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Rapid Report

Relationship between quenching of maximum and dark-level chlorophyll fluorescence in vivo: dependence on Photosystem II antenna size

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Abstract

The effects of nonphotochemical quenching were quantified separately applying the Stern-Volmer formalism for dark level (SV_0) and maximum chlorophyll fluorescence yield (SV_N) in barley leaves comprising a step-wise altered Photosystem II (PS II) antenna size. Proportions of overall SV_N can be attributed to distinct sites of the photosynthetic apparatus: (i) the bulk light-harvesting complex of PS II (LHC II), (ii) the inner LHC II antenna, and (iii) the reaction center/core complex of PS II. The fraction of SV_N which exerts an effect on SV_0 appeared to arise almost exclusively from the inner LHC II antenna. A strong linear correlation between SV_0 and violaxanthin de-epoxidation points to an intrinsic relationship of both. The results are in line with the notion of a regulatory function of the inner LHC II antenna, thus controlling excitation energy delivery from the bulk LHCII to the PS II-core complex.

Keywords: Chlorophyll fluorescence; Nonphotochemical quenching; Light harvesting complex II; Xanthophyll cycle; Minor LHC II complex

The ability of plants to safely dissipate excess excitation energy which can not be utilized in photosynthesis can be assessed as nonphotochemical quenching of chlorophyll (Chl) fluorescence. Several processes contribute to overall nonphotochemical quenching in vivo [1]. Among these the most important is associated with the build-up of a transthylakoid pH gradient and therefore termed 'high-energy state quenching' (qE). qE is considered to be of paramount importance in plant photoprotection [1]. Two theories concerning the origin of qE are currently discussed: (i) quenching may originate in the Photosystem II (PS II) reaction center (RC) [2,3], and (ii) quenching can occur in the light-harvesting complexes of PS II (LHC II) [4–8]. Consistent with the latter, qE was found to be correlated in many cases with the activity of the xanthophyll cycle, i.e., the reversible conversion of violaxanthin (V) via antheraxanthin (A) into zeaxanthin (Z) upon illumination [9,10]. Horton et al. [4] originally proposed that qE results from aggregation of bulk LHC II. Comparing nonphotochemical quenching in leaves of wild-type (WT) with a Chl *b*-less mutant of barley we have previously provided strong evidence for the bulk LHC II being the origin of a considerable fraction of qE [7,8]. However, substantial qE can be found even in the mutant [7,8,11]. This implies that the remaining qE could originate from either the RC/core complex of PS II [2,3] or the inner LHC II antenna.

In this paper we present results suggesting an important role of in particular the inner LHC II antenna in regulatory excitation energy dissipation. To address the question, nonphotochemical quenching was compared in WT and the Chl *b*-less mutant *chlorina* 3613 of barley grown under continuous light (CL) and intermittent light (IML) conditions. The Chl *b*-less barley mutant *chlorina* 3613 is allelic to the well characterized *chlorina* f2 mutant and displays an identical PS II antenna polypeptide pattern [12]. In Chl *b*-less mutants, the bulk (or peripheral) LHC II complex (comprising the polypeptides Lhcb1 and Lhcb2) is completely absent [13,14]. Out of the manifold of the inner (or so called 'minor') LHC II antenna polypeptides only the *Lhcb3* gene product (a 25 kDa polypeptide) is

Abbreviations: A, antheraxanthin; AL, actinic light; Chl, chlorophyll; CL, continuous light; DTT, dithiothreitol; EPS, epoxidation state; F_0, F'_0, dark-level fluorescence yield in the dark- and light-adapted state, respectively; F_m, F'_m, maximal fluorescence yield in the dark- and light-adapted state, respectively; IML, intermittent light; LHC II, Chl *a*/*b* light harvesting complexes of PS II; PS II, Photosystem II; qE, high-energy state quenching; RC, reaction center; SV_0, Stern-Volmer type nonphotochemical quenching of F_0; ΔSV_0, rapidly reversible SV_0; SV_N, Stern-Volmer type nonphotochemical quenching of F_m; ΔSV_N, rapidly reversible SV_N; V, violaxanthin; WT, wild-type; Z, zeaxanthin.

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present at WT levels [14,15]. All other inner LHC II polypeptides (Lhcb4, Lhcb5, Lhcb6) are present at reduced levels [13–16]. To avoid ambiguities, all considerations in this study refer to the recently introduced model of higher plant light-harvesting antenna organization and the therein adopted gene-related nomenclature [17].

Primary leaves of 8-day-old WT barley (*Hordeum vulgare* L. cv. Donaria) and the Chl *b*-less mutant *chlorina* 3613 were used that had been grown either under CL as described previously [7], or for 6 days in the dark followed by a 48-h exposure to IML (20 μmol photons m^{-2} s^{-1}, 2 min light/118 min dark). Thus cultivated IML-plants displayed Chl contents of about 6% (WT) and 10% (mutant) of the respective CL-grown variants. For dithiothreitol (DTT) treatment, attached leaves were submersed in a 4-mM solution. The leaves were allowed to take up DTT for a minimum of 90 min, the last 60 min in darkness.

Nonphotochemical quenching was induced by actinic light (AL) of 1300 μmol m^{-2} s^{-1} and quantified according to the Stern-Volmer equation: $SV_N = F_m/F'_m - 1$ and $SV_0 = F_0/F'_0 - 1$ for F_m and F_0 quenching, respectively. Such an approach allows one to differentiate quenching effects on F_m and F_0 regardless of model assumptions on excitation utilization in PS II; furthermore, the derived parameters are directly proportional to the quencher concentration. F_m and F_0 denote maximum and dark-level fluorescence intensity (dark-adapted), whereas a prime indicates the light-adapted (quenched) state. Modulated Chl fluorescence was measured as previously described [7] with minor modifications: for registration of F'_m only one saturating light pulse was given 10 s prior to the end of illumination. In light-adapted leaves, the F'_0 level was obtained by simultaneously switching off the actinic light (AL) and applying a 5-s far red light pulse (peak wavelength 735 nm) to ensure complete reoxidation of the electron transfer chain. For pigment analysis samples were taken as described in [8], and pigments were determined by high performance liquid chromatography according to [18]. The xanthophyll-cycle activity was assessed as epoxidation state, EPS = [V + 0.5A]/[V + A + Z].

Fig. 1 shows the kinetics of induction and relaxation of Stern-Volmer type SV_N (Fig. 1A, B) and SV_0 (Fig. 1C, D) in barley WT and Chl *b*-less mutant leaves. In both genotypes, SV_N and SV_0 have reached a steady-state level after 15 min of illumination (Fig. 1A, C). Whereas the finally attained SV_N is considerably lower in the mutant as compared to the WT, no significant differences between the SV_0 levels were apparent in the steady state. The latter finding suggests a common mechanism of F_0 quenching in both genotypes. Upon cessation of AL, SV_N relaxed rapidly in both genotypes (Fig. 1B). After about 15 min, most of the SV_N was removed and only very little quenching remained after 180 min in darkness. Striking differences are evident in the relaxation behaviour of SV_0 (Fig. 1D). In the WT, SV_0 reversed rapidly within 5 min to a value close to the initial level and reached the lowest (negative)

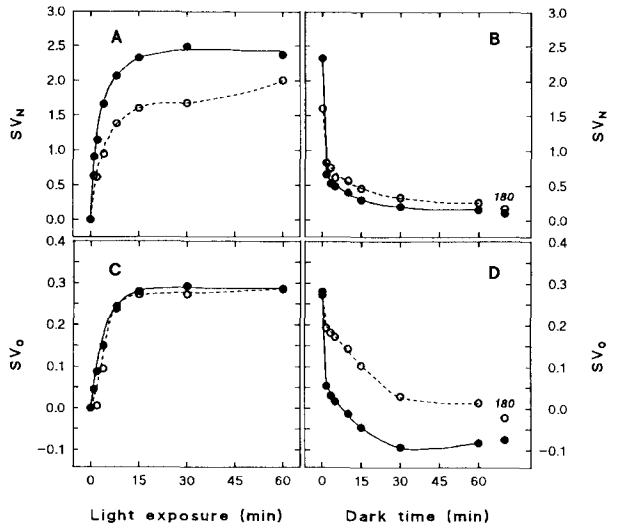

Fig. 1. Induction and dark relaxation kinetics of Stern-Volmer type nonphotochemical quenching of maximum (SV_N; A, B) and dark-level (SV_0; C, D) Chl fluorescence in leaves of wild-type barley (●) and the Chl *b*-less *chlorina* 3613 mutant (○), respectively. Leaves were exposed to actinic light of 1300 μmol m^{-2} s^{-1} after dark adaptation for 60 min. Note that dark relaxation kinetics were followed in leaves that had been preilluminated for 15 min only. Each data point represents the mean of at least five experiments.

value after 30 min. Thereafter, SV_0 appeared to gradually increase again. In the mutant, SV_0 relaxed considerably slower and remained at a higher level than in the WT throughout the 180-min dark relaxation period. We explain the difference in SV_0 relaxation at least in part in terms of a diminished re-epoxidation rate in the mutant (Härtel and Lokstein, unpublished results).

The plot of SV_0 vs. SV_N indicates a linear correlation between both parameters during induction (Fig. 2A). Two features emerge: (i) A similar SV_0 as in mutant leaves is attained in the WT at higher SV_N values. This shift towards higher SV_N points to the bulk LHC II as a source of a proportion of SV_N observed in addition to the one in the mutant. (ii) In mutant leaves, the SV_0 value corresponding to the highest SV_N deviates from the regression line: unchanged SV_0 upon increase in SV_N may indicate photoinhibitory processes becoming important (cf. also Fig. 1A).

To investigate the significance of the inner LHC II complexes for the development of nonphotochemical quenching, in another set of experiments IML-plants were used. As previously shown [14], IML-grown mutants completely lack also the inner LHC II complexes, that still were present in CL-grown mutants. Such plants contain only the PS II-core complex with approximately 37 Chl *a* attached [19]. This is assumed to be the smallest functional Chl antenna stably assembled in vivo. In Fig. 2, the data obtained from IML-grown mutants during the first 15 min of exposure to AL are displayed for comparison (notably, there were virtually no differences between IML-grown

mutant and WT). As compared to the CL-grown mutant, in IML-grown plants the extent of SV_0 and SV_N is further reduced (cf. Fig. 2A). Whereas a significant SV_N (55% of the CL-grown mutant) developed after 15 min AL exposure, SV_0 was almost abolished (attaining less than 13% of the CL-grown mutant level). The slight increase of the latter parameter is ascribed to a 'greening' effect in response to the AL exposure.

Nonphotochemical quenching is accompanied by operation of the xanthophyll cycle, i.e. V de-epoxidation [9,10]. Plotting the EPS vs. corresponding SV_0 data (Fig. 2B) gives for both CL-grown WT and mutant leaves a good linear correlation. Thus an intrinsic relationship between both parameters is conceivable. The difference in the finally attained EPS between both genotypes can be rationalized in terms of the accessibility of V for conversion into A and Z (cf. also the legend to Fig. 2). V appears to be bound more tightly to the LHC-apoproteins in the WT, rendering it less accessible to the V de-epoxidase than in the mutant, where a significant fraction of V might be 'swimming' in the thylakoid membrane lipid phase. The pool size of [V + A + Z] remained constant throughout AL exposure in both genotypes, suggesting that formation of A + Z was exclusively at the expense of V. However, A + Z accumulation alone is not sufficient to cause quenching of F_0. Despite the nearly 10-fold higher pool of [V + A + Z] in relation to Chl *a* in the IML-grown mutant (650 mmol (mol Chl *a*)$^{-1}$) as compared to the CL-grown mutant (76 mmol (mol Chl *a*)$^{-1}$), and the similar efficiency of V de-epoxidation (for minimum attainable EPS, cf. Fig. 2B) a significant SV_0 did not develop in IML-grown plants. The possibility that the disappearance of SV_0 is due to IML-grown plants being incapable of developing a

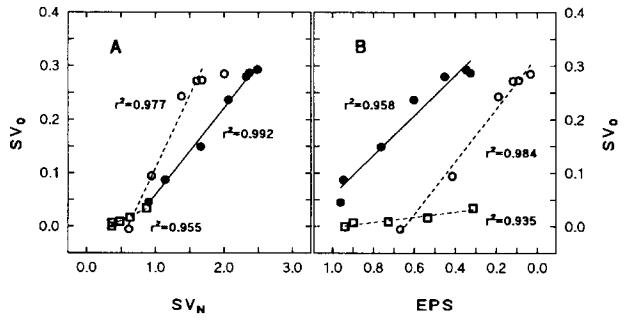

Fig. 2. Dependence of SV_0 on SV_N (A) and the epoxidation state, EPS (B), in leaves of wild-type barley (●), the Chl *b*-less *chlorina* 3613 mutant (○) and IML-grown *chlorina* 3613 mutant (□), respectively, during (0–30 min) exposure to actinic light of 1300 μmol m^{-2} s^{-1}. All leaves were dark-adapted for 60 min prior to light exposure. The maximum EPS obtained after 60-min light exposure was 0.325 (wild-type), 0.032 (Chl *b*-less mutant) and 0.084 (IML-grown Chl *b*-less mutant). The pool sizes of [V + A + Z] were 64 (wild-type), 79 (Chl *b*-less mutant) and 649 mmol (mol Chl *a*)$^{-1}$ (IML-grown Chl *b*-less mutant). CL-grown wild-type and mutant data in Fig. 2A were redrawn from Fig. 1A and C. Each data point represents the mean of at least five experiments. The lines correspond to the first-order linear regression to the experimental data.

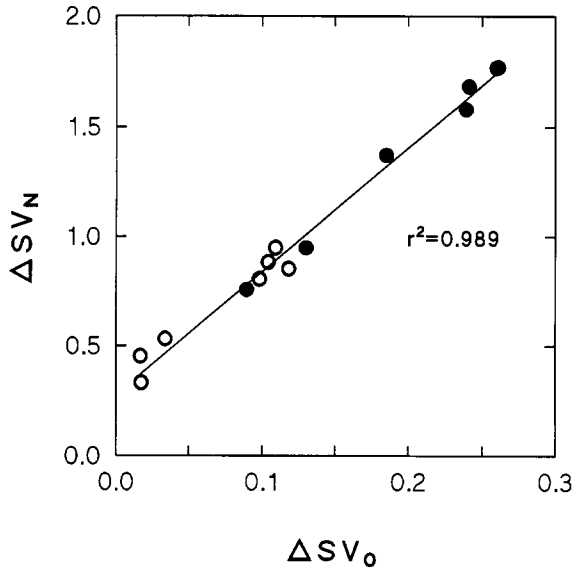

Fig. 3. Relationship between rapidly-relaxing SV_N, ΔSV_N, and rapidly-relaxing SV_0, ΔSV_0, obtained in leaves of wild-type barley (●) and the Chl *b*-less *chlorina 3613* mutant (○), respectively, after exposure to actinic light of 1300 μmol m^{-2} s^{-1}. All leaves were dark-adapted for 60 min prior to light exposure. Each data point represents the mean of at least four experiments. ΔSV_N and ΔSV_0 are the differences between SV_N or SV_0 at the end of the respective light periods minus SV_N or SV_0 after 5 min dark relaxation. The continuous line corresponds to the first-order linear regression to the experimental data of both genotypes.

sufficient ΔpH can be excluded, since comparably high xanthophyll conversion (likewise ΔpH-dependent) occurred. DTT, known to inhibit xanthophyll-cycle activity, completely suppressed SV_0. In addition, a *similar* fraction of SV_N (about 40% of the control WT-level) was found to be retained in DTT-treated CL-grown plants of both genotypes. Thus, we suggest that the operation of the xanthophyll cycle within the inner LHC II antenna is a prerequisite for SV_0 to occur.

To demonstrate whether and to what extent the inner LHC II is involved in the qE mechanism, the rapidly (within 5 min) relaxing fractions of SV_N (ΔSV_N) and SV_0 (ΔSV_0) were assessed [7,20,21]. Fig. 3 shows ΔSV_N plotted vs. the corresponding ΔSV_0. Apparently, the values obtained for WT and mutant closely fit to the same line. This corroborates our previous suggestion [7,8] that qE build-up is a function of bulk LHC II; moreover it also indicates the involvement of the inner LHC II-antenna in the qE mechanism. Consistent with this view, both ΔSV_N and ΔSV_0 were found to be further reduced in IML-grown plants (data not shown). However, in the latter a small proportion of ΔSV_N (about 20% of that found in CL-grown WT plants) still remained. This proportion was considerably smaller than that previously reported for pea plants [11].

In general, because of the unavoidable 'greening' upon AL exposure, results obtained with IML-grown plants have to be interpreted with some caution. However, the fact that

the regression line in Fig. 3 intercepts the y-axis at 0.28 could be indicative for the existence of a minor component of LHC II-independent qE, not causing SV_0. Interestingly, this value corresponds almost exactly to the maximum ΔSV_N obtained after 8 min light exposure of IML-grown mutants (0.29), where virtually no F_0 quenching was detectable. This minor remaining qE component would then, indeed, reflect RC/core antenna related processes [2,3].

As evident from the differences in SV_N between the three variants compared (CL-grown WT; CL-grown mutant and IML-grown mutant; cf. Figs. 1–3), proportions of overall SV_N are assigned to distinct sites of the photosynthetic apparatus: (i) the bulk LHC II, (ii) the inner LHC II and (iii) the RC/core complex of PS II. Since there was no difference in maximum attainable SV_0 between CL-grown WT and mutant (cf. Fig. 1), we conclude that (the component of SV_N leading to) F_0 quenching originates from the inner LHC II. Consistent with this notion, striking differences in SV_0 between CL and IML-grown mutant plants (in the latter almost all SV_0 being absent) strongly point to F_0 quenching originating from the inner LHC II complexes. In both genotypes, SV_0 is related to considerable fractions of overall SV_N (cf. Fig. 2A) and its major component, ΔSV_N (cf. Fig. 3). The latter finding explains well the relatively high qE levels still to be found in the Chl *b*-less mutant [7,8].

Furthermore, LHC II polypeptides are assumed to bind most of the xanthophyll-cycle pigments [13,22,23]. Bassi et al. [22] even assigned 80% of V to the inner LHC II complexes. Thus, it is reasonable to assume that A + Z related nonphotochemical quenching arises mainly from the inner LHC II. Consistent with this view is the observation that nonphotochemical quenching, in particular qE, found under conditions where Z formation was absent, was not accompanied by F_0 quenching [24]. Thus we propose that quenching centers are formed in the inner LHC II in response to V de-epoxidation rendering the basis for a major SV_N (and ΔSV_N) fraction.

Due to the reduced amounts of the inner LHC II complexes coded for by the *Lhcb4, Lhcb5* and *Lhcb6* genes [14–16], it is tempting to assume that mainly the Lhcb3 complex contributes to SV_N resulting in F_0 quenching. The Lhcb3 polypeptide is considered to be the component of the inner LHC II most proximal to the PS II-core complex [14,17]. Due to this location it has been suggested that the Lhcb3 complex is responsible for conducting excitation energy from the bulk LHC II to the PS II-core/RC complex [14]. The results of the present study corroborate this idea. Moreover they suggest that in particular Lhcb3 is involved in regulation of the excitation flow via nonradiative energy dissipation, thus avoiding overexcitation of the RC of PS II. Whether the observed phenomenon is, however, indeed specific for the Lhcb3 complex, or rather the result of a cooperation of all inner LHC II requires further experimental evidence.

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