

Strategies of tomato plants to maintain cellular integrity under
hypoxic stress

Inauguraldissertation

zur

Erlangung des akademischen Grades eines
Doktors der Naturwissenschaften (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Universität Greifswald

vorgelegt von

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Greifswald, 18.09.2024

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Tag der Promotion: 28.01.2025

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1. Abstract

Hypoxic environmental conditions such as flooding and the resulting oxygen limitation for plant roots interrupt oxidative phosphorylation and lead to an energy crisis in plants. The energy shortage eventually results in the breakdown of cellular transmembrane gradients and cell death. The degradation of sucrose and starch and an increase in anaerobic glycolysis are well-described mechanisms to provide energy and survive this crisis. Lactic acid and ethanolic fermentation recycle reducing equivalents to maintain ATP synthesis. However, alternative reducing equivalent oxidation mechanisms (**Chapter I**), alternative energy and carbon sources (**Chapter II**) and regulation of energy consumption (**Chapter III**) can be crucial for survival under low oxygen conditions. To answer this hypothesis, emissions of nitric oxide (NO) under low-oxygen conditions (mechanism – **Chapter I**), changes in lipid composition under hypoxic conditions (energy source – **Chapter II**) and a redox-reactive and highly conserved cysteine residue of a plasma membrane (PM) P-type H⁺-ATPase (energy consumption – **Chapter III**) were investigated.

The thesis confirmed the importance of nitrite (NO₂⁻) as an alternative electron acceptor resulting in NO emissions of plant roots during low-oxygen stress. Firstly, the chemiluminescence-based detection of oxygen-dependent NO emissions in *in vivo* studies for roots of tomato, barley and tobacco is reported using a root reactor (**Chapter I**). The large amounts of low-oxygen NO emission in the root reactor suggest a role in generation of ATP. NO₂⁻ reduction appears to be involved in the mitochondrial electron transfer chain (mETC) and therefore may contribute to ATP generation. In response to oxygen depletion, an escape strategy of the studied species seems likely, including oxygen supply systems. In this strategy, additional ATP is invested to integrate structural changes. However, hypoxic tomato plants seem to rely on glycolysis and fermentation. They do not utilize lipid degradation and β-oxidation as energy source which was confirmed in HPLC-MS-MS analysis studies of hypoxic tomato roots (**Chapter II**). In terms of energy source, neither a depletion of lipids nor an increase in lipid degradation was observed. Triacylglycerol (TG) was more abundant and the degree of unsaturation of fatty acids increased due to hypoxic conditions (**Chapter II**). TGs play a role as intermediates in lipid metabolism. Subsequent utilization of carbon and energy sources during prolonged oxygen scarcity is possible. However, an altered activity of PM P-type H⁺-ATPase due to hypoxic-

induced, posttranslational NO modification was not confirmed. A possible S-nitrosylation site at the conserved cysteine residue was not verified in the biochemical mutagenesis study described in **Chapter III**. Regulation of PM P-type H⁺-ATPase activity via cytoplasmatic ATP-level may be possible in plants.

The maintenance of proteins and cell functions appears to be essential under conditions of low-oxygen and high levels of reactive nitrogen species (RNS) such as NO (**Chapter I**). The induction of antioxidant systems under low-oxygen stress is well known. In this thesis, the highly conserved cysteine near the P-site of the PM P-type H⁺-ATPase was shown to act as an endogenous antioxidant (**Chapter III**). This underlines the importance of this enzyme for the cell. While the changes in lipid composition observed under hypoxia highlight the importance of maintaining membrane integrity and fluidity as in the case of plastids (**Chapter II**). Membrane adaptation results in increased TG abundance which led to the formation of lipid droplets. TG may scavenge toxic intermediates to prevent cell death. Lipid droplets provide a binding site for enzymes involved in adaptation and may even protect unsaturated fatty acids under high reactive oxygen species (ROS) and RNS environments by being incorporated into their core.

Under low-oxygen conditions, various strategies have evolved to provide sufficient energy for adaptation and survival while protecting vital cellular functions in plants. The increase in flooding and heavy rainfall due to climate change poses a challenge to crops and their harvest. Plant-based stress-responses may even be a factor impacting the climate (**Chapter I**). In order to stabilize food production, plant breeding may need to rethink breeding strategies in certain areas. With more heavy rains and same annual rain fall a quiescence strategy via growth retention may sometimes be better than an escape strategy to save crucial energy under time-limited stress conditions. A better understanding of survival mechanisms, as provided in this thesis, may help to develop strategies that strengthen plants under mild low-oxygen conditions.

2. Zusammenfassung

Hypoxische Umweltbedingungen wie Überschwemmungen und die daraus resultierende Sauerstofflimitierung im Wurzelbereich beeinträchtigen die oxidative Phosphorylierung. Die so entstehende Energiekrise in der Pflanze führt, wenn nicht behoben, zum Zusammenbruch der zellulären Transmembrangradienten und schlussendlich zum Zelltod. Ein erhöhter Saccharose - und Stärkeabbau sowie ein Anstieg der anaeroben Glykolyse stellen die benötigte Energie zum Überwinden der Energiekrise bereit. Hierbei recyceln Milchsäure - und alkoholische Fermentation Reduktionsäquivalente, um die ATP-Synthese aufrechtzuerhalten. Allerdings können alternative NADH-Oxidationsmechanismen (**Kapitel I**), alternative Energie- und Kohlenstoffquellen (**Kapitel II**) und die Regulierung des Energieverbrauchs (**Kapitel III**) für das Überleben unter sauerstofflimitierenden Bedingungen entscheidend sein. Um diese Hypothese zu beantworten, wurde die Emission von Stickstoffmonoxid (NO) unter sauerstoffarmen Bedingungen (Mechanismus - **Kapitel I**), die Lipidzusammensetzung unter hypoxischen Bedingungen (Energiequelle - **Kapitel II**) und ein redoxaktive und hochkonservierte Cysteingruppe einer Plasmamembran (PM) P-Typ H⁺-ATPase (Energieverbrauch - **Kapitel III**) untersucht.

Diese Dissertation bestätigt die Relevanz Nitrits (NO₂⁻) als alternativem Elektronenakzeptor unter hypoxischem Stress und der resultierenden Emission von NO in der Pflanzenwurzel. Erstmals werden sauerstoffabhängigen NO-Emissionen in *In-vivo*-Studien an Wurzeln von Tomaten, Gerste und Tabak mittels Chemilumineszenzdetektion nachgewiesen (**Kapitel I**). Die Quantität der hypoxiebedingten NO-Emissionen im Wurzelreaktor lassen auf eine Beteiligung an der ATP-Erzeugung schließen. Die beobachtete NO₂⁻ Reduktion scheint hauptsächlich mitochondrialen Ursprungs zu sein (**Kapitel I**). Als Reaktion auf Sauerstoffmangel scheinen die untersuchten Arten eine Ausweichstrategie, unter Aufbau eines alternativen Sauerstoffversorgungssysteme zu verfolgen. Bei dieser Strategie wird zusätzliches ATP investiert, um strukturelle Veränderungen zu realisieren. Hierzu setzten hypoxische Tomatenpflanzen auf Glykolyse und Fermentation. Lipidabbau und β -Oxidation spielen, wie mittels HPLC-MS-MS gezeigt, bei hypoxischen Tomatenwurzeln eine untergeordnete Rolle in der Energiegewinnung (**Kapitel II**). So wurde weder eine Reduktion der Gesamtlipide noch eine Induktion des Lipidabbaus beobachtet. Sowohl Triacylglycerin (TG) als auch ungesättigten Fettsäuren waren unter hypoxischen Bedingungen abundanter (**Kapitel II**). TGs sind wichtige

Zwischenprodukte im Lipidstoffwechsel. Eine spätere Verwertung als Kohlenstoff- und Energielieferant bei langanhaltender Sauerstoffknappheit ist möglich. Abschließend konnte der Einfluss hypoxisch induzierten, posttranslationalen NO-Modifikation auf die Aktivität der PM P-Typ H⁺-ATPase nicht bestätigt werden. Eine mögliche S-Nitrosylierungsstelle an einer konservierten Cysteingruppe wurde in einer biochemischen Mutagenesestudie nicht verifiziert (**Kapitel III**). Eine Regulierung der PM P-Typ H⁺-ATPase-Aktivität über das zytoplasmatische ATP-Level unter hypoxischen Bedingungen in der Pflanze ist jedoch möglich.

Die Aufrechterhaltung von Protein- und Zellfunktionen scheint unter sauerstofflimitierenden Bedingungen und bei hohen Konzentrationen reaktiver Stickstoffspezies (RNS) wie NO jedoch von wesentlicher Bedeutung zu sein (**Kapitel I**). Die Induktion der antioxidativen Systeme unter hypoxischen Bedingungen ist bekannt. Diese Arbeit identifizierte das hochkonservierte Cystein in der Nähe der P-site der PM P-Typ H⁺-ATPase als endogenes Antioxidans (**Kapitel III**). Dies unterstreicht die Bedeutung dieses Enzyms für die Zelle. Während hypoxischen Veränderungen der Lipidzusammensetzung die Wichtigkeit von Membranintegrität und -fluidität, im Besonderen der Plastiden, hervorheben (**Kapitel II**). Die durch die Membrananpassungen akkumulierten TGs wiederum bilden Lipidkörper. TGs können toxische Zwischenprodukte abfangen und so den Zelltod verhindern. Lipidkörper fungieren aber auch als Bindestelle für in der Stressadaptation involvierten Enzyme. Zusätzlich könnten Lipidkörper durch deren Einlagerung im Inneren ungesättigte Fettsäuren vor reaktiven Sauerstoffspezies (ROS) und RNS schützen.

Unter sauerstofflimitierenden Bedingungen haben sich verschiedene Strategien zur Energieversorgung in Pflanzen entwickelt. Diese stellen nicht nur die nötige Energie für Anpassungen und das Überleben bereit, sondern schützen gleichzeitig lebenswichtige Zellfunktionen. Zum einen stellt die Zunahme von Überschwemmungen und starken Regenfällen aufgrund des Klimawandels eine enorme Herausforderung für die Pflanzen an sich und deren Ertrag dar. Auf der anderen Seite können die pflanzlichen Stressadaptation selbst ein klimabeeinflussender Faktor sein (**Kapitel I**). Bei häufigeren Starkregenereignisse und gleicher jährlicher Gesamtregenmenge muss möglicherweise die Züchtungsstrategie überdacht werden um die Nahrungsproduktion sicherzustellen. Eine Wachstumspause mag unter kurzen aber intensiven abiotischen Bedingungen vorteilhafter denn ein

Entwachsen des Stresses sein. Ein besseres Verständnis der Überlebensmechanismen, wie in dieser Arbeit präsentiert, kann dazu beitragen, diese Strategien zu identifizieren und somit Pflanzen unter sauerstofflimitierenden Bedingungen widerstandsfähiger machen.

3. Introduction

Alternative energy production and NO emissions (**Chapter I**), alternative energy and carbon sources with a focus on lipids (**Chapter II**) and regulation of energy consumption by the PM P-type H⁺-ATPase (**Chapter III**) are discussed with a focus on low-oxygen stress response and regulation. This thesis has contributed to this topic by revealing plant root-based *in vivo* NO emission under low-oxygen conditions and emphasizing the importance of NO₂⁻ reduction for energy production. The observed stress-induced NO emissions may even have an impact on the climate itself (**Chapter I**). The increase in triacylglycerol abundance and degree of unsaturation of fatty acids in hypoxic roots is reported (**Chapter II**) and an endogenous antioxidant residue in the PM P-type H⁺-ATPase is described (**Chapter III**).

Tomato plants were the main species investigated in this thesis. Tomato is a widely grown and important crop plant. It is part of the *Solanaceae* family which includes crops such as potato, eggplants, pepper and tobacco (Gebhardt, 2016). Tomato is a model plant for developmental biology, food science and stress response (Liu *et al.*, 2022). The focus on tomato plants in this thesis based on previous studies on hypoxic adaptations similar to most plant species (Safavi-Rizi *et al.*, 2020; Mignolli *et al.*, 2020). Additionally, tomato's bigger plant size is beneficial for the root reactor measurement and green house growing conditions are similarly to commercial operations.

3.1. Hypoxic stress triggers an energy crisis in plants

Hypoxia occurs when the availability of oxygen for aerobic respiration is insufficient. Oxygen deficiency for plant roots may be caused by heavy rain or flooding (Loreti and Perata, 2020). The soil becomes saturated with water and, depending on the extent, even the entire plant becomes submerged (Sasidharan *et al.*, 2017). The lower diffusion rate of oxygen in water leads to a lack of oxygen in plant tissues (Sojka, 1992). Interestingly, oxygen-limiting niches occur during plant development even without external stressors (Geigenberger, 2003; Weits *et al.*, 2019). Some plants have developed morphological adaptation strategies to survive low-oxygen conditions. The development of aerenchyma, the formation of adventitious roots, but also the elongation of leaves and stems help to circumvent low-oxygen conditions and facilitate oxygen transport within the plant (Loreti and Perata, 2020).

Oxygen scarcity is rapidly achieved in the dark and in non-photosynthetic organs (Mustroph *et al.*, 2014). Limitation of the final electron acceptor for oxidative

phosphorylation leads to an energy crisis in the affected plant organs (Bailey-Serres *et al.*, 2012). The metabolic reconfiguration under low-oxygen stress is necessary to ensure plant survival (summarized in Figure 1). Glycolytic ATP production increases under low-oxygen conditions. The surplus of NADH produced during glycolysis is regenerated by activating lactic acid and ethanolic fermentation (Geigenberger, 2003). As long as sufficient carbon sources are available, anaerobic glycolytic energy production enables cell survival. To ensure this, the degradation of starch and sucrose is enhanced (Bailey-Serres and Voesenek, 2008) while sucrose synthase is favored in sucrose catabolism (Guglielminetti *et al.*, 1995). Additionally, pyrophosphate-dependent enzymes are preferred to be less ATP-dependent (Huang *et al.*, 2008). Via the so-called alanine and 2-oxoglutarate shunt, the amino group is transferred from glutamate to pyruvate to form alanine. Alanine has been shown to reduce the loss of carbon, or acidification of the cytosol, which is associated with the ethanol or lactate produced by fermentation (Ricoult *et al.*, 2006; Limami *et al.*, 2008). A recent study, indicates an additional role of alanine under hypoxic stress. Alanine was highly abundant in xylem and may function a carbon skeleton for glucose production in leaves (Cid *et al.*, 2024). The product of the alanine and 2-oxoglutarate shunt, 2-oxoglutarate can be used to generate additional ATP via a non-circular tricarboxylic acid (TCA) cycle leading to succinate accumulation (Bailey-Serres *et al.*, 2012). The amino group of aspartate can be used to replenish the glutamate pool (Rocha *et al.*, 2010). Thereby, oxalacetate is produced, which can be used to oxidize NADH through a reverse TCA cycle via malate and fumarate, resulting in additional succinate (Vanlerberghe *et al.*, 1989). Alternatively, the γ -aminobutyric acid shunt uses 2-oxoglutarate to regenerate NAD⁺ and potentially lower pH (Bailey-Serres and Voesenek, 2008, Wu *et al.*, 2021). Besides lactate and ethanol as end products of fermentation, alanine, γ -aminobutyric acid and succinate are known to accumulate under low-oxygen conditions, while aspartate levels decline (summarized in Figure 1; reviewed by Bailey-Serres *et al.*, 2012).

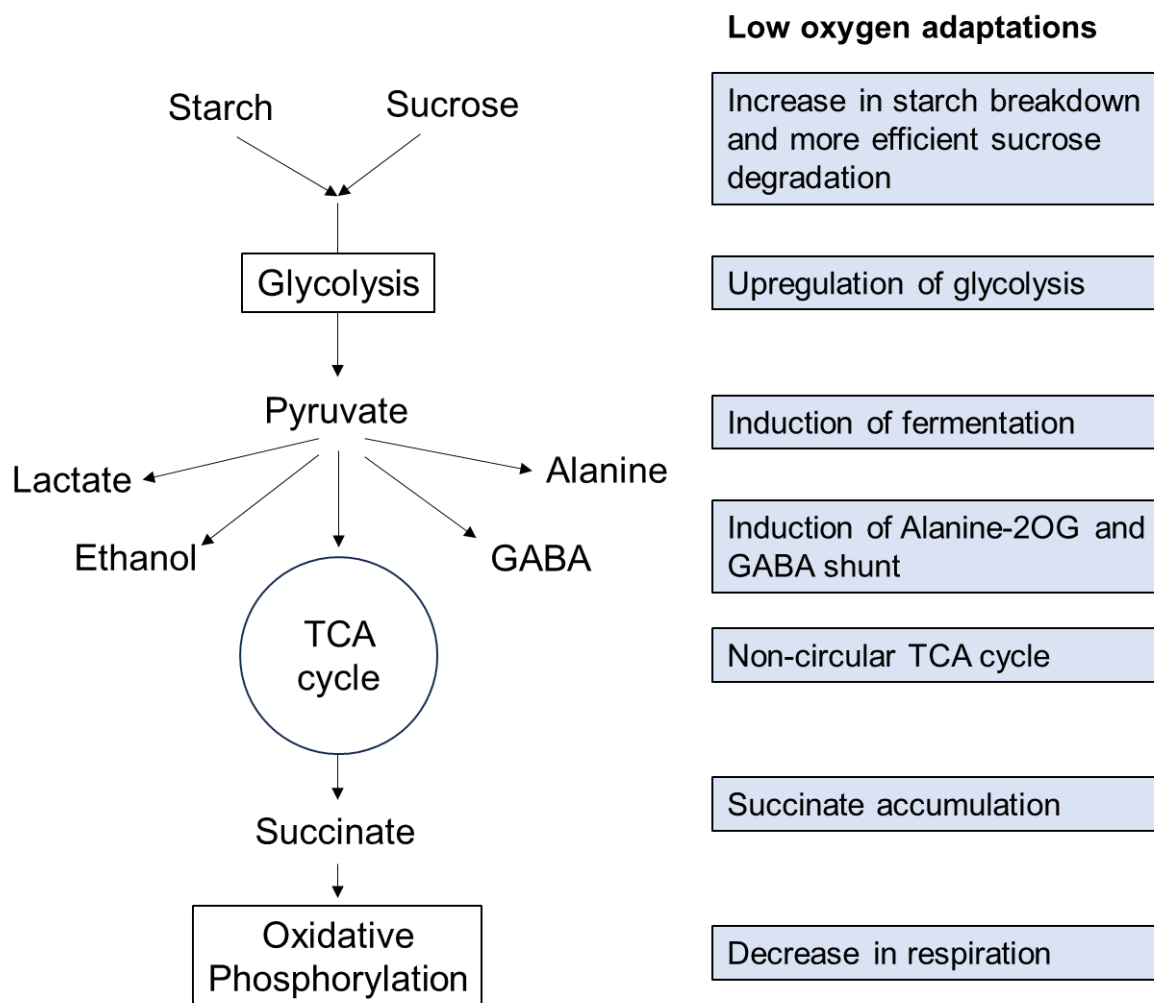


Figure 1: Schemes of metabolic reconfiguration under low-oxygen conditions. The model describes the major known metabolic adaptations in response to reduced oxygen availability. This includes increased sucrose and starch catabolism. Glycolysis and fermentation are induced while a none-circular tricarboxylic acid (TCA) flow is observed. Alanine, 2-oxoglutarate (2OG) shunt and a γ -aminobutyric acid (GABA) shunt are active. Succinate is known to accumulate under low oxygen conditions. Oxidative respiration is reduced due to a lack of oxygen. Blue boxes highlight metabolic adjustments. Black contour indicates metabolic pathways (based on Bailey-Serres et al., 2012).

3.1.1. The role of nitric oxide in hypoxic stress

The beneficial effect of nitrate (NO_3^-) on waterlogged plants is the basis for several hypotheses linking NO_3^- metabolism to plant resilience under low-oxygen stress. However, the mechanism is debated focusing on the reduction of NO_3^- to NO_2^- and to NO (Reggiani et al., 1985; Fan et al., 1988; Igamberdiev and Hill, 2004; Eick and Stöhr, 2012). It has been emphasized that NO_3^- could act as an alternative final electron acceptor under low-oxygen stress (Reggiani et al., 1985; Reggiani et al., 1993). Later studies proposed the potential of the hemoglobin/NO cycle in NADH oxidation (Igamberdiev and Hill, 2004; Igamberdiev et al., 2005). This was followed by the description of a NO_2^- reduction mechanism in mETC that links electron flow, ATP

synthesis and NO emission (Stoimenova *et al.*, 2007; Figure 2). NO_2^- is provided by cytosolic nitrate reductase (NR) by reducing NO_3^- (Botrel and Kaiser, 1997) (Figure 2). The surplus of NADH and succinate from glycolysis and the non-circular TCA cycle is supposed to provide the required electrons. NO_2^- reduction in the mETC is supported by transfer of electrons by calcium-dependent type II NAD(P)H dehydrogenases or complex II to ubiquinol (Stoimenova *et al.*, 2007; Nakamura and Noguchi, 2020). NO_2^- could serve complex III and complex IV as the final electron acceptor. The NO_2^- reduction would enable the electron flow and thereby H^+ pumping that drives ATP synthesis (Stoimenova *et al.*, 2007). The proposed mechanism allowed ATP production on a similar scale to glycolysis in the isolated mitochondria studied (Stoimenova *et al.*, 2007). The product of NO_2^- reduction NO could be scavenged by class 1 hemoglobins (Hebelstrup *et al.*, 2008). The oxidized hemoglobin then oxidizes NO to NO_3^- and further recycles NADH. The hemoglobin/NO cycle is completed by NO_3^- reduction to NO_2^- via NR, which also consumes NADH (Igamberdiev and Hill, 2004; Gupta and Igamberdiev, 2011). Oxidation of NO to NO_3^- and subsequently to NO_2^- decreases reducing equivalents, but oxygen is required in this process (Gupta *et al.*, 2020). This would be consistent with the extremely high oxygen affinity of class 1 hemoglobins (Trevaskis *et al.*, 1997).

Most plant species produce NO under hypoxic stress (Stöhr and Ullrich, 2002; Rockel *et al.*, 2002; Meyer *et al.*, 2005; Gupta *et al.*, 2005; Stöhr und Stremlau, 2006; **Chapter I**). Alternatives to mitochondrial NO production in response to low-oxygen concentrations (Gupta *et al.*, 2005) are NO formation at the plasma membrane by nitrite: NO reductase (Stöhr *et al.*, 2001; Stöhr und Stremlau, 2006) and in the cytosol by NR (Rockel *et al.*, 2002; Meyer *et al.*, 2005). Recent studies have also identified the alternative oxidase (AOX) to produce NO in plants (Vishwakarma *et al.*, 2018). However, mitochondrial reduction of NO_2^- to NO is considered to be the main source of NO when plants suffer from oxygen deficiency (Gupta and Igamberdiev, 2011).

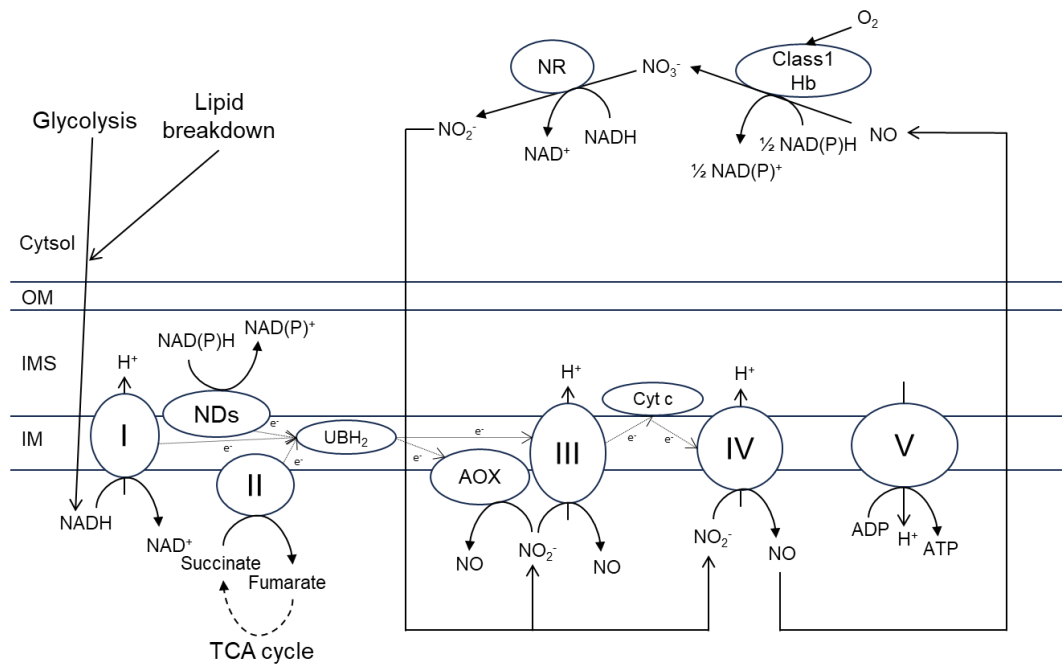


Figure 2: NO_2^- -driven ATP production under low-oxygen conditions. Anaerobic glycolysis and lipid degradation accumulate reducing equivalents under oxygen deficiency. In the mitochondria and in the presence of Ca^{2+} , type II NAD(P)H dehydrogenases (NDs) oxidize reducing equivalents and transfer the electrons to ubiquinol (UQH_2). Succinate, which accumulates under low-oxygen conditions, is oxidized to fumarate while the electrons are transferred to UQH_2 . At low-oxygen levels, NO_2^- can serve as an alternative electron acceptor at complex III (cytochrome bc_1 complex) and VI (cytochrome c oxidase) of the mitochondrial electron transfer chain (mETC). This allows protons (H^+) to be pumped into the intermembrane space to drive ATP synthesis and NO is released. NO is converted to NO_3^- by hypoxically induced Class 1 hemoglobin (Hb) using NAD(P)H and oxygen. Nitrate reductase (NR) converts NO_3^- to NO_2^- using NADH. Filled arrow heads indicate chemical reactions, unfilled arrows indicate transport or diffusion, dotted lines with open arrows electron flow, dotted lines with filled arrows represent the steps of tricarboxylic acid (TCA) cycle. Abbreviations: Ca^{2+} , calcium; AOX, alternative oxidase; Cyt c, cytochrome c; IM, inner mitochondrial membrane; IMS, mitochondrial intermembrane space; OM; outer mitochondrial membrane; I–V; mitochondrial complexes I–V; e^- , electron; NO, nitric oxide; NO_2^- , nitrite; NO_3^- , nitrate; NAD(P), nicotinamide adenine dinucleotide (phosphate) (Based on Stoimenova et al., 2007; Nakamura and Noguchi, 2020; Gupta et al., 2020).

NO is not only a product of NO_2^- reduction but has also been shown to modulate many physiological adaptations to low-oxygen conditions. Hyponasty is a well-described feature of submersion in plants (Gupta *et al.*, 2014). NO is thought to regulate hyponasty via the induction of ethylene synthesis (Hebelstrup *et al.*, 2012). Another adaptational example under flooding and waterlogging is aerenchyma formation which facilitates gas exchange between shoot and root (Drew *et al.*, 2000). In an interplay with ethylene, NO was described to be essential in aerenchyma development (Wany *et al.*, 2017). Additionally, NO and hydrogen peroxide (H_2O_2) contribute to programmed cell death involved in aerenchyma formation (Wang *et al.*, 2013). Under low-oxygen conditions the primary root system may become dysfunctional and is replaced by adventitious roots (Sauter and Seffens, 2013). This adaptation is controlled by auxin

and NO seems to play a role in it in *Suaeda salsa* (Chen *et al.*, 2016). The above-mentioned examples emphasize the importance of NO in physiological adaptations to low oxygen conditions.

NO is also involved in oxygen sensing. In plants, oxygen sensing is mediated by proteasomal degradation of ethylene response factors VII (ERFVII) via N-end rule pathway (Gibbs *et al.*, 2011). Oxygen destabilizes ERFVII via the oxidation of N-terminal cysteine residue. This results in protein arginylation and recognition by an E3 ubiquitin ligase and finally proteasomal degradation (Gibbs *et al.*, 2014b). NO increases ERFVII instability similar to oxygen (Gibbs *et al.*, 2014a; León *et al.*, 2020). However, NO levels are controlled by scavenging systems which allows oxygen sensing under high NO emission under hypoxic stress (Manrique-Gil *et al.*, 2021). The mechanism of the N-end rule pathway also regulates other transcription factors in physiological hypoxic niches (Weits *et al.*, 2019; Labandera *et al.*, 2021) one of which is also controlled by oxygen and NO (Gibbs, *et al.*, 2018). This underlines the importance of the N-end rule pathways and NO in oxygen sensing (Weits *et al.*, 2019). Besides that, oxygen homeostasis is critical under oxygen scarcity to ensure an appropriate internal oxygen concentration. While NO scavenging under normoxic conditions increases respiration but lowers internal oxygen concentration (Gupta *et al.*, 2014), NO inhibits respiration via cytochrome c oxidase (Millar and Day, 1996). A similar mechanism could be in place to regulate ATP homeostasis under low oxygen stress, wherein ATP consumers are inhibited by NO (**Chapter III**). NO-induced post-translational modifications such as tyrosine nitration or S-nitrosylation of cysteine may play a role.

Under low-oxygen conditions, reducing equivalents accumulate and less ATP is generated in plants. According to the research hypothesis of the thesis, the impact of NO₂⁻ reduction in ATP generation and the resulting NO emissions were investigated *in vivo*. NO may thereby not only be a product of NO₂⁻ reduction but also show regulatory properties.

3.2. Lipid degradation and β -oxidation of fatty acids for energy production

Under conditions of energy deficiency, such as hypoxia, plants rely on the degradation of storage compounds such as starch and lipids (Stoimenova *et al.*, 2007; Van Veen *et al.*, 2016). Degradation of membrane and reserve lipids provides carbon skeletons and energy. The process of lipid degradation (lipolysis) occurs in the cytosol and requires specific lipases (Masclaux-Daubresse *et al.*, 2020). Additionally, recent reports indicate that lipids originating from the plasma membrane and organelles, but not from the chloroplasts can be converted by lipophagy (Havé *et al.*, 2019). The free fatty acids are then degraded via peroxisomal β -oxidation cycles to yield acetyl-CoA. Acyl-CoAs undergo multiple cycles of dehydration, hydration, oxidation and thiolysis depending on the chain length (Goepfert and Poirier, 2007). Acetyl-CoA can replenish the TCA cycle or be utilized in gluconeogenesis via the glyoxylate cycle (Graham, 2008). As addressed in this thesis, in the context of hypoxic adaptation via NO_2^- reduction, acetyl-CoA could be utilized in the glyoxylate cycle, producing succinate. Acetyl-CoA could also function as a carbon base in TCA cycle providing reducing equivalents independent of starch degradation.

3.2.1. The central role of triacylglycerols in lipid metabolism

Triacylglycerols are known as classical storage lipids in seeds. However, under stress conditions, TGs accumulate in vegetative tissues (de Vries and Ischebeck, 2020). Cytosolic TGs are assembled in the endoplasmic reticulum (ER) via the Kennedy pathway using *de novo* synthesized fatty acids from plastids (Yu *et al.*, 2021). Glycerol-3-phosphate is acylated to form lysophosphatidic acid (Shockey *et al.*, 2016). Further acylation leads to phosphatidic acid (Kim *et al.*, 2005), which can be converted to diacylglycerol (Pascual and Carman, 2013). Diacylglycerol acyltransferase (DGAT) acylates diacylglycerol to form TG (Ichihara *et al.*, 1988). Alternatively, the fatty acid of phosphatidylcholine can be directly transferred to diacylglycerol by phospholipid:diacylglycerol acyltransferase (PDAT) to form TG (Dahlqvist *et al.*, 2000). Depending on physiological conditions phospholipid DGAT- or PDAT-driven triacylglycerol biosynthesis is favored as has been shown for *Arabidopsis thaliana* (Fan *et al.*, 2013; Tjellström *et al.*, 2015). In plastids, plastid-specific enzymes coordinate the formation of phosphatidic acid (Kunst *et al.*, 1988; Kim *et al.*, 2004), which is converted into diacylglycerol (Nakamura *et al.*, 2007). Diacylglycerol acylation to triacylglycerol is controlled by phytyltransferase 1 and 2 (Lippold *et al.*, 2012). The

different synthesis pathways of TG indicate its central role in conversion of the different lipid species. The implication and TG biosynthesis pathway under hypoxia was investigated in this thesis.

Remodeling of lipid composition during hypoxic stress has been reported (Xie *et al.*, 2021). It is hypothesized that stress induced changes in lipid composition ensure membrane fluidity and integrity (Balogh *et al.*, 2013). It is speculated that membrane fluidity is not altered under hypoxic conditions (Wang *et al.*, 2016b). Therefore, the impact of changes in lipid composition under hypoxic conditions needs to be addressed. TGs sequester free fatty acids and diacylglycerols released during lipid remodeling (Lu *et al.*, 2020). Free fatty acids and diacylglycerols are incorporated into TGs before they are degraded during peroxisomal β -oxidation (Fan *et al.*, 2017). Under heat stress, unsaturated fatty acids are translocated from chloroplast to ER and directed to the phosphatidylcholine pool to be incorporated into TGs via PDAT (Mueller *et al.*, 2017; Higashi *et al.*, 2018). During freezing, diacylglycerols are released and their conversion into TGs via DGAT modulates phosphatidic acid as a freezing signal (Tan *et al.*, 2018). According to recent findings, a TG accumulation mechanism could also be conceivable in salt and drought stress (Wang *et al.*, 2016a; Lu *et al.*, 2020). In this thesis, the mechanism of hypoxic TG synthesis and its importance for protein remodeling was investigated.

TGs accumulated under stress are stored within lipid droplets in the cytosol or plastoglobules of chloroplast (Chapman *et al.*, 2012; Huang, 2018). Cytosolic lipid droplets are composed of non-polar lipids such as TGs and sterol esters and are surrounded by a monolayer of phospholipids derived from ER (Huang, 2018). The lipid droplet proteome is thereby highly specific to tissue and physiological condition (Zhang and Liu, 2019). Plastoglobules are surrounded by thylakoid membranes (Daum and Kühlbrandt, 2011). They have a similar lipid composition to lipid droplets, but also contain phytylestes and carotenoids in their core. The proteins of plastoglobules are associated with plastid metabolism (van Wijk and Kessler, 2017). The above-mentioned variability in lipid droplets and plastoglobulus could allow hypoxic specific TG processing. The intermediate storage of TGs in lipid droplets may be crucial during stress, as it could provide the energy for plant survival investigated in this thesis.

3.2.2. Unsaturated fatty acids in the stress-response

Unsaturated fatty acids are associated with the stress adaptation of plants (He and Ding, 2020). Fatty acid desaturases are the main cause of changes in fatty acid composition under stress (Upchurch, 2008). The major unsaturated fatty acids in higher plants are oleic acid (C18:1), linoleic acid (C18:2) and α -linolenic acid (C18:3) (He and Ding, 2020). In plastids, *de novo* synthesized stearic acid (C18:0) is attached to acyl-carrier protein (ACP), which is converted to oleic acid (C18:1)-ACP by stearyl-ACP desaturase. After esterification in glycerolipids, the C18:1 fatty acid is processed to C18:2 and then to C18:3 fatty acid. This occurs in plastids (“prokaryotic pathway”) or in the ER (“eukaryotic pathway”). Both in the plastid and in the ER, pairs of fatty acid desaturase introduce double bonds into the acyl chains. (reviewed by Ohlrogge and Browse, 1995). Adjusting the degree of fatty acid unsaturation is a well-described strategy in plants (Wallis and Browse, 2002). Under stress, the changes in fatty acid unsaturation are linked to adaptation of membrane fluidity (Upchurch, 2008). Little is known about the function of fatty unsaturation under low-oxygen conditions. Fatty acids are the basis of barrier biopolyesters such as cutin and suberin. As integral components of internal and external barriers, they restrict the flow of water and gases and are involved in abiotic and biotic stress response (Pollard *et al.*, 2008). Such a structural barrier can prevent radical oxygen loss under hypoxic stress (Kotula *et al.*, 2009; Ejiri *et al.*, 2021). Unsaturated fatty acids also play an important role as precursors for bioactive molecules such as jasmonates (Schaller *et al.*, 2004), but can also directly regulate stress signals. C18:3 fatty acids can induce ROS production by enhancing NADPH oxidase activity, the key player in the hypersensitive response (Yaeno *et al.*, 2004), which is also important in aerenchyma formation under hypoxia (Wang *et al.*, 2013). NO production, another feature of low-oxygen adaptation, can be suppressed by C18:1 fatty acid (Mandal *et al.*, 2012). Interestingly, the fatty acid composition of membranes can directly influence enzyme activity. A well-known example is the plasma membrane P-type H⁺-ATPase (Morales-Cedillo *et al.*, 2015). Overall, fatty acids are versatile compounds in the stress response. A closer look could provide insights into low-oxygen adaptations.

Low-oxygen stress reduces the efficiency of energy production and requires a constant flow of energy to ensure survival. According to the research hypothesis lipids may provide the needed energy and carbon via lipid degradation. Thereby, both the central role of triacylglycerol and degree of fatty acids unsaturation were investigated.

3.3. The importance of P-type H⁺-ATPases for plant survival

The plasma membrane P-type H⁺-ATPase is considered to be the powerhouse of plant growth and development. It is therefore no surprise, that the PM P-type H⁺-ATPase is involved in a plethora of physiological and developmental processes in plants (Palmgren, 2001). Well-known is the H⁺ pumping activity of the PM P-type H⁺-ATPase providing an electrochemical gradient across the plasma membrane (Haruta *et al.*, 2010). This PM energization is required for nutrient uptake and loading of xylem and phloem by secondary transport (Sondergaard *et al.*, 2004). Besides that, apoplastic acidification by PM P-type H⁺-ATPase leads to cell elongation (Hager *et al.*, 1991; Hager, 2003) while H⁺ extrusion stabilizes cytosolic pH (Bobik *et al.*, 2010). In terms of stomata opening, activation of ATPase activity hyperpolarizes the plasma membrane, leading to potassium channels opening, water influx, cell swelling and results in stomata opening (Janicka-Russak 2011).

PM P-type H⁺-ATPases are highly conserved among kingdoms (Falhof *et al.*, 2016; Figure 3). AHA2 consists of 10 transmembrane sections. The catalytically active regions are the actuator (A), the nucleotide binding (N) and the phosphorylation (P) domains (Pedersen *et al.*, 2008). In AHA2, aspartate (Asp) 329 is part of the P domain and can be phosphorylated (phosphorylation site). The A domain has a phosphatase function and can dephosphorylates the modified Asp 329 residue. As the A-domain moves away, the N-domain can approach which is required for phosphorylation. The conserved Asp residue of the P-domain is phosphorylated during each pumping process (Pedersen *et al.*, 2008). The C- and N-terminal domains are exposed at the cytosolic site and are involved in the regulation of protein activity. The C-terminal domain inhibits the PM P-type H⁺-ATPases (Palmgren *et al.*, 1991) and is supported by the N-terminal domain (Ekberg *et al.*, 2010). The status of the terminal domains defines the two states of PM P-type H⁺-ATPases in plants and in yeast. In the autoinhibited state, ATP hydrolysis is only loosely coupled to H⁺ pumping while in the upregulated state there is a tight coupling between ATP hydrolysis and H⁺ transport (Falhof *et al.*, 2016). The terminal domains are the most variable among all PM P-type H⁺-ATPases from different species (Falhof *et al.*, 2016; Figure 3). The ubiquitous expression and high conservation of PM P-type H⁺-ATPases emphasizes their importance and raises the question of their involvement in low-oxygen stress.

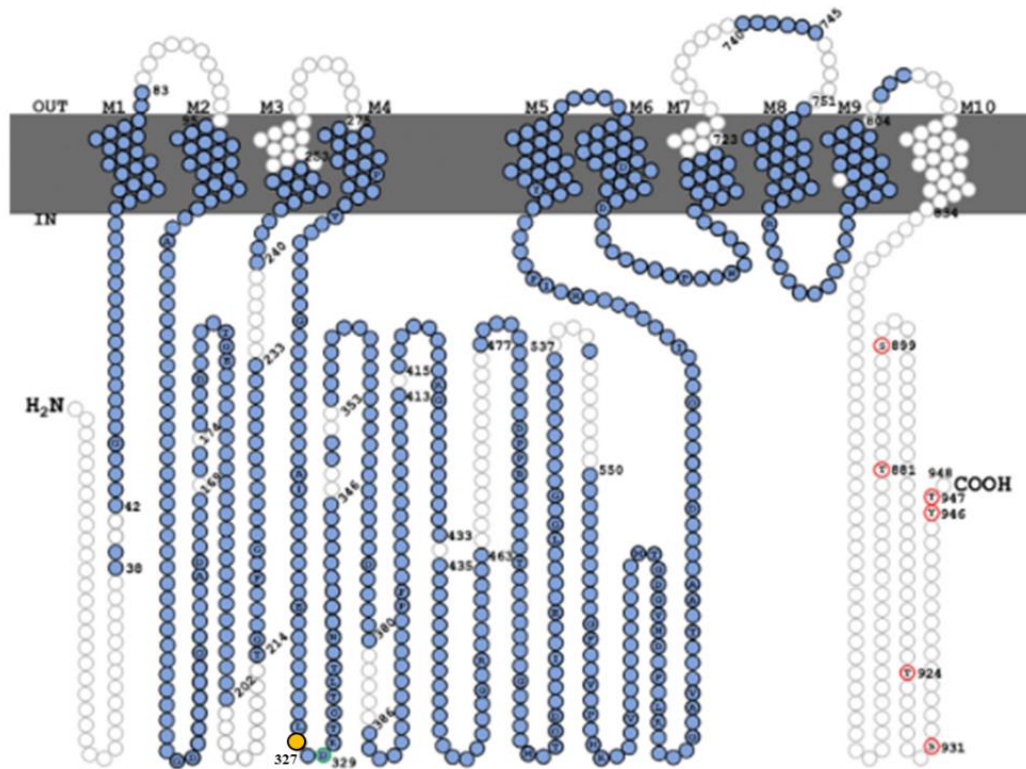


Figure 3: Conservation of amino acid sequence of PM P-type H⁺-ATPases. 135 predicted sequences of PM P-type H⁺-ATPases from green algae, red algae and plants were aligned to the AHA2 2 D model using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) program (Edgar, 2004). Light blue circles represent regions where no deletion or insertion was ever observed. Letters are added if the amino acids were fully conserved in 135 sequences. White circles mark regions where distant sequences do not align. The yellow circle shows the position of the highly conserved Cys-327 described in Chapter III. Red circles show in vivo phosphorylation sites of AHA2 with known function (Rudashevskaya et al., 2012). The green circle marks the Asp-329 of the P domain. The numbering of the residues refers to the AHA2 sequence (adapted from Falhof et al., 2016).

3.3.1. Regulation of PM P-type H⁺-ATPases

PM P-type H⁺-ATPases are major consumers of ATP (Harper *et al.*, 1994), which is scarce under low-oxygen conditions (Bailey-Serres *et al.*, 2012). Rapid regulation of their activity is therefore of great importance. Phosphorylation plays a significant role in the regulation of PM P-type H⁺-ATPases (Duby and Boutry, 2009). 11 phosphorylation sites have been reported for plant PM P-type H⁺-ATPases. Most of them are located in the terminal domain of the protein (Rudashevskaya *et al.*, 2012; Figure 3). The most studied phosphorylation site is the penultimate AHA2 Thr-947 and its homologs. Phosphorylation of these residues allows the binding of 14-3-3 proteins that activate the pump by releasing the autoinhibitory C-terminal domain (Fuglsang *et al.*, 1999). While phosphorylation of AHA2 Thr-881 activates the H⁺-ATPases in a 14-3-3-independent manner (Niittyla *et al.*, 2007; Fuglsang *et al.*, 2014), phosphorylation of Thr-924 and Ser-931 inactivates the pump and interferes with 14-3-3 binding in

Nicotiana tabacum (Fuglsang *et al.*, 2007; DUBY *et al.*, 2009). In addition to phosphorylation, other post-translational modifications may alter the activity of H⁺-ATPases. A redox-related regulation of H⁺-ATPase activity is possible. This is indicated by pump activation of H₂O₂ over NO under salt stress in *Populus euphratica* (Zhang *et al.*, 2007) and supported by the identification of several oxidized cysteine sites in the related sarcoplasmic/endoplasmic-reticulum Ca²⁺-ATPase (Sharov *et al.*, 2006).

PM P-type H⁺-ATPase activity is also regulated by plasma membrane lipid environment in plants (Palmgren *et al.*, 1990). The activity of H⁺-ATPase is lower the higher the unsaturation of fatty acids is and the longer the acyl chains are (reviewed by Morales-Cedillo *et al.*, 2015). The effect of lysophosphatidylcholine on PM P-type H⁺-ATPase activity has been best studied. The derivative of glycerolipid degradation acts as an activator of PM P-type H⁺-ATPases in several species (Palmgren and Sommarin, 1989; Pedchenko *et al.*, 1990). Lysophospholipids relieve autoinhibition of both the N- and C-terminal domains and activate the pump (Wielandt *et al.*, 2015). Stress-specific lipid adaptations raise the question of how and whether stress affects the activity of PM P-type H⁺-ATPases via the lipid environment.

PM P-type H⁺-ATPases are members of a multigene family. Examples are *Arabidopsis thaliana* with 11 PM P-type H⁺-ATPase genes (Falhof, 2016) and *Solanum lycopersicum* with 10 genes (Kalampanayil and Wimmers, 2001). The diversity of different PM P-type H⁺-ATPases has led to speculations whether certain isoforms are expressed in specific tissues or organs and whether they are specialized to environmental conditions (Morsomme and Boutry, 2000). While H⁺-ATPase subfamilies I and II are ubiquitously expressed, subfamilies III, IV and V are only expressed in specific cells and organs (Arango *et al.*, 2003). In *Arabidopsis thaliana*, *AHA1* and *AHA2* are universally expressed, with *AHA1* being mainly transcribed in shoots and *AHA2* in roots (Janicka-Russak, 2011). *AHA6* and *AHA9* are specifically expressed in anthers, while *AHA7* and *AHA8* are only expressed only in pollen (Gaxiola *et al.*, 2007). Transcriptional regulation of PM P-type H⁺-ATPase genes can provide another way to regulate PM energization.

3.3.2. P-type H⁺-ATPases and stress adaptation

Due to their central role in physiology, PM P-type H⁺-ATPases are key players in plant stress (Fuglsang and Palmgren, 2021). The stress-related adaptation mechanisms for PM P-type H⁺-ATPase involvement for salinity and drought are described (Merlot *et*

al., 2007; Janicka-Russak, 2011; Xu *et al.*, 2013). However, little is known about the input of PM P-type H⁺-ATPases under low-oxygen conditions.

Under salt stress, sodium (Na⁺) ions must to be extruded into the apoplastic space or vacuole. The plasma membrane H⁺ gradient and therefore the PM P-type H⁺-ATPase is required to drive Na⁺ apoplastic extrusion via Na⁺/H⁺ antiporter (Janicka-Russak, 2011). PM P-type H⁺-ATPase activation under salt stress is well documented (Janicka-Russak and Kłobus, 2007). PM H⁺-ATPases are also involved in mediating drought response. Water scarcity leads to abscisic acid-mediated stomatal closure to reduce water loss (Merlot *et al.*, 2007). This is achieved by inactivating the PM H⁺-ATPases (Xue *et al.*, 2018). To find remaining water roots need to elongate. This is driven by PM H⁺-ATPases activation (Xu *et al.*, 2013).

Several hypoxic adaptation strategies are linked to PM P-type H⁺-ATPase activity. Upon low oxygen stress a sudden drop in cytosolic pH is observed (Felle, 1996). An impact of H⁺-ATPases on cytosolic acidification is discussed but seems unlikely (Felle, 2005). Cell elongation is another strategy for plants to escape submergence under low-oxygen conditions. In *Potamogeton distinctus* stem elongation was linked to increase PM P-type H⁺-ATPase activity (Koizumi *et al.*, 2011). The availability of ATP, due to the hypoxic energy crisis in plants, is often discussed as inhibiting factor for H⁺-ATPase activity (Felle, 2005; Wu *et al.*, 2021). Recent studies on PM P-type H⁺-ATPase and hypoxic stress indicate the interest in hypoxia and PM P-type H⁺-ATPase (Zhang *et al.*, 2024; Jiang *et al.*, 2024).

As postulated in the research hypothesis of this thesis, ATP scarcity under hypoxic conditions requires regulation of ATP consumers. PM P-type H⁺-ATPase hydrolyses ATP to energize the plasma membrane. How a conserved cysteine residue of PM P-type H⁺-ATPase is affected under stress conditions was investigated.

4. Chapter I: The underestimated role of plant root nitric oxide emission under low-oxygen stress

M. Welle, W. Niether, C. Stöhr (February 2024).

Frontiers in Plant Science, 15, 1290700. <https://doi.org/10.3389/fpls.2024.1290700>



OPEN ACCESS

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RECEIVED 07 September 2023

ACCEPTED 16 January 2024

PUBLISHED 06 February 2024

CITATION

Welle M, Niether W and Stöhr C (2024) The underestimated role of plant root nitric oxide emission under low-oxygen stress. *Front. Plant Sci.* 15:1290700. doi: 10.3389/fpls.2024.1290700

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The underestimated role of plant root nitric oxide emission under low-oxygen stress

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The biotic release of nitric oxide (NO), a greenhouse gas, into the atmosphere contributes to climate change. In plants, NO plays a significant role in metabolic and signaling processes. However, little attention has been paid to the plant-borne portion of global NO emissions. Owing to the growing significance of global flooding events caused by climate change, the extent of plant NO emissions has been assessed under low-oxygen conditions for the roots of intact plants. Each examined plant species (tomato, tobacco, and barley) exhibited NO emissions in a highly oxygen-dependent manner. The transfer of data obtained under laboratory conditions to the global area of farmland was used to estimate possible plant NO contribution to greenhouse gas budgets. Plant-derived and stress-induced NO emissions were estimated to account for the equivalent of 1 to 9% of global annual NO emissions from agricultural land. Because several stressors induce NO formation in plants, the actual impact may be even higher.

KEYWORDS

nitric oxide emissions, root, oxygen deprivation, hypoxia, low oxygen stress

1 Introduction

Climate change causes elevated temperatures, enhanced drought periods, and heavy rainfall and affects all life forms on Earth (Trenberth, 2005). In particular, plants are subjected to increasingly extreme weather conditions because of their sessile lifestyles and are forced to endure stressors with all their consequences (Prasch and Sonnewald, 2015). The impact and response of plants to these stressors have been extensively discussed and investigated from different perspectives (Bigot et al., 2018; Cassia et al., 2018; Agnihotri and Mitra, 2023). However, the influence of stressed plants on climate change remains unclear.

The factors that predominantly influence the climate are the quantity and composition of greenhouse gases. Trace gases in the atmosphere have complex chemistry and may form atmospheric oxidants such as ozone and hydroxyl radicals. Nitric oxide (NO) contributes significantly to this production (Pilegaard, 2013). Interestingly, nitric oxide can also be microbially reduced to nitrous oxide (N₂O) (Cameron et al., 2013; Caranto and Lancaster, 2017), a greenhouse gas with 265-298 times higher global warming potential than that of

carbon dioxide (IPCC, 2014). One major source of nitric oxide is the soil of agricultural and forest systems (Davidson and Kinglerlee, 1997; Stehfest and Bouwman, 2006). Soil NO emissions have been dependent on many factors, such as soil water content, soil temperature, soil pH, ambient NO concentration, soil organic carbon, and nitrogen availability (Ludwig et al., 2001; Pilegaard, 2013). Among the processes of soil NO formation, microbial nitrification and denitrification are considered the major processes (Pilegaard, 2013), whereas abiotic processes are suggested to play a minor role only under low pH conditions. The contribution of soil-derived NO to the total annual tropospheric NO budget has been estimated to be as high as 8% for Saxony (Molina-Herrera et al., 2017).

However, little attention has been paid to the contribution of higher plants to the global NO budget. In plants, NO functions as a signaling molecule and has been well characterized in recent years (Astier et al., 2018). NO plays a crucial role in plant development, including germination, flowering, and leaf senescence, as well as in adaptation to biotic and abiotic stress (reviewed by Nabi et al., 2019). Especially in roots, NO signaling controls several processes in organogenesis (Pagnussat et al., 2002). Under stress conditions, NO induces aerenchyma and adventitious root formation (Chen et al., 2016; Wany et al., 2017).

An increase in plant NO emissions in abiotic stress responses has been described for many stressors such as salt, drought, heavy metals (reviewed by Nabi et al., 2019), and hypoxia (Gupta et al., 2005; Stöhr and Stremlau, 2006; Safavi-Rizi et al., 2020). Hypoxic conditions in terrestrial plants occur primarily after flooding and heavy rainfall (Bailey-Serres et al., 2012). Under these conditions, NO emitted from the soil is thought to be produced through the microbial denitrification process (Ludwig et al., 2001). However, hypoxic conditions in the soil may also lead to a significant increase in NO formation in plant root cells (Gupta et al., 2005; Stöhr and Stremlau, 2006; Liu et al., 2015). A reductive pathway for NO formation triggered by oxygen deprivation has been suggested (Timilsina et al., 2022). First, nitrate is reduced to nitrite by nitrate reductase (Igamberdiev and Hill, 2004). Subsequently, nitrite is thought to be reduced to NO. Among the possible NO-forming enzymes in plants, cytosolic nitrate reductase (Rockel et al., 2002; Meyer et al., 2005), PM-bound nitrite:NO reductase (Stöhr et al., 2001; Stöhr and Ullrich, 2002; Stöhr and Stremlau, 2006), and mitochondrial electron transport chain (mETC) complexes III and IV (Gupta et al., 2005; Gupta and Igamberdiev, 2011) induce NO formation under low-oxygen conditions. Furthermore, NO-forming enzymes containing molybdenum cofactors have also been identified (Wang et al., 2010; Chamizo-Ampudia et al., 2016; Chamizo-Ampudia et al., 2017; Astier et al., 2017). The NO-forming activity of most of these enzymes is assumed to depend on oxygen. The reductive NO formation pathway is thought to fight energy shortages in oxygen-deprived plants, and NO acts as a final electron acceptor (Igamberdiev and Hill, 2004). The extent to which low-oxygen-induced NO emissions contribute to annual global NO emissions is unknown.

In this study, low-oxygen-induced NO emissions were investigated by measuring the entire root system of intact plants (*in vivo*) of different species (tomato, tobacco, and barley).

Protoplasts (*in situ*) and aseptically cultured plants (*in vivo*) were assessed to clarify the origin of NO emission. Based on the obtained data, the contribution of plant-derived NO to the global NO budget during low-oxygen periods was estimated.

2 Plant roots emit NO in an oxygen-dependent manner

The determination of plant NO emissions *in vivo* without impairing organs is an important tool for assessing and evaluating the impact of stress on plants and climate. In this study, the *in vivo* NO emissions of three plant species under low-oxygen conditions were examined. Plant root NO emissions were measured for up to 5 h in intact plants that experienced sufficient oxygen during their growth period (Figure 1). After the initial adjustment to low-oxygen conditions in the reactor, a strong increase in NO emissions was observed. All investigated plant species attained a constant rate of NO formation after 3–5 h, depending on the species. Tobacco roots emitted a maximum of approximately 365 nmol NO*g DW⁻¹*h⁻¹ (Figure 1A), while tomato roots produced up to 1,028 nmol NO*g DW⁻¹*h⁻¹ (Figure 1B). The barley root NO release averaged 407 nmol NO*g DW⁻¹*h⁻¹ (Figure 1C). In addition to the maximum NO emissions, the initial adaptation phase was specific to each species. While barley showed rapid adjustment within 2 h, tobacco required approximately 3 h, and tomato plants required 4 h to attain a constant NO emission rate. All investigated plant species revealed low but determinable NO emissions at the beginning of the measurement, reflecting the state of former normoxia with an explicit increase in response to low oxygen over time.

To investigate the impact of oxygen on NO emissions, the roots of intact tobacco plants were exposed to gas flow at various oxygen concentrations. Only a minor amount of NO was emitted from the tobacco roots at 5.15% oxygen in the nitrogen gas. An increase in NO emissions was observed at oxygen concentrations of < 0.515%. The root systems produced the highest NO emissions under anoxic conditions. A correlation between the oxygen level and relative NO emissions was observed (Figure 2).

3 Plants contribute to global NO emissions under low-oxygen conditions

The sources of NO emissions from agricultural systems are usually soil-borne microbes (Pilegaard, 2013; Yu and Elliott, 2021). However, low-oxygen-induced NO emissions appear to be a general adaptation in higher plants when nitrate is abundant (Figure 1; Rockel et al., 2002; Gupta et al., 2005; Hebelstrup et al., 2012; Mugnai et al., 2012; Oliveira et al., 2013; Liu et al., 2015). The proportion of stress-induced NO emissions by plants to the global annual NO emissions remains elusive.

To facilitate the comparison of data obtained under controlled conditions with field NO emissions, the determined NO emission

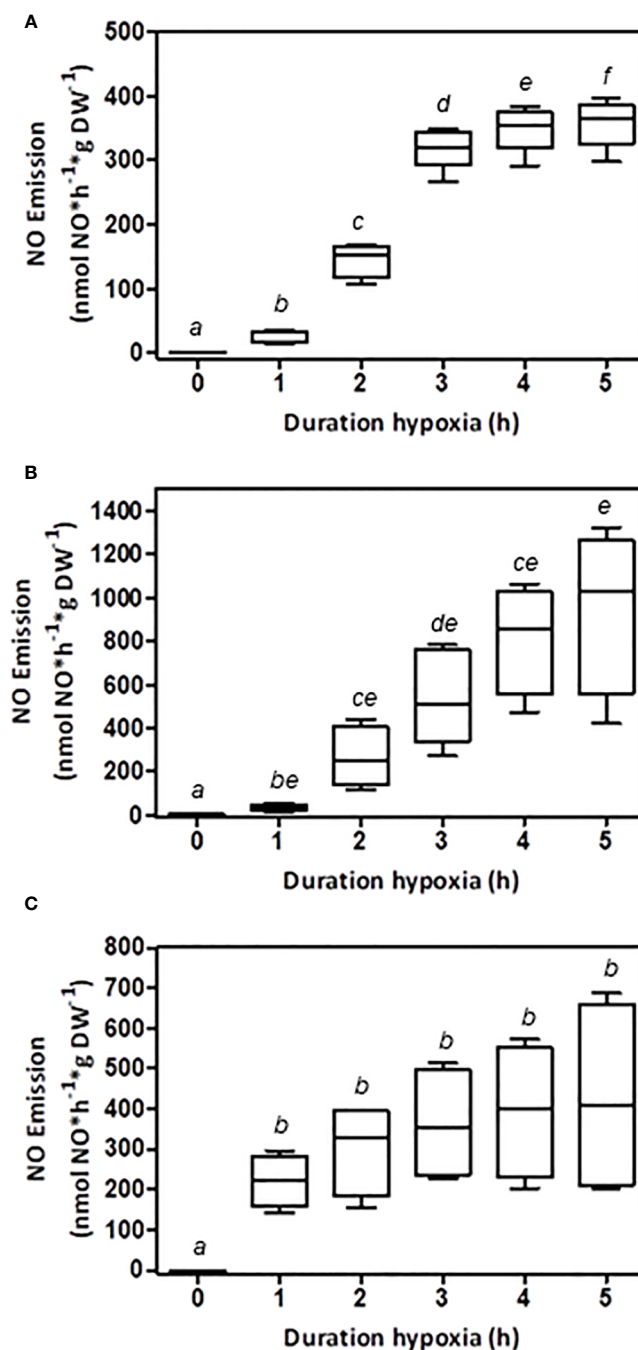


FIGURE 1

Effect of oxygen on NO emission from root systems of different plant species. Hydroponically grown plants were transferred to the root reactor, and NO emissions were measured *in vivo*. The shoot was exposed to ambient air while the root was humidified. An airflow of nitrogen gas was used to achieve low-oxygen conditions inside the sealed root reactor. NO emissions were measured by chemiluminescence. Shown are the values after 0, 1, 2, 3, 4, and 5 hours under low-oxygen conditions. (A) NO emissions from tobacco roots (n=6), (B) NO emissions from tomato roots (n=5), and (C) NO emissions from barley roots (n=5). Boxplots are shown and plotted with median and SD. The significance was tested by multiple paired t-tests with $P \leq 0.05$. Boxes with the same letter are not significantly different.

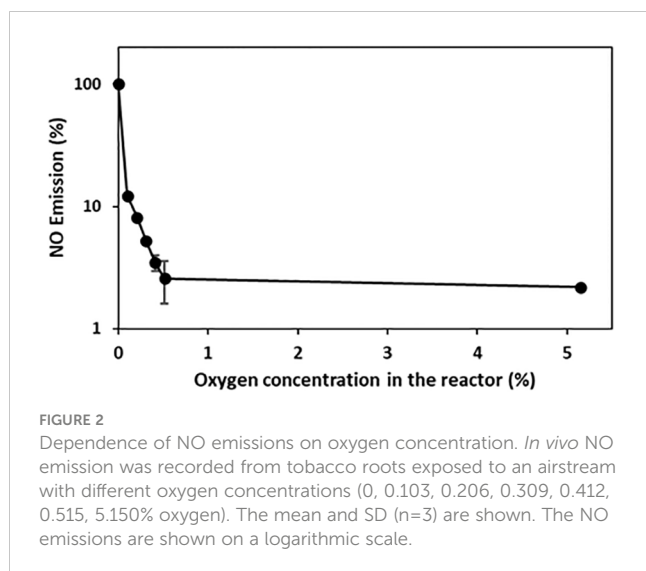
rates were estimated for the annual NO produced per hectare for each of the investigated plant species.

The following assumptions were made:

1. Root DW of single mature plants in the field was taken from literature for tobacco [Wolf and Bates (1964)], tomato

[Brdar-Jokanović et al. (2014)], and barley (Heydari et al. (2019)).

2. Values for plant density per ha were based on the work of Reynolds et al. (2022) for tobacco, Elattir (2002) for tomato, and Paynter and Hills (2009) for barley.
3. The average duration of a flooding period was assumed to be 12 days according to Pendergrass and Knutti (2018),



who found half of annual precipitation falls within the wettest 12 days worldwide. The occurrence of low-oxygen conditions by submergence due to heavy rainfalls or flooding was therefore assumed to be within this time period.

- Flooding results in either anoxic or hypoxic soil conditions that are dependent on soil type and composition and oxygen consumption in the soil (Ponnamperuma, 1972). Based on Figure 2, hypoxic conditions may account for 10% of anoxic NO emissions.
- The detected NO emission rate after 5 h of low-oxygen conditions (Figure 1) was extrapolated for the entire assumed flooding period of 1 year.

Based on these assumptions, the annual NO emissions per hectare planted with different plant species were calculated. Under low-oxygen soil conditions, tobacco roots emitted the lowest NO amount per hectare, followed by tomatoes and barley (Table 1). Under anoxic conditions, the NO emission of each species was 10 times higher than that under hypoxic conditions. In addition to oxygen availability, the main factor that determines NO emissions per hectare is plant density, which depends on the plant size.

4 Discussion

Fossil fuel combustion and microbial emissions are considered to be the main sources of NO emissions into the atmosphere (Pilegaard, 2013). The impact of plants on NO emissions has rarely been acknowledged, although the formation of NO by plants has been known for more than 40 years (Kolbert et al., 2019). Low-oxygen-induced NO formation in plants has been found in a diversity of species such as sunflower (detached leaf) (Rockel et al., 2002), *Arabidopsis thaliana* (rosettes) (Hebelstrup et al., 2012), barley, pea, tobacco (root segments and extracts) (Gupta et al., 2005), poplar (root, stem, and leaf) (Liu et al., 2015), soybean (root) (Oliveira et al., 2013), and maize (seedling

root) (Mugnai et al., 2012). In this study, *in vivo* NO emissions from the root system under low-oxygen conditions were measured in three different plant species. Plants were treated as unaffected as possible to minimize the effects of additional stressors. For all these, a reproducible and similar NO emission course was determined. Based on plant NO emissions, the impact of low-oxygen-stressed plants on global annual NO emissions from agricultural land was assessed. According to our estimation, between one and nine percent of annual NO emissions may be caused by plants under low-oxygen stress.

To ascertain the plant origin of NO formation and exclude microbial contributions, as highlighted by Horchani et al. (2011), root protoplasts and aseptically grown plants were measured. Protoplast isolation can reduce microbial contamination by several washing steps and structural dissolution. NO formation by protoplasts in response to low-oxygen conditions resembled NO emission in hydroponically grown plants (Supplementary Figure 1A). Although the NO emissions from aseptically grown plants were much lower than those from hydroponically grown plants, they also responded to low-oxygen conditions (Supplementary Figure 1B). Differences in NO formation between aseptic- and non-aseptic-cultured plants may be due to the culture conditions on agar medium versus hydroponic culture, as indicated by the reduced growth rate (data not shown). Potential growth limitations owing to a shortage of nutrients, particularly nitrogen, may cause low NO formation under these conditions (Stöhr and Stremlau, 2006; Liu et al., 2015). Overall, there is strong evidence that oxygen-dependent NO formation is directly derived from plant root systems. The detected *in vivo* NO emissions by plant root systems support the results of previous studies. The correlation between low oxygen levels and increased NO emissions has been extensively studied in various plant segments and extracts (Rockel et al., 2002; Gupta et al., 2005; Meyer et al., 2005; Stöhr and Stremlau, 2006). Therefore, NO emissions are highly dependent on oxygen. Anoxic conditions result in the highest NO emissions (Gupta et al., 2005; Stöhr and Stremlau, 2006). A total of 50% of the total NO formation in the plant extracts (I_{50}) was observed at oxygen concentrations between 5% (plasma membrane related: Stöhr and Stremlau, 2006) and 0.05% (mitochondria related: Gupta et al., 2005). The measured I_{50} of *in vivo* root NO emissions was equal to that of mitochondria-derived NO emissions. This is consistent with the view that mitochondria are one of the main sources of NO in response to low-oxygen conditions (reviewed by Gupta and Igamberdiev, 2011).

In accordance with *in vivo* measurements of poplar roots (Liu et al., 2015), NO emissions increased shortly after the onset of low-oxygen conditions. Maximum NO emission was observed after 3–5 h (Figure 1). It is possible that the effect of residual oxygen levels in the root tissue on NO formation must be considered. The maximum NO emissions related to fresh weight ranged from 65 to 20 nmol NO g $FW^{-1} h^{-1}$ for tomatoes, tobacco, and barley (Figure 1). These values were comparable to those recorded in hypoxia-treated soybean roots (60 nmol NO g $FW^{-1} h^{-1}$; Oliveira et al., 2013). In flood-tolerant poplar species, however, hypoxic conditions resulted in root NO emission of 0.05 nmol NO g $FW^{-1} h^{-1}$ (Liu et al., 2015). It has been speculated that flood-tolerant species show lower hypoxic root NO

TABLE 1 Estimation of oxygen-depleted NO emissions per hectare and year from three different plant species based on reactor *in vivo* data.

	Tobacco	Tomato	Barley	Reference
Plant NO emission ($\text{nmol NO} \cdot \text{h}^{-1} \cdot \text{g DW}^{-1}$)	365	1028	407	Figure 1
Root dry weight ($\text{g DW} \cdot \text{plant}^{-1}$)	25 ^a	6.7 ^b	1.5 ^c	^a Wolf and Bates (1964) ^b Brdar-Jokanović et al. (2014) ^c Heydari et al. (2019)
Annual flooding period ($\text{h} \cdot \text{a}^{-1}$)	288 ^g	288 ^g	288 ^g	^g Pendergrass and Knutti (2018)
Planting density ($\text{plant} \cdot \text{ha}^{-1}$)	15000 ^d	30000 ^e	2000000 ^f	^d Reynolds et al. (2022) ^e Elattir (2002) ^f Paynter and Hills (2009)
Annual NO emission – anoxic soil conditions ($\text{g NO-N} \cdot \text{ha}^{-1} \cdot \text{a}^{-1}$)	552	833	4,917	–
NO emissions per ha – hypoxic soil conditions (10%) ($\text{g NO-N} \cdot \text{ha}^{-1} \cdot \text{a}^{-1}$)	55	83	492	Based on Figure 2

The annual NO emission was calculated as: $\text{Annual NO emission} = \text{Plant NO emission} \cdot \text{root dry weight} \cdot \text{annual flooding period} \cdot \text{planting density} \cdot \text{molecular weight NO/molecular weight nitrogen}$. For the NO emissions of the plants, median values of the NO emission data after 5 h shown in Figure 1 were used. Figure 2 shows that residual oxygen in the soil leads to 10% of the anoxic NO emissions.

formation than sensitive species (Liu et al., 2015). These results supported our hypotheses. However, most crop plants are suggested to respond sensitively to flooding stress (reviewed by Mustroph, 2018). In addition to the tested species (tomato, tobacco, and barley), other important crop plants have shown to undergo induction of NO emissions upon oxygen deprivation. Plant materials of maize (Mugnai et al., 2012) and peas (Gupta et al., 2005) showed similar low-oxygen NO formation, which further supports the idea of a ubiquitous plant response. This assumption needs to be confirmed in a range of species and genotypes in future experiments.

Global annual NO emissions from fertilized crops and grasslands were assumed to be 1.8 Tg NO-N (Stehfest and Bouwman, 2006). Yan et al. (2015) measured the NO emissions in cereal fields under different management regimes within the same range. Soil NO emissions have been observed to depend on many factors, including soil water content, soil temperature, and nitrogen availability (Pilegaard, 2013; Ludwig et al., 2001). However, most agriculturally related NO emissions are linked to nitrogen fertilization (Li and Wang, 2008). Among the processes of soil NO formation, microbial nitrification and denitrification are considered the major processes (Pilegaard, 2013). Little attention has been paid to abiotic processes and plants themselves as potential sources of NO. The global area contributing to NO emissions is assumed to be 1906 Mha, including croplands and grasslands (Stehfest and Bouwman, 2006). This amounts to 944 g of NO-N per hectare per year.

To assess an order of magnitude of plant-derived, hypoxia-induced NO emissions, the NO formation rates obtained from laboratory experiments were expanded to the field. Under the assumption that all crop plants show a similar range of low-oxygen-induced NO emissions, NO emitted per ha per year may account for 552–4917 g depending on species and planting density (Figure 1). A critical parameter for the extrapolation of plant NO emissions under low-oxygen stress appears to be the oxygen content of the submerged soils (Figure 2). The rate of oxygen depletion in

soil is dependent on soil structure and composition (Ponnamperuma, 1972). The presence of microbes and plants is critical for consuming remaining oxygen (Pradet and Bomsel, 1978). Reports on the development of anoxic soil conditions as a consequence of flooding range from a few hours (Ponnamperuma, 1972) to days (Scott and Evans, 1955). A recent study identified permanent anoxic microsites in non-flooded soils (Keiluweit et al., 2018). Further increases in the amount of heavy rainfall and flooding periods due to climate change (Tabari, 2020) may increase the frequency of anoxic soil conditions, thereby intensifying the impact of stressed plants on annual NO emissions. With 12 days a year and half of the annual precipitation (Pendergrass and Knutti, 2018), the assumption of low-oxygen conditions in the soil during those days is most likely. Reports on NO transport and storage within plants (Liu et al., 2015; Manrique-Gil et al., 2021), NO uptake by the plant canopy (Bennett and Hill, 1973), and possible adaptations (Wany et al., 2017) add an additional layer of complexity to estimating the impact of plant NO emissions on global NO emissions. The applied method excluded some natural uncertainties such as soil structures and diverse nutrient compositions; however, more species need to be tested to obtain a better picture. Therefore, an understated estimation of plant NO emissions based on the observed hypoxic conditions (0.103% oxygen, Figure 2) may be more realistic. Several other stressors, including drought, salinity, and heavy metal stress (reviewed by Nabi et al., 2019), may also increase NO emissions by plants. However, the impact on global NO emissions may become increasingly important with an increase in extreme weather events.

NO has the potential to be converted into N₂O, a strong greenhouse gas (Cameron et al., 2013; IPCC, 2014; Caranto and Lancaster, 2017). Recent studies have identified plants as producers of N₂O via a pathway involving an NO intermediate under low-oxygen stress. The exact pathways and involvement of soil and plants remain elusive. A protective function to reduce NO

concentrations was discussed by [Timilsina et al. \(2020\)](#). Application of nitrogen isotope-tagged NO to soil and plant settings may help reveal the N₂O conversion mechanism and the involved fractions. NO measurements in the field are primarily performed in chambers spanning the aboveground soil and plants ([Rothardt et al., 2021](#)). Various farmland crops have been cultivated using this technique (reviewed by [Pan et al., 2022](#)). Microbial denitrification emissions have been widely studied. The lack of separate measurements of plant- and soil-derived emissions likely masks the impact of plant NO emissions on global NO emissions. This makes it difficult to evaluate the effect of plants on global NO emissions. [Machacova et al. \(2019\)](#) developed a separate chamber setting for the measurement of N₂O emissions from trees. To date, no fitting system for crop plants has been developed. Isotopic fingerprints for NO sources, similar to those described for N₂O emissions ([Decock and Six, 2013](#)), could be an option. An additional challenge is the application of abiotic stressors such as flooding to a chamber-based measurement system. Separate plant feeding and transferring to the field could overcome these limitations.

5 Methods

Solanum lycopersicum (L.) cv. Moneymaker was grown under greenhouse conditions (14 h light 18°C/10 h darkness 22°C). The tomato plants were grown for 2 weeks on a quartz sand culture with a nutrient solution containing 5 mM NO₃⁻ ([Stöhr and Ullrich, 1997](#); [Wendlandt et al., 2016](#)). The tomato plants were then grown for 5 weeks in a hydroponic culture with the same nutrient solution. The nutrient solution was replaced every 3 days. The hydroponic roots were aerated with ambient air using an ACO-9620 (Hailea) aquarium air pump and an AS 25 air diffuser (Tetra Tec). *Nicotiana tabacum* (L.) cv. Samsun seeds were germinated on filter paper soaked with CaSO₄ solution at room temperature and grown for 2 weeks on a quartz sand culture with a nutrient solution containing 5 mM NO₃⁻ ([Stöhr and Ullrich, 1997](#); [Wendlandt et al., 2016](#)) before being transferred to the same growth regime as described for the tomato plants. *Hordeum vulgare* (L.) cv. seeds were soaked in water. Afterward, the seeds were then sterilized in a 0.5% sodium hypochlorite solution. The seeds were germinated for 8 days on filter paper soaked in CaSO₄ solution. The CaSO₄ solution was replaced with a nutrient solution containing 5 mM NO₃⁻ ([Stöhr and Ullrich, 1997](#); [Wendlandt et al., 2016](#)), and the plants were grown for 7 days. The conditions in the greenhouse corresponded to those described for the tomato plants. For aseptic growth conditions, *Nicotiana tabacum* (L.) cv. Samsun seeds were sterilized in 70% ethanol, followed by a 2% sodium hypochlorite solution containing 0.1% Tween 20. Seeds were germinated on plates containing 1.4% (w/v) bacto-agar, 0.5 mM calcium sulfate, and 2% (w/v) sucrose. Plants were transferred to a 50 ml Erlenmeyer flask containing McCown woody plant medium ([Lloyd and McCown, 1980](#)). Plants were grown for 3 months in the climate chamber (14 h light (28°C) and 10 h darkness (22°C)).

Intact plants were transferred to a custom-built airtight and opaque reactor (4 l, LMS GmbH Ilmenau). Roots were placed into the reactor while the shoot was exposed to light and ambient air. The

reactor was attached to a thermostat (Thermo Electron Corporation, 25°C) and an irrigation system M.R.S Micro Nebeldüse (Micro Rainfall Systems) with a piston pump V1 (Micro Rainfall Systems) to avoid desiccation of the roots. Possible leaks were sealed with Optosil (Heraeus). NO was measured with the chemiluminescence-based NO detector system ANALYZER LCD 88 sp (Eco Physics) with two cold traps [glass bottle on ice (4°C), REFRIGERATED APOR TRAP RVT 100 (Savant) (-50°C)], and a gas hose heater (custom-made, workshop University of Greifswald) upstream of the detector. Humidification of the N₂ carrier gas at a flow rate of 400 mL min⁻¹ was ensured using ultrapure water in a fritted gas-washing bottle gas as described by [Stöhr and Stremlau \(2006\)](#) and depicted in [Supplementary Figure 2](#). The oxygen concentration was adjusted by mixing compressed air (air-liquid) and N₂ gas. The system was calibrated with 86 ppb NO in N₂ gas and adapted to the NO analyzer in accordance with the manufactory guidelines. The addition of a mixture of NO gas and compressed air assured correct NO detection in the presence of O₂. The detected NO concentrations were in accordance with the calculated ones. The baseline was recorded with the empty reactor. The NO production was calculated relative to the root dry weight.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#). Further inquiries can be directed to the corresponding author.

Author contributions

MW: Conceptualization, Investigation, Writing – original draft. WN: Investigation, Writing – review & editing. CS: Conceptualization, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the German Research Foundation (RTG 1947: grant no. 231396381).

Acknowledgments

We thank Stephanie Hasselberg for assistance. We would also like to thank Balázs Grosz, Caroline Buchen-Tschiskale, and Claus Rösemann from Thünen Institute, Institute for Climate-Smart Agriculture for their support in the NO emission calculations.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2024.1290700/full#supplementary-material>

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5. Chapter II: Hypoxia increases triacylglycerol levels and unsaturation in tomato roots

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BMC Plant Biology, tba

1 **Hypoxia increases triacylglycerol levels and unsaturation**
2 **in tomato roots**

3
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17 **Running title:** Hypoxia increases unsaturated triacylglycerols

18 **Keywords:** lipidomics; mass spectrometry; plant stress; stress response

19
20

21 **Abstract**

22 Background: Plants are designed to endure stress, but increasingly extreme weather events
23 are testing the limits. Events like flooding result in submergence of plant organs, triggering an
24 energy crisis due to hypoxia and threaten plant growth and productivity. Lipids are relevant as
25 building blocks and energy vault and are substantially intertwined with primary metabolism,
26 making them an ideal readout for plant stress.

27 Results: By high resolution mass spectrometry, a distinct, hypoxia-related lipid composition
28 of *Solanum lycopersicum* root tissue was observed. Out of 491 lipid species, 11 were
29 exclusively detected in this condition. Among the lipid classes observed, glycerolipids and
30 glycerophospholipids dominated by far (78 %). Differences between the lipidomic profiles of
31 both analyzed conditions were significantly driven by changes in the abundance of
32 triacylglycerols (TGs) whereas sitosterol esters, digalactosyldiacylglycerols, and
33 phosphatidylcholine play a significantly negligible role in separation. Alongside, an increased
34 level of polyunsaturation was observed in the fatty acid chains, with 18:2 and 18:3 residues
35 showing a significant increase. Of note, hexadecatetraenoic acid (16:4) was identified in
36 hypoxia condition samples. Changes in gene expression of enzymes related to lipid
37 metabolism corroborate the above findings.

38 Conclusion: To our knowledge, this is the first report on a hypoxia-induced increase in TG
39 content in tomato root tissue, closing a knowledge gap in TG abiotic stress response. The
40 results suggest that the increase in TGs and TG polyunsaturation degree are common features
41 of hypoxic response in plant roots.

42 **Introduction**

43 The sessile lifestyle forces plants to cope with different stresses during their lifecycle. With
44 increased extreme weather events, the impact on plant growth and productivity becomes more
45 severe [1, 2]. Floods and heavy rainfalls result in waterlogging and submergence of plant
46 organs reducing gas exchange rates. The decline in gas diffusion under these conditions limits
47 oxygen availability and causes an energy crisis due to hypoxia. Therefore, plants evolved
48 several metabolic and morphological adaptations. Energy production is shifted to anaerobic
49 fermentation, and aerenchyma formation facilitates gas transport within the plant [3]. In
50 addition to the toxic metabolites ethanol and lactate, nitric oxide and reactive oxygen levels
51 are highly increased under hypoxia [4].

52 Lipids are crucial for membrane integrity or energy metabolism and are usually well
53 controlled, e.g. to maintain cell membrane functionality [5]. Abiotic stress factors trigger
54 adaptive responses that have been reported to result in alterations of the lipid composition [6].
55 Several recent studies highlight an association between increased triacylglycerol (TG) levels
56 and abiotic stress [7, 8]. These storage lipids are normally found in seeds and fruits. They
57 accumulate in vegetative tissue in lipid droplets under stress. A protective function of the
58 droplets itself or by the sequestration of toxic lipid intermediates has been considered [9, 10].

59 Nevertheless, little is known about the role of TGs and alterations in the fatty acid
60 composition in the response towards hypoxia [11]. Besides their role in membrane fluidity
61 control, polyunsaturated fatty acids are stress hormone precursors and may reveal regulatory
62 activity in stress response [12, 13]. Recent findings indicate the importance of lipids and fatty
63 acids in oxygen sensing. Unsaturated long-chain Acyl-CoAs modulate Ethylene Responsive
64 Factor VII (ERF VII), a key player in hypoxic response in plants [14-16]. Unsaturated very-long-
65 chain ceramide species modulate the ethylene signaling pathway under hypoxia [17]. A closer
66 look at low-abundant and stress-specific lipids can increase our understanding of plant stress
67 adaptation.

68 Being the first organ to experience changes in oxygen availability, the root physiology is
69 important to understand adaptation processes. Therefore, a hypoxia model based on the
70 partial submergence of tomato plants was used to collect root material. Subsequently, lipids
71 were extracted and analyzed by a bottom-up shotgun lipidomics approach using ultra-high
72 performance liquid chromatography coupled to a high-resolution tandem mass spectrometer
73 (UHPLC-MS²). The study provides valuable information about the impact of hypoxic stress on
74 root tissue lipid composition, with a special focus on TG levels and the degree of saturation of
75 their fatty acid chains.

76 **Materials and methods**

77 *Chemicals*

78 Chloroform was of HPLC grade and obtained from Roth. Isopropanol, acetonitrile and
79 methanol had LC-MS grade; water had HPLC grade. Those chemicals were obtained from
80 Th.Geyer. Formic acid was purchased from Honeywell and had LC-MS grade. Ammonium
81 formate was obtained from Sigma-Aldrich and had LC-MS grade. EquiSPLASH Lipidomix was
82 purchased from Avanti Polar Lipids. Magnesium sulfate, calcium nitrate and calcium sulfate
83 were obtained from AppliChem. Other chemicals needed for plant growth were obtained from
84 Merck.

85 *Cultivation of Solanum lycopersicum under root hypoxia and harvest*

86 *Solanum lycopersicum* (L.) cv. Moneymaker was cultivated under greenhouse conditions
87 (14 h light 18 °C / 10 h dark 22 °C). Tomato plants grew on quartz sand culture for four weeks
88 with a nutrient solution containing 5 mM NO₃⁻ [18, 19]. Tomato roots were waterlogged for 48
89 h to apply hypoxic conditions. Therefore, trays were filled with distilled water until the quartz
90 sand substrate was covered. Prior to harvest, the quartz sand mixture was carefully removed
91 from plants using ice-cold water. Two sets of plants were independently grown. Nine control
92 and nine waterlogged plants were harvested per set and prepared separately (N=18 biological
93 replicates).

94 *Lipid extraction*

95 Lipids from tomato roots were extracted with a modified procedure adapted from Shiva and
96 colleagues [20]. In brief, 0.1 g of grinded roots (cooled on liquid nitrogen) were added to a
97 glass reaction tube filled with isopropanol supplemented with 0.01 % BHT. The mixture was
98 incubated for 15 min at 75 °C and cooled on ice afterward. To the cooled-down mixture, 0.5
99 volume of chloroform, 0.2 volume of water and 3 µL of EquiSPLASH Lipidomix were added.
100 Lipids were extracted for 1 h on ice with occasional vortexing in between. Reaction tubes were
101 centrifuged at 5000 x g to induce phase separation, and the organic phase was transferred to
102 another glass reaction tube. To the remaining water phase, 1.33 volumes of a
103 chloroform:methanol mixture (2:1, v/v) supplemented with 0.1 % BHT was added and
104 incubated for 30 min on ice with occasional vortexing. Reaction tubes were again centrifuged
105 at 5000 rpm, and the chloroform phase was transferred to the second glass vial. The
106 chloroform:methanol extraction step was repeated with the remaining water phase 3 times.
107 Afterward, 0.33 volumes of KCl (1 M) were added to the combined lipid extracts and vortexed.
108 The upper water phase was removed, and 0.66 volumes of water were added and vortexed.
109 The upper phase was again removed, and sodium sulfate was added to remove the remaining

110 water. Finally, the lipid extract was dried under constant N₂-flow with a TurboVap sample
111 evaporator (Biotage, Sweden) and frozen at -80°C until MS analysis.

112 *Reversed-phase LC-MS²*

113 Frozen lipid films were rehydrated with chloroform:methanol:isopropanol (1:2:4 v/v/v,
114 supplemented with 5 mM ammonium formate). Lipids were separated on a Vanquish UHPLC
115 equipped with an AccuCore C30 column and guard column (150/10x2.1 mm, Thermo Fisher)
116 at 50 °C. Mobile phases were acetonitrile/water (60:40 v/v, A) and isopropanol/acetonitrile
117 (90:10 v/v, B), both supplemented with 10 mM ammonium formate and 0.1 % formic acid. At a
118 flow rate of 350 µL/min the following gradient was used: 0-3 min 30 % B, 3-8 min 50 % B, 8-
119 22 min 70 % B, 22-29 min 99 % B, 29-37 min hold 99 % B, 37-37.1 min decrease to 30 % B,
120 37.1-41 min hold 30 % B. The LC was connected to a QExactive Plus mass spectrometer
121 (Thermo Fisher), operated in data dependent acquisition mode (top 15) with a dynamic
122 exclusion time of 10 ms. Samples were were injected four times (2x positive and 2x negative
123 mode, two technical replicates per biological replicate). For the full MS survey scans, a range
124 of 100 to 1000 m/z was set and the automatic gain control (AGC) target was 1 x 10⁶ with a
125 maximum ion time (IT) of 80 ms. Resolution used in full MS was 70,000, for MS/MS after
126 higher-energy collisional dissociation 17,500 (HCD, each at 200 m/z). HCD was achieved with
127 a stepped normalized collision energy from 25 to 27 in positive mode and from 20 to 23 in
128 negative mode with an AGC target of 2 x 10⁵ and a maximum IT of 50 ms.

129 *Identification of lipids*

130 MS raw files were analysed with the software LipidSearch (version 4.2.27, Thermo Fisher)
131 using the following parameters: retention time interval 0.01 min, m-score threshold 5, precursor
132 tolerance 5 ppm, product tolerance 8 ppm, ID quality filter A (fatty acid chain and class
133 identified completely) and B (class and some fatty acid chains identified). The resulting text
134 files were processed with an in-house KNIME workflow and filtered for ppm error, peak quality
135 and area score. The area of identical lipids with different ion adducts was summed up. The
136 resulting excel file was processed in R (v 4.0.2) with manual inspection of raw areas (**Figure**
137 **S1A**). Lipids in the blanks or blank extracts were excluded from the data, if not at least 5-fold
138 lower in intensity in blank controls. Lipid areas were normalized to the respective lipids in the
139 EquiSplash standard, and median normalization was applied (**Figure S1B**). Lipids were filtered
140 for entries, that had to be present in both sets and in at least 50 % of the samples within at
141 least one condition. For principal component analyses (PCA), lipid species identified in at least
142 50 % of samples were considered, and the two independent sets were batch-adjusted using
143 the ComBat algorithm. Lipids were annotated on the molecular species level in accordance

144 with the proposed nomenclature by the LIPID MAPS consortium since no information on the
145 sn-position of acyl/alkyl constituents was available [21, 22].

146 *Transcriptome analysis*

147 Transcriptomic raw data (deposited at Sequence Read Archive (SRA) database (bioproject
148 accession PRJNA553994), obtained by NextSeq. 500 Illumina platform (LGC Biosearch
149 Technologies) and processed by CLC Genomics Workbench Software (Qiagen, V. 7.5.5) and
150 published by Safavi-Rizi and colleagues (2020), were reevaluated as described in the same
151 paper with a focus on lipid-related transcriptomic changes [23].

152 *Statistical analysis*

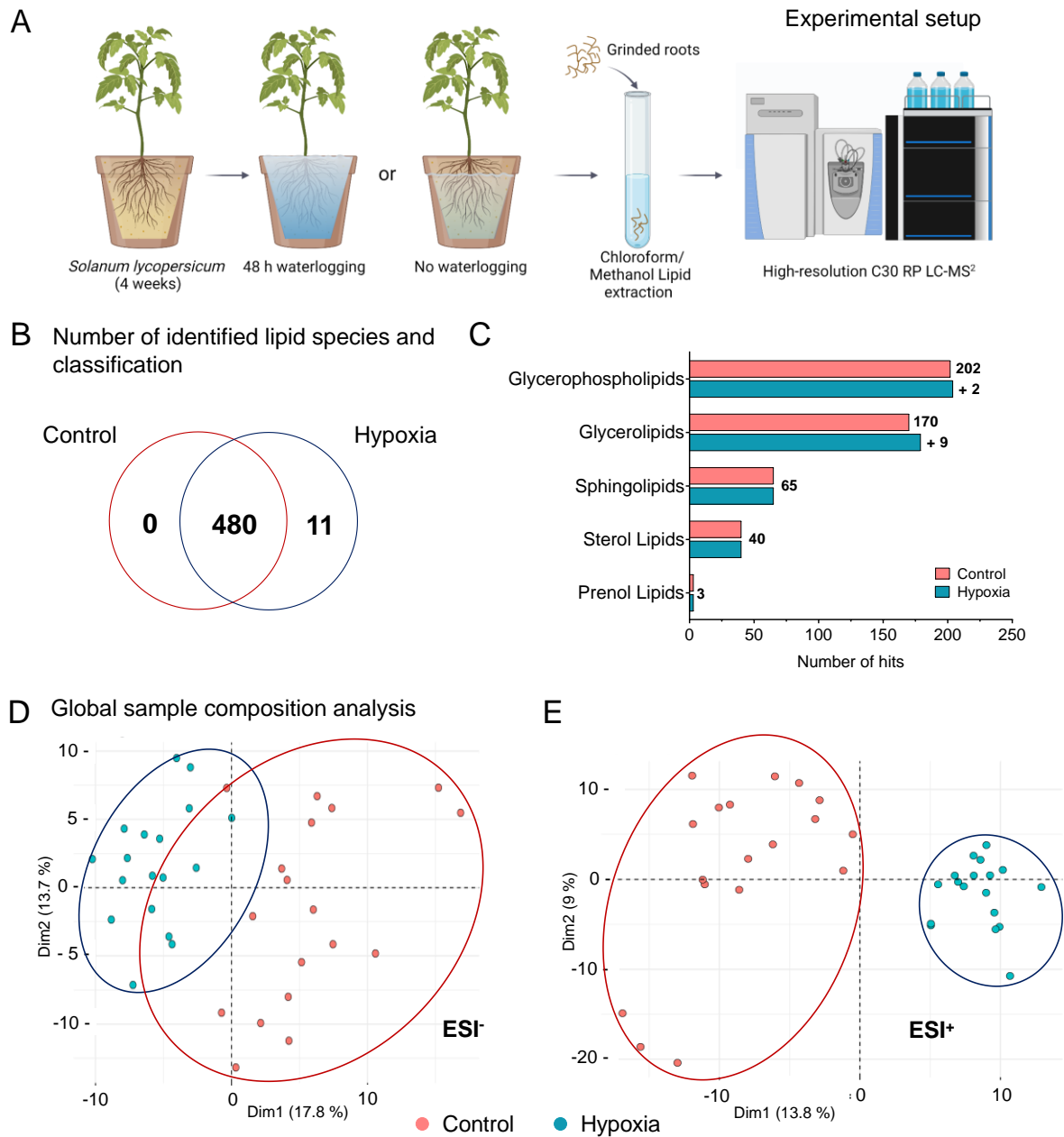
153 To analyze lipid classes contributing to the separation of experimental conditions assessed
154 via PCA, Fisher's Exact test was used. Lipid species contributing more than two-fold the
155 expected weight to the particular dimension were considered to span the dimension. For
156 statistical analysis of TGs, data were tested for normal distribution with the Shapiro-Wilk test
157 and subsequently tested for statistical significance with 2-way ANOVA with Sidak post-hoc test
158 or multiple t-test with Holm-Sidak post-hoc test (tested against control, *** $p < 0.001$; ** $p <$
159 0.01 , * $p < 0.05$).

160

161 Results

162 *Hypoxia alters the tomato root lipidome composition significantly*

163 Little is known about the plant lipidome and its adaptation to hypoxic stress. Therefore, the
164 lipid composition of *Solanum lycopersicum* roots after 48 h of waterlogging was analyzed by
165 high resolution mass spectrometry (**Figure 1A**). With this approach, a total of 491 lipid species
166 were identified. The majority (480) of lipid species were detected under both control and
167 hypoxic conditions, while 11 were exclusively found under hypoxia (**Figure 1B**). Lipids
168 belonging to five categories were detected, with glycerophospholipids and glycerolipids
169 dominating. Together, they comprised 78 % of all identified lipids. Besides, minor amounts of
170 sphingo-, sterol and prenol lipids were found. The lipids exclusively found under hypoxia were
171 among the most frequently detected categories - 9 were glycerolipids, and 2 were
172 glycerophospholipids (**Figure 1C**). While all new glycerolipids belonged to the class of TGs,
173 the two glycerophospholipids were phosphatidylethanolamines (PE). Interestingly, most fatty
174 acid chains of newly identified TG species showed a high degree of unsaturation (**Table S1**).
175 A principal component analysis (PCA) revealed an altered lipidomic profile under hypoxic
176 conditions in both polarities (**Figure 1D, E**). The negative and positive polarity of the first two
177 principal components/ dimensions of the PCAs accounted to 31.5 % (17.8 % and 13.7 %) and
178 22.8 % (13.8 % and 9 %) of the total lipidomic profile variances, respectively, pointing to
179 interindividual sample specific lipidomic profiles. Despite modest variance explanatory power,
180 the control and hypoxia samples were clearly separated along the first dimension. This leads
181 to the suggestion that the hypoxia and control conditions applied to the samples are the main
182 drivers for differences in the lipidomic profiles. Subsequently, to analyze the main condition-
183 separating differences in the lipidomic profiles Fisher's Exact test was used. Enrichment or
184 reduction of lipid class dimension was indicating that TGs are significantly enriched, whereas
185 phosphatidylcholine, digalactosyldiacylglycerol (DGDG) and acyl hexosyl sitosterol esters
186 were reduced in their importance in the separation of the conditions (**Table S2**).



188

189

Figure 1. Experimental workflow and the root lipidome variation under hypoxic conditions.

Tomato plants grew for 4 weeks on quartz sand culture under greenhouse conditions before they were

waterlogged for 48 h, harvested and grinded in liquid nitrogen. After chloroform/methanol extraction,

lipids were analyzed with UHPLC-MS² (A). The detected root lipidome of waterlogged or control tomato

plants consisted of 480 lipid species, 11 species were exclusively identified under hypoxic conditions

(B). The majority of the detected lipid species belonged to five lipid classes, with glycerol- and

glycerophospholipids being the largest group. Hypoxic conditions increased the number of glycerol- and

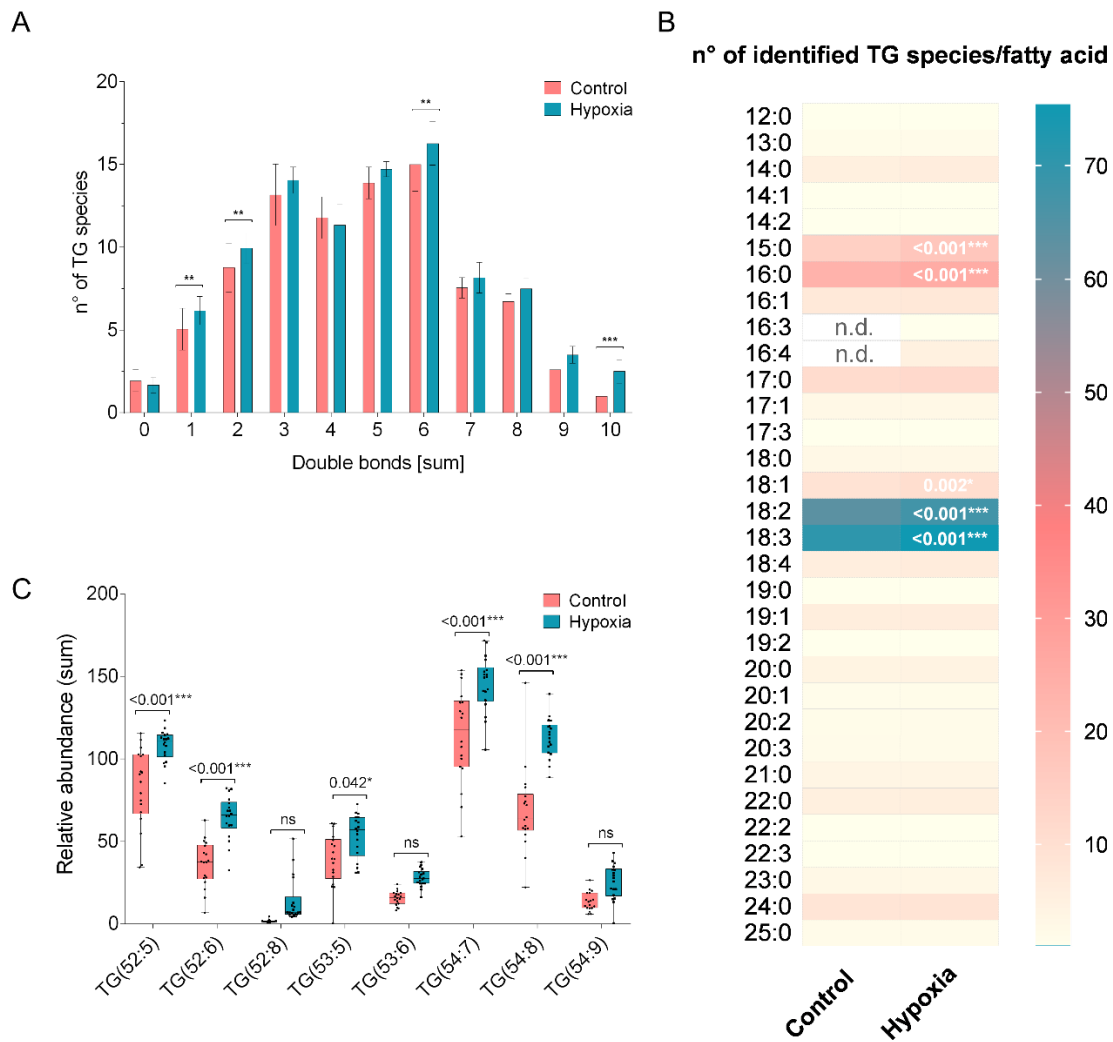
glycerophospholipid species (C). PCA-plots of the first and second dimension calculated based on the

50 percentile lipidome profiles of the samples, which show a separation of samples belonging to the

control and hypoxia condition groups in negative (D) and positive polarity (E) along the first dimension.

200 *Triacylglycerol metabolism is affected under hypoxic conditions*

201 Since TG species were shown [9, 10] to carry information about lipidome separation of the
202 analyzed conditions and most newly detected lipids were TGs, this class underwent further
203 analysis. First, changes in saturation degree were evaluated. Under hypoxic conditions, an
204 increased number of unsaturated TG species was observed while the number of saturated
205 TGs decreased. TG species with one to ten double bonds were increased in number,
206 significant for species with one, two, six and ten double bonds per molecule (**Figure 2A**). In
207 addition to the TG count, the abundance of TG bulk species underwent further analysis. Most
208 abundant TG bulk species across both conditions were 54:7 and 54:8 TGs, while low
209 abundancies were observed for TGs with shorter fatty acid chains (TG 52:5/6 and 53:5). In
210 case of hypoxic conditions, five TG bulk species were significantly increased. Interestingly, no
211 TG species was more abundant under control than hypoxic conditions (**Figures 2C**). Under
212 hypoxic conditions, it was observed that TG species incorporating fatty acids with a higher
213 degree of unsaturation were both more versatile (higher number of identified species) and
214 showed a higher abundancy than the more saturated counterparts. A detailed look at the fatty
215 acid profile may provide information on TG remodeling and origin during hypoxic stress
216 conditions at the root level. Since bulk species abundance does not carry information on
217 individual esterified fatty acids, TG species were categorized and counted in relation to their
218 double bonds. In general, hypoxic samples were richer in fatty acid species. The four most
219 prevalent fatty acid species were 18:2, 18:3, 15:0 and 16:0, all of them component of a higher
220 number of TG species under hypoxia. Interestingly, the fatty acids 16:3 and 16:4 were
221 exclusively detected in new identified TGs (**Figure 2B**). In total, nine TGs were identified that
222 were not present in any of the control plants (**Table S1**). Since the detected compounds carry
223 an unusual fatty acid (hexadecatetraenoic acid, 16:4), they were manually inspected for correct
224 MSMS fragmentation pattern and peak shape (**Figure S2**). Interestingly, also two PE species
225 with the unusual 16:4 fatty acids were identified and manual inspected (**Figure S4**).
226



227

228 **Figure 2. TGs increase their double bonds and the number of species esterified with 15:0, 16:0,**
 229 **18:1, 18:2, and/or 18:3 fatty acids during hypoxic conditions.** Absolute number of TG species were
 230 plotted against their sum of double bonds for control and hypoxic samples (**A**). The number of species
 231 esterified with 15:0, 16:0, 18:1, 18:2, and/or 18:3 fatty acids is significantly increased under hypoxic
 232 conditions (**B**). TG species with significant change in abundance under hypoxic conditions compared to
 233 control, insert shows lower abundant species (**C**). Data show mean and S.D.; statistical analysis was
 234 performed using multiple t-testing with Holm-Sidak post-hoc test (tested against control, * $p \leq 0.05$, ** p
 235 ≤ 0.01 , *** $p \leq 0.001$).

236

237 *The abundance of TG species with 16:0, 18:2 and/or 18:3 fatty acids increase*
 238 *under hypoxia while the abundance of the TG-precursors PA, DG, LPC and*
 239 *MGDG decrease*

240 Beside the number of TG species (**Figure 2C**), also the abundance of TG species
 241 esterified with 16:0, 18:2 and 18:3 fatty acids were significantly increased (**Figure 3A**). Other
 242 long-chain unsaturated fatty acids incorporating TGs (18:1, 18:4) and 17:0 fatty acids showed
 243 a trend to higher abundance (**Figure 3A**). The 16:0, 18:2 and 18:3 fatty acids introduced into
 244 the newly formed TGs in stress conditions may derive from *de-novo* synthesis or have been

245 diverted from the synthesis of other lipid species. Precursors of TG can result from the
246 Kennedy pathway in the ER membrane from conversion of phosphatidic acid (PA) to DG. TG
247 is then formed from diacylglycerol (DG) by diacylglycerol acyltransferase. Another route of TG
248 formation is the phosphatidylcholine (PC) pool as source of acyl species, catalyzed by
249 phosphatidylcholine acyltransferase (PDAT). As further product lysophosphatidylcholine (LPC)
250 is formed (**Figure 3B**). For PA, an increase in abundance under hypoxia was observed (**Figure**
251 **3C**). Interestingly, two PA species with an unusual fatty acid composition accumulated in
252 waterlogged plant roots (**Figure S4A**). According to the MSMS fragmentation spectra (**Figures**
253 **S4D-G**), both species were esterified with 16:4 fatty acids. Out of the detected DGs the DG
254 species 24:0_18:2, 24:0_18:3, 25:0_18:2 and 26:0_18:2 were significantly altered. All were
255 reduced in abundance under hypoxic conditions (**Figure 3D**). The abundance of PC species
256 was decreased in hypoxic conditions. Out of the six detected PC species 3 were significantly
257 decreased in abundance. Higher levels of PC 18:1_18:2, 18:1_18:2 and 16:0_18:1 were
258 observed under control conditions (**Figure 3F**). With a look on fatty acids species in PC lipids,
259 18:1 was more abundant while 18:2 was less abundant under hypoxic conditions (**Figure 3E**).
260 The other fatty acids remained unchanged. Together with PC, LPC species significantly
261 decreased with LPC 17:0 and LPC 18:2 been significantly altered (**Figure 3G**). In accordance
262 with the literature, fatty acid precursors for TG biosynthesis in vegetative tissue may derive
263 from monogalactosyldiacylglycerol (MGDG) and are transferred via PC yielding TGs, catalyzed
264 by PDAT [24]. Corroborating this, the abundance of MGDG species esterified with an 18:3 fatty
265 acid is significantly reduced, and for the fatty acids 16:0 and 16:3 a trend towards a reduced
266 abundance was observed (**Figure 3H**). For individual MGDG species, a reduction was
267 detected for compounds with an 18:2 or 18:3 fatty acid (four and three lipid species,
268 respectively). Only MGDG 18:2_18:2 showed higher abundance under hypoxia. In addition,
269 the abundance of one MGDG species carrying a 16:3 fatty acid was reduced. The MGDG
270 species carrying 16:4 fatty acid were undisturbed by the hypoxia (**Figure 3I**). In plastids,
271 MGDG are metabolized to DGDGs. Under the current conditions, no changes were detected
272 for this lipid class (**Figure S3**). These findings indicate that especially 18:2 and 18:3 fatty acids
273 accumulating in TGs may derive from MGDGs via the PC pool and LPCs formation by DG
274 integration.

275 Safavi-Rizi and colleagues investigated the effects of 48 h hypoxia on the transcriptome
276 of tomato roots (Sequence Read Archive database, bioproject accession PRJNA553994) [23].
277 The raw datasets also contained information on lipid metabolism, which was not addressed so
278 far and therefore were reanalyzed in the frame of this study. Among the five most upregulated
279 genes, two ACB-desaturases, OLEOSIN1, a lysophospholipase and a DGDG synthase
280 (DGD2) were identified (**Table 1**). Strong down-regulation was observed for a DGDG synthase

281 (DGD1), a lipid transfer protein (LP1), an acyl-CoA carboxylase, PMEAMT – an enzyme
 282 involved in choline and phospholipid synthesis [27] – and a TG lipase (**Table 2**).

283 In summary, newly formed TGs incorporate more likely, 18:2 and 18:3 fatty acids in
 284 their molecule while at the same time also abundance of preexisting species carrying the same
 285 fatty acids were decreased.

286

287 **Table 1: Up-regulated gene expression in the biological category *lipid metabolism* after 48 h of**
 288 **water logging (fold change)**

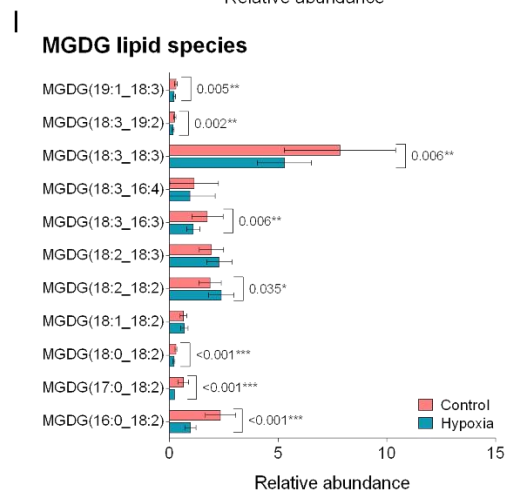
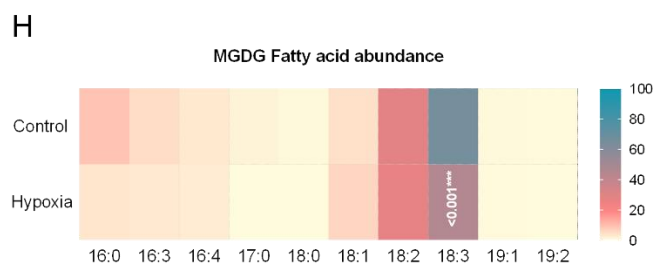
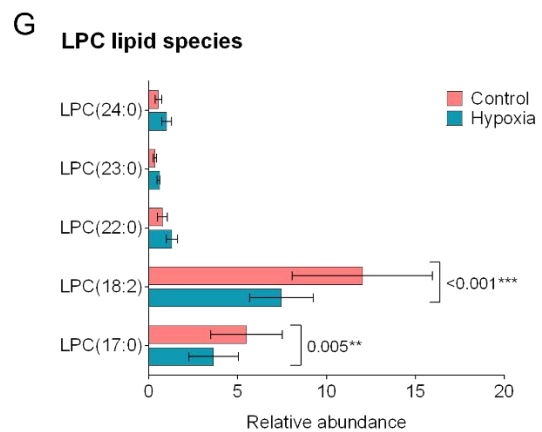
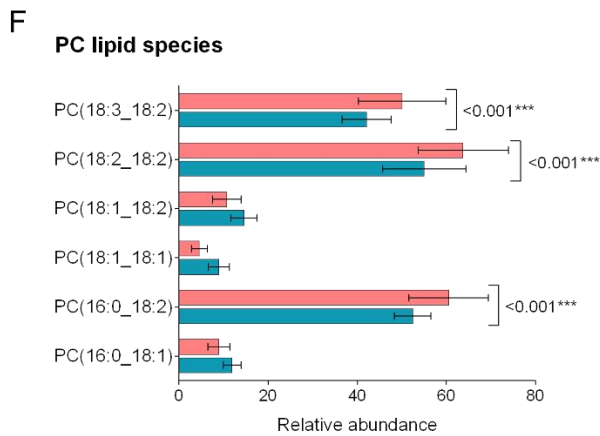
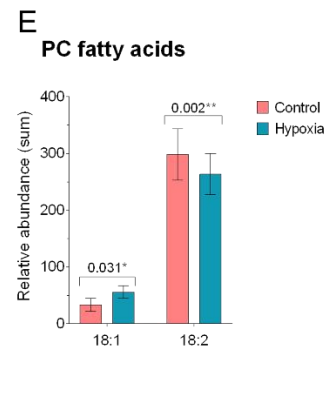
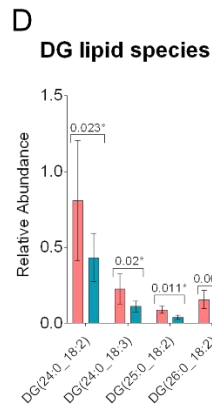
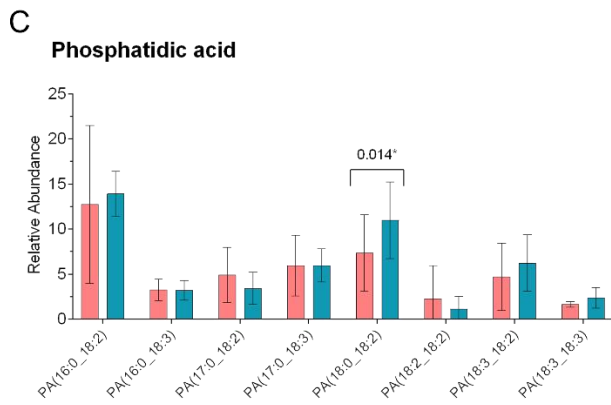
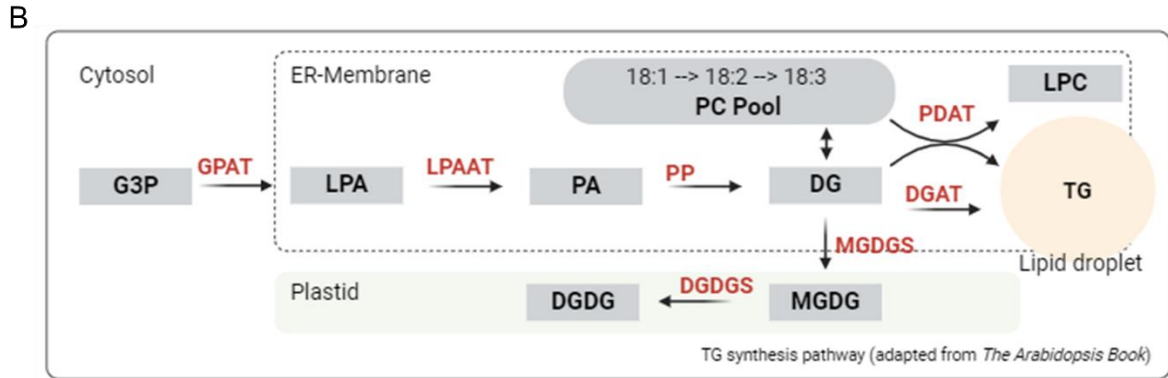
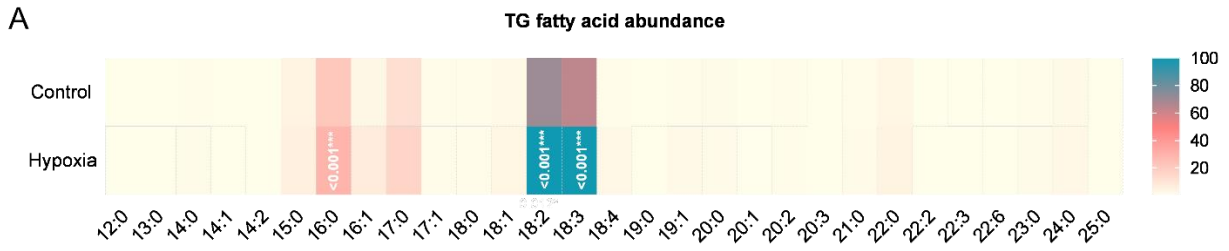
Identifier	Biological category	Arabidopsis homolog	Fold change
solyc11g008680.1.1	FA synthesis and FA elongation; ACP desaturase	AT2G43710.2: SSI2, FAB2, Plant stearyl-acyl-carrier-protein, desaturase (family)	+ 55.1
solyc12g010920.1.1	TAG synthesis	AT4G25140.1, OLEO1, OLE1, oleosin1	+ 43.9
solyc12g042890.1.1	lipid degradation, lysophospholipases, carboxylesterase	AT1G52700.1: α/β -Hydrolase	+ 39.0
solyc01g094170.2.1	glycolipid synthesis, DGDG synthase	AT4G00550.1: DGD2, digalactosyl diacylglycerol deficient 2	+ 10.4
solyc06g053480.2.1	FA synthesis and FA elongation, ACP desaturase	AT2G43710.2: SSI2, FAB2, Plant stearyl-acyl-carrier-protein, desaturase (family)	+ 7.6
solyc02g082910.2.1	FA synthesis and FA elongation, acyl coa ligase	AT1G20560.1: AAE1, Acyl activating enzyme 1	+ 6.2
solyc09g008840.2.1	FA synthesis and FA elongation.pyruvate kinase	AT3G52990.1: Pyruvate kinase family protein	+ 5.6

289

290 **Table 2: Down-regulated gene expression in the biological category *lipid metabolism* after 48 h**
 291 **of water logging (fold change)**

Identifier	Biological category	Arabidopsis homolog	Fold change
solyc01g007100.2.1	glycolipid synthesis, DGDG synthase	AT3G11670.1: DGD1, UDP-Glycosyltransferase	- 21.4
solyc08g067500.1.1	lipid transfer proteins	AT2G38540.1: LP1, LTP1, ATLTP1, lipid transfer protein 1	- 19.7
solyc03g025720.2.1	FA synthesis and FA elongation, acyl CoA ligase	AT3G48990.1: AMP-dependent synthetase and ligase	- 9.9
solyc12g040790.1.1	Phospholipid synthesis	AT1G48600.2: PMEAMT, AtPMEAMT, S-adenosyl-L-methionine-dependent methyltransferase	- 9.3
solyc02g076990.2.1	lipid degradation, lipases, triacylglycerol lipase	AT4G18550.1: α/β -Hydrolase	- 5.7
solyc05g011860.1.1	'exotics' (steroids, squalene)	AT2G03760.1: ST, ATST1, RAR047, ST1, AtSOT1, AtSOT12, SOT12 sulphotransferase 12	- 2.9

292



294 **Figure 3. The abundance of 16:0, 18:2 and 18:3 esterified TG species increases**
295 **while some matching precursor PA, DG, LPC and MGDG decrease significantly under**
296 **hypoxic conditions.** Comparison of TG species (sum of all identified in both conditions)
297 reveals increase in abundance in species incorporating 16:0, 17:0, 18:2 or 18:3 fatty acids (**A**)
298 Pathway of TG synthesis (adapted from Li-Beisson *et al.*, 2013) (**B**). Comparison of TG
299 synthesis precursors phosphatidic acid identified in both conditions (**C**), Significantly changed
300 DG lipid species (24:0_18:3 and DG 26:0_18:3, **D**), PC (sum of 18:1 and 18:2 esterified
301 species, **E** and individual lipid species, **F**), LPC (**G**) and MGDG (abundance of fatty acids (**H**)
302 and individual lipid species (**I**)). Data show mean and S.D.; statistical analysis was performed
303 using multiple t-testing with Holm-Sidak post-hoc test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

304 Discussion

305 By inducing hypoxic conditions in plant roots, a broad stress response is triggered for
306 handling this detrimental condition. Changes in carbon metabolism – including lipid
307 metabolism, signal processes, and anatomical adaptations were found prominent [23, 28]. The
308 alteration in lipid composition is a known feature of stress adaptation. It ensures membrane
309 integrity and energy resources [29], but also the sensing of stress conditions and the
310 subsequent activation of downstream processes [30]. For hypoxia, limited data are available
311 for *Arabidopsis*, where changes in glycerophospholipids, galactolipids, and ceramides were
312 found [17, 31]. The data presented here indicate that hypoxia significantly altered the lipid
313 composition of tomato roots and led to a hypoxia-specific pattern. While phosphatidylcholine
314 (PC) lipid levels decreased in hypoxic tomato roots (**Figure 3C**), confirming observations in *A.*
315 *thaliana* rosette and leaf [17, 31], other glycerophospholipids and ceramides remained
316 unaltered. In contrast, TGs were found to contribute most prominently to differences in the
317 lipidome under hypoxic conditions (**Figure S2**). This observation is in agreement with other
318 studies linking TG accumulation in vegetative tissue to abiotic stress [10]. Under drought, heat,
319 cold, and nutrient starvation, an increase in TG content was observed [32, 33].

320 In vegetative tissue, *de novo* synthesized diacylglycerol (DG) is acylated into TG via
321 diacylglycerol acyltransferase (DGAT) or phospholipid:diacylglycerol acyltransferase (PDAT)
322 (reviewed by [10]). Heat stress led to releasing free fatty acids (FFAs) from MGDG. PDAT used
323 FFAs converted into PC to build TGs [34-36]. Under freezing and water deficiency conditions,
324 DAG derived from MGDG or DGDG and trigalactosyldiacylglycerol (TGDG), respectively.
325 DGAT used the DAG to form TG [33, 37, 38]. Under ozone stress, FFAs and DAG for TG
326 synthesis are derived from MGDG [39, 40]. In this study on hypoxic tomato roots, the content
327 of TG species and their total abundance increased while PC, LPC, DG and MGDG abundance
328 decreased (**Figure 2+3**). The increase in TGs and decrease in PC and MGDG could indicate
329 a similar sensing or management mechanism as under heat stress and point to a role in
330 general stress coping mechanism for TGs.

331 Assessment of the mRNA profile revealed distinct changes due to the hypoxic conditions.
332 The induction of desaturases in stress conditions is consistent with the increase in unsaturated
333 fatty acids (**Figure 2A**). The upregulation of OLEOSIN1, a structural lipid body protein [41],
334 and the reduction in gene expression of a TG lipase may indicate TG accumulation in lipid
335 bodies. The induction of DGD2 but the reduction of DGD1 transcription fits with the functional
336 descriptions of Kelly and Dörmann according to which DGD1 is the major DGDG synthase and
337 DGD2 is active under stress conditions [42]. This is in accordance with observed unaltered
338 DGDG abundance (**Figure S3**) and less importance of DGDGs for differentiation of control and
339 hypoxic lipid profile in the enrichment analysis (**Table S2**). The reduction of PC and LPC
340 (**Figures 3C+G**) are also detectable in the transcriptomic data.

341 The role of accumulated TGs – generally considered as storage lipids – under stress
342 conditions is controversially discussed in the scientific community. One explanation may be
343 the segregation of toxic lipid intermediates. During the adaptational response to abiotic stress,
344 changes in lipid composition ensure membrane integrity [29]. The toxic intermediates DAGs
345 and FFAs are released from thylakoid membranes during this process. As a result of this, TG
346 synthesis might reduce their concentration and attenuating negative effects [43]. In
347 *Arabidopsis*, the key player phytyl ester synthase 1 and 2 (PES1 and 2) involved in this process
348 are induced under nitrogen starvation and senescence [44]. Additionally, the importance of
349 TGs in lipid droplets as a scaffold for several stress-responsive proteins was highlighted [45].
350 Best characterized is caleosin, an abundant lipid droplet-associated protein involved in
351 oxylipins and phytoalexins production [45-48]. OLEOSIN1 is the main protein on lipid body
352 surface and involved in their structural integrity [49]. Interestingly, gene expression of this
353 protein is upregulated upon 48 h hypoxia in tomato roots (**Tabel 1**). The mutation of lipid
354 droplet-associated small rubber particle proteins (SRPs) reduced drought tolerance and
355 emphasized the importance of lipid droplets in stress response [48]. Due to the ubiquitous
356 appearance of TG accumulation in vegetative tissue under stress conditions, a similar function
357 of TG under hypoxia is likely.

358 The hypoxia-relevant TG species showed an increased degree of unsaturation, and higher
359 numbers of double bounds were favored. Unsaturation of membrane lipids plays an important
360 role in response to different stressors such as chilling, freezing, heating, salinity and drought
361 [50-55]. Hypoxia increased fatty acid unsaturation in both *Arabidopsis* leaves and crown galls
362 via an increased expression of desaturases [17, 56]. A recent study even highlighted the
363 importance of polyunsaturated linolenoyl-CoA (18:3-CoA) modulating hypoxic response in *A.*
364 *thaliana* [16]. Under heat stress, 18:3 is removed from thylakoid-derived MGDG and stored in
365 TGs to ensure membrane integrity [36]. An increase in 18:3 fatty acids had also been observed
366 in hypoxic *Arabidopsis* crown galls [56]. The current study showed, that the number TG species
367 with 18:2 and 18:3 fatty acids and their total abundance increased in hypoxic tomato roots
368 compared to normoxic controls (**Figure 3A**). Those fatty acids are known to be enriched in
369 MDGDs and DGDGs [57]. In this study, the origin of the polyunsaturated fatty acids could not
370 be fully solved. Reduced MGDG levels and abundant polyunsaturated C-18 fatty acids (**Figure**
371 **3I**) point to thylakoidal origin from galactosylglycerols, alongside with the PC pool as a central
372 fatty acid buffer. A role of TGs in free fatty acid sequestration is likely.

373 Notably, several polyunsaturated PE and TG lipid species were exclusively identified under
374 hypoxic conditions. All incorporated a 16:4 fatty acid (**Figure S1**) similar to stearidonic acid (ω -
375 3 18:4). TG (16:3_16:4_18:2) additionally showed an unusual 16:3 fatty acid (**Figure S1**). A
376 database search revealed that those lipid species were not included in the general LIPID
377 MAPS and other databases. Additionally, they were not described as part of the plant lipidome

378 of *Solanum lycopersicum* until now. Since no commercial standard for those compounds is
379 available, information on specific MSMS fragmentation behavior is unknown. At large, 16:4
380 fatty acids were considered to occur in pacific krill [25] but mainly in algae species [26] and are
381 used as a taxonomic indicator for algae classes [58]. So far, only a few reports show its
382 presence in higher plants [59-62]. Findings of 16:4 fatty acids in tomato fruit skin are reported
383 [63, 64]. Besides, there are reports of dinor-oxo-phytodienoic acid (dnOPDA, 16:4-O) with
384 respect to MGDG/DGDG acylation and stress response [65-67]. 16:3 fatty acids accumulated
385 in TG species in an *Arabidopsis sdg1* mutant (TG lipase) and was reported to be of
386 MGDG/DGDG origin [68, 69]. In addition, MGDG/DGDG 16:3 fatty acids are linked to several
387 stressors [70, 71]. Our study also identified MGDG containing both 16:3 or 16:4 fatty acids
388 **(Figure 3H+I)**. The appearance of this unusual TG and PE species containing 16:4 and/or 16:3
389 fatty acids in tomato roots seemed to be stress induced. An origin of the fatty acids from MGDG
390 could be possible. In terms of stress induced TG accumulation, *Chlamydomonas reinhardtii*
391 16:4 fatty acids show a similar role as 18:3 fatty acids in higher plants. A relict regulatory
392 function of these hypoxia induced fatty acid would be thinkable.

393 **Conclusion**

394 Hypoxia induced by partial submergence of tomato plants boosts TG content and increases
395 fatty acid polyunsaturation in root tissue, thereby resembling features described for other types
396 of plant stress. The results suggest that the increase in TGs and TG polyunsaturation degree
397 are common features of hypoxic response in plant roots. It may be assumed that those TGs
398 are implemented in lipid droplets which are known to be involved in sequestration toxic lipid
399 intermediates. To our knowledge, this is the first report on a hypoxia-induced increase in TG
400 content in tomato root tissue, closing a knowledge gap in TG abiotic stress response.

401

402 **Supporting information**

403 **Tables**

404

405 **Table S1. Lipid species found exclusively under hypoxic conditions.** Isobar compounds
406 were detected by their different retention times

Lipid species	Bulk ID	Chemical formula	Mass (M-H/M + NH ₄ ⁺)	Adduct	RT 1	RT 2
PE (16:0_16:4)	PE (30:1)	C37H66NO8P	682.4453	-H	10.91	10.10
PE (16:4_18:2)	PE (34:6)	C39H66O8NP	706.4453	-H	9.70	8.97
TG (16:0_16:4_16:4)	TG (48:8)	C51H82O6	808.6450	+NH ₄ ⁺	23.68	22.34
TG (15:0_16:4_18:3)	TG (49:7)	C52H86O6	824.6763	+NH ₄ ⁺	24.68	23.48
TG (15:0_16:4_18:2)	TG (49:6)	C52H88O6	826.6919	+NH ₄ ⁺	25.51	24.61
TG (16:3_16:4_18:2)	TG (50:9)	C53H84O6	834.6606	+NH ₄ ⁺	22.86	21.49
TG (16:1_16:4_18:3) TG (16:2_16:4_18:2)	TG (50:8)	C53H86O6	836.6763	+NH ₄ ⁺	24.23	22.91
TG (16:4_18:2_18:4)	TG (52:10)	C55H86O6	860.6763	+NH ₄ ⁺	23.32	22.06
TG (16:4_18:2_18:3)	TG (52:9)	C55H88O6	862.6919	+NH ₄ ⁺	24.49	23.26
TG (16:4_18:3_19:2)	TG (53:9)	C56H90O6	876.7076	+NH ₄ ⁺	24.84	23.81

407 RT1/2 = retention time batch 1/2

408

409 **Table S2. Enrichment analysis of lipids from the PCAs first dimension.** Statistical analysis
410 was performed using Fisher's exact test (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).

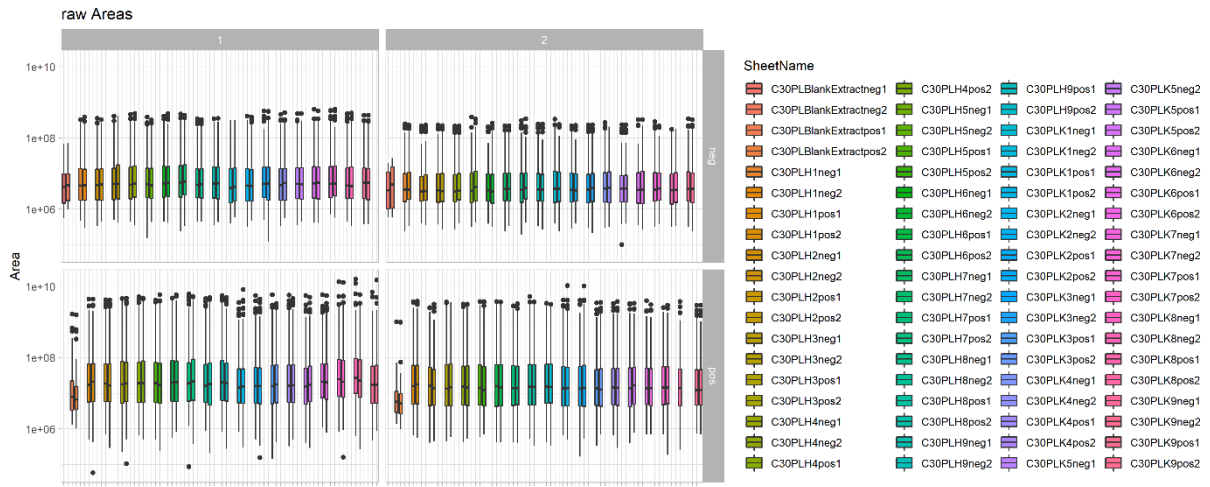
Class	Total species number	Species number contributing to separation of conditions	Accumulation	Polarity	p-value
AcHexSiE	19	0	Reduced	Positive	0.032*
DGDG	27	0	Reduced	Positive	0.008**
PC	47	3	Reduced	Positive	0.029*
TG	168	48	Enriched	Positive	0.00010***

411

412

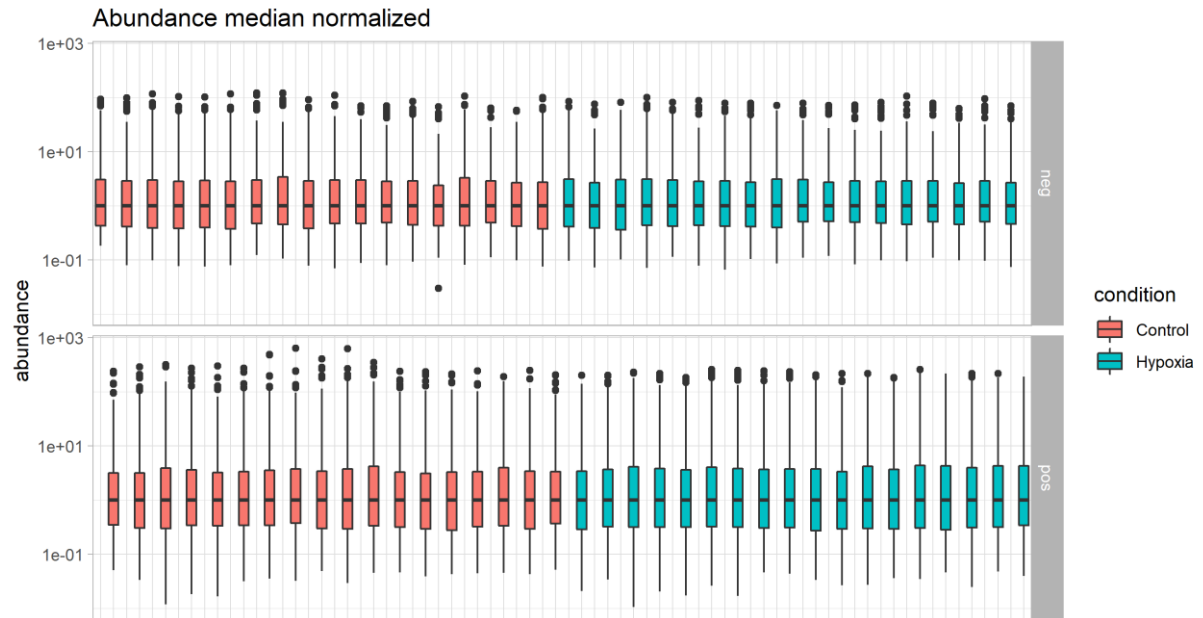
413 **Figures**

414



415

416



417

418

419 **Figure S1.** Acquired raw data plotted from all samples (A). Median-normalized data (B). X-axis
420 (represents each individual sample) was removed for simplicity.

421

422 **Declarations**

423 **Abbreviations**

424 List of abbreviations

AcHexSiE	Acyl hexosyl sitosterol ester
DG	Diacylglyceride
DGAT	Diacylglycerol acyltransferase
DGDG	Digalactosyldiacylglycerol
DGDGS	digalactosyldiacylglycerol synthase
FFA	Free fatty acid
G3P	Glycerol-3-phosphate
GPAT	Glycerol-3-phosphate acyltransferase
LPA	Lysophosphatidic acid
LPAAT	2-lysophosphatidic acid acyltransferase
MGDG	Monogalactosyldiacylglycerol
MGDGS	Monogalactosyldiacylglycerol transferase
PA	Phosphatidic acid
PC	Phosphatidylcholine
PP	Phosphatidate phosphatase
PDAT	Phospholipid:diacylglycerol acyltransferase
PE	Phosphatidylethanolamine
TG	Triacylglycerol
TGDG	Trigalactosyldiacylglycerol

425 **Acknowledgments**

426 The authors acknowledge technical support from Felix Niessner (INP, Greifswald,
427 Germany). We thank Vida Safavi-Rizi (Institute of Botany & Landscape Ecology, Greifswald,
428 Germany) for support with the transcriptomic data.

429 **Funding**

430 This work was supported by the German Research Foundation (DFG) by the Research
431 Training Group (RTG) 1947, and the German Federal Ministry of Education and Research
432 (BMBF; grant numbers 03Z22DN12 to KW and 03Z22Di1 to SB).

433 **Authors contributions**

434 J.S. and M.W. planned the study. J.S. and M.W. carried out the experiments. J.S. and L.M.B.
435 analyzed the data. K.W., S.B. and C.S. supervised the work. M.W., J.S., L.M.B, K.W, C.S. and
436 S.B. wrote the manuscript.

437 **Data availability**

438 The underlying data of this manuscript were deposited at
439 www.metabolomicsworkbench.org under study ID ST002880.

440 **Conflict of interest**

441 The authors declare no conflict of interest.

442 **Ethics approval and consent to participate**

443 Not applicable

444 **Consent for publication**

445 Not applicable

446

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6. Chapter III: A conserved, buried cysteine near the P-site is accessible to cysteine modifications and increases ROS stability in the P-type plasma membrane H⁺-ATPase

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Biochemical Journal, 478 (3), 619-632. <https://doi.org/10.1042/BCJ20200559>

Research Article

A conserved, buried cysteine near the P-site is accessible to cysteine modifications and increases ROS stability in the P-type plasma membrane H⁺-ATPase

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Sulfur-containing amino acid residues function in antioxidative responses, which can be induced by the reactive oxygen species generated by excessive copper and hydrogen peroxide. In all Na⁺/K⁺, Ca²⁺, and H⁺ pumping P-type ATPases, a cysteine residue is present two residues upstream of the essential aspartate residue, which is obligatorily phosphorylated in each catalytic cycle. Despite its conservation, the function of this cysteine residue was hitherto unknown. In this study, we analyzed the function of the corresponding cysteine residue (Cys-327) in the autoinhibited plasma membrane H⁺-ATPase isoform 2 (AHA2) from *Arabidopsis thaliana* by mutagenesis and heterologous expression in a yeast host. Enzyme kinetics of alanine, serine, and leucine substitutions were identical with those of the wild-type pump but the sensitivity of the mutant pumps was increased towards copper and hydrogen peroxide. Peptide identification and sequencing by mass spectrometry demonstrated that Cys-327 was prone to oxidation. These data suggest that Cys-327 functions as a protective residue in the plasma membrane H⁺-ATPase, and possibly in other P-type ATPases as well.

Introduction

All organisms have evolved complex antioxidant defenses that minimize oxidative damage. In proteins, the sulfur-containing amino acids cysteine and methionine are particularly sensitive to oxidation. These amino acids interact with heavy metals. Cysteine residues are involved in metal detoxification reactions [1] and coordination of metals during transport [2].

P-type ATPases constitute a superfamily of ion pumps that are phosphorylated on a conserved aspartate residue during each catalytic cycle. They are divided into five subfamilies, P1–P5, based on phylogeny and substrate specificity. Well-characterized members of this family are sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA) and the Na⁺/K⁺-ATPase, which both belong to the P2 ATPase subfamily, and plasma membrane H⁺-ATPase, which belongs to the P3 ATPase subfamily. Various P2 ATPases are sensitive to oxidative stress [3], and heavy metals have been reported to inhibit the mussel SERCA, the rat Na⁺/K⁺-ATPase, and the plant plasma membrane H⁺-ATPase [4–6]. The mechanism is unknown but it has been suggested that sulfhydryl groups are involved and that interference with Mg²⁺ binding in the phosphorylation (P) domain may play a role [5].

Received: 14 July 2020
Revised: 16 December 2020
Accepted: 8 January 2021

Accepted Manuscript online:
11 January 2021
Version of Record published:
12 February 2021

P-type ATPases all have three cytosolic domains: a nucleotide-binding domain (N-domain), a phosphorylation domain (P-domain), and an in-built de-phosphorylation domain called the actuator domain (A-domain). In the P-domain, two residues upstream of the phosphorylated aspartate residue and close to the Mg^{2+} -binding site, is a cysteine residue that is conserved in all P2 and P3 ATPases. The high conservation of this cysteine residue and its close proximity to functionally important residues have led to several investigations of its role. So far, however, its function in the protein has remained elusive. Hydroxyl radicals inhibit SERCA activity in rabbit, and the corresponding conserved cysteine residue in SERCA (Cys-349) was suggested to be involved in the oxidative response [7]. Cys-349 is also the most sensitive cysteine in SERCA to nitrosylation [8,9], and it showed a high reactivity toward amino acid peroxides [10]. The role of these modifications is unknown. Mutagenesis studies of the yeast plasma membrane H^+ -ATPase Pma1p revealed that substituting alanine for the conserved cysteine did not alter expression level and led to a 7% decrease in ATPase activity. When seven out of the nine cysteines in the Pma1p protein were replaced with alanine, no significant decrease in ATP hydrolysis and proton pumping was observed [11]. However, the substitution of the conserved cysteine with leucine was lethal for yeast. It was proposed that the conserved cysteine has an important structural role rather than a function related to hydrolytic activity [6]. Similar observations were made for rabbit SERCA. When Cys-349 was replaced by alanine, there was no significant change in the rate of ATP-dependent Ca^{2+} transport [12]. In Na^+/K^+ ATPase, the corresponding Cys-376-Ser mutant was wild type with respect to ouabain sensitivity [13]. Substitutions at every cysteine residue in a sheep Na^+/K^+ ATPase caused no changes in ATP binding affinity [14]. Thus, for a variety of P-type ATPases, the substitution of the conserved cysteine residue seems to have only minor effects on protein activity, and no specific functions for this residue have been postulated. This stands in contrast with the high conservation of this residue in P2 and P3 ATPases, and to descriptions in the literature of cysteine's high oxidative reactivity and ability to interact with heavy metals.

The role of the corresponding cysteine residue in plant plasma membrane H^+ -ATPases has so far not been investigated. In this work, a plasma membrane H^+ -ATPase (AHA2) from the model plant *Arabidopsis thaliana* was expressed in yeast. The conserved cysteine residue, Cys-327, was substituted with several amino acids and the resulting proteins were characterized. Our results point to a role for this residue in oxidative stress tolerance and protection against copper.

Materials and methods

Chemicals

Bacto agar and amino acid-free yeast nitrogen base were obtained from BD Biosciences. All other chemicals used were obtained from MERCK. PageRuler™ pre-stained ladder and Pierce™ 1-step transfer buffer were obtained from Thermo Fisher Scientific. Trypsin was obtained from Promega.

Strains and plasmid

The RS72 (MAT a, adel-100 his4–519 leu2–3,112) yeast strain, expressing native PM H^+ -ATPase *PMA1* under the control by a galactose promoter, was used [15]. *Arabidopsis AHA2* was expressed in a plasmid (pmp1625) controlled by the native *PMA1* promoter [16]. PCR fragments for different substitutions were obtained using the primers listed in Supplementary Table S1. Single point mutations were introduced by homologous recombination. Therefore, plasmid and DNA fragment, containing the mutation of interest, were co-transformed in the yeast strain. Yeast cells were transformed with lithium acetate and polyethyleneglycol [17]. All mutants were verified by sequencing.

Cultivation and growth

Yeast cells were transformed, grown, and harvested essentially as described by Villalba et al. [18]. For microsome preparation, transformed yeast was cultured in 2% (w/v) D-galactose selection medium (0.7% (w/v) amino acid-free yeast nitrogen base including adenine (0.4 mg/ml) and L-histidine (0.3 mg/ml)) for 2 days at 30°C with shaking. Then, the cells were diluted 1 : 10 and cultured in 2% (w/v) D-glucose full medium (2% (w/v) bacto-peptone and 1% (w/v) yeast extract) for 20 h at 30°C with shaking. Yeast complementation assays were performed on 2% (w/v) D-glucose selection medium plates with the indicated pH. Then, 3 μ l of OD₆₀₀ 0.1, 0.01, and 0.001 yeast suspension was added to the plates and cultured for 2 days at 30°C [19]. Transformants were cultivated on 2% (w/v) D-galactose selection medium plates pH 5.5.

Membrane preparation

Microsomes were prepared in accordance with Pedersen et al. [20,21]. Briefly, yeast cells were harvested and homogenized at 4°C using glass beads. After centrifugation at 10 000g for 10 min at 4°C, microsomes were obtained from supernatant using ultra-centrifugation. The method from Pedersen et al. was altered as follows: the cells were homogenized in 100 mM MOPS (pH 7.5), 30% (v/v) glycerol, 10 mM EDTA, 40 mM KCl, and 1 mM PMSF. The obtained microsomes were washed and resuspended in 100 mM MOPS (pH 6.5), 20% (v/v) glycerol, 40 mM KCl, and 1 mM PMSF. The samples were frozen in liquid nitrogen and stored at –80°C [22].

ATPase activity

ATP hydrolysis was detected according to Baginski et al. [23], following the modifications by Wielandt et al. [24]. The assay was carried out at 30°C in 20 mM MOPS, 5 mM NaN₃, 0.25 mM Na₂MoO₄, 25 mM KNO₃, 5 mM ATP, and 10 mM MgSO₄ at pH 6.5. Buffer and 0.5 µg protein were mixed and incubated for 45 min at 30°C. Then, equal volume of stop solution (93 mM ascorbic acid, 0.273 M HCl, 0.059% (w/v) sodium dodecyl-sulfate, and 5 mM (NH₄)₂MoO₄) was added. 30 min after stop solution was added, two times volume of arsenite solution (154 mM NaAsO₂, 68 mM trisodium citrate, and 350 mM acetic acid) was added [19]. Absorbance was measured at 860 nm. To determine ATP hydrolysis at different pH values, buffers with pH 4.0, 4.33, 4.87, 5.32, 5.86, 6.26, 6.48, 6.70, 7.12, 7.44, 7.75, and 8.0 were used. To establish the ATP concentration dependence of ATPase activity, the hydrolytic activity was measured using 8 ATP concentrations ranging from 0–1.25 mM. All experiments were performed with at least three biological replicates ± SEM.

AHA2 inhibition

The effect of CuSO₄, CdSO₄, ZnSO₄, HgCl₂, Na₃VO₄, H₂O₂, and ONOO[–] on ATPase activity was tested by ATP hydrolysis. Proteins were mixed with inhibitors and incubated for 15 min at room temperature. Then, the assay was started by the addition of 3 mM ATP and 10 mM MgSO₄ in 20 mM MOPS (pH 6.5) including 5 mM NaN₃, 0.25 mM Na₂MoO₄, and 25 mM KNO₃ (final concentrations) and incubated for 60 min at 30°C. Whereas ONOO[–] was dissolved and diluted in 0.01 M NaOH, the other compounds were dissolved and diluted in H₂O.

Protein reconstitution and proton pumping

Microsomes were reconstituted in soybean (*Glycine max*) lipids [25,26] at a protein/lipid ratio of 1 : 22. The proton pumping activity was measured using 9-amino-6-chloro-2-methoxy-acridin (ACMA) in 50 mM MOPS (pH 6.5), 50 mM K₂SO₄, and 10% (v/v) glycerol (29, modified after 39). The reaction was started by adding 5 mM MgSO₄ and the proton gradient was disturbed by adding 0.5 µM nigericin after 3.5 min.

S-alkylation of protein samples

For iodoacetamide (IAA) alkylation, microsomes were reduced with 10.5 mM DTT in 100 mM ammonium bicarbonate at pH 8.55 for 1 h at 55°C. Afterwards, 18 mM of IAA was added and the sample was incubated for 30 min at RT. After reduction with 10 mM DTT, the alkylation with N-ethylmaleimide (NEM) was carried out in 100 mM HEPES at pH 8, 1 mM EDTA and 0.1 mM neocuproin (HEN buffer) containing 10 mM N-ethylmaleimide and 0.1% (v/v) Triton X100 for 1 h. The samples were washed two times in HEN buffer, centrifuged at 100 000g for 1 h at 4°C and resuspended in HEN buffer. For oxidation detection, native proteins were run on a gel without former treatment.

In gel digestion of protein and sample preparation for mass spectrometry analysis

For IAA alkylated samples and oxidation detection the gels were prepared as described by Shevchenko et al. [27]. In short, excised protein containing gel bands were washed in 50 mM ammonium bicarbonate and then 50% (v/v) acetonitrile. The gel pieces were shrunk in 100% (v/v) acetonitrile and air dried. The washing steps were repeated three times. Gel pieces were reconstituted in 0.025 µg/µl trypsin for 10 min, before the samples were digested in 50 mM ammonium bicarbonate overnight at 37°C. Supernatant was treated in accordance with Gobom et al. [28]. After acidification with 0.25% (v/v) trifluoroacetic acid (TFA) the samples were desalted on a custom-made micro column (plug from an Empore C18 disk (3 M) and 1–2 mm Poros R2 50 µm (Thermo Scientific) in a 10 µl pipet tip). The column was activated and equilibrated in 100% (v/v) acetonitrile and 0.1%

(v/v) TFA, respectively. Following loading of the sample the column was washed twice in 0.1% (v/v) TFA. Peptides were sequentially eluted with 50% (v/v) acetonitrile, 0.1% (v/v) TFA and 70% (v/v) acetonitrile, 0.1% 8 (v/v) TFA, after which the pooled eluate was dried down.

The NEM alkylated samples were prepared as previously described by Bonn et al. [29]. Briefly, gel lanes were fractionated into 10 gel pieces, cut into smaller blocks and transferred into low binding tubes. Samples were and washed until gel blocks were destained. After drying of gel pieces in a vacuum centrifuge, they were covered with trypsin solution. Digestion took place at 37°C overnight before peptides were eluted in water by ultrasonication. The peptide-containing supernatant was transferred into a fresh tube, desiccated in a vacuum centrifuge and peptides were resolubilized in 0.1% (v/v) acetic acid for mass spectrometric analysis.

Liquid chromatography mass spectrometry (LC–MS)

For IAA and oxidation detection, dried samples were dissolve in 0.1% (v/v) formic acid. The column setup was custom-made with a 3.5 cm, 100 µm ID precolumn of Reprosil-Pur 120 C18-AQ, 5 µm (Dr. Maisch) and an 18 cm, 75 µm ID analytical column of Reprosil-Pur 120 C18-AQ, 3 µm (Dr. Maisch). The samples were eluted by a gradient of 3%–45% (v/v) acetonitrile in 0.1% (v/v) formic acid for 45 min followed by a gradient from 45%–95% (v/v) acetonitrile in 0.1% (v/v) formic acid for 5 min. LC–MS/MS analyses was performed on an Q-Exactive HF orbitrap instrument (Thermo Fisher Scientific) using an EASY nLC chromatography system (Thermo Fisher Scientific). MS data was obtained using a top 12 approach, with 120 K mass resolution for MS and 30 K mass resolution for MS/MS, an MS range of *m/z* 350–1600, an isolation window of 1.2 *m/z* unit and dynamic exclusion for 20 s. Data was processed using Proteome Discoverer 2.4, with the search engine being Sequest, (Thermo Fisher Scientific), Xcalibur Qual browser (Thermo Fisher Scientific) and GPMaw (Lighthouse data).

For NEM detection, tryptic peptides were loaded on a self-packed analytical column (OD 360 µm, ID 100 µm, length 20 cm) filled with of Reprosil-Gold 300 C18, 5 µm material (Dr. Maisch) and eluted by a binary nonlinear gradient of 5%–99% (v/v) acetonitrile in 0.1% (v/v) acetic acid over 82 min with a flow rate of 300 nl/min. LC–MS/MS analyses were performed on an LTQ Velos Pro (Thermo Fisher Scientific) using an EASY-nLC II liquid chromatography system. For MS analysis, a full scan in the Orbitrap with a mass resolution of 30 000 was followed by higher-energy collisional dissociation (HCD) of the ten most abundant precursor ions. MS2 experiments were acquired in the linear ion trap. Data was processed using Sorcerer-SEQUEST 4 (Sage-N Research) and Scaffold V4.8.7 (Proteome Software). The MS data were deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD022964.

TCA precipitation and SDS–PAGE

Microsomes were precipitated in 10% (w/v) trichloroacetic acid (TCA) on ice. After centrifugation at 14 000 g for 15 min at 4°C, the sample was dissolved in 5× Laemmli buffer containing 0.3 M TRIS–HCl (pH 6.8), 3.8% (w/v) sodium dodecylsulfate, 50 mM DTT, 13% (w/v) sucrose, 0.25% (w/v) bromphenol blue and 10 mM EDTA. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed on a 10% mini-gel for 2 h at room temperature according to Villalba et al. [18].

Immunoblot

Proteins separated by SDS–PAGE were electrophoretically transferred to a nitrocellulose membrane using Pierce™ 1-step transfer buffer and Pierce™ fast blotter at 25 V for 12 min. The membrane was blocked in 5% (w/v) skim milk powder in 20 mM TRIS–HCl (pH 8.0) and 150 mM NaCl. Primary antibody against the AHA2 C-terminal domain was added. Bands were detected using alkaline phosphatase conjugated to secondary antibody, as described in Villalba et al. [18].

Bradford test

The protein concentration of isolated microsomes was determined using Bradford reagent (0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (v/v) ethanol, and 8.5% (v/v) phosphoric acid) and bovine serum albumin (2–20 µg protein) as standard [30]. The samples were incubated for 15 min and measured at an absorbance of 595 nm.

Table 1 Identified tryptic peptide fragments of AHA2 which were S-alkylated and oxidized following exposure of the native protein to N-ethylmaleimide

191-HPGQEVFSGSTC _{alk} K-203
314-M _{ox} TAIEEM _{ox} AGM _{ox} DVLC _{alk} SDK-330
339-LSVDKNLVEVFC _{alk} K-351

medium. The leucine- and aspartate-substituted proteins did not support growth of the *pma1* yeast strain, but the serine- and alanine-substituted proteins did, indicating that the latter were active proteins (Figure 3).

To further investigate the effects of the substitutions, the mutated genes were expressed in yeast, total microsomes containing the proteins were isolated, and the biochemical properties of the mutant proteins were assayed. In comparison with the wild-type AHA2 pump, the alanine-, serine-, and leucine-substituted proteins had the same V_{\max} and K_M for ATP. Likewise, they had maximum ATPase activity at similar pH (6.2–6.4) and no significant differences in their sensitivity towards the P-type ATPase inhibitor vanadate (Figure 4). Proteins substituted with aspartate or lysine showed low activity, and K_M and V_{\max} could not be determined for them (Supplementary Table S3). Proton transport into lipid vesicles was evaluated for all mutant proteins. Reconstituted protein was added to the cuvette in an amount sufficient to provide the same hydrolytic ATPase activity as 10 μg wild-type protein. Compared with the wild type, the serine- and alanine-substituted mutants had a higher proton pumping rate, measured as a decrease in ACMA fluorescence (Figure 5), indicating that they were able to effectively pump protons.

Thus, the substitution of Cys-327 with the bulky and charged amino acid residues aspartate and lysine severely impacted the function of AHA2, whereas the same residue could be replaced by alanine, serine and leucine without significant loss of function. This indicates that Cys-327 is not essential for the general functionality of the ATPase but rather may have a specialized function.

Substitution of Cys-327 of AHA2 increases Cu^{2+} sensitivity

Cysteine residues interact with heavy metal ions. To test whether Cys-327 is susceptible to heavy metals, the activities of wild-type AHA2 and the alanine and serine substitution mutants were assayed in the presence of various heavy metal ions. Copper (Cu^{2+}), zinc (Zn^{2+}), and cadmium (Cd^{2+}) ions completely inhibited wild-type AHA2 ATPase activity at millimolar concentrations, whereas mercury (Hg^{2+}) ions inhibited this activity in the micromolar range. Hg^{2+} was the most potent inhibitor of wild-type ATPase activity followed by Cu^{2+} , Zn^{2+} , and Cd^{2+} (Figure 6). Among the tested heavy metals, Cu^{2+} had the most divergent effects on the activity of wild-type versus mutant proteins. Both mutants were significantly more sensitive than the wild type to Cu^{2+} inhibition. The IC_{50} for the wild type was 2.1 μM , but was 1.2 μM for the C327S mutant and 1.6 μM for the C327A mutant (Figure 5A and Table 2). Barely any differences were observed between the wild type and the substitution mutants in their sensitivities to Zn^{2+} and Cd^{2+} (Figure 6B,D). In contrast, Hg^{2+} inhibited the ATPase activity of both mutant proteins; however, these mutant proteins were more tolerant to Hg^{2+} than the wild-type protein, and the serine-substituted protein was the more tolerant of the two mutants (Figure 6C). Together, these results show that substitution of Cys-327 with other amino acids increased the sensitivity of the pump to Cu^{2+} but did not have a marked impact on its sensitivity to other heavy metals.

AHA2 Cys-327 mutants show increased sensitivity to reactive oxygen species

Cu^{2+} promotes the formation of reactive oxygen species (ROS), which leads to oxidative stress. Cysteine residues are important redox regulators within cells. To determine the role of C327 in the ROS sensitivity of AHA2, the wild-type and mutant proteins were treated with different ROS. The ATPase activity of wild-type AHA2 was barely affected by hydrogen peroxide (H_2O_2), even at very high concentrations (10 mM) (Figure 7A). In contrast, the alanine and serine substitution mutants were both sensitive to H_2O_2 . At 1 mM H_2O_2 , the alanine substitution mutant was inhibited 28% and the serine substitution mutant 55% relative to the wild type. The wild-type protein and alanine-substituted protein showed an increase in ATP hydrolysis between 0.3 μM and 300 μM H_2O_2 . A complete inhibition of ATP hydrolysis activity was not observed for any genotype.

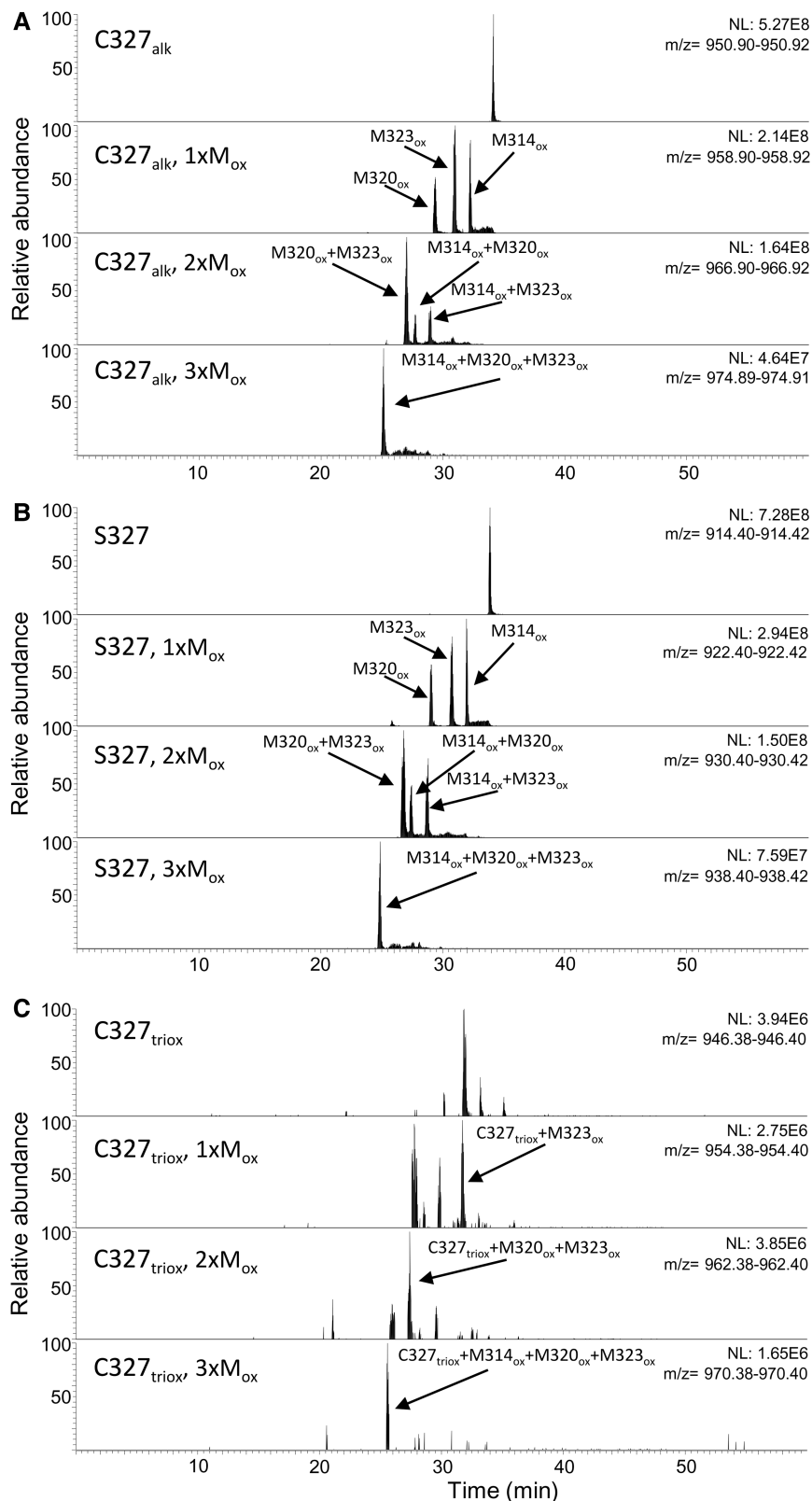


Figure 2. LC-MS/MS analysis of AHA2 wild type and serine mutant accessibility towards S-alkylation by iodoacetamide (IAA) and oxidation.

Part 1 of 2

Shown are the different reconstructed ion chromatograms (RIC) of the tryptic peptide of AHA2 containing the wild-type

Figure 2. LC–MS/MS analysis of AHA2 wild type and serine mutant accessibility towards S-alkylation by iodoacetamide (IAA) and oxidation. Part 2 of 2

Cys-327 or mutant Ser-327 residue. (A) Identified Cys-327 S-alkylation (alk) and Met monooxidations (ox) of AHA2 wild type. (B) Identified Met oxidations of serine mutant. (C) Identified Cys-327 trioxidations (triox) and Met oxidations of untreated AHA2 wild type. M314, M320, M323 are methionine residues in the tryptic peptide. The RIC of C_{triox}, 1xM_{ox} and C_{triox}, 2xM_{ox} may also comprise other combinations involving Cys-327 dioxidation or monooxidation however only the peptide spectrum matches (PSMs) of Cys-327 trioxidation could be verified.

The highly reactive oxidant peroxynitrite (ONOO⁻) completely inhibited the ATPase activity of the wild-type pump (IC₅₀ 0.93 μM) (Figure 7B). Interestingly, both mutants showed a lower IC₅₀ towards ONOO⁻. The serine substitution mutant was the most sensitive to ONOO⁻ (IC₅₀ 0.29 μM) but the alanine substitution mutant also showed increased sensitivity (IC₅₀ 0.59 μM), indicating that the mutant proteins were more susceptible to oxidation. The differences in inhibition between the genotypes were smaller for ONOO⁻ than they were for H₂O₂. Altogether, the substitution of Cys-327 results in a higher ROS sensitivity, especially towards H₂O₂. This idea is supported by the detected oxidations, including cysteine trioxidation, in non-reduced and non-S-alkylated wild-type AHA2 samples (Figure 2C).

Discussion

A conserved cysteine is found close to the DKTGT phosphorylation site in many P-type ATPases. All but a few subfamilies of P-type ATPases have a conserved cysteine at this position, the exceptions being P1B-type heavy metal-transporting ATPases and P4-type phospholipid-flipping ATPases (Figure 1B). In this study, we demonstrated that Cys-327 in the plant P3A-type plasma membrane H⁺-ATPase AHA2 seems to act as a protectant for the pump from Cu²⁺ and H₂O₂, both of which contribute to ROS generation in cells.

Plasma membrane H⁺-ATPases are the powerhouses of plant growth by energizing the cell membrane [32]. These ATPases play an important role under stress conditions, regulate cytosolic pH, and coordinate cell growth. Recently, there has been increased focus on the importance of cysteine and its interplay with ROS sensing and stress responses in plants. External H₂O₂ is sensed by a leucine-rich repeat receptor kinase via modification of a cysteine residue [33]. Inside cells, other reactive species, such as nitric oxide (NO) and peroxynitrite, can oxidize the sulfhydryl group of cysteine [3,34]. Recent studies indicate that plasma membrane H⁺-ATPase is involved in NO-mediated auxin action in cell growth [35,36]. Additionally, NO seems to promote H₂-induced adventitious root formation in cucumber (*Cucumis sativus*) by regulating the expression

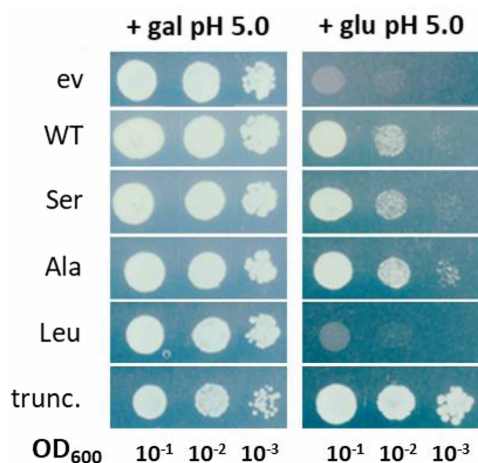


Figure 3. Yeast complementation assay.

Different cysteine substitution mutants of AHA2 were tested for their functionality in a yeast system background. Yeast cultures were diluted (10⁻¹–10⁻³) and plated on D-galactose (+gal) medium as the control and on D-glucose (+glu) medium to assess the function of the AHA2 pump substitution mutants via growth. Empty vector (ev) served as the negative control and AHA2 Δ92 with a truncated C-terminus (trunc.) served as the positive control.

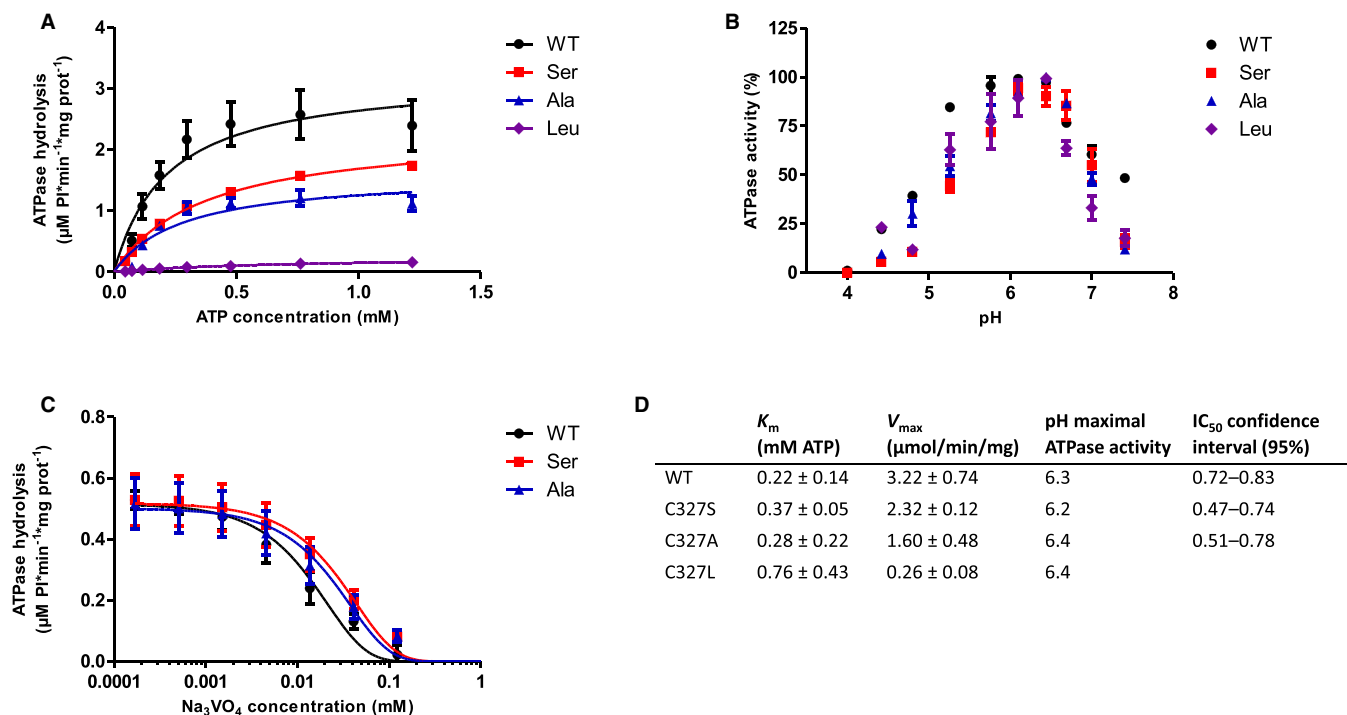


Figure 4. Kinetic analysis of AHA2 Cys-327 substitution mutants.

(A) ATP hydrolytic activity as a function of ATP concentration. (B) ATP hydrolytic activity as a function of pH. (C) ATP hydrolytic activity as a function of vanadate. (D) Kinetic parameters derived from the results in A, B, and C. Shown is two times SEM ($n = 3$).

and interaction of plasma membrane H^+ -ATPase and 14-3-3 protein [37]. Despite these findings, little is known about the effect of redox-active agents on the plant plasma membrane H^+ -ATPase.

Cys-327 is not essential for AHA2 activity and can be substituted with different amino acids without loss of function. Substitution with residues similar to cysteine in size and polarity resulted in functional AHA2 proteins (Figure 4). This finding is in agreement with the results of previous studies in which the corresponding residue was mutagenized in P2-type Na^+/K^+ and Ca^{2+} transporting ATPases [6,11,12,14]. Non-conservative substitutions at this position might result in folding problems and retention of the protein in the endoplasmic reticulum. Such a phenotype has been observed in the *S. cerevisiae* Pma1p C376L mutant, in which the mutated protein accumulates in internal structures of the yeast cell [38]. The ATP affinity and vanadate

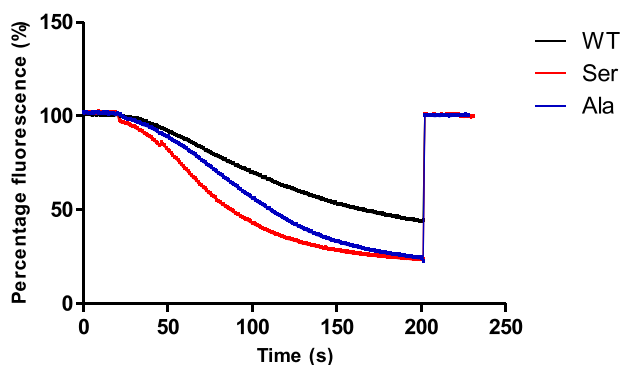


Figure 5. Proton pumping activity of AHA2 cysteine substitution mutants.

Pumping activity was measured over fluorescence quenching of ACMA upon pumping. Prior to measurement, same ATP hydrolytic activity ($0.88 \mu\text{M } P_i \text{ min}^{-1} \cdot \text{mg protein}^{-1}$) was adjusted for the tested genotypes. The assay was started by the addition of ATP and determined using nigericin. Shown is mean ($n = 3$).

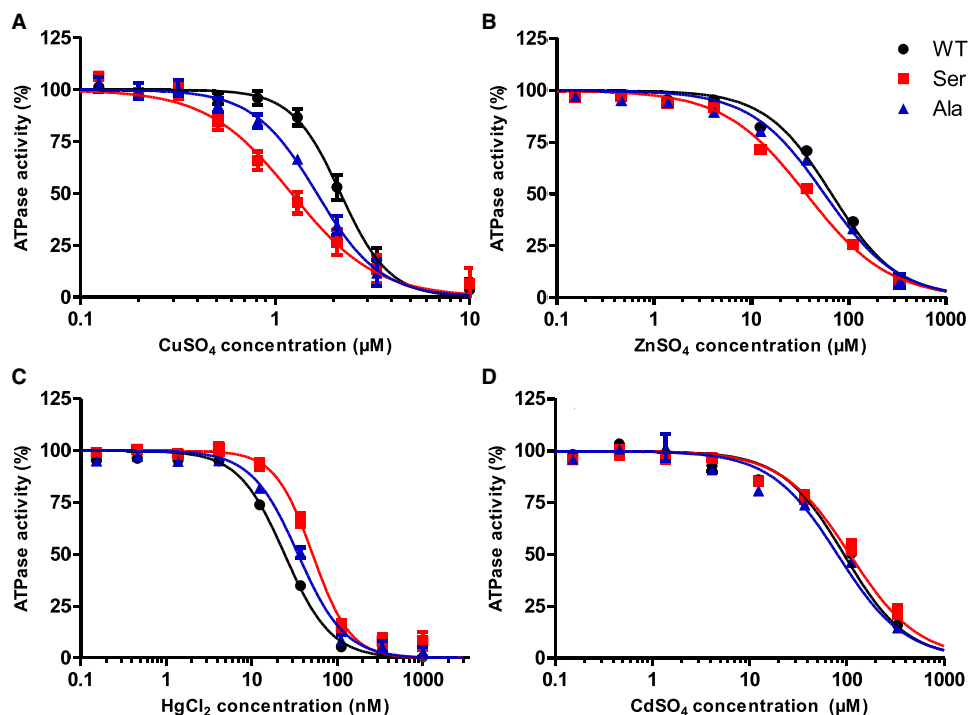


Figure 6. Heavy metal sensitivity of AHA2 substitution mutants.

The sensitivity of the cysteine mutants towards (A) CuSO_4 , (B) ZnSO_4 , (C) HgCl_2 , and (D) CdSO_4 was assessed by quantifying their inhibition on ATP hydrolysis at pH 6.5 and 5 mM ATP. The concentrations of heavy metals were as indicated. Shown is SEM ($n = 3$).

sensitivity of AHA2 were not altered in the tested substitution mutant (Figure 4C), which indicates that binding and hydrolysis of ATP remain unaffected.

Various heavy metals had distinct effects on the Cys-327 substitution mutants. Both substitution mutants tested had an increased sensitivity to Cu^{2+} inhibition, whereas they had decreased sensitivity to Hg^{2+} compared with the wild type. The interactions of proteins with Cu^{2+} and Hg^{2+} have been linked to sulfhydryl residues [5,39]. The wild-type and substitution mutant proteins did not vary significantly in their sensitivity to Cd^{2+} and Zn^{2+} . The observed inhibitory concentrations of heavy metals are in accordance with the heavy metal sensitivities previously reported for plasma membrane H^+ -ATPases [39,40]. In heavy metal-pumping P1B-type ATPases, the heavy metal-binding motif CPX and metal-binding domain CXXC both rely on cysteine to coordinate heavy metals [2,41]. P1B-ATPases might not need a protective cysteine at the active site as the transport site and regulatory domains serve as competing sinks for heavy metals.

Table 2 Inhibition of ATP hydrolysis by different heavy metal

Heavy metal	IC ₅₀ confidential interval (95%)		
	WT	C327S	C327A
CuSO_4 (μM)	2.04–2.31	1.09–1.35	1.51–1.78
ZnSO_4 (μM)	58.41–74.46	32.44–40.47	47.85–64.83
HgCl_2 (nM)	21.86–26.73	45.96–57.29	30.83–39.09
CdSO_4 (μM)	79.59–111.7	92.59–126.8	66.00–98.98
H_2O_2 (mM)	0.156–183.2	0.002–0.009	0.033–0.094
ONOO^- (mM)	0.65–1.33	0.48–0.72	0.22–0.37

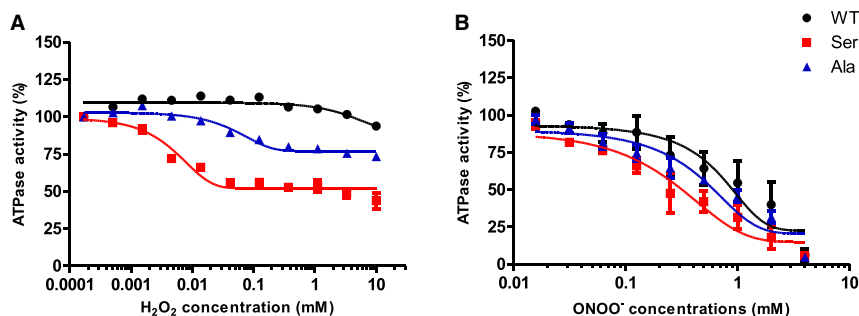


Figure 7. ROS sensitivity of AHA2 C327S and C327A substitution mutants.

The sensitivity of the cysteine mutants towards (A) H₂O₂ and (B) ONOO⁻ was assessed by quantifying their inhibition on ATP hydrolysis at pH 6.5 and 5 mM ATP. The concentrations of ROS were as indicated. Shown is SEM ($n = 3$).

Cu²⁺ is an essential micronutrient for plants, particularly for photosynthesis [42,43]. Because Cu²⁺ can readily gain and lose an electron, it is a cofactor for many oxidases (e.g. amine oxidases, ammonia monooxidase, ceruloplasmin, and lysyl oxidase) and for enzymes reacting with superoxide radicals (e.g. superoxide dismutase and ascorbate oxidase). *In planta* however, excess Cu²⁺ is highly toxic to plants as it catalyzes Fenton-like reactions, which generate hydroxyl radicals, leading to ROS accumulation and oxidative stress [44–46]. *In vitro*, the autoxidation of heavy metal had been shown to have similar effect [47,48]. This increase in ROS leads to changes in the activity of many enzymes involved in antioxidative pathways [46]. Additionally, Cu²⁺ ions from cuprous complexes with thiols, inhibiting protein activity [40]. The plasma membrane H⁺-ATPase can now be added to the list of Cu²⁺-sensitive enzymes.

ROS interact with sulfhydryl residues. The serine and alanine substitution mutants of AHA2 Cys-327 were both inhibited at relatively low H₂O₂ concentrations compared with the wild type; even at 100 μM H₂O₂, wild-type ATP hydrolysis was not affected. Dremina et al. [10] showed that wild-type SERCA activity was also not inhibited by 100 μM H₂O₂. In AHA2, the substitution mutants were inhibited at lower concentrations of ONOO⁻ than the wild type, which was completely inhibited at 4 mM ONOO⁻. The same concentration of ONOO⁻ also completely inhibited SERCA activity [49]. H₂O₂ preferentially reacts with cysteine residues, while ONOO⁻ also favors reactions with tyrosine, tryptophan, phenylalanine, and methionine [50,51]. ONOO⁻ may react with several amino acids and, therefore, may inhibit ATPase activity via a different mechanism than H₂O₂. In SERCA, ONOO⁻ inhibits ATPase hydrolysis via tyrosine nitration and thiol oxidation [49].

In structural models of P-type ATPases, Cys-327 in AHA2 (PDB entry 5KSD) is buried in the P-domain and is not exposed on the surface of the protein (Figure 1A). However, Cys-327 is accessible to external reagents as it could be alkylated by NEM and IAA (Table 1 and Figure 2), in analogy to what has been shown for the corresponding cysteine in a P2A-type ATPase [52]. In addition, Cys-327 has been shown to be prone to oxidation (-SO, -SO₂, -SO₃) even without treatment (Figure 2). Several studies have reported that in native P2A- and P2C-type ATPases the corresponding cysteine residue is modified following exposure to not only reactive oxygen but also nitrogen species [3,8–10] and fatty acids [53]. Taken together, corresponding cysteine residues in P-type ATPases are reachable by several molecules and Cys-327 in AHA2 is most likely accessible to ROS. A cysteine residue directly exposed on the protein surface could be subjected to rapid thiolation and other unwanted reactions, whereas burying a cysteine residue may increase the likelihood of specific interactions [54]. Further inspection of the location of Cys-327 within the structure revealed that its side chain protrudes into an internal cavity that would accommodate a bound Cu²⁺ ion coordinated by the side chains of Cys-327, Met-585, and His-581. While we were not able to observe actual binding of Cu²⁺ to this site, we note that disruption of the potential Cu²⁺-binding pocket by mutagenesis of Cys-329 resulted in increased sensitivity to Cu²⁺ as well as to other agents causing oxidative stress. This increased sensitivity suggests that the site acts to sequester Cu²⁺ and thereby confers protection against ROS. A similar role for a buried Cu²⁺-binding site has been reported for the heat shock protein αB-crystallin [55].

Endogenous thiol residues provide antioxidative protection in proteins. For example, cysteine and methionine residues account for 40%–80% of the antioxidant capacity from human serum albumin (HSA) [56]. Mutagenesis studies revealed that Cys-34 was responsible for 68% of HSA antioxidative activity [57]. Similar to

buried methionine in glutamine synthase, the corresponding conserved cysteine in SERCA had been proposed to function as endogenous antioxidant [3,58]. If AHA2 Cys-327 functions as a protective residue, a substitution mutant should be more sensitive to oxidative inactivation. The results presented in this work are in accordance with this hypothesis; we provide experimental evidence that the cysteine at position 327 in AHA2 could act in a similar way. This protective property could be of importance under stress conditions that are accompanied by increased production of ROS. As a result, other residues around the phosphorylated reaction cycle intermediate, including the Mg²⁺-binding site of the protein, may be protected. The present study supports the previous proposition [58,59] that sulfur-containing amino acids may be key players in metal-chelating and redox-cycling activities that scavenge free radicals. The findings additionally provide the first evidence for redox regulation of plasma membrane H⁺-ATPases and suggest a similar function for the corresponding cysteine in other P-type ATPases.

Competing Interests

The authors declare no conflict of interest.

Funding

This work was supported by the German Research Foundation (RTG 1947: grant no. 231396381) (M.W.). Work in the laboratory of M.P. is supported by the Danish National Research Foundation (PumpKin), the Innovation Fund Denmark (LESSISMORE), the Carlsberg Foundation (RaisingQuinoa; CF18-1113), and the Novo Nordisk Foundation (NovoCrops; 2019OC53580). Proteomics and mass spectrometry research at the laboratory of O.N.J. was supported by generous grants of VILLUM Centre for Bioanalytical Sciences (VILLUM Foundation grant no. 7292) and PRO-MS: Danish National Mass Spectrometry Platform for Functional Proteomics (grant no. 5072-00007B).

CRedit Contribution

Marcel Welle: Conceptualization, Investigation, Writing — original draft. **Jesper Torbøl Pedersen:** Conceptualization, Supervision, Writing — original draft. **Tina Ravnsborg:** Methodology. **Maki Hayashi:** Supervision. **Sandra Maaß:** Methodology. **Dörte Becher:** Methodology. **Ole Nørregaard Jensen:** Methodology. **Christine Stöhr:** Writing — original draft. **Michael Palmgren:** Conceptualization, Supervision, Writing — original draft.

Data Availability

All supporting data are included within the main article and its supplementary files. MS data not included in main article are available at ProteomeXchange Consortium PXD022964.

Acknowledgements

This work was supported by the German Research Foundation (DFG) by the Research Training Group (RTG) 1947 (M.W.) and the Danish National Research Foundation project PUMPKIN (M.P.). We thank Sebastian Grund (University of Greifswald) for assistance in sample preparation and Claudia Hirschfeld (University of Greifswald) for her support with MS analysis. Proteomics and mass spectrometry research at SDU are supported by generous grants to the VILLUM Center for Bioanalytical Sciences [VILLUM Foundation grant no. 7292 to O.N.J.] and PRO-MS: Danish National Mass Spectrometry Platform for Functional Proteomics [grant no. 5072-00007B to O.N.J.].

Abbreviations

ACMA, 9-amino-6-chloro-2-methoxy-acridin; AHA2, H⁺-ATPase isoform 2; HSA, human serum albumin; IAA, iodoacetamide; NEM, N-ethylmaleimide; ROS, reactive oxygen species; SERCA, sarco/endoplasmic reticulum Ca²⁺-ATPase; TFA, trifluoroacetic acid.

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7. Synopsis

Plants are constantly adapting to minor and major changes in the environment. Climate change poses a challenge to the resilience of plants as the frequency of extreme weather events increases (Niu *et al.*, 2014). Adaptations are characterized not only by changes in the structural and functional patterns of plants but also by ensuring the functionality of important mechanisms during these extreme events. Providing the adaptations with the required energy and protecting vital functions is a challenging task. The following chapter summarizes the findings of the thesis when securing plant integrity and highlights the importance of redox-protective mechanisms accompanied by altered redox status due to hypoxic stress.

7.1. Maintaining energy levels under hypoxic stress

Hypoxia interrupts oxidative phosphorylation and leads to an energy crisis, which ultimately leads to the collapse of cellular transmembrane gradients and cell death (Felle, 2005). Plants have evolved strategies for anaerobic energy production to ensure cell survival under low-oxygen stress. Best described are sucrose and starch degradation, enhancement of anaerobic glycolysis for ATP production while lactic acid and alcoholic fermentation oxidize NADH to NAD⁺ to ensure electron flow. However, anaerobic glycolysis yields less ATP than mETC (Bailey-Serres and Voeselek, 2008). Therefore, alternative NADH oxidation mechanisms (**Chapter I**), alternative carbon sources (**Chapter II**) and regulation of energy expenditure (**Chapter III**) may be critical to survive low-oxygen conditions.

The alternative anaerobic NADH oxidation via reduction of NO₂⁻ to NO is one mechanism (Stoimenova *et al.*, 2007; Nakamura and Noguchi, 2020). The increased NO emissions under low-oxygen conditions are well documented in various crop species, as well as in entire organs as in cell extracts (Rockel *et al.*, 2002; Gupta *et al.*, 2005; Stöhr and Stremlau, 2006; Hebelstrup *et al.*, 2012; Mugnai *et al.*, 2012; Oliveira *et al.*, 2013). A chemiluminescence-based detection system is presented to measure NO emissions of plant roots *in vivo* under oxygen-limiting conditions in three plant species for the first time (**Chapter I**). The findings support the hypothesis of a general NO emission mechanism playing a role in plant survival under low-oxygen conditions. The importance of NO emissions under low-oxygen conditions is emphasized by the amount of NO emitted by anoxic plants (**Chapter I**). For comparison, it has been assumed that NO₂⁻ reduction is responsible for 25 % and 11.5 % of the total low-oxygen

ATP turnover in rice and barley, respectively (Stoimenova *et al.*, 2007; Nakamura and Noguchi, 2020). A negative correlation has been observed between oxygen concentration and the amount of NO emitted, with the highest NO emissions detected under anoxic conditions (Gupta *et al.*, 2005; Stöhr and Stremlau, 2006; **Chapter I**). It is assumed that the mainly emitted NO originates from mETC origin (Gupta and Igamberdiev, 2011), which was also indicated in tomato roots, while another mechanism cannot be excluded (**Chapter I**). The species studied exhibited oxygen-dependent NO emissions at levels beyond their regulatory purpose (**Chapter I**), which argue for a role in energy supply under low-oxygen stress. Utilization of NO₂⁻ reduction vary among species. In a recent study on wetland plants, two tolerance mechanisms were refined for different species (Nakamura and Noguchi, 2020). The escape strategy is based on NO₂⁻ reduction, shoot elongation and hyponasty and applies for plants that are able to supply high oxygen quantities during adaptation. The quiescence strategy is based on fermentation and growth management when plants have a low-oxygen supply capacity (Nakamura and Noguchi, 2020). The ability to supply oxygen under low-oxygen conditions may be related to adaptive mechanisms such as aerenchyma formation (Nakamura and Noguchi, 2020). All crop species investigated (**Chapter I**) exhibited similar NO emission profiles under low-oxygen stress. Similar to most crops, barley, tobacco and tomato form aerenchyma under low-oxygen stress (Zhang *et al.*, 2015; Purnobasuki *et al.*, 2018; Mignolli *et al.*, 2020) and 48 hours of hypoxia in tomato roots led to higher transcription of aerenchyma-associated genes (Safavi-Rizi *et al.*, 2020). Taken together, it seems likely that the species studied were able to ensure oxygen availability and avoid a quiescence strategy involving NO₂⁻ reduction as an integral part of energy production under low-oxygen conditions.

Besides sucrose and starch degradation, lipids could be used as a carbon source and energy source under low-oxygen stress. *A. thaliana* and *T. salsuginea* have been shown to rely on β -oxidation and the glyoxylate cycle under prolonged hypoxic stress (Hwang *et al.*, 2020). NO₂⁻ reduction ensures the oxidation of NADPH and functions as an alternative electron acceptor in some species (**Chapter I**). Lipid degradation can promote this process (Stoimenova *et al.*, 2007). Lipid composition changes under low-oxygen conditions in two wheat varieties (Xu *et al.*, 2019) which is also shown in submerged tomato roots (**Chapter II**). The lipidome profiles of control and hypoxic tomato plants were distinguishable, and the differences were made by triacylglycerols. The number and abundance of TG species were increased under hypoxia (**Chapter**

II). TG accumulation in vegetative tissues has been reported for several other stressors (Sakaki *et al.*, 1985, Sakaki *et al.*, 1990; Moellering *et al.*, 2010; Gasulla *et al.*, 2013; Tan *et al.*, 2018), and this study (**Chapter II**) suggests hypoxia as an additional stressor. However, neither did HPLC-MS-MS data show a decrease in lipid levels nor did mRNA data indicate an increase in lipid degradation for hypoxic tomato plants (**Chapter II**). Given the prominent role of TGs in lipid metabolism (Fan *et al.*, 2017) lipids stored in lipid bodies and plastoglobules could be utilized during adaption to reoxygenation (León *et al.*, 2021). Due to the low-oxygen metabolic and structural adaptations sufficient energy and oxygen seems still present. Lipids may be only used as energy source after prolonged low-oxygen conditions as a last resource in tomato roots.

The importance of NO as a signaling molecule under hypoxic stress has been emphasized in recent years (Da-Silva and do Amarante, 2022). Examples include the NO-driven destabilization of group VII ERFs (Gibbs *et al.*, 2014a), the oxygen sensors in plants (Gibbs *et al.*, 2011; Licausi *et al.*, 2011) or the involvement of NO in aerenchyma formation (Wany *et al.*, 2017; Wany and Gupta, 2018). NO signaling can act via post- translational protein modification of cysteine residues (Hess *et al.*, 2005). The increased NO emissions under low-oxygen stress (**Chapter I**) make S-nitrosylated proteins an interesting target for a better understanding of the adaptive response. GPS-SNO 1.0, a software predicting S-nitrosylation, predicted tomato LHA1 cysteine 331 as a possible S-nitrosylation site. Cysteine 331 is located in a prominent position near the P-site of tomato plasma membrane P-type H⁺ ATPase (Table 1). The homologous cysteine residue 327 of *Arabidopsis thaliana* autoinhibited H⁺ ATPase isoform 2 (AHA2) is highly conserved in most subgroups of the P-type ATPase across different kingdoms (**Chapter III**) and also a possible S-nitrosylation site (Tabel 1). However, the cysteine residue is apparently not essential for protein function (**Chapter III**). The possible effect of S-nitrosylation on this important pump is investigated by a mutagenesis study in an autoinhibited AHA2 expressed in a yeast system (**Chapter III**). The addition of various NO-donors caused no difference in ATP hydrolysis activity of AHA2 wild type and mutants (Figure 4). This indicates that NO does not affect AHA2 activity via S-nitrosylation. The possible function of S-nitrosylation of the cysteine residue near the P-site is not clear yet. Similarly, the effect of high NO conditions (**Chapter I**) on the PM P-type H⁺ ATPase is still unclear. In contrast, there are reports of NO-induced activation of PM P-type H⁺ ATPases during

root and shoot elongation. Application of NO led to increased abundance of PM P-type H⁺ ATPase and 14-3-3 protein, H⁺ ATPase phosphorylation and interaction with 14-3-3 protein during adventitious root formation (Li *et al.*, 2020). NO also mediated auxin-induced root development including ATPase activation (Kolbert *et al.*, 2008; Zandonadi *et al.*, 2010). PM P-type H⁺ ATPase-driven shoot elongation was observed in anoxic pondweed (Koizumi *et al.*, 2011). However, low-oxygen stress and the resulting lack of energy can lead to reduced proton pumping activity due to low ATP level (Felle, 2005). This is underlined by the observation of membrane depolarization under hypoxia (Wu *et al.*, 2021). In tomato, this could indicate decreased PM P-type H⁺ ATPase activity in the initial phase of low-oxygen adaptation. When energy becomes more available due to the emplacement of adaptations, PM P-type H⁺ ATPase could be involved in root growth under hypoxic stress.

Table 1: Prediction of S-nitrosylation site of LHA1 and AHA2 using GPS-SNO 1.0 (Chuckoo Workgroup)

Name	Accession	Cys-No site identified by GPS-SNO 1.0	Sequence
LHA1	P22180	331	MAGMDVLCSDKTGTL
AHA2	P19456	327	MAGMDVLCSDKTGTL

It is known that the activity of PM P-type H⁺ ATPase decreases with increasing unsaturation and length of fatty acids (reviewed by Morales-Cedillo *et al.*, 2015). However, among free fatty acids, the addition of C18:3 fatty acids resulted in higher ATPase activity (Palmgren *et al.*, 1988). We see the accumulation of unsaturated fatty acids (C18:2, C18:3) in the TG pool with a decrease in unsaturated phospholipid abundance under hypoxia (**Chapter II**). However, the hypoxic regulation of PM P-type H⁺ ATPase via changes in lipid composition requires future investigation. Neither the lipid composition of the PM nor a suitable setting to the test this hypothesis was applied in this thesis.

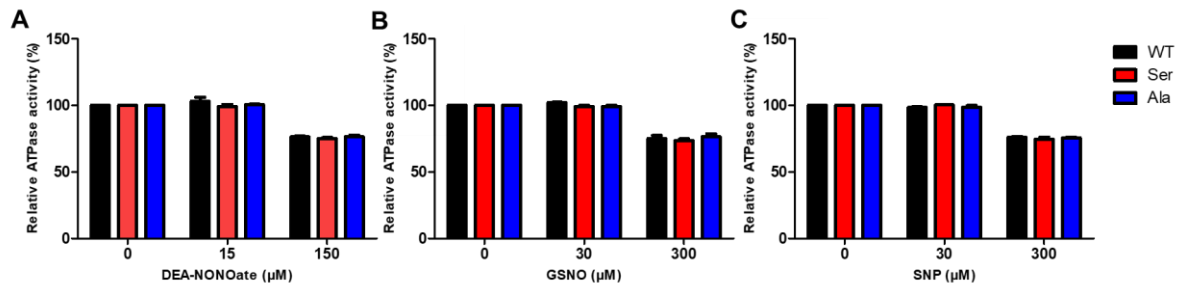


Figure 4: Effect of NO on ATPase activity of AHA2 wild-type and cysteine substitution mutants. Different NO-donors were applied to AHA2 wild-type and cysteine mutants at different concentrations. The sensitivity of the cysteine mutants to (A) DEA-NONOate, (B) GSNO, and (C) SNP was assessed by quantifying their effect on ATP hydrolysis at pH 6.5 with 5 mM ATP. The concentrations of NO-donors were as indicated. Shown is the mean value with SEM ($n = 3$). Abbreviations: WT, AHA2 wild-type; Ser, C327S mutant; Ala, C327A mutant; DEA-NONOate, diethylamine nonoate; GSNO, S - nitrosoglutathione; SNP, sodium nitroprusside.

Taken together (Figure 5), in plants there are general mechanisms for ATP generation under oxygen deficiency. However, the utilization of the different pathways appears to be highly species- and genotype-specific (Xu *et al.*, 2019; Hwang *et al.*, 2020). In tomato roots, NO_2^- reduction appears to be very active (**Chapter I**) and oxidizes NADH produced by anaerobic glycolysis and fermentation driven by starch and sucrose degradation (Safari-Rizi *et al.*, 2020). Changes in membrane lipid composition occur, leading to an increased abundance of TG. Together with the lack of evidence in the transcriptomic data (**Chapter II**), this suggests that the degradation of fatty acids as an energy or carbon source is not increased in tomato under hypoxia. As part of lipid droplets, TGs function as intermediates in lipid metabolism (Fan *et al.*, 2017) which might change with prolonged hypoxia duration. The regulation of one of the most important ATP consumers, the PM P-type H^+ ATPase by S-nitrosylation is still not clear (**Chapter III**). The ability to supply oxygen via aerenchyma (Mignolli *et al.*, 2020) and the usage of NO_3^- as N-source and NO_2^- reduction (**Chapter I**) might indicate a low-oxygen escape strategy, at least for tomato, as has been previously described for wetland species by Nakamura and Noguchi (2020).

7.2. Cellular protective mechanisms – Redox protection

Various abiotic stressors enhance the generation of ROS and RNS in plants (Kapoor *et al.*, 2019), and hypoxic stress is no exception (**Chapter I**, Pucciariello and Perata, 2017). Thereby, the signal molecules NO and H₂O₂ often work in an interplay. This is exemplified by NAD(P)H oxidase activation via NO posttranslational modification which led to an increase in H₂O₂ production (Wany and Gupta, 2018). The generation of ROS and RNS alters the redox homeostasis of the cell and can lead to an increase in oxidative damage to lipids, DNA and proteins (Halliwell and Gutteridge, 1990). However, the negative effects may be more limited than previously thought (Foyer and Noctor; 2005). Antioxidant protection mechanisms maintain cellular functionality at high ROS and RNS concentrations (Kapoor *et al.*, 2019). ROS and RNS are not only harmful, but also act as signaling molecules in the stress-response (Turkan, 2018). For adaptive response, plants need to establish a balance between ROS and RNS signaling and protection against nitro-oxidative stress.

Antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, ascorbate peroxidase, and non-enzymatic molecules such as α -tocopherols, ascorbic acid, glutathione and carotenoids counteract oxidative stress (reviewed by Kapoor *et al.*, 2019). Under hypoxic conditions, hemoglobins are the most important NO scavengers that modulate NO levels (Dordas. *et al.*, 2003; Igamberdiev and Hill, 2004). S-nitrosylation and cytochrome c oxidase are also ways of scavenging NO (Pearce *et al.*, 2002, Gupta *et al.*, 2020). In tomato roots treated with 48 h hypoxia, the gene expression of phytooglobin was increased, while of the total redox-related genes identified, the expression of 22 genes was down-regulated and 25 up-regulated (Safari-Rizi *et al.*, 2020). This underlines the tight regulation of the redox-related protection system.

The increase in triacylglycerol abundance in vegetative tissues reported for hypoxia (**Chapter II**) was also observed under other stressors such as drought, high and low temperatures and nutrient deprivation (de Vries and Ischebeck, 2020). This raises the question of TG synthesis and fatty acid origin. The increase in TG is accompanied by a decrease in PC, LPC, DG and MGDG abundance in hypoxic tomato roots (**Chapter II**). Changes in MGDGs content were also observed under hypoxic stress in *Arabidopsis thaliana* and wheat (Xie *et al.*, 2015; Xu *et al.*, 2019). In addition, C16:3 and C16:4 fatty acids were found in TGs only under hypoxia (**Chapter II**). These fatty

acids have been linked to MGDGs from thylakoid membranes (Barbouch *et al.*, 2012; Vu *et al.*, 2014, Spicher *et al.*, 2016; Shiva *et al.*, 2020) further supporting the MGDG origin of the new TG fatty acids. Similarly, increased TG levels during heat stress are thought to originate from free fatty acids released from MGDG, which is converted to PC by PDAT (Mueller *et al.*, 2017; Higashi *et al.*, 2018). Even though, no photoactive thylakoid membranes are found in roots, galactolipids such as MGDGs and DGDGs are associated with plastidic membranes in general (Solymosi and Schoefs, 2008). A similar mechanism to heat stress seems likely for hypoxia. However, additional studies are needed to confirm TG origin under hypoxic stress. The degradation of MGDG results in the accumulation of toxic lipid intermediates such as free fatty acids (Lu *et al.*, 2020). TGs have a sequestering function for toxic lipid intermediates and thus prevent cell damage and initiation of cell death (He and Ding, 2020). This seems likely to happen under hypoxia as well introducing another protective mechanism under low-oxygen stress.

The degree of unsaturation of fatty acids in membrane lipids plays an important role in maintaining membrane fluidity under various stress factors (He and Ding, 2020). The degree of fatty acid unsaturation increases, and two desaturase genes are induced under hypoxic stress (**Chapter II**). Additionally, C18:3 and C18:2 fatty acids were more abundant in hypoxic TGs (**Chapter II**). Previously an increase in desaturase activity and polyunsaturated linolenoyl-CoA (C18:3-CoA) content was described for *Arabidopsis thaliana* (Xie *et al.*, 2015; Zhou *et al.*, 2020). Hypoxic crown galls in *Arabidopsis* also show an increased level of C18:3 fatty acids (Klinkenberg *et al.*, 2014). The degradation of unsaturated fatty acids from thylakoid membranes appears to be associated with the maintenance of membrane integrity under stress (Higashi *et al.*, 2018). Fatty acids containing double bonds are susceptible to oxidation. This can lead to reactive carbonyl species that contribute to ROS proliferation and result in dysfunctional proteins (Deng *et al.*, 2010). It is known that TGs accumulate in lipid droplets under stress (Lu *et al.*, 2020). The upregulated expression of oleosin 1 in hypoxic tomato roots (**Chapter II**) supports the involvement of lipid droplets in TG storage. A study in *Drosophila* suggests that polyunsaturated fatty acids such as 18:3 fatty acids are less susceptible to oxidative stress when located within the neutral core of lipid droplets than in the membrane (Bailey *et al.*, 2015). Such an antioxidant function has also been suggested in humans (Kuramoto *et al.*, 2012). Nevertheless, the antioxidant function of lipid droplets in plants is still debated. Moreover, lipid droplets

coordinate the stress response by providing a protein binding site (Huang *et al.*, 2018) and the substrates for the biosynthesis of bioactive compounds (Lu *et al.*, 2020). The proposed functions make lipids droplets and TG accumulation under stress an important player for cellular integrity under hypoxic stress.

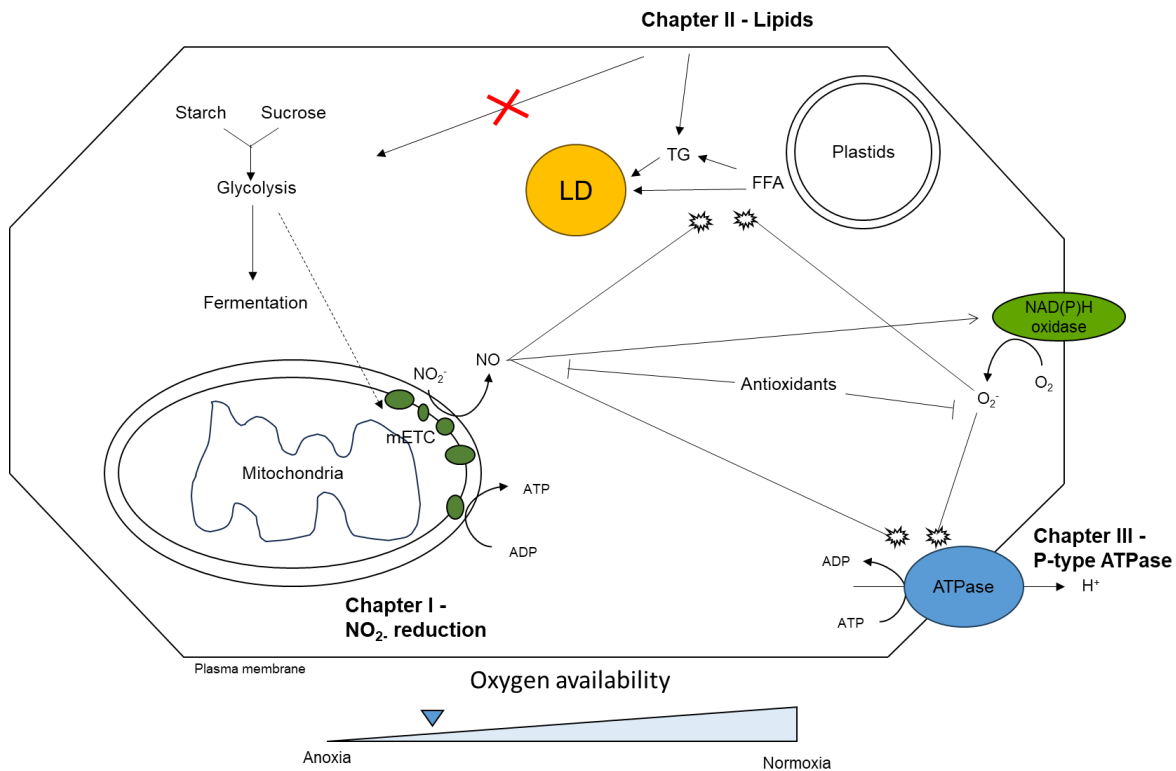


Figure 5: Overview of investigated hypoxic adaptations in tomato plants. The schematic summary reflects the main hypoxic adaptations investigated in this thesis. It shows how tomato plants generate ATP by increasing glycolysis, fermentation and nitrite (NO₂⁻) reduction. Starch and sucrose, but not lipids, serve as energy source for glycolysis. Fermentation and NO₂⁻ reduction recycle reducing equivalents. Additionally, NO₂⁻ reduction at the mitochondrial electron transfer chain (mETC) leads to establishment of a proton gradient for ATP synthesis. Triacylglycerols (TG) accumulate in lipid droplets (LDs) under hypoxic conditions. Plastidic free fatty acids (FFAs) and other lipid species are sequestered in TG or may be integrate in the lipid droplets directly. The low ATP level under hypoxia may inhibit plasma membrane P-type ATPase. Nitric oxide (NO) activates reactive oxygen species producing enzyme NAD(P)H oxidase. The released NO and superoxide (O₂⁻) are partially counteracted by antioxidants. Reactive nitrogen and oxygen species impair protein activity and may damage lipids. The endogenous antioxidant residue of plasma membrane proton P-type ATPase was identified, while lipid droplets may secure unsaturated fatty acids. Filled arrowheads indicate chemical reactions, unfilled arrows indicate induction, and dashed lines with filled arrows provision of reducing equivalents. The partial scavenging of reactive species by antioxidants is shown with blocking lines. Potential oxidative damage is shown by lines with depictions at the end. Cellular compartmentalization of chemical reactions is only schematically indicated.

Proteins are mostly dependent on non-endogenous antioxidant molecules to prevent oxidative damage. Previous studies have shown that methionine residues act as endogenous antioxidants in glutamine synthase (Levine *et al.*, 1996). It has been speculated that cysteine residues, including the conserved AHA2 Cys-327 homolog, of the P-type ATPase of the sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase, may

also function as endogenous antioxidants (Sharov *et al.*, 2006). We provide the first report of a protective cysteine residue for a P-type ATPase. Substitution of the highly conserved cysteine residue 327 in AHA2 led to an increased sensitivity to ROS and copper ions. Mass spectrometry showed that the cysteine residue was accessible and susceptible to oxidation (**Chapter III**). This is supported by several studies on P2A- and P2C-type ATPases showing that the corresponding cysteine residue is modified by RNS, ROS and fatty acids (Viner *et al.*, 1997, Viner *et al.*, 1999; Dremina *et al.*, 2007; Howie *et al.*, 2013). The conserved cysteine of the PM P-type H⁺ ATPase functions as a protein-internal protective residue. This emphasizes the importance of the functionality of PM P-type H⁺ ATPase at elevated ROS concentrations, such as during hypoxic stress conditions. Under salt stress, H₂O₂ is supposed to activate the P-type ATPase (Zhang *et al.*, 2007) which was not observed under hypoxic conditions so far. As apoplastic acidification is needed for hypoxic adaptation via cell elongation (Koizumi *et al.*, 2011) an endogenous antioxidant may help ensure the required ATPase activity also under high RNS and ROS conditions.

Taken together (Figure 5), preventing nitro-oxidative stress and simultaneously allowing ROS and RNS signaling is a challenging task. To mitigate both, the classical enzymatic and non-enzymatic antioxidant systems are induced in tomato (Safari-Rizi *et al.*, 2020). Furthermore, the presented studies show how nitro-oxidative targets such as unsaturated fatty acids (**Chapter II**) and specific proteins (**Chapter III**) can become modulators of RNS and ROS homeostasis and protect themselves.

8. Conclusion

Oxygen depletion is a challenge for the resilience of plants and hinders efficient energy supply. As extreme weather events such as floods and heavy rainfall become more frequent, waterlogging and submergence of plants will become increasingly important in the future. Plants have developed various strategies to overcome oxygen scarcity. The availability of energy is the key to survival and to provide the resources for adaptive responses. This thesis emphasizes the importance of maintaining and protecting cellular functions, which in turn depend on energy.

To obtain energy from anaerobic glycolysis under low-oxygen conditions with an excess of reducing equivalents, plants increase fermentation for its recycling. **Chapter I** has shown that large amounts of NO are released *in vivo* in response to oxygen limitations. NO₂⁻ reduction in mETC appears to be the main source enabling the establishment of an H⁺ gradient and ATP synthesis. Starch and sucrose degradation are the main energy sources, while lipid catabolism does not seem to play a crucial role (**Chapter II**). However, an increase in triacylglycerol indicates storage for later use (**Chapter II**). High NO emissions did not result in posttranslational regulation of PM P-type H⁺-ATPases (**Chapter III**), lead us to assume that plants utilize other strategies to evade oxygen scarcity and increase oxygen availability.

The thesis underlines the importance of maintaining cell functions and protecting them under low-oxygen stress. In addition to the classical antioxidant mechanisms, TG may be used by the species studied to store unsaturated fatty acids in the lipid droplets and thus protect them from an oxidizing environment (**Chapter II**). Membrane integrity is important for the cell, as shown by the observed changes in lipid composition of plastid membranes. TGs also act as scavengers of toxic lipid intermediates. Lipid droplets may protect unsaturated fatty acids and function as the base for stress-related proteins (**Chapter II**). At the protein level, the newly identified endogenous antioxidant of PM P-type H⁺-ATPase (**Chapter III**) emphasizes the importances of protection in adaptation to stress.

For adaptation to low-oxygen stress, plants need both a solid energy base and adequate protection of cellular integrity. As prolonged low-oxygen conditions can threaten crops harvest and plant survival a better understanding of the underlying mechanisms is crucial. Two main survival strategies are described in plants. Plants escape oxygen scarcity by facilitating oxygen flow or reduce growth and stay

quiescent. The frequency of low-oxygen events will increase in the future. But at the same time, we see less and less days responsible for half of the annual rainfall. Short-term, low-oxygen events may cause breeders to shift their efforts to quiescence strategy instead of escape strategy trades in order to obtain optimal plant harvest.

9. References

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10. Author's contributions

Abstract – Solely written by Marcel Welle.

Introduction – Solely written by Marcel Welle.

Chapter I: J. Striesow and **M. Welle** planned the study. J. Striesow and **M. Welle** carried out the experiments. J. Striesow and L. M. Busch analyzed the data. K. Wende, S. Bekeschus and C. Stöhr supervised the work. **M. Welle**, J. Striesow, L. M. Busch, K. Wende, C. Stöhr and S. Bekeschus wrote the manuscript. J. Striesow and **M. Welle** equally contributed as first authors.

Chapter II: **M. Welle** and C. Stöhr planned the study. **M. Welle** and W. Niether carried out the experiments. **M. Welle** and C. Stöhr wrote the manuscript.

Chapter III: **M. Welle**, J.T. Pederson and M. Palmgren planned the study. **M. Welle**, T. Ravensborg, S. Maaß, D. Becher and O. N. Jensen carried out the experiments. J. T. Pederson, M. Hayashi and M. Palmgren supervised the work. **M. Welle**, J. T. Pederson, C. Stöhr and M. Palmgren wrote the manuscript.

Synopsis – Solely written by Marcel Welle.

Conclusions – Solely written by Marcel Welle.

Date

Signature supervisor

Date

Signature doctoral researcher

List of Publications

Welle, M., Pedersen, J. T., Ravnsborg, T., Hayashi, M., Maaß, S., Becher, D., ... & Palmgren, M. (February 2021). A conserved, buried cysteine near the P-site is accessible to cysteine modifications and increases ROS stability in the P-type plasma membrane H⁺-ATPase. *Biochemical Journal*, 478(3), 619-632.

Welle, M., Niether, W., & Stöhr, C. (February 2024). The underestimated role of plant root nitric oxide emission under low-oxygen stress. *Frontiers in Plant Science*, 15, 1290700.

Striesow, J. *, **Welle, M.** *, Busch, L. M., Bekeschus, S., Wende, K. & Stöhr, C (tba). Hypoxia increases triacylglycerol levels and unsaturation in tomato roots. *BMC Plant Biology*, tba.

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Conference Contributions

Poster presentation: Tasdemir, Y., **Welle, M.**, Safavi-Rizi, V., Stöbbe, E- & Stöhr C., Effect of NO and hypoxia on plasma membrane proteome and metabolome of tomato roots, 7th Plant Nitric Oxide International Meeting (2018), Nice, France.

Poster and oral presentation: **Welle, M.**, Pedersen, J. T., Maaß, S., Striesow, J., Biedenweg, D., Tasdemir, Y., Wende K.³, Otto, O., Becher, D., Palmgren, M. G. & Stöhr, C., Hypoxic conditions altered protein expression, cysteine modifications and lipid composition in tomato roots, 1st Baltic Redox Workshop (2020), Greifswald, Germany.

Date

Signature doctoral researcher

11. Acknowledgements

Rückblickend war die Doktorandenzeit sehr schön. Ich habe viel gelernt, gesehen und viele gute neue Leute kennen gelernt. Vielen Dank hierfür.

Mein ganz besonderer Dank geht an Christine für die gute Betreuung während und „zwischen“ der Zeit als Doktorand. Danke für deine Hilfe und Geduld. Ich bin nach wie vor froh, dass du meine meisten Gedanken verstanden hast und mir alle Freiheiten während des Doktors gelassen bzw. mich immer unterstützt hast. Ich komme gerne immer wieder zu den AG-Festen. Da sind wir auch schon bei der AG Pflanzenphysiologie. War sehr schön bei euch. Vielen Dank an Cata für die guten Ideen beim Paper schreiben und die guten Gespräche. Vielen Dank auch an Steffi für die Unterstützung bei der Pflanzenanzucht. Danke Hannah, Andrea und Vida für die schöne Zeit.

Ein großes Dankeschön geht auch an das RTG. Christiane Helm und Christopher Lillig für die gute Organisation. Danke an Uwe Bornscheuer, Oliver Otto, Michael Lalk, Heike Kahlert und Kristian Wende für die gute Kooperation und Hilfe bei meinen Experimenten. Hier geht nochmal ein besonderer Dank an Doreen und die AG Otto welche ich mehrfach mit meiner Protoplastenmessung okkupiert habe. Aber auch an die AG Bornscheuer, AG Lillig, AG Lalk und AG Kahlert. In diesem Zuge auch vielen Dank Sandra Maaß für die Hilfe bei der HPLC und Johanna für die tolle Zusammenarbeit bei unserem „ewigen“ Manuskript.

Was wäre die Doktorandenzeit ohne andere Doktoranden? Ich denke nach wie vor gerne an unsere gemeinsame schöne Zeit zurück. Danke Wini, Danke Faruq, Danke Clara, Danke Flo, Danke Manu, Danke Medi, Danke Christina. War sehr schön mit euch. Danke für ein offenes Ohr und die schönen Abende. Das gleiche gilt auch für die Bornscheuer Clique Sascha, Katja, Simon, Henrik, Aileen und Thomas. Vielen Dank für die tolle Zeit. Ich vermisse die gemeinsamen Abende und unsere sporadischen Städtereisen helfen bedingt.

Ich danke Wini, Sarah, Gesa, Annemarie, Dominik, Katja, Heike und Sascha für Ihr hilfreiches Feedback.

I would like thank Michael Palmgren for hosting me in Copenhagen, Thank you for the great support. Jesper, Jeppe and Maki I am happy that we met and thanks a lot by hosting me in your city and help in the lab. It was a pleasure. Anett, Anton and Emil thanks for the great time. Dominik ein schöner Zufall dich getroffen zu haben. Vielen Dank für dein offenes Ohr und bis bald.

Ganz großer Dank dir Lena für die große Unterstützung in allen Lebenslagen. Ich bin sehr froh euch Drei zu haben. Danke auch an meine Eltern, die Omas und den Opa dafür, dass ihr immer für mich da seid.

Ich danke der DFG, dass sie diese Doktorarbeit im Zuge des RTG 1947 BiOx möglich gemacht hat.