**Dehydroascorbate Reductase Affects Non-photochemical Quenching and Photosynthetic Performance**

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Ascorbic acid (Asc) is a major antioxidant involved in photoprotection and photosynthetic function in plants. Dehydroascorbate reductase (DHAR) catalyzes the regeneration of Asc from its oxidized state and serves as an important regulator of Asc recycling. In this work, we used a molecular biochemical approach to investigate how the efficiency of Asc recycling affects non-photochemical quenching (NPQ). Suppression of DHAR expression resulted in a lower induction of NPQ that correlated with reductions in chlorophyll and xanthophyll pigments, quantum yield of photosystem II, and CO2 assimilation, whereas the level of reactive oxygen species increased. The quickly reversible component of NPQ decreased and the slowly reversible or irreversible component of NPQ increased following a reduction in DHAR expression. Significant photoinhibition was also observed following exposure to high light. Direct feeding with Asc restored the appropriate induction of NPQ in DHAR-suppressed leaves. In contrast, increasing DHAR expression increased the pool size of xanthophyll and chlorophyll pigments as well as the rate of CO2 assimilation, particularly at high light intensities, whereas the level of reactive oxygen species was reduced. Leaves with increased DHAR expression experienced less photoinhibition than did wild-type plants following exposure to high light. DHAR activity, therefore, can affect the appropriate induction of NPQ and level of photoprotection during exposure to high light.

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Despite the efficiency of the reactions that convert solar energy into chemical energy, the capacity of photosynthesis to use absorbed light energy is limited. Excess absorbed light energy can be dangerous because it can result in the production of triplet state chlorophyll (3Chl*) that can transfer energy to ground state O2 to produce singlet oxygen (O2•*) and eventually other reactive oxygen species (ROS), e.g. superoxide anion, hydroxyl radicals, and hydrogen peroxide (H2O2) (1, 2). ROS can damage protein subunits and pigments of photosystem II (PSII), resulting in protein degradation, inactivation of reaction centers, and inhibition of the subsequent repair mechanisms of the reaction center (3, 4).

Thermal dissipation of excitation energy is described as non-photochemical quenching (NPQ) of chlorophyll fluorescence. NPQ is composed of pH-dependent fluorescence quenching (qE or feedback de-excitation), quenching associated with state transition (qT), and quenching caused by photoinhibition (qI). Under many conditions, most of NPQ occurs as qE in the PSII antenna pigment bed and is rapidly reversible (5, 6). qT contributes little to NPQ and is unlikely to be a significant factor in photoprotection. qI is quenching that includes damage to PSII reaction centers (7) and is either irreversible or slowly reversible (8). Under normal growth conditions, qE predominates, dissipating excess excitation energy as heat to prevent photoinhibition and damage to the photosynthetic apparatus. Thus, NPQ plays a key role in regulating light harvesting and photosynthetic performance (5).

Asc, the most abundant antioxidant in plants, is important in maintaining photosynthetic function (9–12). Asc detoxifies ROS and protects the photosynthetic apparatus from oxidative damage (1). Asc can serve as a direct electron donor to PSI and PSII under conditions where the primary electron donor system is impaired, e.g. under high light stress (13–15). Photoreduction of monodehydroascorbate (MDHA), produced following the oxidation of Asc, plays an important role in maintaining the electron transport flow when NAPD+ is limiting by competing with ferredoxin-NADP+ for electrons at the reducing side of PSI (11–12). Asc is also an essential cofactor for violaxanthin de-epoxidase that de-epoxidates violaxanthin to produce zeaxanthin in the xanthophyll cycle, a necessary step reducing side of PSI (11–12). Asc is also an essential cofactor for violaxanthin de-epoxidase that de-epoxidates violaxanthin to produce zeaxanthin in the xanthophyll cycle, a necessary step for the induction of NPQ following exposure to excess light (16, 17). The importance of Asc in protecting photosynthetic function has been shown with vtc mutants of Arabidopsis. The vtc1 mutant, defective in GDP-mannose pyrophosphorylase, accumulates eucharyotic elongation factor 1A; Qo, primary quinone electron acceptor of PSI; qP, photochemical quenching; NPQ, fast relaxing NPQ; NPQs, slow relaxing NPQ; Dss, DHAR-overexpressing; Dss, DHAR-suppressed; WT, wild-type; TdDHAR, wheat DHAR; NiDHAR, tobacco DHAR; chHS70, chloroplast-localized HSP70; V, violaxanthin; A, antheraxanthin; Z, zeaxanthin; Fv/Fm, minimum fluorescence in the dark-adapted state; Fv/Fm, maximum fluorescence in the light-adapted state; Fv/Fm, maximum fluorescence in the light-adapted state; Fv/Fm, maximum fluorescence in the light-adapted state; Fv/Fm, variable fluorescence; Fv/Fm, steady state fluorescence yield during actinic illumination; Fv/Fm, steady state fluorescence yield in the light-adapted state.
mulates 30% of the wild-type level of Asc and is hypersensitive to ozone, sulfur dioxide, or UVB light but exhibits only a moderately slower growth rate under normal growth conditions (18–20). vtc1 plants exhibit no significant difference in light saturation curves for CO₂ assimilation or chlorophyll fluorescence under these growth conditions, suggesting that the effect on growth was not because of decreased photosynthetic capacity or decreased photochemical efficiency (20). In contrast, the vtc2 mutant, which contains only 10–25% of wild-type levels of Asc as a result of reduced GDP-L-galactose-hexose-1-phosphate guanylyltransferase activity, exhibits reduced NPQ and increased photoinhibition when transferred from low to high light (20–22).

Once used, Asc is oxidized to MDHA that can be recycled to Asc by ferredoxin in the chloroplast or by MDHA reductase in the chloroplast or cytosol (23, 24). In the thylakoid lumen, the MDHA produced by violaxanthin de-epoxidase or following donation of electrons from Asc to PSI or PSII is not available for reduction by ferredoxin or MDHA reductase but rapidly disproportionates to Asc and dehydroascorbate (DHA) when the pH of the lumen is low (15, 24). DHAR is then reduced to Asc by dehydroascorbate reductase (DHAR) in a reaction requiring glutathione. DHA undergoes irreversible hydrolysis to 2,3-diketogulonic acid if not rapidly recycled to Asc.

DHAR is expressed in rate-limiting amounts and contributes to the regulation of the symplastic and apoplastic Asc pool size and redox state (25–27). Guard cells in DHAR-overexpressing plants exhibited a reduced level of H₂O₂, decreased responsive- and redox state (25–27). Guard cells in DHAR-overexpressing

**EXPERIMENTAL PROCEDURES**

**Plant Material and Growth Conditions**—Transgenic tobacco (Nicotiana tabacum cv. Xanthi) expressing the His-tagged wheat DHAR from the cauliflower mosaic virus 35S promoter was generated using Agrobacterium tumefaciens as described previously (25). Transgenic tobacco plants suppressed for DHAR were identified following the introduction of the tobacco DHAR cDNA in pBI101. All plants were grown in commercial soil in a greenhouse supplied with charcoal-filtered air. Plants were grown under natural light conditions in a 10-h light and 14-h dark cycle and were watered to saturation twice a day. The average temperature during the day was 25.9 ± 0.6 °C, and the average temperature at night was 20.2 ± 0.5 °C. The average light intensity in the morning (9 a.m.) was 514 ± 206 μmol m⁻² s⁻¹ and in the afternoon (1 p.m.) was 1191 ± 244 μmol m⁻² s⁻¹. Leaves at the 2-week-old stage were taken from 6-week-old plants; leaflets at the 8-week-old stage were collected from 12-week-old plants.

For high light experiments, 2- or 8-week-old leaves that had been dark-adapted were floated on ice-containing water and exposed to a photon flux density (PFD) of 2000 μmol m⁻² s⁻¹ (supplied from high output sodium light) for the times indicated. Fₘ (minimum fluorescence in the dark-adapted state) and Fₘ were measured immediately before the high light exposure and at time points during recovery. For the analysis, leaves with similar initial Fₘ/Fₘ values were used.

For Asc feeding experiments, 2-week-old leaves were floated on 10 mm Asc or water for 2 h in the dark. Following dark adaptation, NPQ was measured following exposure to 1500 μmol m⁻² s⁻¹.

**Gas Exchange and Fluorescence Measurements**—Gas exchange and fluorescence measurements were performed using the LI-COR Li-6400 portable photosynthesis system (LI-COR Biosciences, Lincoln, NE) with the LI-6400-40 leaf chamber, a relative humidity of 50%, and ambient level of CO₂. Fluorescence measurements were taken using overnight dark-adapted leaves. At the start of each experiment, the leaf was exposed to 2 min of far-red illumination (1 μmol of photons m⁻² s⁻¹) for the determination of Fₘ. Saturating pulses (0.8 s) of 7000 μmol of photons m⁻² s⁻¹ were applied to determine the Fₘ or Fₘ values. Actinic light, consisting of 90% red light (λ = 630 ± 20 nm) and 10% blue light (λ = 470 ± 20 nm), was provided by light emission diode sources. Fₘ is the steady state fluorescence yield during actinic illumination. Fₘ (minimum fluorescence in the light-adapted state) was determined in the presence of far-red (λ = 740 nm) light after switching off the actinic light. A total of four to six samples were measured in each experiment. All data presented were calculated from at least three independent measurements. Conventional fluorescence nomenclature was used (30). NPQ was calculated from \((Fₘ - Fₘ)/Fₘ\), the quantum efficiency of PSII (φPSII) was calculated from \((Fₘ - Fₘ)/Fₘ\), qP was calculated from \((Fₘ - Fₘ)/Fₘ\), and the electron transport rate (ETR) was calculated from \(φPSII \times f \times α_{leaf}\) where \(f\) is the fraction of absorbed quanta that is used by PSII and is typically assumed to be 0.5 for C₃ plants; \(α_{leaf}\) is leaf absorbance, and 0.9 was used for tobacco. NPQₙ and NPQₐ were determined as described previously (31).
DHAR Affects Non-photochemical Quenching

H$_2$O$_2$ Assay—Foliar H$_2$O$_2$ concentrations were determined by the method of Gay and Gebicki (32) based on the peroxide-mediated oxidation of Fe$^{2+}$ followed by the reaction of Fe$^{3+}$ with xenylenol orange (o-cresolsulphonphthalein 3′,3′-bis(methyl-limino)diacetic acid, sodium salt). Protein was removed from the leaf extract by adding an equal volume of 25 mM HCl. Protein-depleted extract was mixed with an equal volume of 2X assay reagent (500 μM ammonium ferrous sulfate, 220 mM HClO$_4$, 200 μM xylene orange, 200 mM sorbitol). Absorbance of the Fe$^{3+}$-xenylenol orange complex (A$_{645}$) was detected after 45 min. The specificity for H$_2$O$_2$ was tested by removing H$_2$O$_2$ from the reaction mixture using catalase prior to protein removal. Standard H$_2$O$_2$ curves were obtained for each independent experiment by adding various amounts of H$_2$O$_2$ to 500 μl of assay reagent.

Chlorophyll and Pigment Measurements—Chlorophylls a and b were measured spectrophotometrically as described previously (33). Leaf samples were ground in liquid nitrogen and extracted with 90% (v/v) acetone. The absorbance at 664 and 647 nm was determined and used to calculated chlorophyll a and b content by the equations: Chl a = 11.93A$_{664}$ - 1.93A$_{647}$ and Chl b = 20.36A$_{647}$ - 5.50A$_{664}$ respectively. Each experiment was repeated two to three times, and representative results are presented.

Xanthophyll pigments were extracted with 100% acetone under dim light and were separated on a Spherisorb ODS-1 column (Alltech) as described by Gilmore and Yamamoto (34) using solvent A-1 (acetonitrile, methanol, 0.1 M Tris-HCl pH 7.5 (72:8:3)) and solvent B (methanol:hexane (4:1)). Pigments were identified and quantified by the retention time and amount of standards using a photodiode array detector.

Western Analysis—Protein extracts were resolved using standard SDS-PAGE, and the protein was transferred to 0.22-μm polyvinylidene difluoride membrane by electroblotting. Following transfer, the membranes were blocked in 5% milk in TPBS (0.1% Tween 20, 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.4 mM KH$_2$PO$_4$, pH 7.4) followed by incubation with antiserum raised against DHAR, PsbS, light-harvesting complex protein (LHCP), heat shock protein 70 (HSP70), or eukaryotic elongation factor 1A (eEF1A) diluted 1:1000 in eukaryotic elongation factor 1A (eEF1A) diluted 1:20,000 for 1 h. The blots were washed twice with TPBS and incubated with goat anti-rabbit horseradish peroxidase-conjugated antibodies (Southern Biotechnology Associates, Inc.) diluted 1:20,000 for 1 h. The blots were washed twice with TPBS, and the signal was detected using chemiluminescence (Amersham Biosciences).

Asc and DHA Measurements—Asc was measured as described previously (9). Leaves were ground in 2.5 mM HClO$_4$ and centrifuged at 13,000 rpm for 10 min. Two volumes of 1.25 mM Na$_2$CO$_3$ were added to the supernatant, and following centrifugation, 100 μl was added to 895 μl of 100 mM K$_2$HPO$_4$/KH$_2$PO$_4$, pH 5.6. Asc was determined by the change in absorbance at 265 nm following the addition of 0.25 μM of ascorbate oxidase. The total amount of reduced and oxidized ascorbic acid (i.e., Asc and DHA) was determined by reducing DHA to Asc (in a reaction containing 100 mM K$_2$HPO$_4$/KH$_2$PO$_4$, pH 6.5, 2 mM GSH, and 0.1 μg of recombinant wheat DHAR protein incubated at 25 °C for 20 min) prior to measuring Asc. The amount of DHA was determined as the difference between these two assays.

RESULTS

Photosynthetic Activity and NPQ Decline Concomitantly during Leaf Aging—To characterize the photosynthetic activity of tobacco leaves during development, a light-response curve, representing the CO$_2$ assimilation rate as a function of incident PFD, was determined. 2-week-old leaves, i.e., the first fully expanded leaves, exhibited a high rate of CO$_2$ assimilation with a maximum rate achieved at a PFD of 1500 μmol m$^{-2}$ s$^{-1}$ (supplemental Fig. 1A). A progressive decline in the CO$_2$ assimilation rate and in the light saturation point was observed with leaf age (supplemental Fig. 1A). The substomatal CO$_2$ concentration in 8-week-old leaves (i.e., defined here as the presenescent stage) was higher than in 2-week-old leaves throughout the light-response curve (supplemental Fig. 1C), suggesting that the decline in CO$_2$ assimilation was because of decreased photosynthetic activity.

Chlorophyll fluorescence was also measured in dark-adapted leaves at the same developmental time points to determine which aspects of photosynthetic functioning change during leaf aging. The reduction state of QA, the primary quinone electron acceptor of PSII, was calculated from 1 − qP and plotted as a function of illumination time as an indicator of the efficiency of electron transfer from QA to downstream electron acceptors. During leaf aging, the QA pool shifted toward a more reduced state, i.e., higher 1 − qP values (supplemental Fig. 2A), indicating reduced electron transfer from QA to downstream electron acceptors and inversely correlating with the rate of CO$_2$ assimilation (supplemental Fig. 1A). A reduction in NPQ was also measured during leaf aging (supplemental Fig. 2B), suggesting a reduced capacity to dissipate excitation energy through high energy state quenching, which can lead to over-reduction of QA, production of singlet and triplet state chlorophyll a, and an enhanced production of ROS (3, 35).

Damage to chlorophyll a is indicated by a reduction in the maximum quantum yield of PSII (i.e., $F_v/F_m$) or an increase in the $F_v$ value of dark-adapted leaves. A decrease in the $F_v/F_m$ ratio ($p < 0.01$, $n = 4$) and a significant increase in the $F_v$ value ($p < 0.01$, $n = 4$) was observed in 8-week-old leaves compared with younger leaves (supplemental Table 1), indicating that presenescent leaves may have accumulated photodamage in the light-harvesting antennae chlorophyll as well as in the PSII reaction center at this stage. Fast relaxing quenching (i.e., NPQ$_f$), a measure of qE, declined substantially in 6- and 8-week-old leaves ($p < 0.001$, $n = 4$), whereas slow relaxing quenching (i.e., NPQ$_s$), a measure of qI, increased significantly ($p < 0.05$, $n = 4$) (supplemental Table 1), suggesting a reduction in photoprotection during leaf aging.

Changes in DHAR Expression Affect Photosynthetic Function—To investigate whether the level of DHAR expression affects photosynthetic performance, the rate of CO$_2$ assimilation was measured in DHAR-overexpressing (D$_{xN}$) leaves that exhibit enhanced Asc recycling (25), in DHAR-suppressed (D$_{xK}$) leaves that exhibit impaired Asc recycling (26), and in control leaves. The rate of CO$_2$ assimilation in young (i.e., 2-week-old) D$_{xK}$ leaves was significantly lower than the control ($p < 0.005$, 0.01, $n = 4$) and remained lower than in young D$_{xN}$ leaves throughout development ($p < 0.001$, $n = 4$) (supplemental Table 2), suggesting that sustained accumulation of DHA, as a consequence of DHAR over-expression, can contribute to reduced photosynthesis and photodamage in older leaves.
DHAR expression determines photosynthetic activity. The rates of CO₂ assimilation (A and B), stomatal conductance (C and D), and substomatal CO₂ concentration (E and F) were measured as a function of light intensity (PFD) in 2- and 8-week-old leaves, respectively, of control tobacco (C) and DHAR-suppressed tobacco (DKD) (A). Four to six replicate plants were assayed for each line, and the data were averaged.

Although the rate of CO₂ assimilation was lower in presenescent (i.e. 8-week-old) leaves in all three lines, it remained higher in D₀X leaves than in the control (p < 0.01, paired t test) and lower in DₖD leaves than in the control (p = 0.013, paired t test) (Fig. 1B). The reduction in photosynthetic function in presenescent DₖD leaves was not because of a decrease in Rubisco as its level in DKD leaves is identical to that in the control at this leaf age (28).

The rates of CO₂ assimilation were lower in presenescent DKD leaves than in the control (Fig. 1F). The minimum light intensity that reached a higher level than the control (p < 0.05, paired t test) (Fig. 2A) decreased from 0.78 in young leaves (Fig. 2A) to 0.53 in presenescent leaves (Fig. 2B), a reduction not observed in presenescent control or D₀X leaves (Fig. 2B). Because a reduction in the maximum quantum yield of PSII is indicative of damage, these data suggest that a greater level of photoinhibition accumulates during the aging of leaves with reduced DHAR expression.

Young and presenescent DₖD leaves had a substantially lower ETR (p < 0.001, paired t test), whereas ETR in the corresponding D₀X leaves was consistently higher than in the control (p < 0.05, paired t test) (Fig. 2C and D). The reduction state of QA in 2-week-old DₖD leaves was substantially higher than that in the control, whereas no significant difference was observed between D₀X and control leaves (Fig. 2E). In 8-week-old leaves, the QA pool was more reduced in all lines, but it was even more reduced in DₖD leaves than in the control (Fig. 2F). These data indicate a reduction in electron transport during leaf aging and that reducing DHAR expression results in a consistent increase in the proportion of closed PSII centers and reduced PSII efficiency.

Measurements of NPQ at moderate to high PFD revealed a significant reduction in 2-week-old DₖD leaves relative to the control (p < 0.005, paired t test) (Fig. 2G), suggesting that reducing DHAR expression reduces feedback de-excitation under saturating light conditions. In 8-week-old leaves, NPQ reached a higher level than the control in DₖD leaves at light
DHAR Affects Non-photochemical Quenching

The Asc Pool Size and Redox State of the Chloroplast Are Affected by Changes in DHAR Expression—Changes in DHAR expression alter the foliar Asc pool size and redox state (25–28). The change in photosynthetic function in D_{KD} leaves suggested that suppression of DHAR expression may have affected the Asc pool size and redox state specifically in the chloroplasts of D_{KD} leaves. Therefore, the level of Asc and DHA was measured in the chloroplast fraction of D_{KD} and D_{OX} leaves to determine whether the chloroplast Asc pool size and redox state were altered as a consequence of changes in DHAR expression. In the chloroplast fraction of D_{KD} leaves, the Asc pool size was smaller and the redox state was lower than in wild-type (WT) leaves (Table 1). In contrast, the Asc pool size was larger and the redox state was higher in the chloroplast fraction of D_{OX} leaves than in WT leaves (Table 1). These changes in the Asc pool size and redox state in D_{KD} or D_{OX} leaves correlate with the observed changes in photosynthetic function. The Asc redox state in the chloroplast was more reduced than that of the leaf as a whole, which includes the apoplast (Ref. 28 and see Fig. 7 below), suggesting the importance of maintaining Asc in a reduced state.

The last step in Asc biosynthesis occurs in mitochondria from which it is transported throughout the cell (36–39). Although all members of the gene family encoding DHAR in tobacco have not been identified, DHAR is encoded by a highly conserved, five-member nuclear gene family in Arabidopsis, which includes members targeted to the cytosol and chloroplast (40). DHAR in the chloroplast serves to recycle Asc once it has been oxidized, e.g. during periods of high photosynthetic activity such as high light stress. Because Asc is transported throughout the cell, the observed changes in the chloroplast Asc pool size and redox state of D_{KD} or D_{OX} leaves may have resulted from changes in cytosolic DHAR activity, which would alter Asc levels throughout a cell, or from changes in the level of DHAR present in the chloroplast. Although the expression of

[FIGURE 2. Changes in DHAR expression alter photosynthetic performance. ØPSII (A and B), ETR (C and D), reduction state of Q_{A} (1 - qP) (E and F), and NPQ (G and H) in 2- and 8-week-old leaves, respectively, were measured as a function of light intensity (PFD) of control tobacco (C) ( ), tobacco overexpressing DHAR (D_{OX}) ( ), and DHAR-suppressed tobacco (D_{KD}) ( ) simultaneously with the gas exchange data collected in Fig. 1. ØPSII was calculated as IF_{m} - F_{s}/F_{m} in the absence of actinic light and as IF_{m} - F_{s}/F_{m} in the presence of actinic light. ETR was calculated as ØPSII × PFD × α × 0.5 where α is the fraction of incident light absorbed by the leaf, and 0.9 was used in this experiment. qP was assigned a theoretical value of 1 when the actinic light approaches 0, resulting in a 1 - qP of 0. Four to six replicate plants were assayed for each line, and the data were averaged.

intensities below 200 μmol m^{-2} s^{-1} but reached a markedly lower maximum level at high PFD. This resulted in a unique NPQ curve as a function of light intensity (Fig. 2H), suggesting that a reduction in DHAR expression reduces the maximum capacity of a leaf to induce NPQ.
DHAR Affects Non-photochemical Quenching

TABLE 1
Altering DHAR expression affects the chloroplast Asc pool size and redox state

|          | Asc | DHA | Asc/DHA |
|----------|-----|-----|---------|
| Control  | 520 ± 47 | 88.8 ± 6.9 | 5.86 |
| D_ox     | 730 ± 41   | 107 ± 5.3  | 6.82 |
| D_KD     | 395 ± 40   | 823 ± 6.9  | 4.80 |

the cytosolic wheat DHAR (i.e. TaDHAR) in D_ox leaves would not be expected to result in changes in the level of DHAR in the chloroplast, the suppression of DHAR achieved using a tobacco DHAR gene encoding a cytosolic form of tobacco DHAR (i.e. NtDHAR) to induce RNA silencing might be expected to suppress the expression of chloroplast-targeted as well as cytosolic DHAR proteins. To investigate this, the level of NtDHAR present in the chloroplast of D_KD or D_ox leaves was measured using Western analysis. A substantial reduction in the level of NtDHAR present in the chloroplast and cytosolic fractions of 2- and 8-week-old D_KD leaves was observed relative to the WT (Fig. 3A). Little decrease in the level of LHCP or chloroplast-localized HSP70 (cHSP70), which were used as loading controls, was detected in 2-week-old leaves (Fig. 3A). In 8-week-old leaves, the cHSP70 loading control suggested an even greater reduction in the level of NtDHAR present in D_KD chloroplasts (Fig. 3A). Little change in the level of NtDHAR was observed in the chloroplast fraction of 2- or 8-week-old D_ox leaves, and the cytosolic TaDHAR expressed in the cytosolic fraction of D_ox leaves, which is distinguished from the endogenous NtDHAR by its larger molecular weight, was absent from the chloroplast fraction (Fig. 3A), demonstrating a lack of cytosolic contamination of the chloroplast fraction. These data indicate that the suppression of DHAR expression in D_KD leaves substantially reduces the level of DHAR present in the chloroplast, which may contribute to the smaller Asc pool size and lower redox state observed in Table 1. As the level of NtDHAR present in the chloroplast of D_ox leaves was unchanged, the larger Asc pool size and higher redox state in the chloroplast of these leaves are likely because of the expression of cytosolic TaDHAR, previously shown to increase the Asc pool size and redox state in the cytosol and apoplast (27).

A Reduction in DHAR Expression Results in Increased Photo-inhibition—Because of the effect that reducing DHAR expression had on NPQ in presenescent D_KD leaves, a kinetics analysis of NPQ in

FIGURE 3. Slow NPQ kinetics analysis in tobacco leaves with altered DHAR expression. A, Western analysis of chloroplast-localized DHAR (cDHAR), LHCP, and chHSP70 in the chloroplast fraction and DHAR and eEF1A in the cytosolic fraction of 2- and 8-week old leaves of control tobacco (C), tobacco overexpressing DHAR (D_ox), and DHAR-suppressed tobacco (D_KD). NPQ (B and C) and the reduction state of Q_o (D and E) in 2- and 8-week-old leaves, respectively, were measured every 2.5 min following exposure to actinic light (1500 μmol of photon m⁻² s⁻¹) at time 0 of control tobacco (○), tobacco overexpressing DHAR (●), and DHAR-suppressed tobacco (▲). qP and NPQ were assigned a theoretical value of 0 as dark-adapted leaves have no measurable photochemical or non-photochemical quenching in the absence of light. Consequently 1 – qP at time 0 is 1.
dark-adapted leaves exposed to a saturating level of light (i.e. 1500 μmol m⁻² s⁻¹) was performed. Initially NPQ increased more rapidly in 8-week-old DKD leaves than in the control, but it reached a substantially lower steady state level (p < 0.05, paired t test) (Fig. 3C) similar to the induction of NPQ as a function of light intensity (Fig. 2H). The induction of NPQ was also lower in 2-week-old DKD leaves relative to the control but to a smaller extent (Fig. 3B). Rapid kinetics analysis confirmed the faster initial induction of NPQ in 8-week-old DKD leaves (Fig. 4B). The more rapid initial development of NPQ in 8-week-old DKD leaves suggests either a slower relaxation of NPQ during dark adaptation and/or an enhanced initial ability to induce NPQ following exposure to light, whereas the lower maximum NPQ level achieved suggests a limited capacity to fully induce NPQ. The QA pool remained more oxidized in presenescent D_OX leaves and more reduced in D_KD leaves than in the control (Fig. 3E) as it did in young leaves of the same lines (Fig. 3D), supporting the notion that photochemical efficiency was also affected by a reduction in DHAR expression.

PsbS is essential for feedback de-excitation, and the level of NPQ achieved is proportional to its level of expression (41). To investigate whether changes in NPQ in D_KD leaves correlated with PsbS expression, its level was determined. The level of PsbS in 2-week-old leaves of the three lines was similar to the levels in 8-week-old control and D_OX leaves (Fig. 4C). In contrast, the level of PsbS was elevated in presenescent D_KD leaves, correlating with the faster initial induction of NPQ in these leaves. The level of LHCP in presenescent D_KD leaves relative to the WT was unchanged, demonstrating the increase in PsbS relative to this control protein in D_KD leaves (Fig. 4C).

To determine whether the effect that changes in DHAR expression had on NPQ was because of changes in qE or qI, fast and slow relaxation analysis was performed. 8-week-old D_KD leaves exhibited a significant increase in NPQ_f (p < 0.05, n = 4) and a significant decrease in NPQ_s (p < 0.05, n = 4) relative to the control (Table 2). These data indicate that photoinhibition occurred in D_KD leaves during the period of light adaptation because a smaller fraction of total NPQ relaxed as NPQ_f, whereas a larger fraction relaxed as NPQ_s. Moreover, the significantly higher F_o value in D_KD leaves relative to the control (p < 0.005, n = 4) (Table 2) correlated with a significant reduction in the maximum quantum efficiency of PSII (Fig. 2B), suggesting that reducing DHAR expression increased damage to antenna chlorophyll. The chlorophyll pool size disproportionately decreased in D_KD leaves by 8 weeks of age and was significantly smaller than that in the control (p < 0.005, n = 3) as was the chlorophyll a/b ratio (p < 0.05, n = 3) (Table 3), suggesting that chlorophyll a was preferentially lost in D_KD leaves during aging. The disproportionate decrease in qE as measured by NPQ, and increase in qI as measured by NPQ, in 8-week-old D_KD leaves suggests an accumulation of damage during aging.

As photoinhibition can result from ROS, the level of H₂O₂, a measure of ROS, was determined in D_KD leaves. The level of H₂O₂ was significantly higher in young D_KD leaves than in control or D_OX leaves (p < 0.05 and p < 0.005, respectively; n = 3) (Table 3). An even greater increase in H₂O₂ was observed in presenescent D_KD leaves relative to the control (p < 0.005, n = 3).

![DHAR Affects Non-photochemical Quenching](image)

**FIGURE 4. Fast NPQ kinetics analysis in tobacco leaves with altered DHAR expression.** A and B, NPQ was determined in 2- and 8-week old leaves, respectively, every 10 s following exposure of dark-adapted leaves to actinic light of 1500 μmol m⁻² s⁻¹ at time 0 of control tobacco (C) (●), tobacco overexpressing DHAR (D_OX) (●), and DHAR-suppressed tobacco (D_KD) (●). Each point represents the average and S.D. of four replicates. C, Western analysis of PsbS expression (top panel) and LHCP (bottom panel) in 2- and 8-week old leaves. Samples were loaded on an equal fresh weight basis. Error bars represent S.D.
leaves to light did not result in an increase in the zeaxanthin pool size, resulting in a zeaxanthin pool size in young, light-adapted D_{KD} leaves that was only 89% of that in control leaves (Fig. 5). This indicates that D_{KD} leaves are unable to generate the same amount of zeaxanthin as control leaves in response to light and correlates with the lower level of induction of NPQ in D_{KD} leaves in response to high PFD (Fig. 2G). In contrast, the level of antheraxanthin increased to a level similar to wild-type levels (Fig. 5). With the exception of an 11% increase in the level of lutein, the amount of the remaining pigments in young, light-adapted D_{KD} leaves was little changed relative to control leaves (supplemental Fig. 3).

In contrast to D_{KD} leaves, the pool size of violaxanthin, antheraxanthin, and zeaxanthin was substantially larger in 2-week-old, dark-adapted D_{OX} leaves than in the control (Fig. 5). Following exposure to light, young D_{OX} leaves contained a similar level of antheraxanthin and zeaxanthin as in control leaves (101 and 99%, respectively) despite a 29% larger violaxanthin pool size, indicating that the level of DHAR expression in WT leaves is sufficient for optimal conversion of violaxanthin to antheraxanthin and zeaxanthin.

A decrease in most of the xanthophyll pigments was observed during leaf aging (Fig. 5 and supplemental Fig. 3) consistent with a reduced ability to induce NPQ (supplemental Fig. 2B and supplemental Table 1). The pool size of these pigments was larger in 8-week-old, dark-adapted D_{OX} leaves than in the WT by 19–61% (Fig. 5 and supplemental Fig. 3). Following exposure to light, D_{OX} leaves contained a substantially larger antheraxanthin and zeaxanthin pool size (154 and 140%, respectively) than in the WT, representing increases that were disproportionately greater than the increase in the violaxanthin pool size (111% of the control) (Fig. 5). This suggests that the wild-type level of DHAR expression may become limiting with leaf age and that increasing DHAR expression maintains a higher level of xanthophyll cycle pigments during leaf aging, correlating with a slower, aging-related loss in qE.

In 8-week-old, dark-adapted D_{KD} leaves, the pool size of most of the pigments declined 22–45% relative to the WT with the exception of antheraxanthin and zeaxanthin as in control leaves (101 and 99%, respectively) despite a 29% larger violaxanthin pool size, indicating that the level of DHAR expression in WT leaves is sufficient for optimal conversion of violaxanthin to antheraxanthin and zeaxanthin.

### TABLE 2
Reducing the efficiency of Asc recycling results in greater photoinhibition

| Leaf age | Control | D_{OX} | D_{KD} |
|----------|---------|--------|--------|
| 2 weeks  |         |        |        |
| Chl a     | 139.7 ± 3.9 | 137.7 ± 4.2 | 176.3 ± 20.4 |
| Chl b     | 697.3 ± 11.3 | 692.4 ± 14.8 | 674.1 ± 29.6 |
| 8 weeks   |         |        |        |
| Chl a     | 171.9 ± 3.4 | 173.1 ± 7.1 | 297.8 ± 32.7 |
| Chl b     | 700.7 ± 34.5 | 754.3 ± 21.7 | 768.3 ± 32.7 |

| Leaf age | Control | D_{OX} | D_{KD} |
|----------|---------|--------|--------|
| 2 weeks  |         |        |        |
| Chl a/b ratio | 0.800 ± 0.006 | 0.801 ± 0.004 | 0.736 ± 0.047 |
| Chl b     | 2.201 ± 0.189 | 2.106 ± 0.254 | 2.040 ± 0.149 |
| 8 weeks   |         |        |        |
| Chl a/b ratio | 0.754 ± 0.014 | 0.772 ± 0.010 | 0.613 ± 0.075 |
| Chl b     | 1.354 ± 0.153 | 1.499 ± 0.119 | 0.877 ± 0.073 |

Values were determined from four replicates. The average and S.D. are reported.

### TABLE 3
Changes in the efficiency of Asc recycling affects the pool size of chlorophyll and ROS

FW, fresh weight.

| Leaf age | Control | D_{OX} | D_{KD} |
|----------|---------|--------|--------|
| 2 weeks  |         |        |        |
| Chl a     | 41.86   | 42.38  | 38.71  |
| Chl b     | 0.73    | 0.87   | 0.92   |
| H_{2}O_{2} | 4.05 ± 0.26 | 4.24 ± 0.36 | 4.02 ± 0.31 |
| 8 weeks   |         |        |        |
| Chl a     | 27.18   | 31.09  | 11.42  |
| Chl b     | 0.32    | 0.37   | 0.73   |
| H_{2}O_{2} | 3.25 ± 0.24 | 3.47 ± 0.32 | 2.55 ± 0.17 |

Values were determined from four replicates. The average and S.D. are reported.

Five to six leaves from individual plants of each line were pooled for each replicate, and triplicate replicates were measured. The average and S.D. are reported.
DHAR Is Required for Recovery from High Light Stress—Because the induction characteristics of NPQ were affected by changes in DHAR expression, we examined whether the degree of photoinhibition following high light stress was similarly affected. The degree of photoinhibition was measured by the maximum quantum yield (\( F_v/F_m \)) that serves as a measure of stress-induced damage to PSII reaction centers. 2-week-old, dark-adapted control leaves exposed to high light for 5 h exhibited a substantially greater degree of photoinhibition than the WT (Fig. 6B). As expected, the de-epoxidation status of the xanthophyll cycle pigments (i.e., \( A/(V + A + Z) \)) was low before exposure to the high light and increased substantially following exposure to the high light only to decline again during the subsequent recovery period that occurred in the dark. Only small differences in the de-epoxidation status among the three lines were observed (Fig. 8B). A transient decrease in the pool size of lutein and neoxanthin quickly recovered in control or \( D_{OX} \) leaves, its rate of recovery was substantially slower in \( D_{KD} \) leaves. Exposure to high light stress did not substantially change the level of \( \alpha \)-carotene in control or \( D_{OX} \) leaves, but its level declined in \( D_{KD} \) leaves substantially for the 1st h following exposure to the high light stress, and its

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FIGURE 5. Xanthophyll composition in plants with altered DHAR expression. Violaxanthin (white), antheraxanthin (gray), and zeaxanthin (black) were analyzed in control tobacco (C), tobacco overexpressing DHAR (\( D_{OX} \)), and DHAR-suppressed tobacco (\( D_{KD} \)) in 2- and 8-week old leaves. FW, fresh weight.

FIGURE 6. DHAR expression affects tolerance to high light stress. Dark-adapted 2-week-old (A) and 8-week-old (B) leaves from control tobacco (C) ( ), tobacco overexpressing DHAR (\( D_{OX} \)) ( ), and DHAR-suppressed tobacco (\( D_{KD} \)) ( ) plants were exposed to 2000 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) for 5 and 3 h, respectively, and allowed to recover at low light (1 \( \mu \)mol m\(^{-2}\) s\(^{-1}\)). Tolerance to high light and recovery from the stress were measured by the maximum quantum yield (i.e., \( F_v/F_m \)).
level was slow to recover to the pre-stress amount (Fig. 8E). Exposure to high light did not substantially change the level of β-carotene in the three lines initially, but an increase in the level of β-carotene was observed by 24 h of recovery in control or DOX leaves (Fig. 8F). No similar increase in the level of β-carotene was observed in DKD leaves (Fig. 8F).

As with 2-week-old leaves, the de-epoxidation status of the xanthophyll cycle pigments in 8-week-old leaves was low before exposure to the high light, increased following high light exposure, and declined again during the subsequent recovery period (Fig. 8H). The level of lutein, neoxanthin, and α-carotene declined in all three lines following exposure to the high light, and in each case, recovery of the pigment pool size was most rapid in DOX leaves (Fig. 8I, J, and K, respectively). The level of β-carotene in control or DOX leaves recovered by 24 h following the stress to a level that was greater than that before exposure to the stress, whereas its recovery in DKD leaves was substantially lower (Fig. 8L).

These results indicate that maintenance of the xanthophyll cycle and related pigments following exposure to a high light stress is affected, directly or indirectly, by the level of DHAR expression.

DHAR Expression Affects ROS Production during High Light Stress—To determine whether ROS production correlated with the changes in photoinhibition observed in leaves exposed to high light (Fig. 6), the level of H2O2 was measured in 2- and 8-week-old leaves that had been subjected to high light exposure. Prior to the high light stress, the level of H2O2 was measured in 2- and 8-week-old leaves that had been subjected to high light stress. Prior to the high light stress, the level of H2O2 was similar in 2-week-old leaves of all lines (Fig. 9A). As expected, the level of H2O2 increased in the three lines immediately following exposure to the high light but was lowest in DOX leaves and highest in DKD leaves (Fig. 9B). Following 24 h of recovery, the level of H2O2 had recovered to pre-stress levels (Fig. 9C).
Prior to the high light stress, the level of H$_2$O$_2$ in 8-week-old D$_{KD}$ leaves was significantly higher than in the control (p < 0.01), whereas the level in D$_{OX}$ leaves was similar to that in the control. The level of H$_2$O$_2$ in 8-week-old D$_{KD}$ leaves further increased following exposure to high light, whereas much smaller increases were observed in 8-week-old D$_{OX}$ leaves and WT leaves (Fig. 9B). Following 24 h of recovery, the level of H$_2$O$_2$ remained significantly higher in D$_{KD}$ leaves than in the control (p < 0.05) (Fig. 9C). These results indicate that the level of ROS correlates with the level of photoinhibition following exposure to high light stress, and both inversely correlate with the level of DHAR expression. Moreover, the data indicate that the ability to detoxify ROS following exposure to high light decreases with age in D$_{KD}$ leaves.

Appropriate Induction of NPQ Can Be Restored in D$_{KD}$ Leaves following Direct Asc Feeding—To examine whether the reduction in the Asc pool size was responsible for the defect in NPQ induction in D$_{KD}$ plants, D$_{KD}$ leaves were supplied with 10 mM Asc for 2 h in the dark. As a negative control, D$_{KD}$ leaves were supplied with water for the same period of time. WT leaves were also supplied with 10 mM Asc or water for the same period of time. Following dark adaptation, the induction of NPQ in the treated leaves was measured following exposure to 1500 μmol m$^{-2}$ s$^{-1}$. 2-week-old leaves were used for the analysis to avoid any secondary effects, such as damage, that may have accumulated in aging D$_{KD}$ leaves and that might complicate the analysis. In the absence of exogenous Asc, the induction of NPQ was impaired in D$_{KD}$ leaves relative to WT leaves (Fig. 10), data in good agreement with those in Fig. 3B. Following feeding with Asc, the induction of NPQ in D$_{KD}$ leaves increased substantially to a level that was similar to that in the WT (Fig. 10). Asc treatment did not substantially alter the induction of NPQ in WT leaves. These results demonstrate that the defect in NPQ induction in D$_{KD}$ leaves can be complemented by exogenous Asc, suggesting that it is the decrease in the Asc pool size that is responsible for the impaired induction of NPQ in D$_{KD}$ leaves.

**DISCUSSION**

In this study, we observed that the level of DHAR expression affects the induction of NPQ and the degree of photoinhibition experienced by a plant. Suppressing DHAR expression had a greater effect on feedback de-excitation, photosynthetic activity, and photoinhibition at high light intensities, indicating that DHAR activity is important under conditions of light saturation. The effect that DHAR had on these processes was accompanied by changes in the Asc pool size and redox state of the chloroplast, the pool size of chlorophyll and xanthophyll pigments, ROS, stomatal conductance, and, in presenescent leaves, Rubisco content. The reduction in photosynthetic activity in D$_{KD}$ leaves was not because of a reduction in stomatal conductance as the substomatal CO$_2$ concentration was higher than that in WT leaves. Moreover, although the levels of Rubisco and chlorophyll declined at an accelerated rate in D$_{KD}$ leaves, neither was significantly different from the levels in WT for at least the first 2 weeks of leaf growth (Table 3) (28), and therefore, the reduced photosynthetic activity observed in young D$_{KD}$ leaves could not be explained by changes in Rubisco content or chlorophyll pool size.

NPQ is induced in response to the exposure and absorption of light that exceeds the capacity of photochemistry to utilize the excitation energy (35). NPQ functions as a sensor of the reduction state of the photosystems to prevent the entry of excess light energy into the photosystems where it would result in their over-reduction and the generation of ROS (46, 47). Given the competitive nature of NPQ and qP, a reduction in photosynthetic activity would be expected to result in a greater induction of NPQ to dissipate a larger fraction of the excitation energy. Despite the reduced level of qP in D$_{KD}$ leaves, the induction of NPQ was impaired in response to high PFD and failed to reach the level observed in wild-type leaves (Fig. 2G), suggesting a reduced capacity to induce feedback de-excitation. The impaired induction of NPQ, particularly under conditions of saturating light, did correlate with an increase in ROS (Table 3) and photoinhibition (Fig. 6 and Table 2) in D$_{KD}$ leaves, which, in turn, correlated with a reduction in the maximum quantum yield of PSII, a measure of damage to the photosynthetic machinery (Fig. 2B). The reduced tolerance to high light stress that correlated with the impaired induction of NPQ could not be explained by differences in the maximum quantum efficiency of PSII prior to the onset of the stress particularly in younger leaves (Fig. 6). The failure to induce NPQ to a level that would compensate for the reduced level of photochemistry, however, suggests an impaired response that resulted from the reduction in Asc that accompanied the suppression of DHAR. The observation that the impaired induction of NPQ in D$_{KD}$ leaves could be complemented by exogenous Asc (Fig. 10) supports the notion that the reduced level of Asc in D$_{KD}$ leaves is responsible for the impaired induction of NPQ.

To what extent the impairment in NPQ contributes to the photoinhibition observed in D$_{KD}$ leaves is unknown. Mutants affecting components of NPQ in tobacco, such as zeaxanthin or PsbS, would be necessary to show the extent to which impaired induction of NPQ is responsible for photoinhibition following exposure to high light. Given the multiple roles that Asc plays in photosynthesis, it is likely that changes in the Asc level may affect more than one process. The effect that the reduction in the Asc pool size and redox state in D$_{KD}$ leaves had on these...
processes is similar in many respects to those observed in the vtc2 mutant in which Asc biosynthesis is substantially reduced (8). D_{kD} tobacco (28) and vtc2 Arabidopsis, which contains 10–25% of the Asc level present in wild-type Arabidopsis (47, 48), grow more slowly and accumulate less biomass than the wild type (8). Like D_{kD} plants (26–28), the vtc2 mutant exhibits a lower Asc redox state in addition to smaller Asc pool size particularly when grown under high light (8, 21). The reduced ϕPSII efficiency observed in D_{kD} tobacco (Fig. 2) was also observed in vtc2 plants (8). The light-response curve for NPQ in the vtc2 mutant exhibits the same defects as that in young D_{kD} leaves (Fig. 2G) in that induction of NPQ is normal at low PFD but impaired at higher PFD when light becomes saturating (47). The vtc2 mutant is also deficient in qE, particularly during high light stress (17, 21, 49) as is D_{kD} tobacco, that increases as a function of leaf age (Table 2). The deficiency in qE in vtc2 plants is thought to be a result of substrate (i.e. Asc) limitation of the violaxanthin de-epoxidase enzyme responsible for conversion of violaxanthin to antheraxanthin and zeaxanthin (47). Moreover like D_{kD} tobacco, vtc2 plants exhibit impaired induction of NPQ under saturating light conditions, and both exhibit symptoms of photooxidative stress following exposure to high light as indicated by a lower F_{m’}/F_{m} (Fig. 6 and Refs. 8 and 21, respectively), ϕPSII (Fig. 2 and Ref. 8, respectively), and photosynthetic rate (Fig. 1 and Ref. 8, respectively). A similar decrease in NPQ was observed in vtc1 Arabidopsis at high PFD (20). As observed in D_{kD} leaves (Fig. 10), Asc feeding to vtc2 mutant leaves restored full induction of NPQ (47). vtc2 plants undergo photobleaching and lipid peroxidation when transferred from low to high light (8, 21) similar to the increase in ROS (Fig. 9) and reduction in F_{m’}/F_{m} (Fig. 6) in D_{kD} tobacco, indicating a reduced ability to detoxify ROS produced during high light stress thus resulting in a substantial degree of photo inhibition. Similarly D_{kD} tobacco and vtc1 and vtc2 mutants exhibit greater sensitivity to ozone (18, 27), supporting the notion of a limited ability to detoxify ROS. vtc2 plants grown under high light had a lower chlorophyll a/b ratio similar to that in D_{kD} tobacco even when the latter was grown at moderate

FIGURE 8. DHAR expression affects xanthophyll pigment composition in plants exposed to high light stress. Xanthophyll cycle and related pigments were analyzed in control tobacco (C) (○), tobacco overexpressing DHAR (D_{ox}) (■), and DHAR-suppressed tobacco (D_{kd}) (▲) in 2- and 8-week-old leaves exposed to 2000 μmol m^{-2} s^{-1} for 5 and 3 h, respectively, and allowed to recover at low light (1 μmol m^{-2} s^{-1}).
in the vtc1 mutant is higher than in the vtc2 mutant, accumulating to ~30% of the wild-type level (18–20). Although it is hypersensitive to ozone and exhibits a slower growth rate under normal growth conditions (18–20), little change was observed in the CO2 assimilation or chlorophyll fluorescence of the vtc1 mutant, suggesting that photosynthetic capacity or photochemical efficiency is unaffected in this mutant under normal growth conditions. The vtc1 mutant did not exhibit symptoms of photooxidative stress (20), whereas the vtc2 mutant and DKD tobacco did. The more severe nature of the phenotypes observed in the vtc2 mutant suggests that a greater reduction in Asc than that present in the vtc1 mutant is required before photosynthetic activity, photosynthetic efficiency, and the level of photooxidative stress are affected in Arabidopsis at least under normal growth conditions. Although the level of Asc is not reduced in DKD tobacco to the same extent as in the vtc mutants, the Asc redox state is affected to a greater extent by suppression of DHAR than it is by a reduction in Asc biosynthesis (8), which may account for the effect on photosynthetic processes when demand for Asc is high. It is also possible that photosynthetic processes in tobacco are more sensitive to changes in the level of Asc than they are in Arabidopsis. In fact, the level of foliar Asc in Arabidopsis is substantially higher (4.29 µmol/g fresh weight) than in tobacco (0.96 µmol/g fresh weight) when both are grown under moderate light, supporting the notion that a greater reduction in the level of Asc in Arabi-
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dopsis may be necessary to perturb photosynthetic processes than in tobacco. In fact, taking into account the respective decreases in Asc in $D_{KD}$ and $vtc$ mutant leaves, the level of Asc in $D_{KD}$ leaves would be lower than that in $vtc$ mutant leaves despite the greater percent decrease in the Asc pool size in the $vtc$ mutants relative to the WT. The impaired induction of NPQ in $D_{KD}$ and $vtc$ plants suggests that a reduction in cellular Asc, whether through reduced biosynthesis or recycling, affects this protective process and results in increased photooxidative stress.

Although the npq1 mutant in Arabidopsis, which lacks zeaxanthin, exhibits increased sensitivity to photoinhibition, the npq1/$vtc$ double mutant shows significantly less tolerance to high light than does either mutant alone, demonstrating the synergy between NPQ and Asc in providing protection against photoinhibition (21, 50). The relatively high level of Asc in Arabidopsis compared with tobacco may partially compensate for the loss of qE in the npq1 mutant and thus mask the full effect of high light stress in this species until the level of Asc is substantially reduced, such as in the $vtc$2 mutant.

The failure to induce NPQ fully in response to saturating light suggested possible alterations to the xanthophyll cycle. An abnormally large pool size of zeaxanthin was observed in young, dark-adapted $D_{KD}$ leaves, but a lower pool size was observed in light-adapted leaves relative to control leaves. As zeaxanthin is required for the induction of NPQ, its changes may have contributed to the phenotypes observed in $D_{KD}$ leaves. As zeaxanthin can also function as an antioxidant (51) and functions synergistically with Asc to provide photoprotection (21), the reduction in the zeaxanthin pool size in light-adapted $D_{KD}$ leaves, in conjunction with the reduced Asc pool size, may have contributed to the increase in photooxidative stress observed in these plants. Additional perturbations to other pigments such as lutein and neoxanthin, which are known to be involved in photoprotection (44, 45), may also have contributed to the alterations in NPQ and photooxidative stress.

The observation that a reduction in DHAR expression can affect induction of NPQ was supported by the observation that an increase in DHAR expression had the opposite effect. A larger xanthophyll pigment pool was present in $D_{OX}$ leaves (Fig. 5) as was a reduced oxidative load (Table 3) and a higher tolerance to high light stress (Fig. 6). Although suppression of DHAR in $D_{KD}$ leaves included suppression of DHAR present in the chloroplast, increasing DHAR in $D_{OX}$ leaves was limited to the overexpression of cytosolic DHAR. Following the last step of its biosynthesis in the mitochondrion (36, 39), Asc is transported throughout the cell by means of specific Asc and DHA transporters, and Asc entry into the chloroplast occurs through facilitated diffusion (37, 38). The increase in the Asc pool size and redox state in $D_{OX}$ chloroplasts demonstrated that changes in cytosolic DHAR can affect Asc levels in the chloroplast and is similar to the effect such changes have on apoplastic Asc (27). A reduction in the apoplastic Asc level was also observed in the $vtc1$ mutant (20), further evidence that changes to the level of cytosolic Asc affects the Asc pool size in other cellular compartments.

By the presenescent stage, $D_{KD}$ leaves have a markedly reduced capacity to induce NPQ in response to high light (Fig. 2H). The reduced level of Rubisco observed in presenescent $D_{KD}$ leaves (28) may have contributed to the lower efficiency of photochemical quenching and the greater reduced state of QA. However, such conditions would be expected to induce NPQ. The failure of NPQ to be induced to wild-type levels suggests the notion that the reduction in DHAR expression resulted in an impaired response to high light. qE and qI both contribute to the level of NPQ that is induced during exposure to light. The impaired induction of NPQ in presenescent $D_{KD}$ leaves was largely a result of a reduction in qE as indicated by a lower NPQ$_r$. A significant increase in qI, as indicated by an increase in NPQ$_I$, suggested that photoinhibition had occurred during leaf aging. This conclusion is consistent with the observed reduction in $F_v/F_m$, indicating irreversible damage to PSII centers; higher $F_v$ values suggesting changes in the configuration of the antenna light-harvesting complex that can destabilize chlorophyll binding and disrupt energy transfer from antenna chlorophylls to the PSII core complex, hypersensitivity to high light stress, and a substantial increase in ROS. Impaired induction of NPQ in presenescent $D_{KD}$ leaves in response to high PFD was largely a consequence of high $F_v$ values, indicating that energy transfer to xanthophyll pigments such as zeaxanthin, which is present at near wild-type levels in light-adapted, presenescent $D_{KD}$ leaves, may be impaired. The higher level of PsbT in presenescent $D_{KD}$ leaves (Fig. 4C) may represent an attempted compensatory response to the impaired induction of qE and increase in qI. These results are supported by the lack of change in NPQ$_I$ and NPQ$_r$ in young $D_{KD}$ leaves, indicating little difference in photoinhibition at this developmental stage, a result consistent with the larger Asc pool size present in young leaves and the accumulation of damage over time (26, 38).

During leaf aging, aspects of the observed phenotype in $D_{KD}$ leaves are similar to those observed in WT leaves of significantly older age, suggesting the possibility that loss of DHAR activity may lead to an acceleration of senescence. Conflicting observations concerning whether senescence is accelerated or delayed have been reported for the $vtc$ mutants (20, 52). However, premature senescence of young $D_{KD}$ leaves does not appear to be occurring as their chlorophyll content (Table 3) and level of Rubisco (28) are not substantially different from those in WT leaves, and yet they exhibit impaired induction of NPQ (Fig. 2G). Moreover the ability of exogenous Asc to restore the appropriate induction of NPQ does not support premature senescence as a cause of the impaired induction of NPQ in young $D_{KD}$ leaves. Other aspects of $D_{KD}$ leaves are also not accounted for by accelerated senescence. For example, the level of LHCP declines during leaf aging but remains unchanged in $D_{KD}$ leaves relative to WT leaves of similar age (Fig. 4C). Therefore, not all aspects of an accelerated senescence program appear to be operative in $D_{KD}$ leaves.

Because of the similar phenotypes exhibited by $D_{KD}$, tobacco and $vtc2$ Arabidopsis, those processes affected by reduced DHAR expression are likely to be the result of changes to Asc, which would be expected to have pleiotropic effects. In addition to those processes examined in this study, a reduction in Asc has been reported to affect hormone signaling, the distribution of enzymes involved in ROS scavenging, and gene expression.
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