The Maize *Cornggrass1* miRNA-Regulated Developmental Alterations Are Restored by a Bacterial ADP-Glucose Pyrophosphorylase in Transgenic Tobacco

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Crop-based bioethanol has raised concerns about competition with food and feed supplies, and technologies for second- and third-generation biofuels are still under development. Alternative feedstocks could fill this gap if they can be converted to biofuels using current sugar- or starch-to-ethanol technologies. The aim of this study was to enhance carbohydrate accumulation in transgenic *Nicotiana benthamiana* by simultaneously expressing the maize *Cornggrass1* miRNA (*Cg1*) and *E. coli* ADP-glucose pyrophosphorylase (*glgC*), both of which have been reported to enhance carbohydrate accumulation in planta. Our findings revealed that expression of *Cg1* alone increased shoot branching, delayed flowering, reduced flower organ size, and induced loss of fertility. These changes were fully restored by coexpressing *Escherichia coli* *glgC*. The transcript level of miRNA156 target *SQUAMOSA promoter binding-like* (*SPL*) transcription factors was suppressed severely in *Cg1*-expressing lines as compared to the wild type. Expression of *glgC* alone or in combination with *Cg1* enhanced biomass yield and total sugar content per plant, suggesting the potential of these genes in improving economically important biofuel feedstocks. A possible mechanism of the *Cg1* phenotype is discussed. However, a more detailed study including genome-wide transcriptome and metabolic analysis is needed to determine the underlying genetic elements and pathways regulating the observed developmental and metabolic changes.

1. Introduction

Global energy demand is predicted to grow by 37 percent by the year 2040 [1]. During the same period, the distribution of energy demand will change dramatically, triggered by faster-growing economies and rising consumption in Asia, Africa, the Middle East, and Latin America. To meet this demand, the consumption of petroleum and other liquid fuels is projected to increase from 3.78 billion gallons per day in 2012 to 5.08 billion by 2040 [2]. However, increasing risks of environmental pollution and climate change due to production and use of fossil fuels necessitate the quest for alternative energy sources [3].

Production of biofuels and other chemicals from lignocellulosic biomass has been impeded by biomass recalcitrance (the resistance of plant cell walls to enzymatic deconstruction) largely due to the presence of highly heterogenic polymer lignin, which is a major barrier to cost-effective conversion of biomass to biofuels and useful chemicals [4]. Lignin consists of three major phenylpropanoid units, syringyl, guaiacyl, and hydroxyphenyl units, and can interlock with cellulose and hemicelluloses, limiting the accessibility of these polysaccharides to cellulase and hemicellulase enzymes, respectively [5–7]. Over a period of decades, several pretreatment technologies have been developed to break down lignin in the biomass and increase conversion efficiency [8]. However, these technologies have various limitations and are not being commercialized at the pace needed to address the short-term demand for biofuels. In this context, alternative feedstocks with enhanced carbohydrate yield...
that are easily converted to fuels using current technology have great potential.

Advances in genetic engineering have greatly contributed to the improvement of desirable traits including enhanced biomass yields, polysaccharide content, and modification of the cell wall composition to reduce pretreatment costs [9]. For example, an increase in starch content has been achieved in transgenic potato [10] and cassava [11] tubers overexpressing the Escherichia coli ADP-glucose pyrophosphorylase (AGPase or glgC, EC 2.7.7.27), which catalyzes the first dedicated and rate-limiting step in starch biosynthesis. The glgC gene encodes a major enzyme controlling starch biosynthesis, catalyzing the conversion of glucose 1-phosphate and ATP to ADP-glucose (ADPGlc) and inorganic pyrophosphate, with the ADPGlc subsequently used by starch synthases to incorporate glucosyl units into starch [12, 13]. A mutant form of the enzyme GlgCG336D (the amino acid glycine at position 336 is mutated to aspartic acid), which has less sensitivity to inhibitors and activators [10, 11], was shown to enhance tuber yield in cassava by 260 percent as compared to the nontransformed wild-type plants [11]. This is likely achieved by increasing the GlgC-mediated sink strength for carbohydrates, increasing overall photosynthetic rate, and reducing feedback inhibition of carbohydrate assimilation [10, 11, 14]. Many starch-metabolizing enzymes are redox-regulated [15–17]. Thioredoxins (EC 1.8.1.9) are oxidoreductases that mediate the thiol-disulfide exchange of Cys residues and act as a reductant of the redox-regulated enzymes involved in carbohydrate metabolism [9]. Overexpression of plastidial TrxT gene in transgenic tobacco has been shown to increase carbohydrate biosynthesis (starch and soluble sugars) in leaves [9, 18]. Similarly, a photorespiratory bypass via posttranslational targeting of the E. coli glycolate catabolic pathway (consisting glycolate dehydrogenase (EC 1.1.99.14) subunits E, F, and G) expressed in potato [19] has been shown to increase biomass, rate of photosynthesis, and sugars (glucose, fructose, and sucrose) and transitory starch, suggesting reduced photorespiration [20–22].

In addition to these and other coding genes that produce enzymes, small noncoding RNAs (microRNAs or miRNAs) of approximately 19–24 nucleotides in length can serve as gene regulatory factors and have the potential to improve complex traits including biomass traits [22–24]. Transgenic expression of the maize tandem miRNA Corngrass1, which belongs to miR156, in switchgrass (Panicum virgatum) has been reported to completely inhibit flowering, increase perenni- ality, increase starch content, and improve biomass digestibility with or without pretreatment due to reduced lignin content [25, 26].

miR156 are known to target the SPL transcription factors, which are involved in various physiological processes including promotion of juvenile to adult phase change (heteroblasty), reproductive transition, control of male fertility, and stress responses [27, 28]. The Arabidopsis genome contains 16 SPL genes, the majority of which are targeted by miRNA156 [27, 29]. The SPLs control plant development by directly regulating downstream genes [30]. Based on their conserved DNA-binding domain, the SPLs are grouped into five clades: SPL3/SPL4/SPL5, SPL9/SPL15, SPL2/SPL8/SPL10/SPL11, SPL6, and SPL13A/B [23, 29, 31]. Moreover, gene expression analysis and gain-of-function and loss-of-function studies have revealed several functionally distinct groups [28] including SPL genes regulating control of juvenile-to-adult vegetative transition and the vegetative-to-reproductive transition (SPL2, SPL9, SPL10, SPL11, SPL13, and SPL15) and those that have been reported to play a role in promoting floral meristem identity transition (SPL3, SPL4, and SPL5). One of these genes, SPL8, has been reported to regulate male fertility/seed set, petal trichome production, and root growth [29, 32–34].

In this study, maize Cg1 was expressed in N. benthamiana with or without the E. coli glgC with the purpose of enhancing carbohydrate content in transgenic biomass. Our findings revealed that overexpression of Cg1 alone significantly modulated plant growth and development including delayed flowering, reduced floral organs, and complete loss of fertility. These phenotypes were restored by coexpressing the E. coli glgC. Possible mechanisms of phenotypic alterations in tobacco by Cg1 and the observed antagonistic effect of glgC are discussed.

2. Material and Methods

2.1. Gene Cloning and Generation of Expression Constructs. The sequence of the maize Corngrass1 (Cg1) which encodes two tandem miRNAs [25] (GenBank Acc. number EF541486.1) was synthesized as a gBlocks gene fragment at Integrated DNA Technologies (https://www.idtdna.com) flanked by EcoRI and KpnI for subsequent cloning into the pSAT1 entry vector [35] under the control of the enhanced CaMV 35S promoter. The coding region of E. coli ADPGlc pyrophosphorylase (AGPase or glgC; GenBank Acc. number S58224) was amplified from the pO12 plasmid obtained from Dr. Tony Romeo (University of Florida) using sense (3′-aagg aaaggaCTCGAGatgctttctatatcctcctcgttgacac-3′) and antisense primers, (aaggaCTCCCGGgtggtgatgatgatgtcgctcc tgtgtatgctc) containing XhoI and SmaI, respectively. A 57-amino acid pea chloroplast transit peptide was fused to the N-terminus of the sequence to target protein expression to the amyloplast, a nonpigmented organelle responsible for starch synthesis and storage. Since single-amino acid substitution (Gly336Asp) has been shown to reduce sensitivity of the enzyme to inhibitors and activators [10], the mutation was introduced by site-directed mutagenesis using overlap extension PCR as previously described [36, 37]. glgC was inserted into the pSAT4 entry vector [35] also under the control of an enhanced CaMV 35S promoter. Expression cassettes of Corngrass1 (Cg1) and glgC were assembled into the binary vector pPZP-RCS2 [38] using Ascl and a homing endonuclease I-SceI, respectively, singly or together for coexpression. The resulting binary vectors pPZP-NPTII-Cg1, pPZP-NPTII-glgC, and pPZP-NPTII-Cg1-glgC (Figure 1) were introduced into Agrobacterium strain LBA4404 for subsequent transformation of tobacco (N. benthamiana).

2.2. Tobacco Transformation and Generation of Transgenic Lines. Leaf explants (~0.5 mm2) of 4–6-week-old tobacco were infiltrated with Agrobacterium harboring the expression
vectors pPZP-NPTII-Cg1, pPZP-NPTII-\(\text{glgC}\), and pPZP-NPTII-Cg1-\(\text{glgC}\) or the empty vector pPZP-NPTII for five minutes in the presence of 200 \(\mu\text{M}\) acetylsyringone. Handling of transformed tissues, selection and regeneration, and maintenance of transgenic lines were performed based on Ligaba-Osena et al. [39].

2.3. Validation of Transgene Insertion. To verify the insertion of transgenes, genomic DNA was isolated from 100 mg of fresh leaves of wild-type or transgenic lines (Table S1). The PCR products were analyzed by agarose gel electrophoresis.

2.4. RNA Extraction. Total RNA was extracted from wild-type and independent transgenic lines (Cg1, Cg1-\(\text{glgC}\), or \(\text{glgC}\)). Fully expanded leaves of two-month-old plants were collected and immediately frozen in liquid N\(_2\) and ground to a fine powder using a mortar and pestle. Total RNA was isolated using Spectrum Plant Total RNA Kit (Thermo Scientific). The DNA was used as a template in PCR reactions to amplify the selectable marker gene (neomycin phosphotransferase, \(nptII\)) and Cg1 and \(\text{glgC}\) genes using sense and antisense primers in Supplementary Table S1. The PCR products were analyzed by agarose gel electrophoresis.

2.5. Identification of Cg1 Target Genes. Given that miRNA156 has been implicated in the regulation of flowering via its downstream targets known as SPL transcription factors [22], we studied the expression of putative Cg1 target genes in tobacco. By searching genome database and publications, we identified four SPL contiguous sequences (TC20466, EH36899 (GenBank Acc. number EH368993), and TC9706 and TC7909 [40]. EH36899 showed high homology with SPL11, while TC20466 is annotated as SPL12 (https://solgenomics.net). TC9706 (SPL15) and TC7909 (SPL9) were reported in Tang et al. [40]. To analyze the expression of target genes, primers were designed based on the contiguous sequences (Table S1). Potential Cg1-binding sites in the sequences of SPL genes were determined using the targetfinder.pl software previously developed at the Carrington lab [41].

2.6. Quantitative Real-Time RT-PCR. Expression of transgenes (Cg1 and \(\text{glgC}\)) and putative Cg1-targets was studied by quantitative real-time RT-PCR (qPCR) as described previously [39]. Primers used for gene expression analysis are listed in Table S1; 18S RNA was used as an internal control. Relative expression level was calculated using the \(\Delta\Delta CT\) method available on SDS software (Applied Biosystems).

2.7. Determination of Starch Content. The shoot biomass was ground to 1 mm particle size, and 500 mg was used for the starch assay. The biomass was preextracted to remove free sugars by incubation at 40°C water bath and filtration using Whatman 41 filter paper. The starch content in the biomass was determined according to the Dairy One procedures (Dairy One Forage Laboratory, Ithaca, NY).

2.8. Biomass Saccharification. To see whether coexpression of the Cg1 and \(\text{glgC}\) improves saccharification efficiency, the biomass was harvested at maturity, dried in an oven at 60°C for two days, and ground to powder. One gram of ground biomass was weighed into 15 mL Falcon tubes containing 9 mL of 50 mM sodium acetate pH 5.5, and the samples were vortexed for 2 min and then centrifuged at 9000 g for 10 min. The supernatant was recovered to determine initial sugar content, and the pellet was washed twice with 50 mM sodium acetate buffer and centrifuged again. The final pellet was suspended in 9 mL of the 50 mM sodium acetate buffer to which 50 \(\mu\text{L}\) of each \(\text{Trichoderma reesei}\) cellulase, \(\text{Aspergillus niger}\) glucosidase, and \(\text{Bacillus licheniformis}\) a-amylase (Sigma) [42] was added, with 100 \(\mu\text{L}\) of sodium azide (from 2% stock) added to suppress microbial growth [43]. The reactions were then incubated at 45°C for three days while shaking at 250 rpm. After three days, the samples were centrifuged at 10,000 g for 10 min and the resulting supernatant hydrolysate was filtered (0.22 \(\mu\text{m}\)). This hydrolysate was subsequently analyzed to determine the total reducing sugar yield or the specific sugar species released from the biomass.
2.9. Sugar Quantification. Sugars in the hydrolysate obtained after biomass saccharification were characterized filtered (0.22 μm) for quantifying sugars using Dionex Ion Exchange Chromatography 3000 equipped with an electrochemical detector (Dionex, Sunnyvale, USA) as previously described [39]. The sugar concentration from the IC reading was converted to milligrams of sugar per gram of dry matter or milligrams of sugar per plant. Sugar yield was measured from four replicates for each treatment.

2.10. Statistical Analysis. Experiments were conducted in a complete randomized design in at least three replicates, and each experiment was repeated twice. Data were analyzed using one-way ANOVA using the PROC GLM procedure [44]. After the significant F-tests, the Tukey multiple comparison procedure was used to separate the means (P < 0.05).

3. Results

3.1. Modulation of Vegetative Growth by Cg1. Ectopic expression of Cg1 has been shown to alter growth and development in various plant species [25]. In this study, we investigated whether Cg1 expressed in tobacco alone or with glgC affects plant growth and development. At least six independent lines were generated for each construct. Transgene insertion of at least two independent transgenic lines was validated by PCR using genomic DNA as template (Figure 2(a)).

After one month of growth, transgenic (T₁) lines coexpressing Cg1 and glgC (Cg1-glglC) and glgC were not different from the empty vector and nontransgenic control lines (Figure 2(b)). Because Cg1 lines did not produce seeds, we compared regenerated (T₂) Cg1 plants with the WT, the empty vector control (NPTIII), and Cg1-glglC. Interestingly, Cg1 lines exhibited a distinct phenotype. The Cg1 plants develop smaller leaves and grow slower than the controls and Cg1-glglC (Figure 2(c)). Likewise, in two-month-old plants (Figure 2(d)), transgenic lines expressing Cg1-glglC and glgC were not different from the nontransgenic (WT) and empty vector control lines, suggesting that coexpression of Cg1 and glgC or glgC alone may not interfere with plant growth and development. The Cg1 lines exhibited a bushy phenotype with increased branching and leaf number and reduced leaf size as compared to the WT and the other transgenic lines, which is more evident in two-month-old plants (Figure 2(c)) as compared to the one-month-old seedling, and is more pronounced in Cg1L2 (Figure 2(d)).

3.2. Cg1-Altered Flower Development Is Restored by glgC. In this study, expression of Cg1 singly delayed flowering (Figures 2(c) and 3). Moreover, because the Cg1 plants have more branching, the number of flowers per plants was higher than in the WT, and floral parts were reduced in size when visually observed (Figure 3). The flowers bore smaller petals as compared to WT, whereas there was no marked difference in flowering time (Figures 2(c) and 2(d)) and floral organ development (Figure 3) between the WT control, Cg1-glglC, or glgC. Moreover, the number of flowers per plant in Cg1-glglC and glgC does not appear to be different from that in WT. Intriguingly, none of the flowers of a total of six generated Cg1 lines were fertile; therefore, no seed was recovered from these lines. On the contrary, the flowers of Cg1-glglC and glgC lines were fertile and produced normal seeds same as the WT or empty vector control lines. These findings suggest that coexpression of glgC with Cg1 restores normal flower development and fertility.

3.3. Transgene Expression and Possible Regulation of Putative N. benthamiana SPL Genes. Given that mR156 has been shown to modulate flowering via suppression of SPL transcription factors, we analyzed the transcript level of putative homologs of the Arabidopsis SPL genes including SPL1, SPL9, SPL12, and SPL15 using quantitative PCR in WT and constitutively expressing Cg1, Cg1-glglC, or glgC lines. To distinguish from Arabidopsis SPL genes, the SPL genes analyzed in this study are denoted as NbSPL (for N. benthamiana SPL genes).

As shown in Figure 4, transcripts of both Cg1 and glgC were accumulated at higher levels in the transgenic lines as compared to the nontransgenic control (WT). In Cg1-glglC lines, the transcript levels of Cg1 increased by up to 8000-fold (Figure 4(a)) as compared to the WT while the level of Cg1 transcript accumulation was over 120-fold higher than in WT. Intriguingly, the transcripts of Cg1 in Cg1-glglC lines were over 60-fold higher than in Cg1 lines. Likewise, the transcripts of GlgC were abundantly accumulated in the Cg1-glglC and glgC lines (Figure 4(b)). The increase in transcript abundance ranged from about 120- to 16,000-fold as compared to the WT. The highest increase in glgC transcript was detected in glgC5.

On the other hand, transcript levels of all putative NbSPL genes analyzed were downregulated in most of the transgenic lines. This decrease in transcript levels was more severe in Cg1 lines. Expression of NbSPL1, NbSPL9, NbSPL12, and NbSPL15 was severely downregulated in the Cg1 lines as compared to the WT, Cg1-glglC, or glgC (Figures 4(a)–4(d)). Expression of NbSPL15 was more severely suppressed in the Cg1 lines (Figures 4(c) and 4(e)). The expression of NbSPL9 and NbSPL15 was also downregulated in Cg1-glglC coexpressing lines, but less severely compared to Cg1 lines. Expression of NbSPL1 and NbSPL12 was not markedly affected in the Cg1-glglC lines (Figures 4(d) and 4(f)). Similarly, expression of the SPL genes in glgC lines was not markedly affected (Figure 4). These findings suggest that coexpression of glgC restores expression of NbSPL genes that was suppressed by Cg1.

3.4. Identification of Cg1 and Putative NbSPL Complementary Sites. Since the observed phenotype of the transgenic lines and the gene expression data suggest regulation of the AtSPL paralogue genes in tobacco by Cg1, we searched for the Cg1-binding sites in the putative SPL sequences using the targetfinder.pl software [41]. Putative Cg1-binding sites with high complementarity were identified in all the SPL genes (Supplementary Figure S1). While the complementary site was detected in the ORF of NbSPL1,
Figure 2: Transgenic expression of \( C_g1 \) and \( \text{glg}C \) in tobacco. (a) Integration of \( C_g1 \) and \( \text{glg}C \) into the tobacco genome as confirmed by PCR using genomic DNA as template. Primers specific to \( \text{NPTII} \), \( C_g1 \), or \( \text{glg}C \) were used. (b) Phenotypes of transgenic (T1) and nontransformed tobacco lines after one month. (c) Phenotypes of transgenic (T0) lines regenerated from transgenic callus and nontransformed tobacco lines after one month. Arrows indicate initiation of flowering in WT, \( \text{NPTII} \), and \( C_g1-\text{glg}C \) lines. (d) Phenotypes of transgenic (T1) and nontransformed tobacco lines obtained from seeds, and \( C_g1 \) transgenic (T0) lines regenerated from transgenic callus (right panel) after two months of growth in the soil. Phenotypic alteration is observed in \( C_g1 \)-expressing lines alone.

Figure 3: Comparison of T\(_0\) transgenic lines after flowering. (a) \( C_g1 \)-expressing lines appeared more bushy than did lines expressing \( \text{glg}C \) or coexpressing \( C_g1 \) and \( \text{glg}C \). (b) Close-up pictures to compare floral organs between transgenic and nontransgenic wild-type and empty vector control.
3.5. Overexpression of glgC with or without Cg1 Increases Shoot Dry Matter Yield. To see the effect of glgC on biomass accumulation, dry matter yield of Cg1-glglC- and glgC-expressing T1 lines and the WT was determined. Because the Cg1 lines failed to produce seeds, we were not able to compare the biomass yield of Cg1 lines with that of the WT and lines expressing Cg1-glglC and glgC. As shown in Figure 5(a), coexpressing lines Cg1-glglCL1 and glgCL5 showed between 9 and 48% increase in shoot biomass. In lines Cg1-glglCL1 and glgCL5, biomass yield was increased by 48% and 42%, respectively, followed by glgCL3 (28%) and Cg1-glglCL2 (22%), while biomass yield of Cg1-glglCL3 was only 9% higher than that of the WT.

3.6. Carbohydrate Content in WT and Transgenic Lines. To understand whether overexpression of glgC modulates carbohydrate content, we determined starch and sugar content in mature WT and transgenic lines. The analysis showed that the starch content at maturity was slightly reduced in the transgenic lines overexpressing glgC alone or significantly reduced in a line coexpressing glgC and Cg1 as compared to the WT (Figure 5(b)). The starch content in the Cg1-glglC-
expressing line was reduced by up to threefold. This could be due to the age at which starch content was determined. However, whether decrease in starch content is due to age-dependent changes in carbohydrate dynamics or experimental procedures needs to be determined in detail through future studies.

The concentration of sugars was determined using IC-Dionex in the presence of standards of known sugar concentration. As shown in Figure 6 and Supplementary Table S2, glucose, fructose, and sucrose were the major sugars detected in the samples prior to saccharification by a cocktail of α-amylase, cellulase, and glucosidase. As compared to the WT, slightly more fructose and sucrose were released from the glgC lines (Figure 6(a), I). The amount of sugars released from Cg1-glgC lines was not different from that in the WT. However, the sugar content calculated based on total dry matter per plant in transgenic lines (Cg1-glgCL1, GlgCL3, and glgCL5) was slightly higher than in the WT (Figure 6(a), II) because these lines produced more biomass. Wild-type and transgenic biomasses were subjected to saccharification by a cocktail of α-amylase, cellulase, and glucosidase enzymes to release more sugars. After three days of saccharification, hexose (glucose, galactose, and mannose), and pentose (xylose and arabinose) sugars were released (Figure 6(b), Supplementary Table S3). Glucose was the dominant sugar released from all the tobacco lines (transgenic as well as control lines) (51–66 mg/g DM) accounting for 87% of the total sugars released. The release of glucose was enhanced by saccharification by about fivefold. There was no marked difference in the amount of sugars released from most of the transgenic lines and the WT control, while only glgCL3 released slightly more sugars (66 mg/g DM) than the WT did (51 mg/g DM), as well as the rest of the transgenic lines (Figure 6(b), I). Moreover, GlgCL3 released at least 20% more glucose than the other transgenic lines did. The amount of glucose released from the Cg1-glgC-expressing lines was not significantly different from that in WT plants. There was no marked difference among the lines in the amount of galactose, mannose, xylose, and arabinose released. However, the total amount of sugars (mg/plant) released from most of the transgenic lines (Cg-glgCL1, GlgCL2, glgCL3, and glgCL3) was significantly higher than in the WT control (Figure 6(b), II). Moreover, the total amount of sugars produced in lines overexpressing glgC was slightly higher than in WT and Cg1-glgC lines.

3.7. Discussion. Overexpression of the maize Cg1 in various plant species has been shown to enhance sugar and starch content [25, 26]. Likewise, expression of glgC has been shown to increase sink strength and starch accumulation in transgenic potato [47] and cassava [11]. Therefore, this study was conceived to see whether simultaneous expression of the two genes could modulate carbohydrate metabolism in the transgenic biomass.

3.8. Overexpression of Cg1 Alters Vegetative Growth. In this study, the growth of Cg1-expressing lines was significantly altered. At the early stage, the growth of Cg1 lines was slower than that of the nontransgenic control (Figure 2(b)). Moreover, Cg1 lines produced smaller and more leaves and branches as compared to the WT control. The observed increase in lateral growth could be due to a decrease in apical dominance [48] as reported previously in various plant species expressing Cg1 [25, 26] or miRNA156 [49, 50]. The maize Cg1 expressed in Arabidopsis, Brachypodium, switchgrass [25], and poplar [51] has been shown to produce plants with extra branches and leaves, while in corn, Cg1 has been shown to increase the number of tillers [52]. Similarly, overexpression of the rice stem-loop fragment of the OsmiR156b precursor in switchgrass has been shown to increase tiller

![Figure 5: Determination of biomass and starch content. (a) Matured plants were harvested, and dry matter was determined after drying in an oven at 60°C for two days. (b) Starch content in dried biomass was determined according to the Dairy One procedures as described in the Material and Methods. Bars represent means and standard error of three replicates. Experiments were repeated twice. Bars bearing the same letter are not significantly different (P < 0.05).](image-url)
Reduced leaf size and increased leaf number and alteration of other morphological traits have also been observed in tobacco (*N. tabacum*) overexpressing the *Arabidopsis* miR156A hairpin structure [50].

As reported by Muller and Leyser [53], variation in shoot architecture depends on the formation of axillary meristems and the subsequent regulation of their activation, which depends on the genotype, developmental stage, and environment, which in turn is mediated by hormonal signals. Axillary bud outgrowth and branching are mainly controlled by apical dominance and the crosstalk between plant hormones auxin, cytokinin, and strigolactone [53–55]. In this study, the *Cg1* lines exhibited a bushy phenotype with decreased plant height and increased branching (Figures 2 and 3). This suggests that *Cg1* may affect hormone balance, for example, decreasing the level of auxin and strigolactone or increasing the level of cytokinin; however, this remains to be studied.

### 3.9. Overexpression of Cg1 Alters Reproductive Development.

In this study, we observed a delay in the transition from vegetative to reproductive phase in *Cg1* lines. As compared to the WT and transgenic lines (*NPTII, Cg1-glgC, and glgC*), flower initiation was delayed in *Cg1* lines. Initiation of flowering was observed in less than two months in the former while it was delayed for about two weeks in the latter (Figures 2(c) and 2(d)). This observation is consistent with previous reports on the prolonged juvenile phase in various plant species expressing miRNAs [48, 49, 51, 52].

It is well-established that members of the miRNA miR156 have been shown to prolong juvenile cell identities and delay flowering by targeting the transcripts of the SPL transcription factors, which in turn activate the expression of flowering regulators such as *LEAFY* and *APETALA1* [56] and a different microRNA, *miR172* [57]. Overexpression of *Cg1* in switchgrass has been shown to downregulate the expression of four SPL homologs [25] as compared to the nontransgenic control.

![Figure 6: Biomass saccharification efficiency.](image-url)

Biomass was harvested at maturity and dried in an oven at 60°C for two days. One gram of ground biomass was used for the analysis as described in Material and Methods. Sugars released before (a) or after (b) saccharification with a cocktail of α-amylase, cellulase, and glucosidase were quantified using IC DIONEX. Bars represent means and standard error of four replicates, and measurement was repeated twice. Bars bearing the same letter are not significantly different (P < 0.05).
In this study, we analyzed the gene expression levels of NbSPL1, NbSPL9, NbSPL12, and NbSPL15 (Figure 4). Expression of the NbSPL genes was more severely suppressed in Cg1- and Cg1-glgC-expressing lines as compared to the WT and glgC lines. The reduction in transcript level was more severe in Cg1, particularly for NbSPL15, which was downregulated by nearly a hundred fold, whereas the expression of closely related paralogue NbSPL9 is suppressed by fivefold. In Arabidopsis, both SPL9 and SPL15 have been shown to redundantly regulate juvenile to adult phase transition [58]. AtSPL15 has been implicated in the coordination of basal floral promotion pathways required for flowering in noninductive environments. AtSPL15 has been shown to directly activate transcription of the MADS-box floral activator FRUITFULL (FUL) and miR172b in the shoot apical meristem and during floral induction, whereas AtSPL9 is expressed later in flanks of the shoot apical meristem [59] and has also been implicated in the regulation of miR172b [60]. In contrast to miR156 which delays flowering by suppressing the expression of SPLs, miR172 activates flowering by facilitating the degradation of its target transcription factors related to the APETALA2 (AP2) gene, including TARGET OF EAT1 (TOE1), TOE2, TOE3, SCHLAFMUTZE (SMZ), and SCHNARCHZAPFEN (SNZ), which are implicated in repressing the floral inducer Flowering Locus T (FT) [61], whereas SPL9 has been shown to induce flowering through activating MADS-box genes APETALA1 (AP1), FRUITFULL (FUL), and SUPPRESSOR OF OVEREXPRESSION OF CO1 (SOC1) [56, 62]. The expression level of NbSPL1 and NbSPL12 is also suppressed in Cg1-expressing lines as compared to the WT (Figures 4(d) and 4(f)), suggesting the presence of interaction between Cg1 and NbSPL1/NbSPL12 while the transcript level was not markedly affected in Cg1-glgC and glgC lines. AtSPL1, AtSPL12, and AtSPL14 are expressed most strongly in cauline leaves (growing on the upper part of the stem), flowers, and latest-age shoot apices [63]. The role of SPL1 and SPL12, both lacking negative regulation by miR156 and miR157 [2], in flowering is not well understood. However, recent reports suggest that SPL1 and SPL12 control the expression of many genes and regulate multiple biological processes in Arabidopsis inflorescence upon heat stress [30]. Given that miRNA156 has been implicated in various developmental processes including flowering time, flower fertility, alteration of cell wall composition, and biotic and abiotic stress responses [64], downregulation of NbSPL1 and NbSPL12 homologs in this study may suggest the presence of developmental processes regulated by the interaction of Cg1-NbSPL1/NbSPL12 which needs to be identified in the future.

### 3.10. Overexpression of Cg1 Induces Flower Sterility

Seed production is a key step in the survival of flowering plants; however, its success depends on favorable genetic and environmental factors supporting optimum flower development and fertility. Our findings revealed that flowers of the Cg1-expressing lines were fully sterile, and no seed was recovered from these lines. Although the mechanism of observed sterility in tobacco expressing Cg1 is yet to be understood, it is likely that flower fertility is regulated by Cg1 and its target SPL parologue genes as previously reported. In Arabidopsis, fully fertile flowers require the action of AtSPL8 which functions redundantly with multiple miR156/7-targeted SPL genes including AtSPL2, AtSPL9, and AtSPL15 [33]. In the current study, flower development and fertility were not affected in Cg1-glgC-coexpressing lines (Figures 2 and 3). The Cg1-glgC lines were fully fertile and produced normal seeds, which suggests that a pathway regulated by coexpression of glgC restores floral fertility that was suppressed by Cg1. AtSPL8 is required for proper development of sporogenic tissues in Arabidopsis as early anther development has been shown to be affected in AtSPL8 mutants (spl8-I) overexpressing miR156b, resulting in the development of small and fully sterile anthers [33]. Furthermore, AtSPL8 and miR156-targeted SPL genes also regulate gynoecium development by interfering with auxin homeostasis and signaling [34]. Therefore, the absence of seed formation in Cg1-expressing lines could be due to suppression of a yet to be identified AtSPL8 parologue in tobacco. While it hampers...
plant fecundity, Cg1-induced pollen sterility has a significant biotechnological implication, for example, in eliminating the risk of transgene escape, which is one of the major concerns associated with GM crops [25].

3.11. Possible Role of Carbohydrates in Flower Initiation and Fertility. Our findings revealed that coexpression of Cg1 and glgC does not have a marked effect on flowering time and flower fertility. The phenotype of Cg1-glgC was not different from the nontransgenic control, despite the up to sixtyfold higher Cg1 transcript level in Cg1-glgC lines as compared to those expressing Cg1 alone (Figure 4(b)). This suggests that besides Cg1(miR156)-SPL interaction, there is likely a glgC-sensitive pathway that is involved in the regulation of various aspects of flowering including flower initiation, flower organ development, and fertility. Therefore, since AGPase is a major enzyme controlling starch biosynthesis [13], reconstitution of normal flowering in Cg1-glgC lines as compared to Cg1 lines could be due to glgC-mediated metabolic changes. Involvement of carbohydrates in developmental transitions has been reported before [64–69].

Trehalose-6-phosphate (T6P), which is an indicator of carbohydrate status in plants, has been implicated in the regulation of flowering [65]. A reduction in Tre6P by the loss-of-function mutation of Trehalose-6-phosphate Synthase 1 (TPS1), an enzyme which converts UDP-glucose to Tre6P, has been shown to delay flowering in Arabidopsis, even under flower inductive environmental conditions [65, 70]. Wahl et al. [66] further showed that the Tre6P pathway controls expression of SPL genes via or independently of miR156. Tre6P has also been shown to regulate starch metabolism in plants [71–73]. For example, exogenous application of trehalose in Arabidopsis induced accumulation of starch by increasing the activity of AGPase [71], which has been suggested to be via a thioredoxin-mediated redox reaction [74]. Furthermore, rising sucrose levels in plants are accompanied by increases in the level of Tre6P, redox activation of AGPase, and stimulation of starch synthesis in vivo [71]. Although it remains to be studied, the lack of a normal phenotype in glgC-expressing tobacco lines in the current study could be due to Tre6P-mediated stimulation of AGPase activity and starch synthesis and alteration of carbohydrate dynamics during flower initiation and early floral development. Various genetic and physiological approaches have demonstrated the involvement of starch in the control of floral induction [75] and fertility [76–78].

3.12. Overexpression of glgC Increases Biomass and Sugar Yield. The biomass yield of transgenic lines expressing Cg1-glgC and glgC was higher than in the WT control. This increase in yield is likely due to glgC. glgC has been shown to increase biomass yield in cassava [11] and potato [46] by increasing sink strength for assimilates and releasing possible feedback inhibition on overall photosynthesis or carbon fixation [11]. On the contrary, starch content was not enhanced in glgC-expressing lines (Figure 5) as determined from biomass that was harvested after physiological maturity, which could be due to plant age. Therefore, a more detailed study is needed to determine the level of starch and sugars at different developmental stages. The amount of sugars released from the Cg1-glgC lines before and after saccharification was not different from the WT control. On the other hand, the amount of sugars released from glgC lines was slightly higher than in the WT (Figures 6(a-I) and 6(b-II)). However, the total sugar yield per plant was higher in both Cg1-glgC and glgC lines (Figures 6(a-II) and 6(b-IV)), suggesting that overexpression of glgC with or without Cg1 has a potential to increase overall sugar production as biofuel feedstocks. Taken together, overexpression of glgC only increased total sugar production and sugar release while this was not observed in Cg1-glgC lines. Enhanced total sugar content per plant was observed for glgC and Cg1-glgC expressers as compared to the nontransgenic control, suggesting a potential of the transgenic approach to increase sugar production.

4. Conclusion

Cg1 expression in transgenic tobacco altered vegetative growth, delayed flowering, and led to loss of fertility. Coexpression of glgC with Cg1 restored wild-type phenotype. Cg1-induced changes in vegetative and reproductive growth are likely regulated via suppression of its target SPL genes. The antagonistic effect of glgC in restoring the Cg1 phenotype may suggest involvement of changes in carbohydrate dynamics in flower initiation and fertility. Based on the gene expression analysis and reviewed literature, we propose a model summarizing how Cg1 could modulate flowering and fertility by downregulating the expression of its target SPLs, as well as possible involvement of carbohydrates in flower initiation and fertility (Figure 7). Overexpression of Cg1 leads to down-regulation of SPL transcription factors, which in turn regulates flower initiation, organ growth, and fertility. Likewise, carbohydrates including sugars, trehalose, and AGPase-mediated enhanced starch biosynthesis may be involved in the regulation of flower development and fertility. Future studies will focus on deciphering genetic and physiological mechanisms regulating the observed phenotype. Global transcriptome and metabolomic analysis as well as biomass composition analysis will help in identifying key genetic elements and pathways regulating the observed phenotypes.

Data Availability

All relevant data are available from the corresponding authors upon request.

Conflicts of Interest

The authors declare no competing interests.

Authors’ Contributions

Ayalew Ligaba-Osena and Bertrand Hankoua conceived and designed the study. Ayalew Ligaba-Osena generated and characterized the transgenic lines, analyzed the data, and wrote the manuscript. Kay DiMarco and Tom L. Richard performed the sugar analysis. Tom L. Richard and Bertrand
Hankoua edited the manuscript. All authors have read and approved the final manuscript.

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Supplementary Materials

Supplementary Table S1: primers used for validation of transgenic lines and for gene expression analysis. Supplementary Table S2: composition of free carbohydrates prior to biomass saccharification. Supplementary Table S3: composition of free carbohydrates after biomass saccharification. Supplementary Figure S1: identification of Cgl-binding sites in SPLs. (Supplementary Materials)

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