SCIENTIA FORESTALIS

n. 73, p. 9-18, março 2007

# 14-3-3: Defense and regulatory proteins coding by *Eucalyptus* genome

# 14-3-3: Proteínas de regulação e defesa codificadas pelo genoma do eucalipto

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## *Resumo*

A busca de ESTs do genoma do *Eucalyptus* resultou em quatro clusters de proteínas da família 14-3-3 (EGCEST2257E11.g, EGBGRT3213F11.g, EGBFCL1245H11.g e EGCCFB1223H11.g) altamente conservados e que englobam diversos processos celulares. Os alinhamentos múltiplos foram construídos a partir de vinte e quatro seqüências das proteínas 14-3-3 recuperadas das bases de dados do GenBank e de quatro conjuntos do genoma do *Eucalyptus*. O alinhamento revelou duas regiões altamente conservadas nas seqüências correspondente aos motifs de fosforilação da proteína e nove regiões altamente conservadas na seqüência correspondentes às regiões de ligação de uma estrutura do tipo alfa-hélices propostos no modelo tridimensional da estrutura do dimero funcional. As diferenças do aminoácido dentro dos domínios funcionais e estruturais das proteínas 14-3-3 da planta foram identificadas e podem explicar a diversidade funcional das diferentes isoformas. As árvores filogenéticas da proteína foram construídas usando o valor máximo de parcimônia e vizinhos mais próximos, alinhadas pelo Clustal X e analisadas pelo software PAUP. Suspeita-se que este grupo de proteínas participe dos componentes da resistência do eucalipto a estresses bióticos e abióticos, que serão testados em projeto futuro de genoma funcional.

**Palavras-chave**: Estresses, Proteina de resistência, Proteínas filogenéticas

## *Abstract*

The data mining of *Eucalyptus* ESTs genome finds four clusters (EGCEST2257E11.g, EGBGRT3213F11.g, and EGCCFB1223H11.g) from highly conservative 14-3-3 protein family which modulates a wide variety of cellular processes. Multiple alignments were built from twenty four sequences of 14-3-3 proteins searched into the GenBank databases and into the four pools of *Eucalyptus* genome programs. The alignment has shown two regions highly conservative on the sequences corresponding to the motifs of protein phosphorylation and nine highly conservative regions on the sequence corresponding to the linkage regions of alpha helices structure based on three dimensional of dimer functional structure. The differences of amino acid into the structural and functional domains of 14-3-3 plant protein were identified and can explain the functional diversity of different isoforms. The phylogenic protein trees were built by the maximum parsimony and neighborjoining procedures of Clustal X alignments and PAUP software for phylogenic analysis.

**Keywords**: Stress, Resistance protein, phylogenic proteins

## INTRODUCTION

In the last few years the 14-3-3 protein family has received much attention in literature, because it is believed to be an important participant pathway in disease resistance, associated with a number of different signaling proteins, including Raf-1, Bad, the epithelial keratins K8/K18, Cdc25, telomerase (LIAO and OMARY, 1996; MUSLIN *et al.*, 1996; ZHA *et al.*, 1996; PENG *et al.*, 1997; SEIMIYA *et al.*, 2000) and growth control during the life-cycle of a plant. Based on their interaction with various ligands, the 14-3-3 proteins have been proposed as being important in controlling intracellular signaling pathways (AITKEN, 1996). In addition, they work as molecular chaperones or

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regulate intracellular localation of their binding partners (LIAO and OMARY, 1996; KUMAGAI and DUNPHY, 1999; YANG *et al.*, 1999). They are a family of 30 kDa acidic proteins that form dimerous via their N-terminal regions, which can be homo- and heterodimers; these have been demonstrated both *in vivo* and *in vitro* (JONES *et al.*, 1995; WU *et al.*, 1997).

There is a high degree of sequence identity and conservation between all the 14-3-3 isotypes, particularly in the regions which form the dimmer interface or line the central ligand binding channel of the dimeric molecule. There is a monomer of the nine helices organized in an antiparallel manner, forming an L-shaped structure. These four helices form the concave amphipathic groove that interacts with target peptides (FERL, 1996).

Stemming from the finding that the 14-3-3s regulate plant nitrate assimilation, many techniques have used 14-3-3-affinity chromatography to identify phosphorylated *Arabidopsis* proteins in sugar and amino acid synthesis, vesicle trafficking and cell signaling, that bind the phosphopeptide-binding site of 14-3-3s. It seemed odd that such diverse types of proteins bind 14-3-3s in plants, because most known mammalian 14-3-3-interacting proteins are signaling molecules. Others techniques applied to human cells show a wide range of novel and known human 14-3-3-interacting proteins; regulators of chromatin function, enzymes of metabolism, proteins of protein and vesicle trafficking, a phosphodiesterase, and protein kinases. Our findings show 14-3-3 regulation of cytosolic fructose 2, 6-biphosphate, and mitochondrial ATP synthesis in both Kingdoms. Using 14-3-3 overlays, they found that the phosphorylation and 14-3-3-binding of sub-sets of target proteins are regulated by the survival hormone abscisic acid and nutrients in plant cells, and growth factor, insulin and nutrient signaling pathways in human cells. Overall, our findings add to the impression that many 14-3-3 functions are connected with cell protection and survival. In plants, 14-3-3 proteins include the H+-ATPase regulator in the plasma membrane (KORTHOUT and DE BOER, 1994; MARRA *et al.*, 1994; OECKING *et al.*, 1994) and a protein that specifically inhibits nitrate reductase activity from spinach cells (BACHMANN *et al.*, 1996; MOORHEAD *et al.*, 1996).

The plant 14-3-3 proteins are also found as part of a transcriptional DNA binding complex. 14-3-3 proteins have been reported to associate with G-box DNA binding complexes (LU *et al.*, 1992; SCHULTZ *et al.*, 1998), TATA box binding proteins from plants and eukaryotes, and to activate GAL4-dependent *β*-glucuronidase reporter gene expression when translation ally fused with the GAL4 DNA binding domain in a plant transient expression system (PAN *et al.*, 1999). They also have been found to interact with the transcription factors involved in abscisic acid signaling in plants. Schultz *et al.* (1998) found that 14-3-3 proteins interact with other proteins, while EmBP1 and VP1, both of which activate Em gene of the abscisic acid response element Em1a in the Em promoter.

Brandt *et al.* (1992) reported that pathogens and low temperatures induce some isoforms, this suggests 14-3-3 protein family involvement in the signal transduction pathway, which is related to stress response. Lapointe *et al.* (2001) demonstrated that after treatment with chitosan, salicylic acid, and jasmonic acid, the response is the same as wounding, (ROBERTS and BOWLES, 1999) showed that after treatment with fusicoccin, 14-3-3 is like the gene-for-gene resistance response.

In this paper were present the results of data mining where we identify ESTs from the *Eucalyptus* Genome database that have a high similarity with 14-3-3 proteins from other organisms and specific dicotyledonous plants.

## METHODOLOGY

The *Eucalyptus* genome project (FORESTs) was developed by a consortium of four Brazilian Forestry Companies and with FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo). It was built by 20 laboratories from the São Paulo network and AEG (https://forests.esalq.usp.br), obtaining 123,889 reads, starting with ESTs labels from cDNA derived libraries mainly of different *E. grandis* organs under different growth and climatic conditions. The libraries were of wood (WD), bark (BK), floral button (FB), leaf (LV), seedling (SL), stem (ST), root (RT), and callus (CL). The cDNAs were prepared from poly adenine polyA+ mRNA using the SuperScript plasmid system kit (Gibco-BRL USA) or the ZAP-cDNA synthesis kit (Stratagene USA). Double-stranded cDNA was fractionated and fragments >1kb were used. Sequencing was made using BigDye terminators on an ABI Prism 3700 DNA Analyzer multi-capillary electrophoresis (Perkin Elmer USA). EST clusters were built by alignment

using the CAP3 program. The search for 14-3-3 proteins was made by using a stringent basic local alignment search tool (BLAST) threshold value of E<10–143. The 14-3-3 proteins from the National Center for Biotechnology Information (NCBI – http://www.ncbi.nlm.nih.gov) were used as an analysis driver and aligned by the TBLASTN program (ALTSCHUL *et al.*, 1997) against the forests EST clusters. Once the corresponding 14-3-3 protein forests cluster were obtained, they were aligned by the BLASTX program to the NCBI protein to check the sequence. Nucleotide and protein sequence alignments were built using the Clustal X (v.1.81) multiple sequence alignment program (THOMPSON *et al.*, 1997) and the phylogenetic analyses were built with the neighbor joining method (SAITO and NEI, 1987) in the PAUP program.

## RESULTS AND DISCUSSION

We found four EST clusters (EGCEST2257E11.g, EGBGRT3213F11.g, EGBFCL1245H11.g and EGCCFB1223H11.g) similar to 14-3-3 proteins in the *Eucalyptus* Genome project database. These four clusters are composites of 60 reads from different libraries; 37% are expressed in ST, 22% in SL, 14% in RT 11% in WD, 8 % in LV, 6% in FB, and 2% in CL, as per Figure 1.

The Cluster EGCEST2257E11.g was similar to the 14-3-3-like protein of *Glycine max* (gi|3023194) with 89.4% similarity, whereas clusters EGBGRT3213F11.g and EGCCFB1223H11.g were similar to the 14-3-3-like protein of *Arabidopsis tlaliana* (gi|18396217) with 89.5% and 92.2% similarity, respectively, and the cluster EGBFCL1245H11.g was similar with the 14-3-3-like protein of *Glycine max* (gi|3023194) and *Arabidopsis tlaliana* (gi|18396217) both with 91.4% similarity (Table 1).

The Clusters EGCEST2257E11.g, EGBGRT3213F11.g, EGBFCL1245H11.g and EGCCFB1223H11 were 1,271 bp, 1,167 bp, 1,122 bp and 1,110 bp long, respectively. Cluster EGCEST2257E11.g had a single open reading frame (ORF) of 789 bp encoding a polypeptide of 263 amino acids while clusters EGBGRT3213F11.g and EGBFCL1245H11.g both had an ORF of 765 bp encoding 255 amino acid residues.

There were few differences in the deduced sequences of the four clusters (92.3% similarity). There were close relationship between clusters EGCEST2257E11.g and EGBGRT3213F11.g (95.3% similarity), and clusters EGBFCL1245H11.g and EGCCFB1223H11.g (98.4% similarity). In the phylogenetic tree we showed that the *Eucalyptus* 14-3-3 protein was more related with the *A. thaliana* and *G. max* that *Populus* specie, but showed that the 14-3-3 proteins from tree species, with *Populus* and *Eucalyptus*, was more related with this species (Figure 2).

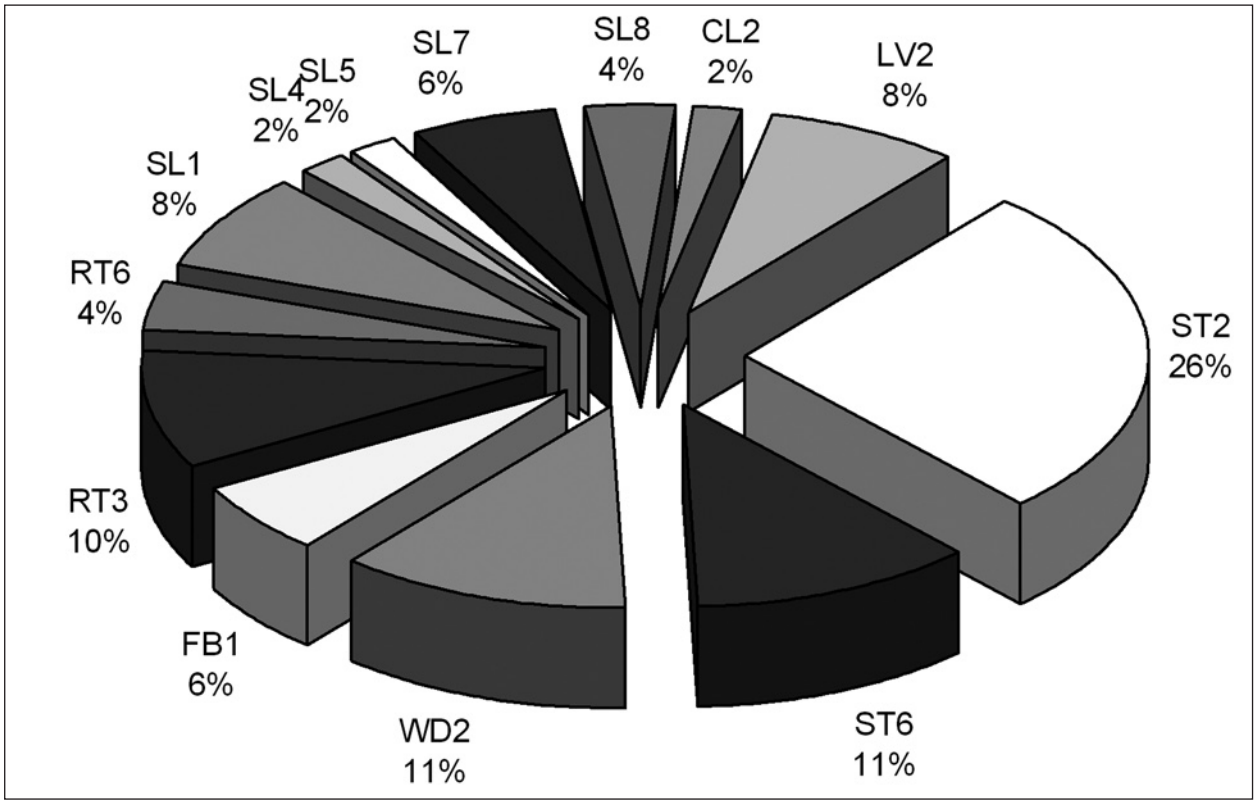


**Figure 1**. Read distributions from tissue which make up the 14-3-3 protein clusters distributed in the sequenced libraries. (Gráfico com a distribuição das seqüências estudadas que compõem os agrupamentos das proteínas 14-3-3 distribuídos pelas bibliotecas seqüenciadas).

**Table 1**. Organism selection for analysis, NCBI - accession number for to GenBank Nucleotide Sequence Database, four clusters of the *Eucalyptus* Genome comparison with other sequences and showing similarities. (Seleção de organismos para a análise, NCBI - número de acesso à base de dados da seqüência de nucleotídeos no GenBank, quatro clusters do genoma de *Eucalyptus* comparando com outras seqüências e apresentando a similaridade).

| Organism | NCBI | *Eucalyptus* clusters | | | |
|---|---|---|---|---|---|
| | | EGCCFB1223H11.g | EGBFCL1245H11.g | EGBGRT3213F11.g | EGCEST2257E11.g |
| *Hordeum vulgare* | gi\|112684 | 86.5 | 85.8 | 81.8 | 82.4 |
| *Arabidopsis thaliana* | gi\|18396217 | 92.2 | 91.4 | 89.5 | 88.0 |
| *Spinacia oleracea* | gi\|112687 | 82.1 | 81.6 | 80.1 | 80.9 |
| *Thellungilla halophila* | gi\|20340229 | 81.6 | 81.0 | 81.7 | 82.1 |
| *Helianthus annuus* | gi\|5902676 | 81.1 | 80.5 | 79.3 | 80.0 |
| *Vicia faba* | gi\|1168189 | 82.4 | 81.7 | 81.0 | 80.9 |
| *Oenothera elata* subsp. *hookeri* | gi\| 1168195 | 83.1 | 82.5 | 81.3 | 82.1 |
| *Cucubita pepa* | gi\|481556 | 83.3 | 83.3 | 82.5 | 82.4 |
| *Vigna angularis* | gi\|13928452 | 85.1 | 84.5 | 82.5 | 83.6 |
| *Glycine max* | gi\|3023194 | 91.4 | 91.4 | 89.1 | 89.4 |
| *Nicotiana tabacum* | gi\|15778152 | 85.2 | 84.2 | 83.0 | 84.1 |
| *Brassica napus* | gi\|13447104 | 84.4 | 83.1 | 81.5 | 81.7 |
| *Pisum sativum* | gi\|4850247 | 84.7 | 84.1 | 82.1 | 83.3 |
| *Picea glauca* | gi\|8050247 | 86.4 | 85.3 | 84.5 | 84.9 |
| *Oryza sativa* | gi\|7435022 | 88.2 | 87.9 | 85.1 | 85.1 |
| *Populus* x *canescens* | gi\|8515888 | 91.7 | 90.9 | 89.2 | 88.7 |
| *Zea mays* | gi\|1345587 | 86.1 | 85.4 | 82.6 | 81.5 |
| *Lycopersicon esculentum* | gi\|1168191 | 91.8 | 91.0 | 88.8 | 89.1 |
| *Solanum tuberosum* | gi\|3766535 | 85.5 | 84.5 | 81.7 | 82.9 |
| *Fritillaria agrestis* | gi\|2921512 | 90.0 | 89.8 | 87.4 | 87.3 |
| *Lilium longiflorum* | gi\|12229593 | 89.6 | 89.4 | 87.4 | 86.9 |
| *Triticum aestivum* | gi\|9798603 | 82.6 | 81.4 | 79.8 | 80.4 |
| *Lentinula edodes* | gi\|11262436 | 72.3 | 72.2 | 71.9 | 72.3 |
| *Bos taurus* | gi\|4557913 | 64.5 | 64.3 | 62.7 | 62.4 |

Figure 3 is a sequence alignment (Clustal X) of the four complete ESTs from *Eucalyptus grandis* and other organisms (Table 1), with these organisms has a zeta isoform 14-3-3 proteins of *Bos taurus* (gi|4557913|), that has 64,3% similarity with the *Eucalyptus* clusters, this 14-3-3 proteins of *Bos taurus* crystallized by Liu *et al.* (1995) has nine antiparallel alpha-helices, and a dimeric structure which forms a groove for a potential interaction with a substrate; other studies have demonstrated that the dimeric structure of 14-3-3 proteins have many protein-binding sites, for many these sites can be specific to their target's phosphorylation motifs conserved within the sequence.

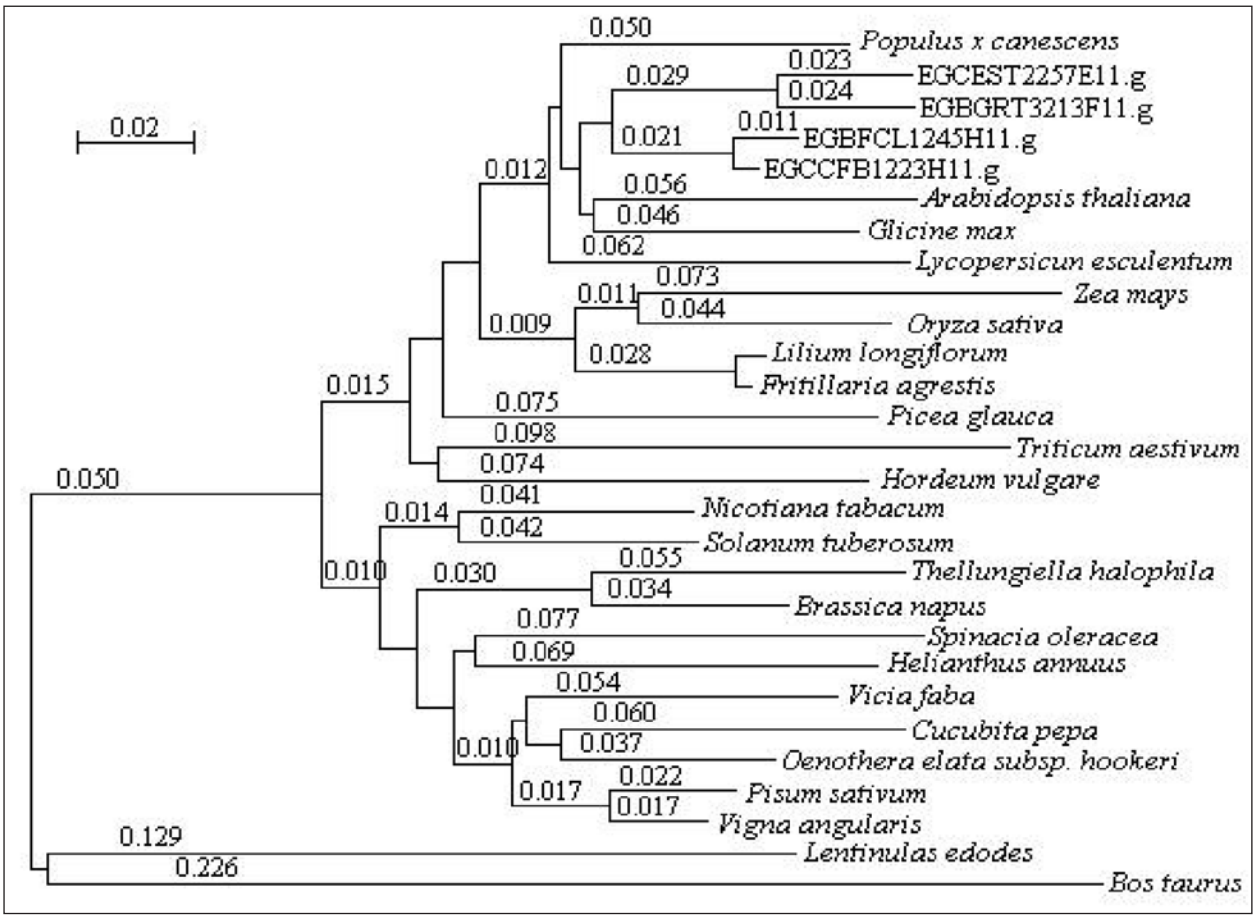


**Figure 2**. Analysis of 14-3-3 protein amino acid sequences. The sequences taken from the EMBL GeneBank (showed in table1) and four isoforms of *Eucalyptus grandis* (EG) was analyzed in the Phylogenetic tree. Branch lengths reflect sequence diversity counted as the number of substitutions per site. The scale is corrected for multiple substitutions. The numbers indicate percentage support for each branch, computed from 1000 bootstrap resamplings (the bar corresponds to 0.1 substitutions per site). The tree was constructed by the neighbors-joining method using PAUP program and visualized by TreeView. (Análise das seqüências de aminoácidos da proteína 14-3-3. O alinhamento do aminoácido selecionado da proteína 14-3-3 arranjada em seqüência. As seqüências do EMBL GeneBank (apresentadas na tabela 1) e quatro isoformas de *Eucalyptus grandis* (EG) foram examinadas na árvore filogenética. Os comprimentos da filial refletem a diversidade da seqüência contada como o número das substituições por local. A escala é corrigida para substituições múltiplas. Os números indicam a sustentação da porcentagem para cada filial, computada de 1000 réplicas de re-amostragem (a barra corresponde a 0.1 substituição por local). A árvore foi construída pelo método vizinho mais próximo, usando o programa PAUP e visualizada pelo TreeView).

In Figure 3, the box represents the phosphorylation motif region where we can see all four EG clusters; in Box 1 we can see that the *Eucalyptus* clusters have an alanine, whereas the 14-3-3 protein *Bos taurus* (gi|4557913) has a serine.

In the α-1 region, at position 11, showing that clusters EGCCFB1223H11.g and EGBFCL1245H11.g had an asparagines, whereas clusters EGBGRT3213F11.g and EGCEST2257E11.g had a tyrosine.

Clusters EGBGRT3213F11.g and EGCEST2257E11.g had a aspartic acid at position 29 and 91, a threonine at position 83 and 114, a serine at position 98 and 109, a glutamic acid at position 105, and an isoleucine at position 116, whereas clusters EGCCFB1223H11.g and EGBFCL1245H11.g had an asparagine at position 29 and 114, a glutamic acid at positions 83, 91, and 98, a valine at position 116, a threonine at position 105, and an aspartic acid at position 109 (Figure 3). In *Arabidopsis thaliana*, Van Heusden *et al.* (1996) reported that differences in amino acid composition and sequence could explain the 14-3-3 isoforms.

Many studies have demonstrated a possible function for 14-3-3 proteins in inducing a defense response from wounding, attack, or any stress induction, Lapointe *et al.* (2001) demonstrated that 14-3-3 proteins are expressed in a plant when this plant is attacked or has a chemical elicitor, suggesting a possible role for these 14-3-3 proteins in the tree's pathogen defense response.

Many studies show that the 14-3-3 protein family are highly conserved, but occur as distinct isoforms (Ferl, 1996); these isoforms can be responsible for different responses, for instance, a wounding response or a salt stress. In *Populus tremula* x *P. alba* clone after chitosan, jasmonic acid, and salicylic acid treatment, there was gene expression of many 14-3-3 proteins isoforms, this may be a reflection of the relative abundance of different 14-3-3 proteins isoforms and their link with different stress processes (LAPOINTE *et al.*, 2001).

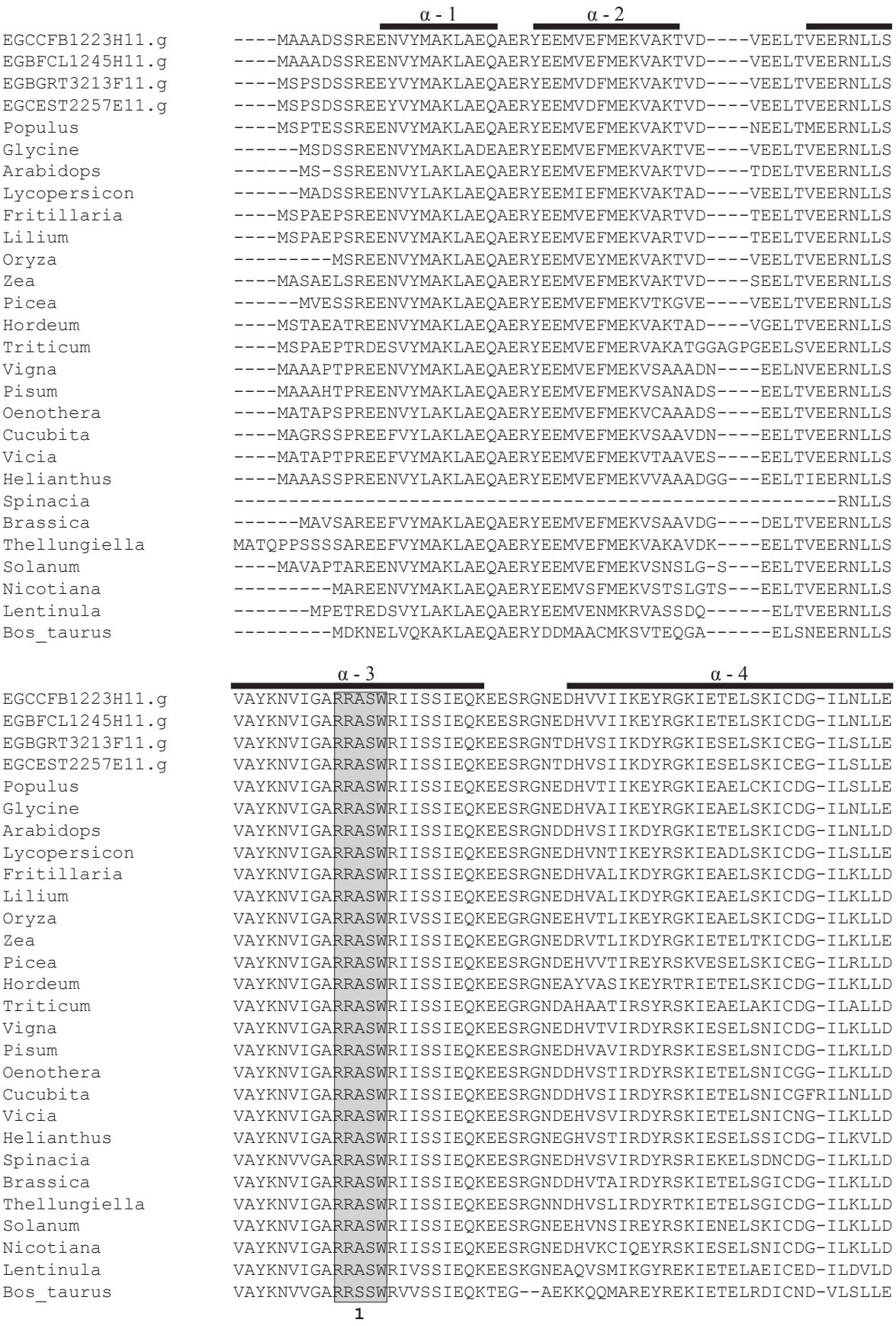


**Figure 3**. Comparative alignment (Clustal X) of deduced amino acid sequences for *Eucalyptus* expressed sequence tag clusters (EG) and others 14-3-3 proteins sequences from GenBank NCBI (Showed table 1).The solid black bar indicates the positions of the nine alpha helices in the three-dimensional structure. Box 1 and 2 indicate the position of the probable site of the phosphorylation motifs. (Alinhamento comparativo (Clustal X) das seqüências deduzidas de aminoácidos dos agrupamentos das seqüências alvos expressas de *Eucalyptus* (EG) e outras seqüências de proteínas 14-3-3 do banco de dados GenBank NCBI (Apresentadas na tabela 1). As barras pretas contínuas indicam as posições das nove alfa-helices na estrutura tridimensional. A caixa 1 e 2 indica a posição do local provável dos motivos de fosforização).

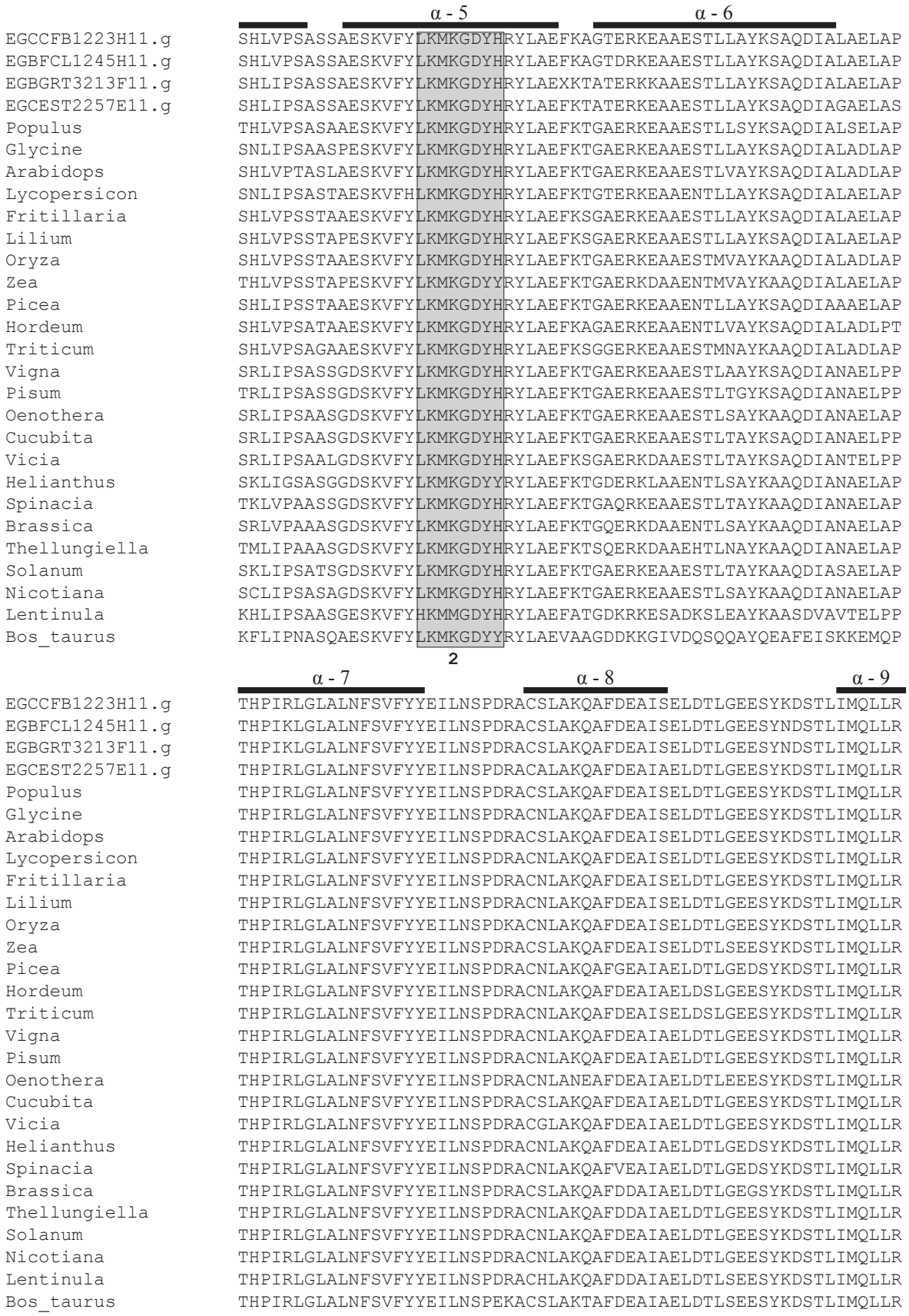


**Figure 3 - Continuation**. Comparative alignment (Clustal X) of deduced amino acid sequences for *Eucalyptus* expressed sequence tag clusters (EG) and others 14-3-3 proteins sequences from GenBank NCBI (Showed table 1).The solid black bar indicates the positions of the nine alpha helices in the three-dimensional structure. Box 1 and 2 indicate the position of the probable site of the phosphorylation motifs. (Alinhamento comparativo (Clustal X) das seqüências deduzidas de aminoácidos dos agrupamentos das seqüências alvos expressas de *Eucalyptus* (EG) e outras seqüências de proteínas 14-3-3 do banco de dados GenBank NCBI (Apresentadas na tabela 1). As barras pretas contínuas indicam as posições das nove alfa-helices na estrutura tridimensional. A caixa 1 e 2 indica a posição do local provável dos motivos de fosforização).

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                                α - 9
EGCCFB1223H11.g      DNLTLWTSDVTDEAG-DEIKESSKR-ESGEG--------------
EGBFCL1245H11.g      DNLTLWTSDVTDEAG-DEINESSK--------------------
EGBGRT3213F11.g      DNLTLWTSDVTDEAG-DEINESSK--------------------
EGCEST2257E11.g      DNLTLWTSDLTDEAG-DDIKEASKL-ESGEGQQ------------
Populus              DNLTLWTSDITDDAG-DEIKEASKR-ESGDGPQ------------
Glycine              DNLTLWTSDITDIAG-DEIKETSKQ-QPGE---------------
Arabidops            DNLTLWNSDINDEAGGDEIKEASKH-EPEEGKPAETGQ-------
Lycopersicon         DNLTLWTSDNADDVG-DDIKEASKP-ESGEGQQ------------
Fritillaria          DNLTLWTSDINEEAG-DEIKEASK---AGEGQ-------------
Lilium               DNLTLWTSDINEEAG-DEIKEASK---AVEGQ-------------
Oryza                DNLTLWTSDLTEDGG-DEVKEASKG-DACEGQ-------------
Zea                  DNLTLWTSDISEDPA-EEIREAPKR-DSSEGQ-------------
Picea                DNLTLWTSDMQEDAG-DEIKETSKR-DEGEEQ-------------
Hordeum              DNLTLWTSDNAEEGG-DEIKEAASK-PEGEGHS------------
Triticum             DNLTLWTSDTNEDDV-DEIKEAPAPKESGDGQ-------------
Vigna                DNLTLWTSDMQDDGA-DEIKEAAPKQD-----DQ-----------
Pisum                DNLTLWTSDMQDDGA-DEIKEAAPKAD-----EQQ----------
Oenothera            DNLTLWTSDMQDDGG-DEIKEAAPKPD-----EQY----------
Cucubita             DNLTLWTSDIRGSR--DDIKEAAPKRDCTNSSEQIDLHVQDSTSP
Vicia                DNLTLWTSDMQDDGA-DEIKEAAPKGN-----DEPQ---------
Helianthus           DNLTLWTSDMQDDTA-EEVKEAP-KPD-----DQ-----------
Spinacia             DNLTLWTSDMQDEAA-DEITEEAAKQQKAVNNNKIAY--------
Brassica             DNLTLWTSDMQDDAA-DEIKEAS-----APKPTEEQQ--------
Thellungiella        DNLTLWTSDMQDDD--DVIKEAAAAAPAAPKPAEEQQQS------
Solanum              DNLTLWTSDMQDDGA-DEIKEDPKPEE-----KN-----------
Nicotiana            DNLTLWTSDMQDDGA-DEIKETKADNE-----QQ-----------
Lentinula            DNLTLWTSDMQDSADKPAEKDEAADAPADE---------------
Bos_taurus           DNLTLWTSDTQGDEA--EAGEGGEN--------------------
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**Figure 3 - Continuation**. Comparative alignment (Clustal X) of deduced amino acid sequences for *Eucalyptus* expressed sequence tag clusters (EG) and others 14-3-3 proteins sequences from GenBank NCBI (Showed table 1).The solid black bar indicates the positions of the nine alpha helices in the three-dimensional structure. Box 1 and 2 indicate the position of the probable site of the phosphorylation motifs. (Alinhamento comparativo (Clustal X) das seqüências deduzidas de aminoácidos dos agrupamentos das seqüências alvos expressas de *Eucalyptus* (EG) e outras seqüências de proteínas 14-3-3 do banco de dados GenBank NCBI (Apresentadas na tabela 1). As barras pretas contínuas indicam as posições das nove alfa-helices na estrutura tridimensional. A caixa 1 e 2 indica a posição do local provável dos motivos de fosforização).

## CONCLUSIONS

In this data mining, four full length 14-3-3 proteins were found in the *Eucalyptus* Genome, the cluster showing a closely relation with others dicotyledonous plants, in question: *A. thaliana* with 90.2%, *G. max* with 90.3% *Populus* X *canescens* with 90.1% and *L. esculentum* with 90% and in the phylogenetic analysis was observed that *Eucalyptus* 14-3-3 proteins was more related with the *A. thaliana* and *G. max*.

## ACKNOWLEDGEMENTS

The authors would like to express their thanks to Ripasa S.A. Celulose e Papel, Duratex, Votorantim Celulose e Papel and Suzano/Bahia Sul; to the Professor Edwards K. Collin, for the revision of the English text. This work was supported by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo).

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Recebido em 16/11/2005
Aceito para publicação em 07/12/2006