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Review article

Manipulating microRNAs for improved biomass and biofuels from plant feedstocks

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Summary

Petroleum-based fuels are nonrenewable and unsustainable. Renewable sources of energy, such as lignocellulosic biofuels and plant metabolite-based drop-in fuels, can offset fossil fuel use and reverse environmental degradation through carbon sequestration. Despite these benefits, the lignocellulosic biofuels industry still faces many challenges, including the availability of economically viable crop plants. Cell wall recalcitrance is a major economic barrier for lignocellulosic biofuels production from biomass crops. Sustainability and biomass yield are two additional, yet interrelated, foci for biomass crop improvement. Many scientists are searching for solutions to these problems within biomass crop genomes. MicroRNAs (miRNAs) are involved in almost all biological and metabolic process in plants including plant development, cell wall biosynthesis and plant stress responses. Because of the broad functions of their targets (e.g. auxin response factors), the alteration of plant miRNA expression often results in pleiotropic effects. A specific miRNA usually regulates a biologically relevant bioenergy trait. For example, relatively low miR156 overexpression leads to a transgenic feedstock with enhanced biomass and decreased recalcitrance. miRNAs have been overexpressed in dedicated bioenergy feedstocks such as poplar and switchgrass yielding promising results for lignin reduction, increased plant biomass, the timing of flowering and response to harsh environments. In this review, we present the status of miRNA-related research in several major biofuel crops and relevant model plants. We critically assess published research and suggest next steps for miRNA manipulation in feedstocks for increased biomass and sustainability for biofuels and bioproducts.

Introduction

Global energy infrastructure relies upon dwindling fossil fuel supplies. To address this problem, researchers are investigating sustainable, environmentally friendly fuels that can be used in current infrastructure. The most pertinent need, arguably, is a partial replacement for liquid transportation fuels (Masjuki *et al.*, 2013). A combination of new technology and alternative energy sources, like renewable electricity and lignocellulosic biofuels, could halve fleet greenhouse gases emissions by 2050 (Bastani *et al.*, 2012). This decline in greenhouse gases could then slow the progression of climate change to minimalize long-term environmental effects. Advanced lignocellulosic biofuels derived from biomass crops fit the above criteria and could play a major role in mitigating climate change over time. These biofuels are produced by subjecting plant biomass to pretreatment, fermentation, liquidification and pyrolysis techniques (Nigam and Singh, 2011).

Switchgrass (*Panicum virgatum*), poplar (*Populus* spp.), maize (*Zea mays*), sugarcane (*Saccharum ssp.*), sorghum (*Sorghum bicolor*), cassava (*Manihot esculenta*) and *Jatropha curcas* are examples of plant feedstock species for bioenergy and bioproducts under various degrees of development. They also represent plants that grow in a range of habitats and have various life history strategies. Maize, sugarcane and sorghum feedstocks are currently used to produce ethanol from starch or sucrose, whereas perennial plants such as switchgrass and poplar are suitable to produce lignocellulosic biofuels (Yuan *et al.*, 2008). In addition, biodiesel can be produced from the seeds of feedstocks such as canola (*Brassica napus*), soya bean (*Glycine max*) and *J. curcas* (Yue *et al.*, 2013). Plant biotechnology is a useful approach to maximize feedstock development and address challenges of plant-derived biofuels and bioproducts. We believe this is especially the case for lignocellulosic biofuel feedstocks given their level of recalcitrance to cell wall digestion, for which plant breeding is unlikely to sufficient address (Baxter and Stewart, 2013).

The role of miRNAs in plant biology

MicroRNAs (miRNAs) are an extensive class of small (i.e. 20–24 nt long) regulatory RNAs (Jin *et al.*, 2013; Zhang *et al.*, 2006). miRNAs originated in the early plant evolutionary lines (Figure 1) (Taylor *et al.*, 2014). Emerging evidence shows that miRNAs participate in the regulation of a wide range of plant development and growth processes (Martin *et al.*, 2010). As shown in Figures 1 and 2, many miRNA families seem to be conserved among all plants, whereas others are found only in certain taxa in

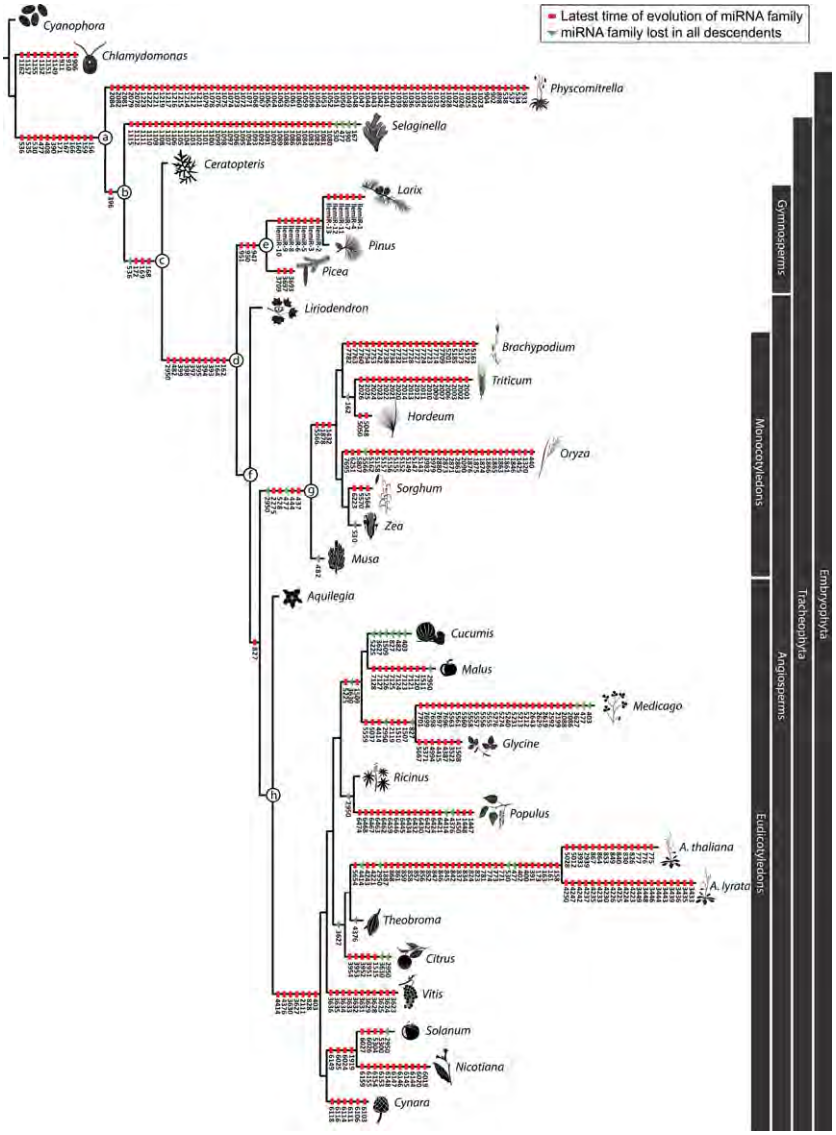

Figure 1 Validated miRNA families in 31 taxa of the plant kingdom. Plant phylogeny terms are used to describe groups of taxa with similar evolutionary characteristics. Biofuel feedstocks are represented by genera such as *Sorghum, Zea* and *Populus*. Several of these taxa hold miRNA that have been lost in all descendents, as indicated by the green arrow depicted in the figure key. Others contain a high number of recently evolved miRNA, such as *Populus*. Taxa including a high number of recently identified species will be particularly useful for future miRNA manipulations. It appears that the abundance of novel miRNAs follows no distribution, as more recently evolved taxa could potentially hold the same number as older taxa (Taylor *et al.*, 2014). Reprinted by permission.

which patterns of conservation seem to be taxonomically related (Zhu *et al.*, 2011b). miRNA-mediated gene regulation is facilitated by miRNAs selectively targeting protein-coding genes at the post-transcriptional level; the result is target transcript cleavage and decreased translation. Thus, miRNAs function through their targeted genes (Naqvi *et al.*, 2012). In the past decade, miRNA-related research has become one of the hottest topics in plant biotechnology, particularly in combination with ever-increasing deep sequencing of plant genomes and transcriptomes. miRNAs have become a new target for improving plant biomass, quality and tolerance to environmental stresses (Zhang and Wang, 2014). miRNAs have been identified and characterized within a variety of plants, including many potential biofuel species and their models (Figure 1) (Taylor *et al.*, 2014). miRNAs with similar sequences (usually less than a 3 nt difference) and common functions are classified into the same miRNA family. miRNAs appear to be inherited via descent, which explains similar functionality among related taxa (Figure 2) (Zou *et al.*, 2014). miRNA families are named sequentially in the order in which they are published. In addition, the 'miR' prefix signifies that a member is known to produce a mature miRNA (Ambros *et al.*, 2003; Griffiths-Jones *et al.*, 2006).

While in the past it was thought that miRNAs typically regulate the gene expression of a single target, recently miRNA–miRNA interactions have been documented. In this scenario, one miRNA regulates the expression of another miRNA, rather than a conventional target gene (Guo *et al.*, 2012). miRNA can have synergistic relationships that change regulatory outcomes in plants when two miRNAs form a complementary duplex structure consisting of a miRNA pair (Lu *et al.*, 2009). Single miRNA interactions sometimes do not capture the breadth of gene regulation that occurs within an organism. Instead, miRNA–miRNA relationships can be observed within an overall network of gene interaction and regulation. Most miRNA–miRNA network research has focused on animal rather than plant systems (Xu *et al.*, 2011). Nonetheless, Xu *et al.* (2011) constructed a miRNA network for soya bean (*Glycine max*), that can be applied to other plant species. This network depicts functions within a genomic context to predict mechanisms related to stress, nitrogen fixation and other plant processes (Xu *et al.*, 2014). Similar networks have

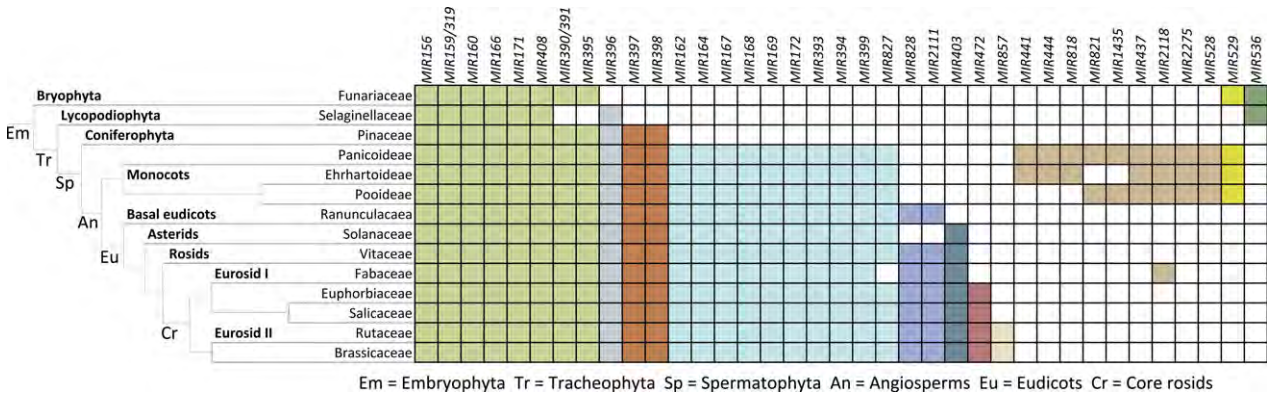

Figure 2 Conserved plant miRNA species. The columns display miRNA families that are shared among the plant families, as rows. Highlights indicate that the miRNA family has been located in at least one species within a plant family. Various colours indicate taxonomic range of miRNA families. (Cuperus *et al.*, 2011). Reprinted by permission.

been constructed for *Physcomitrella patens,* which identified many shared gene targets among miRNAs that were related to drought stress (Wan *et al.*, 2011). This approach should be applied to other plant species in order to understand miRNA functions in global gene regulatory networks.

Pressing problems for biofuel feedstocks

Recalcitrance (i.e. the difficulty of extracting sugars from cell walls), bioconfinement (i.e. the prevention of unwanted gene flow), and low nitrogen and water use efficiencies are some of the most important issues of bioenergy feedstocks (Börjesson and Tufvesson, 2011; Joyce and Stewart, 2012; Phitsuwan *et al.*, 2013). Bioenergy feedstocks also share 'normal' stress problems common to many crops that limit productivity, such as abiotic and biotic stress (Table 1) (Goel and Madan, 2014; Ndimba *et al.*, 2013; Zeng *et al.*, 2014b). Of these issues, one of the most obvious challenges of lignocellulosic bioenergy production from dedicated biomass crops is recalcitrance, which translates into low biofuel yield. In turn, decreasing recalcitrance translates to increased biofuel per unit of biomass (Phitsuwan *et al.*, 2013). Recalcitrance is thought to be caused by occlusion of cellulose and hemicellulose in cell walls by lignin in addition to other factors that are not completely understood (Jung *et al.*, 2012; Kim *et al.*, 2011). Therefore, lignin has been a prominent target to alter in lignocellulosic feedstocks (Neutelings, 2011; Zeng *et al.*, 2014b). On the biorefinery end, pretreatment breaks apart cell walls to increase biofuel yields, but pretreatment increases the total biofuel cost per litre by at least 20% (Kumar *et al.*, 2009; Yang and Wyman, 2008). Therefore, it is imperative to decrease lignin and recalcitrance in feedstocks. This would reduce or eliminate the need for biomass pretreatment (Jin *et al.*, 2013; Zhou and Luo, 2013).

Some feedstocks, such as those used to produce oil (e.g. *Jatropha),* do not have the recalcitrance problem, but they might still need to be genetically engineered for other reasons (Gressel, 2010; Yue *et al.*, 2013). For example, it is likely that all engineered feedstocks will need transgene bioconfinement (Kausch *et al.*, 2010; Liu *et al.*, 2013b). Bioconfinement becomes important especially for large-scale growth of transgenic crops with wild relatives; transgenic pollen and seeds will likely be released into the environment. Thus, adventitious presence of transgenes in the original host species and hybridization and introgression into wild relatives create potential ecological and regulatory problems (Ellstrand *et al.*, 2013; Kwit *et al.*, 2011). One bioconfinement strategy is gene use restriction technology (GURT). GURTs typically take the form of engineering conditional male and female sterility to control when and where plants

Table 1 Pressing problems for biofuel production and commercialization. These issues could be addressed through manipulation of miRNA expression. The description and impact of potential issues are included.

Issue	Description	Impact	Sources
Recalcitrance	Limited sugar availability	Low energy yield Low commercial potential	Joyce and Stewart (2012), Jung *et al.* (2012), Kim *et al.* (2011), Kumar *et al.* (2009), Ndimba *et al.* (2013), Neutelings (2011), Phitsuwan *et al.* (2013), Zeng *et al.* (2014b)
Stress	Abiotic and biotic	Low energy yield Low commercial potential	Goel and Madan (2014), Ndimba *et al.* (2013), Phitsuwan *et al.* (2013), Zeng *et al.* (2014b)
Lack of bioconfinement	Gene flow into native species	Ecosystem degradation Regulatory issues	Ding *et al.* (2014), Gressel (2010), Kausch *et al.* (2010), Liu *et al.* (2013b), Sang *et al.* (2013)
Low nitrogen use efficiency	High nitrogen fertilizer use	Environmental degradation Limited commercial application	Börjesson and Tufvesson (2011), Hirel *et al.* (2007), Liska *et al.* (2009), Phitsuwan *et al.* (2013)
Low water use efficiency	High water use	Environmental degradation Limited commercial application	Börjesson and Tufvesson (2011), de Fraiture *et al.* (2008), Liska *et al.* (2009), Phitsuwan *et al.* (2013)

reproduce (Ding *et al.*, 2014; Kausch *et al.*, 2010; Sang *et al.*, 2013).

Another important sustainability issue for biofuel feedstocks is the efficient use of water and nutrients. Nitrogen use efficiency and water use efficiency are important to optimize in feedstocks as it will not be economically or ecologically sustainable to apply much fertilizer or irrigation to bioenergy crops. These factors are important with regard to practical yield and carbon sequestration that can be obtained under practical agronomic growth conditions (Hirel *et al.*, 2007).

Taken together, recalcitrance, bioconfinement, and nitrogen and water use efficiencies must be considered when engineering feedstocks to maximize biofuel yield per hectare in a sustainable fashion (Table 1). Fortunately, all of these challenges can be, at least partially, addressed by regulating miRNA expression in feedstock crops, which will be the focus of the remainder of the review.

Key miRNA families in the improvement of biomass and biofuel yields

miRNAs are thought to be genomic adaptations to fluctuating environments (Covarrubias and Reyes, 2010). Manipulation of miRNA expression levels could provide an effective strategy for improving plant biomass in response to various biotic and abiotic stresses (Zhang and Wang, 2014). In this review, we focus on *Brachypodium distachyon*, cassava *(Manihot esculenta), Jatropha curcas,* poplar *(Populus* spp.*),* sorghum *(Sorghum bicolor)*, sugarcane *(Saccharum* spp.*),* maize (*Zea mays*) and switchgrass (*Panicum virgatum*) as a representative range of bioenergy feedstocks and models (Khraiwesh *et al.*, 2012; Le *et al.*, 2013; Somerville *et al.*, 2010). While *Brachypodium* is not a biofuel crop, it is a well-studied model grass species that shares characteristics with many biofuel feedstocks (Opanowicz *et al.*, 2008). miRNA surveys have been conducted in several biofuel plant species. These surveys illustrate taxonomic patterns among miRNA distributions. This review is centered around taxonomic divisions of miRNA families (Figures 1 and 2). While there are over thirty highly conserved plant miRNA families, most of our focus is on selected miRNA families that play an important role in plant growth and development, phase change, response to abiotic and biotic stress and recalcitrance (Table 1, Figure 2). Therefore, the least bioenergy-relevant miRNAs among the highly conserved miRNA families are omitted entirely from the review (Figure 2). Where there is a lack of experimentally validated miRNA data for biofuel feedstock species, we will speculate upon the relevant potential miRNA application to biofuel feedstocks based upon our knowledge of miRNA studies in *Arabidopsis* and other plant species (Vishwakarma and Jadeja, 2013; Xie *et al.*, 2010). Further research to experimentally validate target identification and miRNA–miRNA interaction in feedstock crops is necessary to corroborate such speculation.

Each relevant miRNA family typically affects various traits simultaneously. Furthermore, changes in gene expression can affect the suite of phenotypes and pleiotropic effects (Bartel, 2009; He and Hannon, 2004; Schwab *et al.*, 2005). Therefore, we organize the first portion of this review on the effects of widely conserved miRNA families rather than trait to reduce redundancy and increase clarity with regard to genes that should be most relevant to bioenergy crops (Table 2). In addition, we also note that the organization of the paper focuses on the number of species in which the miRNAs are conserved. Accordingly, the miRNAs that are highly conserved among the selected biofuel species are discussed first. We follow this discussion with analysis of minor and unconserved families organized by the processes and traits that they affect. We chose this strategy because there is less overlap in the function of these miRNA families compared to highly conserved miRNA families (Table 2). In addition, we know of no published research on miRNA–miRNA interactions in any biofuel feedstock species, although this is likely to change as technology becomes more accessible. Therefore, the review includes only single known miRNA–miRNA interactions in plants.

miR156 plays a versatile role in development, recalcitrance, bioconfinement and stress response

miR156 is an evolutionarily conserved miRNA that appears to be ubiquitous among vascular plant species (Figure 2, Table 2). miR156 functions by repressing the transcripts that encode for the *squamosa promoter binding protein-like family* (*SPL*) class of transcription factors in many plant species, including *Arabidopsis* (Gandikota *et al.*, 2007; Wang, 2014; Wei *et al.*, 2012). In addition, *SPL* positively regulates organ size through cell number in *Arabidopsis* (Horiguchi and Tsukaya, 2011; Wang *et al.*, 2008). miR156 is associated with a variety of other positive roles within plant development and phase transition, including floral meristem formation and the morphology of juvenile cell walls and leaves (Chuck *et al.*, 2007; Jeong *et al.*, 2013a; Unver and Budak, 2009). miR156 also regulates leaf number and apical dominance as shown in research by Schwab *et al.* (2005). miR156 expression levels are high in young seedlings (Zhou and Luo, 2013). The expression level of miR156 tends to decrease as a plant matures, during which miR172 expression increases (Rubinelli *et al.*, 2013; Wu *et al.*, 2009). Thus, when miR156 expression decreases, *SPL* expression increases (Yang *et al.*, 2011). *SPL* then promotes flowering through two distinct pathways: miR172 and MADS-box genes (Wang *et al.*, 2009; Wu *et al.*, 2009). This phenomenon has been observed in *Arabidopsis* and several other plant species (Wu and Poethig, 2006; Zhou and Wang, 2013). This further supports the conclusion that miR156 is a major regulator of plant development.

Recent studies show that miR156 is an excellent candidate for increasing plant biomass and altering lignin content (Fu *et al.*, 2012; Rubinelli *et al.*, 2013; Schwab *et al.*, 2005). The *cg1* gene, which is targeted by miR156, appears to positively influence gene expression of phenotypic characteristics suggestive of ancestral grasses, such as increased tiller number and perennialism. These characteristics are also important biofuel feedstock traits (Chuck *et al.*, 2007). Overexpression of miR156 significantly increases plant biomass in transgenic model plant species *Arabidopsis* as well as biofuel crop switchgrass (Fu *et al.*, 2012; Schwab *et al.*, 2005). Overexpression of miR156, even at a relatively low level, increases plant biomass yield in switchgrass by 58%–101% (Fu *et al.*, 2012). Overexpression of miR156 also changes lignin content and composition for limiting recalcitrance (Chuck *et al.*, 2007). Overexpression of miR156 in poplar also results in a 30% decrease in lignin content (Rubinelli *et al.*, 2013). The syringyl-to-guaiacyl monolignol ratio is significantly lower than that in the wild-type plant (Rubinelli *et al.*, 2013). In addition to plant architecture, plant development is also an important element in biofuel improvement. Phase change and flowering are critical processes in plants. Phase change represents the transition between plant developmental stages from vegetative growth to

Table 2 miRNA candidates for biofuel improvement. A summary of the potentially important miRNA families for biofuel and biomass improvement. Each family is categorized under a particular species, and computationally identified target(s) are listed. The main role and hypothesized application of each miRNA family is then stated. miRNAs are coded by colour when conserved among taxa. White-coded miRNA are not conserved among selected species

Plant Species	miRNA	Target Annotation	Role	Application Area	Hypothesis basis	Source(s)
Brachypodium distachyon	miR156	*SPL* TFs, *Cg1*	Metabolism, development	Recalcitrance	Flowering time, adaptive stress response	Baev *et al.* (2011), Bertolini *et al.* (2013), Jeong *et al.* (2013a), Lucas *et al.* (2014), Unver and Budak, (2009), Wei *et al.* (2009), Zhang *et al.* (2009)
	miR160	*ARF* TFs	Development	Recalcitrance	Plant architecture, adaptive stress response	Baev *et al.* (2011), Bertolini *et al.* (2013), Jeong *et al.* (2013a), Unver and Budak, (2009), Zhang *et al.* (2009)
	miR167	*ARF* TFs	Development	Bioconfinement	Plant architecture, adaptive stress response	Baev *et al.* (2011), Bertolini *et al.* (2013), Jeong *et al.* (2013a), Lucas *et al.* (2014), Unver and Budak (2009), Wei *et al.* (2009), Zhang *et al.* (2009)
	miR169	*NFY* TF	Development, stress response	Bioconfinement, abiotic stress	Nitrate response, adaptive stress response	Baev *et al.* (2011), Bertolini *et al.* (2013), Jeong *et al.* (2013a), Lucas *et al.* (2014), Unver and Budak (2009), Zhang *et al.* (2009)
	miR172	*AP2* TF, *Cg1* gene	Development, stress response	Bioconfinement, abiotic stress	Flowering time, floral organ development regulation	Baev *et al.* (2011), Jeong *et al.* (2013b), Lucas *et al.* (2014), Unver and Budak (2009), Wei *et al.* (2009), Zhang *et al.* (2009)
	miR397	LAC enzyme	Stress response	Abiotic stress	Lignin metabolism	Baev *et al.* (2011), Bertolini *et al.* (2013), Jeong *et al.* (2013a), Lucas *et al.* (2014), Unver and Budak (2009), Wei *et al.* (2009), Zhang *et al.* (2009)
	miR398	*Superoxide dismutase Cytochrome C, SBP* TF	Development, stress response	Bioconfinement, abiotic stress	Regulation of plant architecture, superoxide radical detoxification	Baev *et al.* (2011), Jeong *et al.* (2013a), Zhang *et al.* (2009)
	miR414	*CBL*-interacting serine, Threonine-protein kinase 1	Development	Bioconfinement	Regulation of plant development, morphology and flowering time	Unver and Budak (2009)
	miR5200	*FT-like* gene	Development	Bioconfinement	Regulation of flowering time	Jeong *et al.* (2013a)
Cassava (*Manihot esculenta*)	miR156	*SPL* TFs, *SPB* TFs Liguleless1 protein, *Cg1* gene	Metabolism, development	Recalcitrance	Regulation of flowering time, adaptive stress response	Ballen-Taborda *et al.* (2013), Patanun *et al.* (2013), Quintero *et al.* (2013)
	miR160	*ARF* TFs	Development	Recalcitrance	Regulation of plant architecture, adaptive stress response	Ballen-Taborda *et al.* (2013), Patanun *et al.* (2013), Perez-Quintero *et al.* (2012)
	miR164	NAC domain-containing protein	Stress response	Abiotic stress	Adaptive stress responses	Ballen-Taborda *et al.* (2013)
	miR172	*AP2* TF, Protein A INTEGUMENT A	Development	Bioconfinement	Regulation of flowering time, floral organ development regulation	Ballen-Taborda *et al.* (2013), Patanun *et al.* (2013), Perez-Quintero *et al.* (2012)

Table 2 Continued

Plant Species	miRNA	Target Annotation	Role	Application Area	Hypothesis basis	Source(s)
	miR395	LAC enzyme, LRR proteins, Sulphur adenylyltransferase	Stress response	Abiotic stress	Regulation of flowering time, adaptive stress response	Ballen-Taborda *et al.* (2013), Patanun *et al.* (2013), Perez-Quintero *et al.* (2012)
	miR482	LRR proteins	Stress response	Biotic stress	Protein interactions, adaptive stress responses	Ballen-Taborda *et al.* (2013), Patanun *et al.* (2013), Perez-Quintero *et al.* (2012)
Jatropha curcas	miR004	*UBC22* TF, *PAG1, ARF7* TF	Development, metabolism	Recalcitrance, abiotic stress	Regulation of plant architecture, adaptive stress response expressed in leaf	Galli *et al.* (2014), Wang *et al.* (2012), Yue *et al.* (2013)
	miR156	PPR protein, *PGA1, Gene 29269.m000250*	Development, stress response	Recalcitrance, abiotic stress	Regulation of flowering time, adaptive stress response	Galli *et al.* (2014), Zeng *et al.* (2010)
	miR172	*AP2* TF	Development	Bioconfinement	Regulation of flowering time, floral organ development regulation, expressed in L1 seeds	Galli *et al.* (2014), Zeng *et al.* (2010)
	miR395	Not yet identified	Development, stress response	Bioconfinement, abiotic stress	Regulation of flowering time, adaptive stress response	Zeng *et al.* (2010)
	miR398	HD-ZIP	Stress response	Abiotic and biotic stress	Regulation of abscisic acid signalling and many adaptive biotic and abiotic stress responses	Zeng *et al.* (2010)
	miR5201	T8K14.20 protein, Ceramidase familyprotein, *Br FatA1*	Development	Recalcitrance	Metabolic regulation	Vishwakarma and Jadeja (2013)
Maize (*Zea* spp.)	miR156	*SBP, Cg1* gene, *TGA1*	Development, stress response	Recalcitrance, abiotic stress	Regulation of domestication, juvenile development	Chuck *et al.* (2007), Kang *et al.* (2012), Wei *et al.* (2009), Zhao *et al.* (2013)
	miR160	*ARF* TFs	Development	Recalcitrance	Regulation of plant architecture, adaptive stress response	Kang *et al.* (2012), Wei *et al.* (2009)
	miR164	*MYB* TFs	Development, stress response	Bioconfinement, abiotic stress	Regulation of leaf development, adaptive stress response	Kang *et al.* (2012), Wei *et al.* (2009), Zhao *et al.* (2013)
	miR166	*AGO10* gene	Development	Bioconfinement	Meristem regulation	Kang *et al.* (2012), Wei *et al.* (2009), Zhao *et al.* (2013)
	miR167	*ARF* TFs	Development	Bioconfinement	Regulation of seed dispersal	Kang *et al.* (2012), Wei *et al.* (2009), Zhao *et al.* (2013)
	miR169	NFY-A-	Stress response	Abiotic stress	Regulation of nitrate Response	Kang *et al.* (2012), Wei *et al.* (2009), Zhao *et al.* (2013)
	miR172	*AP2 TF*, *Cg1* gene	Development, stress response	Recalcitrance, bioconfinement, abiotic stress	Leaf development, seed size and yield	Chuck *et al.* (2007), Kang *et al.* (2012), Wei *et al.* (2009), Zhao *et al.* (2013)
	miR319	*MYB* TFs, TCP TFs	Development	Bioconfinement	Regulation of seed size	Kang *et al.* (2012), Wei *et al.* (2009)
Poplar (*Populus* spp.)	miR156	*SBP* and *SPL* TFs, *Cg1* gene, Nitrate transporter, Glucose-6-phosphate/phosphate	Metabolism, development	Recalcitrance, abiotic stress	Regulation of flowering time, adaptive stress response, stem lignin content and composition	Barakat *et al.* (2007), Khraiwesh *et al.* (2012), Liu *et al.* (2013c), Lu *et al.* (2005, 2008), Rubinelli *et al.* (2013), Shuai *et al.* (2013)
	miR159		Development, stress response	Bioconfinement, abiotic stress		

Table 2 Continued

Plant Species	miRNA	Target Annotation	Role	Application Area	Hypothesis basis	Source(s)
		MYB TFs, Aspargine synthase, (1–4)-b-mannan endohydrolase, Protein kinase			Regulation of leaf development, flowering time, adaptive stress response	Barakat *et al.* (2007), Khraiwesh *et al.* (2012), Liu *et al.* (2013c), Lu *et al.* (2005), Shuai *et al.* (2013)
	miR160	*ARF* TFs	Development, stress response	Recalcitrance, biotic and abiotic stress	Regulation of plant architecture, adaptive stress response	Barakat *et al.* (2007), Liu *et al.* (2013c), Lu *et al.* (2005, 2008)
	miR164	*NAC1* TF, Protein kinase	Development, stress response	Recalcitrance, abiotic stress	Regulation of leaf development, lignin metabolism	Barakat *et al.* (2007), Liu *et al.* (2013c), Lu *et al.* (2005), Rubinelli *et al.* (2013), Shuai *et al.* (2013)
	miR166	HD-ZIPII	Development, stress response	Bioconfinement, abiotic stress	Regulation of leaf development	Barakat *et al.* (2007), Lu *et al.* (2008), Shuai *et al.* (2013)
	miR169	NFY, *MtHAP2-1* TF	Stress response	abiotic stress	Adaptive stress response	Barakat *et al.* (2007), Liu *et al.* (2013c), Lu *et al.* (2008), Shuai *et al.* (2013)
	miR172	*AP2* TF	Development, stress response	Bioconfinement, abiotic stress	Regulation of leaf development, flowering time, sterility	Barakat *et al.* (2007), Liu *et al.* (2013c), Lu *et al.* (2005, 2008), Rubinelli *et al.* (2013), Shuai *et al.* (2013)
	miR319	*TCP/MYB* TFs	Development, stress response	Bioconfinement, abiotic stress	Regulation of leaf development, flowering time, adaptive stress response	Barakat *et al.* (2007), Lu *et al.* (2005, 2008)
	miR472	Pathogen resistance protein	Pathogen response	Disease resistance	Adaptive stress response	Lu *et al.* (2005, 2008), Shuai *et al.* (2013)
	miR1445	Dihydropyrimidinase	Cold response, stress response	Biotic and abiotic stress	Adaptive stress response	Khraiwesh *et al.* (2012), Lu *et al.* (2008)
	miR1446	GNAT family protein, Gibberellin response modulator-like protein	Cold response, stress response	Biotic and abiotic stress	Adaptive stress response	Khraiwesh *et al.* (2012), Lu *et al.* (2008)
Sorghum (*Sorghum bicolor L.*)	miR156	*SBP/SPL* TFs	Metabolism, development	Recalcitrance, bioconfinement	Increased biomass, regulation of development, expressed in many areas	Calvino and Messing (2012) Calvino *et al.* (2011), Katiyar *et al.* (2012), Yan *et al.* (2011)
	miR159	*SPL/MYB* TFs	Development	Bioconfinement	Regulation of flowering time, adaptive stress response	Calvino *et al.* (2011), Yan *et al.* (2011)
	miR164	*NAC* TFs	Development, stress response	Abiotic stress	Regulation of leaf development, lignin metabolism	Calvino *et al.* (2011), Pasini *et al.* (2014), Yan *et al.* (2011)
	miR169	NFY	Stress response, development	Abiotic stress	Upregulated during drought stress	Calvino and Messing (2012), Calvino *et al.* (2011), Katiyar *et al.* (2012), Paterson *et al.* (2009), Yan *et al.* (2011)
	miR172	*AP2, IDS1*	Development	Bioconfinement	Regulation of flowering time	Calvino and Messing (2012), Calvino *et al.* (2011), Yan *et al.* (2011)
	miR395	*ATP, APS1* and *Sultr1*	Development, stress response	Bioconfinement, abiotic stress	Regulation of flowering time, upregulated with water/sulphate deprivation	Calvino *et al.* (2011), Katiyar *et al.* (2012), Pasini *et al.* (2014)
	miR399	UBC24 enzyme	Stress response	Abiotic stress	Upregulated with water deprivation	Calvino *et al.* (2011), Katiyar *et al.* (2012)
	miR444		Development, metabolism	Bioconfinement		

Table 2 Continued

Plant Species	miRNA	Target Annotation	Role	Application Area	Hypothesis basis	Source(s)
		MADS-box, Ferredoxin-sulfite reductase precursor			Regulation of sulphur metabolism, flowering time	Calvino *et al.* (2011), Katiyar *et al.* (2012), Paterson *et al.* (2009)
	miR528	*Not yet identified*	Stress response	Abiotic stress	Downregulated during water stress	Calvino *et al.* (2011), Yan *et al.* (2011)
Sugarcane (*Saccharum* spp.)	miR156	*SBP/SPL* TFs	Development, stress response	Recalcitrance, abiotic stress	High expression in axillary meristems	Ferreira *et al.* (2012), Ortiz-Morea *et al.* (2013), Zanca *et al.* (2010)
	miR159	*SsGAMYB, MYB* protein-like	Development	Bioconfinement	Regulation of abscisic acid signalling	Ortiz-Morea *et al.* (2013), Zanca *et al.* (2010)
	miR164	*NAC* TFs	Stress response	Abiotic stress	Upregulated during drought stress	Ferreira *et al.* (2012), Ortiz-Morea *et al.* (2013)
	miR397	laccase-23-like	Stress response	Abiotic stress	Upregulated during drought stress	Ferreira *et al.* (2012), Ortiz-Morea *et al.* (2013)
	miR399	Inorganic pyrophosphatase 2-like	Stress response	Abiotic stress	Upregulated during drought stress	Ferreira *et al.* (2012), Ortiz-Morea *et al.* (2013)
	miR528	UBX domain-containing protein, Cu2 + -binding domain-containing protein	Stress response	Abiotic stress	Upregulated during drought stress, monocot specific	Ferreira *et al.* (2012), Zanca *et al.* (2010)
Switchgrass (*Panicum virgatum L.*)	miR156	*SPL*, *Cg1* gene, *MYB*, Heat shock protein-binding protein	Development & stress response	Bioconfinement, biofuel yield, abiotic Stress	Upregulation prevents flowering, decreases recalcitrance, increases sugar content, upregulated during drought, salt stress	Chuck *et al.* (2011), Fu *et al.* (2012), Matts *et al.* (2010), Sun *et al.* (2012b), Xie *et al.* (2010, 2014)
	miR164	Sucrose synthase 2	Metabolism & stress response	Biofuel yield, abiotic stress	Sucrose metabolism and carbon fixation regulation, downregulated during salt stress	Matts *et al.* (2010), Xie *et al.* (2010, 2014)
	miR166	Cellulose synthase 3	Development & stress response	Biofuel yield, abiotic stress	Cellulose synthesis regulation, downregulated during salt stress	Matts *et al.* (2010), Xie *et al.* (2010)
	miR167	*ARFs*, Glycosyl transferase-like protein	Development & stress response	Biofuel yield, recalcitrance, bioconfinement, abiotic stress	Upregulated during salt stress, downregulated during drought stress, glycosylation regulation	Matts *et al.* (2010), Sun *et al.*, (2012b), Xie *et al.* (2010)
	miR172	*AP2, SPL3*	Development & stress response	Bioconfinement, abiotic stress	Juvenile cell wall identity and fatty acid metabolism regulation, flowering prevented, downregulated during salt and drought stress	Chuck *et al.* (2011), Fu *et al.* (2012), Matts *et al.* (2010), Sun *et al.* (2012b), Xie *et al.* (2014)
	miR398	Fiber protein Fb2	Development & stress response	Recalcitrance, abiotic stress	Downregulated during drought stress, superoxide radical detoxification	Matts *et al.* (2010), Sun *et al.* (2012b), Xie *et al.* (2010)
	miR414	Triacylglycerol lipase, Glycosyltransferase,	Metabolism, development	Biofuel yield, recalcitrance	Regulation of biological synthesis of cellulose, and plant architecture	Xie *et al.* (2010)

Table 2 Continued

Plant Species	miRNA	Target Annotation	Role	Application Area	Hypothesis basis	Source(s)
		Lipid-binding protein, Sucrose phosphate synthase1				
	miR444	Glucose phosphate isomerase	Metabolism	Biofuel yield, recalcitrance	Gluconeogenesis, glycolysis, starch and sucrose metabolism regulation	Matts *et al.* (2010), Xie *et al.* (2010)
	miR477	Xylanase inhibitor protein 1	Metabolism, development	Biofuel yield, recalcitrance	Adaptive stress regulation	Xie *et al.* (2010)
	miR528	Lipid-binding protein	Metabolism, development and stress response	Biofuel yield, recalcitrance	Carbohydrate metabolic regulation	Matts *et al.* (2010), Xie *et al.* (2010)
	miR531	Glycosyltransferase, Carboxylic ester hydrolase, Sucrose non-fermenting-related protein kinase 1b	Metabolism, development	Biofuel yield, recalcitrance	Regulation of biological cellulose synthesis	Xie *et al.* (2010)
	miR854	Glycoside hydrolases, Carboxylic ester hydrolase	Metabolism, development	Biofuel yield, recalcitrance	Carbohydrate metabolic processes and glycolysis regulation	Xie *et al.* (2010)
	miR1535	Lectin receptor-type protein kinase	Metabolism, development	Biofuel yield, recalcitrance	Starch and sucrose metabolism regulation	Xie *et al.* (2010)
	miR1848	Glucan endo-1,3-beta-glucosidase GVI precursor, AF331854_1 UDP-glucosyltransferase BX8	Metabolism, development	Biofuel yield, recalcitrance	Starch and sucrose metabolism regulation	Xie *et al.* (2010)
	mi2102	Sucrose transporter, Switch/sucrose nonfermenting 3C, Lipid-binding protein, glycosyltransferase	Metabolism, development	Biofuel yield, recalcitrance	Regulation of biological cellulose synthesis, starch and sucrose metabolism	Xie *et al.* (2010)
	miR2118	Lipid-binding protein	Metabolism, development	Biofuel yield, recalcitrance	Regulation of fatty acid metabolism	Xie *et al.* (2010)

TF, transcription factor.

reproductive growth (Huijser and Schmid, 2011; Poethig, 1990) For biofuel crops, delayed flowering may result in high plant biomass, which is a desirable biofuel trait. For example, transgenic switchgrass with miR156 overexpression displays delayed flowering and phase change from vegetative growth to reproductive growth in both field-grown and greenhouse plants of the studies discussed above (Chuck *et al.*, 2011). Low overexpression of miR156 results in delayed flowering and further increased switchgrass biomass (Fu *et al.*, 2012). Switchgrass never flowers when miR156 overexpression is moderate to high. After a certain overexpression level, the plants have decreased biomass and are stunted (Fu *et al.*, 2012). miR156 overexpression yields no signs of flowering and limited decrease in tiller number; higher miR156 expression levels result in stunted growth and shortened internode length, which decreases overall biomass significantly as stated in this study by Fu *et al.* (2012). Therefore, it will be critical to 'tune' the optimal expression of miR156 for flowering and biomass traits. Obviously, plants that never flower would also render bioconfined transgenes.

The manipulation of miR156 expression could be particularly useful when combined with developmental changes that are regulated by other miRNAs (Jin *et al.*, 2013; Zhou and Luo, 2013). For more traditional bioenergy crops such as maize, where seed starch is used for ethanol production, the manipulation of flowering would not be desirable, even if more biomass could be gained for the 'stover' used to produce cellulosic biofuels (Cook *et al.*, 2014). Plant sugar content also influences flowering time in *Arabidopsis* by regulating miR156. A recent study shows that in young leaf primordia, miR156 expression is repressed by sugar in pre-existing leaves to trigger juvenile-to-adult phase transition (Yu *et al.*, 2013). This repression is dependent upon signalling activity of glucose sensor *hexokinase1* and can be observed using exogenous glucose as well (Yang *et al.*, 2013). Another study illustrating the importance of carbohydrates in flowering shows *trehalose-6-phosphate synthase 1* downregulation with miR156 constitutive expression (Wahl *et al.*, 2013).

miR156 is also associated with abiotic stress responses including drought and low nitrogen levels in several important biofuel crops, including *Jatropha,* maize, poplar, sugarcane and switchgrass (Ferreira *et al.*, 2012; Khraiwesh *et al.*, 2012; Sun *et al.*, 2012b; Zeng *et al.*, 2010; Zhao *et al.*, 2013). miR156 is upregulated during recurring heat stress through *SPL* expression in *Arabidopsis* (Stief *et al.*, 2014). Thus, miR156 transgenics should be studied relative to nitrogen and water use efficiencies in the field. Indeed, miR156 might be the best single gene candidate to manipulate to obtain many of the traits desirable in bioenergy feedstocks: increased plant biomass, decreased lignin content, improved response to abiotic stress and bioconfinement.

miR160 could be manipulated for response to environmental stress and recalcitrance

miR160 is another conserved miRNA family playing an important role in plant response to abiotic stress and plant development in many plant species, including *Brachypodium,* cassava, maize, poplar and sugarcane (Table 2) (Jeong *et al.*, 2013a; Perez-Quintero *et al.*, 2012). miR160 is involved in regulating plant physiology and metabolism (Khraiwesh *et al.*, 2012; Liu *et al.*, 2013c; Lu *et al.*, 2008). In switchgrass, miR160 and miR167 are predicted to control differentiation of lateral root cells and root cap development by targeting the transcripts that encode for *auxin response factors (ARF) 10 and 16* (Matts *et al.*, 2010; Sunkar and Zhu, 2004; Wang *et al.*, 2005; Xie *et al.*, 2010). *ARFs* represent a class of conserved targets for transcripts regulated by miR160 in all embryophytes (Finet *et al.*, 2013; Luo *et al.*, 2013). miR160-uncoupled expression of the *ARF16* yields pleiotropic effects, including limited lateral roots and changes in root and cell morphology. A recent study using *Arabidopsis* mutants shows that ARF10 and 16 regulate aluminum-induced root growth inhibition through cell wall synthesis and assembly (Yang *et al.*, 2014). When miR160 is knocked out in *Arabidopsis*, *ARF17* is upregulated, resulting in lower levels of adventitious root cell division and growth, which could be a negative effect for biofuel crop development (Gutierrez *et al.*, 2009; Marin *et al.*, 2010). In addition, miR160 is upregulated in response copper deficiency and the presence of sucrose in *Arabidopsis* (Ren and Tang, 2012). In poplar, miR160 is upregulated during heat stress, UV radiation, sulphate and pathogen infection (Khraiwesh *et al.*, 2012). miR160 could be a potential target to manipulate of biofuel feedstocks because of its involvement with biotic and abiotic stresses, and plant development in multiple species; however, like miR156, its expression may need to be tuned to minimize unintended effects.

miR164 could play a role in plant responses to abiotic stress and bioconfinement

miR164 is a conserved miRNA identified in many plant species, including several biofuel plant species, such as cassava, maize, poplar, sorghum, sugarcane and switchgrass (Barakat *et al.*, 2007; Kang *et al.*, 2012; Ortiz-Morea *et al.*, 2013; Patanun *et al.*, 2013). miR164 functions by regulating transcripts that encode for *NAC (NAM, ATAF1,2, CUC2)* and MYB *(Johnson et al.)*transcription factors (Johnson *et al.*, 2012; Nakashima *et al.*, 2012). The *NAC* and *MYB* transcription factors, as well as other predicted miR164 transcript targets, are related to drought response, early plant development and metabolic processes (Wei *et al.*, 2009). It is also reported that miR164 regulates lateral rooting, which may contribute to positive stress response and plant biomass in model plant species as well biofuel crops (Wei *et al.*, 2009). In addition, the *ORE* transcription factor from the NAC family, which positively regulates aging-induced cell death and senescence in *Arabidopsis* leaves, is negatively regulated by miR164 (Kim *et al.*, 2009). The ETHYLENE-INSENSITIVE3 transcription factor, which stimulates leaf senescence, also represses miR164 transcription (Li *et al.*, 2013). Leaf senescence is significant because it reactivates nutrients from dying leaves to developing organs (Kim *et al.*, 2014). The redirection of nutrients allows the plant to boost its fitness. miR164 could aid biofuel development because delaying senescence could potentially increase yield (Liu *et al.*, 2010).

More importantly, in switchgrass miR164 is predicted to target the transcript that codes for *sucrose synthase2*, which could influence both biofuel yield and recalcitrance (Xie *et al.*, 2010). In cassava, miR164 responds to the hormone abscisic acid, which influences plant developmental processes (Patanun *et al.*, 2013). In contrast, miR164 is associated with drought response in sugarcane, which could improve water use efficiency in transgenic feedstocks (Ballen-Taborda *et al.*, 2013; Ferreira *et al.*, 2012). In maize, miR164 is upregulated in salt stress conditions (Khraiwesh *et al.*, 2012). This evidence suggests that miR164 could be a potential marker and target for improvement and selection of stress-tolerant and high-yield feedstocks for biofuel purposes.

miR166 and miR167 could improve recalcitrance, bioconfinement and abiotic stress

miR166 and miR167 target genes encoding transcription factors, which are associated with plant development, morphology and metabolism in *Brachypodium,* maize, sugarcane and switchgrass (Unver and Budak, 2009; Wei *et al.*, 2009). While their targets are different, the potential biotechnological application of miR166 and miR167 is similar. Because of this, miR166 and miR167 have been lumped together into the same section for this review. The predicted transcript target of miR167, like miR160, is the *ARF* family of transcription factors. miR166 has various predicted targets depending upon the species, including the gene which encodes for protein argonaute 10 (AGO10) (Ji *et al.*, 2011). In *Arabidopsis*, miR166 regulates shoot apical meristem development and floral development (Rubio-Somoza and Weigel, 2013; Zhu *et al.*, 2011a). miR166 is in *B. distachyon,* among other plant species suggesting conserved function (Baev *et al.*, 2011; Bertolini *et al.*, 2013; Wei *et al.*, 2009). miR166 and miR167 are highly expressed in maize seeds during periods of development, which displays their positive role (Kang *et al.*, 2012). miR166 is downregulated and miR167 is upregulated during salt stress in switchgrass (Sun *et al.*, 2012b; Xie *et al.*, 2014). miR167 is predicted to target transcripts encoding for glycosyl transferase-like proteins in switchgrass, which might contribute to improved biofuel yield through reduced recalcitrance (Xie *et al.*, 2010). miR166 and miR167 likely have a widespread effect on the phenotype wherever they are found. Therefore, miR166 and miR167 might have potential applications in biofuel yield, recalcitrance, bioconfinement and abiotic stress because of their involvement in early development.

miR169 could be applied to problems of recalcitrance, bioconfinement and abiotic stress

miR169 is expressed in many plant species, including *Brachypodium,* maize, poplar and sorghum (Kang *et al.*, 2012; Kidner and Martienssen, 2005; Lucas *et al.*, 2014; Yan *et al.*, 2011). miR169 regulates plant development and stress responses by targeting the transcript that encodes for NFYA transcription factors. One study predicts that miR169 is upregulated in young *Brachypodium* leaves under drought stress where cell number and size are increased (Bertolini *et al.*, 2013). This is a positive effect to consider for the engineering of biomass feedstocks. In poplar, miR169 is predicted to associate with transcription factors related to stress responses (Khraiwesh *et al.*, 2012). Similarly, miR169 is associated with adaptation to low soil nitrogen levels in maize. miR169 is predicted to target the transcript that encodes for a *nuclear transcription factor Y subunit A* (NFY-A) mRNA and acts through plant stress pathways (Zhao *et al.*, 2013). In *Arabidopsis*, miR169 is upregulated during heat stress and downregulated with the addition of exogenous abscisic acid (Li *et al.*, 2010). Resistance to abiotic stresses is associated with miR169 in sorghum (Qazi *et al.*, 2012). These data suggest that miR169 could influence, recalcitrance, response to stress, and biomass in a variety of species. Perhaps, alteration of miR169 expression in maize could lead to increased nitrogen use efficiency (Zhao *et al.*, 2013). In addition, its correlation with sugar content could be particularly useful in increasing the biomass of biofuel plants such as sorghum.

miR172 might be manipulated for bioconfinement, improving recalcitrance and abiotic stress tolerance and delaying flower development

It has been well documented that miR172 regulates flower development and phase change in *Arabidopsis*, rice and maize. This role contributes to miR172's potential for improving biofuel biomass through delaying progression through developmental stages similar to miR156. miR172 is in *Brachypodium,* maize, poplar, switchgrass and sorghum according to recent studies, although it will likely be found in more species as research is continued due to its role in plant development (Chuck *et al.*, 2007; Ferreira *et al.*, 2012; Kidner and Martienssen, 2005; Lucas *et al.*, 2014; Rubinelli *et al.*, 2013; Sun *et al.*, 2012b; Yan *et al.*, 2011). In certain cases, miR172 and miR156 co-regulate plant development through *SPL* pathways as discussed previously. miR156 regulates *SPL* transcription factors that promote miR172 transcription (Wu *et al.*, 2009). miR172 expression is positively associated with adult epidermal identity (Wu *et al.*, 2009). The ABC model for floral development places the *apetala 2 (AP2)* gene in a crucial role in flower pattern (Fan *et al.*, 2013; Zhuang *et al.*, 2008). Several studies demonstrate that *AP2* expression is regulated by miR172 (Liu *et al.*, 2013c; Mehrpooyan *et al.*, 2012). Overexpression of miR172 apparently leads to early flowering and disrupted specification of floral organ identity in *Arabidopsis* (Aukerman and Sakai, 2003; Chen, 2004; Zhou and Wang, 2013). Chuck *et al.* (2007) speculate that relative levels of miR156 and miR172 could regulate juvenile-to-adult phase transitions. Specifically, low miR172 and high miR156 expression promotes juvenility, while high miR172 and low miR156 expression promotes the adult reproductive phase (Chuck *et al.*, 2007). Additionally, the expression level of miR172 is negatively correlated with flowering time in sorghum (Calvino *et al.*, 2011). These studies demonstrate that certain miRNAs play important roles in all stages of plant development, which have huge potentials for improving biofuel crops (Galli *et al.*, 2014; Jeong *et al.*, 2013a; Wei *et al.*, 2009; Zeng *et al.*, 2010).

miR319 and miR395 could contribute to bioconfinement

Compared with the highly conserved miRNAs such as miR156, many miRNAs are identified in three or fewer of our selected biofuel species (Table 2). While these miRNAs have yet to be identified in other species, they may be in the near future as technology improves and miRNA research continues to expand and deepen. miR319 is currently identified in poplar and maize only. miR319 is predicted to target a transcript that encodes for transcription factors related to stress responses in poplar (Lu *et al.*, 2008; Wei *et al.*, 2009). In contrast, miR319 is highly expressed in maize seeds during periods of development (Kang *et al.*, 2012). Therefore, it targets transcripts that encode for transcription factors associated with plant development and metabolism which could positively influence the ability of biomass crops to produce sugars or respond to stress (Wei *et al.*, 2009). Another miRNA of interest for transgenic biofuel species bioconfinement is miR395. It is expressed in cassava, sorghum and *Jatropha* (Perez-Quintero *et al.*, 2012). miR395's expression level is negatively correlated with flowering time in sorghum (Calvino

et al., 2011). Delayed flowering increases the potential for bioconfinement of transgenic biofuel plants.

Other conserved miRNAs that could contribute to reduced recalcitrance

Many other conserved miRNAs are associated with plant processes that could be exploited for decreased recalcitrance to increase the ability to convert cell walls to biofuel (Table 2). For example, miRNAs function in 527 biological processes in switchgrass, of which 25 regulate metabolic pathways related to biofuel production according to computational methods (Xie *et al.*, 2010). miR166 potentially targets transcripts encoding for cellulose biosynthesis proteins in switchgrass (Xie *et al.*, 2010). In contrast, miR398 regulates recalcitrance by targeting the transcript that encodes for fiber protein Fb2 in switchgrass, according to computational predictions (Vishwakarma and Jadeja, 2013; Xie *et al.*, 2010). The transcript that miR414 regulates in switchgrass is predicted to target cellulose biosynthesis proteins. In sorghum and switchgrass, miR444 regulates plant development and metabolism by targeting the predicted transcript that encodes for a gene in the *MADS-box* family, which has been a focus of research in *Brachypodium* (Katiyar *et al.*, 2012; Wei *et al.*, 2014; Xie *et al.*, 2010). miR528 targets the transcript that encodes for a lipid-binding protein in switchgrass according to computational research (Xie *et al.*, 2010). Lipid-binding proteins can function in many ways, including energy storage and cell structure and compartmentalization (Marion *et al.*, 2007). Therefore, lipid-binding proteins could improve many potential agronomic traits, such as biofuel yield and recalcitrance.

Another miRNA of interest is miR159. miR159 has been found in poplar, sorghum and sugarcane but not in the remaining feedstocks. miR159 appears to be associated with regulating bud outgrowth and development in sugarcane, which could influence this feedstock's reproduction efficiency (Khraiwesh *et al.*, 2012). In sorghum and sugarcane, miR159 is predicted to target the transcript that encodes for a *MYB transcription factor* (Yan *et al.*, 2011; Zanca *et al.*, 2010). miR159 is abundant in young leaf primordia and is predicted to regulate transcripts that encode for a *SPL* transcription factor to promote bud outgrowth (Ortiz-Morea *et al.*, 2013). This miRNA could influence plant development and architecture for the optimization of biofuel yield and recalcitrance.

Other conserved miRNAs that could contribute to abiotic stress

Many miRNA families are moderately conserved among the selected biofuel species (Table 2). For example, miR159 is involved in stress responses in poplar (Khraiwesh *et al.*, 2012). In cassava, miR395 processes several trans-acting small interfering RNAs, which are associated with plant response to bacterial infection (Quintero *et al.*, 2013). This miRNA is also reported in *Jatropha* where it appears to be related to abiotic stress responses, although no target genes have been identified by experimental or computational approaches (Zeng *et al.*, 2010). Future techniques will likely improve miRNA and target identification (Kuhn *et al.*, 2008). miR397 has been associated with drought stress response in sugarcane and *Brachypodium* (Bertolini *et al.*, 2013; Ferreira *et al.*, 2012). It is predicted to regulate transcripts that encode for similar *Laccase* targets in each species. This suggests that miR397 could be a potential marker for improvement and selection of stress-tolerant sugarcane cultivars.

Another miRNA of interest, miR398, is present in switchgrass, *Jatropha* and *Brachypodium*. Its predicted targets are transcripts that encode for *superoxide dismutase (SOD)* and *cytochrome c* (Baev *et al.*, 2011). These genes are involved in response to environmental stress (Kidner and Martienssen, 2005). In *Jatropha*, miR398 appears to regulate stress response by potentially targeting the HD-Zip transcript. In *Arabidopsis,* the expression of miR398 is induced by the presence of sucrose. Copper deficiency also induces production of miR398 by *SPL7* (Ren and Tang, 2012).

In comparison, miR399 is present in sorghum and sugarcane. In each species, it appears to regulate abiotic stress responses by predicted targets PHOSPHATE 2 (PHO2) and phosphate transporter, respectively (Ferreira *et al.*, 2012; Zhang *et al.*, 2011). Additionally, in *B. distachyon* and *Arabidopsis*, miR414 is predicted to target the transcript that encodes for the *MYB110* transcription factor, which is involved in abiotic stress regulation (Li *et al.*, 2014; Su *et al.*, 2013; Unver and Budak, 2009). Additionally, miR528 is found in switchgrass, sorghum and sugarcane. miR528 is downregulated in young sorghum leaves during drought stress, and the target has not yet been identified in biofuel crops (Pasini *et al.*, 2014). miR528 is also downregulated under experimental drought treatment in sugarcane (Ferreira *et al.*, 2012). Other conserved miRNAs can be found in Table 2.

Unconserved miRNAs that could contribute to bioconfinement

Many important miRNAs are not shared among biofuel feedstock species (Figures 1 and 2). These miRNAs could be important for the regulation of species-specific traits. miRNAs related to development are important because of possible bioconfinement applications. A recent study predicts that miR5200 regulates plant flowering and phase change in *Brachypodium* by targeting the transcript that encodes for *flowering locus T* (Wu *et al.*, 2013). *FT* is a highly conserved florigen gene that plays a key role in plant flower development. Wu *et al.* (2013) demonstrates that *FT* is regulated by a Pooideae-specific miRNA, miR5200. miR5200 is highly expressed in plants grown under short-day conditions, but its expression is repressed under long-day conditions (Wu *et al.*, 2013). Overexpression of miR5200 results in severely delayed flowering time in short-day conditions, but does not affect plant flowering in long-day conditions. This result suggests that miR5200 functions in photoperiod-mediated flowering time regulation in *B. distachyon* (Wu *et al.*, 2013).

Unconserved miRNAs that could contribute to stress responses

As we have seen above, miRNAs could be quite useful as a tool to engineer stress resistance in plants. There are various unconserved miRNAs that appear to be important in bioenergy feedstock responses to abiotic and biotic stress. Studies show that miRNAs are involved in cassava's defence against the bacterial pathogen *Xanthomonas axonopodis* pv. *manihotis* (Perez-Quintero *et al.*, 2012). In addition, miR482 negatively regulates resistance genes that are involved in pathogen response

(Perez-Quintero *et al.*, 2012). Similarly, miR472 potentially inhibits the expression of transcripts that encode for pathogen resistance proteins, which are involved in pathogen resistance in poplar (Shuai *et al.*, 2013). Several miRNAs, including miR1445 and miR1446, are predicted to associate with cold response as well different biotic stress in poplar, such as pathogen infection (Lu *et al.*, 2008). miR399 is also associated with drought stress response in sugarcane according to computational research (Ferreira *et al.*, 2012). A recent study also shows that many other switchgrass miRNAs are significantly induced or inhibited by drought and/or salinity stress treatment (Xie *et al.*, 2014). These results suggest that miRNAs could be useful as markers and candidates for selection and improvement of stress-tolerant feedstocks.

Unconserved miRNAs that could contribute to biofuel yield

Various unconserved miRNAs might be useful to decrease biomass recalcitrance or otherwise boost biofuel yields. A new miRNA, miR004, appears to increase the levels of linoleic acid in *Jatropha*, which suggests involvement in fatty acid metabolism pathway (Galli *et al.*, 2014). It is expressed throughout the plant tissues and is involved in oil composition, which could significantly impact biofuel production and potentially be used to produce drop-in oil-based fuels (Galli *et al.*, 2014; Wang *et al.*, 2012). Cellulose biosynthesis proteins and other compounds related to metabolism and development are encoded by transcripts regulated by miR477, miR531, miR854 and other miRNA shown in Table 2 in switchgrass (Xie *et al.*, 2010). Increasing cellulose biosynthesis in switchgrass could prove useful in decreasing recalcitrance by releasing higher amounts of substrate for hydrolysis. Another miRNA of interest, miR5201, which has been identified in *J. curcas,* is predicted to target transcripts that encode T8K14.20 proteins, Ceramidase family proteins and *Br FatA1*, which influence plant development. In particular, miR5201 could be considered to be a candidate for biofuel improvement because it could limit recalcitrance through metabolic processes in biofuel feedstocks (Vishwakarma and Jadeja, 2013).

The future impact of manipulating miRNAs in biofuel feedstocks

While plant miRNA research began over a decade ago, miRNA research in biofuel feedstock is still in its infancy (Zeng *et al.*, 2014a). The biofuel feedstock miRNA area is replete with opportunities for further research (Table 3). miRNA identification is the first step to understanding the range of roles that miRNAs play in biofuel feedstock species. Although there have been many studies in the past decade on miRNA identification and functional analysis, the majority of these studies were focused on model plant species, such as *Arabidopsis* and rice (Bartel, 2004). Few miRNA studies have been performed in bioenergy feedstocks because of the effort required to survey species with often complex and partially characterized genomes (Wang *et al.*, 2012). In addition, many miRNAs are expressed at variable developmental stages, stresses, and among tissues and cell types (Jin *et al.*, 2013; Sun *et al.*, 2012b; Xie *et al.*, 2010). Computational prediction and experimental methods to assess gene expression and targeting can be applied to bioenergy plants (Moqadam *et al.*, 2013). Deep sequencing and other technologies that make genome analysis more accessible with larger data sets will usher in a new era for the small RNA field and biofuel-related research, which opens the doors for experimental validation (Williamson *et al.*, 2013). Researchers can then take full advantage of miRNA regulation to design the next generation of switchgrass and other feedstocks for improved biomass that yields high volumes of biofuel.

While new tools are available, there remain challenges in miRNA discovery research. For example, sequencing bias that arises through the use of different protocols can hinder the ability to compare results from different studies (van Dijk *et al.*, 2014). Unequal sequencing depths can influence apparent miRNA abundance among samples and studies (Soneson and Delorenzi, 2013). This is especially true for RNA-seq because it is more technically challenging than DNA-seq. Chief among these challenges is inconsistent RNA preparation among species and tissues (Johnson *et al.*, 2012; Tian *et al.*, 2010). There are several ways to limit the effects of sequencing bias. New protocols for optimizing PCR amplification and other techniques can increase

Table 3 Future plant miRNA research areas for biofuel improvement. A compilation of many key areas of research in the miRNA field that will contribute to the improvement of biofuel crops in the future. The table includes potential methods by which information can be gained in each area

Future research areas	Approaches	Sources
miRNA targets	Omics data and bioinformatics Technological innovations	Moqadam *et al.* (2013), Williamson *et al.* (2013)
Interactions between miRNAs	Experimental manipulation Network construction	Liu *et al.* (2014b), Meng *et al.* (2011)
Pleiotropic effects	Precise transgene integration Synthetic DNA promoters	Hammell (2010), Jeong and Green (2012), Liu *et al.* (2013a, 2014b), Meng *et al.*, (2011)
Variable expression	Precise transgene integration Synthetic DNA promoters	Hammell (2010), Jeong and Green (2012), Liu *et al.* (2013a, 2014b), Meng *et al.* (2011)
Sequencing bias and false positives	Consistent protocol Optimized techniques Novel miRNA identification and verification	Aird *et al.* (2011), van Dijk *et al.* (2014), Kuhn *et al.* (2008), Tian *et al.* (2010), Xiao *et al.* (2012)
Microbial conversion and drop-in fuels	Synthetic biology Microbial miRNA engineering	Bhalla *et al.* (2013), Lin and Xu (2013), Liu *et al.* (2014a), Menon and Rao (2012), Peralta-Yahya *et al.* (2012), Shi *et al.* (2014)

throughput without requiring high DNA input, although to limit bias more thoroughly one would have to avoid library amplification by PCR (Aird *et al.*, 2011). False positives in target identification can also be remedied through creating and standardizing new computational protocols and criteria (Kuhn *et al.*, 2008; Soneson and Delorenzi, 2013; Xiao *et al.*, 2012).

While a substantial amount of research has identified and characterized potential miRNAs in a variety of plants, the interaction between particular miRNAs and their related transcripts is generally unknown. The many pleiotropic effects associated with overexpression of miRNAs indicate that we need to perform more systems-level research before precise feedstock improvement can be made (Table 3) (Chuck *et al.*, 2011; Fu *et al.*, 2012; Rubinelli *et al.*, 2013). Consequently, the most difficult aspect of miRNA manipulation for biofuel feedstock optimization will be the application of knowledge to reach a particular practical goal. The positive effects of manipulating any miRNA must overshadow the negative or unintended effects. Even then, variable unintended effects might not be acceptable in agriculture. Therefore, scientists must discover the nuances of interaction between miRNAs and their specific activity (Zeng *et al.*, 2014a).

A related issue is the pleiotropy among biologically relevant bioenergy traits such as increased biomass and decreased recalcitrance. Quantitative trait loci (QTL) mapping is one way to identify the relationship between particular traits of interest and genetic factors. One study has located 28 QTL for 11 interrelated growth and seed traits in *Jatropha* (Sun *et al.*, 2012a). In switchgrass, another study has found 4 biomass QTL and 5 plant height QTL in 11 genomic regions (Serba *et al.*, 2014). QTL are important because the co-localization suggests pleiotropy among traits. Therefore, if one trait is altered in a plant by genomic manipulation, it is likely to affect other traits. In addition, traits themselves are likely to be linked biologically to one another. One example of potential pleiotropic effects is disturbance of plant stress responses from changes in lignin biosynthesis (Poovaiah *et al.*, 2014). Experiments have demonstrated that altering lignin pathways can have positive and negative effects on the vulnerability of transgenic plants to stress (Baxter and Stewart, 2013). If one engineers the cell walls of a biofuel crop to be less recalcitrant, therefore allowing it to release sugars more efficiently, structural elements would be altered. This change in plant structure could then decrease the plant's ability to withstand extreme wind. Such relationships should be considered when selecting bioenergy traits because they are likely to be interrelated.

We also need to consider IsomiRs, which are miRNA variants that originate from the same miRNA precursor and share sequences that can display differential expression and function (Jeong and Green, 2012; Xie *et al.*, 2015). Indeed, conserved miRNAs do not necessarily target the same genetic elements among plant species (Table 2). They can target transcripts that encode for multiple products within a particular plant genome. These products can play a variety of roles in an organism (Hammell, 2010). Therefore, it might be more difficult to achieve directed goals without altering other plant functions. To this end, it will be critical to regulate miRNA expression for precise temporal and spatial control to minimize off-effects.

Notably, unintended effects could be more common in highly expressed miRNA when multiple miRNA are altered in the same plant (Jovelin, 2013). This could be a challenge for researchers attempting to employ high constitutive overexpression of miRNAs fundamental to biofuel and biomass improvement. Even so, perhaps high expression of dose-dependent miRNAs would not be desirable (Chuck *et al.*, 2011). We have observed, for example, a few-fold difference in miR156 transcription using a constitutive promoter yields a range of phenotypes. These extend from a transgenic switchgrass plant that resembles turfgrass with very low biomass to a switchgrass with increased biomass and delayed flowering (Fu *et al.*, 2012). Advanced plant biotechnology tools, such as the use of precise transgene integration and synthetic promoters, have the prospect of decreasing off-target effects of miRNAs and transcription factors (Liu *et al.*, 2013a). For example, there are a plethora of design tools and methodologies to create novel synthetic promoters for precise transgene expression in space and time using omics data, network diagrams and bioinformatics, which we believe will be important to realize the full potential of manipulating powerful genetic elements like miRNAs in plants (Liu *et al.*, 2014b; Meng *et al.*, 2011).

Although miRNAs have been identified in biofuel feedstock species that have the potential to increase biofuel and biomass yield, miRNAs could also be manipulated for the anaerobic digestion of biomass. One potential area of research in the future could explore the role of miRNAs in the factors of digestion, including potential manipulation of miRNAs in microbial inoculation communities to improve levels of biodegradability (Liu *et al.*, 2014a; Shi *et al.*, 2014). Thermophilic microbes increase the efficiency of lignocellulosic biomass conversion into fuel. The catalytic microbes could potentially be optimized through manipulation of miRNA expression (Bhalla *et al.*, 2013). In addition, the production of potential plant-based drop-in fuels, which take advantage of plant metabolites, could potentially benefit from the application of miRNA manipulation. For example, plants produce isoprenoids, which can be used to create biofuels. In the future, perhaps the pathways that produce this chemical compound can be altered through manipulation of miRNA expression (Peralta-Yahya *et al.*, 2012). In this way, plants could produce a viable drop-in fuel source, or one that can work with our existing energy infrastructure without modifications (Lin and Xu, 2013). These pathways could also be exploited in bacteria and other micro-organisms that grow much faster than plants. Synthetic biology is currently used to engineer optimal microbes for biomass pretreatment (Menon and Rao, 2012). In the future, miRNA could be another means to the end of highly efficient biofuel production mechanisms (Richter, 2008).

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