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Correlation between linolenic-acid deficiency in chloroplast membrane lipids and decreasing photosynthetic activity in barley

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Barley plants having chloroplasts with a gradually diminished linolenic and a gradually increased linoleic acid content (a gradually reduced linolenic-to-linoleic acid ratio) were grown by the application of sublethal doses of the pyridazinone compound SAN 9785. The photosynthetic properties of the leaves and of the chloroplasts isolated from the leaves were studied. SAN 9785 caused only minor changes in the chlorophyll and total lipid contents of the chloroplasts. The liposomes prepared from the chloroplast membrane lipids exhibited a lower level of fluidity, as revealed by fluorescence polarization measurements using diphenylhexatriene. A strong correlation was established between the modified chemical composition of the thylakoid membranes (altered linolenic-to-linoleic acid ratio) and (i) the relative intensity of the short-wavelength fluorescence bands of the low-temperature emission spectra; (ii) the normalized increment of fluorescence $(F_i - F_0)/(F_m - F_0)$ from fluorescence induction curves of intact leaves; (iii) the CO_2-fixation activity of intact leaves; (iv) the Hill reaction rate of isolated chloroplasts; and (v) the degree of coupling between electron transport and photophosporylation. The results suggest that the presence of a sufficient amount of linolenic acid associated with chloroplast membrane lipids is essential for the functioning of the electron-transport chain and coupled phosphorylation. There seems to be a linolenic-to-linolenic acid ratio threshold value of 2 for satisfying coupling. SAN 9785 treatment also caused the chlorophyll-protein complex of the reaction centre of Photosystem I to decrease in quantity, suggesting a stabilizing role of the lipid environment in the assembly and organization of Photosystem-I particles.

Introduction

Chloroplast membranes are known to have a unique lipid composition in that galactolipids predominate among the glycerolipids [1,2]. The fatty-acid profile of chloroplast membrane lipids is also different from those of other animal or microbiological membranes, since polyunsaturated fatty acids (mainly linolenic acid) constitute the majority of the fatty acids associated with the chloroplast lipids [3,4]. The structural role and the functional significance of this lipid and fatty-acid constitution have not been satisfactorily elucidated so

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Abbreviations: SAN 9785, 4-chloro-5-(dimethylamino)-2-phenyl-3(2H)-pyridazinone, also known as BASF 13.338; DPH, 1,6-diphenyl-1,3,5-hexatriene; 18:2, linoleic acid (number of carbon atoms/number of double bonds); 18:3, linolenic acid; MGDG, monogalactosyl diacylglycerol; CP1, chlorophyll-protein complex of the reaction centre of Photosystem I; CPa, chlorophyll-protein complex of reaction centre of Photosystem II; LHCP, light-harvesting chlorophyll-protein complex; PS I, Photosystem I; PS II, Photosystem II; F_0, fluorescence of constant yield; F_i, initial yield of fluorescence; F_m, maximal yield of fluorescence; F-687, F-695, F-743, fluorescence bands at 687, 695 and 743 nm, respectively; Q, first stable electron acceptor of Photosystem II; PQ, plastoquinone; I_{50}, half-inhibitory concentration (in μM); D_{9785}, dose of SAN 9785 in mg/100 seedlings; Chl, chlorophyll.

far. Attempts have been made to study the galactolipid [5,6] and phospholipid [7] requirements of various photosynthetic processes. As concerns the fatty acid composition of the chloroplast membranes, it has been established that exogenously added linolenic acid acts as an inhibitor of the electron-transport chain [8,9] and destroys the structure of the inner membrane system of the chloroplasts [10]. Little is known, however, about the in vivo role of linolenic acid. It has been shown that chloroplast thylakoid membranes possess a uniquely high level of fluidity [11,12], and polyunsaturated fatty acids are thought to be involved in the maintenance of this high level, which is very possibly required for the full activity of electron transport and associated processes. Our present knowledge seems to be insufficient for a complete understanding of the interrelationship of the lipid environment and the photosynthetic competence. In our present work we set out to study this problem by 'producing' plants whose chloroplasts are deficient in linolenic acid to different degrees.

For this purpose, the pyridazinone compound SAN 9785 was applied from the onset of germination to barley seedlings. This compound has been shown to be a specific inhibitor of the desaturation reaction between linoleic and linolenic acids [13,14]. Attention was paid to the elimination of further phytotoxic action, since 9785 in higher concentration can interfere with the electron transport of chloroplasts per se [15]. In this paper we show that the photosynthetic activities of barley leaves and of the chloroplasts isolated from the leaves decrease parallel with the decrease of the linolenic to linoleic acid ratio.

Materials and Methods

Barley (*Hordeum vulgare* L., var. Horpácsi kétsoros) plants were grown on moistened filter paper for 7 days, illuminated in 16/8 h light/dark cycles with white light of 0.8 mW · cm^{-2} intensity. SAN 9785 was applied to the filter papers in acetone solution; the exact quantity given was varied between 0 and 2 mg (see Table I). After evaporation of the solvent, 100 seeds were placed on each Petri dish; the germination activity was found to be practically 100%.

Chloroplasts were isolated from the leaves as described previously [16].

For the separation and quantification of chloroplast lipids, the isolated chloroplasts were transferred to a mixture of chloroform/methanol 2 : 1 (v/v) [17]. Lipids were separated by silicic-acid column chromatography [18], followed by thin-layer chromatography [19]. Fatty acids were transesterified, and the methyl esters were separated and determined quantitatively by gas-liquid chromatography as described elsewhere [16]. The chlorophyll contents of the samples were determined according to Ref. 20.

DPH fluorescence polarization measurements on liposomes prepared from total lipid extracts of chloroplasts were performed with a Perkin-Elmer MPF 44/A instrument, as described previously [21,22].

The low-temperature fluorescence spectra of the leaves were recorded at 77 K with the same apparatus from 660 to 780 nm with 430 nm excitation. The fluorescence spectra were corrected for the spectral sensitivity of the apparatus, and were normalized to unit area.

The fluorescence induction curves of the intact leaves were recorded with the laboratory-built apparatus described in Ref. 23.

In vivo $^{14}CO_2$-fixation measurements were carried out as described in [24].

Solubilization of thylakoid membranes with SDS and separation of chlorophyll-protein complexes by SDS-polyacrylamide gel electrophoresis were according Ref. 25. The proportions of the individual bands were estimated from the areas under the bands of densitograms measured at 671 nm. The chlorophyll contents of the leaves were determined according to Ref. 20.

The in vitro photochemical activity of chloroplasts was followed with a Clark-type oxygen-electrode, as light-induced oxygen evolution in the presence of potassium ferricyanide. Isolated chloroplasts were resuspended in a medium containing 0.05 M sorbitol/2 mM EDTA/1 mM $MgCl_2$/1 mM $MnCl_2$/50 mM Tricine (pH 7.8). The 3.1 ml reaction mixture contained 0.33 M sorbitol/50 mM Tricine/5 mM $MgCl_2$/0.5 mM $K_3[Fe(CN)_6]$/(pH 7.8). For phosphorylating conditions, 2.2 mM ADP and 2.2 mM KH_2PO_4 were added [26]. Samples containing chloroplasts equivalent to

40 μg chlorophyll were illuminated with white light of 10 mW · cm^{-2} intensity at a constant temperature of 25°C.

Statistical treatment of the data was carried out by linear regression analysis. The following values were computed: r, Bravais correlation coefficient; m, regression coefficient (slope of line); $\pm s_m$, standard error of m; p, level of significance in Fisher F-test.

Results

The pyridazinone compound SAN 9785 was applied in increasing doses to barley seedlings from the onset of germination. The fatty acid composition of the chloroplast membrane lipids was affected by the changes caused in the proportions of linolenic and linoleic acids by the treatment (Table I). It is clearly seen that increasing concentrations of SAN 9785 led to a gradual decrease of the linolenic acid content, from 71% to as low as 14%. This was accompanied by an increase in the linoleic acid content. Accordingly, there were marked changes in the 18 : 3/18 : 2 ratio. It is of great importance that SAN 9785 left the amounts of the individual lipids practically unchanged, and the observed alterations in fatty-acid composition were of equal magnitude in all lipid classes (data not shown).

TABLE I

EFFECT OF SAN 9785 TREATMENT ON THE PROPORTIONS OF LINOLEIC (18:2) AND LINOLENIC (18:3) ACIDS IN TOTAL LIPIDS OF CHLOROPLASTS ISOLATED FROM BARLEY LEAVES

The applied doses (D_{9785}) and the corresponding concentration values (in μM) are also presented. The fatty-acid values are means of six determinations from three experiments.

D_{9785} (mg/100 seedlings)	Concn. (μM)	18:2 (%)	18:3 (%)	$\frac{18:3}{18:2}$
0	–	16	71	4.44
0.05	1.0	21	63	3.00
0.1	2.0	30	56	1.87
0.2	4.0	35	52	1.49
0.5	10.0	42	46	1.10
1.0	20.0	69	17	0.25
2.0	40.0	72	14	0.19

The relative intensity of the long-wavelength fluorescence band F_{743} displayed decreasing values in response to SAN 9785 treatment. When the peak ratios F_{743}/F_{687} and F_{743}/F_{695} were plotted as functions of the corresponding 18 : 3/18 : 2 value, straight lines were obtained (Fig. 1). Statistical analysis of the data (Table II) showed the existence of a strong correlation between the 18 : 3/18 : 2 ratio and the relative intensity of F_{743}. In other words, lower 18 : 3/18 : 2 ratios give rise to greater proportions of the short-wavelength components F_{695} and F_{687}, originating from PS II and LHCP, respectively [27].

The in vivo fluorescence induction characteristics of dark-adapted leaves have been shown to be sensitive indicators of light-utilization efficiency and of the rate of electron transport between PS II and the PQ pool [28,29]. The ratio $(F_i - F_0)/(F_m - F_0)$ for the fluorescence induction curves recorded in the time range of several seconds, also known as the normalized increment of fluores-

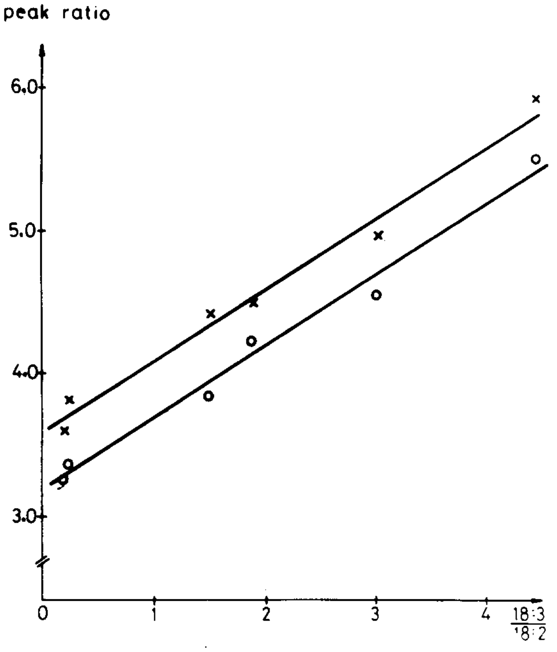

Fig. 1. Peak ratios F_{743}/F_{687} (O——O) and F_{743}/F_{695} (×——×) of the low-temperature fluorescence spectra of barley leaves as functions of the 18 : 3/18 : 2 ratio in total lipids of chloroplasts isolated from barley plants grown in the presence of increasing amounts of SAN 9785. Each value is the mean of nine determinations from three experiments.

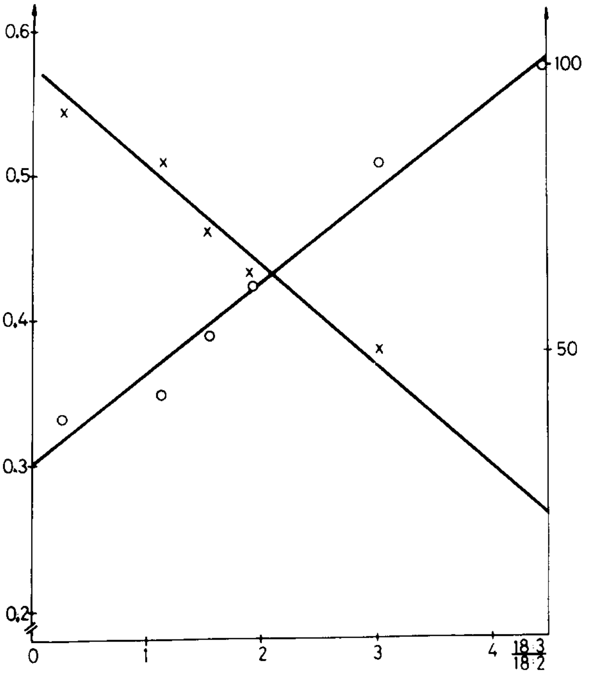

Fig. 2. Values of the $(F_i - F_0)/(F_m - F_0)$ ratio (× —— ×) (left ordinate) and of the $^{14}CO_2$-fixation activity (O —— O) (right ordinate, in % of control) of intact barley leaves as functions of the 18 : 3/18 : 2 ratio in total lipids of chloroplasts isolated from barley plants grown in the presence of increasing amounts of SAN 9785. Each value is the mean of nine determinations from three experiments.

cence yield [30], proved to be a useful quantity for the evaluation of these processes [31]. Fig. 2 shows that increasing SAN 9785 doses (i.e., decrasing 18 : 3/18 : 2 values) resulted in a gradual increase of $(F_i - F_0)/(F_m - F_0)$, indicating that the re-oxidation of Q^- by the PQ pool became progressively more hindered. In vivo $^{14}CO_2$-fixation activity of treated leaves showed gradually decreasing values in response to decreasing 18 : 3/18 : 2 ratio of chloroplast lipids (Fig. 2). Statistical analyses of the data reveal a strict correlation between the fatty acid composition of chloroplast membranes and the in vivo functional properties of the leaves (Table II).

For study of the sensitivity of the photosynthetic electron transport to a linolenic acid deficiency in the thylakoid membranes, the in vitro Hill reaction activities of chloroplasts isolated from leaves grown in the presence of increasing SAN 9785 doses were also investigated. The rate of the ferricyanide-mediated Hill reaction, particularly when the cofactors of photophosphorylation were present, proved to be extremely sensitive to treatment. It is also clear that the stimulatory effect of the phosphorylation cofactors gradually decreased with incraesing SAN 9785 doses. Plots of the 'basal' and 'phosphorylating' electron-transport rates and their ratio against the 18 : 3/18 : 2 ratio for the chloroplast membrane lipids (Fig. 3) indicate a strong correlation between the 'phosphory-

TABLE II

VALUES OF LINEAR REGRESSION ANALYSES AFTER STATISTICAL TREATMENTS OF THE DATA

x, variable x; y, variable y; r, Bravais correlation coefficient; $m \pm s_m$, value ± standard deviation of the regression coefficient (slope of the regression line); n, number of data pairs; p, level of significance in Fischer F-test.

x	y	r	$m \pm s_m$	n	p
18:3	18:2	−1.0023	−1.0009 ± 0.0216	7	0.001
$\frac{18:3}{18:2}$	$\frac{F_{743}}{F_{695}}$	+0.9930	+0.5148 ± 0.0307	6	0.001
$\frac{18:3}{18:2}$	$\frac{F_{743}}{F_{687}}$	+0.9934	+0.5121 ± 0.0297	6	0.001
$\frac{18:3}{18:2}$	$\frac{F_i - F_0}{F_m - F_0}$	−0.9931	−0.0656 ± 0.0039	6	0.001
$\frac{18:3}{18:2}$	$^{14}CO_4$ fixation	+0.9873	+16.0653 ± 1.6694	6	0.001
$\frac{18:3}{18:2}$	$H_2O \rightarrow FeCy$ $(+ADP, +P_i)$	+0.9983	+66.7887 ± 2.2227	5	0.001

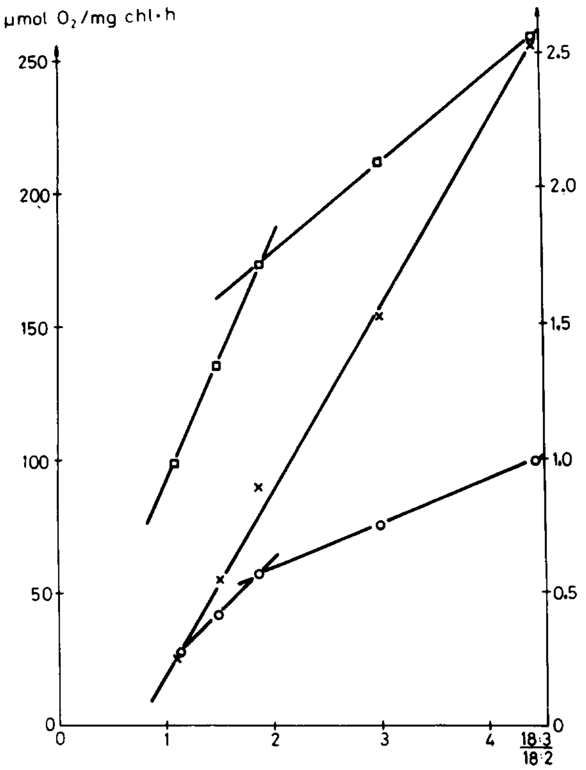

Fig. 3. Rates of 'basal' (○———○) and 'phosphorylating' (×———×) electron transport from water to potassium ferricyanide (both on the left ordinate) and their ratio (phosphorylating/basal) (□———□) (right ordinate) of chloroplasts isolated from barley plants as functions of the 18 : 3/18 : 2 ratio in total lipids of chloroplasts isolated from barley plants grown in the presence of increasing amounts of SAN 9785. Each value is the mean of six determiantions from three experiments.

lating' electron transport and the fatty-acid ratio. Statistical analysis showed the strongest correlation in this case (Table II). As concerns the 'basal' or 'non-phosphorylating' electron transport and also the 'phosphorylating' to 'basal' ratio, a discontinuity of the lines was observed at a 18 : 3/18 : 2 ratio of around 2 (Fig. 3).

To study whether the observed changes induced by SAN 9785 treatment in the fatty-acid composition of the thylakoid membranes were accompanied by alterations in the fluidity characteristics of the lipids constituting the chloroplast membranes, DPH fluorescence polarization measurements were carried out. For this purpose, barley plants grown in the presence of 1 mg of SAN 9785 (corresponding to roughly 20 μM concentration) were used. This dose was chosen because it is sufficiently high to exert a profound change in the 18 : 3/18 : 2 ratio (Table I), but not high enough to cause lethal phytotoxic actions. Fig. 4 shows that liposomes from SAN 9785-treated samples proved to be less fluid in the whole temperature range (0–45°C). It is also seen that the two break-points in the line occur at considerably different temperatures than in the control, indicating a marked alteration of the phase behaviour in the treated samples as compared to that of the controls.

The changes in the low-temperature fluorescence spectra in response to different doses of SAN 9785 (Table I, Fig. 2) led us to investigate the chlorophyll-protein complexes in control and treated plants. For this purpose, plants grown on 1 mg SAN 9785 were used. The treated plants exhibited a 20% loss of total chlorophylls on a fresh weight basis and had a slightly lower Chl *a*/*b* ratio as compared to the control (Table III).

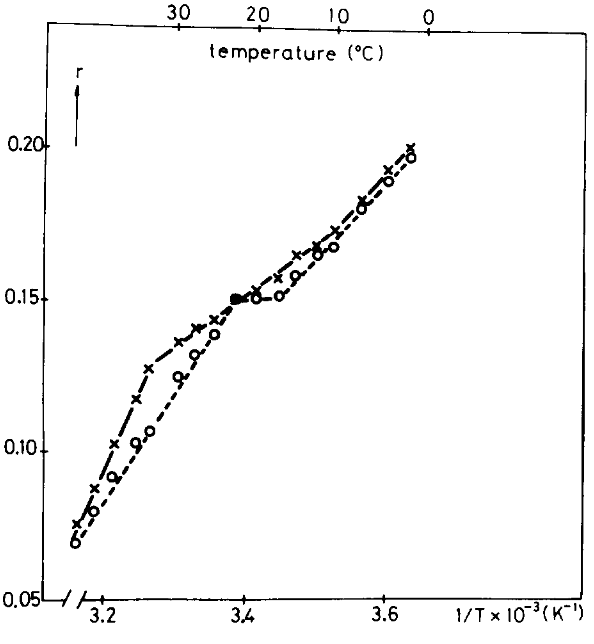

Fig. 4. Anisotropy (*r*) of DPH fluorescence in liposomes prepared from total lipids of chloroplasts isolated from barley plants grown in the presence (○———○) or absence (×———×) of 1 mg (corresponding to 20 μM) SAN 9785 as a function of the reciprocal absolute temperature. Arrows indicate the position of discontinuities in the lines. Values represent the means of three determinations from three independent experiments.

TABLE III

PROPORTION OF CHLOROPHYLL (IN % OF TOTAL) IN CHLOROPHYLL-PROTEIN COMPLEXES OF CHLOROPLASTS ISOLATED FROM BARLEY PLANTS GROWN IN THE PRESENCE OR ABSENCE OF 1 mg (CORRESPONDING TO 20 μM) SAN 9785

Total chlorophyll contents were 1990 ± 210 μg and 1610 ± 175 μg/g fresh weight; the chlorophyll *a/b* ratios were 2.99 and 2.63 for control and SAN 9785-treated leaves, respectively. Each value is the mean of the results of three determinations from three experiments.

	CP1	LHCP I	LHCP II	CPa	LHCP III	Free chlorophyll
Control	28.8	9.9	2.7	9.2	24.7	25.1
San 9785-treated	22.2	10.4	trace	9.6	29.8	28.9

Separation of the chlorophyll-protein complexes by SDS-polyacrylamide gel electrophoresis revealed that the same complexes were present in the thylakoids of the control and the treated plant chloroplasts. The amount of CP1 in the treated samples, however, was decreased by about 25%, and only a trace amount of LHCP II was present in the thylakoids of treated plants (Table III).

Discussion

The results presented in this paper clearly document that the photosynthetic competence of the leaves and chloroplasts is decreased when the plants are grown on increasing quantities of SAN 9785. Since this compound can interfere directly with the photosynthetic electron transport [15], we have to be convinced that the observed action originates solely from the modified lipid environment. The 'prompt' effects of this compound on the various parameters were also investigated, therefore, and the I_{50} values for 'prompt' and 'long-term' inhibition of photosynthetic parameters are presented in Table IV. For 'prompt' inhibitory action (without change in the fatty-acid composition) it is seen that far higher SAN 9785 concentrations are required than those applied during long-term experiments. It seems unlikely that such high concentration could accumulate within the chloroplasts during the growth period, and we conclude that the observed reduction of the photosynthetic activity is indeed a consequence of the modification of the thylakoid fatty-acid composition by SAN 9785.

The reduction of the 18:3/18:2 ratio led to incrased proportions of F_{687} and F_{695} being observed in the fluorescence spectra of leaves (Fig. 1). It follows that either the relative amounts of LHCP and PS II with respect to PS I are higher, or the efficiency of excitation energy channelling towards PS I is decreased. The first alternative was indicated by the analysis of the chlorophyll-protein complexes of thylakoid membranes (Table III). The observed reduction of the CP1 content of the chloroplast membranes is very possibly the cause of the lower Chl *a/b* ratio of the leaves (Table III). It has recently been shown [32,33] that SAN 9785 can be used to modify the stoichiometry between PS II and PS I in vivo in wheat. Our

TABLE IV

HALF-INHIBITORY I_{50} VALUES (IN μM) OF THE 'PROMPT' AND 'LONG-TERM' ACTIONS OF SAN 9785 FOR PARAMETERS RELATED TO PHOTOSYNTHESIS IN BARLEY

'Prompt' effects of SAN 9785 on the $(F_i - F_0)/(F_m - F_0)$ ratio and the $^{14}CO_2$ fixation were determined after 20 min infiltration of 7-days old barley leaves with increasing concentration of SAN 9785 solution; on the Hill reaction rate, SAN 9785 was added in ethanol solution to barley chloroplasts isolated and resuspended by the same procedure, than in the 'long-term' experiments.

Parameter	I_{50} (μM)	
	'prompt'	'long-term'
$\frac{F_i - F_0}{F_m - F_0}$	51	6.1
$^{14}CO_2$ fixation	56	6.6
$H_2O \rightarrow$ FeCy (+ADP, +P_i)	20	1.2

results seem to be consistent with this, but it should be emphasized that (i) barley is much more sensitive to SAN 9785 treatment than wheat in this respect, and (ii) our results suggest a shortage of PS I with unchanged level of PS II.

The linolenic acid deficiency led to a gradual inhibition of the reoxidation of Q^- by the PQ pool, as revealed by fluorescence induction measurements, and of the in vivo $^{14}CO_2$ fixation capacity of the leaves (Fig. 2). This inhibition very possibly occurs at the level of the electron-transport chain, as indicated by in vitro investigations. The Hill reaction activity of isolated chloroplasts was found to be the most sensitive parameter to the modified fatty-acid composition (Fig. 3). Stimulation of the 'basal' electron transport by phosphorylation cofactors is known to be a consequence of the coupling of photophosphorylation to electron transport [34,35]. This means that, under otherwise equal conditions, a higher stimulation reflects a larger degree of coupling. Our results demonstrate that the coupling between electron transport and non-cyclic phosphorylation became progressively less tight as the SAN 9785 dose incrased, and so the proportion of linolenic acid decreased. This finding indicates the existence of a correlation between the sufficient amount of linolenic acid present in the chloroplast membrane lipids and the degree of coupling between electron transport and phosphorylation. There seems to be a threshold value of around 2 for the 18:3/18:2 ratio, above which a satisfactory degree of coupling is observed (Fig. 3). The degree of coupling of ATP synthesis by chloroplasts and the rate of electron transport has been found to correlate well with the MGDG/phospholipid ratio [36]. A negative correlation has been demonstrated between the relative proportion of neutral lipids present in the thylakoids and the degree of coupling [37]. Since linolenic acid is mainly associated with galactolipids, it is reasonable to suppose that the appropriate lipid environment (a high MGDG content with a high linolenic-acid proportion) plays an important role in regulation of the interrelationship between the electron transport and energy conservation.

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