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ORGANIZATION OF THE PHOTOSYNTHETIC APPARATUS OF THE *chlorina-f2* MUTANT OF BARLEY USING CHLOROPHYLL FLUORESCENCE DECAY KINETICS

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The time-resolved chlorophyll fluorescence emission of higher plant chloroplasts monitors the primary processes of photosynthesis and reflects photosynthetic membrane organization. In the present study we compare measurements of the chlorophyll fluorescence decay kinetics of the chlorophyll-*b*-less *chlorina-f2* barley mutant and wild-type barley to investigate the effect of alterations in thylakoid membrane composition on chlorophyll fluorescence. Our analysis characterizes the fluorescence decay of *chlorina-f2* barley chloroplasts by three exponential components with lifetimes of approx. 100 ps, 400 ps and 2 ns. The majority of the chlorophyll fluorescence originates in the two faster decay components. Although photo-induced and cation-induced effects on fluorescence yields are evident, the fluorescence lifetimes are independent of the state of the Photosystem-II reaction centers and the degree of grana stacking. Wild-type barley chloroplasts also exhibit three kinetic fluorescence components, but they are distinguished from those of the *chlorina-f2* chloroplasts by a slow decay component which displays cation- and photo-induced yield and lifetime changes. A comparison is presented of the kinetic analysis of the *chlorina-f2* barley fluorescence to the decay kinetics previously measured for intermittent-light-grown peas (Karukstis, K. and Sauer, K. (1983) Biochim. Biophys. Acta 725, 384–393). We propose that similarities in the fluorescence decay kinetics of both species are a consequence of analogous rearrangements of the thylakoid membrane organization due to the deficiencies present in the light-harvesting chlorophyll *a*/*b* complex.

Introduction

The in vivo chlorophyll fluorescence of higher plant chloroplasts is typically characterized by time-resolved decay kinetics which reflect a heterogeneous fluorescence origin [1–5]. To determine the nature of this heterogeneity, the chlorophyll fluorescence decay has been monitored as a function of (1) the photochemical state of Photosystem II (PS II) reaction centers as altered by excitation light intensity [1,4] and reduction potential of the suspending medium [6,7], and (2) photosynthetic membrane organization as controlled by divalent ion concentration [8], membrane protein phosphorylation [9], and mode of illumination during plant growth [10]. Three exponential phases best describe the fluorescence decay kinetics of higher plant chloroplasts observed at room temperature using low-intensity laser excitation pulses and picosecond-resolution single-photon timing systems. A ‘fast’ phase of approx. 50–130 ps and a ‘middle’ phase of approx. 300–750 ps constitute the major components of the F_0 level of fluorescence with PS II reaction centers open to photochemistry. A

Abbreviations: PS I, II, Photosystem I, II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Chl, chlorophyll; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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'slow' phase of variable lifetime (800 ps–2 ns) is the major fluorescence component in closed PS II reaction centers at the F_{max} level of fluorescence. A long-lived (2–4 ns) fluorescence decay is present under some non-physiological conditions, such as for chloroplasts prepared from intermittent-light-grown plants [10] or for chloroplasts poised at low reduction potentials [6]. The variable kinetics and multiexponential character of the fluorescence emissions measured in these investigations suggest a complex model for the photosynthetic apparatus.

Variations in photosynthetic membrane composition as the result of highly specific mutations affecting identifiable thylakoid polypeptides also alter chlorophyll fluorescence decay kinetics. We present here one such study (also Green, B.R., Karukstis, K.K. and Sauer, K., unpublished data) of the time-resolved chlorophyll fluorescence emission of the viable *chlorina-f2* mutant of barley. This nuclear mutant [11] has normal PS I and PS II activities [11,12], yet lacks chlorophyll *b* [11–15] and is deficient in several major polypeptides [13–21]. Of the three major components of the light-harvesting chlorophyll *a*/*b*-protein complex [15,21–25], the *chlorina-f2* mutant lacks the 27 kDa polypeptide, contains only trace amounts of the 25 kDa polypeptide, and possesses 50% of the amount of 24 kDa polypeptide present in wild-type membranes [21]. It also lacks the three polypeptides in the 20–22 kDa range which are constituents of the PS I peripheral antenna complex normally comprised of approx. 45 chlorophyll molecules bound per P-700 [21,26,27]. The presence of a partial complement of light-harvesting complex in the *chlorina-f2* mutant alters the quantitative cation requirement for structural and functional changes as evidenced by the degree of grana stacking and variable fluorescence [15]. Partial grana stacking is evident by electron microscopy [18,28], although the extent of thylakoid appression is only 50% of that of wild-type chloroplasts [28], even in the presence of high concentrations of divalent ions [15]. The degree of variable fluorescence at room temperature is also cation-controlled [15,29,30]; however, higher concentrations of cations are required to elicit a response in mutant membranes than in wild-type chloroplasts, and the magnitude of the fluorescence increase effected is considerably reduced in the mutant chloroplasts [15,30].

Measurements of the *chlorina-f2* and wild-type barley fluorescence decay kinetics at room temperature are presented here for comparison with the characteristic three-exponential chlorophyll fluorescence emission observed for other higher plant chloroplasts [1–10]. We note that Searle et al. [31] previously used picosecond time-resolved fluorescence spectroscopy to study the organizational and functional differences between chloroplasts from *chlorina-f2* and wild-type barley. However, we believe that a reexamination is warranted as a consequence of the present availability of single-photon timing techniques which permit the use of much lower pulse energies and the resolution of multiple decay components.

Materials and Methods

Broken chloroplasts were prepared from growth-chamber wild-type (*Hordeum vulgare*) and *chlorina-f2* mutant barley as previously described [6,7,10]. For fluorescence measurements the isolated chloroplasts were suspended in a buffer solution of 0.1 M sucrose/50 mM Hepes-NaOH (pH 7.5)/5 mM NaCl ($\pm MgCl_2$ as indicated) at a concentration of 10 μg Chl per ml.

The fluorescence excitation source was a Spectra Physics synchronously pumped mode-locked dye laser (SP 171 argon ion laser, SP 362 mode locker and modified SP 375 dye laser). Chloroplast samples were excited with pulses of 8 ps half-maximum full-width duration at 620 nm. The maximum laser pulse intensity (10^7 photons per cm^2) was attenuated with neutral density filters for intensity dependence experiments. Fluorescence was detected at right angles at 680 nm. The single-photon timing system and numerical analysis methods have been described previously [1,32–34]. All fluorescence decay data were resolved into a sum of exponential decays with a lifetime resolution limit of 50 ps. The degree of variability in the deconvoluted lifetimes for replicate samples measured during the course of a single experiment is generally ± 10 ps for a fluorescence decay component in the 50–200 ps range, ± 25 ps in the 500 ps–1 ns range, ± 50 ps in the 1–2 ns range and ± 150 ps in the 2–4 ns range.

Results

Table I presents the analysis of *chlorina-f2* fluorescence decays measured at the F_0 level of fluorescence (dark-adapted chloroplasts + low excitation laser intensity) and the F_{max} level of fluorescence (high excitation laser intensity + 5 μM DCMU) for chloroplasts isolated in the presence of 5 mM Mg^{2+} from *chlorina-f2* plants of various ages. Three decay components can be resolved: a long-lived component ($\tau = 1600–2300$ ps) of very small amplitude at both F_0 and F_{max}; an intermediate decay component ($\tau = 370–525$ ps) making the largest contribution to the variable fluorescence upon photoreduction; and a fast decay component ($\tau = 90–125$ ps) of largest amplitude at both F_0 and F_{max}. Although the F_0 and F_{max} fluorescence levels decrease with age (data not shown), the average ratio of the F_{max} level of fluorescence to that at F_0 increases with age from 1.5 to 2.1, reaching a plateau after 21 days of growth. The F_0 yields in Table I have been normalized to the same value to demonstrate more effectively the increase in the level of variable fluorescence with plant age.

Fig. 1 summarizes the effect of added divalent cation at several concentrations on the fluorescence decay of chloroplasts from 21-day-old *chlorina-f2* plants. Measurements of the fluorescence decay kinetics were made at F_0 (dark-adapted chloroplasts + low excitation intensity) and F_{max} (high excitation intensity + 5 μM DCMU). Added Mg^{2+} increased the total F_{max} fluorescence yield, with a saturation in fluorescence enhancement at approx. 10 mM and with the most pronounced effect on the slow decay component yield. Variations in fluorescence lifetimes evident upon Mg^{2+} addition are within the experimental error of our measurements and are not considered significant.

Tables II and III compare the fluorescence decay kinetics of wild-type and mutant barley measured in the presence and absence of added cation (5 mM Mg^{2+}) at F_0 (dark-adapted chloroplasts + low excitation intensity) and F_{max} (high excitation intensity + 10 μM DCMU). Three exponential components were resolved for the fluorescence decay of wild-type barley. In the absence of added Mg^{2+}, the decay components are characterized by fluorescence lifetimes of 60, 235 and 600 ps at F_0 and 100, 310 and 730 ps at F_{max}. The fluorescence lifetimes exhibit more variation with photoreduc-

TABLE I

CHARACTERISTICS OF THE FLUORESCENCE DECAY OF *chlorina-f2* BARLEY CHLOROPLASTS AS A FUNCTION OF PLANT AGE

Chlorina-f2 barley chloroplasts were isolated from plants of various ages as indicated and suspended in a buffer medium of 0.1 M sucrose/10 mM HEPES-NaOH (pH 7.5)/5 mM NaCl/5 mM $MgCl_2$. Amplitudes are normalized such that $\Sigma\alpha = 1$ at each fluorescence level. Fluorescence yields are normalized to the same ϕ_{total} at F_0 for each sample.

Fluorescence levels	12-day-old			15-day-old			21-day-old			38-day-old		
	α	τ (ps)	ϕ	α	τ (ps)	ϕ	α	τ (ps)	ϕ	α	τ (ps)	ϕ
F_0												
	0.02	1900	12	0.02	2300	13	0.02	1900	11	0.01	1700	7
	0.29	390	34	0.30	370	34	0.32	370	38	0.26	350	33
	0.69	100	22	0.68	90	21	0.66	90	19	0.73	100	28
			——+			——+			——+			——+
			68			68			68			68
F_{max}												
	0.03	1600	18	0.04	1900	23	0.03	1970	27	0.02	1650	18
	0.38	390	56	0.41	520	74	0.39	425	81	0.33	400	81
	0.59	110	26	0.55	125	24	0.58	120	34	0.65	120	46
			——+			——+			——+			——+
			100			121			142			145

tion in the presence of added cation: $\tau = 35$, 270 and 530 ps at F_0 and $\tau = 110$, 400 and 1200 ps at F_{max}. The wild-type chloroplasts exhibit the usual cation-induced increase in fluorescence yield (2.3-fold at F_{max}) [35–38] and an $F_{max} : F_0$ ratio (= 3.6) upon photoreduction in the presence of 5 mM Mg^{2+} which is typical for higher plant chloroplasts [39]. The F_{max} fluorescence level of the *chlorina-f2* chloroplasts is 60% of that of wild-type chloroplasts in the absence of Mg^{2+} and only 29% of the wild-type F_{max} level in the presence of 5 mM Mg^{2+}. Similar observations have been made for steady-state measurements of the maximum fluorescence level of wild-type and *chlorina-f2* barley [15,30,31].

Discussion

Chlorophyll fluorescence of wild-type and chlorina-f2 mutant barley

The present investigation confirms the results of previous steady-state fluorescence studies which observed pronounced differences in the Mg^{2+}-induced increases in Photosystem II fluorescence at room temperature of wild-type and *chlorina-f2* mutant barley [22,29–31]. These results are consistent with the well-documented observations that the degree of thylakoid membrane stacking is directly related to both the proportion of the major polypeptides of the light-harvesting chlorophyll *a/b* complex present [40–44] and the cation composition of the suspending medium [45–50].

A comparison of the kinetics of the chlorophyll fluorescence decay of wild-type and *chlorina-f2* barley chloroplasts also reveals several significant differences. Most notably, the slow decay component of 500–1200 ps, which is present in wild-type membranes and exhibits a photo-induced differentiation in yield and a cation-induced differentiation in lifetime, is absent in *chlorina-f2* mem-

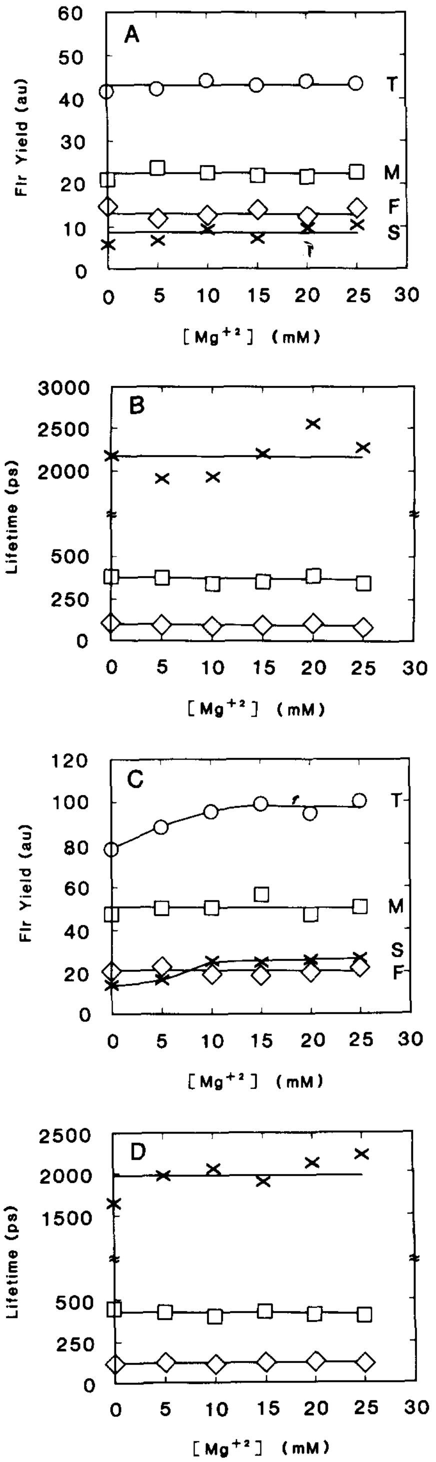

Fig. 1. (A) Total fluorescence yield and the yields of the components of the fluorescence decay in *chlorina-f2* barley chloroplasts at F_0 (dark-adapted chloroplasts + low excitation intensity) as a function of Mg^{2+} concentration. (B) Lifetimes of the components of the fluorescence decay in *chlorina-f2* barley chloroplasts at F_0. (C) Total fluorescence yield and yields of the components of the fluorescence decay in *chlorina-f2* barley chloroplasts at F_{max} (high excitation intensity + 5 μM DCMU) as a function of Mg^{2+} concentration. (D) Lifetimes of the components of the fluorescence decay in *chlorina-f2* barley chloroplasts at F_{max}. The symbols are defined as follows: (○) total fluorescence yield, (x) yield or lifetime of the slow phase, (□) middle phase and (◇) fast phase.

TABLE II

SUMMARY OF FLUORESCENCE DECAY KINETICS OF CHLOROPLASTS OF 21-DAY-OLD *chlorina f-2* BARLEY

Fluorescence levels											
F_0, no added Mg^{2+}			F_{max}, no added Mg^{2+}			F_0, +5 mM Mg^{2+}			F_{max}, +5 mM Mg^{2+}		
α [a]	τ (ps)	ϕ [b]	α [a]	τ (ps)	ϕ [b]	α [a]	τ (ps)	ϕ [b]	α [a]	τ (ps)	ϕ [b]
0.02	2500	3.5	0.02	2200	5	0.02	2800	3.5	0.02	2700	7
0.26	400	8	0.35	490	14	0.25	425	5.5	0.33	490	14
0.72	100	5.5	0.63	140	7	0.73	130	6	0.65	150	8
		——+			——+			——+			——+
		17			26			17			29

[a] Amplitudes are normalized such that $\Sigma\alpha = 1$ at each fluorescence level.

[b] Fluorescence yields are normalized such that $\phi_{total} = 100$ at F_{max} for wild-type barley chloroplasts in the presence of 5 mM Mg^{2+} (see Table III).

branes. This correlation is strong evidence that the slow fluorescence decay component of wild-type chloroplasts is kinetically controlled by the decay processes of the light-harvesting chlorophyll *a/b*-protein complex [1,8]. The origin of this slow phase has been attributed [1,4,5] to PS II fluorescence following charge separation in a closed reaction center ($\text{P-680 IQ}^- \xrightarrow{h\nu} \text{P}^+\text{-680 I}^-\text{Q}^-$) and a subsequent radical pair recombination of the oxidized primary electron donor, P^+-680, and the reduced pheophytin primary electron acceptor, I^-. Excitation arising from the repopulation of the excited singlet state of chlorophyll ($\text{P}^+\text{-680 I}^-\text{Q}^- \rightarrow \text{P}^*\text{-680 IQ}^-$) may be transferred from P*-680 to the antenna chlorophyll molecules and emitted as the slow decay component of fluorescence. In general, the increase in variable fluorescence upon chemical reduction or photoreduction of wild-type PS II reaction centers reflects an increase in the fluorescence yield of the slow decay component. The lifetime of the slow phase is determined by the kinetics of the charge recombination bteween P^+-680 and I^- and by the rates of fluorescence quenching (e.g., radiationless decay, energy transfer to Photosystem I, etc.) from the PS II antenna [8]. In *chlorina-f2* chloroplasts the major contribution to the variable fluorescence is exhibited by the middle decay component. Thus, at least a portion of this component in *chlorina-f2* chloroplasts likely arises from the charge recombination of P^+-680 and I^-. We would explain the decrease in lifetime of this charge-recombination component from the

TABLE III

SUMMARY OF FLUORESCENCE DECAY KINETICS OF CHLOROPLASTS OF WILD-TYPE BARLEY

Fluorescence levels											
F_0, no added Mg^{2+}			F_{max}, no added Mg^{2+}			F_0, +5 mM Mg^{2+}			F_{max}, +5 mM Mg^{2+}		
α [a]	τ (ps)	ϕ [b]	α [a]	τ (ps)	ϕ [b]	α [a]	τ (ps)	ϕ [b]	α [a]	τ (ps)	ϕ [b]
0.05	600	4	0.15	730	17	0.10	530	19	0.46	1200	79
0.42	235	15	0.45	310	21	0.31	270	15	0.30	400	17
0.53	60	4	0.40	100	6	0.59	35	4	0.24	110	4
		——+			——+			——+			——+
		23			44			28			100

[a] Amplitudes are normalized such that $\Sigma\alpha = 1$ at each fluorescence level.

[b] Fluorescence yields are normalized such that $\phi_{total} = 100$ at F_{max} in the presence of 5 mM Mg^{2+}.

1–2 ns of wild-type chloroplasts to approx. 400 ps in *chlorina-f2* barley as a consequence of increases in the rates of fluorescence quenching in the PS II antenna arising from the modified antenna structure. A change in the kinetics of charge recombination is not predicted.

By contrast, the mutant chloroplasts possess a small amount of a long-lived decay component of 1600–2800 ps, characteristic of uncoupled chlorophyll *a* as observed in chloroplasts of intermittent-light-grown plants [10]. The origin of this unorganized chlorophyll *a* may be an improper assembly of the light-harvesting complex and PS I antennae complex in the absence of chlorophyll *b*. Bellemare et al. [21] have demonstrated that all of the polypeptides that are either missing from or depleted in the membranes of mutant thylakoids are in fact synthesized in vivo. They have further proposed that chlorophyll *b* is required for the stabilization and incorporation of these polypeptides and that, in the absence of chlorophyll *b*, rapid turnover of the polypeptides occurs [21]. Improperly associated chlorophyll *a* may result from the incomplete assembly of the light-harvesting chlorophyll *a/b* complex and the PS I antenna, giving rise to the long-lived fluorescence decay component. An abnormal antenna arrangement of chlorophyll *a* around PS-II reaction centers in *chlorina-f2* barley has also been proposed by Brown et al. from analyses of absorption and steady-state fluorescence emission spectra [51,52].

Wild-type and mutant chloroplasts also differ in the lifetime of the fast phase at F_0, with slower decay kinetics observed for the fast component in mutant membranes. In spinach chloroplasts, this fast component of fluorescence has been proposed to arise from excitation lost from the chlorophyll *a* antennae closely coupled with the PS-I and PS-II reaction centers [1–4,10], and thus represents a composite of PS-I and PS-II antenna decays. As a consequence, lack of the PS-I antenna complex in *chlorina-f2* chloroplasts [21,26,26] may result in the different (slower) kinetics observed for the fast component.

In their time-resolved fluorescence study of *chlorina-f2* membranes, Searle et al. [31] obtained a two-exponential fit to the fluorescence decay, with $\tau = 40$ and 194 ps at F_0 and $\tau = 60$ and 424 ps at F_{max}. These components are similar to our two shorter lifetime components, but the limited time range of analysis (1 ns) used in the study of Searle et al. precluded the observation of the long-lived fluorescence component of approx. 2 ns present in our work. The reported absence of any effect of $MgCl_2$ on the fluorescence decay kinetics of *chlorina-f2* chloroplasts in the Searle et al. investigation may be a consequence of the limited concentration range (0–5 mM) studied, because our investigation revealed measurable effects of $MgCl_2$ at higher concentrations. Finally, the earlier kinetic measurements [31] may include distortions from biexcitonic annihilation processes induced by the high excitation intensities employed [53,54].

As a general observation of our measurements of the fluorescence decay kinetics of higher plant chloroplasts, we note that the lifetimes of the three fluorescence decay components in wild-type chloroplasts vary with the species. The most pronounced variation is observed for the lifetime of the slow decay component at F_0 and F_{max} in the presence of Mg^{2+}. The shortest range of lifetimes for this component is exhibited by barley chloroplasts (530–1200 ps), intermediate lifetimes by pea chloroplasts (800–1600 ps) [10], and the longest lifetimes by spinach chloroplasts (1–2 ns) [1,6–9]. Despite the slight variations in decay kinetics, these fluorescence components behave similarly from one wild-type species to another, and we therefore feel that these components have similar origins regardless of species.

Chlorophyll fluorescence of chlorina-f2 barley and intermittent-light-grown plants

Chloroplasts of intermittent-light-grown plants contain unstacked, primary thylakoids [55] devoid of chlorophyll *b* and deficient in the light-harvesting chlorophyll *a/b* complex [55–58] and thus are similar in thylakoid composition to the *chlorina-f2* mutant. The chlorophyll fluorescence decay kinetics of both types of photosynthetic membranes exhibit similar characteristics. In both systems the absence of a slow fluorescence decay component analogous to that resolved in wild-type chloroplasts is suggestive of a specific consequence of the deficiency in the light-harvesting chlorophyll *a/b* complex. Improper chlorophyll *a* antenna arrangements in both species [51,52] appear to give rise t a long-lived fluorescence component [10] reflecting abnormal chlorophyll-protein organization. The partial complement of light-harvesting complex in the mutant provides for limited cation-induced grana stacking and fluorescence yield changes which are not evident for the chloroplasts of intermittent-light-grown seedlings [10]. These results are consistent with the concept that the light-harvesting complex is the primary membrane constituent which is responsible for cation-mediated structure-function processes [15,59,60]. The absence of a differentiation in the kinetics of the slow decay component of intermittent-light plastids at F_0 and F_{max} in the presence of Mg^{2+} has been associated with the absence of grana [10]. An analogous association can be suggested for the similar slow decay kinetics of *chlorina-f2* membranes. Furthermore, the lack of differentiated PS II units (α- and β-centers) in the barley mutant chloroplast (as a consequence of the light-harvesting chlorophyll *a/b* complex deficiency [43,44]) is a likely explanation for the similar kinetics of the middle decay component at F_0 and F_{max}, as also observed in the fluorescence studies of intermittent-light seedlings [10].

The increase in the level of variable fluorescence with the age of the *chlorina-f2* barley plant, as demonstrated in Table I, reveals the known dependence of chloroplast development on the age of the plant tissue [10,61]. A similar dependence was previously observed in our study of protochloroplast to chloroplast differentiation of intermittent-light-grown plants [10], where the rate and extent of chloroplast development, as revealed through measured fluorescence lifetime and yield variations during plant growth, was controlled by the age of the etiolated tissue. The increase in the ratio of F_{max}/F_0 with age observed in this study for *chlorina-f2* chloroplasts most likely parallels the development of the partial complement of light-harvesting complex and the extent of grana formation [10,62].

Conclusion

Structural and organizational changes associated with the deficiencies in the light-harvesting chlorophyll *a/b* complex of the *chlorina-f2* barley mutant have been correlated with the measured kinetic parameters of chrorophyll fluorescence emission. Our results confirm earlier studies which demonstrate that the variable kinetics and multiexponential character of the fluorescence emission of higher platns reflect the heterogeneous population of fluorescing chlorophyll *a* molecules and the variations in organization of photosynthetic units [1–10]. A comparison of the fluorescence decay kinetics of *chlorina-f2* barley chloroplasts and plastids from intermittent-light-grown peas, with comparable molecular compositions of photosynthetic pigments, suggests an analogous structural assembly of chlorophyll-protein complexes. Gene mutations in higher plants which result in a highly specific polypeptide deficiency in thylakoid composition will provide further tests of the correlations between chlorophyll fluorescence kinetic parameters and photosynthetic membrane organization.

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