

Research Article

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

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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 semiquantitative 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 conserva-

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. tion 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 suboptimal 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 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 embryoexpressed 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, semiquantitative 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 μ g of total RNA digested with DNase I (Fermentas, USA), 500 ng of oligo-dT₂₅-ancored primer (5'-T(25)VN-3'), and the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA), in a 20 μ L 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 μ L, containing 0.3 μ M of primers and 2 μ L 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 AAM81249.1	
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 COTYLEDONI	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. aCloned in another work.

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

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.

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 AaAGO 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 (*Aac* 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*

100

		*	20	*	40	*	60	*	80	*	100		
PtZLL	:	KRICETDLG	LITOCCLSKHVFKI	SK	OYLANLSIKINVK OYLANVSIKINVK	GGRNTVI	LASCRIN	LVSDIPTIIR	GADVTHEEN CEDS-	SPSIAAVV	SODWPEVTKY	A :	98
PtZLL1	:	KRICETDLC	LETOCCLSKHVFKI	SK	OYLANVSLKINKK	MGGRNTVI	LIAISCRIF	LVSDIPTIIE	GADVEREN EDS-	SPSIAAVV	SODVPEVTKY	A :	98
RCZLL	:	KRICETDLG	LISOCCLTKHVFKI	SR	OYLANVSLKINWK	MGGRNTVI	LIAISCRIF	LVSDIPTIIE	GADVTHPEN BEDS-	SPSIAAVV	SODWPEVTKY	A :	98
ZLL	:	KRICETELG	LISOCCLTKHVFKI	SK	OYLANVSLKINMK	MGGRNTVI	VEAUSCRIF	LVSDIPTII	GADVEHEENCEES-	SPSTAAVV	SODVPEVEKY	A :	98
BnZLL	:	KRICETELG	LISOCCLTKHVF	SK	OYLANVSLKINNK	IGGRNTVI	LAUSCRIE	LVSDIPTIIE	GADVTHEENCEES-	SPSICAVV	SODWPEVTKY	A :	98
OsZLL	:	KRICETDLC	LISOCCLTKHVFKI		OYLANVSLKINVK		LATSWRIE	LVSDIPTIIE	GADVTHEET CEDS-	SPSIAAVV	SODWPEVTKY	A :	98
VVZLL	:		LISOCCLTKNVYKI		OYLANVSLKINVK				GADVTHEET CDDS-	CPSIAAVV	SODWPEVTKY	A :	98
PtAG01	:		LVSOCCLTKHVFKM		OYLANVALKINVK								98
AG01	:	KRICETELG	IVSOCCLTKHVERM		OYMANVALKINWK	VGGRNTVI	VDATSRRIEI	LVSDRPTIIE	GADVTHEHPCEDS-	SPSIAAVV	SODWPEITKY	A :	98
PptAGO	:	KKOCETVLC	VVSOCELTKHVERM	SK	OYLANVALKINWK	VGGRNTVI	VALTRE	LVSDEPTIE	GADVTHEHPCEDS-	SPSIAAVV	SODWPEVTKY	A :	98
AaAG01	:	KRIICE/IDUC	IVSOCCLAKHVLKK		OYMANVALKINWK	VGGRNTVI	VDAIRRRSS	LVSDVPTIIE	GADVTHPHPGEDT-	SPSIAAVV	SODWPEVTKY	1 :	98
RCAG05	:		IVSOCCOPROAAKL		OYFENVALKINVK		NEAVORRES	LVTDCPTIIN	GADVTHPPPCEDS-	SPSIAAVV	SMOWPEVTKY	R :	98
PtAGOS	:	KRICETELG	IVSOCCOPOOAKKI	sK	OY ENVALKINVK	AGGRNTVI	NEAHORRIEN	VTDEPTIIE	GADVTHEORCEDS-	SPSIAAVV	SMOWPEVTKY	R :	98
AG05	:	KRICETELG	IVSOCCOPROVNED	NR	OYMENVALKINWK	TGGRNTVI	NEALRRNIE	LITORPTIIM	GADVTHPOPCEDS-	SPSIAAVV	SMDWPEINKY	R :	98
VvAG05	:	KRICETELG	IVSOCCOPSOASKI	NK	OYFENVALKINWK	VGGRNTVI	FUALORKIE	LVSDEPTIIE	GADVTHPOPCEDS-	SPSIAAVV	SMOWPEVTKY	R :	98
AGO3	:	KWIAETKLC	LVTOCFLTISAIKG	ETVSE	OYDANLALKINAK	GGTNVEL	VONEFSFFK	-KEK-VMFI	GADVNHEAAHDNM-	SPSIVAVV	TLNW PEANRY	A :	99
AG02	:	KWIAETKLC	LVTOCFLTGPATEG	GD	OYRANLALKMNAK	GGSNVEL	ME-TFSFFK	-KEEE-VMFI	GADVNHEAARDKM-	SPSIVAVV	TLNWPEANRY	A :	95
AGO7	:	KRISETRIG	VVTOCCLY PNITKL	ss	OFVSNLALKINAK	GGSMEEL	YNSIPSHIERL	RPEEVIEM	GADVEREHPFDDC-	SPSVAAVV	SINWPEANRY	V : 1	100
AGO9	:	KKKDLVDLC	IVTOCIAPTRD	ND	OYIITNVLLKINAK OYIITNLLLKINAK	LGGLNSLI	AMERSPAME	KVT OVPTIIV	MDVSHGSPEOSD-	IPSIAAVV	SROWPLISKY	к :	95
AGO4	:	KKNLEFE	IVT QOMAPTROP	NE	OYLTNLLKINAK	LGGLNSMI	SVERTPAFT	VISKVPTIIL	GMDVSHGSPCOSD-	VPSIAAVV	SREWPLISKY	R :	96
AG08	:	KNSDVYEKSCSM	WNCECIVPPON		OYLTNLLLKINAK	LGGLNSVI	DME SGT ME	LVMRVPTIII	GMDVSHGSPGQSDH	IPSIAAVV	SREMPLISKY		100
PgAGO	:	KRKFLADLC	VINCCIAPPNMRKV										98
AG06	:	KKICLIEEC	HEOCICPIKI	SD	OYLTNVLLKINSK	LGGINSLI	GIEYSYNIE	IINKIPTLIL	GMDVSHGPPERAD-	VPSVAAVV	SKCMPLISRY	R :	95
		+ 100		1.10		1.00							
		* 120	DIMATWODPVRGTV	140	Total a March	160	TTEYRDGVS	G : 159					
	:	CT ACADAU POR	DIVISTWODPVRGTV	CONT	SDINING SPRANCON-		IIFYRRVLDGVS	G : 162					
	:		DIVETWODPVRGTV					G : 159					
			DINUTWODPVRGTV		DIMMIS RKANGON-	PLS	TIFYRDGVS	G : 159					
	:		DLWNTWODPVRGTV				IIFYRDGVS	G : 159					
	:	GLUCAGAHROBUT	DIMETWHDPORGTV'	GGMI	BELLIS RKALGON-	PL	IIFYRDGVS	G : 159					
	:		ODLWNTWKDPQGGTV			BL	IIFYRDGVS	G : 159					
	:		ODIANS TWODPVRGTV:				IIFYRDGVS	G : 159					
	:		ODIER EWKDPQKGVV			PL	IIFYRDGVS	G : 159					
	:	GLUCAGAHROEDI	ODIWNEWRDPQKGTM	GGMI	KEULISERCATGQK-		IIFYRDGVS						
	•	GLWSACALROBIL	SDINEVFODPRRGAV	Gent	DIMESSIRNS GQ3-		IIFYRDGVS IIFYRDGVS	G : 159					
	:	GIVSAGAREETI	CDDVX KYQDPQKGLVI	15 GNL	CEPTIA RRENGA-		IIFYRDGVS	G : 159 G : 159					
	:	CTACACALINERT	DIVISLVODPORGLVI	IC CLAT	PUPUANPPANCOT-		IIFYRDGVS						
		GLUSACHHEREIT	DIMATTADPHKGVT	Gent	RIAD ASRESOCYU-		IIFYRDGVS						
		ARVKAOSTRKEET	GF	GETC	BINEAHSQAPEKS-	RNR	IVIFRDGVS						
	:	ARVIAOPURKEEL	GF	GDAC	LEWKAHVQA GKS-	PN		DA : 143					
	:	ARMIACPHRKEET SRMRSOTHROBIT			BULDD YKAVKKL-		IIFFRDGVS	T : 148					
	:		DNILER PVNGKDI					s: 152					
	:		SLVERNGTEDI					s : 153					
	:		DSDESPVSDKD					s : 157					
	:		BANNEPLPSGK					SS : 161					
		AARKTESPELO	DS POPIENTEKG	JIN ETHON	NEELESEIKTERAE	KEKC	IIIFRDGVS	S : 155					

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

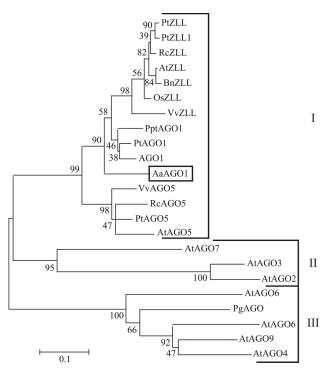


Figure 2 - Phylogenetic tree of *ARGONAUTE* proteins. Multiple alignments were done using ClustalW (version 1.74) software. MEGA software was used in bootstrap analysis and tree construction. Bootstrap percentages are indicated at each fork. Accession numbers: *Arabidopsis thaliana* - *AGO1*: NP_849784, *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_002523757. *Araucaria angustifolia* - *AaAGO*: GW924721.

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 (Cai-

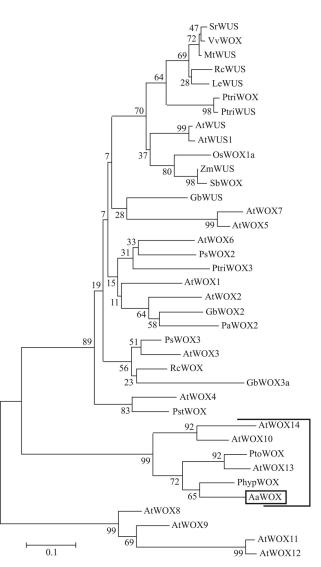


Figure 3 - Phylogenetic tree of WOX proteins. Multiple alignments were done using ClustalW (version 1.74) software. MEGA software was used in bootstrap analysis and tree construction. Bootstrap percentages are indicated at each fork. Accession numbers: Arabidopsis thaliana - AtWOX1: Q6X7K0, AtWOX2: Q6X7K1, 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.

rney 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

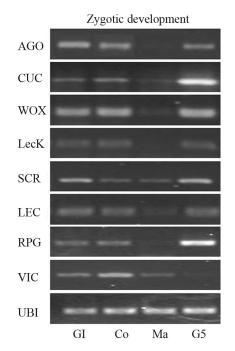


Figure 4 - Expression pattern of embryogenesis-regulating genes (ARGONAUTE (AaAGO), CUP-SHAPED COTYLEDON1 (AaCUC), wushel-related WOX (AaWOX), S-locus lectin protein kinase (AaLecK), SCARECROW-like (AaSCR), VICILIN 7S (AaVIC), LEAFY COTYLEDON 1 (AaLEC), and a Reversible glycosylated polypeptide (AaRGP)) during Brazilian pine zygotic embryogenesis. RT-PCR reactions were carried out on reverse-transcribed total RNA samples from late globular (GI), 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.

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).

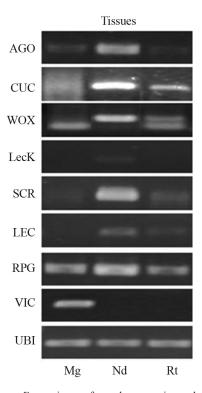


Figure 5 - Expression of embryogenesis-regulating genes (ARGONAUTE (*AaAGO*), CUP-SHAPED COTYLEDON1 (*AaCUC*), wushel-related WOX (*AaWOX*), S-locus lectin protein kinase (*AaLecK*), SCARECROW-like (*AaSCR*), VICILIN 7S (*AaVIC*), LEAFY COTYLEDON 1 (*AaLEC*), and a Reversible glycosylated polypeptide (*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.

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 postembryogenic 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.

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Figure S1A

		*	20	*	40	*	60	*	80		
NAMP×H	:	NKCEPW <mark>E</mark> LPEKAKMG	EKEWYFFSLR	DRKYPTGLRTI	NRATEAGYWK	TGKDREIVS	SKTSA <mark>LV</mark> GMKKT	LVFYRGRAP	kgeks <mark>N</mark> WVMHeyr	:	89
CUC2At	:	NKCEPW <mark>QLP</mark> GRAKMG	EKEWYFFSLR	DRKYPTGLRTI	NRAT <mark>EA</mark> GYWK	ATGKDRE I <mark>F</mark> S:	SKTCA <mark>LVGMKKT</mark>	LVFYKGRAP	kgeks <mark>n</mark> wvmheyr	:	89
CUC1At	:	NKSEPWELPEKAKMG	EKEWYFFTLR	DRKYPTGLRTI	NRATEAGYWK	ATGKDRE I <mark>KS</mark>	SKTKS <mark>LLGMKKT</mark>	LVFYKGRAP	kgeks <mark>c</mark> wvmheyr	:	89
CUCVv	:	NKCEPWDLPAKASMG	EKEWYFFSLR	DRKYPTGIRTI	NRATEAGYWK	TGKDKD I YR-	AGI <mark>LVGMKKT</mark>	LVFYKGRAP	kgeksnwvnheyr	:	87
CUCPp	:	NKCEPW <mark>DLP</mark> VKAKMG	EKEWYFFSLR	DRKYPTGMRTI	NRAT <mark>DA</mark> GYWK	a tgkdrdvf a i	HGRSH <mark>LVGMKKT</mark>	LVFYRGRAP	KGEKTNWIMHEYR	:	89
CUCGm	:	NKCEPW <mark>DLP</mark> KKAKMG	EK <mark>DWYFF</mark> CQR	DRKYPTGMRTI	NRAT <mark>QS</mark> GYWK	ATGKDKE IFK-	-GKNN <mark>LV</mark> GMKKT	LVFYRGRAP	kgektNwvmhefr	:	88
CUCCm	:	NKCEPW <mark>DLP</mark> HKAKMG	EKEWYFF <mark>CQ</mark> R	DRKYPTGMRTI	NRAT <mark>QT</mark> GYWK	ATGKDKE I <mark>LK</mark> -	-grtv <mark>l</mark> agmkkt	LVFYKGRAP	kgektNwvmhefr	:	88
CUCZm	:	NKCEPW <mark>E</mark> LP <mark>GKAKMG</mark>	EKEWYFYSLR	DRKYPTGLRTI	NRAT <mark>VA</mark> GYWK	atgkdre i <mark>rs</mark> (GRTGA <mark>LV</mark> GMKKT	LVFYRGRAP	KGQKT <mark>H</mark> WVMHE YR	:	89
CUCAang	:	NRSEPWDLPAKATMG	EKEWYFFSLR	DRKYPTGLRTI	NRAT <mark>QA</mark> GYWK	ATGKDRE IFK	GRTNI <mark>LV</mark> GMKKT	LVF YKGRAP	kgekt <mark>n</mark> wvmheyr	:	89
CUC3At	:	NRCEPWELPEMAKMG	EREWYFYSLR	DRKYPTGLRTI	NRAT <mark>T</mark> AGYWK	TGKDKEVFS	GGGGQ <mark>LV</mark> GMKKT	LVFYKGRAP	rg <mark>lkt</mark> kwvmheyr	:	89

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

			*	20	*	40	*	
WOX14	:	WTPTSTQI	LQILESIYE	ECSGTEN	RRRIREIATELS	EHGQITETNV	YN <mark>WFQN</mark> RRA :	54
WOX10	:	WTPTTTQI	LQILDNIYK	EGSGTEN	PRRIKEITMELS	EHGQIMEKNV	YH <mark>WFQN</mark> RRA :	: 54
WOX13	:	WT PT PVQI	LQI <mark>L</mark> ERIFE	QGTGTPS	KQKIKDITEELS	QHGQTAEQNV	YN <mark>WFQN</mark> RRA :	: 54
WOXAang	:	WTPSQTQI	LQILERLFE	QGTATEN	KQKIKEITMELS	QH <mark>G</mark> QI SETNV	YYN <mark>WFQN</mark> YKA :	: 54
WOX3	:	WCPTPEQI	LMILEEMYR	SGIRTPN	AVQIQQITAHLA	FY <mark>G</mark> RIEGK <mark>N</mark> V	/FY <mark>WFQN</mark> HKA :	: 54
WOX6	:	WNPTPEQI	[TT <mark>le</mark> elyr	SGTRTPT	TEQIQQIASKLR	KY <mark>G</mark> RIEGKNV	/FY <mark>WFQN</mark> HKA :	: 54
WUS	:	WTPTTEQI	KI <mark>l</mark> kelyy	NAIRSPT	ADQIQKITARLR	QF <mark>G</mark> KIEGKNV	/FY <mark>WFQN</mark> HKA :	: 54
WOX4	:	WNPTQEQI	IGI <mark>le</mark> mlyk	GGMRTPN	AQQIEHITLQLG	KY <mark>G</mark> KIEGKNV	/FY <mark>WFQN</mark> HKA :	: 54
WOX7	:	WNPTVEQV	/KL <mark>L</mark> TDLFK	AGLRTPS	TDQIQKISMELS	FYGKIESKNV	/FY <mark>WFQN</mark> HKA :	54
WOX5	:	WNPTVEQI	LKILTDLFR	AGLRTPT	TDQIQKISTELS	FYGKIESKNV	/FY <mark>WFQN</mark> HKA :	: 54
WOX2	:	WNPTKDQI	TL <mark>LE</mark> NLYK	EGIRTPS	ADQIQQITGRLR	AY <mark>G</mark> HIEGKN\	/FY <mark>WFQN</mark> HKA :	: 54
WOX1	:	WNPTPDQI	LRV <mark>LE</mark> ELYR	QGTRTPS	ADHIQQITAQLR	RY <mark>G</mark> KIEGKN\	/FY <mark>WFQN</mark> HKA :	: 54
WOX11	:	WSPKPEQI	LILESIFH	ISGMVNPP	KEETVRIRKMLE	KF GAVGDAN V	FY <mark>WFQN</mark> RRS :	: 54
WOX12	:			and the second se	KDETVRIRKMLE	CARDON ACTOR CONTRACTOR C		: 54
WOX9	:	WNPKPEQI	IRILEAIFN	ISGMVNPP	REEIRRIRAQLQ	EY <mark>G</mark> QVGDANV	/FY <mark>WFQN</mark> RKS :	: 54
WOX8	:	WNPKPEQI	IRILESIFN	ISCTINPP	REEIQRIRIRLQ	EY <mark>G</mark> QIGDANV	FY <mark>WFQN</mark> RKS	: 54

Supplemental figure 1 B - Alignment of WOX proteins from Arabidopsis and Parana pine.

ArabidopsisThaliana 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

			* :	20	*	40	*	60	*	80		
VvLPK	:	LVYDYMP-	NGSIDA	HLFHEKDS	SEVLDWKK	YQTALGTA	RGLTYLHEKC	RDCINHCDIN	PENILLD	AELCPKVADF	GLAK :	78
RCLPK	;	lvydymp-	NGSLDS	FLFQGNKI	LIVLDWKT	CNIALGTA	kglaylhekc	KDCIIHCDIH	(PENILLD)	SEFCPKVTDF	GLAK :	78
Cllpk	:	lvydfmp-	NGSLDS	HLFTEKD:	SDFLDWKT	YQIALGTA	rglaylhekc	RDCIIHCDIN	VPENILLD.	AEFCPKVSDF	GLAK :	78
PpLPK	:	LVYDYMP-	NGSLDS	LLFSEKNT	rkv <mark>l</mark> dwkti	YSTALGTA	rg <mark>l</mark> nylhekc	RDC <mark>IIH</mark> CDIH	(PENILLD)	QFCPKVADF	GLAK :	78
Atlpk	:	LVYDYMP-	NGSLDSHL	FLNQVEEH	KIV <mark>L</mark> GWKLE	FQIALGTA	rglaylhdec	RDCITHCDIN	PENILLD:	BQFCPKVADF	GLAK :	80
SblPK	:	lvy <mark>dyme-</mark>	NGSLAS	VLSGHSH	FRLLDWRA	FGIMAGVA	RGLAYLHEQC	QERIVHCDVH	VPENILLD.	AGFCPKVADF	3MAK :	77
OslPK	:	lvybyme-	NGSLDS	HPFSETSI	r-V <mark>l</mark> gwnlf	HQIVGIA	rglaylheec	RDSIIHCDIH	VPENILLD.	AEFCPKIADF	SMAK :	77
AaLPK	:	lvybfmpi	SGSPNT	FLSQES-	-KVLDWKT:	FEIALGVA	rgllylheec	RDRIIHCDIH	VPENILLN:	BDFCPKVADF	SIAQ :	77
VVCLVL	:	lvybymb-	NGSLGD	LTHSNKO	ggl <mark>l</mark> dwpt:	YKTALDAA	EGLSYLHHDC	VPPIVHRDVH	SNNILLD	GDFGARVADF	GVAK :	77
Sblrrpk	:	lvfefmp-	NGSLNHWLHI.	-				100 March 100 Ma	2015	2DMSARV <mark>G</mark> DF		82
PpCLVL	:	TAABAWB-	NGSLGD	LTHSSKO	GGLLDWPT	YKIVVDAA	EGLSYLHHDC	VPPIVHRDVH	SNNILLD	GDFGARVADF	GVAK :	77
AtHSL1	:	l aabaand-	NGSLGD	LLHSSKO	ggm <mark>l</mark> gwqt:	FKIILDAA	EGLSYLHHDS	VPPIVHRDIN	SNNILIDO	5DYGARVADF	GVAK :	77
PpCLVL	:	lvybymb-	NGSLGD	LLHSSKO	3GLLDWPT:	YKTALDAA	EGLSYLHHDC	VPPIVHRDVH	SNNILLD2	AEFGARVADF	GVAK :	77
RCCLVL	:	lvybymb-	NGSLGD	LTHSSK:	3GLLDWPT:	YKTALDAA	EGLSYLHHDC	VPPIVHRDVH	SNNILLDO	SEFGARVADF	GVAK :	77
PgCLVL	:	PANBAWB-	NGSLGD	LLHGPK	ASVLDWPI	YKIALGAA	QGLAYLHHGC	VPAIVHRDVH	SNNILLD	SDYVAHVADF	GVAK :	77
MtCLVL	:	l aabaad		649	100 M	100 CO. 100	10 III III	10 Million		EDFSARVADF	Concession in which the local division in which the local division in the local division	77
AaLRRPK	:	MVYBFMP-	NGSLYNCLHA'	-								82
AaCLVL1	:	mmybfmp-	and the second s		100			100		ADFNARVADF	1000000	77
AtCLV1		LLYBYMP-		Enter .				man and a second	100	BDFEAHVADF	10000	77
Pt2LRRPK		100	NGNLEEWLHP'	-	100			- 80 - 80	10	El and		84
PCLRRPK			NGSLESWLHP:				_					85
Oslrrpk		1000	NGSLYQWLHQ.		1213					/EFKARIADL		81
MaLRRPK	:	IVFDFMP-	NGSLESWLHP		en:			100 Mar 100		SNMTARVGDF	1000000	82
VvLRRPK	:	LVYDFMP-	NGSLENWLHP	VPTPDEINDVI	LRILSLPO	LNTAIDVA	SALDYLHHHC	HKPICHCDLI	PSNILLD	VDMTAHVGDF0	JLAR	85

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

		*	20	* 40	*	60	*	80	*	100	*	
PsSCR	:	EAIHYYSALFDSLGAS	YPEDSHDRHLVE	QQLLSREIKNILAV	GGPARTGEIKFDN	WRDQLKOTGF	OPISLAGNAAN	QATLLL-GMFP0	QGYTLME	NGULKLGWK <mark>G</mark> LCL	L :	109
PpSCR	:	EALHYYSAL FDSLGAS	YKADSPORHMVE	QQLLS <mark>C</mark> EIKNILA <mark>F</mark>	GGPARTGDAKFDO	WRDELGKR-FI	KPVSLSGKAAH	QAALLLQGLFPO	EGYTLLE	RGTLKLGWKDLYL	F :	109
AaSCR	:	EAIHYYSA <mark>F</mark> FDSLGAS				The second se			The second second second			109
RCSCR	:	EAIHYYSALFDSLGAS	YGEESEERHVVE	QQLLSREIRNVLAV	GGPSRSGDVKFHI	WREKLROSGE	GISLAGNAAN	QATLLL <mark>-</mark> GMFP:	DGYTLVEI	NGULKTCMKDFCF	ī. :	109
PtSCR		EAIHYYSALFDSLGAS										109
MtSC	:	EAIHYYSALFDSLG <mark>S</mark> S										109
AtSCR	:	EAIHYYSALFDSLGAS	YGEESEERHVVE	QQLLSKEIRNVLAV	GGPSRSGEVKFES	WREKMOOCGE	K <mark>G</mark> ISLAGNAAN	QATLLL <mark>-</mark> GMFP:	DGYTL	NGTLKLGWKDLSL	i :	109
PsaSCR	:	EAIHYYSALFDSLG <mark>S</mark> S	YGEESEERHVVE	QQLLSREIRNVLAV	GGPSRSGEIKFHN	WREKLOOCGE	R <mark>G</mark> VSLAGNAAT	QASLLL <mark>-</mark> GMFP:	EGYTLVEI	DNG <mark>I</mark> LKLGWKDLCL	1 :	109
CsSCR	:	EAIHYYSALFDSLG <mark>V</mark> S	YGEESEERHLVE	QQLLSREIRNVLAV	GGPSRSGEVKFON	WREKLOOSGE	GISLAGNAAN	QATLLL-GMFP:	DGYTLVEI	NGTLKLGWKDLCL	il :	109
InSCR	:	EAIHYYSALFDSLGA <mark>C</mark>	YGEESEERHAVE	QQLLSREIRNVLAV	GGPSRSGEVKFM	WREKFOOSGFE	RGVSLAGNAAA	QATLLL-GMFH	DGYTLADI	NG <mark>A</mark> LKLGWKDLCL	i :	109
SbSCR	:	EAIHYYSALFDSL <mark>D</mark> AS	YGODSPORHVVE	QQLLSREIRNVLAV	GGPARTGDVKFGS	WREKLAQSGE	RAASLAGSAAA	QASLLL <mark>-</mark> GMFP:	DGYTLVE	ENG <mark>A</mark> LKLGWKDLCL	1 :	109
ZmSCR	:	EAIHYYSALFDSL <mark>D</mark> AS	YGODSPORHVVE	QQLLSREIRNVLAV	GGPARTGDVKFGS	WREKLAQSGE	RAASLAGSAAA	QASLLL <mark>-</mark> GMFPS	DGYTLVE	NG <mark>A</mark> LKLGWKDLCL	1 :	109
VvSCR	:	EAIHYYSALFDSLGAS	YGEESEORHAVE	QQLLSREIRNVLAV	GGPSRSGDVKFNN	WREKLOOSGE	RVVSLAGNAAN	QATLLL-GMFP:	DGYTLVEI	NGTLKLGWKDLCL	ī. :	109
OSSCR	:	EAIHYYSA <mark>L</mark> FDSL <mark>D</mark> AS	YSODSPORHVVE	QQLLSREIRNVLAV	GGPARTGDVKFGS	WREKLAQSGF	RVSSLAG <mark>S</mark> AAA	QAVLLL-GMFP:	DGYTLIE	NGALKLGWKDLCL	L :	109

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:Ipomoeanil, 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

	*	20	*	40	*	60		
LECBo :	lpianv <mark>s</mark> rimkk <mark>a</mark> i	PANAKI SKDAK	ETMQECVS	EFISFVTGEA <mark>S</mark>	DKCQKEKRK!	TINGDDLLWA	: 6	53
LECPs :	lpianv <mark>s</mark> rimkk <mark>a</mark> l	PANAKI SKDAK	ETVQECVS	EFISFITGEA <mark>s</mark>	DKCQREKRK!	ringddllwa	: 6	53
LECAang:	MPIANV <mark>S</mark> RIMKK <mark>A</mark> I	PANAKI SKDAK	ETVQECVS	EFISFITGEA <mark>s</mark>	DKCQREKRK!	ringddllwa	: 6	53
LECPs1 :	lpiani <mark>s</mark> rimkk <mark>a</mark> l	PANGKIAKDAK	ETVQECVS	EFISFIT <mark>S</mark> EA <mark>S</mark>	DKCQRE <mark>K</mark> RK	ringdllma	: 6	53
LECSt :	lpiani <mark>g</mark> rimkk <mark>a</mark> l	PANGKIAKDSK	DTVQECVS	EFISFIT <mark>s</mark> EA <mark>s</mark>	DKCQKEKRK!	ringddllsa	: 6	53
LECPp :	lpiani <mark>s</mark> rimkk <mark>a</mark> i	PANAKIAKDAK	ETVQECVS	EFISFIT <mark>s</mark> EA <mark>s</mark>	DKCQRE <mark>K</mark> RK!	ringddll@A	: 6	53
LECVv :	lpianv <mark>s</mark> rimkk <mark>a</mark> l	PANAKI SKDAK	ETVQECVS	EFISFVTGEA <mark>S</mark>	DKCQRE <mark>K</mark> RK!	FINGDDLLWA	: 6	53
LECPs ₂ :	lpianv <mark>g</mark> rimkk <mark>a</mark> l	PANGKVSKDAK	ETVQECVS	EFISFIT <mark>GEA</mark> S	DKCQREKRK!	ringddllwa	: 6	53
LECVv2 :	lpianv <mark>g</mark> rimkk <mark>v</mark> i	P <mark>GNG</mark> KI SKDAK	ETVQECVS	EFISFVTGEA <mark>S</mark>	DKCQRE <mark>K</mark> RK!	FINGEDIIWA	: 6	53
LEC1Gm :	MPIANV <mark>I</mark> RIMRK <mark>I</mark> I	PPHAKI SDDAK	ETIQECVS	EYISFITGEA <mark>N</mark>	ERCQREQRK!	TITAEDVLWA	: 6	53
LEC1Bn :	MPIANV <mark>I</mark> RIMRK <mark>I</mark> I	PPHAKI SDDAK	ETIQECVS	EYISFVTGEA <mark>N</mark>	ERCQREQRK!	FITAEDILWA	: 6	53
LEC1Zm :	MPIANV <mark>I</mark> RIMRR <mark>V</mark> I	PA <mark>HAKI S</mark> DAK	ETIQECVS	EYISFITGEA <mark>n</mark>	ERCQREQRK!	FITAEDVLWA	: 6	53
LECTa :	MPIANV <mark>I</mark> RIMRR <mark>A</mark> I	PAHAKI S <mark>D</mark> DAK	E <mark>A</mark> IQECVS	EFISFVTGEA <mark>N</mark>	erc <mark>rm</mark> qhrk'	IVNAEDIVWA	: 6	53
LECAt :	MPIANV <mark>I</mark> RIMRR <mark>I</mark> I	PAHAKISDDSK	ETIQECVS	EYISFITGEA <mark>N</mark>	ERCQREQRK'	FITAEDVLWA	: 6	54

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

		*	20	*	40	*	60	*	80		
RPGAang	:	YTIDDDCFVAKDPSG	KDINALEQHIK	NLLCPSTR	FFFNTLYDPYR	GADFVRGYP	FSLRHGTPTA	VSHGLWLNI	PDYDAPT <mark>L</mark> L	:	83
RPGPs	:	FTIDDDCFVAKDPSG	KD INALEQHIF	NLL <mark>S</mark> PSTF	FFFNTLYDPYRI	DGADFVRGYP	FSLR <mark>H</mark> GTPTA	VSHGLUMNI	PDYDAPTQL	:	83
RPGVv	:	YTIDDDCFVAKDPSG	KD INALEQHIF	NLLAPSTR	FFFNTLYDPYR	DGADFVRGYP	FSLREG <mark>V</mark> PTA	VSHGLWLNI	PDYDAPTQL	:	83
RPGZm	:	YTIDDDCFVAKDPSG	KD INALEQHIF	NLLSPSTF	FFFNTLYDPYR	7 <mark>GADFVRGYP</mark>	FSLREG <mark>V</mark> PTA	VSHGLWLNI	PDYDAPTQL	:	83
RPGRC	:	YTIDDDCFVAKDPSG	KD INALEQHIF	INLLCPSTR	FFFNTLYDPYR	I <mark>GADFVRGYP</mark>	FSLREG <mark>V</mark> PTA	VSHGLWLNI	PDYDAPTQL	:	83
RPGVu	:	YTIDDDCFVAKDPSG	KD INALEQHIK	INLLCPATE	FFFNTLYDPYR	GADFVRGYP	FSLREG <mark>A</mark> PTA	VSHGLWLNI	PDYDAPTOL	:	83
RPGGM	:	YTIDDDCFVAKDPSG	KD INALEQHIF	NLLCPSTR	FFFNTLYDPYR	GADFVRGYP	FSLREG <mark>A</mark> PTA	VSHGLWLNI	PDYDAPTQL	:	83
RPGS1	:	YTIDDDCFVAKDPSG	KD INALEQHIF	NLLCPSTR	H <mark>FFNTLYDPYR</mark> I	DGADFVRGYP	FSMREG <mark>A</mark> PTA	VSHGLWLNI	PDYDAPTQL	:	83
RPGOS	:	YTIDDDCFVAKDPSG	KD INALEQHIK	NLL <mark>N</mark> PSTF	FFNTLYDPYR	DGADFVRGYP	FSLREG <mark>A</mark> PTA	VSHGLWLNI	PDYDAPTOL	:	83
RPGTa	:	FTIDDDCFVAKDPSG	KD INALEQHIF	NLL <mark>S</mark> PSTF	FFFNTLYDPYR	GADFVRGYP	FSLREG <mark>A</mark> PTA	VSHGLWLNI	PDYDAPTOM	:	83
RPG1AT	:	FTIDDDCFVAKDPSG	K <mark>A</mark> VNALEQHIF	NLLCPSTR	FFFNTLYDPYR	E <mark>GADFVRGYP</mark>	FSLREG <mark>VS</mark> TA	VSHGLWLNI	PDYDAPTQL	:	83
RPGPt	:	FTIDDDCFVAKDPSG	K <mark>E</mark> INALQQHIR	NLLAPSTE	FFFNTLYDPYR	E <mark>GT</mark> DFVRGYP	FSLREG <mark>VP</mark> TA	VSHGLWLNI	PDYDAPTOL	:	83

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;

SI: 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.