Table 2
Summary of the purification scheme of casein kinase IIB from *A. thaliana*

Step	Volume (ml)	Protein (mg)	Activity (U)	Specific activity (U/mg)	Purification-fold
Homogenate	1000	18 670	21 700	1.16	1.00
$(NH_4)_2SO_4$ ppt	56	920	1487	1.61	1.39
DEAE-Sepharose	18	20	107	5.35	4.62
Protein-Pak Q	0.5	0.075	6.85	91.33	78.73

One unit incorporates 1 pmol of phosphate into β-casein in 1 min at 30°C.

and regulatory subunits, respectively, as deduced from the *Arabidopsis* cDNA sequences [10,11] (Fig. 2). From these results we conclude that CKIIB is a form composed only by catalytic subunit, and CKIIA is an oligomeric form composed by both catalytic and regulatory subunits.

Two different β-subunits had been cloned from *Arabidopsis thaliana* being the only organism, except for yeast, where a heterogeneity for the β-subunit has been found [11]. Their deduced molecular mass is a little higher than what we found by Western blot analysis (32 versus 27 kDa). This can be explained by an anomalous mobility of the protein on SDS/PAGE, due to its structure. In fact, β- and β'-subunits from *Arabidopsis* have an unusual amino-terminal extension with no homology with other β-subunits. An anomalous mobility on SDS/PAGE has been also described for a β-subunit in yeast [19].

In order to corroborate our inmunological data showing a seemingly monomeric structure for the CKIIB form, we performed gel filtration chromatography in a Protein Pak 125 column of a fraction of maximal activity from the DEAE-Sepharose chromatography. Fractions collected were assayed by activity toward β-casein and analyzed by SDS/PAGE followed by autoradiography. The casein kinase II activity eluted at an apparent molecular weight of 55 000 Da (Fig. 3). Assuming a globular shape of the protein, this result agrees with a monomeric structure for the CKIIB form, made of a single α-subunit.

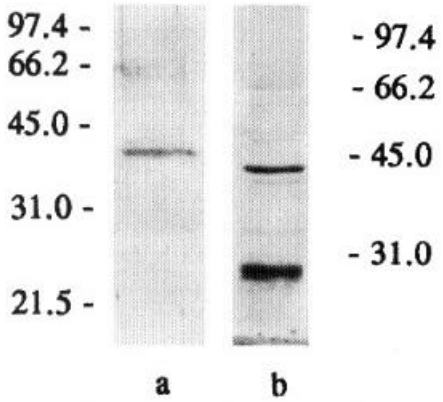

Fig. 2. Immunoblot analysis of *A. thaliana* CKIIA and CKIIB forms. Fractions showing maximal CKII activity from the last step of purification were subjected to SDS/PAGE, transblotted and developed with antibodies raised against bovine oligomeric CKII (dilution 1:500). Lane a: CKIIB form, electrophoresed on a 12.5% SDS/PAGE; Lane b: CKIIA form electrophoresed on a 10% SDS/PAGE. Markers size is indicated in kDa.

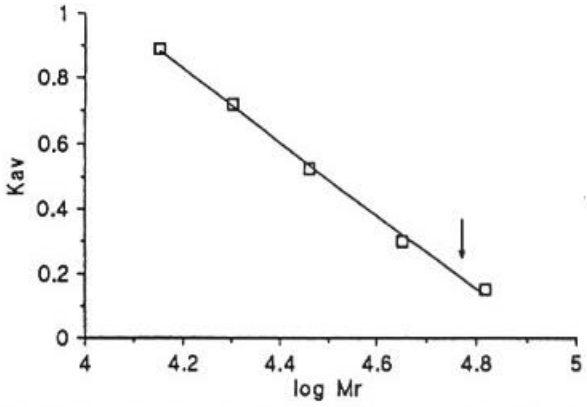

Fig. 3. Determination of native molecular mass of *Arabidopsis* CKIIB form by gel filtration. CKIIB form after DEAE-*Sepharose* chromatography was subjected to gel filtration analysis and the elution peak identified by kinase activity using β-casein as substrate. Molecular weight reference markers used were: bovine serum albumin (66 kDa), ovoalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14 200 Da). The position of CKIIB peak is indicated by an arrow.

Table 3
Summary of the main properties of casein kinases IIA and IIB from *A. thaliana*

Property	CKIIA	CKIIB
Subunit composition	α and β	α
Autophosphorylation	No	No
Km β-casein (mg/ml)	0.068	0.175
Vmax β-casein (U/ml)	3.7	18.2
Phosphorylation rate of 0.32 mM peptide substrate RRRDDDSDDD (U/ml)	60.83	634.7
Effect of 5 μg/ml heparin	90% inhibition	100% inhibition
Effect of 0.2 mg/ml polylysine	3 times stimulation	25 times stimulation

3.3. Enzymatic properties

In order to establish differences and similarities between CKIIA and CKIIB, we investigated the enzymatic properties characteristic of casein kinase II, such as utilization of GTP as phosphate donor, inhibition by heparin, stimulation by polybasic polypeptides, phosphorylation of acidic substrates and autophosphorylation on the β-subunit. Table 3 shows a summary of the results obtained.

Both forms are able to use GTP instead of ATP to phosphorylate casein. Experiments of competition showed that the addition of unlabelled GTP to the assay reduces to a 34% the incorporation of radioactive phosphate from ATP (experiments not showed). This observation is consistent with a preference of casein kinase II for ATP as phosphate donor, as described for other species [1].

Both forms efficiently phosphorylate casein as well as the acidic synthetic peptide RRRDDDSDDD (Boehringer) which is a specific substrate for CKII. Their activity toward β-casein is 90% (CKIIA) or 100% (CKIIB) inhibited at 5 μg/ml heparin. K_m and V_{max} values for β-casein calculated for the CKIIA form are 0.068 mg/ml and 3.7 U/ml respectively, and for CKIIB form 0.175 mg/ml and 18.2 U/ml, respectively. Moreover, CKIIA activity toward β-casein was weakly increased in the presence of 0.2 mg/ml polylysine (two times) while CKIIB activity was strongly stimulated (25 times) in the same conditions (Table 3 and Fig. 4, lanes 1–4).

Calmodulin is a well-characterized substrate for casein kinase II. It is efficiently phosphorylated by the α-subunit alone but not by the holoenzyme, which needs the presence of polybasic compounds to achieve a significant phosphorylation of that protein [20]. In order to explore the effect of the presence of the β-subunit on substrate specificity, we have determined the relative abilities of CKIIA and CKIIB forms of *Arabidopsis* to phosphorylate calmodulin. Fig. 4 (lanes 5–7) shows that CKIIB is more active toward calmodulin than CKIIA, in the absence of any effector, and that in the presence of polylysine there is a significant stimulation of the CKIIA activity. According to the units of enzymatic activity (measured by ^{32}P-incorporation into β-casein) loaded in the reaction for each isoform, we estimate that CKIIB is at least four times more efficient phosphorylating calmodulin than CKIIA. These results demonstrate that calmodulin is a substrate for *Arabidopsis* CKII but that the β-subunit plays an inhibitory role in the activity of the enzyme toward this substrate, as had been reported for other species [20].

Autophosphorylation on the β-subunit is a known property of the oligomeric CKII. The presence of some polycations in the reaction (polylysine, polyarginine, protamine) provokes a shift in the incorporation of phosphate from the β- to the α-subunit, while spermine or spermidine causes an increment in the phosphate incorporation on the β-subunit. We investigated the behaviour of CKIIA and CKIIB and neither form was autophosphorylated nor responsive to protamine and spermine as used as modulators of the autophosphorylation reaction. However, a phosphorylated band of 15 kDa was detected in the CKIIB reaction (Fig. 5), which most likely represents an endogenous substrate that co-purify ex-

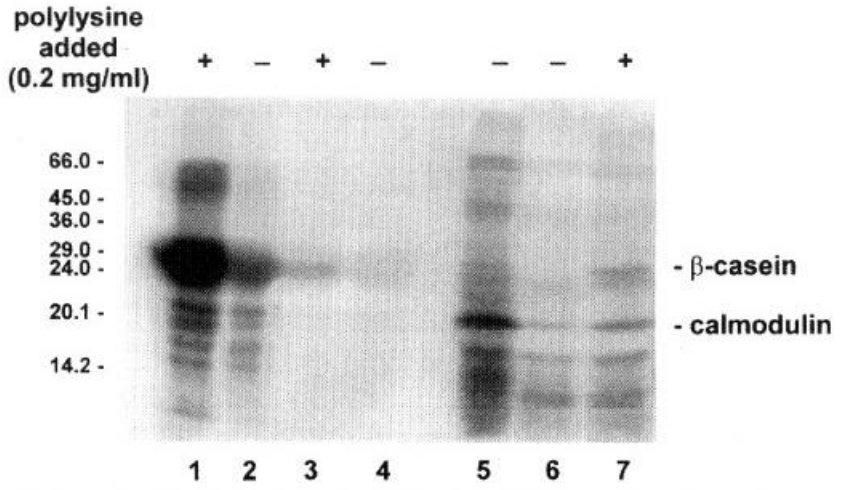

Fig. 4. Substrate specificity of *Arabidopsis* CKIIA and CKIIB. Radiolabelled phosphorylated substrates were separated by SDS-PAGE and visualized using a GS-525 Molecular Imager System (BioRad). Lanes 1, 2 and 5 correspond to CKIIB form and lanes 3, 4, 6 and 7 to CKIIA form. The presence or absence of polylysine in the reaction mixture is indicated by '+' or '-'. Lanes 1–4 used β-casein (1 mg/ml) as substrate; lanes 5–7 used calmodulin (1 mg/ml) as substrate. The same volume of the respective enzymatic isoform was used to perform the reaction with either β-casein or calmodulin as substrates. This allowed us to quantify the relative abilities of CKIIA and CKIIB to phosphorylate calmodulin, by comparing the signal ratio of ^{32}P-incorporation into β-casein between the two isoforms versus that of ^{32}P-incorporation into calmodulin. The position of the molecular mass markers are shown on the left in kDa.

tensively with this form. Incorporation of radioactive phosphate to the 15 kDa-band was inhibited by heparin, supporting the casein kinase II's substrate nature of this component. Association of the catalytic subunit of casein kinase II with polypeptides different from the β-subunit have been reported in different systems [21] and, particularly, with monomeric forms from broccoli [6] and maize [7].

4. Discussion

Over the past few years, *Arabidopsis* has become a model plant for molecular geneticists, but few biochemical studies are currently being done with it, in part due to the inconvenience of its small size. However, biochemical data obtained from plant material are important in order to confirm genetic findings and complement them. Until now, few examples of the casein kinase II protein family have been isolated from plant material and much of the published biochemical data concerning them come from recombinant proteins using DNA technology. However, important biochemical differences have been observed between the monomeric CKII-like activities isolated from plant material and those from recombinant catalytic monomers expressed in bacteria [12,22], suggesting the existence of either separate genes or posttranslational modifications. Moreover, no examples of any kind of protein kinase have been isolated from *Arabidopsis*, in spite of the increasing number of genes belonging to this protein family that are currently being cloned from this organism. Only in broccoli, a closely related plant, has a partial purification of CKII been achieved [6] but no biochemical and enzymatic properties have been determined.

We report here the existence of two different molecular forms, CKIIA and CKIIB, of casein kinase II from *Arabidopsis thaliana*. Our data show that CKIIA is composed by two different subunits cross-reacting with antibodies against whole CKII from calf thymus whereas CKIIB is composed by a single subunit related to the catalytic α/α' subunits of animal CKII. These results suggest an oligomeric nature for the CKIIA form,

with the $\alpha_2\beta_2$ structure typical of most CKII, and a monomeric nature for the CKIIB form, with only one catalytic subunit. Determination of native molecular weight of CKIIB by gel filtration confirmed that this form is composed by a single α-subunit.

Using the protocol described above we purified CKIIA and CKIIB enzymes up to 89- and 79-fold respectively, reaching specific activities of 855 and 91 U/mg. Our preparations were highly enriched in casein kinase II-type activity, according to the commonly accepted enzymatic criteria for this kinase family (see below). The preparations were free of other interfering casein-phosphorylating kinase activities, as shown by the 90–100% inhibition of casein phosphorylation by heparin.

The enzymatic properties of casein kinase II from *Arabidopsis thaliana* reported here present great similarities to those of enzymes purified from other sources. They include phosphorylation of casein, use of either ATP or GTP as nucleoside triphosphate donor, heparin sensitivity, and recognition of the acidic sequence RRRDDDSDDD as a efficient substrate for phosphorylation. All of these properties distinguish the enzyme from casein kinase I and at least three—*sensitivity* to low concentrations of heparin, use of GTP and phosphorylation of the acidic substrate—*appear* to be unique to casein kinase II. Moreover, the K_m values for β-casein are similar to those reported for other casein kinase II preparations.

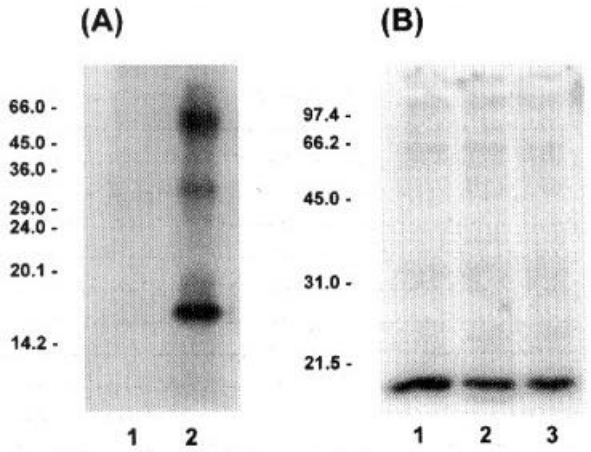

Fig. 5. Autoradiography of CKIIB isoform after incubation with [γ-^{32}P]ATP. (A) CKIIB isoform after DEAE-Sepharose chromatography was subjected to a 15% SDS/PAGE following an autophosphorylation reaction. Lane 1: reaction in the presence of 5 μg/ml heparin, an inhibitor of CKII activity. Lane 2: control reaction, in the absence of heparin. (B) CKIIB isoform after DEAE-Sepharose and heparin-agarose chromatographies was subjected to a 10% SDS/PAGE following an autophosphorylation reaction either in the absence or the presence of effectors. Lane 1: control reaction without effectors. Lane 2: reaction in the presence of 1 mg/ml protamine, a stimulator of the α-subunit phosphorylation. Lane 3: reaction in the presence of 2 mM spermine, a stimulator of the β-subunit phosphorylation. Results were visualized using a GS-525 Molecular Imager System (BioRad). Size of markers is indicated in kDa.

In contrast, there are several biochemical features of the *Arabidopsis* CKII that are distinct from some of the classical properties of this enzyme from other sources. These concern chromatographic behaviour, stability of the enzyme, responsiveness to polylysine and autophosphorylation, as discussed below.

CKIIB shows an atypical chromatographic behaviour in that it does not bind to a phosphocellulose column in the presence of moderate amounts of salt (250 mM NaCl), whereas CKIIA does. Since binding of casein kinase II to phosphocellulose depends on the amount of salt [23], one possible explanation for this anomalous behaviour is a higher requirement of salt for this form in order to adopt the adequate conformation to bind the resin. The absence of β-subunit could be responsible for this fact.

CKIIA is very unstable as a purified enzyme. That makes it very difficult to proceed through the last purification steps, since activity, though not protein, is lost rapidly. A similar observation has been made by Klimczak et al. [12] regarding the oligomeric CKII from broccoli. This is rather surprising since β subunit has been described in animal systems to increase the stability and the

overall catalytic activity of CKIIα toward canonical substrates [24].

Both CKIIA and CKIIB are responsive to the peptide polylysine, which improves their ability to phosphorylate β-casein. However, the CKIIB form is much more stimulated by polylysine than the CKIIA form, despite the absence of β-subunit in its structure. The stimulation by polylysine in animal CKII seems to be mediated by the β subunit, at least for the recombinant α-subunit, although monomeric CKIIB from maize shows a behaviour that is similar to the one reported here [5] but different from the recombinant enzyme [22]. This different behaviour between maize CKIIB and recombinant CKII-α, has been ascribed to either the existence of additional α-subunit genes or to posttranslational modifications of the protein. An additional hypothesis that we want to report here is that it can be due to the interaction of the α-subunit with other proteins, like the endogeneous substrate showed above, that are present in the plant cell but not in bacteria, and that might result in structural arrangements of the catalytic subunit.

Autophosphorylation on the regulatory subunit (β-subunit) is a characteristic property of casein kinases II. Two different cDNAs with an overall 55% homology to animal β-subunits has been cloned in *Arabidopsis thaliana* [11]. This is the only eukaryotic organism, apart from yeast, where a heterogeneity for the β-subunit has been clearly demonstrated. However, the amino acidic sequence deduced from these cDNAs show that there are important differences with other β subunits in regions that are rather conserved and potentially involved in functionally significant events. One striking feature is the presence of a long amino-terminal extension totally absent in other CKII. This is especially significant because it can drastically affect to the tridimensional structure of the whole chain and particularly the autophosphorylation site (typically located on Ser^2 and Ser^3 of the β-subunit) which is, in addition, poorly conserved in the *Arabidopsis* β- and β′-sequences. In our many tentatives, we could never achieve an in vitro phosphorylation of the *Arabidopsis* enzyme, neither in the β-subunit nor in the α-subunit. The importance of the tridimensional structure of the CKIIβ chain in relation to the process of autophosphorylation has been reported [25] and mutational studies have demonstrated the importance of an acidic amino acid stretch at position 55–64 [26]. *Arabidopsis* β-subunits contain many changes in this region, and the Pro-58, an important position within it [27], is not conserved. Autophosphorylation, on the other hand, is not required for the reconstitution of a fully active heterotetrameric CKII holoenzyme [28] and its physiological significance is far from being understood.

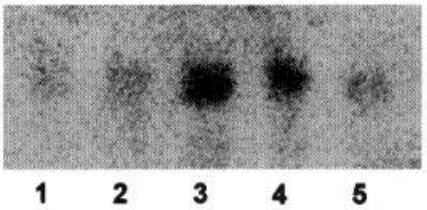

Fig. 6. Northern analysis of the β-subunit expression in different organs of *A. thaliana*. 10 μg of total RNA, isolated from shoots (lane 1) and 15 μg of total RNA from roots (lane 2), leaves (lane 3), flowers (lane 4) and 2-week old whole seedlings (lane 5), were loaded. A radiolabelled probe derived from the CKB1 insert [12] was used. The signal ratio (corrected for the amount of mRNA loaded) is 1:1.05:2.9:2.29:0.9. Results were visualized and quantified using a GS-525 Molecular Imager System (BioRad)

The extensive differences in the structure of the *Arabidopsis* CKIIβ-subunits, the only known incidence in plants so far, together with the existence of free α-forms, raises the question of whether a distinct form of regulation of CKII activity is used in plants. The cdc2 site for phosphorylation and the 'destruction box' on β-subunit sequence, are also missing in the *Arabidopsis* genes. On the other hand, mRNA corresponding to the β-subunit is present in all the organs of an *Arabidopsis* adult plant, but not at the same level (Fig. 6). Much higher expression in flowers and leaves than in roots and shoots is observed, with a ratio of 2.3:2.9:1.05:1. This result is in contrast with the transcript levels reported for *Arabidopsis* catalytic subunits [10] that are as high in roots as in flowers and leaves. It can be speculated that this different pattern of tissue expression of catalytic and regulatory subunits of *Arabidopsis* CKII might reflect the distribution of different molecular forms in a

tissue- or cell-specific manner. That would allow functional specializations of the isoenzymes through the interaction with different polypeptides (β-type and others) that modulate the substrate specificity. A more detailed study of the expression of the different subunits at the cell-type level is needed in order to prove this hypothesis.

CKII is a multifunctional enzyme with a puzzling regulation and with no precisely known physiological function. The availability of CKII forms isolated from plants offers the opportunity of investigating new variants present in the living world that can help to elucidate many 'dark' points on the structure-function relationship of this enzyme.

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