ion release from mitochondria was shown to occur during the course of PT in wheat mitochondria (**II**) while complete rupture of the outer membranes was measured in the potato tuber mitochondria (Arpagaus *et al.* 2002). Studies on pea stem mitochondria show divergent results since no swelling of mitochondria neither cytochrome *c* release were detected in the course of PT (De Col *et al.* 2018), regardless of the facts that PT of pea stem mitochondria have several shared characteristics with the mammalian PT such as membrane depolarization and Ca^{2+}-ion release during the course of PT.

There are several common features in plant and mammalian mitochondrial permeability transition but plant PT show also features which are opposite to those observed in mammalian PT. Some studies on plant PT show similarities to yeast PT such as lack of Ca^{2+}-ion uptake of mitochondria observed in potato tuber (Fortes *et al.* 2001) and pea stem (De Col *et al.* 2018) mitochondria. There are also features which are estimated to be specific to plant PT such as dithioerythritol (DTE) dependence of CsA-induced inhibition of PT which has been shown in PT of potato tuber mitochondria (Arpagaus *et al.* 2002) and pea stem mitochondria (Vianello *et al.* 1995). The DTE dependency of the CsA-induced inhibition was, however, not detected in the studies on PT of wheat root mitochondria (**II**), despite the similarities of the experimental set-up, and hence, the specificity of the DTE dependency is not likely to be common among different plant species or plant tissues of different origin.

At the moment, there are few studies on the characteristics of plant PT but nevertheless, variety of features detected in the studies show great divergence in different plant species or tissues. There are two studies for example on potato tuber PT (Fortes *et al.* 2001; Arpagaus *et al.* 2002) demonstrating opposing results on the CsA-induced inhibition of PT. PT of potato tuber mitochondria was fully inhibited by CsA in the study by Arpagaus and colleagues (2002) but no CsA-induced inhibition could be shown in the study by Fortes and colleagues (2001). Divergent results can arise from using different potato cultivars but also differencies in the experimental procedures may lead to contrasting results. Altogether, scarcity of plant PT studies hinder the analysis of the conflicting results. Several aspects of plant PT are still either poorly studied such as PT of different plant organs and tissues, or completely unknown such as identity of PTP in plants.

4.3 Isolated root mitochondria of yellow flag iris (*Iris pseudacorus*) and wheat (*Triticum aestivum*) consume ATP at different rate under short term anoxia

ATP use of isolated wheat root and yellow flag iris mitochondria was determined under 30 minute anoxic incubation (**IV**). Results of the study show statistically a very significant difference in the ATP use under anoxia since mitochondria of yellow flag iris contained approximately three times higher amount of ATP at the end of the experiment in comparison to the amount measured in wheat mitochondria (**IV**). Enzyme activity of the mitochondrial F_1F_o-ATPase was measured under normoxic conditions, and the results demonstrated that F_1F_o-ATPase of wheat root mitochondria hydrolyze ATP statistically at significantly higher rate when compared to the rate measured in yellow flag iris mitochondria (**IV**). Respiration of isolated mitochondria and intactness of the mitochondrial outer membranes were measured to evaluate the quality of the freshly isolated mitochondria. Results of the respiration studies showed that the respiration rates of wheat mitochondria were very significantly higher at states 2, 3 and 4 than those measured in yellow flag iris (**IV**). Instead, there was no statistical difference in the respiratory control ratios (RCR) between the species studied (**IV**). Intactness of the mitochondrial outer membranes were determined by measuring succinate:cytochrome *c* oxidoreductase activity of freshly isolated mitochondria. According to the results higher amount of wheat mitochondria had intact outer

membranes when compared to the results of yellow flag iris the difference being statistically rather significant (**IV**).

The amount of studies on the function of plant mitochondria or plant F_1F_o-ATPase under anoxia stress are at the moment minor (Shingaki-Wells *et al.* 2014). Nitrite-driven ATP synthesis in anoxic rice and barley root mitochondria is one of the rare studies on the anaerobic function of plant mitochondria (Stoimenova *et al.* 2007). In addition among the few publications there are a few studies on the anaerobically functioning mitochondria of barnyard grass (*Echinocloa* spp.) species (Kennedy *et al.* 1987; Fox and Kennedy 1991; Fox *et al.* 1994). At the moment there is one study (**IV**) in which the ATP use of the F_1F_o-ATPase under anoxia stress has been investigated in mitochondria of anoxia-tolerant and -intolerant plant species demonstrating very significant difference in the anaerobic ATP use between the species studied. Results of the F_1F_o-ATPase activity studies performed under normoxic conditions and the values of respiratory control ratios (RCR) also support the results of the anoxic ATP consumption studies (**IV**).

While the anoxic F_1F_o-ATPase activities of anoxia-tolerant freshwater turtles (Gomez and Richards 2018) and frogs (St-Pierre *et al.* 2000) were demonstrated to be inhibited up to 80-87% of the enzyme activity measured under normoxia, activity of the F_1F_o-ATPase in yellow flag iris mitochondria was not inhibited under anoxia in similar quantity (**IV**). Instead, ATP content of yellow flag iris mitochondria after 30 minute anoxia was approximately 16% of the amount measured before anoxia started while more than 80% of the ATP was consumed during the anoxic incubation (**IV**). The difference in the ATP use of anoxia-tolerant animals and yellow flag iris mitochondria might result from fundamental differencies in the functional and regulatory properties of animal and plant mitochondria. Earlier it has been shown that the inhibitory mechanism of the F_1F_o-ATPase activity under anoxia differs substantially in different animal species (Gomez and Richards 2018). S-nitrosylation of the Complex V inhibits the ATPase activity in mice mitochondria under anoxia but not in mitochondria of anoxia-tolerant freshwater turtles. Whether results of plant mitochondria can be directly compared to the results of animal mitochondria is highly questionable since plant and animal mitochondria have several functional and structural differencies such as the four

NAD(P)H dehydrogenases and alternative oxidase (AOX) found only in the inner membranes of plant mitochondria (Møller 2001).

Mitochondrial F_1F_o-ATPase activity under normoxia was also measured demonstrating that wheat mitochondria hydrolyze ATP at significantly higher rate when compared to the rate in yellow flag iris mitochondria (**IV**). There are several earlier studies in which the rate of plant mitochondrial F_1F_o-ATPase activity has been investigated (O'Rourke and Wilson 1992a; O'Rourke and Wilson 1992b; Valerio *et al.* 1993a; Valerio *et al.* 1993b; Valerio *et al.* 1994). Overall, measured ATPase activites of plant mitochondria are generally low but there are also exceptions such as the pea leaf mitochondria which show high ATP hydrolyzing activity (Valerio *et al.* 1994). According to the studies by O'Rourke and Wilson (1992b) and Valerio and colleagues (1993b), it has been suggested that results on plant mitochondrial ATPase activity refer to the existence of an ATPase inhibitor protein. Mitochondrial ATPase inhibitor protein (IF1) in plant mitochondria was first characterized in 1990 by Norling and colleagues. However, it has not been studied whether the IF1 binding to the F_1F_o-ATPase or some other putative inhibitory mechanism causes the observed inhibitions in the mitochondrial ATPase activity found in the earlier plant mitochondrial studies.

Altogether, results of the study demonstrate very significant difference in the anaerobic ATP use between the mitochondria of wheat and yellow flag iris, and the results correlate well with the anoxia tolerances of the species. The results of the F_1F_o-ATPase activity studies both under anoxia and normoxia might refer to fundamental differences in the regulatory system of Complex V activity between the species. Results indicate also divergent survival strategies of the studied species under oxygen deprivation stress. Anoxia-tolerant yellow flag iris mitochondria consume ATP at a sparing rate under anoxia which may be a part of the survival strategy of the species under stress but instead, wheat mitochondria hydrolyze ATP molecules at high rate under anoxia which leads rapidly to depletion of the ATP pool and abrupt cell death.

5 CONCLUSIONS

In the thesis, several aspects of plant responses under anoxia stress have been demonstrated in tissues or mitochondria of anoxia-sensitive and -tolerant plant species.

Changes in the redox state of the small molecular mass antioxidant glutathione under anoxia-reoxygenation stress were shown in the study to correlate with the anoxia-tolerances of yellow flag iris (*I. pseudacorus*) and garden iris (*I. germanica*). In conclusion, the redox state of the glutathione pool in the tissue of the anoxia-tolerant yellow flag iris was unchanged under anoxia-reoxygenation stress which might have a significant role in the anoxia-tolerance of the species.

Anaerobic ATP use of both yellow flag iris (*I. pseudacorus*) and wheat (*Triticum aestivum*) root mitochondria was investigated in the thesis. Results of the study indicate that mitochondria of the anoxia-sensitive wheat hydrolyze ATP molecules at a high rate while the mitochondria of anoxia-tolerant yellow flag iris show substantially lower ATP hydrolyzing rate. In conclusion, the results correlate well with anoxia-tolerances of the species studied and the sparing rate of ATP consumption of yellow flag iris mitochondria under anoxia may be a fundamental factor of the anoxia-tolerance of the species.

Permeability transition of wheat (*Triticum aestivum*) root mitochondria was characterized in the study demonstrating several similarities with the properties of the mammalian permeability transition. However, several features of the permeability transition in wheat mitochondria were shown to diverge from the properties of the permeability transition observed in mitochondria of different plant species or origin of plant tissue.

In the future, more studies on the mitochondrial function of anoxia-tolerant plant species should be performed in order to understand anoxia-tolerance of plants. Especially function and properties of the plant F_1F_o-ATPsynthase should be studied in order to understand its involvement in the physiological responses of plants.

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