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Variability in Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Small Subunits and Carboxylation Activity in Fern Gametophytes Grown under Different Light Spectra [1]

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ABSTRACT

Two distinct ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) small subunit (SSU) populations were observed in *Pteris vittata* gametophytes grown under different illumination conditions. Exposure of the fern gametophytes to continuous red light (R) resulted in Rubisco SSUs that were not recognized by polyclonal antibodies raised against SSUs from spinach. Unlike the R-induced SSUs, blue light (B) induced SSUs were well recognized. This difference in SSU composition also reflected in Rubisco activity. *In vitro*, B-induced Rubisco exhibits a significantly higher carboxylation activity as compared to the R-induced Rubisco. Approximately a two- to threefold increase in the V_{max} value of the B-induced carboxylase as compared to the R-induced one was measured. It thus seems very likely that certain domains in the SSU molecule affect enzyme activity.

Rubisco[2] (EC 4.1.1.39) is an abundant enzyme in all photosynthetic organisms. It catalyses both photosynthetic CO_2 fixation via the carboxylation of RuBP in the Calvin cycle and oxygenation of the same substrate in photorespiration (3). In C_3 plants, which lack a CO_2 concentrating system, photosynthesis does not reach maximal efficiency due to the relatively low ratio of CO_2/O_2 in the atmosphere. Possible modification of the holoenzyme structure, leading to a preferential increase in carboxylation activity, could thus increase plant growth.

The Rubisco holoenzyme is composed of eight chloroplast-encoded LSUs and eight nuclear-encoded SSUs. The *rbc*L, encoding the LSU, is present as a single copy per chloroplast genome. Due to reiteration of genomes per chloroplast and chloroplasts per cell, there are thousands of copies of *rbc*L in every leaf cell (18). These genes are posttranslationally photoregulated and very conserved throughout evolution (8, 16). Only small differences have been found in the amino acid sequence of the LSUs from different plants (3).

In contrast to the LSUs, the Rubisco SSUs are encoded by a nuclear gene-family, whose members (*rbc*Ss) are usually all expressed (7, 28). However, individual gene members can show maximal expression in different organs and at different developmental stages (7, 14). Although there is divergence in the nucleotide sequence of *rbc*S coding regions, the amino acid composition of the various mature subunits is very conserved and only slight differences amongst the mature proteins have been identified (7). Transcription of *rbc*S is light-regulated through phytochrome and a blue-light photoreceptor, which act in concert (12, 14, 25). This regulation has been shown to be linked to light-responsive elements located upstream to the consensus sequences of the *rbc*S promoter (12).

Plant Rubisco requires the presence of both the LSUs and the SSUs for catalysis (1). The activation site of the enzyme by CO_2 and Mg^{2+}, as well as the RuBP binding site, are located on the LSU, which also determines the degree of partitioning between carboxylation and oxygenation (2). There is relatively little known about the SSU role. However, based on crystallography studies of L_2 and L_8S_8 Rubisco, Schneider *et al.* (24) recently suggested that the SSU modulates substrate binding to the LSU by introducing conformational changes in LSU active site.

The present work further supports the idea that SSUs are functionally involved in governing carboxylation activity of Rubisco. Using a unique system of fern gametophytes grown under blue or red illumination it was possible to show the occurrence of two distinct light-induced SSU populations. The diversity in SSUs was found to be correlated with different catalytic properties of Rubisco as a carboxylase.

MATERIALS AND METHODS

Materials

[^{14}C]$NaHCO_3$ (50 mCi/mmol) and [^{125}I] donkey anti-rabbit IgG were purchased from the Radiochemical Center, Amersham. Spinach RuBP carboxylase and mol wt markers were purchased from Sigma. All chemicals were of analytical grade.

Germination and Illumination of Ferns

Fresh spores of *Pteris vittata* L. were collected in the Botanical Gardens of Tel-Aviv University, sterilized, and grown axenically as described previously (30). Following 48 h imbi-

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[2] Abbreviations: Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose-1,5-bisphosphate; LSU, large subunit; SSU, small subunit; R, red light; B, blue light; W, white light; *rbc*L and *rbc*S, genes encoding LSUs and SSUs respectively; $^{A}CO_2$, activator CO_2.

bition in the dark, synchronized germination was induced by exposing the cultures for 2 h to red light. The spores were kept for an additional 48 h in darkness and then transferred to continuous R (600–660 nm), B (400–550 nm), or W as described before (30). All cultures were illuminated at an equal energy of 1 W m^{-2}.

Anti-LSU and Anti-SSU Antibodies

Preparation of antibodies was carried out essentially according to Zemel and Gepstein (29). Spinach Rubisco subunits were separated on SDS-PAGE gels and the LSU and SSU bands were eluted. Rabbit anti-LSU and anti-SSU polyclonal antibodies were prepared according to Nelson (23).

Preparation of Gametophyte Extracts

Axenically grown fern gametophytes were collected, frozen and ground in liquid N_2, and then ground and homogenized in a freshly prepared buffer containing 200 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, 10 mM $MgCl_2$, 10 mM ascorbate, 2% PVP (M_r 360,000), and 5 mM DTT at 4°C. Since Rubisco is a stromal enzyme, its relative concentration in gametophyte extracts could be increased by using an extraction buffer devoid of detergent, thus avoiding the apparent destruction of thylacoid membranes. Following centrifugation at 12,000*g* for 1 min the supernatant ("Rubisco enriched" extract) was collected and used for the different measurements. Extraction of total proteins was done as above except that the homogenizing buffer contained 60 mM Tris-HCl (pH 6.8), 3% SDS, 10% glycerol and 0.7 M β-mercaptoethanol.

Evaluation of Rubisco Content in Extracts

Total protein content as determined according to Marder *et al.* (21) was found to vary between 2 and 7 mg protein/mL. Evaluation of Rubisco amounts in each extract was performed by dot blotting of increasing amounts of Rubisco extracts (up to 3 μg) and then probing first with rabbit anti-LSU and then with ^{125}I-donkey anti-rabbit IgG. Following overnight exposure to RP-2 (Agfa) film, the dots were excised from the nitrocellulose sheet and counted. Purified spinach Rubisco protein was used as standard. Linear correlation between Rubisco concentration and ^{125}I-counting enabled accurate estimation of Rubisco content in the enzyme assays.

Separation of Proteins by Polyacrylamide Gels

The total protein pattern was analyzed on SDS-polyacrylamide gradient gels (5–15% or 10–20%) (4). Rubisco holoenzyme was first separated on 5 to 25% nondenaturing polyacrylamide gels. The holoenzyme band was eluted in 25 mM Tris, 0.2 M glycine, 12.5% glycerol, and 2 mM PMSF, at 4°C overnight, and then electrophoresed on SDS-PAGE. Purified spinach Rubisco and/or proteins of known mol wt were included as markers.

Rubisco Carboxylation Activity

Rubisco carboxylase activity was assayed in closed vials, at pH 8.0 and 25°C, by measuring RuBP-dependent incorporation of $^{14}CO_2$ into acid-stable products. The total assay volume was 0.55 mL and contained 0.3 mL assay buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 10 mM $MgCl_2$, and 5 mM DTT), 0.1 mL activated enzyme extract and various amounts of [^{14}C]$NaHCO_3$ or RuBP to give final concentrations as indicated in the legends of Figures 6 and 7. Full Rubisco activation was achieved by preincubating the enzyme extract at 4°C for 10 min with 10 mM Mg^{2+} (present in the extraction buffer) and 10 mM $NaHCO_3$. Catalysis was initiated by addition of the activated enzyme to the reaction mixture. The reaction was terminated after 0.5 min of incubation by the addition of 0.1 mL 6 N HCl. Samples were dried for 2 h at 60°C under an air stream, and then counted for radioactivity in Hydroluma. Exact [CO_2] of the assay solutions were calculated, given the total [$NaHCO_3$] injected (including that added with reactants and buffers as well as CO_2 present in the gas phase before closing the vials, altogether 0.46 μmol), as the distribution of various carbon forms in the assay mixture at the given pH (=0.7% CO_2), temperature and ionic strength, and taking into account the distribution of CO_2 between the aqueous and gas phase of the closed vial (the molar fractionation ratio being 0.88). Preexperiments had shown that CO_2 equilibria with the gas phase were completed well within the activation period.

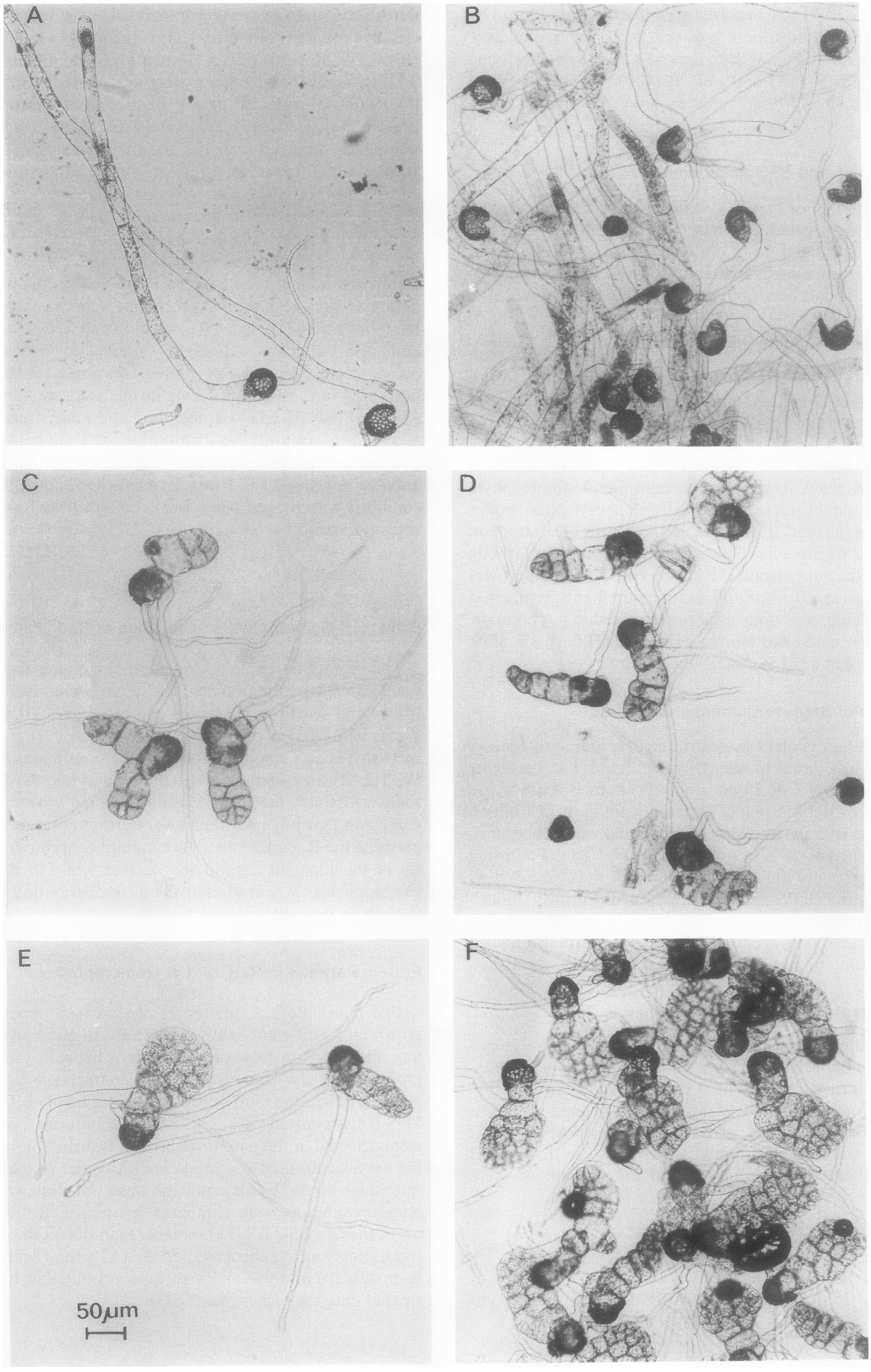
RESULTS

Growth of Gametophytes under Blue or Red Illumination

The free-living haploid gametophytes of the fern *Pteris vittata* are easy to manipulate and their growth can be controlled and synchronized by red and blue light (30). Figure 1 shows the different pattern of development of *P. vittata* gametophytes as a result of growth under continuous R, B, or W. The R gametophytes are filamentous while the B gametophytes exhibit the usual two-dimensional growth pattern similar to that observed under W. Furthermore, the chloroplasts of the R gametophytes are mainly concentrated at the tip of the filament around the nucleus while in B and W gametophytes there is a relatively even distribution of chloroplasts in the cells.

Protein Patterns in R, B, and W Gametophytes

The morphological differences observed in gametophytes grown under different light spectra led us to question whether any alterations in total protein pattern between these three groups could also be detected. Crude extracts prepared from each illumination group were electrophoresed on a 10 to 20% SDS-polyacrylamide gel (Fig. 2). No significant differences were detected in the pattern of protein distribution in any of the extracts. Similarly, no apparent difference in the relative intensities of the bands could be observed. But when fern Rubisco subunits were compared to spinach Rubisco subunits, the fern SSU is 1.3 kD smaller than that of the spinach. Interestingly, in cyanobacterial SSUs a 12 amino acid portion is missing (3) and this accounts for a peptide that is 1.4 kD smaller than the higher plant SSUs.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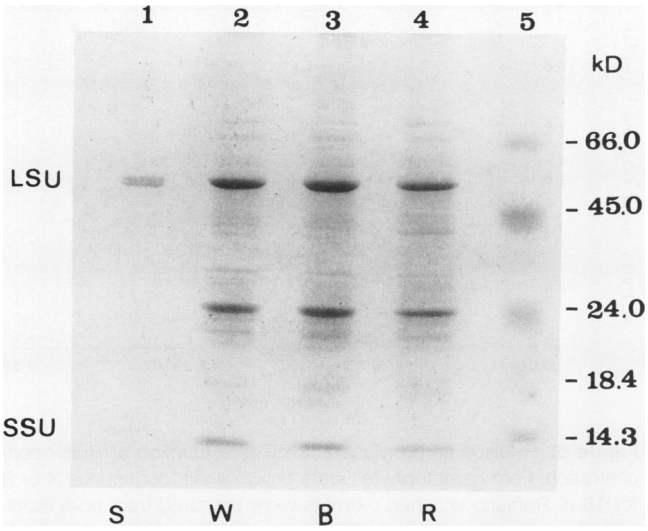

Figure 2. Total protein pattern of extracts prepared from W- (lane 2), B- (lane 3), and R-gametophytes (lane 4). Total proteins extracted from 19 d old gametophytes in the presence of SDS were separated on a 10 to 20% SDS-PAGE gel. Each lane (2–4) contains 51 μg protein. Purified spinach (S) Rubisco (12 μg) was used as a marker to identify the fern LSUs and SSUs (lane 1).

Identification of Rubisco Holoenzyme and Subunits in Fern Gametophytes

To specifically identify Rubisco LSUs and SSUs in the R-, B-, and W-gametophytes, equal amounts of protein extracts were separated on SDS-PAGE gels. Thereafter the gels were blotted onto nitrocellulose filters and probed with polyclonal antibodies raised in rabbit against either SDS-treated spinach LSU or SSU. Figures 3 and 4 show typical immunoblots using anti-LSU or anti-SSU, respectively, as probes. LSUs in extracts from each light treatment are equally recognized by anti-LSU sera (Fig. 3). However, R-induced SSUs are not recognized by antibodies raised against SSU from spinach, while B- and W-induced SSUs are well recognized (Fig. 4). Application of a double amount of protein extract did not improve detection of R-induced SSUs (10). Because R and B LSUs are equally well recognized by spinach anti-LSU sera, the addition of a small amount of anti-LSU, as marker, to the anti-SSU served to verify that equal amounts of Rubisco, from each treatment, were indeed loaded on gels. The surprising lack of reactivity of the anti-SSU antibodies with the SSUs of the R-gametophytes raised the possibility that perhaps the SSUs were entirely missing in the R-induced gametophytes. Therefore, the three types of protein extracts, isolated under nondenaturing conditions, were electrophoresed on native PAGE gels (Fig. 5, panel A). No difference could be detected in the migration pattern of the holoenzymes in the R, B, or W extracts. Furthermore, when the Rubisco holoenzyme bands were excised and eluted from the gel and further separated on SDS-PAGE gels, the SSUs could be visualized clearly and evenly in all light treatments (Fig. 5, panel B). Thus, our results suggest that, in contrast to the homogenous appearance of LSUs in every light treatment, the SSUs of Rubisco from the different photoinduced types of gametophytes (R and B) probably represent two different types of antigens. Since white spectrum consists of both blue and red lights, the SSU population of the W treatment appears to be a mixture of both SSU types and its antigenic response reflects the blue induced SSUs.

Comparison of Rubisco Carboxylase Activity in Extracts of R- and B-Gametophytes

The presence of SSUs have been shown to be essential for proper catalysis. However, their exact function remains questionable. Thus, the natural occurrence of two distinct SSU populations in gametophytic cells while LSUs are identical enabled us to examine the possible effect of different SSUs on carboxylation activity of naturally produced holoenzymes.

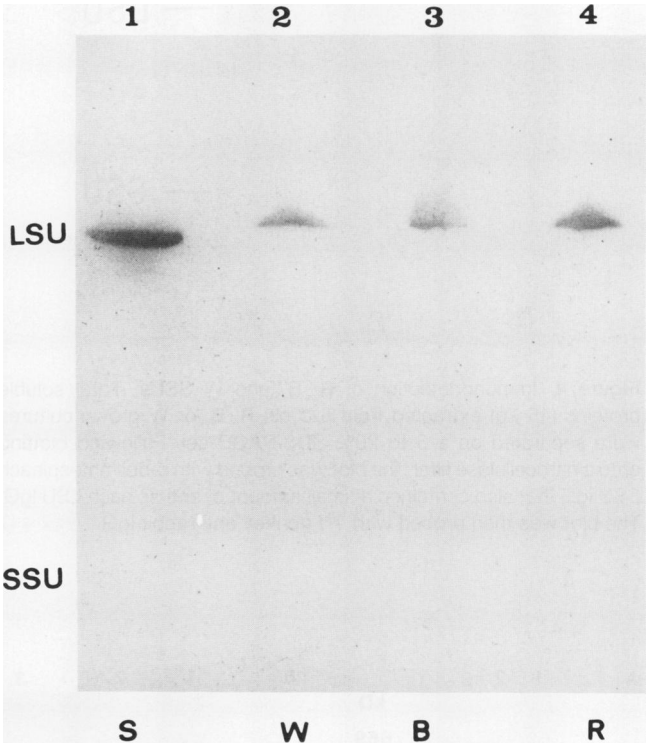

Figure 3. Immunodetection of R, B, and W LSUs. Rubisco enriched extracts (50 μg) from 6 d old R, B, and W grown cultures were separated on a 10 to 20% SDS-PAGE gel similarly to that in Figure 3. Following blotting onto a nitrocellulose filter, the blot was probed with polyclonal antibodies raised in rabbit against SDS-treated spinach LSU and then with ^{125}I-donkey anti-rabbit IgG. Purified spinach (S) Rubisco (10 μg) was used as a marker for the LSU of the enzyme.

Figure 1. Morphology of fern gametophytes grown under different light spectra. Fern gametophytes were germinated and synchronized as described in "Materials and Methods." After additional 48 h in dark, the gametophytes were exposed to continuous R (panel A and B), B (panel C and D), or W (panel E and F) light for 18 d.

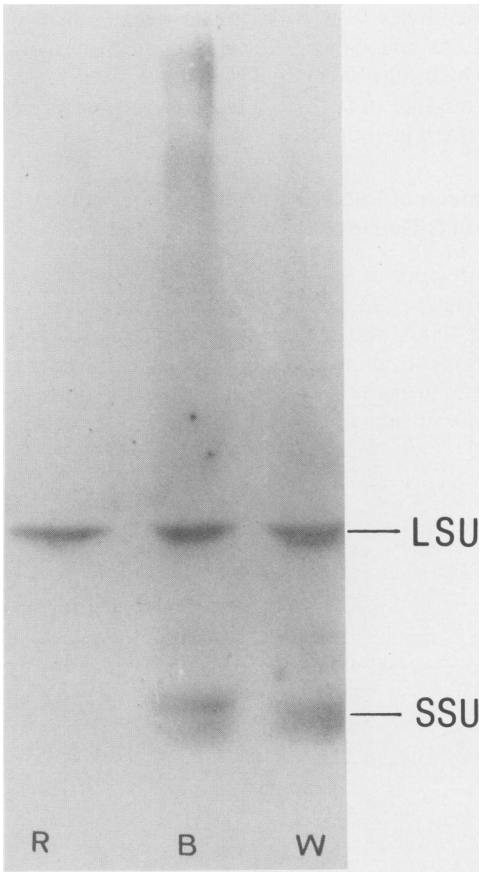

Figure 4. Immunodetection of R, B, and W SSUs. Total soluble proteins (45 μg) extracted from 6 d old R, B, or W grown cultures were separated on a 5 to 20% SDS-PAGE gel. Following blotting onto a nitrocellulose filter, the blot was probed with rabbit anti-spinach SSU IgG that also contained a small amount of anti-spinach LSU IgG. The blot was then probed with ^{125}I-donkey anti-rabbit IgG.

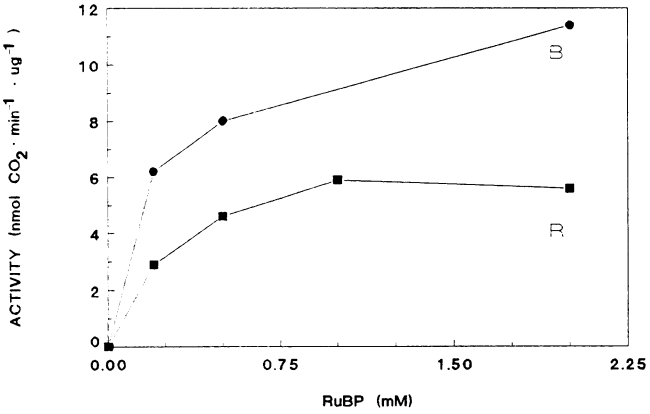

Figure 6. Rubisco carboxylase activity as a function of RuBP concentration. Fern gametophytes were grown under continuous R or B for 18 d. Rubisco enriched extracts were prepared from both illumination groups as described in "Materials and Methods." Carboxylation activity was assayed using 27 μg R and 39 μg B Rubisco protein in the presence of 18.2 mM $NaHCO_3$ (22.5 μM CO_2). Data are average of three replicates; coefficient of variation was less than 15%.

We chose to compare the activity in the R- and B-gametophytes only, since these represent homogenous populations of SSUs. Rubisco enriched extracts were prepared from both R- and B-gametophytes. The maximal rate of carboxylation of Rubisco was determined in the presence of increasing concentrations of either of the two substrates (RuBP or CO_2). Figure 6 depicts carboxylation activity as a function of increasing concentrations of RuBP. It shows clearly that the carboxylation activity of B-gametophytes is significantly higher than that of R-gametophytes. Similarly, when Rubisco carboxylation activity was determined in the presence of increasing concentrations of $NaHCO_3$ (CO_2) (Fig. 7), again an approximately threefold higher maximal rate of carboxylation is observed in the B-gametophyte extract. Evidently, a certain correlation exists between differences in SSU primary structure as revealed by its antigenicity and the holoenzyme carboxylation activity.

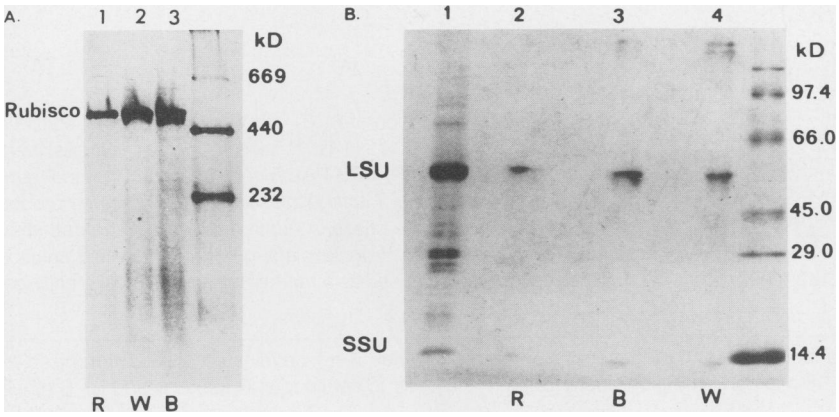

Figure 5. Characterization of Rubisco holoenzyme and subunits in fern gametophytes. A, Rubisco enriched extracts (18 μg) from 21 d old R, B, and W grown cultures were loaded onto 5 to 25% nondenaturating acrylamide gel and electrophoresed to equilibrium parallely to sizemarkers. Following electrophoresis, the gel was stained with Coomassie brilliant blue. B, Separation on 10 to 20% SDS-PAGE gel of Rubisco bands excised from the nondenaturating gel (A) and eluted overnight in SDS-PAGE loading buffer. After electrophoresis, the gel was stained as above. The left lane represents the pattern of 60 μg total proteins extracted from R-gametophytes in the presence of SDS.

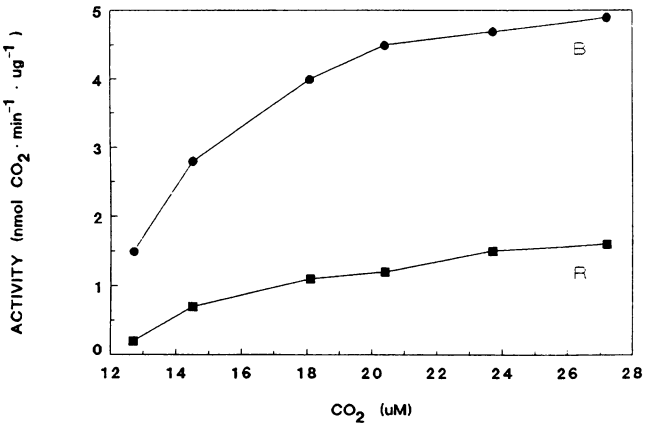

Figure 7. Rubisco carboxylase activity as a function of CO_2 concentration in the assay. Fern gametophytes were grown in continuous R or B for 24 d. Rubisco enriched extracts were prepared from both illumination groups as described in "Materials and Methods." Carboxylation activity was assayed using 25 μg R and 46 μg B Rubisco protein in the presence of 2 mM RuBP. Data are average of three replicates; coefficient of variation was less than 13%.

DISCUSSION

Growth of fern gametophytes under continuous B or R results in the appearance of two distinct types of SSUs which can be distinguished by their antigenicity. Although the involvement of R and B in the regulation of transcription of different *rbc*Ss has been reported (14), we know of no reports indicating the presence of significantly different translation products in the same species (22). The natural occurrence of two SSU populations in the same plant grown under different illumination conditions enabled us to study the possible correlation between specific SSU type and catalytic properties of Rubisco. Indeed, it was found that Rubisco carboxylase activity *in vitro* is significantly higher in extracts from fern gametophytes grown under B as compared to R. The enhanced activity could not be accounted for by differential A_{CO_2} * Mg^{2+} activation kinetics of the enzyme from both sources, since both types of carboxylases were found to be fully activated after 10 min preincubation with HCO_3^- and Mg^{2+} (data not shown). In addition, the influence of a possible R induced inhibitor, analogous to the nocturnal one (17), was ruled out by measuring carboxylation activity of enzyme extracted from R grown gametophytes that were transferred to W 24 h prior to the experiment. No increase in carboxylation activity could be detected in these extracts (data not shown). It seems, therefore, that the enhanced Rubisco activity in B-gametophytes compared to R-gametophytes is dictated by the enzyme molecule. The presence of two distinct SSU populations in the R- and B-gametophytes suggests that a correlation exists between the SSU primary structure revealed by its antigenicity and the holoenzyme activity.

The function of SSU has been a subject of dispute over the past years. It is well agreed that the carbamate formation site (active site) as well as substrate binding sites of the enzyme are located on the LSU (3). However, recent work by Schneider *et al.* (24) suggests that the SSU can modulate substrate binding by inducing conformational changes of the active site. The degree of partitioning between carboxylation and oxygenation (τ value) of Rubisco from higher plants as compared to that of less evolved photosynthetic organisms (τ = 80 for higher plants, τ = 60 for algae, and τ = 50 for cyanobacteria) on one hand and the conservation of LSU amino acid sequences throughout evolution on the other hand suggest that differences in SSUs may play an important role in increasing Rubisco net carboxylation activity (3, 19). Nevertheless, no change in the τ value was revealed by Andrews and Lorimer (2) when comparing catalytic properties of a hybrid between cyanobacterial LSUs and higher plant SSUs of Rubisco with those of a homologously reassembled cyanobacterial enzyme. Similar studies using higher plant LSUs have not been performed yet due to the irreversible denaturation of LSUs in the absence of SSUs and the need for a chaperon for a correct assembly of the LSU octamer (11, 15, 26). Using a different approach, the contribution of SSUs to Rubisco activity has also been studied in *Anacystis nidulans* by Voordouw *et al.* (27). They performed site-directed mutagenesis of two Trp residues in one of the conserved regions of cyanobacterial *rbc*S. The mutated enzyme had a similar τ value and therefore in this respect it confirms the results of Andrews and Lorimer (2). However, whereas the K_m (CO_2) was unchanged, the V_{max} (CO_2) was significantly lower for the mutated enzyme as compared to the wild-type one, demonstrating that single amino acid replacements in the so called "noncatalytic" SSU influence the catalytic rate of the enzyme. These results suggest an activating rather than regulating role for the SSU.

The fern gametophytes are a unique system for the study of the contribution of SSUs to Rubisco activity. This system enables us to examine naturally modified sources of *rbc*Ss by using the special photoinduced occurrence of two distinct SSU populations. Although we have not determined the τ values of the R- and B-gametophytes, our results clearly suggest a functional role for the SSUs. Hence, comparison of SSU amino acid sequences in R- and B-gametophytes might indicate specific amino acid differences affecting carboxylation activity. A computer prediction (9) of the antigenic index of tobacco SSU shows that the evolutionary conserved region, composed of amino acids 40 to 70 in the mature polypeptide, is expected to be the most antigenic. Interestingly, the point mutations in *Anacystis rbc*S affecting carboxylation activity, introduced by Voordouw *et al.* (27), are also located in this region. Thus, it is plausible that the differential immunorecognition observed in the fern gametophyte SSUs could be due to changes in amino acid sequences in this particular region.

In contrast to the *rbc*L, transcription of *rbc*Ss is known to be light-regulated, both through the phytochrome system and via blue light receptor (12, 13). Quantitative regulation of *rbc*S has been attributed mainly to sequences 5′ and/or 3′ to the coding region (5, 6, 20). Differences between expression of *rbc*S genes do exist between different organs and at various developmental stages (7). Thus, it is possible that under various light spectra different *rbc*Ss from the multigene family are expressed. Currently the fern *rbc*S gene-family is being studied in order to point out possible differences in *rbc*S exons that could affect carboxylase activity.

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