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Overexpression of *GA20-OXIDASE1* impacts plant height, biomass allocation and saccharification efficiency in maize

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Summary

Increased biomass yield and quality are of great importance for the improvement of feedstock for the biorefinery. For the production of bioethanol, both stem biomass yield and the conversion efficiency of the polysaccharides in the cell wall to fermentable sugars are of relevance. Increasing the endogenous levels of gibberellic acid (GA) by ectopic expression of *GA20-OXIDASE1* (*GA20-OX1*), the rate-limiting step in GA biosynthesis, is known to affect cell division and cell expansion, resulting in larger plants and organs in several plant species. In this study, we examined biomass yield and quality traits of maize plants overexpressing *GA20-OX1* (GA20-OX1). GA20-OX1 plants accumulated more vegetative biomass than control plants in greenhouse experiments, but not consistently over two years of field trials. The stems of these plants were longer but also more slender. Investigation of GA20-OX1 biomass quality using biochemical analyses showed the presence of more cellulose, lignin and cell wall residue. Cell wall analysis as well as expression analysis of lignin biosynthetic genes in developing stems revealed that cellulose and lignin were deposited earlier in development. Pretreatment of GA20-OX1 biomass with NaOH resulted in a higher saccharification efficiency per unit of dry weight, in agreement with the higher cellulose content. On the other hand, the cellulose-to-glucose conversion was slower upon HCl or hot-water pretreatment, presumably due to the higher lignin content. This study showed that biomass yield and quality traits can be interconnected, which is important for the development of future breeding strategies to improve lignocellulosic feedstock for bioethanol production.

Introduction

Bioenergy grasses are defined as members of the grass family (*Poaceae*) that employ C4 metabolism, and are capable of producing high amounts of lignocellulose, fermentable juice or fermentable grain (Feltus and Vandenbrink, 2012; Vermerris *et al.*, 2007). Perennial grass species are considered to have the highest potential as dedicated biomass crops, as they require a low cultivation input (Muylle *et al.*, 2015; Van der Weijde *et al.*, 2013). In more moderate climates, also C3 forage grasses such as perennial ryegrass can form a potential feedstock for the production of bioenergy (Tonini *et al.*, 2012; Wang and Brummer, 2012).

Zea mays (maize) is often regarded as a bioenergy grass, despite its annual growth behaviour and the need for significant water and fertilizer input. At present, the first generation of biofuels is produced from the easily accessible sugars in the maize grain, but is considered unsustainable (EASAC, 2012; IPCC, 2014). However, the maize stover represents an abundant source of biomass for cellulosic ethanol production (Vermerris *et al.*, 2007), even when sufficient amount of biomass is left on the field to prevent a reduction in soil fertility (Van der Weijde *et al.*, 2013). Approximately 1 billion tonnes of stover is produced annually as crop residue from maize grain production (FAO Statistics Division, 2013), based on an average harvest index of 50% (dry weight of the seed divided by the total dry weight, from Lorenz *et al.*, 2010 and Pennington, 2013). Thus, the well-established production chains for maize have the potential to supply the next generation of biorefineries with large amounts of agricultural residues (Van der Weijde *et al.*, 2013; Waclawovsky *et al.*, 2010).

The improvement of lignocellulosic crops, including maize, as feedstock for the production of bioethanol currently focuses either on enhanced biomass yield or on improved biomass quality. However, focusing on improving one of these traits holds the risk of unwillingly negatively affecting another trait, with no net benefit as result. Indeed, in the past decades, breeding efforts in Europe achieved a great yield improvement in maize through the introduction of hybrids with favourable alleles for grain production and forage yield, pest resistance and stalk breakage resistance. However, this has led to unfavourable effects on plant digestibility. Cell wall digestibility has declined steadily since the 1950s, leading to a substantially reduced feeding value of elite forage maize hybrids (Barriere *et al.*, 2006), until cell wall digestibility was included as evaluation criterion during official variety testing trials (Boon *et al.*, 2012; Brenner *et al.*, 2010; Jung, 2011; Pichon *et al.*, 2006; Riboulet *et al.*, 2008).

The improvement of biomass quality for processing to fermentable sugars, determined by the saccharification efficiency, can also negatively affect biomass yield (Casler *et al.*, 2003; Pedersen *et al.*, 2005; Van Acker *et al.*, 2014). Lignin content is one of the main factors determining saccharification efficiency

(Chen and Dixon, 2007; Van Acker *et al.*, 2013; Zeng *et al.*, 2014), but perturbation of lignin biosynthesis often affects other biochemical routes (Bonawitz and Chapple, 2013; Vanholme *et al.*, 2012a,b; Vogt, 2010; Zabala *et al.*, 2006). Depending on the genotype and the environmental conditions, potential effects of lignin biosynthesis perturbation on agricultural fitness include reduced grain yield, reduced dry matter yield, reduced early season vigour and increased propensity to lodging (Chen, 2011; Pedersen *et al.*, 2005; Van Acker *et al.*, 2014).

Plant biomass that is used for cellulosic biofuel production is primarily from vegetative tissues, such as stems and leaves. The effect of gibberellic acid (GA) on stem height and whole-plant growth rates under different environmental conditions has been extensively studied in several plant species (Baker, 1987; Cleland and Briggs, 1969; Juska, 1958; Lambers *et al.*, 1995; Lester *et al.*, 1997; Norcia *et al.*, 1964; Paleg *et al.*, 1965; Peng *et al.*, 1999; Sasaki *et al.*, 2002; Srivastava, 2002; Zawaski and Busov, 2014). The reduction in endogenous GA levels was an important factor in the green revolution, when mutants in GA biosynthesis or signalling were used for breeding wheat and rice varieties (Evenson and Gollin, 2003; Peng *et al.*, 1999; Sasaki *et al.*, 2002). These GA biosynthesis mutants had shorter stems with improved harvest index (Flintham *et al.*, 1997; Walcott and Laing, 1976). On the other hand, enhanced endogenous GA levels can result into taller plants and larger organs, which was demonstrated in Arabidopsis (Coles *et al.*, 1999; Huang *et al.*, 1998), tobacco (Biemelt *et al.*, 2004), rice (Oikawa *et al.*, 2004), potato (Carrera *et al.*, 2000), tomato (García-Hurtado *et al.*, 2012), citrus (Fagoaga *et al.*, 2007) and poplar (review by Dubouzet *et al.*, 2013). Notably, GA levels also influence cell wall composition. For instance, increased GA levels induced xylem lignification in tobacco (Biemelt *et al.*, 2004), and an increased syringyl/guaiacyl (S/G) ratio in the lignin of poplar (Israelsson *et al.*, 2003). A reduction in endogenous GA levels leads to lower lignin levels in tobacco (Biemelt *et al.*, 2004) and rapeseed (Zhao *et al.*, 2010). Furthermore, in eucalyptus and poplar, GA levels affect the length of xylem fibres, which are a major constituent of lignocellulosic biomass (Eriksson *et al.*, 2000; Israelsson *et al.*, 2005; Mauriat and Moritz, 2009; Ridoutt *et al.*, 1996).

In maize and rice, it has been suggested that variation in endogenous GA levels is responsible for the variation in plant growth rates in hybrids (Ma *et al.*, 2011; Rood *et al.*, 1990). The overexpression of *GA20-OX1,* a key gene in GA biosynthesis (Claeys *et al.*, 2013; Yamaguchi, 2008), under the control of a constitutive promoter in maize resulted in increased endogenous GA levels (Nelissen *et al.*, 2012). This resulted in longer stems and leaves (Nelissen *et al.*, 2012; Voorend *et al.*, 2014). The latter has been assigned to a larger cell-division zone (Nelissen *et al.*, 2012). These plants were not yet evaluated for whole-plant biomass production in greenhouse or field conditions.

In this study, we evaluate the potential of *GA20-OX1* overexpression in maize as a strategy for the improvement of feedstock for bioethanol production. Plant morphology and vegetative plant biomass of *GA20-OX1*-overexpressing (hereafter GA20-OX1) and control maize plants were determined in greenhouse and field conditions. In addition, biomass quality traits relevant to saccharification efficiency were examined. Lignin and cellulose deposition as well as lignin biosynthetic gene expression were monitored during stem development to obtain insights into the mechanisms that can mediate the interaction between GA accumulation and cell wall properties.

Results

GA20-OX1 overexpression alters plant morphology in greenhouse and field conditions

Maize plants that overproduce GA were obtained by overexpression of *GA20-OX1* under the control of the maize *UBI* promoter (Nelissen *et al.*, 2012). Greenhouse-grown hemizygous transgenic and control plants were evaluated for plant morphology and biomass yield. Already in the earliest investigated stage, when ten leaf collars are visible (V10), the GA20-OX1 plants were 45% taller than the nontransgenic control plants (Figure 1a). At maturity (silking stage, S), the GA20-OX1 plants were on average 37% taller than control plants. In contrast, the transgenic plants had a reduced stem diameter throughout development, with a reduction of 21% at S stage (Figure 1b).

Because the transgenic plants had taller but more slender stems, we also estimated the stem volume. No significant differences were detected between GA20-OX1 and control plants when internodes along the stem of mature plants were compared ($P > 0.05$) (Table S1). Despite the comparable stem volumes, the dry weight (DW) of the stem was increased (+32%) as well as the DW of the cob without seeds (+37%) in GA20-OX1 plants (Table 1). In contrast, the DW of the leaves was reduced (−17%). Together, the stover yield was 14% higher in GA20-OX1 plants as compared to that in control plants (Table 1). In terms of second generation bioethanol production, the increased stover yield, the major source of cellulose, is a desirable feature.

To investigate whether the results obtained in the greenhouse were reproducible under field conditions, homozygous transgenic and nontransgenic control plants were evaluated in a field trial. The fields were established in 2012 and 2013 in Belgium, at slightly different locations on the same parcel, and consisted of three and nine randomized blocks per line (transgenic, control), respectively. In both growing seasons, the mature fourth leaf was significantly longer in GA20-OX1 plants as compared to control plants (Table 2). Also total plant height was significantly increased in GA20-OX1 plants as compared to control plants. In contrast, differences in DW per plant were not consistent over the years. In 2012, DW was significantly higher in the GA20-OX1 plants, but unaltered in 2013. In both years, the difference in percentage of dry weight (%DW) appeared not significant (Table 2). We checked for *genotype* x *year* effects for four traits (plant height, leaf#4 length, %DW and DW per plant) using a factorial ANOVA with *year* and *genotype* as factors. Three traits showed significant *genotype x year* effects: leaf#4 length, plant height and DW per plant (Table S2). For leaf#4 length and plant height, GA20-OX1 plants were superior to the control line in both years, but the difference was more pronounced in 2012 than in 2013. For DW per plant, *genotype* was not significant. However, significant effects were detected for *year* and *genotype x year*, because GA20-OX1 plants were superior in 2012, while no significant difference was observed in 2013 (Table S2). For %DW, only year effects were observed, with higher values in 2013 than in 2012 (Table S2).

Overexpression of *GA20-OX1* affects the saccharification efficiency of stem biomass by altering the cell wall composition

To assess whether changes in plant morphology were accompanied by changes in cell wall composition, we determined the cell wall fraction, cellulose content, lignin content and lignin compo-

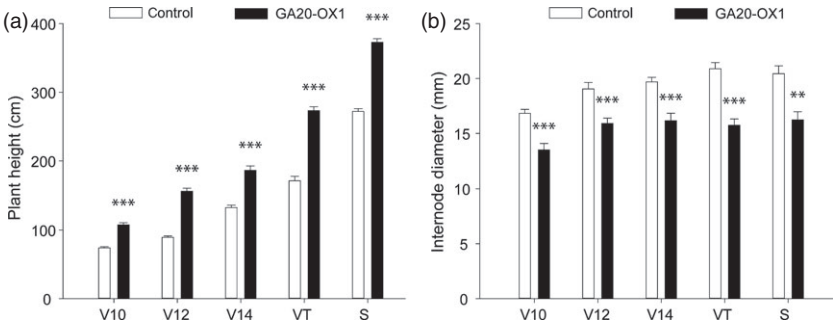

Figure 1 Plant height (a) and internode diameter (b) of GA20-OX1 and control plants over development grown in the greenhouse. V10, V12, V14: 10, 12 and 14 leaf collars visible, respectively; VT: male flowering (tasseling); S: female flowering (silking); Plant height is measured as height of the tip of youngest leaf in V10, V12 and V14 stages and as height of the tassel in VT and S stages. Internode width is the width of the ninth internode, counted from the bottom, **: $0.01 > P \geq 0.001$, ***: $0.001 > P$. Error bars represent standard errors for 9 biological repeats in V10, V12, V14 and VT stages and 7 biological repeats for S stage.

Table 1 Dry weight measurements of senesced plant parts of GA20-OX1 and control plants grown in the greenhouse. Stover yield was calculated as the sum of stem, leaves and cob without the seeds

Parameter	Control (mean ± SEM)	GA20-OX1 (mean ± SEM)	% difference	t-test
Stover (g)	156.7 ± 5.3	178.0 ± 5.5	14	**
Stem (g)	69.1 ± 1.9	91.1 ± 3.3	32	***
Leaf (g)	61.1 ± 2.1	50.5 ± 2.1	−17	**
Cob without seeds (g)	26.5 ± 1.4	36.4 ± 1.3	37	***

SEM, standard error of the mean.
Values are means for 13 biological repeats for control plants and 15 biological repeats for GA20-OX1 plants.
$0.01 > P \geq 0.001$, *$0.001 > P$.

Table 2 Leaf length, total plant height, dry weight (DW) and DW% measurements in 2012 and 2013 field trials with control and GA20-OX1 maize plants

Year	Parameter	Control (mean ± SEM)	GA20-OX1 (mean ± SEM)	% difference	t-test
2012	Leaf#4 length (mm)	242.7 ± 5.6	388.2 ± 12.1	60	***
	Plant height (cm)	104.6 ± 2.0	145.1 ± 16.4	39	**
	DW per plant (g)	53.4 ± 1.8	68.5 ± 1.1	28	**
	%DW (g/100 g FW)	20.1 ± 0.5	21.8 ± 0.7	8	NS
2013	Leaf#4 length (mm)	190.7 ± 13.0	296.7 ± 7.0	56	***
	Plant height (cm)	127.6 ± 0.9	147.7 ± 0.9	16	***
	DW per plant (g)	114.5 ± 1.1	93.5 ± 4.2	−18	NS
	%DW (g/100 g FW)	22.4 ± 1.7	24.4 ± 1.7	9	NS

SEM, standard error of the mean.
$0.01 > P \geq 0.001$, *$0.001 > P$, NS: not significant ($P > 0.05$).

sition of milled senesced stems of greenhouse-grown GA20-OX1 and control plants.

The cell wall fraction or cell wall residue (CWR) per unit DW as well as the cellulose and lignin content were significantly higher in GA20-OX1 plants (Figure 2). The higher lignin content was confirmed by a phloroglucinol staining of stem sections of the ear internode of GA20-OX1 plants 14 days after silking (S + 14d). The staining was more intense in the sclerenchyma surrounding xylem and phloem in the vascular bundle and in the collenchyma in sections of GA20-OX1 plants (Figure 3). On the other hand, no apparent difference in the number of xylem cells was found. Next, thioacidolysis was used to determine possible shifts in lignin composition. The relative abundance of thioacidolysis-released H, G and S units was not different and was about 2%, 36% and 62%in both the lignin of control and GA20-OX1 plants, respectively (Figure S1).

To examine whether the compositional changes of the cell wall of GA20-OX1 plants affect the cell wall processing efficiency, we investigated the saccharification efficiency. Milled stem biomass of senesced greenhouse-grown GA20-OX1 and control plants was first pretreated, after which enzymatic glucose release was measured. Using either hot water (98 °C, 3 h), acid (1 M HCl, 80 °C, 2 h) or alkali (0.5 M NaOH, 90 °C, 2 h) as pretreatment, GA20-OX1 plants yielded 7%, 11% and 6%, respectively, more residual biomass, as compared to control plants (Figure S2). In control plants, pretreatment with hot water, acid or alkali resulted in an increased release of glucose (+25%, +123% or +578%, respectively) as compared to non-pretreated biomass (Figure S3).

After an alkaline pretreatment, 11% more glucose could be released per gram DW when comparing the saccharification efficiency of GA20-OX1 plants with that of control plants after 12 h of hydrolysis (Figure 4a). There was no significant difference in glucose release per DW between control and GA20-OX1 plants after 12 h of hydrolysis, following acid or hot-water pretreatment. The cellulose-to-glucose conversion appeared slower in GA20-OX1 plants as compared to control plants that were pretreated with hot water or with acid (Figure 4b). This was visible by significantly less glucose that was measured at 2 h, 4 h and 8 h of hydrolysis following hot-water pretreatment, and 2 h and 4 h of hydrolysis following acid pretreatment (Figure 4b). On a *per stem* basis, GA20-OX1 plants yielded significantly more glucose than control plants using either pretreatment (Figure 4c). The increase was highest for alkali (+53%), followed by acid (+51%) and hot-water (+35%) pretreated biomass.

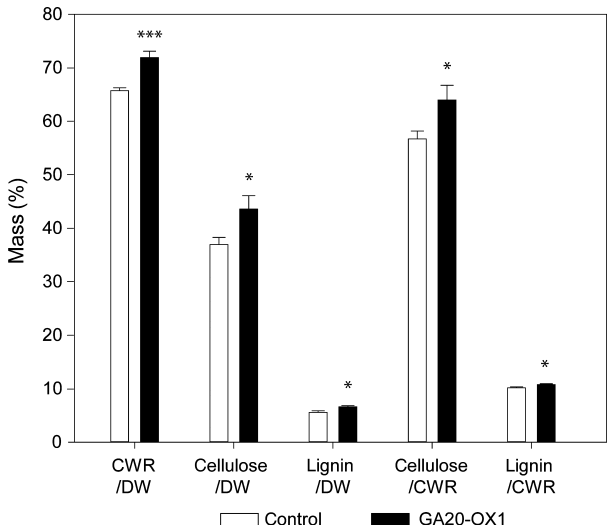

Figure 2 Lignin and cellulose levels in senesced stems of GA20-OX1 and control plants. CWR: cell wall residue, DW: dry weight. Error bars represent standard errors of ten biological repeats. *: $0.05 > P \geq 0.01$, ***: $0.001 > P$.

Cell wall compositional changes arise early in secondary cell wall biosynthesis

To obtain a more detailed view on the cell wall compositional changes, the cell wall composition of GA20-OX1 plants was studied in a developmental series. More specifically, we investigated the ninth internode of greenhouse-grown plants at five developmental stages from early vegetative stage until 14 days after silking.

The cell wall residue per unit of dry weight (CWR/DW) was significantly higher in the internode of GA20-OX1 plants as compared to control plants at all investigated stages, except V14 (Figure 5a). In internodes of control plants, relative cellulose content, expressed as percentage of the CWR, increased from 25% in V10 stage (10 visible leaf collars) to 63% in the V12 stage (12 visible leaf collars; Figure 5b). At later stages, the relative cellulose content decreased slightly and remained constant at around 55% of CWR from the V14 stage onwards. Thus, the major relative accumulation of cellulose in the cell wall of the investigated internode occurred between the V10 and V12 stage. Strikingly, in transgenic plants, the cellulose fraction constituted already 52% of the CWR at V10, which is about twofold as compared to control plants (Figure 5b). On the other hand, no differences were detected in the relative cellulose content between transgenic and control in V12, V14, S and S + 14d stages. In control plants, the lignin content as percentage of the CWR increased rapidly between V12 (4%) and V14 (9%) and further towards S stage (10%) (Figure 5c). Consistent with the cellulose measurements, the lignification of the cell wall occurred earlier in development in the GA enhanced plants (Figure 5c). The relative lignin content was already 6% in GA20-OX1 plants at V10, compared to 4% for the control (Figure 5c).

Expressed per unit of DW, cellulose and lignin levels were significantly higher in the internode of GA20-OX1 plants as compared to control plants at all investigated stages, except cellulose per DW at 14 days after silking (S + 14D) (Figure S4). For example, at V10, the cellulose and lignin per DW in the GA20-OX1 internode were five and three times higher than those in the control internode, respectively (Figure S4).

To further study the cause of increased lignin accumulation in the internodes of GA20-OX1 plants, the expression pattern of several genes involved in monolignol biosynthesis was investigated by RT-qPCR. Higher transcript levels of *ZmPAL1*, *ZmC4H1*, *ZmHCT2*, *ZmC3H1*, *ZmCCoAOMT3*, *ZmCCR1*, *ZmF5H1*, *ZmCOMT* and *ZmCAD2* were detected in V10 and V12 stages in greenhouse-grown GA20-OX1 plants, while their transcript levels were in general similar to or lower than those in the control samples at later stages (Figure 6). The only notable exception is *ZmF5H1*, which is involved specifically in the formation of syringyl (S) units and stayed more expressed throughout development in the transgenics as compared to that in the control (Figure 6). Taken together, these results suggest that the maximal expression

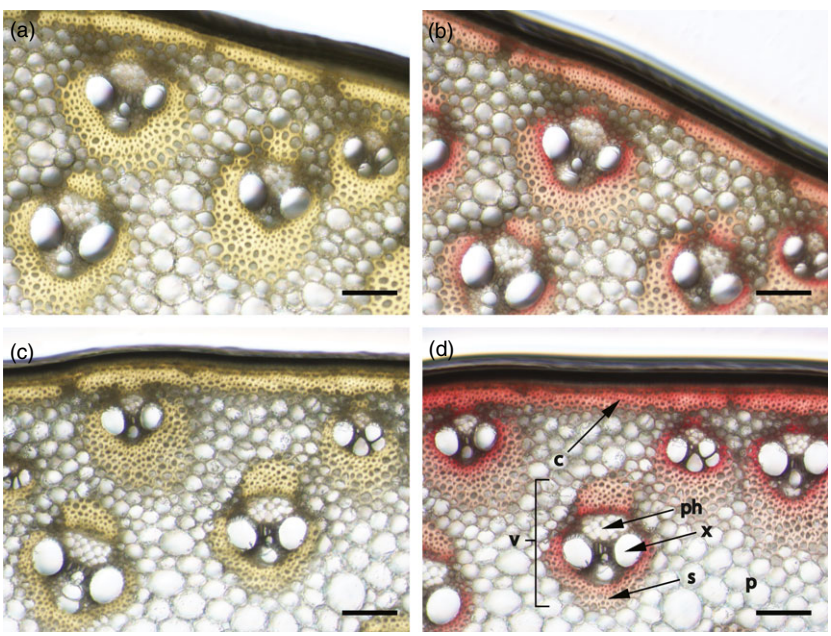

Figure 3 Stem sections of control (a,b) and GA20-OX1 plants (c,d) at 14 days after silking (S+14d), unstained (a and c, respectively) and stained with phloroglucinol (b and d, respectively). v, vascular bundle; x, xylem vessel; ph, phloem; s, sclerenchyma fibers; p, parenchyma; c, collenchyma. Scale bar is 100 μm.

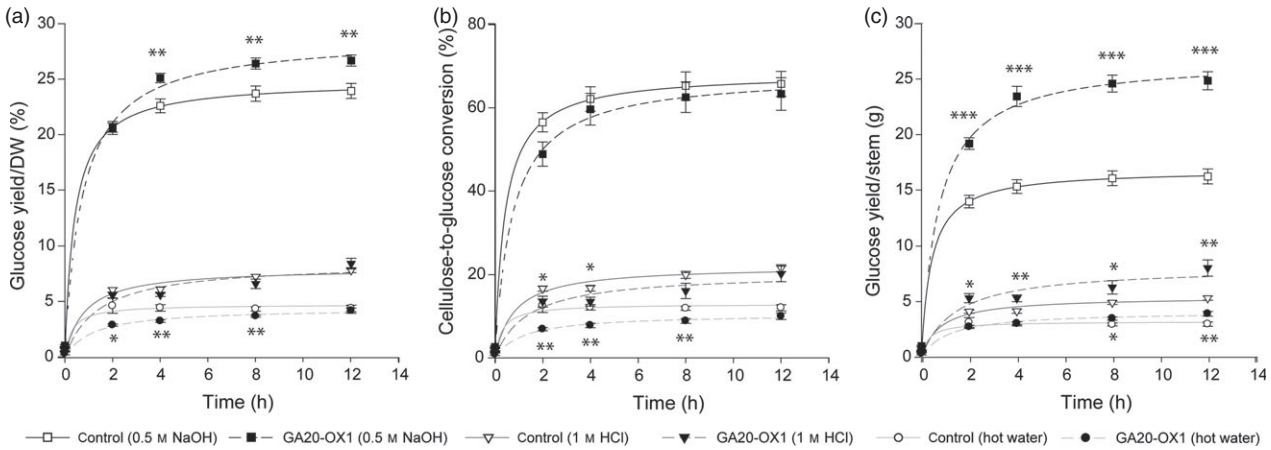

Figure 4 Saccharification efficiency in senesced stems of GA20-OX1 and control plants. The biomass was pretreated either with 0.5 M NaOH, 1 M HCl or hot water, and the saccharification efficiency was expressed as glucose yield per DW (a), per cellulose (b) or per stem (c). CWR: cell wall residue, DW: dry weight. Error bars represent standard errors of ten biological repeats. *: $0.05 > P \geq 0.01$,**: $0.01 > P \geq 0.001$, ***: $0.001 > P$.

of lignin biosynthesis genes was shifted to an earlier developmental stage in GA20-OX1 plants compared to control plants.

Discussion

We investigated whether *GA20-OX1* overexpression in maize is a valuable strategy to improve biomass feedstock for second generation bioethanol production. With maize as genetic model for the improvement of biomass quality and yield of bioenergy crops (Carpita and McCann, 2008; Riedelsheimer *et al.*, 2012), the generated knowledge can also be applied to related grass species, such as Miscanthus, Sorghum and switchgrass (Vermerris, 2011).

Our results showed that the greenhouse-grown GA20-OX1 maize plants are taller, but also more slender. On a plant-by-plant basis, the greenhouse-grown GA20-OX1 plants already produced more vegetative biomass, a highly desirable trait for lignocellulosic crops (Feltus and Vandenbrink, 2012). An increased yield of stem biomass was previously also observed in *GA20-OX1*-overexpressing poplar and tobacco plants (Biemelt *et al.*, 2004; Eriksson *et al.*, 2000). However, the slight increase in the fraction of leaf biomass observed in poplar (Eriksson *et al.*, 2000) contrasts to the reduction in leaf biomass in GA20-OX1 maize plants observed in this study. Perhaps, the elongating stem of GA20-OX1 maize plants formed a strong sink, leading to altered biomass allocation (from leaves to stem), as compared to control plants. Alternatively, the stem might be more responsive to GA, in terms of induction of cell wall biosynthesis, than the leaf. Interestingly, the longer internodes had a reduced diameter and the resulting volume was not altered in GA20-OX1 plants as compared to control plants. This compensatory effect might be induced by a control mechanism that maintains organ size. Although the mechanism underlying such a compensation effect is largely unknown, such effects have been observed in many cases when plant or organ shape was altered (Gonzalez *et al.*, 2012; Hisanaga *et al.*, 2015). Another 'compensation mechanism' might exist at the level of the stem. The slender stem of GA20-OX1 plants has an increased cell wall content. These reinforced cell walls, consisting of polysaccharides and lignin, could provide the necessary rigidity and strength to the slender GA20-OX1 plants.

The increased leaf length (Nelissen *et al.*, 2012) and increased plant height, observed in greenhouse-grown GA20-OX1 maize, were also observed in field conditions. In contrast, the increase in DW observed in greenhouse-grown GA20-OX1 maize was not consistently observed in the two years of the field trial. The inconsistency of particular phenotypes between the greenhouse and field experiments can be attributed to the environmental conditions that differed substantially between the different experiments. Indeed, phenotypes appear not only dependent on

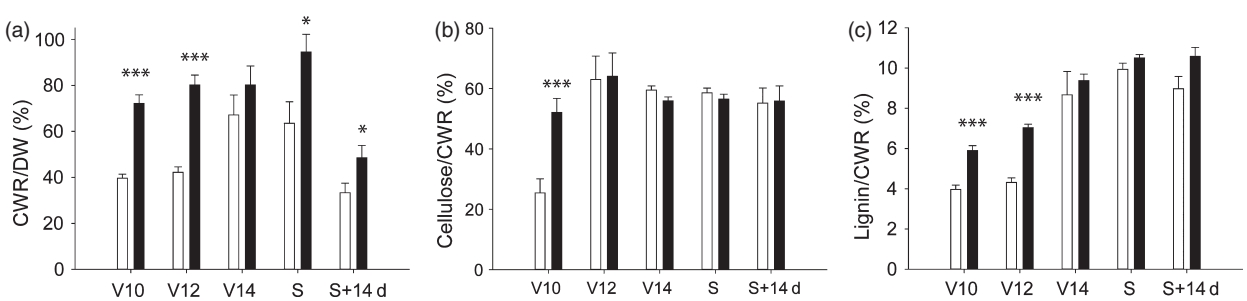

Figure 5 Cell wall composition in the developing ninth internode of control (white bars) and GA20-OX1 plants (black bars). Cell wall residue (CWR) per dry weight (DW) (a); cellulose per CWR (b); lignin per CWR (c); V10, V12, V14 (10, 12 and 14 leaf collars visible, respectively), silking (S) and 14days after silking (S + 14d) indicate the different developmental stages; Error bars represent standard errors of 9, 9, 8, 7 and 6 biological replicates in stages V10, V12, V14, S and S+14d respectively; *: $0.05 > P \geq 0.01$, ***: $0.001 > P$.

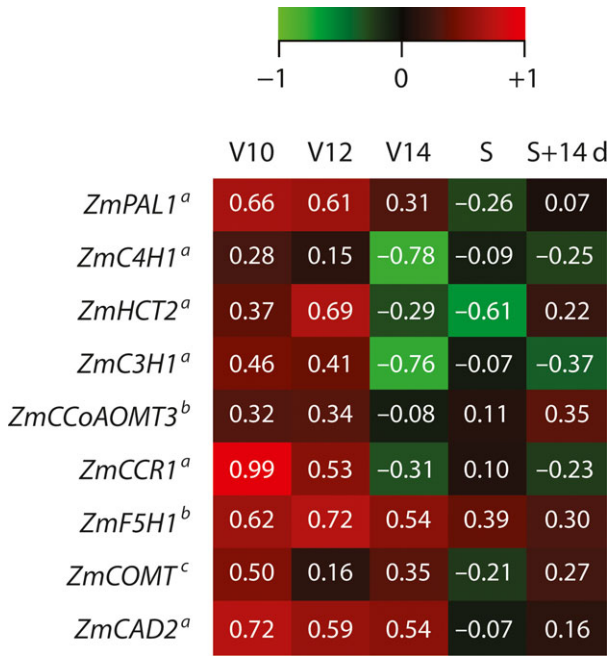

Figure 6 Fold changes (log2) of lignin biosynthetic gene expression in GA enhanced plants versus control plants in the developing maize ninth internode. V10, V12, V14 (10, 12 and 14 leaf collars visible respectively), silking (S) and 14d after silking (S+14d) indicate the different developmental stages.. [a], significant interaction effect. [b], significant genotype effect. [c], non-significant genotype effect.

the genotype and the environment, but also on *genotype* x *environment* interaction effects (Malmberg *et al.*, 2005). For example, in the field, we observed a significant *year* effect for all four investigated traits and significant *genotype* x *environment* interaction effects were observed for three of four investigated traits. In the greenhouse, the growth conditions were highly controlled, with a minimum temperature of 25 °C during day and 23 °C during night, in a 16-h light and 8-h dark photoperiod. On the other hand, the spring of 2012 was wet and cold, which caused a delay in the sowing (25 May 2012 versus 29 April 2013). In addition, the temperature and radiation was lower in July 2012 as compared to July 2013. The differences in temperature and radiation between 2012 and 2013 might logically explain why the DW of both control and GA20-OX1 lines was considerably lower in 2012 as compared to 2013. Overall, biomass yield in the field trials was relatively low as compared to the greenhouse, as the inbred line B104 was not adapted to the local climate. The presence of environmental and genotype x environment interaction effects urges to caution when generalizing greenhouse-based conclusions (reviewed in Nelissen *et al.*, 2014). In particular, for multigenic and environmentally regulated traits, such as biomass yield, translation of greenhouse results to field appeared difficult (Casati *et al.*, 2011; Van Acker *et al.*, 2014; Viswanath *et al.*, 2011; Voelker *et al.*, 2011; Witt *et al.*, 2012). Taken together, these observations also stress the importance of field trials. For instance, by comparing both greenhouse and field studies, we can conclude that the increase in leaf length and total plant height of GA20-OX1 plants are more robust traits than the increase in stem biomass production.

Since long, the involvement of GA in fibre differentiation and lignin formation in phloem fibres and xylem is known (Aloni, 1979; Aloni *et al.*, 1990; Roberts *et al.*, 1988). Furthermore, GA can affect the length of xylem fibres, which become highly lignified after cell death (Israelsson *et al.*, 2005; Mauriat and Moritz, 2009; Ridoutt *et al.*, 1996). In tobacco plants overproducing GA, the increase in DW has been attributed to a higher degree of xylem lignification (Biemelt *et al.*, 2004). In our analysis, whole stems of *GA20-OX1*-overexpressing senesced plants contained more lignin and cellulose and had a higher CWR. In addition, stem cross sections of GA20-OX1 maize plants did not indicate an increased number of xylem cells, as was shown previously for tobacco plants overexpressing *GA20-OX* (Biemelt *et al.*, 2004). Perhaps, the role of GA in xylem differentiation differs between monocots and dicots.

A direct effect of GA on lignin biosynthesis has been shown by spraying GA on wild-type tobacco hypocotyls, which resulted in increased lignin formation (Biemelt *et al.*, 2004). However, in the latter experiment, no increased expression of lignin biosynthesis genes was observed (Biemelt *et al.*, 2004). In our experiments with GA20-OX1 maize, the total cell wall content was increased, rather than lignin alone.

To obtain deeper insight into the cause of the changed cell wall properties, the cell wall composition of GA20-OX1 and control plants was studied in a developmental series. We have measured cellulose and lignin levels in the ninth internode of plants from V10 to S + 14d. Consistent with literature (Morrison *et al.*, 1994; Zhang *et al.*, 2014), we found that the onset of secondary cell wall formation in a developing internode of control plants involved cellulose deposition first (V10 to V14), followed by lignin biosynthesis (V12 to S). The cell wall fraction (CWR/DW) first increased (V10 to V14) and then was stable towards silking (between V14 and S). Remarkably, the CWR/DW of both control and transgenic plants dropped in the S + 14d stage, most likely due to sucrose accumulation at this stage in the investigated internode (De Souza *et al.*, 2013; Dwyer *et al.*, 1995; Jung and Casler, 2006; Setter and Meller, 1984). Already early in development, the stem of GA20-OX1 maize plants, grown in greenhouse conditions, accumulated more CWR and higher relative amounts of cellulose and lignin than control plants. The earlier developmental onset of lignin biosynthetic gene expression also indicated that secondary cell wall formation starts earlier in stems of GA20-OX1 plants as compared to control plants.

Stems of greenhouse-grown senesced GA20-OX1 maize plants contain more cellulose and therefore were evaluated for their saccharification potential following either hot-water, acid or alkaline pretreatments, which have different mode of actions (reviewed in Hendriks and Zeeman, 2009 and Alvira *et al.*, 2010). Alkaline pretreatment removes hemicellulose and part of the lignin in the cell wall, making the cellulose highly exposed to enzymatic hydrolysis. This generally results in high enzymatic glucose release (Pedersen *et al.*, 2011). In contrast, the acid and hot-water pretreatments remove part of the hemicellulose but leave the lignin in the cell wall intact (Hendriks and Zeeman, 2009). The altered cell wall composition of GA20-OX1 maize plants resulted in a higher glucose release from dry whole-stem material pretreated with alkali but not when pretreated with hot water or acid. The conversion of the cellulose to glucose appeared slower in the acid and hot-water-pretreated GA20-OX1 maize samples as compared to control plants. It has been shown previously that recalcitrance to acid pretreatment and enzymatic digestion is directly proportional to lignin content (Chen and Dixon, 2007). Thus, the higher lignin content probably negatively influenced the cellulose-to-glucose conversion of stem biomass of

GA20-OX1 maize plants, when left intact by the pretreatment method. When the increased stem biomass of greenhouse-grown GA20-OX1 plants is taken into account, the saccharification efficiency on a stem basis appeared positive for each pretreatment tested.

Experimental procedures

Plant material

Constitutive overexpression of *GA20 oxidase* was attained by cloning the *GA20-OX1* gene (At4 g25420) behind the maize UBI1 promoter (Christensen and Quail, 1996) in pMBb7Fm21GW-UBIL (http://gateway.psb.ugent.be) and introduction into the maize B104 inbred line by *Agrobacterium tumefaciens*-mediated transformation of immature embryos as described (Nelissen *et al.*, 2012). From the five described transgenic lines (Nelissen *et al.*, 2012), the strongest overexpression line was selected for analysis.

Greenhouse experiments

A segregating population derived from a backcross of a transgenic plant heterozygous for the *GA20-OX1* overexpression construct with a wild-type B104 plant was used. The greenhouse was kept at minimum 25 °C during the day and 23 °C during the night in a 16 h/8 h rhythm. Supplementary light was added when natural light intensity was below 200 W/m² using high-pressure sodium vapour lamps. Fertilizer was added with the water supply; conductivity Ec = 1mS/cm; and water-soluble fertilizer Poly-feed (Haifa, Belgium) (N, P_2O_5, K_20; 20:5:20 + 3 MgO). Transgenic and control plants in the segregating population were identified based on Basta resistance using Basta leaf painting (Yao *et al.*, 2006).

A total of 30 progeny plants (set GH-a) were grown until maturity. Fertilization was achieved by shaking the stems for pollen shed every morning until no more pollen was produced. The plants were allowed to dry off completely whereupon leaves, stems, cobs and seeds were weighted separately. Whole stems were milled using a cutting mill (Fristch, Lelystad, Netherlands) with sieve of 0.5 mm and used for cell wall characterization and saccharification analysis.

Another set of five transgenic and five control plants (set GH-b) were grown for measurements of stem volume. For this, the length (L) of internodes 2, 4 and 6 as well as the width along their broadest (A) and slimmest (B) diameters were measured using a vernier caliper (0.01 mm accuracy). The final plant height (P) was measured from soil level using a foldable meter. The volume per internode was then calculated as π*(A/2)*(B/2)*L, the formula for an ellipsoid cylinder. The total stem volume was then calculated as π*(A/2)*(B/2)*(P), with average values for A and B over the three internodes per plant.

Another set of 160 progeny plants (set GH-c) were used for the analysis of plant morphology, cell wall characterization and expression analysis over development. Nine transgenic and nine control plants were selected randomly in V10, V12, V14 and VT (10, 12 and 14 visible leaf collars and tasseling, respectively) stage and seven in S and S + 14d (silking and 14 days after silking, respectively) stage. The height of the tip of the youngest visible leaf in V10, V12 and V14 stage and height of the tassel in VT, S and S + 14d were measured from soil level using a foldable meter. The width of the ninth internode, counted from the base, was measured along its broadest diameter using a vernier caliper (0.01 mm accuracy). The ninth internode was dissected from the stem and cut transversally in three parts of similar lengths, immediately frozen in liquid nitrogen and stored at −80 °C. The top part of the internode (developmentally older than the bottom part; Scobbie *et al.* 1993) was used for biochemical cell wall characterization and the bottom part for expression analysis. This was considered convenient for our purpose as in this way, expression analysis of lignin biosynthetic genes can be evaluated earlier on in development than cell wall characteristics.

Field trial

Homozygous transgenic and azygous seed stocks were used, resulting from selfing maize plants heterozygous for the *GA20-OX1* overexpression construct. The seeds were sown in two independent sites, but on the same parcel, in Wetteren, Belgium (50.979340, 3.829451) (25 May 2012 and 29 April 2013). The sowing densities were 100 000 plants per hectare. In 2012, the field consisted of three randomized blocks per line (transgenic and nontransgenic control), resulting in a total amount of 239 and 336 plants, respectively. In 2013, the field consisted of nine randomized blocks per line, resulting in 890 transgenic and 1211 nontransgenic control plants. The blocks measured 16 m², in which the seeds were sown in 4 rows. Commercial hybrids (Ronaldinho in 2012 and Sago in 2013) were used as border plants surrounding the blocks. Fertilizer was applied at rates equivalent to 130, 22 and 150 kg/ha/year $N_{plant\ available}$, P and K, respectively. Weed was controlled manually and by herbicides applied after maize emergence (June 26 in 2012 and June 14 in 2013) in accordance with good agricultural practice. The final length of leaf 4 (on 13 July 2012 and 26 June 2013) and leaf 7 (on 6 August 2012) was measured from tip to soil level, using a foldable meter. Total plant height was measured from soil level to the implementation of the highest leaf, and not the tassel as the plants were emasculated as demanded by the Biosafety Advisory Council. Total plant height was measured just prior harvest, which was on 17 October 2012 and 11 October 2013, respectively. Fresh weight (FW) and DW (dried in a ventilated oven) were determined on individual plants. Then, %DW was calculated as g DW per 100 g FW.

Expression analysis using RT-qPCR

Expression analyses were performed on plants from 'set GH-c'. Several genes involved in lignin biosynthesis were selected from the MAIZEWALL database (Guillaumie *et al.* 2007; http://www.polebio.lrsv.ups-tlse.fr/MAIZEWALL/) (Table S2). Gene-specific primers from MAIZEWALL were tested for specificity and primer efficiency >1.7 in qRT-PCR. If not satisfactory, new primers were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) with standard settings (Table S2). Internode samples were milled with a Mixer Mill MM 400 and Tungsten carbide 25 mL grinding jars (Retsch, Haan, Germany). RNA was extracted using RNeasy kit (Qiagen, Valencia, CA), and a DNase treatment was performed using DNA-free™ (Ambion, Life technologies, Carlsbad, CA). Extracted RNA was quantified using the nanodrop® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE), and 400 ng RNA was used for cDNA synthesis using the first-strand cDNA synthesis kit (Thermo Scientific, Thermo Fisher Scientific, Waltham, MA). A ten times diluted cDNA sample was used for RT-qPCR using a sensiFAST SYBR No-ROX-kit (Bioline, London, UK) on a Lightcycler 480 (Roche, Basel, Switzerland). Samples were run in technical triplicate on the LC480 with following protocol: 1 activation cycle of 10 min at 95 °C; 45 amplification cycles of 10 s at 95 °C, 10 s at 60 °C and 10 s at 72 °C; and 1 melting curve cycle measuring from 65 to 95 °C. Fluorescence values were exported from the lightcycler

program whereupon Ct values, normalization factors and primer efficiencies were calculated according to Ramakers *et al.* (2003) using *ZmEF1a* and *Zm18S* as reference genes.

Lignin analyses

Aliquots of 5 mg milled stem material (set GH-a and GH-c) were subjected to a sequential extraction to obtain a purified CWR: water (98 °C), ethanol (76 °C), chloroform (59 °C) and acetone (54 °C), 30 min each. The remaining CWR was dried under vacuum. The lignin was quantified according to a modified version of the acetyl bromide method (Dence, 1992), optimized for small amounts of plant tissue. The dried CWR was dissolved in 0.1 freshly made 25% acetyl bromide in glacial acetic acid and 4 μL 60% perchloric acid. The solution was incubated for 30 min at 70 °C while shaking (850 rpm). After incubation, the slurry was centrifuged at 20 000 ***g*** for 15 min. To the supernatant, 0.2 mL of 2 M sodium hydroxide and 0.5 mL glacial acetic acid was added. The pellet was washed with 0.5 mL glacial acetic acid. The supernatant and the washing phase were combined, and the final volume was adjusted to 2 mL with glacial acetic acid. After 20 min at room temperature, the absorbance at 280 nm was measured with a nanodrop® ND-1000 spectrophotometer (Thermo Scientific). The lignin concentrations were calculated by means of the Bouguer–Lambert–Beer law: $A = \varepsilon \times l \times c$ (A = absorbance, ε = extinction coefficient, l = path length, c = concentration), with ε = 20.48 L/g/cm (Fukushima and Hatfield, 2004) and l = 0.1 cm.

The lignin composition (set GH-a) was investigated with thioacidolysis as previously described (Robinson and Mansfield, 2009). The monomers involved in β-O-4 ether bonds, released upon thioacidolysis, were detected with gas chromatography (GC) as their trimethylsilyl (TMS) ether derivates on a Hewlett–Packard HP 6890 Series system (Agilent, Santa Clara, SA) coupled with a HP-5973 mass-selective detector. The GC conditions were as described (Robinson and Mansfield, 2009). Data processing was performed using MS Quantitative Analysis (B.06.00 Agilent). The quantitative evaluation was based on the specific prominent ions for each compound (adapted from Yue *et al.*, 2012). A summary of the specific ions for each specific compound can be found in Table S3.

Cellulose analysis

Aliquots of 5 mg milled stem material (set GH-a and GH-c) were subjected to a sequential extraction to obtain a purified CWR, as described above. To estimate the amount of cellulose, we used a colorimetric method (based on DuBois *et al.*, 1956; Leplé *et al.*, 2007). The CWR was incubated with 2 M trifluoroacetic acid and 20 μL inositol (5 mg/mL) for 2 h at 99 °C while shaking (750 rpm). After incubation, the remaining pellet was washed three times with water and twice with acetone and dried under vacuum. Concentrated sulphuric acid (150 μL) and 30 μL 5% (w/v) phenol (freshly made in water) were added to the dried pellet and incubated for 1 h at 90 °C with gentle shaking (500 rpm). After centrifugation for 3 min at 23 477 g, a 50 μL aliquot of the supernatant was diluted 20 times with MilliQ water (Millipore, Billerica, MA) to measure the absorbance at 493 nm. The amount of cellulose was calculated based on a standard curve of Avicel®PH-101 (FMC BioPolymer, Philadelphia, PA).

Saccharification assay

The saccharification assay was performed as described in Van Acker *et al.* (2013), with modifications. Aliquots of 10 mg of dry stem material (set GH-a) were used. The biomass was pretreated with 1 mL of either hot water (98 °C, 3 h), 1 M HCl (80 °C, 2 h) or 0.5 M of NaOH (90 °C, 2 h), while shaking (850 rpm). The supernatant was removed, and the pellet containing pretreated material was washed three times with water to obtain a neutral pH. Subsequently, the material was incubated in 1 mL 70% (v/v) ethanol overnight, 55 °C. The remaining biomass was washed three times with 1 mL 70% (v/v) ethanol, once with 1 mL acetone, dried under vacuum for 45 min and weighed. The pretreated ethanol-extracted residue was dissolved in 1 mL acetic acid buffer solution (pH 4.8) and incubated at 50 °C. Accelerase® 1500 (Genencor, Denmark) enzyme mix was first desalted over an Econo-Pac 10DG-column (Bio-Rad, Hercules, CA), stacked with Bio-gel® P-6DG gel (Bio-Rad) according to the manufacturer's guidelines. The activity of the enzyme mix was measured with a filter paper assay (Xiao *et al.*, 2004). To each sample, the enzyme mix with an activity of 0.014 filter paper units, dissolved in acetic acid buffer (pH 4.8), was added. Twenty microlitres of aliquots was taken after 0 h, 4 h, 7 h and 24 h incubation at 50 °C and 10-fold diluted with acetic acid buffer (pH4.8). The concentration of glucose was measured indirectly with a spectrophotometric colour reaction (glucose oxidase–peroxidase; GOD-POD). A 100 mL aliquot of the reaction mix from this colour reaction contained 50 mg ABTS, 44.83 mg GOD (Sigma-Aldrich, St. Louis, MO) and 173 μL of 4% (w/v) POD (Roche Diagnostics, Brussels, Belgium) in acetic acid buffer (pH 4.5). To measure the concentration of glucose, 50 μL of the diluted samples was added to 150 μL GOD-POD solution and incubated for 30 min at 37 °C. The absorbance was measured spectrophotometrically at 405 nm. The concentration in the original sample was calculated with a standard curve based on known D-glucose (Sigma-Aldrich) concentrations. Curves were added to the graphs by the use of the hyperbolic function in Sigmaplot (Systat Software Inc, San Jose, CA).

Cell wall residue measurements of internodes over development

For the biochemical analysis of cell wall components such as cellulose, lignin and cell wall residue (CWR), fresh internode samples from 'set GH-c' were frozen in liquid nitrogen for use. To obtain a correction factor that allowed to estimate the CWR per unit dry matter, the dry-to-fresh weight ratio was determined for three stages over development (V10, V14 and S + 14d) using the samples that were reserved for expression analysis (bottom part of the same 9th internode). The DW accumulation in the transgenics and nontransgenics was modelled using a polynomial, and the respective equations were used to convert the CWR values per fresh weight into CWR per DW values.

Microscopy on stem sections

Stem sections of the ear internode of two randomly chosen nontrangenic and two transgenic plants (set GH-b) were used. Stem pieces of 5 cm were fixated using freshly made fixating agent (2.5% formaldehyde in 0.05 M acetic acid buffer) for 2 days at 4 °C while shaking. The fixated stems were dehydrated by removing the fixating agent and replacing with increasing concentrations of ethanol (10%, 30%, 50% and 70%), each time incubated for 2 h at 4 °C while shaking. At least three sections of 200 μm per stem were made using a vibroslicer. Stem sections were stained using Wiesner staining reagent (1% phloroglucinol w/v in 100 mL 95% ethanol (v/v) and 16 mL 37% (v/v) HCl). Stained and unstained sections were visualized using Olympus BX51 microscope (Shinjuku, Tokyo, Japan).

Statistical analysis

Statistical analyses consisted of Student's t-tests for comparisons of greenhouse-grown transgenic/control samples for lignin, cellulose, CWR and saccharification efficiency measurements. Analyses of the field trial also comprised a Student's t-test for comparison of transgenic and control plants, and factorial ANOVA to test for block, year and genotype effects and interactions. These analyses were carried out in the software package STATISTICA version 11 (Statsoft Inc., Tulsa, Oklahoma, USA). For the RT-qPCR data, a two-way interaction general linear model (intercept + genotype + time + genotype*time) was applied and a reduced model (intercept + genotype + time) on the genes that did not show a significant interaction ($P < 0.05$) in the software package SPSS Statistics 22 (IBM, Armonk, New York, USA).

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Conflicts of interest

No conflict of interests are to be declared.

References

Aloni, R. (1979) Role of auxin and gibberellin in differentiation of primary Phloem fibers. *Plant Physiol.* **63**, 609–614.

Aloni, R., Tollier, M.T. and Monties, B. (1990) The role of auxin and gibberellin in controlling lignin formation in primary phloem fibers and in xylem of Coleus blumei stems. *Plant Physiol.* **94**, 1743–1747.

Alvira, P., Tomás-Pejó, E., Ballesteros, M. and Negro, M.J. (2010) Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. *Bioresour. Technol.* **101**, 4851–4861.

Baker, J. (1987) *Experiment With the Plant Growth Hormone Gibberellin*. Honor Theses Paper 282.

Barriere, Y., Alber, D., Dolstra, O., Lapierre, C., Barrière, Y., Alber, D., Dolstra, O., Lapierre, C., Motto, M., Ordás Pérez, A., Van Waes, J., Vlasminkel, L., Welcker, C. and P., M.J. (2006) Past and prospects of forage maize breeding in Europe. II. History, germplasm evolution and correlative agronomic changes. *Maydica*, **51**, 435–449.

Biemelt, S., Tschiersch, H. and Sonnewald, U. (2004) Impact of altered gibberellin metabolism on biomass accumulation, lignin biosynthesis, and photosynthesis in transgenic tobacco plants. *Plant Physiol.* **135**, 254–265.

Bonawitz, N.D. and Chapple, C. (2013) Can genetic engineering of lignin deposition be accomplished without an unacceptable yield penalty? *Curr. Opin. Biotechnol.* **24**, 336–343.

Boon, E.J.M.C., Struik, P.C., Engels, F.M. and Cone, J.W. (2012) Stem characteristics of two forage maize (*Zea mays* L.) cultivars varying in whole plant digestibility. IV. Changes during the growing season in anatomy and chemical composition in relation to fermentation characteristics of a lower internode. *NJAS Wageningen J. Life Sci.* **59**, 13–23.

Brenner, E.A., Zein, I., Chen, Y., Andersen, J.R., Wenzel, G., Ouzunova, M., Eder, J., Darnhofer, B., Frei, U., Barrière, Y. and Lübberstedt, T. (2010) Polymorphisms in O-methyltransferase genes are associated with stover cell wall digestibility in European maize (*Zea mays* L.). *BMC Plant Biol.* **10**, 27.

Carpita, N.C. and McCann, M.C. (2008) Maize and sorghum: genetic resources for bioenergy grasses. *Trends Plant Sci.* **13**, 415–420.

Carrera, E., Bou, J., García-Martínez, J.L. and Prat, S. (2000) Changes in GA 20-oxidase gene expression strongly affect stem length, tuber induction and tuber yield of potato plants. *Plant J.* **22**, 247–256.

Casati, P., Campi, M., Morrow, D.J., Fernandes, J. and Walbot, V. (2011) Transcriptomic, proteomic and metabolomic analysis of maize responses to UV-B: comparison of greenhouse and field growth conditions. *Plant Signal. Behav.* **6**, 1146–1153.

Casler, M.D., Pedersen, J.F. and Undersander, D.J. (2003) Forage yield and economic losses associated with the brown-midrib trait in Sudangrass. *Crop Sci.* **43**, 782–789.

Chen, Y. (2011) *Pleiotropic effects of genes involved in cell wall lignification on agronomic characters*. Grad Theses Diss Paper 10415.

Chen, F. and Dixon, R.A. (2007) Lignin modification improves fermentable sugar yields for biofuel production. *Nat. Biotechnol.* **25**, 759–761.

Christensen, A.H. and Quail, P.H. (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Res.* **5**, 213–218.

Claeys, H., De Bodt, S. and Inzé, D. (2013) Gibberellins and DELLAs: central nodes in growth regulatory networks. *Trends Plant Sci.* **19**, 231–239.

Cleland, C. and Briggs, W. (1969) Gibberellin and CCC effects on flowering and growth in the long-day plant *Lemna gibba* G3. *Plant Physiol.* **50**, 3–507.

Coles, J.P., Phillips, a.L., Croker, S.J., García-Lepe, R., Lewis, M.J. and Hedden, P. (1999) Modification of gibberellin production and plant development in Arabidopsis by sense and antisense expression of gibberellin 20-oxidase genes. *Plant J.* **17**, 547–556.

De Souza, A.P., Arundale, R.A., Dohleman, F.G., Long, S.P. and Buckeridge, M.S. (2013) Will the exceptional productivity of Miscanthus x giganteus increase further under rising atmospheric CO 2? *Agric. For. Meteorol.* **171-172**, 82–92.

Dence, C.W. (1992) The Determination of Lignin. In *Methods in Lignin Chemistry* (Stephen, Y.L. and Dence, C.W., eds), pp. 33–61. Berlin Heidelberg: Springer.

DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **28**, 350–356.

Dubouzet, J.G., Strabala, T.J. and Wagner, A. (2013) Potential transgenic routes to increase tree biomass. *Plant Sci.* **212**, 72–101.

Dwyer, L.M., Andrews, C.J., Stewart, D.W., Ma, B.L. and Dugas, J.A. (1995) Carbohydrate levels in field-grown leafy and normal maize genotypes. *Crop Sci.* **35**, 1020–1027.

EASAC (2012) *The current status of biofuels in the European Union, their environmental impacts and future prospects; EASAC policy report 19*.

Eriksson, M.E., Israelsson, M., Olsson, O. and Moritz, T. (2000) Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. *Nat. Biotechnol.* **18**, 784–788.

Evenson, R.E. and Gollin, D. (2003) Assessing the impact of the green revolution, 1960 to 2000. *Science*, **300**, 758–762.

Fagoaga, C., Tadeo, F.R., Iglesias, D.J., Huerta, L., Lliso, I., Vidal, A.M., Talon, M., Navarro, L., García-Martínez, J.L. and Peña, L. (2007) Engineering of gibberellin levels in citrus by sense and antisense overexpression of a GA 20-oxidase gene modifies plant architecture. *J. Exp. Bot.* **58**, 1407–1420.

FAO Statistics Division (2013) *FAOSTAT 2013*. http://faostat3.fao.org/. Accessed 16 February 2015

Feltus, F.A. and Vandenbrink, J.P. (2012) Bioenergy grass feedstock: current options and prospects for trait improvement using emerging genetic, genomic, and systems biology toolkits. *Biotechnol. Biofuels* **5**, 80.

Flintham, J.E., Angus, W.J. and Gale, M.D. (1997) Heterosis, overdominance for grain yield, and alpha-amylase activity in F1 hybrids between near-isogenic Rht dwarf and tall wheats. *J. Agric. Sci.* **129**, 371–378.

Fukushima, R.S. and Hatfield, R.D. (2004) Comparison of the acetyl bromide spectrophotometric method with other analytical lignin methods for determining lignin concentration in forage samples. *J. Agric. Food Chem.* **52**, 3713–3720.

García-Hurtado, N., Carrera, E., Ruiz-Rivero, O., López-Gresa, M.P., Hedden, P., Gong, F. and García-Martínez, J.L. (2012) The characterization of transgenic tomato overexpressing gibberellin 20-oxidase reveals induction of parthenocarpic fruit growth, higher yield, and alteration of the gibberellin biosynthetic pathway. *J. Exp. Bot.* **63**, 5803–5813.

Gonzalez, N., Vanhaeren, H. and Inzé, D. (2012) Leaf size control: complex coordination of cell division and expansion. *Trends Plant Sci.* **17**, 332–340.

Guillaumie, S., Pichon, M., Martinant, J.-P., Bosio, M., Goffner, D. and Barrière, Y. (2007) Differential expression of phenylpropanoid and related genes in brown-midrib bm1, bm2, bm3, and bm4 young near-isogenic maize plants. *Planta* **226**, 235–250.

Hendriks, A.T.W.M. and Zeeman, G. (2009) Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresour. Technol.* **100**, 10–18.

Hisanaga, T., Kawade, K. and Tsukaya, H. (2015) Compensation : a key to clarifying the organ-level regulation of lateral organ size in plants. *J. Exp. Bot.* **66**, 1–9.

Huang, S., Raman, A.S., Ream, J.E., Fujiwara, H., Cerny, R.E. and Brown, S.M. (1998) Overexpression of 20-oxidase confers a gibberellin-overproduction phenotype in Arabidopsis. *Plant Physiol.* **118**, 773–781.

IPCC. (2014) *Climate Change 2014: Synthesis Report*. Geneva, Switzerland: IPCC.

Israelsson, M., Eriksson, M.E., Hertzberg, M., Aspeborg, H., Nilsson, P. and Moritz, T. (2003) Changes in gene expression in the wood-forming tissue of transgenic hybrid aspen with increased secondary growth. *Plant Mol. Biol.* **52**, 893–903.

Israelsson, M., Sundberg, B. and Moritz, T. (2005) Tissue-specific localization of gibberellins and expression of gibberellin-biosynthetic and signaling genes in wood-forming tissues in aspen. *Plant J.* **44**, 494–504.

Jung, H. (2011) Forage digestibility: the intersection of cell wall lignification and plant tissue anatomy. *Int. Adv. Rumin Nut. Res.* **16**, 2–174.

Jung, H.G. and Casler, M.D. (2006) Maize stem tissues: cell wall concentration and composition during development. *Crop Sci.* **46**, 1793–1800.

Juska, F. (1958) *Some effects of gibberellic acid on turf grasses*. Golf Course Report 25–28.

Lambers, H., Nagel, O. and Arendonk, J.Van.. (1995) The control of biomass partitioning in plants from favourable''and stressful''environments: a role for gibberellins and cytokinins. *Bulg. J. Plant Physiol.* **21**, 24–32.

Leplé, J.-C., Dauwe, R., Morreel, K., Storme, V., Lapierre, C., Pollet, B., Naumann, A., Kang, K.-Y., Kim, H., Ruel, K., Lefèbvre, A., Joseleau, J.-P., Grima-Pettenati, J., De Rycke, R., Andersson-Gunneràs, S., Erban, A., Fehrle, I., Petit-Conil, M., Kopka, J., Polle, A., Messens, E., Sundberg, B., Mansfield, S.D., Ralph, J., Pilate, G. and Boerjan, W. (2007) Downregulation of cinnamoyl-coenzyme A reductase in poplar: multiple-level phenotyping reveals effects on cell wall polymer metabolism and structure. *Plant Cell* **19**, 3669–3691.

Lester, D.R., Ross, J.J., Davies, P.J. and Reid, J.B. (1997) Mendel's stem length gene (Le) encodes a gibberellin 3 beta-hydroxylase. *Plant Cell* **9**, 1435–1443.

Lorenz, A.J., Gustafson, T.J., Coors, J.G. and de Leon, N. (2010) Breeding maize for a bioeconomy: a literature survey examining harvest index and stover yield and their relationship to grain yield. *Crop Sci.* **50**, 1–12.

Ma, Q., Hedden, P. and Zhang, Q. (2011) Heterosis in rice seedlings: its relationship to gibberellin content and expression of gibberellin metabolism and signaling genes. *Plant Physiol.* **156**, 1905–1920.

Malmberg, R.L., Held, S., Waits, A. and Mauricio, R. (2005) Epistasis for fitness-related quantitative traits in *Arabidopsis thaliana* grown in the field and in the greenhouse. *Genetics* **171**, 2013–2027.

Mauriat, M. and Moritz, T. (2009) Analyses of GA20ox- and GID1-over-expressing aspen suggest that gibberellins play two distinct roles in wood formation. *Plant J.* **58**, 989–1003.

Morrison, T., Kessler, J., Hatfield, R.D. and Buxton, D.R. (1994) Activity of two lignin biosynthesis enzymes during development of a maize internode. *J. Sci. Food Agric.* **65**, 133–139.

Muylle, H., Van Hulle, S., De Vliegher, A., Baert, J., Van Bockstaele, E. and Roldán-Ruiz, I. (2015) Yield and energy balance of annual and perennial lignocellulosic crops for bio-refinery use: a 4-year field experiment in Belgium. *Eur. J. Agron.* **63**, 62–70.

Nelissen, H., Rymen, B., Jikumaru, Y., Demuynck, K., Van Lijsebettens, M., Kamiya, Y., Inzé, D. and Beemster, G.T.S. (2012) A local maximum in gibberellin levels regulates maize leaf growth by spatial control of cell division. *Curr. Biol.* **22**, 1183–1187.

Nelissen, H., Moloney, M. and Inzé, D. (2014) Translational research: from pot to plot. *Plant Biotechnol. J.* **12**, 277–285.

Norcia, L.N., Evans, J.D., Faulkner, D.N. and Rohrbaugh, L.M. (1964) Growth changes in pea plants induced by gibberellic acid and their effects on the concentrations of tissue lipids. *Biochem* **J90**, 633–637.

Oikawa, T., Koshioka, M., Kojima, K., Yoshida, H. and Kawata, M. (2004) A role of OsGA20ox1, encoding an isoform of gibberellin 20-oxidase, for regulation of plant stature in rice. *Plant Mol. Biol.* **55**, 687–700.

Paleg, L., Kende, H., Ninnemann, H. and Lang, A. (1965) Physiological effects of gibberellic acid. 8. Growth retardants on barley endosperm. *Plant Physiol.* **40**, 165–169.

Pedersen, J.F., Vogel, K.P. and Funnell, D.L. (2005) Impact of reduced lignin on plant fitness. *Crop Sci.* **45**, 812–819.

Pedersen, M., Johansen, K.S. and Meyer, A.S. (2011) Low temperature lignocellulose pretreatment: effects and interactions of pretreatment pH are critical for maximizing enzymatic monosaccharide yields from wheat straw. *Biotechnol. Biofuels* **4**, 11.

Peng, J., Richards, D.E., Hartley, N.M., Murphy, G.P., Devos, K.M., Flintham, J.E., Beales, J., Fish, L.J., Worland, A.J., Pelica, F., Sudhakar, D., Christou, P., Snape, J.W., Gale, M.D. and Harberd, N.P. (1999) "Green revolution" genes encode mutant gibberellin response modulators. *Nature*, **400**, 256–261.

Pennington, D. (2013) *Harvest index: A predictor of corn stover yield*.

Pichon, M., Deswartes, C., Gerentes, D., Guillaumie, S., Lapierre, C., Toppan, A., Barrière, Y. and Goffner, D. (2006) Variation in lignin and cell wall digestibility in caffeic acid O-methyltransferase down-regulated maize half-sib progenies in field experiments. *Mol. Breed.* **18**, 253–261.

Ramakers, C., Ruijter, J.M., Lekanne Deprez, R.H. and Moorman, A.F.M. (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Lett.* **339**, 62–66.

Riboulet, C., Lefevre, B., Denoue, D., Barrière, Y. and Barriere, Y. (2008) Genetic variation in maize cell wall for lignin content, lignin structure, p-hydroxycinnamic acid content, and digestibility in set of 19 lines at silage harvest maturity. *Maydica*, **53**, 11–19.

Ridoutt, B.G., Pharis, R.P. and Sands, R. (1996) Fibre length and gibberellins A1 and A20 are decreased in Eucalyptus globules by acylcyclohexanedione injected into the stem. *Physiol. Plant.* **96**, 559–566.

Riedelsheimer, C., Czedik-Eysenberg, A., Grieder, C., Lisec, J., Technow, F., Sulpice, R., Altmann, T., Stitt, M., Willmitzer, L. and Melchinger, A.E. (2012) Genomic and metabolic prediction of complex heterotic traits in hybrid maize. *Nat. Genet.* **44**, 217–220.

Roberts, L.W., Gahan, P.B. and Aloni, R. (1988) *Vascular Differentiation and Plant Growth Regulators*. Berlin, Heidelberg: Springer.

Robinson, A.R. and Mansfield, S.D. (2009) Rapid analysis of poplar lignin monomer composition by a streamlined thioacidolysis procedure and near-infrared reflectance-based prediction modeling. *Plant J.* **58**, 706–714.

Rood, S.B., Buzzell, R.I., Major, D.J. and Pharis, R.P. (1990) Gibberellins and Heterosis in Maize: quantitative relationships. *Crop Sci.* **30**, 281.

Sasaki, A., Ashikari, M., Ueguchi-Tanaka, M., Itoh, H., Nishimura, A., Swapan, D., Ishiyama, K., Saito, T., Kobayashi, M., Khush, G.S., Kitano, H. and Matsuoka, M. (2002) Green revolution: a mutant gibberellin-synthesis gene in rice. *Nature*, **416**, 701–702.

Scobbie, L., Russell, W., Provan, G.J. and Chesson, A. (1993) The newly extended maize internode: A model for the study of secondary cell wall formation and consequences for digestibility. *J Sci Food Agric* **61**, 217–225.

Setter, T.L. and Meller, V.H. (1984) Reserve carbohydrate in maize stem : C14-glucose and C14-sucrose uptake characteristics. *Plant Physiol.* **75**, 617–622.

Srivastava, L.M. (2002) *Plant Growth and Development: Hormones and Environment, Reprint*. Amsterdam: Academic Press.

Tonini, D., Hamelin, L., Wenzel, H. and Astrup, T. (2012) Bioenergy production from perennial energy crops: a consequential LCA of 12 bioenergy scenarios including land use changes. *Environ. Sci. Technol.* **46**, 13521–13530.

Van Acker, R., Vanholme, R., Storme, V., Mortimer, J.C., Dupree, P. and Boerjan, W. (2013) Lignin biosynthesis perturbations affect secondary cell wall composition and saccharification yield in *Arabidopsis thaliana*. *Biotechnol. Biofuels* **6**, 46.

Van Acker, R., Leplé, J.-C., Aerts, D., Storme, V., Goeminne, G., Ivens, B., Légée, F., Lapierre, C., Piens, K., Van Montagu, M.C.E., Santoro, N., Foster, C.E., Ralph, J., Soetaert, W., Pilate, G. and Boerjan, W. (2014) Improved saccharification and ethanol yield from field-grown transgenic poplar deficient in cinnamoyl-CoA reductase. *Proc. Natl Acad. Sci. USA* **111**, 845–850.

Van der Weijde, T., Alvim Kamei, C.L., Torres, A.F., Vermerris, W., Dolstra, O., Visser, R.G.F. and Trindade, L.M. (2013) The potential of C4 grasses for cellulosic biofuel production. *Front Plant Sci.* **4**, 107.

Vanholme, R., Morreel, K., Darrah, C., Oyarce, P., Grabber, J.H., Ralph, J. and Boerjan, W. (2012a) Metabolic engineering of novel lignin in biomass crops. *New Phytol.* **196**, 978–1000.

Vanholme, R., Storme, V., Vanholme, B., Sundin, L., Christensen, J.H., Goeminne, G., Halpin, C., Rohde, A., Morreel, K. and Boerjan, W. (2012b) A systems biology view of responses to lignin biosynthesis perturbations in Arabidopsis. *Plant Cell* **24**, 3506–3529.

Vermerris, W. (2011) Survey of genomics approaches to improve bioenergy traits in maize, sorghum and sugarcane. *J. Integr. Plant Biol.* **53**, 105–119.

Vermerris, W., Saballos, A., Ejeta, G., Mosier, N.S., Ladisch, M.R. and Carpita, N.C. (2007) Molecular breeding to enhance ethanol production from corn and sorghum stover. *Crop Sci.* **47**, S–142.

Viswanath, V., Ma, C., Etherington, E., Dharmawardhana, P., Pearce, D.W., Rood, S.B., Busov, V.B. and Strauss, S.H. (2011) Greenhouse and field evaluation of transgenic poplars with modified gibberellin metabolism and signaling genes. *BMC Proc.* **5**, O22.

Voelker, S.L., Lachenbruch, B., Meinzer, F.C. and Strauss, S.H. (2011) Reduced wood stiffness and strength, and altered stem form, in young antisense 4CL transgenic poplars with reduced lignin contents. *New Phytol.* **189**, 1096–1109.

Vogt, T. (2010) Phenylpropanoid biosynthesis. *Mol. Plant* **3**, 2–20.

Voorend, W., Lootens, P., Nelissen, H., Roldán-Ruiz, I., Inzé, D. and Muylle, H. (2014) LEAF-E: a tool to analyze grass leaf growth using function fitting. *Plant Meth.* **10**, 37.

Waclawovsky, A.J., Sato, P.M., Lembke, C.G., Moore, P.H. and Souza, G.M. (2010) Sugarcane for bioenergy production: an assessment of yield and regulation of sucrose content. *Plant Biotechnol.* **J8**, 263–276.

Walcott, J. and Laing, D. (1976) Some physiological aspects of growth and yield in wheat crops: a comparison of a semidwarf and a standard height cultivar. *Aust. J. Exp. Agric.* **16**, 578.

Wang, Z.Y. and Brummer, E.C. (2012) Is genetic engineering ever going to take off in forage, turf and bioenergy crop breeding? *Ann. Bot.* **110**, 1317–1325.

Witt, S., Galicia, L., Lisec, J., Cairns, J., Tiessen, A., Araus, J.L., Palacios-Rojas, N. and Fernie, A.R. (2012) Metabolic and phenotypic responses of greenhouse-grown maize hybrids to experimentally controlled drought stress. *Mol. Plant* **5**, 401–417.

Xiao, Z., Storms, R. and Tsang, A. (2004) Microplate-based filter paper assay to measure total cellulase activity. *Biotechnol. Bioeng.* **88**, 832–837.

Yamaguchi, S. (2008) Gibberellin Metabolism and its Regulation. *Annu. Rev. Plant Biol.* **59**, 225–251.

Yao, Q., Cong, L., Chang, J.L., Li, K.X., Yang, G.X. and He, G.Y. (2006) Low copy number gene transfer and stable expression in a commercial wheat cultivar via particle bombardment. *J. Exp. Bot.* **57**, 3737–3746.

Yue, F., Lu, F., Sun, R.C. and Ralph, J. (2012) Syntheses of lignin-derived thioacidolysis monomers and their uses as quantitation standards. *J. Agric. Food Chem.* **60**, 922–928.

Zabala, G., Zou, J., Tuteja, J., Gonzalez, D.O., Clough, S.J. and Vodkin, L.O. (2006) Transcriptome changes in the phenylpropanoid pathway of Glycine max in response to *Pseudomonas syringae* infection. *BMC Plant Biol.* **6**, 26.

Zawaski, C. and Busov, V.B. (2014) Roles of gibberellin catabolism and signaling in growth and physiological response to drought and short-day photoperiods in populus trees. *PLoS ONE* **9**, e86217.

Zeng, Y., Zhao, S., Yang, S. and Ding, S.-Y. (2014) Lignin plays a negative role in the biochemical process for producing lignocellulosic biofuels. *Curr. Opin. Biotechnol.* **27**, 38–45.

Zhang, Q., Cheetamun, R., Dhugga, K.S., Rafalski, J.A., Tingey, S.V., Shirley, N.J., Taylor, J., Hayes, K., Beatty, M., Bacic, A., Burton, R.A. and Fincher, G.B. (2014) Spatial gradients in cell wall composition and transcriptional profiles along elongating maize internodes. *BMC Plant Biol.* **14**, 27.

Zhao, X., Zhu, D., Zhou, B., Peng, W. and Lin, J. (2010) Over-expression of the AtGA2ox8 gene decreases the biomass accumulation and lignification in rapeseed (*Brassica napus* L.). *J. Zhejiang* **11**, 471–481.

Supporting information

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

Figure S1 Thioacidolysis lignin composition in senesced stems of control (white bars)and GA20-OX1 (black bars) plants.

Figure S2 Residual biomass after only ethanol wash (without) or treatment with hot water, acid (1 M HCl) or alkali (0.5 M NaOH) of GA20-OX1 and control stem material.

Figure S3 Saccharification efficiency in milled senesced stem material of control plants after alkali (0.5 M NaOH), acid (1 M HCl), hot water or no pretreatment (only ethanol wash), expressed as glucose yield per dry weight (DW). Error bars represent standard errors of ten biological replicates.

Figure S4 Cellulose (A) and lignin (B) per DW in the developing ninth internode of control (white bars) and GA20-OX1 (black bars) plants.

Table S1 Internode and stem volumes of GA20-OX1 and control plants at maturity.

Table S2 Results of ANOVA analysis taking year and genotype as factors for 4 traits measured in 2012 and 2013

Table S3 Selection of genes for qRT-PCR.

Table S4 List of specific prominent ions used to extract the ion-specific chromatograms and quantify the different lignin units, released during thioacidolysis.

