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The miR156: A Master Regulator to Harmonize Complex Biofuel Traits in Lignocellulosic Biomass Crops

***Singh, A. P**

*Genomics and Proteomics Lab, Department of Biochemistry, GGDSD College (Panjab University), Chandigarh, India Email: akhlash@ggdsd.ac.in

Abstract

Currently, energy security and environmental degradation are the two biggest challenges before humanity that can be surmounted with the use of green and sustainable biofuels produced from lignocellulosic crops. In the future, to ensure adequate and cost-effective supply of biofuels, it requires a sufficient amount of amenable and quality lignocellulosic feedstocks. Therefore, agricultural yields of lignocellulosic biomass crops should be substantially increased by intense genetic maneuvering of key gene regulatory mechanisms and signaling pathways that control plant biomass yield. Recently, numerous miRNAs families are identified, characterized, and validated across the plant kingdom. Plant microRNAs (miRNAs) are 21 to 24 nucleotides long, non-coding small RNAs, act as regulators of their target genes via inducing modifications in transcription, translation, and epigenome. MiRNAs represent many hallmark characteristics like sequence-specific regulation, tissue, and species-specific expression, evolutionary conservation, and functional diversity. They coordinate well physiological and life cycle processes in plants under adverse environmental conditions. Hence, miRNAs offer accurate, precise, and efficient regulatory switches in the miRNA-targeted genetic networks. It is evident from the study of the miR156 family and its target *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL)* genes network that controls highly significant agronomic traits in crop plants. The miR156/SPL module acts as a master circuit that synchronizes many intricate complex biological functions such as growth and development, and metabolic processes by sensing internal and external environmental signals in plants. Therefore, miR156 can prove a potential target for miRNAs based plant biotechnology to harmonize complex biofuel traits and improve biomass yield in lignocellulosic biomass crops.

Highlights

- Lignocellulosic biomass crops are highly significant to retain marginal lands and to provide feedstocks for bioenergy industries.

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- Lignocellulosic based SGBs have the potential to offset current hydrocarbon-based fuels used in heavy industries and transports, hence, these crops are important to meet Sustainable Development Goals set by the United Nations.
- Supply of biomass feedstocks is the biggest challenge to produce biofuels and bio-products in biorefineries that can be achieved through genetic improvements by using miRNAs based plant biotechnology.

Keywords: Lignocellulosic biomass crops, biofuels, plant miRNAs, miR156, miR156/*SPL*-system, plant biotechnology, abiotic and biotic stresses, and bio-confinement.

Word count: 12,651

Abbreviations: AMF, Arbuscular mycorrhizal fungi; ARF, AUXIN RESPONSE FACTOR; BNF, Biological nitrogen fixation; FGBs, First Generation of Biofuels; HSP, Heat shock proteins; LRR, Leucine-rich repeat; MAMP, Microbe-associated molecular pattern; MRE, MiRNAs responsive elements; NBS, Nucleotide-binding site; NGS, Next Generation Sequencing; NLS, Nuclear localization signal; PAMP, Pathogen-associated molecular patterns; RISC, RNA-Induced Silencing Complex; SAM, Shoot apical meristematic; SBPs, Squamosa-Promoter Binding Proteins; SGBs, Second Generation of Biofuels; *SPLs*, SQUAMOSA PROMOTER BINDING PROTEIN-LIKE; SRC, Short rotation coppice; TYMV, Turnip yellow mosaic virus; WUE, Water-use-efficiency; BECCS, bioenergy with carbon capture and storage.

1. Introduction

Currently, 85% of global energy and fuel demands are coming from non-renewable hydrocarbon-based reserves that caused serious environmental degradation and economic crisis particularly, in developing countries. The consequences of burning fossil fuels in current infrastructures including heavy machinery and automobiles release a huge amount of CO_2 and other greenhouse gases (GHGs), which are mainly responsible for climate changes on the earth [1]. To overcome these problems of climatic emergency, researchers have shifted their current attention to discover various climate mitigation strategies that include exploration of many renewable energy resources including, the production of alternative, sustainable, and green energy systems, such as next-generation biofuels (NGBs) like, bioethanol, biodiesels, biobutanol, gasoline, ethyl levulinate, and gaseous biofuels. The NGBs are non-hydrocarbon based fuels, contain high-energy-density (Net energy value; 2.63 to 6.96 (MJ m−2), diverse in nature, and derived from organic materials, which are produced in a highly renewable manner by plants via photosynthesis process e.g., plant biomass [1, 2].

In the beginning, the first generation biofuels (FGBs) including bioethanol and biodiesel were produced using edible feedstocks obtained from food and non-edible oil crops, respectively [3]. But, FGBs are affected by several shortcomings, such as un-sustainability, and being highly water-energy intensive thus

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require extra water and energy inputs for their manufacture. Moreover, continuous production of FGBs can lead to the "food vs. feed conflict", and cause serious food inflation due to the production from food biomass that often used for human and animal consumptions [4]. Therefore, now research investigations are more focused to overcome demerits of FGBs, and thus, second-generation biofuels (SGBs) created by using non-food crops/dedicated bioenergy crops e.g., switchgrass, Miscanthus species, and poplar, etc. The SGBs offer many benefits like manufactured from carbon-neutral, sustainable, and most plentiful lignocellulosic biomass provided by perennial grasses and woody plants. Lignocellulosic biomass is mainly composed of cellulose, hemicellulose, lignin, and other biopolymers that produced in a renewable manner and available most abundantly on the earth. Moreover, a plethora of studies clearly showed that SGBs are more promising, cost-effective, and sustainable to support other renewable energy sources like solar, wind, hydro, tidal, nuclear and geothermal energy endeavor to ensure energy security in the future [4, 5]. Simultaneously, SGBs are capable to offset hydrocarbon-based liquid fuels, which are currently used in large scale heavy industries and global transport systems. Although SGBs are sustainable and better than FGBs in many aspects, there are several unresolved technical issues afflicted them [6]. These are a lack of sufficient supply of raw material/feedstocks, less amenability, and low agricultural yield of lignocellulosic crops. Moreover, various physio-chemical and biological downstream processes involved in biofuels productions which ultimately contribute to the high cost of liquid transport fuels [4, 7]. It is worth mentioning that lignocellulosic biomass is now converted into electricity, liquid transport fuels, biogas production and high-value bioproducts (enzymes, acids, and biochemicals) that can greatly reduce the biofuel cost [8-10]. But the supply of quality feedstocks is still a major limiting factor. Hence, there is an urgent need to address these problems to get sufficient, amenable, and cost-effective supply of lignocellulosic feedstock for biorefinery or lignocellulosic industries.

In order to improve yields and quality of feedstocks, many desirable biofuel traits are incorporated in lignocellulosic feedstocks crops by using many conventional genetic improvement strategies like genetic engineering and molecular breeding. But these efforts could not yield expected results because of plant genomes complexities, gene pleiotropism, and appearance of undesirable traits in transgenic cultivars [11]. Therefore, it requires target based gene manipulation methods, for example, miRNA based next-generation RNA and genome editing technologies to incorporate suitable bioenergy feedstock traits in dedicated bioenergy crops, [12-14]. The ideal lignocellulosic crops (ideotypes) must carry complex agronomic traits including high water and nutrients use efficiency, resilient to climate change, and high yield potential, therefore, these crops can substantially contribute to fulfilling biomass supply for industrial applications as well as in the climate mitigation endeavors [15-17]. To achieve these goals, complex agronomic traits must harmonize in a synergetic and coordinated manner, hence, the overall

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physiology of crops can improve plant biomass yield. This can be achieved by fine-tuning of key genetic networks through master gene regulators, such as plant miRNAs in fast-growing lignocellulosic biomass crops which are the most suitable candidates to become ideal feedstocks crops [18]. Currently, several studies have shown that microRNAs (miRNAs) are better candidates to synchronize desirable agronomic and biofuels traits in the bioenergy ideotypes. Since, plant miRNAs are implicated in virtually all biological functions in plants especially, to control growth and development under adverse environmental conditions [14]. Recently hundreds of miRNAs families have been identified in various crop plants [19-21] that deposited in various microRNAs repositories [22, 23]. These databases provide a great wealth of knowledge related to miRNAs, and their biogenesis, structures, functions, and evolutionary origins.

MicroRNAs are the most abundant, single-stranded, non-coding small RNAs (with 18 to 24 nucleotides) present in animals and plant kingdoms. They negatively regulate expressions of their target genes in a sequence-specific manner by an extremely conserved molecular mechanism in plants. For example, the miR156 family regulates functions of large *SPL* gene family members by cleaving the transcripts (mRNAs) of SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SBPs/SPLs) transcription factors and regulatory box proteins in flowering plants [24]. Plant miRNAs represent two major hallmark characteristics, i.e. diversity and conservation in terms of sequences, members, species and functions, which help plants to coordinate multiple biological processes very efficiently, for instance, growth and development, embryogenesis, molecular signaling, and response to adverse [25], climatic conditions [17]. Hence, miRNAs can prove better regulatory points for genetic manipulation to improve the overall yield of plant biomass under extreme climatic conditions.

Phylogenetic analysis of the miR156 family in 51 plant species indicates that the miR156a is an ancient subfamily that is extremely conserved in the whole plant kingdom (fig.1). In plants, the miR156a and miR156c play very significant roles in the plant aging pathway. Conversely, other candidates of the miR156 family such as miR156m/n/o/p/q/r/s/t/u/v/w/x/y/z, occur only in few plant species [26] e.g., *Glycine max* and *Malus domestica*, therefore, the miR156a has emerged as a potential target for plant biotechnology. Moreover, the miR156 family and its target miR156/SPL circuit are evolutionarily conserved, and play important regulatory roles across the plant kingdom [27-30]. Recently, it has been revealed that the miR156/SPL module [24, 25, 31] controls many plant morphological traits including cell size and numbers, trichome, stomata [32], leaves, and flower development, shoots maturation, which also collectively called “heteroblastic change” in plants [33].Conclusively, these morphological changes affect overall plant growth, and ultimately lignocellulosic biomass yield [30, 34] can regulate by targeting miR156/SPL module in plants.

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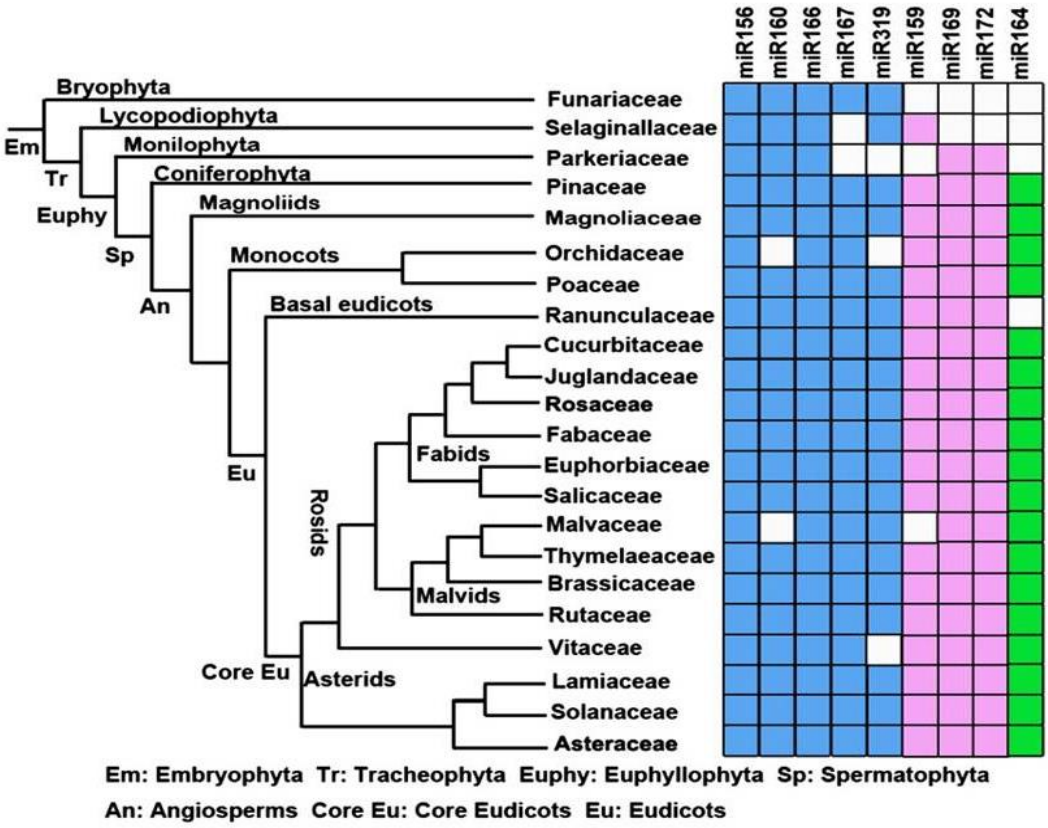

Fig.1: Phylogenetic conservation of miR156 across the plant Kingdome (produced with permission of publisher, Elsevier Ltd.)

It already proved that miRNAs are involved in the various layers of hierarchical regulatory systems that offer more sophistication and regulatory flexibility at the organism/plant level. Hence, miRNAs are better switch “to fine-tune” systems via transcriptional cleavage of target mRNA or reversibly control biological processes by using “on or off” scheme e.g., translational repression of a gene in the response of a feedback signal that originates due to endogenous and exogenous cues [35, 36]. These types of regulatory properties of miRNAs have been observed in the case of miR156 family which regulates expressions of the *SPLs* genes that govern the vegetative phase transitions in plants [24]. Therefore, the miR156/SPL circuit provides opportunities to improve the yield potential of the bioenergy plants by RNA based synthetic biology (Table 1). The current review article briefly describes the potential regulatory roles of the miR156, to improve yields, composition, and amenability of lignocellulosic biomass that can be used for biofuel production.

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2. Lignocellulosic energy crops and their desirable characteristics

Lignocellulosic biomass crops are more photosynthetic efficient and fast-growing plants that require minimal agricultural inputs for their farming. These crops are mainly divided into two major categories. (1) Lignocellulosic crops (non-woody); the high biomass herbaceous plants belong to the grass family and mainly having C_4 type of photosynthesis, thus, are highly productive plants on the earth. Lignocellulosic crops mainly include switchgrass (*Panicum virgatum*), Napier grass (*Pennisetum purpureum*), (*Miscanthus. Sinensis*, *M. sacchariflorus*, and their hybrid *M. ×giganteus*, or Mxg), *Brachypodium distachyon*, Setaria species (*Setaria viridis*), indigo bush (*Amorpha fruticosa*), reed canary grass (*Phalaris arundinacea*), smooth cordgrass (*Spartina alterniflora*), *Sorghum bicolor* L (sorghum), and Indian grass [4, 37, 38].

(2) Woody biomass crops; that carry C_3 type of photosynthesis, also produce high energy packed biomass (upto 30 tons ha−1 y−1). These include *Populus* (poplar), *Salix* (willow), and *Eucalyptus*, also known as short rotation coppice (SRC). Currently, these dedicated bioenergy crops are under intensive scientific investigations for the improvement of SGBs yields. In the recent past, studies have shown that herbaceous lignocellulosic plants offer more advantages over woody trees as raw material that are generally used for the SGBs production [3,18,39-42].

However, many advantages are linked with the SGBs because of their sustainability, carbon neutrality and high potential to reduce GHGs [43]. According to estimation, about 43 % GHGs can displaced by using switchgrass based biofuels that replaced with hydrocarbon based fuels. But current challenges hinder complete exploitation of cellulosic crops in the complex integrated biorefinary. These challenges include: (1) lack of suitable and economically viable lignocellulosic crops; (2) less and unstable biomass yield (average 20 tonnes Mg ha^{-1} yr^{-1}) per unit area of land, hence, there is significant yield gap of 15 tonnes ha^{-1}year-1 [4,18, 37]; (3) high level of biomass recalcitrance; (4) lignocellulosic crops are not able to grow optimally on the degraded lands with minimum agricultural inputs [44]; (5) to make crops more eco-friendly in the local niche; (6) to improve water and nutrients-use-efficiency or high C: N ratio in biomass; (7) to enhance resistance capability of cellulosic crops against abiotic and biotic stresses; (8) to improve biocontainment capability of transgenic lignocellulosic crops [45] (9) to make lignocellulosic crops more photosynthetic efficient so that, can fix more solar energy into biomass[5,37,46,47]; (10) simultaneously, crops system must also improve overall soil fertility; (11) bioenergy crops can be used as cost-effective, eco-friendly phytoremediation agents to retain degraded land, therefore, lignocellulosic crops must able to grow in diverse ecological and geographical locations [8,42,48]. Currently, about 2 billion hectares of degraded lands are available worldwide, therefore, that can be used for lignocellulosic

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crop farming to produce biomass feedstocks without encroaching arable land used for food crops [18, 42, 49-51]. But, why lignocellulosic biomass crops are the most suitable candidates for energy farming and climate mitigation programme.

Lignocellulosic biomass crops are efficient energy converters that change solar energy into plant biomass, and relatively more resistant to emerging unfavorable climatic conditions. Therefore, these crops are becoming the most prominent candidates to incorporate desirable biofuels traits by using the latest miRNAs editing techniques. Lignocellulosic crops also offer a high output/input energy ratio, for example, switchgrass offers up to 540% output energy than what it is required to grow [50-52]. But, so far, no single lignocellulosic crop is available that contains multiple desirable biofuel traits. It is also well known that the current cost of cellulosic biofuel is two to three folds higher than hydrocarbon-based fuels due to lack of enough supply of amenable feedstocks. Therefore, there is an urgent need to develop such ideotypes that can act as BECCS (bioenergy with carbon capture and storage), and simultaneously, also provide amenable feedstocks for the production of biofuels, electricity, biogas, and hydrogen production. Recently, it is also proposed that biomass crops must also produce endogenous value-added products that can further be processed along with biofuels in biorefineries [53-56]. So the current cost of biofuels can reduce substantially. In view of these problems, the miR156 based plant biotechnology and synthetic biology tools will play significant roles to improve biomass yield and further expedite molecular breeding and domestication of lignocellulosic crops in the future.

3. Discovery of the miR156 in lignocellulosic plants

In the past two decades, microarrays, high-throughput sequencing (HTS) especially, deep sequencing techniques, bioinformatics, and others important tools are applied to analyze whole genomes and transcriptomes for miRNAs discoveries in plants. Consequently, a large number of plant miRNAs families have been identified, characterized, and validated by using RT-PCR, northern blotting, and RNA-Seq [57–65]. So far, 30,424 mature miRNAs from different 206 species have been deposited in the plant microRNA database (http://www.mirbase.org ;) [22, 23]. More recently, artificial intelligence-based feature selection algorithms are also applied to identify the working pattern of many miRNAs species and their contribution to resilience against abiotic stresses in model plants. The regression-based machine learning models like decision tree (DT), support vector machines (SVMs), and Naïve Bayes (NB) prove effective to determine the precise role of a particular mRNA species in the specific biological process [63, 64], through screening of big data sets housed in various miRNAs databases [22, 23].

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Currently, scientists have put more emphasis to understand the regulatory roles of various miRNAs in endogenous gene expression processes related to specific plant tissue development, e.g., root, stem, leaf, flower, fruit, and seeds. Therefore, various miRNAs based manipulating strategies can be applied [13, 14, 64] to enhance crucial genetic traits that are controlled by different miRNAs and their target genes in lignocellulosic crops. So far several conserved and novel miRNAs families are identified which are involved in the "fine-tuning" of significant feedstock traits in many bioenergy crops like switchgrass, *Miscanthus* species [65-67] *Medicago sativa* [68,69] S. *bicolor* [70,71] *Brachypodium distachyon* [72, 73] *Populus* [74, 75] Setaria species [76] Cordgrass, giant reed [77] Cordgrass [78], *Arundo donax.L* [79] *Sorghastrum nutans* (L.), Pennisetum [80], and other biofuel crops. However, numerous miRNAs families are identified and characterized but few of them have clearly emerged as master regulators, and thus, play a versatile role in controlling complex plant traits, for example, miR156 and miR164 (bio-recalcitrance), miR159, miR172, miR395, miR444 (flower development), miR169, miR395, miR399 and miR528 (water stress) in lignocellulosic plants [8,27,45,67,81-85]. But among them, the miR156 family has emerged as the master regulator in bioenergy plants, if compare its diverse regulatory roles with other miRNAs families in the respect of multiple physiological processes [26, 86, 87]. The miR156 family regulates phase transitions, biomass development, and signaling cascades [28, 45, 88, 89], under biotic and abiotic stresses in crop plants. Substantial shreds of evidence show that miR156 is a better single gene candidate for precise genetic manipulations to incorporate a set of desirable agronomic traits in cellulosic energy crops.

4. Biogenesis and action of the miR156

In plants, the miR156 biogenesis is an evolutionarily conserved process across the plant kingdom. Currently, the miR156 is well characterized in numerous models as well as crop plants, but for the sake of simplicity, a common biogenesis pathway is mentioned here [35, 90]. Plant miRNAs transcribed from *MIR* genes by RNA polymerase II (Pol II) as a long single-stranded primary miRNAs (pri-miRNAs, 60 to 500 ntd), that subsequently turn into a hairpin-like structure containing polyadenylated cap at 5' end. It is noteworthy to mention here that, *MIR* genes transcription follows all rules as by mRNA transcription such as the requirement of locus-specific transcription factors and several effector proteins that interact with different components of biogenesis machinery (fig.2). The pri-miRNAs are identified and processed at Dicing-bodies (D bodies) by DCL1 proteins (Dicer Like- RNA III endonuclease) into a stem-loop structure, also called precursor miRNAs (pre-miRNAs) [91]. Afterward, pre-miRNAs processed by the combinatorial actions of DCL1, double-stranded RNA-binding protein Hyponastic Leaves 1 (HYL1), the zinc-finger protein Serrate (SE) and Cap-binding protein (CBC) to give rise a mature miRNA duplex (miRNA/miRNA*) also known as guide/passenger strands* inside the D bodies. Next duplex is

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methylated by HUA ENHANCER 1 (HEN1) protein at 2'-O-methylated at both 3' ends [36, 92]. Inside the nucleus, miRNA duplex is associated with a nuclear membrane bound transport protein HST1 exportins 5 (Exp 5) that transport miRNA duplex to the cytoplasm (P bodies).

In the cytoplasm, one strand of mature duplex loaded on the ARGONAUTE (AGO) proteins and incorporated into RNA-Induced Silencing Complex (RISC) in the cytoplasm [93]. The RISC complex further binds to multiple target sites known as miRNAs responsive elements (MRE) like 5'UTR or 3'UTR and coding regions of targeted mRNA transcripts, e.g., transcription factor (TFs) mRNAs [94]. The miRNA-linked AGO protein in the RISC complex examines mRNA molecules for perfect base complementarity in coding regions of target molecules, and subsequently, takes different actions. For example, miRNA cause protein repression by using (Figure 2) three different molecular mechanisms during or after transcription via (1) degradation of mRNA transcripts, regulated by RISC or AGO1[21, 92] ; (2) suppression of the translational process; and (3) inhibition of transcription by methylation of nitrogenous (epigenetic changes) via recruiting the additional cofactors like General Control Non-repressed Protein 5 (GCN5) which acetylates to H3K14 that further controls expression of *MIR156a* and *MIR156c* genes [21, 95-97]. It also reported that miRNAs [98, 99] further lead to the synthesis of secondary siRNAs called phasiRNAs and/or easiRNAs.

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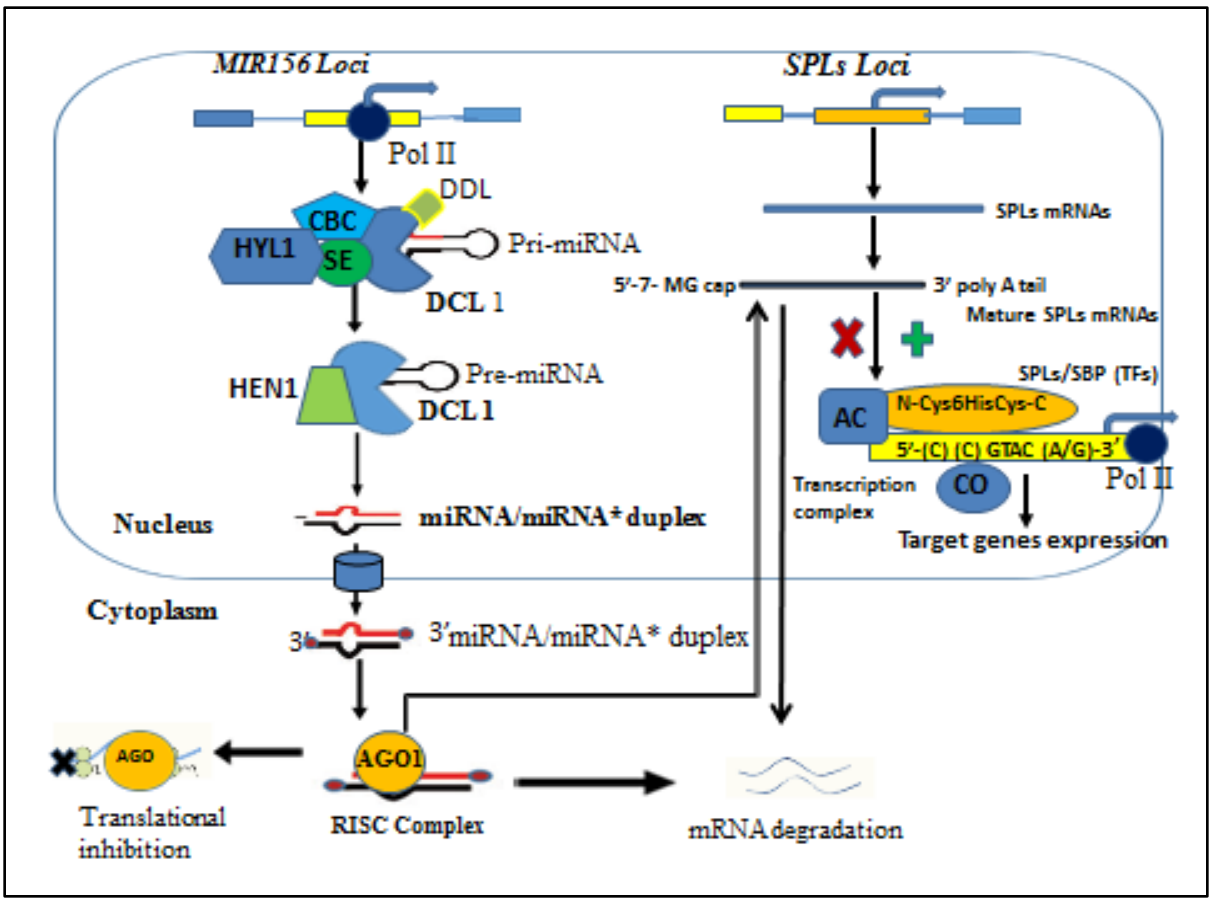

Fig.2: Biogenesis and general mechanism of miR156 mediated regulation of *SPLs* genes, which are involved in various plant functions. In order to inhibit SPL gene, the overexpressed miR156 binds with mRNA of SBP transcription factor, hence no synthesis of TF, no gene expression (shown by **x**). While downregulation of miR156 allow expression of SPLs genes that affect various plant functions (indicate by **X**) (Abbreviations - DCL1, Dicer-Like 1 ribonuclease; DBR1, Double-Stranded RNA Binding-1; HYL1, Hyponastic Leaves-1; SE, Serrate; HEN1, Hua-Enhancer1; AGO1, Argonaute; RISC, RNA-induced silencing complex; AC, activators; CO, Coactivator)

In recent studies, it observed that the multistep process of miRNA biogenesis and degradation plays a crucial role in the "fine-tuning" of plant response to growth and development under environmental stresses [100,101]. Furthermore, it is also noticed that individual components of the miR156 biogenesis pathway such as D-body proteins like DCL1, AGO (Argonaut), HYL 1, SE, DRB2 (Double-stranded RNA binding proteins-2) and miRNA–miRNA* duplex, and their interactome also plays a crucial role to produce a final response at the plant level to external as well as internal perturbations, [102]. Given that the miR156s mediated gene regulatory mechanisms affect activities of target genes (*SPLs*), transcription factors (SBPs/SPLs), regulatory/effector proteins as well as enzymes, and the activities of Pol II in the

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miR156 based *SPL*s gene circuit, which is involved in the regulation of many important biological functions and metabolic pathways in lignocellulosic plants [24, 31, 83, 103, 104].

Molecular studies also support this theory that the miR156 biogenesis pathway is mainly responsible for structural and functional diversities of the *SPLs* mediated downstream processes that are regulated under internal and external cues. As an example, the cellular concentration of HYL1 is regulated by light and dark cycles. In the dark, the HYL1 is degraded by an unknown protease activity in the cytoplasm. Conversely, constitutive expression of COP, a Ring finger E3 ligase can prevent the HYL1 degradation by halting the activity of the protease. Moreover, phosphorylation and dephosphorylation of HYL1 by both Mitogen-Activated Protein Kinase 3 (MPK3) and SNF1-related Protein Kinase 2 (SnRK2) in the response of light affect its transport from the nucleus to the cytoplasm. But finer points related to miRNAs based signal cascades are still poorly understood [21, 87, 92, 96,102,105]. Currently, several good review articles describe the recent developments in the area of miRNA biogenesis, and its related newly discovered proteins, enzymes, transporters, and small nuclear RNAs (tasiRNAs and phasiRNAs). These components get modified in the response of external or internal molecular signaling, and ultimately create a systems-level of response against the environmental changes [99,105-109].

The secondary structure of pre-miR156, one of the major components of the biogenesis pathway, also plays a very significant role in the regulatory process which is endorsed by the SNP associated mapping studies in the natural population of poplar plants. The modification, interactions, and binding of the duplex with target mRNA transcripts at the time of biogenesis provide more regulatory potential to the miR156 in the terms of phenotypic variations. Simultaneously, these studies have also proved a very significant contribution of natural allelic variation in *SPL* genes and their interactions with target miRNA/miRNA* [92,110-116], but this aspect is still poorly described. Although most of the miR156 related studies are widely conducted only in the model plants, it will also provide a conceptual foundation for genetic manipulation of lignocellulosic plants. This can be achieved by constructing artificial genetic circuits by applying RNA based technologies such as artificial microRNAs and micro proteins, genome editing, and synthetic biology [[13,64,117]. The role of several major components of miR156-SPLs based genetic networks such as miRNAs, miRNA coded peptides (mipeps), mRNA transcripts, relay proteins, ions, and phytohormones are still unknown. But the recent discovery of more sophisticated genetic manipulation tools such as Meganucleases, ZNF proteins, TALENs, and CRISPR/Cas9 [118] will offer more opportunities by creating accurate mutations to unravel the roles of miR156-moulded-SPL modules in lignocellulosic crops.

5. The MiR156 and its target genes

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In the case of plants, miR156 family members (miR156a to miR156i) were initially discovered in the *A. thaliana* genome [119]. Like other plant miRNAs, the miR156 also targets mRNA (transcripts) that encode for a phylogenetically conserved network of SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SBPs/SPLs) transcription factors (TFs) and other regulatory proteins which are involved in various physiological processes in crop plants. Structurally, SPL proteins contain highly conserved DNA binding SBP (Squamosa-Promoter Binding Protein) domain of 79 amino acids. The SBP domain is divided into three functionally active motifs, including two zinc fingers each comprising 10 Cys or His residues, and a nuclear localization signal (NLS) element at the C terminal (figure.2). These Cys or His residues are arranged in a specific sequence that constitutes a motif structure ($Cys_3HisCys_2HisCys$ or $Cys_6HisCys$). The SBP domain is highly basic and binds to consensus region 5'-(C) (C) GTAC (A/G)-3' in the promoter of target genes [69,120,121]. The miR156 is not only affected the expressions of *SQUAMOSA-promoter binding like (SPL*) gene family but also the expression of non-*SPL* genes like, *DIHYDROFLAVONOL-4-REDUCTASE (DFR)* regulating *WD40-1*, *TEOSINTE GLUME ARCHITECTURE1 (TGA1), COLORLESS NON-RIPENING (CNR),* and *LIGULELESS1 (LG1)* genes families which regulate important growth and development functions in plant biology [120,122,123] . Therefore, the miRNA156 is an important potential switch that regulates or coordinates the operation of multiple physiological functions in plants, and thus proposed as the most promising target for genetic manipulation.

A large number of molecular level scrutiny has revealed that the miR156 family does not negatively control the downstream expression of its target *SPLs* genes, but it also affect its mode of action that will be discussed in different sections of the same paper. The miR156 also targets sub-families of *SPLs* genes, as an example, miR156 affects the expression of 11 subfamily genes out of 17 *SPLs* in Arabidopsis [24]. The regulatory mechanisms may be different in the case of individual genes, e.g., *SPL3* and *SPL9* genes to get turn off through transcript cleavage, and translational inhibition respectively. Another example, two closely miR156 targeted *SPL1* and *SPL2* genes effectively regulate tiller formation and internode elongation but not affect developmental transition and internode initiation in switchgrass, which reflect redundant genes functions [83]. It is well known that miR156 targeted SBPs/SPLs TFs bind to *cis*-acting elements in the promoter region of target genes (*SPLs*), and thus, involved in the formation of transcription complex at initiation sites, consequently, TFs can control the pace of genes expressions via binding to promoter site. In this manner the miR156 indirectly can also act as a positive and negative modulator of target genes involved in the complex genetic networks [124-126]. In switchgrass, the miR156 positively regulates *SPL9* expression, whereas it negatively controls *SPL7* and *SPL8* switchgrass [103]. Hence, it requires a detailed study about the structure and functions of the miR156/SPL module

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and associated down regulatory components that are involved in the regulation of biofuel related traits in bioenergy plants.

6. The miR156, master regulator to synchronize complex biological functions

As already given that the miR156 biogenesis pathway is highly responsive to different developmental stages, nutrient status, metabolic flux, and feedback from the expression of the corresponding genes in plants. Therefore, the miR156 acts as a developmental timer and its expression regulate many complex plant processes by sensing internal (hormones, metabolites, and nutrients) as well as external signals (temperature, CO_2, and photoperiods) that can better explain by considering a classic example of the flowering process (reproductive phase change). Flowering is also an inevitable part of growth and development and aging in all plants. The flowering process is widely studied, and well elucidated by using various loss or gain of function experiments and natural mutations (e.g., *hyl1-2*) in diverse plant species [19,122,127]. The overexpression of miR156 causes a substantial delay in the development of the flower, and simultaneously, also improves biomass yield by downregulating several key genes, e.g. *SPL1*, *SPL2, SPL3, SPL4, SPL5, SPL7, SPL8* and *SPL10* that further control expression of downstream MADS-box genes such as LEAFY *(LFY), FRUITFULL (FUL) APETALA2 (AP2) SUPPRESSOR OF OVEREXPRESSION OF CO (SOC1), SEPALLATA3 (SEP3), MADS32*, and AGAMOUS-*LIKE 42 (AGL42)*, which are key floral meristem identity genes. This whole network of genes is highly conserved in a wide variety of plant species. In the shoot apical meristems (SAM) of switchgrass plants, the interactome of these *SPLs* genes affects the function of the *APETALA1* (*AP), FLOWERING LOCUS (FT)* and *SOC1*, three major signal integrator genes that control flowering process in the transgenic lines. Importantly, both *SPL 7* and *SPL8* genes not only intensely halt flowering events but also promote reversion of inflorescence during vegetative-to-reproductive phase transition [103]. Owing to the complexities of the flowering pathway, several metabolic pathways, and their crosstalks play crucial roles to determine the flowering time. For instance, the miR156-SPLs-miR172-regulated pathway, a crucial regulator of the flowering pathway (table 1), also plays an important role in the vegetative phase (change of juvenile to mature stage), panicles initiations, and root development processes [28,29,47,103,122].

Both Chromatin immunoprecipitation (ChiP) and GUS assays studies indicated that adult plants having high expression of miR172 gene transcripts, while the amount of miR156 declined gradually. Because, a transcriptional repressor, DELLA1 that suppressed the expression of *SPL9* gene in the favor of miR172. It has been thought that *SPL 9, SPL13* and *SPL15* are expressed consistently throughout the developmental

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transition in the shoot apical meristem (SAM) [47, 128, 129]. In view of these facts that the miR156 and miR172 both act as a sequential antagonist of each other in young and mature plant tissues, respectively. The high concentration of miR172 affects negatively a group of TFs, also known as flower repressors like TARGET OF EAT 1 (TOE1), TOE2, TOE3, SCHLAFMUTZE (SMZ), SCHNARCHZAPFEN (SNZ), and APETALA2 (AP2) via translational suppression that causes flowering initiation. These TFs are also promoted by the up-regulated activities of targeted *SPL*s genes and further coordinate actions of flower-making genes, and finally, cause suppression of the *FT* gene involved in the floral development process in poplar (see. Table 1) [19].

In Arabidopsis plants, mutation-based studies show that the level of miR156 is also regulated by both sugars concentration and Cyclin-Dependent Kinase 8 (CDK8) network genes (*CENTER CITY (CCT)/MED12* and *GRAND CENTRAL (GCT MED12)/CCT MED13),* these all act in a highly synchronized manner in leaves and vegetative tissues that ultimately control phase transition process [130]. More recently, it is found that the lower level of *MIR156*a/*MIR156c* is controlled by the expression of a histone *H3 lysine 27 methylation (H3K27me3)* gene [131,132]. Earlier, the down-regulation of miR156 is also mediated with sugars like glucose, Trehalose-6-phosphate, and sensing proteins like hexokinase1 that proved experimentally by adding glucose exogenously, and by the study of *cao*/*ch1* mutant. This mutant shows faulty chlorophyll b and a reduced level of photosynthesis activities [130-132]. The relationship between sugar and miR156 level is highly conserved, both act as an antagonist to each other, for example, a high level of sugar breaks the primary mRNA transcripts of miRNA156 via HEXOKINASE-1 (HXK1), known as a glucose sensor. However the complete pathway of how sugar influences the miR156 expression is still unknown. The pivotal role of miR156 in the flowering and vegetative phase transition has been confirmed by many artificial miR156 target mimics (*35S::MIM156*) expressions and STTM techniques in the model as well as in poplar plants. Apart from the flowering development, the miR156-SPLs-miR172-regulated module is also implicated in the adventurous root development in Eucalyptus species [83,128].

Based on the study of the miR156/SPL module in a wide variety of plant species, it can be inferred that the miR156 predominantly targets mRNAs of TFs, and other key effectors proteins, [79,133,134] are involved in a variety of significant agronomic and biofuel traits [60,125]. These traits or morphological changes include flowering, inflorescence meristem, increased plastochron, tiller number, phase transition which are responsible for high cellulosic biomass growth (Table 1). Additionally, the miR156 also offered a high level of resistance against abiotic and biotic stresses [45, 60,135]. Despite enormous structural diversities among plants, the miR156/SPL- system shows extreme functional conservation. Thus, it makes

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the miR156 family a potential target for bioengineering and synthetic biology to fine-tune agronomic traits in the case of potential bioenergy crops.

Table 1: Various targets of the miR156 and their regulatory functions in different lignocellulosics biomass crops

Name of crop	**Target genes in miR156-SPL module and their expression level**	**Downstream target gene/ proteins/ metabolites**	**Functions**	**Reference**
Switchgrass (*Panicum virgatum* L.)	*SPL1(D)* *SPL2(D)* *SPL3(D)* *SPL4(D)* *SPL5(D)* *SPL7(D)* *SPL8(D)* *SPL10(D)*	Flower regulators repressed MADS-box genes such as *LFY*, *FUL*, *AP*, *FT*, *SOC1*, *SEP3*, *MADS32*, and *AGL42* miR172 Transcriptional repressor, DELLA1	Delay flowering process Internode elongation, Promote new leaves and tillers prolonged juvenile phase reversion of inflorescence Improves biomass yields Increase saccharification Help in gene biocontainment	[3, 28,29,47, 83,103,122, 128, 129 139]
	SPL 9 (U/C) *SPL 13(C)* *SPL 15(C)*	MYB transcription factor, various heat shock protein, proline, HSPs, GABA and anthocyanin miR172 suppress the floral repressors genes *TOE1*, *TOE2*, *TOE3*, *SMZ* , and *SNZ*	Resistance against abiotic stress Resistance against abiotic stress Promote flowering and vegetative phase transition	[60,215,217,226, 152]
Brachypodium Distachyon	*SPL (D)* by miR156	*SPL* transcription factors ATP binding	Decreases lignin content Improve biomass yield	[139]

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		and nutrient regulatory proteins	Reduced bio-recalcitrance	
Spartina alterniflora	*SPL2 (D)* *SPL3(D)* by miR156 *SPL13(C)*	Cell wall hydrolyzing proteins e.g., laccases MYB33/62, MYB33 TFs via miR156/SPL network	Provide salinity resistance Improve plant resilience against the polycyclic aromatic hydrocarbon	[78, 81 154]
Medicago sativa	*SPL6(D)* *SPL12 (U)* *SPL13 (U)*	auxin response factor (ARF) via SPL based transcription factors *MYB2* gene activity and phosphate transporters such as PHO1 and PHT miR172c–AP2-1 system, *ENOD40* and *SYMPK, POLLUX, CYCLOPS, CERBERUS*, and *NODULATION-SIGNALING PATHWAY1* gene	Decreases lignin content Improve biomass yield Reduced bio-recalcitrance Regulate root development Enhance Pi uptakes by regulating salinity resistance BNF pathway and nodulation formation Help in the plant-microbial symbiosis	[136, 175] [178,179]
Setaria italica	*SPLs* (D) by miR156c	SBP family TFs, MYB, ARF17, HD-Zip , AP2, HSPs and laccase	Resistance against the abiotic stresses	[205,206]
Miscanthus	*SPL s (D)* by	NA	Regulates the growth of	[168]

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species	miR156		root Improve natural N_2 supply	
Bermudagrass (*Cynodon dactylon) (L).Pers.*	NA	Enzymes like CDC-like kinase, beta-glucosidase and coniferyl-aldehyde dehydrogenase	Affect carbohydrate metabolism	[195]
Populus species	*SPL3 (D)* *SPL9(D)* *SPL15(D)* *SPL20* (D) *SPL25(D)* by miR156c and miR156i	*MYB2* gene activity and phosphate transporters such as PHO1 and PHT1 FT protein via NaKR1	Promote leaf initiation Enhance leaf sizes and internode elongations Improve plant height Regulate K, and Na transport Enhance wood formation Enhance total biomass yield	[142, 143]. [144]
Eucalyptus species	*SPL3(D)* by miR156e *SPL5 (U)* *SPL9(U)* *SPL10 (U)*	miR172 and *AP2*, Increase miR172 expression	To improve heteroblastic characteristics Prolong the vegetative phase Delayed flowering, and control the development of the adventitious roots under abiotic stresses	[145] [127]

7. The MiR156 as a potential target for miRNAs based plant biotechnology

Currently, researchers have started to exploit the regulatory potential of miR156s and their targets *SPLs* genes family, which regulates a wide range of biological functions in bioenergy as well as crop plants. Currently, a lot of investigations are going on to unravel the potential roles of individual *SPL* genes and

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their interactions with miRNAs in a genetic network. Moreover, new regulatory functions of the miR156 are continuously emerging like, nodulation formation in nitrogen-fixing plants [136], regulation of stomata, and synthesis of secondary metabolites, which act as protective agents for plants under adverse environmental conditions [32]. The miR156/SPL system contains a variety of potential targets [25, 36] that are influenced by the miR156 expression. Hence, it offers a single gene target to improve quality as well as quantity of lignocellulosic bioenergy feedstocks under extreme climatic conditions (see table 1).

7.1 Role of miR156 in biomass production

The improvement of cellulosic biomass includes three major aims; (1) to improve yields of feedstocks (2) changes in composition and amenability; and (3) production of value-added products endogenously in lignocellulosic plants [53]. A core objective of these three major aims is to ensure the proper supply of cost-effective feedstocks for biofuels and bio-products manufactured in biofuel industries. Because the lack of a sufficient quantity of cellulosic raw material is the major factor that substantially enhances the production cost of biofuels. Therefore, it requires to increase plant biomass yield in terms of apical dominance and vegetative growth in lignocellulosic plants through various genetic manipulation techniques that can positively modify plant architecture (e.g., branching, internodes, and shoot branching). In this direction, the miR156-SPL system is a better target for miRNAs based editing technologies. Because this single genetic network controls multiple structural and physiological properties of leaves like, the vegetative transition from juvenile to adult phase, inflorescence, tillering number, shoot apical meristematic (SAM), tissue structure, plant height, lateral organs and root formations [24, 134,135]. These factors are very crucial to enhance total biomass growth, and ultimately, it will improve the yield of feedstocks in bioenergy crops [24, 86].

7.1.1 Herbaceous lignocellulosic crops

During the growth phase transition, the miR156 governs several morphological changes in plants like, an increased in petiole length and length to width ratio of lamina that are mainly responsible for overall enhanced plant biomass [24, 28, 81]. Since, the miR156 acts as a key developmental switch in the synchronization of metabolism and plant growth at various stages of vegetative phase transitions under the influence of several factors, such as hormones, regulatory proteins, and climatic conditions [86,136-138].

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It is suggested that if delaying the reproductive process, for example, the flowering process subsequently, the energy that used to reproductive activities can channelize [83] to support the vegetative growth of plants [127]. As proof of concept, this strategy is applied to many plant species to get more vegetative growth through delaying flowering development [24, 25, 27, 28, 45, 69]. It is well established that the miR156-*SPL*-miR172 network and other regulatory proteins/ TFs are crucial to regulating vegetative phase transition, and finally cellulosic biomass growth (Figure 2). Hence, it will prove an effective strategy to improve the yield of feedstock [9]. But the miR156-SPL-miR172 module is well characterized only in a few lignocellulosic crops and model plants such as *Arabidopsis*, rice, switchgrass, *Eucalyptus grandis* and *Populus* × *canadensis*is [24, 88,127]. Conclusively, the miR156-SPL-miR172 is an important regulatory pathway to control the vegetative phase transition in both herbaceous and woody lignocellulosic crops. Moreover, being an evolutionarily conserved module in a wide variety of model plants e.g., Arabidopsis and rice that makes it an important potential target for the conceptualization of fundamental knowledge transfer from model plants to cellulosic bioenergy crops.

To further test the efficacy of miR156 based regulation strategy in bioenergy plants, a genetic map-based cloning study was conducted on the maize *Corngrass1* (*Cg1*) dominant mutant. This neoteny mutant allows constitutively over-expression of miR156 in the SAM and lateral organs during vegetative phase transition, subsequently, it promotes initiation of new tillers and leaves. The Agrobacterium-mediated transformation of *Cg1*cDNA with a 35S promoter was carried out in *Brachypodium*, *Panicum virgatum*, and *Arabidopsis* plants. It was found that the moderate to severe miR156 expression results in both improved quantity and amenability of feedstocks in transgenic plants. Simultaneously, *Cg1*gene also offers high saccharification efficiency of biomass that is attributed to more axillary branches (tillers) and young leaves due to prolonged juvenile phase in switchgrass plants [139]. The up-regulation of miR156 also offers similar results in cotton plants also [134]. Furthermore, the overexpression of the rice *OsPIL1* gene also causes biomass growth in switchgrass, but no correlation could establish with the miR156 expression [139,140]. In the same plant, the miR156 and other miRNAs families are identified which govern inflorescence and developing tillers. But the miR172 expression was five times more in inflorescence than tillers [135,139] because both miR156 and miR172 are crucial for phase transition characteristics such as inflorescence and tiller development.

The effect of miR156 based strategy is quantitatively significant that reflects from various results. In field-grown plants, the over-expression of miR156 influenced cell wall chemistry in a positive manner, consequently, about 25% to 56% more biomass was produced. Subsequently, it enhanced the total biofuel yield by up to 30% in transgenic switchgrass [27, 28]. These results suggest that the miR156 overexpression influenced plant architecture in terms of the double, tiller, and shoot numbers that offer

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higher growth of feedstocks [27]. In the same plants, low to moderate overexpression of miR156 was linked with improved biomass (58–101%) than control plants, these results further endorsed the efficacy of this approach [89,123]. The high biomass growth in a wide variety of plants is attributed to the overexpression of miR156 that substantially suppressed the expression of *SPL* genes and important flowering genes that promote the juvenile stage, which are endorsed by the status of over or down expression of various homologs of *AP2 MADS-box* genes in plants.

In order to know the exact effect of miR156 on individual *SPL* genes in the miR156-SPL module, several reverse genetics-based experiments clearly show that some *SPLs* genes are not targeted and influenced by the expression level of miRNA. Moreover, it is also observed that few *SPLs* genes perform opposite functions in respect to their paralogs. In the case of switchgrass transgenic lines that expressed a high level of miR156 down-regulate both *SPL1* and *SPL2* genes but do not affect flower time and internode initiation. This report is extremely inconsistent with previous reports [83]. Furthermore, a genome-wide study shows that the individual expression of *SPL2* increases tiller numbers, and finally improves quality as well as quantity of biomass yields as mentioned above [83]. Therefore, miR156s do not squarely influence the functioning of all *SPLs* genes (see table 1). With similar aims, to unravel the role of miR156 in the regulation of *SPLs*, two artificial microRNAs (amiR) constructs were used to down-regulate *SPL7* and *SPL8* genes in switchgrass, and expressions of *SPLs* genes observed individually as well simultaneously. In both conditions, the miR156 overexpression makes an extreme delayed flowering and phase transition, and inflorescence inversion without dwarfing of plants [103,141]. Hence, it suggests that *SPL 7*and *SPL8* can be better candidates for breeding purposes than other *SPLs* genes. So far, the roles of the *SPLs* gene family are studied at a broader level. In the light of above reports, it can conclude that the miR156-SPL system must operate at the appropriate level to achieve optimum growth and development in plants. Therefore, it becomes essential to study the detailed functioning of individual *SPL* family and its subfamily members at the molecular level, thus, *SPLs* genes can be utilized for the speed breeding and domestications of cellulosic crops.

7.1.2 Woody viable plants

Woody perennial trees are also a major source of lignocellulosic biomass feedstocks. The woody plants also underwent vegetative changes in their life cycles, and it is mainly controlled by the miR156/SPLs module [83, 142]. In Canadian poplar, the overexpression of miR156 negatively regulates two *SPL3* and *SPL9* that substantially improve morphological parameters such as leaf initiation, leaf sizes, internode elongations that ultimately improve plant height [142, 143]. In the same plant species, 12 members of the miR156 family were identified in the natural plant population by using transcript profiling, furthermore,

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the effect of miR156s overexpression on total wood formation was also studied. The results indicated that the miR156s expression show variable effects on their target genes in different tissues such as phloem, cambium, and xylem. For example, miR156c negatively affects *SPL20* and *SPL25*, whereas positively promotes *SPL15* gene expression, but the overall outcome was increased in wood formation [143,144]. The SNP based characterization and interactome of miRNAs in tension wood formation were studied that further implicates the role of miR156i, miR156j, miR396a, and miR6445b in biomass enhancement [115,144]. The *E. globulus* is an important plant that provides substantial amounts of cellulosic feedstocks for biofuel production. In this plant, the moderately expressed miR156e, down-regulates SPL3 TF, and plays a positive role to improve heteroblastic characteristics that prolong the vegetative phase and improves biomass substantially. It is also given that, Quantitative Trait Loci (QTL) linked MiR156 based regulatory network is regulated by natural variations that help *E. globulus* to regulate vegetative phase change under extreme environmental conditions[145,146]. The QTL-miR156 combination is the best candidate to exploit in breeding and diversification of crops. In the view of the above investigations, it can be concluded that the miR156 is a conserved master switch of the vegetative phase transition in both herbaceous and woody crops, hence, that can improve the yield of lignocellulosic feedstock.

7.1.3. The role of miR156 in the reduction of bio-recalcitrance

Currently, most of the scientific efforts are aiming to change the composition of cellulosic feedstocks, simultaneously, it must also improve amenability, saccharification, and reduce bio-recalcitrance [147]. To increase fermentable sugar or yield of biofuels, it requires to improve the relative amount of cell wall components, like cellulose or non-structural carbohydrates, reduced lignin content, and expression of endogenous hydrolyzing proteins that support more saccharification process [148,149]. Recently, large numbers of miRNAs and genes have been examined, which regulate cell wall synthesis, compositions, and bio-recalcitrance in a wide variety of plants [129,148,150]. It is well known that bio-recalcitrance is a major limiting factor that is mainly responsible for up to 45-55% total cost of biofuel production [108,151]. In most of the studies, the up-regulation of miR156 is positively correlated with the quality and amenability of lignocellulosic biomass, consequently, it substantially decreases lignin content in switchgrass, *Medicago sativa*, *Brachypodium,* and Sorghum crops [27, 28]. The moderate expression of miR156 caused reduced lignin amount up to 12.2–16.0 % with less acetyl bromide (AcBr) in transgenic plants. Simultaneously, the composition-wise amount of both guaiacyl (G) and syringyl (S), lignin monomers also reduced significantly in the same plants [152]. Similar results are also reported where the role of miR156 is well appreciated in the reduction of bio-recalcitrance and fermentable sugars yields(see table 1) [27, 28,139,153]. The main endogenous natural cell wall hydrolyzing proteins e.g., laccases also synthesized in the high expressing miR156 *Spartina alterniflora* and switchgrass plants afflicted with

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water-stress [154]. Summarily, it is inferred that the recalcitrance of feedstocks can be reduced significantly by rewiring the miR156–SPL system via synthetic biology tools [155] in the bioenergy plants. Interestingly, why the miR156 overexpressing plants show the dwarf and short morphology? Therefore, it needs to deeply investigate the crosstalks of sub-family genes of the miR156- modulated-SPL module.

7.1.4. The Role of miR156 in root development

The root system is an extremely important organ that provides structural support to the aerial part of plants. It also plays a very significant role in growth and development, water holding capacity, and nutrition supply. Simultaneously, the root system also protects plants from various kinds of abiotic stresses including drought, high salinity, and exposure to pollutants. The role of various miRNAs families in root initiation and development has been well investigated at physiological, cellular, and molecular levels [66]. This is well established that the plant root system is of great significance, particularly in bioenergy crops, because of long and well-developed, healthy, and extensive root system improves water-use-efficiency (WUE), nutrient recycling including, nitrogen absorption and assimilation. Simultaneously, both root-microbiome mediated nutrients recycling and biological nitrogen fixation (BNF) also significantly improve plant growth as seen in the case of e.g. Sorghum, *Miscanthus x giganteus*, and poplar [156]. Therefore, the appropriate availability of nutrients to roots supports the overall improvement of plant biomass yield.

During embryonic development, the overexpression of miR156 regulates the activities of two crucial *SPL3, SPL9,* and *SPL10* genes resulting in better roots development. Reversely, plants lacking the miR156 expression show features like defective roots and embryo. Thus, these results reflect the fundamental regulatory roles of the miR156-SPL system in roots growth and development. More recently, it has been reported that overexpression of miR156 decreased root meristem size, whereas downregulation of miR156 improves root size under the influence of cytokinin that endorsed by the miR156 (*p35S::MIR156*), *rSPL10* and *SPL10* loss of function experiment in Arabidopsis [66]. In two woody plants, *Eucalyptus grandis* and *Eucalyptus brachyphylla*, the interaction of miR156 and miR172 turn on activities of their two target genes *SPL5* and *APETALA 2*, as a result, control the development of the adventitious roots, but there was no interrelationship between the actions of miR156 and miR172 in stem and root tissue. However, this process is orchestrated similarly as seen in the case of flowering and growth phase transition [127, 157-159]. Here, the variable response of the miR156 is mainly based on its spatiotemporal and species-specific expression, and that needs to be satisfying accurate quantitative ratio of the miR156: SPL. Moreover, it would be noteworthy that roots development is a complex process that

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governed by complex signaling pathways that involve phytohormones, a large number of miRNAs (miR399, miRNA160, miRNA166, and miRNA167) and small RNAs like, tasiRNA long noncoding (lnc) RNAs and small regulatory peptides, called miPEPs [160]. Therefore, the role of individual components involved in roots development needs to be deeply investigated in bioenergy plants. Based on NGS methods, the genome-wide response of miR156s hyper-accumulation on target genes and their down streaming effects on root growth were studied in transgenic alfalfa. The miR156 suppressed the expressions of target genes, especially *SPL6*, *SPL12*, and *SPL13* subsequently; control variable responses of 132 classes of important down streaming genes, which further regulate root development [136]. From these considerations, it becomes clear that the miR156 overexpression targets miR156/SPL circuit that further also influenced expression of many down streaming genes involved in the synthesis of phytohormones like auxin, cytokinin, gibberellin, and their mediated signaling pathways affect root growth and nodulations[161]. Therefore, the miR156/SPL circuit emerges as an apparent target for bioengineering to improve the root system for better nutrition and water use efficiency and sustainable production of lignocellulosic crops.

7.2. The role of MiR156 in Nutrient Use Efficiency (NUE)

For proper growth and development, plants require appropriate ratios of major macronutrients like nitrogen (N), phosphorus (Pi), sulfur (S), copper (Cu), and potassium (K), etc. Since micronutrients are precursors of vital cellular components [162]. The role of different 80 families of plant miRNAs has been investigated in diverse plant species. These miRNAs are highly significant in the assimilation of macronutrients, their transport mechanisms, and metabolisms, under different growing conditions [162,163,170]. The roles of miRNAs related to nitrogen metabolism are widely studied in bioenergy plants also including, sorghum [70], and *Populus* [164]. This wealth of emerging knowledge can apply in the marker-assisted breeding and farming of sustainable bioenergy crops. Hence, lignocellulosic crops must be able to grow on degraded land without applying expensive nitrogen and phosphate-based fertilizers during their cultivation.

7.2.1 The role of miR156 in nitrogen-use-efficiency

Nitrogen (N) is closely linked with high plant growth and development, and reproduction because of its essentiality for the biosynthesis of important cellular components including, amino acids, chlorophyll, ATP, nucleotides, and metabolites [164-166]. The bioenergy crops must carry high growth potential with low agricultural inputs that mean carry out their farming with minimum addition of pesticides, irrigation, and chemical fertilizers. So plants must require inherent capacity of high nitrogen use efficiency and biological nitrogen fixation (BNF) through rhizosphere or nodulation as seen in switchgrass, poplar,

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elephant grass (*Pennisetum purpureum*, Schumach), and alfalfa. The miR156/SPL module and related TFs, which engaged in regulation, absorption, and transport of N, P, and S, are well documented in Arabidopsis, rice, and maize plants [44, 68,129, 167].

In nitrogen stressed *Populus tomentosa* plantlets, the miR156 was hyper accumulated as a most conserved family of miRNAs, as in the case of *Populus trichocarpa*. But the expressions of miR393, miR395, and miR396 was high, while miR169 and miR390 were very low [116,164]. Likewise, in the previous investigations, the miR156 regulates the DIHYDROFLAVONOL-4-REDUCTASE (DFR)/WD4-1 pathway that involved in the controlling of plant growth and development and anthocyanin biosynthesis in the response of nitrogen availability [123,137]. Thus, the miR156 not only regulates the vegetative growth of the root but also plays a role in the physiological process that provides natural nitrogen supply to nitrogen-fixing plants although this process is relevant only to selected promising bioenergy crops e.g., *Miscanthus*. By using ^{15} N enriched N_2 isotopic tracer techniques, the Associative Nitrogen Fixation (ANF) was investigated that shows the substantial level (up to 42 %) of nitrogen is fixed in various tissues of switchgrass through inoculations of diazotrophic endophytes [168-170]. Therefore, it needs to identify the role of miR156 in the cultivars, which promote nitrogen fixation in plant tissues, and thus, promote biomass growth and branch initiations through endophyte inoculations, these plants must be selected for breeding programs.

Given above, nitrogen supply is also achieved by a symbiotic relationship between plant's roots, and diverse types of nitrogen-fixing bacteria live in the nodules or rhizosphere as part of the plant microbiome [170]. Currently, a large number of bacteria like, *Bradyrhizobium*, *Rhizobium helanshanense*, S. *meliloti*, and other 190 bacterial varieties including many fungal species such as *Serendipita vermifera* [68, 69,170] are involved in BNF process. These microorganisms act as endophytic diazotrophs, and live in between extracellular space of root cells and not in nodules. Such types of plant-microorganisms relationships are identified in different lignocellulosic crops like switchgrass, poplar, Eucalyptus, elephant grass, and Miscanthus species [168 -173]. Symbiotic relations of plants with arbuscular mycorrhizal fungi (AMF) and bacteria generally reduce nutrients requirements in soil because they act as biofertilizers [168].

The roles of miRNAs in BNF are studied very well in pulses [136]. But the involvement of miR156/SPL module reported in the early stages of nitrogen fixation processes, which is very crucial [174]. In the case of *Medicago sativa* L. and *L. japonicus*, the miRNA172 overexpression targets miR172c–AP2-1 system that further regulates the expression of *ENOD40* and *SYMPK*, *POLLUX*, *CYCLOPS*, *CERBERUS*, and *NODULATION-SIGNALING PATHWAY1* genes. Hence, this module affects nodulation formation and

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efficiency in *Rhizobium etli* and *B. japonicum* based plant-microbial symbiosis [175]. Currently, the role of the miR156-SPL system is poorly studied in lignocellulosic crops. In addition to the detailed investigation of miR156/SPL module, other possibilities may be explored, e.g. transfer of BNF pathway to bioenergy crops from nitrogen-fixing plants by using synthetic biology techniques. By improving the plant-root-microbiome relationship in cellulosic biomass crops can substantially enhanced nutrients availability to plants, and thus ameliorate the adverse effects of nitrogen deficiency.

7.2.2. The role of miR156 in phosphorus use efficiency

Phosphorus (Pi) is another most essential macronutrient that is required for plant growth, development, physiology, and reproduction [68,176] because it is an inevitable part of nucleic acids, biomembranes, and energy transport reactions in cells. Phosphorus actively participates in basic metabolism and many regulatory processes, hence, its deficiency caused up to a 21% decline in the photosynthesis process and resulting in about 19.2% decrease in total biomass production [176,177]. Regulatory roles of various miRNAs in the phosphorus absorption, its transportation, and metabolism have already been described in a variety of crop plants [116,126,162]. In the case of phosphorus-deficient alfalfa plants, the miR156 reduced substantially with other 43 miRNAs families, particularly miR160, (miR172b, miR172c), and miR398 and miR399. The miR156 regulates the activity of auxin response factor (ARF) via SPL based transcription factors, hence regulating lateral root development. Simultaneously, it also promotes Pi uptakes during its scarcity, by moving itself to the target site and acting in coordination with the miR399-PHOSPHATE2 (PHO2) system. This interaction modulates the *MYB2* gene activity and ultimately turns on phosphate transporters such as PHO1 and PHT1 [178,179]. The expression of miR156 found to further regulate the expression and transport of FLOWERING LOCUS T (FT) protein via SODIUM POTASSIUM ROOT DEFECTIVE1 (NaKR1) in the phloem by regulating the *SPL3* gene activity in response to K+ availability in soil [126, 164,180]. It would be noteworthy that both miR172 and miR156 target the AP2-like family of transcription factors that regulates vegetative growth, therefore, this network helps in maintaining whole-plant nutrient homeostasis (table 1). The role of miR156 is approved by using a target mimicry mediated 35S: *MIM156* in Arabidopsis plants indicates that it down regulates *SPL3* gene in Pi-deficient plants that further turn on the activities of PLDZ2, PHT1;5 and mobile miR399 family to regulate phosphorus metabolism [181]. Therefore, miRNAs can also act as a mobile signal molecule. Moreover, various miRNAs families like miR156, miR167, miR171, miR394, miR778, miR828, miR897 miR169, miR395, miR398, and miR399 also play significant roles in phosphorus metabolism have been studied in shoots and roots of *Populus tomentosa* [180]. Among them, miRNA399 affects the expression of Pi transporter (PHT1; 7) via cleaving UBC24 transcript in *Panicum virgatum* [177,182], these results are consistent with the earlier reports in *Medicago sativa L*. Therefore, miR156 plays an important role in

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maintaining homeostasis between endogenous phosphorus concentration and externally available nutrients during different developmental stages of plants under abiotic and biotic stress.

Taken together with several reports highlighted that the miR156 performs multiple roles in plants, hence, there is needs to study, the regulatory capability of miR156 and its antagonist miR172 mediated genetic network in broad perspective like vegetative growth, flowering, abiotic stresses, and nitrogen-use-efficiency and other biological functions [159] (Table 1). Therefore, biomass yield in bioenergy plants can be sufficiently increased by targeting the miR156/SPL module consisting of several regulatory points. But it requires detailed scrutiny related to cross-talk functions in bioenergy plants. For example, miRNA family miR156, 159, 164, 166, 172, 319, 393, 396, and 414 are possibly participating in cross-talk between biomass productions under abiotic stresses so that a common protective mechanism can be devised [183-184]. The complete knowledge of the interactome involved in the response to nutrient signaling, which is very complex, is still lacking in bioenergy plants. But, a better understanding of these pathways will help to design such plants that can integrate external and internal cues under various nutrient conditions.

7.3. The role of miR156 in combating environmental stresses

The unfavorable environmental conditions such as biotic and abiotic stresses are major threats to plant growth and development. According to estimation, nearly 70% of agricultural productivity is lost due to the cumulative impact of biotic and abiotic stresses [182, 185] thus significantly hinder agricultural yields of plant biomass. Currently, the effects of various adverse climatic conditions on crops and lignocellulosic crops are the most relevant issue because it will adversely affect crop systems and overall yield in the near future. Moreover, experts have proposed that lignocellulosic crops must grow on marginal lands including degraded, saline, alkaline, and sodic soils [185]. Such land soils are always inflicted with various types of abiotic stresses that make crop plants more susceptible to pests and diseases. Recently, a wide variety of miRNAs has been discovered, which help in the acclimatization of bioenergy plants against the combined effect of biotic and abiotic stresses. Abiotic stress is a group of adverse environmental conditions that negatively affect vital cellular activities, ionic balance, and photosynthesis in plants, thus cause substantial loss of plant productivity [186-189]. Therefore, cellulosic crops must have a great level of resilience against environmental stresses.

7.3.1. Salinity stress

Salt stress is one of the major limiting factors that cause a high level of osmotic imbalance (excessive Na^+ and Cl^- ions) inside the plant cells or tissue. It adversely affects metabolism, physiology, and cellular

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homeostasis, and finally causes low plant growth [84, 133]. According to a rough estimation, salinity will affect almost 50% arable land world over by the mid of 21 century [190]. So, next-generation crops (NGC) have endurance to salinity stress, particularly lignocellulosic biomass crops. Moreover, to reap maximum economic and environmental benefits from the lignocellulosic crop system thus a crop supposed to be growing on marginal lands/degraded soils that are generally afflicted with extreme saline conditions [42, 48]. Currently, several investigations conducted in many crop plants clearly indicate that plants often employ a miRNAs based intricate gene network system that provides sufficient resistance against the salinity stress in salt-tolerant genotypes [77,191]. Therefore, it becomes essential that the roles of miRNAs families under salinity stress must investigate thoroughly across the plant species and identify the potential targets for suitable genetic manipulations. Hence, the loss of agricultural yields in lignocellulosic crops can be minimized.

Currently, the roles of various miRNAs families including, miR156 under saline conditions are reported in various cellulosic crops such as switchgrass, sorghum, poplar, Setaria species, smooth cordgrass, *Brachypodium distachyon*, giant reed (*Arundo donax* L.), Bermuda grass, and alfalfa [60,76,77,81,137,192-198]. Based on the above investigations, to know the precise roles of potential miRNAs candidates from complex miRNAs networks, therefore, the current focus is shifted to know the regulatory role of miR156 in the salinity affected wild as well as manipulated plants. The transgenic plants with hyper-accumulation of miR156 offered a substantial improvement in the form of fresh and dry biomass under the salt stress that manifestation with late flowering, improved apical dormancy, and more lateral branching and roots formations [185]. The response of salinity on the miR156/SPL module was studied in a grass halophyte (*Spartina alterniflora* Loisel), a model plant that shows a high level of salinity resistance [77, 78]. In the same plant, the miR156 was the most abundant among the most conserved miRNA species along with miR397 that regulate abiotic stress [154,199].

Recently, differential expression of various miRNAs is investigated in the leaves of five species of *Spartina alterniflora* grown in high salt containing soils, and the over-expression of miR156 correlated with a high level of salt concentration present in the plant cells [77, 137, 200]. Furthermore, both leaves and roots were investigated, almost 57.4% to 72.4% more miR156 was expressed in salt-affected than control poplar plants. But, the expression of miR156 was higher in root as compared to leaves [77, 78]. This type of findings may be due to the mobile nature of miR156, because it moves at the most prominent site of stress i.e. root, and acts at the organismal level. Saline stress also affects the expression of miR156 in Bermudagrass [*Cynodon dactylon (L)* Pers.], and mainly regulates carbohydrate metabolism [195]. The role miR156-targeted SPLs module is also identified in *Tamarix chinensis* (*T. chinensis*), a highly resistant plant to salinity. The main target of overexpressed miR156 are two *MREs* in *SPL5*gene,

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moreover, *SPL6*, *SPL7*, *SPL8* and *SPL9* are mainly involved in protein to protein interactions after being down-regulated [123]. Although a total of 14 *SPLs* genes with conserved SBP-boxes were identified that show functional redundancy in *T. chinensis* plants. Moreover, the tissue-specific expression of miRNAs is mainly responsible for the regulation of various metabolic pathways in their fine-tuning in stressed plants. In sorghum, the miR156 observed the most dominant miRNAs with other miRNAs species in salinity as well as drought-affected plants [71, 201]. In the view of above studies, the hyper-accumulation miR156 not only makes plants more resistant against salt stress via reducing expression of *SPL*s gene family but also provides plasticity to plants via regulating other gene networks.

7.3.2. Water stress

In the view of recent climate changes, drought stress is another growth-limiting factor in the crop plants [120, 202]. A plethora of reports shows that a substantial level of resistance is offered by the miR156 based SPLs system in drought-affected plants. Consequently, a high-level of antioxidants, ABA, DNA repair proteins, and another compatible solute/metabolites (e.g., proline, galactinol, raffinose, and GABA) are produced inside the different plant tissues [185, 203]. These biomolecules protect plants from harmful effects of water deficiency [123] although, this is still not known how the miR156 promotes formation of secondary metabolites in plants. In many model plants, the overexpression of miR156 mainly targets ATPase E1-E2 type protein family through the miR156/157-SPL circuit [60, 204]. In case of switchgrass, it found that the over synthesis of miR156 led to the improvement in biomass yield [34, 36,142,154]. Furthermore, genome level investigations show that the miR156 overproduction mainly influences activities of transcription factors belonging to the SBP family (table 1), in *Setaria italica* [205,206]. In order to confirm the regulatory role of miR156/SPL module in water-stressed plants, genome-wide degradome sequencing studies were also carried out that also support aforesaid reports [137, 207]. The ABA level and miR156 expression are well documented in various plant species affected with abiotic stresses [133]. Several experiments show that the up-regulation of the miR156 gene and high level of ABA protect plants from a group of abiotic stresses including water scarcity, by activating the downstream dehydration-responsive element-binding protein/C-repeat binding factors (DREB/CBFs) based signaling pathway [208]. The DREB mediated cascade includes activations of a large number of structural and regulatory genes and proteins/enzymes and transcription factors, which enhance antioxidant activities and osmolytes synthesis, thus finally improve the overall yield of plant biomass under water stress [188]. The contribution of miR156 in DREB based signaling is already conformed in model plants by using the target artificial mimicry method [131,135] but still poorly described in lignocellulosic biomass crops.

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The anthocyanin biosynthesis provides a substantial level of protection to plants under water stress conditions. This process is mainly governed by the expression of miR156 targeted *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 9 (SPL9)* and *DIHYDROFLAVONOL-4-REDUCTASE (DFR)* pathway which are well studied in model plants [87,123]. Although it also requires the participation of several other genes like *ANS, F3'H, UGT75C1*, and *UGT78D2*, and their roles are proven by Pro35S: *MIR156* target mimicry in plants. Moreover, it is also reported that the miR156 suppresses the activities of *SPL*9, consequently, prevents further interaction of PAP1 with TTT8 and TTG1 that activate the MYB-bHLH-WD40 mediated transcription activation complex that promotes formation of anthocyanin and proanthocyanidins [123, 209]. A similar type of response is also observed in transgenic switchgrass plants. Interestingly, the miR156 expression was substantially higher in water-stressed than salinity affected plants [60,139]. Recently in the case, the sorghum [70, 73] many trans-acting small interfering RNAs (tasi-RNAs) isolated, which are regulated under the miR156 biogenesis pathway in drought susceptible (DS) plants. The same study also shows a variable expression of miR156 family members, for example, more than 50% expression of miR156a and miR156* noticed in DT species, whereas miR156a down-regulated in DS. In drought-affected Setaria plants, miR156c was down-regulated, while up-regulated in susceptible cultivars with other miRNAs such as miR160d, and miR6248a species [76, 205, 206, 208]. These results show the functional diversification of miRNAs is quite evident for fine-tuning of biological functions in plants.

To develop such lignocellulosic cultivars that can perform optimally under multiple abiotic stress conditions. A large scale microRNAs profiling is conducted in switchgrass leaves under multiple abiotic stresses e.g., drought and heat stress. Consequently, 29 conserved including miR156 and miR398 with 62 other novel miRNA families and their targets were identified. These miRNAs species further target *SPL*, MYB, ARF17 (auxin response factor), HD-Zip (homodimer leucine zipper), AP2 (activator ptotein2), heat shock proteins (HSPs) and laccase. But it is palpable that the over-accumulation of miR156 provides a high level of thermo-tolerance against moderate to severe heat stress through downregulating the miR156/SPLs module [66, 137]. However, several classic works already reported the role of miR156 overexpression and its positive role in the improvement of plant biomass in major bioenergy crops [60, 76, 77, 81, 137, 192-198]. But to trace out a common gene regulatory mechanism that involves miR156 needs to unravel hidden interactions among crucial genes, proteins, and metabolites, which operate under water stress.

7.3.3. Cold stress

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Generally, C4 perennial lignocellulosics are well adapted to temperate regions, and hence, prove highly productive plants due to having a highly efficient photosynthesis system. But growth and development, and productivity of C4 plants are adversely affected by extreme cold temperatures. To enhance plant biomass yield, bioenergy plants must be resilient to extremely cold conditions that mainly prevailed in the Europe, USA and other parts of the world [44]. The genome-wide expression of cold-responsive miRNAs is investigated in *Brachypodium* seedlings, which reports a differential expression of 3 conserved miRNAs and 25 other miRNAs families. Though the role of miR156 could not be determined, its expression increased up to 5 folds with other miRNAs like miR169, miR393, miR396, miR394, and miR398 that mainly target eight different genes such as *SBP, ARF*, and *AUX/IAA TFs*. Furthermore, these genes regulate an intricate network of regulatory proteins (NAC, NAM, ATAF, and CUC), and nearly 105 other genes, which mostly encode for various transcription factors in both cellulosic and crop plants [196, 201-212]. Recently in Bermudagrass, the miR156 showed down-regulation and linked with an enzyme β-glucosidase and coniferyl-aldehyde dehydrogenase regulates carbohydrate metabolism under alone cold or combined cold and saline stress conditions [195]. These reports are also confirmed, where the miR156 is suppressed by the overexpression of BdVIL4 (VERNALIZATION INSENSITIVE 4) protein during flowering under low temperature. To further investigate the role of the miR156 across the different lineages in the plant kingdom under cold stress, a cloning based study is carried out in Arabidopsis, rice, and pine [213].The observed overexpression of the OsmiR156 mainly targets *SPL3*, a positive effector of *OsWRKY71* that further negatively regulates the expression of the two most important OsMYB2 and OsMYB3R-2 proteins. It is well known that the MYB transcription factor is responsible for creating a substantial level of resistance against cold stress in *Brachypodium distachyon* and *Panicum virgatum* [204, 214-216]. In the same experiment, the OsmiR156 expressing cell lines having considerable over expressions of *OsKNOLLE2, OsCTP1, OsCycB1.1, OsCycB2.1*, and *OsCDC20.1* genes, which are involved in the cold stress controlling pathway [217]. In light of the above reports, the variable response of miR156 family in different crops is attributed to species and tissue-specific expression of miRNAs that target different mRNAs in plants under diverse abiotic stresses.

7.3.4. Heavy metal stresses

Some researchers have suggested that lignocellulosic biomass crops can be exploited as cost-effective phytoremediation as well as biomass supplying agents. Therefore, it becomes essential that dedicated bioenergy plants must be able to grow on non-arable, barren, and contaminated soils containing a high concentration of heavy metals. To exploit lignocellulosic crops in the process of environmental purification, climate mitigation, and restoration of degraded land, that would be an additional benefit linked with the energy crop system [42, 218]. But it needs to study the molecular response of bioenergy

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crops to metal toxicities. Although numerous NGS based deep-sequencing studies unravel the roles of metal-regulated miRNAs involved in sulfate allocation and assimilation, phytohormone signaling, antioxidation pathways in many crop plants [219-221]. So far, very little knowledge is available related to miRNAs expression and their regulatory role under heavy metals stresses in cellulosic biomass crops. Generally, plants require physiological concentrations of several trace metal ions, for instance, iron (Fe), copper (Cu), Zn (zinc) and manganese (Mn) to maintain ions homeostasis inside cells for proper plant growth and development, and production [222]. But, the extreme amount of the ions in the rhizosphere disturbs cellular structural, physiological, and molecular homeostasis inside cells, therefore, this significantly reduce agricultural productivity of crops [219–221].

Many genome-wide studies have identified the up-regulation of miR156, miR393, and miR395, while miR159, miR162, miR166, miR171, miR390, and miR396 were down-regulated under Arsenic metal stress. The higher expression of various miRNAs act on their targets like miR156 (SPL-TF), miRNA162 (DCL), miR390 (SRK) miRNA396 (GRFTFs, rhodenase-like proteins, kinesin-like protein B), and miR397b (LACs) in the model and well as crop plants grow on soils affected with heavy metal stress, such as Cd, Hg and As [148,153]. In a highly significant secondary data scrutiny [149,154] has reported that the overexpression of miR156 counters to high concentrations of Mn, As, Al, conversely, its expression got a decrease in the case of Hg and Cd. The miR156 mainly targets the 5'CCG sequence in *SPL7* gene that encodes glutathione-g-glutamylcysteinyl transferase, a protein which further activates PC based metal chelation/detoxification mechanism in Brassica. In *Medicago truncatula* plants, both miR156 and miR395 target mainly to heme/steroid binding domain protein that affects the plant response to cadmium ion.

In addition to the above-mentioned implications of the miR156/SPL module, it also plays a very significant role in additional abiotic stresses such as heat, UV-B stress, recurring stress memory, ozone stress, and mechanical stress, etc. in *Populus tremula,* and other plants [137, 182, 223]. However, the biogenesis of stress-responsive miRNAs species is evolutionarily conserved across the plant kingdom, but their response to environmental stresses mainly depends on types of miRNA species, stresses, tissues, and genotypes. It might be due to great sequence variability among the mRNA/mRNA* duplex and target transcripts (mRNAs), and interactome of regulatory proteins, such as DCL1 and AGO 1, which are involved in the biogenesis and target pathway.

Currently, xenobiotic detoxification for phenanthren is studied in the leaf of two interspecific hybridization and allopolyploid *Spartina* species, (i.e. hybrid *S. x townsendii* and the allopolyploid S. *anglica)*. Using *MIR159* and *MIR156* mutants indicate the involvement of miR156-SPL- miR159

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regulatory module in plants that inflicted with aromatic pollutants. The overexpression of miR156 affects *SPL2* and *SPL3* but not *SPL13* genes, simultaneously, it also down-regulate the expression of miR159 and MYB33/62, MYB33 TFs via miR156/SPL network [78, 81]. These findings are very significant because MALs would be proposed sites for farming of cellulosic biomass crops. It is worth mentioning that MALs are affected by various types of pollutants that affect plant biomass growth adversely. Therefore, the miR156-SPL module can be a potential target to improve plant resilience against the polycyclic aromatic hydrocarbon that adversely affects plant biomass growth.

8. The role of miR156 in the development of plant immunity

It is already stated that lignocellulosic crops must be able to grow on the marginal lands that are more prone to biotic stresses. There are several biological stressors like, viruses, bacteria, fungi, pests, and nematode that adversely affect crop production by up to 30% [224]. Plants respond to these pathogens by expressing a variety of miRNAs, which regulate the gene functions related to disease resistance [90, 225]. Various species of miRNAs regulate plant immunity under pathogenic attacks through manipulating pathogen-associated molecular patterns (PAMPs) and by employing many regulatory proteins [226, 227]. Based on detailed investigations in many plants, the expression of conserved miRNAs families are noticed in the response to biotic stress. These are miR156, miR159, miR160, miR166, miR398, miR1511, miR1514, miR2118 miR358, which overexpressed, and further regulate the activities of other genes such as DNA binding *Auxin response factor (ARF)*, a gene that regulates Auxin metabolism, enzymes superoxide dismutase (SOD), homeodomain and C_2H_2 zinc fingers, and also target a network of AP2/ERF, bZIP, MADS-box, MYB, NAC and WRKY finger (transcription factors) and various metabolites in the case of viral infection [90, 226]. Interestingly, these TFs also participate to generate plant response against abiotic stress, and immunogenic response to cope with biotic stresses as well.

In switchgrass, the overexpression of miR156 makes transgenics more susceptible up to 195% against rust (*P. emaculata*) infection by affecting MYB4-TFs (myeloblastosis-4 transcription factors) [27, 28,41]. In the case of foliar fungal infection caused by the *Melampsora laricipopulina* in *Populus szechuanica* seedlings, the high level of miR156 (up to 60%) was observed with miR166, in the affected plants. Furthermore, the up-regulated miR156 mainly turns the activities of the nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains that offer disease resistance by manipulating the activity of ubiquitin C. [227, 228]. It is well known that ubiquitin C based cascade is involved in the DNA repair and miRNA biogenesis pathways against the various types of abiotic stresses.

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Although similar kinds of results are also reported in various strains of poplar plants, the appearance of miRNAs species is changed with types of infectious agents, for example, during plant-microorganisms interactions microbe-associated molecular pattern (MAMP) rewire miRNA based genetic networks [226, 229]. Recently, the amiRNA technology has been applied as a potential tool to enhance resistance in plants against various biotic stresses. In Arabidopsis, an artificial miR159 based strategy applied to develop resistance against both Turnip yellow mosaic virus (TYMV) and Turnip mosaic virus (TuMV) [90, 226, 230]. In search of common regulatory mechanisms to improve the resistance potential of bioenergy crops against the pathogens, therefore, we need systems biology approaches that examine the cumulative effects of abiotic and biotic stresses in affected plants, and put forth a common molecular response in terms of miRNAs expressions.

9. The role of miR156 in the gene biocontainment

In order to fulfill future energy demands, it requires to genetically develop such ideal lignocellulosic plants, which carry suitable commercial biofuel traits. In this direction, scientists have applied genetic engineering and synthetic biology techniques to bioenergy plants resulting in the production of transgenic crops or high yielding varieties. But grass crops transgenics posed a great ecological challenge of transgene gene flow in the native vegetation. This is a big regulatory concern for the farming of genetically manipulated lignocellulosic crops that ultimately hindered their adaptation, breeding, domestications, and commercialization [45]. To overcome this pressing problem, several traditional approaches like, pollen removal, plant separation distance, and crop surroundings are used [231]. Simultaneously, a genetic method of pollen inactivation by using *Bxb1/att* recombination system also applied in switchgrass, but could not prove more effective [232].

The miRNAs based genetic strategy that improves biofuel feedstocks simultaneously, it also reduce flowering. Therefore, miRNAs based crop manipulation is a more suitable option for bio-contentment (figure 2). This could be achieved by the moderate overexpression of miR156 under the constitutive maize ubi-1 promoter in switchgrass plants. The miR156 plays a significant role in the flowering process, which is discussed above [27, 28]. Currently, two full flowering cycles were observed in switchgrass plants where moderate to overexpressed miR156 transgenics that grow on non-agricultural land. The results of two seasons clearly show that medium level miR156 expression phenotypes) show late flower or reduced flowering up to 70.6% with almost 96% fewer seeds production, and panicles as compared to control plants [27-29].

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Currently, the constitutive overexpression of miR156 by using suitable promoters or enhancers can prove a better strategy for bioconfinement in the case of transgenic cellulosic crops (table1). The synthetic biology approaches like, the introduction of artificial amino acids, micro proteins, and the nucleotide in proteins and genetic material respectively, by using synthetic protein and gene design strategies [233], hence, biocontainment can achieve in the future. Since, several experiments have already tested the efficacy of artificial RNAs based biocontainment methods.

10. Conclusion and future prospects

Recent advances in the RNA detection technologies, particularly in NGS-based deep sequencing and bioinformatics tools have revolutionized the area of miRNAs profiling in animals and plants. It is well known that miRNAs control virtually all aspects of plant's life. Currently, a significant progress has been made in the identifications of specific miRNAs species, and their roles in flowering, phase transitions, and aging pathways in crop plants. Plant miRNAs are deeply involved in the fine-tuning and controlling of various metabolic pathways under various adverse environmental conditions that can be endorsed by the miR156/ SPLs module that can play very significant regulatory roles in lignocellulosic crops. But in the view of several conflicting reports related to a different mechanism of the same miRNA family within the same plant or the conserved functions of a miRNA species across a wide variety of plant species e.g. miR156. This reflects the two important characteristics of plant miRNAs i.e. functional conservation and diversity, hence, it can be concluded that miRNAs regulate complex gene networks in a hierarchical and context-based manner to efficiently regulate plant functions at transcriptional, translation, and epigenome levels. These types of regulatory strategies provide plants an enormous opportunity to efficiently manage their energy and resources under unfavorable climatic conditions. Simultaneously, it also offers to identify precise and potential targets for genetic manipulation in plants. But it also requires a meticulous study of miRNAs and their relationships with biofuel traits at the molecular level in cellulosic biomass plants. So far the knowledge related to miRNAs, and their targets genes, small RNAs, regulatory proteins, and their post-translational modifications in plants, is still far from perfect.

To exploit the full potential of the miR156/SPL system to enhance the agronomic traits like biomass yields, and ultimately improved biofuel supply. It requires a more detailed investigation of *SPL*s genes functions owing to their enormous molecular diversity prevailing in the plant system. So that molecular level details about the miR156s and their targets SPLs gene family including its subfamily members are required, therefore, the actual regulatory roles of each component could be assigned without redundancy. In these endeavors latest tools of RNA biology, including transgenesis, cisgenesis, intragenesis, artificial *MIR* genes, endogenous and artificial target mimicry, short tandem target mimic technology (STTM) can play very significant roles. A piece of enormous knowledge has been gained about the miR156s biology

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in crops and model plants that can provide a solid foundation for bioengineering of this module by using genome editing and pathway engineering techniques. Therefore, bioengineering tools, for instance, Meganucleases, ZNF proteins, TALENs, and CRISPR/Cas9 or CRISPR/Cpf1, CRISPR/dCas9, or dCpf1, CRISPR13a can introduce precise mutations or base changes thus will help in the breeding and domestications of bioenergy crops. Since next-generation genome editing tools are more precise and accurate to manipulate miRNA based gene networks. As already mentioned that miRNAs regulate processes all three working levels, such as transcriptional, post-transcriptional levels and epigenetic levels that can be manipulated by CRISPR/Cas9 technologies. But, there are several challenges in the manipulation of bioenergy crops due to their complex nature of genome structure, which leads to the off-target manipulation that can be rectified with the availability of quality whole genome sequences and complete annotation of gene products very soon. The lack of sufficient germplasm and genomic resources are another pressing problem in these crops.

The sustainable supply of lignocellulosic feedstocks will ensure climate change mitigation and low-cost supply of biofuels and bio-products inside the integrated biorefineries in the future. To achieve this goal, therefore, lignocellulosic crops must grow on the about 2 billion Mha of MALs/non-agricultural lands, available worldwide. But the agriculture of lignocellulosic crops on degraded soils, and to get a sufficient supply of feedstocks sustainably is an uphill task. In this endeavor, the exploration of the multitasking miR156-SPL module can be a viable option that provides opportunities to increase biomass yield by improving nutrients and water use capabilities of plants under extreme environmental conditions. Therefore, the current yield gap per hectare (15 tonnes ha-1year-1) can be fulfilled by improving lignocellulosic biomass harvest through genetically manipulating the miR156/SPL module. Simultaneously, it also reduces biomass recalcitrance of feedstocks, and finally provides a supply of profitable biofuels, consequently, the future energy demands can be achieved, In the future, it may be possible that the complete miR156/SPL module can be transformed into lignocellulosic plants from a well-characterized plant species e.g., *Arabidopsis*. But there is still a lack of information about the miR156/SPL module, and its relationship with physiological changes in plants, for example, how it regulates the synthesis of primary and secondary metabolites in the response of internal and external cues. Moreover, there is an urgent need for species-specific investigations of miRNAs families in crops as well as in lignocellulosic crops.

Recently, single domain-containing proteins, also known as microproteins are used as a major modifier of key regulatory proteins, which are involved in the biogenesis pathway and working mechanisms of miRNAs. These microproteins can bring substantial changes in the activities of proteins, such as transcription factors, ARGONAUTE (AGO) proteins, and small regulatory peptides (miPEPs). But, the

complete interactome of all components of miRNAs biogenesis pathways is not yet fully known, because it does not involve only DCL1, DRB1, SE, and AGO1 but also includes many unknown factors and their post-translational modifications. Recently, several *cis*-acting regulatory elements, including the miR156-SPL system have been identified, which are involved in the domestications of agronomic traits in crops plants, therefore, miR156 can exploit for rapid domestication and speed breeding of lignocellulosic crops by using new synthetic biology tools.

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