DISSERTATION

VARIATION IN CELL WALL COMPOSITION AND BIOENERGY POTENTIAL OF RICE STRAW

Submitted by

Paul Tanger

Department of Bioagricultural Sciences and Pest Management

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2014

Doctoral Committee:

Advisor: Jan E. Leach

Asa Ben-Hur
Daniel R. Bush
John K. McKay

Copyright by Paul Tanger 2014

All Rights Reserved

ABSTRACT

VARIATION IN CELL WALL COMPOSITION AND BIOENERGY POTENTIAL OF RICE STRAW

In most grain crops the leaf and straw is often under-utilized. This biomass is largely plant cell wall, whose heterogeneous composition and recalcitrance limits end uses such as forage or bioenergy. I review the desirable traits for several bioenergy pathways from this biomass and identify traits in biomass that need to be optimized for enzymatic or thermochemical conversion of the biomass to energy. Sufficient variation exists across species and varieties for improving these traits through breeding. I assess variation in cellulose, lignin, hemicellulose, ash, total glucose, total xylose, mixed linkage glucan, saccarification yield and efficiency, hydroxyproline content and bulk density across two environments in the leaf and stem tissue of five rice varieties. Environment and tissue type are highly influential on the composition and yield phenotypes, and some traits perform better than others at predicting bioenergy yield in the field environment.

Optimizing specific bioenergy-related phenotypes in isolation is not sufficient as overall crop health relies on many components. The plant cell wall serves an important function in crop health as a critical barrier against pests and diseases. I investigate the role of a family of putative broad spectrum defense response genes in rice, *OsOXOs*, that degrade oxalic acid: a pathogenicity factor. When expression of these genes is modified, I find a large impact on disease resistance to *Sclerotinia sclerotiorum* but little impact in the presence of *Rhizoctonia solani*. *OsOXOs* must play an important role in defense against *S. sclerotiorum* which relies on oxalic acid as a pathogenicity factor, because *OsOXOs* can degrade oxalic acid. *R. solani*

utilizes a broader range of enzymes and compounds, limiting the effectiveness of *OsOXOs* against *R. solani*.

With the bioenergy phenotyping methods optimized above, I assess saccharification yield of a rice mapping population, along with other agronomic traits including total biomass, flowering time, grain yield, and plant height. Transgressive segregation is apparent for all traits and quantitative trait loci (QTL) mapping approaches are presented. With the methods and populations evaluated here, we are closer to identifying the conditions and genes that can maximize biomass tailored for many purposes.

ACKNOWLEDGEMENTS

I thank my committee for their guidance and direction over the years. I especially thank my advisor Dr. Leach for advice on all things professional and personal, motivation in the face of obstacles, and patience as I failed and excitement when I succeeded. Dr. Leach never paused to introduce me to new collaborators and new ideas and I thank her for the opportunity to meet and work with amazing people in amazing places all over the world. Dr. Leach encouraged me to pursue interesting opportunities, even if they did not involve working in the lab and I am forever grateful for this. I also thank all my co-authors on published, and hopefully soon to be published manuscripts for their comments and advice.

I thank all of the present and former members of the Leach lab without whom I would still be grinding straw or extracting DNA. Jillian Lang: not only the best lab manager, but an amazing person; thanks for your optimism that we would eventually obtain overexpression lines among countless other help you provided. Courtney Jahn, Myron Bruce, Rene Corral, Paul Langlois, Sam Vazquez, Jesse Ellgren, Meghan Ferguson, Lysa DuCharme, Ashanti Robinson, Adam Overton, Brian Hadley, Jacob Snelling, as well as Amanda Broz, Bettina Broeckling, Julius Mojica, Leon Van Eck, and anyone else I omitted to mention by name. You are all amazing and I'm so lucky to have worked with you. Michael Joyce: thanks for your amazing job shooting and putting together our video documentary, and for your interview coaching tips as well; I needed them! I thank Mawsheng Chern for providing the overexpression vector pUbiNC1300-RFCA, Marty Dickman for providing the *Sclerotinia sclerotiorum* strains and Anna McClung for providing Lemont and Jasmine-85 seed. Leif Anderson, for creating the LaTeX document class I adapted to typset this document. I thank Beth Grabau for answering detailed questions enabling me to optimize the OXO enzyme

activity assay. Jesse Poland for saving our genotyping, and introducing me to the wonders of R. Jim zumBrunnen for many conversations about statistics; I'll always have more to learn.

My collaborators and field crews at IRRI: you were always patient with my many questions and my inability to navigate deftly through the rice fields. Thank you for tackling field trials with mind-boggling numbers of plants, I certainly couldnt do any of this without you. I should especially mention Mayette Baraoidan, Ramil Mauleon, and Hei Leung for hosting me and for your guidance over the years. Janice, Josie & Dang: I think I learned more lab tricks in the weeks I was there than the years at CSU. Salamat Po!

I especially thank John Field, Sam Evans, Scott Fulbright, Stevan Albers, Barb Gibson and the rest of the IGERT crew for great conversations and great times over the years and helping me appreciate the many facets and angles of bioenergy research and commercialization. I also appreciate the organizations that supported my research including the CSU Clean Energy Supercluster, NSF IGERT program, USDA, USAID, and DOE.

Finally, but not least, I thank my family, and especially my mom who is one of the smartest people I know and who raised me to be independent, ask questions, and never quit. I'd also like to thank my grandma who kept telling me to stick with it (whatever 'it' was). My God for who watches over me and provides everything I need. My friends who tried to made sure I had enough distractions. Christine, my beautiful and patient girlfriend for her love, support and just being amazing. I never would have survived without her, and I'm sure she is as happy as I am that we're on to the next chapter.

TABLE OF CONTENTS

Abstract ii

Acknowledgements iv

Chapter 1. Introduction 1

1.1. Bioenergy as a sustainable energy solution 1

1.2. Biomass and the plant cell wall 2

1.3. Rice: an important crop and scientific tool 3

1.4. Breeding for bioenergy use must consider many factors 4

1.5. Targeting known genes with molecular genetics 5

1.6. Novel gene discovery through QTL mapping 5

1.7. Scope of dissertation 6

References 9

Chapter 2. Biomass for thermochemical conversion: targets and challenges 12

2.1. Introduction 13

2.2. Feedstock properties for thermochemical conversion 16

2.3. Genetic control of traits related to feedstock properties 24

2.4. Potential for high-throughput phenotyping 30

2.5. Box 1: Silica in grasses: example and opportunity 37

2.6. Conclusion 40

References 47

Chapter 3. Rice straw compositional variation between varieties, tissue types, and environments: impacts on bioenergy potential 78

3.1. Introduction 79

3.2. Materials and methods 81

3.3. Results & discussion 88

3.4. Conclusion 100

References 125

Chapter 4. Characterization of *OXO* genes in rice 135

4.1. Introduction 136

4.2. Materials and methods 139

4.3. Results 146

4.4. Discussion 149

References 168

Chapter 5. Conclusion 175

Appendix A. QTL for biomass and bioenergy traits in a rice mapping population 180

A.1. Introduction 180

A.2. Materials and methods 182

A.3. Results 187

A.4. Discussion 189

References 196

Appendix B. Supplementary material 203

B.1. Chapter 3 supplementary material 203

B.2. Chapter 4 supplementary material 220

B.3. Video documentary of research 230

References 231

CHAPTER 1

Introduction

1.1. Bioenergy as a sustainable energy solution

Developing sustainable alternatives to the limited supply of fossil fuels while mitigating the drivers of climate change is one of the greatest challenges society faces today. Alternatives that are inexpensive, energy-dense, sustainable and able to produce energy at the commercial scale will require major technological advances. Many alternative energy sources exist and more are being developed. Some are better suited for certain end uses and regions of the world; it is likely a combination of these sources will be utilized. For example, wind, solar, and nuclear energy can only be sited in certain areas but are excellent sources of renewable, low carbon electricity. Nuclear power has a questionable future due to high capital costs and an uncertain policy environment, but emits no carbon and provides baseload power.

Recent policy, along with advances in plant genetics, has renewed interest and research in bioenergy as a local, sustainable source of energy with the potential to reduce greenhouse gas emissions. Bioenergy is derived from plants that use sunlight to assimilate CO_2 into biomass. When the biomass is burned as a fuel, the carbon in the biomass is re-released into the atmosphere as CO_2; a net carbon neutral process. Even when other emissions from growing and converting the biomass to energy are included, emissions are still lower than most fossil fuels [1, 2]. However, first generation biofuels are derived from crop grains typically produced as food sources and the sustainability of these technologies has been questioned [3, 4]. Development of second generation bioenergy to utilize the cellulosic portion of biomass avoids many of these issues, but second generation technologies are limited by the recalcitrance of the cellulosic biomass, which is mostly composed of the plant cell wall.

1.2. Biomass and the plant cell wall

The recalcitrance of plant cell walls is the key factor responsible for the success of plants in environments full of pathogens and environmental stressors. The cell wall provides structural support, a barrier between the plant and abiotic and biotic stresses, and regulates the flow of materials through the plant. These roles are possible because the cell walls are composed of heterogeneous networks that can handle the various stresses plants encounter. Four networks within the cell wall are responsible for the characteristics the cell wall exhibits: a network of cellulose, composed of α(1-4) linked D-glucose, hemicellulose, composed of heterogeneous branched sugars, a pectin network composed of charged sugar residues, extensins and other proteins, and in secondary cell walls a network of lignin, composed of branched phenolic monolignols. The cell wall is divided into three layers moving from the cell membrane out: these are the secondary cell wall, primary cell wall, and middle lamella. Major differences exist in plant lineages; dicots have type I cell walls that contain greater amounts of pectin and xyloglucan than type II cell walls of monocots, which replace xyloglucan with glucoronoarabinoxylan [5].

While the composition of plant cell walls is well characterized, the synthesis, transport, and assembly of the components is an active area of research. The genes and transcription factors that orchestrate this complex system are only now being characterized. For example, the transportation of lignin from the cytosol to the cell wall has only recently been described [6].

The composition and architecture of the plant cell walls influence the bioenergy characteristics, and these characteristics vary among plant species and are different for different conversion technologies. Thus, a better understanding of the cell wall will aid development

of improved conversion and processing technologies, but also enable the classical breeding or engineering of cell wall composition optimized for bioenergy.

1.3. Rice: an important crop and scientific tool

With complex biological systems, it is useful to have model species to investigate how parts of the systems interact. Plant biology has greatly benefited from the model crop species *Oryza sativa* (rice) [7]. Rice was one of the first plant species to have the genome completely sequenced, and the genome assembly remains one of the most complete to date. It is primarily selfing and has a relatively small diploid genome. It is amenable to genetic transformation, and several mutant populations exist to enable functional genetics. There is a large collection of diverse germplasm, as well as several closely related species from which useful traits can be discovered and transferred to rice. All these characteristics enable detailed genetic and functional studies, which, because many other crops are related cereal species, can be translated from rice to other important crops.

Not only is rice a useful model species, it is an extremely important crop worldwide. It is grown on over 150 million hectares, and over 700 million tons of grain are produced per year. This grain supplies 21% of the global human caloric intake, and for 3 billion people, rice supplies over 50% of their caloric intake [8]. Rice has been cultivated for over 10,000 years and over 100,000 varieties exist with diverse genetic backgrounds as well as different agronomic and morphological traits.

Rice straw can represent 50-70% of the crop biomass, but has little economic value and end use. Often it is burned or tilled back into the field, practices which reduce local air quality or carryover of disease from season to season respectively. This straw could be a vast source of agricultural residues, and since the crop is already grown for food, no additional

inputs or land are required. Improving rice straw for forage or bioenergy would add value to a crop with real world impact, especially in parts of the world with lower incomes.

1.4. Breeding for bioenergy use must consider many factors

Through recurrent selection for useful traits, cultivated plant species are highly modified from related but wild species. As with all breeding strategies, the trait of interest must be identified, and variation in that trait must exist—either naturally occurring variation, or induced variation from biotechnology or mutant approaches. Then plants with the optimal combination of traits are selected and used to develop improved varieties.

In the history of agriculture, breeding for bioenergy is a relatively recent strategy and the optimal plant archetype is not well defined. In fact, one universal archetype does not exist as it will depend on the intended conversion technology. Understanding what traits to optimize, and how to measure these traits in large numbers of plants in breeding populations is the current frontier of bioenergy feedstock research. Only after these parameters and quantification techniques are clearly defined can the difficult task begin of identifying the network of genes that control the traits of interest for bioenergy.

Many traits are highly influenced by environmental conditions as plants have adapted to respond to different environments; this is referred to as genetics by environment (GxE). In addition to optimizing traits for different conversion processes, one must be aware of the environment in which the traits are being measured, and how these traits change in different environmental conditions. How influential environment and thus GxE is on a trait is hard to predict and usually must be experimentally tested. The better we can characterize bioenergy phenotypes in multiple environments, the more effectively we can develop models to predict how influential different parameters are on bioenergy yields.

1.5. Targeting known genes with molecular genetics

Two major approaches to link genotype to phenotype are reverse genetics, and the association approach (genome wide association studies, GWAS; and quantitative trait loci mapping, QTL mapping). The candidate gene approach starts with an *a priori* hypothesis about which genes might be involved. The role of the gene(s) is validated through mutation or transgenic manipulation. GWAS and QTL mapping approaches work by measuring the phenotype of many individuals either in a population of diverse plant varieties (GWAS), or a mapping population from a bi-parental cross or a multi-parental MAGIC or NAM population [9, 10].

The plant cell wall is the first line of defense against pathogens, and many defense-related genes are active in the cell wall. Some of these defense-related genes act by remodelling the cell wall through callose or lignin formation [11]. One of these gene families, oxalate oxidase, could be an early response and broad spectrum resistance gene since it catalyzes the conversion of oxalic acid, a pathogenicity factor, into H_2O_2, a signaling molecule for disease response [12]. This product, H_2O_2, could also drive the deposition of lignin, and the release of Ca^{2+}, another signaling molecule [13, 14]. In addition to overlap between disease resistance and modifications in the cell wall, simply improving the disease resistance of a crop will improve bioenergy yield because yield per area will be higher as plants suffer less disease.

1.6. Novel gene discovery through QTL mapping

Functional genetics has benefited from the integration of knowledge of several fields—from biochemical characterization of protein targets, and protein domain databases, to the comparative genetics between homologous genes in different species. However, there is often

a need to identify novel genes responsible for important traits. As mentioned above, QTL and GWAS enable the association between a measured phenotype from a population and the genetic region that may contain the underlying genes responsible for the phenotype. The populations are individually genotyped, most recently through genotyping by sequencing (GBS) [15], and statistical associations between the phenotypic values and the genetic regions are revealed [16–18].

1.7. Scope of dissertation

This dissertation aims to develop the tools necessary to measure important bioenergy related traits in rice straw, and characterize some of the variation in these traits that exist across varieties and environments. I then proceed to link a candidate gene with a functional role in disease resistance, and phenotype a mapping population for several bioenergy traits. These aims are accomplished with the following objectives:

1) (In Chapter 2) To identify and review the important feedstock traits for thermochemical conversion of biomass, and examine existing literature for variation (breeding potential) and genetic control of these traits. I review the two major bioenergy pathways: enzymatic and thermochemical conversion. I discuss how these pathways are similar and different, and how different factors are important for each pathway. I focus on the thermochemical pathway, which has enormous potential; however feedstock parameters are not as well understood as those for enzymatic pathways. My approach is to explore the desired traits, and specifically identify those which are possible targets for breeding and biotechnology. This vertical integration of knowledge and understanding from the field to the engine is critical

for plant biotechnology with real-world impact. A key feature of this chapter is to link common terminology used by engineers and biologists. I conclude with a review of methods for measuring these traits.

2) (In Chapter 3) To measure environmental and genetic variation in the stem and leaf tissue of five varieties of rice for cell wall composition and bioenergy yield. I focus on the relationships between composition and sugar yield for enzymatic conversion. Characterizing these relationships is critical for bioenergy research to move from the greenhouse to the field. Often, plants are characterized in the greenhouse without a clear understanding of whether the cell wall composition will change in the field, and how it will change. I conclude with an examination of whether composition or architecture are the primary drivers of variation in bioenergy yield.

3) (In Chapter 4) Examine the role of the *OXO* family of genes in broad host disease resistance and cell wall composition. I generate silenced and overexpression lines in rice and measure how these lines respond to challenge with two pathogens (*Sclerotinia sclerotiorum*, *ScS*; *Rhizoctonia solani*, *Rs*). I challenge these rice lines with a pathogen that generates oxalic acid as a pathogenicity factor (*ScS*), as well as a mutant strain of this pathogen lacking the ability to generate oxalic acid and quantify the importance of oxalic acid, and *OXOs* in rice resistance to this pathogen. I contrast this with resistance to the rice pathogen, *Rs*.

4) (In Appendix A) Phenotype and genotype by sequencing a large mapping population of rice for biomass and bioenergy traits. Traits important for bioenergy are also important for increased grain yield (total biomass) as well as forage feed for animals and plant defenses against pathogens (cell wall composition). Using a recombinant inbred population (RIL) of rice developed from two parents diverging for biomass traits, I design and carry out a

field experiment over two seasons to measure bioenergy traits and straw tissue for cell wall composition analysis. These traits include biomass, height, grain weight, flowering time, glucose yield and pentose yields from straw tissue. QTL mapping approaches are discussed.

REFERENCES

[1] Borrion, A. L., McManus, M. C., and Hammond, G. P., 2012. Environmental life cycle assessment of lignocellulosic conversion to ethanol: A review. *Renewable and Sustainable Energy Reviews*, 16, 7:4638–4650

[2] Hsu, D. D., Inman, D., Heath, G. A., Wolfrum, E. J., Mann, M. K., and Aden, A., 2010. Life Cycle Environmental Impacts of Selected US Ethanol Production and Use Pathways in 2022. *Environmental Science & Technology*, 44, 13:5289–5297

[3] Tilman, D., Socolow, R., Foley, J. A., Hill, J., Larson, E., Lynd, L., Pacala, S., Reilly, J., Searchinger, T., Somerville, C., and Williams, R., 2009. Beneficial BiofuelsThe Food, Energy, and Environment Trilemma. *Science*, 325, 5938:270 –271

[4] Searchinger, T., Heimlich, R., Houghton, R. A., Dong, F., Elobeid, A., Fabiosa, J., Tokgoz, S., Hayes, D., and Yu, T.-H., 2008. Use of U.S. Croplands for Biofuels Increases Greenhouse Gases Through Emissions from Land-Use Change. *Science*, 319, 5867:1238–1240

[5] Scheller, H. and Ulvskov, P., 2010. Hemicelluloses. *Annual Review of Plant Biology*, 61:263–289

[6] Miao, Y.-C. and Liu, C.-J., 2010. ATP-binding cassette-like transporters are involved in the transport of lignin precursors across plasma and vacuolar membranes. *Proceedings of the National Academy of Sciences*, 107, 52:22728–33

[7] Bush, D. R. and Leach, J. E., 2007. Translational Genomics for Bioenergy Production: There's Room for More Than One Model. *The Plant Cell Online*, 19, 10:2971 –2973

[8] FAO, 2011. FAOSTAT. `http://faostat.fao.org/`. [Accessed 2014-01-10]

[9] McMullen, M. D., Kresovich, S., Villeda, H. S., Bradbury, P., Li, H., Sun, Q., Flint-Garcia, S., Thornsberry, J., Acharya, C., Bottoms, C., Brown, P., Browne, C., Eller, M., Guill, K., Harjes, C., Kroon, D., Lepak, N., Mitchell, S. E., Peterson, B., Pressoir, G., Romero, S., Rosas, M. O., Salvo, S., Yates, H., Hanson, M., Jones, E., Smith, S., Glaubitz, J. C., Goodman, M., Ware, D., Holland, J. B., and Buckler, E. S., 2009. Genetic Properties of the Maize Nested Association Mapping Population. *Science*, 325, 5941:737–740

[10] Cavanagh, C., Morell, M., Mackay, I., and Powell, W., 2008. From mutations to MAGIC: resources for gene discovery, validation and delivery in crop plants. *Current Opinion in Plant Biology*, 11, 2:215–221

[11] Malinovsky, F. G., Fangel, J. U., and Willats, W. G. T., 2014. The role of the cell wall in plant immunity. *Frontiers in Plant Science*, 5, May:178

[12] Carrillo, M. G. C., Goodwin, P. H., Leach, J. E., Leung, H., and Vera Cruz, C., 2009. Phylogenomic Relationships of Rice Oxalate Oxidases to the Cupin Superfamily and Their Association with Disease Resistance QTL. *Rice*, 2:67–79

[13] Bhuiyan, N. H., Selvaraj, G., Wei, Y., and King, J., 2009. Gene expression profiling and silencing reveal that monolignol biosynthesis plays a critical role in penetration defence in wheat against powdery mildew invasion. *Journal of Experimental Botany*, 60, 2:509 –521

[14] Lane, B., 1994. Oxalate, germin, and the extracellular matrix of higher plants. *The FASEB Journal*, 8, 3:294 –301

[15] Elshire, R. J., Glaubitz, J. C., Sun, Q., Poland, J. A., Kawamoto, K., Buckler, E. S., and Mitchell, S. E., 2011. A Robust, Simple Genotyping-by-Sequencing (GBS) Approach for High Diversity Species. *PLoS ONE*, 6, 5:e19379

[16] Mackay, T. F., 2001. The genetic architecture of quantitative traits. *Annual Review of Genetics*, 35:303–39

[17] Collard, B., Jahufer, M., Brouwer, J., and Pang, E., 2005. An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. *Euphytica*, 142, 1:169–196

[18] Takeda, S. and Matsuoka, M., 2008. Genetic approaches to crop improvement: responding to environmental and population changes. *Nature Reviews Genetics*, 9, 6:444–457

CHAPTER 2

Biomass for thermochemical conversion: targets and challenges[1]

Overview

Bioenergy will be one component of a suite of alternatives to fossil fuels. Effective conversion of biomass to energy will require the careful pairing of advanced conversion technologies with biomass feedstocks optimized for the purpose. Lignocellulosic biomass can be converted to useful energy products via two distinct pathways: enzymatic or thermochemical conversion. The thermochemical pathways are reviewed and potential biotechnology or breeding targets to improve feedstocks for pyrolysis, gasification, and combustion are identified. Biomass traits influencing the effectiveness of the thermochemical process (cell wall composition, mineral and moisture content) differ from those important for enzymatic conversion and so properties are discussed in the language of biologists (biochemical analysis) as well as that of engineers (proximate and ultimate analysis). We discuss the genetic control, potential environmental influence, and consequences of modification of these traits. Improving feedstocks for thermochemical conversion can be accomplished by the optimization of lignin levels, and the reduction of ash and moisture content. We suggest that ultimate analysis and associated properties such as H:C, O:C, and heating value might be more amenable than traditional biochemical analysis to the high-throughput necessary for the phenotyping of large plant populations. Expanding our knowledge of these biomass traits will play

[1]Published as"Biomass for thermochemical conversion: targets and challenges" in Frontiers in Plant Science, 2013 Jul 1;4:218. doi: 10.3389/fpls.2013.00218 by Paul Tanger, John L. Field, Courtney E. Jahn, Morgan W. DeFoort and Jan E. Leach.

a critical role in the utilization of biomass for energy production globally, and add to our understanding of how plants tailor their composition with their environment.

2.1. Introduction

2.1.1. Multiple pathways from feedstock to energy

Our society and economy rely heavily on energy from fossil fuels. Most (84%) of the worlds energy comes from fossil fuels and demand will increase as world energy consumption is expected to increase 53% by 2035 [1]. As prices rise, unconventional fossil resources (tar sand oil, shale gas, arctic and deepwater oil) may become economically viable to extract, but they are ultimately a limited resource and carry risks to our health and environment [2–4].

Bioenergy, derived from plants that use sunlight and CO_2 to assimilate carbon into biomass, has emerged as a potentially sustainable energy source with low climate impact. The Renewable Fuel Standard, enacted in 2005 and expanded in 2007, mandates liquid biofuel production in the US [5]. The majority of the fuel produced today to support this mandate is derived from either ethanol fermented from corn grain, or biodiesel from soybean oil, but by the year 2022, 58% of the legislated 36 billion gallons is required to be produced from cellulosic or advanced cellulosic biomass. Technological advances and commercialization have not occurred as quickly as expected, and several barriers must be overcome to achieve these targets [6].

One of these barriers is the production of high quality biomass that can be economically converted into useful energy products. Biomass quality depends on the plant composition — cellulosic biomass is primarily comprised of cellulose, hemicellulose, lignin, and lesser amounts of other extractable components such as pectins, proteins, etc. that make up the plant cell wall. Cellulose is a polymer of D-glucose. Hemicellulose is a general term for

heterogeneous branched five and six carbon sugars. Lignin is a complex branched polymer of phenolics, and is classified as three major types, based on the monomers present: sinapyl (S) coumaryl (H) and coniferyl (G) [7]. The proportions and specific chemical composition of these components varies greatly among species [8–12]. Furthermore, significant compositional variation has been observed within a species [11, 13], within tissue type [14–17], as well as between developmental stages [16], cell types, and even regions of the cell wall [7]. Additional variability is observed throughout the growing season and as plants senesce [18–23], as well as across different environments [22, 24–27].

Variation, either naturally existing variation or driven with biotechnology, is the ultimate source of improved crop varieties. Most feedstock improvement efforts have focused on the enzymatic conversion pathway, and how to increase the availability of components of plant biomass that can readily be converted into simple sugars and fermented into alcohols; i.e., maximizing cellulose and minimizing lignin. Other articles in this research topic address challenges and advances in enzymatic conversion, as have multiple recent reviews [28–31].

A promising alternative form of bioenergy production is via thermochemical conversion the controlled heating or oxidation of biomass [32, 33]. The term covers a range of technologies including pyrolysis, gasification, and combustion which can be configured to produce outputs of heat, electricity, or gaseous or liquid precursors for upgrading to liquid fuels or chemical feedstocks (Figure 2.1 and [34–38]). Thermochemical technologies show great promise for the production of renewable electricity, both in the context of biomass co-firing in existing coal powerplants [39, 40], and for decentralized electrification projects in developing countries [41–43]. Thermochemical produced electricity could help fulfill standards enacted in many US states that require a certain percentage of electricity be produced from renewable sources [44–46]. In some cases, thermochemical production of renewable electricity or

liquid fuels and associated co-products is the most effective use of biomass for fossil energy displacement [47–51].

A well-functioning system requires the pairing of appropriate feedstocks and conversion technologies [52], but optimization of biomass for thermochemical conversion has received little attention. The paradigm within which plant biologists discuss and analyze biomass is different than that of engineers analyzing feedstocks for thermochemical systems. While there is overlap between the paradigms, thermochemical feedstock development could focus on traits or approaches that provide the most direct path to optimized feedstock composition. In this chapter, we discuss how, through collaboration of biologists and engineers, optimized biomass composition and process engineering might result in reduced transport and pre-processing costs and maximized energy yields via thermochemical utilization of biomass.

We begin with a review of thermochemical conversion technologies with an emphasis on the feedstock properties that are important for each technology and relate these properties back to biomass traits that are commonly measured by biologists. This is followed by a discussion of the natural variation in plant traits that can be exploited for optimization of these properties, including what is known of the genetics governing those traits, and the potential impacts of modifying these traits at a systems level. We end with a discussion of how best to measure these properties and traits, and offer a perspective on which approaches might be useful for high-throughput phenotyping. To help relate the different biomass traits that biologists and engineers measure, we provide a brief list of terms and definitions (Table 2.1). Areas where there are large gaps in knowledge are highlighted as future research needs. Our focus is on cellulosic biomass from herbaceous crops because (1) herbaceous agricultural residues comprise a large potential resource [53], (2) a large fraction of the US biofuel mandate is expected to be dedicated herbaceous bioenergy crops [54, 55], and, (3)

herbaceous crops can be grown in more regions than woody crops, and allow more flexibility in year to year land allocation.

2.2. Feedstock properties for thermochemical conversion

2.2.1. Thermochemical conversion technologies

Thermochemical conversion is the controlled heating and/or oxidation of biomass as part of several pathways to produce intermediate energy carriers or heat (Figure 2.1). Included is everything from biomass combustion, one of the simplest and earliest examples of human energy use, to experimental technologies for the production of liquid transportation fuels and chemical feedstocks. Thermochemical conversion technologies are classified by their associated oxidation environment, particle size and heating rate, ranging from heating biomass in an oxygen-free environment (endothermic) to full exothermic oxidation of biomass (Figure 2.1).

Pyrolysis is the thermal decomposition of biomass into highly heterogeneous gaseous, liquid, and solid intermediates in the absence of oxygen; the process is endothermic. The liquid product (pyrolysis oil) is a heterogeneous mixture characterized by high oxygen content and alkalinity, which can be upgraded to fuels or chemicals. The solid product (char) can be used as a fuel or soil amendment [56]. Pyrolysis is differentiated between slow pyrolysis, with residence times ranging from minutes to days and optimized for the production of char whereas fast pyrolysis, with residence times on the order of seconds to minutes, is optimized for the production of pyrolysis oil [57]. On the engineering front, research is focused on optimizing process variables (temperature, heating rate, oxidation environment) and product upgrading via catalytic and thermal processes to produce infrastructure-compatible liquid transportation fuels [58].

Gasification is the exothermic partial oxidation of biomass with process conditions optimized for high yields of gaseous products (syngas or producer gas) rich in CO, H_2, CH_4, and CO_2. The gas can be cleaned and used directly as an engine fuel or upgraded to liquid fuels or chemical feedstocks through biological fermentation [59] or catalytic upgrading via the Fischer-Tropsch process [60–62]. One of the challenges of gasification is the management of higher molecular weight volatiles that condense into tars; these tars are both a fouling challenge and a potential source of persistent environmental pollutants such as polycyclic aromatic hydrocarbons [63].

The direct combustion of biomass is still the dominant bioenergy pathway worldwide [64](Gaul, 2012). Complete combustion involves the production of heat as a result of the oxidation of carbon- and hydrogen-rich biomass to CO_2 and H_2O. However, the detailed chemical kinetics of the reactions that take place during biomass combustion are complex [57, 65] and imperfect combustion results in the release of intermediates including environmental air pollutants such as CH_4, CO, and particulate matter (PM). Additionally, fuel impurities, such as sulfur and nitrogen, are associated with emission of SO_X and NO_X [52].

Other thermochemical technologies include carbonization, the production of charcoal via the partial oxidation of woody feedstocks with long residence time [66], and hydrothermal approaches, which utilize an aqueous environment at moderate temperatures (200-600° C) and high pressures (5-40 MPa) to decompose biomass into solid, liquid, and gaseous intermediates [67, 68]. Another technology, torrefaction, is the low temperature (200-300° C) pyrolysis of biomass in order to remove water and volatiles, increasing its energy density and susceptibility to mechanical pretreatment [69]. The remainder of this chapter will focus on pyrolysis, gasification, and combustion, as these are the most fully developed modern bioenergy pathways with the most clearly defined feedstock requirements.

2.2.2. Relationships between feedstock properties

The performance of these thermochemical conversion pathways relies on the use of appropriate biomass feedstocks. The mass balance of a kilogram of biomass is commonly conceptualized in three different ways, via either biochemical, proximate, or ultimate analysis (Figure 2.2). Biochemical analysis refers to the relative abundance of various biopolymers (e.g., cellulose, lignin, etc) in the biomass, whereas ultimate analysis refers to the relative abundance of individual elements (e.g., C, H, O, N, and S). Proximate analysis involves the heating of biomass to quantify its thermal recalcitrance via the relative proportions of fixed carbon (FC) and volatile matter (VM), a method originally designed for the characterization of coal (e.g., American Society for Testing and Materials, ASTM standard D3172). These different conceptualizations are alternate ways to describe the same biomass; for example, a higher lignin:cellulose ratio (biochemical) also implies lower H:C and O:C ratios (ultimate) [70]. Moisture and elemental ash complete the mass balance of a unit of freshly-harvested biomass, and are universal across these different conceptualizations. Different combinations of these mass-based properties (summative properties) result in different bulk properties (intensive properties) such as grindability (comminution), density and heating value.

Feedstock properties that affect thermochemical conversion effectiveness include heating value, ash content, moisture level, and others discussed next. While thermochemical conversion engineers typically describe biomass in terms of proximate or ultimate analysis, biologists and breeders are more accustomed to the terminology of biochemical analysis. Thus, important properties are introduced in the context of proximate/ultimate analysis, and then related back to their biochemical equivalents. Current knowledge of the genetic and environmental control of these biochemical properties are then described in detail in Section 2.3.

2.2.3. Heating value and ratios of C, H & O

Heating value, also known as calorific value, is the energy available in the feedstock as estimated from the heat released during complete combustion to CO_2, H_2O (gaseous H_2O for lower heating value, LHV, or liquid H_2O for higher heating value, HHV), and other minor products (N_2, ash, etc.), and is a primary measure of quality of a feedstock. Moisture content impacts the useful energy of freshly harvested biomass as heat liberated during combustion is wasted evaporating this moisture [71]. Since HHV is a mass based measurement, high mineral content leads to a decrease in HHV, because minerals contribute little energy during biomass oxidation [72, 73]. This is particularly important for grasses and other herbaceous feedstocks that can consist of up to 27% ash by mass (Table 2.2).

Biomass feedstocks are also described in terms of ultimate analysis based on the relative content of individual elements such as C, H, and O. The overall ratios of these elements are directly related to the biochemical components of the cell wall. Cellulose has a higher H:C and O:C ratio than lignin [70]. Lignin has a higher HHV than cellulose or starch [74, 75], consistent with the idea that oxygenated fuels release less heat on combustion [76]. This is an example of divergent feedstock requirements for enzymatic versus thermochemical conversion pathways: while minimizing lignin improves hydrolysis and fermentation yields, high lignin is beneficial for the energy balance of thermochemical systems.

Upgrading gaseous pyrolysis and gasification products to liquid fuels also requires a specific H:C stoichiometry [59, 103]. Biomass has a low H:C ratio (ranging from 0.7-2.8 in Table 2.2) relative to that of the desired liquid products (2-4 for alcohols and alkanes), so full conversion requires adding supplemental hydrogen in the form of steam or H_2, or removing carbon as CO_2 [104, 105]. High lignin levels may be advantageous for thermochemical

conversion pathways targeting liquid fuels, as it may move the process closer to overall stoichiometric balance.

2.2.4. Proximate analysis and conversion product yields

Proximate analysis separates the biomass into four categories of importance to thermal conversion: moisture, volatile matter (VM, gases and vapors driven off during pyrolysis), fixed carbon (FC, non-volatile carbon), and ash (inorganic residue remaining after combustion) [65, 106, 107]. The measurement is a proxy for thermochemical conversion performance, and the relative proportions of fixed carbon versus volatile matter are related to the relative yields and composition of solid, liquid, and gaseous products generated during pyrolysis and gasification [36]. Even for combustion, the FC:VM ratio may significantly change the emissions profile of products of incomplete combustion [108]. Biomass generally contains high levels of volatile matter (ranging from 64-98%, Table 2.2) compared to fossil coal (typically below 40% [82]).

In addition to impact on heating value, the relative concentrations of cellulose and lignin also affect the yields of thermochemical conversion products. The different biochemical constituents of biomass have different levels of thermal stability, and as pyrolysis temperatures increase hemicellulose reacts first, followed by cellulose and then lignin [109, 110]. This is consistent with studies that show isolated lignin extracts having a higher FC content than pure cellulose [70], a strong positive correlation between FC and lignin across multiple biomass samples [111], and increasing lignin levels associated with low gas yields and high char yields during fast pyrolysis [112]. However, several studies suggest the opposite, showing cases where increasing lignin is associated with lower fixed carbon [96, 109], or increasing yields of pyrolysis oils [113].

Clear relationships between FC:VM and lignin:cellulose content in biomass samples are likely confounded by the presence of minerals, some of which exert a strong influence on the yields and qualities of thermochemical conversion products due to catalytic activity [70, 112]. For pyrolysis, high mineral content reduces oil yield and increases char and gas products [70, 109, 113]. Relationships between VM and lignin are confounded by ash content [114]. In addition, ash exerts a catalytic effect on the liquid fraction, encouraging cracking of high molecular weight species into lighter ones [96]. The catalytic activity of ash changes the dynamics of combustion and gasification; reducing the ash content of biomass by washing has been shown to increase the temperature of peak combustion rate [109] but decrease the temperature of peak gasification mass loss rate [112]. Many studies show a negative correlation between mineral content and lignin across many types of biomass [96, 109, 112]. Thus, the relationship between ash, lignin, and pyrolysis product yield is complex and careful experimental manipulation will be necessary to determine the causality underlying the observed correlations of low ash, high lignin, and high yields of heavy liquid products [70, 96].

2.2.5. Other effects of mineral content

Besides lowering the heating value of biomass and changing the distribution of conversion products, mineral and elemental ions that plants accumulate can interfere with the operation of thermochemical conversion equipment. The elements in plant biomass volatilize during combustion and form a liquid slag or solid deposits as they cool [106]. The elements Na, K, Mg, Ca as well as Cl, S and Si are the most problematic for thermochemical processes [106], and the combination of alkali metals with silica can form alkali silicates [115] see Section 2.5 for more information regarding silica. The Cl in biomass can also be a significant problem because it interacts with vaporized metals, shuttling them to boiler surfaces where they

form sulfates [10]. Cl can also lead to elevated HCl and dioxin emissions [116]. As volatile gases combine, they form corrosive deposits that degrade components of the boiler. Other interactions can occur between the elements in biomass and coal when co-fired [117]. Since gasification can occur at lower temperatures, the severity of these issues might be reduced with that process; however, other issues can become more severe ([118] and see [112] for discussion). Although difficult to generalize due to the complex and unique interactions that occur in each feedstock, ash content above 5% is probably unacceptable [119] and element specific recommendations are listed elsewhere [120]. The alkali index (kg K_2O and Na_2O per GJ energy) can be used to predict performance in a thermochemical setting [65]. With an alkali index above 0.17 kg/GJ, fouling is probable, and above 0.34 kg/GJ, it is almost certain. Several other indices exist, but were created for coal, so may not be good predictors for biomass [121]. High feedstock mineral content can be mitigated to a certain extent by using newer alloys to construct components that can minimize and withstand some corrosion, and controlling the temperature of the reaction [65, 96].

2.2.6. Moisture content

Moisture content is a measure of the amount of water in biomass and is usually expressed as percent mass (wet basis). In addition to reducing the net heating value as discussed previously, high moisture content can reduce the effectiveness of individual thermochemical conversion processes. For combustion or co-firing, low moisture content, preferably around 5%, is desired because incomplete combustion can occur when the moisture content is too high. Some systems such as fluidized bed combustors are more flexible, and allow up to 35% moisture [71]. For gasification, acceptable moisture content can be as high as 20% or 30% [108], but more commonly is around 15% moisture. For pyrolysis, initial moisture

content contributes to the water content in the pyrolysis oil and above around 10% moisture, the oil produced will separate into two phases [36, 38]. For hydrothermal conversion, wet biomass can be used without drying, but these technologies are still in the development stages [67, 105, 122, 123].

2.2.7. Other considerations

In general, biomass has low amounts of S relative to fossil fuels, which minimizes SO_X pollution from gasification or combustion systems and avoids catalyst poisoning in fast pyrolysis systems [68]. It can have similar or higher N, which contributes to NO_X emissions, but this can be mitigated to some extent through engineering in the process, e.g., by the use of exhaust scrubbers [121]. High levels of nitrogen can also be problematic for the quality of liquid fuel products from fast pyrolysis [124]. For combustion processes, lignin is associated with PM emissions [125], a factor that must be balanced against the associated increase in feedstock HHV from a systems perspective.

In addition to direct effects on thermochemical conversion performance, biomass properties are also relevant to the upstream logistics associated with biomass transport and mechanical pre-treatment. Minimizing moisture reduces weight during transport from the field, and maximizing dry bulk density allows more cost effective transport of biomass. It has been estimated that reducing moisture content from 45% to 35% in biomass can lead to a 25% increase in the net present value of a thermochemical project producing ethanol from cellulosic biomass mostly by reducing the energy and cost of drying the biomass [126]. Grindability relates to many other properties including moisture content and composition [127]. Beyond impacts on biomass transport costs, bulk density can influence how easily biomass can be ground for processing [128].

2.3. Genetic control of traits related to feedstock properties

As highlighted in Figure 2.2 and introduced in the previous section, feedstock properties are related to biochemical traits that have been the focus of research by the forage, pulp and paper industry, as well as enzymatic bioenergy research for many years. These biochemical traits are more easily explained in the context of genes that encode the proteins that synthesize and deliver the components of the cell wall as well as the enzymes responsible for assembly of the wall components into complex structures.

For breeding or biotechnology approaches to improve cell wall composition, a major constraint is understanding which genes or gene pathways are important. Relating genotype to phenotype, i.e., to assign a gene responsible for a particular phenotype, allows identification, functional analysis, and modification of the gene (or its regulation) to improve the phenotype. For example, experiments that modify genes individually and in combination show the effect of a given gene on the composition of the biomass [129–131]. This information can be the basis for development of molecular markers to improve the phenotype by breeding or to design gene constructs for improvement through biotechnology. This knowledge, frequently gained from model plants can be applied even to distantly related species by using comparative genomics approaches [132]. This is important because for some species, notably several emerging energy grasses, genetic tools are just being developed. As with all breeding efforts, agronomic considerations must be considered; that is, the plants must still be able to survive and produce an acceptable yield. In the following sections, we discuss the genetic and environmental control of traits related to thermochemical conversion properties.

2.3.1. Cellulose and lignin

Often comprising more than 50% of the cell wall, cellulose and lignin have been well-studied and the enzymes involved in their synthesis are well understood [133, 134]. However, how these components are linked within the cell wall, and how the synthesis and modification are regulated are not well understood [135]. There is a complex balance between cellulose and lignin levels, and the manipulation of genes involved in their biosynthesis sometimes leads to unexpected results [136]. Plants are surprisingly flexible, and can utilize a diverse set of precursors to build their cell walls. For example, Jensen and coworkers modified the native form of xyloglucan (a hemicellulose) in Arabidopsis without any apparent phenotypic consequences [137]. Yang and colleagues engineered plants to have thicker cell walls with more polysaccharides, but less lignin without negative consequences [131].

Research has focused on genes controlling the wall composition of the model dicot, Arabidopsis, or woody crops like poplar. However, to apply knowledge of these genes to more feedstocks, the findings will need to be validated in new crops. For example, lignin monomer composition differs between woody and herbaceous crops [138, 139]. Gymnosperms have mostly G lignin while dicots have G and S and monocots generally have all three types. These monomers have different properties, including different estimated HHV [140], and may influence the thermochemical properties of the biomass [141]. It has been found that coniferous (mostly G) lignin is more thermally-stable than deciduous (mostly S) lignin [142], and this is likely because G lignins contain more resistant linkages than S lignins [133]. Approaches to fine-tune lignin composition have been suggested [143]. The ratio of these monomers, as well as the soluble phenolics, may have consequences as important as cellulose and lignin ratios [110, 144–146]. Because lignin biosynthesis genes vary across plant families, and between dicots and monocots, [147], it is likely that other unexamined differences in

lignin composition in crop species might exist [147]. In addition to the three major lignin monomers, monocots contain relatively large amounts of soluble phenolics and the genes controlling these might be useful targets to modify cell wall composition [148–150].

Beyond genetically controlled variation of wall composition within and between species, growth environment plays a large role. Adler and colleagues observed that lignin content increased from 10% to 33% between a fall and spring harvest of the same crop of switchgrass [22]. Monono and colleagues observed differences in total yield, composition, and ethanol yield in switchgrass between locations and seasons [26]. Miscanthus also displays variation in composition across environments [25]. Switchgrass S, G, and H monomer ratios show major differences when grown in the growth chamber, greenhouse or field [24], which is consistent with strong genotype by environment interactions [151, 152]. Sugarcane internode composition changes over the growing season [153]. Thus, although a viable focus, optimization of biomass through manipulation of wall lignin and cellulose composition and content will require not only an understanding of the genetic controls for these components, but also significant knowledge of the environmental component.

2.3.2. Mineral content and elemental ash

Elements commonly found in biomass ash are profiled in Table 2.2. There are major differences in the concentrations of these elements between woody and herbaceous crops, and herbaceous crops generally have more N, Cl, and K, but less Ca than woody crops [82, 83]. Though not essential for survival, Si is accumulated to high levels in many grasses, up to 10% dry weight [154]. Vassilev and colleagues find that levels of elements seem to exist in five associated groups in biomass, and these associations may have underlying biological

significance: C-H; N-S-Cl; Si-Al-Fe-Na-Ti; Ca-Mg-Mn; and K-P-S-Cl [82]. Therefore, attempting to modulate Ca levels for example, might also impact Mg and Mn levels and it might be difficult to breed away from these associations. In addition to individual elemental associations, there is also evidence of a relationship between total ash content and biochemical constituents, with total ash content inversely proportional to lignin [96, 109], and total ash proportional to cellulose [112]. It has been hypothesized that this relationship is due to overlap in the roles of lignin and mineral fraction with regard to mechanical stability and resistance to attack [109].

While the uptake, transport and roles of several of these mineral elements in plants are well understood [155], little is known about the genes controlling variation for these traits [156–158]. Uptake and distribution of these elements through the plant occurs via many different pathways, including uptake from the rhizosphere, transfers from roots to shoots, and remobilization among organs. These transport pathways can be both shared and opposing between elements, as indicated by positive and negative correlation of mineral and micronutrient phenotypes (reviewed in [159]). For example, Si is negatively correlated with Ca in some species [160], and reducing Si may simply increase Ca in plant tissues (and the Ca associated thermochemical issues). Cl content varies between stems and leaves of miscanthus [116], and Cl and Ca variation has been observed in the bark, needles, and wood of various tree species [161]. Tissue specific differences in other elements probably exist indicating genetic control. Heritability for mineral content ranges from 10-90%, so breeding for some elements will be more difficult than others [159]. Understanding variation for these traits among cultivars of switchgrass is complicated by strong environmental interactions [152, 162], as is probably the case for other feedstocks.

Elemental concentrations also vary widely between and within species, by tissue type, and across harvest time and environments [18–22, 163–165]. Of considerable importance when focusing on crop improvement in elemental composition is that any attempt at improvement will be complicated by the interaction of these gene pathways with other traits essential for crop productivity, i.e., agronomic traits such as drought and salt tolerance, disease or pest resistance [166–169]. Because the genetics is complex and the potential implications on agronomic traits are serious, focus has been on reducing the impacts of these elements by other solutions, such as adjusting harvest time [15], allowing the minerals to leach out in the field before collection [72], and adding compounds to minimize reactions during thermochemical conversion [120].

2.3.3. Moisture content

Wet biomass from the field can contain greater than 50% moisture on a wet basis, but this can vary greatly (Table 2.2), and intrinsic moisture (water tightly bound to biomass) is much lower. Although moisture content is an important component of the energy content, the literature on genetic variation and alteration of traits governing moisture content are sparse. In several species of willow, differences in moisture content of up to 16% exist and almost 40% of this variation is due to genotype [170]. In rice, moisture content between 20 diverse varieties varied from 43-74% and broad sense heritability was found to be 0.6 [91].

It is well known that species and varieties of plants vary in their ability to cope with drought stress [171, 172]. One strategy that plants employ is to manipulate the osmotic potential of their cells, and thus allow water to be maintained under drought conditions [173]. It is through this mechanism that genetic control of the moisture content of the cells exists, and thus possibly the plant as a whole at harvest time. Many of the genes involved

in these processes have been characterized [174]. There may also be significant correlations between moisture content and mineral content, since minerals ions are utilized to modulate the osmotic potential of the cells [175, 176]. In rice varieties studied by Jahn *et al.* [91], a correlation between leaf ash but not stem ash and moisture content was observed, although these relationships have yet to be directly examined.

Clearly there is evidence that selection for moisture content is feasible but application of genetic approaches to improving biomass crops for moisture content has remained largely unexplored. As for mineral content, agronomic solutions to minimizing moisture content have been employed. For example, post-senescence drying reduced moisture content by 30% in miscanthus stems [23].

2.3.4. Other important traits

Other traits highlighted in Figure 2.2 but not discussed thus far in this section include HHV, grindability, bulk density, as well as components of proximate and ultimate analysis. While some information exists about their relationship with biomass composition, little information exists about the genetic control of these traits. Bulk density may be influenced by cell wall changes [177] and variation in grindability has been observed among corn stover, straw, and hardwood [178]. The first steps towards studying these might be to measure their variation across a species (a genome wide association mapping study, GWAS), or study their segregation in a genetic mapping population (a quantitative trait loci, QTL study) [179–182]. A critical component of both of these approaches is the ability to measure these traits in large numbers of plants in a high-throughput manner.

2.4. Potential for high-throughput phenotyping

We have identified many of feedstock traits important for the thermochemical conversion and discussed the relationships between traits. In this section, we review how these traits are measured, and in cases where several methods exist, we highlight those methods which might be amenable to high-throughput phenotyping of many individual plants.

2.4.1. Biochemical analysis

The most complete approach to quantifying the cell wall content is quantitative saccharification (also referred to as dietary fiber, Uppsala method, or NREL method). Water and ethanol soluble fractions are isolated, followed by hydrolysis and quantification of the component sugars, sugar degradation products, and organic acids by high performance liquid chromatography (HPLC) or gas chromatography mass spectroscopy (GC/MS) and acid soluble lignin with UV-vis spectroscopy. Starch is quantified and subtracted from cellulose, since it would contribute glucose monomers and inflate the cellulose component. Protein, ash and acid insoluble lignin (Klason lignin) are quantified from the remaining residue [183, 184]. Another common method originally developed to determine forage quality is called detergent fiber or the Van Soest method, and involves treating biomass with various concentrations of acids and bases to sequentially hydrolyze [185, 186]. Each method highlighted here assumes the monomeric sugars are derived from certain polymers in the cell wall, and each method has its own set of biases [187, 188].

While any method is probably feasible for high-throughput given enough investment in lab time, equipment or automation (such as robotics), we highlight recent approaches in lignin quantification and monomer composition with pyrolysis molecular beam mass spectroscopy (pyMBMS) [24, 145] or thioglycolic acid lignin [189]. Cellulose, hemicellulose and

lignin have been estimated with a thermogravimetric analyzer (TGA) which is essentially a microbalance inside a controlled-atmosphere furnace [190]. High-throughput glycome profiling of cell wall extracts detects presence or absence of specific polysaccharides but does not quantify the various components [191]. Pretreatment and saccarification approaches [192, 193] or ethanol yield [194] directly test how amenable biomass is to enzymatic conversion, and indirectly provide information about the cell wall composition.

2.4.2. Proximate analysis

Proximate analysis separates the biomass into moisture, VM, FC, and ash. This is accomplished through controlled heating of a ground sample in a furnace and observing mass lost during heating. VM and FC are determined after correcting for moisture and ash content. Proximate analysis can also be conducted in a single operation using a TGA. Heating value is also typically measured in the course of proximate analysis using bomb calorimetry, in which a biomass sample is fully combusted in a pure oxygen environment within a reaction vessel suspended in a water jacket; calorific value of the fuel is inferred from changes in the water temperature. HHV includes the energy released when the H_2O produced during the combustion process condenses. An adjustment can be made since the energy due to water condensing is not captured in some systems the adjusted value is the LHV.

While moisture content is part of standard proximate analysis procedure, it can also be evaluated by itself. The simplest, yet most time consuming method to assess moisture content is the oven dry method moisture is removed by drying and the difference in mass is assumed to be moisture loss. These methods assume that the sample has been stored in an airtight container; otherwise moisture gain or loss (due to varying relative humidity of the storage

environment) will have occurred between sample collection, and moisture determination. Often as received moisture is referred to in the literature—this is a meaningless value as it depends on the conditions that the sample underwent between the field and the lab and varies with humidity in the environment, and how long the plant was allowed to senesce in the field. Moisture content can also be estimated on a wet basis with handheld moisture meters [195]. These meters work by testing the conductance or capacitance of the material or various chemical means but only work in certain ranges of moisture [196]. Biomass moisture is conceptually simple to understand and measure, but often goes unmeasured or unclearly reported, hampering our knowledge of the underlying genetic and environmental control.

2.4.3. Ultimate analysis

Profiling the individual elements is accomplished with approaches that measure electronic properties of elements (absorption, emission, and fluorescence spectroscopy) or techniques that measure nuclear properties (radioactivity, mass spectroscopy). Elemental analyzers available from many manufacturers either flash oxidize or pyrolyze the biomass and measure products such as CO_2, H_2O, NO_X in the exhaust gas via gas chromatography and thermal conductivity in order to stoichiometrically back-calculate the initial concentrations in the biomass (see standards in Figure 2.2). Profiling elements in the ash fraction has traditionally been accomplished by solubilizing the ash and detection with atomic absorption spectroscopy (AAS). This involves ionizing atoms using a flame and measuring the portion of light absorbed by the elements as they pass through the detector [197, 198]. When coupled with autosamplers, these instruments can be relatively high-throughput.

Recently, profiling the inorganic fraction in whole biomass (ionomics), has improved with advances in Inductively Coupled Plasma (ICP) techniques. These techniques ionize atoms in

a plasma gas and measure emissions using Optical Emission Spectroscopy as the atoms fall to their ground state (ICP-OES), or the ionized atoms are passed to a mass spectrometer (ICP-MS) [199]. ICP-OES can also be called ICP-AES (Atomic Emission Spectroscopy). Advantages with these approaches include sensitivity, small sample size, and the ability to quantify many elements from the same sample but quantification of some elements (notably Si; see Section 2.5 for further discussion) require special equipment and additional sample preparation.

2.4.4. Other traits

Grindability is measured by recording the energy consumption of the equipment used to grind a sample to specified size [79, 200]. A standard procedure does not appear to exist but would be essential to develop before larger studies are undertaken because the trait is influenced by many factors including moisture content, particle size, and how tightly the biomass is packed before measurement [81, 88]. It should be possible to adapt the existing standard for testing and comparing different types of grinding equipment (ASTM E959) to compare different types of biomass using a standardized piece of equipment. Standard procedures exist for bulk density (Figure 2.2), but are highly dependent on the initial particle size. Particle density, which excludes the air space between particles, is another technique to estimate density of biomass. This can be measured with a gas pycnometer that displaces the air between biomass particles with a known volume of gas [88].

2.4.5. High-throughput phenotyping: automation and indirect measurements

Phenotyping biomass to distinguish between genetic and environmental controls on individual bioenergy traits requires the characterization of large populations of plants, and some of the techniques described above are more appropriate for analyzing large sets of samples

than others. Detergent fiber analysis has been somewhat automated with filter bag systems [201]. Robotic systems that can grind and weigh many samples at once exist to determine properties important for enzymatic conversion [193]. Traditionally, protein is quantified with dyes (Bradford, Lowry, etc), with UV-vis spectroscopy, or other techniques reviewed in [202] or [203], but indirect methods that quantify N (such as the Kjeldahl method or elemental analyzers) simply use a conversion factor to estimate crude protein [204]. There are a number of automated proximate analyzers, elemental analyzers, and calorimeters available [205–207], in which multiple samples can be loaded into racks and then analyzed automatically by the instrument.

Another approach to high-throughput phenotyping is the identification of correlations between the trait of interest and others traits that are more easily measured. For example, heating value can be estimated based on biochemical, proximate, or ultimate analysis through various equations, summarized in [73]. Interestingly, ultimate analysis is the most reliable approach maybe in part due to variation in estimating biochemical or proximate properties. It should be highlighted that like many regression approaches, the sample set that is used to build the equation is critical and thus the equations may be plant species specific. Since grindability is ultimately a function of properties like moisture and composition, equations can be used to predict it in various types of biomass [127, 208].

A variety of properties can also be predicted from non-destructive high-throughput spectroscopic methods, particularly infrared (IR), often measured with an instrument capable of utilizing a Fourier Transform approach (FTIR), or raman spectroscopy which provides information complementary with FTIR, and Near Infrared (NIR) methods. IR spectroscopy measures the absorption of IR radiation by functional groups within compounds and may be used to directly fingerprint the compound, or in complex samples (such as biomass) a

predictive model can be developed to quantify the biomass composition. NIR spectroscopy provides information through the combinations of fundamental bond vibrations (harmonics and overtones) in many compounds that absorb different wavelengths of NIR radiation depending on their resonance structure and penetrates deeper into the sample than IR [209]. Because of the complex interactions in the NIR spectra, it is generally necessary to develop a predictive model to correlate spectra with a primary analytical method to predict composition and may not be as sensitive as IR methods. Spectra and primary analytical quantification of the trait of interest is collected on a diverse set of representative samples and this is used to derive a calibration equation using multivariate statistical methods such as partial least squares (PLS) or principal component analysis (PCA) to correlate the spectra with the primary analytical methods. An excellent example of the range of assays that can be utilized as analytical methods to build NIR models is presented by [210]. The equation is tested on another subset of samples to ensure that it accurately predicts the trait of interest basely solely on the spectra obtained [211, 212].

While there is a large initial investment in developing a model, the ability to predict composition of new samples based only on quickly capturing spectral information makes these methods an attractive option. Consequently, spectroscopic methods have been used to estimate almost all the properties previously discussed. Based on detergent fiber calibration, NIR has predicted biochemical composition of sugarcane [17], rice [213], corn stover and switchgrass [214], miscanthus [23] and several other species. Dietary fiber calibration has also been used to predict detailed monomeric sugar composition of corn [215] and miscanthus [216]. Proximate and ultimate analysis and heating value have been estimated for rice straw using NIR [217, 218]. FTIR models have successfully been used to estimate N content, heating value and alkali index of switchgrass and reed canary grass [219], and lignin

and heating value in poplar [220]. NIR has been used to estimate moisture, ash and heating value of spruce [221] as well as miscanthus and willow [222] and heating value in sorghum and miscanthus [223, 224]. Lestander *et al.* [221] also show that NIR can even predict the energy required to pelletize sawdust, and NIR would likely have similar success in predicting the energy required to grind biomass. Though often omitted in methodological discussions, sample preparation can become the limiting step for any high-throughput phenotyping method. From this perspective, these may be less attractive due to necessary sample preparation steps. Both IR and NIR can utilize small sample sizes; <10 mg for IR and <100 mg for NIR [225] and while commonly the samples are ground, this is not always necessary [226]. NIR is non-destructive, and through the use of various techniques (Attentuated Total Reflectance (ATR), Diffuse Reflectance (DR)), FTIR can also be non-destructive.

While research exploring the genetic basis for variation among these traits is often conducted in conditions that minimize the environmental variability (growth chambers and greenhouses), assessing the genetic and environmental interplay in field environments is essential to improving desirable traits in the new energy feedstocks. Recent efforts have begun to assess field populations with sensors that use various spectra of light and correlate with phenotypes such as plant height, biomass, drought tolerance and others [227–229]. These efforts need to be expanded to other bioenergy relevant traits. Nondestructive spectral imaging could be adapted from current applications in precision fertilizer application [230] to other compositional properties. These approaches might be used to predict heating value or even moisture content from spectral-based elemental composition [231].

In summary, several primary analysis techniques might be amenable to high-throughput, mostly by automating the steps involved or multiplexing to process many samples at once. However there appears to be real promise in leveraging regression or multivariate approaches

to predict key properties like heating value and ash based on data from elemental analyzers or spectral approaches.

2.5. Box 1: Silica in grasses: example and opportunity

We present silica here as a practical matter—in grasses it can represent a large proportion of ash content—and as an example of how existing genetic knowledge might be leveraged to optimize a thermochemical trait in feedstocks. Silica does not provide energy during thermochemical conversion, hence it lowers the energy density. Furthermore, silica reacts with other alkali metals such as potassium and forms alkali silicates that have a lower melting point, thereby increasing the slagging and deposition rates at lower temperatures [62]. Manageable silica levels are difficult to estimate, since it depends on the levels of other alkali metals in the biomass. However, for many grasses, lowering silica levels at least below the 5% ash threshold would improve the thermochemical potential of these grasses.

Some have argued to include silicon as an "essential" element [154] due to its important and diverse roles. Silica serves as a structural element, keeping leaves erect and stems from lodging. Its physiological roles include detoxifying Al, Mn, and Fe by binding with them and regulating P uptake [232], decreasing transpiration and reducing water stress [154, 233] and in its protective role, it may provide a mechanical barrier that hinders diseases and pests [232, 234–236]. These roles of silica have been validated in many diverse species such as rice, sugarcane, barley, jute, tomato, cucumber and strawberry [237].

Although the second most abundant element in the world's soils, silicon is not always in a form available to plants [238]. Soil water concentrations of monosilicic acid (H_4SiO_4), the plant available form of silica, vary from 0.1 to 0.6 mM in most soils [237]. Silica deficiency is rare, but in sandy and highly weathered soils, and intensely cultivated soils, silica application

can improve yields [237, 239]. In most plants, silica, an uncharged molecule in biological conditions, is taken up with the water stream and diffuses through membranes, following the transpiration stream up the xylem [240]. It is deposited as opal or phytoliths, more accurately called amorphous silica ($SiO_2 \bullet nH_2O$), usually where transpiration has caused the solution to become saturated—in the intercellular spaces and the bulliform cells. Deposition also occurs frequently in silica bodies, xylem cells, root endodermis cells, and in the cuticle silica double layer along the epidermis of leaf blades [241, 242]. It is becoming clear that silica deposition can be a carefully engineered process directed by the plant, as temporal control of silica deposition in silica bodies demonstrates [243].

Silica content of plants ranges from trace (less than 0.5%) to small (0.5 to 1%, roughly corresponding to the amounts in the soil water), to high (1 to 15%) amounts [237, 239, 244]. Accumulation of high levels seems to require an active system of transporters. For example, rice accumulates high levels of silica via characterized transporters, including an aquaporin in root cells, an antiporter that uses the proton gradient to load silica into the xylem, and a passive transporter that moves silica from the xylem to the leaf [245]. Several bioenergy feedstocks accumulate high silica levels (see Table 2.2) but the specific transporters are yet to be identified and the effects of modifying their production or activity are unknown. It is important to note for practical purposes additional silica may be introduced into the feedstock with soil contamination of the biomass.

While there is usually a correlation between soil-available silica and amounts of silica taken up by plants, there is large variation for the amount accumulated, even within a species. When grown the same soil, some varieties of rice always accumulate more silica than other varieties [246]. In general, japonica rice varieties take up more silica than indica varieties, maybe because the japonica types were domesticated on silica deficient soils [237].

Plants that are non-accumulators (corresponding to the trace levels discussed above) do not take up silica—even under high soil silica conditions. It is unclear why plants have adapted to maintain such different levels of silica. Cell specific deposition indicates that silica is under genetic control [15, 241, 247]. Quantitative trait loci (QTL) have been mapped for silica concentration in various tissues [248, 249] and there are hints that some disease resistance genes may actively modify silica levels [250] and different types of silica deposition may have different roles [251].

Silica content is estimated in plant tissue by hydrofluoric acid extraction and a molybdenum blue assay [252] or by gravimetric techniques [237]. Measurement is also possible with ICP-OES [162] and distribution within a tissue can be assessed by X-ray fluorescence spectroscopy [237]. In all cases, care must be taken to avoid glassware that could introduce additional Si into the sample. Since the large majority of ash in many grasses is silica, crude measures of ash analysis can correlate with silica content. Additionally, ash can be predicted via NIR spectroscopy and these indirect methods (crude ash and NIR) might be optimized for high-throughput measurement of silica.

In conclusion, the observations of natural variation in silica content and the discovery of specific targets, the silica transporters, indicate the potential of silica levels as a target for biomass crop improvement. Indeed, in a study of ash levels across 144 species, Tao *et al.* identified silica content as a good target for optimized biomass [83]. A targeted approach might be to simultaneously decrease silica content: possibly by downregulating silica transporters, while upregulating lignin production to compensate for the loss of silica. However, it will be important to monitor plant performance as silica levels are manipulated because silica can be critically important for plant growth and yield.

2.6. Conclusion

Thermochemical conversion of biomass is an increasingly viable way to use bioenergy crops and agricultural residues to fulfill energy needs. Plant biologists and engineers both have important roles to play in the design of thermochemical bioenergy systems that result in appropriate pairings of biomass feedstocks and conversion technologies, though each group is limited by the constraints of their respective sub-systems. For engineers, this involves the design of efficient supply chains and conversion technologies that are robust to natural variations in biomass properties while minimizing energy use, material costs, and harmful emissions. Preprocessing technologies such as baling straw [253] or torrefaction [52] can also contribute to feedstock standardization. For biologists, this involves optimizing favorable biomass traits without compromising the plant's ability to survive in a sometimes hostile ecosystem. Natural genetic variation is a powerful resource for the improvement of bioenergy traits (both enzymatic and thermochemical) in feedstock plants, and the biological community has made great progress in understanding and manipulating the genetic pathways behind various relevant plant traits. Conversely, if natural variation for a trait is low, it is likely that modifications would incur serious consequences for the plant.

We find sufficient variation in lignin and evidence for potential genetic manipulation and several relatively high-throughput measurement methods. Unlike enzymatic systems where lignin is highly problematic, its role is more nuanced in thermochemical conversion systems where it is associated with higher HHV feedstocks, but changes to product distributions that may or may not be desirable. From an agronomic standpoint, increased lignin might be more feasible than reduced lignin due to the important roles it plays in physical stability and protection against pathogens. While less clear how amenable ash content is to genetic manipulation, we find larger variation in ash and variation in many of the minerals

that contribute to ash and suitable methods to phenotype them. Silica is a special problem with grasses, but could be addressed by exploiting the huge diversity observed within and among species, particularly using knowledge of the transporters with which its deposition might be controlled. Reducing silica in grasses and increasing lignin to mitigate the associated agronomic impacts might lead to an optimal thermochemical feedstock. However, the relationship between lignin, ash, and thermochemical conversion products is still not well understood, and additional systematic experimentation or meta-analysis will be necessary to confirm these strategies. The most biologically interesting traits may not be the traits that will have the greatest economic and lifecycle impact. Efforts to determine heritability in more abstract traits such as moisture content, grindability and bulk density would be valuable next steps based on observations of genetic variation seen in rice and other species, though high-throughput methods to measure grindability and density do not currently exist.

Biochemical and proximate/ultimate analysis are both equally valid paradigms for describing a kilogram of biomass, though the latter does present two distinct advantages in the context of feedstocks for thermochemical bioenergy production. Predicting biomass properties such as HHV from biochemical analysis results is challenging (likely because of biases associated with different measurement methods), while regressions based on ultimate analysis appears to work even across diverse data sets [73]). Secondly, ultimate analysis may prove to be more amenable to high-throughput phenotyping efforts, with automated elemental analyzers and spectroscopy as promising direct and indirect methods for the measurement of many important properties. While initial investments in equipment and model development can be high, the establishment of core facilities and modeling equations for thermochemical characterization of biomass can make these approaches more accessible.

While this chapter has focused on genetic approaches to crop improvement, agricultural management (including what fertilizers to apply, when to harvest, and how to store the biomass) is critical and will impact the characteristics of the biomass, and ultimately, the lifecycle of the system [52, 124, 254]. Teasing out genetic variation and environmental effects has been and will continue to be a major challenge. Careful observation of all key traits, including agronomic traits related to sustainable crop production, will need to be done—some pathways are common to some molecules or elements, and plants may need to compensate for composition changes in unexpected ways. It is critical to analyze results in the context of the environment and avoid sweeping generalizations attributed to a certain species or specific transgenic plant [255]. Moving from the individual plant in the greenhouse to a field of plants will present new challenges and new surprises. Can we make valid conclusions from biomass composition at the field level, or will understanding genetic control require phenotyping at the resolution of individual plant organs or even cell types? Large-scale high-throughput phenotyping is the next frontier in plant science, and this chapter can help biologists and engineers prioritize traits for next generation bioenergy crop improvement. Beyond bioenergy, the food, forage, pulp, and paper industries will benefit as we fine-tune all aspects of biomass composition.

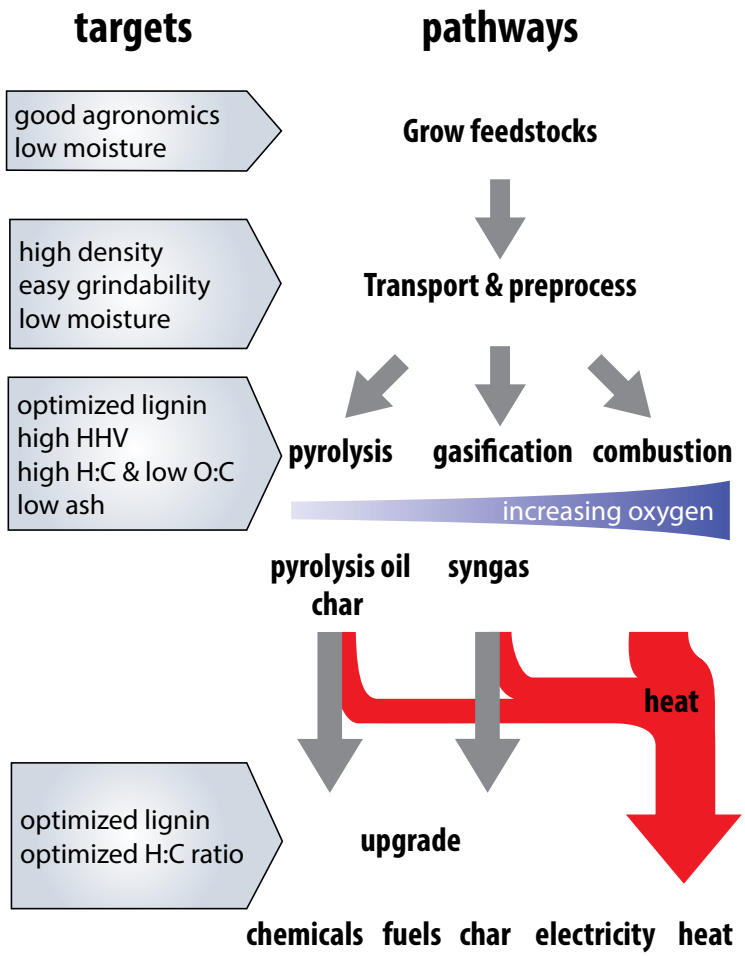
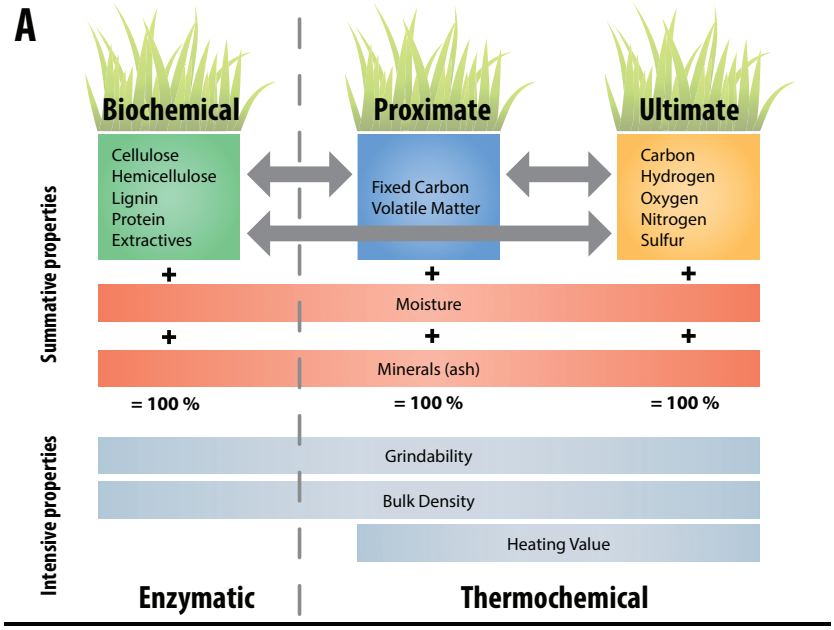


FIGURE 2.1. Overview of the steps involved in growing, transporting, processing, and converting biomass into thermochemical energy products. Pyrolysis, gasification and combustion take place under conditions of increasing oxygen availability during the reactions. Particle residence time and temperature may be optimized to yield different proportions and types of products. Boxes represent the properties important for each step (growing, transport and processing, conversion, upgrading). The primary products of each process and the potential end uses are highlighted. Note that intermediate products such as syngas and pyrolysis oil can be upgraded to chemicals or liquid transportation fuels or converted to obtain electricity and heat. Agronomic traits include those traits that allow the plant to survive and produce acceptable yields.



B

property	primary methods	ASTM standards
Biochemical		E1821, E1758
Cellulose	quantititve saccharification, detergent fiber, TGA	D5896
Hemicellulose	quantititve saccharification, detergent fiber, TGA	D5896
Lignin	detergent fiber, Klason lignin, others (see text)	D1106, D1721
Protein	quantification of N content with a conversion factor	
Proximate		D3172, D7582
Fixed Carbon (FC) Volatile Matter (VM)	mass loss after heating (VM), by subtraction (FC)	E872
Ultimate		D3176
C, H, O	GC of combustion products (O by subtraction)	E777
N	acid digestion to ammonia salts, GC of combustion products	E778
S	precipitation of ash, GC of combustion products	E775
Moisture	mass loss after oven drying	E871
Total ash	mass remaining after complete combustion	E1755
Elemental ash	atomic absorption spectroscopy (AAS), ICP-OES, ICP-MS	D3682
Grindability	energy consumption of grinding equipment	D409
Bulk Density	mass per volume of sample ground to certain size	E873
Heating Value	bomb calorimetry	D5865

FIGURE 2.2. Overview of the relationships between biomass traits and properties, and common methods of quantification. **A**. Biomass characterization in terms of summative properties (shown in green, blue, orange, and red) and intensive properties (shown in grey). Three common paradigms for describing biomass are inter-related: biochemical, proximate, and ultimate. While enzymatic conversion has focused on characterizing biomass in a biochemical paradigm, two alternatives more appropriate for thermochemical conversion are proximate and ultimate analysis. Moisture and minerals (ash) are common across all paradigms. **B**. Examples of common primary (direct) methods of quantifying each component identified in **A**. Note that this list is not complete, and note that proximate analysis necessitates moisture and total ash quantification. Elements that remain in the ash when biomass is combusted are referred to as minerals before combustion and ash afterwards. Examples of relevant ASTM standards for biomass, wood, refuse, or coal are listed. These direct methods are contrasted with indirect methods described in the text but not shown here.

Table 2.1. Common terms used in this chapter in the context of biomass for bioenergy.

Term	Definition
Biochemical analysis	characterization of biomass in terms of structural and nonstructural carbohydrates, lignin, protein and extractives (pectins, lipids, etc)
Enzymatic conversion	use of microorganisms or pure enzymes to transform feedstocks into energy products and co-products, e.g., fermentation, anaerobic digestion
Fixed Carbon (FC)	mass remaining as a solid after proximate analysis, excluding ash
Higher Heating Value (HHV)	energy released as biomass undergoes complete combustion to CO_2, H_2O (condensed), and other minor products at standardized conditions
Intensive properties	non-separable traits that are independent of the mass of a sample
Property	trait or parameter in the context of a certain bioenergy conversion pathway or engineering systems
Phenotype	observable or measurable characteristic specific to a given environment
Proximate analysis	characterization in terms of the mass volatilized (as moisture and volatile matter) and mass remaining (fixed carbon and ash) during a standardized heating regime
Summative properties	traits that describe specific separable components of the biomass and sum to 100% in the context of a mass balance
Thermochemical conversion	controlled heating or oxidation of feedstocks to produce energy products and/or heat e.g., pyrolysis, gasification, combustion
Trait	genetic or physical characteristics (physical characteristics are also referred to as phenotypes)
Ultimate analysis	characterization of biomass in terms of its individual constituent elements (C, H, O, N, S, etc.)
Volatile Matter (VM)	mass loss as gaseous products (excluding moisture) during proximate analysis

Table 2.2. Ranges of key thermochemical properties in several biomass feedstocks as summarized from literature.[a]

	corn stover[b]	corn cob[c]	wheat straw[d]	rice straw[e]	sugarcane bagasse[f]	reed canarygrass[g]	switchgrass[h]	miscanthus[i]	poplar[j]
Biochemical									
cellulose (%)	28-51	26-36	25-51	28-41	32-43	26-39	30-50	41-58	39-49
lignin (%)	11-21	6-17	8-30	10-23	19-28	4-14	5-23	8-22	18-32
Proximate									
fixed carbon (%)	15-20	17-19	15-22	15-25	12-20	16-24	13-27	5-26	12-28
volatile matter (%)	72-85	80-83	71-85	64-98	74-88	73-83	73-87	74-94	72-86
moisture (%)	11-33	12-55	8-15	3-74	16-50	15-25	40-70	20-52	8-59
total ash (%)	4-10	1-9	1-23	8-26	1-13	3-13	2-10	1-9	0.4-4
Ultimate									
C (%)	40-51	41-50	42-53	35-60	38-55	44-50	42-53	40-52	47-52
H (%)	4.7-6.3	5-7.4	3.2-9.8	3.9-7	5.3-6.7	5.2-6.5	4.9-6.5	4.4-6.5	5.6-6.3
O (%)	34-50	44-51	29-52	31-50	33-50	39-49	36-49	39-49	40-46
O:C molar ratio	0.50-0.94	0.66-0.93	0.43-0.93	0.38-1.07	0.58-0.99	0.59-0.84	0.51-0.88	0.56-0.92	0.58-0.74
H:C molar ratio	1.10-1.91	1.21-1.95	0.73-2.83	0.79-2.42	1.23-2.13	1.26-1.79	1.12-1.87	1.02-1.97	1.30-1.62
Mineral (ash) composition									
Al_2O_3 (% ash)	0.1-5	0.8-5	0.1-12	0.1-3.39	5-21	0.2-2	0.12-7	0.1-3	0.2-3
CaO (% ash)	5-15	0.5-15	3-17	0.7-10	2-19	0.5-10	5-14	3-14	29-61
Cl (% ash)	0.3-1.9	—	0-7.2	0.6**	0.03**	0.06**	0.1-0.6	0.03-7	0.01-0.03
Fe_2O_3 (% ash)	0.4-2.5	0.2-7	0.7-2.2	0.1-3	2-16	0.2-1.7	0.35-3.6	0.08-2.6	0.3-1.4
K_2O (% ash)	15-21	2-20	6-37	6-25	0.15-20	2-23	5-28	2-34	10-34
MgO (% ash)	1.9-10	2.5-6	0.8-4	0.8-5.8	1.9-12	0.01-5	2.6-6.5	0.9-12	0.1-18
Na_2O (% ash)	0.2-1.5	0.2-1.8	0.1-17	0.2-4	0.4-1.6	0.03-2.3	0.1-1.9	0.1-2.3	0.1-0.4
P_2O_5 (% ash)	1.9-9	0.7-10	1.2-8	0.7-9	0.9-3.2	0.4-14	2.6-15	1.5-29	0.9-8
SiO_2 (% ash)	50-69	40-75	27-73	50-82	46-58	37-95	46-70	26-86	3-9
SO_3 (% ash)	0.8-13	1.4-13	1.2-8	0.7-6	0.4-3.8	0.02-2.1	0.4-9	0.6-5	2-3.8
TiO_2 (% ash)	0.2-0.3	—	0.01-.22	0.01-0.09	2.6-3.8	0.05-5	0.09-.37	0.02-0.05	0.3**
alkali index (kg alkali oxide /GJ)[k]	—	—	1.1-1.7	1.4-1.6	0.06**	—	0.6**	—	0.14**
Other properties									
higher heating value (MJ/kg)	18-20	16-19	12-22	15-20	19-20	18-21	17-20	17-22	17-21
bulk density (kg/m^3)	66-131	195**	51-97	63-75	50-75	—	65-105	70-100	—

[a]Ranges are combinations of species and/or hybrids, and include different environments, soils, treatment conditions, contamination, experimental error, etc. Values <4 were rounded to 1 decimal place, values >4 were rounded to whole numbers (except for ratios, and values used to calculate ratios. O:C and H:C were calculated by taking the C, H, and O, and dividing by the atomic masses for each element to give molar mass, then dividing the min by the max to get the global min, and the max by the min to get the global max. Where possible, values reported are on a dry matter basis, and using similar methods. Comparing values across methods is especially problematic for bulk density, moisture, cellulose and lignin as standardized methods are not always practiced or described, and some methods are more accurate than others. ** indicates only individual values were located in the literature search rather than a range.
[b][11, 12, 77–83]
[c][11, 12, 77, 83–86]
[d][9, 11, 65, 77, 79–81, 83, 86–89]
[e][10–12, 65, 77, 82, 83, 89–93]
[f][11, 65, 77, 78, 82, 83, 86, 94, 95]
[g][10, 11, 77, 82, 83, 96, 97]
[h][65, 79–81, 83, 87, 88, 98]
[i][10, 11, 77, 78, 83, 99, 100]
[j][9, 11, 12, 65, 77, 78, 82, 83, 101, 102]
[k]Alkali index is a ratio calculated from the relative amounts of K_2O and Na_2O. See text or [65] for detailed explanation.

REFERENCES

[1] EIA, 2011. *International Energy Outlook 2011.* Energy Information Administration, Office of Integrated Analysis and Forecasting, US Department of Energy, Washington, DC

[2] Kelly, E. N., Schindler, D. W., Hodson, P. V., Short, J. W., Radmanovich, R., and Nielsen, C. C., 2010. Oil sands development contributes elements toxic at low concentrations to the Athabasca River and its tributaries. *Proceedings of the National Academy of Sciences*, 107, 37:16178 –16183

[3] Osborn, S. G., Vengosh, A., Warner, N. R., and Jackson, R. B., 2011. Methane contamination of drinking water accompanying gas-well drilling and hydraulic fracturing. *Proceedings of the National Academy of Sciences*, 108, 20:8172–8176

[4] Frohlich, C., 2012. Two-year survey comparing earthquake activity and injection-well locations in the Barnett Shale, Texas. *Proceedings of the National Academy of Sciences*, 109, 35:13934–13938

[5] EISA, 2007. Energy independence and security act of 2007 pub. l. no. 110-140, 121 stat. 1492, 1783-84

[6] National Research Council, 2011. *Renewable Fuel Standard: Potential Economic and Environmental Effects of U.S. Biofuel Policy.* Washington, D.C.: The National Academies Press

[7] Albersheim, P., Darvill, A., Roberts, K., Sederoff, R., and Staehelin, A., 2010. *Plant Cell Walls.* New York: Garland Science, 1 ed.

[8] Pauly, M. and Keegstra, K., 2008. Cellwall carbohydrates and their modification as a resource for biofuels. *The Plant Journal*, 54, 4:559–568

[9] Carroll, A. and Somerville, C., 2009. Cellulosic Biofuels. *Annual Review of Plant Biology*, 60, 1:165–182

[10] Allison, G. G., Robbins, M. P., Carli, J., Clifton-Brown, J. C., and Donnison, I. S., 2010. Designing biomass crops with improved calorific content and attributes for burning: a UK perspective. In P. N. Mascia, J. Scheffran, and J. M. Widholm, eds., *Plant Biotechnology for Sustainable Production of Energy and Co-products*, vol. 66, pp. 25–55. Berlin, Heidelberg: Springer Berlin Heidelberg

[11] Tao, G., Lestander, T. A., Geladi, P., and Xiong, S., 2012. Biomass properties in association with plant species and assortments I: A synthesis based on literature data of energy properties. *Renewable and Sustainable Energy Reviews*, 16, 5:3481–3506

[12] Zhao, X., Zhang, L., and Liu, D., 2012. Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose. *Biofuels, Bioproducts and Biorefining*, 6:465–482

[13] Jin, S. and Chen, H., 2006. Structural properties and enzymatic hydrolysis of rice straw. *Process Biochemistry*, 41, 6:1261–1264

[14] Summers, M., Jenkins, B., Hyde, P., Williams, J., Mutters, R., Scardacci, S., and Hair, M., 2003. Biomass production and allocation in rice with implications for straw harvesting and utilization. *Biomass and Bioenergy*, 24, 3:163–173

[15] Monti, A., Di Virgilio, N., and Venturi, G., 2008. Mineral composition and ash content of six major energy crops. *Biomass and Bioenergy*, 32, 3:216–223

[16] Rancour, D. M., Marita, J. M., and Hatfield, R. D., 2012. Cell wall composition throughout development for the model grass Brachypodium distachyon. *Frontiers in Plant Biotechnology*, 3:266

[17] Sabatier, D., Thuriès, L., Bastianelli, D., and Dardenne, P., 2012. Rapid prediction of the lignocellulosic compounds of sugarcane biomass by near infrared reflectance spectroscopy: comparing classical and independent cross-validation. *Journal of Near Infrared Spectroscopy*, 20, 3:371

[18] Nassi o Di Nasso, N., Angelini, L., and Bonari, E., 2010. Influence of fertilisation and harvest time on fuel quality of giant reed (Arundo donax L.) in central Italy. *European Journal of Agronomy*, 32, 3:219–227

[19] Singh, M., Erickson, J., Sollenberger, L., Woodard, K., Vendramini, J., and Fedenko, J., 2012. Mineral composition and biomass partitioning of sweet sorghum grown for bioenergy in the southeastern USA. *Biomass and Bioenergy*, 47:1–8

[20] Zhao, Y. L., Steinberger, Y., Shi, M., Han, L. P., and Xie, G. H., 2012. Changes in stem composition and harvested produce of sweet sorghum during the period from maturity to a sequence of delayed harvest dates. *Biomass and Bioenergy*, 39:261–273

[21] Landström, S., Lomakka, L., and Andersson, S., 1996. Harvest in spring improves yield and quality of reed canary grass as a bioenergy crop. *Biomass and Bioenergy*, 11, 4:333–341

[22] Adler, P. R., Sanderson, M. A., Boateng, A. A., Weimer, P. J., and Jung, H.-J. G., 2006. Biomass Yield and Biofuel Quality of Switchgrass Harvested in Fall or Spring. *Agronomy Journal*, 98, 6:1518

[23] Hodgson, E. M., Lister, S. J., Bridgwater, A. V., Clifton-Brown, J., and Donnison, I. S., 2010. Genotypic and environmentally derived variation in the cell wall composition of Miscanthus in relation to its use as a biomass feedstock. *Biomass and Bioenergy*, 34, 5:652–660

[24] Mann, D. G. J., Labbé, N., Sykes, R. W., Gracom, K., Kline, L., Swamidoss, I. M., Burris, J. N., Davis, M., and Stewart, C. N., 2009. Rapid Assessment of Lignin Content and Structure in Switchgrass (Panicum virgatum L.) Grown Under Different Environmental Conditions. *BioEnergy Research*, 2, 4:246–256

[25] Allison, G. G., Morris, C., Clifton-Brown, J., Lister, S. J., and Donnison, I. S., 2011. Genotypic variation in cell wall composition in a diverse set of 244 accessions of Miscanthus. *Biomass and Bioenergy*, 35, 11:4740–4747

[26] Monono, E. M., Nyren, P. E., Berti, M. T., and Pryor, S. W., 2013. Variability in biomass yield, chemical composition, and ethanol potential of individual and mixed herbaceous biomass species grown in North Dakota. *Industrial Crops and Products*, 41:331–339

[27] Serapiglia, M. J., Cameron, K. D., Stipanovic, A. J., Abrahamson, L. P., Volk, T. A., and Smart, L. B., 2013. Yield and Woody Biomass Traits of Novel Shrub Willow Hybrids at Two Contrasting Sites. *BioEnergy Research*, 6, 2:533–546

[28] Vermerris, W., 2011. Survey of Genomics Approaches to Improve Bioenergy Traits in Maize, Sorghum and SugarcaneFree Access. *Journal of Integrative Plant Biology*, 53, 2:105–119

[29] Feltus, F. A. and Vandenbrink, J. P., 2012. Bioenergy grass feedstock: current options and prospects for trait improvement using emerging genetic, genomic, and systems biology toolkits. *Biotechnology for Biofuels*, 5, 1:80

[30] Jordan, D., Bowman, M., Braker, J., Dien, B., Hector, R., Lee, C., Mertens, J., and Wagschal, K., 2012. Plant cell walls to ethanol. *Biochemical Journal*, 442, 2:241

[31] Nookaraju, A., Pandey, S. K., Bae, H.-J., and Joshi, C. P., 2013. Designing Cell Walls for Improved Bioenergy Production. *Molecular Plant*, 6, 1:8–10

[32] Demirbas, A., 2004. Combustion characteristics of different biomass fuels. *Progress in Energy and Combustion Science*, 30, 2:219–230

[33] Goyal, H., Seal, D., and Saxena, R., 2008. Bio-fuels from thermochemical conversion of renewable resources: A review. *Renewable and Sustainable Energy Reviews*, 12, 2:504–517

[34] Butler, E., Devlin, G., Meier, D., and McDonnell, K., 2011. A review of recent laboratory research and commercial developments in fast pyrolysis and upgrading. *Renewable and Sustainable Energy Reviews*, 15, 8:4171–4186

[35] Wang, M. Q., Han, J., Haq, Z., Tyner, W. E., Wu, M., and Elgowainy, A., 2011. Energy and greenhouse gas emission effects of corn and cellulosic ethanol with technology improvements and land use changes. *Biomass and Bioenergy*, 35, 5:1885–1896

[36] Brar, J. S., Singh, K., Wang, J., and Kumar, S., 2012. Cogasification of Coal and Biomass: A Review. *International Journal of Forestry Research*, 2012:1–10

[37] Bridgwater, A., 2012. Review of fast pyrolysis of biomass and product upgrading. *Biomass and Bioenergy*, 38, 0:68–94

[38] Solantausta, Y., Oasmaa, A., Sipilä, K., Lindfors, C., Lehto, J., Autio, J., Jokela, P., Alin, J., and Heiskanen, J., 2012. Bio-oil Production from Biomass: Steps toward Demonstration. *Energy & Fuels*, 26, 1:233–240

[39] Demirbas, A., 2003. Relationships between lignin contents and fixed carbon contents of biomass samples. *Energy Conversion and Management*, 44, 9:1481–1486

[40] Baxter, L., 2005. Biomass-coal co-combustion: opportunity for affordable renewable energy. *Fuel*, 84, 10:1295–1302

[41] Yin, X. L., Wu, C. Z., Zheng, S. P., and Chen, Y., 2002. Design and operation of a CFB gasification and power generation system for rice husk. *Biomass and Bioenergy*,

23, 3:181–187

[42] Hiloidhari, M. and Baruah, D. C., 2011. Rice straw residue biomass potential for decentralized electricity generation: A GIS based study in Lakhimpur district of Assam, India. *Energy for Sustainable Development*, 15, 3:214–222

[43] Shackley, S., Carter, S., Knowles, T., Middelink, E., Haefele, S., and Haszeldine, S., 2012. Sustainable gasificationbiochar systems? A case-study of rice-husk gasification in Cambodia, Part II: Field trial results, carbon abatement, economic assessment and conclusions. *Energy Policy*, 41:618–623

[44] DOE, 2012. DSIRE. `http://www.dsireusa.org/summarymaps/index.cfm?ee=0&RE=0`. [Accessed 2012-11-03]

[45] Carley, S., 2009. State renewable energy electricity policies: An empirical evaluation of effectiveness. *Energy Policy*, 37, 8:3071–3081

[46] EIA, 2012. Most states have Renewable Portfolio Standards. `http://www.eia.gov/todayinenergy/detail.cfm?id=4850`. [Accessed 2012-11-03]

[47] Botha, T. and von Blottnitz, H., 2006. A comparison of the environmental benefits of bagasse-derived electricity and fuel ethanol on a life-cycle basis. *Energy Policy*, 34, 17:2654–2661

[48] Campbell, J. E., Lobell, D. B., and Field, C. B., 2009. Greater Transportation Energy and GHG Offsets from Bioelectricity Than Ethanol. *Science*, 324, 5930:1055–1057

[49] Cherubini, F., Bird, N. D., Cowie, A., Jungmeier, G., Schlamadinger, B., and Woess-Gallasch, S., 2009. Energy- and greenhouse gas-based LCA of biofuel and bioenergy systems: Key issues, ranges and recommendations. *Resources, Conservation and Recycling*, 53, 8:434–447

[50] Searcy, E. and Flynn, P. C., 2010. A criterion for selecting renewable energy processes. *Biomass and Bioenergy*, 34, 5:798–804

[51] Giuntoli, J., Boulamanti, A. K., Corrado, S., Motegh, M., Agostini, A., and Baxter, D., 2013. Environmental impacts of future bioenergy pathways: the case of electricity from wheat straw bales and pellets. *GCB Bioenergy*, 5, 5:497–512

[52] Robbins, M. P., Evans, G., Valentine, J., Donnison, I. S., and Allison, G. G., 2012. New opportunities for the exploitation of energy crops by thermochemical conversion in Northern Europe and the UK. *Progress in Energy and Combustion Science*, 38, 2:138–155

[53] DOE, 2011. *U.S. Billion-Ton Update: Biomass Supply for a Bioenergy and Bioproducts Industry*. Oak Ridge, TN: Oak Ridge National Laboratory

[54] DOE, 2009. Biofuels Strategic Plan. `http://science.energy.gov/~/media/ber/pdf/biofuels_strategic_plan.pdf`. [Accessed 2012-12-10]

[55] USDA, 2010. A USDA Regional Roadmap to Meeting the Biofuels Goals of the Renewable Fuels Standard by 2022

[56] Field, J. L., Keske, C. M. H., Birch, G. L., DeFoort, M. W., and Francesca Cotrufo, M., 2012. Distributed biochar and bioenergy coproduction: a regionally specific case study of environmental benefits and economic impacts. *GCB Bioenergy*, 5:177–191

[57] Babu, B. V., 2008. Biomass pyrolysis: a state-of-the-art review. *Biofuels, Bioproducts and Biorefining*, 2, 5:393–414

[58] Demirbas, A., 2007. Progress and recent trends in biofuels. *Progress in Energy and Combustion Science*, 33, 1:1–18

[59] Datar, R. P., Shenkman, R. M., Cateni, B. G., Huhnke, R. L., and Lewis, R. S., 2004. Fermentation of biomass-generated producer gas to ethanol. *Biotechnology and*

Bioengineering, 86, 5:587–594

[60] Boerrigter, H. and Rauch, R., 2005. Chapter 10: Syngas production and utilisation. In *Handbook Biomass Gasification*, pp. 211–230. The Netherlands: Biomass Technology Group (BTG), 1 ed.

[61] Huber, G. W., Iborra, S., and Corma, A., 2006. Synthesis of transportation fuels from biomass: chemistry, catalysts, and engineering. *Chemical Reviews*, 106:4044–4098

[62] Wang, L., Weller, C. L., Jones, D. D., and Hanna, M. A., 2008. Contemporary issues in thermal gasification of biomass and its application to electricity and fuel production. *Biomass and Bioenergy*, 32, 7:573–581

[63] Milne, T. A., Abatzoglou, N., and Evans, R. J., 1998. *Biomass gasifier "tars": their nature, formation, and conversion.* Golden, CO: National Renewable Energy Laboratory

[64] Gaul, M., 2012. An analysis model for small-scale rural energy service pathways applied to jatropha-based energy services in sumbawa, indonesia. *Energy for Sustainable Development*, 16, 3:283 – 296

[65] Jenkins, B. M., Baxter, L. L., Miles Jr, T. R., and Miles, T. R., 1998. Combustion properties of biomass. *Fuel Processing Technology*, 54, 1-3:17–46

[66] Bailis, R., Cowan, A., Berrueta, V., and Masera, O., 2009. Arresting the Killer in the Kitchen: The Promises and Pitfalls of Commercializing Improved Cookstoves. *World Development*, 37, 10:1694–1705

[67] Peterson, A. A., Vogel, F., Lachance, R. P., Fröling, M., Michael J. Antal, J., and Tester, J. W., 2008. Thermochemical biofuel production in hydrothermal media: A review of sub- and supercritical water technologies. *Energy & Environmental Science*, 1, 1:32–65

[68] Brown, R. C., ed., 2011. *Thermochemical Processing of Biomass: Conversion into Fuels, Chemicals and Power*. Chichester, West Sussex, United Kingdom: Wiley, 1 ed.

[69] van der Stelt, M. J. C., Gerhauser, H., Kiel, J. H. A., and Ptasinski, K. J., 2011. Biomass upgrading by torrefaction for the production of biofuels: A review. *Biomass and Bioenergy*, 35, 9:3748–3762

[70] Couhert, C., Commandre, J.-M., and Salvador, S., 2009. Is it possible to predict gas yields of any biomass after rapid pyrolysis at high temperature from its composition in cellulose, hemicellulose and lignin? *Fuel*, 88, 3:408–417

[71] Bridgwater, A., Toft, A., and Brammer, J., 2002. A techno-economic comparison of power production by biomass fast pyrolysis with gasification and combustion. *Renewable and Sustainable Energy Reviews*, 6, 3:181–246

[72] Jenkins, B., Bakker, R., and Wei, J., 1996. On the properties of washed straw. *Biomass and Bioenergy*, 10, 4:177–200

[73] Sheng, C. and Azevedo, J., 2005. Estimating the higher heating value of biomass fuels from basic analysis data. *Biomass and Bioenergy*, 28, 5:499–507

[74] Helsel, Z. R. and Wedin, W., 1981. Direct combustion energy from crops and crop residues produced in Iowa. *Energy in Agriculture*, 1:317–329

[75] Demirbas, A., 2001. Relationships between lignin contents and heating values of biomass. *Energy Conversion and Management*, 42, 2:183–188

[76] Vermerris, W. and Saballos, A., 2013. Genetic Enhancement of Sorghum for Biomass Utilization. In *Genomics of the Saccharinae*, vol. 11, pp. 391–425. New York: Springer

[77] Energy research Centre of the Netherlands, 2012. Phyllis2, database for biomass and waste. `http://www.ecn.nl/phyllis2`. [Accessed 2012-12-22]

[78] Oak Ridge National Laboratory. Appendix B - Biomass Characteristics

[79] Mani, S., Tabil, L. G., and Sokhansanj, S., 2004. Grinding performance and physical properties of wheat and barley straws, corn stover and switchgrass. *Biomass and Bioenergy*, 27, 4:339–352

[80] Carpenter, D. L., Bain, R. L., Davis, R. E., Dutta, A., Feik, C. J., Gaston, K. R., Jablonski, W., Phillips, S. D., and Nimlos, M. R., 2010. Pilot-Scale Gasification of Corn Stover, Switchgrass, Wheat Straw, and Wood: 1. Parametric Study and Comparison with Literature. *Industrial & Engineering Chemistry Research*, 49, 4:1859–1871

[81] Chevanan, N., Womac, A. R., Bitra, V. S., Igathinathane, C., Yang, Y. T., Miu, P. I., and Sokhansanj, S., 2010. Bulk density and compaction behavior of knife mill chopped switchgrass, wheat straw, and corn stover. *Bioresource Technology*, 101, 1:207–214

[82] Vassilev, S. V., Baxter, D., Andersen, L. K., and Vassileva, C. G., 2010. An overview of the chemical composition of biomass. *Fuel*, 89, 5:913–933

[83] Tao, G., Geladi, P., Lestander, T. A., and Xiong, S., 2012. Biomass properties in association with plant species and assortments. II: A synthesis based on literature data for ash elements. *Renewable and Sustainable Energy Reviews*, 16, 5:3507–3522

[84] Smith, R. D., Peart, R. M., Liljedahl, J. B., Barrett, J. R., and Doering, O. C., 1985. Corncob property changes during outside storage. *Transactions of the ASAE*, 28, 3:937–942

[85] Coovattanachai, N., 1989. Biomass gasification research and field developments by the Prince of Songkla University, Thailand. *Biomass*, 18, 34:241–271

[86] Spokas, K. A., 2010. Review of the stability of biochar in soils: predictability of O:C molar ratios. *Carbon Management*, 1, 2:289–303

[87] McKendry, P., 2002. Energy production from biomass (part 1): overview of biomass. *Bioresource Technology*, 83, 1:37–46

[88] Lam, P. S., Sokhansanj, S., Bi, X., Lim, C. J., Naimi, L. J., Hoque, M., Mani, S., Womac, A. R., Narayan, S., and Ye, X. P., 2008. Bulk density of wet and dry wheat straw and switchgrass particles. *Applied Engineering in Agriculture*, 24, 3:351–358

[89] Wu, Y., Wu, S., Li, Y., and Gao, J., 2009. Physico-Chemical Characteristics and Mineral Transformation Behavior of Ashes from Crop Straw. *Energy & Fuels*, 23, 10:5144–5150

[90] Kargbo, F., Xing, J., and Zhang, Y., 2010. Property analysis and pretreatment of rice straw for energy use in grain drying: A review. *Agriculture and Biology Journal of North America*, 1, 3:195–200

[91] Jahn, C. E., Mckay, J. K., Mauleon, R., Stephens, J., McNally, K. L., Bush, D. R., Leung, H., and Leach, J. E., 2011. Genetic Variation in Biomass Traits among 20 Diverse Rice Varieties. *Plant Physiology*, 155, 1:157–168

[92] Liu, Z., Xu, A., and Zhao, T., 2011. Energy from Combustion of Rice Straw: Status and Challenges to China. *Energy and Power Engineering*, 3, 3:325–331

[93] Zhang, Y., Ghaly, A., and Li, B., 2012. Physical properties of rice residues as affected by variety and climatic and cultivation conditions in three continents. *American Journal of Applied Sciences*, 9, 11:1757–1768

[94] Kaar, W., Gutierrez, C., and Kinoshita, C., 1998. Steam explosion of sugarcane bagasse as a pretreatment for conversion to ethanol. *Biomass and Bioenergy*, 14, 3:277–287

[95] Tsai, W., Lee, M., and Chang, Y., 2006. Fast pyrolysis of rice straw, sugarcane bagasse and coconut shell in an induction-heating reactor. *Journal of Analytical and Applied Pyrolysis*, 76, 12:230–237

[96] Fahmi, R., Bridgwater, A., Donnison, I., Yates, N., and Jones, J., 2008. The effect of lignin and inorganic species in biomass on pyrolysis oil yields, quality and stability.

Fuel, 87, 7:1230–1240

[97] Lindh, T., Paappanen, T., Rinne, S., Sivonen, K., and Wihersaari, M., 2009. Reed canary grass transportation costs Reducing costs and increasing feasible transportation distances. *Biomass and Bioenergy*, 33, 2:209–212

[98] Sokhansanj, S., Mani, S., Turhollow, A., Kumar, A., Bransby, D., Lynd, L., and Laser, M., 2009. Large-scale production, harvest and logistics of switchgrass (Panicum virgatum L.) - current technology and envisioning a mature technology. *Biofuels, Bioproducts and Biorefining*, 3, 2:124–141

[99] Clifton-Brown, J. and Lewandowski, I., 2002. Screening Miscanthus genotypes in field trials to optimise biomass yield and quality in Southern Germany. *European Journal of Agronomy*, 16, 2:97–110

[100] Arabhosseini, A., Huisman, W., and Müller, J., 2010. Modeling of the equilibrium moisture content (EMC) of Miscanthus (Miscanthus x giganteus). *Biomass and Bioenergy*, 34, 4:411–416

[101] Klasnja, B., Kopitovic, S., and Orlovic, S., 2002. Wood and bark of some poplar and willow clones as fuelwood. *Biomass and Bioenergy*, 23, 6:427–432

[102] Spinelli, R., Nati, C., and Magagnotti, N., 2009. Using modified foragers to harvest short-rotation poplar plantations. *Biomass and Bioenergy*, 33, 5:817–821

[103] Wright, M. M. and Brown, R. C., 2007. Comparative economics of biorefineries based on the biochemical and thermochemical platforms. *Biofuels, Bioproducts and Biorefining*, 1, 1:49–56

[104] Borgwardt, R. H., 1999. Transportation fuel from cellulosic biomass: a comparative assessment of ethanol and methanol options. *Proceedings of the Institution of Mechanical Engineers, Part A: Journal of Power and Energy*, 213, 5:399–407

[105] Pereira, E. G., da Silva, J. N., de Oliveira, J. L., and Machado, C. S., 2012. Sustainable energy: A review of gasification technologies. *Renewable and Sustainable Energy Reviews*, 16, 7:4753–4762

[106] Miles, T. R., Miles Jr, T. R., Baxter, L. L., Bryers, R. W., Jenkins, B. M., and Oden, L. L., 1996. Boiler deposits from firing biomass fuels. *Biomass and Bioenergy*, 10, 23:125–138

[107] Riley, J. T., 2007. *Manual 57 Routine Coal and Coke Analysis: Collection, Interpretation, and Use of Analytical Data*. West Conshohocken, PA: ASTM International

[108] Cummer, K. R. and Brown, R. C., 2002. Ancillary equipment for biomass gasification. *Biomass and Bioenergy*, 23, 2:113–128

[109] Fahmi, R., Bridgwater, A., Thain, S., Donnison, I., Morris, P., and Yates, N., 2007. Prediction of Klason lignin and lignin thermal degradation products by PyGC/MS in a collection of Lolium and Festuca grasses. *Journal of Analytical and Applied Pyrolysis*, 80, 1:16–23

[110] Gani, A. and Naruse, I., 2007. Effect of cellulose and lignin content on pyrolysis and combustion characteristics for several types of biomass. *Renewable Energy*, 32, 4:649–661

[111] Demirbas, A., 2003. Sustainable cofiring of biomass with coal. *Energy Conversion and Management*, 44, 9:1465–1479

[112] Lv, D., Xu, M., Liu, X., Zhan, Z., Li, Z., and Yao, H., 2010. Effect of cellulose, lignin, alkali and alkaline earth metallic species on biomass pyrolysis and gasification. *Fuel Processing Technology*, 91, 8:903–909

[113] Tröeger, N., Richter, D., and Stahl, R., 2013. Effect of feedstock composition on product yields and energy recovery rates of fast pyrolysis products from different straw

types. *Journal of Analytical and Applied Pyrolysis*, 100:158 – 165

[114] Raveendran, K., Ganesh, A., and Khilar, K. C., 1995. Influence of mineral matter on biomass pyrolysis characteristics. *Fuel*, 74, 12:1812–1822

[115] McKendry, P., 2002. Energy production from biomass (part 2): conversion technologies. *Bioresource Technology*, 83, 1:47–54

[116] Lewandowski, I. and Kicherer, A., 1997. Combustion quality of biomass: practical relevance and experiments to modify the biomass quality of Miscanthus x giganteus. *European Journal of Agronomy*, 6, 34:163–177

[117] Dayton, D. C., Belle-Oudry, D., and Nordin, A., 1999. Effect of Coal Minerals on Chlorine and Alkali Metals Released during Biomass/Coal Cofiring. *Energy & Fuels*, 13, 6:1203–1211

[118] Mansaray, K., Ghaly, A., Al-Taweel, A., Hamdullahpur, F., and Ugursal, V., 1999. Air gasification of rice husk in a dual distributor type fluidized bed gasifier. *Biomass and Bioenergy*, 17, 4:315–332

[119] McKendry, P., 2002. Energy production from biomass (part 3): gasification technologies. *Bioresource Technology*, 83, 1:55–63

[120] Van Loo, S. and Koppejan, J., 2008. *The handbook of biomass combustion and co-firing*. Earthscan/James & James

[121] Yin, C., Rosendahl, L. A., and Kæ r, S. r. K., 2008. Grate-firing of biomass for heat and power production. *Progress in Energy and Combustion Science*, 34, 6:725–754

[122] Yoshida, T., Oshima, Y., and Matsumura, Y., 2004. Gasification of biomass model compounds and real biomass in supercritical water. *Biomass and Bioenergy*, 26, 1:71–78

[123] Waldner, M. H. and Vogel, F., 2005. Renewable Production of Methane from Woody Biomass by Catalytic Hydrothermal Gasification. *Industrial & Engineering Chemistry Research*, 44, 13:4543–4551

[124] Wilson, D. M., Dalluge, D. L., Rover, M., Heaton, E. A., and Brown, R. C., 2013. Crop Management Impacts Biofuel Quality: Influence of Switchgrass Harvest Time on Yield, Nitrogen and Ash of Fast Pyrolysis Products. *BioEnergy Research*, 6, 1:103–113

[125] Williams, A., Jones, J., Ma, L., and Pourkashanian, M., 2012. Pollutants from the combustion of solid biomass fuels. *Progress in Energy and Combustion Science*, 38, 2:113–137

[126] Gonzalez, R., Daystar, J., Jett, M., Treasure, T., Jameel, H., Venditti, R., and Phillips, R., 2012. Economics of cellulosic ethanol production in a thermochemical pathway for softwood, hardwood, corn stover and switchgrass. *Fuel Processing Technology*, 94, 1:113–122

[127] Ghorbani, Z., Masoumi, A., and Hemmat, A., 2010. Specific energy consumption for reducing the size of alfalfa chops using a hammer mill. *Biosystems Engineering*, 105, 1:34–40

[128] Cabiles, D. M., Angeles, O. R., Johnson-Beebout, S. E., Sanchez, P. B., and Buresh, R. J., 2008. Faster residue decomposition of brittle stem rice mutant due to finer breakage during threshing. *Soil & Tillage Research*, 98:211–216

[129] Jung, H.-J. G., Samac, D. A., and Sarath, G., 2012. Modifying crops to increase cell wall digestibility. *Plant Science*, 185-186:65–77

[130] Wang, H.-Z. and Dixon, R. A., 2012. OnOff Switches for Secondary Cell Wall Biosynthesis. *Molecular Plant*, 5, 2:297–303

[131] Yang, F., Mitra, P., Zhang, L., Prak, L., Verhertbruggen, Y., Kim, J.-S., Sun, L., Zheng, K., Tang, K., Auer, M., Scheller, H. V., and Loqué, D., 2012. Engineering secondary cell wall deposition in plants. *Plant Biotechnology Journal*, 11, 3:325–335

[132] Ficklin, S. P. and Feltus, F. A., 2011. Gene Coexpression Network Alignment and Conservation of Gene Modules between Two Grass Species: Maize and Rice. *Plant Physiology*, 156, 3:1244

[133] Boerjan, W., Ralph, J., and Baucher, M., 2003. Lignin biosynthesis. *Annual Review of Plant Biology*, 54:519–546

[134] Endler, A. and Persson, S., 2011. Cellulose Synthases and Synthesis in Arabidopsis. *Molecular Plant*, 4, 2:199–211

[135] Zhao, Q. and Dixon, R. A., 2011. Transcriptional networks for lignin biosynthesis: more complex than we thought? *Trends in Plant Science*, 16, 4:227–233

[136] Gallego-Giraldo, L., Jikumaru, Y., Kamiya, Y., Tang, Y., and Dixon, R. A., 2011. Selective lignin downregulation leads to constitutive defense response expression in alfalfa (Medicago sativa L.). *New Phytologist*, 190, 3:627–639

[137] Jensen, J. K., Schultink, A., Keegstra, K., Wilkerson, C. G., and Pauly, M., 2012. RNA-Seq Analysis of Developing Nasturtium Seeds (Tropaeolum majus): Identification and Characterization of an additional Galactosyltransferase Involved in Xyloglucan Biosynthesis. *Molecular Plant*, 5, 5:984–992

[138] Grabber, J. H., Ralph, J., Lapierre, C., and Barrière, Y., 2004. Genetic and molecular basis of grass cell-wall degradability. I.Lignincell wall matrix interactions. *Comptes Rendus Biologies*, 327, 5:455–465

[139] Buranov, A. U. and Mazza, G., 2008. Lignin in straw of herbaceous crops. *Industrial Crops and Products*, 28, 3:237–259

[140] Amthor, J. S., 2003. Efficiency of lignin biosynthesis: a quantitative analysis. *Annals of Botany*, 91, 6:673–695

[141] Shen, D., Gu, S., Luo, K., Wang, S., and Fang, M., 2010. The pyrolytic degradation of wood-derived lignin from pulping process. *Bioresource Technology*, 101, 15:6136–6146

[142] Müller-Hagedorn, M., Bockhorn, H., Krebs, L., and Müller, U., 2003. A comparative kinetic study on the pyrolysis of three different wood species. *Journal of Analytical and Applied Pyrolysis*, 6869:231–249

[143] Weng, J.-K., Li, X., Bonawitz, N. D., and Chapple, C., 2008. Emerging strategies of lignin engineering and degradation for cellulosic biofuel production. *Current Opinion in Biotechnology*, 19, 2:166–172

[144] Shen, H., Fu, C., Xiao, X., Ray, T., Tang, Y., Wang, Z., and Chen, F., 2009. Developmental Control of Lignification in Stems of Lowland Switchgrass Variety Alamo and the Effects on Saccharification Efficiency. *BioEnergy Research*, 2, 4:233–245

[145] Studer, M. H., DeMartini, J. D., Davis, M. F., Sykes, R. W., Davison, B., Keller, M., Tuskan, G. A., and Wyman, C. E., 2011. Lignin content in natural Populus variants affects sugar release. *Proceedings of the National Academy of Sciences*, 108, 15:6300–6305

[146] Elumalai, S., Tobimatsu, Y., Grabber, J. H., Pan, X., and Ralph, J., 2012. Epigallocatechin gallate incorporation into lignin enhances the alkaline delignification and enzymatic saccharification of cell walls. *Biotechnology for Biofuels*, 5, 1:59

[147] Xu, Z., Zhang, D., Hu, J., Zhou, X., Ye, X., Reichel, K., Stewart, N., Syrenne, R., Yang, X., Gao, P., Shi, W., Doeppke, C., Sykes, R., Burris, J., Bozell, J., Cheng, M. Z.-M., Hayes, D., Labbe, N., Davis, M., Stewart, C. N., and Yuan, J., 2009. Comparative genome analysis of lignin biosynthesis gene families across the plant kingdom. *BMC*

Bioinformatics, 10, Suppl 11:S3

[148] Ishii, T., 1997. Structure and functions of feruloylated polysaccharides. *Plant Science*, 127, 2:111–127

[149] Bartley, L. E., Peck, M. L., Kim, S.-R., Ebert, B., Maniseri, C., Chiniquy, D., Sykes, R., Gao, L., Rautengarten, C., Vega-Sánchez, M. E., Benke, P. I., Canlas, P. E., Cao, P., Brewer, S., Lin, F., Smith, W. L., Zhang, X., Keasling, J. D., Jentoft, R. E., Foster, S. B., Zhou, J., Ziebell, A., An, G., Scheller, H. V., and Ronald, P. C., 2013. Overexpression of a BAHD Acyltransferase, OsAt10, alters rice cell wall hydroxycinnamic acid content and saccharification. *Plant Physiology*, 161, 4:1615–1633

[150] Molinari, H. B., Pellny, T. K., Freeman, J., Shewry, P. R., and Mitchell, R. A., 2013. Grass cell wall feruloylation: distribution of bound ferulate and candidate gene expression in Brachypodium distachyon. *Frontiers in Plant Biotechnology*, 4:50

[151] Hopkins, A. A., Vogel, K. P., Moore, K. J., Johnson, K. D., and Carlson, I. T., 1995. Genotype Effects and Genotype by Environment Interactions for Traits of Elite Switchgrass Populations. *Crop Science*, 35, 1:125–132

[152] Lemus, R., Brummer, E., Moore, K. J., Molstad, N. E., Burras, C., and Barker, M. F., 2002. Biomass yield and quality of 20 switchgrass populations in southern Iowa, USA. *Biomass and Bioenergy*, 23, 6:433–442

[153] Lingle, S. E. and Thomson, J. L., 2011. Sugarcane Internode Composition During Crop Development. *BioEnergy Research*, 5, 1:168–178

[154] Epstein, E., 1999. Silicon. *Annual Review of Plant Physiology and Plant Molecular Biology*, 50, 1:641–664

[155] Taiz, L. and Zeiger, E., 2006. *Plant Physiology*. Sunderland, MA: Sinauer Associates, 4th ed. ed.

[156] Mäser, P., Thomine, S., Schroeder, J. I., Ward, J. M., Hirschi, K., Sze, H., Talke, I. N., Amtmann, A., Maathuis, F. J. M., Sanders, D., Harper, J. F., Tchieu, J., Gribskov, M., Persans, M. W., Salt, D. E., Kim, S. A., and Guerinot, M. L., 2001. Phylogenetic Relationships within Cation Transporter Families of Arabidopsis. *Plant Physiology*, 126, 4:1646–1667

[157] Raboy, V., 2003. myo-Inositol-1,2,3,4,5,6-hexakisphosphate. *Phytochemistry*, 64, 6:1033–1043

[158] Ghandilyan, A., Vreugdenhil, D., and Aarts, M. G. M., 2006. Progress in the genetic understanding of plant iron and zinc nutrition. *Physiologia Plantarum*, 126, 3:407–417

[159] Ghandilyan, A., Ilk, N., Hanhart, C., Mbengue, M., Barboza, L., Schat, H., Koornneef, M., El-Lithy, M., Vreugdenhil, D., and Reymond, M., 2009. A strong effect of growth medium and organ type on the identification of QTLs for phytate and mineral concentrations in three Arabidopsis thaliana RIL populations. *Journal of Experimental Botany*, 60, 5:1409–1425

[160] Nishimura, K., Miyake, Y., and Takahashi, E., 1989. On silicon, aluminium, and zinc accumulators discriminated from 147 species of Angiospermae. *Memoirs of the College of Agriculture - Kyoto University*, 133:23–43

[161] Werkelin, J., Skrifvars, B.-J., and Hupa, M., 2005. Ash-forming elements in four Scandinavian wood species. Part 1: Summer harvest. *Biomass and Bioenergy*, 29, 6:451–466

[162] El-Nashaar, H., Banowetz, G., Griffith, S., Casler, M., and Vogel, K., 2009. Genotypic variability in mineral composition of switchgrass. *Bioresource Technology*, 100, 5:1809–1814

[163] Christian, D. G., Yates, N. E., and Riche, A. B., 2006. The effect of harvest date on the yield and mineral content of Phalaris arundinacea L. (reed canary grass) genotypes screened for their potential as energy crops in southern England. *Journal of the Science of Food and Agriculture*, 86, 8:1181–1188

[164] Boateng, A., Hicks, K., and Vogel, K., 2006. Pyrolysis of switchgrass (Panicum virgatum) harvested at several stages of maturity. *Journal of Analytical and Applied Pyrolysis*, 75, 2:55–64

[165] Baxter, I., Hermans, C., Lahner, B., Yakubova, E., Tikhonova, M., Verbruggen, N., Chao, D., and Salt, D. E., 2012. Biodiversity of mineral nutrient and trace element accumulation in Arabidopsis thaliana. *PloS one*, 7, 4:e35121

[166] Egilla, J. N., Jr, F. T. D., and Drew, M. C., 2001. Effect of potassium on drought resistance of Hibiscus rosa-sinensis cv. Leprechaun: Plant growth, leaf macro- and micronutrient content and root longevity. *Plant and Soil*, 229, 2:213–224

[167] Dordas, C., 2008. Role of nutrients in controlling plant diseases in sustainable agriculture. A review. *Agronomy for Sustainable Development*, 28, 1:33–46

[168] Baxter, I., Hosmani, P. S., Rus, A., Lahner, B., Borevitz, J. O., Muthukumar, B., Mickelbart, M. V., Schreiber, L., Franke, R. B., and Salt, D. E., 2009. Root Suberin Forms an Extracellular Barrier That Affects Water Relations and Mineral Nutrition in Arabidopsis. *PLoS Genetics*, 5, 5:e1000492

[169] Baxter, I., Brazelton, J. N., Yu, D., Huang, Y. S., Lahner, B., Yakubova, E., Li, Y., Bergelson, J., Borevitz, J. O., Nordborg, M., Vitek, O., and Salt, D. E., 2010. A Coastal Cline in Sodium Accumulation in Arabidopsis thaliana Is Driven by Natural Variation of the Sodium Transporter AtHKT1;1. *PLoS Genet*, 6, 11:e1001193

[170] Mosseler, A., Zsuffa, L., Stoehr, M. U., and Kenney, W. A., 1988. Variation in biomass production, moisture content, and specific gravity in some North American willows (Salix L.). *Canadian Journal of Forest Research*, 18, 12:1535–1540

[171] Zhu, J.-K., 2002. Salt and Drought Stress Signal Transduction in Plants. *Annual Review of Plant Biology*, 53:247–273

[172] Golldack, D., Lüking, I., and Yang, O., 2011. Plant tolerance to drought and salinity: stress regulating transcription factors and their functional significance in the cellular transcriptional network. *Plant Cell Reports*, 30, 8:1383–1391

[173] McCann, S. E. and Huang, B., 2008. Evaluation of Drought Tolerance and Avoidance Traits for Six Creeping Bentgrass Cultivars. *HortScience*, 43, 2:519–524

[174] Bartels, D. and Sunkar, R., 2005. Drought and salt tolerance in plants. *Critical Reviews in Plant Sciences*, 24, 1:23–58

[175] Patakas, A., Nikolaou, N., Zioziou, E., Radoglou, K., and Noitsakis, B., 2002. The role of organic solute and ion accumulation in osmotic adjustment in drought-stressed grapevines. *Plant Science*, 163, 2:361–367

[176] Arjenaki, F. G., Jabbari, R., and Morshedi, A., 2012. Evaluation of Drought Stress on Relative Water Content, Chlorophyll Content and Mineral Elements of Wheat (Triticum aestivum L.) Varieties. *International Journal of Agriculture and Crop Sciences*, 4, 11:726–729

[177] Wang, H., Avci, U., Nakashima, J., Hahn, M. G., Chen, F., and Dixon, R. A., 2010. Mutation of WRKY transcription factors initiates pith secondary wall formation and increases stem biomass in dicotyledonous plants. *Proceedings of the National Academy of Sciences*, 107, 51:22338–22343

[178] Cadoche, L. and López, G. D., 1989. Assessment of size reduction as a preliminary step in the production of ethanol from lignocellulosic wastes. *Biological Wastes*, 30, 2:153–157

[179] Mackay, T. F., 2001. The genetic architecture of quantitative traits. *Annual Review of Genetics*, 35:303–39

[180] Collard, B., Jahufer, M., Brouwer, J., and Pang, E., 2005. An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. *Euphytica*, 142, 1:169–196

[181] Takeda, S. and Matsuoka, M., 2008. Genetic approaches to crop improvement: responding to environmental and population changes. *Nature Reviews Genetics*, 9, 6:444–457

[182] Zhu, C., Gore, M., Buckler, E. S., and Yu, J., 2008. Status and prospects of association mapping in plants. *The Plant Genome*, 1, 1:5–20

[183] Theander, O., Aman, P., Westerlund, E., Andersson, R., and Petersson, D., 1995. Total dietary fiber determined as neutral sugar residues, uronic acid residues, and Klason Lignin (The Uppsala method): Collaborative study. *Journal of AOAC International*, 78, 4:1030–1044

[184] Sluiter, J. B., Ruiz, R. O., Scarlata, C. J., Sluiter, A. D., and Templeton, D. W., 2010. Compositional Analysis of Lignocellulosic Feedstocks. 1. Review and Description of Methods. *Journal of Agricultural and Food Chemistry*, 58, 16:9043–9053

[185] Van Soest, P., Robertson, J., and Lewis, B., 1991. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. *Journal of Dairy Science*, 74, 10:3583–3597

[186] Uden, 2005. Use of detergent system terminology and criteria for submission of manuscripts on new, or revised, analytical methods as well as descriptive information on feed analysis and/or variability. *Animal Feed Science and Technology*, 118, 3-4:181–186

[187] Moxley, G. and Zhang, Y.-H. P., 2007. More Accurate Determination of Acid-Labile Carbohydrates in Lignocellulose by Modified Quantitative Saccharification. *Energy & Fuels*, 21, 6:3684–3688

[188] Wolfrum, E., Lorenz, A., and DeLeon, N., 2009. Correlating detergent fiber analysis and dietary fiber analysis data for corn stover collected by NIRS. *Cellulose*, 16, 4:577–585

[189] Suzuki, S., Suzuki, Y., Yamamoto, N., Hattori, T., Sakamoto, M., and Umezawa, T., 2009. High-throughput determination of thioglycolic acid lignin from rice. *Plant Biotechnology*, 26, 3:337–340

[190] Serapiglia, M. J., Cameron, K. D., Stipanovic, A. J., and Smart, L. B., 2009. Analysis of Biomass Composition Using High-Resolution Thermogravimetric Analysis and Percent Bark Content for the Selection of Shrub Willow Bioenergy Crop Varieties. *BioEnergy Research*, 2, 1-2:1–9

[191] Pattathil, S., Avci, U., Miller, J. S., and Hahn, M. G., 2012. Immunological Approaches to Plant Cell Wall and Biomass Characterization: Glycome Profiling. In M. E. Himmel, ed., *Biomass Conversion*, pp. 61–72. Totowa, NJ: Humana Press

[192] Gomez, L. D., Whitehead, C., Barakate, A., Halpin, C., and McQueen-Mason, S. J., 2010. Automated saccharification assay for determination of digestibility in plant materials. *Biotechnology for Biofuels*, 3, 1:1–12

[193] Santoro, N., Cantu, S. L., Tornqvist, C.-E., Falbel, T. G., Bolivar, J. L., Patterson, S. E., Pauly, M., and Walton, J. D., 2010. A High-Throughput Platform for Screening Milligram Quantities of Plant Biomass for Lignocellulose Digestibility. *BioEnergy Research*, 3, 1:93–102

[194] Lee, S. J., Warnick, T. A., Pattathil, S., Alvelo-Maurosa, J. G., Serapiglia, M. J., McCormick, H., Brown, V., Young, N. F., Schnell, D. J., Smart, L. B., Hahn, M. G., Pedersen, J. F., Leschine, S. B., and Hazen, S. P., 2012. Biological conversion assay using Clostridium phytofermentans to estimate plant feedstock quality. *Biotechnology for Biofuels*, 5, 1:5

[195] Jensen, P. D., Hartmann, H., Böhm, T., Temmerman, M., Rabier, F., and Morsing, M., 2006. Moisture content determination in solid biofuels by dielectric and NIR reflection methods. *Biomass and Bioenergy*, 30, 11:935–943

[196] Bala, B. K., 1997. *Drying and storage of cereal grains*. Enfield, NH: Science Publishers

[197] Dean, J. R., Ando, D. J., and Metcalfe, E., 1997. *Atomic absorption and plasma spectroscopy*. Chichester, New York: Published on behalf of ACOL (University of Greenwich) by J. Wiley, 2nd ed. ed.

[198] Hoenig, M., Baeten, H., Vanhentenrijk, S., Vassileva, E., and Quevauviller, P., 1998. Critical discussion on the need for an efficient mineralization procedure for the analysis of plant material by atomic spectrometric methods. *Analytica Chimica Acta*, 358, 1:85–94

[199] Salt, D. E., Baxter, I., and Lahner, B., 2008. Ionomics and the Study of the Plant Ionome. *Annual Review of Plant Biology*, 59, 1:709–733

[200] Abdullah, H. and Wu, H., 2009. Biochar as a Fuel: 1. Properties and Grindability of Biochars Produced from the Pyrolysis of Mallee Wood under Slow-Heating Conditions.

Energy & Fuels, 23, 8:4174–4181

[201] Vogel, K. P., Pedersen, J. F., Masterson, S. D., and Toy, J. J., 1999. Evaluation of a Filter Bag System for NDF, ADF, and IVDMD Forage Analysis. *Crop Science*, 39, 1:276–279

[202] Sapan, C. V., Lundblad, R. L., and Price, N. C., 1999. Colorimetric protein assay techniques. *Biotechnology and Applied Biochemistry*, 29, 2:99–108

[203] Noble, J. E. and Bailey, M. J., 2009. Chapter 8 Quantitation of Protein. In *Methods in Enzymology*, vol. 463, pp. 73–95. San Diego, CA: Elsevier

[204] Mosse, J., 1990. Nitrogen-to-protein conversion factor for ten cereals and six legumes or oilseeds. A reappraisal of its definition and determination. Variation according to species and to seed protein content. *Journal of Agricultural and Food Chemistry*, 38, 1:18–24

[205] Fu, C., Mielenz, J. R., Xiao, X., Ge, Y., Hamilton, C. Y., Rodriguez, M., Chen, F., Foston, M., Ragauskas, A., Bouton, J., Dixon, R. A., and Wang, Z.-Y., 2011. Genetic manipulation of lignin reduces recalcitrance and improves ethanol production from switchgrass. *Proceedings of the National Academy of Sciences*, 108, 9:3803 –3808

[206] Kumar, R., Pandey, K., Chandrashekar, N., and Mohan, S., 2011. Study of age and height wise variability on calorific value and other fuel properties of Eucalyptus hybrid, Acacia auriculaeformis and Casuarina equisetifolia. *Biomass and Bioenergy*, 35, 3:1339–1344

[207] Smets, K., Adriaensens, P., Reggers, G., Schreurs, S., Carleer, R., and Yperman, J., 2011. Flash pyrolysis of rapeseed cake: Influence of temperature on the yield and the characteristics of the pyrolysis liquid. *Journal of Analytical and Applied Pyrolysis*, 90, 2:118–125

[208] Miao, Z., Grift, T., Hansen, A., and Ting, K., 2011. Energy requirement for comminution of biomass in relation to particle physical properties. *Industrial Crops and Products*, 33, 2:504–513

[209] Reich, G., 2005. Near-infrared spectroscopy and imaging: Basic principles and pharmaceutical applications. *Advanced Drug Delivery Reviews*, 57, 8:1109–1143

[210] Vogel, K. P., Dien, B. S., Jung, H. G., Casler, M. D., Masterson, S. D., and Mitchell, R. B., 2010. Quantifying Actual and Theoretical Ethanol Yields for Switchgrass Strains Using NIRS Analyses. *BioEnergy Research*, 4, 2:96–110

[211] Sanderson, M. A., Agblevor, F., Collins, M., and Johnson, D. K., 1996. Compositional analysis of biomass feedstocks by near infrared reflectance spectroscopy. *Biomass and Bioenergy*, 11, 5:365–370

[212] Burns, D. A. and Ciurczak, E. W., 2001. *Handbook of Near-Infrared Analysis*. Boca Raton, FL: CRC Press

[213] Jin, S. and Chen, H., 2007. Near-infrared analysis of the chemical composition of rice straw. *Industrial Crops and Products*, 26, 2:207–211

[214] Liu, L., Ye, X. P., Womac, A. R., and Sokhansanj, S., 2010. Variability of biomass chemical composition and rapid analysis using FT-NIR techniques. *Carbohydrate Polymers*, 81, 4:820–829

[215] Wolfrum, E. and Sluiter, A., 2009. Improved multivariate calibration models for corn stover feedstock and dilute-acid pretreated corn stover. *Cellulose*, 16, 4:567–576

[216] Hayes, D. J., 2012. Development of near infrared spectroscopy models for the quantitative prediction of the lignocellulosic components of wet Miscanthus samples. *Bioresource Technology*, 119:393–405

[217] Huang, C. J., Han, L. J., Liu, L., and Yang, Z. L., 2008. Proximate analysis and calorific value estimation of rice straw by near infrared reflectance spectroscopy. *Journal of the Energy Institute*, 81, 3:153–157

[218] Huang, C., Han, L., Yang, Z., and Liu, X., 2009. Ultimate analysis and heating value prediction of straw by near infrared spectroscopy. *Waste Management*, 29, 6:1793–1797

[219] Allison, G. G., Morris, C., Hodgson, E., Jones, J., Kubacki, M., Barraclough, T., Yates, N., Shield, I., Bridgwater, A. V., and Donnison, I. S., 2009. Measurement of key compositional parameters in two species of energy grass by Fourier transform infrared spectroscopy. *Bioresource Technology*, 100, 24:6428–6433

[220] Zhou, G., Taylor, G., and Polle, A., 2011. FTIR-ATR-based prediction and modelling of lignin and energy contents reveals independent intra-specific variation of these traits in bioenergy poplars. *Plant Methods*, 7, 1:9

[221] Lestander, T. A. and Rhén, C., 2005. Multivariate NIR spectroscopy models for moisture, ash and calorific content in biofuels using bi-orthogonal partial least squares regression. *Analyst*, 130, 8:1182–1189

[222] Fagan, C. C., Everard, C. D., and McDonnell, K., 2011. Prediction of moisture, calorific value, ash and carbon content of two dedicated bioenergy crops using near-infrared spectroscopy. *Bioresource Technology*, 102, 8:5200–5206

[223] Everard, C. D., McDonnell, K. P., and Fagan, C. C., 2012. Prediction of biomass gross calorific values using visible and near infrared spectroscopy. *Biomass and Bioenergy*, 45:203–211

[224] Roberts, C. A., Houx, J. H., and Fritschi, F. B., 2011. Near-Infrared Analysis of Sweet Sorghum Bagasse. *Crop Science*, 51, 5:2284

[225] Laurens, L. M. L. and Wolfrum, E. J., 2011. Feasibility of Spectroscopic Characterization of Algal Lipids: Chemometric Correlation of NIR and FTIR Spectra with Exogenous Lipids in Algal Biomass. *BioEnergy Research*, 4, 1:22–35

[226] Penning, B. W., Hunter, C. T., Tayengwa, R., Eveland, A. L., Dugard, C. K., Olek, A. T., Vermerris, W., Koch, K. E., McCarty, D. R., Davis, M. F., Thomas, S. R., McCann, M. C., and Carpita, N. C., 2009. Genetic Resources for Maize Cell Wall Biology. *Plant Physiology*, 151, 4:1703 –1728

[227] Montes, J., Technow, F., Dhillon, B., Mauch, F., and Melchinger, A., 2011. High-throughput non-destructive biomass determination during early plant development in maize under field conditions. *Field Crops Research*, 121, 2:268–273

[228] Normanly, J., ed., 2012. *High-Throughput Phenotyping in Plants*, vol. 918. New York: Humana Press

[229] White, J. W., Andrade-Sanchez, P., Gore, M. A., Bronson, K. F., Coffelt, T. A., Conley, M. M., Feldmann, K. A., French, A. N., Heun, J. T., Hunsaker, D. J., Jenks, M. A., Kimball, B. A., Roth, R. L., Strand, R. J., Thorp, K. R., Wall, G. W., and Wang, G., 2012. Field-based phenomics for plant genetics research. *Field Crops Research*, 133:101–112

[230] Haboudane, D., Miller, J. R., Tremblay, N., Zarco-Tejada, P. J., and Dextraze, L., 2002. Integrated narrow-band vegetation indices for prediction of crop chlorophyll content for application to precision agriculture. *Remote Sensing of Environment*, 81, 2-3:416–426

[231] Seelig, H., Hoehn, A., Stodieck, L. S., Klaus, D. M., Adams III, W. W., and Emery, W. J., 2008. The assessment of leaf water content using leaf reflectance ratios in the visible, near, and shortwaveinfrared. *International Journal of Remote Sensing*, 29,

13:3701–3713

[232] Richmond, K. E. and Sussman, M., 2003. Got silicon? The non-essential beneficial plant nutrient. *Current Opinion in Plant Biology*, 6, 3:268–272

[233] Ahmad, R., Zaheer, S. H., and Ismail, S., 1992. Role of silicon in salt tolerance of wheat (Triticum aestivum L.). *Plant Science*, 85, 1:43–50

[234] Winslow, M. D., Okada, K., and Correa-Victoria, F., 1997. Silicon deficiency and the adaptation of tropical rice ecotypes. *Plant and Soil*, 188, 2:239–248

[235] Cotterill, J. V., Watkins, R. W., Brennon, C. B., and Cowan, D. P., 2007. Boosting silica levels in wheat leaves reduces grazing by rabbits. *Pest Management Science*, 63, 3:247–253

[236] Keeping, M. G., Kvedaras, O. L., and Bruton, A. G., 2009. Epidermal silicon in sugarcane: Cultivar differences and role in resistance to sugarcane borer Eldana saccharina. *Environmental and Experimental Botany*, 66, 1:54–60

[237] Datnoff, L. E., Snyder, G. H., and Korndrfer, G. H., eds., 2001. *Silicon in Agriculture, Volume 8*. New York: Elsevier Science, 1 ed.

[238] Sommer, M., Kaczorek, D., Kuzyakov, Y., and Breuer, J., 2006. Silicon pools and fluxes in soils and landscapesa review. *Journal of Plant Nutrition and Soil Science*, 169, 3:310–329

[239] Ma, J. F. and Takahashi, E., eds., 2002. *Soil, Fertilizer, and Plant Silicon Research in Japan*. Amsterdam: Elsevier Science, 1 ed.

[240] Mitani, N., Ma, J. F., and Iwashita, T., 2005. Identification of the silicon form in xylem sap of rice (Oryza sativa L.). *Plant and cell physiology*, 46, 2:279–283

[241] Yoshida, S., Ohnishi, Y., and Kitagishi, K., 1962. Chemical Forms, Mobility and Deposition of Silicon in Rice Plant. *Soil Science and Plant Nutrition*, 8, 3:15–21

[242] Prychid, C. J., Rudall, P. J., and Gregory, M., 2003. Systematics and Biology of Silica Bodies in Monocotyledons. *The Botanical Review*, 69, 4:377–440

[243] Zhang, C., Wang, L., Zhang, W., and Zhang, F., 2013. Do lignification and silicification of the cell wall precede silicon deposition in the silica cell of the rice (Oryza sativa L.) leaf epidermis? *Plant and Soil*, 372, 1-2:137–149

[244] Raven, J. A., 1983. The Transport and Function of Silicon in Plants. *Biological Reviews*, 58, 2:179–207

[245] Ma, J. F., Yamaji, N., and Mitani-Ueno, N., 2011. Transport of silicon from roots to panicles in plants. *Proceedings of the Japan Academy, Series B*, 87, 7:377–385

[246] Deren, C. W., Datnoff, L. E., and Snyder, G. H., 1992. Variable silicon content of rice cultivars grown on everglades histosols. *Journal of Plant Nutrition*, 15, 11:2363–2368

[247] Duan, B. W., Zhuang, J. Y., Zhang, K. Q., Cai, R., Dai, W. M., and Zheng, K. L., 2005. Genetic dissection of silicon content in different organs of rice. *Crop Science*, 45, 4:1345–1352

[248] Wu, Q. S., Wan, X. Y., Su, N., Cheng, Z. J., Wang, J. K., Lei, C. L., Zhang, X., Jiang, L., Ma, J. F., and Wan, J. M., 2006. Genetic dissection of silicon uptake ability in rice (Oryza sativa L.). *Plant Science*, 171, 4:441–448

[249] Dai, W. M., Zhang, K. Q., Wu, J. R., Wang, L., Duan, B. W., Zheng, K. L., Cai, R., and Zhuang, J. Y., 2008. Validating a segment on the short arm of chromosome 6 responsible for genetic variation in the hull silicon content and yield traits of rice. *Euphytica*, 160, 3:317–324

[250] Li, W., Shao, M., Zhong, W., Yang, J., Okada, K., Yamane, H., Zhang, L., Wang, G., Wang, D., Xiao, S., Chang, S., Qian, G., and Liu, F., 2012. Ectopic Expression of Hrf1 Enhances Bacterial Resistance via Regulation of Diterpene Phytoalexins, Silicon and

Reactive Oxygen Species Burst in Rice. *PLoS ONE*, 7, 9:e43914

[251] Isa, M., Bai, S., Yokoyama, T., Ma, J., Ishibashi, Y., Yuasa, T., and Iwaya-Inoue, M., 2010. Silicon enhances growth independent of silica deposition in a low-silica rice mutant, Lsi1. *Plant Soil*, 331, 1:361–375

[252] Saito, K., Yamamoto, A., Sa, T., and Saigusa, M., 2005. Rapid, Micro-Methods to Estimate Plant Silicon Content by Dilute Hydrofluoric Acid Extraction and Spectrometric Molybdenum Method. *Soil Science & Plant Nutrition*, 51, 1:29–36

[253] Lötjönen, T. and Paappanen, T., 2013. Bale density of reed canary grass spring harvest. *Biomass and Bioenergy*, 51:53–59

[254] Davis, S. C., Boddey, R. M., Alves, B. J. R., Cowie, A. L., George, B. H., Ogle, S. M., Smith, P., van Noordwijk, M., and van Wijk, M. T., 2013. Management swing potential for bioenergy crops. *GCB Bioenergy*, 5, 6:623–638

[255] Voelker, S. L., Lachenbruch, B., Meinzer, F. C., Jourdes, M., Ki, C., Patten, A. M., Davin, L. B., Lewis, N. G., Tuskan, G. A., Gunter, L., Decker, S. R., Selig, M. J., Sykes, R., Himmel, M. E., Kitin, P., Shevchenko, O., and Strauss, S. H., 2010. Antisense Down-Regulation of 4CL Expression Alters Lignification, Tree Growth, and Saccharification Potential of Field-Grown Poplar. *Plant Physiology*, 154, 2:874–886

CHAPTER 3

Rice straw compositional variation between varieties, tissue types, and environments: impacts on bioenergy potential [2]

Overview

Breeding has transformed wild plant species to maximize the proportion of their photosynthetic assimilate into grain, fiber, and other products for human use. Despite progress in increasing the harvest index, much of the biomass of crop plants is not utilized. Potential uses for these large amounts of agricultural residues that accumulate are animal fodder or bioenergy, though these may not be economically viable without additional efforts such as targeted breeding or improved processing. We characterized leaf and stem tissue from a diverse set of rice germplasm grown in two environments and report bioenergy-related traits across these variables. We measured cellulose, hemicellulose, mixed linkage glucan (MLG), lignin, ash, cell wall structural proteins (HRGPs), bulk density, as well as total glucose and xylose, and glucose and pentose yields after pretreatment of the biomass. For cellulose, hemicellulose, lignin, ash, total glucose, and glucose yield we find large variation between environments, irrespective of the germplasm. We confirm previously observed positive relationships between total glucose and hemicellulose and glucose yield, as well as negative correlations between lignin and ash with glucose yield. Trends in our data suggest that

[2]This chapter will be published as a collaborative effort with these authors: Paul Tanger, Miguel Vega-Sanchez, Margaret Fleming, Kim Tran, Seema Singh, James B. Abrahamson, Courtney E. Jahn, Nicholas Santoro, Elizabeth B. Naredo, Marietta Baraoidan, John M.C. Danku, David E. Salt, Hei Leung, Pamela C. Ronald, Daniel R. Bush, John K. McKay and Jan E. Leach. Author contributions: Conceived and designed the experiments: PT M-VS CEJ PCR DRB JKM JEL. Performed the experiments: PT M-VS MF KT SS JBA CEJ NS JMCD DES. Managed field trials: EBN MB. Analyzed the data: PT M-VS.

greenhouse studies may overestimate the bioenergy potential of biomass. Glucose yield from greenhouse grown plants predicts glucose yield in field samples and could serve in greenhouse studies as an indicator of potentially lower cost conversion of field biomass for bioenergy. Efforts to improve bioenergy traits must examine both stem and leaf tissues as they may be under separate genetic control.

3.1. Introduction

Bioenergy is a potential alternative to fossil fuel energy resources. Several obstacles have thus far prevented widespread commercialization of bioenergy resources [1]. One major obstacle is the high cost of processing plant biomass to efficiently break down the cell walls into fermentable substrates used for biofuel production [2]. The recalcitrance and heterogeneity of the biomass usually requires several pretreatment steps and the use of enzyme cocktails to significantly degrade the biomass. The variability in types of biomass requires fine-tuning the process with the expected biomass source. Methods are being developed to both improve the biomass [3–6], and the technologies to more effectively process the biomass for bioenergy [7]. However, this research is often performed in only one experimental environment, with biomass from one source.

The majority of biomass is comprised of plant cell walls, which in turn are comprised of mostly cellulose, hemicellulose, lignin, pectins, and proteins [8] and it is the variation in these components that influence bioenergy yield. Plant cell wall composition varies between [9, 10] and within species [11] and tissue type [12, 13], as well as between developmental stages [14]. Adaptation to various biotic and abiotic pressures has resulted in very different cell wall compositions in different plant lineages [15, 16] and these compositional differences vary greatly, even within closely related species. For example, in a recent study mapping

QTL for hemicellulose, a variety of *O. rufipogon*, the progenitor of rice, had 84% more glucose and 31% less xylose in the stem cell walls compared to an indica variety of rice [17]. While there is evidence that composition varies across environments for many plant species [18–20], the relationships between genetics and the environment are not well studied except in a few cases, for example in switchgrass, wheatgrass, maize and sorghum [21, 22].

Variation in biomass composition has a direct impact on bioenergy potential [23, 24]. Lignin content in sugarcane [7, 25], and hemicellulose in miscanthus [26] have been negatively correlated with overall enzymatic bioenergy yields. In addition, grasses in general have high levels of ferulic acids and mixed linkage glucans (MLG) in their cell walls and these may play a role in the efficiency of enzymatic conversion [27–29]. MLG content varies between tissue types in miscanthus and rice [27, 30] as well as between rice and other grasses [31]. Another component of interest are structural cell wall proteins, particularly hydroxyproline rich glycoproteins (HRGPs), as they become covalently linked into the mature cell wall [32]. Biomass pretreatment commonly uses either dilute acid or base, which are not strong enough to extract the majority of the covalently bound HRGPs [33–35]. Beyond just composition, other parameters such as the energy required to transport the biomass are important. The cost of transport of biomass depends on the bulk density which has been found to vary between switchgrass, wheat and corn stover [36].

It is still unclear if the ratios of cellulose, hemicellulose, and lignin are critical, or if the cell wall architecture (how these components are assembled together) is more important. In rice and wheat straw samples with similar amounts of cellulose and hemicellulose, cellulose crystallinity state and hemicellulose side chains were correlated with enzymatic conversion efficiency [37]. Previously proposed and accepted models of how cellulose and hemicellulose are linked are probably incorrect [38] and new models must be validated.

In this chapter, we use an array of compositional assays to examine a selected group of rice germplasm grown in two environments with the goal of identifying composition trends between varieties and tissue types across environments. We then ask how these compositional parameters influence the yield from a pretreatment and enzymatic digestion: a measure of how easily biomass can be converted to bioenergy. We conclude with an assessment of which assays are the most reliable and most likely to translate successfully from lab and greenhouse conditions to the field.

3.2. Materials and methods

3.2.1. Plant materials

Twenty genetically and agronomically diverse varieties, lines, and landraces (hereafter collectively referred to as varieties) of *O. sativa* were selected from across the world's germplasm collection as described in [39–41] and Table 3.1. In addition, the variety Kitaake was included in some assays because it is commonly used in genetic transformation studies to assess gene contributions to biomass traits.

3.2.2. Growth conditions and sample preparation

The complete set of 20 varieties was grown from February-October 2012 with n=3 per variety in a completely randomized design in a greenhouse (GH) with controlled conditions at Colorado State University (lat 40°34'17.5"N long 105°04'52.5"W, elevation 1519 m) with a mean temperature of 27°C and 76 % RH and supplemental high-intensity discharge lighting to maintain a 16-h-light/8-h-dark photoperiod at an irradiance of 20-55 mW/m^2. Rice seeds were germinated in 1:1000 dilution of Maxim XL fungicide (Syngenta) for 3 days prior to planting in a custom potting mixture (4:4:1 Pro-Mix BX Mycorrhizae: Canadian sphagnum peat: Quikrete play sand) in 7.6 L pots in standing water. Plants were fertilized twice

weekly with Peters Excel 15-5-15 Cal-Mag (Scotts), starting at 1 month post transplanting at a rate of 300 mg/L, and initially at 1 month, and as needed with an iron chelator, Sprint 330 (BASF). To induce flowering in Aswina and Pokkali, plants were moved to a growth chamber with 28/24°C day/night temperature and constant 80% RH under a 8-h-light/16-h-dark photoperiod at a light intensity of 800 µE/m^{2}s. Plants were harvested 5 cm above soil level as they matured over several months between 14:00-18:00 each day to minimize variation in nonstructural carbohydrates and other effects of circadian rhythms, and to capture plant straw tissue as it would realistically be collected (after grain maturity and start of senescence). Plants were separated into panicles, leaf (the leaf blade), and stem or culm (including the leaf sheath and mature stem: nodes and internodes). All samples were oven dried at 50°C until no further change in mass was observed. They were then stored at room temperature (approximately 22°C and 21 % RH).

The complete set of 20 varieties was also grown from June-November 2012 at the Experiment Station at the International Rice Research Institute (IRRI), Los Baños, Laguna, Philippines (lat 14°10' 11.69" N, long 121°14' 38.63" E, 21m elevation). Seeds were incubated at 50°C for 7 d to break dormancy and germinated in June 2012. Seedlings were transplanted in July at the IRRI demonstration field into plot sizes of 3.5 x 1.5 m^2 at 20 x 20 cm spacing (8 rows x 18 rows), without replication. Soil type at this location is Aquandic Epiaquoll according to USDA classification system [42]. Pre-emergence herbicides (SOFIT 300 EC (Pretilachlor + safener, Syngenta), at 1 L/ha), molluscicide (Snailkill (Metaldehyde, Agasin Pte. Ltd) at 1 L/ha), and carbofuran (Furadan 3G, FMC Corp. at 25 kg/ha) were applied right after transplanting. Fertilizer rate was 90-30-30 in split application of N, which was 30 kg N per hectare from complete fertilizer (14-14-14) applied basally during the final leveling of the field, and 30 kg N per hectare at 30 and 60 days after transplanting from

UREA (45-0-0). No diseases or pests were noted during the growing season. Tissues were harvested from inner rows in November 2012, oven dried, then shipped to Colorado State University where they were stored in the same location as the GH tissues.

Of the 20 varieties grown, five varieties (Aswina, Azucena, IR64-21, LTH & Zhenshen 97B) were subjected to more detailed analyses, described below. For GH conditions, individual plants represent biological replicates, and their stems and leaves were kept separate. For field conditions, pools of 3-29 plants were created as biological replicates; the stems and leaves of the pooled plants were ground separately. Each samples was ground to 6 mm or 2 mm using a knife mill (SM2000, Retsch GmbH, Haan, Germany or Model 4 Wiley mill, Thomas Scientific, Swedesboro, NJ) followed by grinding to a fine powder using a bead mill (Tissuelyzer II, Qiagen, Valencia, CA). This ground tissue was used in all subsequent analysis, described below.

3.2.3. Forage analysis (cellulose, hemicellulose, lignin, ash)

Samples (10 g) were analyzed for cellulose, hemicellulose, lignin and ash at the U.C. Davis Analytical Laboratory. Methods are available at http://anlab.ucdavis.edu and described previously [43]. Briefly, Neutral Detergent Fiber (NDF) was determined by treating biomass with a neutral detergent solution and heat. Sodium sulfite was used to aid in the removal of some nitrogenous matter. Heat-stable amylase was used to allow for the removal of starch and to inactivate potential contaminating enzymes that might degrade fibrous constituents. A hot, acidified detergent solution was used to dissolve soluble components, hemicellulose and soluble minerals leaving a residue of cellulose, lignin, and heat damaged protein and a portion of cell wall protein and minerals (ash). Acid Detergent Fiber (ADF) was determined gravimetrically as the residue remaining after acid detergent extraction. Lignin

was determined gravimetrically after the ADF residue was extracted with 72% H_2SO_4 and ashed. Cellulose was determined by subtracting the pre-ash lignin value from the ADF value. Hemicellulose was determined by subtracting ADF from NDF.

3.2.4. Quantification of Klason lignin, total glucose, total xylose and ash

Samples were analyzed for glucose, xylose, lignin, and ash based on the National Renewable Energy Laboratory (NREL) protocol [44]. Briefly, samples (100 mg) were weighed into 50 mL glass serum vials. One mL 72% H_2SO_4 was added. The samples incubated for 1 h, then 28 mL DI water was added and they were autoclaved for 1 h. The samples were filtered (15 µm porous bottom porcelain crucibles) and the liquid fraction was analyzed with HPLC (Agilent 1260 with a Biorad Aminex HPX-87H column) with D-glucose and D-xylose standards (Supelco), while the solid fraction was heated to 105°C followed by 575°C overnight. The difference between the crucible + sample mass after heating to 105°C and the mass after heating to 575°C overnight is the Klason lignin, and the difference between the crucible mass and the mass after 575°C overnight is the Klason ash. Each sample was measured at least twice, however some data was omitted because 1) it was not possible (percent less than 0 or greater than 100) or 2) the studentized residuals were outside the distribution of the data (Bonferroni $p<0.05$). This filtering resulted in only one technical replicate of five samples. Total glucose was corrected for free glucose by subtracting the free glucose measured in the digestibility assay from the total glucose measured.

3.2.5. Digestibility assay

Samples were processed in a pretreatment and digestibility assay described in [35]. Samples were divided into six aliquots: three were processed with only hot water (90°C, 3 h), and three were processed with a dilute base solution (6.25 mM NaOH, 90°C, 3 h). After

pretreatment, samples were digested with Accellerase1000 (Genecor, Rochester, NY), and glucose and pentose concentrations determined with enzyme based colorimetric assays. To a separate aliquot distilled water was added and assayed for free glucose without any pretreatment. This free glucose value was subtracted from the glucose values obtained after hot water pretreatment to allow direct comparison with glucose after base pretreatment, since the dilute base would have degraded free glucose in those samples.

3.2.6. Quantification of MLG

MLG quantification was modified from [27]. Five mg of ground biomass sample was treated with 4 M NaOH at 4°C overnight with vigorous shaking (1400 RPM) for extraction of MLG and other hemicellulose. Base extracted samples were neutralized with glacial acetic acid and total sugar content in the supernatant was measured with a DNS assay. Samples were normalized by subtracting the free glucose from the total sugar content to account for variation in soluble sugars. Samples were then diluted to a concentration of 100 µg/ml antigen in distilled water for ELISA. The ELISA was performed as described in [45] using an monoclonal anti-MLG antibody (1:1000 dilution) with the following modifications: antibody incubations were done in 5% milk dissolved in 1X TBS buffer, and detection was performed by using PNPP substrate (1 mg/ml in dieathanolamine buffer) and an alkaline phosphatase-conjugated anti-mouse secondary antibody. The reaction was allowed to develop for 20 min, and absorbance was measured at 405 and 490 nm with a spectrophotometer. The MLG values are reported as the ratio of Abs 405/490.

3.2.7. Quantification of hydroxyproline

Hydroxyproline-rich glycoproteins (HRGPs) were quantified by measuring the concentration of hydroxyproline. Ground biomass (45 mg) was hydrolyzed in 6 N hydrochloric acid for

18 h at 100°C. The hydrolysate was adjusted to pH 3.0 (± 0.1) with 12 M sodium hydroxide and the hydroxyproline was quantified with the colorimetric assay of [46]. Each sample was measured in triplicate.

3.2.8. Bulk density

A graduated cylinder containing 2-5 g of finely ground biomass was tapped once from 1 cm high onto four paper towels on a hard surface, and the mass and volume of the biomass in the graduated cylinder was recorded. This measurement was repeated three times for each sample.

3.2.9. Statistical analysis

Data was processed in Microsoft Excel and compiled in Access 2010 (Microsoft Corporation, Redmond, WA), JMP Pro 11 (SAS Institute Inc., Cary, NC) and R Statistical Computing version 3.0.2 [47]. R was used to calculate the n, mean, SD, SE, and CV for each variety / tissue / environment parameter combination. JMP was used to calculate Spearman's correlations (ρ) between each phenotype (genotypic correlations). The Principal Components Analysis (PCA) using REML on the covariance matrix was also generated in JMP. Density was left in the original units of g/ml^3 to allow a PCA of covariance matrix rather than a correlation matrix (since the units are similar to the units of the other phenotypes).

Normality was checked for each phenotype and a box-cox transformation was performed when Shapiro-wilk test $p < 0.05$. Transformed data was used only for contrasts, heritability estimates, R^2 estimates, and glucose yield prediction models as described next. A linear model was fitted to calculate the main effects of variety and environment. Let y_{ij} be the response parameter estimated for the ith variety in the jth environment and assume the

following linear model: $y_{ij} = \mu + \alpha_i + \beta_j + \alpha_i * \beta_j + \epsilon_{ij}$, where μ is the overall mean, α_i is the fixed effect of variety for the ith variety, β_j is the fixed effect of environment for the jth environment and ϵ_{ij} is the residual variance with mean 0 and variance σ^2. This model was also used to calculate the p value of the contrasts between environments for the least square means (LSmeans) of each variety. The means and LSmeans were similar, so means were used in the remainder of the analysis. This model was run with and without Azucena (since n=1 in the GH) but the results were similar, so Azucena was left in the data. Separately, covariance estimates were obtained to calculate the percent variation due to variety (broad-sense heritability, H^2). The model is the same as described above without the interaction term $(\alpha_i * \beta_j)$, and both variety and environment are considered random effects. Variances were also calculated separately for each environment and tissue type. In this case, the only effect was variety, and variety was considered random. R^2 was calculated in R Statistical Computing with the appropriate linear model using phenotypic data from GH to predict glucose yield after base pretreatment, only from phenotypes with significant Spearman's ρ ($p<0.05$). To determine relationships between glucose yield after base pretreatment overall, across both environments, linear models with all possible combinations of compositional parameters were generated in JMP with glucose yield after base treatment as the response variable. Since parameters were highly correlated this was done in two sets: both sets had MLG, HRGP, and density, and one set additionally contained the forage phenotypes (cellulose, hemicellulose, lignin, ash) and the other set additionally contained the NREL acid hydrolysis phenotypes (Klason lignin, Klason ash, total glucose, total xylose) as possible parameters. Models were sorted for minimum Akaike Information Criteria corrected for small sample sizes (AICc) and models <10 ΔAICc from the model with the lowest AICc were selected.

3.3. Results & discussion

3.3.1. overview

Twenty rice varieties had previously been selected that represented the agronomic and genetic diversity in rice [41]. Preliminary experiments were completed on the 20 varieties in the GH, and subsequent studies in two environments focused on five of these twenty varieties. For each variety in each environment, samples were separated into leaf and stem tissues. The preliminary study results with the 20 varieties are presented first, followed by results of the five varieties. Sixteen phenotypes were measured on the five varieties and the phenotypes are divided into sets (Table 3.2). Each set of phenotypic data is presented together. Variation within environment, tissue type, and variety are summarized through both PCA and heritability estimates. Relationships between compositional phenotypes and bioenergy phenotypes are discussed, especially with a focus on phenotypes in the GH that can predict bioenergy yield in the field, and overall relationships between composition and bioenergy yield. Model selection of phenotypes as predictors of glucose yield is presented.

3.3.2. Cell wall composition and bioenergy phenotypes of 20 varieties

In a preliminary analysis, four sets of samples of the 20 varieties were analyzed as described in Appendix B.1.1. AcBr lignin, MLG, saccharification and cell wall monosaccharide composition were quantified on a set of stem samples from plants grown in the GH, and digestibility of whole plant, leaf, and stem was measured on another set of GH grown samples (Supplemental Table B.1 and B.2). Stem wall thickness was measured on a third set of GH grown samples and elemental composition of leaf, stem, and grain was quantified on a fourth set of plants grown in the field (Supplemental Table B.3 and B.4).

From these collective data, we observed that the means of the varieties were different for all assays (F test, $p < 0.05$), except for arabinose composition ($p = 0.09$) and pentose yield from stem tissue ($p = 0.09$). Often the same varieties appeared at the extremes for the various measurements (Figure 3.1). For example, variety LTH had high glucose yield after base pretreatment (Supplemental Table B.2) as well as high AcBr lignin (Supplemental Table B.1), while variety Zhenshan 97B had low glucose yield but high pentose yield after base pretreatment (Supplemental Table B.2) and high saccharification efficiency, but low MLG (Supplemental Table B.1). The unique combinations of cell wall composition in these varieties could provide insight into the best combination of traits from a bioenergy perspective. It is unclear how LTH had high glucose yield and high lignin, as it has been reported there is a negative relationship between these parameters [7, 25]. Another relationship, between forage lignin [reported in [41] but on the same set of varieties grown in the same greenhouse] and AcBr lignin (Supplemental Table B.1), were both inversely correlated with MLG (Supplemental Table B.1 and Supplemental Figure B.1). This could indicate that MLG and lignin might serve similar roles in the cell wall of different varieties. We also observed positive correlations between MLG, saccharification, and glucose yield after base treatment (Supplemental Figure B.1), so increasing MLG content could be a new strategy for improving bioenergy yield.

3.3.3. A closer look: composition and sugar yield of five varieties in two environments

To further examine the relationships observed in the data from the 20 varieties, and how these relationships might change in different environments, five varieties were selected that consistently appeared in the top or bottom of the rankings for cell wall composition,

digestibility, stem wall thickness, and elemental composition, based on Fisher's Least Significant Difference (LSD). In addition to Azucena, Zhenshan 97B and LTH (three varieties that appeared at the extremes for several phenotypes), IR64-21 and Aswina were included because they are parental varieties of a Recombinant Inbred Line (RIL) mapping population that is a focus for other bioenergy traits (Appendix A).

3.3.3.1. *Large differences in cell wall composition between environments*

Since the selected varieties represented very diverse rice germplasm, we expected to observe large differences in composition among the varieties; this was generally not the case. To demonstrate this and more easily visualize the differences in composition that were present, means of all varieties for each of the composition phenotypes are plotted in Figure 3.2. These phenotypes include the four components of the detergent fiber (forage) compositional assay: cellulose, hemicellulose, lignin and ash, as well as the four components of the NREL acid hydrolysis assay: total glucose, xylose, Klason lignin and Klason ash. The four components of each assay are approximating the same parts of biomass: total glucose and cellulose, hemicellulose and total xylose, lignin and Klason lignin, ash and Klason ash. In these figures, the standard deviation (SD) bars represent the variation between the varieties and this variation is generally small compared to the differences between the dots, which represent the means in the two environments.

Cellulose and total glucose were higher in the GH than the field, though these trends were not consistent for individual varieties (plots of the separate varieties are in Figure 3.3 and 3.4). Hemicellulose and total xylose were also higher in the GH than the field, though the difference between environments was larger for hemicellulose than total xylose (Figure 3.2). Lignin content was higher in all field samples compared to the corresponding GH samples, and remained in the same range in the field for all varieties in all tissues (4-6%

for lignin and 9-16% for Klason lignin) (Figure 3.2). We observed the largest differences between environments for ash content, more than doubling between environments (Figure 3.2). Variety-specific differences in ash content exist just as for lignin and hemicellulose content, and leaf samples always had more ash than stem (shown separately in Figure 3.3 and Figure 3.4). After environment, variation in tissue types was sometimes observed. For example, stems had more cellulose and total glucose than leaves, and leaves had more ash than stems.

In general, the trends for cellulose, hemicellulose, lignin, and ash observed here between tissue types support previous studies [48]. In contrast to other studies on switchgrass and wheat [12, 13], we observed higher lignin levels in the leaf in the field than in the GH. Liu *et al.* [49] also report higher lignin in the leaf tissue than stem of switchgrass, so other factors may be involved in this trait in the leaf. Of the lignin differences that did exist in the field, the trends were generally consistent for varieties across tissue types, e.g., Aswina had the least lignin in both leaf and stem (Figure 3.3).

The differences in lignin content between environments could be due to less exposure in the GH to UV light or other environmental stresses such as wind. These stresses, present in the field, would trigger lignin and phenolic production in plants [50]. Specifically, some enzymes involved in lignin biosynthesis respond directly to UV light [51], and overall lignin biosynthesis responds to many other environmental stimuli such as temperature and wind [52, 53].

Ash levels were higher in the field than the GH for all samples tested. While this could be due to soil contamination from muddy field conditions, we do not believe this to be the case. First, the minerals that contribute to ash content follow the transpiration stream from the stem to the leaf; thus we observe higher ash in leaf samples. If the higher ash

observed in the field was due to soil contamination, we would expect to see higher ash in all stem samples compared to the respective leaf samples, since the stem tissue is closest to the soil. Furthermore, Jin and Chen [11] report that after the panicle, the rice leaf contains the highest levels of ash. Thus, the high ash content in field samples is very likely due to the growth of the plant in the field environment.

3.3.3.2. *Sugar yields and efficiency varies among environment, tissue, variety and pretreatment*

As a direct measure of bioenergy potential, glucose and pentose monomers were measured after a pretreatment (either dilute base or hot water) and enzymatic digestion of the biomass. We tested both pretreatments to see if there was a difference, as hot water pretreatment is inexpensive and less hazardous, but generally does not perform as well as dilute base treatment. Since yield is relative to the total amount of sugars available in the biomass, we calculated efficiency as a percent of total glucose or total xylose in each sample. As with the compositional assays, means of sugar yields for all varieties are compiled in Figure 3.5 to reveal differences between pretreatments, environments, and tissues.

Glucose yield and efficiency are always less from plants in the field, and base pretreatment always releases more sugar than hot water pretreatment. Less variation in pentose yield was observed among the different environments, tissues, and treatments. In contrast to the compositional assays, some large differences between varieties existed. For example, the variation in glucose yield in stem tissue was quite wide (presented separately for each variety in Figure 3.6). Notably, in stem tissue in the GH for glucose yield after base pretreatment, Aswina yields 30% glucose while Azucena and LTH yielded approximately 20%. Rankings of varieties based on glucose yield are the same regardless of pretreatment but only for samples grown in the greenhouse (Aswina>IR64-21>Zhenshan 97B>Azucena>LTH for

stems; Azucena>Aswina>IR64-21>Zhenshan 97B>LTH for leaves). These rankings change in the stem and the differences are less evident for field samples, especially following base pretreatment. It is clear that there are differences in glucose yield that depend on tissue type and environment; but in general, Aswina and LTH consistently rank as the highest and lowest in glucose yields, respectively, regardless of pretreatment, tissue type or growth environment. Why these varieties may have the highest yields is discussed in more detail in section 3.3.5. While Aswina generally had the highest glucose yields, Zhenshan 97B had the highest efficiency, as a percent of total glucose and xylose content in all conditions and tissue types (Figure 3.7).

We measured free glucose simply to correct for glucose differences between samples due to variation in free sugars throughout the day as well as other factors [54]. However, we observed that some varieties had consistently higher free glucose levels than others (Figure 3.8). Although high non-structural carbohydrates have been previously reported in rice straw [55], the large differences we observed between varieties merit further study. As noted in the methods, Aswina took longer to flower in the GH, which might have influenced the higher free glucose values, celluloses, and hemicellulose, but Aswina also had some of the highest values for these three components in the field where Aswina flowered at the same time as the other varieties.

3.3.3.3. *Variation in MLG, HRGPs, and bulk density are less influenced by environment*

We measured MLG because in the analysis of 20 varieties it was associated with high sugar yields. Variation in MLG did not vary between environments and tissue types consistently for the five varieties (Figure 3.9). Notably, Zhenshan 97B, which had the highest glucose yield, also had highest MLG of all varieties in the GH but not in the field.

Hydroxyproline content is a measure of structural proteins in the cell wall such as hydroxyproline rich glycoproteins (HRGPs). We hypothesized there might be a negative relationship between HRGPs and bioenergy yield, and the relationships we found are discussed in section 3.3.3.7. HRGPs varied between environments for some varieties, but not all (Figure 3.10). The differences between the mean values for leaf tissue were larger than for stem tissue in the two environments. The biomass samples were not boiled to degrade soluble HRGPs, so some variation observed here may be due to the variation in soluble HRGPs in the samples.

Beyond simply higher sugar yields, other important phenotypes affect the logistics of a bioenergy production system. We quantified variation in bulk density because higher density biomass would allow more biomass to be transported at lower cost and less energy used in transport. For leaf tissue, bulk density does not vary much between environments or varieties, but trends indicate higher density in field tissue (Figure 3.11). For stem tissue, GH samples have higher density, and for some varieties (Aswina, Azucena, and IR64-21), the differences could become significantly important if extrapolated to the large amounts that would be transported for bioenergy or forage.

3.3.3.4. *Environment and tissue type play the largest role in composition and bioenergy traits*

Principal Component Analysis (PCA) is an approach to reduce variation in phenotypes to components that explain the largest amount of variation in the data. The first component, explaining 65% of the variation in the data, is mostly represented by environment, as demonstrated by the separation on the x-axis (Figure 3.12). The second component, explaining 20% of the variation, likely represents the tissue type, as demonstrated by the separation on the y-axis; however, the separation is not as clear for the field samples as for the GH samples. On the PCA, phenotypes associated with each other are clustered together.

For example, samples with high lignin and ash are clustered together, and correspond to the leaf samples in the field.

To more directly quantify the variation attributable to variety (broad-sense heritability, H^2) and environment, we estimated the percent of variance attributed to each component (genotype, environment, or residual variance). The heritability varies among phenotypes, and even for each phenotype in leaf vs stem tissue ranging from 64% for total glucose in the leaf, to 0% for others (Table 3.3). Sometimes, the reason for low genotypic variance is because it is driven by environment, e.g., ash phenotypes in which most of the variance is explained by environment. In other cases, for example MLG and HRGP in the stem, most of the variance is not explained by either genotype or environment; variation between individual plants is quite high for these phenotypes. Since breeding efforts focus on one environment, calculating the genotypic variation within each environment separately can provide a clearer picture of breeding potential. In this calculation, the heritability is much higher for all phenotypes (Table 3.4). Notably, the heritability of glucose yield in the field is much higher: 54% for leaf and 63% for stem.

3.3.3.5. *Few parameters in GH can predict glucose yield in the field*

Many studies are performed first in a greenhouse before being brought into the field. Parameters in the GH that could predict performance in the field would be useful, especially to differentiate them from parameters that are not as consistent between environments. Glucose yield was relatively consistent between environments: varieties that yielded the highest in the GH were also the highest in the field (Figure 3.13). Since glucose yield between base and water treatments were also correlated regardless of environment (Figure 3.14), we focus on glucose yield after base treatment for the remainder of our analysis. Based on Spearman's correlations, only five compositional phenotypes in the GH were associated with

glucose yield after base pretreatment in the field: total glucose, Klason lignin and Klason ash, HRGPs and density. The predictive power of these parameters was estimated with linear models and high R^2 values were observed from GH samples in linear models to predict glucose yield after base treatment from field samples (Table 3.5). These five phenotypes in the GH were correlated with each other (Spearman's ρ $p<0.05$), thus measuring any one of these phenotypes might be sufficient to accurately predict glucose yield.

3.3.3.6. *Ash and lignin are negatively correlated with glucose yield across all variables*

Correlations between compositional parameters and glucose yield, regardless of environment, could indicate causal relationships. Low lignin, ash (both forage ash and Klason ash) and high hemicellulose were associated with high glucose yield (Figure 3.14). Since lignin, ash, and hemicellulose covary, it is not clear whether individually or together they are affecting the efficiency of the pretreatment. However, a negative correlation between lignin and glucose yield has been well established [2, 19, 56, 57] so it is likely that lignin is directly interfering with enzymatic processes. The correlation with ash is likely an artifact of the mass-based approach, rather than an indication that ash interferes with glucose yield. Since ash is not sugar but contributes to the mass, as ash is reduced, glucose as a percent of the total mass increases, giving rise to a negative correlation between ash and total glucose and a positive relationship between low ash, and high total glucose and glucose yield. The relationships observed here are not extremely strong, but other factors likely play a role. For example, the type of lignin (S vs G) in switchgrass influences the relationship with glucose yield [58], and more than just lignin is influencing yield in switchgrass mutants, although exactly which parameters was not clear [59]. In addition, simple chemical or physical removal of lignin alone does not significantly increase glucose yields [60].

3.3.3.7. *Relationships among composition and sugar yield change with environment*

Several relationships were different between the two environments (Supplemental Table B.6). Since MLG has a glucose backbone it could contribute to glucose yields and we would expect to see a positive relationship but this relationship was weak, regardless of environment. Another parameter, HRGP, was not correlated with glucose yield after base treatment when the two environments are plotted together (Spearman's $\rho = 0.00$ $p = 0.99$), but if data from each environment was plotted separately, it was negatively correlated with glucose yield (GH $\rho = -0.67$ $p = 0.03$, field $\rho = -0.44$ $p = 0.20$). These differences between the environments could be due to other factors we did not measure, such as the cell wall architecture and this could influence glucose yield.

Cellulose in the field was positively correlated with glucose yield in the field ($\rho = 0.70$ $p = 0.03$), but there was no relationship with glucose yield in the GH ($\rho = -0.02$ $p = 0.96$). Some studies have established positive correlations between cellulose and glucose yield [23], but others [61] observe little relationship between composition and yield. Since glucose yield is derived from the glucose in cellulose microfibrils, we would have expected a strong positive relationship between cellulose and glucose yield; which we observed in the field. Because total glucose and glucose yield were positively correlated with each other in the GH ($\rho = 0.87$ $p<0.01$), our estimates of total glucose and glucose yield are probably correct, but it is possible that there were some issue with the cellulose estimates in the GH samples. We were not able to identify a specific cause.

3.3.3.8. *Model selection supports lignin and ash as most relevant parameters*

One useful approach to predicting bioenergy yield would be to determine the minimum number of parameters that could be measured and accurately predict glucose yield. Since

one would likely not measure both forage and NREL hydrolysis composition, phenotypes as predictors were grouped into two sets: one with forage composition, and the other with NREL hydrolysis composition. The other compositional phenotypes (MLG, HRGPs, density) were included in both sets. Models with all possible combinations of parameters that could predict glucose yield after base pretreatment were sorted by AICc values. In this way, one could choose which parameters to measure and predict glucose yield. For the set including forage compositional parameters, the model with the best fit included ash, hemicellulose and MLG (Table 3.6). Hemicellulose and MLG appeared in almost all of the top models, and there was very little difference in the predictive power of the models: they ranged from 0.85-0.90. For the set including the NREL hydrolysis compositional parameters, the model with the best fit included total glucose, xylose, Klason ash, density and HRGPs (Table 3.7). As with the forage models, all the top models in this set performed equally well and ranged from 0.88-0.92.

Since many of these parameters are correlated with each other (Figure 3.14), it is not surprising that they appear in all of the models. For example, total glucose and Klason ash appear in all of the top NREL hydrolysis composition models, and samples with high total glucose had low Klason ash (ρ= -0.63 p <0.01). Almost as effective as top models in either set is simply measuring ash (R^2 = 0.88 for forage ash and 0.80 for Klason ash). The ash content reduces the mass that could be sugar as discussed in the previous section. The less ash a sample has, the more potential mass could be sugar. Because we were interested in compositional factors that influence glucose yield, not just mass closure relationships (ash), we included the top model without ash in Tables 3.6 and 3.7. It is not surprising that these models without ash for both sets include cellulose and total glucose respectively; glucose yield is derived from the cellulosic portion of the biomass.

3.3.4. A new role for silica?

Silica is the largest component of ash content in rice, often comprising 60% or more of total ash [62]. Recently, several papers have investigated the relationship between silica and other components of the plant cell wall and offer some insight into the correlations we observed between ash and lignin. Silica soil amendments reduces cellulose and phenolic acid levels in rice leaves [63], but at least in *Phragmites australis*, a wetland grass, changes in cellulose and lignin levels in leaf blades, leaf sheaths, and stems were not consistent between various silica treatments [64]. Rice mutants deficient in silica uptake have more lignin compared to wild-type plants, and it was observed in wild-type plants that as silica availability was decreased, lignin increased [65]. In another study of 16 aquatic and wetland plant species [66], the relationship between cellulose and silica was negative for aquatic plant species, but positive for wetland species. In the same study, a slight positive correlation was observed between silica and lignin in aquatic species but the relationship was negative in wetland species. It has been proposed that, like lignin, plants may utilize silica to protect themselves from UV radiation [67]. Silica and lignin could play a role in pest and disease resistance as well [68]. The factors that control lignin and silica levels are not fully understood, and could be responsible for the conflicting relationships reported across species and environments.

3.3.5. Varieties may represent unique combinations of cell wall components

As pointed out earlier, Aswina yielded the highest glucose, but Zhenshan 97B had the highest efficiency as a percent of it's total sugars. So different varieties could be targeted with different strategies; Aswina has alot of sugar, but this is not as easily accessible as the sugar from Zhenshan 97B. From a processing standpoint, one must balance total yield and efficiency to maximize each kilogram processed. LTH did poorly, and also had the highest

lignin. Both Zhenshan 97B and LTH had high ash, raising the possibility that the cell wall architecture of these varieties is unique, and that this drives the variation in bioenergy phenotypes.

3.4. Conclusion

Recent literature has examined environmental trends in cell wall composition, though not often with the same varieties in multiple environments. One such example is [69], in which transgenic switchgrass lines downregulated in a lignin biosynthesis gene (*COMT*) with lower lignin were developed and tested in the GH, and then tested in the field. They observe that while saccharification yields after hot water pretreatment go down in the field, the difference is not as great as some of the differences we observe. However, transgenic approaches with constitutive gene silencing are probably less susceptible to environmental influence of the phenotype than natural variation as we have tested here. Mann *et al.* [18] found few differences in cell wall composition of one variety of switchgrass grown in the GH and field but did observe lower lignin S/G ratio in switchgrass grown in growth chambers compared to GH and field. From a thermochemical processing standpoint, Couhert *et al.* [70] report little correlation between composition and gas yield from biomass and while Rath *et al.* [71] demonstrate it is possible to construct an equation to predict biogas yield in maize, they did not validate these predictions with new samples that were not used to construct the model equation.

It is often assumed by researchers that findings from small-scale greenhouse or growth chamber studies would be relevant once taken to the field. In this study, we demonstrate that multiple factors could influence whether these assumptions are valid. It is clear from the

results here that environment plays a large role in cell wall composition and bioenergy phenotypes. We confirmed that many of the relationships previously observed still held across the same varieties grown in two environments, but the composition and bioenergy phenotypes sometimes changed drastically between environments. Little correlation was observed between assays performed on GH- and field-grown rice plants. Glucose yield did correlate between environments, and furthermore, glucose yield after hot water pretreatment was almost as high with some varieties in the field as with base pretreatment. This information could be used to develop varieties that respond to hot water pretreatment, which is less expensive than dilute base pretreatment. Beyond the correlation of glucose between environments, it is possible that simple relationships between individual components of the cell wall do not capture the complexity and the architecture of the cell wall, much of which is not well understood. Furthermore, while there may be genetic control of cell wall composition and architecture [72], environmentally-triggered expression may play a larger role than anticipated for the phenotypes we measured here, along with such environmentally dependent factors such as soil type. Our results stress the need for more genotype by environment (GxE) studies in the field of biomass improvement for biofuel production.

In general, our results confirm the well-reported relationships between cellulose (and total glucose), lignin, and glucose yield. A negative correlation between HRGP proteins and glucose yield exists, which to our knowledge has not been previously reported and could represent a new direction for improving biomass for bioenergy. However, the low broad-sense heritability, and the differences between leaf and stem indicate that, at least in these rice varieties, breeding to improve these traits will be difficult and environment- and tissue-specific.

While many high throughput systems are being developed to examine the bioenergy potential of biomass, plant composition may not be the best or easiest phenotype to measure. It is likely that cell wall architecture plays a large role in enzymatic efficiency. In the absence of a fast, inexpensive direct measure of bioenergy yield, such as glucose yield, at least in the varieties studied here, it is almost as effective to simply measure ash, and try to minimize ash in breeding efforts. This has the added benefit of increasing bioenergy potential from a thermochemical processing standpoint, as ash negatively affects thermochemical processes [62]. Bulk density is even simpler to measure and correlates with glucose yield, and has an added benefit of minimizing the energy and cost expended on transport of the biomass.

These data represent the first step towards characterizing cell wall and bioenergy traits of certain genotypes in more than one environment and provides baseline numbers for further study. It is clear from the observed variability that more work needs to be done but this data can serve as a foundation to detailed investigation of the relationship between cell wall composition and bioenergy yield, and what environmental factors trigger genetic changes across these parameters.

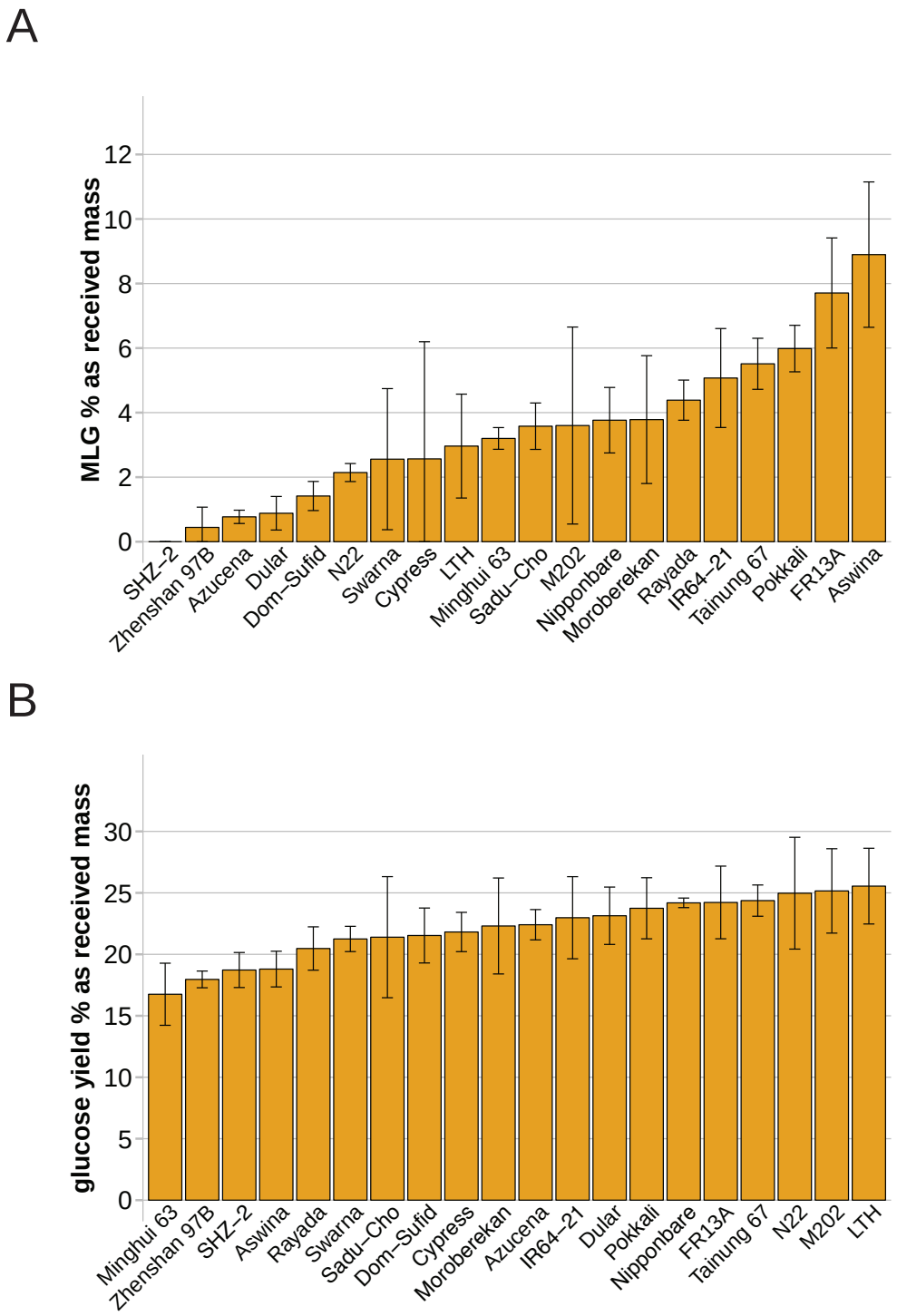


FIGURE 3.1. Bar graph of MLG and glucose yield after dilute base pretreatment. Means for stem tissue from twenty varieties grown in the GH. **A.** MLG assay. **B.** glucose yield assay. Bars ±SD.

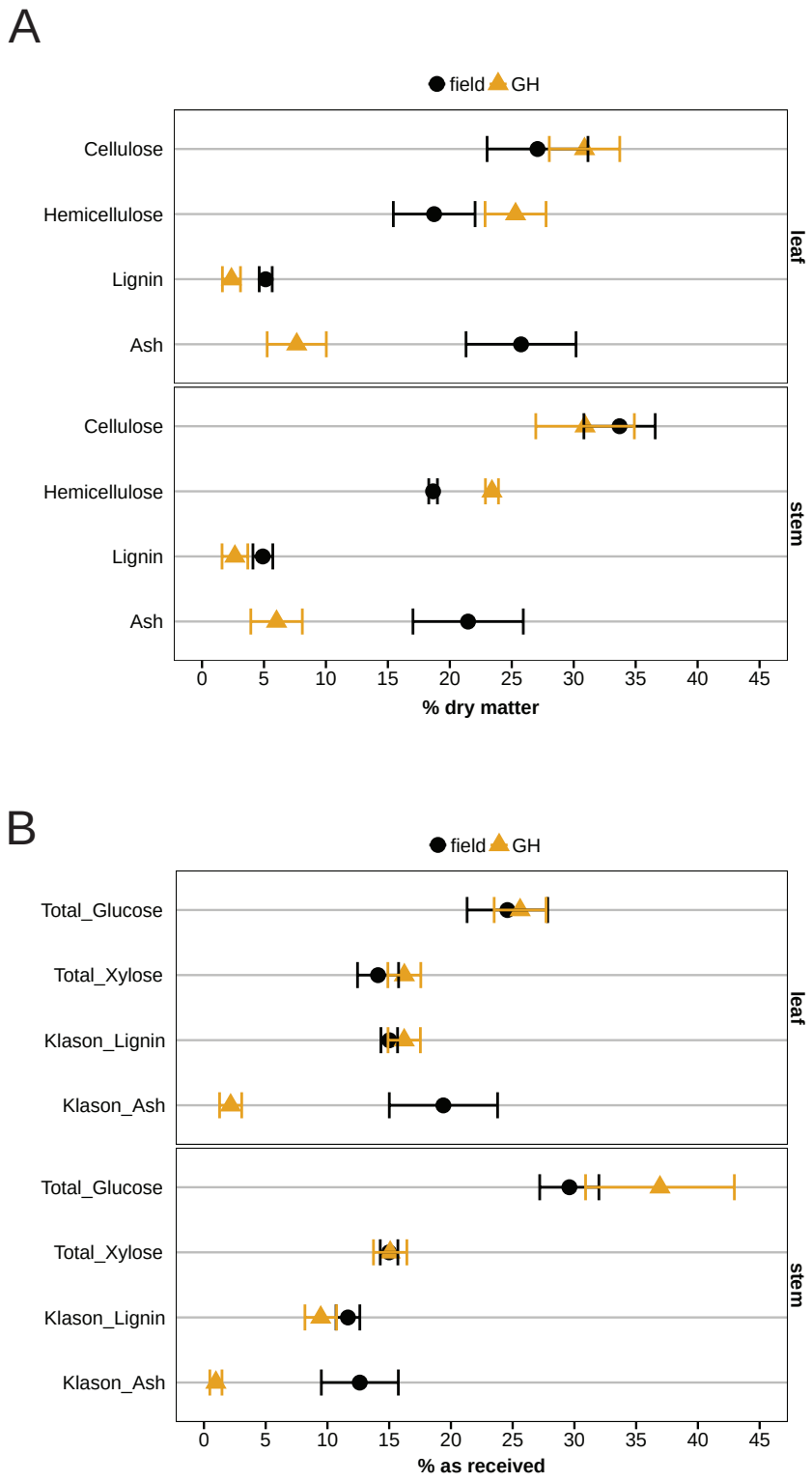


FIGURE 3.2. Dotplots of variation across five varieties in forage (**A**) and NREL hydrolysis (**B**) compositional assays. Means for leaf and stem tissue in either greenhouse (GH) or field environment. Bars ±SD of five varieties.

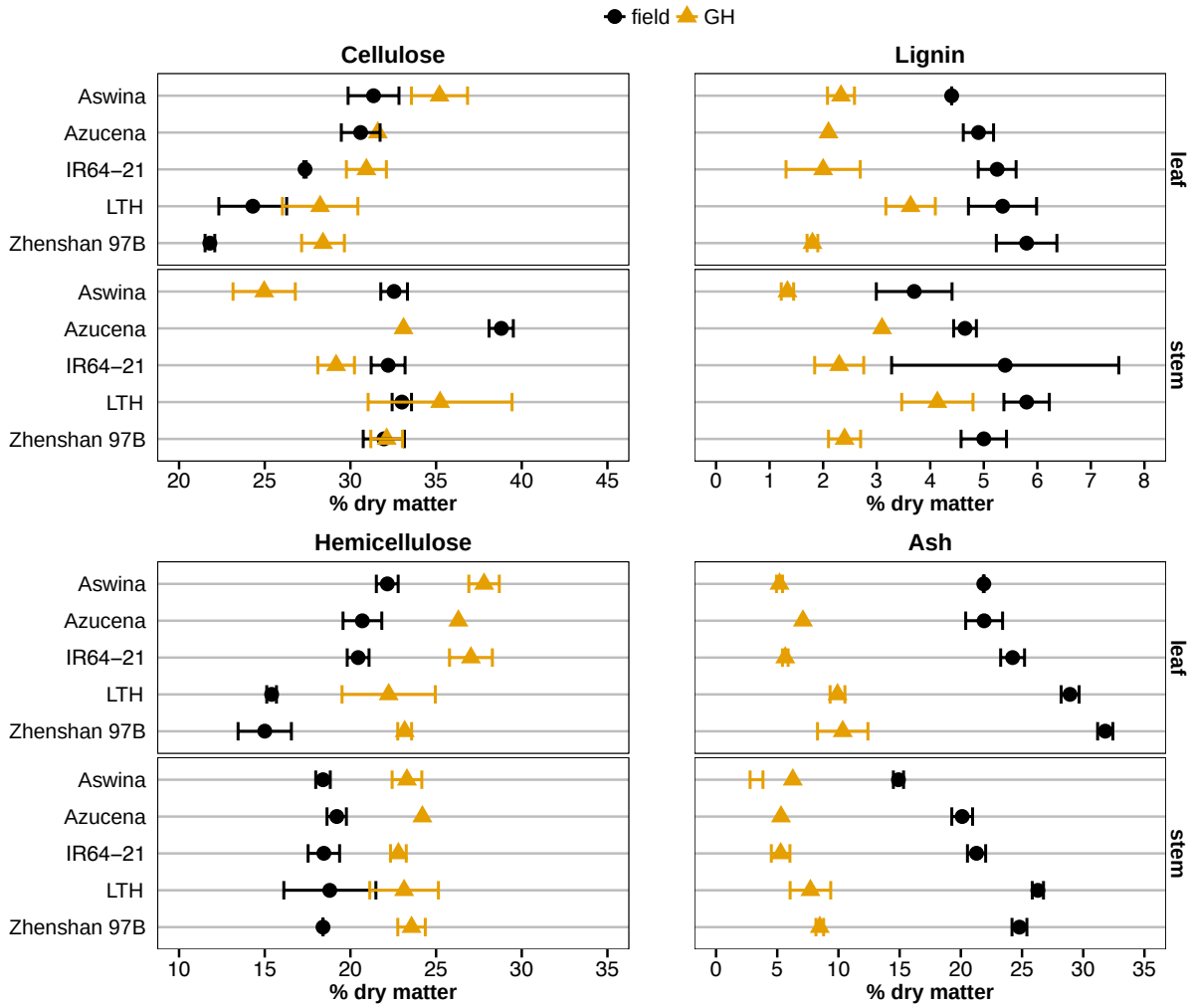


FIGURE 3.3. Dotplots of cellulose, lignin, hemicellulose and ash. Means for leaf and stem tissue from five varieties in either greenhouse (GH) or field environment. Bars ±SD.

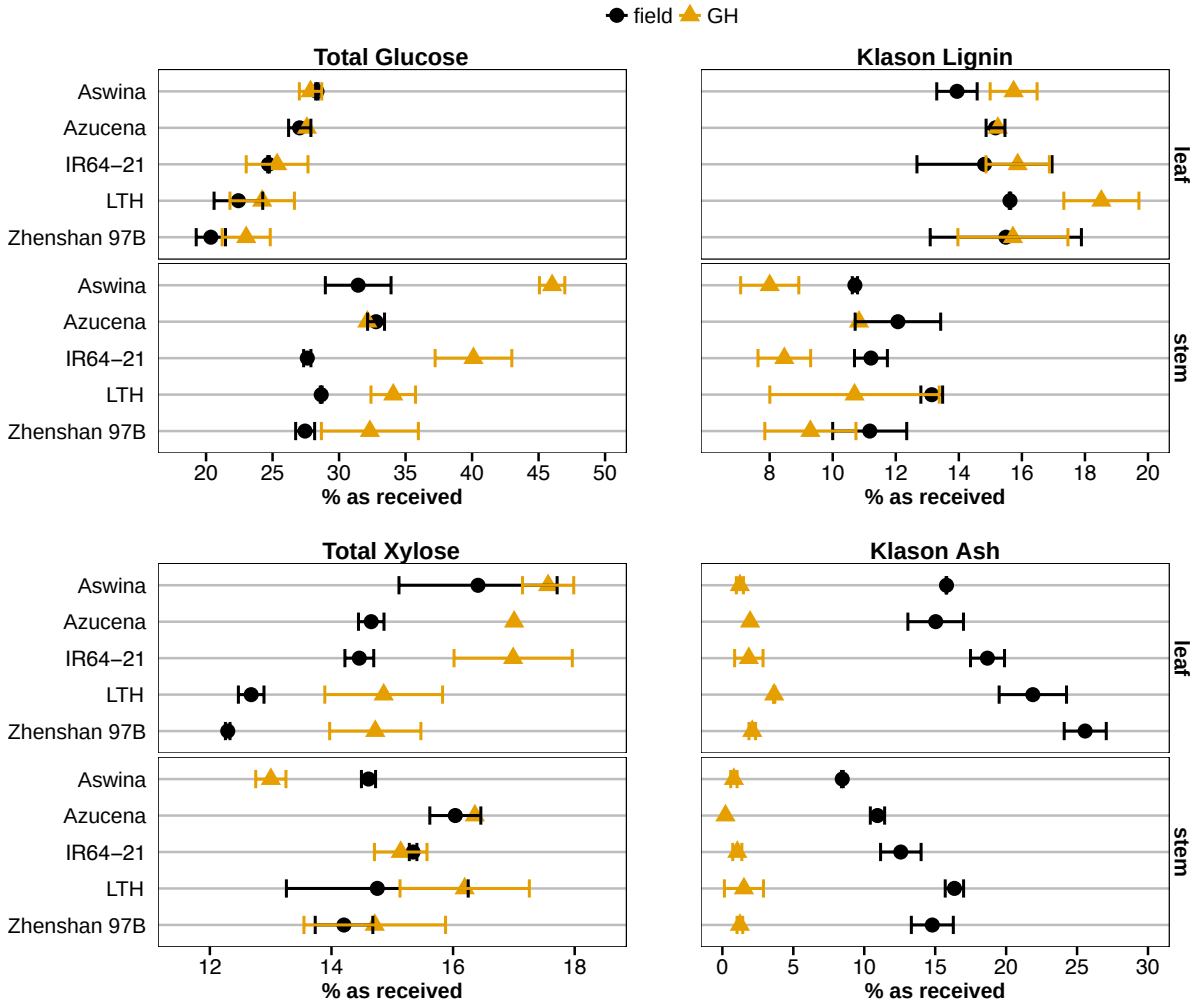


FIGURE 3.4. Dotplots of components of NREL hydrolysis of biomass as total glucose, total xylose, Klason lignin and Klason ash. Means for leaf and stem tissue from five varieties in either greenhouse (GH) or field environment. Bars ±SD.

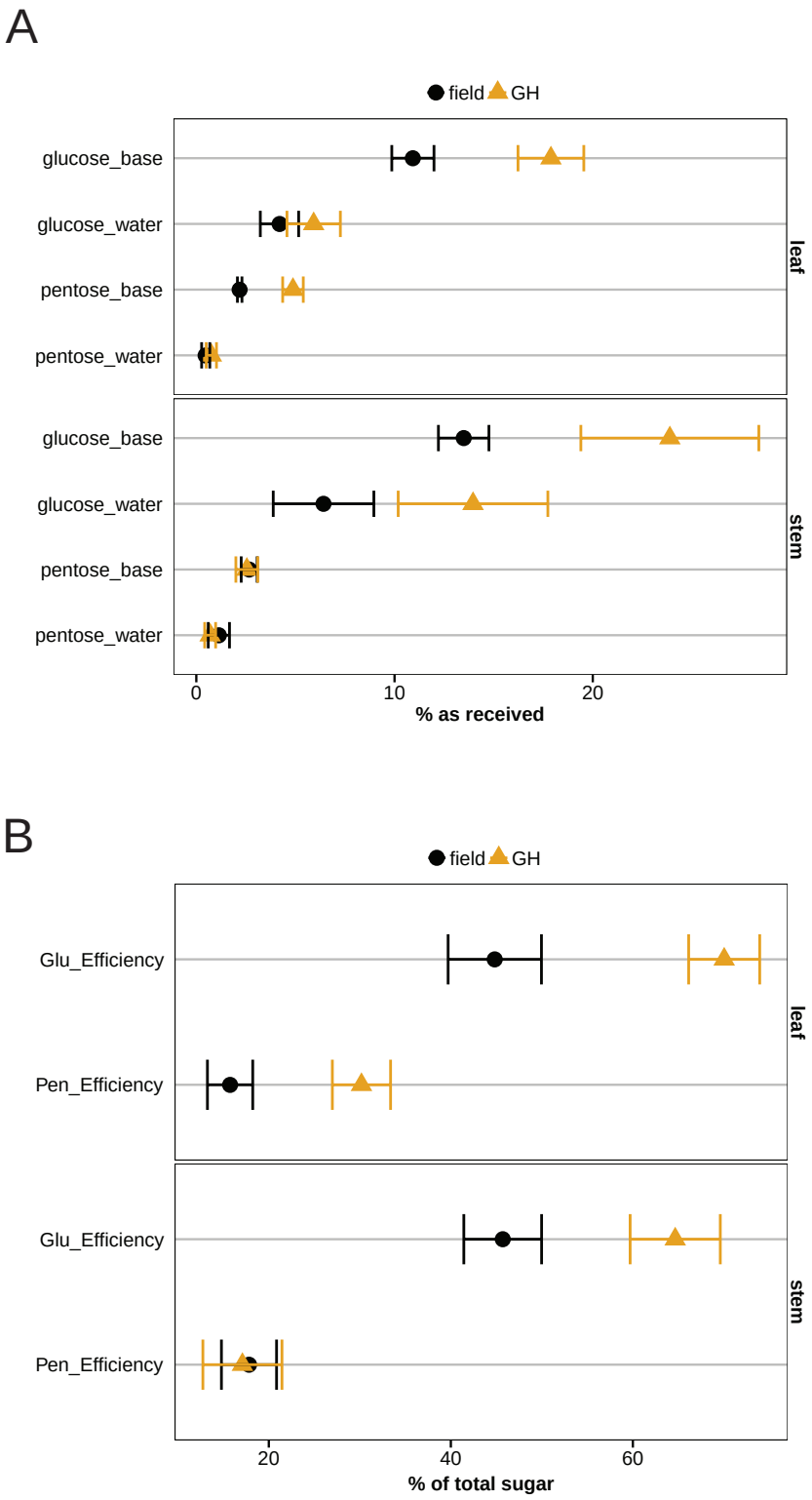


FIGURE 3.5. Dotplots of variation across five varieties for sugar yield (**A**) and efficiency (**B**) phenotypes. Means for leaf and stem tissue in either greenhouse (GH) or field environment. Bars ±SD of five varieties.

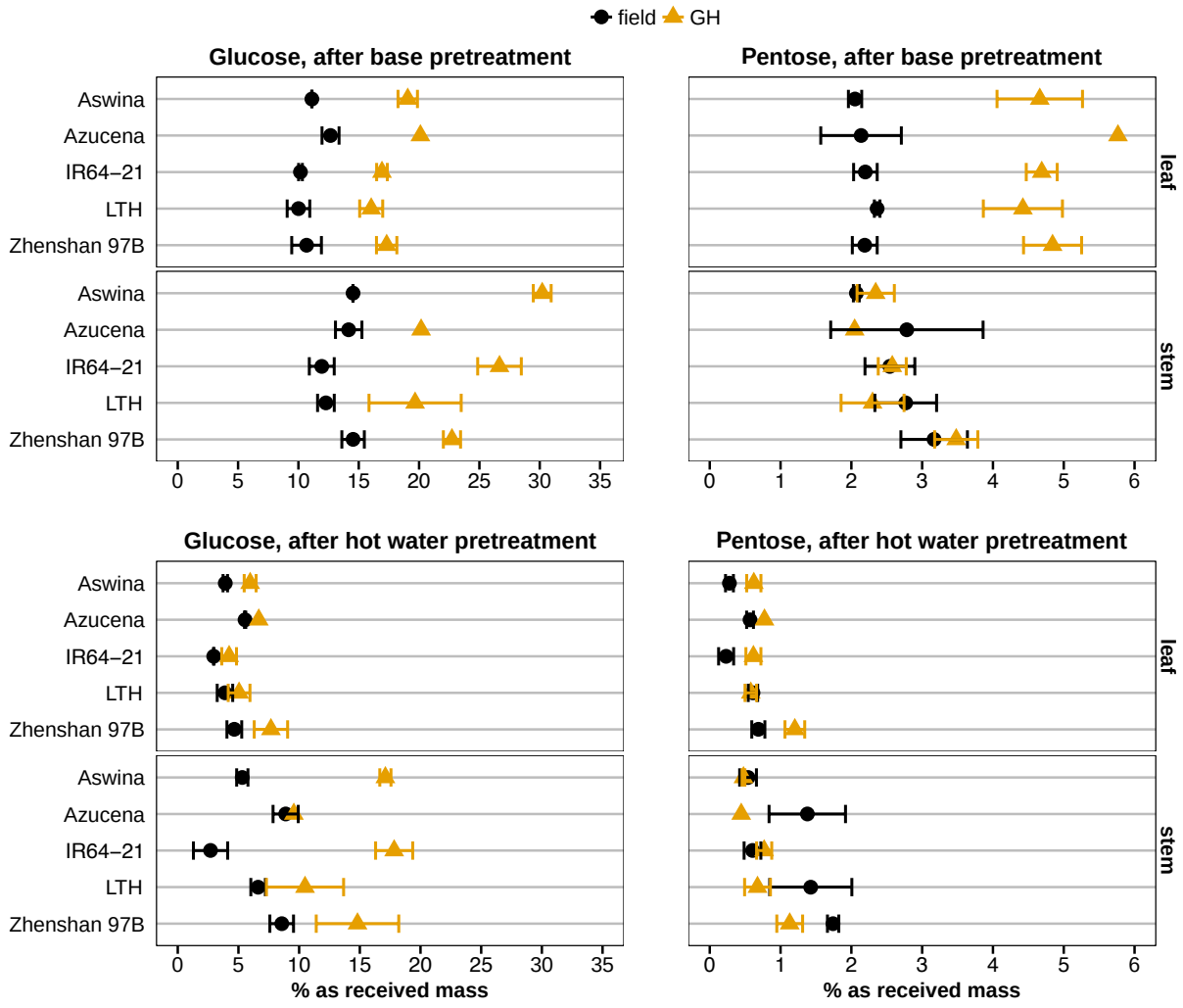


FIGURE 3.6. Dotplots of glucose and pentose yields following either dilute base pretreatment or hot water pretreatment. Means for leaf and stem tissue from five varieties in either greenhouse (GH) or field environment. Bars ±SD.

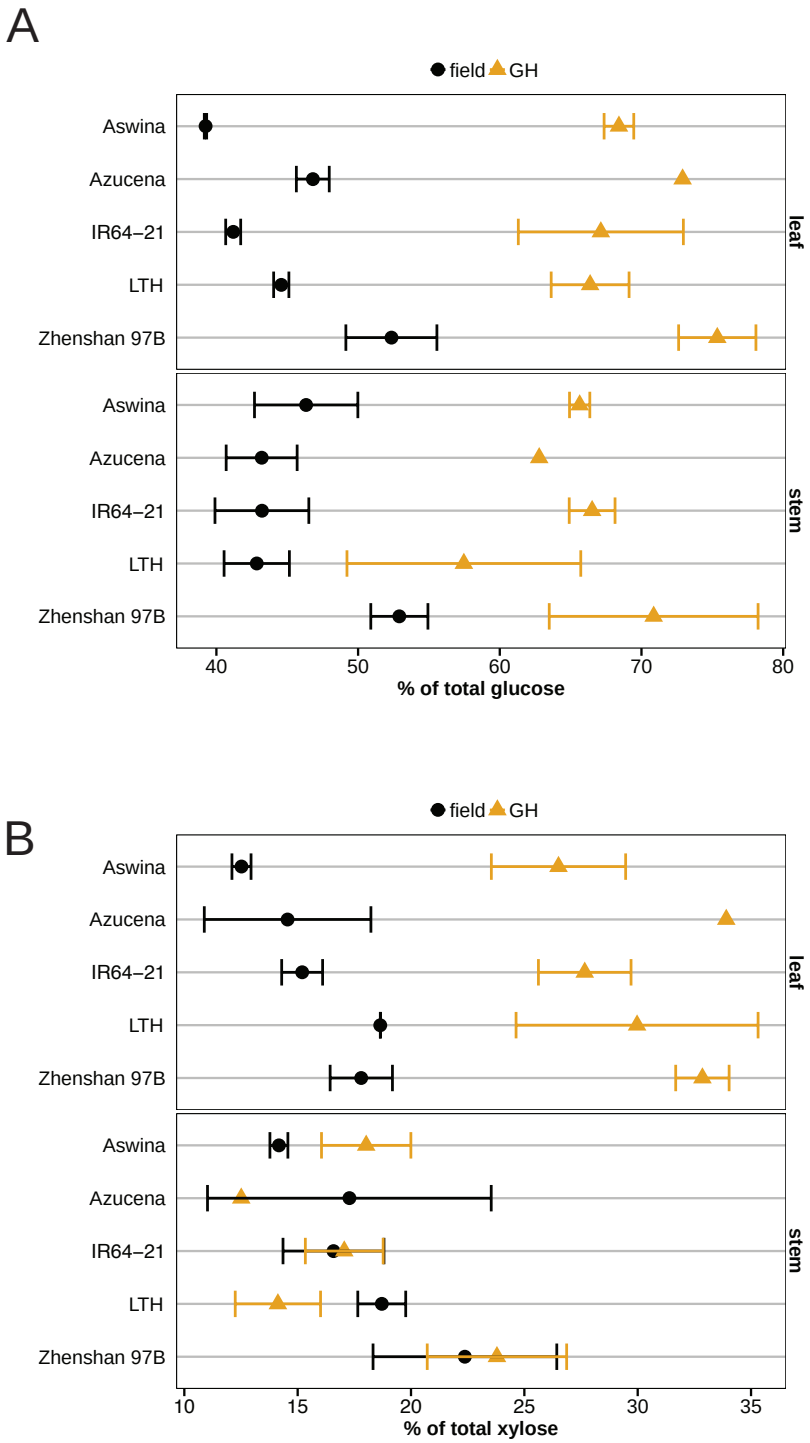


FIGURE 3.7. Dotplots of glucose (**A**) and pentose (**B**) yield efficiency, as percent of total glucose and xylose content. Means for leaf and stem tissue from five varieties in either greenhouse (GH) or field environment. Bars ±SD.

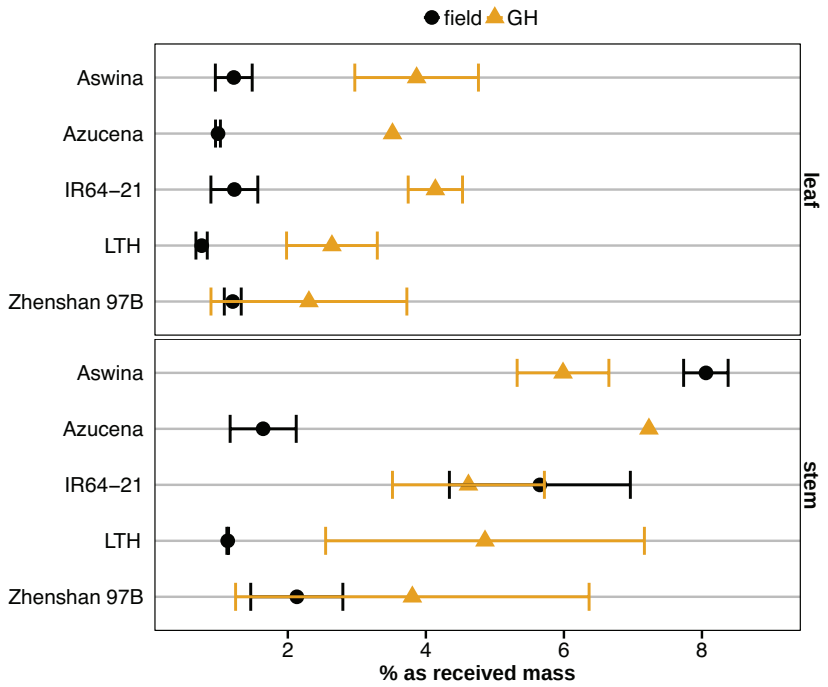


FIGURE 3.8. Dotplots of soluble free glucose measured with no pretreatment. Means for leaf and stem tissue from five varieties in either greenhouse (GH) or field environment. Bars ±SD.

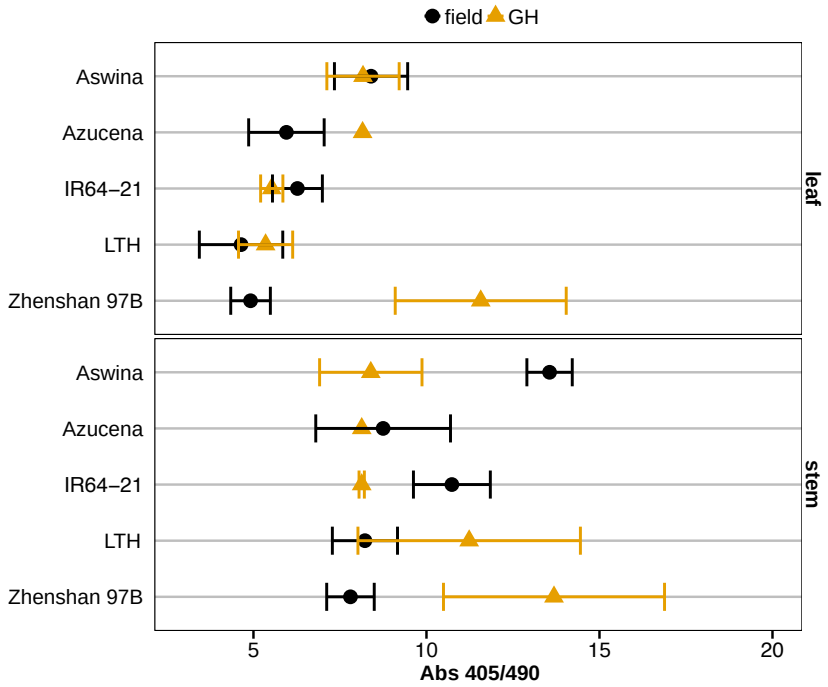


FIGURE 3.9. Dotplots of Mixed Linkage Glucan (MLG). Means for leaf and stem tissue from five varieties in either greenhouse (GH) or field environment. Bars ±SD.

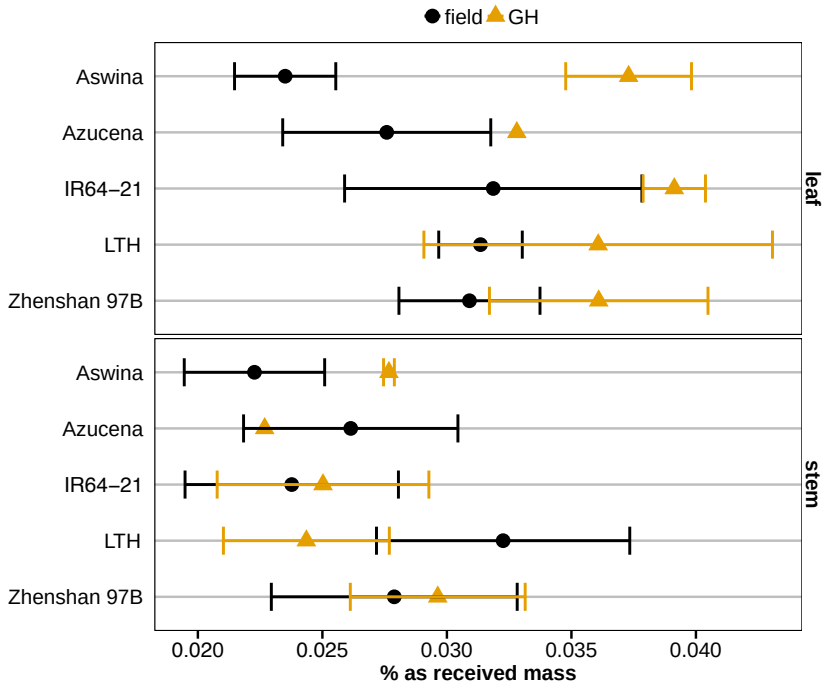


FIGURE 3.10. Dotplots of hydroxyproline rich glycoproteins (HRGP). Means for leaf and stem tissue from five varieties in either greenhouse (GH) or field environment. Bars ±SD.

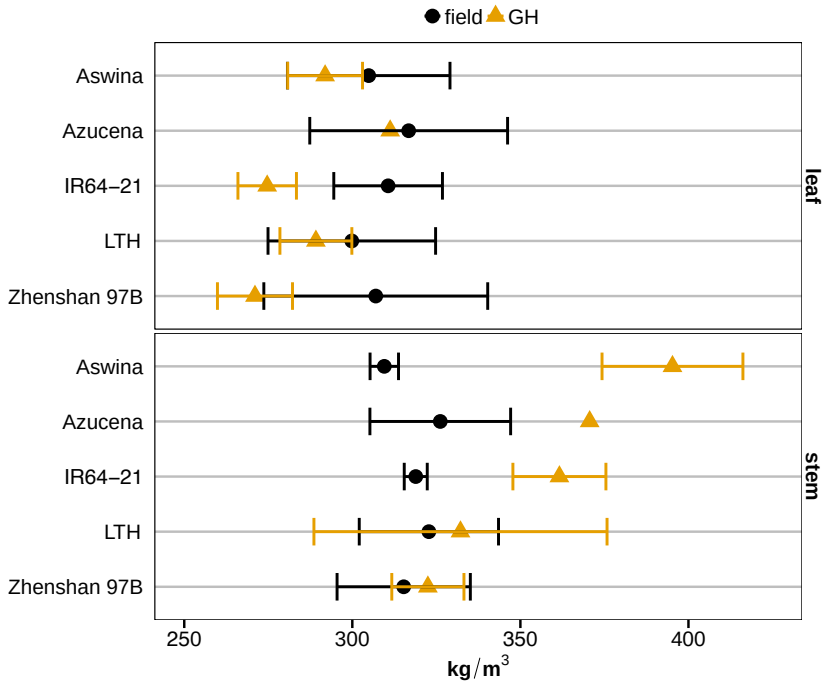


FIGURE 3.11. Dotplots of bulk density of finely ground biomass. Means for leaf and stem tissue from five varieties in either greenhouse (GH) or field environment. Bars ±SD.

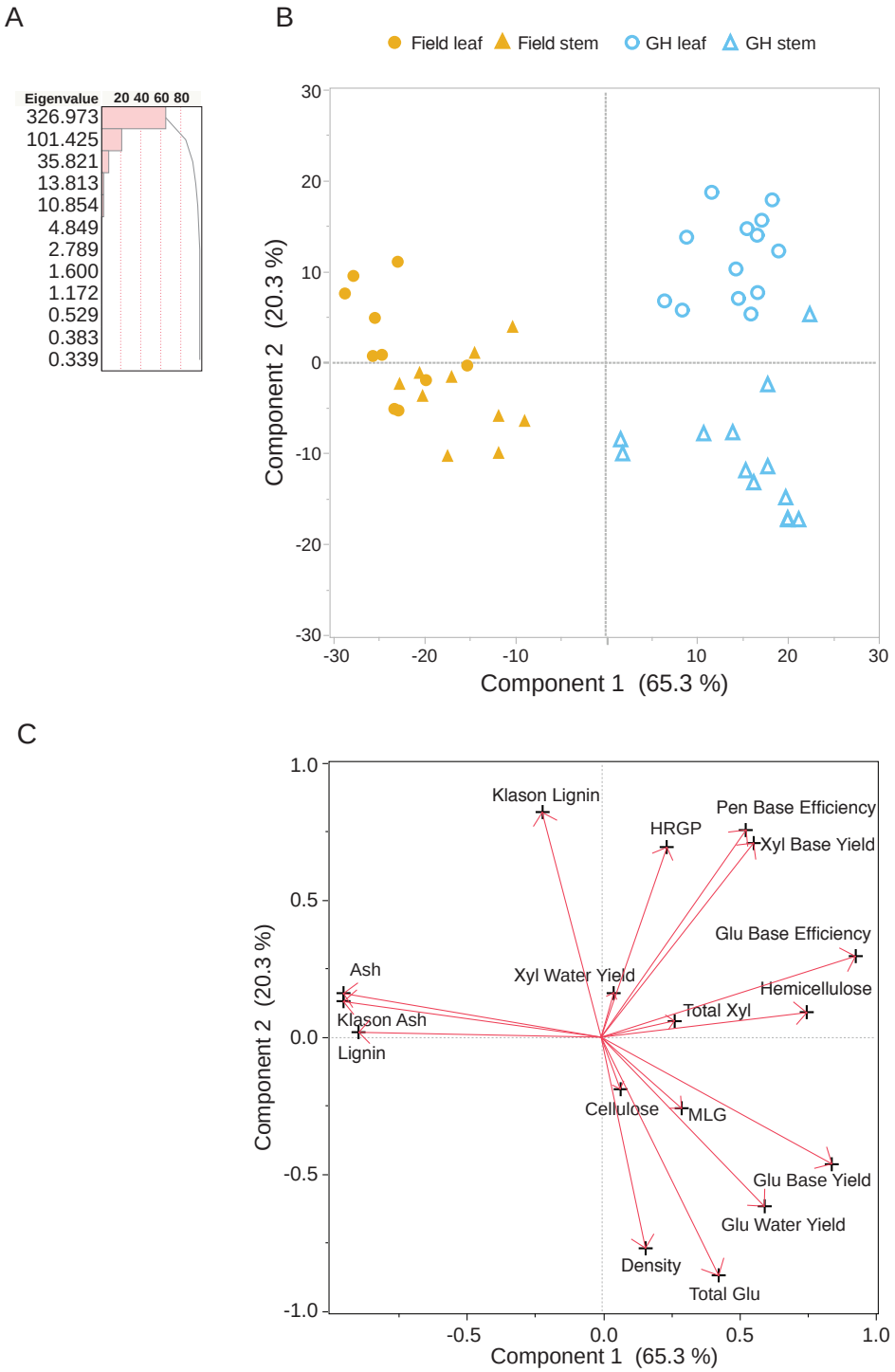


FIGURE 3.12. PCA of covariance matrix of all data. **A.** Eigenvalues calculated for first 12 components. **B.** Loadings plot where solid orange circles denote field leaf samples, solid orange triangles denote field stem samples, open blue circles denote GH leaf samples, and open blue triangles denote GH stem samples. **C.** Biplot with each phenotype labeled.

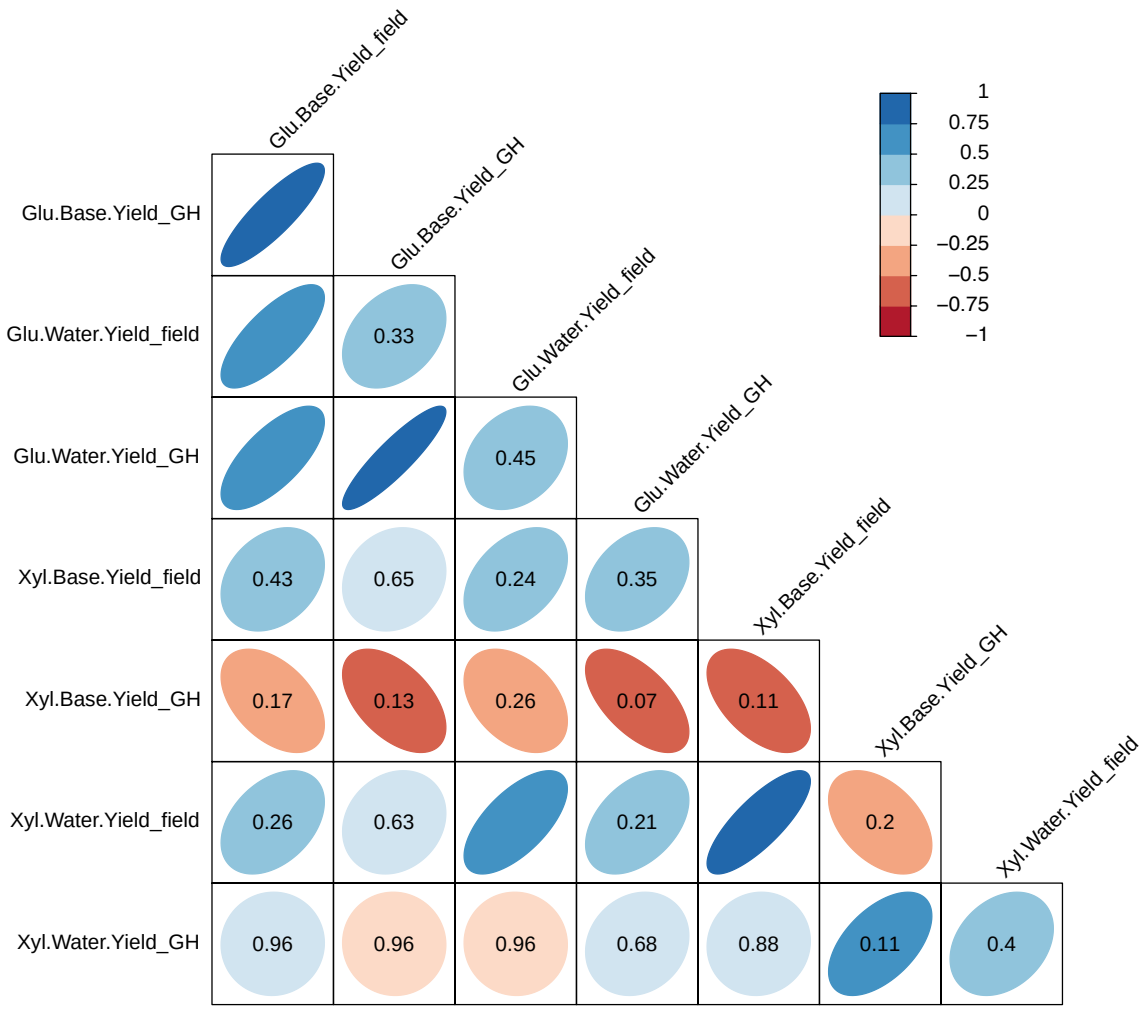


FIGURE 3.13. Matrix of correlations between the field and GH. Colors represent the Spearman's ρ rank correlation coefficients, and p values are reported in the boxes if p>0.05.

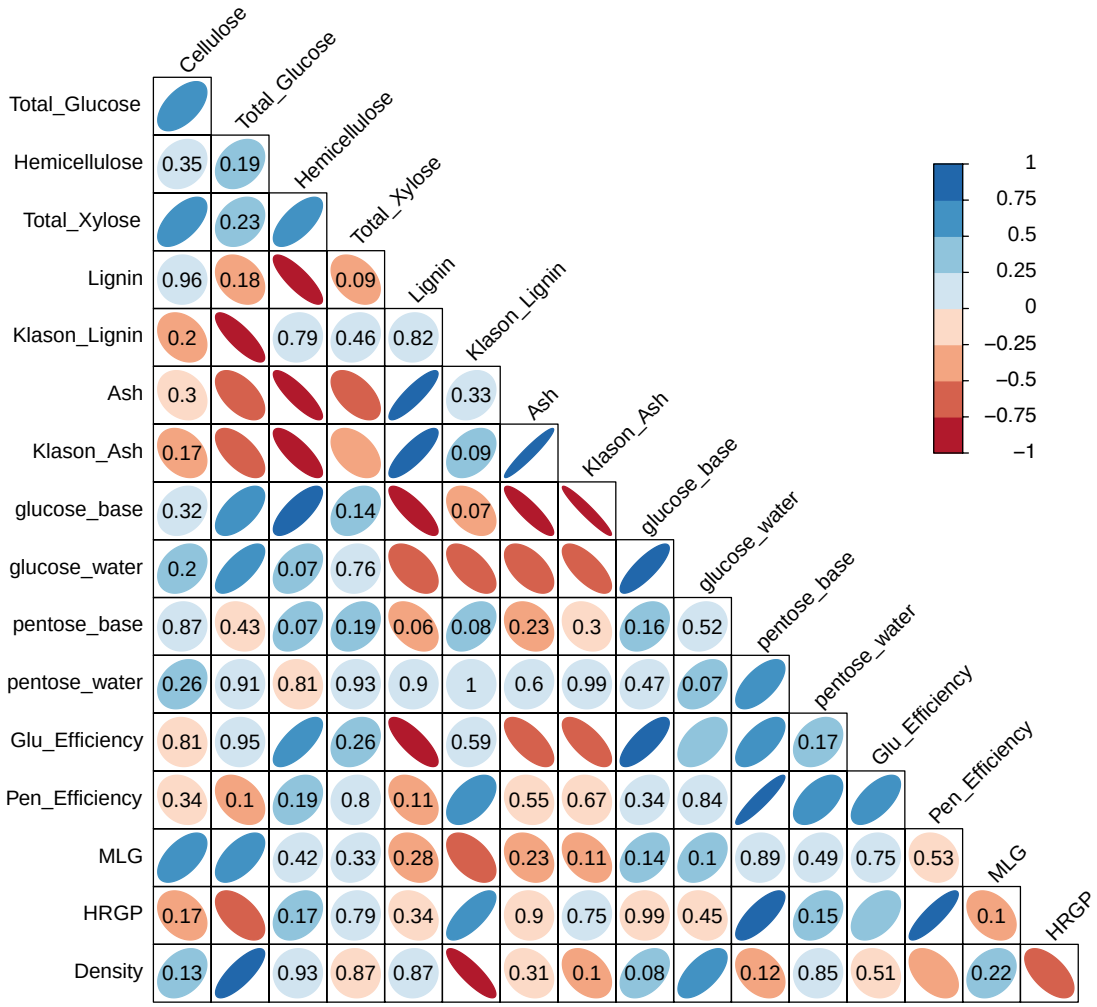


FIGURE 3.14. Matrix of correlations between phenotypes on five varieties in field and GH. Colors represent the Spearman's ρ rank correlation coefficients, and p values are reported in the boxes if p>0.05. The complete correlation matrix is presented in Supplemental Table B.5.

Table 3.1. List of varieties used in this study.

Name used in text	IRGC	Full name	Country of origin	Varietal group	Variety class
Aswina	117281	Aswina	Bangladesh	indica	Landrace
Azucena	117264	Azucena	Philippines	japonica	Landrace
Cypress	117282	Cypress	United States	japonica	Advanced
Dom-Sufid	117265	Dom-Sufid	Iran	japonica	Landrace
Dular	117266	Dular	India	aus	Landrace
FR13A	117267	FR13A	India	aus	Landrace
IR64-21	117268	IR64-21	Philippines	indica	Advanced
LTH	117269	Li-Jiang-Xin-Tuan-Hei-Gu	China	japonica	Advanced
M 202	117270	M 202	United States	japonica	Advanced
Minghui 63	117271	Minghui 63	China	indica	Advanced
Moroberekan	117272	Moroberekan	Guinea	japonica	Landrace
N22	117273	N22	India	aus	Landrace
Nipponbare	117274	Nipponbare	Japan	japonica	Advanced
Pokkali	117275	Pokkali	India	indica	Advanced
Rayada	117283	Rayada	Bangladesh	aus	Landrace
Sadu-Cho	117276	Sadu-Cho	Korea	indica	Landrace
SHZ-2	117277	Shan-Huang Zhan-2	China	indica	Advanced
Swarna	117278	Swarna	India	indica	Advanced
Tainung 67	117279	Tainung 67	Taiwan	japonica	Advanced
Zhenshan 97B	117280	Zhenshan 97B	China	indica	Advanced

Table 3.2. List of phenotypes and methods used in this study.

Type	Phenotypes	Methodology	Purpose	Units
Composition	Cellulose, lignin, hemicellulose, ash	forage (detergent fiber) components	compositional estimates	% dry matter (corrected for moisture)
	HRGP proteins	hydroxyproline extraction, colorimetric assay	structural cell wall proteins	% as received weight
	Mixed linkage glucan (MLG)	base extraction, ELISA assay	potentially important hemicellulose	405/490 abs ratio
	Total glucose, Klason lignin, total xylose, Klason ash	two stage acid hydrolysis (NREL method)	compositional estimates	% as received weight
Bioenergy	Bulk density	mass per volume of ground biomass	important logistical phenotype	kg/m^3
	Saccharification (glucose, pentose)	dilute base pretreatment, enzymatic digestion	bioenergy yield	% as received weight
	Saccharification (glucose, pentose)	hot water pretreatment, enzymatic digestion	less expensive & less hazardous bioenergy yield	% as received weight
	Free glucose	no pretreatment, enzymatic digestion	to correct for variation in free glucose	% as received weight

Table 3.3. Variance of phenotypes due to variety (H^2), environment and residual variance.

Phenotype	Tissue	H^2	% environment var	% residual var
Cellulose	leaf	51%	37%	12%
Cellulose	stem	46%	15%	39%
Lignin	leaf	4%	86%	10%
Lignin	stem	21%	68%	12%
Hemicellulose	leaf	24%	70%	5%
Hemicellulose	stem	0%	91%	9%
Ash	leaf	7%	92%	2%
Ash	stem	9%	89%	2%
Total Glucose	leaf	64%	4%	32%
Total Glucose	stem	18%	62%	20%
Total Xylose	leaf	43%	46%	11%
Total Xylose	stem	49%	0%	51%
Klason Lignin	leaf	18%	25%	57%
Klason Lignin	stem	16%	52%	31%
Klason Ash	leaf	3%	95%	3%
Klason Ash	stem	0%	91%	9%
glucose_base	leaf	6%	92%	2%
glucose_base	stem	5%	87%	7%
glucose_water	leaf	40%	47%	13%
glucose_water	stem	0%	68%	32%
pentose_base	leaf	0%	95%	5%
pentose_base	stem	39%	0%	61%
pentose_water	leaf	42%	39%	19%
pentose_water	stem	35%	26%	39%
Glu Efficiency	leaf	5%	93%	3%
Glu Efficiency	stem	8%	83%	9%
Pen Efficiency	leaf	4%	88%	8%
Pen Efficiency	stem	41%	0%	59%
MLG	leaf	31%	17%	52%
MLG	stem	0%	0%	100%
HRGP	leaf	0%	63%	37%
HRGP	stem	1%	0%	99%
Density	leaf	0%	45%	55%
Density	stem	8%	41%	51%

Table 3.4. Variance of phenotypes due to variety (H^2), separately for each environment and residual variance.

		Field		GH	
Phenotype	Tissue	H^2	% residual var	H^2	% residual var
Cellulose	leaf	91%	9%	74%	26%
Cellulose	stem	91%	9%	71%	29%
Lignin	leaf	52%	48%	70%	30%
Lignin	stem	12%	88%	86%	14%
Hemicellulose	leaf	92%	8%	69%	31%
Hemicellulose	stem	0%	100%	0%	100%
Ash	leaf	95%	5%	88%	12%
Ash	stem	98%	2%	84%	16%
Total Glucose	leaf	87%	13%	36%	64%
Total Glucose	stem	80%	20%	76%	24%
Total Xylose	leaf	88%	12%	71%	29%
Total Xylose	stem	31%	69%	69%	31%
Klason Lignin	leaf	0%	100%	45%	55%
Klason Lignin	stem	44%	56%	15%	85%
Klason Ash	leaf	86%	14%	56%	44%
Klason Ash	stem	92%	8%	0%	100%
glucose_base	leaf	54%	46%	75%	25%
glucose_base	stem	63%	37%	73%	27%
glucose_water	leaf	84%	16%	67%	33%
glucose_water	stem	75%	25%	60%	40%
pentose_base	leaf	0%	100%	0%	100%
pentose_base	stem	7%	93%	61%	39%
pentose_water	leaf	87%	13%	85%	15%
pentose_water	stem	63%	37%	77%	23%
Glu Efficiency	leaf	91%	9%	47%	53%
Glu Efficiency	stem	64%	36%	35%	65%
Pen Efficiency	leaf	54%	46%	25%	75%
Pen Efficiency	stem	17%	83%	78%	22%
MLG	leaf	58%	42%	80%	20%
MLG	stem	72%	28%	43%	57%
HRGP	leaf	29%	71%	0%	100%
HRGP	stem	23%	77%	20%	80%
Density	leaf	0%	100%	51%	49%
Density	stem	0%	100%	48%	52%

Table 3.5. Predictive power of selected phenotypes measured on GH samples to predict glucose yield after base pretreatment in the field.

predictor	R^2
Density	0.68
Klason Lignin	0.66
Total Glucose	0.57
HRGP	0.54
Klason ash	0.49

Table 3.6. Summary of model selection statistics for glucose yield after base treatment from all possible combinations of forage compositional phenotypes. Models are sorted by AICc. Phenotypes also included density, HRGP, and MLG. Models with ΔAICc <10 from the best model are included. In addition, the best model with one parameter and a model with only ash was also included.

Parameters	R^2	AICc	ΔAICc
Ash, Hemicellulose, MLG	0.88	-164.05	0.00
Ash, Hemicellulose, Density, MLG	0.89	-163.60	0.44
Ash, Hemicellulose, MLG, HRGP	0.88	-162.33	1.72
Cellulose, Ash, Hemicellulose, MLG	0.88	-162.28	1.76
Lignin, Ash, Hemicellulose, Density, MLG	0.89	-161.69	2.36
Lignin, Ash, Hemicellulose, MLG	0.88	-161.69	2.36
Cellulose, Ash, Hemicellulose, Density, MLG	0.89	-161.55	2.49
Ash, Density, MLG	0.87	-161.04	3.00
Ash, Hemicellulose, Density, MLG, HRGP	0.89	-161.00	3.05
Cellulose, Lignin, Ash, Hemicellulose, Density, MLG	0.90	-160.92	3.12
Cellulose, Lignin, Ash, Hemicellulose, MLG	0.89	-160.73	3.32
Lignin, Ash, Hemicellulose, MLG, HRGP	0.89	-160.54	3.51
Cellulose, Ash, Hemicellulose, MLG, HRGP	0.89	-160.08	3.96
Cellulose, Lignin, Ash, Hemicellulose, Density	0.89	-160.08	3.96
Cellulose, Lignin, Ash, Hemicellulose, HRGP	0.89	-159.76	4.29
Lignin, Ash, Hemicellulose, Density, MLG, HRGP	0.89	-159.38	4.67
Ash, Density, MLG, HRGP	0.88	-159.34	4.71
Lignin, Ash, Density, MLG	0.88	-159.30	4.74
Cellulose, Lignin, Ash, Hemicellulose, MLG, HRGP	0.89	-159.24	4.80
Cellulose, Lignin, Ash, Hemicellulose	0.88	-158.89	5.15
Cellulose, Lignin, Ash, Hemicellulose, Density, HRGP	0.89	-158.87	5.18
Cellulose, Ash, Hemicellulose, Density, MLG, HRGP	0.89	-158.67	5.38
Cellulose, Ash, Density, MLG	0.87	-158.48	5.56
Ash, MLG, HRGP	0.87	-158.35	5.70
Lignin, Ash, Density, MLG, HRGP	0.88	-158.34	5.71
Cellulose, Lignin, Ash, Hemicellulose, Density, MLG, HRGP	0.90	-158.27	5.78
Cellulose, Ash, Hemicellulose	0.87	-158.14	5.91
Cellulose, Ash, Hemicellulose, Density	0.87	-157.81	6.24
Cellulose, Ash, Hemicellulose, HRGP	0.87	-157.61	6.43
Ash, Hemicellulose, HRGP	0.86	-157.18	6.87
Lignin, Ash, Hemicellulose, HRGP	0.87	-157.15	6.90
Lignin, Ash, MLG, HRGP	0.87	-157.04	7.01
Cellulose, Ash, Density, MLG, HRGP	0.88	-156.69	7.35
Cellulose, Lignin, Ash, Density, MLG	0.88	-156.52	7.53
Lignin, Ash, Density, HRGP	0.87	-156.35	7.69
Cellulose, Ash, MLG, HRGP	0.87	-156.23	7.82
Ash, MLG	0.85	-156.14	7.90
Lignin, Ash, HRGP	0.86	-156.03	8.02
Cellulose, Ash, Hemicellulose, Density, HRGP	0.88	-155.94	8.11
Ash, HRGP	0.85	-155.90	8.15
Lignin, Ash, Hemicellulose, Density, HRGP	0.87	-155.70	8.35
Ash, Density, HRGP	0.86	-155.66	8.39
Cellulose, Lignin, Ash, Density, MLG, HRGP	0.88	-155.43	8.62
Ash, Hemicellulose, Density, HRGP	0.87	-155.39	8.65
Cellulose, Lignin, Ash, Density, HRGP	0.87	-155.20	8.85
Ash, Hemicellulose, Density	0.86	-154.88	9.16
Ash, Hemicellulose	0.85	-154.42	9.63
Cellulose, Lignin, Ash, MLG, HRGP	0.87	-154.34	9.71
Cellulose, Ash, MLG	0.85	-154.19	9.86
Ash	0.82	-148.30	15.75
Cellulose, Lignin, Density	0.76	-131.681	32.36

Table 3.7. Summary of model selection statistics for glucose yield after base treatment from all possible combinations of NREL hydrolysis compositional phenotypes. Models are sorted by AICc. Phenotypes also included density, HRGP, and MLG. Models with ΔAICc <10 from the best model are included. In addition, the best model with one parameter and a model with only Klason ash was also included.

Parameters	R^2	AICc	ΔAICc
Total Glucose, Total Xylose, Klason Ash, Density, HRGP	0.92	-175.80	0.00
Total Glucose, Total Xylose, Klason Ash, HRGP	0.91	-174.71	1.09
Total Glucose, Total Xylose, Klason Ash, Density, MLG, HRGP	0.92	-172.95	2.86
Total Glucose, Total Xylose, Klason Lignin, Klason Ash, Density, HRGP	0.92	-172.88	2.92
Total Glucose, Total Xylose, Klason Lignin, Klason Ash, HRGP	0.91	-172.43	3.37
Total Glucose, Total Xylose, Klason Ash, MLG, HRGP	0.91	-172.28	3.52
Total Glucose, Total Xylose, Klason Ash, Density	0.90	-171.16	4.64
Total Glucose, Total Xylose, Klason Lignin, Klason Ash, MLG, HRGP	0.91	-170.49	5.31
Total Glucose, Total Xylose, Klason Ash	0.90	-169.95	5.86
Total Glucose, Total Xylose, Klason Lignin, Klason Ash, Density, MLG, HRGP	0.92	-169.84	5.96
Total Glucose, Klason Ash, HRGP	0.90	-169.44	6.36
Total Glucose, Total Xylose, Klason Lignin, Klason Ash	0.90	-169.02	6.78
Total Glucose, Total Xylose, Klason Ash, Density, MLG	0.91	-168.72	7.08
Total Glucose, Total Xylose, Klason Lignin, Klason Ash, Density	0.91	-168.66	7.14
Total Glucose, Klason Ash	0.88	-167.40	8.40
Total Glucose, Total Xylose, Klason Ash, MLG	0.90	-167.38	8.42
Total Glucose, Klason Lignin, Klason Ash, HRGP	0.90	-167.07	8.73
Total Glucose, Klason Ash, Density, HRGP	0.90	-166.93	8.87
Total Glucose, Klason Ash, MLG, HRGP	0.90	-166.80	9.01
Total Glucose, Total Xylose, Klason Lignin, Klason Ash, MLG	0.90	-166.36	9.44
Total Glucose, Total Xylose, Klason Lignin, Klason Ash, Density, MLG	0.91	-165.83	9.97
Klason Ash	0.80	-145.44	30.36
Total Glucose, MLG, HRGP	0.65	-114.34	61.46

TABLE 3.8. Mean ±SD of assays done on five selected varieties grown in GH and field. Pairwise F test p values of contrasts between environments: * p<0.05, ** p<0.01. F tests and SD are not shown for Azucena in the GH since n=1. If SD<0.05 it was rounded to 0 for this table. This is also attached to this dissertation at the Colorado State University Libraries' Digital Repository as an excel file.

	Aswina				Azucena				IR64-21				LTH				Zhenshan 97B			
	leaf		stem		leaf		stem		leaf		stem		leaf		stem		leaf		stem	
	GH	Field	GH	Field	GH	Field	GH	Field	GH	Field	GH	Field	GH	Field	GH	Field	GH	Field	GH	Field
Cellulose % DM	35.20 ± 1.6 *	31.35 ± 1.5 *	24.97 ± 1.8 **	32.55 ± 0.8 **	31.60 ± na	30.60 ± 1.1	33.10 ± na	38.80 ± 0.7	30.93 ± 1.2 *	27.35 ± 0.1 *	29.17 ± 1.1	32.20 ± 1.0	28.23 ± 2.2 *	24.30 ± 2.0 *	35.23 ± 4.2	33.00 ± 0.6	28.40 ± 1.2 **	21.80 ± 0.3 **	32.12 ± 0.9	31.95 ± 1.2
Hemicellulose % DM	27.80 ± 0.9 **	22.15 ± 0.6 **	23.30 ± 0.9 **	18.40 ± 0.4 **	26.30 ± na	20.70 ± 1.1	24.20 ± na	19.20 ± 0.6	27.03 ± 1.2 **	20.45 ± 0.6 **	22.80 ± 0.5 **	18.45 ± 0.9 **	22.23 ± 2.7 **	15.40 ± 0.3 **	23.13 ± 2.0 **	18.80 ± 2.7 **	23.17 ± 0.4 **	15.00 ± 1.6 **	23.57 ± 0.8 **	18.40 ± 0.0 **
Lignin % DM	2.33 ± 0.2 **	4.40 ± 0.0 **	1.33 ± 0.1 **	3.70 ± 0.7 **	2.10 ± na	4.90 ± 0.3	3.10 ± na	4.65 ± 0.2	2.00 ± 0.7 **	5.25 ± 0.3 **	2.30 ± 0.5 **	5.40 ± 2.1 **	3.63 ± 0.5 **	5.35 ± 0.6 **	4.13 ± 0.7 *	5.80 ± 0.4 *	1.80 ± 0.1 **	5.80 ± 0.6 **	2.40 ± 0.3 **	5.00 ± 0.4 **
Ash % DM	5.19 ± 0.2 **	21.88 ± 0.0 **	3.31 ± 0.5 **	14.90 ± 0.4 **	7.10 ± na	21.91 ± 1.5	5.31 ± na	20.11 ± 0.8	5.66 ± 0.2 **	24.23 ± 1.0 **	5.28 ± 0.8 **	21.29 ± 0.7 **	9.92 ± 0.6 **	28.93 ± 0.7 **	7.71 ± 1.7 **	26.30 ± 0.5 **	10.35 ± 2.1 **	31.80 ± 0.6 **	8.47 ± 0.3 **	24.79 ± 0.6 **
Total Glucose % AR	27.86 ± 0.8	28.33 ± 0.1	46.02 ± 0.9 **	31.44 ± 2.5 **	27.58 ± na	27.04 ± 0.8	32.13 ± na	32.78 ± 0.6	25.34 ± 2.3	24.70 ± 0.1	40.10 ± 2.9 **	27.61 ± 0.3 **	24.22 ± 2.4	22.43 ± 1.8	34.07 ± 1.7 *	28.66 ± 0.1 *	23.02 ± 1.8	20.36 ± 1.1	32.32 ± 3.6 *	27.45 ± 0.7 *
Total Xylose % AR	17.56 ± 0.4	16.41 ± 1.3	13.00 ± 0.2 *	14.61 ± 0.1 *	17.00 ± na	14.65 ± 0.2	16.36 ± na	16.04 ± 0.4	16.99 ± 1.0 **	14.46 ± 0.2 **	15.14 ± 0.4	15.34 ± 0.1	14.86 ± 1.0 *	12.68 ± 0.2 *	16.19 ± 1.1	14.75 ± 1.5	14.72 ± 0.8 **	12.29 ± 0.0 **	14.71 ± 1.2	14.21 ± 0.5
Klason Lignin % AR	15.74 ± 0.7	13.94 ± 0.6	8.00 ± 0.9	10.70 ± 0.1	15.24 ± na	15.16 ± 0.3	10.83 ± na	12.07 ± 1.4	15.87 ± 1.0	14.81 ± 2.1	8.46 ± 0.8	11.21 ± 0.5	18.52 ± 1.2 *	15.62 ± 0.0 *	10.69 ± 2.7	13.14 ± 0.3	15.72 ± 1.7	15.49 ± 2.4	9.29 ± 1.4	11.17 ± 1.2
Klason Ash % AR	1.23 ± 0.2 **	15.79 ± 0.0 **	0.81 ± 0.2 **	8.46 ± 0.1 **	1.95 ± na	15.04 ± 2.0	0.22 ± na	10.93 ± 0.5	1.86 ± 1.0 **	18.69 ± 1.2 **	1.05 ± 0.3 **	12.58 ± 1.4 **	3.65 ± 0.1 **	21.88 ± 2.4 **	1.52 ± 1.4 **	16.35 ± 0.7 **	2.11 ± 0.2 **	25.58 ± 1.5 **	1.23 ± 0.2 **	14.80 ± 1.5 **
MLG abs 405/490	8.17 ± 1.0	8.40 ± 1.1	8.39 ± 1.5 *	13.56 ± 0.7 *	8.15 ± na	5.95 ± 1.1	8.13 ± na	8.75 ± 1.9	5.53 ± 0.3	6.27 ± 0.7	8.13 ± 0.1	10.73 ± 1.1	5.35 ± 0.8	4.64 ± 1.2	11.23 ± 3.2	8.22 ± 0.9	11.57 ± 2.5 **	4.92 ± 0.6 **	13.69 ± 3.2 *	7.80 ± 0.7 *
HRGP % AR	0.04 ± 0.0 **	0.02 ± 0.0 **	0.03 ± 0.0	0.02 ± 0.0	0.03 ± na	0.03 ± 0.0	0.02 ± na	0.03 ± 0.0	0.04 ± 0.0	0.03 ± 0.0	0.03 ± 0.0	0.02 ± 0.0	0.04 ± 0.0	0.03 ± 0.0	0.02 ± 0.0 *	0.03 ± 0.0 *	0.04 ± 0.0	0.03 ± 0.0	0.03 ± 0.0	0.03 ± 0.0
Density kg/m^3	291.91 ± 11.1	304.88 ± 24.2	395.25 ± 21.0 **	309.51 ± 4.2 **	311.26 ± na	316.77 ± 29.4	370.61 ± na	326.17 ± 20.9	274.67 ± 8.7 *	310.65 ± 16.2 *	361.61 ± 13.8	318.87 ± 3.4	289.16 ± 10.7	299.85 ± 24.9	332.18 ± 43.6	322.78 ± 20.7	271.04 ± 11.2 *	306.98 ± 33.3 *	322.48 ± 10.7	315.29 ± 19.8
glucose_base % AR	19.06 ± 0.8 **	11.12 ± 0.0 **	30.21 ± 0.7 **	14.52 ± 0.0 **	20.11 ± na	12.66 ± 0.7	20.17 ± na	14.17 ± 1.1	16.92 ± 0.4 **	10.17 ± 0.2 **	26.67 ± 1.8 **	11.93 ± 1.0 **	16.03 ± 0.9 **	10.00 ± 0.9 **	19.67 ± 3.8 **	12.28 ± 0.7 **	17.31 ± 0.8 **	10.68 ± 1.2 **	22.72 ± 0.7 **	14.53 ± 0.9 **
glucose_water % AR	5.96 ± 0.5 **	3.92 ± 0.2 **	17.11 ± 0.5 **	5.32 ± 0.5 **	6.67 ± na	5.55 ± 0.1	9.56 ± na	8.89 ± 1.0	4.23 ± 0.6 *	2.96 ± 0.0 *	17.83 ± 1.5 **	2.70 ± 1.4 **	5.05 ± 0.9	3.89 ± 0.6	10.49 ± 3.2	6.62 ± 0.6	7.68 ± 1.4 **	4.66 ± 0.6 **	14.81 ± 3.4 *	8.56 ± 1.0 *
pentose_base % AR	4.66 ± 0.6 **	2.05 ± 0.1 **	2.34 ± 0.3	2.07 ± 0.0	5.77 ± na	2.14 ± 0.6	2.05 ± na	2.78 ± 1.1	4.69 ± 0.2 **	2.20 ± 0.2 **	2.58 ± 0.2	2.54 ± 0.3	4.42 ± 0.6 **	2.36 ± 0.0 **	2.30 ± 0.4	2.77 ± 0.4	4.84 ± 0.4 **	2.19 ± 0.2 **	3.48 ± 0.3	3.17 ± 0.5
pentose_water % AR	0.62 ± 0.1 **	0.28 ± 0.1 **	0.48 ± 0.0	0.54 ± 0.1	0.77 ± na	0.57 ± 0.1	0.44 ± na	1.38 ± 0.5	0.62 ± 0.1 **	0.23 ± 0.1 **	0.77 ± 0.1	0.60 ± 0.1	0.58 ± 0.1	0.61 ± 0.1	0.67 ± 0.2 *	1.43 ± 0.6 *	1.20 ± 0.1 **	0.69 ± 0.1 **	1.13 ± 0.2 *	1.74 ± 0.1 *
Glu Efficiency % Total Glucose	68.41 ± 1.1 **	39.23 ± 0.1 **	65.64 ± 0.7 **	46.34 ± 3.6 **	72.90 ± na	46.80 ± 1.2	62.78 ± na	43.19 ± 2.5	67.13 ± 5.8 **	41.19 ± 0.5 **	66.52 ± 1.6 **	43.21 ± 3.3 **	66.38 ± 2.8 **	44.58 ± 0.5 **	57.47 ± 8.2 **	42.85 ± 2.3 **	75.35 ± 2.7 **	52.35 ± 3.2 **	70.86 ± 7.4 **	52.91 ± 2.0 **
Pen Efficiency % Total Xylose	26.51 ± 3.0 **	12.52 ± 0.4 **	18.02 ± 2.0	14.17 ± 0.4	33.91 ± na	14.56 ± 3.7	12.51 ± na	17.28 ± 6.3	27.66 ± 2.0 **	15.20 ± 0.9 **	17.06 ± 1.7	16.58 ± 2.2	29.97 ± 5.3 **	18.65 ± 0.0 **	14.13 ± 1.9	18.71 ± 1.1	32.86 ± 1.2 **	17.81 ± 1.4 **	23.79 ± 3.1	22.38 ± 4.0

REFERENCES

[1] National Research Council, 2011. *Renewable Fuel Standard: Potential Economic and Environmental Effects of U.S. Biofuel Policy.* Washington: The National Academies Press

[2] Torres, A. F., Weijde, T. v. d., Dolstra, O., Visser, R. G. F., and Trindade, L. M., 2013. Effect of Maize Biomass Composition on the Optimization of Dilute-Acid Pretreatments and Enzymatic Saccharification. *BioEnergy Research*, 6, 3:1038–1051

[3] Lindedam, J., Andersen, S., DeMartini, J., Bruun, S., Jø rgensen, H., Felby, C., Magid, J., Yang, B., and Wyman, C., 2012. Cultivar variation and selection potential relevant to the production of cellulosic ethanol from wheat straw. *Biomass and Bioenergy*, 37:221–228

[4] Carpita, N. C., 2012. Progress in the biological synthesis of the plant cell wall: new ideas for improving biomass for bioenergy. *Current Opinion in Biotechnology*, 23, 3:330–337

[5] Nookaraju, A., Pandey, S. K., Bae, H.-J., and Joshi, C. P., 2013. Designing Cell Walls for Improved Bioenergy Production. *Molecular Plant*, 6, 1:8–10

[6] Yang, F., Mitra, P., Zhang, L., Prak, L., Verhertbruggen, Y., Kim, J.-S., Sun, L., Zheng, K., Tang, K., Auer, M., Scheller, H. V., and Loqué, D., 2012. Engineering secondary cell wall deposition in plants. *Plant Biotechnology Journal*, p. doi: 10.1111/pbi.12016

[7] Lv, S., Yu, Q., Zhuang, X., Yuan, Z., Wang, W., Wang, Q., Qi, W., and Tan, X., 2013. The Influence of Hemicellulose and Lignin Removal on the Enzymatic Digestibility from Sugarcane Bagasse. *BioEnergy Research*, 6, 4:1128–1134

[8] Vogel, J., 2008. Unique aspects of the grass cell wall. *Current Opinion in Plant Biology*, 11, 3:301–307

[9] Sarkar, P., Bosneaga, E., and Auer, M., 2009. Plant cell walls throughout evolution: towards a molecular understanding of their design principles. *Journal of Experimental Botany*, 60, 13:3615–35

[10] Tao, G., Lestander, T. A., Geladi, P., and Xiong, S., 2012. Biomass properties in association with plant species and assortments I: A synthesis based on literature data of energy properties. *Renewable and Sustainable Energy Reviews*, 16, 5:3481–3506

[11] Jin, S. and Chen, H., 2006. Structural properties and enzymatic hydrolysis of rice straw. *Process Biochemistry*, 41, 6:1261–1264

[12] Bhandari, H. S., Walker, D. W., Bouton, J. H., and Saha, M. C., 2013. Effects of ecotypes and morphotypes in feedstock composition of switchgrass (Panicum virgatum L.). *GCB Bioenergy*, 6:26–34

[13] Zhang, H., Fangel, J. U., Willats, W. G., Selig, M. J., Lindedam, J., Jø rgensen, H., and Felby, C., 2014. Assessment of leaf/stem ratio in wheat straw feedstock and impact on enzymatic conversion. *GCB Bioenergy*, 6, 1:90–96

[14] Rancour, D. M., Marita, J. M., and Hatfield, R. D., 2012. Cell wall composition throughout development for the model grass Brachypodium distachyon. *Frontiers in Plant Biotechnology*, 3:266

[15] Fangel, J. U., Ulvskov, P., Knox, J. P., Mikkelsen, M. D., Harholt, J., Popper, Z. a., and Willats, W. G. T., 2012. Cell wall evolution and diversity. *Frontiers in Plant Science*, 3, July:152

[16] Popper, Z. A., Michel, G., Hervé, C., Domozych, D. S., Willats, W. G., Tuohy, M. G., Kloareg, B., and Stengel, D. B., 2011. Evolution and Diversity of Plant Cell Walls: From Algae to Flowering Plants. *Annual Review of Plant Biology*, 62, 1:567–590

[17] Zhang, S.-J., Song, X.-Q., Yu, B.-S., Zhang, B.-C., Sun, C.-Q., Knox, J. P., and Zhou, Y.-H., 2012. Identification of quantitative trait loci affecting hemicellulose characteristics based on cell wall composition in a wild and cultivated rice species. *Molecular Plant*, 5, 1:162–75

[18] Mann, D. G. J., Labbé, N., Sykes, R. W., Gracom, K., Kline, L., Swamidoss, I. M., Burris, J. N., Davis, M., and Stewart, C. N., 2009. Rapid Assessment of Lignin Content and Structure in Switchgrass (Panicum virgatum L.) Grown Under Different Environmental Conditions. *BioEnergy Research*, 2, 4:246–256

[19] Serapiglia, M. J., Cameron, K. D., Stipanovic, A. J., Abrahamson, L. P., Volk, T. A., and Smart, L. B., 2013. Yield and Woody Biomass Traits of Novel Shrub Willow Hybrids at Two Contrasting Sites. *BioEnergy Research*, 6, 2:533–546

[20] Hopkins, A. A., Vogel, K. P., Moore, K. J., Johnson, K. D., and Carlson, I. T., 1995. Genotype Effects and Genotype by Environment Interactions for Traits of Elite Switchgrass Populations. *Crop Science*, 35, 1:125–132

[21] Monono, E. M., Nyren, P. E., Berti, M. T., and Pryor, S. W., 2013. Variability in biomass yield, chemical composition, and ethanol potential of individual and mixed herbaceous biomass species grown in North Dakota. *Industrial Crops and Products*, 41:331–339

[22] Barriere, Y., Guillet, C., Goffner, D., and Pichon, M., 2003. Genetic variation and breeding strategies for improved cell wall digestibility in annual forage crops. A review. *Animal Research*, 52, 3:193–228

[23] Ioelovich, M., 2014. Correlation analysis of enzymatic digestibility of plant biomass. *Biomass Conversion and Biorefinery*

[24] Studer, M. H., DeMartini, J. D., Davis, M. F., Sykes, R. W., Davison, B., Keller, M., Tuskan, G. A., and Wyman, C. E., 2011. Lignin content in natural Populus variants affects sugar release. *Proceedings of the National Academy of Sciences*, 108, 15:6300–6305

[25] Bottcher, A., Cesarino, I., Santos, A. B., Vicentini, R., Mayer, J. L. S., Vanholme, R., Morreel, K., Goeminne, G., Moura, J. C. M. S., Nobile, P. M., Carmello-Guerreiro, S. M., Anjos, I. A., Creste, S., Boerjan, W., Landell, M. G. A., and Mazzafera, P., 2013. Lignification in sugarcane: biochemical characterization, gene discovery and expression analysis in two genotypes contrasting for lignin content. *Plant Physiology*, p. pp.113.225250

[26] Xu, N., Zhang, W., Ren, S., Liu, F., Zhao, C., Liao, H., Xu, Z., Huang, J., Li, Q., Tu, Y., Yu, B., Wang, Y., Jiang, J., Qin, J., and Peng, L., 2012. Hemicelluloses negatively affect lignocellulose crystallinity for high biomass digestibility under NaOH and H2SO4 pretreatments in Miscanthus. *Biotechnology for Biofuels*, 5, 1:58

[27] Vega-Sánchez, M. E., Verhertbruggen, Y., Christensen, U., Chen, X., Sharma, V., Varanasi, P., Jobling, S. A., Talbot, M., White, R. G., Joo, M., Singh, S., Auer, M., Scheller, H. V., and Ronald, P. C., 2012. Loss of Cellulose synthase-like F6 function affects mixed-linkage glucan deposition, cell wall mechanical properties, and defense responses in vegetative tissues of rice. *Plant Physiology*, 159, 1:56–69

[28] Bartley, L. E., Peck, M. L., Kim, S.-R., Ebert, B., Maniseri, C., Chiniquy, D., Sykes, R., Gao, L., Rautengarten, C., Vega-Sánchez, M. E., Benke, P. I., Canlas, P. E., Cao, P., Brewer, S., Lin, F., Smith, W. L., Zhang, X., Keasling, J. D., Jentoft, R. E., Foster, S. B., Zhou, J., Ziebell, A., An, G., Scheller, H. V., and Ronald, P. C., 2013. Overexpression of a BAHD Acyltransferase, OsAt10, alters rice cell wall hydroxycinnamic acid content

and saccharification. *Plant Physiology*

[29] Buanafina, M. M. D. O., Langdon, T., Hauck, B., Dalton, S., and Morris, P., 2008. Expression of a fungal ferulic acid esterase increases cell wall digestibility of tall fescue (Festuca arundinacea). *Plant Biotechnology Journal*, 6, 3:264–80

[30] Xue, J., Bosch, M., and Knox, J. P., 2013. Heterogeneity and Glycan Masking of Cell Wall Microstructures in the Stems of Miscanthus x giganteus, and Its Parents M. sinensis and M. sacchariflorus. *PLoS ONE*, 8, 11:e82114

[31] Vega-Sanchez, M. E., Verhertbruggen, Y., Scheller, H. V., and Ronald, P. C., 2013. Abundance of mixed linkage glucan in mature tissues and secondary cell walls of grasses. *Plant Signaling & Behavior*, 8, 2

[32] Lamport, D. T. A., Kieliszewski, M. J., Chen, Y., and Cannon, M. C., 2011. Role of the Extensin Superfamily in Primary Cell Wall Architecture. *Plant Physiology*, 156, 1:11–19

[33] Heath, M. F. and Northcote, D. H., 1971. Glycoprotein of the wall of sycamore tissue-culture cells. *The Biochemical Journal*, 125, 4:953–61

[34] Lamport, D. T. A., 1980. Structure and function of plant glycoproteins. In P. Stumpf, ed., *The Biochemistry of plants: a comprehensive treatise (USA)*, vol. 3, pp. 501–541. New York: Academic Press, 3 ed.

[35] Santoro, N., Cantu, S. L., Tornqvist, C.-E., Falbel, T. G., Bolivar, J. L., Patterson, S. E., Pauly, M., and Walton, J. D., 2010. A High-Throughput Platform for Screening Milligram Quantities of Plant Biomass for Lignocellulose Digestibility. *BioEnergy Research*, 3, 1:93–102

[36] Chevanan, N., Womac, A. R., Bitra, V. S., Igathinathane, C., Yang, Y. T., Miu, P. I., and Sokhansanj, S., 2010. Bulk density and compaction behavior of knife mill chopped switchgrass, wheat straw, and corn stover. *Bioresource Technology*, 101, 1:207–214

[37] Wu, Z., Zhang, M., Wang, L., Tu, Y., Zhang, J., Xie, G., Zou, W., Li, F., Guo, K., Li, Q., Gao, C., and Peng, L., 2013. Biomass digestibility is predominantly affected by three factors of wall polymer features distinctive in wheat accessions and rice mutants. *Biotechnology for Biofuels*, 6, 1:183

[38] Cosgrove, D. J. and Jarvis, M. C., 2012. Comparative structure and biomechanics of plant primary and secondary cell walls. *Frontiers in Plant Physiology*, 3:204

[39] McNally, K. L., Bruskiewich, R., Mackill, D., Buell, C. R., Leach, J. E., and Leung, H., 2006. Sequencing multiple and diverse rice varieties. Connecting whole-genome variation with phenotypes. *Plant Physiology*, 141, 1:26

[40] McNally, K. L., Childs, K. L., Bohnert, R., Davidson, R. M., Zhao, K., Ulat, V. J., Zeller, G., Clark, R. M., Hoen, D. R., Bureau, T. E., and Others, 2009. Genomewide SNP variation reveals relationships among landraces and modern varieties of rice. *Proceedings of the National Academy of Sciences*, 106, 30:12273

[41] Jahn, C. E., Mckay, J. K., Mauleon, R., Stephens, J., McNally, K. L., Bush, D. R., Leung, H., and Leach, J. E., 2011. Genetic Variation in Biomass Traits among 20 Diverse Rice Varieties. *Plant Physiology*, 155, 1:157–168

[42] Dobermann, A., Dawe, D., Roetter, R. P., and Cassman, K. G., 2000. Reversal of Rice Yield Decline in a Long-Term Continuous Cropping Experiment. *Agronomy Journal*, 92:633–643

[43] Van Soest, P., Robertson, J., and Lewis, B., 1991. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. *Journal of Dairy Science*, 74, 10:3583–3597

[44] Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., and Crocker, D., 2008. *Determination of structural carbohydrates and lignin in biomass.* National

Renewable Energy Laboratory: Golden, Colorado, USA

[45] Verhertbruggen, Y., Marcus, S. E., Haeger, A., Ordaz-Ortiz, J. J., and Knox, J. P., 2009. An extended set of monoclonal antibodies to pectic homogalacturonan. *Carbohydrate Research*, 344, 14:1858–62

[46] Kivirikko, K. I. and Liesmaa, M., 1959. A colorimetric method for determination of hydroxyproline in tissue hydrolysates. *Scandinavian Journal of Clinical & Laboratory Investigation*, 11, 2:128–133

[47] R core Team, 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria

[48] Ghasemi, E., Ghorbani, G. R., Khorvash, M., Emami, M. R., and Karimi, K., 2013. Chemical composition, cell wall features and degradability of stem, leaf blade and sheath in untreated and alkali-treated rice straw. *Animal*, 7, 7:1106–12

[49] Liu, L., Ye, X. P., Womac, A. R., and Sokhansanj, S., 2010. Variability of biomass chemical composition and rapid analysis using FT-NIR techniques. *Carbohydrate Polymers*, 81, 4:820–829

[50] Rozema, J., van de Staaij, J., Björn, L. O., and Caldwell, M., 1997. UV-B as an environmental factor in plant life: stress and regulation. *Trends in Ecology & Evolution*, 12, 1:22–28

[51] Hahlbrock, K. and Grisebach, H., 1979. Enzymic Controls in the biosynthesis of lignin and Flavonoids. *Annual Review of Plant Physiology*, 30, 105

[52] Moura, J. C. M. a. S., Bonine, C. A. V., de Oliveira Fernandes Viana, J., Dornelas, M. C., and Mazzafera, P., 2010. Abiotic and biotic stresses and changes in the lignin content and composition in plants. *Journal of Integrative Plant Biology*, 52, 4:360–76

[53] Cipollini Jr., D. F., 1997. Wind-induced mechanical stimulation increases pest resistance in common bean. *Oecologia*, 111, 1:84–90

[54] Haydon, M. J., Bell, L. J., and Webb, A. A. R., 2011. Interactions between plant circadian clocks and solute transport. *Journal of Experimental Botany*, 62, 7:2333–48

[55] Park, J.-y., Kanda, E., Fukushima, A., Motobayashi, K., Nagata, K., Kondo, M., Ohshita, Y., Morita, S., and Tokuyasu, K., 2011. Contents of various sources of glucose and fructose in rice straw, a potential feedstock for ethanol production in Japan. *Biomass and Bioenergy*, 35, 8:3733–3735

[56] Dien, B. S., Sarath, G., Pedersen, J. F., Sattler, S. E., Chen, H., Funnell-Harris, D. L., Nichols, N. N., and Cotta, M. A., 2009. Improved Sugar Conversion and Ethanol Yield for Forage Sorghum (Sorghum bicolor L. Moench) Lines with Reduced Lignin Contents. *BioEnergy Research*, 2, 3:153–164

[57] Chen, F. and Dixon, R. A., 2007. Lignin modification improves fermentable sugar yields for biofuel production. *Nature Biotechnology*, 25, 7:759–761

[58] Shen, H., Fu, C., Xiao, X., Ray, T., Tang, Y., Wang, Z., and Chen, F., 2009. Developmental Control of Lignification in Stems of Lowland Switchgrass Variety Alamo and the Effects on Saccharification Efficiency. *BioEnergy Research*, 2, 4:233–245

[59] Sarath, G., Dien, B., Saathoff, A. J., Vogel, K. P., Mitchell, R. B., and Chen, H., 2011. Ethanol yields and cell wall properties in divergently bred switchgrass genotypes. *Bioresource Technology*, 102, 20:9579–9585

[60] Rollin, J. a., Zhu, Z., Sathitsuksanoh, N., and Zhang, Y.-H. P., 2011. Increasing cellulose accessibility is more important than removing lignin: a comparison of cellulose solvent-based lignocellulose fractionation and soaking in aqueous ammonia. *Biotechnology and Bioengineering*, 108, 1:22–30

[61] Ray, M., Brereton, N., Shield, I., Karp, A., and Murphy, R., 2012. Variation in Cell Wall Composition and Accessibility in Relation to Biofuel Potential of Short Rotation Coppice Willows. *BioEnergy Research*, 5, 3:685–698

[62] Tanger, P., Field, J. L., Jahn, C. E., DeFoort, M. W., and Leach, J. E., 2013. Biomass for thermochemical conversion: targets and challenges. *Frontiers in Plant Biotechnology*, 4::218

[63] Goto, M., Ehara, H., Karita, S., Takabe, K., Ogawa, N., Yamada, Y., Ogawa, S., Sani, M., Yahaya, M. S., and Morita, O., 2003. Protective effect of silicon on phenolic biosynthesis and ultraviolet spectral stress in rice crop. *Plant Science*, 164, 3:349–356

[64] Schaller, J., Brackhage, C., and Dudel, E. G., 2012. Silicon availability changes structural carbon ratio and phenol content of grasses. *Environmental and Experimental Botany*, 77:283–287

[65] Suzuki, S., Ma, J. F., Yamamoto, N., Hattori, T., Sakamoto, M., and Umezawa, T., 2012. Silicon deficiency promotes lignin accumulation in rice. *Plant Biotechnology*, 29, 4:391–394

[66] Schoelynck, J., Bal, K., Backx, H., Okruszko, T., Meire, P., and Struyf, E., 2010. Silica uptake in aquatic and wetland macrophytes: a strategic choice between silica, lignin and cellulose? *The New Phytologist*, 186, 2:385–91

[67] Schaller, J., Brackhage, C., Bäucker, E., and Dudel, E. G., 2013. UV-screening of grasses by plant silica layer? *Journal of Biosciences*, 38, 2:413–416

[68] Datnoff, L. E., Snyder, G. H., and Korndörfer, G. H., eds., 2001. *Silicon in Agriculture, Volume 8*. New York: Elsevier Science, 1 ed.

[69] Baxter, H. L., Mazarei, M., Labbe, N., Kline, L. M., Cheng, Q., Windham, M. T., Mann, D. G. J., Fu, C., Ziebell, A., Sykes, R. W., Rodriguez, M., Davis, M. F., Mielenz,

J. R., Dixon, R. A., Wang, Z.-Y., and Stewart, C. N., 2014. Two-year field analysis of reduced recalcitrance transgenic switchgrass. *Plant Biotechnology Journal*

[70] Couhert, C., Commandre, J.-M., and Salvador, S., 2009. Is it possible to predict gas yields of any biomass after rapid pyrolysis at high temperature from its composition in cellulose, hemicellulose and lignin? *Fuel*, 88, 3:408–417

[71] Rath, J., Heuwinkel, H., and Herrmann, A., 2013. Specific Biogas Yield of Maize Can Be Predicted by the Interaction of Four Biochemical Constituents. *BioEnergy Research*

[72] Oh, C.-S., Kim, H., and Lee, C., 2013. Rice cell wall polysaccharides: Structure and biosynthesis. *Journal of Plant Biology*, 56, 5:274–282

CHAPTER 4

Characterization of *OXO* genes in rice [3]

Overview

It has been proposed that the class of oxalate oxidase proteins (OXO, EC 1.2.3.4) have evolved in monocots as broad host defense genes, especially against pathogens such as *Sclerotinia sclerotiorum* (*ScS*) that secrete oxalic acid as a major virulence factor. *ScS* is a major pathogen of over 400 plant species, but generally not a pathogen of cereal species. In rice, *OXO* genes reside in quantitative trait loci (QTL) associated with resistance to other generalist necrotrophic pathogens such as *Rhizoctonia solani* (*Rs*). Silencing *OXO* genes increases susceptibility of rice to *ScS*, and overexpression of *OsOXO*4 increases resistance to *ScS*. In contrast, a mutant *ScS* strain lacking the ability to produce oxalic acid is unable to produce disease on silenced or overexpression lines. Responses to *Rs* were more variable between two assay methods. In *Rs* microchamber assays, overexpression lines demonstrated enhanced resistance, but there was no difference between silenced lines and wild type. In detached leaf assays, neither silenced nor overexpression lines responded differently to *Rs* compared to wild type. These studies confirm that expression of oxalate oxidase in monocots restricts accessibility to pathogens that depend on oxalic acid for virulence, and suggest responses to rice pathogens (*Rs*) may have been tempered through plant-pathogen evolution, varietal differences, environmental effects, and other factors that define resistance and susceptibility.

[3]This chapter will be published as a collaborative effort with these authors: Paul Tanger, Rebecca M. Davidson, Paul Langlois, Seweon Lee, Elysa M. Ducharme, Maria Gay C. Carrillo, Casiana M. Vera Cruz, Hei Leung, and Jan E. Leach. Author contributions: Conceived and designed the experiments: PT, RMD, SL, MGCC, CVC, HL, JEL. Performed the experiments: PT, RMD, PL, SL, LMD, MGCC. Analyzed the data: PT, RMD, PL, SL, MGCC.

4.1. Introduction

4.1.1. OXOs convert oxalate to H_2O_2

The plant cell wall is the first line of defense against biotic invasion and attack. Many components of the cell wall are associated with a role in disease resistance [1]. One such component is the family of oxalate oxidase genes (*OXOs*), widely found in monocot species but not in dicots [2]. These genes have been associated with disease resistance QTL, and when overexpressed in some species, they confer additional disease resistance [3–8]. Initially isolated and characterized from barley seedling roots, OXOs catalyze the reaction of oxalic acid (oxalate) to H_2O_2 and CO_2 [9]. This family of *OXO* genes contain a cupin domain and are considered part of a larger family of germin like proteins thought to be important for seedling growth. OXOs also contain an N terminal secretion signal and are detected in soluble and cell wall protein extracts [10, 11], and it has been shown in rice that they localize to the cell wall [12].

The substrate and products of the reaction catalyzed by OXO support a role in disease resistance. Some pathogens, especially necrotrophic fungi, secrete oxalic acid as a pathogenicity factor [13, 14]. When OXOs in the cell wall encounter and convert this oxalic acid to H_2O_2, it could trigger an oxidative burst, a well-known disease defense response, as well as trigger more complex downstream defense pathways [15]. H_2O_2 may also induce callose and lignin synthesis and facilitate the deposition of lignin into the cell wall [16]. When dicots, which lack *OXO*s, are transformed with monocot *OXO* genes, increased disease resistance is observed across many species [3–8]. Despite all this evidence, the exact role of *OXO* genes in disease resistance has not been determined.

4.1.2. OXOs may contribute to broad host resistance in monocots

While pathogens have evolved virulence effectors to facilitate disease, plants have developed ways to recognize the pathogens and stop disease development. Generally plant defense pathways are referred to as immune responses, and the pathogen molecules that activate them are grouped into two classes: microbe (or pathogen) associated molecular patterns (PAMPs) and effectors. PAMPs are associated with a basal defense response, called pathogen-triggered immunity (PTI), and effectors are associated with a more specific gene-for-gene interaction, effector triggered immunity (ETI) [17].

Breeding for gene for gene resistance can sometimes only be effective for several years before the pathogens overcome the resistance. Breeding for broad spectrum host resistance is an alternative strategy, and might be more difficult for pathogens to evade. Since oxalic acid is produced by many pathogens and is an important virulence factor for some, and H_2O_2 triggers many defense pathways, OXOs may play a role in PTI: broad spectrum host resistance. In rice, a cluster of four *OXO* homologs are located on chromosome 3 (MSU/TIGR Rice Genome Annotation v7: Os03g48750, Os03g48760, Os03g48770, and Os03g48780) which are termed *OsOXO*1 through *OsOXO*4, and including 1 kb upstream, span 27779000-27793100 bp). This region was associated with several disease resistance QTL (summarized in Table 4.1). The protein sequence of these four genes have 90% similarity [18]. Recently, two studies investigated the role of *OXO* genes in disease resistance through overexpression [19] and overexpression as well as silencing of *OsOXO* genes [12] followed by challenge with several rice pathogens. Results of these two studies are contradictory—while Molla *et al.* [19] found that overexpressing *OsOXO* enhances resistance to sheath blight, Zhang *et al.* [12] found no difference in resistance to bacterial blight, and less or no resistance to rice blast

and sheath blight. The authors did not address if differences in the varieties, environments and/or strains of pathogens influenced the role of *OXO* in disease resistance.

4.1.3. A tail of two pathogens

Sclerotinia sclerotiorum (*ScS*) is a major pathogen on many crops and the causal agent of white mold disease [20]. Under rice cultivation conditions, it is not a pathogen of rice and it has been hypothesized that this is because the pathogen relies on oxalic acid as a pathogenicity factor [13]. Mutant lines of *ScS* exist which do not produce oxalic acid and have been used to investigate the role of oxalic acid in plant-pathogen interactions. In contrast to *ScS*, the host range of another necrotrophic pathogen, *Rhizoctonia solani* (*Rs*), includes both dicots and monocots. *Rs* is the causal agent of sheath blight of rice, a major disease to which little resistance is available [21]. So while *Rs* infects rice, it does not rely soley on oxalic acid as a pathogenicity factor [22]. Thus, understanding the mechanism of broad host disease resistance against one or more of these diverse pathogenic strategies could enable identification of additional genes that allow plant survival.

In this study, we use transgenic rice either overexpressing or silenced for expression of rice oxalate oxidase genes to understand the role of *OXO* in resistance to a non-host fungal pathogen, *ScS*, as well as a known rice pathogen, *Rs*. The importance of oxalic acid as a pathogenicity factor for *ScS* is examined in disease assays on rice leaves, and compared to disease from *Rs*. The cell wall composition of several trangenic lines is examined for differences compared to the wild-type.

4.2. Materials and methods

4.2.1. Generation of overexpression and silenced lines

To generate overexpression lines, genomic DNA (gDNA) was extracted from *O. sativa* ssp. *indica* cv. Moroberekan. The coding sequence of *OsOXO*4 (Os03g48780), which does not contain any introns, was amplified by PCR using a high fidelity polymerase (Phusion Hot Start F-540S, Thermo Scientific, Pittsburgh, PA USA) from gDNA extracted with Easy-DNA (Invitrogen, Carlsbad, CA USA). The primers (Forward: 5' caccATGGAGCACAGCTTCAAAACCA 3'; Reverse: 5' TTAGTACCCGCCGGTGAATTT 3') were designed with an overhang on the forward primer to allow cloning into the vector. The fragment was gel-purified (Wizard SV, Promega, Madison, WI USA), sequenced, and ligated into pENTR/dTOPO (Invitrogen, Carlsbad, CA USA); the plasmid was then transformed into *E. coli* DH5-α. Plasmid DNA was purified from positive transformants (DNA Miniprep, Qiagen, Valencia, CA USA), and the purified plasmid was recombined using LR Clonase (Invitrogen, Carlsbad, CA USA) into a gateway compatible version (pUbiNC1300-RFCA) of the overexpression vector pUbiNC1300 [23]. pUbiNC1300-RFCA was created by ligating Reading Frame Cassette A (RFCA) into the *smaI* site of the MCS of the pUbiNC1300 vector. *A. tumefaciens* strain LBA4404 [24] was transformed with the clone, and correct sequence and insert orientation were confirmed by sequencing. The vector contains a maize ubiquitin promoter that drives the constitutive expression of the *OsOXO*4 gene.

Rice plants were transformed using a protocol modified from [25]. Immature green *O. sativa* ssp. *japonica* cv. Kitaake rice seeds were dehulled and sterilized in 40% bleach with 1 drop of Tween20 (Sigma) for 30 min, followed by four distilled water rinses. Dehulled seeds were placed on Calli Induction (CI) media in large petri dishes and grown for 15 days

at 29°C and 25 µE/m^{2}s light intensity and 16 h day/8 h night cycle. The CI media consisted of 4.4 g MS salts+vitamins (Caisson Labs, North Logan, UT USA), 30 g sucrose, 4 g Gelrite (Research Products International Corp., Mt. Prospect, IL USA), and 2 mg 2-4-D (#D295 Phytotechnology Labs, Shawnee Mission, KS USA) in 1 L water, pH 5.8 with KOH then autoclaved. *A. tumefaciens* strain LBA4404 harboring the *OsOXO*4 binary construct was grown on YM media with 100 mg/L streptomycin, and 50 mg/L kanamycin. A sterile loopful of the strain was added to 5 mL liquid TY media with 0.2 mM acetosyringone, and incubated for 2 h at 25°C at 250 RPM; the culture was adjusted to 0.1 OD_{600} using a spectrophotometer. Calli were inoculated with the diluted culture and incubated on an orbital shaker at 30°C and 30 RPM for 30 min. After blotting on several layers of sterile filter paper, the calli were plated on co-cultivation (CC) media (same as CI media but containing 50 g/L sorbitol, 0.2 mM acetosyringone and 6 g/L Gelrite instead of 4 g/L) and sealed with micropore tape. Co-cultivation was done in the dark at 25°C for 3 d. Calli were then gently washed and rinsed with a solution of 4.4 g/L MS salts+vitamins, 30 g/L sucrose and 400 mg/L carbenicillin to remove excess *A. tumefaciens*; rinses continued until washes were clear. The calli were plated on selection media (same as CI media but with 250 mg/L carbenicillin, 50 mg/L hygromycin, and 500 µL/L Preservative for Plant Tissue Culture Media (Plant Cell Technology, Washington DC, USA), and plates were sealed with micropore tape and incubated at 30°C under continuous light for 4 wks, with calli being replated on fresh selection media a total of 3 times. The surviving putative resistant/transformed microcalli were then transferred to regeneration media (same as CC media but no 2-4D and with 3 mL/L BAP (Phytotechnology Labs #B130), 0.5 mL/L NAA (Phytotechnology Labs #N605), 125 mg/L carbenicillin and 50 mg/L hygromycin) on petri dishes and sealed with parafilm to prevent dehydration and incubated under continuous light at 30°C to allow leaf primordia

formation. Calli were placed on regeneration media for a total of 10 wks and transferred 3-4 times. After significant leaf formation, the 'plantlets' (T0) were separated and plated on rooting media (same as CI media but no 2-4D and with 2 g/L Gelrite instead of 4 g/L and with 50 mg/L carbenicillin and 50 mg/L hygromycin) in individual plastic cups (Dart, Mason, MI USA) with humidity domes. After 1 wk plants were transplanted into a custom potting mixture (4:4:1 Pro-Mix BX Mycorrhizae: Canadian sphagnum peat: Quikrete play sand). The plants were tested for presence of the transgene using primers (Forward: 5' GGCCTCCAGAAGAAGATGTTG 3'; Reverse: 5' GAGCCTGACCTATTGCATCTC 3') that amplify a 456 bp fragment of the *hygromycin phosphotransferase* (*hpt*) resistance gene in the vector from gDNA extracted with a rapid DNA extraction protocol [26].

Rice lines silenced for *OXO* expression were generated by RNAi. Briefly, a fragment of the conserved region between the four *OXO* genes in rice (Os03g48750, Os03g48760, Os03g48770, Os03g48780) was amplified from *O. sativa* ssp. *japonica* cv. Kitaake gDNA, ligated into pENTR/dTOPO (Invitrogen, Carlsbad, CA USA), and recombined into the pANDA silencing vector [27] using Gateway LR Clonase (Invitrogen, Carlsbad, CA USA). The vector contains a maize ubiquitin promoter which drives the hairpin dsRNAi silencing complex, designed to silence all four *OXO* genes. *A. tumefaciens* strain EHA105 [28] was transformed with purified plasmid and used for transformation of *O. sativa* cv. Kitaake as described for the overexpression lines.

4.2.2. Plant growth

Seeds were germinated in a 1:1000 dilution of Maxim XL fungicide:water (Syngenta, USA) for 5 d, and then were transplanted to a custom potting mixture (4:4:1 Pro-Mix BX Mycorrhizae: Canadian sphagnum peat: Quikrete play sand) in 7.6 L pots in standing water.

Three seeds of a given line were planted in each of the pots and the pots were set up in a completely randomized design in a greenhouse (GH) with controlled conditions at Colorado State University (lat 40°34'17.5"N long 105°04'52.5"W, elevation 1,519 m) with approximately 27°C and 76% RH and supplemental high-intensity discharge lighting to maintain a 16-h-light/8-h-dark photoperiod at an irradiance of 20-55 mW/m^2. 30 T4 plants of each silenced line, and 15 T3 plants of each overexpression line were germinated. After transplantation, the pots and plants had only numerical labels to prevent any scoring bias.

4.2.3. Microchamber assay for resistance to sheath blight

R. solani Kühn (teleomorph: *Thanatephorus cucumeris* (A. B. Frank) Donk) isolate RR0140-1, anastomosis group 2 (AG2) [29], the causal agent of sheath blight, was grown by placing sclerotia in the center of petri dishes with 1/2 strength Potato Dextrose Agar (Difco PDA, Becton Dickinson, Franklin Lakes, NJ USA) sealed with parafilm and incubated for 1-2 d at 27°C until the mycelia reached the edge of the petri dishes. Three plugs with *Rs* mycelia or PDA agar plugs (mock) were placed at the base of rice plants 26 days after transplantation and the plants covered with soda bottles as part of the microchamber assays described in [30]. Ten days later, plants were measured for lesion height, node height, and plant height. Rice cultivars Lemont (IRGC 66756) and Jasmine-85 (IRGC 32591) were used as susceptible and resistant controls. Disease was calculated as lesion height as a percent of height of the tallest node on the main tiller.

4.2.4. Detached leaf assays for resistance to white mold and sheath blight

S. sclerotiorum (Lib.) de Bary isolate N 1980, the causal agent of white mold, and an oxalate-deficient mutant (A-2) of this strain [31] were grown by placing sclerotia (for the wt) or filter paper with mycelia (for the mutant, since it does not make sclerotia) in the center

of petri dishes with Potato Dextrose Agar (Difco PDA, Becton Dickinson, Franklin Lakes, NJ USA) for several days at 22°C and 21% RH. Agar plugs were excised from petri dishes containing *ScS* mycelia using the inverted end of a 1 mL pipette tip and forceps and placed mycelia-side-up on sections of the last fully expanded ("youngest") leaf from the main tiller of plants, taped with micropore tape (3M, St. Paul, MN USA) abaxial side up onto filter paper soaked with sterile water in petri dishes as part of the detached leaf protocol described in [32]. For assays of plant responses to *Rs*, plugs of mycelia were prepared as above. Each leaf was divided into sections and sections were randomly assigned a plug of wt *ScS*, A-2 *ScS*, *Rs* or mock (plain agar plug). The plates were sealed with micropore tape or parafilm and incubated at 22°C and ambient light (or 29°C in the dark for *Rs*) until disease was almost to the ends of the control leaf sections (2-4 d). The leaf sections were imaged with a dissecting microscope at the same focus for each leaf section and for the first detached leaf assay with the filter paper background and the second assay using a piece of blue plastic as a background to assist in scoring. The background was removed using the magnetic lasso tool in Photoshop CS6 (Adobe Systems, Mountain View, CA USA) and image files containing only the plant number and letter code for the leaf section were scored for percent lesion using Assess 2.0 (APS Press, St. Paul, MN USA). The *ScS* assay was performed twice; once on the same day as the microchamber assay scoring to prevent any wounding influence from the scoring and sampling of leaves, and again 33 days later along with a detached leaf assay for *Rs*. In the first experiment, leaves were divided into four sections, and two leaf sections were used for wt *ScS*, and one for mock and one for A-2. In the second experiment, leaf sections were divided into six sections, four for *ScS* as above, and two for *Rs*. In the second experiment, the *Rs* had progressed very fast, and the adaxial side of the leaves was imaged instead of the abaxial.

4.2.5. Quantification of OXO enzyme activity

Quantification of oxalate oxidase activity was determined with an enzymatic assay modified from [6]. Briefly, a 5 mm diameter corer (Uni-Core, Ted Pella, Inc, Redding, CA USA) was used to excise a leaf disc from the second to last fully expanded leaf on the main tiller and placed in 15 µL of water in a 96 well microtiter plate. The corer was rinsed in water between samples. Effort was made to ensure that the leaf disc was taken from the middle of each leaf, containing the main vein, and that the disc was completely circular thus maintaining the same circumference between samples. To each sample was added 200 µL assay buffer solution consisting of 5 ml 25 mM succinic acid solution (succinic acid (#S-5047, Sigma) buffered with 3.5 mM EDTA to pH 4), 9 mg oxalic acid (#O-0376, Sigma), and 45 ml water. The plates were allowed to incubate at 37°C for 15 min. 100 µL of the solution was transferred to a new plate with 70 µL of developer solution and allowed to incubate for 15 min. The developer solution consisted of 3 mg of aminoantipyrene partially dissolved in 15 µL of N,N-dimethylaniline, which was added to 4.8 mL 1 M NaH_2PO_4 and 0.2 mL 1 M Na_2HPO_4 in 45 mL water. Horseradish peroxidase (Type VI HRP, #P8375, Sigma, St. Louis, MO USA) was dissolved in water at 140 mg/mL and 4 µL was added to developer solution. After incubation, absorbance at 550 nm was quantified with a plate reader (Powerwave HT, BioTek, Winooski, VT USA). The blank wells consisted of 15 µL of water and absorbance of all other wells were subtracted from the mean blank readings. A positive control consisted of 2 µL of oxalate oxidase extracted from *H. vulgare*. It was demonstrated in separate testing that variation from leaf discs taken on different parts of leaves, and different leaves was minimal (data not shown). This assay was only sensitive enough to detect enzyme activity from overexpression lines; both wild type and silenced lines result in absorbance values the same as blank controls, even leaf discs next to active disease from *Rs* or *ScS* (data not shown).

Groups of plants were assayed as described above. The pre-inoculation group was assayed 27 days after transplantation using 2 µL instead of 4 µL HRP and incubated for 20 min instead of 15 min. One leaf disc was quantified per plant. All plants were assayed on the same day as disease scoring for the microchamber assay and one leaf disc was quantified per plant. All plants were assayed again on the same day as the second detached leaf experiment and three leaf discs from the same leaf were quantified per plant and incubated for 20 min instead of 15 min.

4.2.6. Quantification of *OXO* expression

On the day of the *Rs* microchamber assay scoring, the first ("youngest") leaf of the main tiller was collected to obtain RNA unless that leaf was too diseased, in which case the second leaf was taken and immediately separated into leaf and sheath and flash frozen in liquid nitrogen and stored at -80°C. RNA was obtained using a column based kit (Spectrum Plant Total RNA, Sigma, St. Louis, MO USA) from 100 mg of frozen tissue after grinding with a TissueLyser II (Qiagen, Valencia, CA USA) and treated with a DNAse (Turbo DNA-free, Ambion, Grand Island, NY USA). RNA quantity was assessed with spectrophotometer (Nanodrop, Thermo Scientific, Pittsburgh, PA USA) and RNA integrity was assessed with 250 ng of each sample on 1% agarose gel, 80V, 90 min. Lack of gDNA contamination was confirmed with PCR with ef1α primers (F: 5' TTTCACTCTTGGTGTGAAGCAGAT 3' and R: 5' GACTTCCTTCACGATTTCATCGTAA 3') [33]). cDNA was synthesized using a mix of random hexamer and oligo(dT) primers (iScript, Bio-Rad, Hercules, CA USA). qPCR was performed in triplicate using primers to amplify a 88 bp fragment from the 3' end of the *OsOXO*4 gene (F: 5' GAACAGGTACTACTCCAAGGTGGT 3'; R: 5' CTTGC-CAACGTTGAACTGGAAGTG 3') in 25 uL reactions with 12.5 uL of SsoFast EvaGreen

(Bio-Rad, Hercules, CA USA). Amplification was performed with 30 cycles of a two-step protocol at 95°C for 12 sec and 60°C for 40 sec (after initial 95°C for 2 min), and followed by a final 95°C for 10 sec and melt curve determination from 60-95°C in 0.5°C increments for 5 sec each. Expression was normalized using the same ef1α primers described above as a reference gene and relative expression calculated from the mean expression of wt Kitaake with the ΔΔct method [34], after confirming that PCR efficiency was 90-110% with a standard curve of wt Kitaake cDNA for both sets of primers (ef1α and *OXO*4).

4.2.7. Cell wall compositional analysis

Stem tissue of plants from two T1 overexpression lines (UbiOxo-10 and UbiOxo-17) that were confirmed to have a hygromycin insert and OXO activity, and one plant of Kitaake were ground to a fine powder and analyzed at the CSU soil testing lab for cellulose, lignin, hemicellulose and ash as described in Chapter 3 section 3.2.3.

4.2.8. Statistical analysis

Data was compiled in Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA) and relevant statistical tests and figures were generated in R Statistical Computing 3.0.2 [35].

4.3. Results

4.3.1. Characterization of overexpression and silenced lines

Ten T0 overexpression lines ("UbiOxo-", followed by the plant number of the T0 plant: 1,2,3,5,6,9,10,12,14,17) that were confirmed to have the *hygromycin phosphotransferase* (*hpt*) transgene (Supplemental Figure B.2), were advanced to the T1 generation. We confirmed that enzymatic activity (Supplemental Figure B.3) was associated with plants containing the *hpt* insert in these T1 lines (Supplemental Figure B.4). At the same time, we tested all

T1 plants for resistance to *Rs* in a preliminary experiment (Supplemental Figure B.5), and 13 T1 lines were chosen that represented a distribution of enzymatic activity and resistance to *Rs*. 15 T2 plants of each of the 13 T1 lines were used in further experiments.

Based on reduced expression of *OsOXO*4 as assessed by RT-PCR of 53 T0 plants, four silenced lines were selected (oxo-3, oxo-5, oxo-14, oxo-38). T1 lines from the oxo-14 T0 were selected because they showed resistance to *Rs*, rice blast, and bacterial blight. T2 lines (oxo-14-19-23 and oxo-14-19-30) were selected because they exhibited silencing of the *OXO* gene and increased sheath blight disease. 30 T3 plants of both T2 lines were used in the experiments.

While the activity assay was quantitative (Supplemental Figure B.6), we observed variation between leaf positions and leaf discs (Supplemental Figure B.7 and Figure B.8), so only absorbance values above 0.02 were considered overexpression. No detectable activity from wild type or silenced lines was ever observed (Supplemental Figure B.9). The activity assay was performed twice and the correlation between runs was very high (Supplemental Figure B.10), so absorbance from the scoring day is used unless otherwise noted. To determine if inoculation with *Rs* would induce higher expression in the overexpression lines, we collected activity samples on the day of inoculation and the scoring day (12 days later). However we observed that variation existed between leaf discs and leaves of the same plant (described above, Supplemental Figure B.8), and we cannot separate this variation from variation that may be due to OXO protein levels.

*OsOXO*4 expression of a subset of plants used in the detached leaf assays has been quantified (Figure 4.1). All silenced lines tested were found to be silenced compared to wild type except plant oxo-14-19-23-187, which was omitted from further analysis. All overexpression lines tested were found to have higher expression than wild type. There was

a strong relationship between the amount of OXO activity quantified and *OXO* expression level in the overexpression lines (Figure 4.2), so while expression of all overexpression lines has not been completed, if the lines had measurable OXO activity, they were not omitted from analysis.

4.3.2. White mold disease response

In detached leaf assays on silenced, wt, and overexpression rice lines, wt *ScS*, which produces oxalate, induced more disease on all lines than did the oxalate-deficient A-2 mutant (two-tailed unequal variance t-test, $p<0.03$) (Figure 4.3). After inoculation with wt *ScS*, the mean disease of the silenced lines was higher than Kitaake (19.6% vs 10.3%), and the mean of the overexpression lines was the lowest (7.7%). The differences between Kitaake and the transgenic lines after wt inoculation were not significant (Kitaake and silenced $p=0.11$, Kitaake and overexpression $p=0.47$) but silenced were more diseased than overexpression ($p=0.03$).

4.3.3. Sheath blight disease response

In microchamber assays with *Rs*, responses of silenced and overexpression lines to *Rs* were highly variable (Figure 4.4). Mean disease responses of Kitaake and the *OsOXO* silenced lines were not significantly different, however the overexpression lines had less disease than Kitaake controls (two-tailed unequal variance t-test, $p=0.01$).

In contrast to the *Rs* microchamber results, the detached leaf *Rs* inoculation (Figure 4.5) revealed silenced lines were more susceptible to *Rs* compared to Kitaake controls (two-tailed unequal variance t-test, $p=0.11$). Responses of overexpression lines in *Rs* detached leaf assays were more variable, but together trended toward more susceptible than Kitaake controls (two-tailed unequal variance t-test, $p=0.34$).

4.3.4. Cell wall composition of transgenic lines

To determine if there was an effect of *OsOXO4* overexpression on cell wall composition, stem tissue of T1 plants were analyzed. Ash content of the overexpression line was lower than Kitaake (Figure 4.6) but cellulose, hemicellulose and lignin were similar in both overexpression and control lines.

4.3.5. Relationships between *OXO* expression and disease resistance

I hypothesized that there might be a relationship between the level of *OXO* expression and disease resistance. To investigate this, linear regression models were generated for the detached leaf assays, since differences in disease resistance was observed in these assays. For the *ScS* detached leaf assay (Figure 4.7), there is a negative relationship between the amount of *OXO* expression and disease susceptibility. While a negative trend is evident between disease susceptibility with the *Rs* detached leaf assay, the relationship is weak (Figure 4.8), and characterized by high variability in disease response.

4.4. Discussion

While *ScS* has a broad host range, it rarely infects monocots. It is a necrotrophic pathogen that depends on the production of oxalic acid to induce programmed cell death and allow the pathogen to invade the dead cells. In contrast, a mutant *ScS* strain lacking oxalic acid elicits a hypersensitive response in normally susceptible plants [13]. Monocot species, which contain oxalate oxidases, can degrade oxalic acid and it was believed this is the mechanism which confers resistance in all monocots to pathogens like *ScS*.

This study confirms the hypothesis that rice is resistant to oxalic acid generating pathogens because it harbors broad host resistance oxalate oxidases. We have demonstrated this by silencing or overexpressing *OsOXO4* and challenging with *ScS*, an oxalate producing pathogen

that does not normally infect rice. We have shown that when the *OXO* gene is silenced, rice is more susceptible to this pathogen, presumably because it cannot break down the oxalic acid produced by the pathogen. Conversely, we have demonstrated that when *OXO* is overexpressed, rice is more resistant to *ScS*, even when wild type plants are symptomatic. Furthermore, when these same lines are challenged with a *ScS* strain unable to produce oxalic acid, there is little disease, and no detectable difference between silenced, wild type, or overexpression lines, indicating that oxalic acid is a necessary component of a compatible interaction.

The importance of *OXO* genes in resistance to *Rs*, a pathogen of rice, is less clear. With one method, the microchamber assay, overexpression lines are more resistant than Kitaake controls, but there was no significant difference between silenced lines and Kitaake controls. In the detached leaf assay, silenced lines were more susceptible than Kitaake controls, but there was no difference between Kitaake controls and overexpression lines. While OXO enzymatic activity can be detected in the overexpression lines, we were not able to detect activity in wild type and silenced lines and thus determine if all silenced lines used in the microchamber assay are truly silenced. Confirmation of silencing in these lines is currently underway by measurement of gene expression, which could reveal differences between lines with less *OXO* expression and lines where silencing did not occur.

In the detached leaf assay with *Rs* the trend towards susceptibility was not consistent among the overexpression lines (Figure 4.9), and there could be several reasons for this. First, the lines could have different levels of *OXO* expression, either due to the presence of multiple inserts or the location of insertion, and it is possible that constitutive expression of *OXO* could, through the ROS generated, cause more cell death for the pathogen to colonize. This could be accelerated in the detached leaf assay compared to the microchamber assay

method. Second, the insertion construct could have disrupted the function of other defense genes, though we did not observe any visible phenotypic differences between the lines. While *Rs* does produce oxalic acid, *Rs* produces many cell wall degrading enzymes [22] and does not rely on oxalic acid for virulence like *ScS* does [31].

We also sought to investigate whether silencing or overexpressing *OXO*s leads to changes in cell wall composition. When chestnut (*Castanea dentata*) calli were exposed to exogenous oxalic acid, altered cell wall composition was observed; the calli had less lignin and more cellulose [8]. This, along with more general evidence that OXOs may play a role in cell wall remodeling as part of a defense response, led us to test the straw composition of some of lines in our study. New approaches to modifying the cell wall composition of rice straw could allow the utilization of straw tissue for bioenergy. If OXOs were found to enhance disease resistance, and enhance the bioenergy potential of the straw cell wall, this would be advantageous. We did not observe strong evidence that the cell wall composition was different from wild type, however the three *OXO* overexpression lines tested had less ash than wildtype (overexpression mean: 12%, Kitaake: 19%). Along with our results that indicate *OXO* overexpression increases susceptibility to *Rs*, more detailed studies must be undertaken before *OXO* genes would be considered a viable bioenergy target.

Many examples have demonstrated the effectiveness of overexpressing *OXO* in dicots to enhance resistance to pathogens like *ScS*. Here, we demonstrate at least one of the native roles of *OXOs* in monocots may be defense against these pathogens that depend on oxalate as a virulence factor. Another role could be cell wall modification, although this role has not been validated. Possibly *OXOs* first evolved to aid in cell wall loosening to allow plant growth, and expanded as roles in broad host defense response evolved. Or maybe *OXOs* evolved from a common ancestral germin-like protein shared between dicots and monocots after the

divergence of these lineages as a component of the oxidative burst response, and subsequently diversified into cell wall remodeling roles. It is not clear why convergent evolution has not led to proteins with similar modes of action in dicots. In rice, *OXOs* have been shown to be under purifying selection, so an important functional role must exist for these genes [18]. It is unlikely that the function is resistance to a specific pathogen because if that were the case, the genes would be under diversifying selection as the "arms race between the pathogen and the *OXO* genes would occur [36]. If, as Zhang *et al.* [12] speculates, *OXOs* do not have a role in disease resistance, what is their role, and why would families of these genes be maintained and actively expressed?

The role of each of the four genes in the *OXO* family in rice is still not clear. If through sequence comparison, the ancestral gene could be determined that would provide insight into the original roles of the *OXO* genes in cereals. Possibly these gene have adapted to tissue-specific roles. For example, *OsOXO*1 appears to be expressed only in the flower, while expression of *OsOXO*2 was not observed in any tissue, and *OsOXO*3 and *OsOXO*4, which have the same signal peptide sequence, were observed in leaf and root tissue [2]. In *OsOXO*1 and *OsOXO*4 constitutive overexpression lines, Zhang *et al.*, [12] observed changes in expression in the other *OXO* genes relative to wild-type and variation in disease response between *OsOXO*1 and *OsOXO*4 overexpression lines, so perturbing one of the genes may affect the expression of the other genes. *OsOXO*1 overexpression lines had more disease than wild-type in response to leaf and panicle blast [12]; similar to our results with the *Rs* detached leaf assay. They also observed no difference between *OsOXO*4 overexpression lines and controls in leaf and panicle blast disease assays. With a different rice variety and different strain of *Rs*, there was enhanced resistance to *Rs* in lines overexpressing *OsOXO*4 with a green tissue promoter as demonstrated by fewer infection cushions in a detached

leaf assay and less disease in a percent disease index assay [19]. This assay is similar to the microchamber assay we performed, but was done on plants at maximum tillering stage, and the inoculum was a rice hull mixture. They also observed that *PAL* and *RC*24 genes were upregulated in *OsOXO*4 overexpression lines. Generalizations between these studies are difficult because many factors are different, but there are likely interactions between the transgenic *OXO* gene and the other *OXO* genes, as well as other defense response genes; these interactions may vary between rice varieties.

4.4.1. Variation in disease response to *Rs*

The variation observed within and between the *Rs* assays is not unusual. The accurate measurement of disease depends on environmental factors such as humidity and canopy temperature as well as morphological factors such as plant height and heading date [21]. A diverse array of methods have been developed to measure the disease phenotype in rice in response to *Rs*. Many methods exist possibly because the phenotype is variable and difficult to measure.

It has been observed that taller plants are less susceptible to *Rs* [21, 37, 38]. Both silenced lines (oxo-14-19-23, oxo-14-19-30) and several overexpression lines (UbiOxo-17-106, UbiOxo-14-56, UbiOxo-17-93, UbiOxo-17-76) germinated faster than the other lines which could affect the scoring of the *Rs* microchamber assay in Figure 4.4. However, the lesion length is calculated as a percent of the plant height so this should control for that factor. Additionally, there is a positive relationship between plant height and lesion length ($r = 0.27$, $p<0.01$), and if taller plants are less susceptible, a negative relationship would have been found.

Variation in disease responses of *OsOXO*4 overexpression lines was high, with some lines exhibiting more disease, and others with less. This was true with the detached *Rs* assay (Figure 4.9) and the microchamber assay (Figure 4.10). One line, UbiOxo-17-106, was segregating for the overexpression activity, but this did not translate into different responses to the *Rs* pathogen in the microchamber assay. One overexpression line, UbiOxo10-41, was significantly different than Kitaake (Tukey adjusted F-test of all lines, p=0.03) in the microchamber assay. In addition, the variation in Kitaake response to *Rs* was quite high in both the *Rs* microchamber (45 ±12% SD) and *Rs* detached leaf assays (38 ±26% SD). Conducting a power analysis to estimate the sample size needed to detect a 27% effect size—the best case scenario from previous work on the QTL with which *OXO* is associated—proposes a sample size of 22 per group (silenced, control, overexpression) in order to determine if significant differences exist with a power of 0.80. This sample size is larger than used in this study, but this information could be used to plan follow-up studies.

Interest in the *OXO* family of genes centered around several studies summarized in Table 4.1 which reported QTL in the region of rice chromosome 3 where the *OXO* genes are located. However the phenotypic variance in response to various pathogens ranged from 3-27% of the total phenotypic variance. In fact, the two studies at the ends of this range are with the same population (Lemont x Jasmine85) with the same pathogen (*Rs*); the major difference being the environments. In the field, Zou *et al.* [39] report a phenotypic variance of 27% while in the GH, Liu *et al.* [40] report phenotypic variances of 3.0-3.7% depending on the markers used. Differences in temperature and humidity conditions also influence disease [41], so taken together, we cannot rule out that our findings for *Rs* are related to differences in the genetic background of the rice cultivars, pathogens, or due to the environmental conditions in which the assays were conducted. The *OsOXO*4 allele in Kitaake is not the most 'effective' allele, as

it does not contain a promoter insert associated with increased OXO activity and expression found in varieties such as Moroborekan with enhanced resistance [18]. Thus, when comparing disease resistance of silenced lines to *Rs*, the quantitative differences might be more difficult to detect compared to a population with and without the promoter insert. Furthermore, it is reasonable that the virulent strain of *Rs* used in the current study, through close evolution with rice, adapted to overcome defense responses related to *OsOXO*4 expression. We did not evaluate other rice strains or *Rs* from other hosts.

4.4.2. Future directions

Variation in the promoter sequence influences the effectiveness of defense response genes [2, 42] and this is an area for future research with *OXO* genes. Silencing and overexpressing *OXO* genes in different varieties of rice has recently been reported [12, 19], but the role of the endogenous *OXO* genes in the varieties used in these studies has not been established; this variation might impact gene expression. If, for example, in the varieties used in [12, 19], the promoters are identical to Kitaake, we would expect the *OXO* genes to have little overall contribution to defense. On the other hand, if the promoter includes the insertion associated with the enhanced resistance of Moroborekan [18], we would expect enhanced defense function in wild type, and more pronounced differences in silenced lines of those varieties.

4.4.3. Conclusion

Silencing of *OsOXO*s in rice cultivar Kitaake rendered the rice more susceptible to the non-host, oxalate-producing pathogen *ScS*, while overexpression of the *OsOXO*4 gene led to plants that were more resistant to *ScS*. In contrast, silencing of *OsOXOs* did not significantly increase susceptibility and overexpression did not increase resistance of Kitaake to the rice

sheath blight pathogen, *Rs*, an aggressive necrotrophic pathogen of rice that does not rely on production of oxalate as a virulence factor. However there are many factors to consider, and *OXOs* may play a minor role in *Rs* disease.

Understanding the roles of the *OXO* gene family will enable focused breeding and biotechnology efforts to enhance disease resistance as well as possibly optimize cell wall composition. More generally, crops with enhanced disease resistance traits allow more grain and more straw production which is necessary to support a growing population with growing energy needs.

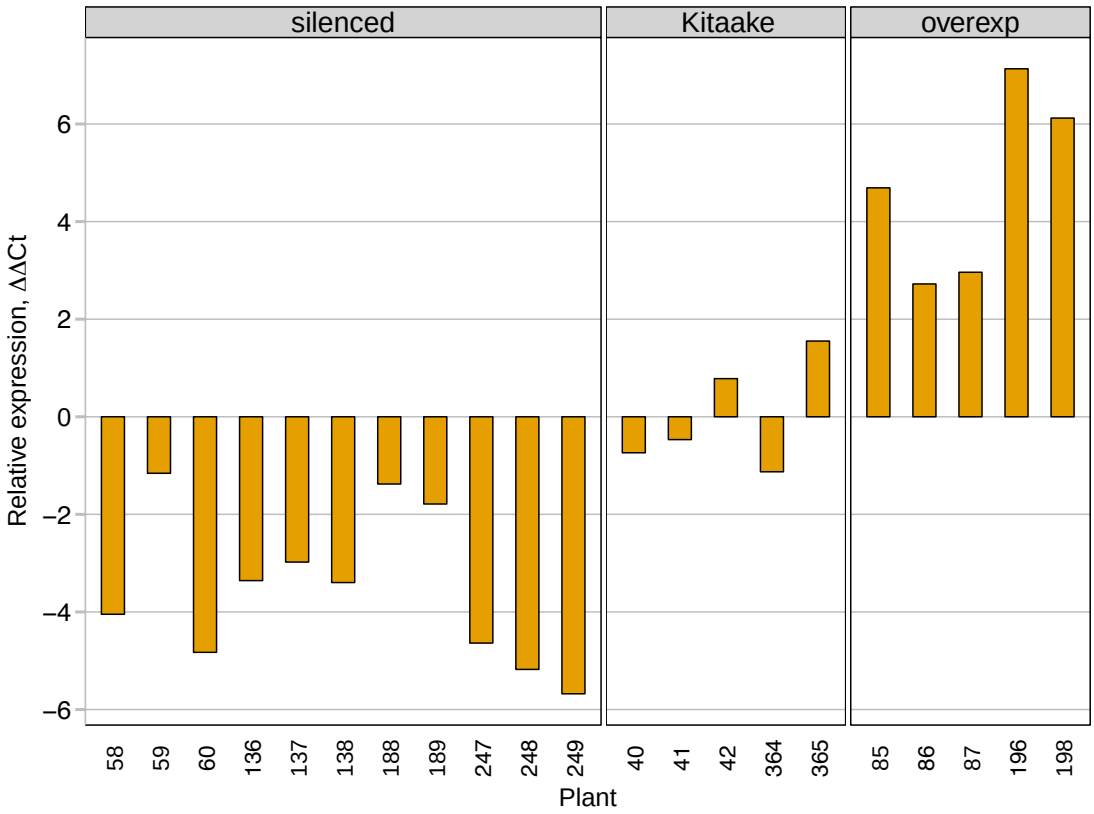


FIGURE 4.1. Expression of *OXO* in transgenic lines relative to the mean expression of wild type Kitaake plants. Each number represents a plant from different transgenic lines used in the studies.

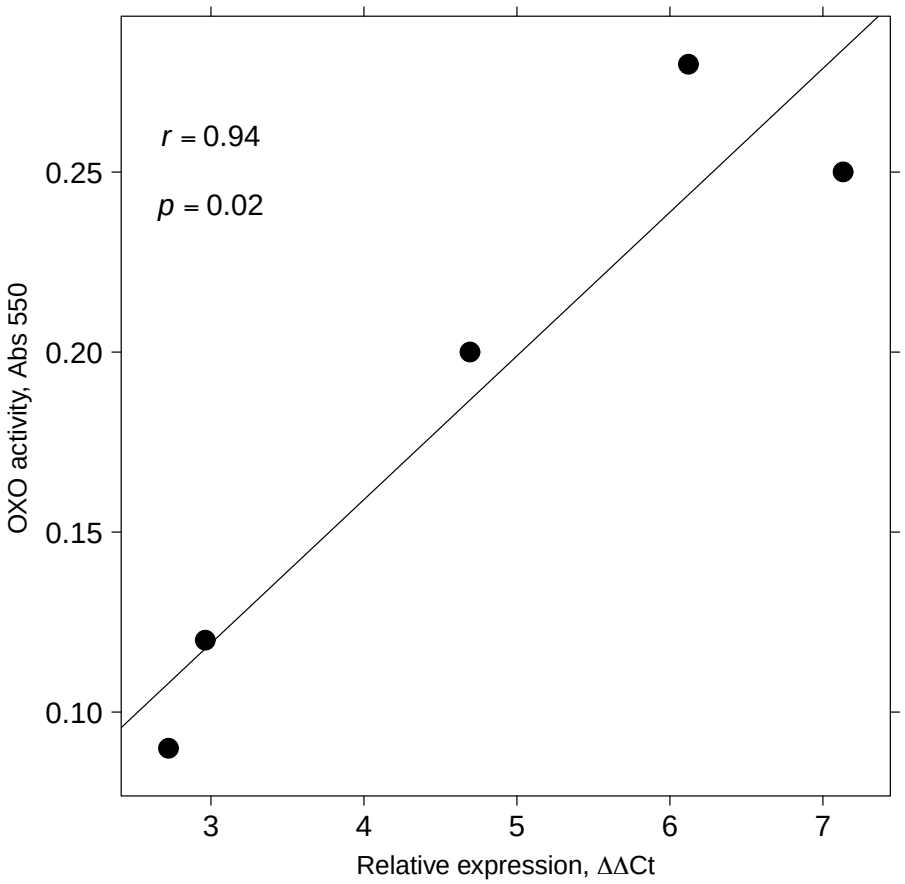


FIGURE 4.2. Scatterplot and Pearson's correlations of OXO activity and relative *OsOXO*4 expression measured on the five overexpression lines from Figure 4.1.

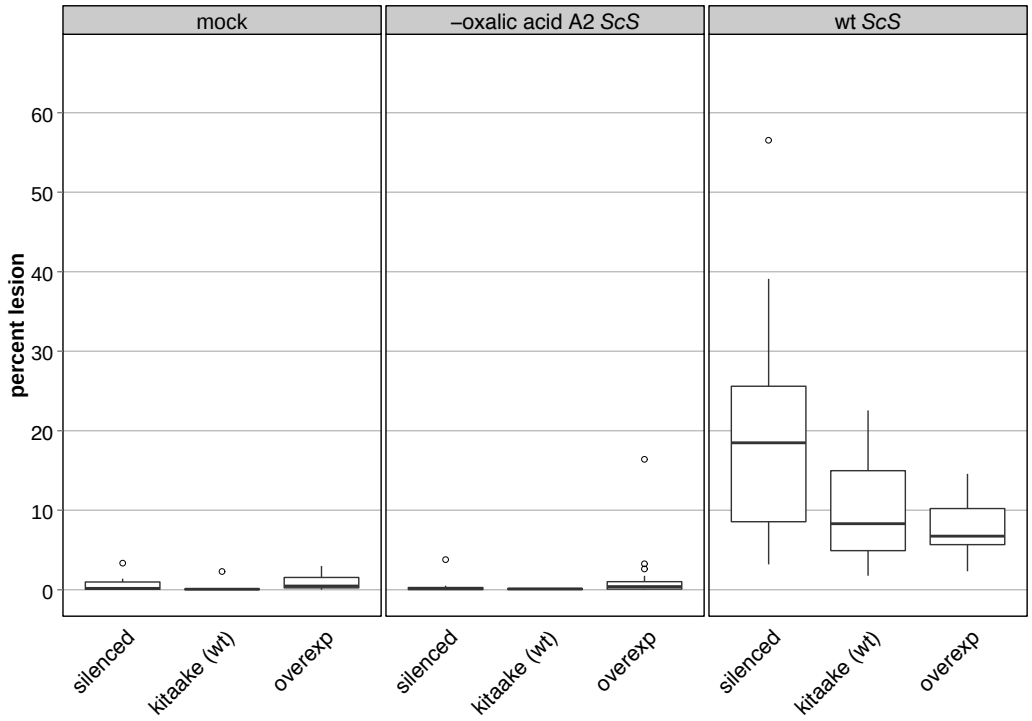


FIGURE 4.3. Boxplots comparing percent lesion development from detached leaf assays for Kitaake control, *OsOXO* silenced lines, and *OsOXO*4 overexpression lines after inoculation with *ScS* isolate N 1980 (wt) or the A-2 mutant, or a mock inoculation (agar plug). Kitaake n=6 plants, overexpression lines n=20 T2 plants, silenced lines, n=11 T3 plants. The disease of silenced and overexpression groups were significantly different between the A2 mutant and the wt *ScS* (two-tailed unequal variance t-test, $p<0.03$).

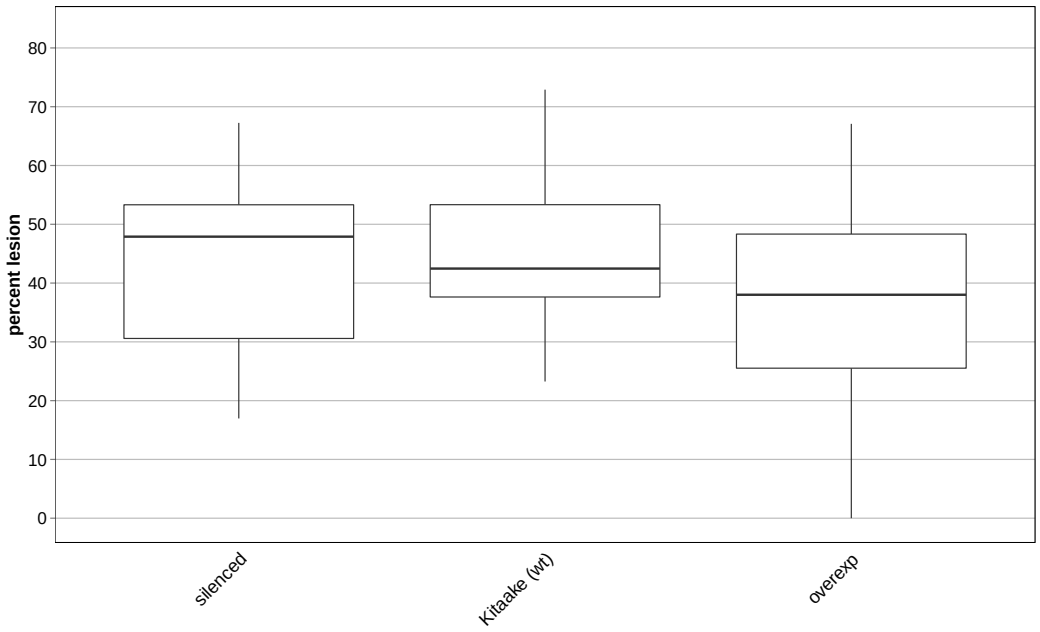


FIGURE 4.4. Boxplots comparing percent lesion development in microchamber assays for Kitaake control, *OsOXO* silenced lines, and *OsOXO*4 overexpression lines after inoculation with *Rs*. Kitaake n=19, silenced lines n=39, overexpression lines n=65. Mock treatments exhibited no lesion development and are not shown. Silenced lines were not different than Kitaake control (p=0.75) with a two-tailed unequal variance t-test, but overexpression lines were different than Kitaake control (p=0.01).

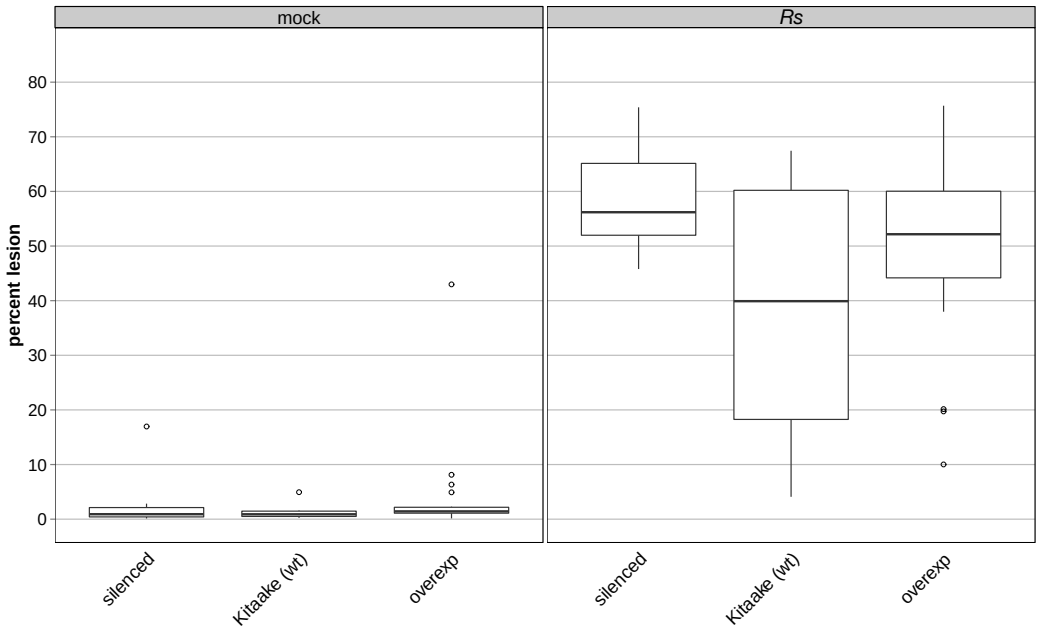


FIGURE 4.5. Boxplots comparing percent lesion development in detached leaf *Rs* assays for Kitaake control, *OsOXO* silenced lines, and *OsOXO*4 overexpression lines after inoculation with *Rs*. Kitaake n=6, Silenced n=10, overexpression n=19. Neither silenced nor overexpression lines were different than controls (two-tailed unequal variance t-test, silenced p=0.11 and overexpression p=0.34).

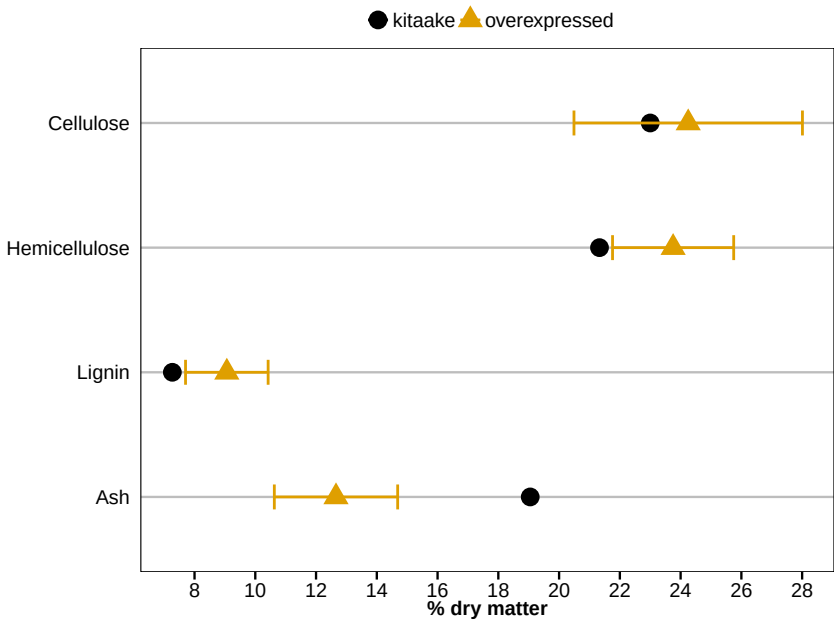


FIGURE 4.6. Composition of stem tissue of Kitaake (n=1) and UbiOxo-17 T1 plants (n=3). Bars ±SD of overexpression plants.

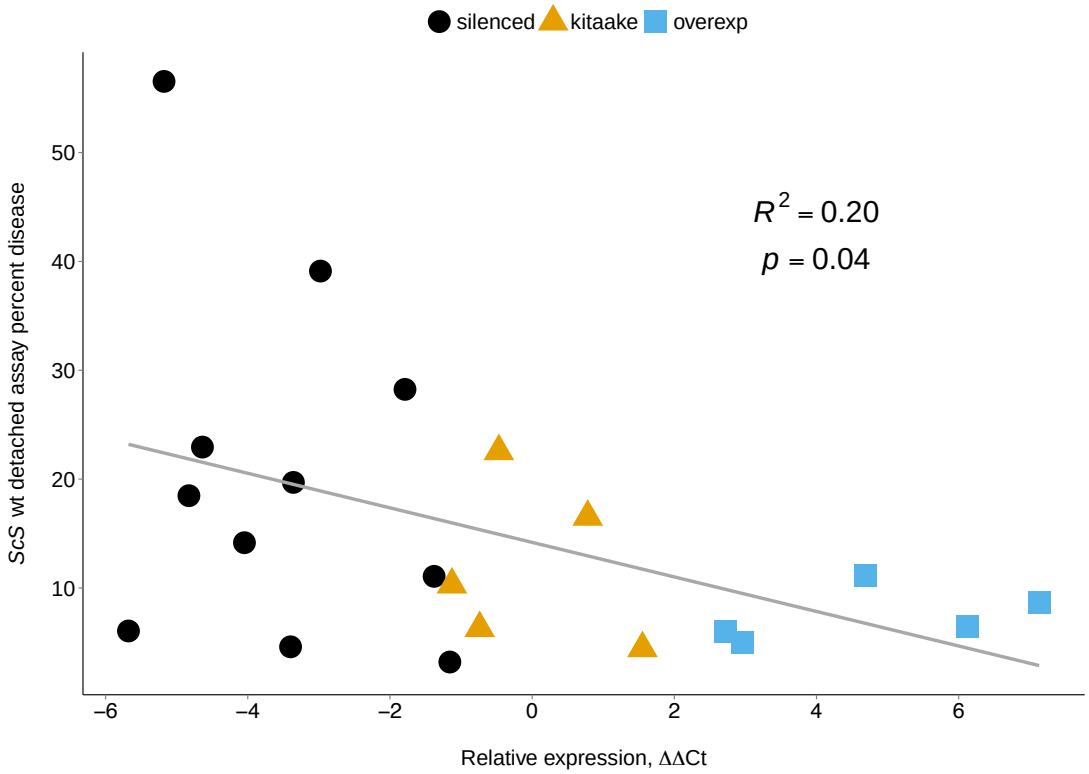


FIGURE 4.7. Relationship between *OXO* expression and detached leaf *ScS* percent lesion disease.

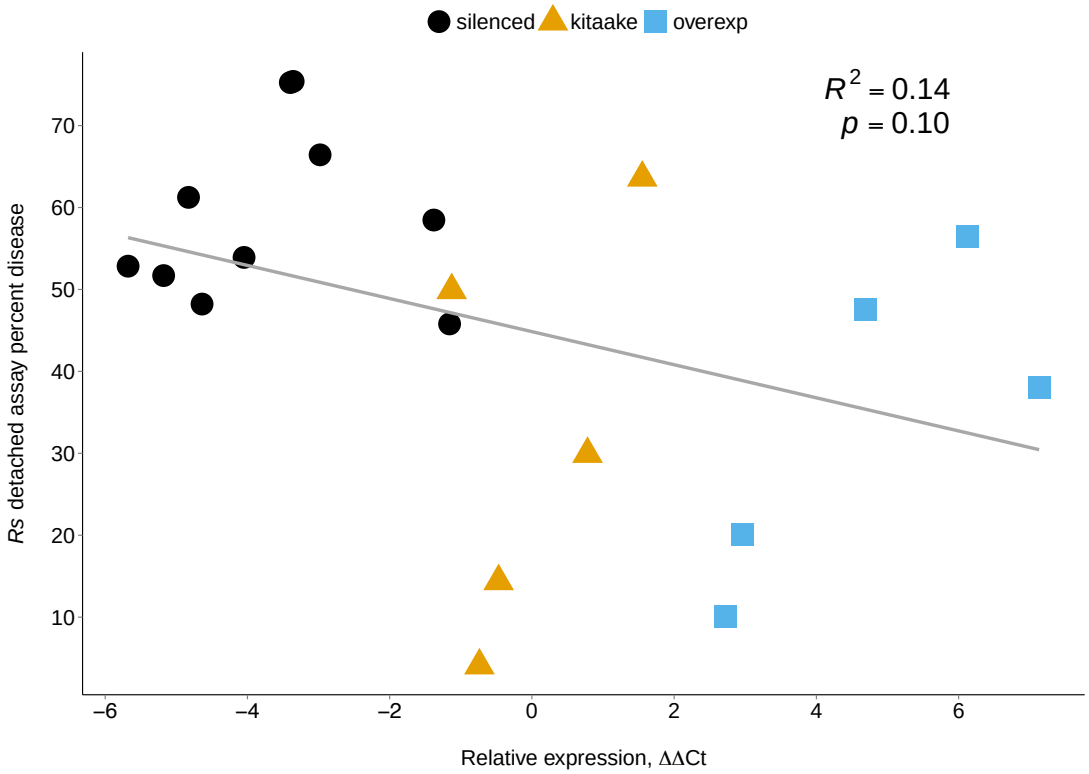


FIGURE 4.8. Relationship between *OXO* expression and detached leaf *Rs* percent lesion disease.

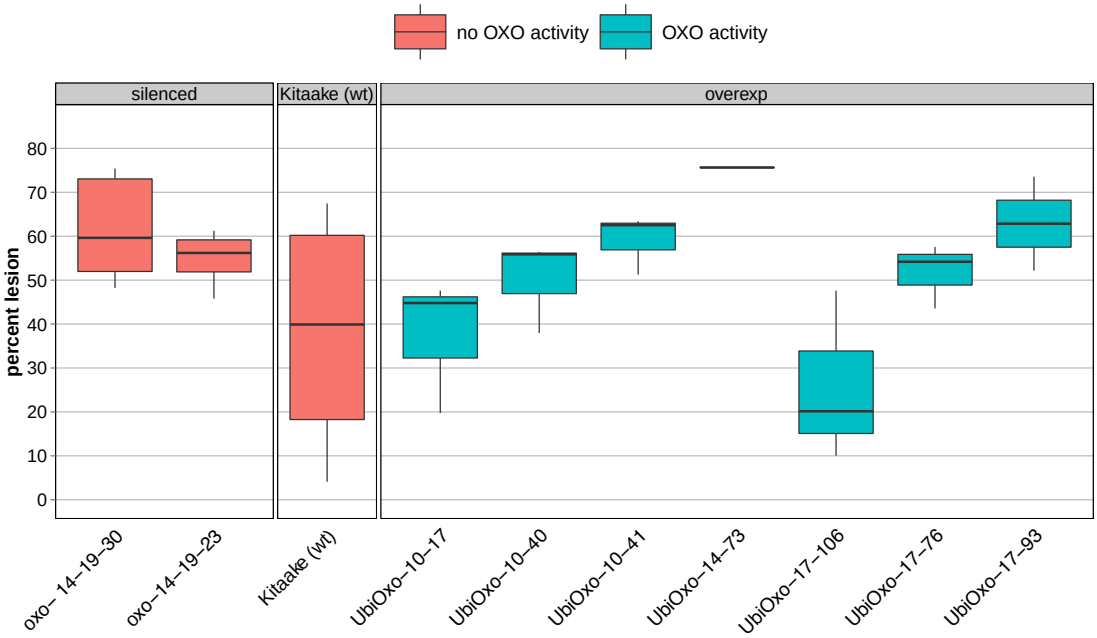


FIGURE 4.9. Sheath blight disease development with detached leaf *Rs* inoculation plotted for each line. n=2-3 per overexpression line.

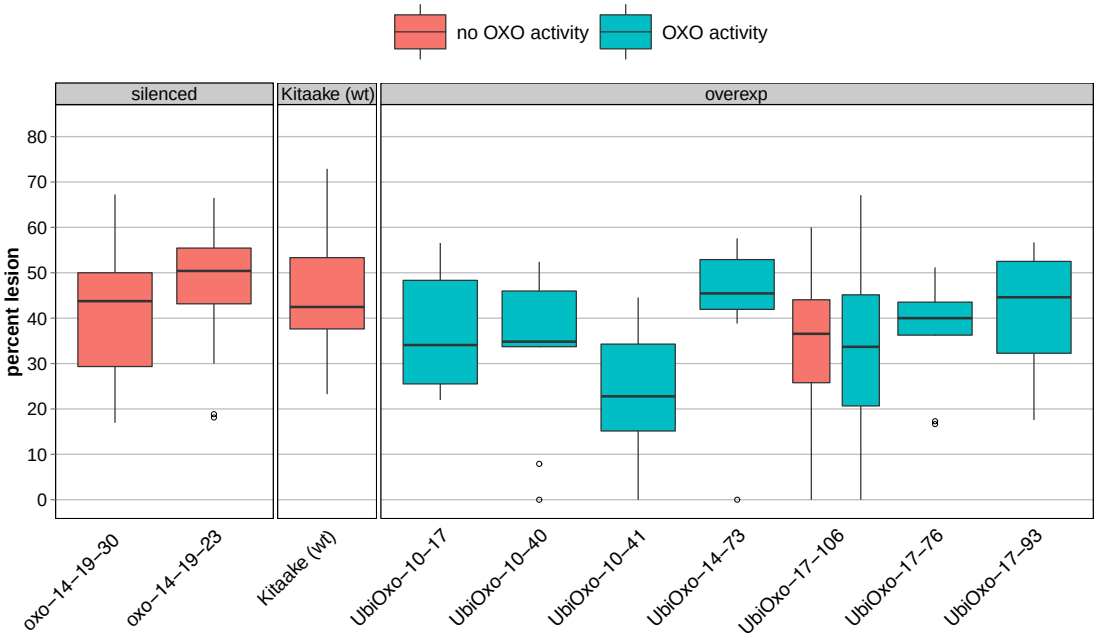


FIGURE 4.10. Sheath blight disease development with microchamber *Rs* inoculation plotted for each line. n=9-11 per overexpression line.

Table 4.1. Summary of QTL reported in literature in the region of rice chromosome 3 that contains the *OsOXO* genes.

Disease	Population	R^2 (%)	Marker interval	Reference
bacterial blight	Lemont x Teqing	not reported	RG482-CDO795	[43]
sheath blight	Lemont x Jasmine85	26.5	C746-R250	[39]
rice blast	CT9993 x Khao Dawk Mali 105	6	RM16-RM168	[44]
rice blast	Lemont x Teqing	10.9-12.9	RZ474	[45]
rice blast	Zhenshan97 x Minghui 63	3.2	R19-RZ403	[46]
bacterial blight	IR64 x Azucena	10.3	CDO337-OXO (Acc.Y14203)	[47]
rice blast	Vandana x Moro	10.73	RM168	[48]
sheath blight	Lemont x Jasmine85	3.7, 3.3, 3	RM16-RM426, RM5626-RM426, RM514-RM85	[40]
sheath blight	Lemont x Teqing	10	RZ474	[37]
sheath blight	Lemont x Jasmine85	26.1	C746-R250	[49]
sheath blight	Zhai Ye Qing 8 x Jing Xi 17	10-10.5	G249-G164	[50]
sheath blight	WSS2 (Tetep) x Hinohikari	19.4	RM3856	[51]

REFERENCES

[1] Malinovsky, F. G., Fangel, J. U., and Willats, W. G. T., 2014. The role of the cell wall in plant immunity. *Frontiers in Plant Science*, 5, May:178

[2] Davidson, R. M., Reeves, P. A., Manosalva, P. M., and Leach, J. E., 2009. Germins: A diverse protein family important for crop improvement. *Plant Science*, 177:499–510

[3] Donaldson, P. A., Anderson, T., Lane, B. G., Davidson, A. L., and Simmonds, D. H., 2001. Soybean plants expressing an active oligomeric oxalate oxidase from the wheat gf-2.8 (germin) gene are resistant to the oxalate-secreting pathogen Sclerotina sclerotiorum. *Physiological and Molecular Plant Pathology*, 59, 6:297–307

[4] Hu, X., Bidney, D. L., Yalpani, N., Duvick, J. P., Crasta, O., Folkerts, O., and Lu, G., 2003. Overexpression of a gene encoding hydrogen peroxide-generating oxalate oxidase evokes defense responses in sunflower. *Plant Physiology*, 133, 1:170–181

[5] Liang, H., Maynard, C. A., Allen, R. D., and Powell, W. A., 2001. Increased Septoria musiva resistance in transgenic hybrid poplar leaves expressing a wheat oxalate oxidase gene. *Plant Molecular Biology*, 45, 6:619–629

[6] Livingstone, D. M., Hampton, J. L., Phipps, P. M., and Grabau, E. A., 2005. Enhancing Resistance to Sclerotinia minor in Peanut by Expressing a Barley Oxalate Oxidase Gene. *Plant Physiology*, 137, 4:1354–1362

[7] Thompson, C., Dunwell, J. M., Johnstone, C. E., Lay, V., Ray, J., Schmitt, M., Watson, H., and Nisbet, G., 1995. Degradation of oxalic acid by transgenic oilseed rape plants expressing oxalate oxidase. *Euphytica*, 85, 1-3:169–172

[8] Welch, A., Stipanovic, A., Maynard, C., and Powell, W., 2007. The effects of oxalic acid on transgenic Castanea dentata callus tissue expressing oxalate oxidase. *Plant Science*,

172, 3:488–496

[9] Lane, B., 1994. Oxalate, germin, and the extracellular matrix of higher plants. *The FASEB Journal*, 8, 3:294 –301

[10] Dumas, B., Freyssinet, G., and Pallett, K. E., 1995. Tissue-Specific Expression of Germin-Like Oxalate Oxidase during Development and Fungal Infection of Barley Seedlings. *Plant Physiology*, 107, 4:1091–1096

[11] Berna, A. and Bernier, F., 1997. Regulated expression of a wheat germin gene in tobacco: oxalate oxidase activity and apoplastic localization of the heterologous protein. *Plant Molecular Biology*, 33, 3:417–429

[12] Zhang, X. Y., Nie, Z. H., Wang, W. J., Leung, D. W. M., Xu, D. G., Chen, B. L., Chen, Z., Zeng, L. X., and Liu, E. E., 2013. Relationship between Disease Resistance and Rice Oxalate Oxidases in Transgenic Rice. *PLoS ONE*, 8, 10:e78348

[13] Williams, B., Kabbage, M., Kim, H.-J., Britt, R., and Dickman, M. B., 2011. Tipping the Balance: Sclerotinia sclerotiorum Secreted Oxalic Acid Suppresses Host Defenses by Manipulating the Host Redox Environment. *PLoS Pathology*, 7, 6:e1002107

[14] Nagarajkumar, M., Jayaraj, J., Muthukrishnan, S., Bhaskaran, R., and Velazhahan, R., 2005. Detoxification of oxalic acid by pseudomonas fluorescens strain pfMDU2: implications for the biological control of rice sheath blight caused by Rhizoctonia solani. *Microbiological Research*, 160, 3:291–298

[15] Torres, M. A., Jones, J. D., and Dangl, J. L., 2006. Reactive oxygen species signaling in response to pathogens. *Plant Physiology*, 141, 2:373

[16] Bhuiyan, N. H., Selvaraj, G., Wei, Y., and King, J., 2009. Gene expression profiling and silencing reveal that monolignol biosynthesis plays a critical role in penetration defence in wheat against powdery mildew invasion. *Journal of Experimental Botany*, 60, 2:509

–521

[17] Thomma, B. P., Nürnberger, T., and Joosten, M. H., 2011. Of PAMPs and Effectors: The Blurred PTI-ETI Dichotomy. *The Plant Cell*, 23, 1:4 –15

[18] Carrillo, M. G. C., Goodwin, P. H., Leach, J. E., Leung, H., and Vera Cruz, C., 2009. Phylogenomic Relationships of Rice Oxalate Oxidases to the Cupin Superfamily and Their Association with Disease Resistance QTL. *Rice*, 2:67–79

[19] Molla, K. A., Karmakar, S., Chanda, P. K., Ghosh, S., Sarkar, S. N., Datta, S. K., and Datta, K., 2013. Rice oxalate oxidase gene driven by green tissue-specific promoter increases tolerance to sheath blight pathogen (Rhizoctonia solani) in transgenic rice. *Molecular Plant Pathology*, 14, 9:910–922

[20] Bolton, M. D., Thomma, B. P., and Nelson, B. D., 2006. Sclerotinia sclerotiorum (Lib .) de Bary : biology and molecular traits of a cosmopolitan pathogen. *Molecular Plant Pathology*, 7:1–16

[21] Srinivasachary, Willocquet, L., and Savary, S., 2010. Resistance to rice sheath blight (Rhizoctonia solani Kühn) [(teleomorph: Thanatephorus cucumeris (A.B. Frank) Donk.] disease: current status and perspectives. *Euphytica*, 178, 1:1–22

[22] Zheng, A., Lin, R., Zhang, D., Qin, P., Xu, L., Ai, P., Ding, L., Wang, Y., Chen, Y., Liu, Y., Sun, Z., Feng, H., Liang, X., Fu, R., Tang, C., Li, Q., Zhang, J., Xie, Z., Deng, Q., Li, S., Wang, S., Zhu, J., Wang, L., Liu, H., and Li, P., 2013. The evolution and pathogenic mechanisms of the rice sheath blight pathogen. *Nature Communications*, 4, May 2012:1424

[23] Chern, M., Fitzgerald, H. A., Canlas, P. E., Navarre, D. A., and Ronald, P. C., 2005. Overexpression of a Rice NPR1 Homolog Leads to Constitutive Activation of Defense Response and Hypersensitivity to Light. *Molecular Plant-Microbe Interactions*, 18,

6:511–520

[24] Hoekema, A., Hirsch, P. R., Hooykaas, P. J. J., and Schilperoort, R. A., 1983. A binary plant vector strategy based on separation of vir- and T-region of the Agrobacterium tumefaciens Ti-plasmid. *Nature*, 303, 5913:179–180

[25] Cheng, X., Sardana, R. K., and Altosaar, I., 1997. Rice Transformation by Agrobacterium Infection. In C. Cunningham and A. J. R. Porter, eds., *Methods in Biotechnology, Vol. 3: Recombinant Proteins from Plants: Production and Isolation of Clinically Useful Compounds*, vol. 3 of *Methods in Biotechnology*. Totowa, NJ: Humana Press

[26] Chen, D. and Ronald, P. C., 1999. A Rapid DNA Minipreparation Method Suitable for AFLP and Other PCR Applications. *Plant Molecular Biology Reporter*, 17:53–57

[27] Miki, D. and Shimamoto, K., 2004. Simple RNAi vectors for stable and transient suppression of gene function in rice. *Plant & cell physiology*, 45, 4:490–5

[28] Hood, E. E., Gelvin, S. B., Melchers, L. S., and Hoekema, A., 1993. NewAgrobacterium helper plasmids for gene transfer to plants. *Transgenic Research*, 2, 4:208–218

[29] Wamishe, Y. A., Yulin, J., Singh, P., and Cartwright, R. D., 2007. Identification of field isolates of Rhizoctonia solani to detect quantitative resistance in rice under greenhouse conditions. *Frontiers of Agriculture in China*, 1, 4:361–367

[30] Jia, Y., Correa-Victoria, F., McClung, A., Zhu, L., Liu, G., Wamishe, Y., Xie, J., Marchetti, M. A., Pinson, S. R. M., Rutger, J. N., and Correll, J. C., 2007. Rapid Determination of Rice Cultivar Responses to the Sheath Blight Pathogen Rhizoctonia solani Using a Micro-Chamber Screening Method. *Plant Disease*, 91, 5:485–489

[31] Godoy, G., Steadman, J., Dickman, M., and Dam, R., 1990. Use of mutants to demonstrate the role of oxalic acid in pathogenicity of Sclerotinia sclerotiorum on Phaseolus vulgaris. *Physiological and Molecular Plant Pathology*, 37, 3:179–191

[32] Singh, A., Rohilla, R., Singh, U. S., Savary, S., Willocquet, L., and Duveiller, E., 2002. An improved inoculation technique for sheath blight of rice caused by Rhizoctonia solani. *Canadian Journal of Plant Pathology*, 24, 1:65–68

[33] Jain, M., Nijhawan, A., Tyagi, A. K., and Khurana, J. P., 2006. Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. *Biochemical and Biophysical Research Communications*, 345, 2:646–651

[34] Livak, K. J. and Schmittgen, T. D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2ΔΔCT Method. *Methods*, 25:402–408

[35] R core Team, 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria

[36] Liu, J., Zhang, Y., Lei, X., and Zhang, Z., 2008. Natural selection of protein structural and functional properties: a single nucleotide polymorphism perspective. *Genome Biology*, 9, 4:R69

[37] Pinson, S. R. M., Capdevielle, F. M., and Oard, J. H., 2005. Blight Resistance in Rice Using Recombinant Inbred Lines. *Crop Science*, 45, 2:503–510

[38] Sharma, A., McClung, A. M., Pinson, S. R. M., Kepiro, J. L., a. Robert Shank, Tabien, R. E., and Fjellstrom, R., 2009. Genetic Mapping of Sheath Blight Resistance QTLs within Tropical Rice Cultivars. *Crop Science*, 49, 1:256

[39] Zou, J. H., Pan, X. B., Chen, Z. X., Xu, J. Y., Lu, J. F., Zhai, W. X., and Zhu, L. H., 2000. Mapping quantitative trait loci controlling sheath blight resistance in two rice cultivars (Oryza sativa L.). *Theoretical and Applied Genetics*, 101, 4:569–573

[40] Liu, G., Jia, Y., Prado, G. A., Yeater, K. M., Mcclung, A., and Correll, J. C., 2009. Mapping Quantitative Trait Loci for Resistance to Sheath Blight in Rice. *Phytopathology*, 99:1078–1084

[41] Webb, K. M., Oña, I., Bai, J., Garrett, K. a., Mew, T., Vera Cruz, C. M., and Leach, J. E., 2010. A benefit of high temperature: increased effectiveness of a rice bacterial blight disease resistance gene. *The New Phytologist*, 185, 2:568–76

[42] Manosalva, P. M., Davidson, R. M., Liu, B., Zhu, X., Hulbert, S. H., Leung, H., and Leach, J. E., 2009. A germin-like protein gene family functions as a complex quantitative trait locus conferring broad-spectrum disease resistance in rice. *Plant Physiology*, 149, 1:286

[43] Li, Z. K., Pinson, S. R. M., Marchetti, M. A., Stansel, J. W., and Park, W. D., 1995. Characterization of quantitative trait loci (QTLs) in cultivated rice contributing to field resistance to sheath blight (Rhizoctonia solani). *Theoretical and Applied Genetics*, 91, 2:382–388

[44] Sirithunya, P., Tragoonrung, S., Vanavichit, A., Pa-In, N., Vongsaprom, C., and Toojinda, T., 2002. Quantitative trait loci associated with leaf and neck blast resistance in recombinant inbred line population of rice (Oryza sativa). *DNA Research*, 9, 3:79–88

[45] Tabien, R. E., Li, Z., Paterson, A. H., Marchetti, M. A., Stansel, J. W., and Pinson, S. R. M., 2002. Mapping QTLs for field resistance to the rice blast pathogen and evaluating their individual and combined utility in improved varieties. *Theoretical and Applied Genetics*, 105, 2-3:313–324

[46] Chen, H., Wang, S. P., Xing, Y. Z., Xu, C. G., Hayes, P. M., and Zhang, Q. F., 2003. Comparative analyses of genomic locations and race specificities of loci for quantitative resistance to Pyricularia grisea in rice and barley. *Proceedings of the National Academy of Sciences*, 100, 5:2544–2549

[47] Ramalingam, J., Cruz, C. M. V., Kukreja, K., Chittoor, J. M., Wu, J. L., Lee, S. W., Baraoidan, M., George, M. L., Cohen, M. B., Hulbert, S. H., Leach, J. E., and Leung, H.,

2003. Candidate Defense genes from rice, barley, and maize and their association with qualitative and quantitative resistance in rice. *Molecular Plant-Microbe Interactions*, 16, 1:14–24

[48] Wu, J. L., Sinha, P. K., Variar, M., Zheng, K. L., Leach, J. E., Courtois, B., and Leung, H., 2004. Association between molecular markers and blast resistance in an advanced backcross population of rice. *Theoretical and Applied Genetics*, 108, 6:1024–1032

[49] Pan, X., Zou, J., Chen, Z., Lu, J., Yu, H., Li, H., Wang, Z., Pan, X., Rush, M. C., and Zhu, L., 1999. Tagging major quantitative trait loci for sheath blight resistance in a rice variety, Jasmine 85. *Chinese Science Bulletin*, 44, 19:1783–1789

[50] Kunihiro, Y., Qian, Q., Sato, H., Teng, S., Zeng, D.-L., Fujimoto, K., and Zhu, L.-H., 2002. [QTL analysis of sheath blight resistance in rice (Oryza sativa L.)]. *Yi chuan xue bao = Acta genetica Sinica*, 29, 1:50–5

[51] Sato, H., Ideta, O., Ando, I., Kunihiro, Y., Hirabayashi, H., Iwano, M., Miyasaka, A., Nemoto, H., and Imbe, T., 2004. Mapping QTLs for Sheath Blight Resistance in the Rice Line WSS2. *Breeding Science*, 54, 3:265–271

CHAPTER 5

Conclusion

Sustainable bioenergy will require careful pairing of optimized crops and processing technologies. This dissertation assesses the potential of rice straw as a bioenergy resource by characterizing the diversity of cell wall composition, validating the function of a potentially important cell wall-modifying protein in plant defense, and characterizing a mapping population with useful bioenergy traits. Viable bioenergy systems will be defined by available land, resources, and the type of fuels needed in each region. Breeding crops optimized for bioenergy processing first requires knowledge about the important traits. Understanding the genetic basis of these traits would allow targeting of genes and transcription factors that influence the trait. In the absence of this knowledge, an assessment of the natural variation in the trait would allow traditional breeding approaches to select the best lines or varieties. While much progress has been made, improving crops for bioenergy is still in its infancy and enormous potential exists.

A diverse set of technologies exists to convert biomass into usable energy. Each technology has advantages and disadvantages and different parameters that can be optimized. These technologies have certain requirements of biomass feedstocks, and these requirements are best understood in the context of what types of biomass can be produced. For example, enzymatic processing requires biomass in which the enzymes can easily access their substrates, while thermochemical processing requires minimal ash levels or the ash can foul and clog the equipment at high temperatures.

In chapter 2, the two major types of bioenergy conversion technologies are reviewed and the important parameters for thermochemical conversion technologies are defined. While enzymatic conversion seeks to minimize lignin content and maximize convertible sugars in the biomass, ideal biomass for thermochemical conversion has high lignin and bulk density and low moisture and ash. These traits, and their variation across several potential bioenergy feedstocks are described and the genetic control of these traits is reviewed. Large variation exists for most of these traits. Biologists and engineers essentially measure the same traits, but use different methods and language. Biologists discuss biomass in terms of biochemical molecules such as cellulose, hemicellulose, and lignin, while process engineers consider biomass as ratios of C, H, O, and minerals. Because biologists think in terms of molecules, most genetic characterization of traits is ratios of cellulose and lignin. However, the more relevant traits for thermochemical conversion are abstract traits such as higher heating value (HHV) and C:O ratios. Methods are reviewed for efficiently measuring these traits, each with certain advantages and disadvantages. Nondestructive methods such as infrared (IR) and near-infrared reflectance (NIR) hold great potential to predict a range of compositional parameters, as well as elemental analyzers that measure C and O content of the biomass. Applying these methods to phenotype biomass will help identify the genes that regulate these traits.

The diversity of processing technologies is dwarfed by the diversity of biomass produced by different species in the plant kingdom. The heterogeneity of the plant cell wall demands flexibility in the conversion technologies, and some types of biomass are more compatible with certain conversion technologies. Understanding the diversity in biomass composition will enable the pairing of the most compatible biomass crops with each technology. Furthermore,

with the knowledge of what parameters to optimize, plant breeders will have well-defined targets for new varieties.

In chapter 3, some of the methods discussed in chapter 2 are applied to a diverse set of rice varieties grown in two environments to assess the variation in bioenergy traits of rice straw. The focus of this study is understanding the relationships between composition and enzymatic bioenergy potential, and to estimate how influential the environment and tissue type (leaf vs stem) are on these parameters and relationships. For cellulose, hemicellulose, lignin, ash, total glucose and glucose yield, I find a large influence of both environment and tissue type, and while varietal differences existed, the variation was relatively small. I confirm the negative relationships between lignin and glucose yield, as well as a negative relationship between ash content and glucose yield. Measurements made in the greenhouse environment may overestimate the bioenergy potential of the same varieties in field conditions. Thus, it is critical to assess bioenergy potential in more than one environment. Beyond direct measures of bioenergy yield, such as glucose yield, the best approach, at least in rice, might be to measure and breed for reduced ash content. Few significant relationships between composition and bioenergy yield were maintained across both environments, and I therefore hypothesize that cell wall architecture is more important than composition *per se* for bioenergy. Clearly, composition needs to be assessed in multiple environments and new techniques to assay the cell wall architecture should be employed at the same time.

Notwithstanding the importance of the cell wall in bioenergy production, it is not the only factor. The viability of the crop depends on a suite of agronomic traits to allow plants to maximize the use of available resources such as light, water, and nutrients while avoiding damage from environment stress, pests, and diseases. Traits important for all crops include predictable flowering time, optimized plant height to avoid lodging, and increasing total

biomass. Additionally, disease resistance traits minimize losses and help understand the complex interactions that drive plant-pathogen relationships.

In chapter 4, a family of four oxalate oxidase *OsOXO* genes proposed to play a role in broad spectrum disease resistance are characterized using transgenic approaches. *OsOXOs* are predicted to enhance resistance by enhancing cell wall structure and/or degrading oxalic acid used by some fungi as a pathogenicity factor. A unique system was developed to assess how oxalic acid influences susceptibility and resistance in rice lines silenced for the *OXO* genes, and rice lines constitutively overexpressing *OsOXO*4. This system consisted of a wild type (wt) *ScS* pathogen which produced oxalic acid, and a mutant strain unable to produce oxalic acid. A high throughput method for quickly assessing the OXO activity in the overexpression lines was optimized using rice leaf discs. Silenced lines were more susceptible to the wt *ScS* because they lacked the ability to degrade oxalic acid, while overexpression lines were more resistant because they were able to more quickly degrade oxalic acid. In the absence of oxalic acid (when inoculated with the mutant *ScS* strain), the pathogen was unable to produce disease on any of the lines, and there was no difference between silenced, wt, and overexpression lines. To test the broad spectrum resistance of this family of genes, disease assays were performed with *Rs*, another necrotrophic fungal pathogen of rice, but one that does not rely on oxalic acid. Differences between the transgenic lines and wt were less clear in response to *Rs*, but many factors could be influencing these results. The line, the strain, the environmental conditions, and the close evolution of plant and pathogen could influence the resistance or susceptibility. Overall, this study confirms the hypothesis that monocots are resistant to pathogens like *ScS* because they maintain *OXOs* that degrade the essential pathogenicity factor, oxalic acid. This system could be the basis of further studies that investigate how each *OXO* gene functions in different tissues and timepoints.

In Appendix A, phenotyping and genotyping of a large mapping population reveal potential for QTL mapping for several bioenergy traits. These bioenergy traits were observed to vary in the parents of this population, and transgressive segregation in the population is evident for biomass, height, grain weight, flowering time, glucose yield and pentose yield. This phenotyping was performed in the field, over two seasons, and high correlations between seasons were observed, indicating a high degree of genetic control of these traits. The QTL mapping is currently underway.

In summary, this work provides the tools to assess and breed for improved bioenergy traits in rice straw. This basic knowledge is currently lacking, not only for established crops like rice, but for species proposed as dedicated bioenergy crops such as switchgrass and miscanthus. With the methods used here, the conclusions outlined here can be validated in more varieties and species, and across more environments. The QTL will lead to the identification of important genes controlling these traits and through comparative genomics these genes can be identified in other species. At the same time, pathways to utilize agricultural wastes like rice straw and corn stover will minimize resources and maximize agriculture: a complete harvest. Expanding and generalizing the lessons learned will enable the development of targeted breeding programs and economical, sustainable bioenergy.

APPENDIX A

QTL FOR BIOMASS AND BIOENERGY TRAITS IN A RICE MAPPING POPULATION [4]

Overview

As rice yields plateau, new strategies are needed to develop the next generation of rice varieties. These strategies include new plant architecture, and larger plants that resist lodging to allow optimal photosynthesis and grain yields. Potential uses of agricultural residues such as rice straw, corn stover and wheat straw are animal fodder or bioenergy—both end uses benefit from increased enzymatic digestibility, which ultimately depends on the composition of the plant cell walls in the biomass. We sought to identify quantitative trait loci (QTL) for total aboveground biomass and enzymatic digestibility of rice straw from a cross between *Oryza sativa* ssp. *indica* varieties IR64-21 and Aswina. We report the phenotyping, genotyping by sequencing and QTL for biomass and bioenergy traits in a large recombinant inbred line (RIL) population of over 1,500 individuals.

A.1. Introduction

Recent trends indicate that crop yields are either reaching a plateau or on a linear trajectory [1–3]. At the same time, world population is increasing exponentially, and agriculture is not projected to meet demand [4]. While there is still uncertainty about the impact of climate change on rice yields, several studies indicate it could have a negative impact on

[4]This chapter will be published as a collaborative effort with these authors: Paul Tanger, Marietta Baraoidan, Stephen Klassen, Ma. Elizabeth B. Naredo, Julius Mojica, Kenneth L. McNally, John K. McKay, Daniel R. Bush, Hei Leung, and Jan E. Leach. Author contributions: Conceived and designed the experiments: PT, MB, SK, EBN, KLM, JKM, DRB, HL, JEL. Performed the experiments: PT, MB, SK, EBN, JM. Managed field trials: MB. Analyzed the data: PT, SK, JM.

yield [5–7]. Over 40% of arable land is already dedicated to crop and pastureland and further expansion negatively impacts other ecosystem services [8], so yield increases must come from improvements in crop production and delivery systems [9].

Rice is the staple food for over half of the world's population, and in many countries, rice accounts for 60% or more of total caloric intake. In many systems, agronomic practices to maximize rice yields have been implemented and yields are close to theoretical maximums [3]. Advances in the architecture of the plant, such as fewer but more productive tillers [10], creating C4 rice [11] as well as larger plants with more biomass will enable the future gains.

For every ton of grain produced, there is at least one ton of rice straw produced, and often this rice straw has little economic value and is burned or contributes to greenhouse gas emissions as it decomposes. Converting straw into usable energy has been proposed [12–16], but improvements in the composition of the straw could greatly facilitate the feasibility of these scenarios [17, 18]. For all these approaches, large agronomic and genetic diversity exists in rice germplasm from which new varieties can be developed with both increased grain yields and value added traits in the rice straw [19, 20].

Most important agronomic traits are quantitative traits: traits spanning a range of values and include yield, biomass, plant height and flowering time. These complex traits are generally controlled by many genes. One approach to identifying these genes is to first detect the quantitative trait loci (QTL) underlying these traits through statistical association between phenotype and genotype [21, 22]. While this approach has its limits (described in [23]), QTL mapping has been relatively successful at leading to gene discovery with the advancements in genome sequencing [24].

Several studies have examined QTL from diverse varieties in search of genes to improve biomass, yield, and straw traits. For example, QTL have been identified for plant height [25],

biomass [26–28], plant height and heading date [29–32] and cell wall traits [33–36]. Many of the QTL from these studies are collected and annotated on a genome browser [37] and comparisons can be made between studies to identify large effect QTL detected in multiple studies. However, many of these studies were performed with relatively low coverage of the genotypes and with few individuals in the population, reducing the power of the studies.

We describe here the phenotyping and genotyping of a large RIL population with increased biomass and bioenergy yield potential. We identify QTL for plant height from a cross between a modern high yielding semidwarf variety (IR64), and a landrace (Aswina) with higher biomass and higher energy yields from straw tissues. We discuss the potential for identification of additional QTL for the other phenotypes measured in this study.

A.2. Materials and methods

A.2.1. Plant materials

F7 RILs (1,751) were derived from eight F1 plants from a cross between two *Oryza sativa* ssp. *indica* varieties, IR64-21 (IRGC 117268) x Aswina (IRGC 117281), by single seed descent at the International Rice Research Institute (IRRI), Philippines. IR64-21 is a popular advanced semi-dwarf variety developed at IRRI, and Aswina is a deep-water landrace from Bangladesh with high biomass.

A.2.2. 2012 DS field trial

In the Spring 2012 Dry Season (DS) a subset (300 lines) of the 1,751 F7 RILs were grown. These 300 lines were composed of 100 lines stratifying biomass measurements from the F5 generation, and 200 lines randomly selected from the full population. Total aboveground biomass (excluding panicles) was measured on the F5 generation in Fall 2011 DS and lines were divided into 10 bins based on the biomass. Ten lines were selected from each bin (100

lines). 200 lines were randomly selected from the complete F7 population without regard to biomass bins. Seeds from these 300 lines were germinated in December 2011 and transplanted January 2012 at the Experiment Station at the International Rice Research Institute (IRRI), Los Baños, Laguna, Philippines (lat 14°10' 11.69" N, long 121°14' 38.63" E, 21 m elevation). Each line was planted in a 0.8 m^2 plot of 5 x 4 plants separated by 25 cm each. The plots were arranged in an incomplete block design with 5 blocks of 70 plots each. Each block was augmented with one plot of each parental variety. The entire planting (50 rows of 7 plots) was bordered by one row of IR64-21. 260 lines had one plot each, while 40 lines with sufficient seed had two plots and this design was replicated in two additional plantings in adjacent fields, offset by a week between each. Molluscicide (Snailkill (Metaldehyde), Agasin Pte. Ltd) was applied at a rate of 1L/ha immediately after transplanting to control snails. The fertilizer rate was 106-14-14 in split application of N which was 14 kg N per hectare from complete fertilizer (14-14-14) applied right after transplanting and 46 kg N per hectare each at 20 and 50 days after transplanting from UREA (46-0-0). Days to heading from date of transplanting was recorded when 80% of the plants in a plot had flowered. The plant height was measured as the height of the tallest leaf blade 1-2 wks before grain maturity on the six plants from the middle of each plot. These six plants were harvested at grain maturity and total fresh aboveground biomass (excluding panicle) and panicle mass were recorded, and a tiller from each plant collected for compositional analysis of the rice straw. Plant height was not measured in the second planting due to time constraints.

A.2.3. 2013 DS field trial

All 1,751 lines were grown in Fall 2012 Wet Season (WS) and lines that were visually segregating were culled and seed was bulked at F7 for each of the remaining 1,526 lines.

These 1,526 lines were grown in the 2013 DS field trial; this set included 227 of the 300 lines grown in 2012 DS. Seed was germinated December 2012 and transplanted January 2013 at IRRI in a row column design with eight blocks into which lines were assigned based on mean height of each line from the Fall 2012 WS. Each line was planted in a 1.2 m^2 plot which consisted of 5 x 6 plants, separated by 20 cm each. Extra plots of some lines or the parental varieties were included in each block (based on height) to estimate within block effects. No common entry was included in all blocks. Together, the 8 blocks comprised 49 rows of 16 plots wide in two adjacent fields. This design was repeated in two additional plantings in adjacent fields, offset by two weeks between each. Molluscicide was applied immediately after transplanting to control snails. The fertilizer rate followed the recommendations for yield trials and was 160-30-30 with split application of N which was 30 kg N per hectare from complete fertilizer (14-14-14) and 30 kg N per hectare from UREA (46-0-0) applied right after transplanting then 40 kg N and 60 kg N per hectare applied at 20 and 50 days after transplanting from UREA (46-0-0). Days to flowering was recorded when the first plant from a plot was flowering and days to heading was recorded when 80% of the plants in the plot had flowered. Three plants from the middle of each plot were randomly sampled for plant height 1-2 weeks before harvest, and total aboveground biomass (excluding panicle) and panicle mass were recorded at grain maturity. Panicle mass was only collected on the 3rd planting due to time constraints.

A.2.4. Compositional analysis of rice straw

Straw samples of the tillers from each of six plants from the 2012 DS field trial were separated into leaf blade and stem and oven dried. The stems were ground together using a knife mill with a 2 mm screen (Digital ED-5 Wiley Mill, Thomas Scientific, Swedesboro,

NJ USA) to produce one pooled stem straw sample per plot. 101 lines were selected to be analysed based on available genotyping data (61 lines), interesting phenotype data (such as lines with high or low biomass, or high or low ^{13}C isotope ratios; 5 lines), and the remaining (35 lines) randomly selected. For each selected line, 100 mg were analyzed in a pretreatment and digestibility assay described in [38]. Briefly, samples were divided into three technical replicates and treated with a dilute base solution (6.25 mM NaOH, 90° C, 3 h). After pretreatment, samples were digested with Accellerase1000 (Genecor, Rochester, NY USA), and glucose and pentose concentrations were determined with enzyme based colorimetric assays.

A.2.5. Statistical analysis

For 2012 DS, for each phenotype, plot means were calculated for each line per planting date. Least Square means (LSmeans) were estimated for each line using a model where line and planting were fixed effects. A random effects model where line and planting were random effects was used to calculate broad-sense heritability, H^2, as the percentage of variance due to line, according to the following equation: $H^2 = \sigma_G^2/(\sigma_G^2 + \sigma_P^2 + \sigma_\epsilon^2)$, where σ_G^2 is the genetic variance, σ_P^2 is the variance of the planting date and σ_ϵ^2 is the residual variance. Normality and residual variance was checked for each phenotype and outliers were examined. The biomass measurement of one plant and the grain measurement of another plant were excluded from the data because they appeared to be data entry errors. All data manipulation and statistical testing was completed in R Statistical Computing 3.0.2 [39].

For 2013 DS, data from plots with less than 13 plants that survived through harvest were discarded and the means of each line was calculated per planting for each phenotype. LSmeans, BLUPs, and H^2 were calculated as described for 2012 DS.

A.2.6. Genotyping

Leaf samples were collected (five plants per RIL for the 300 line subset; one plant per RIL for remainder of the lines) and DNA was extracted from these leaf samples using a column based kit (DNAeasy Plant Mini Kit, Qiagen, Valencia, CA USA). We performed genotyping by sequencing using the restriction digest approach according to [40]. DNA quantity was normalized, digested with *PstI* and *MspI*, ligated to adaptors, purified, amplified and sequenced on the Illumina platform. Sequence was processed in TASSEL 3, build date October 3 2013 [41] by alignment to the Nipponbare reference sequence version 7 [42] using bwa version 0.7.5a-r405 and bowtie2 version 2.1.0 [43]. We identified 3,854 SNP markers after filtering for lines with calls in at least 80% of markers, less than 10% heterozygous markers, and markers between 30-70% frequency. Genetic maps were constructed in Joinmap 4 (www.kyazma.nl) using the Kosambi mapping function [44] with the threshold recombination frequency of less than 0.25 and log of the odds ratio (LOD) scores greater than 2.0 and minimum LOD score of 10 and produced 16 linkage groups.

A.2.7. QTL mapping

To this point, 121 of the lines have been genotyped, and as a preliminary demonstration of the capability to do QTL mapping, a linkage group representing part of chromosome 1 was examined. QTL mapping was performed with R/qtl 1.30-4 [45] a package for R Statistical Computing 3.0.2. In R/qtl, single QTL scans were completed with standard interval mapping (EM) [46], Haley-Knott (HK) [47], Extended Haley-Knott (EHK) [48] and multiple imputation (IMP) [49] algorithms. Genotype probabilities were estimated at 1 cM intervals, and for the IMP algorithm, estimated with 256 draws using the Kosambi mapping function.

Significance LOD thresholds were derived from 10,000 permutations according to a genome-wide type I error rate of 5%. Bayes credible intervals, additive effect, and percent of variance were estimated for each QTL.

A.3. Results

A.3.1. Phenotypes display transgressive segregation

Phenotypic variation in the RILs (IR64 x Aswina) displays a large range and transgressive segregation for each of the agronomic phenotypes in the field (Figure A.1). The distribution of days to heading (DTH) was wide (range: 62-101 days) but most lines matured around the same time as IR64 (80 days ±2 SD). This population was developed in part because Aswina has more total biomass than IR64 and this remains valid in our study (220 g vs 162 g), though biomass of the RIL population appears normally distributed around the biomass of IR64 with a mean of 168 g. The grain yield of Aswina was low (18 g ±8 SD), but the distribution of grain yield of the population was normally distributed and closer to the mean of IR64 (45 g ±9 SD). Plant height displays a bimodal distribution around the means of the two parents (IR64 97 cm ±4 SD and Aswina 158 cm ±14 SD). This suggests that this trait is highly influenced by one gene (likely the semidwarf gene), as discussed in more detail below.

In addition to generally important agronomic traits, we collected rice straw samples and analysed the capacity to generate bioenergy from rice straw. Glucose and pentose available in the straw was measured on a subset of the 300 lines (Figure A.2). Both glucose and pentose yield are normally distributed, and transgressive segregation, although evident, is not as extensive as with the agronomic phenotypes. Some lines yield more sugars than either parent, and the majority of the lines yield more pentose than the parents.

A.3.2. Phenotypes are highly heritable

Genotypic correlations between phenotypes sometimes existed, though no one phenotype was correlated with all others (Figure A.3). There was a strong positive correlation between DTH and biomass with height and a weak negative correlation between DTH and grain. Positive correlations between glucose yield and biomass and height supports data presented in Chapter 3 that Aswina, and lines with Aswina characteristics, yield higher glucose from the straw tissue.

Across the two seasons, the performance of each line was similar. This was especially evident for DTH and height, despite several differences in the seasons, notably different fields and fertilizer rates (Figure A.4). Broad-sense heritability (H^2) is an estimate of the genetic component of a phenotype. Estimates for each phenotype measured in 2012 DS or 2013 DS are presented in Table A.1. In both seasons, H^2 is high for DTH (0.81) and height (0.85), and 0.32–0.46 for biomass. H^2 is low for grain yield (0.20). Broad-sense heritability was similar in 2013 DS.

A.3.3. A QTL for plant height is present on chromosome 1

While a complete linkage group for chromosome 1 has not been constructed for this population, a linkage group containing 61 markers and 48 cM was examined for the plant height phenotype. QTL mapping using the EHK algorithm revealed a putative QTL for plant height at 30 cM with an LOD score of 24 ($p<0.004$) (Figure A.5). This QTL had an additive effect from Aswina of 10.5 $\pm$1.5 SD and represented 14% of the total variance for plant height in the population.

A.4. Discussion

Distributions of phenotypes display transgressive segregation and potential for useful lines to be identified and bred from this population. The large differences observed in some traits will result in identification of large effect QTL. The genetic correlations and broad-sense heritability support the conclusion that much of the observed variation is driven by the genotypes of the individuals in the population.

IR64-21 harbors the *semidwarf1 (sd1)* allele for the gibberellin (GA) 20-oxidase enzyme (OsGA20ox2, Os01g66100) located on the long arm of chromosome 1 resulting in the semidwarf stature [50]. I hypothesize that Aswina does not carry this allele, based on several pieces of evidence. First, Aswina is 61 cm taller than IR64 on average. Second, the bimodal distribution and the high broad-sense heritability of the plant height phenotype support the hypothesis that lines in the RIL population will be short or tall largely dependent on which allele they have at this locus. The QTL reported on chromosome 1 falls between the nearest markers at 29 cM (37,975,782 bp) and 35 cM (39,261,073 bp) and where the *SD1* gene on the Nipponbare reference genome would be located (38,382,382 - 38,385,504 bp). Furthermore, the additive effect of Aswina is 10.5 cm.

The phenotypic data measured on this population should serve as a solid foundation for QTL mapping. With the large number of lines, and high marker coverage, we expect to fine map some QTL. While we did not investigate root biomass in this study, this could be a useful population to perform such a study and determine if Aswina-like lines also have higher root biomass.

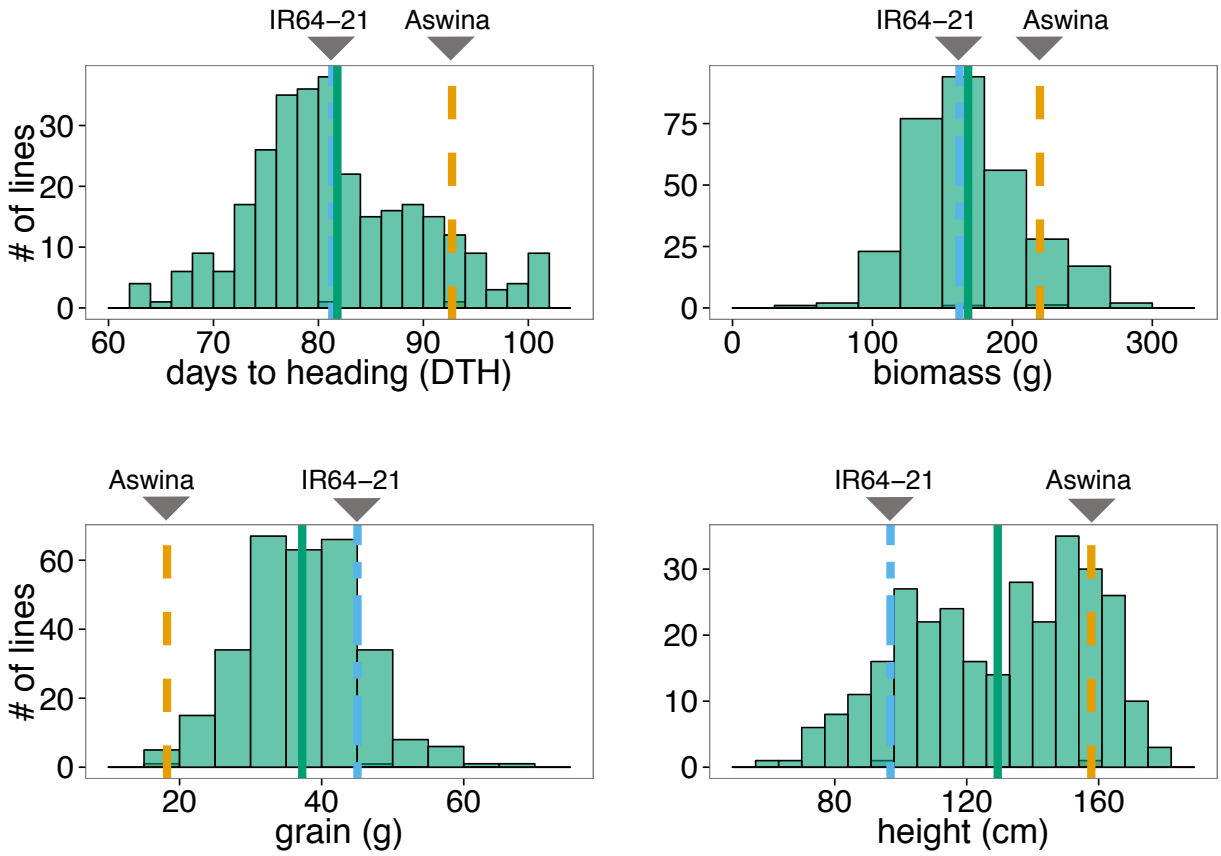


FIGURE A.1. Distribution of LSmeans of DTH, biomass, grain, and height of 300 lines from 2012 DS field trial. Phenotypes distributions in 2013 DS were similar. Mean of Aswina is shown as a dashed vertical line, mean of IR64-21 shown as a long-short dashed line, and mean of lines shown as a solid line.

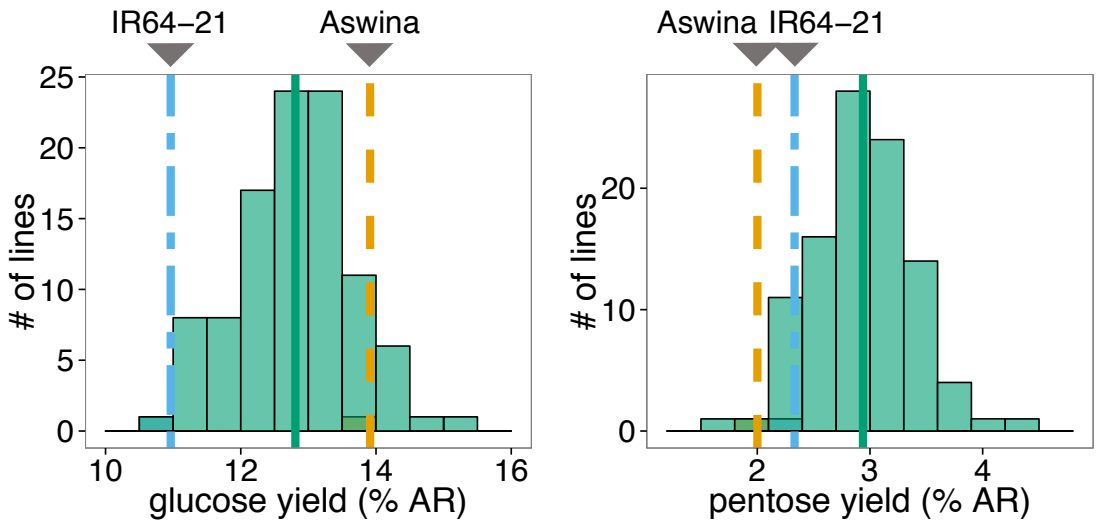


FIGURE A.2. Distribution of LSmeans of glucose and pentose yield after dilute base pretreatment from stem tissue of 101 lines from 2012 DS field trial in percent of As Received (AR) mass. Mean of Aswina is shown as a dashed vertical line, mean of IR64-21 shown as a long-short dashed line, and mean of lines shown as a solid line.

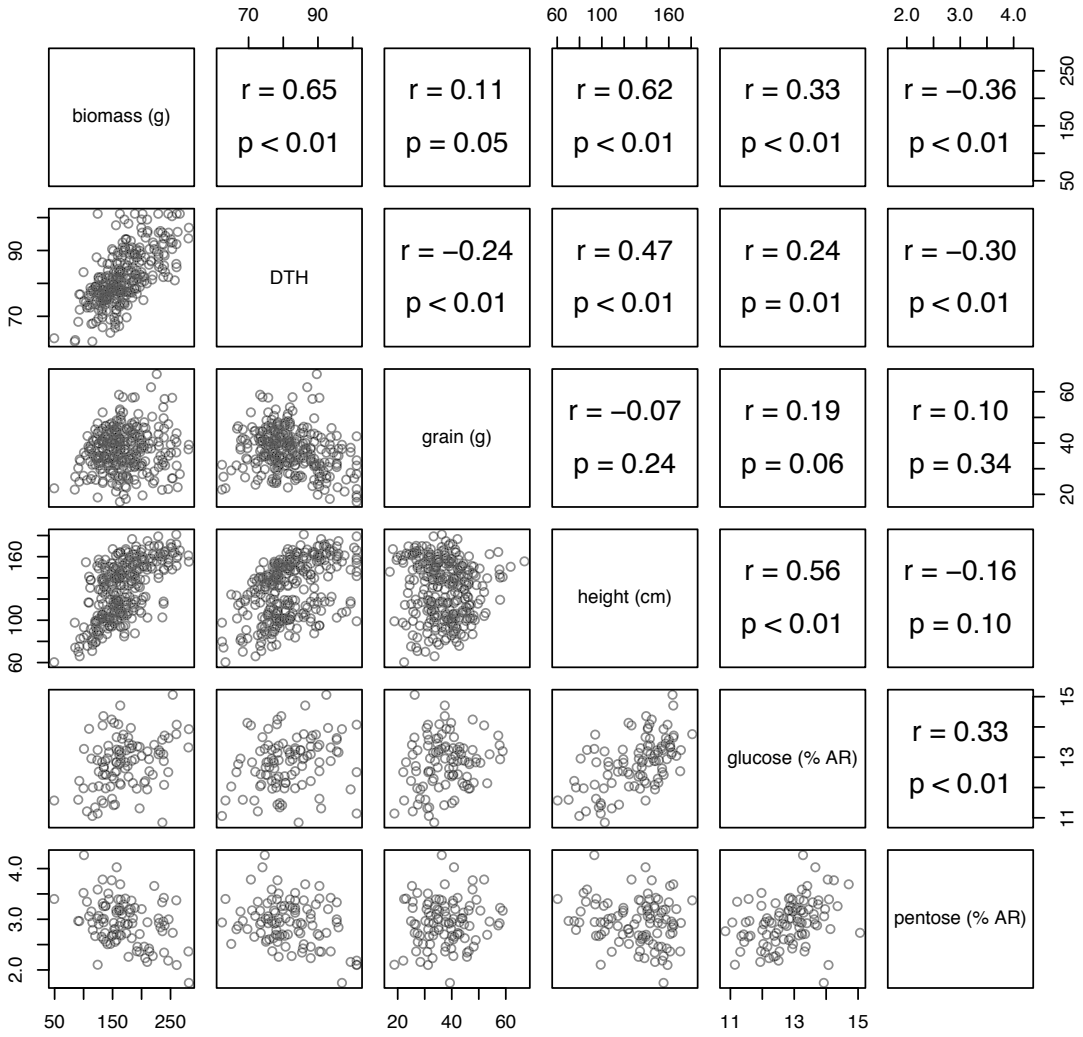


FIGURE A.3. Pearson's correlation between phenotypes measured from 2012 DS field trial on 300 lines. LSmeans are shown and calculated from all three plantings except height, which was only measured in plantings 1 and 3. Correlations were similar in both magnitude and direction for 2013 DS.

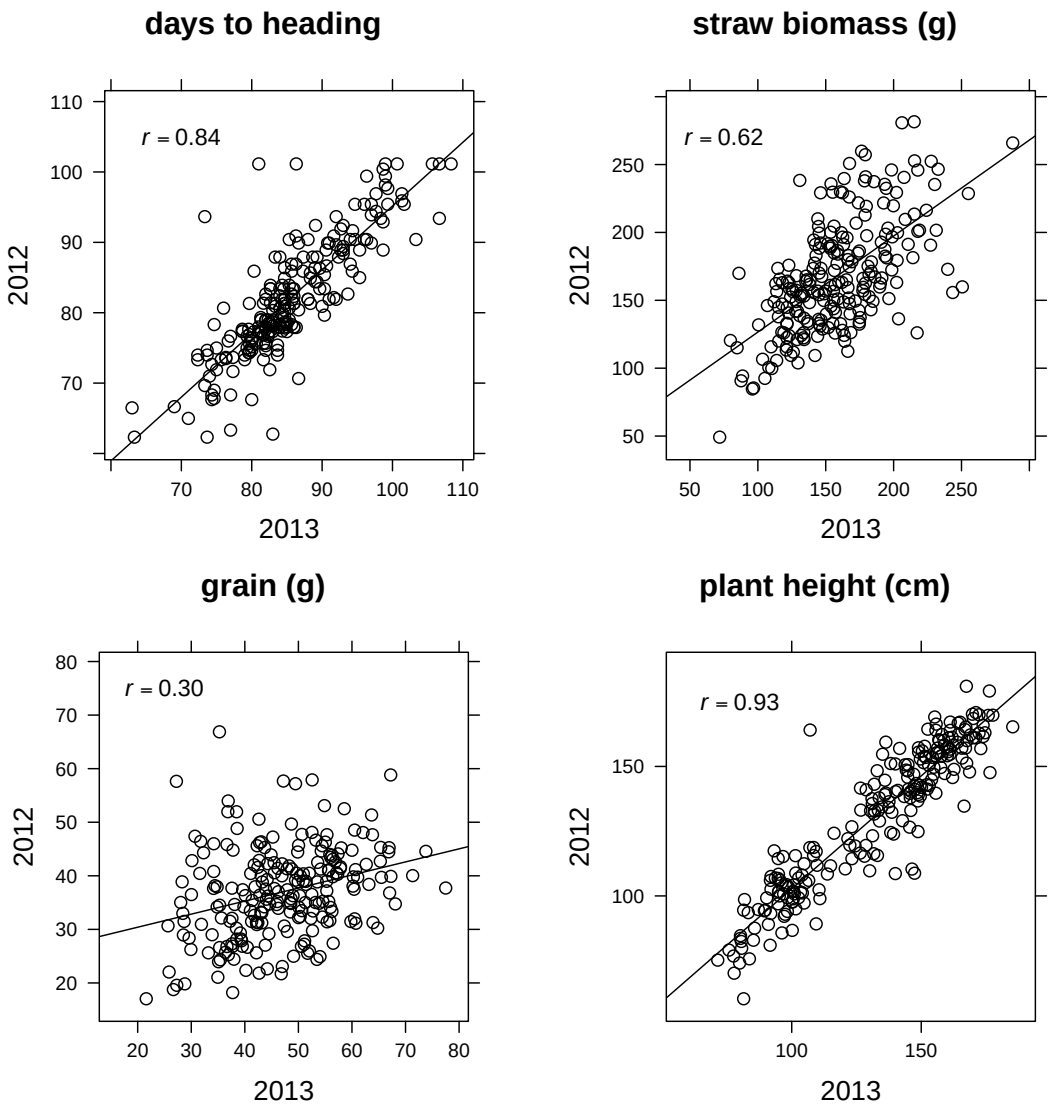


FIGURE A.4. Pearson's correlation coefficient and scatterplots of phenotypes measured in both 2012 DS and 2013 DS. LSmeans of 225 lines grown in both seasons are shown. All correlations are significant at p <0.001.

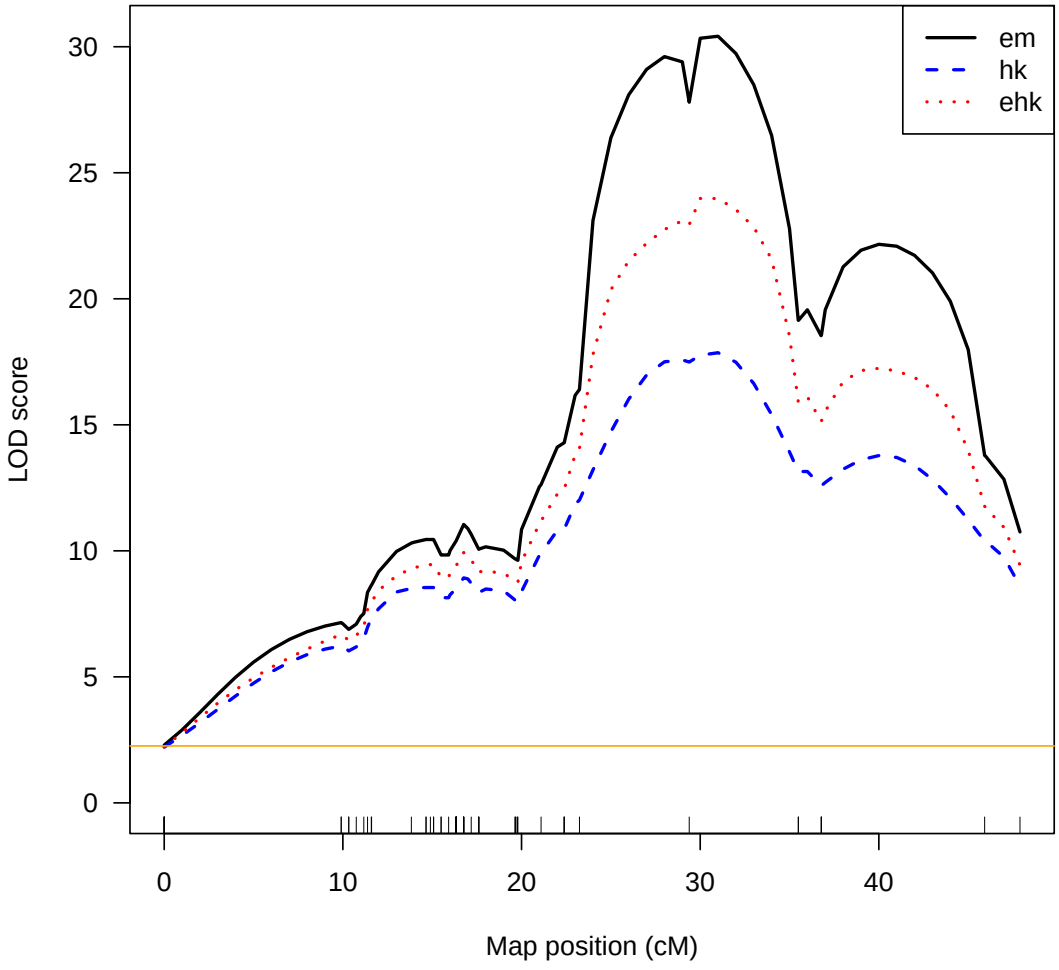


FIGURE A.5. QTL profile of plant height LSmeans in 2012 DS represented by a LOD peak (y axis) along the genetic distance of a linkage group from chromosome 1 (x axis), using three different algorithms. The tick marks on the x axis represent the marker locations. The solid horizontal orange line represents the LOD threshold (1.6), calculated from 10,000 permutations.

Table A.1. Broad-sense heritability estimates of grain, DTH, biomass, and plant height. Percent variance due to the 3 plantings and the residual error is also shown.

phenotype	H^2	% var planting	% var residual
grain	20%	20%	60%
DTH	81%	7%	12%
biomass	32%	12%	56%
height	85%	2%	13%

REFERENCES

[1] Grassini, P., Eskridge, K. M., and Cassman, K. G., 2013. Distinguishing between yield advances and yield plateaus in historical crop production trends. *Nature Communications*, 4:2918

[2] Ray, D. K., Ramankutty, N., Mueller, N. D., West, P. C., and Foley, J. A., 2012. Recent patterns of crop yield growth and stagnation. *Nature Communications*, 3:1293

[3] van Wart, J., Kersebaum, K. C., Peng, S., Milner, M., and Cassman, K. G., 2013. Estimating crop yield potential at regional to national scales. *Field Crops Research*, 143:34–43

[4] Ray, D. K., Mueller, N. D., West, P. C., and Foley, J. a., 2013. Yield Trends Are Insufficient to Double Global Crop Production by 2050. *PloS one*, 8, 6:e66428

[5] Challinor, A. J., Watson, J., Lobell, D. B., Howden, S. M., Smith, D. R., and Chhetri, N., 2014. A meta-analysis of crop yield under climate change and adaptation. *Nature Climate Change*, 4, 4:287–291

[6] Peng, S., Huang, J., Sheehy, J. E., Laza, R. C., Visperas, R. M., Zhong, X., Centeno, G. S., Khush, G. S., and Cassman, K. G., 2004. Rice yields decline with higher night temperature from global warming. *Proceedings of the National Academy of Sciences*, 101, 27:9971–5

[7] Kim, H.-Y., Ko, J., Kang, S., and Tenhunen, J., 2013. Impacts of climate change on paddy rice yield in a temperate climate. *Global Change Biology*, 19, 2:548–62

[8] Foley, J. a., Defries, R., Asner, G. P., Barford, C., Bonan, G., Carpenter, S. R., Chapin, F. S., Coe, M. T., Daily, G. C., Gibbs, H. K., Helkowski, J. H., Holloway, T., Howard, E. a., Kucharik, C. J., Monfreda, C., Patz, J. a., Prentice, I. C., Ramankutty, N., and

Snyder, P. K., 2005. Global consequences of land use. *Science*, 309, 5734:570–4

[9] McClung, C. R., 2014. Making Hunger Yield. *Science*, 344, 6185:699–700

[10] Jiao, Y., Wang, Y., Xue, D., Wang, J., Yan, M., Liu, G., Dong, G., Zeng, D., Lu, Z., Zhu, X., Qian, Q., and Li, J., 2010. Regulation of OsSPL14 by OsmiR156 defines ideal plant architecture in rice. *Nature Genetics*, 42, 6:541–4

[11] Karki, S., Rizal, G., and Quick, W. P., 2013. Improvement of photosynthesis in rice (Oryza sativa L.) by inserting the C4 pathway. *Rice*, 6, 1:28

[12] Kadam, K. L., Forrest, L. H., Jacobso, A., and Jacobson, W., 2000. Rice straw as a lignocellulosic resource: collection, processing, transportation, and environmental aspects. *Biomass and Bioenergy*, 18, 5:369–389

[13] Hiloidhari, M. and Baruah, D. C., 2011. Rice straw residue biomass potential for decentralized electricity generation: A GIS based study in Lakhimpur district of Assam, India. *Energy for Sustainable Development*, 15, 3:214–222

[14] Kim, S. and Dale, B. E., 2004. Global potential bioethanol production from wasted crops and crop residues. *Biomass and Bioenergy*, 26, 4:361–375

[15] Liu, Z., Xu, A., and Zhao, T., 2011. Energy from Combustion of Rice Straw: Status and Challenges to China. *Energy and Power Engineering*, 3, 3:325–331

[16] Matsumura, Y., Minowa, T., and Yamamoto, H., 2005. Amount, availability, and potential use of rice straw (agricultural residue) biomass as an energy resource in Japan. *Biomass and Bioenergy*, 29, 5:347–354

[17] Tanger, P., Field, J. L., Jahn, C. E., DeFoort, M. W., and Leach, J. E., 2013. Biomass for thermochemical conversion: targets and challenges. *Frontiers in Plant Biotechnology*, 4::218

[18] Jensen, J. W., Magid, J., Hansen-Mø ller, J., Andersen, S. B., and Bruun, S., 2011. Genetic variation in degradability of wheat straw and potential for improvement through plant breeding. *Biomass and Bioenergy*, 35, 3:1114–1120

[19] Tanksley, S. D. and McCouch, S. R., 1997. Seed Banks and Molecular Maps: Unlocking Genetic Potential from the Wild. *Science*, 277, 5329:1063–1066

[20] Jena, K. K., 2010. The species of the genus Oryza and transfer of useful genes from wild species into cultivated rice, O. sativa. *Breeding Science*, 60, 5:518–523

[21] Mackay, T. F., 2001. The genetic architecture of quantitative traits. *Annual Review of Genetics*, 35:303–39

[22] Ashikari, M. and Matsuoka, M., 2006. Identification, isolation and pyramiding of quantitative trait loci for rice breeding. *Trends in Plant Science*, 11, 7:344–50

[23] Nelson, R. M., Pettersson, M. E., and Carlborg, O., 2013. A century after Fisher: time for a new paradigm in quantitative genetics. *Trends in Genetics*, 29, 12:669–76

[24] Feuillet, C., Leach, J. E., Rogers, J., Schnable, P. S., and Eversole, K., 2011. Crop genome sequencing: lessons and rationales. *Trends in Plant Science*, 16, 2:77–88

[25] Salas Fernandez, M. G., Becraft, P. W., Yin, Y., and Lübberstedt, T., 2009. From dwarves to giants? Plant height manipulation for biomass yield. *Trends in Plant Science*, 14, 8:454–461

[26] Liu, G.-F. G.-F., Yang, J., and Zhu, J., 2006. Mapping QTL for biomass yield and its components in rice (Oryza sativa L.). *Acta Genetica Sinica*, 33, 7:607–16

[27] Babu, R. C., Nguyen, B. D., Chamarerk, V., Shanmugasundaram, P., Chezhian, P., Jeyaprakash, P., Ganesh, S. K., Palchamy, A., Sadasivam, S., Sarkarung, S., Wade, L. J., and Nguyen, H. T., 2003. Genetic Analysis of Drought Resistance in Rice by Molecular Markers : Association between Secondary Traits and Field Performance.

Crop Science, 43:1457–1469

[28] Hittalmani, S., Huang, N., Courtois, B., Venuprasad, R., Shashidhar, H. E., Zhuang, J.-Y., Zheng, K.-L., Liu, G.-F., Wang, G.-C., Sidhu, J. S., Srivantaneeyakul, S., Singh, V. P., Bagali, P. G., Prasanna, H. C., McLaren, G., and Khush, G. S., 2003. Identification of QTL for growth- and grain yield-related traits in rice across nine locations of Asia. *Theoretical and Applied Genetics*, 107, 4:679–90

[29] Li, Z. K., Yu, S. B., Lafitte, H. R., Huang, N., Courtois, B., Hittalmani, S., Vijayakumar, C. H. M., Liu, G. F., Wang, G. C., Shashidhar, H. E., Zhuang, J. Y., Zheng, K. L., Singh, V. P., Sidhu, J. S., Srivantaneeyakul, S., and Khush, G. S., 2003. QTL x environment interactions in rice. I. heading date and plant height. *Theoretical and Applied Genetics*, 108, 1:141–53

[30] Matsubara, K., Kono, I., Hori, K., Nonoue, Y., Ono, N., Shomura, A., Mizubayashi, T., Yamamoto, S., Yamanouchi, U., Shirasawa, K., Nishio, T., and Yano, M., 2008. Novel QTLs for photoperiodic flowering revealed by using reciprocal backcross inbred lines from crosses between japonica rice cultivars. *Theoretical and Applied Genetics*, 117, 6:935–945

[31] Nonoue, Y., Fujino, K., Hirayama, Y., Yamanouchi, U., Lin, S. Y., and Yano, M., 2008. Detection of quantitative trait loci controlling extremely early heading in rice. *Theoretical and Applied Genetics*, 116, 5:715–722

[32] Maheswaran, M., Huang, N., Sreerangasamy, S., and McCouch, S., 2000. Mapping quantitative trait loci associated with days to flowering and photoperiod sensitivity in rice (Oryza sativa L.). *Molecular Breeding*, 6, 2:145–155

[33] Barrière, Y., Mechin, V., Lefevre, B., and Maltese, S., 2012. QTLs for agronomic and cell wall traits in a maize RIL progeny derived from a cross between an old Minnesota13

line and a modern Iodent line. *Theoretical and Applied Genetics*, pp. 1–19

[34] Zhang, S.-J., Song, X.-Q., Yu, B.-S., Zhang, B.-C., Sun, C.-Q., Knox, J. P., and Zhou, Y.-H., 2012. Identification of quantitative trait loci affecting hemicellulose characteristics based on cell wall composition in a wild and cultivated rice species. *Molecular Plant*, 5, 1:162–75

[35] Barrière, Y., Thomas, J., and Denoue, D., 2008. QTL mapping for lignin content, lignin monomeric composition, p-hydroxycinnamate content, and cell wall digestibility in the maize recombinant inbred line progeny F838F286. *Plant Science*, 175, 4:585–595

[36] Dai, W. M., Zhang, K. Q., Wu, J. R., Wang, L., Duan, B. W., Zheng, K. L., Cai, R., and Zhuang, J. Y., 2008. Validating a segment on the short arm of chromosome 6 responsible for genetic variation in the hull silicon content and yield traits of rice. *Euphytica*, 160, 3:317–324

[37] Yonemaru, J.-i., Yamamoto, T., Fukuoka, S., Uga, Y., Hori, K., and Yano, M., 2010. Q-TARO: QTL Annotation Rice Online Database. *Rice*, 3, 2-3:194–203

[38] Santoro, N., Cantu, S. L., Tornqvist, C.-E., Falbel, T. G., Bolivar, J. L., Patterson, S. E., Pauly, M., and Walton, J. D., 2010. A High-Throughput Platform for Screening Milligram Quantities of Plant Biomass for Lignocellulose Digestibility. *BioEnergy Research*, 3, 1:93–102

[39] R core Team, 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria

[40] Poland, J. a., Brown, P. J., Sorrells, M. E., and Jannink, J.-L., 2012. Development of high-density genetic maps for barley and wheat using a novel two-enzyme genotyping-by-sequencing approach. *PloS one*, 7, 2:e32253

[41] Bradbury, P. J., Zhang, Z., Kroon, D. E., Casstevens, T. M., Ramdoss, Y., and Buckler, E. S., 2007. TASSEL: software for association mapping of complex traits in diverse samples. *Bioinformatics*, 23, 19:2633–5

[42] Kawahara, Y., Bastide, M. d. l., Hamilton, J. P., Kanamori, H., McCombie, W. R., Ouyang, S., Schwartz, D. C., Tanaka, T., Wu, J., Zhou, S., Childs, K. L., Davidson, R. M., Lin, H., Quesada-Ocampo, L., Vaillancourt, B., Sakai, H., Lee, S. S., Kim, J., Numa, H., Itoh, T., Buell, C. R., and Matsumoto, T., 2013. Improvement of the Oryza sativa Nipponbare reference genome using next generation sequence and optical map data. *Rice*, 6, 1:4

[43] Langmead, B. and Salzberg, S. L., 2012. Fast gapped-read alignment with Bowtie 2. *Nature Methods*, 9, 4:357–9

[44] Kosambi, D. D., 1943. The estimation of map distances from recombination values. *Annals of Eugenics*, 12, 1:172–175

[45] Broman, K. W., Wu, H., Sen, S., and Churchill, G. A., 2003. R/qtl: QTL mapping in experimental crosses. *Bioinformatics*, 19, 7:889–890

[46] Lander, E. S. and Botstein, D., 1989. Mapping Mendelian Factors Underlying Quantitative Traits Using RFLP Linkage Maps. *Genetics*, 121, 1:185–199

[47] Haley, C. S. and Knott, S. A., 1992. A simple regression method for mapping quantitative trait loci in line crosses using flanking markers. *Heredity*, 69, 4:315–324

[48] Feenstra, B., Skovgaard, I. M., and Broman, K. W., 2006. Mapping quantitative trait loci by an extension of the Haley-Knott regression method using estimating equations. *Genetics*, 173, 4:2269–82

[49] Sen, S. and Churchill, G. A., 2001. A statistical framework for quantitative trait mapping. *Genetics*, 159, 1:371

[50] Spielmeyer, W., Ellis, M. H., and Chandler, P. M., 2002. Semidwarf (sd-1), "green revolution" rice, contains a defective gibberellin 20-oxidase gene. *Proceedings of the National Academy of Sciences*, 99, 13:9043–8

APPENDIX B

Supplementary material

B.1. Chapter 3 supplementary material

B.1.1. supplemental methods

B.1.1.1. *Growth conditions and sample preparation*

The complete set of 20 varieties were grown in two greenhouses with controlled conditions at Colorado State University for several experiments between 2009-2012. Samples described as grown before 2010 were in a greenhouse with approximately 25°C and 55% RH. Samples described as grown after 2010 were in the same greenhouse as described in Chapter 3 section 3.2, with 27°C and 76% RH. All experiments in the greenhouses were a completely randomized design and samples were collected at grain maturity stage. Other details of growth conditions are identical to those described in Chapter 3 section 3.2.

B.1.1.2. *AcBr lignin, MLG, saccharification, cell wall monosaccharide sugar on all 20 varieties*

Plants grown in the greenhouse from January 2008 - October 2009 with n=2 per variety were separated into leaf, sheath, and stem and oven dried at 93°C until a constant mass was achieved. Tissue was ground into a fine powder with a coffee grinder (Braun Aromatic KSM2). Stem samples were analyzed for AcBr lignin, MLG, saccharification, and cell wall monosaccharide sugar composition as described in [1]. Two technical replicates were performed per sample.

B.1.1.3. *Elemental analysis of leaf, stem, grain on all 20 varieties*

Each of the 20 varieties were grown at IRRI in a row in plots consisting of 7 x 15 hills, separated by 25 cm. Seeds were sown in June 2011 in plastic trays in the greenhouse and transplanted July 2011. The whole field plot was bordered with two rows of purple variety and a single row between each plot. Molluscicide (Snailkill (Metaldehyde), Agasin Pte. Ltd at 1 L/ha) was applied right after transplanting to control snail. The fertilizer rate was 106-14-14 in split application of N which was 16 kg N per hectare from complete fertilizer (14-14-14) applied right after transplanting and 46 kg N per hectare each at 20 and 50 days after transplanting from UREA (46-0-0). Rice tungro bacilliform virus (RTBV) disease was observed during the growing season. Grain samples and two leaf samples from n=4 plants per variety were collected and separated into leaf blade and leaf sheath, and after rinsing briefly with DI water, were dried at 90°C overnight. Samples were transferred individually into Pyrex test tubes (16 x 100 mm). After weighing the appropriate number of samples (these masses were used to calculate the rest of the sample masses), trace metal grade nitric acid (Baker Instra-Analyzed; Avantor Performance Materials) spiked with indium internal standard was added to the tubes (1.5 mL) and the samples digested in dry block heaters for 5 h at 115°C. After dilution to 10 mL with 18.2 MΩcm Milli-Q water, subsamples of solutions (0.75 mL leaf and sheath; 1.0 mL seed) were transferred to new tube sets and nitric acid (0.9 mL) added and digested the second time as before. The digested samples were diluted to 10 mL as before and aliquots transferred to 96-well deepwell plates for analysis. Elemental analysis was performed with an ICP-MS (NexION 300D equipped with ESI autosampler and Apex HF; PerkinElmer) for the 22 elements. Liquid reference materials composed of pooled samples of digested leaf/sheath and seed materials were prepared and analyzed after every 9th sample in all ICP-MS sample sets to correct for variation between and within ICP-MS

analysis runs [2]. Sample concentrations were calculated using external calibration method with the instrument software.

B.1.1.4. *Digestibility of 20 varieties*

Plants grown in the greenhouse between 2008-2009 with n=3 were sampled as whole plant, leaf or stem and ground to 2mm with a knife mill (Model 4 Wiley mill, Thomas Scientific, Swedesboro, NJ). Digestibility methods were performed as described in detail in Chapter 3 section 3.2.5.

B.1.1.5. *Stem wall thickness of 20 varieties*

Stem samples were taken from plants grown in the greenhouse between Fall 2011 and spring 2012, n=1 per variety. Wall thickness was measured for 5-10 stems, at 4 points along each stem: 5, 10, 15 and 20 cm from the soil line.

B.1.1.6. *Statistical analysis*

Means were compiled, one-way ANOVA was performed and Fisher's Least Significant Differences (LSD) were calculated and compiled in JMP Pro 11 (SAS Institute Inc., Cary, NC).

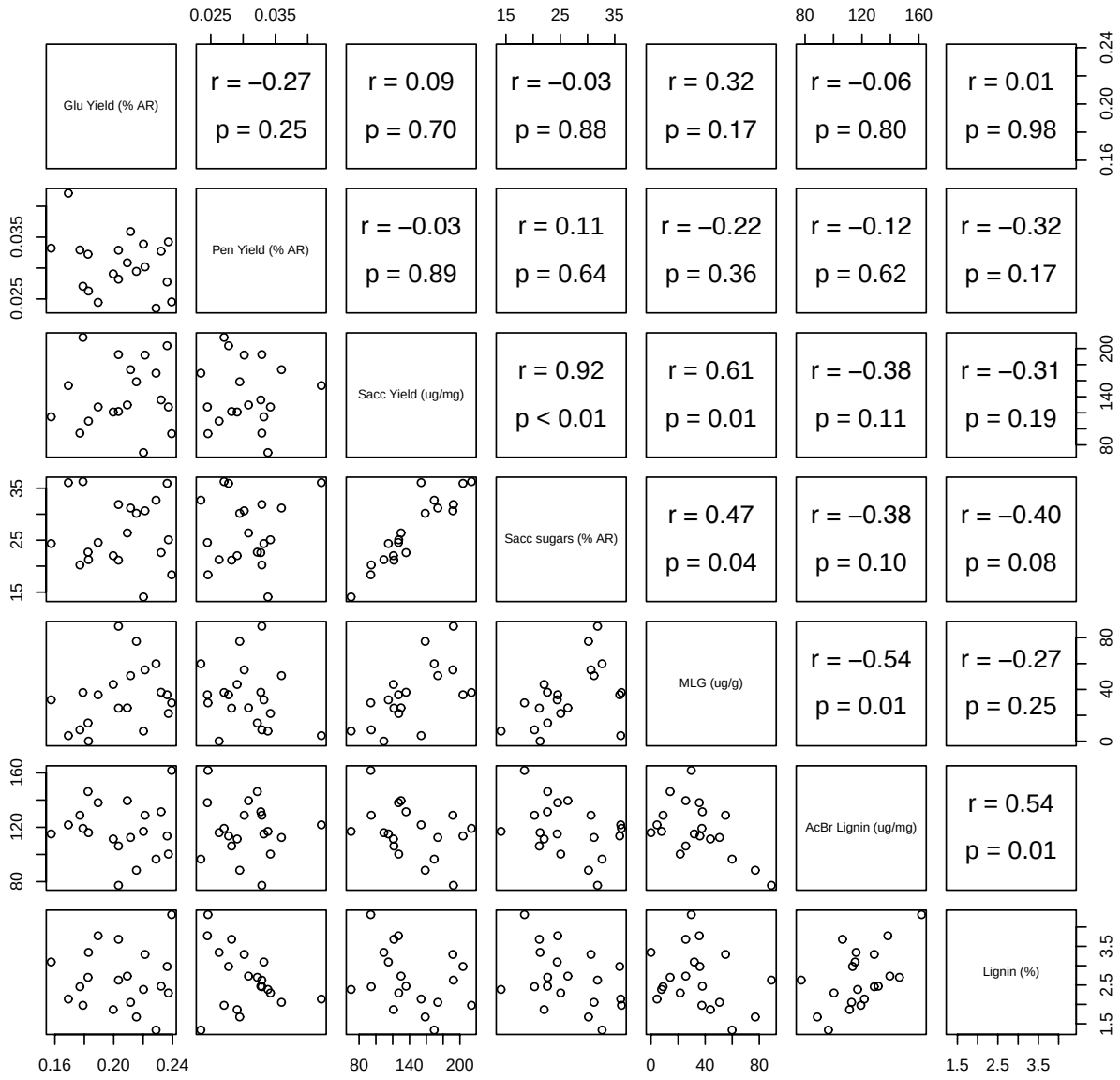


FIGURE B.1. Pearson's correlations and scatterplots of selected data from 20 rice varieties in the GH. Glucose (Glu) and Pentose (Pen) yield is from whole plant samples, and saccarification (Sacc) yield, Sacc percent and MLG is from stem samples, from different plants. Sacc percent is sugars as a percent of as received (AR) dry weight. Lignin data is from a different set of samples from [3].

Table B.1. MLG, AcBr lignin, cell wall monosaccharide composition of 20 rice varieties grown in the GH. All samples are stem tissue. This is attached to this dissertation at the Colorado State University Libraries' Digital Repository as an excel file.

Assay	Variety	LSD letter group									Mean
MLG (ug/mg)	Aswina	A									88.96
MLG (ug/mg)	FR13A	A	B								77.07
MLG (ug/mg)	Pokkali	A	B	C							59.85
MLG (ug/mg)	Tainung		B	C	D						55.13
MLG (ug/mg)	IR64		B	C	D	E					50.72
MLG (ug/mg)	Rayada			C	D	E	F				43.86
MLG (ug/mg)	Moroberekan			C	D	E	F	G			37.84
MLG (ug/mg)	nipponbare			C	D	E	F	G			37.65
MLG (ug/mg)	M202			C	D	E	F	G	H		36.00
MLG (ug/mg)	Sadu-Cho			C	D	E	F	G	H		35.79
MLG (ug/mg)	Minghui			C	D	E	F	G	H	I	32.00
MLG (ug/mg)	LTH			C	D	E	F	G	H	I	29.63
MLG (ug/mg)	Cypress				D	E	F	G	H	I	25.67
MLG (ug/mg)	Swarna				D	E	F	G	H	I	25.58
MLG (ug/mg)	N22					E	F	G	H	I	21.43
MLG (ug/mg)	dom_sufid						F	G	H	I	14.16
MLG (ug/mg)	Dular							G	H	I	8.82
MLG (ug/mg)	Azucena							G	H	I	7.70
MLG (ug/mg)	Zhenshan-97B								H	I	4.43
MLG (ug/mg)	SHZ-2									I	0.00
AcBr lignin (ug/mg)	LTH	A									161.83
AcBr lignin (ug/mg)	dom_sufid	A	B								146.25
AcBr lignin (ug/mg)	Cypress	A	B	C							139.50
AcBr lignin (ug/mg)	Sadu-Cho	A	B	C							138.20
AcBr lignin (ug/mg)	Moroberekan		B	C	D						131.35
AcBr lignin (ug/mg)	Tainung		B	C	D	E					128.77
AcBr lignin (ug/mg)	Dular		B	C	D	E					128.77
AcBr lignin (ug/mg)	Zhenshan-97B		B	C	D	E	F				121.71
AcBr lignin (ug/mg)	nipponbare		B	C	D	E	F				119.23
AcBr lignin (ug/mg)	Azucena		B	C	D	E	F	G			116.98
AcBr lignin (ug/mg)	SHZ-2			C	D	E	F	G			115.99
AcBr lignin (ug/mg)	Minghui			C	D	E	F	G			115.06
AcBr lignin (ug/mg)	M202			C	D	E	F	G			113.49
AcBr lignin (ug/mg)	IR64			C	D	E	F	G			112.57
AcBr lignin (ug/mg)	Rayada			C	D	E	F	G			111.34
AcBr lignin (ug/mg)	Swarna				D	E	F	G	H		106.31
AcBr lignin (ug/mg)	N22					E	F	G	H		100.36
AcBr lignin (ug/mg)	Pokkali						F	G	H		96.53
AcBr lignin (ug/mg)	FR13A							G	H		88.45
AcBr lignin (ug/mg)	Aswina								H		77.15
saccharification effic (%)	nipponbare	A									36.23

Table B.1 (continued). MLG, AcBr lignin, cell wall monosaccharide composition of 20 rice varieties grown in the GH. All samples are stem tissue.

Assay	Variety	LSD letter group							Mean
saccharification effic (%)	Zhenshan-97B	A							36.11
saccharification effic (%)	M202	A							35.93
saccharification effic (%)	Pokkali	A	B						32.70
saccharification effic (%)	Aswina	A	B	C					31.87
saccharification effic (%)	IR64	A	B	C					31.18
saccharification effic (%)	Tainung	A	B	C					30.67
saccharification effic (%)	FR13A	A	B	C	D				30.20
saccharification effic (%)	Cypress	A	B	C	D				26.37
saccharification effic (%)	N22	A	B	C	D	E			25.08
saccharification effic (%)	Sadu-Cho	A	B	C	D	E			24.55
saccharification effic (%)	Minghui	A	B	C	D	E			24.38
saccharification effic (%)	dom_sufid		B	C	D	E			22.68
saccharification effic (%)	Moroberekan		B	C	D	E			22.62
saccharification effic (%)	Rayada		B	C	D	E			21.99
saccharification effic (%)	SHZ-2		B	C	D	E			21.24
saccharification effic (%)	Swarna		B	C	D	E			21.14
saccharification effic (%)	Dular			C	D	E			20.23
saccharification effic (%)	LTH				D	E			18.38
saccharification effic (%)	Azucena					E			14.09
saccharification (ug/mg)	nipponbare	A							213.70
saccharification (ug/mg)	M202	A	B						203.43
saccharification (ug/mg)	Aswina	A	B	C					192.31
saccharification (ug/mg)	Tainung	A	B	C	D				191.55
saccharification (ug/mg)	IR64	A	B	C	D	E			173.47
saccharification (ug/mg)	Pokkali	A	B	C	D	E	F		169.19
saccharification (ug/mg)	FR13A	A	B	C	D	E	F		158.68
saccharification (ug/mg)	Zhenshan-97B	A	B	C	D	E	F		153.86
saccharification (ug/mg)	Moroberekan		B	C	D	E	F	G	136.09
saccharification (ug/mg)	Cypress		B	C	D	E	F	G	129.80
saccharification (ug/mg)	N22		B	C	D	E	F	G	127.21
saccharification (ug/mg)	Sadu-Cho		B	C	D	E	F	G	127.10
saccharification (ug/mg)	Swarna			C	D	E	F	G	121.50
saccharification (ug/mg)	Rayada			C	D	E	F	G	120.71
saccharification (ug/mg)	Minghui				D	E	F	G	114.99
saccharification (ug/mg)	Dular					E	F	G	114.33
saccharification (ug/mg)	SHZ-2					E	F	G	109.56
saccharification (ug/mg)	Dular						F	G	94.57
saccharification (ug/mg)	LTH						F	G	94.08
saccharification (ug/mg)	Azucena							G	70.46
Rhamnose (mol%)	Dular	A							0.71
Rhamnose (mol%)	SHZ-2	A	B						0.68
Rhamnose (mol%)	Zhenshan-97B	A	B						0.66
Rhamnose (mol%)	Minghui		B	C					0.61

Table B.1 (continued). MLG, AcBr lignin, cell wall monosaccharide composition of 20 rice varieties grown in the GH. All samples are stem tissue.

Assay	Variety	LSD letter group										Mean
Rhamnose (mol%)	Swarna			C	D							0.55
Rhamnose (mol%)	Azucena			C	D	E						0.54
Rhamnose (mol%)	N22			C	D	E	F					0.52
Rhamnose (mol%)	nipponbare				D	E	F	G				0.51
Rhamnose (mol%)	M202				D	E	F	G	H			0.49
Rhamnose (mol%)	Cypress				D	E	F	G	H	I		0.49
Rhamnose (mol%)	IR64				D	E	F	G	H	I		0.48
Rhamnose (mol%)	Moroberekan				D	E	F	G	H	I		0.47
Rhamnose (mol%)	Sadu-Cho				D	E	F	G	H	I		0.47
Rhamnose (mol%)	Pokkali					E	F	G	H	I		0.45
Rhamnose (mol%)	Tainung						F	G	H	I	J	0.44
Rhamnose (mol%)	Rayada							G	H	I	J	0.43
Rhamnose (mol%)	Aswina								H	I	J	0.41
Rhamnose (mol%)	FR13A								H	I	J	0.40
Rhamnose (mol%)	dom_sufid									I	J	0.40
Rhamnose (mol%)	LTH										J	0.36
xylose (mol%)	Dular	A										67.73
xylose (mol%)	N22	A	B									60.99
xylose (mol%)	Zhenshan-97B	A	B									60.03
xylose (mol%)	SHZ-2	A	B	C								59.29
xylose (mol%)	M202	A	B	C								59.18
xylose (mol%)	LTH	A	B	C	D							57.13
xylose (mol%)	Minghui		B	C	D	E						55.21
xylose (mol%)	Cypress		B	C	D	E						54.15
xylose (mol%)	Azucena		B	C	D	E						53.86
xylose (mol%)	Sadu-Cho		B	C	D	E						53.33
xylose (mol%)	Swarna		B	C	D	E						52.06
xylose (mol%)	dom_sufid		B	C	D	E						51.89
xylose (mol%)	nipponbare			C	D	E	F					48.67
xylose (mol%)	Moroberekan				D	E	F					47.82
xylose (mol%)	Aswina				D	E	F					47.58
xylose (mol%)	IR64				D	E	F					47.43
xylose (mol%)	FR13A					E	F					45.95
xylose (mol%)	Tainung					E	F					45.77
xylose (mol%)	Rayada					E	F					45.53
xylose (mol%)	Pokkali						F					39.29
glucose (mol%)	Pokkali	A										38.53
glucose (mol%)	Rayada	A	B									37.22
glucose (mol%)	Tainung	A	B	C								34.84
glucose (mol%)	FR13A	A	B	C	D							34.37
glucose (mol%)	Aswina	A	B	C	D	E						32.46
glucose (mol%)	Moroberekan	A	B	C	D	E						32.36
glucose (mol%)	IR64	A	B	C	D	E						31.96

Table B.1 (continued). MLG, AcBr lignin, cell wall monosaccharide composition of 20 rice varieties grown in the GH. All samples are stem tissue.

Assay	Variety	LSD letter group							Mean
glucose (mol%)	dom_sufid	A	B	C	D	E			30.27
glucose (mol%)	nipponbare	A	B	C	D	E			29.48
glucose (mol%)	Sadu-Cho	A	B	C	D	E	F		27.10
glucose (mol%)	Swarna	A	B	C	D	E	F		25.62
glucose (mol%)	Cypress	A	B	C	D	E	F		25.46
glucose (mol%)	Azucena		B	C	D	E	F	G	24.27
glucose (mol%)	LTH			C	D	E	F	G	23.38
glucose (mol%)	Minghui				D	E	F	G	21.69
glucose (mol%)	M202				D	E	F	G	21.34
glucose (mol%)	N22					E	F	G	19.61
glucose (mol%)	Zhenshan-97B						F	G	15.72
glucose (mol%)	SHZ-2						F	G	15.23
glucose (mol%)	Dular							G	11.34
GlcA (mol%)	Zhenshan-97B	A							0.73
GlcA (mol%)	Sadu-Cho	A							0.73
GlcA (mol%)	Dular	A	B						0.72
GlcA (mol%)	SHZ-2	A	B	C					0.70
GlcA (mol%)	Minghui	A	B	C					0.70
GlcA (mol%)	Pokkali	A	B	C	D				0.68
GlcA (mol%)	N22	A	B	C	D	E			0.66
GlcA (mol%)	M202	A	B	C	D	E			0.65
GlcA (mol%)	Cypress	A	B	C	D	E			0.65
GlcA (mol%)	IR64	A	B	C	D	E			0.65
GlcA (mol%)	nipponbare	A	B	C	D	E			0.65
GlcA (mol%)	Aswina	A	B	C	D	E	F		0.62
GlcA (mol%)	Azucena	A	B	C	D	E	F		0.59
GlcA (mol%)	Rayada		B	C	D	E	F		0.57
GlcA (mol%)	Moroberekan			C	D	E	F		0.55
GlcA (mol%)	LTH				D	E	F		0.53
GlcA (mol%)	FR13A				D	E	F		0.53
GlcA (mol%)	Tainung					E	F		0.52
GlcA (mol%)	dom_sufid					E	F		0.50
GlcA (mol%)	Swarna						F		0.48
Galactose (mol%)	SHZ-2	A							7.08
Galactose (mol%)	Pokkali	A	B						6.86
Galactose (mol%)	Azucena	A	B	C					6.01
Galactose (mol%)	nipponbare		B	C	D				5.87
Galactose (mol%)	Tainung		B	C	D	E			5.78
Galactose (mol%)	Minghui		B	C	D	E			5.76
Galactose (mol%)	Swarna		B	C	D	E			5.71
Galactose (mol%)	Moroberekan		B	C	D	E	F		5.70
Galactose (mol%)	Aswina			C	D	E	F	G	5.31
Galactose (mol%)	IR64			C	D	E	F	G	5.26

Table B.1 (continued). MLG, AcBr lignin, cell wall monosaccharide composition of 20 rice varieties grown in the GH. All samples are stem tissue.

Assay	Variety	LSD letter group										Mean
Galactose (mol%)	FR13A			C	D	E	F	G	H			5.11
Galactose (mol%)	Zhenshan-97B				D	E	F	G	H	I		4.81
Galactose (mol%)	dom_sufid				D	E	F	G	H	I		4.75
Galactose (mol%)	Cypress					E	F	G	H	I		4.68
Galactose (mol%)	M202						F	G	H	I	J	4.53
Galactose (mol%)	Sadu-Cho							G	H	I	J	4.40
Galactose (mol%)	LTH							G	H	I	J	4.34
Galactose (mol%)	N22								H	I	J	3.97
Galactose (mol%)	Rayada									I	J	3.76
Galactose (mol%)	Dular										J	3.49
GalA (mol%)	Zhenshan-97B	A										3.77
GalA (mol%)	SHZ-2		B									2.91
GalA (mol%)	Dular		B	C								2.87
GalA (mol%)	Minghui		B	C	D							2.63
GalA (mol%)	Swarna		B	C	D	E						2.47
GalA (mol%)	LTH			C	D	E	F					2.44
GalA (mol%)	M202				D	E	F					2.42
GalA (mol%)	Azucena				D	E	F	G				2.35
GalA (mol%)	Cypress				D	E	F	G				2.31
GalA (mol%)	IR64				D	E	F	G				2.29
GalA (mol%)	Aswina				D	E	F	G				2.28
GalA (mol%)	N22				D	E	F	G				2.27
GalA (mol%)	Sadu-Cho				D	E	F	G				2.18
GalA (mol%)	nipponbare					E	F	G				2.12
GalA (mol%)	Tainung					E	F	G				2.10
GalA (mol%)	Moroberekan					E	F	G				2.09
GalA (mol%)	FR13A					E	F	G				2.07
GalA (mol%)	dom_sufid						F	G				2.00
GalA (mol%)	Pokkali							G				1.94
GalA (mol%)	Rayada							G				1.92
fucose (mol%)	Zhenshan-97B	A										0.24
fucose (mol%)	SHZ-2	A	B									0.22
fucose (mol%)	M202	A	B	C								0.20
fucose (mol%)	Minghui	A	B	C	D							0.19
fucose (mol%)	Swarna	A	B	C	D							0.18
fucose (mol%)	Dular	A	B	C	D							0.18
fucose (mol%)	Pokkali	A	B	C	D							0.18
fucose (mol%)	LTH	A	B	C	D							0.17
fucose (mol%)	Cypress		B	C	D							0.16
fucose (mol%)	Azucena		B	C	D							0.16
fucose (mol%)	dom_sufid		B	C	D							0.16
fucose (mol%)	IR64			C	D							0.15
fucose (mol%)	nipponbare			C	D							0.15

Table B.1 (continued). MLG, AcBr lignin, cell wall monosaccharide composition of 20 rice varieties grown in the GH. All samples are stem tissue.

Assay	Variety	LSD letter group					Mean
fucose (mol%)	Sadu-Cho			C	D		0.15
fucose (mol%)	N22			C	D		0.14
fucose (mol%)	FR13A			C	D		0.14
fucose (mol%)	Tainung				D		0.13
fucose (mol%)	Rayada				D	E	0.13
fucose (mol%)	Aswina				D	E	0.13
fucose (mol%)	Moroberekan					E	0.07
Arabinose (mol%)	Zhenshan-97B	A					14.04
Arabinose (mol%)	SHZ-2	A	B				13.89
Arabinose (mol%)	Minghui	A	B	C			13.21
Arabinose (mol%)	Dular	A	B	C			12.95
Arabinose (mol%)	Swarna	A	B	C			12.94
Arabinose (mol%)	nipponbare	A	B	C	D		12.54
Arabinose (mol%)	Azucena	A	B	C	D	E	12.22
Arabinose (mol%)	Cypress	A	B	C	D	E	12.10
Arabinose (mol%)	Pokkali	A	B	C	D	E	12.06
Arabinose (mol%)	N22	A	B	C	D	E	11.85
Arabinose (mol%)	IR64	A	B	C	D	E	11.76
Arabinose (mol%)	LTH		B	C	D	E	11.65
Arabinose (mol%)	Sadu-Cho		B	C	D	E	11.63
Arabinose (mol%)	FR13A			C	D	E	11.43
Arabinose (mol%)	Aswina			C	D	E	11.21
Arabinose (mol%)	M202			C	D	E	11.18
Arabinose (mol%)	Moroberekan			C	D	E	10.95
Arabinose (mol%)	Rayada				D	E	10.44
Arabinose (mol%)	Tainung				D	E	10.44
Arabinose (mol%)	dom_sufid					E	10.04

Table B.2. Sugar yield of 20 rice varieties grown in the GH. Combined tissue is the whole plant. This is attached to this dissertation at the Colorado State University Libraries' Digital Repository as an excel file.

Assay	Variety	LSD letter group						Mean	Tissue
glucose release (%)	LTH	A						0.255	stem
glucose release (%)	M202	A						0.252	stem
glucose release (%)	N22	A						0.250	stem
glucose release (%)	Tainung	A	B					0.244	stem
glucose release (%)	FR13A	A	B					0.242	stem
glucose release (%)	nipponbare	A	B					0.242	stem
glucose release (%)	Pokkali	A	B					0.237	stem
glucose release (%)	Dular	A	B	C	D			0.231	stem
glucose release (%)	IR64	A	B	C				0.230	stem
glucose release (%)	Azucena	A	B	C	D			0.224	stem
glucose release (%)	Moroberekan	A	B	C	D			0.223	stem
glucose release (%)	Cypress	A	B	C	D			0.218	stem
glucose release (%)	dom_sufid	A	B	C	D			0.215	stem
glucose release (%)	Sadu-Cho	A	B	C	D			0.214	stem
glucose release (%)	Swarna	A	B	C	D			0.213	stem
glucose release (%)	Rayada		B	C	D	E		0.205	stem
glucose release (%)	Aswina			C	D	E		0.188	stem
glucose release (%)	SHZ-2			C	D	E		0.187	stem
glucose release (%)	Zhenshan-97B				D	E		0.180	stem
glucose release (%)	Minghui					E		0.168	stem
glucose release (%)	LTH	A						0.239	combined
glucose release (%)	N22	A						0.237	combined
glucose release (%)	M202	A						0.236	combined
glucose release (%)	Moroberekan	A	B					0.232	combined
glucose release (%)	Pokkali	A	B	C				0.229	combined
glucose release (%)	Tainung	A	B	C	D			0.221	combined
glucose release (%)	Azucena	A	B	C	D			0.220	combined
glucose release (%)	FR13A	A	B	C	D			0.215	combined
glucose release (%)	IR64	A	B	C	D	E		0.211	combined
glucose release (%)	Cypress	A	B	C	D	E		0.209	combined
glucose release (%)	Aswina	A	B	C	D	E	F	0.203	combined
glucose release (%)	Swarna	A	B	C	D	E	F	0.203	combined
glucose release (%)	Rayada	A	B	C	D	E	F	0.200	combined
glucose release (%)	Sadu-Cho	A	B	C	D	E	F	0.189	combined
glucose release (%)	SHZ-2		B	C	D	E	F	0.183	combined
glucose release (%)	dom_sufid		B	C	D	E	F	0.183	combined
glucose release (%)	nipponbare			C	D	E	F	0.179	combined
glucose release (%)	Dular		B	C	D	E	F	0.177	combined
glucose release (%)	Zhenshan-97B				D	E	F	0.169	combined
glucose release (%)	Minghui						F	0.157	combined
pentose release (%)	Zhenshan-97B	A	B					0.042	combined
pentose release (%)	IR64		B	C				0.036	combined

Table B.2 (continued). Sugar yield of 20 rice varieties grown in the GH. Combined tissue is the whole plant.

Assay	Variety	LSD letter group					Mean	Tissue
pentose release (%)	N22		B	C	D		0.034	combined
pentose release (%)	Azucena		B	C	D		0.034	combined
pentose release (%)	Minghui		B	C	D	E	0.033	combined
pentose release (%)	Dular		B	C	D	E	0.033	combined
pentose release (%)	Aswina		B	C	D	E	0.033	combined
pentose release (%)	Moroberekan		B	C	D	E	0.033	combined
pentose release (%)	dom_sufid		B	C	D	E	0.032	combined
pentose release (%)	Cypress		B	C	D	E	0.031	combined
pentose release (%)	Tainung			C	D	E	0.030	combined
pentose release (%)	FR13A			C	D	E	0.029	combined
pentose release (%)	Rayada			C	D	E	0.029	combined
pentose release (%)	Swarna			C	D	E	0.028	combined
pentose release (%)	M202			C	D	E	0.028	combined
pentose release (%)	nipponbare			C	D	E	0.027	combined
pentose release (%)	SHZ-2			C	D	E	0.026	combined
pentose release (%)	LTH				D	E	0.025	combined
pentose release (%)	Sadu-Cho				D	E	0.024	combined
pentose release (%)	Pokkali					E	0.023	combined
pentose release (%)	Zhenshan-97B	A					0.038	stem
pentose release (%)	Dular	A	B				0.032	stem
pentose release (%)	IR64	A	B	C			0.030	stem
pentose release (%)	Moroberekan	A	B	C			0.030	stem
pentose release (%)	Aswina		B	C	D		0.029	stem
pentose release (%)	Tainung		B	C	D		0.029	stem
pentose release (%)	Swarna		B	C	D		0.028	stem
pentose release (%)	Minghui		B	C	D		0.028	stem
pentose release (%)	M202		B	C	D		0.027	stem
pentose release (%)	dom_sufid		B	C	D		0.026	stem
pentose release (%)	N22		B	C	D		0.026	stem
pentose release (%)	Cypress		B	C	D		0.025	stem
pentose release (%)	Pokkali		B	C	D		0.024	stem
pentose release (%)	Sadu-Cho		B	C	D		0.024	stem
pentose release (%)	SHZ-2		B	C	D		0.023	stem
pentose release (%)	Rayada		B	C	D		0.023	stem
pentose release (%)	nipponbare		B	C	D		0.023	stem
pentose release (%)	Azucena		B	C	D		0.023	stem
pentose release (%)	FR13A			C	D		0.023	stem
pentose release (%)	LTH				D		0.021	stem

Table B.3. Stem wall thickness of 16 rice varieties grown in the GH, from the base of the plant. This is attached to this dissertation at the Colorado State University Libraries' Digital Repository as an excel file.

Stem Position (cm)	Variety	LSD letter group									Mean
base	Aswina	A	B								0.117
base	Pokkali	A									0.114
base	Azucena	A	B	C							0.106
base	Minghui	A	B	C	D						0.092
base	FRI3A		B	C	D	E					0.086
base	Tainung			C	D	E	F				0.077
base	IR64				D	E	F	G			0.066
base	Cypress					E	F	G	H		0.057
base	Dom Sufid					E	F	G	H		0.054
base	N22						F	G	H	I	0.046
base	M202							G	H		0.045
base	Dular							G	H	I	0.038
base	SHZ							G	H	I	0.034
base	LTH								H	I	0.031
base	Zhenshan97B									I	0.023
base	Kitaake									I	0.021
5.08	Aswina	A									0.097
5.08	Pokkali	A	B								0.084
5.08	Azucena		B	C							0.068
5.08	Tainung		B	C							0.067
5.08	FRI3A		B	C							0.067
5.08	Minghui			C	D						0.051
5.08	Dom Sufid				D	E					0.043
5.08	IR64				D	E					0.042
5.08	Cypress				D	E	F				0.039
5.08	N22				D	E	F				0.037
5.08	M202				D	E					0.036
5.08	LTH				D	E	F				0.030
5.08	SHZ				D	E	F				0.029
5.08	Dular					E	F				0.028
5.08	Kitaake					E	F				0.026
5.08	Zhenshan97B						F				0.021
10.16	Aswina	A									0.090
10.16	Pokkali	A									0.086
10.16	Minghui		B								0.062
10.16	Tainung		B	C							0.052
10.16	Azucena		B	C							0.052
10.16	FRI3A		B	C	D						0.047
10.16	Dom Sufid			C	D	E					0.036
10.16	N22			C	D	E					0.035
10.16	LTH			C	D	E					0.034
10.16	IR64			C	D	E					0.034

Table B.3 (continued). Stem wall thickness of 16 rice varieties grown in the GH, from the base of the plant.

Stem Position (cm)	Variety	LSD letter group							Mean
10.16	Cypress			C	D	E			0.031
10.16	SHZ			C	D	E			0.027
10.16	M202				D	E			0.027
10.16	Dular				D	E			0.023
10.16	Zhenshan97B					E			0.018
10.16	Kitaake					E			0.013
15.24	Aswina	A							0.078
15.24	Pokkali	A	B						0.071
15.24	FRI3A	A	B	C					0.068
15.24	Azucena	A	B	C	D				0.051
15.24	Dom Sufid		B	C	D	E			0.049
15.24	Minghui			C	D	E	F		0.048
15.24	Tainung			C	D	E	F		0.046
15.24	N22				D	E	F	G	0.037
15.24	Cypress				D	E	F	G	0.029
15.24	IR64				D	E	F	G	0.028
15.24	SHZ				D	E	F	G	0.026
15.24	M202						F	G	0.026
15.24	LTH				D	E	F	G	0.022
15.24	Dular					E	F	G	0.022
15.24	Zhenshan97B							G	0.018
15.24	Kitaake							G	0.017
20.32	Aswina	A							0.069
20.32	Pokkali	A							0.066
20.32	FRI3A	A	B						0.053
20.32	Tainung	A	B						0.048
20.32	Azucena	A	B	C					0.047
20.32	Minghui		B	C	D				0.045
20.32	Dom Sufid		B	C	D	E			0.032
20.32	Cypress		B	C	D	E			0.029
20.32	N22		B	C	D	E			0.029
20.32	LTH			C	D	E			0.024
20.32	M202					E			0.023
20.32	IR64				D	E			0.022
20.32	Dular				D	E			0.021
20.32	SHZ				D	E			0.021
20.32	Zhenshan97B					E			0.016

Table B.4. Elemental composition of leaf, stem, and seed of 20 varieties of rice. This is also attached to this dissertation at the Colorado State University Libraries' Digital Repository as an excel file.

Tissue	Element	Aswina	Azucena	Cypress	Dom Sufid	Dular	FR13A	IR64-21	LTH	M2O2	Minghui 63	Moroberekan	N22	Nipponbare	Pokkali	Rayada	Sadu-Cho	SHZ2	Swarna	Tainung 67	Zhenshan 97B
leaf	B11	36.66	32.72	36.44	38.32	56.61	41	33.59	33.5	50.45	27.3	46.32	45.46	23.94	44.34	39.54	35.44	47.96	28.68	37.28	33.38
leaf	Na23	76.31	68.11	71.36	62.75	58.43	72.62	109.4	85.99	132.3	46.67	69.96	62.73	98.04	188.18	62.7	56.98	62.19	87.6	57.49	48.03
leaf	Mg25	1082.12	1184.94	802.9	817.21	802.46	1912.79	1607.84	1289.79	1143.79	1370.7	743.45	553.55	690.03	1338.97	1285.18	2161	1050.54	803.75	1203.98	1040.97
leaf	P31	1918.45	2093.87	2056.33	1583.09	2047.75	1417.96	1487.56	1612.12	1649.74	1983.05	1535.83	1321.99	1748.15	1276.95	1933.28	2098.84	1343.49	1699.19	1747.39	2016.14
leaf	S34	1298.1	1525	1471.88	1328.03	1489.91	1195.01	1304.81	1531.01	1329.5	1444.88	1371.27	1063.59	1398.46	1230.8	1295.3	1440.53	1197.03	1107.85	1590.27	1272.11
leaf	K39	10230.41	17001.78	14176.57	9450.05	8898.1	12180.89	8875.89	9758.41	10252.41	13553.9	19900.6	11374.79	12692.47	17197.95	9722.8	8323.29	10623.32	13909.87	16898.85	9555.71
leaf	Ca44	2231.19	2595.43	2269.03	3331.07	2932.29	3856.05	3856.73	3200.4	4818.35	3734.52	3189	2117.16	2930.86	3288.62	3017.96	3792.81	5751.27	1553.66	3581.53	4071.49
leaf	Cr52	0.6	0.57	0.54	0.56	0.62	0.56	0.58	0.63	0.54	0.61	0.53	0.63	0.76	0.6	0.64	0.59	0.69	0.58	0.59	0.59
leaf	Mn55	224.12	128.53	204.33	208.13	178.56	398.07	346.38	256.89	321.39	501.65	312.64	120.09	225.8	492.21	196.15	344.44	609.81	212.75	368.95	506.15
leaf	Fe57	63.07	57.82	66.95	58.47	83.62	89.01	65.44	53.72	133.85	50.61	96.4	75.1	172.96	78.06	70.97	66.63	56.31	93.98	72.73	86.48
leaf	Co59	0.16	0.15	0.15	0.1	0.15	0.21	0.21	0.14	0.32	0.1	0.22	0.15	0.36	0.49	0.14	0.1	0.06	0.22	0.37	0.12
leaf	Ni60	0.17	0.14	0.15	0.14	0.1	0.13	0.15	0.17	0.16	0.11	0.2	0.17	0.23	0.13	0.15	0.14	0.16	0.31	0.12	0.14
leaf	Cu65	3.28	2.2	2.4	2.97	1.69	1.63	2.8	2.58	1.45	3.79	2.46	1.98	2.07	2.19	1.96	2.52	2.99	4.19	3.51	3.84
leaf	Zn66	12.77	15.67	12.67	12.23	13.94	9.5	10.29	8.21	9.13	13.25	11.58	13.27	11.39	13.86	12.83	11.02	11.06	15.76	21.92	9.94
leaf	As75	0.8	1.2	1.99	1.29	1.54	1.79	1.17	1.75	2.33	1.04	2.36	1.18	1.97	1.36	1.43	1.26	1.49	0.67	1.4	1.69
leaf	Se82	2.56	2.82	2.87	3.47	3.53	3.61	2.83	3.52	2.75	2.84	3.13	4.09	3.69	3.1	3.63	3.03	3.36	2.84	2.66	3.12
leaf	Rb85	11.75	21.2	17.44	11.17	13.29	23.56	9.95	12.5	11.25	19.53	16.39	13	16.8	26.82	13.6	12.32	10.98	17.52	22.52	12.71
leaf	Sr88	10.6	13.26	12.89	16.86	16.83	20.5	19.74	17.55	26.27	18.92	18.26	11.14	17.89	16.26	16.61	17.95	31.25	8.76	17.44	23.7
leaf	Mo98	7.69	7.89	6.56	7.4	2.73	4.34	4.21	6.34	7.97	5.92	10.96	3.3	6.04	5.81	2.51	5.46	5.39	2.8	4.21	8.21
leaf	Cd111	0.01	0.01	0.01	0.02	0.02	0.01	0.02	0.01	0.02	0.01	0.01	0.02	0.02	0.02	0.01	0.01	0.02	0.01	0.01	0.02
leaf	Al27	25.68	11.55	30.66	15.89	29.76	34.26	25.54	18.35	68.6	8.91	12.02	37.14	151.3	42.94	14.2	11.99	15.34	78.62	12.18	19.88
seed	B11	17.96	18.08	20.25	16.92	12.17	12.85	9.44	12.36	10.75	19.73	14.08	12.46	11.97	13.07	15.31	18.64	20.96	17.49	9.45	20.17
seed	Na23	450.12	515.52	428.06	346.94	456.53	358.85	515	448.86	485.67	516.05	463.48	418.61	467.56	436.54	311.21	362.65	455.64	492.51	544.47	430.51
seed	Mg25	1177.34	1048.21	1337.92	2061.3	1109.96	1400.63	1142.48	1198.11	1300.92	892.24	1242.5	1091.97	1303.37	1289.74	1568.16	1513.38	1062.89	1062.8	1047.14	1200.83
seed	P31	1592.89	1578.64	1943.08	2932.36	1709.45	2020.87	1451.46	1711.82	2150.56	1246.79	1515.46	1670.82	1996.35	1644.64	2213.5	2149.76	1388.32	1233.34	1479.27	1520.14
seed	S34	6920.14	6815.88	5869.25	5131.98	8105.29	6759.16	7298.64	6390.26	6571.11	7578.6	5696.84	7754.37	6538.2	7229.24	6245.22	6156.77	6007.11	6591.18	7441.34	7106.77
seed	K39	2717.79	3373.74	3411.28	4814.18	3759.59	3621.3	3171.82	2932.13	4829.25	2518.28	3621.11	3195.24	4545.69	3838.21	3084.67	3880.19	2270.82	2007	3252.33	3053.05
seed	Ca44	289.5	324.44	336.86	487.57	319.75	320.77	315.18	298.3	365.64	296.42	435.03	307.64	312.7	346.84	336.37	335.68	304.25	315.01	330.91	322.49
seed	Cr52	0.48	0.5	0.58	0.72	0.4	0.45	0.39	0.42	0.48	0.32	0.58	0.49	0.52	0.42	0.54	0.5	0.46	0.45	0.42	0.42
seed	Mn55	8896.49	10134.59	8604.65	5241.91	10198.96	7020.88	11033.69	9931.41	9482.43	11407.8	8583.57	9170.1	9160.54	8821.2	6164.1	6762.51	9268.79	10203.36	11652.39	8747.91
seed	Fe57	16.21	16.1	15.69	19.34	16.67	17.37	11.45	13.35	15.09	9.14	18.4	16.49	17.27	29.36	15.22	13.33	11.94	48.43	11.53	12.92
seed	Co59	9.87	11.61	8.85	5.79	10.65	8.05	11.63	10.57	10.1	12.55	9.4	10.05	9.9	10.54	6.61	7.78	9.73	11.08	13.12	9.6
seed	Ni60	4.95	6.04	4.41	2.86	6.56	4.61	6.12	5.25	4.79	6.93	4.76	5.94	4.92	4.99	4	3.8	5.07	5.63	6.11	5.16
seed	Cu65	2.36	1.25	1.35	2.97	0.92	1.94	1.71	2.1	0.96	2.1	1.5	1.36	1.3	1.65	1.61	2.04	2.01	1.71	1.24	2.03
seed	Zn66	1727.43	2101.75	1098.66	810.59	2227.99	1137.93	2634.85	2398	1854.49	2624.21	1091.95	1472.7	1716.29	1962.15	762.65	1173.37	1962.1	2297.87	2948.66	1853.19
seed	As75	0.2	0.21	0.26	0.27	0.28	0.26	0.2	0.21	0.24	0.17	0.24	0.28	0.25	0.2	0.36	0.26	0.22	0.22	0.23	0.22
seed	Se82	4.08	2.73	3.23	1.68	4.42	4.03	3.79	3.31	3.09	3.87	3.59	3.29	3.12	3.54	4.4	3.17	4.69	3.99	3.91	3.97
seed	Rb85	9.46	15.74	14.41	25.97	17.42	22.07	12.08	14.09	19.91	11.98	12.8	11.75	21.1	22.32	14.21	18.73	8.64	8.08	16.43	14.56
seed	Sr88	1.03	1.21	1.41	2.29	1.28	1.54	1.19	1.27	1.48	0.94	2.4	1.31	1.39	1.44	1.58	1.51	1.18	1.35	1.14	1.4
seed	Mo98	1.7	0.83	1.42	2.32	0.7	0.92	0.72	1.48	0.85	1.42	0.77	0.82	0.83	0.76	0.81	0.91	0.85	0.54	0.57	0.85
seed	Cd111	0.54	0.52	0.46	0.28	0.42	0.38	0.63	0.5	0.49	0.65	0.42	0.44	0.45	0.58	0.28	0.4	0.51	0.58	0.58	0.53
seed	Al27	52.69	26.56	24.52	28.55	32.74	40.35	18.34	12.43	24.9	13.51	23.13	50.93	50.41	96.1	19.62	22.6	16.89	167.49	17.19	22.31
sheath	B11	30.9	25.21	34.46	34.66	42.26	31.15	65.62	24.99	42.23	31.26	26.14	39.23	14.15	27.59	46.85	31.17	50.3	24.94	22.47	25.16
sheath	Na23	138.44	338.55	209.59	77.56	75.8	285.28	90.16	282.91	507.21	150.56	555.57	233.47	513.87	193.92	115.08	83.07	96.34	554.78	122.69	133.39
sheath	Mg25	1585.82	1201.75	940.97	1427.35	997.44	1388.02	1675.33	909.8	1079.86	1315.51	1406.51	705.01	813.31	987.25	1105.27	1862	1050.81	876.6	1662.86	1341.4
sheath	P31	1114.84	1170.19	1160.75	838.42	1227.87	842.37	868.23	1134.53	1077.85	944.17	824.7	1237.33	1155.6	779.49	1176.7	1209.09	1062.85	1478.34	839.96	859.81
sheath	S34	1232.26	854.74	1123.18	993.27	1104.25	761.99	931.3	1322.83	989.01	822.21	689.99	1024.44	1189.8	891.84	1040.03	1068.01	942.33	859.85	867.7	829.84
sheath	K39	15244.64	26475.87	21271.58	16058.53	15151.99	20896.45	12842.94	15288.14	15536.65	19321.68	21315.17	21999	13427.77	18120.61	14372.05	15687.75	12084.23	25551.31	24601.93	22034.5
sheath	Ca44	997.38	906.23	741.54	832.65	953.49	714.57	2434.79	727.77	974.31	1068.35	947.23	748.26	803.68	2128.5	858.67	1049.54	1157.67	761.41	795.44	1003.66
sheath	Cr52	0.58	0.37	0.44	0.46	0.47	0.5	0.61	0.53	0.41	0.43	0.31	0.58	0.8	0.46	0.52	0.53	0.62	0.53	0.42	0.44
sheath	Mn55	149.27	85.51	125.7	175.35	99.78	161.36	252.65	160.79	132.94	226.04	156.16	102.54	180.21	400.81	104.15	173.39	212.67	204.23	322.11	184.91
sheath	Fe57	147.43	46.23	49.26	65.4	50.52	111.68	53.12	70.53	165.61	43.56	71.28	65.24	1128	169.77	56.65	50.23	38.25	214.38	99.59	77.56
sheath	Co59	0.55	0.58	0.68	0.67	0.99	0.87	0.53	1.11	1.61	0.54	0.76	0.88	1.65	0.61	0.71	0.62	0.45	0.65	1.64	0.55
sheath	Ni60	0.17	0.09	0.12	0.11	0.14	0.11	0.14	0.14	0.17	0.11	0.1	0.15	0.37	0.13	0.14	0.12	0.14	0.21	0.13	0.1
sheath	Cu65	2.2	1.12	1.32	1.54	0.89	1.16	2.2	1.39	1.12	2	1.59	1.05	3.92	1.89	0.8	1.53	2.2	3.23	2.23	2.89
sheath	Zn66	15.36	8.51	9.7	14.6	8.43	11.14	11.25	8.03	12.93	10.49	9.6	9.88	9.28	13.75	9.09	8.96	16.09	13.27	19.11	7.59
sheath	As75	0.5	0.68	0.7	0.58	0.87	0.78	0.95	0.73	0.73	0.45	0.78	0.68	0.82	0.91	0.7	0.55	0.52	0.39	0.66	0.54
sheath	Se82	2.2	2.34	2.29	3.13	3.28	2.98	2.77	3.06	2.79	2.26	2.17	3.15	6.18	2.13	3.17	2.49	2.54	2.63	2.17	2.65
sheath	Rb85	22.75	41.85	33.67	30.99	29.59	45.74	16.34	29.88	26.22	35.11	37.97	28.58	26.73	31.3	28.3	29.23	19.1	42.89	56.58	45.58
sheath	Sr88	5.66	6.14	5.35	5.33	6.83	5.35	13.95	5.28	6.56	6.77	6.79	5.42	6.99	13.52	6.08	6.06	7.29	5.45	5.6	7.11
sheath	Mo98	2	1.6	1.36	1.38	1.06	0.84	2.28	2.14	1.58	0.82	1.83	0.79	1.07	3.71	0.38	1.33	1.04	0.54	1.03	2.14
sheath	Cd111	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.03	0.01	0.01
sheath	Al27	177.92	39.01	51.25	73.06	45.63	103.47	32.13	69.12	197.78	35.83	66.57	68.59	1699.15	213.84	42.3	35.92	28.32	285.24	91.45	87.11

TABLE B.5. Matrix of Spearman's correlations coefficients for all phenotypes measured. * p<0.05, ** p<0.01. This is also attached to this dissertation at the Colorado State University Libraries' Digital Repository as an excel file.

Spearman's correlation coefficients, * p<0.05, ** p<0.01

	Cellulose	Total_Glucose	Hemicellulose	Total_Xylose	Lignin	Klason_Lignin	Ash	Klason_Ash	glucose_base	glucose_water	pentose_base	pentose_water	Glu_Efficiency	Pen_Efficiency	MLG	HRGP
Cellulose																
Total_Glucose	0.57 **															
Hemicellulose	0.22	0.30														
Total_Xylose	0.60 **	0.28	0.67 **													
Lignin	0.01	-0.31	-0.83 **	-0.39												
Klason_Lignin	-0.30	-0.83 **	0.06	0.17	0.05											
Ash	-0.24	-0.56 *	-0.86 **	-0.55 *	0.87 **	0.23										
Klason_Ash	-0.32	-0.63 **	-0.80 **	-0.45 *	0.83 **	0.39	0.95 **									
glucose_base	0.23	0.61 **	0.75 **	0.34	-0.83 **	-0.42	-0.88 **	-0.95 **								
glucose_water	0.30	0.70 **	0.41	0.07	-0.51 *	-0.62 **	-0.56 *	-0.71 **	0.83 **							
pentose_base	0.04	-0.18	0.42	0.31	-0.43	0.41	-0.28	-0.24	0.33	0.15						
pentose_water	0.27	0.03	-0.06	0.02	0.03	0.00	0.12	0.00	0.17	0.42	0.64 **					
Glu_Efficiency	-0.06	0.01	0.71 **	0.26	-0.79 **	0.13	-0.68 **	-0.70 **	0.78 **	0.49 *	0.64 **	0.32				
Pen_Efficiency	-0.23	-0.38	0.31	0.06	-0.37	0.52 *	-0.14	-0.10	0.23	0.05	0.92 **	0.55 *	0.64 **			
MLG	0.58 **	0.60 **	0.19	0.23	-0.26	-0.53 *	-0.28	-0.37	0.34	0.38	0.03	0.16	0.08	-0.15		
HRGP	-0.32	-0.56 *	0.32	0.07	-0.22	0.73 **	-0.03	0.08	-0.00	-0.18	0.76 **	0.33	0.46 *	0.86 **	-0.38	
Density	0.35	0.79 **	0.02	-0.04	0.04	-0.85 **	-0.24	-0.38	0.41	0.64 **	-0.36	0.04	-0.15	-0.48 *	0.29	-0.60 **

Table B.6. Matrix of Spearman's correlations coefficients for all phenotypes measured, separately for each environment. * p<0.05, ** p<0.01. This is also attached to this dissertation at the Colorado State University Libraries' Digital Repository as an excel file

Spearman's correlation coefficients, * p<0.05, ** p<0.01

	Ash_field	Ash_GH	Cellulose_field	Cellulose_GH	Density_field	Density_GH	Glu.Base.Efficiency_field	Glu.Base.Efficiency_GH	Glu.Base.Yield_f	Glu.Base.Yield_G	Glu.Water.Yield_field	Glu.Water.Yield_H	Hemicellulose_f	Hemicellulose_G	HRGP_field	HRGP_GH	Klason.Ash_field	Klason.Ash_GH	Klason.Lignin_fa	Klason.Lignin_G	Lignin_field	Lignin_GH	MLG_field	MLG_GH	Total.Glu_field	Total.Glu_GH	Total.Xyl_field	Total.Xyl_GH	Xyl.Base.Efficiency	Xyl.Base.Efficiency	Xyl.Base.Yield_f	Xyl.Base.Yield_G	Xyl.Water.Yield_	
Ash_field																																		
Ash_GH	0.93 **																																	
Cellulose_field	−0.65 *	−0.55																																
Cellulose_GH	−0.01	−0.06	0.44																															
Density_field	−0.34	−0.16	0.73 *	0.50																														
Density_GH	−0.76 *	−0.61	0.92 **	0.10	0.60																													
Glu.Base.Efficiency_field	0.26	0.49	−0.25	−0.46	−0.08	−0.01																												
Glu.Base.Efficiency_GH	0.34	0.34	−0.72 *	−0.15	−0.39	−0.64 *	0.48																											
Glu.Base.Yield_field	−0.52	−0.31	0.70 *	0.24	0.54	0.76 *	0.39	−0.14																										
Glu.Base.Yield_GH	−0.71 *	−0.55	0.71 *	−0.02	0.52	0.87 **	0.30	−0.20	0.85 **																									
Glu.Water.Yield_field	−0.08	0.14	0.53	0.48	0.49	0.42	0.31	−0.18	0.74 *	0.34																								
Glu.Water.Yield_GH	−0.41	−0.28	0.65 *	−0.06	0.49	0.77 **	0.36	−0.20	0.78 *	0.89 **	0.27																							
Hemicellulose_field	−0.44	−0.54	0.25	0.64 *	0.30	0.08	−0.64 *	−0.02	0.06	−0.01	0.04	−0.32																						
Hemicellulose_GH	−0.34	−0.39	−0.01	0.43	0.01	−0.14	−0.32	0.33	0.16	−0.04	0.18	−0.41	0.77 **																					
HRGP_field	0.81 **	0.76 *	−0.33	0.18	0.06	−0.54	−0.04	−0.04	−0.44	−0.66 *	0.01	−0.43	−0.18	−0.25																				
HRGP_GH	0.39	0.19	−0.85 **	−0.22	−0.77 **	−0.85 **	−0.10	0.65 *	−0.64 *	−0.67 *	−0.55	−0.74 *	0.11	0.41	0.18																			
Klason.Ash_field	0.89 **	0.72 *	−0.78 **	−0.04	−0.50	−0.92 **	−0.07	0.36	−0.81 **	−0.92 **	−0.36	−0.69 *	−0.21	−0.14	0.73 *	0.65 *																		
Klason.Ash_GH	0.83 **	0.72 *	−0.85 **	−0.21	−0.54	−0.85 **	0.15	0.46	−0.71 *	−0.82 **	−0.34	−0.67 *	−0.20	−0.18	0.64 *	0.64 *	0.90 **																	
Klason.Lignin_field	0.67 *	0.60	−0.78 **	−0.12	−0.46	−0.83 **	−0.06	0.37	−0.79 **	−0.89 **	−0.32	−0.81 **	−0.02	−0.04	0.53	0.59	0.85 **	0.90 **																
Klason.Lignin_GH	0.53	0.41	−0.71 *	0.02	−0.54	−0.83 **	−0.32	0.27	−0.81 **	−0.95 **	−0.34	−0.96 **	0.16	0.22	0.47	0.72 *	0.78 **	0.73 *	0.88 **															
Lignin_field	0.75 *	0.69 *	−0.31	−0.01	0.10	−0.42	0.10	0.06	−0.47	−0.40	−0.26	0.01	−0.44	−0.62	0.73 *	−0.01	0.64 *	0.52	0.41	0.16														
Lignin_GH	0.26	0.28	0.33	0.59	0.28	0.13	−0.27	−0.49	−0.02	−0.20	0.32	−0.06	0.08	−0.28	0.39	−0.41	0.10	0.02	0.06	0.15	0.25													
MLG_field	−0.85 **	−0.85 **	0.82 **	0.14	0.43	0.84 **	−0.30	−0.50	0.56	0.79 **	0.12	0.69 *	0.24	0.10	−0.69 *	−0.56	−0.84 **	−0.93 **	−0.87 **	−0.74 *	−0.47	−0.12												
MLG_GH	0.19	0.20	0.16	0.26	0.08	0.10	0.47	0.27	0.56	0.34	0.58	0.46	−0.24	0.07	−0.06	−0.15	−0.08	−0.15	−0.36	−0.47	0.04	−0.12	0.10											
Total.Glu_field	−0.76 *	−0.71 *	0.95 **	0.42	0.55	0.88 **	−0.36	−0.69 *	0.65 *	0.66 *	0.48	0.53	0.36	0.16	−0.58	−0.71 *	−0.79 **	−0.87 **	−0.74 *	−0.61	−0.52	0.22	0.87 **	0.14										
Total.Glu_GH	−0.66 *	−0.64 *	0.84 **	0.14	0.50	0.90 **	−0.07	−0.54	0.70 *	0.87 **	0.22	0.83 **	0.09	−0.14	−0.48	−0.70 *	−0.82 **	−0.81 **	−0.92 **	−0.89 **	−0.29	0.06	0.88 **	0.24	0.79 **									
Total.Xyl_field	−0.69 *	−0.73 *	0.67 *	0.61	0.48	0.54	−0.65 *	−0.39	0.26	0.37	0.12	0.20	0.75 *	0.37	−0.49	−0.39	−0.54	−0.61	−0.38	−0.25	−0.41	0.26	0.69 *	−0.15	0.76 *	0.50								
Total.Xyl_GH	−0.14	−0.25	−0.03	0.68	0.16	−0.24	−0.67 *	0.08	−0.30	−0.36	−0.12	−0.56	0.90 **	0.62	0.04	0.26	0.15	0.13	0.37	0.49	−0.16	0.19	−0.08	−0.39	0.07	−0.27	0.61							
Xyl.Base.Efficiency_field	0.65 *	0.77 **	0.02	0.14	0.22	−0.09	0.38	−0.13	0.06	−0.14	0.34	0.18	−0.58	−0.58	0.66 *	−0.32	0.30	0.20	0.06	−0.04	0.65 *	0.59	−0.38	0.27	−0.22	−0.09	−0.47	−0.46						
Xyl.Base.Efficiency_GH	0.58	0.46	−0.52 **	−0.38	−0.64 *	−0.78 **	0.36	0.78 **	−0.47	−0.55	−0.33	−0.01	−0.07	0.16	0.28	0.78 **	0.61	0.82 **	0.73 *	0.68	0.18	−0.41	−0.78 **	−0.08	−0.86 **	−0.76 *	−0.58	0.13	−0.18					
Xyl.Base.Yield_field	0.19	0.38	0.39	0.31	0.68	0.26	0.16	−0.33	0.28	0.16	0.41	0.33	−0.26	−0.37	0.42	−0.58	−0.13	−0.25	−0.27	−0.25	0.48	0.62	0.01	0.07	0.14	0.20	−0.07	−0.26	0.83 **	−0.53				
Xyl.Base.Yield_GH	0.43	0.34	−0.88 **	−0.22	−0.53	−0.81 **	0.22	0.87 **	−0.47	−0.52	−0.39	−0.60	0.12	0.33	0.16	0.83 **	0.59	0.73 *	0.67 *	0.58	0.11	−0.47	−0.69 *	−0.06	−0.81 **	−0.72 *	−0.42	0.38	−0.27	0.95 **	−0.54			
Xyl.Water.Yield_field	0.38	0.58	0.31	0.38	0.42	0.21	0.46	−0.16	0.39	0.18	0.65 *	0.43	−0.47	−0.48	0.33	−0.68	−0.82	−0.89	−0.16	−0.31	0.42	0.59	−0.12	0.49	0.10	0.13	−0.19	−0.43	0.88 **	−0.36	0.79 **	−0.44		
Xyl.Water.Yield_GH	0.54	0.53	−0.42	0.09	−0.02	−0.42	0.53	0.74 *	0.02	−0.02	−0.02	0.15	−0.20	−0.08	0.22	0.22	0.36	0.39	0.18	−0.08	0.52	−0.16	−0.39	0.55	−0.54	−0.21	−0.34	−0.10	0.31	0.44	0.06	0.54	0.30	

THIS PAGE INTENTIONALLY LEFT BLANK

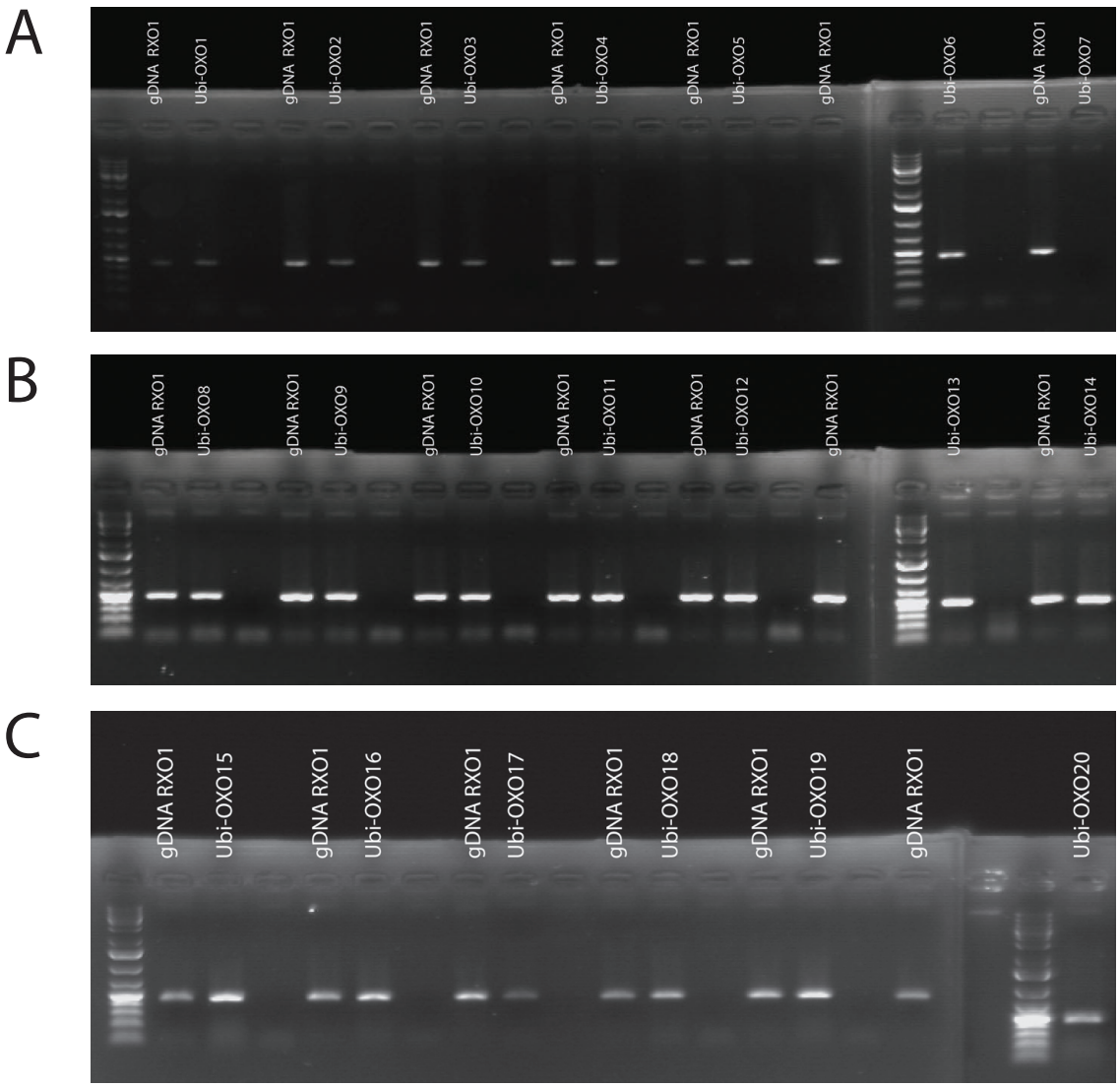


FIGURE B.2. Gel image of PCR of *hygromycin phosphotransferase* fragment (456 bp) from gDNA of T0 plants.

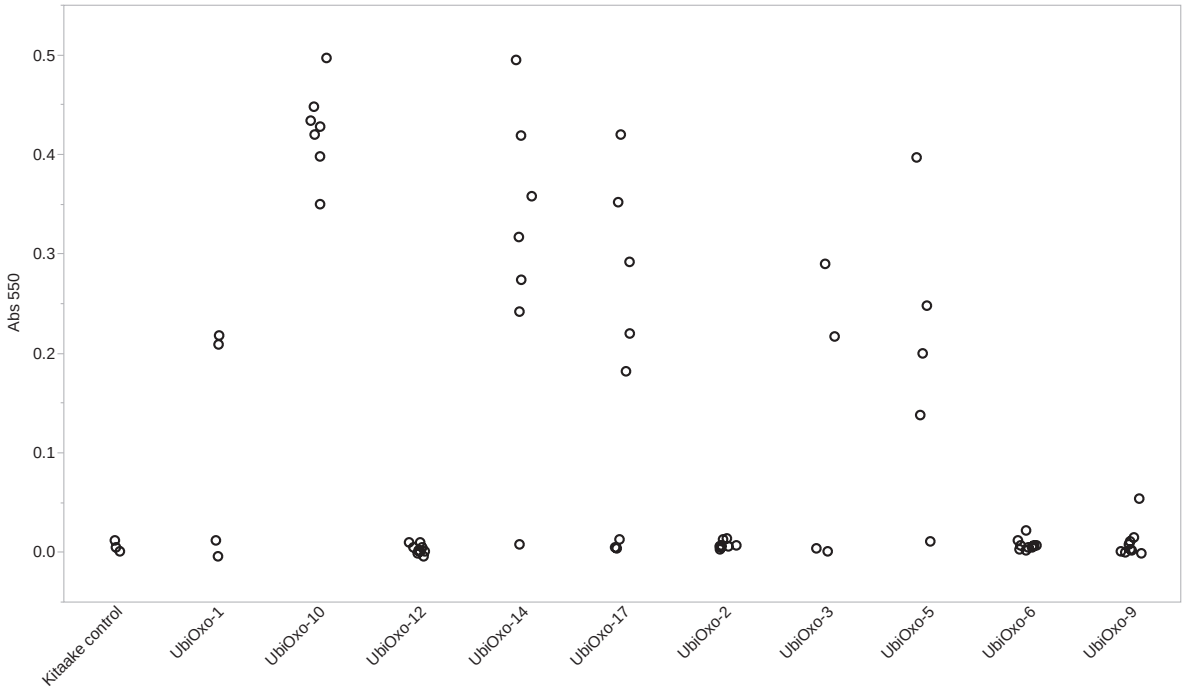


FIGURE B.3. OXO activity of leaf discs excised from T1 plants.

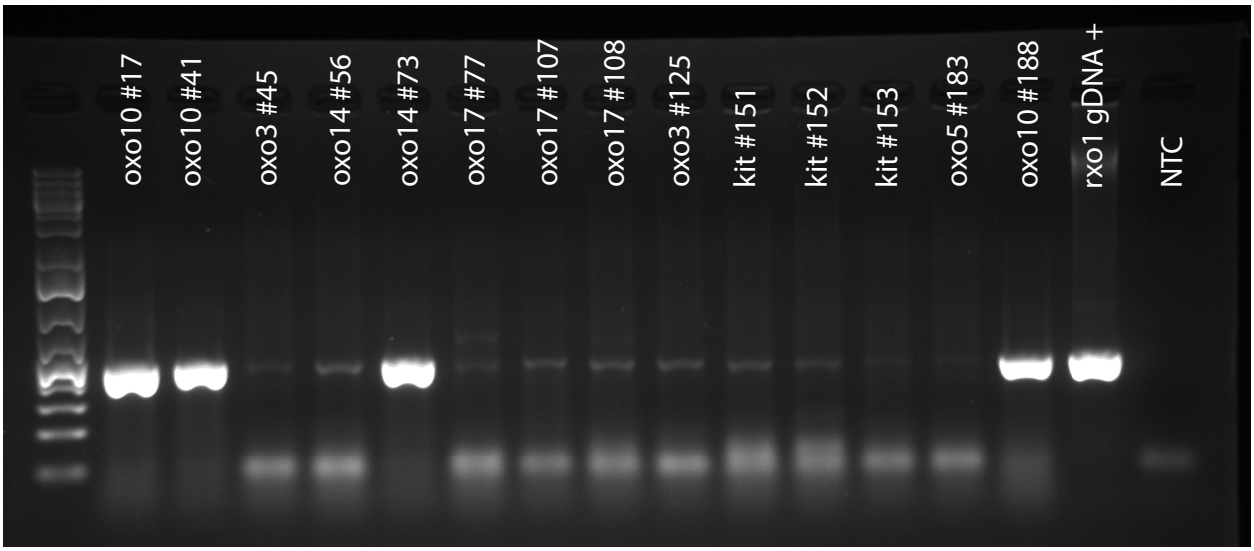


FIGURE B.4. Gel image of PCR of *hygromycin phosphotransferase* fragment (456 bp) from gDNA of T1 plants.

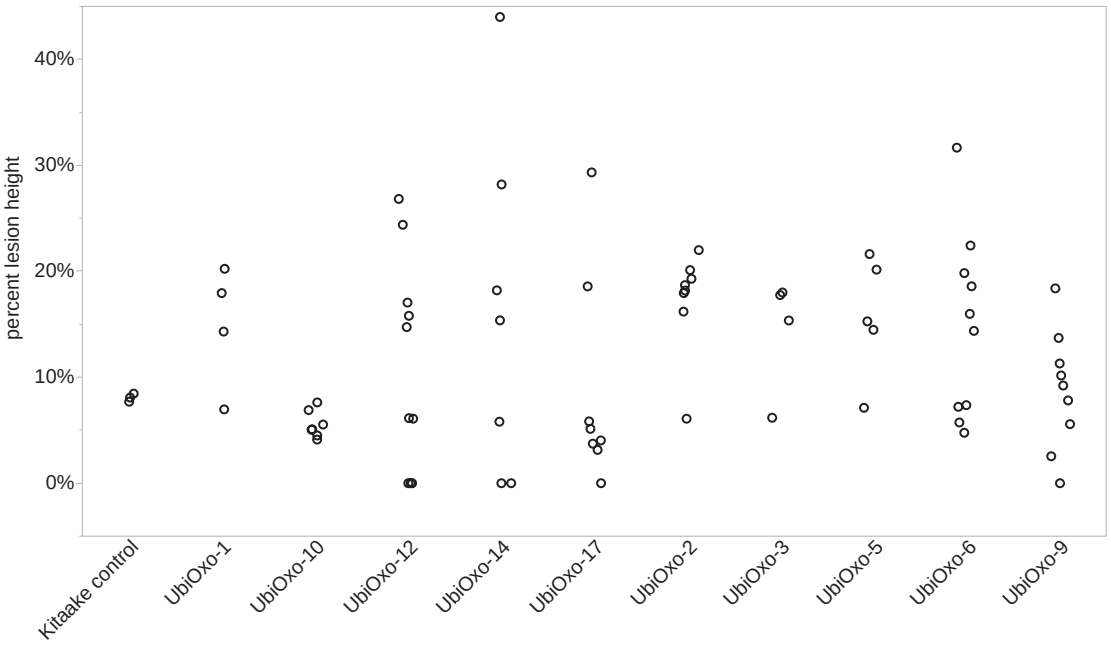


FIGURE B.5. Lesion height as a percent of total plant height from *Rs* microchamber assays on T1 plants.

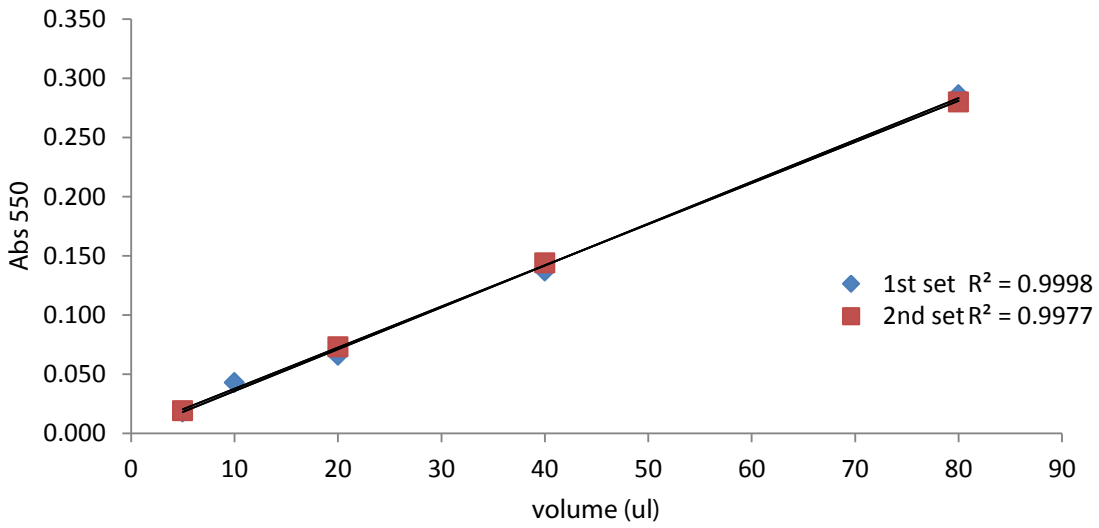


FIGURE B.6. Four point standard dilution of the OXO activity assay with two technical replicates. A linear regression was fit to each set of technical replicates.

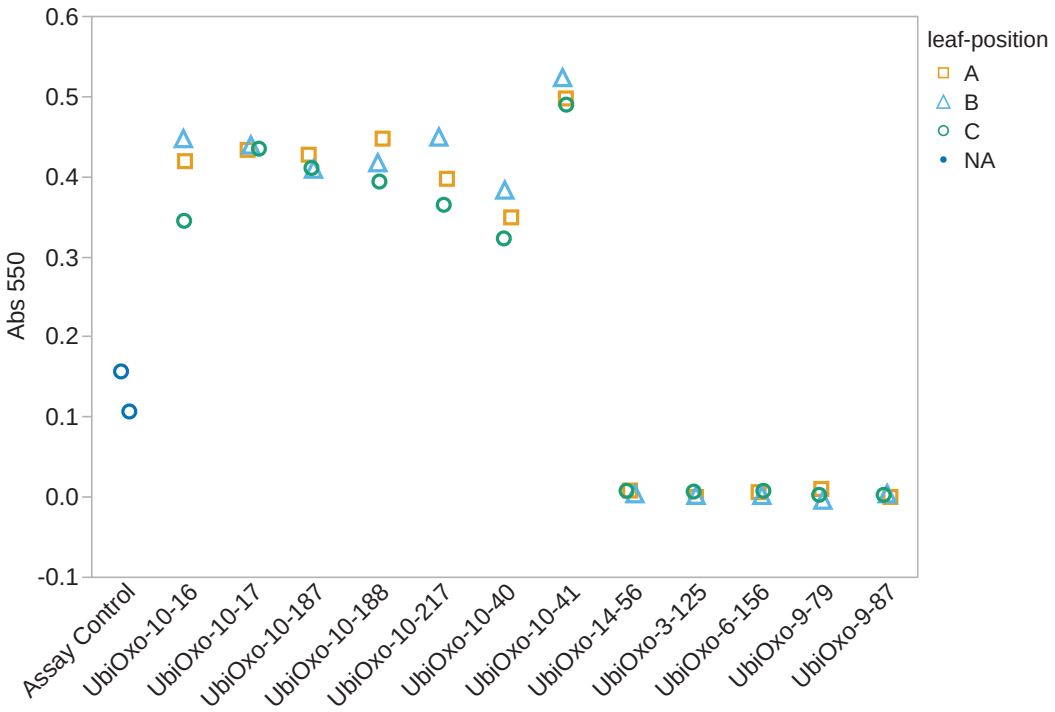


FIGURE B.7. Variation of OXO activity assay on leaf discs from different positions along leaf. Positions A and B were from leaf discs excised close to each other on the same leaf of each plant, and position C was on a different leaf. Leaf used was second fully expanded leaf.

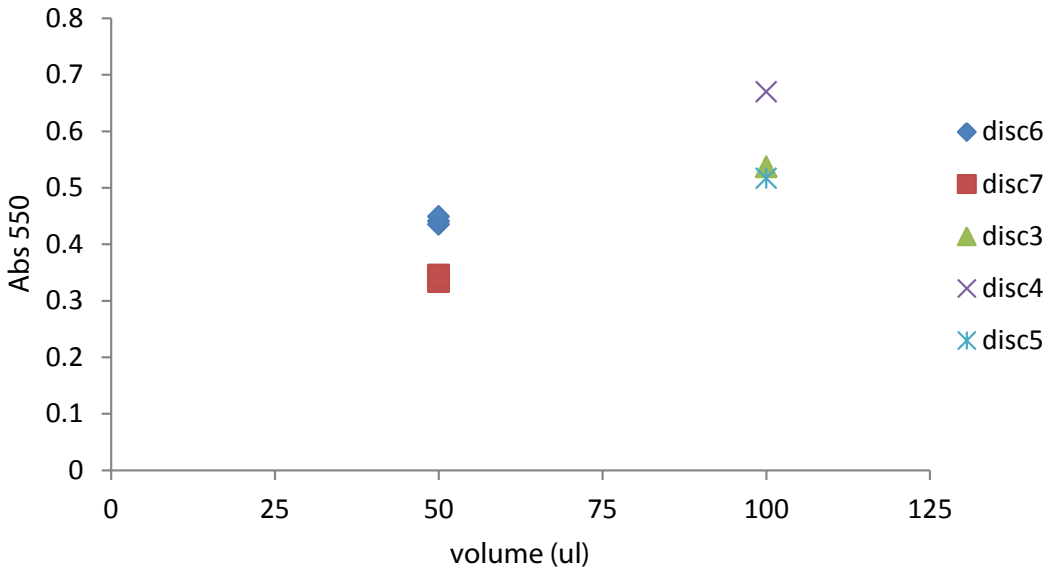


FIGURE B.8. Variation of OXO activity assay of 7 leaf discs taken from the second fully expanded leaf from plant UbiOxo-10-41-229. All discs were taken in a row from the middle the leaf, as close as possible next to each other. Discs 6 and 7 served as technical replicate controls, and 3 aliquots of 50 µL of supernatant was taken from the well containing either disc 6 or disc 7, to determine any variation in the assay. Discs 3-5 served to determine any variation between leaf discs from the same leaf, and 100 µL of supernatant was used.

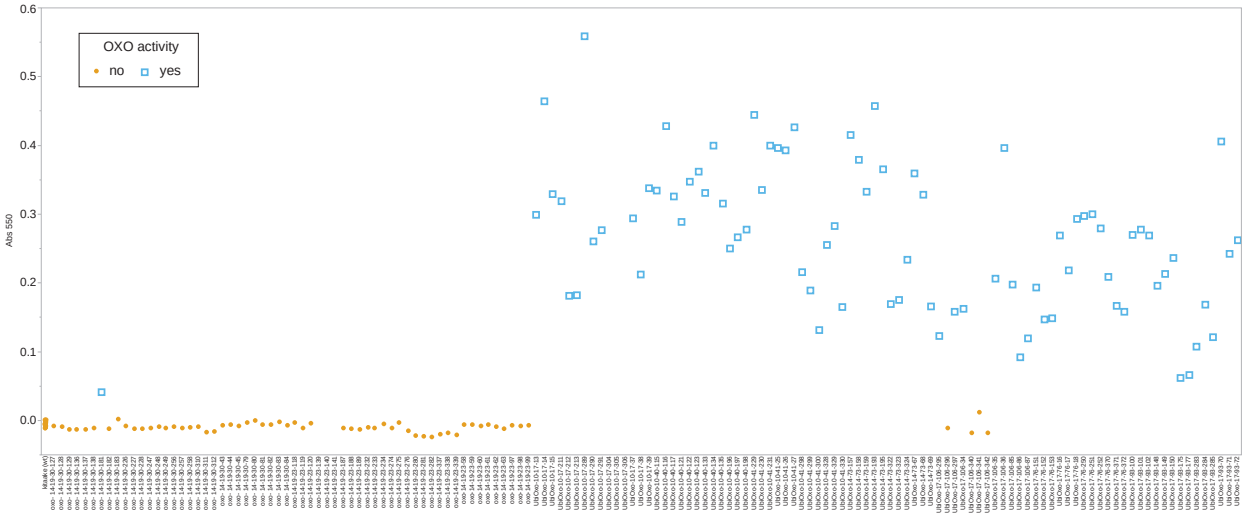


FIGURE B.9. OXO activity of silenced, control, and overexpression plants. On the left side is kitaake controls and silenced plants (orange circles), and on the right is the overexpression plants (blue squares).

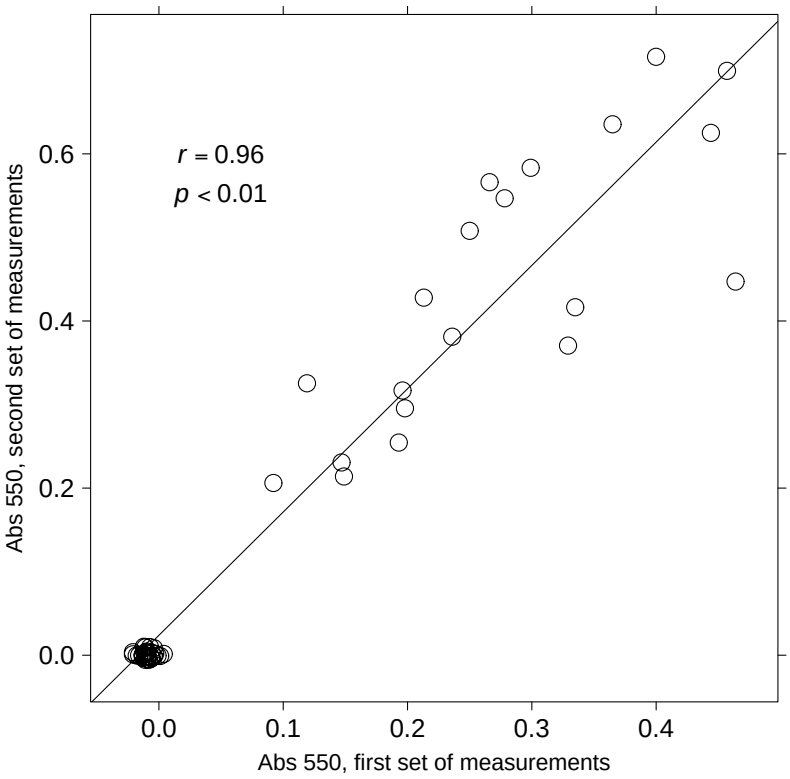


FIGURE B.10. Scatterplot and Pearson's correlations of OXO activity from the day that the microchamber assay was scored, and day of leaf collection for the second detached leaf experiment of leaf discs from the same plants. The cluster of points around zero are Kitaake and silenced lines, which never exhibited OXO activity.

B.3. Video documentary of research

Communicating science to different groups of society is a critical need. Beyond basic research which seeks knowledge, applied research attempts to address real world issues; both aspects of scientific research can be highlighted. In order to give our research a broad audience and highlight the motivation, collaboration, and potential benefit of our research, I conceived and directed a short documentary of one of my research projects. Footage was shot both at the field site in the Philippines, and on the Colorado State University campus, along with interviews of some of the key collaborators.

Colorado State University and the International Rice Research Institute have outfitted a farm tractor with GPS positioned sensors that allow the measurement of several plant characteristics (plant height, temperature, water use, biomass, among others) on thousands of plants at once. We are using this technology to identify plant genes that control biomass production—the leaves and stems, and enable development of rice varieties with not only increased grain production, but better utilization of the leaves and the stems for animal feed or bioenergy.

The video is attached to this dissertation at the Colorado State University Libraries' Digital Repository.

REFERENCES

[1] Vega-Sánchez, M. E., Verhertbruggen, Y., Christensen, U., Chen, X., Sharma, V., Varanasi, P., Jobling, S. A., Talbot, M., White, R. G., Joo, M., Singh, S., Auer, M., Scheller, H. V., and Ronald, P. C., 2012. Loss of Cellulose synthase-like F6 function affects mixed-linkage glucan deposition, cell wall mechanical properties, and defense responses in vegetative tissues of rice. *Plant physiology*, 159, 1:56–69

[2] Danku, J. M., Lahner, B., Yakubova, E., and Salt, D. E., 2013. Large-scale plant ionomics. In *Methods Mol Biol*, vol. 953, pp. 255–276. New York, NY: Humana Press

[3] Jahn, C. E., Mckay, J. K., Mauleon, R., Stephens, J., McNally, K. L., Bush, D. R., Leung, H., and Leach, J. E., 2011. Genetic Variation in Biomass Traits among 20 Diverse Rice Varieties. *Plant Physiology*, 155, 1:157–168