Article

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BENNOUN, Pierre, *et al*.

Abstract

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BENNOUN, Pierre, *et al*. Characterization of photosystem II mutants of *Chlamydomonas reinhardii* lacking the psbA gene. *Plant Molecular Biology*, 1986, vol. 6, no. 3, p. 151-160

DOI : 10.1007/BF00021484

Plant Molecular Biology 6: 151–160, 1986

Characterization of photosystem II mutants of *Chlamydomonas reinhardii* lacking the psbA gene

Pierre Bennoun[1], Muriel Spierer-Herz[2], Jeanne Erickson[2], Jacqueline Girard-Bascou[1], Yves Pierre[1], Monique Delosme[1] & J.-D. Rochaix[2]
[1] *Institut de Biologie Physico-chimique, 75005 Paris, France*
[2] *Departments of Molecular and Plant Biology, University of Geneva, 1211 Geneva 4, Switzerland*

Summary

We have examined 78 chloroplast mutants of *Chlamydomonas reinhardii* lacking photosystem II activity. Most of them are unable to synthesize the 32 Kdalton protein. Analysis of 22 of these mutants reveals that they have deleted both copies of the psbA gene (which codes for the 32 Kdalton protein) in their chloroplast genome. Although these mutants are able to synthesize and to integrate the other photosystem II polypeptides in the thylakoid membranes, they are unable to assemble a stable functional photosystem II complex. The 32 Kprotein appears therefore to play an important role not only in photosystem II function, but also in stabilizing this complex.

Introduction

The chloroplast psbA gene coding for the 32 Kdalton thylakoid membrane polypeptide in higher plants and green algae has recently received considerable attention. This integral membrane polypeptide which is associated with photosystem II (2, 10) has not only a highly conserved sequence in various plants and algae (cf ref 16 for review), it is also related to the reaction center L and M subunits of purple photosynthetic bacteria (38, 40). A characteristic feature of this protein is its high turnover in the light as compared with other thylakoid polypeptides (26). It has been proposed that this polypeptide binds the secondary quinone Q_B of photosystem II, near the external face of the thylakoid (2). The 32 Kdalton protein appears to be the target for a wide class of herbicides (2, 28). Analysis of mutants resistant to atrazine [2-chloro-4-ethylamino-6 (isopropylamino)-s-triazine] and DCMU (3-3-,4 dichlorophenyl, -1,1-dimethyl urea) has revealed point mutations in the psbA gene which result in single amino acid substitutions in the 32 Kdalton polypeptide (14, 15, 20, 22). In most cases, herbicide resistance is accompanied by a slowing down of electron transfer between the primary (Q_A) and secondary (Q_B) quinones of the photosystem II reaction centers (1, 14, 17).

In higher plants the psbA gene exists as a single uninterrupted gene near one end of the inverted repeats of the chloroplast genome (41). In contrast, four introns have been found in the psbA genes of *Euglena gracilis* (23, 24) and *C. reinhardii* (13). In the latter case the psbA gene is contained within the chloroplast inverted repeat and it is therefore present in two copies per chloroplast genome. The exact role of the psbA gene product in photosystem II function and assembly is not yet known. In order to gain more insights into this question we have examined several chloroplast mutants of *C. reinhardii* lacking PSII activity which are unable to synthesize the 32 Kdalton membrane polypeptide. Here we show that all the mutants examined have deleted both copies of the psbA gene. Examination of the synthesis and stability of photosystem II polypeptides in these mutants has revealed that the 32 Kdalton protein plays a role in the assembly of a stable, functional photosystem II complex.

Materials and methods

Strains and genetic characterization

C. reinhardii wild-type strain 137c and several uniparental mutants deficient in photosystem II activity were used. Uniparental mutants 11-1A, 11-4D and 8-36C were isolated and described by Spreitzer and Mets (36). Mutants FuD7, FuD112, FuD13 and sixteen other photosystem II mutants were obtained as described (6). A preliminary characterization of FuD7 has been published (3). Genetic analysis showed that the mutations in FuD7, FuD112 and FuD13 are transmitted uniparentally by the mating type + parent and that these mutations and those of 11-1A, 11-4D and 8-36C do not complement each other in young zygotes (4).

Growth

C. reinhardii cells were grown in Tris-acetate phosphate (TAP) medium (21) at 25 °C at 250 lux.

Pulse labeling

A cell culture of 200 ml (2×10^6 cells/ml) was centrifuged at 1500 g for 5 min and washed in minimal medium. Cells were resuspended in the same medium at the initial cell density and illuminated at 250 lux for 1 h on a rotary shaker at 25 °C. Cells were then preincubated with anisomycin (3.75×10^{-4} M) for 10 min at 250 lux and labelled for 45 min with ^{14}C acetate (specific activity 55 mCi/mmole, 0.5 μCi/ml) at 250 lux. At the end of the labelling period an excess of non-radioactive sodium acetate was added. The cell suspension was divided into two aliquots: 150 ml were directly processed for thylakoid membrane purification as described (7), 50 ml were centrifuged at 1500 g for 10 min, the cells were washed with 20 mM Hepes pH 7.5, resuspended by adding 80 μl of 0.1 M Na_2CO_3, 0.1 M dithiothreitol and stored at −80 °C. Subsequently the extract was fractionated by electrophoresis either on 12–18% polyacrylamide gels containing 8 M urea (29) or on 7.5–15% SDS polyacrylamide gels as described (7).

Fluorescence yield measurements

Fluorescence induction kinetics of chlorophyll *in vivo* was performed as described (6).

DNA and RNA isolation in C. reinhardii

The strains were grown in 500 ml cultures in TAP medium in the dark to stationary phase and DNA was isolated as described (33).

DNA from 10 ml stationary cultures was prepared by a modification of the method of Davis *et al.* (9). After centrifugation the cell pellet was frozen and resuspended into 0.35 ml 20 mM Tris-HCl pH 8.0, 0.1 M NaCl, 50 mM EDTA and transferred into an Eppendorf tube. The cells were lysed with 1% SDS and 1 mg/ml pronase and incubated at 50 °C for 2 h. The lysate was adjusted to 0.05% diethylpyrocarbonate and incubated 15 min at 70 °C. The lysate was made 0.5 M K-acetate, left in ice for 30 min and spun 15 min in an Eppendorf centrifuge. The supernatant was extracted with an equal volume of phenol and the aqueous phase was recovered after centrifugation in a new Eppendorf tube which was then filled with ethanol. The precipitate was pelleted (1 min centrifugation), washed with 70% ethanol, dried and resuspended in 50 μl 10 mM Tris HCl pH 7.5, 1 mM EDTA.

RNA was isolated by a modified procedure (19) (M. Goldschmidt-Clermont, personal communication). Cells were grown in TAP medium in the dark to a concentration of $1-2\times10^6$ cells/ml. The cells were centrifuged for 5 min at 5000 g, resuspended into 1 ml of TAP medium and transferred into sterile Eppendorf tubes. After centrifugation the pellet was resuspended into 0.4 ml A buffer (6 M guanidinium chloride, 0.1 M Na acetate pH 5.2) and glassbeads (previously washed in 0.1 NHCl) were added. The mixture was vortexed for several minutes and centrifuged 1 min. The supernatant was transferred to a new tube, 0.75 vol of ethanol was added and the mixture was left at −20 °C for several hours. After centrifugation, the pellet was resuspended into 0.5 ml of A buffer, heated 1 min at 65 °C and precipitated with 0.25 ml ethanol. After several hours at −20 °C the extract was centrifuged, resuspended into 0.25 ml 1 mM EDTA pH 7.5, heated 1 min at 65 °C and centrifuged 1 min. Ten μl 4 M NaCl and 625 μl ethanol were added to the supernatant. The mixture was left at −20 °C for several hours and centrifuged. The pellet was washed with 70% ethanol, dried and resuspended into 100 μl 1 mM EDTA. The RNA was fractionated on denaturing formaldehyde agarose gels as described (30) and transferred onto nitrocellulose filters (37).

Plasmids

Several plasmids containing parts of the psbA gene and of its flanking regions were used as probes. Plasmid pSoc S15 consists of pBR322 and a 8.7 kb spinach chloroplast SalI fragment containing the entire psbA gene (41). A 1.2 kb XbaI-SmaI fragment containing psbA coding sequences and 220 bp of 5′ sequences, was excised from the SalI fragment and used as a spinach psbA probe (41). All other plasmids contain chloroplast DNA fragments from *C. reinhardii*. Plasmid pcp58 contains the EcoRI fragment R24 in the plasmid vector pCRI (31, cf Fig. 4). Plasmids pDCSR6 and pCWR14 contain the EcoRI fragments R6b and R14, respectively in the vector pBR328. Plasmids pCWHB 2.5 and pCDSHR 1.5 consist of pBR322 and the 2.5 kb HindIII-BamHI or the 1.5 kb EcoRI-HindIII fragments that flank the psbA gene (cf Fig. 4).

Hybridizations

DNA was digested with restriction endonucleases and fractionated by agarose gel electrophoresis. The DNA fragments were transferred to nitrocellulose filters (35) and hybridized with probes specific for the psbA gene and its flanking regions. DNA-DNA hybridizations were done in 5×SSC, 1×Denhardt, 0.1% SDS, 25 μg/ml salmon sperm DNA. DNA-RNA hybridizations were performed at 42 °C in 5×SSPE, (1×SSPE: 0.18 M NaCl, 10 mM NaH_2 PO_4 pH 7.0, 1 mM EDTA), 50% formamide, 5×Denhardt, 200 μg/ml salmon sperm DNA.

Results and discussion

Pulse labelling of thylakoid polypeptides in wild type cells and photosystem II mutants

Thylakoid membranes consist of several macromolecular complexes which contain polypeptides encoded both by the nuclear and chloroplast genomes. It is observed that mutants deficient in a specific activity associated with the photosynthetic membrane generally lack the corresponding complex responsible for this function (5, 18, 29). This is due to the fact that the correct assembly of a given complex in the membrane usually requires the presence of all its components in an intact form. The analysis of thylakoid membrane polypeptides in mutants of this sort will therefore not allow one to determine the primary effect of the mutation. It is, however, possible to overcome this problem with respect to polypeptides of the thylakoid membrane that are synthesized within the chloroplast. When whole cells are pulse-labelled with $_{14}$C-acetate in the presence of an inhibitor of 80S ribosome translation, solubilized with detergent and fractionated by polyacrylamide gel electrophoresis, the most abundant polypeptides which are detected by autoradiography are the large subunit of ribulose 1,5 bisphosphate carboxylase and the thylakoid membrane proteins which are synthesized within the chloroplast (10). The other soluble chloroplast products and the mitochondrial proteins are synthesized at a considerably lower rate. It is therefore possible to measure specifically the synthesis of the thylakoid membrane polypeptides made within the chloroplast even in mutants in which these polypeptides are not stablely integrated in the thylakoid membranes.

Using this approach we have examined several mutants deficient in photosystem II activity. Fig. 1A compares the coomassie blue staining of the thylakoid polypeptides of wild type cells and the mutant FuD7, FuD112 and FuD13 fractionated by SDS-urea polyacrylamide gel electrophoresis. It is apparent that the major photosystem II polypeptides 5, 6, 19, 24, 34 and 36 are missing or strongly diminished in these mutants. The lower part of the figure shows this more clearly for polypeptide 5 which can be separated from the α subunit of ATP synthase when electrophoresis is performed on an SDS polyacrylamide gel. While polypeptides 5, 6 and 36 are encoded by the chloroplast genome, the genes of polypeptides 19, 24 and 34 are located in the nucleus (10). A different picture is obtained when the cells are pulse labelled for 45 min in the presence of anisomycin and the labelling pattern of the chloroplast-encoded thylakoid membrane polypeptides is examined. Two other chloroplast-encoded phototsystem II polypeptides, D1 and D2, which stain poorly with coomassie blue (Fig. 1A), are heavily labelled with C^{14} acetate in wild-type cells (Fig. 1B, 1C). Fig. 1B shows that the chloroplast-encoded polypeptides 5, 6 and 36 (L8 in Fig. 1B) of the photosystem II complex, which

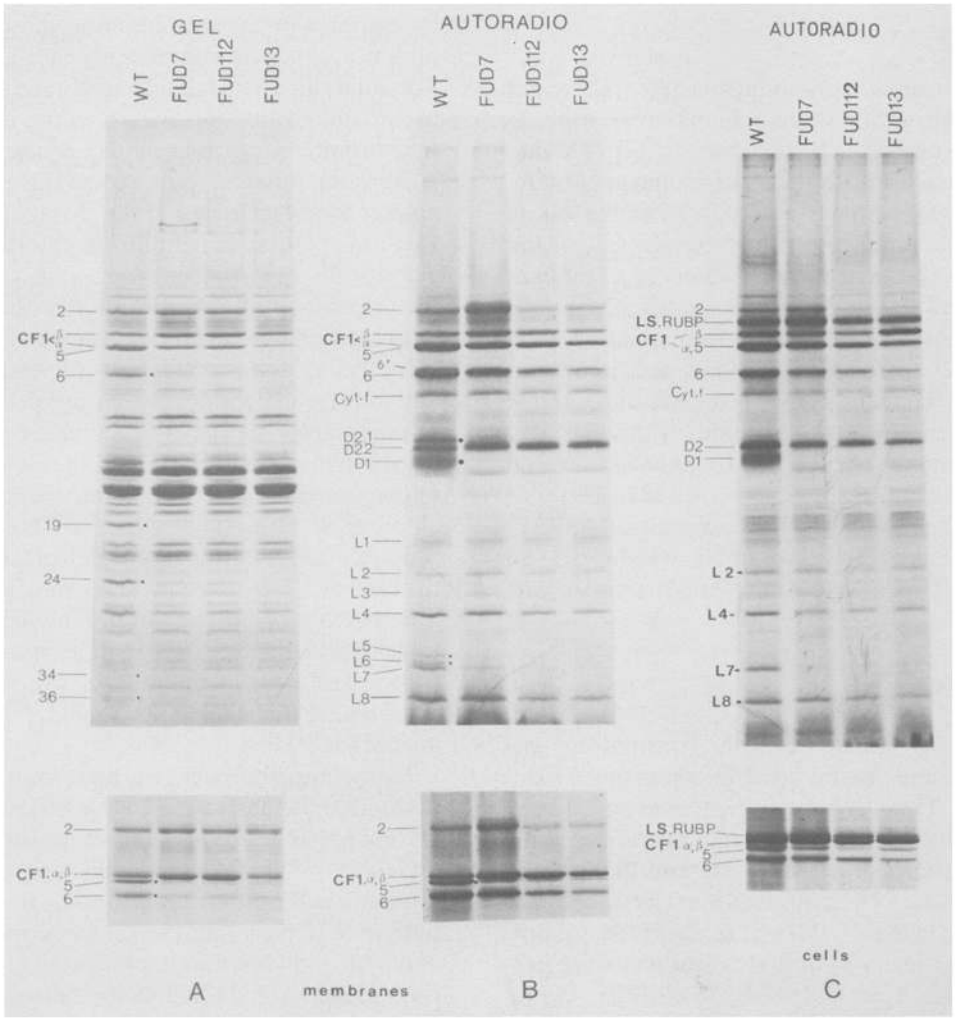

Fig. 1. Gel electrophoretic fractionation of thylakoid membrane polypeptides (A, B) and of whole cell protein (C) from wild-type cells and photosystem II mutants of *C. reinhardii* labelled for 45 min with ^{14}C acetate in the presence of anisomycin. Stained gel is shown in panel A, autoradiograms in panels B and C. Upper part: 12–18% SDS polyacrylamide gels with 8 M urea. Lower part: 7.5–15% SDS polyacrylamide gels; only the region of polypeptide 5 is shown because this band comigrates with the α subunit of ATP synthase in the SDS urea gel shown above. The bands that are missing or strongly reduced in the mutants are indicated by dots. The nomenclature of thylakoid polypeptides is according to Chua and Bennoun (7), Bennoun *et al.* (3), and Delepelaire (10).

are barely detectable by coomassie blue staining, are synthesized and incorporated into the thylakoid membrane. Similar findings were reported previously by Delepelaire (10) with F34, a nuclear mutant deficient in PSII activity. Fig. 1B also shows that the bands, D2.1, L5 and L6 are not labelled in the mutants. The former is a phosphorylated form of polypeptide D2.2, and the two latter are processed, phosphorylated forms of L3 (12). The most striking difference between wild-type cells and the three mutants is that there is no D1 synthesis in the latter. The D1 polypeptide is the psbA gene product of *C. reinhardii* (13). The same feature can also be observed with whole cell extracts (Fig. 1C). The labelling patterns obtained from whole cells and thylakoid membranes are very similar, suggesting that the polypeptides of the PSII complex that are synthesized in the chloroplast are incorporated in the membrane of the mutants. The three mutants display very similar patterns of label-

ling, with respect to each other, except for FuD7 in which an additional chloroplast product of small size is synthesized (between L8 and L9 in Fig. 2).

The stationary state polypeptide profiles of thylakoid membranes of the mutants indicate that both nuclear and chloroplast DNA encoded polypeptides of the photosystem II complex are nearly undetectable. However, the results of pulse-labelling studies show that, even in the absence of D1, most of the chloroplast products belonging to the photosystem II complex can still be synthesized and incorporated into the membrane. Moreover, these polypeptides appear to be firmly anchored in the membrane since they are not removed by washing with 6 M guanidine (data not shown), a treatment known to remove peripheral membrane polypeptides (29). In spite of their integration into the membrane, these proteins, which are only detected by pulse labelling, are subsequently degraded, possibly because they do not assemble into a functional complex.

Fluorescence induction curves of the FuD7 mu-

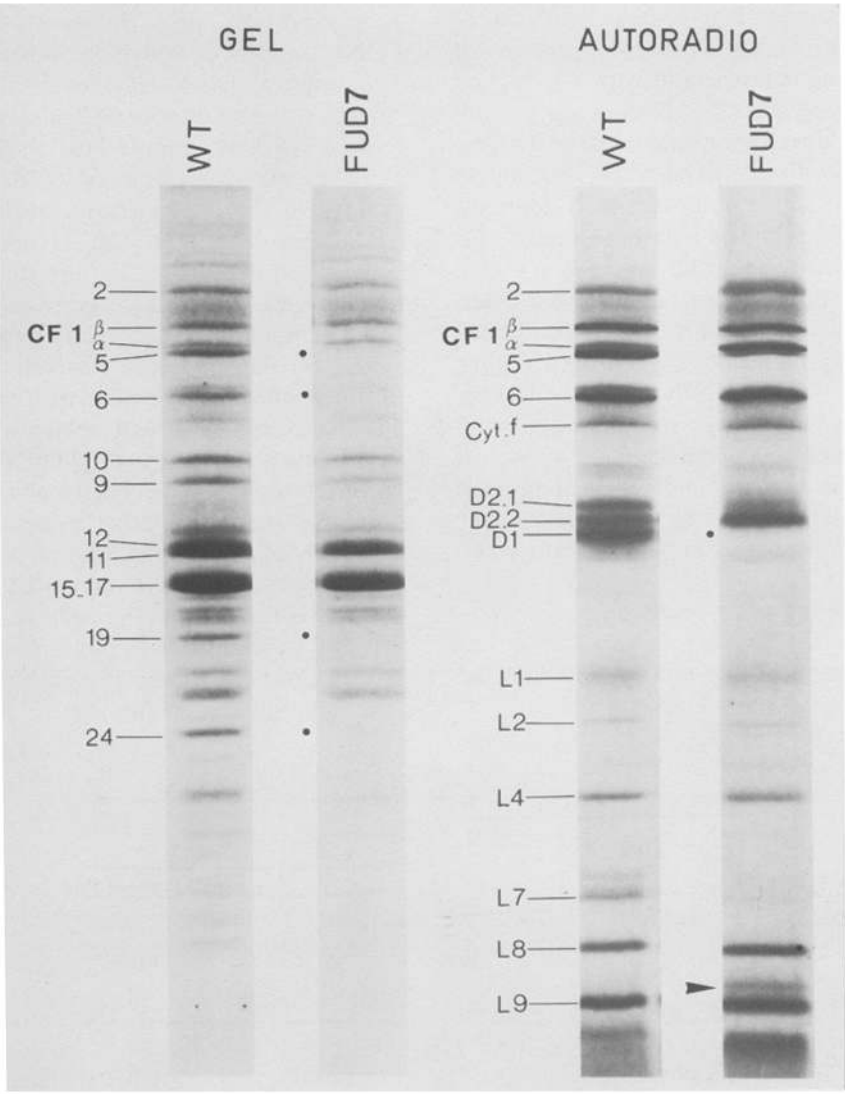

Fig. 2. Gel electrophoretic fractionation of thylakoid membrane polypeptides from wild-type cells and FuD7 mutant of *C. reinhardii* labelled as described in fig. 1. The staining patterns and the autoradiograms of 12–18% SDS polyacrylamide gels with 8 M urea are shown. Dots indicate polypeptides missing in FuD7.

tant cells following different pretreatments are shown in Fig. 3. The variable fluorescence observed in the wild type is associated with photosystem II activity since the fluorescence yield is mainly controlled by the redox state of the primary quinone Q_A of photosystem II centers. This variable fluorescence is absent in the FuD7 mutant confirming an earlier report (3). The same is true for the other photosystem II mutants examined. It has been shown previously that in the nuclear mutant F34, which lacks most of the polypeptides associated with photosystem II, State I to State II transitions are still observed. This process is associated with the phosphorylation of the polypeptides of the light harvesting complex and with a reduction of the fluorescence yield (39). Thus it can be concluded that the plastoquinone mediated redistribution of light energy to photosystem I still occurs in the absence of an active photosystem II complex. However, the F34 mutant is able to synthesize D1 (10). In order to test whether D1 may play a role in this process, the fluorescence kinetics were determined in FuD7 mutant cells under conditions where the plastoquinone pool is oxidized (light), partially reduced (dark) or fully reduced (dark + anaerobiosis). It can clearly be recognized in Fig. 3 that fluorescence changes characteristic of State I to State II transitions occur as the plastoquinone pool becomes reduced. Thus we conclude that the D1 polypeptide is not necessary for this process.

Molecular analysis of psbA mutations

The previous section has shown that when the photosystem II mutants are pulse-labelled with ^{14}C acetate, the D1 polypeptide is conspicuously absent among the newly synthesized chloroplast proteins. This observation suggested that the mutation was affecting the psbA gene or its expression. In order to test these possibilities, the DNAs of the mutants described above and of three other characterized photosystem II mutants were isolated. DNAs digested with EcoRI were fractionated by agarose gel electrophoresis and hybridized with a spinach psbA gene probe (see Materials and methods). The latter, which is highly homologous to its *C. reinhardii* counterpart, was used because the introns of the psbA gene of *C. reinhardii* contain repetitive DNA sequences which hybridize with numerous chloroplast DNA fragments, thus making the hybridization patterns difficult to interpret (Erickson & Rochaix, unpublished results).

The two psbA genes of *C. reinhardii* are contained in the EcoRI fragments R14 (or R16) and R24 (Fig. 4, ref. 13). The R14 and R16 fragments each span one end of the inverted repeat and one end of the large single copy region (31). Fig. 5 shows that the spinach psbA probe hybridizes to the R14, R16 and R24 fragments from wild type cells. It does not hybridize with any of the mutant DNAs except for 12–3C which was originally isolated as a photosystem II mutant (36) and has since reverted to wild type. The results indicate that the mutants lack both psbA genes. This conclusion agrees with the observation that no psbA transcript is detectable in these mutants when the RNA of the strains is fractionated by agarose gel electrophoresis and hybridized with the same psbA probe (Fig. 6). Control experiments reveal that the mRNA of the large subunit of ribulose 1,5 bisphosphate car-

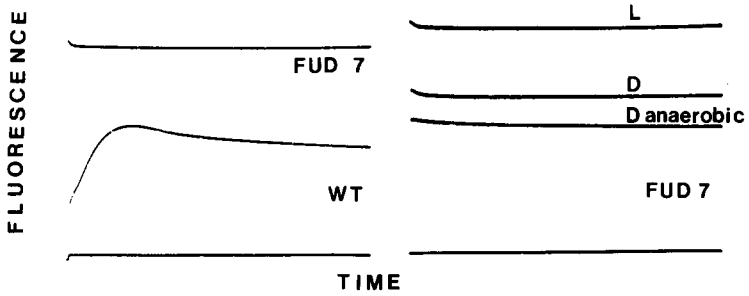

Fig. 3. Left. Fluorescence induction kinetics of dark adapted cells from wild-type and FuD7 mutant. Whole sweep is 2 sec. Right. Fluorescence induction kinetics of cells of FuD7 mutant. Cells were incubated 10 min in the light (L), in the dark (D) and in the dark under anaerobic conditions (D anaerobic); anaerobiosis was achieved by adding 20 mM glucose and 2 mg/ml glucose oxidase to the culture; whole sweep is 2 sec.

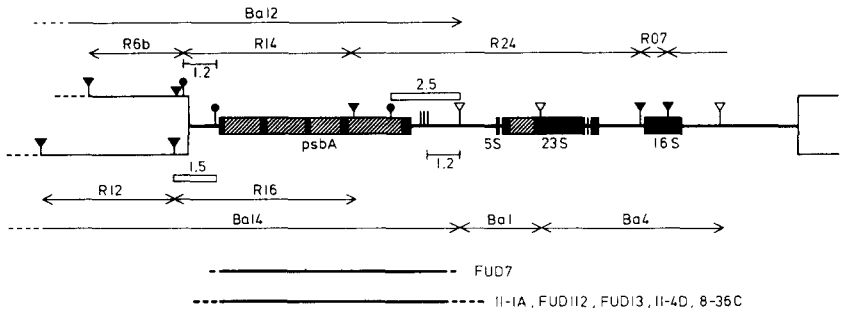

Fig. 4. Localization of the psbA deletions of *C. reinhardii* photosystem II mutants. The entire inverted repeat with the psbA gene and the ribosomal operon is drawn (13). ■ exons or coding regions, ▨ introns. The two ends of each single copy region are also shown. Only restriction endonuclease sites used for mapping the deletions are indicated: EcoRI, ▼ HindIII ▼, BamHI ▽, KpnI |. The 1.5 kb EcoRI-HindIII and 2.5 kb HindIII-BamHI fragments used as probes are marked with open bars. The 1.2 kb HindIII and 1.2 kb KpnI-BamHI fragments used for determining the ends of the deletions are indicated by ⊢—⊣. The restriction fragments are labelled as in ref. 31. Deletions are drawn as horizontal lines at the bottom. Dotted lines indicate the regions where the ends of the deletions have been mapped.

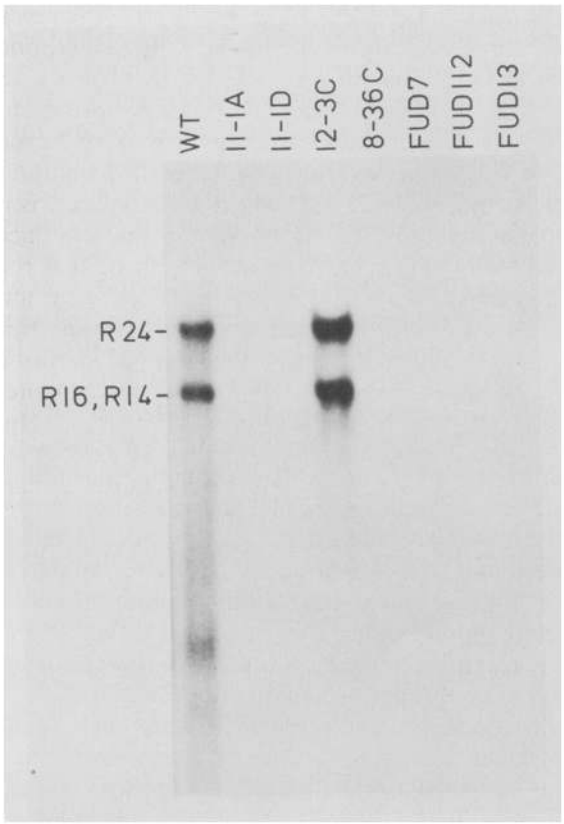

Fig. 5. Autoradiogram showing wild-type and mutant DNAs after hybridization with the ^{32}P-labeled spinach psbA probe. The DNAs were digested with EcoRI, fractionated by electrophoresis on a 0.8% agarose gel, transferred to nitrocellulose and hybridized (35).

boxylase is present in each of these mutants (Fig. 6).

In order to map the endpoints of the deletions the chloroplast EcoRI digests of the mutants were hybridized separately with the R6b and R12 fragments, which are the neighbouring fragments of R14 and R16, respectively, in the large single copy region (Fig. 4). In all six mutants examined, the R6b and R12 fragments are still intact (data not shown), indicating that one end of the deletion must be located between the 5′ end of the psbA gene and the nearby EcoRI site. Agarose gel electrophoresis and Southern transfer of EcoRI-HindIII digested DNA from the mutant FuD7, followed by hybridization with the labelled 1.5 kb EcoRI-HindIII fragment of R14 (containing the 5′ flanking sequences of psbA) (Fig. 4) reveals two hybridizing fragments of 1.5 and 1.2 kb. This indicates that in this mutant the deletions are entirely contained within the inverted repeat. Since the deletions of FuD7 start on the right hand side of the HindIII site (Fig. 4) which is located in the 5′ non-translated region of psbA, this mutant has maintained the promoter and at least a portion of the 5′ non-translated region of psbA (13). It is therefore possible that the small polypeptide which appears among the newly synthesized proteins of FuD7 (Fig. 3) originates from this region.

Similar hybridizations of BamHI digested DNA from the mutants with labelled R24 fragment revealed that the mutants FuD7, FuD13 and 8-36C

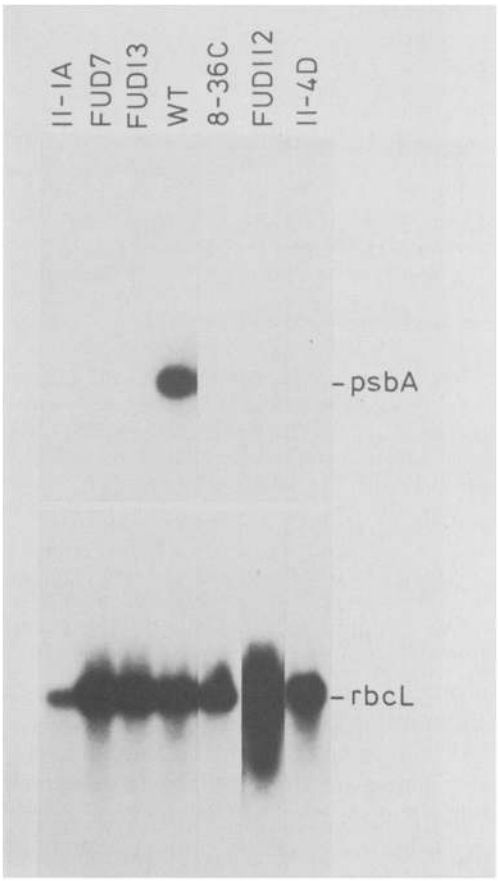

Fig. 6. Autoradiogram showing wild-type and mutant RNAs. The RNAs were run on a denaturing agarose formaldehyde gel (30), transferred to nitrocellulose and hybridized first with a ^{32}P-labelled spinach probe (upper), and then with a rbcL probe (lower).

contain at least one copy of the Bal fragment (see Fig. 4) whereas mutants 11-1A, and 11-4D lack this fragment (data not shown). The DNAs of the first three mutants were also digested with KpnI and BamHI and hybridized with the labelled 2.5 kb HindIII-BamHI fragment which contains the 3′ region of psbA (Fig. 4). Since no signal was detectable, it can be concluded that the deletion end points in these mutants are very close to the BamHI site which separates fragments Ba12 and Ba1 (Fig. 4). Therefore, each of the deletions in FuD7 removes close to 8 kb. In the other mutants, the deletions range between 8 and 10 kb. The data do not allow us to determine whether the double deletions which have occurred in the 6 mutants are perfectly symmetrical. The chloroplast DNA of the FuD7 mutant was isolated and digested with EcoRI which generates 37 fragments (31). Examination of the restriction pattern revealed no DNA changes other than the psbA deletions. Since the deletions cover a region of 1 to 1.5 kb beyond the 3′ end of the psbA gene, the possibility that a second gene has been deleted and that its absence causes the mutant phenotype has to be considered. However no major transcript from this region can be detected in wild type cells when the 1.2 kb Kpn-I-BamHI fragment (cf. Fig. 4) is hybridized with total RNA (data not shown). In contrast all photosystem II chloroplast coding regions give rise to abundant transcripts (32, 34).

DNA mini extractions were performed on 16 additional photosystem II mutants unable to synthesize D1. Hybridization of BamHI digests of the DNA of these mutants with the spinach psbA probe revealed that all of them had double deletions of the psbA genes. It is noteworthy that among 78 chloroplast photosystem II mutants examined, 68 were unable to synthesize D1. 22 of these mutants were tested and found to lack the psbA genes.

The high proportion of psbA mutations found among photosystem II deficient mutants might be due to the large size, nearly 7 kb, of the psbA gene and to an efficient mismatch correction mechanism in the inverted repeat. It is surprising that all the mutants examined so far lack both copies of the entire psbA gene or at least a very large portion of it. In no case have we observed small deletions within this gene. Similar findings have been reported by Palmer *et al.* (27) on five mutants (including FuD7). Our earlier work (31) and recent results by Palmer *et al.* (27) show that the chloroplast inverted repeat of *C. reinhardii* contains several repetitive elements that are interspersed throughout the chloroplast genome. Since the psbA gene is flanked by these repetitive elements, it is possible that the deletions arise through recombination (27).

In conclusion, the genetic, biochemical and molecular analysis of several photosystem II mutants has shown that they all contain deletions at the psbA locus. In at least one case, in mutant FuD7, we have verified that the only detectable chloroplast DNA rearrangement occurs in the psbA gene region. Since all these mutants are unable to assemble a stable and functional photosystem II

complex, it can be concluded that the 32 Kdalton protein is required for both these processes. This protein has therefore multiple functions: it appears to be involved in herbicide resistance (15, 22, 28), electron transfer (2, 14, 17, 26) photoinhibition (25) and now also in stabilizing the photosystem II complex.

Acknowledgements

We thank O. Jenni for drawings and photography. This work was supported by a CNRS grant (ATP, Biologie Moléculaire Végétale) to P.B. and Swiss National Foundation grant 3.587-0.84 to J.D.R.

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Received 25 July 1985; in revised form 11 November 1985; accepted 19 November 1985.