ASL/LBD Phylogeny Suggests that Genetic Mechanisms of Root Initiation Downstream of Auxin Are Distinct in Lycophytes and Euphyllophytes

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Abstract

Paleobotanical studies suggest that roots evolved at least twice independently during land plant diversification, once in lycophytes and once in euphyllophytes. Auxin promotes postembryonic root initiation in both groups but from different cell types. In several euphyllophytes, such as *Arabidopsis*, rice, and maize, AS2/LOB-domain (ASL/LBD) proteins act directly downstream of auxin and are conserved elements necessary for root initiation. It is currently unknown whether similar or different genetic mechanisms act downstream of auxin for root initiation in lycophytes and euphyllophytes. We searched for ASL/LBD proteins in genome sequences spanning the tree of life to retrace their evolutionary history. We performed a phylogenetic analysis of ASL/LBD proteins and mapped the functions of all characterized ASL/LBD onto the phylogenetic trees. We identified a clade specifically associated with root development, which includes no lycophyte sequence. This points toward the existence of distinct genetic mechanisms downstream of auxin for root initiation in lycophytes and euphyllophytes.

The successful colonization of terrestrial environments by plants was accompanied by the evolution of rooting structures. Although the bryophyte-like earliest land plants had only filamentous cells called rhizoids, lycophytes (clubmosses) were the first plants to evolve roots in the Lower Devonian, approximately 415 Ma (Raven and Edwards 2001). No evidence of roots has been found in euphyllophyte fossils of the Lower Devonian. The earliest occurrence of roots in this group, represented today by ferns, gymnosperms, and angiosperms, is dated as Middle Devonian (~390 Ma), suggesting that roots evolved independently in lycophytes and euphyllophytes (Pires and Dolan 2012). Moreover, cellular processes underlying root initiation are different in these two lineages. Roots of the lycophyte *Selaginella martensii* initiate by division of cells located below the epidermal layer at the tip of the rhizophore, a leafless and capless organ arising from stem branching points (Imaichi and Kato 1991; Lu and Jernstedt 1996). In contrast, angiosperm roots develop from groups of initial cells in either the ground meristem in shoots (Itoh et al. 2005) or the pericycle, or occasionally the endodermis, in pre-existing roots (Peret et al. 2009; Coudert et al. 2010).

Despite these differences, there is evidence that some molecular mechanisms underlying root organogenesis are conserved. In angiosperms, postembryonic root initiation involves a core regulatory pathway activated by auxin and dependent on ASL (asymmetric leaves-2-like)/LBD (LOB-domain) transcription factors (TFs). The *Arabidopsis lbd16/lbd29* double mutant has very few lateral roots compared with wild type (Okushima et al. 2005, 2007), and the rice *crown rootless1* mutant defective in the *OsCRL1/LBD3-2* gene cannot initiate crown roots (Inukai et al. 2001, 2005). Auxin activates *AtLBD16*, *AtLBD29*, and *OsCRL1* expression but cannot restore root formation in corresponding mutants. The induction of root initiation by exogenous auxin application is conserved in *Selaginella* (Williams 1937; Webster 1969; Wochok and Sussex 1975), but downstream molecular mechanisms, such as the possible involvement of ASL/LBD TFs, are unknown. The origin and evolutionary history of the ASL/LBD protein family remain unresolved: the last phylogenetic study included only *Arabidopsis*, rice, and maize sequences (Majer and Hochholdinger 2010). To investigate whether root initiation-related ASL/LBD proteins are present in lycophytes, we analyzed the phylogenetic distribution of ASL/LBD proteins across land plant diversification and mapped functional information onto the trees where available.

Protein sequences containing the conserved AS2/LOB domain were retrieved from genomic data of extant species of major groups that diverged during land plant evolution: the moss *Physcomitrella patens* (26 sequences), the lycophyte *Selaginella moellendorfii* (11), and the angiosperms *Vitis vinifera* (42), *Populus trichocarpa* (54), *Arabidopsis* (42) (Iwakawa et al. 2002), rice (35) (Yang et al. 2006), and maize (43) (Majer and Hochholdinger 2010) (supplementary methods and data S1-1, Supplementary Material online). We aligned the AS2/LOB domains with the MAFFT program to obtain a broad view of ASL/LBD protein diversity (supplementary methods, Supplementary Material online). Two classes corresponding to the previously described class I and class II were distinguishable in all species examined, which suggests both were likely present in the land plant common ancestor and persisted

through evolution (supplementary data S1-2, Supplementary Material online). To investigate when these proteins appeared, we searched for ASL/LBD sequences in available genomic data sets of charophyte green algae, the closest living relatives of land plants. Single partial ASL/LBD proteins, with class I primary structure, were found in *Coleochaete orbicularis* and *Spirogyra pratensis* expressed sequence tag data sets (supplementary methods and data S1-3, Supplementary Material online). We unsuccessfully searched for complete or partial AS2/LOB-domain sequences in more than 1,500 sequenced genomes (Dievart et al. 2011) including genomes of chlorophyte algae (*Volvox carteri*, *Chlamydomonas reinhardtii*, and *Ostreococcus taurii*) that are estimated to have diverged from streptophytes

(charophyte algae and land plants) approximately 1.2 billion years ago (Yoon et al. 2004). We conclude that ASL/LBD TFs are streptophyte-specific proteins and likely appeared in charophyte algae.

To understand the evolutionary relationships between ASL/LBDs in land plants, we computed phylogenetic trees for both class I and class II proteins using the maximum likelihood method with the PhyML 3.0 program (fig. 1, supplementary methods and data S2, Supplementary Material online). Partial charophyte algal sequences could not be accurately aligned and were not included in the analysis. Seven subclasses were defined for class I and two for class II based on tree topology and branch lengths. These subclasses fitted well with those defined in previous studies (fig. 1).
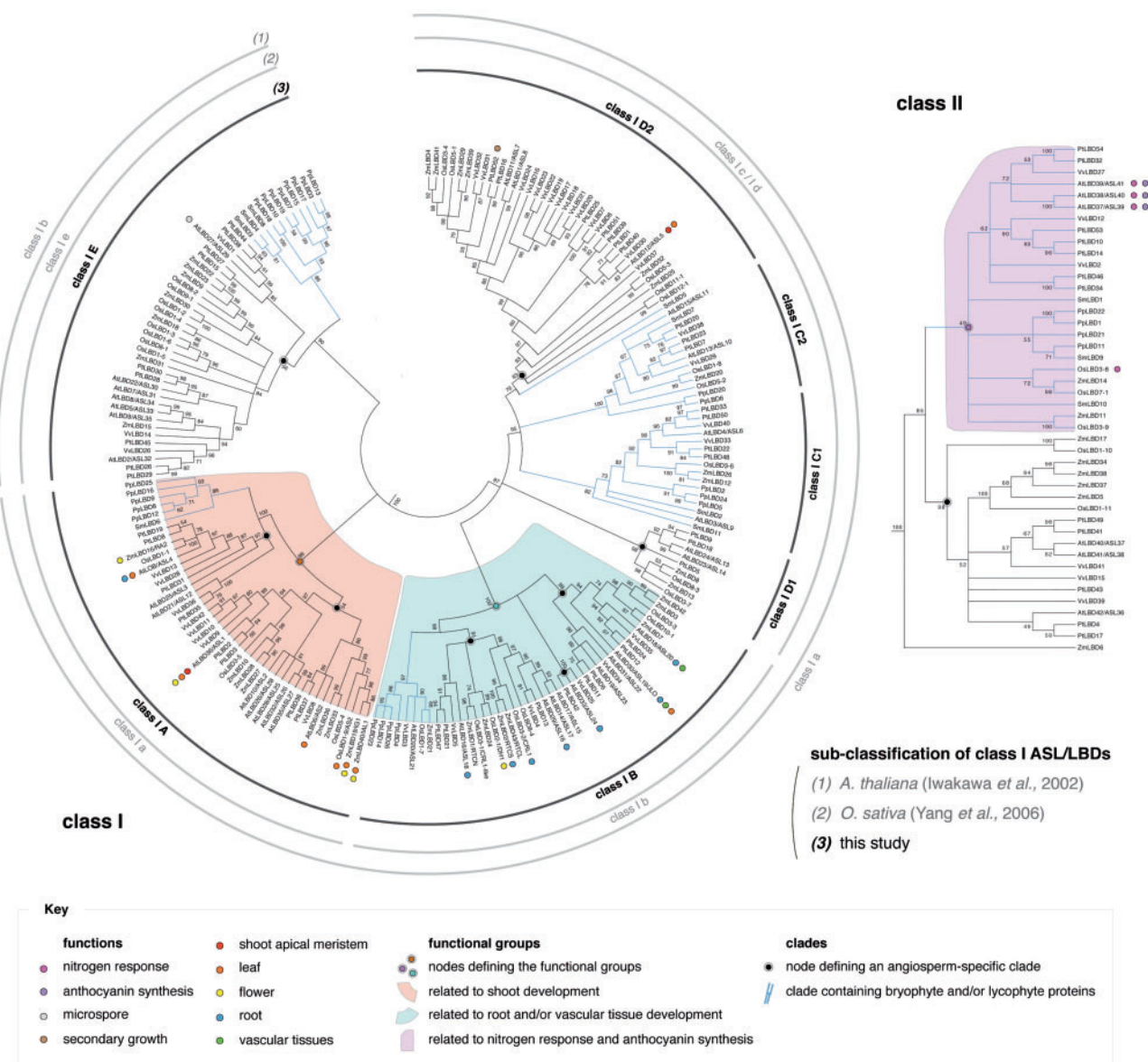
Fig. 1. Maximum likelihood trees representing phylogenetic relationships between ASL/LBD proteins in *Physcomitrella patens* (Pp), *Selaginella moellendorffii* (Sm), *Oryza sativa* (Os), *Zea mays* (Zm), *Populus trichocarpa* (Pt), *Arabidopsis thaliana* (At), and *Vitis vinifera* (Vv). Phylogenetic trees were generated by aligning full-length protein sequences. Class I and class II sequences were used to define outgroups for class II and class I phylogenetic trees, respectively. The numbers at the nodes are posterior probability values. Clades with less than 49% support have been collapsed.
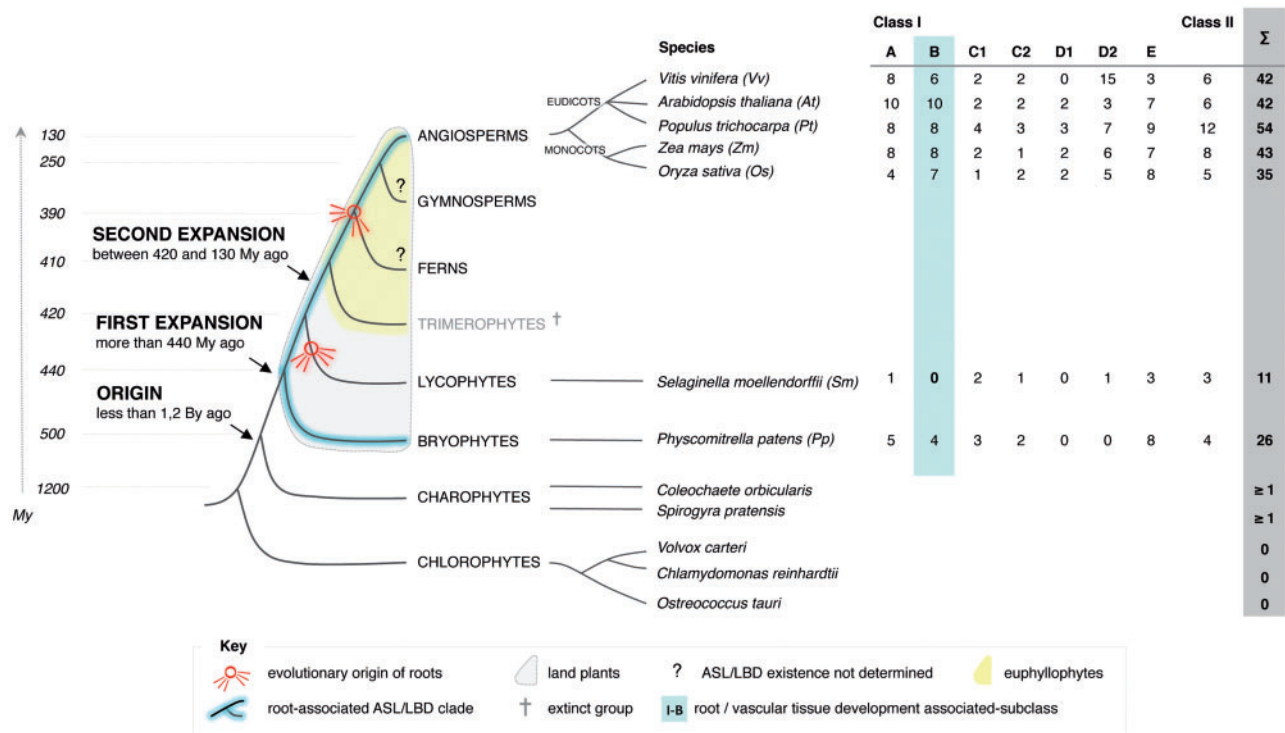
Fig. 2. Root evolution and the evolutionary history of ASL/LBD proteins in plants. Synthetic monophyletic tree representing phylogenetic relationships between the main groups of plants in the green lineage. Time scale and thin dashed lines on the left indicate approximate radiation dates in million years (My) for the different groups. Hypothesized major evolutionary events undergone by the ASL/LBD protein family are indicated. ASL/LBD protein number is given for each species in each subclass on the right of the tree. Σ, total protein number.

Within each subclass, one or several clades can be distinguished. Six clades contain both moss and lycophyte, as well as angiosperm proteins implying that ASL/LBDs may have undergone an initial expansion before the divergence of mosses and lycophytes more than 440 Ma (fig. 2). This expansion event likely contributed to the diversification of gene regulatory networks necessitated by significant changes in lifestyle, which accompanied the transition of plants from an aquatic to a terrestrial habitat (McCourt et al. 2004; Bowman et al. 2007). The nine angiosperm-specific clades probably appeared after the last common ancestor of lycophytes and angiosperms that lived in the Silurian period approximately 420 Ma. All these clades include monocot and eudicot proteins suggesting that a second expansion event occurred before their radiation approximately 130 Ma (Willis and McElwain 2002; Cleal and Thomas 2009) (fig. 2). This is reminiscent of important expansions that occurred at the same time within other TF families such as MICK-type MADS-box TFs or euANT-type AP2 TFs (Floyd and Bowman 2007). This second expansion of ASL/LBD TFs may be associated with the acquisition of new developmental functions during a major period of plant diversification such as the Carboniferous for ferns (between 350 and 290 Ma), the Triassic and Jurassic for gymnosperms (between 250 and 150 Ma), or the Cretaceous for angiosperms (approximately 130 Ma) (Cleal and Thomas 2009). Collecting genome sequences from ferns and gymnosperms would help explore this point. Nevertheless, this hypothesis is supported by the

fact that most ASL/LBDs studied so far play a role in plant development (supplementary data S1-4, Supplementary Material online).

We analyzed the distribution of functions of all characterized genes in the phylogenetic trees. Nearly all genes having similar functions were included in a single clade. Three functional groups, each containing proteins from a single clade, were thus defined (fig. 1). The first group includes class II proteins that are related to environmental responses, including nitrogen response and anthocyanin synthesis. The second comprises proteins from class I A involved in shoot-specific processes such as leaf, flower, and shoot apical meristem (SAM) development. In euphyllophytes, leaf initiation necessitates KNOX, ARP, and AS2/LOB-domain proteins (Byrne 2012), whereas leaves evolved independently in lycophytes and euphyllophytes, a KNOX/ARP module was also recruited for leaf formation in *Selaginella* (Harrison et al. 2005). The SmLBD6 protein identified in the shoot development clade might be a third key component of this conserved developmental mechanism. The third group encompasses class I B proteins that control root and/or vascular tissue development such as AtLBD16, AtLBD29, OsCRL1, and ZmRTCS, which are necessary for postembryonic root initiation in angiosperms. This group comprises four *Physcomitrella* ASL/LBDs despite the fact that bryophytes have no roots but rhizoids. The increase in morphological complexity of the sporophyte during land plant evolution was accompanied in some cases by the co-option of genes that originally regulated

morphogenesis in the gametophyte of early land plants. For example, *AtRHD6*, a bHLH TF controlling root hair development in the *Arabidopsis* sporophyte, is orthologous to *PpRSL1* and *PpRSL2*, which are induced by auxin and control rhizoid development in the *Physcomitrella* gametophyte. *PpRSL1* can functionally complement an *Arabidopsis rdh6* loss-of-function mutant (Sakakibara et al. 2003; Menand et al. 2007; Jang et al. 2011). Interestingly, this phylogenetic analysis points out the absence of *Selaginella* proteins in the root-associated clade (fig. 2). Waiting for further genomics data and functional analysis in other lycophyte species, this result weighs in favor of the independent evolution of molecular mechanisms downstream of auxin sustaining the initiation of lycophyte and euphyllophyte roots, besides their distinct cellular origins.

Supplementary Material

Supplementary methods and data S1-1 to S1-4 and S2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

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