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A point mutation in the gene for the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase affects holoenzyme assembly in *Nicotiana tabacum*

Adi Avni, Marvin Edelman, Ivan Rachailovich, Dvora Aviv and Robert Fluhr

Department of Plant Genetics, The Weizmann Institute of Science, 76100 Rehovot, Israel

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In photosynthetic eukaryotes, the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is composed of eight large and eight small subunits. Chloroplast-coded large subunits are found in association with chaperonins (binding proteins) of 60-61 kd to form a high mol. wt pre-assembly complex (B-complex). We have isolated a heterotrophic, maternally-inherited mutant from *Nicotiana tabacum* var. *Xanthi* which accumulates the B-complex but contains no Rubisco holoenzyme. The B-complex of the mutant dissociates in the presence of ATP, as does that of the wild-type. Processing of the nuclear-coded small subunit takes place in the mutant and neither large nor small subunits accumulate. The large subunt gene from mutant and wild-type plants was cloned and sequenced. A single nucleotide difference was found between them predicting an amino acid change of serine to phenylalanine at position 112 in the mutant. Based on the resolved structure of *N.tabacum* Rubisco, it is argued that the alteration at position 112 prevents holoenzyme assembly by interfering with large subunit assembly.

Key words: binding protein/chaperonins/chloroplast mutant/Rubisco

Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyzes two competing reactions: the fixation of carbon dioxide in the process of photophosphorylation and fixation of oxygen in the process of photorespiration (Andrews and Lorimer, 1987). In eukaryotes, the holoenzyme is composed of eight large subunits (LS) of ~52 kd and eight small subunits (SS) of ~15 kd (Rabinowitz *et al.*, 1975). LS is encoded by the chloroplast *rbc*L gene, whereas SS is encoded by the nuclear genome, synthesized in a precursor form on cytoplasmic ribosomes and processed following uptake by the chloroplast (Smith and Ellis, 1979). Holoenzyme assembly occurs in the chloroplast stroma (Chua and Schmidt, 1978; Smith and Ellis, 1979). The mechanism of assembly involves imported, nuclear coded proteins, called binding proteins (BP) or chaperonins (Hemmingsen *et al.*, 1988), which were recently shown to be homologs of the bacterial *gro*E gene product (Hemmingsen *et al.*, 1988; Goloubinoff *et al.*, 1989) and which are not part of the final structure of the enzyme (Barraclough and Ellis, 1980; Bloom *et al.*, 1983; Hemmingsen and Ellis, 1986). Newly synthesized LS are bound to BP to form an oligomer of 12-14 BP and one LS (Musgrove and Ellis, 1986; Hemmingsen *et al.*, 1988). The structure is called the binding complex (B-complex).

A maternally-inherited nonsense mutation producing a truncated LS protein and affecting holoenzyme assembly was characterized in *Chlamydomonas* (Spreitzer *et al.*, 1985). We have combined seed mutagenesis with tissue culture propagation to obtain maternally-inherited point mutations in tobacco chloroplasts (Fluhr *et al.*, 1985). A heterotrophic *Nicotiana tabacum* mutant (XV1) that lacks Rubisco holoenzyme but synthesizes full-length subunit proteins and accumulates the B-complex has been isolated and is characterized here. This mutant is shown to have a changed nucleotide in a region of *rbc*L involved in LS assembly.

Results

Mutant XV1 lacks Rubisco subunits

Mutant XV1 was selected by screening nitrosomethylurea-mutagenized seedlings for the appearance of light green sectors on a normal green background. Mutant heterotrophic tissue was cloned by tip culture and seeds were obtained by grafting on wild-type *N.tabacum* var. *Xanthi* stock. The XV1 phenotype is maternally inherited. This was established in reciprocal crosses between *N.tabacum* var. *Xanthi* wild-type and the mutant line. Approximately 300 seeds were germinated on Nitsch agar (Nitsch, 1969). When wild-type

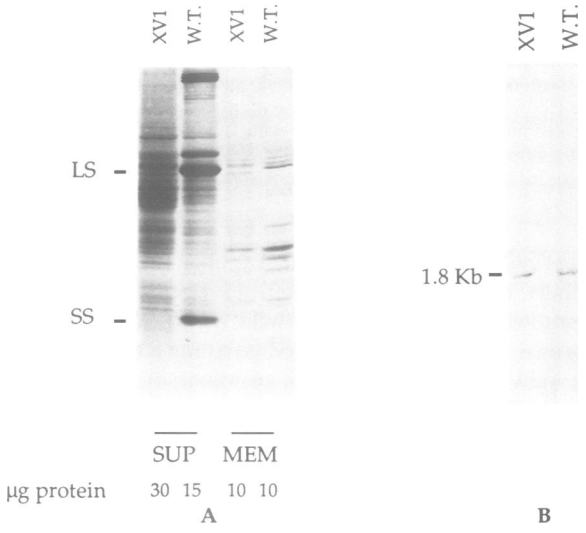

Fig. 1. (**A**) Polypeptide profiles of XV1 and wild-type extracts. Leaves were extracted as described in Materials and methods. Supernatant and membrane protein fractions were resolved on an SDS 10-20% polyacrylamide gel and stained with Coomassie brilliant blue. (**B**) Analysis of total RNA transcripts from XV1 and wild-type. Total cell RNA from XV1 and wild-type plants was glyoxylated, electrophoretically fractionated on 1% agarose gels (7 μg RNA/lane) and immobilized on nitrocellulose filters. Hybridization of nick-translated LS-specific probe, pBI-20 (Fluhr, 1985) was carried out as described (Fluhr *et al.*, 1983).

Table I. Photosynthetic parameters of *N.tabacum* var. *Xanthi* wild-type and mutant line XV1

Reaction	Wild-type	XV1 mutant
CO_2 fixation (c.p.m./mg chlorophyll/h)		
1. ^{14}C incorporation	8000	96
O_2 uptake (μeq/mg chlorophyll/h)		
2. H_2O to methyl viologen	28	33
3. Reaction 2 + (ADP + Pi)	64	51
4. Reaction 2 + Atrazine	14	4
5. Dichlorophenol indolephenol + ascorbate to methyl viologen	104	75

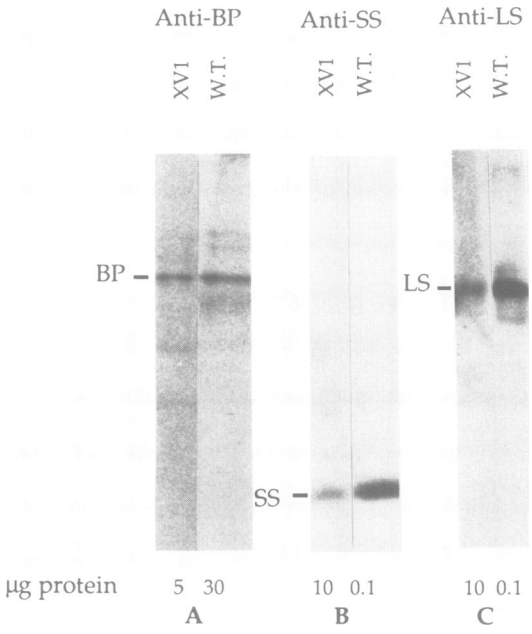

Fig. 2. Immunoblots of XV1 and wild-type extracts. Leaf extracts were fractionated on SDS–polyacrylamide gels and transferred to nitrocellulose. Strips of nitrocellulose were probed with antisera as designated and subsequently incubated with ^{125}I-labeled protein-A. Varying amounts of supernatant proteins as indicated in the figure were loaded in order to obtain signals of approximately similar intensity.

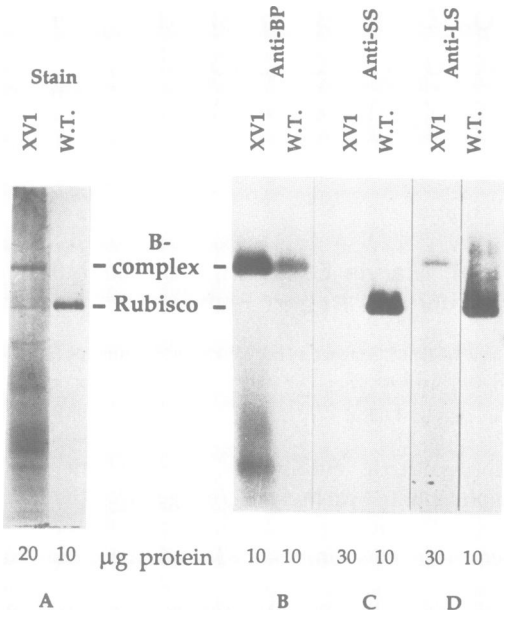

Fig. 3. Polypeptide profiles of XV1 and wild-type extracts on non-denaturing polyacrylamide gels. (**A**) Leaves were extracted as described in Materials and methods and supernatant protein fractions resolved on a 4–10% polyacrylamide gel following electrophoresis. Staining was with Coomassie brilliant blue. (**B–D**), XV1 and wild-type extracts were fractionated as in **A** and transferred to nitrocellulose as described in Materials and methods. Strips of nitrocellulose were probed with antisera as designated, incubated with ^{125}I-labeled protein-A and autoradiographed.

flowers were crossed with XV1 pollen all germinated seedlings (F_1) were dark green. However, when XV1 flowers were pollinated with wild-type pollen (i.e. the reciprocal cross) all germinated seedlings (F_1 progeny) were light green and unable to grow on soil.

The SDS–polyacrylamide gel pattern of extracted polypeptides of XV1 and wild-type plants is shown in Figure 1A. Several quantitative changes in the membrane fraction, especially in photosystem II components, are apparent; the reasons for these changes are unknown. However, the lack of Coomassie blue-stained amounts of LS and SS in the supernatant fraction is the outstanding feature of mutant tissue.

Wild-type and mutant tissues were compared for several photosynthetic parameters. In XV1 tissue, CO_2 fixation was $<0.1\%$ of normal tissue (Table I). However, O_2 uptake was unaffected using methylviologen as acceptor in isolated chloroplast membranes. Sensitivity to the electron transport inhibitor atrazine, as well as electron flow enhancement by the addition of ATP, were similarly unchanged in mutant tissue (Table I). The chlorophyll content of XV1 was ~30% that of wild-type for plants grown over a range of light intensities (2–150 μmol/m^2/s). Taken together, the data suggest that XV1 is an oligate heterotroph due to lack of Rubisco.

Analysis of total RNA from XV1 using the LS gene as hybridization probe (pBI-20; Fluhr, 1985) revealed no change in the size, nor a decrease in the abundance, of LS RNA compared to wild-type (Figure 1B). Therefore, assuming that the cytoplasmically inherited mutation is in the LS gene, these data indicate that the lesion in XV1 is unlikely to be at the level of transcription.

Translation of SS, LS and BP in mutant XV1

Soluble proteins from XV1 and wild-type were separated by SDS–polyacrylamide gel electrophoresis, blotted onto nitrocellulose and probed with antibodies against BP, SS and LS (Figure 2). Immunoreaction to all three antibodies was obtained. The relative mobilities of immunoreactive BP, SS and LS in XV1 were indistinguishable, respectively, from those in wild-type. Thus, LS, SS and BP subunits are synthesized in the XV1 mutant and SS is processed to its mature size. However, the steady-state amounts of the Rubisco-related proteins in the XV1 mutant are vastly different from those in wild-type: <2% for SS and LS, but >500% for BP (Figure 2). The reduction in steady-state amounts of LS is not a result of reduced rate of synthesis, as a comparable amount of radioactive LS-specific protein is immunoprecipitated in short-term labeled mutant and normal leaves (data not shown).

The high level of binding proteins (chaperonins) in XV1 led us to search for mutant and wild-type B-complexes by

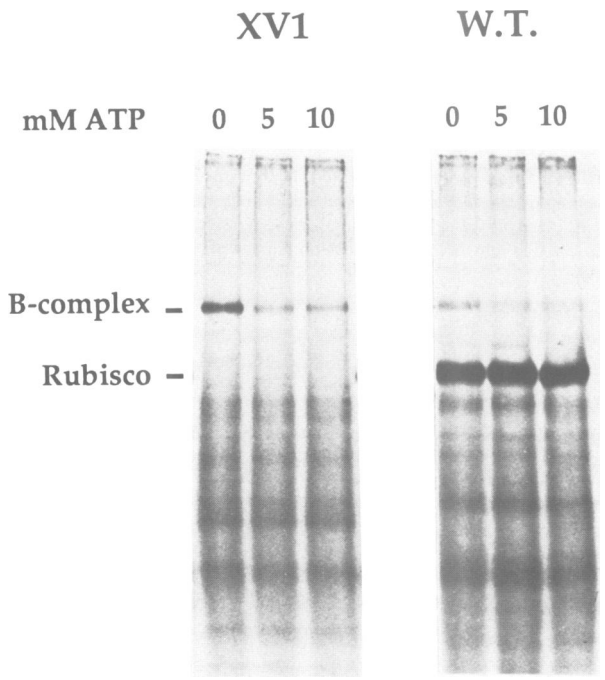

Fig. 4. The effect of ATP on the stability of the B-complex of XV1 and wild-type. Leaves were extracted as described in Materials and methods. Extracts were dialyzed against buffer containing 6 mM $MgCl_2$ and 220 mM KCl (Bloom *et al.*, 1983) and incubated for 30 min at 4°C with various amounts of ATP as indicated in the figure. Samples were fractionated on a 4–10% non-denaturing polyacrylamide gel and stained with Coomassie brilliant blue.

fractionating extracts on a non-denaturing polyacrylamide gel. XV1 contained a major staining band of high mol. wt (Figure 3A) which reacted with antibodies to BP (Figure 3B) and LS (Figure 3D) but not SS (Figure 3C). An immunoreactive complex with the mobility of Rubisco holoenzyme was not detected (Figure 3C, D). Wild-type showed a much lower level of B-complex and contained instead a major Coomassie-stained, immunoreactive Rubisco band.

The B-complex is known to dissociate in the presence of Mg–ATP (Bloom *et al.*, 1983; Lennox and Ellis, 1986; Roy *et al.*, 1988b). Incubation of mutant extracts with Mg–ATP indeed caused dissociation of the XV1 B-complex (Figure 4). The accumulation of the B-complex *in vivo* in XV1 is reminiscent of its accumulation in isolated chloroplasts (Barraclough and Ellis, 1980; Bloom *et al.*, 1983; Musgrove and Ellis, 1986; Roy *et al.*, 1988a). However, while in isolated chloroplasts this accumulation might result from limiting amounts of nuclear-coded nascent SS molecules, in maternally-inherited XV1 we suspected the cause to lie with the LS gene.

Sequence analysis of the XV1 rbcL gene

The complete nucleotide sequence of the LS gene (*rbc*L) from wild-type *N.tabacum* var. *Xanthi* and mutant line XV1 was determined. The nucleotide sequence from wild-type *Xanthi* was identical to that published for *N.tabacum* var. *BY4* (Shinozaki and Sugiura, 1982). However, a single nucleotide change was found in *rbc*L from mutant XV1. This consisted of a C to T transition in the mRNA-like strand predicting a change from serine to phenylalanine at position 112 in the LS protein (Figure 5). The transition is consistent with the mutagenic base-specific properties ascribed to nitrosomethylurea (Zarbl *et al.*, 1985).

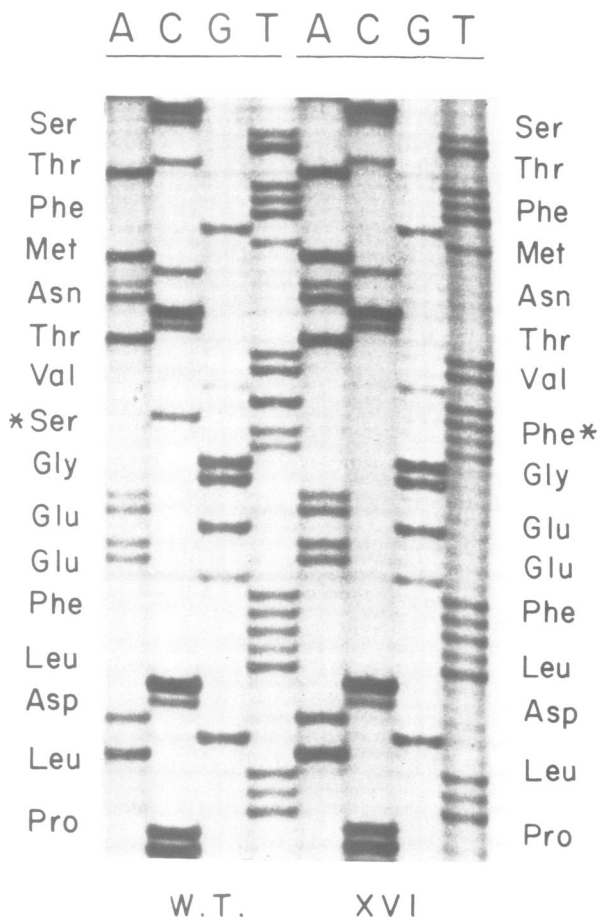

Fig. 5. Nucleotide sequencing of the *rbc*L gene in XV1 and wild-type. Nucleotide sequencing was carried out on a 6% urea–polyacrylamide gel as described in Materials and methods. The sequence of the mRNA-like strand is shown. The nucleotide sequence shown for the wild-type is identical to nucleotides 310–357 of Shinozaki and Sugiura (1982). The C to T transition in the mRNA-like strand of the XV1 *rbc*L gene predicts a serine to phenylalanine change at position 112 of the mutant LS polypeptide. Asterisk indicates the amino acid change.

Discussion

The serine to phenylalanine change at position 112 does not inhibit assembly of LS and BP into B-complexes. Nor is it likely to affect LS–SS interaction since such interactions seem to be limited to the carboxy domain of the LS molecule (Chapman *et al.*, 1987, 1988). Serine is conserved at position 112 in all Rubisco sequences of the L_8S_8 type published (Andrews and Lorimer, 1987). However, this serine and the residues surrounding it are not conserved in the L_2-type Rubisco from *Rhodospirillum rubrum* (Nargang *et al.*, 1984). Chapman *et al.* (1988) have shown that serine 112 is close to the two-subunit LS pocket that forms the active site of Rubisco. Indeed, serine 112 is deep in a region where strong interaction between two LS subunits takes place. In addition, serine 112 participates in intramolecular interactions involving a single LS subunit (Chapman *et al.*, 1988). In either case, the change from serine to a bulky amino acid such as phenylalanine in XV1 might prevent the formation of a properly-aligned LS dimer (the basic building unit of the holoenzyme) and, consequently, higher order oligomerization. In agreement with the above, site-directed mutagenesis of serine 109 of *Synechococcus rbc*L to phenylalanine (which is equivalent to the serine 112 to

phenylalanine change in *Nicotiana*) prevented assembly of L_8S_8 Rubisco holoenzyme but not L_2 dimer-like structures (P.Goloubinoff, A.A.Gatenby and G.H.Lorimer, in preparation).

B-complex accumulation occurs both in isolated chloroplasts and the XV1 mutant. The former case suggests the importance of SS availability while the latter suggests the importance of LS conformation in causing this phenotype. A hypothesis which would reconcile both observations would have all Rubisco assembly steps in a dynamic equilibrium. We note that accumulation of B-complex in XV1 is substantial, allowing for its preparative isolation. Structural analysis of isolated B-complex should lead to the identification of the molecular domain(s) on higher-plant LS molecules interacting with chaperonins.

Materials and methods

Plant material and mutagenesis

Seeds of *N.tabacum* var. *Xanthi* were mutagenized during imbibition using nitrosomethylurea as described by Fluhr *et al.* (1985). Mutagenized seeds were sown on Nitsch agar (Nitsch, 1969). About 20% of the plants scored positive for light green variegation. Leaf tissue from the light green variegated plants was extracted and assayed for the presence of LS by SDS–polyacrylamide gel electrophoresis (Hoffman-Falk *et al.*, 1982). Out of ~50 variegated plants screened, two lacked LS and were grown to maturity. Seeds from pods yielding pure mutant phenotypes were used to establish vegetatively-propagated clonal lines. One line, named XV1, was analyzed. Tip cultures were maintained in MS medium (Murashige and Skoog, 1964) containing 2 μg/ml indole acetic acid and 0.2 μg/ml kinetin, in 15 $\mu mol/m^2/s$ of light.

Preparation of plant extracts and gel fractionation

100–500 mg of plant tissue from clonal line XV1 were homogenized in a tight-fitting glass homogenizer in 100–500 μl of 2.5 mM Tris-glycine pH 8.5 and centrifuged at 100 000 *g*. The supernatant was adjusted to 10% glycerol and immediately applied to a non-denaturing or denaturing gel. Transfer of fractionated proteins to nitrocellulose blots and hybridization with antibodies followed by linkage to iodinated protein-A (Amersham) was carried out as described by Gershoni (1988). SDS 10–20% polyacrylamide gel electrophoresis was performed as described (Hoffman-Falk *et al.*, 1982). Non-denaturing 4–10% polyacrylamide gel electrophoresis was similarly carried out but without SDS. Fluorography was performed according to Bonner and Laskey (1974). Preparation of plant RNA, fractionation in agarose gels and subsequent probing with nick-translated probes were performed as described by Fluhr *et al.* (1983).

Measurement of photosynthetic parameters

Fixation of CO_2 was measured by exposing 200 mg of intact leaves under 120 $\mu mol/m^2/s$ cool-white light to 35 nCi of $NaH[^{14}C]O_3$ (20.6 mCi/mmol) per liter air for 15 min. Leaves were soaked overnight in 5 ml of *N,N'*-dimethylformamide to remove pigments and the extracts were counted in PPO-toluene (Lewinsohn and Gressel, 1983).

Thylakoids were isolated as described (Avron, 1960) in a buffer containing 200 mM sucrose, 100 mM NaCl, 50 mM Tricine pH 7.8 and 1 mg/ml bovine serum albumin (BSA) and washed once in the same media containing 0.2 mg/ml BSA. Reactions were in 2 ml of 30 mM KCl, 30 mM Na Tricine pH 8.0, 5 mM $MgCl_2$, 5 mM NaN_3 and thylakoids equivalent to 25 μg of chlorophyll. Additions were 1 mM ADP, 5 mM phosphate, 5 mM ascorbate, 0.02 mM atrazine, 0.1 mM dichlorophenol indolephenol or 0.1 mM methyl viologen. Oxygen uptake was measured with a Clark oxygen electrode (Yellow Spring Instrument) using a $CuSO_4$ filter at saturating light.

Chloroplast DNA

Chloroplast DNA was isolated and fragmented according to Fluhr and Edelman (1981). Restriction enzymes were from New England Biolabs.

Cloning of rbcL gene and sequencing

An 8.8 kbp *Xho*I fragment (X8; Fluhr *et al.*, 1983) containing the entire *rbc*L gene was cloned from *N.tabacum* var. *Xanthi* and mutant line XV1 into the *Sal*I site of a pUC19 vector. A 3.3 kbp *Hin*dIII–*Eco*RI fragment containing the entire *rbc*L gene was subcloned into M13mp19 phage. Sequencing was performed by the dideoxy chain termination method (Sanger *et al.*, 1977) using [^{35}S[dATP and 17mer oligonucleotide primers specifically complementary to the *N.tabacum rbc*L gene.

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