Pergamon 0031-9422(95)00913-2 *Phytochemistry*, Vol. 42, No. 3, pp. 589–594, 1996

0031-9422/96 $15.00 + 0.00

CHLOROPLAST ADENYLATE KINASE FROM TOBACCO. PURIFICATION AND PARTIAL CHARACTERIZATION

UWE SCHLATTNER,*† EDGAR WAGNER,‡ HUBERT GREPPIN and MARC BONZON

Laboratoire de Biochimie et Physiologie Végétales, Université de Genève, Place de l'Université 3, CH-1211 Genève 4, Switzerland; ‡Institut für Biologie II, Universität Freiburg, Schänzlestr. 1, D-79104 Freiburg i. Br., Germany

(*Received in revised form* 23 *October* 1995)

Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; adenylate kinase; chloroplast isoform; purification; substrate specificity; inhibition.

Abstract—A soluble isoform of adenylate kinase (AK, EC 2.7.4.3) from tobacco leaves (*Nicotiana tabacum* L.) was purified about 60-fold by a protocol using ammonium sulphate fractionation, anion exchange chromatography, affinity chromatography and gel filtration. The purified protein was homogeneous, as judged by SDS–PAGE, IEF–PAGE and Mono Q ion exchange chromatography, and had a specific activity of 500 nkat mg^{-1}. Its M_r was determined as 28 000 and 30 000 by SDS-PAGE and gel filtration, respectively. It is therefore monomeric and belongs to the long-variant-type adenylate kinases. The isoelectric point of *ca* 4.45, as measured by IEF–PAGE and the elution profile of the Mono Q column, is characteristic for a chloroplast AK isoform. Like the chloroplast AK of maize, the activity with ATP/AMP as substrates was about two times higher than with ADP and the apparent K_m was about 10-times higher for ATP/AMP than for ADP. In contrast to the maize enzyme and many other eukaryotic AKs, both substrate binding sites showed an exceptionally high specificity for all three adenylate substrates, together with a rather low affinity, as judged by the apparent K_m-values. These differences at the substrate-binding sites are confirmed by a low sensitivity of the enzyme to the competitive AK inhibitor diadenosine pentaphosphate, i.e. high K_i-values.

INTRODUCTION

Adenylate kinase (AK, EC 2.7.4.3) catalyses a freely reversible transphosphorylation between adenine nucleotides ($MgADP + ADP \rightleftarrows MgATP + AMP$) [1]. This reaction is essential for biosynthesis of ADP from AMP [2] and for buffering of adenylate pools. It maintains a defined equilibrium between the three adenylates [3, 4] and acts as thermodynamic buffer [5]. More recently, AK was also implicated in high-energy phosphoryl transfer [6, 7], dynamic compartmentation of adenylates [8] and functional coupling to other proteins, such as the adenylate carrier at the mitochondrial envelope [9]. The enzyme has been extensively purified, characterized, sequenced and structurally analysed from animal tissues, yeast and some prokaryotes (e.g. [10, 11]). It was found to occur as a family of generally monomeric isoforms, consisting of short variants of M_r 21–23 k (cytosolic isoforms in vertebrates) and long variants of 24–31 k (mitochondrial isoforms in vertebrates and yeast, bacterial enzymes).

In our previous studies [12, 13] we showed that AK isoforms in cell cultures from tobacco (*Nicotiana tabacum* L. cv. Havana 425) occur in cytosol, mitochondria and chloroplasts. In this paper we report the purification to homogeneity of one of these isoforms and its catalytic properties. To date, several attempts have been made to purify AK from higher plants [14–16]. However, only two studies reported purification to homogeneity, mainly of a chloroplast isoform from the C_4-plant maize [17, 18]. This AK, which has recently been sequenced [19], is 60-times more abundant than AK of C_3-plants like tobacco [20]. As the maize enzyme has a specific function in C_4-metabolism and is immunologically different from chloroplast AK of C_3-plants [21], the tobacco enzyme is supposed to have other molecular and catalytic properties. Furthermore, AK isoforms are considered to play an important role in the energy metabolism of higher plants and their activity was found to be regulated by a number of different factors [13, 22, 23]. Therefore, we were interested in devising a purification protocol to permit structural and molecular studies.

*Author to whom correspondence should be addressed.
†Present address: Inst. für Zellbiologie, ETH, Zürich Hönggerberg, HPM F44, CH-8093 Zürich, Switzerland.

RESULTS AND DISCUSSION

Purification of adenylate kinase, molecular characteristics and subcellular localization

A soluble tobacco AK isoform was purified from 2-month old vegetative tobacco plants by a five-step procedure, including ammonium sulphate precipitation, three preparative and one analytical column chromatographic separations. A fraction of the crude extract, precipitated between 30 and 70% ammonium sulphate saturation and containing the entire AK activity, was separated by a DEAE-Sephacel anion-exchange column into three main activity peaks (Fig. 1a). AK activity of peak 1 was further purified by Blue Sepharose affinity chromatography, using a specific elution with the AK inhibitor diadenosine pentaphosphate (Ap_5A, Fig. 1b), gel filtration with Sephadex G-100 (Fig. 1c,d) and FPLC-based anion-exchange chromatography with Mono Q (Fig. 1e). A single activity peak was eluted from all these columns and only trace amounts of other proteins, probably copurified AK isoforms, were co-eluted from Mono Q. In a representative purification procedure (Table 1), the final preparation represented a 58-fold enrichment and had a specific activity of about 500 nkat mg^{-1} (ATP/AMP as substrates).

Denaturing SDS–PAGE of the pure enzyme revealed a single protein band of about M_r 28 k (Fig. 2, lane e). Thus, chloroplast AK from tobacco belongs, like mitochondrial AK (24–30 k, [10]) and chloroplast maize AK (24.8 k, [19]), to the large AK variants. Elution of the native enzyme from a calibrated Sephadex G-100 column showed a M_r of 30 k (Fig. 1d), demonstrating the monomeric structure of the protein. The homogeneity of the obtained AK preparation, especially the good separation from other AK isoforms, was confirmed by the detection of only a single AK activity band in native PAGE (results not shown) and IEF–PAGE separations (Fig. 3). The pure protein had a pI of about 4.45 by IEF–PAGE (Fig. 3, lane a) and corresponded to the second of three isoforms present in the crude leaf extract (Fig. 3, lane b). This isoform was detected in a previous study in extracts from highly purified chloroplasts (band 5 in [12]). The purified isoform also elutes in Mono Q separations at about the same salt concentration as an isoform present in extracts of purified chloroplasts (Fig. 1e).

Kinetic analysis of the purified enzyme

As observed for all nucleoside monophosphate kinases [24], the enzymatic reaction of AK in both directions was strictly dependent upon the concentration of magnesium. In our standard assays, using 1.8 mM ATP/2 mM AMP or 4.2 mM ADP [12], no activity was detectable without Mg^{2+} (in the presence of 0.1 mM EDTA), while activity reached a maximum at 3 mM in both assays and declined at higher Mg^{2+} concentrations, especially with ADP as substrate. Under conditions of maximal AK activity (3 mM Mg), 98% of ATP, 4% of AMP and 58% of ADP and were Mg-bound, as calculated by the corresponding stability constants [25]. This suggested that the true substrates of tobacco chloroplast AK are the MgATP/AMP and MgADP/ADP couples, as previously determined also in more detail for the maize chloroplast AK [17, 26].

The K_m-values of the purified enzyme (Table 2) determined for the three adenylate substrates were up to 15-times higher than those reported for chloroplast AK from maize [26], partially purified AK isoforms of *Chenopodium rubrum* [16] and most other AKs [1]. Two further characteristics were very similar to maize AK but different from many other eukaryotic AKs. First, the K_m for ATP was slightly lower than for AMP and both K_m values were about 10 times higher than that for ADP. Second, the maximal rate with ATP/AMP as substrates (about 500 nkat mg^{-1}) was at least two times higher than that with ADP (about 240 nkat mg^{-1}-corrected value, see Experimental). These kinetic parameters have to be considered together with intrachloroplastic adenylate concentrations, e.g. those measured for *Avena sativa* in the dark: 200 μM AMP, 600 μM ADP and 320 μM ATP [27]. Under these conditions, the AK is only saturated with ADP ($[ADP] \gg K_m$) but not with ATP ($[ATP] < K_m$) and therefore not yet working at the maximal ATP/AMP-rate, which could reach twice the rate with ADP. Upon illumination, AK could therefore efficiently remove ATP to keep the phosphorylation potential inside the chloroplast at a low level compared with the cytosol [28], thus allowing efficient photophosphorylation to take place.

A special characteristic of the purified tobacco AK was the high specificity for its natural substrates, the adenylate nucelotides (Table 3). Of the numerous other nucleotides tested, only CMP allowed a low but significant AK activity. The high substrate specificity may ensure compartmentation of the AK reaction separate from other potential nucleotide substrates. Furthermore, affinity for the competitive AK inhibitor Ap_5A was relatively low: inhibitor constants were 25 times higher than for maize AK (Table 2). This is unexpected, as substrate specificity for adenylates is high and Ap_5A is known to mimic the natural substrate couple ATP/AMP [29, 30]. The contradiction may be explained by a specific steric arrangement of the two substrate binding sites, enhancing substrate specificity but diminishing binding of Ap_5A, in which the adenine moieties are stereochemically limited in their flexibility.

EXPERIMENTAL

Materials. Tobacco plants (*N. tabacum* L. cv. Havana 425) were grown at a constant temperature of 22° with short days of 12 hr light (20 W m^{-2}, supplied by white fluorescent tubes type 244332, 40 W, Sylvania). Leaves were harvested from 3-month old plants, frozen and stored in liquid N_2 until use. Enzymes and substrates for activity tests were from

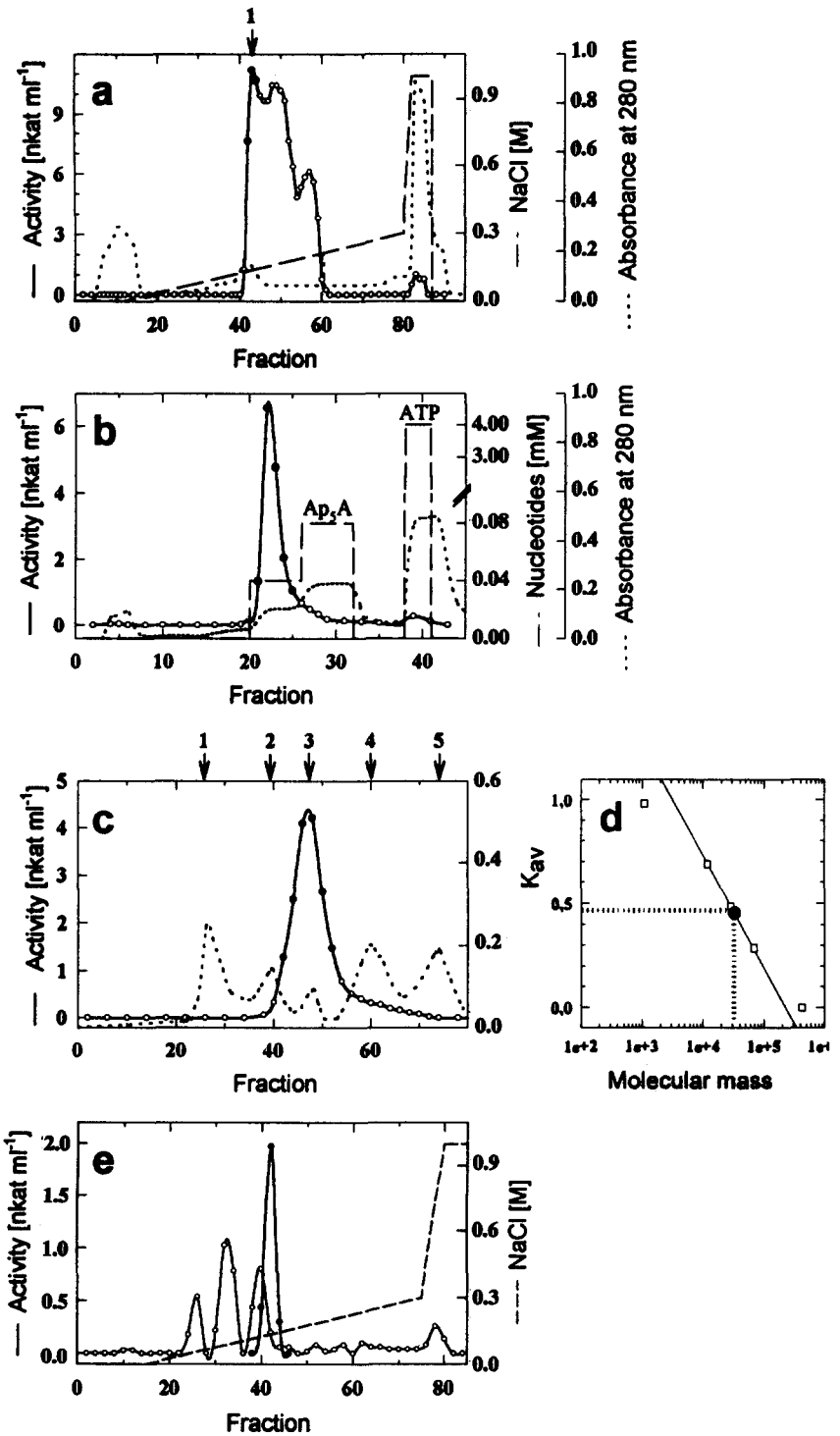

Fig. 1. Elution profiles of AK activity (bold lines) and protein (dotted lines) from columns used for AK purification: (a) DEAE Sephacel, elution with a linear salt gradient (dashed line), fraction size 3 ml; (b) Blue Sepharose, elution with an Ap_5A step gradient and ATP (dashed lines), fraction size 3 ml; (c) Sephadex G-100, fraction size 2 ml and the corresponding standard curve (d) for M_r determination and (e) Mono Q, elution with a linear salt gradient (dashed line), fraction size 2 ml. Active fractions indicated by filled circles, e.g. peak in (a), were pooled for the following chromatography step. The protein elution profile in (c) shows the M_r standards: (1) apoferritin, 443 k; (2) bovine serum albumin, 66 k; (3) carbonic anhydrase, 29 k; (4) cytochrome C, 12 k; (5) bradykinin, 1.06 k. The activity elution profile in (e, open circles) shows AK isoforms present in a purified chloroplast preparation. Columns (a) to (c) were run with 30 mM Tris-HCl (pH 8), 5 mM DTT, 5 mM EDTA and protease inhibitor mix, column (e) with 20 mM ethanolamine-HCl (pH 9.5).

Table 1. Purification summary of chloroplast AK from tobacco leaves

Fractions	Protein (mg ml^{-1})	Volume activity (nkat ml^{-1})	Specific activity (nkat mg^{-1})	Enrichment (-fold)	Yield (%)
1. Crude extract	0.52	4.2	8.8	1.0	100
2. Ammonium sulphate (30–70%)	5.80	63.1	10.9	1.2	110
3. DEAE-Sephacel (peak 1)	3.50	134.0	38.3	4.3	14
4. Blue Sepharose	0.045	17.5	389	44.2	8
5. Sephadex G-100	n.d.	2.9	n.d.	n.d.	n.d.
6. Mono Q	0.015	7.6	507	57.6	5

Data of a representative purification. Values are given for the pooled fractions, in steps 3 and 6 for concentrated pooled fractions of the indicated purification step. Note, that in steps 3 to 6, the values for yield correspond only to peak 1 of the DEAE-Sephacel separation. Activities were measured with ATP/AMP as substrates.

n.d., Not determined.

Boehringer Mannheim, protease inhibitors from Sigma, all other chemicals from Fluka, and chromatographic media and PAGE gels from Pharmacia.

Extraction and purification. The whole purification procedure was performed at temperatures between 0 and 4°. About 50 g fr. wt was reduced to a fine powder with mortar and pestle and extracted in 150 ml buffer containing 250 mM Tris-HCl, pH 7.4, 8 mM $MgSO_4$ 5 mM EDTA, 14 mM 2-mercaptoethanol, insoluble (PVPP, Polyclar AT) and soluble polyvinylpyrrolidone (PVP K30), both at 10% (w/w) relative to sample wt, and protease inhibitors (1 mM benzamide, 1 mM benzamidine, 5 mM α-amino-caproic acid and 1 μg ml^{-1} leupeptin). Purified chloroplasts were prepared and lysed in hypo-osmotic buffer as described [12]. Crude extract and lysed chloroplasts were centrifuged 20 min at 39 000 *g*, the supernatant prptd at 30% $(NH_4)_2SO_4$ saturation and centrifuged as before. After a second prptn at 70% $(NH_4)_2SO_4$ saturation and centrifugation, the pellet was resuspended in 20 ml run buffer (30 mM Tris-HCl, pH 7.4, 5 mM EDTA, 5 mM DTT and protease inhibitors) and desalted using different PD-10 columns (Pharmacia, Switzerland) equilibrated with the same buffer. The sample was then applied to a DEAE-Sephacel column (14 × 180 mm) and eluted at a flow rate of 0.33 ml min^{-1} in a linear salt gradient (0–0.3 M NaCl in run buffer, fr. size 3 ml). Three active fractions of activity peak 1 (88 nkat) were pooled and applied to a Blue Sepharose column (10 × 200 mm) and eluted at a flow rate of 0.33 ml min^{-1} with an Ap_5A step gradient (0–40–80 μM Ap_5A in run buffer, fr. size 3 ml). No significant AK activity was eluted at 80 μM Ap_5A or 4 mM ATP. The five most active fractions, eluting at 40 μM Ap_5A, were each concd with

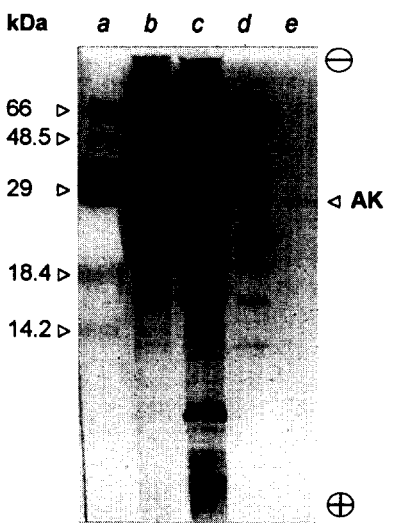

Fig. 2. SDS–PAGE from different AK purification steps. Crude extract (lane b), resuspended and desalted pellet of ammonium sulphate precipitation (lane c), pooled fraction of peak 1 from DEAE-Sephacel separation (lane d) and pooled fraction from the final Mono Q separation (lane e). The arrow indicates the purified protein. M_r standards (lane a) were: bovine α-lactalbumin, 14.2 k; bovine β-lactalbumin, 18.4 k; bovine carbonic anhydrase, 29 k; porcine heart fumarase, 40.5 k; bovine serum albumin, 66 k.

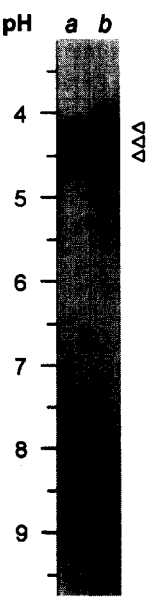

Fig. 3. IEF-PAGE (pH 4–5) of purified, concentrated tobacco AK (lane a) and concentrated, desalted leaf extract (lane b). Arrows indicate three AK isoforms with pI's at 4.5, 4.45 and 4.35. The first two are located in chloroplasts [12].

Table 2. Apparent kinetic constants of tobacco chloroplast AK

Substrates	K_m (Michaelis constant; μM)					K_i (inhibitor constant for Ap_5A; μM)				
	Tobacco	Maize [24]	*Chenopodium rubrum* [16]			Tobacco	Maize [24]	*Chenopodium rubrum* [16]		
	chl.	chl.	chl.	chl.	mit.	chl.	chl.	chl.	chl.	mit.
ATP/AMP (ATP varied)	954	25	760	620	650	n.d.	0.011	0.36	0.36	0.55
ATP/AMP (AMP varied)	1170	69	310	310	150	7.6	0.031	n.d.	n.d.	n.d.
ADP (ATP varied)	88	3–6	n.d.	n.d.	n.d.	1.7	0.080	n.d.	n.d.	n.d.

Mean values of two independent experiments.
n.d., Not determined.
Localization of the isoforms: chl., chloroplasts; mit., mitochondria.

Centricon-10 tubes (Amicon) to a final vol. of 2 ml. Pooled frs were applied to a Sephadex G-100 column (10 × 1000 mm) and eluted at 0.07 ml min^{-1} with run buffer (without protease inhibitors, fr. size 2 ml). The active frs were pooled, adjusted with NaOH to pH 9.5, applied to a Mono Q HR 5/5 column and eluted at 1 ml min^{-1} with a linear salt gradient (0–0.3 M NaCl in Q buffer: 20 mM ethanolamine-HCl, pH 9.5, 14 mM 2-mercaptoethanol).

Activity assay and protein determination. The AK forward reaction (ADP as substrate) was measured by the reduction of NADP with a coupled hexokinase-glucose-6-phosphate dehydrogenase system; the backward reaction (ATP/AMP as substrate) was measured by the oxidation of NADH with the coupled lactate dehydrogenase-pyruvate kinase system, against a reference without AMP to exclude phosphatase activities [12]. The amount of enzyme activity required to reduce 1 nmol NADP sec^{-1} or oxidize 1 nmol NADH sec^{-1} has been defined as 1 nkat. However, as two molecules ADP are formed from one ATP/AMP couple but only one molecule ATP from one ADP/ADP couple, activity with ADP as substrate has to be corrected by a factor of two. Protein was quantified according to ref. [31] using the Bio-Rad reagent microassay.

Analysis of kinetic properties. Concs of free and complexed adenylates were calculated by a computer program using algorithms published in ref. [32] and normalized stability constants suggested in ref. [25]. Apparent K_m values were determined with 0.1–7 mM ATP or AMP and 0.1–2 mM ADP as substrates under conditions where all ATP and half of ADP were Mg-complexed (concn ratios: $[MgSO_4]/[ATP] = 1.5$, $[MgSO_4]/[ADP] = 0.7$). K_i values were determined using two different inhibitor concns (10 μM and 20 μM Ap_5A). All K_m and K_i values were calculated by the ENZFIT software (Elsevier [33]).

Gel electrophoresis and staining. Ready-made IEF–PAGE plates (Pharmacia) were run and stained for AK activity as described in ref. [12]. SDS–PAGE with Excel gels (Pharmacia) according to the manufacturer's

Table 3. Substrate specificity of chloroplast AK isoforms

Substrate	Activities [%]	
	Tobacco	Maize [24]
ADP	100	100
CDP	2	7
GDP	0	0
UDP	0	0
ATP + AMP	100	100
+ CMP	7	10
+ GMP	0	0
+ UMP	0	0
CTP + AMP	1	13
GTP + AMP	0	3
UTP + AMP	0	12

Activities measured in the standard assay [12]. 100% corresponds to 3.8 nkat with ATP/AMP and 1.8 nkat with ADP (corrected value, see Experimental).

instruction, and gels were stained with silver stain plus reagents (Bio-Rad, Switzerland).

Acknowledgements—This investigation was supported by grant 31-9093.87/3.044-0.87 from the Swiss National Science Foundation. U.S. is recipient of a fellowship from the foundation E. & L. Schmidheiny (Geneva, Switzerland). We thank Dr R. Cassada (University of Freiburg, Germany) for English text correction.

REFERENCES

1. Noda, L. H. (1973) in *The Enzymes Vol. VIII, Group Transfer Part A* (Boyer, P. D., ed.) Vol. 3, p. 279. Academic Press, New York.
2. Kit, S. (1970) in *Metabolic Pathways* (Greenberg, D. M., ed.) Vol. 3, p. 70. Academic Press, London.
3. Atkinson, D. E. (1968) *Biochemistry* **7**, 4030.
4. Pradet, A. and Raymond, P. (1983) *Annu. Rev. Plant Physiol.* **34**, 199.
5. Stucki, J. W. (1982) in *Metabolic Compartmentation* (Sies, H., ed.) p. 39. Academic Press, London.
6. Bessman, S. P. and Carpenter, C. L. (1985) *Annu. Rev. Biochem.* **54**, 831.
7. Zeleznikar, R. J., Dzeja, P. P. and Goldberg, N. D. (1995) *J. Biol. Chem.* **270**, 7311.
8. Gellerich, F. N. (1992) *FEBS Letters* **297**, 55.
9. Fricaud, A.-C., Walters, A. J., Whitehouse, D. G. and Moore, A. L. (1992) *Biochim. Biophys. Acta* **1099**, 253.
10. Schulz, G. E. (1987) *Cold Spring Harb. Symp. Quant. Biol.* **52**, 429.
11. Schricker, R., Magdolen, V. and Bandlow, W. (1992) *Mol. Gen. Genet.* **233**, 363.
12. Schlattner, U., Wagner, E., Greppin, H. and Bonzon, M. (1993) *Plant Physiol. Biochem.* **31**, 815.
13. Schlattner, U., Wagner, E., Greppin, H. and Bonzon, M. (1994) *J. Plant Physiol.* **144**, 400.
14. Rodionova, M. A., Kuz'min, A. N., Kholodenko, N. Y. and Makarov, A. D. (1976) *Fiziol. Rast.* **23**, 847.
15. Kholodenko, N. Y., Kartashov, I. M. and Makarov, A. D. (1983) *Biokhimiia* **48**, 411.
16. Haertlé, U. (1977) Ph.D. thesis, Albrecht Ludwig University, Freiburg i. Br., Germany.
17. Kleczkowski, L. A. and Randall, D. D. (1986) *Plant Physiol.* **81**, 1110.
18. Deppert, W. R. and Wagner, E. (1994) *J. Plant Physiol.* **145**, 17.
19. Schiltz, E., Burger, S., Grafmüller, R., Deppert, W. R., Haehnel, W. and Wagner, E. (1994) *Eur. J. Biochem.* **222**, 949.
20. Hatch, M. D. (1982) *Aust. J. Plant Physiol.* **9**, 287.
21. Kleczkowski, L. A. and Randall, D. D. (1987) *J. Exp. Bot.* **38**, 1440.
22. Schlattner, U., Wagner, E., Greppin, H. and Bonzon, M. (1994) *Biol. Plant.* **36**, S367.
23. Schlattner, U., Wagner, E., Greppin, H. and Bonzon, M. (1994) *Cell Biol. Int.* **18**, 569.
24. Kleczkowski, L. A. and Randall, D. D. (1991) *J. Exp. Bot.* **42**, 537.
25. O'Sullivan, W. J. and Smithers, G. W. (1979) *Methods Enzymol.* **63**, 294.
26. Kleczkowski, L. A., Randall, D. D. and Zahler, W. L. (1990) *Z. Naturforsch.* **45c**, 607.
27. Hampp, R., Goller, M. and Ziegler, H. (1982) *Plant Physiol.* **69**, 448.
28. Stitt, M., Lilley, R. M. and Heldt, H. W. (1982) *Plant Physiol.* **70**, 971.
29. Egner, U., Tomasselli, A. G. and Schulz, G. E. (1987) *J. Mol. Biol.* **195**, 649.
30. Lienhard, G. E. and Secemsky, I. I. (1973) *J. Biol. Chem.* **248**, 1121.
31. Bradford, M. M. (1976) *Analyt. Biochem.* **72**, 248.
32. Goldstein, D. A. (1979) *Biophys. J.* **26**, 235.
33. Leatherbarrow, R. J. (1990) *TIBS* **15**, 455.