BRIEF COMMUNICATION

Endogenous abscisic acid and protein contents during seed development of *Araucaria angustifolia*

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Abstract

This paper describes a proteome analysis and changes in endogenous abscisic acid (ABA) contents during seed development of *Araucaria angustifolia* (Bert.) O. Ktze. Megagametophytes and embryonic axis tissues exhibited a similar ABA variation pattern during seed development, reaching maximum values at the pre-cotyledonary stage. The embryonic axis protein content increased until the cotyledonary stage with following stabilization at mature seed. The two-dimensional electrophoresis at the torpedo developmental stage showed approximately 230 polypeptides against 340 in the mature stage. Peptide mass fingerprinting analyses identified three polypeptides, corresponding to an AtSAC4, a late embryogenesis abundant (LEA) and a storage protein, respectively.

Additional key words: comparative proteomic, conifer, LEA, plant proteome, somatic embryogenesis, storage protein, zygotic embryo.

Araucaria angustifolia (Bert.) O. Ktze. is the only native conifer of economic importance in Brazil. Nowadays, only relicts of natural vegetation are found, representing from 1 to 2 % of the original area (Guerra *et al*. 2000). Somatic embryogenesis protocol has been performed for *A. angustifolia*, although, up to now only somatic embryos in early developmental stages have been obtained (Guerra *et al*. 2000, Silveira *et al*. 2002). Seed development is a good system for studying the molecular and physiological basis of embryogenesis, and results can be used in system manipulation for *in vitro* multiplication *via* somatic embryogenesis. Micropropagation through somatic embryogenesis is an alternative pathway for the propagation of conifers with high rates of multiplication (Salajová and Salaj 2005, Silveira *et al.* 2006, Vooková and Kormuťák 2006). In order to increase the efficiency of embryo development, an improved understanding of the biochemical and molecular events that occur during embryo development is essential (Silveira *et al.* 2006). New data on seed structure and metabolism would be important for future biotechnology efforts in this context.

Plant growth regulators play a crucial role in the processes of seed development and germination (Atici *et al.* 2005). It is well documented that after complete embryonic formation, ABA concentrations rise while the embryo establishes dormancy and acquires storage reserves. The pattern content variation observed either by applying the compound onto tissues and evaluating their effect, or by measuring the endogenous content in specific tissues and correlating this with physiological responses (Bonetta and McCourt 2002), has led to attempts to understand ABA action. ABA is involved in gene expression of storage proteins and LEA (late embryogenesis abundant) proteins that are synthesized in the seed after embryo differentiation (Bonetta and McCourt 2002, Wise and Tunnacliffe 2004).

In this paper we compared changes in contents of proteins and abscisic acid (ABA) during seed development of *A. angustifolia*.

Seeds of *Araucaria angustifolia* (Bert.) O. Ktze were

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Abbreviations: ABA - abscisic acid; f.m. - fresh matter; HPLC - high performance liquid chromatography; LEA - late embryogenesis abundant protein; PMF - peptide mass fingerprinting.
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collected in Santa Catarina State, Brazil (27º47'S, 49º29'W), from January to March (2003) at the following dates: 26th January, 5th February, 18th February and 26th March, representing the different stages of embryo development, such as torpedo, pre-cotyledonary, cotyledonary and mature, respectively.

ABA determination was performed for two samples of embryonic axis and two samples of tissue of megagametophytes at each developmental stage. ABA contents were extracted and determined according to Silveira *et al*. (2004). [^{3}H]ABA was added to the sample as internal radioactive standards. ABA content was determined and analyzed by reverse phase HPLC (*Shimadzu*, Tokyo, Japan), using a 5 µm C_{18} column using a UV-VIS detector at 254 nm. Fractions containing ABA were collected and analyzed in the *Packard Tri-Carb* (*Perkin Elmer*, Waltham, USA) liquid scintillation counter to estimate losses.

Proteomic analysis was performed for three samples of embryonic axis and three samples of megagametophytes at the torpedo and mature stages. Buffer-soluble proteins were extracted according to Silveira *et al*. (2004). Samples containing 100 µg of protein were precipitated in trichloroacetic acid (10 %) and washed three times with cold acetone. Proteins were solubilized in 0.37 cm^3 of 2D-sample buffer 2D (Paba *et al*. 2004) and applied to 18 cm IPG gel strips with a linear separation range of pH 4 - 7 (*GE Healthcare*, Uppsala, Sweden). After 12 h of rehydration, IEF was carried out using an *IPGphor* unit (*GE Healthcare*). Before second-dimension electrophoresis, the IPG gel strips were subjected to reduction and alkylation steps (Paba *et al*. 2004). SDS-PAGE was performed on 10 % polyacrylamide gels run in a *Protean II* system (*Bio-Rad*, Hercules, USA) connected to a *Multitemp II* cooling bath (*GE Healthcare*). Gels were silver-stained (Paba *et al*. 2004) and analyzed with the *Image Master 2D* elite software (*GE Healthcare*).

Eight polypeptides were digested with a sequencing grade modified porcine trypsin (*Promega*, Madison, USA), according to Paba *et al*. (2004). The resulting peptides were analyzed in a *MALDI-TOF* mass spectrometer (*Bruker Daltonics*, Billerica, USA) and protein identification was performed using the *MASCOT* software. Peptide mass lists were searched against the NCBI (National Center for Biotechnology Information) nonredundant protein database. Mass tolerance was allowed at 0.005 - 0.010 %, protein mass restriction values being set according to Mr values experimentally determined for each 2-DE spot.

ABA and protein contents data were analyzed by *ANOVA*, followed by the Tukey test ($P < 0.05$). Statistical tools provided by *MASCOT* as well as the candidate proteins returned by the software, were used for result of peptide mass fingerprinting (PMF) interpretation and protein identification.

ABA content in the megagametophyte and embryonic axis tissues of *A. angustifolia* exhibited the same pattern of variation throughout development, reaching the maximum in the pre-cotyledonary stage (Fig. 1*A*). As observed for *A. angustifolia* zygotic embryos, in our work (Fig. 1*A*), ABA contents reach the maximum between initial development and the beginning of zygotic embryo maturation in most species (Garello *et al*. 2000, Silveira *et al*. 2004). The highest ABA level in cotyledonary embryos of *A. angustifolia* might be necessary for the synthesis of storage and LEA proteins present in late embryogenesis. It was hypothesized that ABA acts through a standard signal transduction pathway, in which the binding of the hormone to a receptor elicits a transduction cascade (Bonetta and McCourt 2002).

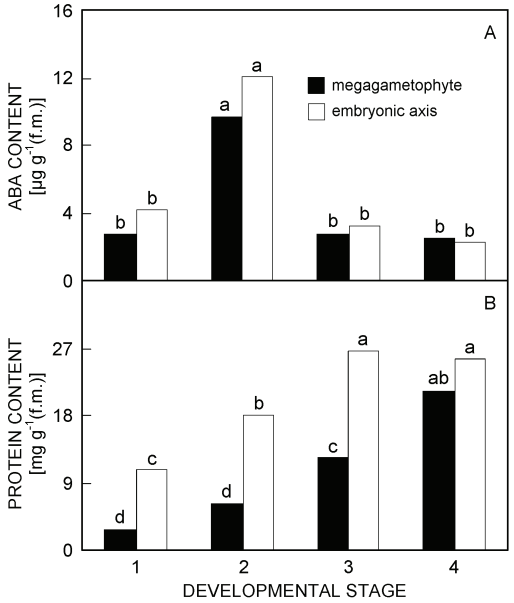

Fig. 1. Abscisic acid (ABA) (*A*) and soluble protein (*B*) contents in megagametophytes and embryonic axis tissues during seed development of *Araucaria angustifolia*. Torpedo (1), pre-cotyledonary (2), cotyledonary (3) and mature (4) developmental stages. Means followed by the same letters are not significantly different at $P < 0.05$ according to the Tukey test. Coefficient of variation $(CV)_{ABA} = 22.3$ %, $n = 2$, $CV_{protein} = 8.7$ %, $n = 3$.

Protein content in the embryonic axis increased until the cotyledonary stage followed by stabilization in the mature seed. A continuous increase throughout seed development occurred in megagametophyte tissue (Fig. 1*B*). During zygotic embryogenesis, a protein content increase could be interpreted as a result of storage and LEA proteins synthesis of those that have a great affinity with water molecules, thus acting in seed dehydration protection (Wise and Tunnacliffe 2004). Studies of seed storage proteins with different coniferous species have shown that the protein bodies are composed of a protein matrix containing water insoluble crystalloid and globoid proteins (for review see Attree and Fowke 1993). However, previous studies with *A. angustifolia* seeds showed that buffer-soluble proteins are the most important storage proteins with insoluble protein (crystalloid) representing less than 2 % of total storage

proteins (Fernandez 2001).

To understand protein variation during seed development, we analyzed the protein pattern in different embryonic axis stages: torpedo and mature. The 2-DE pattern maps were different for these two developmental stages (Fig. 2). At the torpedo developmental stage, approximately 230 polypeptides against 340 in the mature stage, with 90 matched spots, were detected. High molecular mass proteins, observed at the torpedo stage, reduced their expression in the maturity (Fig. 2). However, new proteins of low molecular mass were specifically synthesized in mature embryos (Fig. 2). Eight differentially expressed polypeptides were selected for mass spectrometry and PMF analyses (Fig. 2). The polypeptides 203, 208 and 327 were positively identified as AtSAC4, late embryogenesis abundant (LEA), and storage proteins, respectively (Table 1).

The polypeptide 203 was identified as an AtSAC4 protein, a phosphoinositide phosphatase family protein in *Arabidopsis* (Zhong and Ye 2003) (Fig. 2; Table 1). Phosphoinositide phosphatases modulate the contents of phosphatidylinositols, these being a group of phospholipids that are key regulators of a number of signal transduction processes (Despres *et al*. 2003). Studies with the AtSAC1 family genes in *Arabidopsis* showed that they are responsible for the control of phosphatidylinositol content during vegetative growth (Despres *et al*. 2003). AtSAC4 and AtSAC1 proteins exhibit 75 %

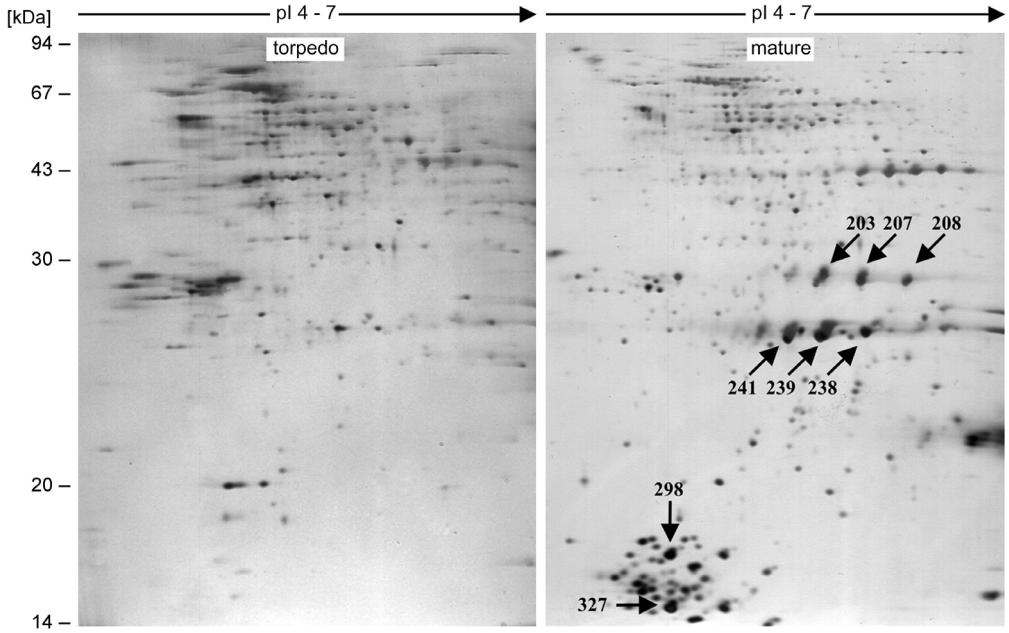

Fig. 2. Two-dimensional electrophoresis (2-DE) pattern maps from *A. angustifolia* zygotic embryos at torpedo (*A*) and mature (*B*) stages. Numbered spots correspond to polypeptides differentially expressed, selected for mass spectrometry and peptide mass fingerprinting (PMF) analyses.

Table 1. Protein identification by peptide mass fingerprinting (PMF) in *A. angustifolia* mature embryos 2-DE map. Proteins were analyzed according to probability based mowse score, using the *MASCOT* algorithm against the NCBI nonredundant database. Protein scores higher than 65 are significant ($P < 0.05$). p*I* t/o - isoelectric point, theoretically/experimentally observed by 2-DE; Mr t/o - molecular mass, theoretically/experimentally observed by 2-DE; S - protein score: probability based Mowse score using the *MASCOT* algorithm against the NCBI nonredundant database; M.P. - number of matched peptides; C - % of sequence coverage.

Spot	Accession number	Protein	Species	pI t/o	M_r t/o [kDa]	S [%]	M.P.	C [%]
203	gi 30688003	AtSAC4	*A. thaliana*	6.5/5.8	95/28	84	13	22
207	gi 7387829	LEA3	*Z. mays*	8.8/6.0	23/28	58	6	33
208	gi 7387829	LEA3	*Z. mays*	8.8/6.3	23/28	72	7	38
238	gi 34905350	QJ9990_A01.23	*O. sativa*	8.7/5.8	12/24	40	4	62
239	gi 6688636	ATP synthase β subunit	*M. alnifolia*	5.3/6.0	54/24	62	7	18
241	gi 42408183	hypothetical protein	*O. sativa*	10.5/5.6	16/24	42	5	48
298	gi 2495170	δ-aminolevulinic acid dehydratase	*C. reinhardtii*	7.7/5.0	43/19	58	6	24
327	gi 21913852	vicilin-like storage protein	*A. angustifolia*	7.7/5.0	54/18	71	8	13

similarity in their gene sequence (Zhong and Ye 2003), suggesting that the AtSAC4 protein might be responsible for the regulation of phosphatidylinositol pools and requirement of phosphoinositide phosphatase activities during embryo development in *A. angustifolia*.

The polypeptide 208 was identified as a late embryogenesis abundant protein, group 3 (LEA) in maize (White and Rivin 1995) (Fig. 2; Table 1). This group 3 LEA mRNAs has been reported in several plant species and in some cases with homology over 95 %, indicating that a common mechanism for desiccation protection may be present (for review see Ried and Walker-Simmons 1993). LEA protein expression from angiosperm species in conifers was previously observed in *Picea glauca* (Leal and Misha 1993). These authors observed that there are sequence similarities between the *LEA* genes of angiosperm and gymnosperm, and that these genes share a common ancestral origin.

The polypeptide 327 was identified as a vicilin-like storage protein in *A. angustifolia* (Fernandez 2001) (Fig. 2; Table 1). The S-vicilin-type globulins group is the most important storage proteins group observed in *A. angustifolia* (Fernandez 2001). The globulins are the most widely distributed group of storage proteins, and can be divided into two groups based on their sedimentation coefficients: the 7S vicilin-type globulins and the 11S legumin-type globulins (Shewry *et al*. 1995). Storage proteins are deposited in the embryo during seed development and their breakdown, in the embryo and megagametophyte after seed germination, provide nitrogen for the development of seedlings in the form of free amino acids (Silveira *et al*. 2004).

Seed development is a complex process, involving physiological and biochemical factors including ABA accumulation and synthesis of specific proteins such as LEAs and storage proteins. In this work we were able to describe unpublished results related to the metabolism of developing seeds of *A. angustifolia*. The *A. angustifolia* genome is not sequenced, thus hindering the protein identification by PMF, but our results showed that, even with PMF, it is possible to identify proteins differentially expressed from the zygotic embryo proteome map that might be used as biochemical markers of embryo development.

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