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Chloroplast fructose-1,6-bisphosphatase: the product of a mosaic gene

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ABSTRACT

We show here that light stimulates the expression of nuclear genes in wheat leaves for chloroplast fructose-1,6-bisphosphatase (FBPase) and describe a sequence of amino acids in this enzyme which may be responsible, via thioredoxin, for the light regulation of its activity. This data results from (a) our isolation and characterization of a cDNA of this enzyme which contains its entire coding sequence, and (b) our use of this cDNA as a probe to detect mRNA levels in wheat plants subjected to different light regimes. The similarity in amino acid sequence of the encoded enzyme from diverse sources suggests that the FBPase genes all had a common origin. However, their control sequences have been adjusted so that they are appropriately expressed and their coding sequences modified so that the enzymic activity of their products are suitably regulated in the particular cellular environment in which they must function. The light-activated regulatory sequences in the gene for the chloroplast protein have probably come together by a shuffling of DNA segments.

INTRODUCTION

Fructose-1,6-bisphosphatase (FBPase) is both an interesting and important enzyme for although it always catalyses the hydrolysis of fructose-1,6- bisphosphate to fructose-6-phosphate and inorganic phosphate, it is involved in several quite different metabolic pathways. For example, in *Escherichia coli* and *Saccharomyces cerevisiae* it is necessary for growth on substances such as glycerol, succinate and acetate, in bumblebee flight muscles it is involved in a shuttle between fructose mono- and bisphosphate which liberates heat, while in mammalian tissues it catalyses a reaction essential for gluconeogenesis.

In plants two FBPase isoenzymes are necessary for photosynthesis to take place. One form, localised in the cytosol, is involved in sucrose synthesis from triose phosphates exported from the chloroplasts. The other, found within chloroplasts, takes part in the regeneration of ribulose bisphosphate in the photosynthetic carbon reduction cycle. These enzymes have potentially

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regulatory roles as their estimated activities in vitro are little more than sufficient to account for the observed rates of CO_2 fixation and sucrose biosynthesis (1). In addition, both catalyse a reaction which is essentially irreversible.

One striking feature of FBPases is the way in which they are regulated. The chloroplast enzyme is unique in this respect as its activity is stimulated in the light through pH changes, Mg^{2+} levels and also by light-modulated reduction of essential disulphide groups via the ferredoxin-thioredoxin f system (for review see (2)). Conversely the cytosolic form of the plant enzyme is similar to that of mammals and yeast in that it is inhibited by metabolic affectors such as 5' AMP (3). The enzyme from E. coli is also 5' AMP sensitive but is less sensitive than mammalian and yeast enzymes to fructose-2,6-bisphosphate.

In view of this, it was of particular interest to identify the structural features of the chloroplast FBPase which could be involved in the regulation of its activity. To date the complete amino acid sequence of two mammalian FBPases, pig kidney (4) and sheep liver (5) have been determined and sequence comparison shows a 90% homology. In addition, partial amino acid sequence has been obtained from spinach chloroplast FBPase and some homology found with mammalian gluconeogenic FBPase (6,7). Therefore, the primary aim of this study was to isolate and determine the coding sequence for the wheat chloroplast enzyme so that the complete amino acid sequence of the protein could be deduced. Comparison of the derived amino acid sequence of this protein with those available from other organisms highlights areas of the enzyme likely to be of importance for catalysis and regulation. We show also that the synthesis of chloroplast FBPase mRNA is induced by light.

MATERIALS AND METHODS

cDNA library construction

Double-stranded cDNA was synthesised from 5 μg of wheat leaf poly A^+ RNA using the RNase H method of Gubler and Hoffman (8) (Amersham Kit). The double-stranded cDNA was cloned into a λgt 11 vector (Stratagene) using essentially the methods described by Huynh et al. (9) with the exception that the cDNA was size fractionated on a sucrose gradient and only cDNA larger than 500 bp was used in the ligation. The recombinant λgt 11 was packaged in vitro (Stratagene) and the initial library contained 1.25×10^6 individual recombinants. The library was amplified before use in screening (see (9)) giving a final titre of 3×10^{10} PFUs ml^{-1}.

Screening of cDNA library

The λgt 11 library was screened (10) using polyclonal antibodies raised against spinach chloroplast fructose-1,6-bisphosphatase (a gift from N.-H. Chua, Rockefeller Institute, New York). The positive plaques were picked and purified to homogeneity after which phage DNA was prepared by standard techniques. For sequencing, the largest inserts were subcloned from λgt 11 into the plasmid vector pUBS1 (a version of pUC19 containing the polylinker from the Bluescript plasmid of Stratagene).

Sequencing

Dideoxy sequencing (11) of the wheat chloroplast FBPase was carried out using a double-stranded plasmid sequencing method as described by Murphy and Kavanagh (12).

Northern and Southern analyses

Poly A^+ RNA was isolated as described by Baulcombe and Buffard (13). The RNA was electrophoresed in formaldehyde-MOPS gels using standard procedures and blotted onto Zetaprobe (Bio-Rad).

High molecular weight DNA was prepared (14) from dark grown wheat shoots (var. Chinese Spring). Restriction enzyme digests of the DNA were run in 0.8% agarose and blotted onto Zetaprobe (Bio-Rad) according to Southern (15). Probes were labelled by random priming (16).

Western blotting

Total protein was extracted from wheat leaves by homogenisation in an ice cold buffer (Tricine, 20 μM; NaCl, 10 μM and $MgCl_2$, 2 μM, pH 7.0). This extract was centrifuged for 15 min at 15000 rpm (Sorvall RC5B, SS 34 rotor) and the proteins in the supernatants were separated on 12.5% SDS-polyacrylamide gels (17) and transferred to nitrocellulose (18). Immunodetection was performed using polyclonal antibodies to spinach chloroplast FBPase followed by horseradish peroxidase-conjugated goat anti-rabbit antisera (Bio-Rad) and the peroxidase activity was detected by staining with chloro-1-napthol (Sigma Chemical Co.).

RESULTS

The nucleotide sequence

The nucleotide sequence encoding the chloroplast FBPase enzyme along with its deduced amino acid sequence is shown in Figure 1. This cDNA clone contains an insert of 1399 nucleotides comprising a 70 nucleotide 5' non-coding region, a 100 nucleotide 3' untranslated region and a 1230 base pair open reading frame. Although no poly A^+ tail was found size estimations

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        10                  30                  50                  70                  90                 110
GGGCCACCACCACGGTGCGCGCCAAGACAAGGCAGGGGAGAGAAATTCGTCAATCCGCAGCACCAAAGCAATGGCCGCCGCGACCACCACCACCTCCCGCCCGCTTCTGCTGTCCCGCCA
                                                                       M  A  A  A  T  T  T  T  S  R  P  L  L  L  S  R  Q

       130                 150                 170                 190                 210                 230
GCAGGCGGCGGCTAGCTCCCTCCAATGCCGCCTCCCCAGGAGGCCCGGAAGCAGCCTCTTTGCCGGCCAGGGCCAGGCGTCGACTCCGAATGTGCGGTGCATGGCAGTCGTGGACACGGC
  Q  A  A  A  S  S  L  Q  C  R  L  P  R  R  P  G  S  S  L  F  A  G  Q  G  Q  A  S  T  P  N  V  R  C  M  A  V  V  D  T  A

       250                 270                 290                 310                 330                 350
CTCGGCGCCGGCGCCGGCGGCGGCTAGGAAGAGGAGCAGCTACGACATGATCACGCTGACGACGTGGCTGCTGAAGCAGGAGCAGGAGGGGGTCATCGACAACGAGATGACCATCGTGCT
  S  A  P  A  P  A  A  A  R  K  R  S  S  Y  D  M  I  T  L  T  T  W  L  L  K  Q  E  Q  E  G  V  I  D  N  E  M  T  I  V  L

       370                 390                 410                 430                 450                 470
GTCCAGCATATCCACGGCGTGCAAGCAGATCGCCTCGTTGGTGCAGCGCGCGCCCATCTCCAACCTCACCGGCGTCCAGGGCGCCACCAACGTGCAGGGCGAGGACCAGAAGAAGCTCGA
  S  S  I  S  T  A  C  K  Q  I  A  S  L  V  Q  R  A  P  I  S  N  L  T  G  V  Q  G  A  T  N  V  Q  G  E  D  Q  K  K  L  D

       490                 510                 530                 550                 570                 590
CGTCATCTCCAACGAGGTGTTCTCGAACTGCCTGAGGTGGAGTGGCCGCACCGGCGTGATCGCATCGGAGGAGGAGGACGTGCCGGTGGCGGTGGAGGAGAGCTACTCGGGCAACTACAT
  V  I  S  N  E  V  F  S  N  C  L  R  W  S  G  R  T  G  V  I  A  S  E  E  E  D  V  P  V  A  V  E  E  S  Y  S  G  N  Y  I

       610                 630                 650                 670                 690                 710
CGTGGTGTTCGACCCGCTCGACGGCTCCTCCAACATCGACGCCGCCGTCTCCACCGGCTCCATCTTCGGCATCTACAGCCCATCCGACGAGTGCCACATTGGCGACGACGCAACCCTTGA
  V  V  F  D  P  L  D  G  S  S  N  I  D  A  A  V  S  T  G  S  I  F  G  I  Y  S  P  S  D  E  C  H  I  G  D  D  A  T  L  D

       730                 750                 770                 790                 810                 830
CGAAGTGACGCAGATGTGCATAGTGAACGTGTGCCAGCCAGGGAGCAACCTGCTCGCCGCCGGCTACTGCATGTACTCGAGCTCGGTCATCTTCGTGCTCACCATCGGCACCGGGGTGTA
  E  V  T  Q  M  C  I  V  N  V  C  Q  P  G  S  N  L  L  A  A  G  Y  C  M  Y  S  S  S  V  I  F  V  L  T  I  G  T  G  V  Y

       850                 870                 890                 910                 930                 950
CGTGTTCACGCTGGACCCGATGTACGGCGAGTTCGTGCTGACGCAGGAGAAGGTGCAGATCCCAAAGTCGGGCAAGATCTACTCCTTCAACGAGGGCAACTACGCGCTCTGGGACGACAA
  V  F  T  L  D  P  M  Y  G  E  F  V  L  T  Q  E  K  V  Q  I  P  K  S  G  K  I  Y  S  F  N  E  G  N  Y  A  L  W  D  D  K

       970                 990                1010                1030                1050                1070
GCTCAAGAAGTACATGGACAGCCTCAAGGAGCCCGGCACCTCCGGCAAGCCCTACTCCGCGCGCTACATCGGCAGCCTCGTCGGCGACTTCCACCGCACCATGCTCTACGGCGGCATCTA
  L  K  K  Y  M  D  S  L  K  E  P  G  T  S  G  K  P  Y  S  A  R  Y  I  G  S  L  V  G  D  F  H  R  T  M  L  Y  G  G  I  Y

      1090                1110                1130                1150                1170                1190
CGGGTACCCCAGCGACCAGAAGAGCAAGAACGGCAAGCTGCGGCTGCTCTACGAGTGCGCGCCCATGAGCTTCATCGCCGAGCAGGCCGGCGGCAAAGGCTCCGACGGCCACCAGAGGGT
  G  Y  P  S  D  Q  K  S  K  N  G  K  L  R  L  L  Y  E  C  A  P  M  S  F  I  A  E  Q  A  G  G  K  G  S  D  G  H  Q  R  V

      1210                1230                1250                1270                1290                1310
ACTCGACATCATGCCCACAGCGGTCCATCAGAGAGTGCCTCTGTACGTCGGGAGCGTGGAGGAAGTGGAGAAGGTGGAGAAATTCTTGTCTTCAGAGTAGAACAAGAACGAGGGAGGGAT
  L  D  I  M  P  T  A  V  H  Q  R  V  P  L  Y  V  G  S  V  E  E  V  E  K  V  E  K  F  L  S  S  E  *

      1330                1350                1370                1390
ACACAGGCTGTTTCTTCCAAGAAATTATTGTAACTAATATATAATGTAGCCCTTTTCTTGTGATGCGGAAAATATATTT
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Figure 1. Nucleotide sequence and deduced amino acid sequence of the chloroplast FBPase gene. Nucleotides are numbered above the line.

by Northern blot analysis suggest that the cDNA clone is close to full length. The DNA sequence is GC rich (62%) with a pronounced bias in codon usage towards G and C residues in the 3rd position; this bias has also been found in all the nuclear genes for chloroplast proteins of higher plants studied so far (19; Dyer, unpublished finding).

Wheat FBPase protein - primary structure

The FBPase protein encoded by this clone is 409 amino acids in length and comprises a mature protein and a presequence transit peptide which is necessary for directing the protein into the chloroplast. A putative cleavage site between these components at methionine-51 (Figure 1) has been identified by comparison with known cleavage site sequences from ribulose bisphosphate carboxylase small subunit (SSU), light-harvesting chlorophyll *a*/*b*-protein complex (LHCII) and ferredoxin (20). Assuming that methionine-51 is the cleavage site then the transit peptide would be 51 residues and the mature protein 358 residues long. This would give a molecular weight of

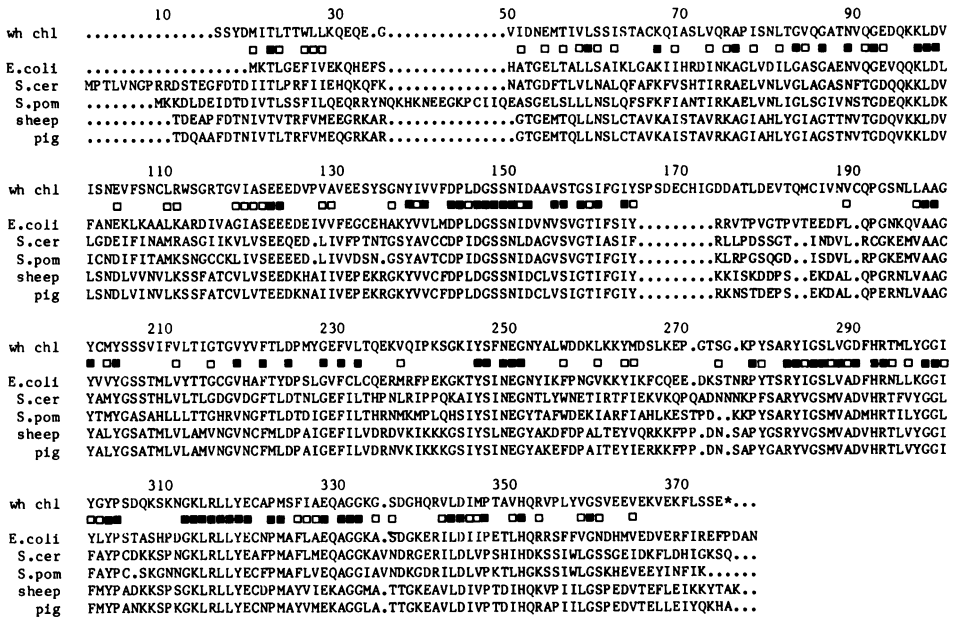

Figure 2. Sequence alignment comparison of the amino acid sequence of chloroplast FBPase with that from E. coli (Hamilton and Dyer, unpublished results), Saccharomyces cerevisiae and Schizosaccharomyces pombe (31), sheep (5) and pig (4). This comparison does not show the transit peptide of the chloroplast FBPase but otherwise includes the complete sequence of each protein. Amino acids showing exact homology in all five proteins are indicated with a closed box (■) and those with conservative amino acids with an open box (□). Spaces (shown by dots) have been introduced to give optimal alignment.

39,778 Da for the mature FBPase protein which is within the range of estmates given for this enzyme (35-44,000) (21).

Alignment of FBPase amino acid sequences

We have compared the wheat FBPase amino acid sequence as deduced from the cDNA sequence with FBPases from diverse sources and the resulting sequence alignments are shown in Figure 2. A high level of homology exists between these enzymes, the chloroplast showing exact homology with the yeast, mammalian and bacterial sequences at about 45% of amino acid positions. In about a further 20% of positions the substitutions found are conservative changes. The highly conserved region of eight amino acids at residues 311-319 contains the lysine residue L-314 thought to be the active site (4). The most significant observation to be made from these alignments is that the

chloroplast FBPase contains an extra 12 residues (165-190) within which are three cysteines (C-170, C-185 and C-190) not found in other FBPases.

Developmental regulation of FBPase synthesis

We have made use of the natural gradient of cell development, including chloroplast development, which exists from the base to the tip of a wheat leaf to study the developmental regulation of FBPase synthesis (22,23). A significant level of FBPase mRNA is observed in the basal leaf section (section 1, Figure 3) which then increases in section 2 to a maximum, after which the level decreases slightly in section 3 and then more appreciably in the sections (4, 5 and 6) towards the leaf tip.

Parallel analysis of FBPase protein levels is shown in Figure 3. These results contrast with those obtained from the mRNA analysis in that very little FBPase protein is detectable until section 2 and that this level remains constant in all other sections to the tip. These results suggest that the FBPase protein is relatively stable.

Light induction of FBPase synthesis

The effect of light on FBPase mRNA and protein in wheat leaves grown under different light regimes has been investigated. Etiolated, 5 day old tissue contains a barely detectable level of FBPase mRNA (see Figure 4B) which was found to increase after only 2 min exposure to light (Figure 4A). A further significant increase in FBPase mRNA levels occurred between 4-24 hrs exposure of etiolated plants to light.

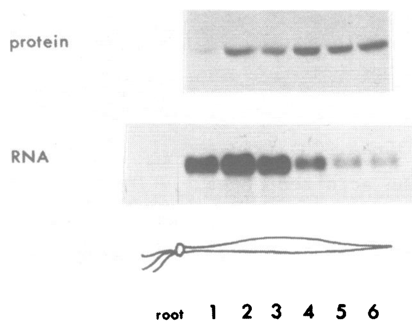

Figure 3. Wheat leaves from 5 day old plants were cut into 6 sections of 2 cm (1-6) numbered from the base as depicted above. RNA and protein was prepared from pools of the leaf sections and from roots. Northern blot analysis of the mRNA samples (10 μg per lane) was performed using a ^{32}P-labelled EcoRI, fragment of the FBPase cDNA EcoRI insert as a probe. Western blot analysis of total protein samples (10 μg) was carried out using FBPase specific polyclonal antibodies.

In order to separate the influence of light from that of chloroplast development on the expression of the FBPase gene, these light induction experiments were repeated using mature green seedlings. FBPase mRNA levels in fully green plants were found to drop, after 40 hr darkness, to that observed in etiolated plants which had only been illuminated briefly. On this basis, green 5 day old plants were placed in the dark for 40 hr and subsequently transferred into the light for up to 24 hr. In this case, mRNA levels increased appreciably between 1-4 hrs re-illumination (Figure 4B) in contrast 4-24 hrs light was required for etiolated plants to attain these same levels.

On illumination of etiolated tissue the increase in the amount of FBPase protein was found to correlate well with that of the mRNA (Figure 4A). However, in contrast to mRNA the relative amount of FBPase protein present in mature green leaves did not decrease even after 40 hrs darkness and consequently no effect was seen on re-illumination of this tissue (Figure 4B). This finding, together with the developmental study (Figure 3) suggests that the FBPase protein is stable once produced and that continuous light is not required to maintain the steady state level found in mature green leaves.

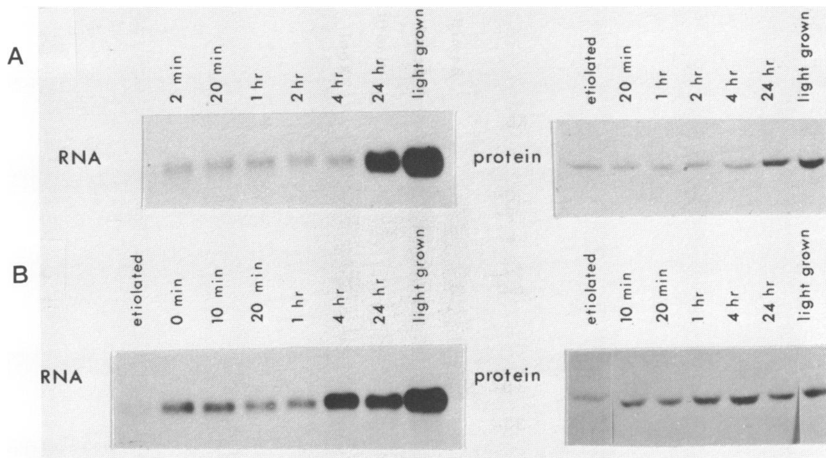

Figure 4. Northern and Western blot analyses of wheat leaves which had been grown under different light regimes. (A) 5 day old etiolated wheat leaves were illuminated for increasing periods of time and the mRNA and total protein then extracted. (B) Mature green leaves put into the dark for 40 hrs then re-illuminated over the time course indicated and then mRNA and total protein extracted. 10 μg of mRNA and 10 μg protein were loaded (details as Figure 3).

Gene copy number

Southern blot analysis of wheat genomic DNA using the FBPase specific cDNA probe has shown that three bands are present for each restriction digest of this genomic DNA (Figure 5). The genome of cultivated hexaploid wheat (*Triticum aestivum*) arose by polyploidisation involving 3 diploid wheats whose genomes are designated A, B and D. This result suggests that the FBPase protein is not encoded by a large multigene family and that the gene may be present as a single copy with each diploid genome contributing an FBPase gene to the hexaploid genome. Further support for this proposal has come from Southern blot analysis using restriction fragment length polymorphism (RFLP) analyses of DNA isolated from addition and substitution lines of wheat (Chao *et al*., in preparation).

DISCUSSION

FBPase is an ubiquitous enzyme with four identical subunits and a molecular weight of approximately 160,000 Da. In all of the situations where this enzyme functions, it is under some form of regulation and the comparison of the aligned sequences (Figure 2) shows some interesting features relating to this aspect. The chloroplast enzyme exclusively has an insertion of 12

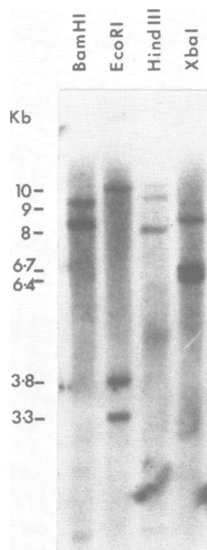

Figure 5. Southern blot analysis of restriction digests of wheat genomic DNA (10 µg per lane). The probe was as indicated in Figure 3.

extra amino acids in the variable region (residues 165-190) of the protein and also within this region there are 3 cysteine residues not found in other FBPases. This feature of the chloroplast FBPase may be important regarding the light regulation of this enzyme with 2 of these cysteine residues contributing to the disulphide bond which is reduced by thioredoxin to sulphydryl groups during light activation (2,24,25). In yeast (S. cerevisiae) a different strategy for controlling enzyme activity has been adopted and protein degradation is part of the regulatory process. This yeast FBPase has an amino terminal extension which contains the sequence motif RRXS (Figure 2, residues 9-12) which is believed to be the recognition site required for phosphorylation by cAMP-dependent protein kinase (26). This phosphorylation causes deactivation of the yeast FBPase and is also thought to be part of a signalling mechanism for the degradation of this enzyme when it is no longer needed. This is in contrast with the chloroplast enzyme which is relatively stable (see result in Figures 3 and 4) possibly because in this case activation and deactivation are regular events mediated by light. In both of these FBPases the regulation of the enzyme has been brought about by the introduction of additional residues to the basic FBPase sequence.

More subtle changes may also have occurred with regard to AMP inhibition. The mammalian and yeast FBPases are sensitive to AMP whilst the chloroplast enzyme is insensitive. Both of the mammalian enzymes but not the chloroplast have a lysine residue at position 174 within the variable region of the protein and this residue is thought to be involved in AMP inhibition (4).

Another unique feature of the chloroplast FBPase, as compared to FBPases from other sources, is that it is synthesized in the cytosol as a precursor with a transit peptide and is subsequently transported to the chloroplast where it is processed to the mature protein. The deduced amino acid sequence presented here shows the sequence of the transit peptide sequence of the chloroplast FBPase (Figure 1). The chloroplast transit peptides studied so far do not show large degrees of homology with each other (20). However, close inspection of SSU, LHCII and ferredoxin transit sequences has revealed three possible homology blocks (27). By comparison it can be seen that the FBPase transit sequence also shares some of these characteristics: (i) it begins with a number of uncharged amino acids and has the consensus MA as its first two residues (interestingly the sequence of the first few residues resembles most closely those from wheat (28) and petunia LHCII (29)

presequences); (ii) adjacent to the cleavage site we have proposed (M-51) a number of residues are in common with the wheat Rubisco SSU cleavage site; and (iii) although the FBPase transit peptide does not have the central conserved block of homology suggested by Karlin-Neumann and Tobin (27), it does have a P...FAG motif observed in a number of LHCII gene transit sequences. These observations suggest that, unlike mitochondrial presequences, chloroplast transit peptides share some homology with each other and it may be that they have had a common evolutionary origin.

In this paper we show that the expression of the FBPase gene is complex and besides being regulated by light it also has developmental factors superimposed. Illumination of etiolated tissue resulted in a two step increase in the level of FBPase mRNA; firstly there was a rapid induction of synthesis of mRNA resulting in the appearance of a low but detectable level and this effect is brought about by illumination for less than 2 minutes. Secondly, a more significant increase occurs between 4 and 24 hr illumination raising the mRNA level close to that found in fully greened, mature leaves (see Figure 4A). In darkened mature tissue the time scale for light-induction is condensed and the greatest increase in accumulation of FBPase mRNA occurs between 1 and 4 hr illumination in contrast to between 4 and 24 hr in etiolated leaves (see Figures 4A and B). This disparity may reflect the time required for chloroplast development to reach a stage such that photosynthesis can take place. This argument is supported by the correlation which we observed between the levels of FBPase mRNA and the gradient of chloroplast development in the wheat leaf. This pattern of light-induced accumulation of FBPase mRNA is similar to that found for SSU and LHCII genes where the initial rapid response is attributed to phytochrome and is observed in immature tissues and the second more significant increase to a blue light receptor and is observed in mature leaves (see review (30)). These data suggest that the transcription of the FBPase gene is light-regulated. However, it does not rule out the possibility that the FBPase transcript level increases due to stabilisation by light. This latter proposal seems less likely in view of the data obtained for other nuclear encoded photosynthesis genes SSU and LHCII which have been shown, both by run-off transcription and analyses of genomic sequences in transgenic plants, that these genes are activated by light (see review (30)).

These results taken together lead us to propose that the chloroplast FBPase gene is a genetic mosaic in which specific control sequences are joined to a basic, relatively uniform coding region common to a wide range of

organisms to give it distinctive properties. The specific elements which have been brought together in the gene for the chloroplast protein are a coding region for a transit peptide which facilitates the transport of the protein precursor into the chloroplast from its cytosolic site of synthesis, the incorporation of a nucleotide sequence into the main coding region which renders its product susceptible to light regulation and possibly a light-activated promoter. We have yet to isolate and sequence the cDNA for the cytosolic version of this enzyme but suspect that it will resemble that of yeasts and mammals more closely than the gene for the chloroplast protein.

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