Zeaxanthin Retention and Biomass Production in Two Ecotypes of *Arabidopsis thaliana*

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Undergraduate Honors Thesis

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Abstract

Zeaxanthin is a carotenoid produced by plants for protection against photo-damage and supports human vision and health when consumed with the human diet. Zeaxanthin in plants is accumulated and retained most strongly (i) under harsh, growth-retarding conditions and (ii) by inherently slow-growing plants. By selecting for maximal biomass production, modern agriculture may have inadvertently selected for nutritionally suboptimal plants. This thesis explores whether zeaxanthin retention can be triggered by mild light stress without concomitant decreases in biomass production, and whether different plant varieties respond differently. Two ecotypes of *Arabidopsis thaliana* adapted to biogeographic extremes of this species' distribution (Italy and Sweden) were grown in the presence of mild light stress and assayed for zeaxanthin content and retention, plant photo-protection capacity against damage by intense light, and biomass. When grown under mild light stress, only the Swedish ecotype retained zeaxanthin, suggesting heightened responsiveness of the Swedish ecotype to subtle environmental triggers. In addition, the Swedish ecotype demonstrated a greater ability than the Italian ecotype to rapidly form additional zeaxanthin when exposed to an experimental treatment with very high light levels. It can be concluded that both moderate changes in environmental conditions and selection of plant variety can serve to augment plant zeaxanthin content without compromising biomass production.

Introduction

Plants produce nutrients essential for human health, such as the carotenoid zeaxanthin that protects the human eye against damage by intense light and also supports other aspects of human health susceptible to oxidative damage (Mares-Perlman et al. 2002). Zeaxanthin is presumably also an indicator for overall plant content of other beneficial antioxidants, all of which are typically up-regulated together (Garcia-Plazaola et al. 2004). Due to synergistic reactions among phytochemicals (plant chemicals) found in whole foods, consumption of whole plant-based food is more beneficial to human health than antioxidant supplements (Liu 2003). Plants accumulate zeaxanthin for their own protection against damage by intense light (Demmig et al. 1988; Demmig-Adams and Adams 1990) and consumption of plant-based foods allows humans to acquire this antioxidant defense since humans are unable to synthesize zeaxanthin themselves (Mares-Perlman et al. 2002; Demmig-Adams and Adams 2002). However, leaf zeaxanthin content is typically very low since leaves removed from otherwise unstressed plants begin to remove zeaxanthin as soon as they are no longer exposed to intense light (see background section below; Demmig Adams and Adams 1994; Bilger and Björkman 1994). Lasting maintenance (retention) of high zeaxanthin levels in leaves has been observed predominantly under environmental conditions severely inhibiting plant growth (Demmig et al. 1988; Adams et al. 1995, 2002, 2006; Demmig-Adams et al. 1998, 2006; Adams & Demmig-Adams 2004). Growth of crop plants under conditions that severely lower plant productivity is, of course, undesirable.

This thesis addressed the question of whether or not conditions and/or plant varieties can be identified that allow zeaxanthin retention under mild light stress conditions with no or minimal negative impact on plant growth and productivity. To address this question, two different varieties ("ecotypes") of the plant model species *Arabidopsis thaliana* from contrasting geographic origin (Sweden and Italy) were chosen as experimental specimens. The ecotype from Italy had been shown to be significantly less capable of surviving the native environment of the Swedish ecotype due to harsh winter conditions (Ågren and Schemske 2012) that induce similar physiological responses as excessive growth light levels (Demmig-Adams and Adams 2006). This thesis employed a carefully chosen growth condition, representing only very mild light stress, to explore whether or not this latter condition (mildly excessive light) would trigger zeaxanthin retention in one or both ecotypes without large negative impacts on plant biomass production. I predicted that the ecotype from Sweden would be more likely than the Italian ecotype to respond to a mild light stress treatment (low background growth light with several moderately high light pulses added every day) with higher levels of zeaxanthin formation and retention.

Background

Dual nature of light: energy source for growth as well as potentially destructive force

Light energy absorbed by plants fuels photosynthesis and is the foundation of virtually all food chains on Earth. However, when absorbed in greater quantities than can be immediately utilized in photosynthesis, this light energy becomes

"excessive" and can cause cellular damage and death to the photosynthetic organism (Demmig-Adams and Adams 1994). As depicted in Figure 1 (modified after Demmig-Adams and Adams 1994), light absorption by chlorophyll (Chl) causes an electron in the chlorophyll molecule to move from the ground state (Chl) to the energized singlet-excited state (^{1}Chl*), followed by utilization of ^{1}Chl* for photosynthesis (photochemistry). When more light is absorbed than can expediently be used in photochemistry, ^{1}Chl* accumulates and converts to the triplet-excited state of Chl (^{3}Chl*) that can, unlike ^{1}Chl*, pass excitation energy on to ever-present oxygen by converting ground state (triplet) oxygen to highly destructive excited singlet oxygen ($^1O_2$*). To avoid $^1O_2$* formation, plants employ an alternative route (alternative to either photochemistry or triplet chlorophyll formation) that safely dissipates excess excitation energy by thermal de-excitation of ^{1}Chl* directly back to ground state Chl (in thermal energy dissipation that can be quantified as non-photochemical fluorescence quenching). A small portion (only about 2%) of ^{1}Chl* furthermore reverts back to ground state by converting excitation energy to another form of radiation, i.e. chlorophyll fluorescence that can be used to probe how much excitation energy goes into photochemistry (from photo-chemical fluorescence

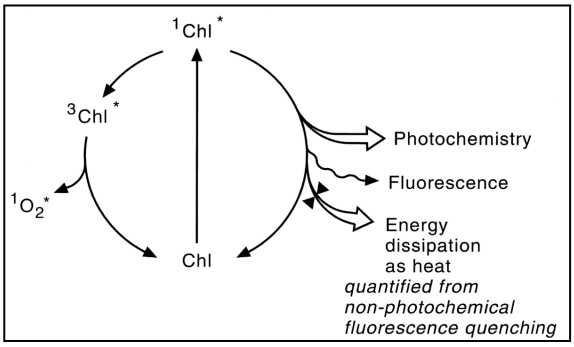

Figure 1. Schematic depiction of the different pathways for absorbed light (excitation energy). Figure modified after Demmig-Adams and Adams (1994; courtesy of B. Demmig-Adams). Light energy excites chlorophyll from the ground state (Chl) to the singlet-excited state (^{1}Chl*). As the excited chlorophyll falls back to ground state, the energy released leads to either photosynthesis, thermal dissipation, chlorophyll fluorescence, or the generation of reactive oxygen species.

quenching) and into thermal dissipation (from non-photochemical fluorescence quenching; see section below on "Use of chlorophyll fluorescence to probe the fate of absorbed energy").

This duality of light, as both necessary for plant growth but detrimental in excess, has favored the evolution of physiological processes protecting plants either against ***absorption*** of too much light in the first place or against any ***damage*** caused ***by already absorbed excess light*** (Demmig-Adams and Adams 2006; Jahns and Holzwarth 2012). Protection from damage by excessive excitation energy is facilitated by a group of carotenoid pigments, chiefly involving the xanthophyll zeaxanthin under natural conditions (Demmig-Adams and Adams, 1994) as well as, possibly, minor contributions from other xanthophylls (Jahns and Holzwarth 2011; Ruban et al. 2012).

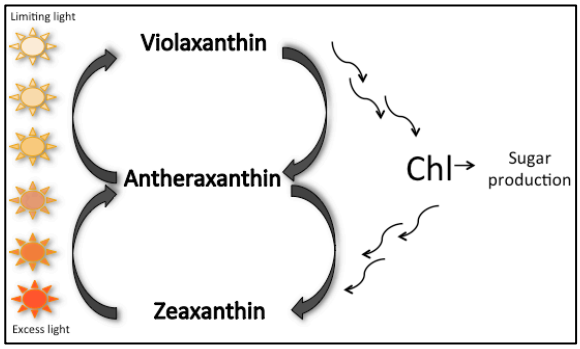

Figure 2. Schematic depiction of the xanthophyll cycle. Zeaxanthin and antheraxanthin facilitate thermal energy dissipation (act as "dissipaters") in the presence of excessive light, while the xanthophyll violaxanthin does not act as a dissipater. An enzyme activated by the presence of excessive light converts violaxanthin to zeaxanthin (via the intermediate antheraxanthn); another enzyme, active in the presence of non-excessive light, converts zeaxanthin (and antheraxanthin) to violaxanthin. Violaxanthin levels are greater in light-limited environments, where energy from excited chlorophyll is used for sugar synthesis. Conversely, energy from excited chlorophyll is quenched by zeaxanthin, and thus unavailable for photosynthesis, in the presence of excessive light.

The xanthophyll cycle as a facilitator of plant photo-protection

Plants' chlorophyll-binding light-harvesting complexes (antennae) absorb light and are composed of chlorophyll and various carotenoids, including the xanthophylls studied here. It is in these antennae that the fate of incoming electrons is decided

(Fig. 1; Salin 1987; Apel and Hirt 2004). In the presence of excess light, the most common photo-protective response is thermal dissipation (Fig. 1) that relies on zeaxanthin quickly produced from a precursor (violaxanthin) in the cyclic (xanthophyll cycle) reactions detailed in Figure 2 (Demmig et al. 1987). Plants depend on the xanthophyll cycle (i.e. rapid enzymatic conversion between the non-dissipating violaxanthin and the dissipaters antheraxanthin [an intermediate] and zeaxanthin) to assure both (i) prompt formation of the energy dissipaters under exposure to excessive light and (ii) rapid removal of the dissipaters upon return to low light levels to avoid loss of any excitation energy for photosynthesis. Leaves quickly remove zeaxanthin (and antheraxanthin) during the portions of the day with less-than-maximal light levels (for example, morning and afternoon, and, of course, night) as long as plants are rapidly growing and thus depend on efficient energy allocation for photosynthesis (Demmig-Adams et al. 2012). In contrast, plants typically maintain/retain continuously high levels of zeaxanthin (and antheraxanthin) 24-hours-a day when severe stress arrests plant growth, and efficient light collection is no longer beneficial at any time of day (Demmig-Adams et al. 2012). To optimize both photo-protection and efficient light utilization as needed, formation of zeaxanthin (and antheraxanthin) is carefully regulated by several physiological factors serving as excellent indicators of the presence of excessive (or limiting) light (see below).

Absorption of light by the chlorophyll antennae leads to the build-up of a pH gradient across the photosynthetic membrane that sharply increases as soon as photosynthesis ***no longer utilizes*** all absorbed excitation energy (Müller et al.

2001), which triggers (i) activation of the xanthophyll cycle enzyme that converts violaxanthin to zeaxanthin and antheraxanthin (Demmig-Adams and Adams 1992) and (ii) protonation of a specialized light-harvesting protein, PsbS, that engages already-produced zeaxanthin in active thermal dissipation (Li et al. 2004).

The advantage of removing the dissipaters zeaxanthin and antheraxanthin under low light conditions has been demonstrated by showing that lasting retention of high levels of zeaxanthin (in a mutant of *Arabidopsis* lacking the enzyme for re-conversion of zeaxanthin to violaxanthin) decreases photosynthetic efficiency and plant growth at very low growth light intensities (Bassi et al. 1993). It is thus likely that the two-step regulatory process, comprised of the xanthophyll cycle conversions and engagement of actual dissipation via the PsbS protein, serves to optimize both light utilization for plant growth and photo-protection. Furthermore, this two-step process may enable retention of zeaxanthin without corresponding thermal dissipation of excitation energy as engaged by PsbS, which may permit efficient photosynthetic activity even in the presence of retained, albeit non-engaged, zeaxanthin.

Human nutrition and plant xanthophyll production

Carotenoids and various other antioxidants produced by plants in response to harsh environmental conditions serve as essential dietary nutrients for humans (Demmig-Adams and Adams 2002, 2010; Maccarrone et al. 2005). Zeaxanthin is especially important for the function and protection of human vision as it promotes visual acuity and lowers the risk for cataracts and age-related blindness (Seddon et al.

1994; Richer et al. 2011). Furthermore, zeaxanthin and other plant antioxidants lower the risk of chronic diseases like cancer and heart disease (Mares-Perlman et al. 2002; Demmig-Adams and Adams 2002, 2010).

However, modern agriculture may have inadvertently selected for crop varieties with minimal zeaxanthin/antioxidant content by the practice of growing crops under conditions virtually free of environmental stress and by selecting crop varieties with the highest rates of growth and biomass production. While plants accumulate more zeaxanthin, and are thus nutritionally superior, when grown under harsh environmental conditions, these harsh conditions simultaneously inhibit plant growth and efficient light utilization (Demmig-Adams and Adams 2006). Even under identical growing conditions, fast-growing (and rapidly photosynthesizing) plants thermally dissipate much less light and accumulate much less zeaxanthin than slow-growing, slowly photosynthesizing, plants (reviewed in Demmig-Adams et al. 2012). Since modern agriculture favors fast-growing varieties, these latter plants are typically less nutritionally dense but more economically viable when compared to hardy crop varieties resistant to unfavorable environmental conditions and exhibiting elevated antioxidant production (Fernie et al. 2006). As stated above, the question explored in the present thesis is whether or not it is possible to trigger retention of zeaxanthin without cutting deeply into plant biomass production.

Arabidopsis *ecotypes*

A previous study on two ecotypes (from Sweden and Italy) of the plant model species *A. thaliana* had employed reciprocal transplant experiments to assess plant response to the environmental extremes of this species' geographic distribution (Ågren and Schemske, 2012). When the ecotype from Sweden was transplanted to Italy, and *vice versa*, it was clear that neither ecotype performed as well as in their respective native climates (Ågren and Schemske 2012). Plant genetic adaptations thus seem to favor each ecotype in its native range; in particular, the ecotype from Italy was less able to survive in Sweden while the Swedish ecotype survived but did not thrive in Italy (Ågren and Schemske 2012). The latter finding suggests that the Swedish ecotype is hardier and better able to tolerate extreme physical (abiotic) environments than the Italian ecotype (Ågren and Schemske 2012). As stated above, it has been shown that winter conditions involve high levels of light stress (especially under sunny conditions with simultaneously low temperatures, and thus slow

Figure 4: Photograph of a plant of the Italian ecotype two days prior to sampling after 44 days of growth under low background light in the presence of moderately high light pulses in a growth chamber.

Figure 3: Photograph of plants of the Swedish ecotype two days prior to sampling after 44 days of growth under low background light in the presence of moderately high light pulses in a growth chamber.

photosynthetic rates) that triggers increased photo-protective thermal dissipation of excess absorbed light energy (Demmig-Adams and Adams 2006). It is likely that adaptation to Swedish conditions (or similar conditions) increases protective response to abiotic stress in general.

Use of chlorophyll fluorescence to probe the fate of absorbed energy

As described above (see Fig. 1), a very small percentage of singlet-excited chlorophyll releases its energy as fluorescence (Demmig-Adams and Adams 2000). Chlorophyll fluorescence is highest when no other pathways are open to drain excitation energy, such as during periods when neither photosynthesis nor thermal dissipation of singlet-excited chlorophyll occurs (Adams et al. 1990). A characteristic reduction in fluorescence (photo-chemical fluorescence quenching) can serve as an indicator of the rate of photochemistry and a different type of reduction of fluorescence (non-photochemical fluorescence quenching, NPQ) as an indicator of photo-protective thermal dissipation. Given that NPQ is typically positively correlated with zeaxanthin levels (Demmig et al. 1987), I utilized fluorescence measurements to gain further insight into the Swedish and Italian ecotypes' biochemical responses to excess light.

Materials and Methods

Plant material

Two ecotypes of *Arabidopsis thaliana* from the extremes of the species' geographic range, one from northern Sweden and the other from southern Italy (Ågren &

Schemske 2012), were germinated in standard six-plug trays (cell-volume of 50 mL) in temperature- and humidity-controlled growth chambers at 25°C, ambient CO_2, and low light levels of 200-250 μmol photons m^{-2} s^{-1} (provided by fluorescent and incandescent light bulbs over a nine-hour photoperiod). For reference, the light intensity of full sunlight is between 1500 and 2000 μmol photons m^{-2} s^{-1} (Demmig-Adams and Adams 1996). Plants were watered daily and received liquid nutrients every other day. Seedlings were transferred to larger (3.35-L) pots and kept under the conditions described above for a re-adjustment period of two days. Half of individuals remained under control conditions (25°C, ambient CO_2, 200 μmol photons m^{-2} s^{-1}), while the other half were transferred to a chamber with 200 μmol photons m^{-2} s^{-1} baseline light intensity and six added 5-min moderately high light pulses during the nine-hour photoperiod. During pulses, light level was increased instantly from 200 to 800 μmol photons m^{-2} s^{-1}. The latter moderately high light pulses were evenly spaced over the nine-hour photoperiod with an hour of 200 μmol photons m^{-2} s^{-1} each at the beginning and end of light period, i.e. before and after the first and last pulses, respectively.

Chlorophyll fluorescence measurements

Two non-self-shaded, mature and fully expanded leaves from each plant of the two ecotypes were selected for characterization by chlorophyll fluorescence approximately six weeks after germination. Leaves were placed in a leaf-disc oxygen electrode (Model LD2/3 equipped with an LS-2 halogen light source providing a very high light intensity of 2050 μmol photons m^{-2} s^{-1} Hansatech, King's Lynn,

Norfolk, UK) under a humidified stream of 98% N_2 and 2% O_2 and non-photochemical fluorescence quenching (NPQ), as a measure of the level of thermal dissipation of excess absorbed light, was assessed with an XE-PAM fluorometer (Waltz, Effeltrich, Germany). Data were recorded as fluoresence traces using a BD40 1-pen strip-chart recorder set to 10 mm/min and 1000 mV (Kipp and Zonen, Delft, the Netherlands). Baseline measurements were taken in the dark at 1.6 kHz. Initial fluoresence (F_o), representing the state of photosystem II with all reaction centers open (oxidized) and ready to accept excitation energy, was excited with a low-intensity beam of 1.6 kHz intensity. Maximal fluoresence (F_m) levels, representing the state of photosystem II with all reaction centers closed (reduced) and unable to accept excitation energy, were recorded by quickly pulsing leaf discs with two saturating light pulses (measured at 100 kHz). Following the F_m measurement, the intensity of the light measuring beam was set to 100 kHz for more accurate fluorescence measurments. While still at 100 kHz, the measuring light and chamber halogen light source were turned on to record fluoresence over the high-light period. Saturating pulses for determining F_m' values were given at regular times over a twenty minute duration (at 1, 2, 3, 6, 9, 12, 15 and 20 min). Final F_o' was determined after fluorescence was stable by turning on continuous far red light, switching from 100 kHz back to 1.6 kHz and turning off the chamber halogen light. Leaves were immediately combined with the rest of the plant for dry biomass determination.

High performance liquid chromatography. Leaf discs of 0.325 cm^2 were collected from fully light-exposed leaves for HPLC analysis. Collection occurred before onset, and at the end, of the photoperiod in the growth chamber approximately 60 min after the final light pulse, as well as after 20 min of high light treatment in the treatment chamber described above. To prevent changes in pigment levels post-collection, each sample was immediately dropped into an aluminum envelope and submersed in liquid nitrogen until biochemical analysis. To extract pigments, leaf discs were masticated in a cold glass cylinder with a small amount of $MgCO_3$ and 0.2 mL of an 85% acetone:water solution. The sample was then transferred to an Eppendorf tube and combined with the volume of acetone used to rinse the glass grinder (twice with 0.2 mL and once with 0.1 mL 85% acetone). The resultant slurry was centrifuged at 10,000 x g for 5 min at 4°C. The supernatant was decanted to a new Eppendorf tube, and the remaining pellet washed with 0.2 mL 100% acetone, centrifuged and decanted into the second Eppendorf tube. This second process was repeated once more. The total volume of the combined supernatant was determined with a syringe, passed through a filter (Cameo Disposable Syringe Filters, 0.45 um pore size, No. DDN0400300, MSI, Westboro, MA, USA) and then bubbled with gaseous nitrogen before capping the tube. Each sample was assayed via HPLC (Shimadzu Corporation, Kyoto, Japan). The HPLC column used to separate carotenoids was a bonded silica Carotenoid YMC™ 5 mm column from Waters, Inc (Milford, MA, USA). A gradient of solvent A (86.7% acetonitrile, 9.6% methanol, 3.7% 0.1 M Tris-HCL pH 8.0, all HPLC grade) to solvent B (80% methanol, 20% hexane, both HPLC grade) was used to elute all pigments except b-carotene. The

gradient was followed by an isocratic elution of β-carotene using solvent B. Injection volume for each elution was 20 μL. Peak areas in the HPLC trace were converted from mAu to μmol carotenoid/ m^2 leaf area based on a calibration standard.

Biomass. Leaf tissue samples and aboveground fresh biomass of each plant was weighed immediately after harvesting. Leaf discs were removed from plants prior to harvest but included in fresh weight measurements before being taken to the NPQ chamber for testing. All fresh biomass measurements were taken prior to light exposure. After testing, combined aboveground biomass for each plant was dried for one week at 60°C, and then weighed again for dry biomass. Leaf weight was measured using an analytical balance (Denver Instrument Company, Denver, CO, USA).

Statistical Analysis. Data were recorded in Microsoft Excel (2011) and analyzed using statistical software JMP (Pro 10.0.1, SAS Institute Inc., Cary, NC, USA). Student's t tests were used for comparison of means between treatment and control groups. ANOVA and Tukey-HSD tests compared variance of means for all ecotype/treatment groups in conjunction.

Results

Continuous zeaxanthin retention

Continuous retention of zeaxanthin (throughout the entire 24-hour light-dark cycle in the growth chambers) was significantly greater in the Swedish ecotype grown

with light pulses (grown under low background light with several moderately high light pulses) as compared to the consistent low levels of zeaxanthin retention in the ecotypes from either Sweden or Italy grown in the absence of light pulses (Fig. 5). The minimal level of zeaxanthin seen in the Swedish and Italian ecotypes grown in the absence of light pulses was negligible (Fig. 5). Significant differences between carotenoid and chlorophyll content among ecotypes should be tested further in future (Table 1), but for the purposes of this research the statistical trends are clear enough to consider the data normalized.

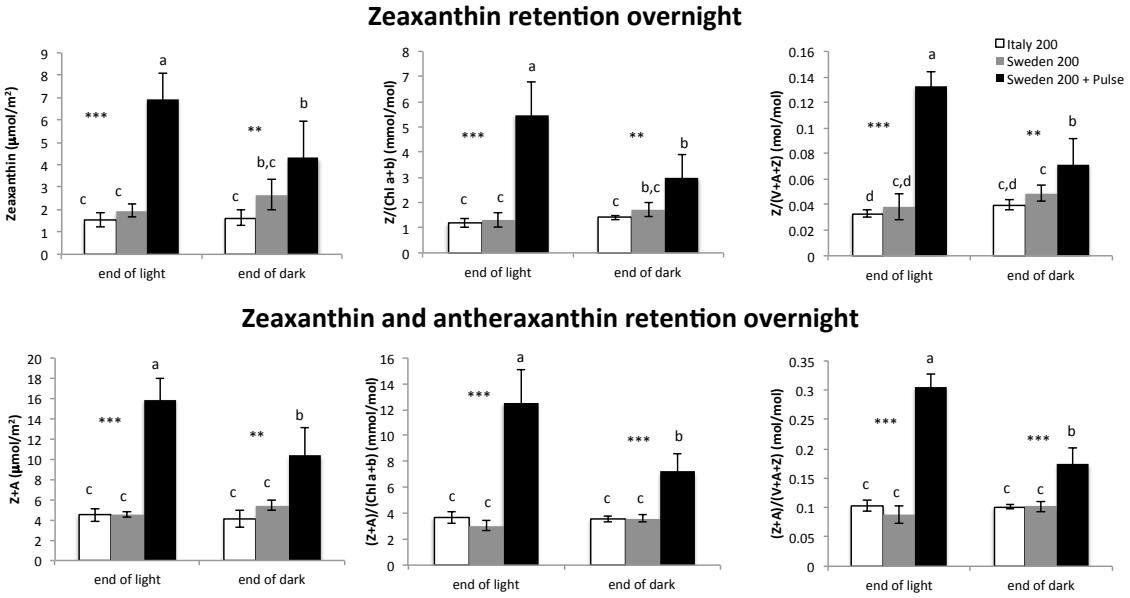

Figure 5. Zeaxanthin (Z) and zeaxanthin+antheraxanthin (Z+A) levels for the two ecotypes grown in the presence and absence of mild light stress (in the form of pulses of moderately high light), respectively. Samples were collected prior to the photoperiod (end of dark period) and at the end of the photoperiod (end of light period) from the growth chambers. Levels of Z and Z+A are expressed per leaf area (left panels), relative to total leaf chlorophyll (middle panels), and as a fraction of the total xanthophyll cycle pool (right panels), respectively. The mean ± standard deviation is shown for each ecotype and treatment group (N=4-5). Significant differences between Sweden 200+pulse and Italy 200 are indicated by asterisks and are based on Student's t-tests for paired means. Two asterisks indicates significance at α =0.01 and three asterisks indicates significance at α=0.001. Comparison among all mean values per panel, as indicated by lower case letters, are based on Tukey HSD tests of significance.

Ecotype/treatment	Chlorophyll a+b	Chlorophyll a/Chlorophyll b	Neoxanthin/(Chlorophyll a+b)	Lutein/(Chlorophyll a+b)	β-carotene/(Chlorophyll a+b)	(V+A+Z)/(Chlorophyll a+b)
	mol	mol/mol	mol/mol	mol/mol	mol/mol	mol/mol
Italy 200	1197 ± 62 (b)	3.0 ± 0.02 (a)	37.5 ± 1 (a)	125.6 ± 2 (a)	77.3 ± 1 (a)	35.3 ± 1 (b)
Sweden 200	1513 ± 49 (a)	3.1 ± 0.02 (a)	37.4 ± 0.9 (a)	127.8 ± 2 (a)	77.0 ± 1 (a)	34.7 ± 1 (b)
Sweden 200+Pulse	1358 ± 55 (ab)	3.0 ± 0.03 (a)	37.5 ± 1 (a)	123.2 ± 3 (a)	76.5 ± 1 (a)	41.0 ± 1 (a)
p-value	*0.0005****	*0.2*	*0.99*	*0.44*	*0.93*	*0.002***

Table 1. Mean values ± standard deviation of chlorophyll (chl a/b) and chlorophyll to pigment content for neoxanthin, lutein, beta-carotene and the xanthophyll cycle pool. Samples were collected prior and following a 9-hour photoperiod. Letters in parenthesis indicate Tukey HSD analysis for differences in means (N=4-5) while asterisks describe the degree of statistically significant difference. Two asterisks corresponds with significance at α=0.01 and three asterisks indicates significance at α=0.001.

Probing the maximal potential for zeaxanthin-associated thermal dissipation via short, experimental exposure to very high light

In addition to the above assessment of zeaxanthin retention under the prevailing growth light conditions, I also characterized the maximal potential of photo-protection (zeaxanthin-associated maximal NPQ) in the two ecotypes grown under the two respective growth conditions. I performed short experimental treatments with very high light on excised leaves, sampled prior to the onset of the photoperiod in the growth chambers (end of dark).

The high experimental light levels (2050 μmol photons m^{-2} s^{-1}) greatly exceeded not only the background growth light intensity but also the light intensity of the daily treatment pulses (800 μmol photons m^{-2} s^{-1}). Sudden exposure to very high light levels allow an assessment of maximal NPQ capacity as an indicator of the maximal ability of each ecotype to harmlessly dissipate excess absorbed light (Fig. 6). Saturating light levels were chosen as approximately equivalent to natural sunlight, which is around 2,200 μmol photons m^{-2} s^{-1} at high noon.

Each ecotype exhibited a significantly higher maximal NPQ capacity when grown with versus without light pulses (Fig. 6). Growth of the Swedish ecotype with light pulses resulted in the highest maximal NPQ capacity seen among all ecotypes and growth conditions used here (Fig. 6).

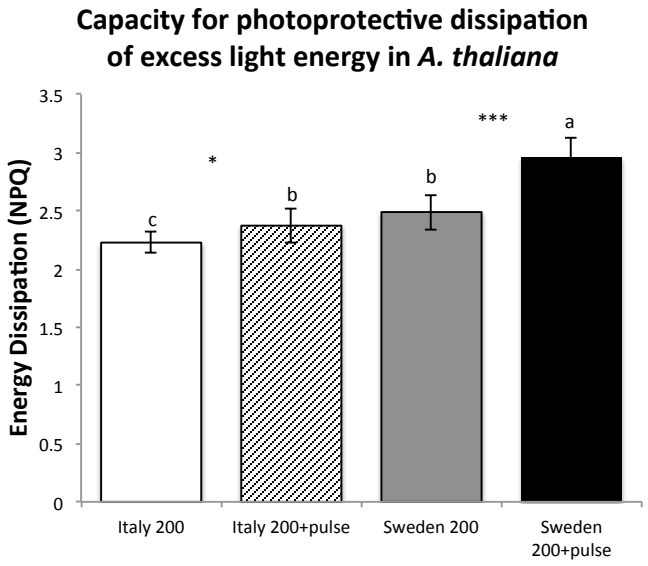

Figure 6. Capacity for dissipation of excess light energy, expressed as nonphotochemical quenching (NPQ=(F_m/F_m') -1), for the two ecotypes grown in the presence and absence of mild light stress (in the form of pulses of moderately high light), respectively. The mean ± standard deviation is shown for each ecotype and treatment group (N=5). Significant differences in variance indicated by letters are based on ANOVA tests among all means. Statistically significant differences between pairs indicated by asterisks are based on Student's t-tests for paired means within each ecotype. One asterisk corresponds with significance at α=0.05 and three asterisks indicates significance at α=0.001. For Student's t-tests between pairs p-values Italy=0.048, Sweden<0.0001. ANOVA R^2=0.62, p-value<0.0001.

Consistent with its greater capacity for thermal dissipation (Fig. 6), the Swedish ecotype also exhibited significantly greater maximal levels of zeaxanthin (and of the sum of zeaxanthin+antheraxanthin, Z+A) than the Italian ecotype irrespective of reference base (relative to leaf area, chlorophyll content or total xanthophyll cycle pool size; Fig. 7).

It is noteworthy that the Swedish ecotype, when grown in the ***presence*** versus absence of light pulses, exhibited the ***same*** maximal levels of zeaxanthin (or zeaxanthin + antheraxanthin) (Fig. 7) and yet significantly ***lower*** maximal NPQ

capacities (Fig. 6). Underlying reasons for this effect will be addressed in the Discussion.

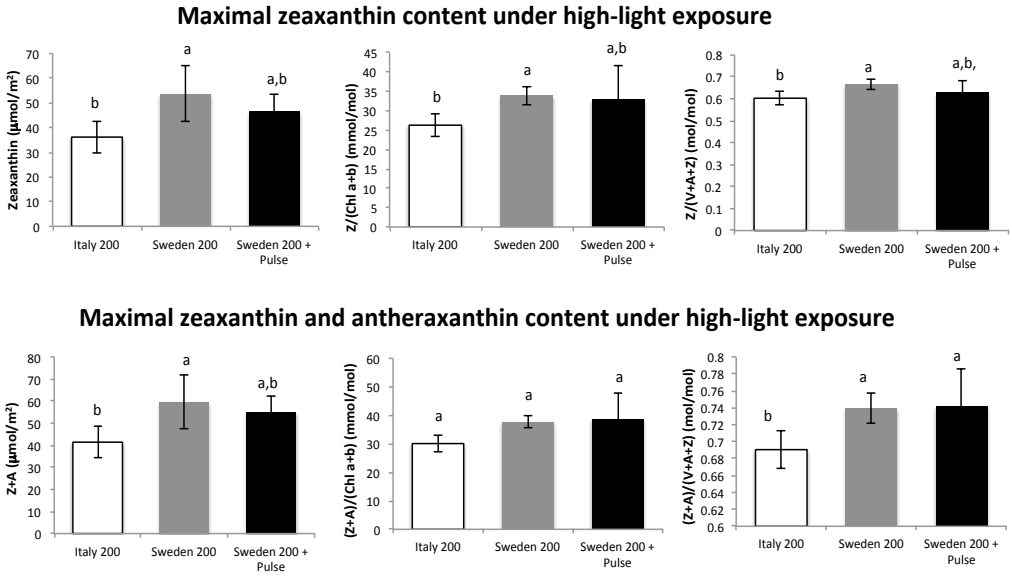

Figure 7: Zeaxanthin and zeaxanthin+antheraxanthin levels for the two ecotypes grown in the presence and absence of mild light stress (in the form of pulses of moderately high light), respectively, on multiple bases. Data were collected after a twenty-minute period of high light. The mean ± standard deviation is shown for each ecotype and treatment group (N=4-5). Levels of Z and Z+A are expressed per leaf area (left panels), relative to total leaf chlorophyll (middle panels), and as a fraction of the total xanthophyll cycle pool (right panels), respectively. Comparisons of mean values, as indicated by lower case letters, are based on Tukey HSD tests of significance (N=4-5).

Biomass production and photosynthetic performance

Aboveground fresh biomass (Fig. 8, left panel) was not significantly different between light-pulse-treated and control plants for either ecotype. In the ecotype from Sweden, aboveground dry biomass accumulation was even ***higher*** in the presence versus absence of light pulses (Fig. 8, right panel). Finally, both fresh and dry biomass accumulation was somewhat lower in the ecotype from Sweden compared to that from Italy, irrespective of growth conditions (Fig. 8).

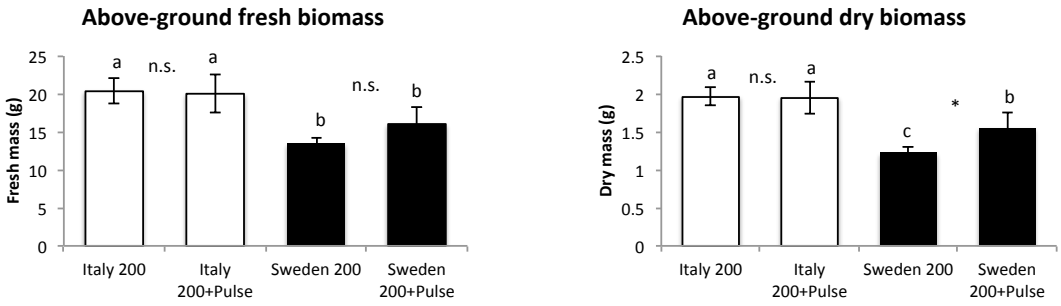

Figure 8. Aboveground fresh (left) and dry (right) biomass for the two ecotypes grown in the presence and absence of mild light stress (in the form of pulses of moderately high light), respectively. Significance levels between all means were determined by ANOVA tests and are indicated by lower-case letters. Student's t test results for significant differences between treatments within ecotypes are indicated by asterisks. One asterisk corresponds with significance at α=0.05 (n.s.=no statistical difference).

In-situ chlorophyll fluorescence measurements of non-photochemical quenching (NPQ) under the actual low background light conditions in the growth chambers revealed that there were no significant differences in reduction state of photosystem II, which is a measure of the accumulation of excess excitation energy (Fig. 9, left panel; 1-qP of 1.0 would indicate that 100% of the absorbed light is excessive and cannot be used in photosynthesis), nor in the rate of photosystem II electron transport between ecotypes or treatments (Fig. 9, middle panel). Similarly, there were no differences in non-photochemical quenching under the actual low growth light regime (Fig. 9, right panel). *In-situ* NPQ levels (Fig. 9 right panel) were low in all cases compared to NPQ levels measured under experimental high light exposure (Fig, 9, right panel; cf. Fig. 6); since the growth light level is unlikely to represent excess excitation energy, *in-situ* NPQ in the growth chamber may not represent thermal dissipation.

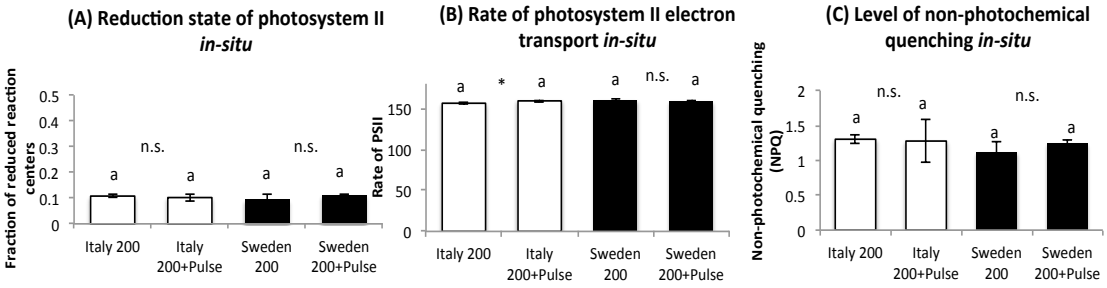

Figure 9. *In-situ* (A) photosystem II reduction state, (B) photosynthetic (photosystem II) electron transport rate, and (C) NPQ directly all measured directly in the growth chambers on young plants of the two ecotypes grown in the presence and absence of mild light stress (in the form of pulses of moderately high light), respectively. Mean values ± standard deviation shown. Significant differences between pairs indicated by asterisks are based on Student's t-tests. One asterisk corresponds to significance at α =0.05 (n.s.=no statistical difference). ANOVA tests were run to determine differences in variance between ecotypes and treatments (N=5).

Discussion

The present study was successful in defining a novel growth condition able to trigger lasting retention of some levels of the carotenoid zeaxanthin without negatively impacting plant biomass production in a hardy variety well-adapted to environmental stress (the Swedish ecotype of *Arabidopsis*). This finding is of considerable interest for the production of nutrient-rich plants under conditions that also favor plant productivity. Furthermore, the present study shows that the Swedish ecotype's superior propensity for quick formation of high levels of zeaxanthin, as probed under experimental high-light exposure, was only associated with actual engagement of the highest levels of thermal dissipation when plants had been grown in the presence of light pulses. It can be concluded that both moderate changes in environmental conditions ***and*** selection of plant variety/ecotype may serve to simultaneously optimize plant nutrient content and plant growth. It should

be noted that future research should assess zeaxanthin retention in the Italian ecotype to discern possible differences in thermal dissipation capacity between *Arabidopsis* ecotypes.

Plant nutritional quality for human health

As stated above, zeaxanthin supports vision acuity, overall eye health, and immune function in humans (Mares-Perlman et al. 2002). A problem for the dietary acquisition of plant-based zeaxanthin is that, by virtue of xanthophyll cycle operation, plants typically remove zeaxanthin quickly upon return to low light conditions favorable for growth (e.g. absence of drought or extreme temperatures) (Demmig-Adams et al. 2012). It would be advantageous to produce leafy greens that continuously retain at last some zeaxanthin under growth conditions without reducing plant productivity. The present finding (i.e. the Swedish ecotype's propensity for quick formation and continuous retention of zeaxanthin without losses in biomass under growth conditions with mild light pulses) suggests that adaptive characteristics of a plant ecotype/variety may determine its propensity for responding to excess light by producing zeaxanthin, while subtle environmental triggers are apparently sufficient to elicit zeaxanthin retention. Plants that accumulate and retain zeaxanthin typically also possess greater pools of other antioxidants important for plant photo-protection (e.g. vitamins C and E, due to general concomitant antioxidant up-regulation; Seddon et al. 1994; Garcia-Plazaola et al. 2004) as well as for human nutrition (Demmig-Adams and Adams 2002). It can thus be concluded that selecting crop varieties with greater stress responsiveness

may be a promising approach to improving the nutritional quality of leafy greens. This conclusion is consistent with views expressed in the literature that locally adapted, highly stress-tolerant crop varieties ("landraces") may possess a higher nutritional quality than common elite (fast-growing) strains of crop plants bred for high biomass yield (Zeven 1998). Close attention should be paid to selection of crop varieties with a higher propensity for antioxidant accumulation that comes without high costs in terms of losses in plant yield.

Results of the present research suggest that simultaneous accumulation of zeaxanthin (and possibly other antioxidants) without loss of biomass is possible, and that crop varieties from high latitudes (and potentially other hardy varieties) should be considered. Furthermore, mild environmental stress during plant development may prompt retention of zeaxanthin for human consumption, thus providing a method for growing nutrient-dense crops. The mild light pulse treatment employed here presents such an opportunity to induce lasting zeaxanthin retention without losses in plant yield. This same approach (using light pulses) would be suitable for plant production under artificial light, e.g. in greenhouses or small-scale urban agriculture systems. Our results are thus relevant and potentially valuable particularly for use with controlled lighting systems, as those used by astronauts who, in fact, require extra protection against cataracts caused by high-level radiation experienced during deep-space missions (Lett et al. 1994; Cucinotta et al. 2001). Mild light pulse treatment of crop plants grown in space should increase zeaxanthin availability for astronauts who otherwise rely on less-effective antioxidant supplements for nutrition (Liu 2003; Pezzoti et al. 2012). Our results

may encourage further research into the development of "smarter" lighting systems designed for maximal zeaxanthin (and antioxidant) production during deep space missions, as well as for terrestrial systems that integrate food production into built environments (Renalds et al. 2010).

Plant adaptation to the environment

The finding that the Swedish ecotype is apparently more responsive to mild light stress than the Italian ecotype, with respect to induction of zeaxanthin formation and thermal dissipation under high light, has implications for the understanding of plant adaptation to the environment. A higher sensitivity to subtle light stress may play an important role in plant adaptation to, and survival in, harsh environmental conditions at high latitudes, characterized by highly variable light and temperatures (see Ågren and Schemske 2012). Furthermore, the present evidence of zeaxanthin retention in the Swedish ecotype is consistent with the responses typically seen in overwintering plants under field conditions (Demmig-Adams et al. 2012). Our remarkable finding that the Swedish ecotype exhibits zeaxanthin retention in response to mild light stress in the absence of any temperature stress is consistent with the view that light stress is a key challenge for overwintering plants (Demmig-Adams and Adams, 2006).

The observation in the present study that the Swedish ecotype can, irrespective of growth conditions, form high levels of zeaxanthin more quickly than the Italian ecotype under experimental probing with very high light, suggests that

the Swedish ecotype may ***constitutively*** express higher levels of enzymes involved in zeaxanthin synthesis.

On the other hand, our finding that actual engagement in NPQ (i.e. in photo-protective thermal energy dissipation) of zeaxanthin formed under experimental probing with very high light was dependent upon the presence of light pulses during plant growth is consistent with the well-known involvement of ***two*** conditions required for NPQ (see Demmig-Adams and Adams 1992). Niyogi's group demonstrated that NPQ is largely abolished (1) in mutants that cannot form zeaxanthin (Niyogi et al. 1998) and (2) in mutants that do not possess a certain light-stress-associated protein, the PsbS protein (Li et al. 2000) from the family of light-harvesting proteins (Jansson 1999). Furthermore, leaves of an evergreen plant species growing in full sunlight, and exhibiting high maximal NPQ levels, possessed both larger xanthophyll cycle pools (V+A+Z pools) and greater levels of PsbS than leaves of the same species grown under low light (Demmig-Adams et al. 2006).

Several molecular mechanisms have been proposed to explain the requirement of ***two*** conditions (rather than only a single condition) for thermal energy dissipation. While some authors favor the view that PsbS and zeaxanthin facilitate ***separate*** thermal energy dissipation events (Holzwarth et al. 2012; Ruban et al. 2012), others have proposed a ***sequence*** in which zeaxanthin is first formed at a small distance from chlorophyll molecules, and another factor (such as PsbS) then causes a conformational change in chlorophyll- and xanthophyll-binding protein complexes, which moves zeaxanthin close enough to chlorophyll for engagement of the actual dissipation of excess light (Demmig-Adams and Adams 2006). Our

present results suggest that the Swedish ecotype readily forms zeaxanthin irrespective of growth conditions, but only readies the second condition (protonation of PsbS) for actual engagement of NPQ when some form of light stress is present during plant growth (Li et al. 2000). It can be speculated that the Swedish ecotype grown in the absence of light pulses possesses lower levels of the PsbS protein than the Swedish ecotype grown under light pulses, and that synthesis of additional PsbS protein takes longer than the 20-minute experimental high-light treatments utilized in this study. There is precedence for leaves under tree canopies retaining zeaxanthin without showing continuous actual thermal dissipation, yet maintaining the capacity to instantly engage thermal dissipation when struck by a shaft of light penetrating the forest canopy (Adams et al. 1999).

In addition, or perhaps as an alternative to invoking PsbS as being involved in the lower maximal NPQ level in the Swedish ecotype grown in the absence versus presence of mild light stress, it should also be noted that the location of zeaxanthin (and antheraxanthin) among the many chlorophyll-binding protein complexes may vary in plants grown under different conditions and exhibiting differential engagement of zeaxanthin (and antheraxanthin) in NPQ. It has been shown that the distribution of violaxanthin, antheraxanthin and zeaxanthin among chlorophyll/xanthophyll-binding complexes varies, and that growth light environment can affect this distribution pattern (e.g. Betterle et al. 2012; Fuciman et al. 2012). While there is agreement that quickly-inducible and quickly-reversible engagement of NPQ involves PsbS protonation as light stress augments the pH-gradient across the photosynthetic membrane (Li et al. 2004), recent additional

research is suggesting that yet other factors may also play a role in the engagement of high levels of NPQ (Demmig-Adams and Adams 2006; Paul Suman, Onno Muller, Tobias Schumann, Barbara Demmig-Adams, William W. Adam III, Peter Jahns, Alfred R. Holzwarth, unpublished data). Future research should address these latter processes in the Swedish ecotype grown under different conditions.

The present findings, that mild pulses do not lower, but rather ***increase*** biomass production of the Swedish ecotype, are consistent with earlier findings that light pulses can provide usable additional energy to plants grown under low background light intensity limiting to photosynthesis (Pearcy 1994). However, the fact that aboveground biomass production was slightly lower in the Swedish versus the Italian ecotype under either growth condition used here does suggest that the greater stress responsiveness of the Swedish ecotype may come at a cost in terms of above-ground productivity. It is clear that this cost is not associated with zeaxanthin retention since the Swedish ecotype exhibited lower aboveground biomass irrespective of zeaxanthin retention. Furthermore, we cannot exclude the possibility that the Swedish ecotype allocates more resources to belowground biomass than the Italian ecotype. For agricultural purposes, which emphasize the use of aboveground leafy biomass, it is important to recognize this possible trade-off between zeaxanthin retention and biomass production. Future research should address the eco-physiological factors involved in differential stress responsiveness, and potential methodologies to improve crops accordingly.

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