Investigation of NADPH-Dependent Thioredoxin Reductase-C (NTRC) In Chloroplast Lipid Metabolism

By

Shannon Li Donnelly

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Director of Thesis: Patrick J. Horn

Major Department: Biology

Abstract

Redox reactions are ubiquitous within plant metabolism and critical for the sustainment of photosynthesis within thylakoid membranes. Specialized glycerolipids that make up these membranes in turn require redox reactions for the synthesis and modification of their fatty acyl chains. Previous research has shown that plants subjected to oxidative stress (e.g., high light) often exhibit conditions associated with an unbalanced flow of electrons coupled with changes in lipid metabolism ad structure. However, it remains unclear how chloroplast membranes are impacted and subsequently repair their membranes to maintain photosynthesis. This project explores the potential role of NADPH-Dependent Thioredoxin Reductase-C (NTRC), a reactive oxygen species (ROS)-scavenging enzyme, in chloroplast lipid metabolism. NTRC has been implicated in many diverse pathways; however, *ntrc* also displays an altered lipid phenotype that has yet to be investigated. We are beginning to understand how the photosynthetically starved state of *ntrc* mutants reacts with the additional loss of key lipids through development of higher order *ntrc* mutants. We have characterized these novel mutants, in addition to their single mutant parents, through measurements of growth and photosynthetic parameters, lipid profiles, and lipid stress-recovery assays. Ultimately, we predict that through an improved understanding of chloroplast membrane dynamics our results can inform engineering strategies that target enhanced plant resilience subjected to adverse environmental conditions.

Investigation of NADPH-Dependent Thioredoxin Reductase-C (NTRC) In Chloroplast Lipid Metabolism

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Shannon Li Donnelly

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By

Shannon Li Donnelly

APPROVED BY:

Director of Thesis _____

Patrick J. Horn, PhD

Committee Member _____

Beth Thompson, PhD

Committee Member _____

Adam Offenbacher, PhD

Chair of the Department of Biology _____

David Chalcraft, PhD

Dean of the Graduate School _____

Paul J. Gemperline, PhD

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CHAPTER 1: THE CHLOROPLAST UNDER ABIOTIC STRESS

1.1 REDOX BIOLOGY AND PHOTOSYNTHESIS

Reduction-oxidation (redox) reactions are ubiquitous in all organisms and serve critical roles in maintaining cellular homeostasis. Redox may be broadly defined as the exchange of electrons between molecules leading to a change in oxidation state. Plants use oxidoreductase enzymes to facilitate the delicate redox balance between light absorption and energy consumption to maintain photosynthesis (Horn, 2021). However, this balance may be disrupted under abiotic stress requiring the recruitment of redox protective mechanisms to maintain photosynthetic efficiency and reduce photooxidative damage (Nikkanen and Rintamaki, 2014; Rizhsky *et al.*, 2003). A classic example of a photoprotective mechanism coupled to photosynthetic electron flow is the splitting of water in the thylakoid membrane.

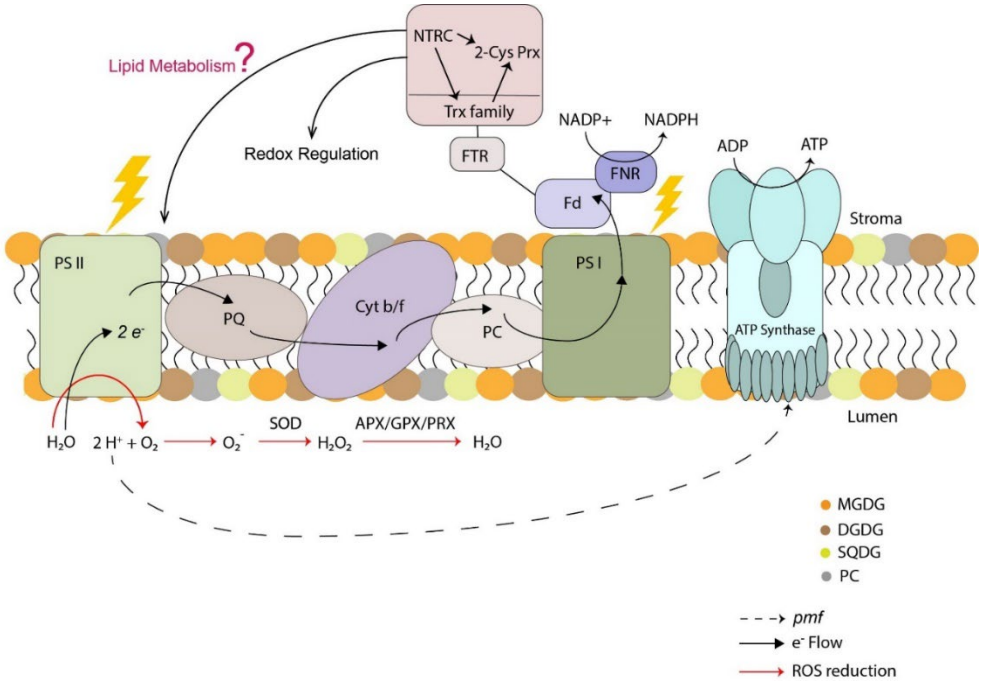


Fig. 1. Classical model whereby oxygenic photosynthesis generates reactive oxygen species via the splitting of water and undergoes subsequent reduction steps. The splitting releases electrons to begin the electron transport chain and production of NADPH and ATP. PS II = Photosystem II; PQ = Plastoquinone complex; Cyt b/f = Cytochrome b6f complex; PC = Plastocyanin complex; PS I = Photosystem I; Fd = Ferredoxin; FNR = Ferredoxin-NADP Oxidoreductase; FTR = Ferredoxin-Trx System; Trx = Thioredoxin; NTRC = NADPH-Dependent Thioredoxin Reductase-C; 2-Cys PRX = 2-Cysteine Peroxiredoxin; SOD = Superoxide dismutase; APX = Ascorbate peroxidase; GPX = Glutathione peroxidase; PRX = peroxiredoxin

The light-induced splitting of water is necessary as it provides the energy required to fuel the electron transport chain (Fig. 1). This essential process inevitability leads to the production of molecular oxygen (and protons) within the thylakoid lumen, which is quite reactive as an electron acceptor due to a reduction potential of +1.21 V (Pegis *et al.*, 2015). Reduction potentials indicate the likeliness of a molecule to be reduced by nearby electrons. Thus, because of oxygen's relatively high reduction potential it has a high affinity to participate in downstream redox reactions (Baranowska *et al.*, 2018). For example, oxygen can react in some cases enzymatically or spontaneously to form a wide variety of ROS including superoxide radicals ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) (Rizhsky *et al.*, 2003). ROS in low quantities can act as vital signaling molecules allowing the plant to quickly adapt and begin metabolic repair processes (Foyer *et al.*, 2017). However, unmanaged accumulation of ROS can lead to severe oxidative damage resulting in the breakdown of pigments, proteins, and membranes eventually causing cell death (Das and Roychourhury, 2014; Dat, *et al.*, 2000; Levine *et al.*, 1994).

Plants have evolved to manage the inevitable production of ROS through a collection of enzymes known as ROS-scavengers (e.g., superoxide dismutase (SOD), ascorbate peroxidases (APXs), peroxiredoxins (PRXs)). Referring back to the example of the water splitting, ROS-scavengers are recruited to reduce molecular oxygen (e.g., O_2 to H_2O_2) and its ROS derivatives to eventually generate water again (which may then provide additional electrons for photosynthetic processes). This reduction series is known as the water-to-water cycle and involves a specific set of scavengers (Fig. 1) (Asada, 2006). This process provides an excellent example when describing how redox reactions, via ROS-scavengers, have become necessary for photosynthetic function under basal conditions as well as adverse molecular conditions.

Scavengers may differ in their cellular localization and target molecule but overall exhibit similar functions in reducing oxidative damage (Yu and Chan, 2015).

Despite natural ROS response mechanisms, plants subjected to constant and/or frequently fluctuating abiotic stressors can lead to an unmanageable accumulation of ROS (Sachdev *et al.,* 2021). The anticipated climate changes we shall face in the future may require an enhancement of these natural mechanisms such that crops may better handle drastic changes in their environment (Medina *et al.*, 2021). One route to accomplish this goal may be found by studying the thioredoxin system, a chloroplast localized, highly versatile, and light activated redox network (Nikkanen and Rintamaki, 2014; Mata-Peréz and Spoel, 2019).

1.2 Light Protective Mechanisms

While ROS are naturally occurring molecules in the chloroplast, they may also accumulate under light stress (Xiong, *et al*., 2021). To handle fluctuating light conditions, plants can use a protein oxidoreductase family known as Thioredoxins (Trxs). Trxs participate in electronic exchange through the generation then cleavage of disulfide bonds with two nearby cystine residues (Nikkanen, *et al*., 2017). Oxidized Trxs are then reactivated by a thioredoxin reductase (TR). The Trx system may participate in ROS scavenging due to their reduction of 2-Cys Peroxiredoxins (2-Cys Prxs), which is one of the key enzymes involved in the reduction of hydrogen peroxide into water in the final step of the water-water cycle (Fig. 1) (Nikkanen *et al*., 2017; Asada, 2006).

There are two thioredoxin systems where the use of either is dependent on light exposure. The classical model for Trx activation, prior to the discovery of a second system, relied on photoactivated ferredoxin (Fd) generated in the electron transport chain (Fig. 1). At the onset of

illumination and/or under higher light intensities, the Ferredoxin-Thioredoxin system (FTR) begins with the reduction of ferredoxin in photosystem I (Nikkanen, *et al.*, 2017). The FTR system will use a FT reductase (FTR) to establish a disulfide bridge with 2-Cys Prxs. In a similar manner, the second system now known as NADPH-dependent thioredoxin reductase-c (NTRC) is activated to reduce Trxs and act as the primary reductant of 2-Cys Prxs specifically under low light irradiances. Whereas Fd requires photoactivation, NTRC does not. NTRC uses the reducing power of NADPH to form the disulfide bond (Gonzalez *et al.*, 2019). Under light restriction, plants generate NADPH for energy, rather than ATP, through the pentose phosphate pathway. While NADP+ also acts as the final electron acceptor in the electron transport chain, it is believed that NADPH production via the PPP regulates NTRC activation specifically at low irradiances when the NADPH pool has accumulated and photosynthesis begins (Peréz-Ruiz *et al.*, 2006). Also of importance, NTRC does not interact with a separate thioredoxin reductase like the FTR system. Instead, NTRC contains an internal thioredoxin domain within its tertiary structure that potentially allows for quicker Trx activation (Peréz-Ruiz *et al.*, 2006).

1.3 MEMBRANE LIPID METABOLISM

Photosynthetic complexes such as photosystem I/II and cytochrome b6f are embedded in the thylakoid membrane making these membranes critical in maintaining a functional photosynthetic apparatus (Andersson and Anderson, 1980; Jarvi *et al.*, 2013). Disruptions in the electron transport chain during abiotic stress and ROS accumulation induces a remodeling of the thylakoid membrane (in addition to other broader cellular changes in lipid metabolism) (Higashi *et al.*, 2015; Moellering *et al.*, 2010; Vijayan *et al.*, 2021; Yu *et al.*, 2021). Remodeling can induce breakdown of glycerolipid backbones which may increase fluidity, alter desaturation enzymatic activity, and replace key lipids for functionality (Cook, 2021). While this process is

disruptive to photosynthesis, it is required to maintain photosynthetic efficiency under rapidly changing environments.

The thylakoid membrane is composed primarily of galactolipids, a lipid class containing acyl chains at the *sn-1* (Carbon 1) and *sn-2* (C2) position and a galactose moiety at C3 (Li-Beisson *et al.*, 2013). The four major membrane lipids are monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), phosphatidylglycerol (PG), and sulfoquinovosyl diacylglycerol (SQDG), in order of abundance (Table 1, Fig. 1). Knockout (and in some cases knockdown) mutants within the biosynthetic pathways for any of these essential polar lipids result in impaired growth and photosynthetic response indicating their necessary role for a functional photosynthetic apparatus (Jarvis *et al.*, 2000; Kelly *et al.*, 2003; Du *et al.*, 2018).

There are two pathways for membrane lipid biosynthesis in *Arabidopsis thaliana*, the plastid ("prokaryotic") and endoplasmic reticulum (ER, "eukaryotic") pathway (Roughan and Slack, 1982; Browse *et al.*, 1986). These pathways are spatially divided within different organellar compartments in the generation of the lipid precursor, phosphatidic acid (PA). This lipid precursor is further biochemically modified in both pathways to generate the full set of membrane lipids required for plant growth and development.

1.31 THE PLASTID PATHWAY

In the plastid, or "prokaryotic", pathway, PA is commonly delineated from the ER, or "eukaryotic", pathway by a 16C acyl chain at the *sn-2* position (Roughan and Slack, 1982; Heinz and Roughan, 1983). Glycerolipid modification and assembly will occur within the chloroplast, primarily at the inner envelope membrane (IEM). ACYLTRANSFERASE 1 (ATS1) is an IEM protein responsible for the initial acylation step of 16:0 to generate Lyso-PA (from glycerol 3-

phosphate) which then will undergo a second acylation using ATS2 to generate PA (Kunst *et al.*, 1988; Xu *et al.*, 2006).

A knockdown in *ats1* results in a ~95% reduction of plastid-derived lipids and provides an excellent tool to understand metabolic contributions under abiotic stress (Table I and II) (Kunst *et al.*, 1988, Xu *et al.*, 2006; Dormann *et al.*, 1999; Yu *et al.*, 2021). Despite this genetically driven alteration in lipid metabolism, the *ats1* mutant does not display altered growth phenotypes under most conditions tested (Kunst, 1988). The lack of phenotypic variation from wildtype in the *ats1-1* mutant is unique considering its drastic metabolic disruption. For example, loss of ATS2 is embryo lethal whereas *ats1*, as mentioned, is not and does not display severe growth phenotypes (Bin *et al.*, 2014). This implies there may be more to explore regarding the plastid pathway but nonetheless due to its depletion of the 16:2 and 16:3 fatty acids *ats1-1* can provide valuable insights on plastid lipid metabolism *in vivo* (Table II).

Table I. Total Polar Lipids in Leaves of Wildtype, *ats1-1*, and *tgd-1*

	WT [1]	*ats1-1**	*tgd1*[1]
MGDG	44.1 +/- 0.02	39.2 +/- 0.01	39.6 +/- 0.3
DGDG	12.6 +/- 0.006	18.6 +/- 0.03	12.9 +/- 0.1
SQDG	3.2 +/- 0.001	8.1 +/- 0.001	3.4 +/- 0.7
PG	12.0 +/- 0.01	10.9 +/- 0.01	12.6 +/- 0.1
PC	17.7 +/- 0.01	13.2 +/- 0.01	16.8 +/- 1.2
PE/PI	10.3 +/- 0.006	10.0 +/- 0.01	12.7 +/- 0.4

[1] Xu, et al., 2005 mole % (mean +/- s.d, n=4*, n=10)

Table II. Fatty Acid Composition in Leaves of Wildtype, *ats1-1*, and *tgd1*

	WT [1]	*ats1-1*[1]	*tgd1*[1]
16:0	14.1	12.6	14.2
16:1	3.2	3.4	9.3
16:2	0.5	0.5	1.4
16:3	11.4	1.5	11.8
18:0	1.7	0.8	3.3
18:1	3.0	8.4	11.9
18:2	13.4	17.8	16.1
18:3	52.5	55.1	32.2

[1] Kunst, et al., 1988

1.32 THE ENDOPLASMIC RETICULUM PATHWAY

The ER, or "eukaryotic", pathway receives glycerolipid precursors from the chloroplast, imports and modifies in the ER, then exports back into the chloroplast for assembly or further modification (Browse *et al*., 1986). In *Arabidopsis,* 40% of chloroplast lipids are exclusively plastid-derived and ~60% are exported for synthesis in the ER, thus highlighting the importance of ER-lipid trafficking in membrane metabolism (Xu *et al*., 2009). Similar to how we have a mutant indicative of lipid contributions via the plastid pathway, researchers who aim to understand ER-lipid contributions may use the TRIGALACTOSYLDIACYLGLYCEROL1 (TGD1) mutant, known as *tgd1* (in particular the *tgd1-1* mutant line) (Fan and Xu, 2011).

TGD1 is part of a lipid trafficking system allowing for the influx of ER precursors into the chloroplast inner envelope for final synthesis (Xu, 2003). Unlike *ats1-1*, *tgd1* displays severe growth phenotypes and very interestingly an embryo lethality around 50% due to a developmental error in the heart phase of embryonic maturation (Table 3) (Xu, 2005). In *tgd1*

plants, lipid metabolism primarily relies on the plastid pathway causing an influx of 16 Carbon species; however, the mutation is leaky leaving some metabolism of ER lipids (Table I and II) (Xu *et al.*, 2005).

By using either *ats1* or *tgd1*, researchers can study contributions made by either the plastid- or ER-derived lipids, respectively. This mutant set has been useful when determining metabolic flux under varying growth conditions (Falcone *et al.*, 2004; Klaus *et al.*, 2002). One interesting contribution from the use of these mutants relates to membrane response to light induced stress. Recently, it was shown that plants subjected to low light will increase the production of plastid-derived lipids. Alternatively, plants under high light will increase production of ER-derived lipids (Yu *et al.*, 2021). This finding not only added to pathway contributions under suboptimal growth conditions, but also showed that in addition to redox-protective light mechanisms lipid membranes are also capable of responding to address abiotic light stress.

1.4 ROS Scavenging and Fatty Acid Metabolism

How the thylakoid membrane senses an abiotic stressor such as light, remains unknown. One theory is that a redox-mediated signal is involved in sensing the stressor and initiating membrane response. This is based on the knowledge that some ROS-scavenging enzymes possess unique lipid phenotypes, which could imply a role in a lipid metabolism pathway. Therefore, by studying ROS-scavenger mutants with lipid phenotypes one may be able to answer the question of how membrane lipids sense abiotic stress and remodel accordingly.

Peroxiredoxin-Q (PRXQ) is a 2-Cys Prx localized in the chloroplast (Kong *et al.*, 2000). It is a reductant of hydrogen peroxide and thus is considered an antioxidant, or ROS scavenger.

During a large lipidomic screen of redox-associated mutants, *prxq* was discovered to have 75% less 16:1t fatty acid relative to wildtype (Horn, *et al*., 2020). This is a unique chloroplast-specific fatty acid due to its *trans* double bond configuration, exclusive localization with the PG lipid, and presence in almost all plantae (Gao *et al*., 2009). Later, it would be shown that PRXQ was required to stimulate the fatty acid desaturase-4 (FAD4), which is responsible for 16:1t synthesis (Horn, *et al*., 2020). The 16:1t lipid was hypothesized to be a key indicator lipid for chloroplast redox status, or broadly redox health, because of this close connection with an ROS-scavenging enzyme. This discovery poses the question of whether there are more redox-lipid relationships that have yet to be discovered. By studying ROS-scavenging mutants displaying altered lipid metabolism phenotypes, we may begin to identify previously undescribed biochemical relationships and set the foundation for elucidating their mechanisms.

CHAPTER 2: CHARACTERIZATION OF *NTRC* MUTANTS

INTRODUCTION

Similar to the PRXQ/FAD4 relationship discovered due to the *prxq* lipid phenotype, we identified that *ntrc* displays an alteration in lipid metabolism (Fig. 2). NTRC has been implicated in many diverse pathways, yet there is still much unknown about its role *in vivo*. Prior to the publication of this thesis, the lipid phenotype of *ntrc* was unpublished and therefore unexplored in the literature. This information, taken alongside other known NTRC functions, positions NTRC to be a powerful player in chloroplast redox status. By studying NTRC, this thesis begins to ask if chloroplast redox status, or broadly chloroplast redox health, affects membrane lipid metabolism. One way to investigate this hypothesis is to understand current proposed functions for NTRC (described below) and ask how these roles may affect the disrupted fatty acid metabolism in *ntrc*.

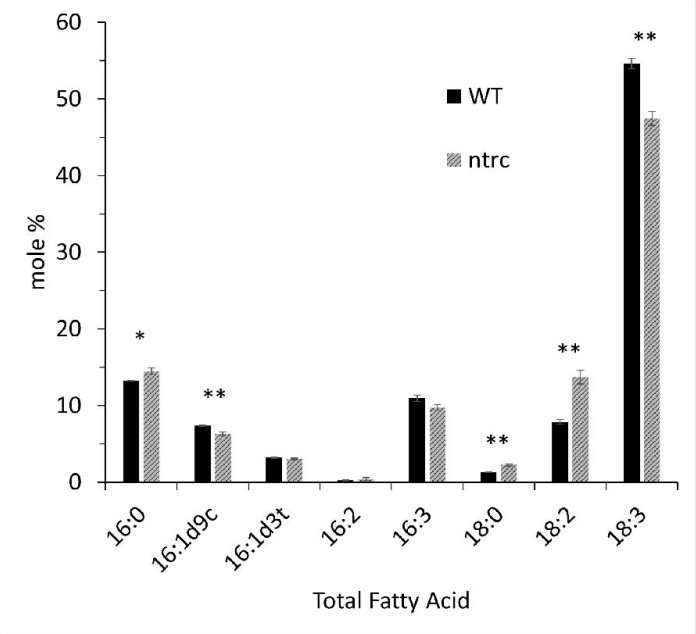


Figure 2. The *ntrc1* mutant displays an altered lipid profile compared to wildtype (Col-0). Rosette leaves were harvested from 4-week old plants with at least six replicates. Asterisk indicates a p-value <0.05. Double asterix indicates p-value <0.005.

2.1 2-Cysteine Peroxiredoxin Reduction

NTRC's main function as an antioxidant is due to its reduction of 2-Cys Prxs (Perez-Ruiz, et. al., 2006). Under low light 2-Cys Prxs are reduced by NTRC through a thiol-disulfide electron exchange (see *Chapter. 1.3*) (Fig. 3).

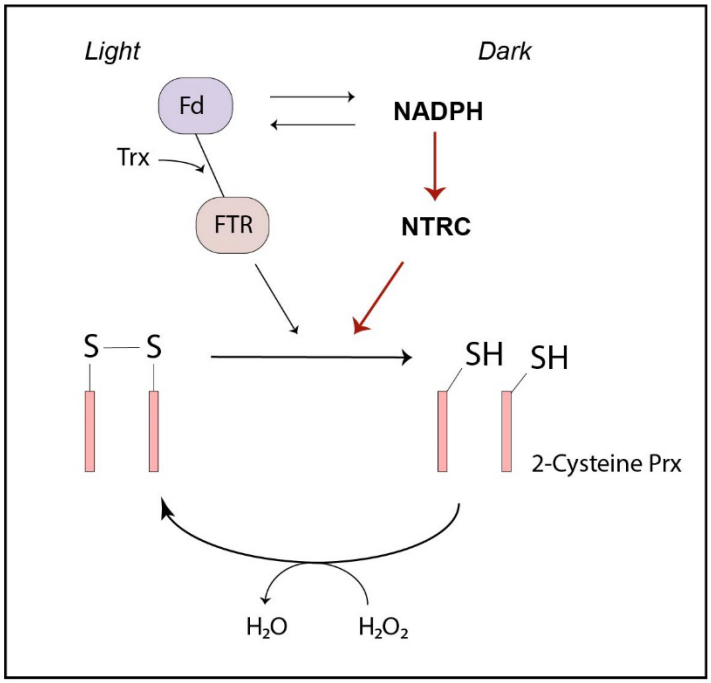


Figure 3. NTRC is the preferred reductant of 2-Cys Prxs under low light irradiance or darkness. 2-Cys Prxs undergo a thiol-disulfide reduction in order to reduce hydrogen peroxide. Trx = Thioredoxin Fd = Ferredoxin FTR= Fd reductase NTRC = NADPH dependant thioredoxin reductase. Adapted from Puerto-Galán et al., 2013.

In 2006, Perez-Ruiz *et. al* was interested in the photosynthetic efficiency of the *ntrc* mutant. To understand how light intensity affected the rate of photosynthesis, the group measured photosynthetic active radiation (PAR) for wildtype (Col-0) and *ntrc* at various light intensities. At a low light intensity of 100 µmol m^{-2} s^{-1} *ntrc* had a negative PAR reading. This indicated respiration was occurring at a higher rate than photosynthesis. Respiration occurs when Rubisco binds to molecular oxygen rather than carbon dioxide due to its higher availability in the chloroplast (Jetva *et al*., 2022). Perez-Ruiz *et al*. attributed the accumulation of molecular oxygen in *ntrc* to the loss of effective 2-Cys Prx activation. This would mimic respiration conditions explaining the poor photosynthetic performance of *ntrc* under low light. This study was the first to show NTRC to be the preferred reductant for 2-Cys Prx at low light and thus initiate ROS scavenging under these specific conditions (Perez-Ruiz *et al*., 2006).

2.2 ATP SYNTHASE ACTIVATION

Non-photochemical quenching (NPQ) is a natural defense mechanism activated under high light exposure to avoid the over production of chemical energy through the dissipation of light energy as heat (Demming-Adams *et al.*, 2014). NPQ is triggered via an acidification (i.e., buildup of protons) in the thylakoid lumen. In 2016, Naranjo *et. al* investigated various photosynthetic parameters of *ntrc* under low light. They found the mutant to be in a permanent state of NPQ, which resulted in a "photosynthetically starved state" due to the constant dissipation of electrons as heat (Naranjo *et al.*, 2015). At this time, NTRC was only known for its antioxidant abilities and this role was attributed to be the cause of elevated NPQ. However, it would very shortly after shown that the permanent state of NPQ was unrelated in NTRC's role as a 2-Cys Prx reductant. Instead, another research group proposed that NTRC directly regulates NPQ through a modulation of the ATP synthase γ subunit (Carrillo, *et al.*, 2016). The γ subunit contains reactive cysteine residues sensitive to redox modification, which would allow for NTRC interaction (Richter 2004). This research led to a model proposing that a light trigger, after a period of darkness, acidifies the lumen and triggers NTRC to turn on the ATP synthase (summarized in Fig. 4). Thus, in *ntrc* with the loss of γ subunit activation, the ATP synthase remains inactive forcing a permanent state of NPQ under low light (Fig. 6). On the

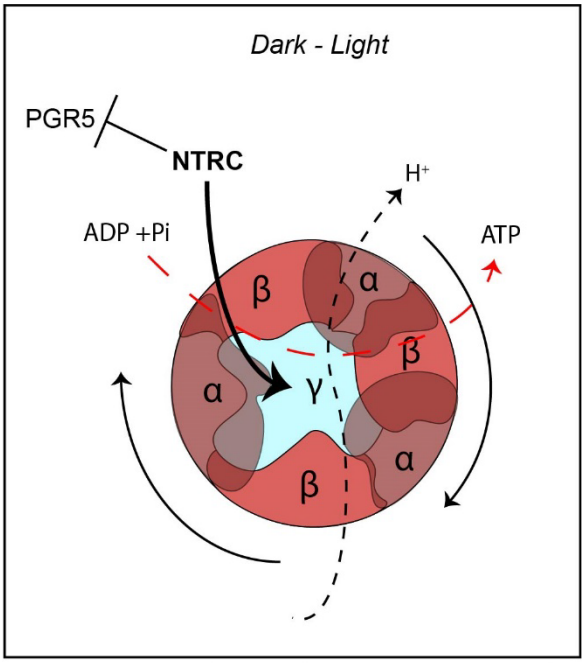


Figure 4. NTRC modulates (activates) the γ-subunit of ATP synthase via thiol-disulfide exchange at the onset of illumination while inhibiting PGR5 activity.
PGR5 = Proton gradient regulation 5

return to light, NPQ recovers to normal levels highlighting the importance of fluctuating light mechanisms in *ntrc* (Carrillo, *et al*., 2016).

2.3 Cyclic Electron Flow

Another well-known involvement of NTRC is its involvement in cyclic electron flow (CEF). Whereas linear electron flow (LEF) is the typical flow of electrons during photosynthesis whereby electrons generate ATP and NADPH, CEF utilizes unused electrons generated by the ferredoxin complex to produce additional ATP. This is completed as CEF will shuttle these electrons into the plastoquinone (PQ) eventually generating a *proton motive force* (*pmf*) that is specific for ATP generation (Suorsa, 2015) (summarized in Fig. 5). The process of CEF generates ATP without the production of NADPH. Therefore, CEF can be broadly defined to assist in balancing the ratio of ATP:NADPH (Nikkanen, *et al*., 2018).

CEF has two pathways for which it may begin. They are known as the antimycin-A sensitive pathway and the antimycin-A NADH-dehydrogenase like complex (Niu *et al*., 2022). The preference for either system remains unknown, but it has been proposed the NDH-complex is initiated under lower light intensities and activated via thioredoxin modification (Yamori, et al., 2015; Courteille *et al*., 2013). Also interestingly, and relevant for my study, the NDH-complex has been proposed to act in response to oxidative stress (Shikanai, 2007). Knowing that one CEF pathway relies on the reducing power of NADH and is specifically involved during low light, one may surmise that NTRC may act within the NDH-complex pathway. This was later demonstrated by Nikkanen *et al*. in 2018 when an *NTRC* overexpression line enhanced the NDH-dependent CEF pathway and *ntrc* accumulated reduced ferredoxin in darkness and during dark to light transitions. Researchers noted that NTRC regulation of the NDH-complex of CEF alone was not enough to explain the altered *pmf* leading to elevated NPQ in the mutant. Recently, a

separate group responded to this theory by showing NTRC may inhibit the activity of PROTON GRADIENT REGULATION 5 (PGR5), which is the key protein involved in the other CEF pathway, the antimycin sensitive pathway (Naranjo *et al.*, 2021) (Fig. 4). If this hypothesis is true, it would mean that in the absence of NTRC, PGR5 and the NDH-complex are not regulated and would begin a *pmf* of electrons from Fd to the lumen resulting in the initiation of NPQ regardless of abiotic conditions.

All the information gathered from mutant studies of NTRC regarding its NPQ phenotype leads to a few hypotheses that have been summarized in Fig. 6. At a loss of NTRC, the ATP synthase remains inactive due to loss of γ subunit activation. At the same time, PGR5 activity is at a loss of regulation and thus initiates CEF to occur; however, due to the loss of ATP synthase activity, *ntrc* CEF results in the buildup of a *pmf* that is forced to be expelled as heat by the Psbs protein at Photosystem II and via the xanthophyll cycle (Fig. 6). The proposed mechanisms would cultivate into the photosynthetically starved state of *ntrc* leading to elevated NPQ when ntrc is most required: at low light or dark-light transitions.

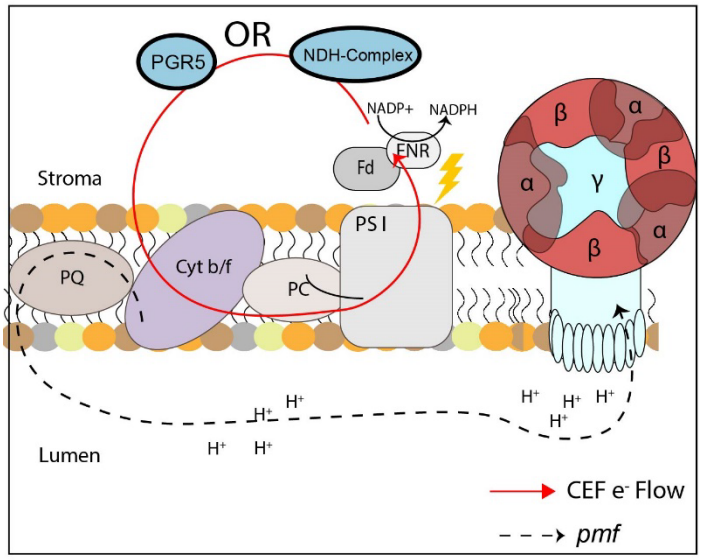


Figure 5. Cyclic electron flow utilizes either the PGR5-mediated pathway (antimycin-A sensitive) or the NADH-dehydrogenase like complex to reuse electrons generated by ferredoxin. The cyclic flow shuttles electrons through cytochrome b.f and plastocyanin while generating a pmf through plastoquinone in order to generate ATP.

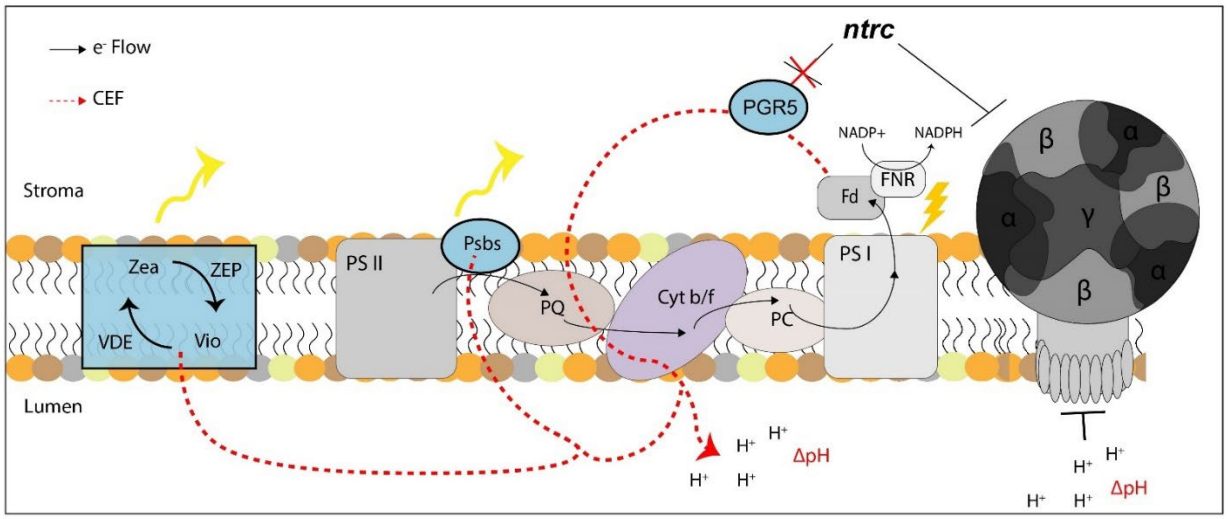


Figure 6. Overview of potential explanations for elevated NPQ in ntrc. The ntrc mutant experiences elevated NPQ due to inactivation of ATP synthase (Carrillo et al., 2016), loss of PGR5 regulation (Naranjo et al., 2021), and activation of the Psbs and VDE-mediated energy dissipation (Nikkanen et al., 2019). PGR5 = Proton gradient regulator 5, VDE = Violaxanthin de-epoxidase; ZEP = Zexanthin epoxidase; Zea = Zexanthin; Vio = Violaxanthin.

2.4 Plastid Metabolism

Variegated, or reticulate, mutants are defined as those having unique patterns of chlorosis due to an alteration in chloroplast development (Yu *et al.*, 2007). This specific leaf phenotype has been associated with disruptions in the development of mesophyll and/or bundle sheath cells but most interesting, reticulate mutants indicate an alteration in plastid metabolism (Lundquist *et al.*, 2014). *Ntrc* is a reticulate mutant (Fig. 10) exhibiting the strongest phenotype under short-day conditions (Lepisto *et al.*, 2009). Thus, it is possible to surmise that *ntrc* may play a role in plastid lipid metabolism and this disruption to a central plastid metabolic pathway could be causing the reticulate phenotype.

Reticulate mutants typically have disrupted aromatic amino acid synthesis that may be recovered with media supplements (Lundquist *et al.*, 2014). This is true of for *ntrc* and has been hypothesized to be responsible for its reticulate patterning; however, this has yet to be conclusively shown (Lepisto *et al.*, 2009, Lundquist *et al.*, 2014).

2.5 Specific Environmental Conditions Trigger A Response in *ntrc*

Plants in natural environments are subjected to fluctuating light conditions regularly and as such they have developed short-term solutions to manage these changing light stresses (Roeber *et al.,* 2020). Short-term responses can include thylakoid membrane alterations (see *Chapter 1.2*), NPQ (see *Chapter 2.2*), alteration in carbon fixation rates, as well as changes in CEF (see *Chapter 2.3*). NTRC is an oxidoreductase enzyme which has, to date, been implicated in most of these short-term light stress response mechanisms. All the information presented indicates NTRC may act to maintain photosynthetic performance under fluctuating light conditions, especially during a low light oscillation or transition by altering the redox state of the chloroplast, as it has been shown to do with 2-Cys Prxs and the ATP Synthase (Perez-Ruiz, et. al., 2006; Naranjo, 2016). This presents NTRC as a key in maintaining redox homeostasis.

3. Thesis Aims

Previous evidence has strongly suggested that NTRC is a key redox mediator in maintaining chloroplast health. This may be surmised from several mutant studies whereby there is a dysregulation of metabolic function particularly under low light conditions (Perez-Ruiz, et. al., 2006; Naranjo *et al.*, 2016; Carrillo *et al.*, 2016; Nikkanen *et al.*, 2018). The significance of *ntrc's* lipid phenotype has yet to be investigated.

At the beginning of this project, I wanted to understand if either perturbations in the plastid and/or ER metabolic pathway was responsible for the *ntrc* lipid phenotype. To do this, I generated the higher-order mutants *ntrc ats1-1* and *ntrc tgd1* to understand potential contributions of the plastid- and ER-pathways of lipid metabolism, respectively. Furthermore, because the *ntrc* lipid phenotype had previously been observed only under normal conditions I aimed to understand the impact of low light on the *ntrc* membrane composition.

I also aimed to understand why *ntrc* mutant alleles have varied phenotypes and if the lipid phenotype was retained in multiple mutant lines. To accomplish this, I began protein and transcript analysis on two well studied *ntrc* alleles to understand if either of these mutants are knockdowns rather than knockouts. I also performed lipid profiling on three mutant alleles to determine if the *ntrc* lipid profile was due to a pleiotropic affect. Both objectives (*ntrc* single and double mutants) led to the following aims:

1. Are the differences in *ntrc* allelic phenotypes due to partial protein and/or transcript production?
2. How does the *ntrc* membrane remodel under low light?
3. Does the *ntrc* lipid phenotype rely on either the plastid or ER metabolic pathway?

4. METHODS

Plant and Growth Conditions

The NTRC (*AT2G41680*) T-DNA mutant allele used to generate all double mutants is known as *SAIL115_E08* and will be referred to as *ntrc1*. The second *ntrc* mutant allele analyzed alongside *ntrc1* for protein and transcript data was *SALK_096776*, hereby known as *ntrc2*. The third allele used only for lipid profiling was *SALK_114293*, known are *ntrc3.* All mutants were acquired from Arabidopsis Biological Resource Center.

Seeds were stratified at 4C for two days before planting on 1.5 x 1.5-inch pots in SunGro soil. Plants were fertilized once a week using Peter's 15-5-15 fertilizer at a final concentration of 50 ppm. For normal light conditions, plants were grown in a growth room under long-day (16 hr. light/8 hr. dark) at approximately 100 $\mu mol^{-2}s^{-1}$ with standard fluorescent lighting. For the low light growth conditions, plants were grown under long-day at 25 $\mu mol^{-2}s^{-1}$. Short-day light

conditions were grown under 6 hr. light/18 hr. dark. Age of plants at time of measurement are recorded in figure legends and described below, where appropriate.

DNA Isolation and Molecular Identification of ntrc mutants

DNA isolation was performed according to Edwards, Johnstone, and Thompson, 1991 with a few modifications. Around 0.5 cm of plant tissue was flash frozen in liquid nitrogen and then homogenized using silica beads. 500 µL of extraction buffer (200 mM Tris-HCl, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, pH 7.5) was added to the homogenized tissue and centrifuged at max speed for 5 minutes. To a new tube containing 500 µL of isopropanol, the supernatant was added, inverted several times, then incubated at -20°C for 10 minutes. After cold incubation, the samples were centrifuged at max speed for 5 minutes. The supernatant was discarded leaving only the pellet. Cold 70% ethanol was added (500 µL) to the pellet and softly inverted as to not disturb the pellet before centrifugation at max speed for 5 minutes. All of the ethanol was removed via pipette and dried with the tube open at 37°C for 15 minutes. The pellets were resuspended in 40 µL of ddH_2O and stored in -20°C prior to genotyping.

To confirm the *ntrc1* T-DNA insertion for both single and double mutant analysis, primers were designed such that only *ntrc1* mutant allele would amplify (Left Primer or LP (specific for *NTRC*): 5'-TCCCTTGGTGTTGCTCAATAC, Border Primer (specific for T-DNA): 5'-TAGCATCTGAATTTCATAACCAATCTCGATACAC). To test for wildtype (Col-0) NTRC, the following primers were used (LP: 5'-TCCCTTGGTGTTGCTCAATAC, Right Primer or RP: 5'-GGTCCCGATTTAATGGAGAAG).

To confirm the *ntrc2* insert (SALK 096776), the following primers were used to detect the T-DNA insertion and wildtype *NTRC*, respectively (LP: 5'-

TCAGAAGTGCAACGATCACTG, BP: 5'-ATTTTGCCGATTTCGGAAC, LP+ RP: 5'-CAAGCATTTTCTCTGCCTCAC).

NTRC *Protein Analysis*

Plants were grown to 5-weeks old and extracted according to Martínez-García and Quail, 1999. PhytoAB's anti-NTRC antibody (#PHY0792A) was used to detect the NTRC protein at a 1:2,000 dilution. After blocking with 5% w/v non-fat dry milk for 12 hours, the membranes were incubated with anti-rabbit conjugated with HRP (Biorad #1706515) at a 1:20,000 dilution for 1 hour in 5% w/v non-fat dry milk. The blots were developed using Clarity Western ECL Substrate kit (Biorad ##1705061) and imaged on a BioRad Chemidoc.

NTRC *Whole Transcript Analysis by Semi-Quantitative RT-PCR*

Total RNA from leaves of 5-week-old plants was extracted with the Qiagen RNAeasy Plant Mini Kit (#74904) according to manufacturer's instructions. cDNA was generated using Thermofisher's SuperScript IV First-Strand Synthesis kit (#18091050) according to manufacturer's instructions using oligo dTs. To amplify the entire *NTRC* sequence, two primers were designed that span a codon region (LP: 5'-ATGGCTGCGTCTCCCAAG, RP: 5'ATTTATTGGCCTCAATGAATTCT) (Figure 6). ETF1 was used as a quality control (LP: 5'-TGAGCACGCTCTTCTTGCTTTCA, RP: 5'GGTGGTGGCATCCATCTTGTTACA). The PCR ran for 35 cycles and its product was analyzed on a 1% (w/v) agarose gel at 120V in sodium borate buffer for 30 minutes and visualized with ethidium bromide staining using a BioRad Chemidoc system.

Generation of higher-order mutants

All double mutants were generated using traditional manual crossing methods for *Arabidopsis* (Table 4) (Weigel and Glazebrook, 2002). After each generation (F_1, F_2, and F_3), DNA was extracted, and the mutants were analyzed via PCR to confirm the genotype.

Photosynthetic Parameters

All photosynthetic parameters such as PhiNPQ, Phi2, and LEF were measured using the MultiSpecQ, a handheld fluorometer capable of measuring multiple photosynthetic parameters simultaneously (Sebastian *et al.,* 2016). Results were analyzed using the program Photosynthesis RIDES.

Photosynthetic Pigment Determination

To determine total chlorophyll, 4-week-old leaves were weighed before submersion in 2 mL of 80% acetone for 24 hours. Absorbances were read at 646 nm and 663 nm. Total chlorophyll was quantified according to Lichtenthaler and Welburn, 1983. To determine anthocyanins, ~100 mg of leaf tissue from 4-week-old plants was extracted following Wang and Benning, 2011 for lipid analysis (see below). Within this extraction, the aqueous layer was removed, and an absorbance was recorded at 520 nm. For both chlorophyll and anthocyanins, absorbances were normalized using tissue fresh weight.

Dry Weight Determination

Whole rosettes were taken from 5-week-old plants. All flowering stems were cut at the base such that only the rosette was weighed. Plants were dried for 5 days at 60$^{\circ C}$ and normalized according to fresh weight.

Fatty Acid Methyl Ester Total Lipid Extraction

To analyze total fatty acids, one rosette leaf from 4-week-old plants was extracted in 1 mL of 1 N methanolic hydrochloric acid with 100 µL of the 15:0 FFA-TAG internal standard (Avanti Polar Lipids # 791648). Samples were tightly capped and heated at 80˚C for 30 mins. The organic and aqueous layers were separated with 1 mL of 1% sodium chloride (NaCl) and 1 mL of mixed hexanes after centrifugation for 3 minutes at 1500 rpm. The organic layer was removed and dried under a constant stream of nitrogen gas. FAMEs were resuspended in 100 µL of hexane and analyzed via GC (Agilent #7890B). This is an adapted protocol from Wang and Benning, 2011.

Thin-Layer Chromatography

To analyze specific polar lipid classes, ~100 mg of tissue from 4-week-old plants was extracted in 600 µL of organic solvent (20:10:1 v/v/v; methanol:chloroform:formic acid) within microcentrifuge tubes. Samples were vortexed continuously for 5 minutes. Next, 300 µL of the aqueous solution (0.2 M phosphoric acid, 1 M potassium chloride) was added and vortexed for 5 seconds. The samples were centrifuged for 1 minute at 13,000xg before loading onto silica plates.

Two days prior to sample extraction, Macherey-Nagel G-25 HR silica plates (#809043) were dipped in 0.15 M ammonium sulfate for 30 seconds and air dried in a fume hood for at least 48 hours. On the day of the experiment, the plates were activated by heating at 120˚C for 2.5 hours. Total lipid extracts were spotted onto the plates at 20 µL for total fatty acid (at far end of plate, i.e., does not undergo separation) and 40 µL for lipid class determination (at origin, i.e., for

separation). Separation was carried out in an acetone, toluene, and water solvent (91 mL: 30 mL: 7.5 mL). Separation of the TLC plate lasted for ~1 hour, or until samples neared the top of the plate (>1cm). After the plate had air dried, it was placed in an iodine chamber for no longer than 45 seconds for lipid class visualization. After tracing the location of each class with a pencil, each lipid spot corresponding to a specific class was scraped and transferred to a screw top glass tube. The protocol then proceeded as described in FAME analysis (see *Methods 2.25*). This is also an adapted protocol from Wang and Benning, 2011.

5. RESULTS

5.1 Characterization of *ntrc* alleles

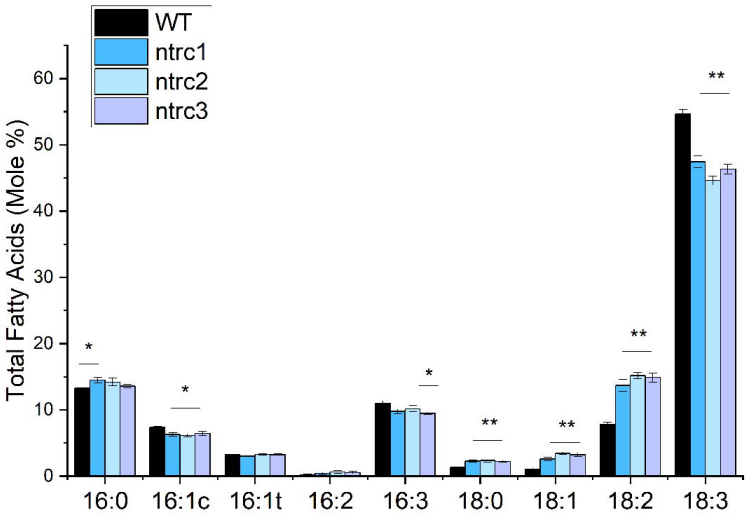


Figure 7. Lipid profile of three *ntrc* mutant alleles. Asterick indicates a p-value <0.05 for all alleles compared to wildtype (Col-0). Rosette leaves extracted from 4-week old plants with at least three replicates. *Ntrc1=SAIL115_E08, ntrc2=SALK096_776, ntrc3= SALK_114293.* Asterisk indicates p-value <0.05. Double asterix indicates p-value <0.005

The *ntrc* mutant has been reported as pleiotropic based on allelic differences in *ntrc* T-DNA lines (Carrillo *et al.*, 2016; Nikkanen *et al.*, 2018). To be sure the lipid phenotype was consistent amongst all alleles, I analyzed three *ntrc* alleles and found the phenotype of altered total fatty acid composition in leaves. This indicated the *ntrc* alteration in lipid metabolism

was not due to a random T-DNA insertion.

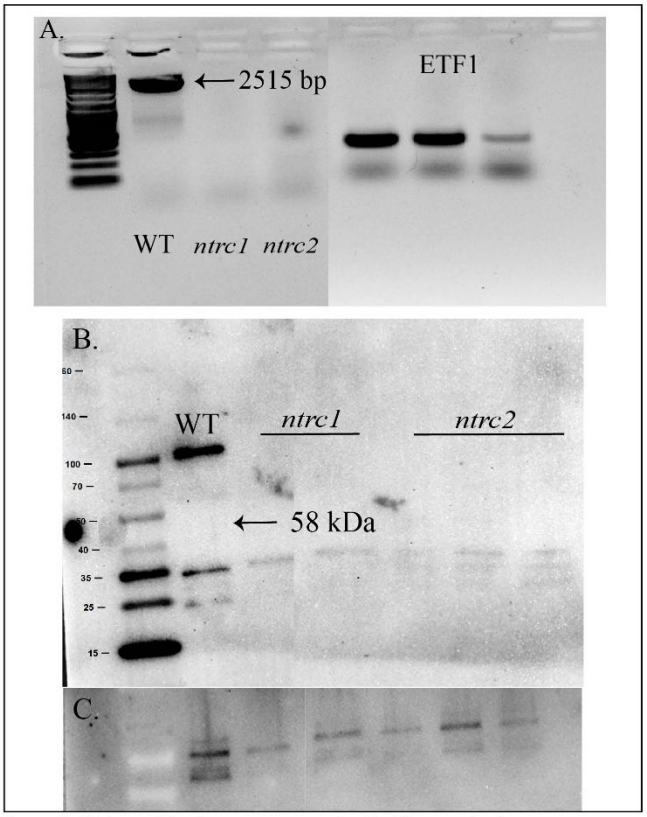


Figure 8. Pleiotrophic phenotypes associated with *ntrc* single mutants are not due to full-length transcript production in two lines tested. **A**. Whole transcript amplifaction of NTRC in wildtype (Col-0), *ntrc1*, and *ntrc2*. Primer location described in Fig. 9A. ETF1 was used as a cDNA quality control. **B.** Protein analysis of NTRC in wildtype (Col-0), *ntrc1*, and *ntrc2*. **C.** Protein samples were detected with plastocyanin as a quality control. Both trials were run twice with at least two replicates per genotype.

Why and/or how *ntrc* alleles display varied phenotypes (i.e., not related to lipid composition) has yet to be answered in the literature. *Ntrc1*, used in the generation of the *ntrc ats1-1* and *ntrc tgd1* doubles, needed to be confirmed as an effective NTRC knockout to accurately predict hypotheses about potential *ntrc* involvement in lipid metabolism. To do this, total protein and RNA were extracted from *ntrc1* and *ntrc2* to determine if the allelic differences were due to a partial protein and/or transcript production (See *Methods*). Using primers that span the entire NTRC transcript (Fig. 8) (see Appendix B), it was determined that neither mutant is producing a full-length transcript (Fig. 8). The results displayed in figure 8 are from the most recent RT-PCR and Western blots performed.

The protein data was inconclusive as the primary antibody did not bind to the NTRC protein at the expected size (58kDa) (Figure 8). It is possible that the higher molecular weight band (~100kDa) is an aggregation or dimerization of the NTRC protein; however, at this time this possibility remains unknown.

5.2 *Ntrc* HIGHER-ORDER MUTANTS

All *ntrc* double mutants were generated using the *ntrc1* mutant allele. After each crossing and replication cycle, mutants were screened via PCR and gas chromatography (GC) to test for the *ntrc* insert and lipid phenotypes associated with the lipid parents (Fig. 9) (see *Methods*). GC was used to determine the mutant lipid phenotype as *ats1-1* and *tgd1* are single point mutations and thus difficult to scan via PCR. Primers set used for genotyping may be found in Appendix B.

Although the focus of this thesis will be the *ntrc ats1-1* and the *ntrc tgd1* mutants, *ntrc* was also crossed with other lipid metabolism-associated and ROS-scavenging enzymes such as *fatty acid desaturase 4, 5, 6,* and *7/8*, *catalase 1 and 2*, and *prx- q, a,* and *b* (see Appendix A)

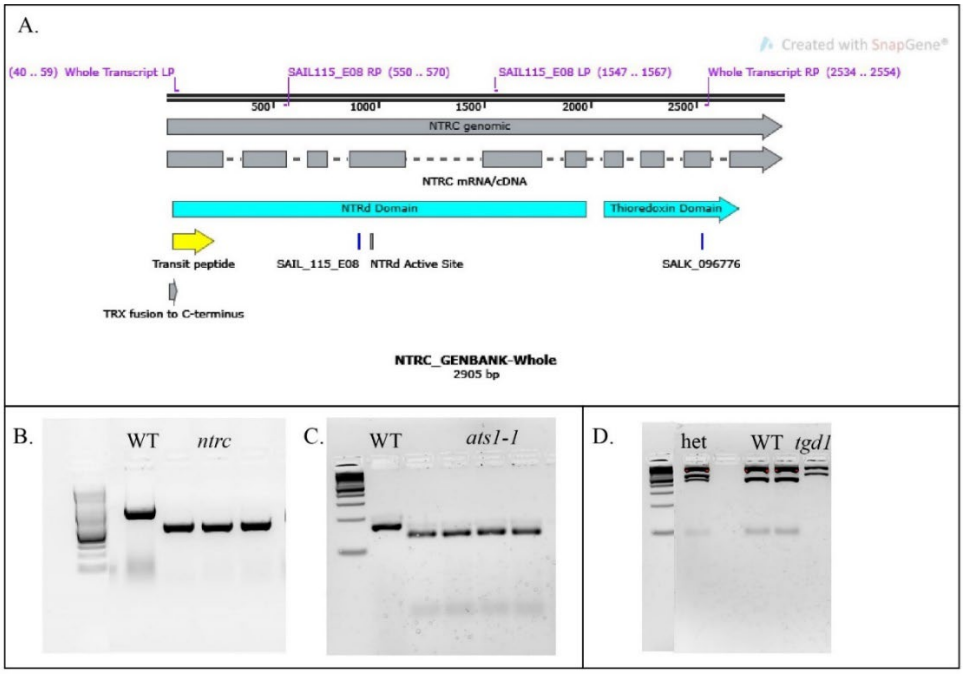


Figure 9. Molecular characterization of *ntrc* mutant alleles and double mutants. A. NTRC sequence obtained through Genbank. Genotyping primers for wildtype SAIL115_E08 *ntrc* allele are denoted as right primer (RP) and left primer (LP). Primers used for RNA transcript analysis are denoted as Whole Transcript RP and LP. B. PCR amplication of NTRC and *ntrc*. C. PCR amplication of ATS1 and *ats1-1* using the enzyme DdeI. D. PCR amplication of TGD, heterozygous *tgd1*, and *tgd1*.

5.3 CHARACTERIZATION OF *ntrc ats1-1*

Overall, the *ntrc ats1-1* double displays a stunted growth phenotype compared to wildtype and *ntrc* (Fig. 10A). This growth phenotype, compared to *ntrc*, is not dependent on light availability as it was visualized under both normal and light limiting conditions (Fig. 10A and Fig. 11 B, E).

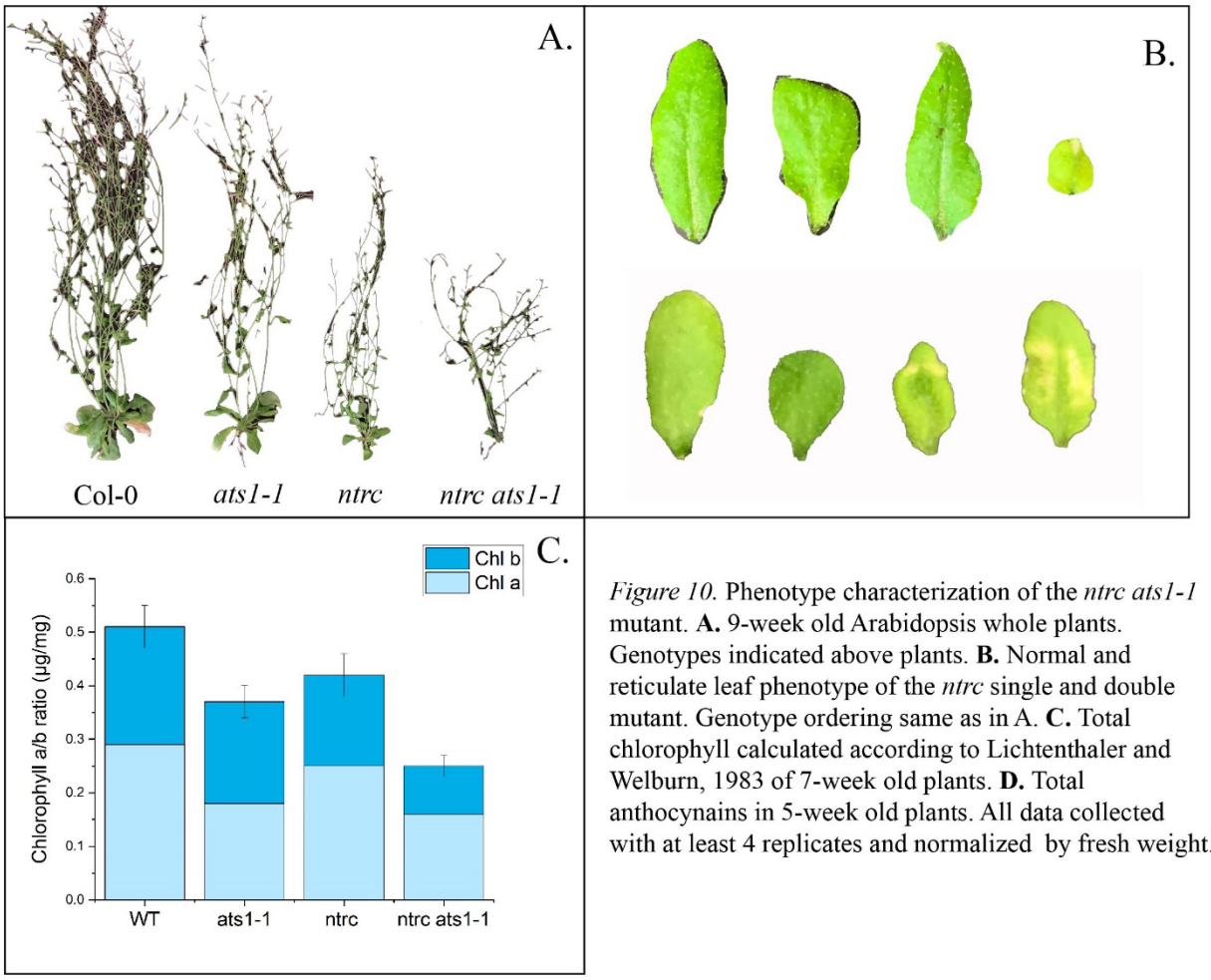


Figure 10. Phenotype characterization of the *ntrc ats1-1* mutant. **A.** 9-week old Arabidopsis whole plants. Genotypes indicated above plants. **B.** Normal and reticulate leaf phenotype of the *ntrc* single and double mutant. Genotype ordering same as in A. **C.** Total chlorophyll calculated according to Lichtenthaler and Welburn, 1983 of 7-week old plants. **D.** Total anthocynains in 5-week old plants. All data collected with at least 4 replicates and normalized by fresh weight.

Leaves of young plants (4-weeks, pre-inflorescence), but not older (7-weeks, post-inflorescence with several siliques established), of *ntrc ats1-1* exhibit an exaggeration of the reticulate phenotype associated with *ntrc* (Fig. 10B). This presumably led to a decrease in overall chlorophyll content (µg/mg) for *ntrc ats1-1* (Fig. 10C). The gradual "greening", or loss of the

reticulate phenotype during aging, has been visualized in *ntrc* and published by Lepisto *et al.* in 2009 (See *Section 2.4*). Due to the heightened reticulate phenotype of *ntrc ats1-1* compared to *ntrc*, a disruption in plastid lipid metabolism could be the central metabolic pathway leading to the reticulate phenotype. This disruption may also be specific to a developmental time point for *Arabidopsis*.

When comparing photosynthetic data of *ntrc ats1-1,* the double mutant displays an elevated PhiNPQ phenotype that exceeds *ntrc* under normal and recovery conditions (Fig. 11A and 11D). To determine the PhiNPQ levels under low light stress, *ntrc ats1-1* was grown alongside wildtype (Col-0), *ats1-1*, and *ntrc* under normal light conditions (100 µmol m^{-2} s^{-1}) for two weeks. The plants were then subjected to a low light stressor (25 µmol m^{-2} s^{-1}) for another two weeks before placement back into the normal light irradiance (i.e., the recovery period) (Fig, 11C). One notable phenotype observed during these assays was the *ntrc ats1-1* retainment of elevated PhiNPQ one week after light recovery (Fig. 11D).

Whereas *ntrc* returned to normal PhiNPQ levels shortly (24 hours) after the stress removal, *ntrc ats1-1* was unable to recover and return PhiNPQ to wildtype levels even given 1 week of recovery (Fig. 11D). It is interesting to note the *ats1-1* single mutant also displayed slightly elevated PhiNPQ under low light when compared to the wildtype; however, this was not a significant difference. Regardless, this slight yet consistent phenotype was observed in all three stress-recovery trials. A NPQ phenotype for *ats1-1* has not been previously reported nor studied. Lastly, a decrease in rosette size was observed for *ntrc ats1-1* regardless of growth conditions. Rosette sizes for all genotypes under normal and recovery conditions can be seen in Fig. 11 B and E, respectively.

Under normal growth conditions *ntrc ats1-1* displays an elevated levels of the 18:2 fatty acid, known as linoleic acid; however, this was the only substantial difference in lipid profile compared to either mutant parent. This degree of elevated 18:2 synthesis was not observed in either *ntrc* or *ats1-1* despite both single mutants displaying alterations in 18:3 metabolism, which could lead to an increase in less desaturated species (i.e., 18:2, 18:1).

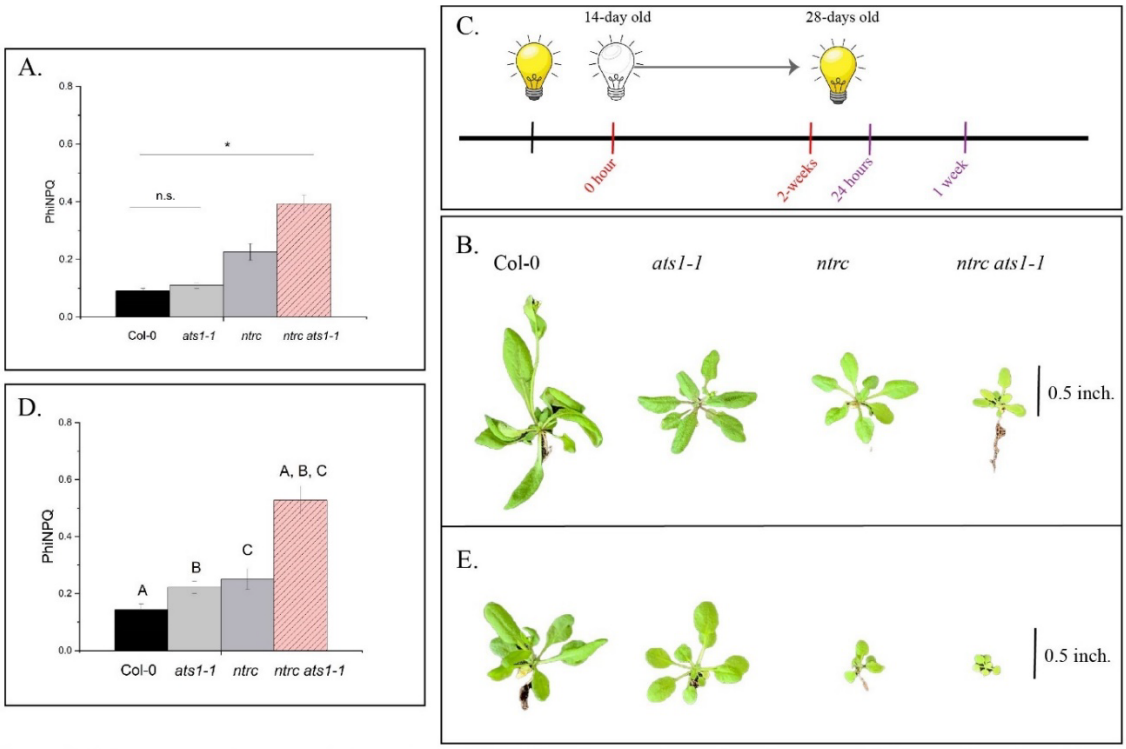


Figure 11. The *ntrc ats1-1* mutant displays elevatd PhiNPQ and stunted growth under normal (100 µmol^{-2}sec^{-1}) and low light (25 µmol^{-2}sec^{-1}) growth conditions. **A.** PhiNPQ is elevated in *ntrc ats1-1* under normal conditions. **B**. Rosette size comparison under normal growth conditions. **C.** Experimental design for stress-recovery assay. Stress data points are indicated in red. Recovery data points indicated in purple. **D.** Seven days after a return to normal growth conditions the *ntrc ats1-1* mutant maintains the elevated PhiNPQ phenotype. **E**. Rosette size comparison after 1-week low light recovery. Rosettes leaves from 5-week old plants were analyzed using the MultiSpecQ handheld fluormeter. PhiNPQ was avergaed from three trials using at least 3 replicates per genotype.

To see if the fatty acid profiles would change under known *ntrc* stressors (short-day, low light), the *ntrc ats1-1* double was subjected to both light limiting scenarios to determine how composition of fatty acids (FAs) was affected (Fig. 12). Although exact molar percent between

treatments differed, the overall trend between genotypes remained consistent to its respective control groups. Thus, the *ntrc ats1-1* lipid phenotype is not subjected to drastic changes in metabolism under light limiting conditions. Similarly based on this data, the *ntrc* lipid phenotype is not dependent on light conditions like many other *ntrc* phenotypes. One more stress-recovery trial will be performed so that I may obtain cleaner data for a publication.

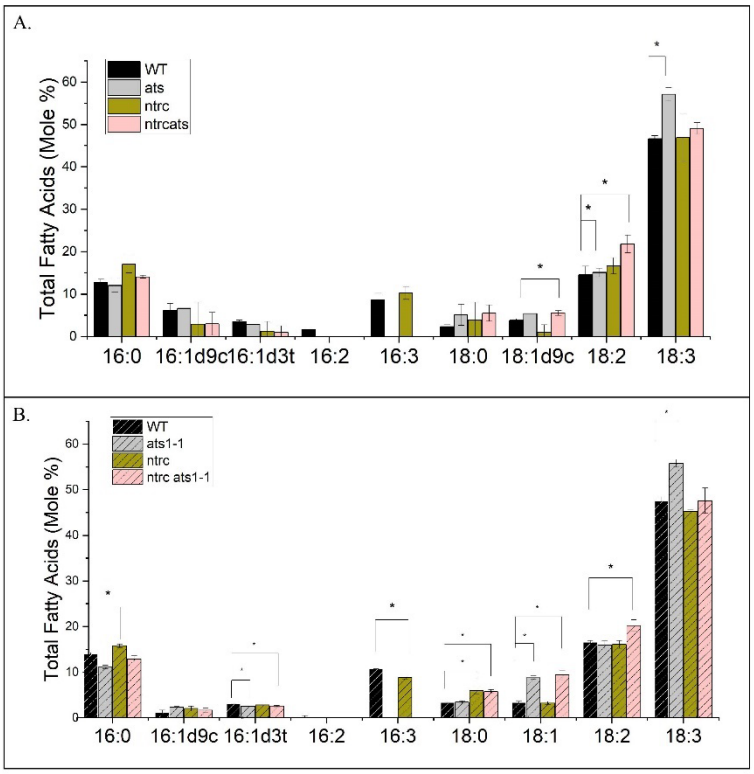


Figure 12. Total fatty acids under short-day and low light growth conditions. **A.** Short day (8hrs light/16hrs dark) total fatty acids from 4-week old plants. **B.** Low light (25 µmol $m^{-2}s^{-1}$) total fatty acids from 4-week old plants. Stress trial was completed twice with at least four replicates per genotype.

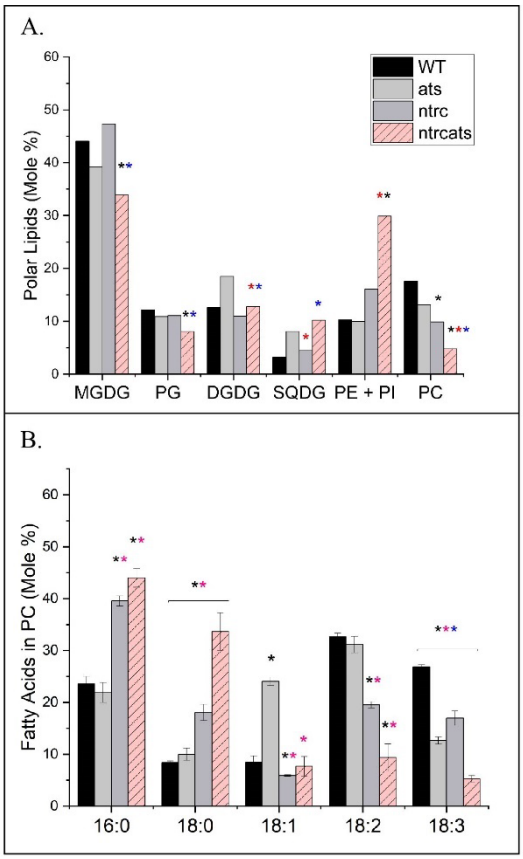


Figure 13. Lipid profiling of the *ntrc ats1-1* mutant reveals a disruption in PC metabolism. A. Percent of total polar lipids. B. Total fatty acids associated with PC. Whole rosettes were collected from 5-week old plants. Black asterisk indicates signifance of <0.05 from the wildtype, red asterix is signifance from *ats1-1*, blue from *ntrc*.

After analyzing the percent of each lipid class in *ntrc ats1-1*, it was discovered that *ntrc ats1-1* displays a severe reduction in phosphatidylcholine (PC) (Fig. 12B). Whereas wildtype levels of PC are ~17%, the *ntrc ats1-1* displays levels ~8%. PC is found primarily in the outer envelope membrane (OEM) of the chloroplast as well as non-chloroplast membranes such as the

ER, where it is synthesized (Li-Beisson *et al.,* 2013) (Fig. 12A). When analyzing fatty acids associated with PC, I found that the 18:3 fatty acid was severely decreased in *ntrc ats1-1* (Fig. 13B). This indicated that the overall decrease in PC content may be due to a reduction in 18:3 synthesis.

5.4 CHARACTERIZATION OF *ntrc tgd1-1*

Tgd1-1, as a single mutant, exhibits a severe decrease in ER-derived lipids due to a disruption in their transport (Xu *et al.,* 2005). Thus, the *ntrc tgd1-1* double would be a plant deficient in ER-derived lipids and forced to rely primarily on plastid-derived species for chloroplast membrane structure. Research on *ntrc ats1-1* has begun to provide insights into the potential involvement of NTRC in plastid lipid metabolism. However, the most revealing piece of data in support of this hypothesis was not found in *ntrc ats1-1*, but in *ntrc tgd1-1* due to its high embryonic lethality.

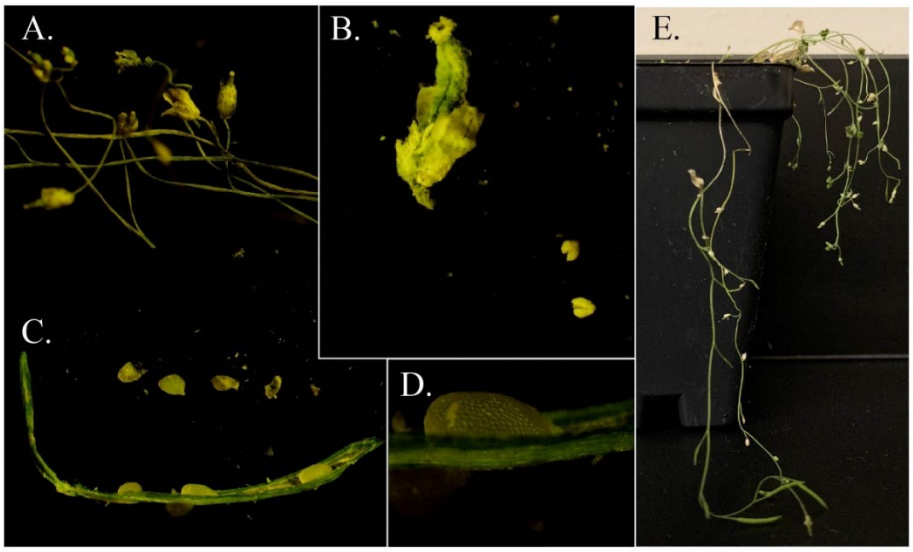


Figure 14. The *ntrc tgd-1* double mutant expresses n high embryo abortion rate alongside stunted silique growth and weak stem support. **A.** Stunted silique growth after pollination. **B.** Pollen grains remain intact in mutant siliques. **C**. High embryo abortion and maturation arrest. **D.** Developing embryo in mutant silique. **E.** Weak stem support displayed in the mutant.

Embryo abortions (~50%) are associated with the *tgd1-1* mutant due to a disruption during the heart phase of seed development (Xu *et al.,* 2005). The homozygous *ntrc tgd1-1* exceeds *tgd1-1* by exhibiting an abortion rate of 94% (Table 3). In addition to its embryo viability phenotype, *ntrc tgd1* is a very sick plant displaying little to almost no stem support as well as stunted and aborted silique growth (Fig.14). Like *ntrc ats1-1*, the total fatty acid profile of *ntrc tgd1-1* follows that of its lipid parent, *tgd1-1* (Fig. 15).

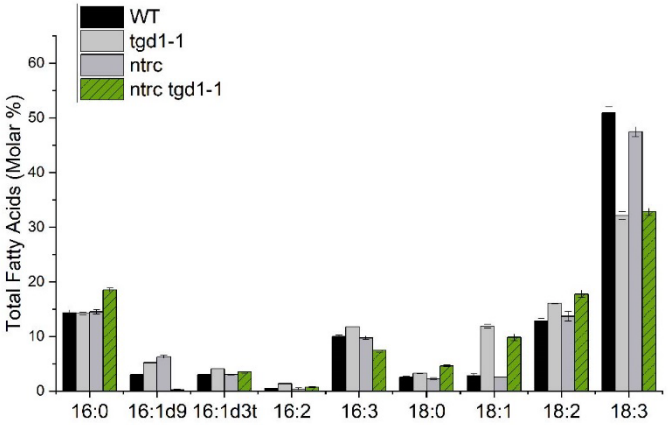


Figure 15. Lipid profile of *ntrc tgd1-1* compared to single mutant parents. Rosette leaves were extracted at 5-weeks with at least three replicates per genotypes.

Table 3. Germination rate (%) of *ntrc tgd1-1* exceeds that of *tgd1-1*. *Ntrc tgd1-1* % was quantified once. Wildtype (Col-0), *tgd1*, and *-/- ntrc +/- tgd1-1* was quantified twice.

WT	100 %
tgd1-1	50 %
-/- ntrc +/- tgd1-1	96 %
ntrc tgd1-1	6 %

Obtaining the homozygous *ntrc tgd1-1* was difficult due to its high lethality, so there is limited data on the double at this point as we continue to gather seeds from various genetic backgrounds including homozygous *ntrc*/heterozygous *tgd1-1* and vice versa. Despite this timing setback, a main takeaway from the generation of this double mutant is evidence for NTRC involvement in plastid lipid metabolism. It was previously shown that the loss of both metabolic pathways (i.e., *ats1 tgd1-1)* is lethal (Xu *et al.,* 2005). the *ntrc tgd1-1* double is unable to synthesis ER-derived lipids due to *tgd1-1* and is coupled with an alteration in plastid lipids due to *ntrc*, it would make sense for the offspring to be highly

abortive. Thus, it is hypothesized that NTRC is a critical redox regulator in plastid lipid metabolism.

6. Discussion & Future Directions

Allelic differences in *ntrc* lines are not due to whole transcript production. Although neither *ntrc1* nor *ntrc2* tested produced a full-length transcript, the fact remains that there are distinct phenotypic differences between the two. Because the NTRC antibody did not bind to the expected size of the protein in our control (wildtype, Col-0), it is unknown if the allelic differences are due to the production of a partial NTRC protein. To troubleshoot this issue, we are currently expressing NTRC in yeast so that we may obtain a blotting control for future protein analysis.

NTRC Affects Younger Leaf Plastid Metabolism Leading to the Reticulate Phenotype of *ntrc ats1-1*. Regarding the *ntrc ats 1*-1 double, its reticulate phenotype was steadily lost as the plant aged indicating that there may be a disruption in plastid metabolism during development of young plants (4-weeks, pre-inflorescence), but not older (7-weeks, post-inflorescence with several siliques established) (Fig. 10B). This was originally discovered by Lepisto *et al.* in 2009 and was coined as a "greening" effect due to chlorophyll content increasing with age. The greening of *ntrc* presupposes that the reticulate phenotype can repair itself with age and/or may be accompanied by a switch away from NTRC-mediated metabolism at some developmental time point. To quantitatively test this, it may be advantageous to measure growth stages of *ntrc* and *ntrc ats1-1* using developmental stage descriptors for *Arabidopsis* as outlined by Boyes *et al.*, 2001. This would reveal if the lessening of the *ntrc ats 1-1* reticulate phenotype is associated with a particular stage in vegetative and/or reproductive growth.

Nonetheless, the increased chlorosis patterning in *ntrc ats1-1* compared to *ntrc* may be indicative in understanding how the phenotype arises. The reticulate phenotype of *ntrc* is heightened under short-day, or light limiting conditions (Lepisto *et al*., 2009). Reticulate mutants represent mutations in a chloroplast metabolic pathway although the exact pathway disruptions remains unknown (Lundquist *et al.,* 2014). Understanding that metabolism of plastid-derived lipids are preferred under low light (Yu *et al*., 2021) and that *ntrc ats1-1* exhibits a higher level of variegation than *ntrc* (Figure 10B), it may be that the disruption causing the reticulate phenotype in *ntrc* lies within a lipid metabolic pathway. This would explain reticulation in *ntrc* and its amplification in *ntrc ats1-1* due to an almost complete loss of plastid-derived lipids. This theory would presuppose that ER-lipids are more critical at older developmental stages than plastid-derived. This hypothesis has not been explored, yet I believe it warrants further investigation.

***Ntrc* and *ats1-1* exhibit a synergistic relationship regarding elevated levels of NPQ.** While there seems to be a recovery of the reticulate phenotype, the same cannot be said for elevated NPQ in *ntrc ats1-1.* Under normal conditions, the double mutant displays a PhiNPQ phenotype exceeding that of *ntrc* by two-fold (Figure 11A). Additionally, *ntrc ats1-1* is unable to return to normal PhiNPQ levels given 7-days of recovery where it remains three-fold higher than *ntrc* subjected to the same conditions (Figure 11D). This indicates there is a lack of repair in the double mutant related to the NPQ phenotype previously associated only with *ntrc.* This information, coupled with the slight yet consistent increase observed in the *ats1-1* single mutant may prove to be indicative of a synergistic, and therefore functional, relationship between *ntrc* and *ats1-1* regarding NPQ downregulation.

A genetic interaction is defined as any deviation of a double mutant from the expected neutral, or wildtype, phenotype (Mani, *et al.,* 2008). Synergy is a type of genetic interaction that can be assumed of two genes when their combined double mutant exceeds a phenotype associated with either parent genes (Peréz- Peréz *et al*., 2009). Synergy typically results from one of two genetic possibilities: 1) Both genes converge at a pathway node and thus the pathway is disrupted in the double mutant, or 2) The presence of one gene mutation enhances the sensitivity of the other mutation (Peréz- Peréz *et al*., 2009). In the case of *ntrc ats1-1*, synergy was observed regarding elevated NPQ as the double mutant displays a phenotype exceeding both single parents. This indicates that *NTRC* and *ATS1*, or some other plastid lipid metabolism gene disrupted downstream of *ATS1*, have a functional relationship that is most likely relevant in NPQ downregulation. Lastly, a functional relationship between NTRC and plastid lipid metabolism may explain the lethality of *ntrc tgd1-1* (Table 3).

Ntrc exhibits elevated 18:2 synthesis that is enhanced in ntrc ats1-1. The increase of linolic acid (18:2 FA) remained consistent throughout all conditions tested (normal, short-day, and low light) indicating that there may be a relationship between *ntrc* and plastid lipid metabolism related to the 18:2 fatty acid (Figure 7, 12, and 13). In 2004, Falcone *et al*. described an increase in 18:2 synthesis to be associated with membrane acclimatization after a heat stress. Natural activation of NPQ initiates under high light environments, often accompanied with a heat stressor (Tang *et al.,* 2007). It may be that in response to the constant, elevated NPQ levels in *ntrc* and *ntrc ats1-1* that 18:2 synthesis is favored when the membrane is duped into believing it requires a high light, heat stress remodeling event. This idea requires extensive research in order to be properly discussed but this may propose a new function for linoleic acid in membrane acclimation to light while also unraveling the *ntrc ats1-1* lipid phenotype. In support of this goal,

it would be advantageous to extract total fatty acids from plants displaying a deficiency in NPQ. This could be accomplished by studying *npq1*, which is unable to complete the xanthophyll cycle, and *npq4*, lacking the critical PsBs protein (Niyogi, 1999; Li *et al.,* 2000). Both of these mutants display a decrease in NPQ efficiency. Alternatively, it would also be good to measure the lipid profile of *npq2*, which overexerts NPQ (Niyogi *et al.,*1998).

Alternatively, the *ntrc* phenotype could be explained through a stimulating relationship of NTRC and Fatty acid desaturase 7/8 (FAD7/8), which is responsible for the third desaturation event generating both 16:3 or 18:3 from 16:2 or 18:2, respectively. This hypothesis would be derived from the same logic in discovering the PRXQ/FAD4 relationship. If NTRC acts to stimulate FAD7/8, in *ntrc* you would see a decrease in trienoic acids, which is what is observed (Figure 2). I have generated the *ntrc fad7/8* mutant to test these interactions(see Appendix). So, future studies on the *ntrc* lipid phenotype may want to start by studying *ntrc fad7/8*. Additional information may be gained by using protein-protein interaction assays to determine if the two interact.

NTRC Interacts with a Phosphatidylcholine-specific phospholipase. While there does appear to be a synergistic relationship between NTRC and ATS1, *ats1-1* disrupts the initial step of the pathway. Therefore, it is more likely that NTRC has a functional relationship with a downstream gene that is disrupted in *ats1-1* but is not ATS1 itself, per se. One possibility is that NTRC acts on plastid-derived PC through the actions of a PC-specific phospholipase.

González, *et al*. in 2016 found NTRC to interact with a phospholipase *in vivo* after a tandem affinity purification assay. Although the interaction was not explored then, the data presented here may provide insights into this interaction while providing a framework for future directions to explore the *ntrc* lipid phenotype. NTRC supposedly interacts with two lipid

metabolism proteins *in vivo*: ACCase β-subunit and PLDα1. The β -subunit of ACCase acts to initiate the first enzymatic step in fatty acid synthesis via carboxylation of acetyl-CoA producing malonyl-CoA (Yasaki *et al.,* 2004). PLDα1 is a phospholipase which will hydrolyze phosphatidylcholine (PC) and phosphatidylethanol (PE); however, PC is the favored substrate (Wang, X;, 2000). After hydrolysis, the phospholipid is de-acylated into its precursor, phosphatidic acid (PA) (Wang, X; 1999). The benefit of this process is an efficient remodeling system as PA is the precursor for all membrane lipids (Li-Beisson *et al*., 2013). Phospholipases are activated under abiotic and biotic stresses such as drought, wound inducement, and ROS accumulation (Maarouf *et al.,* 1999; Wang, C *et al*., 2000; Sang *et al.,* 2001). When phospholipases generate PA, the membrane may favor the production of fatty acids required to defend and/or repair the current stressors. For NTRC to interact with PLDα1, a phospholipase with substrate specificity for PC, may explain the lipid class profile for *ntrc* and *ntrc ats1-1* (Figure 13).

Ntrc and its double mutant displayed decreased levels of PC, indicating that there was a disruption in the metabolism of PC. While PC is a critical chloroplast membrane lipid, it is solely found in the outer envelope of the plastid where it is the primary component (Botella *et al*., 2016). In the outer envelope PC will undergo what is known as acyl editing where linolenic acid will be cleaved allowing for free fatty acids to be utilized in either the eukaryotic or prokaryotic synthesis pathways (Bates *et al.,* 2013; Li-Beisson *et al*., 2013). A complete disruption in PC synthesis is lethal (Chen *et al*., 2019).

Like other membrane lipids, PC may be synthesized via the plastid and/or eukaryotic pathway. Due to a severe depletion of PC in *ntrc ats1-1* and the high lethality of *ntrc tgd1-1*, NTRC may act on plastid-derived PC via an interaction with a PC-specific phospholipase. This

theory may be tested by quantifying the amount of PA in *ntrc* and its doubles. If an interaction between NTRC and PLDα1 is causing the *ntrc* lipid phenotype, the mutant should display increased levels of PA, which is the reaction product of PLDα1. This would be a relatively simple way to determine if the phospholipase is responsible for the severe decrease of PC in *ntrc*.

REFERENCES

Andersson B., Jan M. Anderson. Lateral heterogeneity in the distribution of chlorophyll-protein complexes of the thylakoid membranes of spinach chloroplasts. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, Volume 593, Issue 2, 1980, Pages 427-440, ISSN 0005-2728, https://doi.org/10.1016/0005-2728(80)90078-X.

Asada K. (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant physiology, 141(2), 391–396. https://doi.org/10.1104/pp.106.082040

Bates PD, Fatihi A, Snapp AR, Carlsson AS, Browse J, Lu C. Acyl editing and headgroup exchange are the major mechanisms that direct polyunsaturated fatty acid flux into triacylglycerols. Plant Physiol. 2012 Nov;160(3):1530-9. doi: 10.1104/pp.112.204438. Epub 2012 Aug 29. PMID: 22932756; PMCID: PMC3490606.

Baranowska Monika, Klaudia SµLiborska, Wojciech Chrzanowski, Barbara Kusznierewicz, Jacek Namieśnik, Agnieszka Bartoszek. The relationship between standard reduction potentials of catechins and biological activities involved in redox control. *Redox Biology*,Volume 17, 2018, Pages 355-366, ISSN 2213-2317, https://doi.org/10.1016/j.redox.2018.05.005.

Bin Y., Wakao S., Fan J.L., Benning C. Loss of plastidic lysophosphatidic acid acyltransferase causes embryo-lethality in Arabidopsis. Plant Cell Physiol. 2004;45:503–510.

Browse J, N Warwick, C R Somerville, C R Slack; Fluxes through the prokaryotic and eukaryotic pathways of lipid synthesis in the '16:3' plant Arabidopsis thaliana. Biochem J 1 April 1986; 235 (1): 25–31. doi: https://doi.org/10.1042/bj2350025

Botella César, JµLiette Jouhet, Maryse A Block. Importance of phosphatidylcholine on the chloroplast surface. Progress in Lipid Research, Volume 65, 2017, Pages 12-23, ISSN 0163-7827, https://doi.org/10.1016/j.plipres.2016.11.001.

Boyes, D. C., Zayed, A. M., Ascenzi, R., McCaskill, A. J., Hoffman, N. E., Davis, K. R., & Görlach, J. (2001). Growth stage-based phenotypic analysis of Arabidopsis: a model for high throughput functional genomics in plants. The Plant cell, 13(7), 1499–1510. https://doi.org/10.1105/tpc.010011

Chadee Avesh, Alber Nicole A., Dahal Keshav, Vanlerberghe Greg C. (2021) The Complementary Roles of Chloroplast Cyclic Electron Transport and Mitochondrial Alternative Oxidase to Ensure Photosynthetic Performance. Frontiers in Plant Science, 12 1664-462X https://doi.org/10.3389/fpls.2021.748204

Changcheng Xu, Jilian Fan, John E. Froehlich, Koichiro Awai, Christoph Benning, Mutation of the TGD1 Chloroplast Envelope Protein Affects Phosphatidate Metabolism in Arabidopsis , The Plant Cell, Volume 17, Issue 11, November 2005, Pages 3094–3110, https://doi.org/10.1105/tpc.105.035592

Chen, W., Taylor, M. C., Barrow, R. A., Croyal, M., & Masle, J. (2019). Loss of Phosphoethanolamine N-Methyltransferases Abolishes Phosphatidylcholine Synthesis and Is Lethal. Plant physiology, 179(1), 124–142. https://doi.org/10.1104/pp.18.00694

Cook, R.; Lupette, J.; Benning, C. The Role of Chloroplast Membrane Lipid Metabolism in Plant Environmental Responses. Cells 2021, 10, 706. https://doi.org/10.3390/cells10030706

Courteille, A. , Vesa, S. , Sanz-Barrio, R. , Cazale, A. , Becuwe-Linka, N. , Farran, I. , … Rumeau, D. (2013). Thioredoxin m4 controls photosynthetic alternative electron pathways in Arabidopsis . Plant Physiology, 161, 508–520. 10.1104/pp.112.207019

Dat, J., Vandenabeele, S., Vranová, E. *et al*. Dual action of the active oxygen species during plant stress responses . CMLS, Cell. Mol. Life Sci. 57, 779–795 (2000). https://doi.org/10.1007/s000180050041

Detlef Weigal and Jane Glzebrook. Adapted from "Genetic Analysis of Mutants," Chapter 3 in Arabidopsis by Detlef Weigel and Jane Glazebrook. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 2002.

Demmig-Adams B, Garab G, William Adams III, Govindgee (2014) Non-photochemical quenching and energy dissipation in plants, algae and cyanobacteria, Advances in Photosynthesis and Respiration 40. Springer Science+Business Media Dordrecht

Dormann, P., Balbo I., C. Benning (1999). *Arabidopsis Galactolipid Biosynthesis and Lipid Trafficking Mediated by DGD1*. Science, Vol. 284, Issue 5423, p. 2181-82184. DOI: 10.1126/science.284.5423.2181

Dörte Klaus, Heiko Härtel, Lynda M. Fitzpatrick, John E. Froehlich, Jamie Hubert, Christoph Benning, Peter Dörmann, Digalactosyldiacylglycerol Synthesis in Chloroplasts of the Arabidopsis dgd1 Mutant, Plant Physiology, Volume 128, Issue 3, March 2002, Pages 885–895, https://doi.org/10.1104/pp.010780

Falcone, D.L., Ogas, J.P. & Somerville, C.R. RegμLation of membrane fatty acid composition by temperature in mutants of Arabidopsis with alterations in membrane lipid composition. BMC Plant Biol 4, 17 (2004). https://doi.org/10.1186/1471-2229-4-17

Jilian Fan & Changcheng Xu (2011) Genetic analysis of Arabidopsis mutants impaired in plastid lipid import reveals a role of membrane lipids in chloroplast division, Plant Signaling & Behavior, 6:3, 458-460, DOI: 10.4161/psb.6.3.14715

Falcone DL, Ogas JP, Somerville CR. RegμLation of membrane fatty acid composition by temperature in mutants of Arabidopsis with alterations in membrane lipid composition. BMC Plant Biol. 2004 Sep 17;4:17. doi: 10.1186/1471-2229-4-17. PMID: 15377388; PMCID: PMC524174.

Foyer Christine H., Alexander V. Ruban, Graham Noctor; Viewing oxidative stress through the lens of oxidative signalling rather than damage. Biochem J 15 March 2017; 474 (6): 877–883. doi: https://doi.org/10.1042/BCJ20160814

González Maricruz, Víctor Delgado-Requerey, JµLia Ferrández, Antonio Serna, Francisco Javier Cejudo, Insights into the function of NADPH thioredoxin reductase C (NTRC) based on identification of NTRC-interacting proteins in vivo, Journal of Experimental Botany, Volume 70, Issue 20, 15 October 2019, Pages 5787–5798, https://doi.org/10.1093/jxb/erz326

Heinz, E. and Roughan, G. (1983) Similarities and differences in lipid metabolism of chloroplasts isolated from 18:3 and 16:3 plants. Plant Physiol. 72, 273–279.

Higashi Y, Okazaki Y, Myouga F, Shinozaki K, Saito K. Landscape of the lipidome and transcriptome under heat stress in Arabidopsis thaliana. Sci Rep. 2015 May 27;5:10533. doi: 10.1038/srep10533. PMID: 26013835; PMCID: PMC4444972.

Horn PJ, Smith MD, Clark TR, Froehlich JE, Benning C. PEROXIREDOXIN Q stimµLates the activity of the chloroplast 16:1Δ3trans FATTY ACID DESATURASE4. Plant J. 2020 May;102(4):718-729. doi: 10.1111/tpj.14657. Epub 2020 Jan 31. PMID: 31856363.

Jarvis, P. , Dörmann, P. , Peto, C.A. , Lutes, J. , Benning, C. , Chory, J. (2000). Galactolipid deficiency and abnormal chloroplast development in the Arabidopsis MGD synthase 1 mutant. Proc. Natl. Acad. Sci. USA 97: 8175–8179.

Jethva, J.; Schmidt, R.R.; Sauter, M.; Selinski, J. Try or Die: Dynamics of Plant Respiration and How to Survive Low Oxygen Conditions. Plants 2022, 11, 205. https://doi.org/10.3390/plants11020205

Kelly, A. A., Froehlich, J. E., & Dörmann, P. (2003). Disruption of the two digalactosyldiacylglycerol synthase genes DGD1 and DGD2 in Arabidopsis reveals the existence of an additional enzyme of galactolipid synthesis. The Plant cell, 15(11), 2694–2706. https://doi.org/10.1105/tpc.016675

Kong W, Shiota S, Shi YX, Nakayama H, Nakayama K. 2000. A novel peroxiredoxin of the plant Sedum lineare is a homologue of Escherichia coli bacterioferritin co-migratory protein (Bcp). Biochem. J. 351:107–14.

Kunst L, Browse J, Somerville C. Altered regµLation of lipid biosynthesis in a mutant of Arabidopsis deficient in chloroplast glycerol-3-phosphate acyltransferase activity. Proc Natl Acad Sci U S A. 1988 Jun;85(12):4143-7. doi: 10.1073/pnas.85.12.4143. PMID: 16593939; PMCID: PMC280382.

Lepistö A., Saijaliisa Kangasjärvi, Eeva-Maria Luomala, Günter Brader, Nina Sipari, Mika Keränen, Markku Keinänen, Eevi Rintamäki, Chloroplast NADPH-Thioredoxin Reductase Interacts with Photoperiodic Development in Arabidopsis , Plant Physiology, Volume 149, Issue 3, March 2009, Pages 1261–1276, https://doi.org/10.1104/pp.108.133777

Levine A, Tenhaken R, Dixon R, Lamb C (1994) H2O2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79: 583–593

Li, X.P., Bjorkman, O., Shih, C., Grossman, A.R., Rosenquist, M., Jansson, S., and Niyogi, K.K. (2000). A pigment-binding protein essential for regulation of photosynthetic light harvesting. Nature 403, 391–395.

Lithenhaler, Hartmut and Alan Wellburn. Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochem Soc Trans 1 October 1983; 11 (5): 591–592. doi: https://doi.org/10.1042/bst0110591

Lundquist PK, Rosar C, Bräutigam A, Weber AP. Plastid signals and the bundle sheath: mesophyll development in reticulate mutants. Mol Plant. 2014 Jan;7(1):14-29. doi: 10.1093/mp/sst133. Epub 2013 Sep 17. PMID: 24046062.

Mata-Pérez, Capilla and Steven H. Spoel. *Thioredoxin-mediated redox signalling in plant immunity*, Plant Science, Volume 279, 2019, Pages 27-33, ISSN 0168-9452, https://doi.org/10.1016/j.plantsci.2018.05.001.

Mani R, St Onge RP, Hartman JL 4th, Giaever G, Roth FP. Defining genetic interaction. Proc Natl Acad Sci U S A. 2008 Mar 4;105(9):3461-6. doi: 10.1073/pnas.0712255105. Epub 2008 Feb 27. PMID: 18305163; PMCID: PMC2265146.

Maarouf, H.E.; Y. Zuily-Fordil, M. Gareil, A. d'Arcy-Lameta and A.T. Pham Thi. Plant Mol. Biol, 39 (1999), pp. 1257-1265

Medina, E., Kim, S. H., Yun, M., & Choi, W. G. (2021). RecapitµLation of the Function and Role of ROS Generated in Response to Heat Stress in Plants. Plants (Basel, Switzerland), 10(2), 371. https://doi.org/10.3390/plants10020371

Michael L. Pegis, John A. S. Roberts, Derek J. Wasylenko, Elizabeth A. Mader, Aaron M. Appel, and James M. Mayer. Inorganic Chemistry 2015 54 (24), 11883-11888 DOI: 10.1021/acs.inorgchem.5b02136

Moellering, E.R. , Muthan, B. , Benning, C. (2010). Freezing tolerance in plants requires lipid remodeling at the outer chloroplast membrane. Science 330: 226–228.

Naranjo B, Penzler J-F, Rühle T, Leister D. NTRC Effects on Non-Photochemical Quenching Depends on PGR5. Antioxidants. 2021; 10(6):900. https://doi.org/10.3390/antiox10060900

Nikkanen, L., Guinea Diaz, M., Toivola, J., Tiwari, A., & Rintamäki, E. (2019). MµLtilevel regµLation of non-photochemical quenching and state transitions by chloroplast NADPH-dependent thioredoxin reductase. Physiologia plantarum, 166(1), 211–225. https://doi.org/10.1111/ppl.12914

Nikkanen, L., Toivola, J., Trotta, A., Diaz, M. G., Tikkanen, M., Aro, E. M., & Rintamäki, E. (2018). RegµLation of cyclic electron flow by chloroplast NADPH-dependent thioredoxin system. Plant direct, 2(11), e00093. https://doi.org/10.1002/pld3.93

Niyogi, K.K. (1999). Photoprotection revisited: Genetic and molecular approaches. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 333–359.

Niyogi, K.K., Grossman, A.R., and Bjo¨rkman, O. (1998). Arabidopsis mutants define a central role for the xanthophyll cycle in the regulation of photosynthetic energy conversion. Plant Cell 10, 1121–1134.

Pérez-Pérez JM, Candela H, Micol JL. Understanding synergy in genetic interactions. Trends Genet. 2009 Aug;25(8):368-76. doi: 10.1016/j.tig.2009.06.004. Epub 2009 Aug 6. PMID: 19665253.

Pérez-Ruiz JM, Spínola MC, Kirchsteiger K, Moreno J, Sahrawy M, Cejudo FJ. Rice NTRC is a high-efficiency redox system for chloroplast protection against oxidative damage. Plant Cell. 2006 Sep;18(9):2356-68. doi: 1

Roeber, VM, Bajaj, I, Rohde, M, Schmülling, T, Cortleven, A. Light acts as a stressor and influences abiotic and biotic stress responses in plants. Plant Cell Environ. 2021; 44: 645– 664. https://doi.org/10.1111/pce.13948 0.1105/tpc.106.041541. Epub 2006 Aug 4. PMID: 16891402; PMCID: PMC1560923.

Roughan, P.G. and Slack, C.R. (1982) CellµLar organization of glycerolipid metabolism. Annu. Rev. Plant Physiol. 33, 97–132.

Sachdev, S., Ansari, S. A., Ansari, M. I., Fujita, M., & Hasanuzzaman, M. (2021). Abiotic Stress and Reactive Oxygen Species: Generation, Signaling, and Defense Mechanisms. Antioxidants (Basel, Switzerland), 10(2), 277. https://doi.org/10.3390/antiox10020277

Sang, Y., Cui, D., & Wang, X. (2001). Phospholipase D and phosphatidic acid-mediated generation of superoxide in Arabidopsis. Plant physiology, 126(4), 1449–1458. https://doi.org/10.1104/pp.126.4.1449

Kuhlgert Sebastian, Austic Greg, Zegarac Robert, Osei-Bonsu Isaac, Hoh Donghee, Chilvers Martin I., Roth Mitchell G., Bi Kevin, TerAvest Dan, Weebadde Prabode and Kramer David M. 2016MultispeQ Beta: a tool for large-scale plant phenotyping connected to the open PhotosynQ networkR. Soc. open sci.3160592160592 http://doi.org/10.1098/rsos.160592

Suorsa M (2015) Cyclic electron flow provides acclimatory plasticity for the photosynthetic machinery under various environmental conditions and developmental stages. Front. Plant Sci. 6:800. doi: 10.3389/fpls.2015.00800

Tang, Y., Wen, X., Lu, Q., Yang, Z., Cheng, Z., & Lu, C. (2007). Heat stress induces an aggregation of the light-harvesting complex of photosystem II in spinach plants. Plant physiology, 143(2), 629–638. https://doi.org/10.1104/pp.106.090712

Toshiharu Shikanai . Cyclic Electron Transport Around Photosystem I: Genetic Approaches. Annual Review of Plant Biology 2007 58:1, 199-217

Vijayan, Jithesh; Nishikant Wase, Kan Liu, Chi Zhang, Wayne R. Riekhof (2021). Reactive oxygen species mediate thylakoid membrane remodeling and triacylglycerol synthesis under nitrogen starvation in the alga Chlorella sorokiniana. bioRxiv 2021.05.15.444036; https://doi.org/10.1101/2021.05.15.444036

Wang, Z., & Benning, C. (2011). Arabidopsis thaliana polar glycerolipid profiling by thin layer chromatography (TLC) coupled with gas-liquid chromatography (GLC). Journal of visualized experiments : JoVE, (49), 2518. https://doi.org/10.3791/2518

Wang X. (2000). MµLtiple forms of phospholipase D in plants: the gene family, catalytic and regµLatory properties, and cellµLar functions. Prog Lipid Res.;39:109–149

Wang C, Zien CA, Afitlhile M, Welti R, Hildebrand DF, Wang X. Involvement of phospholipase D in wound-induced accumµLation of jasmonic acid in arabidopsis. Plant Cell. Nov;12(11):2237-46. doi: 10.1105/tpc.12.11.2237. PMID: 11090221; PMCID: PMC150170.

Wang, X. (1999). The role of phospholipase D in signaling cascades. Plant Physiol. 120, 645–651

Xiong H., Hua L., Reyna-Lorens I., Shi Y., Chen K.M., Smirnoff N., Kromdijk J., and Hibberd J. (2021). Photosynthesis-independent production of reactive oxygen species in the rice bundle sheath during high light is mediated by NADPH oxidase. PNAS, 118 (25). https://doi.org/10.1073/pnas.2022702118

Xu C., Fan J., Riekhof W., Froehlich J., and Benning C. (2003). A permease-like protein involved in ER to thylakoid lipid transfer in Arabidopsis. The EMBO Journal, 22, 2370-2379. https://doi.org/10.1093/emboj/cdg234

Xu, C., Fan, J., Froehlich, J. E., Awai, K., & Benning, C. (2005). Mutation of the TGD1 chloroplast envelope protein affects phosphatidate metabolism in Arabidopsis. The Plant cell, 17(11), 3094–3110. https://doi.org/10.1105/tpc.105.035592

Yamori, W., Shikanai, T. & Makino, A. Photosystem I cyclic electron flow via chloroplast NADH dehydrogenase-like complex performs a physiological role for photosynthesis at low light. Sci Rep 5, 13908 (2015). https://doi.org/10.1038/srep13908

Y. Sasaki, Y. Nagano, Plant acetyl-CoA carboxylase: structure, biosynthesis, regµLation, and gene manipµLation for plant breeding, Biosci. Biotechnol. Biochem. 68 (2004) 1175–1184

You, J., & Chan, Z. (2015). ROS RegµLation During Abiotic Stress Responses in Crop Plants. Frontiers in plant science, 6, 1092. https://doi.org/10.3389/fpls.2015.01092

Yu, L., Fan, J., Zhou, C., & Xu, C. (2021). Chloroplast lipid biosynthesis is fine-tuned to thylakoid membrane remodeling during light acclimation. Plant physiology, 185(1), 94–107. https://doi.org/10.1093/plphys/kiaa013

Yu, F.E.I.; A. Fu, M. Aluru, S. Park, Y. Xu, H. Liu, X. Liu, A. Foudree, M. Nambogga, S Rodermel. Variegation mutants and mechanisms of chloroplast biogenesis. Plant Cell Environ, 30 (2007), pp. 350-365

Yuxi Niu, Dušan Lazár, Alfred R. Holzwarth, David M. Kramer, Shizue Matsubara, Silvia D. Schrey, Ladislav Nedbal. The dynamics of non-photochemical quenching and cyclic electron transport in A.thaliana exposed to harmonically oscillating light. bioRxiv 2022.02.09.479783; doi: https://doi.org/10.1101/2022.02.09.479783

Zhi-Yan Du, Ben F. Lucker, Krzysztof Zienkiewicz, Tarryn E. Miller, Agnieszka Zienkiewicz, Barbara B. Sears, David M. Kramer, Christoph Benning, Galactoglycerolipid Lipase PGD1 Is Involved in Thylakoid Membrane Remodeling in Response to Adverse Environmental Conditions in Chlamydomonas, *The Plant Cell*, Volume 30, Issue 2, February 2018, Pages 447–465, https://doi.org/10.1105/tpc.17.00446

Xu, C., Yu, B., Cornish, A.J., Froehlich, J.E. and Benning, C. (2006), Phosphatidylglycerol biosynthesis in chloroplasts of Arabidopsis mutants deficient in acyl-ACP glycerol-3-phosphate acyltransferase. The Plant Journal, 47: 296-309. https://doi.org/10.1111/j.1365-313X.2006.02790.x

APPENDIX A: *NTRC* HIGHER-ORDER MUTANTS FOR FURTHER INVESTIGATION

Although the focus of this project was the *ntrc ats1-1* and *ntrc tgd1-1* double mutants, I have also generated a much larger set of redox-lipid mutants (Table 4). This mutant toolbox may be helpful in exploring future redox-lipid relationships in addition to follow-up NTRC research. Except for *catalase 1 and 2 (cat1, cat2)*, which are localized in the cytosol and peroxisome, respectively, all mutants crossed with *ntrc* are chloroplast localized. This may prove insightful when further teasing apart NTRC involvement in chloroplast metabolism.

To follow-up with the data presented in this thesis, it may be advantageous to study the *ntrc fad6* and *ntrc fad7/8* mutants, first. FAD6 is responsible for desaturating plastidial 18:1 generating 18:2. Similarly, FAD7/8 desaturases 18:2 to generate 18:3. By studying these two *ntrc* double mutants, future studies may be able to determine why/how the *ntrc* background over synthesizes 18:2.

Table 4. Generation of *ntrc* double mutants. Status of crosses are indicated by color. *Ntrc = NADPH dependent thioredoxin reductase c (*AT2G41680*). Prx-q, a, b, IIE= peroxiredoxin- Q (*AT3G26060*), A (*AT3G11630*), B (*AT5G06290*), IIE. Cat1 = catalase 1 (*AT1G20630*). Cat2 = catalase 2 (*AT4G35090*). Flu1 = fluorescent in blue light (*AT3G14110*). Ats1-1= acyltransferase 1 (*AT1G32200*). Tgd1 = trigalactosyldiacylglyercol (*AT1G19800*). Fad = fatty acid desaturase.*

	ats1-1	*tgd1*	*fad4*	*fad6*	*fad7/8*
ntrc					
prxq					
cat1					
cat2					
flu1					

F3
F2
F1
Unconfirmed

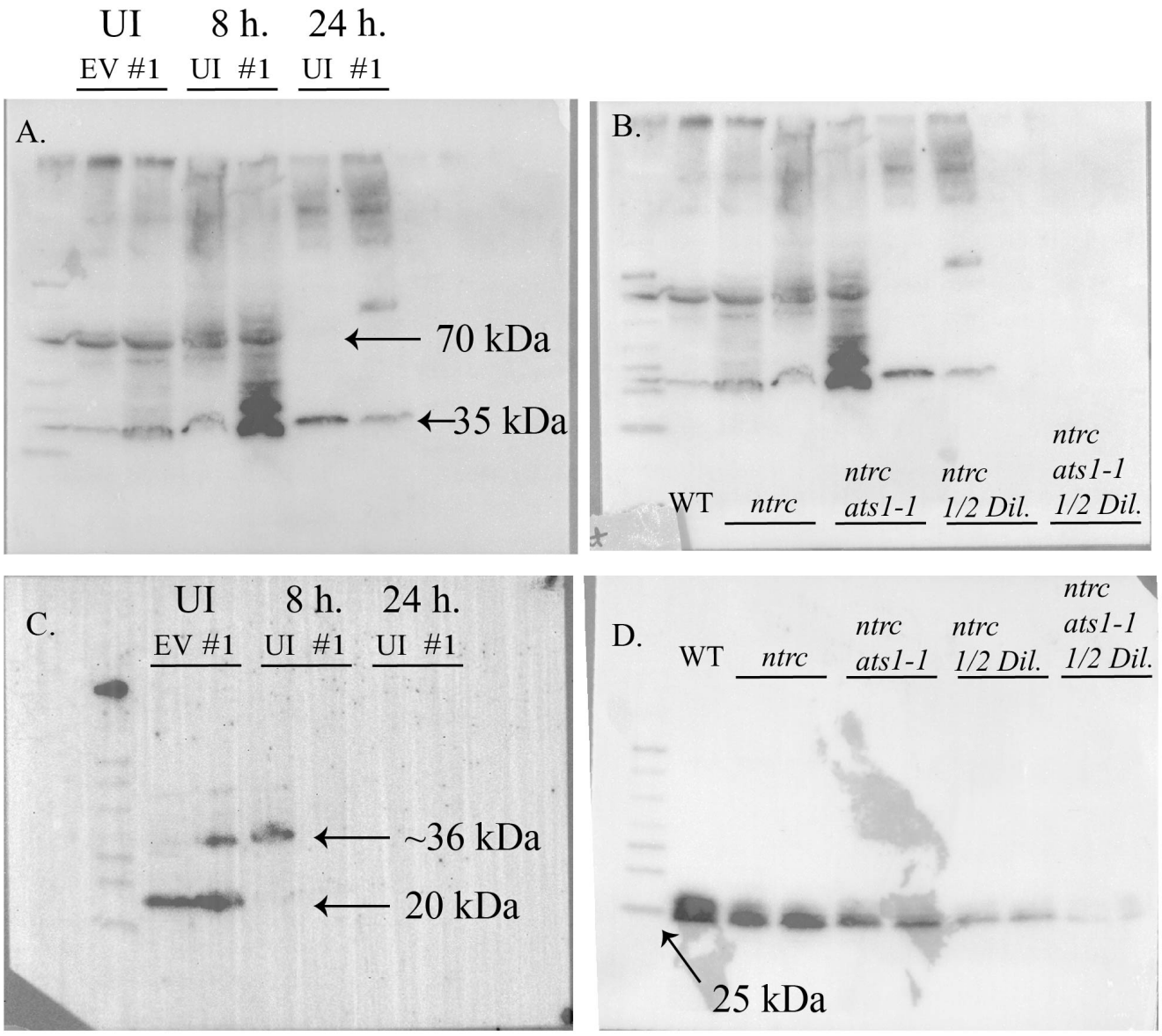
	PRXA	*PRXB*	*PRXIIE*	*PRXQ*
prxa	-			
prxb		-		
prxIIE			-	
prxq				-

	fad4	*fad6*	*fad7/8*
fad5			
fad6		-	
fad7/8		-	-

APPENDIX B: LIST OF PRIMERS

Primer Name	5' to 3' Primer Sequence
SALK_096776 Genotyping Primers	TCAGAAGTGCAACGATCACTG CAAGCATTTTCTCTGCCTCAC
SAIL115_E08 Genotyping Primers	TCCCTTGGTGTTGCTCAATAC GGTCCCGATTTAATGGAGAAG
SALK Border Primer	ATTTTGCCGATTTCGGAAC
SAIL Border Primer	TAGCATCTGAATTTCATAACCAATCTCGATACAC
Tgd1 Genotyping Primers + BsmAI Digestion	ATGATGCAGACTTGTTGTATCCA CACAGTTCTTCAAAGAATCTCC
Ats1 Genotyping Primers + PsiI/DdeI Digestion	CAGGACTTTAATACTCAACTGTTTAATTATA GCTCAGAACAAGGCTCGAAGG
NTRC Whole Transcript Amplification	GTCTCCCAAGATAGGCATCG CTTGAAGAACTGCACACATGG
Failed NTRC Whole Transcript Amplification	ATGGCTGCGTCTCCCAAG ATTTATTGGCCTCAATGAATTCT
Failed Salk_096776 End Sequence Amplification	GAAATGCTCAGGTTAGGAAACG GAATCATGATAAGATTTGGCATCTGCC
Failed SIN2 Contamination Test	CGTTTTGAATTGGGGTGGTC GGACACGGAGGATGAGAATG
NTRC Upstream Amplification	GGTATTGCCTCCGTCTCATC CGGAGGGAATCAGAAGAGC
NTRC Middle	TGGTTGGAGGAGGAGATACG GTCCCTTGGTGTTGCTCAAT
NTRC Downstream 1	AAACTGAGCTGGAGGCAAAA GCTTGTCTCCATTCGTGGTC
NTRC Downstream 2	AAAGCACAAGGGACAGTATGC CCCATAATTCCAGCTGCTTC
pESC-NTRC-pMDC32 Amplification	GGGGGCCGCAAAAATGGATTACAAGG AAGAGCTCCTACTTATTGGCCTCAATGAATTCTC
ETF1A cDNA Quality Control	TGAGCACGCTCTTCTTGCTTTCA GGTGGTGGCATCCATCTTGTTACA

APPENDIX C: PREVIOUS PROTEIN ANALYSIS OF *NTRC* ALLELES



Supplemental 2. A. Yeast expressing NTRC blotted with an NTRC antibody. UI = uninduced, or negative control. EV = empty vector (here EV is pESC-HIS). #1 = pESC-HIS-NTRC Colony. B. Total protein from *Arabidopsis* leaves blotted with an NTRC antibody. C. Yeast expressing NTRC blotted to yeast FLAG tag. D. Total protein from *Arabidopsis* leaves blotted by plastocyanin (PC).