Chapter 5

Genetic Strategies to Enhance Plant Biomass Yield and Quality-Related Traits for Bio-Renewable Fuel and Chemical Productions

Massimiliano Lauria, Francesco Molinari and Mario Motto

Additional information is available at the end of the chapter

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Abstract

Owing to the increasing concerns on the environment, climate change, and limited natural resources, there are currently considerable efforts applied to produce chemicals and materials from renewable biomass. While initial emphasis has been placed on biofuel production from food plant sugars, the competition between crop usage for food and non-food applications has promoted research efforts to genetically improve yield and quality-related traits for biorefining applications. This chapter summarizes the potential of genetic and biotechnological strategies for improving plant biomass yields and quality-related traits and for breeding varieties more suitable to meet biorefining applications. Attempts were also made to provide a description on the genetic and molecular mechanisms affecting starch, cell wall composition and architecture, and oils synthesis and deposition, including genetic strategies to modify these traits. Similarly, the chapter covers the genetic strategies to improve yields by emphasizing the efforts done to identifying genetic variation and gene(s) governing critical morphological, structural, and physiological traits that in turn influence biomass yields. Finally, in the chapter it is suggested that knowledge of plant biosynthetic pathways will eventually provide valuable opportunities for metabolic engineering, as well as access to chemical transformations unique to plants for breeding varieties with built-in new traits.

Keywords: Starch biosynthesis, cell wall and compositions, gene regulation, signal transduction network, genome editing, yield genes, sink strength, transgenic plants, metabolic engineering

1. Introduction

Current society and economy are largely dependent on petroleum as a source of many industrial products ranging from fuels to commodity and specialty chemicals. However, petroleum feed-stocks are limited and nonrenewable, and their broad use is also deeply contributing to unwanted increases in atmospheric CO_2 concentrations [1]. Therefore, there is at present increasing demand to develop and implement strategies for production of chemical commodities or platform molecules (see Glossary) from biomass instead of using petroleum. The drive towards bio-based products (such as fuels, chemicals, and plastics), which seeks to replace the conventional petrochemical processes with new technologies, must be economically competitive, if not advantageous [2].

Advances in genetics, biotechnology, process chemistry, and engineering are leading to a new manufacturing concept to convert complex biomass into value-added products. In this context, emphasis has been placed upon the genetic improvement of plant biomass as a sustainable source of organic carbon (C) for the large-scale production of chemicals and materials. Accordingly, in this chapter we focus our attention on the potential of genetic and biotechnological strategies for improving plant biomass yields and quality-related traits to develop dedicated and highly specialized plant varieties that meet targeted applications and end-uses, maximizing the value throughout the whole bio-based value chain. Future perspectives in this field of research are also described.

2. Biomass feed-stocks for biorefinery applications

Notably, biomass—organic matter that has stored energy through the process of photosynthesis—accounts for over 10% of global primary energy supply and is the world's fourth largest source of energy [3]. Thus, biomass in a variety of forms (solid stock, herbaceous matter, seeds, algae, biowaste, and crop residues) represents an abundant C-neutral renewable resource for the production of bioenergy and biomaterials [4].

While initial emphasis on biomass for biorefinery applications has been placed on biofuel production from fermentable feed-stocks, such as starch and sugar, resulting in an increasing demand for agricultural crops, such as maize and sugarcane, the drive to reduce the competition between crop usage for food and non-food applications has promoted research efforts to access the less digestible saccharides in cell walls (lignocellulosics) [5]. This 'biorefining technology' of using cellulosic biomass as the feed-stock has not yet been fully commercialized because of high production cost. In addition, the bulky biomass harvested seasonally in rural areas poses a challenge to feed-stock logistics and storage. It is worth noting that the biorefinery technology has the same goals as today's petroleum refineries, namely the conversion of a raw material source (in this case biomass or bio-derived feed-stocks) into bio-based products, most commonly via microbial conversion of fermentable sugars derived from cellulose and, ideally, hemicelluloses [reviewed in 6]. Figure 1 outlines a scheme of the biorafinery concept.

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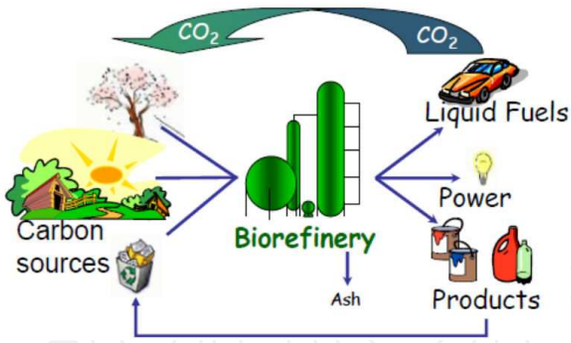

Figure 1. Illustration of the biorefinery concept.

3. Genetic strategies to modify plant biomass properties

The potential for improvement of plant biomass production has not yet been extensively explored because traditional breeding of crop plants (e.g., maize, rice, and soybean) has been focused on selection of the high grain-yield traits. Thus, to convert biomass effectively into fuels, fine chemicals, and commodity materials, a range of approaches using genetic strategies have been explored. This can be addressed from at least two points of view: modifying biomass properties to reduce processing costs or increasing biomass yield and reducing agricultural inputs. An example of these possibilities is given in Figure 2.

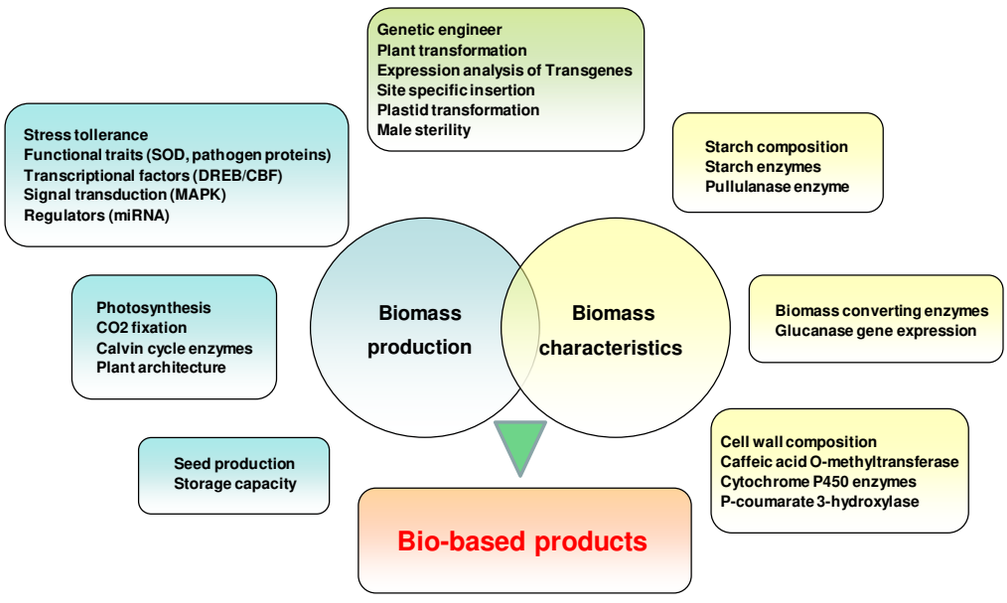

Figure 2. Possible approaches to enhance productivity of a crop biomass. Diagram indicating the two main routes for enhancing plant biomass yield and quality-related traits via genetic strategies. The first objectives to increase the biomass yield per land area (*i.e.* the biomass yield and its stability). The second objectives to modify biomass characteristics and composition to generate conversion process-friendly products for fuels and biochemicals.

3.1. Genetic strategies to modify biomass quality-related traits

The quality-related traits herein considered refer to sugar- and starch-based and lignocellulosic feed-stocks. In addition, we will also consider in this section plant oils. Bio-based polymers can be generated, besides from polysaccharides and lignin of biomass crops, also from lipids, oils, and fatty acids (FAs) synthesized in oil crops (e.g., oil palm, canola, soybean).

3.1.1. Sugar- and starch-based feedstocks

The prevalence of metabolic fluxes inside plant cells is focalized on the production and usage of sugars, the primary products of the photosynthetic process, and their conversion into storage and structural carbohydrates, namely starch and cellulose.

Starch is the dominant constituent of many harvestable organs (e.g., tubers or grain) and is the second-largest form of biomass produced by vascular plants. It is a versatile and useful biopolymer not only because it is a cheap, natural material, but because of the ease with which its physicochemical properties can be altered through chemical or enzyme modifications and/or physical treatments. Starch can be efficiently hydrolyzed by α-amylases that break starch mainly into oligosugars and glucoamylases that yield glucose monomers in biotechnological production processes [7].

3.1.1.1. Biosynthesis and genetics of starch

In recent years, our understanding on the nature and starch accumulation has largely increased, resulting in a vast body of published literature. The reader is referred to several reviews on these topics for detailed information [e.g., 8, 9]. The main findings emerging from these studies indicate that in chloroplasts, starch is accumulated for only short periods of time and thus is named 'transitory starch'; whereas in amyloplasts, starch is accumulated for long-term storage and therefore termed 'reserve starch'. In storage tissues starch is deposited as insoluble, semi-crystalline granules but also occurs to a lesser extent in most vegetative tissues of plants. It is composed of two distinct fractions: amylopectin (highly branched, 75–80% of starch dried mass) and amylose (mostly linear, 20–25% of dried mass). Both are made of α-1,4 glucosidic bond glucose residues branched via α-1,6 glucosidic linkages. The clustered nature of the α-1,6 branch points allows glucan side-chains to form double-helical structures, compacting large amounts of glucose. Consequently, extremely large structures can be synthesized and packaged in an insoluble state.

Advances in genetics and biochemistry have led to significant discoveries in how starch is synthesized in plants [10]. Three enzymes in this pathway, localized in the plastids, are playing a cardinal role in the synthesis of amylose and amylopectin: ADP-glucose pyrophosphorylase (AGPase, involved in the initiation of starch biosynthesis), starch synthase (SS, involved in elongation and granule formation), and branching enzyme (BE, involved in branching and granule formation). Further studies across plant species have indicated that these enzymes carrying out starch synthesis are encoded by well-conserved families of genes. Moreover, a number of mutations that cause defects in various steps in the pathway of starch biosynthesis were described and used to clone genes involved in this biosynthesis. Table 1 gives a list of

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the enzymes and genetic loci of cloned genes involved in the pathway of starch synthesis in maize endosperm. Furthermore, these mutants have provided information to achieve modified natural starches by reshaping endogenous processes by using, for instance, antisense RNA technology, ectopic expression or mutant enzymes, or by introducing or modifying enzymes or molecules that are implicated indirectly in starch synthesis [12].

Enzyyme	Class	Mutation[a]	Mayor biochemical changes[b]	Structural changes in mutant
Sucrose synthase	SuSy-SH1	*sh1*	↑ Sugars, ↓ Starch	None/minimal
AGPP	Cytosol. SS	*bt2*	↑ Sugars, ↓ Starch	None/minimal
AGPP	Cytosol. LS	*sh2*	↑ Sugars, ↓ Starch	None/minimal
AGT	AGT	*bt1*	↑ Sugars, ↓ Starch	None/minimal
Starch synthase	GBSSI	*wx*	↑ 100% Amylopectin	Low-amylose
Starch synthase	SSI	-	-	-
Starch synthase	SSII	*su2*	↑ Sugars, ↓ Starch	Lacks intermediate chains in amylopectin
Starch synthase	SSIII	*du1*	↑ Apparent amylase	Lacks longer chains in amylopectin
Starch synthase	SSIV	-	-	-
Branching enzyme	BEI	*sbe1*	None/minimal	None/minimal
Branching enzyme	BEIIb	*ae*	↑ Apparent amylose ↑ Loosely branched polysaccharide 60%, 70%, 45%	High-amylose, long chain amylopectin
Debranching enzyme	ISAI	*su1*	↑ Sugars, ↓ Starch	Compound granules, phytoglycogen
Debranching enzyme	ISAII	-	-	-
Debranching enzyme	ISAIII	-	-	-
Debranching enzyme	ZPUI	*pu1*	None/minimal	None/minimal

a) ae = Amylose extender; bt1 = brittle1; bt2 = brittle2: du1 = dull1; sbe1= starch branching enzyme1; sh1= shrunken1; sh2= shrunken2; su1= sugary1; su2= sugary; wx = waxy. pu1= pullulanase1.

b) Changes relative to normal. ↑↓= increase or decrease, respectively. Sugars = the alcohol-soluble sugars.

Table 1. Summary of mutant effects in specific maize endosperm mutants where an associated enzyme lesion has been reported. [Modified from Motto et al., 2011, [11].

Notably, starch biosynthesis is a remarkable regulated process, both at transcriptional and post-transcriptional level; it is also interconnected with an ample variety of cellular processes

and metabolic pathways [10]. Its regulation involves a complex and an as yet not well clear assemblage of factors that are adjusted to the physiological status of the cell. For example, marked regulatory properties were found for enzymes involved in starch biosynthesis, especially for AGPases, which is subject to multilevel regulation. AGPases are heterotetramers that contains two large (51 kD) and two slightly smaller (50 kD) subunits that are both required for optimal enzyme activity but have nonequivalent roles in enzyme function; the large subunit plays more of a regulatory role, while the small subunit has both catalytic and regulatory properties.

Evidence from comprehensive expression profiling in various plant species has revealed a pathway-wide regulation of the expression of genes affecting sucrose-starch interactions [13]. Furthermore, the coordinated regulation of gene expression in source and sink tissues appears, to a large extent, orchestrated by the sugar status of the cell. Although the sensing and signaling mechanisms mediating these processes are largely unknown, studies have suggested the presence of members of the WRKY(term derived from the most prominent feature of these proteins the WRKY domain), or WRKY domain) and AP2/EREBP (APETALA 2/ethylene response element binding protein) families of transcription factors (TFs) [14,15] and of an ethylene receptor participating in source-sink communication and sucrose-mediated regulation of starch synthesis [16]. Additional research has shown that starch biosynthesis may also be regulated by post-translational protein modification, including allosteric regulation by metabolites, redox regulation, protein–protein interactions and reversible protein phosphorylation [17]. It is suggested that some of the newly discovered aspects of fine control of the starch biosynthetic pathway may apply to many other proteins that are directly and indirectly involved in polymer synthesis and degradation. Thus, to achieve a significant progress in the rate of starch synthesis, it would be important to increase the expression of a set of enzymes and transporters in the starch pathway.

3.1.1.2. Modification of starch-specific properties

Altering the quality of the starch by plant breeding and molecular biology has already been achieved via the commercial exploitation of some starch mutants that include types that cook to form clear colloidal solutions rather than opaque gels (e.g., waxy maize or wheat) or others that are useful industrially (e.g., amylose extender maize), and finally others that accumulate less starch and more sugar (e.g., sweet maize, sweet potato). However, the industrial applications of starch to formulate commercial products are yet limited due to poor reactivity of glucose, which is the elementary unit of starch. The addition, during starch biosynthesis, of glucose residues possessing reactive side-chains or charged groups would expand the number of commercially usable chemical alterations and, consequently, enlarge the future uses of starch industrial applications [12]. Thereby, the need for starch possessing specific properties by diverse industries is fueling starch biotechnology research [18]. Figure 3 gives examples of possible genetic and biochemical modifications directed for improving starch characteristics more adapted to industrial end uses.

Another strategy to improve the efficiency of starch as a feed-stock is to reduce the energy requirements for the starch in the biorefining conversion process of plant biomass to chemicals

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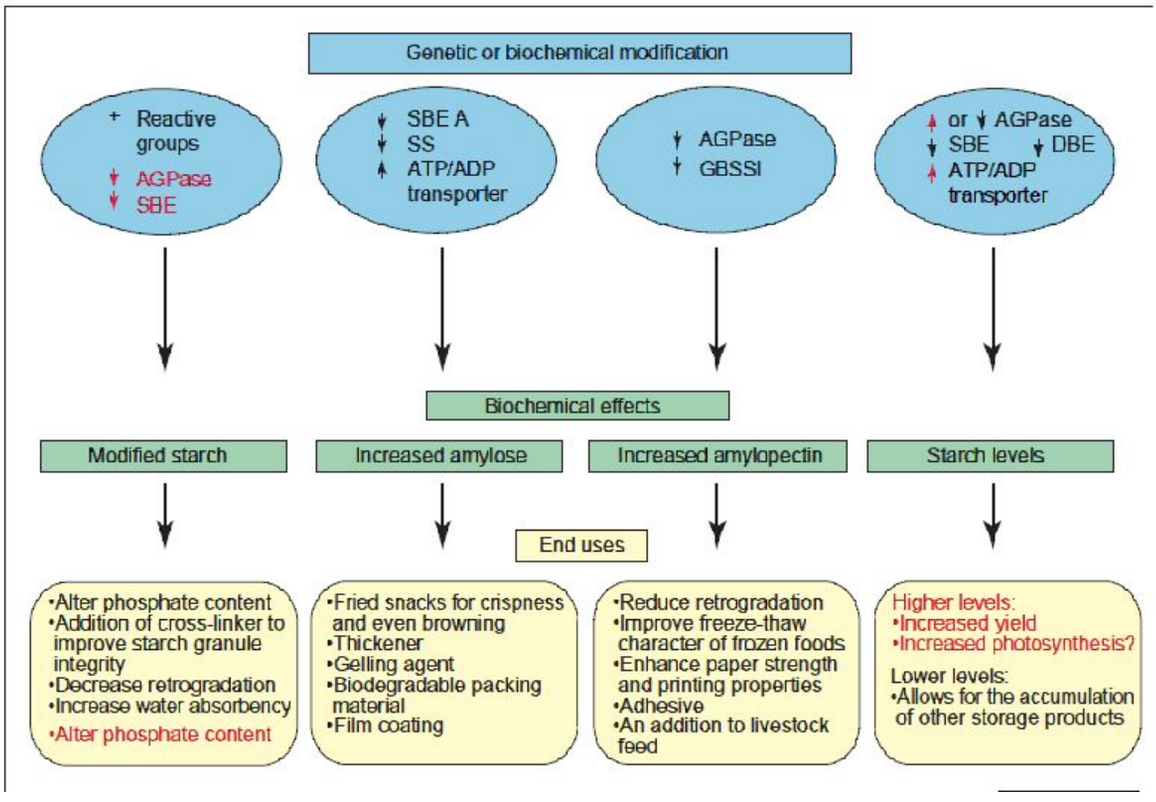

↑: an enhancement in the level of an enzyme;
↓: a decrease in the level of an enzyme;
+: addition of reactive groups.
DBE: debranching enzyme; GBSSI: granular-bound starch synthase I; SBE: starch branching enzyme; SBE A: class A SBE; SS: starch synthase.

Figure 3. Alteration of starch and their enduses. The makeup, modification and levels of starch can be modified through genetic and biochemical strategies. The resulting variations may modify the characteristics and applications of starch (Reproduced with permission from Slattery et al., 2000, [12]).

including *in planta* production of enzymes useful for starch degradation. For instance, gelatinization is the first passage for bioethanol production from starch. It is reliable that a modified starch possessing a lower gelatinization temperature might need a minor supply of energy in the biorefining conversion process. Rice research has highlighted that the expression of a recombinant amylo-pullulanase-formed starch that when heated to 85°C was perfectly transformed into soluble sugars [19].

In addition to altering starch quality, it is also possible to modify starch quantity via biotechnological approaches by increasing starch content, and thus yield, in storage organs of crops [10]. These strategies include enhancement of AGPase activity, extending the supply of starch precursors to the amyloplast, increasing the supply of sucrose to heterotrophic cells, expanding sucrose breakdown within the heterotrophic cell, enhancing the expression of starch synthase class IV, blocking starch breakdown, altering the expression of global regulators, enhancing trehalose-6-phosphate content, blocking the activity of ADPG breakdown enzymes, and increasing starch content in heterotrophic organs.

Altering contents of molecules or enzymes that are not directly affecting starch biosynthetic processes may also be a useful strategy to change positively starch quality and quantity. Production of starches possessing novel properties might permit to maintain natural starch properties that could be damaged by post-harvest operations. Surely, the generation of these starches might abolish the necessity for post-harvest changes [9].

Additional investigations are required to unveil how the different levels of regulation (e.g., transcriptional, allosteric, and post-translational) interact to control the subtle structure of starch and starch granules. Only when this level of knowledge will be achieved, the complete capacity for the comprehensive arrangement of starch molecules with specific functionality will be practicable.

3.1.2. Lignocellulose feed-stocks

Lignocellulosic biomass, derived from crops and agricultural residues, is a promising renewable source for the production of fuels and bio-based materials. It is estimated that there is an annual worldwide production of 10–50 billion tons of dry lignocellulose, accounting for about half of the global biomass yield [20]. Thus global availability and unsuitability for human nutrition have promoted lignocellulosic feed-stocks into the focus for biorefinery applications, as a fundamental source of fermentable carbohydrates for biofuel productions and for a broad array of chemicals and biodegradable compounds. Nevertheless, the generation of fermentable sugars from lignocellulose is one of the major constraints for the industrialization of lignocellulose biorefining. This is attributable to the compact and rigid structure of lignocellulose commonly referred as biomass recalcitrance [21], a distinguishing feature closely related to the chemical and physical characteristics of the cell walls that is crucial for plant survival [22].

Biomass crops, either woody species (e.g., pine, poplar, eucalyptus) or grasses (e.g., sugarcane, sorghum, miscanthus, maize stover) consist primarily of cell walls. These are formed by intricate assemblages of celluloses, hemicelluloses, pectins, lignins, and proteoglycans [23]. A diagrammatic illustration of the framework of lignocellulose structure forming cell walls can be seen in Figure 4.

3.1.2.1. Cell wall composition and architecture

The main component of cell walls is cellulose, which makes up 15–30% of the dry biomass of primary cell walls and up to 40% of the secondary cell walls. Cellulose is a β(1- 4)-linked chain of glucose molecules, with a degree of polymerization varying in length between 8000–15,000 residues [24]. Its building blocks, qualified as elementary fibrils, which are supposed to accommodate approximately 36 β-D-glucan chains, are covered with other non-cellulosic polysaccharides to form microfibrils; these are subsequently cross-linked with hemicellulose/pectin matrixes to generate macrofibrils that confer structural stability at the cell wall. Hemicelluloses (20–30%), the second most abundant constituent of lignocellulosic biomass, are heterogeneous polymers derived from varying combinations of both hexoses (D-glucose, D-mannose, D-galactose) and pentoses (D-xylose and L-arabinose), including heteromannans, xyloglucan, heterxylans, and mixed-linkage glucan [25]. In contrast to cellulose, the polymer

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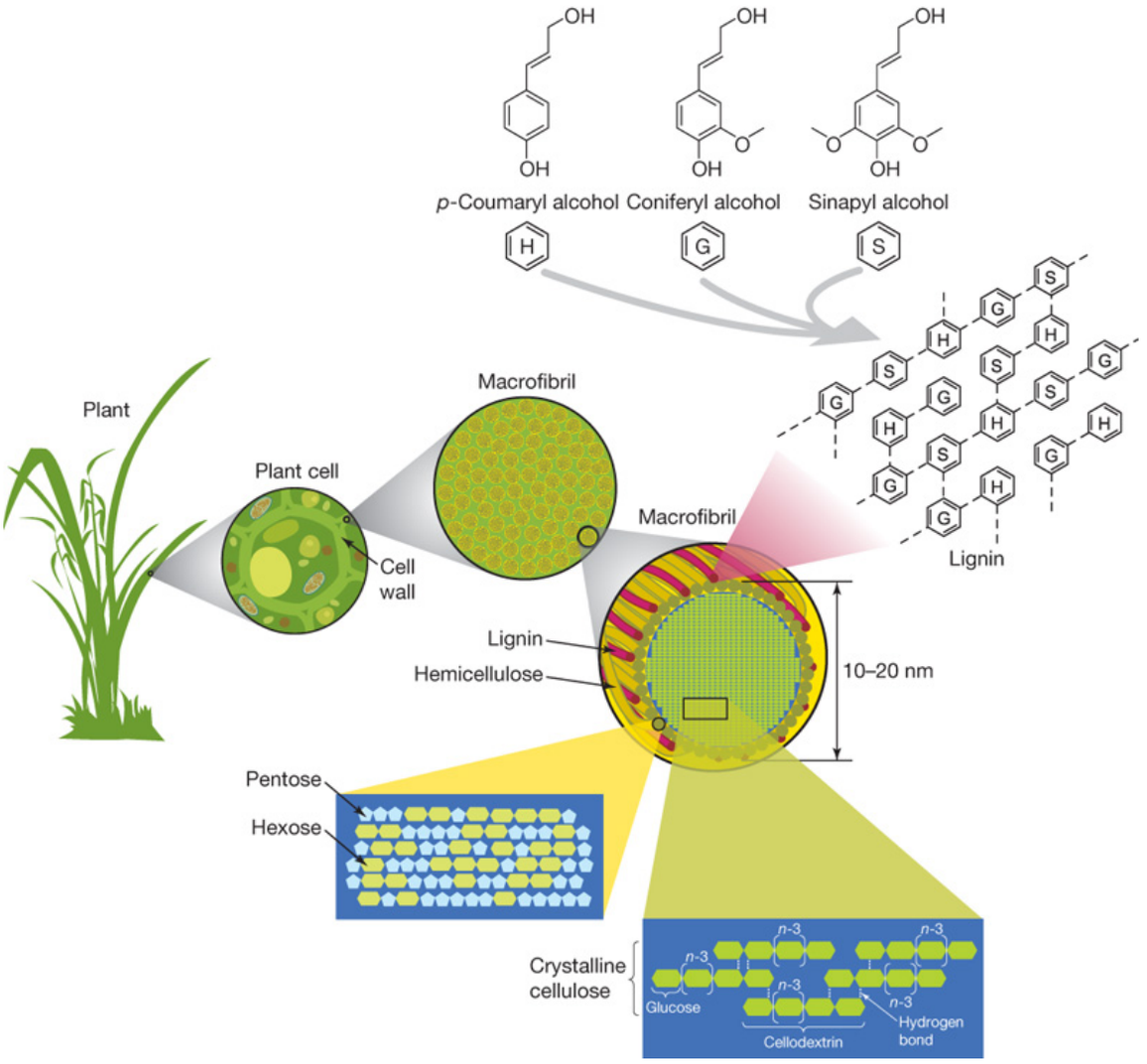

Figure 4. Structure of lignocellulose. Cellulose, hemicellulose, and lignin form structures called microfibrils, which are organized into macrofibrils that mediate structural stability in the plant cell wall. (Reproduced with permission from Rubin 2008, [23].

chains are branched and achieve comparatively short molecular lengths (500–3000 residues). The utilization of hemicellulose, in general, and its main component xylose, in particular, are at the center of research efforts in metabolic engineering to optimize lignocellulosic feed-stocks for biorefining technologies. Lignin (15–25%) is the third most-abundant biopolymer in cell walls and the largest available resource of natural aromatic polymers [26]. It is a heteropolymer mainly composed of three major phenolic monomers, namely p-coumaryl alcohol (H), coniferyl alcohol (G), and sinapyl alcohol (S). Combinations of these structural units or monolignols are incorporated into lignins with species, tissue, and developmental specificity. Lignin performs an important role in strengthening cell walls by cross-linking polysaccharides, thus providing support to structural elements in the overall plant body and conferring an exceptionally resistant to biological attacks, properties that interfere, however, with enzymatic conversion of polysaccharide components.

3.1.2.2. Biogenesis and genes involved in cell wall assembly

Genetic progress to improve cell wall composition and structure is a crucial objective for two motives: i) cell walls constrict cell size and shape and thus have a remarkable role in plant growth, affecting biomass production and ii) cell walls are recalcitrant to degradation by microorganisms to liberate sugars for fermentation, consequently affecting biomass quality [27].

According to Carpita and McCann [28], plants devote approximately 10% of their genome (i.e., ≈2,500 genes) to construction and dynamic rearrangement of their cell walls during growth. Specifically, the previous authors have grouped ≈1200 cell wall-related genes that are implicated in the synthesis, assembly, and disassembly of the plant cell walls into six categories/stages of cell wall biogenesis consisting of substrate generation, polysaccharide synthesis, membrane trafficking, assembling and turnover, secondary cell wall formation, and signaling. What emerges from this study is that the differences among angiosperms in cell wall compositions are reflected in the structure of these gene families.

In higher plants, cellulose is synthesized by large multimeric plasma membrane-associated cellulose synthase rosettes, termed CESA [29]. The subunits are encoded by the *CESA* genes represented by multiple, usually 10 or more, members, the majority of which appear to be involved in primary wall formation as judged from mutational genetic studies and gene expression profiling. In addition to *CESA* genes, chemical and genetic screens have also identified various genes that indirectly contribute to cellulose biosynthesis, such as *COBRA* (encoding a protein anchored to the membrane through glycophosphatidylinositol, GPI), *KOBITO (*encoding a membrane associated protein of unknown function), and *KORRIGAN* (encoding a membrane-anchored β-glucanase) [29]. A member of the *COBRA* gene family, *CobL4* from Arabidopsis, and its orthologs *Brittle culm-1* (*Bc1*) from rice, and *Brittle stalk-2 (Bk2*) from maize, have been shown to specifically affect cellulose formation in secondary cell walls.

The complexity of events contributing to the activation of *CESA* at the plasma membrane and its motility suggest that the list of players in this biosynthesis is far from complete and might include accessory proteins and cell wall-sensing mechanisms that appear to affect cell wall biogenesis [29]. Furthermore, gene expression studies have revealed that CESA proteins are expressed spatially and temporally throughout plant development, indicating that specific transcription factors belonging to NAC (no apical meristem), MYB (myeloblastosis), WRKY, and leucine zipper families, play a role in cell wall biogenesis. Therefore, a better understanding of the regulation, activation, and assembly of the CESA complex as well as discovery and characterization of CESA accessory proteins and plant-specific TFs will further clarify targets for genetic manipulation.

The biosynthesis of hemicelluloses requires the coordinated expression of several glycan synthases and glycosyltransferases (GTs) for polymer backbone and side-chain formation, respectively [25]. In this context, it was found that several *CELLULOSE SYNTHASE-LIKE F* (*CSLF*) genes encoding Golgi-localized GLs, are involved in hemicellulose biosynthesis. More specifically, evidence indicates that 25 *xylem-specific GT* genes from 7 GT families support this

biosynthesis [30]. Furthermore, it was clarified that several *GT* gene families (i.e., *GT43*, *GT47*, and *GT61*) cooperate in xylan biosynthesis. Additionally, the identification of Arabidopsis *IRX* mutants has implicated that *GT8*, *GT43*, and *GT47* families as potential *glucuronoxylan* (*GX*) biosynthetic genes. Further research has documented that the biosynthesis of GAXs would require at least three GTs: xylosyl-transferase (XylT), arabinosyl-transferase (AraT), and glucuronosyl-transferase (GlcAT) [31]. Similarly, it was reported that in xylan synthesis glycosyl hydrolases may also play a role, as well as a number of transcription factors [32]. These include, in particular, master switches such as Secondary Cell Wall Associated NAC Domain 1 (SND1) and other TFs directly downstream, such as multiple MYB factors and a KNOTTED1-like homeodomain protein. Furthermore, in maize, *UDP-glucose 6-dehydrogenase* genes were found to encode central enzymes of hemicellulose biosynthesis and appear essential for cell wall formation in young organs. Although, the information reported above has pointed out the importance of the hemicelluloses in plant growth and development, future research is required to combine these single components and their assemble. This will permit to improve our understanding on the biosynthesis of this important class of plant cell wall components. It is expected that altering the expression of those genes will very likely change the amounts and properties of hemicellulose, which in turn, may lead to decrease recalcitrance.

Lignin is one of the most relevant objectives for genetic improvement of cellulosic biomass adapted for biofuel production: modifications in its chemical composition and quantity directly influence the pretreatment costs presently used in biofuel production starting from cellulosic biomass as feed-stock [33]. Biosynthesis of lignin involves two major processes: the monolignol pathway (via the phenylpropanoid pathway) in the cytosol and polymerization of the monomers into the cell wall [34]. The enzymes needed for monolignol biosynthesis are well described and comprise ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H), 4-(hydroxy)cinnamoyl CoA ligase (4CL), hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase (HCT), p-coumaroylshikimate 3′-hydroxylase (C3′H), caffeoyl CoA O-methyltransferase (CCoAOMT), (hydroxy)cinnamoyl CoA reductase (CCR), ferulic acid 5-hydroxylase (F5H), caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT), and (hydroxy) cinnamyl alcohol dehydrogenase (CAD) [35]. Similarly, the genes involved in the synthesis from phenylalanine to hydroxycinnamates and monolignol substrates of lignin biosynthesis are well established [30]. Furthermore, it was found that many of the genes encoding key lignin biosynthetic enzymes belong to multigene families [36]. Thus, specific isoforms may be expressed in different cell types at different developmental stages or in response to changing environmental conditions, complicating attempts to alter lignin accumulation.

3.1.2.3. Genetic strategies to improve lignocellulosic components

In recent years, genetic modification of the lignin biosynthesis pathway has received great attention because of the use of model plants to dissect the biosynthetic pathway and because its content in biomass is inversely correlated with its forage digestibility and quality value in the pulping industry [33]. Moreover, these findings indicate that lignin cannot be simply removed from growing plants without causing negatives developmental effects. In several plant species (e.g., maize, switchgrass, poplar, and pine), efforts using natural mutants or

silencing (RNAi) strategies directed at the down-regulation of a number of genes encoding lignin biosynthesis enzymes were not successful. This is likely due to the fact that those interventions drastically reduced lignin content in a non-selective way. Nevertheless, there are cases in which mild genetic manipulations have been used to moderately reduce lignin content or modify its composition in biomass, modestly improving saccharification efficiency, forage digestibility, and pulping yield [37].

The recent development of targeted genome-editing technologies, such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered, regularly interspaced, short palindromic repeats (CRISPR) or CRISPR-associated (CAS) systems, offers exciting potential to resolve the issues of highly specific genome modifications with great efficiency and specificity [38,39]. These technologies make use of sequence-specific designer nucleases that cleave targeted loci, enabling creation of small insertions and deletions, insertion of novel DNA, or even replacement of individual alleles. A simplified model summarizing the emerging techniques for plant engineering of lignin proposed by Eudes and coworkers [33] is reported in Figure 5. According to the previous authors, this strategy will eventually offer the opportunity to design crops with optimized lignin composition and distribution while retaining all other traits related to the phenylpropanoid pathway.

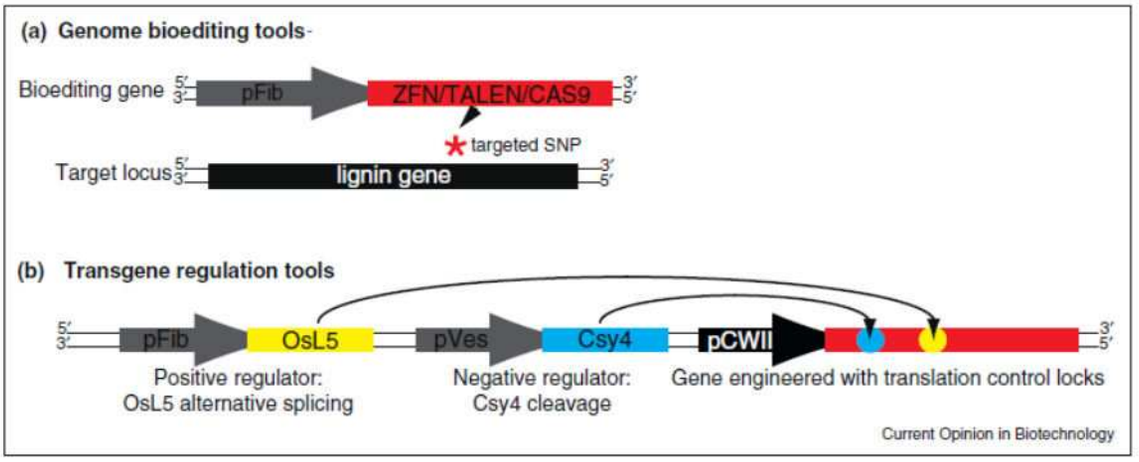

Figure 5. Cases of novel strategies for multifaceted genetic engineering of plants. (a) Genome bioediting techniques. Black box, endogenous lignin locus (target of editing); grey arrow, fiber specific promoter used to drive the expression of the bioediting gene; red box, bioediting gene: ZFNs, TALENs or CRISPR/CAS9; red star, SNP generated when the genome bioediting gene is expressed. (b) Transgene regulation techniques. Grey arrow, fiber (pFib) or vessel (pVes) specific promoter; yellow box, gene encoding the OsL5 protein with the alternative splicing cassette shown in the same color inserted in transgenes (yellow circle); blue box, gene encoding the Cys4 protein with its cognition sequence shown in the same color inserted in transgenes (blue circle); black arrow, secondary cell wall promoter (pCWII); red box, engineered gene: gene used to manipulate lignin composition which has been engineered with transgene regulation tool (yellow circle, OsL5 alternative splicing cassette; blue circle, Cys4 cognition sequence) (Reproduced with permission from Eudes et al., 2014, [23]).

Besides traditional lignin reduction methods that directly target genes from the lignin biosynthetic pathway, novel dominant approaches are currently in development. This new trend for lignin engineering focuses on the redirection of C flux to the production of related

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phenolic compounds and on the replacement of monolignols with novel lignin monomers to improve biophysical and chemical properties of lignins such as recalcitrance, or industrial uses [33]. Alternatively, although lignocellulosic feedstocks might be employed for conversion to biomaterials, two principal drawbacks in the producing systems are the costs of transport and processing of biomass. A solution to this problem is to produce directly in the plant cells the microbial cellulase enzymes. This will promote directly *in planta* the conversion of fermentable sugars during the biomass transportation to bioraffineries [40]. In maize, the expression of the catalytic domain of the thermostable 1,4-b-endoglucanase of *Acidothermus cellulolyticu*s [41] corroborates the idea that plant may be used as a biofactory for cellulose-degrading enzymes. Alternatively, although lignocellulosic feed-stocks might be used for conversion to biomaterials, two major limitations for this process are the costs of transport and biomass processing. A solution is to produce microbial cellulase enzymes in the plant cells to facilitate the conversion of fermentable sugars *in planta* during the biomass to biorefinery conversion process [40]. Expression of the catalytic domain of the thermostable 1,4-b-endoglucanase of *Acidothermus cellulolyticu*s in maize [41] supports the opinion that maize can be used as a biofactory for cellulose-degrading enzymes.

3.1.3. Lipids

Oil from crop plants represents the bioproduct that is chemically more similar to petrol and consequently has the highest aptitude to substitute it in the chemical industry. Petrol is considered to be originated from ancient, lipid-rich organic compounds, namely spores and planktonic algae (sedimented and transformed) under high pressure and temperature, throughout millions of years [42].

3.1.3.1. Biogenesis and genes involved in oil production

Virtually, all plant seeds contain storage lipids in the form of triacylglycerol esters (TAGs) containing three FAs with chain lengths of C8–C24, with C16 and C18 (i.e., palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid) being the most commons [43]. A number of comprehensive reviews on characteristics of structure and enzymes that are involved in oil biosynthesis and deposition in seeds have been recently published. Again the reader is referred to several excellent in-depth reviews and books for detailed information [e.g., 44, 45]. Briefly, studies in this field have indicated that plant oil is synthesized from glycerol-3-phosphate and fatty acyl-CoA in the endoplasmic reticulum as TAGs, and esters of FAs acids and glycerol. Moreover, FAs are synthesized in plastid from acetyl-CoA and then transported to the cytoplasm in the form of fatty acyl-CoA. In the ER, FAs are employed for the acylation of the glycerol-3-phosphate backbone either by the Kennedy pathway or by acyl exchange between lipids. Then, the resulting TAGs are deposited in specialized structures termed oil bodies. A schematic outline of the biosynthesis of storage lipids in seeds and in vegetative tissues is depicted in Figure 6.

Additional studies have indicated that some plants can produce, besides the common five FAs above-cited, also an array of unusual or 'novel' FAs exhibiting a wide diversity of FA structures, including various functionalities such as hydroxylation, epoxidation, acetylation, and

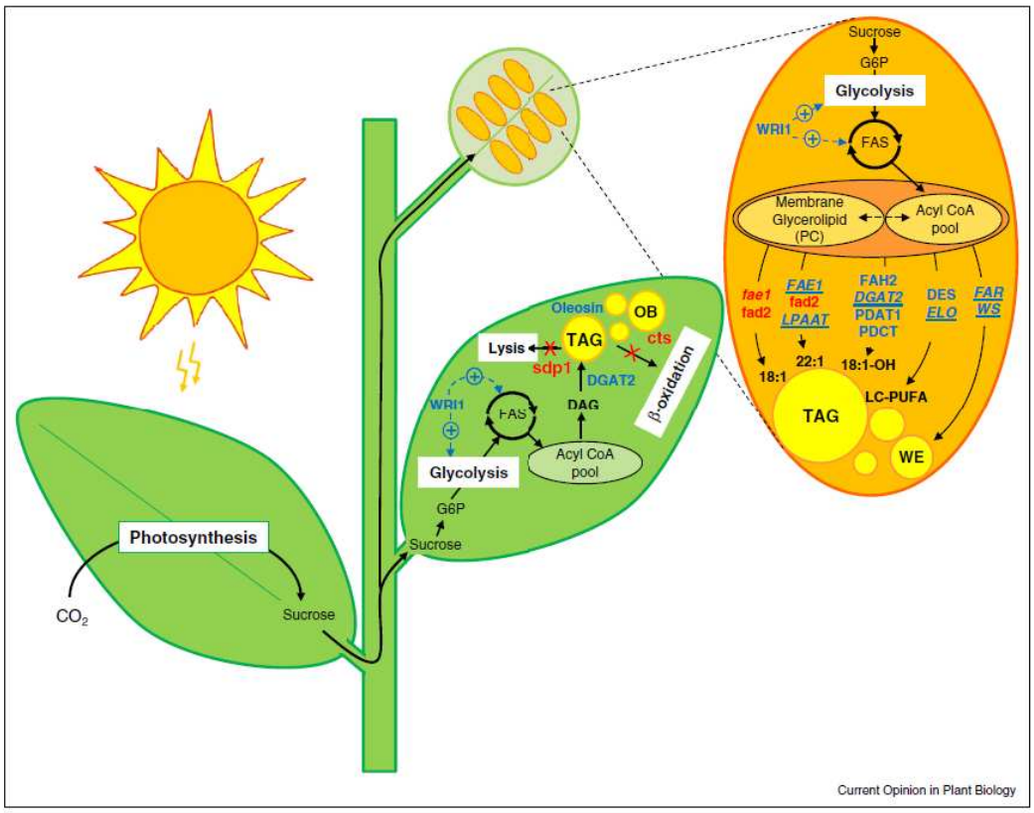

Figure 6. Schematic representation of metabolic engineering strategies for manipulation of oil content and composition in vegetative and seed tissues. Blue: target genes suitable for overexpression; Red: target genes for inactivation by mutation or RNAi constructs. Genes encoding enzymes using acyl-CoA substrates are underlined. FAS = plastid localised fatty acid synthase (Reproduced with permission from Napier et al., 2014, [46]).

conjugation that impart properties required for specific industrial uses [46, 47]. Furthermore, studies in this field have discovered a large diversity occur in the function of various FA-modifying enzymes, such as FA desaturase2 (FAD2), thioesterases, fatty-acid elongases, cytochrome P450s, acyl-CoA desaturases, acyl-ACP desaturases, sphingolipid-Δ8 desaturases, and S-adenosylmethionine methyltransferases [48]. The properties of many of these enzymes showing new functionality have recently been reviewed in detail by Voelker and Kinney [49], while for gene required for lipids synthesis visit http://www.canr.msu.edu/lgc/.

3.1.3.2. Genetic strategies to modify oil content and composition

At present, the industrial value of the current seed oils is limited by their FA composition that is not well suited for the manufacture of specialty chemicals and polymers. Therefore, due to complex inheritance of oil content and composition in seeds, several metabolic engineering approaches have been employed. Although, several examples of transgenic plants with altered oil composition were obtained, none of these plants had a high level of an unusual FAs necessary for industrial application (≈ 90–95% of the total FAs) [48]. These results suggest that plants vary considerably in the ability of their background metabolic machinery to handle the newly synthesized FAs. It is probable that further research will uncover genes for specialized

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forms of the various acyltransferase and TAG assembly enzymes capable of efficiently handling the unusual FAs. It is suggested that co-expression of such genes along with the previously introduced FAs acid biosynthetic pathways should contribute to further increases in accumulation of novel FAs transgenic plants in the future and lead to the development of economically viable crop sources of industrial raw materials.

In addition, to search for naturally occurring enzymes that can be used for the transgenic production of industrially useful FAs, other strategies have been considered such as diverting carbon flow from starch to TAG, up-regulating FA synthesis, modifying expression of individual TAG biosynthetic enzymes, and the use of TFs [43, 46]. In this context, genetic investigations have found various seed-specific regulatory genes that may play an important role by controlling oil deposition in the seed. For instance, the *WRINKLED1 (WRI1*), an AP2/EREBP transcription factor initially described in Arabidopsis, plays a regulatory function [50]. These studies have also identified several motifs that are important for WRI1 binding and transactivation. This is further highlighted by the study of Shen and co-workers [51] in which the maize ortholog of *LEAFY COTYLEDON1 (LEC1*) (another TF involved in the regulation of oil accumulation, upstream of *WRI1*) was expressed in transgenic maize under the control of an oleosin promoter. The resulting *ZmLEC1* overexpression lines of maize manifested outstanding levels of seed oil (up to 48% greater than null segregants), mainly due to extended storage lipids in the embryo. Although this high-oil phenotype was stable over various generations, it was also unfortunately associated with detrimental agronomic traits, such as reduced seed germination and plant growth, suggesting unacceptable and unpredictable pleiotropic effects caused from impaired seed-specific expression of *ZmLEC1*. To overcome these negative effects, the previous workers performed analogous experiments by overexpressing ZmWRI1. This research resulted in a high oil phenotype (≈ 31% higher than nulls) without the negative side effect on germination and growth. Notably, the strength of the promoter adopted to drive their expression of *ZmWRI*1 gave a favorable impact on the enhancement in seed oil deposition. Alternatively, the use of a weaker promoter produced only an accumulation in the seed approaching 17%.

The possibility of producing TAGs in leaves and other vegetative tissues has recently attracted considerable attention [46]. A schematic representation of metabolic engineering strategies for manipulation of oil content and composition in vegetative and seed tissues is given in Figure 6. A number of reports have documented that TAG accumulation can be increased by ectopic expression of individual biosynthetic enzymes, TFs that control seed development and maturation, or by mutating genes involved in TAGs and FAs turnover [46]. However, in the majority of these investigations enhancement of TAGs concentration in leaf was low and/or dependent on carbohydrates supply. Because key enzymes for both oil synthesis and breakdown are expressed in vegetative tissues, it was suggested that attaining significant amounts of storage lipid in the leaf will be essential in the re-orientation of C flux into TAGs [46]. Nevertheless, a remarkable increase in TAG levels (exceeding 15% of dry weight in vegetative tissue) has only been realized by integrated metabolic strategies directed to improve FAs and TAGs synthesis while inhibiting lipolysis. Additionally, the detection of non-seed proteins affecting the binding and stabilization of lipid-rich molecules in the cytosol of plant cells has

highlighted a new angle of the cellular machinery influencing TAGs packaging in plant vegetative tissue. It will be attractive to clarify if oil accumulation in green biomass can be further improved without severely impairing photosynthesis and plant development. A possibility for achieving this target is the use of senescence-induced promoters to engineer plants in which TAG accumulations will be initiated only after leaves have reached their maximum size [52]. Another strategy that might be devised is to directly connect C fixation to FAs biosynthesis by introducing a functional glycolytic pathway that is efficient to transform 3-phosphoglycerate to phosphenolpyruvate. Independently from the strategy that will be employed, the challenge of using photosynthetic cells to accumulate very high amounts of oil is an attractive objective. However, reaching levels of oil accumulation exceeding those currently found in seed oil crops, namely, superior to 35% of TAGs (% dw), remains a noteworthy metabolic engineering goal.

3.2. Genetic strategies to improve biomass yield

Plant breeding is driven by the need to continually increase sustainable yield and quality of crop plants and by meeting projected increases in global food demand. Targeted genetic improvement in yield for developing new varieties suitable as biorefinery feed-stocks will depend on identifying genetic variation in critical morphological, structural, and physiological traits affecting biomass production. This involves manipulating complex traits, such as those associated with plant growth and development. Biomass yield can also be enhanced by manipulation of additional pathways such abiotic and biotic stress. These topics are outside the scope of this chapter and have been reviewed elsewhere [e.g. 53, 54].

3.2.1. Yield genes

To accomplish maximal biomass yield in the development of new biomass crops, it is relevant to: i) identify genes and genetic pathways that are crucial to biomass production, ii) recognize the selective forces that have molded the frequencies of these genes in current varieties, and iii) establish which morphological and physiological traits may eventually lead to more efficient plants in yield performance. Although there is genetic variability in yield traits, many of them (yield, yield stability, nutrient, and water use) that are important in crop productivity are multigenic traits and are often difficult to breed for. Quantitative trait loci (QTLs) mapping approaches are common genomic tools to dissect the genetic architecture underlying complex traits and to identify QTLs [55]. Furthermore, the development of high-throughput sequencing and genotyping technologies has greatly improved the accuracy of QTL analysis. In this respect, biomass QTL mapping has been conducted in several crops with the purpose of identifying genomic regions and genetic loci underlying biomass feedstock yield (e.g., poplar, maize, switchgrass, perennial ryegrass) or sugar yield in sugarcane [56]. These studies carried out in different species and population types have manifested the prevalence of additive main effects, digenic epistasis, QTL x environment interactions, multiple minor effects, and QTL distributed over several genomic regions; moreover it was shown that both parents were contributing favorable and unfavorable alleles irrespective of their biomass yield potential.

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3.2.2. Molecular biology approaches for increasing biomass yield

Although genetic dissection of yield components, such as those affecting biomass productions, can help to elucidate the physiological route from gene to phenotype, current progresses in our knowledge of how plants function and develop can expand potential and efficiency of plant breeding programs devoted to yield improvement. Insights into gene and genome sequences, the regulation of gene expression and the molecular and cellular mechanisms and pathways underlying plant architecture, development, and function, may offer new options to plant geneticists to comprehensively devise novel breeding programs.

These strategies include molecular approaches to increasing biomass yield and transgenic research directed toward increasing biomass yield through genetic modification of different plant traits.

3.2.2.1. Photosynthesis

Photosynthesis provides the primary energy and C input for plant growth. Improving photosynthesis has been identified as a key strategy for the production of crop plants with higher biomass yield [57]. Molecular targets were identified by the study of bottlenecks of photosynthesis) and approaches to overcome these bottlenecks were mostly based on the up-regulation or down-regulation of single genes [58]. In some instances, synthetic pathways were used to overcome limitations of the endogenous pathways. A list of potential targets to improve photosynthesis is given in Table 2.

1. Improving Rubisco function
i. Improving Rubisco catalytic activity
ii. Altering Rubisco amount per leaf area
2. Increasing thermostability of Rubisco activase to sustain Rubisco activity at high temperature
3. Enhancing CO_2 concentration around Rubisco to maximize catalytic rate and minimize photorespiration
i. Turning C3 plants into C4 plants
ii. Installing algal or cyanobacterial CCM into C3 plants
iii. Redesigning photorespiratory metabolism
iv. Improving CO2 transfer pathways via stomata and/or mesophyll cells
4. Enhancing chloroplast electron transport rate
i. Improving whole chain electron transport
ii. Modifying light-harvesting systems
5. Enhancing enzyme activity of Calvin cycle (*e.g.*, FBPase, SBPase)
6. Enhancing the capacity of metabolite transport processes and C utilization

Table 2. Potential targets for improving plant photosynthesis.

An obvious target for increasing the source strength is the production of photoassimilates during photosynthesis. In this respect, research has been undertaken to introduce a more efficient, C4-like photosynthesis in C3 plants [58]. These last workers indicated that the introduction of single C4 enzymes (i.e., phosphoenol pyruvate carboxylase, PEPC, and pyruvate orthophosphate dikinase, PPDK) in C3 plants has until now not generated improvement in photoassimilate accumulation. This is probably attributable to disturbances in the fluxes of C4 intermediates for metabolic pathways excluding the C4 cycle. Notably in rice, the joined expression of two C4 cycle enzymes was found to raise photosynthetic ability up to 35% and grain yield up to 22%. In this research, the maize genes were transferred to the rice genome together with their corresponding promoters, which might have turned out in a superior spatial and temporal expression of the C4 cycle enzymes than the more expected expression of transgenes by using constitutive promoters. Additionally, work in transgenic tobacco has also showed that increased levels of fructose-1, 6-bisphosphatase (FBPase) and sedoheptulose-1,7-bisphosphatase (SBPase), two Calvin cycle enzymes, significantly increased dry weight [59].

Besides the above strategies based on C4 photosynthesis, other approaches have been taken to improve the efficiency of photosynthetic C assimilation. One of these strategies is focused on the enzyme RUBISCO ACTIVASE, a key regulator of RUBISCO (Ribulose 1,5-bisphosphate carboxylase/oxygenase essential component of the photosynthetic process of fixing CO_2 into organic C) activity [60]. Transgenic Arabidopsis plants expressing a heat-tolerant version of RUBISCO ACTIVASE showed a significant improvement in photosynthesis and leaf growth when exposed to heat. Other efforts to improve the photosynthetic efficiency of plants have been focused on: i) increasing electron flow by overexpressed CYTOCHROME C6 (CYTC6), a protein involved in the photosynthetic electron transport chain [61]; ii) engineering new pathways into the chloroplast that bypass photorespiration and release CO_2 directly into the chloroplast stroma [62] ; and iii) using various 'add-ons' or CO_2-concentrating mechanisms (CCMs) to elevate CO_2 levels in the vicinity of RUBISCO. These CCMs were pursued by improving mesophyll CO_2 conductance via overexpressing aquaporin [63] or stomatal CO_2 conductance via manipulating stomatal characteristics [64]. In this respect, more recently Lin and co-workers [65] have successfully engineered tobacco plants containing a functioning RUBISCO from a cyanobacterium. The cyanobacterial enzyme has a greater catalytic rate than any 'C3' plant. The lines generated in this research open the way for future addition of the remaining components of the cyanobacterial CCM, an important step towards enhancing photosynthetic efficiency and improving crop yields. Furthermore, it has been shown that the triose-phosphate/phosphate translocator (TPT) strongly limits photosynthesis under high CO_2 conditions [65]. The TPT provides a regulatory link between CO_2 assimilation and cytosolic C metabolism. In this context an approach for increasing plant yield was performed by overexpressing sucrose transporters in sink cells, thereby enhancing sink demand and inducing an increase in photosynthesis and assimilates export. When overexpresssing a potato sucrose symporter (*StSUT1*) in storage parenchyma cells of pea seeds, there was enhanced sucrose influx into cotyledons and greater cotyledon growth rates [67]. In addition, it has been shown that enhancement of sucrose synthase activity represents a useful strategy for increasing starch accumulation and yield in potato tubers. In a future world of higher CO_2 concen-

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tration, enhancing the capacity for sucrose export and carbon utilization would be an important component for maximizing photosynthesis and yield. While altering transport capacity alone is unlikely to change photosynthetic capacity, enhancing photosynthetic capacity as well as transport capacity could lead to improved plant growth and yield.

Technology for nucleus or chloroplast genome transformation has been advancing and it would enable easier and more precise manipulation of the photosynthesis process. It is expected that such plants could exhibit more efficient photosynthesis under controlled conditions; the plant factory in which plants are produced in an optimized growth environment would have potential advantages of high productivity. In the future, the combined uses of several strategies would greatly help to improve photosynthetic capacity and thus plant growth and ultimately yield.

3.2.2.2. Plant architecture

Considerable progress has been realized over the past decade in revealing the molecular mechanisms that underlie the formation of plant architecture. Dissection of plant architecture with plant morphological mutants enabled plant scientists to gain insides into various aspects affecting plant architecture as exemplified by the shot apical meristem (SAM) maintenance and differentiation, the initiation of axillary meristems (AMs), the formation and outgrowth of axillary buds, the elongation of stems, and the number or angle of branches (tillers) [68].

Studies carried out in model plants (i.e., Arabidopsis and petunia) and crop plants (e.g., tomato, maize, and rice) served as platforms for understanding the molecular basis of plant architecture. In this respect, RNA interference (RNAi) technology was used to improve crop productivity by modifying plant architecture. For example, in rice over-expression or suppression of the *OsPIN1* (a member of the gene family of auxin transporters in plants) expression through a transgenic approach resulted in changes of tiller numbers and shoot/root ratio, suggesting that *OsPIN1* played a role in auxin-dependent adventitious root emergence and tillering [69]. In general, these studies have provided insights on the molecular mechanisms affecting each specific aspect that collectively specializes a particular type of plant architecture, among which the signaling pathways that regulate plant height and control the maintenance and differentiation of the SAM have been particularly well analyzed. Although our current understanding of how a particular plant specializes its architecture is still poor, the findings obtained so far in studying plant architecture have already allowed us to open a way for optimizing the plant architecture of crops by molecular design and improving biomass productivity.

3.2.2.3. Regulation of plant biomass production

In plants, the potential to improve biomass production has not yet been largely investigated. This is because the conventional breeding of crop plants (e.g., maize, rice, wheat, and soybean) has been centered on the selection of the superior grain-yield traits. However, current molecular and genetic research has singled out a number of regulators affecting plant biomass production. A diagram depicting various factors affecting plant biomass production reported by Demura and Ye [70] is given in Figure 7. It is evident from this graphical representation

that an increase in biomass yield could be achieved by an enhancement of the vegetative apical meristem activity by KNOX proteins (i.e., KNOX are homeodomain TFs) and of the vascular cambium activity by KNOX and cytokinin, an inhibition of the transition into reproductive growth by activating FLC (protein encoded by the *FLOWERING LOCUS C,FLC,* affecting flowering time) and by repressing SOC1/FUL/FT (encoded respectively by *FLOWERING LOCUS T, FT*, and *SUPPRESSOR OF OVEREXPRESSION OF CONSTAxlink (SOC1*), both involved in regulation of the indeterminacy of meristems and the longevity of plants), an increased cell elongation by gibberellin, an elevated photosynthetic efficiency, and an increase in secondary cell wall biosynthesis. An improved cellulose digestibility by modifying cell wall properties could contribute to increased biofuel production. Therefore, flowering-time genes could potentially be manipulated for generation of grass crops with increased biomass yield. Overexpression of some members of NAC family (a plant-specific family of TFs) also induces increased biomass production. Arabidopsis transgenic lines overexpressing *NAC1* are bigger, with larger leaves, thicker stems, and more abundant roots compared with control plants [71]. Similarly, overexpression in Arabidopsis of another NAC-domain transcription factor, ATAF2, leads to increased biomass, mainly by production of bigger leaves with larger cells [72].

Additionally it is obvious that genes regulating cell number and organ size in plants could potentially contribute to yield increases. For example in maize, Guo et al. [73] have isolated and described a family of genes termed *Cell Number Regulators (CNRs)*. Insight into their function was achieved by ectopically expressing one of this members, namely *CNR1.* It was shown that *CNR1* reduced overall plant size when ectopically overexpressed, while plant and organ size increased when its expression was co-suppressed or silenced. Leaf epidermal cell counts showed that the increased or decreased transgenic plant and organ size was due to changes in cell number, not cell size supporting the idea that *CNR*s function as cell number regulators in maize. This suggests the potential for application to crop improvement via generation of more vigorous and productive plants.

3.2.2.4. Phytohormones

Phytohormones are known to play important roles in plant growth and development, including the regulation of meristematic activities and cell elongation, both of which are crucial for biomass yield. For example, the plant hormones auxin and brassinosteroids (BRs) are important regulators of plant growth, stimulating both cell division and cell elongation. Arabidopsis plants that are unable to synthesize or perceive BRs are dwarfs with rounded leaves and reduced pollen fertility that show sizeable delayed flowering time and leaf senescence [74]. STEROID 22a HYDROXYLASE, encoded by the *DWF4* gene, is a key enzyme for BR biosynthesis. Overexpression of *DWF4* in *A. thaliana* produced plants that grew 35–47% taller and produced 33% more seed. The rice mutant *dwf4-1* had depressed levels of BRs and an ideotype characterized by slight dwarfism and erect leaves. Although individual *dwf4-1* plants had reduced biomass yield, their ideotype allowed high-density planting that led to increased grain yield per unit area [75].

A way by which auxin controls final plant organ size is through the transcription factor *ARGOS* (*AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE*) which acts upstream of *AINTE-*

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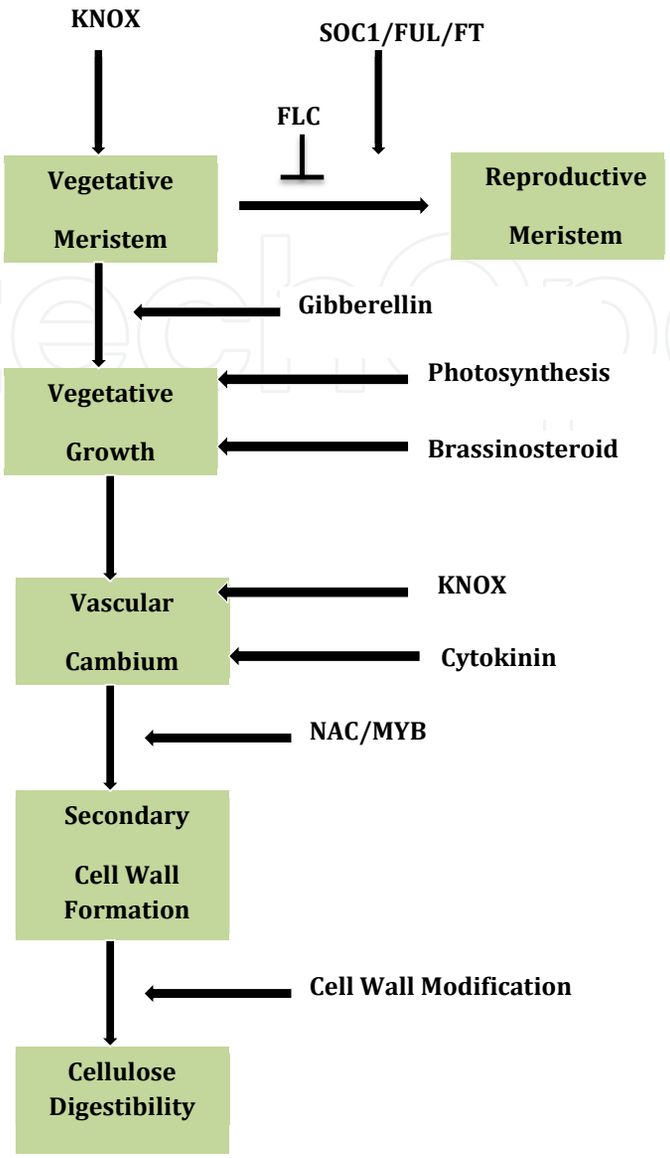

Figure 7. Schematic representation indicating several factors influencing plant biomass yield (Redrawn from Demura and Ye, 2010, [70]).

GUMENTA (ANT), a member of the AP2/ERF family of TFs [76]. For both *ANT* and *ARGOS*, transgenic plants overexpression resulted in an increased plant and organ size. These last genes promote growth through prolonging the cell proliferation period (but not the rate), and the final organ size change is mainly due to an increase in cell number, not cell size. Notably, Arabidopsis *ARGOS-LIKE*, another growth promoting factor, enlarges organ size by enlarging cell size not cell number. *EBP1*, a putative Arabidopsis ortholog of human *ErbB-3* epidermal growth factor receptor binding protein, is another key regulator promoting plant and organ growth. In contrast with ARGOS and ANT, transgenically overexpressing *EBP1* accelerates plant growth and development by simultaneously stimulating cell growth, proliferation, and development, resulting in both increased cell number and cell size [77]. For all three of these growth-promoting genes, not only does transgenically increasing expression promote plant

and organ growth, but also down-regulation of gene expression or loss-of-function via mutation reduces the plant and organ growth. Among these key players are growth repressors, an example is Arabidopsis *AUXIN RESPONSE FACTOR2* (*ARF2*). Loss of *ARF2* function causes enhanced organ size, such as thicker stems and larger seeds, floral organs, and leaves [78]. Physiological studies have suggested synergistic roles for auxin and BRs in cell elongation and genome-wide microarray expression studies identified a large number of genes that respond to both auxin and BRs [79]. Integration of auxin and BR signaling has been suggested to occur at the level of ARFs because the auxin-response element (TGTCTC) is enriched in the promoters of BR-responsive genes [80].

There is information indicating that cytokinins also promote organ growth by stimulating cell proliferation with cytokinin depletion or overproduction resulting in smaller or larger leaves and flowers, respectively [76]. Likewise, gibberellins promote growth through expansion and/or proliferation, acting to repress the activity of the growth-restraining DELLA proteins; DELLA factors may be important in adjusting growth in response to environmental influences. In addition to these classical phytohormones, a novel mobile signal whose synthesis depends on the related cytochrome P450 enzymes KLUH/CYP78A5 and CYP78A7 promotes leaf and floral-organ growth. Investigations on chimaeric plants indicate that the presumed signal is integrated across flowers, suggesting that it may be used to coordinate growth within and between individual organs [76].

In conclusion, it is worth noting that a better understanding of genes affecting hormonal metabolism and signaling should help to design strategies to increase plant growth and organ size and, ultimately, crop yield.

3.2.2.5. Enhancing sink strength

To increase sink strength, credible biochemical targets are represented by enzymes directly or indirectly involved in the conversion of sucrose into starch. One approach to increase sink strength has been devoted to modify the adenylate pools in potatoes; adenylate levels was found to be relevant for starch content in their tubers [81]. In this respect, it was shown that down-regulation of the plastidial isoform of adenylate kinase induced a 60% upgrade of starch in potatoes and, interestingly, a 39% gain in tuber production [82].

Other approaches for yield improvement are more specifically directed towards starch synthesis. Many of these attempts showed only limited success [83]. On the contrary, positive results were obtained in different plant species by acting on AGPase that catalyses a rate-limiting step in the starch biosynthetic pathway. As mentioned above, these enzymes, subject to allosteric control by orthophosphate (P_i) and 3-phosphoglycerate (PG) effectors, have received the most attention [84]. For example, in maize overexpression of wild-type *Sh2* (that encodes the endosperm large subunit of AGPase suscettible to P_i inhibition) and *Bt-2* (*Bt2*, which encodes the endosperm small subunit of AGPase stimulated by PG) increased individual seed weight to 15% by increasing starch content [85]. More recently, Hannah and co-workers [86], by studying the expression of a transgenic form (*HS33*/*Rev6 Sh*2) of AGPase with enhanced heat stability and reduced P_i inhibition, reported an increased maize yield up to 64%. Interestingly, as previously found in wheat and rice, this transgene increases maize yield by

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increasing seed number. Moreover. genetic, physiological, and molecular data provided by Hannah et al. [86] point to *HS33/Rev6 Sh2* expression in maternal tissue, rather than in the seed, as the cause of enhanced seed number. Therefore, these workers concluded that the transgene does not increase ovary number; rather, it increases the probability that a seed will develop. Furthermore, the addition of allosterically altered AGPases and increased Arabidopsis leaf transitory starch turnover improved its growth characteristics [87] and increased fresh weights of aerial parts of lettuce plants ([88].

3.2.2.6. Other strategies

An approach to remodel plant growth is to vary the expression of cell cycle-related genes. Cockcroft et al. [89], by overexpressing the Arabidopsis *CYCD2* gene in tobacco, obtained plants that were 35% higher in comparison to controls. These transgenic plants also manifested normal cell and meristem size associated with an exalted overall growth rates, an enhanced rate of leaf initiation, and a faster rate of growth at all stages of plant development considered in the experiment. Similarly, the overexpression of another Arabidopsis D-type cyclin, *CYCD3;1*, a rate-limiting gene affecting the G1/S transition phase, was found to promote ectopic cell divisions, generating leaves with more but smaller cells [90]). On the contrary, the ablation of *ABAP1*, an Arabidopsis gene that controls the cell proliferation rate by limiting mitotic DNA replication, resulted in larger leaves containing more cells [91]. Further studies on developing leaves revealed that during early leaf development, cell division rates were smaller in *ABAP1*-overexpressing plants in comparison with controls. On the other hand, cell division was faster in plants possessing a defective copy of *ABAP1* gene. Another gene whose variation increases biomass is *CDC27a* [92]. This gene encodes a protein that is a component of the ligase Anaphase-promoting Complex (APC). Overexpression of Arabidopsis *CDC27a* in tobacco produced plants that were up to 30% higher at flowering time, with slight alterations of apical meristems [92]. These last authors concluded that it is likely that the growth promotion mechanism observed in *CDC27a* overexpressing plants is due to the APC, rather than CDC27a protein itself, because the overexpression of at least two other subunits of the APC displays similar enhanced growth.

Another mechanism promoting cell proliferation and ultimately organ growth involves TFs of the *TCP* (*TEOSINTE BRANCHED1, CYCLOIDEA, PCFs) and GROWTH-REGULATING FACTOR (GRF*) classes, two redundant multi-gene families in Arabidopsis. The importance of the TCP family in growth control became evident from the *cincinnata (cin*) mutant in snapdragon (Antirrhinum) and the jaw-D activation-tagged mutant of Arabidopsis [93,94]. In these mutants, leaves overgrow to a highly-wrinkled shape because of excess cell proliferation particularly at the leaf margins. It was found that *CIN* encodes a member of the TCP family and in *cin* mutants, the size of the proliferative region at the leaf base appears enlarged and its distal boundary is concave. In this respect cells at the leaf margin still proliferate at positions where cells in the center have already arrested proliferation. In the *jaw-D* mutant, overexpression of the microRNA miR319a down-regulates five genes of the TCP family. Down-regulation of another three gene family members causes even more severe phenotypes of overproliferation in leaves, while miR319-resistant versions of TCPs and loss-of-function mutations in

miR319a reduce organ size and cause premature cell differentiation [93, 94]. In fact, promoting cell differentiation has been proposed as the primary function of TCPs, rather than directly arresting proliferation [95].

A novel gene regulation mechanism was recently discovered in metazoans, the RNA silencing or RNAi, or microRNA (miRNAs) [96]. Rodriguez et al. [97] found that overexpression of miR396 in Arabidopsis had a negative impact on cell proliferation in developing leaves through the repression of GRF activity and a decrease in the expression of cell cycle genes. Accordingly, disruption of the recognition of *GRF2* by miR396 resulted in larger leaves with more cells than control plants. Similarly in Arabidopsis, it was reported that over-expression of miR156 promotes a vegetative-phase transition delay and an enhancement in leaf initiation rate [98], while over-expression of miR172 induces adult leaf traits and flowering [99]. The regulation of both miRNAs is closely connected as their expression is influenced by age, temperature, and light acting in a contrasting way [100]. At a molecular level, miR156 acts through the down regulation of genes coding for the *SQUAMOSA PROMOTER BINDING-LIKE (SPL*) and the *AP2-like transcription factor*, respectively, are subjected to feedback regulation by their targets [101].

Recently, miRNAs have also emerged as key regulators of phytohormone response pathways *in planta* by affecting their metabolism, distribution, and perception [100,102] have demonstrated that gibberellic acid (GA) promotes flowering in Arabidopsis through a miR156-dependent pathway, indicating a connection between miRNA and phytohormone signaling pathways in the control of shoot development. In general, it appears evident from this data that miRNAs as growth regulators are interesting targets for gene manipulation for improving biomass yield.

4. Conclusions and future perspectives

Due to the increasing concerns on the environment, climate change, and limited natural resources, there is considerable effort applied to produce chemicals and materials from renewable biomass. While initial emphasis was reversed on biofuel production from food plant sugars, the competition between crop usage for food and non-food applications has stimulated research efforts to genetically improve yield and quality-related traits of plants for biorefining applications.

Targeted genetic improvement of biomass for biorefining applications depends on identifying genetic variation in critical morphological, structural, biochemical, and physiological traits affecting biomass yield and its chemical composition. This involves manipulating complex traits, such as those associated with plant growth and development or tolerances to abiotic and biotic stresses, usually in production environments that are highly variable and unpredictable, as well as to gain insights into the less digestible carbohydrates in cell walls components. This last aspect, in turn, has promoted inspections on the use of other plant-derived metabolites for chemical productions embracing the high-value specialty segments via platform intermediates needed for bulk production.

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Although there is genetic variability in the traits sustaining biomass yield, many of them important in crop productivity and sustainability are complex multigene traits, often difficult to breed for. Nevertheless, advances in plant genetics and genomic technologies are contributing to the acceleration of gene discovery for product development. In this respect, several new genomics technologies such as next-generation sequencing and high-throughput marker genotyping can be used not only for developing high-density genetic and physical maps but also for generating transcriptome or sequence data [55]. These approaches together with –omics technologies (e.g., transcriptomics, genetical genomics, metabolomics, and proteomics) have emerged as powerful tools for understanding genome variation in crop species at DNA, RNA, as well as protein level and for identifying genomic regions or genes affecting the expression of trait(s) that are of interest to improving plants for biorefining applications or for breeding varieties with in-built new traits such as creating higher value-added products. Furthermore, a range of novel genetic techniques, particularly techniques collectively referred to as 'genome editing', have been developed that allow targeted changes to be made to genomes [103]. Changes can include adding or removing DNA at a specified location in the genome or replacing a specified segment of DNA with a different one. It is also expected to make epigenetic changes (histone modifications and DNA methylation), where the DNA sequence remains unchanged but gene expression is altered because of chromatin changes that may be heritable [104].

Knowledge of plant biosynthetic pathways will also provide valuable opportunities for metabolic engineering, as well as access to chemical transformations unique to plants. It is expected that genomics will corroborate plant biochemistry as researchers seek to understand the metabolic pathways for the synthesis of high value-added products. Identifying rate-limiting steps in their synthesis could provide targets for genetically engineered biochemical pathways to produce augmented amounts of useful compounds and new compounds in plant cells. Although, plant-based production of novel compounds such as biopolymers (e.g., polyhydroxyalkanoates) in plant tissues has been documented [105], plant metabolic pathways are complex and often feature multiple levels of regulation suggesting that it will be difficult to target the best intervention points and accurately predict the outcome [106]. However, recent developments in knowledge-based metabolic engineering strategies, that is the storing and mining of genomic, transcriptomic, proteomic, and metabolomic data, might permit to generate models of metabolic pathways useful to define and refine optimal intervention strategies for synthesis of specific chemicals for industrial applications [107].

The recent emergence of synthetic biology as the basis for metabolic engineering in plants promises to positively change the way in which different intervention strategies are selected and implemented, as is already the case in microbes [108,109]. In this context, metabolic engineering has contributed successfully in the last two decades towards broadening the product portfolio containing various value-added and commodity chemicals and materials from renewable resources [108,110]. Obviously, the attempts of engineering plants using the strategies employed for microbes are definitively more cumbersome. However, plant

scientists have already established efficient methods for the routine genetic transformation of the majority of our principal crops. Using these technologies, they have also achieved substantial progress in understanding and manipulating plant primary and secondary metabolism. Moreover, further research on metabolic regulation and genetic information and on novel tools for genetic modifications may help to overcome the limitations for breeding varieties that meet targeted applications and end-uses. This will certainly help maximize their value throughout the whole bio-based value chain for biorefining applications or eventually breeding varieties with in-built new traits that are creating higher value-added products.

5. Glossary

Biofuel: Fuel produced from crop-derived carbohydrates. Includes bioethanol produced from fermentable sugars and biodiesel produced from plant oil.

Biomass: Biological materials used for fuel or industrial production. Here, we refer to the sum of plant harvestable tissues.

Bio-based (or bio-derived) platform molecule: A chemical compound whose constituent elements originate wholly from biomass (material of biological origin. excluding fossil C sources), and that can be utilized as a building block for the production of other chemicals.

Biorefinery feedstocks/Platform chemical feedstocks: Biorafineries are typically differentiated into polvsaccharides, lignin, protein, and extractives (e.g., triglycerides and terpenes) as all are found as constituent parts within typical biomass in varying amounts.

Building block chemicals: Molecules with multiple functional groups that possess the potential to be transformed into new families of useful molecules. According to the US Department of Energy the top 12 sugar-based building blocks are: 1,4-diacids (succinic, fumaric and malic), 2,5-furan dicarboxylic acid, 3-hydroxy propionic acid, aspartic acid, glucaric acid, glutamic acid, itaconic acid, levulinic acid, 3-hydroxybutyrolactone, glycerol, sorbitol, and xylitol/arabinitol.

Metabolic engineering: Describes the targeted modification of endogenous metabolism to control the accumulation of one or more specific products.

Sink strength: Ability of a sink organ (any organ, e.g., roots, developing seeds, or immature leaves, that imports photosynthetic assimilates) to competitively mobilize assimilates toward itself.

Synthetic biology: Aims at creating novel functional parts, modules, circuits, and/or organisms using synthetic DNA and mathematical/logical methodologies, and has been shown to be practical and useful in various biotechnological applications.

Author details

Massimiliano Lauria[1], Francesco Molinari[2] and Mario Motto[3*]

*Address all correspondence to: mariomotto@libero.it

1 Consiglio Nazionale delle Ricerche, Istituto di Biologia e Biotecnologia Agraria, Milano, Italy

2 Dipartimento di Scienze per gli Alimenti, la Nutrizione e l'Ambiente, Univerisità degli Studi di Milano, Milano, Italy

3 Fondazione Istituto Tecnico Superiore per le Nuove Tecnologie della Vita, Bergamo, Italy

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