

**Mitochondrial alternative NAD(P)H  
dehydrogenases A1 and A2 control levels of  
reactive oxygen species and improve plant  
survival during reoxygenation**

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# Abbreviation

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## Abbreviation

|       |   |
|-------|---|
| AA    | Antimycin A   |
| ABI4  | ABSCISIC ACID INSENSITIVE 4   |
| ADH1  | ALCOHOL DEHYDROGENASE 1   |
| ANAC  | NO APICAL MERISTEM/ARABIDOPSIS TRANSCRIPTION ACTIVATION<br>FACTOR/CUP-SHAPEDCOTYLEDON |
| AOX   | ALTERNATIVE OXIDASES  |
| AP2   | APETALA2  |
| APX2  | ASCORBATE PEROXIDASE 2  |
| AT    | 3-amino-1,2,4-triazole  |
| CAT   | CATALASE  |
| CDKE  | CYCLIN DEPENDENT KINASE E   |
| cDNA  | Complementary DNA   |
| Cyt c | Cytochrome c  |
| DAB   | 3,3'-diaminobenzidine   |
| DRE   | Dehydration responsive element  |
| EIN3  | ETHYLENE INSENSITIVE 3  |
| EMSA  | Electrophoretic mobility shift assays   |
| EPR   | Electron paramagnetic resonance   |
| ERF   | ETHYLENE RESPONSE FACTOR  |
| ETC   | Electron transport chain  |
| FRET  | Fluorescence resonance energy transfer  |

## Abbreviation

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|                               |   |
|-------------------------------|---|
| GDH                           | GLUTAMATE DEHYDROGENASE                       |
| gDNA                          | Genomic DNA                                   |
| GR1                           | GLUTATHIONE REDUCTASE 1                       |
| H <sub>2</sub> DCF-DA         | 2',7'-dichlorodihydrofluorescein diacetate    |
| H <sub>2</sub> O <sub>2</sub> | Hydrogen peroxide                             |
| HRE                           | HYPOXIA RESPONSIVE ERF                        |
| HRU1                          | HYPOXIA-RESPONSIVE UNIVERSAL STRESS PROTEIN 1 |
| JA                            | Jasmonic acid                                 |
| LB                            | Luria-Bertani                                 |
| LRs                           | Lateral roots                                 |
| MPK                           | Mitogen-activated protein kinase              |
| MS                            | Murashige & Skoog                             |
| MSD1                          | MANGANESE SUPEROXIDE DISMUTASE 1              |
| MV                            | Methyl viologen                               |
| NASC                          | Nottingham Arabidopsis Stock Center           |
| NBT                           | Nitro blue tetrazolium chloride               |
| NDA                           | INTERNAL TYPE II NAD(P)H DEHYDROGENASES A     |
| NERP                          | N-end rule pathway                            |
| NPQ                           | Non-photochemical quenching                   |
| OD                            | Optical density                               |
| PCD                           | Programmed cell death                         |
| PCR                           | Polymerase chain reaction                     |

## Abbreviation

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|          |   |
|----------|---|
| PPDK     | PYRUVATE PHOSPHATE DIKINASE   |
| PS II    | Photosystem II  |
| qRT-PCR  | Quantitative real-time PCR  |
| RAP2     | RELATED TO APETALA 2  |
| RBOHD    | RESPIRATORY BURST OXIDASE HOMOLOG D   |
| RCJ      | Root cap junction   |
| ROS      | Reactive oxygen species   |
| rpm      | Revolutions per minute  |
| RT       | Room temperature  |
| SDS      | Sodium dodecyl sulphate   |
| SOD      | SUPEROXIDE DISMUTASE  |
| TCA      | Tricarboxylic acid  |
| T-DNA    | Transfer DNA  |
| TMTH-HCL | N-(1-Hydroxy-2,2,6,6-tetramethylpiperidin-4-yl)-2-methylpropanamide-<br>Hydrochloride |
| UCP      | Uncoupling proteins   |
| UPLC     | Ultra performance liquid chromatography   |
| UQ       | Ubiquinone  |
| X-Gluc   | 5-Bromo-4-Chloro-3-Indolyl- $\beta$ -D-Glucuronide                                    |
| YEP      | Yeast extract peptone   |



## Zusammenfassung

Für Pflanzen stellen Überflutungen einen in zwei Phasen unterteilten Stress dar. Anfangs kommt es während der Überflutung zu einer Verknappung des molekularen Sauerstoffs, woran sich eine Phase der Reoxygenierung anschließt. Für das Überleben dieser sehr unterschiedlichen Stresse, ebenso wie für die Versorgung mit Energie bei Entwicklungs- oder Wachstumsprozessen, ist es für Pflanzen unerlässlich, die Entstehung von reaktiven Sauerstoffspezies (ROS) zu kontrollieren und ein Gleichgewicht dieser aufrechtzuerhalten. Mitochondrien spielen eine zentrale Rolle bei der zellulären Energieversorgung sowie dem Aufrechterhalten des Redox-Gleichgewichts. Ist genügend Sauerstoff vorhanden, dient die mitochondriale Elektronentransportkette (mitochondrial electron transport chain, mETC) der ATP-Produktion. Da bei einer Verknappung von Sauerstoff eine Übertragung von Elektronen auf Sauerstoff nicht möglich ist, liegen die Komponenten der mETC dann in einem reduzierten Zustand vor. Dies kann bei der Reoxygenierung der Pflanze zu einer verstärkten Bildung von ROS führen. Pflanzliche Mitochondrien verfügen über eine alternative ETC, zu welcher die auf der Matrixseite der inneren Mitochondrienmembran lokalisiert liegenden ALTERNATIVEN NADH DEHYDROGENASEN A1 und A2 (NDA1 und NDA2) gehören. Bei diesen handelt es sich um pflanzenspezifische Proteine, welche Elektronen transportieren ohne einen Protonengradienten zu erzeugen. Zusammen mit der mitochondrialen alternativen Oxidase (AOX) agieren NDAs, um die in NADH gespeicherte Energie in Wärme umzuwandeln. Basierend auf der Hypothese, dass die alternative ETC dazu beiträgt, eine übermäßige ROS-Produktion zu verhindern, untersuchten wir die Rolle der NDAs während der Reoxygenierung nach Sauerstoffmangel in *Arabidopsis thaliana*. Die Ergebnisse zeigen, dass NDAs die Bildung von ROS verringern, da ein *Knockout* der NDAs in *nda1 nda2*-Pflanzen in einer erhöhten ROS-Bildung resultiert und zu einer geringeren Überlebensrate im Vergleich zum Wildtyp führt. Im Gegensatz dazu konnte bei der Überexpression von NDAs eine verringerte ROS-Produktion und eine höhere Überlebensrate beobachtet werden. Dies weist darauf hin, dass der alternativen ETC eine wichtige Funktion für das Überleben der Pflanze bei Reoxygenierung zukommt. Die ATP-Produktion oder das NADH/NAD<sup>+</sup>-Redox-Gleichgewicht unterschieden sich nicht in Wildtyp, *nda1-1 nda2-2*, *NDA1ox3* oder *NDA2ox2* Pflanzen, weder unter anoxischen Bedingungen noch während der Reoxygenierung. Ein geringeres Fv/Fm-Verhältnis im Spross von *nda1-1 nda2-2* Keimlingen im Vergleich zum Wildtyp nach 8 h Reoxygenierung deutet auf

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eine Funktion der mitochondrialen NDAs für die Stabilität der plastidären Photosysteme während der Reoxygenierungsphase hin. Andererseits spielt die Photorespiration keine entscheidende Rolle in der Reoxygenierungsphase, da die Überlebensrate der Keimlinge in 1% CO<sub>2</sub> oder in Luft mit 0,039% CO<sub>2</sub> gleich war. Untersuchungen der *NDA*-Expression zeigten, dass der HYPOXIA-RESPONSIVE ETHYLENE RESPONSE FACTOR 2 (*HRE2*) als Repressor von *NDA1* und *NDA2* fungiert. Die Expression von *NDA1* und *NDA2* war nach 2 h Reoxygenierung im Wildtyp das 5- und 4-fache, in den Linien *erfVII* und *hre2-2* um das 7-fache erhöht. Umgekehrt blieb die Expression von NDAs in *HRE2ox1*- und *HRE2ox5*-Keimlingen auf Kontrollniveau. Es wurde gezeigt, dass die Transkriptionsfaktoren NO APICAL MERISTEM/ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR/CUP-SHAPED COTYLEDON 013 (*ANAC013*), *ANAC016* und *ANAC017* an das mitochondriale dysfunktionale Motiv (MDM) im Promotor von *NDA1* binden und die Expression des Gens aktivieren. Darüber hinaus war die Expression von *NDA1* und *NDA2* in der Linie *anac017* im Vergleich zum Wildtyp während der Reoxygenierung reduziert, was auf eine positive Regulation der NDAs durch *ANAC017* und möglicherweise auch *ANAC013* und *ANAC016* hinweist. Diese Ergebnisse zeigen, dass während Anoxie und Reoxygenierung die Expression von *NDA1* und *NDA2* durch *HRE2* und *ANAC017* antagonistisch reguliert wird. Zusammengefasst wurde in dieser Studie eine zentrale Funktion der mitochondrialen NDAs bei der Regulierung von ROS und dem Überleben der Pflanze während Reoxygenierung gezeigt. Auf der Ebene der Transkription werden NDAs positiv von *ANAC017* und negativ von *HRE2* reguliert. Basierend auf diesen Ergebnissen schlussfolgern wir, dass während der Erholungsphase von geringer Sauerstoffverfügbarkeit das Kontrollieren der mitochondrialen ROS-Konzentration von zentraler Bedeutung für das Überleben der Pflanzen bei Überflutungsstress ist.

### Summary

Flooding is a biphasic stress with an initial reduction of molecular oxygen during submergence followed by reoxygenation post-flooding. Survival of this compound stress and growth vigor of the plant rely on efficient control of reactive oxygen species (ROS) homeostasis. Mitochondria are central to cellular energy supply and redox homeostasis. The mitochondrial electron transport chain (mETC) produces ATP under oxygen-sufficient conditions while under limited oxygen level, the mETC gets reduced as electrons are not transferred to molecular oxygen. Upon reoxygenation of flooded plants, the reductive power can lead to excess ROS formation. Plant mitochondria possess an alternative ETC. The alternative NADH dehydrogenases A1 and A2 (NDA1 and NDA2) are plant-specific mitochondrial non-proton pumping respiratory bypass proteins located at the matrix side of the inner mitochondrial membrane. NDAs act in concert with the mitochondrial alternative oxidase (AOX) to dissipate energy conserved in NADH as heat. Based on the hypothesis that the alternative ETC helps avoid excessive ROS production, we studied the role of NDAs in post-anoxic recovery in *Arabidopsis thaliana*. The results show that NDAs limit ROS formation, and their knockout in *nda1 nda2* plants resulted in elevated ROS levels and a much reduced survival rate compared to wild type while overexpression of NDAs led to reduced ROS and a higher survival rate indicating that the alternative ETC is important for plant survival during post-anoxic recovery. The energy and redox metabolism of the cells, in terms of the ATP production and NADH/NAD<sup>+</sup> redox balance showed no obvious differences during anoxia and reoxygenation in wild type and *nda1-1 nda2-2*, *NDA1ox3* and *NDA2ox2*. A lower Fv/Fm ratio in shoots of *nda1-1 nda2-2* seedlings after 8 h of reoxygenation indicated that NDAs are essential for photosynthetic recovery during reoxygenation. On the other hand, reoxygenation in 1% CO<sub>2</sub> and in ambient air with 0.039% CO<sub>2</sub> resulted in the same survival rate indicating that photorespiration was not a crucial factor. Analysis of *NDA* gene regulation revealed that the hypoxia-responsive ethylene response factor 2 (HRE2) acts as a transcriptional repressor of *NDA1* and *NDA2*. The expression of *NDA1* and *NDA2* increased 5-fold and 4-fold after 2 h of reoxygenation in wild type and 7-fold in *erfVII* and *hre2-2*. Conversely, expression of NDAs remained at control levels in *HRE2ox1* and *HRE2ox5* seedlings. The Arabidopsis transcriptional regulators No apical meristem/Arabidopsis transcription activation factor/ Cup-shaped cotyledon (ANAC013), ANAC016, and ANAC017 were shown to bind to the mitochondrial

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dysfunctional motif (MDM) in the *NDA1* promoter and activated the *NDA1* gene. More so, the *anac017* mutant showed reduced expression of *NDA1* and *NDA2* during reoxygenation in comparison to wild type indicating positive regulation of *NDA*s by *ANAC017* and possibly *ANAC013* and *ANAC016*. The results indicate that HRE2 and *ANAC017* antagonistically regulate expression of *NDA1* and *NDA2* during anoxia and reoxygenation. Taken together, this study revealed a central function of mitochondrial *NDA*s in ROS regulation and plant survival of reoxygenation stress. Based on our findings, we propose that control of mitochondrial ROS levels during recovery from low oxygen stress is central to plant survival of flooding.

## 1 Introduction

### 1.1 Flooding and its impact on global crop production

In recent years, there has been a significant increase in the number of severe weather events like floods, droughts, and extreme temperature due to global warming (Lesk *et al.* 2016). Flooding is defined by excessive amounts of water that harm agricultural production by means of delayed planting, reduced vigor, altered plant development, and increased vulnerability to diseases (Voesenek & Bailey-Serres 2015). As stated by the Food and Agriculture Organization of the United Nations (FAO), almost 65% of global crop loss and damage measured from 2006 to 2016 was caused by flooding. With the number of flooding events expected to increase in the future especially in Southeast Asia, Southern India, Northern parts of South America and East Africa, global food security is under serious threat, particularly in developing countries (Hirabayashi *et al.* 2013). The demand for global agricultural crop production is increasing to meet the requirements of the rising world's population. To meet the needs of the world's population in the future, it is essential to develop strategies to find better-adapted varieties or look for key genes that facilitate better endurance of plants under severe abiotic stress events (Tester & Langridge 2010).

### 1.2 Submergence and post-submergence stress

Flooding encompasses root inundation or waterlogging where underground parts like roots and rhizomes are underwater or partial or complete submergence marked by the condition when aerial tissue is underwater partially or completely. The severity of the flooding stress is dependent on water depth and water conditions like fresh, stagnant, or saline water and the duration of the stress. In nature, submergence events can occur due to an increase in water levels at low elevation due to heavy rainfall or tsunami and can also be due to storm surges (Bailey-Serres *et al.* 2012b). The inundation results in limited availability of gases like oxygen (O<sub>2</sub>) and carbon dioxide (CO<sub>2</sub>) and also affects the soil ecology that might affect nitrogen fixation and nutrient availability to the plant. The aerobic respiration and photosynthesis are also reduced underwater due to the limited availability of O<sub>2</sub>, CO<sub>2</sub> and light. The limitation of O<sub>2</sub> availability, either anoxia (0% O<sub>2</sub>) or hypoxia (O<sub>2</sub> levels below 21%), creates an energy crisis

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inside the cells. Hence, to meet the energy demand of the cell, the submerged plants usually switch to anaerobic respiration or catabolic processes (Zabalza *et al.* 2009). However, ATP production under low oxygen conditions is much lower in comparison to aerobic respiration, but it ensures cell survival as long as carbohydrates are available.

The re-exposure of the plants to normal oxygen conditions after floodwater retreat, termed post-submergence or reoxygenation, imposes more challenges on the plant which may be as severe as or even more severe than the oxygen deficiency itself. Hence, flooding can be regarded as a compound stress as the plant encounters distinct stresses upon submergence and post-submergence (Yeung *et al.* 2019). During submergence, plant tissues adapt to low oxygen and low light in turbid water, while the re-exposure to the atmospheric oxygen after the low-oxygen phase results in post-anoxic injuries specifically due to the excessive formation of reactive oxygen species (ROS) (Rasmusson & Møller 2011; Yeung *et al.* 2018). Prolonged hypoxia or anoxia during submergence leads to a saturated electron transport chain, low adenylate energy charge, and high levels of reducing equivalents which can have a direct effect on intracellular ROS production once oxygen becomes available during reoxygenation (Rasmusson *et al.* 2008).

The reoxygenation restores the aerobic metabolism in plant cells to meet the high energy demand, but the accelerated activity of mitochondrial respiration also stimulates the production of ROS (Monk *et al.* 1987; Biemelt *et al.* 1998; Pavelic *et al.* 2000). ROS are produced inside the plant cell continuously and are a major byproduct of aerobic metabolism. The chloroplast and mitochondria are the primary sites of ROS production in photosynthetically active tissues, predominantly due to their high metabolic activities and increased rate of electron transfer. The PSI and PSII reaction centers are major sites of ROS production in the chloroplast (Asada 2006). The electron transport chain (ETC) is at the central position for mitochondrial energy production, and also it is a primary site for ROS production. Complex I and III of the mitochondrial ETC are major sites for mitochondrial superoxide ( $O_2^{\cdot-}$ ) production. Roughly 2–5% of the total oxygen consumed by mitochondria is converted to  $O_2^{\cdot-}$  (Taylor *et al.* 2009).

## 1.3 Plant survival under low oxygen and reoxygenation

Flood-tolerant plants employ common and different strategies to adapt to submergence. Among the conserved traits is the formation of aerenchyma for better gas exchange, adventitious root development to replace the main root system, and leaf gas film formation for a better gas exchange (Jackson & Armstrong 1999; Mommer *et al.* 2004; Sauter 2013; Steffens & Rasmussen 2016). However, under complete submergence, flood-tolerant plants exploit one of two opposite strategies, namely: the escape strategy or the quiescence strategy (Bailey-Serres & Voeselek 2008). In the escape strategy, plants utilize their carbon and nutrient reserves to support petiole, leaf or internodal elongation to escape from the water and keep the shoot in contact with air. Examples of plant species that use this strategy are deepwater rice (*Oryza sativa* L) and *Rumex palustris* (Métraux & Kende 1983; Jackson & Osborne 1989; Voeselek *et al.* 2006). By contrast, some plant species become dormant upon submergence to minimize their energy expenses and maintain the basic metabolism until the water recedes. Lowland rice varieties, *Rumex crispus* and *Ranunculus repens* are also utilizing this strategy to survive flooding (Voeselek *et al.* 1990; Setter & Laureles 1996).

The post-submergence injuries are mainly associated with excessive production of ROS, that results in oxidation of lipid membranes, proteins, nucleic acids, and carbohydrates (Blokhina *et al.* 2000; Santosa *et al.* 2007). One of the primary survival strategies of plants during recovery from anoxia or hypoxia is to upregulate antioxidant defense mechanisms to minimize the oxidative stress. There have been several reports of different plant species upregulating the antioxidant defense system during reoxygenation. In Arabidopsis, a high level of ascorbate and glutathione levels were observed in the genotypes exhibiting better recovery during reoxygenation (Yuan *et al.* 2017; Yeung *et al.* 2018). In soybean roots and hypocotyl, the enzymatic activities of peroxidases were suppressed during flooding, but were elevated significantly during recovery (Khan *et al.* 2014; Khan & Komatsu 2016).

Plants that are better adapted to submergence and de-submergence also exhibit a better antioxidant defense capacity. *Alternanthera philoxeroides* is a submergence-resistant wetland species that uses the escape strategy during submergence via rapid stem elongation. Upon reoxygenation, *A. philoxeroides* leaves exhibit increased ascorbic acid, catalase (CAT), and superoxide dismutase (SOD) activities for ROS scavenging (Luo *et al.* 2010). The M202(Sub1),

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rice cultivar containing the SUB1A submergence-tolerance gene, displayed reduced ROS accumulation and less lipid peroxidation upon reoxygenation (Fukao *et al.* 2011). The better antioxidant defense or enhanced ROS scavenging upon reoxygenation might also be useful for photosynthesis recovery. Efficient ROS scavenging reduces damage of photosystem II (PS II), which in turn limits the photoinhibition and allows for faster refueling of energy reserves through photosynthetic carbon fixation during recovery. In comparison to the submergence-sensitive Arabidopsis accession Bay-0, the tolerant Arabidopsis accession Lp2-6 exhibited an optimum Fv/Fm ratio within three days after reoxygenation (Yeung *et al.* 2018). The flooding-tolerant M202 (*Sub1*) rice also displayed better maintenance of the non-photochemical quenching (NPQ) and showed faster recovery of the Fv/Fm on reoxygenation than submergence-sensitive M202 (Alpuerto *et al.* 2016).

## 1.4 Low-oxygen and reoxygenation stress signaling in Arabidopsis

The plant's response to low oxygen conditions has been extensively studied in the past and has provided some insight into the molecular mechanisms that help cope with this stress. The low oxygen response in Arabidopsis is regulated by members of the ETHYLENE RESPONSE FACTOR (ERF) family of transcription factors (TFs). The Arabidopsis group VII ERFs include three *RELATED TO AP-2* (*RAP2.2*, *RAP2.3*, and *RAP2.12*) and two *HYPOXIA RESPONSIVE ERF* (*HRE1* and *HRE2*) genes (Francesco *et al.*; Hinz *et al.* 2010; Gasch *et al.* 2016). The ERF-VII TFs have a conserved APETALA2 (AP2) domain for protein-DNA interaction and the N-terminal motif MCGGAI/V for proteasomal degradation through the N-end rule pathway (Gibbs *et al.* 2011; Licausi *et al.* 2011). Under low oxygen conditions, these ERF-VII TFs are stabilized and direct the regulation of downstream anaerobic response genes (Mustroph *et al.* 2009). Apart from Arabidopsis, ERF-VII homologs have also been identified in rice and barley (*Hordeum vulgare*) (Xu *et al.* 2006; Mendiondo *et al.* 2016). The conserved N-terminal motif of ERF-VIIs in different species point to plant-specific oxygen sensing mechanism that is different from that in animals (Sasidharan & Mustroph 2011). Besides oxygen-dependent destabilization of ERF-VII by the N-end rule pathway as an O<sub>2</sub> sensing mechanism, ROS produced from the mitochondrial ETC and from plasma membrane-associated NADPH oxidases may act as an additional signaling mechanism (Pucciariello & Perata 2012). The RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD) has been described to act together with the HYPOXIA-



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RESPONSIVE UNIVERSAL STRESS PROTEIN 1 (HRU1) that is induced by RAP2.12. The data suggest a central mediator role of HRU1 between low O<sub>2</sub> sensing and ROS signaling during hypoxia (Gonzali *et al.* 2015).

The post-submergence response in Arabidopsis is mediated by hormonal cross-talk between ethylene and jasmonic acid (JA). Ethylene signaling during reoxygenation is required for replenishing the tricarboxylic acid (TCA) cycle intermediates (Tsai *et al.* 2014). During reoxygenation, ethylene signaling mediated by ETHYLENE INSENSITIVE3 (EIN3) enhances the expression of GLUTAMATE DEHYDROGENASE (GDH) that catalyzes glutamate into 2-oxoketoglutarate. 2-Oxoketoglutarate reacts with alanine to allow the refueling of pyruvate in the TCA cycle via transaminase reaction. The enzyme PYRUVATE PHOSPHATE DOKINASE (PPDK) is controlled by ethylene during reoxygenation. The PPDK enzyme catalyzes the conversion of phosphoenolpyruvate to pyruvate, which is further converted into oxaloacetate. Overall, ethylene supports TCA cycle replenishment and carbohydrate metabolism during reoxygenation (Tsai *et al.* 2014; Tsai *et al.* 2016). JA signaling in Arabidopsis is involved in maintaining the oxidative stress response. Reoxygenation results in the induction of JA biosynthesis genes and in the accumulation of JA in Arabidopsis rosettes within a few hours. Mutants deficient in JA signaling or biosynthesis exhibited a weak recovery phenotype. Moreover, pre-treatment of JA prior to submergence results in better survival after reoxygenation. The JA-driven reoxygenation response is under the control of MYC2, a basic helix-loop-helix leucine-zipper transcription factor, that promotes the expression of genes involved in ascorbate and glutathione biosynthesis (Yuan *et al.* 2017). In general, the reoxygenation response in Arabidopsis is maintained by fine-tuning between ethylene and JA, and consequently by balancing oxidative stress and energy metabolism (Yeung *et al.* 2019).

## 1.5 Mitochondrial response under anoxia and reoxygenation

Better survival under low oxygen also depends upon enhanced mitochondrial adaptation. Being a primary site of energy production and one of the major organelle exhibiting high redox changes, regulation of mitochondrial metabolism is critical for anoxia survival (Shingaki-wells *et al.* 2014). In recent years, the understanding of low-oxygen survival has increased considerably. However, the understanding of mitochondrial metabolism under anoxia and reoxygenation is still not clear.

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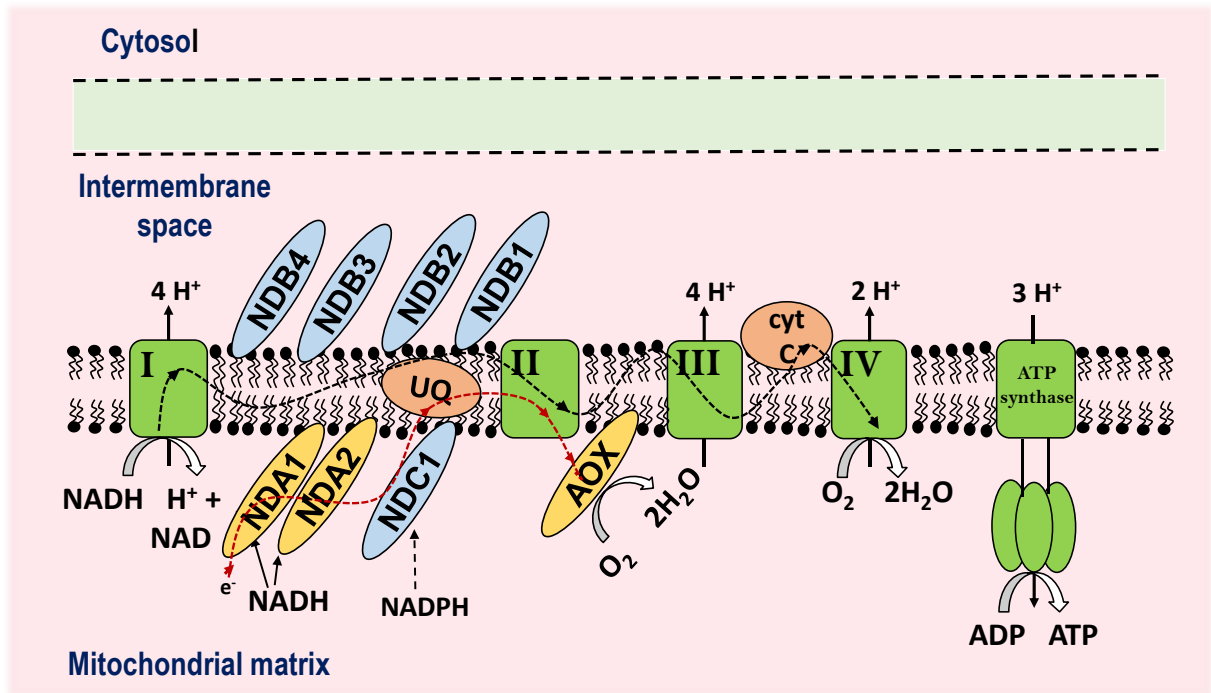
Changes in morphology of plant mitochondria under hypoxia or anoxia and reoxygenation is well documented by Shingaki-wells *et al.*, 2014. There is also evidence of mitochondria disintegration in the absence of oxygen in anoxia-intolerant wheat (Oliveira 1977). However, anoxia-tolerant rice and *Echinochloa phyllopogon* were able to maintain the ultrastructure and shape of the mitochondria (Kennedy *et al.* 1980). Besides changes in ultrastructure, mitochondrial ROS production is a key element in anoxia and reoxygenation stress signaling. The inhibition of mitochondrial ETC (mETC) by ETC inhibitors results in the activation of MITOGEN-ACTIVATED PROTEIN KINASE 3 (MPK3) and MPK6, similar to their activation during anoxia and reoxygenation. The MPKs confer better survival under anoxia in *Arabidopsis*. Given that MPKs are induced by ROS produced in the mETC points to a regulatory role of the mETC during anoxia and reoxygenation (Chang *et al.* 2012). Additionally, the involvement of respiratory chain components in NO and ROS signaling under low oxygen has also been reported (Blokhina *et al.* 2003; Gupta *et al.* 2011). Thus, apart from playing a major role in energy production, the mETC could have a signaling role in anoxic and post-anoxic survival.

## 1.6 Mitochondrial Electron Transport Chain (mETC)

Because of the sessile nature of plants, the energy metabolism in plants has to be adaptable to acclimatize to a wide range of environmental conditions. In comparison to mammalian mitochondria, the mETC of plant mitochondria is multifaceted, having unique components like the alternative NAD(P)H dehydrogenases and alternative oxidases that provide flexibility to mitochondrial energy metabolism (Vanlerberghe & McIntosh 1997; Rasmusson *et al.* 2004). The ATP producing mETC consists of four multi-subunit protein complexes, complex I, also called NADH dehydrogenase complex, complex II, or succinate dehydrogenase complex, complex III, also known as cytochrome bc1 complex, and complex IV, the cytochrome oxidase. Complex I oxidizes NADH while complex II oxidizes succinate and passes the electron to the mobile electron carrier ubiquinone (UQ) that forwards the electron to complex III. The electrons are passed to a second mobile electron carrier, cytochrome c (Cyt c), which ultimately delivers the electrons to cytochrome oxidase, where it is used to reduce oxygen and protons to generate water. The electron transport activities by complex I, II, and IV are coupled to the translocation of a proton across the inner mitochondrial membrane. The

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electrochemical proton gradient generated across the inner mitochondria membrane is utilized by the ATP synthase to generate ATP (Figure 1-1).



**Figure 1-1: The schematic representation of the electron transport chains of plant mitochondria.**

The basal electron transport chain is marked by a black dashed line and black arrows while an alternative electron transport chain is marked by red dashed line and red arrows. Roman numerals highlight complex I to IV. The type II NAD(P)H dehydrogenase A, B, C are abbreviated as NDA, NDB, NDC; AOX, alternative oxidase; UQ, ubiquinone; Cyt c, cytochrome c.

Apart from the above described universal mETC, plant mitochondria also possess an alternative respiratory chain composed of type II NAD(P)H dehydrogenases (NDs), alternative oxidases (AOX) and uncoupling proteins (UCP) (Rasmusson & Møller 1991; Vanlerberghe & McIntosh 1997; Vercesi *et al.* 2006). The alternative NAD(P)H dehydrogenases (NDs) can bypass complex I and act as an alternative entry point for electrons for the ETC while AOX bypasses complex III and IV and acts as an alternative electron exit point of the ETC as it can transfer electrons from UQ to molecular oxygen (Figure 1-1). However, electron transport activities by NDs and AOX are not used to pump protons across the membrane and hence reduce respiratory ATP yield. Roughly, the respiratory ATP yield is reduced by 30-60 % by NDAs, located at the matrix side, and AOX depending on the substrates used for respiration (Rasmusson *et al.* 2008). UCP acts downstream of the mETC and bypasses ATP synthase by allowing proton flow back to the mitochondrial matrix. Thus, UCP shares the function of plant-energy balance adjustment with the energy-bypass proteins of the alternative mETC.

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## 1.6.1 Type II NAD(P)H dehydrogenases

The complex I of the mETC is a major dehydrogenase for the oxidation of mitochondrial NADH in most eukaryotes. Type II NAD(P)H dehydrogenases in plants, fungi, protists, and many bacteria also oxidize NADH, however, without pumping protons across the membrane (Friedrich *et al.* 1995; Joseph-Horne *et al.* 2001; Melo *et al.* 2004; Rasmusson *et al.* 2004). Type II NAD(P)H dehydrogenases are flavoproteins made up of single polypeptide of 50-60 kDa with a covalently bound FAD as a cofactor. Type II NAD(P)H dehydrogenases are categorized in internal and external type II NAD(P)H dehydrogenases based on their localization. The internal type II dehydrogenases, NDA and NDC, are located at the inner surface of the inner mitochondria membrane and oxidize matrix NADH, while the external type II NAD(P)H dehydrogenases, NDB, are located at the outer surface of the inner mitochondrial membrane and oxidize cytosolic NADH (Elhafez *et al.* 2006). In *Arabidopsis thaliana*, seven type II NAD(P)H dehydrogenase have been identified, that are grouped into three gene families; *NDA*, *NDB*, and *NDC* (Michalecka *et al.* 2003). The genes *NDA1*, *NDA2*, and *NDC1* are internal type II NAD(P)H dehydrogenases while *NDB1-4* are external type II NAD(P)H dehydrogenases. Homologs of all three gene families also exist in monocotyledonous plants (Rasmusson *et al.* 2004). The protein sizes of NDB genes are close to 60-65 kDa, while NDA and NDC proteins have sizes of 50-55 kDa (Melo *et al.* 2004). Dual targeting of ND proteins was observed in *Arabidopsis* where *NDA1*, *NDA2*, and *NDB1* are targeted to mitochondria and peroxisomes while *NDC1* was targeted to mitochondria and chloroplasts (Carrie *et al.* 2008).

The correlation between enzyme activity and gene expression in potato and *Arabidopsis* indicated that *NDA1* use NADH as a substrate (Moore *et al.* 2003). However, the substrate specificity for *NDA2* has not been determined yet, but high sequence similarity between *NDA1* and *NDA2* with highly similar active sites indicates that both oxidize NADH (Michalecka *et al.* 2004). Internal type II NAD(P)H dehydrogenases are supposed to act under high matrix NADH concentration based on their high  $K_m(\text{NADH})$  values (Rasmusson *et al.* 2004). *Arabidopsis* *NDA1* and *NDA2* exhibit 82% amino acid sequence identity, may have evolved from a gene duplication and may have overlapping functions (Vision *et al.* 2000; Rasmusson *et al.* 2004). Although, the transcriptional regulation of both genes is different with *NDA1* exhibiting light-dependent expression and increased activity in photosynthetically active leaves (Michalecka *et al.* 2003; Elhafez *et al.* 2006). In contrast, the expression of *NDA2*

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is not light-dependent and is expected to have a function in heterotrophic metabolism (Elhafez *et al.* 2006). Thus, it is possible that the two genes may not entirely complement each other under defined physiological conditions.

In the absence of functional respiratory complexes like complex I or IV, the plants show considerably reduced growth and altered metabolomes and transcriptomes (Noctor *et al.* 2007). In contrast, plants lacking proteins from the alternative respiratory pathway display subtle phenotypes. For example, suppression of NAD(P)H DEHYDROGENASES B1 (NDB1) in *Nicotiana sylvestris* showed no consequences for biomass accumulation, while in *Arabidopsis*, suppression of NDA1 and NDA2 results in a slightly reduced biomass (Liu *et al.* 2009; Wallström *et al.* 2014). Conversely, no differential growth phenotype was observed in an *NDA1* knockout (Moore *et al.* 2003). Besides, the suppression of NDB4 in *Arabidopsis* showed a reduced leaf area during early developmental stages (Smith *et al.* 2011). Moreover, an *Arabidopsis* mutant deficient in AOX1a showed no difference in growth under standard growth condition but exhibited growth defects upon cold and drought stress (Fiorani *et al.* 2005; Giraud *et al.* 2008). The moderate phenotypes of mutants lacking components of the alternative respiratory pathway under standard growth condition point towards mild redox changes under this condition. However, these proteins are highly conserved groups of protein across the plant kingdom and expected to have a function during adaptation to adverse environmental conditions.

## 1.6.2 Regulation of alternative ETC under stress

The components of the alternative mETC are induced by many stress conditions in different plant species at the mRNA and protein level. An increased activity of alternative respiratory chain components was observed under cold and drought stress (Vanlerberghe & McIntosh 1992; Ribas-Carbo *et al.* 2005). Moreover, it was reported that hypoxia-induced activation of AOX controls ROS production (Gupta *et al.* 2009). Aside from abiotic stress, the alternative respiratory chain components are induced by pathogen effectors and response intermediates and by metabolic perturbation (Lennon *et al.* 1997; Huang *et al.* 2002; Clifton *et al.* 2005). Furthermore, mutants lacking subunits of the respiratory complex I and complex IV in maize and plants with a defective complex I in *Nicotiana sylvestris* showed induction of alternative mETC components (Sabar *et al.* 2000; Karpova *et al.* 2002). Overall, the alternative mETC

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components are activated whenever the function of the basal mETC is compromised either via mutation or because of biotic or abiotic stress. In general, under different stress conditions, mETC is prone to produce ROS due to a dysbalance in input and output components either by increased production of respiratory substrates or compromised mETC capacity (Rasmusson & Møller 2011). The physiological function(s) of the alternative mETC are not yet understood. However, it is well-known that it acts as an overflow mechanism and provides metabolic plasticity to the cell to survive under environmental stress conditions. It also prevents overreduction of the mETC and in turn, minimizes excess ROS production (Rasmusson & Møller 1991; Rasmusson *et al.* 2004, 2008). Besides, It has been reported that AOX can lower the oxygen concentration in the cell to minimize ROS formation (Gupta *et al.* 2009). Furthermore, several reports describe coexpression of AOX1 and type II NAD(P)H dehydrogenases. In Arabidopsis, NDA2, NDB2, NDB3, and NDB4 are coexpressed with AOX1 (Ho *et al.* 2007; Rasmusson *et al.* 2009). Besides, qPCR expression analysis under various external stimuli results in a coordinated expression pattern of AOX1 and type II NAD(P)H dehydrogenases (Clifton *et al.* 2005; Elhafez *et al.* 2006). The induction of alternative mETC components under various stresses has been well documented, although there are very few reports on their regulation at the molecular level. The AOX1a protein was studied extensively and shown to be regulated by the CYCLIN-DEPENDENT KINASE E;1 (CDKE1), ABSCISIC ACID INSENSITIVE4 (ABI4), and the transcription factors NO APICAL MERISTEM/ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR/CUP-SHAPED COTYLEDON13 (ANAC013) and ANAC017 (Giraud *et al.* 2009; Ng *et al.* 2013; De Clercq *et al.* 2013). However, there is not much information about the regulation of type II NAD(P)H dehydrogenases. A screen of the *NDA1* and *NDA2* promoters revealed cis-acting elements that bind ERF-VII and ANAC transcription factors. The presence of a GCC-box, dehydration responsive element (DRE), and ATCTA motif, known as a cis-binding element for ERF-VIIs in promoter regions of type II dehydrogenases point to regulation by these TFs but has yet to be experimentally tested. The stability of ERF-VII proteins is controlled by molecular oxygen. In normoxia, they undergo degradation in the N-end rule pathway (NERP) and are stabilized under hypoxic conditions (Licausi *et al.* 2011). This oxygen-sensing mechanism via NERP is highly conserved among plant species (Bailey-Serres *et al.* 2012a). ERF-VIIs act as a major regulators of hypoxic gene expression in plants and activate hypoxic responses (Mustroph *et al.* 2009). However, not much is known about their role in regulating early reoxygenation events. Even though there are recent reports

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which showed the stabilization of ERF-VII proteins during reoxygenation (Gibbs *et al.* 2014; Kosmacz *et al.* 2015), much research is needed to better understand the regulation of reoxygenation responses by ERF-VIIs. Besides ERF-VIIs, ANACs could also be involved in reoxygenation stress adaptation as they are involved in the regulation of mitochondrial retrograde signaling and oxidative stress response. ANACs are plant-specific transcription factors that are present in land plants and are one of the largest transcription factor families with diverse functions in plant development, defence and abiotic stress responses (Hegedus Yu and Baldwin, 2003; Xie *et al.* 2000; Takada *et al.* 2001; Vroemen *et al.* 2003; Fujita *et al.* 2004; Tran *et al.* 2004). ANAC013, ANAC016, and ANAC017 are involved in mitochondrial retrograde signalling with ANAC013 and ANAC017 regulating the expression of *AOX1a* (De Clercq *et al.* 2013; Van Aken *et al.* 2016). However, their role during reoxygenation and in regulating other members of the alternative respiratory chain is still to be examined.

## 1.7 Objectives

The primary goal of this study is to understand the mechanisms by which plants adapt to reoxygenation following a period of low oxygen as occurs during flooding. Specifically, the work aims to understand the function of the ALTERNATIVE NAD(P)H DEHYDROGENASES A1 and A2 (NDA1 and NDA2) during oxygen deficiency and subsequent reoxygenation in model plant *Arabidopsis thaliana*. Reduction in cellular O<sub>2</sub> is one of the major consequences of flooding and oxygen deficiency impairs respiration. Lack of a suitable electron acceptor results in a saturated redox chain, accumulation of NAD(P)H and excessive formation of reactive oxygen species (ROS, Rasmusson *et al.* 2004). The present work is set out to analyze ROS accumulation during low oxygen conditions and during reoxygenation using different cytological methods. To this effect, gene knockout plants lacking NDA1 or NDA2 or both as well as plants overexpressing *NDA1* or *NDA2* will be generated and analyzed for ROS accumulation. In addition, changes in ATP production and in the NADH/NAD<sup>+</sup> redox balance will be analyzed during low oxygen and reoxygenation in wild type and NDA mutant lines to more completely understand the function of NDAs. For an overall physiological evaluation of the importance of NDA activity for flooding resistance, plant survival of reoxygenation will be analyzed in wild type and NDA mutants. Since photosynthetic recovery during reoxygenation is a crucial factor for plant survival, I will further examine a possible cross-talk between

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chloroplasts and mitochondria by analyzing photosynthetic activity in wild type and NDA mutants and the impact of photorespiration in these genotypes by exposing plants to low or high CO<sub>2</sub> during reoxygenation. Finally, the project aims to understand transcriptional regulation of *NDA* genes which will be achieved by qPCR analysis during different phases of low oxygen treatment and reoxygenation. In addition, regulation of *NDA* genes by candidate transcription factors of the ERF and ANAC families, that were identified based on literature search and the bioinformatic identification of *cis*-elements in the promoters of *NDA1* and *NDA2*, will be analyzed. Taken together, the study intends to elucidate the role of NDAs in reoxygenation and the regulatory mechanisms that control their activity.



## 2 Materials & Methods

### 2.1 Materials

#### 2.1.1 Chemicals & Enzymes

All chemicals used for this work were obtained from the companies Roth (Karlsruhe, Germany), Sigma-Aldrich (Munich, Germany), Duchefa Biochemie (Haarlem, The Netherlands), Merck (Darmstadt, Germany), QIAGEN (Hilden, Germany), or Enzo life sciences (Lörrach, Germany), and were available in the purity grade for analysis (p.a). The enzymes and molecular biology kits (GeneJET Gel Extraction Kit, GeneJET Plasmid Preparation, and Gateway LR Clonase II Enzyme Mix Kit) used were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Unless stated otherwise, the protocols were followed according to the instructions of the manufacturer provided with the kits.

#### 2.1.2 Primers

The primers used for polymerase chain reaction (PCR) analysis were synthesized by Eurofins Genomics (Ebersberg, Germany). The primers were ordered in desalted quality as 100  $\mu$ M stock solutions. Dilutions were prepared according to the manufacturer's instructions. The primers were stored at -20°C. The list of primers used can be found in Table 2-1.

#### 2.1.3 Media & Solutions

For the production of media and solutions, distilled water was used. The distilled water was obtained from an ultrapure water system plant (SG water treatment and regeneration GmbH, Hamburg, Germany). The media and solutions were either steam-sterilized or filter-sterilized. For steam sterilization, solutions and media were heated at 125°C under 200 KPa pressure for 20 minutes while filter sterilization was done using a 0.2  $\mu$ m or 0.45  $\mu$ m filter. The composition of the solutions used is described in the respective methods section.

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### 2.1.3.1 Medium for bacteria growth

*Escherichia coli* cells were cultured in liquid or solid Luria-Bertani (LB) medium while *Agrobacterium tumefaciens* cells were grown in liquid or on solid Yeast extract peptone (YEP) medium.

#### LB-Medium

|                             |                     |
|-----------------------------|---------------------|
| (Luria-Bertani, autoclaved) | 10 g/l Trypton      |
|                             | 5 g/l Yeast extract |
|                             | 10 g/l NaCl         |
|                             | pH 7.0              |

#### YEP-Medium

|                                     |                      |
|-------------------------------------|----------------------|
| (Yeast extract peptone, autoclaved) | 10 g/l Pepton        |
|                                     | 10 g/l Yeast extract |
|                                     | 5 g/l NaCl           |
|                                     | pH 7.0               |

For solid media, 15 g/l agar were added. Media were stored at room temperature (RT). Transformed bacteria were selected by adding one or more antibiotics to the culture medium. An overview of the antibiotics used and the final concentration used is shown in Table 2-3.

**Table 2-1:** List of primers and their application.

| Sequence (5' → 3')                  | Name      | Application                   |
|-------------------------------------|-----------|-------------------------------|
| <i>RT-PCR Arabidopsis thaliana</i>  |           |                               |
| CAGACCACTTCTTCGTCT                  | NDA1 F4   | <i>AtNDA1</i><br>(At1g07180)  |
| TGGCTTCAATGTGCT                     | NDA1 R4   |                               |
| TGCGTGACCTTCAGTGCAA                 | NDA2 F5   | <i>AtNDA2</i><br>(At2g29990)  |
| CCAGCTCACGAAACCAGTCA                | NDA2 R5   |                               |
| ATGGACTAGAGCTCCGACGA                | AOX1a-F   | <i>AtAOX1a</i><br>(At3g22370) |
| AACGTCTGAAGCGATTTGCAG               | AOX1a-R   |                               |
| <i>qRT-PCR Arabidopsis thaliana</i> |           |                               |
| GATTCTACAATGGCTGACAAGAAGA           | GAPC-Q-F1 | <i>AtGAPC1</i><br>(At3904120) |
| ATGAAGGGGTTCGTTGACAGC               | GAPC-Q-R1 |                               |

## Materials & Methods

|                          |                |                 |
|--------------------------|----------------|-----------------|
| ACATTCCAGCAGATGTGGATCTC  | ACTIN-Q-F1     | <i>AtACTIN2</i> |
| GATCCCATTTCATAAAACCCCAGC | ACTIN-Q-R1     | (At3g18780)     |
| CTCCGTGAGAGCAAGGAAGG     | qNDA1-F        | <i>AtNDA1</i>   |
| CCAAAGACAAAGGTAGTGAGCC   | qNDA1-R        | (At1g07180)     |
| TTGACGATGGCACAGAGGTT     | qNDA2-F        | <i>AtNDA2</i>   |
| CGTCAATGCCAATCCTTCCAG    | qNDA2-R        | (At2g29990)     |
| ACTGTTTCAGGCCATGGGAA     | qAOX1a-F       | <i>AtAOX1a</i>  |
| ATCGGTAGGCCAACGAAGAG     | qAOX1a-R       | (At3g22370)     |
| GATCAAGGTGGCTGTTTACCC    | RBOHD-F        | <i>AtRBOHD</i>  |
| TTCCTCGTACACACTCGTGC     | RBOHD-R        | (At5g47910)     |
| AATCAACGGCGGAGGTCA       | <i>MSD1-qF</i> | <i>AtMSD1</i>   |
| AGCGTCAATGGCACTACCAA     | MSD1-qR        | (At3g10920)     |
| AAGTATCCAACCTCCGCTGC     | CAT2-qF        | <i>AtCAT2</i>   |
| TCGTTCTTGCCTCTCTGGTG     | CAT2-qR        | (At1g58030)     |
| CACAGGGATGAGGAGGTCAAT    | CAT1-qF        | <i>AtCAT1</i>   |
| CTCCCCTGGTTGCTTGAAG      | CAT1-qR        | (At1g20630)     |
| TCAGGACTTCCAGACCGAGT     | GR2-F          | <i>AtGR2</i>    |
| CAACCCCAAATCCCTGGATGA    | GR2-R          | (At3g54660)     |
| TCCTGGAGAAGTAATGGCGTC    | qPCRHRE1for    | <i>AtERF73</i>  |
| CCATCTGATGCTGAGCCTGAA    | qPCRHRE1rev    | (AT1G72360)     |
| GGCTCTGTTAGCAGCAGGAA     | qPCRHRE2for    | <i>AtERF71</i>  |
| CGAATCTCCGCTGCCATTT      | qPCRHRE2rev    | (AT2G47520)     |
| AAGAGGAAAACCCAGTGGAGC    | RAP2.2-qF      | <i>AtRAP2.2</i> |
| CCCCAACAGCAAATCCAACAC    | RAP2.2-qR      | (AT3G14230)     |
| CCGATTATGCCCCTCTCGTC     | RAP2.3-qF      | <i>AtERF72</i>  |
| ACGTAACTTGTTGGTGGGA      | RAP2.3-qR      | (AT3G16770)     |
| GCTGCGGAAGGTTTCAGTTTTT   | RAP2.12-qF     | <i>AtERF74</i>  |
| CACGTATCTCAGCAGCCCAT     | RAP2.12-qR     | (AT1G53910)     |
| CGTTACCCGGTTTGGTGAGG     | qPCRNAC13for   | <i>AtNAC013</i> |
| CTCGTCTTTTGACGGGTCATCT   | qPCRNAC13rev   | (AT1G32870)     |
| CTGGTTATTGGAAGGCGACAGG   | qPCRNAC16for3  | <i>AtNAC016</i> |

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|   |                     |   |
|---|---------------------|---|
| CTCCCTAGCTCTTCTTCATCC                       | qPCRNAC16rev3       | (AT1G34180)   |
| CTCGTCGTGTGGATGAGAGG                        | qPCRNAC17for        | <i>AtNAC017</i>   |
| CCAACGTA CTCTGCAGCTCT                       | qPCRNAC17rev        | (AT1G34190)   |
| Genotyping and cloning                      |                     |   |
| GGTGAAGATGAGAAGAAGAGGC                      | NDA1-F3             | SALK T-DNA  |
| CCAGGTGGAAGGATTGGTATTG                      | NDA1-R3             | insertion<br>check  |
| CACTCCTCTTCTTGCTTCTACTT                     | NDA2-R3_Gk29        | GK T-DNA  |
| ACTCAACACCAGTTGGTCCAC                       | NDA2-F3_GK29-<br>15 | insertion check   |
| GGATCCATGTTTCATGATCAAAAACCTC                | NDA2-BamH1-F1       | Amplification   |
| GCGGCCGCGAGATACGGCTAATGTCACG                | NDA2-Not1-R1        | of <i>AtNDA2</i> CDS  |
| GCGCGCGGATCCATGTTGTGGATCAAAAACCTAGC         | Pentra1A-NDA1-F     | Amplification   |
| GGTCGGGACATTAGCCGAATCTGGCGGCCGCTAAACT<br>AT | Pentra1A-NDA-R      | of <i>AtNDA2</i> CDS  |
| CACCCGTTGTGATCTCCTCTGTTC                    | NDA1- us-F1         | <i>AtNDA1</i>   |
| GATTCTTTAGGTACTCTTCTTC                      | NDA1-gus-R1         | promoter<br>amplification<br>(1052 bp<br>before<br>transcriptional<br>start site) |

**Table 2-2:** Primers used for generating EMSA probes

|                     |                                   |
|---------------------|-----------------------------------|
| NDA1emsaMDMlabF     | ATTGTCGTTTCTTGCGCATCAAGTTCTGCTGAG |
| NDA1emsaMDMlabR     | CTCAGCAGAACTTGATGCGCAAGAAACGACAAT |
| NDA1emsaMDMcompF    | ATTGTCGTTTCTTGCGCATCAAGTTCTGCTGAG |
| NDA1emsaMDMcompR    | CTCAGCAGAACTTGATGCGCAAGAAACGACAAT |
| NDA1emsaMDMmutcompF | ATTGTCGTTTATTACGCATCAATTTCTGCTGAG |
| NDA1emsaMDMmutcompR | CTCAGCAGAAATTGATGCGTAATAAACGACAAT |



## 2.2 Methods

### 2.2.1 Plant Material and growth condition

*Arabidopsis thaliana* L. ecotype Columbia-0 was used for all analyses and as a genetic background for mutations and transgene expression. Seed stocks for the transfer DNA (T-DNA) insertion lines *nda1-1* (SALK\_054530), *nda2-1* (GK-293E05) and *nda2-2* (GK-151F10) were obtained from the Nottingham Arabidopsis Stock Centre (University of Nottingham, Loughborough, United Kingdom). The double knock-out line *nda1-1 nda2-2* was created by crossing the single knock-out lines *nda1-1* and *nda2-2*. The crossing was done by Dr. Melanie Demuth, a former doctoral student in the group of Prof. Margret Sauter. The T-DNA insertion lines were analyzed for homozygosity and absence of transcripts was confirmed as described in chapter 2.2.6.

Plants were grown on a 1:1 sand/humus mixture or under sterile conditions on the standard MS medium described in chapter 2.1.3.2 at 22°C with an average irradiance of 90  $\mu\text{mol photons} \times \text{sec}^{-1} \times \text{m}^{-2}$  under long day conditions with 16 h light and 8 h darkness. For seedling growth on medium, seeds were sterilized in a 2% (v/v) sodium hypochlorite solution with continuous shaking at 1400 rpm (Thermomixer comfort, Eppendorf) for 20 minutes and subsequently washed four times with autoclaved water before planting on sterile MS medium. For specific growth experiments, 50  $\mu\text{M}$  rotenone were added to the growth medium from filter-sterilized stocks. The sterile plates and pots with seeds were stratified at 4°C for two days before placing them in the climate chamber.

### 2.2.2 Isolation of genomic DNA

For the isolation of genomic DNA, one or two young leaves from *Arabidopsis* were homogenized in 400  $\mu\text{l}$  DNA extraction buffer (200 mM Tris pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8 and 0.5% (w/v) SDS) in 1.5 ml reaction tube using a micropistill. The subsequent centrifugation step (5 min, RT, 12000 x g) was done to pellet out the cell debris while 300  $\mu\text{l}$  supernatant were transferred to a new 1.5 ml reaction tube. To precipitate DNA, 300  $\mu\text{l}$  of isopropanol were added to the supernatant and mixed several times by inverting the tube followed by another centrifugation step (5 min, RT, 12000 x g). After that, the pellet was washed with 500  $\mu\text{l}$  of 70% (v/v) ethanol (5 min, RT, 12000 x g). After drying, the pellet was

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dissolved in 100 µl of autoclaved water. The genomic DNA was stored at 4°C.

### 2.2.3 Isolation of total RNA

For isolation of total RNA, the plant material was harvested in 0.5 ml of Tri-reagent (Sigma-Aldrich) in 1.5 ml reaction tube and either homogenized immediately with a micropistill or frozen at -80°C to be processed later. After homogenization, samples were incubated for 5 min at RT. Subsequently, 100 µl of chloroform were added to the samples followed by vigorous mixing of samples by vortexing for 15 seconds. The samples were incubated at RT for 10 min and subsequently centrifuged at 4°C, 12500 x g for 15 min. The upper aqueous phase up to 300 µL was transferred to a new 1.5 ml reaction tube followed by RNA precipitation using 125 µL isopropanol and 125 µl high salt solution (1.2 M sodium chloride, 0.8 M sodium citrate). The samples were incubated for 10 min at RT, followed by another centrifugation step at 4°C, 12500 x g for 10 min. Thereafter, the supernatant was removed, and the pellet was washed with 500 µL 70% (v/v) ethanol (4°C, 12500 x g) for 5 min and then dried for 5-10 min at RT. RNA was dissolved in 20-50 µL of RNase-free water at 55°C for 10 min. The concentration of RNA was determined using the NanoDrop™ 2000 (Thermo Fisher Scientific), and the quality of the RNA was checked by agarose gel electrophoresis (chapter 2.2.5). The RNA was stored at -80° C.

### 2.2.4 Reverse transcription of RNA

Prior to complementary DNA (cDNA) synthesis, RNA samples were treated with RNase-free DNase I (Thermo Fisher Scientific) in order to avoid possible contamination with genomic DNA. The following reaction mixture was used for DNase treatment:

- 1 µg total RNA
- 1 U DNase I, RNase-free
- 2 µL 10x DNase buffer (100 mM Tris-HCL, 25 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>)

The distilled water was added up to 20 µl. The reaction mix was incubated at 37°C for 60 min. After that, DNase was inactivated at 75°C for 15 min. The subsequent cDNA synthesis was carried out using the following approach.

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1 µg total RNA (DNase I-treated)  
0.2 mM dNTP mix  
0.2 mM oligo-dT primer  
100 U RevertAid reverse transcriptase (Thermo Fisher Scientific)  
20 µl 5x reaction buffer (250 mM Tris-HCL, 250 mM KCL, 20 mM MgCl<sub>2</sub>,  
50 mM DTT)

The distilled water was added up to 20 µl. The cDNA synthesis was carried out at 42°C for 75 min. Subsequently, enzymes were inactivated by incubation at 70°C for 10 min.

### 2.2.5 Polymerase chain reaction (PCR) & gel electrophoresis

The polymerase chain reaction (PCR) was used for the targeted amplification of gene-specific DNA fragments. DreamTaq™ DNA polymerase (Thermo Fisher Scientific) was used for PCR analysis except for cloning. For cloning, the DNA fragments were amplified via Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific) according to manufacturers' instructions. The PCR reaction was performed with an Applied BiosystemSTM 2720 Thermal Cycler (Thermo Fisher Scientific). The standard approaches are described below.

#### DreamTaq™ DNA Polymerase

|          |                            |
|----------|----------------------------|
| 1-100 ng | template DNA               |
| 0.5 mM   | dNTP mix                   |
| 0.5 µM   | 5'-primer                  |
| 0.5 µM   | 3'-primer                  |
| 0.25 U   | DreamTaq™DNA polymerase    |
| 2 µl     | 10x DreamTaq™ green buffer |

The distilled water was added up to 20 µl.

#### Phusion™ High- Fidelity DNA Polymerase

|           |              |
|-----------|--------------|
| 50-250 ng | template DNA |
| 0.5 mM    | dNTP mix     |
| 0.5 µM    | 5'-primer    |
| 0.5 µM    | 3'- primer   |



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1 U Phusion™ high-fidelity DNA polymerase

2 µl 5x Phusion™ HF buffer

The distilled water was added up to 50 µl.

The respective PCR program used for the amplification as follows:

### DreamTag™ DNA Polymerase

|                      |          |         |       |
|----------------------|----------|---------|-------|
| Initial denaturation | 5 min    | 94°C    |       |
| Denaturation         | 30 sec   | 94°C    | } 35x |
| Annealing            | 30 sec   | 50-60°C |       |
| Elongation           | 1 min/kb | 72°C    |       |
| Final elongation     | 10 min   | 72°C    |       |

### Phusion™ High- Fidelity DNA Polymerase

|                      |           |         |       |
|----------------------|-----------|---------|-------|
| Initial denaturation | 5 min     | 98°C    |       |
| Denaturation         | 30 sec    | 98°C    | } 35x |
| Annealing            | 30 sec    | 50-68°C |       |
| Elongation           | 30 sec/kb | 72°C    |       |
| Final elongation     | 10 min    | 72°C    |       |

The annealing temperature was chosen depending on the primers used. The information about the primers used in the study can be found in Table 2-1.

Agarose gel electrophoresis was used for size-dependent separation of DNA and RNA fragments. Depending upon the size of the products, 1 to 3% (w/v) agarose gels in TAE buffer (40 mM Tris, 1 mM EDTA pH 8, 20 mM acetic acid) were used. To detect the nucleic acids under UV light, 1 µg/ml ethidium bromide was added to the gels. For fragment size

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determination SmartLadder (Eurogentec), GeneRuler 100 bp or Ultra Low Range DNA Ladder (Thermo Fisher Scientific) were used as markers.

### 2.2.6 Genotyping of T-DNA insertion lines

To check the insertion of T-DNA in both the alleles of the gene, PCR with two gene-specific and a gene- and T-DNA-specific oligonucleotide was performed (for primers see Table 2-1). Template DNA for PCR was isolated as mentioned in chapter 2.2.2. To test whether T-DNA insertion resulted in loss of transcripts, RNA was isolated from seeds of individual plants (2.2.3) and the presence of transcripts was analyzed by reverse transcription-PCR using appropriate primers (Table 2-1).

### 2.2.7 Quantitative real-time PCR (qPCR)

The PrimerBlast tool from the National Center for Biotechnology Information (NCBI) was used to design primers that anneal at 60°C and yield gene-specific amplicons of 115-150 base pairs (bp). Primers were designed either at the exon-exon junction or at sites in the gene of interest that are separated by at least one intron. Prior to qPCR, the primers were tested via RT-PCR and gel electrophoresis for the calculated product size. For quantitative expression analysis using real-time PCR (qPCR), the Rotor-Gene SYBR® Green PCR Kit (QIAGEN) was used. The reactions were always set up with a technical replicate as follows.

10 ng cDNA template  
7.5 µl Rotor-Gene SYBR® Green PCR master mix (QIAGEN)  
0.5 µM 5'-primer  
0.5 µM 3'-primer  
5.3 µl water

The Rotor-Gene® Q real-time PCR cycler (QIAGEN) was used for PCR reactions with the following program:

|                      |       |      |       |
|----------------------|-------|------|-------|
| Initial denaturation | 5 min | 95°C |       |
| Denaturation         | 05 s  | 95°C | } 45x |
| Elongation           | 10 s  | 60°C |       |

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For the evaluation of the relative expression ratio (R), the values of real-time PCR efficiency (E) and Cycle thresholds (Ct) were obtained from the Rotor-Gene® Q software (QIAGEN). The Ct values of the samples were normalized by using *Arabidopsis thaliana* *ACTIN2* (*ACT2*, At3g18780) and *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C SUBUNIT* (*GAPC*, At3g04120) as reference genes. The calculation of the relative expression ratio R was carried out in accordance with the AACT method using the following formula:

$$R = E_{target}^{\Delta Ct} / \sqrt{E_{GAPC}^{\Delta Ct} * E_{ACT2}^{\Delta Ct}}$$

### 2.2.8 Production of chemically competent *Escherichia coli* cells

In this work, chemically competent *E. coli* strains *DH5α* and *DB3.1* were used. For the production of the chemically competent *E. coli* cells, a single colony, grown on solid LB medium at 37°C, was inoculated in 5 ml of LB liquid medium. The preculture was incubated overnight at 37°C and 250 revolutions per minute (rpm) (incubator shaker G25, New Brunswick Scientific). To obtain competent cells, 100 ml of LB medium were inoculated with 400 µl of preculture. The medium was incubated at 37°C and 250 rpm (incubator shaker G25, New Brunswick Scientific) until the optical density (OD) at 600 nm (OD600) was 0.3. All subsequent steps were processed at 4°C. At first, the bacterial cells were pelleted by centrifugation at 3500 x g. The pellet was resuspended in 80 ml transformation buffer I (30 mM potassium acetate, 50 mM manganese (II) chloride, 100 mM potassium chloride, 10 mM calcium chloride, 15% (v/v) glycerol) and incubated on ice for 5 minutes followed by another centrifugation (5000 g x 5 min) step to pellet the cells. The cells were dissolved in 4 ml transformation buffer II (10 mM MOPS, 75 mM calcium chloride, 10 mM potassium chloride, 15% (v/v) glycerol). The cells were then aliquoted and immediately frozen in liquid nitrogen and stored at -80°C until further use. The cultivation of *E. coli* strains *DH5α* and *DB3.1* was done on LB medium at 37°C.

### 2.2.9 Transformation of chemically competent *E. coli* cells

For transformation, the chemically competent *E. coli* cells were thawed on ice before inoculating with 1-5 µl of plasmid. The mixture was incubated on ice for at least 30 min followed by heat shock at 42°C for 40-50 sec. The cells were again transferred to the ice for 2-3 min. Thereafter, 800 µl of LB medium were added to the cells with subsequent incubation

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for 1 h at 37°C and 250 rpm (Incubator shaker G25, New Brunswick Scientific). The cells were then plated on LB solid medium containing the appropriate antibiotic and incubated overnight at 37°C.

### 2.2.10 Production of chemically competent *A. tumefaciens* cells

In this work, the *A. tumefaciens* strain GV3101 was used. To prepare chemically competent *A. tumefaciens* cells, a single colony was inoculated in 2 ml of YEP medium and incubated overnight at 28°C at 250 rpm. Thereafter, 50 ml of YEP medium were inoculated with the preculture and incubated at 28°C at 250 rpm until the OD<sub>600</sub> reached 0.5. The subsequent steps were performed at 4°C. The cells were pelleted by centrifugation at 2000 g x min<sup>-1</sup> for 5 min and subsequently resuspended in 10 ml of 0.15 M NaCl. The cells were pelleted by another centrifugation at 1000 x g for 5 min and resuspended in ice-cold 20 mM CaCl<sub>2</sub>. Then, 15% (v / v) glycerol was added. The cells were divided in 200 µl aliquots and quickly frozen in liquid nitrogen and stored at -80°C until further use.

### 2.2.11 Transformation of chemically competent *A. tumefaciens* cells

For the transformation, chemically competent *A. tumefaciens* cells were thawed on ice and inoculated with 1 µg of plasmid DNA. The mixture was incubated on ice for 30 min and flash frozen in liquid nitrogen for 1 min. The cells were thawed for 2 to 3 minutes at 37°C. (Thermomixer comfort, Eppendorf). Thereafter, 1 ml of YEP medium was added to the cells and incubated for 2 to 4 h at 28°C at 250 rpm. The cells were pelleted by centrifugation, resuspended in 100 µl of fresh YEP medium and then plated on solid YEP medium containing an appropriate antibiotic. The plates were incubated at 28°C for 2 to 3 days.

### 2.2.12 Isolation of plasmid DNA

For plasmid DNA isolation, a single colony of *E. coli* cells was inoculated in 5 ml of LB medium containing the appropriate antibiotic and incubated overnight at 37°C and 250 rpm (Incubator shaker G25, New Brunswick Scientific). All centrifugation steps were done at 12000 x g at RT. From the overnight culture, 2 ml of the culture were centrifuged for 2 min to collect the cells as pellets. The pellets were resuspended in 100 µl of solution I. Subsequently, 200 µl of solution II were added and the tube was mixed 4-5 times and incubated at RT for 3 min.

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Thereafter, solution III was added to the samples and the mixture was incubated on ice for 10 minutes and then centrifuged for 5 min. The supernatant was transferred to a new 1.5 ml reaction tube and 1 ml of isopropanol was added to precipitate the nucleic acids followed by centrifugation for 10 min. The pellet was washed with 500  $\mu$ l of 70% (v/v) ethanol and subsequent centrifugation for 10 min. The pellet was dried at RT, resuspended in 50  $\mu$ l autoclaved distilled water and stored at -20° C.

Solution I: 50 mM Glucose

25 mM Tris, pH 8.0

10 mM EDTA

3 ng/ml RNase A (*Thermo Fisher Scientific*)

Solution II: 400 mM NaOH

2% (w/v) SDS

Solution III: 3 M Sodium acetate, pH 4.8

### 2.2.13 General cloning techniques

For cloning, DNA fragments were synthesized using Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific) and checked by agarose gel electrophoresis for the expected size (2.2.5). The desired DNA fragments were then eluted from the gel using GeneJET Gel Extraction Kit (Thermo Fisher Scientific) according to the manufacturers' instructions. For restriction-digestion and DNA ligation, restriction endonucleases (Thermo Fisher Scientific) and T4 DNA ligase (Thermo Fisher Scientific) were used according to manufacturers' instruction. For cloning, the *Gateway*™ cloning technology was used. pENTR™/D-Topo® and pENTR1A™ (Thermo Fisher Scientific) were used as entry vectors. Information about destination vectors is given in Table 2-4.

**Table 2-4:** Destination vectors and their application.

| Destination vector | Application                              |
|--------------------|--|
| pBGWFS7,0          | <i>AtNDA1<sub>pro</sub>: GUS</i>         |
| pB7WG2,0           | NDAox ( <i>35S<sub>pro</sub>:AtNDA</i> ) |

### 2.2.14 Sequencing and sequence analysis

*Lightrun*<sup>TM</sup> sequencing (GATC Biotech AG, Köln, Germany) was used to verify the nucleotide sequence cloned into the entry plasmids. For sequencing, plasmids were isolated by GeneJet Miniprep Kit (Thermo Fisher Scientific, Darmstadt, Germany) according to manufacturers' instructions. The sequences were analyzed using the *Bio Edit v7.0.5* (Carlsbad, USA) software to ensure proper amplification and cloning.

### 2.2.15 Stable transformation of *Arabidopsis thaliana*

The floral dip method (Clough & Bent, 1998) was used for stable transformation of *A. thaliana* using the agrobacteria strain GV3101. The plants were grown on soil under long day conditions (16 h light / 8 h dark, 21°C) at 60% humidity until the shoot reached a length of 2 to 10 cm. The plants were well watered one day before the transformation, and the flowers of stage 13 or older (Alvarez-Buylla *et al.*, 2010) were removed from the plants before the first transformation. The day before the transformation, 5 ml of YEP medium containing the appropriate antibiotic were inoculated with a single colony of transformed GV3101 cells and incubated overnight at 28°C and 250 rpm (Incubator shaker G25, New Brunswick Scientific). On the following day, 20 ml YEP culture containing the appropriate antibiotic were inoculated with 500 µl of preculture from the previous day and incubated for 5-6 h at 28°C and 250 rpm (Incubator shaker G25, New Brunswick Scientific). After that, cells were centrifuged for 5 min, at 4°C, and 2000 x g to pellet the cells followed by resuspension in 10 ml of fresh YEP medium without antibiotic. To this medium, 0.5 g sucrose, and 5 µl Silwett L-77 were added, and the bacterial suspension was distributed dropwise on the inflorescences. The plants were kept in the dark at high humidity for 24 h before transferring them to controlled conditions (16 h light/8 h dark, 21°C) at 60% air humidity. After one week, the transformation procedure was repeated to increase the number of transformants.

### 2.2.16 Histochemical GUS-analysis

To detect, β-glucuronidase activity in stably transformed *Arabidopsis* and rice, the tissue to be analyzed was immersed in X-Gluc solution and incubated for 8 to 24 h at 37°C. The reaction was stopped by placing the plant material in 70% (v/v) ethanol. For the analysis of leaves and stems, shoots were placed in 70% (v/v) ethanol until the chlorophyll was completely removed.

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To make cross sections of Arabidopsis seedlings, the root tissue was embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) according to manufacturers' instructions and cut into 10-50  $\mu\text{m}$  thin sections with a Leica RM 2255 microtome. For rice stems, sections were carried out by hand using a razor blade. The tissue was then placed in clarifying solution for better documentation. Staining of the tissue was visualized with a binocular (Nikon SMZ25, Prague, Czech Republic) or microscope (Olympus BX41, Hamburg, Germany) and documented with a camera.

### X-Gluc solution:

50 mM sodium phosphate, pH 7.2

0.2% (v/v) Triton X-100

2 mM potassium hexacyanoferrate

2 mM potassium hexacyanoferrate (III)

2 mM 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronic acid dissolved in DMF

### Clarifying solution:

67% (w/v) chloral hydrate

11% (v/v) glycerol

### 2.2.17 Low oxygen treatment and reoxygenation

Anoxia and hypoxia treatments were carried out in transparent, airtight acrylic boxes with a volume of 7 liters. A maximum of three plates without lids was placed in the boxes and flushed at a flow rate of 2 L/min  $\text{N}_2$  or 2 L/min of  $\text{N}_2$  with 40 mL/min  $\text{O}_2$  (AIR LIQUIDE, Düsseldorf, Germany) to equilibrate the atmosphere at 0% or 2% oxygen, respectively. The oxygen concentration within the acrylic boxes was checked by gas chromatography at the beginning and end of the first treatment to ensure an adequate oxygen concentration inside the box (Sasidharan *et al.*, 2017). Controls were placed in acrylic boxes in ambient air in the dark. The plates were positioned upright leaning backward at an angle of 6-9° to the wall of the box. The low oxygen treatments were always performed at 9:00 a.m. in order to nullify the effect of

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the circadian clock on the treatments. The subsequent reoxygenation was carried out in the light with an average irradiance of  $90 \mu\text{mol photons} \times \text{sec}^{-1} \times \text{m}^{-2}$  under long day conditions (16 h light/ 8 h dark  $21^\circ\text{C}$ ) at 60% air humidity. For experiments with reoxygenation in a high  $\text{CO}_2$  environment, the reoxygenation was done in the same transparent, airtight acrylic boxes flushed with pressurized air containing 1%  $\text{CO}_2$ . The boxes were then closed and placed in a climate chamber under long day conditions (16 h light/ 8 h dark  $21^\circ\text{C}$ ) at 60% air humidity. The controls were kept in the similar transparent acrylic boxes flushed with pressurized air.

### 2.2.18 Survival assays

For survival assays, *Arabidopsis* seedlings were grown on 0.5 X MS containing 0.1% sucrose for 7 days. The seedlings were exposed to anoxic conditions for 16 h as described in 2.2.17 and subsequently reoxygenated for 7 d in ambient air. After that, the percentage of alive, dead, and damaged seedlings was determined. The seedlings that grew new leaves were considered as alive while seedlings with complete leaf chlorosis were considered as dead. The seedlings with damaged cotyledons were counted as damaged. The percentage of survival was calculated for each plate and genotype. A minimum of five plates were analyzed for each biological replicate.

### 2.2.19 Treatment with chemicals

For chemical treatments, *Arabidopsis* seedlings were submerged in a 25 ml solution containing the specific chemical (rotenone, antimycin A, 3-amino-triazole and methyl viologen) in a 100 ml conical flask. The flasks were placed on a rotatory shaker at 125 rpm for 3 h in order to maintain proper aeration inside the flask. The flasks were placed in the controlled environment at  $22^\circ\text{C}$  with an average irradiance of  $90 \mu\text{mol photons} \times \text{sec}^{-1} \times \text{m}^{-2}$ . The controls were placed in a mock solution containing only the solvent without chemical.

### 2.2.20 Detection of reactive oxygen species (ROS)

#### 2.2.20.1 DAB staining for the detection of $\text{H}_2\text{O}_2$

To detect  $\text{H}_2\text{O}_2$  in *Arabidopsis* seedlings, 3,3'-diaminobenzidine (DAB) staining was used (Thordal-Christensen *et al.* 1997). Treated seedlings were immediately transferred to 10 mM



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$\text{Na}_2\text{HPO}_4$  DAB (1 mg/ml), vacuum infiltrated for 5 min and incubated for 4-5 h in the dark on a rotatory shaker (80-100 rpm). To prepare 10 mM  $\text{Na}_2\text{HPO}_4$  DAB, 50 mg DAB powder (Sigma-Aldrich) was dissolved in 45 ml of sterile distilled water and the pH was adjusted to 3.0 using 0.2 M HCL. After that, 2.5 ml of 200 mM  $\text{Na}_2\text{HPO}_4$  and 0.05% (v/v) Tween 20 were added to the DAB mixture for better penetration of the stain into the tissue. After the staining, the staining solution was removed, seedlings were washed with distilled water and placed in 70% ethanol to remove the chlorophyll. A SMZ25 binocular (Nikon, Prague, Czech Republic) equipped with a DS-Fi3 camera (Nikon, Prague, Czech Republic) was used for documentation of dark brown stain formed inside the tissue. Approximately 15 seedlings were analyzed for each treatment and genotype in three independent biological experiments. For quantification of the staining intensity, the method described by Nguyen *et al.* (2013) was used.

### 2.2.20.2 NBT staining for the detection of $\text{O}_2^-$ detection

The nitro blue tetrazolium chloride (NBT) staining was used to detect  $\text{O}_2^-$  anions in Arabidopsis seedlings as described previously (Dunand *et al.* 2007), with some modifications. After the treatment, seven-day-old Arabidopsis seedlings were quickly transferred to 2 mM NBT in 20 mM K-phosphate at pH 6.1 and incubated for 5 min in the dark. Subsequently, the stain was removed, and seedlings were washed thoroughly with distilled water and transferred to 70% ethanol in order to remove chlorophyll. The dark blue precipitate formed by the reaction between  $\text{O}_2^-$  anions and NBT was documented using a SMZ25 binocular (Nikon, Prague, Czech Republic) equipped with a DS-Fi3 camera (Nikon, Prague, Czech Republic). About 15 seedlings per treatment and genotype were analyzed in three independent biological experiments. The method described by Nguyen *et al.* (2013) was used to quantify the staining intensity.

### 2.2.20.3 $\text{H}_2\text{DCF-DA}$ staining for $\text{H}_2\text{O}_2$ detection

To detect  $\text{H}_2\text{O}_2$  in roots, 2',7'-dichlorodihydrofluorescein diacetate ( $\text{H}_2\text{DCF-DA}$ ) staining was used. Briefly, 7-day-old seedlings were incubated in 10  $\mu\text{M}$   $\text{H}_2\text{DCF-DA}$  stain in 1 X PBS (phosphate-buffered saline) for 5 min in the dark at 22°C. After that, the staining solution was removed, and seedlings were washed three times in 1 X PBS and analyzed immediately with a fluorescence microscope BX41 (Olympus, Germany). Approximately 20 seedlings were

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examined per treatment and genotype in three independent biological experiments. To determine the staining intensity, the Fiji program (Schindelin *et al.* 2012) for biological image analysis was used.

### 2.2.20.4 Electron Paramagnetic Resonance (EPR) spectroscopy

To quantify oxygen anion radicals ( $O_2^{\cdot-}$  anions) in shoots during reoxygenation, Electron Paramagnetic Resonance (EPR) spectroscopy was used. Seven-day-old seedlings were exposed to anoxia as described in 2.2.17. The shoot samples were harvested at defined times during reoxygenation and immediately frozen in liquid nitrogen. 150  $\mu$ L of 2 mM N-(1-Hydroxy-2,2,6,6-tetramethylpiperidin-4-yl)-2-methylpropanamide-Hydrochloride (TMT-H) spin probe dissolved in 1 mM EDTA were added to each sample. The samples were incubated at 40°C in a water bath for 90 min, and 25  $\mu$ L of supernatant were drawn up in a capillary glass tube for measurement. The measurement was carried out on a Bruker Elexsys E500 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). Measurements were performed at room temperature with the following acquisition parameters: resonance frequency 9.585 GHz, modulation frequency 100 kHz, modulation amplitude 0.13 mT, microwave power 0.2 mW, receiver gain 60 dB, time constant 81.92 ms, conversion time 40 ms, center field 351.15 mT, and a sweep width of 8 mT. Using the XEPR software of Bruker BioSpin GmbH, the concentration of TMT radicals was calculated by spin counting of the absolute spins using the double integral of the entire spectrum. ROS concentration is therefore given as TMT radical equivalents as spins per  $mm^3$ .

### 2.2.21 Metabolite measurements

#### 2.2.21.1 Measurement of ATP and NADH using FRET-based sensors

For real-time analysis of ATP and NADH inside living cells, the fluorescence resonance energy transfer (FRET)-based genetically encoded sensors ATeam for ATP, and Peredox-mCherry for NADH were used. The constructs pH2GW7\_cATeam1.03nD/nA (ATeam) and pSS02\_c-peredox-mCherry (Peredox-mCherry) were obtained from Prof. Markus Schwarzländer (University Münster, Germany). Wildtype arabidopsis and *nda* mutants were transformed with the constructs described above as described in 2.2.15. A multiwell plate reader-based

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fluorimeter analysis was used to analyze ATP and NADH levels during anoxia and reoxygenation. The assays were performed in the laboratory of Prof. Schwarzländer by his student Sophie Lichtenauer. Briefly, 7-day-old Arabidopsis seedlings were submerged individually in wells of a 96 well multiwell plate (Sarstedt, Nümbrecht, Germany) filled with assay medium consisting of 10 mM MES, 10 mM MgCl<sub>2</sub>, 10 mM CaCl<sub>2</sub>, 5 mM KCL, pH 5.8. The plate was moved to a BMG Labtech CLARIOstar microwell plate reader (BMG Labtech, Ortenberg, Germany) and the fluorophores were excited at a specific wavelength. The ATeam sensor for the measurement of ATP consists of  $\epsilon$  subunit of the bacterial FoF1-ATP synthase flanked by the cyan (mseCFP) and yellow (cp173-mVenus) fluorescent proteins. The ATeam sensor was excited at a wavelength of  $435 \pm 10$  nm while emission was recorded at  $483 \pm 9$  nm for mseCFP and  $539 \pm 6.5$  nm for cp173-mVenus. The Peredox-mCherry sensor for the NADH-NAD<sup>+</sup> redox state consists of a circularly permuted GFP T-Sapphire interlaced between the two NADH-binding Rex subunits with a C-terminal RFP mCherry. The T-Sapphire and mCherry fluorophores were excited at  $400 \pm 5$  and  $570 \pm 10$  nm, respectively. The emissions were read at  $520 \pm 5$  (T-Sapphire) and  $610 \pm 5$  nm (mCherry). A transparent qPCR film was used to block oxygen supply to the seedlings. Residual oxygen in the wells was consumed by the respiratory activity of the seedlings. The plate was kept in the dark except for the light flashes necessary to excite the fluorophores. The fluorescent background of Col-0 plants and of the incubation medium was recorded and subtracted from all data before analysis. For reoxygenation, the transparent qPCR film was removed, and the measurement was done as described above.

### 2.2.21.2 Analysis of metabolites using LC-MS

The adenylate status (ATP, ADP, and AMP) and the NADH-NAD<sup>+</sup> redox status were analyzed by metabolite fingerprinting using a ultra performance liquid chromatography (UPLC) system. The 7-day-old Arabidopsis seedlings were treated as described in 2.2.17 and shoot samples were harvested at 0 h, 0.5 h, 2 h, 4 h, and 8 h after reoxygenation. Approximately 60-70 mg shoot material was harvested and immediately frozen using liquid nitrogen in 2 ml safe lock Eppendorf Micro test tubes (Hamburg, Germany). The samples were sent to the Metabolomics Core Technology Platform (MCTP, University of Heidelberg, Germany). Measurements were carried out by Dr. Gernot Poschet. Briefly, the samples were ground in

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liquid nitrogen to a fine powder by using the tissue grinder and extracted with 1 ml 0.1 M HCl by vortexing at 4°C for 15 min. After that, samples were centrifuged twice at 4°C and 12,000 x g for 5 min and the supernatant was transferred to a new 1.5 ml safe lock Eppendorf Micro test tube (Hamburg, Germany). The separation of metabolites was carried out by reversed phase chromatography on an Acquity BEH C18 column (150 mm × 2.1 mm, 1.7 µm, Waters) connected to an Acquity H-class UPLC system and detected by fluorescence (Acquity FLR detector, Waters, excitation: 280 nm, emission: 410 nm, gain: 100). Quantification was carried out using ultrapure standards (Sigma) as described (Weger *et al.* 2018).

### 2.2.22 The maximal quantum yield of PSII (Fv/Fm) measurements

To determine the maximal quantum yield of PSII, the ratio of variable to maximum fluorescence (Fv/Fm) was measured during reoxygenation in 11-day-old seedlings. The seedlings were exposed to anoxic condition for 16 h as described in 2.2.17 and the measurements were taken at different time points after reoxygenation using Imaging PAM IMAG-MAX/K (Walz, Effeltrich, Germany). Data were analyzed by Imaging Win V2.41a (Walz, Effeltrich, Germany).

### 2.2.23 Transactivation assay

Preparation and transformation of Arabidopsis shoot protoplasts was performed according to Zhang *et al.*, (2011). The truncated CDS of *ANAC013*, *ANAC016* and *ANAC017* lacking the C-terminal transmembrane domain in p2GW7,0 were co-expressed with *pNDA1* in p2GWL7,0 using 5 µg for each plasmid (Karimi *et al.*, 2002). The promoter was placed upstream of the firefly *LUC* gene, and a normalization vector containing *35S:RLUC*. Dual luciferase promoter assays (Promega) with 4 biological replicates were performed according to Licausi *et al.*, (2011) and luminescence was detected using the plate-reader Biotek Synergy MX.

### 2.2.24 Electrophoretic mobility shift assays

The truncated CDS of *ANAC013*, *ANAC016* and *ANAC017* lacking the C-terminal transmembrane domain was fused with a FLAG tag by PCR and cloned into the pF3A WG Flexi vector (Promega). Subsequently, protein expression was done by using TNT SP6 High Yield Wheat Germ protein expression system (Promega) according to the manufactures protocol

## Materials & Methods

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with 4 µg of plasmid DNA. Successful expression of proteins was ensured by performing a western blot against the FLAG-epitope tag. For gel-shift assays, IR-dye680-labelled probes were generated based on 33 bp long regions derived from the NDA1 promoter. DNA probes were generated by annealing forward and reverse oligonucleotides in equal-molar ratios and slowly cooling them down from 95 to 30 °C. Probes were diluted to a final concentration of 500 fmol and kept in amber tubes until use. Binding reactions were performed using the Odyssey EMSA Buffer kit (Pierce, Rockford, USA) for 2 hours in the dark at 4 °C. Subsequently, the reaction mixture was run on a 5% native polyacrylamide gel in TBE buffer. Visualization of the gel after the run was done by using ChemiDoc system from Biorad. Primers used for EMSA is mentioned in Table 2-2.

### 2.2.25 Statistical evaluation of the data

MINITAB Release 14.20 (Minitab Inc., Pennsylvania, USA) was used for the statistical analysis. The data were first checked for normal distribution and equal variances of single values by Anderson-Darling and Bartlett's test, respectively. The statistical difference of means was evaluated by Students t-test, variances with an ANOVA (Tukey-test) and Kruskal-Wallis test. The probability of significance was set to  $P \leq 0.05$  or  $P \leq 0.001$ . The information about the applied test and P values are indicated in figure legends.

### 2.2.26 Databases and computer programs used for bioinformatics

The following databases were used to obtain nucleotide and protein sequences, search T-DNA insertion lines, and get knock out mutant seeds and expression data.

1. National Center for Biotechnology Information (NCBI)  
<https://www.ncbi.nlm.nih.gov/>
2. The Arabidopsis Information Resource (TAIR)  
<https://www.arabidopsis.org/>
3. The Nottingham Arabidopsis Stock Center (NASC)  
<http://arabidopsis.info/>
4. Salk Institute Genomic Analysis Laboratory (SIGnAL)  
<http://signal.salk.edu/>
5. Rice Genome Annotation Project (RGAP)

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<http://rice.plantbiology.msu.edu/>

The software and online tools used for bioinformatics were as follow:

Genevestigator for expression analysis, BioEdit Version 7.2.5 for sequence alignment, SnapGene for in silico cloning, *ExPASy* translate tool to translate nucleotide sequences to amino acid sequences, Fiji for image processing and analysis, and NCBI-primer blast for qRT-PCR primer design.

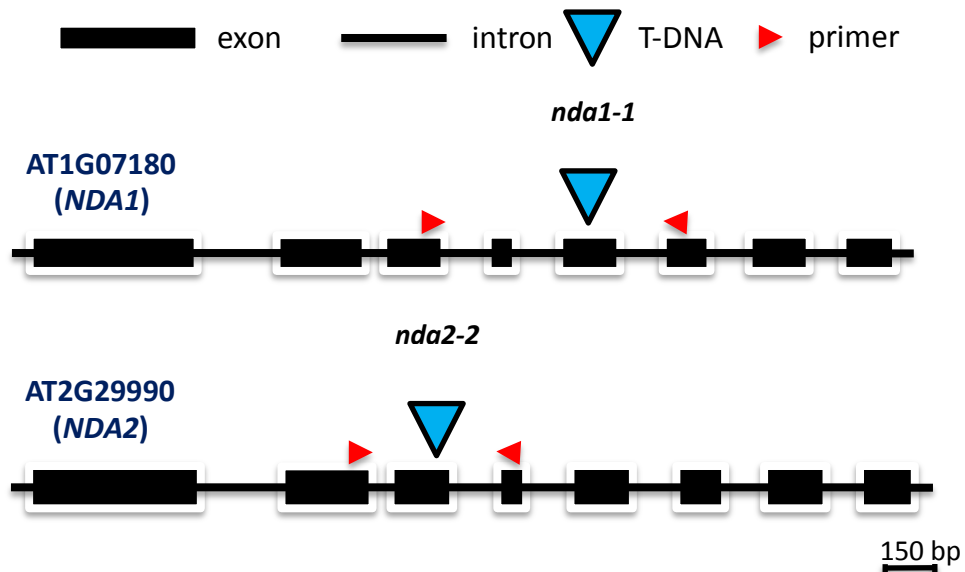
## 3 Results

### 3.1 Identification of *NDA* knockout and generation of *NDA* overexpression lines

Mutagenesis is one of the essential tools used in genetics to study gene function or the contribution of a gene to defined biological traits. For *Arabidopsis thaliana*, a vast collection of T-DNA insertion mutants is available that is a valuable resource for research in plant biology. To study the function of *NDA1* and *NDA2*, T-DNA insertion lines of the *A. thaliana* ecotype Columbia-0 were selected. The T-DNA insertional lines were identified using T-DNA Express, an *Arabidopsis* gene-mapping tool, and homozygous seed stocks were ordered from the Nottingham *Arabidopsis* Stock Center (NASC). The schematic representation of the gene structures of *AtNDA1* and *AtNDA2* with the position of the T-DNA insertion is depicted in Figure 3-1. Lines which have the T-DNA insertion in an exon were chosen to ensure gene disruption. The T-DNA insertion lines were first tested for homozygosity of the T-DNA, and after that, the lines were tested via RT-PCR for mRNA abundance to check the suppression of the target gene. *Actin2* was used as a reference gene to control the input of cDNA generated from mRNA. In, the *NDA* single knockout lines *nda1-1* and *nda2-2* and the double knock-out line *nda1-1 nda2-2*, no transcripts were detected by RT-PCR (Figure 3-2A). Homozygous lines were identified and used for functional and phenotypic analysis.

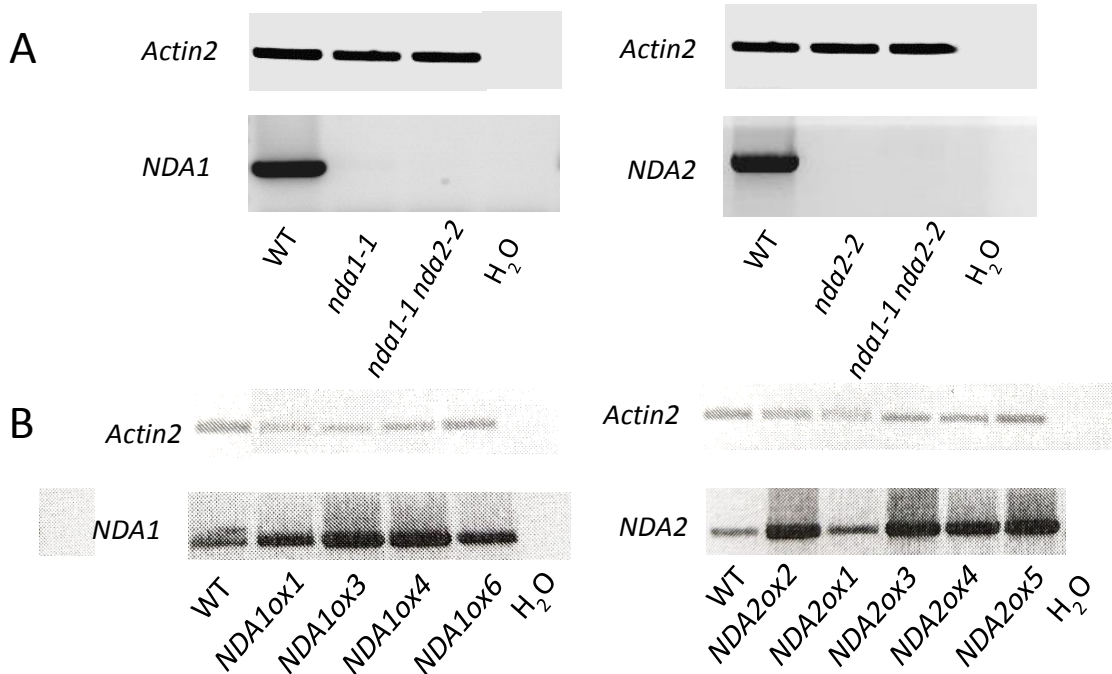
Apart from loss-of-function mutations, gain-of-function mutations are frequently used in plant science to study the function of a gene. A gain-of-function mutation can be obtained by expressing the gene under the influence of a strong promoter. In the current study, *NDA1* and *NDA2* overexpression lines driven by the 35S promoter were created. Several individual lines were tested for the overexpression of the target gene via RT-PCR with *Actin2* as a reference gene. Expression analysis of several *NDA1ox* and *NDA2ox* lines verified the overexpression of *NDA1* and *NDA2* transcripts (Figure 3-2B). The lines with the highest transcripts levels of the particular gene were selected for the phenotypic analysis.

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**Figure 3-1: Schematic representation of the gene structures of *AtNDA1* and *AtNDA2*.**

The exons are shown as black boxes while introns are indicated as a black line. The position of the primers used for genotyping are marked by red arrows while the position of the T-DNA insertion is indicated by a blue triangle.



**Figure 3-2: Expression analysis of *NDA* transcripts in *NDA* mutants.**

A) RT-PCR analysis with gene-specific primers confirmed the loss of *NDA1* and *NDA2* transcripts in the respective *nda* single and double gene knockout lines. B) Relative transcript levels of *NDA1* and *NDA2* in wt and in independent *NDA1ox* and *NDA2ox* lines were analyzed in 7-day-old seedlings by RT-PCR. *ACTIN2* was used as a reference gene.

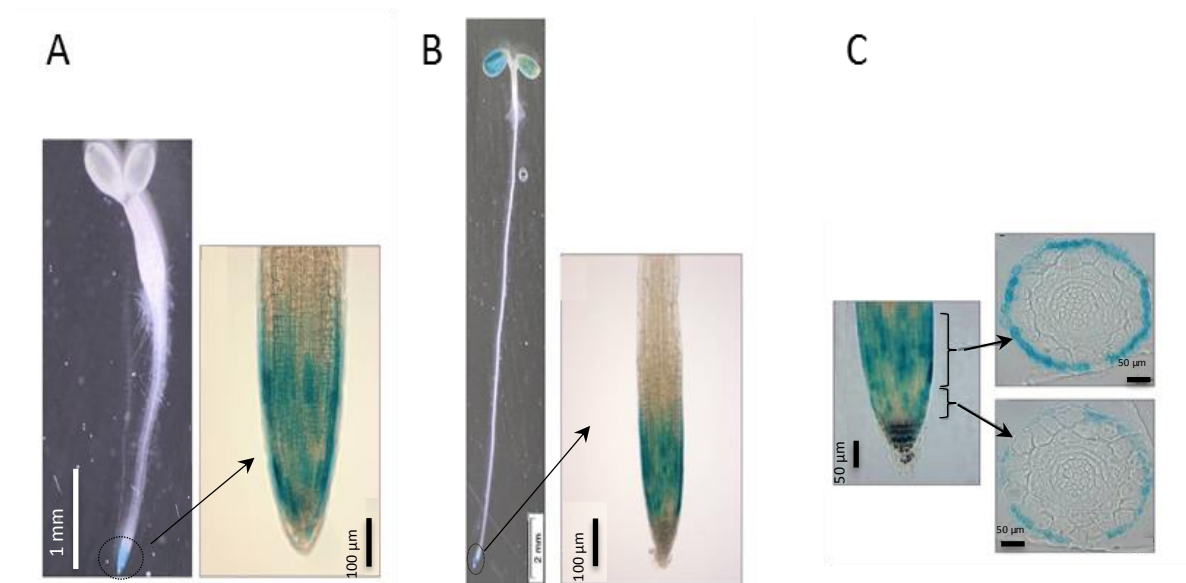


# Results

## 3.2 Expression of *NDA*s in roots

### 3.2.1 Promoter activity of *NDA1* in roots

To know the precise spatial and temporal expression of a gene is one of the critical aspects of functional genomics. The GUS reporter system is widely used in plant biology to study gene expression patterns. The promoter activity of *NDA1* was analyzed in three-day-old and seven-day-old *AtNDA1:GUS* seedlings. In 3-day-old seedlings with expanded cotyledons and a short primary root, *AtNDA1* promoter activity was visible in the root apex cells (Figure 3-3A), whereas in 7-day-old seedlings with photosynthetically active cotyledons and a longer primary root, the *AtNDA1* promoter was active in root apex cells as well as in cotyledons (Figure 3-3B). To analyze the spatial expression pattern in root apex cells in more detail, cross-sections were taken from the GUS stained area at the root apex. The analysis of cross-sections revealed that *AtNDA1* is expressed explicitly in the primary root cap (Figure 3-3C).



**Figure 3-3: *NDA1* is expressed in the root cap.**

A) Three-day-old and B) 7-day-old light-grown *AtNDA1:GUS* seedlings were stained to visualize promoter activity. C) The primary root tip and cross-sections of the root tip as indicated from 7-day-old seedling.

### 3.2.2 Promoter activity of *NDA1* under low oxygen and reoxygenation

To gain insight into the role of *NDA1* under low oxygen conditions, *AtNDA1:GUS* seedlings were subjected to normoxic (21% O<sub>2</sub>) or hypoxic (2% O<sub>2</sub>) conditions for 6 h and the GUS activity

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in roots was observed. The results showed that hypoxia did not change spatial distribution or the strength of *NDA1:GUS* activity in roots (Figure 3-4). Seedlings exposed to anoxic conditions for 6 h showed reduced *NDA1:GUS* expression (Figure 3-5). By contrast, *NDA1:GUS* activity was substantially increased by reoxygenation following anoxia (Figure 3-5). Within 2 h of reoxygenation, both, the range and the strength of *NDA1:GUS* activity at the root apex increased.



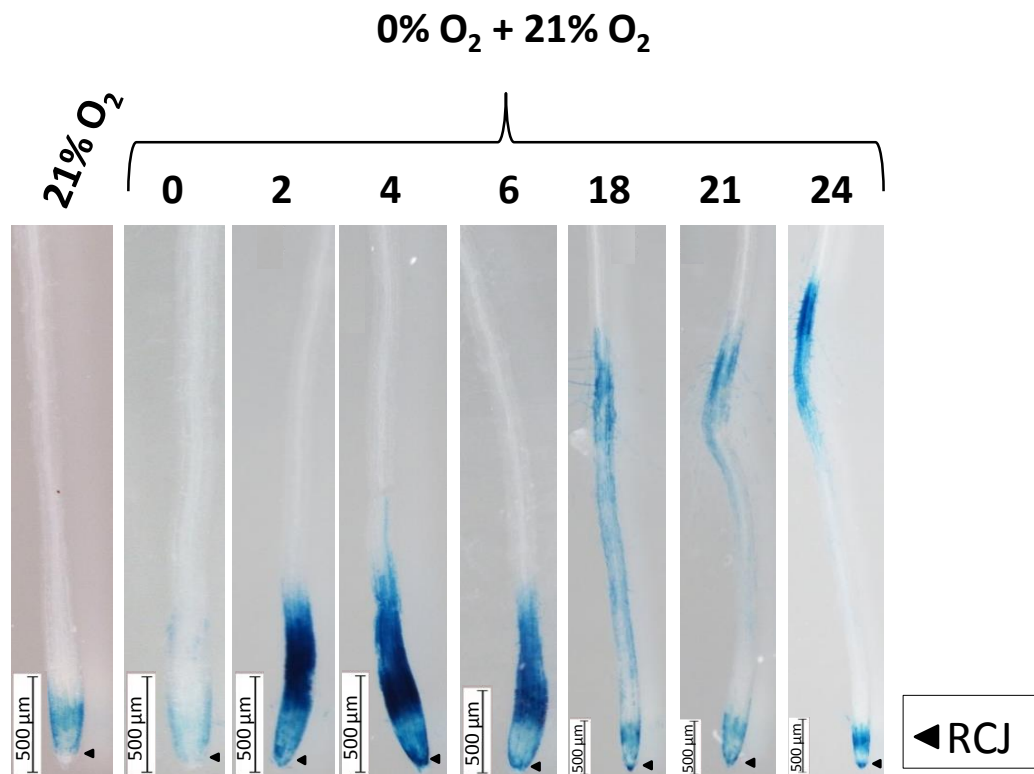
**Figure 3-4: Hypoxia does not induce *NDA1:GUS* expression.**

*NDA1:GUS* activity in the primary root of 7-day-old light-grown seedlings exposed to air (21% O<sub>2</sub>) or hypoxia (2% O<sub>2</sub>) for 6 h in the dark. The scale bar indicates 500  $\mu$ M. RCJ: Root Cap Junction

The Arabidopsis primary root apex has four distinct growth zones. A meristematic zone, which is 200  $\mu$ m away from the root cap junction (RCJ), a transition zone (200-500  $\mu$ m from RCJ), an elongation zone (520-850  $\mu$ m) and a growth terminating zone (850-1500  $\mu$ m) (Verbelen *et al.* 2006). Under normoxia, GUS activity is observed in the meristematic zone (MZ) and transition zone (TZ) of the root apex. After 2 h of reoxygenation, GUS activity not only increased but the staining extended to the elongation zone (EZ). The GUS activity at 4 h of reoxygenation remained higher, and the expression pattern was quite similar to the pattern observed at 2 h with staining visible in all three zones (MZ, TZ, EZ). After 6 h of reoxygenation, the *NDA1:GUS* activity was marginally reduced at the root apex cells and was further reduced

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after 18-24 h of reoxygenation at the apical root zones. After 18 h the staining appeared at the distal root zone called growth terminating zone (Figure 3-5).



**Figure 3-5: Expression of *AtNDA1:GUS* increases in the primary root during reoxygenation.**

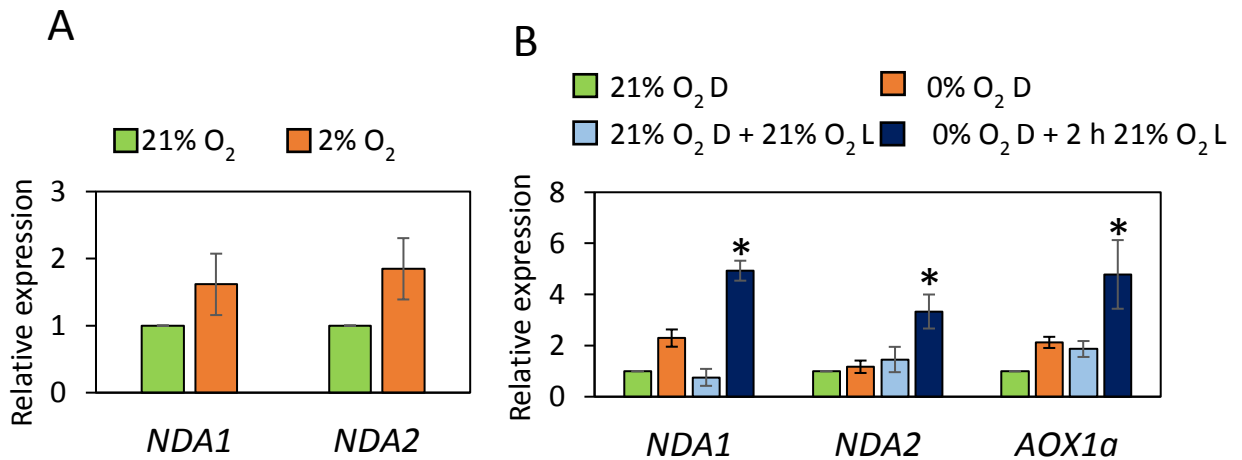
Seven-day-old *NDA1:GUS* seedlings were exposed to air (21% O<sub>2</sub>) or anoxia (0% O<sub>2</sub>) for 6 h in the dark and reoxygenated (21% O<sub>2</sub>) for 0-24 h in the light. The scale bar indicates 500 µm. RCJ: Root Cap Junction.

### 3.2.3 Expression of *NDA*s and *AOX1a* increases during reoxygenation

The histochemical GUS analysis showed increased activity of *NDA1* during reoxygenation. In order to quantify changes in the expression of *NDA*s in roots during low oxygen conditions and during reoxygenation real-time quantitative PCR (qPCR) was performed. No significant changes in *NDA1* and *NDA2* expression were observed under hypoxia (2% O<sub>2</sub>) or anoxia (0% O<sub>2</sub>) (Figure 3-6). However, after 2 h of reoxygenation (0% O<sub>2</sub> + 21% O<sub>2</sub>), there was a significant increase in transcript abundance of *NDA1*, *NDA2*, and *AOX1a*. The transcript levels of *NDA1*, *NDA2*, and *AOX1a* were increased by 4.9 fold, 3.3 fold, and 4.8 fold, respectively (Figure 3-6). The results obtained were consistent with the GUS expression data in the root of *NDA1:GUS* seedlings. The induction of these genes during reoxygenation indicated that the internal

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alternative respiratory chain might be involved in root adaptation to reoxygenation following anoxia.



**Figure 3-6: Reoxygenation increases the expression of *NDA1*, *NDA2*, and *AOX1a* in roots.**

A) Seven-day-old seedlings were exposed to air (21% O<sub>2</sub>) or hypoxia (2% O<sub>2</sub>) for 6 h in the dark. The expression level of *NDA1* and *NDA2* in roots was analyzed by qPCR.

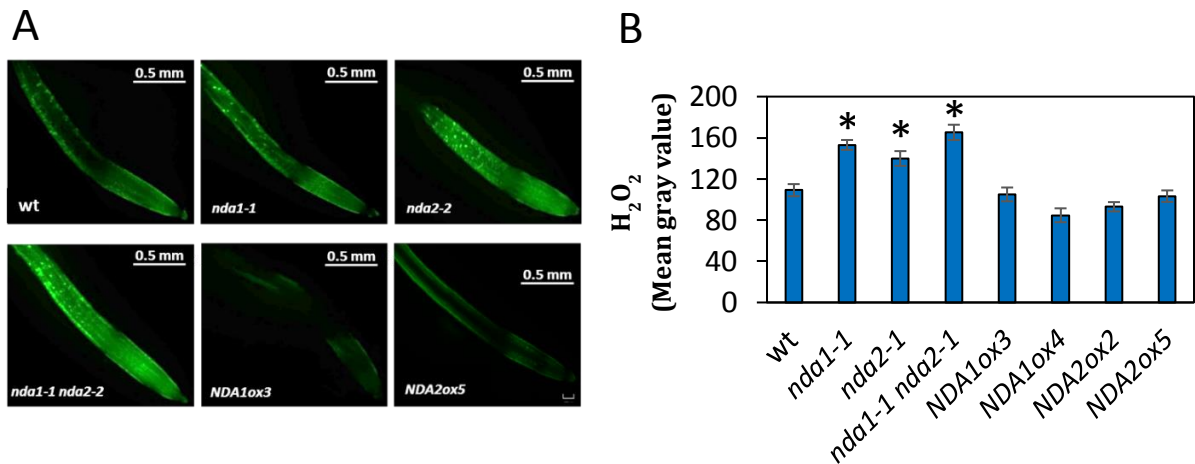
B) Expression of *NDA1*, *NDA2* and *AOX1a* was analyzed in seedling roots exposed to air (21% O<sub>2</sub>) or anoxia (0% O<sub>2</sub>) for 6 h in the dark (D), or exposed to air (21% O<sub>2</sub>) or anoxia (0% O<sub>2</sub>) for 6 h in the dark plus recovery in 21% O<sub>2</sub> for 2 h in the light (L). Results are means ( $\pm$  SE) of three independent biological replicates with two technical repeats each (Mann-Whitney test,  $P < 0.05$ ). The asterisks indicate significantly different values.

### 3.3 Accumulation of reactive oxygen species (ROS) in roots

#### 3.3.1 NDAs control H<sub>2</sub>O<sub>2</sub> accumulation in the primary root

To analyze the accumulation of H<sub>2</sub>O<sub>2</sub> in the primary root, 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCF-DA) staining was used. The staining assays reveal that untreated single and double *NDA* knockout seedlings accumulated more H<sub>2</sub>O<sub>2</sub> than wild-type (wt) in the primary root (Figure 3-7). In contrast, overexpression of NDAs in *NDA1ox* and *NDA2ox* lines did not significantly change H<sub>2</sub>O<sub>2</sub> levels in comparison to wt. The quantification of staining intensity was done via Fiji program (Schindelin *et al.* 2012), and a significant difference was observed between *NDA* knock-outs and wt. These observations suggest that *NDAs* might be involved in H<sub>2</sub>O<sub>2</sub> control under standard growth condition.

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**Figure 3-7: NDAs limit H<sub>2</sub>O<sub>2</sub> accumulation.**

A) H<sub>2</sub>O<sub>2</sub> was detected with a H<sub>2</sub>DCF-DA stain in 7-day-old light-grown seedlings.

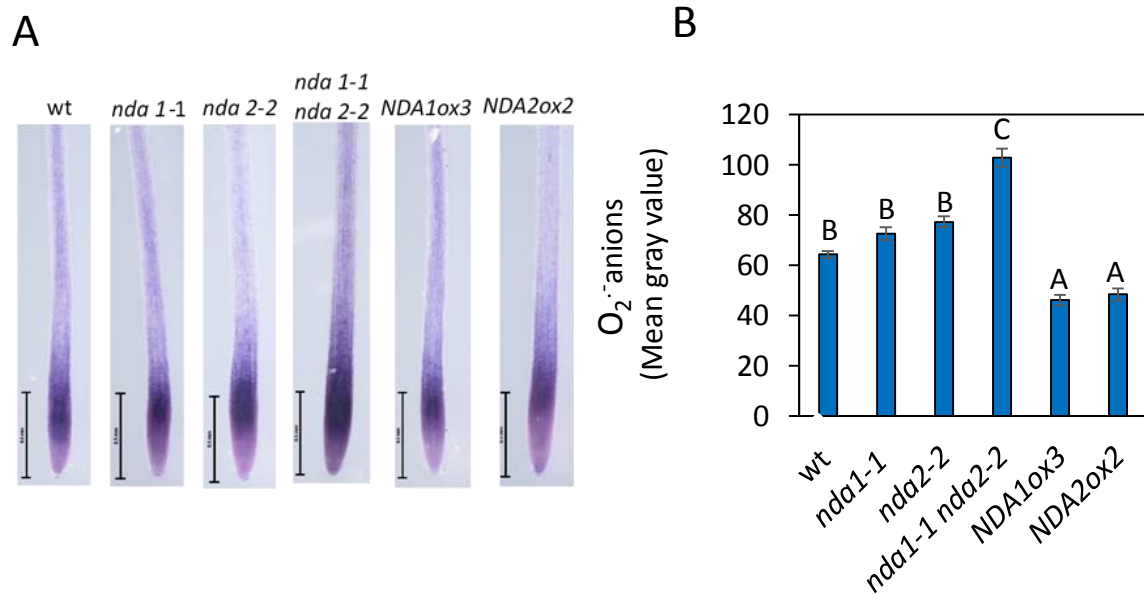
B) The H<sub>2</sub>DCF-DA staining intensity in primary roots was quantified by Image J. Results are means ( $\pm$  SE, n=35 to 42) of three biological replicates. \* Indicates a significant difference to wildtype as analyzed by a Kruskal-Wallis test,  $P < 0.05$ .

### 3.3.2 NDAs limit oxygen anion radical accumulation in the primary root

Apart from H<sub>2</sub>O<sub>2</sub>, we investigated the relative level of O<sub>2</sub><sup>-</sup> using nitro blue tetrazolium chloride (NBT) staining. The primary roots of *nda1-1 nda2-2* seedlings were stained more extensively by NBT stain than those of wt, indicating higher O<sub>2</sub><sup>-</sup> anions accumulation. In contrast, *NDAox* lines exhibited significantly lower O<sub>2</sub><sup>-</sup> levels (Figure 3-8A,B) as quantified according to Nguyen *et al.* (2013). Thus, we observed a difference in accumulations of H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> in primary roots. *NDA* overexpression did not reduce H<sub>2</sub>O<sub>2</sub>, but it did reduce O<sub>2</sub><sup>-</sup>. By contrast, knockout of both *NDAs* in *nda1-1 nda2-2* seedlings elevated both types of ROS (Figure 3-7 and Figure 3-8).

The data show that in terms of ROS homeostasis, one of the *NDA* might compensate for the function of the other. However, the absence of both *NDAs* resulted in increased ROS accumulation and possibly, higher oxidative stress. These data indicate that under standard growth conditions, *NDAs* control ROS accumulation. The mitochondria is a primary site of ROS production inside the living cell and complex I and complex III are major sites of mitochondrial ROS production. *NDAs* together with the alternative oxidase dissipate energy conserved in NAD(P)H as heat and avoid leakage of electrons to O<sub>2</sub> thereby preventing ROS formation.

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**Figure 3-8: NDAs limit accumulation of O<sub>2</sub><sup>-</sup> anions.**

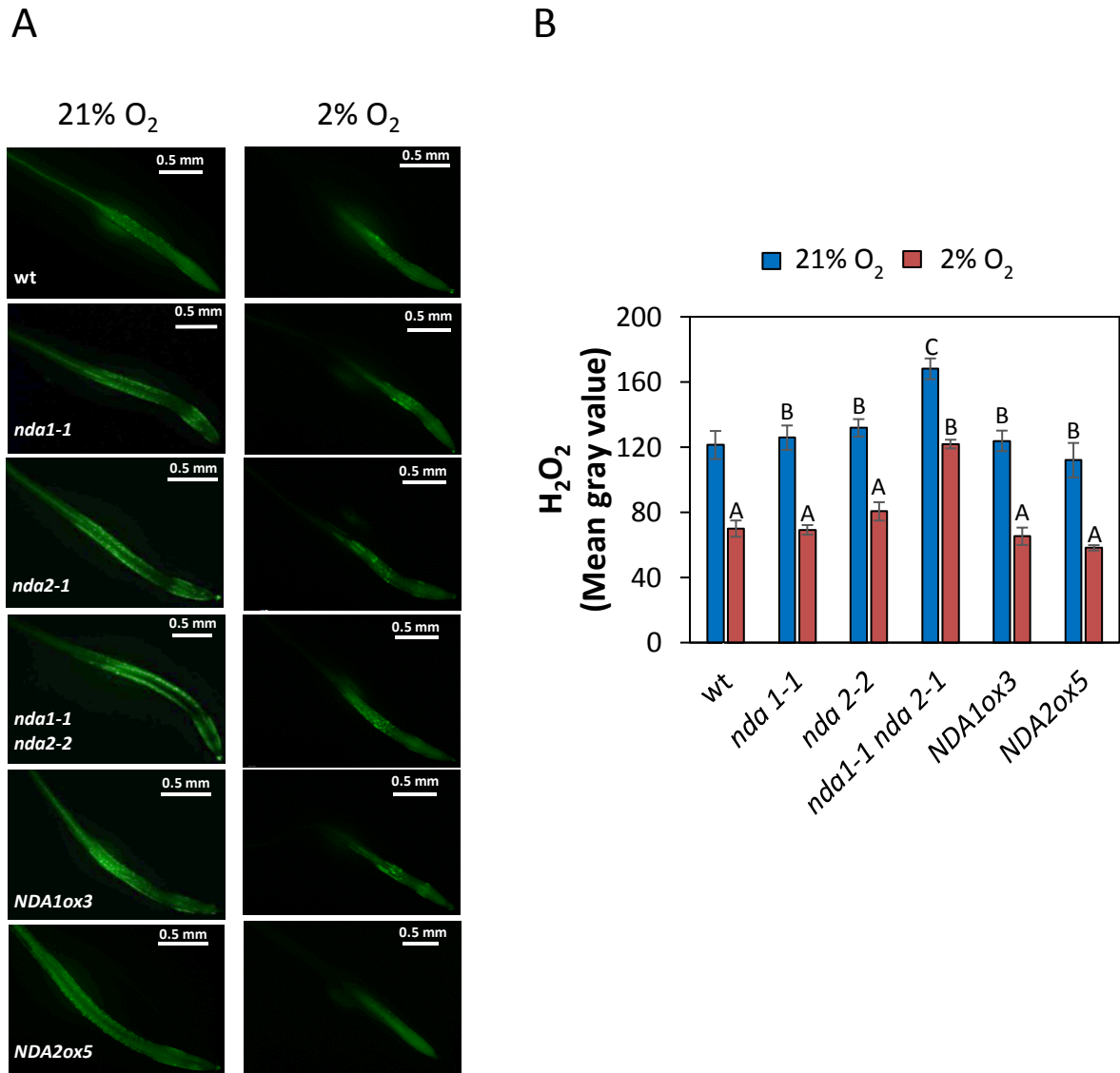
The primary roots of *nda1-1 nda2-2* exhibit increased O<sub>2</sub><sup>-</sup> while NDA overexpression reduced O<sub>2</sub><sup>-</sup> in comparison to wt. A) Detection of O<sub>2</sub><sup>-</sup> using NBT staining in 7-day-old light-grown seedlings. The scale bar in the figure indicates 500  $\mu$ M. B) The staining intensity of NBT in roots was determined with Image J. Results are means ( $\pm$ SE) of three biological replicates. Kruskal-Wallis test ( $n=27$  to  $34$ ;  $p < 0.05$ ).

### 3.3.3 Hypoxia lowers the ROS levels in the primary root independent of NDAs

We next analyzed ROS accumulation in the primary root tip during hypoxia. As mentioned before, we employed H<sub>2</sub>DCF-DA and NBT staining to detect H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> anions, respectively. In all genotypes analyzed, exposure to hypoxia for 16 h resulted in decreased ROS (H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup>) accumulation. However, *nda1-1 nda2-2* seedlings displayed higher ROS accumulation than wild type under normoxic and hypoxic conditions (Figure 3-9 & Figure 3-10).

A possible reason for that might be the ROS accumulation observed under control conditions. While the O<sub>2</sub><sup>-</sup> accumulation was decreased to the similar level in wt and *NDAox* lines after 16 h of hypoxia. The accumulation of O<sub>2</sub><sup>-</sup> was lowest in *NDAox* lines under control conditions and it did not change even after 16 h of hypoxia. The difference in H<sub>2</sub>O<sub>2</sub> accumulation under 21% O<sub>2</sub> was observed in comparison to the Figure 3-7 could be because of incubation of seedlings in dark for 16 h. The *NDA1* has shown to be regulated by light and there are also evidences of differential ROS production in the light and dark (Escobar *et al.* 2004; Elhafez *et al.* 2006).

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**Figure 3-9: Hypoxia reduces H<sub>2</sub>O<sub>2</sub> levels in primary roots.**

A) H<sub>2</sub>O<sub>2</sub> was detected with H<sub>2</sub>DCF-DA staining in the primary roots of 7-day-old light-grown seedlings exposed to air (21% O<sub>2</sub>) or hypoxia (2% O<sub>2</sub>) for 16 h in the dark.

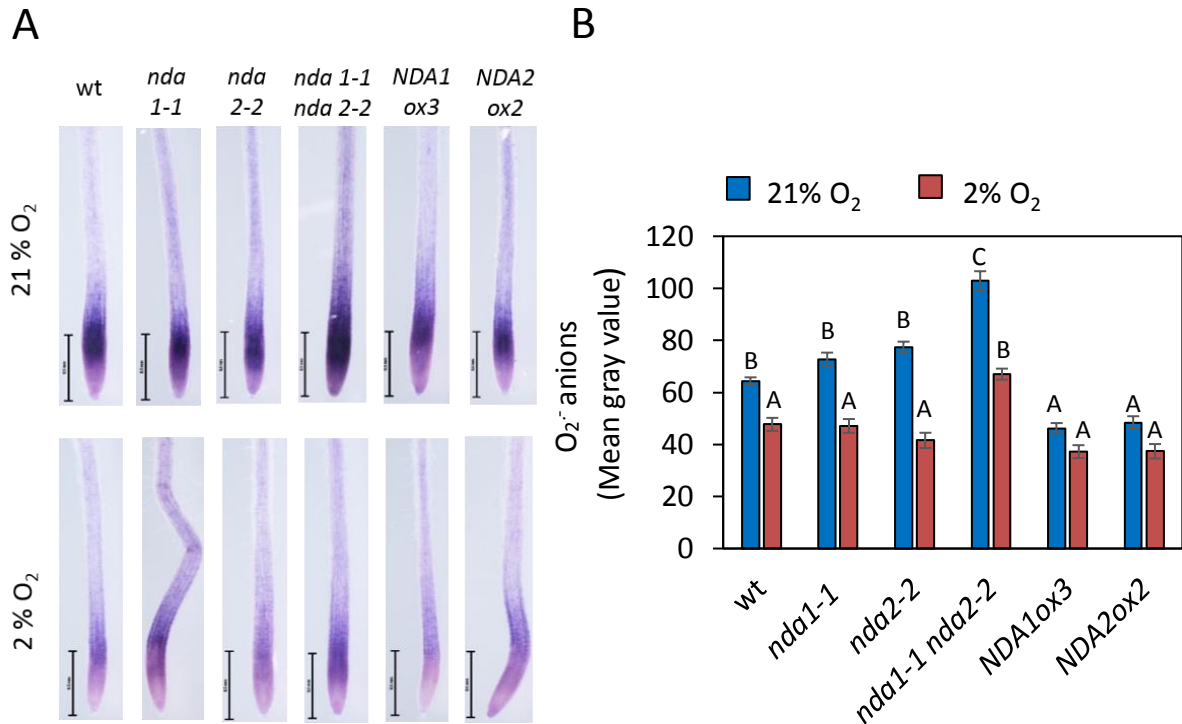
B) Quantification of the H<sub>2</sub>DCF-DA staining intensity in primary roots of wild type and *NDA* mutants by Image J. Results are means ( $\pm$  SE, n=35-37) of three biological replicates, analyzed by one-way ANOVA with Tukey's test,  $P < 0.05$ .

However, the lower ROS accumulation after hypoxia in all genotypes may be explained by the limited availability of oxygen. As O<sub>2</sub> is limited during hypoxia, fewer electrons can be leaked to molecular oxygen resulting in reduced ROS formation. However, the reduction in H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> during hypoxia was independent of NDAs, as knockout of NDAs did not alter it. Nevertheless, the data are in accordance with the expression of *NDAs* that is not elevated during hypoxia (Figure 3-6). Ectopic *NDA* expression did reduce O<sub>2</sub><sup>-</sup> levels at normoxic conditions. Since *NDA* expression was induced by reoxygenation, we decided to analyze ROS



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levels during reoxygenation in NDA knockout and overexpression lines.



**Figure 3-10: Hypoxia reduces O<sub>2</sub><sup>-</sup> anion levels in the primary roots.**

A) Visualization of O<sub>2</sub><sup>-</sup> anions by NBT staining in primary roots of 7-day-old light-grown seedlings exposed to air (21% O<sub>2</sub>) or hypoxia (2% O<sub>2</sub>) for 16 h in the dark. The scale bar in the figure indicates 500 μM.

B) The NBT staining intensity in the primary root was quantified by Image J. Results are means (± SE, n=32 to 36) of three biological replicates analyzed by one-way ANOVA with Tukey's test, *P* < 0.05.

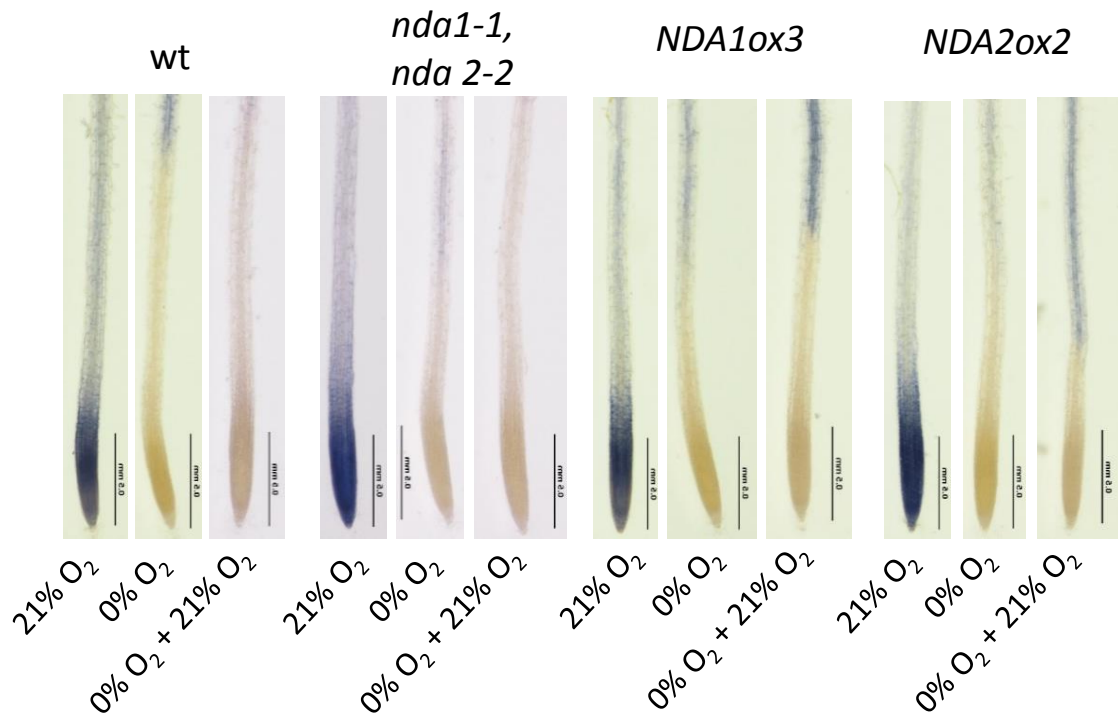
### 3.3.4 O<sub>2</sub><sup>-</sup> levels in the root tip during anoxia and reoxygenation

Results described above showed that expression of *NDA*s was induced during reoxygenation and that *NDA*s limit ROS accumulation in roots under normoxic conditions. To deduce the role of *NDA*s in ROS homeostasis under reoxygenation, we employed NBT staining in order to detect O<sub>2</sub><sup>-</sup> under normoxia, anoxia, and reoxygenation. O<sub>2</sub><sup>-</sup> levels were almost undetectable in anoxia and after 2 h of reoxygenation in the root tip of all genotypes (Figure 3-11). Since reoxygenation imposes massive oxidative stress on plants, this finding was quite astounding. Contrast, the primary root of all genotype did not show any sign of oxidative stress. Possibly due to the reduced growth and metabolic activity during low oxygen condition results in lower electron leakage from electron transport chain (ETC) to O<sub>2</sub>, which might lead to diminished ROS production. Besides, we observed that the ROS accumulation in roots was independent of *NDA*s during hypoxia. Hence, it might also be possible that *NDA*s are also not involved in



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ROS homeostasis during anoxia and reoxygenation in roots.



**Figure 3-11: Levels of O<sub>2</sub><sup>-</sup> at the primary root tip are reduced by anoxia and remain low after 2 h of reoxygenation independent of NDAs.**

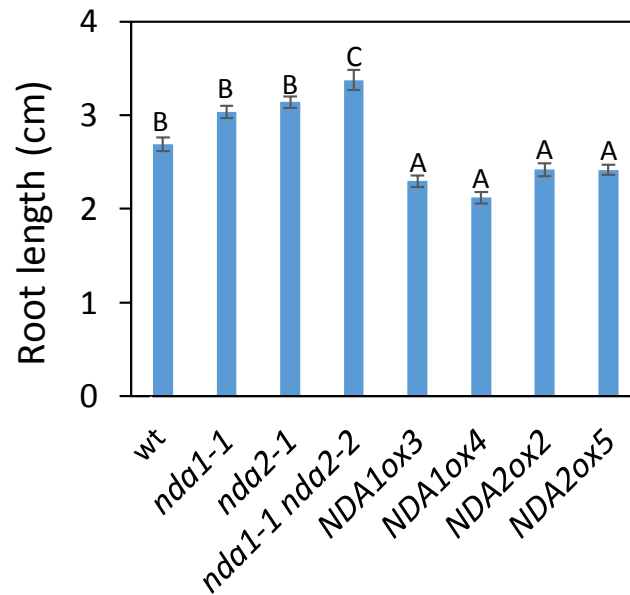
O<sub>2</sub><sup>-</sup> were detected by NBT staining in the primary root of 7-day-old seedlings exposed to 21% O<sub>2</sub> or 0% O<sub>2</sub> for 6 h in the dark or to 0% O<sub>2</sub> for 6 h in the dark followed by recovery in 21% O<sub>2</sub> for 2 h in the light. The scale bar indicates 500 μM in each picture.

### 3.4 Primary and lateral root growth under normoxia and hypoxia

#### 3.4.1 NDAs limit primary root growth under normoxia

To study primary root growth under unstressed conditions, 7-day-old seedlings grown under long-day (LD) conditions were used. The primary root length of wt, *NDA* knock-out, and over-expression mutants were compared. The data revealed that NDAs decrease the primary root elongation as *NDA* over-expression lines showed reduced primary root length while *nda1-1 nda2-2* exhibited increased root length in comparison to wt (Figure 3-12). In contrast, the primary root length of the *nda1-1* and *nda2-2* single knockout seedlings was similar to that of wt, which indicated that both NDAs contribute to root growth inhibition. These results illustrate that under non-stressed conditions, NDAs restrict root growth.

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**Figure 3-12: NDAs inhibit primary root growth at normoxic conditions.**

Average primary root lengths ( $\pm$  SE) of 7-day-old light-grown seedlings were determined in three independent experiments. Different letters indicate significantly different values (Kruskal-Wallis test,  $n = 45-48$ ,  $P < 0.05$ ).

### 3.4.2 The influence of hypoxia and subsequent reoxygenation on primary root growth

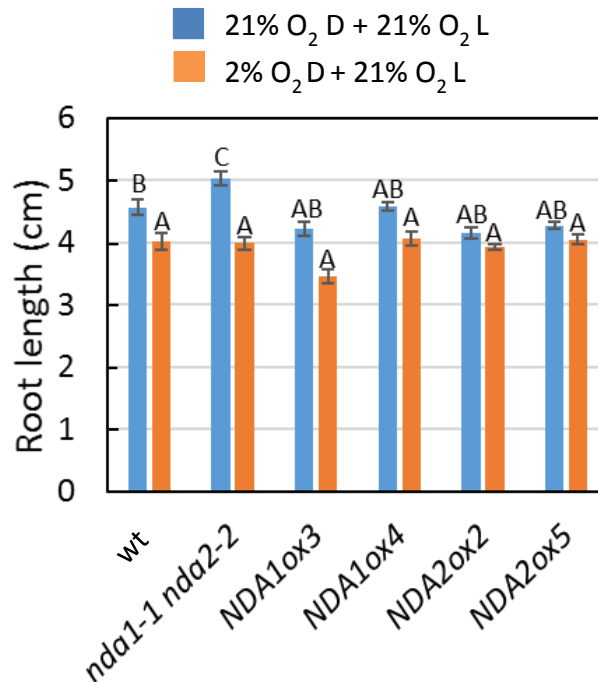
The results from expression analysis in roots gave us a clue that NDAs might have a regulatory role during reoxygenation. To analyze this role, 7-day-old light-grown seedlings were subjected to hypoxia for 2 days and allowed to recover for 2 days, and primary root length was measured. Hypoxia inhibited root growth in wt and *nda1-1 nda2-2* in comparison to control. In contrast, *NDAox* lines did not show a reduction in primary root length in comparison to normoxia grown seedlings upon hypoxia plus reoxygenation. The data suggest that over-expression of NDAs do help plant to resume the normal root growth during reoxygenation. However, we did not observe an opposite effect in the absence of NDAs in *nda1-1 nda2-2*. Moreover, to get a clear idea about root growth during hypoxia followed by reoxygenation, the lateral root growth should also be considered.

### 3.4.3 The effect of hypoxia plus reoxygenation on lateral root growth

To analyze the possible influence of NDAs on root growth during reoxygenation, we also studied lateral root growth in wt and NDA mutants. Root branching plays a fundamental role

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in plants ability to enhance water and nutrient uptake efficiently from the soil. In Arabidopsis, lateral roots (LRs) originate from a pair of pericycle cells at the xylem pole (Himanen *et al.* 2002; Dubrovsky *et al.* 2006). Cells undergo a series of cell divisions until they form a lateral root primordia.



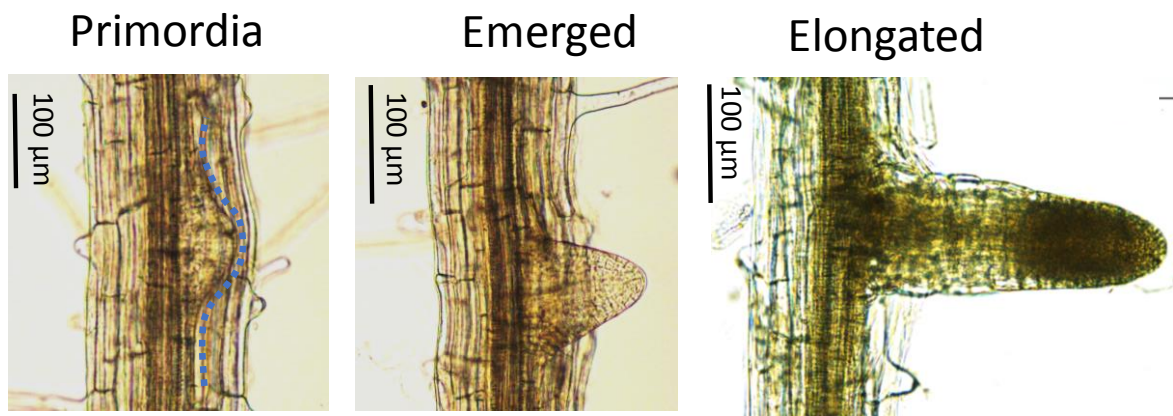
**Figure 3-13: The effect of hypoxia plus reoxygenation on primary root growth.** Six-day-old light-grown seedlings were transferred to the dark in the air (21% O<sub>2</sub>) or hypoxia (2% O<sub>2</sub>) for 2 days and allowed to recover in the air for 2 days in the light. The primary root length was measured at the end of the recovery period. Results are averages ( $\pm$  SE) from three independent experiments. Different letters indicate significantly different values (One-way ANOVA with Tukey's test,  $n = 24$ ,  $P < 0.05$ ).

The development of lateral root primordia can be divided into seven developmental stages, with stage VIII being emerged LRs which elongate into fully grown LRs (Malamy & Benfey 1997). In the current study, we divided LRs into three different categories, LRs from stages III-VII were classified as primordia, LRs from stage VIII were grouped as emerged, and the LRs beyond stage VIII were termed elongated LRs. The three categories of LRs are depicted in Figure 3-14. Six-day-old light-grown seedlings were subjected to hypoxia for 2 days and allowed to recover for 2 days after which the number of primordia, emerged and elongated LRs were counted. The lateral root density was also determined. LR density was not significantly different between control and hypoxia plus reoxygenated seedlings in all genotypes (Figure 3-15A). The number of primordia decreased in wt after hypoxia plus

## Results

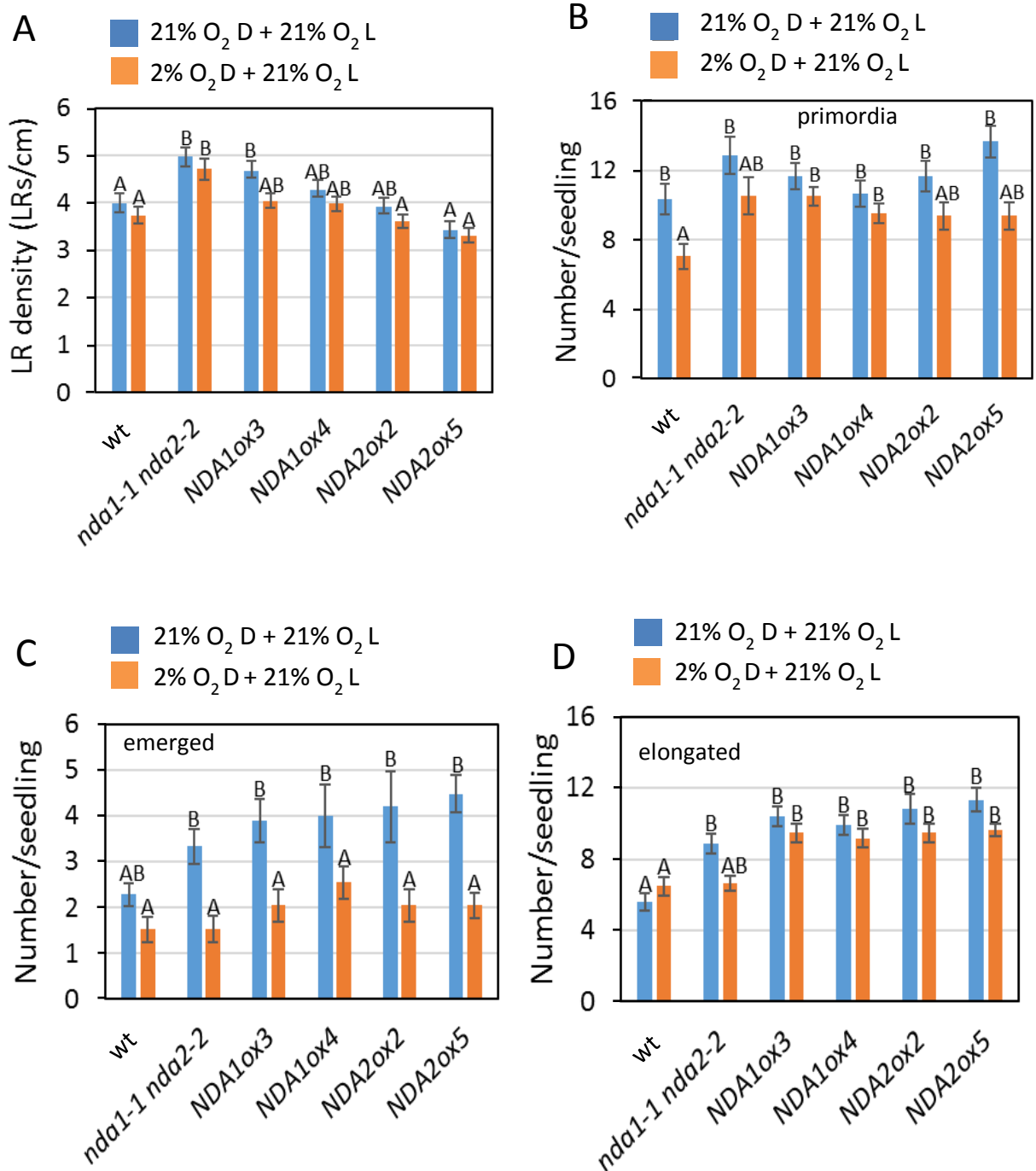
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reoxygenation while in NDA mutants it was similar to the control (Figure 3-15B). On contrary, the number of emerged LRs was considerably decreased in *nda1-1 nda2-2* and *NDAox* lines during hypoxia and subsequent reoxygenation in comparison to normoxia grown roots, while it was unchanged in wt (Figure 3-15). The number of elongated LRs was unaffected by hypoxia followed by reoxygenation in all genotypes in comparison to normoxia-grown seedlings. Concerning LR growth, we could only see the difference between wt and NDA mutants in the number of primordia and emerged LRs. In the wt the number of primordia was reduced while in NDA knock-outs and over-expression lines the number of emerged LRs were decreased during hypoxia-reoxygenation. However, we could not see an antagonistic effect of reduced and elevated NDA expression as *nda1-1 nda2-2* and *NDAox* lines behaved similarly during hypoxia plus reoxygenation. Overall, the data suggest that NDAs might not be involved in primary or lateral root development during hypoxia and subsequent reoxygenation.



**Figure 3-14: Examples of primordia, penetrated and elongated LRs.**

## Results



**Figure 3-15: NDAs are not involved in lateral root elongation during reoxygenation following hypoxia.**

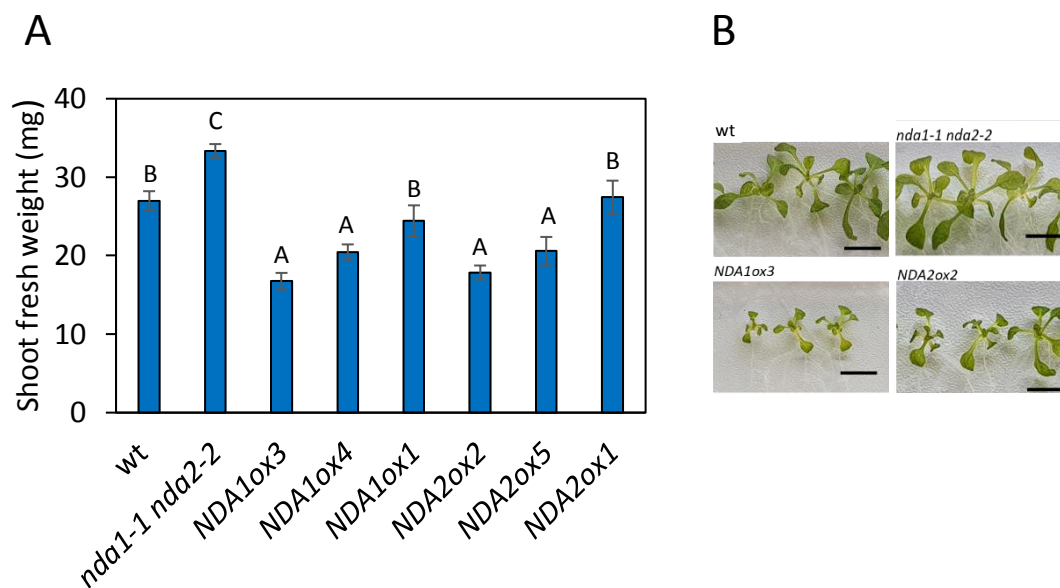
Six-day-old light-grown seedlings were transferred to the dark in air (21% O<sub>2</sub>) or hypoxia (2% O<sub>2</sub>) for 2 days and allowed to recover in 21% O<sub>2</sub> for 2 days in the light. The number of primordial, penetrated, and elongated lateral roots were counted at the end of the recovery period. The density was calculated by dividing the number of total lateral roots by primary root length. Results are averages ( $\pm$  SE) from three independent experiments. Different letters indicate significantly different values (One-way ANOVA with Tukey's test,  $n = 24$ ,  $P < 0.05$ ).

- A) Lateral root density.
- B) Number of lateral root primordia.
- C) Number of emerged lateral roots.
- D) Number of elongated lateral roots after the reoxygenation phase.

## Results

### 3.5 NDAs limit shoot growth under normoxic conditions

To analyze shoot growth under standard growth conditions, shoot fresh weights of 14-d-old wt and NDA mutant seedlings grown under long-day (LD) conditions were determined. *nda1-1 nda2-2* plants had a higher biomass in comparison to wt. In contrast, the NDA over-expression lines *NDA1ox3*, *NDA1ox4* and *NDAox2*, *NDA2ox5* exhibited a reduced biomass in comparison to wt whereas shoot fresh weights of *NDA1ox1* and *NDA2ox1* were similar to the wt in accordance with a lower transcript abundance in comparison to other over-expression lines (Figure 3-2). Hence, the data indicated that under standard growth conditions NDAs limit shoot growth (Figure 3-16). A similar effect was observed for root growth (Figure 3-12). In general, under non-stressed condition NDAs reduce the seedling growth. Therefore, the plant must control the expression of NDAs to achieve optimal growth.



**Figure 3-16: NDAs limit growth under non-stressed conditions.**

A) Shoot fresh weight of 14-day-old seedlings grown in 16 h light/8 h dark on vertically oriented plates. Results are means ( $\pm$  SE, n=35-42) of three biological replicates analyzed by one-way ANOVA with Tukey's test,  $P < 0.05$ .

B) Representative pictures of wt, *nda1-1 nda2-2*, *NDA1ox3*, and *NDA2ox2* seedlings. Scale bars in the figures indicate 1 cm.

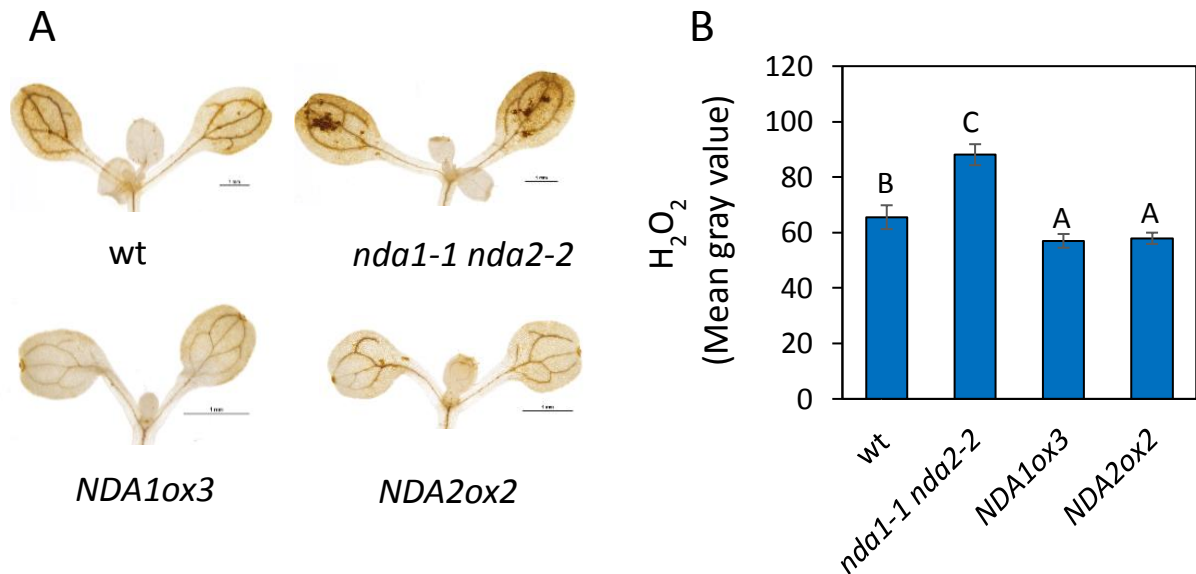
### 3.6 Control of reactive oxygen species (ROS) in shoots by NDAs

#### 3.6.1 NDAs control H<sub>2</sub>O<sub>2</sub> accumulation in shoots

In order to analyze H<sub>2</sub>O<sub>2</sub> accumulation in shoots, we used 3,3'-diaminobenzidine (DAB)

## Results

staining. As observed in primary roots, shoots of *nda1-1 nda2-2* accumulated higher amounts of  $H_2O_2$  in comparison to wt, while the *NDA* overexpression lines *NDA1ox3* and *NDA2ox2* accumulated less  $H_2O_2$  than wt (Figure 3-17). The quantification of  $H_2O_2$  staining as mean gray value confirmed the significant differences in  $H_2O_2$  between wt, *nda1-1 nda2-2* and *NDAox* lines suggesting that *NDA*s prevent oxidative stress under normal growth condition.



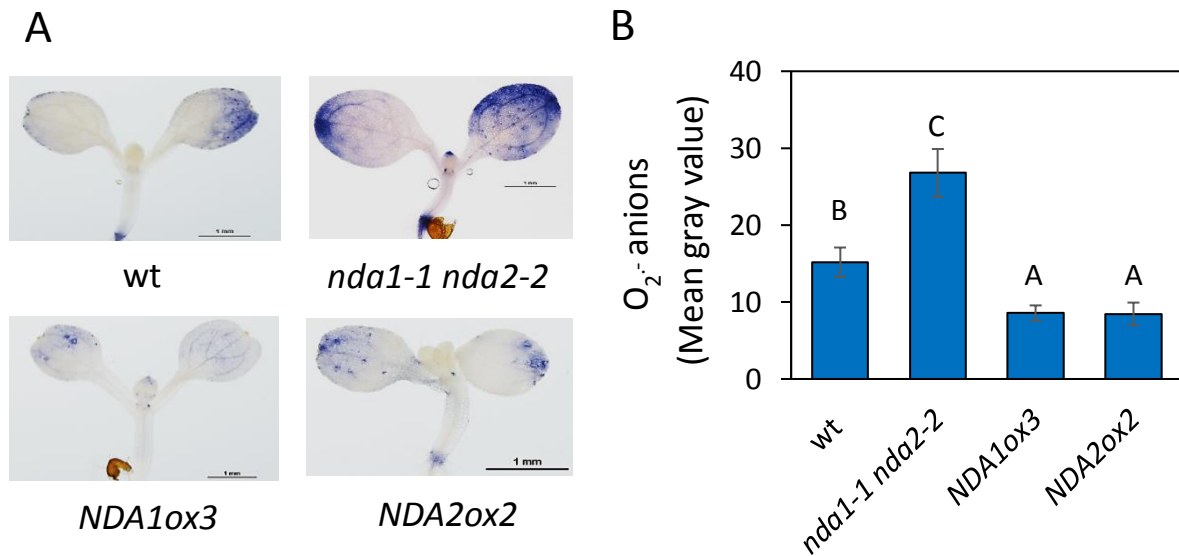
**Figure 3-17: NDAs control  $H_2O_2$  levels under nonstress conditions.**

A) Detection of  $H_2O_2$  with DAB in shoots of 7-day-old light-grown seedlings. Scale bars indicate 1 mm. B) The staining intensity of DAB stained leaves was determined with Image J. Results are means ( $\pm$  SE,  $n=32-35$ ) of three biological replicates analyzed by Kruskal-Wallis test,  $P < 0.05$ .

### 3.6.2 NDAs maintain $O_2^{\cdot-}$ anion accumulation in shoots

We next investigated the presence of  $O_2^{\cdot-}$  anions using nitro blue tetrazolium chloride (NBT) staining. The data was consistent with the  $O_2^{\cdot-}$  anions accumulation data in primary roots. Shoots of *nda1-1 nda2-2* were stained more heavily than wt indicating higher  $O_2^{\cdot-}$  anions accumulation. In contrast, *NDA1ox3* and *NDA2ox2* shoots exhibited significantly less  $O_2^{\cdot-}$  anions (Figure 3-18), as quantified via the method described by Nguyen *et al.* (2013). Taken together, more  $H_2O_2$  and  $O_2^{\cdot-}$  anions accumulated in leaves of *nda1-1 nda2-2* and fewer in leaves of *NDAox* lines compared to wt indicating that under normal growth conditions ROS homeostasis in the shoot is controlled by *NDA* activity.

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**Figure 3-18: NDAs control the levels of  $O_2^{\cdot -}$  under unstressed conditions.**

A)  $O_2^{\cdot -}$  anions detected with NBT in shoots of 7-day-old light-grown seedlings. Scale bars indicate 1 mm.

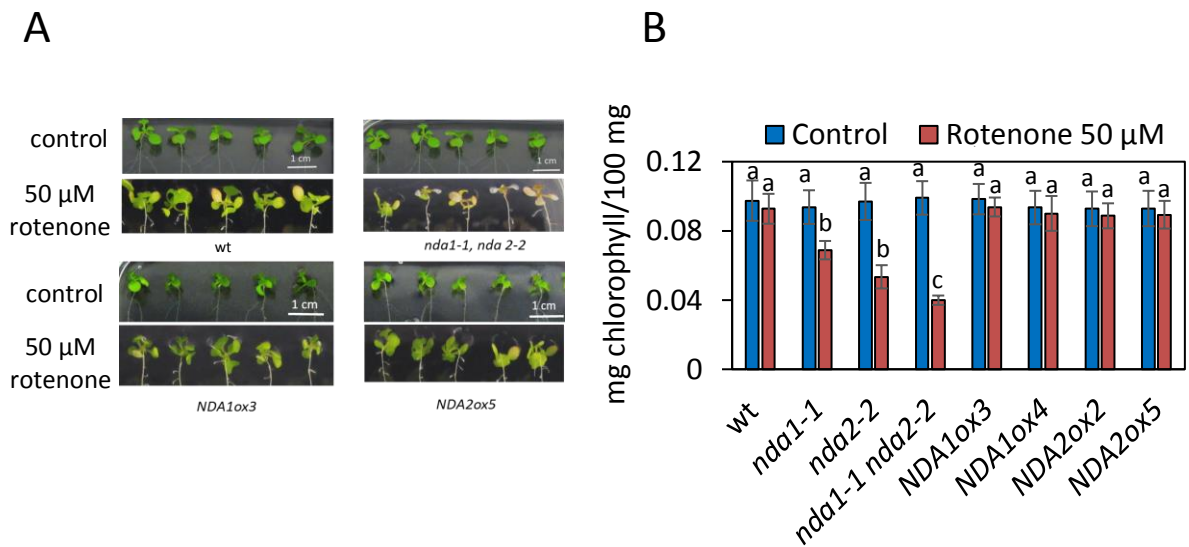
B) The staining intensity of NBT stained leaves was determined with Image J. Results are means ( $\pm$  SE,  $n=28$  to 32) of three biological replicates analyzed by Kruskal-Wallis test,  $P < 0.05$ .

### 3.7 NDAs are essential when complex I is inhibited

Complex I of the mitochondrial ETC is a proton-pumping type I NADH(P) dehydrogenase that is inhibited by rotenone. In contrast, the internal type II NADH(P) dehydrogenase (NDAs) are insensitive to rotenone. The rotenone-insensitive NDAs bypass complex I and oxidize mitochondrial matrix NADH (Rasmusson and Møller 1991). To assess the role of NDAs in the absence of a functional complex I, we transferred seven-d-old seedlings to plates with 50  $\mu$ M rotenone and grew them for seven more days. After seven days on rotenone, the seedlings of all genotype showed an abnormal growth phenotype in comparison to the untreated seedlings including signs of leaf chlorosis. The chlorophyll content was determined as a measure for plant fitness. Under control conditions, it was comparable in all genotypes. However, after rotenone treatment, *nda1-1*, *nda2-2*, and *nda1-1 nda2-2* seedlings showed a considerable reduction in chlorophyll content. *nda1-1 nda2-2* seedlings showed the lowest amount of chlorophyll among the genotypes tested. In contrast, the chlorophyll content did not decline in wt, *NDA1ox*, and *NDA2ox* lines after seven days of rotenone treatment (Figure 3-19). The data indicate that in the absence of complex I, NDAs maintain chloroplast function likely by restricting ROS formation in mitochondria.



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**Figure 3-19: Knock-out of *NDA* genes renders seedlings susceptible to the inhibitor of complex I in the mitochondrial ETC, rotenone.**

Seven-day-old light-grown seedlings were treated with 50  $\mu$ M rotenone or left untreated for seven days.

A) Representative pictures of control and rotenone-treated seedlings.

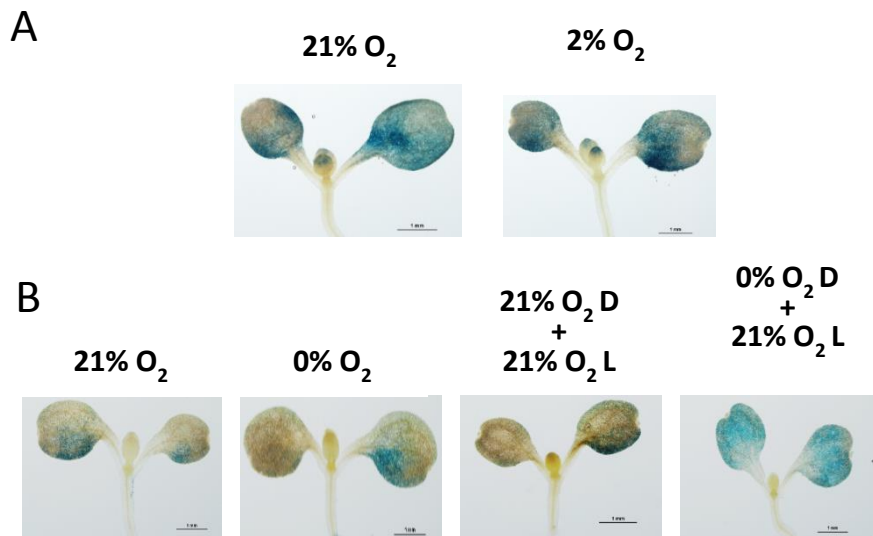
B) Average ( $\pm$  SE,  $n=3$ ) chlorophyll content. Asterisks indicate a significant difference to the control analyzed by Kruskal-Wallis test,  $P < 0.05$ .

### 3.8 Expression of NDAs under hypoxia and reoxygenation in shoots

#### 3.8.1 *NDA1* promoter activity under hypoxia, anoxia and reoxygenation

To investigate the role of *NDA1* in shoot adaptation to low oxygen and reoxygenation, *NDA1:GUS* seedlings were subjected to 2%  $O_2$  or 21%  $O_2$  and histochemical GUS staining was carried out. No differences in GUS activity in shoots was observed between seedlings subjected to normoxia and hypoxia (Figure 3-20). These results were comparable to the observations made in roots where GUS activity did not change in response to hypoxia (Figure 3-4). Similarly, GUS activity was also unchanged during anoxia (0%  $O_2$ ) in comparison to normoxia. In contrast, *NDA1* promoter activity considerably increased in shoots during reoxygenation (0%  $O_2$  + 21%  $O_2$ ) (Figure 3-20) pointing to a role of *NDA1* under this stress.

## Results



**Figure 3-20: Reoxygenation induces *NDA1:GUS* expression in the shoot.**

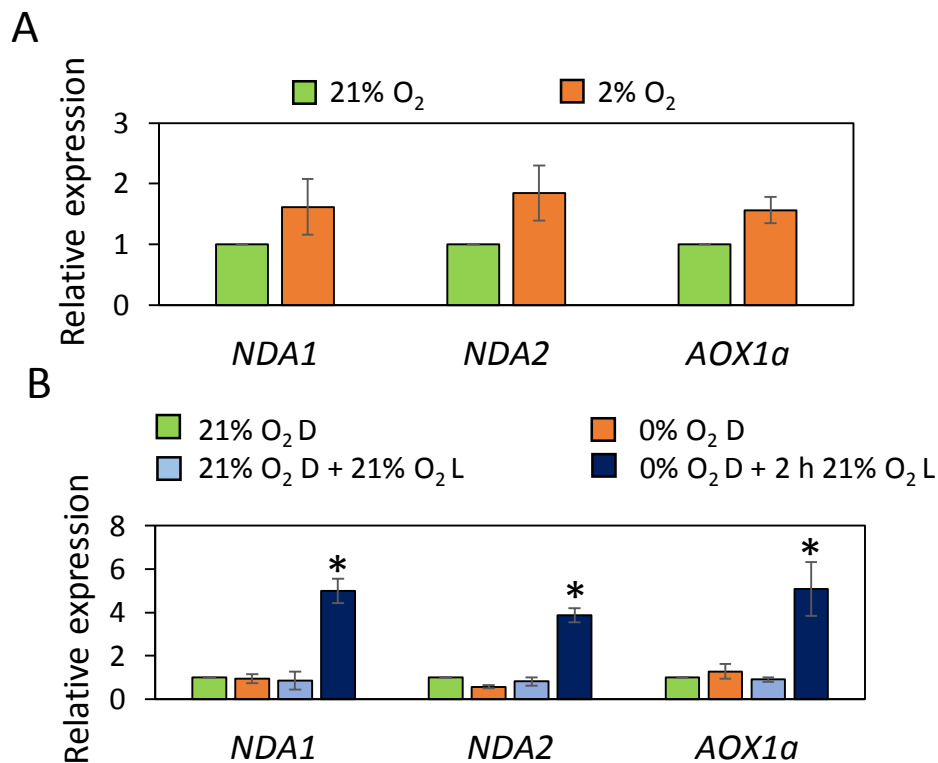
A) *NDA1:GUS* activity in shoots of 7-day-old-seedlings exposed to air (21% O<sub>2</sub>) or hypoxia (2% O<sub>2</sub>) for 6 h in the dark.

B) Seedlings subjected to air (21% O<sub>2</sub>) or anoxia (0% O<sub>2</sub>) for 6 h in the dark and exposed to air (21% O<sub>2</sub>) or anoxia (0% O<sub>2</sub>) for 6 h in the dark followed by re-/oxygenation in air (21% for 2 h in the light). The scale bar indicates 1 mm.

### 3.8.2 Expression of *NDA*s and *AOX1a* increases during reoxygenation

In order to quantify the gene expression changes in shoots during reoxygenation, we employed qRT-PCR. The expression of *NDA1*, *NDA2* and *AOX1a* was analyzed under normoxia, hypoxia, anoxia, and during reoxygenation in shoots. The expression on *NDA*s was not induced during hypoxia (2% O<sub>2</sub>) and anoxia (0% O<sub>2</sub>) confirming the GUS expression data. The expression of *NDA1* and *AOX1a* was increased close to 5-fold, while expression of *NDA2* was induced close to 4-fold during reoxygenation (0% O<sub>2</sub> + 21% O<sub>2</sub>) compared to normoxia in accordance with the GUS data (Figure 3-21). The results obtained from GUS and qRT-PCR expression analysis suggested a significant role of the alternative electron transport pathway via *NDA*s and *AOX1a* in reoxygenation stress adaptation in the shoot. During low oxygen conditions, the electron transport chain is in a highly reduced state due to the lack of O<sub>2</sub>. Hence, the alternative respiratory chain is needed the most when O<sub>2</sub> is present after a period of low oxygen to prevent electron leakage to molecular oxygen.

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**Figure 3-21: Reoxygenation induces expression of *NDA1*, *NDA2*, and *AOX1a* in the shoot.**

A) Relative transcript levels of *NDA1*, *NDA2*, and *AOX1a* were analyzed by qPCR in shoots of 7-day-old wild-type seedlings exposed to air (21% O<sub>2</sub>) or hypoxia (2% O<sub>2</sub>) for 6 h in the dark

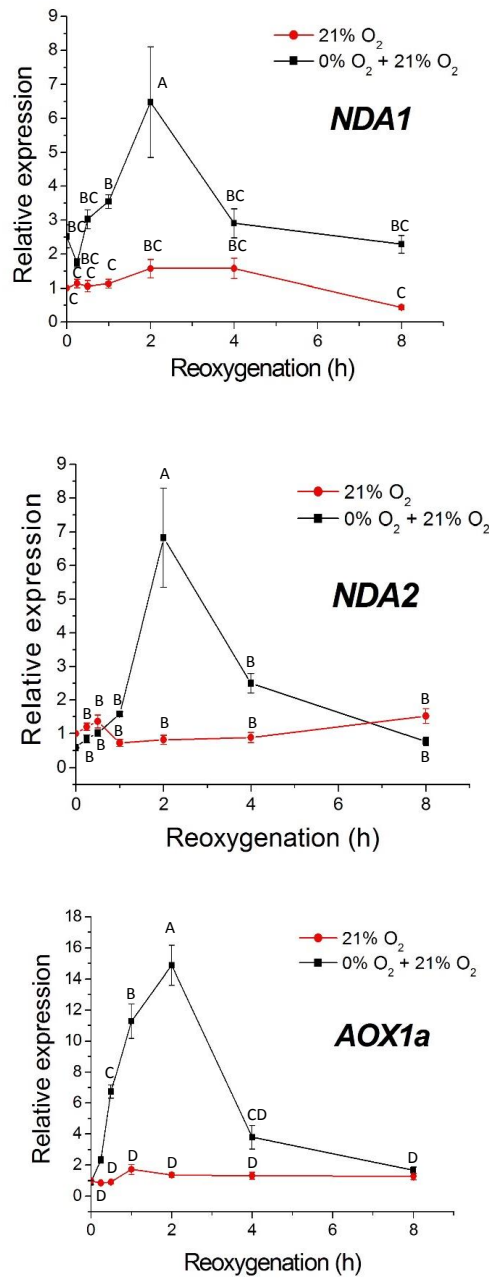
B) Relative transcript levels in seedlings exposed to air (21% O<sub>2</sub>) or anoxia (0% O<sub>2</sub>) for 6 h in the dark followed by exposure to 21% O<sub>2</sub> for 2 h in the light. Results are means ( $\pm$  SE) of three independent biological replicates with two technical repeats each (Mann-Whitney test,  $P < 0.05$ ).

### 3.8.3 Expression of *NDA*s and *AOX1a* peak after 2 h of reoxygenation

To study the regulation of the internal alternative ETC during reoxygenation in more detail, we used qRT-PCR to study the expression of *NDA*s and *AOX1a* from 0 h to 8 h of reoxygenation after 6 h of anoxia. *NDA1*, *NDA2* and *AOX1a* showed maximum expression after 2 h of reoxygenation. In the case of *NDA1* and *AOX1a*, the expression level gradually increased from 0 h until 2 h whereas expression of *NDA2* increased rapidly between 1 h and 2 h of reoxygenation. The expression level of all three genes decreased after 4 h and reached a minimum level after 8 h of reoxygenation (Figure 3-22). The change in expression was due to O<sub>2</sub> rather than light as seedlings analyzed as a control in normoxia were also shifted from dark to light for the duration of the treatment. At this point, it is not clear whether *NDA*s and *AOX1a* are controlled by oxygen or by the oxidative stress that occurs as a consequence of reoxygenation. It is clear however that the internal alternative ETC plays a significant role

# Results

during adaptation to early reoxygenation stress in shoots.



**Figure 3-22: Expression of *NDA1*, *NDA2*, and *AOX1a* peaks after 2 h of reoxygenation.**

qPCR expression analysis of *NDA1*, *NDA2*, and *AOX1a* in 7-day-old light-grown seedlings exposed to air (21% O<sub>2</sub>) or anoxia (0% O<sub>2</sub>) for 6 h in the dark followed by a recovery phase in air for 0-8 h in the light. Results are means ( $\pm$  SE) of three independent biological replicates with two technical repeats each (one-way ANOVA with Tukey's test,  $P < 0.01$ ).

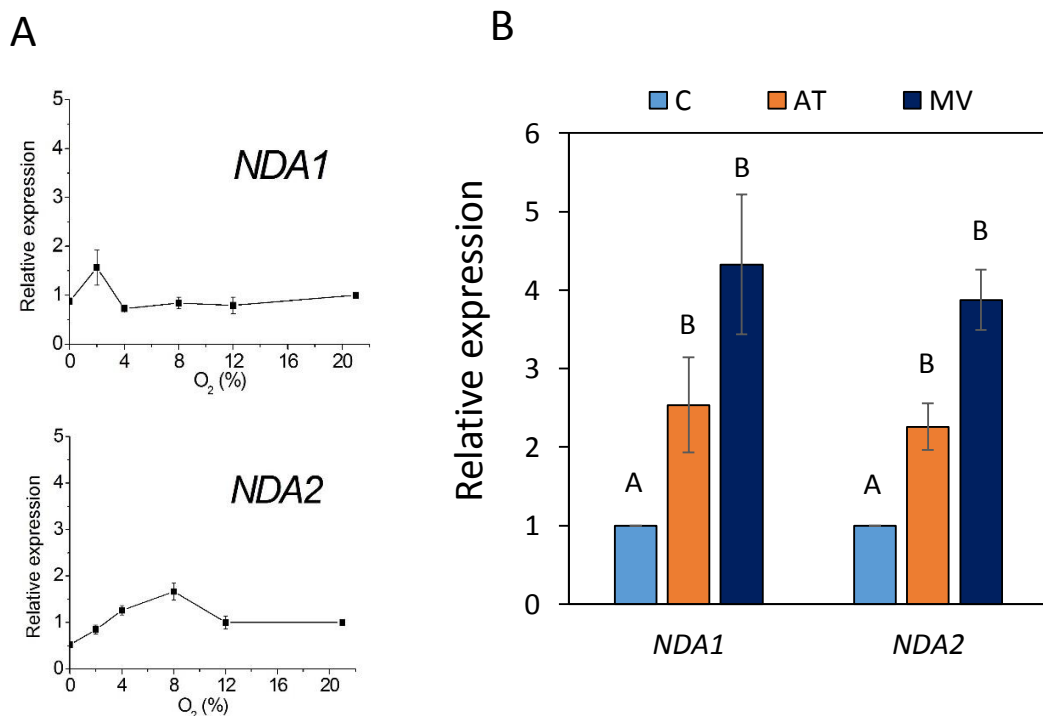
## 3.8.4 The expression of NDAs is regulated by internal ROS

To test whether the expression of *NDAs* is controlled by the atmospheric oxygen concentration, we subjected 7-d-old seedlings to the gradient of oxygen concentration for 6 h

## Results

in the dark. We used 2% to 21% of oxygen gradient and analyzed the change in the expression level of *NDA*s. Although the expression level of *NDA1* and *NDA2* was unchanged by the percentage of atmospheric oxygen present (Figure 3-23). The results indicated that both *NDA1* and *NDA2* expression did not seem to have any relationship with the amount of oxygen present in the surroundings.

To check whether endogenous ROS accumulation altered *NDA* expression 7-d-old seedlings were treated with 3-amino-1,2,3 triazole (AT) that inhibits catalase activity and with methyl viologen (MV) that inhibits electron transfer in chloroplasts to induce ROS accumulation inside the cell. In comparison to mock-treated seedlings, the expression levels of *NDA1* and *NDA2* we increased significantly after exposure to AT and MV. Upon AT treatment, the expression of *NDA*s was induced close to 2.5 fold, while upon MV treatment, which is known to accumulate ROS inside the cells rapidly, *NDA*s expression increased up to 4 fold (Figure 3-23). The data indicated that expression of *NDA1* and *NDA2* is controlled by endogenous ROS rather than oxygen.



**Figure 3-23: *NDA* expression is controlled by ROS rather than low oxygen.**

A) qPCR expression analysis of *NDA1* and *NDA2* in 7-day-old light-grown seedlings exposed to different oxygen concentrations for 6 h in the dark.

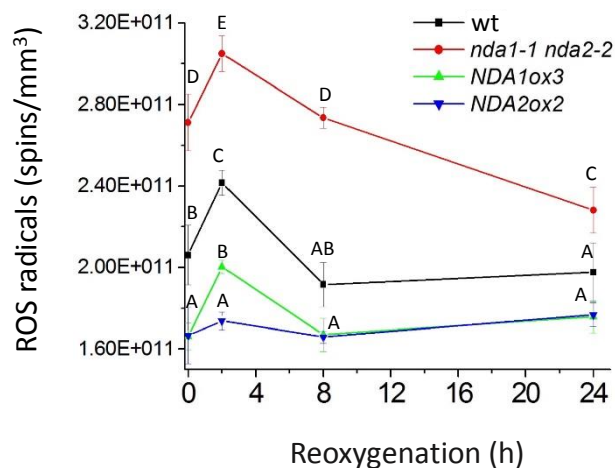
B) Expression of *NDA1* and *NDA2* in seedlings treated with 3-amino-1,2,3 triazole (AT) or methyl viologen (MV) or left untreated (C) for 3 h in the light. Results are means ( $\pm$  SE) of three independent biological replicates with two technical repeats each (one-way ANOVA with Tukey's test,  $P < 0.05$ ).

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### 3.9 The impact of NDAs on ROS homeostasis during reoxygenation

#### 3.9.1 NDAs maintain ROS homeostasis

From previous experiments, we learned that the expression of *NDAs* is stimulated by ROS formed during reoxygenation. For a better time resolution, we analyzed ROS accumulation at different time points of reoxygenation. Electron paramagnetic resonance (EPR) spectroscopy was used to detect the presence of oxygen anion radicals ( $O_2^{\cdot-}$ ) in shoots. There was a significant difference in terms of accumulation of ROS between *wt*, *nda1-1 nda2-2*, and *NDAox* lines. *nda1-1 nda2-2* shoots accumulated the highest  $O_2^{\cdot-}$  levels between 0 and 8 h of reoxygenation. In contrast, *NDA1ox3* and *NDA2ox2* seedlings produced the least  $O_2^{\cdot-}$  during reoxygenation. In *wt*,  $O_2^{\cdot-}$  accumulated at an intermediary level between *nda1-1 nda2-2* and *NDAox* lines. In all genotypes the maximum  $O_2^{\cdot-}$  level was observed after 2 h of reoxygenation (Figure 3-24). The results from EPR spectroscopy further supported the finding that NDAs limit oxidative stress in shoots in particular during the early reoxygenation phase.



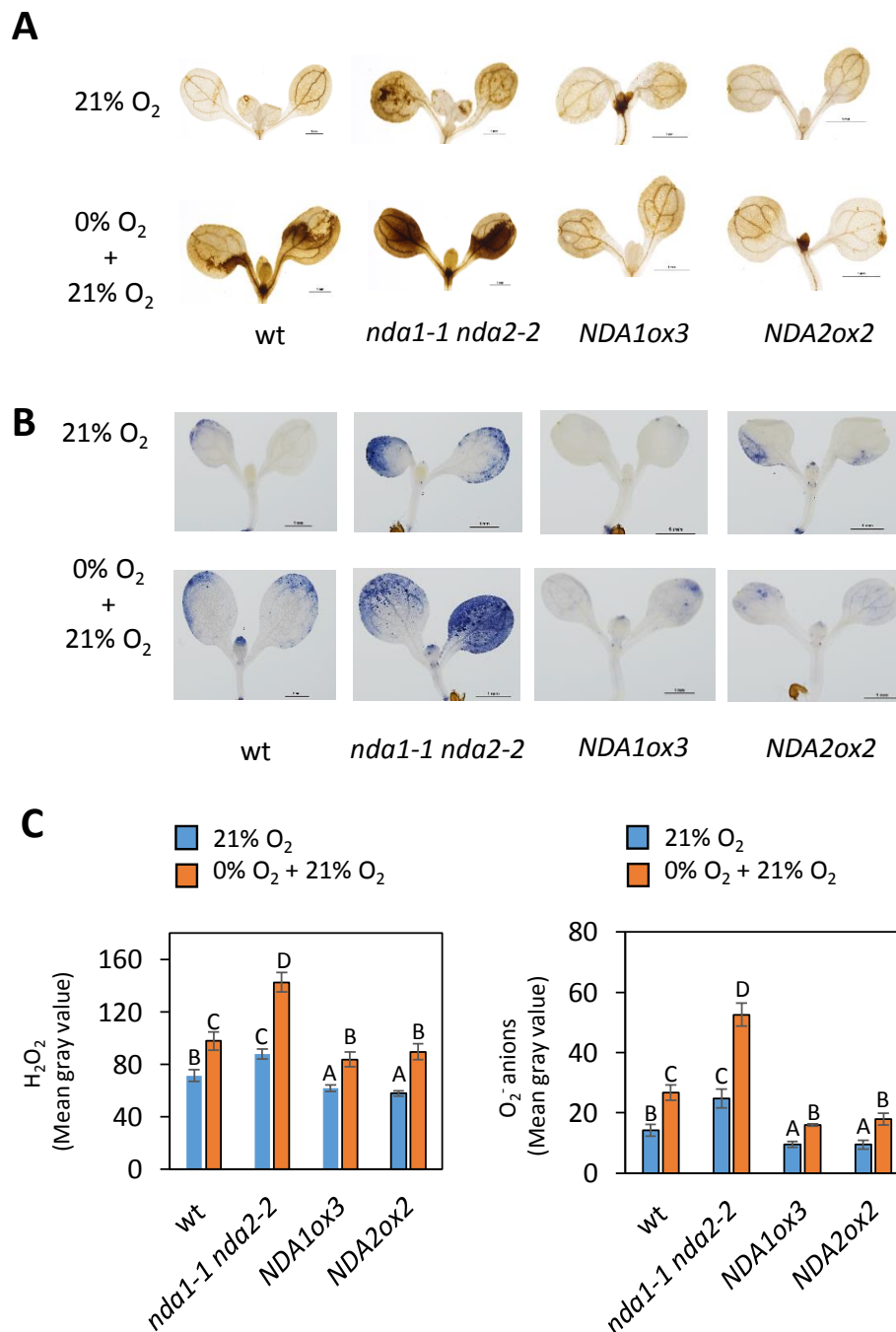
**Figure 3-24: NDAs control ROS homeostasis.**

ROS were quantified by electron paramagnetic resonance (EPR) spectroscopy in shoots of 7-day-old light-grown seedlings exposed to 0%  $O_2$  for 16 h in the dark followed by recovery in air for 0-24 h in the light. Results are means ( $\pm$  SE) of three biological replicates (one-way ANOVA with Tukey's test,  $P < 0.05$ ).

#### 3.9.2 NDAs constrain $H_2O_2$ and $O_2^{\cdot-}$ anions accumulation in shoot

To specify the ROS that accumulate in shoots during reoxygenation and their time course of accumulation, we used DAB and NBT staining methods were used as described earlier. The

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**Figure 3-25: NDAs control H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> levels.**

A, B) DAB staining of H<sub>2</sub>O<sub>2</sub> and NBT staining of O<sub>2</sub><sup>-</sup> in 7-day-old light-grown seedlings exposed to 21% O<sub>2</sub> or 0% O<sub>2</sub> for 16 h in the dark followed by recovery in 21% O<sub>2</sub> for 2 h in the light. The scale bar indicates 1 mm.

C) The staining intensity of DAB and NBT-stained areas in leaves was determined with Image J. Results are means (± SE, n=25-32) of three biological replicates (Kruskal-Wallis test, *P* < 0.05).

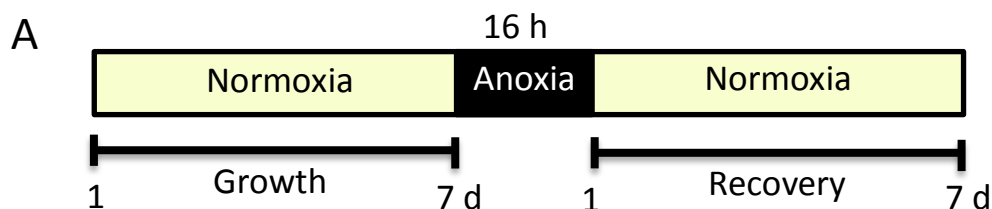
results showed that more H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> accumulated after 2 h of reoxygenation in all genotypes in comparison to seedlings subjected to normoxia. In support of the results

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obtained by EPR spectroscopy *nda1-1 nda2-2* seedlings accumulated the highest amounts of H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> during normoxia and reoxygenation while *NDA1ox3* and *NDA2ox2* showed the lowest H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> levels. As observed with EPR spectroscopy, shoots of wt showed intermediate ROS accumulation under normoxia as well as after 2 h of reoxygenation (Figure 3-25). In summary, NDAs are important players in maintaining ROS homeostasis in shoots, particularly during reoxygenation.

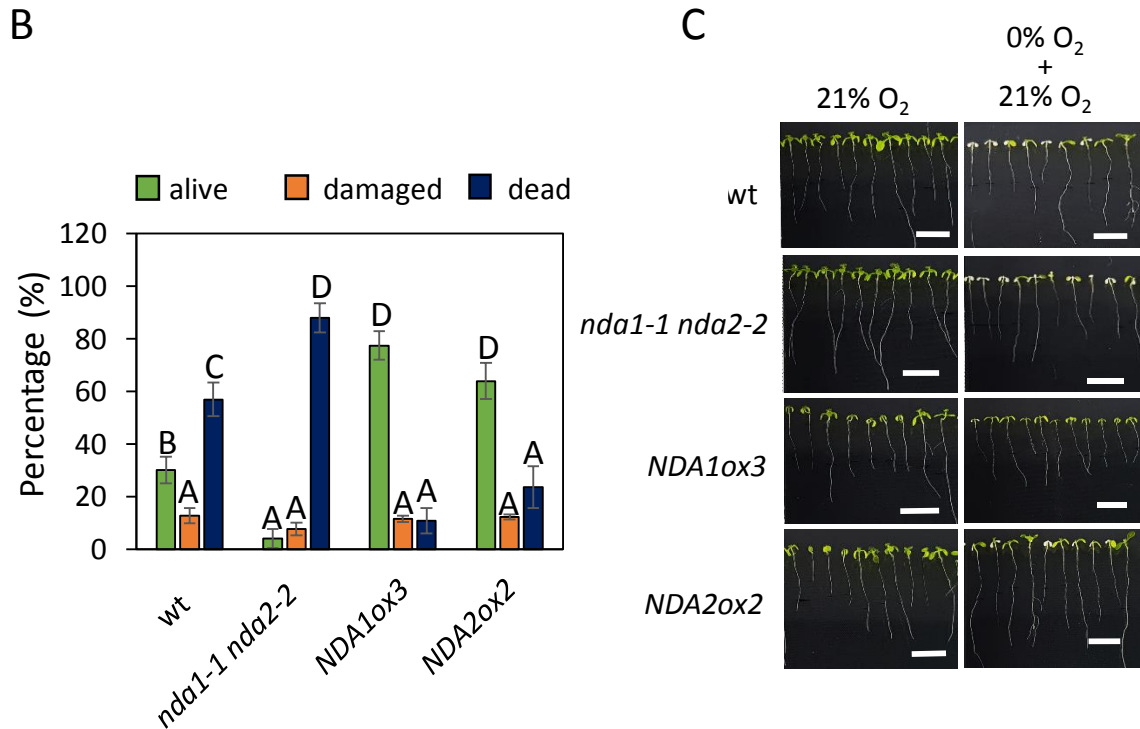
### 3.10 NDAs are essential for seedling survival during reoxygenation

The results of *NDA* expression analysis and ROS measurements in *NDA* mutant lines suggested a significant role of NDAs in shoot resilience to reoxygenation. In order to test such a role seven-d-old seedlings were subjected to either control (21% O<sub>2</sub>) or anoxia (0% O<sub>2</sub>) in the dark and allowed to recover in ambient air for 7 d (Figure 3-26) and seedling survival was calculated. The phenotypes observed after 7 d of recovery were categorized as shown in Figure 3-26. After 7 d of reoxygenation, close to 50% wt seedlings were dead. In comparison *nda1-1 nda2-2* seedlings showed a 90% death rate. The *NDA1ox3* and *NDA2ox2* lines performed better during reoxygenation with death rates of about 20% and 30%, respectively (Figure 3-26). The percentage of damaged seedlings were almost equal in all genotypes analyzed. In correlation with ROS accumulation in shoots, we can say that seedlings under higher oxidative stress during early reoxygenation exhibited a low survival rate while seedlings with low ROS accumulation during early reoxygenation displayed better survival rates. On the other hand, ROS accumulation during reoxygenation is directly dependent on NDAs providing a direct link between mitochondrial alternative electron transport and reoxygenation stress resistance.





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**Figure 3-26: NDAs improve seedling survival during reoxygenation.**

A) Seven-day-old light-grown seedlings were exposed to air (21% O<sub>2</sub>) or anoxia (0% O<sub>2</sub>) for 16 h in the dark and returned or maintained in air for 7 days in a light/dark cycle.

B) The percentage of live, damaged, and dead seedlings was determined after 7 days of recovery. Results are means ( $\pm$  SE) of three biological replicates. Different letters indicate significantly different values (Kruskal-Wallis test,  $P < 0.05$ ).

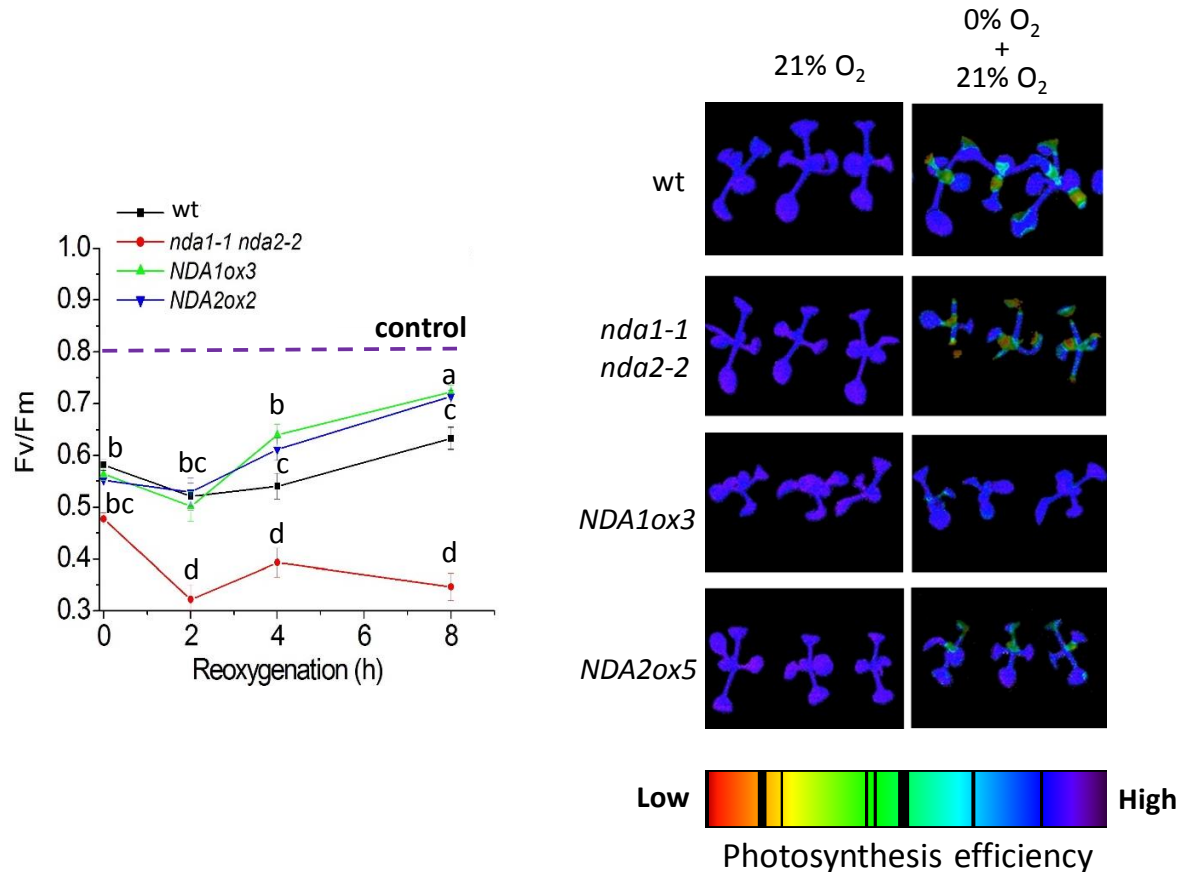
C) Plant phenotypes observed at the end of the 7 day recovery phase.

### 3.11 NDAs are vital to photosynthetic recovery during reoxygenation

In order to check photosynthetic capacity during reoxygenation, the maximum quantum efficiency of photosystem II was measured by analyzing Fv/Fm (variable fluorescence/ maximal fluorescence) ratio. The Fv/Fm ratio was quite low around 0.5-0.6 in all genotypes at 0 h of reoxygenation in comparison to seedlings kept in normoxia with a maximum photosynthetic efficiency (Fv/Fm ratio) of around 0.8. The Fv/Fm ratio in wt, *NDA1ox3*, and *NDA2ox2* remained low up to 2 h of reoxygenation and increased thereafter in all three genotypes. The Fv/Fm ratio reached 0.7 in *NDA1ox3* and *NDA2ox2* and 0.6 in wt after 8 h of reoxygenation indicating photosynthetic recovery. In *nda1-1 nda2-2* seedlings, the Fv/Fm ratio was around 0.3 at the end of the anoxic phase and remained low at around 0.35 after 8 h of reoxygenation (Figure 3-27). In conclusion, mitochondrial NDAs are crucial for photosynthetic recovery during reoxygenation. The low Fv/Fm ratio in *nda1-1 nda2-2* revealed

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significant damage to photosystem II, which prevents carbohydrate supply during reoxygenation. In addition to the enhanced ROS formation limited carbohydrate resources may render seedlings prone to damage or even death.



**Figure 3-27: Photosynthetic recovery during reoxygenation requires NDAs.**

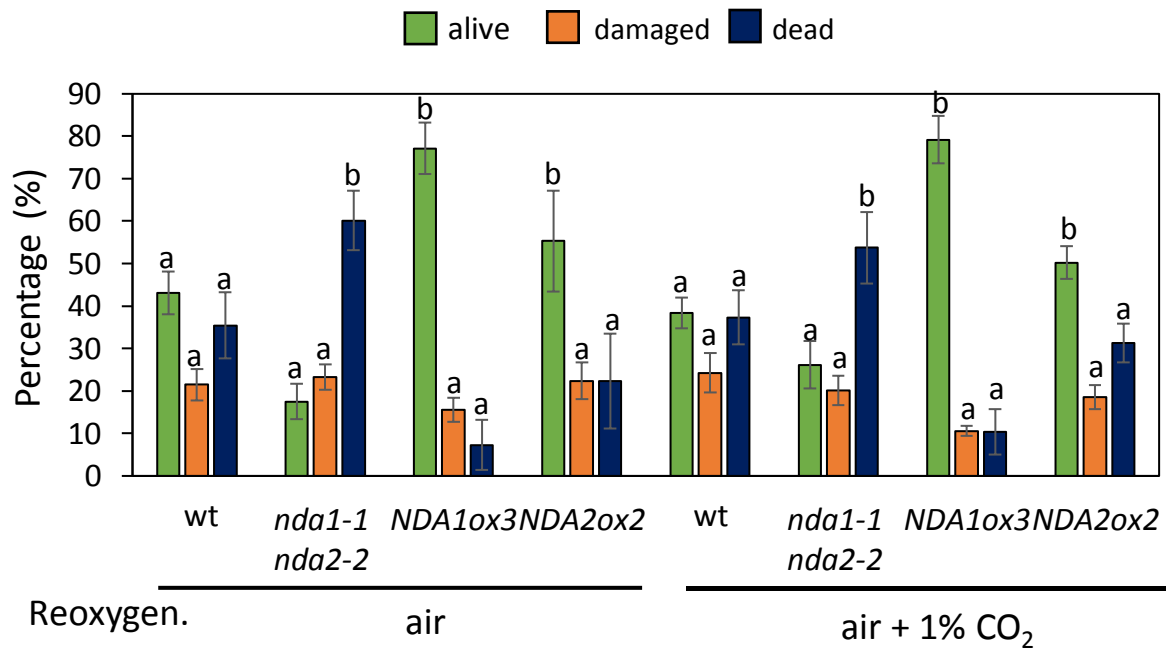
The maximum quantum efficiency of photosystem II (Fv/Fm) of 11-d-old light-grown seedlings is given as means ( $\pm$ SE,  $n=30-35$ ) of 3 biological replicates. Different letters indicate significantly different values (Kruskal-Wallis test,  $p<0.05$ ).

### 3.12 Recovery from anoxia is independent of photooxidation

During reoxygenation, apart from ROS and reduced photosynthetic ability, seedlings may encounter photorespiration due the shift from dark to light that accompanies reoxygenation. We therefore analyzed if photorespiration contributed to leaf chlorosis and seedling death. In order to evaluate the contribution of photorespiration, seedling survival was compared after reoxygenation in high  $\text{CO}_2$  versus reoxygenation in low  $\text{CO}_2$ . High  $\text{CO}_2$  favors the carboxylation reaction over the oxygenation reaction of Ribulose 1,5-biphosphate carboxylase (RuBisCO) in the Calvin-Benson cycle. Seven-d-old seedlings were subjected to 0%  $\text{O}_2$  for 16 h in the dark with subsequent reoxygenation in either ambient air (low  $\text{CO}_2$ ) or air supplemented with 1%

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CO<sub>2</sub> (high CO<sub>2</sub>). The rate of seedling survival was comparable for both conditions (Figure 3-28). Again, *nda1-1 nda2-2* seedlings showed the lowest survival rate while *NDA1ox3* and *NDA2ox2* seedlings showed the highest survival rate with an intermediate value for wt. The observation demonstrates that recovery during reoxygenation depends on the alternative mitochondrial ETC and is independent of photorespiration.



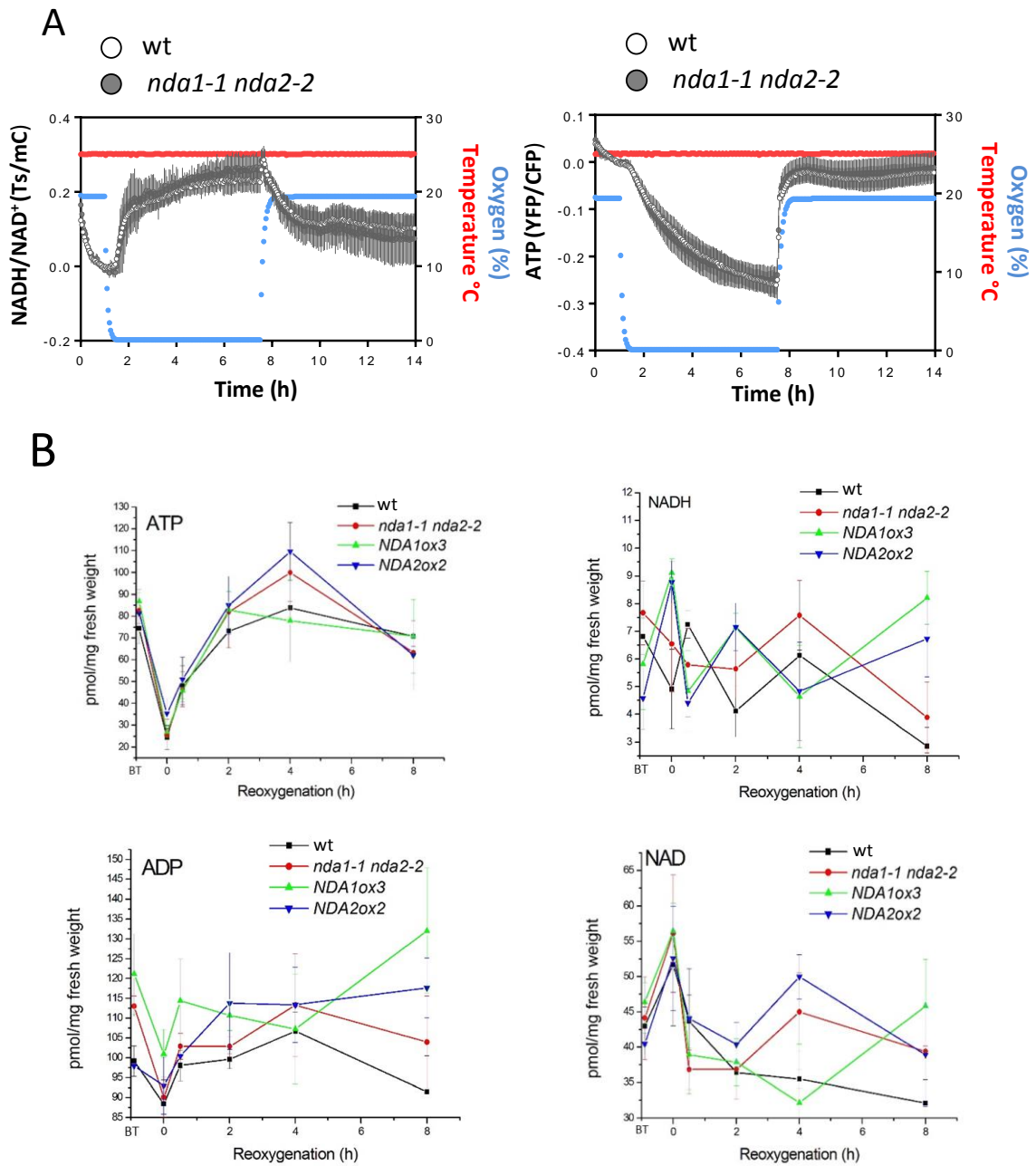
**Figure 3-28: Recovery from anoxia is dependent on the mitochondrial alternative ETC and independent of photorespiration.**

Survival of 7-d-old light-grown seedlings exposed to 0% O<sub>2</sub> in the dark followed by 7 days of recovery in a light/dark cycle under ambient air or air supplemented with 1% CO<sub>2</sub> (means ±SE of 3 biological replicates, One-Way ANOVA, *P* < 0.05).

### 3.13 NDAs do not alter ATP levels and NADH redox balance

It was shown that low oxygen reduces production of ATP and alters the NADH/NAD<sup>+</sup> redox balance (Gibbs & Greenway 2003; Wagner *et al.* 2019). To analyze a role of NDAs in ATP production and redox balance, we stably expressed the C-Peredox-mCherry sensor to measure changes in the NADH/NAD<sup>+</sup> ratio and the ATeam 1.03 sensor to measure changes in ATP *in planta* using a multiwell plate reader-based fluorimetric assay. The NADH/NAD<sup>+</sup> ratio was considerably increased as the level of oxygen became zero, and it increased further during the anoxia phase.

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**Figure 3-29: NDAs do not affect ATP levels and NADH redox balance.**

A) Analysis of cytosolic NADH/NAD<sup>+</sup> and ATP in 7-day-old seedlings assessed by stably expressed C-Peredox-mCherry (NADH/NAD<sup>+</sup>) and ATeam 1.03 (ATP) sensors using multiwell plate reader-based fluorimetry (means  $\pm$ SE, n=8).

B) UPLC-based quantification of ATP, NADH, ADP, and NAD<sup>+</sup>. Results are means ( $\pm$  SE) of four biological replicates. No significant differences were detected between genotypes or treatment (one-way ANOVA with Tukey's test,  $P < 0.01$ ). BT= before treatment

During reoxygenation, the NADH/NAD<sup>+</sup> ratio went down reaching the level of normoxia within few hours. No significant differences in NADH redox balance were observed between wt and *nda1-1 nda2-2* at any treatment (Figure 3-29). The level of ATP significantly decreased during

## Results

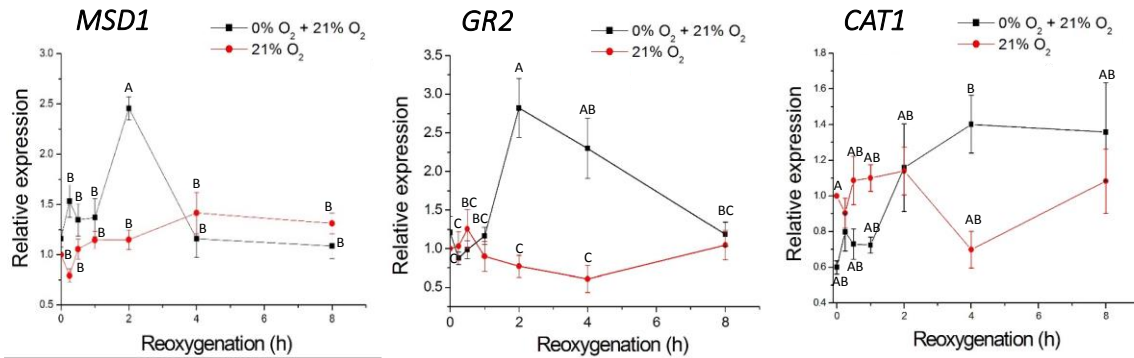
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anoxia treatment (Figure 3-29) and increased again within 1 h of reoxygenation until it reached the level observed during normoxia, as was observed for the NADH/NAD<sup>+</sup> redox balance. No difference in ATP levels was observed between wt and *nda1-1 nda2-2*. The data was confirmed by UPLC-based quantification of ATP, ADP, NADH and NAD<sup>+</sup> in wt, *nda1-1 nda2-2*, *NDA1ox3*, and *NDA2ox2* with similar results as with *in planta* fluorimetry. There was no difference in terms of ATP, NADH, ADP, and NAD<sup>+</sup> levels between the genotypes. While we observed increased NADH and decreased ATP levels in anoxia, these changes were independent of NDAs. In summary, the data indicated that the level of ATP and NADH redox balance were not dependent on NDAs. Nevertheless, it was evident that the NADH redox balance and ATP levels quickly recovered during reoxygenation. Hence, during reoxygenation, seedlings can effectively maintain energy demand and NADH redox balance whereas maintenance of ROS homeostasis and reduction of oxidative stress are a major challenge during reoxygenation.

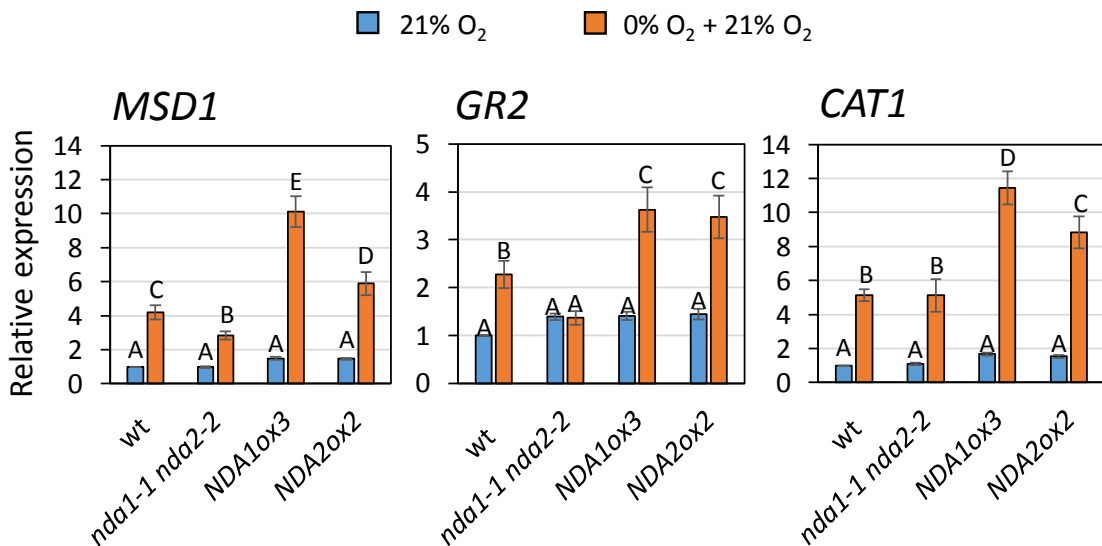
### 3.14 NDAs regulate reoxygenation-induced production of antioxidant enzymes

The data described above revealed that maintaining ROS homeostasis was a most crucial factor for seedling survival and that NDAs substantially contribute to it. Therefore, we decided to next study the expression of antioxidant enzymes during reoxygenation. We analyzed the expression of *manganese superoxide dismutase1 (MSD1)*, *glutathione reductase 2 (GR2)* and *catalase1 (CAT1)* during reoxygenation. All of these enzymes are highly active in mitochondrial matrix in antioxidant defense response. However, GR2 and CAT1 also have chloroplastic and cytosolic activity. The relative transcript levels of *MSD1* and *GR2* were increased to highest levels after 2 h of reoxygenation, and decreased thereafter to the level of normoxic seedlings after 4 h and 8 h, respectively, while the expression of *CAT1* did not differ between reoxygenation and control (Figure 3-30). To evaluate a role of NDA activity for the transcriptional regulation of antioxidant enzymes, we compared the expression of *CAT1*, *MSD1*, and *GR2* in response to reoxygenation in wt, *nda1-1 nda2-2*, *NDA1ox3*, and *NDA2ox2* seedlings. As illustrated in Figure 3-31, the expression levels of these genes were considerably higher in *NDAox* lines than in the wt and *nda1-1 nda2-2* under reoxygenation. In contrast, the expression levels of *CAT1*, *MSD1*, and *GR2* in wt and *nda1-1 nda2-2* were higher during reoxygenation than in normoxia, but remained lower than in *NDAox* lines.

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**Figure 3-30: Expression of *MSD1*, *GR2*, and *CAT1* during reoxygenation.** Seven-day-old light-grown seedlings were exposed to air (21% O<sub>2</sub>) or anoxia (0% O<sub>2</sub>) for 6 h in the dark and subsequently transferred for recovery in 21% O<sub>2</sub> for 0-8 h in the light. qPCR results are means ( $\pm$  SE) of three independent biological replicates with two technical repeats each (one-way ANOVA with Tukey's test,  $P < 0.01$ ). *MSD1*=Manganese superoxide dismutase1, *GR2*=Glutathion reductase 2, *CAT1*=Catalase1.



**Figure 3-31: The expression of genes encoding mitochondrial antioxidant enzymes is induced by reoxygenation and regulated by NDAs.**

Expression of *CAT1*, *MSD1*, and *GR2* was analyzed by qPCR in shoots of 7-day-old seedlings exposed to air (21% O<sub>2</sub>) or anoxia (0% O<sub>2</sub>) for 6 h in the dark followed by recovery in 21% O<sub>2</sub> for 2 h in the light. Results are means ( $\pm$ SE) of three independent biological replicates with two technical repeats each (Kruskal Wallis test,  $P < 0.05$ ). *MSD1*=Manganese superoxide dismutase1, *GR2*=Glutathion reductase, *CAT1*=Catalase1.

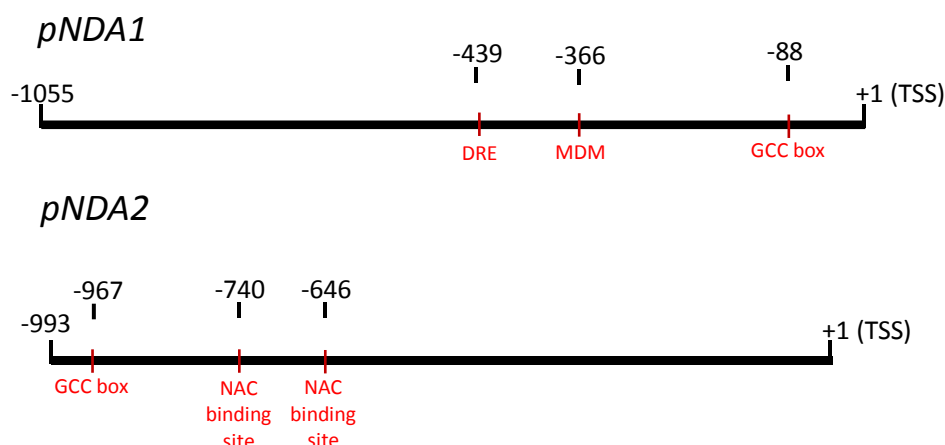
The *CAT1*, *MSD1*, and *GR2* are amongst the enzymes which are in the first line of defense in preventing the formation of ROS. The expression of *CAT1* and *GR2* during reoxygenation was lower in *nda1-1 nda2-2* than in wt and *NDAox* lines. Taken together, these findings indicate that the antioxidant capacity during reoxygenation is in part regulated independent of NDA

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activity. On the other hand, NDA activity is positively coupled to antioxidant gene activation during reoxygenation.

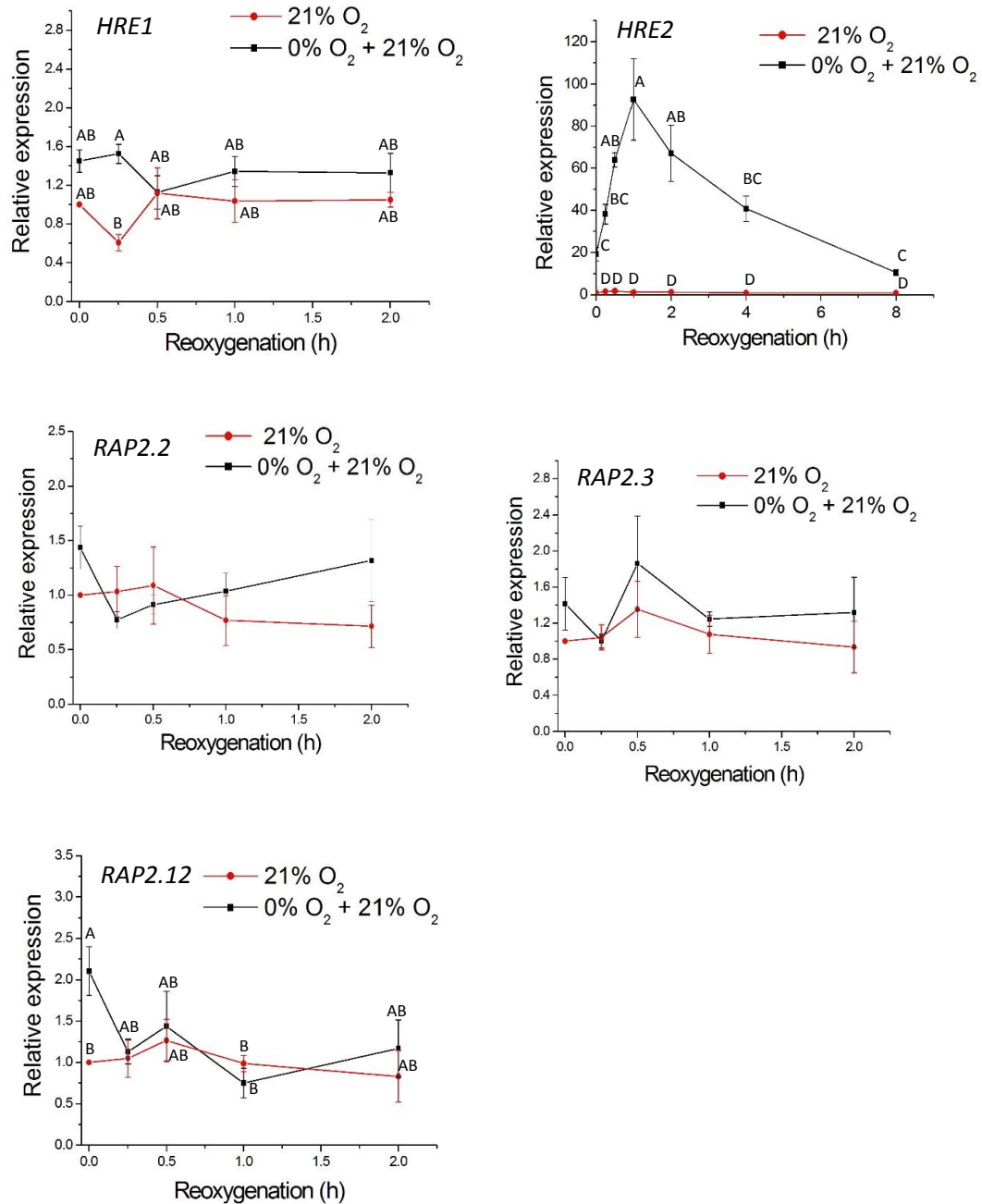
### 3.15 The expression of *HRE2* is induced by reoxygenation

Group VII ethylene response transcription factors (ERFVIIs) act as major regulators of hypoxic gene expression in plants and confer hypoxia and flooding tolerance. ERFVIIs are plant-specific transcription factors that bind to the GCC box or dehydration responsive element (DRE) of an ethylene response element in the promoter of the gene regulated by them (Ohme-Takagi & Shinshi 1995; Sun *et al.* 2008). Analysis of promoter sequences of *NDA1* and *NDA2* revealed the presence of a GCC box. The *NDA1* promoter also contains a dehydration responsive element (DRE) (Figure 3-32). In addition to their established role in regulating low oxygen responses, we hypothesized that ERFVIIs might regulate *NDA*s during reoxygenation. The *Arabidopsis* group VII ERFs consist of three *Related to AP-2* (*RAP2.2*, *RAP2.3*, and *RAP2.12*) and two *Hypoxia Responsive ERF* (*HRE1* and *HRE2*) genes. Out of these five, *RAP2.2* and *RAP2.12* were identified as major activators of hypoxia-responsive genes, whereas *RAP2.3*, *HRE1*, and *HRE2* were appeared to play minor roles (Gasch *et al.* 2016). Hence, it might be possible that these genes might have regulatory role during other phase of the low oxygenation stress such as reoxygenation. However, there is not much information available about their expression during reoxygenation.



**Figure 3-32: Position of the cis-regulatory element of ERFVIIs and ANACs transcription factors in *NDA1* and *NDA2* promoters.** TSS: Transcriptional start site, DRE: Dehydration responsive element, MDM: Mitochondrial dysfunctional motif.

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**Figure 3-33: Expression of *HRE2* but not *HRE1*, *RAP2.2*, *RAP2.3*, and *RAP2.12* is induced by reoxygenation.**

Seven-day-old light-grown seedlings were exposed to air (21% O<sub>2</sub>) or anoxia (0% O<sub>2</sub>) for 6 h in the dark followed by recovery in 21% O<sub>2</sub> for 0-8 h in the light. Results are means ( $\pm$ SE) of three independent biological replicates with two technical repeats each (one-way ANOVA with Tukey's test,  $P < 0.01$ ).

To study the expression of *ERFVIIs* during reoxygenation, seven-d-old seedlings were subjected to air (21% O<sub>2</sub>) or anoxia (0% O<sub>2</sub>) for 6 h in the dark with subsequent recovery in



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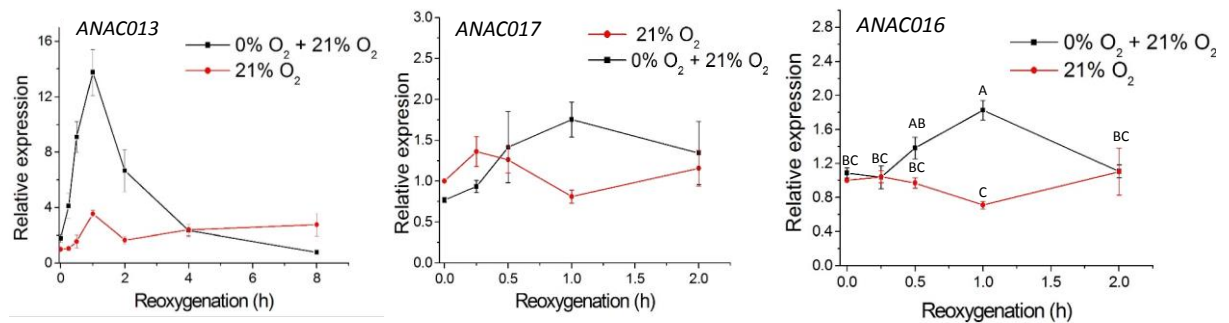
21% O<sub>2</sub> for 0 to 8 h in the light. Of the five genes analyzed, only *HRE2* was upregulated during reoxygenation while the expression of *HRE1*, *RAP2.2*, *RAP2.3*, and *RAP2.12* were unaffected during reoxygenation in comparison to seedlings subjected to normoxia (Figure 3-33). During normoxia, the expression of *HRE2* was 20-fold lower than in anoxia. After reoxygenation, expression of *HRE2* further increased and reached 100-fold higher transcript levels after 1 h of reoxygenation compared to normoxic seedlings (Figure 3-33). Between 1 h and 8 h, transcript levels gradually declined. These results might indicate a specific role of *HRE2* in regulating early reoxygenation responses.

### 3.16 The expression of *ANAC013* and *ANAC016*, *ANAC017* during reoxygenation

The NO APICAL MERISTEM/*ARABIDOPSIS* TRANSCRIPTION ACTIVATION FACTOR/CUP-SHAPED COTYLEDON (NAC) transcription factors are plant-specific transcription factors and it is one of the largest transcription factor families consist of 105 members in *Arabidopsis* (Ooka *et al.* 2003). The NAC proteins involved in diverse functions such as development, defence and abiotic stress responses (Hegedus, Yu and Baldwin 2003; Xie *et al.* 2000; Takada *et al.* 2001; Vroemen *et al.* 2003; Fujita *et al.* 2004; Tran *et al.* 2004). *ANAC013*, *ANAC016*, and *ANAC017* were shown to be involved in mitochondrial retrograde signaling (De Clercq *et al.* 2013; Van Aken *et al.* 2016). In *Arabidopsis*, the NAC proteins bind to cis-regulatory elements termed Mitochondrial Dysfunctional Motif (MDM) or NAC binding site in the promoter of the target gene that they activate (De Clercq *et al.* 2013; Ng *et al.* 2013). The promoter region of *NDA1* contains an MDM site at position -366 from the transcriptional start site while the *NDA2* promoter has two NAC binding sites at positions -646 and -740 (Figure 3-32). To analyze the expression of *ANAC016* and *ANAC017* during reoxygenation, seven-day-old seedlings were subjected to either ambient air (21% O<sub>2</sub>) or nitrogen (0% O<sub>2</sub>) for 6 h in the dark with subsequent recovery in ambient air in the light. The expression of *ANAC017* did not change between 0-2 h of reoxygenation in comparison to seedlings exposed to 21% O<sub>2</sub> while expression of *ANAC016* was induced 1.5-fold within 1 h of reoxygenation compared to seedlings kept at normoxic conditions. The mRNA level of *ANAC013* was 11-fold higher after 1 h of reoxygenation and eventually returned to the level at normoxia after 8 h of reoxygenation (Figure 3-34). In summary, *ANAC017* was not transcriptionally regulated while

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*ANAC013* and *ANAC016* were induced during reoxygenation.



**Figure 3-34: Expression of *ANAC013*, *ANAC016* and *ANAC017* during reoxygenation.**

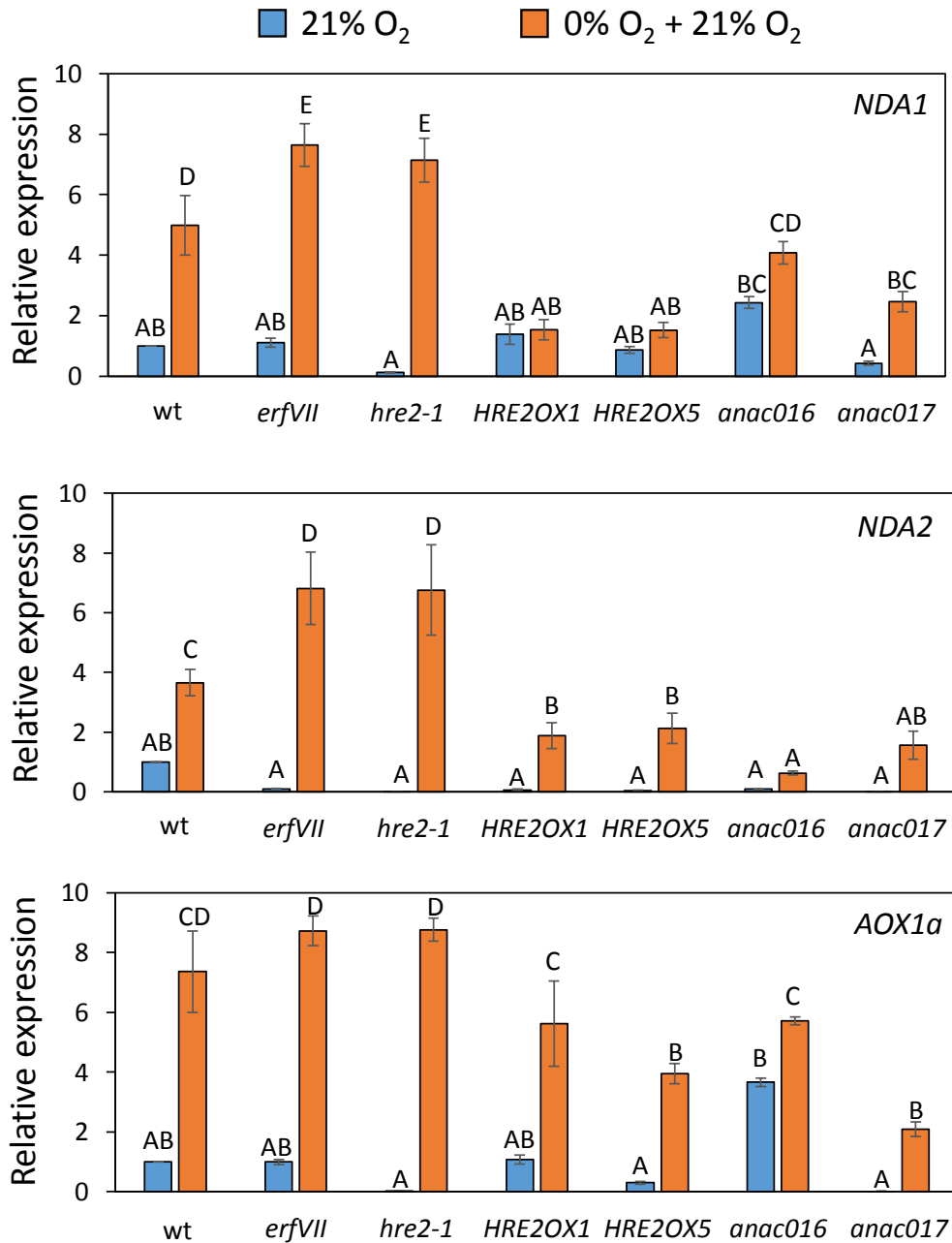
Seven-day-old light-grown seedlings were exposed to air (21% O<sub>2</sub>) or anoxia (0% O<sub>2</sub>) for 6 h in the dark followed by recovery in 21% O<sub>2</sub> for 0-8 h in the light. Results are means ( $\pm$  SE) of three independent biological replicates with two technical repeats each (one-way ANOVA with Tukey's test,  $P < 0.01$ ).

### 3.17 *ANAC016* & *ANAC017* positively regulate *NDA*s while *HRE2* inhibit the expression of *NDA*s during reoxygenation

After analyzing the expression of *ERFVII*s and *ANAC*s during reoxygenation, *ERFVII* and *ANAC017* and *ANAC016* mutants were used to further deduce their role in the regulation of *NDA*s. The *erfVII* quintuple knockout mutant, the single knockout mutants *hre2-2*, *anac017*, and *anac016* and two *HRE2ox* lines were used for the analysis. Seven-day-old mutant seedlings were subjected to either 21% O<sub>2</sub> or 0% O<sub>2</sub> in the dark for 6 h followed by 2 h of reoxygenation in ambient air in the light and the expression of *NDA1*, *NDA2*, and *AOX1a* was analyzed after 2 h of reoxygenation. The expression of *NDA*s increased considerably after 2 h of reoxygenation in wt as shown previously (Figure 3-21). In *erfVII* and *hre2-2*, *NDA* expression was even higher compared to wt (Figure 3-35). Conversely, *NDA* expression remained low in *HRE2ox1* and *HRE2ox5* seedlings (Figure 3-35) supporting the conclusion that *HRE2* act as a repressor of *NDA*s during reoxygenation. Knockout of *HRE2* enhances expression of *NDA*s while overexpression suppresses it. On the other hand, the *anac017* mutant showed reduced expression *NDA1* and *NDA2* during reoxygenation in comparison to wt indicating positive regulation of *NDA*s by *ANAC017* (Figure 3-35). Surprisingly, the expression pattern of *AOX1a* in different mutants differed from that of *NDA1* and *NDA2*. Expression of *AOX1a* in wt, *erfVII* and *hre2-2* were induced to a similar level during reoxygenation and *AOX1a* transcripts were likewise induced during reoxygenation in *HRE2ox1* lines indicating that *AOX1a* is regulated

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independent of HRE2. Furthermore, *AOX1a* transcripts were elevated in *anac016* and repressed in *anac017* during reoxygenation (Figure 3-35) indicating differential regulation of the internal alternative respiratory chain components.



**Figure 3-35: HRE2 and ANAC16 and ANAC017 antagonistically regulate NDAs during reoxygenation.** The relative expression levels of *NDA1*, *NDA2*, and *AOX1a* were analyzed in shoots of 7-day-old seedlings of wildtype, *erfVII*, *HRE2*, *ANAC016* and *ANAC017* knockout mutants and *HRE2* overrepressors exposed to air (21% O<sub>2</sub>) or anoxia (0% O<sub>2</sub>) for 6 h in the dark followed by recovery in 21% O<sub>2</sub> for 2 h in the light. Results are means ( $\pm$ SE) of three independent biological replicates with two technical repeats each (analyzed one-way ANOVA with Tukey's test,  $P < 0.05$ ).

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### 3.18 ANAC013, ANAC016, and ANAC017 bind to and activate the *NDA1* promoter

To elucidate if the regulation of *NDA1* by ANAC013, ANAC016, and ANAC017 was direct, binding of all three proteins to the MDM motif of the *NDA1* promoter was tested. The analysis of *NDA1* promoter revealed the consensus MDM sequence CTTGNNNNNCA[AC]G previously reported as ANAC013 protein binding site (De Clercq *et al.* 2013). As mentioned in Figure 3-36, an MDM is present at position -366 (Figure 3-36). To validate binding of ANAC013, ANAC016, and ANAC017 to the *NDA1* promoter, an electrophoretic mobility shift assay (EMSA) was used. To confirm the binding of the NAC transcription factors to the MDM, The DNA probes were synthesized that contained 33 bp MDM-surrounding regions of *NDA1* promoter. The ANAC013, ANAC016, and ANAC017 were successfully produced in TNT SP6 High Yield Wheat Germ protein expression system. Due to binding of IR-dye680-labelled DNA probe containing the wildtype MDM and ANAC017, ANAC016 or ANAC013 proteins, a clear band shift was visible in the gel. Hence it was evident that, all three ANACs showed specific binding to the MDM in the *NDA1* promoter. Furthermore, when an unlabelled MDM competitor probe was used the binding of labelled probe was abolished resulting in no band. While, the probe mixed with an unlabelled competitor containing mutated MDM was used, the band for the labelled probe was visible on the gel (Figure 3-36). Hence, the EMSA assay indicated that ANAC013, ANAC016, and ANAC017 bind specifically to the MDM in the *NDA1* promoter.

To test whether the ANAC013, ANAC016, and ANAC017 can indeed regulate the expression of *NDA1*, a trans-activation assay was used. The 1055 bp long *NDA1* promoter was fused to the luciferase reporter. ANAC013, ANAC016, and ANAC017 were co-expressed with *NDA1:LUC* reporter in Arabidopsis shoot protoplast and luciferase luminescence was detected. All three ANACs induced the expression of the luciferase reporter gene (Figure 3-36). Luminescence from luciferase activity driven by the *NDA1:Luc* expression was increased 9-fold, 36-fold and 102-fold, respectively by ANAC013, ANAC016, and ANAC017 supporting the conclusion that *NDA1* is activated by ANAC013, ANAC016, and ANAC017.



## 4 Discussion

The mitochondria are considered the power-house of the cell, and the mitochondrial electron transport chain (mETC) is central to energy production. Aside from the mETC, plant mitochondria possess an alternative ETC that uses up reductive power without the production of ATP. In the present work, we have comprehensively analyzed the function of the energy bypass proteins termed INTERNAL TYPE II NAD(P)H DEHYDROGENASE A1 (NDA1) and NDA2 in particular for plant survival of anoxia followed by reoxygenation as commonly occurs in flooding stress. During flooding, due to a reduced gas exchange, the availability of oxygen is limited, which limits mitochondrial respiration resulting in an energy crisis. However, when floodwater retreats, the plants that have acclimated to hypoxia or anoxia are confronted with a sudden surge in oxygen, which poses an additional stress on the plants, that is sometimes even more detrimental than oxygen deficiency itself (Alpuerto *et al.* 2016; Yeung *et al.* 2018). In the current study, we used knock-out and overexpression mutants of *NDA1* and *NDA2* to study the impact of the alternative respiratory chain in low oxygen and reoxygenation conditions. NDAs are hypothesized to prevent over-reduction of the respiratory chain, which in turn could minimize the generation of ROS (Rasmusson *et al.* 2008). In the current work, we tested this hypothesis and analyzed the role of NDAs in ROS homeostasis in anoxia and reoxygenation and for plant survival of reoxygenation. To more fully understand the role of NDAs in energy and redox metabolism, the ATP production and the NADH/NAD<sup>+</sup> redox balance were also investigated during anoxia and reoxygenation in wild type and in NDA mutants. Since reoxygenation comes with a sudden exposure to high light after a period of low light, the impact of photorespiration and photosynthetic recovery during reoxygenation were also examined as a first insight into the cross-talk between chloroplasts and mitochondria. In the last part, the regulation of NDAs was studied with the aim to ultimately decipher the reoxygenation signaling pathway.

### 4.1 NDAs provide resistance to the complex I inhibitor rotenone

The aim of this work was to characterize the role of the mitochondrial bypass proteins NDA1, and NDA2 in conditions where the mETC is compromised. The internal type II dehydrogenases act as a bypass for complex I and allow for electron flux from NAD(P)H to Ubiquinone to

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alternative oxidase (Rasmusson & Møller 1991). We showed that inhibition of complex I by rotenone in wild type and NDAox lines does not change seedling fitness measured as leaf chlorophyll content. The activity of NDAs in wild type or NDAox lines helped the plant to adapt better in comparison to NDA knock-outs in the absence of complex I (Figure 3-19). Similarly, minor changes in respiration, oxygen consumption and mitochondrial redox levels were observed in Arabidopsis seedlings treated with rotenone (Schwarzlander *et al.* 2009). These observations can be explained by the presence of respiratory bypass proteins. Blockage of one of the mETC complexes and of the bypass proteins resulted in elevated mitochondrial ROS production and a disturbed mitochondrial redox balance (Popov *et al.* 2003; Schwarzlander *et al.* 2009). The alternative NAD(P)H dehydrogenases and alternative oxidases were also induced in cultured Arabidopsis cells treated with rotenone (Clifton *et al.* 2005; Garmier *et al.* 2008) and the alternative respiratory chain components were also up-regulated in plants deficient of complex I, such as the CMSII mutant of *Nicotiana tabacum* (tobacco) and the Arabidopsis *otp43* mutant (Sabar *et al.* 2000; de Longevialle *et al.* 2007). In the absence of the complex I and its bypass proteins NDA1 and NDA2, the seedlings exhibited a significant reduction in leaf chlorophyll content (Figure 3-19) indicating that NDAs become important in the absence of complex I as they act as entry points for electrons to mETC and thereby, in part, replace complex I.

### 4.2 NDAs limit growth under non-stressed conditions

The biochemical function of NDAs has been well established, and it was shown in various studies that NDAs are targeted to mitochondria (Rasmusson & Møller 1991; Michalecka *et al.* 2003; Moore *et al.* 2003; Elhafez *et al.* 2006; Carrie *et al.* 2008). There is previous evidence of the importance of mitochondrial proteins in plant growth. For example in *Nicotiana sylvestris*, the cytoplasmic male sterile II (CMSII) mutant known to have altered respiratory ETC shown reduced shoot and root growth under high nitrogen condition. (Pellny *et al.* 2008). Similarly, the double knock-out mutant of mitochondrial malate dehydrogenase 1 and 2 showed reduced shoot growth in Arabidopsis (Tomaz *et al.* 2010). In this study, we observed a significant increase in shoot fresh weight and root length in *nda1-1 nda2-2* seedlings in comparison to wild type (Figure 3-12 and Figure 3-16) while an Arabidopsis line with reduced *NDA1* and *NDA2* expression did not show induced growth (Wallström *et al.* 2014) possibly due

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to residual NDA activity. In the single knock-out lines *nda1-1* and *nda2-2*, the root length was not altered (Figure 3-12). Hence, it is likely that both NDAs can compensate for each other. A similar observation was made by Moore *et al.* (2003) who did not observe a growth phenotype in an Arabidopsis *NDA1* knock-out mutant. Because of gene duplication events, a number of genes are present in two or more isoforms (Vision *et al.* 2000). The large section of chromosome 1 that contains *NDA1* exhibits synteny with a part of chromosome 2 containing *NDA2* and both genes show a similar gene structure (Blanc *et al.* 2000). The high sequence similarity with 82% amino acid identity and the conserved N-terminal region indicate that both genes have a similar function (Michalecka *et al.* 2003; Moore *et al.* 2003; Elhafez *et al.* 2006; Rasmusson *et al.* 2008). Over-expression of *NDA1* or *NDA2* resulted in a reduced shoot fresh weight and a shorter primary root (Figure 3-12 & Figure 3-16). Similarly, a decrease in shoot growth was also observed in Arabidopsis plants overexpressing *ALTERNATIVE OXIDASE 1A* (*AOX1a*, Smith *et al.* 2009). A shorter inflorescence stem was observed in some transgenic *Manihot esculenta* (cassava) lines overexpressing Arabidopsis *AOX1a* (Zidenga *et al.* 2012). The increased growth in the absence of NDAs and the decreased growth in plants overexpressing NDAs suggests that the non-ATP producing pathway is not of advantage in non-stressed conditions but is nonetheless maintained (Rasmusson *et al.* 2008). It has been reported that the plant growth rate is proportional to the respiration rate and its efficient coupling with ATP synthesis (Hansen *et al.* 2002). By modulating the energy yield, the alternative respiratory chain can be involved in optimizing the respiration rate and energy demand of the cells by controlling high-energy consuming processes (Rasmusson *et al.* 2008; Vanlerberghe *et al.* 2009). Furthermore, it is possible that the alternative respiratory chain plays a major role in stress tolerance as AOX is known to be induced under different stress conditions (Fiorani *et al.* 2005; Smith *et al.* 2009). Hence, the alternative ETC might be of importance under specific stress conditions.

### 4.3 NDAs contribute to maintenance of ROS homeostasis

The mitochondria are one of the major sites for ROS production due to the electron leakage from the mETC to molecular oxygen during aerobic respiration. Roughly, 2-5 % of the total O<sub>2</sub> consumed in mitochondria under normal growth conditions is converted into ROS (Corpas *et al.* 2015). The mETC complexes I and III are the primary sites for superoxide anion (O<sub>2</sub><sup>•-</sup>)



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production (Møller 2001; Sweetlove & Foyer 2004; Gupta & Igamberdiev 2015). These conditions prevail at high levels of reducing equivalent in the matrix, which results in a highly reduced ubiquinone pool and elevated  $O_2^{\cdot-}$  levels. To avoid ROS production due to electron overflow during high metabolic activities, the plant mitochondria possess the bypass proteins type II NADH dehydrogenase and alternative oxidases (Rasmusson *et al.* 2008; Corpas *et al.* 2015). A role of AOX in oxidizing the highly reduced ubiquinone pool to reduce  $O_2^{\cdot-}$  production has been reported (Maxwell *et al.* 1999; Corpas *et al.* 2015). However, a role of NDA1 and NDA2 in maintaining ROS homeostasis has not been studied previously. *nda1-1 nda2-2* seedlings deficient in NDAs produced elevated hydrogen peroxide ( $H_2O_2$ ) and  $O_2^{\cdot-}$  levels in the shoot and in the primary root tip (Figure 3-7, Figure 3-8, Figure 3-17 and Figure 3-18). Likewise, the inhibition of AOX in pea and soybean mitochondria resulted in strong induction of  $H_2O_2$  production (Popov *et al.* 1997). ROS also accumulated in the single knock-out lines *nda1-1* and *nda2-2* was similar to the levels of wt (Figure 3-7 & Figure 3-8), indicating that both NDAs have similar functions and can compensate for each other (Michalecka *et al.* 2003; Elhafez *et al.* 2006; Rasmusson *et al.* 2008). In contrast, the overexpression lines *NDA1ox3* and *NDA2ox2* exhibited reduced  $O_2^{\cdot-}$  levels in the primary root and in the shoot in comparison to wild type (Figure 3-8 & Figure 3-18). Similarly, transgenic cultured cells of tobacco over-expressing AOX had half as much ROS as control cells, while cells with reduced expression of AOX by antisensing exhibited five times more ROS than control cells (Maxwell *et al.* 1999). Moreover, the heterologous expression of internal NADH dehydrogenase (Ndi1) from *Saccharomyces cerevisiae* in mammalian cells results in reduced ROS accumulation (Seo *et al.* 2006). Taken together, the data suggest that the bypass proteins for complex I of the mETC, NDA1 and NDA2, play a significant role in controlling ROS levels under standard growth conditions.

### 4.4 NDAs improve survival of reoxygenation

Complete submergence severely reduces the light intensity and gas exchange which compromises photosynthesis and respiration and ultimately brings about an energy shortage and carbon imbalance. In the recent years, the research on flooding tolerance has primarily centered on the submergence phase. However, the recovery phase marked by subsidence of water, has been examined to a much lesser extent. During the recovery phase, the plants acclimated to hypoxia or anoxia encounter sudden exposure to light and air, which poses an

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additional stress that can be even more harmful than oxygen deficiency itself. It is well-known that reoxygenation results in excessive ROS formation due to electron leakage from the reactivated mETC (Elstner & Osswald 1994; Smirnov 1995; Huang *et al.* 2016). Despite of a few recent reports on the effects of reoxygenation in rice and Arabidopsis (Fukao *et al.* 2011; Tsai *et al.* 2014; Tsai *et al.* 2016), the mechanisms regulating reoxygenation stress tolerance are not understood.

Here, we identified a role of the two mitochondrial bypass proteins NDA1 and NDA2 in reoxygenation survival in Arabidopsis. The involvement of respiratory chain components complex I, complex III and AOX in NO and ROS signaling under low oxygen has been well-documented (Blokhina *et al.* 2003; Gupta *et al.* 2011). In the present work, we observed that the expression levels of *NDA1*, *NDA2*, and *AOX1a* were significantly increased in the shoot and primary root during reoxygenation (Figure 3-6 & Figure 3-21). Similarly, the alternative ETC components AOX and NDA were induced at the protein and transcript levels in rice seedlings during the reoxygenation phase following 24 h of anoxia (Millar *et al.* 2004; Narsai *et al.* 2009). The reoxygenated rice coleoptiles were able to respire in the absence of complex IV due to the induction of AOX protein (Millar *et al.* 2004). In addition, the activity of a *Aspergillus niger* alternative dehydrogenase was induced under oxidative stress in oxygen-enriched cultures of the fungus (O'Donnell *et al.* 2011). The setup mimics reoxygenation in plants where the activity of the mETC is highly enhanced, such that the mitochondria need alternative ways for electron transport to avoid electron leakage to O<sub>2</sub> and excessive ROS formation. We hypothesized that the specific induction of *NDA1*, *NDA2*, and *AOX1a* during reoxygenation might have a function in maintaining a ROS balance. This hypothesis was strengthened by the observation that the expression levels of *NDA1* and *NDA2* substantially increased upon treatment with 3-amino-1,2,3 triazole (AT), the inhibitor of the antioxidant enzyme catalase and it is known to elevate cellular ROS levels (Figure 3-23). Furthermore, the treatment with methyl viologen (MV) known to produce excessive ROS in mitochondria and chloroplast by blocking the electron transport chain in both organelles also significantly induced the expression of *NDA1* and *NDA2* (Figure 3-23). Similarly, *AOX* expression was induced upon treatment of seedlings with H<sub>2</sub>O<sub>2</sub> or antimycin A (AA) (Vanlerberghe & McIntosh 1996; Zarkovic *et al.* 2005; Ng *et al.* 2013). *NDA1*, *NDA2*, and *AOX1a* were induced early during reoxygenation with a peak after 2 h followed by a decline to control levels after 8 h of reoxygenation (Figure 3-22). Previous experiments suggest that both NDAs and AOX are

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induced by ROS, which were reported to accumulate during reoxygenation (Elstner & Osswald 1994; Smirnoff 1995; Huang *et al.* 2016). Hence, the specific induction of alternative respiratory chain components early during reoxygenation support their role in restricting ROS formation. The flood-tolerant *Arabidopsis* accession Lp2-6 showed restricted ROS accumulation during the first hour of reoxygenation, and an improved survival of reoxygenation. Hence, tolerance of reoxygenation is related to controlled ROS accumulation during the early recovery phase (Yeung *et al.* 2018). However, the sensitive Bay-0 accession was unable to control ROS production during reoxygenation ultimately leads to reduced survival after desubmergence (Yeung *et al.* 2018). *Arabidopsis* seedlings overexpressing the transcription factor MYC2 showed better survival of reoxygenation and efficient control of ROS production during reoxygenation while the *myc2-2* knockout mutant was unable to limit ROS production during reoxygenation and also exhibit reduced survival in comparison to overexpression line (Yuan *et al.* 2017). In accord with this conclusion, the the genes encoding antioxidant enzymes ascorbate peroxidase 2 (APX2) and glutathione reductase 1 (GR1) were induced in *Arabidopsis* within 3 h of reoxygenation (Yuan *et al.* 2017).

ROS accumulate during submergence and more so during reoxygenation (Blokhina *et al.* 2003; Fukao *et al.* 2011; Chang *et al.* 2012; Yuan *et al.* 2017; Yeung *et al.* 2018). ROS are signaling molecules that help plants to sense their environment and trigger adaptive responses (Bailey-Serres *et al.* 2012a; Voeselek & Sasidharan 2013). During hypoxia, H<sub>2</sub>O<sub>2</sub> controls the activity of the alcohol dehydrogenase (ADH), that contributes to energy supply via fermentation (Yang 2014). ROS are highly reactive and can cause irreversible oxidative damage to e.g. lipids, proteins, and nucleic acids and trigger programmed cell death (PCD) (Pérez-Pérez *et al.* 2012). To survive reoxygenation, plants needs to maintain the balance between ROS production and ROS scavenging (Sharma *et al.* 2012). We hypothesize that NDAs and AOX maintain physiological ROS levels during reoxygenation and thereby avoid post-anoxic injuries, ultimately improving the survival rate. We showed that NDAs control ROS homeostasis, and other reports demonstrated a role of AOX in controlling ROS levels (Zarkovic *et al.* 2005; Cvetkovska and Vanlerberghe 2012; Ng, Giraud, *et al.* 2013; Corpas *et al.* 2015).

In summary, better survival of reoxygenation was linked with the efficient containment of ROS during this phase. NDA overexpressors were also able to maintain the antioxidant status of the cells by elevating gene expression of *manganese superoxide dismutase1 (MSD1)*, *glutathione reductase 2 (GR2)* and *catalase1 (CAT1)* during reoxygenation (Figure 3-30). The

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respective enzymes act as a first line of defense under oxidative stress. The cellular homeostasis during reoxygenation requires fine-tuning between ROS and antioxidant status of the cells (Yeung *et al.* 2019). A controlled ROS production during the initial phase of the reoxygenation is necessary, but it also needs to be proficiently balanced by the antioxidant defense system (Yeung *et al.* 2018). As reoxygenation results in increased ROS accumulation in both tolerant Lp2-6 and sensitive Bay-0 accessions but Bay-0 accession was unable control prolonged and excessive ROS production during reoxygenation. In contrast, the Lp2-6 accession exhibits restricted ROS accumulation during first hour of reoxygenation followed by substantial increase in antioxidant status (Yeung *et al.* 2018).

The acclimation of the shoot to reoxygenation is also influenced by photosynthetic recovery. Photosynthetic recovery is essential to prevent leaf senescence and cell death during reoxygenation (Kato *et al.* 2014). In many plant species, a significant reduction in maximal quantum yield of PSII (Fv/Fm) and nonphotochemical energy quenching (NPQ) was observed during reoxygenation (Luo *et al.* 2009, 2010, 2018; Sone & Sakagami 2017). We also found a significant reduction in the Fv/Fm ratio in wildtype and NDA mutants during the early phase of reoxygenation. Photosynthetic recovery was observed in wildtype and in NDA overexpressors as Fv/Fm ratio reached 0.7 in *NDA1ox3* and *NDA2ox2* and 0.6 in wildtype after 8 h of reoxygenation. In contrast, *nda1-1 nda2-2* seedlings exhibited an Fv/Fm ratio of 0.5 at the end of the anoxic phase that remained low around 0.35 after 8 h of reoxygenation (Figure 3-27). Similarly, the submergence sensitive Arabidopsis accession Bay-0 exhibited a lower Fv/Fm ratio close to 0.4 than submergence tolerant accession Lp2-6 having optimum Fv/Fm ratio, three days after reoxygenation (Yeung *et al.* 2018). The submergence tolerant rice accession Sub1A also displayed faster recovery of the Fv/Fm ratio upon reoxygenation (Alpuerto *et al.* 2016). An efficient ROS management through the alternative mETC in mitochondria during reoxygenation might minimize the damages to PSII, which allows refueling of energy reserves through photosynthesis and an increased survival rate during recovery. Furthermore, the light responsiveness and diurnal regulation of *NDA1* and *AOX1* point to a link of the alternative mETC with photosynthetic metabolism (Svensson & Rasmusson 2001; Escobar *et al.* 2004; Elhafez *et al.* 2006; Rasmusson & Escobar 2007). Future research will specify the contribution of the alternative respiratory pathway for chloroplast function.

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Besides that, Photoinhibition, marked by excessive light, causes a reduction in photosynthetic capacity due to excessive ROS production that leads to inactivation of the reaction centers of photosystem I and II (Pospíšil 2009). Photorespiration helps plants to dissipate the surplus of photochemical energy and to prevent photo-oxidative damage (Kozaki & Takeba 1996). This study showed that seedling survival of reoxygenation was independent of photorespiration as there was no difference in survival percentage between reoxygenation in low or high CO<sub>2</sub> (Figure 3-28). This was true for wild type as well as NDA mutants. Similarly, the glycine/serine ratio was unaffected in NDA-suppressed lines indicating no effect of NDAs on photorespiration in high light (Wallström *et al.* 2014). Overall, the survival of seedlings was similarly affected by photooxidative damage in wildtype and NDA mutants indicating that control of ROS homeostasis in mitochondria has a significant impact on seedling survival during reoxygenation independent of photorespiration.

An insufficient replenishment of energy reserves and a redox imbalance are other factors that might impede plant survival during low oxygen conditions and reoxygenation. It has been reported that suppression of *NDA1* and *NDA2* affects the NAD(P)H redox balance (Wallström *et al.* 2014). However, *in planta* fluorimetry and ultra performance liquid chromatography (UPLC)-based quantification of ATP, ADP, NADH, and NAD<sup>+</sup> in wildtype, *nda1-1 nda2-2*, *NDA1ox3*, and *NDA2ox2* showed no differences between genotypes (Figure 3-29). A significant increase in NADH and a decrease in ATP levels were observed during anoxia, but these were independent NDA activity (Figure 3-29). A similar observation was made by Wagner *et al.* (2019), who found a 8-fold reduction in ATP and an 4-fold increase in the NADH/NAD<sup>+</sup> ratio during anoxia. Likewise, Arabidopsis seedlings showed a significant decrease in ATP levels within 4 h of anoxia (Tsai *et al.* 2016). In this study, the NADH/NAD<sup>+</sup> ratio went down within a few hours of reoxygenation to the level of normoxia, while the level of ATP significantly increased within 1 h of reoxygenation to the level observed during normoxia (Figure 3-29). A prior study (Tsai *et al.* 2016) reported recovery of ATP production within 1 h of reoxygenation, but the seedlings were unable to recover NADH/NAD<sup>+</sup> redox balance during reoxygenation. In the current study, quick recovery of the NADH redox balance and ATP levels indicated that seedlings could effectively maintain energy demand and NADH redox balance during reoxygenation, whereas maintenance of ROS homeostasis is a major challenge during reoxygenation. While evidence for ROS accumulation and induction of antioxidant defense

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responses during reoxygenation has been accumulating, few studies were dedicated to understand the underlying regulatory mechanisms (Fukao *et al.* 2011; Tsai *et al.* 2014; Yuan *et al.* 2017; Yeung *et al.* 2018).

### 4.5 HRE2 and ANAC016/ANAC017 antagonistically regulate *NDA*s during reoxygenation

The regulation of type II NAD(P)H dehydrogenases has yet to be understood. So far, no transcription factors regulating *NDA*s have been identified. A forward genetic promoter screen with of *NDA1* and *NDA2* revealed cis-elements for GROUP VII ETHYLENE RESPONSE FACTORS (ERFVII) and NO APICAL MERISTEM/ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR/CUP-SHAPED COTYLEDONS (ANAC) transcription factors. ERFVIIs have previously been reported to regulate hypoxic response genes that are involved in the metabolic shift from respiration to fermentation (Francesco *et al.* 2010; Hess *et al.* 2011; Bui *et al.* 2015). Arabidopsis ERFVIIs consist of five members. RAP2.2 and RAP2.12 were identified as major activators of hypoxia-responsive genes, whereas RAP2.3, HRE1, and HRE2 appeared to play minor roles (Gasch *et al.* 2016). The differential expression of ERFVII genes in darkness, hypoxia, or in response to ethylene indicates distinct physiological functions (Hinz *et al.* 2010; Licausi *et al.* 2010; Hess *et al.* 2011). The minor role of HRE1 and HRE2 in activating hypoxia core response genes suggests functions that are different from those of RAP2.2, RAP2.3 and RAP2.12.

The transcript level of *HRE2* increased 100-fold within 1 h of reoxygenation possibly indicating a role of HRE2 in reoxygenation. In contrast, the increased expression level of *NDA*s in *hre2-2* and *erfVII* while reduced expression in *HRE2ox* lines during reoxygenation indicating negative regulation of *NDA*s by HRE2 transcription factor. A prior report stated that *HRE2* transcripts are also unstable in air and may undergo mRNA decay (Licausi *et al.* 2010). Besides, the ERFVII proteins are degraded in the presence of oxygen by the N-end rule pathway (NERP) (Licausi *et al.* 2011). Hence, to support a role of HRE2 during reoxygenation, possibly in controlling postanoxic ROS levels, the presence of the HRE2 protein in the nucleus during reoxygenation will have to be analyzed. As RAP2.12 was shown to regulate the *HYPOXIA RESPONSIVE UNIVERSAL STRESS PROTEIN1 (HRU1)* via H<sub>2</sub>O<sub>2</sub> produced from NADPH oxidase (Gonzali *et al.* 2015) revealing a link between ERFVIIs and ROS. However, the protein stability of HRE2 and ROS levels in *hre2-2*, *HRE2ox* lines and *erfVII* mutants should also be analysed in

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future to deduce their role during reoxygenation. Furthermore, the increased expression of *HRE2* under mitochondria produced ROS by Antimycin A treatment (Ng *et al.* 2013b) suggesting their role in ROS regulation and mitochondrial retrograde signalling.

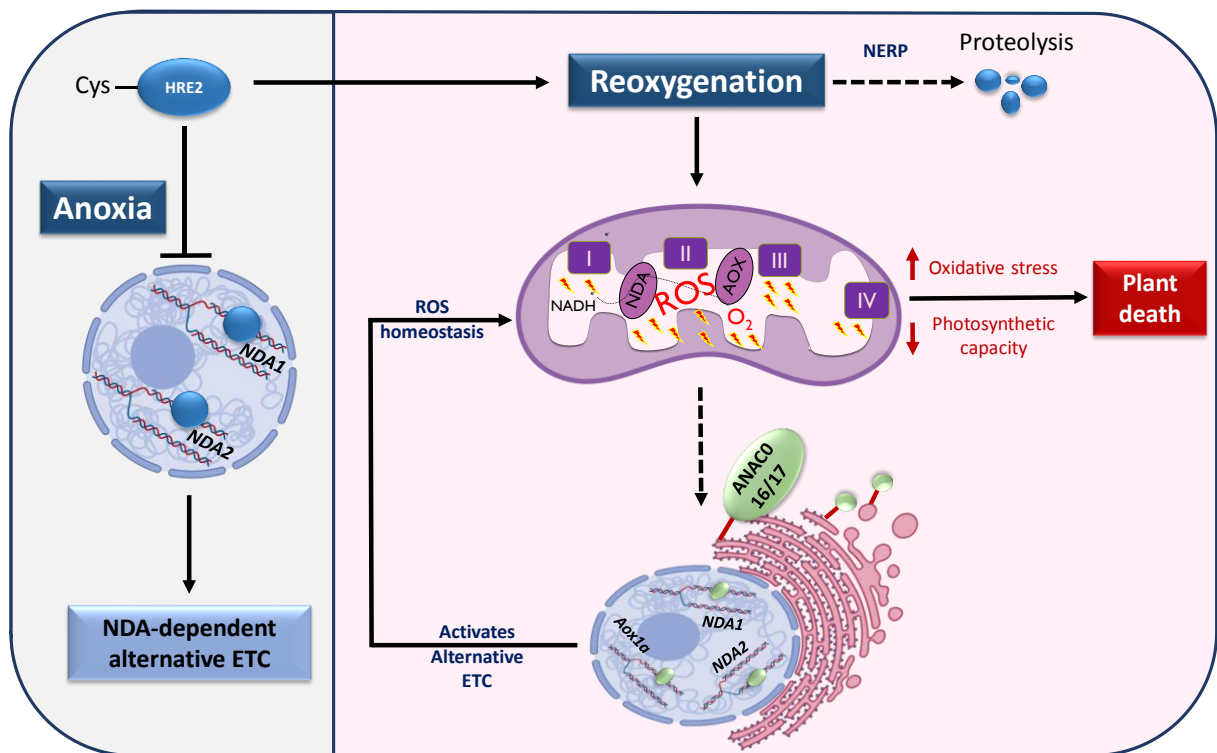
ANACs are plant-specific transcription factors involved in the regulation of mitochondrial retrograde signaling and oxidative stress response (De Clercq *et al.* 2013; Van Aken *et al.* 2016). This study showed that ANAC013, ANAC016, and ANAC017 bind to the *Cis*-regulatory sequence mitochondrial dysfunctional motif (MDM) in *NDA1* promoter and activate luciferase activity driven by the *NDA1:LUC* expression (Figure 3-36). ANAC017 induced *NDA1:LUC* expression 102-fold (Figure 3-36) and expression of *NDA1* during reoxygenation was lower in *anac017* compared to wildtype, indicating positive regulation of *NDA1* by ANAC017 (Figure 3-35). ANAC017 was previously shown to also regulate *AOX1a*, and ANAC013 was described to maintain oxidative stress tolerance (Ng *et al.* 2013b; De Clercq *et al.* 2013). In *anac017* seedlings, expression of *NDA2* and *AOX1a* during reoxygenation was lower compared to wildtype, indicating positive regulation of *NDA2* and *AOX1a* by ANAC017 (Figure 3-35). ANACs are membrane-associated transcription factors that are attached to the endoplasmic reticulum (ER). The transcription factors are released via proteolysis and subsequently translocated to the nucleus under various stresses to initiate downstream transcriptional responses (Kim *et al.* 2006; Seo, Kim *et al.* 2008). ROS are involved in mitochondrial retrograde signaling (Murphy 2009). During reoxygenation, ROS produced from mitochondria might act as a signal initiating cleavage of ANAC017 to allow translocation of ANAC017 to the nucleus to induce the expression of *NDA*s and *AOX1a*. As it was shown previously that under AA treatment the ROS produced from mitochondria acts as a signal to initiate the cleavage of ANAC017 by rhomboid proteases which in turn induces the expression of *AOX1a* (Ng *et al.* 2013b).

There have been several examples of co-expression of type II dehydrogenases and alternative oxidases (Escobar *et al.* 2004; Clifton *et al.* 2005; Rasmusson *et al.* 2009). They may work as a fully functional alternative respiratory chain to reduce the formation of ROS. Besides, the MDM *cis*-regulatory element is also present in promoters of ANAC013 and ANAC016, indicating the regulatory interaction between the ANACs. There has been previous reports of interaction of ANAC013 with ANAC017 (De Clercq *et al.* 2013). Hence, it might be possible that ANAC017 act as a principal regulator of the alternative respiratory chain during



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reoxygenation, but ANAC016 and ANAC013 might have specific second-line of regulation in optimizing expression of specific alternative respiratory chain components. The expression of the ANAC013 one of the highly regulated ANACs under mitochondrial perturbations (De Clercq *et al.* 2013) showed reduced expression upon AA treatment in *anac017* (Ng *et al.* 2013b). Furthermore, induction of *HRE2* expression under AA treatment was also not visible in *anac017* mutants (Ng *et al.* 2013b), which further points towards principle role of ANAC017 in mitochondrial retrograde regulation. However, further research should be carried out to prove the pivotal role of ANAC017 and understand the differential regulation of alternative respiratory chain components during reoxygenation.



**Figure 4-1: The signaling network regulating reoxygenation response in *Arabidopsis thaliana*.**

The anoxia results in stabilization of HRE2 which then move to nucleus to repress the NDAs and carry out hypoxia gene response. The NDA-dependent alternative ETC is repressed during anoxia in order to save energy. The reoxygenation might also lead to degradation of HRE2 via N-end rule pathway (NERP). However, it needs to be tested and indicated as a dashed arrow. The reoxygenation also results in increased oxidative stress and ROS production inside the cell. The excessive ROS may cause damage to cause severe cellular damage, but it can also act as a signal to trigger downstream reoxygenation response. The ROS might serve as a regulatory signal to cleave ANAC016 & ANAC017 proteins. The cleaved ANACs move to the nucleus to regulate the expression of *NDAs* and *AOX1a*. However, the signal triggering ANACs and movement of ANACs to the nucleus is still to be determined and indicated as a dashed arrow. The ANACs induced activity of *NDAs* and *AOX1a* results in efficient ROS regulation, which in turn reduces the oxidative stress and increases the photosynthetic capacity to increase the survival rate of the plant during reoxygenation.



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HRE2 acts as a repressor of *NDA*s possibly indicating that the *NDA*-dependent alternative mETC is repressed by HRE2 during low oxygen conditions. However, *HRE2* expression is highly induced upon reoxygenation when *NDA* expression increases, leaving the role of HRE2 in *NDA* regulation an open question. *NDA*s limit ROS formation specifically during the reoxygenation phase when the alternative mETC is essential for plant survival. On the other hand, seedlings rapidly restore ATP levels and the NADH/NAD<sup>+</sup> redox balance following reoxygenation independent of *NDA*s suggesting that the main function of *NDA*s is to restrict mitochondrial ROS formation. Seedlings lacking *NDA*s have a lower Fv/Fm ratio, indicative of a damaged PSII, revealing that mitochondrial ROS production during reoxygenation impairs chloroplast function. The Arabidopsis transcriptional regulators ANAC013, ANAC016, and ANAC017 were shown to bind to the MDM *cis*-element in the *NDA1* promoter and to activate the *NDA1* gene. The *anac017* mutant exhibited reduced expression of *NDA1* and *NDA2* during reoxygenation in comparison to wild type indicating positive regulation of *NDA*s by ANAC017 and possibly ANAC013 and ANAC016. The results indicate that HRE2 and ANAC017 antagonistically regulate expression of *NDA1* and *NDA2* during anoxia and reoxygenation. At the transcriptional level, *NDA*s are positively controlled by ANAC017 and negatively controlled by HRE2. Whether a mitochondrial ROS burst during reoxygenation leads to release of ANACs has yet to be verified. Taken together, this study revealed a central function of mitochondrial *NDA*s in ROS regulation and plant survival of reoxygenation stress.

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## **Declaration of authorship**

I hereby declare that I authored the presented thesis “Mitochondrial alternative NAD(P)H dehydrogenases A1 and A2 control levels of reactive oxygen species and improve plant survival during reoxygenation” independently and that it is original and the result of my own investigation, apart from the advice given by Prof. Dr. Margret Sauter on the manuscript and during experimentation. I did not use any other sources and aids than the indicated references. I declare that, to the best of my knowledge and belief, I followed the rules of Good Scientific Practice of the Deutsche Forschungsgemeinschaft. This thesis has not been submitted, either in part or whole, at this or any other University. I did not withdraw from any academic degree.

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## Curriculum Vitae

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