

## Review

# Bioengineering and Molecular Biology of Miscanthus

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**Abstract:** *Miscanthus* is a perennial wild plant that is vital for the production of paper and roofing, as well as horticulture and the development of new high-yielding crops in temperate climates. Chromosome-level assembly of the ancient tetraploid genome of *Miscanthus* chromosomes is reported to provide resources that can link its chromosomes to related diploid sorghum and complex polyploid sugarcane. Analysis of *Miscanthus sinensis* and *Miscanthus sacchariflorus* showed intense mixing and interspecific hybridization and documented the origin of a high-yielding triploid bioenergetic plant, *Miscanthus × giganteus*. The *Miscanthus* genome expands comparative genomics functions to better understand the main abilities of *Andropogoneae* herbs. *Miscanthus × giganteus* is widely regarded as a promising lignocellulosic biomass crop due to its high-biomass yield, which does not emit toxic compounds into the environment, and ability to grow in depleted lands. The high production cost of lignocellulosic bioethanol limits its commercialization. The main components that inhibit the enzymatic reactions of fermentation and saccharification are lignin in the cell wall and its by-products released during the pre-treatment stage. One approach to overcoming this barrier could be to genetically modify the genes involved in lignin biosynthesis, manipulating the lignin content and composition of *Miscanthus*.

**Keywords:** *Miscanthus*; genome; chromosomes; patterns; bioengineering; monolignol; biofuels



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## 1. Introduction

Humanity uses natural gas, coal, peat and oil as the main sources of energy [1,2]. All these resources are nonrenewable, and their reserves are being depleted. In this regard, the issue of finding alternative renewable energy sources is acute, and plant raw materials are of particular interest. Such renewable plant raw materials as trees are a slowly renewable energy resource. Although 23% of the territory of the Russian Federation is covered with forest, wood accounts for more than 71.4 million hectares of the forested territory—81.5 billion m<sup>3</sup> [3].

*Miscanthus* is used in landscape design, for the manufacture of paper and roofing. This is a promising biomass resource that can replace fossil hydrocarbon fuels [4]. It belongs to the *Andropogoneae* herb family, which includes corn, sorghum and sugar cane—these crops are high yielding and important all over the world, and they can be grown as sources of food, feed and biofuels. *Miscanthus* is unpretentious and easy to grow. It can thrive in

marginal areas, requires only limited fertilizer, tolerates drought and low temperatures well and uses a more efficient form of C4 photosynthesis [5].

In addition to its historical role as an ornamental plant and in paper production, wild miscanthus can also produce highly productive biomass, thereby protecting and potentially increasing the carbon content in the soil [6]. The increase in the carbon content in the soil is associated with the absorption of light, the absorption of nutrients and the efficient use of water, which makes miscanthus a promising bioenergy crop. Bioenergy raw materials (miscanthus) are constantly being genetically improved to increase yields [6]. Before continuous biotic and abiotic stress, it is necessary to guarantee the yield and stability of these crops. This is particularly true for perennial grasses, as the economic and environmental sustainability of perennial grasses, compared to annual crops, depends on the life expectancy of the plants [7]. Kosolapov et al. demonstrated that perennial crops have a huge potential to increase yields and minimize environmental impacts, which makes them a promising, affordable and environmentally friendly resource for biofuels [7]. Dubouzet et al. established that genetic engineering can accelerate the development and reproduction of varieties, but it is not essential to increase biomass yield [8].

It is reported that the global availability of hydrocarbons is increasing due to the modern hydraulic fracturing of rock formations [9]. However, studies of Pärt et al. showed that these isolation methods, which ensure the production of fuel and other biological products, use toxic chemical additives, allergens, mutagens and carcinogens and cause the decomposition of radioactive materials [10]. Miscanthus can provide a more environmentally friendly solution for the extraction of bioenergy from the second-generation crops miscanthus provides as it is a potential supplier of sustainable biomass [11]. Miscanthus is the most environmentally friendly fuel because miscanthus plants can grow in one location for more than 20 years, reducing the need for circulating capital inputs significantly. It is resistant to pests and diseases and can be grown without the use of chemicals. Furthermore, miscanthus successfully performs ecological and environmental improvement functions: it protects landscapes from erosion, promotes organic matter accumulation in the soil and significantly reduces CO<sub>2</sub> emissions [12].

Kukk and Söber demonstrated in their study that due to miscanthus's high-biomass yield, long-term growth, soil carbon sequestration potential, reduced soil erosion and lower fertilizer requirements, the most related varieties of miscanthus are used, including *M. sinensis*, *M. sacchariflorus* and *M. × giganteus* [10]. In the UK, miscanthus is typically planted in the spring and harvested after rooting for 15 years. New shoots usually appear in mid-spring and grow quickly in the next few months, depending on the genotype, they can reach a height of several meters in mid-summer [13]. Arnoult and Brancourt-Hulmel established that autumn frost causes aging, and as the miscanthus ages, nutrients are transferred from the aboveground organs to the rhizomes [14]. Therefore, usually, only fully aging plants are harvested, which guarantees the resumption of crop growth in the next season [14].

Rosen and Kishawy found that the efficient and sustainable development of the world economy requires mandatory and comprehensive consideration of environmental aspects [15]. The constant increase in global energy consumption, as well as the limited availability of fossil raw materials and energy, necessitate the replacement of existing industrial technologies with energy-, nature-, and resource-saving biotechnologies [16]. Environmental pollution caused by plastic packaging materials and products made from synthetic polymers is a serious global environmental problem of our time [17]. This necessitates the urgent development and increase in the production of biodegradable and biocompatible packaging materials, including those derived from renewable raw materials, in order to contribute to the environmental stabilization of the natural environment [18]. Bhatia and Goli demonstrated that there are countries in the world that are actively developing the direction of biotechnology based on the production of valuable substances and products with high added value from plant materials (biomass of energy crops with intensive rates of photosynthetic activity) [19].

According to Baibakova et al., one of the most promising plants in this regard is miscanthus (*Miscanthus* spp.), which has a relatively high adaptive potential (it can be grown on unproductive degraded lands, on fields with a slope of up to 7°, with soil acidity pH 5.5–7.5). The largest energy yield per unit area was 13.55 GJ/ha [20].

Its aboveground cellulose-containing biomass belongs to non-traditional renewable sources of raw materials and energy, the production of which does not require significant capital investments [21].

The study of miscanthus bioengineering and molecular biology is a vital task in this regard [10]. For the first time, this review gathered information on miscanthus species, their properties, and their application in bioengineering to obtain a substantial amount of plant biomass for biofuel production. This work aimed to summarize the research of the world's leading scientists on the methods of bioengineering and molecular biology of miscanthus to obtain promising sustainable biofuels.

## 2. The Importance of the Cell Wall in Miscanthus Bioengineering

Although there are many potential applications in bioprocessing, lignocellulose biomass is still largely untapped due to the stability of the cell wall, i.e., resistance to deconstruction, relative abundance and interaction between cell wall components [22]. Therefore, as demonstrated by Ochoa-Villarreal et al., in order to effectively use the cell wall as a renewable source of useful molecules, it is important to increase knowledge on how to assemble the wall in terms of composition and structure [23]. The cell walls of commelinoid monocots, including grasses, differ from other plant groups. In addition to lignin, these cell walls also contain a high percentage of cellulose, a low percentage of xyloglucan, mixed bonds and a high content of 4- $\beta$ -xylan [24]. Xylanes usually contain acetyl substituents, arabinose and/or glucuronic acid attached to certain xylose residues in the main chain, hence, the name arabinoxylan (AX) and glucuronic acid arabinoxylan (GAX) [25]. The walls of miscanthus cells also contain a small amount of pectin. The studies of da Costa demonstrated that these pectins are polysaccharides rich in  $\alpha$ -galacturonic acid. Polysaccharides are thought to be composed of three domains (homogalacturonan, rhamnogalacturonan-I and rhamnogalacturonan-II), linked by glycosidic bonds [26]. Cellulose was determined by the Kürschner method. The fiber content was determined by the Kürschner method, which is based on the oxidative destruction of all substances, except fiber, in a mixture of acetic and nitric acids. Cellulose fibers were separated and dried. The percentage to the weight of a raw or anhydrous sample was determined as a result of weighing [27]. In Table 1, the chemical composition of samples [28,29] of various miscanthus types are presented.

**Table 1.** Chemical composition of various miscanthus types (4 years of age).

| Type of Miscanthus       | Mass Fraction of the Component, % |             |              |              |              |                       |
|--------------------------|-----------------------------------|-------------|--------------|--------------|--------------|-----------------------|
|                          | Adipose Fraction                  | Ash         | Lignin       | Pentosans    | Cellulose *  | Extractive Substances |
| <i>M. × giganteus</i>    | 4.37 ± 0.16                       | 5.12 ± 0.19 | 22.14 ± 0.66 | 23.62 ± 0.69 | 42.00 ± 1.26 | 5.7 ± 0.1             |
| <i>M. sinensis</i>       | 4.81 ± 0.14                       | 6.05 ± 0.18 | 23.79 ± 0.67 | 23.17 ± 0.69 | 43.63 ± 1.30 | 2.8 ± 0.1             |
| <i>M. sacchariflonis</i> | 5.05 ± 0.15                       | 6.20 ± 0.18 | 22.11 ± 0.66 | 25.47 ± 0.69 | 41.98 ± 1.26 | 4.1 ± 0.1             |

\* according to Kürschner.

Miscanthus plants (Table 1) of the species *M. × giganteus*, *M. sinensis* and *M. Sacchariflonis* were grown in open ground in the conditions of the Siberian Forest steppe, in a temperate climate with a sufficient amount of heat and solar energy, at a moisture level of up to 500–600 mm of precipitation per year. Miscanthus was planted in open ground in sunny locations in the spring, when the temperature is high and the earth heats up to +20–25 °C. No more than 1–2 plants were planted per 1 m<sup>2</sup>. The soil for growing miscanthus was well drained, but not waterlogged. Miscanthus plants grew just as well on acidic and heavy clay soils as it did on black or fertilized soils. A well-developed root

system makes the plant suitable for growing on various types of soils, from sands (with a low level of groundwater) to soils with a high organic matter content [30]. *Miscanthus* grew well in a wide range of soil pH, but the ideal pH was between 5.5 and 7.5. The fallen leaves of the plant itself were an excellent fertilizer. Nitrogen fertilizers were applied in autumn and organic fertilizers were applied several times during the growing season. It is critical not to overuse nitrogen fertilizers, as this will affect the color of the leaves as well as the quality of the biomass. Fertilizers, pesticides and inter-row tillage were applied in the same manner as for traditional crops, but mostly in the first year of growth. Such measures are very limited in subsequent years (if appropriate, they are carried out only in early spring, at the beginning of the growing season). Weed management was essential for *miscanthus* plantings, particularly in the first and second years of cultivation. After that, it was not necessary, since the *miscanthus* plants were already well rooted and competed with weeds. *Miscanthus* is cut-harvested as biomass grows (2–3 times per season). *Miscanthus* samples were chemically analyzed after four years of cultivation [30].

Galacturonans (HG) makes up most of the cell wall pectin and includes unbranched chains of  $\alpha$ -galacturonic acid residues connected by methylated bonds. In addition, rare ingredients, such as hydroxycinnamate (HCA) and structural proteins, can be found [31]. The complexity of cell wall composition is complicated by the fact that various components of the cell wall are linked together by a process, much like the molecular structure that maintains the integrity of plant tissue and resists external attacks. Therefore, according to Marowa et al., it is not easy to understand these complex connections, especially considering that about 10% of plant genomes are related to the assembly/disassembly of cell walls [32].

Research into improving lignocellulose biomass for biological purification purposes has stimulated the structure study of plant cell walls [32]. Determining the required characteristics of the cell wall and cultivating cultures containing such characteristics are key steps to optimize the bioremediation of lignocellulose [32]. However, the study of [30] showed that since the composition of all cell walls is different, often, there are difficulties in the complex assessment of the quality of different raw materials, especially throughout different studies that consider the profile of the reference cell wall to increase the biomass of leaves and stems of *Miscanthus* [30]. In this material, particular attention is paid to the composition of cell wall glycans [31].

Research on the structural composition and distribution of glycans in *miscanthus* organs and/or tissues is important not only for optimizing the use of lignocellulose biomass as a raw material for renewable biological products and bioenergetic solutions, but also for understanding the significance of the cell wall [31]. Studies based on mid-infrared Fourier transform spectroscopy in the mid-infrared region show that structural polysaccharides are a major factor in composition variability during trunk development and between organs [32].

The study of [33] identified factors causing this variation in components (temperature, humidity, pH, organic and mineral composition of the soil, type and age of the plant and its productivity) and discussed some important observations about possible interactions between components and cell wall structures. It has been shown that various monoclonal antibodies targeting plant glycans can be markers of changes in characteristics of glycans in the matrix (distribution, structure and extractability) [33]. This set of molecular markers of *Miscanthus* has sufficient variability to identify most types of non-cellulose polysaccharides in the plant cell wall [19]. This was the first time that researchers used a complex of glycan-targeted antibodies to comprehensively characterize cell wall glycome variation across cultures to create a representative sample of *Miscanthus* wall biomass data (reference map) [32]. Several global breeding programs are known to genetically improve the biomass and yield characteristics of *Miscanthus*, based on the use of genotypic and phenotypic differences between and within the species diversity of *Miscanthus* [34].

The detailed *miscanthus* cell wall portrait in these studies contributes to genetic transformation, providing important information for the formation of the necessary charac-

teristics in different types of miscanthus, which are planned to be processed into biofuels and other biological materials [34].

Thus, this section discussed some important observations about possible interactions between the components and structures of cell walls and presented the factors that contribute to the transformation of miscanthus into a renewable source of biofuel. The complexity of the miscanthus cell wall composition, which affects miscanthus bioconversion into biofuel, was revealed.

### 3. Study of the Miscanthus Genome

In the studies of [35], the miscanthus genome was assembled on  $n = 19$  chromosomes by combining data from the target genome (WGS) and affinity for the phosphoramidite end and chromatin in vitro and in vivo. The control sample is a duplicate of the previously characterized sample [36]. It was established that it is a homozygous haploid DH18. The genome assembly attributes a contig of 1.68 GB to a chromosome with a contig length of N50 33.1 bp [35]. The length up to HiC N50 is 190 bp. An additional 0.20 GB contig sequence in the frame was not placed in the pairing group. Mitros et al. confirmed that compared to an integrated genetic map with 4298 labeled markers, the assembly occurs at the chromosomal scale [35].

Based on various pieces of evidence, the structure of 67,967 protein-coding genes was predicted, including homology with other crops and deep transcriptome data for Miscanthus and sugarcane [35]. These predicted genes accounted for 98% and 94% of protein coding genes, respectively, and were assigned to chromosomal positions. These genes are embedded in moving element chains and other repeating sequences (making up 72.4% of the Miscanthus genome assembly). Mitros et al. established that the most common type of transposon to be assembled is the retrotransposon with a long terminal repeat (LTR) [35].

After considering the fusion of chromosomes 4 and 7 of the ancestral sorghum chromosomes, each sorghum chromosome corresponds to a pair of Miscanthus chromosomes, so the ancient Miscanthus tetraploid was discovered at the sequence level [35]. This makes the karyotype  $n = 20$ ,  $kn = 19$ . As expected (Mitros et al.), the genomes of Miscanthus and sorghum showed extensive conservative collinear synthesis of 2:1, which corresponds to complete duplication of genomes in overlapping lines of Miscanthus origin [35]. Although it is believed that duplication is typical for sugar cane, a comparison of the of *M. sinensis* and *S. spontaneum* genomes found that duplication in these two strains differs. Although studies of Miscanthus genomics have shown its allotetraploid origin, the mechanism of the ancient tetraploid has not been described, partly due to the lack of known diploid progenitor cells [35].

The studies of Mitros et al. demonstrated that *M. × giganteus* is a perennial plant with a triploid rhizome obtained naturally as a result of natural crossing of diploid *M. sinensis* and tetraploid *M. sacchariflorus* [35]. Unique characteristics (high biomass production, low environmental impact, the potential for cultivation on poor soils) of *M. × giganteus* determine the prospects for use of the crop for the production of lignocellulose biomass for bioethanol [35]. However, as established by Zeng et al., the persistent nature of lignocellulose raw materials increases the cost of pretreatment, saccharification and fermentation processes, which slows down the current commercialization of lignocellulose bioethanol [37].

Lignin, among the biopolymers of lignocellulose biomass, is one of the main factors determining the stability of organisms, due to the significant content of secondary cell walls [38]. Moreover, by-products released at the pretreatment stage act as the main inhibitors of enzymatic reactions in the process of fermentation or saccharification [39]. Zeng et al. observed a similar correlation in natural samples of Miscanthus. In addition, sterility of *M. × giganteus* increases the ecological safety of genetic engineering. These results demonstrated that the genetic manipulation in the process of lignin biosynthesis is





In some species, it can convert caffeine-CoA into caffeic acid, but there are no orthologs in the genomes of *B. distachyon*, *Z. mays* and *S. bicolor* [48]. Devi et al. showed that this diversity underscores the necessity to comprehend monolignol biosynthetic pathways in all lignocellulose cultures [21].

In essence, some studies concentrated on the genetics and regulation of the monolignol biosynthesis way in certain bioenergetic herbs [40]. However, comprehensive studies are still lacking for *Miscanthus* species, and a comprehensive model has not yet been created. To fill this gap, the main genes of monolignol biosynthesis in *M. × giganteus* were identified and possible transcription factors that directly regulate these genes were predicted [40]. These studies provide a list of potential target genes for genetic modification of lignocellulose biomass *M. × giganteus*. In addition, they demonstrated the genetic, regulatory and transcriptional methods of monolignol biosynthesis in *M. × giganteus* [40].

MgCCR1 and MgCCR2 are two of the genes generated in the genus-specific part of WGD. Installed, compared to MgCCR2, MgCCR1 rapidly accumulates mutations in MgCCR3 and is independent of MgCCR4 for a short period of evolution [40]. This indicates that the evolution of asymmetric monolignol biosynthesis genes may accelerate to an early stage after WGD, and then gradually decreases. This conclusion is consistent with the results of observation of yeast WGD [40].

Wang et al. established that data on transcription of various organs of *M. × giganteus* allow us to study the main mechanisms of regulation of gene transcription in the border regions of genes. Firstly, the functional interrelationships of the co-expressed genes are indicated [49]. Genes showing a statistically significant pattern of positive correlation expression (Spearman correlation coefficient  $\geq 0.4$ ,  $p < 0.05$  value) were considered co-expressed genes. In monolignol biosynthesis, for each major gene, the co-expressed genes constitute from 3.19% to 18.42% of the total number of expressed genes [40]. Analysis of the accumulation of GO and KEGG shows that these genes are more representative in the GO and KEGG pathways [50]. According to Zeng et al., these pathways are associated with the formation of secondary cell walls or have overall intermediate products with monolignol biosynthesis (Fischer's exact test using the Benjamin-Hochberg multiple criteria to correct  $p < 0.05$ ) [40].

The co-expressed MgHCT1 gene was associated with "the process of lignin biosynthesis", "the process of phenylpropane biosynthesis", "the process of phenylpropane metabolism", "secondary generation of plant-type cell wall" and "phenylalanine". The KEGG pathway is associated with GO enrichment, from the side of "phenylalanine metabolism", "flavonol biosynthesis", "suberin blocking and paraffin biosynthesis". The conformity between gene expression and gene function instructs that the main genes of monolignol biosynthesis are closely related to these genes. It is jointly regulated by *M. × giganteus*, as demonstrated by Zeng et al. [40].

The breeding of improved *Miscanthus* for biomass production in order to obtain biofuels may depend on the abundance and ploidy level of wild genetic material of various species [35,51]. Therefore, according to Schaefer et al., the *Miscanthus* genetic variety and the distribution of interspecific and intraspecific variability in mixed populations were investigated. Using the new WGS sequencing technology, WGS sequencing was performed on 18 different ploidic attachments (including triploid biomass of the *M. × giganteus* «Illinois») [35] in combination with genotyping data, mainly from wild *Miscanthus* species in the north and southeast of China, Japan and Russia. Genome-wide mixing and PPSH easily identify differences between two species (*M. sinensis* and *M. sacchariflorus*) [52]. Hodgkinson et al. established that *Miscanthus* species, such as *M. transmorrisonensis* and *M. floridulus*, belong to the genetic variation in *M. sinensis*, indicating that these taxa should be more properly considered subtypes of *M. sinensis* [53]. However, the specimen called *M. junceus* is significantly different and appears to be less related to *Miscanthus* than to sugarcane. It is African and sometimes belongs to a separate *Miscanthidium* and is distinctly separated from *M. sinensis* [53]. The data obtained prove that the high-yield

triploid *M. × giganteus* “Illinois” is an interspecific hybrid of the tetraploid *M. sacchariflorus* and the diploid *M. sinensis* [54].

The main allele ratio of *M. sacchariflorus*: *M. sinensis* is 2:1. This hypothesis supports the genome; however, it was observed that the ancestors of *M. sacchariflorus* are interspecific mixed, indicating that the greatest productive genotype of *M. sinensis* currently cultivated penetrates into *M. sacchariflorus*. Hybrids of *M. sacchariflorus* and *M. sinensis* are often very active and highly productive, regardless of whether they are diploid, triploid or tetraploid [40]. Therefore, Zeng et al. showed that understanding the previous gene infiltration of *M. sinensis* (and *M. sacchariflorus*) alleles and the genetic background affecting the potential yield of subsequent interspecific hybrids will be very important for optimizing breeding strategies [40].

Specifically, *M. × giganteus* combines the genes of its parent *M. sinensis* (many stems per unit area; short rhizomes) with the habitus of the spreading rhizome of its parent *M. sacchariflorus* (several stems per unit area; long rhizomes); usually, an average number of stems per unit area is necessary for highly productive reproduction of *M. × giganteus* [52]. Productivity indicators of different *Miscanthus* species [55], depending on their ploidy, are presented in Table 2.

**Table 2.** Productivity indicators of different *Miscanthus* species depending on their ploidy.

| Type of <i>Miscanthus</i> | Ploidy     | Productivity      | Amount of Biomass: Raw/Dry, t/ha | Sources |
|---------------------------|------------|-------------------|----------------------------------|---------|
| <i>M. × giganteus</i>     | triploid   | highly productive | 10.71/3.53                       | [56]    |
| <i>M. sacchariflorus</i>  | tetraploid | highly productive | 9.72/2.97                        | [52]    |
| <i>M. sinensis</i>        | diploid    | productive        | 7.76/2.36                        | [52]    |

A recently collected [57] sample of the Japanese triploid *M. × giganteus* “Ogi80” is similar to “Illinois” and contains several short chains, including two or three alleles of *M. sinensis*. These regions may be the result of segmental transformation or loss of a gene during reproduction of this sterile triploid, or the result of interspecific infiltration before the formation of a triploid [58]. Another natural triploid, “Ogi63”, showed a unique pattern, emphasizing the variety of *Miscanthus* natural polyploid hybrids of *Miscanthus*, as reported by St Charles et al. [58].

Polyploidy is also common in group C4 of closely related and high-yielding perennial grasses of the subfamily Saccharinae, including sugarcane (*Saccharum* spp.) and *Miscanthus*. By crossing *Miscanthus* hybrids with a sugarcane hybrid [59], *Miscanthus* hybrids can be obtained, which indicates that the natural genetic variability in the two genera can be combined to mix the desired traits (such as cold resistance and disease resistance), as demonstrated by Kar et al. [59].

In the study of [60], it was found that the ratio of C/N in the collected biomass of *M. × giganteus* steadily increased as the season moved into winter, while in spring and summer, minimum levels of the C/N ratio were observed (Table 3).

**Table 3.** The ratio of carbon to nitrogen content in aboveground biomass of *M. × giganteus* from August to February in various parts of Illinois.

| Month     | C/N Ratio |       |       |
|-----------|-----------|-------|-------|
|           | 1         | 2     | 3     |
| August    | 95.2      | 97.5  | 99.2  |
| September | 103.1     | 109.5 | 120.3 |
| October   | 108.7     | 121.8 | 160.3 |
| November  | 113.6     | 148.9 | 204.1 |
| December  | 129.8     | 188.4 | 264.0 |
| January   | 135.4     | 201.7 | 300.5 |
| February  | 140.1     | 212.0 | 322.4 |

1—northern part of the state; 2—central part of the state; 3—southern part of the state.



To describe the seasonal dynamics of gene expression and regulatory programs associated with perennial *Miscanthus*, RNA sequencing was performed from the same tissue samples selected for the nitrogen cycle profiling [40]. According to Guo et al., Principal Component Analysis detected two main sources of tissue-type variation, followed by sampling time [61]. Using gene catalogs to compare tissues, it was found that the genes preferred for leaves are present in significant numbers in genes associated with carbon fixation and metabolism, and it is preferable that stem genes include genes associated with amino acid metabolism and biosynthesis of the phenylpropane [40]. Samson et al. reported that gene expression in rhizomes is more like expression in stems than in leaves, which corresponds to the genetic origin of the modified stems [40]. Rhizomes, unlike stems and leaves, mainly express transcription to the genes that respond to stimuli, such as water and stress and factors that regulate growth and metabolic processes. As such, 35 genes were identified, mainly expressed in rhizomes, including homologues of such genes as *Giganteus* (GIK) and *Short Internode* (SHI), which are involved in the structure, differentiation and elongation of cells [40]. In plants, they are compact, with short stem internodes, [41], which correspond with morphological differences between rhizomes and stems of *Miscanthus*.

The *Miscanthus* yield was demonstrated to be dependent on harvest time as well as the biosynthesis pathway of monolignols. Genetic studies were described to establish the dependence of the yield of *Miscanthus* on the genotype.

#### 4. Cloning of M1ARF-GEP, M1KHCP, M1SERK1, M1SERK2 and M1TypA

In the study of Zhao et al., full-length ORF cDNA sequences of three genes, M1ARF-GEP, M1SERK1 and M1SERK2 (GenBank access numbers KU640196, KU640198 and KU640199), obtained from KU640199, were cloned by amplification of degenerate primers designed for sorghum and maize sequences [62]. However, for Zhao et al., based on the resulting cDNA, the other two genes accept only partial open frames for reads in the cDNA sequence and are referred to as M1KHCP and M1TypA (GenBank access numbers KU640197 and KU640200) [62]. The length of the complete M1ARF-GEP ORF sequence is 5385 bp and encodes a protein of 1794 amino acids. The length of the M1SERK1 cDNA sequence is 1956 bp. It contains an open reading frame measuring 1872 bp, which encodes a protein of 624 amino acids. The length of 5'-UTR and 3'-UTR is 9 and 75 base pairs, respectively [62]. The length of the M1SERK2 cDNA sequence is 2119 bp. It contains an ORF of size 1878 bp, which encodes 626 amino acids. Zhao et al. determined that the length of the incomplete ORF M1TypA sequence is 1962 bp and encodes a protein of 654 amino acids [62].

Compared to other monocots (*B. distachyon*, *O. brachyantha*, *O. sativa*, *S. italica*, *S. bicolor* and *Z. mays*) and dicots (*A. thaliana*, *B. napus*, *M. domestica* and *R. communis*), the sequence and length of the M1ARF-GEP protein are quite conservative [62,63]. It is determined by multiple comparison analysis [62]. Compared to M1ARF-GEP, the highest identity is 99% (SbARF-GEP). The phylogenetic tree constructed by the MEGA 5.1 program shows that M1ARF-PVA is divided into monocotyledonous and dicotyledonous groups [62]. According to Zhao et al., this means that M1ARF-GEP evolved irrespective after differentiation of monocots and dicots. Furthermore, M1ARF-GEP found the closest genetic interrelation with SbARF-GEP [62]. Multiple alignment analysis of M1KHCP persisted in both monocots and dicots plants, and adjacent connecting trees showed characteristics similar to M1ARF-GEP. M1KHCP has shown a close genetic interrelation with SbKHCP [62]. In addition, it has been confirmed that it is compatible with monocotyledons (*O. sativa* Indica group, *S. bicolor*, *T. aestivum* and *Z. mays*), dicotyledons (*A. thaliana*, *C. sinensis*, *G. max*, *G. hirsutum*, *N. benthamiana*, *S. lycopersicum* and *V. vinifera*), mosses (*P. patens*) and ferns (*S. moellendorffii*) [62]. M1SERK1 and M1SERK2 are conserved in protein sequence and length. The phylogenetic tree constructed using MEGA 5.1 shows that M1SERK1 and M1SERK2 are divided into four key groups (monocots, dicots, mosses and ferns). M1SERK1 shows the closest genetic interrelation with SbSERK2, and M1SERK2 demonstrates the closest genetic relationship with ZmSERK2 and SbSERK3 [62].

According to Zhao et al., the phylogenetic tree of five genes showed that M1ARF-PVA ORF and ARF-GEPS are dicotyledonous and monocotyledonous plants. ORF M1KHCP and other KHCPs form dicotyledonous and monocotyledonous plants. MISERK1, MISERK2 ORF and SERK form dicotyledonous and monocotyledonous plants. ORF M1TypA and other types form dicotyledonous and monocotyledonous plants [62].

Thus, *Miscanthus* genes were cloned by amplification of degenerate primers. Compared to M1ARF-GEP, the highest gene identity was 99% (SbARF-GEP). It was found that M1ARF-GEP developed independently after the differentiation of monocots and dicots.

### 5. Analysis of Polymorphism of SSR Markers of 5 Genes

Zhao et al. reported that embryogenic callus from 37 specimens of *M. lutarioriparius* was induced in only 9 specimens [62,64]. In the sorghum genome database (based on five genes), the CCP locus was not detected in the M1ARF-GEP and M1TypA genes using the CCP Hunter 1.3 analysis method [65]. Although five SSR loci were found in M1KHCP and one SSR locus was found in MISERK2, polymorphism was not found. It turned out that two MISERK1-3 and MISERK1-4 CCP loci were amplified by three alleles in 37 *M. lutarioriparius*. The MISERK1-3 mutation shows the importance of the induction response but is independent of MISERK1-4 [62]. All individuals that can be induced by embryogenic *Miscanthus* callus were observed in part of heterozygotes, and during amplification of MISERK1-3, all homozygotes were induced by nonembryogenic *Miscanthus* callus [66].

Results obtained by Glowacka et al. demonstrated that explants in tissue culture of *Miscanthus* species tissues from immature inflorescences are often used [67]. However, the genotype of the material, the conditions of cultivation of callus *Miscanthus* and the concentration of plant regulators in the environment will affect the growth and development of embryogenic *Miscanthus* callus simultaneously [67]. Similar results were obtained for rice [67]. The inflorescence length is one of the causes for the induction of embryogenic *Miscanthus* callus [67]. In this study, 37 specimens of *M. lutarioriparius* were used for callus induction. A variety of induction frequencies has been studied [62]. The results of induction are influenced by various genotypes [62]. Some genes, such as SERK, can play an important role in embryogenesis. In our study, two SERK MISERK1 and MISERK2 genes were evaluated. According to Zhao et al., they showed similar or different expression patterns at different stages of development of embryogenic *Miscanthus* callus [62,68]. In addition to the above results, MISERK1 showed significant differences between the genotype and characteristics of callus. Zhao et al. confirmed that all genotypes that can be induced from embryogenic *Miscanthus* callus are heterozygotes of MISERK1 [62]. This discovery may mean that MISERK1 heterozygotes are easier to induce than homozygous ones. This study shows that the MISERK1 protein plays an important role in embryogenic *Miscanthus* callus formation, and more research is needed to determine its exact role [62]. Zhao et al. demonstrated that the relative expression of MISERK1 and MISERK2 increased significantly in the third week, which may be due to the accumulation of hormones in the medium [62]. Relative expression dropped sharply at week 4, possibly due to a weakening of hormones. Many reports indicate that *Miscanthus* species are insufficiently cultured in tissue culture every 3 weeks [69], so the expression patterns of MISERK1 and MISERK2 confirm the above method at the molecular level [70].

According to available data, ZmARF-GEF belongs to the ADP ribosylation factors (ARF); when alternating between PIB and GTP, it acts as a relay [71]. In maize, ZmARF-GEF is associated with auxin flux and polarization, which also affects the forming of embryogenic *Miscanthus* callus [62]. Huyen et al. proved that M1ARF-GEP is tightly related to ZmARF-GEF. Furthermore, depending on the expression patterns, in two types of callus M1ARF-GEP, this means that M1ARF-GEP in *M. lutarioriparius* and ZmARF-GEF in *Z. mays* may play similar roles [62,72].

Results of Zhao et al. demonstrated that KHCP can be a type of protein similar to the heavy chain of kinesin, which can spread through the fibers of the cytoskeleton, hydrolyzing

ATP for energy production [62]. Analysis of the alignment of homologous sequences shows that MlTypA is very similar to TypA protein (protein phosphorylated with tyrosine) of other species [62]. Phylogenetic analysis established that MlTypA is close to SbTypA and ZmTypA. TypA is a new type of ribosome-binding protein from the GTPase superfamily, which participates in a variety of regulatory pathways [73]. Dou et al. confirmed that the AtTypA gene is involved in the growth of pollen tubes of Arabidopsis [74]. A fragment of cDNA TypA was isolated from the callus of a corn embryo. It is assumed that ZmTypA may play a similar role in the regulation of other species [62,75].

Thus, the possibility of Miscanthus microclonal reproduction was studied, and it was demonstrated that tissues of immature inflorescences are often used as explants in tissue culture of Miscanthus species. It was shown that the genotype of the material, cultivation conditions of the Miscanthus callus, and the concentration of plant increase regulators in the environment have an effect on the increase and development in the Miscanthus callus.

## 6. Conclusions

In this study, MgPAL1, MgPAL5, Mg4CL1, Mg4CL3, MgHCT1, MgHCT2, MgC3'H1, MgCCoAOMT1, MgC3'CCo and MgCCoAOMT1, and MgC3'CCoH1s were discussed as the major genes for monolignol biosynthesis in *M. × giganteus*. ERF families can directly regulate the expression of these major genes by associating with their promoters for the biosynthesis of monolignols [62]. Based on the presented results, an integrated model and a potential regulatory mechanism of the monolignol biosynthesis pathway were generalized. This work presents important information for understanding the genetic and transcriptional regulation of the monolignol biosynthesis pathway in *M. × giganteus* [40]. In addition, by combining the content and structure of lignin, the complexity of potential candidate genes for a genetic increase in the quality of lignocellulose biomass was identified [76].

Miscanthus is a promising source of long-term biomass and a promising candidate for biofuel crops (it has highly efficient C4 photosynthesis), which is easily adaptable. Its high resistance to drought and cold and its ability to grow in areas with limited resources make it suitable for both tropical and temperate climates [77]. Optimization of Miscanthus productivity and resistance is determined by systematic improvements based on the reviewed genome sequences and their analysis. It is expected that a comparative analysis of *Andropogoneae* [78], in which Miscanthus is combined with corn, sorghum and sugar cane, will form the genetic basis of innovations, resulting in high yields and wide adaptation of this plant species [79].

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