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Review

Multi-Omics and Genome Editing Studies on Plant Cell Walls to Improve Biomass Quality

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Abstract: Biomass is one of the most important sources of renewable energy and plays an important role in reducing our reliance on fossil fuels. Efficient biomass production is essential to obtain large amounts of sustainable energy with minimal environmental cost. However, the biochemical and molecular processes behind the synthesis of the main components of biomass are still not fully understood. This review provides a comprehensive summary of the most relevant studies on cell wall biosynthesis and degradation mechanisms, focusing on the lignocellulosic component, in which the conversion process to fermentable sugars is expensive, due to its recalcitrant nature. A focus is placed on multi-omics research involving genomics, transcriptomics, proteomics, metabolomics, and phenomics, since multi-omics approaches offer a unique opportunity to investigate the biological pathways underlying the genotype traits characterizing cell wall energy crops. Furthermore, our study highlights the advances in genome editing approaches and proposes the modification of the genes that are involved in the complex cell wall structure as a feasible solution to an efficient biomass production. Several key points for future research activities based on these emerging technologies are also discussed, focusing on the combination of multi-omics and gene editing approaches, which offer potential for improved biomass valorization and the development of tangible bioproducts.

Keywords: energy crops; genome editing; genomics; metabolomics; phenomics; proteomics; transcriptomics

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1. Introduction

The global increase in the price of fossil fuels and the need to decrease carbon dioxide (CO_2) emissions and achieve energy security have increased the importance of using biomass for energy production [1]. Biomass is a renewable, abundant, and easily generated source of energy, and it contributes to decrease the level of greenhouse gases in the environment [2]. Biomass can be directly used as a source of energy, or it can be converted into biofuels in order to increase the efficiency of energy production or to facilitate transport and storage. However, careful consideration must be given to the source of biofuels, as the greatest environmental benefits can be achieved by using waste products and land that is too poor to grow food crops on [2].

In industrialized countries, the economic importance of bioenergy has been recognized for many years, and several initiatives, such as the "Biomass Action Plan" and the "Multi-Year Plan", have been undertaken [3]. The former plan highlighted the need of reducing carbon dioxide (CO_2) emissions, according to the Kyoto Protocol regulations. The latter details how agricultural and energy policies are handled among different countries by identifying, in research and development and market behaviors, the strategic activities that are required to meet the energy and sustainability challenges [4]. Effective

biomass conversion into tangible energy products is a critical key factor to facilitate sustainable development and to obtain ecological and socio-economic benefits. Further research is required in order to develop biorefining technologies for an efficient utilization of these resources. To make this possible, it is necessary to have a thorough understanding of the biochemical and molecular processes in both the synthesis and the degradation of major biomass components.

The composition of biomass is extremely diverse, varying widely and depending on the species of plant and the tissue from which it is harvested. Broadly speaking, plant biomass predominantly consists of cellulose, hemicellulose, and lignin [5]. These are the main components of secondary cell walls (SCWs), which give plant cells their structural integrity (the lignocellulosic component). SCWs are strong, rigid, thick cell walls that are deposited after cell expansion in the sclerenchyma. Most SCWs are associated with woody tissue and constitute the major source of plant biomass [5]. Cellulose and hemicellulose make up wood fibers, and lignin binds them together, providing rigidity [6]. Therefore, the extraction of the cellulose from the plant requires the lignin to be broken down first. Lignin is insoluble in acids and is resistant to bacterial degradation, as it has very low biomass digestibility. Therefore, the extraction process may require complex methods, which are chosen according to the type of lignin [7]. It can include biological approaches, aimed to depolymerize lignin through enzymatic oxidation, or microbial conversion by using bacteria that are involved in wood decomposition, or fungi belonging to *white-rot fungi* or *brown-rot fungi* groups [7,8]. However, given the high availability of lignin in nature and its production worldwide, which reaches 70 million tons per year, innovative technologies for lignin decomposition are still being investigated [9].

A deep understanding of the biochemical and molecular processes in both the synthesis and the degradation of major biomass components can be obtained through the use of multi-omics approaches for investigating the cell walls of energy crops. They allow us to elucidate the biological pathways behind traits characterizing biomass, and they provide crucial data for the selection of cultivars that are suitable for biofuel production. Therefore, methods and studies involving the use of genomics, transcriptomics, and proteomics/metabolomics are described here, with particular attention to the lignocellulosic component of cell walls. Furthermore, the final utilization of sugar derived from lignin is limited, due to the cell wall structure, which only allows a small area to be subjected to enzymatic and/or chemical hydrolysis processes [10]. As a consequence, we believe that several approaches, in addition to chemical pre-treatments, are needed in order to improve biomass saccharification, such as the design of genetically modified plants that are characterized by less recalcitrant cell walls. Therefore, we discuss some relevant studies that aimed to reduce lignin content, modifying the cell wall composition by downregulating or knocking-out specific target genes or by acting on related transcription factor mechanisms.

Specifically, we describe the main gene families that are involved in the biosynthesis, growth, development, and degradation of cell walls. Such families are then further described and contextualized in regard to multi-omics approaches and genome editing methods.

Furthermore, in order to describe multi-omics approaches for increasing biomass yield and improving quality, we discuss genetic studies on the phenotypical traits of energy crops. Genetic mapping and quantitative trait loci (QTL) identification approaches, as well as the detection of candidate genes that are associated with biomass characteristics of interest [11], are examined.

Next, we focus on studies on multi-omics applications resulting in biomass quality improvement. To date, these applications are most commonly used in plants, compared to multi-omics data integration-based applications, which are hard to process on the very heterogeneous data deriving from the different omics layers.

Genome editing studies aimed to achieve cell wall biosynthesis characterization and degradation, and to selectively edit target genes, are thus described, with particular attention to their applications for lignin, cellulose, and hemicellulose biosynthesis, and their degradation [12].

Finally, some key points for future research activities based on these emerging technologies are discussed, focusing on the potential provided from the combination of omics approaches and gene editing methods.

The abbreviations that have been used throughout the manuscript are also listed in Table 1.

Table 1. List of abbreviations used in this manuscript.

Abbreviation	Definition
4CL	4-coumarate:CoA ligase
C3H	p-Coumaroylshikimate 3′-hydroxylase
C4H	Cinnamate 4-hydroxylase
CAD	Cinnamyl alcohol dehydrogenase
Cas9	CRISPR-associated protein 9
CAZy	Carbohydrate active enzyme
CCoAOMT	Caffeoyl-CoA O-methyltransferase
CCR	Cinnamoyl-CoA reductase
CESA	Cellulose synthase complexes
COMT	Caffeic acid O-methyltransferase
CRISPR/	Clustered regularly interspaced short palindromic repeats
Csl	Cellulose-synthase-like
F5H	Ferulate 5-hydroxylase
GBS	Genotyping by sequencing
GH	Glycoside hydrolase
GT	Glycosyltransferase
GWAS	Genome-wide association study
HCT	Quinate/shikimate p-hydroxycinnamoyltransferase
MAS	Marker-assisted selection
NBDI	Network based on data integration
NGS	Next generation sequencing
PAL	Phenylalanine ammonia-lyase
QTL	Quantitative trait loci
RILs	Recombinant inbred lines
SCWs	Secondary cell walls
SNP	Single-nucleotide polymorphism
WAK	Wall-associated kinase

2. Cell-Wall-Related Molecular Investigations

The analysis and identification of cell-wall-related genes and enzymes is a convenient approach to study the role of cell wall components in bioenergy crops. The main gene families that are explored in biomass investigations are those that are involved in the processes of cell wall biosynthesis, growth, development, and degradation. They are described in this section and summarized in Table 2.

Cell wall biosynthesis involves large enzyme families, characterizing the different cell wall components. Specifically, cellulose synthase (CESA) complexes consist of proteins that are involved in the synthesis of cellulose [13]. CESAs are located in the plasma membrane and synthesize cellulose in three steps, beginning with the initiation of the β-1,4-glucan chain, followed by an elongation phase, and then the termination of the polymer chain [14]. The *CESA* gene family has been characterized in several plant species used for biofuels including rice and barley [15,16].

Hemicellulose biosynthesis mechanisms are still poorly understood, but our understanding has improved after the application of genetic approaches. For instance, it has been reported that the hemicellulose polysaccharides named mannans are synthesized from guanosine diphosphate mannose (GDP-mannose), guanosine 5′-diphosphoglucose (GDP-glucose), and uridine diphosphate galactose (UDP-galactose) [17]. These activated nucleotide sugars are then utilized by highly specific glycosyltransferases (GTs), which allows the synthesis of the polymer.

The enzymes that are involved in callose biosynthesis and hydrolysis include the 1,3-β-glucan synthases and the 1,3-β-glucan hydrolases, respectively. These enzymes have historically been associated with pathogen response, cell division, and plant reproduction [18].

Lignin is produced by the phenylalanine/tyrosine metabolic pathway in plant cells. In this phenylpropanoid pathway, three enzymes, namely, phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate:CoA ligase (4CL), catalyze the initial steps to provide the precursors for all of the downstream metabolites [19]. Other phenylpropanoid enzymes, such as quinate/shikimate p-hydroxycinnamoyltransferase (HCT), p-coumaroylshikimate 3′-hydroxylase (C3H), caffeoyl shikimate esterase (CSE), caffeic acid O-methyltransferase (COMT), and caffeoyl-CoA O-methyltransferase (CCoAOMT), working downstream of 4CL, are also essential for lignin biosynthesis [20]. In addition to these enzymes, others that are specific for lignin biosynthesis have been identified, including cinnamoyl-CoA reductase (CCR), ferulate 5-hydroxylase (F5H), and cinnamyl alcohol dehydrogenase (CAD) [19]. After biosynthesis and transport, lignin is generally polymerized by the enzymes peroxidase (POD) and laccase (LAC) in the secondary cell walls with three types of monolignols, namely sinapyl alcohol, coniferyl alcohol, and p-coumaryl alcohol [19].

Cell wall growth and development incorporate large families of enzymes, including glycosyltransferases (GTs), glycosylhydrolases (GHs), methyltransferases, and acetylesterases, part of the carbohydrate active enzymes, or CAZymes, classified in the CAZy database [21]. Despite their importance, many *CAZy* genes are still uncharacterized [21]. Furthermore, the cellulose-synthase-like (*Csl*) gene superfamily appears to be crucial in regulating β-glucan synthesis during plant development [22]. For instance, the *CslF6* gene is expressed in many plant tissues during development [23]. However, further investigation is necessary in order to define the precise role of 1,3;1,4-β-glucan and the *CslF* gene family in cell wall composition.

Other factors influencing plant development are the wall-associated kinases (*WAK*s), which are required for cell wall expansion, as shown in Arabidopsis, where leaves expressing an antisense *WAK* transcript have lower WAK protein levels and show a loss of cell expansion [24].

The ERULUS (ERU) protein, which is part of the FERONIA (FER) kinase family, is required for correct root hair formation and regulates cell wall composition through the negative control of pectin methylesterase (PME) activity [21]. Interestingly, *ERU* transcription is downregulated in several mutants showing pectin-related changes in cell wall composition. This trend suggests the existence of a feedback mechanism from the wall itself to regulate pectin composition [21].

The endogenous degradation process of the cell wall involves several enzymes. It is a step-by-step reaction that starts with the expansion and subsequent separation of cells in which pectins are targeted, followed by hydrolysis of the cell wall components, and degradation of hemicellulose and cellulose [25]. Among the enzymes that are involved in cell wall degradation, there are members of the glycoside hydrolase 9 (GH9) family, the endo-β-1,4-glucanases, which cleave the β-1,4-glycosidic bonds with monomers of glucose, contributing to the cellulose deconstruction. Furthermore, *GH10* and *GH11* xylanase genes are also known to control the hemicellulose degradation [26]. Therefore, by overexpressing these key enzymes, it may be possible to modify the cell wall structure of energy crops and thus to drive improvements in the technologies for biofuel production.

Table 2. Main enzyme families involved in cell wall biosynthesis, growth, development, and degradation.

Enzyme Families	Cell Wall Process	References
CesA/cellulose-synthase-like (Csl)—GTs	Cellulose biosynthesis	[13–16]
CesA/cellulose-synthase-like (Csl)—GTs	Hemicellulose biosynthesis	[17]
GTs	Callose biosynthesis	[18]
PAL, C4H, 4CL, HCT, C3H, CSE, COMT, CCoAOMT	Lignin biosynthesis	[19]
CCR, F5H, CAD, POD, LAC	Specific lignin biosynthesis enzymes	[19]
CesA/cellulose-synthase-like (Csl)	Cell wall growth and development	[21]
CAZy family (GTs and GHs), ERU, PME	Cell wall growth and development	[21]
WAK	Cell expansion	[24]
Endogenous degradation process—GHs	Cell wall degradation	[25,26]

3. Applications of Molecular Markers and QTL Mapping in Major Energy Crops

Molecular markers are extensively used in innovative breeding programs due to their independence from environmental conditions and plant growth stages, and, likewise, QTL studies were undertaken for their use in marker-assisted selection (MAS) programs [11]. Furthermore, technological progress has allowed the scientific community to improve the knowledge on genetic mapping and QTL identification, making it possible to adopt strategies for the detection of candidate genes that are related to crop characters of interest for biomass yield and feedstock quality improvement [11]. These advancements, combined with high quality re-sequencing, can be used to further investigate bioenergy agronomic traits. In fact, re-sequencing has contributed to enrich the availability of tailored single-nucleotide polymorphism (SNP) resources, which were utilized for genomics-based studies, such as the genome-wide association study (GWAS) and the QTL-seq for mapping biomass crop traits [27].

A large number of QTL-based investigations were evaluated by using molecular markers on several energy crops, while those purely based on biomass traits are less numerous [11,27–29]. These studies focused on the QTL mapping of traits such as plant height and stem thickness, which are vital for bioethanol production, sugar content, plant maturity, and brix.

Here, and in Table 3, we summarize the molecular markers and QTL mapping research resources for relevant energy crops.

Table 3. Molecular markers and QTL mapping resources for energy crops.

Energy Crops	Resources and Research Findings	References
Populus trichocarpa × *P. deltoides*	Identification of 45 QTL associated to eight stem and biomass traits	[28]
Panicum virgatum L.	Availability of 11 genomic regions to control biomass yield and/or plant height	[29]
Oryza sativa L.	Definition of genetic traits related to biomass yield, plant weight, and stem and leaf weight	[30]
Sorghum bicolor L. *Moench*	Identification of QTL traits for brix, maturity, height, and other biomass-related QTLs-SNPs from the GBS approach	[31]
Miscantus sinensis	Development of SNP-based genetic map–Over 80 QTLs for biomass quality properties	[32]
Cannabis sativa L.	Definition of 16 QTLs associated to glucose, mannose, xylose, and lignin content—12 candidate genes involved in polysaccharide and lignin biosynthesis	[33]
Zea mays L.	Findings about biomass quality, water deficit, and yield traits	[34]

Populus is a genus of fast-growing trees, and understanding their interaction with the environment is essential in order to develop new high-yielding genotypes. For instance, an analysis of 210 genotypes from an F2 population that was derived from a cross between *Populus trichocarpa* and *P. deltoides*, originating in southern UK, central France, and northern

Italy, led to the identification of 45 QTL associated to bioenergy traits, allowing a detailed understanding of the genetic nature of biomass yield [28]. Furthermore, taking into account environment factors, such as climate change, the results provided very important insights for future breeding applications.

In switchgrass (*Panicum virgatum* L.), a perennial grass identified as a promising feedstock for bioenergy production, the study of biomass yield is a priority. Switchgrass biomass yield and plant height have been associated to 11 genomic regions [29], in which the QTL presented pleiotropic effects, making it possible to select for plant height as a trait contributing to biomass yield. The markers linked to the identified QTL became candidates to be used in MAS in order to improve switchgrass breeding, leading to faster genetic improvement of the cultivars and by offering a good alternative selection approach compared to the conventional plant breeding methods.

QTL pleiotropic effects were also investigated in rice (*Oryza sativa* L.), a crop with high amounts of cellulose (32–47%) and hemicellulose (19–27%) [30]. Specifically, by using a cultivar from a cross between two high-yielding Japanese genotypes, a QTL-based selection approach was applied to explore the genetic basis of biomass yield, the plant, and the stem and leaf weight [30]. Four QTLs were identified and mapped for plant weight, three for grain weight, and five for stem and leaf weight, with some overlapping traits. Furthermore, multiple QTLs correlated with phenotypic plant traits related to biomass yield.

Other interesting correlations among biomass traits were investigated in sorghum (*Sorghum bicolor* L. *Moench*), which, due to its abiotic stress tolerance and its diverse genetic base, is considered to be a good candidate for efficient and low-cost biofuel production. For instance, a biomass QTL validation analysis using over 200 recombinant inbred lines (RILs) that was derived from a cross between two sweet sorghum lines showed the presence of QTLs associated to plant height, total soluble solids and sucrose, fibers, fresh biomass yield, juice extraction yield, and sugars [31].

Miscanthus (*M. sinensis*) is another yielding grass species with great potential as a bioenergy feedstock. An interesting study [32] was undertaken where, for the first time, the genetic mapping of the cell wall composition and the bioconversion traits were investigated. A new SNP-based genetic map was developed using a genotyping by sequencing (GBS) approach, and over 80 QTLs for biomass quality properties were identified, 20 of which were related to several efficiency aspects of the conversion processes. Marker sequences have also been aligned to the sorghum reference genome, with the aim of comparing different energy crops. These results were considered to be reliable and applicable in MAS programs to improve miscanthus biomass quality [32].

Bast fiber traits were investigated in hemp (*Cannabis sativa* L.). A panel of over 100 phenotypically different hemp accessions was used to investigate the genetic characteristics of their cell wall and bast fiber traits [33]. This panel was genotyped, and the obtained SNP markers were used for a GWAS. Given the lack of a complete hemp genome sequence, QTL detection was performed on the known traits. Petit et al. [33] identified 16 QTLs that were associated to glucose, mannose, xylose, and lignin content, as well as 12 candidate genes that were involved in the monosaccharide, polysaccharide, and lignin biosynthesis, showing their fundamental function in hemp fiber quality.

Other recent studies have explored known issues about the negative impact of water deficit on biomass quality. In one of these works, the mapping effectiveness of a maize (*Zea mays* L.) RIL population analysis was combined with chemical methods based on near infrared spectroscopy [34]. The findings showed that cell wall degradability and β-O-4-linked H lignin subunits increased due to water deficit, while lignin and p-coumaric acid contents decreased. They also demonstrated that only half of the identified responsive QTLs co-localized with the biomass yield QTLs, suggesting the existence of specific genetic factors related to biomass quality and water deficit, that are not linked to yield traits.

4. Multi-Omics Approaches to Study Plant Cell Walls

In plant genomics, next generation sequencing (NGS) has played a very important role and has provided opportunities in the field of functional genomics due to the availability of reference genomes for several model crop and woody plant species. For instance, the genome of *Populus trichocarpa* (Torr. and Gray) was released in 2006 [35] and, after initial sequencing, the genome assembly has gone through several revisions, which are available on Phytozome [36]. It is relatively small and is considered to be a model species for trees and woody plant species [36]. Subsequently, in 2014, the genome of *Eucalyptus grandis* was sequenced and was used as the reference genome for eucalypts, providing essential insights to investigate important crop biomass traits [37]. In the same year, despite the difficulties to assemble the complex conifer genomes, research identified a promising candidate to use as a reference genome for *Pinus* species, opening potential avenues for improving biomass production in this genus [38]. The genome sequencing process of some herbaceous species, including switchgrass (*Panicum virgatum* L.), has been challenging and only recently a highly continuous genome assembly of a lowland switchgrass genotype AP13 has been developed [39], allowing the study of genes that underlie biomass productivity [40].

The progress in plant genomics, combined with advances in metabolomics, provides an effective means for elucidating the underlying molecular mechanisms that are involved in plant growth and development, as well as in cell wall biosynthesis. The advances in the omics technologies have led to the discovery of genes and biomolecules with remarkable precision, and, as a consequence, to the development of specific plant resources and databases [41].

Biomass crop genomes, omics, and genome editing research have been used to gain a deep understanding of the regulatory networks underlying cell wall pathways with the end goal of contributing to create a less recalcitrant form of biomass [41]. Many of these solutions used multi-omics approaches [42,43], which are becoming a mainstream tool to explore the biological pathways underlying complex genotype traits and to improve our knowledge about the roles of the genes that are involved in biomass component biosynthesis. In fact, they allow us to link the genotype to the phenotype, and to identify or confirm the candidate genes that are involved in complex biological pathways, contributing to enhancing our knowledge about each considered phenotype [43]. The candidate genes can be used in genome engineering approaches for several aims, for instance, to obtain a lignocellulosic biomass that is richer in cellulose [44], or less rich in lignin [45], as well as to reduce its recalcitrance [46], with the final aim to improve the biomass quality and yield, as well as to optimize the conversion process.

A large set of omics studies have focused on microbial biomass breakdown, and many candidate strains have already been detected. Such progress in omics has made it possible to achieve impressive advances in the characterization of the microbiota/microbiome involved in cell wall deconstruction, and the combination of metaproteomics and metatranscriptomics has provided a multidimensional analysis of how the microbes react to a changing environment [47]. Studies have explored *Clostridia* species' ability to degrade cellulose [48] and fungi that express genes that are involved in the decomposition of the most recalcitrant features of lignin [49]. The enzymatic mechanisms of lignocellulose degradation have been described in individual microbial species, and, consequently, the majority of industrial approaches for lignocellulose degradation use mixtures that are composed of a single bacterial/fungal species, which unfortunately are only able to hydrolyze biomass after pre-treatments [50]. Therefore, the study of microbial communities offers information on the microbial digestion of biomass [50]. The recent advances in transcriptome sequencing have allowed us to explore the behavior of these communities under specific growth conditions, and whole metagenome shotgun sequencing has been employed successfully to investigate this [51]. Several omics investigations have explored the interaction between the microbial communities and external factors, such as those related to the gut microbiome [52], soil [53], and marine ecosystems [54]. However, only a limited number of multi-omics studies have been carried out on microbial community interactions within the

context of the lignocellulose degrading processes [53]. Consequently, the complex enzymatic mechanisms of microbial communities that efficiently breakdown biomass in nature have not been well studied, despite their potential to optimize the biomass production process [50].

4.1. Energy Crop Multi-Omics Studies

Populus, being the first woody plant species to be sequenced, and being characterized by a small and easily genetically modifiable genome, has acquired importance as a woody plant model organism. Consequently, many studies have been undertaken on *Populus*, and resources have been developed to aid future research [55]. From this, a good genetic and biochemical basis for adaptive traits, such as biomass production, were gained, and this has helped to inform the development of more resilient and high-yielding germplasms. A population of ~1000 natural *P. trichocarpa* accessions has been re-sequenced in order to provide high-throughput data for SNP identification [35]. These have been extensive projects, with ~450 individuals from the *P. trichocarpa* population studied, looking at ~34,000 SNPs and GWAS performed on 40 different traits, including biomass phenotypes such as height and volume, as well as eco-physiological traits such as leaf shape and chlorophyll content [56]. This large body of resources on poplar has allowed us to focus on a crucial challenge: to understand the regulatory network controlling the cell wall biosynthesis and to identify candidate genes to validate in biomass genome editing and innovative breeding programs for bioenergy use.

Due to the availability of high-throughput genotyping and high-resolution linkage maps in several bioenergy crops, such *Populus* [57] genetic QTL approaches and their association mapping became great tools to study woody biomass traits in perennial crops. However, these methods provided little information about how the genes interact in the biological pathways to affect trait variation. Studies based on inbred mapping pedigrees, where QTL size is a limiting factor in breeding crop populations, have now addressed to omics investigations with extensive natural populations, taking into account their increased genetic variation [57]. Multiple layers of biological complexity, based on transcripts, proteins, and metabolites data, are recognized to be effective to elucidate the genetics of complex traits such as wood density and chemistry [57].

Recently, researchers have reported that regulatory mechanisms of the lignin biosynthesis pathway of many woody plant species are broadly homologous to those that are found in *Populus* [58]. As a consequence, the results of the studies about this species could be successfully applied to other perennial woody plants, facilitating the understanding of biochemical and molecular mechanisms regulating SCWs, possibly impacting biomass conversion and its valorization.

Moreover, innovative multi-omics methods based on comparative de novo approaches have been carried out in order to analyze plant genetic variation and agronomic traits, including those impacting on biomass improvement [59]. For instance, intergenomic comparisons identified over 20 million sequence variants in rice, which will further promote functional studies in this crop [59]. Other recent multi-omics approaches have discussed how genotyping, combined with high throughput phenotyping platforms, could achieve valuable genetic evidence for complex traits in crops with standardization and high reproducibility [60]. This method was used in rapeseed (*Brassica napus*; *canola*) to analyze the genetic architecture of plant growth and yield. Following this workflow, an automatic image analysis pipeline to quantify 43 dynamic traits across multiple developmental stages, with 12 time points, was developed [60].

4.2. Plant-Degrading Microorganism Multi-Omics Studies

The composition and the structure of cell walls impact both the quantity and the yield of fermentable sugars from biomass for biofuel production. Its degradation is a function of how polymers crosslink and aggregate within the walls [61]. Microorganisms such as ascomycetes and basidiomycetes are predominantly responsible for lignocellulosic degradation in nature. A large number of enzyme typologies, such as cellulases and

hemicellulases, are known to be able to enzymatically break down plant cell walls, leading to their deconstruction [62]. These microorganisms have been a key topic of research interest from the industry, because of the need for renewable fuels.

Clostridium thermocellum was grown on switchgrass to evaluate changes in metabolism and proteome during the conversion of lignocellulosic biomass into ethanol [48]. Hemicellulose-derived sugars and sugar alcohols were found to rise over time in association with an increase in the abundance of enzymes involved in C5 sugar metabolism, suggesting that *C. thermocellum* has a key role in these mechanisms, leading to lignocellulose breakdown [48]. Today, *C. thermocellum* is a noteworthy bacterium that contributes to the breakdown of lignin, and it is capable of both saccharification and fermentation, which are crucial processes to convert lignocellulosic biomass to ethanol without using an external enzyme source [63]. Another study combined metabolomic and proteomic approaches and provided insight into the cellular responses of *Clostridium acetobutylicum* to the cytotoxic inhibitors that are released during the deconstruction of lignocellulose [64]. A metabolomic analysis based on the main inhibitors (acids, furans, and phenols) characterizing lignocellulose hydrolysates and limiting the conversion efficiency has revealed that these inhibitors triggered the cellular response of *C. acetobutylicum*, and a proteomic analysis based on peptide MS further supported this theory [64]. This microorganism produces substantial amounts of butanol and constitutes a good solution for biofuel production using consolidated bioprocessing [65], and therefore is now recognized as a commercially valuable bacterium.

Regarding fungi, we have selected three multi-omics studies that investigate the metabolism of a specific fungal species, which show great potential for improving biofuel production.

Recently, a multi-omics approach, including genomics, transcriptomics, and proteomics, yielded a comprehensive understanding of the *Laetiporus sulphureus ATCC 52,600* mechanism behind the degradation of lignocellulosic material [66]. The multi-omics approach showed that the fungus has a higher efficiency to assimilate glucose than brown rot fungi and confirmed its oxidative-hydrolytic metabolism, leading to lignocellulose hydrolysis [66]. *L. sulphureus* has acquired remarkable biotechnological interest due to its cellulose-degrading ability, and its potential for polysaccharide and secondary metabolite biosynthesis needs further investigation.

In the same year, it was shown that a fungus, *Parascedosporium putredinis NO1*, isolated from a mixture of wheat straw, secretes a large set of CAZymes during its growth on lignocellulosic substrates and that its oxidase activity cleaves the major β-ether units in lignin, enhancing the degradation process [49]. The study, which was based on a combination of transcriptomics by RNA-sequencing and proteomics analysis using liquid and gas chromatography–mass spectrometry, demonstrated that *P. putredinis NO1*-based treatments can increase the digestibility of lignocellulosic biomass.

Microbial/fungal communities are more complex to investigate compared to a single microbe species, due to the interactions and the combination effects on plant cell wall degradation. Recently, a study investigated the deconstructive abilities of a microbial community including species with different functions during biomass breakdown in sorghum varieties with different lignin contents [67]. Here, the network reconstructions of gene expression allowed the identification of key deconstructive communities within the adapted sorghum group, including *Actinotalea, Filomicrobium*, and *Gemmatimonadetes* populations, while a functional analysis of gene expression confirmed that the microbiomes are linked to enzymes that degrade plant cell wall polymers. The combined use of network and functional analysis allowed us to underline the role of cellulose-active *Actinobacteria* in characterizing the performance of the examined microbiomes by providing new insights about the release of sugars and aromatics in the biomass and their subsequent conversion to biofuels. The multi-omics approaches that have been discussed above are summarized in Table 4.

Table 4. Multi-omics approaches to study plant cell wall biosynthesis and degradation.

Omics Technological Approaches	Crops Species	Subject	References
Genomics, Phenomics	*Brassica napus; canola*	Genotyping combined with high throughput phenotyping platforms to analyze crop yield	[60]
Genomics, Phenomics	*Oryza sativa*	Pan-genome analysis on cultivated and wild rice.	[59]
Transcriptomics/microarray, Genomics/SNPs, Phenomics	*Populus trichocarpa*	A large set of genetic, genomic, and phenotypic evidence was used in an integrative approach to predict wood properties	[55]
Metabolomics, Transcriptomics	*Populus*	The genetic architecture underlying the lignin biosynthesis pathway in *Populus*.	[58]
Metabolomics, Proteomics	*Panicum virgatum*	Metabolic and protein changes associated to the plant growth of *Clostridium thermocellum*	[48]
Transcriptomics, Proteomics	*Triticu*	Lignocellulose degradation by microbial communities	[50]
Metabolomics, Proteomics	-	*Clostridium acetobutylicum* and deconstruction of lignocellulose	[64]
Metagenomics, Metatranscriptomics	*Sorghum*	The identification of key deconstructive microbial communities (Actinotalea, Filomicrobium, and Gemmatimonadetes populations)	[67]
Genomics, Transcriptomics, Proteomics		*Laetiporus sulphureus ATCC 52,600* strategies in the degradation of lignocellulosic substrates	[66]
Transcriptomics, Proteomics	*Triticum*	*P. putredinis NO1*-based treatments and the increase in the digestibility of lignocellulosic biomass	[49]

4.3. Integration of Multi-Omics Data to Improve Biomass Yield and Quality

Different levels of data integration can be considered, from pair-wise correlations to the use of advanced integration models using multivariate correlations. Regardless of the method that has been used, it is complex to integrate heterogeneous omics data obtained in crop species, due to their large, poorly annotated genomes and the presence of diverse secondary metabolites in many of them. Here, we introduce some studies where data integration modeling was successfully applied in plants. Lignin biosynthesis in poplar (*Populus trichocarpa*) was successfully modeled using the ordinary differential-equations-based approach, obtaining mutant plants for 21 target genes of the monolignol pathway, on which transcriptomics and proteomics methods were applied [68].

Mathematical models can also be used to build a genome-scale model, starting with experimental evidence [69]. A multi-omics analysis of lignocellulosic carbon utilization in *R. toruloides* and a genome-scale metabolic network of this yeast demonstrated that *R. toruloides* was able to metabolize the cellulose, hemicellulose, and lignin of lignocellulosic biomass [69].

Furthermore, a network-based data integration (NBDI) method for a genetic analysis and the pathways underlying biomass and bioenergy-related traits was applied to *Eucalyptus*, showing a correlation between biologically significant sets of genes and complex wood properties [70]. Moreover, an integration and correlation of metabolomic and transcriptomic datasets allowed us to identify the processes that were impacted by K-fertilization and water limitation in *Eucalyptus*, revealing that the genes and metabolites that were correlated to wood complex traits were strongly involved in stress responses and may have affected biomass production [71].

The combination of genomics, transcriptomics, and phenomics data was also used to identify and characterize the genes involved in the lignin pathway in *Populus deltoides* by using a population of over 260 individuals [72]. The findings showed that the *R2R3-MYB* transcription factor *MYB125* was directly connected to all of the genes involved in the lignin biosynthesis pathway [72].

Recently, machine learning approaches have been used to identify the genes that are responsible for a specific metabolism that is important for plant–environment interactions [73,74], as well as precision breeding for energy traits of interest. The development of effective machine learning algorithms could become a future direction in plant omics integration data research.

Table 5 summarizes the above discussed studies.

Table 5. Integration multi-omics data-based studies to improve biomass yield and quality.

Omics Technological Approaches	Crop Species	Subject	Reference
Transcriptomics, Proteomics	*Populus trichocarpa*	Modeling of lignin biosynthesis using an ordinary differential-equation-based approach	[68]
Metabolomics, Transcriptomics, Proteomics	Fungus	The construction of a *R. toruloides* metabolic network using a genome-scale approach	[69]
Genomics/Genotyping, Transcriptomics	*Eucalyptus* hybrid population	An NBDI method for a systems-level analysis of genes and pathways underlying bioenergy-related traits was applied	[70]
Metabolomics, Transcriptomics	*Eucalyptus*	An integrated network-based approach allowed us to identify processes impacted by K-fertilization and water limitation	[71]
Genomics, Transcriptomics, Phenomics	*Populus deltoides*	Systems genetics approach to characterize genes involved in the lignin pathway	[72]
Genomics, Transcriptomics, Proteomics, Metabolomics		An integrated multi-omics platform for green systems biology and plant breeding	[74]

5. Gene Editing Approaches to Improve Biomass Quality

The design of genetically modified plants to synthetize less recalcitrant cell walls has been applied to improve biomass saccharification. Recently, genetic engineering approaches have been applied to modify the genes that are involved in the cell wall structure [12]. The modification of the cell wall composition by downregulating or knocking-out the lignin biosynthetic genes, or by acting on related transcription factor mechanisms, has been attempted with the aim of reducing lignin content [75]. However, the success rate of these approaches was limited, due to undesirable traits in plants with mutations in lignin biosynthesis, such as reduced biomass yields, low germination frequency, decreased height, and increased sensitivity to pathogens [75].

Gene overexpression is another approach that is applied to enhance a target trait. For instance, glycoside hydrolase (GH) overexpression increased the accessibility of polysaccharides [76]. Furthermore, changes in the pectin content, and/or its modification pattern, led to an increased saccharification, and in several crops the overexpression of plant pectinases led to an increased release of simple sugars [77].

However, overexpressing or mutating just a single gene to decrease the lignin content does not necessarily promote saccharification [75]. Since these processes involve several cell wall modifications, a decreased recalcitrance can arguably be obtained as a result of an enhanced and optimized modification of the entire catabolic pathway. Therefore, a proper understanding of the metabolic pathways and the genetic mechanisms through a combination of different omics analyses can be essential for gene editing success.

To this respect, the modification of lignocellulosic biomass was carried out with bioengineering technologies [78], such as gene silencing methods, for entire gene family members [79], or the latest genome editing methods based on targeted gene manipulation, such as clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) systems [80]. In metabolic engineering, this tool allowed an easier discovery and evaluation of the relevant genes and pathways and has become the first choice for the genetic improvement of many organisms, including industrially relevant ones [80].

CRISPR-based methods were applied successfully in several woody plants to effectively alter the lignocellulosic composition in order to facilitate the extractability of its components, including sugar, and to improve the pulping quality [81].

In the next section, we will focus on the gene editing approaches that are used in energy crops to achieve the following: (i) slow down the lignification process by modifying the lignin biosynthetic pathway; (ii) increase carbohydrates; and (iii) decrease cell wall recalcitrance through the modification of the cellulose and hemicellulose biosynthesis and their degradation pathways.

Gene Editing in Energy Crops

The CRISPR/Cas9 approach was tested in the woody perennial poplar by editing three 4-coumarate:CoA ligase targeted genes (*4CL1*, *4CL2*, and *4CL5*), focusing on lignin and flavonoid biosynthesis [45]. The results showed that mutations in the *4CL1* gene slow down the lignification process, and mutagenesis in the *4CL2* gene lead to an overall 20% decrease in lignin content, indicating that *4CL1* and *4CL2* can play a primary role in this biosynthesis pathway. In addition, a CRISPR-based application was carried out in poplar to decrease the lignin content by targeting the *PtoMYB156* transcription factor [82]. *MYB156* knock-out in poplar resulted in the deposition of lignin, xylan, and cellulose during SCW formation, showing how this gene may repress phenylpropanoid biosynthesis and how it negatively regulates SCW development [82]. Despite the negative effects on plant growth, this provided useful directions for future research.

Regardless of the advances in plant genomics, a crucial limitation to the genetic improvement of some bioenergy crops is still the complexity of their genomes, which slows down the use of modern breeding approaches.

O. sativa has a compact diploid genome [83] of approximately 500 Mb and several gene editing investigations have been carried out on this crop. Here, we report one of the most significant studies [84], in which the *C3H* transcription factor knockdown mutant led to an altered lignin composition that resulted in enriched p-hydroxyphenyl components, with a strong reduction in cell wall cross-linking ferulates. Such structural alterations led to an important discovery: the reduction in cell wall recalcitrance and enhanced biomass saccharification [84].

In *Panicum virgatum*, its allotetraploid genome (2n = 4x = 36) represented an impediment to generate homozygous knock-out plants. However, in one study [85], the development of genome-editing technologies made it possible to successfully apply the CRISPR/Cas9 method. This technique was used to mutate a key gene involved in the lignin biosynthesis, the *Pv4CL1* gene, which was selected as the gene target because of its preferential expression in highly lignified stem tissues. The results showed less lignin and significantly higher glucose and xylose content in the knock-out plants compared to the wild type.

Recently, pioneering efforts have been made to genetically modify *Arundo donax* L. [86], an energy crop that is able to grow under resilient conditions that is characterized by a complex genome. Since this crop is polyploid, it is very difficult to induce and select trait promising mutations. To the best our knowledge, no transgenic *A. donax* crops with improved biomass characteristics have been developed yet. However, by investigating the lignin biosynthetic pathway of *A. donax*, a high copy number of *PAL* and *C4H* genes were found giving target genes for *A. donax* biomass quality improvement [87].

Increasing cellulose biosynthesis is another important aim in biomass improvement because cellulose entirely consists of C6 sugar glucose, which is useful for saccharification. Therefore, the overexpression of cellulose synthase genes (*CESA*s) is often used to obtain transgenic plants that are enriched in cellulose [81]. However, attempts to overexpress *CESAs* in secondary cell walls of aspen and barley have resulted in decreased cellulose content and reduced plant growth [88].

Recently, the cellulose biosynthesis *CESA* gene family was manipulated to increase the cellulose production in poplar. Transgenic plants were obtained by overexpressing the *PmCesA2* gene from *Pinus massoniana* through an *Agrobacterium*-mediated transformation [89]. The transgenic poplar showed an enhanced growth performance and an improved cellulose production, but also an increase in lignin content, due to changes in the cell wall polysaccharide composition.

Other studies have focused on the overexpression of genes belonging to the sucrose synthase (*SUS*) gene family, observing a general increased plant growth and cellulose and starch content [90]. For instance, in hybrid poplar (*Populus alba* × *grandidentata*), a small increase in cellulose was found, as well as an increase in cellulose crystallinity, which contributes to increase biomass recalcitrance [44]; however, in tobacco, such findings led to ~20% thicker cell walls, 18% more cellulose, and 9–11% less cellulose crystallinity [91].

In 2020, the *COBRA*-like gene, which is important for cellulose biosynthesis, has been proposed as a possible target for creating transgenic plants that are rich in cellulose [81]. The *GhCOBL9A*, a *COBRA*-like gene from cotton (*Gossypium hirsutum*) that is overexpressed in *Arabidopsis*, led to a notable increase in the total biomass and cellulose content (59%). Furthermore, the *CESA* gene expression of the transgenic plants measured in the SCW showed a significant increase, suggesting the involvement of a *COBRA*-like gene in the *CESA* pathway of the transgenic Arabidopsis [92]. The cell walls of cotton fibers almost entirely consist of cellulose and are an interesting model for high-level cellulose production. The approach that was adopted in this study could be a great strategy to increase the cellulose content in bioenergy crops.

Furthermore, a gene that is not directly involved in cellulose biosynthesis has been demonstrated to influence its content. Particularly, the overexpression of the rice *OsMYB103L* gene, encoding the *R2R3-MYB* transcription factor and controlling leaf development, caused a rise in the expression of *CESA* genes and an increase in cellulose content [93]. Conversely, knocking down this gene led to a lower expression of *CESA* genes.

Several investigations have focused on the reduction in C5 sugars, such as xylose, which form linkages with cell wall hemicelluloses [94]. Mutants in xylan biosynthesis have been generated, however, the complexity of the genome of several bioenergy crops has hindered gene editing studies. In Chen et al. [95], the inactivation of the rice *OsIRX10* led to a decrease in xylan content in the cell walls and an improved biomass saccharification. Furthermore, the simultaneous knockdown expression of two glycosyltransferase genes (*GAUT*), *PtGAUT12.1* and *PtGAUT12.2*, in *P. deltoides* has been shown to reduce the xylan content during wood formation and reduce the recalcitrance of cell walls [46].

Among the genome editing investigations looking at hemicellulose, we focus here on two particularly promising studies with regards to the improvement of biomass yield and quality. In the first study, the silencing of *GH10* genes, which are known to control the hemicellulose degradation and are highly expressed during secondary wall deposition, led to alterations in the regulation of stress-responsive genes, releasing tensional stresses [96]. These changes could enhance primary growth and consequently result in an improved biomass yield. In the second study, endoglucanases genes from poplar (*PtGH9B* and *PtGH9C*) were expressed in *Arabidopsis*. The transgenic lines showed changes in the sugar content and differences in cell wall crystallinity compared to the wild type, suggesting that these endoglucanases impact secondary cell wall development by contributing to the cell wall crystallization process [97].

Table 6 summarizes the main results that have been discussed in Section 5, highlighting a set of potential target genes for the improvement of energy crops.

Table 6. Potential target genes for the improvement of energy crops.

Energy Crops	Target Genes	Effect on Cell Wall and Biomass	References
Populus tremula × *alba clone*	*4CL1-4-coumarate:CoA ligase* gene Biallelic mutation	Slow down the lignification process	[45]
Populus tremula × *alba clone*	*4CL2-4-coumarate:CoA ligase* gene Biallelic mutation	~20% decrease in lignin content	[45]
Populus tomentosa	*PtoMYB156* gene Genes overexpression Gene mutation	Influence on SCW thicknesses of xylem fibers and in cellulose lignin and xylose content	[82]
Populus deltoides × *Populus euramericana*	*PmCesA2* Gene overexpression	Enhanced growth performance with improvement of biomass production	[89]
Populus trichocarpa	*PtGAUT12.1* and *PtGAUT12.2*. Knockdown expression of both genes simultaneously	Reduction in xylan content and recalcitrance	[46]
Populus tremula × *tremuloides*	*PtxtXyn10A* Gene Downregulation	Changes in stress-responsive genes, increase in primary growth, and consequently increase in biomass yield	[96]
Panicum virgatum	*Pv4CL1-4-coumarate:coenzyme A* ligase 1 gene knock-out plants	Decrease in lignin content in knock-out plants and improvement in sugar release	[85]
Populus alba × *grandidentata*	Cotton (*Gossypium hirsutum*) SuSy Gene overexpression	Increase in cellulose content but also in cellulose crystallinity	[44]
Nicotiana tabacum	Hybrid poplar (*Populus simonii* × *Populus nigra*) gene *PsnSuSy2* Gene overexpression	~20% thicker cell walls, 18% more cellulose, and 9–11% less cellulose crystallinity	[91]
Arabidopsis	*COBRA-like* gene from cotton (*Gossypium hirsutum*) Gene overexpression	Notable increase in total biomass and cellulose content (59%)	[92]
Arabidopsis	*PtGH9B5* and *PtGH9C2* expression in Arabidopsis via *AtCesA8* promoter	Decrease in plant height and rosette diameter, which suggest that this gene can affect cell expansion	[97]
Oryza sativa L.	*p-Coumaroyl ester 3-hydroxylase (C3'H* gene Gene Downregulation	Alteration of cell wall structures and improvement of biomass saccharification	[84]
Oryza sativa L.	*OsMYB103L* gene Gene Downregulation	A lower expression of *CESA* genes, with cellulose content reduced by 13%	[93]
Oryza sativa L.	*OsIRX10* (GT47) Gene Inactivation	Reduced ratio of xylose and arabinose, influence on plant growth	[95]

6. Conclusions

NGS advances have led to an increase in multi-omics studies, using data coming from different layers of biological complexity, such as metabolomics, genomics, transcriptomics, and phenomics. Enough research evidence is available regarding the detection of candidate genes, which have been used, and can be still used, in biomass genome engineering approaches and/or to enhance the biomass traits of bioenergy crops.

Progress in the omics field has also made it possible to achieve notable results in the characterization of the microbiota/microbiome involved in cell wall deconstruction, and the combination of different omics approaches has been recognized to provide a multidimensional analysis of how the microbes react into a given changing environment.

However, while reliable progress has been registered when a single microbial species was analyzed, more complex enzymatic mechanisms of microbial communities that efficiently breakdown biomass in nature have not yet been fully clarified, despite their potential to optimize the biomass production process.

Genome editing has been applied in woody plants to effectively alter the lignocellulosic composition and to facilitate the extractability of its components, including sugar, and also to improve the pulping quality, leading to the detection of a significant set of target genes. These genes can be used to obtain plants with specific characteristics and bioenergy traits that are associated to the improvement of the effectiveness of biomass conversion processes and their valorization.

However, despite promising results, an effective engineering genome editing strategy for major bioenergy crops has not been fully established, and there are contradictory results among some studies, which is possible due to differences in the physiology of different crops species. Furthermore, to best of our knowledge, large-scale gene mutant resources for energy crops are not available yet.

Finally, it is important to reiterate that cell wall recalcitrance can be managed through enhanced and optimized modifications in catabolic pathways. Since these processes involve several cell wall modifications, a deep understanding of the underlying genetic and metabolic pathways is crucial, and combined omics approaches can successfully address this issue. Therefore, innovative applications based on the use of omics and gene editing methods are a promising direction to take in order to generate tangible bioproducts and could be proposed as a novel strategy for energy crop improvement in breeding programs.

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