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# The chl *a* fluorescence intensity is remarkably insensitive to changes in the chlorophyll content of the leaf as long as the chl *a*/*b* ratio remains unaffected

Emine Dinç [a,1], M. Georgina Ceppi [b,1], Szilvia Z. Tóth [a], Sándor Bottka [a], Gert Schansker [a,b,1,*]

[a] Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, H-6701 Szeged, Hungary
[b] Laboratory of Bioenergetics, University of Geneva, CH-1211 Geneva, Switzerland

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ABSTRACT

The effects of changes in the chlorophyll (chl) content on the kinetics of the OJIP fluorescence transient were studied using two different approaches. An extensive chl loss (up to 5-fold decrease) occurs in leaves suffering from either an $Mg^{2+}$ or $SO_4^{2-}$ deficiency. The effects of these treatments on the chl *a*/*b* ratio, which is related to antenna size, were very limited. This observation was confirmed by the identical light intensity dependencies of the K, J and I-steps of the fluorescence rise for three of the four treatments and by the absence of changes in the $F_{685\ nm}/F_{695\ nm}$-ratio of fluorescence emission spectra measured at 77 K. Under these conditions, the $F_0$ and $F_M$-values were essentially insensitive to the chl content. A second experimental approach consisted of the treatment of wheat leaves with specifically designed antisense oligodeoxynucleotides that interfered with the translation of mRNA of the genes coding for chl *a*/*b* binding proteins. This way, leaves with a wide range of chl *a*/*b* ratios were created. Under these conditions, an inverse proportional relationship between the $F_M$ values and the chl *a*/*b* ratio was observed. A strong effect of the chl *a*/*b* ratio on the fluorescence intensity was also observed for barley *Chlorina f2* plants that lack chl *b*. The data suggest that the chl *a*/*b* ratio (antenna size) is a more important determinant of the maximum fluorescence intensity than the chl content of the leaf.



## 1. Introduction

Chlorophyll (chl) *a* fluorescence is the light emitted by chl *a* molecules upon excitation by light. Extending this observation it could be argued that if the antennae of all photosystem II (PSII) reaction centers would contribute to the chl *a* fluorescence emitted by that leaf, a strong relationship between the chl content of a leaf and the intensity of the fluorescence emitted by that leaf would be expected. However, there are several factors that potentially complicate the relationship between chl content and chl *a* fluorescence intensity. The palisade parenchyma cells act like light guides for the incoming light, whereas the spongiform parenchyma cells diffuse the light that passes through [1]. As the light penetrates deeper into the leaf the light intensity declines rapidly [2,3], although it has to be noted that the light profile as a function of the depth inside the leaf is wavelength dependent. Green light, for example, is less efficiently absorbed by chl and as a consequence its light gradient is not as steep as that of blue or red light [4–6]. Since the chloroplasts located deeper in the leaf perceive a lower photon flux they will also emit less fluorescence since the fluorescence intensity is linearly related to the intensity of excitation light (see e.g. the inset of Fig. 5 in ref. [7]). Numerous studies on the light acclimation of leaves to their light environment have shown that chloroplasts located deeper in the leaf are acclimated to lower light intensities (e.g. [5,8–10]). This means that their photosystems have larger antenna sizes (lower chl *a*/*b* ratios) to make optimal use of the lower internal light intensity [9,11,12]). Chloroplasts are also known to respond to changes in their light environment. It has been shown that an increase of the photon flux density will induce an acclimative response of the chloroplasts optimizing their photosynthetic system, i.e. adjusting their antenna size, the PSII density, etc. (e.g. [13,14]). However, changes in the leaf chl content due to certain stress conditions will also change the photon flux density perceived by the chloroplasts in the leaf and one would expect this to lead to an acclimative response as well. Re-absorption of fluorescence by photosystem I (PSI) is another factor determining the fluorescence intensity that has been considered in the literature (e.g. [15]). In contrast, Hsu and Leu [16]) demonstrated that a stack of two leaves emit more fluorescence (at wavelengths at which fluorescence is recorded by commercial fluorimeters) than a single leaf, suggesting

*Abbreviations:* C, control = complete solution; CAB, chlorophyll *a*/*b* binding protein; chl, chlorophyll; DMF, N,N-dimethylformamide; $F_0$ and $F_M$, fluorescence intensity measured when all photosystem II reaction centers are open or closed respectively; FR, far red; $F_V$, variable fluorescence; HS, half-$SO_4^{2-}$ solution; ODN, oligodeoxynucleotide; OJIP-transient, fluorescence induction transient defined by the names of its intermediate steps: $O$ = 20 μs, $J$ = 3 ms, $I$ = 30 ms and $P$ = the maximum fluorescence intensity; PQ, plastoquinone; PSII and PSI, photosystems II and I, respectively; $Q_A$ and $Q_B$, primary and secondary quinone electron acceptors of photosystem II respectively; TS, traces of $SO_4^{2-}$ solution; $V_J$ and $V_I$, height of the J and I steps as a fraction of the variable fluorescence; WM, without magnesium and half-$SO_4^{2-}$ solution

* Corresponding author at: Institute of Plant Biology, Biological Research Center Szeged, Hungarian Academy of Sciences, H-6701 Szeged, Hungary. Tel.: +36 62 599 722; fax: +36 62 433 434.
*E-mail address:* gert.schansker@gmail.com (G. Schansker).
[1] These authors contributed equally to the work.

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that cells located deep in the leaf still make a significant contribution to the fluorescence intensity.

The factors discussed above make it difficult to predict what the effect of chl loss on the intensity of the measured chl *a* fluorescence intensity will be. In this study our aim was to resolve two points: 1. what is the relationship between chl content and the measured $F_0$ or $F_M$ intensities and 2. what is the effect of changes in the chl content on the fluorescence kinetics. To this end we have evaluated the effect of the leaf chl content on the amplitude and kinetics of the OJIP-transient using two approaches: 1. the induction of mineral deficiencies ($SO_4^{2-}$ and $Mg^{2+}$ deficiencies) in hydroponically grown sugar beet plants leading over time to a massive chl loss in the deficient leaves without changes in the chl *a*/*b* ratio and without a strong effect on the functionality of the remaining photosynthetic electron transport chains and 2. the creation of a wide range of chl *a*/*b* ratios (i.e. differences in the antenna size) using etiolated wheat leaves treated with 10 different antisense oligodeoxynucleotides (ODNs) that were allowed to green for either 8 or 24 h. These antisense ODNs were specifically designed to interfere with the translation of mRNA of genes coding for chl *a*/*b* binding proteins ([17] and references therein). Our data suggest that changes in the chl *a*/*b* ratio (= differences in antenna size) have much more effect on the shape and amplitude of the measured OJIP-transients than changes in chl content that do not affect the chl *a*/*b* ratio.

## 2. Materials and methods

### 2.1. Plant material and growth conditions

Sugar beet—For a description of the growth conditions of the sugar beet plants see ref. [18].

Barley *Chlorina f2*—Wild type and *Chlorina f2* plants were grown on perlite in a greenhouse, under short day conditions. The temperature was about 20 °C during the day and 18 °C during the night. Seedlings were watered twice with Knopp's solution and they were used when they were 7 to 10 days old. The lack of chl *b* in the barley *Chlorina f2* was confirmed by a chl-content determination (see below).

Wheat—Wheat seeds (*Triticum aestivum*, genotype CY-45) were sown in pots filled with commercial soil. The pots were kept in a growth chamber in total darkness for 11 days.

### 2.2. Mineral deficiency treatments of sugar beet plants

For a description of the hydroponic solutions used for the mineral deficiency experiments see ref. [18]. The effects of the mineral deficiency on the sugar beet plants were followed for 1 month.

### 2.3. Design and synthesis of antisense ODNs

Oligodeoxynucleotide sequences were selected based on a search for freely accessible single stranded loops in the mRNA secondary structure (M. Zuker—Mfold web server for nucleic acid folding and hybridization prediction; http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form [19]). Synthesis of ODNs was performed using an Expedite 8909 DNA synthesizer (Applied Biosystems, Foster City, CA) by standard cyanoethyl phosphoramidite chemistry. All the other reagents for the automated ODN synthesis were purchased from Applied Biosystems as well. The ODNs were purified on Poly-PAK cartridges (Glen Research, Sterling, VA) yielding a population of >97% full sized ODNs as shown by analytical ion exchange HPLC. For further details see ref. [17].

### 2.4. ODNs synthesized

In wheat (*Triticum aestivum*) ODN(1) 5′-TATGGTGTGTTGTCCCTGT-3′ (18 mer), ODN(2) 5′-ACTTATGGTGTGTTGTCC-3′ (18 mer), ODN(3) 5′-GGAAAGAGACATGGTGG-3′ (17 mer), ODN(4) 5′-AGTTCTT-GACGGCCTTGC-3′ (18 mer), ODN(5) 5′-GACGAGGGCAAGTTCTT-3′ (17 mer), ODN(6) 5′-AGAGCACACGGTCAGAG-3′ (17 mer), ODN(7) 5′-GAAGGTCTCAGGGTCAG-3′ (17 mer), ODN(8) 5′-TCGCTGAAGATCT-GAGAG-3′ (18 mer), ODN(9) 5′-ACGGCCCCCATGAGCACAA-3′ (19 mer), ODN(10) 5′-GCGAGGCGGCCATTCTTG-3′ (18 mer) chl *a*/*b*-binding protein (*cab*) antisense ODNs were used. ODNs were complementary to a deposited wheat *cab* mRNA sequence (Gen Bank accession number M10144.1) at the positions 42–24, 46–28, 82–65, 115–97, 126–109, 227–210, 331–314, 476–458, 568–549 and 716–698. As control, a 17-mer random-nonsense 5′-GGCGGCTAACGCTTCGA-3′ ODN was used.

### 2.5. Antisense ODN treatment of wheat leaves

Etiolated wheat leaves were detached and the lower 1–2 cm parts were submerged in water containing 10 μM of one of the ten *cab* antisense ODNs, a random-nonsense ODN (control) or distilled water (control-water). After 12 h of dark-incubation leaves were illuminated (100 μmol photons $m^{-2}\,s^{-1}$; white light of fluorescent tubes Philips TL-D 18W/33-640) for 24 h. Data from three independent experiments were used and for each experiment four leaves were treated with each antisense ODN. OJIP-transients were measured after 8 and 24 h of illumination. Following the fluorescence measurements the leaf segments were harvested and stored at −80 °C until further use.

### 2.6. Measuring equipment

Chl *a* fluorescence measurements were made using a PEA Senior instrument or a Handy-PEA instrument (Hansatech Instruments Ltd, King's Lynn, UK); for further details on the PEA Senior see ref. [20,21] and for the Handy-PEA see ref. [22]. Leaf samples were illuminated with 1800 μmol photons $m^{-2}\,s^{-1}$ produced by four 650-nm LEDs in the case of experiments with the PEA Senior and 3500 μmol photons $m^{-2}\,s^{-1}$ produced by three 650-nm LEDs in the case of experiments with the Handy-PEA.

### 2.7. Determination of Chl content

To follow changes in the chl content of the leaves during the mineral deficiency treatment, each measuring day leaves from the different treatments were measured with a chl content meter CL-01 (Hansatech Instruments Ltd, King's Lynn, UK). The instrument estimates the chl content on the basis of the absorbance at 620 and 940 nm. To calibrate the instrument, parallel measurements were made on spots on the leaves that were subsequently extracted with 80% acetone (see ref. [23] for further details) after which the chl content was determined according to ref. [24], using a Lambda 3 UV/Vis spectrophotometer (Perkin Elmer, Massachusetts, US). The plants were dark-adapted for 1 h before the measurements with the CL-01 were made, which led to more consistent results (MG Ceppi, unpublished observations). A good correlation between the chl $(a+b)$ content of the leaves expressed per $cm^{-2}$ and the readout of the CL-01 was found (Fig. 1). The correlation was approximately linear between 20 and 100 μg chl $(a+b)$ $cm^{-2}$. This is in agreement with the measurements in ref. [25] where a linear correlation between the CL-01 readings and chl content for 4 different plant species was found.

For the *Chlorina f2* and the greening experiments the chl content was determined following N,N-dimethylformamide (DMF) extraction [26]. The absorbance was measured at 647 and 664.5 nm using a Hitachi U-2900 spectrophotometer. The chl content was determined according to ref. [27].

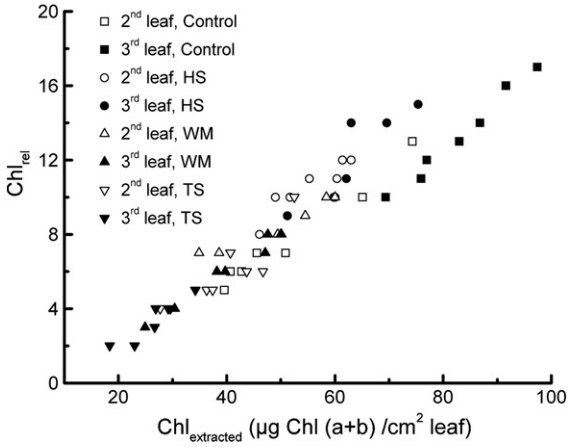


**Fig. 1.** Correlation between relative chl content determined by a chl meter (CL-01) and the chl content based on an 80% acetone extraction. Plants had been grown for 29 days on different hydroponic solutions when the chl contents were determined.

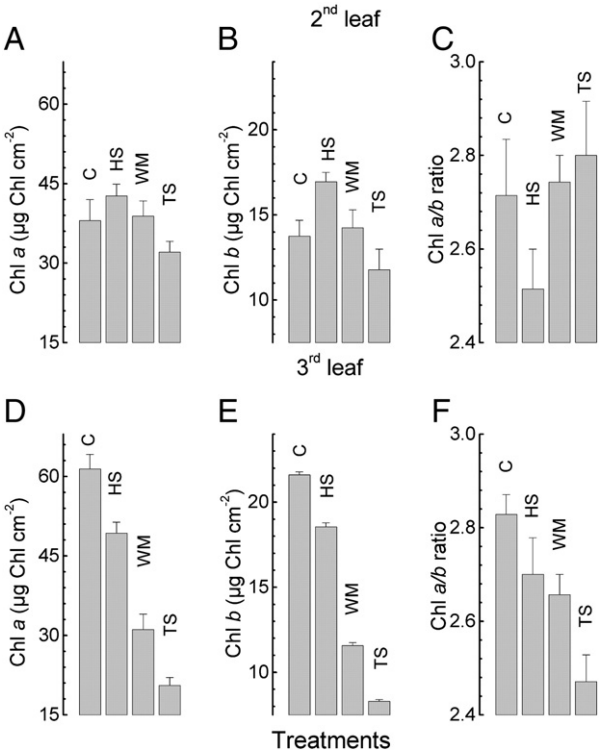


**Fig. 2.** Chl *a* and chl *b* contents and chl *a*/*b* ratios of 2nd and 3rd leaves of sugar beet plants grown for 29 days on different hydroponic solutions. The leaves were extracted with 80% acetone as described in ref. [23]. The values represent the averages of 7 independent measurements with their standard error.

### 2.8. 77K-measurements

Low temperature (77 K) chl *a* fluorescence spectra were determined between 600 and 800 nm. Leaf discs of 0.9 cm diameter were taken from dark-adapted leaves, placed in the sample holder and frozen rapidly in liquid nitrogen. The leaf discs were excited at 633 nm with a He/Ne laser (20 $Wm^{-2}$). A multi-branched fiber-optic bundle was used to guide the excitation and fluorescence emission light to and from the leaf disc. The emitted fluorescence was detected by an S-20 Hamamatsu photomultiplier (R928; Hamamatsu, Japan). The excitation light was passed through a monochromator (H 10 VIS, 0.5-nm slit; Jobin Yvon SAS, Longjumeau, France) equipped with a scan controller. The photomultiplier was protected by a red cut-off filter (CS 2-64; Corning, USA).

## 3. Results

### 3.1. Modification of the Chl content upon $Mg^{2+}$ and/or $SO_4^{2-}$ deficiency

Sugar beet plants were grown for more than 4 weeks on hydroponic solutions that differed in their $Mg^{2+}$ and $SO_4^{2-}$ concentrations. Fig. 1 demonstrates that 29 days after transferring sugar beet plants from a complete solution to one containing half the $SO_4^{2-}$ concentration (HS), a combination of half the $SO_4^{2-}$ concentration and no $Mg^{2+}$ (WM), only traces (2.1 µM) of $SO_4^{2-}$ (TS), or the same complete solution (C) resulted in leaf chl contents ranging from 18 to 97 µg chl $cm^{-2}$ corresponding to 1100 to 3500 µg chl/g FW. Fig. 1 also shows that the non-invasive absorbance measurements made with a CL-01 chl content meter correlated nearly linearly with the chl $(a+b)$ contents determined by 80% acetone extraction expressed per $cm^2$. For a more complete description of the effects of $SO_4^{2-}$ and $Mg^{2+}$ deficiency on sugar beet plants see ref. [18,23].

Fig. 2 shows that the effect of the treatments on the chl content was rather limited (less than 10%) if the leaves had already developed before the start of the treatment (second leaves). In general, the chl *a*/*b* ratios changed only slightly in response to a $SO_4^{2-}$ or $Mg^{2+}$ deficiency but in the case of the second leaves of plants grown under HS conditions a relatively large decrease in the chl *a*/*b* ratio was found (2.5 compared with 2.7–2.8 for the other three treatments, Fig. 2). For the third leaves, which developed after the start of the treatment, the chl content of the plants grown under WM and TS conditions was considerably lower than the chl content of leaves grown under C and HS conditions. In the case of TS plants this resulted also in a lower chl *a*/*b* ratio. The lower chl *a*/*b* ratio may have been due to a relatively stronger PSI loss in these leaves [18] since PSI is known to have a higher chl *a*/*b* ratio than PSII [27,28].

Information on the antenna size of PSII and the electron transport processes was obtained by determining the light intensity dependencies of the fluorescence intensity at 300 µs (the position of the K peak, that probably represents one charge separation at the light intensity used [22]), and the J and I steps (Fig. 3). Since the fluorescence rise follows the stepwise reduction of the electron transport chain [21,29], the light intensity dependence of the I step gives also information on the rate with which the PQ pool is reduced. Fig. 3 shows that light intensity dependencies are nearly identical for the C, HS and WM treatments. For the TS treatment the light intensity dependencies were quite different, which might be due to the fact that plants suffering from a severe $SO_4^{2-}$ deficiency have a more reduced PQ pool (see Discussion and [23]). The strong heterogeneity of the leaf material grown under TS conditions was responsible for the much stronger variability between measurements when compared with the data of the other treatments.

In Fig. 4 the relationship between chl content (measured with the CL-01 chl content meter) and $F_0$ and $F_M$ values was determined for the second and third leaves. Fig. 4 shows that the treatments strongly affected the chl content but had nearly no effect on the $F_0$ and $F_M$ values with two exceptions. A decrease in the $F_M$ values of the third leaves of the $Mg^{2+}$ deficient plants was observed that was not accompanied by a change in their $F_0$ value (Fig. 4C) and an increase of the $F_0$ values of the third leaves of the $SO_4^{2-}$-deficient plants was observed that was not accompanied by a change in the $F_M$ value (Fig. 4D).

Double normalization of the fluorescence transients between O and P eliminates amplitude information and focuses attention on changes in the fluorescence kinetics. In Fig. 5 the $V_J$ $((F_J-F_0)/(F_M-F_0))$ and $V_I$ $((F_I-F_0)/(F_M-F_0))$ values are given as a function of the chl content. For the plants grown under HS conditions almost

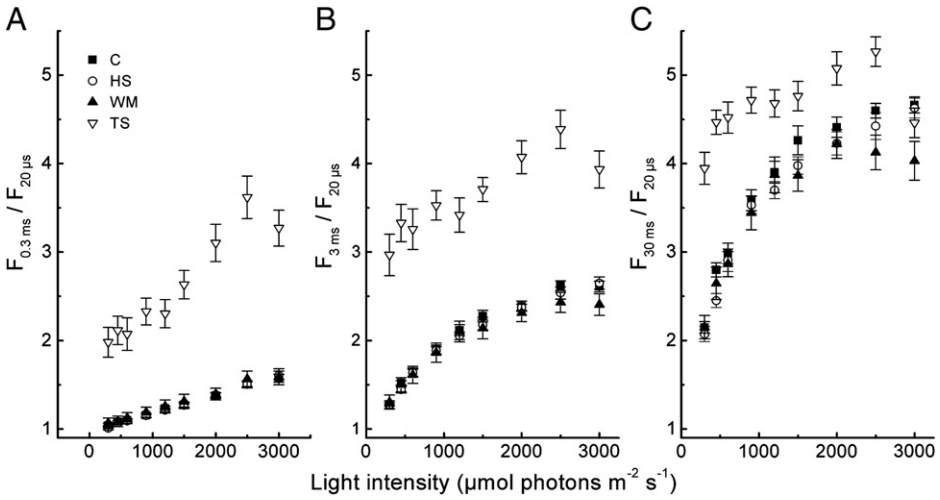


**Fig. 3.** Light intensity dependencies of the $F_{300\ \mu s}$ ($F_K$) shown in panel A, $F_{3\ ms}$ ($F_J$) shown in panel B and $F_{30\ ms}$ ($F_I$) shown in panel C for the four different treatments made on the 3rd leaves of the sugar beet plants after 24 days of the mineral deficient treatments. Each measuring point represents the average of 10–12 measurements with their standard error.

no change in the chl content and in the $V_J$ and $V_I$ values was observed. For the control and $Mg^{2+}$ deficient plants a slight increase of both $V_J$ and $V_I$ was observed as the chl content decreased, which could point towards a higher level of saturation of these fluorescence steps. The plants grown under TS conditions form a special case. There, the higher $V_J$ values were due to a more reduced PQ pool in darkness (see Discussion and [23]) and the higher $V_I$ values were due to a relatively stronger loss of PSI [18].

At the end of the deficiency treatments 77 K fluorescence emission spectra were determined on leaf discs of the third leaf pair (Table 1). The $F_{685\ nm}/F_{694\ nm}$ ratio was very similar for all four treatments (Table 1). The ratio $F_{685\ nm}/F_{694\ nm}$ is sensitive to the antenna size of PSII as shown e.g. in ref. [30] for greening pea leaves and the unchanged ratio confirmed that the treatments had little effect on the antenna size of the PSII. A point worth noting is that for leaves grown under WM conditions the $F_{694\ nm}/F_{730\ nm}$ did not decrease, which one might have expected in the case of increased spillover [31], instead it even increased somewhat. For plants grown under TS conditions a clear increase of the $F_{694\ nm}/F_{730\ nm}$ ratio was observed, which confirmed the PSI loss reflected by the chl *a*/*b* ratio (see above) and the loss of 820 nm transmission signal in these leaves [23].

### 3.2. The effect of changes in the antenna size on the fluorescence levels $F_M$ and $F_0$

The mineral deficiencies strongly affected the chl content of the leaves, but had relatively little effect on the chl *a*/*b* ratio and, therefore, on the antenna size, as shown by the unchanged dependence of the $F_{300\ \mu s}$ values on the light intensity and the 77 K fluorescence data. *Chlorina f2* is a well known mutant of barley that lacks chl *b*, and as a consequence has a reduced antenna size (e.g. [32]). The reduction of the antenna size led to a lower chl content as well: WT = 768.0 ± 43.7 μg chl/g FW and *Chlorina f2* = 374.2 ± 45.1 μg chl/g FW. In Fig. 6, OJIP transients of *Chlorina f2* and wild-type barley plants are compared. The kinetics of the fluorescence rise were quite similar, but the $F_0$ value of the mutant is 74% of the wild type and the $F_M$

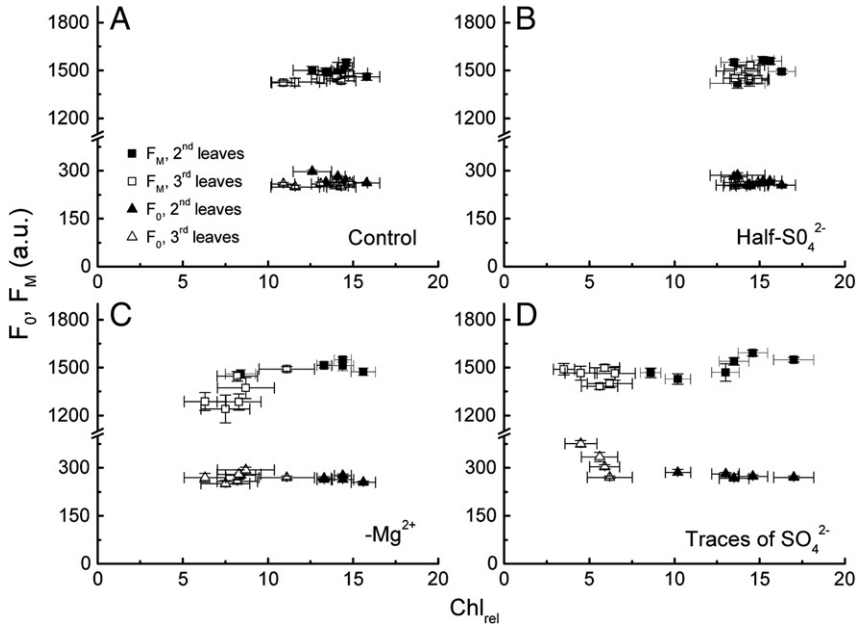


**Fig. 4.** Correlation between the $F_0$ and $F_M$ levels and the chl content of sugar beet leaves for the control treatment (panel A), the half-$SO_4^{2-}$ treatment (panel B), the -$Mg^{2+}$ treatment (panel C) and the traces of $SO_4^{2-}$ treatment (panel D). Each measuring point represents the average of a set of measurements made on a specific day ($n$ = 7–20) with its standard error.

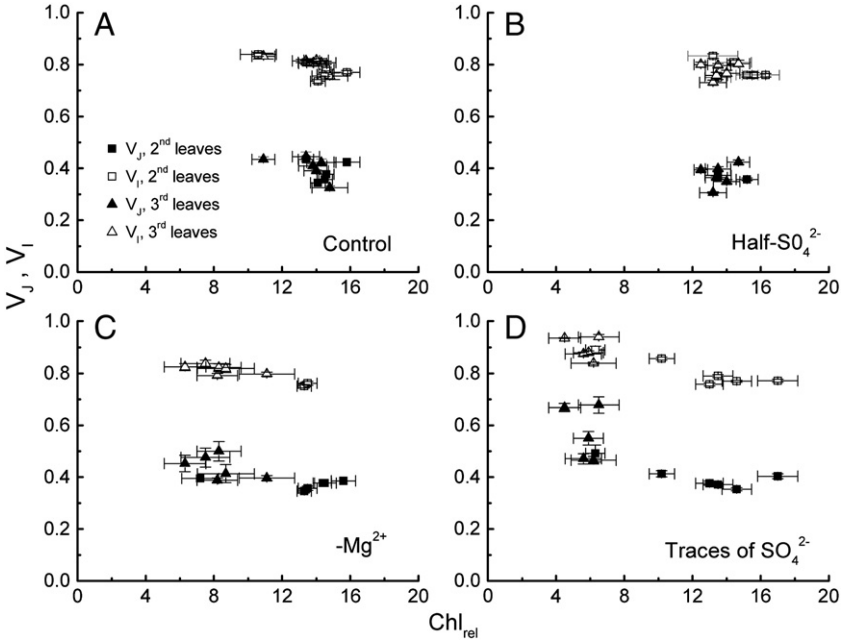


**Fig. 5.** Relationship between the parameters $V_J$ and $V_I$ and the chl content of sugar beet leaves for the control treatment (panel A), the half-$SO_4^{2-}$ treatment (panel B), the -$Mg^{2+}$ treatment (panel C) and the traces of $SO_4^{2-}$ treatment (panel D). Each measuring point represents the average of a set of measurements made on a specific day ($n = 7$–20) with its standard error.

value of the mutant is 54% of the wild type value. The difference between the effect on the $F_0$ and $F_M$ values may in part be due to the contribution of PSI fluorescence to the $F_0$ intensity [33,34].

### 3.3. Greening and cab antisense ODNs

To study the effect of antenna size on the fluorescence intensity in more detail a greening experiment was carried out on wheat seedlings, using antisense ODNs specifically designed to interfere with the synthesis of CAB proteins. Antisense ODNs are short synthetic strands of DNA or analogs that consist of 15–20 nucleotides. They specifically target their complementary stretches of RNA by duplex formation and inhibit protein biosynthesis. In principle, they are able to interfere with each step of nucleic acid metabolism, preferentially with transcription, splicing and translation [35–39]. Dinç et al. have shown that antisense ODNs can be used in plant leaves as well and that they can be used to modulate carotenoid content and antenna size during greening [17]. In the present study, ten antisense ODNs were used that differed in their ability to inhibit the synthesis of the CAB proteins. By inhibiting the synthesis of the CAB proteins to different extents, biological variability was induced. Detached wheat leaves were treated with antisense ODNs for 12 h in darkness, which was followed by a 24 h illumination period (100 μmol photons $m^{-2}$ $s^{-1}$ of white light).

In Fig. 7A and B, OJIP transients of wheat leaves treated with antisense *cab* ODNs after 8 h (panel A) and 24 h (panel B) of greening, respectively, are shown. After 8 h of greening, the transients of the untreated wheat leaves and the leaves treated with the antisense *cab* ODNs that had had the least effect on the synthesis of the CAB proteins already showed the typical OJIP kinetics. Transients measured on wheat leaves treated with the antisense ODNs that had the strongest effect on the $F_M$ value did not only have a smaller fluorescence amplitude, they also lacked the IP phase. The IP phase has been shown to reflect electron flow through PSI [21] and its amplitude has been associated with PSI content [23,40]. Double normalizing the transients between O and I demonstrates that the OJ rise is much slower for the antisense ODN-treated leaves indicating that these had a smaller effective antenna size (inset of Fig. 7A). After 24 h of greening the fluorescence kinetics both for the control and antisense ODN-treated leaves had changed considerably. There were still differences in the amplitudes of the transients between the different ODN treatments but the OJIP kinetics had become qualitatively very similar and the IP phase had become considerably more prominent. In addition, the JI phase had a small relative amplitude compared to mature green leaves.

The combination between the treatments with the different antisense ODNs and the two time points at which the measurements

**Table 1**
Fluorescence parameters derived from 77 K spectra measured on leaves of sugar beet plants that had been grown for 31 days on the different hydroponic solutions. Each value represents an average of 4–5 measurements with its standard error.

| | $F_{685\ nm}$ | $F_{694\ nm}$ | $F_{730\ nm}$ | $F_{685\ nm}/F_{694\ nm}$ | $F_{694\ nm}/F_{730\ nm}$ |
|---|---|---|---|---|---|
| C | 787.8 ± 30.0 | 1633.0 ± 48.1 | 3436.8 ± 114.3 | 0.482 | 0.475 |
| HS | 673.3 ± 30.6 | 1436.5 ± 59.2 | 3055.5 ± 89.3 | 0.469 | 0.470 |
| WM | 928.6 ± 35.2 | 2005.6 ± 74.4 | 3808.4 ± 75.0 | 0.463 | 0.527 |
| TS | 864.5 ± 62.0 | 1774.3 ± 95.7 | 2898.0 ± 103.4 | 0.487 | 0.612 |

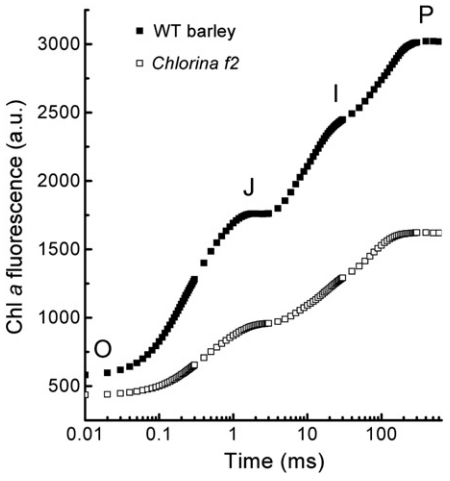


**Fig. 6.** OJIP transients measured on leaves of WT barley plants and the *Chlorina f2* mutant of barley.

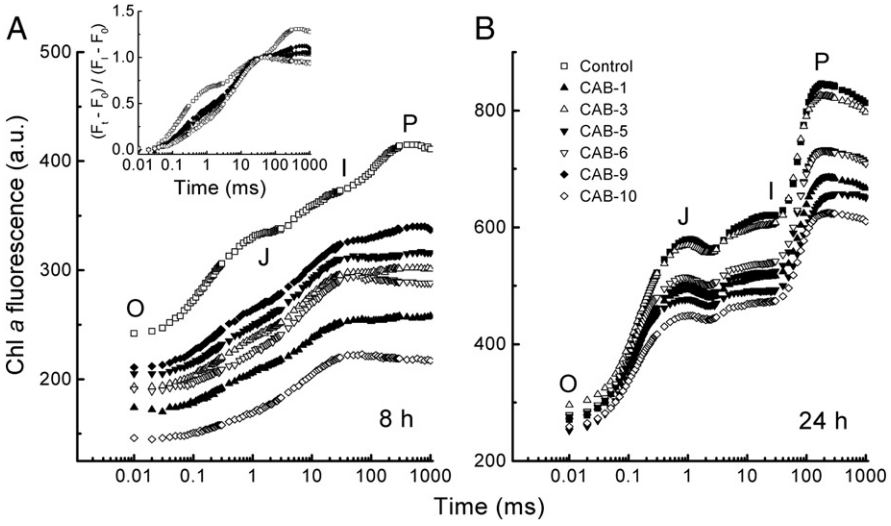


**Fig. 7.** Effects of 8 and 24 h of greening on ODN-treated etiolated wheat leaves. A selection of fluorescence transients measured on wheat leaves treated with *cab* ODNs after 8 (panel A) and 24 h (panel B) of greening. In the inset of panel A, the fluorescence transients were double normalized between O and I to allow a comparison of the kinetics of the OJIP transients. The chl *a*/*b* ratios associated with the measurements, following the order given by the legend, were 7.6, 13.6, 12.2, 10.1, 12.6, 11.8 and 14.7, respectively after 8 h of greening and 4.4, 5.6, 4.8, 4.3, 5.0, 5.2 and 5.7, respectively after 24 h of greening. Each transient represents the average of four independent measurements.

were made yielded a broad range of chl contents. For the chl *b* content a ten-fold difference between the leaves most affected by the antisense ODNs and the controls was obtained (Fig. 9B). In Figs. 8 and 9 the variability induced by the antisense ODN treatments was used to analyze the relationship between fluorescence intensity ($F_0$, $F_M$) and chl content (chl *a*, chl *b* and chl *a*/*b* ratio). Fig. 8A demonstrates that after 8 h of greening an increase of the chl $(a+b)$ content correlated with an increase of the $F_0$ and $F_M$ intensities. After 24 h of greening the correlation between the $F_0$ value and the chl $(a+b)$ content was lost (Fig. 8A and B). The dependence of the $F_M$ on the chl content after 24 h of greening is more easily interpreted on the basis of Fig. 8B, where the $F_M$ values are shown as a function of the chl *a* content. For both the 8 h and 24 h data an approximately linear relationship is observed between the $F_M$ and chl *a* values. However, after 24 h of greening a given $F_M$ value correlates with a higher chl *a* value than after 8 h of greening, i.e. the points have shifted towards the right side of the panel. To illustrate the dependence of the $F_0$ and $F_M$ values on the greening time (8 or 24 h), the parameter $F_M/F_0$ is given as a function of the $F_0$ value in Fig. 8C. After 8 h of greening there was an approximately linear relationship between the $F_0$ values and the $F_M/F_0$ ratio (Fig. 8C). This relationship was completely lost after 24 h of greening where changes in the $F_M$ value were no longer accompanied by changes in the $F_0$ value. Fig. 9A demonstrates that, although the relationship between the $F_M$ value and the chl *a*/*b* ratio as a whole may be best described by a hyperbole, the relationship can be described also quite well by a linear regression for chl *a*/*b* ratios up to 10. It is worth noting that the naturally observed range of chl *a*/*b* ratios is quite limited. For example for pea plants grown at different light intensities values ranging from 2.1 for pea plants grown at 10 μmol photons $m^{-2}\,s^{-1}$ to 3.2 for pea plants grown at 840 μmol photons $m^{-2}\,s^{-1}$ were observed [41]. The data in Fig. 9A suggest that the antenna size is an important determinant for the $F_M$ value. The Lhcb1-3 proteins contain 8 chl *a* and 6 chl *b* molecules [42,43]. On the basis of the LHCII-trimers a slope of 0.75 would be expected if chl *b* is presented as a function of chl *a*. However, this is an upper limit and the slope for the average of all Lhca and Lhcb complexes together will be probably be somewhat lower although 0.5 is a likely under limit since, as noted above, a chl *a*/*b* ratio of 2.1 was observed for pea leaves grown under low light conditions [41]. Fig. 9B shows that initially the chl *a* content

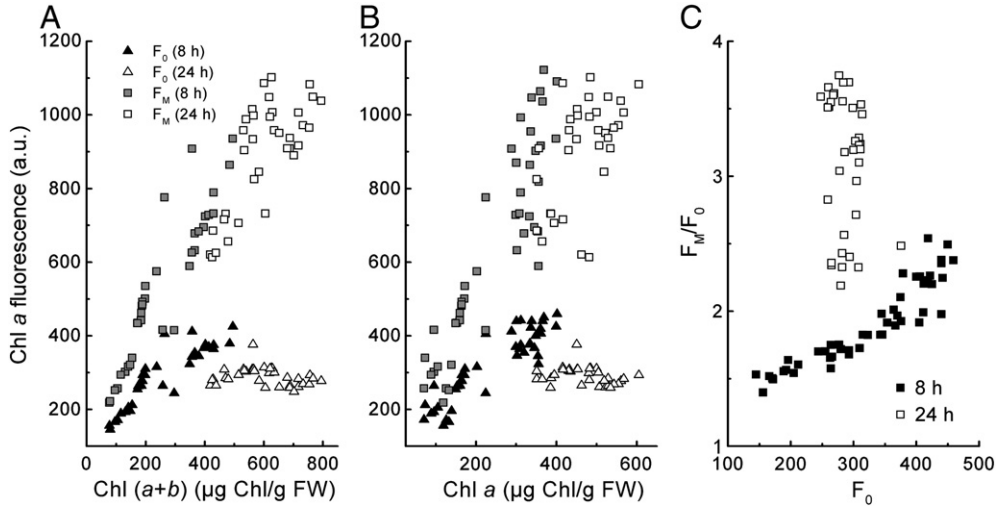


**Fig. 8.** Relationship between the chl content and chl *a*/*b* ratio and the fluorescence intensity ($F_0$ or $F_M$) for greening wheat leaves treated with ten different antisense ODNs designed to interfere with the translation of *cab* mRNA, measured after 8 and 24 h of greening: relationship between chl $(a+b)$ content and the $F_M$ and $F_0$ values (panel A); relationship between chl *a* content and the $F_M$ and $F_0$ values (panel B); relationship between the parameters $F_0$ and $F_M/F_0$ (panel C). The data were derived from three independent experiments.

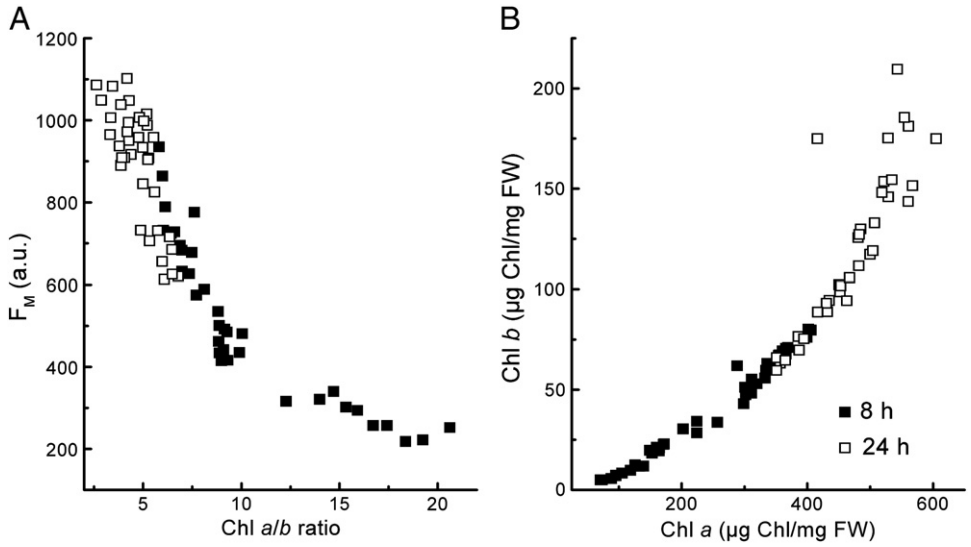


**Fig. 9.** Relationship between the $F_M$ value and the chl *a*/*b* ratio (panel A) and the relationship between the chl *b* and chl *a* content of antisense ODN-treated wheat leaves after 8 and 24 h of greening (panel B).

increases much faster than 1.33 to 2 × chl *b* indicating that after 8 h of greening the relationship is still dominated by the synthesis of new PSI and PSII complexes. Only after 24 h of greening for the least inhibited samples the slope of the data in Fig. 9B is becoming steeper suggesting that in these leaves the enlargement of the antenna started to dominate the increase of the chl content.

## 4. Discussion

### *4.1. Experimental system to test the impact of changes in the chl content on chl a fluorescence measurements*

Chl loss is a common effect of stress but occurs also during senescence. Hsu and Leu [16] suggested that chl loss by itself affected the chl *a* fluorescence kinetics complicating the interpretation of fluorescence transients under stress conditions. The evaluation of the issue raised in ref. [16] is not straightforward, though. To study the effects of changes in the chl content the optical properties of the experimental system used are very important. In ref. [16] an approach was used that did not take into account changes in the leaves that would normally accompany changes in the chl content (cf. [13,14]): they removed the spongiform parenchyma or added an extra leaf. The same is true for the approach of Sušila et al. [43] who changed the concentration of isolated thylakoid membranes. The random orientation of the thylakoid membranes in a suspension has optical properties that differ considerably from the optical properties of the well-ordered thylakoid membranes in chloroplasts inside the well-ordered palisade parenchyma cells (e.g. [1]). To assess the effects of changes in chl content a dynamic system is needed in which the chloroplasts are allowed to acclimate to changes in their light environment. A developing stress condition meets this requirement best. A second requirement is that the stress condition should not have too much effect on the functionality of the photosynthetic electron transport chain. The mineral deficiencies studied here, where plants during the treatment became more and more severely affected by the $Mg^{2+}$ and $SO_4^{2-}$ deficiencies, form a good model system in this respect. These treatments caused a severe loss of chl over time (Figs. 1, 2 and 4), but as shown e.g. by Fig. 3, this had only a limited effect on the integrity of the electron transport chain. Our data suggest that the treatment mainly caused a loss of whole photosynthetic electron transport chains and/or chloroplasts. This allowed us to separate effects of the chl content on the fluorescence measurements from other stress-related effects. Another consideration is the measuring equipment used. We worked here with PEA-instruments and probe light intensities of either 1800 or 3500 µmol $m^{-2}$ $s^{-1}$. For the PAM the intensity of the probe light it is at least a factor 1000 lower than the probe light intensity of PEA instruments. However, if we assume that the individual fate of a photon of a given wavelength does not depend on the presence of other photons, both light sources should probe equally deep and both instruments should yield, with respect to the type of measurements presented here, approximately the same results. The main difference will be that a good signal to noise ratio will be more of a challenge in the case of a PAM instrument.

### *4.2. Chl a fluorescence and chl content*

Somewhat surprisingly, for the mineral-deficiency-induced chl loss, there was no correlation between chl content and chl *a* fluorescence intensity in the range studied (Fig. 4). There are several factors that may play a role in this effect: 1. There is a screening effect, i.e. chloroplasts in the upper cell layers prevent the light from reaching the chloroplast on the other side of the leaf, which means that these chloroplasts become, from a fluorescence emission point of view, invisible; 2. Re-absorption, i.e. fluorescence emitted by chloroplasts deep in the leaves is absorbed by chloroplasts in the upper cell layers reducing the fluorescence detected by the fluorimeter. The consequence of these two factors is that the excitation light does not probe the whole leaf. The results of the mineral deficiency experiment, which demonstrated that a lower chl content by itself does not affect the fluorescence intensity, allow us to suggest that the lower fluorescence intensities observed for *Chlorina f2* leaves (Fig. 6) are due to its smaller antenna size. The results from the greening experiment (Figs. 7–9) are in agreement with the results of the mineral deficiency experiment. The relationship between $F_M$ and the chl *a* content (Fig. 8B) shifted on going from 8 to 24 h of greening where more chl had accumulated in the leaves, suggesting that chloroplasts in the upper cell layers of the greening leaf started to hinder the passing of light through these cell layers, effectively screening the excitation light and the fluorescence. The same screening effect, but then in reverse, can explain as well the insensitivity of $F_M$ and $F_0$ to the chl content during the stress-induced chl loss (Fig. 4). The data in Figs. 2 and 3 indicate that the chl loss had little effect on the antenna size. This can be explained by a continuous acclimation of the photosynthetic apparatus of the chloroplasts to their light environment (otherwise the chl *a*/*b* ratio would gradually decrease since chloroplasts located deeper in the leaf have a lower chl *a*/*b* ratio [9]). This would mean that the excitation light used for the fluorescence measurements

probes at all times a similar population of chloroplasts. The greening experiment suggests, though, that below a certain value of the chl content (in the greening case high chl *a*/*b* ratios) the absorption of the leaves at 650 nm becomes so low that the excitation light can probe all PSII reaction centers (Fig. 8). Below this absorption value the $F_0$ and $F_M$ intensities become dependent on the chl content. However, even an 80% loss of the chl content of sugar beet leaves (Fig. 1) was not enough to reach this domain suggesting that it is irrelevant for the range of chl losses that would in most experiments be induced by stress factors.

In ref. [16] it was claimed that a change in the chl content would affect the fluorescence kinetics. The fluorescence intensity at the J-step increased slightly as the leaves lost more chl suggesting that the light was becoming somewhat more saturating (see Fig. 5, control and $Mg^{2+}$-deficient plants). Therefore, our measurements do confirm the observation made in ref. [44] that chl loss shifts the light intensity where saturation occurs to lower values, but this effect is small under our conditions and would have been even smaller if we had used higher light intensities for the fluorescence measurements.

### 4.3. Stacking

Magnesium plays several roles in the chloroplast. Each chl molecule contains a bound $Mg^{2+}$ ion (reviewed in ref. [45]) and $Mg^{2+}$ is important as well for ATP-synthase activity (e.g. [46]). Terry and Ulrich [47] observed that sugar beet leaves could lose 80% of their $Mg^{2+}$ content without an effect on their photosynthetic activity. Magnesium also plays an important role in the stacking of thylakoid membranes (e.g. [31,48,49]) and $Mg^{2+}$ deficiency is known to cause destacking of granal membranes (e.g. [50]). In Fig. 4C ($-Mg^{2+}$) a loss of $F_M$ intensity that is not accompanied by a change in the $F_0$-intensity is observed for the third leaf pairs. This symptom is well explained by destacking. Destacking allows a better mixing of PSII and PSI and leads to an increase of spillover ([48] and see also the hypothesis presented in ref. [51]), which results in a lowering of the (variable) fluorescence intensity. Increased levels of spillover should show up in 77 K fluorescence spectra in the form of smaller peaks associated with PSII and a bigger peak associated with PSI [31]. Kirchhoff et al. [31] observed on destacking of thylakoid membranes that the $F_{PSII}/F_{PSI}$ ratio ($\sim F_{694\ nm}/F_{730\ nm}$) dropped from 0.53 to 0.23. This is not observed in Table 1 where the $F_{694\ nm}/F_{730\ nm}$ ratio of the WM leaves was even somewhat higher than for the C and HS leaves. This discrepancy can be explained if an increase of spillover would be compensated by a lower re-absorption by PSI antennae of fluorescence emitted by the PSII complexes due to the loss of PSI and PSII complexes [52]. That the destacking in the case of the leaves growing under WM-conditions was only partial can also be deduced from the $F_M/F_0$ ratio. In ref. [31] it was observed for thylakoid membranes that this parameter fell from 5.05 to 2.02 on destacking. Here, the $F_M/F_0$ ratio dropped from 5.85 to 4.33 after 24 days of growth under WM conditions.

A correlation between destacking and the decline of $F_M$ in plants grown under WM conditions would also imply that there is no correlation between the loss of chl and the $F_M$ level in these plants.

### 4.4. PQ pool redox state

In Fig. 4, it was observed that $F_0$ increases in the third leaves of plants grown under TS conditions whereas the $F_M$ remained unchanged. In Fig. 5 the same observation was made for the $V_J$ value. Ceppi et al. [18] observed that upon an FR preillumination a considerably lower $F_J$ and $F_0$ intensity was obtained for the plants grown under TS conditions, suggesting that these plants had a more reduced PQ pool in darkness (see e.g. [53]). This is in agreement with work by the group of Godde [54,55] who observed that a combined $SO_4^{2-}$ and $Mg^{2+}$ deficiency resulted in plants with a more reduced PQ pool in darkness. Also in this case the observed changes in the $F_0$ and $V_J$ values are in all likelihood related to changes in the redox state of the electron transport chain in darkness and not to changes in the photosynthetic apparatus. This suggests that for all the treatments applied here, no correlation between the $F_0$ and $F_M$ values and the chl content exists. As the leaves turned more yellow, the remaining PSII complexes acclimated to their new light environment (chloroplast located deeper in the leaf have lower chl *a*/*b* ratios [9]) and as a consequence the actinic light probed a similar number of PSII complexes with similar antenna sizes (chl *a*/*b* ratios).

### 4.5. Lack of antenna size changes in response to the mineral deficiencies

The light intensity dependencies of the K, J and I steps of the fluorescence rise measured on the 3rd leaves at the end of the treatment (Fig. 3) were nearly identical for the C, HS and WM treatments suggesting that the effective antenna size in all three cases was nearly identical. The data on the extracted chl and the 77 K data (Table 1) gave also little indication of an effect on the antenna size of PSII. The ratio $F_{685\ nm}/F_{695\ nm}$ ranged from 0.463 to 0.487, values routinely observed for e.g. untreated pea leaves (SZ Tóth, unpublished observations). In Fig. 2F the chl *a*/*b* ratio of the 3rd leaves of plants grown under TS conditions was considerably lower than the chl *a*/*b* ratios of the other treatments. As reviewed in ref. [27,28], PSI has a higher chl *a*/*b* ratio than PSII and as a consequence a loss of PSI will lead to a lowering of the overall chl *a*/*b* ratio. This agrees with the observation made in ref. [18] that a sulphur deficiency leads to a stronger loss of PSI. The observed decrease of the chl *a*/*b* ratio for the 2nd leaves of plants grown under HS-conditions is probably better explained by an increase in the antenna size.

### 4.6. Antisense ODNs

This study demonstrates that by treating plants with specifically designed antisense ODNs it is possible to induce a considerable amount of biological variability, allowing the study of the relationships between parameters like fluorescence intensity and chl content. The peripheral antennae of PSII and PSI consist of approximately ten different members of the *cab* gene family. Dinç et al. [17], on the basis of RT-PCR and Western blots, showed for two of the antisense ODNs used here that these antisense ODNs targeted simultaneously the mRNA of several *Lhcb* and *Lhca* genes. We expect that also the other antisense ODNs had multiple targets. A potential complication of the Western blot analysis that was not considered in ref. [17] is the possibility that the treatments with the antisense ODNs give rise to a fraction of free antenna subunits not energetically connected to a reaction center. However, with respect to the two antisense ODNs analysed in ref. [17], which were used in this study as well, we did not observe in Fig. 8C any unexpected decreases of the $F_M/F_0$ values. The present study is a global study in which we ignored the precise effects of individual ODNs. The fact that we obtained meaningful correlations between the different parameters that we studied implies that the approach worked. However, two effects described in the literature are worth mentioning here. The knockout of Lhcb6 (CP24) and the antisense suppression of Lhcb4 (CP29) have been shown to have a strong effect on exciton migration through the antenna due to their importance for the connection between the LHCII trimers and the reaction center [56]. ODNs that would strongly affect these two members of the CAB-family could have a much stronger effect on the fluorescence intensity than would have been expected on the basis of the effect on the chl content. The other effect worth mentioning is the ability of the chloroplast to compensate the inhibition of some of the CAB-members. Using antisense mutants it was shown that for PSII in the absence of Lhcb1 and Lhcb2, trimers consisting of either two Lhcb5 (CP26) and one Lhcb3 [57,58] or three Lhcb5 [58] were formed. A loss of Lhcb3 was shown to be compensated for by increases in Lhcb1 and Lhcb2 [59]. The organization of the antenna of PSI on the other hand was shown to be more rigid with the only

exchange described being the replacement of Lhca4 by Lhca5 [60]. The fact that we obtained a range of antenna sizes indicates that these compensatory mechanisms did not overcome all the inhibitory effects of the antisense ODNs and this may be due to the fact that the antisense ODNs used targeted several members of the CAB-family.

### 4.7. Greening

In Fig. 8A and B it is shown that after 8 h and 24 h of greening the $F_M$ value changes proportionally to the chl content but after 24 h the relationship between $F_M$ and chl content had shifted to higher chl contents. This suggests that on the one hand there is an increase of the fluorescence intensity reflecting the maturation of the chloroplasts and the photosystems. On the other hand, the data also seem to suggest that as the chl content increases, no longer all chl molecules are monitored by the measurement (screening effect). This would be in agreement with the insensitivity of the $F_M$ and $F_0$ values to changes in the chl content as observed for the mineral deficient sugar beets plants (Fig. 4). The Lhcb1–3 complexes have been shown to bind 8 chl *a* and 6 chl *b* each [42,43]. The literature on the chl *a*/*b* ratio of the other Lhca and Lhcb protein complexes is contradictory. With respect to Lhca1–4 the crystal structure of PSI of pea plants suggests that all 4 protein complexes bind 14 chls just like Lhcb1–3 [61]. *In vitro* studies suggest that Lhca1–4 bind fewer chls (e.g. [62,63]), but this may also be due to a loss of more loosely bound chls during the preparation of these protein complexes. Despite these uncertainties it is possible to associate a ratio of 1.33 to 2 chl *a* per chl *b* with a predominant increase of the antenna size and much higher values with an important contribution of the synthesis of PSII and PSI reaction center to the greening process. Since the chl *a* content initially increased more than the 1.33 to 2 × chl *b* which would have been expected for a predominant increase of the antenna size (Fig. 9B), the data suggest that two phases can be distinguished during the greening process: 1. increase of the number of PSI and PSII complexes and 2. enlargement of the antenna. This observation was also made by Srivastava et al. [30] who used intermittent light grown plants instead of etiolated leaves as starting material for their greening experiment. If we interpret Fig. 9B in this way, then the data of Fig. 8C suggest that the $F_0$ intensity is sensitive to the increase of the number of PSII complexes (dominant process after 8 h of greening), but essentially insensitive to the antenna size (dominant process after 24 h of greening). In contrast, the increase of the antenna size was an important factor determining the $F_M$ intensity.

### 4.8. Chl a fluorescence intensity and antenna size

The data presented in this paper suggest that especially the $F_M$ intensity depends much more on the antenna size of PSII (cf. Fig. 4 with Figs. 6 and 8) than on the actual chl content of the leaf. Two possible explanations for this observation present themselves. More LHCII trimers bound to PSII (i.e. a larger PSII antenna size) lead to more extensive stacking of the thylakoid membranes [64]. As observed for the $Mg^{2+}$ deficient leaves (Fig. 4) and thylakoid membranes (e.g. [31,48,49]) unstacking leads to a decrease of the $F_M$ value. The second explanation has to do with the transfer of the excitation energy within the PSII-antenna. The probability that excitation energy is lost as fluorescence depends on the lifetime of the exciton and if this lifetime would be sensitive to the antenna size it can also explain some of the observations made here. Work on PSII core particles with a small antenna size led to the exciton/radical pair model [65] which assumes that PSII is trap limited. This means that the transfer of excitons within the antenna does not impose a rate limitation on the trapping of the energy. As research is shifting to more intact PSII reaction centers it is becoming clear that the energy migration component to the total trapping time is becoming more important as the antenna size increases and as a consequence PSII reaction centers with a full complement of antenna subunits are partially diffusion limited with the migration component contributing up to 50% to the total trapping time [56,66–68]. All the published data were obtained under $F_0$ conditions and there is less information with respect to measurements made under $F_M$ conditions. In the *Chlorina* mutant of barley it was observed that the lifetime of an excited state under $F_M$ conditions is 13–23% shorter than in wild type leaves [69]. A larger antenna may, therefore, also mean a higher probability that absorbed photons are re-emitted as fluorescence.

In summary, our data indicate that fluorescence measurements are remarkably insensitive to changes in the chl content of the leaf as long as the chl *a*/*b* ratio remains unaffected. This has two consequences: 1. the $F_0$ and $F_M$ values cannot be used as measures for the chl content of the leaf as, for example, has been proposed in ref. [70] and 2. a loss of chl, by itself, does not complicate the interpretation of the kinetics of OJIP fluorescence transients.

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