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# Evaluating the performance of low-lignin transgenic bioenergy feedstocks in the field

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# Evaluating the performance of low-lignin transgenic bioenergy feedstocks in the field

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# ABSTRACT

Lignin in the cell walls of lignocellulosic biomass limits the accessibility of carbohydrates for breakdown into fermentable sugars and subsequently biofuels. The resistance of plant biomass to enzymatic or microbial deconstruction, known as biomass recalcitrance, can be overcome by reducing lignin content or modifying its composition through genetic modification of the lignin biosynthetic pathway. However, few studies to date have assessed the performance of low-lignin biofuel feedstocks under field conditions. Because lignin plays a vital role in several developmental and stress-related processes, characterization of these plants under the appropriate agronomic conditions is necessary to confirm that the improved biofuel-related traits can be maintained under field conditions without compromising plant growth or susceptibility to stresses. The general goal of this thesis project was to gain a better understanding of how lignin-modified feedstocks might perform in the field. The first chapter provides an introduction on the use of lignocellulosic biomass for biofuel production, the significance of lignin engineering for improving biofuel yields, and the importance of field trials to validate greenhouse results in a more realistic environmental setting. Chapter two is a review of the consequences of altered lignin biosynthesis on plant susceptibility to biotic and abiotic stresses. Chapter three reports the results of a two-year field evaluation of reduced recalcitrance transgenic switchgrass for chemical composition, sugar release, ethanol yield, agronomic performance, and disease susceptibility.

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# CHAPTER 1
# INTRODUCTION

The use of plant biomass as a renewable energy source has the potential to benefit the environment by reducing greenhouse gas emissions compared to conventional energy sources, while simultaneously promoting energy independence and security [1]. First generation biofuels, produced from the sugars or starches of food crops, are used in low percentage blends with conventional fuels to reduce carbon dioxide emissions. However, there is concern that the use of food crops for biofuel production could raise food commodity prices by creating competition between food and fuel crops for the use of agricultural land. A more sustainable route for fuel production from plant biomass lies in the development of second-generation biofuels, which are derived primarily from cellulose and hemicellulose in the cell walls of woody or herbaceous lignocellulosic plants. Ideally, these plants should be high-yielding, require minimal inputs, and grow well on lands not suitable for food crop production. The use of nonfood crops as a biofuel source could significantly reduce greenhouse gas emissions without interfering with food production [2].

Despite the advantages of second-generation biofuels, the complex cell walls that comprise lignocellulosic feedstocks are significantly more difficult to break down relative to the easily-digestible sugars and starches of first generation biofuel source materials. Lignin, an aromatic polymer comprised of phenylpropanoid units, contributes structural stability to the body of the plant, aids in water transport and cell wall hydrophobicity, and protects cell wall polysaccharides from bacterial and fungal degradation [3]. Because it interferes with enzymatic access to polysaccharides, the presence of lignin in the cell wall

is a primary contributor to the resistance of biomass to conversion into biofuels, also known as biomass recalcitrance [4]. Overcoming the recalcitrant structure of lignocellulosic cell walls necessitates the use of thermochemical pretreatments prior to fermentation in order to improve the accessibility of cell wall polysaccharides for enzymatic conversion into fermentable sugars. This pretreatment step is a major limiting factor in cost-effective fuel production from lignocellulosic biomass, as it adds significant costs to the conversion process.

Genetic engineering of enzymes in the lignin biosynthetic pathway to reduce lignin content or modify its structure can improve the release of sugars from the cell wall, and therefore has the potential to lower conversion costs by reducing or eliminating the pretreatment step [5]. In alfalfa, the downregulation of individual lignin biosynthetic enzymes nearly doubled the amount of sugars released from the cell walls in some transgenic lines relative to their controls [6]. More recently, lignin pathway modifications have been successful in enhancing cell wall deconstruction in several candidate bioenergy feedstocks, including switchgrass (*Panicum virgatum* L.) [7, 8, 9, 10], poplar (*Populus* spp.) [11], and sugarcane (*Saccharum* spp.) [12]. Switchgrass, a warm-season C4 perennial grass native to North America, is a particularly promising biofuel candidate due to its high biomass production, minimal water and nutritional requirements, widespread adaptability, and high net energy gain [13]. As a lignocellulosic plant, the carbohydrates in switchgrass cell walls are not readily available for enzymatic hydrolysis. Manipulating lignin biosynthesis, either by downregulating individual lignin genes or by

overexpressing a transcriptional regulator of lignin biosynthesis, significantly increases saccharification and ethanol yield in switchgrass plants grown under greenhouse conditions [7, 8, 9, 10]. The first of these studies found that the downregulation caffeic acid O-methyltransferase (COMT), an enzyme involved in monolignol biosynthesis, produced switchgrass plants with a reduction in total lignin content (11.4-13.4%) and the S/G lignin monomer ratio (44-46%). COMT-downregulated plants exhibited an increase in the enzymatic release of sugars (17-22%) and a higher ethanol yield (30-38%) relative to controls. Additionally, improvements in sugar release and ethanol yield were observed whether or not a pretreatment was performed [7].

While lignin engineering has produced promising results in greenhouse experiments with switchgrass and other biofuel feedstock candidates, only a few studies to date have extended these experiments into the field. Due to environmental effects, knowledge acquired from greenhouse studies with transgenic plants may not necessarily be translatable to the field. Lignin biosynthesis is strongly influenced by environmental factors, and exposure to biotic and abiotic stresses can result in modifications to lignin content and composition [14]. Therefore, characterization of lignin-modified plants in the field is necessary to ensure that the improved biofuel-related traits are maintained under both greenhouse and field conditions. Another relevant concern is that the reduced lignin content could compromise plant susceptibility to fungal and bacterial pathogens in the field, since plants accumulate lignin as a defense mechanism against pathogens to strengthen the cell wall and prevent further infection [15, 16]. Additionally, modifying

genes involved in lignin biosynthesis can have indirect effects on the synthesis of stress-related secondary metabolites, as well as the expression of defense-related genes [17, 18, 19], which could further influence how these plants respond to stresses in the field.

The general goal of this research project was to gain an improved understanding how feedstocks with altered lignin biosynthesis might perform in an agronomic setting. Chapter two reviews the effects of lignin pathway modifications on plant stress metabolism, and how such changes could ultimately influence how plants respond and adapt to stresses in a field environment. Chapter three describes a two-year field evaluation of COMT-downregulated switchgrass for chemical composition, sugar release and ethanol yield, agronomic performance, and disease susceptibility.

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# CHAPTER 2
# EFFECTS OF ALTERED LIGNIN BIOSYNTHESIS ON PHENYLPROPANOID METABOLISM AND PLANT STRESS

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## Abstract

Modification of lignin in bioenergy feedstocks has become a common strategy to increase saccharification and biofuel yield. The lignin biosynthetic pathway in several plant species has been dissected and key enzymes have been manipulated in transgenic plants. Recent analyses of lignin-modified plants have shown that decreasing lignin biosynthesis can alter carbon flow within the phenylpropanoid pathway and indirectly affect the synthesis of other secondary metabolites, many of which can play important roles in plant-environment interactions. In addition, lignin modifications have also been shown to induce the expression of various stress response-related genes. Examining and understanding these indirect effects of lignin modification on stress-related processes are essential since they could ultimately impact the performance of low-lignin bioenergy feedstocks under agronomic field conditions. Recent efforts to characterize such effects will be discussed in this review.

# Introduction

While lignocellulosic feedstocks represent a promising renewable and sustainable alternative to petroleum-based fuels, high production costs associated with conversion processes currently prevent them from being economically viable for large-scale implementation [1]. The production of biofuels from lignocellulosic feedstocks requires the depolymerization of cell wall carbohydrates into simple sugars that can be utilized during fermentation. However, the desired cellulose microfibrils are surrounded by a matrix of lignin and hemicellulose, which greatly inhibits their accessibility to hydrolytic enzymes [1, 2]. Lignin is a phenolic polymer that reinforces the secondary cell wall, confers structural integrity to the plant, aids in water transport, and also plays an important role in plant responses to various environmental stresses. The presence of lignin in plant cells has been identified as a major contributor to the resistance of converting cell walls into fuel precursors [3]. Expensive and energy-intensive thermochemical pretreatments are generally required to disrupt the lignin-polysaccharide barrier and allow better access of the cellulose to hydrolysis prior to fermentation. An alternative approach for improving the accessibility of cell wall sugars and reducing the need for pretreatment is through genetic engineering of the lignin biosynthetic pathway. Reducing lignin content and modifying its composition can be achieved by downregulating or over-expressing genes involved in either lignin biosynthesis or its regulation [3, 4, 5].

The early studies from a decade or more ago that contributed to our current understanding of lignin biosynthesis and its manipulation have focused on poplar (*Populus* spp.) to improve pulping performance [6, 7], and also forage species, such as alfalfa (*Medicago sativa* L.) and tall fescue (*Festuca arundinacea*), for improving digestibility [8, 9, 10]. A great deal of insight into lignin engineering has also been achieved through studies with the model species *Arabidopsis thaliana* [11] and tobacco (*Nicotiana tabacum*) [12]. Because the lignin biosynthetic pathway appears to be highly conserved among plant species, many of the genetic engineering strategies that have proven to be successful in model species can also be applied to lignocellulosic feedstocks [4]. Of particular importance to biofuel-related studies, *A. thaliana* has been identified as a relevant model system for studying the effects of lignin modification on cell wall recalcitrance. Cell wall phenotypes in *Arabidopsis* have been translatable to commercial dicot species such as alfalfa (*Medicago sativa*) and poplar (*Populus* spp.) [13]. Currently, considerable research is focused on developing strategies for the genetic improvement of bioenergy feedstock candidate species including switchgrass (*Panicum virgatum* L.), sorghum (*Sorghum bicolor*), willow (*Salix* spp.), *Miscanthus*, sugarcane (*Saccharum* spp.), and poplar [14]. Transgenic modifications are likely to be the most direct way for improving the quality of lignocellulosic biomass for cost-effective conversion into biofuels [15], and manipulation of the lignin biosynthetic pathway has thus far proven to be an effective route for increasing saccharification efficiency and/or ethanol yield in greenhouse-grown switchgrass, field-grown poplar, and greenhouse- and field-grown sugarcane [16, 17, 18, 19, 20, 21, 22, 23]. Additionally, opportunities for genetic engineering of lignin in other

potential biofuel species might also exist. Sorghum mutants with low lignin content from brown mid-rib (bmr) mutations are known for having enhanced digestibility and saccharification [24, 25]. The lignin biosynthetic genes underlying some of these mutations have been identified [26, 27, 28], and may represent potential targets for downregulation.

The majority of studies with lignin-modified plants have primarily focused on the effects of lignin pathway modifications on lignin content and composition, and the associated changes in pulping performance, digestibility, or recalcitrance to saccharification. More in-depth characterization of lignin-modified plants at the transcript, protein, and metabolite level have revealed that these modifications can have unexpected effects on other metabolic processes beyond lignin biosynthesis. Monolignols, the lignin monomers, are synthesized through the lignin-specific branch of the phenylpropanoid metabolic pathway. Phenylpropanoid metabolism encompasses a network of metabolic pathways responsible for synthesizing a wide variety of secondary metabolites that play various roles in developmental and stress-related processes [29, 30], and several studies show that suppressing lignin biosynthesis can alter metabolic channeling within the phenylpropanoid pathway and differentially affect the synthesis of other secondary metabolites [31, 32, 33, 34].

In addition to altering phenylpropanoid metabolism, modifications to lignin biosynthesis can also be associated with changes in how plants respond to biotic and abiotic stresses.

The accumulation of lignin in response to pathogen infection, wounding, and mechanical damage is an important defense mechanism in plants [35], and it has long been presumed that a reduction in lignin content would render plants more susceptible to such stresses. However, recent studies suggest that changes in stress susceptibility in lignin-modified plants are not necessarily direct results of reducing lignin content, but can be influenced by indirect effects of lignin pathway perturbations on stress-related metabolism and gene expression. Some reports with low-lignin mutant and transgenic plants indicate that the accumulation or suppression of certain secondary metabolites resulting from the alterations to phenylpropanoid metabolism can affect plant stress responses [34, 36, 36, 38]. Additionally, recent studies have revealed an increase in the expression of stress-related genes in lignin-modified plants, which some reports hypothesize to be triggered by the altered structural organization of the cell wall [31, 39, 40]. Evaluation of these plants under stress in controlled greenhouse or laboratory environments have indicated that these modifications to secondary metabolism and/or stress-related gene expression can interfere with or enhance the ability of the plant to tolerate stresses.

Because of the influence of lignin modification on plant stress, it is possible that altering lignin biosynthesis in second-generation bioenergy feedstocks could impact their performance in the field. In order to be commercially competitive, bioenergy crops should possess a number of desirable agronomic traits, including high biomass productivity under minimal agronomic inputs, high water and nutrient use efficiency, a relative resistance to pathogen and insect pests, and tolerance to a wide range of abiotic

conditions [41, 42, 43]. While modifying lignin content through transgenic approaches provides a promising route for improving saccharification efficiency and ethanol production, information regarding the impact of such modifications on the ability of these feedstocks to tolerate biotic and abiotic stresses when grown under natural environmental conditions is limited. This is an important consideration since the commercial viability of low-lignin bioenergy feedstocks will depend on their growth and fitness in an agronomic setting, which could be enhanced or debilitated from pleiotropic effects of lignin modification. This review will provide an overview of efforts to characterize the effects of lignin pathway modifications on phenylpropanoid metabolism and plant stress-related processes, including what has been learned from studies in model plants, as well as recent knowledge gained from studies with species that have been identified as potential bioenergy feedstocks. Since most of these studies have been performed in controlled greenhouse environments, the possible implications of altered stress-related metabolism for the field performance of low-lignin bioenergy feedstocks will also be addressed in this review.

## Alterations to phenylpropanoid metabolism

Phenylpropanoid metabolism in plants is an intricate network of pathways responsible for the synthesis of a broad range of secondary metabolites including flavonoids, isoflavonoids, sinapate esters, and lignin. Intermediate and end-products of phenylpropanoid metabolism function as antimicrobial and antiherbivory compounds, antioxidants, pigments, and UV-protectants [29, 44]. The compounds that specifically aid

in plant responses to stress range from simple precursor metabolites such as hydroxycinnamic acids, to more complex compounds such as flavonoids, isoflavonoids, and stilbenes [45]. Phenylpropanoid metabolic pathways can be developmentally induced in specific tissues, or activated in response to biotic and abiotic stress. Initiation of the phenylpropanoid pathway begins with the conversion of phenylalanine, an amino acid product of the shikimate pathway, to cinnamic acid via phenylalanine ammonia-lyase (PAL). The next two steps are catalyzed by cinnamate 4-hydroxylase (C4H) and 4-coumaroyl CoA ligase (4CL) to produce *p*-coumaroyl CoA, a metabolite that serves as a branch point from which other metabolic pathways in the phenylpropanoid network diverge [44]. The enzymes and enzyme families that control metabolic channeling among the major classes of phenylpropanoid products have been discussed in reviews [44, 46], as well as the transcription factors and signaling networks that regulate gene expression among the various secondary metabolic pathways [47, 48].

As the second most abundant natural polymer after cellulose, lignin is a major end product of phenylpropanoid metabolism. The lignin-specific branch of the phenylpropanoid pathway is responsible for the synthesis of *p*-coumaryl, coniferyl, and sinapyl alcohol monolignols. These monolignols serve as precursors for the production of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) monomeric lignin subunits, respectively, which undergo polymerization by peroxidases to form the lignin polymer [49]. More recently, the discovery of a cachetyl lignin polymer (C-lignin), comprised solely of caffeyl alcohol monomers, has been observed in the seed coats of some species

[50]. While many of the enzymes that participate in lignin biosynthesis can independently utilize multiple compounds as substrates, mixed substrate reactions reveal strong enzyme preferences for specific substrates and a high degree of competitive interactivity among enzymes for particular substrates [51, 52, 53]. This gives rise to a main metabolic stream toward the production of monolignols, with each substrate being primarily catalyzed by a specific enzyme [4, 54]. Characterization of mutant and transgenic plants with altered lignin biosynthesis has helped elucidate the influence of individual enzymes on lignin content and composition [49, 55].

While the effects of altered lignin biosynthesis on total lignin content and lignin polymer composition have been studied in great detail, the consequences of lignin pathway perturbations on other metabolic processes are not as well understood. Recent transcriptomic, proteomic, and metabolomic profiling of such plants have helped reveal some of these broader effects. Several studies have indicated that downregulating single or multiple lignin pathway genes can alter the metabolic flux through the phenylpropanoid pathway, and differentially affect the biosynthesis of other secondary compounds (Table A.1). Depending on the lignin biosynthetic gene(s) being suppressed, plants can undergo various metabolic changes in response to the reduced carbon flow into lignin biosynthesis. In the next sections, the known biochemical effects of altering the expression of characterized lignin biosynthetic genes will be examined.

***Hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase***

Hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (HCT) is a member of a large gene family encoding for acyltransferases, a group of enzymes that catalyze the acylation of secondary metabolites. HCT catalyzes an early step in the phenylpropanoid pathway and initiates the carbon flux toward the monolignol-specific branches by catalyzing the conversion of *p*-coumaroyl-CoA into p-coumaroyl shikimate [56]. *P*-coumaroyl-CoA is a metabolite located at a branching point in the phenylpropanoid pathway, where the monolignol and flavonoid biosynthetic pathways diverge. If catalyzed by HCT, *p*-coumaroyl-CoA is converted into shikimate and quinate esters, and carbon is directed toward the synthesis of monolignols [56, 57]. Alternatively, *p*-coumaroyl-CoA can also serve as a substrate for chalcone synthase (CHS), an enzyme that catalyzes the initial step toward flavonoid biosynthesis. If catalyzed by CHS, the metabolic flux will instead be directed toward the production of flavonoids, including flavonols, anthocyanins, and tannins [58, 59]. Thus, competition between HCT and CHS for the same substrate makes these two enzymes highly influential in controlling the metabolic flux toward either monolignol or flavonoid biosynthesis.

Lignin downregulation by RNAi-mediated gene silencing of HCT in *A. thaliana* resulted in a hyperaccumulation of flavonoid products [32]. The observed increase in flavonoid biosynthesis was suggested to be a consequence of the metabolic flux being redirected away from the lignin pathway in the absence of HCT, and into the flavonoid pathway through CHS activity. HPLC profiling revealed that flavonoid products, most notably

flavonols and anthocyanins, accumulated in higher amounts in the HCT-downregulated plants compared to control plants. HCT-downregulated plants also exhibited a dark purple coloration of the leaves relative to the controls as a result of the anthocyanin accumulation. Similarly, downregulation of HCT in alfalfa (*Meticago sativa* L.) also resulted in an increase in flavonoid biosynthesis and an accumulation of anthocyanins, which were suggested to be a result of metabolic spillover from suppressed lignin biosynthesis into the flavonoid pathway [31]. In addition to the evidence supporting a metabolic spillover into flavonoid metabolism, the expression of several flavonoid biosynthetic pathway genes were induced in the HCT-silenced plants; the activation of these genes could also be contributing to the observed flavonoid accumulation. A significant increase in coumaric acid was also observed in HCT-silenced plants; researchers hypothesized that the accumulating HCT substrate, *p*-coumaroyl-CoA, may either undergo hydrolysis into coumaric acid or be redirected into the flavonoid biosynthetic pathway.

***P-coumarate 3-hydroxylase***

After being synthesized by HCT, p-coumaroyl shikimate is converted into caffeoyl shikimate by *p*-coumarate 3-hydroxylase (C3H) [60, 61]. Downregulation of C3H in hybrid poplar (*Populus grandidentata* × *Populus alba)* resulted in the accumulation of soluble secondary metabolites, the majority of which were p-coumaric acid-derived phenylglucosides, including *O*-*p*-coumaroyl-β-D-glucoside and populoside, grandidentatin, and trichocarposide. The researchers hypothesized that the decreased C3H activity could cause p-coumarate to be diverted into ester-linked glucosides, which

would prevent a potentially toxic accumulation of this substrate by allowing it to be mobilized to the phloem [33].

### *Caffeoyl-CoA O-methyltransferase (CCoAOMT)*

Caffeoyl-CoA *O*-methyltransferase (CCoAOMT) methylates caffeoyl-CoA to feruloyl-CoA, as well as 5-hydroxyferuloyl-CoA, to sinapoyl-CoA. This enzyme, along with caffeic acid 3-O-methyltransferase (COMT), is involved in catalyzing the methylation of the monolignol precursors [62, 63]. Downregulation of CCoAOMT in poplar resulted in a redirection of the metabolic flux away from monolignol biosynthesis, and into the pathway leading from caffeic acid to sinapic acid. The increased flux into this pathway resulted in an increased synthesis and accumulation of phenolic acid glucosides in transgenic plants relative to controls, including $O^3$-β-D-glucopyranosyl-caffeic acid , $O^4$-β-D-glucopyranosyl-vanillic acid, and $O^4$-β-D-glucopyranosyl-sinapic acid [64]. Similarly, downregulation of CCoAOMT in alfalfa resulted in an accumulation of caffeoyl glucosides [65].

### *Cinnamoyl-CoA reductase*

Cinnamoyl-CoA reductase (CCR) catalyzes cinnamoyl-CoA esters to their corresponding cinnamaldehydes, which is considered to be the first step in the monolignol-specific branches of the lignin biosynthetic pathway [66, 67, 68].For most species, the predominant role of CCR in lignin biosynthesis is the reduction of feruloyl-CoA into

coniferaldehyde [49]. In *A. thaliana*, poplar, and tobacco, suppressed CCR activity has been shown to affect the soluble phenolics composition, most notably by increasing the accumulation of various ferulate derivatives. In studies with *A. thaliana* mutants deficient in CCR, a decreased flow of metabolites into lignin biosynthesis was accompanied by a reduction in sinapoyl malate biosynthesis, and an increased flow toward the synthesis of feruloyl malate, as well as an incorporation of ferulic acid into the lignin polymer [69]. Two possible mechanisms employed by plants to avoid the accumulation of monolignol precursors is by sequestering them into storage metabolites or incorporating them into the cell wall structure [70]. It was hypothesized that the CCR-deficient mutants avoid a potentially toxic buildup of feruloyl-CoA, the preferred substrate of CCR, via one or both the following redirection mechanisms: by hydrolyzing feruloyl-CoA into ferulic acid, which is further processed into feruloyl malate, or by transferring feruloyl-CoA-derived ferulic acids to the cell wall and incorporating them into the lignin structure [69]. Transgenic poplar and tobacco plants with CCR-downregulation exhibited modifications to the soluble phenolics composition similar to those observed in *A. thaliana* CCR mutants [39, 40, 71]. In poplar, CCR downregulation was associated with an overall increase in the soluble phenolics content, most notably in the levels of ferulic and sinapic acid esters, relative to control plants [40]. In addition, the concentration of ferulic acid in transgenic tissues increased relative to coniferaldehyde. It was suggested that the decreased conversion of feruloyl-CoA into coniferaldehyde in CCR-downregulated plants caused a diversion of the metabolic flow away from lignin biosynthesis, which was compensated for by an increased flow toward the synthesis of ferulic acids. Similar to

what was observed in the CCR mutants of *A. thaliana*, NMR and thioacidolysis analyses of CCR-downregulated poplar indicated that some of the accumulating ferulic acid was transported to the cell wall and cross-coupled with lignin. Transgenic tobacco with suppressed CCR activity also exhibited an increased metabolic flux toward the production of ferulic acid and sinapic acid relative to control plants [39, 71]. In one of these studies, an increase in glycosylated and quinylated derivatives of feruloyl-CoA was observed in transgenic plants relative to their controls [39]. It was proposed that the accumulating feruloyl-CoA could be quinylated or redirected toward the synthesis of ferulic acid. The ferulic acid could then be partially converted to sinapic acid, and detoxification of these acids could occur via glycosylation. Increased transcript levels of 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP), a regulator of the carbon flux into shikimate metabolism, indicated that the metabolic flux may also be partially redirected toward the shikimate pathway.

***Caffeic acid 3-O-methyltransferase***

Caffeic acid 3-O-methyltransferase (COMT) is a member of the *o*-methyltransferases, a family of enzymes which catalyze the methylation of various secondary metabolites in the phenylpropanoid pathway [72]. In lignin biosynthesis, COMT is primarily responsible for catalyzing the *O*-methylation of the 5-hydroxyl group of 5-hydroxyconiferaldehyde to produce sinapaldehyde [37]. A suppression of COMT enzyme activity has been shown to affect the phenolics profiles of *A. thaliana*, tobacco, and switchgrass [73, 36,36, 74]. *A. thaliana* mutants deficient in COMT activity accumulate significantly less sinapoyl

malate relative to controls [36, 73]. One of these reports also indicated a significant increase in hydroxyferuloyl malate in COMT mutants compared to controls; this compound is a derivative of the COMT substrate, 5-hydroxyconiferaldehyde [36]. In switchgrass, RNAi-mediated gene silencing of COMT resulted in an accumulation of phenolic acids and aldehydes, most notably in ferulic acid, 5-hydroxyferulic acid, and ferulic acid-glycoside conjugates, vanillin, and 5-hydroxyconiferaldehyde. Additionally, a novel monolignol-like metabolite was observed in the transgenic plants, identified as trans-3, 4-dimethoxy-5-hydroxycinnamyl alcohol (iso-sinapyl alcohol) [74].

***Cinnamyl alcohol dehydrogenase***

Cinnamyl alcohol dehydrogenase (CAD) catalyzes the last step in the monolignol-specific pathway by reducing cinnamaldehydes into their corresponding cinnamyl alcohols [68]. In switchgrass, downregulation of CAD resulted in a 40-170% increase in the level of chlorogenic acid (caffeoyl quinic acid) in most of the transgenic lines relative to the control [17]. Downregulation of CAD in flax (*Linum usitatissimum* L.) led to an increase in total phenolic content, including a 2-fold increase in ferulic acid and thirty percent increase in *p*-coumaric acid compared to the control plants [75]. In CAD-downregulated tobacco, an increased metabolic flux toward the production of soluble phenolics was also observed, particularly in levels of syringic, ferulic, *p*-coumaric, and sinapic acids [76]. Additionally, ferulic acid increased by 58-fold in the transgenic plants relative to the wild-type controls. Another study with CAD-downregulated tobacco reported an increase in the accumulation of the two substrates of CAD, coniferaldehyde

and sinapaldehyde, in the transgenic plants relative to controls [39]. Additionally, it was suggested that CAD downregulation may also result in a partial flux toward the shikimate pathway, as indicated by increased transcript levels of DAHP synthase [39].

### *MYB transcription factors*

Rather than silencing the expression of individual lignin biosynthetic genes, an alternative approach for decreasing lignin in cell walls is to manipulate the activity of transcription factors that act as repressors of lignin genes. MYB proteins comprise a diverse family of transcription factors that play important regulatory roles in various plant functions. The R2R3-MYB family of transcription factors has been described as playing an important role in the regulation of lignin biosynthetic genes [5]. The activities of R2R3-MYB transcription factors are generally not restricted to lignin biosynthesis, but rather have an influence on gene expression in multiple pathways within the phenylpropanoid network, including phenolics, anthocyanins, lignins, and flavonols [77]. *A. thaliana* plants with over-expression of ZmMYB42, an R2R3-MYB transcription factor derived from maize (*Zea mays*), exhibited a suppression of several lignin biosynthetic genes and a corresponding decrease in total lignin content relative to control plants [38]. Additionally, these plants were reported to have a 66% reduction in total phenolic content, mainly from a significant reduction in flavonols. Suppression of flavonoid biosynthesis was attributed to the negative regulation of ZmMYB42 on two major flavonoid pathway genes. In addition to flavonoid and lignin suppression, these plants also exhibited lower levels of sinapoyl malate compared to controls. This reduction

in sinapoyl malate biosynthesis could be related to the negative regulation of ZmMYB42 on the expression of C4H, ferulate 5-hydroxylase (F5H), and COMT, as a reduced expression of these genes in mutant plants has been associated with a decrease in sinapoyl malate synthesis [73, 78]. Additionally, this reduction could also be explained by the negative regulation of ZmMYB42 on aldehyde dehydrogenase, the enzyme catalyzing the initial step toward sinapoyl malate biosynthesis [38]. *A. thaliana* plants expressing another R2R3-MYB transcription factor, ZmMYB31, also exhibited a significant decrease in lignin content and a reduction in sinapoyl malate biosynthesis relative to control plants [34]. However, in contrast to the ZmMYB42 plants, there was an increase in flavonoid biosynthesis in ZmMYB31 over-expressing plants relative to controls. This increase in flavonoid biosynthesis was accompanied by an accumulation of anthocyanins in transgenic tissues. The researchers concluded that the ZmMYB31 transcription factor strongly influence carbon partitioning among various branches of the phenylpropanoid pathway, and that over-expression of this transcription factor in *A. thaliana* results in a reduced flux of carbon into lignin and sinapoyl malate biosynthesis, and redirects the flux into flavonoid biosynthesis [34].

## Effects of lignin modification on plant stress

As discussed above, suppressing single or multiple lignin biosynthetic steps can lead to the accumulation or suppression of other secondary metabolites. Many of these compounds play important roles in plant-environment interactions, and altering their biosynthesis can influence the susceptibility of plants to stresses. Additionally,

transcriptomic and proteomic studies have indicated that lignin modifications can trigger an increase in the expression of stress response-related genes [31, 34, 39, 40], and in one report there was a broad upregulation of defense-related genes that was associated with in an enhanced tolerance to biotic and abiotic stresses [31]. The exact mechanisms triggering changes in gene expression in lignin-modified plants are not yet clearly understood. The reduction in lignin content may trigger signaling pathways that cause the activation of stress genes [34]. One hypothesis is that the activation of stress-related genes could be triggered by changes to the structural organization of the secondary cell wall, either by mimicking cell wall wounding or pathogen damage [39,40], or by facilitating the release of primary cell wall polysaccharide components that act as elicitors of defense responses [31]. In addition there is accumulating evidence that altering cell wall integrity can induce long-term modifications to gene expression, including the constant activation of defense-related genes [79, 80].

***Biotic stress-related effects***

Several phenolic compounds play essential roles in mediating plant-microbe interactions [81], and modifications to the phenolic profile resulting from altered phenylpropanoid metabolism have been shown to influence such interactions in mutant and transgenic plants with suppressed lignin biosynthesis. Hydroxyferuloyl malate, a derivative of the COMT substrate, accumulated in *A. thaliana* mutants deficient in COMT activity [36]. Elevated levels of this compound were correlated with an enhanced resistance to the downy mildew-causing oomycete pathogen, *Hyaloperonospora arabidopsidis*, and an

inhibition of asexual sporulation was observed in the COMT mutant plants compared to wild-type controls. *In vitro* assays showed that application of hydroxyferuloyl malate to the oomycete pathogen enhanced sexual reproduction and weakened mycelium vigor [36]. Additionally, transgenic tobacco plants with downregulated COMT activity developed significantly smaller tumors than control plants when inoculated with *Agrobacterium tumefaciens*, a soil bacterium that causes crown gall disease. These plants had lower *Agrobacterium* virulence (*vir*) gene-inducing activities and lower levels of soluble phenolics including acetosyringone, a known elicitor of *vir* expression in *Agrobacterium* [36].

An upregulation of pathogen stress-related genes in absence of an actual infection has been observed in poplar, *A. thaliana*, and alfalfa plants with modified lignin biosynthesis. In CCR-downregulated poplar, increased transcript levels of a U-box domain protein resembling the CMPG1 protein triggered by fungal elicitors in *Petroselinum crispum* were observed relative to control plants [40]. In *A. thaliana* plants over-expressing the ZmMYB31 transcription factor, a proteome analysis showed increased levels of osmotin relative to control plants [34]; osmotin is a pathogenesis-related (PR) protein that primarily functions as a plant defense protein by providing resistance against a variety of fungal pathogens [82]. Downregulation of HCT in alfalfa resulted in a constitutive activation of defense responses, and a transcriptome analysis of stem tissue revealed that the expression of various PR transcripts was 5- to 56-fold higher in transgenic plants than in controls [31]. It was suggested that this constitutive PR gene expression could be

related to the altered cell wall integrity. Fragments of pectin, a galacturonic acid-rich component of plant primary cell walls, were shown to be more easily released from the secondary cell wall in transgenic alfalfa compared to controls [31]. Previous studies have shown that galacturonides can trigger defense responses in several plant species [83, 84, 85]. In addition, these plants had higher levels of salicylic acid (SA) relative to controls, a biotic defense-related phytohormone that has previously been shown to stimulate the upregulation of PR proteins in response to infection and enhance resistance to a wide range of pathogens [31, 86]. Levels of SA were correlated with the amount of extractable pectin in the cell wall. SA can be synthesized either from cinnamate by PAL activity, or from chorismate by isochorismate synthase activity, and the production of SA through the isochorismate pathway has been observed in *A. thaliana* plants during defense response [87]. Therefore, it was hypothesized that the continuous leaching of these pectic elicitors from the cell wall as a result of reduced lignification could trigger the production of SA through the isochorismate pathway, and high levels of SA could then stimulate the production of PR proteins responsible for the observed constitutive defense response [88]. Since constitutive expression of PR proteins in plants has previously been shown to confer resistance to a wide range of pathogens [89, 90], transgenic alfalfa plants were exposed to alfalfa anthracnose (*Colletotrichum trifolii*) to test whether elevated levels of PR transcripts would translate into enhanced pathogen resistance. Incidence and severity of necrotic legions were significantly lower in HCT-downregulated plants compared to the controls [31].

### *Abiotic stress-related effects*

#### *Oxidative stress, photorespiration, and UV-sensitivity*

Lignin modifications have been shown to be associated with the activation of genes involved in oxidative stress responses in tobacco, poplar, and *A. thaliana*. In tobacco, transgenic plants had elevated transcript levels of metallothionein and glutathione S-transferase (GST), two enzymes involved in detoxifying oxidative stress metabolites, along with heat shock transcription factors that promote transcription of genes involved in protection against oxidative stress damage [39, 91, 92]. In addition to oxidative stress-related transcripts, an increase in the abundance of photorespiratory-related transcripts and metabolites were observed in transgenic plants relative to controls, and gas-exchange analyses confirmed that the transgenic plants exhibited elevated photorespiration. This elevated photorespiration was suggested to be caused by an enhanced efficiency of photosystem II (PSII), the first protein complex in the light-dependent reactions of photosynthesis. Gas exchange analyses indicated that the increased efficiency of PSII in the transgenic plants was not associated with a change in photosynthetic $CO_2$ assimilation. Therefore, researchers hypothesized that the transgenic plants absorbed more light energy than could be used for photosynthesis, and the observed increase in photorespiration could be a protective mechanism against light-induced damage to the photosynthetic apparatus [39]. An accumulation of $H_20_2$ was also observed in the leaves of the CCR-downregulated plants, and lesions developed that resembled those observed in catalase-deficient tobacco grown under high-light conditions. It was hypothesized that photorespiration, specifically the elevated levels of photorespiratory $H_20_2$, could be

triggering the oxidative stress phenotype. An alternative hypothesis for the observed oxidative stress phenotype suggested that the altered cell wall structure could be actively inducing the expression of oxidative stress-related genes as part of a wound-like response. In support of this hypothesis, metabolomic profiling showed an increase in the levels of feruloyl tyramine in CCR-downregulated plants compared to the controls [39]. In solanaceous plants, feruloyl tyramine has been shown to accumulate at the site of wounding or pathogen attack in order to reinforce the cell wall [93]. Similar to that observed in tobacco, CCR-downregulation in poplar appeared to trigger a wound-like stress response and a corresponding increase in the expression of oxidative stress-related transcripts, also hypothesized to be induced by a defective cell wall [40]. Additionally, these plants had significantly higher levels of transcripts encoding for a PSII reaction center protein and a glutamine synthetase compared to controls, indicating a possible connection between oxidative stress and photorespiration similar to the one described in CCR-downregulated tobacco [40]. In *A. thaliana*, proteome analyses indicated that plants with over-expression of ZmMYB31 had increased levels of enzymes involved in protecting against oxidative stresses compared to control plants. ZmMYB31 also downregulates the expression of aconitase, an enzyme that plays a role in mediating oxidative stress and associated cell death, and it has been reported that mutant *A. thaliana* plants deficient in this enzyme are more tolerant to oxidative stress [34, 94].

The suppression of lignin biosynthesis has also been shown to affect the production of secondary metabolites that function as UV-protectants. UV light-induced stress can

induce the synthesis of flavonoids in epidermal tissues of plants, where they function as a protective screen against UV damage and subsequent cell death by absorbing light in the UV-B range [59, 95]. Sinapoyl malate and other sinapate esters have also been recognized as important UV protectants in *A. thaliana* [95]. In CCR-deficient mutant plantlets of *A. thaliana*, a substantial reduction in sinapoyl malate biosynthesis was observed relative to control plants [69]. Exposure to UV light resulted in slower growth of the mutant plantlets relative to controls, and their leaves were yellow, indicating an increased sensitivity to UV light. Similarly, ZmMYB42 over-expression in *A. thaliana* resulted in the downregulation of several phenylpropanoid pathway genes including those involved in sinapoyl malate and flavonoid biosynthesis [38]. As a result, transgenic plants were more sensitive to UV light, as indicated by a yellowing of the leaves relative to control plants [38]. *A. thaliana* plants with over-expression of another MYB transcription factor, ZmMYB31, also had lower levels of sinapoyl malate compared to controls [34]. However, unlike the ZmMYB42 plants, ZmMYB31 over-expression resulted in an increase in levels of UV-protecting flavonoids. Despite this increase in flavonoids, transgenic *A. thaliana* plants were still highly sensitive to UV radiation compared to controls, displaying an upward leaf curling phenotype resembling those observed in mutant plants deficient in UV-protectant compounds [34, 95]. The researchers concluded that the over-expression of this transcription factor in *A. thaliana* resulted in a reduced flux of carbon into lignin and sinapoyl malate biosynthesis, and a redirection of the flux into flavonoid biosynthesis [34].

*Water stress*

In HCT-downregulated alfalfa, transcriptome profiling revealed an upregulation of abiotic stress-related transcripts, the majority of which were heat and drought stress-related [31]. Metabolomic analyses of transgenic plants also showed elevated levels of abscisic acid (ABA), a phytohormone that plays a central role in sensing water deficiency and activating the expression of drought stress-related genes [31, 96]. To investigate whether the observed changes in drought stress-related gene expression were associated with an enhanced tolerance to drought, plants were deprived of water for a period of nine days. Transgenic plants exhibited fewer symptoms of drought stress than controls and were able to recover completely after five days of rehydration, while control plants showed extensive damage and did not survive. Leaf water potential measurements showed that water potential in the transgenics decreased at a slower rate than the controls throughout the experiment. Additionally, changes that are commonly associated with adaptation to drought stress, including reduced leaf transpiration rate and increased number of stomatal cells, were observed in transgenic leaf tissue [31, 97].

## Perspectives and conclusion

The use of systems biology-based approaches that incorporate information at the transcript, protein, and metabolite level are continually improving our understanding of how plants respond to altering lignin. It is important to examine and understand these broader effects of lignin modification since the commercial viability of altered-lignin bioenergy feedstocks will depend not only on improved biofuel traits, but also on their

ability to perform at least as well as their non-transgenic counterparts in the field. This performance includes optimal growth potential in the absence of stress, the ability to tolerate abiotic stresses, and the ability to resist pests. Vulnerability on any of these fronts would likely make modified feedstocks an undesirable risk to farmers. Therefore, field-based experiments to characterize lignin-modified feedstocks in these crucial areas are absolutely required.

To date, most of the research with transgenic low-lignin feedstocks grown in the field has been done in trees and forage crops for evaluating traits related to improved pulping or digestibility, respectively [6, 98]. More recently, saccharification efficiency has been assessed in field-grown bioenergy crop species [21, 23, 99]. Such studies have provided valuable insight into the impacts of altered lignin content on susceptibility to biotic and abiotic stresses in a field setting. In a four-year field evaluation of poplar with reduced lignin content for improved pulping performance, there was no effect of lignin modification on the susceptibility to insect herbivory or rust (*Melampsora sp.*) [6]. Similarly, field-grown COMT-downregulated sugarcane was not more susceptible to orange rust (*Puccinia kuehnii*) compared with non-transgenic controls [21].On the other hand, COMT-downregulation in field-grown perennial ryegrass plants led to an increased susceptibility to rust (*Puccinia* species) relative to control plants [100 ]. In addition to examining biotic stress interactions in lignin-modified crops, abiotic stress-related effects have also been assessed in the field. A two-year field study of low-lignin poplar with downregulation of 4CL found that the transgenic events with the strongest reduction in

lignin content were associated with significantly impaired xylem transport efficiency, resulting in frequent shoot dieback despite being watered regularly [101]. Interestingly, further studies with these plants found that the impaired xylem water conductivity in the lignin-downregulated lines was a result of accumulating phenolics and tyloses that were being deposited in xylem vessels and impeding water transport [102]. In addition to impairment of water transport, the reduction in lignin content in transgenic poplar lines was also associated with decreased wood strength and stiffness, and an increase in the formation of tension wood [103]. Taken together, the outcomes of these field trials suggest that some types of lignin modifications may be more preferable than others for preserving the growth and viability of low-lignin crops in the field, and this will likely vary from species to species. More extensive field studies are necessary in order to gain a better idea of the types of lignin modifications that will be best tolerated by plants in their environment. Particularly, the indirect effects of modified lignin biosynthesis on secondary metabolism and stress-related gene expression should be further explored under field conditions, as these factors could significantly impact the susceptibility of plants to stresses in an agronomic setting.

In absence of pertinent field data, we can speculate on the potential agronomic effects of altered stress-related metabolism in low-lignin bioenergy feedstocks. As previously discussed, modified lignin biosynthesis can lead to the differential accumulation or suppression of various phenylpropanoid pathway intermediates, some of which have been shown to influence plant-microbial interactions under controlled environmental conditions. Therefore, it is possible that this altered biochemistry in feedstocks could

influence interactions between the plant and bacterial or fungal pests in the field. In particular, levels of lignin precursors such as p-coumaric acid, ferulic acid, and sinapic acid were shown to accumulate in plants with suppressed lignin biosynthesis; *in vitro*, these metabolites have been shown to have antibacterial and antifungal properties [104]. In switchgrass, the downregulation of CAD led to an accumulation of chlorogenic acid [17]; higher levels of chlorogenic acid have been shown to correlate with increased plant resistance to bacterial pathogens and insect herbivory [105, 106]. In addition to influencing plant-microbial interactions, biochemical changes in the plant tissue as a result of altered secondary metabolism could influence the palatability of plants to insects. While the relationship between total lignin content and susceptibility to insect herbivory is unclear [107], a recent field study comparing switchgrass lines with naturally varying lignin levels suggested that other factors, such as the accumulation of compounds that interfere with nutrition, may be more important than overall lignin content in explaining resistance to insect herbivory [108]. In maize, higher levels of p-coumaric and ferulic acids were strongly associated with an increased resistance to infestations by maize weevil (*Sitophilus zeamais*) and stem borer (*Sesamia nonagrioides*), respectively, in the field [109, 110]. In transgenic poplar with downregulation of C3H, an accumulation of phenolic glucosides was observed. These compounds have previously been shown to deter the activity of fungi and insect pests of poplar, and it was suggested that the elevated levels could potentially enhance defense responses of transgenic poplar toward biotic pests [33]. A constitutive expression of PR genes has also been shown to influence stress interactions, and was shown to increase the tolerance of transgenic alfalfa

plants to anthracnose infection [31]; thus, a constitutive activation of biotic defense-related genes could potentially render plants more prepared for defending themselves against pathogens in a field setting.

Modifications to the lignin biosynthetic pathway have also been shown to suppress or enhance levels of secondary compounds that could influence plant acclimation to abiotic stresses. Plants have been shown to accumulate anthocyanins and/or flavonoids as a protective mechanism against salinity stress, UVB damage, cold temperatures, and water stress [111, 112]. As previously discussed, downregulation of lignin pathway genes can result in an increased or decreased synthesis of these compounds. Altered levels of these compounds could influence plant sensitivity to abiotic stresses in the field. A field study comparing salt-tolerant and salt-susceptible clones of sugarcane found that the accumulation of soluble phenolics, anthocyanins, and flavones were 3-fold higher in the tolerant clone, and these compounds were suggested to promote salinity tolerance by protecting cytoplasmic structures and chloroplasts from the damages of salinity stress [111]. In addition to their roles in salinity tolerance, levels of anthocyanins and some phenolic acids might play an important role in the acclimation of plants to cold stress, as they have been shown to accumulate in the leaf mesophyll cells in plants exposed to low temperatures [113]. *A. thaliana* with suppressed CCR activity, or with over-expression of MYB transcription factors, were shown to be more sensitive to UV damage due to the consequences of suppressed lignin on sinapoyl malate and/or flavonoid biosynthesis, as previously discussed. A recent study found that UV-B radiation, in addition to inhibiting

plant growth, can alter plant tissue biochemistry and result in a significant reduction in cell wall digestibility and the enzymatic release of sugars from the biomass [114]. Although this study was performed in a greenhouse, the results could have significant implications for ethanol production from field-grown low-lignin feedstocks that have been made more susceptible to the effects of UV radiation.

Given this knowledge, it is conceivable that alterations to phenylpropanoid metabolism and/or the upregulation of stress-related genes resulting from lignin modification could positively or negatively influence the agronomic performance of low-lignin transgenic feedstocks. It is also important to note that there can be significant metabolic costs associated with an increased allocation of energy into the production of defense-related secondary metabolites and constitutive expression of defense-related genes, such that a higher resistance to stresses through either these mechanisms could be associated with stunted growth phenotypes and consequent reductions in biomass yield [115, 116 ]. This potential trade-off between stress-resistance and biomass yield should be considered, since high biomass production is also an important trait for bioenergy feedstocks. In conclusion, extensive field evaluations and an improved understanding of the relationship between lignin and stress-related metabolism are necessary to ensure the sustainable growth of transgenic low-lignin bioenergy feedstocks in agronomic field environments.

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# Appendix A

## *Tables*

**Table A.1**. Summary of the effects of altered lignin biosynthesis in transgenic or mutant plants on the synthesis of phenylpropanoid-derived secondary metabolites (↑ = accumulation; ↓ = suppression).

| Gene | Type of modification | Plant | Effect on phenylpropanoid metabolism | Ref. |
|---|---|---|---|---|
| HCT | Downregulation | *A. thaliana* | ↑ flavonoids | 32 |
| | Downregulation | Alfalfa | ↑ flavonoids | 31 |
| | | | ↑ coumaric acid | 31 |
| C3H | Downregulation | Poplar | ↑ phenolic glucosides | 33 |
| CCoAOMT | Downregulation | Poplar | ↑ phenolic glucosides | 64 |
| | Downregulation | Alfalfa | ↑ caffeoyl glucosides | 65 |
| CCR | Mutant | *A. thaliana* | ↑ feruloyl malate | 69 |
| | | | ↓ sinapoyl malate | 69 |
| | Downregulation | Poplar | ↑ ferulic acid | 40 |
| | | | ↑ sinapic acid | 40 |
| | Downregulation | Tobacco | ↑ ferulic acid | 39, 71 |
| | | | ↑ sinapic acid | 39,71 |

**Table A.1**. Continued.

| Gene | Type of modification | Plant | Effect on phenylpropanoid metabolism | Ref. |
|---|---|---|---|---|
| COMT | Mutant | *A. thaliana* | ↓ sinapoyl malate | 36, 73 |
| | | | ↑ hydroxyferuloyl malate | 36 |
| | Downregulation | Switchgrass | ↑ ferulic acid | 74 |
| | | | ↑ 5-hydroxyferulic acid | 74 |
| | | | ↑ vanillin | 74 |
| | | | ↑ 5-hydroxyconiferaldehyde | 74 |
| | | | ↑ iso-sinapyl alcohol | 74 |
| CAD | Downregulation | Switchgrass | ↑ chlorogenic acid | 17 |
| | Downregulation | Flax | ↑ ferulic acid | 75 |
| | | | ↑ *p*-coumaric acid | 75 |
| | Downregulation | Tobacco | ↑ syringic acid | 76 |
| | | | ↑ ferulic acid | 76 |
| | | | ↑ *p*-coumaric acid | 76 |
| | | | ↑ sinapic acid | 76 |
| ZmMYB42 transcription factor | Overexpression | *A. thaliana* | ↓ flavonoids | 38 |
| | | | ↓ sinapoyl malate | 38 |
| ZmMYB31 transcription factor | Overexpression | *A. thaliana* | ↑ flavonoids | 34 |
| | | | ↓ sinapoyl malate | 34 |

# CHAPTER 3
# TWO-YEAR FIELD ANALYSIS OF REDUCED RECALCITRANCE TRANSGENIC SWITCHGRASS

A version of this chapter will be published in Plant Biotechnology Journal by Holly Baxter, Mitra Mazarei, Nicole Labbé, Lindsey Kline, Qunkang Cheng, Mark Windham, David G.J. Mann, Chunxiang Fu, Angela Ziebell, Robert Sykes, Miguel Rodriguez, Mark Davis, Jonathan Mielenz, Richard A. Dixon, Zeng-Yu Wang, C. Neal Stewart, Jr., under the following title:

Two-year field analysis of reduced recalcitrance transgenic switchgrass

This version of the manuscript was written by Holly L. Baxter, with the exception of the following sections in Materials and Methods: 'Cell wall composition by wet chemistry' and 'Fourier transform infrared spectroscopy' (written by Lindsey Kline), 'Analysis of COMT transcript levels' (written by Chunxiang Fu and Zeng-Yu Wang), and 'Rust Evaluation' (written by Qunkang Cheng). This manuscript was edited and revised by C. Neal Stewart, Jr. and Mitra Mazarei.

Holly Baxter performed the majority of the work involved in the field trial, including general field maintenance and observation, collection and preparation samples for all experiments, agronomic performance evaluations, ethanol fermentation experiments, and statistical analyses for all experiments.

## Abstract

Switchgrass (*Panicum virgatum* L.) is a leading candidate for a dedicated lignocellulosic feedstock due to its high biomass production, wide adaptation, and low agronomic input requirements. One major limitation of such bioenergy feedstock is the recalcitrance of the biomass to conversion into biofuels. The presence of lignin in the secondary cell wall is known to be a primary contributor to recalcitrance, limiting the accessibility of cell wall carbohydrates to enzymatic breakdown into fermentable sugars. Low-lignin transgenic switchgrass events produced by the downregulation of caffeic acid *O*-methyltransferase (COMT), a lignin biosynthetic enzyme, were analyzed in the field over a period of two growing seasons for cell wall composition, sugar release efficiency, ethanol yield, agronomic performance, and disease susceptibility. COMT transcript abundance, total

lignin content, and the syringyl to guaiacyl lignin monomer ratio were consistently lower in the COMT-downregulated plants throughout the duration of the field trial. At the end of year two, sugar release was improved by up to 34% and ethanol yield by up to 28% in both field-grown transgenic events relative to their controls. Additionally, these results were obtained using senesced plant material harvested at the end of the growing season, compared to the young, green tissue that was used in the greenhouse experiments. Furthermore, transgenic plants were not more susceptible than their controls to rust (*Puccinia emaculata*). The results of this study suggest that, after an initial period of establishment, improvements in sugar release and ethanol yield in low-lignin transgenic switchgrass can be achieved in the field with no negative impact on biomass yield or disease susceptibility.

# Introduction

Switchgrass (*Panicum virgatum* L.), a warm-season C4 perennial grass native to the prairies of North America, has been identified as a promising lignocellulosic biofuel feedstock due to its high biomass production, minimal water and nutritional requirements, widespread adaptability, and high net energy gain. In addition, switchgrass is relatively tolerant to a range biotic and abiotic stresses and provides numerous environmental benefits including reduced soil erosion, improved surface water quality, and the potential to reduce greenhouse gas emissions due to a high rate of carbon sequestration [1, 2, 3, 4]. Despite these advantages, high costs associated with the conversion of plant biomass into biofuels currently prevent lignocellulosic feedstocks such as switchgrass from being economically competitive fuel sources [5].

A major barrier to cost-efficient biofuel production from lignocellulosic plants is the resistance of the cell wall to chemical, microbial, or enzymatic deconstruction into fermentable monosaccharides, also referred to as cell wall recalcitrance [5]. The primary structural components of switchgrass cell walls are cellulose (30-40%), hemicellulose (25-35%) and lignin (15-20%) [6]. The cellulose microfibrils are integrated into a highly cross-linked matrix of lignin and hemicellulose [7]. Lignin is a complex phenolic polymer composed of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) lignin units, which are derived from the monolignols p-coumaryl, coniferyl, and sinapyl alcohol, respectively. Lignin contributes structural stability to the plant, aids in water transportation in vascular tissues, and protects the cell wall from pathogen penetration. Because it inhibits enzymatic and microbial access to fermentable cell wall

polysaccharides, lignin is known as a primary contributor to biomass recalcitrance. Recalcitrance can be influenced by lignin content as well as the relative abundance of the S and G lignin monomers [9, 10, 11, 12]. Expensive and energy-inefficient thermo-chemical pretreatment processes are generally required to disrupt the lignin-polysaccharide matrix and increase the accessibility of carbohydrates for hydrolysis prior to fermentation [13]. In addition to being costly, many pretreatment processes also generate degradation products that can be inhibitory to downstream microbial fermentation [14].

One approach to improve biofuel yields from lignocellulosic plants is by genetically modifying cell wall characteristics to develop feedstocks that are more amenable to enzymatic hydrolysis. Manipulating genes in the lignin biosynthetic pathway can decrease the total lignin content and/or modify its composition, which could reduce or eliminate the need for pretreatment prior to fermentation [9, 15]. The downregulation of lignin biosynthetic enzymes has been successful in improving saccharification in lignocellulosic grass species including Brachypodium (*Brachypodium distachyon*), sugarcane (*Saccharum* spp. hybrids), and switchgrass [16, 17, 18, 19, 20].

Caffeic acid *O*-methyltransferase (COMT) is an enzyme functioning late in the lignin biosynthetic pathway, catalyzing the *O*-methylation of the 5-hydroxyl groups of monolignol precursors. Studies with monocot and dicot species have demonstrated that the preferred substrates of COMT are 5-hydroxyconiferyl aldehyde and 5-hydroxyconiferyl alcohol, two metabolites involved in the synthesis of the S-monomer subunit sinapyl alcohol [21, 22]. Reduced COMT activity results in a decreased flux of

lignin precursors toward the synthesis of sinapyl alcohol, thus reducing the S/G lignin monomer ratio and the overall lignin content. These characteristics have been observed in COMT-deficient brown midrib mutants of maize (bm3) and sorghum (bmr12), as well as in transgenic plants with antisense or RNAi-mediated downregulation of COMT [17, 21, 23, 24, 25]. Consistent with these studies, greenhouse-grown $T_1$ generation transgenic COMT-RNAi switchgrass exhibited a reduction in total lignin content (11.4-13.4%) and the S/G lignin monomer ratio (44-46%). These plants, without pretreatment, also exhibited an increase in the enzymatic release of sugars (29-38%) and produced up to 42% more ethanol yield per unit of biomass than controls. With pretreatment, a 17-22% increase in enzymatic sugar release and 30-38% increase in ethanol yield per unit of biomass compared to controls was observed. Additionally, the reduced lignin content had no effect on morphology or biomass yield [18].

While the results of the greenhouse study are promising, no field trials of transgenic switchgrass with modified lignin biosynthesis have been reported to date. Given the important roles of lignin in plant development and stress-related processes, evaluating the performance of low-lignin plants in the field is essential. The synthesis and deposition of lignin is influenced by a range of biotic and abiotic stresses, including herbivory, pathogen infection, temperature stress, light and oxidative stress, water deficiency, and mechanical injury [26]. As lignin-modified plants respond and adapt to stresses in the field, the consequent physiological changes could cause results to vary from those observed in plants grown under greenhouse conditions. Considering this potential for variation, field trials are necessary to ensure that the altered lignin profiles and associated

gains in saccharification and ethanol yield can be maintained under field conditions. Additionally, these improvements need to be demonstrated in senesced biomass harvested at the end of the growing season, as this is the material that is used in commercial biofuel production [27].

There is currently limited knowledge available regarding the downstream effects of lignin modification on plant response to biotic stresses in a field environment. Therefore, another goal of this study was to assess disease susceptibility in COMT-downregulated switchgrass plants. Increased production and accumulation of lignin or lignin-related phenolic compounds at specific sites to protect the cell wall from penetration by insects and pathogens is a well-documented defense mechanism in plants [28, 29], and inhibiting the expression of genes involved in lignin biosynthesis has been shown to negatively interfere with plant stress responses to fungal and bacterial pathogens [30, 31]. Of particular relevance, the fungal pathogen *Puccinia emaculata* has been reported to cause rust disease in switchgrass and has recently been observed in agronomic fields in Tennessee [32] and Arkansas [33]. Switchgrass rust epidemics typically start at varying times of the season, but there has been a trend over the past four years for disease outbreaks to occur earlier each year [34]. Resistant switchgrass plants exhibit a hypersensitive response to inhibit the spread of rust infection [35], and previous studies have indicated that lignin and its precursors are essential for activating this response in several plant species [36]. Moerschbacker *et al.* [37] found that rust-resistant wheat plants treated with lignification inhibitors had increased growth of *Puccinia graminis* as a result of the reduced hypersensitive response to infection. Additionally, RNAi-mediated

transient gene silencing of COMT in perennial ryegrass led to an increased susceptibility of the plants to *Puccinia* rust infections under field conditions compared to control plants [38]. Therefore, downregulating COMT in switchgrass may increase sugar conversion efficiency and ethanol yields, but could simultaneously increase susceptibility to rust and cause stunted growth and biomass yield reductions.

The objectives of this study were: (i) to verify that RNAi-mediated silencing of COMT was stable in transgenic tissues and that the expected modifications to lignin content and composition were retained in field-grown plants, (ii) to characterize the chemical composition of the cell wall, and to assess whether the improved sugar release efficiency and ethanol yield were maintained in field-grown plants, and (iii) to investigate potential impacts of COMT-downregulation on agronomic performance and disease susceptibility.

## Materials and Methods

### *Generation of plants used in the experiments*

Generation of the plants used in this experiment is described in detail by Fu et al. [18]. Briefly, transgenic COMT events were generated by propagating seed-derived callus from the switchgrass cv. Alamo and transforming it using *Agrobacterium*-mediated transformation with an RNAi-hairpin loop construct containing a DNA sequence specifically designed to target suppression of the *COMT* gene. The most promising plants were regenerated from tissue culture and selected based on down-regulation of COMT expression level, reduced lignin content and S/G ratios. Each first generation transgenic

(T0) event was outcrossed to wild-type Alamo plants to obtain second generation transgenic (T1) plants containing the transgenic insert and null segregants which were used as non-transgenic controls. Plants were replicated by vegetative propagation from tillers in a greenhouse at the Noble Foundation, Ardmore, OK. Plants were shipped from the Noble Foundation to the University of Tennessee in September of 2010 following USDA-APHIS transportation guidelines, and maintained in D40 Deepots (16.4 cm diameter × 25 cm height) (Stuewe and Sons, Inc., Corvallis, Oregon, USA) in an environmentally controlled growth chamber under standard growing conditions (16/8-h photoperiod, 25-29°C) for 6 months prior to transplantation to the field.

***Field design and maintenance***

Transgenic, non-transgenic control, and border plants at approximately the E2-E4 growth stage (growth stages determined using the criteria described by Moore *et al.* [38]) were transplanted from Deepots (approximately 2-3 tillers per plant) to the field on May 11, 2011. The field was located at the University of Tennessee Plant Sciences Unit of the East Tennessee Research and Education Center (ETREC). The field layout consisted of a 22.9 m x 25.9 m plot (607.0 $m^2$) containing two independent transgenic events (COMT2 and COMT3) and two corresponding null-segregant control plants for each of these events. Ten replicates for each transgenic event and five replicates for each control (30 replicates in total) were distributed throughout the plot in a completely randomized design (Figure B.1; all figures and tables are located in appendix B). Nine vegetatively-propagated clones were planted within each replicate. Replicates were spaced 152.4 cm

apart with 76.2 cm between the nine plants within each single replicate. The investigated plants were surrounded by border plants to reduce any shading effects.

The field trial was conducted for two consecutive growing seasons in years 2011 and 2012 (pictures of field: Figure B.2). A soil pH and fertility test was performed by the Soil, Plant and Pest Center (University of Tennessee) and no fertilizer application or other modifications were recommended for either year. During the first growing season, newly transplanted plants were irrigated as needed, depending on the amount of rainfall, to ensure successful establishment. Irrigation was not used during the second growing season. No herbicides were applied; all weeds were removed from the field by hand or by tilling for both years. Weeding was required less frequently in the second growing season after the plants had become more established. Following USDA-APHIS guidelines, plants were monitored daily during the reproductive stage and emerging panicles were removed from all plants (transgenic, non-transgenic control, and border plants) at the R0-R1 growth stage by cutting the plant below the top node containing the inflorescence.

### *Analysis of COMT transcript levels*

*Sample collection*. Samples were collected from green plants in August in 2011 and 2012. All samples were collected at the same date and time for each year analyzed. Analyses were performed on four randomly selected replicates for each transgenic event, and four randomly selected replicates for each corresponding control. A single tiller at the R0 growth stage was chosen at random within each selected replicate. The tiller was cut below the top internode, and the top portion (with the top two leaves intact) was

immediately flash frozen in liquid nitrogen in the field, transported back to the laboratory on dry ice, and stored at -80°C until quantitative RT-PCR analysis.

*Quantitative RT-PCR analysis*. Total RNA was isolated from leaves using Trizol reagent (Promega, Madison, WI) and subjected to reverse transcription using an Omniscript® Reverse Transcription Kit (Qiagen, Valencia, CA) after incubation with RNase-free RQ1 DNase (Promega, Madison, WI) at 37°C for 10 min. The cDNA from reverse transcription was used for quantitative RT-PCR analysis. The gene-specific primers used for the qRT-PCR were: ACGTCGTCCGGTGCCAGATG (forward) and GTACGCCTTGTTGAACGGGAT (reverse). SYBR Green (Applied Biosystems, Foster City, CA) was used as the reporter dye. The cycle thresholds were determined using the ABI PRISM 7900 HT sequence detection system (Applied Biosystems, Foster City, CA). The qRT-PCR data were normalized using the levels of *ELF1A* transcripts.

***Green and senesced sample collection and preparation***

*Green tissue samples*. Samples were collected mid-growing season from green plants at the same developmental stage. The tallest tiller from each plant was selected and the whole tiller (including stem and leaves) was cut from 10-15 cm above the soil surface. For first year analyses, green tissue samples were collected in October of 2011 from plants at the E6 growth stage. For second year analyses, green tissue samples were collected in August of 2012 from plants at the R0 growth stage.

*Senesced tissue samples*. Whole aboveground senesced biomass was harvested after the killing frost in December of each year. After determination of dry weight yield, sub-samples were taken from each replicate to be used for the senesced tissue analyses.

All green and senesced tissue samples were oven-dried at 43°C for 96 h. The dried samples were allowed to equilibrate at ambient conditions prior to size reduction. The switchgrass samples were milled with a Wiley mill (Thomas Scientific, Model 4, Swedesboro, NJ) through either a 20-mesh (1.0 mm) or 40-mesh (0.425 mm) screen. The milling process was monitored closely for generation of heat and subsequent damage to the biomass. For the senesced tissue samples, the milled material was divided into smaller 100 g bags with a PT100 sample divider (Retsch, Hann, Germany) to ensure the composition of each fraction analyzed in this study was representative of that of the original material and without loss of material.

***Determination of lignin content and composition by py-MBMS***

For determination of lignin content and composition by py-MBMS, all 10 replicates for each transgenic event and all 5 replicates for each corresponding control were assessed. Green and senesced biomass samples were dried, ground to a 20-mesh (1.0 mm) particle size, and shipped to the National Renewable Energy Laboratory (NREL) for determination of lignin content and composition (S/G monomer ratio) by pyrolysis molecular beam mass spectrometry, following the protocol described by Sykes *et al.* [40]. Briefly, cell wall residues of the biomass were prepared by subjecting samples to a

hot water and ethanol extraction to remove soluble extractives and starch. Approximately 4 mg of biomass per sample were loaded in 80 ul stainless steel cups and pyrolyzed at 500˚C in a quartz reactor using a Frontier py2020 autosampler. The resulting pyrolysis vapors were analyzed using a custom Extrel single quadrapole molecular beam mass spectrometer. The relative intensities of the peaks identified as lignin precursors were summed to estimate total lignin content. S/G lignin monomer ratio was determined by summing the intensity of the syringyl peaks and dividing by the sum of the intensity of the guaiacyl peaks.

***Cell wall composition by wet chemistry***

For cell wall compositional analyses, all 10 replicates for each transgenic event and all 5 replicates for each corresponding control were assessed. Senesced samples were dried and ground to a 40-mesh (0.425) particle size. Sample fractions to be analyzed for chemical composition were first extracted in a Dionex (Sunnyvale, CA) Accelerated Solvent Extractor (ASE) 350, following the method described in National Renewable Energy Laboratory (NREL) Laboratory Analytical Procedure (LAP) "Determination of Extractives in Biomass" to remove non-structural extractives. In this process, 5 g of raw biomass containing <10% moisture were added to a 33mL extraction cell and sequentially extracted by pressurized water then ethanol under 1500 psi, 100°C, 5 min heating time, 7 min static time, at three static cycles. The material was then allowed to air dry to less than 10% moisture content by weight and a change in weight <1% in 24 h, using a sub-sample that was dried in a 105°C convection oven for a minimum of 4h to

determine the % of total solid. The extractives-free material was stored in polyethylene bags at ambient temperatures until further analyses were performed.

The quantification of cellulose, hemicellulose, lignin, and structural ash in the switchgrass was performed using the protocols developed by NREL, "Determination of Structural Carbohydrates and Lignin", "Determination of Total Solids in Biomass", and "Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples", using three replicates. The procedure is suitable for samples that do not contain extractives. A two stage acid-catalyzed hydrolysis was performed to fractionate the sample into soluble and insoluble matter, and the two fractions were separated through vacuum filtration and ceramic fine porosity filtering crucibles. The insoluble solids fraction consisted of acid-insoluble lignin and ash. The acid-insoluble lignin was quantified gravimetrically after combustion of the residue at 575°C for 24 h. The monomeric units of polysaccharides within the soluble liquid fraction were quantified via a Flexar high pressure liquid chromatography (HPLC) (Perkin Elmer, Shelton, CT) with a refractive index (RI) detector. The system was equipped with an Aminex HPX-87P carbohydrate column (300 x 7.8 mmID, 9 µm particle size) and deashing guard column (125-0118) from Bio-Rad (Hercules, CA), using deionized water at 0.25 mL/min at 85°C. Acetyl quantification was performed using a Bio-Rad Aminex HPX-87H organic acid column (300 x 7.8 mm ID, 9 µm particle size) at 55°C, using 0.1N sulfuric acid at 0.6 mL/min. The acid-soluble lignin content of the soluble liquid fraction was measured using a two-beam Lambda 650 series spectrometer (Perkin

Elmer), and this value combined with the gravimetric value for acid-insoluble lignin provided the total lignin content. The structural ash content of the extracted sample was determined gravimetrically after combustion of the non-ash materials at 575°C for 24h in a muffle furnace. A total of eight primary components were measured: glucan, xylan, galactan, arabinan, mannan, acetyl, structural ash, and lignin.

***Fourier transform infrared spectroscopy***

FTIR analyses were performed on all 10 replicates for each transgenic event and all 5 replicates for each corresponding control. Senesced samples were dried and ground to a 40-mesh (0.425) particle size. FTIR was utilized to collect spectral data on the raw biomass via a Spectrum One FTIR spectrometer (Perkin Elmer). Spectra from 4000 to 600 $cm^{-1}$ were collected in absorbance mode with 8 scans per spectrum at 1 $cm^{-1}$ resolution using an ATR attachment. Ten spectra were collected for each sample. An ATR correction was employed to account for less than optimal sample/crystal contact, and normalization was performed in the Spectrum software. The index of crystallinity of each sample was calculated by the intensity ratio between the bands of 1422 and 899 $cm^{-1}$, assigned to $CH_2$ bending mode and deformation of anomeric CH, respectively [41].

***Sugar release***

For the sugar release assays, all 10 replicates for each transgenic event and all 5 replicates for each corresponding control were assessed. Green and senesced biomass samples were

dried, ground to a 20-mesh (1.0 mm) particle size, and shipped to NREL for determination of sugar release efficiency following the high throughput pretreatment and enzymatic hydrolysis protocol described by Studer *et al*. [42]. Briefly, cell wall residues were prepared by removing soluble extractives and starch. Samples were then loaded in triplicate into a custom-made 96 well metal plate. A hot water pretreatment at 0.5-1%w/w solids loading was conducted at 180°C for 17.5 min, using condensing steam for heating. After pretreatment, enzymatic hydrolysis was performed in the well plate on the pretreated slurry by incubation with Ctec2 enzyme cocktail (70 mg protein/g biomass) at 40°C for 72 h. Following a 72-hour hydrolysis, the amount of glucose and xylose released to liquid was measured by HPLC. Sugar release data were reported in grams of sugars released per gram of cell wall residues.

***Quantitative saccharification, pretreatment, and fermentation***

Ethanol analyses were performed on senesced samples. Five randomly selected replicates for each transgenic event and all five replicates for each corresponding control were dried and milled to a 20-mesh (1.0 mm) particle size. Prior to fermentation, all samples were pretreated at 180°C for 7.5 min in 0.5% $H_2SO_4$ following the procedure described by Fu *et al*. [18]. Briefly, dry milled biomass was soaked in a nine-fold excess of 0.5% $H_2SO_4$ for 18 h and centrifuged at 8000 rpm, 30 min, and 4°C in a Sorvall RC-5B (DuPont instruments) centrifuge. After removing the supernatant, the biomass was loaded at approximately 2.5 g dry biomass per cylinder into 10 cm x 1 cm hastelloy steel tubular pretreatment reactors (Industrial Alloys Plus, Inc.). Cylinders were placed in boiling

water for 2 min before heating to 180°C in a sand bath (Omega FSB1: Techne Co.) for 7.5 min. Directly after removing from the sand bath, biomass was cooled by placing cylinders in an ice bath for 2 min. Pretreated biomass was washed with 100 mL Milli-Q water per gram dry biomass and stored in -20°C until fermentation. Following the protocol described in ASTM E 1758–01 (ASTM 2003) and HPLC method (NREL/TP 51–42623), quantitative saccharification was performed on the pretreated biomass in triplicate to obtain the cellulose contents. Simultaneous saccharification and fermentation was conducted in triplicate for each replicate following the procedure described by Fu *et al*. [18], using *Saccharomyces cerevisiae* D5A (ATCC200062) and enzymes Spezyme CP and Accellerase BG provided by Genencor International. The procedures used to monitor weight loss, and a description of the bottles used for the fermentation, have been previously described by Mielenz *et al*. [43].

***Agronomic performance***

Since plants were at varying growth stages after being transplanted to the field in May 2011, all above-ground biomass was cut back in August to synchronize growth and allow for uniform comparisons. After a sufficient period of re-growth, tiller height and plant width were measured in October of 2011. Tiller height was measured by selecting the tallest tiller (approximately E6 growth stage) from each individual plant and measuring from the base of the soil to the tip of the top leaf. Plant diameter was determined by measuring around the mid-section of each whole plant. In the second year, new shoots began to emerge in March of 2012. Tiller height and plant width were measured in

August of 2012, following the same criteria described above for first year measurements, on plants at the R0 growth stage.

For both growing season, plants remained green until mid-to-late November, after which they began to senesce. The first frost occurred on November 1st for both 2011 and 2012. Tiller number and dry weight biomass yield were determined after plants had completely senesced in December. All aboveground senesced biomass was harvested and transported back to the laboratory following USDA-APHIS transportation guidelines. The biomass was oven-dried at 43°C for 96 h. After equilibration at ambient temperature, aboveground dry biomass was weighed to determine total dry weight biomass yield.

***Rust evaluation***

Plants were evaluated for rust susceptibility once per week during the second growing season, from June 22, 2012 through September 20, 2012. Three plants within each replicate were selected at random, and a single tiller from each plant was rated for disease each week. All live leaves on the selected tillers were rated using a numerical system to visually estimate the percentage of the top of the leaf surface covered by rust uredia, where 0=0%, 1≤5%, 2≤10%, 3≤25%, 4≤50%, 5>50%. To evaluate rust susceptibility differences at each individual time point, a *t*-test was used to compare each transgenic event to its corresponding control.

### *Statistical analyses*

For each independent event, the transgenic group was compared to its corresponding control group using the PROC TTEST procedure in SAS version 9.3 (SAS Institute Inc., Cary, NC). The data for each analysis fit the assumptions of a normal distribution (Shapiro-Wilk test >0.05), and variances were equal (Levene test >0.05). Differences were considered significant where p-values (based on a two-sided *t*-test) were less than 0.05.

## Results

### *Analysis of COMT transcript levels*

COMT transcript abundance for transgenic T1-generation field-grown events, COMT2 and COMT3, were analyzed by qRT-PCR. COMT transcript levels were reduced by up to 97% in both transgenic events for 2011 and 2012 (Figure B.3).

### *Lignin content and composition*

Pyrolysis molecular beam mass spectrometry (py-MBMS) of cell wall residues were used to estimate the total lignin content and the S/G lignin monomer ratio for whole tillers at the same developmental stage harvested mid-growing season (green tissue), and whole above-ground biomass harvested at the end of the growing season (senesced tissue) each year. COMT-downregulation resulted in 10.1-14.5% less lignin in biomass harvested at the green-tissue stage for both years (Figure B.4a, c). The green-tissue S/G ratio was 22-

27% less than that of controls in year one, and 33-36% less in year two (Figure B.4b, d). For biomass harvested after senescence, an 8.4-10.6% decrease in lignin content was observed in transgenic events relative to their controls in year one (Figure B.5a), accompanied by an 19-20% reduction in the S/G ratio (Figure B.5b). In year two, senesced tissue from transgenic events had an 11.6-12.0% decrease in lignin content and a 35-39% reduction in the S/G ratio compared to the controls (Figure B.5c, d).

***Cell wall composition and index of cellulose crystallinity***

To investigate potential changes in the content and/or structure of other cell wall constituents, the chemical composition of the cell wall and the index of cellulose crystallinity were determined for field-grown samples harvested after senescence each year. Sugar profiles were similar between transgenic and control plants harvested in the first year. Cellulose and hemicellulose comprised approximately 39% and 28-30% of the cell wall, respectively. A 16.4% increase in structural ash was observed in event COMT3 compared with its control (Table B.1). No differences in cellulose structure, as estimated by the index of cellulose crystallinity, were observed between transgenic and control plants (Figure B.6).

In second-year harvested samples, cellulose and hemicellulose accounted for 38-39% and 30-32% of the cell wall. Similar to the first year results, no differences in cellulose content were detected; however, transgenic events had slightly higher hemicellulose levels (6.4-7.4%), primarily due to an increase in xylose. Additionally, an 11.6% increase

in acetyl content was observed in event COMT3 (Table B.1). The index of cellulose crystallinity was reduced by 18.6% in event COMT2, and 16.0% in event COMT3, compared with controls (Figure B.6).

***Sugar release efficiency***

To evaluate the impact of COMT-downregulation on cell wall saccharification, sugar release by enzymatic hydrolysis was determined for whole tillers at the same developmental stage harvested mid-growing season (green tissue), and whole above-ground biomass harvested at the end of the growing season (senesced tissue) each year. Sugar release assays were performed by subjecting cell wall residues to a hot water pretreatment, followed by a 72 hr incubation with hydrolyzing enzymes. The amounts of glucose, xylose, and total sugars (glucose and xylose combined) released per gram of dry biomass were determined for each sample. In year one, transgenic plants harvested at the green-tissue stage exhibited a 9.0 to 11.7% increase in total sugar release. Similarly, the total sugar release for green-tissue transgenic plants harvested in the second growing season was improved by 14.7-18.7% relative to the controls. Sugar release improvements in transgenic plants harvested after senescence were only observed in event COMT2 in year one. In year two, sugar release of senesced tissues were improved by 32.0% and 34.2% in events COMT2 and COMT3, respectively (Table B.2).

### *Ethanol yield*

The bioconversion efficiency of field-grown senesced biomass was tested by performing a pretreatment followed by yeast-based simultaneous saccharification and fermentation (SSF). SSF integrates enzymatic hydrolysis and microbial fermentation within the same vessel, allowing the hydrolyzed glucose molecules to be quickly converted into ethanol by the fermentation microorganism. Following the same methods used in the greenhouse study [18], samples were pretreated under moderate dilute acid conditions, followed by SSF with glucose-fermenting *Saccharomyces cerevisiae*. Weight loss from the bottles due to $CO_2$ release was used to monitor the progression of the fermentation over time. In year one, event COMT3 exhibited a faster fermentation rate based on weight loss curves, and had a 21.2% higher endpoint ethanol yield per gram of biomass than its corresponding control. The endpoint ethanol yield for event COMT2 was not different from its control (Figure B.7). In year two, both transgenic events fermented more quickly than their controls and exhibited ethanol yield improvements of 28.2% (COMT2) and 21.4% (COMT3) (Figure B.8).

### *Agronomic performance*

In order to assess the growth and productivity of COMT-downregulated switchgrass under field conditions, morphological traits including tiller height and plant diameter were measured mid-growing season of each year, and tiller number and above-ground dry weight biomass yield were determined at the end of the season. In the first year, event COMT2 was not significantly different than its corresponding control in tiller height, plant diameter, tiller number, or dry weight biomass yield. However, event COMT3

exhibited a decrease in tiller height (15.3%), plant diameter (8.1%), and dry weight yield (50.5%) relative to its control. In the second year, event COMT2 had an increase in tiller height and plant diameter, and a corresponding 18.2% increase in dry weight biomass yield relative to its control, whereas event COMT3 was not different from its control in morphology or dry weight biomass yield (Table B.3).

***Disease susceptibility***

All plants were continually monitored throughout both growing seasons for any deleterious effects caused by pathogens or insect pests. Ladybugs, grasshoppers, and spiders were observed on all plants. Insect damage to the leaves of transgenic and nontransgenic plants was minimal. The most prevalent diseases observed were switchgrass rust caused by *Puccinia emaculata*, and leaf spot caused by a *Bipolaris* species. For both growing seasons, rust was observed from mid-to-late June until the plants began to senesce toward the end of November. Disease severity of rust infection was rated weekly during the second growing season. Rust severity, as the percentage of the leaf covered in rust uredia, ranged from 3-5% in mid-to-late June, to 25-30% in late September. COMT-downregulation did not affect rust susceptibility in either transgenic event relative to its control at any of the time points assessed (Figure B.9). The occurrence of *Bipolaris* was not quantified since the symptoms were relatively minor, but the severity of infection between transgenic and control plants did not appear to differ based on visual observation.

## Discussion

The current study evaluated the field performance of switchgrass with reduced recalcitrance over the course of two years: the first year (2011), in which the plants were newly transplanted, and the second year (2012), in which the plants were well-established and had reached a more steady-state growth pattern. Experiments with established transgenic plants harvested in the second growing season demonstrate that improved saccharification and ethanol yields can be achieved under field conditions with no adverse effects on biomass yield or disease susceptibility. To our knowledge, this is the first reported field study evaluating the potential of transgenic switchgrass with reduced lignin content for use as a bioenergy crop.

Brown midrib (bmr) mutant and transgenic grasses with reduced COMT enzyme activity are characterized by significant reductions in S-lignin, resulting in a lower S/G monomer ratio, as well as a decrease in the overall lignin content [18, 21, 23, 24, 38, 44, 24]. In agreement with these studies, COMT downregulation in field-grown switchgrass was associated with a reduction in the S/G ratio and lower lignin levels. RNAi-mediated gene silencing of COMT gene expression was maintained in the transgenic events for the duration of the field experiment, showing a reduction of 82-97% compared to the control plants. COMT-downregulation resulted in an 8-15% decrease in lignin content in green- and senesced-harvested biomass from transgenic events in years one and two. While differences in lignin levels between transgenic and control plants were relatively consistent across both years, the reduction in the S/G ratio was significantly more

pronounced in the second growing season, where a 35-39% reduction was observed in both green and senesced tissues from transgenic events relative to their controls. Similar to the greenhouse study [18], field-grown COMT-downregulated plants exhibited a brownish coloration at the basal internode of the stems, a phenotype that is attributed to the reduced lignin content and/or altered lignin composition [45, 46].

Understanding the broader effects of lignin downregulation on the synthesis of other cell wall components is important, as any unintended changes in composition could ultimately affect the quality of the feedstock for bioconversion. Although the mechanisms are not yet well understood, previous studies have demonstrated that downregulating lignin genes can affect other metabolic processes, including primary metabolism and the synthesis of cell wall carbohydrates [47, 48, 49]. Some studies suggest that plants compensate for a reduction in lignin content by shifting carbon into the synthesis of cell wall carbohydrates to maintain structural integrity. For example, it has been hypothesized that observed increases in cellulose content [48, 50] or cellulose crystallinity [51] in lignin-deficient plants may be compensatory mechanisms for maintaining mechanical strength. In the present study, cellulose content remained unchanged between field-grown transgenic and control plants for both years. This is consistent with the greenhouse study, which reported minor or negligible impact of lignin reduction on cellulose content in these two events [18]. However, hemicellulose content was increased by 6.4-7.4% in the field-grown transgenic events in the second year, primarily due to an increase in xylose, the major monosaccharide component of hemicellulose in grasses. A slight increase in

xylose content was also observed in these plants when grown under greenhouse conditions [18]. Similarly, a field trial with COMT-downregulated switchgrass reported an increase in xylose content for the transgenic event that displayed the strongest reduction in lignin content [21]. In addition to altered hemicellulose levels, the transgenic events in this experiment exhibited a 16.0-18.6% decrease in the index of cellulose crystallinity relative to their controls, suggesting that COMT-downregulation in field-grown switchgrass might also have an indirect influence on structural characteristics of cellulose. A lower ratio of crystalline to amorphous cellulose is considered to be a desirable trait in bioenergy feedstocks since it can enhance enzymatic digestion of the cell wall [5].

Greenhouse experiments with transgenic switchgrass have demonstrated the advantages of lignin downregulation for improving sugar release and ethanol yield in live, green plants [18, 19, 20]. However, the improved traits observed in young green plants grown in low-stress environments may or may not be retained in mature field-grown plants due to environmental and age-related effects. Additionally, the use of senesced biomass harvested after the killing frost at the end of the growing season is preferable for most commercial biofuel conversion technologies due to its reduced mineral and moisture content [27]. For these reasons, greenhouse studies need to be validated in a realistic growth environment using biomass harvested after senescence in order to truly assess the economic potential of lignin-modified switchgrass as a bioenergy feedstock. In the first year analyses, the release of glucose and total sugars (glucose and xylose combined) from

field-grown biomass were significantly higher in both transgenic events relative to their corresponding controls, but only in plants that were harvested while green. For plants harvested after senescence, only event COMT2 exhibited a higher release of sugars relative to its control. More consistent results for sugar release were observed in the established plants harvested in the second year; both transgenic events exhibited a higher release of glucose, xylose, and total sugars relative to their controls in both green- and senesced- harvested biomass. Most important, at the end of year two, senesced-tissues of COMT2 and COMT3 events had 32% and 34% higher sugar release, respectively, compared with their controls. The altered lignin and improved sugar release in the transgenic events harvested in year two translated to a faster rate of fermentation and ethanol yield improvements of 28.0% (COMT2) and 21.4% (COMT3) in senesced plants relative to their controls.

While modifications to lignin biosynthesis can significantly improve the accessibility of fermentable sugars, some can be associated with developmental abnormalities and yield reductions which could potentially offset the positive gains in saccharification and ethanol yield [52]. In particular, the downregulation of genes that occur early in the lignin biosynthetic pathway frequently result in dwarfing phenotypes and biomass yield reductions. This dwarfing could be a direct consequence of the lignin deficiency, as lignin plays an essential role in developmental and growth-related processes in plants. Growth abnormalities could also be triggered indirectly, possibly through disruptions to phenylpropanoid metabolism or through the accumulation of lignin pathway

intermediates that could be inhibitory to growth [52]. One advantage of silencing genes that occur later in the pathway is that it tends to have less impact on plant growth and productivity. In this respect, COMT downregulation in grass species has been shown to have minimal effects on growth and development, including greenhouse-grown switchgrass and maize, and field-grown perennial ryegrass [18, 21, 38]. In sugarcane (*Saccharum* spp. hybrids), COMT downregulation was shown to negatively impact biomass yield in the transgenic event that showed the greatest reduction in lignin content, whereas all other transgenic events in the study had no growth abnormalities or yield penalties. These observations were consistent under both greenhouse and field conditions [44, 17]. In this study, event COMT2 was phenotypically similar to its control and had no difference in dry weight biomass yield in the first growing season. In the second growing season, event COMT2 exhibited an increase in tiller height and plant width, as well as an 18% increase in dry weight biomass yield relative to its control. In contrast, event COMT3 yielded less biomass than its control during the first growing season, but was not significantly different than its corresponding control in the second growing season. Because this event showed normal growth and development under greenhouse conditions [18] and under field conditions once adequately established, it is likely that the apparent yield reduction observed in the first growing season was an effect of establishment year. Examples from literature suggest that switchgrass can be slow to establish and can require multiple years to reach their full yield potential [53, 54]; consequently, switchgrass stands that exhibit poor growth during the establishment year often produce significantly higher yields in the following growing seasons [54]. From this, it can be

inferred that measures of biomass yield obtained from plants harvested in the subsequent years following establishment may be more realistic and reliable than those obtained in the initial year of planting.

In addition to potential effects on biomass yield, another concern when evaluating the sustainability of plants with reduced lignin content is the effect of altered cell wall integrity on susceptibility of the plant to biotic stresses. Lignin and its precursors play a central role in defense against pathogens, both as a physical barrier against pathogen entry and as an induced response to infection [28, 29]. Given the potential threat posed by fungal pathogens and insect pests to the successful establishment and sustainability of bioenergy feedstocks [55], the possible consequences of reduced lignin on plant susceptibility to such stresses must be thoroughly evaluated in a field setting. Rust disease, caused by *Puccinia emaculata*, has been identified as being potentially damaging to the quality and yield of switchgrass grown for biofuel production [56].The downregulation of COMT has been shown to have varying effects on the susceptibility of field-grown transgenic crops to different rust species; COMT downregulation in perennial ryegrass increased the susceptibility of field-grown transgenic plants to rust [38], but had no effect on rust susceptibility in COMT-downregulated sugarcane or poplar grown in the field [44, 57]. In this study, the most common pathogens observed in the field were *Puccinia emaculata* and a *Bipolaris* species. The severity of *Bipolaris* was minimal and rust susceptibility did not differ in COMT-downregulated switchgrass plants

relative to their non-transgenic controls. Additionally, no major insect damage to the leaves of control or transgenic plants was observed.

The results obtained from second year analyses were generally in agreement with the findings of the greenhouse study. In summary, lignin content remained relatively constant for both years of the field study, while the reduction in the S/G ratio in the transgenic events relative to their controls was much greater in second-year harvested plants. Similarly, sugar release and ethanol yield results were better in second-year harvested transgenic events, and no negative impacts on biomass yield or disease susceptibility were observed (results summarized in Table B.4). Inconsistent and variable results obtained in the first year may be attributable to the newly-transplanted plants acclimating to the stressful transition from greenhouse to field. This underlines the need for field trials of at least two years in order to fully evaluate the biofuel feedstock potential of transgenic switchgrass with altered lignin biosynthesis. Considering the important developmental and stress-related roles of lignin, plants deficient in lignin biosynthesis could initially be more susceptible to this critical transition period than the non-transgenic controls. If so, this could have perhaps be avoided by transplanting earlier in the growing season to allow the plants more time to acclimate before end-of-season harvest.

Taken together, observations from this study suggest that improvements in saccharification and ethanol yield can be maintained in transgenic switchgrass plants grown under agronomic field conditions once plants are fully established. This first two-

year field trial of a transgenic herbaceous cellulosic feedstock shows that genetic engineering of an appropriate lignin target, in this case decreasing the expression of caffeic acid *O*-methyltransferase, can confer significant real-world improvements in biofuel yield without negatively impacting biomass yield or disease susceptibility.

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# Appendix B

## *Figures*

**Figure B.1.** COMT switchgrass field design. The field layout consisted of a 22.9 m x 25.9 m plot (607.0 $m^2$) containing two independent transgenic events (COMT2 (+) and COMT3 (+)) and corresponding null-segregant control plants for each of these events (COMT2 (-) and COMT3 (-)). Ten biological replicates for each transgenic event and five biological replicates for each control (30 replicates in total) were distributed throughout the plot in a completely randomized design (CRD). Nine vegetatively-propagated clones were planted within each replicate. Replicates were spaced 152.4 cm apart with 76.2 cm between the nine plants within each single replicate. The investigated plants were surrounded by border plants (switchgrass genotype Alamo 2) to reduce any shading effects.

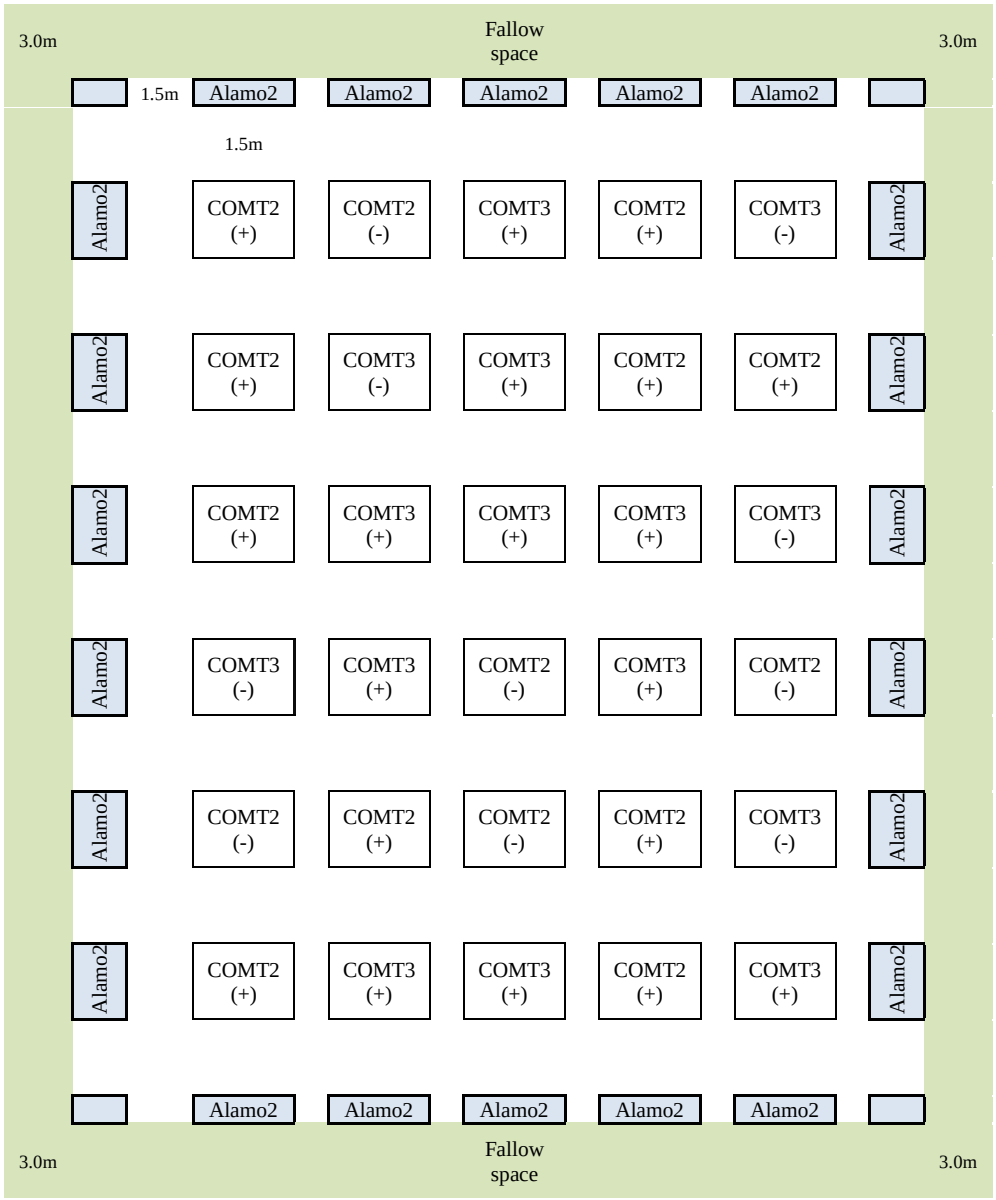



**Figure B.1.** Continued.

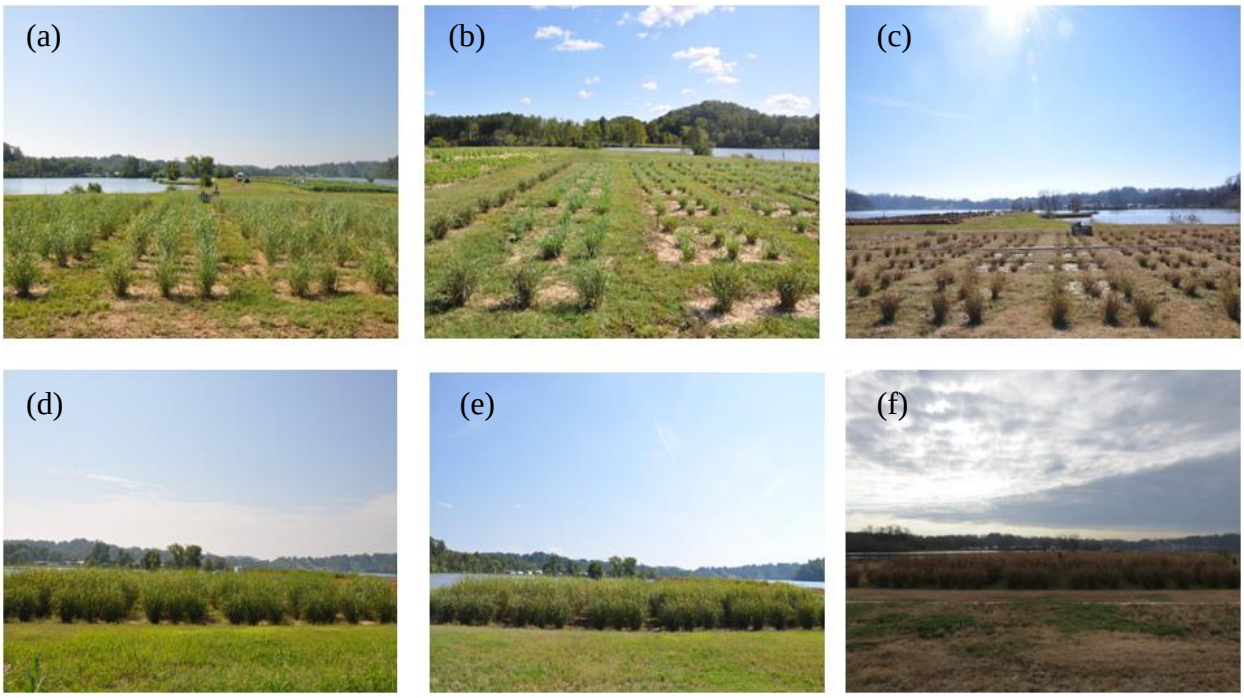



**Figure B.2.** Pictures of the COMT field site at different time points in 2011 and 2012. First year pictures include: (a) August 2011 (3 months after transplantation), (b) October 2011, and (c) December 2011 (prior to harvesting). Second year pictures include: (e) August 2012, (d) October 2012, (e) December 2012 (prior to harvesting).

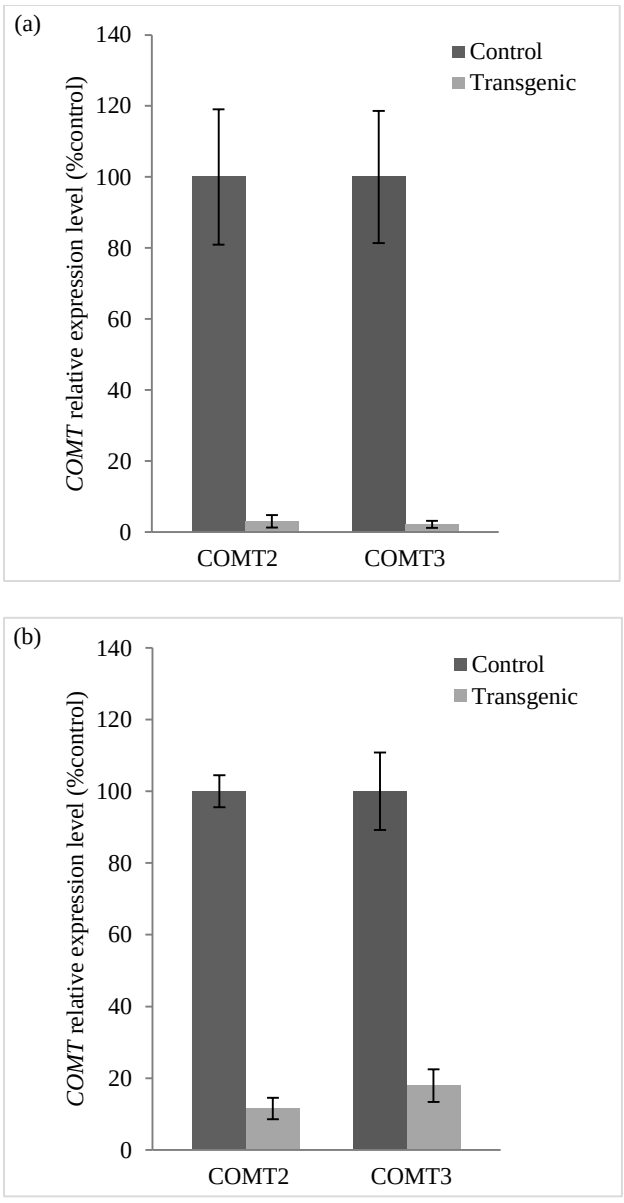



**Figure B.3.** Analysis of COMT transcript levels for 2011 (a) and 2012 (b). Four biological replicates were randomly selected from each control and transgenic group for the analyses. After RNA isolation from the leaves, cDNA from reverse transcription was used for quantitative RT-PCR. Bars represent the average of the biological replicates within each group ± standard error.

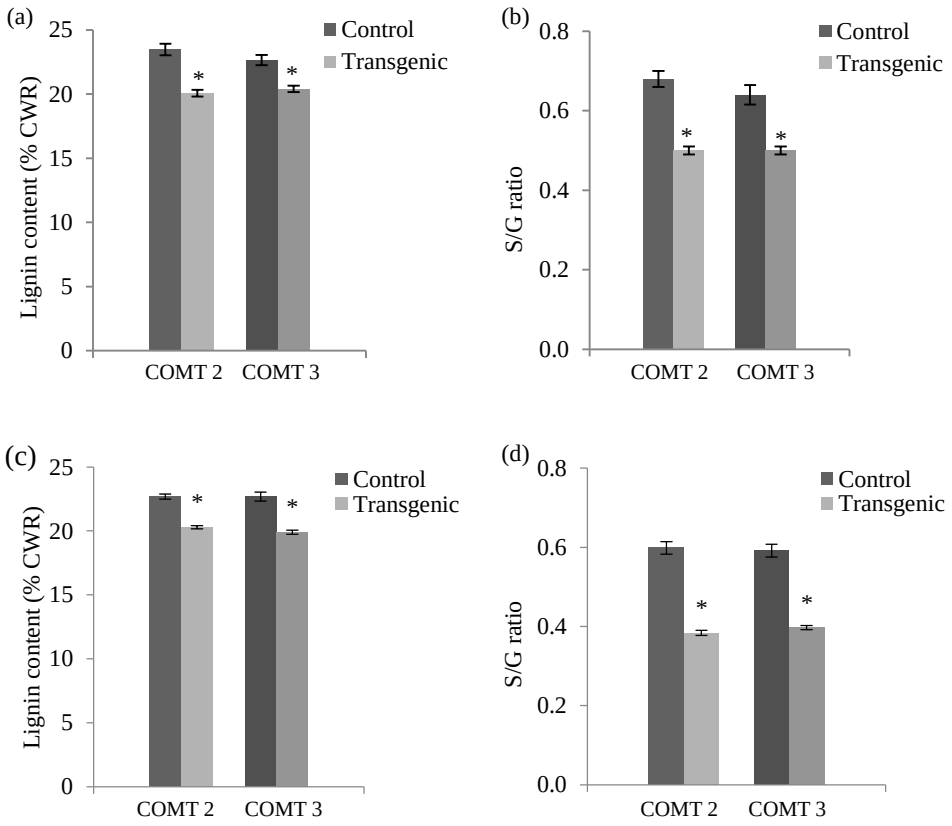



**Figure B.4**. Lignin content and S/G ratio of green tissue samples, as estimated by pyrolysis-molecular beam mass spectrometry, for 2011 (a-b) and 2012 (c-d). All five biological replicates from each control group, and all 10 biological replicates from each transgenic group, were used in the analyses. Bars represent the average of the biological replicates within each group ± standard error. Each transgenic event was compared to its respective control. An asterisk indicates a significant difference as determined by a *t*-test ($P < 0.05$). CWR, cell wall residues.

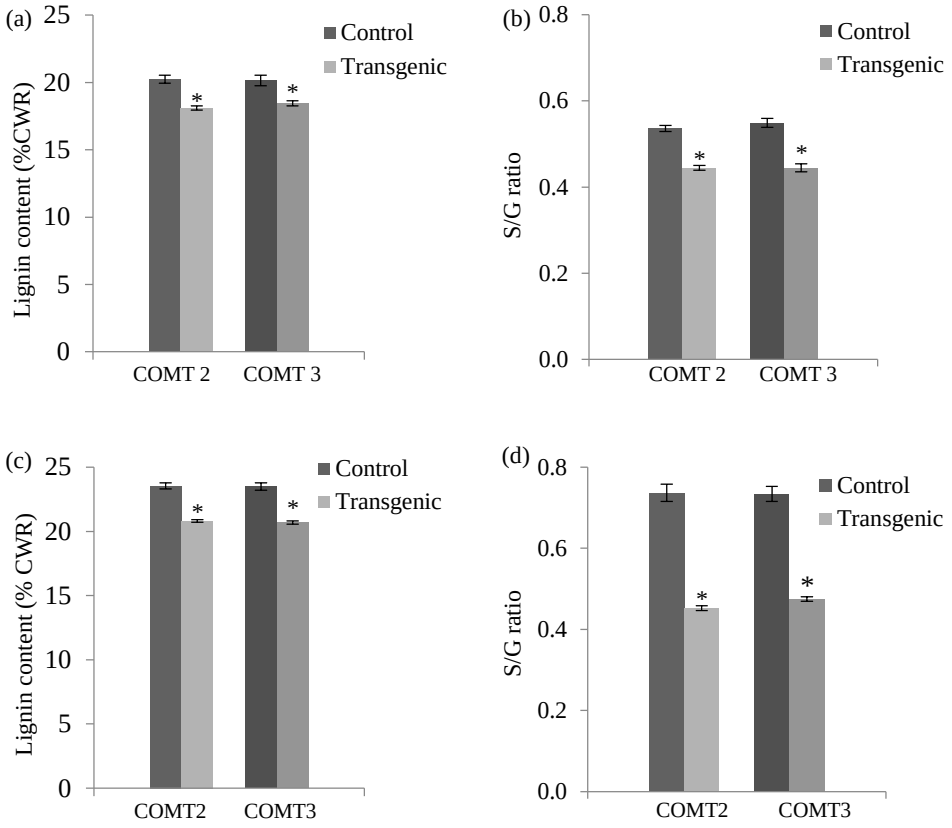



**Figure B.5**. Lignin content and S/G ratio of senesced tissue samples, as estimated by pyrolysis-molecular beam mass spectrometry, for 2011 (a-b) and 2012 (c-d). All five biological replicates from each control group, and all 10 biological replicates from each transgenic group, were used in the analyses. Bars represent the average of the biological replicates within each group ± s.e.m. Each transgenic event was compared to its respective control. An asterisk indicates a significant difference as determined by a *t*-test ($P < 0.05$). CWR, cell wall residues

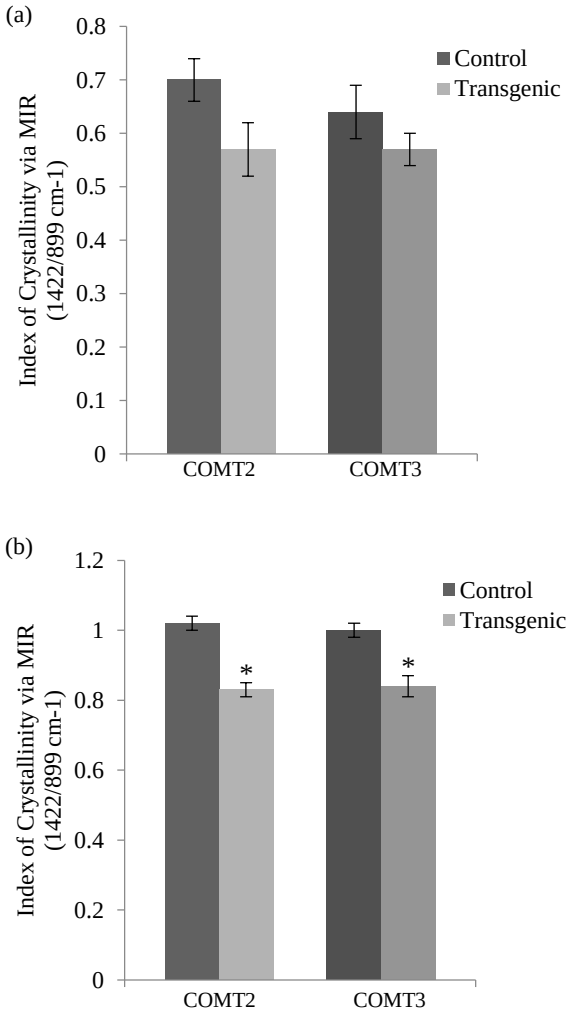



**Figure B.6**. Index of cellulose crystallinity as determined by Fourier transform infrared spectroscopy of senesced tissue samples. MIR, mid-infrared. All five biological replicates from each control group, and all 10 biological replicates from each transgenic group, were used in the analyses. Bars represent the average of the biological replicates within each group ± standard error. Each transgenic event was compared to its respective control. An asterisk indicates a significant difference as determined by a *t*-test ($P < 0.05$).

**Figure B.7**. Ethanol yield as determined by simultaneous saccharification and fermentation of whole aboveground senesced biomass in 2011. All five biological replicates from each control group, and five randomly selected samples from each transgenic group, were used in the analyses. All samples were pretreated (180°C for 7.5 min, 0.5% $H_2SO_4$) prior to fermentation. (a, b) Weight loss of fermentation bottles due to $CO_2$ release over time for events COMT2 (a) and COMT3 (b); data points represent the average of the biological replicates within each group ± standard error. (c) Final ethanol yield after 17d; bars represent the average of the biological replicates within each group ± standard error. Each transgenic event was compared to its respective control. An asterisk indicates a significant difference as determined by a *t*-test ($P < 0.05$).

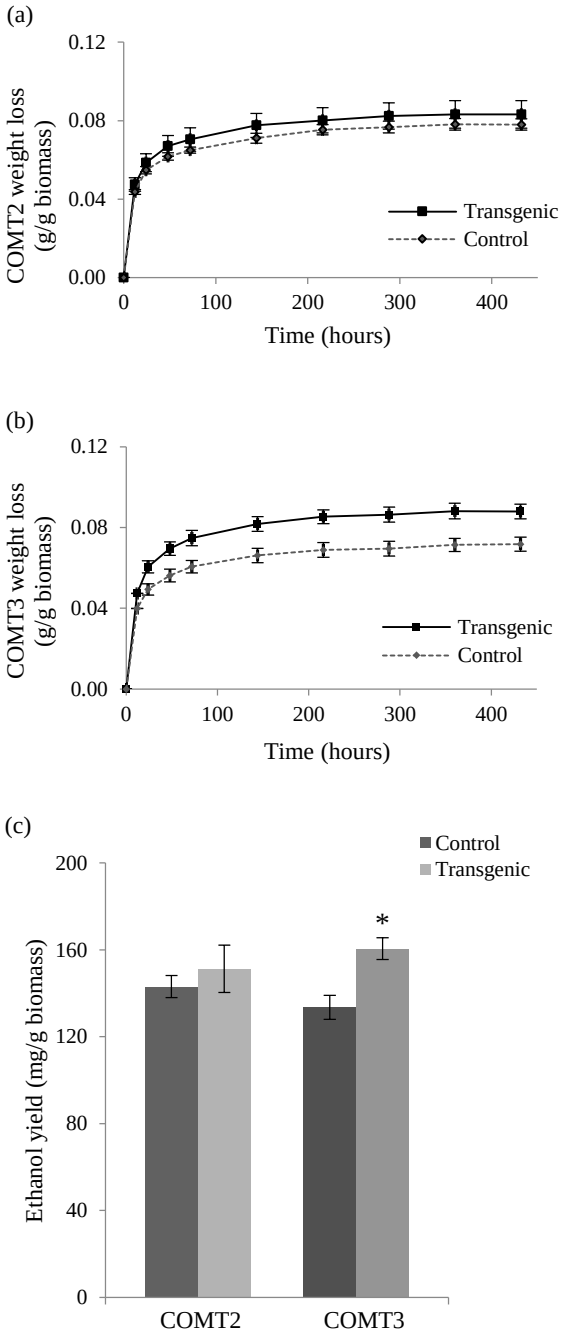



**Figure B.7**. Continued.

**Figure B.8**. Ethanol yield as determined by simultaneous saccharification and fermentation of whole aboveground senesced biomass in 2012. All five biological replicates from each control group, and five randomly selected samples from each transgenic group, were used in the analyses. All samples were pretreated (180°C for 7.5 min, 0.5% $H_2SO_4$) prior to fermentation. (a, b) Weight loss of fermentation bottles due to $CO_2$ release over time for events COMT2 (a) and COMT3 (b); data points represent the average of the biological replicates within each group ± standard error. (c) Final ethanol yield after 17d; bars represent the average of the biological replicates within each group ± standard error. Each transgenic event was compared to its respective control. An asterisk indicates a significant difference as determined by a *t*-test ($P < 0.05$).

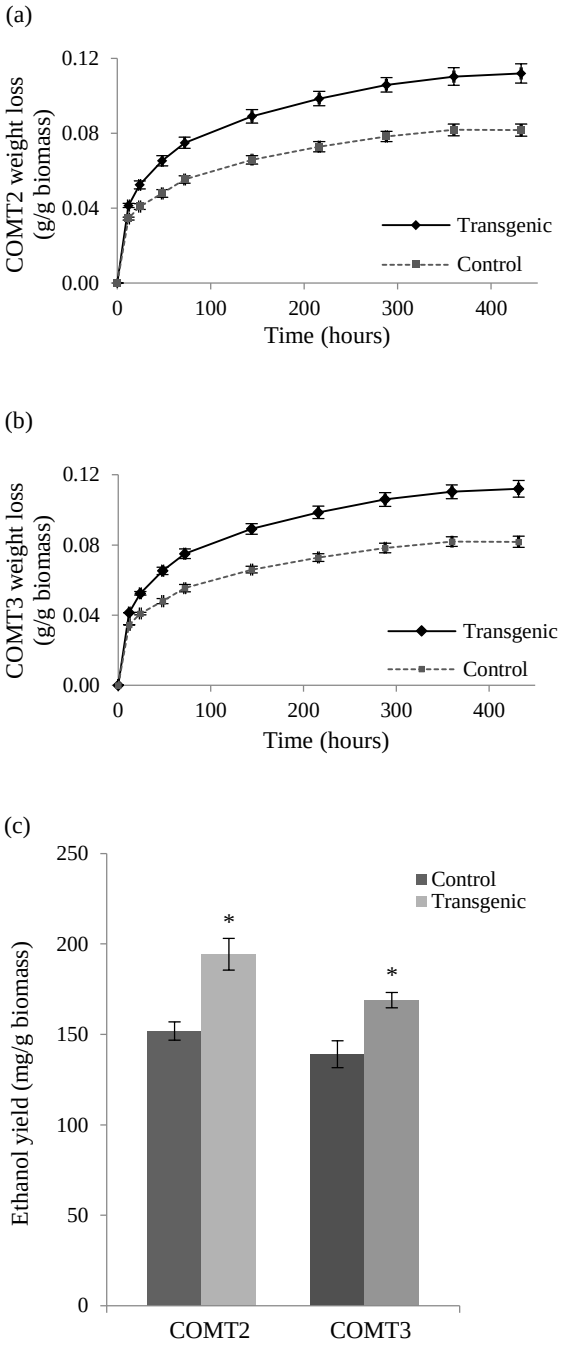
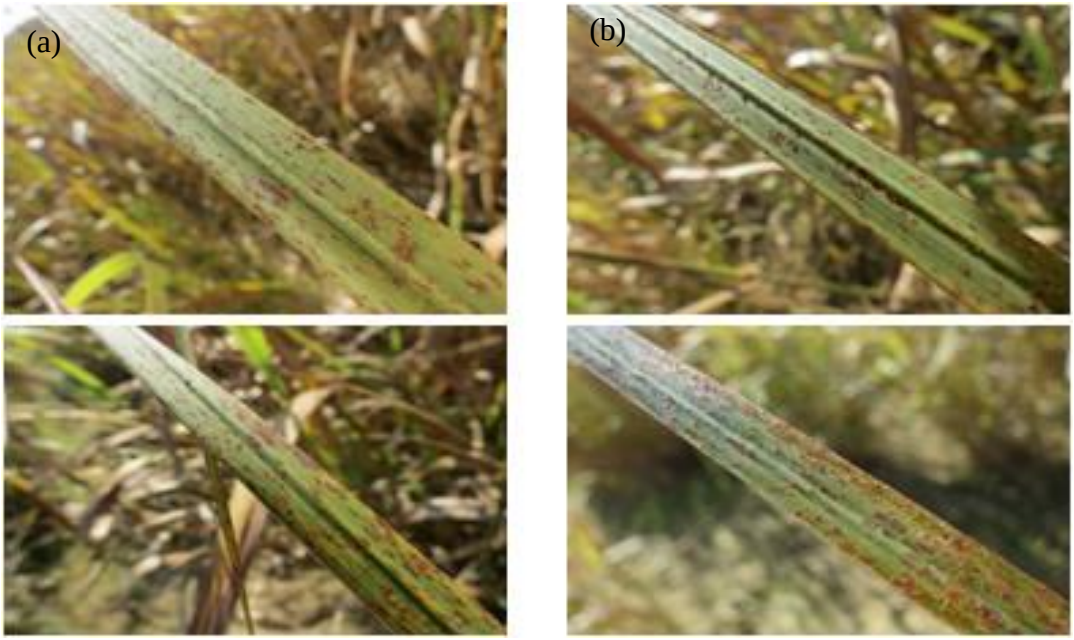



**Figure B.8**. Continued.

**Figure B.9**. Rust susceptibility of field-grown transgenic plants. (a, b) Pictures of rust-infected leaves on four randomly selected leaves from transgenic (a) and control plants (b), taken in November of 2012. (c) Severity of rust infection in transgenic and control plants at various time points across the growing season in 2012. All five biological replicates from each control group, and all 10 biological replicates from each transgenic group were used in the analysis. Bars represent the average of the biological replicates within each group ± standard error. For each time point, each transgenic event was compared to its respective control using a *t*-test at the 5% level of significance. No significant differences were detected.



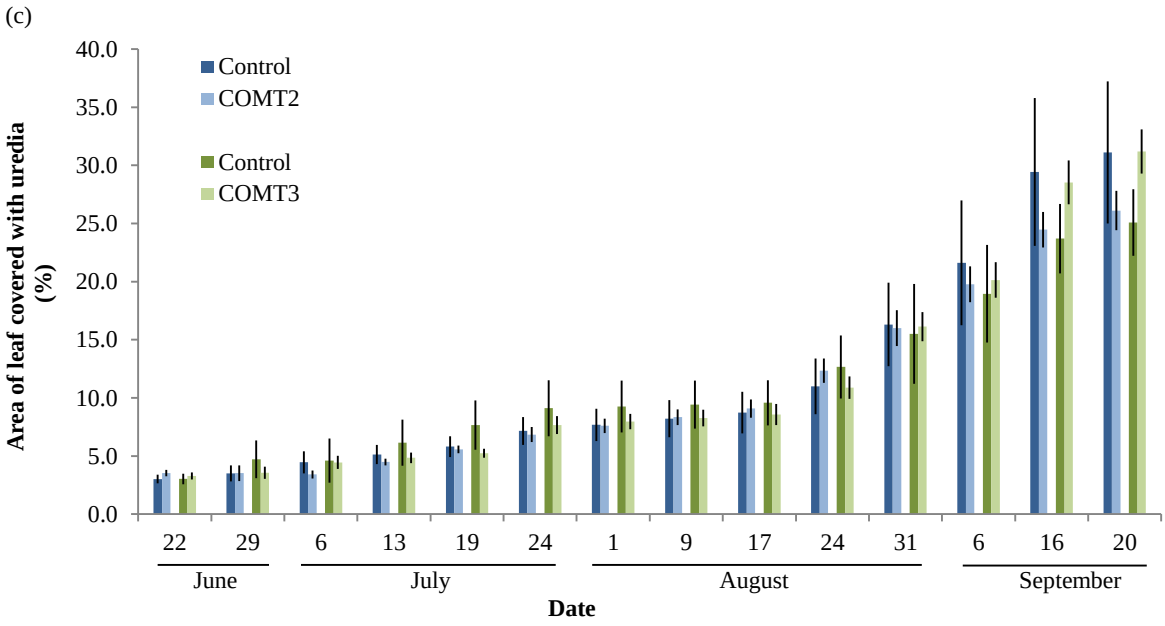




**Figure B.9**. Continued.

## *Tables*

**Table B.1**. Chemical composition of senesced biomass on an extractives-free basis in 2011 and 2012.

| | Control | COMT2 | Control | COMT3 |
|---|---|---|---|---|
| Year 1 (2011) | | | | |
| Structural ash | 5.1 ± 0.3 | 5.3 ± 0.2 | **4.6 ± 0.3** | **5.6 ± 0.2*** |
| Lignin | **22.2 ± 0.4** | **20.9 ± 0.4*** | **22.4 ± 0.3*** | **21.3 ± 0.2*** |
| Acetyl | 2.6 ± 0.1 | 2.4 ± 0.1 | 2.7 ± 0.1 | 2.6 ± 0.1 |
| Total carbohydrates | 67.0 ± 1.4 | 67.5 ± 0.7 | 70.1 ± 0.5 | 69.2 ± 1.0 |
| Cellulose | 39.0 ± 0.7 | 39.4 ± 0.4 | 39.7 ± 0.4 | 39.3 ± 0.5 |
| Hemicellulose | 28.0 ± 0.8 | 28.1 ± 0.5 | 30.4 ± 0.1 | 29.9 ± 0.6 |
| Xylan | 22.9 ± 0.7 | 22.7 ± 0.5 | 25.3 ± 0.1 | 24.4 ± 0.5 |
| Galactan | 1.9 ± 0.1 | 2.0 ± 0.1 | **1.7 ± 0.0** | **2.1 ± 0.1*** |
| Arabinan | 2.9 ± 0.3 | 3.0 ± 0.1 | 2.8 ± 0.3 | 3.0 ± 0.2 |
| Mannan | 0.3 ± 0.0 | 0.4 ± 0.0 | 0.4 ± 0.0 | 0.3 ± 0.0 |
| Year 2 (2012) | | | | |
| Structural ash | 2.2 ± 0.2 | 2.4 ± 0.1 | 2.6 ± 0.1 | 2.3 ± 0.2 |
| Lignin | **22.1 ± 0.1** | **20.5 ± 0.1*** | **22.4 ± 0.3** | **20.5 ± 0.1 *** |
| Acetyl | 4.6 ± 0.1 | 4.6 ± 0.1 | **4.3 ± 0.1** | **4.8 ± 0.1*** |
| Total carbohydrates | 68.9 ± 0.5 | 70.9 ± 0.8 | **67.5 ± 0.9** | **69.9 ± 0.5*** |
| Cellulose/Glucan | 39.0 ± 0.4 | 39.1 ± 0.4 | 37.8 ± 0.6 | 38.0 ± 0.4 |
| Hemicellulose | **29.9 ± 0.2** | **31.8 ± 0.4*** | **29.7 ± 0.4** | **31.9 ± 0.2*** |
| Xylan | **26.1 ± 0.3** | **27.8 ± 0.3*** | **26.0 ± 0.3** | **28.0 ± 0.2*** |
| Galactan | 0.9 ± 0.1 | 0.9 ± 0.1 | 0.9 ± 0.2 | 0.8 ± 0.1 |
| Arabinan | 2.7 ± 0.1 | 2.9 ± 0.1 | **2.6 ± 0.1** | **2.9 ± 0.1*** |
| Mannan | 0.2 ± 0.1 | 0.2 ± 0.0 | 0.2 ± 0.1 | 0.3 ± 0.0 |

**Table B.1**. Continued. Chemical composition data are presented on a percent dry weight basis. All five biological replicates from each control group, and all 10 biological replicates from each transgenic group, were used in the analyses. Values represent the average of the biological replicates within each group ± standard error. Each transgenic event was compared to its respective control. An asterisk indicates a significant difference as determined by a *t*-test ($P < 0.05$).

**Table B.2**. Sugars released by enzymatic hydrolysis from green and senesced samples in 2011 and 2012.

| | | Green tissue | | | Senesced tissue | | |
|---|---|---|---|---|---|---|---|
| Year | | Glucose release (g/g CWR) | Xylose release (g/g CWR) | Total release (g/g CWR) | Glucose release (g/g CWR) | Xylose release (g/g CWR) | Total release (g/g CWR) |
| 2011 | Control | **0.241 ± 0.009** | 0.181 ± 0.008 | **0.421 ± 0.018** | **0.234 ± 0.007** | 0.162 ± 0.005 | **0.396 ± 0.010** |
| | COMT2 | **0.277 ± 0.003*** | 0.194 ± 0.003 | **0.471 ± 0.004*** | **0.255 ± 0.004*** | 0.163 ± 0.003 | **0.418 ± 0.004*** |
| | Control | 0.249 ± 0.006 | 0.183 ± 0.006 | **0.432 ± 0.011** | 0.230 ± 0.012 | 0.163 ± 0.005 | 0.393 ± 0.015 |
| | COMT3 | 0.269 ± 0.006 | 0.201 ± 0.005 | **0.471 ± 0.010*** | 0.243 ± 0.005 | 0.170 ± 0.002 | 0.413 ± 0.006 |
| 2012 | Control | **0.151 ± 0.007** | **0.158 ± 0.002** | **0.309 ± 0.010** | **0.126 ± 0.004** | **0.163 ± 0.002** | **0.289 ± 0.005** |
| | COMT2 | **0.178 ± 0.005*** | **0.176 ± 0.003*** | **0.354 ± 0.007*** | **0.182 ± 0.004*** | **0.200 ± 0.003*** | **0.382 ± 0.006*** |
| | Control | **0.147 ± 0.005** | **0.157 ± 0.003** | **0.304 ± 0.006** | **0.132 ± 0.002** | **0.180 ± 0.008** | **0.300 ± 0.006** |
| | COMT3 | **0.183 ± 0.005*** | **0.177 ± 0.003*** | **0.360 ± 0.009*** | **0.195 ± 0.003*** | **0.208 ± 0.002*** | **0.403 ± 0.004*** |

All five biological replicates from each control group, and all 10 biological replicates from each transgenic group, were used in the analyses. Values represent the average of the biological replicates within each group ± standard error. Each transgenic event was compared to its respective control. An asterisk indicates a significant difference as determined by a *t*-test ($P < 0.05$). CWR, cell wall residues.

**Table B.3.** Morphology and dry weight biomass yield of field-grown transgenic events during the 2011 and 2012 growing seasons.

| Year | | Tiller height (cm) | Plant diameter (cm) | Tiller number | DW biomass yield ($g/m^2$) |
|---|---|---|---|---|---|
| 2011 | Control | 69.5 ± 4.2 | 73.5 ± 3.6 | 71.5 ± 6.7 | 38.5 ± 14.0 |
| | COMT2 | 68.4 ± 1.9 | 75.5 ± 1.8 | 85.4 ± 3.2 | 44.4 ± 3.4 |
| | Control | **77.3 ± 2.8** | **77.9 ± 1.5** | 81.3 ± 2.5 | **75.8 ± 9.5** |
| | COMT3 | **65.5 ± 1.8*** | **71.6 ± 1.1*** | 88.8 ± 3.2 | **37.7 ± 3.9*** |
| 2012 | Control | **184.9 ± 3.0** | **200.5 ± 9.9** | 180.8 ± 10.7 | **2150.7 ± 169.4** |
| | COMT2 | **197.2 ± 1.7*** | **232.9 ± 3.5*** | 196.1 ± 9.1 | **2628.5 ± 92.6*** |
| | Control | 188.2 ± 5.1 | 225.4 ± 7.1 | 186.2 ± 4.3 | 2720.2 ± 318.3 |
| | COMT3 | 192.1 ± 2.8 | 217.9 ± 5.4 | 192.5 ± 10.0 | 2160.4 ± 123.6 |

All five biological replicates from each control group, and all 10 biological replicates from each transgenic group, were used in the analyses. Values represent the average of the biological replicates within each group ± standard error. Each transgenic event was compared to its respective control. An asterisk indicates a significant difference as determined by a *t*-test ($P < 0.05$). DW, dry weight.

**Table B.4.** Summary of the differences between each transgenic event and its corresponding control for years one (2011) and two (2012) in the field experiment.

| | | | Year 1 (2011) | | Year 2 (2012) | |
|---|---|---|---|---|---|---|
| **Analysis** | **Tissue** | **Trait** | **COMT2** | **COMT3** | **COMT2** | **COMT3** |
| Lignin content, composition (MBMS) | Green | Lignin | **-14.5%** | **-10.1%** | **-10.6%** | **-12.3%** |
| | | S/G ratio | **-26.5%** | **-21.9%** | **-35.9%** | **-32.9%** |
| | Senesced | Lignin | **-10.6%** | **-8.4%** | **-11.6%** | **-12.0%** |
| | | S/G ratio | **-18.5%** | **-20.0%** | **-38.6%** | **-35.3%** |
| Cell wall characterization | Senesced | Structural ash | NS | **+16.4%** | NS | NS |
| | | Acetyl | NS | NS | NS | **+11.6** |
| | | Lignin | **-5.9%** | **-4.9%** | **-7.2%** | **-8.5%** |
| | | Cellulose | NS | NS | NS | NS |
| | | Hemicellulose | NS | NS | **+6.4%** | **+7.4%** |
| | | Xylan | NS | NS | **+6.5%** | **+7.7%** |
| | | Arabinan | NS | NS | NS | **+11.5%** |
| | | Galactan | NS | **+21.4%** | NS | NS |
| | | Mannan | NS | NS | NS | NS |
| | | Index of crystallinity | NS | NS | **-18.6%** | **-16.0%** |
| Sugar release | Green | Total sugar release | **+11.7%** | **+9.0%** | **+14.7%** | **+18.7%** |
| | Senesced | Total sugar release | **+5.6%** | NS | **+32.0%** | **+34.2%** |
| Fermentation | Senesced | Ethanol yield | NS | **+21.2%** | **+28.2%** | **+21.4%** |
| Agronomic performance | | Tiller height | NS | **-15.3%** | **+6.7%** | NS |
| | | Plant diameter | NS | **-8.1%** | **+16.2%** | NS |
| | | Tiller number | NS | NS | NS | NS |
| | | Biomass yield | NS | **-50.5%** | **+18.2%** | NS |
| Rust susceptibility | | | -- | -- | NS | NS |

Values are the percent difference between each transgenic event and its corresponding control. Significance was determined by a *t*-test ($P < 0.05$). A negative sign (-) indicates a decrease relative to the control; a positive sign (+) indicates an increase relative to the control. NS, not significant.

# CHAPTER 4
# CONCLUSION

Transgenic modification of lignin biosynthesis in lignocellulosic feedstocks offers a direct and effective route for increasing the availability of sugars for fermentation. However, modifications to the lignin pathway can affect stress-related pathways, which could ultimately influence plant performance in a field setting. The development of sustainable low-lignin bioenergy feedstocks requires an improved understanding of the possible downstream effects of lignin modification on plant growth, fitness, and stress tolerance in an agronomic setting.

In the study presented in Chapter three, COMT-downregulated switchgrass harvested during the second year of the field trial produced results that were comparable to those which were observed in greenhouse experiments. Furthermore, the reduced lignin content and altered lignin composition had no negative impact on disease susceptibility or biomass yield in fully-established plants. An 18% increase in biomass yield was observed in one transgenic event, which, when combined with the gains in ethanol yield, resulted in over 50% more liters of ethanol per hectare than its control. These results validate the previously conducted greenhouse research in a more realistic setting, further demonstrating the feasibility of using a transgenic approach to improve lignocellulosic feedstock quality for biofuel production. Further research is needed to evaluate the growth and resilience of transgenic switchgrass, and other low-lignin transgenic feedstocks, when exposed to more extreme environmental conditions not present in this study.

# VITA

Holly Lauren Baxter was born in Knoxville, Tennessee on December 15, 1983. She completed a Bachelor of Science degree in Ecology and Evolutionary Biology from the University of Tennessee in August of 2007. After two years of working in the department of Plant Sciences as a research technician, she began her graduate studies in Plant Sciences under Dr. Neal Stewart in January of 2011.