

LIGNIN: A DEFENSIVE SHIELD HALTING THE ENVIRONMENTAL STRESSES – A REVIEW

KHAN, P. – TONG, L. – KHAN, S. U. – ZHANG, C. – WANG, W.*

*Biotechnology Research Institute, Chinese Academy of Agricultural Sciences
Beijing 100081, China*

**Corresponding author
e-mail: wangweixuan@caas.cn*

(Received 10th Sep 2021; accepted 23rd Nov 2021)

Abstract. Plants are exposed to various environmental stresses, like mineral deficiency, heavy metals, high and low temperature, and drought, causing adverse effects on plant growth and productivity. Currently, various strategies are employed to generate plants that can withstand these environmental stresses. To this end, the induction of phenylpropanoid pathways are triggered by the signaling network of extracellular ATP (eATP) and dinucleotide polyphosphates (NpnN's) yielding several metabolites including lignin. Depending upon the stress, lignin plays protective, sustaining or disruptive roles in addition to its involvement in plant growth, development and defense responses. Aside from its involvement in plant development, lignin is also active in the response to numerous abiotic and biotic stress, and hence plays a significant role in plant adaptation to their environment. To differentiate it from developmental lignin, the lignin polymer generated in response to stress is referred to as 'stress lignin' or 'defense lignin.' In this review, we analyzed the role of lignin as a defensive shield under environmental stress. The physiological and molecular aspects of lignin biosynthesis were also summarized. The analysis and information presented in this review article will help in understanding and the future investigation of lignin in plants with respects to its defensive role under environmental stressed conditions.

Keywords: *abiotic stress, plant hormones, drought, minerals, toxic metals*

Introduction

Lignin is an important secondary metabolite produced by the tyrosine-phenylalanine metabolic pathway in all plants. It is the second most abundant biopolymer, accounting for 31% of the organic carbon mass in the biosphere (Fleming et al., 2002). Classically, lignin is a three-dimensional phenolic polymer formed by the oxidation of hydroxycinnamyl alcohols, namely coniferyl alcohol, p-coumaryl alcohol, and sinapyl alcohol, even though other monomers can also be integrated (Fleming et al., 2002). This class of molecules is very important in plant development as it acts as a defense barrier to both biotic and abiotic stress (Fleming et al., 2002). The hydrophobicity of lignin, for example, waterproofs xylem conducting cells, and the stiffness they give to cell walls, reinforces supporting fiber cells in xylem and phloem tissues. The chemical structure of these developmental lignins differs depending on various factors, including plant community, organ, tissue, and even cell wall layer.

Gymnosperm lignin is mostly comprised of G-units (guaicyl units resulting from coniferyl alcohol). In contrast, angiosperm lignin comprises guaicyl-syringyl lignin (G and S units, derived from coniferyl and sinapyl alcohol) (Nawawi et al., 2016). Plants use lignins to protect themselves from a variety of environmental stresses (Thompson, 1984). The lignin synthesized in this case (in response to environmental stress) is known as defense lignin (Xie et al., 2018), which differs from developmental lignin. The formation of cell walls with such material confers an impermeable barrier protecting the healthy plant tissues from dehydration and fungal infection (Lourenço and Pereira, 2018).

Defense lignin formation has also been involved in plant responses to various abiotic stresses (Nicholson and Hammerschmidt, 1992). Previous studies suggest that the defense lignin is chemically different from developmental lignin (Stange et al., 2001; Cesarino, 2019). For example, defense lignin containing an abundance of p-coumaraldehyde units (H-units), contrary to developmental lignin in cucurbit that is composed of angiosperm guaiacyl-syringyl (G–S) lignin (Xie et al., 2018).

Similarly, defense lignin in almond trees and wounded poplar has G-lignin contrary to the normal G–S developmental lignin (Chezem et al., 2017). While the enzymatic reaction for both developmental and defense lignin appears to be the same but the signal transduction pathways are different. For example, phenylpropanoid ammonia lyase (PAL) and O-methyltransferase genes are differentially regulated during development against external stimuli (Gallego et al., 2018). Thus, such regulation presumably allows the plant to direct its phenolic metabolite pool to where it is needed. *Arabidopsis cinnamoyl* CoA reductase genes AtCCR1 and AtCCR2 (Xie et al., 2018) showed a differential expression pattern under pathogen attack, suggesting that the production of defense lignin is also controlled at the level of the bio-synthetic pathway.

In this respect, lignin production under biotic and abiotic stress has been demonstrated to be the outcome of a complex genetic network involving multiple enzymes that respond differentially to abiotic and biotic impacts (Hawkins and Boudet, 1996). In this context, the literature is replete with examples of plants exhibiting enhanced lignin concentration or a variation in chemical composition under stress conditions, implying complicated 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. Here in this review, we summarize the progress in lignin for its structural features, regulatory mechanisms, and interaction with plant hormones in response to abiotic or environmental stresses.

Structure and regulation of lignin under environmental stress

Lignin polymer is produced by the phenylpropanoid pathway via oxidative coupling of three hydroxycinnamyl alcohols or monolignols, i.e., p-coumaryl, coniferyl and sinapyl (*Fig. 1*). Their assimilation produces different units of lignin with varying degrees of methoxylation. These units are p-hydroxyphenyl (H), syringyl (S) and guaiacyl (G) units. The composition, contents, and linkage frequency of developmental lignin are different among different cell types, growth stages, and plant species. The gymnosperm and angiosperm have different compositions and units of lignin. For example, gymnosperm contains G lignin with a few H units, and angiosperm contains mostly G and S units (Logemann et al., 1995). The physical characteristics and structural features of defense lignin are different from developmental lignin (Lauvergeat et al., 2001; Bonawitz and Chapple, 2010). There is limited knowledge about stress induced lignin and its chemical nature needs to be explored. The structure of lignin polymer varies among plant species and stress. Some of the common responses regarding stress and lignin are shown in (*Fig. 2*).

Angiosperms and gymnosperms have been demonstrated to synthesize more condense lignin with a higher inter C-C bond and H units in response to several environmental stresses, including ozone exposure, high nitrogen fertilization, and mechanical stimuli (Bonawitz and Chapple, 2010; Hawkins and Boudet, 2003). Lignin observed in Norway

spruce, and Jack pin shows the same features when treated with fungal elicitor (Lauvergeat et al., 2001). Compression wood has an increased amount of H units in response to gravistimulation. It was predicted that compression wood might have a higher proportion of condense linkage, but a recent report showed that compressed wood and normal wood have no statistical difference (Sato et al., 2011). Lignin induction by stress might cause the enrichment of H units that ultimately exhaust the precursors of coniferyl and sinapyl alcohol, whose incorporation in lignin monomer built a biosynthetic pathway with faster response (Lauvergeat et al., 2001). The condensation degree might be enhanced by the incorporation of H units resulting formation of C-C bonds (Sato et al., 2011; Cabané et al., 2004). In addition, it has been reported that stress always triggers monolignols comprising β -5 and β - β condensed bonds through dehydrodimerization reactions (Cabané et al., 2004). Interestingly, these structural characteristics of lignification in gymnosperm and angiosperm, in which lignin is formed in middle lamella with enriching condensed H units. The S lignin has been proven to show resistance against various microbes (Nanayakkara et al., 2011). In other studies, S lignin preferentially accumulates in infected tissues (Saito and Fukushima, 2005; Barber et al., 2000).

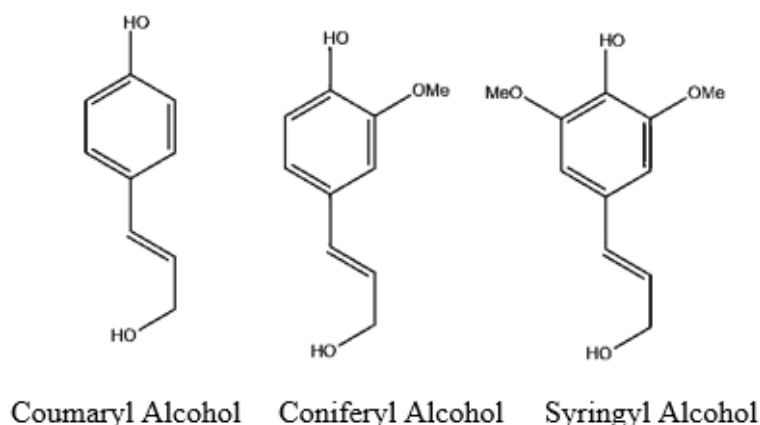


Figure 1. Structure of lignin monomers

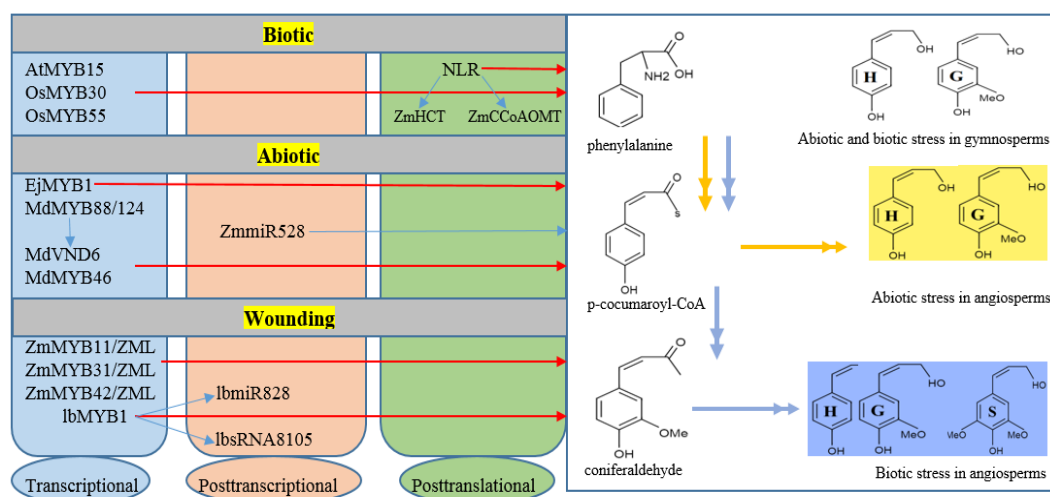


Figure 2. Structure and regulation of lignin during stress

Moreover, it was observed that the engineered S lignin showed increased resistance against environmental stresses (Hano et al., 2006; Menden et al., 2007). The expression of monolignol genes is activated by the transcription factor AtMYB58 by binding to the AC elements in their promoter region (Eynck et al., 2012). The promoter of FERULATE 5- HYDROXYLASE (F5H) contain no AC element to synthesize S lignin (Wuyts et al., 2006); instead, (F5H) is directly regulated by SND1, but it cannot activate other lignin genes. It is a NAC master switch that activates the whole program of secondary cell wall deposition (Gallego et al., 2018; Zhou et al., 2009).

Lignin deposition under environmental dares

Lignification is an irreversible asset of energy and carbon. Developmental lignin deposition is regulated at transcriptional (Raes et al., 2003), post-transcriptional, post-translational level (Zhao et al., 2010; Cesarino, 2019). Recently, it was reported that stress related lignin is controlled at several regulatory levels, similar to that for developmental lignin. The H₂O₂ signal transduction contributes to extracellular lignin and flavonoid biosynthesis and the extracellular lignin production; on the contrary, it is severely hindered by H₂O₂ scavenging (Rao and Dixon, 2018). As a result, changes in cellular redox status caused by the accumulation of reactive oxygen species (ROS) in response to biotic and abiotic stresses are likely to be the first layer of regulation during lignin formation.

Ascorbate peroxidase stimulates the formation of the monolignol radicals in the cell wall, catalyzed by peroxidases, and detoxify hydrogen peroxide in the cytosol during monolignol formation representing a possible correlation between monolignol synthesis and detoxification of hydrogen peroxide. This enzyme exists in a diverse cellular compartment in plants, with a key function of detoxifying hydrogen peroxide produced upon abiotic stress (Gou et al., 2018).

In a monolignol pathway, coumarate 3-hydroxylase (C₃H) is the third enzyme with a secondary function associated with ROS, being caffeoyl shikimate esterase (CSE) and cinnamoyl CoA reductase (CCR). During the evolution of the monolignol pathway, the earliest land plants acquired phenylalanine ammonia-lyase (PAL) from soil bacteria through horizontal gene transfer with a primary function of defense against various environmental stresses (Gou et al., 2018; Lin et al., 2015).

The Arabidopsis lysophospholipase 2 (CSE/LPL2) binds to acyl CoA-binding protein 2 (ACBP2) through the ankyrin repeat (Laitinen et al., 2017; Caverzan et al., 2012). Hydrogen peroxide triggers the expression of lysophospholipase 2/CSE, and its loss of function and overexpression alter its sensitivity to hydrogen peroxide, in turn (Laitinen et al., 2017). Characteristics of this enzyme complex need to explore further regarding its function in monolignol formation.

The final steps in monolignol biosynthesis are sequential declines in an acyl CoA (for G units, feruloyl CoA) catalyzed via cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) (Weng et al., 2010). In multiple species, mathematical modeling (Gao et al., 2010) and protein interaction experiments (Miao et al., 2019) have indicated that CCR and CAD are localized in a membrane associated complex whose behavior seems dynamic. In theoretical models, the membrane cavity was previously defined as the endoplasmic reticulum (ER) outer surface (Gao et al., 2010), but it may also be the plasma membrane's inner surface. As such, previous research had demonstrated that the interaction of OsCCR2 with

OsRAC1, a member of the Rac/Rop family of small membrane bound GTPases that is a positive regulator of hydrogen generation via the NADPH oxidase (N.O.X.) (Zhuo et al., 2019).

The interaction between OsRAC1 and OsCCR2 resulted in a 10-fold increase in OsCCR2's catalyzed feruloyl CoA to coniferyl aldehyde; additionally, OsCCR2 bound to OsRAC1 primarily in the presence of GTP, which was also required for OsRAC1's activation of NOX (Zhuo et al., 2019). Stimulation of NOX induces the hydrogen peroxide needed for monolignol oxidation and polymerization via peroxidases (PX).

It is worth noting that OsCCR2 and OsRAC1 expression is directly linked to lignification through plant defense, and many plants have at least two CCR genes, each with specialized and redundant functions.

The loss of function of Arabidopsis CCR1, which is involved in the regulation of developmental lignin, results in a significant decline in reactive oxygen species (ROS) (Faraji et al., 2018). Of the several classes of enzymes that convert superoxide into hydrogen peroxide, few Cu/Zn SODs are widely distributed in the apoplast synthesizing hydrogen peroxide for lignification (Yan et al., 2019); hydrogen peroxide (H₂O₂) diffuse through membranes poorly and is mainly transported primarily through aquaporins water channels (AqP) (Kawasaki et al., 2006).

In addition, concentration gradients of hydrogen peroxide within the apoplast leading to an inward movement of H₂O₂ into the cell membrane. It is likely that this could occur even after activation of NOX (RBOH) in plant protection, even though it is less clear whether these gradients arise throughout developmental lignification (Möller et al., 2019).

Lignin in stress tolerance

Lignin and minerals

A few studies were performed to determine the effect of nitrogen fertilization on wood properties, showing lignin was increased by nitrogen due to high phenylalanine ammonia lyase (PAL) activity (Möller et al., 2019). The impact of nitrogen N seems to vary regarding plant type, degree of development, and the tissue conducted. Excess N fertilization decreased lignin in the roots but did not affect the aerial parts in pine (*P. palustris*) seedlings (Table 1). The nitrogen-phosphorus-potassium (NPK) fertilization in red pine (*Pinus resinosa*) decreased the lignin content of the branches (Dixon et al., 2019) but increase lignin contents in the main stem of *Picea abies* (Gellerstedt and Henriksson, 2008). *Populus* plants receiving high N (10 mM NH₄NO₃) had lowered lignin content, decreased β -O-4 binding, and increased (Möller et al., 2019) β -hydroxyphenyl unit frequency, leading affinity towards low S/G ratios. At the molecular level, pot171 gene in *Populus trichocarpa* deltoid hybrids, encoding a protein with similarity to CCoAOMT was regulated negatively in response to N (Möller et al., 2019).

The influence of calcium (Ca) on phenylpropanoid biosynthesis is not clear enough. Few studies demonstrate that the activity of guaiacol-POD and PAL increased by (Ca) (Luo et al., 2004); whereas, a decrease in phenolic compounds has also been observed (Blodgett et al., 2005). The reduction in the activity of phenylalanine ammonia-lyase (PAL) and peroxidase (POD) as well as a reduction in phenolic compound and lignin has been reported (Kostiainen et al., 2004). No significant variations in the amount of these compounds have been reported (Kolupaev et al., 2005). In *Populus tremula* and *Populus tremuloides*, wood deformation was caused by Ca deficiency (Table 1) (Obeso

et al., 2003). Likewise, Ca is essential for lignin biosynthesis in plants. However, Ca is necessary to link certain PODs with the galacturonic pectin domains, and such a relationship exists only in the pectin chain known as Ca-pectates (Teixeira et al., 2006). Middle lamella and cell corners were reported to be the first places for lignification as they have a higher level of Ca-pectates (Tomás et al., 1997).

Table 1. Lignin response to environmental stress (mineral stress)

Env. Stress	Concentration	Species	Plant organs	Effect on lignin	Ref
N	High	Maize	Stem	↓	Sun et al., 2018
	High	<i>P. palustris</i>	Roots	↓	Entry et al., 1998
	High	<i>P. palustris</i>	Aerial parts	---	Entry et al., 1998
	High	Populus	Xylem	9-10% decrease	Pitre et al., 2007
NPK	High	<i>P. resinosa</i>	Branches	↓	Blodgett et al., 2005
	High	<i>Picea abies</i>	Main stem	↑	Kostiainen et al., 2004

Lignin and toxic metal

Heavy metal toxicity may have several consequences, including blocking functional groups of important molecules (e.g., enzymes, secondary metabolites, and polynucleotides), disrupting the plant's transport mechanism for critical nutrients and ions and inactivating or triggering an excess of antioxidant enzymes by displacing essential ions from cellular sites (Lautner et al., 2007). The effects of some of the key heavy metals stress on the lignin content of plants are summarized in *Table 2*. Heavy metals like manganese (Mn), copper (Cu), cadmium (Cd), and zinc (Zn) ions are taken up by the plants through roots, where the cell wall becomes the first barrier for those ions (Penel et al., 1996). These heavy metal ions (Cu²⁺, Cd²⁺, Pb²⁺, etc.) trigger the pathway of phenolic compounds, resulting in increased lignin accumulation in the secondary cell wall (Carpita et al., 1993). As the polymer of lignin comprises a variety of functional groups (hydroxyl, carboxyl, and methoxyl), it can bind various heavy metal ions such as copper (Cu), cadmium (Cd), zinc (Zn), and manganese (Mn), avoiding heavy metals entry into the cytoplasm (Anwar et al., 2018). Aluminum (Al) is an important heavy metal that causes toxicity and inhibits plant growth, most commonly in root growth inhibition available in acidic soil (Wuana and Okieimen, 2011).

Root endoderm cell walls depositing sufficient lignin may prevent the entry of heavy metal into or out of vascular bundles (Anwar et al., 2018). Recently, it was reported that Al induces 4CL, CAD, C3H, and PAL gene expression in the lignin pathway (Anwar et al., 2018). In an experiment with a tea plant observed under a high concentration of Al, the activity of phenylalanine ammonia-lyase (PAL) and peroxidase (POD) in the cell wall decreased, causing reduction of lignin up to a significant level (Emamverdian et al., 2015). Similarly, Copper (Cu) caused harmful effects on plants; its higher concentration induces the expression of POD, caffeic acid, CAD, and PAL genes. The growth of soybean root was adversely affected by treating with cadmium, inducing peroxidase (POD) and Lacase (LAC) activity, accompanied by increase content of lignin (Liu et al., 2018).

Zinc (Zn) is considered vital for plants, but higher concentration leads to detrimental effects. The roots of *Thlaspi caerulescens* accumulate more Zn/Cd than *Arabidopsis* where Zn causes the induction of genes responsible for lignin, the reason being

endodermis cell wall in the roots of *T. caeruleascens* accumulate more lignin (Fang et al., 2020). These findings suggest that lignification of xylem in roots is potentially involved in the reduction of the transports of Cu, Cd, Zn and Mn to shoots, thus reduced the toxic effect of stress and stabilize ROS accumulation.

Table 2. Lignin response to environmental stress (toxic metals)

Env. stress	Concentration	Species	Plant organs	Effect on lignin	Ref
Ca	Low	Lemon	Seedling	↑	Castaiqeda and Perez, 1996
	High	Soybean	Roots	↓	Teixeira et al., 2006
	Low	Populus	Xyleum	↓	Lautner et al., 2007
Al	High	Maize	Roots	↑	Vardar et al., 2011
	High	Wheat	Roots	↑	Sasaki et al., 1996
	High	<i>Eucalyptus camaldulensis</i>	Root tip	↑	Tahara et al., 2005
	High	Rice	Root	↑	Ma et al., 2012
Cd	High	<i>Phragmites australis</i>	Roots	↑	Ederli et al., 2004
	High	<i>C. sinensis</i>	Callus	↑	Zagoskina et al., 2007
Zn	High	<i>Thlaspi caeruleascens</i>	Roots	↑	Mortel et al., 2006
	High	<i>A. thaliana</i>	Roots	↑	
Cu	High	<i>Capsicum annuum</i>	Hypocotyls	↑	Diaz et al., 2001
	Low	<i>Raphanus sativus</i>	Roots	↑	Chen et al., 2002
	High	<i>Panax ginseng</i>	Roots	↑	Ali et al., 2006
	Low	Wheat	Leaf	↓	Robson et al., 1981
	High	Soybean	Roots	↑	Lin et al., 2005

Lignin and drought

Among various abiotic stresses, water deficit or drought is a critical environmental factor threatening the throughput and yield of various crops. It adversely affects the anti-oxidant systems, chlorophyll contents, photosynthetic activity, and stability of membrane (Ghanati et al., 2005). A key gene of the lignin pathway, Phenylalanine ammonialyase (PAL), is a key enzyme in the lignin pathway that catalyzes phenylalanine's deamination transscinnamate. In rice, drought stress caused upregulation of PAL (Yang et al., 2007). Furthermore, several adverse physiological changes have been considered because of drought stress (Table 3). In maize plants, the activity of anionic peroxidase is reduced by the water deficit and may reduce the synthesis of lignin (Klein et al., 2008). Lignin deposition occurred in a particular region of roots (Fahad et al., 2017; Pandey et al., 2010).

In rice plants, genes responsible for cell development and root extension were induced after exposure to 16 h water stress (Pandey et al., 2010). Similarly, water stress causes lignin deposition in *Citrullus lanatus* sp. (Pandey et al., 2010). In another study, PAL and ascorbate peroxidase activity was found to be enhanced after 28 days of exposure to water deficit in *Trifolium repens* with increased lignin biosynthesis (Alvarez et al., 2008; Fan et al., 2006). During drought stress, the yield and physiological traits were observed in *Z. mays* L. var. TWC647 by spraying amino acids

leading to a significant increase in lignin contents with an increased accumulation of proline (Yoshimura et al., 2008). In another study, soybean seeds were examined under the condition of water deficit and discovered that phenol, isoflavone and lignin contents were higher in the treated seeds, and found that all these compounds play a role in biotic and abiotic stress tolerance (Lee et al., 2007).

Under drought stress, soybean significantly enhances the expression on the CCoA-OMT gene typically elongated region of roots with enhanced lignin accumulation (*Table 3*) (Komatsu et al., 2010). CAD and COMT, two key lignin genes, were upregulated under drought stress, showing a positive response in maize (Kasraie et al., 2012). Under low water stress, the Cinnamyl CoA reductase (CCR) gene upregulated significantly in maize roots (Bellaloui et al., 2012). Drought stress had a significant effect on the lignin and CCR protein expression in *Leucaena* stem development, suggesting the role played by lignin in drought stress tolerance (Yamaguchi et al., 2010).

Table 3. Lignin response to environmental stress (drought)

Species	Plant organs	Effect on lignin	Ref
Maize	Xylum sap	↓	Alvarez et al., 2008
Water melon	Roots	↑	Yoshimura et al., 2008
Trifolium	Leaves	↑	Bok-Rye et al., 2007
Soybean	Seeds	↑	Bellaloui et al., 2012
Soybean	Roots	↑	Al-Hakimi et al., 2006

Lignin and extreme temperature

Temperature is major abiotic stress-causing considerable damage to physiological processes and limiting plant growth and development worldwide (Bellaloui et al., 2012). High and low temperature exacerbates normal plant metabolism, including photosynthesis, nucleic acid and protein, membrane lipid peroxidation, and flower bud abortion—alteration in any of these parameters threatening agriculture by significant yield losses (Hu et al., 2009).

Likewise, other abiotic stresses, lignin, play a fundamental role in temperature (both low and high temperature) stress and improve plant growth and development (*Table 4*) (Fan et al., 2006). C3H (p-coumarate 3-hydroxylase) is a major gene involved in the lignin biosynthesis pathway. Recently, cold acclimation induced (C3H) gene expression in the Rhododendron plant leading to enhanced lignin contents in its leaves. In Rhododendron, C3H might affect the water permeability and rigidity of call wall by altering the S/G ratio during cold stress (Srivastava et al., 2015). In *Betula platyphylla*, the BpMADS12 gene regulates lignin metabolism against brassinosteroid (BR) signaling, endeavoring to elaborate the mechanisms responsible for development and wood formation (Le et al., 2015). The author reported that BpMADS12 regulates several Brassinosteroid biosynthesis genes.

Brassinosteroid (BR) is a plant steroid hormone, which is potentially involved in chilling and heat stress in a plant (Liu et al., 2018). The lignin content of the cad1 mutant was significantly lower than that of the wild type in *Medicago truncatula*; while, there was no visible growth difference between the mutant and wild types at normal temperatures. These results represented that Cinnamyl Alcohol Dehydrogenase 1 (CAD1) is involved in lignin in this MtCAD1 mutant (Liu et al., 2018).

Table 4. Lignin response to environmental stress (temperature)

Species	Plant organs	Effect on lignin	Ref
Low temperature			
<i>Populus tremula</i>	Seedlings	↑	Hausman et al., 2000
<i>Brassica napus</i>	Seedlings	----	Solecka et al., 1999
<i>Glycine max</i>	Roots	↑	Janas et al., 2000
<i>Triticum aestivum</i>	Leaves	----	Olenichenko and Zagoskina, 2005
High temperature			
<i>Panicum maximum</i>	Fifth Leaf	↑	Ford et al., 1979
<i>Setaria anceps</i>	-----	↑	
<i>Heteropogon contortus</i>	Fifth Leaf	↑	
<i>Chloris gayana</i>	Fifth Leaf	↑	
<i>Paspalum dilatatum</i>	Fifth Leaf	↑	
<i>Sorghum almum</i>	Fifth Leaf	↑	
<i>Cenchrus ciliaris</i>	Fifth Leaf	↑	
<i>Themeda australis</i>	Fifth Leaf	↑	
<i>Panicum coloratum</i>	Fifth Leaf	↑	
<i>Melinis minutiflora</i>	Fifth Leaf	↑	
<i>Cynodon dactylon</i>	Fifth Leaf	↑	
<i>Digitaria smutsii</i>	Fifth Leaf	↑	
<i>Astrebla lappacea</i>	Fifth Leaf	↑	
<i>Triticum vulgare</i>	Fifth Leaf	↑	
<i>Bromus unioloides</i>	Fifth Leaf	↑	
<i>Festuca arundinacea</i>	Fifth Leaf	↑	
<i>Avena sativa</i>		↑	
<i>Lolium rigidum</i>	Fifth Leaf	↑	
<i>Dactylis glomerata</i>	Fifth Leaf	↑	
<i>Phleum pratense</i>	Fifth Leaf	↑	
<i>Phalaris tuberosa</i>	Fifth Leaf	↑	
<i>Danthonia caespitosa</i>	Fifth Leaf	↑	
<i>Hordeum leporinum</i>	Fifth Leaf	↑	
<i>Lolium perenne</i>	Fifth Leaf	↑	
<i>Panicum maximum</i>	Fifth Leaf	↑	

Lignin and elevated CO₂

Currently, CO₂ concentrations have risen up to 400 ppm due to industrialization and are expected to double in the 2100 century (Wei et al., 2006). It is well known that an elevated level of CO₂ has profound effects on the morphology and physiology of both C₃ and C₄ plants. According to the previous experiments conducted in maize, elevated CO₂ causes stomatal closure and reduces its conductance but increases biomass, growth, and yield (Li et al., 2016). The growth and biomass stimulation are often concomitant

with higher carbon assimilation (Zhao et al., 2013) depending on the response of the species considered. Moreover, elevated CO₂ has little effect on C4 species than herbaceous species, but trees are more responsive to CO₂. Similarly, nitrogen (N) availability was found to CO₂-induced stimulation of photosynthesis. According to the source-sink balance hypothesis, it is predicted that elevated CO₂ escalates the concentration of secondary metabolites (Ainsworth and Long, 2005). The carbon availability is relatively increased by the elevated CO₂, or nutrient stress that ultimately leads to the source leaves, being rich in carbon-based secondary or structural compounds. There is a general tendency towards increasing levels of secondary compounds shown by the various authors, but the chemical nature of lignin varies depending on the species or genotype (Ainsworth and Long, 2005). There are many controversial studies about the contents of lignin and elevated CO₂. Numerous studies have shown that elevated CO₂ enhances Lignin content in tree leaves (Raven et al., 2012). However, in each case, the effect of CO₂ was shown to be highly dependent on N supply (Bidart-Bouzat and Imeh Nathaniel, 2008). Leaf lignin content was higher under the effect of elevated CO₂ in N-limited plants, whereas in plants grown with high nutrient supply, there was no change or decrease in lignin contents by elevated CO₂ (Table 5) (Norby et al., 2001).

Table 5. Lignin response to environmental stress (elevated CO₂)

Env. stress	Species	Plant organs	Effect on lignin	Ref
CO ₂	Herbaceous/woody plants	Leaf liter	↑	Norby et al., 2001
	<i>N. tabacum</i>	Root	----	Matros et al., 2006
	<i>N. tabacum</i>	Leaves	-----	Schlimme et al., 2002
	<i>Populus tremul</i>	Stem	↑	Richet et al., 2012
	<i>Betula pendula</i>	Leaves	↑	Oksanen et al., 2005
	<i>Glycine max</i>	Leaves	↑	Booker et al., 2005

The recent transcriptomic analyses show that elevated CO₂ causes stimulation of the phenylpropanoid pathway. For example, birch trees accumulated phenylpropanoid pathway transcripts during the month of August under elevated CO₂ levels cultivated for six years. In this study, the authors suggested that this stimulation would ultimately give rise to phenolic compounds (Porteous et al., 2009). The same pattern of stimulation regarding gene expression was also observed in *Arabidopsis* grown under elevated CO₂. Among other cell wall related genes, PAL1 and LAC4 genes were upregulated.

During metabolite profiling, it was observed that elevated CO₂ affects most of the amino acids except phenylalanine, histidine, and tryptophan. In another study, genes involved in secondary metabolism, particularly phenylpropanoid pathway genes, also show increased expression in soybean grown for 40 days under elevated CO₂. However, response to elevated CO₂ was genotype dependent, as demonstrated earlier (Blaschke et al., 2002). In a study, two clones of *Populus tremuloides*, CO₂ responsive and unresponsive, were grown in long term FACE experiments. These two genotypes showed similar physiological responses, including leaf area index, photosynthesis, and stomatal conductance. It is well known that leaves are the entrance and source organs, so studies regarding the effect of elevated CO₂ on plants have been focused on leaf physiology and biochemistry. In *Populus tremuloides*, gene expression profiles of

leaves and stems reflect a higher abundance of the gene in stems suggesting a higher readjustment to elevated CO₂ levels in stems (Dong et al., 2018). Most genes were found to be upregulated in leaves regarding shikimate and flavanol synthesis; whereas phenylpropanoid and lignin synthesis (C3H, COMT and CAD) genes were found to be upregulated in stems. In free air concentration enrichment (FACE) experiments, it was shown that lignin content was enhanced in the wood from coppices of both *Populus euramericana* and *Populus nigra* (Kontunen et al., 2010) and in birch as well (Cseke et al., 2009). However, other studies found contradictory results with reduced lignin (Bidart-Bouzat and Imeh Nathaniel, 2008), and no change in lignin contents was found (Druart et al., 2006).

Lignin and ozone

Among other pollutants, tropospheric ozone is well known pollutants having a detrimental effect on plants (Luo et al., 2009). The ozone concentration has been increasing since the beginning of the industrial era and is predicted to be more than double in the future (IPCC, 2007). Ozone has many adverse effects on plants' growth and yield (Mattson et al., 2005). Additionally, ozone has been shown to cause visible damage to leaves as plants respire ozone through stomata generating reactive oxygen species (ROS) after reacting with apoplast. It has also been suggested that ozone may induce plant defense reactions such as programmed cell death (Kostiainen et al., 2008). Many reports present the role of ozone in stimulating phenylpropanoid metabolism in many species both at an acute and chronic level. The PAL enzyme activity was shown to increase by ozone in *Pinus sylvestris* (Booker et al., 2009), soybean, *Glycine max* (Wittig et al., 2009) poplar (*Populus* spp.) and grape (Rosemann et al., 1991). There are numerous transcriptomics studies available representing that ozone control lignification through transcriptional regulation, for example, PAL, 4CL, C3H, CCoAOMT, and CCR in rice (Booker et al., 1998). COMT in beech *Arabidopsis* (Di Baccio et al., 2008) and CCR in birch responded to ozone treatment (Porteaus et al., 2009). Beyond the stimulation of the phenylpropanoid pathway, it was found interesting that ozone also upregulates shikimate pathway that provide substrate to phenylpropanoid pathway (Hawkins et al., 2003; Sgarbi et al., 2003).

It was found quite interesting that experiments conducted in open top chambers and natural conditions such as free air ozone fumigation facilities; plants show the same response and trend in both cases (Frei et al., 2011). Conclusively, it is obvious that the phenylpropanoid pathway is upregulated in leaves under ozone treatment so it might be involved in defense and acclimation mechanisms. However, until recently, it is not clear how does ozone affect lignin in leaves as no modifications of lignin content were observed in soybean (Wittig et al., 2009), barley (Olbrich et al., 2009), *Pinus taeda* (Janzik et al., 2005), yellow poplar (Betz et al., 2009), cotton (Plessl et al., 2005) and *Quercus ilex* (Booker et al., 1996). On the other hand, there are several contradictory reports that show an increase in Lignin contents after ozone treatments in poplar, *Trifolium* spp. (Boerner et al., 1995) sugar maple (Betz et al., 2009), *Briza maxima* (Booker et al., 2000), and rice (Booker et al., 1998).

Two possibilities are there supporting these contradictory results. The first possibility is that the ozone effect might be species dependent, so some species respond well and others not. For example, increasing contents of lignin has never been found in conifer (Wittig et al., 2009) and another possibility is the use of various techniques applied and the potential errors made in handling those techniques, i.e., Klason, LTGA, used to

determine lignin contents (Baldantoni et al., 2011), especially in those cases where there are weak variations between control and experimental samples.

Extracellular ATP and uncommon nucleotide in lignin biosynthesis

Plants have established a signaling network enabling them to maintain homeostasis against external environmental stimuli. These signals are perceived by the receptors in the plasma membrane that ultimately respond to the external stimulus in the form of altered metabolism. Here, we focus on the role of extracellular ATP (eATP) and uncommon nucleotides such as mono- (pnNs) and dinucleotide polyphosphates (NpnN's) and their role as signaling molecules throughout lignin biosynthesis under abiotic stress.

Involvement of eATP in responses to abiotic stresses

Text Adenosine 5'-triphosphate (ATP), along with the other nucleotide triphosphates, has been identified as a source of energy in various cell reactions in both animal and plant species (Muntiferi et al., 2006). The role of nucleotides as signaling molecules that functioned similarly in plants and animals was regarded with skepticism for decades. The discovery of a membrane receptor protein and its high affinity for extracellular nucleotides was a real breakthrough in this area (Sanz et al., 2011). In contrast, the mechanism of ATP transfer from the cytoplasm into the extracellular matrix in plants was discovered earlier (Dence, 1992). ATP can be released in a variety of ways in plants; environmental stimuli can cause the release of ATP by the exocytosis (Tripathi et al., 2018), injured cell membrane (Choi et al., 2014), and by the p-glycoprotein (PGP1) transporters (Dark et al., 2011), and plasma membrane localized nucleotide transporters (PM-ANT1) (Song et al., 2006). The interaction between eATP and receptors triggers a series of downstream physiological changes that protect the plant from environmental stresses ensuring appropriate growth and development (Kim et al., 2006). In plants, eATP, on binding to the P2K1 receptor, playing a role as an active messenger stimulating signaling pathways. Cytosolic secondary messenger, including reactive oxygen species (ROS), Ca²⁺, and nitric oxide (NO) exist in various signaling pathways. These secondary messengers cause the phosphorylation of mitogen activated protein kinases (MAPK), and induce the expression of defense related genes (Thomas et al., 2000). Apart from eATP-triggering and signaling molecules, the classical protective hormones, namely jasmonate, ethylene, and salicylic acid, induce plant resistance to pathogens and abiotic stresses (Rieder et al., 2011).

Very few studies demonstrate the role played by eATP against different environmental stresses. Apart from mechanical stress, ATP is also generated in the presence of molecules such as L-glutamate and abscisic acid (Lim et al., 2014). Osmotic and salt stress can also cause similar reactions (Tanaka et al., 2014) and cadmium treatment (Jewell et al., 2019). Therefore, eATP accumulation induces a plant defense system against various abiotic and biotic stresses. In response to environmental stimuli, it enables rapid leaf stomatal closure (Clark et al., 2011), probably seedling viability improvement (Jeter et al., 2004) and alterations of root growth while undergoing hindrance (Hou et al., 2017).

Likewise, hypertonic salt stress disrupts the photosynthesis machinery by lowering photosystem II's maximum efficiency and depleting the photochemical quenching method (Jewell et al., 2019). Furthermore, cadmium induced abiotic stress enhances

lipid peroxidation as well as antioxidant and lipoxygenase activities in *Arabidopsis thaliana* cells (Jewell et al., 2019). All of these reactions cause jasmonic acid to be synthesized, a molecule involved in a variety of stress responses (Chen et al., 2017). These reactions are linked to increased jasmonic acid synthesis, essential molecules involved in stress responses.

Cadmium is a major anthropogenic contaminant today that poses a serious threat to human health today (Kim et al., 2009). In plants, it disrupts photosynthesis and respiration, resulting in a decline in crop biomass (Tanaka et al., 2010). Cadmium, in turn, encourages oxidative stress by synthesizing reactive oxygen species (ROS) (Kim et al., 2009), alterations in gene expression (Wasternack et al., 2018; Rizwan et al., 2017) and changes in enzyme activity as well (Fargaová et al., 2018) that ultimately lead to activating plant defense.

Uncommon nucleotides as a signaling molecule and lignin biosynthesis

Although mono and dinucleoside polyphosphates were discovered in the late twentieth century, our understanding of their biological role, especially in plants, is still limited. Mononucleoside polyphosphates (PnNs) encompass a nucleoside and oligophosphate chain, e.g., adenosine 5'tetraphosphate (p4A) and adenosine 5'-pentaphosphate (p5A). In higher plants, despite the lack of awareness about the nature and accumulation of mononucleotide polyphosphates, several enzymes have been identified that can synthesize pnNs; 4-coumarate: CoA ligase (4CL2) from *Arabidopsis thaliana* was the first plant enzyme synthesizing pnNs, and it mediates the reaction of p4A and p5A synthesis (Sarry et al., 2006). In the phenylpropanoid pathway, this enzyme contributes to flavonoids, lignin, and stilbenes biosynthesis as a branch point. Plants show responses against various environmental changes in terms of phenylpropanoid pathway activation (Herbette et al., 2006).

In *Arabidopsis thaliana*, amino acid synthetase (JAR1) is another enzyme that synthesizes p4A and triggers the function of jasmonic acid (JA) (Sanità et al., 1996). JAR1 plays a role in the action of jasmonic acid (JA) as a plant hormone, as well as catalyzing the synthesis of other JA-amido conjugates, the most important of which is jasmonic acid isoleucine. Either of the plant enzymes listed above is a member of the acyl adenylate-forming firefly luciferase superfamily (Pietrowska et al., 2003) catalyzes a two-step reaction. Firstly, ATP and acyl combine to synthesize an acyladenylate intermediate, releasing pyrophosphate simultaneously (PPi). Secondly, 4CL mediates the synthesis of uncommon mononucleoside polyphosphates, including p4A and p5A, in the absence of CoA (Herbette et al., 2006).

Only a few papers have been published so far describing the function of uncommon nucleotides in plants. Exogenous uncommon nucleotides have been shown to affect the phenylpropanoid pathway (Dixon et al., 1995). According to the most recent report, in juvenile *Arabidopsis thaliana* seedlings, diadenosine 5',5''' P1, P3-triphosphate (Ap3A, ApppA) and diadenosine 5',5''' P1, P4-tetraphosphate (Ap4A, AppppA) regulate gene expression and enzyme activity implicated in the phenylpropanoid pathway (Guranowski et al., 2007). Similarly, Ap3A and Ap4A was reported to stimulate the expression of (about 70-fold) PAL2 genes that significantly enhanced PAL activity (about 9-fold). Furthermore, it was reported that seedlings treated with Ap3A and Ap4A showed higher expression of the 4CL gene (Staswick et al., 2002). These findings also suggest that 4CL has a dual role in the plant's stress response. The activation of the phenylpropanoid pathway by NpnNs, which increases the development of various

phenylpropanoid compounds, including flavonoids, lignin, anthocyanins, and salicylic acid, is recognized as one of higher plants' defensive strategies against environmental stresses (Fig. 3) (Pietrowska et al., 2011).

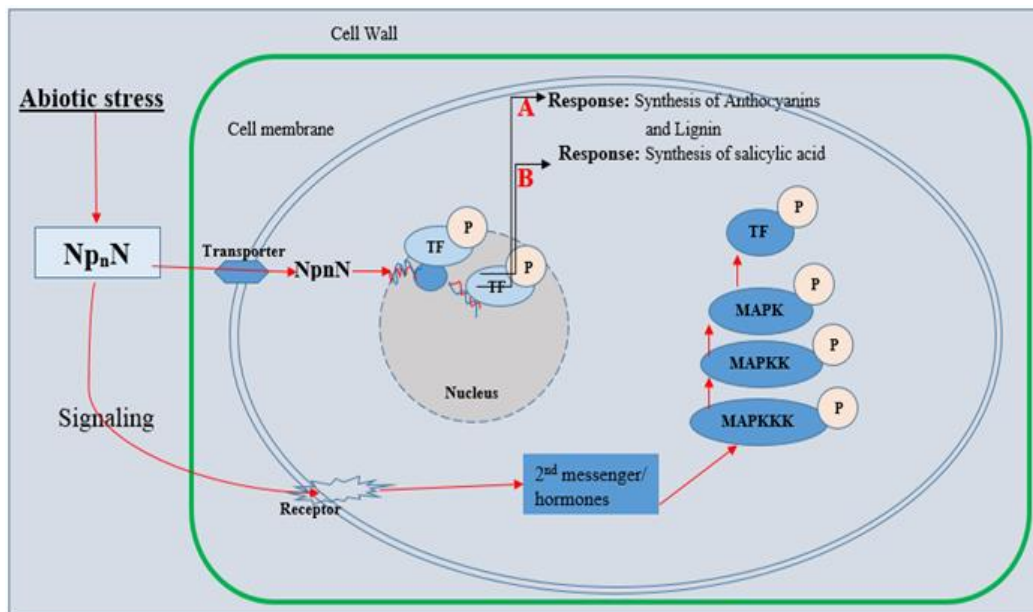


Figure 3. Theoretical model showing *NpnN* signaling network of *NpnN* in plant cell

Conclusion

Recently, considerable research has been done to understand the molecular and physiological mechanism of lignin to define its regulatory mechanism, signaling transduction pathway, and transcription factors involved in stress responses. Lignin is a polymer that plays a variety of roles in the plant kingdom. It is one of the most abundant renewable raw materials. Lignin biosynthesis can be influenced by various abiotic and biotic environmental influences, reducing the harmful effects as described above. Numerous studies reported various candidate genes (QTLs) and transcription factors potentially involved in lignin biosynthesis under abiotic stresses. In this review, we addressed different aspects of the lignin signaling transduction pathway to clarify how lignin induces stress tolerance and biosynthesis mechanism, but the underlying mechanism needs to be explored. For better understanding, researchers are looking into the role of the lignin-signaling pathway, hormone interactions, and crosstalk at the organ, tissue, and cell levels. Future research will explore the possibility of genetically engineering crop varieties and improving nutrient acquisition to reduce fertilizer usage.

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