An Intensive Examination of Chloroplast Movement and NPQ in Arabidopsis thaliana Wild type and Mutants Grown Under Different Light Conditions.

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An Intensive Examination of Chloroplast Movement and NPQ in *Arabidopsis thaliana* Wild type and Mutants Grown Under Different Light Conditions.

Andrea J. Bae

Advisor: Professor Martina Königer

Neuroscience Program


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Abstract

Light is a taxing environmental challenge to plants. Although it is necessary for photosynthesis, light in excess of what can be used can inflict cellular damage, reduce photosynthetic ability, decrease growth, and strain fitness. Despite their immobility, plants manage to tolerate an impressive range of environmental light conditions through a variety of sophisticated acclimation responses. One mechanism plants utilize to acclimate to the drastically changing light variations of the natural world is chloroplast movement. Chloroplast movement involves the physical rearrangement of chloroplasts within the cell to either avoid or maximize light absorption. In addition to chloroplast movement, plants rely on non-photochemical quenching (NPQ), also known as heat dissipation, to minimize light-induced damage through the conversion and subsequent dissipation of excess light into heat. The present study evaluated how well various Arabidopsis thaliana wild type and mutants with impaired chloroplast movement or NPQ performed these photoprotection mechanisms, and whether plants compensated for their genetic deficiencies by upregulating the other acclimation mechanism. In addition, we examined how different light conditions during growth affected chloroplast movement and NPQ. We pursued these questions by utilizing % transmission and confocal microscopy as measures of chloroplast movement, and chlorophyll a fluorescence as an indirect measure of NPQ. Our results showed that mutants exhibited chloroplast movement and NPQ abilities that well reflected their genetic limitations. Additionally, confocal images of chloroplast arrangement in various genotypes also revealed surprising details of each mutant’s response to pre-treatments in different light conditions. Interestingly, both mechanisms acclimated in environmental light conditions. Higher light conditions during growth attenuated chloroplast movement in all plants, but upregulated NPQ in some plants. At the same time, this study revealed that mutants did not compensate for their genetic impairments. Therefore plants incapable of chloroplast movement did not upregulate their NPQ abilities and vice versa. Overall the findings of this study compose a better picture of various genotypes of A. thaliana in their NPQ and chloroplast movement abilities, and attests to the impressive flexibility of these mechanisms to light conditions during growth.
Introduction

Light is a taxing environmental challenge to plants. Although plants need to maximize light absorption under low light conditions, light in excess of what can be used in photosynthesis can inflict cellular damage, reduce photosynthetic ability, decrease growth, and strain fitness. In order to respond appropriately to the drastically changing light variations of the natural environment, plants have evolved several impressive mechanisms.

Light utilization during photosynthesis

Under optimal light conditions, the light reactions of photosynthesis efficiently absorb and convert light energy into chemical energy (Taiz and Zeiger 2010). These reactions generate ATP and NADPH to provide chemical energy for carbon fixation during the Calvin cycle.

Pigment-protein complexes within the thylakoid membrane of chloroplasts, called the light harvesting complex (LHC), initiate the light reactions by absorbing light. Several different pigment molecules, like chlorophylls and carotenoids, reside within the LHC. Chlorophyll a and chlorophyll b make up the majority of the pigment composition in the LHC and absorb most of the light for photosynthesis within the 650 nm and 670 nm light spectrum. However accessory pigments, like carotenoids, also maximize light absorption in spectrums of light that are not efficiently absorbed by chlorophylls (450 nm), and transfer this energy to chlorophylls. Some carotenoids, like the xanthophylls and lutein, also serve photoprotective functions, which will be discussed in further detail later on. When excited by optimal light intensities, these pigment molecules funnel light energy to the reaction center, a light absorbing protein complex, through an energetically favorable gradient via resonance transfer (carotenoids ➔ chlorophyll b ➔ chlorophyll a ➔ reaction center chlorophyll) (Taiz and Zeiger 2010).

Once in the reaction centers, light energy is utilized to initiate a series of redox reactions that will ultimately result in the reduction of NADP⁺ into NADPH. In addition, energy from the
captured electron is utilized to establish a proton motive force for the production of ATP by ATP synthase. The first photochemical event occurs in the reaction center of protein complex called, photosystem II, when an electron emitted from the excited chlorophyll in the reaction center is captured by a nearby acceptor molecule. At the same time, the oxygen evolving complex (OEC) within photosystem II breaks down H₂O into H⁺ and O₂ to donate an electron to the electron deficient reaction center (Taiz and Zeiger 2010).

Meanwhile, the captured electron will flow through the electron transport chain (ETC). The ETC enables the transport of free-floating protons in the stroma across the thylakoid membrane into the lumen and establishes a transthyakoid proton gradient (ΔpH). This ΔpH then provides a proton motive force for the production of ATP from ADP by ATP synthase. Eventually, the electron reaches photosystem I where it reduces NADP⁺ into NADPH. Both ATP and NADPH, which are the final end products of the light reactions, then provide the chemical energy necessary for the Calvin cycle (Taiz and Zeiger 2010).

However excess light in the environment challenges the plant’s photosynthetic mechanisms with the excessive generation of reactive oxidative species (ROS) (Niyogi 1999). ROS are dangerous free radicals that can interfere with and inflict damage on photosynthetic mechanisms. Recent studies have demonstrated that the oxygen evolving complex and the D1 protein within the reaction center of photosystem II exhibit constant turn-over due to photodamage (Takahashi and Badger 2011). Although ROS are produced at all light intensities as a consequence of photosynthesis, high light exacerbates ROS production and strains the plant’s repair mechanisms. One circumstance in which excess light exacerbates ROS production is when the Calvin cycle does not use NADPH fast enough and hence NADP⁺ becomes limiting. When this occurs, NADP⁺ cannot act as the final electron acceptor and thus the electrons within the ETC reduce nearby O₂; thereby generating ROS (Niyogi 1999).
Although ROS production is inevitable during photosynthesis, photodamage in optimal light conditions is manageable because the plant’s repair mechanisms can counteract the photodamage (Takahashi and Badger 2011). In excess light, the balance between rate of repair and the rate of photodamage is offset and the plant can experience photoinhibition, or a decrease in the efficiency and/or maximum rate of photosynthesis. Because of the serious consequences of high light exposure, plants possess sophisticated photoprotective mechanisms to avoid photoinhibition (Niyogi 1999).

**Heat dissipation**

Non-photochemical quenching (NPQ), or heat dissipation, is one efficient mechanism that minimizes the production of ROS and subsequent cellular damage in excess light. NPQ, which occurs within minutes of incident high light, involves the dissipation of excess light into heat (Demmig-Adams and Adams III 1992). Previous studies have demonstrated that NPQ is an effective photoprotective mechanism that ultimately preserves fitness in the face of environmental light variations (Kulheim et al. 2002).

Most observations on NPQ can be made indirectly through measurements of chlorophyll $a$ fluorescence through a fluorometer (Krause and Weis 1991; Henriques 2009). When a chlorophyll $a$ molecule absorbs light and becomes excited, it transfers its light energy to either photosynthesis or heat dissipation. Light that is not utilized for either process is emitted as chlorophyll $a$ fluorescence. In the end, all of the light that the leaf absorbs must equal the sum of the light that is utilized for photosynthesis, heat dissipation, and emitted as chlorophyll $a$ fluorescence. Given that these three parameters occur in proportion to each other, scientists have determined equations to make inferences about complex biochemical processes like heat dissipation and photosynthesis from relatively simple measurements of chlorophyll $a$
fluorescence (Bilger and Bjorkman 1990b; Krause and Weis 1991; Henriques 2009; Bilger and Bjorkman 1990a).

Although NPQ is a complex multifaceted mechanism, the $\Delta pH$-dependent component of NPQ ($qE$) makes up the majority of the total NPQ ($qN$). This component of NPQ occurs rapidly within the first 5-15 minutes of high light exposure and relaxes just as quickly in the dark upon the elimination of the light-induced $\Delta pH$ (Horton et al. 1994; Yamamoto 1979). By monitoring changes in NPQ upon exposure to high light and darkness, observations on the induction and relaxation kinetics of NPQ can be made. When a leaf is exposed to light, NPQ will rise like a saturation curve and plateau to a max NPQ value. When leaves are subsequently acclimated to the dark, NPQ will drop quickly to a sustained NPQ value and then to zero. Monitoring NPQ kinetics provides information on how fast the plant can respond to light variation. Observations on maximum NPQ provide an evaluation of the plant’s maximum NPQ abilities. Sustained NPQ is also an informative parameter that reflects the degree of sustained photoinhibition after NPQ due to long-standing protein degradation from photodamage (Horton et al. 1994).

The mechanisms behind $qE$, which will now be referred to as NPQ, relies on three factors: 1) A transthylakoid pH gradient ($\Delta pH$), 2) the xanthophyll cycle which results in the production of zeaxanthin, and 3) the PsbS protein.

NPQ is not triggered by the direct perception of light intensities within the environment, but rather by the detection of indirect changes in pH that occur in response to high light (Demmig-Adams and Adams III 1996; Yamamoto 1979). In high light, the limiting availability of NADP+ creates a backlog in the electron transport chain, which resultanty induces an extreme $\Delta pH$. When the $\Delta pH$ reaches a certain threshold, the LHC will change conformation to engage NPQ (Horton et al. 1996). In this way, a large $\Delta pH$ is an excellent indicator of high light
in the environment because it signals that photosynthetic electron transport is overwhelmed (Horton et al. 1996; Demmig-Adams and Adams III 1996).

The ΔpH is also a trigger for the xanthophyll cycle, which refers to the interconversion of the carotenoid pigments violaxanthin, zeaxanthin, and antheraxanthin (Demmig-Adams and Adams III 1996, 1992). Most accessory carotenoids reside alongside chlorophyll pigments within the LHC to aid light absorption. However when exposed to excess light, violaxanthin converts into zeaxanthin through the intermediate antheraxanthin. Interestingly, this reaction is catalyzed by pH-sensitive enzyme, violaxanthin de-epoxidase. In the presence of zeaxanthin, light energy is converted and dissipated as heat by the direct transfer of energy from excited chlorophyll pigments to the lower energy zeaxanthin pigments (Demmig-Adams and Adams III 1996).

It is important to note that NPQ ultimately downregulates photosynthesis by reducing light that is utilized for photosynthesis (Horton et al. 1996). Although NPQ is helpful in preventing cellular damage from high light, it is inefficient for the plant under low light conditions. Luckily, maximal light absorption for photosynthesis is preserved under low light by the enzyme zeaxanthin epoxidase, which catalyzes the conversion of zeaxanthin to violaxanthin and the attenuation of NPQ (Horton et al. 1996).

Much of our knowledge of the xanthophyll cycle and NPQ comes from impairing NPQ with chemical inhibitors and from mutants. Initial studies involving the chemical inhibition of violaxanthin de-epoxidase with dithiothreitol (DTT) demonstrated that preventing the formation of zeaxanthin resulted in the inhibition of a major component of NPQ (Demmig-Adams et al. 1990; Bilger et al. 1989; Winter and Koniger 1989). Later the discovery of several NPQ impaired A. thaliana mutants enabled researchers to further investigate the specific mechanisms of NPQ (Baroli and Niyogi 2000).
Characterization of *npq1*, which lacks violaxanthin de-epoxidase, revealed that the accumulation of zeaxanthin is important in regulating normal NPQ kinetics and max NPQ (Niyogi et al. 1997a; Niyogi et al. 1998). Although *npq1* exhibits severe compromises to NPQ because it cannot convert violaxanthin into zeaxanthin, it is still capable of performing NPQ to some degree. In fact, NPQ may also rely on the presence of another carotenoid, lutein (Niyogi et al. 2001; Pogson et al. 1996). Double mutant *npq1lut2*, which lacks zeaxanthin and lutein, exhibit severely impaired NPQ. In addition, characterization of *szl1npq1*, which lacks zeaxanthin but accumulates higher levels of lutein, has demonstrated that higher expression of lutein partially restores NPQ, and even suggests that lutein may have the ability to substitute zeaxanthin (Li et al. 2009).

Lastly, discovery of PsbS protein, which is a pH sensitive protein within the LHC, suggests that PsbS plays an important role in regulating NPQ (Li et al. 2000). Characterization of *npq4-1*, demonstrated that PsbS is responsible for binding protons, chlorophylls, and xanthophylls. These observations suggest that PsbS may provide a site for pH-dependent and xanthophyll-dependent NPQ within the LHC (Li et al. 2000). Additional studies further corroborated the importance of PsbS in regulating NPQ by demonstrating that over expression of PsbS resulted in a faster rate of NPQ induction and relaxation (Zia et al. 2011). Further investigation of the biochemical properties of PsbS showed that PsbS allosterically regulates NPQ by modulating the conformation of the LHC in photosystem II (Kiss et al. 2008).

**Chloroplast movement**

In addition to NPQ, plants also depend on chloroplast movement as a fast acclimation response to light variation. Several studies have demonstrated that chloroplast movement is an effective mechanism that enables plants to adjust to their environmental light variations and prevent photodamage (Kasahara et al. 2002; Koniger et al. 2008; Sztatelman et al. 2010).
Chloroplast movement involves the physical rearrangement of chloroplasts to various patterns within the cell to either minimize or maximize light absorption. For example, in *Arabidopsis thaliana*, three distinct chloroplast arrangement patterns are observed in dark, low, and high light. When leaves are acclimated in the dark, chloroplasts settle to the bottom of the cell (Suetsugu and Masamitsu 2012). However when leaves are exposed to low light intensities, chloroplasts exhibit the accumulation response and aggregate to the periclinal walls of the cell to maximize light absorption. On the other hand, when exposed to high light intensities, chloroplasts exhibit the avoidance response and rearrange to the anticlinal walls of the cell to minimize light absorption and subsequent photodamage. These three chloroplast arrangement patterns are the result of chloroplast photorelocation, which is specifically induced by blue light (Banas et al. 2012; Suetsugu and Masamitsu 2012).

Observations on various chloroplast arrangement patterns have been documented since the 19th century using simple light microscopy on fixed-cell sections (Wada 2013). However, recent advancements in high resolution imaging techniques have enabled researchers to visualize the dynamic cellular mechanisms underlying chloroplast movement behavior. While these methods are powerful, the widespread use of these imaging techniques has been limited for some time by the costly and limited availability of the tools. Because of these limitations, methods of measuring % light transmission through a leaf after exposure to various intensities of blue light have been popularized as a cost-effective technique in studying chloroplast movement (Berg et al. 2006; Walczac and Gabrys 1980). % transmission is an excellent measure of chloroplast arrangement because it reflects the degree to which light from one side of the leaf is impeded by the distribution of chloroplasts. Typical transmission apparatuses shine red light onto one surface of the leaf and measure the amount of light that passes through to the other side. The degree of change in % transmission relative to the dark is an indication of chloroplast movement.
Additionally, the speed of chloroplast movement can be inferred from calculating the change in % transmission over time. Scientists often complement measurements of % transmission with confocal microscopy to confirm that the transmission measurements correspond to the predicted chloroplast arrangement patterns at that light intensity (Wada 2013).

Different species of plants exhibit chloroplast movement to varying degrees (Koniger and Bollinger 2012). Luckily, *A. thaliana* is a well-suited model organism for investigating chloroplast movement due to its very distinct chloroplast arrangement patterns in dark, low light, and high light. In addition, *A. thaliana*’s genome, which comprises 25,500 genes, has been sequenced completely (Arabidopsis Genome 2000; Rhee et al. 2003). As a powerful genetic tool, *A. thaliana* has enabled researchers to elucidate the underlying genetic basis and molecular mechanisms behind chloroplast movement (Meinke et al. 1998; Rhee et al. 2003). In fact, mutant variants of *A. thaliana* with impaired chloroplast movement abilities have been invaluable in identifying important components of this behavior.

Typical wild type *A. thaliana* cells hold about 100 chloroplasts that are about 50 μm² (Koniger et al. 2008). *A. thaliana* leaves have four distinct cell layers: the adaxial epidermis, palisades layer, spongy mesophyll layer, and abaxial epidermis (Fig. 1). The adaxial epidermis refers to the top surface of the leaf, and the abaxial epidermis refers to the bottom surface of the leaf. Chloroplasts rearrangement has been observed most thoroughly in the regular, columnar shaped cells of the palisades layer, and the irregularly shaped globular cells of the spongy mesophyll layer (Bollinger & Königer 2012).
Fig. 1. Schematic diagram of accumulation and avoidance in different cell layers of *A. thaliana* leaves. (A) depicts the accumulation response where chloroplasts aggregate towards the top and bottom periclinal surfaces of the cell to maximize light absorption. (B) depicts the avoidance response where chloroplasts aggregate towards the side walls of the cell to minimize light absorption. Top-down views from the adaxial surface of the leaf provide visualization of chloroplast arrangement in the palisades cell layer and bottom-up views from the abaxial surface of the leaf provide visualization of chloroplast arrangement in the spongy mesophyll layer.
Three fundamental mechanisms underlie chloroplast movement: 1) light sensitive receptors at the plasma membrane that detect signals for movement, 2) an internal signal transduction cascade that relays the external stimulus to the chloroplast, and 3) a motility system that physically moves the chloroplasts. This review will mainly focus on the role of phototropins, the light sensitive receptors, and the chloroplast unusual positioning protein (chup), involved in chloroplast movement.

Phototropins are blue light sensitive proteins that mostly localize to the plasma membrane of cells, but have also been observed along chloroplast outer membrane to a lesser extent (Kong et al. 2013). Extensive studies on two mutants that lack phototropins have established that phototropins trigger the appropriate chloroplast movement response (Jarillo et al. 2001; Kagawa et al. 2001; Banas et al. 2012). *A. thaliana* relies on two phototropins, phototropin1 (phot1) and phototropin2 (phot2), to regulate both the avoidance and accumulation response.

Physiological comparisons between *phot1* and *phot2* mutants revealed that *phot1* operates to respond to a broader range of blue light intensities while *phot2* responds specifically to high intensity blue light (Kagawa et al. 2001; Sakai et al. 2001; Jarillo et al. 2001). In this way *phot1* mediates both the accumulation and avoidance response, whereas *phot2* mainly induces the avoidance response (Luesse et al. 2010). Functional characterization of *phot1/phot2* further confirms that phototropins are necessary for chloroplast movement because this double mutant lacks both varieties of phototropins and thus cannot detect the blue light signals necessary to initiate chloroplast movement (Sakai et al. 2001).

While blue light perception through phototropins is important for the initiation of movement through a not yet clearly defined signaling pathway, actin filaments are also required for chloroplast movement. Studies involving chemical inhibitors first demonstrated that
chloroplast movement relied on actin filaments, rather than microtubules (Kandasamy and Meagher 1999).

Functional characterization of the chloroplast unusual positioning1 (chup1) demonstrated that CHUP1 is necessary for photorelocation, chloroplast anchorage, and chloroplast positioning (Oikawa et al. 2003; Oikawa et al. 2008). In these mutants, chloroplasts aggregate to the bottom of the cell and fail to photorelocate (Oikawa et al. 2003). Subsequent investigation of CHUP1 revealed that this protein localized to the periphery of the outer chloroplast membrane, and provided a site for actin polymerization on the chloroplast. Additionally, CHUP1 was found to play an important role in anchoring the chloroplast to the plasma membrane of the cell wall (Oikawa et al. 2008). Interestingly the cytoplasmic actin cables were not compromised in this mutant, suggesting that impairments in the mechanisms responsible for polymerizing actin on chloroplasts are independent of the mechanisms that govern actin functionality for the larger cytoplasmic framework of the cell (Oikawa et al. 2003).

While initial studies suggested that chloroplasts migrated along major actin cables with the aid of myosin-related motor proteins (Kandasamy and Meagher 1999), recent investigations suggest that chloroplasts utilize a unique motility system that involves light-activated reorganization of short actin filaments (Kandasamy and Meagher 1999; Kadota et al. 2009; Tsuboi and Wada 2012, 2011; Tsuboi et al. 2009).

These short actin filaments, also known as chloroplast actin (cp-actin), surround the outer envelope of the chloroplast. When the chloroplast is stationary, cp-actin is equally distributed throughout the chloroplast periphery. However, during chloroplast movement, cp-actin filaments reorganize along the chloroplast envelope to direct movement (Kadota et al. 2009; Kong and Wada 2011; Suetsugu and Masamitsu 2012). When chloroplasts were exposed to an adjacent microbeam of weak blue light, the cp-actin filaments aggregated to the leading edge proximal to
the light before moving towards the illuminated area (Kadota et al. 2009). The leading edge of the chloroplast was determined to be the area of the chloroplast that directed movement away or toward the light. However, when chloroplasts were exposed to an adjacent microbeam of strong blue light, the cp-actin filaments proximal to the light first transiently disappeared and then aggregated to the leading edge before moving away from the illuminated area (Kadota et al. 2009). This active reorganization of cp-actin during chloroplast movement relies on phototropins because the phot1/phot2 did not display this behavior (Kadota et al. 2009).

Further analysis demonstrated that the velocity of chloroplast movement is dependent on the degree of biased cp-actin distribution between the leading edge and the rear end of the chloroplast (Kadota et al. 2009; Kong and Wada 2011). Therefore the greater the difference in cp-actin concentration between the leading and rear of the chloroplast, the faster the chloroplast movement (Kong and Wada 2011).

Although extensive studies on phot1, phot2, and chup1 have well documented the importance of phototropins and cp-actin in chloroplast movement, recent discovery of several other mutants with compromised chloroplast movement highlights the complexity of the mechanisms underlying this behavior. Studies suggest that while CHUP1 is necessary to the polymerization of cp-actin filaments for chloroplast movement, two kinesin-like-proteins, KAC1 and KAC2, may also play an important role in either maintaining or contributing to the polymerization of actin that is important for chloroplast movement and plasma membrane anchorage (Oikawa et al. 2003; Oikawa et al. 2008; Suetsugu et al. 2010). In addition, mutants that are unable to express THRUMIN1, an actin bundling molecule that localizes on the plasma membrane, exhibit slower avoidance and accumulation behavior (Whippo et al. 2011).

Additionally, the WEB1 (weak chloroplast movement under blue light1) and PMI2 (plastid movement impaired2) mutants, which also exhibit impaired avoidance response and
slower-than-normal accumulation abilities, suggest that chloroplast movement involves protein-protein interactions between these two molecules (Kodama et al. 2011; Luesse et al. 2006; Kodama et al. 2010). Lastly, though the precise molecular signal cascade from blue light perception to chloroplast movement remains to be elucidated, discovery of the JAC1 (J-domain protein required for chloroplast accumulation response 1) mutant suggests that the avoidance response and accumulation response are mediated by either the activation or suppression of JAC1 (Suetsugu et al. 2005).

**The present study**

The current body of literature features several studies that attempt to elucidate the mechanisms underlying chloroplast movement and NPQ. While both mechanisms are presumed to have vital functions for the plant’s survival and fitness, few studies validate this claim (Kasahara et al. 2002; Kulheim et al. 2002). The general observation has been made that sun-acclimated plants (sun plants) are capable of tolerating higher light intensities than shade-acclimated plants (shade plants). While this faculty in sun plants is partially due to their ability to utilize more light for photosynthesis, it is also known that sun plants upregulate their photoprotective mechanisms. For example, NPQ capabilities of leaves vary flexibly depending on environmental light conditions during growth (Demmig-Adams and Adams III 1992). In fact, the size of the xanthophyll pool is smaller in shade plants than in sun plants, suggesting that sun plants are capable of performing NPQ to a greater degree than shade plants (Thayer and Bjorkman 1990). Like NPQ, the degree to which plants rely on chloroplast movement is also flexible (Trojan and Gabrys 1996). Plants grown in high light exhibit slight differences in chloroplast distribution compared to plants grown in low light. The initial dark arrangement of plants grown in high light is biased toward an avoidance response in plants grown under low light. These subtle arrangement differences may be associated to a greater degree of chloroplast
movement in low light acclimated plants than high light acclimated plants (Trojan and Gabrys 1996). Though these studies insinuate that plants strategize their utilization of these photoprotective mechanisms depending on their environmental conditions during growth, even these investigations do not address the plant’s photoprotective mechanisms holistically. In fact, most investigations evaluate NPQ and chloroplast movement separately, when in reality, these two mechanisms are recruited simultaneously as the plant’s most active, fast acclimation responses. The current study attempts to validate the importance of NPQ and chloroplast movement and determine the relative importance of each mechanism to establish a better knowledge of how plants strategize their photoprotective mechanisms.

In this investigation, we investigated the flexibility of these photoprotection mechanisms by examining whether mutants compensated for their genetic deficiencies by upregulating other acclimation mechanisms. In addition, we investigated how different light conditions during growth, low light (LL) and intermediate light (IL), affect chloroplast movement and NPQ to determine how these mechanisms are influenced by environmental conditions. Overall, this multifaceted study attempts to paint a more accurate portrait of the plant’s utilization of NPQ and chloroplast movement in tolerating their environmental light conditions.
Materials and Methods

Plant materials and growth conditions

Wildtype (ecotype Columbia) and various mutants of *Arabidopsis thaliana* with impaired NPQ (*npq1*) or chloroplast movement (*phot1, phot2, phot1/phot2*, and *chup*) were acquired from the Arabidopsis Biological Resource Center (Table 1). Plants were grown under either 100 μmol photons m$^{-2}$ s$^{-1}$ with 12 hrs light and day/night temperatures of 23/20°C or 400 μmol photons m$^{-2}$ s$^{-1}$ with 12 h light and day/night temperatures of 21/20°C under fluorescent bulb light sources. Plants were fertilized weekly with all purpose fertilizer. Leaf discs for experimentation were taken from mature leaves.
Table 1. List and description of various Mutants studied in this investigation.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Mechanism of Impairment</th>
<th>Impairment</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>phot1</em></td>
<td>Lacks phototropin 1</td>
<td>Performs the accumulation response to a lesser degree than Wild type</td>
</tr>
<tr>
<td><em>phot2</em></td>
<td>Lacks phototropin 2</td>
<td>Cannot perform avoidance response</td>
</tr>
<tr>
<td><em>phot1/phot2</em></td>
<td>Lacks both phototropins</td>
<td>Cannot perform any chloroplast movement</td>
</tr>
<tr>
<td><em>chup</em></td>
<td>Lacks chup</td>
<td>Cannot perform any chloroplast movement</td>
</tr>
<tr>
<td><em>npq1</em></td>
<td>Lacks violaxanthin de-epoxidase</td>
<td>Impaired NPQ</td>
</tr>
</tbody>
</table>
Confocal microscopy of chloroplast arrangement

In order to evoke accumulation and avoidance arrangement of chloroplasts, light intensity during pre-treatment was maintained at either 1.5 μmol photons m$^{-2}$ s$^{-1}$ (LL) or 1000 μmol photons m$^{-2}$ s$^{-1}$ (HL) with a fluorescent bulb for 1.5 hrs. Then at the same light intensity, leaf discs fixed by floating in 2.5% glutaraldehyde (EMS grade, Polysciences, Inc.) in phosphate buffer (100 mM KCl, 1 mM CaCl$_2$, 5 mM KH$_2$PO$_4$) for an additional 2 hrs. Afterwards, discs were vacuum infiltrated until discs sunk, then kept in glutaraldehyde solution overnight at 4°C. Leaves were washed in buffer solution the following morning. All images of chloroplasts were taken with a 63x oil immersion objective on a Leica TCS-NT Confocal Laser microscope (Wetzlar, Germany). Chloroplasts were visualized by their autofluorescence (chlorophyll a fluorescence) using an Argon laser (excitation 450 nm, absorption 680-750 nm). Images of chloroplasts in the cells of the palisades layer were taken from the adaxial surface (upper surface) of the leaf, and images of chloroplasts in the cells of the spongy mesophyll layer were taken from the abaxial surface (bottom surface) of the leaf. Optical sections were spaced every 0.5 μm and averaged over 6 frames. The total number of optical sections varied between leaf samples depending on leaf thickness and the depth of laser penetration through the leaf. Final images in this study are overlay pictures of all optical sections. 4-5 images were taken for each plant genotype grown under the different light conditions.

Quantifying Chloroplast Movement

Whole plants were acclimated to darkness overnight prior to experimentation. Mature leaves were then excised from the plant and placed in a specially built microcontroller based photometric instrument (also referred to as a transmission apparatus) (Berg et al. 2006). The microcontroller within the transmission apparatus controlled the intensity and duration of blue
light exposure and the photometric component of the apparatus measured % transmission of red light through the leaf, which is a measure of chloroplast arrangement.

In a typical experiment, leaves were acclimated in the dark for 4 hrs, then 3 hrs to 0.1 μmol photons m^{-2} s^{-1} of blue light. Afterwards, leaves were exposed to increasing intensities of blue light (0.2, 0.4, 0.8, 1.6, 5, 10, 30, 40, 50, 60, 90, and 100 μmol photons m^{-2} s^{-1}) every hour. Transmission of light through the leaf was measured every minute throughout the experiment.

Since the maximum accumulation and avoidance were reached at 10 and 19 hrs in wild type, we calculated maximum degrees of accumulation and avoidance by taking the % transmission values at 0.8 (10 hrs) and 100 μmol photons m^{-2} s^{-1} (19 hrs) respectively. Maximum accumulation and maximum avoidance values were normalized and calculated relative to the end of dark transmission at 4hrs. Therefore, maximum degree of accumulation was calculated as:

\[
\frac{\%\text{transmission}_{0.8} - \%\text{transmission}_{\text{dark}}}{\%\text{transmission}_{\text{dark}}} \times 100
\]

Similarly, maximum degree of avoidance was calculated as:

\[
\frac{\%\text{transmission}_{100} - \%\text{transmission}_{\text{dark}}}{\%\text{transmission}_{\text{dark}}} \times 100
\]

Measuring NPQ

Leaf discs from mature leaves were floated in phosphate buffer solution at 1.5 μmol photons m^{-2} s^{-1} for 1 hour to restore chloroplast positioning to the accumulation response and relax NPQ. Then, leaf discs were floated in a 0.1% detergent (Tween® 20) in phosphate buffer for an additional 1.5 hrs at the same light intensity. Afterwards, leaves were vacuum infiltrated.

Leaf samples were then acclimated to the dark for 10 minutes and exposed to a brief 5 sec pulse of far red light to clear out any remnant electrons in photosystem II. In order to observe NPQ induction and relaxation kinetics, leaf discs were exposed to 1000 μmol photons m^{-2} s^{-1}.
for 12 minutes, followed by darkness for 15 minutes. During each trial, changes in NPQ were calculated from monitoring changes in chlorophyll \( a \) fluorescence, which were taken every minute using a pulse amplitude modulation fluorometer, PAM-2000 (Heinz Walz GmbH, Effeltrich, Germany). Chlorophyll \( a \) measurements were utilized in the Stern-Vollmer relationship to calculate NPQ (Krause and Weis 1991).

\[
NPQ = \frac{Fm - Fm'}{Fm'}
\]

Fm, or the maximal fluorescence value, was determined immediately after dark acclimation. Afterwards, fluorescence measures taken every minute thereon were designated as Fm’, which reflected the decreases in chlorophyll fluorescence as more of the absorbed light was utilized for photosynthesis and NPQ.

From the NPQ traces, we acquired max NPQ and sustained NPQ values to compare and contrast each plant’s NPQ abilities. Max NPQ, taken as the last NPQ value of the high light exposure, was understood as an indication of the plant’s maximal ability to carry out NPQ. Sustained NPQ, taken as the last NPQ value of the dark exposure, was understood as an indication of whether the plant retained its elevated levels of zeaxanthin and antheraxanthin in favor of sustaining NPQ and reduced photosynthetic efficiency.

Statistics

All statistics for this study were determined using one-way ANOVAs followed by Tukey-Kramer HSD (JMP; SAS, Cary, NC, USA).
Results

\textit{The effect of light conditions during growth on chloroplast arrangement}

Light conditions during growth did not affect chloroplast arrangement patterns considerably in all plants. But it seemed as though plants grown under higher light conditions during growth exhibited greater accumulation in wild type (Fig. 2A and 2B). The descriptions below will not distinguish between chloroplast arrangement patterns in plants of the same genotype grown under different light conditions because light conditions during growth did not impact pre-treatment induced chloroplast arrangement patterns.

\textit{Wild type plants exhibit typical accumulation and avoidance chloroplast arrangement patterns on both the adaxial and abaxial surfaces of leaves.}

We used confocal microscopy to characterize chloroplast arrangement across wild type and mutant \textit{A. thaliana} grown under different light conditions. Chloroplast accumulation was induced with pre-treatment in 1.5 $\mu$mol photons m$^{-2}$ s$^{-1}$ and chloroplast avoidance was induced with pre-treatment in 1000 $\mu$mol photons m$^{-2}$ s$^{-1}$.

Confocal images of the adaxial surface provided top-down views of chloroplasts that reside in the uniform, column-shaped cells of the palisades layer. Because penetration of the laser during confocal microscopy attenuates with increasing leaf depth, the intensity of chlorophyll $a$ fluorescence from chloroplasts was greatest for chloroplasts closest to the epidermis. Typical images of all wild type leaves grown under any light condition and pre-treated in 1.5 $\mu$mol photons m$^{-2}$ s$^{-1}$ showed several chloroplasts spread out in the face position. Although it was not possible to visualize the precise boundaries of the cell wall, chloroplasts were often nicely organized and confined within circle-like boundaries (Fig. 2A, a). In this way, it was easy to differentiate areas in the images that were due to the absence of chloroplasts in the extracellular space from the absence of chloroplasts in the intracellular space.
However, fewer chloroplasts were imaged when wild type leaves were pre-treated in 1000 μmol photons m\(^{-2}\) s\(^{-1}\) (Fig. 2A, c). Images from this light treatment showed chloroplasts that clung to the sides of the edges of the cell. Because chloroplasts re-arrange themselves toward the anticlinal walls of the cell under high light, only chloroplasts closest to the epidermis could be seen; though more chloroplasts were most likely lining the cell wall.

On the other hand, confocal images of the abaxial surface provided bottom-up views of chloroplasts that resided in the irregularly shaped cells of the spongy mesophyll layer (Fig. 2A, b). Images of wild type leaves incubated in 1.5 μmol photons m\(^{-2}\) s\(^{-1}\) showed chloroplasts that were maximally spread out and organized in globular frames. However, unlike images from the palisades layer, images of the spongy mesophyll layer showed that there was more space unoccupied by chloroplasts, in-between cells. When pre-treated in 1000 μmol photons m\(^{-2}\) s\(^{-1}\), chloroplasts in the mesophyll cells seemed to respond with less avoidance than palisades cells on the adaxial surface (Fig. 2A-L, a,b). Unique to the abaxial surface were smaller guard cell chloroplasts that reside throughout the abaxial epidermis.

Under most circumstances, noticeable changes in chloroplast arrangement were best observed on the adaxial surface. The following description will primarily focus on differences in chloroplast arrangement on the adaxial surface.

*Chloroplast arrangement patterns in mutants are similar and dissimilar from wild type depending on their genotype.*

The greatest aberrations in chloroplast arrangement from wild type were observed in *chup*, whose chloroplasts clumped to the sides of the cells (Fig. 2I and 2J). Images of the abaxial surface of *chup*’s leaves further confirmed that the chloroplasts in these mutants tended to aggregate at the bottom of the cells (Fig. 2I b,d and 2J b, d). Light during pre-treatment did not impact chloroplast arrangement in *chup*.
Full accumulation arrangements were only observed in wild type, phot2, and phot1/phot2 plants (2A-B a,b and 2E-H a,b), but limited accumulation was observed in phot1, chup, and npq1 (2C-D a,b, 1I-J a,b, and 2K-L a,b).

Phot1 does not exhibit the full accumulation response, even in pre-treatment under 1.5 μmol photons m\(^{-2}\) s\(^{-1}\) (2C-D). Phot2, on the other hand, exhibits the full accumulation, but fails to exhibit avoidance arrangement when pre-treated in 1000 μmol photons m\(^{-2}\) s\(^{-1}\) (2E-F).

Interestingly, the phot1/phot2’s chloroplasts assume the accumulation arrangement at all times (2G-H).

npq1’s chloroplast arrangement patterns in response to different light intensities during pre-treatment were especially surprising because npq1 does not accumulate its chloroplasts fully (Fig. 2K-L, a,b).
Wild type grown under 100 μmol photons m$^{-2}$s$^{-1}$
Wild type grown under 400 μmol photons m⁻² s⁻¹

LL

adaxial

HL

2B

abaxial
*phot1* grown under 100 μmol photons m⁻² s⁻¹
*phot1* grown under 400 μmol photons m$^{-2}$ s$^{-1}$

**a**

**b**

**c**

**d**

LL

HL

adaxial

2D

abaxial
phot2 grown under 100 μmol photons m\(^{-2}\) s\(^{-1}\)

- LL
- HL
- adaxial
- abaxial
- 2E
phot2 grown under 400 μmol photons m$^{-2}$ s$^{-1}$
phot1/phot2 grown under 100 μmol photons m⁻² s⁻¹

LL

HL

a

b

c

d

adaxial

2G

abaxial
phot1/phot2 grown under 400 μmol photons m⁻² s⁻¹
*chup* grown under 100 grown under μmol photons m\(^{-2}\) s\(^{-1}\)
chup grown under 400 μmol photons m$^{-2}$ s$^{-1}$

LL

HL

adaxial  2J  abaxial
npq1 grown under 100 μmol photons m$^{-2}$s$^{-1}$

LL

HL

adaxial  2K  abaxial
Fig. 2. Chloroplast arrangement in *A. thaliana* wild type and mutants exposed to different light treatments and grown under different light conditions. Plants were grown under either 100 μmol photons m\(^{-2}\) s\(^{-1}\) or 400 μmol photons m\(^{-2}\) s\(^{-1}\). Leaf discs from plants were acquired and pre-treated in either 1.5 μmol photons m\(^{-2}\) s\(^{-1}\) to induce accumulation or 1000 μmol photons m\(^{-2}\) s\(^{-1}\) to induce avoidance. After pre-treatment leaves were fixed in glutaraldehyde for imaging. All chloroplasts were visualized by their autofluorescence (chlorophyll a fluorescence) on a confocal microscope using an Argon laser. The column on the left (a&c) shows chloroplasts from the palisades cells of the adaxial surface and the column on the right (b&d) shows chloroplasts in the spongy mesophyll cells of the abaxial surface. The top row represents chloroplasts treated under 1.5 μmol photons m\(^{-2}\) s\(^{-1}\) (LL) and the bottom row displays images taken from plants pre-treated under 1000 μmol photons m\(^{-2}\) s\(^{-1}\) (HL). Images are representative examples of overlay pictures from several optical sections.
Quantifying chloroplast movement through transmission provides a means to analyzing both chloroplast arrangement and movement.

While confocal microscopy provides an informative snap-shot of how chloroplast arrangement changes in response to different light treatments, it is difficult to quantitatively study dynamic chloroplast movement with this technique. The % transmission of light through the leaf, on the other hand, provides a convenient measure of how chloroplast arrangement changes over time in response to increasing blue light intensities. Additionally, transmission provides a quantitative measure of chloroplast arrangement averaged across a portion of the entire leaf.

Prior to experimentation, whole plants were acclimated in the dark over night to restore chloroplast arrangement to the dark position. Mature leaves were then excised from the plant and placed in individual chambers between an LED that exposes the adaxial surface of the leaf to blue or red light and a photometric device that measures the percentage of red light that penetrates through the abaxial side (% transmission).

Typical transmission experiments began by exposing dark-acclimated leaves to darkness for 4 hrs. During this time, % transmission values were constant at about 6-7% in A. thaliana wild type and mutants (Fig. 3). % transmission when chloroplasts are in the dark is an important reference frame for when the % transmission changes due to changes in chloroplast arrangement in response to blue light.

After initial darkness readings, leaves were exposed to increasing intensities of blue light. First, plants were exposed to 3 hrs of 0.1 μmol photons m$^{-2}$ s$^{-1}$ to induce accumulation. During this time, % transmission exhibited a strong initial drop and then approached some stable value. Afterwards, leaves were exposed to incremental increases in blue light intensities every hr for 1 hr (0.2, 0.4, 0.8, 1.6, 5, 10, 30, 40, 50, 60, 90, 100 μmol photons m$^{-2}$ s$^{-1}$ ).
When leaves were exposed to light intensities between 0.1-30 μmol photons m⁻² s⁻¹ (0-10 hrs), % transmission values in wild type plants incrementally dropped to 4-5%, and indicated that less light penetrated through to the abaxial side as chloroplasts rearranged into accumulation. However, when leaves were exposed to 40-100 μmol photons m⁻² s⁻¹ (11-19 hrs), % transmission increased and surpassed initial dark values; thus indicating that more light was penetrating through to the abaxial surface of the leaf as chloroplasts were rearranging into avoidance. Chloroplast sensitivity to light surprisingly responded to changes in light intensity as small as 0.2 μmol photon m⁻² s⁻¹.

**General trends in transmission traces reflect plant genotype.**

As the only plants with unimpaired chloroplast movement abilities, wild type and npq1 exhibited transmission traces that demonstrate evidence of accumulation at lower blue light intensities and avoidance at higher blue light intensities. However, it is important to note that mutants with chloroplast movement impairments did not exhibit this stereotypical transmission trace. General patterns from the transmission traces reflected the phenotypic defects associated with each plant genotype (Fig. 3).

The phototropin mutants provided a clear example of this claim. As the phototropin mutant with the least chloroplast movement impairment, phot1’s transmission traces resembled wild type the most. Though phot1 exhibited less of a decrease in % transmission associated with accumulation, it exhibited increases in % transmission similar to wild type. On the other hand, phot2’s transmission trace exhibited evidence of accumulation that were similar to wild type, but as blue light intensity increased, phot2’s transmission value remained constant, and did not indicate avoidance (Fig. 3).
Chup and phot1phot2, which are mutants incapable of all chloroplast movement, exhibit flat line transmission traces that were unresponsive to increasing blue light intensity.

Quantitative analysis of accumulation and avoidance across genotypes.

We quantitatively compared chloroplast movement abilities between different plants by calculating the % change in transmission relative to dark at 10 hrs and 19 hrs. We determined the % change in transmission at 10 hrs and 19hrs because these time points best reflected instances during the experiment when the blue light intensity at 0.8 and 100 μmol photons m$^{-2}$ s$^{-1}$ would evoke maximal accumulation and maximal avoidance respectively. Therefore, plants that are capable of accumulation will exhibit the greatest negative change in % transmission at 10 hrs due to accumulation. On the other hand, plants that are capable of avoidance will exhibit the greatest positive change in % transmission at 19 hrs due to avoidance.

We additionally confirmed that differences in percent transmission were not influenced by differences in initial dark % transmission values. Dark arrangement, as indicated by % transmission during dark acclimation at 4 hrs, was comparable between most plants (Table 2). Only significant differences in % transmission values associated with dark arrangement were observed between chup grown under 400 μmol photons m$^{-2}$ s$^{-1}$ and phot1, phot2, and npq1 grown under 100 μmol photons m$^{-2}$ s$^{-1}$ (Table 2).

As expected, the greatest negative % change in transmission at 10 hrs was observed in plants capable of performing accumulation. This observation held true whether plants were grown in either 100 (LL) or 400 (IL) μmol photons m$^{-2}$ s$^{-1}$ (Fig. 4, Table 3,4). Phot2 (LL: -16.4 ± 2.4, IL: -10.2 ± 2.2) exhibited a greater negative % change in transmission than any other genotype (Fig. 3, Table 2,3). Wild type (LL: -12.1 ± 1.9, IL: -8.4 ± 2.1) and npq1 (LL: -12.0 ± 2.5, IL: -9.1 ± 2.2) displayed evidence of accumulation, and exhibited comparable degrees of accumulation. Phot1 (LL: -6.8 ± 1.7, IL: -5.2 ± 1.8) exhibited lower accumulation than wild type
or npq1 but significantly greater than chup (LL: -0.5 ± 1.1, IL: -0.2 ± 0.9) and phot1/phot2 (LL: 1.5 ± 1.8, IL: 0.9 ± 1.3). As predicted, % change in transmission was negligibly small in mutants that were incapable of all chloroplast movement (i.e. chup and phot1/phot2). Even still, chup exhibited a significantly greater negative % change in transmission than phot1/phot2 (Fig. 4, Table 3,4).

As expected, the greatest positive % change in transmission was observed in plants that were capable of performing avoidance (Fig. 4, Table 3,4). This observation held true whether plants were grown in 100 (LL) or 400 (IL) μmol photons m⁻² s⁻¹. Wild type (LL: 19.2 ± 4.9, IL: 10.4 ± 3.4), npq1 (LL: 20.1 ± 4.1, IL: 9.4 ± 4.4), and phot1 (LL: 18.2 ± 3.5, IL: 10.1 ± 3.6) exhibited the greatest positive changes in transmission than any other genotype (Fig. 4, Table 3,4). The next greatest positive changes in transmission were observed in the two mutants that were incapable of all chloroplast movement, chup and phot1/phot2. Although both chup’s and phot1/phot2’s changes in transmission were negligibly small, chup (LL: 2.8 ± 2.5, IL: 3.2 ± 2.1) exhibited a significantly greater positive change than phot1/phot2 (LL: -3.6 ± 3.0, IL: -2.0 ± 3.6). Lastly, unlike all the other genotypes, phot2 (LL: -16.9 ± 2.6, IL: -9.9 ± 2.6) exhibited the least positive change in % transmission that reflected how its transmission trace seemed to be trapped in accumulation (Fig. 4, Table 3,4).

**Greater light intensity during growth attenuates chloroplast movement.**

Interestingly, the transmission traces indicated that light conditions during growth impacted the degree to which each plant exhibited chloroplast movement (Fig. 3,4, Table 3,4). Growth under higher light attenuated all chloroplast movement responses, as observed in the lower absolute % changes in transmission in plants grown under 400 μmol photons m⁻² s⁻¹ when compared to plants grown under 100 μmol photons m⁻² s⁻¹ (Fig. 3,4, Table 3,4).
Light conditions during growth impacted the % change in transmission at 10 hrs for plants that were capable of unimpaired accumulation, namely wild type, phot2, and npq1. In these genotypes, plants grown under 100 μmol photons m\(^{-2}\) s\(^{-1}\) exhibited greater negative % changes in transmission than plants grown under 400 μmol photons m\(^{-2}\) s\(^{-1}\). As expected, no significant differences were observed between plants grown under different light conditions in *chup, phot1/phot2*, or *phot1*.

Similarly, light conditions during growth only impacted plants that were capable of avoidance, like wild type, *phot1*, and *npq1*. Plants grown under 100 μmol photons m\(^{-2}\) s\(^{-1}\) exhibited greater positive % changes in transmission than plants grown under 400 μmol photons m\(^{-2}\) s\(^{-1}\) in these genotypes. No significant differences were observed between plants grown under different light conditions in *chup, phot1/phot2*, and *phot2*. 
Fig. 3. Changes in chloroplast arrangement (as measured by % transmission) due to increasing blue light intensity in A. thaliana wild type and mutants grown under either 100 or 400 μmol photons m$^{-2}$ s$^{-1}$. Whole plants were acclimated in the dark overnight prior to experimentation. Leaves were then excised and placed in a transmission apparatus. In a typical experiment, leaves were acclimated in the dark for 4 hrs, then 3 hrs of 0.1 μmol photons m$^{-2}$ s$^{-1}$ of blue light. Afterwards, leaves were exposed to increasing intensities of blue light (0.2, 0.4, 0.8, 1.6, 5, 10, 30, 40, 50, 60, 90, and 100 μmol photons m$^{-2}$ s$^{-1}$) every hour. Transmission of light through the leaf was measured every minute throughout the experiment. The column on the right represents transmission traces from plants grown under 100 μmol photons m$^{-2}$ s$^{-1}$ and the column on the left represents transmission traces from plants grown under 400 μmol photons m$^{-2}$ s$^{-1}$ (n = 25 leaves from different plants for each genotype). Traces are averages of 25 trials for each mutant and growth condition.
Table 2. Effect of genotype and light conditions during growth on chloroplast arrangement as measured by % transmission at the end of the dark period (4hrs) in *A. thaliana* wild type and mutants. Table below reports the % transmission at 4 hrs as mean ± SD, and the results from a one way-ANOVA followed by a Tukey-HSD Kramer analysis. Plants represented by letters that are different are statistically different from each other. ANOVA, F = 2.9420, df = 11, 316, p < 0.0010.

<table>
<thead>
<tr>
<th></th>
<th>% transmission at 4 hrs in plants grown under 100 μmol photons m² s⁻¹</th>
<th>% transmission at 4 hrs in plants grown under 400 μmol photons m² s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>6.9 ± 1.7 (AB)</td>
<td>6.1 ± 1.3 (AB)</td>
</tr>
<tr>
<td>phot1</td>
<td>7.3 ± 1.6 (A)</td>
<td>6.3 ± 1.6 (AB)</td>
</tr>
<tr>
<td>phot2</td>
<td>7.3 ± 2.1 (A)</td>
<td>6.1 ± 1.5 (AB)</td>
</tr>
<tr>
<td>phot1/phot2</td>
<td>6.6 ± 1.6 (AB)</td>
<td>6.1 ± 1.5 (AB)</td>
</tr>
<tr>
<td>chup</td>
<td>6.2 ± 2.1 (AB)</td>
<td>5.7 ± 1.3 (B)</td>
</tr>
<tr>
<td>npq1</td>
<td>7.3 ± 2.2 (A)</td>
<td>6.2 ± 1.8 (AB)</td>
</tr>
</tbody>
</table>
Fig. 4. % transmission changes during maximal accumulation and avoidance in *A. thaliana* wild type and mutants. Whole plants were acclimated in the dark over night prior to experimentation. Leaves were then excised and placed in a transmission apparatus where leaves were exposed to increasing intensities of blue light and darkness, and % transmission of red light through the leaf was measured. (A) A typical transmission trace in wild type indicates the % change in transmission relative to dark at which leaves were exposed to light intensities that would induce maximal accumulation (10 hrs) and maximal avoidance (19 hrs). (B) represents % transmission relative to dark at 0.8 μmol photons m\(^{-2}\) s\(^{-1}\) (10 hrs). (C) represents % transmission relative to dark at 100 μmol photons m\(^{-2}\) s\(^{-1}\) (19 hrs). Dark bars represent % change for plants grown under 100 μmol photons m\(^{-2}\) s\(^{-1}\) and white bars represent % change for plants grown under 400 μmol photons m\(^{-2}\) s\(^{-1}\). Data represent Means ± SD. n=25.
Table 3. Effect of genotype and light condition during growth on changes in chloroplast arrangement as measured by % change in transmission relative to dark at 10 and 19 hrs. % change in transmission were determined at 10 hrs and 19 hrs because these time points reflect instances during the experiment when chloroplasts in wild type would be exposed to light intensities that evoke accumulation and avoidance respectively. Columns under LL represent plants grown under 100 μmol photons m\(^{-2}\) s\(^{-1}\) and columns under IL represent plants grown under 400 μmol photons m\(^{-2}\) s\(^{-1}\). Data is represented as means ± SD. Data also reports the results from a one way ANOVA followed by a Tukey-HSD Kramer analysis. (10 hrs : ANOVA, F = 271.9, df = 11, 319, p < 0.0001; Avoidance at 19 hrs : ANOVA, F = 315.8, df = 11, 319, p < 0.0001). Values not connected by the same letter are significantly different.

<table>
<thead>
<tr>
<th></th>
<th>% change in transmission at 10 hrs</th>
<th>% change in transmission at 19 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Grown under LL</td>
<td>Grown under IL</td>
</tr>
<tr>
<td>Wild type</td>
<td>-12.1 ± 1.9 (G)</td>
<td>-8.4 ± 2.1 (DE)</td>
</tr>
<tr>
<td>phot1</td>
<td>-6.8 ± 1.7 (CD)</td>
<td>-5.2 ± 1.8 (C)</td>
</tr>
<tr>
<td>phot2</td>
<td>-16.4 ± 2.4 (H)</td>
<td>-10.2 ± 2.2 (F)</td>
</tr>
<tr>
<td>phot1/phot2</td>
<td>1.5 ± 1.8 (A)</td>
<td>0.9 ± 1.3 (AB)</td>
</tr>
<tr>
<td>chup</td>
<td>-0.5 ± 1.1 (B)</td>
<td>-0.2 ± 0.9 (B)</td>
</tr>
<tr>
<td>npq1</td>
<td>-12.0 ± 2.5 (G)</td>
<td>-9.1 ± 2.2 (EF)</td>
</tr>
</tbody>
</table>
Table 4. Summary of statistical results from comparing accumulation and avoidance in different *A. thaliana* genotypes and plants grown under different light conditions. (\(<\)) indicates values that are significantly smaller and (\(\approx\)) indicates values that are statistically similar. Plants marked with LL represent plants grown under 100 μmol photons m\(^{-2}\) s\(^{-1}\) and plants marked with IL represent plants grown under 400 μmol photons m\(^{-2}\) s\(^{-1}\).

<table>
<thead>
<tr>
<th>% change in transmission at 10 hrs</th>
<th>Does genotype affect chloroplast movement?</th>
<th>Do light conditions during growth affect chloroplast movement?</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Growth under 100 μmol photons m(^{-2}) s(^{-1}) (LL):</strong></td>
<td>(\text{phot 2} &lt; \text{WT} = \text{nqpl} &lt; \text{phot1} &lt; \text{chup} &lt; \text{phot1/phot2})</td>
<td>(\text{phot1/phot2}<em>{\text{LL}} = \text{phot1/phot2}</em>{\text{IL}})</td>
</tr>
<tr>
<td><strong>Growth under 400 μmol photons m(^{-2}) s(^{-1}) (IL):</strong></td>
<td>(\text{phot2} &lt; \text{nqpl} = \text{WT} &lt; \text{phot1} &lt; \text{chup} &lt; \text{phot1/phot2})</td>
<td>(\text{WT}<em>{\text{LL}} &lt; \text{WT}</em>{\text{IL}})</td>
</tr>
</tbody>
</table>

| % change in transmission at 19 hrs | **Growth under 100 μmol photons m\(^{-2}\) s\(^{-1}\) (LL):** | \(\text{nqpl} = \text{WT} = \text{phot1} > \text{chup} > \text{phot1/phot2} > \text{phot2}\) | \(\text{WT}_{\text{LL}} > \text{WT}_{\text{IL}}\) |
| **Growth under 400 μmol photons m\(^{-2}\) s\(^{-1}\) (IL):** | \(\text{WT} = \text{phot1} = \text{nqpl} > \text{chup} > \text{phot1/phot2} > \text{phot2}\) | \(\text{phot1}_{\text{LL}} > \text{phot1}_{\text{IL}}\) | \(\text{chup}_{\text{LL}} = \text{chup}_{\text{IL}}\) |
|                                  | **phot1/phot2\(_{\text{LL}}\) = phot1/phot2\(_{\text{IL}}\)** | \(\text{phot1}_{\text{LL}} > \text{phot1}_{\text{IL}}\) | \(\text{phot1/phot2}_{\text{LL}} = \text{phot1/phot2}_{\text{IL}}\) |
|                                  | **\(\text{phot2}_{\text{LL}} > \text{phot2}_{\text{IL}}\)** | | \(\text{phot2}_{\text{LL}} > \text{phot2}_{\text{IL}}\) |
The effects of genotype and light conditions during growth on NPQ.

In our comprehensive evaluation of light tolerance mechanisms in wild type and mutant A. thaliana, we first observed how NPQ induction and relaxation kinetics differed across plants of different genotype and growth condition (Fig. 5). Prior to experimentation, all leaves were dark adapted for 10 minutes to relax NPQ. Then, plants were exposed to high light (1000 μmol photons m\(^{-2}\) s\(^{-1}\)) in order to induce NPQ. The typical NPQ induction trace in wild type and mutants with unimpaired NPQ resembles a saturation curve. The greatest changes in NPQ are observed within the first few minutes of light exposure, but then level off to some maximum value (maximum NPQ). Subsequent relaxation of NPQ upon acclimation to the dark resembles exponential decay where NPQ relaxes drastically within the first few minutes and then levels off to a sustained NPQ value.

*Only npq1 exhibits clear differences in its NPQ abilities from wild type and other genotypes.*

The npq1 mutant, grown in either 100 or 400 μmol photons m\(^{-2}\) s\(^{-1}\), exhibits a drastic aberration in NPQ kinetics. NPQ induction in these mutants does not resemble a saturation curve. Instead, NPQ induction seems to rise more linearly. Similarly, NPQ relaxation kinetics are also defunct in that NPQ relaxation also seems to fall linearly (Fig. 5).

Comparisons of max and sustained NPQ between different genotypes revealed that there were no clear differences between genotypes (Fig. 6A,B, Table 5, 6). Surprisingly, despite npq1’s impairment with zeaxanthin cycle dependent NPQ, npq1 (1.57 ± 0.11) exhibited similar max NPQ values to wild type (2.00 ± 0.1). Additionally, npq1 exhibited significantly greater sustained NPQ than any other plant genotype (Fig. 6B, Table 5,6).

For plants grown under 100 μmol photons m\(^{-2}\) s\(^{-1}\), the only significant differences in max NPQ were observed between chup, phot1/phot2, and npq1 (Fig 6A, Table 4,5). Chup (2.20 ±
exhibited significantly greater max NPQ than phot1/phot2 (1.62 ± 0.11) and npq1 (1.57 ± .11) (Fig. 6A, Table 5,6).

For plants grown under 400 μmol photons m⁻² s⁻¹, significant differences in max NPQ values were observed in wild type, phot1/phot2, and npq1 (Fig 6, Table 5,6). Wild type exhibited a significantly greater max NPQ than phot1/phot2. Additionally, npq1 exhibited the lowest max NPQ value of all plants (Fig. 6A, Table 5,6). There were no significant differences in sustained NPQ across all plants (Fig. 6B, Table 5,6).

*Light conditions during growth impact max NPQ and sustained NPQ.*

Interestingly, light conditions during growth do not impact the general shape of the NPQ traces. Upon closer observation of the effects of light conditions during growth on NPQ, we examined max NPQ, determined as the NPQ value at the end of the 12 minute high light exposure, and sustained NPQ, determined as the last NPQ value at the end of the 15 minutes dark recovery, across plants. In general, max NPQ in wild type, phot2, and phot1/phot2 plants were greater in plants grown under intermediate light than plants grown under low light (Fig. 6A, Table 5,6). Sustained NPQ was greater in npq1 mutants grown under low light than mutants grown under high light (Fig. 6B, Table 5,6).
Fig. 5. Non-photochemical quenching kinetics in wild type and mutant *A. thaliana* grown under different light conditions. Plants were grown in either 100 μmol photons m⁻² s⁻¹ or 400 μmol photons m⁻² s⁻¹. Leaf discs were collected from the mature leaves of several plants for each plant genotype. Prior to experimentation, leaf discs were acclimated in the dark for 10 min. and exposed to a brief pulse of far red light to oxidize photosystem II. Leaves were then exposed to high light (1000 μmol photons m⁻² s⁻¹) for 12 minutes to induce NPQ, followed by 15 minutes of dark acclimation to relax NPQ. Data represent Means±SD, n=7-13 discs from several plants per genotype.
Fig. 6. **Max NPQ and Sustained NPQ in Wild type and mutant A. thaliana.** Plants were grown in either 100 μmol photons m$^{-2}$ s$^{-1}$ (Low light, grey bars) or 400 μmol photons m$^{-2}$ s$^{-1}$ (Intermediate light, white bars). Leaf discs were collected from the mature leaves of several plants for each plant genotype. Prior to experimentation, leaf discs were acclimated in the dark for 10 min. and exposed to a brief pulse of far red light to oxidize photosystem II. Leaves were then exposed to high light (1000 μmol photons m$^{-2}$ s$^{-1}$) for 12 minutes to induce NPQ, followed by 15 minutes of dark acclimation to relax NPQ. Max NPQ, taken as the last NPQ value of the high light exposure, was understood as an indication of the plant’s maximal ability to carry out NPQ. Sustained NPQ, taken as the last NPQ value of the dark exposure, was understood as an indication of whether the plant retained its elevated levels of zeaxanthin and antheraxanthin in favor of sustaining NPQ and reducing photosynthetic efficiency. Means±SD, n=7-13 discs from several plants per genotype. (A) represents mean max NPQ values of various genotypes, and (B) represents mean sustained NPQ values of various genotypes.
Table 5. Tukey-HSD Kramer results from comparing Max NPQ and Sustained NPQ in *A. thaliana* wild type and mutants. Max NPQ: ANOVA, $F = 17.4$, df = 11, 97, $p < 0.0001$; Tukey-HSD test, $p < 0.05$ & sustained npq: ANOVA, $F = 5.78$, dF = 11, 97, $p < 0.0001$; Tukey-HSD test, $p < 0.05$). Columns under LL represent plants grown under 100 μmol photons m$^{-2}$ s$^{-1}$ and columns under IL represent plants grown under 400 μmol photons m$^{-2}$ s$^{-1}$. Data represent means ± SD. Data not connected by the same letters are significantly different.

<table>
<thead>
<tr>
<th></th>
<th>Max NPQ</th>
<th>Sustained NPQ</th>
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<tr>
<td></td>
<td>LL</td>
<td>IL</td>
</tr>
<tr>
<td>Wild type</td>
<td>2.0 ± 0.2 (DEF)</td>
<td>2.7 ± 0.4 (A)</td>
</tr>
<tr>
<td><em>phot1</em></td>
<td>2.1 ± 0.3 (CDE)</td>
<td>2.4 ± 0.5 (ABCD)</td>
</tr>
<tr>
<td><em>phot2</em></td>
<td>1.8 ± 0.3 (EF)</td>
<td>2.5 ± 0.3 (ABC)</td>
</tr>
<tr>
<td><em>phot1/phot2</em></td>
<td>1.6 ± 0.2 (FG)</td>
<td>2.2 ± 0.5 (BCDE)</td>
</tr>
<tr>
<td><em>chup</em></td>
<td>2.2 ± 0.4 (ABCDE)</td>
<td>2.6 ± 0.2 (AB)</td>
</tr>
<tr>
<td><em>npq1</em></td>
<td>1.6 ± 0.2 (FG)</td>
<td>1.3 ± 0.2 (G)</td>
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Table 6. Summary of statistical results from comparing max NPQ and sustained NPQ in different *A. thaliana* genotypes and plants grown under different light conditions. Plants marked with LL represent plants grown under 100 μmol photons m$^{-2}$ s$^{-1}$ and plants marked with IL represent plants grown under 400 μmol photons m$^{-2}$ s$^{-1}$.

<table>
<thead>
<tr>
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<th>Does genotype affect NPQ?</th>
<th>Do light conditions during growth affect NPQ?</th>
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<tr>
<td>Max NPQ</td>
<td><strong>Growth under 100 μmol photons m$^{-2}$ s$^{-1}$ (LL)</strong>: <em>chup</em> &gt; <em>phot1/phot2=npq1</em></td>
<td><em>WT</em>$<em>{IL}$ &gt; <em>WT</em>$</em>{LL}$</td>
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<td></td>
<td><strong>Growth under 400 μmol photons m$^{-2}$ s$^{-1}$ (IL)</strong>: <em>WT</em> &gt; <em>phot1/phot2 &gt; npq1</em></td>
<td><em>phot2</em>$<em>{II}$ &gt; <em>phot2</em>$</em>{IL}$</td>
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<td></td>
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<td><em>phot1/phot2</em>$<em>{II}$ &gt; <em>phot1/phot2</em>$</em>{IL}$</td>
</tr>
<tr>
<td>Sustained NPQ</td>
<td><strong>Growth under 100 μmol photons m$^{-2}$ s$^{-1}$ (LL)</strong>: <em>npq1</em> &gt; <em>WT</em> = <em>phot1</em> = <em>phot2</em> = <em>phot1/phot2=chup</em></td>
<td><em>npq1</em>$<em>{II}$ &gt; <em>npq1</em>$</em>{IL}$</td>
</tr>
<tr>
<td></td>
<td><strong>Growth under 400 μmol photons m$^{-2}$ s$^{-1}$ (IL)</strong>:</td>
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<tr>
<td></td>
<td><em>WT</em> = <em>phot1</em> = <em>phot2</em> = <em>phot1/phot2=chup</em> = <em>npq1</em></td>
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Strong correlation between a plant’s accumulation and avoidance response is affected by light condition during growth.

In order to investigate whether correlations exist between accumulation and avoidance, we explored the relationship between % change in transmission relative to dark at 10 and 19 hrs (Fig. 7). The average values from these time points were understood as indirect measures of the plant’s accumulation and avoidance abilities. However, phot2 was excluded from this investigation because the nature of our transmission experiments does not accurately reflect phot2’s inability to exhibit avoidance.

There is a strong negative correlation between % change in transmission relative to dark at 19 hrs and % change in transmission relative to dark at 10 hrs (R² = 0.85) (Fig. 7A). Therefore plants that exhibit greater negative changes in % transmission at 10 hrs, exhibit greater positive changes in % transmission at 19 hrs.

The results from this investigation also revealed that light condition during growth may be affecting the relationship between accumulation and avoidance. Interestingly, this correlation was stronger in plants grown under 400 μmol photons m⁻² s⁻¹ (R² = 0.91) than in plants grown under 100 μmol photons m⁻² s⁻¹ (R² = 0.82) (Fig. 7B,C).

There is no relationship between NPQ and chloroplast movement.

There were no correlations between max NPQ and % change in transmission at 10 hrs (R² = 0.06) or max NPQ and % change in transmission at 19 hrs (R² = 0.02) (Fig. 8).

There is a relationship between max avoidance and sustained NPQ in plants grown under 400 μmol photons m⁻² s⁻¹ but not 100 μmol photons m⁻² s⁻¹.

Interestingly, a strong correlation existed between % change in transmission at 19 hrs, an indicator of maximum avoidance, and sustained NPQ in plants grown under 400 μmol photons m⁻² s⁻¹ (R² = 0.86) (Fig. 9B). However, this observation did not hold true for plants grown under lower light conditions at 100 μmol photons m⁻² s⁻¹ (R² = 0.18) (Fig. 9A).
Fig. 7. Relationship between accumulation and avoidance in *A. thaliana* wild type and mutants grown under different light conditions (excluding *phot2*). We determined the relationship between % change in transmission at 10 and 19 hrs as an indication of the plant’s ability to maximally accumulate and avoid respectively. (A) represents the correlation between maximum avoidance and maximum accumulation in all plants, excluding *phot2*. (B) represents the correlation between maximum avoidance and maximum accumulation in plants grown under 100 μmol photons m\(^{-2}\) s\(^{-1}\), but excluding *phot2*. (C) represents the correlation between maximum avoidance and maximum accumulation in plants grown under 400 μmol photons m\(^{-2}\) s\(^{-1}\).
Fig. 8. Relationship between chloroplast movement and NPQ in A. thaliana wild type and mutants. (A) represents the graph of % change in transmission relative to dark at 10 hrs, indicator of max accumulation, versus max NPQ. (B) represents the graph of % change in transmission relative to dark at 19 hrs, indicator of max avoidance, versus max NPQ.
Fig. 9. Relationship between maximum avoidance and sustained NPQ in A. thaliana wild type and mutants grown under either 100 or 400 μmol photons m$^{-2}$ s$^{-1}$. (A) represents correlations between % change in % transmission at 19hrs, an indicator of maximum avoidance, and sustained NPQ, in plants grown under 100 μmol photons m$^{-2}$ s$^{-1}$. (B) represents correlations between maximum avoidance and sustained NPQ in plants grown under 400 μmol photons m$^{-2}$ s$^{-1}$. 
Discussion

The present investigation evaluated chloroplast movement behavior and NPQ through an extensive survey of several different mutants.

*A. thaliana mutants behave accordingly to their genotypic defects.*

The general patterns in the transmission traces that were observed across plants reflected each plant’s genotype. For example, mutants with either no or minimal impairments with chloroplast movement, like wild type, *phot1*, and *npq1*, exhibited transmission traces that demonstrated clear evidence of the accumulation response at lower light intensities and avoidance response at higher light intensities. On the other hand, mutants with chloroplast movement impairments exhibited transmission traces that reflected their defects. For example, *phot2*’s leaf transmission traces lacked the upwards shift in % transmission that is characteristic of the avoidance response at higher light intensities. *phot2*’s inability to exhibit avoidance is attributed to its lack of blue light sensitive protein, phototropin 2 (Kagawa et al. 2001; Sakai et al. 2001). Transmission traces of *phot2* leaves from Jarillo et al., 2001 closely resembled the transmission traces observed in our experiment where % transmission dipped lower than initial dark transmission values at low intensities of blue light, and remained low even with increasing intensities.

Lastly, mutants that lacked all chloroplast movement, namely *phot1/phot2* and *chup*, exhibited flat line % transmission traces that accurately reflected no changes in chloroplast arrangement in response to any light intensity. Previous investigations of *phot1/phot2* and *chup* utilized microbeam light microscopy where single cells were exposed to a finely focused microbeam of blue light to induce chloroplast movement. These studies confirmed that *phot1/phot2* and *chup* were incapable of all chloroplast movement because chloroplasts in these
mutants showed no response toward the microbeam (Oikawa et al. 2003; Oikawa et al. 2008; Sakai et al. 2001; Kagawa et al. 2001). Our investigation is the second study, aside from König & Bollinger 2012, to corroborate that phot1/phot2 and chup cannot perform chloroplast movement by measuring changes in % transmission.

It is important to note that changes in % transmission in our study were not impacted by differences in the initial dark positioning of the leaves because analysis of % transmission values at 4 hrs was similar across most genotypes. These findings are different from Luesse et al., 2010’s findings that compared chloroplast movement through transmission in phot2, phot1, and wild type. Transmission analysis of phot2 in their study revealed that initial % transmission values in phot2 were 2 values lower than wild type; suggesting that phototropin 2 also influences normal dark positioning. Given these findings, we should have observed phot2 and phot1/phot2 to exhibit different transmission start values than other plants. Although our study did not find these differences, it is important to note that Luesse et al., 2010 took their dark transmission values for 60 minutes, while we measured dark transmission values for 4 hrs. Additionally, plants utilized in Luesse et al., 2010’s study were grown under much lower light conditions than our plants. While their study grew plants under 60-70 μmol photons m\(^{-2}\) s\(^{-1}\), the lowest light conditions that our plants were grown under was 100 μmol photons m\(^{-2}\) s\(^{-1}\).

We were also able to make interesting observations on the NPQ abilities of different genotypes. In order to examine NPQ induction and relaxation kinetics, vacuum infiltrated leaf discs were exposed to high light for 12 minutes, and then acclimated to darkness for 15 minutes. While the NPQ kinetics in most plants resembled saturations curves, and were similar in most mutants as compared to wild type, npq1 exhibited distinctly different NPQ kinetics. However, despite differences in kinetics, max NPQ was only significantly lower than wild type when plants were grown under 400 μmol photons m\(^{-2}\) s\(^{-1}\).
Literature descriptions of npq1 report that the mutant’s inability to perform zeaxanthin dependent NPQ results in different kinetics and significantly lower max NPQ values compared to wild type (Niyogi et al. 1997a, b; Li et al. 2000). While our study also observed impaired NPQ kinetics in npq1, we were unable to observe differences in max NPQ between npq1 and wild type when plants were grown under 100 μmol photons m⁻² s⁻¹. This observation is interesting because the max NPQ values observed in our wild type plants (2.0-2.7) are consistent with literature values (2.0-2.5) (Demmig-Adams and Adams 2006; Niyogi et al. 1997b). Nevertheless, npq1 exhibited lower max NPQ than any other genotype when plants were grown under 400 μmol photons m⁻² s⁻¹. These findings suggest that the impairments associated with npq1 are most obvious when plants are grown under higher light.

Confocal images of chloroplast arrangement in A. thaliana wild type and mutants provide a different perspective to our understanding of chloroplast movement.

Prior to this investigation, relatively few studies utilized confocal microscopy to characterize chloroplast arrangement on a range of cells from both leaf surfaces (Davis et al. 2011; Kaiserli et al. 2009; Wada et al. 2003; Oikawa et al. 2008; Trojan and Gabrys 1996; Koniger and Bollinger 2012). The few studies that did characterize chloroplast arrangement often relied on cross-sectional images of leaves to characterize chloroplast arrangement in different cell layers. Although cross sectional images are informative, they fail to capture how chloroplasts are distributed within each cell, particularly when chloroplasts are in avoidance. In fact, the information provided from cross sectional images is limited because it provides such a cursory glance of chloroplast distribution.

Our study provides an intensive examination of chloroplast arrangement patterns in several mutants using confocal microscopy. Typical A. thaliana leaves are about 200 μm thick,
with 4 different cell layers: adaxial epidermis, palisades layer, spongy mesophyll layer, and abaxial epidermis (Davis et al. 2011). Because optical sectioning through all the leaf layers is difficult with confocal microscopy, due to the fact that the intensity of light attenuates with increasing leaf depth, we had to image top-down views of the palisades layer from the adaxial surface and bottom-up views of the spongy mesophyll layer from the abaxial surface. On average, we were able to image chloroplasts through 60 μm of the leaf on both sides; thereby excluding about 80 μm of the leaf during imaging. Despite this shortcoming, confocal microscopy still provided new details on chloroplast arrangement in various mutants that were overlooked from cross-sectional images.

In our experiment, images of the wild type leaves pre-treated in 1.5 μmol photons m\(^{-2}\) s\(^{-1}\) revealed just how closely packed chloroplasts were arranged along the periclinal walls of the cell in order to maximize light absorption by minimizing intracellular space unoccupied chloroplasts on the upper surface of the cell. On the other hand, confocal images of wild type leaves pre-treated in 1000 μmol photons m\(^{-2}\) s\(^{-1}\) revealed just how drastically chloroplasts aggregated to the anticlinal walls of the cell to minimize light absorption while maximizing intracellular space unoccupied by chloroplasts.

In addition, we observed that changes in chloroplast arrangement were best observed in the palisades layer cells of the adaxial leaf surface. Most studies that utilize cross sections do not address differences in chloroplast arrangement in cells from the spongy mesophyll layer compared to cells in the palisades layer. In fact, descriptions on chloroplast arrangement are typically focused on the extent of chloroplast movement in the palisades layer. Only two studies have attempted to differentiate and characterize chloroplast movement in these layers, and have suggested that changes in chloroplast arrangement is comparable between both layers in *A. thaliana* and four different species of ferns (Koniger and Bollinger 2012; Augustynowicz and
Gabry’s 1999). In general, it is difficult to describe and quantify chloroplast arrangements in the spongy mesophyll layers since there are several cell layers and the cells are irregularly shaped. However, given that light hits the adaxial surface of the leaves, chloroplasts that reside in the spongy mesophyll layer may not receive very high light after the light has passed through the other layers.

Another striking observation we were able to make from our confocal images was the differences in chloroplast arrangement patterns across genotypes. Consistent with literature reports, chup exhibited the greatest irregularity in chloroplast arrangement patterns compared to wild type. chup lacks crucial anchorage proteins involved in enabling actin polymerization during chloroplast movement, and is identified by its unusual chloroplast arrangement where chloroplasts clump towards the bottom of the cells (Oikawa et al. 2003; Oikawa et al. 2008; Kasahara et al. 2002). While, confocal images of chup from the present study also observed chloroplast patterns that were consistent with literature reports, we also observed that chup chloroplasts were also capable of aggregating onto the top surfaces of the cell. This was somewhat surprising because previous studies that featured cross sectional images of chup only showed chloroplasts that completely aggregated to the bottom of the cell (Kasahara et al. 2002). Although chup is incapable of chloroplast movement, our images suggest that chup’s chloroplast arrangement is flexible, and may in part be the result of passive movement due to cytoplasmic streaming.

For the most part, mutants exhibited changes in chloroplast arrangement patterns that were consistent with their predicted genotype defects. For example, as mutants incapable of avoidance, phot2, phot1/phot2, and chup’s chloroplast arrangement was not affected by pre-treatment in high light. But, close observations on chloroplast arrangement patterns in the phototropin mutants also revealed surprising subtleties in chloroplast arrangement across
different genotypes. While full accumulation was only observed in wild type, phot2, and phot1/phot2, incomplete accumulation was observed in phot1, chup, and nq. phot1’s inability to fully accumulate emphasizes the importance of phototropin 1 in ensuring a proper accumulation response. Interestingly, even nq1, which has no impairments related to chloroplast movement, did not exhibit full accumulation. At the same time, it was interesting to observe full accumulation in phot2 and phot1/phot2, two mutants that are incapable of avoidance because of their lack of the phototropin 2 blue light sensor. This observation further confirms that plants that lack phototropin 2 does not affect the preservation of the accumulation response, but when plants lack both phototropins, as is the case in the double mutant, they will accumulate as a default position.

These observations on the degree of accumulation response in phot1 and phot1/phot2 are inconsistent with the findings of the only other study that investigated chloroplast arrangement in different mutants using confocal microscopy (Kaiserli et al. 2009). Images from this study suggest that phot1 is capable of full accumulation and that phot1/phot2’s chloroplasts exhibit “random localization” where chloroplasts primarily position themselves to the anticlinal walls. It is worth noting that Kaiserli et al., 2009 induced different chloroplast arrangements in live cells by exposing plants to either blue light or darkness for 3 hrs, whereas the current study pre-treated leaves in white light for 1.5 hrs before fixation. Despite these differences in methodology, discrepancies between our observations and Kaiserli et al., 2009’s findings stress the importance of further investigations on these differences in chloroplast arrangement between mutants, and the importance of confocal imaging in contributing new perspectives to our proper characterization of mutants.
While NPQ and % transmission data reflect the impact of light conditions during growth, confocal images of chloroplast arrangement do not.

All plants except chup that were grown under 400 μmol photons m$^{-2}$ s$^{-1}$ exhibited higher max NPQ values than plants grown under 100 μmol photons m$^{-2}$ s$^{-1}$. This observation is not surprising given our current understanding of NPQ in shade plants (those plants that prefer lower light conditions during growth) and sun plants (those plants that prefer higher light conditions during growth). Several papers have corroborated that plants grown under higher light are capable of greater max NPQ because they also tend to have larger pools of xanthophylls (Alter et al. 2012; Demmig-Adams and Adams 2006; Demmig-Adams 1998). Recent studies also suggest that light conditions during growth may impact NPQ by regulating basal levels of the PsbS protein involved in positioning the xanthophyll pigments in the light harvesting complex for NPQ (Demmig-Adams et al. 2006). In this way, several changes may be facilitating the upregulation of max NPQ in plants grown under high light to help the plant deal with higher light intensities.

We also observed that between the npq1 plants grown under different light conditions, only the mutants grown under 100 μmol photons m$^{-2}$ s$^{-1}$ exhibited evidence of elevated sustained NPQ. Given our understanding from the literature that plants grown under lower light are less equipped with the pigment composition to tolerate high light stress, perhaps npq1 plants grown under low light are less equipped to tolerate high light stress, and thus also exhibit a smaller pool of lutein, another carotenoid that has been implicated with NPQ (Demmig-Adams and Adams 2006; Demmig-Adams et al. 2006; Li et al. 2009).

Interestingly, there was a strong negative relationship between maximum avoidance and sustained NPQ in plants grown under 400 μmol photons m$^{-2}$ s$^{-1}$. These findings suggest that plants capable of avoidance tend to exhibit lower sustained NPQ values. Given our
understanding that plants exhibit elevated sustained NPQ levels when stressed, we can infer from this correlation that plants that exhibit the most avoidance also tend to be less stressed (Demmig-Adams and Adams 2006). However, this observation only holds true for plants grown under higher light conditions, which may reflect an interesting interaction between the environment and the plant’s NPQ mechanisms. Perhaps elevated sustained NPQ at the cost of reduced photosynthetic efficiency is worth the investment when the environmental light condition is sufficiently stressful; especially for plants unable to exhibit avoidance and growing under high light conditions.

Confocal images revealed that light conditions during growth did not impact chloroplast arrangement considerably. However, investigations on chloroplast movement from % transmission revealed that plants grown under 400 μmol photons m⁻² s⁻¹ exhibited chloroplast movement to a lesser degree than plants grown under 100 μmol photons m⁻² s⁻¹. Trojan and Gabryś (1999) observed this similar trend in their investigation on wild type plants grown under extremely low light intensities at 15-20 μmol photons m⁻² s⁻¹ and plants grown under 300 μmol photons m⁻² s⁻¹ where plants grown under higher light exhibited lesser degrees of chloroplast movement. Although Trojan & Gabryś (1999) examined three different plant groups grown under weak light (15-20 μmol photons m⁻² s⁻¹), standard light (130-200 μmol photons m⁻² s⁻¹), and high light (300 μmol photons m⁻² s⁻¹), their investigation did not observe differences between plants grown under the light conditions that the present study investigated (standard light and high light). This discrepancy between our study and Trojan & Gabryś (1999) may be attributed to the fact that Trojan & Gabryś collected % transmission values within shorter time periods (0-200 minutes for the entire transmission run, and 30 minutes for each light intensity). Our study collected transmission values within a 19 hr experimental time frame and exposed leaves to each light intensity for a minimum of 1 hour.
Nevertheless, Trojan & Gabryś (1999) suggested that the attenuation of chloroplast movement in plants grown under higher light is a result of changes in dark positioning between plants grown under different light conditions. Their study reported that plants grown under lower light exhibited a higher percentage of chloroplasts in the face position than plants grown under higher light. Although we attempted to investigate their claims from our % transmission values at 4 hrs, an indicator of dark positioning, we did not find any significant differences between plants grown under different light conditions. In order to investigate this question further, our future study will characterize dark positioning in different plants with confocal microscopy.

It is also difficult to rule out the possibility that light conditions during growth may be impacting the anatomical features of the leaf. Davis et al., (2011) explored the relationship between leaf anatomy, light absorption, and chloroplast movement by correlating cell diameter to the leaf’s light absorption properties in 24 plant species know for their varying degrees of chloroplast movement abilities. Their findings suggested that shade plants with broader, spherical cells could accommodate greater capacities for chloroplast movement than sun plants with narrower columnar cells. Although the present study did not attempt to characterize differences anatomical differences between plants, further investigations on this matter may be necessary to establish a better understanding of how chloroplast movement is affected by anatomy.

Possible interactions between chloroplast movement and NPQ.

One of the major goals of this study was to investigate the possible interactions between chloroplast movement and NPQ in plants grown under different light intensities. Although several studies have painstakingly characterized these fast high light tolerance mechanisms, only one study has attempted to explore possible interactions between these mechanisms. In real
circumstances of high light stress, NPQ and chloroplast movement are recruited simultaneously as the plant’s most dynamic and fast-responding mechanisms toward high light stress.

Cazzaniga et al., (2013) was the first study to find that NPQ kinetics and max NPQ in *A. thaliana*, were influenced by chloroplast movement during NPQ induction. The researchers of this study showed that *phot2* exhibits NPQ curves with flat tops and lower max NPQ values because they specifically lacked the slow induction phase of NPQ. Interestingly, the slow induction phase of the NPQ curve could be eliminated from wild type traces by inducing NPQ with red light, or light that is incapable of evoking chloroplast movement. Given these observations, the authors suggested that chloroplast movement was influencing the slow induction phase of NPQ by changing the optical properties of the leaf with different chloroplast arrangement patterns. For example, the shift in chloroplast arrangement from an accumulation to avoidance during NPQ induction with high light can impact chlorophyll *a* fluorescence measures, and thus subsequent NPQ values that are derived from them.

The NPQ kinetics of *phot2* from our experiments seem to exhibit similar characteristics as those noted by Cazzaniga et al., 2013. However, these findings are somewhat harder to see in our curves because while Cazzaniga et al., 2013 monitored NPQ for 120 minutes and induced NPQ with 350-400 μmol photons m⁻² s⁻¹, our experiments monitored NPQ for 30 minutes and induced NPQ with 1000 μmol photons m⁻² s⁻¹ white light. It is also worth noting that the observations made by Cazzaniga et al., 2013 were not seen in our experiments in other mutants with impaired chloroplast movement, like *phot1/phot2* or *chup*. But, differences between our study and Cazzaniga et al., 2013 may have also been attributed to the fact that we conducted our experiments on vacuum infiltrated leaves, which may have affected chloroplast movement.

We also attempted to investigate whether plants with lower NPQ abilities could upregulate chloroplast movement or vice versa. Tlalka et al., 1999 investigated the possible
relationship between the xanthophyll cycle and chloroplast movement in *Lemna trisulca* L. by observing % transmission traces after leaves were treated with DTT, an inhibitor of the violaxanthin de-epoxidase enzyme important for the xanthophyll cycle (Tlalka and Fricker 1999). Although the study observed that chloroplast movement responses to weak blue light and strong blue light were attenuated after DTT incubation, xanthophylls were only partial effectors of chloroplast movement.

Our study concluded that no compensation responses were observed in various mutants to overcome their genetic limitations. Therefore, *npq1* plants did not upregulate their avoidance response despite their inherent vulnerability to high light damage from NPQ impairments. Similarly, mutants with impaired chloroplast movement did not upregulate their NPQ abilities. However, our study showed that despite the fact that *npq1*’s impairments do not affect chloroplast movement, confocal images of *npq1* revealed that they were incapable of the full accumulation response. This finding provokes further investigations on the possible interactions and overlaps between NPQ and chloroplast movement mechanisms.

**Conclusion**

In this intensive survey of chloroplast movement and NPQ in *A. thaliana* wild type and mutants, we have furthered our understanding of each genotype in their abilities to perform these two mechanisms. We also explored the effects of light conditions during growth on NPQ and chloroplast movement. Transmission analysis of leaves from our different plants revealed that mutants exhibited transmission traces that well reflected their genetic impairments, further confirming that transmission is a powerful tool in investigating chloroplast movement. Confocal images of chloroplast arrangement across different genotypes added interesting details to our understanding of various mutants, and highlighted the need for further exploration of chloroplast
arrangement through confocal microscopy. Additionally, we observed that light conditions during growth attenuate chloroplast movement in genotypes capable of chloroplast movement; although it is unclear why the degree of chloroplast movement is lower with high light conditions. Results from our evaluation of NPQ revealed that NPQ kinetics, max NPQ, and sustained NPQ were comparable between most mutants and wild type. Only npq1 exhibited noticeable differences in NPQ kinetics from wild type. Interestingly, only npq1 plants grown under higher light seemed to show significantly lower max NPQ and higher sustained NPQ. Lastly, light conditions during growth impacted NPQ in that some plants grown under higher light exhibited higher max NPQ values. Although we did not observe any evidence of compensation in mutants dealing with genetic impairments, we did observe that npq1 exhibited different chloroplast arrangement responses to low light than wild type; suggesting some mechanistic interaction between chloroplast arrangement and zeaxanthin. Overall the findings of this study establish a better picture of various genotypes of A. thaliana in their NPQ and chloroplast movement abilities, and attests to the impressive flexibility of these mechanisms to light conditions during growth.
References


