Assessing the Viability of Recovery of Hydroxycinnamic Acids from Lignocellulosic Biorefinery Alkaline Pretreatment Waste Streams

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The hydroxycinnamic acids p-coumaric acid (pCA) and ferulic acid (FA) add diversity to the portfolio of products produced by using grass-fed lignocellulosic biorefineries. The level of lignin-bound pCA in Zea mays was modified by the alteration of p-coumaroyl-CoA monolignol transferase expression. The biomass was processed in a lab-scale alkaline-pretreatment biorefinery process and the data were used for a baseline technology analysis to determine where to direct future research efforts to couple plant design to biomass utilization processes. It is concluded that future plant engineering efforts should focus on strategies that ramp up accumulation of one type of hydroxycinnamate (pCA or FA) predominantly and suppress that of the other. Technoeconomic analysis indicates that target extraction titers of one hydroxycinnamic acid need to be > 50 g kg\(^{-1}\) biomass, at least five times higher than observed titers for the impure pCA/FA product mixture from wild-type maize. The technical challenge for process engineers is to develop a viable process that requires more than 80% reduction of the isolation costs.

Introduction

Hydroxycinnamic acids p-coumaric acid (pCA) and ferulic acid (FA) are high-value chemicals that could be sold as coproducts from the conversion of lignocellulosic biomass into materials, commodity chemicals, and liquid fuels. These phenolic acids are present in all plants,[1, 2] albeit rarely in the free acid form; usually they are ester- or ether-linked to mono- and disaccharides,[3] betacyanins,[4] glycoproteins,[5] long chain fatty acids (waxes),[6, 7] sterols,[8] plant cell wall lignin and/or polysaccharides,[9–11] and many other soluble and insoluble components. Hydroxycinnamic acids have antioxidant, antimicrobial, anti-inflammatory, antithrombosis, and anticancer activities; they protect against coronary disease and can reduce obesity.[12–14]

They are also important chemical building blocks for dyes, sunscreen, cosmetics, nutraceuticals, and other materials appli-
With their large array of beneficial physiological activities, both pCA and FA are used widely across the food, health, chemical, pharmaceutical, and cosmetics industries.

The amount of phenolic acids in fruits and vegetables (e.g., oranges, apples, pears, grapes, bananas, and spinach) is generally <0.1 wt % of the biomass, whereas that of corn stover is approximately 0.9 wt % and that of the cobs and bran is much higher at 1–2 wt %. Commercially, pCA and FA are both produced from the sapinification of phenolic esters found in extracts or oils (metabolites) isolated from plant biomass. FA is harvested from the oil extracted from rice bran.[20] pCA is isolated in a similar fashion from soluble metabolites in *Hedyotidis diffusae* (a species of buckwheat). The production of pCA and FA is limited primarily by the abundance of the source biomass and the economics of extraction, ester hydrolysis, and isolation of the pure phenolic acids.

Linking the production of phenolic acids to the purification of lignocellulosic biorefinery waste streams reduces the minimum product selling price (MPSP) of the product(s) greatly by eliminating the cost of feedstock and the extraction of the crude product mixture. The targeting of such phenolic coproducts from biomass pretreatments (upstream of hydrolysis conversion) is often referred to as a “lignin-first” strategy.[25] Depending on the operating conditions and chemical loading, alkaline pretreatment (AP) and acid pretreatment could be optimized as a lignin-first process.[22–24] Alkaline pretreatment is used to extract a substantial fraction of the biomass into an alkaline pretreatment liquor (APL). This APL contains most of the metabolites, some of the hemicelluloses, and, from grasses, a large portion of the lignin.[25] Alkaline pretreatment hydrolyzes esters to release monomeric hydroxycinnamates, such as pCA and FA, by “clipping” them off cell-wall polymers, such as lignin and hemicelluloses.[26]

Many lignocellulosic biorefineries are designed to process grasses (e.g., sugarcane, corn stover, and energy sorghum) that contain FA bound predominantly to the hemicelluloses,[27] pCA bound to the hemicelluloses and lignins,[28,29] and esters of the hydroxycinnamates in the soluble metabolite pool.[21,23,24] As mentioned previously, the design of biorefineries is often based on potentially lignin-first approaches, for example, the physical separation of the sugarcane bagasse from the hydrolysate stream from acid or alkaline pretreatment (corn stover and energy sorghum). Even without modification of the current operating conditions, a pure hydroxycinnamate could be isolated from some of the waste streams, although an economically viable method still needs to be developed. If multiple hydroxycinnamates are present, the purification strategy cannot be based solely on (re)crystallization, as mixtures of pCA and FA co-crystallize. Previous studies have shown that the separation costs, and especially capital costs, increase sharply with lower titers.[30–35] Thus, to keep the capital investment for the envisioned biorefinery low (2000 Mg day⁻¹), it is crucial to structure the downstream processes to funnel the complex mixture to products that can be isolated economically.[26,36–42]

An alternative approach is to engineer bioenergy feedstocks to produce high levels of a single hydroxycinnamate. This would require expressing, overexpressing, or coexpressing genes to increase the level of one hydroxycinnamate and suppress or knock out genes associated with accumulation of the other hydroxycinnamates simultaneously. There are two main categories of these acyl-CoA transferases (AT) that are directly responsible for the amount of cell-wall-bound pCA and FA in monocots. These are: 1) transferases (PMT and FMT) that attach hydroxycinnamoyl-CoA thiosters to monolignols (p-hydroxycinnamyl alcohols), which in turn become incorporated into the lignin, and 2) those that attach hydroxycinnamoyl-CoA thiosters to arabinosyl units in the hemicellulosic arabinoxylans.

P-Coumaroyl-CoA monolignol transference (PMT), also known as pCAT was the first of these transferases identified, originally in rice (*Oryza sativa*)[43] and maize (*Zea mays*).[44] The analogous transferences for FA-CoA proved more elusive and, in the case of feruloyl-CoA monolignol transference (FMT), more diverse.[45] Two different classes of FMT genes have been identified, the first from *Angelica sinensis* (AsFMT)[46] and the second from rice (*OsaFMT, also referred to as OsATS*).[47] The enzyme that acylates hemicelluloses with pCA has been identified in rice (*OsaAT10*)[47] and reported more recently in switchgrass (*Panicum virgatum*).[46] The AT that forms the arabinose-ferulate linkages found in grass hemicelluloses, and leads subsequently to diferulates during the crosslinking of cell walls,[46] has only been identified putatively in *Setaria viridis* and *Brachypodium distachyon*,[50] but the quest to identify orthologs is underway. There are many other acyltransferase genes that encode enzymes that form plant metabolites that contain pCA and FA esters, such as glucoside esters that plants produce as a means of detoxification.

The second factor that limits the economic viability is that pretreatment [alkaline, acidic, organosolv, ammonia fiber expansion (AFEX), and even steam explosion] extracts other biomass constituents, such as ash, carbohydrates, soluble sugars, lignin oligomers, proteins, metabolites, and waxes. These additional constituents render the extraction liquor into a black solution of viscous waxy sludge. The first step to recover pCA and FA from these pretreatment liquors is, therefore, to separate them from the dissolved wax and oligomer sludge. In an industrial-scale biorefinery, potential processes that could be used to isolate pCA and FA include distillation, membrane separation, adsorption, and extraction.[33,31,52] pCA and FA are (hot-)water-soluble, nonvolatile chemicals; distillation of the water of the APL would, therefore, yield a concentrated mixture of pCA, FA, and waxes/oligomers. The distillation of pCA and FA to pure products is not viable as they decarboxylate thermally to form 4-vinylphenol and 4-vinylgualacol.[53] Furthermore, the distillation of an APL would require the evaporation of a large portion of water, which would increase energy costs significantly. Alternatively, membranes can be used to separate pCA and FA as permeates or to concentrate them by the dehydration of the APL.[54] However, viscous waxes and oligomers in the APL could plug the membrane pores to reduce the operating life of the membranes, which would increase membrane regeneration and replacement costs substantially. The energy costs of pervaporation or vapor permeation processes to dehydrate dilute streams becomes prohibitively expensive.[36,56] Activated carbon (AC) loaded at 0.2 kg AC kg⁻¹ of stover removes waxes/
oligomers from the APL; however, the AC also removes approximately 15% of the pCA and FA, which is then not recoverable. There is also a significant capital and energy cost to regenerate the large load of AC.

Switching from dedicated pCA/FA production facilities to a lignin-first lignocellulosic biorefinery introduces several factors that are currently prohibitive to the economic viability of the production of pCA and FA as part of the biorefinery’s product portfolio. The first factor is that pCA and FA represent a small fraction of the biomass, which leads to low concentrations (titer) of these chemicals in the various waste streams. Conceptually, the simplest solution to these low titers is to use better feedstocks that contain pCA or FA levels similar to those found in *H. diffusae* and rice bran, respectively. The feedstocks would also need to retain the biomass composition required for the rest of the biorefinery’s processes (i.e., abundant biomass that is high in cellulose, hemicelluloses, starch, and glucose and low in toxins that impact downstream processes negatively).

One of the most economically viable processes for the recovery of pCA or FA from APL, if it produced a fairly pure product, would be direct precipitation as a result of the cooling of the APL to room temperature, precipitation during acid-neutralization, or a combination of the two, before wastewater treatment. Unfortunately, the dilute nature of the APL and the presence of other impurities prevents this from working. Alternative approaches are, therefore, needed to isolate pure products. Solvent extraction is one of the least expensive in terms of capital equipment, residence time, and scalability. Within this area of purification there are a lot of different approaches, which include variation of the extracting solvent(s), pH of the solution, and the sequence of the steps involved. Variations of the extraction strategies revolve around some general principles: 1) higher solution alkalinity increases the solubility of hydroxycinnamic acids and waxes/oligomers; 2) sugars have poor solubility in alcohol (ethanol or methanol); 3) hydroxycinnamic acids are less soluble at lower temperatures. Here, we designed a process (Figure 1) based on several isolation strategies reported previously\[^{20,21,24,51,52,54}\] based around these general principles and assessed the economic viability of the approach. The process includes: 1) neutralization of the alkaline supernatant using sulfuric acid; 2) ethanol extraction to separate the impurities (waxes and oligomers) from the pCA and FA; 3) recovery and recycling of the ethanol; 4) acidification of the neutral aqueous solution to precipitate pCA and FA; and 5) acid neutralization using caustic soda before the chemically cleaner waste stream is sent to wastewater treatment. Neutralization is mandatory to prevent the disruption of the function of microbes in the anaerobic and aerobic digesters of the wastewater treatment facility.

In this study, we ignore the purity of pCA and FA and assume that in a viable system the plants would be engineered to have primarily pCA or FA, not both. We focused on changing the expression level of PMT and the subsequent role that lignin-bound pCA has on the technoeconomics of the production of pCA and FA from an APL stream. Three *Zea mays* lines were engineered to have altered PMT expression: *ZmPMT* RNAi suppression (*Zm7*), *ZmPMT* overexpression (*Zm9*), and ectopic expression of *Brachypodium distachyon* BdPMT1 (*Zm10*). These lines were field-grown and subjected to lab-scale biorefinery conditions coupled with pCA and FA recovery. We assessed changes in viability (extreme differences in fungal or insect susceptibility), changes in the fermentation toxins produced during pretreatment, toxicity of the resulting hydrolysate, and the amount of pCA and FA recovered from the APL streams. The data from this plant-forward study was then used to establish a baseline technoeconomic analysis for this lignin-first strategy to determine where to direct our future research efforts. The results of the technoeconomic analysis provided a blueprint for which factors to change for the process to advance from a > $5.05 kg\(^{-1}\) minimum product selling price (MPSP) to a more cost-competitive < $1 kg\(^{-1}\) MPSP.

**Figure 1.** Process flowsheet for pCA and FA recovery from alkaline pretreatment liquor.
Results and Discussion

Genetic engineering of Zea mays

As a starting point to merge research efforts in plant engineering, biorefinery design, and product portfolio composition, we focused on the genetic engineering of corn (Zea mays) stover to enhance the production of high-value chemicals from the pretreatment liquor, all while still converting the sugar into a liquid fuel (ethanol). As most biorefineries will operate on dried biomass (stored) over the course of the year, our plant engineering efforts focused on the cell-wall-bound components and not the soluble metabolites that might degrade upon long-term storage. We up-regulated the ZmPMT gene to increase the amount of pCA bound to the lignin, with the goal to increase the amount pCA produced per ton of corn stover. As a control, we used the RNAi suppression of ZmPMT to reduce the amount of lignin-bound pCA, a strategy demonstrated previously.\(^4\) In another study, Petrik et al. demonstrated that the amount of lignin-bound pCA in Brachypodium distachyon could be either increased (overexpression of BdPMT) or almost completely eliminated (Bdpmt knockout mutant), without significant impact on biomass yield, digestibility, or hemicellulose composition (arabinoxylan-bound pCA and FA)\(^5\). In addition to the augmentation of the expression of the native ZmPMT, we generated transgenic maize plants that express BdPMT, the transferase homolog from Brachypodium. The plants were grown in outdoor field trials that exposed them to pathogens, insects, drought, and flood stresses and the changing of the seasons to ensure that the biomass processed in our lab-scale biorefinery was representative of stover that would be used industrially.

We identified the “best” lines from the progeny of ten different transformation events for each of the three genetic constructs [ZmPMT RNAi (Zm7), ZmPMT overexpression (Zm9), and BdPMT expression (Zm10); all driven by the ZmUBI1 promoter] after they were backcrossed into the inbred B73 line. The best transgenic lines were first flagged by Basta selection (wild type versus transgenic; see the Supporting Information, Figure S2), followed by real-time semi-quantitative polymerase chain reaction (RT-qPCR) of leaf tissue harvested after tasseling (Figure S3). The top two candidate events for each construct were screened by performing the mild-alkaline hydrolysis of biomass (with extractives) for changes in total pCA in the rind tissue of the third internode from the bottom of the stem. The difference in the amount of pCA (or FA) detected by using mild-alkaline hydrolysis between the transgenic and wild-type (WT) plants was smaller than that observed for extract-free biomass previously on similar maize lines grown in the greenhouse.\(^6\)

We hypothesize that the smaller change in pCA between the lines may relate to the growth of the plants under field conditions (replete with insects, pathogens, wind, and unfiltered sunlight) and the corresponding production of metabolites for defense, structural lignification, and UV protection. For example, the expression of PMT is suppressed upon long-term attack by corn borer caterpillars (Sesamia nonagrioides),\(^7\) and yet the amount of cell-wall-bound pCA increases.\(^8\) This stress response results in less pCA on the lignin and more arabinose-bound pCA in the hemicellulosic fraction. A similar stress response was observed in Brachypodium distachyon callus grown hydroponically and stimulated with methyl jasmonate, a plant stress-response hormone.\(^9\)

Biomass and APL compositional analysis

To determine the change in lignin-bound pCA, we assayed isolated lignin from the “best” lines by using 2D HSQC NMR spectroscopy (Table S4). The assays showed a strong correlation between the level of PMT expression and the amount of lignin-bound pCA. To quantify the changes, we performed derivatization followed by reductive cleavage (DFRC) lignin compositional analysis on the best expression line for each of the constructs Zm7, Zm9, and Zm10 (Figure 2). The suppression and overexpression lines (Zm7 and Zm9) performed as expected, whereas the introduction of BdPMT resulted in a stark decrease in lignin-bound pCA. To our knowledge, this is the first example in which the introduction of a second PMT, or any monolignol transferase gene, resulted in a decrease of monolignol p-coumarates. We hypothesize that this might be caused by triggering an unintended pathway that detoxifies the cell by removing or decomposing excess ML-pCA.

After we identified lines with differences in pCA on the lignin, we pooled the rest of the above-ground plant biomass (stover without the cob, tassel, third internode, or seventh leaf) for each engineered line and their associated wild-type siblings (six independent pools). These pools of dried plants were ground, homogenized, characterized, and used in our lab-scale biorefinery study with no further preparation.

The first step of the lab-scale biorefinery was the AP step.\(^2\) The AP process hydrolyzes ester bonds to release free pCA and FA from metabolites such as monosaccharides, oligosaccharides, glycerol, organic acids, and polymers such as arabinoxylans and lignin. We used moderate AP conditions to remove approximately 40% of the biomass without degrading the remaining polysaccharides (Table S5 and Figure S4). Compositional analysis of the corn stover before and after AP indicated that the majority of the biomass loss was from extracted lignin (43% decrease) and ash (65% decrease) content; however, approximately 7% of the sugars was also removed. Surprisingly, the AP stover contained only slightly lower amounts of total pCA and approximately the same amount of total FA as the untreated stover (Figure 2 and Table S6). DFRC analysis of the AP stover was used to reveal an approximately 50% reduction in lignin-bound pCA, and the wild-type, ZmPMT RNAi, and ZmPMT overexpression lines all reached fairly similar levels (2.7–3.2 g kg\(^{-1}\) stover) of lignin-bound pCA (Figure 2 and Table S6). The lignin-bound pCA content of the BdPMT expression line also decreased similarly by approximately 50% to 1.5 g kg\(^{-1}\) stover half the amount of the three other lines and reflective of the lower initial pCA content of the BdPMT expression line. This indicated that most of the AP-extracted pCA and FA could be attributed to the free and lignin-bound fractions.

The APL from the wild-type plants lines contained approximately 19 g kg\(^{-1}\) stover of a mixture of extractables (Table 1). The

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purification of this mixture by silica-gel chromatography yielded pure pCA (4.9 ± 0.2 g kg⁻¹ stover), FA (4.3 ± 0.3 g kg⁻¹ stover), and a large amount of a mixture of other compounds (which includes diferulates, waxes, oligosaccharides, and polysaccharides; Table S5 and Figure S4). The isolated yield of pCA was fairly consistent between the wild-type, ZmPMT RNAi, and ZmPMT overexpression lines, with levels of 4.6–5.2 g pCA kg⁻¹ stover. By contrast, the yield of pCA in the BdPMT expression line decreased to 3.3 g pCA kg⁻¹ stover (Table 1), which is reflected in the decrease in cell-wall-bound pCA (Figure 2). The recovered FA levels were much more variable with wild-type lines in the range of 3.7–4.6 g FA kg⁻¹ stover and the ZmPMT overexpression and BdPMT expression lines that released 2.7 and 3.7 g FA kg⁻¹ stover. The “purity” of pCA, determined by the ratio of pCA/FA, in the APL streams measured from the isolated yield (NMR yield of APL in parentheses) followed the trend in cell-wall-bound pCA for the plant lines. Plant lines with low levels of pCA (ZmPMT RNAi and BdPMT expression) had 58% (59%) and 47% (53%) pCA, whereas the wild-type pCA level was 53% (61%), and the ZmPMT overexpression line was the purest at 65% (67%) pCA (Table S5).

Changes in the pCA and FA content in lignin can have an effect on the hydrodynamic volume (molecular weight profile) of the polymers. Cleavage of the lignin-bound pCA and fragmentation of monolignol ferulate crosslinks should reduce the hydrodynamic volume and shorten the average chain length of the lignin polymer.[46] Enzyme digestion further decreases the polymer chains by chopping up the lignin-bound polysaccharides. The enzyme lignins (produced as described in the Experimental Section) from each stage of the processing were pooled and changes in their molecular weight were analyzed. As expected, the average trend from the molecular weight profiling was untreated > alkaline pretreated > enzyme residue (Figure S5 and Tables S5, S6, and S8). There was no significant difference in the molecular weight profiles of the enzyme lignins between the different plant lines (Zm7, Zm9, Zm10, or the three WTs) at any stage of processing. The untreated biomass had the largest polydispersity followed closely by the alkaline pretreated samples. The alkaline pretreated samples had a slightly lower molecular weight as expected as a result of the clipping-off of pCA and some fragmentation of the polymer chains. The enzyme residue had the smallest polydispersity and the highest peak molecular weight, which indicates that this tissue (the recalcitrant rind of the stover) had the largest amount of lignin polymers.

Viability of hydrolysates

Although the presence of pCA and FA in plant metabolites and the cell wall represents a potential opportunity to extract extra value from the plant in a biorefinery, pCA and FA are known toxins to yeast during fermentation.[64] The efficiency of the alkaline pretreatment step to remove these toxins is, therefore, potentially integral to fermentation success, especially in plant lines with higher levels of pCA. LC–MS analysis of the hydrolysates produced by enzymatic digestion of the pretreated corn stover was used to detect low levels of fermentation toxins (e.g., pCA and FA), compared to similar hydrolysates produced from AFEX-pretreated corn stover[65] (which, in contrast, produced toxic compounds, such as p-coumaroyl amide and feruloyl amide; Table S7). The hydrolysate product was approximately 6% glucan for all plant lines. The relative abundance of the toxins was similar across the plant lines, which indicates that AP was effective at extracting the pCA- and FA-containing metabolites from the biomass but was only partially able to
Table 1. APL composition determined from the isolated yield following ethyl acetate extraction and silica-gel column chromatography. Primary sugar composition of the hydrolysate derived from AP-corn stover and the result of the subsequent fermentation. Values represent the average of two technical replicates.

|          | WT (average) | ZmPMT (RNAi) | ZmPMT (OX) | BdPMT (OX) |
|----------|--------------|--------------|------------|-------------|
| p-CA [g kg⁻¹] | 4.9 ± 0.2 | 5.1 | 4.9 | 3.3 |
| FA [g kg⁻¹] | 4.3 ± 0.3 | 3.7 | 2.7 | 3.7 |
| Wax, oligomers, and diferulates [g kg⁻¹] | 10.0 ± 0.4 | 4.6 | 4.4 | 11.9 |
| Total black sludge [g kg⁻¹] | 19.3 ± 0.6 | 13.4 | 11.9 | 18.9 |
| [pCA]:[FA] | 53:47 | 58:42 | 65:35 | 47:53 |
| (isolated product) | 61:39 | 60:40 | 67:33 | 53:47 |
| (NMR of APL) |  |  |  |  |

Hydrolysate composition

|          | WT (average) | ZmPMT (RNAi) | ZmPMT (OX) | BdPMT (OX) |
|----------|--------------|--------------|------------|-------------|
| Glucose [mm] | 359 ± 6 | 360 ± 5 | 352 ± 22 | 348 ± 11 |
| Xylose [mm] | 166 ± 3 | 179 ± 1 | 147 ± 4 | 172 ± 4 |
| Pyruvate [mm] | 15 ± 6 | 23 ± 16 | 25 ± 18 | 24 ± 17 |
| Lactate [mm] | 21 ± 5 | 57 ± 7 | 8 ± 6 | 37 ± 17 |
| Acetate [mm] | 13 ± 1 | 11 ± 1,0 | 10 ± 0,1 | 11 ± 0,5 |
| Ethanol [mm] | 7 ± 1 | 8 ± 2 | 6 ± 0,4 | 7 ± 0,1 |

Fermentation results

|          | WT (average) | ZmPMT (RNAi) | ZmPMT (OX) | BdPMT (OX) |
|----------|--------------|--------------|------------|-------------|
| Growth rate [h⁻¹] | 0.07 ± 0.00 | 0.08 ± 0.02 | 0.06 ± 0.01 | 0.07 ± 0.03 |
| Glucose rate of consumption [g L⁻¹ (OD600 = 1) h⁻¹] | 2.26 ± 0.15 | 2.42 ± 0.23 | 2.18 ± 0.09 | 2.23 ± 0.22 |
| Xylose rate of consumption [g L⁻¹ (OD600 = 1) h⁻¹] | 0.14 ± 0.01 | 0.16 ± 0.04 | 0.14 ± 0.04 | 0.17 ± 0.02 |
| Process ethanol yield [%] | 79 ± 5 | 79 ± 3 | 77 ± 6 | 79 ± 4 |

Technoeconomic analysis

To determine how economically competitive our biorefinery and engineered plant lines are, we performed a technoeconomic analysis of our system. To provide the most conservative economics for the recovery of a mixture of solid pCA and FA (referred to as “product” hereafter) from the APL, we modeled an independent plant that produces >7 Mg day⁻¹ of product (Figure 1). The MSP, which can be viewed as the break-even price, consists of a combination of the total capital investment (TCI) and operating costs and is reported as per kg of product (Table 2). Our process was calculated to have a MSP of $5.05 kg⁻¹ product. The ethanol distillation column was the main capital investment at $5 million (86%) of the TCI (Table S2). The TCI contributes $0.37 kg⁻¹ product to the MSP, which is relatively small compared to the cost of utilities and raw materials (Figure 3). The operating costs (fixed and variable costs; Table S3) show that utilities and raw materials have the largest impact on the MSP at $3.1 and 1.4 kg⁻¹ product (Figure 3).

Our simulations found that a biorefinery that processes 2000 Mg day⁻¹ of dry corn stover required a flow of 89850 kg (113835 kL) of ethanol per hour to achieve the 30%
ethanol concentration in the APL needed to precipitate waxes/oligomers. If we consider the high ethanol flow rate at the 2014 wholesale price of $2.22 per gallon ($586.46 kL),\textsuperscript{66} efficient recycling of the ethanol is essential. Therefore, minimization of the ethanol losses to less than 0.1% requires a large boil-up ratio in the reboiler of the distillation column. This increases the total heat requirement in the reboiler to 85.5 MW, which is approximately twice as much as the total heat required to dehydrate ethanol to 99.5% from fermentation broth in the National Renewable Energy Lab’s biorefinery design.\textsuperscript{67} The simulation results also show that low-pressure steam at $16.8 million per year ($3.0 kg\textsuperscript{-1} product) contributes to 99% of the total utility cost at the ethanol distillation column. The remaining utility cost comes from electricity purchase at $0.18 million per year ($0.03 kg\textsuperscript{-1} product) required for air-cooled condensers.

The main raw material costs are attributed to caustic soda, sulfuric acid, and ethanol, which are $4.1, 3.3, and 0.56 million per year, respectively. The caustic soda neutralizes acids in the second stage acidification reactor before the liquid stream is sent to wastewater treatment, a step that is required to maintain efficient operation of digesters in the wastewater treatment facility. Approximately half of the total sulfuric acid is consumed in the first acidification reactor to neutralize the APL and the other half in the second acidification reactor to form pCA and FA crystals.

The current market price of pCA and FA starts from $1 kg\textsuperscript{-1}.\textsuperscript{68} It is expected that the price of these chemicals will decrease upon the successful production of pCA and FA in biorefineries. Therefore, given that the costs of the further refinement and separation of pCA and FA crystals are not considered in this study, the recovery of pCA and FA from APL using the proposed process is not economically attractive. However, the cost (or even the need) for further refinement can be reduced or eliminated by engineering plants to produce only one type of phenolic compound rather than both pCA and FA, such as in Hedyothis diffusa.

In our model, we chose a conservative approach in which the economics of pCA and FA recovery were independent from the biorefinery and thus the raw materials and utilities were purchased separately. However, it is likely that significant savings are available through integration of the utilities with the rest of the biorefinery. These synergies would be specific to a given biorefinery and thus were not modeled. We considered scenarios in which significant waste heat from other parts of the biorefinery are available to envision a significant decrease of the utility cost for the recovery of the ethanol. As this cost alone represents $3.0 kg\textsuperscript{-1}, significant savings could be realized with a corresponding reduction in the MPSP. Similarly, if we double the amount of product in the APL, the MPSP is decreased by approximately 50% to $2.53 kg\textsuperscript{-1}. To better understand the impact of the separation cost and product concentration on the MPSP, single-point sensitivity analyses were performed from the results of this study (base recovery cost: $7.41 kg\textsuperscript{-1} product, base concentration: 0.0035 kg\textsuperscript{-1} APL). The product concentration should be increased by more than five times, if the total separation cost remains the same as that in the base case, to reach a MPSP below $1 kg\textsuperscript{-1} (Figure 4). Alternatively, the separation cost must be reduced by approximately 80% to reach a MPSP below $1 kg\textsuperscript{-1} for the base concentration. This would require a change of the isolation technique to eliminate the expensive ethanol precipitation of waxes/oligomers and the subsequent distillation and recovery of ethanol. Perhaps with some method development, higher titers, and process optimization, a selective countercurrent (liquid–liquid) extraction could be an economically viable replacement for the cost-prohibitive ethanol precipitation and distillation process. Recently, such an approach was demonstrated to isolate pCA, resveratrol naringenin, and naringin from fermentation residue.\textsuperscript{69}
To improve the economic prospect of pCA and FA recovery from APL, we contend that future research should be aimed at: 1) the genetic engineering of biomass to produce a single type of phenolic acid at higher concentrations and stored in the soluble metabolite pool; 2) improvement of the efficiency of hydroxycinnamates extraction without the degradation of the polysaccharides; 3) development of technology that further reduces the cost of phenolic acid recovery and purification.

Conclusions

The presence and ease of clipping the hydroxycinnamates, p-coumaric acid (pCA) and ferulic acid (FA), from grass biomass presents an exciting opportunity to derive additional value from plant material processed within lignin-first biorefineries and facilitate the move away from fossil sources to more sustainable practices. The wealth of genetic resources available currently makes it possible to upregulate and downregulate the production of hydroxycinnamates with relative ease and even to engineer plants that produce only pCA or FA (e.g., through CRISPR/Cas9 knockouts). We demonstrate that alkaline pretreatment removes hydroxycinnamates efficiently from the biomass and generates a product stream that is viable for enzymatic digestion and fermentation without causing toxicity, regardless of the initial level of pCA or FA in the biomass. At this point, the low concentration and high background matrix present in the alkaline pretreatment stream coupled to the low value of bulk chemicals prevents the economic viability of the clip-off process described here as a stand-alone process. However, synergistic savings from the coupling of a biorefinery to the alkaline pretreatment liquor recovery process reduce the production costs greatly. We recommend that future research targets processes that generate streams from the pretreatment or aspects of the biorefinery with hydroxycinnamate titers that are \( > 50 \text{ g kg}^{-1}\text{biomass} \) (five time higher than our current titer of around \( 10 \text{ g kg}^{-1}\text{biomass} \)). Processes should focus on the production of either pCA or FA by engineering plant lines to contain a single type of hydroxycinnamate or by designing a pretreatment processes that separates the hydroxycinnamates, and by designing purification (isolation) processes that cost at least 80% less. Alternatively, processes that produce mixtures of hydroxycinnamates are more suitable to downstream microbial conversion into higher value chemicals and materials. It is our belief that these suggestions and benchmarks should enable the overall process to be cost-competitive at below current market price and add value to the overall biorefinery.

Experimental Section

Expression construct assembly

The cloning of the BdPMT (Bradi2g36910) Open Reading Frame (ORF) into pENTR2B was as described in Petrik et al.\[29\]. Subsequently, the BdPMT ORF was Gateway-cloned into the pANIC6E vector\[30\] by an LR Clonase recombinase reaction as per the manufacturer’s instructions to result in the ZmUBI1 promoter-driven BdPMT expression construct Zm10. PCR amplification of the 1.3kb ZmPMT (Zm00001d024763; LOC100283365; GRMZM2G081049.1) ORF was performed using the maize cDNA clone Zm_BFC0019F21 (Genbank accession number BT042717.1) as template obtained from the Arizonan Genomics Institute\[31\] and primer pair ZmPMT.ORF_BamHI_F and ZmPMT.ORF_XbaI_R (Table S1). These primers contained a BamHI and a Xbal restriction site, respectively, to allow for restriction digestion and ligation of the PCR product into pENTR2B, after which the ZmPMT ORF was Gateway-cloned into the pANIC6E overexpression binary vector to result in ZmUBI1 promoter-driven ZmPMT expression construct Zm9. To generate the ZmPMT RNAi binary construct Zm7, the above-described ZmPMT ORF pENTR2B clone was used as a template to PCR-amplify 251 bp of the ZmPMT ORF using primer pair ZmPMT_3’RNAi_BamHI_F and ZmPMT_3’RNAi_NotI_R (Table S1; the latter primer was located adjacent to the ZmPMT stop codon). These primers contained a BamHI and a NsiI restriction site, respectively, to allow for restriction digestion and ligation into pENTR2B, after which the fragment was Gateway-cloned into two locations that flank a linker in the pANIC6E RNAi binary vector\[30\], which resulted in the ZmUBI1 promoter-driven ZmPMT RNAi construct Zm7. All cloned coding sequences and linker regions were sequence verified. The constructs were transformed into Agrobacterium tumefaciens strain AGL1, cultures of which were used to transform immature embryos of maize hybrid Hi II kernels (described below).

Transformation, growth, selection, and genotyping of the transgenic lines

Immature embryos of hybrid Hi II were transformed using Agrobacterium strain AGL1 as described by Frame et al.\[2\]. The transformed plants were grown in a greenhouse and crossed to the inbred B73 maize line to develop a T1 generation. The T1 plants for ZmPMT RNAi (Zm9) were crossed back to the inbred B73 to develop a T2 generation. T1 (Zm7 and Zm10) and T2 (Zm9) generation seeds were planted in a one-row plot (3.8 m long and 0.76 m apart) in field trials in June 2016 at the West Madison Agricultural Research Station in Madison, WI (Figure S1). Transgenic plants were distinguished from their non-transgenic siblings (WT controls) by resistance to 1% (v/v) Basta herbicide applied to the tip of the seventh leaves with a paintbrush (Figure S2).

Basta-screened samples were further screened by PCR for the presence of the plasmid construct using the REDExtract-N-Amp kit following the manufacturer’s instructions (Sigma, St. Louis, MO). PCR for genotyping was performed by using REDExtract-N-Amp PCR ReadyMix in a 20 µL reaction volume. A 2 µL aliquot of the extract was used as the template, and the cycling conditions were: 94 °C for 3 min, followed by 35 cycles of 94 °C for 20 s, 56 °C for 20 s, and 72 °C for 20 to 40 s (which depended on the size of the amplified product) and final extension for 10 min at 72 °C. The primers utilized for amplification are listed in Table S1. Amplification products were visualized by using electrophoresis in a 2% agarose gel.

The ninth leaves of the plants were harvested as green tissue and flash frozen with liquid nitrogen for the semiquantitative polymerase chain reaction (sqPCR) assay (Figure S3), as described below. The rest of the plant was harvested in October 2016 and dried at 60 °C for 10 days. The second internodes up from the ground were used in an array of analytical assays (mild-alkaline hydrolysis, digestibility, NMR spectroscopy, and DFRC) to identify the “best” plant lines. The remaining stover (without cob or tassel) from the best transgenic line for each RNA construct, as determined from the combination of analytical assays, was Wiley-milled,
homogenized, and used in bulk alkaline pretreatments for the pCA and FA clip-off feasibility study, enzyme digestion, fermentation, DFRc, and gel-permeation chromatography.

RNA extraction, reverse transcription, and sqPCR
Leaf tissue from transgenic plants (RNAi and Overexpression ZmPMT and BdPMT in Z. mays) were screened for expression by using reverse transcription (RT) sqPCR. RNA was extracted from leaf tissue by using a GeneJet Plant RNA Purification Mini Kit (ThermoFisher, NJ). Briefly, leaf tissue (ca. 1 cm) was frozen in liquid nitrogen and ball-milled in 2 mL microfuge tubes that contain three 3 mm steel beads by using a Qiagen TissueLyser (tube holder pre-cooled to −80 °C) for 30 s at 30 Hz then mixed with the Mini Kit lysis buffer. The manufacturer’s instructions were followed to obtain total RNA, and its quality was assessed by using electrophoresis on 1% agarose gel. Total RNA (0.5 µg) was treated with DNase I for 1 h at 42 °C to remove contaminating DNA. Samples were then utilized for cDNA synthesis using oligo (dT)18 and Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT, Promega, WI) following the manufacturer’s instructions.

The prepared cDNA (100 ng) was utilized as the template in each sqPCR reaction. sqPCR was performed using the primers listed in Table S1 and GoTagq Flexible DNA Polymerase according to the manufacturer’s instructions in a 20 µL reaction volume (Promega, WI). The thermal cycling conditions were: 96 °C−1 min followed by 30 cycles of 96 °C for 20 s, 55 °C for 20 s, 72 °C for 20 s, and a final extension at 72 °C for 10 min for the ZmPMT RNAi lines. Similar thermal cycling conditions were used for the other two lines with the number of cycles adjusted to 33 for the ZmPMT over-expression lines and 28 for the BdPMT expression lines. Z. mays GAPDH (GRMZM2G180625) was utilized as the expression control. PCR products were visualized by using electrophoresis in a 2% agarose gel.

Alkaline pretreatment conditions
Corn stover pretreatment
Corn stover was pretreated with alkali according to the method described by Wang and Cheng[63] with slight modification. Briefly, corn stover (approximately 200 g) was mixed with hot (80 °C) aqueous sodium hydroxide (1 wt% NaOH, 2 L) in sealed serum bottles and pretreated at 80 °C for 1 h. After pretreatment, the biomass solids were separated from the alkali liquor by filtration, and the filtrate was collected for lignin analysis. The solid residue was washed thoroughly with water and then dried at 105 °C for 12 h, cooled, and stored until it was converted into hydrolysate by enzymatic digestion.

Biomass solids component analysis
Total solids of the untreated, alkaline pretreated, and enzymatically digested corn stover were determined according to NREL/TP-510-42621.[74] Ash was determined according to NREL/TP-510-42622.[74] Carbohydrates and lignin were determined according to our previously modified method[62] and NREL/TP-510-42618,[62] respectively. The measured components accounted for 70–75% of the biomass.

Quantification of the 4-hydroxycinnamate content of the APL
The APL filtrates were cleared by pelleting the suspended fine particles (3345 × g, 7 h, RT), and the APL was decanted. After acidification to pH 1 with concentrated hydrochloric acid (ca. 35 wt%, ca. 30 mL), the brown solution was again pelleted (3345 × g, 30 min, RT). The acidic water solution was decanted, and the organics were extracted with ethyl acetate (1 × 400 and 2 × 200 mL). The combined organic layers were washed with brine (1 × 100 mL), dried over anhydrous sodium sulfate, filtered, and the solvent was removed under vacuum. The resulting dark-red oil contained a mixture of pCA and FA, which was separated using silica-gel chromatography using a binary gradient of A) toluene with 2% acetic acid and B) ethanol with 2% acetic acid. The gradient was ramped from 2% B to 10% B over 10 column volumes. The isolated product levels for each line are reported in Table 1.

Hydrolysate production by enzyme digestion
Hydrosylates were produced from the AP-corn stover by enzymatic digestion, as described previously for AFEX-pretreated corn stover[65] with the following modification. As a result of the low density of AP-corn stover, two loadings were employed (4% glucan loading for the initial loading and 2% glucan loading for the second loading after about 16–20 h hydrolysis). Approximately 220 mL water and 40 g AP-corn stover (4% glucan) were added into a 1 L DasGip Parallel Bioreactor Systems (DasGip BioTools, LLC., now part of Eppendorf North America, Hauppauge, NY), and then autoclaved at 121 °C for 2 h. After cooling, 47.25 mg 1-glucan of CTe2 and 13.5 mg 1-glucan HTe2 enzymes (Novozymes, Franklinton, NC, USA) were added and mixed by stirring at 700–1000 rpm for 16–20 h at 50 °C. Concentrated hydrochloric acid was added to achieve a pH of 5–5.5, and the second 2% glucan-loading AP-corn stover (ca. 20 g) was added into the bioreactor along with treicycline to control contamination (final concentration of 12.5 µg/mL[77]). The mixture was digested for an additional 6 days at 50 °C, and concentrated hydrochloric acid (total 0.8 mL) was added to achieve a pH of 5–5.5. After enzyme digestion, the solids (ED-solids) were pelleted by centrifugation (8200 × g at 4 °C for 10–12 h). The supernatant was decanted and filter-sterilized sequentially through 0.5 µm glass filters (GVS North America, Sanford, ME) and 0.2 µm 1 L Filter Units (WWR, Radnor, PA), and stored at 4 °C until use. A typical production run used roughly 60 g of AP-pretreated corn stover (at 6% glucan loading) and generated about 200 mL of hydrolysate.

Fermentation
Engineered xylose-utilizing S. cerevisiae Y128[77] was used for comparative fermentations for these different corn stover feedstocks. Starter cultures for inoculation were prepared as described previously.[65] Fermentations were conducted in 0.25 L MiniBio bioreactors (Applikon Biotechnology, Foster City, CA, USA) that contained 100 mL of hydrolysate. The starter cultures were centrifuged, and cells were resuspended and then inoculated into each vessel to give an initial OD600 (optical density at 600 nm) of 0.5 in the bioreactor. Fermentations were conducted at 30 °C with continuous stirring (500 rpm) and sparging (20 mL min−1; 100% N2). During the fermentation, the pH was controlled at 5.0, and samples were removed periodically from the bioreactor for an OD600 measurement to monitor cell growth and for HPLC with refractive index detection (RID) analysis of the concentrations of glucose, xylose, and the
end products as described previously.\textsuperscript{[46]} Process ethanol yields, expressed as the percentage of maximal theoretical ethanol yield (0.51 g\textsubscript{ethanol} g\textsuperscript{-1} \textsubscript{substrate}) produced from the total glucose and xylose present in each hydrolysate, were calculated from the initial sugar, final sugar, and ethanol concentrations for each experiment.

### Chemical analysis of hydrolysates and fermentation supernatants

The hydrolysate and fermentation supernatant samples were sterile-filtered or centrifuged to remove cells and particulates and diluted tenfold with water. The sugar composition was determined by using an Aminex HPX-87H with Cation-H guard column (300 x 7.8 mm, BioRad, Inc. Hercules, CA) by using an Agilent 1260 Infinity HPLC–RID (Agilent Technologies, Inc., Palo Alto, CA). The mobile phase was 0.01 M sulfuric acid with an isocratic flow rate of 0.5 mL min\textsuperscript{-1}, and the column and RID flow cell were maintained at 50 °C. Analyte concentrations were determined by using ChemStation vC.01.08[210] with reference compounds used to generate standard curves (0.01–11.0 g L\textsuperscript{-1}).

Common lignocellulose-derived fermentation toxins were determined by using HPLC with tandem mass spectrometry (MS/MS) adapted from a method reported previously.\textsuperscript{[45]} Samples were dilute 10- to 200-fold with water and analyzed directly by using an Agilent 1200 series quaternary gradient pump with vacuum degasser and thermostatic autosampler coupled to an Agilent 6460A triple-trap quadrupole mass spectrometer operated in a dynamic multi-reactor-monitoring mode (d-MRM). The analytical column was an Ascentis Express C18 (15 cm x 2.1 mm, 2.7 μm particle size, Supelco/Sigma–Aldrich, St. Louis, MO, USA), and the mobile phase was a binary gradient of 0.1% formic acid water and acetonitrile.

### Lignin isolation

Enzyme lignins (EL) were prepared from ball-milled cell wall materials as described previously.\textsuperscript{[46]} Briefly, oven-dried untreated biomass or pretreated biomass solid residues were ball-milled by using a Fritsch pulverisette 7 (1 g, 20 mL ZrO sub, 10 x 10 mm ZrO sub ball bearings, 600 rpm for 10 min, 5 min rest, 47 cycles, reverse on). An aliquot (ca. 300 mg) of this material was put aside for analysis by using whole-cell-wall gel-NMR spectroscopy. The remainder of the ball-milled powder (570–700 mg) was transferred to 50 mL centrifuge tubes, suspended in 40 mL of 50 mM sodium acetate buffer (pH 5.0), and treated with crude cellulases (40 mg, Cellulysin Fritsch pulverisette 7, 1 g, 20 mL ZrO sub, 600 rpm for 10 min, 5 min in rest, 47 cycles, reverse on). An end product was set to 0.86 ms (1/8J, d\textsuperscript{H}) and 0.5 mL min\textsuperscript{-1}, and the column and RID flow cell were maintained at 145 Hz. The total acquisition time for a sample was 6 h. Processing used typical matched Gaussian apodization (GB = 0.001, LB = −0.5) in F2 and squared cosine-bell and one level of linear prediction (32 coefficients) in F1. The central DMSO solvent peak was used as internal reference (δ\textsubscript{H} = 39.5, δ\textsubscript{C} = 2.49 ppm). Peak assignments were made by comparison with spectra assigned previously.\textsuperscript{[80, 81]} Volume integration of contours in HSQC plots used Bruker’s TopSpin 3.5pl6 (Mac version) software and set to a G+5+5*S = 100% basis for aromatic (F1 = 95–170 ppm; F2 = 5.5–9 ppm) using just the G and δ\textsubscript{C} correlations (and logically halving the S integrals) and aldehyde signals (F1 = 185–200 ppm; F2 = 9.5–10 ppm), as well as aliphatics on an 1 +6 +8 +B +4 +C +C = 100% basis for aliphatic signals (F1 = 45–115 ppm; F2 = 2.25–6 ppm), using just the δ\textsubscript{C} correlations for each (and again halving the integrals for C and C because a single such [β]-coupled unit has two equivalent C/H pairs).

### DFRC analysis

Incorporation of monolignol p-coumarates and monolignol ferulates into the lignin was determined by using the ether-cleaving, ester-retaining DFRC method established previously for other monolignol conjugates.\textsuperscript{[46, 82–83]} The DFRC protocol was performed on EL samples (15–25 mg) according to the method described by Regner et al.\textsuperscript{[86]} The internal standards used for quantification were added from a premade standards mixture (which consisted of 370 μg H-d\textsubscript{p} 888 μg G-d\textsubscript{p} 956 μg S-d\textsubscript{p} 351 μg G-DDpCA-d\textsubscript{p} 327 μg S-DDpCA-d\textsubscript{p} 168 μg G-DDDA-d\textsubscript{p} and 115 μg S-DDDA-d\textsubscript{p}). Samples were analyzed by using triple-quadrupole GC–MS/MS (Shimadzu GCMS-TQ8030) operated in MM mode using synthetic standards for authentication using settings described previously.\textsuperscript{[86]}

### Lignin molecular weight profile analysis

The isolated lignins were prepared as 1 mg mL\textsuperscript{-1} solutions for molecular weight profiling analysis by dissolving the enzyme lignins (2–3 mg) in DMF with 0.1 M lithium bromide (2–3 mL) to achieve a final concentration of 1 mg mL\textsuperscript{-1}. The samples were sonicated for 1 h to ensure complete dissolution and then filtered through a 0.2 μm PTFE membrane directly into the HPLC vial. The samples were then submitted for molecular weight profiling analysis.

Molecular weight profiles were determined by usingGPC by using a Shimadzu Prominence HPLC connected to a PSS PolarSil analytical linear S column (8 mm x 30 cm, 5 μm particle size) at 40 °C. The mobile phase was degassed DMF with 0.1 M LiBr (Aldrich), filtered through a 0.45 μm PTFE membrane and then filtered a second time through an in-line 0.2 μm PTFE membrane at 0.3 mL min\textsuperscript{-1}. The GPC signal was acquired by using a Shimadzu SPD-M20A UV/Vis flow cell photodiode array (PDA) detector and a RID-10A refrac-
tive index detector. The data were processed using Wyatt Astra v. 7.1.2. The tubing alignment volumes, band broadening, and detector calibration constants were measured by using a 30 kDa polystyrene standard ($M_w = 30$ kDa, $M_p/M_w = 1.06$). The molecular weight profiles were calculated by using Astra software using a fourth-order conventional calibration curve determined using a PSS polystyrene (low molecular weight, peak molecular weight $M_p = 0.25–70$ kDa) standard ReadyCal set (Sigma–Aldrich, P/N:76552).

Recovery process model

The proposed pCA and FA recovery process was modeled by using Aspen Plus v.10 (Aspen Technology Inc., Massachusetts, USA) for a biorefinery processing 2000 dry Mg day$^{-1}$ corn stover.22,23 We consider the deacetylation process described in the National Renewable Energy Laboratory’s (NREL) biorefinery designs in 201323 and 201525 in which lignocellulosic biomass is treated at 80 °C for 1 h at a caustic loading of 17 mg g$^{-1}$ dry at 20% solids loading. The deacetylation process improves the conversion of oligomeric xylose into xylose and removes ash (75%), xylan (2%), acetate (88%), water-soluble extractives (100%), and part of the lignin (20%). In the NREL biorefinery designs22,24 the APL stream goes directly to the wastewater treatment. Therefore, in this study, we assumed that the pCA and FA recovery process is an intermediate unit and receives the APL from the biorefinery at no cost. The unit separates pCA and FA from the APL and sends the waxes/oligomers and liquid residues to the wastewater treatment (Figure 1).

Technoeconomic model

We assumed that the economics of pCA and FA recovery are independent from the biorefinery and that the raw materials and utilities are purchased separately from those of the biorefinery. A discounted cash-flow analysis was performed based on the energy and mass-balance obtained from the Aspen Plus simulation to calculate a MPSP for a mixture of solid pCA and FA. The MPSP can be used as an economic index to assess whether the pCA and FA recovery would be competitive in the current market. The financial assumptions are consistent with the NREL designs22–24 (Table 2). Installed capital costs are scaled from NREL equipment cost reports using a characteristic scaling exponent and updated to the 2014-dollar value using chemical engineering plant cost index. The methodology used to calculate TCi as a function of installed costs is shown in Table S2. Utility and raw materials costs are calculated using the flowrates obtained from the Aspen Plus model and unit materials costs (Table S3).88–90

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Data and Materials availability: The DNA sequences for the ZmPMT (pCAf, Protein ID GRMZM2G028104) and BdPMT (Protein ID Bradi2g36910) genes can be found in GenBank.

Conflict of interest

The authors declare no conflict of interest.

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