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Acclimation of Ribulose Bisphosphate Carboxylase and mRNAs to Changing Irradiance in Adult Tobacco Leaves

DIFFERENTIAL EXPRESSION IN LSU AND SSU mRNA

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ABSTRACT

The transfer of *Nicotiana tabacum* plants grown in low light (60 micromoles quanta per square meter per second) to higher light (360 micromoles quanta per square meter per second) was previously shown to induce adaptive stimulation of photosynthetic capacities. The variations of ribulose bisphosphate carboxylase/oxygenase (RubisCo) expression in mature leaves was examined as a result of this acclimation. Maximum or initial activities increased markedly after low- to high-light transfer with a maximum effect after 2 to 3 days. The higher activity is mainly explained by RubisCo protein synthesis as shown by immuno-rocket technique. Small subunits of RubisCo (SSU) mRNA relative content determined by hybridization of total RNA with DNA probe by Dot-blot method, followed the same pattern as RubisCo quantity. The magnitude of this response was amplified when more contrasting light conditions (25 *versus* 360 micromoles per square meter per second) were established on the same leaf: RubisCo activity, RubisCo protein, and SSU mRNA contents decreased in the shaded zone and increased in the high-light zone within 1 day. After 2 days the shade/light ratio was 1 to 3 for RubisCo protein and 1 to 4 for SSU-RNA, whereas the ratios remained equal to one in controls. Hybridization of the same RNA extracts with large subunits of RubisCo (LSU) probe showed no variation in LSU-RNA content. So in green adult leaves, the expression of SSU and LSU genes is regulated differently. The observed white light quantitative effect on RubisCo expression was not dependent on the photosynthetic rate or assimilate content since low CO_2 concentration around the leaf after the light shift did not modify the response.

Leaf photosynthesis properties are dependent upon incident white light received during growth. In most plants grown under constant conditions, this acclimation tends to adapt the maximum photosynthetic rate to available light through a series of interrelated modifications from the molecular level to whole leaf gas exchanges: electron transport chains, chloroplast ultrastructure, RubisCo[1] activity, leaf structure, and stomatal diffusion (4, 13). Each adapted state is not fixed and any change in irradiance induces within a few days, biochemical and structural rearrangements tending towards the typical phenotype of the new condition. In such light changes, growing tissue appears more flexible (14, 15, 29) than mature or adult ones (5, 9, 21). RubisCo, a main enzymatic step in CO_2 fixation, is a good index for these adaptive responses: the maximum *in vitro* activity responds in parallel to the light saturated photosynthetic rate. In a preliminary study, we have checked that *Nicotiana tabacum* plant grown under low light and transferred in high light behaved similarly to most other plants by increasing the maximum photosynthetic rate and RubisCo activity. Furthermore, RubisCo quantity as measured by immunoprecipitation with specific antibodies raised against the enzyme was shown to increase (18).

Most experiments on light-regulation of RubisCo expression were performed in etiolated plants submitted to dark-light transition. The large stimulation in synthesis of all the proteins of the photoautotrophic pathway resulted from a combination of transcriptional, translational, and post-translational regulations (26, 27). In the case of the nuclear-encoded SSU the light-dependent expression of the gene was shown to be transcriptionally regulated (7) and phytochrome mediated (25). More recently the light inducible sequence has been identified in the 5′region upstream from the coding sequence (11). The chloroplast encoded LSU is regulated differently since LSU-mRNA level is controlled by gene dosage or chloroplast DNA copy number (10, 20) and the LSU-protein expression is posttranslationally regulated (2, 3).

In the present study, we analysed the expression of RubisCo quantity and activity in tobacco leaves adapted to low-light conditions and upon transfer to high light. Total or initial enzyme activities, protein amount and SSU mRNA increased within 1 or 2 d after light shift. The magnitude of the response was amplified by creating contrasting light conditions in the same leaf. Each leaf part was shown to react independently to the incident light it received. The light response was not influenced by the actual photosynthetic rate. LSU mRNA quantity was constant whatever the conditions whereas total RubisCo protein varied in close agreement with SSU mRNA as a function of prevailing light conditions.

MATERIALS AND METHODS

Plant Conditions. *Nicotiana tabacum* (cv Xanthi) were grown from seeds in a growth chamber under low light (60 μmol quanta m^{-2} s^{-1}, 16 h/d, 24°C d, 18°C night, RH 70%) for 90 to 95 d. Plants, in individual pots, were transferred to higher light (360 μmol quanta m^{-2} s^{-1}) and the adult leaves at the time of transfer were harvested sequentially from 0 to 4 d after light change. Leaf discs 0.5 cm^2 were punched and used for RubisCo measurements, the rest of the leaf was stored at −80°C until RNA extraction. In a second series of experiments one adult leaf in each transferred plant was partially shaded by a paper mask covering one-half of the leaf. This mask created lower light condition (25 μmol quanta m^{-2} s^{-1}) in one leaf half whereas the rest of the plant received

[1] Abbreviations: RubisCo, ribulose bisphosphate carboxylase/oxygenase; SSU and LSU, small and large subunits of RubisCo.

360 μmol quanta m^{-2} s^{-1}. Each half of the leaf was sampled separately. In a third experiment the leaf with the paper mask was enclosed in perspex chamber circulated with compressed air at very low flow rate (about 10 L h^{-1}). Net CO_2 uptake was controlled with an IRGA (ADC MK3) and flow rate was adjusted in order to keep photosynthetic rate at the value observed at 60 μmol quanta m^{-2} s^{-1} in normal air. Air flow for control leaves was sufficient (100 L h^{-1}) to ensure normal high-light photosynthesis. Sampling and light changes were always performed at the same time of day, between 10 and 11 AM, which is 3 to 4 h after the beginning of photoperiod (7 AM).

RubisCo Quantity and Activity. RubisCo was extracted from leaf discs (0.5 cm^2) by grinding in Eppendorf tubes with 100 μl extraction buffer (100 mM Tris HCl [pH 7.9], 20 mM KCl, 2.5 mM EDTA, 2.5 mM phenylmethyl sulfonylfluoride, insoluble polyvinyl-pyrrolidone 5% w:v). The slurry was centrifuged 3 min at 12,000 rpm in a microcentrifuge at 4°C. Initial activity was measured immediately by adding 10 μl extract to 50 μl reaction buffer (40 mM Tris HCl, 40 mM $MgCl_2$, 4 mM DTT, 0.5 mM RuBP, 15 mM Na $H^{14}CO_3$) and total activity by incubating the extract for 10 min in the reaction buffer prior to addition of RuBP. RubisCo protein was determined by the immunorocket technique (1% agarose gel in Tris HCl 3 gL^{-1} and glycine 14 gL^{-1}; run overnight at 4°C, 80 V, 5 mamp) using specific rabbit antibodies raised against the tobacco holoenzyme. Serially diluted purified enzyme from tobacco was used as standard for absolute determination. The extracts used for activity measurements were diluted one-tenth and dispensed in the wells (4 μl).

RNA Isolation and Hybridization. Total RNA was extracted from leaves by a modified method from Chirgwin *et al.* (6); tissue (1–5 g) was powered with liquid nitrogen in a mortar and homogenized in 7 ml extraction buffer (4 M guanidium thiocyanate, 0.2 M Tris HCl [pH 8], 0.1 M β-mercaptoethanol, 0.5% w/v lauryl sarcosinate). The extract was centrifuged 30 min at 12,000*g*. The supernatant was layered on 2.5 ml pads (5.7 M CsCl, 10 mM Na_2 EDTA, 10 mM Tris HCl [pH 8]) and centrifuged overnight at 140,000*g*. The opalescent pellets were dissolved in 10 mM Tris HCl (pH 8), 5 mM EDTA, 1% SDS, and reextracted by chloroform/butanol mixture (4:1). The aqueous phase was precipitated twice by cold-ethanol, the pellets were dissolved in 200 to 500 μl H_2O. Total RNA content was measured spectrophotometrically at 260 nm.

Dot Blots. Aliquots of RNA (10 μg) were denatured by heating at 60°C in 7% formaldehyde, SSC (0.15 M sodium chloride, 0.015 M sodium citrate) × 6 and serially diluted in microtiter plates. Each dilution was applied with suction to 4 mm dots on nitrocellulose using a 96-hold manifold apparatus (Schleicher and Schuell). The air-dried filters were baked 2 h at 80°C in a vacuum oven and then prehybridized in hybridization buffer (50% deionized formamide, SSC × 5, 25 mM Hepes (pH 7.0), 0.05% SDS, 0.5 mM EDTA, 100 μg/ml yeast tRNA, 50 μg/ml Herring sperm DNA, 0.05% polyvinyl-pyrrolidone (mol wt 40,000), 0.05% BSA, 0.05% of ficoll (mol wt 400,000) for 6 to 12 h at 42°C. Hybridization buffer containing the nick-translated DNA probe (about 5.10^7 cpm/μg) was introduced after elimination of prehybridization buffer. Incubation lasted 24 to 50 h. Filters were washed 1 h by SSC × 2 at room temperature followed by SSC × 0.1 + 0.1% SDS for 45 min at 55°C and SSC × 0.1 three times at room temperature. The filter, after air drying, was exposed to Kodak XAR-5 x-ray film at −80°C with intensifying screens. Then, each dot was punched and radioactivity corresponding to DNA hybridized to RNA was measured by scintillation counting. SSU probe from tobacco was kindly provided by Dr. J. Fleck (clone psTV34, IBMC Strasbourg). The hybridization was performed either with the transformed plasmid or with the insert (543bp) excised by Cfo1 restriction enzyme. This DNA fragment is located in the 3′ region of SSU gene (28). LSU probe from tobacco chloroplast DNA was cloned by Shinozaki and Sugiura (24) who are kindly acknowledged; clone ptB 1 containing 1.3 kBp of the 3′ coding region was used.

RESULTS

Low to High Light Transfer. Initial and total RuBP carboxylase activity in adult leaves increased progressively during the 3 d following transfer of plants from 60 to 300 μmol quanta m^{-2} s^{-1}. The percentage of activation (initial/total activity ×100) varied from 51% in control to 68% in transferred leaves (Fig. 1A).

The quantity of RuBPCase protein, as measured by immunorockets, followed a similar pattern as the activities, but the magnitude of variation was smaller. The increase in the controls on the first day probably means that the leaves were not fully matured (Fig. 1B). Another possibility is that the rearrangement of the pots in the growth cabinets at the time of transfer slightly modified the light conditions in the low-light controls.

Total RNAs were extracted by Guanidium-CsCl method which gave a good extraction yield, around 200 μg g^{-1} fresh weight. The variations in total extractable RNAs were not significant whatever the experimental conditions. RNAs were not degradated by the extraction as shown by their good translatability *in*

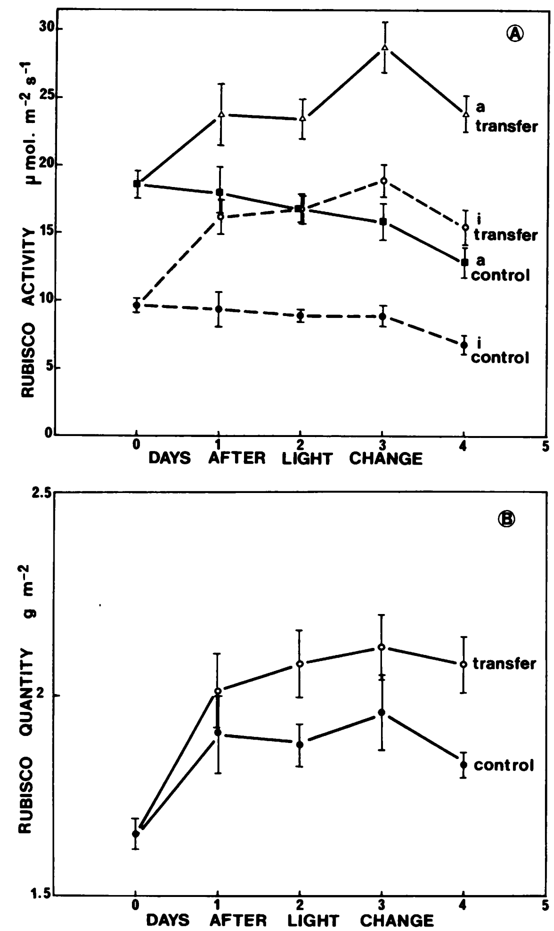

FIG. 1. Effect of changing irradiance during growth from 60 to 360 μmol quanta m^{-2} s^{-1} in *Nicotiana tabacum* plants on (A) initial (i) or fully activated (a) RubisCo activity in adult leaves, (B) RubisCo quantity determined by the immunorocket technique. Controls were maintained under the low light conditions used to grow plants (60 μmol quanta m^{-2} s^{-1}). Means ± SE from four sets of experiments, each experiment with four replicates. Measurements on leaf discs (0.5 cm^{-2}).

vitro and by the fact that the hybridization with SSU-DNA probe (psTV34) to total RNA after gel electrophoresis and transfer to 'Genescreen' (Northern blots) gave one well-defined band corresponding to a mRNA of the correct size (preliminary experiments not shown).

Specific RNAs were quantified by the dot-blot technique: serial one-half dilutions of each sample were applied to nitrocellulose and the radioactivity of each dot, measured by scintillation counting, was plotted against total RNA. The relationship radioactivity/quantity, which was approximately linear, was used to evaluate the relative quantity to a chosen standard (initial control value or final value).

The proportion of SSU-specific mRNA in total RNA rose significantly to a maximum 48 h after the light change (Table I). However, the sample to sample difference was large compared to the effect of treatment; so in order to confirm the influence of incident white light on RubisCo expression we tried both to reduce plant variability and to establish more contrasting light conditions.

Partial Leaf Shading. At the time of transfer from the low-light growth conditions (60 μmol quanta m^{-2} s^{-1}) to higher light the experimental leaves were each shaded with a paper mask covering one-half of the leaf. This mask introduced lower light conditions (25 μmol quanta m^{-2} s^{-1}) in one leaf part whereas the other part was exposed to higher light (360 μmol quanta m^{-2} s^{-1}). The effect of the position of shading in the leaf was examined since we had previously noted that the carbohydrate contents (sucrose, hexoses, and starch) differed depending on whether or not the main vein crossed the shaded zone (18). RubisCo activity and quantity responded, in fact, in the same way if the paper mask was placed at the base of the leaf (so called 'basal shading') or parallel to the main vein on the left or right part of the leaf (so called 'lateral shading'). So, the data from basal and lateral shading were pooled.

One day after light change, RubisCo activity measured immediately after extraction or after activation, increased in the high-light leaf half and decreased in the shaded part as compared to the initial values at day 0. The shade/light difference increased further the second day (Fig. 2A). RubisCo protein quantity varied accordingly but the amplitude of the changes was less than for activities (Fig. 2B).

Total specific activities, expressed on a RubisCo-protein basis, remained constant in the light zone but declined in the shaded zone which means that the light-induced increase in activity was accounted for by the RubisCo-protein synthesis whereas the reduction in the shaded parts was due to both lower protein quantity and lower maximum rate (Fig. 2C). The variation in enzyme activation (55% in shaded zones *versus* 85% in light zones) further amplified the differences in actual rates.

The proportion of SSU mRNA in total RNA in each leaf part also responded very rapidly to the new light conditions: a dramatic decrease in the shaded zone and corresponding increase in the high-light zone (Fig. 3). This response is disymmetrical: at 1 d the increase in the light zone is not significant whereas the decrease is largely significant. This point was confirmed in a specific study of the first 24 h (JL Prioul, A Reyss, unpublished

Table I. *Variation in the Proportion SSU-Specific mRNA in Total RNA from Mature Tobacco Leaves when Increasing Irradiance from 60 to 300 μmol Quanta $m^{-2}s^{-1}$*

Values expressed relative to controls. Means ± SE from five sets of experiments. Total RNA were dot-blotted on nitrocellulose, hybridized with nick-translated SSU-DNA probe and each dot was counted by scintillation. As there were large variations in counting rate from one membrane to another, all the results from one given membrane were expressed relative to the low light control on that membrane.

Low Light Control	Days after Light Change		
	1	2	3
1 (±0.15)	1.43 (±0.26)	1.62* (±0.25)	1.39 (±0.01)

* Significant difference from control (P < 5%).

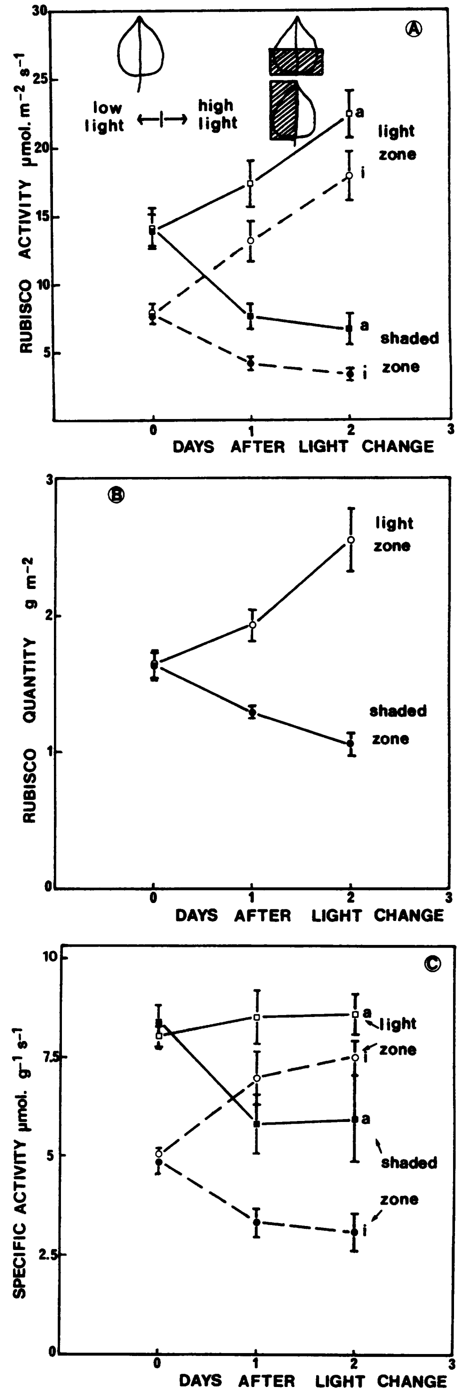

FIG. 2. Effect of contrasting light conditions (25/360 μE m^{-2} s^{-1}) in one adult leaf on (A) RubisCo activity, i: initial activity, a: fully activated; (B) RubisCo protein quantity; (C) Rubisco specific activity. Mean ± SE from five experiments in duplicates ($n = 10$). Measurements were made on leaf discs (0.5 cm^2) punched in the two zones of each experimental leaf for 3 d. Data from basal and lateral shading were pooled. In control, unshaded leaves maintained under low light or transferred in higher light Rubisco activity and quantity remained evenly distributed whatever the leaf zone.

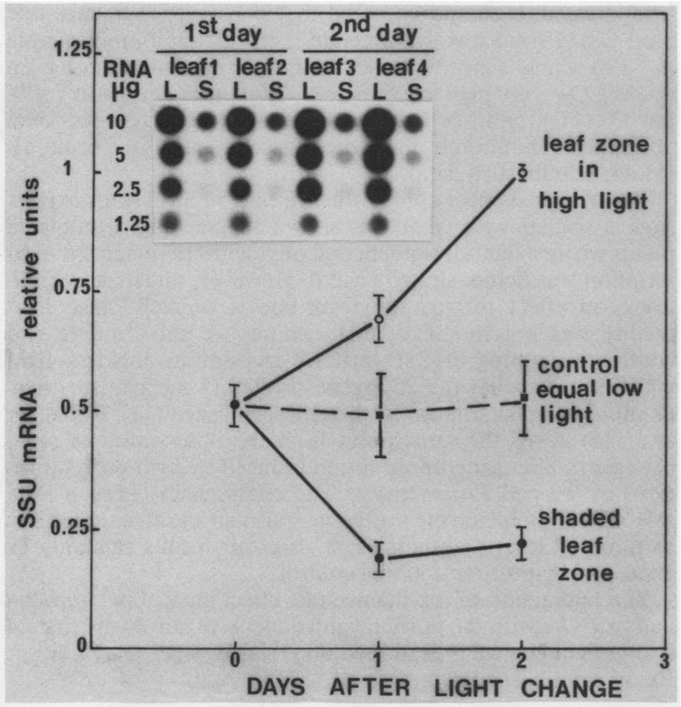

FIG. 3. Effect of contrasting light conditions in one adult leaf on hybridizable quantity of SSU mRNA. Inset, example of autoradiography from serially diluted total RNA hybridized with ^{32}P labeled SSU-DNA, probe; S, shaded zone; L, light zone. Each dot was counted by scintillation. All the data are given relative to the more abundant sample. Mean ± SE; d 0 and 1: 4 repetitions; d 2: 10 repetitions. There were no significant differences between the leaf regions in unshaded low-light control leaves (dashed line).

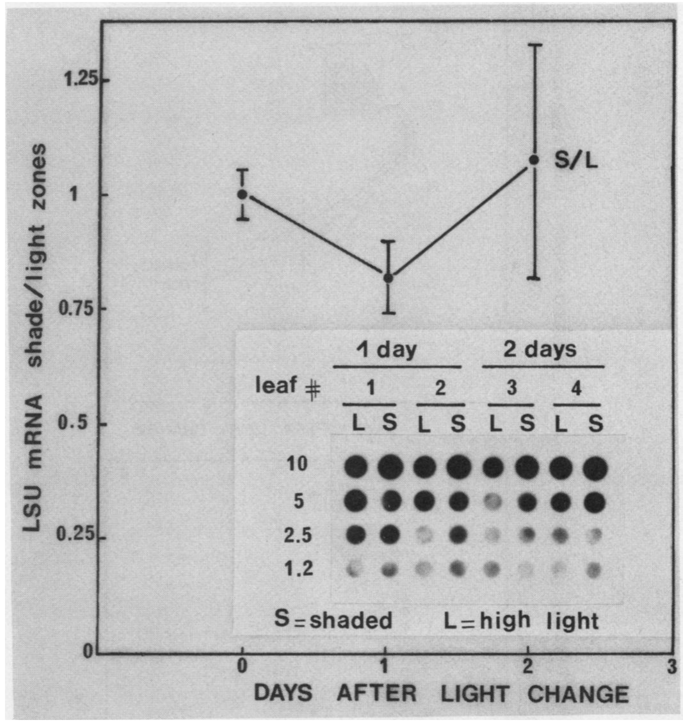

FIG. 4. Effect of contrasting light conditions in one adult leaf on hybridizable quantity of LSU mRNA as expressed by the ratio of shade/light zone contents. Same protocol and extracts as in Figure 3. Mean ± SE (7 repetitions). Inset, autoradiography of dot-blots as in Figure 3.

data). By contrast, LSU mRNA measured from the same extracts showed no significant tip/base differences. However, a large sample to sample variability was noted (Fig. 4). Again the position of shading, basal or lateral, did not introduce differences in mRNA expression. So in each case, RubisCo activity, quantity, and SSU mRNA amounts in a given leaf zone responded specifically and quantitatively to the actual light it received. One question which arose was: does the light act directly through specific receptors or indirectly through its effect on photosynthesis and assimilate level? In order to answer this question complementary experiments were done in which the light-induced increase in leaf photosynthetic rate was counteracted by manipulation of ambient CO_2 concentration.

Partial Shading and Low CO_2. The experimental leaf was enclosed in a small chamber circulated with low CO_2 in order to prevent the increase in photosynthetic rate due to high-light transfer. The control leaf was circulated with ambient air at normal CO_2 concentration. Enclosing the leaf in a chamber tended to increase the variability in the response, due probably to local mechanical stress during the procedure. For this reason, the results were expressed as the ratio shaded zone/light zone. Starting from a value = 1 at d 0, the ratio of RubisCo activities decreased markedly during the two following days (Fig. 5A), the magnitude of changes was less for RubisCo protein (Fig. 5B) but those for SSU mRNA was as important as in the previous experiment (Fig. 5C compared to Fig. 3). LSU mRNA content was steady. Whatever the criterion used, there was no effect of low CO_2-low photosynthetic rate on the observed response.

DISCUSSION

In mature tobacco leaves, RubisCo rapidly responds to changes in light fluence rate received during plant growth. The maximum rate on a leaf area basis tends to increase or decrease in parallel with incident light which leads to a better acclimation to available energy. The response is highly localized since each part of a given leaf responds to the actual light received as previously shown in *Lolium* leaves (14–16).

The acclimation proceeds through two levels of regulation, enzyme activation and RubisCo protein synthesis, which differ on a time scale. On a short-term (minutes), factors acting on the difference between actual and maximum rate are encountered. The activation of the enzyme increases or decreases depending upon the light level as first reported by Perchorowicz *et al.* (12) in wheat leaves. However, in our case, the range of variation is not very high (maximum 25%). The newly discovered phosphorylated light-mediated inhibitor (22, 23) or the so-called RubisCo-activase (19) is a likely candidate for such regulations.

On a longer term basis (day), synthesis or degradation of the RubisCo-protein occurs as clearly demonstrated for the first time by immunological quantification. This observation is consistent with the increase in soluble protein (17, *Lolium*) or fraction 1 protein (9, *Solanum*) reported in other species upon increasing the light fluence rate. Further analysis of the variation in RubisCo expression at the mRNA level enables us to demonstrate for the first time that SSU-RNA pools adjust rapidly and dramatically to incident white light in green mature leaves. The response is apparently quantitative since the effect is related to the magnitude of the light difference (*cf.* Table I and Fig. 4). Under the same conditions, total RNA pool was constant. Another interesting point is that LSU-RNA pool was totally unaffected by the light treatment. So, the SSU and LSU genes are regulated differently and it should be noted that the variations in holoprotein quantity, implying synthesis or degradation, are closely related to the level of SSU-RNA. The light-response is apparently not mediated by photosynthetic rate and associated carbohydrate content as shown by our low CO_2 experiment. Recently, Abbott and Bogorad (1) came to a similar conclusion in green tobacco

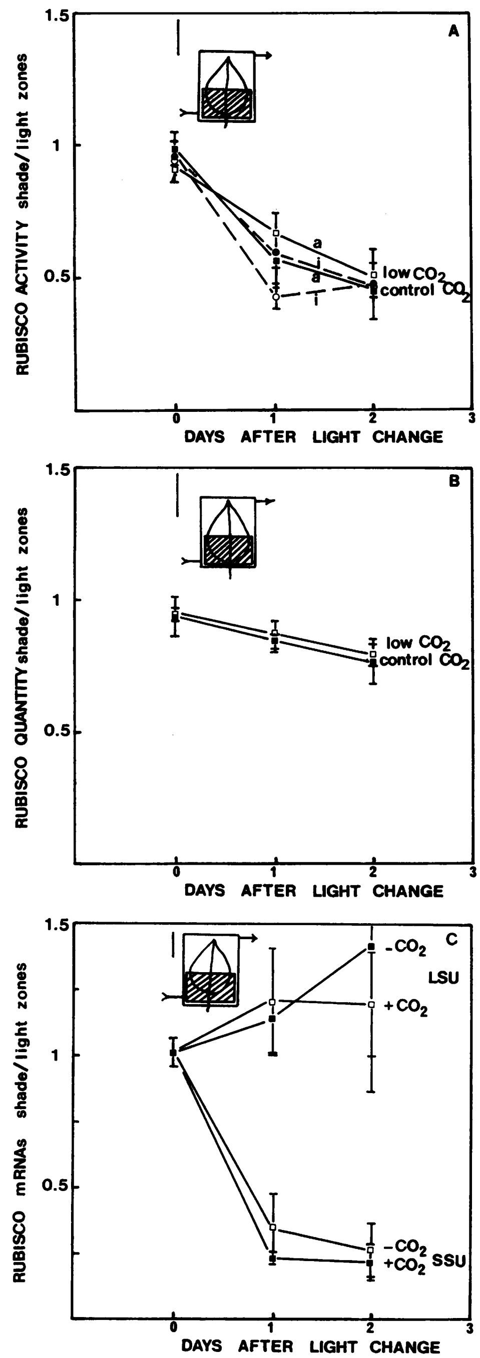

FIG. 5. Effect of CO_2 concentration on the response of the leaf to contrasting light. A, Ratio of the RubisCo activity in shade and light zones; B, ratio of the RubisCo content shade/light zones; C, ratio of LSU-RNA and SSU-RNA shade/light zones. Means ± SE (3 repetitions). The change in CO_2 concentration around the leaf was obtained by enclosing the leaf in a plastic chamber. Air flow rate was controlled, CO_2 concentration and photosynthesis was monitored by an IRGA.

plants grown in test tubes with 1% sucrose: prolonged darkness markedly depressed SSU mRNA while LSU-RNA remained constant.

The question of the photoreceptor of the observed light-quantity effect remains unsolved. If the role of carbon metabolism through assimilate level is discounted, the influence of other light dependent parts of the photosynthetic process such as electron transport chain, amount of ATP, or of reducing equivalents may be addressed. It should be noted that the range of fluence rate used varied from low to moderate levels (25–360 μmol quanta m^{-2} s^{-1}) which is insufficient to obtain any photoinhibition even at low CO_2. Very high irradiances (>2000 μmol quanta m^{-2} s^{-1}), low O_2 (1%), and low CO_2 are necessary to realize a less than 50% photoinhibition in the same tobacco plants (G. Cornic, JL Prioul, unpublished data).

The observed effect of light fluence rate on SSU-RNA expression is similar with what was shown in greening de-etiolated plants where a light-dependent and phytochrome-mediated transcription was demonstrated (7, 25). However, under our conditions, an effect through phytochrome is unlikely since light quality was not modified; furthermore, we show a fine and continuous tuning of SSU mRNA amount by incident light which suggests that the SSU gene regulatory mechanisms may be more complex than those already established (11). Gallagher *et al.* (8) raised the same point from the observation of rapid changes in SSU gene transcription induced by light-dark transitions in greened *Pisum* leaves. The relationship between LSU mRNA and holoenzyme synthesis is also an unsolved question; as those of several authors (2, 3, 10), our results can only be explained by posttranslational control.

The interaction of the fluence-rate effect on RubisCo-protein and mRNAs with the normal light/dark cycle during the first 24 h after light change is examined in a related paper (JL Prioul, A Reyss, unpublished data).

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