REVIEW ARTICLE

Lignin biosynthesis and its molecular regulation*

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Abstract Lignin biosynthesis has become increasingly highlighted because it plays an important role in the growth and development of plant, in the systematic evolution of plant and in the human life. Due to the progress in the field of lignin studies in recent years, the lignin biosynthesis pathway has been revised. Here we discuss some genetic engineering approaches on lignin biosynthesis, and conceive strategy to regulate lignin biosynthesis in order to use lignin resource more efficiently in agricultural and industrial productions.

Keywords: lignin, biosynthesis, molecular regulation, genetic manipulation.

Lignin is a major structural compound of cell wall in vascular plants (pteridophytes, gymnosperms and angiosperms), being incorporated into the cell wall together with the process of thickening plant secondary wall. Distinct from other macromolecules such as protein and cellulose in which all subunits are linked via regular bonds, lignin is derived from dehydrogenative polymerization of three main monolignols, named as *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. Those monolignols are all hydroxycinnamyl alcohols, differing from each other only in the degree of hydroxylation and methylation. These three monolignols are linked together to form high molecular lignin via various kinds of intermonomeric linkages. There are 16 kinds of intermonomeric linkages in theory. This leads to forming hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units in lignin polymers respectively. Now, other uncommon phenylpropanoid ramifications, for example, coniferaldehyde, 5-hydroxyconiferaldehyde and coumaraldehyde, have also been found to be incorporated into lignin[1]. Peroxidases and laccases are proposed to catalyze the polymerization of monolignols into lignin.

Lignin is principally deposited in the secondary wall of certain plant tissues, including xylem, sclerenchyma, phloem fibers and periderm, which are involved in mechanical support of plant stems and hydrophobicity protection of plant cell wall. In plant cell wall, lignin is linked to cellulose, hemicellulose and other cell wall polymers to form extracellular matrix, this structure increases the mechanical intensity and supportable ability of plant tissues. It contributes rigidity and compressive strength to plant stem, which is related to the lodging-resistant phenotype in crop plants such as wheat, rice and maize. Because of its natural property of hydrophobicity, lignin imparts water impermeable to plant cells and tissues. This function is very important, not only in xylem and phloem to transport water and mineral components but also for the successful colonization of land by plants. In fact, the terrestrial vascular plants are proposed to evolve on the earth by the concomitant evolution of lignin biosynthesis. In addition, lignin accumulation in the cell wall forms a physically structural barrier to effectively protect plant from pathogens and lignin synthesis will be induced in response to various kinds of abiotic and biotic stresses such as pathogen infection and wounding.

Second to only cellulose, lignin is the most abundant biopolymer in nature, which accounts for about 30% of carbon (about 4.2×10^{11} kg) fixed into terrestrial plant each year[2]. Lignin content varies among different plants. In trees, lignin content repre-

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sents 27% ~ 32% of the plant dry weight, while it only represents approximately 14% ~ 25% of dry weight in herbaceous plants. As a biodegradable biopolymer in nature, lignin is intimately related to human life. Lignin quantity and composition become a limiting factor that affects not only the quality of paper, but also the digestibility of forage crops. In pulping industry, lignin is an undesirable component that must be removed in the chemical process, which is not only expensive but also leads to severe environmental pollution. Lignin concentration and methoxyl content are negatively correlative with forage digestibility for ruminant animals.

Because of important roles of lignin both in plant growth and development and in agriculture and industry, there is considerable attention to lignin biosynthesis in recent decades. As the application of molecular biology and genetic manipulation in the area of lignin research, understanding of lignin biosynthesis has been deepened and alternation of lignin quantity and composition will become reality.

1 The pathway of lignin biosynthesis

Lignin biosynthesis is a complex physiological and biochemical pathway in plant. This pathway is involved in deamination of *L*-phenylalanine, hydroxylation, methylation, reduction and polymerization of monolignols. Monolignols is synthesized in the cytoplasm, and then transported through the plasma membrane into the cell wall where polymerization of monolignols occurs. There are several literatures in which lignin biosynthesis are summarized. Here we revise some misunderstandings and propose a possible pathway of lignin biosynthesis according to recent progress in this field. Among them, the important progress resulted from the isolation and characterization of coumaric acid 3-hdroxylase (C3H) gene and sinapyl alcohol dehydrogenase (SAD) gene, redefinition of ferulate 5-hydroxylase (F5H) and bispecific caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT) based on kinetic analyses of those two enzymes. Those enzymes have a new position in the successive reactions of hydroxylation and methylation.

Recently, C3H and CYP98A3 from *Arabidopsis* were identified as a P450 protein by the functional genomics approach, which catalyzes the conversion of 5-O-shikimate and 5-O-D-quinate esters of *p*-coumarate into their corresponding caffeic acid conjugates[3]. Franke et al. simultaneously demonstrated that CYP98A3 played a role in lignin biosynthesis. Analysis of *Arabidopsis* mutant termed *ref8* proved that CYP98A3 was C3H. Lignin concentration was reduced dramatically in *ref8* mutant. And also lignin in this mutant contained mainly *p*-coumaryl alcohol, instead of guaiacyl and syringyl monomers in wild-type[4]. Those data showed that shikimate and quinate of *p*-coumaroyl were likely to be lignin biosynthetic intermediates. F5H is another monooxygenase dependent on cytochrome P450 that is thought to catalyze the hydroxylation at the C_5 position of ferulic acid to form 5-hydroxyferulic acid. However, over-expression of F5H in transgenic *Arabidopsis*, tobacco and poplar gave rise to lignin that was composed of almost S units[5]. Feeding experiment with labeled precursors in magnolia provided evidence that sinapyl alcohol came from coniferyl alcohol. Kinetic analysis of F5H enzyme showed that this enzyme had a thousand-fold greater affinity for coniferaldehyde and coniferyl alcohol than for ferulic acid[6]. The similar results derived from kinetic characterization of COMT indicated that 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol should be the suitable substrates of this enzyme, which was the products of coniferaldehyde and coniferyl alcohol hydroxylation catalyzed by F5H. Taken together, F5H and COMT likely act on hydroxylation and methylation at the levels of aldehydes and alcohols, not previously described at free acid levels.

Cinnamoyl-CoA reductase (CCR) catalyzes the reduction of hydroxycinnamoyl-CoA esters into corresponding aldehydes, the first step of lignin branch pathway. This step may be the key point that controls the influx of carbon into lignin biosynthetic pathway. When CCR activity was inhibited in transgenic tobacco, Klason lignin content decreased by approximately 50% with the association of the altered plant development such as reduced size, abnormal morphology of the leaves and collapsed vessels. Recently, a novel gene encoding sinapyl alcohol dehydrogenase (SAD) was identified from poplar. SAD is similar to CAD but with different substrate specificity and expressing localization. SAD catalyzes the reduction of sinapaldehyde, while CAD is responsible for the reduction of coniferaldehyde. SAD, together with F5H and COMT, is expressed and localized in cells and tissues with S units of lignin, however, CAD activity is detected in tissues that has G lignin synthesis[7]. These data suggest that the different monolignols are likely to be reduction via different pathways

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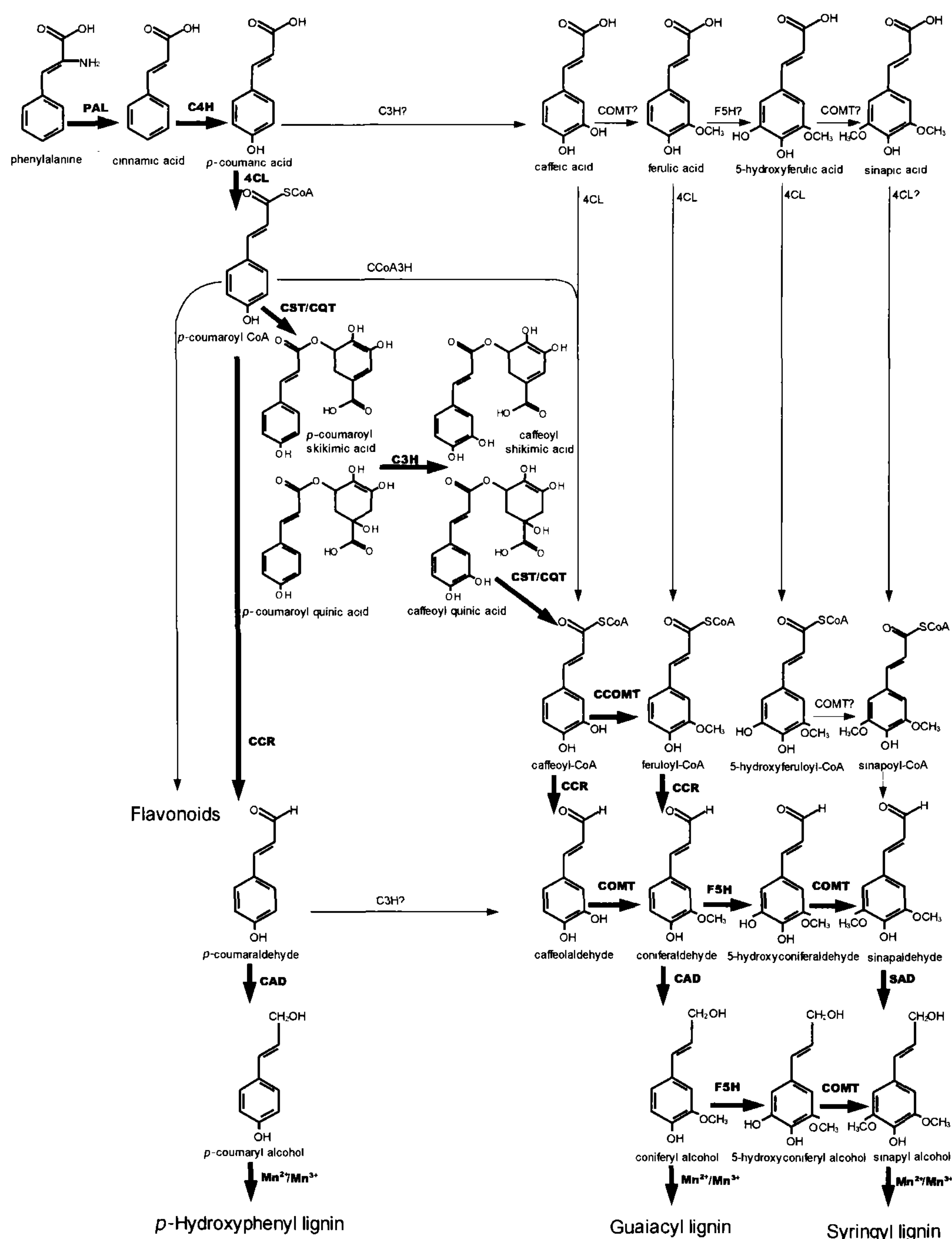

Fig. 1. Pathway of lignin biosynthesis. Black arrows and black enzymes are used to indicate possible channels of lignin biosynthesis, but fine arrows show the revised parts in lignin biosynthesis. CAD, cinnamyl alcohol dehydrogenase; CCR, cinnamoyl-CoA reductase; C3H, coumaric acid 3-hdroxylase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate CoA ligase; COMT, Bispecific caffeic acid/5-hydroxyferulic acid O-methyltransferase; CCOMT, affeoyl-CoA O-methyltransferase; CQT, hydroxycinnamoyl CoA: quinate hydroxycinnamoyltransferase; CST, hydroxycinnamoyl CoA: shikimate hydroxycinnamoyltransferase; F5H, ferulic acid 5-hydroxylase; PAL, phenylalanine ammonia-lyase; SAD, sinapyl alcohol dehydrogenase.

at the last step, and different enzymes will play the roles in those pathways.

As an extraordinary complex process, lignification is involved in polymerization of radicals initiated by redox enzymes such as peroxidases and laccases. Direct evidence of involvement of those enzymes in the monolignol polymerization, however, are still limited. Recently, Hans et al. reported that Mn^{2+}/

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Mn^{3+} redox shuttle played an important role in the lignin formation. The polymers synthesized *in vitro* were very similar to natural lignin in structure. Although it was suggested that peroxidase took part in the lignin synthesis, this enzyme did not contact directly with any lignin monomers in the process of polymerization. Instead, peroxidase oxidized Mn^{2+} to Mn^{3+}, and Mn^{3+} oxidized monolignols to radicals together with reduction of Mn^{3+} to Mn^{2+}. This was called as Mn^{2+}/Mn^{3+} redox shuttle. The monolignols with radicals will form high molecular lignin by various kinds of covalent linkages through an automatic process[8].

Currently, there are several models to explain the lignin formation. The random coupling model was proposed from some early research work. It was suggested that lignin was assembled via coupling of individual monomers in a random fashion. As the results, the amount and type of monolignols at the lignin radicals site and chemical coupling features would determine final lignin formation, and different monolignols could freely exchange in lignin structure[9]. This hypothesis can account for the plasticity of lignin biosynthesis in some mutants and transgenic plants. The dirigent protein model was proposed recently after the discovery of dirigent protein in plants. The dirigent protein was demonstrated to control the formation of individual bonds between special monolignols[10]. According to this model, lignin formation should be a strictly regulated process under special protein termed dirigent protein and its homologues. This model can explain the issue why the majority of bonds in lignin are 8-O-4. In addition, Dixon et al. suggested that metabolic channeling was existed that allowed for G and S lignin formation through independent pathways[11]. In this model, a microsomal-associated compound including most enzymes in special arrangement was involved in the biosynthesis of S lignin. Cinnamic acid 4-hydroxylase(C4H), C3H and F5H, which were all cytochrome P450 enzymes, were combined with this compound through endoplasmic reticulum (ER) system. As a result, the metabolic channel to S monolignol was conducted in the membrane system. Because phenylalanine ammonia-lyase (PAL), caffeoyl-CoA O-methyltransferase (CCOMT), CCR and CAD also exist in a soluble forms, a pathway to G monolignol formation was conducted in cytoplasm, which was different from S lignin channel.

2 Molecular regulation of lignin biosynthesis

Due to the potential economic importance of lignin on paper pulping and forage industry, people have paid much attention to modify lignin quantity and composition in such important resource plants as purple medic, *Eucalyptus gunnii* and poplar using genetic engineering approach. To date, most genes involved in lignin biosynthesis have been isolated from various plants, and many transgenic plants have been constructed (Table 1), including genes of COMT, CCOMT, F5H, CCR and CAD that have the critical role in lignin biosynthesis.

Table 1. Genetic engineering of lignin biosynthesis

Gene	Plant	Technique	Activity reduced (%)	Composition	Content (%)	Ref.
PAL	Tobacco	Cosuppression	80	G reduced	70 decreased	[12]
	Tabacco	Cosuppression	83	Methoxyl unit increased	43 decreased	[13]
C3H	*Arabidopsis*	Mutant (*ref8*)	—	H increased, G, S reduced	40 decreased	[14], [4]
C4H	Tobacco	Antisence	80	S reduced	80 decreased	[13]
F5H	*Arabidopsis*	Overexpression	—	Only S	—	[15]
	Tobacco; poplar	Overexpression	—	S increased	25 decreased	[5]
4CL	*Arabidopsis*	Antisense	92	G reduced	30 decreased	[16]
	Aspen	Antisense	90	Cellulose increased	45 decreased	[17]
	Tobacco	Sense and antisense	99	S reduced	34 decreased	[18]
	Tobacco	Antisense	—	S reduced	No change	[19]
COMT	Poplar	Antisense	90	G increased	No change	[20]
	Tobacco	Antisense	60	G, S reduced	35 decreased	[13]
	Aspen	Cosuppression	75	S reduced	No change	[21]
	Poplar	Antisense	95	S reduced, 5-OHG new	No change	[22]
	Tobacco	Antisense	70	No change	15~58 decreased	[23]
	Alfalfa	Antisense and cosuppression	96	G reduced, S disappear	13~29 decreased	[24]

To be continued

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Continued

Gene	Plant	Technique	Activity reduced (%)	Composition	Content (%)	Ref.
CCOMT	Tobacco	Antisense	70~80	G, S reduced	47 decreased	[25]
	Alfalfa	Antisense	96	G reduced, S no change	17 decreased	[24]
CCR	Tobacco	Antisense	70	G, S reduced, S/G increased	47 decreased	[26]
	Arabidopsis	Mutant (*irx4*)	—	G, S reduced	50 decreased	[27]
CAD	Poplar	Antisense	70	S increased	Little change	[20]
	Alfalfa	Antisense	70	S reduced	No change	[28]
	Maize	Mutant (*bm1*)	60~70	G, S reduced	20 decreased	[29]
	Loblolly pine	Mutant	—	coniferaldehyde increased	9 decreased	[30]
	Sorghum	Mutant	—	—	15~25 decreased	[31]

The Klason lignin decreased dramatically when PAL or C4H activity was down-regulated in transgenic tobacco, which led to alternation of S/G ratio. Down-regulation of C4H resulted in a reduction of S units, similar to the results from down-regulation of COMT and F5H in *Arabidopsis* mutant and transgenic tobacco. Therefore, it is possible that C4H is involved in the poly-enzyme complex directed to S lignin biosynthesis, while G lignin is formed through another pathway bypassing C4H. Over-expression of F5H in *Arabidopsis* contributed to lignin that was mostly composed of S units. Down-regulation of F5H, however, induced a decrease of S units content in lignin. This confirmed that F5H should be a bottle-neck in the pathway to S lignin synthesis. C3H catalyzes the hydroxylation at the C_3 position of *p*-coumaric acid to caffeic acid. The *ref8* mutant defective in the gene encoding C3H had diverse changes in lignin metabolism, for example, the 20% ~ 40% reduction of lignin content, accumulation of lignin which forms primarily from H units, and appearance of uncommon component in the lignin. Furthermore this mutant showed a defective development and sensitivity to fungal attack, which suggests that C3H is necessary for both the growth and development of plant and the reactions of disease resistance[14].

In the transgenic tobacco, poplar and alfalfa, the effects of COMT down-regulation were more significant on S unit than on G unit in lignin composition. This provided the evidence of re-localization of COMT position in the lignin biosynthesis. The brown *midrib3* mutant was resulted from the insertion of a retro-transposon into the COMT gene in maize. This mutant showed 20% reduction of COMT activity comparing with wild plant, and together with decrease in S/G ratio, but without change in lignin content. Directed under a vascular-tissue specific promoter in transgenic alfalfa, down-regulation of COMT resulted in 30% reduction in Klason lignin content that including of decrease in both G and S monomers, but acetylbromide soluble lignin was not reduced[24]. Our recent work indicated that the expression of COMT gene isolated from wheat was related to stem rigidity and lodging-resistant character of wheat[32].

CCOMT also catalyzes methylation reaction in another pathway of lignin biosynthesis. The Klason lignin content was decreased in transgenic tobacco and alfalfa when CCOMT activity was down-regulated. G lignin concentration in the transgenic alfalfa was dropped by 50%, but S lignin concentration did not have any changes when CCOMT activity was almost completely inhibited[13]. These data suggest that CCOMT could participate in the methylation reaction of G lignin synthesis, without the relation to S lignin formation. Consequently, there are different processes in methylation reaction between G and S lignin synthesis.

Down-regulation of 4-coumarate: CoA ligase (4-CL) in different plants, however, had the contradictory results. The inhibition of 4-CL activity in transgenic tobacco resulted in reduction in total lignin levels. The lignin composition was also changed that mainly on S lignin, which led to S/G value from 4.5 to 2.0. The reduction of 4-CL activity only decreased G lignin in transgenic alfalfa, but without influence on S lignin. In transgenic poplar, it was reported that down-regulation of 4-CL led to remarkable reduction in lignin quantity, without influence on lignin composition[17].

Down-regulation of CCR activity up to 70% by antisense technology led to decrease in both lignin content and G unit composition. As a result S/G ratio was increased. The *irx4* mutant, defective in CCR gene in *Arabidopsis*, had only 50% less lignin than wild type plants. The growth of this mutant was slower than wild type plants. This mutant also lacked the upright growth habit and exhibited the collapsed

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xylem phenotype. Its fertility reduced at elevated temperature (31 ℃)[27]. We have analyzed wheat CCR expression in different organs of wheat[33], and obtained some transgenic tobacco lines that had integrated wheat CCR gene. The Klason lignin content decreased about 11.7% in these transgenic tobacco lines. These transgenic lines also grew more slowly than wild type tobacco (data not shown). Down-regulation of CAD resulted in decrease of S units but without changes in actual lignin content. At the same time, some new components were found in these transgenic lines such as cinnamaldehyde[28]. The hybrids, in which CAD and CCR activities were simultaneously down-regulated, displayed a strong decrease down to 50% in lignin content without significant alteration in plant development. These hybrids had low levels of G and S units[34]. It is proposed that CCR and CAD are necessary for both G and S lignin biosynthesis, but F5H and COMT are only responsive for S lignin formation because of their different effects on S/G ratio between CCR/CAD and F5H/COMT.

3 Genetic engineering of lignin biosynthesis

It is obvious that genetic manipulation will play an important role not only in defining the lignin biosynthesis pathway, but also in exploring new plant resources. Almost all of the genes on lignin pathway have been cloned from variety of plants. Consequently, genetic engineering approach will be used to modify lignin profile such as its structure, content and composition to fit for the requirement of human being. Now we sum up the potential strategies of genetic manipulation in this field.

3.1 Genetic engineering of single gene

At present, most transgenic research work pay attention to a single gene in the lignin biosynthetic pathway to regulate the quantity and quality of lignin through antisense and sense RNA technique. Alfalfa, poplar, pine, maize and wheat are primary plants to be investigated. It is delighting that majority of transgenic plants has alternation in lignin content and composition, as well as abnormal development of plant[35]. Because there are differences in the degree of methylation between S and G monolignols that leads to different possibility to form C_5-C_5 covalent bonds among monolignols, it is disadvantageous for paper pulping industry to remove lignin if the amount of G unit in lignin is high. From this consideration, the strategies will be not only on reducing the lignin content, but also on modification of the lignin structure and composition. The traditional techniques of antisense RNA and sense co-suppression depend on a single gene to alter the lignin metabolism. In fact, this strategy lacks versatility because the insertion of heterogenes into a plant genome is a random process that may result in gene silence for the influences of copy number and loci of heterogene in the plant genome[36]. Though some desirable transgenic lines have been selected from different plants, frequently we cannot do it as what we want just through one gene regulation in the metabolism process, especially in the lignin biosynthesis pathway.

Small interfering RNAs (siRNA) is one kind of double-stranded RNA of about 21 ~ 26 nucleotides (nt). It can induce silence of homologous genomic DNA via degrading homologous dsRNA and methylating or suppressing transcription of its homologous gene in genome[37]. siRNA can be used in almost all eukaryotes to suppress the expression of any genes in genome. This technique has been applied in systemic defence reactions of animals and plants by eliminating superabundant and defective mRNA[38]. Now it has been becoming a rapid and effective technique to analyze gene functions. The expressive construct containing a promoter, a terminator and some parts of a gene coding sequence that is linked in reversed direction can be used to transform plants. This coding sequence would form double-stranded siRNA about 21 ~26 nt in length that can suppress expression of endogenous targeted genes. There are many successful reports using T-DNA to generate siRNA[39]. Up to date, it is still an open area in the research of lignin biosynthesis.

3.2 Regulation of multi-gene in lignin biosynthesis

Lignin metabolism is a fairly complex biochemical process in plant. Through different combination of lignin genes in transgenic plants, we may better understand the functions of these genes, at the same time to exploit more useful plant resources for agriculture and industry. Several useful approaches have been applied in co-regulation of multi-gene such as sexual crossing with homozygous transgenic lines, re/multi-transformation using different genes, and co-transformation by several genes. Each method has its advantages and disadvantages, and we should select suitable methods in terms of the different experimental conditions, materials and intentions.

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3.2.1 Suppression of multi-gene in lignin biosythesis Transgenic plants that combined COMT, CCR or CAD were obtained through sexual crossing. The gene silence phenomena were observed in progenies of these transgenic hybrids on all targeting genes including COMT, CCR and CAD. This is probably due to the same promoter, 35S of CaMV, used in the different transgenic plants. The expressions of COMT, CCR and CAD were suppressed in those hybrids, but the impact of suppression on lignin profile was not stable as expected[40]. Transgenic polar of co-suppression of COMT and CAD, as well as transgenic tobacco with co-suppression of COMT and CCOMT by re-transformation approach were reported[41]. The advantage of re/multi-transformation approach is that it can succeed in introducing different genes into plants. This will save time to conduct the sexual crossing. However, this approach will induce gene silence as discussed above, and transgenes in plants will be sexually segregated in the process of plant propagation, especially when the integrated foreign genes are located in a long distance in genome.

A novel approach which uses one chimeric gene to suppress the expression of several genes simultaneously was reported recently. The chimeric gene was constructed by combination of part of different genes together into an expression vector. This chimeric gene can be introduced into plants just like one gene. By this approach, COMT, CCR and CAD can be down-regulation in just one transformation. Using different combinations of COMT, CCR and CAD genes, Abbott et al. obtained the transgenic tobacco plants in which the activity of both or all of the COMT, CCR, CAD was decreased dramatically, together with a decrease of the lignin content and great change in lignin composition. The growth and development of these transgenic plants were also affected[42].

3.2.2 Over-expression of multi-gene in lignin biosynthesis There were few reports concerning over-expression of multi-gene in lignin biosynthesis. Although some transgenic plants have been generated through sense DNA technique, they did not show an increase in the lignin content. In contrast, lignin content, structure and composition in some of these sense transgenic plants were similar to those in transgenic plants with antisense genes. It was supposed that these results were due to the co-suppression of endogenous and heterogenous genes. 2A region is a DNA fragment from aphthovirus, which encodes a peptide of 19 amino acids comprised QLLNFDLLKLAGDVESNPG. This peptide offers a cleavage site of poly-protein dissociation at its C-terminus[43]. At the translation levels 2A can lead to isolation of two or more proteins linking with this region, then produce proteins with the biological functions. At beginning, 2A poly-protein system was used to over-express the proteins of human, mammal, insect, and yeast. Now, it has been applied to plants, especially in the research of complex lignin metabolic pathway.

Franke et al. firstly used this approach to over-express F5H from *Arabidopsis* in transgenic tobacco, which resulted in a significant increase of the S-unit lignin in the stem of transgenic tobacco. They constructed the protein systems of GUS-2A-F5H and GUS-2A-COMT-2A-F5H. After being correctly translated, these systems spliced and separated into functional proteins of GUS-2A, COMT-2A and F5H. Moreover, the spliced F5H protein could be set in the appropriated site of membrane[5]. These results demonstrated that 2A poly-protein system would be a desirable approach to over-express multi-gene in order to regulate the lignin biosynthesis.

4 Prospective

Many models about lignin biosynthesis pathway have been proposed at present, but still these models cannot completely explain all aspects in lignin biosynthesis. For example, the lignin content, composition, structure and metabolic pathway are different from each other in different species, tissues and organs, and at different developmental stages. The reasons for these differences are largely open to investigate. At present it is still difficult to accurately regulate the lignin biosynthesis. To obtain the transgenic plants that are fit for practical application will remain the long-distant goals of research, those include to decrease lignin content and alter lignin composition by down-regulation of lignin genes for pulping paper and forage industry, and to increase lignin content by over-expression of lignin genes in order to improve lodging-resistant ability in crop plants. However, as more and more new approaches are used in this field, such as immunolocalization, *in vivo* labeling and isotope, co-immunoprecipitation, *in vitro* protein expression system, yeast two-hybrid assay and genetic manipulation, more comprehensive knowledge of lignin biosynthesis will be expected and the plant with new lignin characters will be developed which will be greatly benefit for human life and environment in the

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near future.

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