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Identification and overexpression of *gibberellin 2-oxidase* (*GA2ox*) in switchgrass (*Panicum virgatum* L.) for improved plant architecture and reduced biomass recalcitrance

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Summary

Gibberellin 2-oxidases (GA2oxs) are a group of 2-oxoglutarate-dependent dioxygenases that catalyse the deactivation of bioactive GA or its precursors through 2β-hydroxylation reaction. In this study, putatively novel switchgrass C_{20} *GA2ox* genes were identified with the aim of genetically engineering switchgrass for improved architecture and reduced biomass recalcitrance for biofuel. Three C_{20} *GA2ox* genes showed differential regulation patterns among tissues including roots, seedlings and reproductive parts. Using a transgenic approach, we showed that overexpression of two C20 *GA2ox* genes, that is *PvGA2ox5* and *PvGA2ox9*, resulted in characteristic GA-deficient phenotypes with dark-green leaves and modified plant architecture. The changes in plant morphology appeared to be associated with *GA2ox* transcript abundance. Exogenous application of GA rescued the GA-deficient phenotypes in transgenic lines. Transgenic semi-dwarf lines displayed increased tillering and reduced lignin content, and the syringyl/guaiacyl lignin monomer ratio accompanied by the reduced expression of lignin biosynthetic genes compared to nontransgenic plants. A moderate increase in the level of glucose release in these transgenic lines might be attributed to reduced biomass recalcitrance as a result of reduced lignin content and lignin composition. Our results suggest that overexpression of *GA2ox* genes in switchgrass is a feasible strategy to improve plant architecture and reduce biomass recalcitrance for biofuel.

Introduction

Gibberellins (GAs) are a large group of diterpenoid natural products characterized by the presence of tetracyclic 6-5-6-5 ring structure derived from *ent*-gibberellane (Peters, 2012). Approximately 136 GAs have been identified from plants, fungi and bacteria. GAs are structurally classified into two groups, namely C_{20}s and C_{19}s based on the number of carbon atoms in their ring structure. Only C_{19} GAs such as GA_1, GA_3, GA_4 and GA_7 are known to be biologically active (Hedden and Phillips, 2000; Hedden and Thomas, 2012; Peters, 2012). GA plays major roles in the regulation of various developmental and growth processes that have enormous implication for agriculture. One of the common functions of GA includes GA stimulation of shoot elongation that has been extensively utilized for genetic improvement of cereal crops (Harberd *et al.*, 1998; Hedden and Thomas, 2012; Schwechheimer and Willige, 2009). A very well-known example is found in the semi-dwarf and high-yielding Green Revolution varieties of wheat ('Lerma Rojo 64' and 'Sonora 64') and rice (IR8) developed through breeding, which are now attributed to mutations in either GA signalling pathway intermediates (*Reduced height1*, *Rht1*) (Peng *et al.*, 1999) or gibberellin biosynthetic genes (rice *semi-dwarf1, sd1*) (Sasaki *et al.*, 2002). Moreover, recent studies have shown that the level of bioactive GA is negatively correlated with plant tillering and adventitious root development especially among cereal grains (Lo *et al.*, 2008), whereas it is positively correlated with flower and seed formation (El-Sharkawy *et al.*, 2012; Rieu *et al.*, 2008,b; Sakamoto *et al.*, 2003; Schomburg *et al.*, 2003). Additionally, GA has been implicated with increased lignin deposition in eudicots (Biemelt *et al.*, 2004; Zhao *et al.*, 2010). To date, there are no reports on how lignification in monocots is affected by GA.

Bioactive GAs regulation appears to be tightly controlled in plant tissues via rates of GA biosynthesis and deactivation (Olszewski *et al.*, 2002; Yamaguchi, 2008). The biosynthesis of GA has been characterized in several plant species. In general, there are three main enzyme classes that are involved in GA

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biosynthesis. First, the terpene cyclases catalyse the first two cyclization steps from the linear geranylgeranyl diphosphate (GGDP) to the cyclic *ent*-kaurene. Second, the cytochrome P450 mono-oxygenases catalyse the formation of the first GA, GA_{12} (Helliwell *et al.*, 2001). The last step of GA biosynthesis is catalysed by a group of 2-oxoglutarate-dependent dioxygenases, namely GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox), which are localized in the cytosol (Hedden and Thomas, 2012; Olszewski *et al.*, 2002; Sun and Gubler, 2004). A distinct group of 2-oxoglutarate-dependent dioxygenases known as GA2oxs, however, irreversibly catalyse the deactivation of bioactive GA or its precursors via 2-β hydroxylation (Olszewski *et al.*, 2002; Thomas *et al.*, 1999).

Several C_{19} GA-catalysing GA2oxs have been identified and functionally characterized in various plant species including *Arabidopsis*, pea, rice, poplar, runner bean, pumpkin and wheat (Appleford *et al.*, 2007; Busov *et al.*, 2003; Dijkstra *et al.*, 2008; Lester *et al.*, 1999; Lo *et al.*, 2008; Martin *et al.*, 1999; Radi *et al.*, 2006; Rieu *et al.*, 2008a; Sakamoto *et al.*, 2001; Solfanelli *et al.*, 2005; Thomas *et al.*, 1999). Transgenic overexpression of C_{19} *GA2ox* has caused growth and reproductive abnormalities in rice and *Arabidopsis* hindering their potential use in crop improvement without the use of tissue-specific promoters for targeting their expression to nonreproductive organs (Lee *et al.*, 2014; Sakamoto *et al.*, 2003). Alternatively, a small group of C_{20} GA2ox families comprising rice GA2ox6 (Huang *et al.*, 2010; Lo *et al.*, 2008), *Arabidopsis* GA2ox7/8 (Schomburg *et al.*, 2003) and spinach GA2ox3 (Lee and Zeevaart, 2005) were shown to catalyse the 2-β hydroxylation of C_{20} GAs (GA_{12} and GA_{53}) to form inactive GAs (GA_{110} and GA_{97}), respectively (Lee and Zeevaart, 2005). Transgenic expression of the genes coding for these GA2ox proteins was shown to result in not only less severe dwarf phenotypes but also positively affected root growth with less effect on floral and seed development in rice (Lo *et al.*, 2008; Sakamoto *et al.*, 2003). Heterologous overexpression of *AtGA2ox8* in canola resulted in the development of semi-dwarf lines with normal seed yield, but significantly higher seed weight and increased seed oil content (Zhao *et al.*, 2010). Moreover, overexpression of *AtGA2ox1* in tobacco (Biemelt *et al.*, 2004) and *AtGA2ox8* in canola (Zhao *et al.*, 2010) reduced both lignification and the expression of lignin biosynthetic genes via reduction of the bioactive GA in the plant. A very recent study has also shown that *OsGA2ox5* overexpression was associated with improved resistance to high salinity (Shan *et al.*, 2014).

Modifications of plant architecture to develop compact semi-dwarf plants with more tillers per plant, and higher biomass, might have enormous potential for the improvement of bioenergy feedstocks (Jakob *et al.*, 2009), with GA being an obvious phytohormone to target for altering plant architecture (Stamm *et al.*, 2012). One of the major hurdles of lignocellulosic biofuel feedstock development is the biomass recalcitrance (resistance of the cellulose and hemicellulose in the plant biomass to breakdown into fermentable sugars). Reduced lignin content and/or lignin composition through genetic engineering of lignin biosynthetic genes and transcriptional regulators of lignin biosynthesis has shown promising results in addressing the recalcitrance issue (Baxter *et al.*, 2014; Fu *et al.*, 2011; Shen *et al.*, 2012, 2013; Xu *et al.*, 2011). Thus, manipulation of the level of bioactive GA in the plant may also provide an alternative strategy to reduce biomass recalcitrance as GA has already been indicated to play a crucial role in the regulation of plant lignification in several eudicots (Biemelt *et al.*, 2004; Zhao *et al.*, 2010).

Switchgrass (*Panicum virgatum* L.) is a leading candidate for lignocellulosic biofuel feedstocks because of its high biomass yield, resistance to stress conditions, high nutrient-use efficiency, fast growth and ability to thrive on marginal soil conditions (Yuan *et al.*, 2008). To the best of our knowledge, there are no published results on the switchgrass GA catabolic pathways and the genes involved in catalysing these reactions. Despite the enormous potential that manipulation of GA catabolic pathway has on the improvement of bioenergy feedstocks, little effort has been made to exploit these benefits. Therefore, the purpose of this study was to investigate the impact of genetic manipulation of GA catabolic pathway genes via overexpression of the C_{20} *GA2ox* genes on plant architecture, the lignin content and hence the biomass yield and recalcitrance in switchgrass. In this study, we identified two *GA2ox* genes in the lignocellulosic biofuel feedstock switchgrass. Stable transgenic switchgrass plants were produced whereby overexpression of *PvGA2ox* yielded plants with reduced biomass recalcitrance by decreasing lignin content and composition. Our results indicate that plant architecture and other biomass characteristics such as lignification and biomass recalcitrance could be optimized for sustainable energy production by targeting GA biosynthesis.

Results

In silico analysis of *GA2ox* gene family

A total of 10 putative *GA2ox* genes were identified using the respective amino acid sequences of the rice *GA2ox* counterparts as well as the *Arabidopsis AtGA2ox8* sequence to tblastn query the switchgrass expressed sequence tag (EST) databases. The homologous gene variants of the tetraploid switchgrass ($2n = 4x = 36$) *GA2ox* genes were represented by the two subgenomes 'A' or 'B' of switchgrass as in Figure 1. The phylogenetic analysis confirmed the presence of two discrete groups of putative GA2ox proteins that belong to either C_{19} or C_{20} GA classes. The clusters each contained four C_{20} and six C_{19} switchgrass GA2ox proteins along with their subgenomic variants. Multiple sequence alignment (MSA) analysis showed that switchgrass C_{20} GA2ox proteins, namely PvGA2ox5a, PvGA2ox5b, PvGA2ox6a, PvGA2ox6b and PvGA2ox9a shared all three unique motifs with GA2ox proteins of *Arabidopsis* (AtGA2ox7 and AtGA2ox8), spinach (SoGA2ox3), poplar (PtGA2ox9 and PtGA2ox10) and rice (OsGA2ox5, OsGA2ox6 and OsGA2ox9) (Figure S1). However, the predicted amino acid sequences of PvGA2ox9b were very similar to PvGA2ox9a, but the third motif was missing. These motifs were less conserved in PvGA2ox11, OsGA2ox11 and PtGA2ox11 although they were grouped along with the C_{20} GA2ox proteins in the phylogenetic tree. Moreover, the deduced amino acid sequences of the putative switchgrass C_{20} GA2ox variants identified in this study showed less identity among each other than their respective rice homologues (Lo *et al.*, 2008) (Table S1).

Expression patterns of the switchgrass C_{20} *GA2ox* genes

The quantitative reverse transcription-polymerase chain reaction (qRT-PCR) results showed that the putative C_{20} *GA2ox* genes exhibited differential expression patterns according to the developmental stages of the plant as well as type of plant organs (Figure 2). Specifically, the expression of *PvGA2ox5* was high in the seedling and roots whereas it was very low in the other plant organs and stages of development as compared to both *PvGA2ox6* and *PvGA2ox9*. In contrast, *PvGA2ox6* was expressed at very low levels in the seedling stage. Moreover, *PvGA2ox6* and

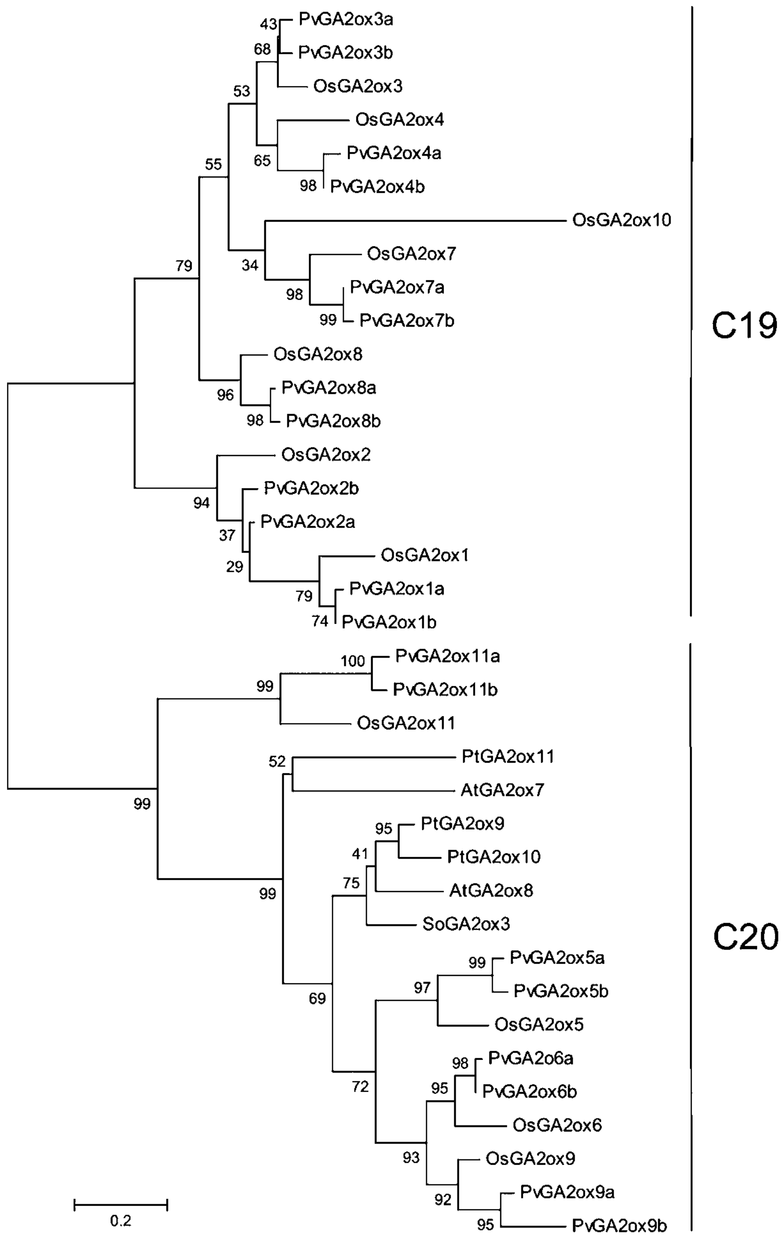

Figure 1 Phylogenetic tree of the C_{19} and C_{20} GA2ox proteins of monocots; switchgrass (*Panicum virgatum*) and rice (*Oryza sativa* L.) and C_{20} GA2ox proteins of eudicots; *Arabidopsis thaliana*, spinach (*Spinacia oleracea*) and poplar (*Populus trichocarpa*) based on the deduced amino acid sequences. The sequences were aligned using MUSCLE program (http://www.ebi.ac.uk/Tools/msa/muscle/), and the alignment was curated by Gblocks at the phylogeny.fr (http://www.phylogeny.lirmm.fr). The tree was constructed by a neighbour-joining method using MEGA 6.0 program (Tamura *et al.*, 2013). Analysis using 1000 bootstrap replicates was performed. The scale bar shows 0.2 amino acid substitutions per site. The locus names of the switchgrass sequences and GenBank accession numbers of the sequences used in this tree are listed in Table S3.

PvGA2ox9 expressions were largely overlapping in almost all plant samples except in the seedling. The level of expression was higher for *PvGA2ox9* in all the plant organs and stages of development examined.

Generation of switchgrass transgenic lines overexpressing *PvGA2ox5* and *PvGA2ox9*

PvGA2ox5b and *PvGA2ox9a* were cloned from cDNA and constitutively overexpressed in switchgrass under the control of maize ubiquitin promoter (Figure S2). Fourteen independent transgenic lines overexpressing *PvGA2ox5* and seven *PvGA2ox9*-overexpressing lines were recovered based on visual screening for expression of the *pporRFP* reporter gene and genomic PCR using primers specific to the transgene and the hygromycin resistance gene (Figure S2). Moreover, the peculiar dwarf and dark-green broad leaf phenotypes of transgenic lines made the screening process facile.

The observed degree of dwarfism between transgenic lines overexpressing the two genes was different (Figure 3). *PvGA2ox5*-overexpressing lines showed an array of dwarfism ranging from extremely dwarf to normal/standard plant height as compared to the nontransgenic control, whereas most of the *PvGA2ox9*-overexpressing lines showed similar degree of dwarfism. The relative expression levels of the transgene in *PvGA2ox9*-overexpressing lines determined by qRT-PCR showed a moderate variation ranging from one to fourfold (Figure 4). However, both types of transgenics exhibited similar GA-deficient phenotypes such as dwarfism and dark-green broad leaves, and slow initial growth as compared to the nontransgenic parent. Therefore, we performed detailed analysis only on the *PvGA2ox5*-overexpressing lines, which appeared to be more promising for bioenergy applications.

Overexpression of *PvGA2ox5* on growth phenotypes in switchgrass

There was a range of relative expression among the *PvGA2ox5* transgenic lines, which appeared to correspond with observed patterns of dwarf phenotypes (Figures 3 and 5). Thus, based on the level of transgene expression and degree of dwarfism, two groups of transgenic lines could be identified: the dwarfs (lines 5, 13 and 14) and the semi-dwarfs (all the remaining lines).

During early developmental stages, transgenic lines had reduced shoot growth while the effect on root growth was less remarkable compared to the nontransgenic controls (Figure 6a). A stark difference in internode length was also observed between the dwarf and the semi-dwarf transgenic lines (Figure 6b). Moreover, the dwarf lines had leafy growth phenotypes with extremely delayed flowering, even after longer vegetative growth.

Additional traits among transgenic plants were also altered, such as tiller height, tiller number and internode length (Table 1). In particular, the dwarf transgenic lines showed 87%–91% reduction in tiller height and 25%–47% reduction in number of tillers per plant relative to the nontransgenic controls. Moreover, these lines exhibited over 96% and 97% reduction in the aboveground fresh and dry biomass, respectively. On the other

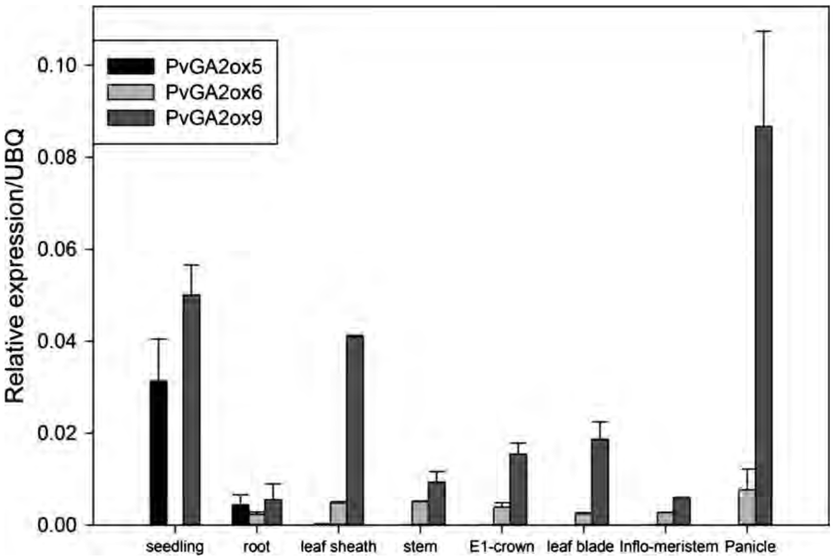

Figure 2 Expression patterns of the three putative C_{20} *GA2ox* genes among tissues and developmental stages in switchgrass as determined by qRT-PCR. The leaf sheath, stem, leaf blade and panicle samples collected from R1 (reproductive stage 1) tillers, samples of inflorescence meristem from E5 (elongation stage with five internode) stage tillers, 2-wk-old seedlings and E1 (elongation stage with one internode) crown were used to obtain the RNA for qRT-PCR. The dissociation curve for the qRT-PCR products showed that the primers were gene-specific. The relative levels of transcripts were normalized to ubiquitin. Bars represent mean values of three replicates ± standard error.

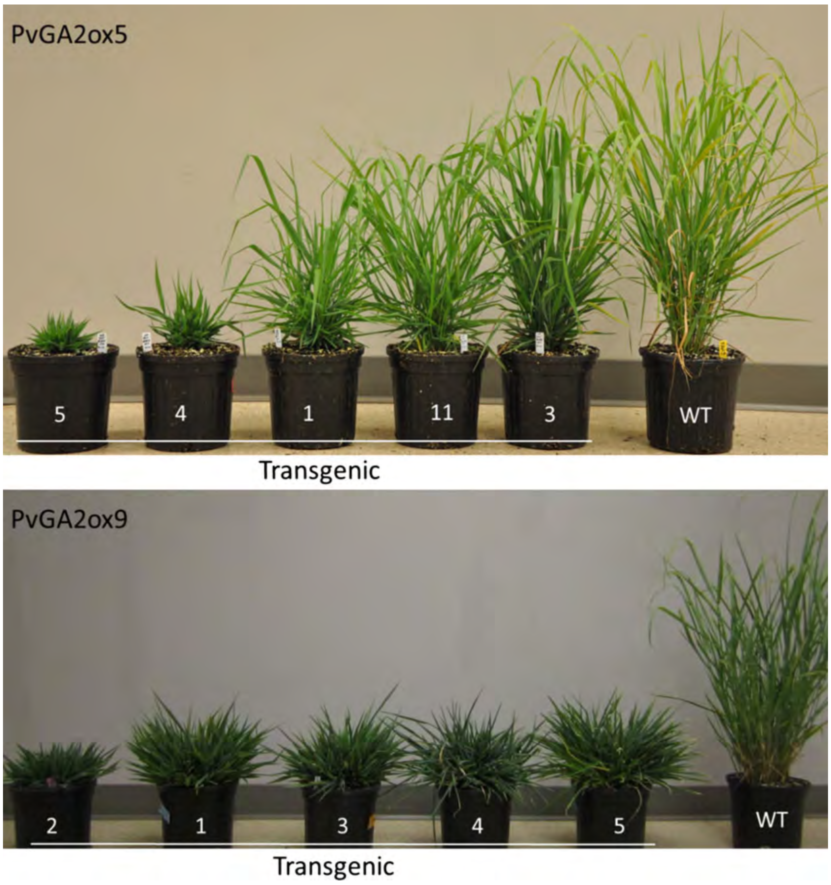

Figure 3 Representative *PvGA2ox5*- and *PvGA2ox9*-overexpressing transgenic lines showing dwarf phenotypes compared to nontransgenic controls (WT).

hand, the semi-dwarf lines displayed a 3%–38% reduction in tiller height, but had a remarkable increase in the number of tillers per plant by 25%–172% as compared to the nontransgenics. Interestingly, up to 35% and 24% increase in fresh and dry biomass weight, respectively, was observed in semi-dwarf transgenic lines relative to controls. More importantly, the dwarf lines exhibited a 54%–83% increase in fresh-to-dry weight ratios, while the semi-dwarf lines also showed up to 41% increase in fresh-to-dry weight ratios. Moreover, heterologous overexpression of *PvGA2ox5* in rice similarly caused extreme dwarfism with severely stunted growth (Figure S3).

Exogenous application of GA on transgenics

To test whether exogenous application of GA_3 could rescue the slow growth phenotypes in the dwarf lines, clones from three of these lines were treated with a 100 μM GA_3 through foliar spray application. Changes in tiller height were recorded weekly for the first 2 weeks followed by a fourth measurement taken 2 weeks later (Figure 7a,b). Foliar spray with 100 μM GA_3 resulted in the recovery of plants as early as 3 days after application (Figure 7a). Further application of GA_3 spray resulted in a rapid recovery in the transgenic lines in a period of about 4 weeks (Figure 7b). Moreover, the growth of transgenic lines was halted when exogenous GA application stopped (Figure 7b).

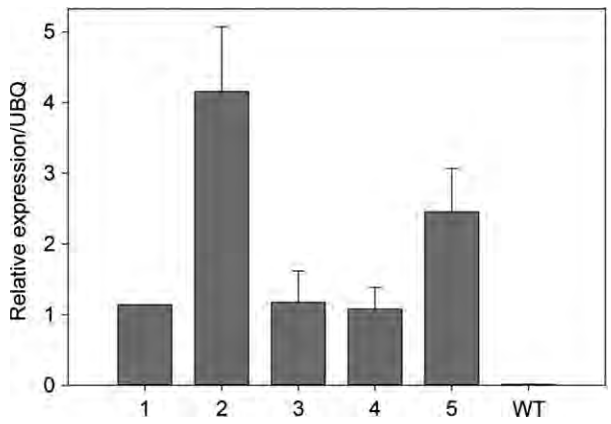

Figure 4 qRT-PCR analysis of *PvGA2ox9*-overexpressing transgenic (1–5) and nontransgenic control (WT) lines from RNA isolated from the top internode of each plant. The relative levels of transcripts were normalized to ubiquitin. Bars represent mean values of three biological replicates ± standard error.

Effect of *PvGA2ox5* overexpression on lignin content and composition

To investigate whether the overexpression of *PvGA2ox5* could have effect on the lignin in switchgrass, histochemical staining (Figure S4) and pyrolysis molecular beam mass spectrometry (py-MBMS) analysis (Figure 8) was conducted. Because of differences in the growth stages between the dwarf and the nontransgenic control lines, we considered only the semi-dwarf transgenic lines and nontransgenic plants from the same developmental stages for these analyses. Accordingly, the phloroglucinol–HCl staining for lignin in the leaves from the 3rd internodes of semi-dwarf lines at R1 (reproductive stage 1) developmental stage revealed a relative reduction in lignin staining relative to nontransgenics (Figure S4). Correspondingly, a quantitative analysis of lignin content in the semi-dwarf transgenic lines by py-MBMS also showed up to 8% reduction in lignin content compared to nontransgenics (Figure 8a). Moreover, analysis of syringyl/guaiacyl (S/G) lignin monomer ratio by py-MBMS also showed up to 23% reduction in transgenic lines overexpressing *PvGA2ox5* as compared to nontransgenic control plants (Figure 8b).

PvGA2ox5 overexpression on the expression of lignin genes

Semi-dwarf lines had significant reduction in expression of most of the lignin biosynthetic genes including *4CL3, CCR, C3H, C4H, CAD, F5H* and *HCT* (Figure 9). Moreover, there were only minor differences in expression levels of lignin biosynthetic genes between dwarf and semi-dwarf transgenic lines except *CCR* (Figure S5).

Effect of *GA2ox* overexpression on the sugar release

Sugar release analysis revealed that there was up to a 15% increase in glucose release from the semi-dwarf transgenic lines as compared to nontransgenics. However, the level of xylose sugar release measured in the same lines showed up to 11%

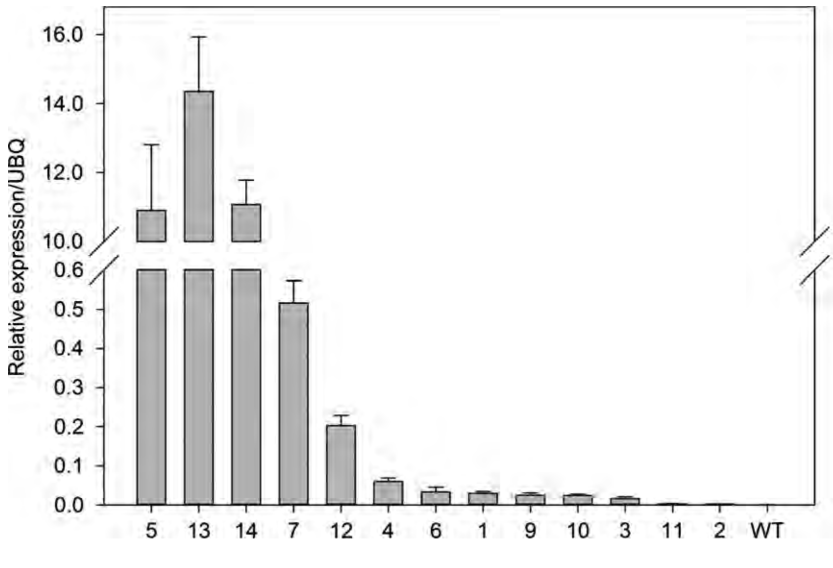

Figure 5 qRT-PCR analysis of *PvGA2ox5*-overexpressing transgenic (1–14) and nontransgenic (WT) lines from RNA isolated from the top internode of each plant. The relative levels of transcripts were normalized to ubiquitin. Bars represent mean values of three biological replicates ± standard error.

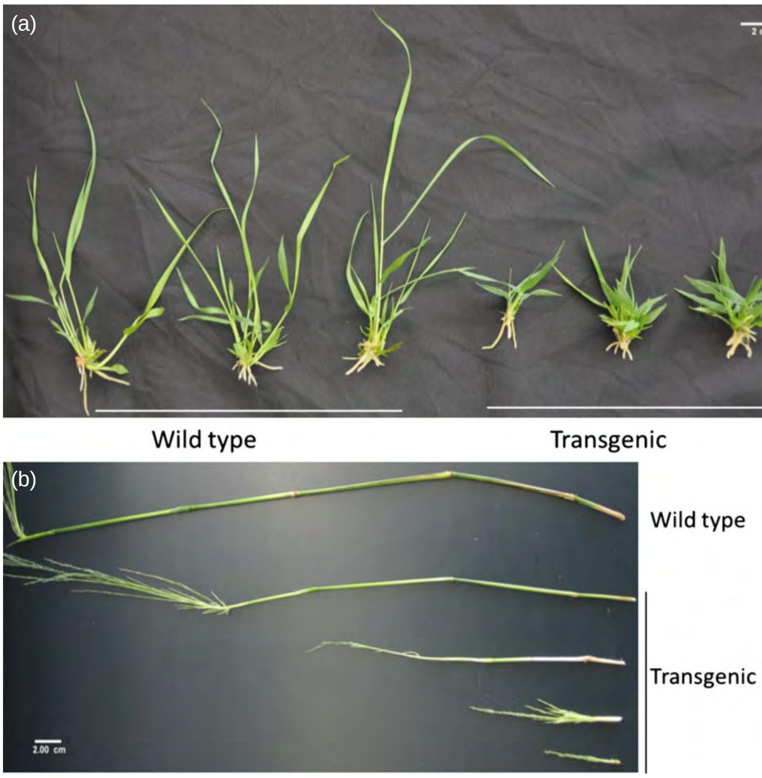

Figure 6 Morphology of plants overexpressing *PvGA2ox5* showing a stunted shoot growth but with less apparent reduction in root growth as compared to the nontransgenic control plant during early stages of development (a). Internode lengths are variably reduced in transgenic lines (b).

reduction relative to controls (Table 2). Consequently, the total sugar release (glucose + xylose) was improved by up to 7% in the semi-dwarf lines relative to controls.

Discussion

In this study, we identified a total of ten GA catabolic *GA2ox* genes comprising six C_{19} and four C_{20} GAs. Of the four switchgrass putative C_{20} GA2ox proteins, three possessed conserved amino acid sequences at all the three motifs shared with the functionally characterized rice, spinach and *Arabidopsis* C_{20} GA2ox proteins (Lee and Zeevaart, 2005; Lo *et al.*, 2008; Schomburg *et al.*, 2003). It has been shown in rice that the C terminal motif is particularly important for C_{20} GA2ox protein activity (Lo *et al.*, 2008). Thus, it can be deduced that the switchgrass GA2ox proteins play similar roles in the GA catabolic pathway. However, the putative switchgrass C_{20} GA2ox, PvGA2ox11, along with its closest homologue in rice, OsGA2ox11, have divergent sequences at these motifs. Whether these GA2ox proteins function in GA catabolism remains to be

Table 1 Morphology and biomass yields of transgenic lines overexpressing *PvGA2ox5* and nontransgenic control (WT) plants

Lines	Tiller height (cm)	Tiller number	Internode length (cm)	Fresh weight (g)	Dry weight (g)	Fresh/dry weight ratio
1	66.7 ± 1.5^{bc}	31.3 ± 2.0^{ab}	7.1 ± 0.2^{bc}	53.1 ± 2.7^{a}	13.9 ± 0.8^{a}	3.8 ± 0.19^{bcdef}
2	78.0 ± 4.6^{ab}	21.3 ± 3.8^{bcde}	10.0 ± 0.4^{a}	50.5 ± 10.3^{a}	16.6 ± 3.6^{a}	3.1 ± 0.06^{f}
3	76.3 ± 1.5^{ab}	25.3 ± 0.9^{bcde}	8.7 ± 0.6^{ab}	52.6 ± 3.3^{a}	14.2 ± 1.4^{a}	3.7 ± 0.15^{bcdef}
4	68.7 ± 1.2^{abc}	28.3 ± 2.7^{abcd}	8.9 ± 0.4^{a}	48.1 ± 5.8^{a}	13.5 ± 1.8^{a}	3.6 ± 0.07^{cdef}
5	10.7 ± 2.3^{e}	10.3 ± 1.5^{de}	–	1.5 ± 0.5^{c}	0.3 ± 0.1^{c}	4.4 ± 0.22^{bc}
6	72.3 ± 0.9^{ab}	25.7 ± 0.9^{bcde}	8.9 ± 0.3^{a}	51.1 ± 2.9^{a}	14.5 ± 1.1^{a}	3.5 ± 0.07^{cdef}
7	59.7 ± 2.2^{cd}	46.3 ± 9.3^{a}	5.4 ± 0.1^{c}	49.6 ± 10.1^{a}	11.8 ± 2.5^{ab}	4.2 ± 0.09^{bcd}
9	76.0 ± 1.2^{ab}	28.7 ± 2.0^{abc}	9.8 ± 0.5^{a}	57.1 ± 2.0^{a}	17.5 ± 0.9^{a}	3.3 ± 0.05^{def}
10	69.0 ± 2.6^{abc}	24.7 ± 1.2^{bcde}	9.2 ± 0.4^{a}	38.5 ± 7.9^{ab}	13.2 ± 2.7^{a}	2.9 ± 0.07^{cdef}
11	75.7 ± 5.2^{ab}	30.0 ± 5.0^{abc}	9.1 ± 0.4^{a}	38.0 ± 0.8^{ab}	11.9 ± 0.2^{ab}	3.2 ± 0.05^{ef}
12	50.3 ± 1.5^{d}	28.3 ± 1.8^{abcd}	5.4 ± 0.2^{c}	16.4 ± 5.3^{bc}	4.0 ± 1.4^{bc}	4.2 ± 0.15^{bcde}
13	7.0 ± 0.6^{e}	12.7 ± 2.7^{cde}	–	0.7 ± 0.2^{c}	0.1 ± 0.02^{c}	5.5 ± 1.09^{a}
14	9.5 ± 0.5^{e}	9.0 ± 2.0^{e}	–	1.1 ± 0.7^{c}	0.2 ± 0.14^{c}	4.6 ± 0.23^{ab}
WT	80.7 ± 2.2^{a}	17.0 ± 3.5^{bcde}	9.9 ± 0.3^{a}	42.3 ± 7.6^{a}	14.2 ± 2.6^{a}	3.0 ± 0.03^{f}

The tiller height was the average of five tallest tillers. The fresh biomass was measured from the aboveground plant biomass cut at similar stages of growth while the dry biomass was measured on fresh biomass dried at 42 °C for 5 days. The plants were grown in 12-L pots in growth chambers under the same conditions for about 9 months before the measurements were taken. Values represented by different letters are significantly different at $P \leq 0.05$.

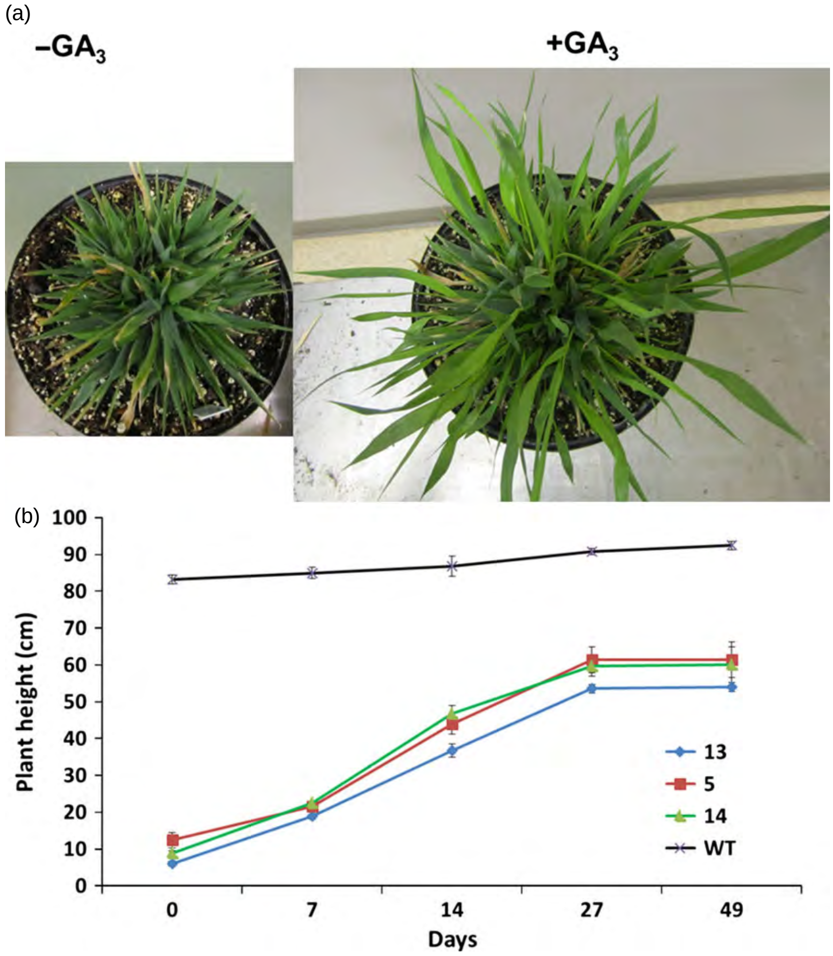

Figure 7 Foliar spray with 100 μM GA_3 application restored normal growth in transgenic lines. Extremely dwarf transgenic plants without treatment with GA_3 (−) and after treatment with 100 μM GA_3 (+) (a). Tiller heights of the transgenic and nontransgenic (WT) plants measured at 0, 7, 14, 27 and 49 day interval with the transgenic plants sprayed three times with 100 μM GA_3 while the WT was sprayed only with water (b). The error bars represent the standard error ($n \geq 3$).

determined. Moreover, the general pattern and number of *GA2ox* genes present in the switchgrass genome correspond with the previous report in rice (Lo *et al.*, 2008). Based on this information and the phylogenetic analysis of putative GA2ox protein sequence in other monocots, GA catabolism is apparently highly conserved among monocots (Figure S6).

The presence of multiple *GA2ox* genes in plants could facilitate differential regulation patterns of gene copies among tissues and organs, which has been documented in rice and poplar (Gou *et al.*, 2011; Lo *et al.*, 2008). The expression pattern analysis of the members of switchgrass C_{20} *GA2ox* genes also indicated the existence of organ-specific differential regulation (Figure 2). Specifically, the nearly exclusive expression of *PvGA2ox5* in the seedling stage as well as in roots highlights the role of this gene in early plant development, including tiller formation and root growth. Consistent with this role, rice *GA2ox5* expressed mainly at seedling and early tillering stage was associated with enhanced tillering and adventitious root formation (Lo *et al.*, 2008). Future studies should shed light on the functional diversification of other switchgrass *GA2ox* genes relative to the C_{20} *GA2ox*s investigated in this work.

Overexpression of the two switchgrass C_{20} *GA2ox* genes, that is *PvGA2ox5* and *PvGA2ox9,* dramatically altered plant architecture resulting in shorter plants with dark-green leaves, extremely reduced internode length, more tillers, and delayed flowering (Figures 3 and 6; Table 1) consistent with previous observation from overexpression of *GA2ox* in numerous other plant species (Appleford *et al.*, 2007; Dijkstra *et al.*, 2008; El-Sharkawy *et al.*, 2012; Lee *et al.*, 2014; Lo *et al.*, 2008). Foliar application of exogenous GA (GA_3) reversed these dwarf phenotypes as expected (Figure 7) (Agharkar *et al.*, 2007; Bhattacharya *et al.*, 2012; Dijkstra *et al.*, 2008; Zhao *et al.*, 2010) indicating that overexpression of *PvGA2ox5* reduced the level of bioactive GA in switchgrass. Moreover, all the observed phenotypes from the overexpression of switchgrass *GA2ox* genes were consistent with that of GA-deficient phenotypes suggesting that the transgenes code for GA catabolic genes.

The level of dwarf phenotype in lines overexpressing *PvGA2ox5* and *PvGA2ox9* observed in this study was in line with the previous observation in rice expressing the corresponding homologues (Lo *et al.*, 2008). Interestingly, overexpression of *PvGA2ox5* in rice also resulted in extremely dwarf plants, indicating the conserved functionality between the two plant species and gene orthologues. Moreover, the observed difference in the relative effect on the shoot and root growth in *PvGA2ox5*-overexpressing lines is indicative of differential regulation of GA levels in the root and shoot by PvGA2ox5 (Figure 6). Similar observation has been reported in rice where overexpression of *OsGA2ox6* showed a reduced shoot growth but not that in roots (Lo *et al.*, 2008). Therefore, based on these results, it could be deduced that *PvGA2ox5* and *PvGA2ox9* genes participate in the deactivation of the C_{20} GA proteins thereby reducing the level of bioactive GA and that these genes may be functional orthologues of the rice *OsGA2ox5* and *OsGA2ox9*, respectively.

Of special note, plant growth was inhibited only when *PvGA2ox5* was highly overexpressed, yielding significantly reduced tiller height (89%) and aboveground fresh (97%) and dry biomass (98%) (Table 1). The semi-dwarf lines with 7.5- to 10-fold lower expression of the transgene than the dwarf lines showed only minor differences in both fresh and dry biomass compared to controls (Table 1). There was a trade-off, in some lines, between tiller number and tiller height (Table 1). The expression of C_{20} *GA2ox* genes in rice was previously reported to promote plant tillering possibly via alteration of GA signalling thereby modulating the expression of transcription factors (TFs) such as the *O. sativa* homeobox1 (OSH1) and TEOSINTE BRANCHED1 (TB1) (Lo *et al.*, 2008). Whether the same pathway is used with similar TFs regulating tillering in switchgrass remains to be investigated. Taken together, these observations further support the assertion that the decreased bioactive GA level in the plant may be from GA2ox-induced GA catabolism as reported in *Arabidopsis*, tobacco, rice and other species (Agharkar *et al.*, 2007; Biemelt *et al.*, 2004; Dijkstra *et al.*, 2008; El-Sharkawy *et al.*, 2012; Huang *et al.*, 2010; Lee and Zeevaart, 2005; Lee *et al.*, 2014; Lo *et al.*, 2008; Zhao *et al.*, 2010). The mechanism behind the decreased bioactive GA level inducing dwarf phenotype, as well as reduced plant biomass, highlights the importance of GA in plant cell elongation and division via elimination of DELLA proteins, the inhibitors of growth promoting factors (Asahina *et al.*, 2002; Cowling and Harberd, 1999; Daviere and Achard, 2013; Digby *et al.*, 1964; Plackett *et al.*, 2011).

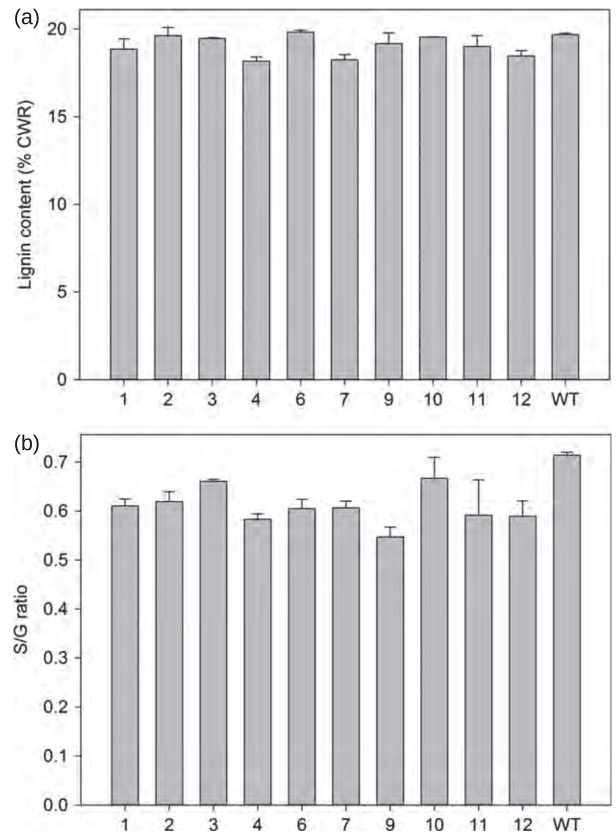

Figure 8 Lignin content (a) and S/G ratio (b) of transgenic and nontransgenic (WT) lines as determined via py-MBMS. Bars represent the average of the replicates ± standard error.

Another intriguing observation in *PvGA2ox5*-overexpressing lines is the relative increase in fresh-to-dry biomass weight ratio (Table 1) accompanied by reduction in lignin content relative to control plants (Figures 8 and S4). Similar observations were previously reported in eudicots such as canola (Zhao *et al.*, 2010) and tobacco (Biemelt *et al.*, 2004) that overexpressed *GA2ox* genes. But to our knowledge, there is no report on how these parameters are affected in monocots such as switchgrass. Here, we hypothesized that lignin reduction could result in decreased dry biomass owing to the fact that lignin normally constitutes over 20% of switchgrass dry biomass; thus, the fresh-to-dry biomass ratio could be increased. Moreover, GA has been shown to directly stimulate lignin accumulation in tobacco petioles (Biemelt *et al.*, 2004). Concurrently, the expression of most lignin biosynthetic genes was shown to be reduced in *PvGA2ox5*-overexpressing lines. This suggests that the mechanism behind the reduction in lignin content might be via reduction in bioactive GA content leading to restricted stimulation of lignin accumulation via its role in the regulation of the lignin biosynthesis pathway. Similar results were reported in canola (Zhao *et al.*, 2010) and tobacco (Biemelt *et al.*, 2004). The observed reduction in S/G ratio in the semi-dwarf transgenic lines might indicate the selective repression of the genes responsible for lignin monomer synthesis although the relative expression level of most of the genes responsible for the synthesis of the two lignin monomers was found to be significantly lower. Reduced S/G ratio in switchgrass has been reported to be associated with improved saccharification efficiency and ethanol yield (Baxter *et al.*, 2014; Fu *et al.*, 2011). Moreover, our analysis demonstrated that

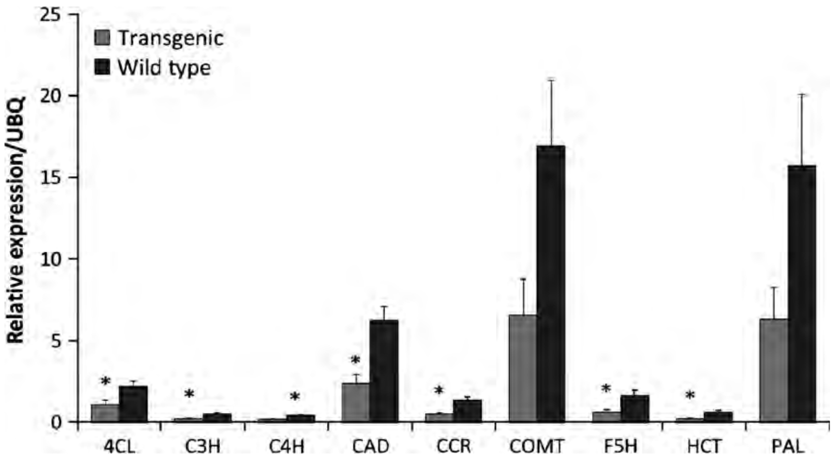

Figure 9 Relative expression of lignin biosynthetic genes in transgenic and nontransgenic lines as determined by qRT-PCR. The relative levels of transcripts were normalized to ubiquitin. Asterisks indicate significant differences from nontransgenic control plants at $P \leq 0.05$. 4CL, 4-coumarate: CoA ligase; C3H, coumaroyl shikimate 3-hydroxylase; C4H, coumaroyl shikimate 4-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; CCR, cinnamoyl CoA reductase; COMT, caffeic acid 3-O-methyltransferase; F5H, ferulate 5-hydroxylase; HCT, hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase; PAL, phenylalanine ammonia-lyase.

Table 2 Sugar release by enzymatic hydrolysis in transgenic and nontransgenic control (WT) lines

Transgenic lines	Glucose release (g/g CWR)	Xylose release (g/g CWR)	Total release (g/g CWR)
1	0.239 ± 0.011	0.172 ± 0.004	0.411 ± 0.025
2	0.207 ± 0.006	0.171 ± 0.002	0.378 ± 0.014
3	0.236 ± 0.006	0.171 ± 0.001	0.408 ± 0.008
4	0.232 ± 0.007	0.169 ± 0.003	0.401 ± 0.013
6	0.238 ± 0.021	0.181 ± 0.011	0.418 ± 0.057
9	0.219 ± 0.003	0.166 ± 0.007	0.385 ± 0.017
10	0.234 ± 0.004	0.175 ± 0.007	0.409 ± 0.020
11	0.227 ± 0.014	0.169 ± 0.011	0.396 ± 0.042
12	0.241 ± 0.003	0.161 ± 0.002	0.401 ± 0.009
WT	0.209 ± 0.007	0.181 ± 0.001	0.390 ± 0.011

CWR, cell wall residues.
All data are means ± SE (n = 3).

GA2ox-overexpressing lines with reduced lignin content have equivalent increase in glucose release efficiency as expected although there is a modest reduction in xylose sugar content. Taken together, these results suggest that manipulation of *GA2ox* gene expression in switchgrass has potential biotechnological applications in the emerging field of bioenergy.

In summary, the switchgrass C_{20} *GA2ox* genes identified in this work have a tremendous potential for the improvement of bioenergy feedstocks for increased biofuel for the following reasons. First, the improved plant architecture characterized by increased tillering and slightly higher plant biomass in the semi-dwarf lines could suit cultivation of switchgrass on marginal lands by providing protection against soil erosion, lodging and weed colonization. Second, the reduced biomass recalcitrance followed by improved sugar release efficiency in these lines could tremendously benefit the lignocellulosic biofuel industry. Additionally, it has recently been reported that genetic engineering for reduced GA levels could enhance plant resistance to pathogens (Qin *et al.*, 2013) and high salinity (Shan *et al.*, 2014). Whether reduced GA levels in switchgrass via overexpression of *GA2ox* play similar roles should be the target of future investigations as this added value may enhance the potential use of these lines in future plant breeding and transgene stacking for various bioenergy traits. Moreover, our findings provide an alternative strategy for genetic engineering of food crops such as cereal grains and fruit trees for semi-dwarfism for the following reasons. First, lines with desirable phenotypes could be selected based on the required level of transgene expression and the degree of dwarfism. Second, the semi-dwarf transgenic lines overexpressing these genes have normal floral and seed development, which are the most desirable traits in these crops (Lee and Zeevaart, 2005; Lo *et al.*, 2008; Schomburg *et al.*, 2003; Zhao *et al.*, 2010). Thus, as initially shown by the first Green Revolution, it is clear that GA biosynthesis biotechnology stands tall as a candidate for manipulation to benefit bioenergy and other crop applications.

Experimental procedures

Plant materials and growth conditions

Plants were grown in growth chambers under standard conditions (16-h day/8-h night light at 24 °C, 390 μE/m^2/s) and watered three times per week, including weekly nutrient supplements with Peter's 20-20-20 fertilizer. Transgenic and nontransgenic 'Alamo' ST1 clone lines were propagated from a single tiller in three replicates for measuring growth parameters. For expression pattern analysis, root, leaf blade, leaf sheath, internode and panicle samples were collected from tillers at R1 developmental stage while the remaining samples were collected from 2-week-old seedlings, E1 (elongation stage with one internode) crown and inflorescence meristem of tillers at E5 (elongation stage with five internodes) stage for assaying transgene transcript abundance (Moore *et al.*, 1991; Shen *et al.*, 2009). Each sample was snap-frozen in liquid nitrogen and macerated with mortar and pestle in liquid nitrogen. Alternatively, samples were stored at −80 °C for subsequent maceration. The macerated samples were used for RNA extraction as described below.

Transgene candidate identification, vector construction and plant transformation

The tblastn program was run to identify the homologous gene sequences in all available switchgrass expressed sequence tag (EST) databases using the amino acid sequences of AtGA2ox8 (At4g21200), OsGA2ox5 (LOC_Os07g01340.1), OsGA2ox6 (LOC_Os04g44150.1) and OsGA2ox9 (LOC_Os02g41954.1). Phylogenetic trees and MSA analysis were used to identify the most closely related genes for cloning. For overexpression of *PvGA2ox5* and *PvGA2ox9*, the open reading frame (ORF) of the genes was isolated from cDNAs of the ST1 clone of switchgrass 'Alamo' cultivar using individual gene-specific primers flanking the ORF of each gene. Both genes were cloned into pCR8 entry vector for sequencing, and subcloned into pANIC-10A expression vector (Mann *et al.*, 2012) by GATEWAY recombination cloning system. The pANIC-10A has the maize ubiquitin 1 (ZmUbi1) promoter driving the expression of the switchgrass *GA2ox* genes. Embryogenic callus derived from ST1 switchgrass genotype was transformed with the expression vector construct through *Agrobacterium*-mediated transformation (Burris *et al.*, 2009). Antibiotic selection was carried out for about 2 months on 30–50 mg/L hygromycin followed by regeneration of orange fluorescent protein (pporRFP; OFP) reporter positive callus sections on regeneration medium (Li and Qu, 2011) containing 400 mg/L timentin. Regenerated plants were rooted on MS medium (Murashige and Skoog, 1962) plus 250 mg/L cefotaxime (Grewal *et al.*, 2006), and the transgenic lines were screened based on the presence of the insert and expression of the transgene. Rice transformation was performed using callus derived from mature seeds of rice variety TP309 as described before (Nishimura *et al.*, 2006).

RNA extraction and qRT-PCR

For transgene transcript analysis, total RNA was extracted from leaf and stem samples of transgenic and nontransgenic lines using Tri-Reagent (Molecular Research Center, Cincinnati, OH). The purified RNA (3 μg) was treated with DNase-I (Promega Madison, WI) to remove any potential genomic DNA contaminants. The DNase-treated RNA was used for first-strand cDNA synthesis using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems Foster city, CA). qRT-PCR analysis was conducted using Power SYBR Green PCR master mix (Applied Biosystems) according to the manufacturer's protocol. All the experiments were conducted in triplicates. The list of all primer pairs used for qRT-PCR is shown in Table S2. Analysis of the relative expression was carried out by the change in C_t method using ubiquitin (UBQ) (Switchgrass Unitranscript ID: AP13CTG25905) as a reference gene (Shen *et al.*, 2009). No amplification product was observed with all the primer pairs when using only the RNA samples or water instead of cDNA.

Phloroglucinol staining

For lignin staining analysis, leaf samples were collected at R1 developmental stage and cleared in a 2 : 1 solution of ethanol and glacial acetic acid for 5 days (Bart *et al.*, 2010). Subsequently, the cleared leaf sample was immersed in 1% phloroglucinol (in 2 : 1 ethanol/HCl) overnight for staining. Low magnification microphotographs were taken using an Infinity X32 digital camera mounted on Fisher Scientific Stereomaster microscope Pittsburgh, PA.

Lignin content and composition by py-MBMS

For the quantification of lignin content and S/G lignin monomer ratio, tillers were collected at R1 developmental stage, air-dried for 3 weeks at room temperature and milled to 1 mm (20 mesh) particle size. Subsequently, lignin content and composition were determined via National Renewable Energy Laboratory (NREL) high-throughput py-MBMS on extractives- and starch-free samples (Baxter *et al.*, 2014; Sykes *et al.*, 2009).

Sugar release

Tiller samples were collected at R1 developmental stage and air-dried for 3 weeks at room temperature before grinding to 1 mm (20 mesh) particle size. Sugar release efficiency was determined via NREL high-throughput sugar release assays on extractives- and starch-free samples (Baxter *et al.*, 2014; Decker *et al.*, 2012; Studer *et al.*, 2010). Glucose and xylose releases were determined by colorimetric assays, and total sugar release is the sum of glucose and xylose released.

Data analysis

Tukey's least significant difference procedure was used to perform multiple comparisons between means of treatments using SAS version 9.3 (SAS Institute Inc., Cary, NC). Different letters next to the numbers in the table indicate a statistically significant difference between values at $P \leq 0.05$ level whereas the asterisk on the bars in the figures shows a significant difference from the controls type at $P \leq 0.05$ level as determined by two-sided *t*-test.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Multiple amino acid sequence alignment of switchgrass C_{20} GA2ox proteins and their closest homologs along with two C_{19} GA2ox proteins.

Figure S2 Molecular characterization of transgenic switchgrass plants overexpressing the *PvGA2ox5* gene.

Figure S3 *PvGA2ox5* overexpressing rice plants showing extremely dwarf phenotypes as compared to the wild type control.

Figure S4 Histochemical detection of lignin in leaves of *PvGA2ox5* overexpressing and wild type lines in light microscopy.

Figure S5 The relative expression of lignin biosynthetic genes in transgenic dwarf, semi-dwarf and non-transgenic (WT) lines as determined by qRT-PCR.

Figure S6 Phylogenetic analysis of putative GA2ox genes from monocots (switchgrass (*Panicum virgatum*), rice (*Oryza sativa*), sorghum (*Sorghum bicolour*), maize (*Zea mays*), foxtail millet (*Setaria italica*) and *Brachypodium distachyon*) and dicots (*Arabidopsis*, poplar (*Populus trichocarpa*) and spinach (*Spinacia oleracea*).

Table S1 Comparison of the deduced amino acid sequences among switchgrass C20 PvGA2ox proteins.

Table S2 List of primers used in this study.

Table S3 List of the locus names and GenBank accession numbers of the sequences used in this study.