Journal of Photoscience, Vol. 4, No. 3, pp. 141–145

CLONING AND LIGHT-DEPENDENT EXPRESSION OF A cDNA FOR PEA CYTOSOLIC FRUCTOSE-1,6-BISPHOSPHATASE

TAE-JONG SON and TAE-RYONG HAHN*
Department and Institute of Genetic Engineering, Kyung Hee University, Suwon, Korea 449-701

(Received 20 *August* 1997; *accepted* 17 *October* 1997*)*

Abstract – Polymerase chain reaction(PCR) was conducted with a pea cDNA library using two primers synthesized from homology analysis of amino acid sequences for animal and plant cytosolic FBPases. A PCR product with 650 bp long was cloned into pGEM-T vector and sequenced. The deduced amino acid sequence of the cDNA fragment was 98, 91, and 85% homologous with those of cytosolic FBPases from spinach, sugarbeet, and sugarcane, respectively. It was 51% homologous with amino acid sequence of FBPase from pea chloroplasts. Northern blot analysis was proceeded with the cDNA clone resulting that 1.2 kb transcript was highly expressed in light-grown pea leaves but almost not expressed in dark-grown etiolated pea seedlings. When peas grown in the light for 10 days were transferred to darkness, the transcript was gradually decreased with dark treatment, indicating that the expression of the enzyme was induced by continuous white light but suppressed by dark treatment. Pea cytosolic FBPase was highly expressed in leaves with trace amounts in stems, but almost not expressed in roots.

INTRODUCTION

Fructose-1,6-bisphosphatase (FBPase) (EC 3.1.3.11) which catalyzes the dephosphoric reaction of fructose-1,6-bisphosphate to fructose-6-phosphate and inorganic phosphate, exists as two isozymes in the cytosol and chloroplasts of higher plants. The cytosolic enzyme is required for the synthesis of sucrose from triose phosphate, which is the major form of reduced carbon exported from chloroplasts. On the otherhand the chloroplastic enzyme is involved in one of the critical steps constituting the Calvin cycle of photosynthetic carbon dioxide fixation. Cytosolic FBPase is regulated by AMP, fructose-2,6-bisphosphate and citrate, as are all other gluconeogenic enzymes from other species.[1-4] Chloroplastic FBPase is distinct from the cytosolic enzyme in several ways, the most striking being that its activity is stimulated by light.[5,6] It is activated by alkaline pH, Mg^{2+}, and reducing agents, such as dithiothreitol *in vitro*, similar to conditions occurring in the stroma of chloroplasts upon light illumination.[6] In contrast to chloroplastic FBPase, the cytosolic enzyme is active at neutral pH and insensitive to reducing agents.[4,7]

cDNA sequences of cytoplasmic FBPase has been reported in spinach,[8] sugarbeet,[9] and sugarcane.[10] The expression of the cytosolic enzyme is down-regulated by water stress,[9] but induced by light illumination.[11,12] Recently we purified and characterized chloroplastic and cytosolic FBPases from pea leaves.[7,13,14] We also cloned and sequenced cDNAs for pea and soybean chloroplastic FBPases.[15,16] In connection with previous works, we report here a cDNA sequence and expression of pea cytoplasmic FBPase in an attempt to elucidate the comparative properties of both enzymes in plant tissues.

MATERIALS AND METHODS

Materials. Peas (*Pisum Sativum* L. Alaska) were grown in a growth chamber with 16 h/8 h light-dark photoperiod at 25°C.

Sephadex G-50 was purchased from Pharmacia Fine Chimical Co.. Nylone membrane (Hybond-N), [α-^{32}P]dATP and sequencing kit (Sequenase version 2.0) were from Amersham International Biotechnology Inc.. Bacto-tryptone, yeast-extract and bacto-agar were from Difco Co.. Phenol, chloroform and alcohol were from Merck Co.. X-gal (5-bromo 4-chloro 3-indolyl β-D-galactopyranoside), IPTG (Isopropyl-β-D-thiogalactosidase) and other chemicals were from Sigma Chemial Co.. Restriction enzymes, T_4 DNA ligase and DNA polymerase were from Poscochem Co.. Specific oligomers for polymerase chain reaction (PCR) or DNA sequencing were from Bioneer Co.. PCR mixture were from TaKaRa Inc. and T-vector for PCR were purchased from Promega Co..

Polymerase chain reaction. Phage DNA was prepared from a cDNA library constructed from 2-week-old green pea leaves.[15] PCR was carried out with phage DNA as template using two oligomers synthesized from homology analysis of amino acid sequences for animal and plant cytosolic FBPase (Fig. 1). Reaction mixtures contained 100 pmol of template,

* To whom correspondence should be addressed.
†*Abbreviations used :* FBPase, fructose-1,6-bisphosphatase ; PCR, polymerase chain reaction

PCF : 5′ -GCCAAGCTCATAGGACTTGCAGG-3′

PCR : 5′ -CCGTTGGGGCTCTTCCTATC-3′

Figure 1. Synthetic primers for PCR experiment. The primer sequences were from analysis of cytosolic FBPase cDNA sequences for plants, yeasts, and animals.

50 pmol of each primer, 500 m*M* KCl, 100 m*M* Tris-Cl, 10 m*M* dNTPs, 250 m*M* $MgCl_2$ and 2.5 U of Taq DNA polymerase.[17] PCR temperature cycling was composed of 3 cycles. First cycle was 5 min at 94 °C, 2 min at 55 °C and 3 min at 72 °C. Second cycle was 1 min at 94 °C, 2 min at 55 °C, 3 min at 72 °C. Third cycle was 1 min at 94 °C, 2 min at 55 °C, 7 min at 72 °C.

Subcloning of PCR product. The 0.7 kb DNA fragment was isolated from PCR products by agarose-gel electrophoresis and then subcloned into pGEM^R-T vector. Recombinant clones were transformed into *E.coli* JM109 (*rec*A1, *end*A1, *gyr*A96, *thr*, *hsd*R17 (r_k^-, m_{k+}), *rel*A1, *sup*E44, Δ (*lac-pro*AB)), (*F*, *tra*D36, *pro*AB, *lacI*^{q}ZΔM15) on LB plate supplied with ampicillin, X-gal and IPTG.

DNA sequencing analysis. Plasmid DNA was prepared by the method of Maniatis *et al.*[17] A partial fragment of the cDNA was sequenced according to the dideoxynucleotide chain termination method using double strand DNA as a template. [18] Sequencing gels were prepared from a stock of 38 % acrylamide and 2% N,N'-bis acrylamide to a final concentration of 6% containing 7 *M* Urea. Nucleotide and amino acid sequence analysis was performed by DNASIS and PROSIS programs.

Northern blot analysis. Northern blot analysis was carried out with total RNA prepared from pea leaves, stems and roots using the cloned cDNA as a probe. Total RNA was extracted with guanidium thiocyanate-phenol-chloroform extraction method.[19] Total RNA was separated by electrophoresis in 1.2% agarose-10% formaldehyde gel before transferring to nylone membrane. The filter was dried briefly, fixed by UV light and then prehybridized in buffer (50% formamide, 5 × SSC, 0.1% SDS, 5 × Denhardt's reagent, salmon sperm DNA) at 42 °C for 3 h. The filter was hydridized with the labeled probe at 42 °C for 16 h , and then washed with buffer (1 × SSC, 0.1% SDS). The filter was exposed to X-ray film with an intensifying screen.

RESULTS AND DISCUSSION

FBPase (EC 3.1.3.11) is a key enzyme for sucrose biosynthesis in higher plants. Two isozymes have been identified in the cytosol and chloroplast.[20,21] In previous studies pea chloroplastic and cytosolic FBPases[7,13,14] were purified and characterized as a preliminary work for elucidating light-modulated activation mechanism of the enzyme. cDNAs for pea and soybean chloroplastic FBPases were also cloned and sequenced.[15,16] In this work cDNA for pea cytosolic FBPase was cloned by PCR method and sequenced.

```
GCC AAG CTC ATA GGA CTT GCA GGG GAG ACT AAC ATT CAG GGT GAA GAG  48
 A   K   L   I   G   L   A   G   E   T   N   I   Q   G   E   E

CAA AAG AAG CTA GAT GTG CTC TCA AAT GAA GTT TTT GTT AAG GCT TTG  96
 Q   K   K   L   D   V   L   S   N   E   V   F   V   K   A   L

ACA AGT AGT GGC CGA ACT TGC ATC CTT GTG TCT GAA GAG GAC GAG GAG 144
 T   S   S   G   R   T   C   I   L   V   S   E   E   D   E   E

GCA ACA TTC ATT GAG CCA TCT CTG CGT GGA AAG TAT TGT GTT GTA TTT 192
 A   T   F   I   E   P   S   L   R   G   K   Y   C   V   V   F

GAC CCT CTG GAT GGA TCT TCC AAC ATT GAC TGT GGT GTT TCA ATT GGG 240
 D   P   L   D   G   S   S   N   I   D   C   G   V   S   I   G

ACG ATA TTT GGG ATT TAC ATG GTG AAA GAT TTT GAA ACT GCG ACT CTT 288
 T   I   F   G   I   Y   M   V*  K   D   F   E   T   A   T   L

GAA GAT GTC CTG CAA CCT GGA AAG AAT ATG GTA GCT GCT GGT TAT TGC 336
 E   D   V   L   Q   P   G   K   N   M   V   A   A   G   Y   C

ATG TAT GGG AGG TCT TGG AGG CTT GTT TTG AGC ACT GGA AGC GGT GTT 384
 M   Y   G   R   S   W   R   L   V   L   S   T   G   S   G   V

AAT GGT TTC ACT CTA GAT CCA TCT CTT GGA GAG TAT ATT TTA ACT CAT 432
 N   G   F   T   L   D   P   S   L   G   E   Y   I   L   T   H

CCA GAC ATC AAG ATT CCA AAC AAG GGT AAG ATC TAT TCA GTG AAT GAA 480
 P   D   I   K   I   P   N   K   G   K   I   Y   S   V   N   E

GGC AAT GCA AAG AAT TGG GAT GGC CCA ACA ACA AAG TAT GTG GAA AAA 528
 G   N   A   K   N   W   D   G   P   T   T   K   Y   V   E   K

TGT AAG TTC CCG ACA GAT GGT TCA TCA CCC AAG TCT CTC AGA TAC ATT 576
 C   K   F   P   T   D   G   S   S   P   K   S   L   R   Y   I

GGA AGT ATG GTA GCT GAT GTT CAT CGC ACT TTA CTA TAT GGT GGC ATC 624
 G   S   M   V   A   D   V   H   R   T   L   L   Y   G   G   I

TTT TTG TAC CCT GGT GAT AGG AAG AGC CCC AAC GG                  659
 F   L   Y   P   G   D   R   K   S   P   N
```

Figure 2. Nucleotide and deduced amino acid sequences of a PCR clone for pea cytosolic FBPase. The synthetic primer sites were underlined.

PCR was carried out with phage DNA as template using two oligomers synthesized from homology analysis of amino acid sequences for animal and plant cytosolic FBPases (Fig. 1). Phage DNA was prepared from a cDNA library constructed with 2-week-old green pea leaves.[15] The PCR products were analyzed by agarose-gel electrophoresis and the expected 0.7 kb DNA fragment was isolated. The cDNA fragment was subcloned into pGEM^R-T vector and sequenced

Figure 2 shows the nucleotide and deduced amino acid sequences of the cloned cDNA. The sequence contained total 660 nucleotides which could encode more than two third of full-length polypeptide. Fig. 3 shows deduced amino acid sequence comparison of pea cytosolic FBPase cDNA with those of cDNAs from spinach,[8] sugarbeet,[9] and sugarcane.[10] The amino acid sequence of pea cytosolic FBPase was 98%, 91%, and 85% homologous with those of spinach, sugarbeet, and

```
Spinach     --HCERKCAS  GEERE-SR-R  -REKRKMDHA  GD-A-M-RTD  LMTITRYVLN  EQSKRPESRG  60
Sugarbeet   ----------  --DK-YS-GR  AKEE-E-EH-  GSCSGSTRTD  LMTITRFVLN  EQSKRPESRG
Sugarcane   --T-TG-K-A  S-PIQVP-A-  GS-E-GE-MD  HAADA-HRTD  LMTITRHVLN  EQSRNPESRG
Pea         ----------  ----------  ----------  ----------  ----------  ----------

Spinach     DFTILLSHIV  LGCKFVCSAV  NKAGLAKLIG  LAGETNIQGE  EQKKLDVLSN  EVFVKALTSS  120
Sugarbeet   DFTILMSHIV  LGCKFVCSAV  NKAGLAKLIG  LAGETNIQGE  EQKKLDVLSN  EVFIKALISS
Sugarcane   DFTILLSHIV  LGCKFVASAV  NKAGLAQLIG  LAGETNVQGE  EQKKLDVLSN  EVFVKALVSS
Pea         ----------  ----------  -----AKLIG  LAGETNIQGE  EQKKLDVLSN  EVFVKALTSS

Spinach     GRTCILVSEE  DEEATFIEPS  LRGKYCVVFD  PLDGSSNIDC  GVSIGTIFGI  YMVKDFETAT  180
Sugarbeet   GRTCILVSEE  DEEATFVEPS  LRGKYCVVFD  PLDGCSNIDC  GVSIGTIFGI  YMVKDLNNAT
Sugarcane   GRTCVLVSEE  DEETTFVDPK  LRGKYCVCFD  PLDGSSNIDC  GVSIGTIFGI  YMIKDKDNVT
Pea         GRTCILVSEE  DEEATFIEPS  LRGKYCVVFD  PLDGSSNIDC  GVSIGTIFGI  YMVKDFETAT

Spinach     LEDVLQPGKN  MVAAGYCMYG  SSCTLVLSTG  SGVNGFTLDP  SLGEYILTHP  DIKIPNKGKI  240
Sugarbeet   LDDVLQPGKN  MVAAGYCMYG  SSCTLVMSTG  SGVNGFTHDP  SLGEFILTHP  DIKIPKKGKI
Sugarcane   LSDVLQPGKD  MLAAGYCMYG  SSCTLVLSTG  TGVNGFTLDP  SLGEFILTHP  DIKIPKKGKI
Pea         LEDVLQPGKN  MVAAGYCMYG  RSWRLVLSTG  SGVNGFTLDP  SLGEYILTHP  DIKIPNKGKI

Spinach     YSVNEGNAKN  WDGPTTKYVE  KCKFPTDGSS  PKSLRYIGSM  VADVHRTLLY  GGIFLYPGDK  300
Sugarbeet   YSVNEGNAKN  WDGPTTKYVE  KCKFPKDGSS  PKSLRYIGSM  VADVHRTLLY  GGIFMYPGDK
Sugarcane   YSVNEGNAKN  WDVPVAKFVE  KCKYPKDGSP  PKSLRYIGSM  VADVHRTLLY  GGVFLYPADQ
Pea         YSVNEGNAKN  WDGPTTKYVE  KCKFPTDGSS  PKSLRYIGSM  VADVHRTLLY  GGIFLYPGDR

Spinach     KSPNGKLRVL  YEVFPMSFLM  EQAGGQAFTG  KQRALDLIPT  K-IHERSPVF  LGSYDDVEDI  360
Sugarbeet   KSPNGKLRVL  YEVFPMSFLM  EQAGGQAFTG  EQRALDLVP-  KNIHDRSPVF  LGSYDDVEDI
Sugarcane   KSPNGKLRVL  YEVFPMSFLM  EQAGGQSFTG  KERALDLVPT  K-IHERSPIF  LGSYDDVEEI
Pea         KSPN------  ----------  ----------  ----------  ----------  ----------

Spinach     KALYAAQEK-  TA-T-S-AVV  -RS-ILKLFL  KLHLSSH-S-  ALQEYF-VL-  LYILHKC-A  420
Sugarbeet   KALYAAEQKN  AASADVRSTV  PSNWI-QIYF  -LHI-T-V-V  T-TE-LCA-R  IY-APMCIT
Sugarcane   KALYAEQAKS  SSASGNPNEV  PGSAV-T-FR  VSDIS-FL-G  -V-VV-S-LH  S-VYIV-GR
Pea         ----------  ----------  ----------  ----------  ----------  ----------
```

Figure 3. Amino acid sequence comparison of the pea cytosolic FBPase cDNA with cytosolic FBPases from spinach, sugarbeet, and sugarcane.

sugarcane, respectively. It has 51% homology with pea chloroplastic FBPase. The present data of cytosolic FBPase were comparable with spinach cytoplasmic[4] and chloroplastic enzymes.[22]

It has been reported that the sugarbeet cytosolic FBPase is modulated by light-dependent manner.[11] The enzyme activity and transcript levels of cytosolic FBPase changes during a diurnal cycle.[11] To test light dependent expression of pea cytosolic FBPase northern blot analysis was conducted. Total RNA was isolated from dark-grown etiolated pea seedlings and light-grown pea leaves and then hybridized with the cloned cDNA as a probe. Fig 4 shows that a 1.2 kb transcript was highly expressed only in pea leaves grown in the light. Peas grown in the light for 10 days were transferred to darkness and then analyzed by northern blot. Fig. 5 shows that the transcript was gradually decreased during dark treatment, indicating that the expression of the enzyme induced by light is suppressed by dark treatment. Pea chloroplastic FBPase is also expressed by light stimulation at transcription level.[15,23] However the fact that the expression of chloroplastic FBPase is induced by red light pulse[23] indicates that the expression of chloroplastic enzyme is mediated by phytochrome system. On the other hands, cytosolic FBPase requires several hours of exposure to white light to observe a

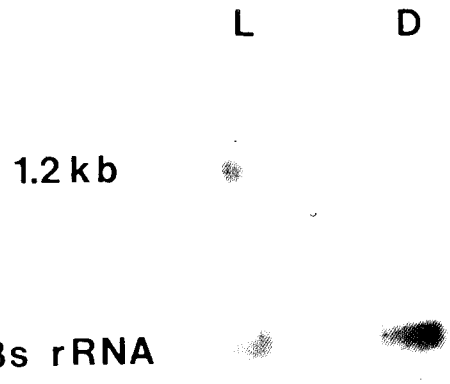

Figure 4. Northern blot analysis of light and dark grown pea tissues. Total RNA was isolated from light grown pea leaves (L) and dark grown etiolated pea hypocotyls(D), and then hybridized with the pea cytosolic FBPase cDNA.

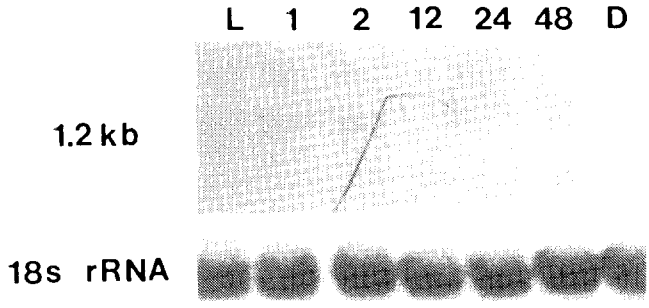

Figure 5. Northern blot analysis for the effect of dark treatment on the light grown pea leaves. Peas grown in the light for 10 days(L) were transferred to dark over the time course(h) indicated. Total RNA was isolated and hybridized with the pea cytosolic FBPase cDNA.

recognized expression of the enzyme[11,12], suggesting that the light-dependent modulation of pea cytosolic FBPase might not be phytochrome-mediated. The enzyme was majorly expressed in pea leaves with trace amounts in stems, but almost not expressed in roots (Fig. 6) as pea chloplastic FBPase.[23]

Acknowledgement—This work was supported by grants from Korea Science and Engineering Foundation (96-0401-12-01-3, 971-0506-030-2), and the academic research fund of Ministry of Education, Republic of Korea (GE 97-32).

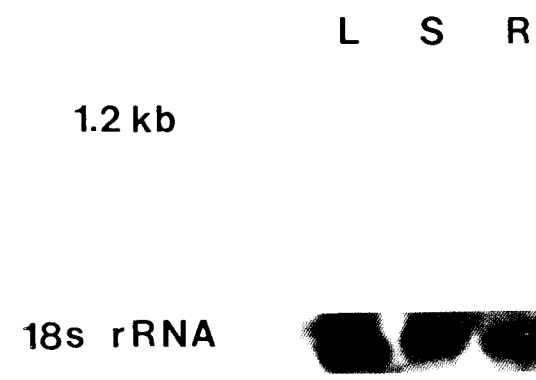

Figure 6. Northern blot analysis for the tissue specific expression of pea cytosolic FBPase. Total RNA was isolated from leaves(L), stems(S), roots(R) of mature green peas grown in the light for 10 days and hybridized with the pea cytosolic cDNA.

REFERENCES

1. Zimmermann, G., G. J. Kelly and E. Latzko (1978) Purification and properties of spinach leaf cytoplasmic fructose-1,6-bisphosphatase. *J. Biol. Chem.* **253**, 5952-5956
2. Harbron, S., C. Foyer, and D. Walker (1981) The purification and properties of sucrose-phosphate synthetase from spinach leaves : The involvement of this enzyme and fructose bisphosphatase in the regulation of sucrose. *Arch. Biochem. Biophys.* **22**, 237-246
3. Stitt, M. and W. P. Quick (1989) Photosynthetic carbon partitioning; its regulation and possibilities for manipulation. *Physiol. Plant.* **77**, 633-641
4. Ladror, U. S., S. P. Latshaw and F. Marcus (1990) Spinach cytoplasmic fructose-1,6-bisphosphatase : Purification, enzyme properties and structure comparison. *Eur.J. Biochem.* **189**, 89-94
5. Buchanan, B. B., P. Wolosiuk and P. P. Kalberer (1971) Ferredoxin-activated fructose disphosphatase of spinach chloroplasts : Resolution of the system, properties of the alkaline fructose disphosphatase component and physiological significance of the ferredoxin-linked activation. *J.Biol. Chem.* **246**, 5952-5956
6. Buchanan, B.B. (1980) Role of light in the regulation of chloroplast enzymes. *Ann. Rev. Plant Physiol.* **31**, 341-394
7. Lee, S.-W., M.-H. Cho, H.-C. Kang and T.-R. Hahn (1994) Purification and characterization of pea cytoplasmic fructose-1,6-bisphosphatase. *Kor. Biochem. J.* **27**, 538-543
8. Hur, Y., E. A. Unger and A. C. Vasconcelos (1992) Isolation and characterization of a cDNA encoding cytosolic fructose-1,6-bisphosphatase from spinach. *Plant Mol. Biol.* **18**, 799-802
9. Harn, C. and J. Daie (1992) Cloning and nucleotide sequence of a complementary DNA encoding the cytosolic fructose-1,6-bisphosphatase of sugarbeet. *Plant Physiol.* **98**, 790-791
10. Grof, C. P. L., D. Glassop, R. C. Bugos and P. H. Moore (1995) Sequence of a cDNA encoding cytosolic fructose-1,6-bisphosphatase from sugarcane. *Plant Physiol.* **109**, 339-340
11. Khayat, E., C. Harn and J. Daie (1993) Purification and light-dependent molecular modulation of the cytosolic fructose-1,6-bisphosphatase in sugar beet leaves. *Plant Physiol.* **101**, 57-64

12. Lee, S.-W. and T.-R. Hahn (1997) White light dependent expression of pea cytosolic fructose-1,6-bisphosphatase. *Plant Physiol.* submitted.
13. Bhoo, S.-H. and T-R. Hahn (1989) Purification and characterization of chloroplast fructose-1,6-bisphosphatase from pea leaves. *Kor. Biochem. J.* **22**, 405-410
14. Cho, M.-H. and T.-R. Hahn (1991) Biochemical characteristics of the purified pea chloroplast fructose-1,6-bisphosphatase. *Kor. Biochem. J.* **24**, 617-624
15. Dong, S.-M., J.-H. Rhim and T.-R. Hahn (1995) Nucleotide sequence analysis of a cDNA encoding chloroplastic fructose-1,6-bisphosphatase from pea (*Pisum sativum* L.). *Plant Physiol.* **107**, 313-314
16. Jeon, Y.-H., S.-H. Bhoo and T.-R. Hahn (1997) Molecular cloning and characterization of a cDNA encoding chloroplastic fructose-1,6-bisphosphatase from soybean (*Glycine max* L.). *Mol. Cell* (In press)
17. Maniatis, T., E. F. Fritsh and J. Sambrook (1989) Molecular Cloning : a laboratory mannual. Cold spring Harbor Laboratory Press, Cold Spring Harbor, NY
18. Sanger, F., S. Nicklen and A. R. Coulson (1977) DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467
19. Chomczynski, P. and N. Sacchi (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Biochem.* **162**, 156-159
20. Kelly, G. J., G. Zimmermann and E. Latzko (1982) Fructose-1,6-bisphosphatase from spinach lesf chloroplast and cytoplasm. *Method Enzymol.* **90**, 371-378
21. Stitt, M., B. Herzog and H. W. Heldt (1984) Control of photosynthetic sucrose synthesis by fructose-2,6-bisphosphate : II. Partitioning between sucrose and starch. *Plant Physiol.* **75**, 548-553
22. Marcus, F. and P. B. Harrsch (1990) Amino acid sequence of spinach chloroplast fructose-1,6-bisphosphatase. *Arch. Biochem. Biophy.* **279**, 151-157
23. Hahn, T.-R., J.-H. Rhim and S.-M. Dong (1997) Red light stimulated expression of pea chloroplastic fructose-1,6-bisphosphatase. *Plant Physiol.* submitted