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Research Article

Expression of Dehydroshikimate Dehydratase in Sorghum Improves Biomass Yield, Accumulation of Protocatechuate, and Biorefinery Economics

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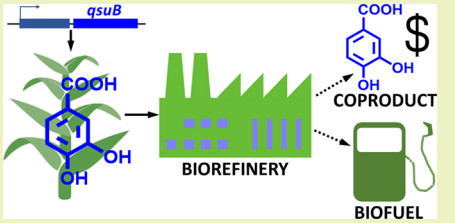
ABSTRACT: Engineering bioenergy crops to accumulate value-added coproducts *in planta* is an attractive approach to increasing the value of lignocellulosic biomass and enabling a sustainable bioeconomy. In this study, we engineered sorghum with a bacterial gene encoding a dehydroshikimate dehydratase (*qsuB*) to convert the endogenous pool of 3-dehydroshikimate into the valuable compound protocatechuate (DHBA). We find that, when grown under field conditions, transgenic sorghum lines can accumulate up to 0.3% DHBA in stover on a dry weight (DW) basis without showing any difference in cell wall composition. An unexpected finding was an increase in yield for all *qsuB*-expressing lines. The grain yield and total biomass yield were 71 and 29% higher in the highest yielding line, respectively. On average, the total biomass yield of the engineered lines was 22.3 t/ha (DW). Moreover, we conducted a techno-economic analysis to investigate the economic impact of coproducing DHBA along with bioethanol in an integrated cellulosic biorefinery. Using engineered biomass sorghum with 0.3 DW% DHBA accumulated *in planta* as the feedstock, the economics of the integrated biorefineries has the potential to be improved. Our data demonstrate an engineering strategy to overproduce DHBA in bioenergy crops to facilitate sustainable manufacturing of biofuels and bioproducts.



KEYWORDS: *bioenergy crops, 3,4-dihydroxybenzoate, biofuels, techno-economic analysis, coproduct*

INTRODUCTION

Lignocellulosic biomass is an important renewable feedstock for the manufacture of advanced biofuels and bioproducts, in part because of the potential for biomass energy crops to achieve lower greenhouse gas emissions relative to conventional starch or sugar feedstocks.[1] However, the important cost associated with deconstructing plant biomass into simple sugars has been one of the obstacles preventing the implementation of economically sustainable advanced biofuels.[2] The in-planta accumulation of value-added coproducts that can be recovered at biorefineries to generate an additional revenue stream has been proposed to improve the economics of advanced biofuels.[3] In this case, engineered bioenergy crops not only provide carbohydrates for biofuel production but also produce valuable compounds such as platform chemicals, polymers, pharmaceuticals, or flavors and fragrances that can be extracted from biomass, purified, and sold.[4]

Sorghum represents an important bioenergy crop because of its low input needs during growth, effective nitrogen recycling, high water use efficiency, tolerance to drought and heat, and high biomass yields.[5,6] Sorghum has been genetically modified to reduce its recalcitrance to biomass deconstruction, but few metabolic engineering studies aiming at accumulating bioproducts in sorghum biomass have been reported.[7] In this study, we engineered sorghum to overproduce protocatechuate (i.e., 3,4-dixydroxybenzoate or DHBA), a promising metabolite for several pharmacological applications because of its antioxidant activity as well as anti-inflammatory, anti-cancer, and anti-viral properties.[8,9] DHBA can also serve as a precursor to performance-advantaged food packaging materials,[10] platform chemicals such as muconate, beta-ketoadipate, and 2-pyrone-4,6-dicarboxylate,[11−13] and other industrially relevant aromatics such as gallate and vanillin.[14,15] The use of DHBA for fuel cell applications has been recently proposed as an alternative to ascorbic acid because it can be derived from lignin and has comparable power output.[16] DHBA is found in several plant species, but the exact metabolic steps and genes involved in its biosynthesis remain unknown.[9] Overproduction of DHBA *in planta* can be achieved by the heterologous expression of plastid-targeted bacterial 3-dehydroshikimate

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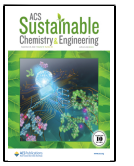

dehydratase (QsuB) to convert endogenous 3-dehydroshikimate into DHBA (Figure 1a).[17−19]

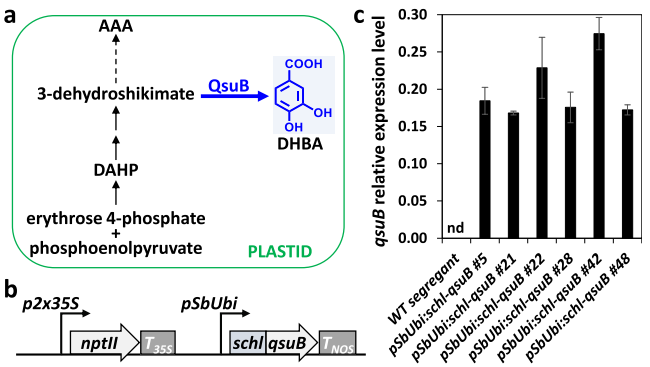

Figure 1. Expression of *qsuB* in sorghum. (a) Schematic diagram of the conversion of 3-dehydroshikimate into DHBA catalyzed by plastid-targeted QsuB. AAA, aromatic amino acids; DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate. (b) Construct used for sorghum transformation. Genetic elements in the T-DNA include a selectable marker gene for kanamycin resistance (*nptII*) placed between a duplicated 35S promoter (*p2x35S*) and a terminator (T_{35S}) from the cauliflower mosaic virus, as well as the *qsuB* synthetic gene located between the sorghum polyubiquitin gene promoter (*pSbUbi*) and the *Agrobacterium tumefaciens* nopaline synthase gene terminator (T_{NOS}). *Schl* encodes a plastid signal peptide. (c) *QsuB* expression in six independent transgenic lines. The level of *qsuB* expression in comparison to that of the *PP2A* gene is shown. cDNA obtained from the wild type (WT) were used as the negative control. Values are means ± SD of four biological replicates (n = 4 plants). nd, not detected.

Here, we transformed sorghum (*Sorghum bicolor* (L.) Moench) with a DNA construct designed for *qsuB* expression to accumulate DHBA *in planta*. DHBA content ranged between 0.13 and 0.30% on a DW basis in transgenic lines grown under field conditions, which represents a 45- to 103-fold increase compared to titers measured in wild-type control plants. An unexpected finding was an increase in both biomass and grain yield in all the lines expressing *qsuB*. Additionally, we developed a techno-economic model to explore the cost feasibility of coproducing DHBA along with bioethanol in cellulosic biorefineries using the engineered sorghum. Techno-economic analysis revealed that accumulation of DHBA at 0.3% DW in sorghum could reduce the minimum selling price of lignocellulosic ethanol and supply DHBA at a price below the current market selling price. We propose that introducing the DHBA coproduct trait into sorghum, mediated by *qsuB* expression, could increase not only the value of sorghum as a bioenergy feedstock but also the production of advanced biofuels.

MATERIALS AND METHODS

Plant Growth Conditions. Plants were grown at the UC Berkeley South greenhouse Oxford facility between September 2020 and March 2021 as previously described.[20] Homozygous transgenic seeds in the second generation (T2) and wild-type segregant seeds were germinated directly on soil and plants were grown until full physiological maturity. Fertilizer (Osmocote Plus 15-9-12) was added to the soil biweekly until the flowering stage. At the end of the growing period, watering was stopped and pots were allowed to dry for three weeks. Plants without their panicles were harvested, further dried in an oven at 50 °C for five days, ground using a Model 4 Wiley Mill equipped with a 1 mm mesh (Thomas Scientific, Swedesboro, NJ), and milled into a fine powder for cell wall and metabolite analyses using a Mixer Mill MM 400 (Retsch Inc., Newtown, PA) and stainless-steel balls. T3 seeds were harvested and used for the field trial.

Design of the *pSbUbi:Schl-qsuB* Construct. The *pSbUbi:schl-qsuB* construct was designed as previously described using the *pSbUbi* promoter[20] and the jStack cloning method.[21] A codon-optimized DNA sequence encoding QsuB from *Corynebacterium glutamicum* (GenBank Accession Number YP_001137362.1) preceded with a plastid targeting signal (SCHL)[22] was synthesized by GenScript (Piscataway, NJ). All the plasmids are listed in Table S1 and the sequences are available at http://public-registry.jbei.org.

Sorghum Transformation and Genotyping. The *Agrobacterium tumefaciens* AGL1 strain was employed to transform sorghum (*S. bicolor*, variety Wheatland) immature embryos using published procedures.[23] The identification of primary transformants with a single-copy event was conducted as previously described.[20] Plants homozygous for the transgene and wild-type segregants were identified for six selected lines in the T1 generation. T2 seeds from one T1 wild-type segregant plant from each line were pooled and used as wild-type segregant controls for greenhouse experiments. T3 seeds from these control plants were used for the field trial.

Analysis of *qsuB* Expression. *QsuB* expression was assessed using reverse transcription quantitative PCR (RT-qPCR) performed on stems from three-week-old plants in the T2 generation as previously described.[18,20] The *PP2A* gene was used as a reference,[24] and the results represent the average from four biological replicates. RT-qPCR primers are listed in Table S2.

Field Trial. Seeds (T3 generation) from lines *pSbUbi:schl-qsuB #05*, *#21*, *#22*, *#42*, *#48*, wild-type segregants, and untransformed wild type (Wheatland variety) were planted in a field trial conducted at the University of California Davis Plant Sciences Research Farm in 2021 under a USDA-APHIS Biotechnology Regulatory Services permit for regulated sorghum (#BRS 20-356-102r). Seeds were planted on a Yolo clay loam soil (fine-silty, mixed thermic Fluventic Haploxerept) on May 28, 2021. Percent germination was taken for each line and adjusted for a target seeding rate of 197,600 seeds ha^{-1}. The experiment was a randomized complete block design with four replicates. Row spacing was 0.76 m. Plots measured 3 × 6 meters and comprised 4 rows. A commercial sorghum variety (NK8828 from S&W Seed Company, Longmont, CO) was also planted as border rows to limit edge effects. Total N-P-K fertilizer was applied at planting at a rate of 224, 91, and 22 kg ha^{-1}, respectively. Sivanto was applied on June 22, 2021, at a rate of 481 g ha^{-1} for sugarcane aphid control. Irrigation water was applied utilizing surface furrow methods to satisfy the fully watered evapotranspiration requirement for sorghum during the growing season. All panicles were covered with bags prior to anthesis to avoid pollen flow. Plot harvest took place on September 21, 2021. Panicles (heads with immature grain) were harvested separately by hand. Remaining stover was harvested using a Wintersteiger Cibus forage chopper (Wintersteiger, Salt Lake City, UT). Subsamples of approximately 500 g of fresh weight stover material were taken from each harvested plot and dried at 55 °C for one week in a forced air oven to determine dry matter and calculate yield. Ten panicles were taken as a subsample to estimate panicle yield. Stover samples were ground into a fine powder for composition analyses.

Metabolite Analysis. Metabolites were extracted from ball-milled biomass samples using 80% (v/v) methanol−water and subsequent acid hydrolysis as previously described.[17] DHBA recovered from hydrolyzed methanol extracts was quantified using high-performance liquid chromatography (HPLC), electrospray ionization (ESI), and quadrupole time-of-flight (QTOF) mass spectrometry (MS) as previously described.[25]

Biomass Composition Analysis. Ball-milled biomass (1 g) was sequentially extracted using a Dionex ASE 350 accelerated solvent extractor set to 7 min static extraction cycles (Thermo Fisher Scientific, Waltham, MA). Solvents (5 mL) were water (two cycles), 80% (v/v) ethanol−water (ten cycles), 50% (v/v) methanol−chloroform (one cycle), and acetone (one cycle). Klason lignin and

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cell wall monosaccharides were determined using the standard NREL biomass procedure.[26] Glucose, xylose, and arabinose were measured by HPLC as previously described.[27] Cell-wall-bound aromatics were released from cell wall residues via mild alkaline hydrolysis[28] and quantified using HPLC-ESI-QTOF-MS analysis.[25]

Techno-Economic Analysis. *SuperPro Designer v12* was used to develop a techno-economic model of DHBA accumulation in sorghum biomass. The simplified process diagram is shown in Figure S1. We designed an integrated cellulosic biorefinery to maximize the utilization of sorghum to produce biofuel and DHBA simultaneously. We assumed that DHBA can be accumulated at 0.3 DW% in biomass sorghum stover (the highest DHBA content reported from the field trial), and this DHBA remains stable during harvest, dry-down, and storage at the biorefinery. During feedstock handling, foreign materials are removed and biomass is routed for short-term storage. The extraction of DHBA from biomass is performed prior to biomass deconstruction and bioconversion. We assume that the metabolite extraction step will preserve the integrity of lignin and polysaccharides in cell walls. Briefly, DHBA is extracted from biomass with 80% (v/v) methanol−water for 6 h at 70 °C using a biomass−solvent ratio of 0.1 g/mL. The extraction process is repeated twice. Methanolic extracts are centrifuged, filtered, and then treated with hydrochloric acid to release the free DHBA from its conjugated form at 95 °C for 3 h.[17] DHBA is further purified after centrifugation, membrane filtration, and drying. The distillation of methanol and recycling back to the extractor is done at a 95% rate. The DHBA extraction and purification process diagram can be found in Figure S2. Once the extraction of the metabolite from sorghum biomass is complete, the remaining biomass is routed to an integrated one-pot high-gravity ionic liquid (IL: [Ch][Lys]) pretreatment process to deconstruct extractive-free lignocellulosic biomass. The IL pretreatment is conducted at 140 °C for 3 h, and IL is assumed to be recycled at 97% after fermentation.[29] The unit price of IL is $2 per kg with a range of $1 to $5 per kg.[30] The leftover lignin and solids as well as the biogas produced in the wastewater treatment section are sent to the heat and power generation section, also known as the utility section, to produce heat and power to fulfill the onsite requirement. Excess electricity produced in the utility section is sold to the grid as a byproduct. The enzymatic hydrolysis breaks down polysaccharides into glucose and xylose, which are then converted to ethanol by fermentation. Ten mg cellulase per g of glucan is used, and the hydrolysis time is three days.[31] Based on the NREL's cellulosic model,[30,31] the conversion rates for glucose-to-ethanol and xylose-to-ethanol are assumed to be 95 and 85% of the stoichiometric theoretical maximum, respectively. Finally, ethanol is recovered and purified for sale as a fuel blendstock, while residual solids and process wastewater are treated on-site. Biogas generated during wastewater treatment is burned for onsite heat and power generation, along with lignin. Onsite energy generation satisfies the heat and electricity demands of the facility, and excess electricity is sold to the grid at a price of $0.068/kWh.[32]

Using the biorefinery process model, we first calculated the mass and energy balance of the process and then conducted the discounted cash flow analysis to obtain (1) the minimum selling price (MSP: $/kg) of DHBA considering bioethanol as the coproduct and (2) the minimum ethanol selling price (MESP: $/gal ethanol) when selling DHBA as the coproduct. The cellulosic biorefinery is assumed to work 24 h per day and 330 days per year (7920 h per year) for 30 years. Biomass sorghum is used as the feedstock (with biomass yields consistent with our field trial results), and the daily processing amount in the biorefinery is 2000 bone-dry tonne.[33] The unit price of biomass sorghum is calculated using the sorghum supply logistic model developed by Baral et al.[1] Specifically, we used the direct-supply bale system for transport to the biorefinery due to the lowest greenhouse gas emission associated with this system relative to other options such as ensiled biomass and densely packed modules; the fertilizer amount applied is consistent with our field experiment: 224 kg ha^{-1} of N, 91 kg ha^{-1} of P, and 22 kg ha^{-1} of K; we also assume that sorghum is cultivated on 5% of the land surrounding the biorefinery, which determines the average transportation distance from farm to biorefinery. Other parameters, including dry matter loss during the entire supply chain and winding factor of the sorghum logistic supply chain, are consistent with the previous study.[1] The market price of DHBA has a large range ($20−60/kg) based on its purity and final application.[34] In the absence of reliable market reports on DHBA prices, we conducted a thorough search of suppliers on Alibaba based on (1) promised ∼99% product purity, (2) an ISO9001 certificate proving their ability to produce such product, and (3) positive customer reviews indicating their ability to deliver the product as specified. In this study, we conservatively used $20/kg as the base price of DHBA and performed the MESP calculation on the highest DHBA price to explore the potential price impact. To better understand the parameters that impact the final selling price, we conducted single-point sensitivity analysis by exploring the maximum and minimum values of the major input parameters. Additional details are provided in Table S3.

RESULTS AND DISCUSSION

Generation of Sorghum Expressing *qsuB*. A construct containing the promoter of a sorghum polyubiquitin gene followed by a codon-optimized *qsuB* open reading frame preceded with a sequence encoding a plastid-targeting peptide was built for *Agrobacterium*-mediated sorghum transformation (Figure 1b). Fifty primary T0 transformants were regenerated from calli and analyzed by real-time PCR to identify lines containing a single copy of the transgene. Six of these single-copy events were grown in the T1 generation, and wild-type segregants and homozygous plants were identified. T2 wild-type segregant seeds from each line (one plant per line) were pooled and used as controls for further experiments. The expression of *qsuB* in each transgenic line was confirmed by qPCR using cDNA synthesized from total RNAs extracted from stems of three-week-old plants in the T2 generation (Figure 1c). Measurements of growth parameters including the number of days to panicle emergence, number of flowering tillers, height of the main tiller, and dry weights from stover and seeds did not reveal any differences between wild type and the transgenic lines, except for line *pSbUbi:schl-qsuB #42*, which was significantly shorter (Table S4).

DHBA Accumulation and Cell Wall Composition in QsuB Sorghum Lines. Methanol-soluble metabolites were extracted from the stover (stem and leaves) of physiologically mature senesced plants (T2 generation) grown in the greenhouse. An acid-hydrolysis step of the methanolic extracts was performed to release the DHBA aglycone form, which was quantified using HPLC-ESI-QTOF-MS analysis. Compared to wild-type control plants, the QsuB sorghum lines showed large increases in the DHBA content (up to 145-fold), which ranged between 841 and 3320 μg/g DW (Figure 2).

The main cell wall components were measured in extractive-free stover biomass. Using the Klason method, no difference could be observed between WT controls and transgenics regarding the amount of insoluble lignin, glucose, xylose, and arabinose, suggesting that *qsuB* expression does not impact lignin deposition and the synthesis of cellulose and hemicellulose in cell walls (Table S5). The amount of *p*-coumarate and ferulate released upon saponification of cell wall samples was similar between WT and transgenic lines, and a small amount of DHBA (5−10 μg/g of cell wall residue) was detected only in cell-wall-bound aromatic fractions obtained from transgenics (Table S6).

Field-Testing of QsuB Sorghum. Five engineered QsuB sorghum lines were grown in the field in the T3 generation to evaluate their performance under natural environment. For this field trial, we again included the pool of WT segregants as

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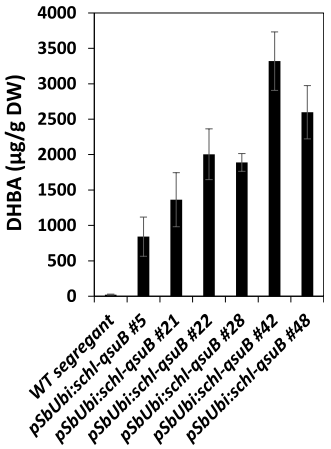

Figure 2. DHBA content in stover biomass from WT and QsuB sorghum lines grown in the greenhouse. Values are means ± SD of five biological replicates (n = 5 plants).

controls, as well as another WT control from the sorghum Wheatland variety that did not go through the transformation process. Plants grown until the soft dough stage did not show any obvious differences in growth patterns, and disease was not observed in any of the genotypes, which is typical for sorghum grown in California. Stover and heads containing immature seeds were harvested separately to evaluate biomass yields at the soft dough stage. Unexpectedly, all the lines had higher grain, stover, and total biomass yields than the two controls, with many of the differences being highly significant (Figure 3). *pSbUbi:schl-qsuB #05*, *#21*, and *#22* had a significant

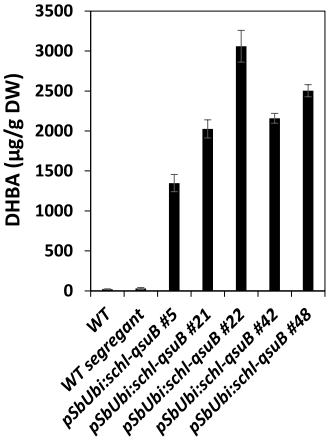

Figure 3. Biomass yields from WT and QsuB sorghum lines grown in the field. Values are means ± SE of four biological replicates (n = 4 plots). Columns with the same letter indicate lines that were not different (multivariate ANOVA and Duncan's test for multiple comparisons, P < 0.05). The letters indicate comparison within each group (grain, stover, total) and not between these groups.

increase in grain yields of 71, 54, and 53%, respectively, and the same lines had 29, 29, and 24% higher total biomass yield. Lines *#5* and *#21* had a total biomass yield of 23.4−23.9 ± 0.4 t/ha. Line *#21* had the highest stover yield (15.1 ± 0.5 t/ha) while line *#5* had the highest grain yield (9.7 ± 0.4 t/ha).

Next, analysis of methanol-soluble metabolites extracted from the stover showed large increases of DHBA content (up to 103-fold) in the QsuB sorghum lines compared to control plants, which ranged between 1348 and 3059 µg/g DW (Figure 4). These DHBA titers are comparable to those obtained from engineered plants harvested at the later

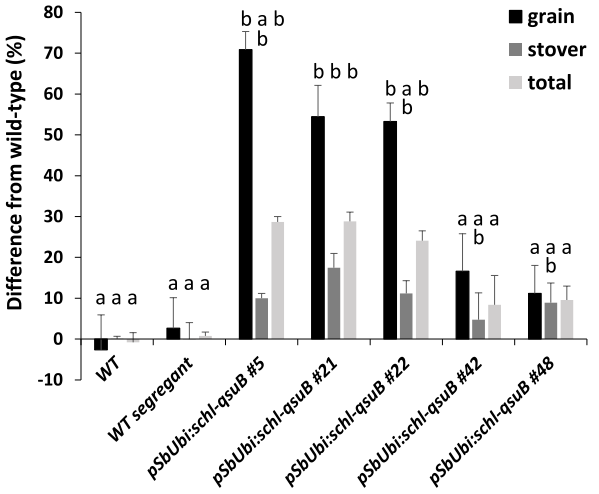

Figure 4. DHBA content in stover biomass from WT and QsuB sorghum lines grown in the field. Values are means ± SD of four biological replicates (n = 4 plots).

senesced mature stage in the greenhouse experiment (Figure 2), suggesting that DHBA remains stable within biomass during plant development. These are important observations considering that sorghum cultivated for the manufacturing of advanced biofuels and bioproducts can be harvested either at full maturity or at an earlier stage that is compatible with the ensiling process.[6,35] Overall, the DHBA titers measured in QsuB sorghum biomass are approximately 10-fold higher than those measured previously in switchgrass modified with the *qsuB* gene, but lower than those from QsuB poplar in which DHBA ranged between 0.7−1.2% on a DW basis in woody biomass.[18,19] Besides differences in crop types, the use of different promoters to drive *qsuB* expression in these crops can explain the variability observed for the DHBA content.

Finally, no difference could be observed between WT controls and transgenics regarding the amount of insoluble lignin, glucose, xylose, and arabinose (Table S7), which is also consistent with the data obtained from the culture in the greenhouse. The amount of cell-wall-bound *p*-coumarate and ferulate was similar between WT and transgenic lines, while small amount of cell-wall-bound DHBA was detected only in cell wall hydrolysates from transgenics (Table S8). The lack of changes in the amount of structural carbohydrates in QsuB engineered sorghum compared to WT control plants is an important observation considering that polysaccharides (i.e., cellulose and hemicellulose) are the source of simple fermentable sugars for production of advanced biofuels and bioproducts. Lignin content is a major factor contributing to biomass recalcitrance given that lignin surrounds polysaccharides in cell walls and protects them from enzymatic hydrolysis. Unlike in Arabidopsis, switchgrass, and poplar in which expression of *qsuB* resulted in reductions of lignin content in biomass,[17−19] engineered QsuB sorghum lines produced the same quantity of lignin compared to controls in both greenhouse and field conditions (Tables S5 and S7). In Arabidopsis, switchgrass, and poplar QsuB lines, the reduction of lignin has been attributed to a reduction of the shikimate pool that is used by hydroxycinnamoyl-coenzyme A:shikimate hydroxycinnamoyl transferase (HCT) during lignin biosyn-

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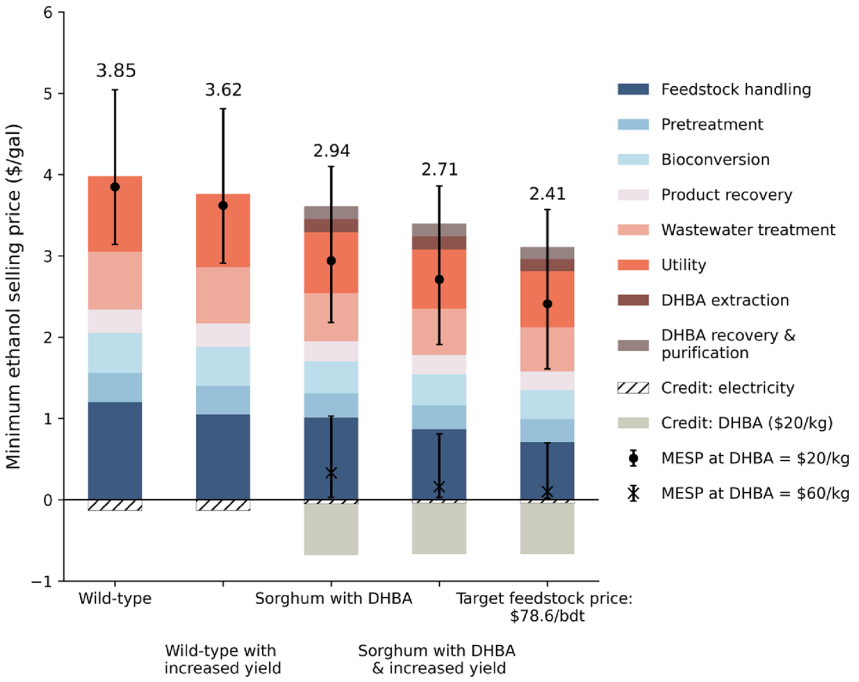

Figure 5. Techno-economic analysis results of integrated cellulosic biorefinery producing DHBA as the coproduct. MESP ($/gal ethanol) when producing DHBA onsite is shown. Black circle is the MESP when selling DHBA (if any) at $20/kg.[34] The cross sign shows the MESP when selling DHBA at its upper market price ($60/kg).[34] Sensitivity bars represent the variation of MESPs with the max and min value of input parameters.

thesis because 3-dehydroshikimate is both the substrate of QsuB and the direct precursor of shikimate in plastids.[17−19] However, recent studies highlighted that HCT may not have a major role for lignin biosynthesis in certain plants including sorghum,[36] which may explain our data on the lignin content in QsuB modified sorghum. Nevertheless, lowering biomass recalcitrance via reducing lignin is an important trait for bioenergy crops to limit the costs associated with pretreatment and enzymatic hydrolysis. Stacking the DHBA coproduct trait with existing low-lignin sorghum, such as recently engineered lines[20] or brown-midrib varieties,[37] could be the next step of crop improvement to further mitigate technical challenges in biomass deconstruction and improve the economics of lignocellulosic biorefineries.

Techno-Economic Analysis. Based on the field experimental results, we observed a significant increase in the biomass yield from 18.41 bone-dry tonne (bdt)/ha in WT sorghum to the highest of 23.89 bdt/ha in line *pSbUbi:schl-qsuB #05*. A higher biomass yield typically results in a lower delivered feedstock price, which is the single largest cost for biorefineries.[3,6,31,33] To explore both the accumulation of DHBA and the sorghum biomass yield increase, we modeled both effects separately and then assessed the impact of the two effects when combined. For the wild-type sorghum in our farm and logistics model, the delivered price is calculated based on the relationship between yield and delivered costs at biorefineries established in a previous study on bioenergy sorghum.[1] We used a calculated baseline delivered price of $113.09/bdt based on the biomass yield from the wild type;[1] and this price could be reduced to $98.03/bdt in the case of the highest yielding line that produced 29% more biomass (Figure 3).[1] With the improved biomass yield and thus lower delivered feedstock price, the yield improvements alone reduce the minimum ethanol selling price (MESP) from $3.85/gal to $3.62/gal (Figure 5). Accumulating a value-added coproduct like DHBA in sorghum biomass at titers obtained in our best QsuB engineered lines (0.3 DW%) has an even more substantial effect on MESP compared to the previously discussed yield improvements, as shown in Figure 5. Without the yield increase, engineered sorghum with 0.3 DW% DHBA reduces the MESP from $3.85/gal to $2.94/gal, assuming DHBA is extracted and purified to sell for $20/kg.[34] Combining the accumulation of DHBA with the observed biomass yield increase in the best-performing engineered line, the MESP can be further reduced to $2.71/gal (a 30% decrease compared to the WT). In the optimal case, where the feedstock price reaches an aggressive target of $78.6/bdt set by the U.S. Department of Energy (DOE)[38] and DHBA is accumulated, the MESP could be further reduced to $2.41/gal. However, to reach this target feedstock price, the biomass yield needs to be further improved to 38.00 t/ha, which is about 60% higher relative to the best engineered line we obtained in field trials. Such higher crop yields could be met by introgressing the QsuB engineering strategy into biomass sorghum varieties that have the capacity to produce >40 t/ha.[5,39] The degree to which such a dramatic cost decrease is achievable may be determined by local market conditions. Another factor that could result in additional reductions in MESP is the DHBA selling price. With a higher DHBA selling price of $60/kg (the high end of our estimated range), the MESP can be dramatically reduced (see black cross signs in Figure 5). As shown in the breakdown of MESP provided in Figure 5, feedstock-related costs and the utility section in the biorefinery are the largest contributors to the total cost. The DHBA extraction and purification process require $16 million and $20 million, respectively, and can supply about 1.7 million kg DHBA on a yearly basis. The revenue gained from selling DHBA at $20/kg is approximately double the costs of extraction and purification.

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The sensitivity analysis shown in Figure 6 indicates that, aside from the DHBA selling price, the discount rate, variations

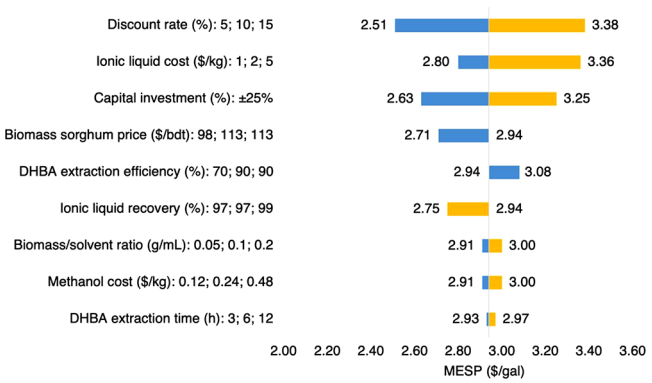

Figure 6. Sensitivity analysis of the MESP (baseline: $2.94/gal ethanol) assuming no yield increment relative to WT sorghum and a DHBA selling price of $20/kg.

in capital costs, and IL costs drive the MESP. Because the DHBA selling price is the single most impactful and uncertain parameter in this analysis, an alternative approach is to fix the MESP and determine the DHBA selling price required to achieve a net present value of zero. This approach treats DHBA as the main product from the cellulosic biorefinery and ethanol as the coproduct.[40,41] We find that the minimum selling prices (MSPs) of DHBA are below the upper bound of the current DHBA selling price ($60/kg)[34] in all three ethanol price scenarios (Figure S3). A higher ethanol selling price results in a lower MSP for DHBA. When ethanol is sold at the base case price ($3.85/gal ethanol or $5.85/gallon of gasoline-equivalent GGE)), DHBA must sell for a minimum of $5.5/kg. To reach a more aggressive target fuel price of $2.5/GGE,[40] the MSP of DHBA must increase to $40.2/kg; even if ethanol sells at a price equivalent to the U.S. historical gasoline rack price of $1.53/GGE,[41] the calculated MSP of DHBA is $50.2/kg, which is still competitive with the current market (Figure S3). Results from this techno-economic analysis are consistent with previous reports, indicating that value-added coproducts can improve the economics of biorefineries.[3,42]

CONCLUSIONS

Using DHBA as a case study, we combined plant metabolic engineering, field testing, and techno-economic analysis to evaluate the impact of accumulating an industrially relevant compound in bioenergy crops on the price of cellulosic ethanol. Results from the techno-economic analysis indicate that feedstock-related costs are the largest contributors to the final cost of biofuel production. It is therefore crucial to ensure that engineered traits in bioenergy crops do not inadvertently reduce biomass yields or introduce other detrimental agronomic traits (e.g., increased lodging or susceptibility to pests and disease). The data show that increasing DHBA to 0.3 DW% in sorghum via the QsuB engineering strategy enables the production of lower-cost biofuels because the revenue generated through the sale of extracted DHBA far exceeds the cost of performing the extraction prior to downstream biomass conversion to biofuel. Previous techno-economic studies indicated that the isolation of valuable compounds from biomass could be technically challenging and affect overall costs if these compounds are strongly attached to cell walls.[43] In the present study, efficient extraction of DHBA from biomass was achieved using aqueous methanol as the solvent, and only a minor fraction was found cross-linked to cell walls (Tables S6 and S8). Nonetheless, in the future, screening a range of solvents that enable efficient extraction of DHBA at reduced cost will improve further the economics of the proposed biorefinery concept. In this regard, reactive extraction appears to be an effective method for the separation and recovery of carboxylic acids such as DHBA from fermentation broth and aqueous streams.[44]

Although the impact on biomass yield had a smaller impact on overall economics relative to the co-production of DHBA, the fact that QsuB sorghum showed no appreciable yield penalty and, in some cases, a yield increase suggests that this engineering strategy could significantly increase the value of sorghum. The physiological basis for these higher yields will need to be investigated as they were not observed under controlled greenhouse conditions and did not correlate with extractable DHBA titers measured in biomass. For example, it would be informative to analyze the composition of root exudates in the various lines and possible changes in the rhizobiome because DHBA is used as a carbon source by several microbes. The QsuB engineering approach has been shown to confer drought tolerance in Arabidopsis,[45] but it is not yet clear if this is also the case in sorghum. The plants in the field trial were not subjected to drought but would still experience abiotic stresses not present in the greenhouse. Monitoring the agronomic performance of the QsuB sorghum lines under reduced irrigation in the field will be important to further investigate these responses as bioenergy crops are expected to be grown on marginal lands that do not compete with food production. It will also be important to determine how the yield increases depend on local climate and soil conditions across a variety of geographic regions, as well as the genetic background in which the trait might be introduced. Additional genetic modifications to the feedstock to enhance DHBA titers are expected to improve the economics of the overall process. For example, a more active promoter could be used to drive *qsuB* expression, and/or a more active 3-dehydroshikimate dehydratase could be employed for higher conversion of 3-dehydroshikimate into DHBA. We previously demonstrated in Arabidopsis and tobacco that co-expression of QsuB with a plastid-targeted feedback-insensitive 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase from *Escherichia coli* (i.e., the first enzyme that condenses erythrose 4-phosphate with phosphoenolpyruvate to yield DAHP in the shikimate pathway; Figure 1a) resulted in up to 350% higher DHBA titers compared to the expression of QsuB alone.[27] It would be therefore worthwhile to introduce such a foreign DAHP synthase in our top QsuB transgenic sorghum lines. Beyond the specific case of QsuB sorghum, this integrated biorefinery concept is potentially applicable to a wide variety of bioenergy crops, different coproducts, and alternative advanced biofuels.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.2c01160.

Table of DNA constructs; table of input parameters for techno-economic modeling and uncertainty analysis; table of sorghum growth parameters; tables of sorghum

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cell wall chemical composition; process diagrams of the integrated cellulosic biorefinery and of the extraction and purification of DHBA; and chart of DHBA minimum selling prices (PDF)

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Author Contributions

Y.T. performed biomass composition analyses; M.Y. conducted the TEA; J.-H.P. and C.-Y.W. generated the sorghum lines; C.-Y.L. performed RT-qPCR analysis; C.M.D.B. conducted the field trial and biomass handling; J.D. provided logistics for the field trial; K.M.V. and P.M.S. contributed to the cloning methods; R.K. and E.E.K.B. performed HPLC-ESI-QTOF-MS analysis; A.E., M.Y., H.V.S., and C.D.S. wrote the manuscript; J.-H.P., E.E.K.B., S.T., D.H.P., H.V.S., C.D.S., and A.E. supervised the research. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

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ABBREVIATIONS

AAA, aromatic amino acids; [Ch][Lys], cholinium lysinate; DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate; DHBA, protocatechuate; DW, dry weight; HCT, hydroxycinnamoyl-coenzyme A:shikimate hydroxycinnamoyl transferase; HPLC-

ESI-QTOF-MS, high-performance liquid chromatography-electrospray ionization quadrupole time-of-flight mass spectrometry; MESP, minimum ethanol selling price; MSP, minimum selling price.

REFERENCES

(1) Baral, N. R.; Dahlberg, J.; Putnam, D.; Mortimer, J. C.; Scown, C. D. Supply cost and life-cycle greenhouse gas footprint of dry and ensiled biomass sorghum for biofuel production. *ACS Sustainable Chem. Eng.* **2020**, *8*, 15855−15864.

(2) Baral, N. R.; Sundstrom, E. R.; Das, L.; Gladden, J.; Eudes, A.; Mortimer, J. C.; Singer, S. W.; Mukhopadhyay, M.; Scown, C. D. Approaches for more efficient biological conversion of lignocellulosic feedstocks to biofuels and bioproducts. *ACS Sustainable Chem. Eng.* **2019**, *7*, 9062−9079.

(3) Yang, M.; Baral, N. R.; Simmons, B. A.; Mortimer, J. C.; Shih, P. M.; Scown, C. D. Accumulation of high-value bioproducts in planta can improve the economics of advanced biofuels. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, *117*, 8639−8648.

(4) Lin, C.-Y.; Eudes, A. Strategies for the production of biochemicals in bioenergy crops. *Biotechnol. Biofuels* **2020**, *13*, 71.

(5) Mullet, J.; Morishige, D.; McCormick, R.; Truong, S.; Hilley, J.; McKinley, B.; Anderson, R.; Olson, S. N.; Rooney, W. Energy sorghum–a genetic model for the design of C4 grass bioenergy crops. *J. Exp. Bot.* **2014**, *65*, 3479−3489.

(6) Yang, M.; Dahlberg, J.; Baral, N. R.; Putnam, D.; Scown, C. D. Identifying forage sorghum ideotypes for advanced biorefineries. *ACS Sustainable Chem. Eng.* **2021**, *9*, 7873−7881.

(7) Vanhercke, T.; Belide, S.; Taylor, M. C.; El Tahchy, A.; Okada, S.; Rolland, V.; Liu, Q.; Mitchell, M.; Shrestha, P.; Venables, I.; et al. Up-regulation of lipid biosynthesis increases the oil content in leaves of *Sorghum bicolor*. *Plant Biotechnol. J.* **2019**, *17*, 220−232.

(8) Kakkar, S.; Bais, S. A review on protocatechuic acid and its pharmacological potential. *ISRN Pharmacol.* **2014**, *2014*, No. 952943.

(9) Song, J.; He, Y.; Luo, C.; Feng, B.; Ran, F.; Xu, H.; Ci, Z.; Xu, R.; Han, L.; Zhang, D. New progress in the pharmacology of protocatechuic acid: A compound ingested in daily foods and herbs frequently and heavily. *Pharmacol. Res.* **2020**, *161*, No. 105109.

(10) Liu, J.; Meng, C.; Liu, S.; Kan, J.; Jin, C. Preparation and characterization of protocatechuic acid grafted chitosan films with antioxidant activity. *Food Hydrocolloids* **2017**, *63*, 457−466.

(11) Okamura-Abe, Y.; Abe, T.; Nishimura, K.; Kawata, Y.; Sato-Izawa, K.; Otsuka, Y.; Nakamura, M.; Kajita, S.; Masai, E.; Sonoki, T.; et al. Beta-ketoadipic acid and muconolactone production from a lignin-related aromatic compound through the protocatechuate 3,4-metabolic pathway. *J. Biosci. Bioeng.* **2016**, *121*, 652−658.

(12) Otsuka, Y.; Nakamura, M.; Shigehara, K.; Sugimura, K.; Masai, E.; Ohara, S.; Katayama, Y. Efficient production of 2-pyrone 4,6-dicarboxylic acid as a novel polymer-based material from protocatechuate by microbial function. *Appl. Microbiol. Biotechnol.* **2006**, *71*, 608−614.

(13) Wu, W.; Dutta, T.; Varman, A. M.; Eudes, A.; Manalansan, B.; Loqué, D.; Singh, S. Lignin valorization: Two hybrid biochemical routes for the conversion of polymeric lignin into value-added chemicals. *Sci. Rep.* **2017**, *7*, 8420.

(14) Chen, Z.; Shen, X.; Wang, J.; Wang, J.; Yuan, Q.; Yan, Y. Rational engineering of *p*-hydroxybenzoate hydroxylase to enable efficient gallic acid synthesis via a novel artificial biosynthetic pathway. *Biotechnol. Bioeng.* **2017**, *114*, 2571−2580.

(15) Kunjapur, A. M.; Prather, K. L. J. Development of a vanillate biosensor for the vanillin biosynthesis pathway in *E. coli*. *ACS Synth. Biol.* **2019**, *8*, 1958−1967.

(16) Homma, T.; Tsurusaki, Y.; Kamimura, N.; Masai, E.; Ang, L. Z. P. Protocatechuic acid fuel cell: A sustainable energy generation system based on microbial metabolism of lignin-derived aromatic compounds. *Biomass Bioenergy* **2021**, *154*, No. 106254.

(17) Eudes, A.; Sathitsuksanoh, N.; Baidoo, E. E. K.; George, A.; Liang, Y.; Yang, F.; Singh, S.; Keasling, J. D.; Simmons, B. A.; Loqué, D. Expression of a bacterial 3-dehydroshikimate dehydratase reduces lignin content and improves biomass saccharification efficiency. *Plant Biotechnol. J.* **2015**, *13*, 1241−1250.

(18) Hao, Z.; Yogiswara, S.; Wei, T.; Benites, V. T.; Sinha, A.; Wang, G.; Baidoo, E. E. K.; Ronald, P. C.; Scheller, H. V.; Loqué, D.; et al. Expression of a bacterial 3-dehydroshikimate dehydratase (QsuB) reduces lignin and improves biomass saccharification efficiency in switchgrass (*Panicum virgatum* L.). *BMC Plant Biol.* **2021**, *21*, 56.

(19) Unda, F.; Mottiar, Y.; Mahon, E. L.; Karlen, S. D.; Kim, K. H.; Loqué, D.; Eudes, A.; Ralph, J.; Mansfield, S. D. A new approach to zip-lignin: 3,4-Dihydroxybenzoate is compatible with lignification. *New Phytol.* **2022**, *235*, 234−246.

(20) Tian, Y.; Lin, C.-Y.; Park, J.-H.; Wu, C.-Y.; Kakumanu, R.; Pidatala, V. R.; Vuu, K. M.; Rodriguez, A.; Shih, P. M.; Baidoo, E. E. K.; et al. Overexpression of the rice BAHD acyltransferase AT10 increases xylan-bound *p*-coumarate and reduces lignin in *Sorghum biolor*. *Biotechnol. Biofuels* **2021**, *14*, 217.

(21) Shih, P. M.; Vuu, K.; Mansoori, N.; Ayad, L.; Louie, K. B.; Bowen, B. P.; Northen, T. R.; Loqué, D. A robust gene-stacking method utilizing yeast assembly for plant synthetic biology. *Nat. Commun.* **2016**, *7*, 13215.

(22) Lebrun, M.; Leroux, B.; Sailland, A. Gène chimère pour la transformation des plantes. European patent application. Patent Application, EP 508909A1, 1992.

(23) Gurel, S.; Gurel, E.; Miller, T. I.; Lemaux, P. G. *Agrobacterium*-mediated transformation of *Sorghum bicolor* using immature embryos. *Methods Mol. Biol.* **2012**, *847*, 109−122.

(24) Reddy, P. S.; Reddy, D. S.; Sivasakthi, K.; Bhatnagar-Mathur, P.; Vadez, V.; Sharma, K. K. Evaluation of sorghum [*Sorghum bicolor* (L.)] reference genes in various tissues and under abiotic stress conditions for quantitative real-time PCR data normalization. *Front. Plant Sci.* **2016**, *7*, 529.

(25) Amer, B.; Kakumanu, R.; Tian, Y.; Eudes, A.; Baidoo, E. E. K. Targeted analysis of phenolic compounds by LC-MS. 2021, DOI: 10.17504/protocols.io.byhcpt2w.

(26) Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J. Determination of structural carbohydrates and lignin in biomass. In *Laboratory Analytical Procedure. Technical Report*, NREL/TP-510-42618; National Renewable Energy Laboratory: Golden, CO, 2008.

(27) Lin, C.-Y.; Vuu, K. M.; Amer, B.; Shih, P. M.; Baidoo, E. E. K.; Scheller, H. V.; Eudes, A. In-planta production of the biodegradable polyester precursor 2-pyrone-4,6-dicarboxylic acid (PDC): Stacking reduced biomass recalcitrance with value-added co-product. *Metab. Eng.* **2021**, *66*, 148−156.

(28) Eudes, A.; George, A.; Mukerjee, P.; Kim, J. S.; Pollet, B.; Benke, P. I.; Yang, F.; Mitra, P.; Sun, L.; Cetinkol, O. P.; et al. Biosynthesis and incorporation of side-chain-truncated lignin monomers to reduce lignin polymerization and enhance saccharification. *Plant Biotechnol. J.* **2012**, *10*, 609−620.

(29) Xu, F.; Sun, J.; Konda, N. V. S. N. M.; Shi, J.; Dutta, T.; Scown, C. D.; Simmons, B. A.; Singh, S. Transforming biomass conversion with ionic liquids: process intensification and the development of a high-gravity, one-pot process for the production of cellulosic ethanol. *Energy Environ. Sci.* **2016**, *9*, 1042−1049.

(30) Klein-Marcuschamer, D.; Simmons, B. A.; Blanch, H. W. Techno-economic analysis of a lignocellulosic ethanol biorefinery with ionic liquid pre-treatment. *Biofuels, Bioprod. Biorefin.* **2011**, *5*, 562−569.

(31) Humbird, D.; Davis, R.; Tao, L.; Kinchin, C.; Hsu, D.; Aden, A.; Schoen, P.; Lukas, J.; Olthof, B.; Worley, M.; et al. *Process design and economics for biochemical conversion of lignocellulosic biomass to ethanol: Dilute-acid pretreatment and enzymatic hydrolysis of corn stover*; National Renewable Energy Laboratory (NREL): Golden, CO (United States), 2011.

(32) U.S. EIA. *Electricity Power Annual 2019*; U.S. EIA, 2021.

(33) Baral, N. R.; Kavvada, O.; Mendez-Perez, D.; Mukhopadhyay, A.; Lee, T. S.; Simmons, B. A.; Scown, C. D. Techno-economic analysis and life-cycle greenhouse gas mitigation cost of five routes to bio-jet fuel blendstocks. *Energy Environ. Sci.* **2019**, *12*, 807−824.

https://doi.org/10.1021/acssuschemeng.2c01160
ACS Sustainable Chem. Eng. 2022, 10, 12520−12528

(34) Protocatechuate price at https://www.alibaba.com/product-detail/Protocatechuic-Acid-Low-Price-Protocatechuic-Acid_1600318469608.html?spm=a2700.galleryofferlist.normal_offer.d_title.5b962dadvGSp0h&s=p (accessed Dec 17, 2021).
(35) Magurudeniya, H. D.; Baral, N. R.; Rodriguez, A.; Scown, C. D.; Dahlberg, J.; Putnam, D.; George, A.; Simmon, B. A.; Gladden, J. M. Use of ensiled biomass sorghum increases ionic liquid pretreatment efficiency and reduces biofuel production cost and carbon footprint. *Green Chem.* **2021**, *23*, 3127−3140.
(36) Barros, J.; Escamilla-Trevino, L.; Song, L.; Rao, X.; Serrani-Yarce, J. C.; Palacios, M. D.; Engle, N.; Choudhury, F. K.; Tschaplinski, T. J.; Venables, B. J.; et al. 4-Coumarate 3-hydroxylase in the lignin biosynthesis pathway is a cytosolic ascorbate peroxidase. *Nat. Commun.* **2019**, *10*, 1994.
(37) Rivera-Burgos, L. A.; Volenec, J. J.; Ejeta, G. Biomass and bioenergy potential of brown midrib sweet sorghum germplasm. *Front. Plant Sci.* **2019**, *10*, 1142.
(38) Davis, R. E.; Grundl, N. J.; Tao, L.; Biddy, M. J.; Tan, E. C.; Beckham, G. T.; Humbird, D.; Thompson, D. N.; Roni, M. S. *Process design and economics for the conversion of lignocellulosic biomass to hydrocarbon fuels and coproducts: 2018 biochemical design case update; biochemical deconstruction and conversion of biomass to fuels and products via integrated biorefinery pathways*; National Renewable Energy Laboratory (NREL): Golden, CO (United States), 2018.
(39) Olson, S. N.; Ritter, K.; Rooney, W.; Kemanian, A.; McCarl, B. A.; Zhang, Y.; Hall, S.; Packer, D.; Mullet, J. High biomass yield energy sorghum: developing a genetic model for C4 grass bioenergy crops. *Biofuels, Bioprod. Biorefin.* **2012**, *6*, 640−655.
(40) Schwa, B. A. *Bioenergy Technologies Office, 'Multi-Year Program Plan'*; U.S. Department of Energy, 2016.
(41) U.S. EIA, *U.S. Regular Gasoline Rack Sales Price by Refiners (Dollars per Gallon)*, 2020.
(42) Yang, M.; Liu, D.; Baral, N. R.; Lin, C.-Y.; Simmons, B. A.; Gladden, J. M.; Eudes, A.; Scown, C. D. Comparing *in planta* accumulation with microbial routes to set targets for a cost-competitive bioeconomy. *Proc. Natl. Acad. Sci. U. S. A.* **2022**, No. e2122309119.
(43) Karlen, S. D.; Fasahati, P.; Mazaheri, M.; Serate, J.; Smith, R. A.; Sirobhushanam, S.; Chen, M.; Tymokhin, V. I.; Cass, C. L.; Liu, S.; et al. Assessing the viability of recovery of hydroxycinnamic acids from lignocellulosic biorefinery alkaline pretreatment waste streams. *ChemSusChem* **2020**, *13*, 2012−2024.
(44) Antony, F. M.; Wasewar, K. Reactive extraction: a promising approach to separate protocatechuic acid. *Environ. Sci. Pollut. Res.* **2020**, *27*, 27345−27357.
(45) Yan, J.; Aznar, A.; Chalvin, C.; Birdseye, D. S.; Baidoo, E. E. K.; Eudes, A.; Shih, P. M.; Loqué, D.; Zhang, A.; Scheller, H. V. Increased drought tolerance in plants engineered for low lignin and low xylan content. *Biotechnol. Biofuels* **2018**, *11*, 195.

https://doi.org/10.1021/acssuschemeng.2c01160
ACS Sustainable Chem. Eng. 2022, 10, 12520−12528