Rethinking the Influence of Chloroplast Movements on Non-photochemical Quenching and Photoprotection

Sam Wilson and Alexander V. Ruban

School of Biological and Chemical Sciences, Queen Mary University of London, London, E1 4NS, United Kingdom

ORCID ID: 0000-0001-8554-0249 (A.V.R).

Under blue light, plant chloroplasts relocate to different areas of the cell. The photoreceptor phototropin2 (phot2) mediates the chloroplast movement mechanism under excess blue light alongside the chloroplast unusual positioning1 (chup1) protein. Recently, it has been proposed that leaf transmittance changes associated with chloroplast relocation affect measurements of nonphotochemical quenching (NPQ), resulting in kinetic differences due to these movements (termed “qM”). We evaluated these claims using Arabidopsis (Arabidopsis thaliana) knock-out mutants lacking either phot2 or chup1 and analyzed the kinetics of both the onset and recovery of NPQ under equivalent intensities of both red and blue light. We also evaluated the photoprotective ability of chloroplast movements both during the early onset of photoinhibition and under sustained excess light. We monitored photoinhibition using the chlorophyll fluorescence parameter of photochemical quenching in the dark, which measures the redox state of QA within PSII without any of the complications of traditional Fv/Fm measurements. While there were noticeable differences between the responses under red and blue light, the chloroplast movement mechanism had no effect on the rate or amplitude of NPQ onset or recovery. Therefore, we were unable to replicate the “qM” component and its corresponding influence on the kinetics of NPQ in Arabidopsis grown under “shade” conditions. Furthermore, chloroplast relocation had no effect on the high-light tolerance of these plants. These data cast doubt upon the existence of a chloroplast movement-dependent component of NPQ. Therefore, the influence of chloroplast movements on photoprotection should be thoroughly reevaluated.

Chloroplasts are cytoplasmic organelles and are the site of photosynthesis in many eukaryotic phototrophic organisms. In plants, the chloroplast originated via primary endosymbiosis of a cyanobacterium ~1.5 billion years ago (Dyall et al., 2004; Keeling, 2013). The chloroplast consists of a double membrane that encloses the thylakoid membrane. The outer chloroplast envelope does not act as a permeability barrier, however, due to the vast amounts of porins located within (Fischer et al., 1994; Joyard et al., 1998). It also accommodates several other proteins and processes that play a significant role in the regulation of signaling activities and other metabolic processes (Inoue, 2007). The intracellular distribution of chloroplasts varies markedly under both different qualities and intensities of light (Wada et al., 2003; Wada, 2013, 2016). Under low-intensity blue light, chloroplasts accumulate at the periclinal walls of the cell, while under high-intensity blue light, the chloroplasts aggregate along the anticlinal cell walls (Wada et al., 2003). These movements occur on the timescale of minutes to hours (Wada, 2016). In angiosperms, chloroplast movements only occur under blue light (Inoue and Shibata, 1973; Sztatelman et al., 2010), however, in ferns, movements also occur under red light (Kawai et al., 2003).

Optically, the leaf differs from a solution of pigments in a number of ways. In the leaf, the pigments are concentrated inside chloroplasts, and due to the structure of leaf tissue virtually all photons are scattered (Osbourne and Raven, 1986; Terashima et al., 2009). The former influences the packet effect, where self-shading of photosynthetic pigments can occur, attenuating the absorption efficiency at wavelengths that are strongly absorbed (Osbourne and Raven, 1986). The latter influences the detour effect, where the path length of an incident photon is lengthened due to scattering (McClenon and Fukshansky, 1990a, 1990b; Terashima et al., 2009). The refractive index for plant cells is ~1.43, relative to a value of 1.33 and 1 for water and air,
respectively. The increased refractive index enhances the detour effect in the leaf that would in turn raise the probability for light to encounter a chloroplast within the leaf (Vogelmann, 1993), particularly for poorly-absorbed wavelengths, such as with green light (Terashima et al., 2009). Chloroplast movements alter the package (McClendon and Fukshansky, 1990a, 1990b) but not the detour effect (Davis et al., 2011). Thus, one of the most frequent methods to assay chloroplast movements has been through monitoring blue-light–induced changes in red light transmittance through the leaf (Wada, 2013). Despite this, the putative chloroplast avoidance only reduces overall leaf transmittance by a small amount, with the mean change in shade leaves being 6.31% ± 4% and in sun leaves being 2.09% ± 1% (Davis et al., 2011).

Despite chloroplast movements being observed over 100 years ago (Senn, 1908), only in the past few decades has any progress been made in understanding the photoreceptors, the signal transduction pathway, and the protein effectors involved (Christie, 2007; Wada, 2013). The blue-light photoreceptors that control chloroplast movements are the phototropins phot1 and phot2 (Sakai et al., 2001), both of which are localized on the outer chloroplast membrane (Kong et al., 2013b). phot1 and phot2 are both autophosphorylating kinases (Matsuoka and Tokutomi, 2005) and contain two light, oxygen, or voltage domains that bind flavin mono nucleotide, which acts as the blue-light–sensing chromophore (Christie et al., 1999; Harper et al., 2003). While both phot1 and phot2 control the low-light chloroplast accumulation response, only phot2 controls the high-light–induced chloroplast movement response (Jarillo et al., 2001; Kagawa et al., 2001). Much less, however, is known about the signal transduction pathway. Calcium has been hypothesized to play a role, but is yet to be fully confirmed (Wada, 2016). Several mutants with impaired chloroplast movements lack particular proteins and are termed plastid movement impaired 1 (pmi1), pmi2, and pmi15 (DeBlasio et al., 2005; Luesse et al., 2006). They have been proposed to play a role in signaling, but the biochemical function of these proteins is yet to be fully elucidated. In contrast, the actual physical mechanism of movement is much better resolved (Suetsugu et al., 2010a). Movement is mediated by actin filaments that are localized to the chloroplast (cp-actin; Kadota et al., 2009; Kong et al., 2013a; Wada and Kong, 2018). phot2 is the primary photoreceptor involved in the organization of these filaments (Kong et al., 2013a). Kinesin-like proteins for actin-based chloroplast movement, KAC1 and KAC2, attach and provide the motive force for the chloroplasts to move along the cp-actin (Suetsugu et al., 2010b), while the chloroplast unusual positioning protein (chup1) is likely involved in polymerization of the cp-actin filaments themselves (Oîkawa et al., 2003; Wada, 2013). Mutations in any of these proteins results in complete impairment in the putative chloroplast avoidance response.

Chloroplast movements have been hypothesized to occur for a number of reasons: firstly, to reduce intraleaf light gradients to allow for more uniform light penetration into the leaf (Osbourne and Raven, 1986); secondly, to maximize CO2 diffusion between chloroplasts within the cell (Evans, 1999); and thirdly, as a photoprotective mechanism against high light (Kasahara et al., 2002). Chloroplast movements only occur in “shade” (or low-light) grown plants (Trojan and Gabryś, 1996; Davis et al., 2011; Gotoh et al., 2018a). In highly light-limited environments, chloroplast movements are of ecological importance for the fine-tuning of photosynthesis (Zurzycki, 1955; Lechoski, 1974; Trojan and Gabryś, 1996). However, when acclimated to higher light, plant cells become more columnar, which heavily restricts any organelle movement, and chloroplast movements become much less physiologically important (Davis et al., 2011; Gotoh et al., 2018a).

While the hypothesized photoprotective value of chloroplast movements involves a putative direct light avoidance, many other photoprotective strategies also exist (Takahashi and Badger, 2011; Ruban, 2015). The fastest and one of the most well-characterized responses is nonphotochemical quenching (NPQ; Ruban, 2015, 2016). qE, the major component of NPQ, involves the direct dissipation of excess absorbed excitation energy as heat (Ruban, 2016) and requires light harvesting complex II, trimers, and ΔpH across the thylakoid (Townsend et al., 2018). The PsbS protein and zeaxanthin act as allosteric modulators, shifting the Pk of qE to a higher value, making the light harvesting complex II trimers more sensitive to ΔpH (Johnson and Ruban, 2011; Johnson et al., 2012; Ruban et al., 2012). The other well-characterized components of NPQ include qI, which is one of the most slowly forming and relaxing components dependent on both photoinhibition (Krause and Weis, 1991) and long-term photoprotective quenching (Ruban and Horton, 1995; Malnoë et al., 2017); qT, which occurs under low light and depends on state transitions involving the redistribution of light-harvesting antenna between PSI and PSII (Ruban and Johnson, 2009); and qZ, which is zeaxanthin-dependent quenching (Nilkens et al., 2010). In 2013, Cazzaniga et al. (2013) identified an additional component termed “qM.” They state that this component is a blue-light–dependent quenching mechanism induced by chloroplast movements, which is absent in phot2 knockout mutants (Cazzaniga et al., 2013; Dall’Osto et al., 2014).

Plants are often exposed to a highly fluctuating light environment (Burgess et al., 2016) and cannot avoid absorbed light as readily as many single-cellular photosynthetic organisms, either through flocculation (Conradi et al., 2019) or phototaxis (Wilde and Mullineaux, 2017), despite the similarity of these latter mechanisms to chloroplast movements. Under excess light, photochemistry cannot keep up with the rate of excitation energy being absorbed by the light-harvesting complexes, and damage can occur to the photosynthetic machinery, particularly PSII (Vass, 2012). This is due to the high reducing power generated by the P680 chlorophyll
complex within the PSII reaction center (RC) necessary to oxidize water (Kato et al., 2016; Kern et al., 2018). This process is termed photoinhibition and results in down-regulation of the photosynthetic efficiency of PSII (Powles, 1984). The traditional method for monitoring photoinhibition is through measurement of $F_{v}/F_{m}$, after light stress (Ruban and Murchie, 2012). However, this method is highly problematic as the long-term decrease in $F_{v}/F_{m}$ is not solely indicative of photoinhibitory processes, but also of photoprotective processes (Ruban and Horton, 1995; Townsend et al., 2017). To combat this, a procedure was recently developed to measure photoinhibition directly through measurement of the redox state of the QA site of PSII (Ruban and Murchie, 2012). This is done through measurement of photochemical quenching in the dark ($q_{Pd}$). $q_{Pd}$ declines linearly with oxygen evolution (Giovagnetti and Ruban, 2015), is sensitive to lincomycin (Ruban and Belgio, 2014), can detect photoinhibition caused by both PSII acceptor- and donor-side limitations (Wilson and Ruban, 2019), and has been validated in a range of plant species (Wilson and Ruban, 2020). See Ruban (2017) for a recent review and materials and methods for more details.

Here, we test the validity of the “$q_{M}$” component of NPQ using Arabidopsis (Arabidopsis thaliana) phot2 and chup1 knockout mutants under red and blue monochromatic actinic light (AL) applied separately. We further test the photoprotective capacity of chloroplast movements through the use of the $q_{Pd}$ parameter under both sustained and changing light conditions.

RESULTS

NPQ Is Upregulated under Blue Light Independent of Chloroplast Movements

To identify the effects of chloroplast movements on the measured NPQ, leaves of wild-type, phot2, and chup1 Arabidopsis were measured using red or blue AL (Fig. 1, A–C). These experiments were designed to replicate those of Cazzaniga et al. (2013), but comparing monochromatic ALs rather than white and red. Figure 1A shows that, similar to Cazzaniga et al. (2013), NPQ was larger in wild-type plants under blue AL compared to red AL ($P < 0.05$). The amplitude of NPQ after 1 h of light was 0.866 ± 0.027 and 0.452 ± 0.073 under blue and red AL, respectively. However, the blue-light-dependent component of NPQ remained in both the phot2 and chup1 knockout mutants (Fig. 1, B and C). Therefore, this component cannot be ascribed to chloroplast movements as in Cazzaniga et al. (2013). NPQ differed after 1 h under blue versus red light in phot2 (0.799 ± 0.018 and 0.466 ± 0.064, respectively; $P < 0.05$) and in chup1 (0.910 ± 0.052 and 0.637 ± 0.036, respectively; $P < 0.05$). There were no significant differences among the lines in their red or blue maximum NPQ induction values (blue AL, $P > 0.05$; red AL, $P > 0.05$). Furthermore, NPQ recovered to lower levels under red AL compared to blue AL after 1 h of darkness across all lines. In wild type, NPQ recovered to 0.243 ± 0.004 and 0.075 ± 0.019 ($P < 0.05$) under blue and red AL, respectively. NPQ in phot2 recovered to 0.186 ± 0.018 and 0.070 ± 0.013 ($P < 0.01$) under blue and red AL, respectively, and NPQ in chup1 recovered to 0.232 ± 0.029 and 0.093 ± 0.004 ($P < 0.05$) under blue and red AL, respectively. As with the induction of NPQ, there were no significant differences among the lines in their NPQ recovery values within either light treatment (blue AL, $P > 0.05$; red AL, $P > 0.05$).

Blue Light Causes Greater Initial Levels of Photoinhibition

We also measured $q_{Pd}$ to identify any differences in photoinhibition between these mutants under red or blue AL of fixed intensity, as shown in Figure 1, D–F. After 5 min of illumination, leaves treated with blue AL showed higher levels of photoinhibition (lower values of $q_{Pd}$) than leaves treated with red AL within each line. In wild-type leaves, $q_{Pd}$ after 5 min was 0.843 ± 0.007 and 0.958 ± 0.003 ($P < 0.001$) under blue and red AL, respectively. In phot2 leaves, $q_{Pd}$ after 5 min was 0.881 ± 0.005 and 0.960 ± 0.007 ($P < 0.001$) under blue and red AL, respectively. In chup1 leaves, $q_{Pd}$ after 5 min was 0.887 ± 0.004 and 0.960 ± 0.005 ($P < 0.001$) under blue and red AL, respectively. After 1-h light treatment, there were no significant differences ($P > 0.05$) between $q_{Pd}$ values under red or blue AL within the phot2 and chup1 lines; however there was in wild type (0.905 ± 0.003 under blue light and 0.949 ± 0.008 under red light; $P < 0.001$). In general, there were no significant differences among lines under red AL ($P > 0.05$), but $q_{Pd}$ in wild type was significantly lower than in phot2 and chup1 under blue AL ($P > 0.05$), with a constant offset of ~4%. While the initial blue-light–induced decline in $q_{Pd}$ is likely due to direct damage to the PSII donor side and an imbalance in the energy delivered to PSII and PSI, relative to red AL (Vass, 2012; Wilson and Ruban, 2019), the constant offset seen here is likely due to a chloroplast position-related effect, as it is absent in the knockout mutants. After the transition to darkness, $q_{Pd}$ recovered to similar levels in each line with no significant differences between each line ($P > 0.05$). Interestingly, the level of $q_{Pd}$ increased from the level seen at 5 min to 60 min here, showing a decrease in photoinhibition over time. The alleviation of the extent of photoinhibition could likely be due to a few different factors, such as induction of downstream electron sinks (Kaiser et al., 2017), stomatal regulation (Lawson et al., 2002; Hetherington and Woodward, 2003; Baker, 2008), and rebalancing of excitation energy between PSII and PSI (Allen et al., 1981; Kim et al., 1993).

Initial Excitation Pressure on PSII Is Wavelength Dependent

We determined the differential excitation pressure induced by red or blue AL by monitoring $1 - q_{P}$ using
the same routine as with \( q_{Pd} \) and \( NPQ \), with \( 1 - q_{P} \) derived from the same saturating pulse (SP) as the \( NPQ \) data (Fig. 1, G–I). Between each line within a light treatment there were no significant differences between either red- or blue-AL–induced excitation pressure (\( P > 0.05 \)), nor were there differences within each line for steady-state excitation pressure between red and blue AL treatments. However, after 5 min of illumination, the excitation pressure was significantly higher after blue AL as compared with red AL in each line. For blue and red AL treatment, wild-type \( 1 - q_{P} \) values were \( 0.428 \pm 0.050 \) and \( 0.252 \pm 0.015 \), respectively (\( P < 0.05 \)). For blue and red AL treatment, \( phot2 \) values were \( 0.411 \pm 0.036 \) and \( 0.244 \pm 0.023 \), respectively (\( P < 0.05 \)), and \( chup1 \) values were \( 0.436 \pm 0.039 \) and \( 0.289 \pm 0.028 \), respectively (\( P < 0.05 \)). This indicated that under blue AL, excitation pressure was initially higher than under red AL across all lines. Again, this would likely be due to the wavelength-specificities of each photosystem, with blue AL causing a greater energetic imbalance between PSII and PSI relative to red AL (Kim et al., 1993). This also likely led to the upregulated \( NPQ \) seen under the blue AL, from which the excitation pressure on PSII recovered to a similar level as under the red AL.

Chloroplast Movements Cause No Difference in \( NPQ \) Levels across the Whole Plant

To assess blue-light–dependent quenching across the whole plant, chlorophyll fluorescence was imaged on wild-type, \( phot2 \), and \( chup1 \) plants (Fig. 2). There were no substantial differences between \( NPQ \) levels reached

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**Figure 1.** \( NPQ \) kinetics, photoprotection, and excitation pressure under different light qualities. A to C, \( NPQ \) induction under red (peak \( \lambda = 635 \) nm) or blue light (peak \( \lambda = 460 \) nm). D to F, \( q_{Pd} \) under red or blue light, measured during short dark periods every 5 min in the light and every 10 min in the dark. The dashed line represents \( q_{Pd} = 0.98 \). G to I, Excitation pressure under red and blue light, shown as \( 1 - q_{P} \), measured from the same SP as the \( NPQ \) measurement. Red and blue light intensities used were 288 and 292 \( \mu \)mol m\(^{-2}\) s\(^{-1}\), respectively. Light phase (white bar) was 1 h, followed by 1-h darkness (black bar). A, D, and G, Wild-type (WT) leaves. B, E, and H, \( phot2 \) leaves. C, F, and I, \( chup1 \) leaves. Error bars represent mean ± se (\( n = 3 \)). Asterisk indicate statistical significance at *\( P < 0.05 \), **\( P < 0.01 \), and ***\( P < 0.001 \), using Student’s t test. ns, No statistical significance.
after light treatment nor in the recovery between lines across comparable leaves after darkness \((P > 0.05)\). However, the maximum \(NPQ\) in each plant reached \(\sim 1.6\). This is higher than in the single leaf experiments (Fig. 1, A–C). This discrepancy is likely due to using a slightly higher AL in the imaging experiments.

Quantiﬁcation of the Photoprotective Eﬃcacy of Chloroplast Movements

To assess any differences in photoinhibition and the protective effectiveness of \(NPQ\) across the plant lines, a series of 5-min light steps of increasing intensity were applied to the leaves. From this, \(NPQ\) was measured alongside RC closure (shown via the \(qPd\) parameter). This is much more speciﬁc and sensitive than using \(Fv/Fm\), as \(qPd\) directly measures the redox state of PSII, bypassing the problematic heterogeneity within the \(qI\) or \(Fv/Fm\) parameters (Ruban and Murchie, 2012; Ruban, 2017; Townsend et al., 2017). Figure 3 shows the relationship among \(NPQ\), PSII yield, and \(qPd\). This displays the antagonistic relationship between \(qPd\) and \(NPQ\) against the overall quantum yield of PSII (\(\PhiII\)). These measurements were done using blue AL to observe any differences in these experiments due to chloroplast movements. There were no signiﬁcant diﬀerences in the maximum \(NPQ\) reached between lines, reaching \(3.13 \pm 0.07, 3.01 \pm 0.09,\) and \(3.13 \pm 0.11\) \((P > 0.05)\) in wild type, \(phot2\), and \(chup1\), respectively. Furthermore, there were also no signiﬁcant diﬀerences between the ﬁnal \(qPd\) reached after light treatment, reaching \(0.873 \pm 0.02, 0.898 \pm 0.09,\) and \(0.865 \pm 0.03\) \((P > 0.05)\), in wild type, \(phot2\), and \(chup1\), respectively.

Figure 4 shows population light tolerance curves for each line (see “Materials and Methods” for more details). This allows quantification of the light tolerance of a plant line over a population of leaves. Each point on these curves represents the percentage of the population of leaves that are photoinhibited, i.e. \(qPd < 0.98\). Curves were then ﬁtted with the Hill equation, represented by the solid lines, with 95% conﬁdence intervals shown with dotted lines. From the ﬁt, the 50% tolerance was extracted, i.e. the light intensity at which 50% of leaves had \(qPd < 0.98\), which acts as a suitable comparison point between lines. The 50% tolerance was \(683 \pm 24 \mu mol\ m^{-2} s^{-1}\) in wild type, \(734 \pm 31 \mu mol\ m^{-2} s^{-1}\) in \(phot2\), and \(689 \pm 9 \mu mol\ m^{-2} s^{-1}\) in \(chup1\). There were no signiﬁcant diﬀerences between these values \((P > 0.05)\), indicating that chloroplast movements played no role in protecting PSII against photoinhibition under steadily increasing light.

While traditional \(NPQ\) assays only show the kinetics and magnitude of \(NPQ\), the \(pNPQ\) procedure allows for subtle and sensitive quantiﬁcation of not only the kinetics and magnitude, but also the photoprotective effectiveness (Ruban, 2017). Any \(NPQ\) value with a corresponding \(qPd > 0.98\) is said to be protective and is therefore termed \(pNPQ\). Figure 5 shows the relationship between \(pNPQ\) and the AL intensity. There were no signiﬁcant diﬀerences between the maximum \(pNPQ\) reached in each line. The maximum \(pNPQ\) per leaf reached \(1.61 \pm 0.25, 1.62 \pm 0.15,\) and \(1.89 \pm 0.64\), in wild type, \(phot2\), and \(chup1\), respectively. Moreover, when fit...
with a simple linear regression ($y = mx + c$), the gradients allowed for normalization of $pNPQ$ against AL, meaning that the protective efficacy of $NPQ$ per $pNPQ$ unit can be readily determined. However, among wild type, $phot2$, and $chup1$, there were no significant differences in the gradients of the lines ($P > 0.05$). Altogether, this indicates that chloroplast movements do not function in tandem with $NPQ$ to protect PSII, nor do they have any effect on the efficiency of the $NPQ$ process.

**DISCUSSION**

The leaf is an optically complex structure and is affected by a range of dynamic changes when exposed to light (Vogelmann, 1993; Ruban, 2015). The fluorescence emission of a leaf is dependent on many of these changes and can be defined as follows, as in Baker (2008):

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**Figure 3.** The relationship between $NPQ$ and yield PSII/$qPd$ determined from the eight-step $pNPQ$ procedure. The solid horizontal line corresponds to $qPd = 1$ and the dashed horizontal line corresponds to $qPd = 0.98$. A, Wild-type (WT) leaves. B, $Phot2$ leaves. C, $Chup1$ leaves. Data shown are mean ± SE ($n = 10$). Blue AL (peak $\lambda = 450$ nm) was used for this experiment.

**Figure 4.** Population light tolerance curves determined from the eight-step $pNPQ$ procedure. Data represent the percentage of probed leaves with photoinactivated RCs (where $qPd < 0.98$) at each light intensity and have been fitted with the Hill equation, shown with the black line. Dotted line indicated 95% confidence intervals of the fit. Dashed line indicates the 50% tolerance. A, Wild-type (WT) leaves. B, $Phot2$ leaves. C, $Chup1$ leaves. Data shown are mean ± SE ($n = 10$). Blue AL (peak $\lambda = 450$ nm) was used for this experiment.
by the leaf, $\text{fraction}_{\text{PSII}}$ is the amount of light that is absorbed by PSII, and $\Phi_F$ is the quantum yield of fluorescence, defined here:

$$\Phi_F = \frac{k_F}{(k_F + k_H + k_p P)}$$

(2)

where $k_F$, $k_H$, and $k_p$ are the rate constants for the decay of excitation through fluorescence, internal conversion, or photochemistry, respectively, and $P$ represents the redox state of QA, and therefore the openness of the PSII RC. At $F_o$, $P = 1$, while at $F_m$, $P = 0$. Thus, $\Phi_{F_o}$ and $\Phi_{F_m}$ can be defined as in the following two equations:

$$\Phi_{F_o} = \frac{k_F}{(k_F + k_H)}$$

(3)

$$\Phi_{F_m} = \frac{k_F}{(k_F + k_H + k_p P)}$$

(4)

where $\Phi_{F_o}$ and $\Phi_{F_m}$ are the minimum and maximum quantum yields of fluorescence, respectively; and $k_F$, $k_H$, and $k_p$ are as defined previously. Consequently, commonly used fluorescence parameters can be defined in light of any optical changes to the leaf. For example, $F_o/F_m$ is as follows:

$$\frac{F_o}{F_m} = \frac{I \times A_{\text{leaf}} \times \text{fraction}_{\text{PSII}} \times \Phi_{F_o}}{I \times A_{\text{leaf}} \times \text{fraction}_{\text{PSII}} \times \Phi_{F_m}} = \frac{\Phi_{F_o}}{\Phi_{F_m}}$$

(5)

In this case, any optical changes that would arise from changes in chloroplast position would cancel out and the $F_o/F_m$ parameter would be solely reliant of changes at the level of the quantum yields. However, for other parameters, such as NPQ, the situation is less clear:

$$\text{NPQ} = \frac{(I \times A_{\text{leaf}} \times \text{fraction}_{\text{PSII}} \times \Phi_{F_o}) - (I' \times A'_{\text{leaf}} \times \text{fraction}'_{\text{PSII}} \times \Phi_{F_o})}{I \times A_{\text{leaf}} \times \text{fraction}_{\text{PSII}} \times \Phi_{F_o}}$$

(6)

where all parameters are as defined as previously, but $I$, $A_{\text{leaf}}$, fraction$_{\text{PSII}}$, and $\Phi_{F_o}$ are in the dark; and $I'$, $A'_{\text{leaf}}$, fraction'$_{\text{PSII}}$, and $\Phi_{F_o}'$ are in the light. $I$ and $I'$ will be unaffected, and fraction$_{\text{PSII}}$ and fraction'$_{\text{PSII}}$ are unchanged by chloroplast movements as there are no differences in excitation pressure between wild-type and chloroplast-movement–deficient mutants, as shown in this and in previous studies (Cazzaniga et al., 2013). However, as $A_{\text{leaf}}$ is prone to dynamic changes from chloroplast movements (Inoue and Shibata, 1973), it could be argued that any changes here could lead to an overestimation of the NPQ parameter (Brugnoli and Björkman, 1992). Therefore, while qM-type quenching is theoretically possible, it is highly unlikely to be caused by chloroplast movements in the leaf. It could however be possible in a highly phototactic photosynthetic microorganism, such as *Synechocystis* sp. PCC

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**Figure 5.** Levels of pNPQ at each light intensity. These data have been fit with a simple linear regression, shown with the black line. The dotted lines indicate 95% confidence intervals from the linear fit. A, Wild-type (WT) leaves. B, Phot2 leaves. C, Chup1 leaves. Data shown are mean ± se ($n = 10$). Blue AL (peak $\lambda = 450$ nm) was used for this experiment.

$$F = I \times A_{\text{leaf}} \times \text{fraction}_{\text{PSII}} \times \Phi_F$$

(1)
Furthermore, with regard to qP or qPd, Brugnoli and Björkman (1992) show that chloroplast movements will not affect photochemical quenching, provided \( F_{\infty} \) is measured. In the case of qPd, the actual \( F_{\infty} \) is measured and then compared to the calculated \( F_{\infty} \), as derived by Oxborough and Baker (1997). This likely indicates that the lower qPd offset under constant blue light (Fig. 1A), is not due to a technical underestimation of qPd, but a functional change in the ability of chloroplast movements to photoprotect. It seems in this case that chloroplasts remaining in their “dark” position, as is the case in phot2 and chup1 under high blue irradiance, is better for photoprotection of PSII than by having any chloroplast “avoidance” mechanism itself. It is also worth noting that, depending on the quality of light, not only is there a difference in the energy of photon received by the photosynthetic apparatus, but also in the depth of the leaf the photon would reach, and depending on the experimental setup, a difference in the area of the leaf being probed (Terashima et al., 2009; Oguchi et al., 2011; Evans et al., 2017). Evans et al. (2017) previously showed that blue light can cause an overestimation in some chlorophyll fluorescence parameters due to the aforementioned factors.

The potential for overestimation of NPQ under blue light led Bassi and colleagues in their identification of a white-light–dependent quenching parameter, which they termed “qM,” which is not present in the phot2 knockout (Cazzaniga et al., 2013). However, in this study, when either phot2 or chup1 were knocked out, there was still a blue-light–dependent upregulation of both the magnitude and kinetics of NPQ. We were unable to replicate the results of Cazzaniga et al. (2013). Therefore, any quenching component that occurs on the timescale of minutes to hours cannot be simply ascribed to chloroplast movements. It is much more likely that this component is a sustained quenching mechanism at the molecular, rather than the cellular level, such as the recently identified plastid lipocalin-related quenching pathway (Malnoë et al., 2017) or zeaxanthin-dependent quenching (Nilkens et al., 2010). Given the data presented in this study, it is likely that any transmittance changes due to rearrangement of chloroplasts are so minor that they are overwhelmed by much larger effective changes at the level of the quantum yields of fluorescence. Moreover, previous studies showing the effectiveness of chloroplast movements in avoiding photoinhibition (Kasahara et al., 2002; Cazzaniga et al., 2013) are highly problematic, as the assays they use are highly flawed. Use of \( F_{\infty} / F_m \) as a parameter of photo-inhibition is invalid, as there are many components within this parameter that are not due to photo-inhibition, but to photoprotective aspects (Townsend et al., 2018). Also, Kasahara et al. (2002), use stress conditions of 1,400 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\) for up to 31 h. These conditions are highly artificial (Burgess et al., 2016) and likely induce a phenotype that is not biologically valid.

Furthermore, in response to Johnson and Ruban (2011), Dall’Osto et al. (2014) stated that qE-like quenching found in the PsbS-knockout npq4 was, in fact, due to chloroplast movements. However, this is highly improbable. Not only do chloroplast movements not affect NPQ, as shown in this study; but Johnson and Ruban (2011) only used red AL, meaning that no chloroplast movement could possibly occur during the measurements. However, any technical artifacts induced by chloroplast movements—such as may be present in absorption measurements to monitor zeaxanthin synthesis (Bilger and Björkman, 1990; Townsend et al., 2018; Wilson and Ruban, 2020) or P700 measurements (Shimakawa and Miyake, 2018)—can be avoided by using red AL.

This then raises important questions about the actual nature of the upregulated NPQ under blue light relative to red light. Recently, Pfündel et al., (2018) argued that the blue-light–dependent chlorophyll fluorescence changes were due to chloroplast movements. However, as shown in this study, this is unlikely to be the case. Pfündel et al., (2018) used plants acclimated to low-light as well as low-temperature and high-light conditions. While there is little to no chloroplast rearrangement in high-light-acclimated leaves (Davis et al., 2011), due to changes in cellular shape (Gotoh et al., 2018a), there are many other acclimative factors in photosynthesis that affect chlorophyll fluorescence greatly. For example, changes in the arrangement and amounts of PSII and its associated light-harvesting apparatus (Ballottari et al., 2007; Ware et al., 2015) and in thylakoid ultrastructure (Schumann et al., 2017) are highly variable and are much more likely to affect fluorescence. Furthermore, under cold acclimation, chloroplast rearrangements still occur (Cazzaniga et al., 2013), while many other photosynthetic processes and enzymatic reactions will be greatly inhibited (Oquist and Huner, 2003). The change in NPQ between red and blue light is likely due to a greater effective absorption of higher-energy blue photons by the photosynthetic apparatus (Osbourne and Raven, 1986). This is confirmed by a greater initial excitation pressure upon PSII under blue light, which would induce a greater 4P and therefore a greater NPQ response (Genty et al., 1989; Ruban, 2016). The greater initial photo-inhibition under blue light is likely due to direct damage to the PSII donor side by blue wavelengths (Vass, 2012; Zavafer et al., 2015, 2017; Wilson and Ruban, 2019). Furthermore, observed differences in qP and qPd between red and blue light would also be likely due to the different light qualities affecting the energy distribution between photosystems (Duyzens et al., 1961; Kim et al., 1993). This difference could also explain the greater excitation pressure and photo-inhibition observed under blue light. Thus, it is highly unlikely that changes shown by Pfündel et al., (2018) between red and blue light in these plants can be due to any chloroplast avoidance mechanism.

Therefore, what is the evolutionary benefit of having adaptive chloroplast positioning? From this study, it is highly unlikely that chloroplast movements offer the
capacity for effective photoprotection. It is likely that they are most important for fine-tuning of light usage under highly light-limiting conditions (Zurzycki, 1955; Lechowski, 1974; Trojan and Gabryś, 1996; Gotoh et al., 2018b) and for regulation of signaling pathways, which rely on much finer-tuning of the light environment than photosynthesis (Christie, 2007). Furthermore, chloroplast movements reduce intraleaf light gradients (Osbourne and Raven, 1986; Vogelmann, 1993), allowing for more effective photosynthetic light utilization throughout the leaf structure as a whole. This, coupled with the observation that chloroplast movements can reduce photoinhibition (Vass et al., 1992; Wilson and Ruban, 2019). This causes Fp, act > Fp, calc and causes qPd < 1. When qPd < 0.98 (i.e. 2% of RCs are inhibited), leaves are said to be photoinhibited and NPQ can no longer be judged to be protective (pNPQ), i.e. any value of NPQ where qPd > 0.98 can be defined as pNPQ. For more details, see Ruban (2017) for a recent review.

**Procedure**

Fixed-intensity NPQ induction and qPd measurements were carried out using a DUAL-PAM-100 fluorometer (Walz), using either a blue (peak $\lambda = 460$ nm) or red (peak $\lambda = 635$ nm) AL. The red and blue ALs were calibrated to 288 and 292 $\mu$mol m$^{-2}$ s$^{-1}$, respectively. The measuring light (ML) was blue (peak $\lambda = 460$ nm) and kept at a low non-actinic intensity, which would not induce the high-light–associated chloroplast movement (Pfündel et al., 2018).

For all experiments, the SP intensity was 4,000 $\mu$mol m$^{-2}$ s$^{-1}$, and lasted for 0.6 s. Measurements consisted of 1 h light followed by 1 h darkness, with short (<15 s) periods of darkness every 5 min in the light phase, and every 10 min in the dark phase for parallel measurement of qPd. For this, there was 7 s of low far-red light, 3 s of darkness, followed by an SP to quantify qPd. This was followed by a further 5 s before the next light phase. For a schematic explanation, see Supplemental Figure S1.

pNPQ imaging measurements were carried out on an IMAGING-PAM M-series MAXI fluorometer (Walz), which was equipped with a blue ML and AL (peak $\lambda = 450$ nm), and the intensity of the blue AL was calibrated to 337 $\mu$mol m$^{-2}$ s$^{-1}$. Measurements here consisted of 1-h light and 1-h darkness with NPQ measured at the end of each phase, so the magnitude of NPQ induction and recovery could be imaged across the whole plant.

pNPQ was assessed using a JUNIOR-PAM fluorometer (Walz) equipped with a blue ML and AL (peak $\lambda = 450$ nm). The AL intensities were calibrated to steps of 0, 120, 253, 380, 560, 833, 1,093, 1,533, and 2,000 $\mu$mol m$^{-2}$ s$^{-1}$. Each light phase lasted 5 min, with NPQ quantified at the end of each phase. Between each light phase, qPd was quantified in the same manner as specified before. Population light tolerance curves were constructed using the percentage of leaves with qPd < 0.98, and NPQ was no longer protective against RC closure, which acts as a suitable comparison between plant lines.

For all measurements, a blue ML was used to ensure that the same depth in the leaf was being probed between measurements (Oguchi et al., 2011; Evans et al., 2017). For each chlorophyll fluorescence measurement, the fluorescence detector measured $\lambda > 700$ nm. Within these experiments, the only light quality variables were in the AL.

**Conclusion**

In this study, we have shown that chloroplast movement has very little effect on the high-light effectiveness of photoprotection in “shade”-grown Arabidopsis. Consequently, the existence of a chloroplast-movement–dependent component of NPQ and the influence of chloroplast movements on photoinhibition should be thoroughly reevaluated.

**Materials and Methods**

**Plant Growth Conditions**

Wild-type Arabidopsis (Arabidopsis thaliana; Col-0), the phot2 knockout (phot2; Jarillo et al., 2001), and the chup1 knockout (chup1; Okawa et al., 2003) were used in this study. Seeds were sterilized in 50% (v/v) ethanol and 0.1% (v/v) Triton X-100 and were stored for 48 h at 4°C before being sown on a 6:6:1 ratio of Levington M3 compost, John Innes No. 3 soil, and Perlite (Scotts). All measurements were carried out on 4- to 5-week-old plants grown at 170 $\mu$mol m$^{-2}$ s$^{-1}$ and 292 $\mu$mol m$^{-2}$ s$^{-1}$, with a 10-h photoperiod at 22°C. Before each measurement, plants were dark-adapted for 45 min.

**Chlorophyll Fluorescence Measurements**

**Theory**

When exposed to light, the FII value is undermined by both NPQ and photoinhibition. The relationship among FII, NPQ, and photoinhibition (i.e. qPd) is shown below:

\[
F_{II} = \frac{F_\infty \times qPd}{1 + (1 - F_\infty) \times NPQ} 
\]

where $F_\infty/F_\infty$ is the maximum quantum efficiency of PSII, calculated as ($F_\infty - F_\infty$)/$F_\infty$, and $F_\infty$ and $F_\infty$ are the maximum and minimum fluorescence yields, respectively. NPQ is calculated as ($F_\infty - F_\infty$)/$F_\infty$, and $qPd$ indicates the redox state of the QA site of PSII. qPd is calculated as follows:

\[
qPd = \frac{F_\infty - F_{act}}{F_\infty - F_{calc}} 
\]

where $F_\infty$ is the maximum fluorescence yield in the dark after light treatment; $F_{act}$ is the actual measured $F_{\infty}$; and $F_{calc}$ is based upon the equation of Oxborough and Baker (1997), shown here:

At low light, $F_\infty$ act $\approx$ $F_\infty$ calc, and therefore, $qPd = 1$. However, under excess light, there is a well-characterized rise in $F_{act}$, due to acceptor-side photoinhibition (Vass et al., 1992; Wilson and Ruban, 2019). This causes $F_\infty$ act > $F_\infty$ calc and causes qPd < 1. When qPd < 0.98 (i.e. 2% of RCs are inhibited), leaves are said to be photoinhibited and NPQ can no longer be judged to be protective (pNPQ), i.e. any value of NPQ where qPd > 0.98 can be defined as pNPQ. For more details, see Ruban (2017) for a recent review.

**Accession Numbers**

The sequence data from this article can be found in The Arabidopsis Information Resource database (https://www.arabidopsis.org/) under the following accession numbers: phot2 (AT5CS58140) and chup1 (AT3G25690).

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** pNPQ chlorophyll fluorescence procedure from a wild-type Arabidopsis plant (Col-0) with eight steps of increasing AL exposure.

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