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MULTIPLE ADENYLATE KINASE ISOFORMS IN HIGHER PLANTS

BY

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ABSTRACT

Multiple adenylate kinase isoforms in higher plants. - A family of adenylate kinase (AK) isoforms with different subcellular localization has been characterized in vertebrates and yeast. In higher plants, however, the number and localization of these isoforms are still controversially discussed. To analyze further plant AK isoforms, we present here an efficient extraction procedure combined with different separation techniques (starch gels, native PAGE, IEF-PAGE, gel filtration and anion exchange chromatography) and detection methods (enzyme activity, Western blot). It is shown that, in tobacco plants, the same AK isoforms occur as in tobacco tissue cultures (*Plant Physiol. Biochem.* 31: 815-815), namely one cytosolic and two ore more chloroplast isoforms. Their activities were organ-specific and varied depending whether the forward or backward reaction was considered. Similar AK activities were separated by IEF-PAGE from several other plant species, indicating that an AK isoform family also exists in higher plants.

INTRODUCTION

Adenylate kinase (AK, ATP:AMP phosphotransferase) catalyzes the interconversion of adenine nucleotides and was found to be a ubiquitous and essential enzyme. It plays a crucial role in the biosynthesis of ADP from AMP (KIT, 1970) and in maintaining homeostasis of cellular adenylate pools (RAYMOND *et al.*, 1987). Recent results indicate that AK isoforms with different subcellular localization also play an active role in cellular energy transduction (FRICAUD *et al.*, 1992; SAVABI, 1994; ZELEZNIKAR *et al.*, 1995). Characterization of AK isoforms is therefore of basic importance for understanding the physiological functions of AK.

AK isoforms have been studied in detail in vertebrates and yeast (SCHULZ, 1987). They are small, monomeric proteins (20 - 30 kDa) localized in the cytosol, the intermembrane space of the mitochondrial envelope and the mitochondrial matrix. In higher plants, the number and localization of AK isoforms is still controversial. AK activity is

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mostly localized in chloroplasts (MURAKAMI & STROTMANN, 1978; HAMPP *et al.*, 1982; HATCH, 1982; DEPPERT & WAGNER, 1994) and characterized as a single isoform (KLECZKOWSKI & RANDALL, 1986, 1987; WENDEL *et al.*, 1988) or variety-dependent allelozymes of one gene locus (SMED *et al.* 1989). Only a few localization studies indicate the presence of AK activity in the cytosol (RODIONOVA *et al.*, 1978; MOORE *et al.*, 1984) or in mitochondria (BIRKENHEAD *et al.*, 1982; STITT *et al.*, 1982). These results are in conflict with studies of AK in *Chenopodium rubrum* (WAGNER *et al.*, 1983; SCHLATTNER, 1990) and tobacco (SCHLATTNER *et al.*, 1993, SCHLATTNER *et al.*, 1996a, b) that permitted the identification of one isoform associated with the cytosol, one with the mitochondrial intermembrane space and at least two in different chloroplast compartments. In addition, recent evidence obtained by cDNA cloning support the existence of multiple AK genes in higher plants (KAWAI *et al.*, 1992; NEWMAN *et al.*, 1994).

Due to these obvious contradictions, it appeared necessary to re-examine the AK isoform activities of different higher plant species, especially in tobacco, where their localization is already known (SCHLATTNER *et al.*, 1993). Since AK isoforms may have remained undetected because of low activities and tissue-specific expression, we improved the extraction procedure, then used activity assays and antibodies for detection and examined different plant organs. Moreover, as the use of a single separation method alone is an imperfect detector of isoforms, we compared several electrophoretic and column chromatographic methods which have proved to be useful in separating vertebrate AKs (e.g. CRISS *et al.*, 1970; KHOO & RUSSELL, 1972; KUROKAWA *et al.*, 1990).

MATERIALS AND METHODS

Materials

Rice (*Oryza sativa*) was grown in a natural environment at the Botanical Garden (University of Geneva) and harvested in late summer. *Chenopodium rubrum* seedlings were germinated as desribed elsewhere (SCHLATTNER *et al.*, 1996a) and harvested after 10 d. All other plants were grown in a growth chamber at a constant temperature of 22°C with a 12 h : 12 h photoperiod (20 W m^{-2}, supplied by white fluorescent tubes type 244332, 40 W, Sylvania, USA). Different plant organs were harvested from about 2-month old plants, being vegetative (*Arabidopsis thaliana*, *Nicotiana tabacum*, *Spinacea oleracea*, *Sinapis alba*), flowering (*Phaseolus vulgaris*) or fruiting (*Glycine max*). Maintenance of the tobacco cell line 275N has been described elsewhere (SCHLATTNER *et al.*, 1994). Nucleotides and enzymes were from Boehringer Mannheim (Switzerland) and protease inhibitors from Sigma (Buchs, Switzerland). All other chemicals were from Fluka (Buchs, Switzerland). Q-Sepharose fast flow, Mono Q for FPLC, IEF-PAGE plates and Clean Gels were purchased from Pharmacia (Dübendorf, Switzerland).

Enzyme assays

The AK forward direction (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 substrates) was measured by the oxidation of NADH with the coupled lactate dehydrogenase - pyruvate kinase system, against a reference without AMP to exclude phosphatase activities, as described by SCHLATTNER *et al.* (1993). The amount of enzyme activity required to reduce (or oxidize) one nmol NADP (NADH) per second is defined as one nkat.

Protein extraction

Protein for native gel electrophoresis was extracted according to the procedure described in SCHLATTNER *et al.* (1993) with slight modifications. 200 mg tissue fresh weight were reduced to fine powder in a microdismembrator (Braun, Melsungen, Germany) and extracted in 200 µl extraction buffer containing 250 mM Tris-HCl, pH 7.4; 8 mM $MgSO_4$; 5 mM EDTA; 10 mM DTT; insoluble and soluble polyvinyl-pyrrolidone (Polyclar AT and PVP K30, respectively), both at 10% (w/w) related to sample weight, and protease inhibitors (5 mM α-amino-caproic acid, 1 mM benzamide, 1 mM benzamidine, 1 µg ml^{-1} leupeptin and 10 µg ml^{-1} pepstatin). The centrifuged crude extract was concentrated to a final volume of 50 µl with Centricon-10 tubes (exclusion limit 10 kDa; Amicon, U.S.A, centrifugation for 30 min at 5,000 x g). Low molecular weight compounds (phenolics, alkaloids, salt) were removed by diluting the extract twice with 150 µl gel running buffer (50 mM Tris-HCl, pH 7.4, further composition as for extraction buffer). Extracts with low volume activities (< 10 nkat ml^{-1}) were reconcentrated up to 50 µl and final activities were between 5 and 40 nkat ml^{-1} using ATP/AMP as substrates. For further prepurification, 200 µl centrifuged crude extract was applied to a Sephadex G-50 column (20 ml bed volume) and eluted with gel running buffer at 0.7 ml min^{-1}. Active fractions were pooled and concentrated with Centricon-10 tubes. For anion exchange chromatography, 2 g tissue fresh weight were extracted in 10 ml extraction buffer as described by SCHLATTNER *et al.* (1996a).

Electrophoretic methods, gel native staining and Western blots

IEF-PAGE and native PAGE were carried out on ready made IEF-PAGE plates as described in SCHLATTNER *et al.* (1993) and on Clean Gels (incubated with buffer at pH 8.9) according to the supplier's instructions. Samples of similar activity were applied by adjusting the sample volume to between 10 and 20 µl. Electrophoresis on starch gels was carried out on small gel slabs (12x12x0.8 mm) containing 14% (w/v) starch and 2% (w/v) sucrose in 8 mM Tris / 3 mM citrate (pH 6.7). 20 µl sample were mixed with application medium (1.17 M sucrose, 5% w/w Sephadex G-200), applied in gel slots and run for 5 h at 150 V with 220 mM Tris / 44 mM citrate (pH 6.4) as electrode buffer. AK activity was detected by native staining with ADP and ATP/AMP as

substrates (SCHLATTNER *et al.*, 1993). For Western blot analysis of IEF-gels, proteins were electrotransferred to reinforced nitrocellulose membranes (BA-S 85, Schleicher & Schuell, Dassel, Germany) with the NovaBlot kit for Multiphor II (Pharmacia) according to the supplier's instructions and TOWBIN *et al.* (1979). AK was detected with polyclonal antibodies to the maize chloroplast AK as described by KLECZKOWSKI & RANDALL (1987).

Column chromatographic methods

Crude extracts were applied twice to PD-10 columns (Pharmacia) for desalting and buffer exchange to Q-buffer (20 mM ethanolamine, pH 9.5, and 14 mM mercapto-ethanol). Samples for Mono Q were treated with RNase (DEPPERT *et al.*, 1992) and further prepurified by a batch run on a DEAE cellulose column (20 ml, elution with 0.5 M NaCl) and subsequent desalting with PD-10 columns. Q-Sepharose fast flow (100 ml bed volume) and Mono Q HR 5/5 (FPLC system) were run with Q-buffer using a flow rate of 1 ml min^{-1}. AK activity was entirely eluted by a linear NaCl-gradient of 0 to 0.4 M NaCl, applied over 450 ml (Q-Sepharose) or of 0 to 0.3 M NaCl applied over 60 ml (Mono Q), and collected in fractions of 10 or 2 ml, respectively.

RESULTS AND DISCUSSION

Extraction of AK

Maintaining the native state of AK in concentrated plant extracts, as used in this study, depends on the efficient elimination of certain low molecular mass compounds (i.e. phenolics and alkaloids) and on the inhibition of protease activities (LOOMIS, 1974; GEGENHEIMER, 1990). Different additives to a basic buffer were examined for their capacity to extract and maintain AK activity (Fig. 1). Most efficient in extracting AK activity (Fig. 1) and in eliminating phenolic compounds (Fig. 2) was PVP. Optimal results were obtained by addition of soluble and insoluble PVP, both at a concentration of 10-15% in relation to sample weight (results not shown). Compared to soluble PVP, other additives to the basic buffer (already containing 10% insoluble PVP) like serum albumin, different compounds reversing quinone formation (sodium metabisulfite, DTT, ascorbic acid) or phenoloxidase inhibitors (sodium tetraborate, diethyldithiocarbamate) had no supplementary effect. Further removal of low molecular weight compounds and salts for subsequent IEF or ion exchange chromatography was obtained by gel filtration (Sephadex G-50) or by repeated ultrafiltration in the final concentration step. Both methods reduced phenolic compounds by 70-95% as judged by 300 nm/280 nm extinction ratio (Fig. 2). Protection from proteolytic degradation was achieved using a protease inhibitor mix containing serine, thiol and acidic protease inhibitors (Fig. 1). Addition of detergents (e.g. Triton X-100, Fig. 1) was avoided because of co-extraction of phenolics which are difficult to remove for subsequent separation techniques.

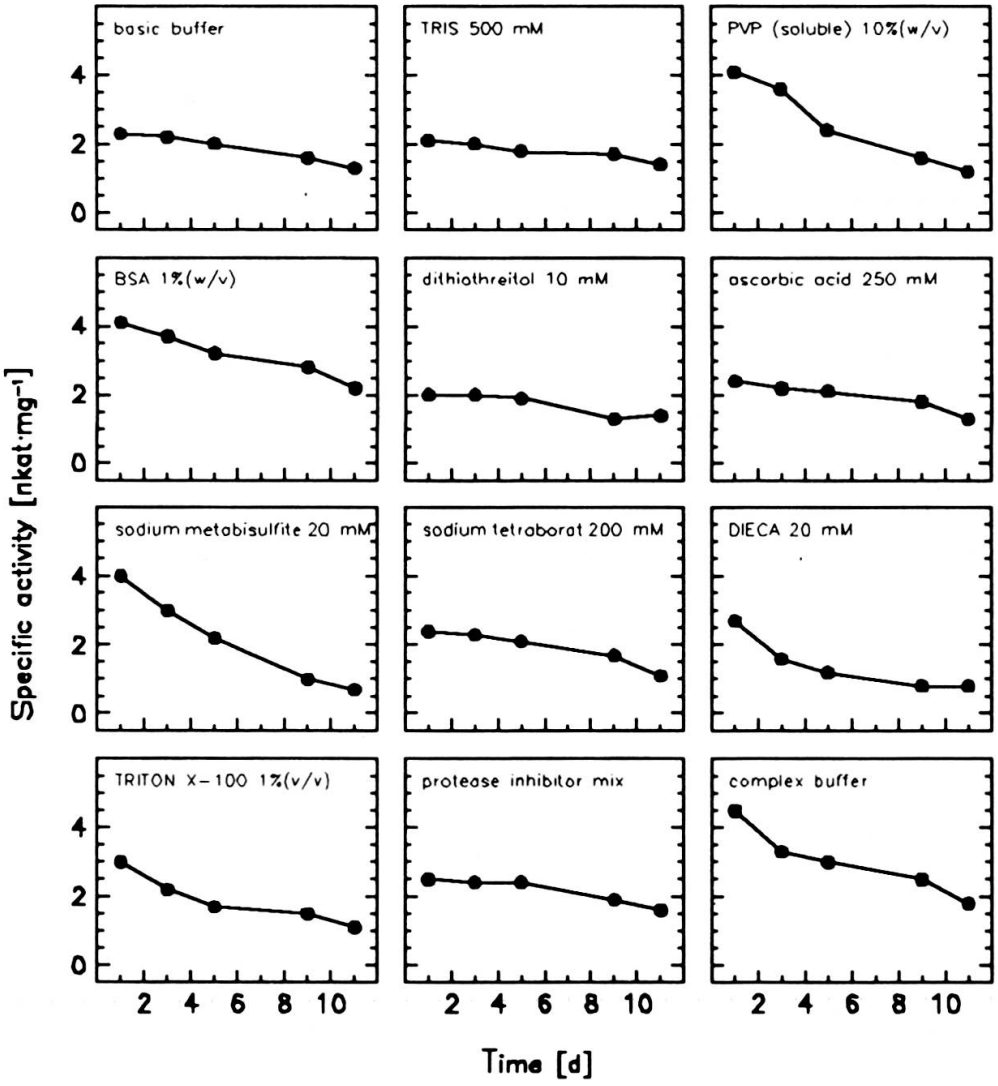

FIG. 1.

Influence of buffer composition on extractability of AK activity and its stability in crude extracts at 4°C. Basic buffer containing 250 mM Tris-HCl (pH 7.4), 8 mM $MgSO_4$, 5 mM EDTA, 1% mercaptoethanol and 10% (w/w sample) insoluble polyvinylpyrrolidone (PVPP, Polyclar AT) was supplemented with different additives. The extraction buffer used in this study (complex buffer) was basic buffer with mercaptoethanol replaced by 10 mM DTT and additional 10% (w/w sample) soluble PVP and protease inhibitor mix. Abbreviations: soluble PVP (soluble polyvinylpyrrolidone, PVP K30); BSA (bovine serum albumin); DTT (dithiothreitol); DIECA diethyldithiocarbamate); protease inhibitor mix (see materials and methods).

Electrophoretic separation of AK from tobacco tissues

Starch gels have been often used for isoform typing because they can be sliced horizontally into several replicates for multiple staining of the same gel (WENDEL & WEEDEN, 1989; KEPHART, 1990) They were successfully applied to separate AK isoforms from vertebrate tissues (e.g. KHOO & RUSSELL, 1972) and are especially useful

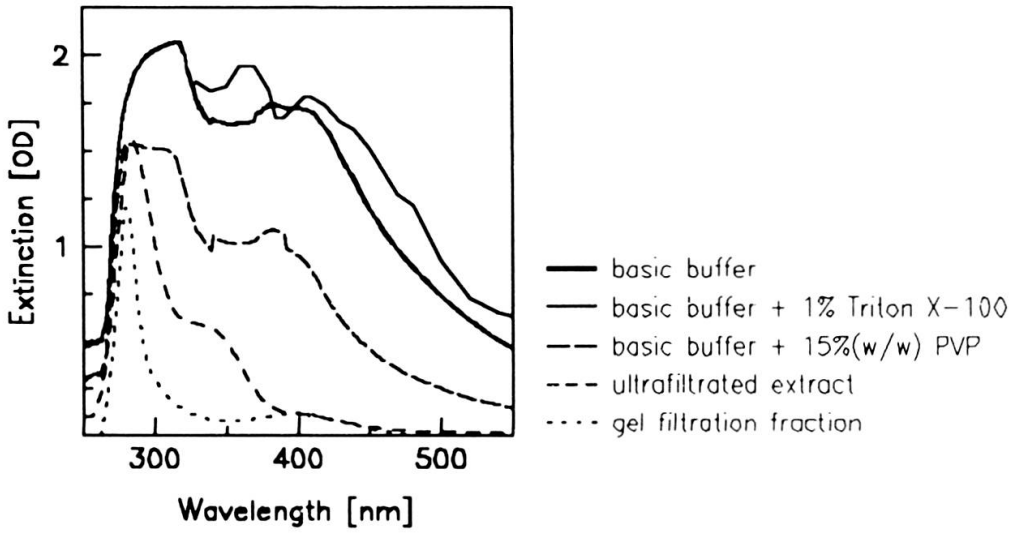

FIG. 2.

Reduction of pigments and phenolic compounds by PVP and ultrafiltration. Absorption spectra of crude extracts between 200 and 800 nm. Basic buffer (see Fig.1) was supplemented either with 15% (w/w) insoluble PVP, 1% Triton X-100 or prepurified by ultrafiltratration or gel filtration with Sephadex G-50 (see materials and methods).

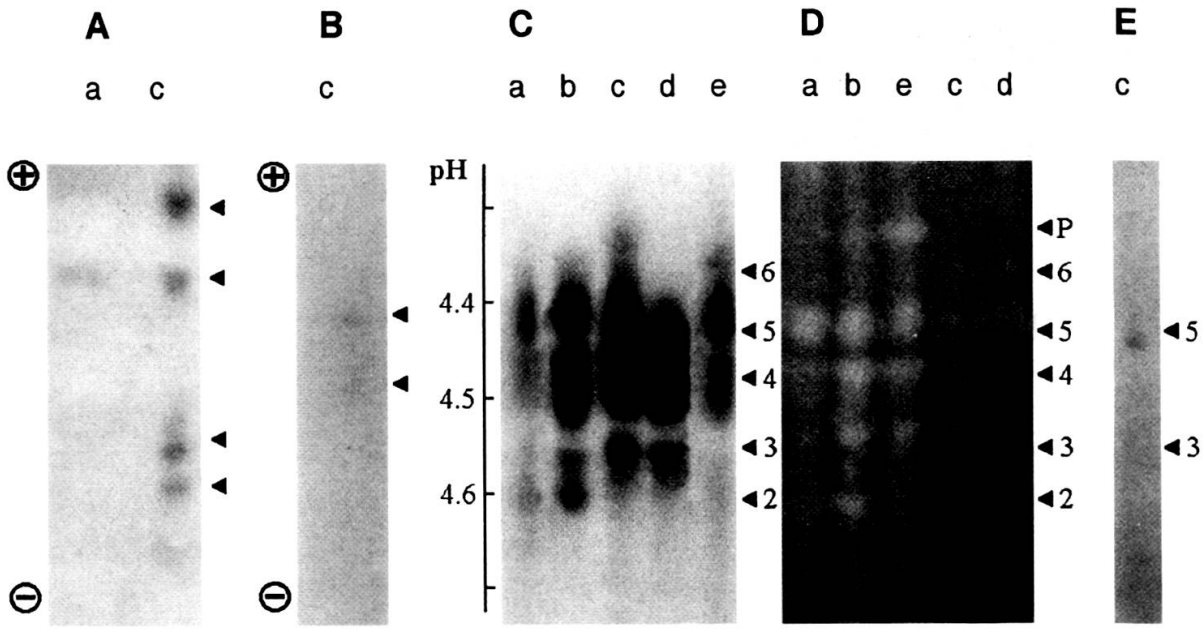

FIG. 3.

Separation of AK isoforms from tobacco by different electrophoretic methods. (A) starch gel electrophoresis, (B) native PAGE, (C-E) IEF-PAGE pH 4.0-5.0. Lanes: (a) 275N tissue culture, (b) 275N suspension culture, (c) leaves, (d) stem, (e) roots. The numbered bands correspond to AK isoforms localized in cytosol (2) and chloroplasts (3-5) and to phosphatase isoforms (P). AK was detected by native staining using ADP (A-C) or ATP/AMP (D) as substrates or by Western blot using maize anti-AK antibodies (E). For details see materials and methods.

for unambiguous identification of AK by four parallel native stainings (ADP and ATP/AMP as substrates, each one in the absence and in the presence of the specific AK inhibitor Ap_5A, see SCHLATTNER *et al.*, 1993). However, the major drawbacks of this method are poor reproducibility and low protein load per gel slice. In starch gels run at pH 7.5, four activity bands were detected in tobacco leaf extracts (40 nkat ml^{-1}), but only one in extracts of low volume activity like roots, stems and tissue cultures (5-10 nkat ml^{-1}, Fig. 3A).

Concentration of samples in the stacking zone and the high resolving power of native polyacrylamide gels (PAGE), run at pH 8.9, were supposed to overcome these limitations. By this method, two weak activity bands could be separated from tobacco leaves (Fig. 3B) and cell culture extracts. Since two AK activity peaks were also eluted from Sephadex G-50 columns (results not shown), two different molecular mass forms of AK may be present in the extracts, corresponding to short and long variants of AK (SCHULZ, 1987) or to free and RNA-bound AK (DEPPERT *et al.*, 1992).

Since vertebrate AK isoforms were frequently separated by their isoelectric points (e.g. CRISS *et al.*, 1970; KUROKAWA *et al.*, 1990; SCHLATTNER *et al.*, 1993), PAGE was combined with the resolving power and reproducibility of isoelectrofocusing (IEF). IEF-PAGE in the range of pH 3.5-9.5 and, especially, in a narrow-range pH-range (pH 4.0-5.0), allowed the detection of more numerous activity bands. Native staining with ADP (Fig. 3C) and ATP/AMP as substrates (Fig. 3D) revealed up to five activity bands in common (cf. numbers 2 to 6) which were also sensitive to Ap_5A (results not shown). Moreover, the activity bands detected for tobacco roots, stems and leaves were identical to those from tissue and suspension cultures of cell line 275N. These bands were already characterized as isoforms localized in either the cytosol (band 2), different chloroplast compartments (bands 3, 4 an 5) or the mitochondrial envelope (band 1, only separated by IEF-PAGE pH 3.5-9.5, SCHLATTNER *et al.*, 1993). However, it is significant that isoform activities varied between ADP- and ATP/AMP-staining as well as among different plant organs (Fig. 3D,E). For example, band 4 was much better stained with ADP as substrate. Chloroplast isoform activities were present, to a different extent, in all tissues and cell cultures. Their presence in roots indicates that they occur in different types of plastids. By contrast, the cytosolic isoform activity was only detectable in cell cultures and roots, in the latter only by ATP/AMP staining (Fig. 3D). Finally, for all examined extracts, we failed to detect the mitochondrial isoform in IEF-PAGE pH 3.5-9.5 by activity staining (results not shown), a negative observation already made for growing tissue cultures in a previous study (SCHLATTNER *et al.*, 1993). However, mitochondrial AK activity was measured in intact and highly purified tobacco mitochondria (SCHLATTNER *et al.*, 1993). Since mitochondrial AK has been shown to be partly membrane-bound (ARRON *et al.*, 1978; SCHLATTNER *et al.*, 1994), the absence of activity in tobacco extracts may be explained by a tight membrane-binding of AK and/or a destabilization effect on solubilized AK analogous to themolabile AK of *E. coli* (GOELZ & CRONAN, 1982).

AK isoforms can also be distinguished by antibodies (KUROKAWA *et al.*, 1990). For plant AK, polyclonal antibodies are available against purified maize leaf chloroplast AK

(KLECZKOWSKI & RANDALL, 1986, 1987). In Western blots of IEF-PAGE (pH 4 to 5), these antibodies identified only bands 3 and 5 (Fig. 3D), thus providing additional evidence for their chloroplast origin, but also confirming the presence of immunologically distinct AK isoforms (KLECZKOWSKI & RANDALL, 1987). However, in cell cultures, roots and stems, an additional protein with an apparent isoelectric point above pH 5 reacted with the maize antibodies (results not shown). This isoform might correspond to enzymatically inactive mitochondrial AK.

Altogether, the results obtained demonstrate the presence of AK isoforms in different tobacco tissues, an organ-specific activity pattern and a variability of the rate of the forward and backward reaction among isoforms as measured by native gel staining.

Column chromatographic separation of AK from tobacco tissues

Frequently used for the purification of vertebrate, yeast and bacterial AK, column chromatographic methods were also applied to separate AK isoforms from plants (WAGNER *et al.*, 1983; DEPPERT & WAGNER, 1994; SCHLATTNER *et al.*, 1996a) and could serve to confirm the tobacco AK isoform pattern separated by electrophoresis. From tobacco leaf extracts, three activity peaks were already separated with DEAE Sephacel (SCHLATTNER *et al.*, 1996b). The strong anion exchangers Mono Q and Q-Stepharose revealed different separation patterns (Fig. 4). Optimal separation conditions for both columns were found at pH 9.5 (binding of the entire AK activity) and an elution with a flat salt gradient (0 to 0.3 M NaCl). However, Q-Sepharose resolved only one mainpeak and one smaller peak (Fig. 4A). By contrast, Mono Q separated at least four activity peaks (Fig. 4B) with the two main peaks corresponding to chloroplast isoforms (SCHLATTNER *et al.*, 1996b). It may be summarized that separations by native PAGE or Q-Sepharose fast flow give an underestimation of existing AK isoforms in tobacco, while starch gels, IEF-PAGE and Mono Q-ion exchange columns have a higher resolving power.

IEF separations from different species

IEF-PAGE (pH 3.5-9.5) was chosen for separating extracts from several higher plants. For all species examined, two to six activity bands were detected although with very different relative activities (Fig. 5). From *Chenopodium rubrum*, where five different isoforms have already been characterized (WAGNER *et al.*, 1983; SCHLATTNER, 1990), four activity bands were separated. Organ-specific patterns were observed in rice (4 bands in leaves, 2 bands in roots), bean (6 bands in leaves, 2 bands in flowers) and soybean (4 bands in leaves, 5 bands in fruits). All detected activities had an isoelectric point between pH 4 and 7. Presumably, these activities correspond, at least in part, to isoforms with different subcellular localization.

Multiple AK isoforms

The existence of multiple AK isoforms in higher plants is in contradiction to results obtained by some localization studies (MURAKAMI & STROTMANN, 1978; HAMPP

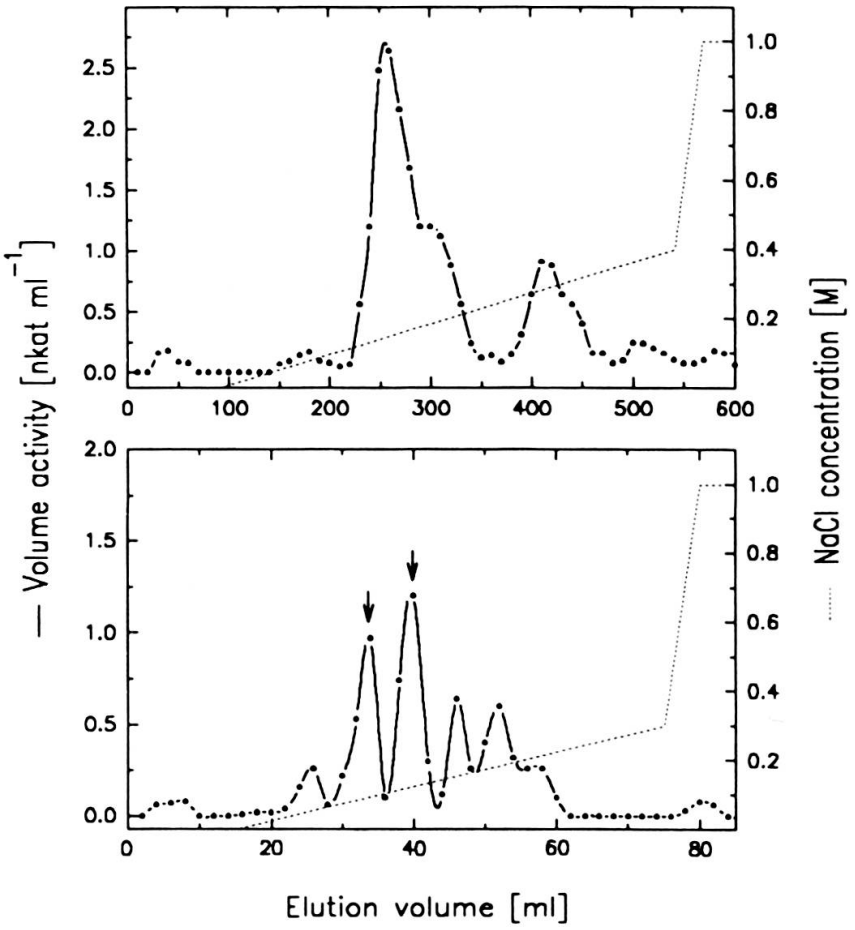

FIG. 4.

Separation of AK isoforms from tobacco leaves by anion exchange chromatography with Q-Sepharose fast flow (A) and Mono Q (B). Mature leaves were from 3-month old plants maintained in short day (vegetative growth). (A) 291 nkat applied, 241 nkat recovered (83%). (B) 15.5 nkat applied, 13.5 nkat recovered (93%); arrows indicate chloroplast isoforms. For further details see materials and methods.

et al., 1982; HATCH, 1982), protein purification and immunodetection (KLECZKOWSKI & RANDALL, 1986, 1987) and population genetic analysis (WENDEL *et al.*, 1988; SMED *et al.*, 1989). These studies indicated the existence of one single AK isoform or allelozymes of one gene, localized in soluble and particulate fractions of chloroplasts. However, different subcellular localization of AK activity has been shown for some plants (RODIONOVA *et al.*, 1978; BIRKENHEAD *et al.*, 1982; STITT *et al.*, 1982; MOORE *et al.*, 1984) and immunologically distinct isoforms were indicated by immunoprecipitation (KLECZKOWSKI & RANDALL, 1987). Finally, similar to this study, different isoforms were separated for *Chenopodium rubrum* (WAGNER *et al.*, 1983; SCHLATTNER, 1990; SCHLATTNER *et al.*, 1996a, b). Very recently, strong support for multiple AK isoforms has been obtained from ongoing EST (expressed sequence tags) sequencing. In *Arabidopsis thaliana*, maize and rice, different AK cDNAs were detected which show significant homology to cytosolic and mitochondrial isoforms of vertebrates and to the maize chloroplast isoform (cf., EST databases). For example, two different AK cDNA

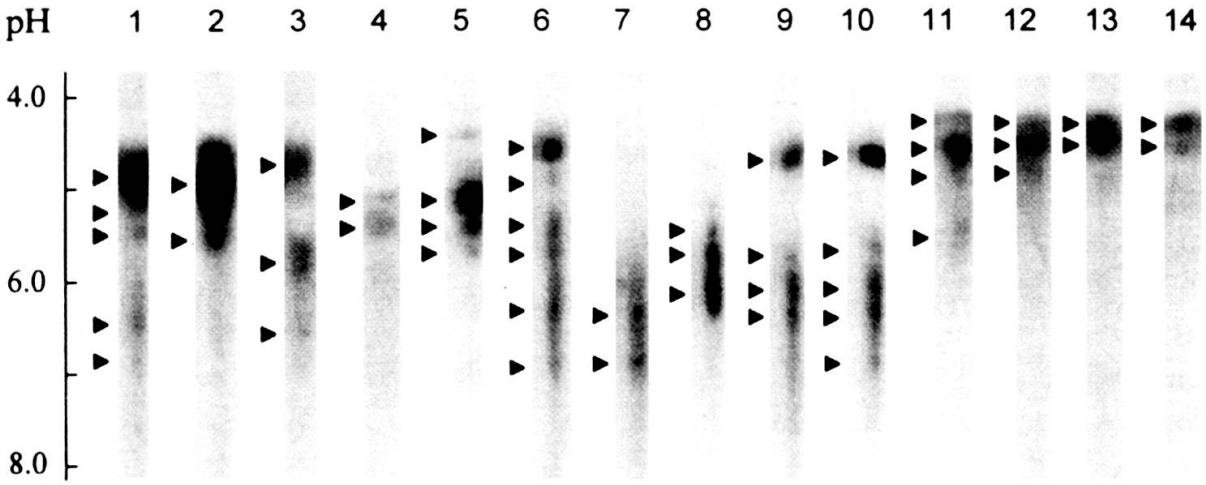

FIG. 5.

Separation of AK isoforms from different higher plants by IEF-PAGE pH 3.5-9.5: (1) *Arabidopsis thaliana leaves*, (2) spinach (*Spinacea oleracea*) leaves, (3) *Sinapis alba* leaves, (4) rice (*Oryza sativa*) roots, (5) rice leaves, (6) bean (*Phaseolus vulgaris*) leaves, (7) bean flowers, (8) *Pharbitis nil* leaves, (9) soybean (*Glycine max*) leaves, (10) developing soybean fruits, (11) *Chenopodium rubrum* seedlings, (12) tobacco (*Nicotiana tabacum*) cell line 275N, (13) tobacco leaves, (14) tobacco roots. AK was detected by native staining with ADP as substrate. For further details see materials and methods.

clones from rice roots show homology to mitochondrial isoforms of vertebrates and are localized mainly in the cytosol (KAWAI *et al.*, 1992; KAWAI & UCHIMIYA, 1995). Furthermore, at least four different AK cDNAs were detected in etiolated seedlings of *Arabidopsis thaliana* (cf. AAtDB cDNA database, internet-address: weeds.mgh. harvard.edu).

The apparent disagreements concerning the number of plant AK isoforms may be simply due to differences in material and methods chosen. For example, most earlier studies exclusively used photosynthetically active leaves or maize plants where one single chloroplast AK is especially abundant because of its specific function in C_4-metabolism (HATCH, 1982). As has been demonstrated in this paper, the number of isoforms is species dependent and varies with the tissue chosen. In addition, physiological and environmental conditions can modify number and activity of isoforms (e.g. WAGNER *et al.*, 1983; SCHLATTNER *et al.*, 1994). As we showed here, an essential point for tracking and distinguishing isoform activities is the choice of suitable extraction and separation methods, since AK isoforms have very similar molecular properties. Further, it is essential to perform activity measurements with ADP and ATP/AMP as substrates. However, AK isoforms may remain undetected due to very low activities (e.g. cytosolic NMP kinase in yeast, SCHRICKER *et al.*, 1992) or to a tight binding to membranes (ARRON *et al.*, 1978).

In the study presented here, the occurrence of multiple AK isoforms with organ-specific activity and different subcellular localization was confirmed for tobacco plants and suggested as a suitable model for other higher plants. This, together with recent molecular evidence on multiple AK genes, supports the existence of an AK isoform family in higher plants.

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RÉSUMÉ

Les isoformes de l'adenylate kinase (AK) ayant des localisations subcellulaires différentes, constituent une famille bien caractérisée chez les vertébrés et la levure. En revanche, chez les plantes supérieures, le nombre et la localisation des isoformes sont encore incertains. Cette étude présente une méthode d'extraction efficace des AK des plantes. Celle-ci est combinée avec différentes techniques de séparation (gels d'amidon, PAGE natif, IEF-PAGE, tamisage moléculaire et chromatographie d'échangeurs d'anions) et quelques méthodes de détection (activité enzymatique, Western blot). Il peut ainsi être démontré que les plantes entières de tabac contiennent les mêmes isoformes que les cultures cellulaires (*Plant Physiol. Biochem.* 31: 815-815), à savoir une isoforme cytosolique et au moins deux isoformes chloroplastiques. Leurs activités dépendent du tissu examiné et diffèrent selon que la réaction directe ou inverse est considérée. Des activités similaires ont été séparées dans plusieurs autres espèces végétales, indiquant ainsi qu'il existe aussi une famille d'isoformes de l'AK chez les plantes supérieures.

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