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ORIGINAL RESEARCH

Class 1 KNOX Gene Expression Supports the *Selaginella* Rhizophore Concept

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Abstract The spikemoss is marked by the unique root-producing pleurogeous rhizophore as well as the lycophytic microphyll. Imaichi and Kato (Bot Mag Tokyo 102:369–380, 1989; Am J Bot 78:1694–1703, 1991) revealed that the exogenous developmental process in the rhizophore is clearly distinguishable from the developmental process in the endogenous root, argued that the axial organ could be coordinate with other fundamental organs including the root and stem, and demonstrated the "rhizophore concept." In this paper, we report on the expression pattern of the spikemoss *Selaginella* class 1 KNOX gene, *SuKNOX1*, in the rhizophore. We show that the *SuKNOX1* mRNA is specifically accumulated at the tip of the rhizophore as well as the shoot apical apex, but not in the root tip. This result supports the "rhizophore concept" at the molecular level.

Keywords Class 1 KNOX · Lycophyte · Microphyll · Rhizophore · *Selaginella* · Spikemoss

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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 long 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 a 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 orthologs 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, we 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 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, we report the class 1 KNOX gene expression in *Selaginella uncinata*, with particular attention to the *Selaginella* rhizophore concept at the molecular level.

Materials and Methods

Cloning of *Selaginella* KNOX Genes

S. uncinata (Desv.) Spring was collected at the Koishikawa Garden of the University of Tokyo, Tokyo, Japan (Fig. 3a). 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.

Phylogenetic Analysis

To construct a phylogenetic tree of KNOX genes, the amino acid sequences shown in Fig. 2 were obtained from EMBL/DDBJ/GenBank DNA databases 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 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 method (Hasegawa and Kishino 1994) are indicated on nodes reconstructed using both the ML and NJ methods.

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′-ATCACCTGAGGTAGCCA CAGTTGA-3′) and SuKN1R1 (5′-AAAAGGATCAATCT CAAACTCCA-3′). The *S. uncinata* ortholog of the ribosomal protein L6 gene (*SuRPL6*, DDBJ accession no. AB521036), which was constitutively expressed in all tissues that we 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).

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 and homeo domains. The sense strand of the *SuKNOX1* mRNA was used as a negative control. The incorporation of DIG-labeled uridine triphosphate 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).

Results

Cloning of *Selaginella* KNOX Genes and Phylogenetic Analysis

We examined the relatively large spikemoss *S. uncinata* because this species provides advantages for histological studies. The *Selaginella* KNOX cDNA, named *SuKNOX1* (DDBJ accession no. AB288208), 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, ELK domain, and TALE-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. 1). In addition, we successfully amplified a class 2 KNOX gene, named *SuKNOX2* (DDBJ accession no. AB288209), 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*.

A

MEINOX domain

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                1                                                                                                  104
SuKNOX1         IKSAIILHPQ YRELVRAHLN CKRIIEAVQD SGETSADSII GELIHKHLLK -FKPAKSSTV GNPELDQFMV AYVNVLNAWG EDLSKTFYGA IECCREMEQE LSNI
STM             VKAKIMAHPH YHRLLAAYVN CQKVGAPPEV VARLEEACSS AAAAAAS--- --MGPTGCLG EDPGLDQFME AYCEMLVKYE QELSKPFKEA MVFLQRVECQ FKSL
KNAT1           MKAKIIAHPH YSTLLQAYLD CQKIGAPPDV VDRITAARQD FEARQQR--- -STPSVSASS RDPELDQFME AYCDMLVKYR EELTRPIQEA MEFIRRIESQ LSML
KNAT2/ATK1      IKSKIASHPL YPRLLQTYID CQKVGAPMEI ACILEEIQRE NHVYKRD--- --VAPLSCFG ADPELDEFME TYCDILVKYK TDLARPFDEA TTFINKIEMQ LQNL
CRKNOX1         IRSKIMSHPT YPRLVMAYVN CHKIGAPPEV ATSLEEISKK YQSFR----- --SSSPAPTG ADPELDNFME TYCNVLQKYH DELMQPYKEA MTFFRKIELQ LNAL
MKN4            LRDAIVDHPL YPELVVAHIS IFKIGAPKGL LIKLDEMEKK FQRFQYGESS WNVLHVTKFG QDPSLDFFMR SYIDLLTKFR EDLENPYNKF AQYKDKVTKD LEDL
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B

ELK domain | Homeo domain

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                1                                                                                  85
SuKNOX1         ELKEMLMCKF GGFIKGLNRE QLQKKKKGKL PKEARDKLFQ WWSEHLDHPY PTEVEKAQLC EITRLDAKQI NNWFINQRKR HWKPS
STM             ELKGQLLRKY SGYLGSLKQE FMKKRKKGKL PKEARQQLLD WWSRHYKWPY PSEQQKLALA ESTGLDQKQI NNWFINQRKR HWKPS
KNAT1           ELKNHLLKKY SGYLSSLKQE LSKKKKKGKL PKEARQKLLT WWELHYKWPY PSESEKVALA ESTGLDQKQI NNWFINQRKR HWKPS
KNAT2/ATK1      DLKDQLLRKF GSHISSLKLE FSKKKKKGKL PREARQALLD WWNVHNKWPY PTEGDKIALA EETGLDQKQI NNWFINQRKR HWKPS
CRKNOX1         KVKEQLLRKY SGYIYKLKQE FLKKKKKGKL PKNAREKLLD WWNQHYKWPY PSEAEKAALA ETTGLDQKQI NNWFINQRKR HWKPS
MKN4            ELKKMLRLKY GKHIAGLKAE FNRVRKKGKL PTNARQILKD WFSRHSYWPY PSEMEKAYLQ RLCGLNLKQI NNWFINERKR HWSCK
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Fig. 1 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 the MEINOX domain (**a**) and the ELK and homeodomains (**b**). The *underlined amino acids* were used in the phylogenetic analysis shown in Fig. 2

To address the evolutionary relationship between *SuKNOX1* and class 1 KNOX genes of land plants, we 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 homeodomains (Fig. 1, 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. 2). 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.

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. 3b). No *SuKNOX1* expression was detected in microphylls, although there was weak detection in juvenile microphyll primordia (Fig. 3b). To examine *SuKNOX1* expression in the rhizophores, we prepared longitudinal sections containing the junctions of branching stems (Fig. 3d). 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. 3f).

We also examined the *SuKNOX1* expression pattern using RT-PCR (Fig. 3g). *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, we could not examine *SuKNOX1* expression patterns in the gametophyte because we 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.

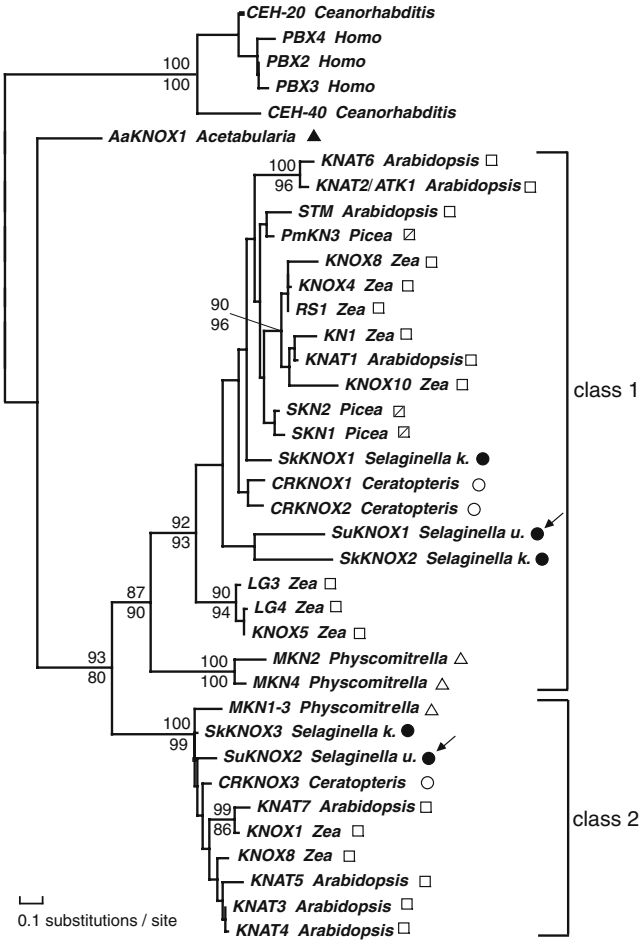
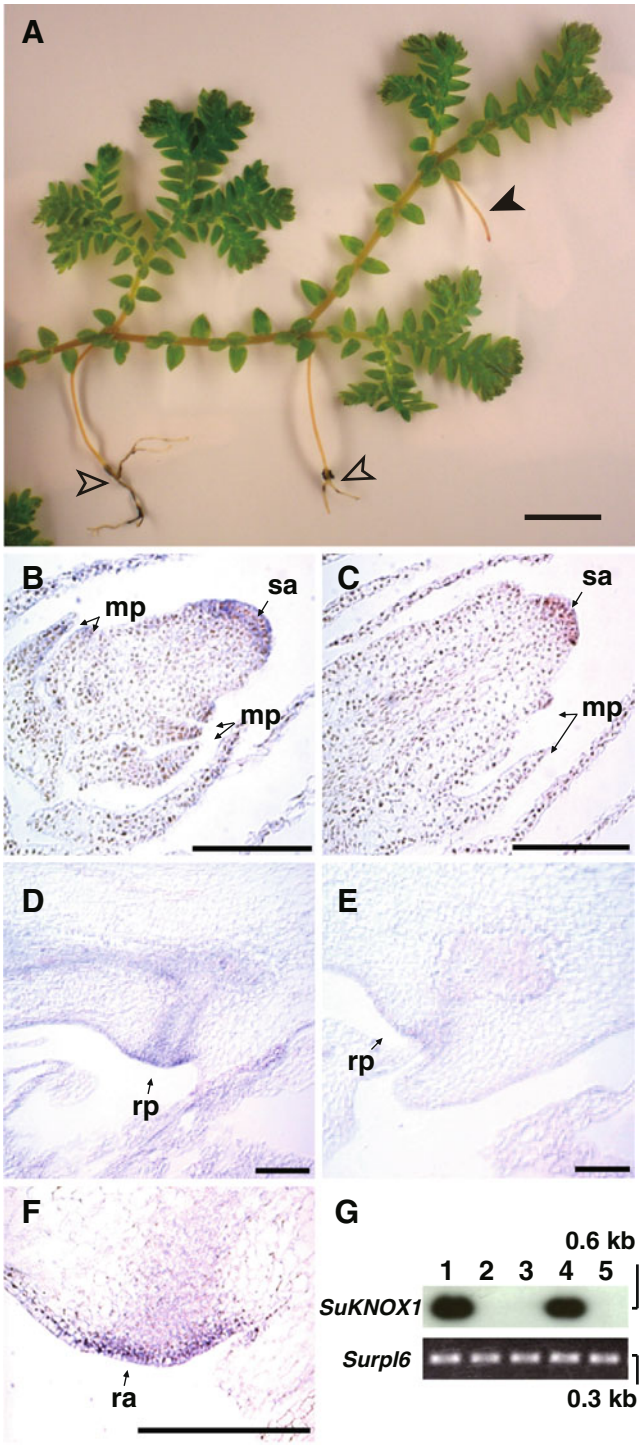


Fig. 2 Maximum likelihood (*ML*) tree showing the phylogenetic relationships among KNOX genes of the spikemoss and representatives from other land plants, a green alga, and metazoans (outgroup). *Symbols following the genus names* represent plant classifications: angiosperms (*open square*), gymnosperms (*square filled with an angled bar*), ferns (*open circle*), spikemosses (*filled circle*), mosses (*open triangle*), green alga (filled triangle). The *S. 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

Discussion

We characterized the *SuKNOX1* cDNA from the spikemoss *S. uncinata*. Based on the gene tree, *SuKNOX1* is clearly included within the class 1 KNOX gene cluster containing *Arabidopsis STM*, *KNAT1*, and *KNAT2*/*ATK1*. As reported by Serikawa and Mandoli (1999), *Acetablaria acetablum*



◀ **Fig. 3** Expression pattern of the *SuKNOX1*. **a** The spikemoss *S. uncinata* has creeping stems with leafy microphylls and rhizophores (*closed arrowhead*), which produce roots (*opened arrowhead*). *Scale bar*, 10 mm. **b–f** Longitudinal sections show the shoot apex (*sa*), microphyll primordium (*mp*), rhizophore primordium (*rp*), and rhizophore apical tip (*ra*). *Scale bars*, 100 µm. **b**, **d**, **f** The locations of *SuKNOX1* mRNA expression were detected using in situ hybridization. **c**, **e** The sense probe was used as a negative control. **g** 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. *SuRPL6* was used as a quantifying control

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 *S. 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. We 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 our 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.

We 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). Our 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 homologs such as HD-Zip genes and NAC genes will reveal additional characters of *Selaginella* appendages. It was found that *Selaginella* HD-Zip III gene expression predicts organ initiation site, similar to that of *Arabidopsis* homologs; however, there have been no reports in rhizophores (Floyd et al. 2006; Prigge and Clark 2006). A BAC library containing ten genome equivalents and ESTs containing 1,301 non-redundant clones have recently been constructed for *Selaginella 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 pleurogeous architectures, which will aid in depicting evolutionary scenarios for early land plants.

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