Gains achieved by molecular approaches in the area of lignification*

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Abstract: In this article we highlight the contribution of molecular biology and lignin genetic engineering toward a better understanding of lignin biosynthesis and spatio-temporal deposition of lignin. Specific examples from the literature and from our laboratory will serve to underline the chemical flexibility of lignins, the complexity of the regulatory circuits involved in their synthesis, and the specific behavior of different cell types within the xylem. We will also focus on strategies aiming to reduce the lignin content or to modify the lignin composition of plants and present their impact on plant development. We will show that the ectopic expression of a specific transgene may have a different impact, depending on the genetic background, and that plants with a severe reduction in lignin content may undergo normal development. Lignification is currently benefiting enormously from recent developments in molecular biology and transgenesis, and the progress made opens the way for future developments to study how the walls of lignified plant cells are built and organized.

INTRODUCTION

Lignification was undoubtedly a crucial process in the development and evolution of land plants. The primary function of lignins is to reinforce cell walls, rendering them rigid, hydrophobic, and impermeable to water. They impart resistance to compressive stresses imposed by water transport and by the mass of the plant. However, despite their importance in the adaptive strategies of tracheophytes (pteridophytes, gymnosperms, and angiosperms), the occurrence of lignins in plants dramatically affects their agro–industrial uses. The digestibility and dietary conversion of grasses are altered by the presence of lignins, and in the pulp industry lignins are undesirable components: the high proportion of this phenolic polymer in woody species entails complex, expensive, and polluting processes to remove it from cellulose, the main component of pulp.

Lignins intrinsically exhibit several specificities that make them rather unique in nature. These complex amorphous chains of phenolic molecules are the second most abundant after cellulose, accounting for nearly 30% of the organic carbon in plant biomass. Their synthesis results from a partially known long series of reactions which represents one of the most expensive biosynthetic processes in plants in terms of energy demand. The final product of this pathway is highly heterogeneous in nature depending on the internal location and on external parameters but always exhibit higher C/H and C/O ratios than the other polymers of the cell wall, resulting in a higher calorific value.

The complexity of the lignification process has been classically associated to the nature of the polymer itself resulting from the nonrepetitive association of different building units through a variety of chemical linkages. Complexity also arises from the occurrence of alternative pathways for the syn-

thesis of the three monolignols, the basic monomeric units of lignins, and to the additional presence of unusual phenolic units in the polymer.

In the plant, as a whole, lignification genes are only expressed in specific tissues, and this target-ed production of individual transcripts is likely controlled by partly characterized transcription factors. Finally, at the cellular and subcellular levels, complex and tight regulation appears to be involved in the deposition of lignins into the different cell types of xylem and the different sublayers of the cell wall.

These various aspects have been highlighted in recent years through the combined use of the tech-niques of molecular biology and transgenesis with the help of genetic studies. Some selected examples will be developed in the following sections.

LIGNIFICATION GENES: EVOLUTIONARY ASPECTS

A preliminary step in the use of molecular approaches for a better understanding of lignification has been the characterization of genes encoding different enzymatic steps along the lignin biosynthesis pathway. At the moment, due to upstream efforts in enzymology and protein isolation, all the genes encoding the enzymes of the basic pathway (with the exception of coumarate hydroxylase) have been cloned and sequenced. That is quite a remarkable result, when compared with the situation for other important biopolymers of the cell wall (cellulose, hemicelluloses) for which very few genes associated with the biosynthetic pathways have been characterized.

The genes associated with the downstream steps of the pathway and which are specific to the lig-nification process are present in pteridophytes, which were the first land plants able to synthesize true lignins. The evolutionary origin of these genes is still unclear, but results obtained in our laboratory sug-gest a potential relationship between certain ancestor genes and these specific lignification genes. We have found interesting homologies in protein sequence but also in the structure of the genes (identical relative locations of introns and exons) between cinnamoyl-CoA reductase (CCR) and dihydroflavonol 4-reductase (DFR) [1]. These observations suggest that either these closely related genes, with differ-ent functions, derive from a common ancestor gene or alternatively that DFR, which is involved in the synthesis of anthocyanins (assumed to be anterior or concomitant to lignins during evolution), was a precursor of CCR.

ALTERNATIVE PATHWAYS IN THE SYNTHESIS OF MONOLIGNOLS

Initially, the enzymatic steps controlling the synthesis of the methylated monolignols (coniferyl alcohol and sinapyl alcohol) were assumed to involve upstream processes in the phenylpropanoid pathways. These included cafeic acid O-methyltransferase (COMT) for the conversion of cafeic acid into ferulic acid and of hydroxyferulic acid into sinapic acid. Ferulic acid hydroxylase (F5H) catalyzing the trans-formation of ferulic acid into 5-hydroxyferulic acid is also implicated.

Recently, other enzymes and other pathways have been shown to be involved in the synthesis of the methylated monolignols. Following the unexpected characteristics of COMT down-regulated tobac-co plants [2,3], which exhibit a decrease in S units but not in G units, and the cloning and in-depth char-acterization of an alternative methylation enzyme cafeoyl CoA O-methyltransferase (CCoAOMT) [4], it was confirmed that several O-methyltransferases participate in the lignin pathway.

CCoAOMT seems to be preferentially associated with the synthesis of G units and COMT with the production of S units. In addition to the methylation of free hydroxycinnamic acids or their corre-sponding cinnamoyl CoA esters, it has been shown that these methyltransferases (or others) can also methylate cinnamaldehydes or cinnamyl alcohols. The target substrates of methylating enzymes are consequently diversified along the lignin biosynthesis pathway suggesting complex regulatory process-es for the control of the monomeric composition of lignins.

Another important step in the synthesis of S units is catalyzed by F5H. The properties of this membrane-located enzyme, belonging to the class of cytochrome P450 dependent monooxygenases,

have been investigated in detail using the recombinant protein. The data obtained show that the affinity of this hydroxylase is much higher for coniferaldehyde and coniferyl alcohol than for ferulic acid [5,6]. These results indicate that the synthesis of sinapyl alcohol is likely controlled at the step where coniferaldehyde or coniferyl alcohol are converted into their methylated products.

CHEMICAL FLEXIBILITY OF LIGNINS

Beyond the three classical monomers of lignins, namely *para*-hydroxycinnamyl alcohol, coniferyl alcohol, and sinapyl alcohol, it has been suggested that other phenolic units are incorporated into the polymer. This is the case for hydroxycinnamaldehydes, which are the reactive residues enabling the use of the Wiesner reagent for the histochemical detection of lignins. However, recent analyses of transgenic plants and plant mutants have revealed that this chemical flexibility is higher than initially expected.

The chemical composition of Arabidopsis lignins in mutant plants lacking F5H or in transformants overexpressing this enzyme highlights this potential flexibility. Indeed, the mutant lignins contain almost exclusively G units, and in the transformed plants the lignins are essentially composed of S units [7].

In addition to these massive compositional shifts in the nature of the monomers, other phenolic compounds have been demonstrated to be an integral part of lignins.

Recently, a lignin isolated from a pine mutant deficient in cinnamyl–alcohol dehydrogenase (CAD; EC 1.1.1.195) was characterized [8]. This lignin had significantly lower coniferyl-alcohol-derived units, compensated for by elevated aldehyde levels, and increased levels of dihydroconiferyl alcohol units. The CAD-deficient mutant appeared able to utilize other phenols as substrates for radical coupling reactions to produce polymers that may function similarly to normal lignins [9,10].

Isolated lignin of antisense-CAD tobacco contained fewer coniferyl and sinapyl alcohol-derived units which were compensated for by elevated levels of benzaldehydes and cinnamaldehydes. Products from radical coupling of cinnamaldehydes, particularly sinapaldehyde, which were barely discernible in normal tobacco, were major components of the antisense-CAD tobacco lignin. Lignin from antisense-CCR tobacco contained fewer coniferyl alcohol-derived units and significant levels of tyramine ferulate. Tyramine ferulate is a sink for the anticipated build-up of feruloyl-CoA, and may be up-regulated in response to a deficit of coniferyl alcohol.

TISSUE- AND CELL-SPECIFIC EXPRESSION OF LIGNIFICATION GENES

When the lignification genes have been available, the use of transgenesis and *in situ* hybridization techniques have demonstrated that the expression of these genes is specifically targeted to individual tissues or cells. The temporal and spatial expression of the genes was essentially investigated using promoter-reporter gene (GUS) fusions in transgenic plants.

Functional analysis of the eucalyptus CAD promoter in transgenic poplar revealed gene expression primarily in regions undergoing active lignification: phloem fibers, differentiating xylem, xylem ray cells, and vascular cambium [11] [12]. This pattern of expression was confirmed by *in situ* hybridization [12] and immunolocalisation techniques [13]. A similar vascular pattern of expression was found in transgenic tobacco plants containing either the tobacco [14] or the eucalyptus CAD promoter (V. Lauvergeat, personal communication), suggesting that annual herbaceous plants such as tobacco can be used to study the expression of genes from perennial woody angiosperms. The developmental regulation of the *Eucalyptus gunnii* EgCCR promoter was also analyzed through the expression of EgCCR-GUS fusions in tobacco. EgCCR promoter activity was strongest in lignified organs (stems and roots) consistent with the EgCCR mRNA level in these organs. Histochemical analysis showed expression in vascular tissues (cambium, young differentiating xylem, ray cells, internal and external phloem) of stems and roots in agreement with *in situ* hybridization data. Promoter deletion

analysis and gain-of-function experiments have identified the sequences between positions -119 and -77 as necessary and sufficient for expression in vascular tissues of stems [15].

More specifically, the lignin composition of each individual cell appears to be controlled by a complex array of gene expression in the differentiating cells or in adjacent cells as suggested, for example, by the recent results of Chen *et al*. [16]. These authors have shown that in transgenic *Populus tremula x P. alba* hybrids, the expression of CCoAOMT promoter–GUS fusions is observed in differentiating xylem preferentially in contact rays adjacent to the vessels but is not detectable in other ray cells or in fibers. This specific expression of the CCoAOMT gene involved in the synthesis of G units could be related to the enrichment of vessels in these units. These data strongly suggest different control of monolignol biosynthesis in the different xylem cell types.

It is clear that the targeted gene expressions are likely due to a specific activation of the transcription machinery by individual or combined transcription factors. The identification of such factors is just starting, and a cDNA clone (Nt lim1) encoding a PAL-box binding protein was recently shown, in antisense tobacco plants, to reduce the level of transcripts for different genes along the phenylpropanoid pathway and the lignin-specific pathway [17].

LIGNIN GENETIC ENGINEERING

Lignin genetic engineering has opened new perspectives concerning the production of improved biomass more suited to different agroindustrial uses and particularly to the pulp industry. Some selected examples will be provided in the next paragraphs.

CAD down-regulated transgenic lines have been obtained by different groups [18–20]. Even though the lignin content is not affected, the transgenic lignins are enriched in hydroxycinnamaldehydes, and these structural changes induce both a pink/red coloration of the xylem and an increased extractability of these lignins as indicated by a decrease in Kappa number during the bleaching of the pulp. Recent results obtained within the TIMBER consortium funded by the E.C. (unpublished results) have shown on a poplar clone of economic interest (OGY) that, for a strong reduction of CAD activity, the lignin content is slightly reduced and that the higher percentage of free phenolic groups makes these lignins particularly soluble in mild alkaline conditions (Lapierre, personal communication). These characteristics are very positive for the pulp industry and will lead to savings in chemicals and energy during the pulping process.

Interestingly, field trials have demonstrated the stability of transgene expression and of changes in lignin profiles in 4-year-old CAD down-regulated poplar trees. In addition, the genetic transformation does not induce significant changes in development or agronomic characteristics, neither does it apparently cause negative environmental impact. Overall, these data show that the strategy of CAD down-regulation may be of immediate utility and can be potentially extended to a large number of woody species.

The down-regulation of other target genes of the common phenylpropanoid pathway 4 CL [21] or of the monolignol specific pathway CCR [9] has demonstrated that the lignin content of the resulting transgenic plants can be strongly decreased (up to 50%). Surprisingly, this reduction in lignin content and (or) some associated processes induce opposite effects on the development of the plants. 4 CL down-regulated aspens appear to grow faster, whereas CCR down-regulated tobaccos display a severe reduction in growth and other alterations in shape and morphology. The explanation for these discrepancies is not clear at the moment. However, recent data [22] suggest that it is not the reduction of the amount of lignins *per se* which is responsible for the altered development of the tobacco plants. Indeed double transformants (down-regulated both for CCR and CAD) display a decrease in global lignin content similar to the CCR down-regulated line but show normal development. In fact, these two kinds of transformants differ by the spatial deposition of lignins. The CCR down-regulated line exhibited a reduction in lignin deposition in the walls of the different xylem cell types, whereas this reduction was selectively targeted to the fibers in the double transformant. Thus, it appears that different events of

transformation leading, in the same species, to a similar reduction of lignin content may induce different patterns of lignin deposition at the cellular and subcellular levels.

These results underline that lignification is tightly regulated in individual cell types and that it should be possible to accurately target lignin modifications to specific cells in the future. Transgenic lines with reduced lignin content and normal (CAD x CCR) or improved (4 CL) development, which have been obtained until now in growth chambers or greenhouses, would be of great interest if it can be demonstrated that they behave as control plants in field conditions.

CONCLUSIONS

It is a sort of paradox to observe that the lignin field, which has for a long time been a typical domain of interest for chemists, has been efficiently explored during recent years with the help of molecular approaches. Indeed, plant science is currently benefiting enormously from recent developments in this area which not only provide new information but also allow the findings to be set in the context of plant function.

It is noteworthy to remark how lignin biosynthesis, a complex aspect of plant secondary metabolism, has been highlighted by these techniques which have led to significant reassessment of certain widespread assumptions concerning lignin composition and the biosynthetic pathways of the polymer.

Recent results have also shown that lignin deposition is tightly and carefully regulated at the cellular and subcellular levels. These data pave the way for future studies of how the walls of lignified plant cells are built and organized. They are also a prerequisite for interesting biotechnological applications.

We assume new aspects of lignification will continue to be discovered through the increased exploitation of molecular approaches and researchers will learn more about the role and impact of lignins in plant biology.

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