Both xanthophyll cycle-dependent thermal dissipation and the antioxidant system are up-regulated in grape (Vitis labrusca L. cv. Concord) leaves in response to N limitation

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Abstract

One-year-old grapevines (Vitis labrusca L. cv. Concord) were supplied with 0, 5, 10, 15, or 20 mM nitrogen (N) in a modified Hoagland’s solution twice weekly for 4 weeks. As leaf N decreased in response to N limitation, leaf chlorophyll (Chl) decreased linearly whereas leaf absorptance declined curvilinearly. Compared with high N leaves, low N leaves had lower quantum efficiency of PSII as a result of both an increase in non-photochemical quenching (NPQ) and an increase in closure of PSII reaction centres at midday under high photon flux density (PFD). Both the xanthophyll cycle pool size on a Chl basis and the conversion of violaxanthin (V) to antheraxanthin (A) and zeaxanthin (Z) at noon increased with decreasing leaf N. NPQ was closely related to A+Z expressed either on a Chl basis or as a percentage of the xanthophyll cycle pool. As leaf N increased, superoxide dismutase (SOD) activity on a Chl basis decreased linearly; activities of catalase (CAT) and glutathione reductase (GR) on a Chl basis increased linearly; activities of ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR) expressed on the basis of Chl decreased rapidly first, then gradually reached a low level. In response to N limitation, the contents of ascorbate (AsA), dehydroascorbate (DAsA), reduced glutathione (GSH), and oxidized glutathione (GSSG) increased when expressed on a Chl basis, whereas the ratios of both AsA to DAsA and GSH to GSSG decreased. It is concluded that, in addition to decreasing light absorption by lowering Chl concentration, both xanthophyll cycle-dependent thermal energy dissipation and the antioxidant system are up-regulated to protect low N leaves from photo-oxidative damage under high light.

Key words: Antioxidant, grape, nitrogen, thermal dissipation, xanthophyll cycle.

Introduction

Nitrogen (N) supply affects both light absorption and light utilization of leaves. Leaf chlorophyll (Chl) concentration decreases in response to N limitation, leading to a decrease in leaf light absorption. However, the decrease in light absorption is not proportional to the decrease in Chl (Cheng et al., 2000). Under high photon flux density (PFD), low-N leaves use only a small fraction of the absorbed PFD in photosynthetic carbon reduction and photosynthetic carbon oxidation because of their low activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39, Rubisco) (Cheng and Fuchigami, 2000). As a result, the amount of excess absorbed PFD is greater in low-N leaves than in high-N leaves under high light. The excess absorbed PFD can potentially lead to the production of singlet oxygen ($^1$O$_2$) and reduced reactive oxygen species, causing damage to photosynthetic apparatus and cell structure. Plants have evolved many photoprotective mechanisms to minimize photo-oxidative damage (Niyogi, 1999, 2000), including (1) xanthophyll cycle-dependent thermal energy dissipation and quenching of $^1$O$_2$, and (2) detoxification of reduced reactive oxygen species generated by photoreduction of oxygen. Excess absorbed light can be dissipated as heat in the antenna pigment complexes of PSII, which involves a xanthophyll cycle and a low lumen pH (Demmig-Adams and Adams, 1996; Niyogi et al., 1998). Thermal
dissipation can safely remove excess excitation energy before it reaches the PSII reaction centres, thereby protecting the reaction centres from photo-oxidative damage (Demmig-Adams and Adams, 1996, 2000). Although N supply did not seem to affect the pool size or the conversion of xanthophyll cycle at midday in Clematis vitalba leaves (Bungard et al., 1997), xanthophyll cycle-dependent thermal dissipation was enhanced in leaves of maize (Khamis et al., 1990), spinach (Verhoeven et al., 1997), and apple (Cheng, 2003) under N limitation. The xanthophyll cycle also protects thylakoid membrane from photo-oxidation by quenching \( ^1 \text{O}_2 \) (Baroli et al., 2003; Havaux and Niyogi, 1999).

The detoxification of reactive oxygen species generated by photoreduction of oxygen is undertaken by an integrated system of enzymatic and non-enzymatic antioxidants that are concentrated in the chloroplast (Asada, 1994). The superoxide anion, the initial product of photoreduction of \( \text{O}_2 \), is dismutated by superoxide dismutase (EC 1.15.1.1, SOD) to \( \text{H}_2\text{O}_2 \) and \( \text{O}_2 \) (Noctor and Foyer, 1998). Although \( \text{H}_2\text{O}_2 \) can be converted to \( \text{H}_2\text{O} \) by catalase (EC 1.11.1.6, CAT) in peroxisomes (Willekens et al., 1995), the main pathway for scavenging \( \text{H}_2\text{O}_2 \) in chloroplasts is the ascorbate–glutathione cycle. In this pathway, ascorbate peroxidase (EC 1.11.1.11, APX) uses ascorbate (AsA) to reduce \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} \), with the concomitant generation of monodehydroascorbate (MAsA). MAsA can be directly reduced to AsA by ferredoxin in the thylakoids (Asada, 1999; Miyake and Asada, 1994) or by monodehydroascorbate reductase (EC 1.6.5.4, MDAR) using NAD(P)H in the stroma (Hossain et al., 1984). If not rapidly reduced, MAsA can spontaneously disproportionate into AsA and dehydroascorbate (DAsA) (Noctor and Foyer, 1998). DAsA is converted to AsA by dehydroascorbate reductase (EC 1.8.5.1, DHAR) using reduced glutathione (GSH) (Noctor and Foyer, 1998). GSH is then regenerated by glutathione reductase (EC 1.6.4.2, GR) (Noctor and Foyer, 1998). Under limiting N, spinach leaves showed higher SOD activity and AsA content, but similar APX and GR activities, on a leaf Chl basis, as compared to N-replete controls (Logan et al., 1999). The activities of CAT, MDAR, and DHAR and the contents of GSH and oxidized glutathione (GSSG), however, were not determined in the study.

Although the effect of N limitation on xanthophyll cycle and thermal energy dissipation has been examined in some detail (Cheng, 2003; Cheng et al., 2000; Khamis et al., 1990; Verhoeven et al., 1997), very little is known about the response of the antioxidant system to limiting N (Logan et al., 1999; Ramalho et al., 1998) and its coordination with thermal dissipation. It is hypothesized that both xanthophyll cycle-dependent thermal dissipation and the antioxidant system are up-regulated to protect low-N leaves from photo-oxidative damage under high light. Responses of thermal dissipation, xanthophyll cycle size and composition, and the enzymatic and non-enzymatic antioxidants in grape leaves to N supply were determined in this study to test the above hypothesis.

Materials and methods

Plant culture and N treatments

Own-rooted one-year-old grapevines (Vitis labrusca L. cv. Concord) were pruned to two nodes and transplanted into 7.6 l plastic pots containing sand before budbreak. Plants were grown outdoors at Cornell Experimental Orchards in Ithaca, NY (42° 26’ N, 76° 29’ W; elevation 500 m). At budbreak in mid-May, extra shoots were removed and only one shoot was allowed to grow on each plant. Beginning from the third week after budbreak, each plant was supplied once weekly with 500 ml of 10 mM N, using Peters’ 20:10:20 (N:P2O5:K2O) water-soluble fertilizer with micronutrients (Scotts-Sierra Horticultural Products, Marysville, OH, USA). When new shoots were approximately 30 cm long, uniform plants were selected for N treatments. Thereafter, they were supplied twice weekly with 500 ml of a modified Hoagland’s solution at an N concentration of 0, 5, 10, 15, or 20 mM (from NH4NO3) (Cheng and Fuchigami, 2000). There were four replications per N treatment with three plants each in a completely randomized design. After 4 weeks, recent fully expanded leaves were chosen for measuring Chl fluorescence, pigments, and antioxidants.

Measurements of leaf absorptance and Chl fluorescence

Leaf reflectance and transmittance were measured with an LI-1800 spectroradiometer and the 1800-125 integrating sphere attachment (Li-Cor Inc., Lincoln, Nebraska). For each leaf, both a reference scan and a sample scan of reflectance or transmittance were made from 400 nm to 700 nm at 1 nm intervals. The sample scan was divided by its corresponding reference scan, and integrated over the wavelength range to obtain the average reflectance or transmittance. Leaf absorptance was calculated as: 1–reflectance–transmittance.

Chl fluorescence was measured with a pulse-modulated fluorometer FMS2 (Hansatech Instruments Ltd., Norfolk, UK) either at predawn or at a photon flux density (PFD) of 1800 ± 50 μmol m\(^{-2}\) s\(^{-1}\) at midday, 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 the predawn and the midday measurements. Maximum fluorescence \( (F_m) \) and minimum fluorescence \( (F_o) \) of dark-adapted leaves were measured at predawn. For the measurements at midday, steady-state fluorescence \( (F_s) \) was monitored to ensure it was stable before a reading was taken. Maximum fluorescence \( (F_m') \) under natural light exposure was obtained by imposing a 1 s saturating flash of approximately 6000 μmol m\(^{-2}\) s\(^{-1}\) PFD at the leaf surface to reduce all the PSII centres. To determine the minimum fluorescence \( (F_o') \) under natural light exposure, a black cloth was used to cover the leaf while a far-red light was switched on to oxidize PSII rapidly by drawing electrons from PSII to PSI.

The maximum PSII efficiency of dark-adapted leaves was calculated as: \( F_s/F_m' = (F_m' - F_o')/F_m' \) (van Kooten and Snel, 1990). Thermal energy dissipation was estimated from non-photochemical quenching (NPQ) as: \( F_o/F_m' - 1 \) (Stern–Volmer quenching; Bilger and Björkman, 1990). The photochemical quenching coefficient \( qP \) was calculated as \( (F_m' - F_o')/(F_m' - F_o) \). The efficiency of excitation transfer to open PSII centres under natural light exposure was \( F_o''/F_m'' = (F_m'' - F_o'')/(F_m'' - F_o) \). PSI quantum efficiency was calculated as: \( (F_m'' - F_o'')/F_m'' \) (Genty et al., 1989).
Analysis of leaf pigments

Immediately before Chl fluorescence measurements, one disc (1 cm²) was punched from the leaf and frozen in liquid N₂. Frozen leaf discs were stored at −80 °C until analysis. Extraction and analysis of the pigments by HPLC followed the same procedure as described previously (Cheng, 2003).

Extraction and assay of antioxidant enzymes

Antioxidant enzymes were extracted according to Grace and Logan (1996). Briefly, two discs (total of 2 cm²) were taken from each leaf under full sun (PFD of 1800 μmol m⁻² s⁻¹) at midday, ground with a pre-cooled mortar and pestle in 2.5 ml extraction buffer containing 50 mM KH₂PO₄-KOH (pH 7.6), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.3% (w/v) Triton X-100, and 4% (w/v) insoluble polyvinylpolypyrrolidone (PVPP). The extract was then centrifuged at 13 000 g for 10 min in an Eppendorf microcentrifuge, and the supernatant was used immediately for enzyme activity assay.

SOD activity was assayed at 550 nm by the cytochrome c method (McCord and Fridovich, 1969). One unit of SOD activity is defined as the amount necessary to produce a 50% inhibition of cytochrome c reduction.

APX activity was determined by following the decrease in absorbance at 290 nm (extinction coefficient of 2.8 mM⁻¹ cm⁻¹) (Nakano and Asada, 1981). The assay mixture (1 ml) contained 50 mM HEPES-KOH (pH 7.6), 0.1 mM EDTA, 0.2 mM H₂O₂, 0.5 mM AsA, and enzyme extract. The reaction was initiated by adding H₂O₂.

CAT activity was determined by following the decrease of absorbance at 240 nm (extinction coefficient of 39.4 mM⁻¹ cm⁻¹). The reaction mixture (1 ml) contained 100 mM potassium phosphate buffer (pH 7.0), 10 μl 10% (w/v) H₂O₂. The reaction was initiated by adding H₂O₂ (Rao et al., 1996).

MDAR activity was assayed at 340 nm (extinction coefficient of 14 mM⁻¹ cm⁻¹) in 1 ml of reaction mixture containing 50 mM HEPES-KOH (pH 7.6), 0.1 mM NADH, 2.5 mM AsA, and 0.3 units AsA oxidase (EC 1.10.3.3). The reaction was initiated by adding AsA oxidase (Miyake and Asada, 1992).

DHAR activity was determined at 265 nm (extinction coefficient of 6.2 mM⁻¹ cm⁻¹) in 1 ml of reaction mixture containing 100 mM HEPES-KOH (pH 7.0), 0.1 mM EDTA, 2.5 mM GSH, and 0.2 mM DAsA. The reaction was initiated by adding DAsA (Dalton et al., 1986).

GR activity was measured at 265 nm (extinction coefficient of 6.2 mM⁻¹ cm⁻¹) in 1 ml of reaction mixture containing 100 mM TRIS-HCl (pH 8.0), 1 mM EDTA, 1 mM GSSG, and 0.2 mM NADPH. The reaction was initiated by adding NADPH (Grace and Logan, 1996).

Extraction and analysis of antioxidant metabolites

Leaf discs were taken under full sun (PFD of 1800 μmol m⁻² s⁻¹) at midday, frozen in liquid N₂, and stored at −80 °C until assay.

Two leaf discs (total of 2 cm²) were ground in 1 ml ice-cold 6% (v/v) trichloroacetic acid (TCA). GSH and GSSG were determined according to Griffith (1980). AsA and DAsA were measured according to Logan et al. (1998). Briefly, one leaf disc (1 cm²) was ground in 1 ml of ice-cold 6% (v/v) HClO₄. The extract was centrifuged at 10 000 g for 10 min at 2 °C. The supernatant was immediately used for the measurements. One hundred μl of extract was neutralized with 30 μl 1.5 mM Na₂CO₃ to raise the pH to 1–2. AsA was assayed spectrophotometrically at 265 nm in 200 mM sodium acetate buffer (pH 5.6), before and after 15 min incubation with 1.5 units AsA oxidase. For total ascorbate, 100 μl of extract was neutralized with 30 μl 1.82 mM Na₂CO₃ to raise the pH to 6–7 and incubated for 30 min at room temperature with equal volume (150 μl) of 20 mM GSH in 100 mM Tricine-KOH (pH 8.5). Total ascorbate was assayed as above. The DAsA was estimated from the difference between total ascorbate and AsA.

Leaf-N analysis

Leaf N was determined by the Kjeldahl method (Schuman et al., 1973).

Results

Leaf N, Chl and absorptance

Leaf-N content increased curvilinearly in response to an increasing N supply (Fig. 1A). Leaf Chl content was linearly correlated with leaf-N content (Fig. 1B). However, leaf absorptance showed a curvilinear relationship to leaf N (Fig. 1C). As leaf N increased from 0.99 to 3.07 g m⁻²,
leaf absorbance increased only from 80.8% to 93.1% (Fig. 1C).

**Xanthophyll cycle, lutein, β-carotene, and neoxanthin in relation to leaf N**

On a leaf area basis, xanthophyll pool size [violaxanthin (V)+antheraxanthin (A)+zeaxanthin (Z)], lutein, β-carotene, and neoxanthin contents, at both predawn and midday, all increased linearly with increasing leaf N (Fig. 2A-D). On a leaf Chl basis, however, xanthophyll cycle pool size and lutein content decreased with increasing leaf N (Fig. 2E, F), whereas neoxanthin content remained relatively constant across the leaf-N range examined (Fig. 2H). β-Carotene content at predawn did not show any significant change over the leaf-N range, but its midday value decreased with increasing leaf N (Fig. 2G). On a leaf area or Chl basis, no significant difference was found in xanthophyll cycle pool size (V+A+Z), lutein, or neoxanthin content at any given leaf-N level between predawn and midday except that the content of β-carotene at midday was lower than that at predawn (Fig. 2A–H).

At midday, leaf A+Z on a Chl basis decreased with increasing leaf N (Fig. 3A). The percentage of xanthophyll cycle pool present as A+Z decreased from 94.2% to 61.3%

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**Fig. 2.** Xanthophyll cycle pool size (A, E), lutein (B, F), β-carotene (C, G) and neoxanthin (D, H) expressed on the basis of leaf area or Chl at predawn and midday in relation to N content of grape leaves. Each point is the mean with standard error (n=4). Regression equations for violaxanthin (V)+antheraxanthin (A)+zeaxanthin (Z) at predawn y=23.39x+20.601 (r²=0.988, P <0.01) and at midday y=26.52x+14.167 (r²=0.982, P <0.01); for lutein at predawn y=34.79x-5.0935 (r²=0.995, P <0.01) and at midday y=35.39x-4.7817 (r²=0.997, P <0.01); for β-carotene at predawn y=24.89x-7.3531 (r²=0.990, P <0.01) and at midday y=9.59x+8.785 (r²=0.831, P <0.05); for neoxanthin at predawn y=12.12x-2.8371 (r²=0.994, P <0.01) and at midday y=11.80x-2.6156 (r²=0.993, P <0.01).
as leaf N increased from 0.99 g to 3.07 g m$^{-2}$ (Fig. 3B). At predawn, A+Z, expressed on a Chl basis, remained unchanged except a slight rise at the lowest leaf N (Fig. 3A). A+Z accounted for less than 10% of the xanthophyll cycle pool (Fig. 3B), with the balance in V.

**Chl fluorescence variables**

As leaf N increased, thermal dissipation, measured as non-photochemical quenching (NPQ) of Chl fluorescence, decreased curvilinearly (Fig. 4A), whereas the efficiency of excitation transfer ($F_v/F_m'$), the photochemical quenching coefficient ($qP$), and the PSII quantum efficiency all increased curvilinearly (Fig. 4B, C, D). The maximal PSII efficiency ($F_v/F_m$) of dark-adapted leaves at predawn remained unchanged except a slight drop at the lowest leaf N (Fig. 4E).

**Relationships between xanthophyll cycle and Chl fluorescence variables**

As leaf A+Z content on a Chl basis increased at midday, NPQ increased linearly (Fig. 5A), whereas $F_v/F_m'$, $qP$, and the PSII quantum efficiency all decreased linearly (data not shown). When A+Z were expressed as the percentage of the xanthophyll cycle pool, NPQ increased curvilinearly (Fig. 5B), whereas $F_v/F_m'$, $qP$, and the PSII quantum efficiency all decreased curvilinearly (data not shown).

$F_v/F_m$ of dark-adapted leaves at predawn decreased linearly with increasing A+Z content at predawn expressed on a Chl basis (Fig. 5C).

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**Fig. 3.** A+Z content on a Chl basis (A) and as a percentage of the xanthophyll cycle pool (B) at predawn and midday in relation to N content of grape leaves. Each point is mean with standard error ($n=4$).}

**Fig. 4.** Non-photochemical quenching, NPQ (A), efficiency of excitation transfer, $F_v/F_m'$ (B), photochemical quenching coefficient, $qP$ (C), PSII quantum efficiency (D), and maximum PSII efficiency, $F_v/F_m$ (E) in relation to N content in grape leaves. Each point is the mean with standard error ($n=4$). Regression equations for (A) $y=0.4204x^2-2.9233x+6.7496$ ($r^2=0.992$, $P<0.01$); (B) $y=-0.0434x^2+0.3039x+0.0473$ ($r^2=0.981$, $P<0.05$); (C) $y=-0.044x^2+0.3204x+0.2426$ ($r^2=0.994$, $P<0.01$); (D) $y=-0.0355x^2+0.2928x-0.0999$ ($r^2=0.980$, $P<0.05$); and (E) $y=-0.018x^2+0.0978x+0.7167$ ($r^2=0.997$, $P<0.01$).
Antioxidant enzymes and metabolites at midday

On a leaf area basis, SOD activity remained relatively constant across the leaf-N range examined (Fig. 6A), whereas the activities of CAT, APX, MDAR, DHAR, and GR all increased with increasing leaf N (Fig. 6B–F). On a leaf Chl basis, SOD activity decreased linearly with increasing leaf N (Fig. 6G); the activities of CAT and GR increased linearly (Fig. 6H, L); and the activities of APX, MDAR and DHAR decreased curvilinearly (Fig. 6I–K).

The contents of AsA and GSH on a leaf area basis showed a curvilinear increase with increasing leaf N whereas the contents of DAsA and GSSG remained relatively constant across the leaf-N range examined (Fig. 7A–D). On a leaf Chl basis, however, the contents of AsA, DAsA, GSH, and GSSG all decreased curvilinearly with increasing leaf N (Fig. 7F–I). As leaf N increased, the ratios of both AsA/DAsA and GSH/GSSG increased (Fig. 7E, J).

Discussion

In response to N limitation, light absorption decreased in grape leaves (Fig. 1C). However, decreasing light absorption alone is not sufficient for low-N leaves to cope with high light as light absorption did not decrease proportionally with decreasing leaf N (Fig. 1C). Low-N leaves had more excess absorbed PFD than high-N leaves as they used only a smaller proportion of the absorbed PFD for photosynthetic electron transport under high light (Fig. 4D). This is consistent with the results obtained on spinach (Verhoeven et al., 1997) and apple (Cheng, 2003). As expected, thermal dissipation of excitation energy, measured as NPQ, increased in response to N limitation under high light (Fig. 4A), to lower the efficiency with which excitation energy is transferred to PSII reaction centres, $F_v/F_m$ (Fig. 4B).

Thermal dissipation of excitation energy is dependent on the accumulation of de-epoxidation products (A+Z) of the xanthophyll cycle (Demmig-Adams and Adams, 1996; Niyogi et al., 1998). Both the xanthophyll cycle pool size on a Chl basis (Fig. 2E) and the conversion of V to A+Z at midday (Fig. 3) increased with decreasing N in grape leaves. Thermal dissipation of excitation energy, indicated by NPQ, was highly correlated with the level of A+Z at midday, expressed on a Chl basis (Fig. 5A) or as a percentage of the xanthophyll cycle pool (Fig. 5B). This is similar to the result of apple leaves in response to N supply (Cheng, 2003). These results indicate that under N-limiting conditions, in addition to decreasing light absorption by lowering Chl concentration, both xanthophyll cycle pool size and the conversion of V to A and Z are up-regulated to dissipate the excess absorbed PFD in low N leaves. Because A and Z are also capable of de-exciting 1$O_2$ (Baroli et al., 2003; Havaux and Niyogi, 1999), the increase in both xanthophyll pool size and the conversion of V to A and Z may also indicate an increased capacity for quenching 1$O_2$ as the production of 1$O_2$ may increase in low N-leaves under high light due to the increased closure of PSII centres (Fig. 4C).

The finding that the predawn $F_v/F_m$ was closely correlated with A+Z levels at predawn (Fig. 5C) suggests that the slightly lower $F_v/F_m$ in low-N leaves may be associated with the sustained xanthophyll cycle-dependent thermal energy dissipation (Fig. 4E). Similar results were observed in leaves of spinach (Verhoeven et al., 1997) and apple (Cheng, 2003) under limiting N. For overwintering plants growing under low temperature stress, very high levels of A+Z were found to be correlated with sustained...
Fig. 6. Activities of antioxidant enzymes at midday in relation to N content in grape leaves. (A, G) Superoxide dismutase (SOD); (B, H) catalase (CAT); (C, I) ascorbate peroxidase (APX); (D, J) monodehydroascorbate reductase (MDAR); (E, K) dehydroascorbate reductase (DHAR); (F, L) glutathione reductase (GR). Each point is the mean with standard error (n=4). Regression equations for (B) $y = 34.604x - 29.061$ ($r^2=0.969$, $P<0.01$); (C) $y = 20.283x^2 + 47.691x + 96.317$ ($r^2=0.997$, $P<0.01$); (D) $y = 2.0306x^2 + 0.5988x + 11.198$ ($r^2=0.999$, $P<0.01$); (E) $y = 0.1951x^2 + 0.4074x + 1.2913$ ($r^2=0.988$, $P<0.01$); (F) $y = 7.6605x - 0.0272$ ($r^2=0.977$, $P<0.01$); (G) $y = -4.639x + 17.047$ ($r^2=0.957$, $P<0.01$); (H) $y = 30.683x^2 + 20.625$ ($r^2=0.938$, $P<0.01$); and (L) $y = 4.6406x^2 + 12.778$ ($r^2=0.885$, $P<0.05$).
low $F_v/F_m$ at predawn (Adams et al., 1994). However, nocturnal retention of Z did not lead to a decrease in $F_v/F_m$ at predawn in Yucca schidigera and Y. brevifolia in summer (Barker et al., 2002). When grape leaves were treated with high light under low temperature, the decrease in $F_v/F_m$ was not only associated with the increase of Z (Chaumont et al., 1995). In rice leaves, the recovery of $F_v/F_m$ from low temperature photoinhibition was not mainly controlled by the change in Z level (Xu et al., 1999). Therefore, a causal relationship between A+Z level and $F_v/F_m$ at predawn can not be inferred based on the correlation observed (Fig. 5C).

Fig. 7. Antioxidant metabolites at midday, expressed on a leaf area or Chl basis, in relation to N content in grape leaves. (A, F) Dehydroascorbate (DAsA); (B, G) ascorbate (AsA); (C, H) oxidized glutathione (GSSG); (D, I) reduced glutathione (GSH); (E) ratio of AsA to DAsA; (J) ratio of GSH to GSSG. Each point is the mean with standard error ($n=4$). Regression equations for (B) $y=0.3104x^2-0.3074x+1.8218$ ($r^2=0.993$, $P<0.01$); (D) $y=0.1413x^2-0.3517x+0.6967$ ($r^2=0.993$, $P<0.01$); (E) $y=0.5554x^2-0.8041x+1.9831$ ($r^2=0.983$, $P<0.05$); (F) $y=0.7724x^2-5.1972x+9.7539$ ($r^2=0.997$, $P<0.01$); (G) $y=1.1565x^2-0.5991x+14.601$ ($r^2=0.995$, $P<0.01$); (H) $y=0.1395x^2-0.8744x+1.6656$ ($r^2=0.998$, $P<0.01$); and (J) $y=0.633x^2-1.6684x+3.6922$ ($r^2=0.995$, $P<0.01$).
Lipophilic β-carotene is present in the core complex of PSII, which quenches $^{1}\text{O}_2$ (Niyogi, 1999). On a leaf area or Chl basis, β-carotene content at midday was lower than that at predawn, especially in high N leaves (Fig. 2C, G). Because there was no difference in xanthophyll cycle pool or neoxanthin between predawn and midday (Fig. 2A, D), the decrease in β-carotene at midday is not a result of hydroxylation of β-carotene to Z under light, as observed in *Chlamydomonas reinhardtii* (Depka et al., 1998) and *Arbutus* (Demmig-Adams, 1990). β-Carotene can be converted to 5,6-epoxide-β-carotene under high light (Sharma and Hall, 1993; Young et al., 1989), but no additional peak was detected on the chromatogram for midday samples. So, the exact cause of the loss of β-carotene at midday remains unknown. The higher β-carotene content on a Chl basis at midday leaves as compared with high-N leaves (Fig. 2G) may suggest a higher capacity for quenching $^{1}\text{O}_2$ to protect thylakoid membranes from photo-oxidative damage.

On a leaf Chl basis, the activities of SOD, APX, MDAR, and DHAR (Fig. 6G, I–K) and the contents of AsA, DAsA, GSH, and GSSG (Fig. 7F–I) all increased in low-N leaves. This rise in enzymatic and non-enzymatic antioxidants on a leaf Chl basis is consist with an increased requirement for scavenging reactive oxygen species in grape leaves with low N under high light. The ratio of Rubisco activity to electron transport activity declines under N limitation (Evans and Terasima, 1987). The resulting surplus electron flow can lead to enhanced photoreduction of oxygen in the chloroplast (Asada, 1994). Under low-N supply, the rate of superoxide formation, estimated by electron spin resonance, was found to increase in * Coffea arabica* leaves when exposed to high light (Ramalho et al., 1999). Therefore, it would be expected that the antioxidant system in chloroplasts of low-N leaves be up-regulated to cope with the increased photoreduction of oxygen. By scavenging reactive oxygen species generated in the photoreduction of oxygen, the antioxidant system also helps to maintain the electron flow from PSII to PSI via the water–water cycle (Asada, 1999), which generates a ΔpH across the thylakoid (Asada, 1999; Niyogi, 1999). In addition, de-epoxidation of V requires AsA as a reductant (Asada, 1999; Niyogi, 1999). Therefore, the high activities of SOD, APX, MDAR, and DHAR (Fig. 6G, I–K) and high contents of GSH and AsA (Fig. 7G, I) on a Chl basis in low-N leaves not only scavenge the products of photoreduction of oxygen, but also help to sustain the xanthophyll-cycle/ΔpH-dependent thermal energy dissipation in the antenna pigment complexes of PSII.

By contrast to SOD, APX, MDAR, and DHAR, GR expressed on a Chl basis increased linearly with increasing leaf N (Fig. 6L). Considering that the activities of DHAR and GR were the lowest among the enzymes in the ascorbate–glutathione pathway, it is possible that the DHAR-catalysed reaction is not the main route for the regeneration of AsA. Activities of DHAR and GR were also much lower than that of MDAR in leaves of soybean (Badiani et al., 1993) and cork oak (Faria et al., 1996). However, the fact that the ratios of GSH to GSSG and AsA to DAsA increased with increasing leaf N (Fig. 7E, J) suggests that the regeneration of GSH and AsA from GSSG and DAsA is not as complete in low-N leaves as in high N-leaves. Under normal circumstances, GSH and AsA account for most of the glutathione pool and the ascorbate pool, respectively, in plants (Foyer, 1997; Smirnoff and Pallanca, 1996). The ratios of AsA to DAsA and GSH to GSSG decrease under oxidative stress (Gossett et al., 1994; Law et al., 1983). However, even the leaves with the lowest N had a predawn $F_v/F_m$ value of approximately 0.8 in this study (Fig. 4E), which indicates that these leaves were fairly well protected from photo-oxidative damage. Therefore, the lower ratios of AsA to DAsA and of GSH to GSSG in low-N leaves compared with high-N leaves (Fig. 7E, J) may indicate that the equilibrium between utilization and regeneration of AsA and GSH was achieved at a lower level of AsA and GSH in the pool, but they do not necessarily imply that the low N-leaves were damaged by the high PFD at midday. On the other hand, low-N leaves have a lower reduction state of both the glutathione pool and the ascorbate pool (Fig. 7E, J) and a higher degree of closure of PSII reaction centres (Fig. 4C), they may be more prone to photo-oxidative damage than high-N leaves when additional stresses arise. Indeed, when the leaf angle of rice plants is altered to increase leaf light exposure in the field, N-deficient leaves are more susceptible to photoinhibition than N-sufficient leaves (Chen et al., 2003).

On a leaf Chl basis, SOD activity exhibited the most pronounced decrease among the antioxidant enzymes with increasing leaf N (Fig. 6G, I–K). Since SOD is present in mitochondria as well as in chloroplasts and elsewhere in the cell, whereas, APX, for example, is primarily localized in chloroplasts (Gillham and Dodge, 1986), it can be speculated that the high SOD activity expressed on a leaf Chl basis under limiting N may be related not only to an increased rate of photoreduction of oxygen in chloroplasts, but also to a rise in respiration and the subsequent scavenging of reduced reactive oxygen species in the mitochondria. N-limited spinach leaves showed an approximately 3-fold higher rate of dark respiration and SOD activity than N-replete leaves expressed on a chlorophyll basis (Logan et al., 1999).

CAT is primarily localized in the peroxisome (Willekens et al., 1995), where it is involved in removing the bulk $\text{H}_2\text{O}_2$ generated by photorespiration. However, CAT is sensitive to photoactivation *in vivo* and *in vitro* (Feierabend and Engel, 1986; Streb and Feierabend, 1996; Willekens et al., 1995). Loss of CAT activity is greatly enhanced under low temperature, heat-shock, salinity, or chemical stress, where protein synthesis is suppressed.
(Feierabend et al., 1992; Streb and Feierabend, 1996; Willekens et al., 1995). The finding that CAT activity, expressed on a leaf area or Chl basis, increased with increasing leaf N (Fig. 6B, H) suggests that photoinactivation of CAT may also be occurring at a higher rate in low-N leaves than in high-N leaves under high light. The increased activities of SOD, APX, MDAR, and DHAR (Fig. 6G, I–K) and the elevated levels of GSH and AsA (Fig. 7G, I) on a Chl basis in N-limited grape leaves may compensate for the low CAT activity (Fig. 6J) to some degree, as has been demonstrated in a CAT-deficient barley mutant (Palatnik et al., 2002).

To conclude, in addition to decreasing light absorption by lowering Chl concentration, both xanthophyll cycle-dependent thermal energy dissipation and the total ability to scavenge reactive oxygen species are enhanced to protect low-N leaves of grapevines from photo-oxidative damage under high light.

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