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Metabolic engineering of sugarcane to accumulate energy-dense triacylglycerols in vegetative biomass

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Summary

Elevating the lipid content in vegetative tissues has emerged as a new strategy for increasing energy density and biofuel yield of crops. Storage lipids in contrast to structural and signaling lipids are mainly composed of glycerol esters of fatty acids, also known as triacylglycerol (TAG). TAGs are one of the most energy-rich and abundant forms of reduced carbon available in nature. Therefore, altering the carbon-partitioning balance in favour of TAG in vegetative tissues of sugarcane, one of the highest yielding biomass crops, is expected to drastically increase energy yields. Here we report metabolic engineering to elevate TAG accumulation in vegetative tissues of sugarcane. Constitutive co-expression of WRINKLED1 (*WRI*1), diacylglycerol acyltransferase1-2 (*DGAT*1-2) and oleosin1 (*OLE*1) and simultaneous cosuppression of ADP-glucose pyrophosphorylase (*AGP*ase) and a subunit of the peroxisomal ABC transporter1 (*PXA*1) in transgenic sugarcane elevated TAG accumulation in leaves or stems by 95- or 43-fold to 1.9% or 0.9% of dry weight (DW), respectively, while expression or suppression of one to three of the target genes increased TAG levels by 1.5- to 9.5-fold. Accumulation of TAG in vegetative progeny plants was consistent with the results from primary transgenics and contributed to a total fatty acid content of up to 4.7% or 1.7% of DW in mature leaves or stems, respectively. Lipid droplets were visible within mesophyll cells of transgenic leaves by confocal fluorescence microscopy. These results provide the basis for optimizations of TAG accumulation in sugarcane and other high yielding biomass grasses and will open new prospects for biofuel applications.

Introduction

Storage lipids in plants, mainly composed of glycerol esters of fatty acids, also known as triacylglycerol (TAG) are one of the most energy-rich and abundant forms of reduced carbon available in nature (Durrett *et al.*, 2008; Thelen and Ohlrogge, 2002). The energy density of TAG is more than twice that of carbohydrates, and it can be easily transformed to biodiesel by converting its acyl chains to fatty acid methyl esters (Ohlrogge and Chapman, 2011). To date, oil seed crops containing high level of TAG in their seeds or fruits have been predominant feedstocks for TAG and biofuel production. Expanding the cultivation area of oil seed crops for biofuel production, however, will be challenging due to negative impact on food supply, environmental sustainability and limited availability of arable land (Ohlrogge and Chapman, 2011). Elevating the lipid content in vegetative tissues of high biomass yielding crops has emerged as a new paradigm for increasing energy density and enhancing biofuel yields in more sustainable ways (Chapman *et al.*, 2013; Ohlrogge and Chapman, 2011).

Because of its high biomass production, sugarcane (*Saccharum* spp. Hybrids) has a great potential as a novel feedstock for TAG production. Sugarcane is the most energy efficient perennial C4 plant and can convert 6–7% of solar energy into biomass (Tew and Cobill, 2008). The sugarcane biomass yield under optimal field conditions is significantly higher than that of other high biomass yielding crops, such as switchgrass or miscanthus (Byrt *et al.*, 2011; Waclawovsky *et al.*, 2010). The theoretical annual maximum yield for harvestable sugarcane biomass is estimated to be 177 Mg/ha on dry weight basis (Waclawovsky *et al.*, 2010). Genetic engineering has the potential to convert sugarcane into a production platform for value-added fuels and chemicals (Altpeter and Oraby, 2010).

Fatty acids synthesis competes with carbohydrate synthesis for newly fixed carbon in the plastids, which is exported and assembled into TAG in the endoplasmic reticulum (ER). TAGs are released from ER and accumulate in the cytosol in form of oil bodies (Chapman and Ohlrogge, 2012). Depending on cellular needs, eventually, TAGs can be recycled for membrane biosynthesis or metabolized in peroxisomes (Chapman *et al.*, 2013). Therefore, metabolic engineering strategies are targeting to divert the carbon flux to lipid biosynthesis and include single or multiple gene manipulations aiming to up-regulate fatty acid biosynthesis (push), TAG assembly (pull) and minimize TAG turnover (protect) (Vanhercke *et al.*, 2014a, b).

*WRINKLED*1 (*WRI*1) is a member of the family of APETELA2/ethylene response element binding protein (AP2/EREBP) transcription factors, which is a positive regulator of fatty acid biosynthesis in seeds (Cernac and Benning, 2004). Overexpression of *WRI*1 resulted in 2.8-fold increase in TAG accumulation in Arabidopsis seedlings (Sanjaya *et al.*, 2011).

The ADP-glucose pyrophosphorylase (*AGPase*) is a critical enzyme in the starch biosynthesis pathway, and its RNAi suppression may increase the availability of carbon for fatty acid biosynthesis. Suppression of *AGP*ase combined with *WRI*1 overexpression drove an 5.8-fold increase in TAG accumulation in Arabidopsis seedling, compared to wild type, while *AGP*ase

suppression alone resulted in only a 1.4-fold increase in TAG accumulation (Sanjaya *et al.*, 2011).

Diacylglycerol acyltransferase (*DGAT*1-2*)* is an enzyme that catalyses the final and only committed step in converting diacylglycerol into TAG (Lehner and Kuksis, 1996). Two independent studies demonstrated that the overexpression of Arabidopsis *DGAT*1 led to up to sevenfold increase in TAG accumulation in tobacco (Bouvier-Navé *et al.*, 2000; Wu *et al.*, 2013). A 25-fold increase of TAG accumulation was reported following constitutive expression of a *DGAT*2 from microalgal origin (Sanjaya *et al.*, 2011).

Increased TAG accumulation has also been achieved by reducing lipid turnover. Oleosins (OLE) are structural proteins that protect oil bodies from coalescence and determine oil body longevity (Jolivet *et al.*, 2004; Sadeghipour and Bhatla, 2002; Siloto *et al.*, 2006). When an engineered version of *OLE*, engineered to contain different numbers of strategically placed Cys residues (Cys-oleosin), was co-overexpressed with *DGAT*1 in Arabidopsis, it facilitated long-term accumulation of fatty acids including TAG and increased TAG content by 44-fold in mature leaves, compared to wild type (Winichayakul *et al.*, 2013). RNAi suppression of genes encoding components in the metabolic breakdown of fatty acids also has the potential to elevate TAG accumulation in vegetative tissues.

A subunit of the peroxisomal fatty acid ABC transporter (*PXA*1) contributes to lipid transport across the peroxisomal membrane for β-oxidation (Hayashi and Nishimura, 2006; Slocombe *et al.*, 2009). The Arabidopsis *PXA*1 mutant displayed a 1.5-fold increase in TAG accumulation in expanding leaf and more drastic increases (10- to 20-fold) in senescent leaf (Slocombe *et al.*, 2009).

Metabolic engineering strategies to elevate TAG accumulation in vegetative tissues have been explored in model plants, Arabidopsis and Tobacco as a proof of concept study (reviewed by Vanhercke *et al.*, 2014b). Both direct effects of single gene manipulation and synergistic effects through multigene engineering strategies on TAG accumulation were reported (Vanhercke *et al.*, 2013, 2014a).

In this study, the potential of co-expressing *WRINKLED1* (*WRI*1), *diacylglycerol acyltransferase*1-2 (*DAGAT*1-2), *oleosin*1 (*OLE*1) in concert with the RNAi suppression of *ADP-glucose pyrophosphorylase* (*AGP*ase) and a subunit of the *peroxisomal ABC transporter*1 (*PXA*1) is investigated with the goal of increasing TAG accumulation in vegetative tissues of sugarcane.

Results

Generation of transgenic sugarcane co-expressing WRI1, DGAT1-2 and OLE1 and suppressing PXA1 and AGPase

Biolistic transfer of a single vector with dual constitutive expression cassettes, *WRI*1/*npt*II, or cotransfer of three vectors with dual constitutive expression cassettes *WRI*1/*npt*II, *OLE*1/*DGAT*1-2, and one dual RNAi inducing hairpin, *PXA*1/*AGP*ase (Figure 1), into sugarcane calli was carried out as described earlier (Taparia *et al.*, 2012).

Sixty-one regenerated plants which expressed the *nptII* gene according to NPTII ELISA analysis were evaluated with endpoint RT-PCR (Figure S1). Of the 61 transgenic lines examined by endpoint RT-PCR, 16 lines showed co-expression of *npt*II, *WRI*1, and unlinked *DGAT*1-2 and *OLE*1 (26% co-expression frequency), while co-expression frequencies of linked *nptII*/*WRI*1 or *OLE*1/*DGAT*1-2 were 41% and 36%, respectively.

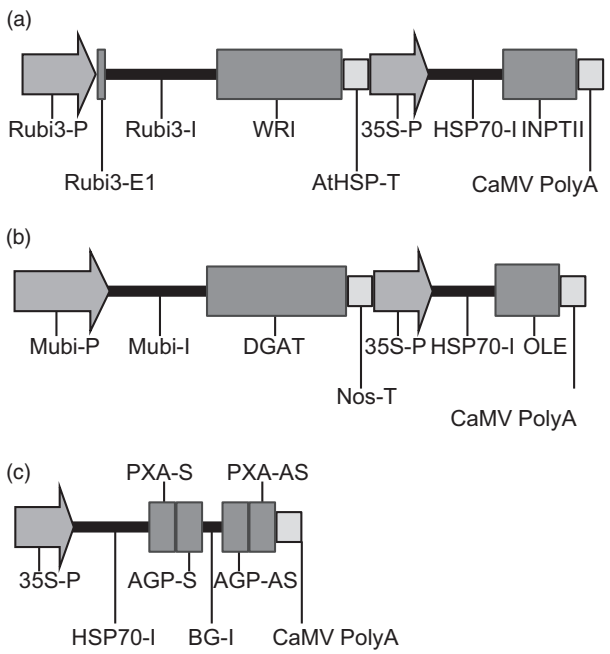

Figure 1 Vector constructs used for genetic transformation. (a) Schematic map of minimal expression cassette for pJK403NPTII. The codon optimized *WRINKLED*1 (*WRI*1) transcription factor under the control of rice ubiquitin 3 promoter (Rubi3-P) with first exon of rice ubiquitin 3 (Rubi3-E1) plus intron (Rubi3-I) and Arabidopsis heat shock protein 18.2 terminator (AtHSP-T) are linked with the *nptII* selectable marker gene under the control of enhanced CaMV 35S promoter (35S-P) with HSP70 intron (HSP-I) and CaMV 35S polyA (CaMV PolyA). (b) Schematic map of the minimal expression cassette for pJK304605. The codon optimized diacyltransferase (*DGAT*1-2) under the control of maize ubiquitin promoter (Mubi-P) with first intron (Mubi-I) and Nos terminator (Nos-T) are linked with Oleosin1 (*OLE*1) under the control of enhanced CaMV 35S promoter (35S-P) with HSP70 intron (HSP-I) and CaMV 35S polyA (CaMV PolyA). (c) Schematic map of minimal expression cassette for pJK60iPXAAGP. The inverted repeats of the 200 bp of the subunit of peroxisomal ABC transporter (sense; *PXA-S* and antisense; *PXA-AS*) and 205 bp of ADP-glucose pyrophosphorylase (sense; *AGP-S* and antisense; *AGP-AS*) fragments separated by 94 bp of *Paspalum notatum* 4CL intron (BG-I) controlled by enhanced CaMV 35S promoter (35S-P) with HSP70 intron (HSP-I) and CaMV 35S polyA (CaMV PolyA).

qRT-PCR was used to quantify gene expression in the transgenic lines (Table 1). There were significant differences in overexpression *of WRI*1, *DGAT*1-2 and *OLE*1, and cosuppression of *PXA*1 and *AGP*ase amongst the transgenic sugarcane lines. Line 19B had the highest co-expression of *WRI*1 and *DGAT*1-2 (Table 1) and showed a similar growth pattern as WT (Figure 2). WRI1 was significantly expressed in lines 233, 25C, 19B, 220E, 5A and 6B. *DGAT*1-2 was significantly expressed in lines 233, 25C, 19B, 220E, 29D and 208F. There was significant expression of *OLE*1 in lines 233, 25C, 19B, 220E, 29D, 204D and 230E (Table 1). The cosuppression levels of *PXA*1/*AGP*ase were analysed in 11 lines, and 9 of these 11 lines showed statistically significant cosuppression of both target genes compared to WT (Table 1). There was statistically significant suppression of *PXA*1 in 10 sugarcane lines compared to *AGP*ase, where 9 of 11 *AGP*ase RNAi lines were significantly suppressed compared to WT (Table 1). However, the level of *AGP*ase suppression was typically stronger than the level of *PXA*1 suppression.

Table 1 TAG accumulation, transgene expression and target gene suppression levels in transgenic sugarcane

	TAG (% DW)		Fold increase to WT		Transgene expression			Target gene suppression (%)	
Line	Stem	Leaf	Stem	Leaf	*WRI*	*DGAT*	*OLE*	*PXA*	*AGP*
WT	$0.02^{d}\pm0.00$	$0.02^{d}\pm0.00$	–	–	$0.00^{d}\pm0.00$	$0.00^{d}\pm0.00$	$0.00^{d}\pm0.00$	$0^{a}\pm0$	$0^{a}\pm0$
233	$0.86^{a}\pm0.11$	$1.90^{a}\pm0.34$	43.0	95.0	$0.07^{ab}\pm0.00$	$0.24^{b}\pm0.01$	$0.30^{c}\pm0.02$	$80^{d}\pm0$	$99^{b}\pm0$
25C	$0.73^{ab}\pm0.07$	$1.53^{ab}\pm0.22$	36.5	76.5	$0.07^{b}\pm0.01$	$0.13^{c}\pm0.01$	$0.27^{c}\pm0.05$	$80^{d}\pm7$	$99^{b}\pm0$
19B	$0.43^{bc}\pm0.10$	$0.61^{bc}\pm0.23$	21.5	30.5	$0.09^{a}\pm0.00$	$0.33^{a}\pm0.03$	$0.83^{b}\pm0.00$	$78^{d}\pm0$	$99^{b}\pm0$
220E	$0.41^{bc}\pm0.06$	$0.85^{bc}\pm0.09$	20.5	42.5	$0.06^{b}\pm0.00$	$0.13^{c}\pm0.01$	$0.23^{c}\pm0.06$	$88^{de}\pm0$	$98^{b}\pm1$
29D	$0.22^{cd}\pm0.04$	$0.65^{c}\pm0.26$	11.0	32.5	$0.01^{cd}\pm0.00$	$0.28^{ab}\pm0.02$	$0.42^{c}\pm0.03$	$79^{d}\pm1$	$99^{b}\pm0$
11	$0.11^{d}\pm0.02$	$0.06^{d}\pm0.01$	5.5	3.0	$0.00^{d}\pm0.00$	$0.00^{d}\pm0.00$	$0.00^{d}\pm0.00$	$76^{d}\pm2$	$98^{b}\pm0$
204D	$0.09^{d}\pm0.04$	$0.08^{d}\pm0.03$	4.5	4.0	$0.00^{d}\pm0.00$	$0.00^{d}\pm0.00$	$1.50^{a}\pm0.27$	$86^{de}\pm3$	$99^{b}\pm0$
208F	$0.07^{d}\pm0.03$	$0.19^{d}\pm0.08$	3.5	9.5	$0.00^{d}\pm0.00$	$0.15^{c}\pm0.03$	$0.00^{d}\pm0.00$	$27^{ab}\pm5$	$0^{a}\pm0$
230E	$0.06^{d}\pm0.01$	$0.09^{d}\pm0.01$	3.0	4.5	$0.00^{d}\pm0.00$	$0.00^{d}\pm0.00$	$1.10^{ab}\pm0.05$	$54^{bc}\pm3$	$0^{a}\pm0$
5A	$0.03^{d}\pm0.00$	$0.04^{d}\pm0.01$	1.5	2.0	$0.02^{d}\pm0.00$	$0.00^{d}\pm0.00$	$0.00^{d}\pm0.00$	$0^{a}\pm0$	$0^{a}\pm0$
9	$0.02^{d}\pm0.00$	$0.02^{d}\pm0.01$	1.0	1.0	$0.00^{d}\pm0.00$	$0.00^{d}\pm0.00$	$0.00^{d}\pm0.00$	$69^{cd}\pm5$	$97^{b}\pm1$
211E	$0.02^{d}\pm0.00$	$0.03^{d}\pm0.00$	1.0	1.5	$0.01^{d}\pm0.00$	$0.00^{d}\pm0.00$	$0.00^{d}\pm0.00$	$78^{d}\pm3$	$99^{b}\pm0$
6B	$0.02^{d}\pm0.00$	$0.04^{d}\pm0.02$	1.0	2.0	$0.07^{d}\pm0.01$	$0.00^{d}\pm0.00$	$0.00^{d}\pm0.00$	$0^{a}\pm0$	$0^{a}\pm0$

WT: wild type. Values are means ± standard error of the mean. Values within one column without common letter were significantly different at $P < 0.05$.

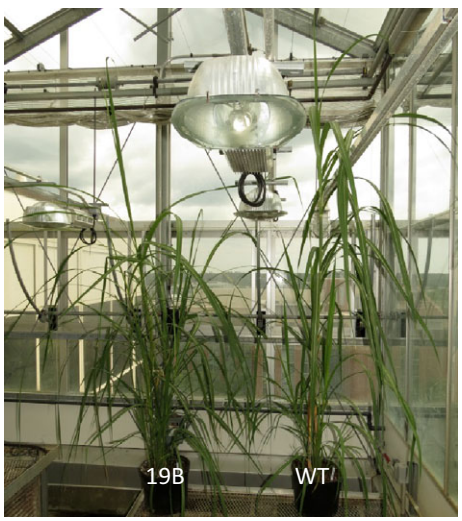

Figure 2 Growth of transgenic sugarcane co-expressing and cosuppressing all target genes in the greenhouse in comparison with wild type. 19B: Transgenic line with co-expression of *WRI*1, *DGAT*1-2 and *OLE*1 and cosuppressing *PXA*1 and *AGP*ase. WT: Wild-type sugarcane plant.

Southern blot analysis to confirm transgene integration

Southern blot analysis confirmed stable integration of *WRI*1, *DGAT*1-2, *OLE*1 and *PXA-AGP*ase in multiple copies (Figure S2a–d). The transgenic line W6, which was cobombarded with a WRI1 and *npt*II expression cassettes, displayed two WRI1 hybridization signals (Figure S2a).

TAG and total FA accumulation in stem and leaf tissues of transgenic sugarcane plants

TAG levels were analysed in stem and leaf tissues of primary transgenic plants and their vegetative progenies. Independent tillers were used for primary transgenics, or different progeny plants were used to allow for replicated analysis. The TAG values for stem and leaf displayed in Table 1 represent the mean of independent and replicated TAG analysis from mature / mid-mature / young internodes or mature / young leaves of primary transgenic lines, respectively. The transgenic sugarcane lines, 233, 25C, 19B, 220E, 29D co-expressing *WRI*1, *DGAT*1-2 and *OLE*1 and cosuppressing *PXA*1 and *AGP*ase, accumulated the highest amounts of TAG in both stem and leaf tissues (Table 1). Transgenic line 233 accumulated the greatest amount of TAG in both stem and leaf (0.86 and 1.90% DW, respectively), which was 43- and 95-fold higher than the TAG value in WT stem (0.02% DW) and leaf (0.02% DW), respectively. Other lines with co-expression of *WRI*1, *DGAT*1-2 and *OLE*1 and cosuppressing *PXA*1 and *AGP*ase (220E 25C, 29D, 19B) accumulated TAG at levels of 0.22–0.73% of DW in stems and 0.65–1.53% of DW in leaves, representing a 11- to 37-fold increase or 31- to 77-fold increase compared to WT stems or leaves, respectively. Transgenic lines (11, 204D, 208F, 230E, 5A) expressing/suppressing one to three of the target genes resulted in 1.5- to 9.5-fold increases of TAG compared to WT.

Vegetative progeny plants of highest TAG accumulating lines 25C and 233 displayed similar TAG levels as the primary transgenic lines. TAG accumulated in these transgenic progenies to levels up to 0.9% of DW in mature stems and up to 1.8% of DW in mature leaves (Table 2, Figure 3).

Mature leaf and mature stem TAG in WT is composed almost exclusively of saturated fatty acids, with palmitic approximately 70% and stearic approximately 30%. In leaves of transgenic lines 25C and 233, the level of saturated fatty acid accumulation is less than half that of WT, and substantial amounts of the unsaturated fatty acids, that is 18:2, 18:3 and 18:1 accumulated (Figure 4a, b). A similar pattern of decreasing saturates and corresponding increase in saturates is seen in the TAG of immature leaves and stems of lines 25C and 233, except that the immature WT leaves and stems contain significant amounts, that is 27% and 20%, respectively, of 18:2 (Figure S4a,b).

Total fatty acid accumulated in transgenic progenies up to 4.6% of dry matter which exceeds WT more than threefold. Comparing the differential between total FA and TAG accumu-

Table 2 Comparison of means of total FA, TAG and total FA-TAG accumulation in different tissues of progeny plants of transgenic sugarcane (25C, 233) in comparison with wild type

Sugarcane line	Tissue	Total FA % of DW	TAG % of DW	Total FA-TAG % of DW
233	Mature leaf	4.65^{A}	1.46^{BC}	3.19^{A}
25C	Mature leaf	4.23^{A}	1.78^{AB}	2.45^{B}
233	Immature leaf	4.03^{A}	2.33^{A}	1.70^{C}
25C	Immature leaf	2.85^{B}	1.28^{BCD}	1.57^{CD}
233	Immature stem	2.35^{BC}	1.05^{CDE}	1.30^{CD}
25C	Immature stem	2.24^{BCD}	0.72^{DE}	1.52^{CD}
233	Mature stem	1.70^{CDE}	0.91^{CDE}	0.79^{E}
25C	Mature stem	1.60^{DE}	0.91^{CDE}	0.69^{E}
Wild type	Mature leaf	1.54^{E}	0.01^{G}	1.53^{CD}
Wild type	Immature leaf	1.31^{E}	0.05^{FG}	1.26^{D}
233	Mid-mature stem	1.30^{EF}	0.64^{EF}	0.66^{E}
25C	Mid-mature stem	1.27^{EF}	0.56^{EFG}	0.71^{E}
Wild type	Immature stem	0.63^{FG}	0.05^{FG}	0.58^{E}
Wild type	Mid-mature stem	0.52^{G}	0.03^{FG}	0.49^{E}
Wild type	Mature stem	0.46^{G}	0.01^{G}	0.45^{E}

Values within one column without common letter were significantly different at $P < 0.05$.

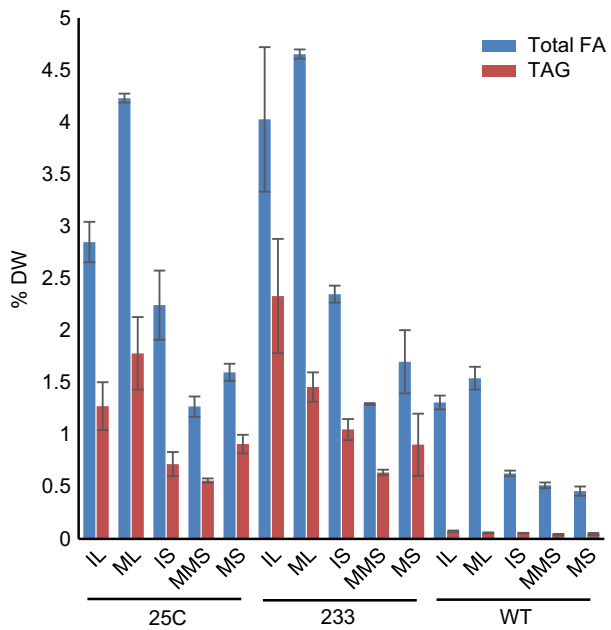

Figure 3 Total fatty acid and TAG contents in different tissues of vegetative progeny plants. IL: immature leaf, ML: mature leaf, IS: immature stem, MMS: mid-mature stem and MS: mature stem. 25C and 233: transgenic lines with co-expression of *WRI*1, *DGAT*1-2 and *OLE*1 and cosuppressing *PXA*1 and *AGP*ase. WT: wild-type plant. Error bar indicates standard error of the mean. Mean represents average of analysis from two independent progeny plants per line.

lation in different lines and tissues (Total FA - TAG % of DW, Table 2) revealed a significant elevation of total FA per percentage of dry matter independent of the TAG accumulation in mature leaves and young stems of both transgenic sugarcane lines.

Lipid accumulation in transgenic leaf tissues was visualized by confocal microscopy following lipid staining (Figure 5). The

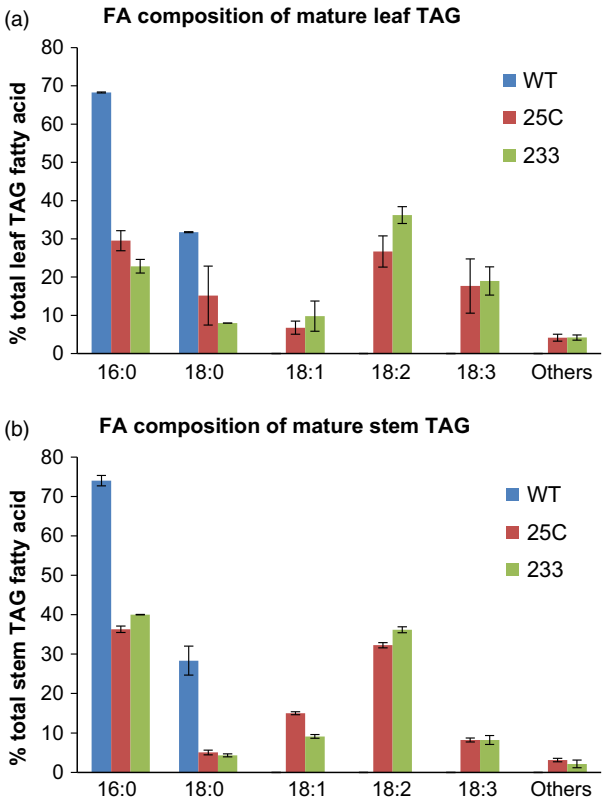

Figure 4 (a) Fatty acid profile of TAG from wild-type and transgenic sugarcane mature leaves. (b) Fatty acid profile of TAG from wild-type and transgenic sugarcane mature stems. 25C and 233: Transgenic lines with co-expression of WRI1, DAGAT1-2 and OLE1 and cosuppressing PXA1 and AGPase. WT: wild-type plant. Error bar indicates standard error of the mean. Mean represents average of analysis from two independent progeny plants per line.

transgenic line 233 clearly showed BODIPY-stained green droplets in confocal microscopy of mesophyll cells indicating the accumulation of lipids including TAG in mesophyll cells in contrast to WT.

Correlation between the levels of gene expression and TAG accumulation

Pearson correlation coefficients indicated that gene expression levels of *WRI*1 and *DGAT*1-2 were significantly correlated with TAG production (Table 3). There was a positive correlation between *WRI*1 and *DGAT*1-2 expression. Statistical analysis of co-expression of *AGP*ase and *PXA*1 showed a highly significant correlation as expected for a linked hairpin, and expression of both was negatively correlated to *WRI*1.

Analysis of sucrose and starch content of transgenic sugarcane plants

TAG accumulating transgenic lines 25C and 233 were used to study the effect of co-expression of *WRI*1, *DGAT*1-2, *OLE*1 and cosuppressing *PXA*1 and *AGP*ase on the starch and sucrose content. Starch content in leaves of transgenic lines (25C and 233) was significantly reduced compared to WT. However, the starch content did not differ significantly from WT in the stem and juice of the transgenic lines 25C and 233 (Table 4). The total soluble solids in the juice (Brix) and/or the juice volume per gram fresh weight were significantly reduced in transgenic lines by 7–15%, respectively, compared to WT (Table 4).

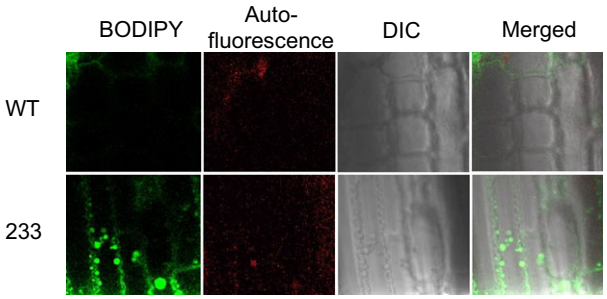

Figure 5 Accumulation of lipid droplets in transgenic sugarcane leaf tissue. Transverse sections of the abaxial side of the first dewlap leaf from transgenic sugarcane (line 233) and untransformed sugarcane (WT). Mesophyll cells are visualized by confocal microscopy with differential interference contrast (DIC). Neutral lipids including TAG, stained with BODIPY493/503, appear as green droplets within the leaf mesophyll cells that contain visible chloroplasts (red autofluorescence).

Discussion

To date, metabolic engineering for TAG production in vegetative tissues has been demonstrated only in model plants, such as Arabidopsis (Fan *et al.*, 2014; Sanjaya *et al.*, 2011) and tobacco (Andrianov *et al.*, 2010; Vanhercke *et al.*, 2014a). We report substantial elevation of TAG accumulation in sugarcane following a multigene metabolic engineering strategy. Sugarcane is a prime biofuel production platform due to its exceptional biomass yields. Stems represent approximately 70% of the total above-ground sugarcane biomass (Tew and Cobill, 2008). In this study, a 43-fold increase of TAG accumulation in sugarcane stems was observed compared to nontransformed sugarcane following constitutive co-expression of WRINKLED1 (*WRI*1), diacylglycerol acyltransferase1-2 (*DGAT*1-2), oleosin1 (*OLE*1) and simultaneous cosuppression of ADP-glucose pyrophosphorylase (*AGPase*) and a subunit of the peroxisomal ABC transporter1 (*PXA*1). The stem accumulation of TAG to 0.9% of DW in transgenic sugarcane is comparable to the maximum observed TAG accumulation in stems of tobacco of 0.9% of DW following co-expression of *WRI*1 *DGAT*1-2 and *OLE*1 (Vanhercke *et al.*, 2014a). Transgenic sugarcane accumulated TAG in leaf tissues up to 1.9% DW, which is 95-fold higher than that in nontransformed sugarcane. Substantial amounts of the unsaturated fatty acids accumulated in transgenic sugarcane on expense of saturated fatty acids. This pattern implies that the maize *DGAT1-2* expressed in these transgenic lines is able to catalyse the esterification of unsaturated fatty acids to diacylglycerol. A similar pattern of increased unsaturated fatty acids was reported upon the expression of *PDAT* in the *tgd1* background (Fan *et al.*, 2013) and both the tgd1sdp1 and *tgd1pxa1* double mutants (Fan *et al.*, 2014). However, a different pattern was observed in tobacco leaves (Vanhercke *et al.*, 2013). TAG in WT tobacco leaves contains a high proportion, that is, more than 55% of 18:3 with 16:0, 18:0, 18:1 and 18:2 comprising the balance. Upon the expression of *WRI1*, *DGAT1-2* and *OLE1*, the levels of 18:3 in TAG dramatically decreased in tobacco to <10%. Also the levels of 16:0, 18:0, 18:1 and 18:2 showed proportionate increases mirroring the decrease of 18:3 in total leaf fatty acids of transgenic versus WT senescing tobacco leaves. The observed differences in FA profiles between tobacco and sugarcane may be caused by the different source of the DGAT enzyme (Arabidopsis vs. maize) or by sampling at different developmental stages. Vanhercke *et al.* (2014a) reported a 76-fold TAG increase in tobacco leaves. Interestingly, nontransformed tobacco leaves have an approximately 10-fold higher TAG content than nontransformed sugarcane, while nontransformed sugarcane and tobacco stems display similar TAG contents. Therefore, it is not surprising that while the relative increases were similar between tobacco and sugarcane, a drastically higher absolute TAG accumulation (16% of DW) was observed in transgenic tobacco leaves (Vanhercke *et al.*, 2014a) compared to transgenic sugarcane leaves in this study. Optimization of transgene overexpression and identification of target genotypes with higher basal TAG content in vegetative tissues may play an important role in attempts to further increase TAG accumulation in sugarcane.

The present study and that of Vanhercke *et al.* (2014a) employed a similar strategy of simultaneously overexpressing the *WRI*1 transcription factor, the *DGAT* acyltransferase and the OLE1 oleosin that coat the surface of the oil body. However, in this study, all three genes were placed under the control of constitutive promoters, whereas in tobacco, the Arabidopsis SSU promoter was used for the expression of the *WRI*1 and *OLE*1 genes which is highly active in leaves, follows a circadian expression rhythm, and

Table 4 Comparison of means of stem juice volume, Brix and starch content in leaf, stem and juice of transgenic sugarcane (25C, 233) in comparison with wild type (WT)

	Juice volume ml/g fresh weight	Brix (fresh stem juice)	Starch mg/g dry weight in leaves	Starch mg/g dry weight in stem	Starch mg/g dry weight in juice
WT	0.57^{A}	16.95^{A}	15.44^{A}	64.37^{A}	92.73^{A}
25C	0.56^{B}	14.45^{B}	10.26^{C}	68.86^{A}	87.15^{A}
233	0.53^{C}	17.02^{A}	13.48^{B}	57.98^{A}	89.07^{A}

Values within one column without common letter were significantly different at $P < 0.05$.

Table 3 Correlation coefficients and level of significance for the variables

	TAG mature leaf	TAG immature leaf	*WRI*	*DGAT*	*OLE*	*PXA*
TAG Immature leaf	0.843 (0.000)					
WRI	0.654 (0.000)	0.647 (0.000)				
DGAT	0.861 (0.000)	0.536 (0.003)	0.565 (0.002)			
OLE	0.235 (0.228)	0.212 (0.280)	0.110 (0.577)	0.279 (0.150)		
PXA	−0.127 (0.518)	−0.194 (0.324)	−0.476 (0.010)	−0.008 (0.967)	−0.081 (0.681)	
AGP	−0.267 (0.170)	−0.287 (0.138)	−0.462 (0.013)	−0.168 (0.392)	0.045 (0.820)	0.818 (0.000)

Cell content: Pearson correlation, (*P*-value).

is subject to feedback regulation. The authors suggested that 'expression of *WRI*1 under SSU promoter might reduce possible unwanted metabolic impacts on leaf function and plant development' (Vanhercke *et al.*, 2014a). As stalks account for the major biomass fraction in sugarcane, it seems that further increases in stem TAG without impacting leaf function could be achieved by placing *WRI*1 under the control of a strong stem-specific promoter. Several candidates for sugarcane stem-specific promoters were recently described (Mudge *et al.*, 2013).

Synergistic effects of multigene overexpression observed for *WRI*1 and *DGAT* on TAG biosynthesis were observed in transgenic sugarcane lines, similar to previous studies conducted in Arabidopsis and tobacco (Kelly *et al.*, 2013; Vanhercke *et al.*, 2013, 2014a). The cotransformation approach taken here is efficient for exploring multiple gene and promoter combinations. By estimating the level of expression of the transgenes and correlating them with TAG accumulation levels, we are able to rapidly screen for combinations of genes that lead to enhanced TAG accumulation. However, using this approach, generating transgenic lines with high level co-expression/suppression of multiple target genes is challenging and may be best accomplished by employing single large vectors that contain all the optimized target gene expression/suppression cassettes. Alternately, additional genes could be sequentially stacked by retransformation of existing transgenics.

A set of lines with different transgene expression and target gene suppression levels revealed the effect of individual and simultaneous gene expression on TAG accumulation. *WRI*1 and *DGAT*1-2 expression levels are highly positively correlated with TAG production. *WRI*1 is a transcription activator of 18 known genes from central metabolism the products of which accumulate in the cytoplasm (Maeo *et al.*, 2009) and fatty acid biosynthesis in the chloroplast, while *DGAT*1-2 contributes to TAG assembly at the endoplasmic reticulum (Vanhercke *et al.*, 2013). A synergistic effect between these two genes was previously reported for TAG production following a transient expression assay in *Nicotiana benthamiana* (Vanhercke *et al.*, 2013). *WRI*1 expression alone (line 6b and 5a, Table 1) resulted in a doubling of TAG accumulation in sugarcane leaves. This impact was achieved with relatively low expression levels (i.e. 0.02-0.07% of GADPH, Table 1) of *WRI*, leaving room for improvement by codon optimization or expression under the control of alternative regulatory elements. Overexpression of the *WRI1* transcription factor in Arabidopsis resulted in a similar (2.8-fold) increase of TAG accumulation in vegetative tissue (Sanjaya *et al.*, 2011).

Transgenic sugarcane line 208F with significant expression of *DGAT*1-2 and nonsignificant suppression of *PXA*1 displayed a 9.5-times elevated TAG accumulation in leaves, similar to tobacco and Arabidopsis plants over expressing *DGAT which* accumulated sevenfold more TAG than nontransformed plants (Bouvier-Nave et al., 2000; Wu *et al.*, 2013).

While *OLE*1 expression and *PXA*1 and AGP suppression were observed in all of the sugarcane lines with high TAG accumulation, they were not significantly correlated to TAG accumulation. For example line 204D which displayed the highest OLE expression, 86% suppression of PXA and 99% suppression of AGP and no expression of *WRI*1 or *DGAT*1-2 resulted only in a fourfold or 4.5-fold increase of TAG in leaves or stems, respectively. Vanhercke *et al.* (2014a) did not evaluate the correlation of *OLE*1 expression with TAG accumulation but suggested that *OLE*1 plays a major role for TAG accumulation in tobacco. *AGPase* was very strongly (98–99%) suppressed in several sugarcane lines, resulting in a moderate suppression of starch content in sugarcane leaves without significant correlation to TAG accumulation. This implies that starch synthesis is not in direct competition with oil synthesis in sugarcane. The effects of suppression of *PXA*1, a strategy designed to reduce FA turnover by decreasing transport into the peroxisome where β-oxidation occurs, are less clear because suppression levels varied between 27 and 86%. Thus, either TAG turnover does not occur to significant levels in sugarcane tissues, or the level of suppression achieved in these experiments was insufficient to reveal a strong effect. To differentiate between these possibilities, a different portion of the *PXA*1 gene could be targeted for RNAi suppression, or other targets of TAG turnover, such as the sugar-dependent 1 lipase (SDP1) (Kelly *et al.*, 2013), or a key enzyme in β-oxidation such as 3-ketoacyl-CoA thiolase (KAT1) (Germain *et al.*, 2001), could be targeted for RNAi suppression.

Total fatty acid accumulated in transgenic sugarcane plants up to 4.6% of dry matter which exceeds WT more than threefold. Moderate reduction of soluble solids in the juice (Brix) of transgenic sugarcane by up to 7% suggests that sufficient carbon reserves remain for boosting lipid accumulation. Interestingly, a significant elevation of between 1.6- and 2.6-fold of total FA per percentage of dry weight independent of the TAG accumulation was detected in mature leaves and young stems of transgenic sugarcane lines 25C and 233. This increase is likely in membrane lipids and suggests that in these tissues, enzymes responsible for moving acyl chains from membranes to TAG, such as DGAT and/or PDAT, may be rate limiting for elevating TAG assembly (Fan *et al.*, 2014). Previous work showed that feeding excess fatty acid can cause feedback down-regulation of ACCase, the rate-limiting step in FA synthesis (Andre *et al.*, 2012). If the bottleneck in sugarcane cells can be relieved by moving more FA through the TAG assembly pathway, then it is possible that yields could be increased by relieving feedback inhibition of ACCase.

The results presented herein clearly demonstrate the feasibility of producing oil in the high biomass crop sugarcane. Due to the large biomass accumulation of sugarcane, we estimate that each percentage of TAG accumulated in sugarcane corresponds to the entire oil yield from the same land area of canola. Elevating the level of TAG accumulation in sugarcane by optimizing expression cassettes and by stacking of additional genes currently in our discovery pipeline has the potential to provide a rich source of sustainable biodiesel.

Experimental procedures

Genetic constructs

Two plasmids, each carrying double, overexpression cassettes, and one plasmid with a dual RNAi suppression cassette were constructed. The native *WRI*1 cDNA from Arabidopsis (Accession no. At3 g54320) was used as a template to design a custom sequence, codon optimized for sugarcane. The codon optimized *WRI*1 was subcloned under the control of a rice ubiquitin 3 promoter with the first exon and intron (Sivamani and Qu, 2006) and terminated by the Arabidopsis heat shock 18.2 (HSP) terminator (Nagaya *et al.*, 2009) following *Sma*I and *Bst*BI restriction digestion. The expression cassette of the *npt*II selectable marker under the control of the 35S promoter from cauliflower mosaic virus (CaMV) (Odell *et al.*, 1985) with the HSP 70 intron of *Zea mays* L. (Rochester *et al.*, 1986) and the CaMV polyA signal (Dixon *et al.*, 1986) was prepared as described by Kim *et al.* (2012). Following digestion with *Not*I, the expression cassette was subcloned into the *WRI*1 expression

vector to form the double expression cassette construct pJK403NPTII (Figure 1a).

The acyl-CoA-dependent diacylglycerol acyltransferase cDNA (*DGAT*1-2) from maize (Zheng *et al.*, 2008) and oleosin synthetase cDNA (OLE1) from Arabidopsis (Yang *et al.*, 2011) were codon optimized according to codon bias of sugarcane. The codon optimized *DGAT*1-2 was subcloned under the control of a maize ubiquitin promoter with first intron (Christensen and Quail, 1996) and NOS terminator (Bevan, 1984). After digestion with *Pme*I and *Not*I restriction enzymes, the expression cassette was inserted into the pUC57 (GenScript, Piscataway, NJ) vector backbone. The codon optimized *OLE*1 was under the regulatory control of the 35S promoter from cauliflower mosaic virus (CaMV) with the HSP 70 intron of *Zea mays* (L.) and the CaMV polyA signal. Following restriction enzyme digestion with *Not*I, the expression cassette was subcloned into the *DGAT*1-2 expression vector to form the double expression cassette construct pJK304605 (Figure 1b).

The dual suppression *PXA*1-*AGP*ase RNAi vector was constructed with custom synthesized sequences (GenScript) derived from highly conserved regions from sugarcane cv. CP88-1762 and including a 200-bp fragment of the subunit of the peroxisomal membrane transporter1 (*PXA1*) followed by a 205-bp fragment of ADP-glucose pyrophosphorylase (*AGP*), the *Paspalum notatum 4CL* intron and the respective antisense sequences of *PXA1* and *AGP*. The *PXA*1/*AGP* RNAi fragment was subcloned under transcriptional control of the 35S promoter from cauliflower mosaic virus (CaMV) with the HSP 70 intron from *Zea mays* (L.) and the CaMV polyA signal. After restriction enzyme digestion with *Hind* III and *Ase* I, *PXA*1/*AGP* RNAi expression cassette was subcloned into the pCAMBIA 2300 to form pJK60iPXAAGP (Figure 1c).

Tissue culture of sugarcane and biolistic gene transfer

Transgenic sugarcane plants were generated using biolistic gene transfer into callus as described by Taparia *et al.* (2012). Immature leaf whorls from sugarcane cv. CP-88-1762 were obtained from the University of Florida, Everglades Research and Education Center in Belle Glade, FL. Plasmids were coprecipitated onto gold particles in a 1:2:2 molar ratio for pJK403NPTII: pJK304605: pJK60iPXAAGP (Figure 1) as described by Altpeter and Sandhu (2010).

NPTII ELISA

ELISAs were conducted according to the manufacturer's instructions (Agdia, Elkhart, IN) as a rapid screen to identify transgenic sugarcane lines. Leaf samples (50 mg) were collected and kept on ice at 4 °C. Samples were ground in a Tissuelyzer II (Qiagen, Valencia, CA) in protein extraction buffer (PEB1) provided with the kit. Protein was quantified with the Coomassie Plus Bradford Assay (Thermo Scientific, Rockford, IL), and the plates for protein quantification were read against a standard curve generated with bovine serum albumin (Fisher, Atlanta, GA) at A595 on a Synergy HI Hybrid Reader (Biotek, Winooski, VT, USA). Total protein (20 μg) was added to each well in the ELISA plate, and the reagents sequentially added according to the manufacturer's instructions. ELISA plates were read against dilutions of the NPTII-positive control provided in the kit at A650.

Endpoint RT-PCR and quantitative real-time RT-PCR (qRT-PCR)

The third leaf (leaf +3) counted from the top visible dewlap leaf (leaf +1) was collected from two tillers of primary transgenic lines and two wild-type plants. Total RNA was extracted with the TRIzol reagent (Invitrogen/Life Technologies, Grand Island, NY) according to the manufacturer's instructions. To prevent genomic DNA contamination, 1 μg of total RNA was treated with RNase-Free RQ1 DNase (Promega, San Luis Obispo, CA). cDNA was synthesized from 500 ng of DNase-treated total RNA using the High Capacity cDNA RT Kit (Applied Biosystems/Life Technologies, Grand Island, NY). The sugarcane glyceraldehyde 3-phosphate (*GAPDH)* was used as a reference gene for the normalization as described in Iskandar *et al.* (2004). Primers for each target gene were described in Table 5. Endpoint RT-PCR was performed in the Mastercycler (Eppendorf, Hauppauge, NY) with Phire Hot Start DNA polymerase (New England Biolabs, Ipswich, MA) with the following conditions: 30 s at 98 °C, followed by 28 cycles of 98 °C for 5 s, 60 °C for 5 s and 72 °C for 20 s, and final elongation at 72 °C for 1 min. PCR products were analysed by 1.5% agarose gel electrophoresis and visualized with ethidium bromide. Quantitative real-time RT-PCR was performed in the CFX Connect Real-Time PCR (Bio-Rad, Hercules, CA) with SsoAdvanced SYBR Green Supermix (Bio-Rad). PCR conditions were as follows: 95 °C for 3 min, 40 cycles at 95 °C for 10 s and 58 °C for 45 s. Amplicon specificity was verified by melt-curve analysis from 65 °C to 95 °C and by agarose gel electrophoresis. The expression level of each target gene was calculated as $2^{[Ct\ (GAPDH)\ -\ Ct\ (target\ gene)]}$ (Iskandar *et al.*, 2004; Livak and Schmittgen, 2001). The suppression levels of *PXA*1 or *AGP*ase expression were the relative expression level of each gene in transgenic lines over that in wild-type plants.

TAG and total FA analysis

Leaf samples from the mid-section of the expanding (immature leaf) or third fully expanded leaf (mature leaf) were collected from two different tillers of the primary transgenic lines and two different plants of their vegetative progenies (V1) growing in soil in the greenhouse. Samples were frozen and ground in liquid N, and freeze-dried prior to analysis. Leaf data in Table 1 represent means of analysis results from immature and mature leaf. Stem data in Table 1 represent means of analysis results from immature internode, mid-mature internode and mature internode. Stem samples were frozen and ground in liquid N, and freeze-dried prior to analysis. Total lipids were isolated from 10 mg of freeze-dried leaf or stem tissue by the addition of 700 μL of extraction solvent (Methanol/ Chloroform/ Formic acid (2:1:0.1, by volume)) and subjected to vigorous mixing for 3 hs with the use of a vortex

Table 5 Primers used for RT-PCR analysis

Target Gene	Primer (5 ′→ 3′)	Product size (bp)	Annealing temperature (°C)
GAPDH	CACGGCCACTGGAAGCA TCCTCAGGGTTCCTGATGCC	101	58
*WRI*1	GTGAAGCAGCAGTACGTGGA CTCCATGATGGTGGAGGAGT	116	58
*DGAT*1-2	TACATCAACCCGATCGTGAA CCACAGGTGGAAGAAGCAGT	138	58
*OLE*1	CGCTGCTGGTGATCTTCTC CCGGTGGCGTACTTGTAGAT	139	58
PXA	CCATCAAATGCACTGTCAGC ATGCTGCCACGGTCTACATT	94	58
*AGP*ase	GCTTTACTCCCTAGTGGAACAG GCAGATCAAGCATGTAACGAGG	98	58

mixer. Total lipid extracts were separated by TLC using a hexane/diethyl ether/acetic acid (70:30:1, by volume) solvent system. Lipids were visualized by spraying 0.05% primuline (in 80% acetone). TAG fractions were identified, excised from the plate and transmethylated into fatty acid methyl esters (FAMEs) by incubation in 1 mL of boron trichloride-methanol at 80–85 °C for 40 min. For total FA analysis, total lipid extracts were transmethylated into fatty acid methyl esters by incubation in 1 mL of boron trichloride-methanol directly. To enable quantification, 5 μg of C17:0 was added as internal standard prior to transmethylation. FAMES were extracted into hexane and dried under nitrogen before dissolving in 100 μL hexane. FAMES were analysed by GC-MS with the use of an Agilent Technologies 7890A GC System equipped with a 5975C mass selective detector.

Southern blots and hybridizations

High molecular weight genomic DNA was extracted from leaves using the CTAB method (Murray and Thompson, 1980). Fifteen micrograms of genomic DNA were digested with the appropriate restriction enzymes, separated by electrophoresis on 0.8% agarose gel and transferred onto the Hybond-N+ membrane using the manufacturer's instructions (GE Healthcare Biosciences, Pittsburgh, PA). Probes were generated by PCR to the promoter region or the coding region of the transformed genes and labelled with 32P-dCTP (Perkin Elmer, Waltham, MA) using the Prime-It II Random Primer Labeling Kit (Agilent Technologies, Santa Clara, CA). Hybridization and washing were performed according to the manufacturer's instructions, and membranes were exposed to Kodak X-ray film (Thermo Fisher Scientific, Waltham, MA) at 80 °C for 2 days.

Lipid staining

The tissues from the first dewlap leaf of transgenic or nontransgenic sugarcane were cut into 8 × 2 mm pieces. For BODIPY staining, fresh tissue was incubated with a solution containing 100 μg/mL BODIPY 493/503 (Invitrogen) in 0.1% Triton X-100 (by dilution from a 10 mg/mL DMSO stock solution). Vacuum was applied for 20 min, before washing twice with PBS to remove excess stain. The BODIPY-stained lipid droplets were imaged using a Leica SP5 confocal laser scanning microscope (Leica, Buffalo Grove, IL) with excitation wavelength set at 488 nm. Lipid droplets were visualized at 63 × magnification, with the gain set to 824 and 1200 for BODIPY stain and chlorophyll, respectively.

Determination of Brix (per cent dissolved solids) of stem juice

Fresh weight of stem was recorded prior to juice extraction using sugarcane juicer (Green Planet Farm, FL). After juice extraction, the juice volume was determined. Brix of freshly pressed juice was measured using ATAGO refractometer (Tokyo, Japan).

Starch content determination

Immature leaf, mature leaf, immature internode, mid-mature internode and mature internode were collected from greenhouse grown transgenic lines. Samples were frozen, ground in liquid N and freeze-dried prior to determination of starch content as described above. Leaf starch data in Table 4 represent means of analysis results from immature and mature leaf, while stem starch data in Table 4 represent means of analysis results from immature internode, mid-mature internode and mature internode. Starch content in the leaf and stem samples was estimated using 250 mg of freeze-dried samples and the protocol provided with the Starch Assay Kit (Sigma-Aldrich, St. Louis, MO).

Statistics

Gene expression for the two double cassette, *npt*II*/WRI1, DGAT1-2/OLE1*, overexpression vectors, and relative gene suppression for *PXA1/AGP*ase RNAi constructs was analysed with simple descriptive statistics and ANOVAs using MINITAB 16. There was a preponderance of '0's in the data; therefore, '0.5' was added to each variable to facilitate analysis. Because the residuals did not follow a normal distribution, each variable was $\log_2$-transformed (Rieu and Powers, 2009), and ANOVAs for randomized complete block design with 2 replications were calculated on the transformed data for primary transgenics and vegetative progeny. Means of gene expression, total FA, TAG and total FA-TAG were compared using Fisher LSD test and Pearson correlation coefficients.

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Supporting information

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

Figure S1 Endpoint RT-PCR analyses of *WRI*1, *OLE*1, and *DGAT* 1-2 expression in transgenic sugarcane**.**

Figure S2 Southern blot analyses of transgenic sugarcane lines.

Figure S3 (a) Fatty acid profile of TAG from wild-type and transgenic sugarcane immature leaves. (b) Fatty acid profile of TAG from wild-type and transgenic sugarcane immature stems.