Article Addendum

Parallels in lignin biosynthesis

A study in *Selaginella moellendorffii* reveals convergence across 400 million years of evolution

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Key words: syringyl lignin, cytochrome P450, convergent evolution, F5H, Selaginella, lycophyte, biofuel

A hallmark of vascular plants is the development of a complex water-conducting system, which is physically reinforced by the heterogeneous aromatic polymer lignin. Syringyl lignin, a major building block of lignin, is often thought to be uniquely characteristic of angiosperms; however, it was demonstrated over fifty years ago that that syringyl lignin is found in another group of plants, known as the lycophytes, the ancestors of which diverged from all the other vascular plant lineages 400 million years ago.[1] To determine the biochemical basis for this common biosynthetic ability, we isolated and characterized cytochrome P450-dependent monooxygenases (P450s) from the lycophyte *Selaginella moellendorffii* and compared them to the enzyme that is required for syringyl lignin synthesis in angiosperms. Our results showed that one of these P450s encodes an enzyme that is functionally analogous to but phylogenetically independent from its angiosperm counterpart. Here, we discuss the evolution of lignin biosynthesis in vascular plants and the role of *Selaginella moellendorffii* in plant comparative biology and genomics.

Introduction

Land plants made their first appearance on land in the Middle Palaeozoic era, between 480 and 360 million years ago.[2] Fossil evidences as well as phylogenetic studies suggest that the earliest land plants were small in stature and simple in morphology, and may have resembled the bryophytes (mosses, liverworts and hornworts) living today.[3] It was not until the Early Devonian that the development of a complex water-conducting xylem structure, strengthened by secondary cell wall lignification, allowed tracheophytes to dominate the flora of the earth during the Carboniferous period.[4] The evolution of lignin biosynthesis, possibly through the elaboration of pathways originally involved in the synthesis of UV-protective pigments, was an essential step towards the successful colonization of terrestrial environments by plants.

Lignin from flowering plants is typically composed of three major building blocks, namely *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) subunits, which are derived from three phenylpropanoid alcohols, *p*-coumaryl, coniferyl and sinapyl alcohol, respectively, also known as monolignols. Research in phenylpropanoid metabolism, mainly in Arabidopsis and other agricultural important dicot species, has revealed that lignin biosynthesis in angiosperms requires three cytochrome P450-dependent monooxygenases (P450s).[5] Whereas cinnamic acid 4-hydroxylase (C4H) and *p*-coumaroylshikimic acid 3'-hydroxylase (C3'H) catalyze aromatic ring 4- and 3-hydroxylation reactions leading to the formation of H and G lignin, ferulic acid/coniferaldehyde/coniferyl alcohol 5-hydroxylase (F5H) diverts G lignin biosynthetic intermediates towards S lignin biosynthesis. It has frequently been considered that F5H is a recent innovation of the angiosperms because S lignin is generally absent in gymnosperms and ferns; however, S lignin has also been found in Selaginella species,[6-10] a genus representing the lycophyte lineage that diverged from other vascular plants 400 million years ago (Fig. 1A and B). This observation raises the intriguing question of how plants separated by 400 million years of evolution possess a comparable lignin biosynthetic repertoire.

Independent Recruitment of a P450 to S Lignin Biosynthesis in Selaginella

The fact that Selaginella deposits S lignin suggests that its genome encodes an enzyme with activity similar to an angiosperm F5H. To determine the identity of this protein, we isolated Selaginella *F5H* candidate genes from a previously reported *Selaginella moellendorffii* cDNA library.[11] Each of these genes was transformed into the F5H-deficient Arabidopsis *fah1-2* mutant but only one complemented all of the mutant's phenotypes,[12] suggesting that the encoded Selaginella P450 is a functional F5H in vivo (*Sm*F5H). When expressed in yeast, the protein's kinetic characterization revealed that *Sm*F5H resembles angiosperm F5Hs in that it favors coniferaldehyde and coniferyl alcohol over ferulic acid as substrates. Interestingly, *Sm*F5H is less than 40% identical to angiosperm F5Hs; whereas, Selaginella C4H and C3'H orthologs share over 60% identity to their angiosperm counterparts. More detailed phylogenetic analysis indicated that *Sm*F5H is not orthologous to angiosperm F5Hs, but rather is a member of a clade of eleven P450s unique to Selaginella. None of the other ten Selaginella P450s in this clade possesses F5H activity. We therefore conclude
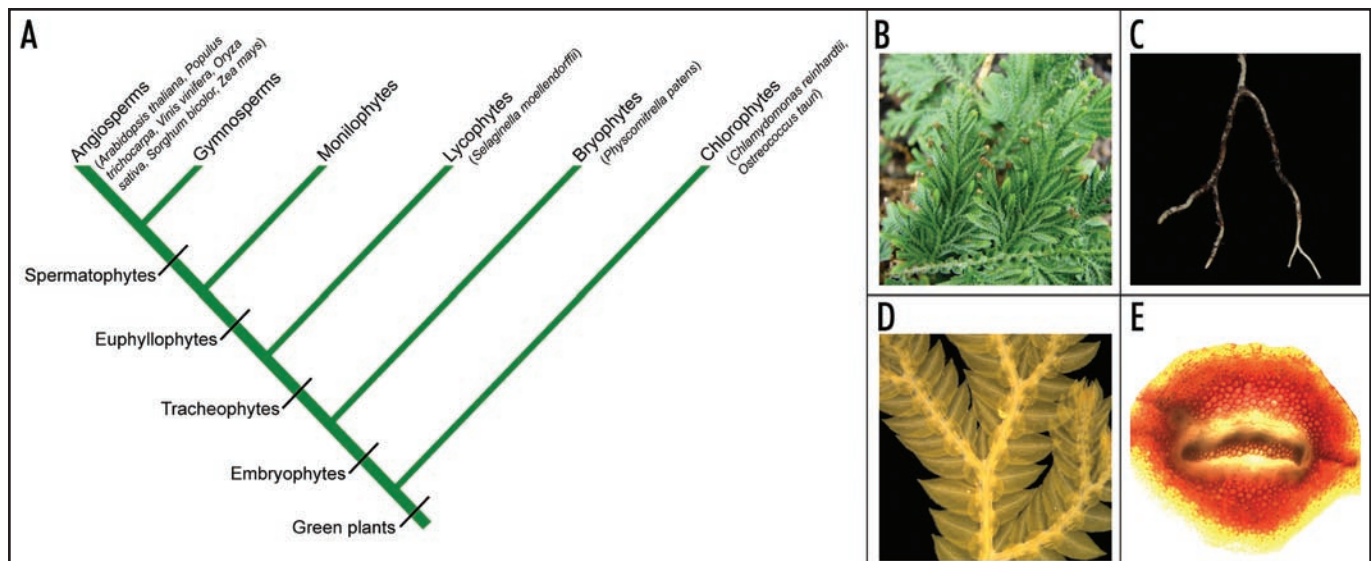
Figure 1. *Selaginella moellendorffii* represents an ancient land plant lineage. (A) A simplified cladogram illustrating the evolutionary position of Selaginella in the plant kingdom. Species with fully sequenced genomes are noted in brackets. (B) The aerial part of *Selaginella moellendorffii* showing dichotomous branching at the shoot. (C) Selaginella root with dichotomous branching pattern. (D) Selaginella microphylls with a single, unbranched vein emerged from the stele. (E) A cross-section of Selaginella stem showing protostelic vasculature.

that the origins of S lignin in Selaginella and angiosperms are due to convergent evolution of P450 activities.

Has S Lignin only Evolved Twice during Plant Evolution?

Reports from the older literature suggest that S lignin might have arisen multiple times in tracheophyte lineages. Using either histochemical staining or, by today's standards, relatively crude chemical degradation methods, S lignin has been detected in fern and gymnosperm species, including cuplet fern (*Dennstaedtia bipinnata*), yew plum pine (*Podocarpus macrophyllus*), sandarac-cypress (*Tetraclinis articulata)*, and all the three extant genera within the division Gnetophyta,[6,13-16] and we have verified the presence of S lignin in *Podocarpus macrophyllus* by the highly diagnostic derivatization followed by reductive cleavage method (DFRC)[17] (Fig. 2). Elucidation of the molecular mechanisms that underlie the deposition of S lignin in these plants will further advance our understanding of the evolution of both P450s and plant phenylpropanoid metabolism.

Selaginella moellendorffii as a Model for Comparative Biology

The evolution of the lycophytes, including extant species like *Selaginella moellendorffii,* has been independent of all the other vascular plants, collectively known as euphyllophytes, since their common ancestors diverged 400 million years ago.[11] Although Selaginella maintains many features that are considered to be primitive for vascular plants, including dichotomous branching pattern of both shoots and roots, microphylls without complex vein networks, and a protostele with xylem surrounded by phloem[18] (Fig. 1B–E), Selaginella may also have evolved developmental and biochemical processes that were uniquely elaborated in the lycophyte lineage. The fact that *Sm*F5H, as well as the other ten Selaginella P450s studied in our research, are not orthologous to any of the known P450s found in other plant lineages suggests these P450s may be representative

of such novel pathways. The recent availability of the *Selaginella moellendorffii* genome sequence makes this species an interesting target for comparative genomics, development and biochemistry.[19]

Using Angiosperm Model Systems to Study Selaginella Gene Function

Although the techniques for gene-knockout, knockdown and overexpression are still to be developed in Selaginella, our research and work from other labs suggests that established model systems such as Arabidopsis can be used as platforms to study Selaginella gene function in planta. For example, Harrison et al., studied the evolution of KNOX-APR interactions in determining leaf formation.[20] They showed that the APR homolog from Selaginella complements the Arabidopsis *APR* mutant *as1-1*, which provided in vivo evidence to support the hypothesis that Selaginella APR is functionally equivalent to its Arabidopsis counterpart.[20] Furthermore, two independent groups studied GID1-DELLA mediated gibberellin (GA) growth regulatory mechanisms in moss and Selaginella.[21,22] Whereas one group showed that both Selaginella GID1 and DELLA homologs can complement the respective mutants in rice,[21] the other group conducted a GA inducible green fluorescent protein (GFP) fluorescence quenching assay to show that transgenic GFP-tagged Selaginella DELLA can interact with Arabidopsis GID1 in vivo.[22]

Future Directions

Our research marks the first step towards understanding lignin biosynthesis, or phenylpropanoid metabolism in general, in Selaginella. Many other genes that encode pathway enzymes or regulatory factors remain to be characterized. Although Arabidopsis mutants for almost all the steps in the pathway are available, thus enabling studies similar to our recent work with *Sm*F5H, development of genetic resources and techniques, such as insertion mutant lines and *Agrobacterium*-mediated transformation, in Selaginella is still needed. This is particularly true when comes to identifying and
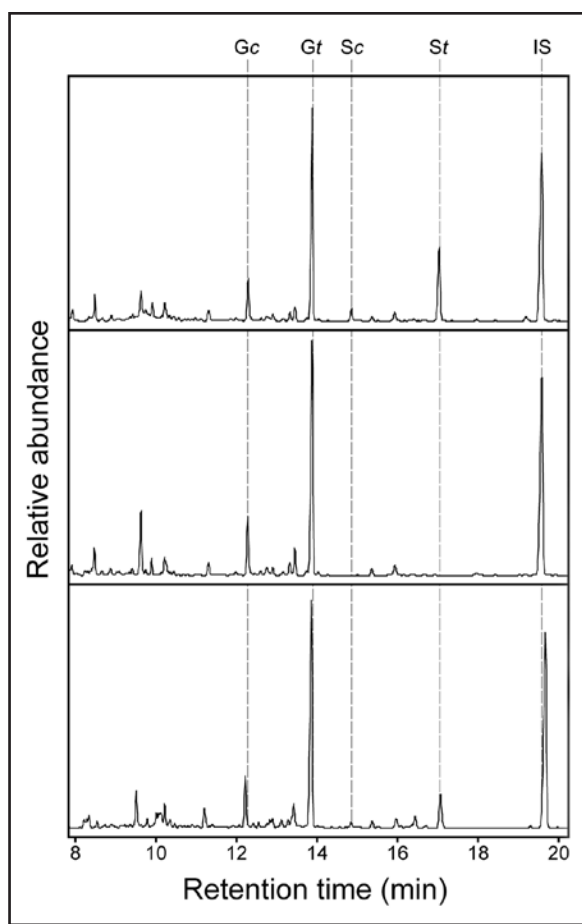
Figure 2. DFRC GC analysis of lignin monomer diversity in vascular plants. Arabidopsis Columbia wild type (top) and the *fah1-2* mutant (middle) serve as positive and negative controls for the presence of syringyl lignin. DFRC lignin analysis was performed as previously described.[17] DFRC analysis of a sample of *Podocarpus macrophyllus* (bottom) collected from the Chicago Botanic Garden reveals the presence of syringyl lignin in this gymnosperm. G/S, guaiacyl/syringyl lignin derivative; *c/t*: cis/trans; IS, internal standard.

characterizing novel pathways that do not exist in flowering plants. From a comparative genomics point of view, the recent completion of genome sequences for *Selaginella moellendorffii* and the moss *Physcomitrella patens*[23] (Fig. 1) has made it obvious that we need sequences for genomes from representative fern and gymnosperm lineages. Approaching gene functions in an evolutionary context, propelled by the development of further genomic resources, will ultimately enable us to understand how plants evolved and flourished on earth.

Acknowledgements

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