Purification, and phosphorylation in vivo and in vitro, of phospho*enol*pyruvate carboxykinase from cucumber cotyledons

Robert P. Walker, Richard C. Leegood*

Robert Hill Institute and Department of Animal and Plant Sciences, University of Sheffield, Sheffield, S10 2UQ, UK

Received 26 January 1995; revised version received 13 February 1995

Abstract Phospho*enol*pyruvate carboxykinase (PEPCK) with a subunit molecular mass of 74 kDa has been purified 450-fold to homogeneity from the cotyledons of cucumber (*Cucumis sativus* L.). This is the first purification of the native form of the enzyme from any plant tissue. Incubation of the purified enzyme with [γ-^{32}P]ATP and either phospho*enol*pyruvate-carboxylase kinase or mammalian cAMP-dependent protein kinase led to labelling of the enzyme in a part of the molecule separate from the active site. This was reversed by incubation with protein phosphatase 2A. Cotyledons of cucumber seedlings were also supplied with $^{32}P_i$. Homogenates of such cotyledons contained a heavily labelled polypeptide which was confirmed as PEPCK by immunoprecipitation. Labelling of PEPCK by $^{32}P_i$ in darkened cotyledons was reversed by illumination.

Key words: Phospho*enol*pyruvate carboxykinase; Protein phosphorylation; Purification; *Cucumis sativus* (cucumber)

1. Introduction

In plants, phospho*enol*pyruvate carboxykinase (PEPCK, EC 4.1.1.49) is important in gluconeogenesis during the germination of fat-storing seeds, such as cucumber [1], and it plays a key role in photosynthetic carbon assimilation in one group of C_4 plants [2], in some Crassulacean Acid Metabolism (CAM) plants [2], and in the CO_2-concentrating mechanism of certain algae [3].

In cucumber, the gene for PEPCK codes for a protein with a predicted molecular mass of about 74 kDa [4]. All previous purifications of the enzyme from C_3 and C_4 plants have yielded an enzyme with a molecular mass of between 62 and 64 kDa [5,6]. These lower molecular mass forms arise in crude extracts by rapid proteolytic cleavage of forms with a higher molecular mass, of about 74 kDa in cucumber cotyledons and 68 kDa in the C_4 grass, *Urochloa panicoides* [5]. The predicted amino acid sequence of PEPCK from cucumber is homologous to those from yeast, *Rhizobium*, *Escherichia coli* and trypanosomes [4], except that the enzyme from cucumber is considerably larger because it possesses an N-terminal extension of about 12 kDa [4], similar in size to the portion of the polypeptide which is lost after proteolysis. Several plant enzymes are known to contain N-terminal extensions when compared to their bacterial or cyanobacterial counterparts and these are susceptible to proteolysis (e.g. [7,8]).

A continuing enigma concerning PEPCK in plants has been the relative lack of regulatory properties of the purified enzyme which would be sufficient to explain its regulatory role and high control coefficient in plant gluconeogenesis [9,10], or the necessity for light-dark regulation in C_4 plants [11] or CAM plants. One explanation is that the portion of the enzyme that is proteolytically-cleaved, either in crude extracts or during purification, confers regulatory properties on the enzyme which are not present in the cleaved smaller form (which is nevertheless catalytically competent [5]). One possible mechanism for such regulation could be phosphorylation, like several other plant enzymes [12].

In this study, the native (74 kDa) form of PEPCK has been purified to homogeneity from the cotyledons of cucumber (*Cucumis sativus* L.). We show that it is reversibly phosphorylated both in vitro and in vivo and that the phosphorylation process is regulated in vivo by light.

2. Materials and methods

2.1. Plant material

Cucumber seeds (*Cucumis sativus* L. cv Masterpiece; W. McNair, Edinburgh) were sown in vermiculite and grown at 25°C in the dark.

2.2. Assay of PEPCK

The carboxylation reaction of PEPCK was assayed as described previously [5].

2.3. Purification of cucumber PEPCK

All procedures except FPLC were performed at 0–4°C. Cotyledons were homogenised using a Polytron in 10 volumes of buffer A (200 mM Bicine-KOH (pH 9.0), 20 mM $MgCl_2$, 5 mM DTT).

2.3.1. $(NH_4)_2SO_4$ fractionation. The homogenate was clarified by centrifugation at 25,000 × *g* for 20 min. Protein in the supernatant that precipitated between 45 and 55% saturation with $(NH_4)_2SO_4$ was collected by centrifugation at 10,000 × *g* for 15 min. The pellet was resuspended in a minimal volume of buffer B (20 mM Bicine-KOH (pH 9.0), 20 mM $MgCl_2$, 5 mM DTT). Insoluble material was removed by centrifugation at 20,000 × *g* for 20 min.

2.3.2. Polyethylene glycol fractionation. An equal volume of 40% (w/v) polyethylene glycol 6000 (PEG) in buffer B was added. After incubation for 30 min, precipitated protein was collected by centrifugation at 15,000 × *g* for 15 min. The pellet was resuspended and subjected to a further cycle of PEG precipitation. The final pellet was resuspended in a minimal volume of buffer B and insoluble material removed by centrifugation at 20,000 × *g* for 10 min.

2.3.3. Superose 12 chromatography. 1 ml of the sample was applied to a Superose 12 HR 10/30 FPLC gel filtration column (Pharmacia) at a flow rate of 0.2 ml · min^{-1} equilibrated with buffer B containing 20% (v/v) glycerol.

2.3.4. Mono P chromatography. 1 ml of sample was applied to a Mono P HR 5/20 FPLC chromatofocussing column (Pharmacia), previously equilibrated with 25 mM Bis Tris-HCl (pH 6.0), 5 mM $MgCl_2$, 5 mM DTT, 20% (v/v) glycerol at a flow rate of 0.5 ml · min^{-1}. Bound

Corresponding author. Fax: (44) (114) 276 0159.

Abbreviations: DTT, dithiothreitol; PEG, polyethyleneglycol; PEP, phospho*enol*pyruvate; PEPC, phospho*enol*pyruvate carboxylase; PEPCK, phospho*enol*pyruvate carboxykinase; PP2A, protein phosphatase 2A.

SSDI 0014-5793(95)00212-X

protein was eluted by applying 10% Polybuffer 74-HCl (pH 4.6), 5 mM $MgCl_2$, 5 mM DTT, 20% (v/v) glycerol.

2.4. Protein determination

Protein was determined using a modified version of the Lowry procedure [13].

2.5. SDS-PAGE

SDS-PAGE was carried out as in [5]. Autoradiography was done as in [14].

2.6. In vitro phosphorylation assay

Unless otherwise stated, the in vitro phosphorylation reaction mixture contained 100 mM Tris-HCl (pH 8.0), 0.5% PEG 6000, 0.1 mM EDTA, 1 mM DTT, 0.01 mM ATP, 1 μCi [γ-^{32}P]ATP (specific activity 3000 Ci·mmol^{-1}, Amersham, UK), 5 mM $MgCl_2$, and 0.5–2 μg PEPC or PEPCK. Reactions were initiated by adding 0.02 μg of a partially purified preparation of PEPC kinase from maize leaves (a gift from Professor Hugh Nimmo, Department of Botany, University of Glasgow) or 0.1 μg of the catalytic subunit of bovine heart cAMP-dependent protein kinase (Sigma Chemicals Ltd). After 30 min at room temperature, proteins were precipitated by acetone and analysed by SDS-PAGE and autoradiography. PEP carboxylase was prepared as in [15]. For reaction with PP2A, ATP was removed after the phosphorylation reaction by incubation with 2 U hexokinase and 2 mM glucose for 5 min. For dephosphorylation, samples were incubated with 0.1 μg PP2A for 15 min. The catalytic subunit of PP2A from bovine cardiac muscle was purified (specific activity 12,000 mU·mg^{-1}) and provided by Bob MacKintosh (MRC Protein Phosphorylation Unit, Dundee, UK). One unit is the amount that catalyses the dephosphorylation of 1 μmol glycogen phosphorylase per min [16].

2.7. In vivo phosphorylation assay

Single 4-day-old cucumber seedlings that had been grown at 20°C under a 12 h photoperiod (150 μmol quanta·m^{-2}·s^{-1}) were placed in 1.5 ml microfuge tubes with their roots immersed in water. One cotyledon was removed and 5 μl (50 μCi) $^{32}P_i$ (specific activity 200 mCi·mmol^{-1}, Amersham, UK) was put on the adaxial surface of the other. Seedlings were left for 90 min under ambient light during which time the $^{32}P_i$ was absorbed. They were then incubated at 25°C in the dark or under lights (450 μmol quanta·m^{-2}·s^{-1}). Cotyledons were then homogenised in 400 μl buffer A, then clarified by centrifuging twice at 20,000 × g for 5 min. 10 μl of the supernatant was taken and protein precipitated by acetone [14] and analysed by SDS-PAGE. For immunoprecipitation, 30 μl of a rabbit polyclonal antiserum against purified cucumber PEPCK (62 kDa form; [5]) was added to 200 μl of the supernatant. After 5 min at 25°C, the immunoprecipitate was collected by centrifugation at 10,000 × g for 5 min. The pellet was washed in 200 μl 20 mM Tris-HCl (pH 7.4), 150 mM NaCl and then analysed by SDS-PAGE.

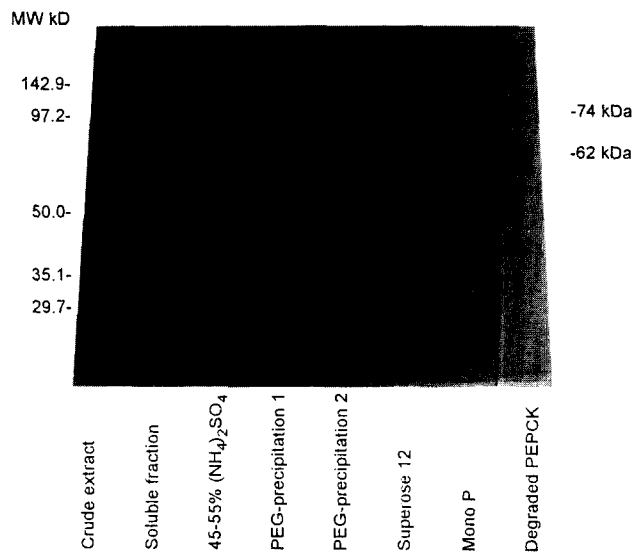

Fig. 1. Purification of PEPCK (74 kDa) from cucumber cotyledons. Material from different stages of purification (see Table 1) was analysed by SDS-PAGE. Degraded PEPCK (62 kDa) was prepared as in [5].

3. Results and discussion

3.1. Purification of native form of PEP carboxykinase

The 74 kDa form of cucumber cotyledon PEPCK was purified about 450-fold to homogeneity (Table 1, Fig. 1). At each step of the purification, the integrity of PEPCK was monitored by SDS-PAGE and immunoblotting. Using the procedure outlined in Table 1, no proteolytic degradation of PEPCK was observed. The enzyme had an isoelectric point of 5.4 as measured by Mono-P chromatography (Table 1). The enzyme had a similar specific activity (41 U·mg^{-1} protein) to the proteolytically-cleaved 62 kDa form isolated from cucumber (48 U·mg^{-1} protein) [5], which supports the view that cleavage does not alter the V_{max}. Gel filtration chromatography of both the purified preparation and rapidly prepared crude extracts showed PEPCK to have a native molecular mass of approximately 400 kDa. We have previously shown that the molecular mass of the

Table 1
Purification of the 74 kDa form of PEP carboxykinase from cucumber cotyledons. 40 g of cotyledons were extracted and the enzyme was purified as described in section 2

Stage	Total activity (μmol·min^{-1})	Protein (mg)	Specific acitivity (U·mg^{-1} protein)	Purification (fold)	Yield (%)
Crude extract	290	3200	0.09	1	100
Soluble fraction	240	2100	0.11	1.2	83
45–55% $(NH_4)_2SO_4$	100	130	0.77	8.6	34
PEG precipitation 1	60	59	1.2	13.3	21
PEG precipitation 2	50	16.6	3.0	33.3	17
Superose 12 chromatography	18	2.5	7.2	80.0	6
Mono-P chromatography	7	0.17	41	456	2.4

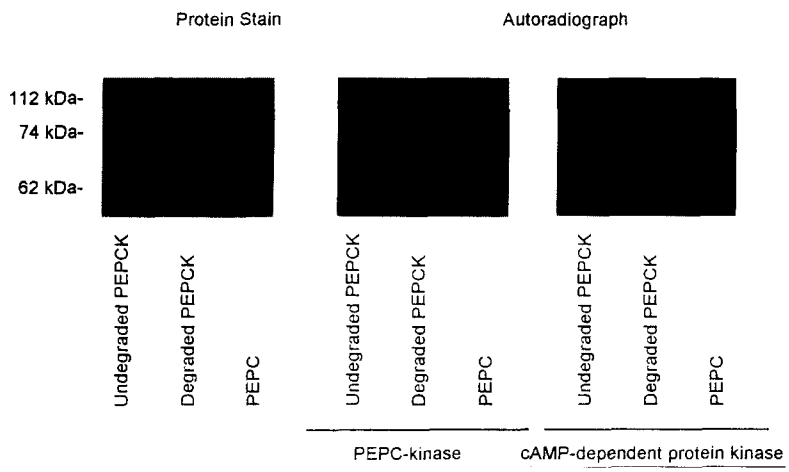

Fig. 2. In vitro phosphorylation of PEPCK from cucumber cotyledons (left-hand lanes) and PEPC from leaves of *Bryophyllum fedtschenkoi* (right-hand lanes) by either PEPC kinase from maize leaves or by the catalytic subunit of bovine heart cAMP-dependent protein kinase. Labelling patterns were analysed by SDS-PAGE and autoradiography. Note that the degraded, 62 kDa, form of PEPCK was not phosphorylated (centre lanes).

proteolytically-degraded form of PEPCK from cucumber was 270 kDa, consistent with PEPCK being a tetramer, in contrast to the hexameric form of the enzyme from C_4 plants [6]. It is well established that gel filtration can only be used to estimate the molecular mass of spherical proteins [17]. The 12 kDa N-terminal extension is likely to project from the molecule and increase its Stokes radius. We therefore suggest that the native 74 kDa form of PEPCK also exists as a tetramer.

3.2. *In vitro phosphorylation of PEPCK*

The native form of PEPCK (purified from dark-grown cucumber cotyledons) was incubated under various conditions, then subjected to SDS-PAGE and autoradiography (Fig. 2). Incubation of PEPCK with [γ-^{32}P]ATP and either PEP-carboxylase kinase from maize or mammalian cAMP-dependent kinase led to labelling of the 74 kDa polypeptide (Fig. 2). Incubation of PEP carboxylase under the same conditions also led to labelling of the 112 kDa subunit of PEP carboxylase (Fig. 2), although incorporation into PEPCK was actually slightly higher than that into PEP-carboxylase, suggesting that PEPCK may be as good a substrate for PEP-carboxylase kinase as PEP carboxylase itself. There was no phosphorylation of PEPCK or PEPC in the absence of added protein kinase (data not shown).

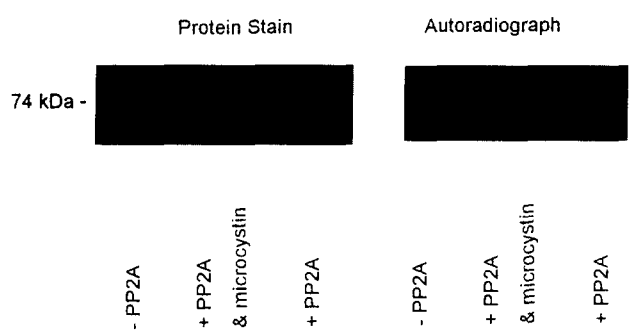

Fig. 3. SDS-PAGE gel and autoradiograph showing that cucumber PEPCK phosphorylated in vitro is dephosphorylated in vitro by PP2A (right-hand lanes). 3 mU (0.25 μg) of pure bovine cardiac muscle PP2A was used per reaction. 1 μM microcystin-LR, an inhibitor of PP2A, prevented dephosphorylation (centre lanes).

Treatments using the 62 kDa, proteolytically degraded, form of PEPCK [5] showed that this polypeptide was not detectably phosphorylated by either kinase (Fig. 2). This demonstrates that phosphorylation was not due to non-specific binding or due to phosphorylation of the enzyme by ATP acting as one of its substrates (which is unlikely in view of the mechanism of PEPCK [18]). It also shows that the part of the enzyme which is cleaved by proteolysis (which may be the N-terminal extension) either contains the phosphorylation site or is necessary for efficient phosphorylation of the remainder of the molecule. Within the N-terminal extension, Ser^{67} and Thr^{68} are likely target residues for phosphorylation because, apart from being within a consensus site for recognition by mammalian cAMP-dependent protein kinase [19], they are also within a sequence of amino acids, Gln-Lys-Lys-Arg-Ser-Thr (residues 63–68) [4], which is similar to a proposed phosphorylation site motif for plant PEP carboxylase-kinase (Glu/Asp-Lys/Arg-X-X-Ser, for review see [12]).

The phosphorylation of PEPCK was reversible. If, following phosphorylation, the ATP was removed by the addition of glucose and hexokinase, PEPCK was readily dephosphorylated by cardiac protein phosphatase 2A (PP2A) (Fig. 3) and this dephosphorylation was inhibited by the characteristic inhibitor of PP2A, microcystin-LR [20].

3.3. *In vivo phosphorylation of PEPCK*

Cotyledons from darkened and illuminated seedlings that had been fed $^{32}P_i$ were extracted at pH 9.0 (to minimise proteolytic degradation [5]) and labelling of the polypeptides assessed by SDS-PAGE and autoradiography. Five strongly labelled bands were present, including a 74 kDa polypeptide (Fig. 4). Immunoprecipitation showed that this polypeptide was PEPCK. Extraction at pH 6.7, which leads to cleavage of PEPCK [5], led to a loss of label from PEPCK (Fig. 5), again indicating that the phosphorylation site in vivo is located in the proteolytically-cleaved portion. The extent to which PEPCK was phosphorylated depended upon the growth conditions of the seedlings. PEPCK was phosphorylated in light-grown seedlings that had been darkened for 16h with a stoichiometry of ca. 0.7 mol P_i per mol PEPCK subunit (Fig. 4). This is a

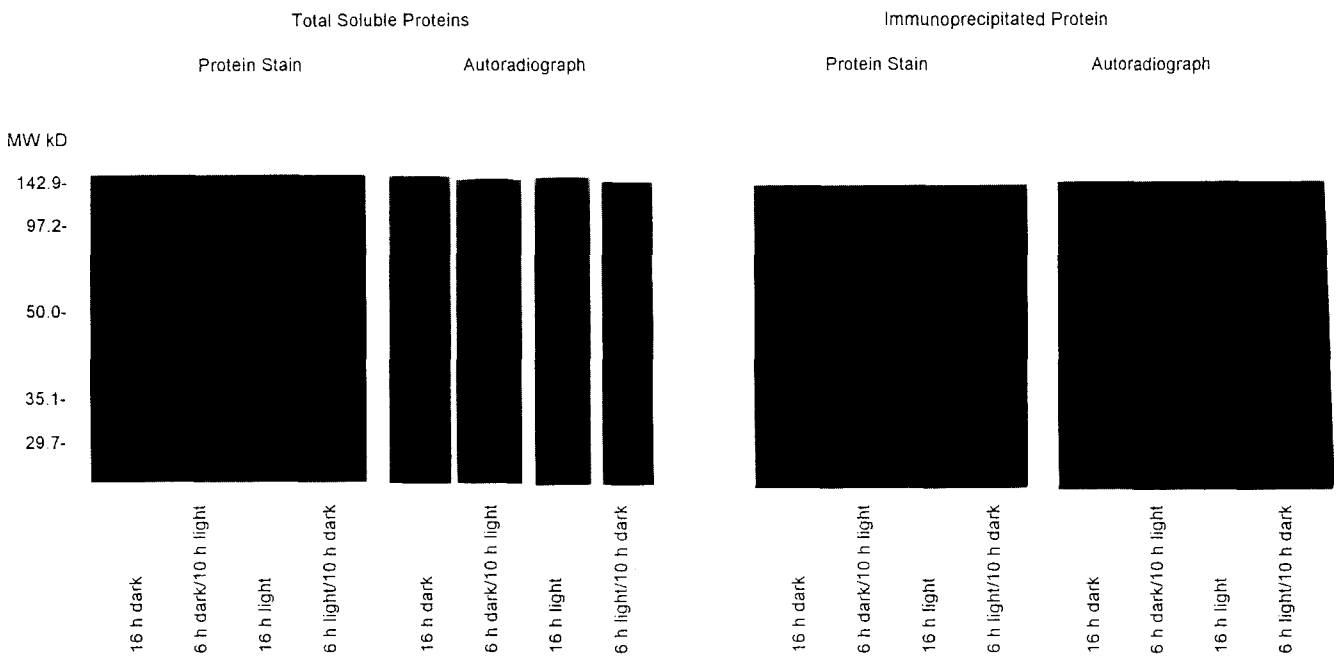

Fig. 4. Analysis of in vivo phosphorylation of cucumber PEPCK by SDS-PAGE and autoradiography. A heavily labelled band of 74 kDa observed in crude extracts of darkened cotyledons was identified as PEPCK by immunoprecipitation (left-hand lanes). Phosphorylation of PEPCK occurred in seedlings darkened for 16 h (left-hand lane). Illumination leads to no phosphorylation, or reverses it (centre lanes), while a period of darkness following illumination led to phosphorylation (right-hand lane).

minimum estimate of the stoichiometry as it does not allow for dilution of the $^{32}P_i$ fed to the leaf by endogenous pools of P_i or adenylates, so in practice the stoichiometry is likely to be even higher. The enzyme was not phosphorylated in cotyledons of illuminated seedlings (Fig. 4). Phosphorylation was reversible since seedlings transferred from darkness to light showed dephosphorylation and seedlings transferred from light to darkness showed phosphorylation (Fig. 4).

3.4. Concluding remarks

The native form of PEPCK has been purified for the first time from any plant tissue. Unlike the proteolytically-degraded form of the enzyme from cucumber, which has a very limited response to possible regulators [9], the native enzyme is phosphorylated both in vitro (by PEP carboxylase-kinase and the catalytic subunit of mammalian cAMP-dependent protein kinase) and this phosphorylation is reversed by incubation with PP2A (we assume that the enzyme purified from darkened cotyledons was not already fully phosphorylated). PEPCK is phosphorylated in vivo with a high stoichiometry in a process which is regulated by light. These data suggest that phosphorylation may be a physiologically important means of regulating the activity of PEPCK and they also raise the intriguing possibility of co-regulation of PEP carboxylase and PEPCK by a common kinase, although it must be recognised that this experiment was done using a partially purified preparation of PEPC kinase. We are currently investigating what effects phosphorylation has on the regulatory properties of PEPCK and whether phosphorylation and any associated changes in properties might also feature in the regulation of photosynthesis in C_4 and CAM plants.

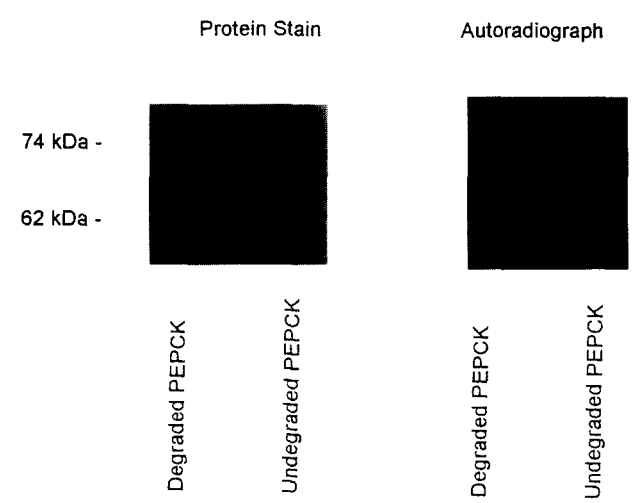

Fig. 5. SDS-PAGE gel and autoradiograph indicating that the fragment that is cleaved from PEPCK by proteolysis following extraction (left-hand lane) contains the site that is phosphorylated in vivo.

Acknowledgements: We are grateful to Prof. Hugh Nimmo (Department of Biochemistry, University of Glasgow) for the gifts of PEP carboxylase and PEP carboxylase kinase and Drs Bob and Carol MacKintosh (MRC Protein Phosphorylation Unit, University of Dundee) for the gifts of cardiac protein phosphatase 2A and microcystin-LR. This research was supported by the joint AFRC/SERC Programme on the Biochemistry of Metabolic Regulation in Plants (PG50/590).

References

[1] Leegood, R.C. and ap Rees, T. (1978) Biochim. Biophys. Acta 524, 207–218.
[2] Leegood, R.C. and Osmond, C.B. (1990) in: Plant Physiology, Biochemistry and Molecular Biology (Dennis, D.T. and Turpin, D.H., Eds.) pp. 274–298, Longman, London.

[3] Reiskind, J.B. and Bowes, G. (1991) Proc. Natl. Acad. Sci. USA 88, 2883–2887.
[4] Kim, D.-J. and Smith, S.M. (1994) Plant Mol. Biol. 26, 423–434.
[5] Walker, R.P., Trevanion, S.J. and Leegood, R.C. (1995) Planta 195, in press.
[6] Burnell, J.N. (1986) Aust. J. Plant Physiol. 13, 577–587.
[7] Lepiniec, L., Keryer, E., Philippe, H., Gadal, P. and Cretin, C. (1993) Plant Mol. Biol. 21, 487–502.
[8] Ocheretina, O., Harnecker, J., Rother, T., Schmid, R. and Scheibe, R. (1993) Biochim. Biophys. Acta 1163, 10–16.
[9] Leegood, R.C. and ap Rees, T. (1978) Biochim. Biophys. Acta 542, 1–11.
[10] Trevanion, S.J, Brooks, A.L. and Leegood, R.C. (1995) Planta, in press.
[11] Carnal, N.W., Agostino, A. and Hatch, M.D. (1993) Arch. Biochem. Biophys. 306, 360–367.
[12] Huber, S.C., Huber, J.L. and McMichael, R.W. (1994) Int. Rev. Cyt. 149, 47–98.
[13] Walker, R.P., Waterworth, W.M. and Hooley, R. (1993) Physiol. Plant. 89, 388–398.
[14] Walker, R.P., Beale, M.H. and Hooley, R. (1992) Phytochemistry 31, 3331–3335.
[15] Nimmo, G.A.,Nimmo, H.G., Hamilton, I.D., Fewson, C.A. and Wilkins, M.B. (1986) Biochem. J. 239, 213–220.
[16] MacKintosh, C. (1993) in: Protein Phosphorylation: A Practical Approach (Hardie, D.G., Ed.) pp. 197–230, IRL Press, Oxford.
[17] Krishnan, H.B., Blanchette, J.T. and Okita, T.W. (1985) Plant Physiol. 78, 241–245.
[18] Urbina, J.A. and Avilan, C. (1989) Phytochemistry 28, 1349–1353.
[19] Bairoch, A. (1993) Nucleic Acids Res. 21, 3097–3103.
[20] MacKintosh, C., Beattie, K.A., Klumpp, S., Cohen, P. and Codd, G.A. (1990) FEBS Lett. 264, 187–192.