博士論文（要約）

論文題目　Study on evolutionary genetics of early morphological evolution in land plants

（陸上植物形態の初期形態進化に関する進化遺伝学的解析）

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Chapter 1.

General Introduction

Land plants originated from the charophyte algae about 450 million years ago (Langdale and Harrison, 2008), and began to diverge in the Silurian and Devonian periods. Land plants consist of two major lineages: Tracheophyta (vascular plants) and Bryophyta (non-vascular plants). Bryophytes, comprising hornworts, mosses, and liverworts, are the early diverging lineages of land plants. The haploid gametophyte is the most familiar part of the life cycle of bryophytes, and the diploid sporophyte is a short stalk without branching that has not been observed in other land plants. Lycophyta was presumed to be a monophyletic group, and one of the earliest diverged lineages of tracheophyta about 400 million years ago (Banks, 2009). Leaves (microphylls and megaphylls) and roots are thought to have evolved independently in the lycophyte and in other vascular plants. Judging from the evolutionary history of land plants, bryophytes and lycophytes have retained some specific features. Thus, these two lineages are worth investigating to obtain answers to the following questions. How were early land plants, bryophytes and/or lycophytes, established? Which genes participated in this evolution? What part in the gene sequence was modified during this evolution?

The evolution of morphology among each lineage in land plants is inferred from the comparison between similarities and differences in the genome structure, gene function, gene system, and so on (Floyd and Bowman, 2007). Many characteristics in plants are estimated to have originated through gene duplication (Moore et al., 2005; Banks et al., 2011). This increase in genes allows for amino acid changes and obtaining new functions, and lineage-specific gene duplication also contributes to lineage divergence and the origins of lineage-

specific features (Moore and Purugganan, 2005; Zhang, 2003). For example, the number of MADS-box genes, which are known to regulate some aspects of morphogenesis including floral organs, is 36 genes in *A. thaliana*, whereas *S. moellendorffii* and *P. patens* have only 3 and 6 genes, respectively. The number of AUX/IAA genes (a component of auxin signaling) is 29 genes in *A. thaliana*, whereas *S. moellendorffii* and *P. patens* have only 4 and 2 genes, respectively (Banks et al., 2011). It has been indicated that an increasing number of genes due to gene duplication leads to more complicated organization and function in land plans. Contrarily, early land plants that maintained a relatively uncomplicated organization and function might retain some peculiar gene functions and systems for specific features in each lineage, in addition to similar gene functions and systems. The function of orthologous genes might differ among angiosperms, bryophytes, and lycophytes as they differ so much in their development and morphology, following positive and purifying selection among paralogous genes (Moore and Purugganan, 2005; Zhang, 2003). Of course, not only different functions but also similar functions must be maintained between orthologous genes.

When we select a candidate gene for gene functional analysis from paralogous genes, the existence of many paralogous genes causes problems in the analysis of molecular biology. It is difficult to distinguish the function of each paralogous gene, and to judge which genes are essential for the study. For example, we cannot state that a target gene has the same function or no function (pseudogene) only from the information derived from the comparison of gene sequences among homologous genes (Ma et al., 1991; Kramer et al., 2004). Since the "molecular evolutionary clock" hypothesis was proposed by Zuckerkandl and Pauling (1962,

1965) and the "neutral theory of molecular evolution" hypothesis was proposed by Kimura (1968, 1983), molecular phylogenetics has progressed with advances in DNA sequencing and computational technologies, and the use of molecular phylogenetic trees has increased as a valuable tool for understanding the diversification of species and genomes (Kumar, 2005). In the study of angiosperms (Ferrario et al., 2003; Kim et al., 2005; Zimmermann and Werr, 2005), molecular phylogenetic analysis including functionally identified genes is a better method to indicate functional relationships between functionally identified genes and unknown genes among homologous genes. Ferrario et al. (2003) revealed that the orthologous gene that closely matched a functionally identified gene in *Arabidopsis* on the molecular phylogenetic tree had the same function as its orthologous gene by a complementation experiment in *Petunia*. In the study, the all genes isolated from *Petunia* were located within a clade including functionally identified genes of *Arabidopsis*, whereas the expressions of the paralogous genes belonging to other subgroups in the same clade were different. Therefore, it is possible to judge which gene would be important for functional analysis, because we could expect that genes in the same clade on a molecular phylogenetic tree will conserve a similar function. In contrast, the validity of this approach between long-distant lineages is uncertain, especially in morphogenetic genes, i.e. the genes to determine organ identity, pattern, and the form of plants. This is because the developmental and morphological differences are so great among bryophytes, lycophytes, and other vascular plants, and since the function of morphogenetic genes might be specialized in different plant lineages, even though the orthologous gene is in the same clade. We need to clarify that molecular phylogenetic analysis,

including functionally identified genes in angiosperms, is also a useful tool for the analysis of molecular biology in early land plants. For example, we will be able to predict that the genes in a clade consisting of angiosperms and bryophytes may retain a function common throughout the land plants and that there will be peculiar behavior in bryophytes if a clade consisting of only bryophytes is shown in the phylogenetic tree of homologous genes. Because we know little about the functions of genes in early diversified land plants and about whether the function of a gene identified in angiosperms is conserved between long-distant lineages (between bryophytes, lycophytes, and other vascular plants) or not (Floyd and Bowman, 2007; Singer and Ashton, 2007), the success of molecular phylogenetic analysis among long-distant lineages for gene functional analysis will be very useful in revealing functionally conserved genes in all land plants and the genes of peculiar to bryophytes and/or lycophytes from among many paralogous genes.

In this study, I will show that molecular phylogenetic analysis, including a gene whose function is known in some model angiosperms, is a better method to select candidate functional genes in early land plants. I demonstrated this approach using the *KNOX* gene family, the homeobox genes involved in shoot apical meristem (SAM) function, (Vollbrecht et al., 1991; Barton and Poethig, 1993) (Chapter 2) and the PIN gene family, i.e. the polar transporter of the plant signaling molecule (phytohormone) auxin (Okada et al., 1991; Gälweiler et al., 1998) (Chapters 3 and 4). I thought that these genes may be functionally conserved genes in all land plants, because SAM and auxin are critical to plant body establishment. At first, in order to reveal that molecular phylogenetic analysis is useful to

search for functionally conserved genes among angiosperms and lycophytes, I performed molecular phylogenetic analysis and gene expression analysis using the KNOX genes (Chapter 2). Next, in order to reveal that molecular phylogenetic analysis is also useful for the functional analysis of peculiar genes in early land plants, I performed molecular phylogenetic analysis including the bryophytes, lycophytes, gymnosperms, and angiosperms (Chapter 3) and gene expression analysis and functional analysis in bryophytes (Chapter 4) using the PIN genes.

Chapter 2.

The expression analysis of the *Selaginella* class 1 *KNOX* gene

2-1 Introduction

All land plants except bryophytes are thought to have evolved from a common dichotomously branching ancestor such as rhyniophytes identified in fossils. Regarding extant vascular plants, the lycophyte group (class Lycopsida) is the sister lineage of the euphyllophytes, which comprise seed plants and fern allies, including ferns, whisk ferns, and horsetails (Kenrick and Crane, 1997; Pryer et al., 2001). Based on the fossil record, the ancient tracheophyte group emerged approximately 400 million years ago (MYA) in the early Devonian, prior to the evolution of leaves and roots in vascular plants, and successfully dominated in the Carboniferous period (Stewart and Rothwell, 1993). The only three surviving modern-day plant orders of this ancient group, i.e., the Lycoposidales (clubmosses), Isoetales (quillworts), and Selaginellales (spikemosses), all of which are monophyletic, are placed in the lycophytes (Raubeson and Jansen, 1992; Kenrick and Crane, 1997). The remarkably common features of lycophytes are the distinguishable microphylls, which are poorly developed, single-veined leaves without a leaf gap, in contrast to the euphylls of ferns and seed plants.

The spikemosses, which are composed of the single genus *Selaginella*, are heterosporous and herbaceous lycophytes. Approximately 700 species belonging to this genus are distributed worldwide (Wochok and Sussex, 1975). In addition to microphylls, *Selaginella* have a unique rhizophore, which is defined as a root-producing, positively geotropic, leafless, and capless axis. For about a century, this axial organ has been a

histologically controversial structure, which had been interpreted as an aerial root or stem-like root (Webster and Jagels, 1977; Gifford and Foster, 1989). However, Imaichi and Kato (1989, 1991) revealed that the exogenous developmental process of the rhizophore is clearly distinguishable from the developmental process of the endogenous root. They demonstrated that the *Selaginella* rhizophore, like the lepidodendrid rhizomorph, could be fundamental axial organ coordinated with the roots and stems.

Molecular genetic and genomic studies have successfully generated much information on the genetic networks that control organ differentiation in higher plants; however, the ancient vascular plant lineage has been little studied at the molecular level, except for the construction of bacterial artificial chromosome (BAC) and expressed sequence tag (EST) libraries (Wang et al., 2005; Weng et al., 2005). The orthologous genes of key regulator genes that are involved in shoot apical meristem and/or lateral architecture differentiation in higher plants could reveal new characters concerning the unique *Selaginella* appendages.

As a first step in examining the molecular characteristics of the spikemoss pleurogeous organs, I focused on the *Knotted1*-like homeobox (*KNOX*) gene family, which was the first homeobox gene identified in plants (Hake et al., 1989; Vollbrecht et al., 1991). Members of the *KNOX* gene family are divided phylogenetically into two classes in land plants, class 1 and 2. Class 1 *KNOX* genes are typically expressed only in the shoot apical meristem, whereas class 2 *KNOX* genes have more diverse expression patterns (Bharathan et al., 1997; Reiser et al., 2000). In simple-leaved angiosperms such as maize, rice,

Arabidopsis, tobacco, and snapdragon, class 1 *KNOX* genes are expressed preferentially in shoot apical meristems and are negatively regulated by *ASYMMETRIC LEAVES1/ROUGHSHEATH2/PHANTASTICA* (*ARP*) genes in the domains in which leaves are expected to develop (Schneeberger et al., 1998; Byrne et al., 2000). In complex-leaved plants such as tomato, class 1 *KNOX* genes are expressed later in leaf development (Bharathan et al., 2002). The loss of the *Arabidopsis SHOOTMERISTEMLESS* (*STM*) gene, a well-characterized class 1 *KNOX* gene, induces a shoot meristem deficiency in the developmental process (Long et al., 1996). The fern *Ceratopteris* class 1 KNOX genes show expression patterns similar to those of their angiosperm counterparts, except in initial leaf formation (Sano et al., 2005). Thus, class 1 *KNOX* genes regulate shoot meristem and leaf formation. In a recent study of class 1 *KNOX* genes in *Selaginella kraussiana*, leaf development was regulated by the interaction of class 1 *KNOX* and *ARP* genes, similar to that in euphyllophytes (Harrison et al., 2005).

Here, I report the class 1 *KNOX* gene expression in *Selaginella uncinata*, with particular attention to the *Selaginella* rhizophore concept at the molecular level.

2-2 Materials and Methods

2-2-1 Cloning of *Selaginella KNOX* genes

Selaginella uncinata (Desv.) Spring was collected at the Koishikawa Garden of the University of Tokyo, Tokyo, Japan (Fig. 1). Total RNA extraction from various organs and 3' and 5' RACE were performed as described by Tanabe et al. (2003). The materials were ground in liquid nitrogen and dissolved completely in extraction buffer (4 M guanidine thiocyanate, 1 M ammonium thiocyanate, 1% lauryl sarcosine, 0.5% PVP, and 1% 2-mercaptoethanol). After three chloroform / isoamyl alcohol (24:1) extractions, the nucleic acids were precipitated in ethanol. The extracted RNA was purified by CTAB precipitation, followed by lithium chloride precipitation or ISOGEN-LS treatment (Nippon Gene).

Complementary DNA was synthesized from the total RNA according to the instructions of the 3' RACE system kit using SuperScript II reverse transcriptase and the universal primer (Invitrogen). Specific degenerate primers were designed to target class 1, KNd41 (5'-$\{CAU\}_4$ AAR AAR AAR GGI AAR YTN CC-3') , and all KNOX genes, KNd2 (5'-$\{CAU\}_4$ AAY AAY TGG TTY ATH AAY CAR MG-3'. The PCR conditions were an initial step at 94°C for 1 min; 35 cycles at 94°C for 1 min, 52°C for 1 min, and 72°C for 1.5 min; and a final step at 72°C for 5 min. The amplified products were cloned into the pAMP1 vector (Gibco-BRL) according to the manufacturer's instructions. The cloned products were sequenced using the DNA Analysis System (Beckman Coulter), using a Dye Terminator Cycle Sequencing kit (Beckman Coulter). The 5' regions of the cloned genes

were also isolated according to the instructions of the 5' RACE system kit (Invitrogen) and then sequenced.

2-2-2 Phylogenetic analysis

To construct a phylogenetic tree of *KNOX* genes, the amino acid sequences shown in Figure 2 were obtained from EMBL/DDBJ/GenBank DNA databases (Table 1) and aligned using the program Clustal W, version 1.8 (Thompson et al., 1994). The maximum likelihood (ML) distances were calculated using the ProtML program with the Jones, Taylor, and Thornton (JTT) model (Jones et al., 1992), and a neighbor-joining (NJ) tree was obtained using the program NJdist (Adachi and Hasegawa, 1992–1996). The trees were analyzed further with a local rearrangement search using the program ProtML to obtain the ML tree. Bootstrap values calculated using the resampling of estimated log-likelihoods (RELL) method (Hasegawa and Kishino, 1994) are indicated on nodes reconstructed using both the ML and NJ methods.

2-2-3 RT-PCR expression analysis

To perform the RT-PCR expression analysis, complementary DNA was synthesized from total RNA extracted from apical tips, including microphyll buds, internodes, microphylls, root tips, and rhizophore tips, as described above. The PCR conditions were an initial step at 95°C for 5 min; 40 cycles at 95°C for 15 s, 52°C for 15 s, and 72°C for 1 min; and a final step at 72°C for 7 min. The PCR amplification test was performed with

SuKNOX1-specific internal primers, SuKN1F1 (5'-ATCACCTGAGGTAGCCACAGTTGA-3') and SuKN1R1 (5'-AAAAGGATCAATCTCAAACTCCA-3'). The *S. uncinata* orthologous gene of the *ribosomal protein L6* gene (*SuRPL6*, DDBJ Accession No. AB521036), which was constitutively expressed in all tissues that I examined, was used as a quantifying control. *SuRPL6* was amplified by PCR with forward SuRPL6F1 (5'-CGTCAACCAGGCGTACGTGAT-3') and reverse SuRPL6R1 (5'-GGACTCGGTCTGCTCGATGA-3') primers. The partial fragments of *SuKNOX1* amplified by internal primers were cloned and used as specific probes for Southern hybridization. Southern hybridization was performed according to the instructions of the AlkPhos Direct Labelling and Detection System (GE Healthcare). The amplified products were fractionated on 1% (w/v) agarose gel and transferred to Hybond N+ nitrocellulose membranes (GE Healthcare) in an alkali transfer buffer (0.008 N NaOH). The membranes were hybridized at 55°C for 16 h in hybridization buffer (GE Healthcare).

2-2-4 *In situ* hybridization

Apical tips and parts producing rhizophores were collected in fixation buffer (4% paraformaldehyde and 50 mM sodium phosphate), dehydrated with tertiary butyl alcohol, and embedded in Paraplast Plus (Oxford Labware). Sections 8 μm thick were prepared. The procedure for in situ hybridization followed that of Jack et al. (1992) using the digoxigenin (DIG)-labeled *SuKNOX1*-specific RNA probe and a DIG RNA labeling kit (Roche), following cloning of the 565 bp of partial *SuKNOX1* cDNA outside of the well-conserved

ELK [glutamic acid (E), leucine (L), and lysine (K)] and homeo domains. The sense strand of the *SuKNOX1* mRNA was used as a negative control. The incorporation of DIG-labeled uridine triphosphate (UTP) into RNA was accompanied by the synthesis of RNA with T7 or SP6 RNA polymerase. Hybridization was performed at 46 °C for 16 h in hybridization buffer (100 mM NaCl, 10 mM Tris-HCl, 10 mM sodium phosphate buffer [pH 6.8], 5 mM EDTA, 50% formamide, 1 mM DTT, 1 mg/ml tRNA, 10% dextran sulfate, and 0.5 U/ml RNase inhibitor). After the washing steps, the signal was detected using a DIG detection kit (Roche).

2-3 Results

2-3-1 Cloning of *Selaginella KNOX* genes and phylogenetic analysis

I examined the relatively large spikemoss *Selaginella uncinata* because this species provides advantages for histological studies. The *Selaginella KNOX* cDNA, named *SuKNOX1* (DDBJ Accession No. AB288208, Fig. 3A), was isolated from *S. uncinata* cDNA using 3' and 5' RACE methods. The *KNOX* cDNA clone was amplified successfully using the described PCR conditions, using the degenerate primer that targets the class 1 *KNOX*-specific sequence KNd41. The deduced amino acid sequence of the *SuKNOX1* contained a MEINOX domain [the amalgam of the animal Myeloid ecotropic viral integration site 1 (MEIS) and plant KNOX domain], ELK domain, and TALE (three amino acid loop extension) type homeo domain with three additional amino acid residues between helix 1 and 2 (Bertolino et al., 1995), all of which are hallmarks of *KNOX* genes (Fig. 3 and Fig. 4). In addition, I successfully amplified a class 2 *KNOX* gene, named *SuKNOX2* (DDBJ Accession No. AB288209, Fig. 3B), using the degenerate primer KNd2, which corresponds to the best-conserved homeobox region. The KNd2 primer can be used to clone both class 1 and 2 *KNOX* genes, but no class 1 *KNOX* genes were amplified, except *SuKNOX1*.

To address the evolutionary relationship between *SuKNOX1* and class 1 *KNOX* genes of land plants, I constructed an ML gene tree that contained 33 *KNOX* genes from a wide range of green plants, including seed plants, ferns, spikemosses, mosses, and ulvophyceans. Metazoan genes were used as an outgroup (Burglin, 1998). The tree was

constructed using 83 amino acid residues covering the ELK and homeo domains (Fig. 2 and Fig. 4, underlined). No reliable alignment was obtained when the set included the MEINOX domain because of the low sequence similarity between metazoan and plant genes; thus, the MEINOX domain was excluded from the alignment data for the phylogenetic analysis. The gene tree indicates that *S. uncinata SuKNOX1* is clearly placed within the class 1 clade and that *SuKNOX2* was placed within the class 2 clade, with high statistical confidence (Fig. 5). Relationships among *S. uncinata SuKNOX1*, *S. kraussiana SkKNOX1* and *2* (Harrison et al., 2005), and the class 1 genes of other vascular plants were not resolved reliably. The gene tree predicts that *S. uncinata* likely possesses another class 1 *KNOX* gene(s) in its genome.

2-3-2 Expression of *SuKNOX1* mRNA

Selaginella has creeping or ascendant stems with leafy microphylls, which are produced by a dome-shaped shoot apex having an apical cell. Rhizophores are initiated exogenously at the junctions of branching stems and endogenously give rise to roots (Imaichi and Kato, 1991). The expression pattern of *SuKNOX1* was investigated using *in situ* hybridization. The longitudinal section shows that *SuKNOX1* mRNA was clearly localized around the shoot apical meristem, including the shoot apical cell (Fig. 6A). No *SuKNOX1* expression was detected in microphylls, although there was weak detection in juvenile microphyll primordia (Fig. 6A). To examine *SuKNOX1* expression in the rhizophores, I prepared longitudinal sections containing the junctions of branching stems (Fig. 6C). These showed clear *SuKNOX1* mRNA accumulation in the outer layer around the

young rhizophore apex and weaker accumulation in the associated vascular bundles. *SuKNOX1* expression in the rhizophore apex was confirmed in another section (Fig. 6E).

I also examined the *SuKNOX1* expression pattern using RT-PCR (Fig. 6F). *SuKNOX1*-specific amplification was detected in the shoot apical tips of microphylls and in rhizophore tips, whereas it was generally not detected in the internodes, microphylls, or root tips. *SuKNOX1* amplification was infrequently detected in the internodes and root tips (data not shown). These results were in accordance with those of in situ hybridization. Unfortunately, I could not examine *SuKNOX1* expression patterns in the gametophyte because I did not have sufficient samples for mRNA extraction. Further assessment of *KNOX* gene expression in the gametophyte will be necessary to infer the comprehensive functions of these genes in the *Selaginella* life cycle.

2-4 Discussion

I characterized the *SuKNOX1* cDNA from the spikemoss *Selaginella uncinata*. Based on the gene tree, *SuKNOX1* is clearly included within the class 1 *KNOX* gene cluster containing *Arabidopsis STM*, *Knotted1-like from Arabidopsis thaliana 1* (*KNAT1*), and *2* (*KNAT2/ATK1*) genes. As reported by Serikawa and Mandoli (1999), *Acetablaria acetablum AaKNOX1*, a *KNOX* gene from an ulvophycean green alga, branched out before the divergence of class 1 and 2 genes. The moss *Physcomitrella* and the fern *Ceratopteris* possess both class 1 and 2 genes (Champagne and Ashton, 2001; Sano et al., 2005), indicating the appearance of the two large *KNOX* gene groups prior to the terrestrialization of green plants approximately 470 MYA (Kenrick and Crane, 1997). Therefore, further studies of *KNOX* genes from charophycean algae, the closest relatives to land plants, will shed light on the ancient split event.

Most class 1 *KNOX* genes exhibit similar expression in shoot apical meristems despite differences in the meristem structures among the diversified euphyllophytes, which include seed plants and ferns. In the euphyllophytes, multicellular and unicellular meristems are regulated similarly by class 1 *KNOX* genes during development (Sano et al., 2005). Lycophytes are the most ancient of the modern vascular plants, branching at approximately 400 MYA, and possess unicellular meristems that are thought to have originated independently in this lineage (Pryer et al., 2001; Sano et al., 2005). With respect to leaf formation, the expression of *KNOX* genes in seed plants is negatively regulated by *ARP*

genes during leaf development, with notable exceptions observed in the compound leaves of tomato (Hareven et al., 1996; Janssen et al., 1998), whereas class 1 *KNOX* genes are expressed in the primordia and immature leaves in the ferns *Ceratopteris* and *Anagramma*, suggesting differences in the leaf developmental mechanisms between seed plants and ferns (Sano et al., 2005). According to paleobotanical evidence and molecular phylogenetic reports of early land plants, seed plant leaves, fern fronds, and spikemoss microphylls originated in parallel (Gifford and Foster, 1989; Stewart and Rothwell, 1993; Kenrick and Crane, 1997; Pryer et al., 2001). Recently, Harrison et al. (2005) reported the detailed expression patterns of the *Selaginella kraussiana* class 1 *KNOX* genes, *SkKNOX1* and *2*, in the shoot apical meristem and in leaf formation. *SkKNOX1* is specifically expressed in the shoot apical meristem, whereas *SkKNOX2* is preferentially expressed in the internode regions; neither is expressed in microphyll primordia. I found clear expression of the *S. uncinata SuKNOX1* in the shoot apex, supporting the evidence that class 1 *KNOX* genes have conserved functions in the primitive sporophytic apices. Reportedly, *SkKNOX1* and *2* originally diverged in the *Selaginella* lineage (Harrison et al., 2005). However, according to my gene trees based on ML and NJ methods, the phylogenetic relationships among *S. uncinata SuKNOX1*, *S. kraussiana SkKNOX1* and *2*, and other vascular plant class 1 genes were not resolved fully, suggesting that the common ancestor of lycophytes and euphyllophytes, a rhyniophyte, possessed at least two or more class 1 genes in the genome.

I also detected *SuKNOX1* expression in the axial apex of the unique spikemoss rhizophore, but not in the root tips. Meanwhile, the expression patterns of *SkKNOX1* and *2*

were not reported by Harrison et al (2005). My expression data suggest that the rhizophore has developmental mechanisms distinct from those of the root. This finding supports the "rhizophore concept" at the molecular level, which postulates that the rhizophore is a fundamental organ distinguishable from the root (Imaichi and Kato, 1989; 1991). Further molecular studies of other developmentally critical regulator homologous genes such as *homeodomain-leucine zipper* (*HD-Zip*) genes and *NO APICAL MERISTEM* (*NAM*)/ *ATAF*/ *CUP-SHAPED COTYLEDON* (*CUC*) (*NAC*) genes will reveal additional characters of *Selaginella* appendages. It was found that *Selaginella class III HD-Zip* gene expression predicts organ initiation site, similar to that of *Arabidopsis* orthologous genes; however, there have been no reports in rhizophores (Floyd et al., 2006; Prigge et al., 2006). A BAC library containing ten genome-equivalents and ESTs containing 1301 non-redundant clones have recently been constructed for *S. moellendorffii* (Wang et al., 2005; Weng et al., 2005). Further studies using genomic resources to construct libraries for other large-sized *Selaginella* species, as well as studies using microarray techniques, will reveal the gene expression profiles of spikemoss pleurogenous architectures, which will aid in depicting evolutionary scenarios for early land plants.

Chapter 5.

General Discussion

It is unclear whether the function of morphogenetic genes identified in angiosperms is conserved between long-distant lineages (e.g., between bryophytes, lycophytes, and other vascular plants) and, especially, which genes are responsible for some traits specific to the bryophytes, lycophytes, and angiosperms (Floyd and Bowman, 2007; Singer and Ashton, 2007; Rensing et al., 2008; Okano et al., 2009; Banks et al., 2011). Because of this redundancy, pseudogenization, subfunctionalization, and neofunctionalization events occurred following gene duplication over a long period of time during evolution (Zhang, 2003; Moore and Purugganan, 2005). It is difficult to identify the gene responsible for the birth of a new trait only by functional analysis. It is also a question whether molecular phylogenetic analysis including morphogenetic genes, whose function is known in some model plants of angiosperms, is a better method to select candidate genes for the responsible genes in early land plants, or not. For example, in gymnosperms, the function of the class B and class C MADS-box genes could partially or fully substitute for their angiosperm orthologous genes in complementation and ectopic expression experiments. This revealed that these gene functions were conserved over more than 300 million years of seed plant evolution, because extant gymnosperms and angiosperms diverged more than 300 million years ago (Sundström and Engström, 2001; Winter et al., 2002; Zhang et al., 2004). This result was consistent with the results of gene phylogenetic analysis. Similarly, in lycophytes and bryophytes, if the selection of genes based on molecular phylogenetic analysis can succeed in functional analysis, such an approach makes a contribution to the study of plant evolutionary genetics. I addressed these questions at the

beginning of the present study, and the results presented in this thesis could serve as a framework for future studies in order to reveal gene function in early land plants.

In Chapter 2, the *SuKNOX1* gene was expressed in the shoot apical meristem but not in the root meristem, as estimated by the molecular phylogenetic tree of *KNOX* genes (Fig. 5 and Fig. 6). The expression of the *SuKNOX1* gene was also detected in the rhizophore meristem (Fig. 6). This suggests that the rhizophore is an organ with stem characteristics, not root characteristics. It was revealed that the *SuKNOX1* gene participates in the development of the rhizophore, which is the peculiar organ to lycophyte. In this study, I indicated that the function of the class 1 *KNOX* gene, expressed in SAM, but not in the root meristem, might be conserved between lycophytes and angiosperms. Here, molecular phylogenetic analysis including the gene identified in angiosperms was shown to be a useful method to select a candidate gene for the functional analysis of molecular biology, also in early land plants. In the present study, two *KNOX*-like genes were isolated from *S. uncinata*. The phylogenetic tree of *KNOX* genes indicated that the *SuKNOX1* and *SuKNOX2* genes belong to class 1 and class 2, respectively (Fig. 5). Most of the class 1 *KNOX* genes exhibit a similar expression pattern in SAM in vascular plants, whereas class 2 *KNOX* genes have more diverse expression patterns (Bharathan et al., 1997; Reiser et al., 2000; Harrison et al., 2005; Sano et al., 2005). The analysis of the expression pattern of the class 2 *SuKNOX2* gene will likely give us a greater understanding of the mechanism of morphogenesis in *S. uncinata* and of the validity of molecular phylogenetic analysis to select a candidate functional gene.

Genes as a subject of research in functional analyses are generally selected based on an expectation of function which is known in some model plant of the angiosperms. In my thesis, the study of the *KNOX* gene family in lycophytes followed this approach. However, the *PpPIND* gene, whose function is probably unknown in some model angiosperms, was selected in expectation of a bryophyte-specific function according to the results of molecular phylogenetic analysis. As a consequence, I suggest that molecular phylogenetic analysis is broadly helpful for functional analysis, not only for functionally conserved genes, but also for functionally peculiar genes in early land plants. Thus, the molecular phylogenetic analysis including a gene whose function is known in some model angiosperm plant would be helpful in the functional analysis of both of functionally conserved and unknown genes in early land plants. In addition, it will be interesting and important if the functional redundancy between clades discriminated by clearly different function exists in bryophytes.

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Table 1. *KNOX* genes

Accession no.	Gene name	Species	Fmily	class	Subdivision	Division
U32344	STM	*Arabidopsis thaliana*	Brassicaceae (Mustard)	Dicotyledoneae (Eudicot)	Angiospermae (Angiosperm)	Spermatophyta (Seed plant)
AF482995	KNAT1	*Arabidopsis thaliana*	Brassicaceae (Mustard)	Dicotyledoneae (Eudicot)	Angiospermae (Angiosperm)	Spermatophyta (Seed plant)
X81353	KNAT2/ATK1	*Arabidopsis thaliana*	Brassicaceae (Mustard)	Dicotyledoneae (Eudicot)	Angiospermae (Angiosperm)	Spermatophyta (Seed plant)
AY086091	KNAT3	*Arabidopsis thaliana*	Brassicaceae (Mustard)	Dicotyledoneae (Eudicot)	Angiospermae (Angiosperm)	Spermatophyta (Seed plant)
X92393	KNAT4	*Arabidopsis thaliana*	Brassicaceae (Mustard)	Dicotyledoneae (Eudicot)	Angiospermae (Angiosperm)	Spermatophyta (Seed plant)
AF306661	KNAT5	*Arabidopsis thaliana*	Brassicaceae (Mustard)	Dicotyledoneae (Eudicot)	Angiospermae (Angiosperm)	Spermatophyta (Seed plant)
AB072361	KNAT6	*Arabidopsis thaliana*	Brassicaceae (Mustard)	Dicotyledoneae (Eudicot)	Angiospermae (Angiosperm)	Spermatophyta (Seed plant)
AF308451	KNAT7	*Arabidopsis thaliana*	Brassicaceae (Mustard)	Dicotyledoneae (Eudicot)	Angiospermae (Angiosperm)	Spermatophyta (Seed plant)
X61308	KN1	*Zea mays*	Poaceae (Grass)	Monocotyledoneae (Monocot)	Angiospermae (Angiosperm)	Spermatophyta (Seed plant)
AAA86287	RS1	*Zea mays*	Poaceae (Grass)	Monocotyledoneae (Monocot)	Angiospermae (Angiosperm)	Spermatophyta (Seed plant)
AF100455	LG3	*Zea mays*	Poaceae (Grass)	Monocotyledoneae (Monocot)	Angiospermae (Angiosperm)	Spermatophyta (Seed plant)
AF457118	LG4a	*Zea mays*	Poaceae (Grass)	Monocotyledoneae (Monocot)	Angiospermae (Angiosperm)	Spermatophyta (Seed plant)
P56659	KNOX1	*Zea mays*	Poaceae (Grass)	Monocotyledoneae (Monocot)	Angiospermae (Angiosperm)	Spermatophyta (Seed plant)
AAB33488	KNOX4	*Zea mays*	Poaceae (Grass)	Monocotyledoneae (Monocot)	Angiospermae (Angiosperm)	Spermatophyta (Seed plant)
P56663	KNOX5	*Zea mays*	Poaceae (Grass)	Monocotyledoneae (Monocot)	Angiospermae (Angiosperm)	Spermatophyta (Seed plant)
AAB33490	KNOX6	*Zea mays*	Poaceae (Grass)	Monocotyledoneae (Monocot)	Angiospermae (Angiosperm)	Spermatophyta (Seed plant)
P56666	KNOX8	*Zea mays*	Poaceae (Grass)	Monocotyledoneae (Monocot)	Angiospermae (Angiosperm)	Spermatophyta (Seed plant)
AAB33489	KNOX10	*Zea mays*	Poaceae (Grass)	Monocotyledoneae (Monocot)	Angiospermae (Angiosperm)	Spermatophyta (Seed plant)
U90091	SKN1	*Picea mariana*	Pinaceae (Pine)	Coniferopsida	Gimnospermae (Gymnosperm)	Spermatophyta (Seed plant)
U90092	SKN2	*Picea mariana*	Pinaceae (Pine)	Coniferopsida	Gimnospermae (Gymnosperm)	Spermatophyta (Seed plant)
AAV63997	PmKN3	*Picea mariana*	Pinaceae (Pine)	Coniferopsida	Gimnospermae (Gymnosperm)	Spermatophyta (Seed plant)
AB043954	CRKNOX1	*Ceratopteris richardii*	Adiantaceae	Pteridopsida		Pteridophyta (fern)
AB043956	CRKNOX2	*Ceratopteris richardii*	Adiantaceae	Pteridopsida		Pteridophyta (fern)
AB043957	CRKNOX3	*Ceratopteris richardii*	Adiantaceae	Pteridopsida		Pteridophyta (fern)
AB288208*	SuKNOX1	*Selaginella uncinata*	Selaginellaceae (Spike moss)	Lycopodiopsida (Club moss)		Lycopodiophyta
AB288209*	SuKNOX2	*Selaginella uncinata*	Selaginellaceae (Spike moss)	Lycopodiopsida (Club moss)		Lycopodiophyta
AY667449	SkKNOX1	*Selaginella kraussiana*	Selaginellaceae (Spike moss)	Lycopodiopsida (Club moss)		Lycopodiophyta
AY667450	SkKNOX2	*Selaginella kraussiana*	Selaginellaceae (Spike moss)	Lycopodiopsida (Club moss)		Lycopodiophyta
AY667451	SkKNOX3	*Selaginella kraussiana*	Selaginellaceae (Spike moss)	Lycopodiopsida (Club moss)		Lycopodiophyta
AF285148	MKN1-3	*Physcomitrella patens* subsp. *patens*	Funariaceae	Bryopsida (moss)		Bryophyta
AF285147	MKN2	*Physcomitrella patens* subsp. *patens*	Funariaceae	Bryopsida (moss)		Bryophyta
AF284817	MKN4	*Physcomitrella patens* subsp. *patens*	Funariaceae	Bryopsida (moss)		Bryophyta
AF170172	AaKNOX1	*Acetabularia acetabulum*	Polyphysaceae	Ulvophyceae		Chlorophyta
CCD63401	CEH-20	*Caenorhabditis elegans*	Caenorhabditis	Secernentea		Nematoda
CAA92154	CEH-40	*Caenorhabditis elegans*	Caenorhabditis	Secernentea		Nematoda
BAA05957	PBX2	*Homo sapiens*	Hominidae	Mammalia	Vertebrata	Chordata
AAH94883	PBX3	*Homo sapiens*	Hominidae	Mammalia	Vertebrata	Chordata
CAC28212	PBX4	*Homo sapiens*	Hominidae	Mammalia	Vertebrata	Chordata

* is isolated from this study.

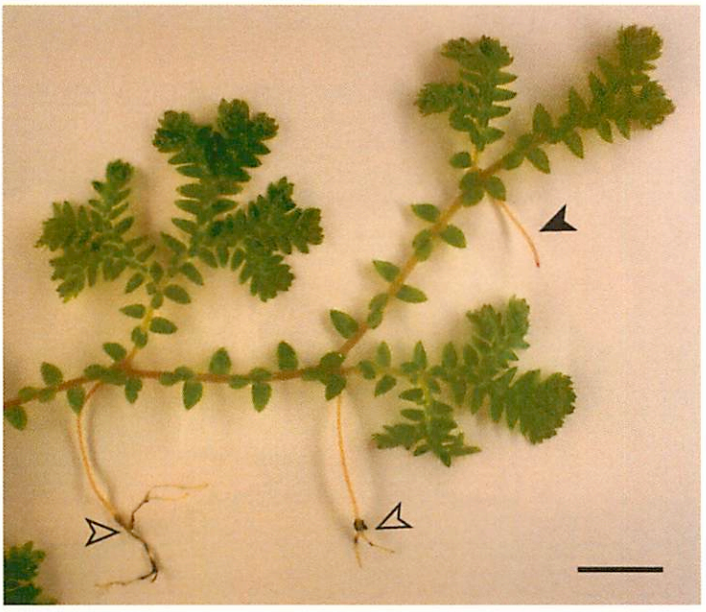
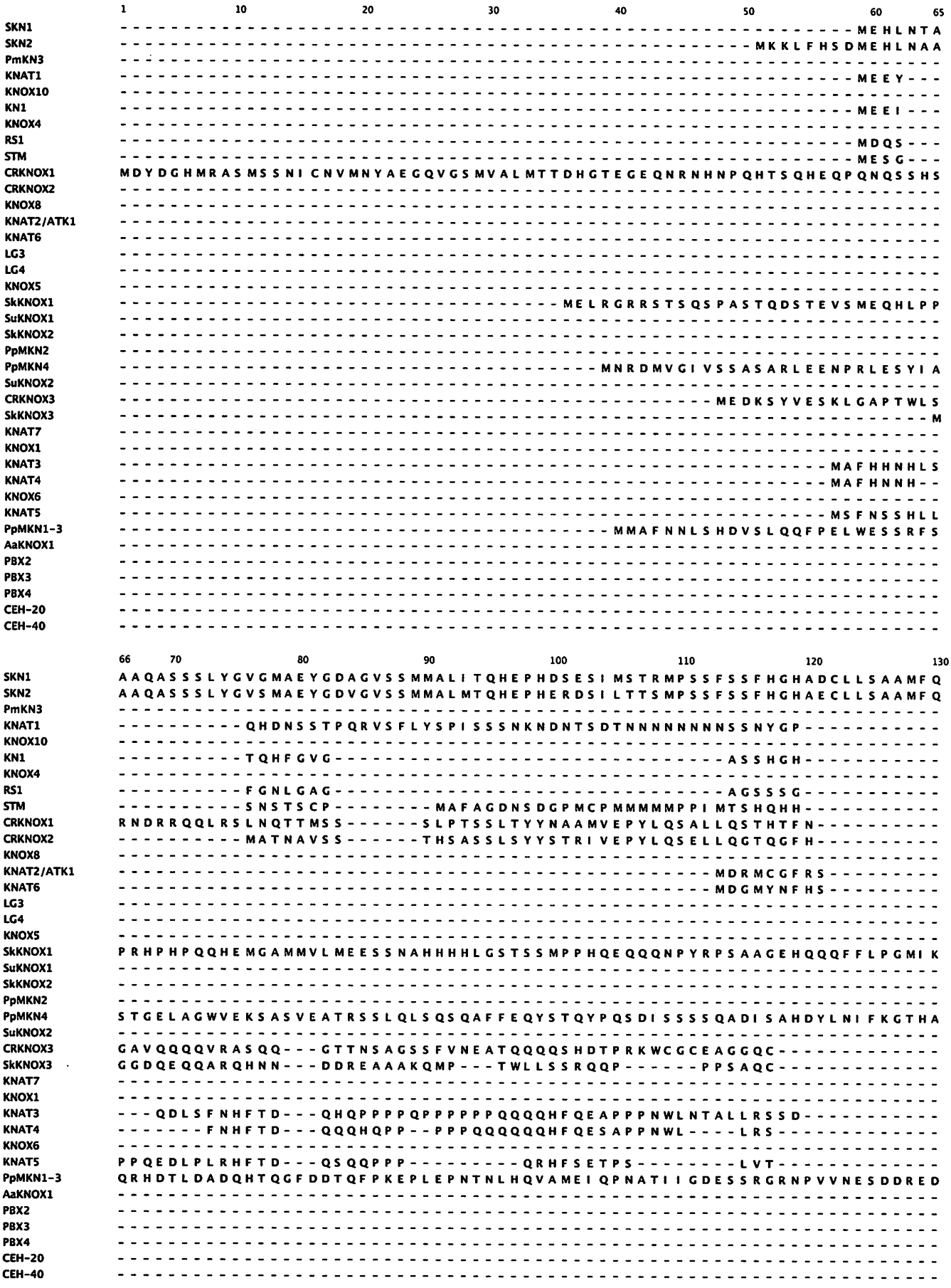


Fig. 1. A photograph of *Selaginella uncinata*.

The spikemoss *Selaginella uncinata* has creeping stems with leafy microphylls and rhizophores (closed arrowhead), which produce roots (opened arrowhead). Scale bar = 10 mm.

Figure 2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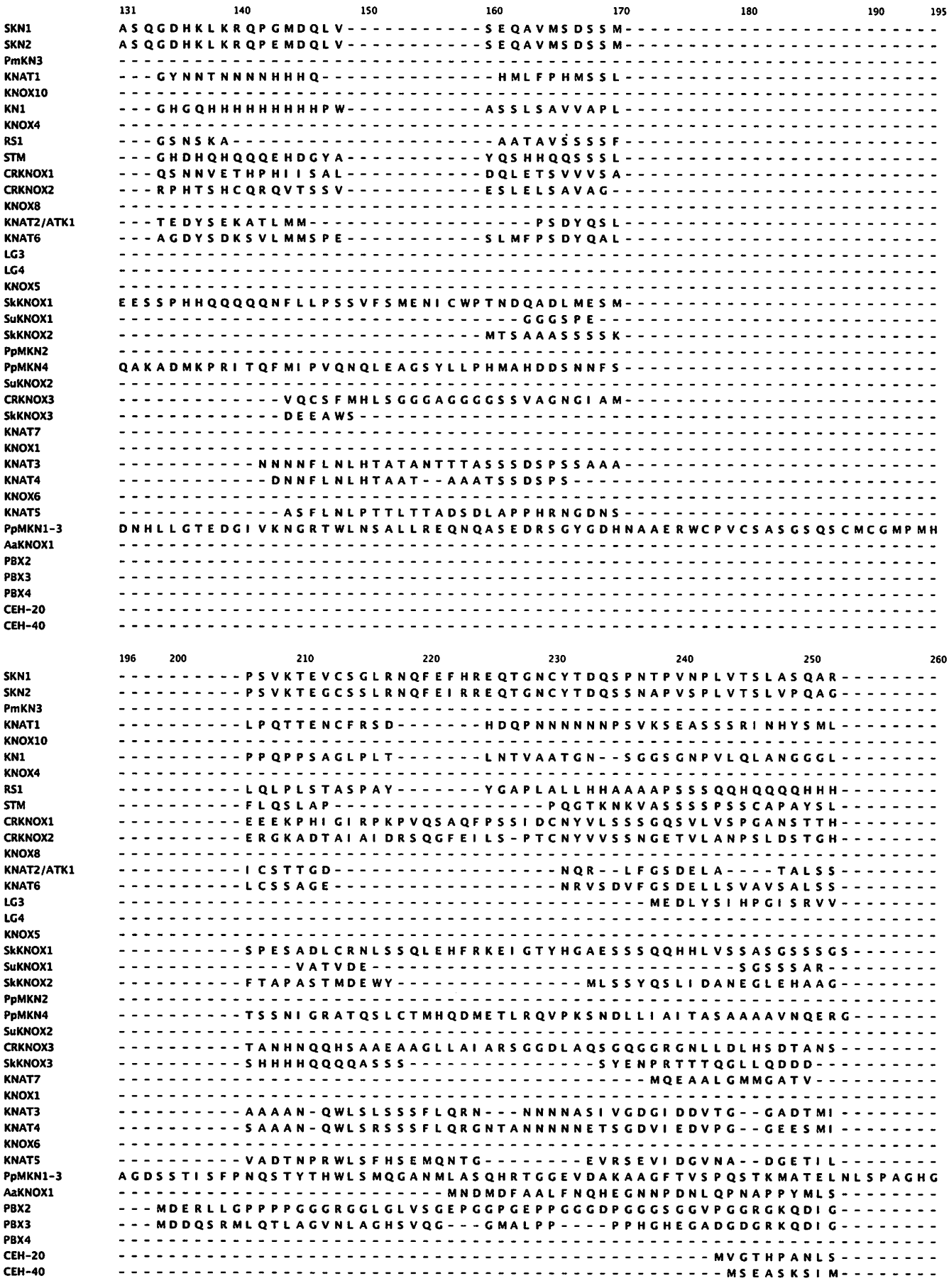
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KNAT1 ---GYNNTNNNNHHHQ----------HMLFPHMSSL----------
KNOX10 ----------
KN1 ---GHGQHHHHHHHHHPW----------ASSLSAVVAPL----------
KNOX4 ----------
RS1 ---GSNSKA----------AATAVSSSSF----------
STM ---GHDHQHQQQEHDGYA----------YQSHHQQSSSL----------
CRKNOX1 ---QSNNVETHPHIISAL----------DQLETSVVVSA----------
CRKNOX2 ---RPHTSHCQRQVTSSV----------ESLELSAVAG----------
KNOX8 ----------
KNAT2/ATK1 ---TEDYSEKATLMM----------PSDYQSL----------
KNAT6 ---AGDYSDKSVLMMSPE----------SLMFPSDYQAL----------
LG3 ----------
LG4 ----------
KNOX5 ----------
SkKNOX1 EESSPHHQQQQQNFLLPSSVFSMENICWPTNDQADLMESM----------
SuKNOX1 ----------GGGSPE----------
SkKNOX2 ----------MTSAAASSSSK----------
PpMKN2 ----------
PpMKN4 QAKADMKPRITQFMIPVQNQLEAGSYLLPHMAHDDSNNFS----------
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SkKNOX3 ----------DEEAWS----------
KNAT7 ----------
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PBX2 ----------
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PBX4 ----------
CEH-20 ----------
CEH-40 ----------
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KNAT1 ----------LPQTTENCFRSD--------HDQPNNNNNNPSVKSEASSSRINHYSML---------
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CRKNOX2 ----------ERGKADTAIAIDRSQGFEILS-PTCNYVVSSNGETVLANPSLDSTGH---------
KNOX8 ----------
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KNAT6 ----------LCSSAGE----------NRVSDVFGSDELLSVAVSALSS---------
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LG4 ----------
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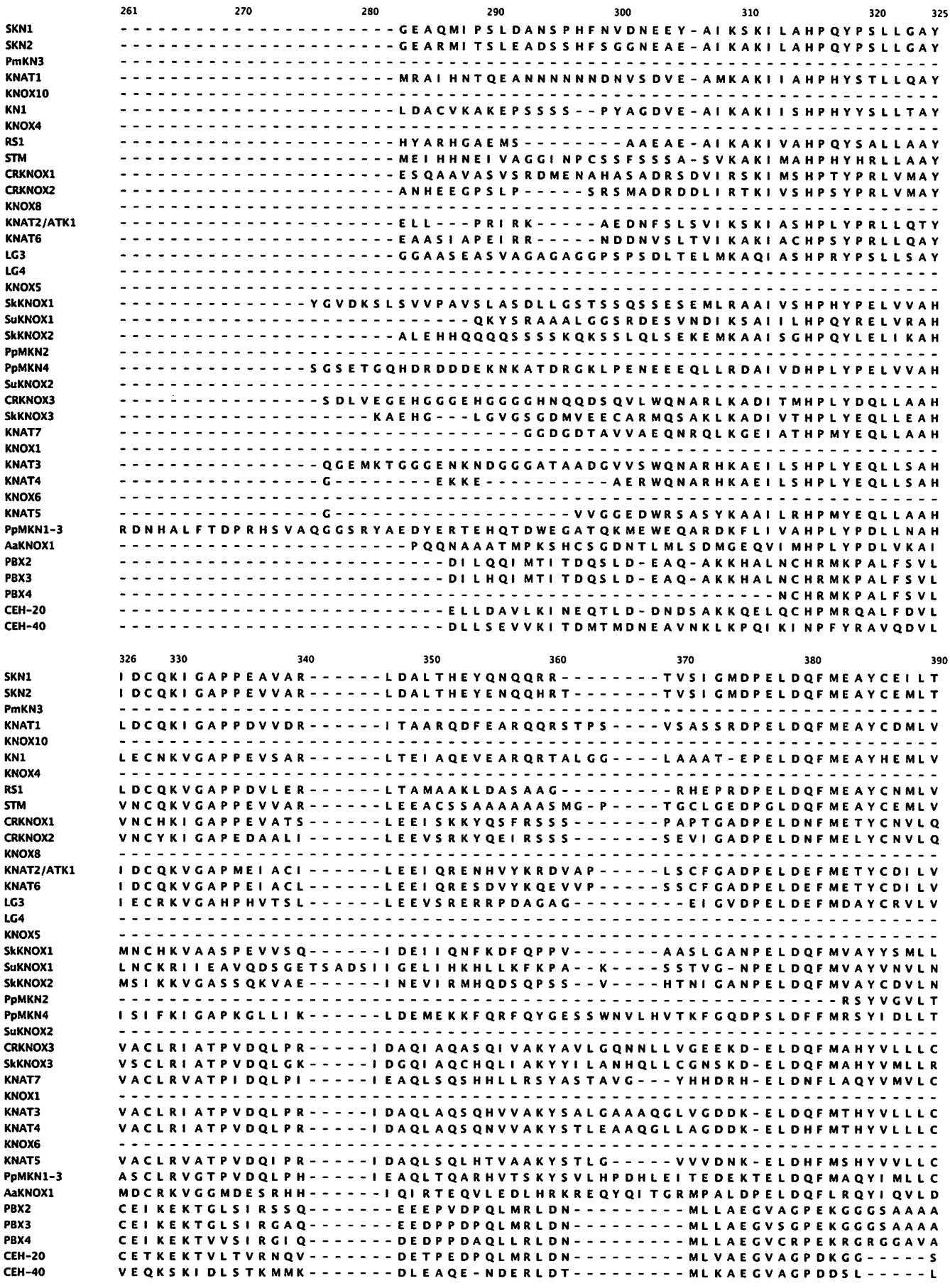
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KNOX4 -
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STM - M E I H H N E I V A G G I N P C S S F S S S A - S V K A K I M A H P H Y H R L L A A Y
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LG4 -
KNOX5 -
SkKNOX1 - - - - - - - - - - - - - - - - Y G V D K S L S V V P A V S L A S D L L G S T S S Q S S E S E M L R A A I V S H P H Y P E L V V A H
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LG4 -
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PpMKN4 I S I F K I G A P K G L L I K - - - - - - L D E M E K K F Q R F Q Y G E S S W N V L H V T K F G Q D P S L D F F M R S Y I D L L T
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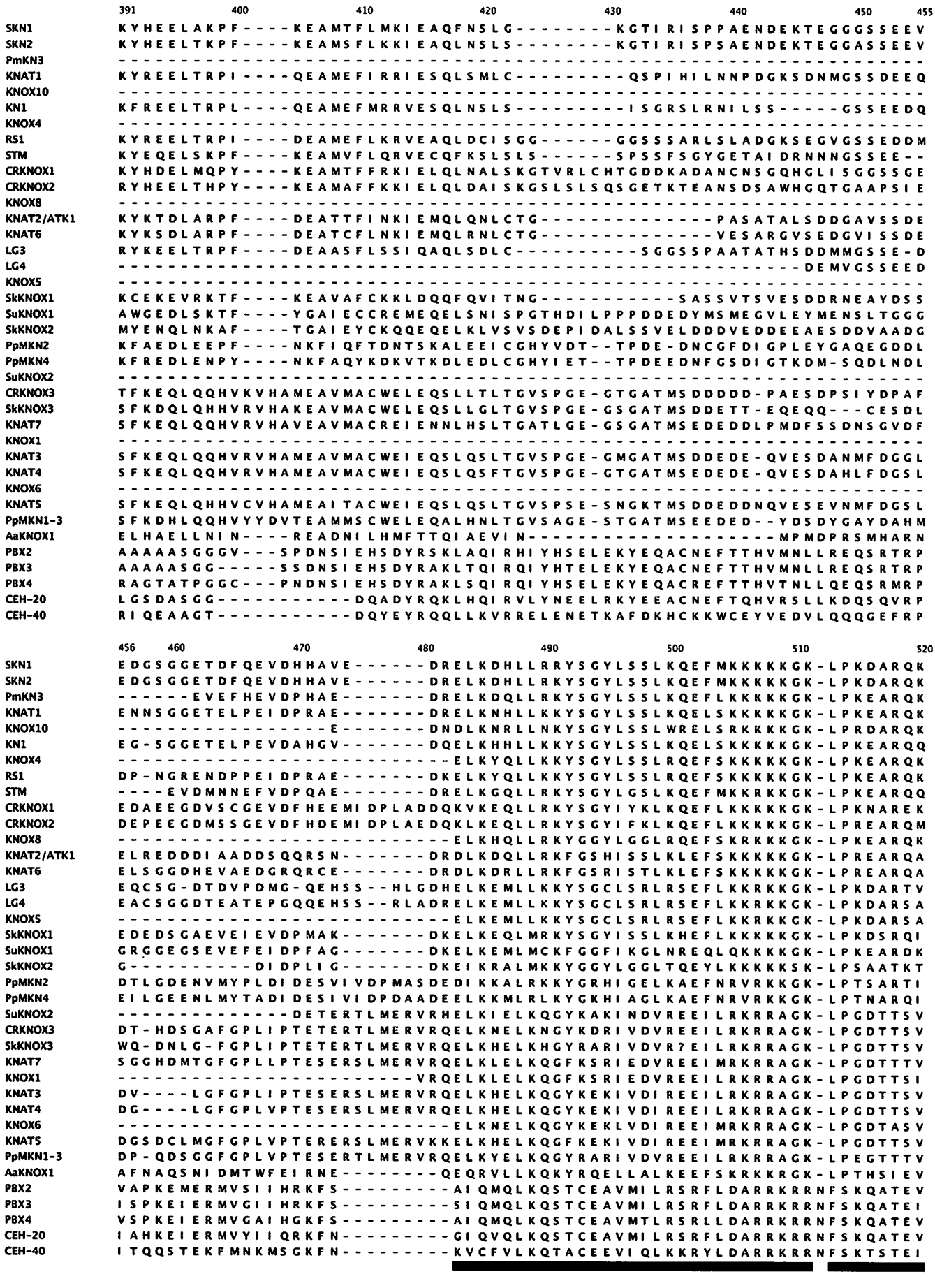
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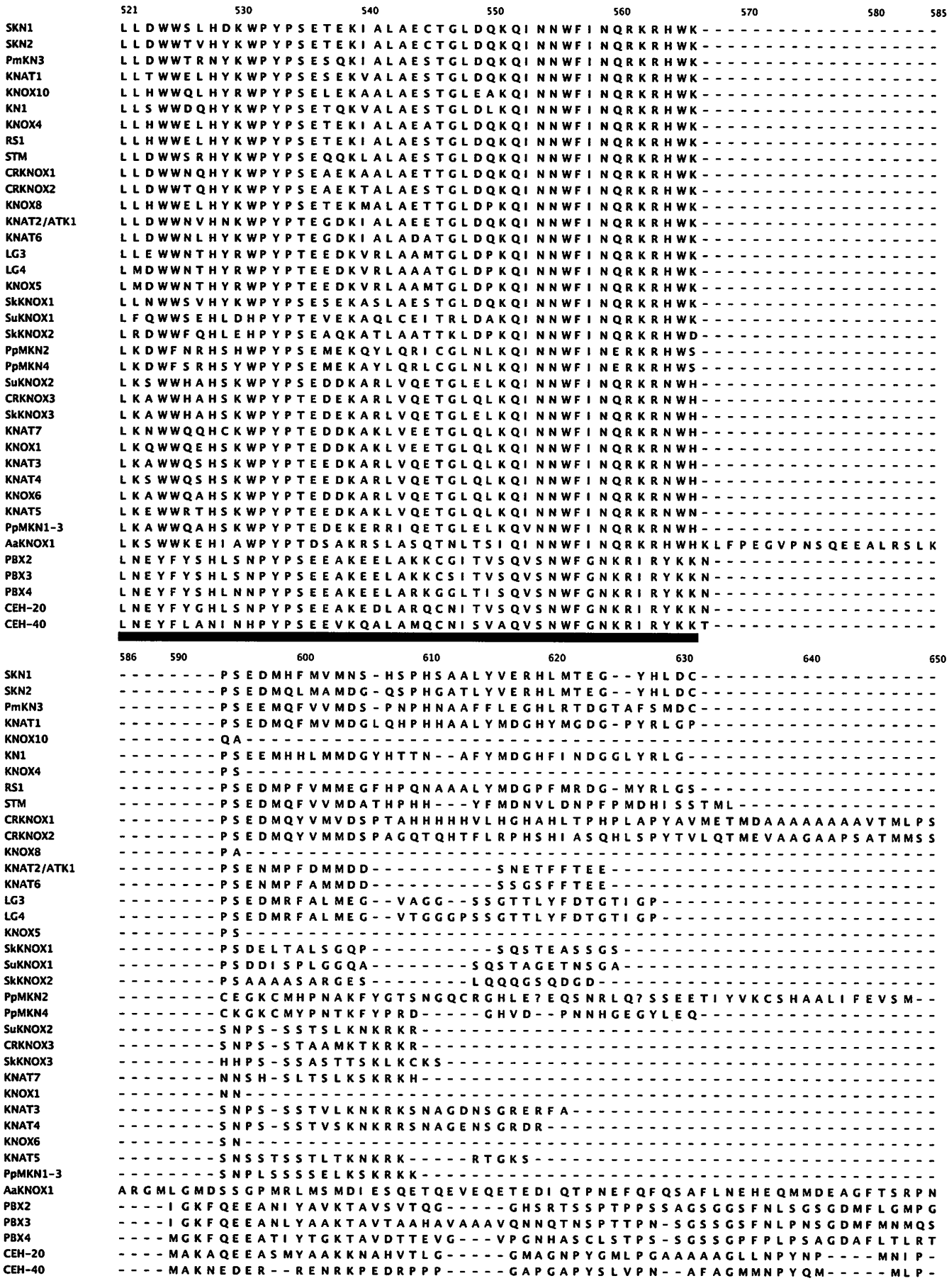
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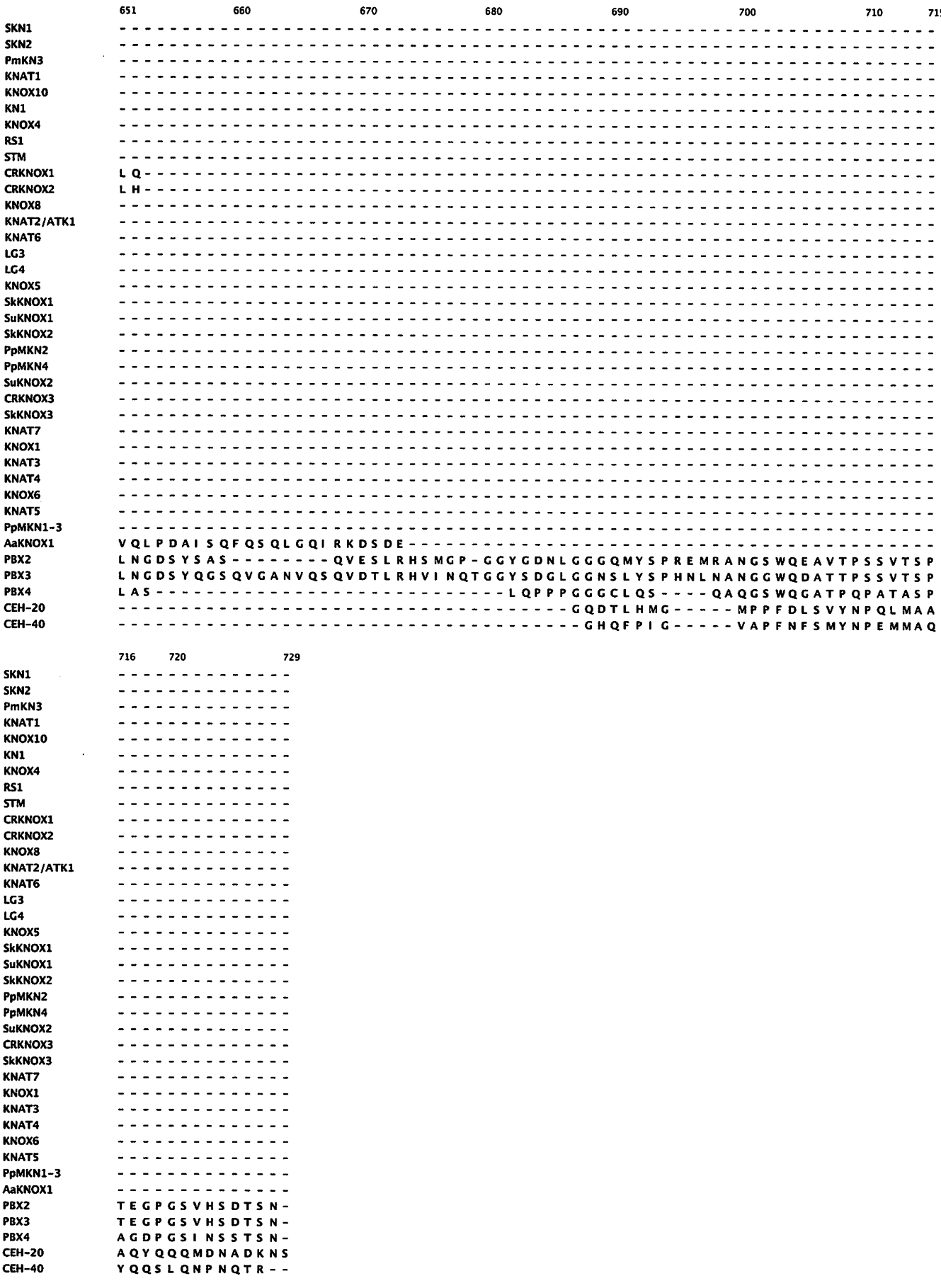
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KNAT6
LG3
LG4
KNOX5
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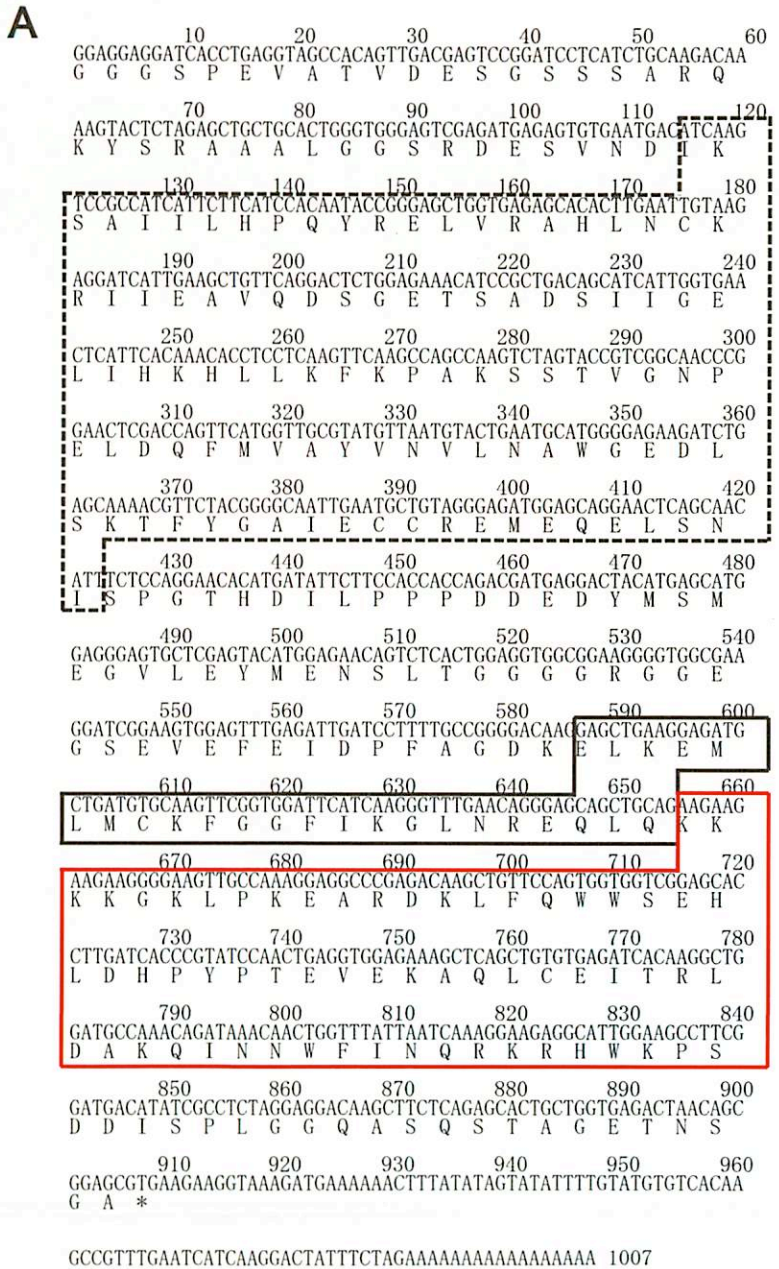

Fig. 3. The nucleotide and deduced amino acid sequences of the *SuKNOX1* (A) and *SuKNOX2* (B) cDNA isolated in this study.

The numberings are nucleotide position when the end of the isolated sequence is 1. The MEINOX domain is broken-line boxed. The ELK domain is solid-line boxed. The homeo domain is red-line boxed.

Figure 3

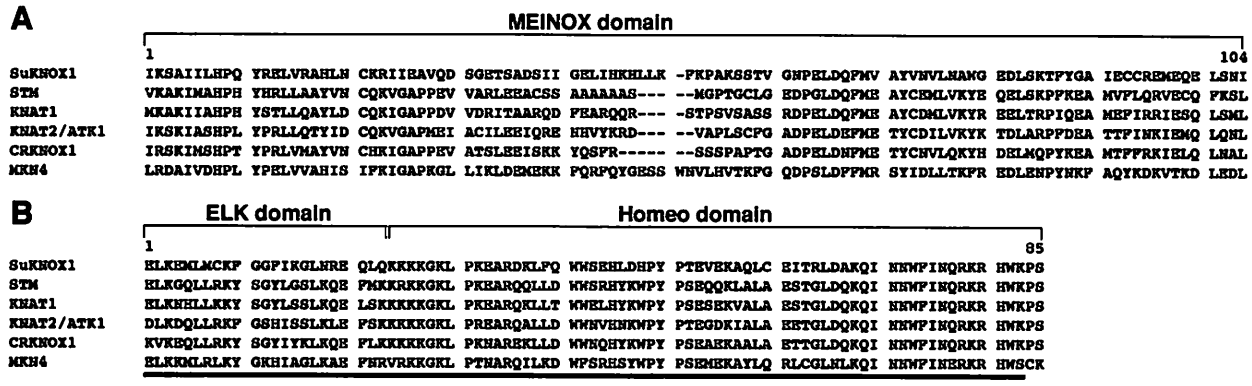

Fig. 4. Alignment of deduced amino acid sequences of *SuKNOX1* and representative class 1 *KNOX* genes from *Arabidopsis thaliana* (*STM*, *KNAT1*, and *KNAT2/ATK*), the fern *Ceratopteris richardii* (*CRKNOX1*), and the moss *Physcomitrella patens* (*MKN4*), including (A) the MEINOX domain and (B) the ELK and homeo domains. The underlined amino acids were used in the phylogenetic analysis shown in Fig. 5.

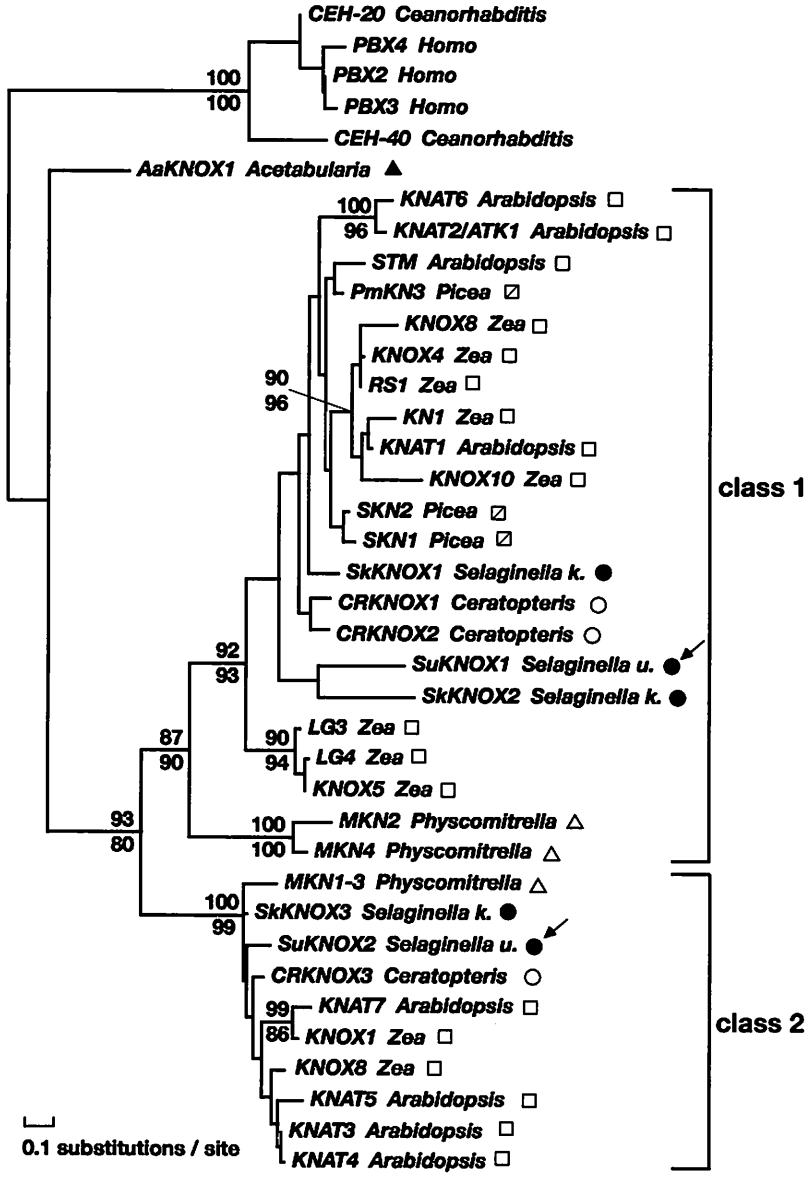

Fig. 5. Maximum likelihood (ML) tree.

ML tree showing the phylogenetic relationships among *KNOX* genes of the spikemoss and representatives from other land plants, a green alga, and metazoans (outgroup). Symbols following the genus names represent plant classifications: open square, angiosperms; square filled with an angled bar, gymnosperms; open circle, ferns; filled circle, spikemosses; open triangle, mosses; filled triangle, green alga. The *Selaginella uncinata* genes are indicated by arrows. Bootstrap values calculated using the ML and neighbor-joining (NJ) methods are indicated above and below the nodes, respectively; only values exceeding 80% and supported by both the ML and NJ topologies are indicated reliably. The length of the bar represents 0.1 amino acid substitutions per residue.

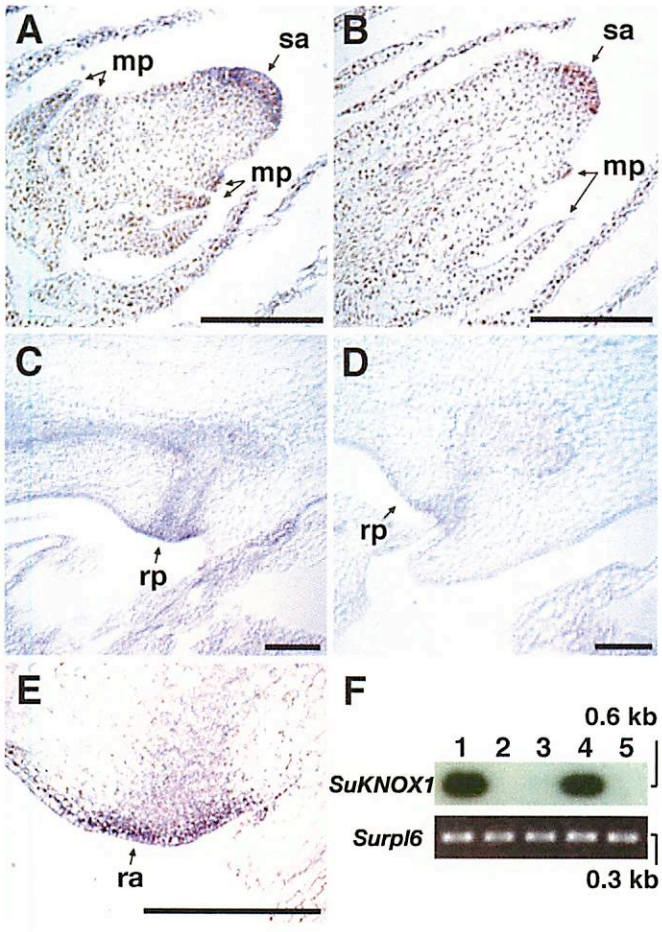

Fig. 6. Expression pattern of the *SuKNOX1*.

(A-E) Longitudinal sections show the shoot apex (sa), microphyll primordium (mp), rhizophore primordium (rp), and rhizophore apical tip (ra). Scale bars = 100 μm. (A, C, E) The locations of *SuKNOX1* mRNA expression were detected using *in situ* hybridization. (B, D) The sense probe was used as a negative control. (F) Amplification of *SuKNOX1* RT-PCR products. Complementary DNA was synthesized from RNA extracted from the apical tips of microphylls (lane 1), internodes (lane 2), microphylls (lane 3), rhizophore tips (lane 4), and root tips (lane 5). *SuKNOX1* PCR products were hybridized with *SuKNOX1*-specific probes. The *SuRPL6* was used as a quantifying control.