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Characterization of the Xanthophyll Cycle and Other Photosynthetic Pigment Changes Induced by Iron Deficiency in Sugar Beet (*Beta vulgaris* L.)[1]

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ABSTRACT

In this work we characterize the changes induced by iron deficiency in the pigment composition of sugar beet (*Beta vulgaris* L.) leaves. When sugar beet plants were grown hydroponically under limited iron supply, neoxanthin and β-carotene decreased concomitantly with chlorophyll *a*, whereas lutein and the carotenoids within the xanthophyll cycle were less affected. Iron deficiency caused major increases in the lutein/chlorophyll *a* and xanthophyll cycle pigments/chlorophyll *a* molar ratios. Xanthophyll cycle carotenoids in Fe-deficient plants underwent epoxidations and de-epoxidations in response to ambient light conditions. In dark adapted Fe-deficient plants most of the xanthophyll cycle pigment pool was in the epoxidated form violaxanthin. We show, both by HPLC and by *in vivo* 505 nanometers absorbance changes, that in Fe deficient plants and in response to light, the de-epoxidated forms antheraxanthin and zeaxanthin were rapidly formed at the expense of violaxanthin. Several hours after returning to dark, the xanthophyll cycle was shifted again toward violaxanthin. The ratio of variable to maximum chlorophyll fluorescence from intact leaves was decreased by iron deficiency. However, in iron deficient leaves this ratio was little affected by light conditions which displace the xanthophyll cycle toward epoxidation or de-epoxidation. This suggests that the functioning of the xanthophyll cycle is not necessarily linked to protection against excess light input.

Plants grown under limited iron supply exhibit marked changes in the structure and function of chloroplasts (15). The most obvious characteristic of Fe-deficient leaves is the reduced amounts of pigments and other chloroplast membrane components per unit area when compared to control plants. Previous studies have indicated that not all photosynthetic pigments are decreased by Fe deficiency to the same extent, xanthophylls being less affected than Chls and β-carotene (4, 14). This has been recently confirmed by HPLC (16).

A specific group of xanthophylls are involved in the so-called xanthophyll cycle. Zeaxanthin (no epoxides) is formed by de-epoxidation of violaxanthin (two epoxides) via antheraxanthin (one epoxide). This reaction is thought to occur in the lumen of thylakoids, catalyzed by violaxanthin de-epoxidase. The back reaction (zeaxanthin to violaxanthin) is catalyzed by an epoxidase thought to be located in the stromal side (12, 18). Zeaxanthin formation has been recently proposed to be the mechanism responsible for part of the non-radiative energy dissipation in case of excessive light energy (3, 6–9). This has been taken as evidence that the pool of pigments within the xanthophyll cycle (VAZ pigments[3]) may constitute a protecting mechanism for PSII (and, in turn, for the whole photosynthetic machinery) in stress situations, when light energy input exceeds the capacity for energy utilization.

The development of new HPLC methodologies, permitting the separation of zeaxanthin, led to the finding that the characteristic relative enrichment in carotenoids induced by Fe deficiency was partially caused by VAZ pigments in pea (1) and pear leaves (2). The aim of the present paper was to characterize the xanthophyll cycle in Fe-deficient plants. Using Fe-stressed sugar beet (*Beta vulgaris* L.) plants, we describe the relative enrichment in VAZ pigments, the kinetics of pigment changes within the xanthophyll cycle as measured by HPLC and light-induced absorbance changes, and some characteristics of the Chl fluorescence induction curve as they relate to the xanthophyll cycle.

MATERIALS AND METHODS

Plant Culture

Sugar beet (*Beta vulgaris* cv Monohill) was grown in a growth chamber in half-Hoagland nutrient solution, with a PFD of 400 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR at a temperature of 25°C, 80% humidity, and a photoperiod of 16 h light/8 h dark.

Pigment Analysis

Pigments were extracted with acetone from liquid-nitrogen frozen leaf discs and stored at −30°C. Pigments were analyzed by the HPLC method described previously (15).

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[3] Abbreviations: VAZ pigments, violaxanthin + antheraxanthin + zeaxanthin; EI, epoxidation index of VAZ pigments; Fo, initial Chl fluorescence emission in the fluorescence induction curve; Fp, Chl fluorescence emission at the peak of the fluorescence induction curve; Fv, variable part of Chl fluorescence (Fp-Fo).

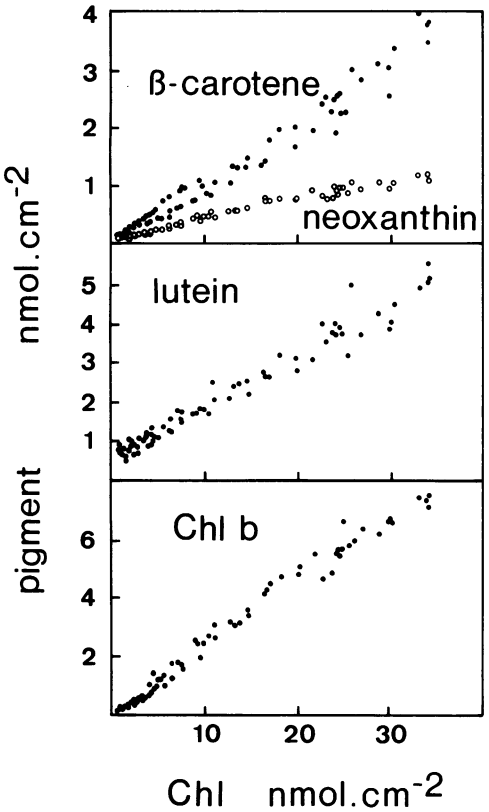

Figure 1. Neoxanthin, β-carotene, lutein, and Chl *b versus* total Chl. All pigments are expressed as nmol of pigment per cm^2 of leaf area. Samples were harvested 2 h after switching on the growth chamber lights.

Induction of Changes Within the VAZ Pool

De-epoxidation of pigments within the xanthophyll cycle pool was induced by switching on the lamps (total PAR 400 $\mu E \cdot m^{-2} \cdot s^{-1}$, 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ incandescent and 350 $\mu E \cdot m^{-2} \cdot s^{-1}$ fluorescent lamps) in the growth chamber at the end of the 8 h dark period. Epoxidation of pigments was induced by switching off the lights. The EI was defined from the relative percents of pigments within the VAZ cycle as (% violaxanthin × 2 + % antheraxanthin)/200. This index is an estimation of the actual number of epoxides over the maximum possible, *i.e.* as if all the VAZ pool was violaxanthin. A large EI (*i.e.* 1.0) would indicate a displacement toward violaxanthin, whereas a low EI (*i.e.* 0.1) would indicate that zeaxanthin and/or antheraxanthin account for a large part of the pool of VAZ pigments.

Measurement of Absorbance Changes at 505 and 535 nm

Light-dependent absorbance changes at 505 and 535 nm were measured in a Shimadzu 3000 double beam, double wavelength spectrophotometer. Absorbance changes shown here were measured in the double wavelength mode, using 560 nm as the reference wavelength. Similar results (not shown) were obtained in the double beam mode, placing neutral density filters in the reference beam. The end-on photomultiplier was protected by a 5 mm Schott blue BG 39 filter. Leaf pieces (2 × 1 cm) were placed in a plastic holder, 45° to the measuring beam. The sample was illuminated with an angle of 45° through a fiber optic with red actinic light (filter Schott RG 665 plus IR filters), entering the sample compartment from the front side. With this set-up, signal noise was 2 to 3.10^{-4} A. Opening of the actinic light (500 $\mu E \cdot m^{-2} \cdot s^{-1}$) produces no detectable transient change in absorbance. Spectra of the red light-induced absorbance changes were measured from 450 to 560 nm in the same apparatus in the double beam mode. Measuring times were kept to a minimum to minimize water loss through the exposed surface of the leaf.

Chl Fluorescence Measurements

Chl fluorescence was measured from intact leaves. Blue light (light from a 150 W tungsten lamp powered with a

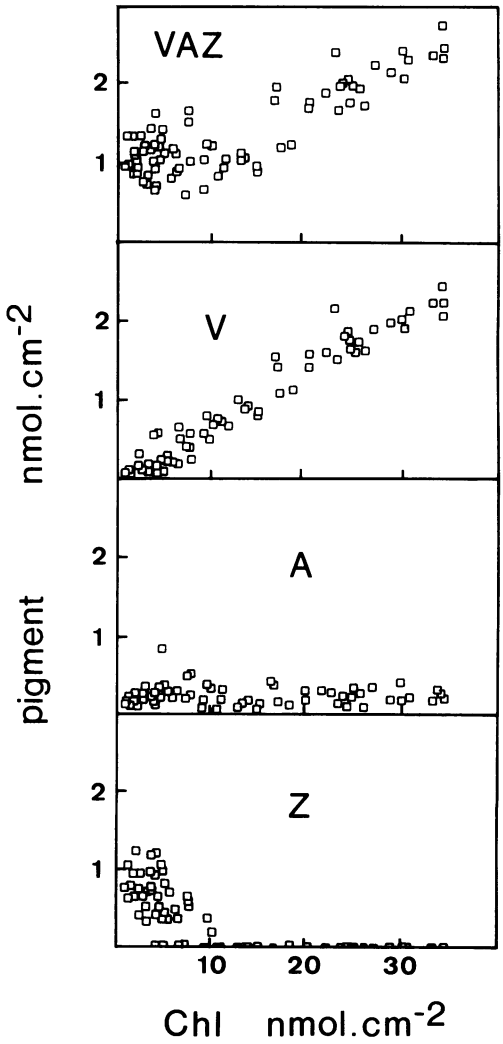

Figure 2. Violaxanthin (V), antheraxanthin (A), zeaxanthin (Z), and VAZ *versus* total Chl. All pigments are expressed as nmol of pigment per cm^2 of leaf area. Samples were harvested 2 h after switching on the growth chamber lights.

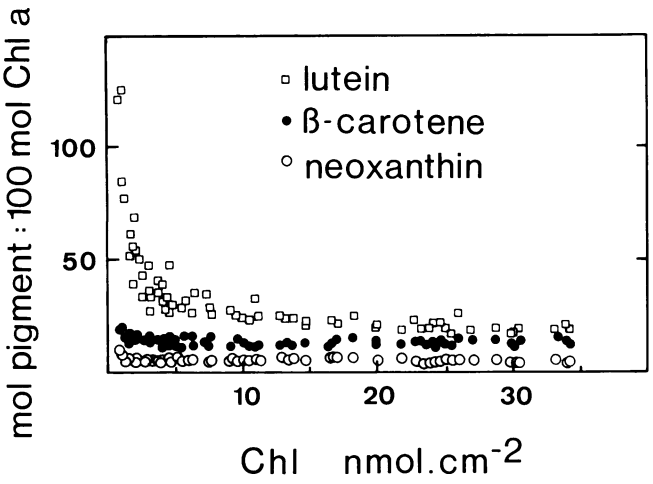

Figure 3. Number of lutein, β-carotene, and neoxanthin molecules per 100 molecules of Chl *a*, plotted *versus* total Chl per area. Samples were harvested 2 h after switching on the growth chamber lights.

stabilized power supply and passing through 1 KG1 and 3 KG3 Schott infrared filters plus a 580 nm cut-off filter) was passed through a Copal photographic shutter (opening time 2 ms) and a Schölly fiber optic guide. Light intensity was 150 $\mu E \cdot m^{-2} \cdot s^{-1}$ at the leaf level. Fluorescence was detected through a 3 mm Schott RG 665 filter and a 680 nm interference filter (10 nm bandpass) with a Hansatech photodiode, and the signal fed to a digital storage oscilloscope. Measurements were made at the end of the 8 h period of darkness and 2 h after switching on the growth chamber lights. Preilluminated plants were kept in the dark for 30 min. In previous experiments, preilluminated plants were kept in the dark for different times, and the Fv/Fp ratio was measured (not shown). This ratio did not increase further after 20 min of dark adaptation, indicating that the short-term fluorescence quenching mechanisms dissappeared by that time.

RESULTS

Iron Deficiency-Induced Changes in Pigment Composition

Iron deficiency decreased the Chl and carotenoid contents of sugar beet leaves (Figs. 1 and 2). Data indicate that the extent of the decrease depends on the specific pigment considered. With extreme iron deficiency sugar beet leaves lost over 95% of their Chls, β-carotene, and neoxanthin (Fig. 1). These chlorotic leaves, however, still conserved around 15% of the lutein (Fig. 1) and 40% of the VAZ pigments pool (Fig. 2) when compared with the controls. The de-epoxidated forms zeaxanthin and antheraxanthin accounted for most of the remaining VAZ pigment pool in Fe deficient sugar beet leaves, violaxanthin accounting only for a small part of the pool (Fig. 2).

When expressed on a per Chl *a* basis, data show that the number of neoxanthin and β-carotene molecules was practically unchanged by Fe deficiency (Fig. 3). The Chl *b*:Chl *a* ratio decreased only below Chl contents of 10 $nmol \cdot cm^{-2}$ (not shown). On the same basis, the number of lutein and VAZ pool molecules increased exponentially below Chl contents of 7 $nmol \cdot cm^{-2}$ (Figs. 3 and 4). The lutein:100 Chl *a* ratio increased from control values of 20 up to values higher than 100. The zeaxanthin:Chl *a* and antheraxanthin:Chl *a* ratios were increased by Fe deficiency, while the violaxanthin:Chl *a* ratio was decreased in most samples (Fig. 4). These relative changes in the pigments within the VAZ pool resulted in increases in the VAZ pigments pool:100 Chl *a* ratio from control values of 10 up to values higher than 100 (Fig. 4).

Epoxidation State of the Xanthophyll Cycle in Fe Deficient Leaves

Since the only difference among the three carotenoids involved in the violaxanthin cycle is the number of epoxides in the molecule, the extent of the pigment interconversions within the VAZ cycle can be described by an EI, defined as

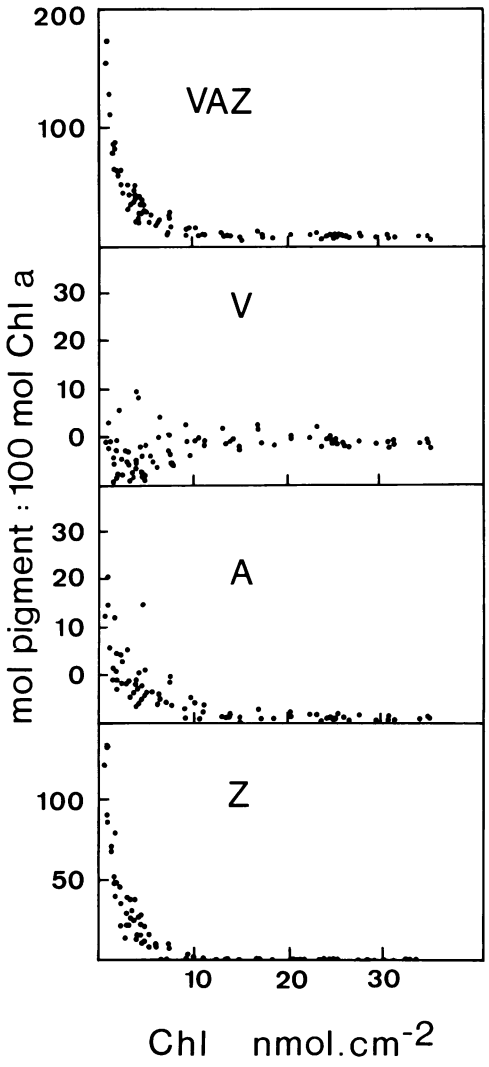

Figure 4. Number of violaxanthin (V), antheraxanthin (A), zeaxanthin (Z), and VAZ molecules per 100 molecules of Chl *a*, plotted *versus* total Chl per area. Samples were harvested 2 h after switching on the growth chamber lights.

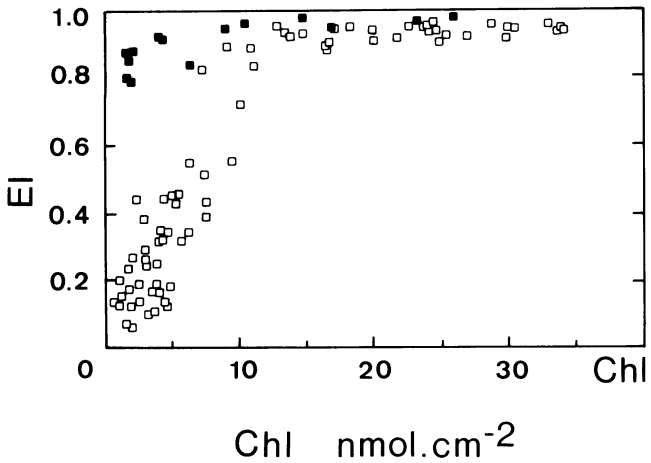

Figure 5. EI of the xanthophyll cycle in sugar beet plants as affected by Fe deficiency. Samples taken in the light (□); samples taken after 8 h dark (■).

indicated in "Materials and Methods." Experiments were run by sampling leaf discs from sugar beet plants in the growth chamber after the 8 h dark period, and then after several h in the light. Dark-sampled control sugar beet leaves exhibited EI values of around 0.9, whereas chlorotic leaves exhibited EIs in the range 0.7 to 0.9. Illumination caused only small decreases in the EI of control and moderately deficient leaves. However, the EI began to decrease significantly in illuminated, Fe-deficient sugar beet leaves when Chl decreases below 12 nmol·cm^{-2}, falling down to values as low as 0.1 in very chlorotic leaves (total Chl 2 nmol·cm^{-2}) (Fig. 5).

Kinetics of De-epoxidation and Epoxidation

The kinetics of de-epoxidation and epoxidation of the VAZ pigments pool was measured under the same light regime used for growing plants. After 8 h in the dark, sugar beet Fe deficient leaves (2–4 nmol Chl·cm^{-2}) had EIs of 0.70 to 0.85 (Fig. 6A). The kinetics of the light-induced, de-epoxidation of the VAZ pool in Fe deficient leaves was very fast; when the growth chamber light was switched on, 95% of the violaxanthin was converted rapidly (half-time less than 2 min) into antheraxanthin and zeaxanthin (Fig. 6A). After this rapid decline the EIs decreased slowly, reaching values of around 0.15 after 4 h of illumination. The de-epoxidation exhibited similar kinetics when other light qualities were used to induce the de-epoxidation (not shown).

The kinetics of the dark-induced epoxidation of the VAZ pool in Fe deficient leaves was much slower. After several hours of light, Fe deficient leaves had EIs of less than 0.2 (Fig. 6B). When lights were switched off, the EIs increased gradually, reaching values of around 0.9 after 11 h of darkness (Fig. 6B). The half-time for epoxidation was 3.5 to 4 h. It is noteworthy that the VAZ pigment pool in some Fe deficient leaves was not completely epoxidated after the normal dark period of 8 h (Fig. 6, A and B).

Light-Induced *in Vivo* Absorbance Changes

Changes in the epoxidation state of VAZ cycle can be measured by following the changes in A_{505} in chloroplasts (17) or leaf pieces (3). To run this experiments, we chose a intensity of the red actinic light (150 μE·m^{-2}·s^{-1}) which induces only small changes in A_{505} in the control plant (Fig. 7B).

In Fe-deficient leaves (Fig. 7A) the actinic light induced very large changes in A_{505}, together with smaller changes in A_{535}. Upon darkening, there was a further increase in A_{505}, an observation previously reported to occur in control leaves (3). The changes in A_{505} were irreversible in the short term in the darkness, as expected from the epoxidation kinetics shown in Figure 6. Although changes in A_{535} were expected to arise from the light-induced proton gradient, at least part of the signal was also irreversible in the darkness, indicating that the changes induced by the violaxanthin cycle were also observed at this wavelength (see below).

Difference spectra (450–560 nm) were run at different times during the measurement, using the spectrum of the dark adapted leaf, before any actinic illumination, as the baseline. Typical difference spectra from a Fe-deficient leaf are shown in Figure 7A (inset). Spectra run after 5 min illumination (light minus dark) showed a peak at 505 nm, a second peak at 467 nm, a shoulder at 535 nm, and troughs at 481 and 451 nm (Fig. 7A, inset, l–d). A significant absorbance change occurred at 535 nm, suggesting that changes in the VAZ cycle will result in changes in A_{535}. The spectrum was characteristic of the displacement of the xanthophyll cycle toward de-epoxidation (3, 17). A second difference spectrum was obtained from the same leaf piece 5 min after switching the red actinic light off, again using the spectrum of the dark adapted

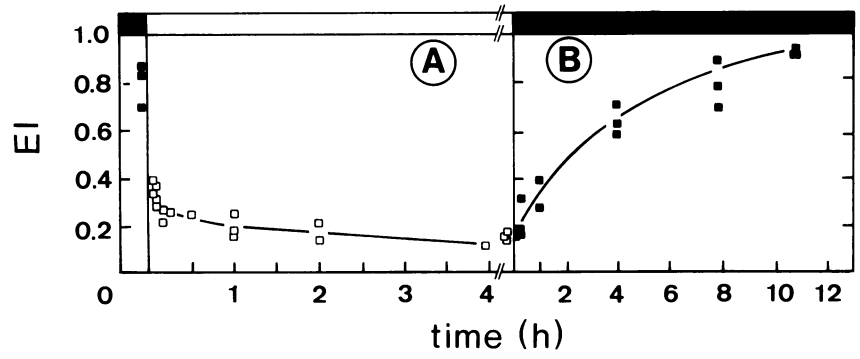

Figure 6. Light-induced changes in the EI of Fe deficient sugar beet plants. A, Changes induced by switching on the growth chamber lights; B, changes induced by switching off the growth chamber lights. Samples taken in the light (□); samples taken in the dark (■).

leaf, before any actinic illumination, as the baseline. This new spectrum exhibited the same maxima and valleys that the light minus dark spectrum, but the shoulder at 535 nm was absent (Fig. 7A, inset, d*–d). This shows that the shoulder at 535 nm in the light difference spectra is reversible upon darkening, and can be attributed to the light-induced proton gradient. This component, with a single maximum at 535 nm, was also observed in the difference spectra actinic light on minus actinic light off (not shown). All these light-induced spectral changes were very similar to those described by Bilger *et al.* (3) for control leaves.

In our control leaves and at this low actinic light intensity, light-dependent changes in A_{505} were small (Fig. 7B). Upon darkening, we observed again an A_{505} increase, similar to that observed by Bilger *et al.* (3). Higher light intensities, similar to that used by Bigler *et al.* in (3), induced much larger A_{505} changes in a control leaf (not shown). At this low actinic light intensity, control leaves exhibited light-induced changes in A_{535} that were partially reversible after darkening, indicative of the light-induced proton gradient (Fig. 7B).

A typical difference spectrum from a control leaf after 5 min illumination showed a broad peak at 509 nm, a second peak at 467 nm, a shoulder at 535 nm, and troughs at 482 and 453 nm (Fig. 7B, inset, l–d). A second difference spectrum was obtained from the same leaf piece 5 min after switching the red actinic light off, which exhibited the same maxima and valleys as the light minus dark spectrum, but the maximum was shifted to 505 nm and the shoulder at 535 nm was absent (Fig. 7B, inset, d*–d). Again, this can be attributed to the reversible light-induced proton gradient.

The difference spectra shown here may indicate that, in spite of the small A_{505} change, a partial displacement of the xanthophyll cycle toward de-epoxidated forms may occur in the control leaves at this low light intensity. Indeed, samples taken from illuminated leaf pieces in experiments similar to that shown in Figure 7 and analyzed by HPLC exhibited small

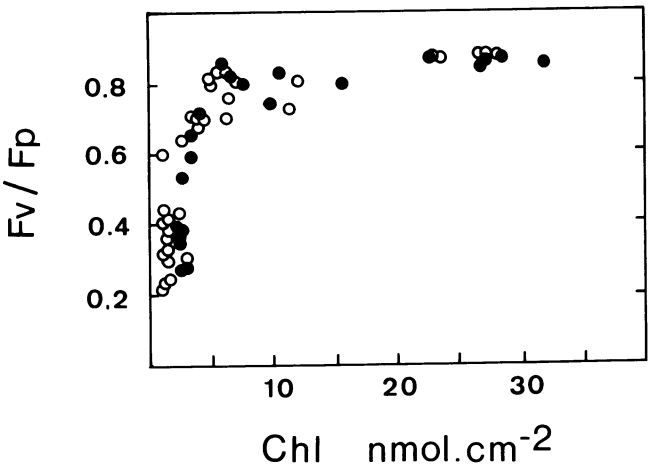

Figure 8. Ratio of variable to maximum fluorescence (Fv/Fp) measured at room temperature from intact sugar beet leaves as affected by Fe deficiency. Leaves were dark adapted overnight (8 h dark) or for 30 min after 2 h light, as explained in the text. Samples taken in the light (○); samples taken after 8 h dark (●).

increases in antheraxanthin (but not zeaxanthin). A detailed analysis of the relationship between absorbance changes and HPLC measurements is under investigation.

Chl Fluorescence Kinetics and Fe Deficiency

Chl fluorescence was measured at two different times during the day, at the end of the 8 h dark period and after 2 h in the light (plus 30 min dark adaptation). Chl fluorescence from control, Fe-sufficient leaves showed Fv/Fp ratios higher than 0.85, both in the dark and after 2 h light (Fig. 8). This was expected, since in these control leaves the xanthophyll cycle is practically unaffected by prolonged darkness and light, being displaced at all times toward epoxidation (Fig. 5). In

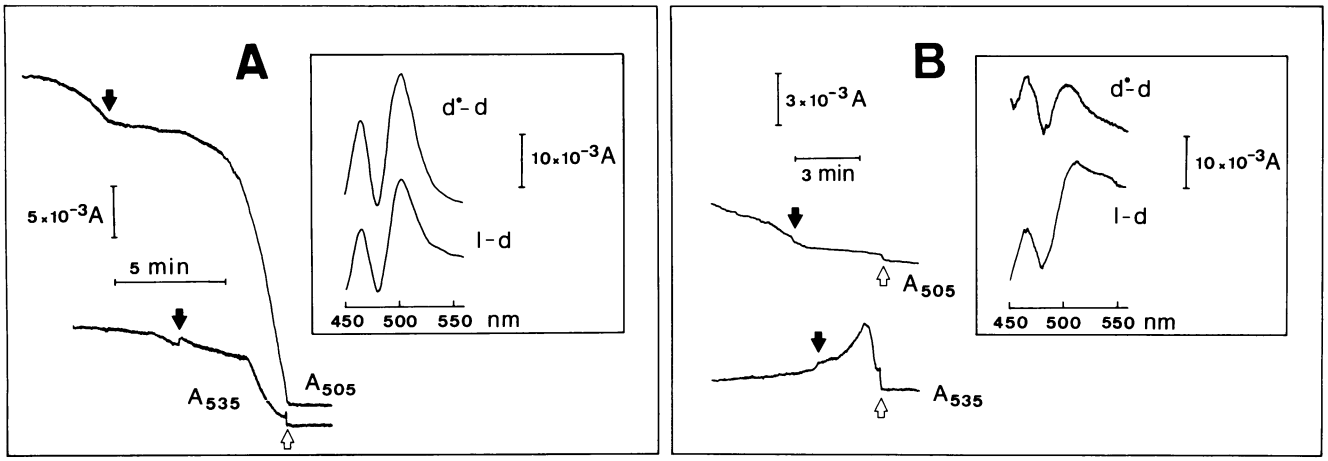

Figure 7. Light-induced changes in absorbance. The apparatus set-up is described under "Materials and Methods." Actinic red light was 150 $\mu E \cdot m^{-2} \cdot s^{-1}$. Open arrows, actinic light on; closed arrows, actinic light off. Inset, difference spectra 5 min darkened leaf after illumination-leaf in the dark before illumination (d*–d) and illuminated leaf-leaf in the dark before illumination (l–d). A, Fe-deficient leaves—different pieces from the same Fe-deficient leaf were used to produce the different curves; B, control, Fe-sufficient leaves—different leaf pieces from the control leaves were used to produce the different curves.

moderately deficient leaves (down to 7 nmol Chl/cm^2) the Fv/Fp ratio exhibited small decreases (down to 0.80), and only when Chl decreased further, iron deficient leaves exhibited major decreases in the Fv/Fp ratio (Fig. 5). Decreases in the Fv/Fp ratio of iron deficient leaves resulted from moderate increases in Fp and relatively larger increases in Fo (not shown). The Fv/Fp ratios in Fe deficient leaves were similar after 8 h dark and after 2 h light (plus 30 min dark adaptation), extreme cases when the xanthophyll cycle is almost completely displaced toward epoxidation and de-epoxidation, respectively (Fig. 5).

DISCUSSION

In this work we characterize the changes induced by iron deficiency in the pigment composition of sugar beet leaves. It was previously known that iron deficiency decreased more the amount of Chls and β-carotene than that of xanthophylls (4, 14). Our data show that iron deficiency decreased photosynthetic pigments to different extents. Among the carotenoids, iron deficiency decreased neoxanthin and β-carotene concomitantly with Chl *a*. Conversely, lutein and the pool of VAZ pigments were much less affected. These pigment changes resulted in major increases—up to 10 times—in the lutein/Chl *a* and VAZ pigments/Chl *a* molar ratios without changing the neoxanthin/Chl *a* and β-carotene/Chl *a* molar ratios. Thus, Fe-deficient leaves exhibited stoichiometries of 1 VAZ molecule per 1 to 2 Chls, compared to 1 VAZ molecule per 13 to 14 Chls in the control, Fe-sufficient plants.

The carotenoids within the xanthophyll cycle in Fe deficient plants undergo epoxidations and de-epoxidations in response to ambient light conditions. In the dark, most of the VAZ pigment pool was in the epoxidated form violaxanthin. In the Fe deficient plants and in response to light, HPLC measurements and *in vivo* absorbance changes reveal that the de-epoxidated forms antheraxanthin and zeaxanthin were rapidly formed, at the expense of violaxanthin. Several hours after returning to dark, the VAZ cycle was shifted again toward violaxanthin. This fully functional VAZ cycle sharply contrasts with the behavior of VAZ pigments in other carotenoid enriched materials. Etiolated or senescent leaves also exhibited large increases in the ratio VAZ pigments/Chl (data not shown), but no interconversions were detectable under short-term (several min) illumination of dark adapted leaves.

When light energy input exceeds the capacity for energy utilization, the light-induced displacement of the VAZ cycle toward de-epoxidation (*i.e.* formation of about 1 molecule of zeaxanthin per 14 Chl molecules) has been recently shown to be paralleled by decreases in the fluorescence yield. In plants affected by several environmental stresses, changes in the Fv/Fp ratios, together with changes within the xanthophyll cycle, have been recently reported (3, 6–9). Although most of these reports indicate a strict kinetic relationship between fluorescence yield loss and zeaxanthin formation, Foyer *et al.* (10), indicated that the kinetics of both phenomena may be different during photoinhibition.

Our experiments indicate that in Fe deficient leaves the increase in the carotenoids to Chl ratio was accompanied by a decrease in the variable to maximum ratio of Chl fluorescence from PSII (Fv/Fp). Decreases in the variable fluorescence have been previously reported in cyanobacteria affected by iron deficiency (11, 13). Our data also indicate that the light-induced full displacement of the xanthophyll cycle toward de-epoxidation in Fe-deficient plants, *i.e.* formation of 1 molecule of zeaxanthin per 1 to 2 Chl molecules, did not produce further significant changes in the Fv/Fp ratio. This indicates that most of the decrease in the Fv/Fp ratio was zeaxanthin independent.

Data obtained with the Fe-deficiency system used here support the idea that zeaxanthin formation is not always connected to significant changes in Chl fluorescence. Bilger *et al.* (3) also showed that leaf discs incubated with ascorbate at pH 5 formed zeaxanthin but did not exhibit the changes in fluorescence usually associated with the cycle. Our data may indicate that zeaxanthin formation only results in these changes if the membrane fulfills some specific requirements which are not met in the Fe-deficient plants. Alternatively, zeaxanthin formation may be just a consequence of the light-induced pH gradient, and may bear no causal relationship to fluorescence changes.

In summary, iron deficient plants exhibit a large pool of xanthophyll cycle molecules, together with a major depletion in Chl. Additionally, this pool of xanthophylls undergoes interconversions similar to those occurring in green leaves under excess light energy input. Because of these reasons, we believe that the Fe deficiency system may provide an excellent tool to study the possible functional implications of the xanthophyll cycle. Further experiments, using modulated fluorescence, are under way to further characterize the molecular basis of Fe deficiency and its associated pigment changes.

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