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

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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 (Muyllle 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 breaking 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.

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.

Keywords: gibberellic acid, bioenergy, lignocellulose, maize, crop yield, plant growth.
et al. (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 in taller plants and larger organs, which was demonstrated in Arabidopsis (Coles et al., 1999; Huang et al., 1998), tobacco (Biemelt et al., 2004), rice (Okawa et al., 2004), potato (Carrera et al., 2000), tomato (García-Hurtado et al., 2012), citrus (Fagoga 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., 2006).

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 (siling 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 S2). 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-
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. **P < 0.01 > P ≥ 0.001, ***P < 0.001 > P.

Altered GA in maize affects 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.
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, 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
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 (Vermeiren, 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 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 ≥ 0.01, **: 0.01 > P ≥ 0.001, ***: 0.001 > P.

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 ≥ 0.01, ***: 0.001 > P.
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 Nielissen 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 pMBb77FM21GW-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; productivity Ec = 1 mS/cm; and water-soluble fertilizer Poly-feed (Haifa, Belgium) (N, P₂O₅, K₂O; 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 separated 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 \( \pi \frac{(A/2)^2(B/2)}{2} L \), the formula for an ellipsoid cylinder. The total stem volume was then calculated as \( \pi \frac{(A/2)^2(B/2)^2}{2} (P) \), 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 tasselling, respectively) stage and seven in S and S + 14d (siling 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; Scobie 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). 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 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 wereemasculated 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.irsrv.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 15 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 = ε × l × 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, CA) 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 µL trifluoroacetic acid and 1 µL ethanol (90 °C), 3 h. After centrifugation, the remaining CWR was dried under vacuum for 45 min and weighed. The treated 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 µL aliquot of the reaction mix from this colour reaction contained 50 µG ABTS, 44.83 µG 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 nontransgenic and two transgenic plants (set GH-b) were used. Stem pieces of 5 cm were fixated using freshly made fixing 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 fixing 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 vibrisscope. 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.

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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.