INTRODUCTION

In the past decades, lignocellulosic biomass has been considered a promising second-generation bioenergy feedstock with a low carbon footprint (Naik et al., 2010). Barley agricultural residues have also become a highly potent resource for bioenergy production. Barley is the fourth most produced cereal crop worldwide after wheat, rice, and maize. The global annual grain production of barley is over 141 million tons on nearly 48 million hectares, predominantly in temperate regions (FAOSTAT, 2020). After harvesting the grains, a significant amount of barley straw is left as a byproduct, which can be utilized for bioenergy production. Barley straw is a lignocellulosic biomass with a high energy content, making it a promising feedstock for biofuel production.

Abstract

Barley is a major cereal crop with a wide ecological range, and its lignocellulosic residues have the potential to be used as a feedstock for various purposes, including biofuel production. Lignocellulosic biomass is an abundant renewable source of carbon energy. However, its heterogeneous properties and intrinsic recalcitrance caused by cell wall lignification have lowered the biorefinery efficiency. The reduced lignin content and/or altered lignin structure have been desirable traits for lignocellulosic feedstock. We report the reduction of lignin content in barley by CRISPR/Cas9-mediated mutagenesis of caffeic acid O-methyltransferase 1 (HvCOMT1), the lignin biosynthetic gene responsible for lignin syringyl unit formation. The transgene-free, homozygous HvCOMT1 mutant was generated and analyzed for its cell wall composition, saccharification efficiency, and bioethanol production performance. The mutant had 14% lower total lignin content and 34% higher fermentable glucose recovery rate, compared to the wild-type (WT). The bioethanol concentration and yield from the hydrolysates of the mutant biomass were 14.3 g/L and 0.46 g/g total sugar, respectively. This result was 34% and 12% higher than those obtained from WT (10.7 g/L and 0.41 g/g total sugar). Under controlled environmental conditions, the overall growth performance of the HvCOMT1 mutant was similar to WT, with no distinct morphological variations between them. The HvCOMT1 mutant barley could offer improved quality lignocellulosic feedstock for efficient lignocellulosic biofuel production.

KEYWORDS
barley, caffeic acid O-methyltransferase, CRISPR/Cas9, lignin modification, lignocellulosic biofuel, lignocellulosic biomass
amounting to approximately 51 million tons per year (Prasad et al., 2007; Tye et al., 2016). Barley straw consists of approximately 33% cellulose, 20% hemicellulose, and 17% lignin in the dry matter (Adapa et al., 2009). Given the annual global production of barley straw, the estimated availability of cellulose that can be fermented into bioethanol is more than 17 million tons per year (Tye et al., 2016).

Lignin, one of the main components in the cell walls of lignocellulosic biomass, is a major factor limiting the efficient bioconversion of lignocellulosic biomass. During the process of enzymatic hydrolysis, lignin protects the accessible surface area of the cellulose and non-specifically adsorbs hydrolytic enzymes, thereby reducing the release of fermentable sugars (De Souza et al., 2015; Mansfield et al., 1999; Oliveria et al., 2020; Rahikainen et al., 2013; dos Santos et al., 2019). Reducing the lignin content and manipulating the lignin composition is a straightforward strategy to make lignocellulosic biomass more amenable for bioenergy production (Halpin, 2019; Sticklen, 2008).

Lignin is a heterogeneous phenolic polymer composed of three major structural monomeric units, p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, which are formed by the incorporation of monolignols, p-coumaryl, coniferyl, and sinapyl alcohols into the lignin polymer, respectively. Monolignol biosynthetic pathways are well conserved across land plants (Bonawitz & Chapple, 2010). Monolignols are synthesized by a series of enzymatic reactions, including hydroxylation, methylation, and side chain reductions catalyzed by more than 10 enzymes (Vanholme et al., 2010). Among lignin biosynthetic enzymes (Figure S1), caffeic acid O-methyltransferase (COMT) is responsible for S unit formation by catalyzing the conversion of 5-hydroxyconiferyl aldehyde and 5-hydroxyconiferyl alcohol to S unit precursors, sinapyl aldehyde and sinapyl alcohol, respectively (Barros et al., 2019; Capellades et al., 1996; Humphreys et al., 1999; Jouanin et al., 2000; Osakabe et al., 1999). COMT has been one of the preferential targets to manipulate lignin biosynthesis and to mitigate lignin-derived biomass recalcitrance. In a wide range of lignocellulosic feedstock (including maize, sorghum, sugarcane, and perennials energy grasses), COMT mutation or RNA interference (RNAi)-mediated suppression of COMT have shown a reduced lignin content and S/G ratio, resulting in increased saccharification efficiency (Dien et al., 2009; Fu et al., 2011; He et al., 2003; Ho-Yue-Kuang et al., 2016; Jung et al., 2012, 2013; Tu et al., 2010). A recent study using RNAi-mediated COMT suppression in barley supported this result (Daly et al., 2019).

Plant genetic engineering tools have drastically improved in recent years. The most revolutionary technology is genome editing, enabling highly efficient and precise site-specific DNA modification (Yin et al., 2017). To date, the clustered regularly interspaced palindromic repeat (CRISPR)/CRISPR-associated 9 (Cas9) is considered a highly efficient and easily applicable platform for genome editing (Ma et al., 2016). In this system, the endonuclease Cas9 and the short non-coding guide RNA (gRNA) induce a DNA double-stranded break (DSB) at targeted genomic locus. Subsequently, DNA DSB repair leads to either site-specific sequence disruption with random nucleotides insertion-deletions (indels) or precise sequence replacement in the presence of a donor DNA fragment (Bibikova et al., 2001; Hsu et al., 2014; Rouet et al., 1994; Sander & Joung, 2014). Since the unveiling of the mechanism of CRISPR/Cas9 (Horvath & Barrangou, 2010; Makarova et al., 2011), CRISPR/Cas9-mediated targeted mutagenesis has been successfully implemented in various crops for improved yield, stress tolerance, and increased nutritional value (Arora & Narula, 2017; Jaganathan et al., 2018; Rodriguez-Leal et al., 2017; Sedeek et al., 2019).

In addition to its superior precision and efficacy, this method enables transgene-free genome modifications, which were not feasible in conventional genetic engineering. Once a genome editing event is induced by CRISPR/Cas9, its expression cassette can be easily removed from the host genome by genetic segregation in subsequent generations. Transgene-free CRISPR/Cas9-mediated engineered plants can thus mitigate the biosafety issues to facilitate regulatory approval for commercial applications.

The present study reports CRISPR/Cas9-mediated targeted mutagenesis of COMT in barley. We aim to reduce the lignin content and improve the efficiency of lignocellulosic biofuel production. Among 10 of the COMT family members found in the barley genome, one putative COMT gene (HvCOMT1) involved in lignin biosynthesis was isolated and targeted for mutagenesis. The elimination of the CRISPR/Cas9 expression cassette was verified. The transgene-free homozygous HvCOMT1 mutant was identified from subsequent generations. The potential use of the mutant as a bioenergy feedstock was evaluated by analyzing cell wall composition, saccharification efficiency, and bioethanol production performance.

2 | MATERIALS AND METHODS

2.1 | Isolation of COMT genes in barley and selecting the gRNA target site

To retrieve the lignin biosynthetic COMT gene from the barley, monocot COMT's, previously characterized to function in lignin biosynthesis from sorghum bmr12, maize bm3, and RNAi lines of switchgrass and sugarcane, were used as queries for the blast search in the IPK Barley BLAST Server (https://webblast.ipk-gatersleben.de/barley_ibsc/; Bout & Vermerris, 2003; Fu et al., 2011; Jung et al., 2012; Vignols et al., 1995). Among the 10 COMT's
found in barley. CAJW010050098 showed the highest similarity and identity to the query COMTs and was designated HvCOMT1. The gene sequence was confirmed in a barley transformation donor cultivar (Golden Promise) by polymerase chain reaction (PCR) using HvCOMT1_CF (5′-CACACACAAAATCAACCCACCA-3′) and HvCOMT1_CR (5′-CGGTTCCAGATGATCCTACTT-3′) primer and their subsequent sequencing.

The gRNA target site with two or more mismatched against the barley genome was selected in the first exon of HvCOMT1 using a web-based gRNA design tool, E-CRISP (http://www.e-crisp.org/E-CRISP/) with the barely reference genome assembly ASM32608v1 (International Barley Genome Sequencing Consortium, 2012). To facilitate mutant selection, the presence of EciI restriction sites in the gRNA selection, the presence of genome assembly ASM32608v1 (International Barley Genome Sequencing Consortium, 2012). To facilitate mutant selection, the presence of EciI restriction sites in the gRNA target site adjacent to the protospacer adjacent motif (PAM) was also considered as demonstrated in Figure 1a.

### 2.2 CRISPR/Cas9 vector construction

The pRGE32 was used as the backbone vector with the following modifications (Addgene plasmid # 63142; http://n2t.net/addgene:63142; RRID:Addgene_63142) (Xie et al., 2015). The promoter of selection marker (hptII) was replaced with a 35 s promoter flanked by maize HSP70 intron isolated from the pTAL-COMT vector (Jung & Altpeter, 2016). The gRNA expression cassette was also replaced with the wheat U6 promoter (TaU6) and the gRNA scaffold (including gRNA spacer cloning site) as described previously (Shan et al., 2013). The gRNA spacer synthesized as double-stranded oligomers was inserted into the vector via BsaI-mediated Golden Gate cloning. The resulting CRISPR/Cas9 vector for HvCOMT1 targeted mutagenesis was named pKCRISPR_HvCOMT1 (Figure 1b). The Cas9 gene in the vector was under the control of the rice ubiquitin promoter (OsUbi) and NOS terminator as described previously (Xie et al., 2015).

### 2.3 Plant materials and transformation

*Hordeum vulgare* cv. “Golden Promise” was used throughout the study. Plant growth and Agrobacterium-mediated

![Figure 1](image-url)
transformation were conducted following the previously described protocol (Harwood, 2014). Briefly, plants were grown under a controlled environment with 15 °C/12 °C (day/night), 16 h photoperiod, and 500 μmol/m²/s light intensity, and 80% relative humidity. Immature embryos were isolated from immature seeds collected 12–15 days after anthesis. The embryo axis was removed from immature embryos, and the remaining scutellum was inoculated with Agrobacterium strain AGL1 harboring the pKCRISPR HvCOMT1 vector. Callus induction and regeneration were carried out using hygromycin (50 mg/L)-containing medium.

Regenerated plantlets were transferred to the soil (Baroker-Horticultural Purpose) in 1 L pots. Selected T0 plants and their progenies (T1, M1, and M2 plants) were grown in a plant growth chamber under 22°C/18°C (day/night), 16 h photoperiod, 500 μmol/m²/s light intensity, and 70% relative humidity. Plants were irrigated with 100 ml of tap water when the soil moisture (volumetric water content) reduced to 15%. After the first tiller emerged (Zadoks stage 21), plants were fertilized with 0.1% Hyponex solution (Hyponex Japan) in 100 ml tap water every 2 weeks until maturity.

2.4 | Selection of CRISPR/Cas9-mediated HvCOMT1 mutants from T0 plants

To select primary transgenic plants (T0 plants), genomic DNA was isolated from the leaves of regenerated plants and the PCR- amplified using T-DNA specific primers PKS2F (5′- ACATGTGTGATGTGCGCTGGT-3′) and PKS2R (5′- GGTGGGGTACTTCTCGTGGT-3′) with an expected product size of 732 bp.

Among the selected T0 plants, CRISPR/Cas9-mediated HvCOMT1 mutants and their zygosity status were determined by PCR/restriction enzyme (PCR/RE) assay with sequencing. Firstly, the partial sequence of HvCOMT1 gene encompassing the CRISPR/Cas9 target site was PCR-amplified with the expected size of 678 bp using HvCOMT1_5UF (5′-GTGAAGCCAGAAAGCCCAT-3′) and HvCOMT1_IR (5′-GCTGCTGGAGCTCAATTTGT-3′) primers, designed from the 5′-flanking region and intron of HvCOMT1, respectively. PCR conditions comprised an initial denaturation step at 98°C for 30 s, followed by 35 cycles at 98°C for 10 s, 65°C for 30 s, and 72°C for 30 s, and then a final extension step of 72°C for 2 min. The amplicon was purified using a DNA Clean & Concentrator Kit (Zymo Research) and directly sequenced using HvCOMT1_5UF primer. Samples showing mixed peak patterns around PAM in the electropherogram were considered putative heterozygous and/or chimeric mutants for HvCOMT1. To further characterize mutation events, the HvCOMT1 amplicon from the putative mutants was cloned into a pCR 2.1 Vector (Invitrogen), and at least 16 clones for each mutant were sequenced. For the RE assay, 100 ng of the purified HvCOMT1 amplicon was digested with 2 units of EcoRI (NEB) at 37°C for 16 h, then separated on 2% agarose gel, and imaged under UV after EtBr staining. Samples presenting undigested (678 bp) or completely digested DNA fragments (330 and 348 bp) were considered homozygous mutant or the unmutated wild type (WT) plants. Samples presenting both undigested and digested DNA fragments were considered heterozygous or chimeric mutants for HvCOMT1.

2.5 | Identification and confirmation of T-DNA-free, homozygous HvCOMT1 mutants from T1 and M2 plants

A total of 30 T1 progenies were produced from the selected T0 plants. To select T-DNA-free plants, genomic DNA from 1-week-old T1 seedlings was PCR-amplified using T-DNA specificprimersPKS2F(5′-ACATGTGTGATGTGCGCTGGT-3′) and PKS2R (5′-GTTGGGGTACTTCTCGTGGT-3′). Samples without PCR product were considered T-DNA-free. Their mutation patterns and zygosity status were also determined using PCR/RE assay and sequencing (as described previously). The T-DNA-free, homozygous T1 mutants were selected and designated as M1 plants. Six M2 plants were produced from each M1 plant, and the absence of T-DNA and the homozygosity for HvCOMT1 mutation were confirmed as described above.

2.6 | Lignocellulosic biomass sample preparation

Above ground biomass below the peduncle was harvested from WT and M2 plants when the developmental stage reached physiological maturity as indicated by the complete loss of any green color on the peduncle and straw (Zadoks stage 92). After leaf removal, samples consisting of three internodes, nodes, and an attached leaf sheath were dried at 45°C until they reached a constant weight. Samples were ground and sieved to pass thorough 0.5 mm mesh. Soluble extract was removed by three successive extractions with 50% ethanol (v/v) at 45°C for 30 min and rinsed twice with distilled water. Extract-free samples were dried at 45°C until a constant weight was reached. The samples were used for the lignin and cell wall carbohydrate analysis and ethanol production.

2.7 | Lignin and cell wall carbohydrate analysis

The total lignin content was measured by the acetyl bromide method as previously described (Jung et al., 2012). The acetyl bromide soluble lignin content was calculated using
the molar extinction coefficient of 17.75 L/g/cm for grass lignin (Foster et al., 2010).

Lignin composition was determined by the thioacidolysis degradative method as previously described (Robinson & Mansfield, 2009). Lignin-derived thioacidolysis monomers, G and S were analyzed using the gas chromatography coupled to mass spectrometry system and quantified by employing response factors of each monomer against an internal standard as previously described (Yue et al., 2012).

Cell wall carbohydrates were analyzed using the National Renewable Energy Laboratory protocol (Sluiter et al., 2008). Liberated monomeric sugars were identified and quantified with Agilent/HP 1200 high-performance liquid chromatography (HPLC) equipped with a refractive index (RI) detector (Agilent Technologies). The HPLC analysis was carried out using a Hi-Plex H column (Agilent Technologies), operating at a flow rate of 0.6 ml/min using 5 mM H2SO4 mobile phase.

The t-tests were performed using R to determine whether the contents of lignin and cell wall carbohydrate were statistically different between WT and the HvCOMT1 mutant.

2.8 Dilute acid pretreatment, enzymatic saccharification, and ethanol fermentation

Lignocellulosic biomass samples harvested from WT and M2 plants were subjected to a dilute acid pretreatment and enzymatic saccharification for lignocellulosic ethanol fermentation. One gram of each biomass was dissolved with 10 ml of 1% (w/v) H2SO4 in a 100 ml bottle. The suspension was autoclaved at 121°C for 30 min and adjusted to pH 5 with 1 M sodium hydroxide.

Enzymatic saccharification was performed using the Ctec2 enzyme (Novozyme) with a cellulose activity of 138 FPU/ml. Fifteen FPU/g of total glucan in biomass was added into each pretreated sample. This was followed by hydrolysis at 50°C for 72 h in a shaking incubator. After saccharification, the liquid hydrolysates were filter-sterilized using a 0.2 μm tube top filter and used for ethanol fermentation without detoxification.

The Saccharomyces cerevisiae strain XUSEA (Tran et al., 2020) was used for ethanol fermentation, as it is capable of co-utilizing glucose and xylose as carbon sources. The yeast synthetic complete (YSC) medium was used for routine culture and fermentation. The medium was composed of 20 g/L glucose (or xylose), 0.79 g/L of complete synthetic medium (CSM; MP Biomedicals), and 6.7 g/L of yeast nitrogen base without amino acids (YNB w/o AA; Difco). The pH of the fermentation media was maintained at 5 with a 100 mM phthalate buffer. For seed culture, XUSEA was inoculated in the YSC medium supplemented with glucose and then transferred to YSC medium with xylose as the sole carbon source for the preculture. Microaerobic fermentation was conducted in a 25 ml serum bottle with a total working volume of 5 ml and initial OD600 20. The fermentation was conducted at 30°C for 48 h in a shanking incubator. Sugar concentrations were measured using Agilent/HP 1200 HPLC equipped with an RI detector (Agilent Technologies). The HPLC analysis was carried out using a Hi-Plex H column (Agilent Technologies), operating at a flow rate of 0.6 ml/min using 5 mM H2SO4 mobile phase. Ethanol was analyzed by GC (Model 6890, Agilent Technologies) equipped with a flame ionization detector and a 30 m × 0.25 μm × 0.25 μm HP-INNOWax polyethylene glycol column (Agilent Technologies).

3 RESULTS

3.1 Generation of CRISPR/Cas9-mediated HvCOMT1 mutant lines

A total of 26 plants were regenerated following their transformation. All plants were primary transgenic lines (T0 plants) harboring T-DNA from the pKCRISPR_HvCOMT1 vector. Eleven out of the 26 T0 plants were found to be CRISPR/Cas9-mediated HvCOMT1 mutants based on direct amplicon sequencing (Figure 1c,d). This result demonstrates a mutagenic efficiency of 42%. All identified mutants appeared to have mono-allelic or chimeric mutations for HvCOMT1 as shown in the PCR/RE assay (Figure 1e).

To further examine the mutation patterns in the mutant lines, HvCOMT1 amplicon containing the gRNA target site was cloned, and 16–20 clones for each mutant line were sequenced. Consistent with the result from the PCR/RE assay, all identified mutants carried mono-allelic or chimeric mutations, while containing the WT HvCOMT1 allele (Figure 1f). The mutation site was located 4 bp upstream of PAM in all cases. The majority of T0 mutants (9 out of 11) including the line C13 had a single-base deletion of C (zygosity: WT/Cd). The line C17 had a single-base insertion of T (zygosity: WT/Ti). The line C24 had chimeric mutations with a single-base insertion of T or three-base deletion of CGG (zygosity: WT/Ti or WT/ CGGd) (Figure 1f). Compared to the WT HvCOMT1 encoding 360 amino acids, Ti and Cd mutations were expected to encode only 145 and 126 amino acids, respectively, due to a premature stop codon from the frameshift mutation. Conversely, the CGGd mutation was expected to encode 359 amino acids with a deletion of a single amino acid, Ala-71 (Figure S2).

3.2 Selection and generation of T-DNA-free, homozygous HvCOMT1 mutants

The mutant lines including C17 and C24 with the Ti and CGGd allele were not further analyzed due to complex
T-DNA integration patterns and limited efficacy of the mutation on disrupting the gene function. In order to select T-DNA-free homozygous mutants, the T0 mutant line of C13 (carrying single T-DNA copy and Cd mutant allele) was used to produce T1 progenies. T-DNA segregation was observed among the T1 progenies following the Mendelian inheritance pattern. Among the 19 T1 plants produced, 6 T1 plants were T-DNA-free based on the T-DNA specific PCR analysis. The subsequent PCR/RE assay and sequencing for HvCOMT1 amplicon isolated the homozygous HvCOMT1 mutants. Two out of 6 T-DNA-free T1 plants were homozygous for the HvCOMT1 mutation (zygosity: Cd/Cd; Figure 2a), and these T-DNA-free, homozygous plants and their seeds were designated as M1 plants and M2 seeds, respectively.

### 3.3 Phenotypes and cell wall characteristics of CRISPR/Cas9-mediated HvCOMT1 mutants

To investigate phenotypic changes and cell wall characteristics in CRISPR/Cas9-mediated HvCOMT1 mutants, M2 progenies were grown to maturity. Homozygosity for the HvCOMT1 mutation was confirmed by PCR/RE assay in all M2 plants (Figure 2b). As provided in Figure S3, the overall growth performance and morphology in homozygous HvCOMT1 mutants were similar to WT throughout the growth period. Furthermore, phenotypes associated with mutations in lignin biosynthetic genes (such as brown midrib, orange lemma, and gold hull) were not distinctively observed in the mutant.

HvCOMT1 mutation altered lignin content and composition of the lignocellulosic biomass (Table 1). The total lignin content was significantly reduced by 14% in the HvCOMT1 mutant compared to WT. The mutant also showed significantly reduced S unit content and S/G ratio by 37% and 51%, respectively. The G unit content was comparable between the HvCOMT1 mutant and WT.

The amount of glucose mostly derived from cellulose in the lignocellulosic biomass did not differ between WT and the HvCOMT1 mutant. The amounts of two major hemicellulose components (xylose and arabinose) were also comparable between them (Table 1).

### 3.4 Enzymatic saccharification and ethanol fermentation of lignocellulosic biomass

Enzymatic saccharification and ethanol fermentation were performed to evaluate the effect of HvCOMT1 mutation on ethanol production from the lignocellulosic biomass. Under given enzymatic hydrolysis conditions, glucose and xylose recovery rates from the lignocellulosic biomass of the mutant were 55.3% and 84.8%, respectively. Glucose recovery rate from the mutant was 34% higher than that from WT (41.2%).

There was no significant difference observed in the xylose recovery rate (Figure 3a).

### Table 1 Comparison of cell wall composition between wild-type and CRISPR/Cas9-mediated HvCOMT1 mutant

| Lines  | Total lignin (mg/g DW) | S unit (µmol/g lignin) | G unit (µmol/g lignin) | S/G ratio | Cell wall carbohydrate |
|--------|------------------------|------------------------|------------------------|------------|------------------------|
|        |                        |                        |                        |            | Glucose (mg/g DW)       | Xylose (mg/g DW) | Arabinose (mg/g DW) |
| WT     | 239.3 ± 1.7            | 223.3 ± 19.6           | 161.4 ± 14.3           | 1.4        | 461.2 ± 7.9            | 246.1 ± 1.4     | 42.7 ± 3.5          |
| C13    | 205.6 ± 1.1**          | 141.5 ± 13.4*          | 206.9 ± 15.3           | 0.7**      | 465.5 ± 5.9            | 252.8 ± 3.1     | 43.9 ± 3.6          |

** and * significantly different from WT at p < 0.01 and p < 0.05 in t-test, respectively.

Note: Values are means ± SE from three biological replications.

Abbreviations: C13, T-DNA free and homozygous CRISPR/Cas9-mediated HvCOMT1 mutant line; WT, Wild-type plant for HvCOMT1 gene.
Microaerobic fermentations of lignocellulosic hydrolysates derived from WT and the mutant were performed using the glucose/xylose co-fermenting *S. cerevisiae* strain XUSEA (Figure 3b). During the first 4 h of fermentation, glucose from the mutant hydrolysates was completely utilized with a total sugar consumption rate of 5 g/L/h. This result was 30% higher than that of WT (3.85 g/L/h). During 48 h of fermentation, the ethanol concentration and yield from the mutant hydrolysates were 14.3 g/L and 0.46 g/g total sugar, respectively. These results were 34% and 12% higher than those obtained from WT (10.7 g/L and 0.41 g/g total sugar).

4 | DISCUSSION

We have utilized CRISPR/Cas9 technology to create a transgene-free, homozygous *HvCOMT1* mutant to improve the use of barley as an efficient lignocellulosic biofuel feedstock. Mutation in the *HvCOMT1* gene reduced the total lignin content, S unit content, and S/G ratio, significantly enhancing saccharification and bioethanol production efficiency from the lignocellulosic biomass.

The overall characteristics of the CRISPR/Cas9-mediated *HvCOMT1* mutant was comparable to a recent study assessing barley RNAi lines with the downregulation of two COMTs (Daly et al., 2019). Similar results have been reported in other crops with reduced COMT activity, including sugarcane, sorghum, maize, rice, and switchgrass (Dien et al., 2009; Fu et al., 2011; Jung et al., 2012, 2013; Lam et al., 2019; Saballos et al., 2008; Vermerris et al., 2007). Our results suggest *HvCOMT1* is a major lignin biosynthetic gene responsible for S unit formation among barley COMT homologs.

Caffeic acid O-methyltransferase family members contain the conserved domains and residues involved in *S*-adenosyl-L-methionine (SAM) binding, phenolic substrate binding/positioning and catalytic activity (Zubieta et al., 2002). A bioinformatic analysis of barley COMTs revealed that three protein homologs (*HvCOMT1*, *HvCOMT2*, and *HvCOMT3*) contain the key conserved sites. These protein homologs share a high degree of similarity with other known monocot lignin biosynthetic COMTs (Daly et al., 2019). However, genes encoding the three homologs exhibit different expression patterns. *HvCOMT1* and *HvCOMT2* are expressed in most of the lignifying tissues, while *HvCOMT1* is more stably expressed in the internodes across different developmental stages. *HvCOMT3* expression is limited to the embryo and root (Daly et al., 2019; Mascher et al., 2017). These genes are located on different chromosomes in barley. In particular, *HvCOMT1* is located in chromosome 7H, which shares syntenic regions with the loci of rice, *brachypodium*, and maize lignin biosynthetic COMT genes (Bennetzen & Chen, 2008; Daly et al., 2019; Vogel et al., 2010). This knowledge further supports a role of *HvCOMT1* in lignin biosynthesis. These previous genetic characterizations of *HvCOMT1* corroborate our blast search result and the rationale for targeted mutagenesis of *HvCOMT1* to manipulate lignin biosynthesis.

Reductions in the lignin content and S unit of CRISPR/Cas9-mediated *HvCOMT1* mutant were significant, but not as dramatic as seen in other plants defective in the COMT gene. The barley lignocellulosic biomass showed a 14% reduction in total lignin content which is largely attributed to a 37% reduction in the S unit (51% reduction in the S/G ratio). Whereas, rice culm of the OsCOMT1 mutant had a 30% reduction in total lignin with an 81% reduction in S/G ratio. Similarly, the stem of *Arabidopsis AtOMT1* mutant had a 46% reduction in total lignin with a 95% reduction in S/G.
ratio (Goujon et al., 2003; Lam et al., 2019). Two possible explanations could be considered for this result: (1) The barley genome contains lineage-specific duplication of several gene families, including COMTs (Daly et al., 2019; Mascher et al., 2017). Their functional relevance might compensate for the lack of HvCOMT1 in lignin biosynthesis, resulting in a relatively moderate reduction in the total lignin and S/G ratio in the HvCOMT1 mutant. Similarly, the barley COMT RNAi lines suppressing HvCOMT1 and HvCOMT2 expression by almost 98%, still maintained 50% of the COMT activity with approximately a 50% reduction in S/G ratio (Daly et al., 2019); (2) Phylogenetic analyses revealed that HvCOMT2 is closely related to a wheat COMT, TaCOMT-3D. In wheat, an overexpression of TaCOMT-3D caused lignin accumulation in stem (Daly et al., 2019; Wang et al., 2018). The expression level of HvCOMT2 is similar to that of HvCOMT1 in stem tissues (Daly et al., 2019; Mascher et al., 2017). Therefore, it might be the case that in barley, HvCOMT2 is as important as HvCOMT1 in S unit formation. Therefore, a disruption of HvCOMT1 results in a relatively moderate lignin reduction and compositional change. The genetic redundancy of COMT could also address other observations of this study. For example, the phenotypic characteristics, such as brown midrib, orange lemma, and gold hull, often associated with reduced lignin biosynthesis in C4 plants (Bout & Vermerris, 2003; Sattler et al., 2010; Tsai et al., 1998; Vignal et al., 1995) were not found in the HvCOMT1 mutant.

The reduced lignin content in the HvCOMT1 mutant induced positive effects on biomass digestibility. Despite no compensatory increases of fermentable sugar content in the cell wall (Table 1), the mutant had 34% and 12% higher glucose recovery from saccharification and ethanol yield, respectively, compared to WT (Figure 3). This result is consistent with previous studies showing a strong negative correlation between lignin content and enzymatic hydrolysis of (hemi)cellulose as well as fermentation in various plants, such as switchgrass, sugarcane, sorghum, and alfalfa (Chen & Dixon, 2007; Chen & Dixon, 2007; Sattler et al., 2010; Tsai et al., 1998; Vignal et al., 1995) were not found in the HvCOMT1 mutant.

In this study, homozygous mutants for HvCOMT1 and segregation of CRISPR/Cas9 construct were identified in the T1 generation. The stable inheritance of the mutation and transgene-free traits were confirmed through subsequent generations. Considering the concerns and regulations related to genetically engineered crops by the random integration of foreign DNA (Hundleby & Harwood, 2019; Kim & Kim, 2016), the transgene-free mutant would be highly favorable for gaining regulatory approval. The newly generated HvCOMT1 mutant barley could offer improved quality feedstock not only for biofuel, but also for other various purposes, including animal feed and green manure (Becker et al., 1994; Frei, 2013). The overall growth performance of the HvCOMT1 mutant was similar to WT. No distinct morphological variations were observed throughout the study (Figure S3). Assessment of the mutant barley performance under field conditions will further assess its feasibility for industrial large-scale production.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**SUPPORTING INFORMATION** Additional supporting information may be found online in the Supporting Information section.

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