Correction

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The authors note that the following statement should be added to the Acknowledgments: “The work conducted by the Joint BioEnergy Institute was supported by the Office of Science, Office of Biological and Environmental Research, of the US Department of Energy under Contract DE-AC02-05CH11231.”

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Overexpression of the maize Corngrass1 microRNA prevents flowering, improves digestibility, and increases starch content of switchgrass

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Biofuels developed from biomass crops have the potential to supply a significant portion of our transportation fuel needs. To achieve this potential, however, it will be necessary to develop improved plant germplasm specifically tailored to serve as energy crops. Liquid transportation fuel can be created from the sugars locked inside plant cell walls. Unfortunately, these sugars are inherently resistant to hydrolytic release because they are contained in polysaccharides embedded in lignin. Overcoming this obstacle is a major objective toward developing sustainable bioenergy crop plants. The maize Corngrass1 (Cg1) gene encodes a microRNA that promotes juvenile cell wall identities and morphology. To test the hypothesis that juvenile biomass has superior qualities as a potential biofuel feedstock, the Cg1 gene was transferred into several other plants, including the bioenergy crop Panicum virgatum (switchgrass). Such plants were found to have up to 250% more starch, resulting in higher glucose release from saccharification assays with or without biomass pretreatment. In addition, a complete inhibition of flowering was observed in both greenhouse and field grown plants. These results point to the potential utility of this approach, both for the domestication of new biofuel crops, and for the limitation of transgene flow into native plant species.

Plant biomass can be broken down to monosaccharides (saccharification) and converted to fuels. Plant cell walls are composed of cellulose microfibrils embedded in a cross-linked network of matrix polysaccharides and copolymerized with lignin. This complex structure inhibits the saccharification of cell wall polysaccharides by cell wall degrading enzymes. Furthermore, byproducts of the harsh pretreatments necessary to enable saccharification inhibit growth of microorganisms used to produce biofuels. Therefore, improving saccharification efficiency is one of the major goals in developing an efficient, cost-effective biofuel industry (1). Because a wealth of genetic and molecular resources exists for maize, it is well suited as a model system for the identification of genes important for biomass accumulation and saccharification in grasses (2).

Plants go through a series of development stages over time in response to a variety of stimuli, both external and internal. Each phase displays unique morphological and physiological characteristics that change when the plant undergoes a transition to the next phase. One such developmental transition is the switch from the juvenile to adult phase of development (3). In general, juvenile plant material is less lignified and displays differences in biomass accumulation and character. These juvenile traits may reduce the recalcitrance of biomass to conversion into fermentable sugars. By controlling the genes that regulate the juvenile to adult phase transition in plants, it may be possible to modify or enhance the biomass properties of a wide range of bioenergy feedstocks.

The dominant maize Corngrass1 (Cg1) mutant fixes plant development in the juvenile phase and affects both biomass accumulation and saccharification efficiency. Cg1 mutants increase biomass due to continuous initiation of axillary branches (tillers) and leaves (4). The resulting biomass has reduced adult characteristics and ectopic juvenile cell identities (5). In addition, Cg1 mutant leaves contain decreased amounts of lignin and increased levels of glucose and other sugars compared with wild type (6), which could provide an improved substrate for saccharification.

We cloned Cg1 and showed that it is an unusual grass-specific tandem microRNA gene that is overexpressed in the mutant (7). This microRNA belongs to the miR156 class that is known to target the SQUAMOSA PROMOTER BINDING LIKE (SPL) family of transcription factors (8). Overexpression of miR156 causes inappropriate cleavage of its targets, demonstrating that the Cg1 mutant phenotype is caused by loss of function of several SPL target genes (7). miR156 is temporally regulated, occurring at high levels during the juvenile phase and gradually disappearing during the adult phase in Arabidopsis and maize (7, 9).

Overexpression of miR156 in Arabidopsis and rice results in phenotypes similar to Cg1 (10, 11), indicating functional conservation of this microRNA across a wide range of plant species. SPL genes have a variety of functions, including control of male fertility (12), copper metabolism (13), plastochron time (14, 15), bract suppression (16), and flowering time (17, 18). Recently, it has been shown that SPL genes directly target and activate expression of flowering regulators such as LEAFY and APETALA1 (19), and a different microRNA, miR172 (17). Expression of miR172 during the adult phase of development promotes flowering by repressing floral repressors such as the SMZ and SNZ genes (20, 21). Thus, the miR156-targeted SPL genes have a positive effect on the floral transition through multiple independent pathways. Consistent with these findings, the Cg1 mutant in maize is slow to flower and has greatly reduced numbers of floral and inflorescence meristems (7).

Because members of the miR156 targeted SPL gene family are conserved in different plant species, it is feasible to transfer the desired biofuel processing properties of Cg1 into any crop of choice simply by overexpressing the Cg1 cDNA. Here we test this hypothesis by constitutively expressing Cg1 in the monocots, Brachypodium distachyon (Brachypodium), Panicum virgatum (switchgrass), and a dicot, Arabidopsis. We show that it is possible to affect biomass composition, digestibility, and flowering through genetic manipulation of miR156 and its targets.

Results

Cg1 Overexpression Promotes Juvenile Leaf Traits. The full-length maize Cg1 cDNA was expressed behind the 35S promoter in Arabidopsis or behind the maize UBQUITIN (UBI) promoter


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(22) in Brachypodium and switchgrass using Agrobacterium-mediated transformation. Similar to the maize Cg1 mutant (Fig. 1A), Arabidopsis 35S::Cg1 transformants (Fig. 1B) displayed increased vegetative branching and increased leaf initiation in line with earlier reports of miR156 overexpression (10). These plants flowered approximately 2 to 3 wk later and had morphologically normal flowers (Fig. 1B). UBI::Cg1 transformants in Brachypodium displayed a similar increase in leaf production, branching, and delayed flowering time (Fig. 1C), but also produced inflorescences with fewer spikelets (Fig. 1C, Inset). The same construct was put into switchgrass with similar results (Fig. 1D), except that the plants never flowered. In general, leaves in all species expressing Cg1 were narrow and more numerous compared with wild-type adult leaves.

Plastic sections of Cg1 overexpressing leaves of grass species revealed numerous differences in cell number, morphology, and identity (Fig. 2A–C). In general, Cg1 overexpressing leaves in maize, switchgrass, and Brachypodium had fewer cell layers, reduced leaf thickness, and fewer hypodermal sclerenchyma cells (Fig. 2), compared with wild-type adult leaves of similar age. Epidermal peels of leaves were performed on switchgrass to determine whether the transformants had juvenile cell identity. In maize, wild-type juvenile waxes stain purple with Toluidine Blue (TBO), whereas adult waxes stain aqua (23), and the same is true in switchgrass (Fig. 2D and E). All leaves of the Cg1 overexpressers displayed juvenile waxes on the basis of TBO staining (Fig. 2F). In addition, epidermal cells were less crenulated and had thinner walls compared with wild type (Fig. 2F). Cg1 overexpressers in both maize and switchgrass also showed increased juvenile biochemical properties such as an increase in the juvenile and grass-specific, cell wall (1,3;1,4)-β-D-glucans (Fig. S1) (24). In summary, the leaves of all species that overexpress Cg1 had both juvenile morphology and cell identities relative to nontransgenic plants, consistent with the maize Cg1 phenotype.

Morphological and Molecular Analysis of Ubi:Cg1 Switchgrass. To fully understand the impact of the Cg1 mutants on the phenotype and composition of switchgrass, 20 independent UBI::Cg1 switchgrass transformants were generated and sorted into three phenotypic classes (severe, moderate, and weak) (Fig. 3A) on the basis of phenotypic severity and expression levels of the transgene (Fig. S2). In general, increasing levels of Cg1 expression were correlated with increasing phenotypic severity. Each class displayed increased vegetative branching, but also possessed unique phenotypes. High transgene expressers were severely dwarfed and had thinner stems with many small needle-like leaves. Moderate expressers had bigger leaves, thicker stems, and were less dwarfed. Both severe and moderate expressers produced long horizontally growing branches that initiated several aerial shoots with roots at each node (Fig. 3B), each capable of producing new plants. The leaves of the low-expressing weak class resembled wild type, except that they were narrower and had juvenile cell identity. In addition, the weak expressers only produced excess branches at nodes located at soil level.

The growth rate of each clone was assessed over the summer in the field (details in Materials and Methods). Transformants from the severe class with high levels of transgene expression displayed very limited growth and often died, whereas the low expressers generally kept pace with wild-type controls (Fig. 3C). None of the transformants ever flowered, even after more than a year in the field (two summers and one winter) or after 2 y in the greenhouse under long day conditions. In contrast, transformants overexpressing a miR156 target mimic construct, designed to inhibit miR156 activity (23), flowered normally.

At the end of the growing season, all of the above ground biomass was harvested, dried, and weighed. Postharvest, the weakly expressing class displayed better regenerative capacity, with each cut shoot forming a new tiller. In contrast, wild-type plants only regenerated new shoots from the periphery of the plant (Fig. 3D). A count of the total number of primary branches postharvest revealed that the weak-expressing class produced four times more branches, whereas the moderate and severe classes had only two to three times more, respectively (Fig. 3F). The average weight of the moderate and severe classes was significantly lower than wild type, but the weak class was not significantly lower (Fig. 3E). These results indicate that high levels...
of Cg1 expression are detrimental to plant growth and biomass accumulation, whereas weak expression can be tolerated.

To confirm that overexpression of Cg1 in switchgrass reduces SPL gene expression, qRT-PCR was performed using oligos corresponding to several switchgrass SPL homologs (Fig. 3G). As expected, the expression levels of four miR156-targeted SPL genes were down by several orders of magnitude in all transformants, the most dramatic found in the moderate and severe classes. Because SPL genes are known activators of AP1 MADS box genes necessary for specifying floral meristem identity (19, 26), the expression of three switchgrass homologs of AP1 was also assayed (Fig. 3H). Two of these genes, FL779848 and FL813474, were down-regulated in all transformants, whereas the third one, FL799727, showed no significant change. Assuming that FL779848 and FL813474 function similarly to AP1, the loss of these genes could explain the lack of flowering in the transformants. Because SPL genes are also known activators of miR172, it was predicted that targets of miR172 would show a converse expression pattern to the SPLs, and therefore be up-regulated in the transformants. This prediction was confirmed in the weakly expressing class with a putative switchgrass homolog of the maize glossy15 gene, FL805250, which functions to repress adult leaf cell characteristics (27) (Fig. 3I). The ectopic expression of this gene may help explain the extended juvenile leaf cell characteristics of the Cg1 transformants in switchgrass, maize, and Brachypodium.

**Biochemical Properties of Ubi:Cg1 Plants.** Composition analysis of dried Ubi::Cg1 switchgrass material was carried out to assess its amenability to biofuel production. Total lignin content of dried whole plants was measured using acetyl bromide assays (28) and showed modest reduction in all three classes of transformants (Fig. S3), compared with both wild type and miR156 target mimic transformants used as a negative control. This finding is consistent with earlier lignin measurements of Cg1 leaves in maize (6). Cellulose levels and the amount of cellulose relative to lignin (Fig. S3 B and C) showed a modest increase in all three classes. In addition, two-step sulfuric acid hydrolysis followed by high-performance anion exchange chromatography (HPAEC) was used to measure monosaccharide levels (Fig. S3D) and revealed that each class of transformants had slightly higher glucose levels and lower xylan levels. These biochemical differences in biomass properties, although slight and significant only in the moderate class, indicated that these plants might be more easily saccharified.

**Ubi:Cg1 Plants Store Starch in Their Stem.** Pretreatment of plant biomass is often used to make it more accessible to enzyme breakdown and fermentation. Saccharification assays using dilute alkali pretreatment demonstrated significantly higher glucose release in the severe switchgrass transformants (Fig. 4A) compared with maize, Brachypodium, and Arachis hypogaea Cg1 plants (Fig. S4). By contrast, saccharification assays on Cg1 switchgrass stems and leaves performed using acid pretreatment showed no significant difference in total glucose release over time in the transformants compared with wild type (Fig. S5). These differences suggest that some fermentable biomass components might be made accessible only by specific pretreatment regimes. Because the switchgrass plants never flower, we hypothesized that this difference might be due to alterations in stored carbohydrates, because many nonflowering Arachidopsis mutants display higher starch levels (29). Indeed, potassium iodide-stained hand sections of field grown leaves and stems demonstrated a striking difference in starch levels in stem segments of Cg1 switchgrass
plants compared with wild-type controls (Fig. 4B). In post-flowering wild-type switchgrass, starch is normally found in the lowermost nodes, whereas in Cg1 lines it is present in every node (Figs. S6 and S7). High levels of starch accumulation were not observed in maize, Brachypodium, and Arabidopsis Cg1 lines using the same potassium iodide assay.

Starch levels were measured directly from the stems of field grown transformants harvested before dawn. The weakly expressing transformants had >250% more starch in the stem, whereas the moderately expressing transformants had up to 189% more (Fig. 4C). The severe class had low starch presumably due to the fact that the tissue was a mixture of stem and leaf. Because the weak class of transformants appeared to grow at near normal rates compared with wild type in the field, they were chosen for further in depth analysis.

Additional saccharification assays of the weakly expressing transformants were performed without biomass pretreatment. Because the weak-expressing class had more starch, α-amylase and amyloglucosidase were either added to the saccharification enzyme mix to digest it (method II) or not (method I). Using method I, only a modest increase in glucose release was seen (Fig. 4D). However, three to four times more glucose was released from stems using method II after 24 h (Fig. 4D). The increase in glucose release from these transformants also displayed improved kinetics when measured over 72 h (Fig. 4D). In fact, the amount of glucose release over time from weakly expressing stems using method II without pretreatment is similar to the amount derived from the same stems pretreated with dilute acid (Fig. S5). This finding indicates that Cg1 overexpression may either reduce or negate the pretreatment requirement for saccharification. Thus, weak Cg1 overexpression appears to transform the stem into a starch-containing storage organ, allowing for significantly higher glucose release in saccharification assays without pretreatment and without severely compromising plant growth.

Discussion

The maize Cg1 gene was overexpressed in several plants to fix them in the juvenile phase of development and determine its impact in the context of biofuel production. Cg1 is a unique tandem miR156 microRNA gene that, to date, has been identified only in grass species (7) and may have been under purifying selection during domestication (30). The unique structure of this gene may play a role in transcript stability because the full-length Cg1 transcript can be easily detected on RNA blots (7) in contrast to many miR156 precursors. In addition, overexpression of Cg1 causes a slightly different suite of phenotypes compared with simple miR156 overexpression in Arabidopsis (10). Because miR156 genes are highly conserved and have been identified in all sequenced plant genomes to date, it is feasible to fix any plant of choice in the juvenile phase simply by overexpressing the Cg1 cDNA. Cg1 overexpression in maize, Arabidopsis, Brachypodium, and switchgrass produced plants with extra branches and leaves that possess juvenile morphology, cell identity, and biochemical properties, confirming that high levels of miR156 are sufficient to induce juvenile development. Previous analysis of juvenile biomass in maize showed that it possessed decreased lignin and increased levels of certain sugars (6), making it a superior substrate for fermentation.

The Departments of Energy and Agriculture have identified switchgrass as a potential bioenergy crop that may reduce our reliance on fossil fuels (31). Ethanol produced from switchgrass biomass is projected to produce more than 500% more renewable energy than the energy consumed on the basis of lifecycle analysis and would emit 94% fewer greenhouse gases than gasoline (32). Switchgrass is a warm season C4 perennial grass with no vernalization requirement and can flower under long days followed by short days (33, 34). It can flourish on marginal cropland, does not compete with food crops, and requires minimal growth inputs. Taking into consideration the ecological impacts such as soil conservation, improvement in water quality, wildlife habitat restoration, and reduction in carbon emissions
genes are required for switchgrass aerial stems store starch and initiate both SPL Arabidopsis genes in controlling "homologs were greatly reduced in expression in the AP1 fl AP2 fl SPL transformants, and in fact, many transformants are ef-

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fl oral repressors (21). Overexpression 

A major ecological problem for growers using transgenic crops 

glossy15 

Cg1 flasher presents a major problem for growers using transgenic crops. 

Although additional studies are needed to determine whether 

the presence of excess starch may make plants more susceptible 

to predation, we show that having extra starch may minimize the 

need for extensive pretreatment of the biomass. Starch is much 

easier to digest compared with cellulose and can simply be re-

leased through saccharification with amylase enzymes, which are 

significantly cheaper that cell wall degrading enzymes. Circum-

venting the pretreatment requirement also results in significant 

energy savings, because this process requires very high tempera-

tures and the use of caustic chemicals. In addition, unlike other 

bioenergy crop plants such as sugarcane, dried starchy biomass 

can be stored long term and is easy to transport. Ultimately, this 

information can be readily transferable to other less genetically 

tractable species such as Miscanthus and sorghum, and may 

improve a variety of different plants for use as biofuel crops.

Materials and Methods

Vector Construction and Transformation. The maize full-length Cg1 cDNA was cloned into the maize Ubiquitin cassette vector pUBI-BASK-Neo cassette, and then blunt-end ligated into the Agro binary vector pWBVec8. This binary vector was transformed into Alamo switchgrass (41) and into Brachypodium Bd21-3 (42). The same cDNA fragment was cloned into pENTR (Invitrogen), recombined into Agrobacterium binary pKGW, containing the 35S promoter NOS 3’ cassette, and then transformed into Arabidopsis Columbia plants by floral dipping.

Histology. For plastic sections, plants were fixed overnight in FAA, dehydrated in an ethanol series, and embedded in JB-4 plastic per the manufacturer’s instructions. Two-micrometer sections were made and stained for 30 s in TBO and then photographed using darkfield optics. For epidermal peels, hole punches of juvenile, adult, and Cg1 overexpressing leaves were made, fixed in 1% formaldehyde in PBS, washed several times with water, and then incubated with 0.1% Pectinolyse overnight (43). The next day, the epidermis was peeled off, dipped into TBO, washed, and photographed using brightfield optics.

Field Trials and Branch Counts. Twenty independent UBI::Cg1 overexpressing switchgrass transformants and four wild-type plants were grown in 4 x 4 inch pots for 1–2 mo in the greenhouse, cut back, and then transplanted into the field in early June. Groups of 10 juvenile, adult, and Cg1 overexpressing leaves biomass from 17 of the surviving lines was harvested in late October 2010 and dried for 3 wk at 37°C in a corn dryer before analysis. Tiller numbers were determined after harvest by counting stem stubs. To date, all Cg1 transformants have still not flowered either in the field or in the greenhouse, whereas target mimic negative control lines flowered after 6 mo.

To calculate significance, dry weight and primary branch number data were analyzed using a two-tailed, Student’s t test with unequal variance. *P < 0.0001.

qPCR Analysis. To quantify transcript levels, qPCR was done on a Bio-Rad CFX-96 real-time PCR machine. Five hundred nanograms of poly(A)* purified RNA was used for each cDNA synthesis reaction. Approximately 5–10 ng of cDNA was included into each PCR with single color real-time detection using Sybr Green. All PCR reactions were done with three biological replicates of each cDNA and two technical replicates against the switchgrass actin gene as a reference using the delta Ct method. All primer sequences are available in SI Materials and Methods.

Analysis of Starch. Freeze dried plant material from two biological replicates of each transformant class and two wild-type plants was ground for 1 min to a fine powder in a Kieco ball mill 8200 (Kieco). Alcohol insoluble residue was prepared by washing the samples with ethanol (70% vol/vol), chloroform/ methanol (1:1 vol/vol), and acetone (once, thrice, and once, respectively). The samples were dried in a speed vac.

Approximately 100 mg of alcohol insoluble residue (AIR) was dispersed into 15 mL conical polypropylene tubes in duplicates. The samples were
wetted with 200 μL ethanol (80% vol/vol). Immediately, 3 mL of thermo-
able α-amylase (100 U/mL in 100 mM sodium acetate buffer, pH 5.0) was added and the tube incubated for 12 min at 100 °C in a Technne DB-3B on the heater with intermittent rigorous mixing after 4, 8, and 12 min. The samples were cooled on ice to room temperature and 100 μL of amylglucosidase (3,300 U/mL) was added. After rigorous mixing, the samples were incubated for 30 min at 50 °C. The volume of the samples was adjusted to 10 mL with distilled water and the samples were centrifuged for 10 min at 3,000 rpm. An aliquot of 1 mL of supernatant was transferred to a 2-mL screw-capped tube and the glucose concentration was measured by the YSI 2700 Select Biochemistry Analyzer (YSI Life Sciences). Two technical replicates were performed for each sample.

Starch staining was done on razor blade hand sections of two biological replicates of field grown stems boiled in 95% ethanol, and then dipped in Lugol’s potassium iodine solution, and then rinsed in water and mounted in glycerol.

**Enzymatic Saccharification of Dilute Base Pretreated Biomass**.

Field grown leaves were freeze dried and treated with dilute base, ground to a fine powder, and treated with Accellerase 1500 enzyme mix (Gencor). Each assay was done with ~2 mg of material in 2-mL screw-cap tubes in 1 mL of 50 mM citrate buffer (pH 4.5) including sodium azide to a final concentration of 0.01 mg/mL and 50 °C under constant (mild) agitation for 20 h. A total of 2 μL of enzyme was used per sample. The Megazyme α-Glucose kit was used to measure the glucose content following the manufacturer’s procedures on six technical replicates on single plants from each transformant class.

**Dilute Acid Pretreatment**.

Switchgrass samples from two biological replicates for each transformant class including two wild-type plants were presoaked in pressurized glass tubes at room temperature in 1.2% (wt/wt) sulfuric acid solution for 24 h. The acidic slurry was heated at 160 °C for 30 min. The acidic slurry was filtered through Whatman filter paper with a Buchner funnel. The recovered solids were washed with deionized water until the pH of the washed water reached 5.0.

**Enzymatic Saccharification of Untreated and Dilute Acid Pretreated Biomass**.

Batch enzymatic saccharification of pretreated and untreated biomass samples from two biological replicates of each class were carried out at 50 °C and 150 rpm in a reciprocating shaker. Two enzyme mixtures were used. One mixture was Accellerase 1500 (50 mL) and the other mixture contained Accellerase 1500 plus α-amylase and amylglucosidase (method II). Each assay was done with ~10 mg of material in 5 mL of 50 mM citrate buffer (pH 4.5), including sodium azide, to a final concentration of 0.01 mg/mL, 10 μL of Accellerase 1500 plus 10 μL of Megazyme thermostable α-amylase, and 10 μL amylglucosidase (only with method II) was added to each sample. Sampling was performed at 0, 0.5, 1, 2, 3, 24, 48, and 72 h. The Megazyme α-Glucose kit was used to measure the glucose content following the manufacturer’s procedures. Two technical replicates were performed for each sample.

Additional methods and references can be found in SI Materials and Methods.

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