

1 **Exploring the evolution of the proteins of the plant nuclear envelope**

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16 **KEY WORDS**

17 Higher plant, Nucleus, Chromatin, LINC complex, SUN domain, KASH domain,  
18 nucleoskeleton

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21 **ABBREVIATIONS AND ACRONYMS**

22 *Arabidopsis thaliana* (*A. thaliana*), Basic Local Alignment Search Tool protein (BLASTp),  
23 Crowded Nuclei (CRWN; also termed LINC for Little Nuclei and NMCP for Nuclear Matrix  
24 Constituent Protein), HMMER (Hidden Markov Model-based sequence alignment tool)  
25 Klarsicht/Anc1/Syne homology (KASH), Lamin B receptor (LBR), Lamin-Emerin-Man1  
26 (LEM), Linker of Nucleoskeleton and Cytoskeleton (LINC), Nuclear Envelope Associated  
27 Protein (NEAP), Reads Per Kilobase of transcript per Million mapped reads (RPKM), Sad1-  
28 Unc84 (SUN), SUN interacting Nuclear Envelope Proteins (SINEs), Toll Interleukin Receptor  
29 domain KASH protein (TIK), trans-membrane (TM), Whole-genome duplication (WGD), WPP  
30 Domain Interacting Proteins (WIPs),

31

32 **ABSTRACT**

33 In this study, we explore the plasticity during evolution of proteins of the higher plant nuclear  
34 envelope (NE) from the most ancestral plant species to advanced angiosperms. The higher  
35 plant NE contains a functional Linker of Nucleoskeleton and Cytoskeleton (LINC) complex  
36 based on conserved Sad1-Unc84 (SUN) domain proteins and plant specific  
37 Klarsicht/Anc1/Syne homology (KASH) domain proteins. Recent evidence suggests the  
38 presence of a plant lamina underneath the inner membrane and various coiled-coil proteins  
39 have been hypothesised to be associated with it including Crowded Nuclei (CRWN; also  
40 termed LINC and NMCP), Nuclear Envelope Associated Protein (NEAP) protein families as  
41 well as the CRWN binding protein KAKU4. SUN domain proteins appear throughout with a  
42 key role for mid-SUN proteins suggested. Evolution of KASH domain proteins has resulted in  
43 increasing complexity, with some appearing in all species considered, while other KASH  
44 proteins are progressively gained during evolution. Failure to identify CRWN homologs in  
45 unicellular organisms included in the study and their presence in plants leads us to speculate  
46 that convergent evolution may have occurred in the formation of the lamina with each  
47 kingdom having new proteins such as the Lamin B receptor (LBR) and Lamin-Emerin-Man1  
48 (LEM) domain proteins (animals) or NEAPs and KAKU4 (plants). Our data support a model  
49 in which increasing complexity at the nuclear envelope occurred through the plant lineage  
50 and suggest a key role for mid-SUN proteins as an early and essential component of the  
51 nuclear envelope.

52

53 **INTRODUCTION**

54 The nuclear envelope is a key component of eukaryotic cells and may be considered to be  
55 composed of three elements, the nuclear membrane, nuclear pore complexes and the  
56 nuclear lamina (Gerace and Burke, 1988; Hetzer, 2010). These structural components are  
57 essential for many processes including nuclear morphology, nuclear migration, chromatin  
58 organisation and regulation of gene expression (Graumann and Evans, 2010a). Significant  
59 progress has been made in describing novel plant nuclear envelope proteins (Parry, 2015;  
60 Tamura et al., 2015; Zhou et al., 2015a). In *Arabidopsis thaliana* (*A. thaliana*), these include  
61 components of the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex for which  
62 functional data is slowly being revealed. *Arabidopsis* contains proteins of the inner nuclear  
63 envelope of the SUN domain family including Cter-Sad1-Unc84 (Cter-SUN) (Graumann and  
64 Evans, 2010b; Graumann et al., 2010; Oda and Fukuda, 2011) as well as mid-SUN domain  
65 proteins in which a SUN-domain homologous to that of the C-ter SUNs is located centrally  
66 within the protein (Graumann et al., 2014). It also contains proteins of the outer nuclear  
67 envelope, of the KASH domain protein family including WPP Domain Interacting Proteins  
68 [WIPs], SUN interacting Nuclear Envelope Proteins [SINEs] and *Arabidopsis thaliana* Toll

69 Interleukin Receptor domain KASH protein [TIK] (Zhou et al., 2012; Graumann et al., 2014;  
70 Zhou and Meier, 2014). In addition, plant proteins proposed to form the nuclear lamina -  
71 Crowded Nuclei (CRWNs; Dittmer et al., 2007; Wang et al., 2013) and CRWN-interacting  
72 proteins such as KAKU4 (Goto et al., 2014) as well as Nuclear Envelope Associated  
73 Proteins (NEAPs), which may be associated with the lamina (Pawar et al., 2016), have been  
74 described in *A. thaliana* and shown to localise to the nuclear periphery.

75

76 Sequence data now available permits comparison of components of the nuclear envelope  
77 between algae, mosses, gymnosperms and angiosperms with the components of *A.*  
78 *thaliana*. Functional analysis of these genes is challenging because they belong to small  
79 gene families as a consequence of gene and whole-genome duplication (WGD) creating  
80 duplicate genes and thus gene redundancy (Gaut and Ross-Ibarra, 2008; Soltis and Soltis,  
81 2016). Whole-genome duplication (WGD) is recognised as an important event for genome  
82 evolution in animals, plants and fungi and to drive key new features, with resulting increased  
83 complexity and speciation (Soltis and Soltis, 2016). Following WGD, massive gene loss can  
84 occur restoring the diploid state for most duplicated loci while few duplicated genes remain  
85 and may provide new evolutionary innovation including structures (e.g. floral organs) and  
86 adaptations (Kellis et al., 2004). Previous analyses of plant genomes have shown that seed  
87 plants share an ancient WGD event, zeta (Jiao et al., 2011). A second WGD, epsilon, has  
88 been detected shortly before the diversification of angiosperms. These two WGDs are  
89 suggested to play a role in the origin and rapid diversification of the angiosperms (Jiao et al.,  
90 2011). Finally, the gamma WGD occurred after eudicot/monocot diversification, followed by  
91 several partial or complete duplication events.

92

93 In this study, we have selected 20 representative species based on the revised classification  
94 of eukaryotes (Adl et al., 2012), their available genome sequences and gene expression  
95 description, in order to explore the evolution of components of the plant nuclear envelope.  
96 Availability of genome sequence data for the core eudicot *Amborella trichopoda* provides an  
97 opportunity to explore the nuclear envelope of a primitive angiosperm. *Amborella*, a New  
98 Caledonian shrub, has been suggested as the sole surviving sister species of all other  
99 angiosperms and is unique in sequenced plant genomes in showing no evidence of recent,  
100 lineage-specific genome duplications (Project et al., 2013). The *Amborella* genome therefore  
101 offers an opportunity to explore the composition of an ancestral plant nuclear envelope and  
102 the effect of genomic changes after polyploidy in other angiosperms (Project et al., 2013).  
103 Finally, RNAseq data for each species were used to describe expression levels within  
104 species to establish that the genes are active and not pseudogenes and to demonstrate  
105 gene activity and when possible tissue specific expression patterns.

106

107 The aim of the work presented in this paper was to explore the evolution of nuclear envelope  
108 proteins in unicellular algae and multicellular plants and to provide evidence for the  
109 composition of the simplest functional plant LINC complex. This study provides valuable  
110 information for mutant and other functional studies by identifying potential redundancy and  
111 specialisation in nuclear envelope and lamina-like components.

112

## 113 **MATERIAL AND METHODS**

### 114 **Homologous LINC complex and lamin-like protein detection**

115 A Perl script was developed and applied to proteomic data to identify KASH domain proteins.  
116 The program tests the presence of the trans-membrane (TM) domain and four specific  
117 amino acids at the C-terminus, which are characteristic for KASH proteins. The position of  
118 the TM domain is variable and the script searches this TM domain up to 40 amino acids  
119 away from the KASH-specific C-terminal motifs detected in *A. thaliana* (either VIPT, VVPT,  
120 AVPT, PLPT, TVPT, LVPT or PPPS; Zhou et al., 2012; Graumann et al., 2014; Zhou et al.,  
121 2014). The identification of the TM domain is based on the Kyte-Doolittle method (Kyte and  
122 Doolittle, 1982). Only proteins, which possess a TM domain and the four KASH specific  
123 amino acids in the C-terminus of the protein, were selected.

124

125 For all proteins of interest a Basic Local Alignment Search Tool protein (BLASTp) was used  
126 with default parameters as well as HMMER (Hidden Markov Model-based sequence  
127 alignment tool; <http://hmmer.org>). The best hits were retained and used for phylogenetic  
128 analysis (Altschul et al., 1990). The proteome of each species was used as reference for the  
129 BLASTp (Figure 1), and the protein sequences of the LINC complex as well as the putative  
130 lamina of *A. thaliana* were used as queries (Supplementary Table 1). BLASTp results are  
131 given as supplementary Table 2 (mid-SUN), 3 (Cter-SUN), 4 (WIP), 5 (SINE), CRWN (6),  
132 NEAP (7) and KAKU4 (8). Reciprocal BLASTp was used to verify the relevance of all  
133 identified orthologs.

134

### 135 **Phylogenetic reconstruction**

136 Selected sequences were first aligned with MUSCLE, a multiple sequence alignment tool  
137 (Edgar, 2004), using default parameters. The alignment was then refined using Gblocks  
138 (Talavera and Castresana, 2007). Fast-Tree was then applied with default parameters, for  
139 the construction of the phylogenetic tree (Price et al., 2010). Fast-Tree infers approximately-  
140 maximum-likelihood phylogenetic trees from alignments. Finally, phylogenetic trees were  
141 drawn using the Interactive Tree Of Life ITOL (Letunic and Bork, 2011).

142

## 143 **RNA sequencing data and analysis**

144 Data used for the RNA-seq analysis was obtained from the NCBI  
145 (<http://www.ncbi.nlm.nih.gov/geo/browse/>) or from the Amborella Genome Database,  
146 respectively (<http://amborella.huck.psu.edu/>). Five different tissues (leaves, roots, flowers,  
147 flower buds, and seeds/siliques) as well as total seedling were chosen for the analysis of the  
148 expression patterns of the genes of interest (Supplementary Table 1). The expression was  
149 analysed for ten species (Supplementary Table 9). Reads from RNA-Seq libraries were  
150 mapped onto the candidate gene sequences allowing no mismatches using TOPHAT v  
151 2.0.14 (Kim et al., 2013) with standard settings and maximum of multihits set at 1, minimum  
152 intron length set at 15 bp, and maximum intron length set as 6,000 bp. Reads were added  
153 together for each gene using HTseq-count with the overlap resolution mode set as  
154 intersection-non empty and with no strand-specific protocol (Anders et al., 2015).  
155 Transcription levels in Reads Per Kilobase of transcript per Million mapped reads (RPKM)  
156 were normalised to *AtSAND* (At2g28390; Czechowski et al., 2005 and Supplementary Table  
157 10). *SAND* was chosen due to its constant gene expression levels across different tissues at  
158 developmental stages in *Arabidopsis thaliana* (Czechowski et al., 2005). For each species,  
159 the *SAND* homologue with the closed sequence identity to *AtSAND* was chosen.  
160 Furthermore, absolute *SAND* expression levels (in RPKM) in different species were  
161 comparable to expression of *Arabidopsis AtSAND*.

162

## 163 **RESULTS AND DISCUSSION**

164 In order to gain an insight into the evolutionary development of known plant nuclear  
165 envelope proteins, we reconstructed the phylogenetic distribution of the LINC complex (SUN  
166 and KASH) and plant lamina (CRWN, NEAP and KAKU4) components by exploring 20  
167 representative species including unicellular photosynthetic algae, lycophytes, mosses,  
168 gymnosperms and angiosperms for which genome sequences and gene expression data are  
169 available (Figure 1; Supplementary Table 11).

170

### 171 **Phylogenetic analysis of inner nuclear membrane proteins**

172

#### 173 *Cter-SUN proteins*

174 The SUNs are divided into two subfamilies according to the position of the SUN domain: the  
175 mid-SUN with a central SUN domain and the Cter-SUNs having a SUN domain at the C-  
176 terminus. The potential origin of the two classes of SUN domain proteins remains obscure  
177 and is discussed in Graumann et al., 2015. The SUNs are key members of the LINC  
178 complex and expressed in all tissues (Murphy et al., 2010; Graumann et al., 2014). Blastp

179 and HMMER analysis revealed thirty-three Cter-SUN proteins across all the 19 species  
180 studied, other than *Chlamydomonas reinhardtii*, where no Cter-SUN protein was detected  
181 (Figure 1). Monocots and eudicots form two paraphyletic groups, with the *Vitis vinifera*  
182 homologue showing greater similarity to the monocot Cter-SUN sequence. The  
183 *Brassicaceae* form a monophyletic group and the duplication of the Cter-SUN gene seems to  
184 have occurred late in evolution because duplicated Cter-SUNs remain grouped within a  
185 given species (Figure 2).

186

187 Expression data for the Cter-SUNs shows a similar transcript level for all the tissue analysed  
188 in different species (Supplementary Figure 1). In some cases, one of the two Cter-SUNs is  
189 more strongly expressed in the seedling (e.g.: AtSUN1 more strongly expressed than  
190 AtSUN2; OsaSUN-a more strongly expressed than OsaSUN-b) (Figure 2). *A. trichopoda*  
191 encodes only one Cter-SUN that is highly expressed in all tissues. The simplest functional  
192 LINC complex may therefore be based on a single Cter-SUN, and strengthens the  
193 suggestion that duplication of the Cter-SUN gene occurred after speciation.

194

195 One or two Cter-SUN proteins were identified in most plants and the moss, although four  
196 close homologues were identified for the club moss *Selaginella molendorffii*. In *A. thaliana*,  
197 SUN1 and SUN2 share almost the same activity and localisation (Graumann et al., 2010).  
198 This is in contrast to mammals, where five Cter-SUN orthologues have clearly differentiated  
199 functions. It appears that the gene duplication resulting in these orthologues occurred earlier  
200 in the evolution of mammals. One likely consequence is the lack of specificity of function of  
201 plant Cter-SUN homologues; for example, a disruption of a single SUN gene results in an  
202 infertility phenotype in animals (Ding et al., 2007), but in *A. thaliana*, a single Cter-SUN  
203 deletion does not affect meiosis or fertility whereas the double mutant *atsun1 atsun2* impacts  
204 fertility and cell division (Varas et al., 2015). This suggests a significant redundancy in Cter-  
205 SUN function in plants and that double knock-out or knock-down mutants are required for  
206 recognisable phenotypes to be obtained.

207

### 208 *mid-SUN proteins*

209 All the species considered contain at least a mid-SUN protein and overall fifty mid-SUN  
210 homologues were identified during our bioinformatic screen. The mid-SUN angiosperm  
211 homologues are clustered in two groups, SUN3/SUN4 and SUN5. In each mid-SUN  
212 homologous group, the basal angiosperm, monocots and eudicots form monophyletic  
213 groups. This suggests that mid-SUN (3, 4) gene duplication occurred after speciation  
214 between angiosperms and gymnosperms (Figure 3). In all tissue analysed the *SUN3/SUN4*  
215 group tends to be more ubiquitously and highly expressed than the *SUN5* group, this is also

216 true for the *A. trichopoda* homologues (Supplementary Figure 1). It has been suggested that  
217 *AtSUN5* has a meiotic function (Graumann et al., 2014) and this is also true for maize with  
218 *ZmaSUN5* (Murphy et al., 2010), although while the double mutants of SUN3, SUN4 and  
219 SUN5 are viable, a *sun3 sun4 sun5* triple mutant is lethal (Graumann et al., 2014). *A.*  
220 *trichopoda* has two mid-SUN proteins, one SUN3/SUN4 homologue and a SUN5  
221 homologue. This suggests that the simplest LINC complex has two mid-SUNs each with a  
222 specific or partially overlapping function.

223

224 In summary, the majority of the 20 species possess at least one mid-SUN and one Cter-SUN  
225 protein except for *Chlamydomonas reinhardtii*, which has only one mid-SUN protein.  
226 Interestingly, in common with Cter SUNs, the club moss *Selaginella* has the highest number  
227 of mid-Sun protein homologues (six). These results are in good agreement with previous  
228 studies that have highlighted the conservation of both Cter- and mid-SUN proteins in most  
229 eukaryotes (Murphy et al., 2010; Graumann et al., 2014) and suggest that the LINC  
230 complex was present in the Last Evolutionary Common Ancestor (LECA; Koreny and Field,  
231 2016). Mid-SUN homologues and Cter-SUN proteins were detected in the unicellular algae  
232 examined, suggesting that SUN emergence pre-dates the evolution of multicellularity. The  
233 evolutionary relationship between Cter-SUN and mid-SUN proteins has yet to be described.  
234 This study suggests that SUN domain proteins may also be among the earliest evolving  
235 components of the plant nuclear envelope and that mid-SUN proteins may have significance  
236 nuclear function in the absence of Cter-SUN.

237

### 238 **KASH protein homologues**

239

240 KASH proteins are diverse in sequence and structure (Zhou and Meier, 2014) but possess a  
241 conserved C-terminal region with a TM domain and a conserved motif of four amino acids at  
242 the extreme C-terminus. For the detection of the KASH domain protein homologues, two  
243 strategies were used. The first was a BLASTp analysis based on known *A. thaliana* KASH  
244 domain proteins (Supplementary Table 1). This analysis permitted detection of KASH protein  
245 homologues in all the organisms studied, except for the unicellular algae where no KASH  
246 protein was detected (Supplementary Table 12). Using this method, 32 SINE homologues  
247 were found, whereas WIP and potential TIK proteins [or TIK-like proteins] (Supplementary  
248 Table 12) were much less common and were found mainly in eudicots. An exception is  
249 *Brassicaceae*, where several potential WIP (3 in *A. lyrata*, 4 in *Brassica rapa*) and TIK (2 in  
250 *A. lyrata* and 1 in *B. rapa*) homologues were identified, these were also detected in *Glycine*  
251 *max* (2 WIP, 1 TIK), *Prunus persica* (1 TIK), *Carica papaya* (1 WIP), *Musa acuminata* (1  
252 WIP), *A. trichopoda* (1 TIK) and the gymnosperm *Picea abies* (1 TIK) (Supplementary Table

253 12). To expand the data collected by Blastp, a script was developed to detect proteins with  
254 the TM domain and C-terminal motif.

255 All the identified plant KASH domain proteins have been divided into three groups: SINEs,  
256 WIPs and TIK (Zhou and Meier, 2014). Six KASH protein clusters were revealed  
257 (Supplementary Figure 2). One includes WIP proteins detected in the monocotyledons and  
258 the basal angiosperms (Supplementary Table 12), as well as seven new putative WIP  
259 proteins to those detected previously by BLASTp. For SINE proteins, three clusters were  
260 detected, for SINE1/2, SINE3 and SINE4 adding respectively two, six and twelve SINE  
261 proteins to those already identified. The high number of proteins in the SINE3 and SINE4  
262 cluster found only by the script was due to weak conservation of these proteins. One much  
263 smaller cluster includes the TIK-like proteins. Only four putative homologues were added but  
264 these were shown subsequently to lack either the TIR domain or the C-terminal TM domain,  
265 therefore suggesting that the TIK protein (Graumann et al. 2014) may be unique to *A.*  
266 *thaliana*. An additional cluster (other) had low sequence similarity and was not included  
267 subsequently. The three WIP proteins in *A. thaliana* show previously described properties  
268 (Xu et al., 2007; Zhao et al., 2008) of a cytoplasmic domain at the N-terminus, with AtWIP1  
269 and AtWIP2 having three coiled-coil domains but AtWIP3 only one. The C-terminal region is  
270 well conserved and the coiled-coil domains align, with all proteins detected as homologues  
271 having a C-terminal predicted TM domain and KASH motif, except AlyWIP1, which lacks  
272 homology at the C-terminal region but is well conserved at the N-terminus.

273

274 All SINEs have a typical KASH TM domain and C-terminal amino acid motif (Zhou et al.,  
275 2014). The SINE gene family comprises four genes in *A. thaliana*, with similarity between  
276 AtSINE1 and AtSINE2, characterised by an Armadillo repeat domain near the N-terminus;  
277 and between AtSINE3 and AtSINE4. The Perl script added only two sequences in the  
278 SINE1/SINE2 group while it added 14 proteins to the SINE3/SINE4 cluster. In this case the  
279 Blastp approach was less efficient than the Perl script because of the absence of well-  
280 conserved domains in the N-terminus. After removal of sequences with the lowest similarity  
281 or without the conserved domain, SINE1/SINE2 proteins are present in all species except  
282 the unicellular algae and club moss, while SINE3/SINE4 were absent (Figure 5). In  
283 summary, the SINE1/SINE2 cluster and WIP proteins are detected in basal angiosperms  
284 whereas the TIK protein is detected only in *A. thaliana*.

## 285 **Phylogenetic analysis of the outer nuclear membrane proteins**

### 286 *WIP proteins*

287 The WIP protein family was the first KASH family detected in *A. thaliana*, (Zhou et al., 2012).  
288 WIP proteins were not detected in unicellular algae, moss, club moss or gymnosperms;  
289 suggesting that they are angiosperm specific proteins. One WIP homologue was detected



290 for *A. trichopoda*. The monocots form a monophyletic group, with one protein for rice , two  
291 and three for maize and *Musa acuminata* suggesting gene duplication (Figure 4). The  
292 eudicots form a paraphyletic group because the WIP homologue of *Nelumbo nucifera* differs  
293 from, and is positioned outside, the WIPs of eudicots. The *Brassicaceae* on the other hand,  
294 form a monophyletic group (Figure 4). This suggests that an ancestral duplication in the  
295 *Brassicaceae* ancestor gave rise to WIP1/WIP2 and WIP3, and then WIP1 and WIP2  
296 resulted from a more recent gene duplication. All three genes are expressed in all the  
297 tissues analysed. In *A. thaliana* *AtWIP3* transcripts are more abundant than *AtWIP1* and  
298 *AtWIP2* in all tissues. This may be due to redundancy in *AtWIP1* and *AtWIP2* function, and  
299 in *A. trichopoda*, the WIP homologue is highly expressed (Supplementary Figure 1).

300

### 301 *SINE proteins*

302 SINEs in *A. thaliana* (Zhou et al., 2014) comprise two groups, SINE1/SINE2 and  
303 SINE3/SINE4. *AtSINE1* is more expressed in guard cells, and its armadillo domain forms F-  
304 actin-associated fibres involved in nuclear positioning while *AtSINE2* is suggested to be  
305 involved in the immunity response of leaves (Zhou et al., 2014). No expression and activity  
306 data was available for *AtSINE3* and *AtSINE4*.

307

308 SINE1/SINE2 proteins were not found in unicellular algae and in club moss, but in contrast  
309 to WIPs, two and three SINE homologues were found in moss and gymnosperms,  
310 respectively (Figure 5). The angiosperms form a monophyletic group and one SINE1/SINE2  
311 homologue was detected for *A. trichopoda* and positioned at the base of the angiosperm  
312 group (Figure 5). The phylogenetic analysis of SINE3 and SINE4 is not possible due to the  
313 low similarity between sequences and a lack of conserved domains. Although SINE3 and  
314 SINE4 are detected in the *Brassicaceae* group, the other sequences are divergent. In the  
315 monocots, two protein homologues were detected for *Musa acuminata*, *Oryza sativa* and  
316 *Zea mays*. However, the phylogeny suggests the presence of recent gene duplication in  
317 *Musa acuminata* (Figure 5). In contrast, the gene duplication between the two other  
318 monocots seems to have occurred before their speciation. All the eudicots possess at least  
319 one SINE1/SINE2 homologue. Four homologues that group together were found in *Glycine*  
320 *max*, suggesting a recent gene duplication. As for WIPs, *Brassicaceae* proteins cluster  
321 together, and one group of homologues is detected for each of SINE1 and SINE2. The  
322 organisation between the two groups suggests a gene duplication to form SINE1 and SINE2.

323 In *A. thaliana*, *AtSINE1* and *AtSINE2* are expressed at the same level in all tissues,  
324 but at a higher level than *AtSINE3* and *AtSINE4*. However, SINE1/SINE2 homologues in  
325 most other species show the lowest level of expression of all KASH proteins for all tissues  
326 analysed expect for maize, rice and *A. trichopoda* (Supplementary Figure 1). In these

327 species WIP and SINE expression is at the same level for all tissues. In *A. thaliana*, *AtWIPs*  
328 are more highly expressed than *AtSINE*.

329

### 330 **Phylogenetic analysis of the putative nuclear lamina and nuclear-envelope associated** 331 **proteins**

332 Proteins of the lamin family are restricted to animals (Cavalier-Smith, 2010). However, to  
333 date, three protein families have been suggested to be components of the putative lamina in  
334 *A. thaliana*, CRWN (Dittmer et al., 2007; Wang et al., 2013), KAKU4, (Goto et al., 2014) and  
335 a novel nuclear envelope associated protein family, NEAP (Pawar et al., 2016).

336

#### 337 *CRWN proteins*

338 The CRWN gene family is imported into the nucleus through an NLS and extensive coiled-  
339 coil domains reminiscent of the animal lamins are hypothesised to allow polymerisation of  
340 the protein to form the plant lamina. Fifty CRWN proteins were detected by BLASTp and  
341 pHMMER in all multicellular plants but are absent from unicellular algae. In most species two  
342 homologues were detected for each species. Two clusters of CRWN proteins were defined  
343 in a previous publication (Ciska and Moreno Diaz de la Espina, 2013) and were also  
344 identified here as two main phylogenetic groups: CRWN1/CRWN2/CRWN3 and CRWN4.  
345 The clusters of CRWN4 homologues constitute monophyletic groups and only one protein  
346 was found for all species except for *Glycine max* (Figure 6). Gymnosperm homologues seem  
347 to have only the CRWN4 lineage while *A. lyrata* has lost the CRWN4 lineage meaning that  
348 some functional redundancy exists between the two monophyletic groups.

349

350 For the second group made up of the homologues of the three other CRWN proteins, the  
351 same organisation was found and only one homologue in *A. trichopoda* was detected  
352 (Figure 6). In the monocot group, only *Musa acuminata* possesses three homologues, the  
353 other monocots possessing only one (Figure 6). In the eudicot group, two clusters can be  
354 distinguished: one for the homologues of AtCRWN1 and the other for AtCRWN2/AtCRWN3.  
355 This reveals a gene duplication, which occurred after the speciation creating monocots and  
356 eudicots. The other duplication, which gave rise to CRWN2 and CRWN3, occurred after  
357 *Brassicaceae* speciation and formed a monophyletic group. The genes belonging to the  
358 cluster CRWN1/CRWN2/CRWN3 show higher expression in comparison to CRWN4. Other  
359 than in the *Brassicaceae*, CRWN2 is less expressed than CRWN1 and CRWN3 for all the  
360 tissues analysed. Surprisingly, no lamin-like proteins were detected in the chlorophyte  
361 unicellular algae. Previous studies have shown the presence of other lamin-like proteins in  
362 unicells like NE81 and NUP1 (DuBois et al., 2012; Krüger et al., 2012). It is likely that several

363 proteins have evolved in different systems to fulfil a similar role and this would reward further  
364 study.

365

#### 366 *NEAP proteins*

367 The NEAP proteins are characterised by a TM region at the C-terminus, a functional NLS  
368 and extensive coiled-coil domains (Pawar et al., 2016). NEAP1, NEAP2, and NEAP3 were  
369 identified in gymnosperms and angiosperms and 28 proteins were detected while NEAPs  
370 are absent from the more ancestral species moss, club moss and unicellular algae  
371 (Supplementary table 11). The monocots form a monophyletic group with two potential  
372 specific gene duplications for *Musa acuminata* and *Zea mays* (Figure 7). As for monocots,  
373 the eudicots form a monophyletic group (Figure 7), and the gene duplication seems specific  
374 to species. So the three NEAP genes in *Brassicaceae* appear to result from a duplication  
375 event during the speciation of *Brassicaceae*. The single NEAP gene in *A. trichopoda* is  
376 expressed at very high level. Lack of expression of *AtNEAP4* and absence of protein  
377 homologues in other species imply that it is a pseudogene. The other *NEAP* genes are  
378 expressed in seedlings and in other tissues but at a low level (Supplementary Figure 1).

379

#### 380 *KAKU4 proteins*

381 KAKU4 homologues are only detected in angiosperms. Only one KAKU4 homologue is  
382 detected in each species except for *Glycine max* and *Brassica rapa*. KAKU4 is therefore a  
383 recent addition specific to angiosperms and as it interacts with CRWN1 and CRWN4, it could  
384 link CRWN proteins to other components at the nuclear periphery. Analysis of KAKU4  
385 phylogeny reveals two monophyletic groups, for the monocot and eudicot homologues  
386 (Figure 8). The protein was not detected in basal angiosperms, gymnosperms, moss, club  
387 moss and unicellular algae. Either KAKU4 is a protein with specific function in angiosperms,  
388 or was not detected due to a high variability between species. KAKU4 homologues are  
389 expressed to comparable levels in all tissues (Supplementary Figure 1).

390

## 391 **DISCUSSION**

392

393 The results presented reveal functional conservation of the proteins of the plant nuclear  
394 envelope with those of other kingdoms, but surprising diversity in protein sequence. Table 1  
395 summarises the occurrence of each of the components in the study, together with their  
396 function. SUN domain proteins, lamina component CRWN and the KASH domain proteins  
397 SINE1-2 involved in actin binding are present before the Zeta WGD (though C-ter SUNs and  
398 CRWN are absent from the *Chlamydomonas*); putative NE- anchored lamina components  
399 NEAPs are first found after the Zeta WGD. Binding of RanGAP to the NE by the KASH

400 proteins designated WIP originates with the gamma WGD of the angiosperms; the  
401 mechanism of anchorage of RanGAP in gymnosperms and mosses therefore warrants  
402 further study. CRWN interacting KAKU4 and KASH protein TIK appear to be of later origin  
403 and were only detected in the *Brassicaceae*, suggesting specialist functions.

404 Data from *A. trichopoda* suggests a minimal angiosperm LINC complex, with two KASH  
405 domain proteins (one WIP and one SINE), three SUN domain proteins (one Cter-SUN and  
406 two mid-SUNs) and putative lamina constituents (two CRWNs) together with one NEAP. The  
407 moss *P. patens* has four SUNs, two KASH and two putative lamina constituents. The  
408 gymnosperm *P. abies* has three SUNs, three KASH (SINEs) and putative lamina  
409 components (two CRWN) together with two NEAPs (Figure 1). Evolution of SUN, KASH and  
410 lamina constituents appears to have accompanied WGD and partial genome duplication  
411 events and to have resulted in a range of homologues during angiosperm speciation. The  
412 results presented also indicate a plant nuclear envelope which has developed significant  
413 complexity and redundancy through gene duplication explaining the need for multiple knock-  
414 out mutants; for instance the double mutant *atsun1 atsun2* (Zhou et al., 2012) or the  
415 quintuple mutant *wifi (atwip1 atwip2 atwip3 atwit1 atwit2)* (Zhou et al., 2015b) before strong  
416 phenotypes are observed.

417

418 The data also suggests evolution from an ancestral LINC complex, in which SUN domain  
419 proteins are multifunctional, to a more multifaceted LINC complex containing an increasing  
420 number of KASH and lamin-like proteins. A key role for mid-SUN proteins is suggested. This  
421 is commensurate with the demonstration that SUNs play a fundamental role in chromatin  
422 interaction with the nuclear envelope during its reformation in plant mitosis (Graumann and  
423 Evans, 2011) and in telomere attachment in meiosis (Varas et al., 2013). KASH domain  
424 proteins appear to be evolving, with SINEs preceding WIPs, with TIK only identified in  
425 *Arabidopsis*. It is suggested that increasing specialisation accompanies the acquisition of  
426 additional KASH homologues and that specific functions of the later evolving proteins (for  
427 instance RanGTP anchorage and nuclear movement in the pollen tube) are undertaken by  
428 other nuclear envelope components in their absence. A similar pattern of evolution of KASH  
429 proteins is suggested in ophisthokonts; Zhou et al., (2014) commenting on novel plant KASH  
430 proteins noted that while some are highly conserved (e.g. Nesprin 1 and 2, ANC-1 and MSP-  
431 300) others are restricted in distribution (e.g. Klarsicht homologues to insects and KDP-1 to  
432 nematodes) suggesting origins after SUN domain proteins and rapid evolution linked to  
433 diversifying function. Finally, higher plants have evolved a lamina-like structure based, like  
434 animal cells, on coiled-coil proteins. This appears to have arisen with the CRWN proteins  
435 present in mosses and clubmosses (lycophytes) and with KAKU4 arising later. These data  
436 are consistent with previous reports suggesting that SUN domain proteins were the first

437 nuclear envelope proteins linking chromatin to the nuclear envelope (Cavalier-Smith, 2010),  
438 predating the evolution of lamins. Indeed lamins are prone to rapid evolution as they interact  
439 with fewer partners than components of the LINC complex (Koreny et al 2016). One of the  
440 striking results of our analysis is the absence of CRWN in unicellular species despite the fact  
441 that the lamina is involved in basic function such as nuclear morphology and chromatin  
442 organisation which are both important for the regulation of gene expression. Although we  
443 cannot exclude if lamins and CRWN are subjected to fast evolution leading to unsuccessful  
444 recovery of homologs by Blast and HMMER analyses, it is tempting to speculate that  
445 convergent evolution occurred in animals and plants, with increasing functionality and  
446 complexity through the introduction of LBR and LEM proteins in animal and KAKU4 in plants.  
447 Similar observations were recently presented for the PRC1 polycomb group complex which  
448 is a conserved function but with poor sequence homology despite the presence of the  
449 conserved RING-domain, again suggesting convergent evolution between plant and  
450 animals. Interestingly, the PRC1 complex is involved in the regulation of gene expression  
451 through the binding of trimethylated Histone H3 at lysine 27 (H3K27me3) a well-known  
452 repressive epigenetic mark also enriched in Lamina-Associated Domains (LADs) (Bickmore  
453 and van Steensel, 2013). Exploring the possible connection between the nuclear envelope  
454 components and the PRC1 repressive complex will lead to a better understanding of the  
455 functions of the nuclear envelope in the regulation of chromatin organisation and gene  
456 expression.

457

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461

462 Table 1

	First appearance	WGD	Function	Location	Reference
C-ter SUN	Moss		LINC complex component; binds KASH; Nuclear shape and size; meiosis	INM	Graumann et al., 2010; Oda and Fukuda, 2011
Mid-SUN	Alga		LINC complex component; binds KASH; Nuclear shape and size; fertility	INM and ER	Graumann et al., 2014
CRWN	Moss		nucleoskeleton; nuclear size and shape; heterochromatin organisation	nuclear periphery and nucleoplasm	Dittmer et al., 2007; Wang et al., 2013
SINE1-2	Moss		LINC complex component; KASH; interacts with actin cytoskeleton; nuclear positioning in guard cells (SINE1); innate immunity response (SINE2)	ONM	Zhou et al., 2012, 2014
NEAP	Basal angiosperm, Gymnosperm	Zeta	NE anchor, SUN binding, chromatin interactor; root growth; nuclear morphology	INM	Pawar, 2015
WIP	Basal angiosperm	Epsilon	SUN binding; anchors RanGAP to NE; nuclear morphology; pollen tube termination; nuclear movement	ONM; RanGAP anchorage	Xu et al., 2007; Zhao et al., 2008
KAKU 4	Monocot	Epsilon	CRWN binding; nuclear size and shape	Nucleoskeleton	Goto et al., 2014
SINE 3-4	Eudicot (Brassicac)	Gamma	LINC complex component; KASH; SUN binding	ONM	Zhou et al., 2014
TIK	Eudicot (Arabidopsis)	Gamma	SUN binding; nuclear size and shape; root growth	ONM	Graumann et al., 2014

463

464 Table 1: protein classes and their origins and function as derived in this study.

465

466

467

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577

578

## 579 **FIGURE LEGENDS.**

580

581 **Figure 1: Distribution of components of plant nuclear envelope in the plant kingdom.**

582 **A)** Selected plant lineages used in this study from left to right: Unicells Algae (pink), Moss  
583 and Club Moss (red), Gymnosperm (orange), Basal Angiosperms (yellow), Monocots (green)

584 and Eudicots (blue). zeta epsilon and gamma WGDs are indicated as arrow heads  
585 respectively in black, grey and purple. **B)** Distribution of the 9 protein families (rows) in the  
586 20 species (columns). Absence (0) of a given protein is highlighted in light orange.

587

588 **Figure 2: Phylogenetic tree of Cter-SUN proteins and gene expression levels.**

589 **Left:** maximum likelihood tree of Cter-SUN protein homologues constructed from an  
590 alignment. Bootstrap values are presented. The colour of the label shows the lineage of the  
591 plant. The gene label is constructed with the three letters from the species name  
592 (supplementary Table 4) and the gene name of the *A. thaliana* homologues. **Right:** red bar  
593 represents the value of the transcription level in seedlings expressed in RPKM, except for  
594 species indicated by \*, the RNA-seq data was obtained from leaf tissue (Supplementary  
595 Table 2).

596

597 **Figure 3: Phylogenetic tree of mid-SUN proteins.**

598 Legend as Figure 2.

599

600 **Figure 4: Phylogenetic tree of WIP proteins.**

601 Legend as Figure 2.

602

603 **Figure 5: Phylogenetic tree of SINE1, SINE2 homologues proteins.**

604 Legend as Figure 2.

605

606 **Figure 6: Phylogenetic tree of CRWN proteins.**

607 Legend as Figure 2.

608

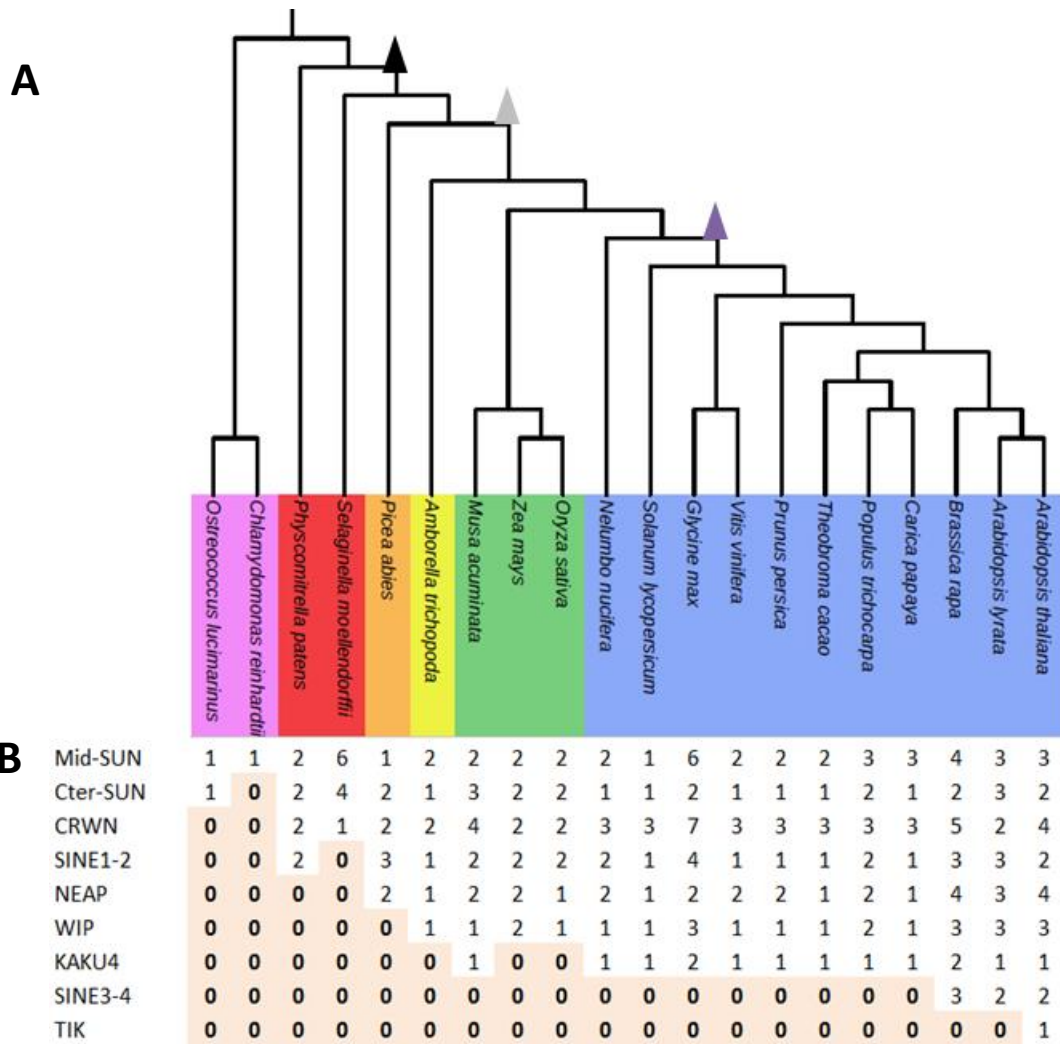
609 **Figure 7: Phylogenetic tree of NEAP proteins.**

610 Legend as Figure 2.

611

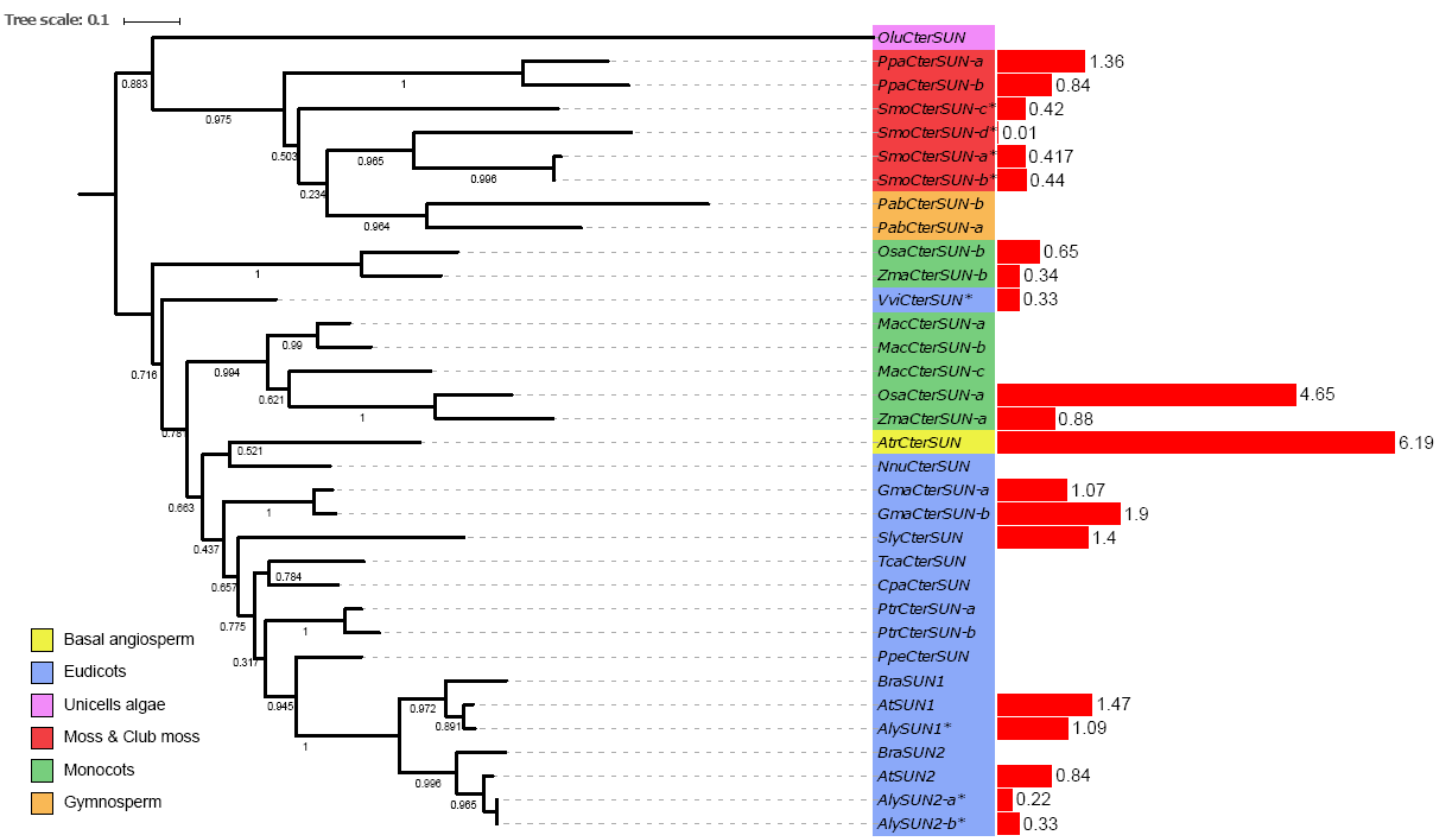
612 **Figure 8: Phylogenetic tree of KAKU4 proteins.**

613 Legend as Figure 2.



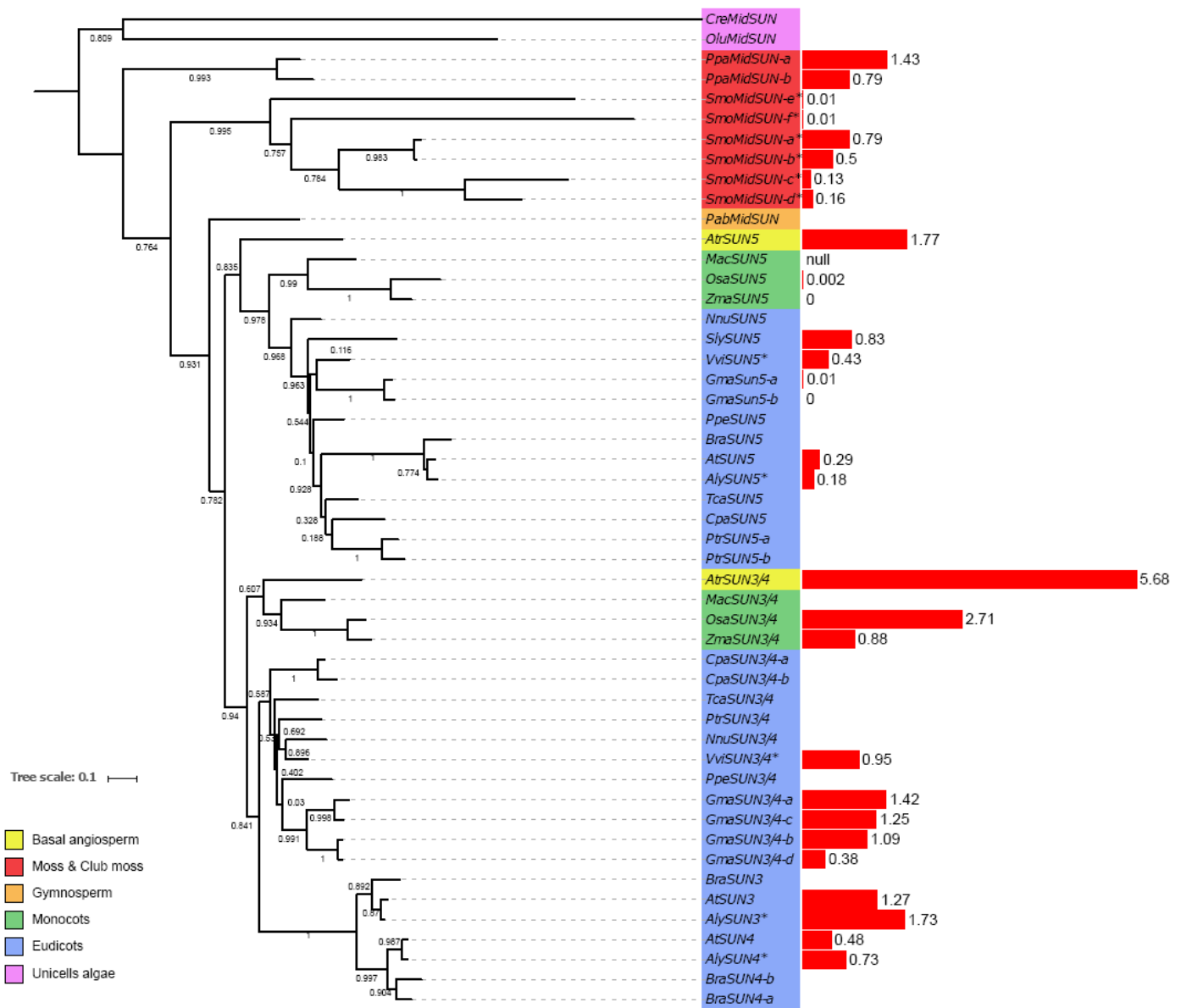
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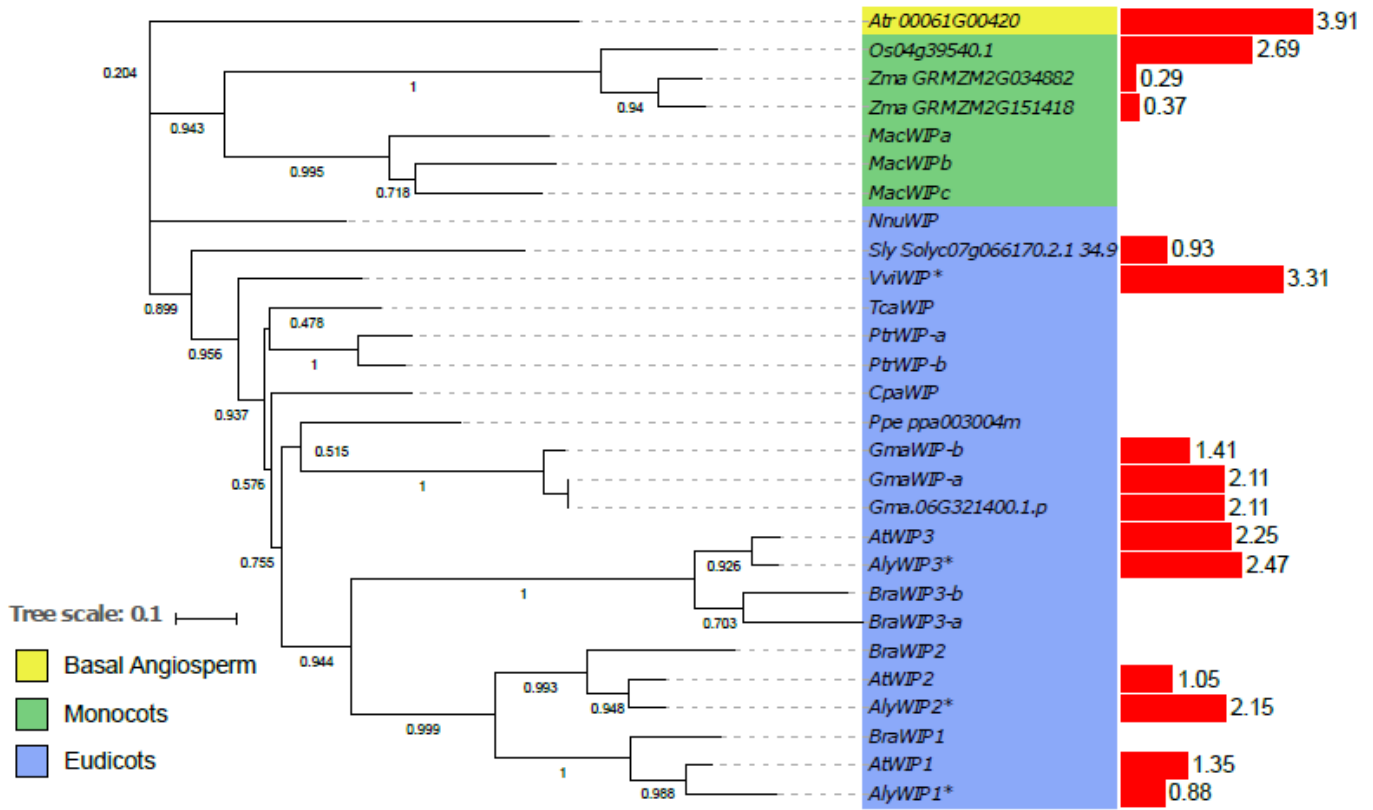


**Figure 2: Phylogenetic tree of Cter-SUN proteins and gene expression levels.**

**Left:** maximum likelihood tree of Cter-SUN protein homologues constructed from an alignment. Bootstrap values are presented. The colour of the label shows the lineage of the plant. The gene label is constructed with the three letters from the species name (supplementary Table 4) and the gene name of the *A. thaliana* homologues. **Right:** red bar represents the value of the transcription level in seedlings expressed in RPKM, except for species indicated by \*, the RNA-seq data was obtained from leaf tissue (Supplementary Table 2).

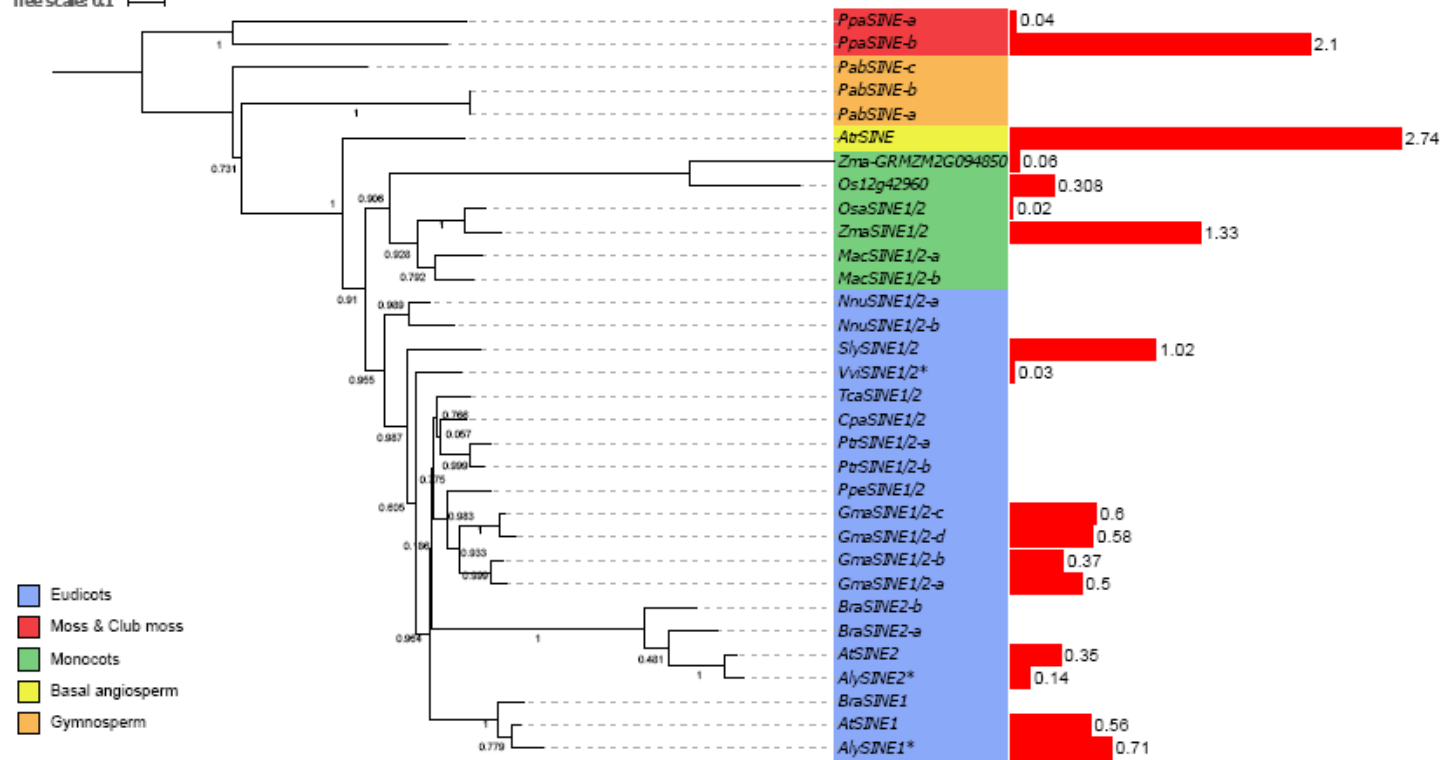


**Figure 3: Phylogenetic tree of Mid-SUN proteins.**  
 Legend as in Figure 2.



**Figure 4: Phylogenetic tree of WIP proteins.**  
 Legend as in Figure 2.

Tree scale: 0.1



**Figure 5: Phylogenetic tree of SINE1, SINE2 homologues proteins.**

Legend as in Figure 2.

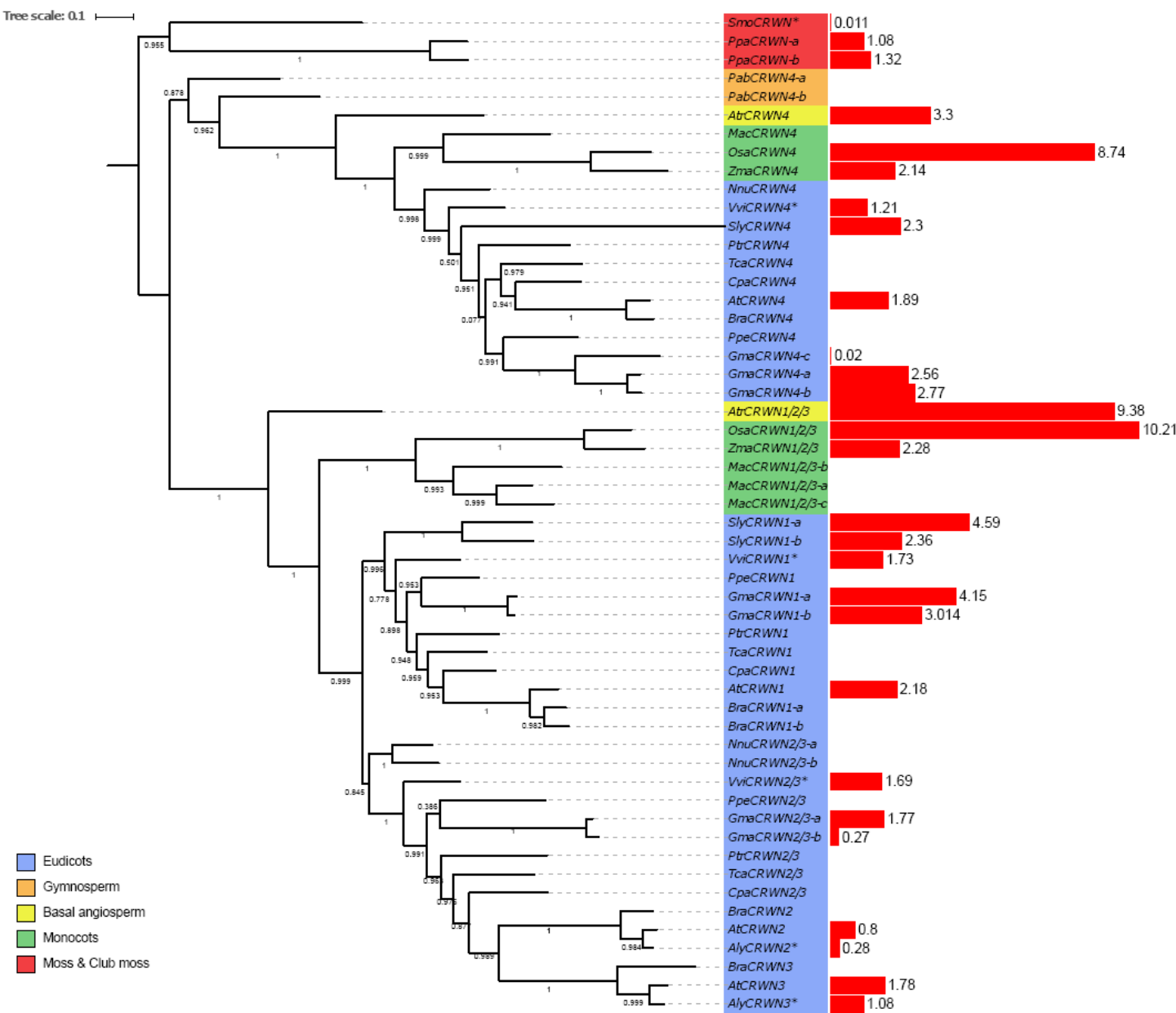
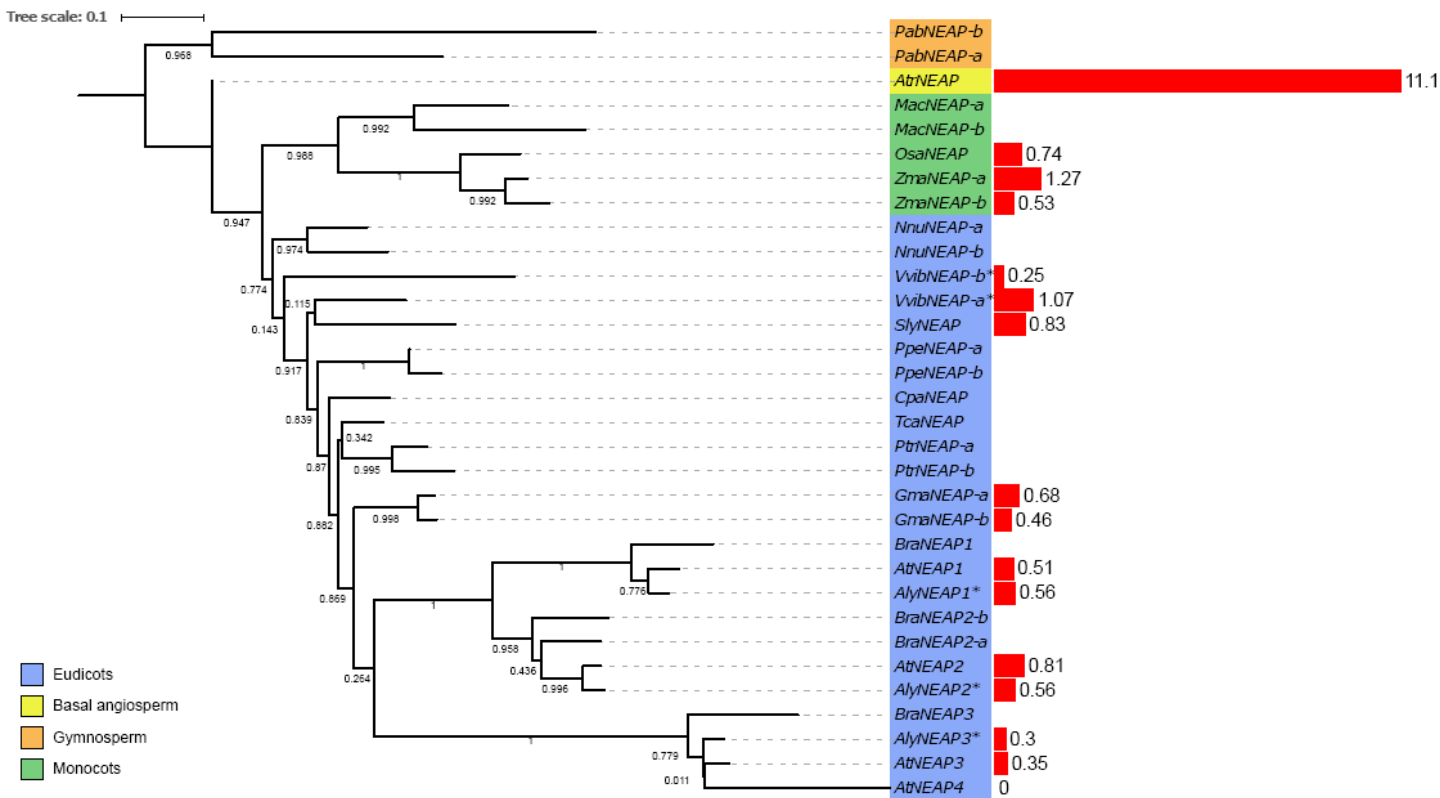


Figure 6: Phylogenetic tree of CRWN proteins.

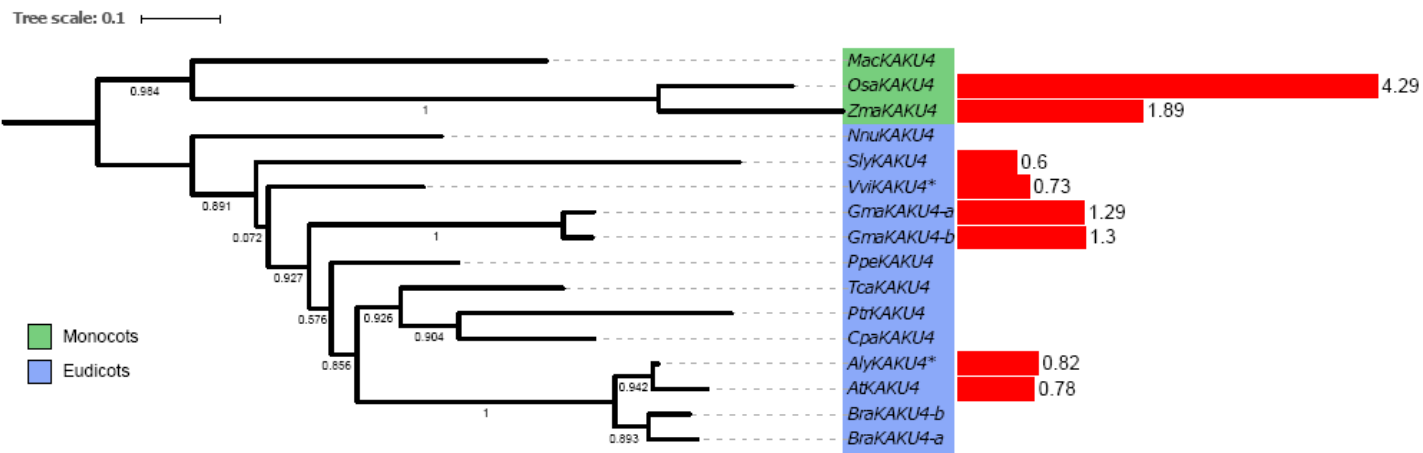
Legend as in Figure 2.





**Figure 7: Phylogenetic tree of NEAP proteins.**

Legend as in Figure 2.



**Figure 8: Phylogenetic tree of KAKU4 proteins.**  
 Legend as in Figure 2.