Expression of ZmGA20ox cDNA alters plant morphology and increases biomass production of switchgrass (Panicum virgatum L.)

Phat T. Do, Joann R. De Tar, Hyeyeong Lee, Michelle K. Folta and Zhanyuan J. Zhang*

Plant Transformation Core Facility, Division of Plant Sciences, University of Missouri, Columbia, MO, USA

Summary
Switchgrass (Panicum virgatum L.) is considered a model herbaceous energy crop for the USA, for its adaptation to marginal land, low rainfall and nutrient-deficient soils; however, its low biomass yield is one of several constraints, and this might be rectified by modulating plant growth regulator levels. In this study, we have determined whether the expression of the Zea mays gibberellin 20-oxidase (ZmGA20ox) cDNA in switchgrass will improve biomass production. The ZmGA20ox gene was placed under the control of constitutive CaMV35S promoter with a strong TMV omega enhancer, and introduced into switchgrass via Agrobacterium-mediated transformation. The transgene integration and expression levels of ZmGA20ox in T0 plants were analysed using Southern blot and qRT-PCR. Under glasshouse conditions, selected transgenic plants exhibited longer leaves, internodes and tillers, which resulted in twofold increased biomass. These phenotypic alterations correlated with the levels of transgene expression and the particular gibberellin content. Expression of ZmGA20ox also affected the expression of genes coding for key enzymes in lignin biosynthesis. Our results suggest that the employment of ectopic ZmGA20ox and selection for natural variants with high level expression of endogenous GA20ox are appropriate approaches to increase biomass production of switchgrass and other monocot biofuel crops.

Introduction
Biofuels are an important component of the energy sources of the planet, and there is great need for developing biofuel feedstock crops (Carroll and Somerville, 2009; Sticklen, 2008). Switchgrass (Panicum virgatum L.) was the first plant selected for bioenergy by U.S. Department of Energy in the 1990s (McLaughlin and Kszos, 2005). This perennial C4 grass has high productivity across different environments and is adapted to marginal land, low rainfall regions and nutrient-deficient soil (Fike et al., 2006). Switchgrass produces net positive renewable energy and has positive environmental benefits (Schmer et al., 2008). U.S. Department of Energy and U.S. Department of Agriculture have projected a national goal for biofuel to supply 20% transportation fuels by 2030. About 1 billion dry tons of biomass will be annually required for the goal, of which one-third of the biomass will come from perennial feedstock such as switchgrass. Therefore, increasing biofuel crop yields is a major goal of U.S. biomass energy research programme (Sanderson et al., 2006). Numerous factors affecting plant biomass production have been studied and applied in attempts to gain higher crop vegetative yields (Demura and Ye, 2010). Of these, manipulation of endogenous plant hormone contents is one of the most effective ways to improve plant growth, development and biomass.

Gibberellins (GAs) comprise a large family of diterpenoid carboxylic acids of more than one hundred compounds currently known in higher plants, fungi and bacteria (Hedden and Phillips, 2000; Olszewski et al., 2002; Yamaguchi et al., 2008). In higher plants, GA1, GA2, GA4 and GA7 are the most common active GAs that control diverse processes of plant growth and development. The complex pathways of bioactive GA biosynthesis in higher plant require three different classes of enzymes and the participation of different cell components (Olszewski et al., 2002; Yamaguchi et al., 2008). In the cytoplasm, the last steps of GA biosynthesis are catalysed by GA20-oxidase (GA20ox) and GA3-oxidase (GA3ox) to form various GA intermediates and mature bioactive GAs (Hedden and Phillips, 2000; Olszewski et al., 2002). GA20-oxidase, a multifunctional enzyme, catalyses several sequential reactions in the formation of inactive gibberellins (GAs, GA20); then, GA3ox introduces a 3β-hydroxyl group to form the mature products (Yamaguchi et al., 2008).

GA20ox is encoded by genes that have been cloned from various dicots (Carrera et al., 2000; Eriksson et al., 2000; Kang et al., 1999; Phillips et al., 1995) and monocots (Du et al., 2009; Toyomasu et al., 1997). The ectopic expression of genes coding for GA20ox has been shown to increase the levels of bioactive GAs and to affect plant growth and morphology. For example, overexpression of GA20ox caused a higher level of GA1 in Arabidopsis thaliana and consequently to accelerate elongated hypocotyls of seedlings, increasing shoot growth and early flowering (Croker et al., 1999). Overexpression of GA20ox in potato resulted in taller plants and longer leaf petioles (Carrera et al., 2000). Ectopic expression of Arabidopsis GA20ox increased bioactive GAs in transgenic tobacco, leading to increased plant growth and biomass production (Biemelt et al., 2004). In citrus, overexpression of GA20ox modified plant architecture. Transgenic citrus plants had much longer thorns and typical organs at juvenile stages. In addition, higher levels of active GA1 were also
observed in these plants (Fagoaga et al., 2007). In hybrid aspen (Erickson et al., 2000), ectopic expression of GA20ox gene increased growth rate and biomass, and caused more and longer fibres compared to wild-type plants.

In rice, Ayano et al. (2014) showed that expression of GA20ox correlated with GA_{1} and GA_{2} content and has a role in internode elongation, but most studies in monocots have focused on down-regulation of GA20ox gene to reduce plant height and increase reproductive yields (Sasaki et al., 2002). Overall, these results indicate that altering the expression of GA20ox changes GA levels, and consequently also plant growth, development and biomass production.

Here, we report that the expression of ZmGA20ox cDNA in switchgrass results in elevation of bioactive GA levels and altered plant architecture with longer internodes, leaves and increased fresh and dry biomass. Moreover, the expression of ZmGA20ox was found to affect expression of genes in lignin biosynthesis. Our results suggested that ectopic ZmGA20ox is a viable approach for increased biomass for biofuel production by switchgrass and possibly other energy monocots.

Results

Generation of transgenic switchgrass plants with ZmGA20ox

Using binary construct for overexpression of ZmGA20ox and Agrobacterium-mediated transformation, more than 20 transgenic switchgrass events were produced and confirmed based on the leaf painting and genomic PCR using specific primers for ZmGA20ox and hptII genes (Figures 1 and S1, and Table S1). After 2 months of growth under glasshouse conditions, phenotypic differences became obvious among the transgenic events compared to WT control plants. All transgenic events exhibited longer tillers (Figures 2, 3 and 4) and could be divided into four groups: group 1—more tillers but the same growth as WT; group 2—more tillers and much faster growth than WT; group 3—fewer tillers and much faster growth than WT; and group 4—very thin leaves and more tillers and much faster growth than WT. Groups 1–4 exhibited 12.7%, 39.8%, 47.1% and 80% increase in tiller height, respectively, as compared to WT (Figure 3). The longer tillers of transgenic plants were the results of longer internodes and leaves, typical of morphological changes caused by GA_{3}. Group 1 plants displayed less change in internode and leaf elongation than remaining groups. Of all groups, group 4 had the largest increase in leaf (42.7%) and internode (approximately 120%) length (Figures 3 and 4).

All transgenic switchgrass plants exhibited a reduction in internode diameter and leaf width. The internode diameter of transgenic plants was reduced to between 2.90 mm and 3.84 mm compared to 4.23 mm of WT plants (Figures 3 and 4). The average leaf width of transgenic plants was between 10 mm (group 4) and 12.5 mm (group 2), whereas that of WT plants was 15.2 mm. There was a correlation between the increase in internode and leaf length and the reduction in internode diameter and leaf width of transgenic plants except for group 1. Transgenic plants of this group showed a significant reduction in internode diameter as compared to group 2. Interestingly, a substantial increase in the number of tillers appears to be compensated by the decreased internode diameter and leaf width. Other groups displayed similar growth phenotypes, that is the longer leaves and internodes were compensated by the narrow leaves and small internode diameters. In addition, group 4 exhibited a weak tiller phenotype that could not stand up well. Curling leaves also occurred in plants of this group.

Effects of ectopic ZmGA20ox on growth rate, biomass and flowering time

ZmGA20ox transgenic switchgrass plants exhibited increased growth rate, especially during vegetative development stage, and faster tiller emergence and elongation were observed in all transgenic lines. For tiller number, groups 2, 3 and 4 transgenic and WT plants had no significant difference in the number of tillers of each plant. By contrast, group 1 transgenic plants had a remarkable increase in the number of tillers (by approximately 130%) compared to WT plants (Table 1). Interestingly, all transgenic groups showed increases in both fresh and dry biomass weight, but a 19.7%–34.8% reduction in fresh to dry weight ratios, respectively. Specifically, groups 1, 2 and 3 displayed a 1.8- to twofold increase in the whole dry biomass as compared to WT plants, whereas group 4 had insignificant increase in dry biomass. To reconfirm the faster growth of transgenic plants, the tillers were cut and the growth rates measured again. One month after cutting back, tillers of ectopic ZmGA20ox plants were 50.5%–86.9% higher than WT plants (Figure S2).

Switchgrass flowering was observed at the R1 stage of individual tiller (Hardin et al., 2013), but flowering times were not correlated with faster growth rate. At 12 weeks under glasshouse condition, wild-type plants exhibited more than 17% flowering tillers, but for transgenic plants, that varied from 0% to 10.6% (Figure S4). Flowering tillers of wild-type switchgrass reached a peak (96%–100%) at 17 weeks. However, the rate of flowering tillers of transgenic switchgrass gradually increased and ranged from 48.1% to 70.2%.

Cell size changed in ZmGA20ox transgenic plants

We examined the effect of ZmGA20ox overexpression on internode cell size of transgenic plants from group 4 using fluorescence microscopy (Figure 5). Both longitudinal and cross sections of transgenic plant (G4-1) showed smaller pith and xylem cells, and transgenic xylem cells displayed 22.8% reduction in average cell size, while the reduction in pith cell size was 36.6% as compared to wild-type plants (Table 2). These data were consistent with decreased tiller thickness and smaller internode diameter. There was no difference in the vascular bundle distribution in stems and the pith cell length between transgenic plants and wild-type control (Figure 5 and Table 2). Both wild-type and transgenic plants exhibited three circles of vascular

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**Figure 1** Schematic of the T-DNA region of the binary construct for switchgrass transformation. 35S Poly A: CaMV35S poly A terminator; hptII: hygromycin phosphotransferase II gene; CaMV35S, CaMV35S promoter; ZmGA20ox, Z. mays Gibberellin (GA) 20-oxidase; OCS poly A, octopine synthase terminator.
bundles in the cross section of fully elongated internodes at the same developmental stage. Therefore, the longer internodes and leaves of ZmGA20ox transgenic plants could be caused by the increase in cell division at the position of leaf divisional zones and intercalary meristems.

Transgenic phenotypes correspond to GA20ox transcript and GAs levels

Transgene integration patterns of various phenotypic groups were analysed. Several events in each group were randomly selected for Southern blot using ZmGA20ox and hptII partial open-reading frames as probes, respectively. Genomic DNA was digested with restriction enzyme BamHI, which cuts once within the T-DNA region, allowing identification of different events and estimation of the number of transgene copies (Figures 1 and 6). When the hptII probe was used, varying banding patterns were shown in transgenic samples, whereas no hybridizing band was detected in wild-type plants (Figure 6a). However, when the ZmGA20ox probe was used, besides varying molecular weight bands corresponding to the transgene, a 4-kb hybridizing band...
was shown in all samples including wild-type plant. This band is believed to be the endogenous GA20ox gene which shares a high degree of sequence homology with ZmGA20ox (Figure 6b).

We analysed transgene expression and GA levels using a representative event from each group. RNA samples isolated from whole tillers at elongation stage E1 of these events were used for cDNA synthesis, and then for both RT-PCR and quantitative real-time PCR (qRT-PCR) with GA20ox primers (Table S1). The RT-PCR and qRT-PCR results were consistent, showing increases in the transcript abundance of transgenic events from the four main groups (Figure 7). Transgenic event G1-3 showed 4.4-fold increase in the transcription level of GA20ox compared to WT control, while that for other events ranged from 16.3- to 17.7-fold. These results agreed with the phenotypic changes of these transgenic groups as described above. No significant difference in the transcript abundances was found between transgenic lines among groups 2, 3 and 4.

We then analysed GA in ZmGA20ox transgenic events, and focused on GA1 and GA4, two bioactive GAs in higher plants (Table 3). The levels of endogenous GAs were correlated to the degrees of altered transgenic phenotypes. Wild-type and G1-3 plants had very low levels of GA1 and GA4 that could not be detected by our current GAs detection system. A high concentration of GA4 (7.4 ng/g) was recorded, whereas GA1 was not detectable in transgenic event G2-2. Both GA1 and GA4 were detected in transgenic events G3-1 and G4-1 at high levels. Importantly, the highest content of bioactive GAs occurred in G4-1 transgenic plant that had the most significant alteration of phenotype. Furthermore, a higher concentration of GA4 than GA1 was found in all GA-detectable transgenic plants.

**Effects of ectopic ZmGA20ox on lignin gene expression**

To explore whether changes in GA20ox expression affected lignin biosynthesis, transcripts of three genes coding for enzymes in lignin biosynthesis pathway were analysed by qRT-PCR. Of these, 4CL (4-coumarate: CoA ligase) is an enzyme in the early steps of this pathway, while cinnamyl alcohol dehydrogenase (CAD) and caffeic acid 3-O-methyltransferase (COMT) catalyse the final steps of monolignol biosynthesis. In group 4 transgenic plants (G4-1), GA20ox expression level correlated with transcript levels of lignin genes, showing a significant increase in the transcript abundance of all three lignin genes (Figure 8). However, remaining groups had only a minor change in the expression levels of these lignin genes. For example, in group 2 plants (G2-2), the expression of 4CL gene was clearly increased, but CAD and COMT expressions had no significant change. The phloroglucinol-HCl staining for lignin was associated with an altered expression of pathway-specific genes (Figure S3). Much higher lignin accumulation was observed in internode cross sections of transgenic group 4, where

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**Table 1** Tiller number and biomass

<table>
<thead>
<tr>
<th>Groups</th>
<th>Tiller number</th>
<th>Dry weight (g)</th>
<th>Fresh/dry ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>23.7</td>
<td>50.7</td>
<td>4.51</td>
</tr>
<tr>
<td>G1</td>
<td>54.5*</td>
<td>108.9*</td>
<td>3.01*</td>
</tr>
<tr>
<td>G2</td>
<td>31.5</td>
<td>103.1*</td>
<td>3.31*</td>
</tr>
<tr>
<td>G3</td>
<td>17.3</td>
<td>94.2*</td>
<td>2.94*</td>
</tr>
<tr>
<td>G4</td>
<td>28.8</td>
<td>80.6</td>
<td>3.62*</td>
</tr>
</tbody>
</table>

*Significance relative to wild-type at $P \leq 0.05$. 

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**Figure 4** T0 plant morphology. (a) Internodes; (b) leaves; (c) tillers. G1-G4, transgenic groups 1–4; WT, wild-type.
Significance relative to wild-type at G4-1 694.62/C6 WT 440.31/C6 bioactive GAs, respectively. Finally, higher biomass and reduced groups were correlated to the different contents and forms of the wild-type control. Furthermore, varied phenotypes of these García (Biemelt et al. 2001) control of the CaMV35S promoter resembled that in dicots of transgenic switchgrass with the ZmGA20ox gene under the control of the CaMV35S promoter resembled that in dicots (Biemelt et al., 2004; Carrera et al., 2000; Croker et al., 1999; García-Hurtado et al., 2012), maize (Voorend et al., 2015) as well as that of exogenous applications of GA₃ to other monocots (Tsai and Arteca, 1985). These architecture changes included longer leaves, internodes and tillers but smaller leaves and internode diameters compared to wild-type control plants. These morphological changes were highly corresponded with the expression of GA20ox and bioactive GA levels, which were higher than the wild-type control. Furthermore, varied phenotypes of these groups were correlated to the different contents and forms of bioactive GAs, respectively. Finally, higher biomass and reduced lignin staining was exhibited not only in sclerenchyma cell walls but also clearly in parenchyma cell walls. Compared to wild-type, transgenic group 1 did not show a clear change in lignin accumulation. In addition, groups 2 and 3 had a slight increase in lignin content.

**Discussion**

GA20 oxidase is a key enzyme in the pathways of bioactive GA biosynthesis in higher plants, and its overexpression has been shown to alter plant phenotypes and to increase relative growth rates in many plant species. Biomass improvement by modulation of GA20ox genes has been achieved in some dicot plants such as tobacco and hybrid aspen (Biemelt et al., 2004; Eriksson et al., 2000). In this study, the observed alteration of plant architecture of transgenic switchgrass with the ZmGA20ox gene under the control of the CaMV35S promoter resembled that in dicots (Biemelt et al., 2004; Carrera et al., 2000; Croker et al., 1999; García-Hurtado et al., 2012), maize (Voorend et al., 2015) as well as that of exogenous applications of GA₃ to other monocots (Tsai and Arteca, 1985). These architecture changes included longer leaves, internodes and tillers but smaller leaves and internode diameters compared to wild-type control plants. These morphological changes were highly corresponded with the expression of GA20ox and bioactive GA levels, which were higher than the wild-type control. Furthermore, varied phenotypes of these groups were correlated to the different contents and forms of bioactive GAs, respectively. Finally, higher biomass and reduced dry-fresh weight ratios were obtained in all transgenic plants. The present work is the first study to report the ectopic expression of GA20ox in switchgrass for improving biomass.

Bioactive GA₁ and GA₄ are produced at the final stage of GA biosynthesis catalysed through two parallel pathways, involving 13-hydroxylation and the non-13-hydroxylation (Vidal et al., 2001). Higher contents of GA₁ than GA₄ were shown in the GA20ox overexpressing transgenic citrus (Citrus sinensis) (Fagoaga et al., 2007) and Populus (Eriksson et al., 2000). In contrast, ectopic GA20ox in potato (Solanum tuberosum) displayed much higher contents of GA₄ in apical shoots (Carrera et al., 2000). The same result was obtained in both shoots and fruits of transgenic tomato (Solanum lycopersicum). The phenotypes of GA20ox transgenic tomato plants were due to the increase of bioactive GA₄ content (García-Hurtado et al., 2012), whereas both GA₄ and GA₁ had function in internode elongation of deepwater rice (Ayano et al., 2014). In the current study, the same altered phenotypes were exhibited by G2-2 and G3-1 lines, even though the lower content of GA₄ but higher level of GA₁ occurred in the G3-1 line. In addition, the largest phenotypic alteration of transgenic line G4-1 was consistent with the highest levels of GA₁ and GA₄ (Table 3). These data indicate that the elongated phenotypes of ZmGA20ox transgenic switchgrass resulted from the activation of both bioactive GAs.

Longer internodes in CcGA20ox1 overexpression citrus could be correlated to cell divisions (Fagoaga et al., 2007). Similar results were observed in the transgenic AtGA20ox Populus trees (Eriksson et al., 2000). By contrast, transgenic AtGA20ox tobacco plants showed longer shoots that resulted from both cell divisions and elongation (Biemelt et al., 2004). The occurrence of cell division and elongation events corresponded to the regions of active GA biosynthesis and signalling (Kaneko et al., 2003). Moreover, in the study of GA biosynthesis and signal transduction in rice, Ayano et al. (2014) found that internode elongation was induced by the accumulation of GA during submergence. The activation of intercalary meristem located in the nodes was proposed as a driving force in internode elongation. GA levels
were suggested to regulate the growth of maize leaves by spatial control of cell division (Nelissen et al., 2012). In our study, no difference in the length of pith cells between transgenic lines and wild-type control plant was found. Therefore, the elongated internodes and leaves of ZmGA20ox transgenic plants were possibly consequences of the increased cell divisions in the leaf divisional zones, leaf primordia and intercalary meristems under higher levels of GAs (Figure 5 and Table 2).

Gibberellin deficiency by ectopic expression of GA2ox promoted earlier tiller formations, and higher tiller number was indicated in rice (Lo et al., 2008) and switchgrass (Wuddineh et al., 2015). However, the number of switchgrass tillers was not correlated with the GA levels. The semi-dwarf switchgrass lines showed an increase in tiller number, whereas dwarf lines displayed a reduction in number of tillers per plant relative to wild-type controls (Wuddineh et al., 2015). On the other hand, GAs were shown to increase tiller numbers of Welsh onion by initiating and promoting axillary bud development (Yamazaki et al., 2015). In addition, Ni et al. (2015) reported that GAs induced the formation of secondary buds as well as promoted shoot branching of some perennial woody plants by synergistically acts with cytokinin. In our study, there was no significant difference in tiller number between transgenic groups showing high levels of GAs and nontransgenic wild-type plants. However, a remarkable increase in the number of tillers was observed in transgenic group 1, which showed a slight increase in GA20ox transcript abundance. To reconcile the effects upon switchgrass tillering by different levels of GA deficiency (semi-dwarf and dwarf switchgrass) (Wuddineh et al., 2015), it is possible that switchgrass tiller formation and development may be impacted by different levels and components of bioactive GAs.

The overexpression of GA20ox in Arabidopsis promoted flowering (Blázquez and Weigel, 2000; Rieu et al., 2008). By contrast, GAs were indicated to inhibit flowering in grapevine (Boss and Thomas, 2002) and a similar observation was made in tomato (García-Hurtado et al., 2012). Recently, Yamauchi et al. (2014) reported that GA inhibits switch flower formation in Arabidopsis by interactions with genes promoting floral fate such as the EUI-LIKE P450 A1 gene (ELA1), LEAFY transcription factor.
GA3 concentration treatments (Biemelt et al., 2004). Interest-ingly, in our study, some transgenic groups showed increased biomass production while displaying no significant change in lignin gene expression.

In summary, this is the first study on the effects of ectopic GA20ox expression on morphology and biomass of switchgrass. ZmGA20ox transgenic plants exhibited drastic alterations in plant phenotypes resulting in longer leaves and internodes. The increased growth rate caused increased fresh and dry biomass, and demonstrates a means to improve the biomass production of this feedstock and possible other cellulosic crops. Furthermore, the insignificant increase of lignin gene expression and lignin contents in those good phenotype groups should be desirable as bioenergy feedstock. Thus, the expression of ectopic GA20 oxidase could be a good experimental approach to benefit biomass production of monocot plants. Results from this study also implies that the use of variation in the natural GA20ox gene expression would be a viable means to select for improved varieties with higher biomass, avoiding the outcrossing risk of transgenic switchgrass pollen.

### Experimental procedures

#### Vector construction and plant transformation

The open-reading frame (1116 bp) of Zea mays GA20 oxidase (ZmGA20ox) from Genbank (NM_00112453.1) coding for 311 amino acids was synthesized by GenScript (Piscataway, NJ, USA). The EcoRI/HindIII fragment encompassing the ZmGA20ox gene and 35S promoter plus TMV Omega enhancer sequence was inserted into binary vector pCAMBIA1300 to generate transgenic T-DNA construct (Figure 1), which was mobilized into Agrobacterium tumefaciens strain AGL1 for switchgrass transformation by the protocol of Li and Qu (2011) with modifications. Embryogenic calli were induced from mature seeds of switchgrass cultivar Alamo. Hygromycin B (Invitrogen™ Life Technologies, Carlsbad, CA, USA) was added to selected medium at 50 mg/L.

#### Growth condition, leaf painting, sample collection and measurement

Transgenic switchgrass events were grown in glasshouses with day/night temperatures of 28/21°C, a photoperiod of 16 h light/8 h dark, in 3-gal pots containing Promix soil supplemented by Osmocote (14-14-14) (Hummergt International, Earth City, MO). Leaf painting was carried out by swiping 1 g/L hygromycin B solution onto the upper surface of a leaf, and results were recorded 1 week later (Figure S1). The phenotypic data including the morphology of leaves, internodes and tillers were collected at R1 stage (Hardin et al., 2013). Internodes (I3 and I4) and their leaves were subjected to phenotypic observations. The above-ground tissues were harvested when 50% of tillers reached R2 stage for biomass measurements. Dried weight was calculated after switchgrass samples were dried in an oven at 45°C for 96 h.

#### PCR and Southern blot

Gene-specific primers (Table S1) were used for PCRs to confirm the presence of transgenes, and Southern blots were used to confirm their integration into the genome. Genomic DNA was extracted from switchgrass leaf tissue using a CTAB procedure modified from (Dellaporta et al., 1983). For Southern blot analysis, 30 μg purified DNA was digested by a restriction enzyme that cut once within the T-DNA region. Digested DNA fragments were fractionated on a 2.0% agarose gel prior to transfer to Zeta-Probe™ GT nylon membrane (Bio-Rad, Hercules, CA, USA). DNA was fixed to nylon membrane by UV cross-link. Hybridization and membrane washing were conducted based on the Zeta-Probe™ GT manufacturer’s instructions at 65°C. Prime-It® RmT Random Primer Labeling Kit (Stratagene, La Jolla, CA, USA) was used to generate 32P-labelled probes of ZmGA20ox (from synthetic transgene template) or hptII (from pCAMBIA1300 vector).

#### Quantitative real-time PCR

Total RNA was extracted from a whole tiller of wild-type and transgenic switchgrass plants at elongation E1 stage (Hardin et al., 2013) using TRIZOL™ Reagent according to the manufacturer’s protocol (Invitrogen™ Life Technologies). The isolated RNA was treated with DNase-I (Invitrogen™ Life Technologies) to remove genomic DNA contamination. The first-strand cDNA was synthesized from the DNase-treated RNA using M-MLV Reverse Transcriptase and Oligo-dT primer (Promega, Madison, WI, USA). RT-PCR was carried out using specific primers for ZmGA20ox gene (Table S1). qRT-PCR was conducted using IQ™ SYBR® Green

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**Figure 8** Relative expressions of lignin genes in transgenic switchgrass and wild-type by qRT-PCR. 4CL, 4-coumarate:CoA ligase; CAD, cinnamyl alcohol dehydrogenase; COMT, caffeic acid 3-O-methyltransferase.

*Significant relative to wild-type (P < 0.05).
Supermix (Bio-Rad). The data were normalized using the levels of switchgrass ubiquitin (UBQ) transcripts (Xu et al., 2011). The primers used for qRT-PCR were the same as described above for RT-PCR (Table S1). Transcript abundance was quantified using three independent biological replicates.

Microscopy and cell size measurement

Images of cross and longitudinal sections of fully elongated internodes were captured under Olympus IX70 Inverted Microscope with ORCA-ER Digital Camera fluorescence optics at 10×. Images were analysed by MetaMorph Microscopy Automation and Image Analysis software to identify cell size, length and number.

Lignin staining

For lignin staining, internode samples were collected at reproduction developmental stage (R1). Internode were cut by Vibratome series 3000 to generate 60 μm cross sections and cleared by ethanol overnight. Cleared sections were immersed in 1% chloroformic staining solution (in 2 : 1 ethanol/HCl) for 2 min (Baum, 2008; Bart et al., 2010; Wuddineh et al., 2015). The cross sections were placed on microscopy slides and covered by coverslip. The edges of the slides were sealed with commercial sealant and examined under Leica DM 5500B Compound Microscope with Leica DFC290 Color Digital Camera at 10×.

GA quantification

Gibberellins were extracted in cold methanol/isopropanol:acetic acid (20:79:1, v/v/v) from samples spiked with deuterium-labelled internal standards of GA1 (D2-GA1; Olkemim Ltd, Olomouc, Czech Republic). After centrifugation at 16 000 g, the supernatants were collected and pellet extraction repeated. The pooled supernatants were evaporated and the resulting pellet redissolved in 200 μL of 30% methanol. Chromatographic separation of metabolites was accomplished using a 3C18-EP-120 column (0.5 mm x 100 mm; Eksigent, Dublin, CA, USA) using a mobile gradient of 85% solvent A (0.1% acetic acid in HPLC-grade water, v/v) to 95% solvent B (0.1% acetic acid in 90% acetonitrile, v/v) in 6 min at a flow rate of 15 μL/min. A 6500-QTRAP (AB Sciex, Foster City, CA, USA) was used to acquire MS spectra. Parameters for analysis were set as follows: ESI in the negative mode (TurbolonSpray), capillary voltage —4500, nebulizer gas 25 arbitrary units (a.u.), heater gas 25 a.u., curtain gas 10 a.u., collision activation dissociation —2 and temperature 250°C. Gibberellins GA1 and GA4 were detected using multiple reaction monitoring (MRM) transitions that were optimized using the standards (GA1 and GA4; Olkemim Ltd) and the deuterium-labelled standard. Concentrations were determined from standard curves of known GA concentrations.

Data analysis

Comparisons between transgenic and wild-type control plants were made by Turkey’s least significant difference procedure using one-way ANOVA and t-test in SPSS software (ver.20; Chicago, IL). Standard errors are provided for statistical diagrams as appropriate. The asterisks on the bars in the figures and the tables indicate a significant difference from the wild-type controls at P < 0.05 or 0.01 levels.

Acknowledgements

The authors thank Dr. William Folk for proofreading of the manuscript and helpful suggestions, and Drs. David Braun and David Mendoza for helpful discussions. Thanks are also extended to Neng Wan (Dr. Zhang’s lab) for his help with glasshouse work, Dr. Sophie Alvarez and the Proteomics Facility at The Donald Danforth Plant Science Center, St. Louis, MO, for assistance with the mass spectrometry (supported by NSF Grant No. DBI-1427521 for acquisition of the QTRAP LC-MS/MS), and the Molecular Cytology Core at University of Missouri for microscopy analysis. P.T. Do was supported by the Vietnam Educational Foundation. The authors have no conflict of interest to declare.

References


Supporting information

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

**Figure S1** Switchgrass leaf painting using hygromycin B.

**Figure S2** Effects of *ZmGA20ox* overexpression on plant growth rate.

**Figure S3** Lignin staining of internode cross sections. (a) Wild-type; (b) G1-3; (c) G2-2; (d) G3-1; (e) G4-1.

**Figure S4** Flowering time of *ZmGA20ox* overexpression plants.

**Table S1** Primers used in this study.