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RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE-DEFICIENT PLASTOME MUTANTS OF *OENOTHERA*

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Summary

In spite of only slightly subnormal pigment contents, two plastome mutants of *Oenothera* (Vα, Iσ) were practically incapable of photosynthetic CO_2 fixation and another one exhibited considerably reduced photosynthesis (IVβ). While other photosynthetic enzymes were present as far as investigated, ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39) activity was very low or missing altogether. As shown by gel electrophoresis, mutant IVβ contained some, though little, fraction I protein. In the other two mutants fraction I protein could not be detected. Also, neither the small nor the large subunit of ribulose-1,5-bisphosphate carboxylase could be found in these mutants. In immunodiffusion experiments with a monospecific antiserum against rye ribulose-1,5-bisphosphate carboxylase, only extracts from wild-type *Oenothera* produced visible precipitation lines. Still, the presence of very low levels of immunochemically reactive antigen was indicated for all three mutants. The highest level was observed in mutant IVβ. The behaviour of the mutant extracts suggested that the antigens of mutant and wild type leaves reacting with the antiserum were not identical. All mutants appeared to have a coupled electron transport system as shown by ATP measurements, light scattering and 515 nm absorption changes. Linear electron transport was possible in the mutants. Still, the photoresponse of cytochrome *f* and fluorescence measurements suggested altered electron transport properties in the mutants. These are interpreted to be secondary lesions of the photosynthetic apparatus caused by primary deficiency in ribulose-1,5-bisphosphate carboxylase activity. From the absence in two mutants (Vα, Iσ) of the small subunit of ribulose-1,5-bisphosphate carboxylase, which is known to be coded for by nuclear DNA and to be synthesized on cytoplasmic ribosomes, it appears that the genetic system of the plastids is

Abbreviations: DCPIP, 2,6-dichlorophenolindophenol; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TEMED, *N,N,N',N'*-tetramethylethylenediamine.

capable of interfering with the genome-controlled synthesis of plastid components.

Introduction

Genetic and biochemical evidences indicate that the large subunit of ribulose-1,5-bisphosphate carboxylase, which catalyzes carboxylation of ribulose bisphosphate in photosynthesis and is thus responsible for carbon fixation, is synthesized on chloroplast ribosomes under the control of chloroplast DNA [1—3]. A nuclear gene is reported to code for the small subunit of the same enzyme. In this case, synthesis occurs outside the chloroplasts on cytoplasmic ribosomes [4].

After import into the chloroplasts, 8 small subunits combine with 8 large subunits to form a functional ribulose bisphosphate carboxylase molecule [5,6]. Obviously, cooperation of the genetic system of the nucleus and the plastids, the genome and the plastome, is required to ensure that the synthesis of the different subunits is synchronized and leads to an ordered assembly of the holoenzyme.

In *Oenothera*, more than 40 plastome mutants are known which are characterized by deficiencies of the photosynthetic apparatus [7]. Some of the mutants are practically devoid of chlorophyll. Others, while possessing chlorophyll, lack functional photosystem I or II [8,9]. Recently, two photosynthesis-deficient plastome mutants were described which did not contain cytochrome *f* [10].

As chloroplast DNA contains the information for the synthesis of the large subunit of ribulose bisphosphate carboxylase, plastome mutants should be expected to exist which cannot photosynthesize because of a defective carboxylating enzyme. Such mutants would be particularly interesting as their analysis might lead to a better understanding of the interaction between genome and plastome.

In this report we wish to describe three mutants with low or lacking ribulose bisphosphate carboxylase activity.

Material

The following paper deals with three photosynthesis-deficient mutants (Iσ, IVβ, Vα) of *Oenothera*. They belong to a collection of plastome mutants, which appeared spontaneously and were selected by W. Stubbe from the 5 existing wild type plastomes I, II, III, IV and V [11].

Biparental transfer of cytoplasm and plastids in *Oenothera* enables the propagation of the mutants by cross-pollination with wild types. The offspring of such a cross will be heteroplastic and variegated as a consequence of somatic segregation of the plastids. Eventually the tissues contain either wild type or mutant plastids only. In such a chimera the heterotrophic tissue containing mutant plastids must be supported by autotrophic tissue containing wild type plastids [7].

Methods

The methods used to measure pigment contents of leaves, their gas exchange, enzyme activities, chlorophyll fluorescence, light scattering by leaves and light-dependent absorption changes have already been described [10].

Gel electrophoresis

Samples ground with sand in 5 vols. of 50 mM Tris-glycine, 2% polyvinylpyrrolidinone 10 000 (Serva), 100 mM mercaptoethanol at 0°C. The supernatant of the filtered and centrifuged (Eppendorf) homogenate was dialyzed against 2.5 mM Tris-glycine, 100 mM mercaptoethanol, pH 8.5. 30% Sucrose was added and the samples were electrophoresed [2] or frozen at −20°C for later analysis. 4—20% gradient gels [12] were polymerized (system Pharmacia) from the following solutions: (a) 4% acrylamide, 0.08% methylenebisacrylamide, 50 mM Tris-glycine, 0.07% potassium persulfate, 0.03% TEMED. (b) 20% acrylamide, 0.4% methylenebisacrylamide, 50 mM Tris-glycine, 0.045% potassium persulfate, 0.015% TEMED.

The gels were equilibrated with the reservoir buffer by electrophoresis (250 V, 1000 pulses/s, 0.1 μF capacitance, system ORTEC) and 10—100 μl of sample with 5 μl of 0.1% Bromophenol Blue were layered onto each gel slot. Electrophoresis was started with 100 pulses/s and continued with 1000 pulses/s at 8°C for 10—14 h. The slabs were stained in Coomassie Blue according to the method of Reisner et al. [13], minor bands were visible after destaining in 5% methanol/5% acetic acid. For gel electrophoresis in sodium dodecyl sulfate Laemmli's buffer system was employed [14]. Samples in 2.5 mM Tris-glycine, 100 mM mercaptoethanol were transferred to sample buffer by mixing with concentrated stock solution. Gradient gels were formed as described above, but with Laemmli's buffer instead of Tris-glycine and with 0.75% agarose instead of polyacrylamide. Electrophoresis was started with 400 V, 50 pulses/s and 0.1 μF capacitance and continued with 800 pulses/s.

The gels were fixed in 15% trichloroacetic acid for 2 h, stained in a solution containing 0.1% Coomassie Blue/50% methanol/1% acetic acid and destained by diffusion in 5% methanol/5% acetic acid.

Immunodiffusion

Oenothera leaves (1 g) were ground with sand in 7 ml of a medium containing 6 mM KCl, 9 mM $MgCl_2$, 0.6 mM EDTA, 6 mM $NaHCO_3$, 30 mM HEPES, 150 mg polyvinylpyrrolidinone (25 000 or 40 000 daltons) and 50 mg Polyclar AT (Serva); pH was 7.8.

After centrifugation and addition of sorbitol to give a final concentration of 300 mM, ribulose bisphosphate carboxylase levels were assayed according to the method of Lilley and Walker [15]. The freeze dried material was dissolved to stock solutions containing 0.3% protein. Assays of ribulose bisphosphate carboxylase activity indicated that little or no loss of enzyme activity had occured during freezing and freeze drying. 20 μl of diluted stock solutions were placed in 3 of 6 peripheral wells surrounding a center well in an agar plate prepared by the method of Khramkova et al. [16]. The fourth well received 20 μl of physiological saline as a control, the two remaining ones each

20 μl extract from rye leaves (dilution from stock 1 : 64, protein concentration 0.0047%). The center well contained 20 μl of a monospecific antiserum against rye ribulose bisphosphate carboxylase diluted so (8-fold) as to give precipitation lines in an equivalence zone approximately the same distance apart from both the wells containing rye enzyme and the well containing antiserum (see Fig. 1). Immunochemically reactive material diffusing from the wells containing *Oenothera* extracts then produced its own precipitation lines or, when concentrations were low, deflections of the precipitation lines between rye enzyme and antiserum, which were used for a semiquantitative estimate of ribulose bisphosphate carboxylase concentration. The sensitivity of the method permits detection of 1 μg of immunochemically reactive ribulose bisphosphate carboxylase per ml. The carboxylase concentration in leaf extracts from wild type leaves containing 0.3% protein and prepared as outlined above may be expected to be about 500 μg/ml.

Results and discussion

1. Pigments

The pigment composition of the *Oenothera* mutants Iσ, IVβ and Vα is similar to that of the wild type, if the plastomes are combined with compatible genomes (Table I). There was usually some slight chlorophyll deficiency, but distinctly less than in other mutants which have been shown to have a defective electron transport chain [8,10].

2. Gas exchange

(a) Infrared absorption measurements. CO_2 measurements in a stream of air which had passed over the leaves showed mutant IVβ to be capable of fixing CO_2 in saturating light, although at a much reduced rate compared with wild type leaves. Maximum rates of photosynthesis were less than one third of the rates of the wild type. Mutant Vα produced like mutant IVβ CO_2 in the dark, but exhibited neither measurable CO_2 uptake nor CO_2 production in the light. As light inhibits mitochondrial respiration in green cells [19–21] this suggests that the mutant had little or no capacity for photosynthesis. The behaviour of mutant Iσ was similar to that of mutant Vα.

(b) Fixation of $^{14}CO_2$. After 10 min illumination in the presence of $^{14}CO_2$, mutant IVβ had taken up about 15%, mutant Vα and Iσ less than 1% of the carbon, which was incorporated by normal leaves into acid-stable products. Interestingly mutants IVβ and Vα incorporated radioactive carbon into sucrose

TABLE I

CHLOROPHYLL AND CAROTENOID CONTENT AND RATIO OF CHLOROPHYLL *a/b* IN MUTANT AND WILD-TYPE TISSUE

	Iσ	IVβ	Vα	Wild type
Carotenoids (% dry weight) after Holm [17]	0.17	0.18	0.16	0.16
Chlorophyll (% dry weight) after Arnon [18]	0.53	0.66	0.56	0.78
Chlorophyll *a/b* after Holm [17]	2.43	2.78	2.04	2.10

TABLE II

INCORPORATION OF ACID-STABLE PRODUCTS AFTER 10 MIN OF PHOTOSYNTHESIS BY ILLUMINATED MUTANT AND WILD-TYPE TISSUE

	IVβ	Vα	Wild type
μmol $^{14}CO_2$ fixed (mg dry wt.)$^{-1} \cdot h^{-1}$	0.069	0.003	0.450
μmol $^{14}CO_2$ fixed (mg chl. *)$^{-1} \cdot h^{-1}$	10.5	0.6	58.0
% of total ^{14}C fixation in sucrose	18.1	5.5	48.5
% of total ^{14}C fixation in sugar phosphates	45.1	18.8	19.8
% of total ^{14}C fixation in aspartate, glutamate, citrate and malate	29.2	72.7	10.8

* chl., chlorophyll.

(Table II). The percentage of the total labelled carbon found in sugar phosphates of these two mutants was also significant. Labelled phosphoglyceric acid occured in a ratio to labelled sugar phosphate which was similar in the mutants and in the wild type. This suggests that reduction of phosphoglyceric acid, which requires ATP and NADPH, was not blocked in the mutants. If compared with total carbon incorporation, products of dark fixation of CO_2 such as aspartate and malate were particularly predominant in mutants Iσ (not shown) and Vα (Table II).

3. Enzymes

Among the enzymes of the photosynthetic carbon cycle, only 3 are specific for photosynthesis. NADP-dependent glyceraldehydephosphate dehydrogenase (EC 1.2.1.13) is such a chloroplast enzyme. Its activity in leaf extracts from the mutants IVβ and Vα by far exceeded the capacity of the mutants for photosynthesis (compare (a) in Table III with Table II). Extracts from wild type leaves contained less activity than extracts from the mutants. It appears that the high tannin content of *Oenothera* leaves is responsible for this. Tannin levels are usually higher in the wild type than in the mutants; they cause enzyme losses even if polyvinylpyrrolidinone is added to the medium used for grinding the leaves to counteract enzyme inactivation.

Phosphoribulokinase (EC 2.7.1.19) is another photosynthesis-specific

TABLE III

RATES OF ENZYME REACTIONS IN EXTRACTS FROM MUTANT AND WILD-TYPE TISSUE IN μmol (mg DRY WEIGHT)$^{-1} \cdot h^{-1}$

Reaction (a) is catalyzed by NADP-dependent glyceraldehyde phosphate dehydrogenase (EC 1.2.1.13) and phosphoglycerate kinase (EC 2.7.2.3), reaction (b) by ribose-5-phosphate isomerase (EC 5.3.1.6) and phosphoribulokinase (EC 2.7.1.19), reaction (c) by ribose-5-phosphate isomerase, phosphoribulokinase and ribulose-1,5-biphosphate carboxylase (EC 4.1.1.39).

	Iβ *	Iσ	IVβ	Vα	Wild type
(a) 3-phosphoglycerate → glyceraldehyde-3-phosphate	0.62	0.96	0.39	0.48	0.23
(b) Ribose-5-phosphate → ribulose-1,5-biphosphate	6.20	8.17	4.26	6.13	3.63
(c) Ribose-5-phosphate → 3-phosphoglycerate	0.61	0.0	0.03	0.02	0.36

* Iβ = electron-transport deficient mutant lacking cytochrome *f*.

enzyme. Rates of the reaction sequence:

ribose-5-phosphate → ribulose-5-phosphate → ribulose-1,5-bisphosphate

which involves both ribose-5-phosphate isomerase (EC 5.3.1.6) and phosphoribulokinase were very high in extracts from both mutant and wild type leaves. Again, wild type extracts contained less functional enzyme than mutant extracts.

In contrast to the situation for glyceraldehydephosphate dehydrogenase (and phosphoglycerate kinase, EC 2.7.2.3, which was measured simultaneously) and the enzyme system ribose-5-phosphate isomerase/phosphoribulokinase, activities of the carboxylase, which is also specific for photosynthesis, were much lower in extracts from mutant tissue than in wild type extracts. Attempts to increase enzyme yields in the mutant extracts by including different concentrations of protective additives such as Polycar AT (Serva), polyvinylpyrrolidinone and mercaptoethanol in the extraction medium failed. The activity of the enzyme in wild type extracts was more than sufficient to account for the observed rates of photosynthesis in wild type leaves. In contrast, the activity of ribulose bisphosphate carboxylase observed in mutant extracts was insufficient to support normal rates of photosynthesis. Other mutants of *Oenothera*, which had a nonfunctional electron transport system (e.g. Iβ, Table III), contained levels of the carboxylase which were much higher than the levels in mutants IVβ, Vα and Iσ.

Although no quantitative information is available, the pattern of radioactive products after photosynthesis in the presence of $^{14}CO_2$ permits the conclusion that other enzymes of the photosynthetic carbon cycle and enzymes en route to sucrose were present in the mutants IVβ and Vα (Table II).

4. Immunochemical estimation of ribulose bisphosphate carboxylase

Leaf extracts from wild type *Oenothera* formed a precipitate with a monospecific antiserum against the enzyme from rye. They reacted also with an antiserum against the large subunit of rye ribulose bisphosphate carboxylase. However, no precipitate was formed and no precipitation lines were seen in immunodiffusion tests, when wild type *Oenothera* extracts were exposed to an antiserum against the small subunit of spinach or rye ribulose bisphosphate carboxylase. Obviously, species-specific differences in the structure of the small subunit of the enzyme were too large to permit a reaction between antibodies against rye or spinach subunits and *Oenothera* antigen. In an attempt to measure immunochemically reactive ribulose bisphosphate carboxylase, immunodiffusion tests were carried out with a monospecific antiserum against rye ribulose bisphosphate carboxylase as antibody and rye and *Oenothera* extracts as antigens. At the same dilution (1 : 64), precipitation lines produced by the reaction between antiserum and wild type *Oenothera* enzyme were more diffuse than lines produced by the corresponding reaction with rye enzyme (Fig. 1A). Still, a clear deflection of the precipitation line between rye enzyme and antiserum was brought about by wild type *Oenothera* extracts as dilute as 1 : 512 from the original stock, a 0.3% protein solution (Fig. 1B, compare with deflection of line by physiological saline). The limiting dilution in the case of rye was 1 : 256 (not shown). None of the mutants produced a precipitation

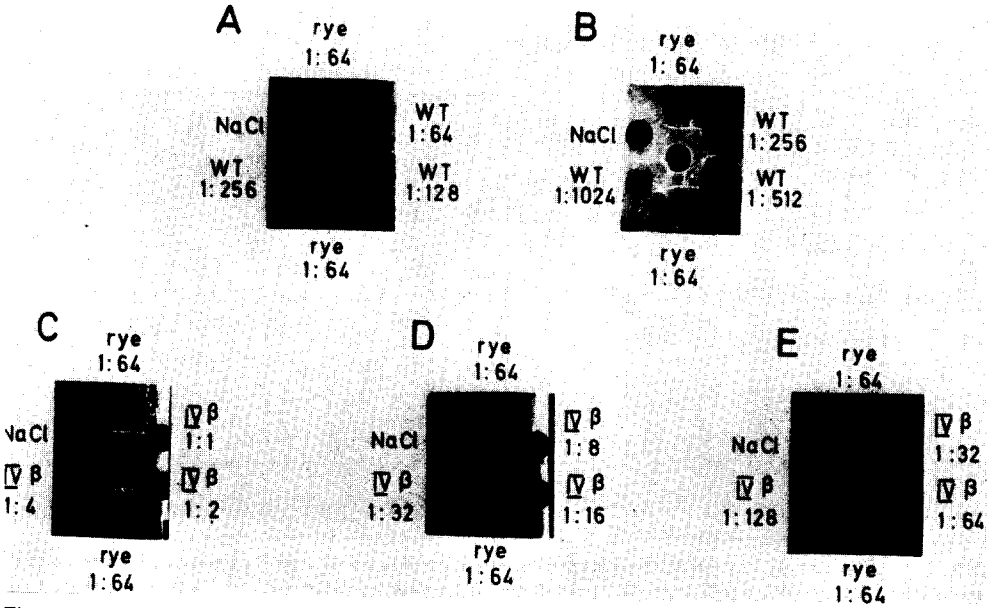

Fig. 1. Reaction between a monospecific antiserum against ribulose bisphosphate carboxylase from rye leaves (A—E), wild type *Oenothera* leaves (WT: A, B) and mutant leaves (IVβ: C—E). Dilutions were made from stock solutions containing 0.3% leaf protein and were filled in the peripheral wells which appear as black circles. The center well contained antiserum. Physiological saline served as control. Note that white precipitation lines are seen only between wells containing antiserum and rye extract (dilution 1 : 64) and antiserum and extract from wild type *Oenothera* (dilutions 1 : 64 and 1 : 128). At higher dilutions wild type extract interfered with the reaction between antiserum and rye extracts causing a deflection of the precipitation line. The same was true for low concentrations of extract from mutant IVβ.

line. However, mutant extracts were capable of deflecting precipitation lines between rye extracts and antiserum. In the case of mutant IVβ (Fig. 1C, D, E), the limiting concentration of the *Oenothera* extract to produce a deflection was between the dilution 1 : 32 and 1 : 64 indicating that the strength of the immunoreaction with mutant antigen was about 10% that of the reaction with wild type antigen. The wild type of *Oenothera* contained, on a unit protein basis, twice as much immunochemically reactive ribulose bisphosphate carboxylase as rye leaves. The carboxylase contents of the mutants as revealed by the strength of the immunoreaction were only a fraction of that of the wild type (1%, 12.5% and 3.5% of the wild type content for mutants Vα, IVβ and Iσ respectively). It is interesting to compare these data with the activity assays (Table III part C). While mutant Iσ showed no detectable carboxylase activity in the enzyme tests, it contained immunologically reactive enzyme, though less than mutant IVβ which was capable of some residual photosynthesis. Mutant Vα, on the other hand, while displaying some enzyme activity, scarcely reacted immunochemically. Another interesting feature: In the wild type *Oenothera* the dilution 1 : 64 from the stock solution still produced a visible precipitation line (Fig. 1A). 1/10 of this concentration barely deflected a precipitation line between rye enzyme and antiserum. In contrast, even the highest protein concentration of mutant IVβ did not cause precipitation, while a dilution of 1 : 64 still deflected the precipitation band between rye and antiserum. These differ-

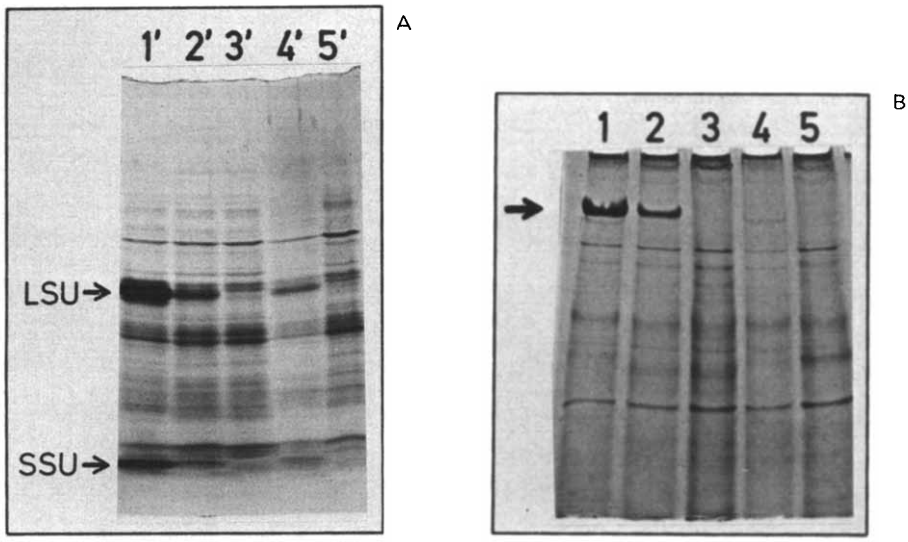

Fig. 2. Gel electrophoresis of crude *Oenothera* extracts. 1: wild type; 2: cohomogenate of 9 parts Vα and 1 part wild type; 3: Vα; 4: IVβ; 5: Iσ. Slots 1 and 2 contain extracts derived from 5 mg, slots 3—5 extracts from 10 mg, and slots 1'—5' extracts from 4 mg of leaf (fresh wt.) each. (A) Electrophoresis under non-denaturing conditions. The arrow indicates the position of Fraction I protein. (B) Electrophoresis under denaturing conditions. LSU = large subunit, SSU = small subunit of Fraction I protein.

ences in behaviour suggest that the mutant enzyme is structurally altered as compared with the wild type enzyme.

5. Gel electrophoresis

Mutant leaves were examined gel electrophoretically for the presence of protein resembling wild type Fraction I. Soluble proteins from crude leaf extracts were run overnight in polyacrylamide gradient gels to a near-stop position. Out of the 38 mutants tested, 35 showed distinct Fraction I bands of varying intensity. A very weak band close to or at the usual Fraction I positions could occasionally be seen in IVβ. In Vα and Iσ no Fraction I bands could be detected (Fig. 2A). Loss of soluble protein due to unspecific aggregation with insoluble cell fractions was ruled out by cohomogenisation of wild type and mutant tissue. In these cases, Fraction I bands in the expected intensities could be demonstrated (Fig. 2A). The electrophoresis was performed with soluble extracts from 5—10 mg of fresh leaf material per gel slot. Since we observed distinct bands with extracts corresponding to 25 μg of wild type material, we may estimate that the concentration of Fraction I in mutants Iσ and Vα is less than 0.2% of the wild type. In comparison, material reacting immunochemically with a monospecific antiserum against ribulose bisphosphate carboxylase was present in the mutants Iσ and Vα at 1—3% of the wild type concentration. Likewise, mutant Vα had more than 1% of the ribulose bisphosphate carboxylase activity of the wild type (Table III).

To determine, whether the mutants contained one or both of the subunits [22] not assembled to Fraction I protein, soluble extracts were denatured with dodecyl sulfate and run in dodecyl sulfate-containing gradient gels. Faint bands

TABLE IV

Cellular levels of ATP and 3-phosphoglyceric acid in the dark and after 20 s illumination with a high light intensity, in nmol · (mg dry weight)$^{-1}$.

		Iσ	IVβ	Vα	Wild type
(a) ATP	Dark	0.46	0.72	0.43	0.78
	Light	1.07	0.83	0.82	1.03
(b) PGA	Dark	—	—	0.28	3.11
	Light	—	—	0.04	2.06

were occasionally observed at the positions of both the large and the small subunit in the mutant VIβ whereas these bands were missing in the mutants Iσ and Vα (Fig. 2B).

6. Electron transport

(a) Production of ATP and NADPH. After 20 s illumination, phosphoglyceric acid drastically decreased in leaf cells of both the wild type and mutant Vα, while ATP increased (Table IV). The decrease in phosphoglyceric acid reflects its light-dependent reduction to triosephosphate [23] which requires ATP and NADPH. While it is already clear from its (limited) capacity for photosynthesis, that mutant IVβ can produce ATP and NADPH in the light, the response of phosphoglyceric acid and ATP levels in mutant Vα to brief illumination indicates that this mutant also can provide ATP and reducing equivalents for photosynthetic substrate reduction. Mutant Iσ also increased ATP levels on illumination.

(b) Reduction of dichlorophenolindophenol and of methylviologen. Reactions of the electron transport chain of chloroplast fragments are difficult to measure in *Oenothera* extracts as destructive effects of tannins in biomembranes are more severe and more difficult to counter than in the case of soluble enzymes. Still, attempts were made to measure the light-dependent reduction of DCPIP and methylviologen in leaf extracts. Table V shows that although observed reduction rates even by the wild type were well below rates of electron transport in photosynthesis in vivo, indicating loss of activity encountered during extraction, DCPIP reduction particularly by the mutants Iσ and Vα was not too different from that by the wild type. Only mutant IVβ exhibited reduced activity. Since this mutant is the one which can still photoreduce CO_2 at significant rates, it is possible that loss of activity during extraction is responsible for reduced electron transport.

TABLE V

REDUCTION OF DCPIP AND METHYL VIOLOGEN IN EXTRACTS FROM MUTANT AND WILD TYPE TISSUE IN μmol · (mg CHLOROPHYLL)$^{-1}$ · h^{-1}

	Iσ	IVβ	Vα	Wild type
(a) reduction of DCPIP	24.9	9.3	30.3	70.1
(b) reduction of methyl viologen	12.8	0	35.5	15.3
(c), (b) after addition of 1,5-diphenylcarbazide	23.9	29.9	22.8	—

Reduction of DCPIP is believed to involve mainly Photosystem II. In view of its low redox potential, methylviologen can be reduced only by Photosystem I [24]. When no electron donor such as diphenylcarbazide is added, oxygen uptake in the presence of methylviologen indicates electron transport from water via Photosystem II and I to methylviologen. In mutant Vα, the rate of electron transport to methylviologen was even higher than the rate shown by the wild type. Oxygen uptake was not stimulated by cyanide which poisons catalase. As in the case of DCPIP reduction, only mutant IVβ did not show much electron transport to methylviologen. The significance of this observation in regard to the block of photosynthesis in this mutant is doubtful, as addition of diphenylcarbazide which donates electrons to Photosystem II [25] induced oxygen uptake in mutant IVβ.

(c) Light-dark difference spectra of leaves. On illumination, intact leaves display small changes in light absorption in different parts of the spectral region. These can be used to identify possible defects in the electron transport chain [8,10,26].

Fig. 3 shows light-dark difference spectra of the wild type and the mutants which were measured in air. There is a comparatively large absorption increase peaking at 520 nm which is accompanied in the wild type and in mutants IVβ and Vα by a negative band with a maximum close to 480 nm. It has been identified as an electrochromic shift in the absorption of lutein/chlorophyll *b* complexes [27] which is caused by a light-generated electrical potential difference across thylakoid membranes. To maintain this membrane potential continued electron transport is required. In mutants with a blocked electron transport system, the 520 nm shift was indeed transient and collapsed after brief illumination (Fig. 4E and ref. 10). In contrast, in the mutants described here

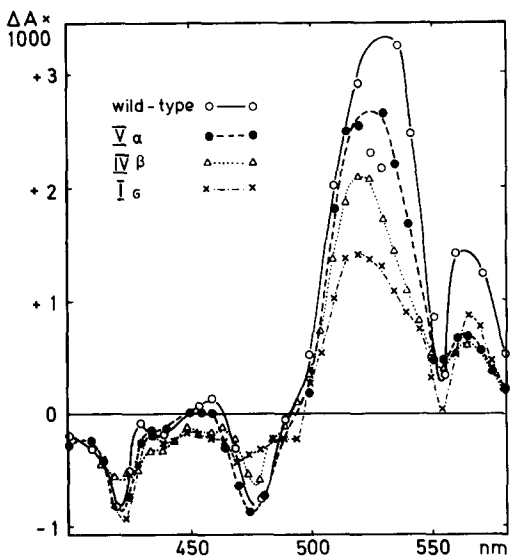

Fig. 3. Difference spectra obtained on illuminating leaves of wild type and mutant *Oenothera* with saturating red light. Differences in ΔA were measured between dark and 1 s in the light. Illuminating times 10 s. Scattering of values in the 520 nm range is caused by different dark times between illumination.

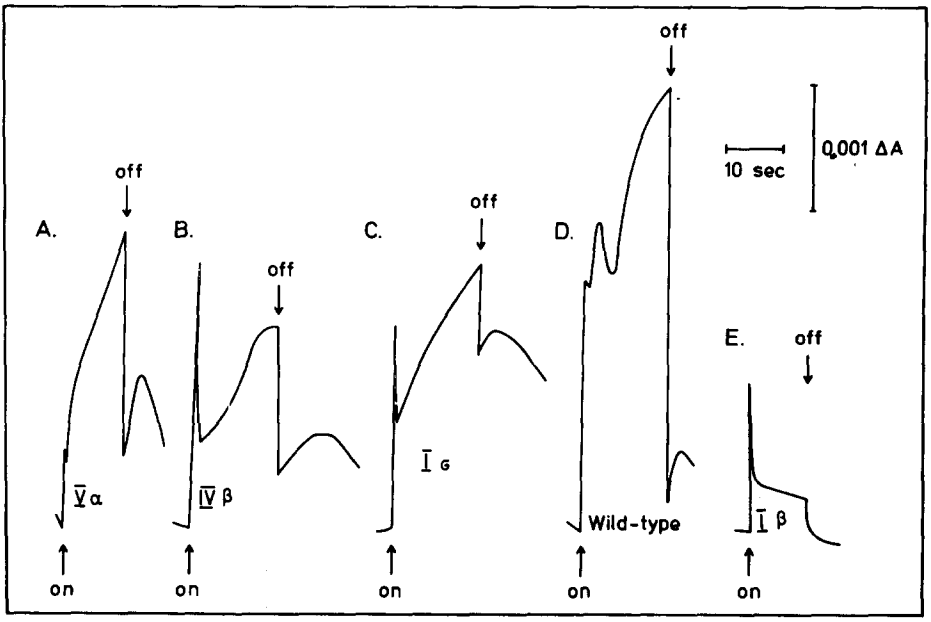

Fig. 4. Light-dependent absorbance changes of wild type and mutant leaves at 515 nm. Illumination with saturating red light. Mutant Iβ lacks cytochrome *f* (cf. ref. 10).

and in the wild type there was, after one or two transients, a secondary increase in the signal (Fig. 4, A—D). Darkening caused a fast decrease in 520 nm absorption, followed by a slow transient increase, which was not seen in electron transport-deficient mutants. The slow secondary increase is attributed to the diffusion potential created during the decay of the light-dependent proton gradient (Vredenberg, W.J., personal communication).

Negative bands in the difference spectra of Fig. 3 with absorption minima close to 420 nm and 555 nm were found both in the mutants and in the wild type. They indicate light-dependent oxidation of cytochrome *f*. A small absorption peak close to 563 nm and a shoulder close to 435 nm are interpreted to show reduction of some cytochrome b_6.

While there was general agreement between the light-induced absorption changes of mutant and wild type leaves, some details differed. A very slow intensity of far-red light was sufficient to oxidize cytochrome *f* in the wild type (Fig. 5A). Much higher intensities were needed to bring cytochrome *f* of the mutants Vα and Iσ into a more oxidized steady state (Fig. 5B for mutant Vα). While in the wild type 651 nm light failed to photooxidize cytochrome *f* (it actually photoreduced the cytochrome when this had been brought into the oxidized state by illumination with far-red light), it caused significant photooxidation in the mutants Vα (Fig. 5B vs. 5A) and Iσ. In consequence, the red/far-red antagonism of cytochrome *f* oxidation, which indicates linkage of the two photosystems of the electron transport chain [28,29] was less clearly seen in mutants Vα and Iσ than in the wild type. Although mutant IVβ exhibited significant residual carbon fixation (Table II), its cytochrome *f* was less photoreactive than that of the other two mutants (not shown).

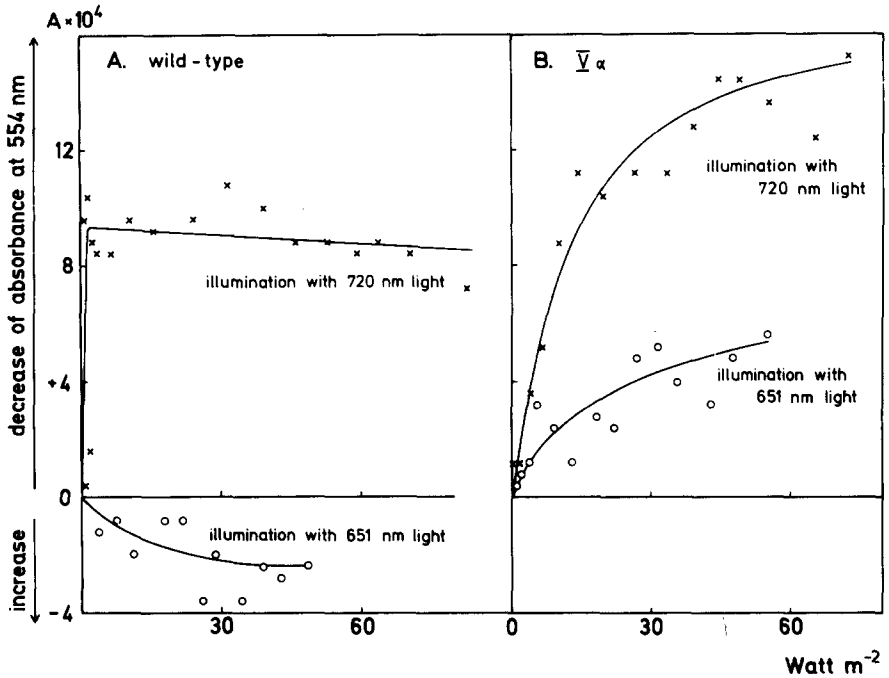

Fig. 5. Absorbance changes at 554 nm in a wild type and a mutant (Vα) leaf brought about by illumination with 720 and 651 nm light. Reference wavelength was 568 nm.

7. Chlorophyll fluorescence and light scattering by leaves

After a long dark time, illumination of both mutant and wild type leaves first caused a very rapid rise in chlorophyll fluorescence which was much faster than the response time of the measuring device. It was followed by a much slower, biphasic fluorescence increase whose slope was intensity-dependent (Fig. 6). After reaching a maximum, chlorophyll fluorescence returned to a lower level (Kautsky effect [30], not shown in Fig. 6, but in Fig. 7). The intensity-dependent biphasic fluorescence rise is interpreted to reflect the reduction of electron carriers between the photosystems [31]. Differences between wild type and mutants in the kinetic of the slow fluorescence rise seen after the fast initial rise (Fig. 6) are due to differences in the number of photosystem II reaction centers and/or the size of the pool of electron acceptors between the photosystems. Those differences are not fundamental. The data indicate a functional photosystem II in the mutants IVβ, Vα and Iσ. Mutants with a non-functional photosystem II do not show slow changes in the fluorescence yield (not shown, see ref. 8).

The secondary decline in chlorophyll fluorescence seen in Fig. 7A is accompanied by and kinetically related to increased light scattering (Fig. 7B, see ref. 32). Both indicate the light-dependent energization of leaf cells, which is driven by coupled electron transport. Krause [33] has shown that in the presence of Mg^{2+} ions formation of the proton gradient across thylakoid membranes is accompanied by a light scattering increase and a fluorescence decline. According to Mitchell [34], ATP formation by chloroplasts is driven by the proton

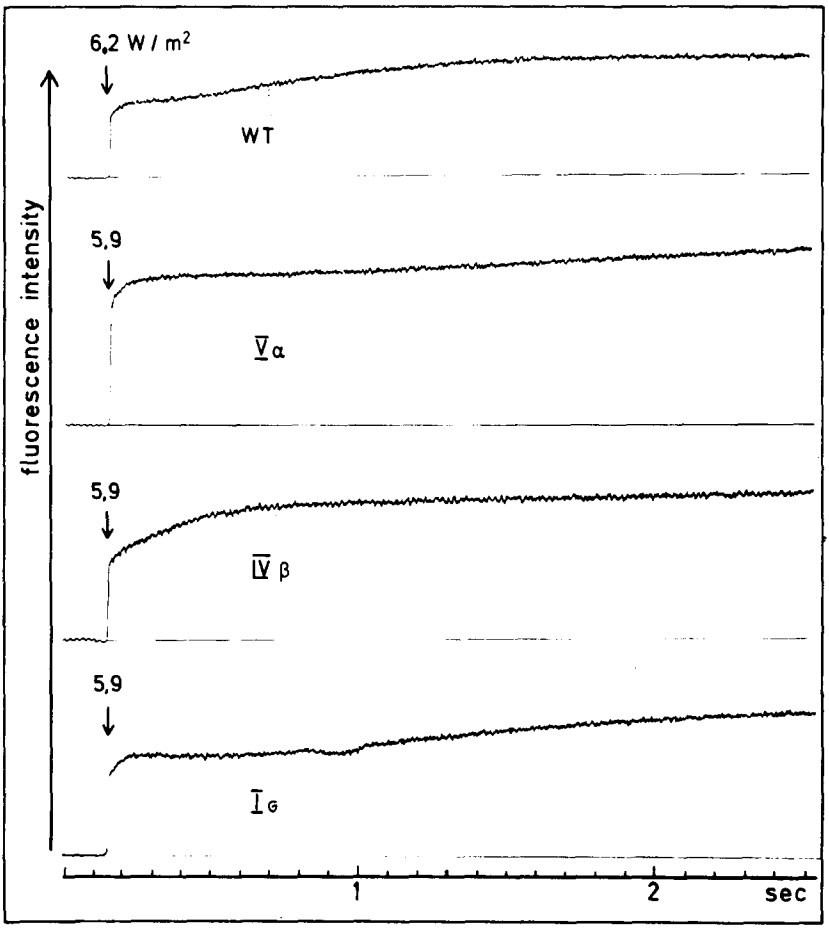

Fig. 6. Kinetics of 742 nm fluorescence of wild type and mutant leaves. Illumination with low-intensity blue light.

motive force which has a ΔpH and a membrane potential component. In leaves, the ATP level may be expected to depend on the magnitude of the proton motive force. The extent of light scattering by leaves and of the fluorescence yield of chlorophyll in the steady state therefore appear to be useful criteria for the energy state of the photosynthetic apparatus. This has indeed been confirmed by measurements of light scattering and ATP in *Dunaliella* cells [35]. Fig. 7 shows simultaneous measurements of 740 nm fluorescence, and of scattering of 535 nm light by wild type and mutant leaves. Illumination with short-wavelength red light of medium intensity (27 $W \cdot m^{-2}$) in a stream of CO_2-free air produced chlorophyll fluorescence with a fast and a slow component during the rise (not shown in Fig. 7, but in Fig. 6). There was a secondary decline which coincided with increased light scattering indicating energization of the system [33]. Addition of 300 ppm CO_2 to the gas stream increased fluorescence and decreased light scattering in a wild type leaf. This can be explained by lowered ATP levels due to ATPconsumption during CO_2 reduction. Removal of CO_2 reestablished high scattering and low fluorescence.

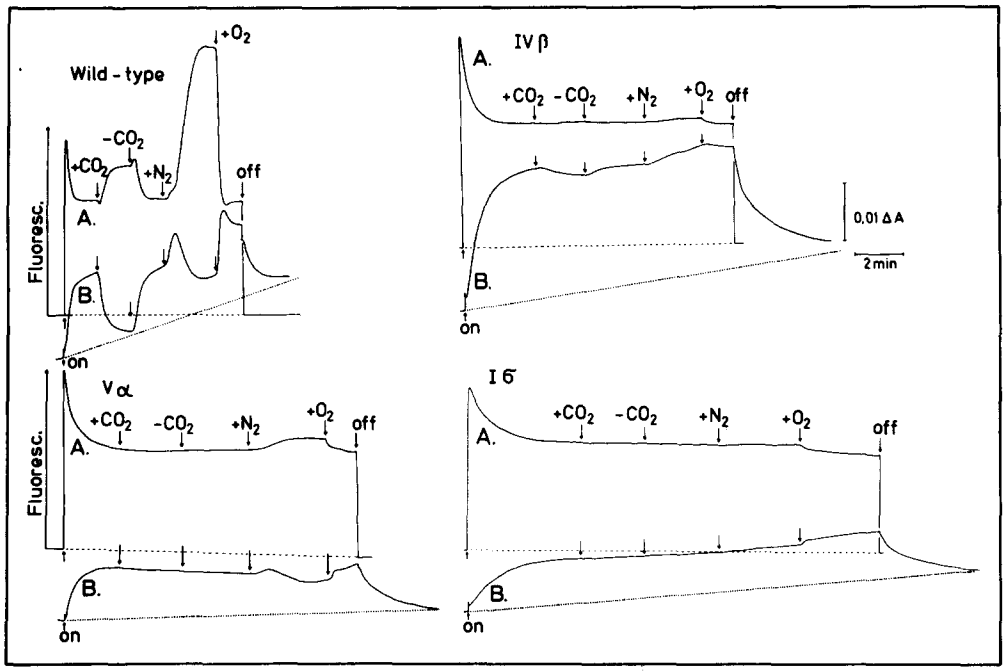

Fig. 7. Simultaneous recordings of 535 nm light transmitted through normal and mutant *Oenothera* leaves (B) and 742 nm chlorophyll fluorescence (A). The leaves were gassed with a stream of CO_2-free air ($40\,l \cdot h^{-1}$) and were illuminated with 27 $W \cdot m^{-2}$ short-wavelength red light. 300 ppm CO_2 or nitrogen were added or withdrawn as indicated. An increase in the apparent absorbance of the leaves (i.e. increased scattering of 535 nm light) is indicative of energy-dependent ion fluxes across thylakoids. The size of light scattering signals is a function of leaf structure and can therefore not directly be compared in different leaves. However, responses to changes in the gas atmosphere in an individual leaf can be compared. Similar relations hold for the fluorescence kinetics.

Removal of oxygen drastically increased fluorescence and decreased scattering. A transient rise in scattering during the transition from 21% O_2 to N_2 is a photorespiratory phenomenon [36,37].

The strong inhibition of light-dependent scattering and the high fluorescence yield under nitrogen in the wild type leaf indicate that illumination was strong enough to fill the electron transport chain with electrons to an extent which restricted cyclic electron flow, which is known to be sensitive to over-reduction of electron carriers [38]. Under a low light intensity, which was insufficient to overreduce the electron transport chain, light scattering was actually increased under nitrogen indicating increased cyclic electron transport. At medium and high light intensities, readmission of oxygen after inhibition of electron transport by nitrogen rapidly led to increased light scattering and reduced the fluorescence yield (Fig. 7, wild type experiment). This indicates energization of the system by electron flow to oxygen.

In contrast to the large response to CO_2 shown by the wild type, mutant IVβ scarcely decreased scattering or increased fluorescence on addition of CO_2 (Fig. 7). The rather small effect complements the observation that this mutant has some residual capacity for photosynthesis (Table II). Also in contrast to the wild type, nitrogen did not decrease but actually increased light scattering in

mutant IVβ. Obviously, Photosystem II of the mutant was unable to over-reduce the electron transport chain and thereby curtail electron transport through Photosystem I. Removal of oxygen rather stimulated cyclic electron flow.

Mutants Iσ and Vα did not respond at all to the addition of CO_2. This confirms the conclusion drawn above that they cannot photosynthesize. Nitrogen decreased light-scattering and increased the fluorescence yield, but much less so than in the wild type. Increasing the light intensity increased inhibition of scattering. Still, the inhibitory effects were much less pronounced than in the case of the wild type (Fig. 7). Even though Photosystem II of the mutants has been shown to be functional, it does not appear to be linked to photosystem I so efficiently as to permit drastic over-reduction of the latter, when no electron acceptor (such as oxygen) is present. This conclusion is supported by the cytochrome *f* data. Photosystem II light was unable to photoreduce cytochrome *f* (Fig. 5). Because of the weak interaction of Photosystem II with Photosystem I, significant cyclic electron transport persisted under nitrogen even under high illumination with short-wavelength red light.

Conclusions

It is always difficult to distinguish primary and secondary effects of a mutation. Quite obviously, a major block in metabolism, which is caused by a mutation, must also lead to secondary defects. This may be illustrated by an example. If photosynthesis cannot proceed because an enzyme of the carbon cycle can no longer be synthesized, physiological energy conservation by the photosynthetic apparatus is impossible. High electron pressure under conditions where the chloroplast-NADP system is largely reduced leads to significant reduction of oxygen. If detoxification of the resulting oxygen radicals by superoxide dismutase and catalase cannot keep pace with production, secondary oxidative destruction is inevitable. In the mutants IVβ, Vα and Iσ ribulose bisphosphate carboxylase activity is very low or missing altogether. Likewise, Fraction I protein was found in the mutants in very small amounts or not at all. While there was an immunoreaction between a monospecific antiserum against ribulose bisphosphate carboxylase and extracts from wild type and mutant *Oenothera* leaves, its extent was dramatically reduced in the mutant extracts.

Loss of or reduction in ribulose bisphosphate carboxylase activity are completely sufficient to account for the low efficiency of the mutants to incorporate carbon from CO_2 into sugars in the light. The mutants were capable of linear electron transport as evidenced by their ability to photoreduce methylviologen. Still, the response of slow chlorophyll fluorescence, light scattering and of cytochrome *f* indicated that Photosystem II interacted with Photosystem I differently in the mutants than in the wild type. Cyclic electron transport appeared to be less tightly controlled by Photosystem II in the mutants than in the wild type. However, this effect cannot explain the almost complete loss of photosynthesis especially in the mutants Vα and Iσ. While it cannot be excluded that the effect on electron transport is, together with the reduction in ribulose bisphosphate carboxylase, directly caused by the mutation, it is

much more likely to be a secondary lesion. In the mutants the plastome, not the genome, is altered. Since the plastome is known to direct the synthesis of the large subunit of ribulose bisphosphate carboxylase, the observed carboxylase deficiency is very probably caused by an alteration of the polypeptide chain of the large subunit. It must be a drastic alteration, as neither Fraction I protein nor a polypeptide resembling in size the large subunit could be found in the mutants Iσ and Vα. Still, polypeptide having antigenic properties of the large subunit is synthesized as shown by the immunoreaction between extracts of the mutants and antisera against the large subunit of *Euglena* carboxylase (data not shown in this manuscript) and rye ribulose bisphosphate carboxylase. Our results cannot easily be reconciled with the 'cytoplasmic control principle' proposed by Ellis et al. [39]. This hypothesis states, that cytoplasmic products control organelle protein synthesis. The experiments of Feierabend and Wildner, who found formation of the small subunit of Fraction I protein even when chloroplast protein synthesis in rye plants was blocked by temperature treatment is compatible with Ellis' view [40,41].

In *Oenothera*, however, plastome mutations prohibited the accumulation of both the large and the small subunit of Fraction I protein totally (Iσ, Vα) or at least partially (IVβ). Thus, if one accepts the current model that the genome codes for the small subunit of Fraction I protein whereas the plastome codes for the large subunit (ref. 39: compare Fig. 8), our data show, that the plastome in turn exerts control on nuclear/cytosolic protein synthesis. The elucidation of this effect of a plastome mutation on the cytosolic protein synthesis of the small subunit will be of great interest.

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