Recruitment and remodeling of an ancient gene regulatory network during land plant evolution

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The evolution of multicellular organisms was made possible by the evolution of underlying gene regulatory networks. In animals, the core of gene regulatory networks consists of kernels, stable subnetworks of transcription factors that are highly conserved in distantly related species. However, in plants it is not clear when and how kernels evolved. We show here that RSL (ROOT HAIR DEFECTIVE SIX-LIKE) transcription factors form an ancient land plant kernel controlling caulonema differentiation in the moss *Physcomitrella patens* and root hair development in the flowering plant *Arabidopsis thaliana*. Phylogenetic analyses suggest that RSL proteins evolved in aquatic charophyte algae or in early land plants, and have been conserved throughout land plant radiation. Genetic and transcriptional analyses in loss of function *A. thaliana* and *P. patens* mutants suggest that the transcriptional interactions in the RSL kernel were remodeled and became more hierarchical during the evolution of vascular plants. We predict that other gene regulatory networks that control development in derived groups of plants may have originated in the earliest land plants or in their ancestors, the Charophycean algae.

bHLH | auxin | protonema | sporophyte | gametophyte

The development of multicellular organisms is controlled by gene regulatory networks (GRNs) and the reorganization of GRN architecture is thought to be a major factor underlying morphological evolution (1–5). GRNs are hierarchic and modular structures where four major component classes can be identified (3): at the periphery of GRNs are differentiation gene batteries encoding proteins that execute cell type-specific functions (such as building a pigmented cell); upstream of these batteries are switches that allow or prevent subcircuits to function in specific developmental contexts, and "plug-ins," small subcircuits that are flexibly and repeatedly used during development (such as signal transduction pathways); at the core of GRNs are kernels, small conserved subcircuits that execute specific developmental functions (such as defining spatial patterns in an embryo). Kernels are comprised of transcription factors that are highly conserved in distantly related species and are unusually stable components of GRNs.

Ancient kernels that regulate body plan and organ development are highly conserved among diverse groups of metazoans (animals) (6–10). In contrast, the core components of plant GRNs are difficult to identify because of the dynamic nature of plant genome evolution and the plastic character of plant development. Floral homeotic genes form a relatively recent kernel controlling flower development (11). Homologs of KNOX and LEAFY transcription factors control shoot development in vascular plants and sporophyte development in mosses (12–15). KNOX/BEL genes also control the development of the diploid phase in unicellular chlorophytes (16) and the haploid-to-diploid transition in mosses (15), suggesting that KNOX and LEAFY genes may be core members of ancient GRNs that control diploid development in plants. Auxin signaling (17, 18), ethylene perception (19), abscisic acid signaling (20), and several small RNAs are conserved between mosses and flowering plants (21, 22), suggesting that many switches and plug-ins of land plant GRNs have been conserved since before the evolution of vascular plants over 440 million y ago. However, the architecture and evolutionary history of these hypothetical ancient GRN kernels are mostly unknown.

In the angiosperm *Arabidopsis thaliana*, root hair development is controlled by the basic helix-loop-helix (bHLH) transcription factors AtRHD6 (*A. thaliana* ROOT HAIR DEFECTIVE 6) and AtRSL1 (*A. thaliana* RHD SIX-LIKE 1); their homologs in the moss *Physcomitrella patens* (Pp), PpRSL1 and PpRSL2, control the development of filamentous rooting structures: caulonema and rhizoids (23–25). This finding suggests that *RSL* genes belong to an ancient land plant GRN that controls the differentiation of cells with a rooting function. In *A. thaliana*, AtRHD6 was also found to form a transcriptional mechanism with two other bHLH transcription factors, AtRSL2 and AtRSL4 (26).

Here we test the hypothesis that the RSL mechanism is an ancient land plant kernel. We show that *RSL* genes form a transcriptional network that controls root hair development in *A. thaliana* and protonema development in *P. patens*. *RSL* genes form two ancient lineages that evolved in charophyte algae or in the first land plants and have been conserved during land plant evolution. Functional and expression analysis of the *RSL* genes in *A. thaliana* and in *P. patens* indicate that the two lineages form a transcriptional regulatory network in both species. Taken together, our results suggest that the *RSL* genes form a kernel that evolved over 450 million y ago and was recruited to control the development of root hairs during the evolution of vascular plants.

Results

RSL Network Controls Root Hair Development in *A. thaliana*. The differentiation of root hairs in *A. thaliana* is controlled by a regulatory mechanism that comprises the bHLH transcription factors AtRHD6, AtRSL1, AtRSL2, and AtRSL4: no root hairs differentiate in *Atrhd6 Atrsl1* or in *Atrsl2 Atrsl4* double-mutants (23, 26), and the transcription of *AtRSL2* and *AtRSL4* is positively regulated by AtRHD6 and AtRSL1 (26). These four genes belong to a phylogenetic group that also includes *AtRSL3* and *AtRSL5* (Fig. 1*B*). To determine if AtRSL3 and AtRSL5 also control root hair development, we characterized the phenotypes of *Atrsl3*, *Atrsl5* and *Atrsl2 Atrsl3* mutants. Root hairs of *Atrsl3* and *Atrsl5* single-mutants were indistinguishable from wild-type,

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