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Competing interests statement

The authors declare that they have no competing financial interests

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Overexpression of β-carotene hydroxylase enhances stress tolerance in *Arabidopsis*

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Plant stress caused by extreme environmental conditions is already a principal reason for yield reduction in crops[1]. The threat of global environment change makes it increasingly important to generate crop plants that will withstand such conditions. Stress, particularly stress caused by increased sunlight, leads to the production of reactive oxygen species that cause photo-oxidative cell damage[2]. Carotenoids, which are present in the membranes of all photosynthetic organisms, help protect against such light-dependent oxidative damage[3]. In plants, the xanthophyll cycle (the reversible interconversion of two carotenoids, violaxanthin and zeaxanthin[4]) has a key photoprotective role[5] and is therefore a promising target for genetic engineering to enhance stress tolerance. Here we show that in *Arabidopsis thaliana* overexpression of the *chy*B gene that encodes β-carotene hydroxylase—an enzyme in the zeaxanthin biosynthetic pathway[6]—causes a specific twofold increase in the size of the xanthophyll cycle pool. The plants are more tolerant to conditions of high light and high temperature, as shown by reduced leaf necrosis, reduced production of the stress indicator anthocyanin and reduced lipid peroxidation. Stress protection is probably due to the function of zeaxanthin in preventing oxidative damage of membranes.

To investigate the protective role of zeaxanthin under stress conditions, we used metabolic engineering to increase the size of the xanthophyll cycle pool in *A. thaliana*. Given the nutritional importance of carotenoids, several groups have manipulated carotenoid biosynthesis in plants[7–9], and overexpression of a bacterial phytoene desaturase gene in tobacco leads to an increase in the xanthophyll cycle pool[10]. Although mutants of *A. thaliana* with an increased xanthophyll cycle pool size are available, they have large alterations in the content of other carotenoids and lesions in the biosynthesis of the plant growth regulator abscisic acid[2,11].

To manipulate specifically the size of the xanthophyll cycle pool, we overexpressed the thylakoid membrane enzyme β-carotene hydroxylase, which catalyses the conversion of β-carotene to zeaxanthin (Fig. 1); antisense inhibition of the encoding gene (*chy*B) is already known to result in a reduction in pool size[12]. We transformed *A. thaliana* (ecotype C24) with the *chy*B gene under the control of a constitutive promoter (Fig. 2a), established several stable lines derived from independent transformation events (Fig. 2b) and checked these for overexpression of β-carotene hydroxylase (Fig. 2c).

Using the published complementary DNA sequence for β-carotene hydroxylase[13], we designed primers specific to +418 to +887 in the carboxy-terminal region and carried out polymerase chain reaction with reverse transcription (RT–PCR) using increasing starting amounts of total RNA from wild-type and transformed plants. Densitometry indicated a linear increase in the amount of wild-type β-carotene hydroxylase messenger RNA over the range shown (Fig. 2c), but for the sense *chy*B transformant the amount of detectable mRNA had almost reached saturation point at the lowest amount analysed (0.25 μg of total RNA), which represented a fourfold increase as compared with wild-type expression.

Lines that were overexpressing the gene were subjected to carotenoid extraction and high performance liquid chromatography (HPLC) to determine their xanthophyll profiles (Table 1). There was a marked (at least twofold) increase in the amount of violaxanthin in the sense *chy*B lines. This would be expected if the conversion of β-carotene to zeaxanthin were enhanced, as the latter would be converted by zeaxanthin epoxidase into violaxanthin (through antheraxanthin) in the dark (Fig. 1). Roughly the same increase was seen in all three lines. This increased pool of viola-

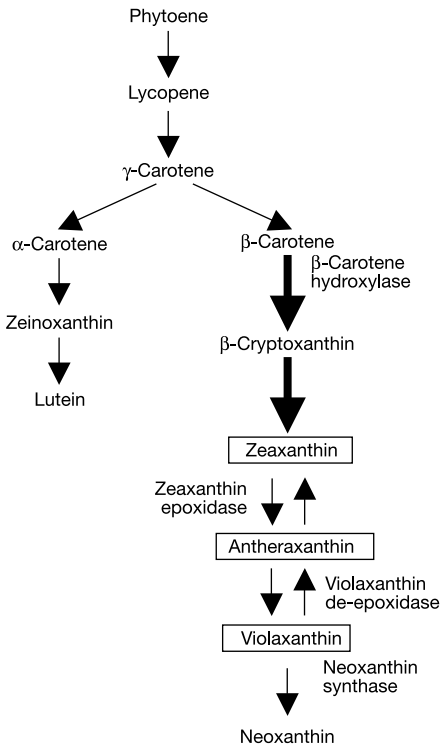

Figure 1 Carotenoid–xanthophyll biosynthetic pathway in plants. The step catalysed by β-carotene hydroxylase is indicated by bold arrows. The xanthophyll cycle carotenoids are shown in boxes.

Table 1 Leaf carotenoid/xanthophyll profiles and photosynthetic parameters of wild-type (C24) and sense *chy*B plants

Line	μg chl *a* per mg dry weight	chl *a*/*b*	Carotenoid (mmol)/chl *a*(mol) Viola	Lutein	β-car	Anth	Neo	Total carot	Fv/Fm	NPQ
C24										
Low-light grown	10.5 ± 1.6	3.05 ± 0.10	49 ± 6	166 ± 8	126 ± 9	8 ± 3	46 ± 4	395	0.81 ± 0.00	2.33 ± 0.09
Moderate-light grown			99 ± 12	199 ± 3	163 ± 11	19 ± 3	66 ± 2	546		
Sense 1										
Low-light grown	10.0 ± 0.7	2.97 ± 0.18	116 ± 10	164 ± 6	110 ± 9	7 ± 3	48 ± 3	445	0.80 ± 0.00	2.25 ± 0.10
Moderate-light grown			257 ± 46	180 ± 4	147 ± 2	25 ± 2	65 ± 3	675		
Sense 2										
Low-light grown	11.1 ± 1.7	3.19 ± 0.26	114 ± 7	154 ± 12	109 ± 6	9 ± 2	44 ± 4	430	0.80 ± 0.01	2.05 ± 0.03
Moderate-light grown			275 ± 30	194 ± 6	155 ± 10	30 ± 7	66 ± 4	720		
Sense 3										
Low-light grown	10.0 ± 2.0	3.00 ± 0.16	117 ± 16	160 ± 11	107 ± 6	9 ± 3	50 ± 6	444	0.81 ± 0.00	2.48 ± 0.22
Moderate-light grown			285 ± 34	186 ± 18	150 ± 10	41 ± 6	65 ± 6	728		

Values are the means ± s.d. of at least three 5-week-old, dark-adapted plants grown in low light (100 μmol photons $m^{-2} s^{-1}$) or in moderate light (400 μmol photons $m^{-2} s^{-1}$). NPQ was determined after illumination with 6,000 μmol photons $m^{-2} s^{-1}$ actinic light. anth, antheraxanthin; β-car, β-carotene; carot, carotenoid; neo, neoxanthin; viola, violaxanthin. Fv (variable fluorescence/Fm (maximum fluorescence) is a measure of photosystem II efficiency.

xanthin was not converted further into neoxanthin. Overexpression of β-carotene hydroxylase did not unduly perturb the rest of the carotenoid biosynthetic pathway; there was only a slight fall in the amount of β-carotene, presumably owing to increased conversion to the xanthophylls.

The total carotenoid content per unit of chlorophyll *a* was increased and, as the chlorophyll content of the plants was unchanged, we concluded that the increase in violaxanthin was not at the expense of other carotenoids. Similar increases in the xanthophyll cycle pool size were obtained regardless of whether the sense *chy*B plants were grown under low (100 μmol photons $m^{-2} s^{-1}$) or moderate (400 μmol photons $m^{-2} s^{-1}$) light (Table 1). Hence, for low-light plants the xanthophyll cycle pool increased from 14% to nearly 30% of total carotenoid for low-light plants, and from 22% to over 40% for moderate-light plants. The latter value is significantly larger than any reported value for wild-type *Arabidopsis*.

To determine where this extra xanthophyll was located, we extracted thylakoid membranes, gently solubilized them with detergent, separated them on a sucrose density gradient and carried out HPLC on the resulting fractions (Fig. 3). The extra xanthophyll was mostly associated with the photosystem II light-harvesting complexes (LHCII), which showed a threefold increase in the amount

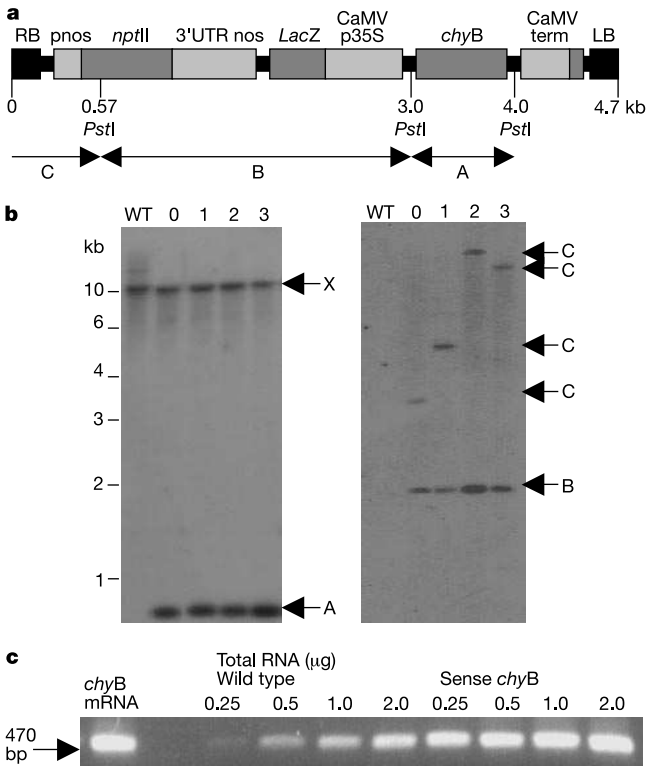

Figure 2 Southern and RT–PCR analysis of 5-week-old sense *chy*B plants grown in low light. **a**, Transfer DNA (TDNA) region of pBin400::*chy*B. LB, left TDNA border; RB, right TDNA border; CaMV p35S, cauliflower mosaic virus 35S promoter; CaMV term, cauliflower mosaic virus 35S terminator; *lac*Z, β-galactosidase gene; *npt*II, kanamycin resistance gene; pnos, nopaline synthase promoter; 3′ UTR nos, nopaline synthase terminator. **b**, Southern blot of *Pst*I-cut DNA probed with *chy*B (left) and *npt*II (right), showing the endogenous (X) and introduced (A) *chy*B genes, a *npt*II-containing internal TDNA fragment (B) and a RB *npt*II fragment (C) containing flanking genomic sequence of varying size because of independent insertions into the genome. WT, wild type; 0–3, T_3 sense *chy*B plants descended from separate T_0 transformants. **c**, RT–PCR specific for *chy*B mRNA carried out on increasing amounts of wild-type and sense *chy*B (line 3) total RNA. The PCR products were separated on a 1% agarose gel (equal loadings per lane). A positive control containing about 10 ng of synthesized full-length *chy*B mRNA was included.

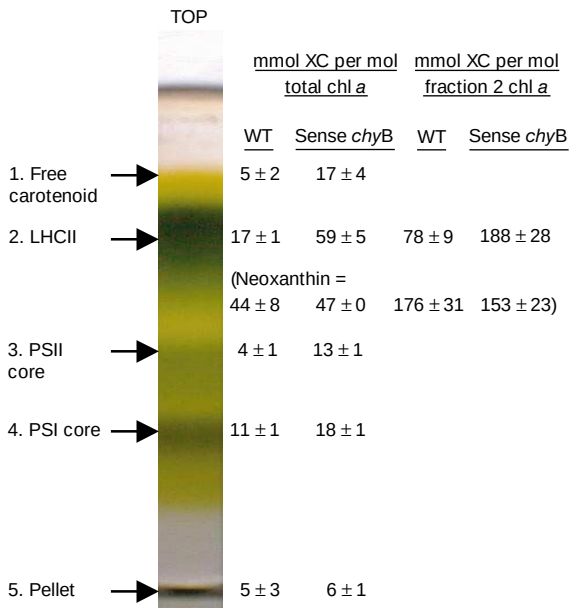

Figure 3 Sucrose density gradient (0.15 M to 1.0 M) profile of thylakoid membranes isolated from dark-adapted 5-week-old, low-light grown wild-type and sense *chy*B plants after solubilization in 1.5% (w/v) dodecyl maltoside. Xanthophyll cycle carotenoids (XC) and neoxanthin (given in parentheses) contents of each fraction are standardized against total moles of chlorophyll *a* (chl *a*) added to the gradient and, where indicated, moles of chl *a* contained in a particular fraction. Values are means ± s.d. ($n = 4$). PS, photosystem; LHCII, photosystem II light-harvesting complex, which includes both the major and minor complexes.

of bound violaxanthin. Previous work has shown that LHCII contains xanthophyll cycle binding sites at the periphery of the complex that are not fully occupied[14]. Thus, the extra violaxanthin associated with LHCII is probably bound to these sites. The ratio of violaxanthin to neoxanthin (1 molecule bound per LHCIIb complex) in the LHCII fraction of the transformed plants (1.2 compared with a wild-type value of 0.4) is consistent with this conclusion.

The extra violaxanthin was available for de-epoxidation when the plants were exposed to high light (15 min, 1,000 μmol photons $m^{-2} s^{-1}$). The amounts of zeaxanthin were at least twice as large in the sense *chy*B lines as in the wild-type, although their average de-epoxidation state (percentage of total xanthophyll pool present as antheraxanthin and zeaxanthin) was the same (52% and 54%, respectively). We conclude that at least some of the extra xanthophyll must be biologically active, which would allow us to test whether an increased xanthophyll cycle pool will confer any improvement in stress tolerance. One of the ways in which plants respond to stress is to increase the size of their xanthophyll pool and so increase their amount of zeaxanthin[2,5]. Engineered plants with a constitutively increased pool size should be 'preprimed' and better able to respond to stress conditions. To investigate this, we switched plants grown for 5 weeks under conditions of low light (100 μmol photons $m^{-2} s^{-1}$) and 20 °C to stress conditions (1,000 μmol photons $m^{-2} s^{-1}$, air temperature, 40 °C; mean ± s.d. leaf temperature: wild type, 36.6 ± 2.4 °C; sense *chy*B, 36.4 ± 2.5 °C; $n = 8$) for 2 weeks. As expected, the amount of zeaxanthin was roughly 2–4 times greater in the sense *chy*B lines than in the wild-type (Fig. 4c).

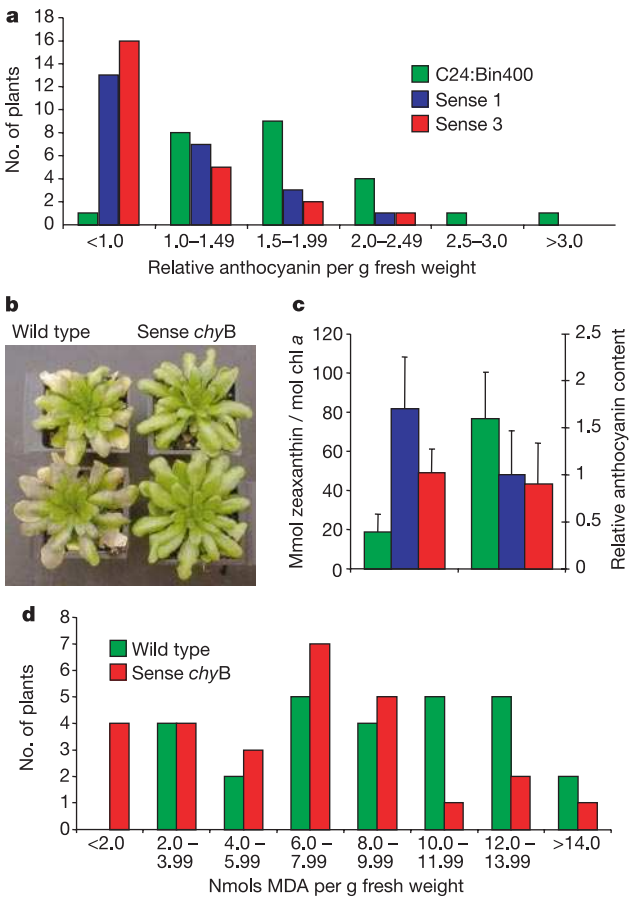

Figure 4 Response of 5-week-old sense *chy*B plants grown in low light subjected to 2 weeks of high-light and high-temperature stress. **a**, Anthocyanin content. C24:Bin400, control wild-type transformed with pBin400. **b**, Physical appearance. **c**, Zeaxanthin content (standardized against chlorophyll *a*) and relative anthocyanin produced per gram of fresh weight. Values are means ± s.d. ($n = 24$). **d**, Malondialdehyde (MDA) content per gram of fresh weight.

An indicator of stress in *A. thaliana* is the presence of the purple flavonoid anthocyanin[15,16]. The flavonoid pathway is distinct from the carotenoid biosynthesis pathway and should not be affected by perturbation of the latter[17]. We analysed 24 stressed plants from the control wild type (transformed with the transformation vector alone) and 2 sense *chy*B lines, and determined their relative anthocyanin content per gram of fresh weight (Fig. 4a). There was no difference between the two sense *chy*B populations (mean ± s.d. 1.0 ± 0.47 and 0.9 ± 0.43), but both these populations had significantly less anthocyanin ($P < 0.0001$, Student's *t*-test) than the wild type (mean 1.6 ± 0.49). This difference in anthocyanin amounts was obvious in the whole plants (Fig. 4b) where the sense *chy*B lines were clearly greener and healthier, and showed less leaf necrosis.

Zeaxanthin is involved in the non-photochemical quenching (NPQ) mechanism used by plants to dissipate excess light energy[18,19]. But increased NPQ is unlikely to be the cause of the observed stress tolerance because we detected no increase in the capacity of NPQ in the sense *chy*B plants (Table 1). This is consistent with observations that constitutive zeaxanthin-accumulating *Arabidopsis* mutants do not show an increase in the maximum extent of NPQ[20,21]. Zeaxanthin also prevents the accumulation of reactive oxygen species that lead to oxidative damage of membranes through lipid peroxidation[22,23]. When zeaxanthin synthesis is inhibited by mutating violaxanthin de-epoxidase, the increase in oxidative stress under high-light conditions does not arise from the inhibition of NPQ but from the removal of this alternative protective function[22]. This observation suggested that the increased stress tolerance of the sense *chy*B plants might arise from an enhancement of this protective role of zeaxanthin. The fact that the extra xanthophyll in the sense *chy*B plants seems to occupy the loose peripheral binding sites on LHCII (which are in contact with membrane lipids) is consistent with this suggestion.

As an estimate of general lipid peroxidation, we determined the amount of malondialdehyde (MDA)—a secondary end product of the oxidation of polyunsaturated fatty acids—in wild-type and sense *chy*B plants exposed to stress. We assayed four populations of plants: wild-type, wild-type transformed with pBin400 and two sense *chy*B lines (1 and 3). There was no significant difference in mean MDA content (nmoles per gram of fresh weight; mean ± s.d.) between the two wild-type populations (wild type, 8.6 ± 4.7; transformed wild type, 9.7 ± 3.5) or the two sense *chy*B lines (line 1, 6.5 ± 4.0; line 3, 6.8 ± 3.6). But there was a difference in the amount of lipid peroxidation between the wild-type and sense *chy*B lines. Figure 4d shows the pooled wild-type and sense *chy*B MDA content values subdivided into groups; the mean values for the two populations (27 plants per population) were 9.2 ± 4.3 and 6.6 ± 3.9, respectively, indicating a significant ~30% drop in the amount of lipid peroxidation in the sense *chy*B lines ($P < 0.02$, Student's *t*-test).

When the production of zeaxanthin is prevented by mutation[22] or antisense suppression[24] of violaxanthin de-epoxidase, plants have an increased sensitivity to light stress. It has been suggested that zeaxanthin also has a role in protecting thylakoid membranes specifically from the effects of heat stress[25]. Our results show that increasing expression of the β-carotene hydroxylase enzyme brings about an increase in the content of the xanthophyll cycle and zeaxanthin in the chloroplast membrane, and notably that this manipulation leads to an improved tolerance of high-light and high-temperature conditions. It is important to point out that these effects on anthocyanin production and lipid peroxidation were observed in plants that had been exposed to stress conditions for 2 weeks; that is, it was a sustained property of the transformed plants. We conclude that genetic manipulation of a single enzyme in carotenoid metabolism can bring about a pronounced increase in the stress tolerance of plants and thus represents a potentially powerful way forward in the production of stress-tolerant crops. □

Methods

Plant material and growth conditions

We grew *A. thaliana* (ecotype C24) on soil:vermiculite:perlite (4:2:1) under a 8 h/16 h light/dark regime at 20 °C and 100 μmol photons $m^{-2} s^{-1}$ (low-light conditions), 20 °C and 400 μmol photons $m^{-2} s^{-1}$ (moderate-light conditions), or 40 °C and 1,000 μmol photons $m^{-2} s^{-1}$ (stress conditions). If selection was required, seed was first germinated on 1 × Murashige–Skoog basal medium supplemented with Gamborgs vitamins, 1% sucrose, 0.5 g l^{-1} MES and 50 μg ml^{-1} kanamycin.

Generation of sense *chy*B plants

The *chy*B gene (GenBank accession code U58919) was isolated by PCR (forward primer, 5′-GCGGATCCTGCAGCCTCAAAACAAGTAGCTCCTCCTTCC-3′; reverse primer, 5′-TTTCTAGACTCGAGTCAAGAACTCGAACTCGACCCGG-3′) on a size-selected cDNA library (derived from 1–2 kilobases of mRNA) supplied by the Arabidopsis Biological Resource Center, Ohio State University. The blunt-ended fragment was inserted into the *Sma*I cloning site located in the 35S CaMV expression cassette of pDH51. The 35S CaMV promoter–*chy*B–terminator cassette was isolated using PCR (forward primer, 5′-GCATCGATAAGCTTCAGCTATGACCATGATTACG-3′; reverse primer, 5′- TAAAAC GACGGCCAGTGCC-3′), confirmed by DNA sequencing and inserted into the *Hin*dIII site of plant transformation vector pBin400 (supplied by M. Bevan, John Innes Institute). The pBin400::*chy*B construct was transformed into *Agrobacterium tumefaciens* C58C1 (pGV2260; rifampicin resistant) by triparental mating, and transformation of *A. thaliana* was carried out in tissue culture using root explants and shoot regeneration as described[26]. We selected primary transformants (T_0) on the basis of resistance to the antibiotic kanamycin and established lines by single seed descent, with selection maintained to the third generation (T_3). Twelve T_3 plants per line were analysed by HPLC for presence of the high xanthophyll pool size phenotype to check for homozygosity.

Southern blotting

Genomic DNA was isolated from plants using the GenElute kit (Sigma). We carried out Southern blotting as recommended by Hybond. Radiolabelled probes were produced using the Ready-to-Go kit (Pharmacia).

RT–PCR

Total RNA was extracted from 5-week-old leaf tissue using the RNeasy kit (Qiagen) and treated with RNase-free DNase (Promega). We synthesized control full-length β-carotene hydroxylase mRNA using an RNA transcription kit (Stratagene). First strand cDNA synthesis was carried out using reverse primer 5′-AGTCAAGAACTCGAACTCGACCC GG-3′ and avian mycloblastosis virus (AMV) reverse transcriptase as recommended by Promega. Second strand synthesis and amplification was carried out with the reverse primer and forward primer 5′-GGGCTCATAGAGCTCTGTGGC-3′ using standard PCR techniques.

Thylakoid membranes, carotenoid extraction and HPLC

We prepared thylakoid membranes as described[27]. Chlorophyll content was measured spectrophotometrically in 80% acetone[28]. Carotenoids were extracted from a single leaf in ethanol:ether (1:2 v/v) under red light, filtered through cotton wool and a 0.2-μm syringe filter, dried under nitrogen gas and stored in the dark at −20 °C before being dissolved in acetone. Samples were separated by reverse-phase HPLC on a Spherisorb 5-μm ODS2 column (4.6 × 250 mm, Waters Corp.) using a 40-min gradient of ethyl acetate (0 to 100%) in acetonitrile:water (9:1 v/v) at a flow rate of 1 ml min^{-1}. Pigments were identified by their retention time and by absorption spectra using a photodiode array detector (model 996, Waters Corp.) and were quantified by integrating peak areas. We converted peak areas to molar concentrations by comparison with carotenoid standards of known concentration run on HPLC and standardized against the molar concentration of chlorophyll *a*. To confirm the determinations of zeaxanthin in those cases where it was not fully separated from lutein, two leaf samples were taken from each plant, one under the ambient light conditions and one after a 1-h dark treatment (to convert zeaxanthin to violaxanthin). Any reduction in the xanthophyll pool size in the former compared with that seen in the latter sample was assumed to be due to zeaxanthin that was undetected owing to masking by lutein.

Anthocyanin extraction and malondialdehyde assay

Anthocyanin was isolated from whole plants as described[29]. The malondialdehyde assay for estimating lipid peroxidation was done on leaf tissue as described[30].

Measurement of non-photochemical quenching

Chlorophyll fluorescence in attached leaves that had been dark-adapted for 15 min was measured by a Walz PAM 101 fluorimeter. We determined NPQ by applying a light saturation pulse after illumination with actinic light over a range of intensities (500, 1,000, 2,000, 4,000 or 6,000 μmol photons $m^{-2} s^{-1}$) for 6 min.

Measurement of leaf temperature

We measured leaf temperature on the underside of the leaf under ambient light and temperature conditions using a thermocouple (type T) and electronic thermometer (model 9009, Comark).

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Competing interests statement

The authors declare that they have no competing financial interests.

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