Xanthophyll cycle pool size and composition in relation to the nitrogen content of apple leaves

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Abstract

The objective of this study was to determine xanthophyll cycle pool size and composition in response to N status and their relationships to non-photochemical quenching in apple leaves. Bench-grafted Fuji/M.26 trees were fertilized with different N concentrations (0–20 mM) in a modified Hoagland’s solution for 6 weeks to create a wide range of leaf N status (1–4.4 g m⁻²). Chlorophyll content, xanthophyll cycle pool size, lutein, total carotene, and neoxanthin on a leaf area basis all increased linearly with increasing leaf N. However, only the ratios of the xanthophyll cycle pool and of lutein to chlorophyll were higher in low N leaves than in high N leaves. Under high light at midday, both zeaxanthin (Z), expressed on a chlorophyll basis, and the percentage of the xanthophyll cycle pool present as Z, increased as leaf N decreased. Thermal dissipation of excitation energy, measured as non-photochemical quenching of chlorophyll fluorescence, was positively related to, whereas efficiency of excitation transfer and photosystem II quantum efficiency were negatively related to, Z, expressed on a chlorophyll basis or on a xanthophyll cycle pool basis. It is concluded that both xanthophyll cycle pool size (on a chlorophyll basis) and conversion of violaxanthin to zeaxanthin are enhanced in response to N limitation to dissipate excessive absorbed light under high irradiance.

Key words: Apple, chlorophyll, excess absorbed light, leaf nitrogen, Malus domestica, non-photochemical quenching, photosystem II quantum efficiency, thermal dissipation, xanthophyll cycle.

Introduction

When light absorption exceeds light utilization in photosynthetic electron transport, excess absorbed light results. This can arise from exposure to a high photosynthetic photon flux density (PFD), or a low requirement for electron transport under environmental stresses. Leaf nitrogen status affects both the capacity of light absorption by antenna pigments and the capacity of light utilization by photosynthetic electron transport. Both chlorophyll content and total Rubisco activity decrease with decreasing leaf N (Cheng and Fuchigami, 2000; Evans 1989). However, the decrease in light absorption is not proportional to the decrease in chlorophyll content (Cheng et al., 2000). In addition, Rubisco decreases more than thylakoid proteins under limiting N (Evans, 1989). As a result, under high PFD, surplus absorbed light is greater in low N leaves than in high N leaves.

Excess absorbed light can be dissipated as heat in the antenna pigment complexes (Demmig-Adams et al., 1995; Genty et al., 1990). Alternatively, if the amount of excess absorbed light exceeds the capacity for thermal dissipation, overreduction of the primary electron acceptors of photosystem II (PSII) occurs. This will lead to the increased formation of triplet state chlorophyll, which has the potential to form toxic singlet oxygen (Asada, 1996). By contrast, thermal dissipation can safely remove excess excitation energy before it reaches the PSII reaction centres, thereby protecting these reaction centres from photo-oxidative damage. For plants grown under limiting N, thermal dissipation is found to be the main pathway for excess absorbed light. In maize (Khamis et al., 1990), spinach (Verhoeven et al., 1997), and apple leaves (Cheng et al., 2000), thermal dissipation, measured as non-photochemical quenching of chlorophyll fluorescence, increased in response to limiting N. As a result of this
up-regulation of thermal dissipation, the proportion of the absorbed PFD that potentially generated singlet oxygen remained unchanged over a wide range of N status in apple leaves (Cheng et al., 2000). The primary electron acceptor of PSII of maize leaves even became more oxidized in response to limiting N (Khamis et al., 1990).

Thermal dissipation of excess excitation energy is closely related to the xanthophyll cycle activity. The diepoxide violaxanthin (V) is de-epoxidized via the monooepoxide antheraxanthin (A) to the epoxide-free zeaxanthin (Z) in response to excess absorbed light. The level of Z or Z+A was highly correlated to non-photochemical quenching, and this relationship was species-independent (Demmig-Adams and Adams, 1996). In maize (Khamis et al., 1990) and spinach (Verhoeven et al., 1997), plants grown under low N versus high N, had a greater proportion of the xanthophyll cycle as Z and A at midday and a higher content of xanthophyll cycle pigments on a chlorophyll basis in their leaves. However, N supply did not affect the pool size and the de-epoxidation status of the xanthophyll cycle at midday in Clematis vitalba (Bungard et al., 1997). The relationship is not known between xanthophyll cycle activity and thermal dissipation of excitation energy of apple leaves in response to N supply. In addition, leaf N status was not determined in most of the studies mentioned above. Therefore, the objectives of this study were to determine (1) xanthophyll cycle pool size and composition in relation to leaf N status; and (2) the relationship between xanthophyll cycle activity and thermal dissipation of excitation energy.

Materials and methods

Plant material and nitrogen treatments

Bench-grafted ‘Fuji’ apple (Malus domestica Borkh) trees on M.26 rootstocks were grown in 3.8 l pots with a medium of 1 peat moss:2 pumice:1 sandy loam soil (v:v) in a lathhouse from 26 March to 5 June. Beginning from budbreak in early May, they were fertilized every 2 weeks with 10.7 mM N, using Plantex 20K2O water-soluble fertilizer with micronutrients (Plantex Corp., Ontario, Canada). During the shoots were approximately 15 cm long, plants were moved to full sunlight. Thereafter, they were fertilized weekly with Plantex® for 3 weeks. Beginning on 30 June, plants were fertilized twice weekly with one of the seven N concentrations (0, 2.5, 5, 7.5, 10, 15 or 20 mM N from NH4NO3) by applying 300 ml of a modified Hoagland’s solution to each pot (Cheng and Fuchigami, 2000). There were four replications for each N treatment in a completely randomized design. Plants were sub-irrigated with a saucer placed at the bottom of each pot. After 6 weeks, recently fully expanded leaves at similar developmental stages were selected for chlorophyll fluorescence measurements and pigment analysis.

Measurements of chlorophyll fluorescence

Chlorophyll fluorescence was measured with a pulse-modulated fluorometer FMS1 (Hansatech Instruments Ltd., Norfolk, UK) either predawn or at a PFD of 1500 ± 50 μmol m−2 s−1 at noon, under natural conditions. The fibre optic of the FMS2 was positioned using the PFD/temperature leaf clip at a 60° angle from the upper surface of the leaf, and the distance between the fibre optic and the leaf surface was kept constant for both predawn and noon measurements. Maximum fluorescence (Fm) and minimum fluorescence (Fo) of dark-adapted leaves were measured predawn. For the measurements at noon, steady-state fluorescence (Fg) was monitored to ensure it was stable before a reading was taken. Maximum fluorescence (Fm') under natural light exposure was obtained by imposing a 1 s saturating flash of approximately 18 000 μmol m−2 s−1 PFD at the end of the fibre optic in order to reduce all the PSI centres. To determine the minimum fluorescence (Fo') under natural light exposure, a black cloth was used to cover the leaf while a far-red light was switched on to oxidize PSI rapidly by drawing electrons from PSI to PSI.

The maximum PSI efficiency of dark-adapted leaves was calculated as: Fm/Fm'=(Fm-Fo)/Fm' (van Kooten and Snel, 1990). Thermal dissipation was estimated from non-photochemical quenching (NPQ) as: NPQ = Fm'/Fm-1 (Stern-Volmer quenching; Bilger and Björkman, 1990). The efficiency of excitation transfer to open PSI centres under natural light exposure was F'v/F'v'=Fv/Fm'. PSI quantum efficiency was calculated as (Fm'−Fo')/Fm' (Genty et al., 1989).

Analysis of leaf pigments

Immediately before chlorophyll fluorescence measurements, one cm2 leaf disc was punched from the leaf and frozen in liquid nitrogen. Frozen leaf discs were stored at −80 °C until analysis.

To extract pigments, a leaf disc was pulverized in liquid nitrogen in a mortar, followed by grinding in 1.5 ml cold 85% acetone. The extract was centrifuged at 12 000 g for 2 min. The supernatant was passed through a 0.2 μm syringe filter into an amber HPLC vial. The extract was then bubbled with N2 for 10 s before sealing the septum.

Pigments were analysed according to Thayer and Björkman (1990) with minor modifications, using a HP1100 Liquid Chromatograph equipped with a diode array detector (Agilent Technology, Palo Alto, CA, USA). The Agilent Technology non-endcapped Zorbax ODS column (4.5×250 mm, 5 μm particle size) was used in the separation, preceded by a C18 Adsorbosphere guard column (Alttech Associates, Inc., Deerfield, IL USA). The pigments were eluted at a flow rate of 1 ml min−1 at a column temperature of 35 °C using 100% solvent A (acetonitrile:methanol 75:25, v/v) for the first 7 min followed by a 2 min linear gradient to 100% solvent B (methanol ethyl acetate 70:30, v/v) which continued isocratically until 23 min. The column was re-equilibrated in solvent A for 5 min before the next injection.

Leaf N determination

After all the above measurements, leaf area was determined with a Li-Cor LI-3000 leaf area meter. Leaves were frozen in liquid nitrogen and stored at −80 °C until freeze-dried. Leaf N content was determined by the Kjeldahl procedure (Schuman et al., 1973).

Results

Leaf pigments in relation to leaf N

On a leaf area basis, chlorophyll content, xanthophyll cycle pool size (V+A+Z), lutein, neoxanthin, and total carotene content all increased linearly as leaf N increased (Fig. 1). On a chlorophyll basis, however, xanthophyll cycle pool size and lutein content decreased with increasing leaf N (Fig. 2A, B), whereas neoxanthin and total
carotene remained relatively constant across the leaf N range examined (Fig. 2C). No significant difference was found in chlorophyll content, total xanthophyll cycle pool size, lutein, neoxanthin, or total carotene content at any given leaf N level between noon and predawn samples (predawn data not shown).

Under high PFD at noon, leaf Z expressed on a chlorophyll basis decreased whereas leaf V increased with increasing leaf N (Fig. 3A). Approximately 93% of the xanthophyll cycle pool was present as Z in leaves with the lowest N content; this percentage then decreased curvilinearly to about 40% at the highest leaf N (Fig. 3B). Correspondingly, only 1–2% of the xanthophyll cycle pool was in V in leaves with the lowest N; this percentage then increased curvilinearly to about 40% (Fig. 3B).

At predawn, both V and Z on a chlorophyll basis decreased with increasing leaf N (Fig. 4A). Approximately 78% of the xanthophyll cycle pool was in V across the leaf N range, whereas only about 12% in Z, except a slight
increase in %Z at the lowest end of the leaf N range (Fig. 4B).

Chlorophyll fluorescence variables in relation to leaf N at noon

As leaf N increased, non-photochemical quenching (NPQ) of chlorophyll fluorescence decreased rapidly first, then stabilized at a low level (Fig. 5A). Correspondingly, the efficiency of excitation transfer ($F_v/F_m$) increased linearly first, then levelled off once leaf N reached approximately 3 g m$^{-2}$ (Fig. 5B). PSII quantum efficiency showed a similar response to leaf N as $F_v/F_m$ (Fig. 5C). Quenching of $F_o$ was closely correlated with NPQ of $F_m$ in leaves with different N levels under high PFD at noon (Fig. 6).

Relationships between xanthophylls and chlorophyll fluorescence variables at noon

As leaf Z expressed on a chlorophyll basis increased, NPQ increased linearly while both $F_v/F_m$ and PSII quantum efficiency decreased linearly (Fig. 7). When Z was expressed as the percentage of the xanthophyll cycle pool, NPQ increased curvilinearly, whereas both $F_v/F_m$ and PSII quantum efficiency decreased curvilinearly as Z increased (Fig. 8).

NPQ was positively correlated with lutein content on a chlorophyll basis, whereas $F_v/F_m$ and PSII quantum efficiency showed a negative relationship (Fig. 9).

$F_v/F_m$ and its relationship to zeaxanthin levels at predawn

Maximum quantum efficiency ($F_v/F_m$) of dark-adapted leaves at predawn increased rapidly with increasing leaf N to a plateau, then was constant over the remaining leaf N range (Fig. 10A). $F_v/F_m$ was highly correlated with leaf Z expressed on a chlorophyll basis (Fig. 10B), but only loosely correlated with Z expressed as % of the xanthophyll cycle pool (Fig. 10C).

Discussion

The data show that xanthophyll cycle pool size on a chlorophyll basis was higher in low N leaves, whereas both...
xanthophyll cycle pool and total chlorophyll on a leaf area basis increased with increasing leaf N (Figs 1, 2). Similar results were obtained in spinach plants grown under a high PFD in response to limiting N (Verhoeven et al., 1997). Under iron deficiency, both sugar beet and pear leaves also exhibited a significant increase in the ratio of xanthophyll cycle pool size to chlorophyll (Morales et al., 1990, 1994). This rise in the ratio of xanthophyll cycle pool to chlorophyll indicates that, in addition to downsizing light-harvesting chlorophyll antennae, increasing the relative concentration of xanthophylls in the light-harvesting complexes of PSII was employed by low N leaves to cope with high PFDs.

Close correlations have been found between Z or Z+A levels and NPQ in different species under a wide range of environmental conditions (Adams et al., 1994; Demmig-Adams and Adams, 1996). Inhibition of violaxanthin de-epoxidase activity in vivo by dithiothreitol resulted in a decrease in NPQ (Demmig-Adams and Adams, 1992). Recent characterization of the Arabidopsis mutant npq1 (Niyogi et al., 1998) and antisense tobacco plants (Chang et al., 2000; Sun et al., 2001), both of which had little violaxanthin de-epoxidase activity, showed that NPQ was greatly suppressed under high PFD, which confirmed that zeaxanthin is necessary for most NPQ. In this context, the finding that NPQ was highly correlated with Z both on a chlorophyll basis and on a xanthophyll cycle pool basis (Figs 7A, 8A) indicates that the increased thermal dissipation of excitation energy in response to limiting N is dependent on the enhanced xanthophyll cycle activity. This is in agreement with the finding, in spinach leaves, that under limiting N supply a greater proportion of the xanthophyll cycle was present as Z+A at midday (Verhoeven et al., 1997). By contrast, the xanthophyll cycle pool size on a chlorophyll basis and de-epoxidation state did not respond to N supply in clematis plants (Bungard et al., 1997). The exact reason for the difference between the two species in response to limiting N is not known. It appears that the clematis plants were able to maintain a delicate balance between light absorption and photosynthetic capacity in response to limiting N under high PFDs (Bungard et al., 1997).

Violaxanthin de-epoxidase, which catalyses the conversion of V to Z via A, is localized in the thylakoid lumen (Bugos and Yamamoto, 1996) and is activated by low lumen pH upon exposure to high PFDs (Eskling et al., 1997). Low lumen pH also results in protonation of proteins in the PSII light-harvesting complexes. The
interaction of Z and/or A with the specific sites of the protonated PSII proteins induces a conformational change that leads to increased quenching of excitation energy (Gilmore, 1997; Müller et al., 2001). Although no attempt was made to determine the response of trans-thylakoid pH to leaf N in this experiment, the close correlation between Z and NPQ (Fig. 7A) suggests that changes in trans-thylakoid pH may be highly co-ordinated with Z formation in vivo. The disassociation of NPQ with Z levels have been observed only under two situations. First, on the forest floor where intermittent sunflecks occur, Z+A of understory leaves reached a high level upon exposure to the first sunfleck of the day, then remained at the elevated level while NPQ still responded to fluctuations of PFD (Adams et al., 1999; Logan et al., 1997). In this case, NPQ was thought to be more dependent on the change of trans-thylakoid pH gradient to rapidly engage or disengage Z+A in response to fluctuating PFD. Second, mutants lacking the PsbS protein in the PSII complex (Li et al., 2000) or defective in sensing trans-thylakoid pH gradient (Peterson and Havir, 2000) maintained the same xanthophyll cycle pool size and composition, but had reduced NPQ, compared with their wild types.

Fig. 7. Relationships of non-photochemical quenching (A), efficiency of excitation transfer (B), and PSII quantum efficiency (C) to leaf zeaxanthin on a chlorophyll (a+b) basis at noon under an incident PFD of 1500 μmol m⁻² s⁻¹. Correlation equations: for NPQ, y=0.334+0.017x (r²=0.93, P < 0.0001); for Fv'/Fm', y=0.725-0.0017x (r²=0.94, P < 0.0001); for PSII quantum efficiency, y=0.635-0.0018x (r²=0.94, P < 0.0001).

Fig. 8. Relationships of non-photochemical quenching (A), efficiency of excitation transfer (B), and PSII quantum efficiency (C) to leaf zeaxanthin expressed as % of xanthophyll cycle pool at noon under an incident PFD of 1500 μmol m⁻² s⁻¹. Correlation equations: for NPQ, y=2.87-0.072x+0.001x² (r²=0.94, P < 0.0001); for Fv'/Fm', y=0.48+0.069x-0.0001x² (r²=0.94, P < 0.0001); for PSII quantum efficiency, y=0.39+0.0068x-0.0001x² (r²=0.93, P < 0.0001).
It is interesting to note that the relationship of NPQ to Z on a xanthophyll cycle pool basis was curvilinear while that on a chlorophyll basis was linear, with very similar \( r^2 \) values (Figs 7, 8). Similar curvilinear relationships have been found when Z+A were expressed as a percentage of V+A+Z under N stress (Verhoeven et al., 1997) and other environmental stresses (Adams et al., 1994). Although the correlation of NPQ with Z or Z+A on a chlorophyll basis was not as high as that on a total xanthophyll cycle pool basis across a wide range of species (Demmig-Adams and Adams, 1996), it appears that expressing Z on a chlorophyll basis is most appropriate when both xanthophyll cycle pool size on a chlorophyll basis and conversion of V to Z changed in apple leaves in response to limiting N. This is also reflected in the tighter relationship of predawn NPQ to Z on a chlorophyll basis compared to a xanthophyll cycle pool basis (Figs 7, 8).

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**Fig. 9.** Relationships of non-photochemical quenching (A), efficiency of excitation transfer (B), and PSII quantum efficiency (C) to leaf lutein content on a chlorophyll \((a+b)\) basis at noon under an incident PFD of 1500 \(\mu\)mol m\(^{-2}\) s\(^{-1}\). Correlation equations: for NPQ, \(y = -4.28 + 0.037x\) \((r^2 = 0.87, P < 0.0001)\); for \(F_v/F_m'\), \(y = 1.21 - 0.0038x\) \((r^2 = 0.91, P < 0.0001)\); for PSII quantum efficiency, \(y = 1.15 - 0.0041x\) \((r^2 = 0.92, P < 0.0001)\).

**Fig. 10.** Maximum quantum efficiency \((F_v/F_m)\) of dark-adapted leaves at predawn in relation to leaf N (A) and the relationship of \(F_v/F_m\) to leaf zeaxanthin expressed on a chlorophyll \((a+b)\) basis (B) or as % of xanthophyll cycle pool (C) in Fuji/M.26 apple trees. Regression equation for (A) \(y = 0.83(1 - e^{-2.295x}) + 0.017\) \((r^2 = 0.92, P < 0.0001)\); correlation equation for (B) \(y = 0.89 - 0.0026x\) \((r^2 = 0.84, P < 0.0001)\); correlation equation for (C) \(y = 0.74 + 0.018x - 0.0008x^2\) \((r^2 = 0.53, P = 0.001)\).
with sustained low high levels of Z+A were found to be closely correlated overwintering plants under low temperature stress, very pumping via ATPase (Gilmore and Yamamoto, 1992). For gradient was sustained by ATP-dependent reverse proton leaves with low N, it may be that a trans-thylakoid pH thylakoid pH gradient is expected to disappear quickly. In thylakoid pH gradient is required for zeaxanthin to be 1997). The mechanism of this sustained operation of the et al in spinach leaves under limiting N (Verhoeven et al, 1993). This may be because the pigment composition and structure of the light-harvesting complexes differ among species, and the same level of NPQ can be achieved at different xanthophyll to chlorophyll ratios, where expressing Z as % of V+A+Z puts all the species on a relative scale.

The close correlation between Z on a chlorophyll basis and \( F_o/F_m \) in predawn leaves (Fig. 10B) and proportional quenching of \( F_o \) and \( F_m \) under light (Fig. 6) suggest that the slightly lower value of \( F_o/F_m \) in low N leaves might be the result of the sustained xanthophyll cycle-dependent thermal dissipation in these leaves. This is different from photoinhibition that normally increases the \( F_o \) level while decreasing the \( F_m \) level (Gilmore et al., 1996). Lower \( F_o/F_m \) values and sustained zeaxanthin levels, along with proportional quenching of \( F_o \) and \( F_m \), were also observed in spinach leaves under limiting N (Verhoeven et al., 1997). The mechanism of this sustained operation of the xanthophyll cycle in the dark remains unclear. A trans-thylakoid pH gradient is required for zeaxanthin to be engaged in thermal dissipation. Upon darkening, a trans-thylakoid pH gradient is expected to disappear quickly. In leaves with low N, it may be that a trans-thylakoid pH gradient was sustained by ATP-dependent reverse proton pumping via ATPase (Gilmore and Yamamoto, 1992). For overwintering plants under low temperature stress, very high levels of Z+A were found to be closely correlated with sustained low \( F_o/F_m \) at predawn (Adams et al., 1994). Recent work suggested that dark-sustained thylakoid protein phosphorylation may keep PSII in a state primed for thermal dissipation in light-stressed leaves (Adams et al., 2001; Ebbert et al., 2001). Although this possibility can not be excluded for low N leaves, they do have a much lower level of zeaxanthin present at predawn compared with overwintering plants.

In addition to xanthophyll cycle pool size (Fig. 2A), lutein is the only other xanthophyll that decreased substantially with increasing leaf N when expressed on a chlorophyll basis (Fig. 2B). Possibly the relationship of NPQ, \( F_o/F_m \), and PSII quantum yield with lutein (Fig. 9) is fortuitous, simply because lutein responded to leaf N in a similar way as Z. Alternatively, the increased ratio of lutein to chlorophyll may suggest a role of lutein in thermal dissipation. The Arabidopsis mutant, npq1, which was unable to convert V to Z, still had residual pH-dependent NPQ (Niyogi et al., 1998), this pH-dependent NPQ was apparently abolished in the double mutant, npq1 lut2 (Niyogi et al., 2001). These results indicated that lutein may play a role in thermal dissipation, but the exact contribution of lutein to thermal dissipation remains to be determined.

In conclusion, both xanthophyll cycle pool size (on a chlorophyll basis) and conversion of V to Z under high light were enhanced in response to limiting N. The increased thermal dissipation of excess absorbed light in low N leaves under high light was closely related to their elevated Z levels.

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**References**


