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The polymorphism of the genes/enzymes involved in the last two reductive steps of monolignol synthesis: what is the functional significance? ☆

Alain-Michel Boudet [a,*], Simon Hawkins [b], Soizic Rochange [a]

[a] *UMR UPS/CNRS 5546, Pôle de biotechnologie végétale, 24, chemin de Borderouge, Auzeville, 31326 Castanet, France*
[b] *Laboratoire de physiologie des parois végétales, UPRES EA 3568/USC INRA, UFR de biologie, bât. SN2, université des sciences et technologies de Lille, 59655 Villeneuve d'Ascq cedex, France*

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Abstract

The polymorphism of genes and enzymes involved in the last two steps of monolignol synthesis is examined in the light of recent data coming from genomic studies and mutant/transformant analyses. The two catalytic activities considered – cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) – are encoded by small multigene families. While some degree of diversification can be noted at the sequence level, it is often difficult to use this information to assign substrate specificities to each member of a gene family. Expression profiles, however, suggest for both CAD and CCR the existence of two sub-families: one devoted to developmental lignification, and the other involved in the synthesis of defence-related compounds. ***To cite this article: A.-M. Boudet et al., C. R. Biologies 327 (2004).***

Résumé

Le polymorphisme des gènes/enzymes impliqués dans les deux dernières étapes de la synthèse des monolignols : quelle signification fonctionnelle ? Le polymorphisme des gènes et enzymes impliqués dans les deux dernières étapes de la synthèse des lignines est examiné à la lumière des données récentes de la génomique et de l'analyse de mutants ou transformants. Une petite famille multigénique correspond à chacune des activités catalytiques considérées : cinnamoyl-CoA réductase (CCR) et alcool cinnamylique déshydrogénase (CAD). Les différences de séquence observées au sein de chaque famille ne permettent pas de prédire les spécificités de substrat de chacun des membres. L'analyse des profils d'expression suggère, en revanche,

☆ This paper is a worthy reminder of our diverse meetings in France and abroad, and, in particular, in memory of the unusual initiation to Molecular Biology given to the pupil Bernard Monties in the rural surroundings of a small East German town.

* Corresponding author.
E-mail addresses: amboudet@smcv.ups-tlse.fr (A.-M. Boudet), rochange@scsv.ups-tlse.fr (S. Rochange).

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l'existence, dans chaque cas, de deux sous-familles, l'une dédiée à la lignification constitutive et l'autre impliquée dans la synthèse de composés de défense. ***Pour citer cet article : A.-M. Boudet et al., C. R. Biologies 327 (2004).***

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Mots-clés : monolignol ; lignine ; phénolique ; paroi ; génomique

1. Introduction

It has been known for decades that lignin synthesis and deposition lies under tight developmental control, since lignin monomer composition varies between cell types, between sub-layers of the wall and along the course of development in one and the same species. In addition, lignins from different species differ in their abundance and constitution. Numerous studies have focused on the determinism of this variability (for reviews, see [1–3]), partly because lignin profiles affect the efficiency of lignocellulose use in downstream agro-industrial processes. At this stage, if there is evidence that lignification is regulated at the levels of monolignol synthesis, polymer assembly, and deposition into the cell wall, the mechanisms underlying this control are still not fully understood.

If we only consider the determinism of lignin monomer composition, an important factor in the pulping process, it is clear that control mechanisms occurring in two compartments, the protoplast and the wall, might be involved. Indeed, the rate of synthesis and availability of individual monolignols and the wall microenvironment are equally important.

The fact that lignin polymerisation is initiated in the middle lamella implies that a monolignol does not undergo dehydrogenative coupling prematurely as it diffuses from the plasma membrane into the lignifying matrix. Rather it presumably must first encounter a growing lignin domain associated with a complementary initiation site. The nature of these initiation sites is a question of central importance. It is not known whether they only facilitate the oxidation and polymerisation of phenoxy radicals or if they guide the synthesis of the lignin polymer through arrays of dirigent proteins [4] or other macromolecules. For example the localization of proline-rich protein (PRP) epitopes has been spatially and temporally correlated with lignin in developing cell walls of maize [5]. These putative dirigent sites may play an important role in determining the nature of the moieties incorporated into the lignin polymer, as well as the type of inter-unit linkages [4]. The polymerisation step is, however, dependent on the oxidation of monolignols into phenolic radicals, which constitutes another level of control, since the corresponding wall-located enzymes can exhibit some degree of specificity. For example, some peroxidases show a substrate preference for sinapyl rather than coniferyl moieties [6]. In addition, Brunow's group has suggested a role for oxidation potential and local concentrations of monolignols in the polymerisation process. They have shown that cross coupling between lignin precursors and the lignin polymer occurs only within a restricted range of oxidation potentials of the phenols and should not be regarded as a random process [7]. The same group [8] has also emphasized the role of local concentrations of coniferyl alcohol at the sites of lignification in the control of lignin structure.

Beyond the importance of the wall microenvironment described above, the relative fluxes of synthesis of the different monolignols are likely a key factor in determining lignin composition. The synthesis of monolignols has been the subject of extensive study (reviewed in [1–3]) leading to the characterisation of most enzymes involved in the process and to the cloning of the corresponding genes. Transgenic plants suppressed for or overexpressing these genes turned out to be a very powerful tool to understand their actual functions. While the impact of induced or natural changes in the activity level of specific biocatalysts along the pathway on the final lignin 'product' has been assessed in detail, enzyme diversity between/within species has received relatively little attention. Recent data coming from genomic studies and in some cases structural biology have shed some light on the polymorphism of several enzymes/genes involved in lignin biosynthesis. In this review, we will try to understand the functional significance of the polymorphism of the enzymes catalysing the last two

reductive steps of monolignol synthesis (cinnamoyl CoA reductase and cinnamyl alcohol dehydrogenase) and to estimate the contribution of this polymorphism to the versatility of lignin synthesis.

2. Cinnamoyl CoA reductase (CCR)

Three monolignols – coumaryl, coniferyl, and sinapyl alcohols – are the precursors of H, G and S lignin units, respectively. They result from the reduction by cinnamoyl CoA reductase of the corresponding hydroxycinnamoyl-CoA esters into hydroxycinnamaldehydes which are further converted into hydroxycinnamyl alcohols by cinnamyl alcohol dehydrogenase. Cinnamoyl CoA reductase has been purified and characterized from cambial sap of spruce (*Picea abies*, [9]), soybean cell cultures (*Glycine max*, [10]), poplar stems (*Populus euramericana*, [11]), and *Eucalyptus gunnii* xylem [12]. The first cDNA-encoding CCR was cloned from *Eucalyptus gunnii* [13]. Consistent with its role in lignin biosynthesis during plant development, expression of the *E. gunnii* gene was demonstrated to be high in lignifying tissue.

2.1. Genes encoding CCR in Arabidopsis thaliana

In Arabidopsis, a homology-based search of EST databases yielded two CCR genes [14]. Their identity was confirmed by the catalytic properties of the corresponding recombinant enzymes. Expression analyses suggested the involvement of AtCCR1 in lignification of vascular tissue. Independently, Jones et al. [15] described the *irx4* mutation, which affected AtCCR1 and resulted in collapsed xylem elements and reduced lignin levels. The role of AtCCR1 in constitutive lignification was further ascertained more recently through an antisense strategy [16].

The other gene, AtCCR2, was shown to be responsive to pathogen attack [14]. Lignins play an important role in plant defence and indeed, one of the characteristic responses of plants to injury and disease is the reinforcement of cell walls in the vicinity of the wound/infection site with lignin, as well as with suberin [17–19]. The role of AtCCR2 in stress response could be twofold: synthesis of defence lignins that help prevent the progression of bacteria, and/or synthesis of other defence-related compounds. It has already been reported that coniferaldehyde and coniferyl alcohol may function as phytoalexins in the incompatible interaction between flax and *Melampsora lini* [20].

More recently, Raes et al. [21] demonstrated by RT-PCR the expression of AtCCR2 in stems at late developmental stages, seedlings, and roots. Moreover, the expression of AtCCR2, but not AtCCR1, was enhanced in cellulose-deficient Arabidopsis seedlings exhibiting ectopic lignification [22]. Hence, AtCCR2 may take part in the synthesis of monolignols in some tissues and in response to developmental cues. It would be of interest to study an AtCCR2 mutant to investigate the involvement of this isoform in defence-related and/or developmental lignification.

After completion of Arabidopsis genome sequencing [23], 11 sequences have been annotated as 'putative CCR', although their identity with AtCCR1 can be as low as 32.8%. As underlined by Costa et al. [24], their catalytic properties have not been assessed biochemically, with the exception of AtCCR1 and AtCCR2. Hence we propose that, based on current evidence, there are only two *bona fide* CCR genes in Arabidopsis. It is clear that conclusions drawn only from sequence homology can lead to misannotation of genes, since enzymes with distinct biochemical functions can share a common ancestor and hence exhibit a strong degree of homology, making it difficult to delimit gene families appropriately *in silico*. A recent analysis of Arabidopsis genes involved in the phenylpropanoid pathway [24] revealed that for most enzyme families considered, gene identification awaits experimental evidence such as the properties of recombinant proteins or the study of mutants.

2.2. Genes encoding CCR in other species

New CCR genes have been isolated on the basis of homology to known CCR clones from a few agronomically important species including maize [25], poplar [26], and tomato (Benoît van der Rest, unpublished data). So far, the number of CCR isoforms identified per species has never exceeded two. In maize, as in Arabidopsis, the two isoforms show differential expression patterns, with one predominantly associated with lignifying cells. So, it appears that CCR polymorphism within species is very limited. Paralogues exhibit distinct expression profiles, which probably re-

late to separate functions of the corresponding genes. There would be one CCR dedicated to constitutive lignification, and in some cases another one potentially involved in defence mechanisms.

2.3. Consequences of a reduced CCR activity

The first transgenics with downregulated CCR activity were obtained in tobacco [27]. Lignin content was decreased down to 50% of the wild-type level for transgenic lines with a dramatic reduction of CCR activity. This represents the strongest reduction of lignin content that can be achieved by downregulation of a single enzyme in the pathway.

In Arabidopsis *irx4* mutants, total lignin content estimated by thioglycolic acid assays was decreased by more than 50% in inflorescence stems, and phloroglucinol or Maüle staining failed to reveal the presence of 'classical' lignin in the xylem (these staining methods indicate the presence of cinnamaldehydes and S units respectively). These mutants most likely lack a functional AtCCR1 [15], meaning that their level of residual CCR activity is presumably very low. Unfortunately, no details of CCR activity or lignin composition in these plants have been published yet. Goujon *et al*. [16] described a set of AtCCR1 antisense lines with residual CCR activities down to about 20% of the wild type. Again, the lignin content was severely reduced in these transformants. Detailed analysis of lignin composition by thioacidolysis showed no consistent change in S/G ratio, while ferulic acid accumulated in lignins to 2–4 fold the level measured in the wild type. The general conclusion we can draw from these studies is that CCR downregulation affects lignin content by blocking the pathway to cinnamaldehydes, which results in the incorporation into lignin of earlier precursors like ferulic acid [28]. The S/G ratio being essentially unaffected, we can hypothesize that CCR does not play an important role in the control of this parameter.

Another important observation coming from antisense studies is that it takes a strong downregulation (a threshold was observed at around $< 30\%$ activity) to bring about a decrease of lignin content [16,27]. Therefore, although the reaction catalysed by CCR is essential for monolignol synthesis, it is unlikely the rate limiting step in the pathway, since the activity is far in excess at least in mature organs.

3. Cinnamyl alcohol dehydrogenase (CAD)

Hydroxycinnamoyl CoAs are converted into hydroxycinnamyl alcohols by cinnamyl alcohol dehydrogenase. This enzymatic conversion was for a long time thought to be performed by a single enzyme with broad specificity (cinnamyl alcohol: NADP(+) dehydrogenase, CAD, E.C. 1.1.1.195), able to catalyse all three reductions, the relative synthesis of each monolignol being dependent on the respective availabilities of the corresponding aldehydes.

In this part of our review, we will detail how subsequent studies showed that (a) this activity is in part shared by two unrelated enzyme families, and (b) extensive diversification has occurred in one of these families and led in some cases to the appearance of divergent members with distinct catalytic properties.

3.1. At least two unrelated enzyme families can catalyse the conversion of cinnamaldehydes into monolignols

CAD activity was first isolated from *Picea abies* and *Glycine max* in 1981 [9]. Several years later, purification of the corresponding enzyme was undertaken from Eucalyptus xylem. This work led to the identification of two major peaks of activity on coniferyl alcohol. The corresponding enzymes, named CAD1 and CAD2, were then purified to apparent homogeneity and their catalytic properties were further analysed. While CAD2 can reduce all three cinnamyl aldehydes into the corresponding alcohols, CAD1 only uses coumaryl and coniferyl aldehydes, and exhibits a lower affinity for these substrates than CAD2. Cloning and sequencing of CAD1 and CAD2 genes revealed that their amino acid sequences did not share any homology. Rather, CAD1 is related to CCR and dihydroflavonol reductase (*DFR*) sequences (48% identity/70% similarity and 43% identity/62% similarity, respectively).

A search for CAD1 homologues in EST databases allowed us to identify closely related proteins in many species. CAD1 seems to be highly conserved in tracheophytes: *Eucalyptus gunnii* CAD1 has 80% identical homologues in angiosperms and 74% identical homologues in some gymnosperms. In non-vascular land plants (e.g., *Physcomitrella patens*), expressed

sequences homologous to CAD1 and CCR could represent a putative common ancestor for these genes. Based on available sequences, the *Physcomitrella* protein would be 78% and 67% similar to *E. gunnii* CAD1 and CCR, respectively.

CAD1 and CAD2 belong to two different families of alcohol dehydrogenases: short chain dehydrogenases and zinc-binding dehydrogenases, respectively. The active form of CAD2 is a dimer while CAD1 is active as a monomer.

Because CAD2 efficiently catalyses the synthesis of all three monolignols, it was considered to be the more relevant enzyme for lignification. The first cDNA-encoding CAD2 was cloned from tobacco [29], and the first transformants carrying antisense CAD2 constructs were also obtained in this species [30]. The analysis of these plants as well as transformants and mutants in other species established the role of CAD2 in lignification in both gymnosperms and angiosperms.

Subsequent search for CAD genes from other species was always homology-based (using CAD2 as a probe), and thus led only to the identification of CAD2 orthologues. Therefore, genes annotated as CAD1 in various species (including Arabidopsis and alfalfa) do not correspond to CAD1 but to CAD2 orthologues.

Tobacco CAD1 was further characterised in our lab [31] and was shown to use a wider range of substrates, including benzaldehyde derivatives. Besides the obvious participation of CAD2 in lignin synthesis, the involvement of CAD1 in lignification cannot be ruled out for the following reasons:

– CAD1 was originally extracted from xylem tissue;
– CAD1 is not able to synthesize S units, but in certain tissues or developmental stages lignins mostly comprise G units (e.g. primary xylem).

In this respect, it is interesting to note that the 'red xylem' phenotype characteristic of CAD2-downregulated plants is confined to secondary xylem: primary xylem cells, which are also lignified, appear normal [32]. Also, lignins enriched in H and G units can be found in the outermost part of the cell wall (middle lamella and cell corners), this region corresponding to the initiation sites for the assembly of lignin polymers.

3.2. Diversity of CAD2-related enzymes

The synthesis of syringyl units of lignin represents an example of pathway diversification in angiosperms since gymnosperms are devoid of such units in their lignins. Li *et al*. [33] have suggested that the reduction of coniferyl and sinapyl aldehydes was achieved by distinct enzymes. The authors postulated that the previously identified CADs were guaiacyl-specific, and identified a new syringyl-specific isoform, termed SAD for sinapyl alcohol dehydrogenase. Interestingly, they went about searching for SAD by probing an aspen cDNA library with a CAD2 clone. Two cDNAs were isolated: one with very high homology with CAD (PtCAD), and the other with only 50% homology (PtSAD). Recombinant enzymes were able to produce all three monolignols, but with a strong preference for coniferyl and for sinapyl alcohol, respectively. The distinct CAD and SAD functions are linked spatiotemporally to the differential biosynthesis of guaiacyl and syringyl lignins in different cell types (vessels enriched in guaiacyl units/phloem fibres enriched in syringyl units). The authors conclude that the last step of the synthesis of G and S units occurs via distinct enzymes in angiosperms. However, sinapaldehyde is abundant in lignins of CAD-deficient poplars [34], which suggests that SAD may not play a substantial role in constitutive lignification in poplar. Here again, studies in Arabidopsis can shed light on the question of CAD polymorphism, since (a) the full genome sequence and a great number of ESTs are available, (b) recombinant proteins have been obtained for all CAD2 homologues and their catalytic properties analysed, and (c) mutants for the main CAD2 isoforms have been described [35]. Among nine genes encoding putative cinnamyl alcohol dehydrogenases [24,36], only two (At3g19450 and At4g34230) possess a significant activity on hydroxycinnamaldehydes, based on the analysis of the corresponding recombinant proteins [37]. Another four (At4g37990, At4g37980, At2g21890, At2g21730) show comparatively little activity, while the last three (At4g39330, At4g37970, At1g72680) did not exhibit any detectable CAD activity. None of them was found to behave like a SAD (i.e. exhibited a preference for sinapaldehyde). Among these genes, three seem to be expressed at a very low level, or not at all, as suggested by the absence of

the corresponding sequences in EST databases [36]: At2g21890, At2g21730 and At4g37970.

These data suggest that there is no specific SAD in Arabidopsis, and that conversion of sinapaldehyde to sinapyl alcohol is achieved by the two main CAD isoforms (At3g19450 and At4g34230). This is confirmed by the analysis of the corresponding mutants [35]: the S/G ratio decreased in both AtCAD-C and AtCAD-D mutants, showing that both isoforms participate in the synthesis of S units.

Looking at the molecular data available for other angiosperms, it is difficult to tell how widely SAD is represented, especially because large EST collections or genomic sequences are only available for very few plants. Many species possess several genes homologous to CAD2. Strong homologies with SAD (in the range of 65 to 75% identity) can be found in several species, notably woody species (e.g., *Citrus sinensis*, *Vitis vinifera*, *Prunus persica*). In many other taxa, the EST that comes closest to SAD is only around 50% identical, and most likely represents another type of CAD variant.

Consistent with these observations, a biochemical approach based on isotope tracer experiments performed on Robinia, Oleander, Magnolia and Arabidopsis revealed that the synthesis of S units is achieved via different pathways in these species [38]. Robinia and Oleander have a pathway that produces sinapyl alcohol from sinapic acid via sinapoyl CoA, while Magnolia and Arabidopsis have a sinapyl alcohol biosynthetic pathway that does not require sinapic acid as an intermediate. Similarly, previous data from Chen et al. [39] obtained on *Magnolia kobus* showed the incorporation of deuterium-labelled coniferyl alcohol into syringyl units. Hydroxylation and methylation steps would then occur at the level of the monolignols themselves. Together, these data strongly suggest that the S lignin biosynthetic pathway varies among Angiosperms.

An in-depth biochemical study of *Eucalyptus gunnii* CAD2 iso-enzymes purified from periderm tissue [40] introduced the idea that the polymorphism of CAD catalytic properties could result in part from distinct combinations of subunits. In that study, it was shown that CAD2 isoforms were dimers of two different subunits. The two CAD subunits might represent allelic differences as previously reported in loblolly pine [41] or the products of two different genes as reported for soybean aliphatic alcohol dehydrogenase [42]. They were differentially combined to give the heterodimer but also two homodimers, resulting in three different combinations associated with distinct enzymatic properties. The high molecular weight homoCAD works most efficiently with sinapaldehyde, whereas heteroCAD works equally efficiently with both sinapaldehyde and coniferaldehyde. These data suggest that beyond the differences in protein sequence, combinatory mechanisms can generate a polymorphism of functional significance.

As in the case of CCR, there seems to exist a subfamily of CAD-related enzymes devoted to defence. For example, two lucerne CAD cDNAs (*Msa*CAD1 and *Msa*CAD2) are differentially regulated during development and plant defence [43]. A significant amount of data [19,44,45] suggests that the chemical composition of defence lignin is different to that of developmental lignin. In Eucalyptus plantlets, for example, defence lignins produced after wounding are poor in syringyl units unlike the usual G/S mixed type. Preliminary approaches indicated that two different types of cinnamyl alcohol dehydrogenase activity are induced and apparently regulated differentially, in response to wounding in Eucalyptus trees [19].

In addition to 'true' CAD cDNAs that have been isolated from a number of species (for a review, see [46]), several other cDNAs showing a homology of approximately 50% with the typical CAD cDNAs have also been characterized. Interestingly, these other cDNAs were all initially isolated as pathogen-related defence genes. For example, the *ELI-3* (for *eli*citor-*i*nduced 3, [47]) transcript was identified in a cDNA library of parsley cells challenged with a fungal elicitor. Later, two ELI3 homologues were identified in Arabidopsis. Sequence analysis first suggested that ELI3 was a mannitol dehydrogenase. Further studies involving ELI3 recombinant protein proved this assumption wrong and revealed that the highest activity of ELI3 was against benzaldehydes. Although ELI3 shares significant homology with CAD2 (60% similarity of the amino acid sequences), it is only weakly active on cinnamaldehydes [48]. It is not clear at the moment whether the proteins encoded by these genes participate in the synthesis of unusual lignin monomers and thus in lignification or in the production of specific phenolics with a putative role in defence.

Hence, it appears that CAD genes have evolved into larger families than CCR (particularly in angiosperms), which resulted in a diversification of biochemical function: some members of the family could be specialized in the synthesis of lignin monomers, while others (like ELI-3) are involved in the production of defence-related compounds outside the scope of lignin synthesis.

4. Conclusions

In contrast to individual adaptive strategies of different plant species involving specific biochemicals, lignification represents a common and basic mechanism in vascular plants or Tracheophytes (Pteridophytes, Gymnosperms and Angiosperms). However, lignins may significantly differ in their composition and structure, depending on the species, organ, tissue and subcellular compartment.

Recent data from genomics and genetic engineering have provided new information on the diversity and functional significance of a variety of enzymes/genes potentially involved in lignin biosynthesis. However, little evidence is available for assigning substrate specificities to each individual gene product, except in cases where biochemical function has been probed experimentally, for example with recombinant proteins. As far as the last two steps of monolignol synthesis are concerned, two main conclusions can emerge: (a) gene polymorphism is limited, (b) expression patterns suggest a function for some members of these small multigene families in defence.

These observations do not support, as sometimes postulated, a key function for CCR and CAD in controlling the monomeric composition of developmental lignin. From the data presently available, it appears that the determinism of lignin monomer composition is more dependent on enzymes involved in the interconversion of lignin precursors at the aldehyde or at the alcohol stages: ferulate 5-hydroxylase (F5H) and caffeic acid *O*-methyltransferase (COMT). However, events such as the combination of different subunits in the case of CAD2 correspond to the creation of diversity at a different level and might contribute to a certain extent to the control of lignin composition.

Furthermore, another level of lignin variability is likely associated to still unknown wall events controlling both composition and structure of the polymer. Altogether, this plasticity may actually be, as underlined by Hatfield and Vermeris [49], an adaptive advantage particularly in the defence against pathogens. Indeed the lack of regularity of the polymer poses a problem to the evolution of hydrolytic enzymes in fungi and insects [50], and lignin types varying in composition but also in structure are likely less susceptible to common degradative mechanisms.

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