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Polymorphism of Lignification Enzymes in Plants: Functional Importance and Applied Aspects

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Abstract—The synthesis of lignin and its deposition in secondary cell walls leads to the formation of sclerenchyma, a tissue having mechanical strength. This biochemical mechanism arose about 400–420 million years ago, in the Early Silurian period, in the first vascular plants (Tracheophyta order). The appearance of sclerenchyma is related to the emergence of plants on land and formation of the terrestrial flora. Lignified tissues of plants also perform conducting and protective functions, in addition to its mechanical (supporting) one. The polymorphic options of the enzymes involved into biosynthesis of lignin and its accompanying substances (PAL (phenylalanine ammonia-lyase), C4H (cinnamate 4-hydroxylase), C3H (*p*-coumarate 3-hydroxylase), F5H (ferulate 5-hydroxylase), COMT (caffeic acid O-methyltransferase), CCoAOMT (caffeoyl-CoA O-methyltransferase), 4CL (4-coumarate:CoA ligase), CCR (cinnamoyl-CoA reductase), CAD (cinnamyl alcohol dehydrogenase), and SAD (sinapyl alcohol dehydrogenase) were considered with the use of a number of plants: corn, millet, sorghum, rice, wheat, fescue, elephant grass, brachypodium (purple false brome), tobacco, alfalfa, arabidopsis, poplar, eucalyptus, pine, spruce, and ginkgo. Mutant variants of enzymes can be found in natural or breeding populations; they may be also obtained with the use of mutagens. In recent decades, T-DNA-mutagenesis is widely used. Mutations can cause changes in the expression of the corresponding genes and (or) disturb the structure of protein molecules. As a result, numerous changes in the phenotype can occur. Among them are changes in the structure and chemical composition of tissues, as well as in growth processes and plant development. The appearance of colored (brown midrib, reddish brown, pink, red-brown, red-wine, etc.) forms of plants was described in many species. Some publications noted an interrelation between the biosynthesis of monolignols and that of flavonoid pigments. Mutant forms are widely used at present to produce forage cultivars and bioethanol, to improve pulp properties (production of paper and viscose fiber), and to sequester atmospheric carbon. The synthesis of mutants from this group of enzymes and their use are considered promising trends of modern plant biotechnology.

Keywords: biosynthesis of monolignols, enzymes, polymorphism, down-regulation, overexpression, tissue properties, cellulose, lignin, bioethanol, paper, energetic plants, renewable resources

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INTRODUCTION

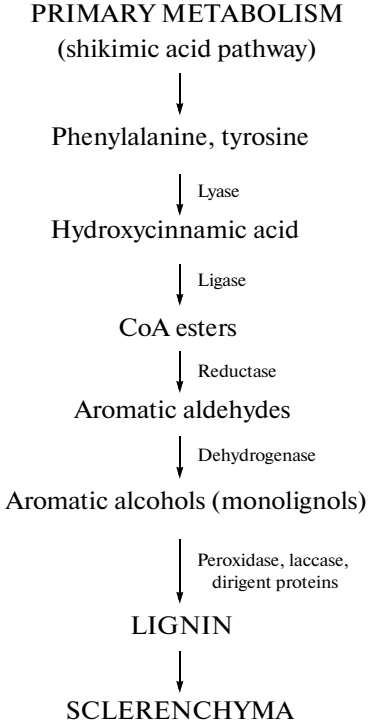
All vascular plants (Tracheophyta division) have a functioning short section of secondary metabolism that begins with the deamination of aromatic amino acids—phenylalanine and tyrosine—and ends with the synthesis of lignin, a three-dimensional polymer that provides stiffness (rigidity) to stem tissues, and its deposition in the secondary cell walls. Lignins are formed by the oxidative polymerization of monolignols—aromatic alcohols, mainly coumaric, coniferyl, and sinapic alcohols, and other aromatic metabolites.

This biochemical mechanism arose about 400–420 million years ago, in the Early Silurian, in the first vascular plants, which were close to modern club mosses (Lycopodiopsida class) (Weng et al., 2008; Guo et al., 2010). The appearance of this metabolic section was associated with the output of plants on land and the formation of ground flora.

With the development of land habitats by plants, at least two additional systems that were absent in algae became necessary—conducting and supporting systems. In mosses (the most primitive land plants, Bryophyta division), a conductive function is provided by diffusion and a supporting one is provided by tissue turgor (osmotic pressure, which bursts cell walls from the inside). These plants can exist only in humid local-

ities and their dimensions do not exceed a few centimeters and several decimeters.

Mechanical tissues were required to form for further evolution. Lignification was the main direction in the formation of stems with mechanical strength. Besides the mechanical (maintenance) function, lignified plant tissues also perform conductive and protective functions. In simplified form, lignification takes place according to the scheme:



Deamination occurs under the action of phenylalanine ammonia lyase (PAL) and tirase. It is not present in all plants; for example, cereals have it and legumes do not.

The regeneration of the carboxyl group of hydroxycinnamic acids into the aldehyde group requires energy (13 kcal/mol), so the conversion of acids into the corresponding aldehydes occurs via intermediates—thioethers (Coenzyme-A esters with macroergic pyrophosphate group) (Goodwin and Mercer, 1983; Heldt, 2005). The formation of CoA esters occurs via the appropriate ligase, and the subsequent formation of an aromatic aldehyde occurs via reductase. Regeneration of the aldehyde group and the formation of alcohols also require energy but less than in the previous case, so the reaction occurs with NADP-H and the CAD or SAD enzyme. Multiple enzyme systems—hydroxylases (EC 1) and transferases (EC 2)—catalyze the interconversion of acids and aldehydes, as well as related reactions, leading to other end-products, in particular, to the flavonoid pigments.

These data show that the formation of monolignols is a fairly expensive process. It requires a significant expenditure of energy and valuable metabolites, proteinogenic aromatic amino acids.

As all metabolic processes in plants, the synthesis of monolignols is under hormonal control, and cytokinins play a significant role (Teutonico et al., 1991).

The conventional nomenclature for monomers, from which lignin is formed, comprises three aromatic alcohols, which are called monolignols (Rabinovitch et al., 2001):

H units: *p*-hydroxyphenyl (*p*-coumaryl alcohol);

G units: guajacyl (coniferyl alcohol);

S units: syringyl (sinapyl alcohol).

The biosynthesis of lignin takes place on the external side of the cell, on a polysaccharide matrix; lignin synthesis without the involvement of the polysaccharide matrix was not found in nature (Bardinskaya, 1964; Zaprometov, 1993; Sharova, 2004; Gorshkova, 2007). The oxidase enzymes peroxidase (so-called basic peroxidase) and laccase (monophenol oxidase) participate in polymerization, but they only contribute to the formation of aromatic radicals and do not control the process of polymerization.

Lignin formation begins at certain positions of the polysaccharide matrix, which contains hydroxycinnamic (*n*-coumaric and ferulic) acids bound to the cell wall; their phenolic oxy-groups serve as "anchors" for the process of lignification. These "anchors," serving as starting points for lignification, are attached to arabinose fragments in the composition of hemicelluloses (Zaprometov, 1993).

Relatively recently, lignification was supposed to be the process of erratic nonenzymatic reactions of aromatic alcohols with their mesomeric free radicals, resulting in a "huge molecule similar to a network that fills the cell wall matrix" (Goodwin and Mercer, 1983, p. 103). According to recent data, lignification process for secondary cell walls (in addition to its mechanical function) plays an important role in forming processes, thus it cannot be erratic. As a result of lignification, specialized tissue structures are related not only to the shape but also to the function of different parts of the plant form.

The exact mechanism of lignin biosynthesis is still unclear. The synthesis process is regulated by extracellular glycoproteins—the so-called dirigent proteins, which, not being enzymes, promote the formation of various polymeric structures in strictly determined parts of the cell wall (Davin and Lewis, 2000; Burlat et al., 2001; Heldt, 2005). Overexpression of one of those proteins in cotton enhances lignification and increases resistance to fungal infection (Shi et al., 2012).

The composition of lignin contains also other aromatic metabolites—aldehydes and acids (Dalimova and Abduazimov, 1994). Aldehydes (monolignans) are included in the lignin in the same manner as monolignols, via peroxidase and laccase (Ros Barceló and Pomar, 2001). The authors believe that the lignin, having aldehydes in its composition, becomes colored red or reddish-brown. The inclusion of aldehydes occurs at low CAD activity or with a lack of NADP cofactor, which is required for the reaction of the aldehyde

reduction to the alcohol. The means of aromatic acid inclusion in lignin have not been fully investigated.

Hydroxycinnamic acid molecules form bridges between blocks of lignin and polysaccharides (Lam et al., 1992). This is due to the fact that phenolic acids (unlike aldehydes and alcohols) are capable of forming two ester bonds: an ether bond via a hydroxyl group and an ester bond via a carboxyl group. The acid molecule binds to a polysaccharide molecule with an ester bond (mainly to arabinose and arabinogalactan, which in turn is bound to the cellulose fibrils), and it binds with an ether bond to a unit of lignin.

Fry has shown that "ferulisation" of hemicelluloses (specifically, arabinoses) occurs intracellularly in the Golgi apparatus, and only after some time, about 25 minutes, do these polymers appear on the external side of the plasma membrane (Fry, 1987). A lignin unit is then formed.

A sclerenchyma (a tissue having mechanical strength that performs other important functions (conductive and protective)) is formed as result.

Phenylpropanoid glycosides, which are found in herbaceous plants, may be appropriate intermediates in the formation of lignin from monolignols (Dalimova and Abduazimov, 1994).

Conifer lignans are guajacyl lignins (Rabinovich et al., 2001). The ratio of units G : S : H for spruce is 94 : 1 : 5; for pine, it is 86 : 2: 13, but the compression regions (under the base of the branches, reaction or compression wood, cross grain) contain lignins with a significant content of *p*-oxyphenylic units. The portion of H-units is up to 70% in the compression wood, i.e. these lignins can be characterized as GH lignins with the highest degree of crosslinking of the structural units.

In flowering woody plants, the compression wood is located above the base of the branches (traction wood). Deciduous trees are guajacyl-syringyl, with a ratio from 4 : 1 to 1 : 2; for beech the ratio of G : S : H is 56 : 40 : 4. The core of the wood contains more syringyl units than the sapwood. Young trees have more guajacyl units, while old ones contain more syringyl units.

Grass lignins differ from the lignins of other plants by their rich content of ester *p*-coumaric acid. This is due to the presence of tirase (TAL), which converts tyrosine into *trans-p*-coumaric acid. In the absence of tirase, the pathway from tyrosine to cinnamic acid is not possible. Tirase is present in all grains (sorghum, wheat, maize, barley, oats, rye, sugarcane), but it is absent in pea, lupine, and clover, as well as in the tissue cultures of coniferous and deciduous plants (*Lignins*, 1971).

There had been reports on the presence of lignin in algae, mosses, club mosses, and horsetails (Manskaya and Codina, 1975). However, it was later established that the mosses and horsetails contain lignans and flavonoids (flavonoids)—substances of aromatic nature that are not lignins (Kalabin et al., 2000). The most likely function of these substances is the protective one.

The enzymes of lignin biosynthesis include the following: TAL, tyrosine ammonia-lyase EC 4.3.1.23 (present in cereals, absent in legumes); PAL, phenylalanine ammonia-lyase EC 4.3.1.24; PAL/TAL, phenylalanine/tyrosine ammonia-lyase EC 4.3.1.25 (previously, all three enzymes were designated as EC 4.3.1.5) (Goodwin and Mercer, 1983); C4H, cinnamate 4-hydroxylase EC 1.14.13.11; (cytochrome P450-dependent) (NIH-shift, in microsomes) (Goodwin and Mercer, 1983); C3H, *p*-coumarate 3-hydroxylase EC 1.14.13.21; F5H, ferulate 5-hydroxylase; EC 1.14.13.88; COMT, caffeic acid O-methyltransferase EC 2.1.1.68; formerly known as O-methyltransferase (COMT; EC 2.1.1.6); 4CL, 4-coumarate: CoA ligase EC 6.2.1.12; (catalyzes the formation of CoA with all acids, *p*-coumaric, ferulic, and sinapic); CCR, cinnamoyl-CoA reductase EC 1.2.1.44; (recovery of thioethers to the corresponding aldehydes); CCoAOMT, caffeoyl-CoA O-methyltransferase EC 2.1.1.104; HCT, *p*-hydroxycinnamoyl-CoA: shikimate/quinate *p*-hydroxycinnamoyltransferase, (EC 2.3.1.99); CHI, chalcone isomerase EC 5.5.1.6; CHS, chalcone synthase EC 2.3.1.212; (branch of monolignolic pathway, leading to the flavonoids); CAD, cinnamyl alcohol dehydrogenase EC 1.1.1.195; SAD, sinapyl alcohol dehydrogenase (nonnomenclature name).

The general scheme of lignin metabolism was given in detail in several reviews (*Lignins*, 1971; Lewis and Yamamoto, 1990; Rabinovich et al., 2001; Anterola and Lewis, 2002; Boerjan et al., 2003; Neutelings, 2011; et al.) and textbooks (Goodwin and Mercer, 1983; Heldt, 2005). However, in recent decades, much evidence was obtained on the differences in this scheme in different taxonomic groups, species, and even individual plant genotypes. A significant role in the study of these differences is played by the polymorphism of the enzymes involved in this process.

These features of the metabolism of different genotypes cannot be separated from the characteristics of the object (i.e. of this plant species), so it is advisable to consider individual cases of polymorphism of enzymes in specific carriers.

MONOCOTYLEDONS

Maize

Maize plants with a brown midrib phenotype (red-brown color of leaf veins) were found in the early 1920s in the United States (Eyster, 1926; Jorgenson, 1931). The trait was controlled by a recessive gene *bm* (*bm1*), which is localized in the fifth chromosome (http://corn.agronomy.wisc.edu/Management/L031.aspx).

It was soon discovered that mutant plants with the *bm* phenotype contained less lignin and, consequently, more polysaccharides, than common plants and that the green mass fed to ruminants had higher fodder value. In the postwar years, these mutants were used for the selection of forage varieties and maize hybrids

(Kuc and Nelson, 1964; Muller et al., 1971; Miku, 1981). However, the mutant plants had also negative traits: increased lodging, susceptibility to fungal infections, and a low grain yield and dry mass.

Other mutants with similar phenotypes and four *brown midrib* genes were later found: *bm1, bm2, bm3*, and *bm4* were located on chromosomes 5, 1, 4, and 9, respectively (Coe and Neuffer, 1977; http://www.maizegdb.org).

The *bm1* mutant proved to be an allelic variant of the *CAD* gene (Halpin et al., 1998). Homozygotes had reduced CAD activity (from 14 to 60% of normal activity, depending on the tissue, the stage of development, and genotypic environment), a low lignin content (15.3% from normal and 12.2% in the mutant), and a changed ratio of lignin subunits G and S.

The *bm3* mutant had a reduced activity of methyltransferase (Lee and Brewbaker, 1984). It was then found that it is the structural gene of this enzyme (caffeic acid O-methyltransferase, COMT; EC 2.1.1.68) (Vignols et al., 1995). Two independently identified mutations were studied—*bm3-1* (from the United States) and *bm3-2* (from France)—that differed in molecular structure: *bm3-1* differed from the wild type by the insertion of the B5 retrotransposon, and *bm3-2* had a deletion in the coding region. The expression was reduced only in *bm3-1*. However, both mutations led to a decrease in enzyme activity, the *brown midrib* phenotype, and a decrease in the degree of lignification.

The *bm2* and *bm4* mutants are poorly studied. According to recent data, the *bm4* gene encodes a folylpolyglutamate synthase enzyme (folylpolyglutamate synthase or tetrahydrofolate synthase, EC 6.3.2.17) in maize (Li et al., 2014).

All four genes *bm1, bm2, bm3*, and *bm4*, cause changes in the expression of not only *CAD* and *COMT* genes but of many other genes (Shi et al., 2006) not limited to the phenylpropanoid metabolism, such as cytochrome P450. Of the 144 genes studied in total, 69 had differences in expression between isogenic genotypes—mutant and normal (Guillaumie et al., 2007). With a library of expressed sequence tag (EST) markers (a kind of label of expressed genes), it was found that *bm* mutants affect the expression of many other genes of the phenylpropanoid metabolism and related pathways. The authors suggested that the *bm* genes perform a regulatory function.

However, mutations in the structural locus *bm1* (in the form of insertions) were later found (Chen, W. et al., 2012): *bm1-das1* with a 3444 bp insert (transposon) and *bm1-ref* with a 2 bp insert (AC). Both mutants had a *CAD2* gene expression that decreased by 91 and 86%, respectively. As a result of residual expression, defective proteins consisting of 48 and 147 amino acid residues, respectively, instead of the normal 367, formed. The lignin content was reduced by 24 and 30%. Both mutants had a well-defined *brown midrib* phenotype. It remains unclear how a mutation in the structural gene of the enzyme that performs a specific function could have an impact on many other genes.

Due to the fact that the *bm* phenotype is accompanied by a series of negative traits that impede its use, researchers obtained *de novo CAD*-mutant genotypes, trying to receive plants with advantageous properties that possibly lacked the negative traits. It was possible for some cases (Fornalé et al., 2012). Maize T-mutant *CAD-RNAi* (downregulation of CAD using RNAi) had a reduced CAD activity (20% of residual activity (as compared with a wild-type) in the roots, 34% in the stem, and 68% in the leaves, wherein the *bm* phenotype was absent).

Mutant plants had an increased number of vascular vessels, but their diameter decreased. The total lignin content did not change and the S/G ratio decreased a little, but cell walls of the stem accumulated higher levels of cellulose and arabinoglucans. On the contrary, the amount of lignin and polysaccharides decreased in leaf veins. The ratio of soluble components, particularly pigments, changed. The phenotype of plants in the field was different from the normal one, but the digestibility of dry matter (without ears) was higher in T-mutants. They provided more bioethanol: 171 g/kg of dry mass against 159 in normal plants and 99 g/m^2 against normal 65 g/m^2 (Fornalé et al., 2012). These results suggest that *CAD* mutants have not only the changed amount and composition of the lignin but also the degree of lignin that is bound to the polysaccharide components from the cell wall. The processing properties of plant biomass changed, and the yield of useful products ultimately increased.

Mutants with similar phenotypes were found repeatedly in breeding materials; some of them were then studied by genetic and molecular genetic methods. Two mutants, *bm5* and *bm6*, which are nonallelic to previous ones, were recently discovered in breeding samples (Chen, Y. et al., 2012). The *bm6* gene is localized on the second chromosome. The mutant plants had reduced height and increased digestibility of green mass. It is assumed that *bm6* is an allelic variant of the *F3H* gene, which controls flavonol synthase/flavanone 3-hydroxylase. This result indicates the general mechanisms of the biosynthesis of aromatic compounds.

An association was found for the biosynthesis of monolignols with the fragments of *COMT, CCoAOMT2, 4CL1, 4CL2, F5H*, and *PAL* genes with molecular methods in normal corn plants without the mutant phenotype; the discovered associations are to be used in the breeding of fodder hybrid (Chen et al., 2010).

Lignin produced artificially from coniferyl aldehyde had a red-wine color (Higuchi et al., 1994), from which the authors concluded that the phenotype of *brown midrib* maize and other cases of reddish-brown coloring in plants with a deficit of lignin-fixing enzymes were caused by the introduction of aldehydes into the lignin. However, not all cases of chemically

modified lignin and introduced aldehydes gave rise to color.

The search for other mutant genes continues. The *Zmccr1* (–) mutant in the *CCR* gene was found among the collection of maize genotypes with insertions of Mu-elements; the mutant had an insertion in the first intron (Tamasloukht et al., 2011). Mutant plants had a residual gene expression of 31% of normal expression. The enzyme activity decreased by 10%, and the lignin content was reduced by approximately the same amount (from 13 to 11.5%). The proportion of syringyl units increased; significant changes in the anatomical structure of the stem, particularly in conducting bundles, were found; and green mass had an increased digestibility. No significant variations were observed in plant development. These mutants had changes in the expression of 36 other genes: a decreased expression was observed in 11 of them; 25, on the contrary, had increased expression. Flavonoid synthesis genes were among the latter. However, there were no reports on color changes.

Sorghum

A series of *brown midrib* mutants were obtained for sorghum (*Sorghum bicolor* (L.) Moench) via treatment with a mutagen (diethyl sulfate) (Porter et al., 1978). They were designated as *bmr*, since the *bm* designation had already been used for the *bloomless* mutant (lack of waxy coating, which gives the typical color to the and leaves). Ten of the 13 obtained mutant lines showed a significant reduction in the degree of lignification to 50% in the stems and 25% in the leaves.

The *bmr6* mutant turned out to be an allelic variant of the *CAD2* gene with the mutation *Gln to STOP* (Bucholtz et al., 1980). The lignin of mutant plants contained large amounts of aldehydes, and the digestibility of green mass increased by 7%. Three alleles were later found in this locus: *bmr6-ref* (null allele with the absence of CAD2 protein), *bmr6-3* (expresses a protein with a violation in the cofactor binding site), and *bmr6-27* (expresses a protein with a violation in the secondary structure). All three alleles gave a *brown midrib* phenotype to homozygous plants but differed in their action (Saballos et al., 2009).

The *bmr2* mutant appeared to be a variant of the *4CL* gene (Saballos et al., 2012). An interesting result was obtained in this paper—the enzyme activity, which was reduced as a result of mutations, leads to increased expression of the mutant gene, i.e., the plant "seeks to compensate" for the negative effects of the mutation. The authors call it an "autoregulatory control." The lignin content was reduced by 20% as compared with the norm.

As in maize, the mutant *bmr* gene was found to have an influence on many other traits (Yan et al., 2012). Furthermore, most *bmr* mutants had a reduced C4H activity (Yan et al., 2013).

Millet

A single mutant in pearl millet (*Pennisetum americanum* (L.) Leeke) with the bmr phenotype (similar maize and sorghum) was obtained after treatment with a mutagen (ethyl methanesulfonate), followed by self-pollination (Cherney et al., 1988). The mutant had 23% less lignin, 4% higher digestibility, and a forage (mouthfeel) preference: cades were in 2.6 times longer in areas with mutant plants than in areas with normal ones (Cherney et al., 1990).

The elephant grass *Pennisetum purpureum* Schumach. is a promising forage and energy crop; therefore, lignin-fixing enzymes, particularly CAD, are being studied (Tang et al., 2014).

Switchgrass (*Panicum virgatum* L.) is one of the most promising "energy plants," a perennial species from the American prairies that has a height of two meters or more. According to the total output of ethanol (grain + straw), it may exceed the maize, since it does not require annual planting. Plants with low CAD activity (downregulation) were obtained with the use of RNAi (Fu et al., 2011). In eight transgenic plants, the activity was 17–39% coniferyl aldehyde and 12–24% sinapic. The digestibility of green mass was 61–71 against 59% for the norm. By sugar yield after pulp hydrolysis, transgenic plants were significantly (up to 43%) better than wild-type plants. Transgenic plants had not only a low lignin content but also a high content of soluble phenolic compounds.

In another study, a reduced CAD activity of switchgrass resulted in an increased content of aromatic aldehydes in lignin (Saathoff et al., 2011). Although plants typically contain several nonallelic *CAD* genes, only one or two of them have regulatory functions (Saathoff et al., 2012).

Rice

In 1917, a *gh2*-mutant form of rice (gold hull and internode 2) was described with a reddish-brown color of internodes, leaf tube base, panicle, and golden-yellow grains. It was found recently that it is a CAD mutant with a reduced activity of aromatic alcohol dehydrogenases: the loss of CAD activity is up to 50%, and SAD activity is lost completely. There is 5–6% less lignin; the ratio of monomers is changed. The amount of G-monomer is especially strongly reduced—by 1.5 times (Zhang et al., 2006). The authors suggest that the use of this and other similar mutants will significantly increase the technological value of rice straw, which is used for fodder and for the production of high-quality paper.

The *flexible culm1* (*fc1*) rice mutant with reduced mechanical properties of the stem also appeared to be an allelic variant of the *CAD* gene (Li et al., 2009).

In total, 12 *CAD* genes were found in rice at nine loci localized in seven chromosomes (two loci in chromosomes 4 and 10). Comparison of the molecular

structure of these genes indicates that the divergence occurred primarily through changes in intron–exon structure (Tobias and Chow, 2005).

Expression of the arabidopsis SHINE transcription factor in the rice genome led to a 45% reduction in lignin in the straw and a 34% increase in the cellulose content (Ambavaram et al., 2011). In addition, the ratio of lignin monomers changed, as well as some sugars in the composition of polysaccharides, in particular, the proportion of glucose and xylose with improved digestibility of green mass. There were also differences in the anatomical structures of the straw. Transgenic plants had the normal phenotype without any deviations in agronomic indicators hindering their growth. The activity of enzymes 4CL, CCR, and CAD were reduced. The authors made a genetic network and concluded that the SHINE factor is essential for the biosynthesis of polysaccharides, primarily cellulose, via suppression of the expression of lignin-fixing enzymes. However, it plays a regulatory role in the synthesis of wax, cutin, and lipids in arabiopsis, in the genome of which this factor was detected.

Brahipodium

Brahipodium *Brachypodium distachyon* (L.) P. Beauv. is a small cereal with a small number of chromosomes (five chromosomes) that is used as a model plant. Two *brown midrib* forms were detected in the population after treatment with mutagens (Bouvier d'Yvoire et al., 2013). Both mutant forms *Bd4179* and *Bd7591* were caused by single nucleotide substitutions in the *BdCAD1* gene and by corresponding changes in the primary structure of the protein. The reduced activity of the enzyme caused a reduction of lignin in the stem. Both mutants had an increased sugar yield after enzymatic hydrolysis of straw.

Tall Fescue

Tall fescue (*Festuca arundinacea* Schreb.), or lipalma, is a forage crop. Transgenic CAD plants were obtained by antisense transgenesis. Plants had a low content of lignin, and the digestibility of green mass increased by 7.2–9.5%. According to other components (cellulose, hemicellulose, sugar-free, ferulic and coumaric acids), no change was observed. The resulting genotypes were intended for use in the breeding of fodder cultivars (Chen et al., 2003).

Barley

It was found recently for barley (*Hordeum vulgaris* L.) that the mutant form *orange lemma* (*rob1*) with colored scales of ear and culm, which was described over 80 years ago, is an allelic variant of the *CAD* gene (Stephens and Halpin, www.hutton.ac.uk/webfm_send/727). Similar mutants were obtained by treatment with a mutagen (ethyl methanesulfonate). The residual enzyme activity in mutant lines was 60–80% of normal, and the results of Western blot hybridization showed that one variant of the enzyme was completely absent. The lignin content in culm was reduced by 10–15% as compared with the norm, and the S/G ratio was also reduced. The mutant *HvCAD2* gene proved to be homologous to one of maize *bm* mutants.

Wheat and Rye

Eleven homologs of the *TaCAD* gene, which were designated *TaCAD1–TaCAD11*, were found in hexaploid wheat (*Triticum aestivum* L.). The *CAD1* gene expression was higher in a sample of wheat resistant to lodging. The authors suggested that genetic analysis can be used in wheat breeding for resistance to lodging (Ma, 2010).

Our research on the study of collections of cereals (various species of rye and wheat) detected a polymorphism in CAD (former name is AADH, aromatic alcohol dehydrogenase) and shikimate dehydrogenase (SKDH, EC 1.1.1.25) (Konovalov et al., 2009). The localization of the relevant genes (Konovalov et al., 2008, 2010) was studied, and it was found that the polymorphism is a functional one, affecting a number of features of plants (Goncharov et al., 2012; Konovalov et al., 2014).

DICOTYLEDONS

Tobacco

The first work to obtain artificial CAD polymorphism was performed on tobacco with the use of transgenic technology (Halpin et al., 1994). The introduction of antisense RNA into the genome of tobacco reduced the enzyme activity; the residual activity was 20 and 7% in two transgenic lines. The activity of O-methyltransferase decreased also in one variant. Transgenic plants were slightly inferior to control ones in their growth and development and had a reddish-brown color of the stem xylem elements resembling a *brown midrib* trait of maize. The lignin content was reduced slightly, but the composition and structure of the lignin components changed significantly, in particular, the S/G ratio decreased and the amount of aldehydes increased. These results showed that it was possible to artificially produce plants with altered (and, to some extent, set) properties of vegetative mass.

Other genes of this pathway were later transformed in tobacco. Plants transformed with an antisense construct of the *C4H* gene had an enzyme activity reduced to 35% of the norm. Plants transformed with a sense structure of the same gene were divided into two classes: those with overexpression (470% of normal) and those with decreased expression (45% of normal). In the latter group, the lignin content was reduced in the different lines: in one of the transgenic lines, it was reduced in almost three times, from 9.7 to 3.4%. The S/G ratio decreased. Overexpression did not lead to a

noticeable enhancement of lignification. Plants transformed with the *PAL* gene were also divided into genotypes with overexpression and downregulation. A twofold decrease in the amount of lignin in the stems was observed in plants with a downregulation (15% from normal activi). The S/G ratio increased, on the contrary. Overexpression did not lead to a noticeable increase in lignification (Sewalt et al., 1997).

Overexpression of C4H in cultured tobacco cells led to an increase in the synthesis of only one phenolic compound—acetosyringone (3,5-dimethoxy-4-hydroxyacetophenone), which appeared to have no direct relationship to lignification (Blount et al., 2002).

These results suggest that the overexpression of individual genes cannot by itself provide the desired metabolic changes.

Double CCR and CAD transformants had a reduced lignin content, even in the heterozygous state; the plants were no different from normal. The changes affected not only the lignin but also polysaccharide cell-wall components, as well as soluble phenolics (Chabannes et al., 2001). Downregulation of CCR caused a reduction in the lignin content, and that of CAD led to the inclusion of aldehydes in the lignin (Dauwe et al., 2007).

Numerous changes in other metabolic pathways, including carbohydrate metabolism, stress, and photorespiration, were revealed in this work, as well as in the previous one (Chabannes et al., 2001). The fact that changes in the secondary metabolism genes cause changes in many areas of the primary metabolism shows the variety of feedback in the process of plant growth and development. One possible mechanism is the action of aromatic derivatives of lignin biosynthesis, e.g., glycoside dehydro coniferyl alcohol as a regulatory factor (Teutonico et al., 1991).

Arabidopsis

Nine *CAD* genes were found in arabidopsis: *CAD 1, CAD A, CAD B1, CAD B2, CAD C, CAD D, CAD E, CAD F*, and *CAD G*; according to their homology, they were distributed into four groups (Sibout et al., 2003): *CAD A, CAD B1*, and *CAD B2* with the poplar *Populus tremuloides SAD* gene; *CAD 1, CAD E*, and *CAD F,* together with *CAD 1* gene from alfalfa *Medicago sativa; CAD C* and *CAD D* in a group with many other CAD genes, including the CAD of pine and spruce, i.e., this was the main evolutionary branch leading from gymnosperms; the *CAD G* gene had no obvious homologs.

The collection of transgenic mutants revealed two null genotypes, *cad-d* and *cad-c*. Only the *cad-d* mutant had a slightly reduced Klason lignin. Double null homozygotes for *cad-c* and *cad-d* alleles were then obtained, and they were significantly different from the norm. These arabidopsis plants had soft stems and fell over in the mature state; the stems had a red-brown color.

There was also growth inhibition in the vegetative state, a delay of bolting, and a total reduction in the size of plants. The lignin content fell to 40%, and the content of some monolignols was 94%. Aldehydes and hydroxycinnamic acids (ferulic and sinapic) were present in the lignin composition instead of the alcohols (Sibout et al., 2005). This result demonstrated the interaction of *CAD* genes.

A triple *cad-c cad-d ccr1* mutant (Thévenin et al., 2011) proved to be a dwarf with a male sterility. The authors suggested that only two of the nine *CAD* genes, *CAD C* and *CAD D*, were involved in lignin biosynthesis. A reduced lignin content was accompanied by an increase in the flavonoid content.

Other mutants of lignin biosynthesis enzymes are known. The *irx4* (irregular xylem4) mutant had impaired fertility at an elevated temperature (31°C) and a 50% reduced lignin content; plants had a shorter stem and were decumbent. The mutant was a variant of CCR enzyme (cinnamoil-CoA reductase) with impaired splicing (Jones et al., 2001).

Many results were obtained in this area in arabidopsis; however, arabidopsis has no practical significance. The mechanisms of genetic control and biosynthesis described for this model plant sometimes coincide with those in other species (having a practical significance), but often there are significant differences.

Alfalfa

Alfalfa *Medicago sativa* L. is widely used as a fodder plant. Antisense transgenesis made it possible to obtain 19 plants with a CAD activity that was reduced by 30% or more compared with the normal one (Baucher et al., 1999). The enzyme activity differed when plants were grown in the greenhouse and in the field (50 and 30% of residual activity, respectively). With a residual activity of 50% or less, plants had a red stem. The lignin content did not decrease, but the ratio of the components changed. This led to an increase in the digestibility of green mass of the two genotypes.

Downregulation of COMT and CCOMT transferases in alfalfa resulted in a reduction of lignin (17.6% in the wild-type and 15.5–12.5% in the transformants) and in changes in its composition (Guo et al., 2001). Downregulation of COMT did not cause any accumulation of soluble aromatic metabolites, whereas downregulation of CCOMT led to the accumulation of caffeic acid glucoside.

A loss of *CAD1* expression was observed in alfalfa *Medicago truncatula* Handbook (small Mediterranean species) after the introduction of retrotransposon *Tnt1* (Zhao et al., 2013). There were changes in the fluorescence of lignin: a strong reduction in blue autofluorescence and a red color of vessels and fibers.

Poplar

Members of the genus *Populus* (poplar, aspen) are widely used for genetic engineering to produce fast-growing trees with a high yield of cellulose and ethanol, as well as carbon sequestration (absorption of CO_2 from the atmosphere). Additional factors of productivity are the use of triploid forms and distant hybridization (e.g., *Populus deltoides* × *P. nigra, Populus tremula* × *P. alba*, etc.).

A series of poplars transformed with antisense technology (hybrid form of *Populus deltoides* × *P. nigra*) with reduced CAD activity had an increased content of free sinapic aldehyde and phenols (Lapierre et al., 2004). The total lignin content decreased slightly, from 20% in the control to 17–18% in the transgenic genotypes, including those in which the residual enzyme activity was only 10% of the norm. Plants in which the residual activity was less than 30% had a red timber. The solubility of lignin in alkali at room temperature increased significantly. Since the proportion of sinapic aldehyde in the lignin in genotypes with low CAD activity increased, the authors concluded that the SAD enzyme played no significant role in lignification, at least, in poplars. The authors suggested that the reduction in lignification was not strong enough, contributing to the maintenance of the normal tree phenotype.

Along with reduced lignification (20–40%), negative traits were observed in 14 transgenic lines of hybrid poplar with varying degrees of 4CL downregulation: dieback, increased mortality, and disorders in the vessel positions. The wood had a brownish color. The authors emphasized the need to maintain a certain level of lignification for normal growth processes: mechanical support, water transport, tree growth, and survival (Voelker et al., 2011).

Analysis of the splitting of 179 QTL (quantitative trait loci) showed that 49 of them correlated with various indicators of lignification in the poplar (Yin et al., 2010). Such a wide polymorphism, the authors believe, allows the performance of breeding in the right direction with the usual selection.

Eucalyptus

Eucalyptus is considered to be a promising target for bioethanol production. The productivity of *Eucalyptus globulus* Labill. can reach 290 liters of ethanol per ton of raw material, which is close to the theoretical limit—320 L/t (Muñoz et al., 2011).

Two genes, *CAD1* and *CAD2*, which are nonhomologous to each other, were found in eucalyptus *Eucalyptus gunnii* F. Hook. *CAD1* is a monomer; it affects coumaric and coniferyl aldehydes and has no effect on sinapic alcohol, but it affects many benzaldehydes; it is dramatically different from any other *CAD* genes and has a homology with another enzyme, cinnamoyl-CoA reductase (CCR), which catalyzes the preceding reaction in the biosynthesis of monolignols, the formation of aldehyde from a thioester. The enzyme has a molecular weight of 35790 Da and a homology to dihydroflavonol 4-reductase (DFR; EC 1.1.1.219, the enzyme of pigment biosynthetic). The expression of the *CAD1* gene was detected in lignified and in nonlignified tissues (parenchyma). The authors suggested that this enzyme was "an alternative" factor in the biosynthesis of monolignols and/or had additional functions. The *CAD1* gene was also expressed in a hybrid poplar, *Populus tremula* × *P. alba* (Goffner et al., 1998).

This enzyme was then studied in tobacco; plants with reduced *CAD1* activity were obtained with RNA interference (Damiani et al., 2005). Transgenic plants did not differ from normal ones in growth and development or lignin content and composition, but significant differences in the content of dimeric and trimeric precursors of lignin were found and an accumulation of 3-*trans*-caffeic and quinic acids (the so-called chlorogenic acid, which is also an intermediate in the synthesis of lignin) was observed.

GYMNOSPERMS

Pine

A *CAD* (*cad-n1*) null mutant was found in the North American pine *Pinus taeda* L., the so-called loblolly pine. Due to its rapid growth, resistance to wood rotting, and a pleasant aroma, it is considered one of the most valuable conifers (Ralph et al., 1997). A heterozygous tree was found during an analysis of megagametophytes by their splitting at a ratio of 1 : 1 by the presence or absence of a CAD-activity zone on a zymogram (MacKay et al., 1997). The *cad-n1* gene leads to noticeable changes in the growth characteristics of seedlings, including a heterozygous state. Mutant trees (homozygous state) have from 0 to 1% of the wild-type activity; a 20-fold decrease in transcription; a slightly reduced lignin content compared to the norm but a significantly modified content; a tenfold increased dehydroconiferol alcohol, a minor component of normal lignin; increased content of aldehydes (vanillin and coniferyl); a reduced content of coniferyl alcohol; and red-brown wood. Homozygous seedlings grew faster and had differences in the stem anatomy (Wu et al., 1999). The *cad-n1* gene has an insertion of two adenosines in the fifth exon, so the frame is shifted. The pseudogene *cad-ps1* was detected in another chromosome, but it is not expressed. According to the authors, this pseudogene was formed through the reintegration of the processed RNA into the genome (Gill et al., 2003).

Transcription of the enzymes PAL, C4H, C3H, 4CL, CCoAOMT, CAD, and CCR in *Pinus taeda* cultured tissues increases coordinately with increased concentrations of sucrose and exogenous phenylalanine, thus changing the ratio of units (Anterola et al., 2002).

Inactivation of the *HCT* gene in cultured tissues of *Pinus radiate* (radiata pine, one of the fastest growing conifers) via RNA interference led to a significant, 42% reduction in the lignification of vascular elements (Wagner et al., 2007).

Spruce

Transgenic coniferous trees were obtained for the first time from Norway spruce (subspecies of *Picea abies* (L.) H. Karst.), one of the most cultivated tree species (Wadenbäck et al., 2008). Five-year-old transgenic plants with an antisense insertion of the *CCR* gene had a somewhat smaller width of the trunk; the rest of the phenotype did not differ from the wild-type. The transcription of the gene was reduced by 35% and the content of lignin fell to 8%, while the portion of H-units decreased to 34%. Changes in the properties of the pulp were observed during the craft cooking (craft process), in particular, the kappa number (the amount of chemicals, required to bleach the pulp) decreased. The expression of other genes of monolignol biosynthesis decreased, which indicates their coordinated expression.

Ginkgo

A correlation between the degree of tissue lignification and CAD expression levels was shown for ginkgo (*Ginkgo biloba* L.) (Cheng et al., 2013). Expression of the *GbCAD1* gene increased under the action of exogenous treatment with abscisic and salicylic acids, ethephon (synthetic plant growth regulator), ultraviolet irradiation, and tissue injury.

POLYMORPHISM OF REGULATORY FACTORS

The transcription factors AmMYB308 and AmMYB330 from the snapdragon (from the family of c-MYB, related to mammalian proto-oncogenes) activate the *PAL* gene (Moyano et al., 1996); however, overexpression of these factors in the genome of tobacco represses the synthesis of both flavonoids and lignin (Tamagnone et al., 1998).

The overexpression of cytokinin in transgenic arabidopsis plants (as well as treatment with an exogenous hormone) leads to different results: in some cases, it leads to growth enhancement, while in others, it results in its oppression. According to the authors, the illumination and the sucrose content in the medium are the determining factors for a given reaction. Anthocyanin synthesis intensified under the influence of illumination and cytokinins (due to the activation of *CHS* gene), and lignin synthesis increased simultaneously (Guo et al., 2005).

However, overexpression of cytokinin in transgenic tobacco in another study resulted in an increase of phenolic metabolites in the vessels, but it did not enhance lignification (Schnablová et al., 2006).

Maize ZmMYB31 factor caused dwarfism and reduced lignification in transgenic plants of arabidopsis; it also indirectly affected anthocyanin synthesis (Fornalé et al., 2010).

Overexpression of TaMYB4 regulatory factor (the repressor of enzymes of phenylpropanoid path), which was transferred from wheat to tobacco, lowered the CAD activity, which led to a decrease in tissue lignification; the amount of flavonoids in the leaves increased (Ma et al., 2011).

These results indicate an association between the metabolism of aromatic compounds and the precursors of lignin and phenylpropanoids. These data are contradictory, and the result of transgenic manipulation is often unpredictable.

The association may ne carried out according to the metabolism scheme that was detected in flax (Preisner et al., 2014), through CHS and CHI enzymes.

The expression of SHINE transcription factor from arabidopsis in the rice genome led to a 45% reduction of lignin in the straw and to a 34% increase in the cellulose content (Ambavaram et al., 2011).

LIGNIFICATION AS A FACTOR OF RESISTANCE TO DISEASES

It was found in the early 1960s that the lignification of plant tissues is a factor of resistance to pathogens (Hijwegen, 1963). A fungus on damaged wheat tissues causes an increase in the activity of PAL, aromatic dehydrogenases (especially, SAD activity), and peroxidase, which is involved in the process of monolignol polymerization (Mitchell et al., 1994).

The protective effect can be expressed as a mechanical barrier to fungal hyphae spread, as well as toxic effects on pathogen aromatic metabolites.

Among the phenylpropanoid components of lignin, the greatest antimicrobial and antifungal activity belongs to aldehydes: the highest is found with coniferyl aldehyde; a somewhat lower activity is found with coumaric and sinapic aldehydes; a lower level is found for phenolic acids; and a much lower level occurs with aromatic alcohols (i.e. monolignols) (Barber et al., 2000). However, these results were obtained from solutions of these compounds, i.e. they were in a monomeric state, and it is unknown to what extent these properties are preserved in the polymeric composition.

Enzymes of the phenylpropanoid path are involved in a range of protective mechanisms, such as the healing of mechanical damage; the induction of multifunctional regulatory and protective substance MeJA (methyl jasmonate); and protection against pathogens (Belhadj et al., 2006; Simons et al., 2008; Tronchet et al., 2010; Li et al., 2012).

Protective ELI3 protein, which was isolated from parsley and controls the resistance to a parasitic fungus *Phytophthora sojae*, proved to be a CAD enzyme (Somssich et al., 1996), the substrates of which are aromatic aldehydes, especially salicylaldehyde. Salicylic acid derivatives are known to be one of the most common factors of plant resistance to pathogens.

In some cases, a decrease in enzyme activity can be a protective plant reaction. Such a mechanism was described for cucumber (Varbanova et al., 2011). Fungal infection was imitated with pectinase treatment. The activity of HCT is decreased, and cucumber CAD enzymes have a low affinity for *p*-coumaric aldehyde. As a result, *p*-coumaric aldehyde accumulates in the tissues and performs the function of phytoalexins (a substance with the protective action).

The *AaCAD* gene, which is expressed in glandular secretory cells and forms hairs on the leaves and stems, was studied in *Artemisia annua* L. (Li et al., 2012). The *AaCAD* gene appeared to be homologous to *AtCAD4* and *AtCAD5* arabidopsis genes, which are involved in monolignol biosynthesis. However, in addition to cinnamon, sinapic, and coniferyl aldehydes, citral (monoterpene acyclic aldehyde) and artemisinic aldehydes are present among its substrates; during the reduction, they form geraniol (terpenoid alcohol) and artemisinic alcohol (also from the group of terpenoids), respectively. These substances are included in the Artemisia extract "artemisinin," which was recommended in 2001 as a cure for malaria by the World Health Organization (WHO).

Phenylpropanoids possess antioxidant activity and are an essential component of food products, especially grains (Kroon and Williamson, 1999; Goncharenko and Timoshchenko, 2014).

PLANTS WITH OTHER TRANSGENIC GENES

The lignin content in plants transformed with other genes that are not associated with lignin biosynthesis can be increased. For example, maize transformed with *cry1Ab* gene, which encodes a toxin from *Bacillus thuringiensis*, made maize resistant to caterpillars of maize moth *Ostrinia nubilalis*; it has a 33–97% higher lignin content in the stem (Saxena and Stotzky, 2001). A similar trend was observed in other plants: rice, tobacco, tomato, cotton, and canola (Flores et al., 2005). The biomass of such plants decompose more slowly in soil; the toxin can be transmitted via food chains and create some problems (Viktorov, 2006).

CONCLUSIONS

Enzyme polymorphism has been intensively studied since the early 1960s of the last century (Schwartz, 1960). Over the next quarter of a century, until the middle 1980s, allelic variants of enzymes were used mostly as formal genetic markers to study such factors as the overall level of variability, the degree of heterozygosity, similarities and differences between populations, migration flows, etc.

This was mainly due to methodological factors—DNA polymorphism was known, but its analysis was very labor-intensive and it was impossible to perform it on a massive scale. Therefore, proteins were used as the closest products of genes to estimate the genetic variability of the formal parameters, although the functional differences of individual enzyme systems were known in this period (Polyakova, 1991).

Since the mid-1980s, when methods of DNA polymorphism analysis became generally available, researchers began to focus on the functional aspects of enzyme polymorphism. Now, the study of allelic variants of proteins (both those natural and artificially produced) and the analysis of various interactions in metabolic chains are major trends in biotechnology.

The data above show that genetic manipulations on enzyme loci are a quite simple and very effective method of producing plants with altered properties. These changes do not always turn out as planned, but there are a few examples of successful changes that are cause for optimism. A typical colored phenotype (brown midrib, reddish brown, pink, red-brown color, red-wine, etc.) was described in many plant species, most often with an insufficient amount of CAD. Plants with this phenotype are easily processed for pulp; wood plants can be used for furniture with no additional coloring (Higuchi et al., 1994). In addition to the decorative effect, such timber is less susceptible to rotting, since aromatic aldehydes have a pronounced toxic effect on the fungi and bacteria (Barber et al., 2000). Lignified portions of paper hamper the development of fungi that can damage the printed product during prolonged storage (Nyuksha, 1994).

The key role of CAD in the biosynthesis of monolignols was noted in many publications, but there is another view that CAD plays no regulatory or key role in lignin biosynthesis (Anterola and Lewis, 2002). According to these authors, monolignol biosynthesis is determined by the presence of phenylalanine and the ratio of active C4H/C3H. Other researchers believe that CCR, which determines the switching of metabolism from monolignol synthesis to flavonoid synthesis, and vice versa, is the key enzyme (Wadenbäck et al., 2008; Tamasloukht et al., 2011). Perhaps there is no single biosynthesis scheme, and the leading role belongs to various factors in different taxa.

The artificial obtainment of genotypes with altered properties (chemical mutagenesis, T-DNA mutagenesis) can be usefully combined with the search for natural mutants and hybridization. The genetic potential of natural variability is far from being exhausted.

Plants with a C_4-photosynthesis, large stem mass, rapid growth, small seeds, preferably perennial, able to grow on not rich, saline, acidic soils or on soils with low water supply often become the objects of transgenic modification.

The use of plant raw material as a renewable source of food and energy products acquired a broad nature in the 1990s due to forecasts on the exhaustion of natural hydrocarbons. Although later it turned out that this was not the case, research on capturing new plant species continues to grow in this area.

The concept of sustainable development of the modern society (which arose in the 1980s and is somewhat similar to the theory of the noosphere proposed by Vernadsky) involves the use of renewable resources, in particular, the widespread use of biofuels (bioethanol and biodiesel) as a replacement of fossil hydrocarbons. For this purpose, "energy plants" with a high content of carbohydrates (for bioethanol) or fat (for biodiesel) are being created. Many natural plant mutants, which were described many decades ago, were suitable for these goals; most of them were mutated variants of enzyme loci. Genotypes that are transgenic for the same loci are obtained by analogy with natural variability. It can be a transgenesis of coding sequences, as well as a transformation of plants with various regulatory elements. The selection of fodder (forage) varieties of plants also remains an important direction.

The production and use of "energy plants," the biomass of which is suitable for producing bioethanol, are encouraged by the FAO (Food and Agriculture Organization), which promotes the production of nongrain bioethanol. Millions of people in the world suffer from malnutrition, and grain consumption on for motor fuel is considered unethical.

Carbon sequestration, the use of fast-growing forms of plants to absorb excessive amounts of CO_2 (which cause the greenhouse effect in the Earth's atmosphere), is one of the future areas of plant biotechnology. The resulting biomass can be buried in geological horizons (used mines, quarries). Carbon can be stored in the form of lignin for the longest time, with a gradual transformation to a carbon-like mass. Plants with a high content of lignin and a biomass with an increased resistance to degradation by soil microflora may be needed for such purposes. Lignin is the most convenient form of long and safe disposal of carbon, while other forms—carbides and carbonates—are less effective for these purposes.

Plant resources are one of the important components of Russia's national wealth, but their use cannot be considered rational. A large quantity of plant raw material is simply combusted with the formation of carbon dioxide, contributing to the greenhouse effect. Modern technology allows the efficient processing of almost any plant material into useful products. The efficiency is significantly improved if plants that were created specifically for a particular purpose are grown for these objectives. It is clear from the citations that these studies are practically absent in our country, but they are necessary, despite the large reserves of hydrocarbon resources.

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