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ISOLATION AND CHARACTERIZATION OF A cDNA ENCODING A SMALL SUBUNIT PRECURSOR OF RUBISCO IN *SPINACIA OLERACEA*

BY

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ABSTRACT

Isolation and characterization of a cDNA encoding a small subunit precursor of Rubisco in *Spinacia oleracea*. - The Rubisco enzyme is a key enzyme that allows the fixation of the atmospheric CO_2 and it is the most abundant protein in plants. This chloroplastic holoenzyme is built with eight large subunits produced in the stroma, and eight small subunits synthesized in the cytoplasm and exported into the plasts. Because of its key role in the carbon cycle, its abundance and dual origin, it has been the subject of many studies in crystallography, enzymology and molecular biology. The small subunit has been well studied in regard of its promoter, its genomic organization and its regulation of expression. We report here on the molecular characterization by cDNA cloning of a Rubisco SSU precursor in spinach and present evidences for the photoperiodic control of the SSU transcripts levels in plants grown in a short-day light cycle.

Key-words: Rubisco, small subunit precursor, photoperiodism, messenger, transit peptide, *Spinacia oleracea*.

Abbreviations: RuBP, Ribulose-1,5-bisphosphate; SSU, Small subunit; LSU, Large subunit; SD, Short days conditions; CL, Continuous light; SDS, Sodium dodecyl sulfate; DIG, Digoxygenin.

INTRODUCTION

Almost all life forms on earth depend on the conversion of solar energy to chemical energy by photosynthesis. Most of this energy is stored by the synthesis and the polymerization of sugar molecules from atmospheric CO_2. The initial step in the photosynthetic assimilation of CO_2, the carboxylation of ribulose-1,5-bisphosphate (RuBP), is catalyzed by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). This enzyme is responsible for the annual net fixation of 10^{11} tons of CO_2 from the atmosphere to the biosphere (our annual net consumption of crude oil is about $3*10^9$ tons).

Rubisco is considered to be the most abundant protein on earth: up to 50% of leaf proteins in plant are Rubisco (SCHNEIDER *et al.*, 1992). Despite the unique and key

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biological role played by the enzyme, this abundance rather reflects the catalytic inefficiency of the Rubisco, which is an heritage of a time when the first photosynthetic ancestors appeared under high atmospheric CO_2 and low O_2 concentrations. The enzyme is not only slow, but it also catalyses a competing oxygenase reaction that leads to a loss of energy by photorespiration. The carboxylation of RuBP yields two molecules of phosphoglycerate: RuBP is regenerated in the Calvin cycle and the fixed carbon is incorporated into carbohydrates. The oxidation of RuBP results in the formation of one molecule of phosphoglycerate which can be metabolized in the Calvin cycle, and one molecule of phosphoglycolate which leads not only to a loss of energy (to metabolize this inhibitor product) but also to a loss of atmospheric CO_2. The carboxylation and the oxygenation of RuBP occur at the same catalytic site of the Rubisco, where both gaseous substrates compete for the second substrate (RuBP). The ratio of carboxylation towards oxygenation is influenced by the relative concentrations of CO_2 and O_2, higher CO_2 concentrations resulting in more efficient photosynthesis and faster production of biomass. It is the key point of the evolutive strategy of the C_4 plants which first assimilate the atmospheric CO_2 in the mesophyll cells (in direct contact with the atmosphere and without chloroplast) into four-carbon molecules (e.g. malate) that are pumped by the bundle sheath cells (further in the leaf and with chloroplasts) where CO_2 is released by reduction of malate. This process increases the efficiency of the CO_2 fixation by the Rubisco, because the elevated concentration of CO_2 in the bundle sheath cells is much higher than in the atmosphere and becomes inhibitory of the oxygenase activity.

Many biochemical and genetics studies of the Rubisco have been carried out in the aim to characterized and/or improved the photosynthetic efficiency of the enzyme (for review, SCHNEIDER *et al.*, 1992, PORTIS, 1992), which presents an important interest for agricultural productivity. Rubisco from higher plants (and most photosynthetic microorganisms) is built up of two different types of subunits: this holoenzyme contains eight large subunits (LSU, ~53 kDa) and eight small subunits (SSU, ~14 kDa). This enzyme represents a very interesting tool to investigate the formation of holomeric protein complexes, integrating organelles targeting, precursors maturation and chaperonin implication. The LSU gene is part of the chloroplast genome, and in most cases there is only one gene copy per genome. The gene product is synthesized on plastid ribosomes and stabilized in the stroma in a high molecular weight complex with 12 or 14 nuclear-encoded chaperonins (Rubisco subunit binding protein). The SSU belongs to a nuclear multigene family (rbcS genes) in both dicot and monocot species. The SSU polypeptides are synthesized on cytoplasmic polysomes as higher molecular mass precursors that are post-translationally imported and processed by the chloroplast.

A large number of SSU nucleotide sequences from various species can be found in the databases and comparisons of predicted amino acid sequences revealed a very high intraspecific conservation and important interspecific homologies.

Studies on gene expression in many plants have shown that the expression of the *rbcS* genes is regulated by light and is under the control of phytochrome (TOBIN &

SILVERTHORNE, 1985; SAWBRIDGE *et al.*, 1996). A blue-light response has also been reported (FLUHR & CHUA, 1986; CLUGSTON *et al.*, 1991; SAWBRIDGE *et al.*, 1994). Furthermore, the *rbcS* genes are apparently transcribed in a cell-specific manner, and the level and tissue-specific pattern of expression may vary among the individual gene family members (FLUHR *et al.*, 1986; SUGITA & GRUISSEN, 1987; DEDONDER *et al.*, 1993; DEAN *et al.*, 1995; SAWBRIDGE *et al.*, 1996).

We report here on the molecular characterization by cDNA cloning of a Rubisco SSU precursor in spinach and present evidences for the photoperiodic of the SSU transcripts levels in plants grown in a short-day light cycle.

MATERIAL AND METHODS

Spinach (*Spinacia oleracea* L. cv. Nobel) was cultivated in a short day photoperiod (SD). The light was switched on at 08 h and off at 16 h (local time). Germination and growth took place in phytotrons at a temperature of 20 ± 0.5°C and at a hygrometry of 70 ± 5% during the day and 50 ± 5% during the night. The illumination was provided by neon tubes and reached 6000 lux at the level of the leaves. Experiments were done with 4-week-old plants.

Isolation of rbcS cDNA

A cDNA library was constructed from poly A+ RNAs extracted from 4-week-old spinach leaves, using the phage Lambda UNI-Zap® system from Stratagene. The *rbcS* cDNA was isolated by serendipity as a "false positive" clone while screening for another gene. The nucleotide sequence was determined by the chain termination method (SANGER *et al.*, 1977) with Sequenase (Amersham Pharmacia Biotech). All sequence analyses were performed with the programs of the Wisconsin GCG software package (DEVEREUX *et al.*, 1984).

Northern blot analysis

Total RNA was extracted from green tissues according to a standard procedure (De Vries et al., 1988). For each sample, five µg of total RNA were loaded on a formaldehyde-denaturing agarose (1.4 %) gel and capillary transferred on a nylon membrane N-Hybond (Amersham Pharmacia Biotech). The transferred RNA was stained with methylene blue (SAMBROOK *et al.*, 1989), and the blots were then hybridized with an antisense DIG-labeled RNA probe and revealed by CSPD® according to the manufacturer's recommendations (Boehringer Mannheim/Roche Diagnostics). Signals of Northern blot hybridizations were measured with a photometer-integrator (Vernon, Paris) and related to the levels of stained ribosomal RNA.

RESULTS AND DISCUSSION

The complete nucleotide sequence of the *rbcS2* cDNA isolated from *Spinacia oleracea* (GenBank accession n° X 97600), including the total amino acid sequence

translated from the ORF is shown in Fig. 1. The shaded boxes indicate the putative presequence acting as an organelle targeting signal (transit peptide). The presequence has been deduced from sequence comparison and is in accordance with the Sigcleave program from the GCG software package. The program also confirms some models of the translocation of the small subunit in the stroma indicating that the transit peptide may be cleaved in two steps by two different endopeptidases (ROBINSON & ELLIS, 1984; MISHKIND *et al.*, 1985). The two differentially shaded boxes represent the two successive elements of the transit peptide.

A multi-alignment has been computed with *rbcS2* and the SSU precursors from various plants (Fig. 2). The boxes enclose similar amino acids in the different sequences. The two elements of the transit peptide are indicated by a bar on top of the alignment. Firstly, a very important conservation of the amino acid sequences of the SSU precursor appears within the plants. This conservation is more important when we consider only the mature peptide. The first part of the transit peptide is less conserved than the second part, which would requiring only chloroplast features for its cleavage. The higher conservation might indicate more specific recognition and processing mechanisms.

The building of an active Rubisco requires the intervention of chaperonin proteins called Rubisco binding protein. These proteins are encoded by the nuclear genome and post-translationally exported into the chloroplasts. The chaperonins stabilize the LSU, and it has been shown that 12 or 14 proteins are necessary to stabilize one LSU (HEMMINGSEN *et al.*, 1988). Light promotes the dissociation of the chaperonins and the dimerization of two LSU, top to bottom (L_2 block) This dimerization allows the formation of two catalytic sites. Then four blocks form a L_8 core that will be stabilized by the SSUs on the top and on the bottom of the core. Each SSU interacts with the two neighbor SSUs and with two different L_2 blocks. The mature Rubisco is a L_8S_8 cubic structure, where four sides are made with the L_2 blocks, and the top and the bottom by S_4 caps. The mature enzyme possesses 8 catalytic sites (ROY *et al.*, 1988; ROY, 1989). The SSUs do not participate to the catalytic site nor in the substrate specificity, they only have a structural role to stabilize the mature enzyme. This role is apparently important in the regulation of the content of active enzyme since it is impossible to purify some intermediates of the formation of the enzyme such as L_2 blocks or L_8 cores. The two only forms which can be isolated are the chaperonins-LSU complexes or the L_8S_8 complexes (ROY, 1989). Furthermore, the SSUs are very little proteins which share an important number of interactions with their neighbors (LSUs or SSUs). This represents an high pressure of conservation of the SSUs and may explain the important conservation within the plant kingdom. Finally, one plant possesses in most cases only one copy of the LSU gene (in the chloroplast genome) but many SSUs genes. In these conditions, all the different SSUs will have to share interactions with the same LSU and with the other SSUs. This implies a pressure of conservation much higher in one species, as can be seen in Figure 3, which displays a phylogenic tree of the SSU mature proteins. The different copies within one species do not diverge from one another and

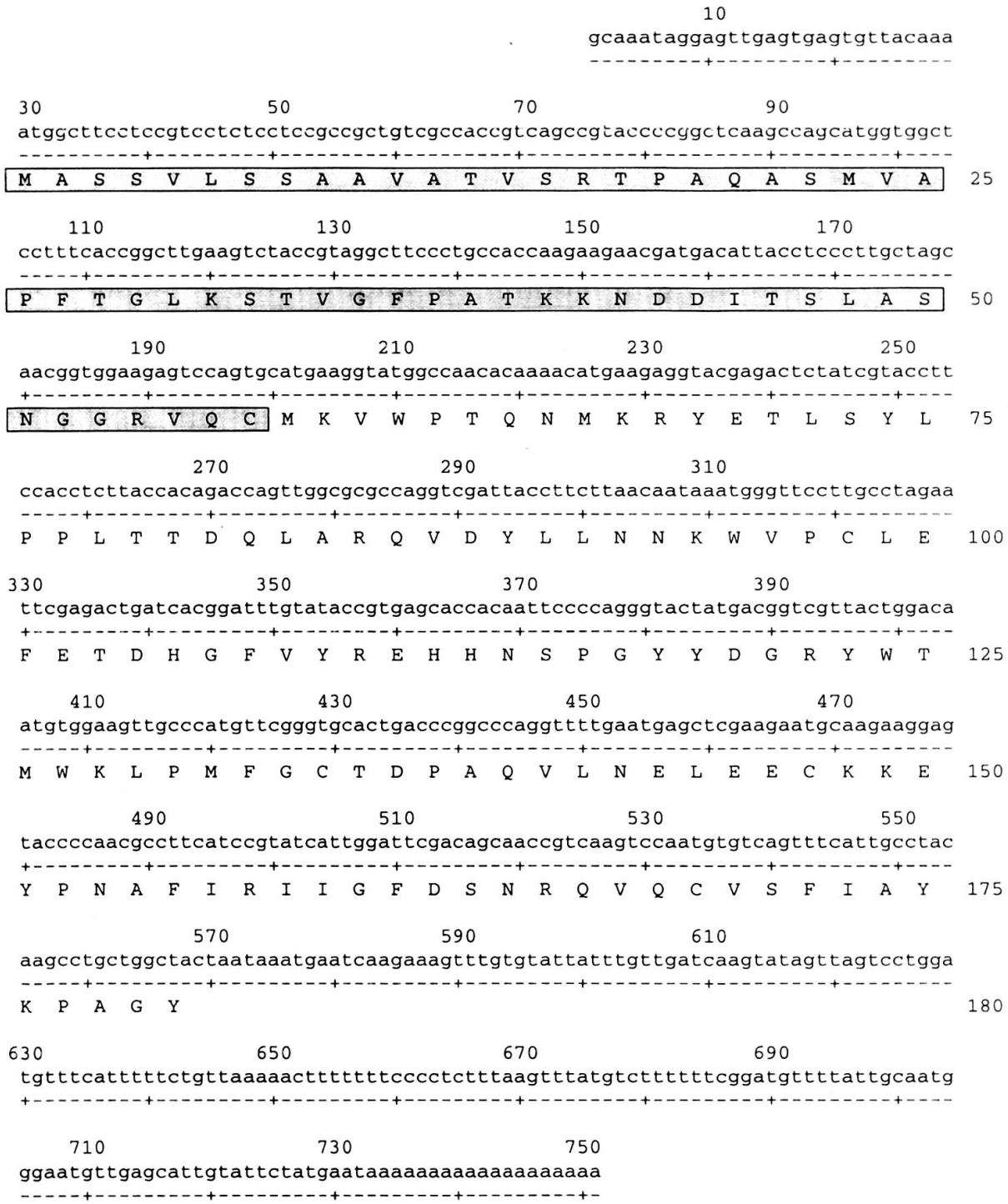

FIG. 1.

Nucleotide sequence of the rbcS cDNA insert. The amino acid sequence translated from the open reading frame is given in the single-letter code under the nucleotide sequence. The putative transit peptide is shaded. The asterisk denotes the stop codon. This sequence is available from the EMBL Nucleotide Sequence Database, accession N° X97600.

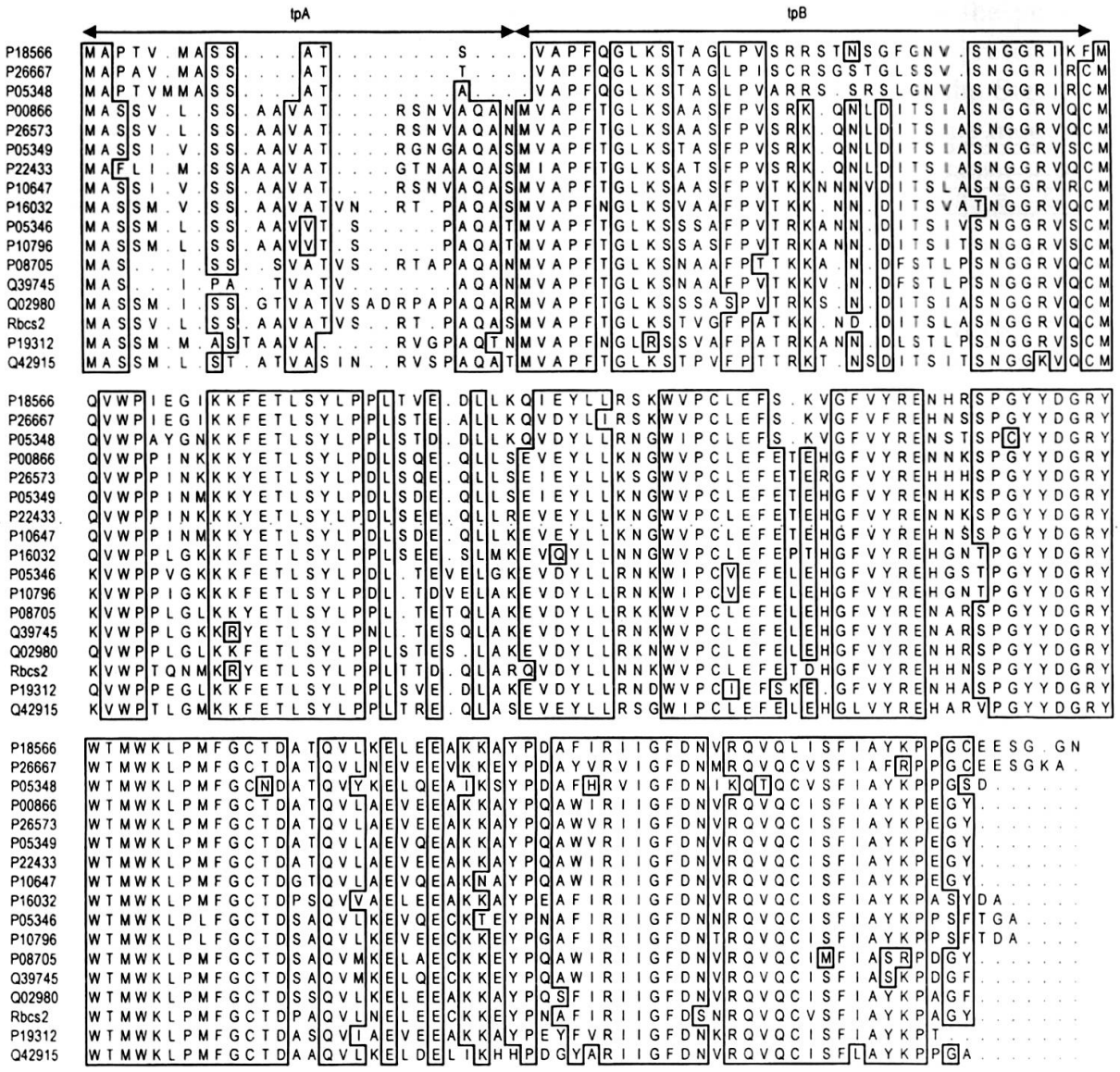

FIG. 2.

Multi-alignment of various *rbcS* amino acid sequences of small subunit precursors of higher plants Rubisco. The two elements of the putative transit peptides are indicated by bar tpA and bar tpB. The boxes enclose similar amino acids. Except for rbcs2, which is the spinach cDNA, the other sequences are named by their database accession number.

the high intraspecific conservation may be the result of gene conversion rather than recent duplications (DEAN *et al.*, 1989). Because of this high conservative pressure, the SSU is not a very good model for phylogenic studies. Only the different plant groups can be discriminated in this tree, but it may happen that some families are dispersed in the tree (e. g. the Fabaceae).

The accumulation of stable *rbcS* messengers was measured during 36 hours in plants maintained in a short day cycle or transferred to continuous light. Because of the intraspecific high degree of homology between the different *rbcS* genes, the use of a labeled antisense probe against the entire mRNA sequence does however not allow the

discrimination of *rbcS2* gene expression alone. It is evident that the accumulation of stable *rbcS* transcripts is directly in relation with the photoperiod (Fig. 4). Under short days, this accumulation follows a rhythmic behavior, increasing continuously during the light period and decreasing slowly during the night. When the plant are submitted to a continuous day, the level of stable *rbcS* mRNA increases to a maximum that is maintained during all the light phase and seems to decrease more rapidly in the dark phase than for plants growing in short days. Transgenic experiments with rbcS promoters indicate significant red-light up-regulation and blue-light down-regulation of the transcriptional activity (PURCELL *et al.*, 1995; BAUM *et al.*, 1997; EWING *et al.*, 1998). The regulation of the amount of stable, or in other words of translationally active *rbcS* messengers, is mainly due to an effect on the transcripts stability (THOMPSON & MEAGHER, 1990) and is controlled by phytochrome, as shown in experiments with red-light pulses (GAMBLE *et al.*, 1989). This effect of light is apparently indirect because the red-light enhanced stabilization is no more effective when protoplasts are pre-treated with a protein synthesis inhibitor (LAM *et al.*, 1989). Light exerts a control not only in the transcriptional step but also in the various steps of protein synthesis, maturation and activation. We can find in the literature various examples of light regulation concerning the enzyme activity in the plant (for review, see PORTIS, 1992). It is also known that *rbcS* translation is enhanced by light (BERRY *et al.*, 1986) and that post-translational export to the chloroplast is directly promoted by light (GROSSMAN *et al.*, 1980). Finally, when mature SSUs compete with the Rubisco binding protein to form mature Rubisco enzymes, light has also a promoting effect (ROY, 1989).

CONCLUSION

The spinach Rubisco has been used as a very popular model to investigate the enzyme properties and constitution. Numerous works have been dedicated to elucidate the catalytical properties of the enzyme and to understand the control of its activity, and the protein has also been one of the most studied plant Rubisco by crystallography (TAYLOR & ANDERSSON, 1997). But surprisingly, the spinach enzyme has not been yet well characterized at the genetic level, probably because spinach is not a popular model in molecular biology studies. *RbcS2* is the second member of the SSU gene family cloned in spinach. Six members, out of 7 estimated, have for the moment been characterized in this family.

In our laboratory, we have chosen spinach as a model system to investigate the light control of the flowering processes, because it is a strict short-day plant that maintains a vegetative growth under short-day conditions and initiates flowers only when the duration of the day is greater than 12 hours. For genetics studies, as for all others, we needed some good controls of various effectors, and the results presented here indicate that the accumulation of stable *rbcS* messengers is a good control of the light effect on the transcript stability and accumulation.

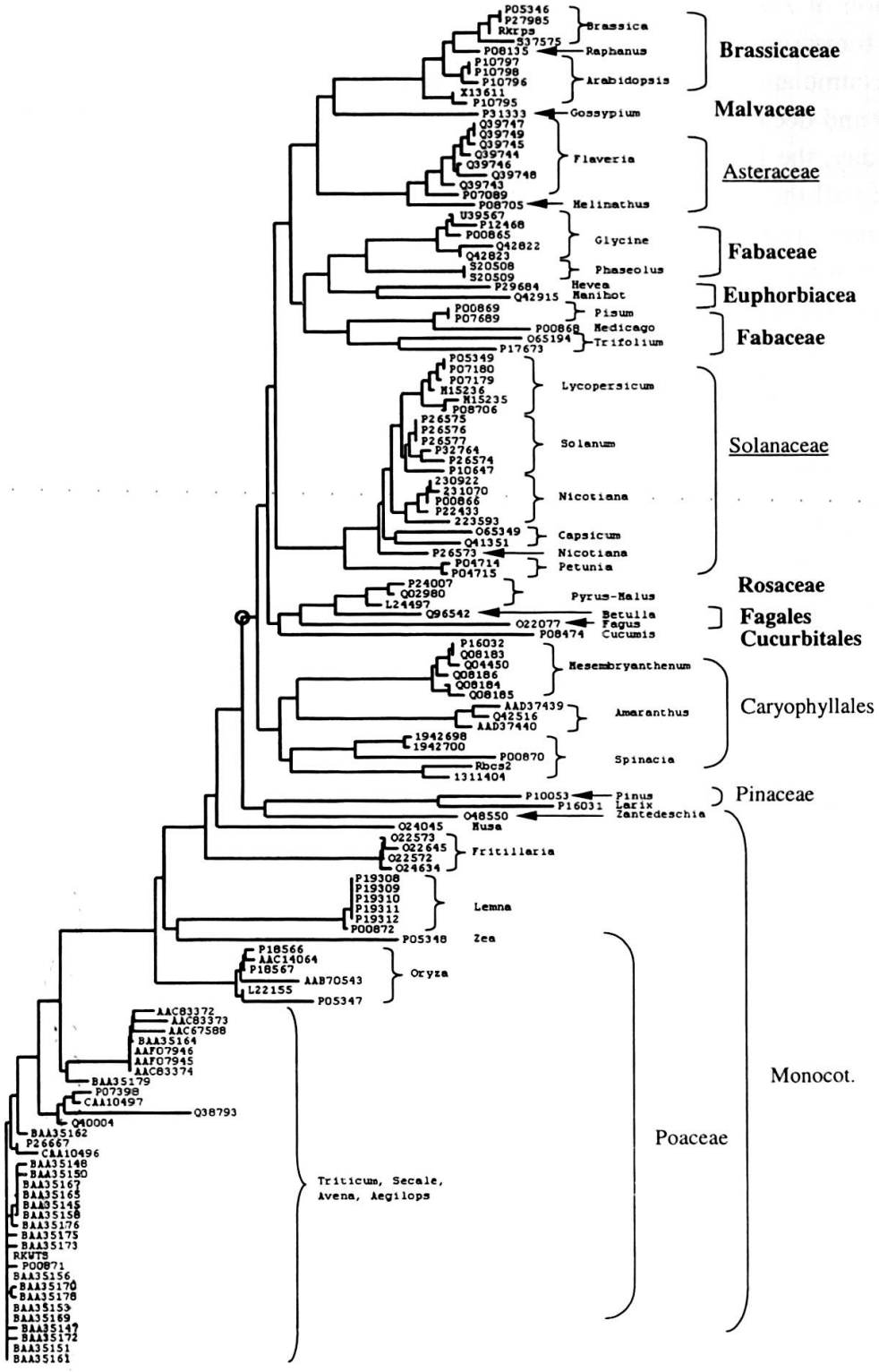

FIG. 3.

Phylogenic tree constructed from the comparison of various mature small subunit amino acid sequences of plants. The sequences are named by their accession number in GenBank. In the dicot part, the bold family names belong to the Rosidae group and the sublined ones to the Asteridae group.

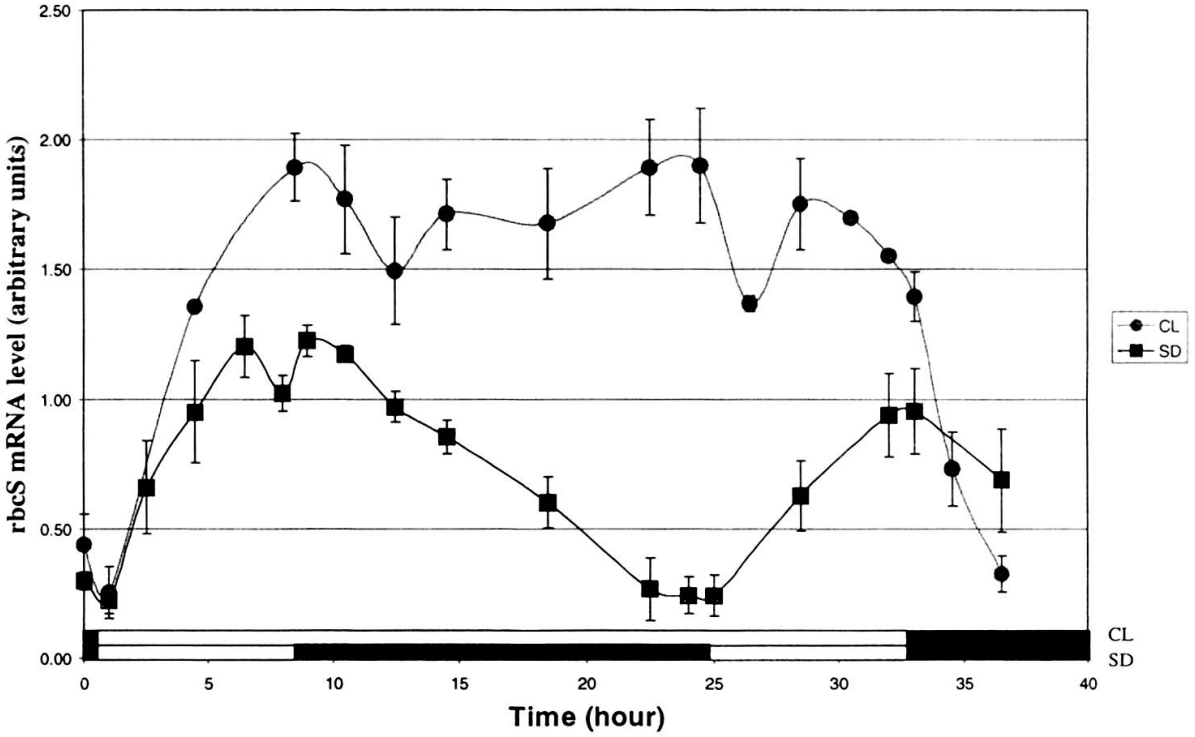

Fig. 4.

Accumulation of *rbcS* mRNA. This figure integrate the results of three independent experiments. The circles represent the values obtained with the plants submitted to one continuous day and the squares, the plants maintained in short days conditions.

RESUME

ISOLATION ET CARACTÉRISATION D'UN ADNc CODANT UN PRÉCURSEUR D'UNE PETITE SOUS-UNITÉ DE LA RUBISCO CHEZ *SPINACIA OLERACEA*

La Rubisco est un enzyme clef qui permet la fixation du CO_2 atmosphérique et représente la protéine la plus abondante chez les plantes. Cet holoenzyme chloroplastique est composé de huit grandes sous-unités produites dans le stroma et de huit petites sous-unités synthétisées dans le cytoplasme et exportées dans les plastes. En raison de son rôle clef dans le cycle du carbone, de son abondance et de son origine mixte, la Rubisco a été le sujet de nombreuses études, en cristallographie, en enzymologie et en biologie moléculaire. Dans le dernier cas, le gène de la petite sous-unité a été largement étudié pour comprendre le fonctionnement de son promoteur, son organisation génique et pour la régulation de l'expression. Nous présentons ici la caractérisation moléculaire par clonage d'ADNc d'une petite sous-unité de la Rubisco chez l'épinard et la mise en évidence d'un contrôle par la photopériode de la quantité des transcripts stables de petites sous-unités.

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