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Endogenous protein phosphorylation and casein kinase activity during seed development in *Araucaria angustifolia*

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Abstract

Protein kinases and phosphatases are responsible for several cellular events mediated by protein phosphorylation and dephosphorylation. Among these events are cell growth and differentiation and cellular metabolism. Casein kinase I (CKI) and casein kinase II (CKII) are involved in the phosphorylation of several substrates. Endogenous protein phosphorylation and casein kinase activity were investigated in the megagametophyte of the native Brazilian conifer *Araucaria angustifolia*, during seed development. It was observed that a number of different polypeptides are phosphorylated in vitro in the three megagametophyte stages of development tested (from globular, cotyledonary and mature embryos, respectively) and the phosphate was incorporated mainly in serine residues. The use of okadaic acid and vanadate in the phosphorylation reactions increased phosphate incorporation in several polypeptides suggesting the presence of serine/threonine as well as tyrosine phosphatases in the megagametophyte. Also, the results obtained in experiments with CKII inhibitor, GTP as phosphate donor, RNA hybridizations, and in-gel kinase assays indicate the presence of CKII in the *A. angustifolia* megagametophyte.

Keywords: *Araucaria angustifolia*; Araucariaceae; Seed development; Protein phosphorylation; Casein kinase II

1. Introduction

Araucaria angustifolia is the only economically important conifer species native to Brazil. Originally, this conifer covered 182,295 km^2 of Brazilian territory. However, due to exploitation, the natural reserves decreased and today represent only 0.7–1% of the original area (Longhi, 1993; Lima and Capobianco, 1997; Guerra et al., 2000). Besides being a good source of high-quality wood, fiber and resin, the seeds from *A. angustifolia* are used by wildlife and in human nutrition (Guerra et al., 2000).

The *A. angustifolia* seeds are big (3–8 cm × 1–2 cm) weighing 6.5–8.5 g (Reitz et al., 1978), and have high nutritious value (Guerra et al., 2000). They are protected by a dense, brown shell that includes the megagametophyte. In conifers, embryo development occurs within the megagametophyte which is responsible for providing nutrients to the embryo and young seedling (Misra, 1994). Though the synthesis and deposition of proteins in the seeds of conifers have been investigated by several authors (Misra, 1994; Krasowski and Owens, 1993; Hakman, 1993; Newton et al., 1992), very little is known about these cellular events in the seed of *A. angustifolia*.

Several cellular events are mediated by protein phosphorylation and dephosphorylation, catalyzed by protein kinases and phosphatases. Phosphorylation is mediated by several groups of protein kinases responsible for the control of cell growth and differentiation, as well as cellular metabolism. In plants, proteins and enzymes like ATPase, histone H1, RNA polymerase, malate synthase, transcription factors and others are phosphorylated (Trewavas and Gilroy, 1991; Ciceri et al., 1997). Among kinases, casein kinase I (CKI) and II (CKII) are responsible for the phosphorylation of several substrates. CKI is usually found in the nucleus, cytoplasm, membranes, ribosomes and mitochondria (Hathaway et al., 1983; Tuazon and Traugh, 1991). CKII was observed in the nucleus and in the cytoplasm

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of several eukaryotes (Hathaway and Traugh, 1983). It is involved in the control of many processes including DNA replication and transcription, gene expression and cell division (Allende and Allende, 1995).

Very little information about protein phosphorylation during seed development is available in the literature. The aim of this work was to investigate endogenous protein phosphorylation and casein kinase activity in the megagametophyte of *A. angustifolia*, during seed development.

2. Results and discussion

2.1. Protein profile and endogenous phosphorylation during megagametophyte development

In conifers, the haploid and maternally derived megagametophyte is the main storage tissue (Tranbarger and Misra, 1996). The development of the megagametophyte occurs before fertilization and it is within the megagametophyte that embryo development occurs (Misra, 1994). Hence, it is interesting to investigate the changes, at the protein level, that occur in the megagametophyte during embryo development. Since very little information about the megagametophyte of the Brazilian native conifer *A. angustifolia* is available, seeds from this conifer, containing embryos in three different developmental stages, globular, cotyledonary and mature, were collected.

The seeds were dissected and the megagametophytes were used to obtain soluble protein extracts. These extracts were analyzed by SDS-PAGE (Fig. 1A). As shown in this figure, several polypeptides were differentially synthesized in the *A. angustifolia* megagametophyte during the different stages of embryo development. For example, the protein bands of 27, 22 and 20 kDa are abundant in the cotyledonary and mature stages, but not in the earliest stage of development. These bands probably represent storage proteins which accumulate as the seed matures. When soluble protein extracts were incubated with a radiolabeled phosphate donor, distinct phosphorylation profiles were also observed for each developmental stage (Fig. 1B–D). Several bands incorporated labeled phosphate in the globular stage, whereas in the cotyledonary and mature stages, a different set of polypeptides were phosphorylated. As expected, the storage proteins which represent the majority of synthesized polypeptides in the later stages of development did not incorporate labeled phosphate. The endogenous phosphorylation reactions were greatly enhanced when protein extracts were diluted in phosphorylation buffer. This might be due to protein kinase inhibitors or regulators present in the developing seeds which must be diluted in order to restore kinase activity. According to Hathaway et al. (1983), the majority of casein kinase activities from certain cell types may be inhibited in crude cell extracts. This inhibition is released only when the cell extracts are diluted or submitted to chromatographic procedures that separate the inhibitors from kinases.

Phosphatase inhibitors were used in order to enhance endogenous protein phosphorylation (Fig. 2). When okadaic acid was added to the endogenous phosphorylation reactions, an increase in phosphate incorporation was observed for almost all the phosphorylated proteins present in the three developmental stages (Fig. 2, lanes 1, 3 and 5). When okadaic acid plus orthovanadate were added to the phosphorylation reactions (Fig. 2, lanes 2, 4 and 6), there was no increase in labeled phosphate incorporation. On the contrary, there was a decrease in phosphorylation of several bands in the cotyledonary and mature stages of development. Okadaic acid is a potent inhibitor of two classes of serine-threonine phosphatases, PP1 and PP2A, and its use lead to increased protein phosphorylation either in vivo or in vitro, in several cell types from mammals, plants and protists (Hardie, 1999; Kuo et al.,

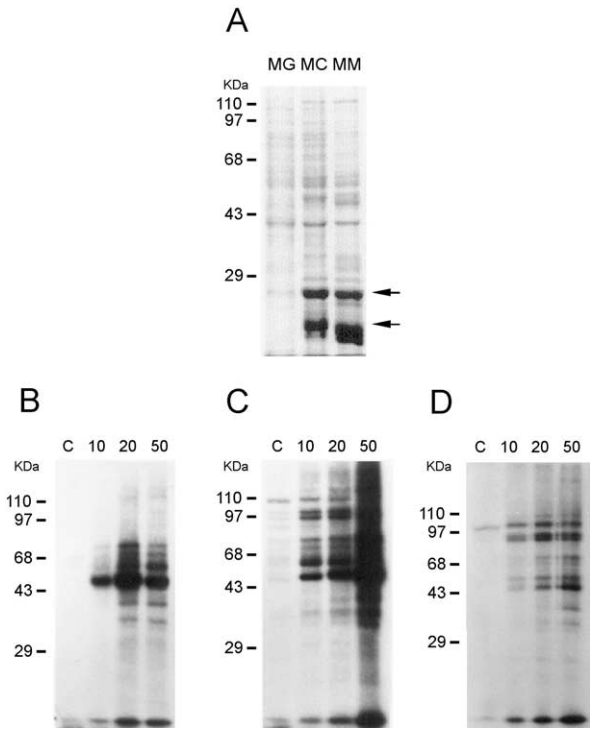

Fig. 1. Endogenous protein phosphorylation profile of three developmental stages of megagametophytes from *Araucaria angustifolia* seeds. Panel A—SDS-PAGE of soluble proteins from the globular (MG), cotyledonary (MC), and mature (MM) stages of embryo development. Arrows indicate polypeptides corresponding to the major storage proteins. Panel B—in vitro phosphorylation of soluble proteins from megagametophytes belonging to the globular (panel B), cotyledonary (panel C) and mature (panel D) stages of development. Numbers above each lane indicate the dilution factor used in the phosphorylation reactions, compared to the undiluted control extract (c).

1996; Smith and Walker, 1996; Linden and Kreimer, 1995). On the other hand, vanadate is a non-selective inhibitor of tyrosine phosphatases and its use promoted an increase in tyrosine phosphorylation (Heffetz et al., 1990). Since phosphorylation and dephosphorylation of tyrosine residues are key steps in signal transduction pathways, inhibition of tyrosine kinases or phosphatases might impair other phosphorylation events in signaling cascades. Taken together, the results obtained with the *A. angustifolia* megagametophytes indicate the involvement of serine/threonine as well as tyrosine phosphatases in the phosphorylation of several polypeptides. Also, inhibition of phosphorylation by vanadate might indicate that dephosphorylation of tyrosine residues is important in the activation of protein kinases present in the later stages of development.

2.2. CKII activity during megagametophyte development

Casein kinase type II is a multifunctional protein kinase with more than 200 known substrates, found in almost every eukaryotic organism examined so far, including plants (Lee et al., 1999; Battistutta et al., 2000). CKII is involved in the control of several cellular mechanisms, such as replication, transcription, RNA processing and signal transduction. Due to its ubiquity, CKII activity was examined in the protein extracts from *A. angustifolia* megagametophytes. One of the criteria used to test for CKII activity was its sensitivity to heparin. Fig. 3 shows the effects of heparin on the endogenous phosphorylation profile of the three seed developmental stages. Several bands had diminished phosphate incorporation in the presence of heparin at concentrations as low as 10 nM (Fig. 3, lane 1), and the label in two bands (97 and 100 kDa) was completely abolished. According to Crute and Buskirk (1992), only CKII is sensitive to this concentration of heparin, whereas 50-fold higher concentrations are necessary in order to inhibit CKI.

Another criterion to test for casein kinase activity in the megagametophyte extracts was the use of dephosphorylated casein as exogenous substrate. Fig. 4 shows phosphorylation of casein by protein extracts from the three seed developmental stages. In the three extracts, casein was phosphorylated with either [γ-^{32}P] ATP or GTP as phosphate donor. According to Pinna (1990), CKII has the ability to use GTP and ATP as phosphate donors, but not CKI. Note that 10 nM heparin also inhibited phosphorylation of casein with ATP or GTP and that the overall pattern of phosphorylation with both phosphate donors for the three developmental stages was basically the same. This suggested that CKII is the major kinase activity present in the megagametophyte of *A. angustifolia*. The phosphoamino acid analysis performed for the three developmental stages gave further evidence to support this hypothesis. Fig. 5 shows clearly that phosphoserine is the major residue phosphorylated in the majority of proteins present in the three stages of megagametophyte development. This is consistent with the preference of CKII for serine over threonine as the phosphoacceptor residue (Pinna and Ruzzene, 1996). Casein was also phosphorylated by the endogenous kinase activity only in serine residues, as expected (Fitzgerald, 1998) (data not shown).

In order to identify polypeptides bearing casein kinase activity, in-gel kinase assays were performed (Fig. 6). In

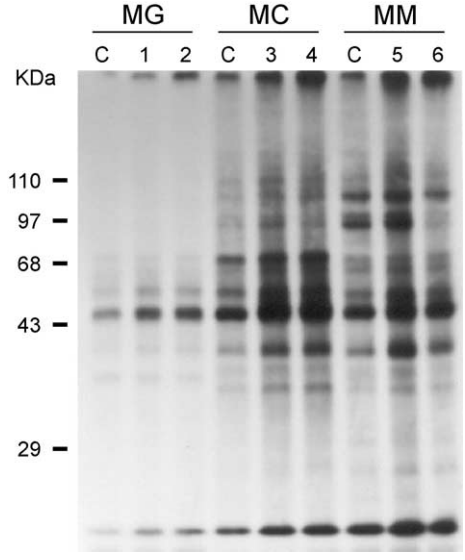

Fig. 2. Effects of phosphatase inhibitors on the endogenous protein phosphorylation profile. Phosphorylation reactions were carried out in the absence of phosphatase inhibiors (C), in the presence of 2 μM okadaic acid (lanes 1, 3 and 5) or in the presence of 2 μM okadaic acid plus 1 mM sodium orthovanadate (lanes 2, 4 and 6).

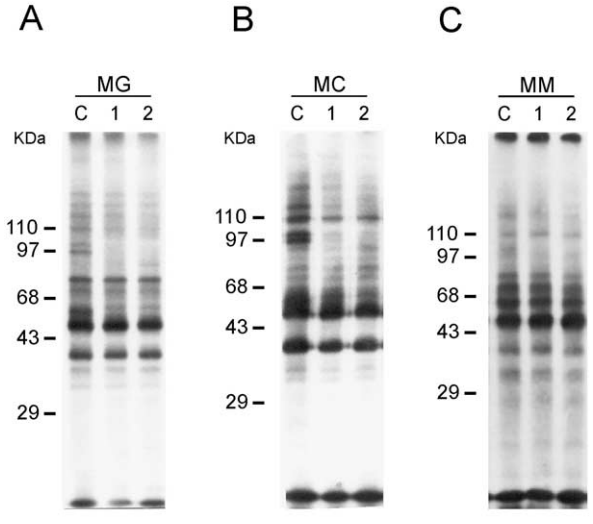

Fig. 3. Effects of heparin on the endogenous protein phosphorylation profile of the three developmental stages of megagametophytes from *Araucaria angustifolia* seeds. Phosphorylation reactions were carried out in the presence of okadaic acid and 10 nM (lane 1) or 100 nM heparin (lane 2). Control reactions (C) contained only the phosphatase inhibitor.

these assays, the substrate (casein) was incorporated into the polyacrilamide gel matrix prior to polymerization. Protein extracts were separated in those gels, and after a series of treatments, the whole gel was incubated with [γ-^{32}P] ATP or GTP. The radiolabeled bands present in the control gels (Fig. 6, panels A and C) indicated the polypeptides which underwent autophosphorylation. The bands present only in the test gels, or showing higher intensity compared to the control, indicated casein kinases. Note that a band with Mr of 35 kDa and three others of higher Mr exhibited more intense labeling when casein was within the gel (Fig. 6, panel B), using ATP as phosphate donor. When GTP was used, only the band of 35 kDa showed increased label incorporation (Fig. 6, panel D), indicating that this polypeptide probably corresponds to the casein kinase type II catalytic subunit. In this assay, the 35 kDa band seems more intense in the latest stages of development, but one must take into account that the amount of total protein in the earliest stage is relatively very low. Therefore the relative activity of casein kinase type II was estimated by measuring the intensity of radioactivity in the 35 kDa band divided by the amount of protein loaded in the gels (Fig. 6, panel E). The results indicated that the CKII relative activity was highest at the globular stage of development, and decreased as the seed matured. A higher protein kinase activity in the earlier stages of development was also observed for seeds from dicots such as winged bean (Mukhopadhyay and Singh, 1997), and monocots such as *Coix* and maize (A. C. da Silva, unpublished data). This might reflect higher rates of metabolic activity in the period prior to maturation when the seeds gradually accumulate reserve proteins and desiccate.

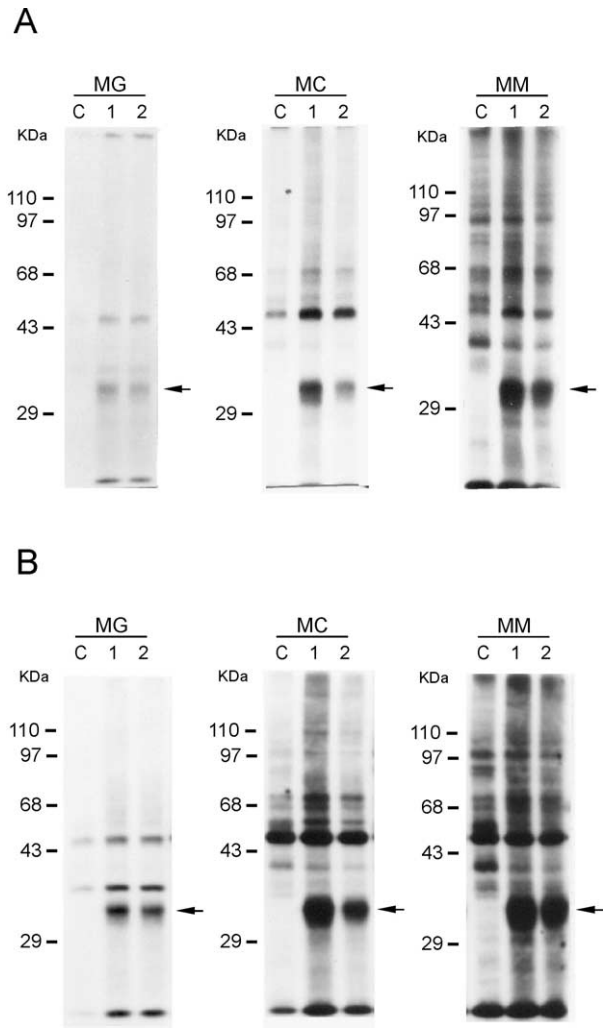

Fig. 4. Phosphorylation of casein by endogenous protein kinases present in the three stages of megagametophytes from *Araucaria angustifolia*. Panel A—Phosphorylation reactions were carried out in the absence (C), or in the presence of 3 μg dephosphorylated casein (lanes 1–6). Heparin was added as casein kinase inhibitor (lanes 2, 4 and 6). [γ-^{32}P]ATP was used as phosphate donor in all reactions represented in panel A, whereas [γ-^{32}P]GTP was used in all reactions represented in panel B. Arrows indicate the band corresponding to casein.

2.3. Expression levels of CKII transcripts

Since a polypeptide bearing casein kinase type II activity was identified in the in-gel kinase assays, RNA slot blot hybridization assays were also performed to test for the presence of CKII transcripts in the three developmental stages of *A. angustifolia* megagametophytes. For these assays, two cDNA probes were used, both derived from sugar cane tissues. One of them, named SCEQAM1039G02.g, was isolated from meristem, and represents a cDNA whose deduced amino acid sequence (128 residues) has strong identity to the N-terminal portion of the casein kinase II alpha subunit from *Zea mays* and *Oryza sativa* (99 and 98%, respectively). The other probe used, SCEQSD1077B01.g, was isolated from sugar cane seeds and represents a cDNA whose deduced amino acid sequence (176 residues) has a

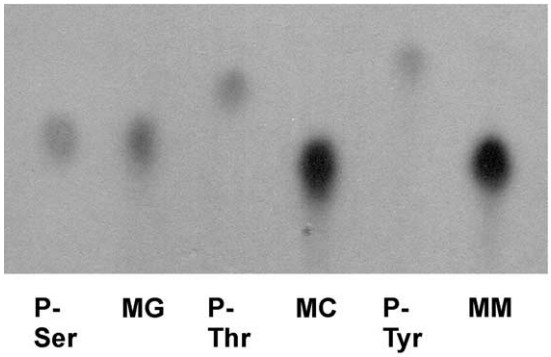

Fig. 5. Identification of ^{32}P-labeled phosphoamino acids from megagametophyte extracts. Whole protein extracts were phosphorylated in vitro, separated by SDS-PAGE and blotted onto Immobilon membranes. The radioactive bands were excised, acid hydrolyzed and separated by cellulose TLC. Lanes with the phosphoamino acid standards show the staining of the TLC plate with ninhydrin. The lanes marked MG, MC and MM show autoradiography of the same plate.

98% identity to the carboxy-terminus of the CKII alpha subunit from *Zea mays*. The results obtained with these assays are presented in Fig. 7.

As shown in Fig. 7, the expression levels of CKII were significantly higher in the globular developmental stage, decreasing progressively in the cotyledonary and mature stages. This was observed using any of the two probes (Fig. 7, panels A and B), representing either the amino- or carboxy-terminus of the protein. These results are corroborated by those obtained with the in-gel kinase assay, where the relative activity of CKII was highest in the earliest stage of development, decreasing as the seed matures.

Altogether the results presented in this work indicate that different phosphorylation events occur during seed development in *A. angustifolia*. A number of different polypeptides in the megagametophyte take up phosphate in the three stages of development tested. The use of okadaic acid and vanadate in the phosphorylation reactions increased phosphate incorporation in several polypeptides suggesting the presence of serine/threonine as well as tyrosine phosphatases in the megagametophyte. Assays using CKII inhibitor, GTP as phosphate donor, phosphoamino acid analysis and RNA hybridization provided strong evidence suggesting that casein kinase type II is the major kinase activity in the megagametophyte.

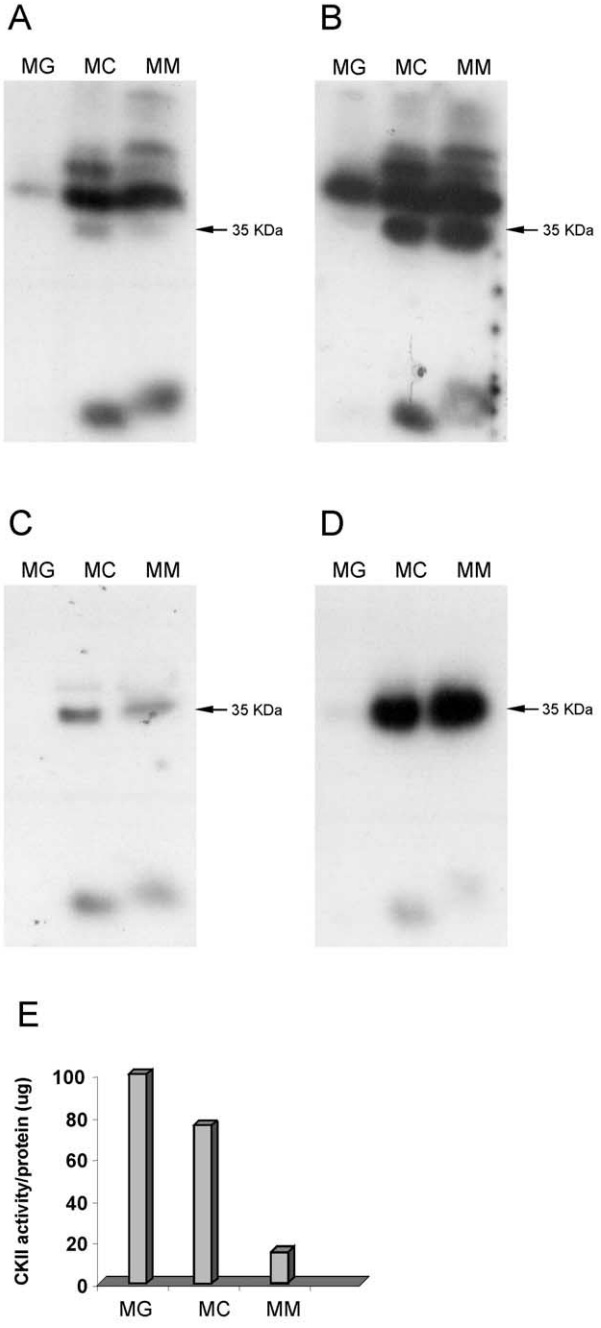

Fig. 6. In-gel casein kinase assay. Protein extracts from the three different developmental stages of megagametophytes were separated by SDS-PAGE in 12% polyacrylamide gels containing casein (panels B and D) or no substrate (control gels, panels A and C). [γ-^{32}P]ATP was used as phosphate donor in the assays presented in panels A and B, and [γ-^{32}P]GTP was used in the assays presented in panels C and D. Panel E represents the relative amounts of casein kinase activity in the three developmental stages of the megagametophyte, expressed as the intensity of label divided by the amount of protein loaded in the gel.

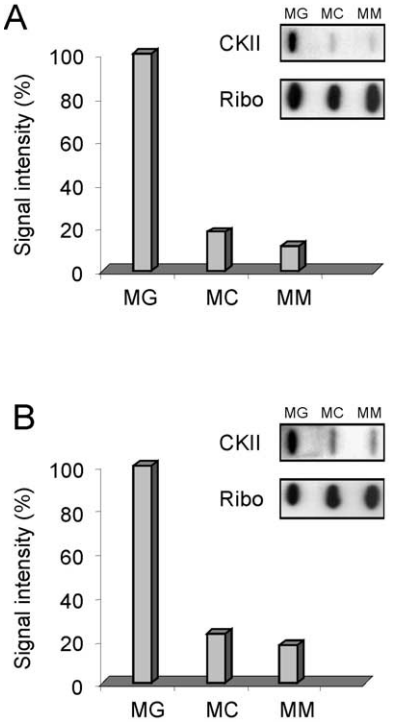

Fig. 7. Expression levels of casein kinase type II. Total RNA was isolated from megagametophytes from the three developmental stages of *Araucaria angustifolia* seeds and transferred to membranes using a slot blot device. The membranes were hybridized with probes derived from two CKII cDNA clones isolated from sugar cane tissues. One of these clones was isolated from meristem (clone SCEQAM1039G02.g) (panel A), and the other, from sugar cane seeds (clone SCEQSD1077B01.g) (panel B). The amount of RNA was normalized by re-hybridizing the membranes with the rDNA 26S from maize (Ribo). Histograms represent the relative amount of hybridization for the CKII probes.

3. Experimental

3.1. Plant material

Female cones from *A. angustifolia* were harvested from native trees in the city of Bom Retiro, State of Santa Catarina, in the southern region of Brazil. The cones were rinsed with EtOH, the seeds were removed and frozen in liquid nitrogen prior to storage at −80 °C. Seeds in three different stages of embryo development, globular (G), cotyledonary (C) and mature (M), were used as criteria for seed collection. The megagametophytes from those seeds were named MG (megagametophyte from seeds with globular embryos), MC (megagametophyte from seeds with cotyledonary embryos) and MM (megagametophyte from seeds with mature embryos). Prior to the experiments, the seeds were dissected in order to separate the megagametophyte from the embryo.

3.2. Protein extraction

Megagametophytes, from seeds containing embryos in the three different stages of development (G, C and M), were homogenized in a mortar containing two volumes of extraction buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM DTT)/g of tissue. After homogenization, the samples were cleared by centrifugation, for 10 min, in a microcentrifuge and the supernatant was transferred to a clean Eppendorf tube. The centrifugation was repeated and the soluble protein content was determined by the Bradford method (Bradford, 1976). All the steps were carried out at 4 °C. The protein extracts were analyzed by electrophoresis in a 10% polyacrylamide gel (Laemmli, 1970).

3.3. Endogenous protein phosphorylation and electrophoresis

In vitro phosphorylation assays were carried out using the megagametophyte protein extracts, diluted in phosphorylation buffer (20 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$ and 1 mM DTT), in the presence of 1 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) per reaction. [γ-^{32}P]GTP was also used as phosphate donor in the reactions for detection of CKII activity. Samples were incubated at 30 °C for 30 min and the phosphorylation reactions were stopped by the addition of Laemmli's sample buffer (Laemmli, 1970) and boiling for 5 min. Proteins were separated by SDS-PAGE using 10% polyacrylamide gels (Laemmli, 1970). The gels were stained with Coomassie brilliant blue R 250, destained and dried over a filter paper. Phosphorylated bands were detected by autoradiography using Hyperfilm (Amersham Pharmacia).

In order to enhance the endogenous phosphorylation reactions in the megagametophyte extracts, sodium orthovanadate (1 mM) and okadaic acid (2 μM) were used as phosphatase inhibitors.

3.4. Phosphoamino acid analysis

The identification of radiolabeled phosphoamino acids was carried out essentially as described by da Silva et al. (1999) and Fazio et al. (1995). Proteins from megagametophyte extracts were first phosphorylated as described above, using [γ-^{32}P]ATP as a phosphate donor, and then separated by SDS-PAGE. The labeled proteins were electroblotted into Immobilon-P^{SQ} membranes (Millipore) and the radioactive bands were excised and submitted to acid hydrolysis with 5.7 N HCl for 2 h at 110 °C. The hydrolysate was lyophilized, dissolved in 10 μl milli-Q water and spotted onto Sigma-Cell type 100 cellulose TLC plates (Sigma Chem. Co.). The solvent system used was isobutyric acid/0.5 M ammonium hydroxide (5:3). Phosphoserine, phosphothreonine or phosphotyrosine standards (8 μg) (Sigma Chem. Co.) were also spotted on the TLC plates and visualized by spraying with 0.25% ninhydrin in acetone. Radiolabed residues were detected by autoradiography.

3.5. Detection of casein kinase activity

The presence of casein kinase activities in the megagametophyte extracts was detected using three different assays. First, dephosphorylated casein (2 μg) (Sigma) were added per reaction mixture as exogenous substrate in the presence of either ATP or GTP as phosphate donors. Second, heparin was used as inhibitor of casein kinases (nanomolar range for CKII and micromolar range for CKI), and third, in-gel kinase assays were performed as follows. Megagametophyte protein extracts were separated by SDS-PAGE in 10% gels. The samples were not boiled before being submitted to electrophoresis. Test gels were cast adding 1 mg/ml of dephosphorylated casein to the acrylamide mixture, prior to polymerization. Control gels, lacking casein in the polyacrylamide matrix, were cast and run simultaneously. After electrophoresis, the gels were washed (2×40 min) with a solution containing 50 mM Tris–HCl pH 8.0, 1 mM DTT and 20% isopropanol, to remove the SDS. Next, the gels were washed with the same solution without isopropanol, for removal of the alcohol. The proteins within the gels were then denatured by soaking the gels in 6 M guanidine–HCl for 1 h. Proteins were renatured by washing the gels several times with 50 mM Tris–HCl pH 8.0, 1 mM DTT and Tween-20 0.4%. The renaturation of the proteins was performed at 4 °C. All the other steps were conducted at room temp. After renaturation, the gels were equilibrated with phosphorylation buffer, and then incubated in 12 ml of the same

buffer containing 50 μCi of [γ-^{32}P]ATP or GTP, for 90 min at room temp. After the phosphorylation step, the gels were washed (5×15 min) with a solution containing 5% TCA and 1% Na-pyrophosphate for removal of the unincorporated label. Radioactive bands were detected by autoradiography of the dried gels.

3.6. RNA slot blots

The expression of CKII in MG, MC and MM was analyzed by RNA slot blots. Total RNA was isolated from MG, MC and MM using the method described by Prescott and Martin (1987). RNA (5 μg) were mixed with *TE*, to obtain a final volume of 12.5 μl, and 37.5 μl of a solution containing deionized formamide (500 μl) formaldehyde 37% (162 μl), MOPS 10× (100 μl) (Sambrook et al., 1989). The samples were incubated at 65 °C for 5 min and 20 × SSC (50 μl) were added. They were transferred in duplicate to nylon membranes (Hybond-N, Amersham) using a slot blot apparatus (Gibco BRL). After incubation at 80 °C for 2 h, the membranes were prehybridized, for at least 2 h, in a solution containing 5 × SSC, 50% deionized formamide, 20 mM Tris–HCl (pH 7.5), 1% sodium dodecyl sulfate (SDS), 100 μg/ml denatured salmon sperm DNA and 10 × Denhardt's solution. Probes were labeled with [α-^{32}P]dCTP, using the Megaprime kit (Amersham) and the hybridization was carried out at 42 °C, overnight, in a hybridization solution containing 5 × SSC, 50% deionized formamide, 20 mM Tris–HCl (pH 7.5), 1% SDS, 5% dextran sulfate, 100 μg/ml denatured salmon sperm DNA, 2 × Denhardt's solution and 10^7–10^8 cpm/ml of the denatured probe. After hybridization, the membranes were washed (2×5 min) at room temp, in a solution containing 2 × SSC and 0.5% SDS. These washes were followed by two 15 min washes at room temp in a solution containing 2 × SSC and 0.1% SDS and a wash at 60 °C in 0.1 × SSC and 0.1% SDS. The membranes were exposed to X-ray films (Amersham). The amount of RNA in the membranes was normalized by hybridization with the rDNA gene 26S from maize. The intensity of the hybridization signals was quantified using the Image Analysis Software (Kodak).

3.7. Probes

Two clones isolated from sugar cane tissues (SCEQAM1039G02.g from meristem, and SCEQSD1077B01.g from seeds) were kindly provided by Dr. Paulo Arruda, CBMEG–UNICAMP.

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