Comparative Immunological Studies on the CF_1-Complex in Mutants of *N. tabacum*, Exhibiting Different Capacities for Photosynthesis and Photorespiration and Different Chloroplast Structures

A. Radunz and G. H. Schmid

Universität Bielefeld, Fakultät für Biologie, Lehrstuhl Zellphysiologie, Postfach 8640, D-4800 Bielefeld 1, Bundesrepublik Deutschland

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Tobacco Mutants, CF_1-Complex, CF_1-Antiserum, Immunochemical Identity, Molar Ratio CF_1: Chlorophyll

We have compared by means of rocket immunoelectrophoresis the amount of CF_1-complex in *N. tabacum* mutants with that in the wild type *N. tabacum* var. John William's Broadleaf. Some of these mutants differ from the wild type not only by higher photosynthetic and photorespiratory activities but also by their chloroplast structure. The amount of CF_1 present was related to the chlorophyll content. However, no correlation between the amount of CF_1 present and a higher photosynthetic and photorespiratory activity was found. On the other hand the molar ratio of CF_1 to chlorophyll seems to depend in these tobacco mutants on the morphological structure of the chloroplasts. In chloroplasts of green phenotypes, in particular in those of the mutants *N. tabacum* var. Consolation, var. NC 95 and var. Xanthi with high grana stacking as well as in those of the wild type *N. tabacum* var. JWB one CF_1-complex occurs per 470–770 chlorophyll molecules. Whereas in chloroplasts of the yellow-green phenotypes with relatively more stroma thylakoids the amount of CF_1 seems to be increased only by a factor of 3–4. The yellow phenotypes contain on the average 1 CF_1-complex per 60 chlorophyll molecules. The CF_1-complex in the investigated chlorophyll-deficient tobacco mutants is immunochemically identical to that of the wild type *N. tabacum* var. John William's Broadleaf as demonstrated by means of the tandem-crossed immunoelectrophoresis. However, between the CF_1-complex of tobacco and that of other higher plants such as spinach *Spinacia oleracea,* only partial identity is observed. Comparative quantitative determinations have shown that one CF_1-complex binds on the average 8–9 CF_1-antibodies which shows that the CF_1-complex of the ATPase is fully exposed in the thylakoid membrane, offering a large adsorption surface to the antibodies. Furthermore, it appears that in the immediate vicinity of the CF_1-complex large amounts of monogalactolipids are located. Thus, we have calculated that in the variegated NC 95 mutant, chloroplasts of the yellow leaf areas, in which only stroma thylakoids occur, exhibiting only photosystem I activity, as well as in chloroplasts of green leaf areas with a normal ratio of grana and intergrana thylakoids, the molar ratio of monogalactolipids to CF_1-complex is the same. It looks as if the functionality and activity of the coupling factor of photophosphorylation depended on a certain quantity of monogalactolipid molecules.

Introduction

The coupling factor of photophosphorylation in chloroplasts (CF_1-complex of the ATPase) has been the object of a series of quantitative determinations [1, 2]. These earlier studies have led to the estimate that the molar ratio of CF_1 to chlorophyll varies between $1/500$ to $1/1200$. These differing values show that the amount of this enzyme, involved in energy conservation is, just as the bifunctional enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase no constant entity in chloroplasts of higher plants but depends on the developmental state and external growth conditions [3].

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Berzborn and co-workers [4] were able to show that the ratio of CF_1 to chlorophyll in spinach chloroplasts from plants cultivated at low light intensities (5000 lux) is considerably lower (1/800) than in chloroplasts from plants grown at high light intensities (12,000 lux), the latter yielding ratios of 1/650.

In the present paper we report on quantitative and qualitative immunological comparative studies on the CF_1-complex in a series of mutants of *N. tabacum*. Analyses were carried out with monospecific antisera in the rocket immuno- and the tandem-crossed immunoelectrophoresis as well as in double immunodiffusion tests. The investigated mutants are suitable objects for the study of interrelationships between morphological structure of the chloroplasts, the amount of CF_1-complex and photosynthetic capacity of the plants. Among these

chlorophyll-deficient mutants we observe on the one hand phenotypes with a photosynthetic and photorespiratory activity distinct from that of the wild type, showing at the same time an altered chloroplast structure [5–11]. Whereas in chloroplasts of the green phenotypes just as in the wild type a normal ratio of grana to intergrana thylakoids is observed, the yellow-green phenotypes have a chloroplast structure with a preponderance of stroma thylakoids and the yellow phenotypes have a reduced number of stroma thylakoids, exhibiting only occasional thylakoid doublings (Table I). The chlorophyll protein ratio is obviously different in these tobacco mutants.

Materials and Methods

Plant material

The studied tobacco plants *(Nicotiana tabacum)* are characterized in Table I. Plants were grown in climatized growth chambers under identical conditions, with a light/dark cycle of 18/6 h. Light intensity was 14,000 lux, temperature in the light 27 °C and in the dark 22 °C. The relative humidity was 60%.

Antisera

The antisera to the CF_1-complex of the ATPase from *N. tabacum* var. John William's Broadleaf and *Spinacia oleracea** were obtained by immunization of rabbits according to earlier described methods [12–14]. All antisera were monospecific. This had the advantage that for the comparative qualitative and quantitative studies just pure chloroplast preparations had to be isolated.

Chloroplast preparations

Leaves of the mutants of *N. tabacum* ground in 0.1 M Tris buffer, pH 8.0 containing 0.6 M sucrose and 4 mM $MgCl_2$ were filtered through a cotton layer. Fractionating centrifugation of the filtrate yielded chloroplast preparations. The thus isolated chloroplasts were resuspended in 24 mM barbiturate buffer, pH 8.6 containing 2% Triton X-100 and treated 3 times for 10 sec in the ultrasonicator (Labsonic 1510, Braun Melsungen, F.R.G.). These chloroplast suspensions were shaken for 2 h at room temperature and subsequently centrifuged for 30 min at $20{,}000 \times g$. The obtained supernatant was directly used for the qualitative and quantitative immunolog-

* The CF_1-complex of *Spinacia oleracea* was prepared by Prof. Dr. H. Strotmann, Botanisches Institut der Universität Düsseldorf, and Prof. Dr. P. Gräber, Max-Volmer-Institut für Biophysikalische und Physikalische Chemie der Technischen Universität Berlin. The CF_0 as well as the CF_0-CF_1-preparation were given to us by Prof. Dr. P. Gräber. We thank for the opportunity to include the preparations in our studies.

Table I. Characterization of the phenotypes of *Nicotiana tabacum* mutants used in the present study.

	Photosynthesis and photorespiration in comparison to the wild type JWB	Structure of chloroplast
1. *Nicotiana tabacum* var. JWB, wild type, green	normal rates of photosynthesis and photorespiration at medium or low light intensities	grana and intergrana regions normal
N. t. Su/su, yellow-green	high rates of photorespiration high rates of photosynthesis at high light intensities	smaller grana, and extended intergrana regions
N. t. Su/su var. Aurea, yellow	high rates of photosynthesis at very high light intensities	thylakoid doublings and extended intergrana regions
2. *N. t.* var. Consolation, green	high rates of photorespiration	grana and intergrana regions normal
yellow-green	low rates of photorespiration	smaller grana
yellow	high rates of photorespiration high rates of photosynthesis at high light intensities	only single isolated thylakoids with occasional thylakoid doublings
3. *N. t.* var. NC 95, green	–	grana and intergrana regions normal
yellow-green	yellow-leaf areas of variegated leaf, contain only photosystem I	only single isolated thylakoids without thylakoid doublings
4. *N. t.* var. Xanthi, green	–	grana and intergrana regions normal
yellow-green	–	–

ical analyses of the coupling factor of photophosphorylation (CF_1-complex of the ATPase).

Immunological tests

Double immunodiffusion tests, tandem-crossed immunoelectrophoresis, as well as the rocket immunoelectrophoresis were carried out in 1% agarose gels. Conditions of the described methods have been detailed in earlier publications [14–17].

Results

The molecular structure of the coupling factor of photophosphorylation (CF_1-complex of the ATPase) is in all tested green, yellow-green and yellow phenotypes of our mutant series immunochemically identical to that of the wild type *N. tabacum* var. John William's Broadleaf (JWB), as demonstrated by the double immunodiffusion test (Fig. 1) and by tandem-crossed immunoelectrophoresis (Fig. 2) in agarose gels. Between the individual chloroplast preparations of the different phenotypes only fusing precipitation bands are observed, one sided spurs are completely lacking.

Moreover, we were able to show, that immunochemical identity also existed between an isolated CF_1-complex and a CF_1-CF_0-complex as demonstrated for an antiserum mixture, consisting of a homologous CF_1-antiserum and a homologous CF_0-CF_1-antiserum (Fig. 3). As the CF_0-complex consists of four hydrophobic subunits different from those of the CF_1-complex, the result permits the conclusion, that the immunization with the CF_0-CF_1-complex in addition should have led to the production of antibodies to the CF_0-complex (Fig. 3) and that the CF_0-portion in the intact isolated ATPase complex remains completely covered by the CF_1-portion [18–20], thus being unable to react with antibodies. This might mean that the quantitative immunological determination of CF_1 in isolated chloroplasts, dissolved by Triton addition might be disturbed by the CF_0-portion.

On the other hand the comparative immunochemical analysis of the CF_1-complex of *N. tabacum* and that of other higher plants such as *Spinacia oleracea*

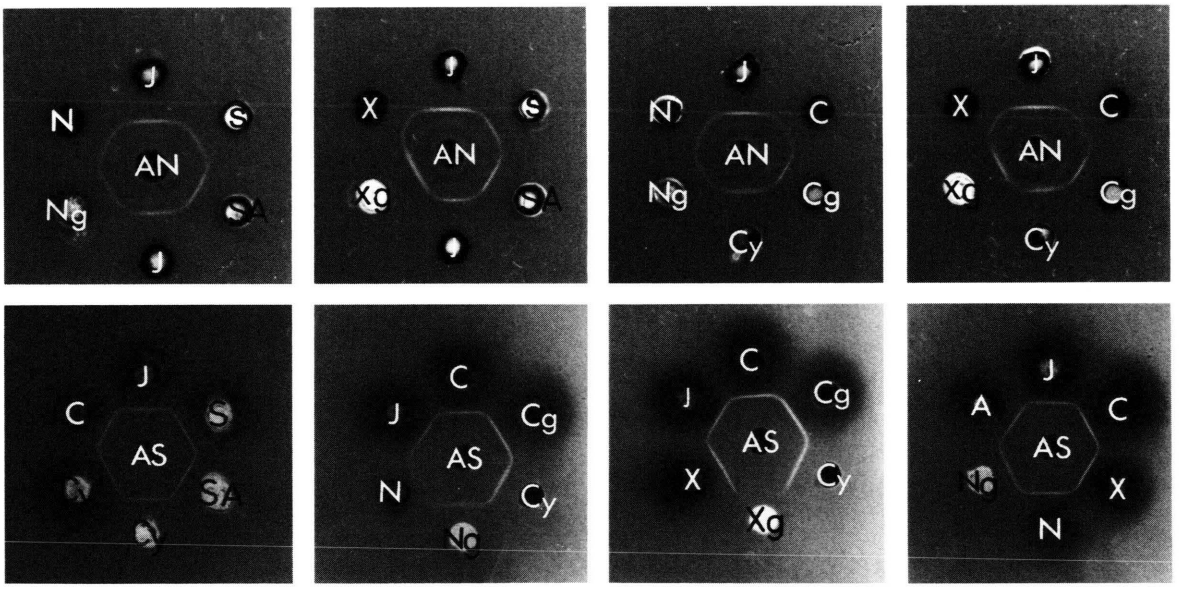

Fig. 1. Immunological comparison of the coupling factor of photophosphorylation of *Nicotiana tabacum* var. JWB with that of various mutants of *N. t.* by means of the double immunodiffusions test. Antisera: AN, antiserum to CF_1 of *Nicotiana tabacum* var. JWB; AS, antiserum to CF_1 of *Spinacia oleracea*. Antigen: Chloroplast preparations of J, *N. tabacum* var. JWB, green; S, *N. tabacum* Su/su, yellow-green; SA, *N. tabacum* Su/su var. Aurea, yellow; C, *N. tabacum* var. Consolation, green; Cg, *N. tabacum* var. Consolation, yellow-green; Cy, *N. tabacum* var. Consolation, yellow; N, *N. tabacum* var. NC 95, variegated, green; Ng, *N. tabacum* var. NC 95, yellow-green; X, *N. tabacum* var. Xanthi, green; Xg, *N. tabacum* var. Xanthi, yellow-green.

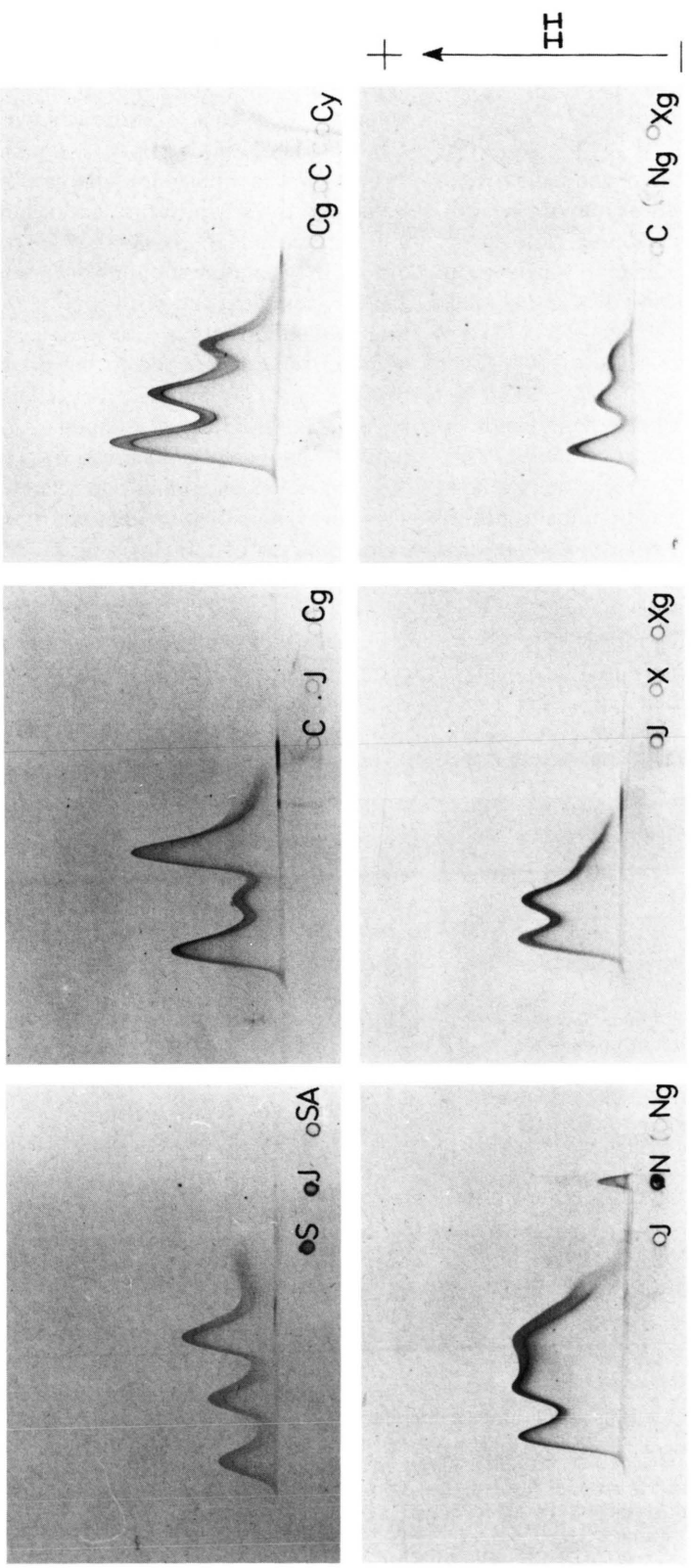

Fig. 2. Demonstration of immunochemical identity of the coupling factor of photophosphorylation between different *Nicotiana tabacum* mutants and the wild type *N.t.* var. JWB by tandem-crossed immunoelectrophoresis. Antisera: 1.5% antiserum of CF_1 in the gel. Antigen: Chloroplast preparations of J, *N. tabacum* var. JWB, green; S, *N. tabacum* Su/su, yellow-green; SA, *N. tabacum* Su/su var. Aurea, yellow; C, *N. tabacum* var. Consolation, green; Cg, *N. tabacum* var. Consolation, yellow-green; Cy, *N. tabacum* var. Consolation, yellow; N, *N. tabacum* var. NC 95, variegated, green; Ng, *N. tabacum* var. NC 95, yellow-green; X, *N. tabacum* var. Xanthi, green; Xg, *N. tabacum* var. Xanthi, yellow-green.

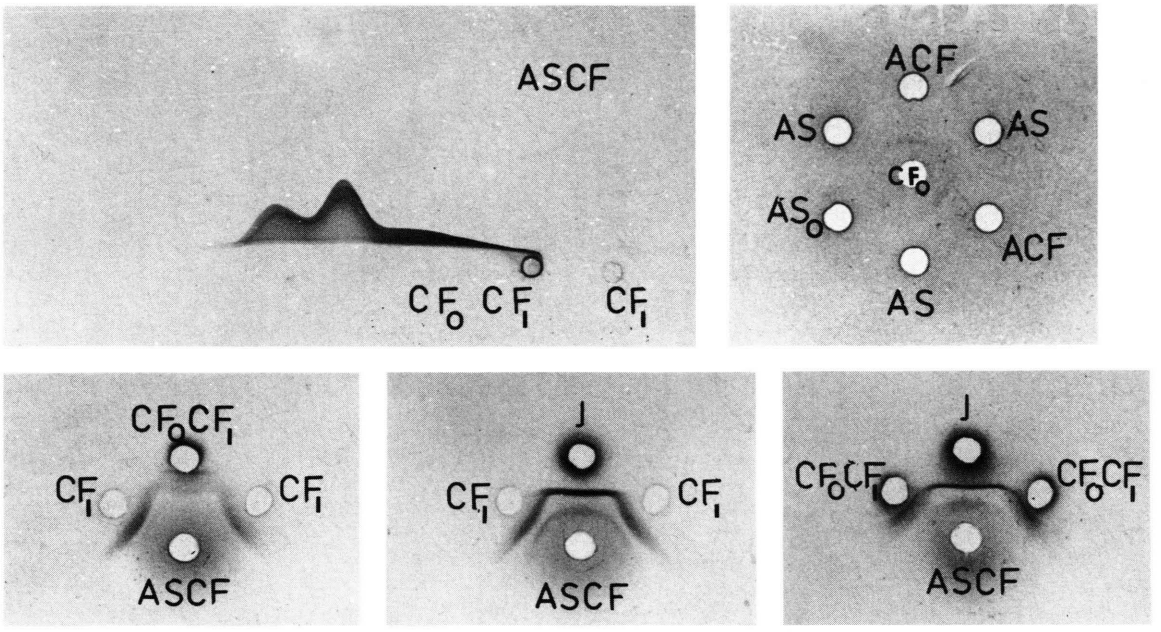

Fig. 3. Demonstration of immunochemical identity of isolated ATPase (CF_0-CF_1-complex) with isolated coupling factor of photophosphorylation (CF_1) from *Spinacia oleracea* by means of tandem-crossed immunoelectrophoresis and double immunodiffusion with different antisera. Antisera: AS, antiserum to CF_1 of *Spinacia oleracea;* ASo, control serum; ACF, antiserum to the CF_0-CF_1-complex of *Spinacia oleracea;* ASCF, mixed antiserum to the CF_1 and CF_0-CF_1-complex. Antigen: Isolated preparations: CF_1, CF_1-complex of *Spinacia oleracea;* CF_0, CF_0-complex of *Spinacia oleracea;* CF_0 CF_1, CF_0-CF_1-complex of *Spinacia oleracea;* J, chloroplast preparations of *N. tabacum* var. JWB.

and *Antirrhinum majus* shows only partial identity as demonstrated by tandem-crossed immunoelectrophoresis as well as by double immunodiffusion test which show one sided spurs (Fig. 4).

The amount of CF_1-complex was determined by rocket immunoelectrophoresis according to earlier described methods. The length of the precipitation bands yielded at CF_1-concentrations of 4 to 0.4 μg in a 1% agarose gel, containing 1.5% antiserum to the CF_1-complex and an electrophoresis duration of 16 and more hours, a linear dependency (Fig. 5). The used antiserum was directed towards CF_1 of *Spinacia oleracea.* The determination of the concentration with this immunochemical method offered in comparison to other methods the advantage, that no isolation of pure CF_1 was necessary, which due to the stability properties of the CF_1-complex would have caused problems. Therefore, it is sufficient to have a pure chloroplast preparation and to solubilize the proteins of the thylakoid membrane by addition of 1–2% Triton. The results of this quantitative immunological determination are summarized in Table II. It is readily seen that the CF_1 portion referred to total protein of the thylakoid membrane is not the same in chloroplasts of the different phenotypes of the tobacco mutants, which as said above, differ with respect to their photorespiratory activity and the lamellar structure of their chloroplasts. Referred to thylakoid membrane protein, CF_1 makes up in all green phenotypes just as in the wild type on the average for 15%. This portion increases in chloroplasts of the yellow-green as well as in those of the yellow phenotypes from ⅕ up to ¼ of all thylakoid membrane proteins.

If the molar ratio of CF_1-complex to chlorophyll is compared in the phenotypes, it is seen that a dependency on the structure of the respective chloroplast lamellar system exists. In chloroplasts of all green phenotypes which have a well balanced ratio of grana and stroma thylakoids, the ratio is one CF_1-molecule per 470 to 770 chlorophyll molecules. In contrast to this, in chloroplasts of the yellow phenotypes, in which practically only single-stranded thylakoids with occasional doubling occur, the same ratio is

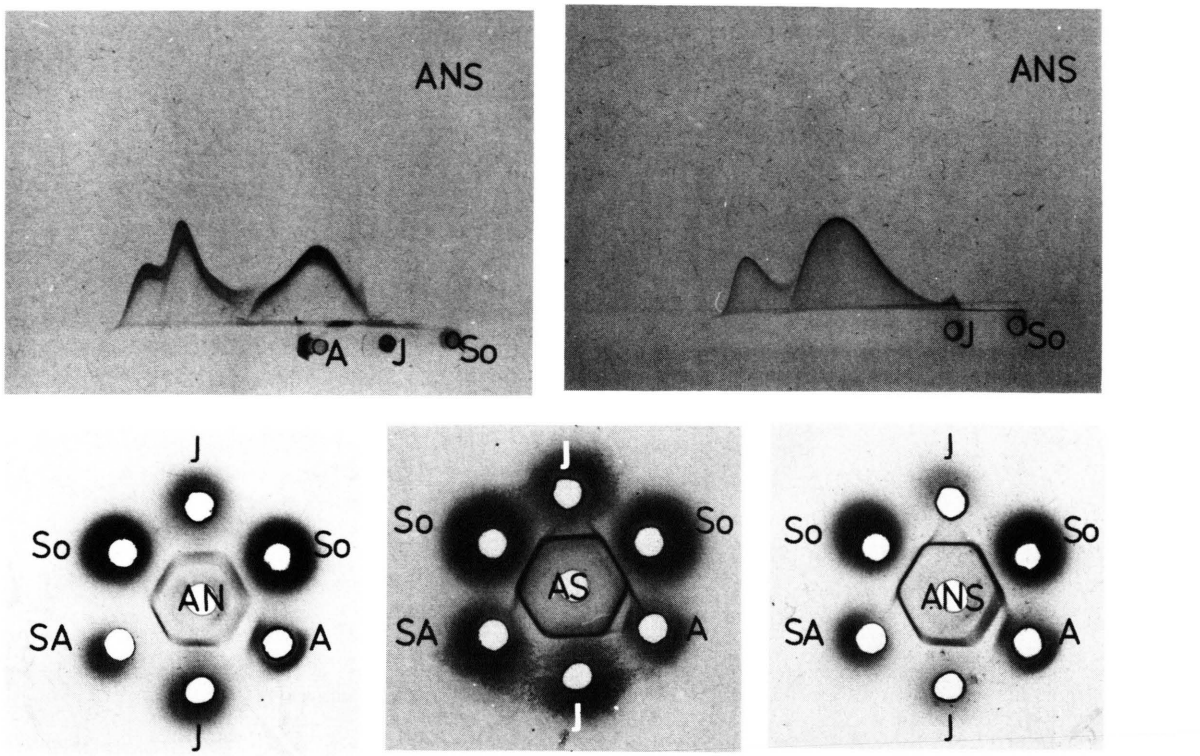

Fig. 4. Demonstration of partial immunochemical identity between the coupling factor of photophosphorylation of the wild type *Nicotiana tabacum* var. John William's Broadleaf, *Spinacia oleracea* and *Antirrhinum majus* by tandem-crossed immunoelectrophoresis and double immunodiffusion with different antisera. Antisera: AN, antiserum to the CF_1 of *N. tabacum* var. JWB; AS, antiserum to the CF_1 of *Spinacia oleracea;* ANS, mixed antiserum to the CF_1 of *N. tabacum* and *Sp. oleracea,* 0.8% in the gel. Antigen: chloroplast preparations of J, *N. tabacum* var. JWB, green; SA, *N. tabacum* Su/su var. Aurea, yellow; A, *Antirrhinum majus;* So, *Spinacia oleracea.*

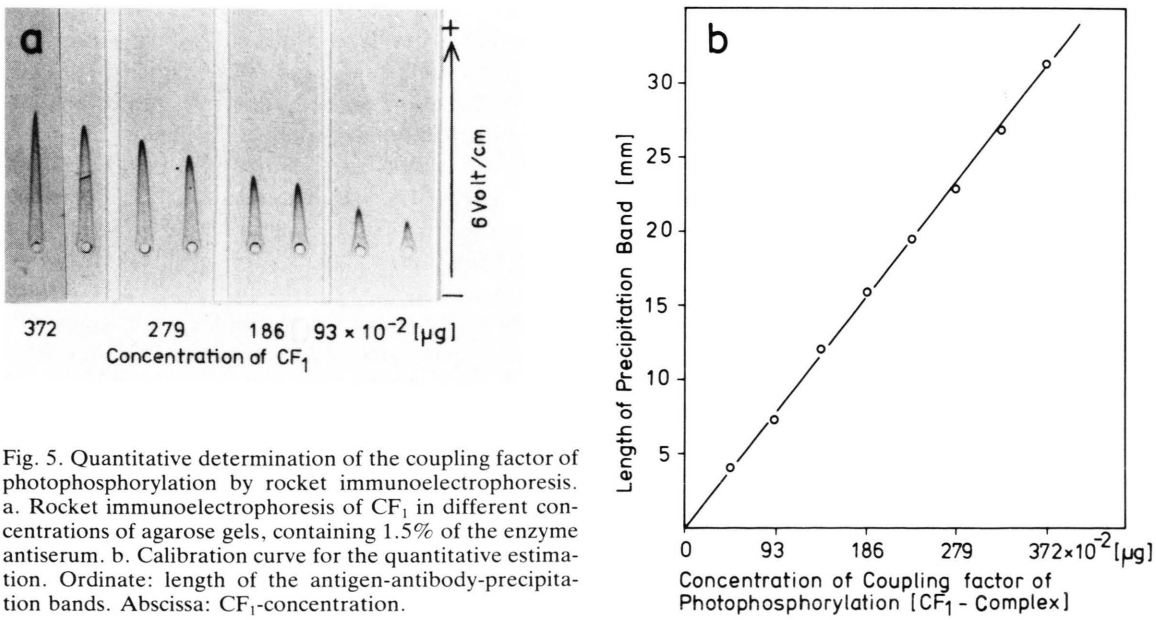

Fig. 5. Quantitative determination of the coupling factor of photophosphorylation by rocket immunoelectrophoresis. a. Rocket immunoelectrophoresis of CF_1 in different concentrations of agarose gels, containing 1.5% of the enzyme antiserum. b. Calibration curve for the quantitative estimation. Ordinate: length of the antigen-antibody-precipitation bands. Abscissa: CF_1-concentration.

Table II. Comparison of the content of coupling factor of photophosphorylation (CF_1) in chloroplasts of green, yellow-green and yellow phenotypes of different mutants of *Nicotiana tabacum*, which are characterized by different chloroplast structures and different capacities for photosynthesis and photorespiration.

	Chlorophyll in % of protein	Chlorophyll *a/b*	CF_1 in % of protein	Molecular ratio chlorophyll/CF_1
Nicotiana tabacum (wild type) var. John William's Broadleaf	21.5	3.1/1	14.6	533/1
N. t. Su/su, yellow-green	12.5	4.0/1	23.5	183/1
N. t. Su/su var. Aurea, yellow	5.7	5.1/1	20.7	58/1
N. t. var. Consolation, green	22.0	3.5/1	14.8	468/1
N. t. var. Consolation, yellow-green	16.9	3.8/1	26.8	225/1
N. t. var. Consolation, yellow	6.0	4.0/1	23.0	64/1
N. t. var. NC 95, variegated plant; green leaf areas	33.4	3.2/1	15.2	774/1
light-green leaf areas	12.1	3.4/1	22.2	193/1
N. t. var. Xanthi, green	23.5	3.3/1	14.5	627/1
N. t. var. Xanthi, yellow-green	1.7	3.2/1	10.5	62/1

high. On the average 60 chlorophyll molecules compare to 1 CF_1-complex. The yellow-green phenotypes, in which in comparison to the green phenotypes relatively more stroma thylakoids than grana thylakoids are observed, are situated somehow in between. Here, an estimate yields 180 to 220 chlorophyll molecules per 1 CF_1-molecule. This means that chloroplasts of different tobacco mutants, exhibiting the same structure of the lamellar system, also exhibit the same molar ratio of CF_1 to chlorophyll. If the structure of the lamellar system changes, this ratio comes out different. Moreover, we can show that the amount of CF_1 or a higher ratio of CF_1/chlorophyll does not correlate with the photosynthetic or photorespiratory activity observed in the mutants. It appears that a high photorespiratory activity does not correspond to a high CF_1-content.

Discussion

Quantitative serological determinations of the CF_1-complex of chloroplast ATPase have led to the result that the ratio of CF_1-molecules to chlorophyll molecules in chloroplasts of the yellow phenotypes in which practically only stroma thylakoids are observed, is higher by a factor of 7 to 10 when compared to chloroplasts of the green phenotypes of the tobacco mutants in which grana and stroma thylakoids form the usual lamellar structure. This data fits observations by electron microscopy [21], which show that CF_1 occurs only in the outer surface of stroma thylakoids and in grana thylakoids only at the surface directed towards the stroma (Staehelin, [21]). Localization experiments with monospecific antisera directed towards the entire CF_1-complex as well as antisera directed towards the five subunits of the CF_1-complex confirm the localization of the coupling factor of photophosphorylation in the outer surface of the thylakoid membrane [22–25]. Quantitative determinations of the maximal binding of CF_1-antibodies onto the outer surface of the thylakoid membrane have led to the result that the CF_1-antibodies bound represent 40% of the maximal amount of antibodies that can be bound onto the outer surface [25]. From the number of bound CF_1-antibody molecules and the number of CF_1-molecules present in *Antirrhinum majus* for example, one can estimate that one CF_1-complex binds 8–9 antibody molecules. From the sterical point of view this number of antibodies bound seems to be realistic, as the CF_1-complex with its molecular weight of 350,000 is more than twice as big as an antibody molecule of the type IgG (150,000). Obviously, one has to assume from this that the CF_1-complex reaches out of the thylakoid membrane, thus offering a large adsorption surface to the antibodies.

The CF_1-complex covers on the outer thylakoid membrane surface vast areas of other antigens and thus impairs binding of other antibodies. Thus, we were able to demonstrate that removal of the CF_1-complex by EDTA-washing permits the binding of approximately 3 times more antibodies to monogalactolipid, which is involved in the membrane structure by 10%. The same is valid for an antiserum to a polypeptide with the apparent molecular weight

25 kDa [26], which makes up for one quarter all polypeptides insoluble in aqueous media. This in turn means that beneath or in the immediate vicinity of the CF_1-complex monogalactolipid and 25,000 Da polypeptide molecules must be located. It was demonstrated for these polypeptide antibodies, that photosynthetic electron transport was only inhibited by these antibodies, if the intact chloroplasts had suffered an ultrasonic treatment [25, 27]. It should be noted that monogalactolipid molecules with a high content of highly unsaturated fatty acids seem to be necessary for the function and activity of the CF_1-complex in the thylakoid membrane. Thus, Pick *et al.* [28] were able to show with vesicle preparations, that the CF_1-activity was substantially enhanced if the membranes of these vesicles were composed of a chloroplast lipid mixture consisting of up to 60% of monogalactolipid with highly unsaturated fatty acids.

In chloroplasts of the yellow-green and the green leaf areas of the variegated tobacco *N. tabacum* NC 95 a quantitative lipid analysis [29] permitted to determine the ratio of CF_1 to monogalactolipid. It was shown that the molar ratio of CF_1 to monogalactosyldiglycerol is the same in both types of chloroplasts despite the fact, that the molar ratio of CF_1 to chlorophyll differs in both chloroplast types by a factor of 4. Thus, the molar ratio of CF_1 to monogalactolipid is in chloroplasts of yellow-green leaf areas which have only stroma thylakoids, 1/1044 and is found to be 1/1054 in chloroplasts of green leaf areas, in which stroma and grana thylakoids occur in a normal ratio.

Beside the variegated tobacco mutant *N. tabacum* Xanthi the tobacco mutants used in this paper were those used in earlier studies on the structure function relationship of the photosynthetic apparatus [5, 7, 30] and on photorespiration [6, 8–11]. The present paper clearly shows that no relationship between CF_1 present and high rates of photorespiration is observed. If one compares the tobacco aurea mutant *N. tabacum* Su/su with the wild type *N. tabacum* var. John William's Broadleaf (JWB) the difference in context with photorespiration is, that the first one has an especially high [5] and the latter a particularly low photorespiration [6]. As Ishii and Schmid have shown, the difference between these mutants is that Su/su eliminates its excess reducing power exclusively *via* carbon metabolism *i.e.* the oxygenase reaction [9, 10] whereas JWB eliminates its major part on the level of photosynthetic electron transport, thus exhibiting a considerable O_2-uptake portion due to a Mehler type reaction [10, 11]. Thus, the total O_2-uptake in JWB was considerably higher than in Su/su. As JWB was under such conditions tested the only fast growing species, it appeared that this O_2-uptake was probably linked to pseudocyclic ATP production [11]. Table II shows that on a chlorophyll basis Su/su and JWB differ considerably whereas they are comparable if the CF_1-content is referred to the protein present.

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