Abiotic and biotic stresses and changes in the lignin content and composition in plants

Running title: Lignin changes caused by stresses

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Abbreviations: PAL - phenylalanine ammonia-lyase; C4H - cinnamate 4-hydroxylase; C3H - cinnamate 3-hydroxylase or p-coumaroyl shikimic acid/quinic acid 3-hydroxylase; OMT - O-methyltransferase; HCT - p-hydroxy cinnamoyl-CoA:shikimate/quininate p-hydroxy cinnamoyl transferase; CCoAOMT - caffeoyl coenzyme A O-methyltransferase; F5H - ferulate 5-hydroxylase; 4CL - 4-coumarate: coenzyme A ligase; CCR - cinnamoyl coenzyme A reductase; Cald5H - coniferaldehyde 5-hydroxylase; AldOMT - 5-hydroxy coniferaldehyde O-methyltransferase; SAD - sinapyl alcohol dehydrogenase; CAD - cinnamyl alcohol dehydrogenase.

Abstract: Lignin is a polymer of phenylpropanoid compounds formed through a complex biosynthesis route, represented by a metabolic grid for which most of the genes involved have been sequenced in several plants, mainly in the model-plants Arabidopsis thaliana and Populus. Plants are exposed to different stresses, which may change lignin content and composition. In many cases, particularly for plant-microbe interactions, this has been suggested as defence responses of plants to the stress. Thus, understanding how a stressor modulates expression of the genes related with lignin biosynthesis may allow us to develop study-models to increase our knowledge on the metabolic control of lignin deposition in the cell wall. This review focuses on recent literature reporting on the main types of abiotic and biotic stresses that alter the biosynthesis of lignin in plants.

Key words: disease, drought, light stress, low temperature, mineral nutrition, plant growth, plant breeding, reaction wood.
Introduction

Lignin (from the Latin word *lignum*, wood) is a highly branched polymer of phenylpropanoid compounds, and a component of the plant cell wall. After cellulose, lignin is the second most abundant organic compound in plants, representing approximately 30% of the organic carbon in the biosphere (Boerjan et al., 2003).

The lignification process seems to have appeared on Earth 430 million years ago (Boudet, 2000) among the possible strategies that allowed plants to adapt to terrestrial life. The functional significance of lignin is associated mainly with the mechanical support allowing plants to stand, with water transport in the xylem vases and as a defence against pests and microorganisms (Boudet, 2000). The first two characteristics are related to the way lignin interacts with other molecules of the cell wall, strengthening the whole structure.

Despite the importance of lignin in plant evolution and development, it represents a problem for the agro-industrial application of several plants, particularly in the papermaking industry. The processes used for the separation of lignin from the cellulose pulp are the most costly and polluting parts of the whole process (Hatfield and Fukushima, 2005). Consequently, several studies have investigated how to improve pulping efficiency (Akgül et al., 2007), as well as alternative pulping methods (Khristova et al., 2006; Huang et al., 2007).

In addition to the chemical studies trying to find more efficient and environmentally friendly methods for lignin extraction, there are also several laboratories around the world seeking to genetically manipulate lignin structure and content. Genetically modified plants with less lignin and/or with a different structural lignin composition may allow for improved cellulose yield during pulping and, at the
same time, reduce the level of pollutants released to the environment. *Arabidopsis thaliana* and *Populus* have been used as model-plants in most of these studies.

Within this context, lignin biosynthesis has been shown to be the result of a complex genetic network (Rogers and Campbel, 2004), where several enzymes are involved and may respond differently to biotic and abiotic effects (Figure 1). In this regard, the literature is rich with examples where lignin content in the plant body is increased or exhibits a change in chemical composition due to stressors, suggesting complex genetic and physiological control. It is possible that understanding how a stressor “modulates” the expression of “lignin genes” would allow us to develop study-models to elucidate genetic control of lignin synthesis and its deposition in the cell wall.

Different types of abiotic stresses, such as mineral deficiency, drought, UV-B radiation and low temperatures, as well as biotic stresses, such as infection by fungi, bacteria and viruses, cause changes in the lignin contents of several plants. Thus, this review focuses on the recent literature reporting on the main types of abiotic and biotic stresses that alter the biosynthesis of lignin in plants.

**Low temperatures**

Low temperature is an important factor limiting the extension of cultivating areas and therefore the productivity of growing several plants. It is known that, in many species, a prior exposure to non-freezing low temperatures allows survival following further exposure to freezing temperatures (Chinnusamy et al., 2007). Understanding which mechanisms are involved in the acclimation to cold would allow researchers to produce genetically modified plants with greater resistance to low temperatures. This
would minimize the impact of cold on the productivity of agriculturally important plants and would also allow the expansion of agricultural areas in several countries.

The acclimation to cold is a process that involves changes in gene expression associated with physiological and biochemical changes. During cold acclimation of Arabidopsis, 3379 genes, including those coding for several transcription factors, exhibit altered expression patterns (Hannah et al., 2005).

In below-zero temperatures, the membranes are the most injured regions of the cell. This damage occurs mainly due to dehydration caused by the formation of extracellular ice (Steponkus, 1984; Steponkus et al., 1993). Other injuries are caused by reactive oxygen species (Suzuki and Mittler, 2006). Therefore, it may be possible to improve cold tolerance by examining changes in lipid composition (Thomashow, 1999), as well as the accumulation of sugars, amino acids and anti-freeze compounds, such as anti-freeze proteins (Atici and Nalbantoglu, 2003) and antioxidant enzymes and compounds (Gratão et al., 2005).

Even though we do not know the role of lignin in the acclimation to cold, low temperatures can cause changes in plant lignin content. Ex vitro poplar (Populus tremula x P. tremuloides L. cv. Muhs1) seedlings grown at 10°C showed an increase in lignin content, but the same was not true for in vitro seedlings (Hausman et al., 2000). The amount of lignin in latewood Picea abies may be positively correlated with the annual average temperature (Gindl et al., 2000). Monocots in temperate climate regions exhibit an increase in the amounts of lignin, cellulose and hemicellulose with increasing temperature (Ford et al., 1979).

Other studies showed variation in the level of phenylpropanoid lignin precursors and in the activities of related enzymes in plants exposed to low temperatures. Brassica napus plants exposed to cold (3 weeks to 2°C followed by a rapid decline to -5°C for
18h, then followed by 6h at 2°C) did not show any change in the levels of lignin or cell
wall-bound phenols (Solecka et al., 1999). However, there was an increase in the levels
of \( \rho \)-coumaric acid, ferulic acid and synapic acid in the cells of the leaf mesophyll, as
well as an increase in the esterified soluble forms of these acids, which may be
associated with an increase in the activity of phenylalanine ammonia-lyase (PAL).

Previous studies showed that \( B. \ napus \) exposed to low temperatures demonstrated
increased PAL activity (Solecka and Kacperska, 1995). The increase in phenolic acid
esterified soluble forms observed by Solecka et al. (1999) might be a mechanism to
avoid the toxic effect on plant cells of free phenols (Whetten and Sederoff, 1995). This
would also allow their transport to the vacuoles (Dixon and Paiva, 1995), where they
could be used as substrates for peroxidase (Takahama, 1988) to protect the cells against
reactive oxygen species, since this enzyme uses peroxide as oxidizing substrate
(Takahama and Oniki, 1997).

\( Glycine \ max \) roots also showed increased PAL activity during acclimation to
cold (10°C), accompanied by a significant increase in the levels of esterified forms of
ferulic, syringic and p-hydroxybenzoic acids (Janas et al., 2000). Such increases were
also suggested to be a mechanism to prevent cell damage, involving transport to the
vacuole and peroxidase activity as scavenger mechanisms to protect against generated
reactive oxygen species.

Peroxidase activity also increased in wheat (Taşgın et al., 2006; Janda et al.,
2007) and \( Saccharum \ spp. \) hybrid (Jain et al., 2007) exposed to low temperature.

Levels of lignin and soluble phenols were closely related in leaves and roots of
\( Triticum \ aestivum \) plants at 2°C (Olenichenko and Zagoskina, 2005). While leaves
showed an increase of soluble phenols and a decrease of lignin, the opposite was
observed in roots. The accumulation of soluble phenolic compounds was argued to
correlate with changes in CO₂ exchange, as there was a smaller reduction in photosynthesis as compared to respiration, since photosynthesis is less sensitive to low temperatures. The increase of these compounds was considered a cellular adaptation to stress, acting as endogenous antioxidant. Other studies with wheat also showed an increase in the accumulation of soluble phenolic compounds in leaves, but no change in lignin content was detected (Olenichenko and Zagoskina, 2005). However, lignin accumulated in tillering nodes; in contrast to other studies where the increased amount of soluble phenolic compounds was correlated with increased PAL activity (Solecka and Kacperska, 1995; Janas et al., 2000), in this case less activity was observed in both tissues with a concomitant increase of free L-phenylalanine.

Curiously, some studies have shown that although no changes in the levels of lignin or its precursors were observed in plants maintained at low temperatures, there was an increase in related enzyme activities as well as an increase in gene expression. During acclimation of *Rhododendron* to cold, there was an increase in the expression of the gene coding for C3H, a cytochrome P450-dependent monooxygenase involved in the biosynthesis of phenylpropanoids and lignin (El Kayal et al., 2006). According to these authors, increased expression of *C3H* could result in changes in the composition of lignin, altering the stiffness of the cell wall.

**Water deficit**

Water deficit occurs in plants when the water supply is insufficient to maintain growth, photosynthesis and transpiration (Fan et al., 2006). This is one of the main problems affecting food production in the world, reducing crop productivity (Vincent et al., 2005).
Very little is known about the effects of drought on lignin biosynthesis. A reduction in the amount of ferulic acid and an increase of p-coumaric and caffeic acids in the xylem sap of maize was observed after 12 days of water upholding (Alvarez et al., 2008). It was also detected decreased anionic peroxidase activity and increased cationic peroxidase activity. According to the authors, the increase of free lignin precursors in the xylem sap as well as the reduced anionic peroxidase activity could be an indication that drought decreases the biosynthesis of lignin in maize (Alvarez et al., 2008).

Different regions of the maize root may respond differently to drought, as the deposition of lignin may be greater in a specific region of the root or at certain times of stress (Fan et al., 2006; Yang et al., 2006; Yoshimura et al., 2008).

It has been shown that the basal part of the roots of maize plants under water stress exhibit a greater reduction in growth than the apical region (Fan et al., 2006). Such a reduction was associated with an increased expression of two genes involved in the biosynthesis of lignin: cinnamoyl-CoA reductase 1 and 2. The reduction was also associated with increased deposition of lignin, which stiffened cell wall extensibility and decreased cell wall expansion. In these plants, reduced growth of the basal root might improve the availability of water, minerals and sugars, factors necessary to maintain minimum growth and survival of young cells in the most apical portion, facilitating renewed growth after rehydration (Fan et al., 2006).

Researches have shown an increase in the expression of genes related to cell growth and extensibility in the roots of rice (Oryza sativa L.) plants during the initial stages (16 hours) of water stress, enabling root growth in these plants (Yang et al., 2006). It was also observed that there was an increased expression of genes involved in lignin biosynthesis during the intermediate and final stages of water stress (from 48 to 72 hours), such as those coding for PAL, C3H, 4CL, CCoAOMT, CAD and peroxidase.
Similar results were obtained with *Citrullus lanatus* sp., which shows an extraordinary resistance to drought (Yoshimura et al., 2008). In the early stages of stress, this plant showed an increase in root growth, which was associated with the induction of the synthesis of proteins involved in morphogenesis (actin, α-tubulin and Ran GTPase) as well as the metabolism of carbon and nitrogen (isoforms of triose-phosphate isomerase, malate dehydrogenase, α-mannosidase, UDP-sugar pyrophosphorylase, NADP-malic enzymes, phosphoglucomutase and UDP glucose-6-phosphate dehydrogenase). In the final stages of the stress, however, there was a reduction in root growth and induction of lignin biosynthesis, with increasing expression of *CCoAOMT* and a large number of isoenzymes comprising class III peroxidases. Growth reduction and tolerance to desiccation were associated with more lignin in the roots.

Other plant organs such as leaves may show marked physiological changes during drought stress. Growing leaves of maize plants accumulated more COMT, mostly at 10 to 20 cm from the point of leaf insertion; drought resulted in a shift of this region of maximal accumulation toward basal regions (Vincent et al., 2005). The lignin content in leaves of maize plants subjected to water stress was lower than in the well-watered control plants, suggesting an adaptation to drought, since the maintenance of high levels of lignin in the region of leaf elongation region might negatively affect growth after rehydration (Vincent et al., 2005).

The biosynthesis of lignin and its functional significance during water stress was studied in the leaves of *Trifolium repens* plants subjected to 28 days of drought (Bok-Rye et al., 2007). Reduced leaf growth occurred concurrently with the increase in lignin biosynthesis. The activities of enzymes involved in the biosynthesis of lignin revealed that the enzyme responses to drought might differ, depending on the period for which plants are exposed to drought. Overall, there was a large increase in the activities of
PAL and ascorbate peroxidase in the early stages of stress (0-14 days), with activity levels decreasing gradually as the period of stress was extended. On the other hand, other enzymes such as guaiacol peroxidase, coniferyl alcohol peroxidase and syringaldazine peroxidase exhibited greater activity during the final stages of stress (14-28 days).

**Light**

Light is essential for plants to ensure normal growth. However, an excess of light has the potential to cause cellular damage, mainly due to the formation of reactive oxygen species (ROS). To cope with this degenerative stress, plants have evolved strategies to protect themselves against ROS, such as increased peroxidase activity, accumulation of anthocyanins and reduction of the antenna systems components to dissipate the energy of absorbed light (Kimura et al., 2003).

Light itself has an effect on lignin biosynthesis. *Ebenus cretica* L. seedlings grown in the light had 2.5 times more lignin than seedlings grown in the dark (Syros et al., 2005).

The control of lignin biosynthesis by light was investigated in 3-day-old soybean seedlings, which were divided in three groups: seedlings kept in the dark, seedlings exposed to light (340 µmol m$^{-2}$ s$^{-1}$) and seedlings exposed to light (340 µmol m$^{-2}$ s$^{-1}$, 16h per day) plus diamine oxidase inhibitors (Andersson-Gunneras et al., 2006). Plants in the light showed increased levels of H$_2$O$_2$ and lignin, as well as higher levels of diamine oxidase and POD activity. The light plus inhibitor condition reduced the amount of H$_2$O$_2$ and lignin.

During the differentiation of cultured calli of *Pinus radiata* into tracheids, it was found a positive effect of light on the activity of the enzymes PAL and CAD, which
resulted in an increase in the concentration of lignin when the calli were transferred from dark to a photoperiod of 16 hours (Möller et al., 2006). Continuous light (16.7 W m$^{-2}$) increased the activity of anionic and cationic PODs and laccases in mungbean hypocotyls, resulting in an increase in the lignin content (Chen et al., 2002).

*Phalaenopsis* orchids maintained for one month at 25ºC ± 2ºC at different light intensities (60, 160 and 300 µmol m$^{-2}$ s$^{-1}$) tended to increase the lignin content in leaves and roots with increasing light intensity, which was associated with the induction of PAL, CAD and POD activities (Akgül et al., 2007). It was concluded that the increase in the biosynthesis of lignin, probably not only provided cell wall rigidity, but also provided protection against radiation stress.

In *Arabidopsis thaliana*, 7,000 genes exhibited altered expression when plants were submitted to high light intensity. Among these, 110 showed increased expression, and several of them are involved in the lignin biosynthesis pathway (Kimura et al., 2003).

The accumulation of gene transcripts coding for enzymes involved in lignin biosynthesis in *A. thaliana* varies during the diurnal cycle, stimulated by light provided that there is a prior period of darkness (Rogers et al., 2005). It was also observed that in the *sex1* mutant, which is deficient in starch metabolism, there was lower expression of genes involved in lignin synthesis during the day, indicating that the availability of carbohydrate may regulate lignin biosynthesis. These mutants accumulated less lignin than wild type; lignin composition was also changed, with a higher proportion of lignin-type S as compared with type G type H (of which there was none). This resulted in lignin that was easier to extract from the cell wall. It was concluded that at least three factors could affect the synthesis of lignin in these plants: light, circadian rhythms and sugar perception.
In recent years, there has been increased interest in the biological effects of UV radiation on living organisms, in part due to the reduction of the atmospheric ozone layer caused by anthropogenic actions, leading to an increase in UV rays reaching the Earth’s surface.

Plants and animals both undergo morphological, anatomical, physiological and biochemical changes following exposure to UV rays. Among the changes caused by UV-B radiation are inhibition of growth, increased thickness of leaf blades, reduced biomass, damage to the photosynthesis apparatus, altered membrane composition and modifications to the balance of phytohormones (Zagoskina et al., 2003). Structural damage to the DNA is also observed, reflected in the formation of cyclobutane pyrimidine dimers (CDPs) and pyrimidine-(6-4)-pyrimidone (Yamasaki et al., 2007).

Thus, to protect themselves from UV damage, plants produce mechanical barriers, such as thicker cuticles and trichomes; they also synthesize protective substances, such as secondary compounds (Yamasaki et al., 2007). UV-B affects the production of several secondary compounds such as flavonoids, tannins and lignin, which are thought to have played an important role in the transition of aquatic plants to land, when they became more exposed to UV-B radiation (Rozema et al., 1997).

UV-B radiation affected the morphogenesis of trichomes in the cotyledons of *Cucumis sativus* L. and induced the accumulation of lignin in these structures (Yamasaki et al., 2007). In a study with two types of *Camellia sinensis* L. cell cultures that differed in their capacity to biosynthesize the phenolic compounds ChS1 and ChS-2, the latter had greater capacity for production of phenolic compounds (Zagoskina et al., 2003). It was observed that under exposure to UV-B radiation, ChS2 demonstrated
greater accumulation of lignin in the cell walls of the parenchyma of tracheid elements and in the intercellular space, in addition to the formation of a layer of lignin-like-material on the surface of the callus. The ChS2 lineage was also more resistant to UV-B, suggesting the involvement of lignin in cell protection against this type of stress.

Cotyledons of *Chenopodium quinoa* exposed to UV-B radiation exhibited increased epidermal cell wall thickness; this was associated with a large accumulation of lignin due to increased peroxidase activity (Hilal et al., 2004). Additionally, changes in the ultrastructure of chloroplasts rendered them similar to the chloroplasts of shaded plants. It was suggested that such alterations in the chloroplast were caused by reduced light penetration in the cells of the mesophyll, due to the protective effect of lignin deposition. Therefore, lignin in epidermal tissue may attenuate radiation.

**Mechanical injuries**

Injuries induce specific reactions in the wood which can be restricted to the bark or might extend to the cambium and xylem (Frankenstein et al., 2006). Several studies, if not most of them, have focused on characterizing histological and anatomical wood changes produced by a particular type of injury, and the obtained information is then used to explain the roles of these changes in adaptation and resistance to stress. In general, attention has been paid to the changes occurring after insect attack or after microorganism infection. Some species have defence mechanisms that range from physical barriers including cuticle formation, lignification, spines and trichomes to the biosynthesis of toxic compounds such as alkaloids and tannins (Delessert et al., 2004).

Electron microscopy has demonstrated that injuries to the stem of *Populus* spp. induce the thickening of the xylem fibre cell wall in three ways: synthesis of an additional S2 layer, thickening of the existing cell wall or synthesis of a sclereid-like
sublayer (Frankenstein et al., 2006). The authors also showed, from the UV-microspectrophotometric determinations, that cell walls of these xylem modified-fibres had a higher content of lignin and uneven distribution of this polymer in the middle lamella and secondary wall. They concluded that the observed changes might enable these plants to resist stress.

Histochemical (phloroglucinol-HCL) and quantitative (acetyl bromide) studies on the accumulation of lignin and ferulic acid in the phloem of *Chamaecyparis obtusa* exposed to injury (injury of the bark near the base of the stem) reported the formation of a parenchymal zone and induction of lignification in the necrotic phloem (Kusumoto, 2005) after 7 days. The formation of a ligno-suberized layer between the necrotic tissue and the parenchymatous area was observed after 14 days. It was also observed the formation of a callus (due to hyperplasia of cells in the parenchyma and cambium), which became lignified. The concentration of lignin measured in the cell walls of necrotic tissues was significantly higher at 28 and 56 days after injury. The concentration of ferulic acid was significantly higher from the seventh day after injury, which suggests that the accumulation of ferulic acid occurred before the accumulation of lignin.

Given the complexity of responses to injury, it is not surprising that genes of different metabolic pathways are induced. Thus, various studies have aimed at identifying which genes are involved in this response and many have already demonstrated the induction of genes related to lignin biosynthesis. The expression of genes of the *At4CL* family (*At4CL1-i*), which codes for the enzyme 4CL, was studied in *Arabidopsis thaliana* in response to injury caused by perforation of the leaf with the tip of a pipette (Soltani et al., 2006). 4CL catalyzes the conversion of hydroxycinnamic acids in CoA esters, which are subsequently reduced to the corresponding monolignol
precursors of lignin. The results showed increased expression of At4CL1 and At4CL2 during two distinct phases, at 2.5 hours (early phase) and 48-72 hours (late) after injury. The level of At4CL4 transcripts also increased during the first 2.5 hours, but was later reduced to lower levels. The expression of At4CL3 was rapidly reduced in response to injury, and returned to initial levels after 4 hours; from that point on, expression gradually increased. The construction of vectors containing At4CL::GUS showed that At4CL1::GUS and At4CL2::GUS were specific to the vascular regions of the root and aerial parts of the plant, unlike AT4CL3::GUS and At4CL4::GUS.

The expression of CaF5H1, which codes for F5H, increased in detached leaf discs of Camptotheca acuminata plants (Kim et al., 2006). F5H is a cytochrome P450-dependent monooxygenase that catalyzes the hydroxylation of ferulic acid, coniferaldehyde and coniferyl alcohol, promoting the biosynthesis of syringyl lignin. The results suggested that such a response represented protection from mechanical stress.

As a strategy to avoid pathogen infection and water loss, injured leaves of Arabidopsis thaliana showed increased expression of genes related to lignin biosynthesis, coding for the enzymes 4CL, CAD and CCR (Delessert et al., 2004). It was suggested that lignin was formed in the cells surrounding the wound site.

The removal of the stem apex in Eucalyptus gunnii caused an increase in the activities of CAD and SAD, enzymes involved in the final steps of lignin biosynthesis (Hawkins and Boudet, 2003). Microscopy and histochemical analyses also detected the formation of a "barrier zone" where the cell walls exhibited a clear intensification of lignin deposition. This lignin was poor in S units and differed from the usual quantity of G-S that has been reported for this species.
Reaction wood: tension wood and compression wood

Reaction wood (RW) is a type of wood that occurs in leaning stems and branches in order to force them into the normal position. Therefore, in some way reaction wood may represent a stressing situation.

In angiosperms, RW develops above the leaning region, pulling it up: in this context, it is called tension wood. In gymnosperms, this wood develops below the leaning region and it is called compression wood; it pushes the plant up (Paux et al., 2005). The wood reaction involves a marked reprogramming of the genes involved in cell wall formation and therefore significantly affects the properties of the wood (Déjardin et al., 2004).

Tension wood: A wide variety of characteristics are observed in the tension wood (TW) of various angiosperms, but the most common type of TW is characterized by having fewer xylem vessel elements, which have a smaller diameter and a higher proportion of fibres with thick walls, more cellulose and less lignin (Wilson and White, 1986). In addition, TW fibres often develop an additional internal layer of the cell wall called the gelatinous layer or G-layer, with higher amounts of cellulose, low microfibril angles (MFA: angle that the cellulose microfibrils form with the longitudinal axis of the fibre) and low levels of lignin (Qiu et al., 2008). Normal wood fibres contain a middle lamella, a thin primary wall and a thicker secondary wall, which is made of 3 sub-layers: S1, S2 and S3 (Clair et al., 2005). In different trees, TW contains fibres with a special morphology and chemical composition due to the development of the G-layer, which replaces the S2-layer and either partially or completely substitutes the S3-layer (Clair et al., 2005).
The lower amount of lignin in TW has been attributed mainly to the reduction of lignin in the G-layer and to changes in the relative proportions of monolignols. During the development of TW in *Eucalyptus viminalis*, there is a reduction in the amount of lignin and an increase in the level of S residues, increasing the ratio of syringyl/guaiacyl monolignol units (Aoyama et al., 2001). Ultrastructural studies on the distribution of lignin in cell walls of TW in *Populus deltoides* using polyclonal antibodies specific to synthetic polymers of lignin showed that, in these plants, the additional G-layer contains low amounts of guaiacyl lignin and greater amounts of syringyl lignin (Joseleau et al., 2004). In contrast, changes in the proportion of monolignols in the lignin have not been observed in the TW of different eucalyptus species (Rodrigues et al., 2001).

In species that do not have the G layer, the S2-layer of the cell wall has similar characteristics to those found in the G-layer. Inclined branches of two angiosperm species of the primitive genus *Magnolia*, which does not form a gelatinous layer, have fewer and smaller vessel elements and no S3-layer in the cell walls of xylem tracheids (Yoshizawa et al., 2000). As expected, the presence of a G-layer was not observed; however, the more internal layer of secondary cell wall (S2-layer) showed characteristics of a G-layer, with smaller cellulose microfibril angles. Additionally it was noted that the amount of lignin stained by the Wiesner reaction was lower in TW fibres as compared with regions of normal wood, suggesting that the amount of lignin (mainly the guaiacyl type) would have been reduced. In *Liriodendron tulipifera*, which does not form a G-layer in TW, it was observed a decrease in the lignin content of secondary cell walls, while the syringyl/guaiacyl proportion increased. Additionally, it was also observed an increase in the amount of cellulose and smaller cellulose microfibril angles (Yoshida et al., 2002).
Eucalyptus nitens, which rarely exhibits a G-layer, was bent to 45°. These plants displayed a TW with high cellulose content, reduced microfibril angle, and less Klason lignin (Qiu et al., 2008).

Laeta procera TW has a peculiar polylaminate secondary wall, formed by thin lignified layers with small microfibril angles alternating with layers that are still thin but exhibit greater lignification (Ruelle et al., 2007).

Most of the work on TW has focused on anatomical and biochemical characterization of the wood, failing to consider the physiological and molecular mechanisms involved. These studies refer only to changes in quantity or quality (syringyl and guaiacyl) of lignin and do not explore which factors are responsible for these changes in the lignifications process, whether hormones, proteins or genes.

Auxin was thought to be the main hormone involved in inducing the formation of reaction wood (Déjardin et al., 2004). For years, experiments with exogenous supplies of auxin and auxin transport inhibitors showed that TW can be induced by a deficiency of auxin (Little and Savidge, 1987). However, reaction wood (from both tension and compression) can be formed without obvious changes in the balance of auxin. This finding indicated the importance of investigating other factors, such as mechanisms of auxin perception or even other signalling molecules (Hellgren et al., 2004). Ethylene appeared as a strong candidate since it was observed that this hormone is distributed asymmetrically in the TW and that expression of a 1-aminocyclopropane-1-carboxylate oxidase gene (PttACO1) controls the process (Andersson-Gunnerås et al., 2003). In addition, there is a connection between ethylene and lignification, as observed in the phenotype of Arabidopsis mutants, where a mutation in a chitinase gene causes the ectopic expression of CCoAOMT, ectopic deposition of lignin and the overproduction of ethylene (Zhong et al., 2002).
In addition to ethylene, gibberellins also seem to be related to the formation of reaction wood. It was observed that the application of gibberellins to the stems of some angiosperms induces the formation of wood with characteristics similar to tension wood: fibres have inner layers similar to a G-layer, with high cellulose content, small microfibril angles and low lignin content (Funada et al., 2008).

In addition to studies of plant hormones, important research has characterized the proteins involved in reaction wood formation. SDS-PAGE comparisons of the pattern of protein accumulation in TW and normal wood showed the presence of at least five proteins induced during the formation of TW (Baba et al., 2000). The extraction conditions used in the protocols led the authors to suggest that these five proteins were cell wall- or membrane-bound proteins. Additionally, a study on peroxidase activity showed that the increase in the S/G ratio, generally observed in TW, could be partly attributed to the increased activity of a syringaldazine peroxidase ionically linked to the cell wall (Aoyama et al., 2001).

The molecular mechanisms involved in the formation reaction wood and their relationship to metabolomics data have been investigated. However, due to the complexity of differential accumulation of lignin in TW, very little is known about the genes involved in the process.

ESTs obtained from different wood tissues, among them TW and opposite wood from the poplar (Populus tremula x P. alba), indicated the presence of some genes specific to these tissues (Déjardin et al., 2004). In tissues under tension, ESTs related to arabinogalactan protein-like (AGP), fructokinase and sucrose synthase were abundant. In contrast, the opposite wood exhibited greater CCoA-OMT expression, which is consistent with the high levels of lignin synthesis observed in this wood.
The increased expression of fasciclin-like arabinogalactan proteins was also observed in G-fibres in another poplar hybrid (*Populus tremula* x *P. tremuloides*) (Andersson-Gunneras et al., 2006). In addition, several genes involved in lignin biosynthesis exhibited reduced expression in the TW: *PttPAL1, PttCCoAOMT1, 4CL, HCT, C3H, CCR, F5H, COMT* and *CAD*. A MYB transcription factor (*PttMYB21a*) previously shown to repress biosynthesis of lignin displayed elevated expression levels.

Branches of artificially inclined *Eucalyptus globulus* showed that the expression of a cellulose synthase gene (*EgCesA*) was correlated with the appearance of the G-layer (Paux et al., 2005). The lignin biosynthesis genes for which expression changed during TW formation included: *4CL, CCR, CAD, COMT*, and *CCoAOMT*. The response of these genes was varied and complex, with different patterns of expression for each gene. The *4CL, CCR* and *CAD* genes exhibited reduced expression during the first 6 h of stress but the transcript levels were similar to those observed in control plants after a week. According to the authors, this variation could not explain the reduced amount of lignin often observed in tension wood; the lower amount of lignin, mainly G-type, was probably due to the differential expression of *CCoAOMT* and *COMT*, genes that encode methylation enzymes.

**Compression wood:** Compression wood (CW) has small and rounded tracheids with thicker cell walls, higher lignin content, less cellulose, large microfibril angles, and typically lacks the S3 layer (McDougall, 2000; Donaldson et al., 2004; Yeh et al., 2005; Yeh et al., 2006). Additionally, the lignin in this wood has high levels of *p*-hydroxyphenylpropane units (Monties, 1989) and alterations in the chemical links between lignin units, when compared with normal wood (Nimz et al., 1981).
As in TW, the chemical and anatomical characteristics of the CW are the results of various altered molecular and physiological factors. Unravelling these factors will be necessary to understand the mechanisms involved and elucidate lignin biosynthesis in wood. Many studies have already identified proteins and genes potentially responsible for the different characteristics of CW, including the high amount of lignin.

The comparison of proteins in CW and in the opposite normal wood showed that the presence of a dioxygenase related to the biosynthesis of anthocyanins (leucoanthocyanidin dioxygenase) might contribute to the colour of this reddish wood (Gion et al., 2005).

Other proteins abundant in this type of wood are: 1-aminocyclopropane-1-carboxylate oxidase, an enzyme that catalyzes the conversion of 1-aminocyclopropane-1-carboxylate to ethylene; glutamine synthetase and fructokinase, enzymes involved in the assimilation of nitrogen and carbon; malate dehydrogenase, an enzyme of the Krebs cycle (Plomion et al., 2000); glyceraldehyde-3-phosphate dehydrogenase (G3PDH), an enzyme related to energy production and metabolism; and α-tubulin, the main component of cortical microtubules (Le Provost et al., 2003).

Other studies have reported on proteins possibly more directly involved in the differential accumulation of lignin. A polypeptide corresponding to a laccase-type of polyphenol oxidase, which is involved in the biosynthesis of lignin, has higher activity in developing xylem in the CW of *Picea sitchensis*, as compared to non-compressed wood (McDougall, 2000).

 Twenty-six proteins whose expression was correlated with an increase in the degree of compression were identified in *Pinus pinaster*, including two methylating enzymes involved in lignin biosynthesis - COMT and CCoAOMT – and members of the S-adenosyl-L-methionine synthetase gene family. In addition to being enzymes
involved in the response to ethylene, this gene family also plays a role in the
methylation of monolignols during the biosynthesis of lignin (Plomion et al., 2000).

Sequenced cDNAs from CW and normal wood of *Pinus taeda* showed that the
genes that were most abundant in the biosynthesis of lignin code for the enzymes PAL,
C4H, OMT, 4CL, CAD, diphenol oxidase (laccase) and POD (Allona et al., 1998).

The R2R3-MYB transcription factors, which have been associated with the
biosynthesis of lignin, also display elevated expression in CW (Bedon et al., 2007).

**Mineral nutrition and heavy metals**

Deficiency disability and abnormally high concentrations of a nutrient can both
cause abnormalities in the accumulation of lignin, where this has been documented by
many studies. Most of these, however, do not include a study of the physiological or
molecular causes of their findings. Recent reports have also shown that lignin content
may be affected by toxic heavy metals.

**Nitrogen (N):** Few studies were conducted to assess the impact of fertilization with N
on the properties of wood (Luo et al., 2005; Liberloo et al., 2006); most of these
investigated grasses used as forage for animal feeding, showing that lignin content was
increased by N due to elevated PAL activity (Pitre et al., 2007).

Moreover, the effect of N seems to vary with the type, degree of development
and tissue examined. In pine (*Pinus palustris*) seedlings, high-N fertilization reduced
the lignin content in roots but had no effect on the lignin concentration in aerial parts of
the plant (Entry et al., 1998).
In red pine (*Pinus resinosa*), combined nitrogen-phosphorus-potassium (NPK) fertilization reduced the lignin content of branches (Blodgett et al., 2005) but had an opposite effect on the main stem of *Picea abies* (Kostiainen et al., 2004). Populus plants receiving high concentrations of nitrogen (10 mM NH$_4$NO$_3$) had less lignin, reduced β-O-4 linkage, increased frequency of ρ-hydroxyphenyl units and a tendency toward low S/G ratios (Pitre et al., 2007).

At the molecular level, the expression of the *pot171* gene, which encodes a protein similar to CCoAOMT, is negatively regulated in response to nitrogen in *Populus trichocarpa x deltoid* hybrids (Cooke et al., 2003).

**Calcium (Ca):** The effect of Ca on the biosynthesis of phenylpropanoids is not sufficiently clear. Some studies have shown that Ca increases the activity of guaiacol-POD and PAL (Castañeda and Pérez, 1996; Kolupaev et al., 2005); however, a reduction in the amount of phenolic compounds has also been reported (de Obeso et al., 2003). Other authors observed a reduction in PAL and POD activity (soluble and cell wall-bound), as well as reductions in the levels of phenolic compounds, including lignin (Teixeira et al., 2006). No substantial changes in the amounts of these compounds was observed (Tomas-Barberan et al., 1997).

In *Populus tremula x Populus tremuloides*, Ca deficiency was responsible for wood deformation, which included smaller diameter of xylem vessels, shorter fibre length and reduced levels of S units in lignin (Lautner et al., 2007). Therefore, it seems that Ca is necessary for the biosynthesis of lignin in plants. It is important to note that several PODs are linked to the galacturonic domains of pectin in the presence of Ca (Penel and Greppin, 1996) and such a link occurs only in the pectin chain that is cross-linked to Ca, known as Ca-pectates (Carpin et al., 2001). Since the middle lamella and
cell corners are rich in Ca-pectates (Carpita and Gibeaut, 1993) and are the first places to be lignified, POD linked to these chains of Ca-pectates might play a role in controlling the spatial deposition of lignin, and changes in the concentrations of Ca would define the location of these PODs (Carpin et al., 2001).

Copper (Cu): It is known that copper has a positive effect on the biosynthesis of lignin. Thus, Cu deficiency results in less lignin (Robson et al., 1981), and excess Cu results in more lignin.

Capsicum annuum hypocotyls grown in a nutrient solution with excess copper (50 mM CuSO₄) showed higher levels of shikimate dehydrogenase and POD activity and greater accumulation of soluble phenolic compounds and lignin (Diaz et al., 2001). Roots of Raphanus sativus grown in the presence of Cu (1-10 mM CuSO₄) showed higher levels of cationic and anionic POD activities and higher levels of lignin in comparison with plants grown in the absence of this element; the increases were dose-dependent (Chen et al., 2002).

Laccases were responsible for the extracellular polymerization of monolignols in the roots of soybean plants during the early stages of Cu exposure. Later, POD activity increases, working cooperatively with laccases in the biosynthesis of lignin (Barnes et al., 1997).

Exposure of Panax ginseng root suspension cultures to increasing concentrations of Cu led to an enhancement of the activities of glucose-6-phosphate dehydrogenase, shikimate dehydrogenase, PAL and CAD and to the accumulation of phenolic compounds and lignin. It also caused an increase in levels of caffeic acid-POD, polyphenol oxidase and β-glucosidase (Akgül et al., 2007).
Thus, lignin accumulates in plant cell walls in the presence of Cu. This process is correlated with enhanced activities of several enzymes, including POD and laccases, which are enzymes that carry out the polymerization of monolignol precursors of lignin. This could be partly explained by the fact that Cu is structurally important for laccases (Claus, 2004). It appears also that Cu has functional importance in terms of peroxidase activity, since an amine oxidase containing Cu that generates H$_2$O$_2$ by oxidising putrescine has been co-located with lignin and with POD activity in tracheid elements of xylem in *Arabidopsis* (Møller and McPherson, 1998).

However, Cu also mediates an increase in the activity of other enzymes of the lignin biosynthesis pathway, such as PAL and CAD. This could be an indirect effect of this metal, which enhances oxidation and polymerization of monolignols via laccases; peroxidase would stimulate (by feedback) the synthesis of these monolignols.

**Boron and Zinc:** Boric acid at 5 mM increased the activities of PAL and syringaldazine-POD and increased the lignin content in soybean (Ghanati et al., 2005). At high concentrations of Zn, both *A. thaliana* and *Thlaspi caerulescens* showed increased expression of genes related to the biosynthesis of lignin (van de Mortel et al., 2006). However, in *T. Caerulescens*, a Zn hyper-accumulator, the expression of these genes was even greater. This could be related to the ability of this species to adapt to higher concentrations of Zn. Among the genes that had higher expression in *T. caerulescens* than in *A. thaliana* were genes related to dirigent proteins, 4CL, CCR, F5H, CAD, CCoAOMT and laccase.

**Metals - Aluminium (Al) and Cadmium:** Al is one of the main factors inhibiting plant growth in acidic tropical soils. A typical symptom of Al toxicity is the inhibition of root
growth (Tahara et al., 2005). Excess of Al causes increased lignin deposition in the cell walls of some plant species (Sasaki et al., 1996; Budíková, 1999). In plants susceptible to Al, lignin accumulation is even higher, dramatically reducing growth; growth inhibition is strongly correlated with the extent to which lignin is deposited in the cell wall, independently of the tolerance to this metal (Sasaki et al., 1996).

Among several species of Myrtaceae, only the most sensitive to Al shows increased lignin content when exposed to high concentrations of aluminium (Tahara et al., 2005).

*Camellia sinensis* is know to be highly tolerant to Al, and its growth is stimulated by high concentrations of this element (Lima et al., 2009). When this species was grown in high concentrations of Al, there was a reduction in the activities of PAL and POD bound to the cell wall, as well as a reduction in lignin content, which might explain enhanced growth of these plants in the presence of Al (Ghanati et al., 2005).

It was also demonstrated that Al induces the expression of genes coding for enzymes of the lignin biosynthesis pathway. Both resistant and sensitive rice (*Oriza sativa*) plants under Al stress exhibited increased expression of 4CL, PAL, CAD and C3H (Chuanzao et al., 2004). Northern blot analysis indicated that the temporal patterns of expression differ between the two varieties and, additionally, that transcripts of PAL accumulated more intensively in the sensitive variety. Al also induces expression of *PAL* in wheat (Snowden and Gardner, 1993).

Cd is a heavy metal pollutant, not essential for plants, which enters the environment primarily through industrial processes and phosphate fertilizers. Among its many effects on plants, Cd may induce the synthesis of lignin. This was observed in roots of *Phragmites australis* (Ederli et al., 2004) and callus tissue culture from the roots and branches of *C. sinensis* (Zagoskina et al., 2007).
Cd concentrations varying from 0.2 mM to 1 mM reduced the growth of soybean roots and increased the lignin content (Bhuiyan et al., 2007). This was accompanied by increased POD (cationic and anionic) activity, elevated laccase activity, and increased expression of genes related to POD. The authors also noted that the laccases are responsible for the biosynthesis of lignin during the initial stages of treatment with Cd and that POD participation become important at later stages.

**Gases (CO₂ and Ozone)**

It is expected that atmospheric concentrations of CO₂ will change from 360 μmol⁻¹ to 550-1000 μmol⁻¹ in the next 100 years. This will probably interfere with the primary and secondary metabolism of plants (Davey et al., 2004).

With respect to secondary metabolism, it is known that high concentrations of CO₂ interfere with the biosynthesis of lignin. But it has been observed both increases in (Koricheva et al., 1998; Richard et al., 2001), reduction in (Knops et al., 2007) or even no effect on the concentration of lignin (Cotrufo et al., 2005) with increasing rates of CO₂. Moreover, the response to high CO₂ concentrations is influenced by fertilization with N (Matros et al., 2006). Accordingly, high concentrations of CO₂ in the roots of *Nicotiana tabacum* cv. Samsun-NN grown in 5 mM of NH₄NO₃ led to an increase in lignin content; however, under 8 mM of NH₄NO₃, no changes in the levels of lignin were observed (Matros et al., 2006).

*Plantago maritima* maintained for a year in an atmosphere of 600 μmol CO₂ mol⁻¹ showed an increase in the biomass of stems and roots and changes in vascularization and lignification when compared with plants kept in normal concentrations of CO₂ (Davey et al., 2004). An atmosphere rich in CO₂ resulted in a
higher concentration of caffeic acid in the leaves, while there was an increase of \( p \)-coumaric acid in the roots.

Both the enzyme activity and gene expression of PAL (Paakkonen et al., 1998), 4CL (Booker and Miller, 1998), COMT (Koch et al., 1998) and CAD (Booker and Miller, 1998; Zinser et al., 1998) are stimulated by exposure to ozone. It was also observed increased shikimate dehydrogenase activity in leaves of *Populus tremula X alba*, as well as elevated lignin content and changes in lignin structure (Cabane et al., 2004).

**Biotic stresses (bacteria, fungi and virus)**

An increase in lignification is often observed in response to attack by pathogen. The response is believed to represent one of a plethora of mechanisms designed to block parasite invasion, thus reducing the susceptibility of the host, since lignin is a non-degradable mechanical barrier for most microorganisms.

Two genes coding for CCR (*AtCCR1* and *AtCCR2*) in *A. thaliana* are differentially expressed upon infection with *Xanthomonas campestris*. *AtCCR1* is preferentially expressed in the normal development of tissues, while *AtCCR2* shows lower expression. However, this relation is reversed in response to the attack by *X. campestris*, indicating that these genes might participate in the hypersensitive response to the pathogen.

*Agrobacterium* induces the production of ferulic acid in wheat. Ferulic acid is a precursor in lignin biosynthesis, suggesting the existence of a defence response preventing infection by the bacteria in these plants (Parrott et al., 2002).

No changes occurred in the lignin content of wheat leaves infected with mosaic virus (Kofalvi and Nassuth, 1995).
Several authors have shown changes in lignin content due to attack by fungi, but not all of these studies have sufficiently demonstrated the importance of lignin during these processes. In addition, fewer still are the works that analyze the changes in plant metabolism and gene expression responsible for this differential accumulation of lignin. The infection of Pinus nigra by Sphaeropsis sapinea induces an increase in the deposition of lignin (Bonello and Blodgett, 2003). Lignification is a defence response in the hypersensitive reaction of wheat to Puccinia graminis (Moerschbacher et al., 1990). During such a response in wheat, lignin rich in syringyl units accumulates (Menden et al., 2007).

The deposition of lignin in necrophylatic periderm in the early stages of infection by Mycosphaerella explains the greater resistance of E. nitens as compared to E. globulus, as this response may prevent the spread of toxins and fungal enzymes to the host, thereby preventing the displacement of water and nutrients from the host cell to the fungus (Smith et al., 2007).

Metabolic studies on the changes in the xylem tissues of Ulmus minor and Ulmus minor x Ulmus pumila after inoculation with Ophiostomis new-ulmi showed that the hybrid has a faster defence response, which is characterized by an increase in the amount of lignin, diminishing the probability of pathogen invasion (Martin et al., 2007).

Regarding physiological and molecular aspects, the differential accumulation of lignin and lignans in cell suspension cultures of Linum usitatissimum was studied, in response to Botrytis cinerea, Fusarium oxysporum and Phoma exigua mycelium extracts (Hano et al., 2006). In this study, genes coding for PAL, CCR and CAD showed elevated expression; PAL activity was enhanced as well.

Finally, COMT expression in diploid wheat (Triticum monococcum) is elevated following inoculation with Blumeria graminis (Bhuiyan et al., 2007).
**Concluding Remarks**

Figure 1 shows the main route of lignin biosynthesis in plants and the enzymes involved in each metabolic step (adapted from Rastogi and Dwivedi, 2008). For most of the genes coding for these enzymes, transgenic plants have been produced using gene-silencing techniques (Anterola and Lewis, 2002). The aim of these works was to reduce lignin content or to change the structure of lignin, goals which have economical and environmental implications: cheaper and more process-amenable trees for pulp and paper manufacture with less pollution, more readily digestible forage for livestock and improved feedstock for fuel/chemical production (Anterola and Lewis, 2002).

The potential applications of changing or silencing the expression of these genes were comprehensively discussed by Anterola and Lewis (2002). Some questions discussed by these authors were, “why do vascular plants consistently produce various types of cell walls with lignins from either two or three monolignols, and how does this occur in vivo? Why are these moieties also differentially deposited into specific regions of developing cell wall types?” Despite the complexity of the lignin biosynthesis network, Anterola and Lewis (2002) used accumulated knowledge obtained with mutants or transgenic plants (in which levels of particular enzymes of the pathway have been modified in some way) to show that monolignol biosynthesis is under fine metabolic and transcriptional coordination.

For several of these genes, repressed expression led to diverse and undesirable impacts on plant physiology due to the multiple products that originate from the phenylpropanoid pathway. Therefore, the *PAL, C4H, C3H* and *4CL* genes (see Figure 1) are not good targets for genetic manipulation aiming to reduce lignin, while *CCoAOMT*,

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COMT and CAD genes can be used to reduce and change lignin composition (Rastogi and Dwivedi, 2008).

The biosynthesis of monolignols in phenylpropanoid metabolism is a metabolic grid; that is, different routes may be used to synthesize a compound. This gives plants the plasticity to produce lignin when a step is inhibited for whatever reason such as when a specific gene is silenced. In addition, enzymes related to lignin biosynthesis usually derive from multigene families; depending on the stressing situation (biotic or abiotic), different genes for different isozymes can be activated. For the sake of complexity, it is also well known that the relative proportions of the monolignols may vary due to environmental influence (Boudet, 1998). It has been suggested that plants are versatile in their ability to tolerate substantial changes in lignin structure and incorporate phenolic compounds other than the three known monolignols (Ralph et al., 2004). Recently, it was shown that acylated monolignols could also serve as lignin precursors (Lu and Ralph, 2003). This can make lignin more recalcitrant to disruption during pulping in the paper industry, for example (Lapierre et al., 1999). However, it does not appear that “non-traditional” phenolics, other than the monolignols, are incorporated into lignin (Anterola and Lewis, 2002).

There are several examples indicating that lignin found in nature may comprise modified molecules (Lu et al., 2004; Morreel et al., 2004). Phenolic amides may also be part of the cell wall, as proven by over-expression of the enzyme responsible for their biosynthesis, which caused an increase in their incorporation into the cell wall matrix (Hagel and Facchini, 2005). Recent research also showed that acetylation of syringyl units seems to be an exclusive characteristic of angiosperms, as the process was observed among 11 angiosperm species but not in 2 gymnosperm species (Del Rio et al., 2007).
This review showed that lignin biosynthesis in nature can increase complexity depending on the stresses and whether the stressor is acting during plant growth and development. Unfortunately, little has been done to elucidate how such stressing situations can change lignin content or alter its composition with regard to the building blocks called monolignols, or other phenolics entering the polymeric structure. Reaction wood has proven to be an important model for understanding lignin formation in plants (Mellerowicz and Sundberg, 2008). Other stresses may also become excellent models that will yield important clues for understanding such complex metabolic pathways, as well as the intricacies of genetic and metabolic control.

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References


Figure 1. Biosynthesis pathway of lignin in plants (adapted from Rastogi and Dwivedi, 2008). PAL - phenylalanine ammonia-lyase; C4H - cinnamate 4-hydroxylase; C3H - cinnamate 3-hydroxylase or p-coumaroyl shikimic acid/quinic acid 3-hydroxylase; OMT - O-methyltransferase; HCT - p-hydroxy cinnamoyl-CoA: shikimate/quinate p- hydroxy cinnamoyl transferase; CCoAOMT - caffeoyl coenzyme A O-methyltransferase; F5H - ferulate 5-hydroxylase; 4CL - 4-coumarate: coenzyme A ligase; CCR - cinnamoyl coenzyme A reductase; Cald5H - coniferaldehyde 5-hydroxylase; AldOMT - 5-hydroxy coniferaldehyde O-methyltransferase; SAD - sinapyl alcohol dehydrogenase; CAD - cinnamyl alcohol dehydrogenase.

Cinnamate  \rightarrow \text{PAL} \rightarrow \text{Phenylalanine}

\[\begin{align*}
\text{Cinnamate} & \rightarrow \text{C4H} \\
\text{4-Coumarate} & \rightarrow \text{C3H (7)} \\
\text{4-Coumarate} & \rightarrow \text{HCT (4-Coumaryl-CoA)} \\
\text{4-Coumaraldehyde} & \rightarrow \text{CAD/SAD (4-Coumaryl alcohol)} \\
\text{p-Hydroxyphenyl} & \rightarrow \text{(H unit)}
\end{align*}\]

\[\begin{align*}
\text{Caffeate} & \rightarrow \text{OMT} \\
\text{Ferulate} & \rightarrow \text{F5H (5-Hydroxylase)} \\
\text{5-Hydroxyferulate} & \rightarrow \text{OMT} \\
\text{Sinapate} & \rightarrow \text{CCoAOMT (5-Hydroxyferuolyl-CoA)} \\
\text{Sinapyl-CoA} & \rightarrow \text{Cald5H (5-Hydroxyconiferaldehyde)} \\
\text{Sinapaldehyde} & \rightarrow \text{AldOMT (5-Hydroxyconiferyl alcohol)} \\
\text{Syringil (S unit)} & \rightarrow \text{OMT (Syringyl alcohol)}
\end{align*}\]

\[\begin{align*}
R_1 = R_2 = H & \Rightarrow \text{p-coumaryl alcohol (p-hydroxy phenyl, H unit)} \\
R_1 = H, R_2 = \text{OCH}_3 & \Rightarrow \text{coniferyl alcohol (guayacyl, G unit)} \\
R_1 = R_2 = \text{OCH}_3 & \Rightarrow \text{sinapyl alcohol (syringyl, S unit)}
\end{align*}\]