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Research Article

Cloning and expression of embryogenesis-regulating genes in *Araucaria angustifolia* (Bert.) O. Kuntze (Brazilian Pine)

Paulo Sérgio Schlögl[1], André Luis Wendt dos Santos[2], Leila do Nascimento Vieira[1], Eny Iochevet Segal Floh[2] and Miguel Pedro Guerra[1]

[1]*Laboratório de Fisiologia do Desenvolvimento e Genética Vegetal, Departamento de Fitotecnia, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil.*
[2]*Laboratório de Biologia Celular Plantas, Departamento de Botânica, Universidade de São Paulo, São Paulo, SP, Brazil.*

Abstract

Angiosperm and gymnosperm plants evolved from a common ancestor about 300 million years ago. Apart from morphological and structural differences in embryogenesis and seed origin, a set of embryogenesis-regulating genes and the molecular mechanisms involved in embryo development seem to have been conserved alike in both taxa. Few studies have covered molecular aspects of embryogenesis in the Brazilian pine, the only economically important native conifer in Brazil. Thus eight embryogenesis-regulating genes, viz.,*ARGONAUTE 1*, *CUP-SHAPED COTYLEDON 1*, *WUSCHEL-related WOX*, *S-LOCUS LECTIN PROTEIN KINASE*, *SCARECROW-like*, *VICILIN 7S*, *LEAFY COTYLEDON 1*, and *REVERSIBLE GLYCOSYLATED POLYPEPTIDE 1*, were analyzed through semi-quantitative RT-PCR during embryo development and germination. All the eight were found to be differentially expressed in the various developmental stages of zygotic embryos, seeds and seedling tissues. To our knowledge, this is the first report on embryogenesis-regulating gene expression in members of the Araucariaceae family, as well as in plants with recalcitrant seeds.

Key words: seed development, Brazilian Pine, embryogenesis-regulating genes, zygotic embryogenesis.

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Introduction

Araucariaceae is one of the most ancient families of gymnosperms. With origins dating back to the Triassic, the family expanded and diversified in the northern and southern hemispheres until the end of the Cenozoic (Kershaw and Wagstaff, 2001). The present restriction to the southern hemisphere alone (Setoguchi *et al.*, 1998) is probably a consequence of angiosperm development in the Mid-Cretaceous. Through the widespread and uncontrolled exploitation of several species for timber, food and ornamental purposes, many are currently considered threatened or endangered.

Araucaria angustifolia, the Brazilian pine, is the only naturally occurring member of this family in Brazil. Until the 1970's, it was the most exploited local timber-source, with the consequential marked depletion of natural populations (Guerra *et al.*, 2000). Nowadays, the priorities set by the Brazilian environmental authorities include conservation of natural remnants and the establishment of breeding programs for the reforestation of exploited areas.

Within this scenario, biotechnological approaches designed to improve somatic embryogenesis, such as the *in vitro* formation of embryos, could potentially pave the way to the development of efficient genetic improvement and germoplasm conservation methods for the Brazilian pine. Although protocols for plant regeneration by somatic embryogenesis have already been obtained for a few conifer species, no such method has been specifically developed in this case, since many of the problems encountered, especially the asynchronous development and misshaping of mature somatic embryos, can be attributed to the sub-optimal conditions used in culture-media. Thus, before starting an investigation, a deeper understanding of the molecular, biochemical, and physiological processes involved during seed development, is a basic requirement (Stasolla *et al.*, 2003), as knowledge thereof could aid in the development of more precise and less empirically based protocols for this specific case (dos Santos *et al.*, 2002).

Seed development can be divided into two phases. In the first, embryo morphogenesis takes place, with the correct establishment of the body plan and the arrangement of

Send correspondence to Miguel Pedro Guerra. Programa de Pós Graduação em Recursos Genéticos Vegetais, Laboratório de Fisiologia do Desenvolvimento e Genética Vegetal, Universidade Federal de Santa Catarina, 88040-900 Florianópolis, SC, Brazil. E-mail: mpguerra@cca.ufsc.br.

cell-types within each tissue of the embryo. It has been shown that, during the second phase (maturation), besides the several signaling pathways integrating information from genetic programs, hormonal and metabolic signals are also required to prepare the embryo for subsequent desiccation and dormancy, as well as to accumulate the necessary nutrients for initial seedling growth (Gutierrez *et al.*, 2007).

When compared to other conifer species, the Brazilian pine reveals unique early zygotic embryogenesis features with a high degree of specialization (Buchholz, 1920; Kaur and Bhatnagar, 1983). Furthermore, the seeds themselves are recalcitrant and mostly orthodox (Attree and Fowke, 1993). Despite recent reports on the accumulation of proteins and abscisic acid (Silveira *et al.*, 2008), as well as polyamines and amino acids (Astarita *et al.*, 2003, 2004), during embryogenesis, nothing has been published so far on gene expression during seed development, not only as regards this species itself, but also other members of the Araucariaceae family as a whole.

Besides the available plant-genome sequences and EST databases, genetic manipulation and the occurrence of mutants also provide a framework for gene expression analysis of embryogenesis in uncharacterized plant species. As angiosperm and gymnosperm plants evolved from a common ancestor around 300 million years ago, subsequent mutual differences in cell anatomy and molecular biology of embryogenesis have obviously been reported to occur (Bowe *et al.* 2000; Cairney *et al.*, 2006; Cairney and Pullman, 2007). Nevertheless, molecular analysis of embryo development in various plant species has revealed that several mutually identical developmental pathways seem to have been maintained. Of the SeedGene database list of the 295 genes that are essential for embryogenesis in *Arabidopsis*, approximately 72% are to be found in the proteome (Cairney and Pullman, 2007). There is ample evidence that transcription factors play a role in those functional polymorphisms affecting growth and development in the different species. Approximately half of the nucleotide polymorphisms account for various phenotypes that occur in regulatory regions (Alonso-Blanco *et al.* 2005). Thus, the resemblance of most coding sequences for embryo-expressed genes in conifers to those in other plants is not surprising (Cairney and Pullman, 2007).

Prior studies of angiosperms have identified some of the molecular components required for forming and maintaining shoot apical (SAM) and root apical (RAM) meristems. The development of both requires the interaction of gene regulatory networks, including *ZWILLE* (*ZLL*), *ARGONAUTE1* (*AGO1*), *NO APICAL MERISTEM* (*CUC1-3*), *WUSCHEL* (*WUS*), *CLAVATA1* (*CLV1*), *SCARECROW* (*SCR*) and others. As yet, the Brazilian pine genome has not been sequenced, the few genes so far characterized during seed development having presented similarities with those found in other conifer genomes and *Arabidopsis*. A leucine-rich-repeat trans-membrane protein that resembles *CLAVATA 1* was shown to be expressed during embryo development in this species, thereby implying that some original embryogenesis-regulating mechanisms have been conserved in the Araucariaceae family (Fernandez HJ (2001) PhD Thesis, Universidade Estadual de Campinas, Campinas, SP, Brazil).

In order to increase current knowledge embryonic gene expression and establish molecular markers for monitoring normal development and/or the detection of abnormalities early in the somatic embryogenesis process, semi-quantitative RT-PCRs (sqRT-PCR) were used in the present study in the analysis of eight embryogenesis-regulating genes, viz., *ARGONAUTE 1*, *CUP-SHAPED COTYLEDON 1*, *WUSCHEL-related WOX*, *S-LOCUS LECTIN PROTEIN KINASE*, *SCARECROW 1*, *VICILIN 7S*, *LEAFY COTYLEDON 1*, and *REVERSIBLE GLYCOSYLATED POLYPEPTIDE*, during seed development in the Brazilian pine. Sequence alignment and phylogenetic reconstruction indicated, not only the shared sequential similarity between angiosperm and gymnosperms species, but also that many embryogenesis-regulating genes have been conserved in both taxa throughout their evolution. In addition, all of the eight selected genes were differentially expressed during zygotic embryo development.

Materials

Plant growth

Seeds were harvested in Santa Catarina State (27°47' S, 49°29' W), Brazil, from December, 2007 to May, 2008, whereupon, embryos at the late globular (Gl), cotyledonary (Co) and mature (Ma) stages were isolated. Due to the very low amount and small size, pro-embryos and early globular stages were excluded from the analysis. *In vitro* seed germination was carried out on basic medium BM basal salts (Gupta and Pullman, 1991), supplemented with 3% (w/v) saccharose and 0.65% (w/v) agar, under a 16/8 (light/dark) photoperiod, at 25 ± 2 °C. Five days after germination, seedling roots and needles were detached, individually weighed and frozen in liquid nitrogen, for subsequent RNA isolation.

Total RNA isolation

Total RNA from late globular and cotyledonary zygotic embryos (200 mg fresh weight) were extracted with Trizol® (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. RNA from mature zygotic embryos, megagametophytes, needles and roots (150-200 mg fresh weight) were extracted as previously described (Preccott and Martim, 1987). RNA quality was monitored by electrophoresis on 1% (w/v) formaldehyde agarose gels, followed by ethidium bromide staining.

Degenerated primer design

Query sequences, comprising full-length *Arabidopsis* gene-sequences of each selected gene, were used for

screening the NCBI database to find homologous cDNAs from various plant species for posterior alignment with Clustal X software (Thompson *et al.*, 1997). The degenerated primers were manually designed, based on the aligned nucleotide sequences thus obtained.

cDNA synthesis, cloning and sequencing

cDNA of each plant was synthesized by using 2 μg of total RNA digested with DNase I (Fermentas, USA), 500 ng of oligo-dT_{25}-ancored primer (5'-T(25)VN-3'), and the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA), in a 20 μL reaction, according to the manufacturers' instructions.

cDNA templates were amplified by PCR, using degenerated primers designed in compliance to the sequences of the selected genes (Table 1). According to nucleotide alignment, cDNA fragments of the expected size were cloned into a TA vector (Invitrogen, Carlsbad, CA), for posterior sequencing with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Alameda, CA) and M13 universal primers.

In order to confirm the identitity of cDNA cloned sequences, each was compared to DNA sequence databases, using BLAST search tools (Altschul *et al.*, 1997). For phylogenetic analysis, deduced protein sequences were aligned using the Clustal X program (Thompson *et al.*, 1997). Phylogenetic and molecular evolutionary analyses were with selected sequences using the MEGA program, version 4 (Tamura *et al.*, 2007). Bootstrap analysis was with 1000 replicates. Generic naming of sequences was according to the denomination of the selected gene used to design the degenerated primer, plus the "*Aa*" prefix, *e.g.*, *AaWOX*.

Semi-quantitative RT-PCR analysis

Semi-quantitative RT-PCR (sqRT-PCR) analysis was to check expression patterns of the selected genes during zygotic embryogenesis and early embryo germination. The design of appropriate primers was based on previously described specific nucleotide sequences of Brazilian pine cDNA fragments. The sequences thus obtained were analyzed by way of Primer 3.0 version 0.4.0 software, with CG Clamp set as 1 or 2. The primer sequences are listed in Table 2. PCR amplifications were carried out to test annealing temperatures, primers and the optimal number of PCR cycles for each of the selected primers (Table 2). Template cDNAs were synthesized as previously described, and dilutions adjusted, with *Ubiquitin-1* (*AaUBI-1*, GW924714) as an endogenous normalization factor.

The following thermal cycle conditions were used for the sqRT-PCR reactions: 94 °C for 3 min, followed by 94 °C for 30 s, a 30 s annealing cycle of each pair of primers, and a 1 min cycle at 72 °C, followed by the respective number cycles for each specific pair of primers and an final 5 min elongation step at 72 °C. The PCR reactions were carried out in a final volume of 25 μL, containing 0.3 μM of primers and 2 μL of cDNA template. The mean length of PCR products ranged from 175 to 200 bp. PCR products, first resolved on 2% (w/v) agarose gels stained with ethidium bromide, were then photo-documented.

sqRT-PCR reactions were carried out in three replicates from total RNA originating from two different biological experiments. An aliquot of the total RNA digested with DNAse I to synthesize the cDNA template, was used as control in separate PCR reactions, to so check for genomic DNA contamination. No signals were obtained,

Table 1 - Homologous genes of angiosperm proteins associated to embryogenesis, and a list of degenerated primers used for cDNA isolation during zygotic embryogenesis and early-seedling growth in the Brazilian pine.

Gene	Function	Primers	Size*
CLAVATA 1	Cell fate	For 5'RTNGGNAWRGGNKSNNYNCC'3 Rev 5'YKVGCVARNCCRAARTCDGC'3	236
WUSCHEL	Meristem maintenance	For 5'ANNDGBHSVMGNTGGAMDCC'3 Rev 5'GRGCYTTRTRRTTYTGRAAC'3	201
UBIQUITIN	Ubiquitination	For 5'HGTBATHTTYGGNCCDGATG'3 Rev 5'CTRWACADBCKDGCDGCTTC'3	270
LEAFY COTYLEDON 1	Maturation	For 5'YWBMTGCCVATHGCNAAYGT'3 Rev 5'ATDGCCCANARVABRTCBTC'3	240
VICILIN 7 S[a]	Seed protein storage	Acess number *AA*M81249.1	
ARGONAUTE 1	Meristem maintenance	For 5'VAARMGNATWTGTGARACTG'3 Rev 5'TGBCCYTCRCTDACWCCATC'3	480
SCARECROW	Cell fate and division	For 5'VATHGAYYTNGACATMATGC'3 Rev 5'RAGHARVSAVAGRTCYTTCC'3	725
CUPSHAPED COTYLEDON1	Organ boundary	For 5'SAACARRTSYGAGCCNTGGG'3 Rev 5'VCKRTAYTCRYGCATNACCC'3	270
AUXIN BINDING POTEIN 1	Auxin flux	For 5'TGAARGAGRTDGARRTDTGG'3 Rev 5'ARYYKBGCWGCWGTRTGDGG'3	250

*PCR product sizes. [a]Cloned in another work.

Table 2 - sqRT-PCR primers, PCR product sizes, melting temperatures, and PCR cycle numbers used in the analysis of gene expression during zygotic embryogenesis and early seedling growth in the Brazilian pine.

Gene	sqRT-PCR primers	TM (°C)	Cycle numbers	Product size (bp)
AaAGO	for 5'TCAAGGTGGGTGGAAGAAAC'3 rev 5'TCAATGATCTCTTGCCGATG'3	50	27	266
AaWOX	for 5'GGCTTTGTGGTTTTGGAAC'3 rev 5'GCCAAGCCAAACTCAACTTC'3	46	35	142
AaLecKIN	for 5'AAGGTGCTGGACTGGAAGAC'3 rev 5'CCACTTTGGGGCAGAAATC'3	50	35	151
AaVIC	for 5'GAGGAGACTCGCTACAGATGC'3 rev 5'CTTCCATCGATTTCTCTTTCC'3	50	27	220
AaCUC	for 5'AAAATGGGGGAAAAGGAATG'3 rev 5'GCATCACCCAGTTGGTCTTC'3	50	35	220
AaUBI	for 5'GTCGGATGTGTTTCATCCTAATG'3 rev 5'CTTCTGGATTTGCAGGACTTG'3	50	27	160
AaLEC	for 5'GCCGATTGCAAACGTGAG'3 rev 5'TGATGGTCTTGCGCTTTTC'3	46	35	164
AaRPG	for 5'AGGGATGTTGAGCCACAAAC'3 rev 5'CATTGATGACGATTGCTTCG'3	50	27	250
AaSCR	for 5'CTTTGCTCGGACCTTGAATC'3 rev 5'TATTTGCATCGGAGCCTGTC'3	50	35	165

thus indicating the absence of any detectable contamination (data not shown).

Results

Identification and sequencing analysis of embryogenesis-regulating genes

Fragments of eight embryogenesis-regulating genes related to morphogenesis, cell signalization, and reserve deposition (*ARGONAUTE 1* (*AaAGO-1*), *CUP-SHAPED COTYLEDON 1* (*AaCUC*), *WUSCHEL-related WOX* (*AaWOX*), *S-LOCUS LECTIN PROTEIN KINASE* (*AaLecK*), *SCARECROW-like* (*AaSCR*), *REVERSIBLE GLYCOSYLATED POLYPEPTIDE 1* (*AaRPG*), *LEAFY COTYLEDON 1* (*AaLEC*) and *VICILIN 7S* (*AaVIC*)) were isolated, cloned and sequenced from cDNA libraries of Brazilian pine embryonic cell cultures.

AaAGO 1 (GW924721) shared high-deduced amino acid sequence identity with not only putative *AGO1* homologues from *Populus trichocarpa* (97%), but also with *AGO1* and *ZLL* (*ZWILLE*) proteins from *Arabidopsis* (91% and 89%, respectively). Sequence alignment with plant *AGO* proteins was to demonstrate the similarity among sequences from the different taxa (Figure 1). The cloned sequence from *AaAGO* represented 15% of the full length *AGO1* sequence from *Arabidopsis*, and was shown to share high similarity within a region between the *PAZ* and *PIWI* domains (amino acid 693-851). In order to define *Aa*AGO evolutionary relationships, alignments of deduced protein sequences of the *AGO* protein family from *Arabidopsis* and other plants were used to construct an unrooted neighbor-joining phylogenetic tree. *AaAGO*, *AGO1*, *ZLL* and *AGO5* proteins from various plant species clustered together in Clade I (Vaucheret, 2007), thereby forming a putative cluster of orthologues with 100% bootstrap support, thus implying that *AaAGO* belongs to the *AGO 1* subgroup (Figure 2).

The putative Brazilian pine *CUC-like* deduced protein (*AaCUC-like*, GW924718) was highly identical with both putative *NAC* proteins from *Solanum lycopersicum* (92%) and *Populus trichocarpa* (87%), and *CUC2* from *Arabidopsis thaliana* (92%). The *AaCUC-like* sequence corresponded to 24% of the entire sequence (*Aac* 55-143), and covered 60% of the *NAC* domain (subdomains N2-N4) of the Arabidopsis *CUC2* protein (Figure S1A).

The deduced *AaWOX-like* protein sequence (GW924719) presented appreciable similarity to a *WOX-like* protein from *Picea sitchensis* (94%), *Physcomitrella patens* (90%), and other related proteins from various plant species (Figure S1B). The protein sequence of *AaWOX* corresponded to 20% of the *WOX13* protein from Arabidopsis (*Aa*c 100-153). Hence, a phylogenetic tree was constructed using the alignment of *AaWOX* and other plant protein sequences of the *WUS/WOX* family. It was observed that *AaWOX* clustered with *WOX10-14* from *Arabidopsis* and *WOX-like* proteins from *Populus tomentosa* and *Physcomitrella patens*. This very close relationship to the *Physcomitrella* sequence possibly implies a very ancient common origin (Figure 3).

Three different sequences of *RECEPTOR-like PROTEIN KINASES* (*RLKs*) were cloned with the degenerated primer for the *CLV1 cytoplasmatic kinase domain*. According to BLAST search, *AaRLK1* (*AaLecKin*, GW924722) was classified within the S-locus lectin protein kinase subfamily and *AaRLK2* (*AaCLVL*, GW924723) - 3 (*LRRPK*, GW924717) as *Leucine-Rich Repeat protein*

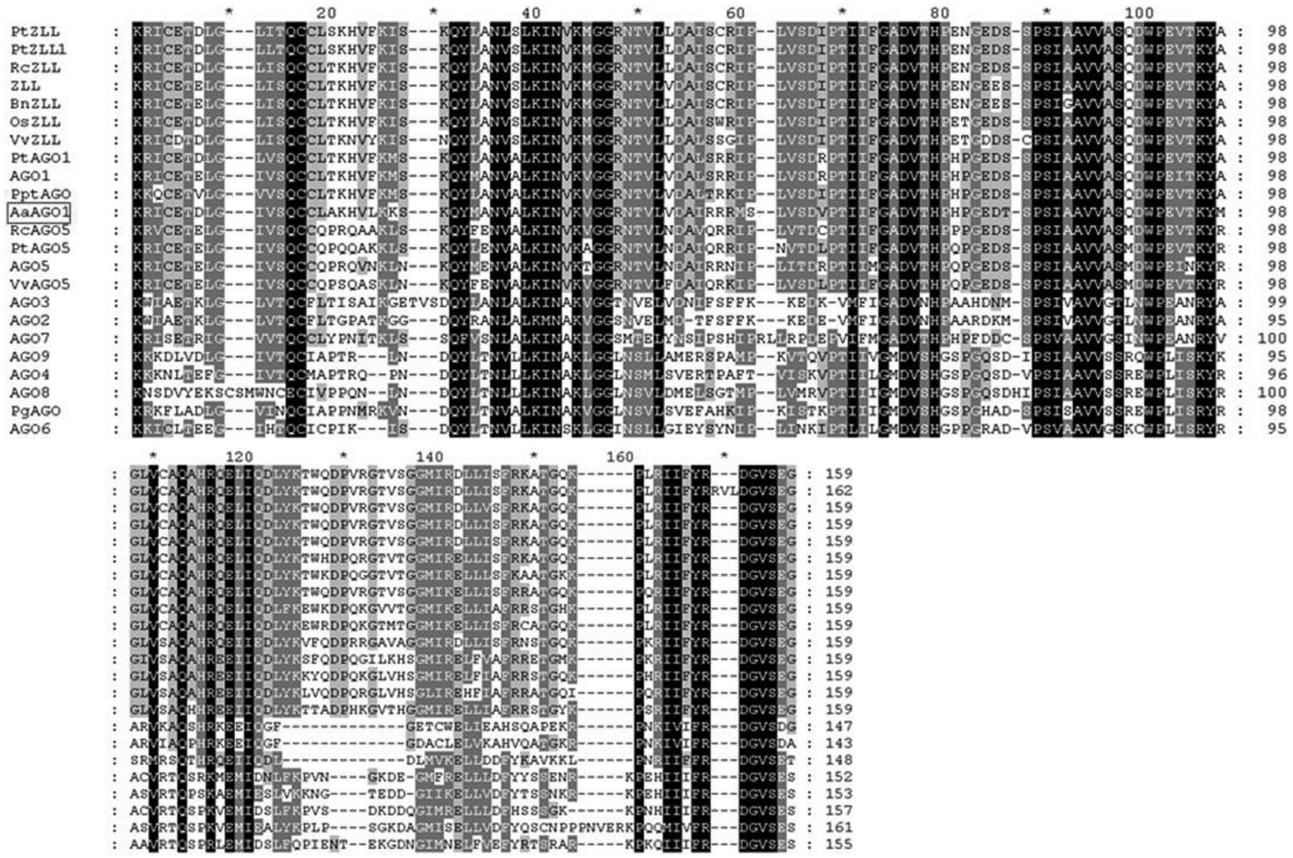

Figure 1 - Alignments of the plant ARGONAUTE protein family. Multiple alignments were done using ClustalW (version 1.74) software. Accession numbers: *Arabidopsis thaliana* - *AGO1*: NP_849784, *AtAGO2*: NP_174413, *AtAGO3*: NP_174414, *AtAGO4*: NP_565633, *AtAGO5*: Q9SJK3, *AtAGO6*: NP180853, *AtAGO7*: NP_177103, *AtAGO8*: NP_197602, *AtAGO9*: CAD66636, *AtZLL*: NP_199194. *Populus trichocarpa* - *PtZLL*: XP_002314663, *PtZLL1*: XP_002312555, *PtAGO1*: XP_002329692, *PtAGO5*: XP_002298162. *Brassica napus* - *BnZLL*: ABY52943. *Vitis vinifera* - *VvZLL*: XP-002281687, *VvAGO5*: XP_002271699. *Physcomitrella patens* - *PptAGO1*: XP_001757611. *Ricinus communis* - *RcZLL*: XP_002517060, *RcAGO5*: XP_002523757. *Araucaria angustifolia* - *AaAGO*: GW924721.

kinase CLV-like. From sequence alignment with different receptor-like protein kinase cytoplasmatic kinase domains (Figure S1C), it was deduced that, as with *Arabidopsis* and other plants, different subfamilies of receptor-like protein kinases participate in the process of cell signalization during Brazilian pine embryogenesis.

AaSCR (GW924716) presented 88% identity with a putative *SCR* deduced protein sequence from *Pinus sylvestris*, and 83% with a putative *SCR* protein from *Populus trichocarpa*. The deduced *AaSCR* sequence covered 16% of the *SCR* protein from *Arabidopsis* (amino acid 534-644), the alignment of protein sequences showing high conservation of the *AaSCR* protein and other plant SCRs (Figure S1D). The *AaSCR* sequence itself encompasses part of the PFYRE domain present in the C-terminal region of *Arabidopsis SCR*s (Pysh *et al.*, 2009).

A putative *AaLEC-like* (CCAAT binding protein) sequence (GW924720) presented 80 amino acids sharing 100% similarity with a homologous protein from *Vitis vinifera* and *Ricinus communis*, besides covering 40% of the full length sequence of the *Arabidopsis* NF-YB3 binding factor. The alignment of LEC-like proteins demonstrated high *AaLEC* similarity to all the proteins analyzed, thereby inferring its role during embryo development (Figure S1E).

By using degenerate oligonucleotides based on the *ABP1* sequence, a *putative reversible glycosylated polypeptide* (*RPG*) was cloned (GW924715). The sequence covered 23% of the full length of the *Arabidopsis RPG 1* protein sequence (amino acid 71-153). Through being involved in the biosynthesis of various plant polysaccharides, such as hemicelullose and starch, there is evidence that *RPG* proteins may play a role in cell-wall biosynthesis (Delgado *et al.*, 1998). BLAST search and the alignment of *RPG-like* sequences demonstrated that *AaRPG* has high amino acid sequence conservation with other *RPG* proteins from plants (Figure S1F).

Expression of embryogenesis-regulating genes during zygotic embryogenesis and initial seedling growth

Semiquantitative RT-PCR was applied in the analysis of selected embryogenesis-regulating gene expression during Brazilian pine embryo development and initial seedling growth (Table 1). Genes related to morphogenesis and cell

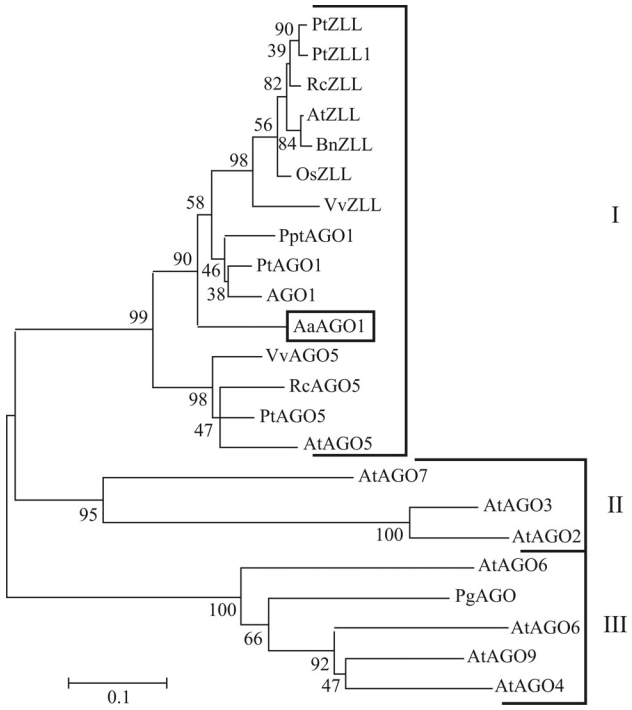

Figure 2 - Phylogenetic tree of *ARGONAUTE* proteins. Multiple alignments were done using ClustalW (version 1.74) software. MEGA software was used in bootstrap analysis and tree construction. Bootstrap percentages are indicated at each fork. Accession numbers: *Arabidopsis thaliana* - *AGO1*: NP_849784, *AtAGO2*: NP_174413, *AtAGO3*: NP_174414, *AtAGO4*: NP_565633, *AtAGO5*: Q9SJK3, *AtAGO6*: NP180853, *AtAGO7*: NP_177103, *AtAGO8*: NP_197602, *AtAGO9*: CAD66636, *AtZLL*: NP_199194. *Populus trichocarpa* - *PtZLL*: XP_002314663, *PtZLL1*: XP_002312555, *PtAGO1*: XP_002329692, *PtAGO5*: XP_002298162. *Brassica napus* - *BnZLL*: ABY52943. *Vitis vinifera* - *VvZLL*: XP-002281687, *VvAGO5*: XP_002271699. *Physcomitrella patens* - *PptAGO1*: XP_001757611. *Ricinus communis* - *RcZLL*: XP_002517060, *RcAGO5*: XP_002523757. *Araucaria angustifolia* - *AaAGO*: GW924721.

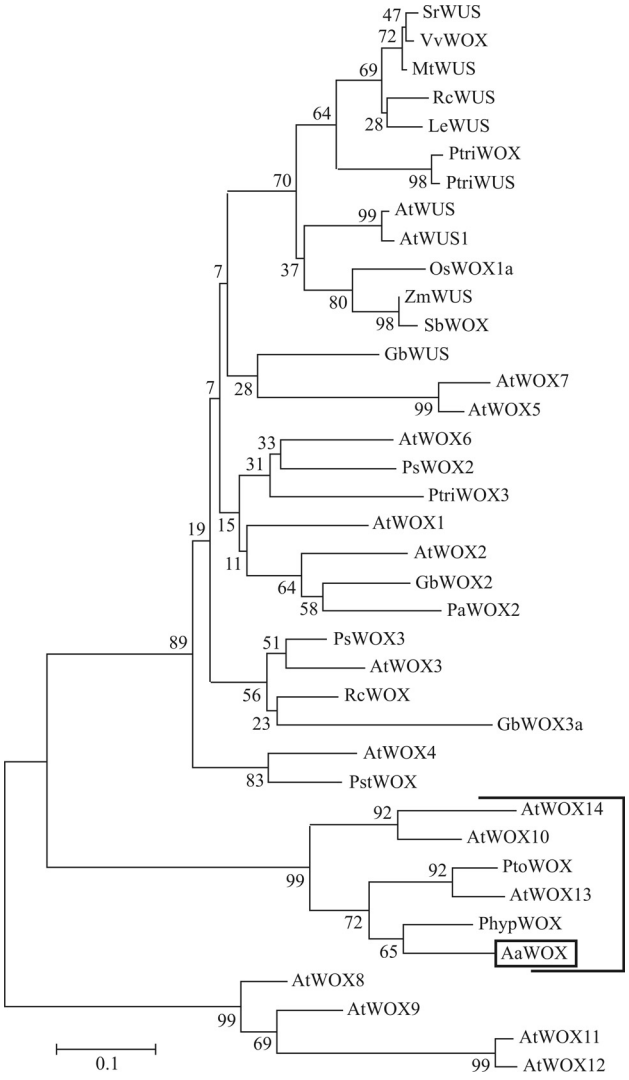

Figure 3 - Phylogenetic tree of *WOX* proteins. Multiple alignments were done using ClustalW (version 1.74) software. MEGA software was used in bootstrap analysis and tree construction. Bootstrap percentages are indicated at each fork. Accession numbers: *Arabidopsis thaliana* - *AtWOX1*: Q6X7K0, *AtWOX2*: Q6X7K1, *AtWOX3*: Q9SIB4, *AtWOX4*: Q6X7J9, *AtWOX5*: AAP37136, *AtWOX6*: Q9ZVF5, *AtWOX7*: Q9FFK0, *AtWOX8*: Q6X7J5, *AtWOX9*: Q6X7J4, *AtWOX10*: Q9LM83, *AtWOX11*: Q6X7J3, *AtWOX12*: Q8GY25, *AtWOX13*: O81788; *AtWOX14*: Q9LM84. *Oryza sativa*: *OsWOX1a*: Q7XM13. *Streptocarpus rexii* - *SrWUS*: B2WSTO. *Vitis vinifera* - *VvWOX*: XP_002266323. *Medicago truncatula* - *MtWUS*: ACK77479. *Ricinus communis* - *RcWUS*: XP_002530735, *RcWOX*: XP_002532820. *Populus trichocarpa* - *PtriWOX*: XP_002327757, *PtriWUS1*: A0AAS8, PtriWOX3:B9HW56. *Zea mays* - *ZmWUS*: NP_001105960. *Sorghum bicolor* - *SbWOX*: XP_002448707. Ginkgo biloba - *GbWUS*: CAT02906, *GbWOX2*: CAT02902, *GbWOX3a*: C3W868. *Pinus sylvestrys* - *PsWOX2*: C3W8A3-1, *PsWOX3*: C3W8A1. *Picea sitchensis* - *PstWOX*: B8LN48. *Picea abies* - *PaWOX2*: Q14FJ6. *Populus tomentosa* - *PtoWOX*: AAR83341. *Physcomitrella patens* - *PhyWOX*: XP_001777634. *Araucaria angustifolia* - *AaWOX*: GW924719.

signaling (*AaAGO*, *AaCUC*, *AaWOX*, *AaLeckin*, *AaLEC*, *AaRPG-like*), and to seed-storage reserve (*AaVIC*), were upregulated until the cotyledonary stage (Co). Subsequently, their expression decreased in mature zygotic embryos (Ma). *AaSCR* levels, high in late globular zygotic embryos (Gl) and seedlings (G5), did not differ at the transcript phase during the Co and Ma stages (Figure 4). Although in mature megagametophytes, *AaAGO* was weakly expressed, *AaCUC*, *AaWOX*, *AaRPG* and *AaVIC* were highly so.

On considering the entire seedling and except for *AaSCR*, genes related to morphogenesis and cell signalization were upregulated, when compared to the Ma stage. *AaVIC* could not be detected in seedlings, isolated needles or roots. In isolated roots, there was little sign of *AaAGO*, *AaSCR*, and *AaLEC*, the contrary to *AaCUC*, *AaWOX*, and *AaRPG* (Figure 4). Whereas *AaLecK* was barely detected in isolated needles, the remaining genes were very much so (Figure 5).

Discussion

The existence of several pine-embryo EST collections containing mRNA sequences from related genes (Carney and Pullman, 2007), and the cloning of the putative homologous cDNAs of these genes in the Brazilian pine, together confirm that like mechanisms govern meristem activity and embryo maturation in gymnosperms as a whole. A putative *ARGONAUTE 1* (*AaAGO*) was expressed and

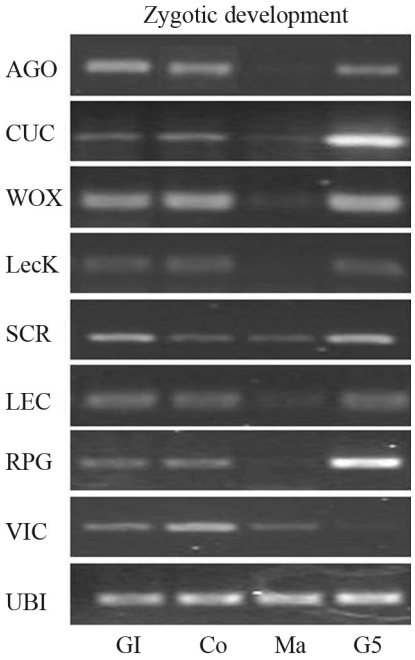

Figure 4 - Expression pattern of embryogenesis-regulating genes (ARGONAUTE (*AaAGO*), CUP-SHAPED COTYLEDON1 (*AaCUC*), wushel-related WOX (*AaWOX*), S-locus lectin protein kinase (*AaLecK*), SCARECROW-like (*AaSCR*), VICILIN 7S (*AaVIC*), LEAFY COTYLEDON 1 (*AaLEC*), and a Reversible glycosylated polypeptide (*AaRGP*)) during Brazilian pine zygotic embryogenesis. RT-PCR reactions were carried out on reverse-transcribed total RNA samples from late globular (Gl), cotyledonary (Co) and mature zygotic embryos (Ma), and mature embryos (G5) after five days of germination. *AaUBI-1* cDNA was used as endogenous normalization factor.

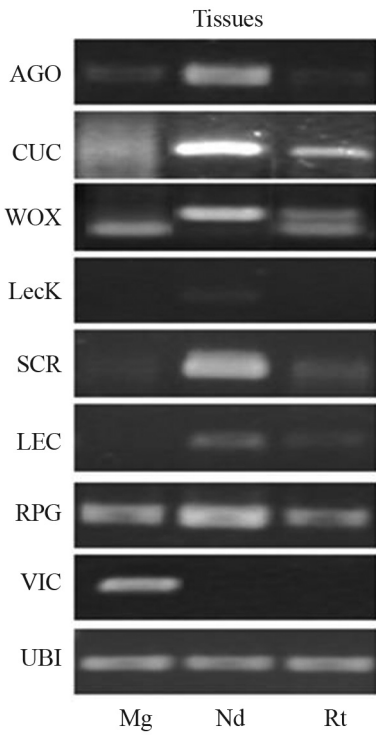

Figure 5 - Expression of embryogenesis-regulating genes (ARGONAUTE (*AaAGO*), CUP-SHAPED COTYLEDON1 (*AaCUC*), wushel-related WOX (*AaWOX*), S-locus lectin protein kinase (*AaLecK*), SCARECROW-like (*AaSCR*), VICILIN 7S (*AaVIC*), LEAFY COTYLEDON 1 (*AaLEC*), and a Reversible glycosylated polypeptide (*AaRGP*)) in young seedling tissues and mature megagamethophytes in the Brazilian pine. RT-PCR reactions were carried out on reverse-transcribed total RNA samples from different tissues: (Mg) mature megagametophytes, (Nd) needles and (Rt) roots. *AaUBI-1* cDNA was used as an endogenous normalization factor.

up-regulated during Brazilian pine early zygotic development and maturation, initial seedling growth (needles and roots) and in mature megagametophytes. Likewise, an *ARGONAUTE* (*PgAGO*) was shown to be up-regulated during meristem formation in the early stages of embryo development in *Picea glauca* (Tahir *et al.*, 2006). As *PgAGO* expression was reportedly restricted to the meristematic cells of both roots and shoots, it was assumed that *AGO* is required for proper embryo development through the specification of stem-fate identity in these cells (Tahir *et al.*, 2006). *AGO1* and *ZLL*, the closest homologues to the *AGO1* protein family, participate in meristem formation, stem-cell fate, and leaf polarity through RNA silencing mechanisms (Lynn *et al.*, 1999; Carmell *et al.*, 2002). The protein sequence similarity with *Arabidopsis* (*AGO1* and *ZLL*) and other plant *AGO1* proteins – in addition to the pattern of *AaAGO* expression – gives to understand that similar mechanisms are capable of regulating SAM formation during early zygotic development and initial seedling development in the Brazilian pine (Figure 4). *AaAGO* was also expressed in mature megagametophytes, thereby indicating the existence of a putative role for *AGO* proteins during seed development in this gymnosperm (Figure 5).

Development of a specific body-plan during embryogenesis requires the coordination of cell fates according to their individual position along embryo axes. A constant stem-cell population indicates that the recruitment of cells into new organs is precisely balanced by the formation of new stem-cell derivatives (Nardmann *et al.*, 2009). These cells are located in stem-cell niches, where signals from the neighboring cells keep them in a pluripotent state. Their undifferentiated state is maintained by signals that depend upon *WUS* expression in a small underlying cell group termed the "organizing center" (OC) (Mayer *et al.*, 1998). In turn, stem cells express *CLAVATA3* (*CLV3*), which acts by restricting *WUS* transcription via *CLV1/CLV2* signaling. This feedback provides a mechanism for controlling the size of the stem-cell pool (Schoof *et al.*, 2000).

In the present work, a putative member of the *WOX* family (*AaWOX*) and three different classes of leucine-rich receptor-like protein kinases, were cloned. In all, gene expression was analyzed, both during the period preceding cotyledon elongation, and in seedling roots and needles (Figures 4 and 5). Phylogenetic analysis of *AaWOX* showed it to belong to a putative cluster of orthologous genes, together with Arabidopsis *WOX10-14*, and a WOX-like pro-

tein from both *Populus tomentosa* and *Physcomitrella patens*, a possible indication that this branch of the *WOX* protein-family is of ancient origin (Figure 3). Several members of the *WOX* family are expressed early during embryo development, and at low levels in vegetative tissues. In *Picea glauca*, *PaWOX2* expression was observed in hypocotyls, apical shoots, and cotyledons, but not in roots (Palovaara and Hakman, 2008). In *Arabidopsis thaliana, WOX5* expressed early during embryogenesis, appears to exert a function in RAMs during stem-cell signaling, analogous to that of *WUS* in SAMs (Sarkar *et al.*, 2007), whereas other *WOX* proteins seem to play more diverse roles in cotyledon and cell division in embryos and suspensors (Wu *et al.*, 2007). The results obtained in the present study attribute a putative role to *AaWOX* in functions associated with the regulation of cell division and/or differentiation during embryogenesis and initial seedling growth in the Brazilian pine.

Through BLAST analysis, three receptor-like protein kinases, cloned in the Brazilian pine, were placed into two RLK subgroups. *AaRLK2-3* was classified as a putative leucine-rich repeat transmembrane CLV-like protein kinase (*CLVL* and *LRRKs*), and *AaLecKin* as a putative S-locus lectin protein kinase. Some cDNAs, homologues to the *receptor kinase CLAVATA1* (*CLV1*) and the *receptor-like protein CLAVATA2* (*CLV2*), were encountered in pine embryo-derived EST-sequences (Cairney *et al.*, 2006). Taken together, these results indicate that the main factors pertaining to the *CLV/WUS* negative feedback loop are present in conifers, and that *AaRLK2-3* might be acting together with *AaWUS-like* in the regulation of meristem maintenance. *AaLecKin* was expressed at low levels during all the stages of zygotic embryogenesis that were analyzed (Figure 3). S-locus lectin protein kinases (lectin RLKs) form a large family of receptor-like kinases with an extracellular legume lectin-like domain that is presumed to be involved in carbohydrate binding activities. Legume lectins are well-known carbohydrate-binding proteins, some members of this family having been shown to be involved in plant development (Wu *et al.*, 2007; Wan *et al.*, 2008). Therefore, it is possible that *AaLecKin*, as with other LecKinases (van Hengel *et al.*, 2002), plays a connecting role in embryogenesis by perceiving the oligosaccharide signal generated in the early stages of embryogenesis, thereby ensuring correct embryo development.

The cotyledon boundary is crucial for postembryonic development during seed formation. The *CUP-SHAPED COTYLEDON* (*CUC*) transcription factors are central regulators of organ boundary structuring in plants, and play a decisive role in the establishment of meristems, through activation of the *SHOOT MERISTEMLESS* (*STM*) gene during embryogenesis (Takada *et al.*, 2001). Of the several factors that have been reported to affect *CUC* gene expression, auxin plays a major role in determining the respective spatial patterns. It is supposed that, together with auxin, miR164c mainly controls the accumulation of *CUC1* and *CUC2* and boundary morphogenesis (Aida and Tanaka 2006). Considering, both the high similarity of *AaCUC* to other *CUC* genes and its expression profile in the Brazilian pine during zygotic embryogenesis and in postembryonic organs, it is possible to suppose that it has developed a similar function to that observed in the other *CUC* genes in model plants, such as *Arabidopsis.*

In the present study, a putative *AaSCR* was expressed, both at the start of embryo development, and in post-embryogenic structures (Figures 4 and 5). In *Arabidopsis*, *SCR* encodes a putative transcription factor that belongs to the *GRAS* family. Radial patterning during embryogenesis and post embryonic development are regulated by *SCARECROW* (*SCR*) in both roots and shoots in *Arabidopsis* (Di Laurenzio *et al.*, 1996). In maize and *Arabidopsis*, *SCR* expression has been observed during the early stages of embryogenesis, thereby implying that radial pattern formation is an early event in both species (Lim *et al.*, 2000). *AaSCR* expression was up-regulated during the globular stage, after five days of embryo germination, and in seedling tissues (Figures 4 and 5). Taken together, members of the *GRAS* family probably play crucial roles in embryo and seedling development in the Brazilian pine.

The *AaLEC-like* sequence was very similar to that of another *LEC*, during protein alignment (Figure S1E). Moreover, its expression was slightly up-regulated during the first two phases of embryo development (globular and cotyledonary) (Figure 4). In *A. thaliana*, three *LEC* genes (*LEC1-2* and *FUS3*) are predominantly expressed during embryogenesis, thereby maintaining embryonic cell fate and specifying cotyledon identity (Braybrook and Harada, 2008). Other *Arabidopsis* LEC-like proteins are expressed at low levels in vegetative organs (Kwong *et al.*, 2003). In the specific case of *AaLEC*, this was found to be expressed in needles and roots (Figure 5). In addition to its importance during embryo morphogenesis, *AaLec* is involved in the regulation of Vicilin accumulation, the main storage protein in the Brazilian pine, the same way as *LEC1* regulates protein accumulation and embryo maturation in *Arabidopsis*. The ectopic expression of *LEC1* demonstrated that seed-storage-protein gene expression is controlled by *LEC1* through the regulation of *ABI3* and *FUS3* expression (Gutierrez *et al.*, 2007). The expression of *AaVicilin* transcripts coincides with the first peak of abscisic acid accumulation during seed development in the Brazilian pine. ABA levels have been shown to reach a peak in the pre-cotyledonary stage, after which a continuous decrease was observed up to the mature stage (Silveira *et al.*, 2008). According to data, *AaVicilin* and *AaLEC-like* expression in the Brazilian pine presumes embryo and seed maturation mechanisms to be similar to those observed in other plant species, this depending on *LEC* TF expression and a fine balance between ABA and GA (Verdier and Thompson, 2008).

The primary cell wall of dicot plants is laid down by young cells prior to cessation of elongation and secondary wall deposition. Reversibly glycosylated polypeptides (RGPs), reportedly involved in polysaccharide biosynthesis, seem to play a role in cell-wall biosynthesis, although their precise function remains unknown (Pysh *et al.*, 1999). On cloning a putative *RPG-like* (*AaRPG*) from the Brazilian pine in the present study, high levels of transcripts were found, not only during the stages preceding cotyledon elongation and embryo germination, but also in all seed and seedling tissues (Figures 4 and 5). *RPG* genes are ubiquitously expressed, reaching the highest levels in actively growing tissues. In *A. thaliana*, *RPG1* and 2 have been shown to be required during microspore development and pollen mitosis, conductive to cell division and/or vacuolar integrity (Drakakaki *et al.*, 2006). As to the Brazilian pine, *RPGs* might present a very similar function to *RPG1* and *2*, seeing that the pattern of mRNA expression is similar, with a highly conserved sequence protein in plants from different species (Figure S1F).

In summary, we analyzed certain important and conserved plant embryogenesis-related genes that participate in the network that regulates meristem formation and regulation, organ specification, cell fate, and embryo maturation in Brazilian pine. Despite the differences observed during embryogenesis, it was noticed that the differential expression of changes at the transcript level of the analyzed genes is similar to that which occurs in other conifers and angiosperm species. The present results, besides providing a basis for further studies of gene expression during embryogenesis in this gymnosperm, may also become a useful tool in the improvement of an *in vitro* embryogenesis protocol.

Acknowledgments

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Internet Resources

Primer3.0 version 0.4.0 (http://frodo.wi.mit.edu/primer3/).

DNA sequence databases used for comparison, http://www.ncbi.nlm.nih.gov (April 15, 2009).

Supplementary Material

The following online material is available for this article:

Figure S1A – Alignments of the plant *CUC* protein family. Multiple alignments were done using ClustalW (ver. 1.74) software.

Figure S1B – Alignments of the plant *WOX* protein family. Multiple alignments were done using ClustalW (ver. 1.74) software.

Figure S1C – Alignments of the plant *LRRK* protein family. Multiple alignments were done using ClustalW (ver. 1.74) software.

Figure S1D – Alignments of the plant *SCR-like* protein family. Multiple alignments were done using ClustalW (ver. 1.74) software.

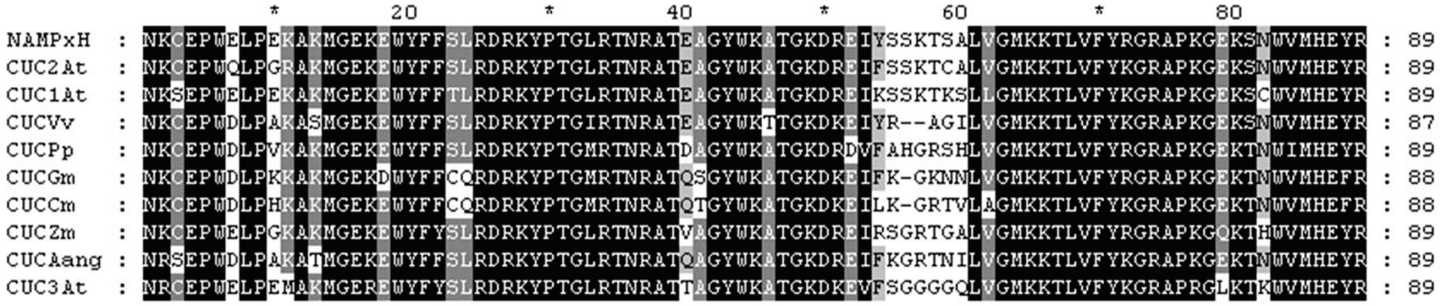
Figure S1E – Alignments of the plant *LEC-like* protein family. Multiple alignments were done using ClustalW (ver. 1.74) software.

Figure S1F – Alignments of the plant *RPG* protein family. Multiple alignments were done using ClustalW (ver. 1.74) software.

This material is available as part of the online article from http://scielo.br/gmb.

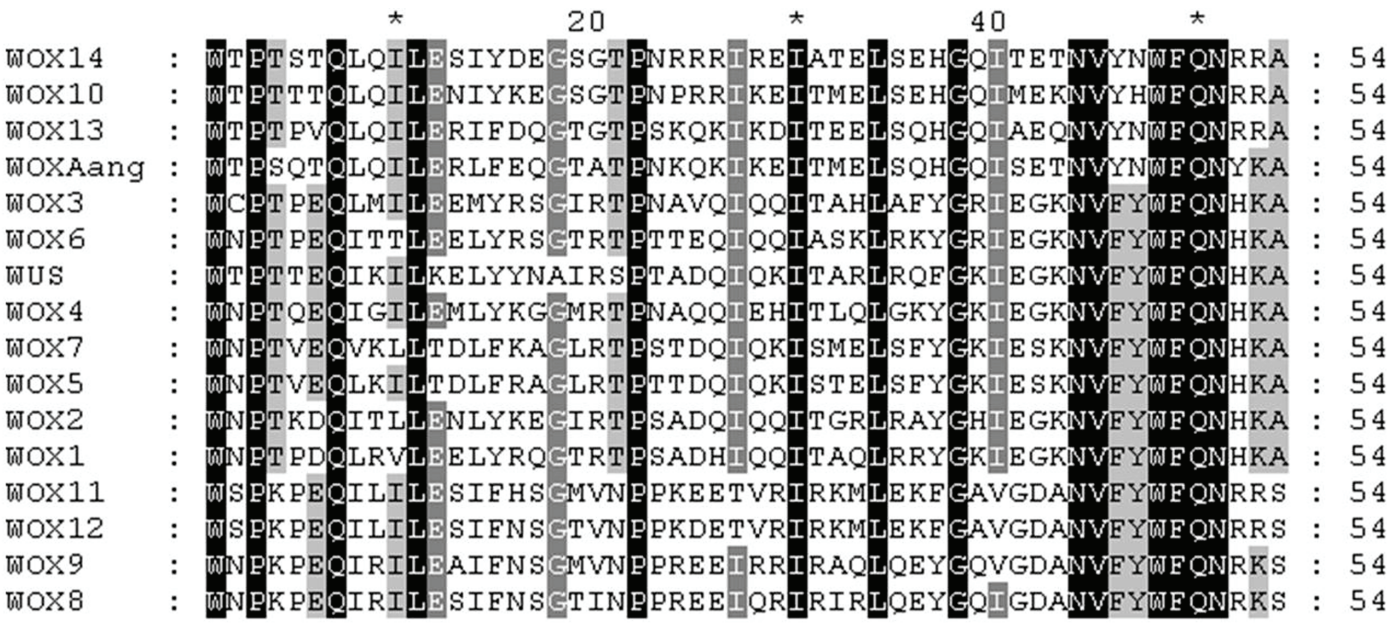
Associate Editor: Adriana S. Hemerly

Figure S1A



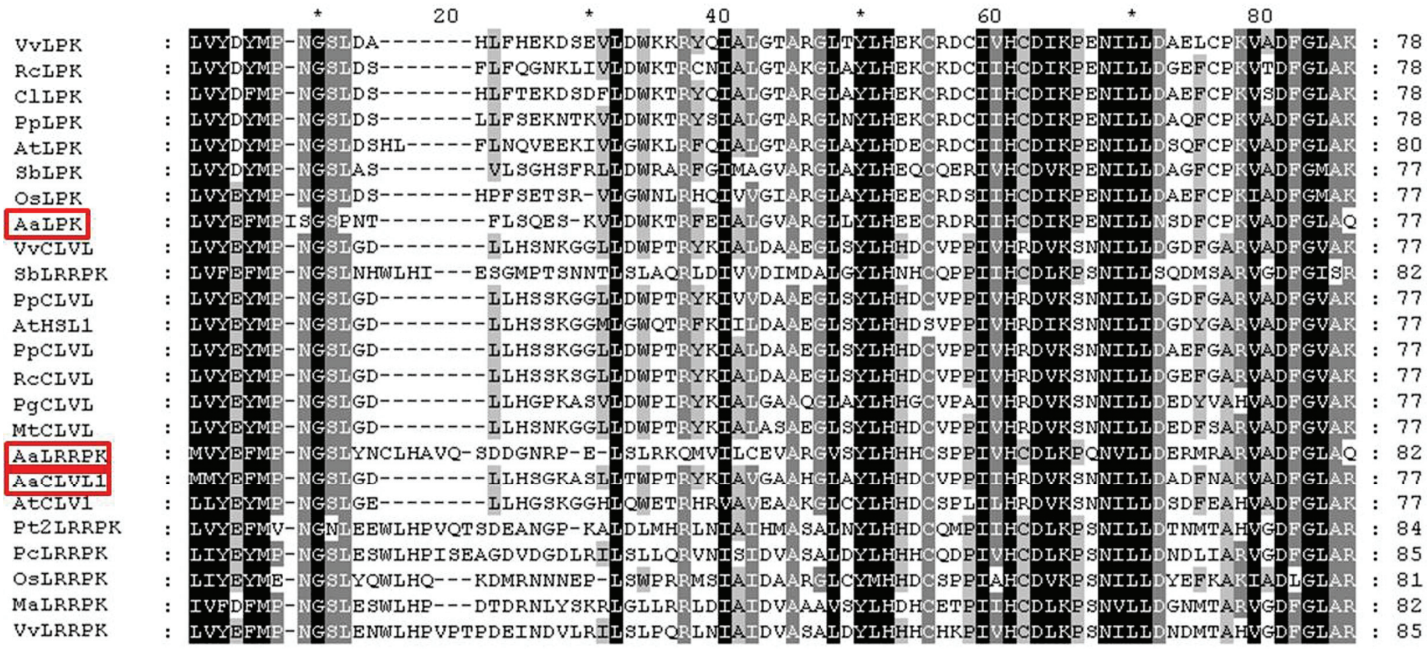
Supplemental figure 1 A - Alignment of CUC proteins from plants.
PxH: *Petunia x hybrida*, NAM-CAA63101;
At: *ArabidopsisThaliana*, CUC1- NP_188135 , CUC2 - NP_200206, CUC3-NP_177768;
Vv: *Vitis vinifera*, CUC- CBI15455;
Pp:*Pscomitrella patens subsp. Patens*, CUC-XP_001777847;
Gm: *Glycine max*, CUC- AAX85982;
Cm: *Cucurbita maxima*, CUC- ACI01723;
Zm: *Zea mays*, CUC- ACG42560 ;
Aang: *Araucaria angustifolia,* GW924718.

Figure S1B



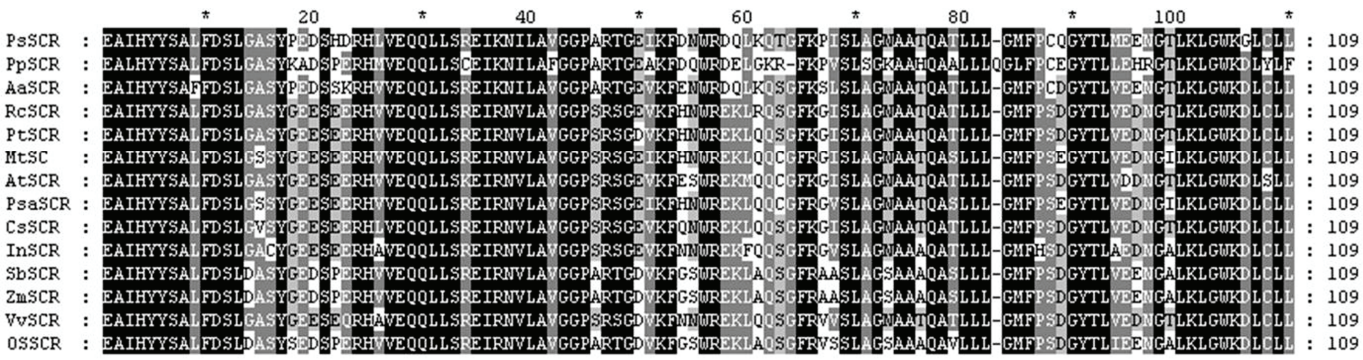
Supplemental figure 1 B - Alignment of WOX proteins from Arabidopsis and Parana pine.
Arabidopsis Thaliana *acess numbers:*
WOX1: Q6X7K0, WOX2: Q6X7K1, WOX3: Q9SIB4, WOX4: Q6X7J9, WOX5:AY25139, WOX6: Q9SB92, WOX7: Q9FFK0, WOX8: Q6X7J5, WOX9: Q6X7J4,WOX10: Q9LM83, WOX11: Q6X7J3,WOX12: Q8GY25,WOX13: O81788 and WOX14: Q9LM84; Aang: *Araucaria angustifolia,* GW924719.

Figure S1C



Supplemental figure 1 C - Alignment of LRRK proteins from plants.
Vv: Vitis vinifera, LPK- XP_002277219, CLVL- CAN77471 ;
Rc: Ricinus communis , LPK- XP_002529278, CLVL- XP_002517850 ;
Cl: Citrus limon , LPK- ACB45099;
Pp: Populus trichocarpa, LPK- XP_002325680 , CLVL - XP_002305776, LRRPK- XP_002329291;
At: *ArabidopsisThaliana* , LPK - NP_179503 , CLV1 - NP_177710 ,HSL1 - NP_177710 ;
Sb: Sorghum bicolor , LPK- XP_002465512, LRRPK- XP_002455198;
Os: Oryza sativa, LPK- BAA94518, LRRPK- EAZ01332;
Pg: Picea glauca, CLVL- ABF73316;
Pc: Prunus cerasus, LRRPK- ABV30769;
Ma: Musa acuminata, LRRPK- ABX56574;
Aa: *Araucaria angustifolia*. In red squares: LPK: GW924722, CLVL1: GW924723 and LRRPK: GW924717.

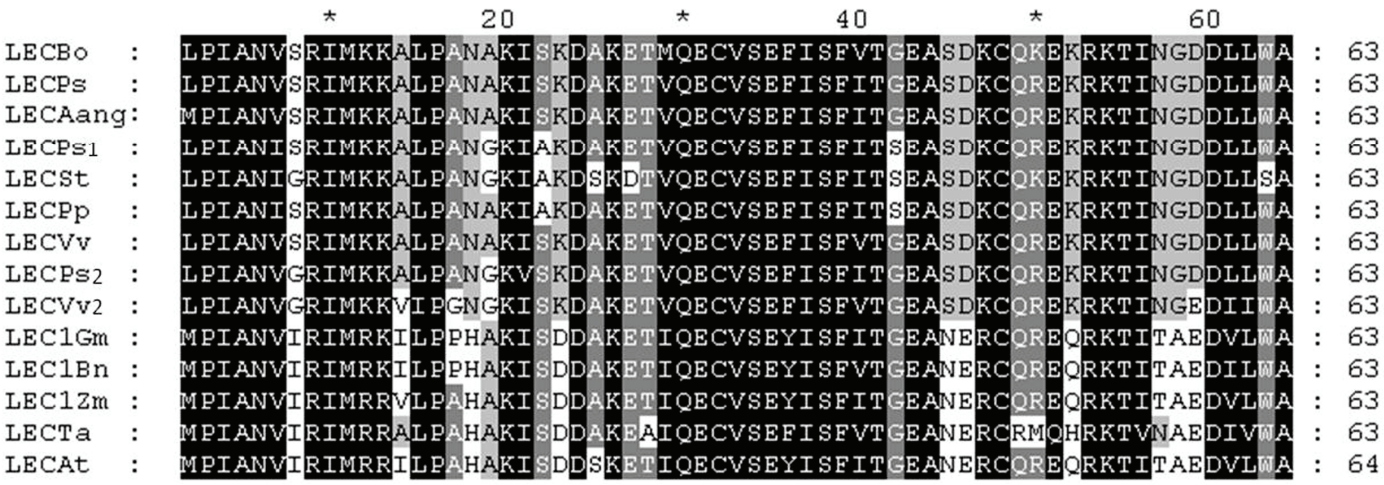
Figure S1D



Supplemental figure 1 D - Alignment of SCR-like proteins from plants.

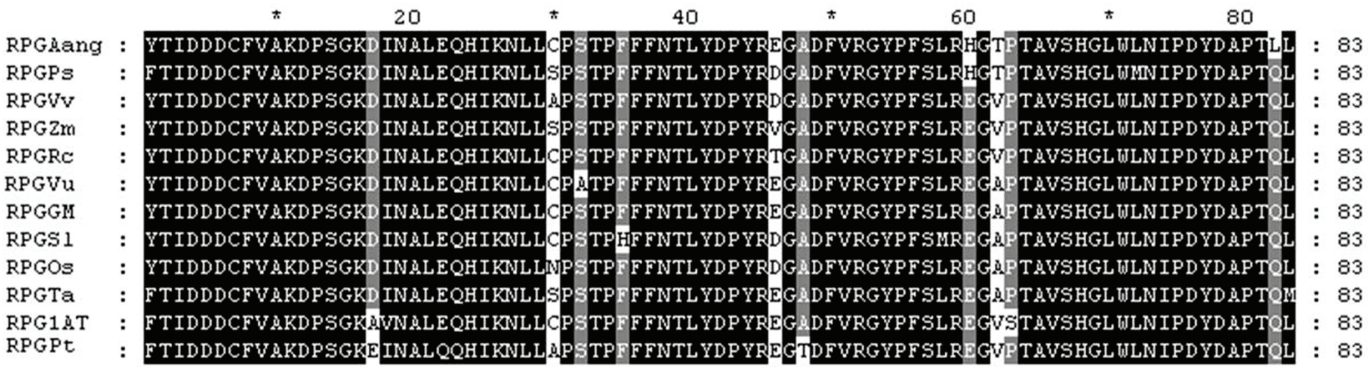
Ps:Pinus sylvestris, SCR-ABH85406;
Pp: Physcomitrella patens subsp. patens, SCR-XP_001786265;
Aa: *Araucaria angustifolia,* GW924716*;*
Rc: Ricinus communis , SCR- XP_002519983;
Pt:Populus trichocarpa, SCR-XP_002323112;
Mt:Medicago truncatula,SCR- ABN08308;
At: *ArabidopsisThaliana*, SCR-AAB06318;
Psa:Pisum sativum,SCR-Q9AVK4;
Cs:Cucumis sativus, SCR-CAI30892;
In:Ipomoea nil, SCR-Q2Z2E9;
Sb:Sorghum bicolor,SCR-XP_002448913;
Zm: Zea mays, SCR-Q9FUZ7;
Vv: Vitis vinifera, SCR -XP_002264349;
Os: Oryza sativa, SCR -BAD22576;

Figure S1E



Suplementar figure 1 E - Alignment of LEC-like proteins from plants.
Bo: Brassica oleracea , LEC - ABD64993
Ps: Picea sitchensis, LEC - ABK21387;
Aa: *Araucaria angustifolia,* GW924720;
Ps1: Picea sitchensis, LEC - ABK27065;
St: Solanum tuberosum, LEC -;
Pp: Physcomitrella patens subsp. patens, LEC - XP_001757004;
Vv: Vitis vinifera, LEC - CAN70795;
Ps2: Picea sitchensis , LEC - ABK23156;
Vv2: Vitis vinifera, LEC - CAN71881;
Gm: Glycine max, LEC - ABW71514;
Bn:, Brassica napus, LEC - ACB12186;
Zm: Zea mays, LEC - NP_001105518;
Ta:Triticum aestivum, LEC - AAL27661;
At: *ArabidopsisThaliana*, LEC -NP_199578;

Figure S1F



Supplemental figure 1 F - Alignment of RPG proteins from plants.
Aang: *Araucaria angustifolia,* GW924715;
Ps: Picea sitchensis, RPG- ABK21236;
Vv: Vitis vinifera, RPG- CAO62439;
Zm: Zea mays, RPG-ACG33746;
Rc: Ricinus communis, RPG-XP_002514626;
Vu: Vigna unguiculata, RPG-AAB61672 ;
Gm: Glycine max, RPG-ACU19796;
Sl: Solanum lycopersicum, RPG-AAT44738;
Os: Oryza sativa, RPG-NP_001060224;
Ta:Triticum aestivum, RPG-CAA77237;
At: *ArabidopsisThaliana*, RPG-NP_186872;
Pt:Populus trichocarpa, RPG-XP_002330138.