ON THE IMPORTANCE OF WATCHING GRASS GROW: PLANT DESIGN AND INTERNODE GROWTH AND DEVELOPMENT IN SORGHUM BICOLOR

A Dissertation

by

JOEL ANDREW OLIVER

Submitted to the Graduate and Professional School of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	John Mullet
Committee Members,	Tatyana Igumenova
	Scott Finlayson
	Dorothy Shippen
Head of Department,	Joshua Wand

August 2021

Major Subject: Biochemistry

ABSTRACT

The old Western idiom “like watching grass grow” implies disinterest, apathy, and boredom. The historical etymology of this phrase is uninspired as grasses are one of the most important family of plants across the globe. They are used for everything from recreation to infrastructure and food; grasses have been manipulated and designed for centuries to fit our needs. One innovative area is the design of dedicated bioenergy grasses to supplement the growing demand for clean energy. *Sorghum bicolor* is a C4 grass that grows to 4-5 meters tall with high biomass accumulation in its thick, sugar-packed stems. This biomass can be easily converted to bioenergy through mechanical, enzymatic, and chemical processing. Because of the importance of the stem in biomass accumulation, understanding stem biology is critical to the future design of *Sorghum* as a dedicated bioenergy grass. Here, we characterize a dwarfing gene responsible for stem growth, Dw2, and further define specific tissues within the stem. Both findings contribute to the understanding of growth and development of grass stems and internodes. As shown later, the AGCVIII kinase Dw2 controls internodal growth through the regulation of lipid signaling, endomembrane trafficking, and reactive oxygen species. Additionally, the research shows that the stem of grasses consists of four major tissues: the Pulvinus, the Nodal Plexus, the Internode, and the White Band. Each of these tissues have specialized roles in the proper development of grass stems and their coordination is required for efficient biomass accumulation. Overall, this research facilitates the underlying knowledge required to design *Sorghum* as an ideal bioenergy grass, draws

connections to other important crops, and highlights the necessary, albeit sometimes tedious, requirement of watching grass grow.

ACKNOWLEDGEMENTS

I would like to thank my committee, Dr. Shippen, Dr. Igumenova, and Dr. Finalyson for their guidance and support throughout this process. Doing this work is not easy, and they were incredibly helpful in pushing me to think to the next level. Thank you to my thesis advisor, Dr. Mullet, for putting up with my, at times, exhausting enthusiasm, my constant need to jump to a model, and always being ready "to see something cool."

The department of TAMU Biochemistry and Biophysics also deserves praise. I learned a staggering amount of information, not only about science, but about myself as well. Many thanks to the professors and staff who helped guide me throughout this process. Special thanks to Terry Lovingshimer and his amazing team of building managers; more than once did I get a call in the middle of the night about growth chambers going down, and he and his people were right there with me, hurling plants from the rapidly warming room.

A huge thanks goes to the Great Lakes Bioenergy Research Center's (GLBRC) Plant Design team. Many fruitful discussions were had regarding my project, and it was an amazing experience to work with such dedicated and brilliant scientists from around the world on a regular basis.

I would also like to thank my friends who I can commiserate and celebrate with on a daily basis; I could not have done it without them. This includes those I only see

rarely or wave at in the hallway. Stay strong and stay tough; I firmly believe great things will come to all of you.

Finally, I would like to thank my family, and most specifically my parents, Lisa Oliver and Charles (“Chuck”) Oliver Jr. Thank you for accepting me as I am and for teaching me that kindness is always cool. I love you so very much.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Professor Dorothy Shippen (Chair), Professor John Mullet (Advisor), Associate Professor Tatyana Igumenova of the Department of Biochemistry and Biophysics and Associate Professor Scott Finlayson of the Department of Soil and Crop Sciences.

Proteomic and Phosphoproteomic data used in Chapter 2 were collected by Dr. Mingzhu Fan and Dr. Curtis Wilkerson of Michigan State University, and I thank you for your input and guidance for the analysis. *In situ* data for Chapter 2 was collected by Starla Zemelis-Durfee and Dr. Federica Brandizzi at Michigan State University. I thank you for your input regarding the analysis. All other work in this document was collected and analyzed by me independently, with collaborative meetings as needed for discussion.

Funding Sources

Graduate study was supported from the Great Lakes Bioenergy Research Center (GLBRC) and the Department of Energy (DOE) grant number DOE BER Office of Science DE-SC0018409. Additionally, this work was funded in part through the Perry L. Adkisson Agricultural Chair account awarded to John Mullet. Its contents are solely the responsibility of the authors and do not necessary represent the views of the United States Department of Energy.

NOMENCLATURE

Most acronyms are spelled out in the text the first time they are used. Listed here are some that are common throughout this document.

TAMU	Texas A&M University
Dw1,2,3	Dwarfing Locus 1,2,3
Pulv	Pulvinus
WB	White Band
NP	Nodal Plexus
Int	Internode
PLD	Phospholipase
SynBio	Synthetic Biology
GE	Genetic Engineering

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

1. INTRODUCTION

The Poaceae family of plants, commonly referred to as grasses, dominate the world, blanketing over 40% of the total land area covering 52.5 million square kilometers (World Resources Institute). Their reach expands through ecosystems. From the humid to the arid, the sweltering to the frigid, grasses have evolved to survive throughout a myriad of conditions (Kellogg 2001). Humans have taken notice of this diversity and resilience and harnessed their growth in order to fuel ourselves and our economies; about half of all metabolic energy needs in the world are achieved through direct consumption of grasses, and grain feed production contributes over $80 billion dollars to the US economy every year (Crop Value List, USDA). However, grasses do not just feed the population. For centuries, East Asian cultures have used the strong lignified stem of bamboo (genus *Bambusoideae*) as a base for infrastructure. Furthermore, grasses contribute to leisure and recreation as many sporting events are played upon grass fields and personal lawns are meticulously trimmed, manicured, and nursed.

Despite their entrenchment into our economy and livelihoods for centuries, grasses are still proving valuable to new avenues of application. One of the most promising new utilizations is the development of dedicated bioenergy grasses that can be used to combat climate change. With the projected increase in population expected to reach 10 billion, coupled with an increase in global affluence that places pressure on the global energy market, agricultural productivity must double by 2050 (Foley et al. 2011). Plant based bioenergy solutions provide a double-edged sword to address the

productivity gap; developing dedicated bioenergy grasses helps solve energy demand problems while also furthering our knowledge of the family of plants most responsible for food across the globe. For example, a sustainable bioenergy crop must require low input and utilize land not already available for food production (marginal land). By engineering or breeding highly productive bioenergy crops, we learn about mitigation pathways for events such as nutrient limitation and drought. These findings in bioenergy grasses can be directly translated to some of the most important food cultivars on the planet, including corn, wheat, and rice (Mullet et al. 2014).

To design the ideal bioenergy grass, many factors that increase productivity must be considered. These include the previously mentioned low input, and growth on marginal land, but also include characteristics such as a broad ecological range, diverse germplasm, amenable genetics, and even farmers' willingness to accept bioenergy crop supplementation. As the world's fifth most produced cereal crop, Sorghum bicolor has emerged as a highly promising dedicated bioenergy crop over the past two decades due to its C4 photosynthesis and high Nitrogen Use Efficiency (Mullet et al. 2014). As expanded upon later, Sorghum also has a large and diverse germplasm that spreads across most continents, which allows for breeders and engineers to harness the natural genetic diversity of Sorghum to accomplish their goals. Additionally, Sorghum has diploid genetics which allows for technological advances in genetic engineering and synthetic biology to be more simply introduced into the Sorghum germplasm. All these factors make sorghum a practical choice for the design, development, and deployment of a dedicated bioenergy grass.

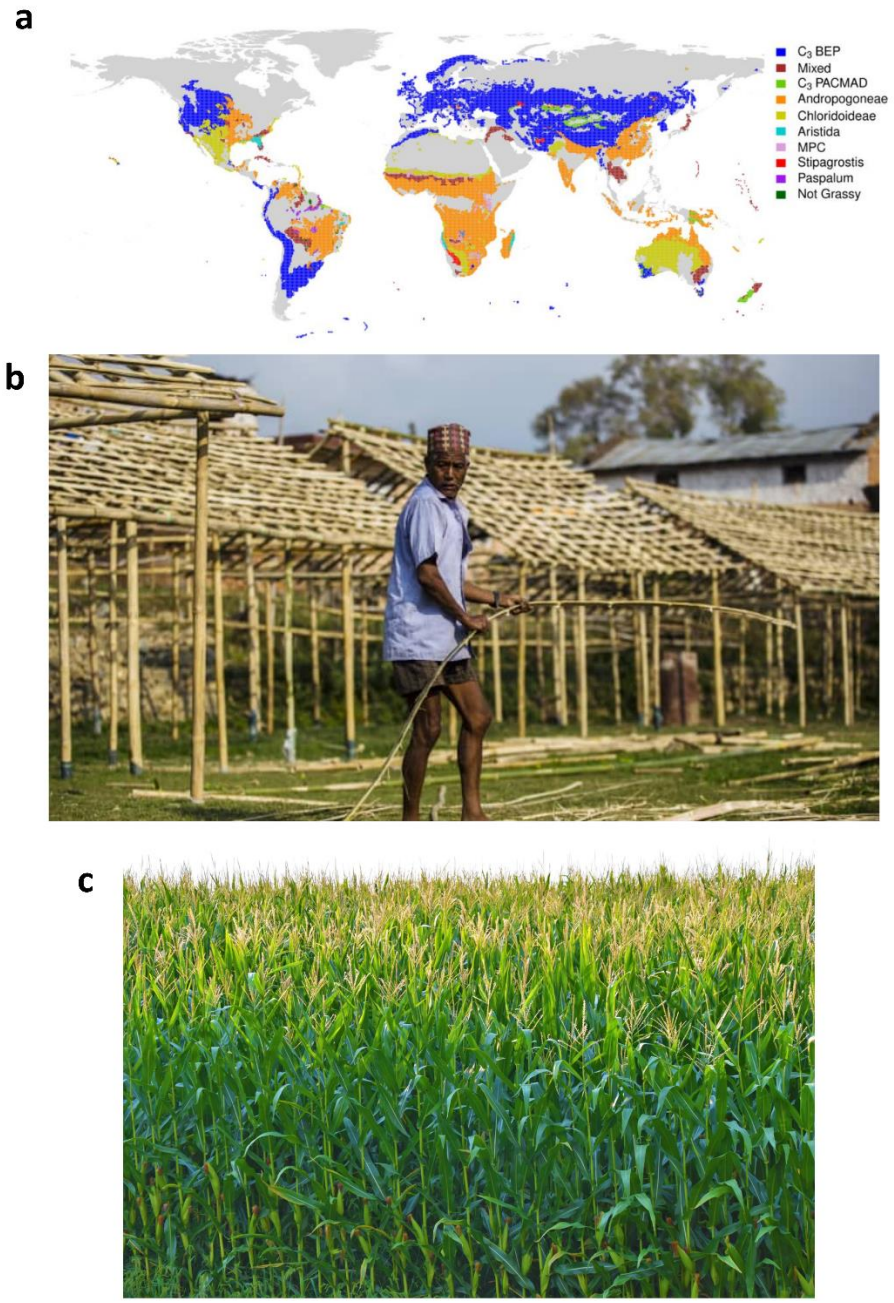


Figure 1.1 Grasses and their diversity a) Range of grass biomes. Grasses are present on almost all major continents and throughout different climates. Figure adapted from (Lehmann et al. 2019) b) Nepalese man using bamboo as an infrastructure for houses. Image from Prashanth Vishwanathan/Bloomberg/Getty Images c) Corn field showing the growth of the grass *Zea mays*, one of the most important food crops in the world. Image credit Waldemar Brandt

1.1. The First Agricultural Revolution and the beginnings of Plant Design

Plant design at its core is not a novel topic. Since the dawn of civilization, people have been tinkering with plants in order to optimize them for their own uses. This began with the domestication of wild plants that led to early agricultural crops in the First Agricultural Revolution. Indeed, domestication has been described as one of the most important events in human evolution (Diamond 2002). In an era dubbed the Neolithic, one of the oldest indications of agriculture comes from southwest Asia between 14,500 and 12,000 before present day (BP) where the Natufians people used small stone tools to gather wild plants such as barley. The first grasses (millet and rice) were probably domesticated around 8500-8000 BP in East Asia by small tribes of people living there (Britannica Origins of Agriculture).

Early agriculture was based around the same principles as it is today: by discouraging growth of non-useful species, and increasing production of designed crops to extract valuable resources from the environment, namely food, fiber, and medicine. This was mostly achieved through directed selection of species with more beneficial traits (Purugganan and Fuller 2009). Generally, speciation forming from human intervention is viewed as a special class apart from natural selection and is often viewed as a plant/animal coevolution (Zeder et al. 2006). This human intervention has been highly proliferative; it is estimated that around 1,000-2,500 semi- and fully domesticated plant species have emerged throughout the course of civilization (Meyer, DuVal, and Jensen 2012). The pace at which these designs emerged is also quite striking; compared

to human evolution, all these species are quite young, and a significant number have only existed for just a few centuries (Milla et al. 2018).

1.2. The Second Agricultural Revolution

Even newer still is the advent of modern agriculture. This was marked by the Second Agricultural Revolution which first took place in England in the seventeenth and early eighteenth centuries before spreading to North America and across the globe. It was largely driven by a few main ideas including crop rotation, improved machinery, ownership of land, and increased transportation infrastructures. As a result, dramatic increases in agricultural productivity, paired with less labor required to produce food, allowed populations to flourish. With the increase in population, and less manpower required to feed them, the mass urbanization of the global workforce drove the innovations attributed to the Industrial Revolution. In turn, the machines invented supplemented the farm by being affordable and reliable, consequently proliferating their use and pushing agricultural productivity even further (Britannica Agricultural Revolution; Overton 1996).

1.3. The Green Revolution

Despite great advances in yield during the Second Agricultural Revolution, there was one major problem: the world population had doubled to over 6 billion people in just 40 years. In the early to middle 20th century, scientists began to worry about the impending “Population Bomb” which would lead to famine and global unrest ((Ehrlich 1971). However, productivity goals were met and exceeded throughout this time by the introduction of high-yielding varieties of wheat and rice and industrial use of fertilizers

and pesticides. This began the Third Agricultural Revolution, also known as The Green Revolution, that spanned the 1950s through the 60s.

Spearheaded by Norman Borlaug and other dedicated scientists, the introduction of dwarfing genes into cereal crops was critical to yield increases seen during this time. The tall stems in use at the time were flimsy and not strong enough to hold up panicles filled with heavy seed. As a result, plants would fall over (or lodge) in high wind or rain and cause severe yield loss (Berry and Spink 2012). By utilizing careful breeding and selection processes, semi-dwarf crops were designed that had short, strong stems that did not lodge and were resistant to pathogens (Borlaug 1958). Additionally, more nutrients could be transported and partitioned up to the seed, which also contributed to yield increases. While the breeders of the time were simply interested in whatever worked most effectively, many of the Green Revolution genes have since had their molecular functions characterized. Interestingly, the Reduced Height (*Rht*) and the semidwarf1 (*sd1*) genes of wheat and rice respectively were both shown to be involved in the gibberellic acid (GA) pathway, which is known to be involved in stem elongation and other developmental processes (Peng et al. 1999; Monna et al. 2002; Sasaki et al. 2002; Spielmeyer, Ellis, and Chandler 2002; Willige et al. 2012). *Rht* was found to have nonsense mutation within the DELLA domain required for GA-dependent proteasomal degradation, which resulted in a truncated protein that functions as GA insensitive repressors of growth (Peng et al. 1999; Dill, Jung, and Sun 2001). In rice, *sd1* mutants could be rescued with GA supplementation and it was subsequently shown to encode a

GA 20-oxidase required for GA biosynthesis (Monna et al. 2002; Sasaki et al. 2002; Spielmeyer, Ellis, and Chandler 2002).

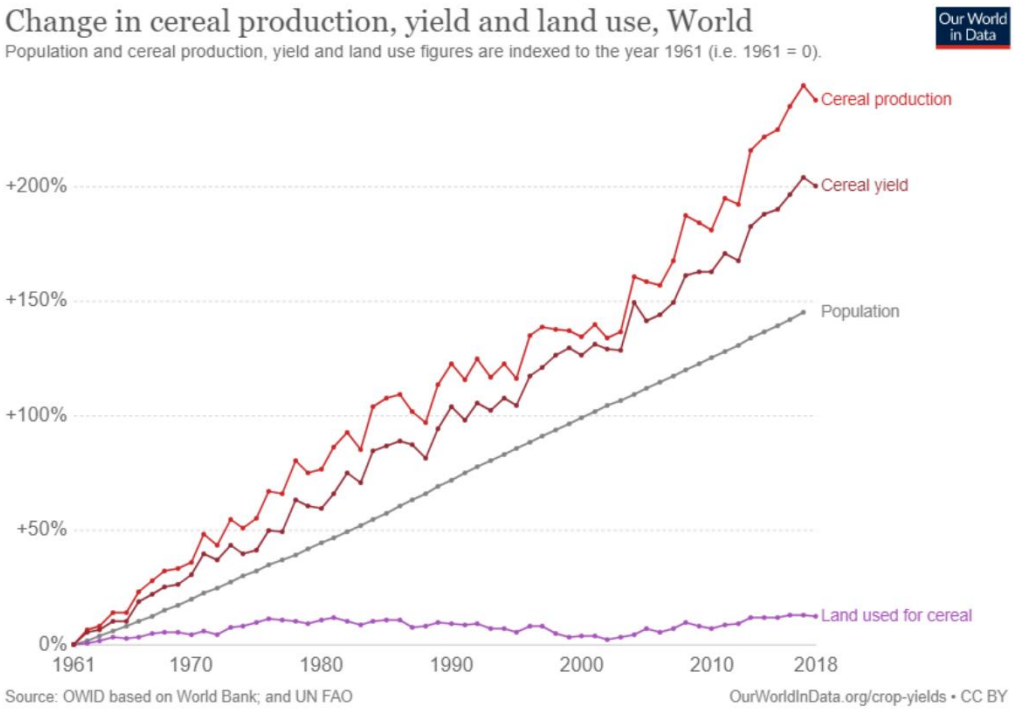


Figure 1.2 Graph depicting the increases in cereal yield brough upon by the Green Revolution. Graph credit from Our World in Data, World Bank, and UN FAO.

1.4. Plant Design at its core: The Source and Sink Relationship

Whether historical farmers knew it or not, plant design in general has been focused around one area of plant biology: the Source and Sink relationship. A source is defined as a tissue where macromolecules are absorbed or synthesized, and a sink is defined as a tissue where those macromolecules are utilized or stored (Paul and Foyer 2001). These types of relationships are critical to the overall health and yield of a plant, and different tissues go through developmental phases of being a source or a sink

depending on the situation (M. R. Smith, Rao, and Merchant 2018). By simply altering which sink tissue gets the most allotment from source tissues, you can vastly change how a plant behaves and its ultimate use (Chang and Zhu 2017). For example, in the dwarf wheat varieties designed during the Green Revolution, by shifting the sink tissue from the stem and toward the seed by breeding, you create strong, short plants with large panicles (Peng et al. 1999). Furthermore, by increasing the amount of source (such as reducing competition from pests and weeds or by supplementing with fertilizer), you also increase the sink capacity as there are simply more resources to go around (Baligar, Fageria, and He 2001). In essence, farmers, breeders, and scientists throughout history have hijacked the source and sink relationship to design plants for their uses.

More modern avenues of Source-Sink manipulation mix both historical breeding and molecular biology. By using knowledge accumulated from breeders over the years, molecular biologists and genetic engineers have begun tweaking expression of important genes known to influence Source and Sink relationships. For example, by overexpressing the Ammonium transporter *AMT1* (an example of increasing source availability), rice was engineered to have better growth, higher grain yield, and increased ammonium uptake under both optimal and suboptimal ammonium conditions (Ranathunge et al. 2014). More innovative methods have also been described; fusions of proteins within the same metabolic pathway, such as sucrose biosynthesis, also have the potential to increase yield. Sucrose is an important source carbohydrate and signaling molecule in plants. Upon arrival to sink tissues, sucrose can be used for cell wall biosynthesis, respiration, to maintain cellular metabolism, or converted into starch

(Sturm 1999). Sucrose synthesis is well characterized and involves two enzymes: sucrose-6-phosphate synthase (SPS), and sucrose-6-phosphate phosphatase (SPP). SPS is a main hub for carbohydrate metabolism and regulates the partitioning of carbon between source (carbohydrate accumulation) and sink tissues (starch biosynthesis) (John E. Lunn and MacRae 2003). SPP catalyzes the final step in sucrose biosynthesis by hydrolyzing the phosphate moiety to produce sucrose (J. E. Lunn and ap Rees 1990). Fusion of these two proteins together led to an increase in growth in both Arabidopsis and hybrid Poplar, which suggests new engineering methods for increasing source carbohydrate in all plants (Maloney et al. 2015).

1.5. New Areas of Plant Design: *Sorghum bicolor* as a Bioenergy Grass

While the designed plants throughout history and in the Green Revolution were largely targeted to increase food production, new applications of plant design have begun to emerge over the past few decades. As mentioned previously, one of the most promising is the design of dedicated bioenergy crops to combat climate change, and in particular the design of bioenergy grasses. Biofuels derived from C4 grasses such as *Miscanthus* (Hillier et al. 2009), switchgrass (Schmer et al. 2008), and *Sorghum* (Olson et al. 2012) can partially offset the rise in atmospheric CO2 levels caused by the burning of fossil fuels, as they are effectively carbon neutral. Deployment of these grasses onto non-arable land can lead to significant accumulations of biomass without having to sacrifice land used for food production (Somerville et al. 2010). Additionally, some of these grasses, such as Miscanthus and sugarcane, are perennial and do not have to be replanted each season, thus discounting the cost of maintenance over the years. Perennial

C4 grasses with the highest bioenergy potential are also polyploid with large genomes. Miscanthus x giganteus is a sterile triploid with 57 chromosomes and a genome size of approximately 2 Gigabases (Joint Genome Institute; Rayburn et al. 2009). These grasses evolved complex genomes and ploidy as they contribute to high productivity and environmental resistance (Rayburn et al. 2009). However despite their potential, such large genomes sizes and complex ploidies make breeding and engineering of these grasses difficult. This ultimately led to the development of new bioenergy models, such as *Brachypodium distachyon* and *Sorghum bicolor*, with smaller genome sizes and more tractable diploid genetics (International Brachypodium Initiative 2010; Paterson et al. 2009).

There are also other genetic traits that make sorghum an ideal candidate for bioenergy plant design. Unlike the other simple C3 grass model, Brachypodium distachyon, Sorghum utilizes C4 photosynthesis and exhibits high nitrogen use efficiency, both of which contribute to the high productivity of Sorghum (Olson et al. 2012). Sorghum breeding began in the early 1900s and has contributed significantly to crop improvement due to the fact the Sorghum is diploid, self-pollinating, and can be crossed produce hybrids (Quinby 1974; W. L. Rooney 2004). Furthermore, Sorghum originated in Africa and has a phenotypically and genetically diverse germplasm that contain a variety of traits to adapt to different regions of the world (Billot et al. 2013; Mace et al. 2013). The genotypes used to develop the various types of sorghum are divided into five clusters based on phenotypic similarity. These are collectively known as the Sorghum races and are Kafir, Durra, Caudatum, Guinea, and Bicolor (Harlan and

Wet 1972). The races also cluster together genetically, and patterns emerge that indicate breeding and selection occurred outside the African continent (Morishige et al. 2013; Mullet et al. 2014). In part due to its simple genetics, many of these ecotypes have been fully sequenced and large amounts of genomic data has been collected that reflects the diversity of phenotypes associated with the Sorghum races (Mace et al. 2013). These types of studies highlight the plethora of genetic diversity that can be mined for useful alleles within the Sorghum germplasm for future design efforts.

1.5.1. Bioenergy *Sorghum* design thus far

Current design efforts for bioenergy Sorghum began in 2003 with breeding (William L. Rooney et al. 2007). Many Sorghum genotypes are tall and late flowering when exposed to long days (>12 hours), meaning they are photoperiod sensitive and only flower under short days (C. W. Smith and Frederiksen 2000). Grain sorghum genotypes, however, are photoperiod insensitive and flower in approximately 60-70 days regardless of day length. While being shorter and easier to cross pollinate, plants that flower early devote most of their resources to filling their grain, which is counterproductive to the biomass accumulation needed in bioenergy grasses; early flowering plants' target sink is the seed whereas the target sink for bioenergy grasses is the shoot (leaves, leaf sheaths, and stems). Bioenergy sorghum hybrids were developed by crossing early flowering genotypes with complementary recessive alleles required for photoperiod sensitivity that generate photoperiod sensitive hybrids. As a result, these bioenergy hybrids have a long vegetative phase and the height of the late flowering genotypes (W. L. Rooney and Aydin 1999; William L. Rooney et al. 2007).

1.5.2. Stem growth as a sink for Bioenergy *Sorghum*

As demonstrated by the design of energy Sorghum hybrids, two of the main sinks in plant biology are the seed and the stem. By selecting for which is the most important, breeders, designers, and engineers can manipulate the plant's physiology to their advantage. For energy sorghum, the stem is by far the most important organ as it generates over 80% of the total biomass (Olson et al. 2012). Additionally, the sorghum stem provides large capacity (sink strength) for storing excess sugars and bioproducts, such as waxes (McKinley et al. 2018; Jordan et al. 1984). The most obvious difference between grain and energy Sorghum genotypes is the height as energy sorghum can reach 4-5 meters tall (Olson et al. 2012). Height in Sorghum is controlled by four dwarfing genes, the functions of which are described in later chapters. In brief, Dw1 is involved in brassinosteroid signaling (J. Hilley et al. 2016; Yamaguchi et al. 2016; K. Hirano et al. 2017) Dw2 is involved in endomembrane trafficking and lipid signaling (Oliver et al. 2021), discussed here as Chapter 2), and Dw3 is an auxin efflux transporter (Multani et al. 2003). Dw4 has yet to be identified but is predicted to exist based on Mendelian segregation done by Quinby (Quinby 1974).

Understanding specifics in the growth and development of sorghum stems is critical for its effective design. These findings are directly translatable to other important grasses such as wheat, corn, and rice. In this dissertation, two manuscripts are reproduced that illuminate some of the knowledge gaps that are present in grass stem biology. Specifically, this dissertation describes two major acomplishment: 1) defines the physiological and molecular function of the dwarfing gene Dw2 and 2) characterizes

the specific tissues within the sorghum stem and includes the identification of a putative boundary layer that determines stem growth and development. Taken together, these data are incorporated into two competing models of internode growth. Additionally, further experiments and design possibilities are discussed in the Future Directions section that future scientists can use to understand the development and ultimate design of Sorghum bicolor as a dedicated bioenergy grass.

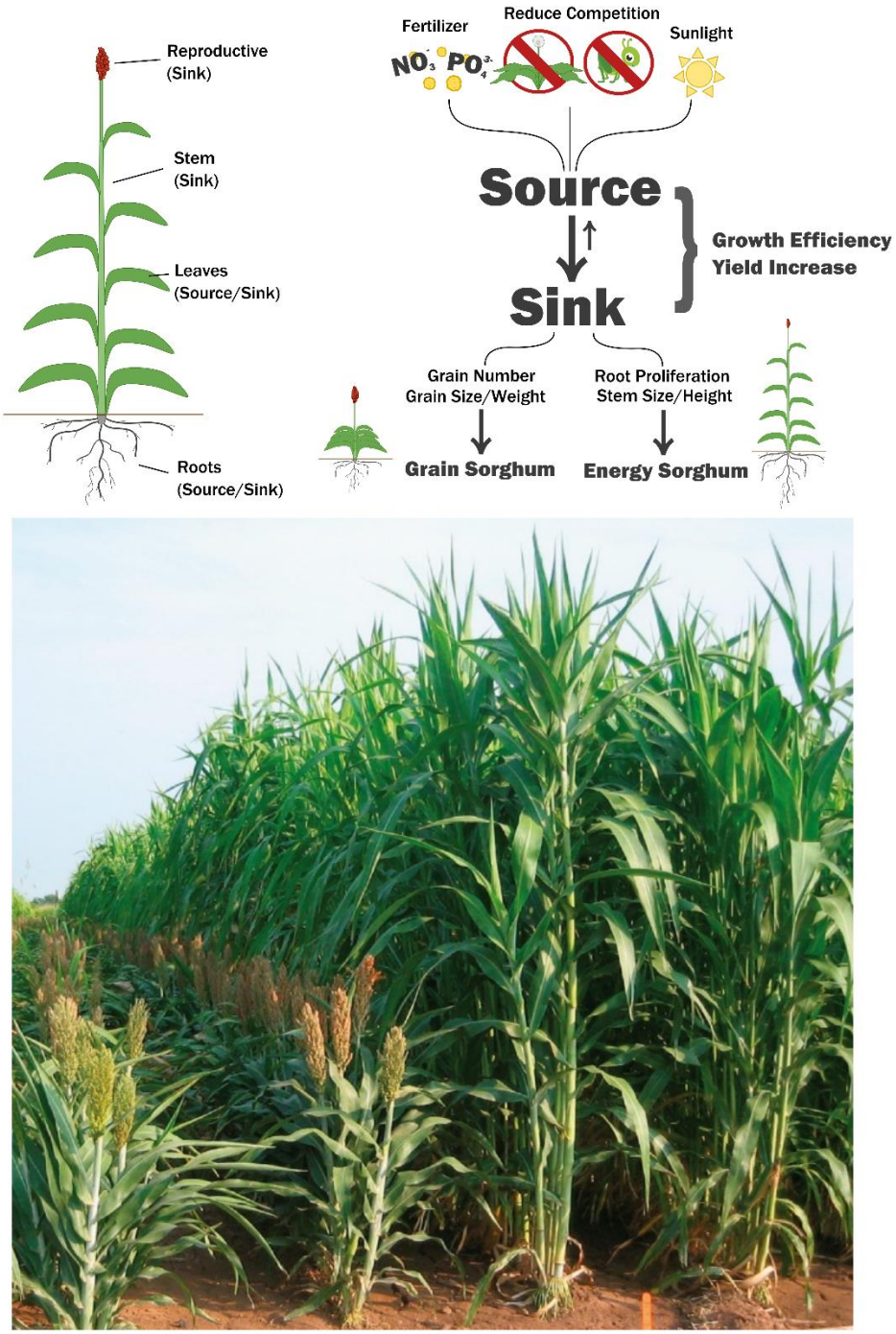


Figure 1.3 Source and Sink (S/S) relationship and plant design. Top left) Sorghum diagram which shows the different tissues and how they exist with the S/S relationship. Some tissues can behave as either a source or sink depending on the age of the plant (for example, young leaves being a sink and older leaves being a source). Top right)

Examples of plant design aspects. By increasing source (through increased light, fertilizer, or reducing competition from pests and weeds) or increasing the sink (grain or stem) plant designers can choose how to grow their plants for particular uses. Bottom) Grain sorghum (left) and Energy sorghum (right) showing the difference in selected sink (grain or stem) utilized in plant design strategies. Image adapted from Olson *et al.* 2012.

2. THE AGCVIII KINASE DW2 MODULATES CELL PROLIFERATION, ENDOMEMBRANE TRAFFICKING, AND MLG/XYLAN CELL WALL LOCALIZATION IN ELONGATING STEM INTERNODES OF *SORGHUM BICOLOR* [1]

2.1. Overview

Stems of bioenergy sorghum, a drought tolerant C4 grass, contain up to 50 nodes and internodes of varying length that span 4-5 meters and account for ~84% of harvested biomass. Stem internode growth impacts plant height and biomass accumulation and is regulated by brassinosteroid signaling, auxin transport, and gibberellin biosynthesis. In addition, an AGCVIII kinase (Dw2) regulates sorghum stem internode growth, but the underlying mechanism and signaling network are unknown. Here we provide evidence that mutation of Dw2 reduces cell proliferation in internode intercalary meristems, inhibits endocytosis, and alters the distribution of heteroxylan and mixed linkage glucan in cell walls. Phosphoproteomic analysis showed that Dw2 signaling influences the phosphorylation of proteins involved in lipid signaling (PLDδ), endomembrane trafficking, hormone, light and receptor signaling, and photosynthesis. Together, our results show that Dw2 modulates endomembrane function and cell division during

[1] Reprinted with permission from "The AGCVIII kinase Dw2 modulates cell proliferation, endomembrane trafficking, and MLG/xylan cell wall localization in elongating stem internodes of Sorghum bicolor Joel Andrew Oliver *et al*., 2021. *The Plant Journal*, 105 (4): 1053-71, Copyright 2021 Joel Andrew Oliver *et al.*

sorghum internode growth providing insight into the regulation of monocot stem development.

2.2. Introduction

The C4 grass Sorghum bicolor is an important drought and heat tolerant crop used for production of grain, forage, sugar, and biofuels (William L. Rooney et al. 2007; Mullet et al. 2014). Grain sorghum was bred to have short stems to reduce lodging, increase grain harvest index, and to simplify mechanical harvesting. In contrast, bioenergy sorghum hybrids have 4-5m long stems containing 40-50 internodes that account for ~84% of harvested biomass (Olson et al. 2012). A subset of the bioenergy sorghum genotypes accumulate high levels of stem sucrose similar to sugarcane (McBee and Miller 1982; Lingle 1987). These 'sweet' sorghum genotypes have increased capacity to accumulate stem sugars and starch (McKinley et al. 2018) due to their elongated stems and inactivation of NAC_D, a gene that stimulates conversion of stem pith parenchyma to aerenchyma (Casto et al. 2018; Xia et al. 2018; L.-M. Zhang et al. 2018).

The sequential production of phytomers containing nascent node-internode tissues by the shoot apical meristem (SAM) is a key early event in stem growth. Nascent internodes initially increase in size through balanced cell division and cell growth followed by a phase of rapid internode elongation (Kebrom, McKinley, and Mullet 2017). Rapidly growing internodes contain a region of cell division in an intercalary meristem (IM) located at the base of the internode and a zone of cell elongation adjacent

to and above the IM. Cells that have stopped elongating accumulate secondary cell walls in the upper portion of the internode (Kebrom, McKinley, and Mullet 2017).

The length of sorghum internodes varies during plant development, in response to environmental factors, and among genotypes. Insight into the biochemical basis of variation in internode length in sorghum has been obtained through analysis of dwarfing loci (Dw) used to shorten the stems of grain sorghum (Quinby 1974). For example, Dw3 encodes an ABCB1 auxin efflux transporter and inactivation of Dw3 reduces internode length and cell elongation (Multani et al. 2003). The maize homolog of Dw3, BR2, was shown to help export IAA from stem nodes indicating that regulated auxin transport is required for normal internode elongation (Knöller et al. 2010). Positional cloning of Dw1 identified a novel membrane protein with predicted plasma membrane localization (J. Hilley et al. 2016). Dw1 was subsequently shown to stimulate internode cell proliferation by binding to and inhibiting the nuclear localization of BIN2, a negative regulator of brassinosteroid signaling (Yamaguchi et al. 2016; K. Hirano et al. 2017).

Dw2, the focus of the current study, was found to encode a member of AGCVIII (cAMP-dependent protein kinases (PKA), cGMP-dependent protein kinases (PKG), and protein kinase C (PKC)) protein kinase family (J. L. Hilley et al. 2017). The closest Arabidopsis thaliana homolog of Dw2 is Kinesin-like Calmodulin Binding Protein Interacting Protein Kinase (KIPK) (J. L. Hilley et al. 2017). In Arabidopsis, KIPK interacts with KCBP, a plant-specific kinesin-like calmodulin-binding protein that regulates trichome development and the organization of cytoskeletal components (Day et al. 2000). AtKIPK also interacted with proline-rich extensin-like receptor-like kinases

(PERK) that modulate growth (T. V. Humphrey et al. 2015). In Arabidopsis, KIPK mutants show only a very mild root phenotype and no stem growth phenotypes (T. V. Humphrey et al. 2015). Other members of the plant AGCVIII kinase family have been reported to regulate phototropism, gravitropism and planar growth (Rademacher and Offringa 2012).

In this work, we investigated the role of *S. bicolor* Dw2 in regulating internode growth. We provide evidence that mutation of Dw2 inhibits cell proliferation in elongating internodes, alters cell morphology in vascular bundles and root hairs, and causes a large number of changes in the phosphoproteome that could affect phospholipid signaling, vesicle trafficking, cytoskeletal functions and cell proliferation. Mutation of Dw2 also disrupts endocytosis and the localization of polysaccharides in cell walls that are trafficked to cell walls by the endomembrane system. Together these results provide insights into how Dw2 regulates internode growth in C4 grass crops, a function not associated with homologs in dicot species.

2.3. Results

2.3.1. Dw2 modifies stem and internode growth

The role of Dw2 in stem and internode growth regulation was investigated using the near isogenic genotypes Dwarf Yellow Milo (DYM, *Dw2*) and Double Dwarf Yellow Milo (DDYM, *dw2*). DDYM encodes a *dw2* allele with a stop codon in the first exon thereby producing a truncated protein of 190 amino acids lacking the kinase domain instead of the 809 amino acids in the full length AGCVIII kinase (J. L. Hilley et al. 2017). DYM and DDYM have similar flowering times and produce the same number

of stem internodes during development. However, DYM plants are taller than DDYM plants because DYM stem internodes are longer than DDYM internodes (Fig. 1b).

2.3.2. Mutation of *Dw2* reduces the number of cells spanning the length of internodes

During vegetative growth, new phytomers comprised of nascent leaves, leaf sheaths, and internodes are produced by the shoot apical meristem (SAM) approximately every 3-4 days. The ~4 nascent internodes below the SAM contain relatively small non-elongated cells. Cell division occurring throughout nascent internodes increases cell number and size of these internodes prior to internode elongation (Fig 2.1a). In elongating internodes, cell division occurs in an intercalary meristem (IM) located at the basal end of the internode. Cells displaced from the intercalary meristem by cell proliferation stop dividing and enter a zone of elongation (ZoE) where an increase in cell length occurs. When internode cells reach full length, cells associated with vascular bundles subsequently develop lignified secondary cell walls in a region called the Zone of Maturation (ZoM). The number of fully elongated cells in the ZoM increases over time during internode growth until the activity of the IM ceases (Fig 2.1a).

The difference in DYM and DDYM internode lengths (Fig 2.1b) could be due to differences in cell number and/or cell length. Cells in internodes are stacked in vertical columns facilitating microscopic analysis and quantification of the number and length of cells that span the length of internodes. Analysis of fully elongated internodes showed that cells in the upper portion of fully elongated DYM and DDYM internodes were similar in length (Fig 2.1c) and that there were ~2.2 times more cells spanning DYM

internodes than DDYM internodes (Fig 2.1d). Therefore, the difference in the overall length of the DYM and DDYM internodes is primarily due to a reduction in cell number in DDYM.

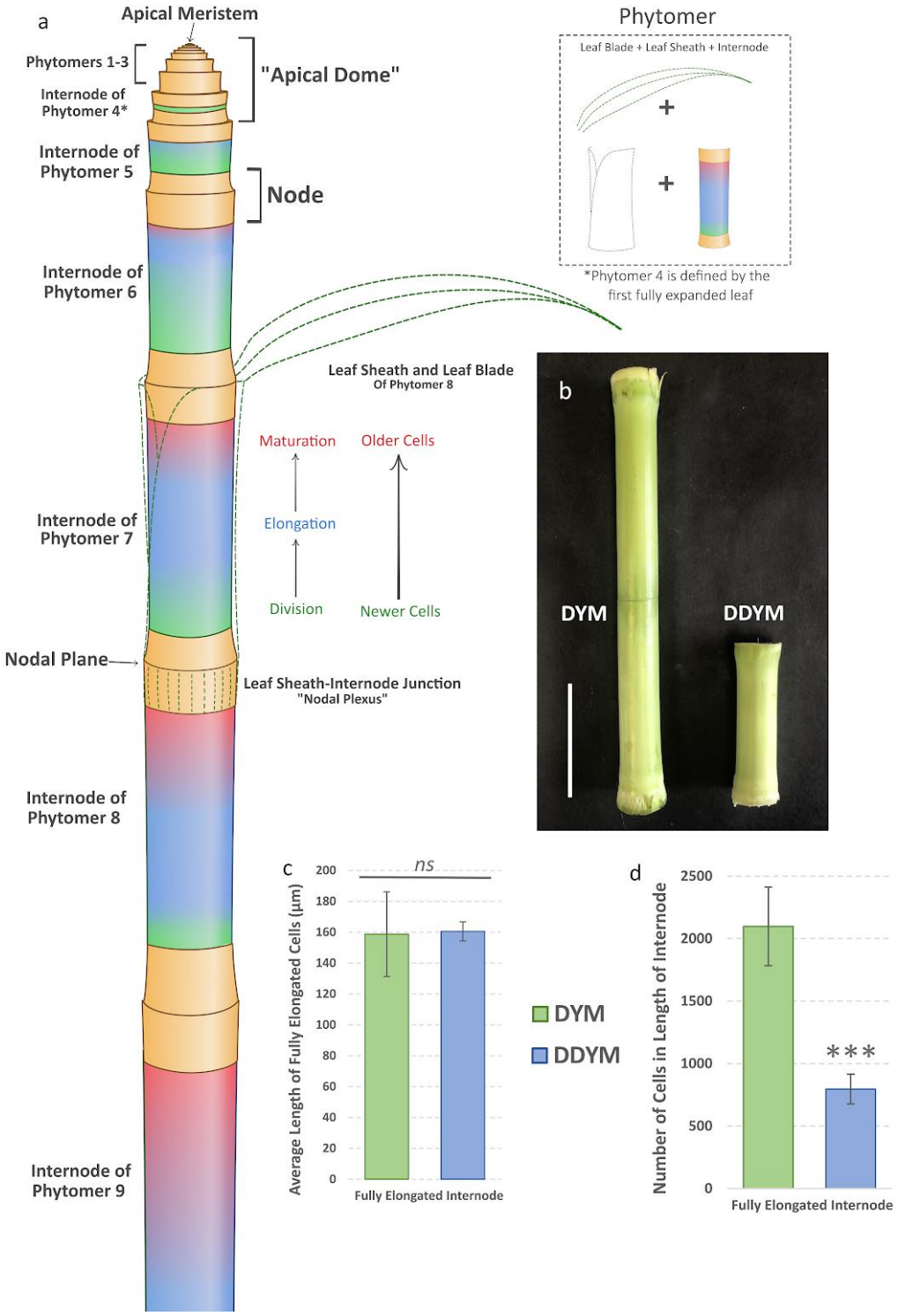

a
Apical Meristem
Phytomers 1-3
Internode of Phytomer 4*
"Apical Dome"
Internode of Phytomer 5
Node
Internode of Phytomer 6
Internode of Phytomer 7
Nodal Plane
Internode of Phytomer 8
Internode of Phytomer 9
Leaf Sheath and Leaf Blade
Of Phytomer 8
Maturation
Older Cells
Elongation
Division
Newer Cells
Leaf Sheath-Internode Junction
"Nodal Plexus"
Phytomer
Leaf Blade + Leaf Sheath + Internode
*Phytomer 4 is defined by the first fully expanded leaf
b
DYM
DDYM
c
ns
Average Length of Fully Elongated Cells (µm)
Fully Elongated Internode
DYM
DDYM
d
Number of Cells in Length of Internode
***
Fully Elongated Internode

Figure 2.1 Diagram of Internode Development in Monocots; Dw2 phenotype (a)Monocots grow stems in discriminate segments called internodes. Each internode is a part of a phytomer which contains the leaf blade and leaf sheath. At the base of elongating internodes is an area of active cell division, Zone of Cell Division ZoD. Above the ZoD are areas of cell elongation (ZoE) and maturation (ZoM) where cell expansion and maturation/lignification occur respectively. The apical meristem is located at the very tip of the growing stem and is where new phytomers are formed. Beneath the apical meristem are small undeveloped internodes of phytomers 1-3 that have yet to elongate. For this study, the apical meristem and Int(P1-4) were grouped together in what we define as the "Apical Dome". (b) Mature DYM and DDYM internodes that are wt and *dw2* respectively. DYM internodes are longer. Scale bar is 5cm. (c) Measure of maximum length of cells in a fully mature internode. There is no difference in maximum length of cells in DYM or DDYM. Error bars represent SD over 3 replicates (d) Number of cells in the length of the internode, measured from nodal plane to nodal plane. DYM internodes have ~2.2x greater cells than DDYM internodes. Errors bars represent SD over 3 replicates.

Mutation of Dw2 in DDYM could reduce the number of cells in the internode by inhibiting cell division prior to internode elongation and/or by reducing rates of cell d ivision in the IM of elongating internodes. These alternatives were evaluated by quantifying the number of cells that span the length of internodes of phytomers 4-6 that correspond to internodes just prior to elongation (Int(P4)), those in an early stage of elongation (Int(P5)), and those at a late stage of elongation (Int(P6)). DYM and DDYM were found to have a similar number of cells in Int(P4) (Fig 2.2d); however more cells accumulated in Int(P5) and Int(P6) in DYM compared to DDYM during the phase of internode elongation. The cells in the IM of Int(P6) were smaller in DYM compared to DDYM consistent with more rapid rates of cell division in the IM of DYM. Fully elongated cells in the ZoM of Int(P6) of the two genotypes were similar in length (Fig 2.2c). Taken together, these results indicate that mutation of Dw2 reduces the extent of cell division in the IM during the phase of internode elongation.

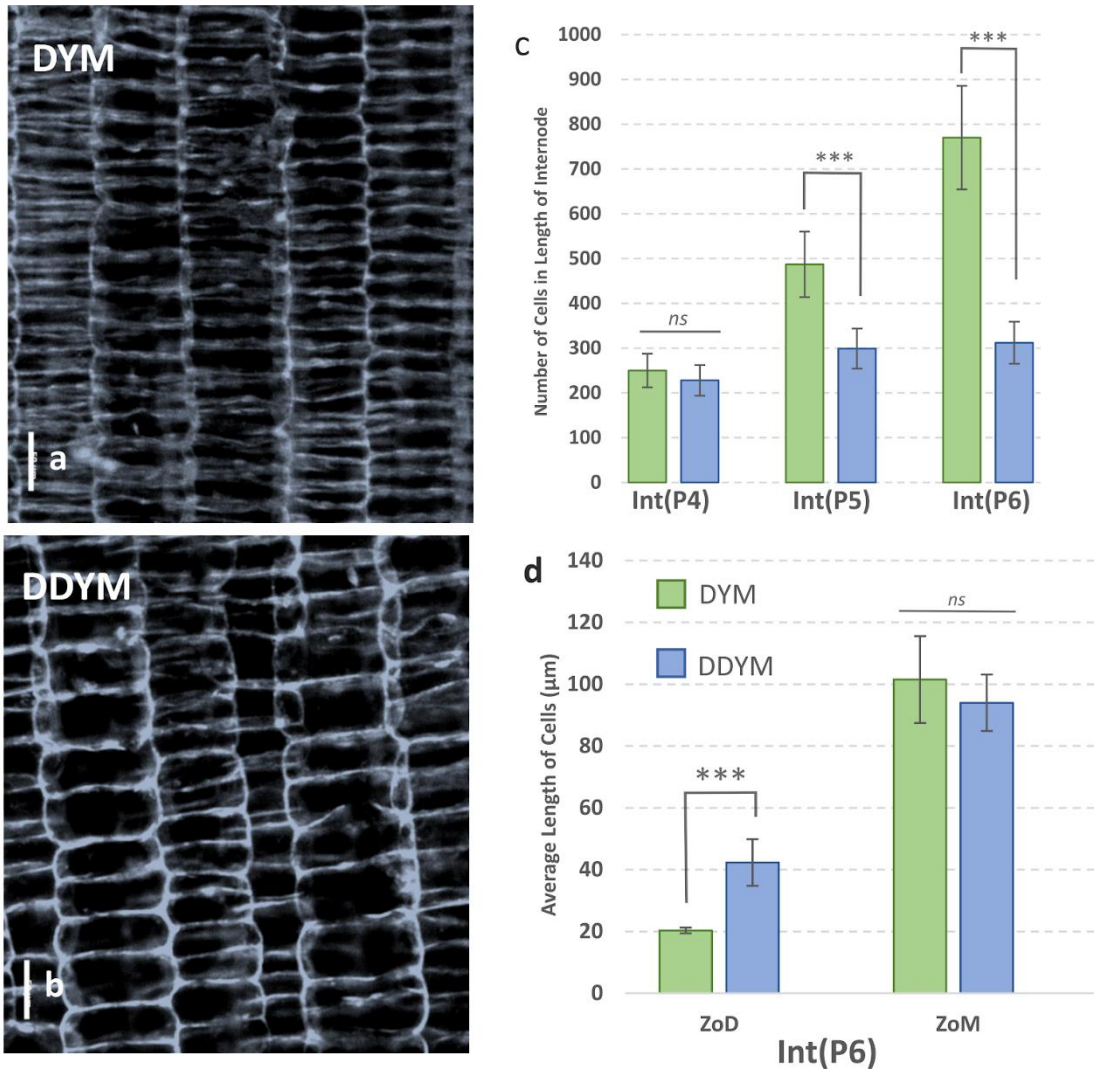


Figure 2.2 Mutation of Dw2 decreases cell proliferation in growing internodes (a,b,d) Cells in the Zone of Division in elongating internodes of DYM (a) and DDYM (b). Cells in DDYM are larger (d). Scale bar is 50μm. Error bars represent StdDev of 3 replicates. (c) Cell number in internodes of phytomers 4, 5, and 6 from nodal plane to nodal plane. Cell numbers in DYM and DDYM Int(P5), Int(P6) and Int(P7) diverge during internode development. Error bars represent SD of 3 replicates. *ns* not significant, pvalue ≤ 0.001 = ***

2.3.3. Internode cell and vascular bundle morphology is altered in the *Dw2* mutant

The morphology of internode cells of fully elongated Int(P8) of DYM and DDYM was examined to determine if mutation of *Dw2* has an impact on cell shape and tissue-level organization in fully elongated internodes. Longitudinal and cross-sections of fully elongated DYM (*Dw2*) internodes (mid-internode) showed well organized columns of cells and vascular bundles with typical monocot morphology (Fig 2.3a, b). In DDYM internodes, longitudinal sections revealed cells having more irregular shape and size and cell columns are less uniform compared to DYM (Fig 2.3c). In addition, cross sections of DDYM internodes showed vascular bundles with irregular shapes and cell numbers sometimes containing more vessel elements compared to DYM stem vascular bundles (Fig 2.3d). Overall, approximately 35% of the vascular bundles in DDYM stems showed abnormal phenotypes. These results indicate that mutation of Dw2 affects cell and tissue morphology of fully elongated internodes, and that the morphology of vascular bundles located in or near the rind/epidermis was perturbed to the greatest extent.

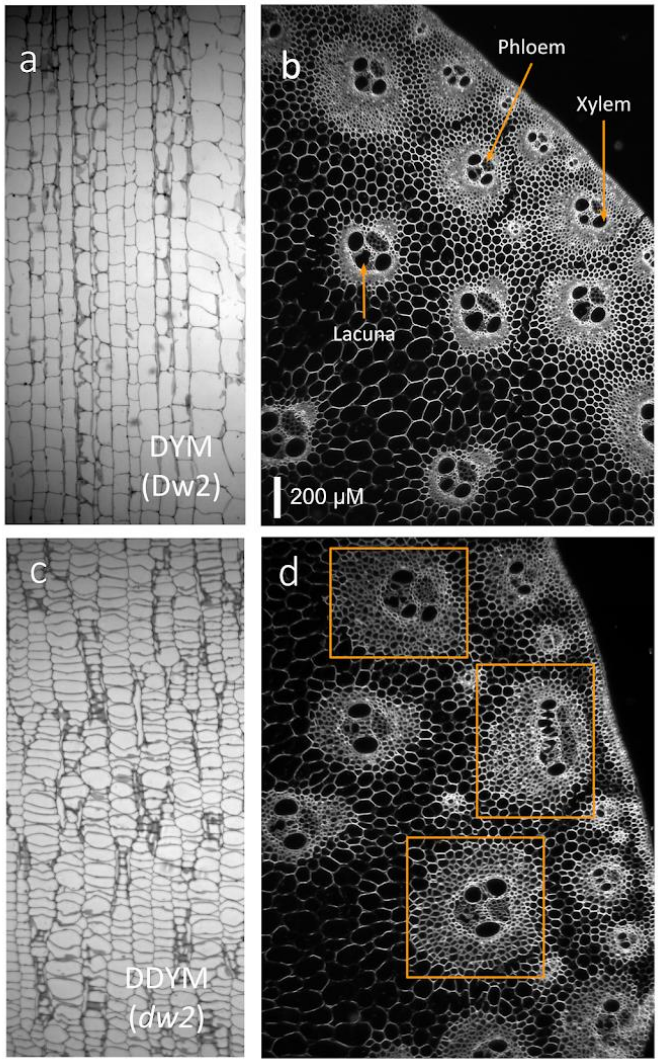


Figure 2.3 Figure 3. Dw2 causes uniform cell morphology and vascular bundles (VB) (a, d) Longitudinal sections of mature internodes in DYM (a) and DDYM (*dw2*) (c) plants. Cells in DYM show uniform cell file and morphology, whereas mutant plants show irregular cell shapes and compromised cell files. (b,d) Horizontal sections of mature internodes in DYM (b) and DDYM (d) plants. DYM plants show typical monocot vascular bundle (VB) morphology, whereas DDYM plants show aberrations in VB morphology (orange boxes). Scale bar is the same for all images.

2.3.4. *Dw2* is expressed in cells associated with vascular bundles and other stem cell types

Dw2 expression in elongating and fully elongated internodes was previously reported (J. L. Hilley et al. 2017). In the current study, the distribution of *Dw2* RNA among internode cells was examined using *in situ* hybridization (Fig 2.4). *Dw2* RNA was detected in most internode cells of Int(P5) (Fig 2.4c). However, *Dw2* expression was elevated in cells associated with nascent vascular bundles near the epidermis (Fig 2.4c) and in parenchyma cells associated with more fully developed vascular bundles located closer to the center of the stem (Fig 2.4f).

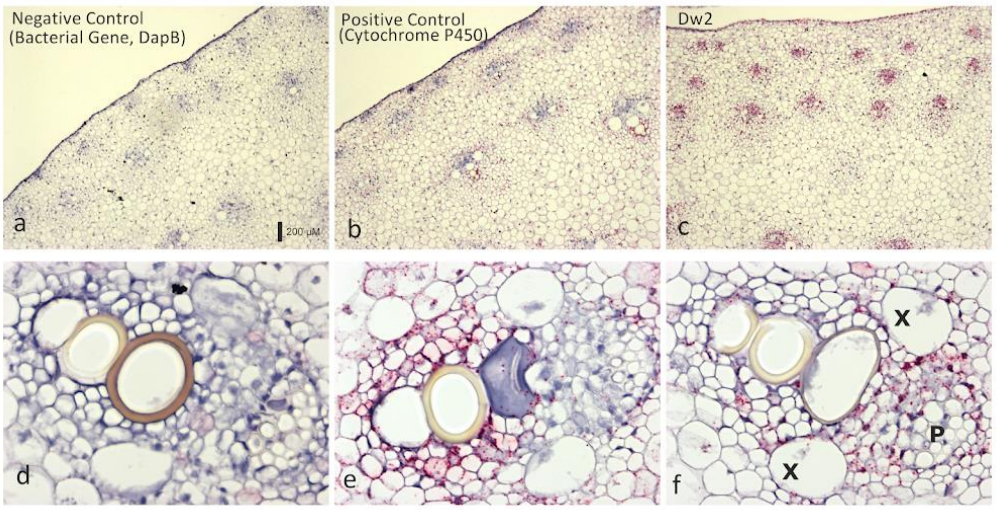


Figure 2.4 Dw2 is expressed in pith and rind, but is concentrated around vascular bundles (a, d) Negative control of RNAscope in situ hybridization of bacterial gene DapB. (b,e) Positive control of in situ hybridization of a cytochrome P450 in DYM plants. (c, f) RNAscope in situ hybridization of Dw2 in Int(P5) of DYM plants. Signal (red spots) is present in pith of the internode (c), but is highly concentrated at the rind, surrounding VB, and within VB (f). X=Xylem, P=Phloem

2.3.5. Phosphoproteomic analysis of DYM and DDYM internodes

To better understand how Dw2 influences internode growth, a phosphoproteomics approach was utilized to identify differences in protein phosphorylation between elongating internodes of DYM (Dw2) and DDYM (dw2). Tissue for phosphoproteomic analysis was collected from internodes of phytomer 5 (Int(P5)) that had just begun to elongate. Phosphoproteomic analysis identified 7138 phosphosites in 2065 proteins in this tissue. The relative phosphorylation of phosphosites within 206 of these proteins differed significantly between the two genotypes (q-value <0.1). The extent of differential phosphorylation at specific phosphosites ranged from <2-fold to >32-fold. To distinguish between differential phosphorylation of a phosphosite and differences caused by variation in protein abundance, data on the proteome was collected from the same samples used for the phosphoproteomic analysis. High-pH reversed-phase chromatography was implemented as a prefractionation strategy and isobaric tagging was used for protein quantitation. This approach quantified the relative abundance of 7390 proteins and showed that 22 proteins were significantly different in abundance in the two genotypes (q-value < 0.1). Only a few of the proteins that were differentially phosphorylated differed in relative abundance in internode 5 of the two genotypes. Dw2 was detected in DYM but not in DDYM as expected due to a mutation that truncates the protein. In DYM, a phosphosite in Dw2 (S628) was located near the insertion domain present in most AGCVIII kinases and a second phosphosite (S657) was located within the T-loop [SxS*FVGTxEYxAPE]

activation segment (Rademacher and Offringa 2012). Dw2 contains a C-terminal FxxF sequence motif present in other ACG kinases that mediates PDK1 binding and phosphorylation of active site amino acids (Rademacher and Offringa 2012). SbPDK1 (Sobic.003G372200) is expressed in sorghum stem internodes, therefore PDK1 is a potential regulator of Dw2 in this tissue. One other differentially phosphorylated protein, psbH, also showed a significant difference (1.4-fold) in abundance in internodes of the two genotypes.

Overall, out of the 206 proteins that were differentially phosphorylated, 23 proteins contained phosphosites that were more highly phosphorylated in DYM compared to DDYM (Fig 2.5a). This subset of the phosphoproteins was enriched in proteins involved microtubule dynamics and maintenance (Fig 2.5b). The remaining 183 proteins showed higher phosphorylation in DDYM internodes indicating that loss of Dw2 has a large indirect effect on protein phosphorylation. This subset of phosphoproteins was enriched in proteins involved in subcellular localization, cytoskeletal organization, exocytosis, and Golgi trafficking (Fig 2.5c).

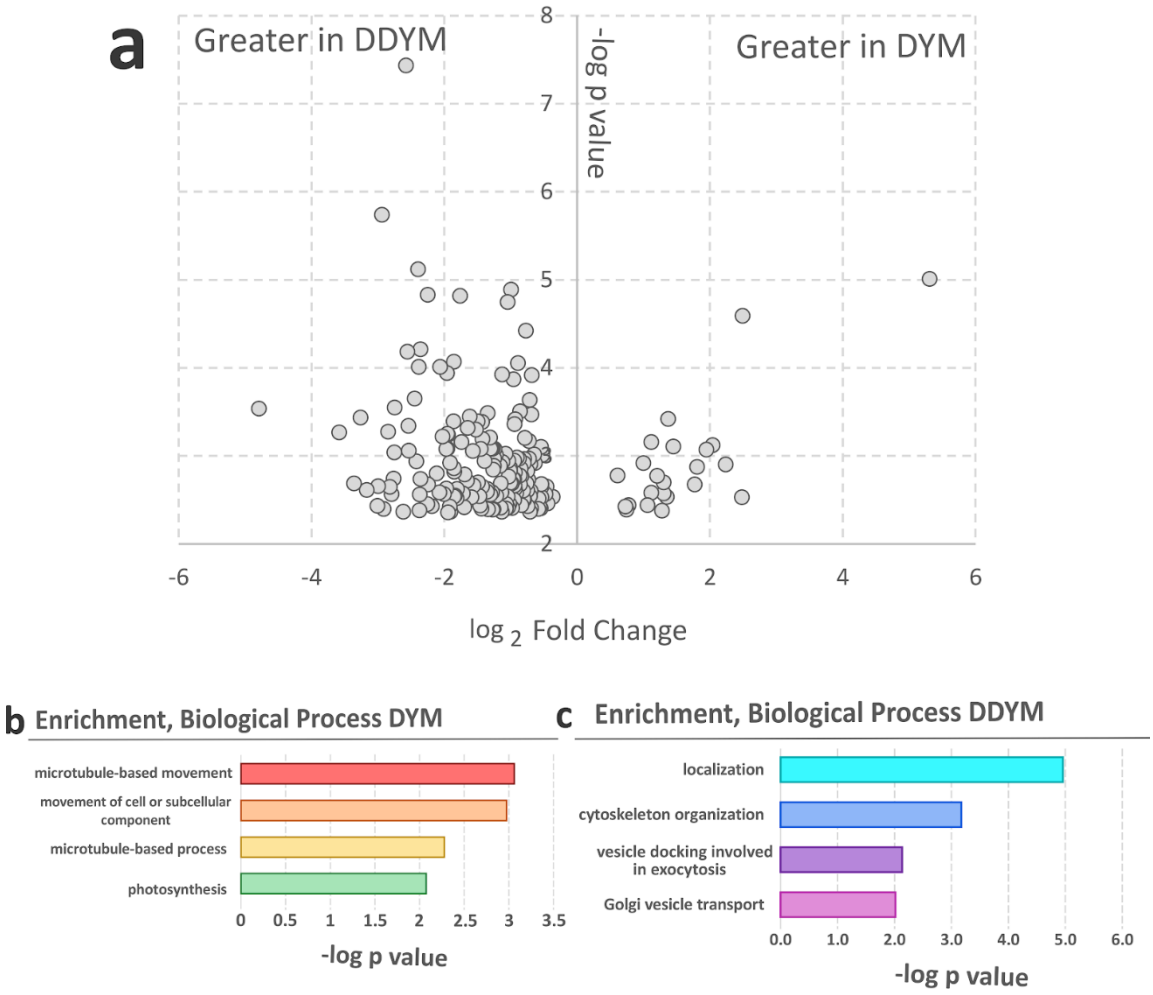


Figure 2.5 Phosphoproteomic of Internode Tissue reveals Endomembrane, Cytoskeleton proteins (a) Volcano plot of phosphoproteomic experiment on growing Int(P5) tissue. Most differential phosphorylation events occur in DDYM tissue. (b) Gene Ontology Enrichment analysis of the phosphoproteins in the right section of (a). There is statistical significance of enrichment in many processes involved in cytoskeletal maintenance. (c) Gene Ontology Enrichment analysis of the phosphoproteins in the left section of (a). There is statistical significance of enrichment in cytoskeletal organization, as well as localization, exocytosis, and vesicle transport. A complete list of enriched terms for both phosphoprotein datasets can be found in the supplementary information.

2.3.6. Proteins with higher phosphorylation in DYM Int(P5)

Proteins with higher levels of phosphorylation in internodes of DYM (*Dw2*) compared to DDYM (*dw2*) could be direct targets of Dw2. Therefore proteins of similar abundance that were differentially phosphorylated in DYM were analyzed further to identify potential direct targets of the AGCVIII kinase. A phosphosite (S684) in phospholipase D (PLDδ) was only detected in DYM although PLDδ protein abundance was similar in internode 5 of the two genotypes. Proteins involved in endomembrane function such as MPK3, a MAP kinase responsible for phosphorylating LIP5 to control multivesicular body (MVB) biogenesis (Fei Wang et al. 2014), and Golgin Candidate 5 (GC5), a protein involved in vesicle tethering to the Golgi apparatus (Latijnhouwers et al. 2007; Kang and Staehelin 2008), were more highly phosphorylated in DYM compared to DDYM (Table 2.1). SIT4, a protein phosphatase required for ER to Golgi trafficking in yeast that plays a role in cell cycle progression (Sutton, Immanuel, and Arndt 1991; Bhandari et al. 2013), also had higher phosphorylation levels in DYM. In addition, a subunit of a vacuolar ATP synthase, the Rho-protein effector RIP5, and a homolog of ARK1, a kinesin involved in microtubule dynamics associated with root hair growth showed differential phosphorylation in DYM (Eng and Wasteneys 2014) (Table 2.1).

Table 2.1 Greater Phosphorylation in DYM

Sorghum ID	Arabidopsis ID	Arabidopsis Symbol	Fold Change	Function	(P)site
Dw2					
Sobic.006G067700	AT2G36350	KIPK	***	Signaling Kinase, unknown specific function	S657, S628
Endomembrane System & Signal Transduction					
Sobic.002G282500	AT4G35790	PLD Delta	***	Phospholipase D, PA Signaling	S684
Sobic.009G229600	AT1G79830	GC5	7.0	Golgin Candidate 5, Vesicle Tethering to Golgi Apparatus	S873
Sobic.001G384000	AT3G14172	GPI-anchored Protein	5.6	Adhesin-like, unknown specific function	S740
Sobic.003G294400	AT1G28280	MPK3/MVQ1	4.1	Oxidative Stress Signaling, Cell Proliferation, Defense signaling	S228
Sobic.009G255700	AT1G30470	SIT4	2.6	Phosphatase, Cell cycle progression, Required for ER to Golgi traffic (Yeast)	S527
Sobic.006G203900	AT4G23710	VHA-G2	2.5	Vacuolar ATP Synthase subunit G2	S8
Sobic.003G301200	At5G60210	RIP5	2.0	ROP interactive partner 5, Rho Protein effector	S115
Kinesins					
Sobic.001G500400	AT3G54870	KUNUC/ARK1	2.5	Plus-end microtubule motor protein, Microtubule Catastrophe	S617
Photosynthesis & Light Signaling					
Sobic.002G338000	AT2G05070	LHCB2.2	***	Light Harvesting Complex II	T109
Sobic.003G168800	ATCG00710	psbH	4.7	Photosystem II, reaction center H	T5, T3

*** No phosphorylation detected in DDYM

Two chloroplast localized proteins, Lhcb2 and psbH showed elevated levels of phosphorylation at threonine phosphosites in DYM compared to DDYM (Table 2.1). In Arabidopsis, Lhcb2 proteins are phosphorylated at an N-terminal threonine residue (T3) by the kinase State Transition 7 (STN7) that results in association of LHCII trimers with PSI in blue light (Longoni, Samol, and Goldschmidt-Clermont 2019). Phosphorylation of sorghum Lhcb2 on T109 could modulate state transitions or protein turnover. Phosphorylation of psbH and PSII core proteins such as D1 increases at high light and is generally attributed to the need for turnover and repair of damaged D1 to maintain PSII function (Fristedt et al. 2012; Levey, Westhoff, and Meierhoff 2014).

2.3.7. Proteins with higher phosphorylation in DDYM Int(P5)

Proteins with higher relative phosphorylation in Int(P5) of DDYM compared to DYM were enriched in functions associated with the endomembrane, cytoskeleton, signaling, transport and regulation (Table 2.2). Numerous sorghum homologs of proteins involved in endomembrane system function were differentially phosphorylated in DDYM Int(P5) including Sec14, KEU, DELTA-ADR and remorins, proteins involved in vesicle trafficking, cytokinesis, and endocytosis (B. C.-H. Lam et al. 2001; Zwiewka et al. 2011; L. Fan et al. 2015; J. Wu et al. 2013) (Table 2.2). Proteins involved in ion transport such as BOR1 were differentially phosphorylated in DDYM/DYM (Table 2.2). BOR1 is trafficked from the plasma membrane (PM) to the vacuole via the endocytic pathway for

degradation to regulate optimal levels of boron in tissues (Takano et al. 2010; Kasai et al. 2011).

Proteins involved in hormone transport, receptor signaling, and light and calcium signaling were phosphorylated at higher levels in DDYM Int(P5). The ABCB1 (Dw3) auxin transporter was differentially phosphorylated at three sites, two of which (S365, S362) are located near S263, a site of PID phosphorylation that activates IAA transport (Henrichs et al. 2012). The STRUBBELIG receptor (Sobic.001G480800) has three phosphosites with elevated levels of phosphorylation in DDYM plants (Table 2.2). Family members of this receptor have been shown to regulate tissue morphogenesis, cell division planes in Arabidopsis, and internode elongation in rice (Chevalier et al. 2005; Vaddepalli et al. 2011). Proteins with homology to NRL5 were differentially phosphorylated in DDYM. Phosphorylation of proteins in the NRL-gene family such as NPH3 are known to modulate interaction with the AGC kinase phototropin 1 (Vaddepalli et al. 2011; Christie et al. 2018). The function of NRL5 is unknown, but these proteins may regulate Dw2 or be regulated by Dw2 signaling. Additionally, Root Hairless 1 (RHL1) shows relatively high differential phosphorylation in DDYM. RHL1 is a component of the DNA topoisomerase VI complex required for endoreduplication and ploidy-dependent cell growth (Sugimoto-Shirasu et al. 2005). Furthermore, phosphorylation of a Sec14p-like family member at S291 was only detected in DDYM. The Sec14 family has many different biological roles such as regulating membrane trafficking and phosphoinositol signaling (J. Huang, Ghosh, and Bankaitis 2016). Sec14p-like proteins have been shown to localize to the cell

plate to coordinate vesicle trafficking to the new cell wall of dividing cells (Zhou et al. 2019; Peterman et al. 2004).

Table 2.2 Greater Phosphorylation in DDYM

Sorghum ID	Arabidopsis ID	Arabidopsis Symbol	Fold Change	Function	(P)site
Endomembrane System					
Sobic.010G187500	AT1G75370	Sec14p-like	***	Phosphatidylinositol/choline transfer, Secretion	S291
Sobic.006G033200	AT1G12360	KEU	3.6	SNARE-interacting Protein KEULE, cytokinesis, vesicular trafficking	S590
Sobic.007G059900	AT1G34220	Regulator of Vps4	2.5	Regulator of Vps4 in MVB pathway	S400
Sobic.010G231200	AT1G13920	Remorin Family	2.1	Microdomain-associated Endocytosis	S13
Sobic.008G072200	AT1G48760	DELTA-ADR	2.0	Clathrin Adaptor complex, Vesicle budding	S182
Cytoskeletal					
Sobic.002G359000	AT5G67470	FH6	***	Formin Homolog, Organization and polarity of actin	S772, S150
Sobic.010G216000	AT4G30160	VLN4	2.1	Villin, Actin Organization	S805
Sobic.001G366700	AT2G41740	VLN2	2.1, 2.0, 1.4	Villin, Actin Organization	S842. S838, S860
Hormone & Receptor Signaling					
Sobic.001G480800	AT3G14350	SRF7	***	STRUBBELIG receptor, Tissue morphogenesis	S373, S351, S390
Sobic.001G013000	AT3G25070	RIN4	6.8	Essential Regulator of Plant Defense	S58
Sobic.007G163800	AT2G36910	ABCB1, *SbDw3*	4.9	Auxin Efflux	S365
Light Signaling					
Sobic.006G120000	AT1G67900	NRL5/NPY1	5.6	Phototropism, Ubiquitination	S372
Sobic.004G187100	AT5G64330	RPT3	5.3	Root Phototropism Protein, Ubiquitination	S23

Sobic.001G076400	AT3G50780	BTB/POZ domain	3.9	Phototropism, Uniquination	S57
Ion Signaling & Transport					
Sobic.002G303300	AT4G11610	*No symbol*	6.1	Calcium/Lipid phosphoribosyltransferase Putative Synaptotagmin	S207
Sobic.008G132300	AT2G47160	BOR1	2.7, 2.5, 2.4, 2.2	Boron Transporter	S672, S654, S668, S668
RNA & DNA Processing					
Sobic.010G277900	AT1G48380	RHL1	27.7	Root Hairless 1, DNA topoisomerase, endoreduplication	S98

*** No phosphorylation detected in DYM

2.3.8. Phospholipase D inhibitor n-butanol phenocopies mutant plants

The phosphorylation of PLDδ in DYM but not DDYM internode tissue indicated that PLDδ could be a direct phosphorylation substrate of Dw2. PLD plays a major role in lipid signaling by releasing phosphatidic acid (PA), a modulator of growth, the cytoskeleton, endocytosis, development and defensive responses(Hong et al. 2016; Pleskot et al. 2013). Dw2 could modify cell proliferation in internodes by phosphorylating and activating PLDδ increasing production of PA that is required for several processes involved in cell proliferation (X. Wang 2005). Further support for this hypothesis was obtained by treating DYM and DDYM internodes with n-butanol, an inhibitor of PLD (Munnik et al. 1995). Treatment of DYM with n-butanol reduced internode length by 50% whereas treatment of the *dw2* mutant DDYM had minimal impact on internode length (Fig 2.6a). In addition, n-butanol treatment of DYM increased the length of cells in the internode ZoD consistent with inhibition of cell proliferation, whereas treatment of DDYM with n-butanol did not alter the length of cells in the ZoD (Fig 2.6). These results indicate that PLD activity is necessary for normal cell proliferation during internode elongation. Analysis of RNAseq data previously collected from developing stems showed that PLDδ is expressed in elongating internodes together with several other genes annotated as encoding PLDs (Sobic.001G34900, Sobic.003G050400, Sobic.010G185600, Sobic.001G320200)

(Kebrom, McKinley, and Mullet 2017). Therefore the specific role of PLDδ in cell proliferation in the internode ZoD will require targeted mutation of this gene.

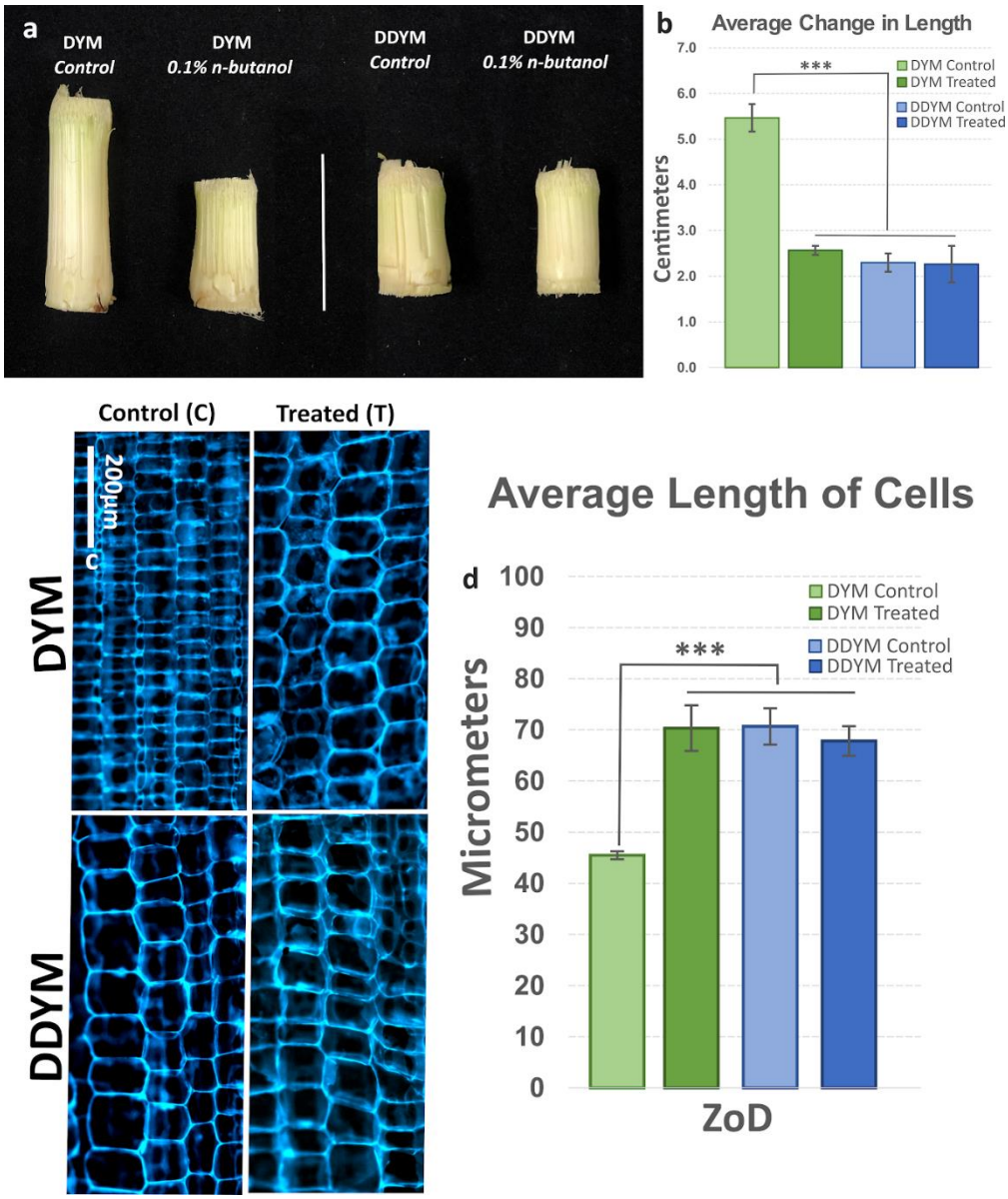


Figure 2.6 Phospholipase D inhibitor n-butanol causes DYM to phenocopy DDYM (a) Internodes from control (C) and treated (T) DYM and DDYM plants. DYM-T internodes are indistinguishable from DDYM internodes from either condition as quantified in (b). (c) Images of cells from the ZoD of DYM-(T/C) or DDYM-(T/C). DYM-(T) internodes have significantly longer cells in the ZoD than DYM-C cells and are indistinguishable from DDYM in either condition as quantified in (d).

2.3.9. Dw2 mutants are susceptible to ROS

PLDδ is a unique family member of the phospholipases and has many specialized roles. It is associated with the plasma membrane and microtubules, has been implicated in ROS signaling, and is activated by oleic acid and H_2O_2 (W. Zhang et al. 2003; C. Wang and Wang 2001). Furthermore, proteomic evidence shows proteins with higher accumulation in *dw2* internodes are enriched in ROS scavenging activity (Fig 2.7a). Taken together, these data formulate the hypothesis that internode elongation is regulated, in part, by ROS signaling and is dependent upon Dw2.

To test the role of ROS in elongation in Dw2 and *dw2* plants, we employed two simple experiments to accumulate ROS within whole plants and internodes. The first was to treat growing plants with the Photosystem II (PSII) inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea, commonly known as DCMU (Joo et al. 2005). Treatment with DCMU blocks the electron transfer within PSII, and thus increases ROS concentration in the chloroplasts (Joo et al. 2005). Dw2 and *dw2* 70 day old plants were watered with 500mL of 500 μM DCMU twice a week for three weeks. Mutant internodes remained underdeveloped and small whereas wt internodes grew more regularly (Fig 2.7 b,c).

Because plants were watered with DCMU solution, we wanted a more direct method of determining the effect of ROS accumulation on Dw2 and *dw2* internodes. To accomplish this, we cut a small window in the leaf sheath of growing internodes and sprayed 3% H_2O_2 directly onto the exposed surface. We then covered the area with foil

and allowed them to grow for two weeks. Mutant internodes remained short and over proliferated their nodal roots whereas wt internodes still elongated and had fewer nodal roots (Fig 2.7 d,e,f). These data suggest that mutant internodes are more sensitive to ROS accumulation, possibly because Dw2 is required to propagate the PLDδ pathway required for detoxification. Interestingly, this experiment also suggests ROS signaling plays a role in the proliferation of nodal roots.

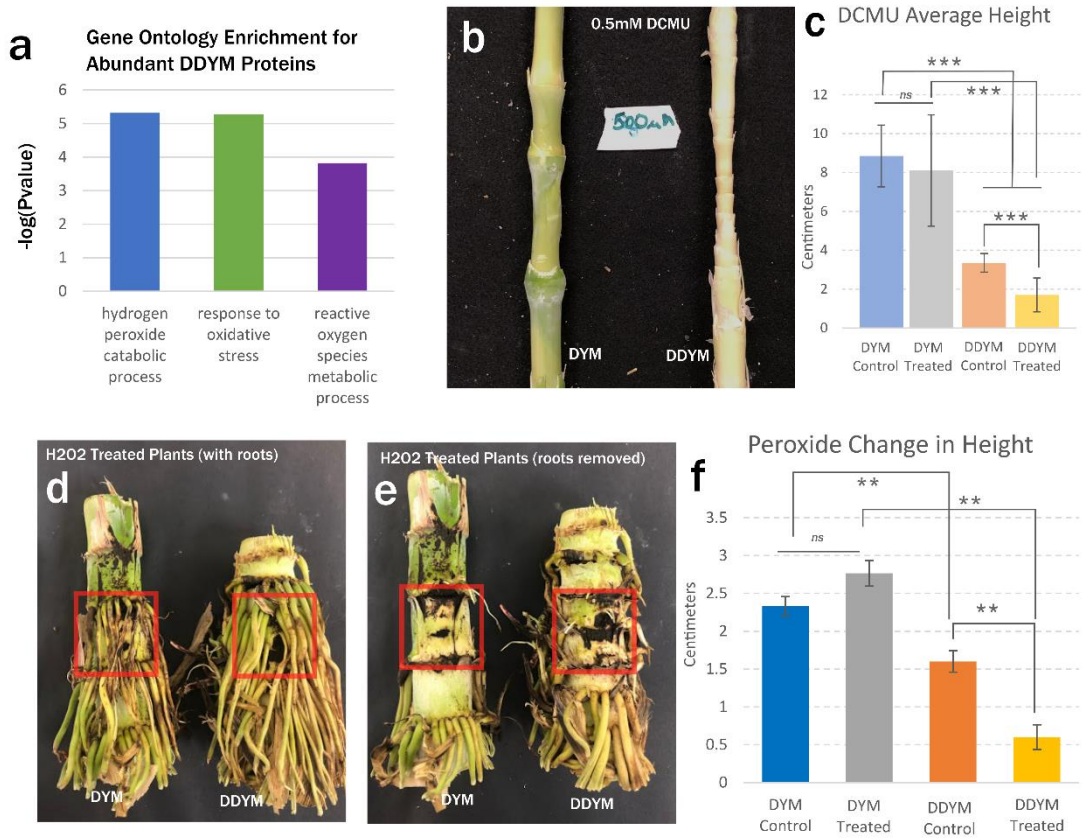


Figure 2.7 Dw2 mutant plants are more sensitive to ROS toxicity. a) Proteomics data from proteins with greater abundance in DDYM internodes b) Picture of DYM and DDYM internodes treated with DCMU to induce ROS formation c) DCMU treated plants average height of internodes. Error bars are SD of 12 internodes d) DYM and DDYM internodes treated with hydrogen peroxide, with (d) and without (e) nodal roots f) Average change in length of internodes treated with hydrogen peroxide. Error bars are SD of three plants. **= pvalue <0.05 ***=pvalue < 0.001

2.3.10. Dw2 signaling regulates endocytosis

Differential phosphorylation of proteins associated with the endomembrane system observed in DYM/DDYM could result in alteration of endomembrane functions that affect internode cell morphology and growth. FM4-64 is a lipophilic dye used to monitor endocytosis because it labels the PM and is internalized via endocytosis to endosomal compartments with subsequent distribution to the tonoplast (Sugimoto-Shirasu et al. 2005; Rigal, Doyle, and Robert 2015). FM4-64 analysis of endocytosis is often carried out on roots and root hairs because cells are readily accessible and they lack chlorophyll. Additionally, root hairs were imaged because they require a complex coordination of vesicular trafficking and cytoskeleton organization to grow (Samaj et al. 2006). This analysis revealed that DYM root hairs were straight whereas DDYM root hairs were wavy or bent, a phenotype that could be linked to altered lipid signaling (T. Hirano et al. 2018) (Fig 2.7a). Exposure of emerging root hairs to FM4-64 for 15 mins resulted in more dye internalization in DYM compared to DDYM (Fig 2.7b). FM4-64 uptake by root cells was examined next. Exposure of DYM root tissue to FM4-64 for 5 minutes resulted in the dye becoming associated with the plasma membrane (Fig 2.7c). After 30 min of treatment, the relative FM4-64 signal associated with internal membranes of the cytoplasm increased. In contrast to DYM, incubation of DDYM roots with FM4-64 resulted in less dye associated with internal membranes after 30 minutes of treatment (Fig 2.7c (bottom row), d), indicating that uptake and trafficking of

the dye occurs more slowly in the mutant (Fig 7e). This was confirmed by quantification of the FM4-64 signal in the PM and the cytosol, which indicated a higher PM/cytosol ratio in DDYM compared to DYM after 30 minutes of treatment (Fig 7d). To extend this observation, Brefeldin A (BFA) was used to disrupt endocytosis. BFA inhibits endosomal cycling which leads to the formation of endosomal aggregates called BFA bodies (S. K. Lam et al. 2009). Mutants with endocytic defects reduce the number of BFA bodies per cell (Stefano et al. 2018). After 1 hour of BFA treatment, DYM root cells show extensive accumulation of BFA bodies (~8 per cell, Figure 2.7e, (left panel) white arrowheads, f). In contrast, DDYM root cells contained fewer BFA bodies (~2 per cell Figure 2.6e, (right panel), white arrowheads, f). Taken together, these data indicate endocytosis is reduced in the *dw2* mutant.

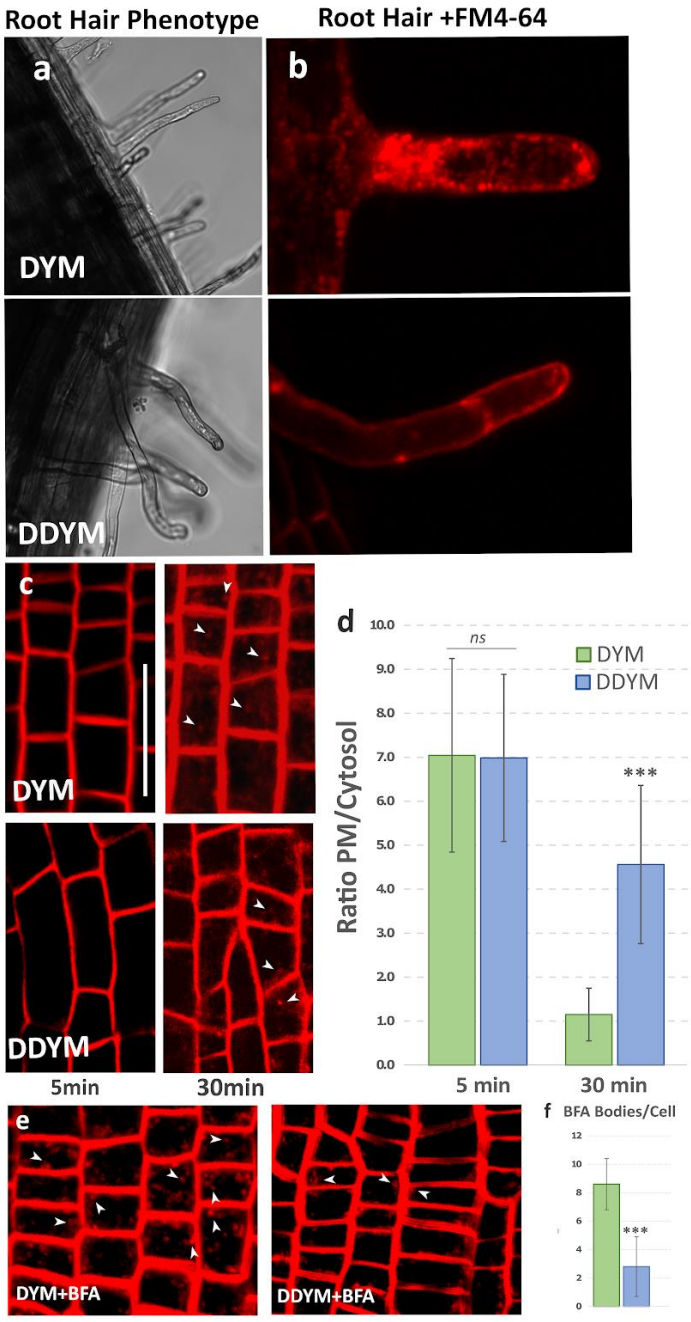


Figure 2.8 Dw2 mutants have altered root hair morphology and reduced endocytosis (a, top) DYM 12 day old seedling straight root hairs. (a, bottom) DDYM 12 day old seedlings have bent or wavy root hairs. (b, top) DYM root hair after 15 minutes of FM4-64 treatment. Most of the signal been taken up into the cytoplasm. (b, bottom) DDYMroot hair after 15 minutes of FM4-64 treatment. Most of the signal is localized to

the plasma membrane.(c) Time course of the endocytic tracking dye FM4-64 of root cells. At 5 minutes after treatment, both genotypes show strong signal in the PM. As time progresses to 30 minutes after treatment, DYM roots show strong internalization of the dye, with DDYM plants showing a higher fluorescent signal in the plasma membrane, indicating slower uptake (d). Error bars represent SD of 50 cells over 5 replicates. (e) DYM roots treated with Brefeldin A (BFA), an inhibitor of endocytosis. After 1 hour of treatment, DYM roots contain many "BFA bodies" per cell (f). DDYM roots contain fewer BFA bodies per cell (e,f). Error bars represent SD of 50 cells over three replicates.0.001 = ***. Scale Bar in (c) 25μm.

2.3.11. Mutation of Dw2 alters deposition of endomembrane trafficked cell wall polysaccharides

Differences in endomembrane phosphoproteomics and activity observed in DYM and DDYM could affect secretion and deposition of cell wall polysaccharides such as heteroxylans (HX) and mixed linkage glucans (MLG) (S. Kim et al. 2018; Oikawa et al. 2013). In DYM, immunolocalization analyses showed that MLG is localized fairly uniformly in pith parenchyma cell walls located near vascular bundles in the ZoD of Int(P5) (Fig 2.8a). In contrast, in DDYM, MLG accumulated in the vertices where adjacent pith parenchyma cells meet and to a much-reduced extent along the sides of adjacent cells (Fig 2.8b). Quantitation showed that signal from MLG staining was differentially localized in cell vertices of DDYM compared to DYM (Fig 8c) (S. Kim et al. 2018; Oikawa et al. 2013).

Heteroxylan (HX) is another component of plant cell walls that is delivered to the cell wall through the endomembrane system. In internodes of DYM, heteroxylan was localized in cell walls that surround pith parenchyma cells near vascular bundles (Fig 2.8d). In contrast, DDYM internode cell walls showed heteroxylan accumulated in a

distinct “dashed-line” pattern of deposition with higher abundance in the faces (or sides) of adjacent cells (Fig 2.8e,f).

Cellulose is synthesized at the plasma membrane by cellulose synthases. Cellulose staining of DYM and DDYM cells in the ZoD by calcofluor white, which preferentially stains cellulose and callose, showed that cellulose was uniformly distributed in cell walls of both genotypes (Fig 2.8 g,h). There was slightly reduced levels of cellulose in the cell walls of DYMM internodes compared to DYM (Fig 2.8i). These results indicate that disruption of signaling propagated by Dw2 affects the deposition of cell wall polysaccharides that are synthesized in the Golgi and transported via vesicles to the cell wall and have less of an effect on polysaccharides synthesized at the plasma membrane.

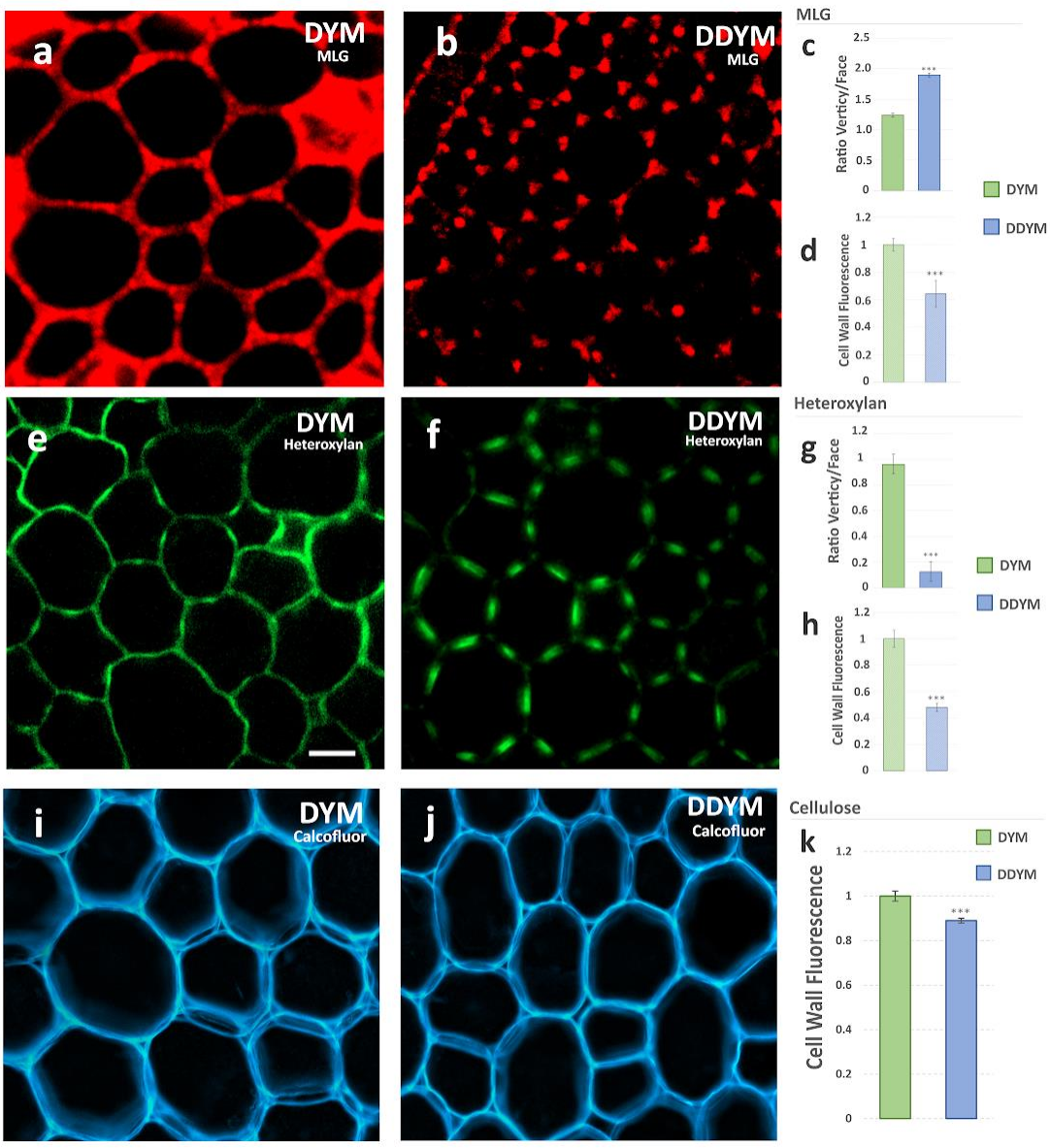


Figure 2.9 Dw2 signaling regulates the localization of cell wall components trafficked by the endomembrane system (a,b,c,d) Immunolocalization of Mixed-link glucan (MLG) in DYM (a) and DDYM (b) internode cells. MLG shows strong localization in the cellular vertices of elongating DDYM internodes (b,c) but shows overall less fluorescence intensity in the entire cell wall (d). DYM plants show uniform MLG localization (a) and show greater fluorescence intensity in the cell wall (d). Cell wall fluorescence is normalized to DYM levels. (e,f,g,h) Heteroxylan (HX) immunolocalization in DYM (e) and DDYM (f) internode cells. In DDYM, HX shows strong localization at the planes where adjacent cells meet (faces) (f, g), but show overall less fluorescence signal in the cell wall (h). DYM plants show uniform HX localization

(e) and show greater fluorescence signal in the cell wall (h). Cell wall fluorescence is normalized to DYM levels. Both MLG and HX are polysaccharides that are trafficked through the endomembrane system. (i,j,k) Calcofluor stain in DYM (i) and DDYM (j) internode cells. Calcofluor preferentially stains cellulose and callose. Both DYM and DDYM cells show uniform signal in their cell walls, with total fluorescence intensity reaching 90% of that in the DYM cells (k). Scale bar is 10μm and error bars are SD of 50 cells over three replicates. pvalue ≤ 0.001 = ***

2.4. Discussion

The plant specific AGCVIII kinase family regulates growth in response to light (phototropism), gravity (gravitropism), developmental and morphogenic signals (Rademacher and Offringa 2012; Barbosa and Schwechheimer 2014). Dw2 is one of 21 AGCVIII kinases encoded by the sorghum genome (J. L. Hilley et al. 2017). Ten of the sorghum AGCVIII kinases are expressed during stem internode development; 4 D6PKs, PHOT1, Dw2 (homolog of KIPK), KIPK-like, AGC1-3a, AGC1-3b, and AGC1-12 (J. L. Hilley et al. 2017) this study). D6PKs, PHOT1, and AGC1-12 regulate phototropic, gravitropic and planar growth by phosphorylating PIN proteins, mediators of polar IAA transport. In the current study, Dw2 was found to regulate the extent of cell proliferation in elongating sorghum internodes. Phosphoproteomic analysis of DYM (Dw2) and DDYM (dw2) elongating internodes did not reveal differences in PIN phosphorylation indicating Dw2's mechanism of action differs from D6PK and PHOT. As discussed below, mutation of Dw2 caused down regulation of cell proliferation in elongating internodes, extensive changes in protein phosphorylation, inhibition of endomembrane activity, altered accumulation of cell wall polysaccharides and vascular bundle morphology.

Mutation of Dw2 causes a significant decrease in the length of stem internodes, a trait that was selected for in grain sorghum breeding programs to reduce lodging (J. L. Hilley et al. 2017). The current study showed that mutation of Dw2 did not alter cell proliferation in nascent internodes located in the apical dome prior to their elongation, but inhibited cell proliferation in the intercalary meristem of elongating internodes. This indicates that Dw2 has a specialized role in the control of cell proliferation in the IM during the elongation of internodes that occurs over a period of ~6-9 days. The regulation of cell proliferation in the IM of elongating internodes is important since the extent of cell proliferation has a significant impact on internode length, plant height, competition for light, and the amount of resources the plant allocates to stem growth (sink strength). The growth of grass stems occurs by the sequential production of phytomers containing internodes that elongate and differentiate during a specific phase of phytomer development. The rate of phytomer production is regulated during grass development and in response to environmental inputs. Likewise the elongation of internodes is regulated so that it occurs following growth of the leaf blade and leaf sheath of the same phytomer. Once internodes begin elongating, polarized growth and differentiation occurs across the length of the internode that is sustained for 6-9 days by continued cell proliferation in the intercalary meristem located at the base of the internode (Kebrom, McKinley, and Mullet 2017). Growth within zones of cell division and elongation requires coordination across tissues (epidermis to the center of the stem) and among different cells types. The unique developmental biology of grass stems may explain why mutations that affect internode growth in grasses often have minimal impact

on stem growth in the dicot Arabidopsis. For example, mutation of KIPK, the closest Arabidopsis homolog of Dw2, does not affect stem growth in Arabidopsis (T. V. Humphrey et al. 2015). Similarly, mutation of the gene corresponding to sorghum ABCB1 (Dw3) has a significant impact on internode growth in grasses (Multani et al. 2003; Knöller et al. 2010) whereas mutation of AtABCB1 has only a minor impact on stem growth in Arabidopsis (Noh, Murphy, and Spalding 2001).

The main phenotype associated with mutation of Dw2 was shorter internode lengths due to reduced cell proliferation, however several additional cellular phenotypes were observed that could contribute to our understanding of how Dw2 regulates cell proliferation and the identification of additional Dw2 regulatory functions. The cellular/sub-cellular phenotypes that distinguish DDYM from DYM include: (1) irregular internode cell shapes and vascular bundle morphology in fully elongated internodes, (2) a wavy root hair morphology, (3) inhibition of endocytosis/endomembrane activity measured by reduced rates of FM4-64 uptake, and (4) modified accumulation and localization of heteroxylan and MLG in cell walls. We observed that Dw2 is expressed in most stem cell types indicating that Dw2 could regulate cellular processes in many cell types and stages of internode development. For example, in situ analysis showed elevated expression of Dw2 in nascent vascular bundles of Int(P5) that persisted in parenchyma cells associated with vascular bundles in elongating internodes. The loss of Dw2 function in these vascular bundle cells could be responsible for the abnormal anatomy of vascular bundles in DDYM by altering endomembrane activity that normally promotes symplastic movement of transcription

factors such as SHORT-ROOT (SHR) that specify cell identity (Koizumi et al. 2011). Elevated expression of Dw2 in developing vascular bundles is interesting because PDK1, a regulator or AGC-kinases, is also expressed in provascular tissues and Arabidopsis pdk1pdk2 mutants are dwarfed plants with abnormal vein morphology (Xiao and Offringa 2020). Dw2 contains a phosphosite (S657) located within the T-loop [SxS*FVGTxEYxAPE] activation segment that is a preferential site of PKD1 phosphorylation and Dw2 has a C-terminal FxxF sequence motif present in AGC kinases that mediates PDK1 binding and phosphorylation of active site amino acids (Rademacher and Offringa 2012). PDK1 mediated activation of Dw2 therefore could stimulate endomembrane activity required for vascular tissue differentiation and cell proliferation. PDK1 activity is modulated by auxin induced production of phosphoinositols (Xiao and Offringa 2020). Mutation of the ABCB1 auxin efflux transporter in sorghum (dw3) (Multani et al. 2003) and maize (br2) (Knöller et al. 2010) causes inhibition of internode elongation demonstrating the importance of auxin in C4 grass internode growth. Mutation of inositol polyphosphate 5-phosphatase in maize bv1 causes inhibition of internode elongation and altered expression of genes involved in cell wall biosynthesis, transmembrane transport and cytoskeletal function (Avila et al. 2016). Mutation of Dw2 affects the expression of genes involved in these same pathways/processes. Taken together, these results indicate that lipid signaling plays an important role in C4 grass internode growth regulation and that Dw2 is an integral part of this pathway.

Phosphoproteomic and proteomic analyses of tissue from elongating internodes of DYM and DDYM were utilized to obtain insight into Dw2 targets and regulatory activity. Phosphoproteomic analysis identified 205 proteins containing phosphosites that were differentially phosphorylated in DYM and DDYM tissue from Int(P5). The majority of these proteins (89%) showed higher phosphorylation in DDYM internodes indicating these changes were indirect effects of loss of Dw2 function. This group included proteins involved in endomembrane function, transport, and hormone/light/calcium signaling, with an enrichment in localization and Golgi trafficking proteins. Of all differentially phosphorylated proteins, only 23 proteins showed higher phosphorylation in Int(P5) of DYM. These proteins could be direct targets of Dw2, however, many showed relatively low levels of differential phosphorylation. A few phosphoproteins were only detected in DYM or DDYM. For example, phosphorylation of Dw2 was only detected in DYM because Dw2 protein did not accumulate in DDYM consistent with the mutation in Dw2 which creates a stop codon in the first exon leading to protein truncation (J. L. Hilley et al. 2017). PLDδ was present in the internodes of both genotypes but phosphorylation was only detected in DYM Int(P5) indicating that this protein could be a direct target of the Dw2 kinase. The treatment of DYM internodes with n-butanol, a PLD inhibitor, inhibited internode elongation to the same extent as observed in DDYM, suggesting that PLD signaling could play an important role in Dw2 regulated internode elongation. In animal systems, phosphorylation of PLD1 by RSK2 results in activation and synthesis of phosphatidic acid (PA) at locations of neurite cell proliferation and growth (Ammar et al. 2013). The

authors proposed that mutations in RSK2 impairing PLD1 activity resulted in reduced vesicle trafficking and membrane synthesis required for growth.

Arabidopsis PLDδ was identified as a tubulin and microtubule-binding protein that also interacts with actin, clathrin heavy chain, and a flotillin homolog (Ho et al. 2009). Clathrin heavy chain is involved in spindle organization and phragmoplasts in tobacco cells (Ho et al. 2009; Tahara et al. 2007) and a tobacco homolog of PLDδ localizes to the mitotic spindle (Marc et al. 1996). Taken together, reduced activation of PLDδ in the IM of DDYM could contribute to lower cell proliferation. KIPK, the Arabidopsis homolog of Dw2, interacts with KCBP (Marc et al. 1996; Day et al. 2000) and PERKs (T. V. Humphrey et al. 2015). Interaction with KCBP is especially interesting because KCBP is a calcium-binding protein implicated in several aspects of cell division that interacts with microtubules and is localized to the cortical division zone (Vos et al. 2000; Vinogradova et al. 2009; Buschmann et al. 2015; J. Tian et al. 2015). If Dw2 also binds KCBP, then this could facilitate localization of Dw2 to cell division zones where regulation of PLDδ could affect vesicle trafficking and other processes involved in cell division.

Two chloroplast localized proteins, Lhcb2 and psbH showed high levels of phosphorylation in DYM compared to DDYM. The subcellular localization of Dw2 has not yet been characterized, but it is likely that elevated phosphorylation of these plastid-localized proteins is an indirect effect of mutation of Dw2. Phosphatidic acid has been shown to activate monogalactosyldiacylglycerol synthase (MGD1) and MGDG is essential for chloroplast development (Dubots et al. 2010). Therefore, it is possible that

mutation of Dw2 affects PLD-mediated PA synthesis causing inhibition of chloroplast development and associated changes in Lhcb2 and psbH phosphorylation. Alternatively, reduced internode growth in DDYM results in the internodes being wrapped in additional leaf sheaths that attenuates and alters the spectral distribution of light reaching the internode surface. This could cause a delay in chloroplast development and alter light-mediated changes in phosphorylation involved in state transitions (Dubots et al., 2010; Longoni et al., 2019).

Lack of Dw2 in DDYM reduced endocytosis/endomembrane activity in seedling roots and emerging root hairs. Altered endomembrane activity in DDYM is likely responsible for altered deposition and localization of heteroxylan and MLG in stem pith parenchyma cells in the ZoD. In DYM, MLG was distributed uniformly in the apoplast-cell wall space. However, in DDYM, MLG was depleted from cell faces and accumulated in locations where several cells meet (edges or interstices). In contrast, heteroxylan accumulated along cell faces and was depleted in cell interstices, possibly because of high MLG accumulation in those regions. Cellulose, which is synthesized by a complex localized to the PM, was uniformly distributed around cells. The altered distribution of MLG and heteroxylan could be a consequence of depletion of these polysaccharides in cell walls (25-50%) which affected their detection (Martin-Tryon and Harmer, 2008; Xue et al., 2013). It is also possible that the altered distribution is a consequence of altered endomembrane activity and localized wall properties that affects the flow of cell wall polysaccharides to different regions of the cell wall. Prior studies have shown that endomembrane trafficking can specifically deliver materials/proteins to

the apical and basal regions of cells and to cell facial and edge domains (Łangowski et al. 2010; Kirchhelle et al. 2016). Based on these studies, it has been proposed that normal cell geometry requires maintenance of wall stiffness at geometric edges and that RAB-A5c, which is localized to unique domains of the trans-Golgi network, mediates endomembrane trafficking to edge domains. Mutation of RAB-A5c results in abnormal cell shapes. In this context it is interesting that mutation of CslF which encodes MLG synthase results in altered cell wall stiffness (Kido et al. 2015). Moreover, lack of Dw2 activity in DDYM results in internode cells that are less uniform in shape compared to DYM (Fig 2.3a, d). Therefore it is possible that mutation of Dw2 which impairs endomembrane activity, directly or indirectly affects cell wall properties (i.e., stiffness) that results in MLG accumulation at the cell edges located in interstices.

Sorghum grain breeders identified and utilized genotypes encoding inactive Dw2 alleles to reduce plant height and lodging (Quinby 1974). During field assessment of the utility of dw2 genotypes for grain breeding, the genotypes were found to have significantly reduced stem length but also decreased panicle length and seed weight without reducing leaf number or altering flowering time (Graham and Lessman 1966). The reduction in panicle length and grain yield was not due to a reduction in leaf area, although reduced leaf spacing along the stem could alter light interception. Reduced stem length could have limited the capacity to store carbohydrates and nitrogen used for grain filling with negative impact on seed weight. The current study also raises the possibility that reduced panicle length and lower grain yield could be due in part to reduced cell proliferation or altered endomembrane activity. Increases in the yield of

grain sorghum have been very slow over the past 30 years (Pfeiffer et al. 2019), suggesting a limitation has been reached with the current dwarf hybrid crop genotypes that are used commercially. This study suggests that developing grain hybrids containing dominant alleles of Dw2 could be useful, while selecting for increased stalk strength similar to the development of bioenergy sorghum.

2.5. Materials and Methods

2.5.1. Plant Material and Growth Conditions

Dwarf Yellow Milo (DYM; Dw2) and Double Dwarf Yellow Milo (DDYM; *dw2*) plants were grown in greenhouses or in growth chambers during 2017-18. For height and cell morphology experiments, plants were grown in a long day greenhouse. For all RNAseq, phosphoproteomic, and immunolocalization experiments, plants were grown in long day growth chambers (14/10 day/night) to minimize biological variance. In all cases, plants were grown in 5 gallon pots and fertilized with osmocote initially and supplemented with Peter's nutrient solution as needed. Images of plants were taken with Apple's iPhone 8 and remained unprocessed other than cropping.

2.5.2. Cell Size, Number, Morphology, and Staining

Cell size and number calculations were obtained by staining cells with Calcofluor White (1:20 in PBS) of various internodes and imaging on a Zeiss Axioplan 2 microscope. Sizes of cells were then measured using ImageJ and averaged for a target tissue using three biological replicates. The length of each cell per micrometer of internode was used to estimate the number of cells in the total length of the internode.

For cell staining and morphology characterization, the center of mature internodes were harvested and stained with either 0.1% Toludine Blue or Saffrinin O to visualize cell wall. Images were obtained using a black and white microscope, which allowed for the greater contrast and discrimination of cell wall and vascular bundle elements than with color images. For the abnormal vascular bundle phenotypes, total vascular bundle numbers were calculated in five images from mature internodes of both DYM and DDYM in three biological reps and were marked as either “normal” or “misshapen”. After summation, the number of misshapen vascular bundles were reported as a total percentage of all vascular bundles counted. Cellulose staining was achieved by staining cells with Calcofluor White (1:20 in PBS) for 2 minutes with a five minute destaining step and imaged using a Zeiss Axioplan 2 microscope. Three replicates were used for this experiment.

2.5.3. In Situ Hybridization

2.5.3.1. Plant materials and paraffin embedding

Plants were grown as previously described. Int(P5) tissue samples were formalin-fixed in 10% NBF for 24hrs at room temperature. Samples were then washed with 1XPBS and dehydrated through a series of graded ethanol washes, cleared with xylene and embedded in paraffin wax according to established protocols (Karlgren et al. 2009) . Embedded samples were stored at 4°C.

2.5.3.2. Probe Synthesis and mRNA in situ hybridization

The sequence of Dw2 (Sobic.006G067700.2) and a Cytochrome P450 (positive control, Sobic.003G324800.1) transcripts were obtained from Phytozome and submitted

to Advanced Cell Diagnostics, Inc. (ACD) for probe design. RNAscope RNA probes were synthesized by Advanced Cell Diagnostics, Inc. (ACD) using a custom probe design service. In general, several double Z probe pairs are produced that hybridize with high specificity to the target RNA. Binding of probes in pairs allows amplified signal generation which is then observed as a dot of red chromogenic precipitate.

Embedded sorghum internodes were thinly sectioned (6μm thick) using a Leica Microtome, sections were placed on Fisherbrand Superfrost Plus slides, Cat. No. 12-550-15, and put on a hot plate at 42°C for a few minutes and then further dried overnight at room temperature. Slides were processed as previously described (Fay Wang et al. 2012). Specifically, RNAscope 2.5 HD Detection Reagent – RED kit was used, Cat. No. 322360. Probed slides were sealed with EcoMount, Cat. No. EM897L. Images were obtained using bright field through a Zeiss Axio Imager.M2 microscope.

2.5.4. Heteroxylan and MLG Immunolocalization

Plant tissue was harvested and lightly vacuum fixed in FAA (5% acetic acid, 3% paraformaldehyde, and 50% ethanol by volume). Tissues were then embedded in 5% agarose blocks and hand sectioned either longitudinally or horizontally depending on the type of imaging desired (Carraro and Peer 2016). Primary antibodies used for heteroxylan (Kerafast, ELD017) or MLG (Biosupplies Australia, 400-3) were diluted to 1:500 in Starting Block Blocking Buffer (Thermo Fisher) then incubated with sections at 4°C overnight with light rocking. After a series of washes, an Alexafluor 488 conjugated mouse anti-rabbit antibody (Thermo Fisher, 1:600 dilution in blocking buffer) was used to visualize immunolocalization signal. Three replicates each were used for each

experiment. Images were taken using a Zeiss Axioplan 2 epifluorescent microscope (HX) or an Olympus FV1000 Laser Confocal microscope (MLG) and processed (increasing contrast, subtracting background, etc.) using the Zen Lite software or NIH's ImageJ.

2.5.5. Phosphoproteomics

Isolation of phosphopeptides was performed as previously described (S. J. Humphrey et al. 2018) with modifications adapted to plant tissues. Frozen sorghum internodes were ground into fine powder using liquid nitrogen with a mortar and pestle. Ground tissues were suspended in cold SDC lysis buffer (100 mM Tris-HCl, pH 8.5, 4% (w/v) sodium deoxycholate). The crude extracts were immediately heat-treated at 95 °C for 5 min and then homogenized with sonication (60 sec pulses with 0.5 sec on/0.5 sec off at level 1.5). 200 ug of protein was applied for trypsin digestion and phosphopeptide enrichment as described (Humphrey et al., 2018) with the following modifications. 10 mg of TiO2 beads (Titiansphere 5 micron;GL Sciences, Inc) were resuspended in bead suspension buffer and incubated with peptides at 40°C for 5 min in ThermoMixer R at 1,400 rpm. Beads were collected by centrifugation and the phosphopeptides were eluted as described in the original protocol. The supernatant from this step was further incubated with another 20 mg of TiO2 beads, and phosphopeptides were eluted, combined with the first, and further processed as one sample. Each sample was injected twice, onto a Q Exactive HF-X mass spectrometer using 90-min LC gradients as indicated. Six biological replicates were performed for each genotype.

LC/MS/MS Analysis performed with isolated phosphopeptides that were re-suspended in 2%ACN/0.1%TFA to 15uL. Injections of 10uL were automatically made using a Thermo (www.thermo.com) EASYnLC 1200 onto a Thermo Acclaim PepMap RSLC 0.1mm x 20mm C18 trapping column and washed for ~5min with buffer A. Bound peptides were then eluted over 125min onto a Thermo Acclaim PepMap RSLC 0.075mm x 500mm resolving column with a gradient of 5%B to 25%B at 90min, ramping to 42%B at 114min, to 100% B at 115min and held at 100%B for the duration of the run (Buffer A = 99.9% Water/0.1% Formic Acid, Buffer B = 80% Acetonitrile/0.1% Formic Acid/19.9% Water) at a constant flow rate of 300nl/min. Column temperature was maintained at a constant temperature of 50oC using an integrated column oven (PRSO-V2, Sonation GmbH, Biberach, Germany). Eluted peptides were sprayed into a ThermoScientific Q-Exactive HF-X mass spectrometer (www.thermo.com) using a FlexSpray spray ion source. Survey scans were taken in the Orbi trap (60,000 resolution, determined at m/z 200) and the top 10 ions in each survey scan are then subjected to automatic higher energy collision induced dissociation (HCD) with fragment spectra acquired at 15000 resolution.

Data files from the LC-MS/MS analysis were processed using MaxQuant (version 1.6.3.4) to identify proteins and calculate isobaric tag intensities using the Andromeda search engine using the default parameters and with an FDR of < 0.01 at the protein and peptide levels. The *Sorghum bicolor* v3.1.1 proteome obtained from Phytozome containing 47121proteins was used for the search. Perseus (version1.6.2.3) was used to compare the two genotypes. Phophosites with location probabilities lower

than 0.75 were removed as were peptides from the reverse databases and probable contaminants. The intensities were log2 transformed and phosphosites with less than 3 valid values in one of the two genotypes were removed. Missing values were imputated using a width of 0.3 and a downshift of 1.8. A two-sided Student's T-test was conducted, and a permutation-based multiple testing correction was applied as implemented in Perseus.

2.5.6. Proteomics

Samples were digested according to Kulack, et.al (Kulak et al. 2014). Briefly, protein samples (100ug) were re-suspended to 270uL in 100mM ammonium bicarbonate supplemented with 4% (wt/v) sodium deoxycholate (SDC). Samples were reduced and alkylated by adding a solution of TCEP and Iodoacetamide (10mM and 40mM, respectively, at pH8) and incubated for 5min at 45C with shaking at 1400 rpm in an Eppendorf ThermoMixer R. The samples were allowed to cool to room temperature and trypsin/lysC enzyme mixture (Promega, V5071), in 100mM ammonium bicarbonate, was added at a 1:100 ratio (wt/wt, enzyme:protein). The mixture was then incubated at 37C overnight in the ThermoMixer R with shaking at 1400rpm. Final volume of each digest was ~300uL. After digestion, SDC was removed by phase transfer and the peptides acidified to 2% trifluoroacetic acid (TFA). Peptides were then subjected to C18 solid phase cleanup using StageTips (Rappsilber, Mann, and Ishihama 2007) to remove salts. Peptide eluates were dried by vacuum centrifugation and stored at -20C. Five biological replicates were performed for each genotype.

2.5.7. Isobaric Peptide Labeling

Peptide samples were then re-suspended in 100uL of 100mM triethylammonium bicarbonate and labeled with TMT reagents from Thermo Scientific (www.thermo.com) according to manufacturers' instructions. Aliquots of 2uL were taken from each labeled sample and reserved for testing the labeling/mixing efficiency by MS. Remaining labeled peptides were mixed 1:1 by volume and purified by solid phase extraction using c18 StageTips. Eluted peptides were dried by vacuum centrifugation to ~2uL and stored at -20C.

2.5.8. High-pH Reversed-Phase Fractionation

The combined peptide sample was re-suspended in 2% acetonitrile (ACN)/0.1%TFA to 50uL and fractionated by high pH reverse phase chromatography. The entire sample was injected onto a Waters Acquity UPLC BEH 1.7um, 2.1mm x 100mm c18 column using a Waters Acquity H-class UPLC. Bound peptides were washed using 0.1% TFA in water for 2min and then separated over 60min using a gradient of 1%B to 25%B at 49min, raised to 60%B at 53min, raised to 70%B at 55min and held at 70%B for the duration of the run (Buffer A = 10mM ammonium bicarbonate, pH10, in water; Buffer B = 10mM ammonium bicarbonate, pH10, in 90% acetonitrile) at a constant flow rate of 0.3mL/min. Column temperature was maintained at 50C using an integrated column heater. Fractions were collected at 1min intervals using a Gilson FC403B fraction collector and then concatenated into 12 total fractions post-run. Fractions were dried by vacuum centrifugation and frozen at -20C.

2.5.9. LC/MS/MS Analysis

Each fraction was re-suspended in 2%ACN/0.1%TFA to 20uL and diluted 1:10 in the same buffer, on-plate. Injections of 5uL were automatically made using a Thermo (www.thermo.com) EASYnLC 1200 onto a Thermo Acclaim PepMap RSLC 0.1mm x 20mm C18 trapping column and washed for ~5min with buffer A. Bound peptides were then eluted over 95min onto a Thermo Acclaim PepMap RSLC 0.075mm x 500mm resolving column with a gradient of 5%B to 8%B at 5min, ramping to 42%B at 83min, to 90% B at 85min and held at 90%B for the duration of the run (Buffer A = 99.9% Water/0.1% Formic Acid, Buffer B = 80% Acetonitrile/0.1% Formic Acid/19.9% Water) at a constant flow rate of 300nl/min. Column temperature was maintained at a constant temperature of 50^{o}C using and integrated column oven (PRSO-V2, Sonation GmbH, Biberach, Germany).

Eluted peptides were sprayed into a ThermoScientific Q-Exactive HF-X mass spectrometer (www.thermo.com) using a FlexSpray spray ion source. Survey scans were taken in the Orbi trap (120,000 resolution, determined at m/z 200) and the top 15 ions in each survey scan are then subjected to automatic higher energy collision induced dissociation (HCD) with fragment spectra acquired at 45000 resolution.

Data files from the LC-MS/MS analysis were processed using MaxQuant (version 1.6.3.4) to identify proteins and calculate isobaric tag intensities using the Andromeda search engine using the default parameters and with an FDR of < 0.01 at the protein and peptide levels. The *Sorghum bicolor* v3.1.1. obtain from Phytozome containing 47121proteins was used for the search. Perseus (version1.6.2.3) was used to compare the two genotypes. The intensities were $\log_2$ transformed and normalized by subtracted the

mean intensity value for that sample from each value. A two-sided Student's T-test was conducted, and a permutation-based multiple testing correction was applied as implemented in Persues. Enrichment analysis was performed using The PlantRegMap's GO enrichment tool (F. Tian et al. 2020).

2.5.10. N-butanol Treatment

DYM and DDYM plants were grown for ~70 days upon which a small window was cut in the leaf sheath to expose the growing internode. Lanolin paste (control) and Lanolin paste+0.1% n-butanol were smeared onto the growing Int(P5). Exposed internodes were then covered with foil to prevent light damage and to promote continued growth. Plants were then allowed to grow for 8 days before imaging and quantification. Cells sizes were quantified as previously described above.

2.5.11. FM4-64 and Brefeldin A Treatment

Growing seedlings of DYM and DDYM plants were placed into water containing 5µM FM4-64 for five minutes. After two quick washes to remove excess dye, seedlings were mounted and imaged using an Olympus FV1000 laser confocal microscope at 5 and 30 minute after staining as described in (Rigal, Doyle, and Robert 2015; Stefano et al. 2018). For inhibitor treatment, seedlings were placed in 5µM FM4-64 for five minutes, washed, and then placed in 3µg/mL BFA solution and incubated for 1 hour before imaging. All images are a projection of Z stack images. Fluorescent signal was quantified by taking the total fluorescence intensity of the plasma membrane and comparing it to the total fluorescence intensity within the plasma membrane. Five replicates were used for the FM4-64 and three replicates were used for the BFA+FM4-

64. All image processing was performed in NIH's ImageJ and all images used for comparisons were treated equally.

2.5.12. Data Availability Statement

Proteomic and phosphoproteomic datasets are deposited in the PRIDE database with the accession number: PXD020160. All other relevant data can be found within the manuscript and its supporting materials.

3. DEVELOPMENT AND GROWTH OF MONOCOT STEMS: INDENTIFICATION OF SPECIFIC TISSUE TYPES WITHIN THE INTERNODE OF SORGHUM BICOLOR

3.1. Overview

The stems of monocots contain internodes that are flanked on either side by nodes. The development of these tissues leads to healthy plants with higher yield potentials. *Sorghum bicolor* is a C4 monocot that has shown promise as a bioenergy crop due to its ability to grow large stems filled with sugars which contain the majority of the biomass. Therefore, stem development is critical to understanding the role of biomass accumulation in bioenergy crops. However, definitive tissues within the stem are largely understudied and overlooked. Here we provide evidence that the Sorghum stem is divided into at least four segments: the Nodal Plexus, the Pulvinus, the Internode, and the "White Band". Moreover, the "White Band" shows characteristics of a boundary layer that separates the Pulvinus from the Internode, and establishment of the White Band is essential for robust internode growth. Together, our results show that stem growth in monocots is directed by the development of the independent tissues. We use our data to describe two competing models of internodal growth which provide insight into the regulation of monocot stem organogenesis.

3.2. Introduction

The C4 grass *Sorghum bicolor* has shown promise as a dedicated bioenergy crop due to its nutrient efficiency, high biomass potential, ability to grow on marginal lands,

and genetic diversity. By providing a renewable source of hydrocarbons for forage, biofuels, and specialty bioproducts, Sorghum can help reduce greenhouse gas emissions through market supplementation (Mullet et al. 2014). The most important organ for increasing biomass and bioenergy is the stem: ~80% of harvested biomass comes directly from stem tissues. Additionally, stem composition impacts conversion efficiency and provides structural integrity of plants that reach 4-5 meters tall (Olson et al. 2012). Despite the significance, detailed knowledge of monocot stem development and stem organogenesis remains poorly understood.

Organogenesis in general is critical for overall health and architecture of plants. For organs to grow, plants must specialize their cells into different tissues and types. This occurs from differentiation of the shoot apical meristem (SAM) into nascent leaves, inflorescence, and stems. Cells of different fates are separated by layers called "boundary layers". Boundary layer formation occurs due to hormone gradients and the accumulation of transcription factors and other marker genes (Richardson and Hake 2018). For example, the transcription factor family LATERAL ORGAN BOUNDARIES (LOB) DOMAIN (LBD) play important roles in many plant developmental processes such as root formation, early leaf formation, and embryo development (Xu et al. 2015) Additionally, CUP-SHAPED COTYLEDON (CUC) transcription factors are important for defining the boundaries of emerging shoot organ primordia and are most highly expressed at an auxin minima adjacent to the SAM (Bilsborough et al. 2011). Within the lower portion of the SAM is an area called the Rib Zone (RZ), which contains the Rib Meristem (RM) and surrounding subapical regions. The exact location of the RM and

RZ are under investigation as it appears there is no clear boundary separating dividing and differentiating cells. However, the RZ and RM work together to form neat and organized cell files associated with stem morphology (McKim 2019). In grasses, leaf primordia remain connected in a “disc of insertion” (DOI) that surround the apex. The current phytomer model establishes the apical end by the upper DOI which develops into the leaf sheath, and the node is established by the lower DOI, which places the internode between two sequential DOIs (Sharman 1942; Johnston, Leiboff, and Scanlon 2015; McKim 2019). While the early development of leaves and organization of the SAM is well studied, the development of the monocot specific internodes and nodes is much less understood.

Stems of monocots are comprised of a series of alternating nodes and internodes. Leaf sheath tissue joins the stem node at the nodal plexus and is established by the DOI early in development. The nodal plexus is a tissue enriched in vascular bundles that have their origins in leaves and stem vascular bundles that traverse internodes and nodes (Shane 2000). Unlike dicots, monocots have an active area of cell division at the base of internodes called the Intercalary Meristem (IM) that spans a Zone of cell Division (ZoD). Below the ZoD is an area called the pulvinus which is a tissue involved in gravitropic responses and where root buds form in grasses. Above the ZoD is an area of rapid cell expansion called the Zone of Elongation (ZoE). The Zone of Maturation (ZoM) is the final developmental stage of internodal growth in grasses and transitions from the ZoE. Cells in the ZoM have stopped elongating and accumulate secondary cell walls (Kebrom, McKinley, and Mullet 2017). The coordination between the three zones

contribute to internode length, strength, and diameter (Kebrom, McKinley, and Mullet 2017). Using this knowledge, the current field in stem development and plant science at large has loosely defined the Intercalary Meristem (IM) as residing "at the base of the internode." However, the exact starting location of the IM has not been characterized.

The growth of sorghum stem tissue has been the focus of numerous previous studies, but in general is influenced by four conditions: (i) regulation of the duration of vegetative growth, which increases the phytomer number (ii) regulation of the rate of phytomer production (phyllochron) (iii) regulation of cell proliferation during stem development and/or (iv) regulation of cell elongation (Mullet et al. 2014; Kebrom, McKinley, and Mullet 2017). Other previous studies have analyzed whole tissue gene expression during vegetative phase stem internode tissue development and characterized pathways involved in stem composition post floral initiation (Kebrom, McKinley, and Mullet 2017; McKinley et al. 2018). However, a more complete understanding of sorghum stem and tissue biogenesis is needed in order to understand when and how stem growth and composition are regulated. The current study focuses on characterizing differences in gene expression in tissues that comprise the different sections of the sorghum stem. This led to the identification of a putative boundary situated at a visible, pale band (henceforth called the "White Band", WB) immediately above the pulvinus tissue that marks the location of the intercalary meristem and has implications for development of monocot stems more broadly. The results were incorporated into a model of the sorghum stem node-internode-phytomer unit.

3.3. Results

3.3.1. Identification of stem tissues that have distinct cell morphologies

Cell morphologies are a classic way to group similar tissues, and we wanted to examine the morphologies of every cell type within the internode. To achieve this, we sectioned internodes from top to base to facilitate microscopic and microCT analysis. Cells within the internode are neat and ordered in uniform cell lines. By contrast, cells within the Nodal Plexus and the Pulvinus are highly disorganized and no clear cell file or regular shape. In microCT images, the pulvinus and nodal plexus are light grey due to the high amount of vascular bundles. In the “white band”, there is a clear transition from the disorganized cells of the pulvinus to the neat and ordered cells of the internode. MicroCT data of the WB shows a distinct opaque area that has less vascular bundles running through it. These results suggested to us the existence of a boundary between these tissues that results in different cellular morphologies in pulvinus (below) and internode (above) (Figure 3.1).

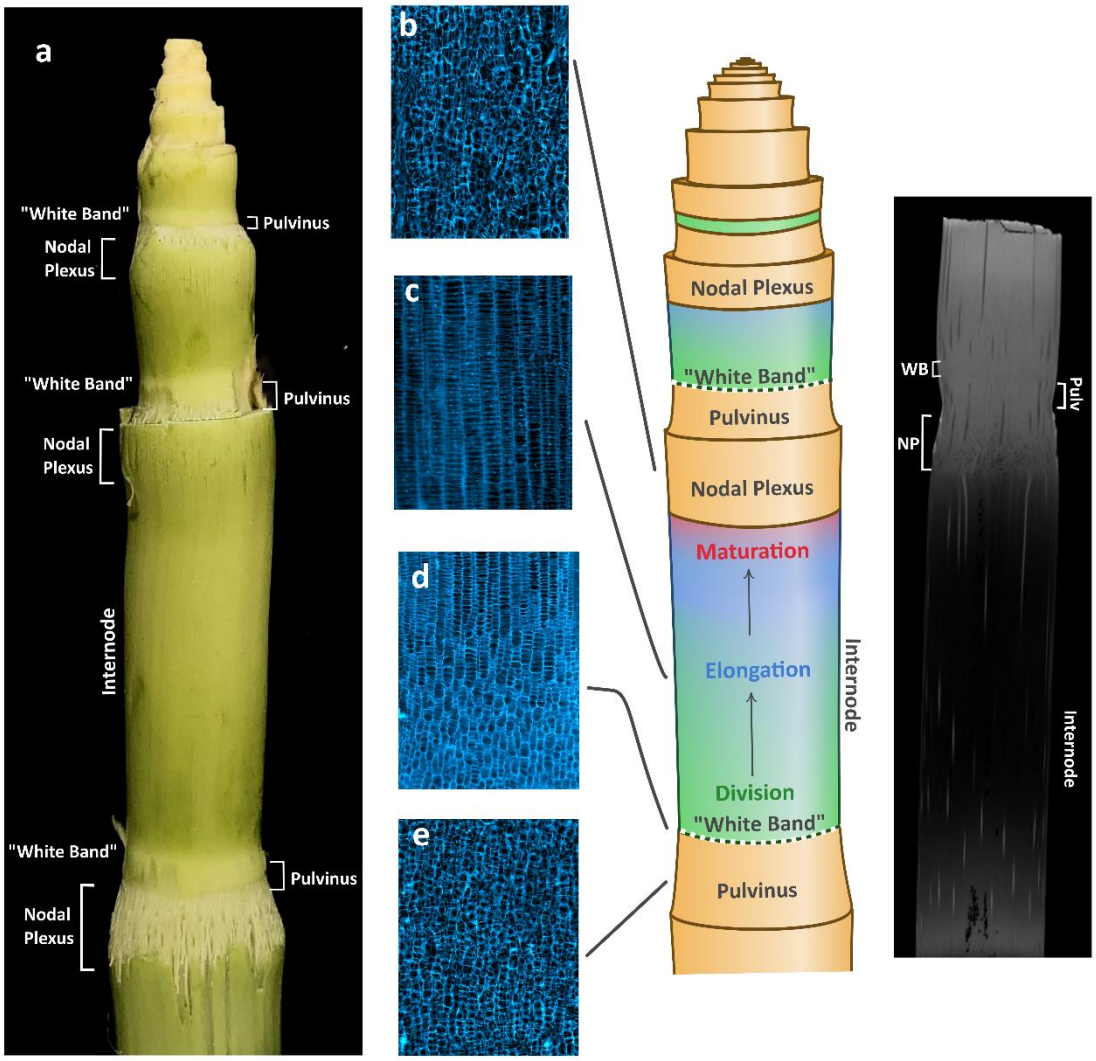
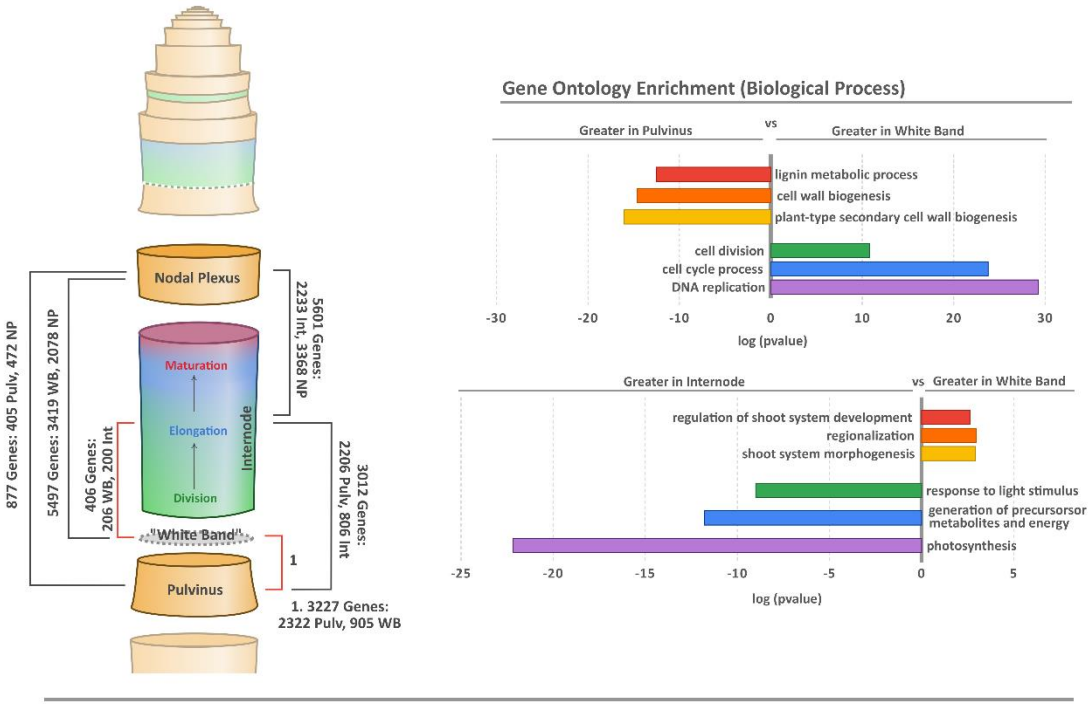
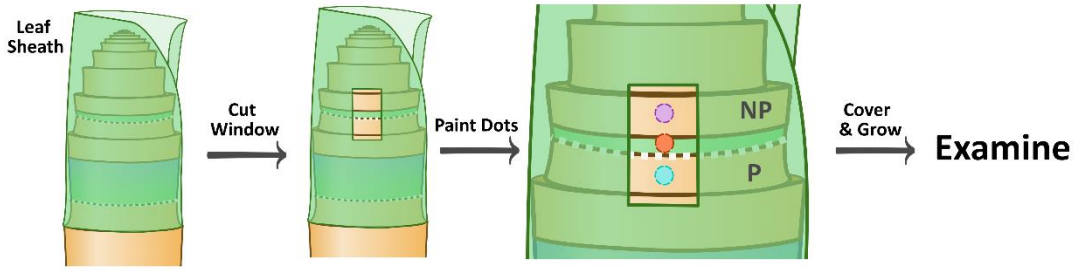


Figure 3.1 Different Types of Tissues within *Sorghum bicolor* Stems. a) Desheathed Sorghum stem showing stacked internodes as they develop and the tissues that are contained within each stem segment. b) Cellular morphology of the Nodal Plexus (NP) of Sorghum stems. Cells are randomly shaped with no clear cell file. c) Cellular morphology of the Internode. Cells are regular, neat, and have uniform cell files. d) Cellular morphology of the White Band, WB. Stark transition between the randomly and uniformly shaped cells surrounding the WB tissue. e) Cellular morphology of the Pulvinus. Similarly to the NP, cells in the pulvinus are randomly shaped with no defined cell file. f) Micro-CT of sorghum stems. The opaque section shows the WB clearly, with the Pulv and NP just below.

3.3.2. Differential Expression in Developing Stem Tissues

To better understand the transcriptional profile of growing internodes, we performed tissue specific RNAseq of the different tissues within an internode. Tissues were divided into the Nodal Plexus, the Pulvinus, the internode, and a small section taken through the visible white band. The greatest difference of 2x DE genes occurred between the White Band and the Nodal Plexus for a total of 5497 genes with 3419 greater in the White Band and 2078 greater in the Nodal Plexus. The fewest number of DE genes occurred between the White Band and the Internode tissue, which contained 406 genes total with 206 up in the White Band and 200 up in the Internode. Since the White Band tissue had shown some interesting characteristics, GO enrichment was done on genes differentially expressed from the surrounding tissues, the Pulvinus and the Internode. When compared to the Pulvinus, White Band tissue had much greater enrichment in cell division and cell cycle genes. Interestingly, the Pulvinus had strong enrichment in secondary cell wall biogenesis and lignin metabolism, a process usually contained within fully mature internodes. In the White Band and Internode comparison, genes with higher expression in the internode contained enrichment in photosynthesis and response to light stimulus. This is likely due to the White Band being pale and lacking chlorophyll. This also suggests that the tissue separation achieved from physical sectioning was precise. Greater in the White Band, there was strong enrichment in genes involved in shoot system development, morphogenesis, and regionalization. All three of these terms have to do with the establishment of boundary layers and the differentiation of cells into new organs (Figure 3.2, top).


Nodal Plexus
Maturation
Elongation
Division
Internode
White Band
Pulvinus
877 Genes: 405 Pulv, 472 NP
5497 Genes: 3419 WB, 2078 NP
406 Genes: 206 WB, 200 int
5601 Genes: 2233 Int, 3368 NP
3012 Genes: 2206 Pulv, 806 Int
1
1. 3227 Genes: 2322 Pulv, 905 WB
Gene Ontology Enrichment (Biological Process)
Greater in Pulvinus
vs
Greater in White Band
lignin metabolic process
cell wall biogenesis
plant-type secondary cell wall biogenesis
cell division
cell cycle process
DNA replication
-30
-20
-10
0
10
20
30
log (pvalue)
Greater in Internode
vs
Greater in White Band
regulation of shoot system development
regionalization
shoot system morphogenesis
response to light stimulus
generation of precursorsor metabolites and energy
photosynthesis
-25
-20
-15
-10
-5
0
5
log (pvalue)


Leaf Sheath
Cut Window
Paint Dots
NP
P
Cover & Grow
Examine

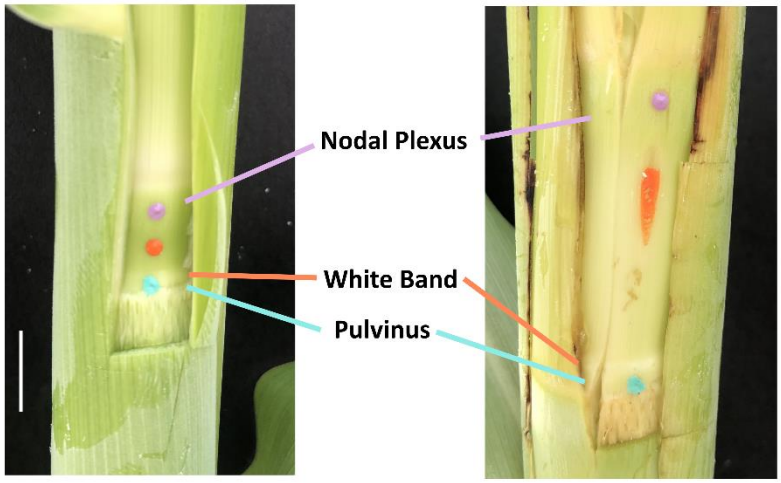

Nodal Plexus
White Band
Pulvinus

Figure 3.2 Differential Expression of Sorghum stem tissues reveals area of cell division Top left) Diagram of the sectioned stem tissue showing the number of genes DE in each comparison. Top right) Gene Ontology (GO) Enrichment showing enriched genes that are differentially expressed when compared to the WB. The top graph compares the Pulvinus to the WB. The bottom graph compares the WB to the Internode. Cell division and related genes are overrepresented in the WB tissue. Bottom diagram) Schematic of the experiment used to determine where the bulk of cell division happens in growing internodes. A window was cut into the Leaf Sheath of Int(P4) and paint dots were placed in the three places shown. The window was covered and allowed to grow before examination. Bottom) Paint dots how they were originally placed on Int(P4). After four days of growth, the NP dot and the Pulvinus dot remain the same, but the dot placed above the WB has moved and smeared, suggesting that cell division is happening above the WB and not below and is limited to the internodal tissue. Scale bar is 2cm.

3.3.3. Internode Tissue accumulates between stem nodes

Because the RNAseq data suggested cell division was occurring at the WB and not in the pulvinus, we next wanted to visually determine the area of the most cell division. During development, tissue just below the SAM that will become a new phytomer becomes visible below the SAM approximately every 3-4 days. Following formation and during the early phase of phytomer biogenesis (phytomer 1-3), the leaf blade-sheath tissue grows out from the newly formed stem node (citations). During this time, delocalized cell division occurs in the nascent stem tissue (Kebrom, McKinley, and Mullet 2017). Onset of internode tissue accumulation and internode elongation occurs between phytomer 3 and phytomer 4. To determine which stem tissue grows during this developmental phase (phy3 to phy4), a small window was cut into phytomer 3 and dots of paint were placed at the pulvinus, just above the white band, and at the nodal plexus. After 4 days of additional growth, the dots in the pulvinus and the nodal plexus remained circular, but the dot placed at the white band had been displaced into the developing internode tissue and had become smeared. Taken together, RNAseq and visual data

suggest that cell division and elongation is occurring in close association (just above) the white band of tissue located at the apical end of the pulvinus (Figure 3.2, bottom)

3.3.4. Tissue Specificity

We next wanted to know which genes had specific expression in a given tissue. The calculation Tau is used to represent the tissue specificity of a given gene in a dataset (Yanai et al. 2005). Data from individual tissues was compared within the same section to determine which genes were more specifically expressed in each tissue. GO enrichment was then performed on each tissue that had genes with a Tau>0.7. In the Nodal Plexus, there was strong enrichment in transport proteins and genes involved in localization. Internode tissue had DNA packaging, and cell differentiation. In the White Band section, many genes involved in DNA replication, microtubule-based movement, and cell cycle were specifically expressed. One particular gene that was specific to the White Band was an LBD protein, which are known to be involved in boundary layer formation. The pulvinus section had enrichment in hormone biosynthesis specifically brassinosteroids and gibberellins. These results suggest that tissues within the internode are specialized with highly divergent functions. Additionally, the White Band section had strong enrichment in cell division related genes, further indicating that cell division happens at the White Band (Fig 3.3 a,b).

After determining specificities and differences within tissues, we next wanted to determine similarities. To accomplish this, we performed a Principal Component Analysis (PCA) on the entire dataset to determine the variance across two principal components that contribute to ~44% of the total variation within each dataset. As

expected, the most mature phytomer has the least variability among itself (red cluster of points, Fig 3.3c), suggesting that as the internode ages, it becomes less specialized. However, none of the younger phytomer units grouped in tight clusters. In fact, groupings among data points in the PCA align more closely with specialized tissues within the phytomer as opposed to the entire phytomer unit. This is especially true for the NP and Pulv tissues (Fig 3.3d) and the WB and Int tissues (Fig 3.3e). WB data points are closer to the Apical Dome section (A) which contains the SAM and generally populate Quadrant II on the graph, suggesting that early WB tissues in particular are similar to the apex. Internode tissues have a wider range of distribution across the graph, suggesting that internode tissues themselves constitute a large amount of variability between growing internodes. Interestingly, NP and Pulvinus tissue cluster tightly together in Quadrants I and IV, with a clear progression rightward toward the mature internode cluster as the tissues age. This type of trend suggests that not only are the Pulvinus and NP tissues more similar to aged tissue, but that they are more similar to each other than they are to other tissues within the internode. Additionally, this suggests that even as early as Phytomer 6, the NP and Pulvinus have begun to differentiate themselves greatly from internode, apex, and WB tissues.

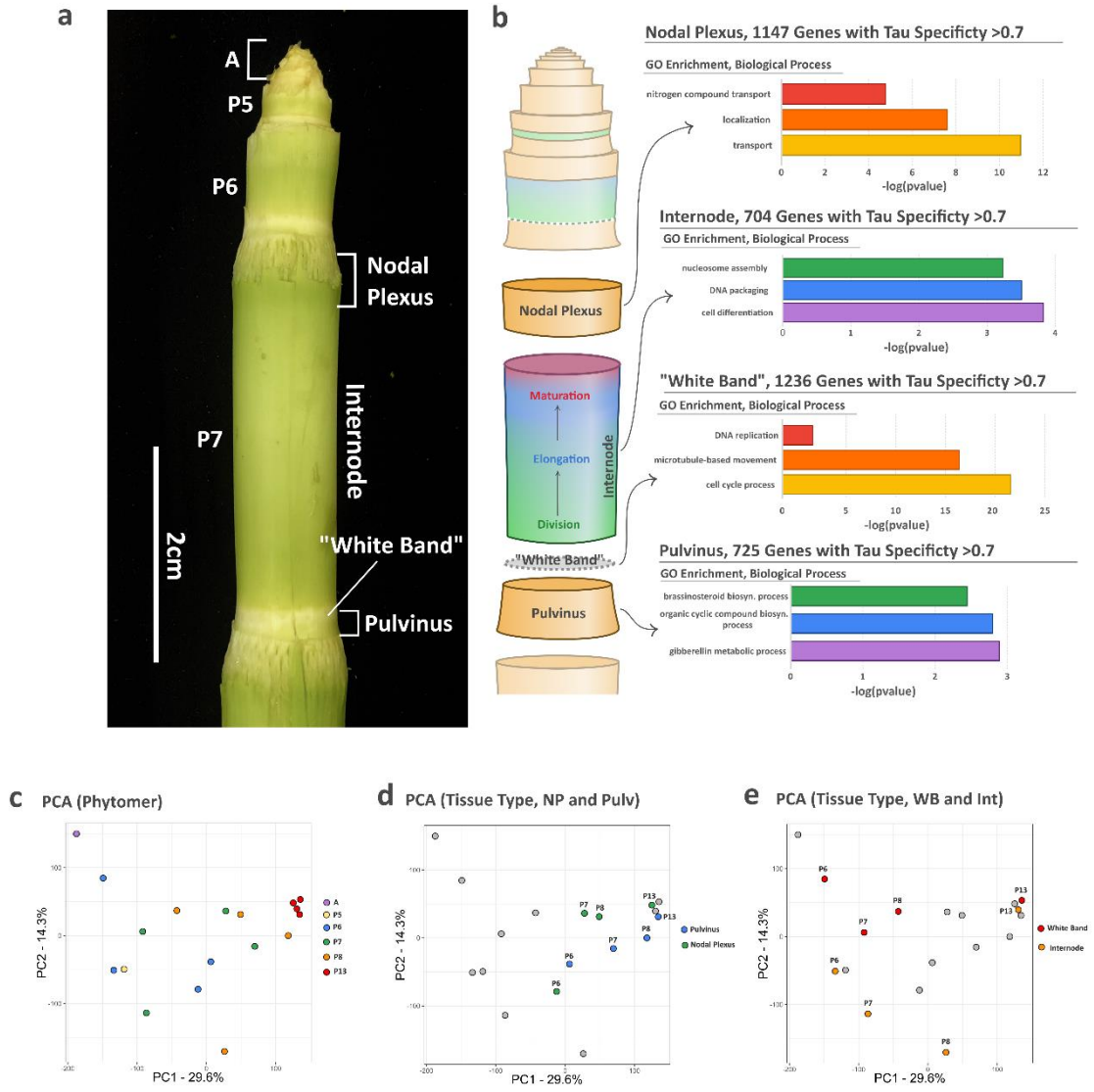


Figure 3.3 Tissue Specificity Analysis (Tau) reveals specialized functions of different stem tissues. a) Desheated Sorghum stem labeled with different tissues and phytomer numbers (P#). b) Diagram showing the tissues used and GO enrichment in Tissue Specificity with a cutoff of Tau>0.7 c) Principal Component Analysis (PCA) of all 47,000 genes in Sorghum when compared to other phytomer datasets within our experiment. Mature internodes cluster tightly together, suggesting little variation among tissues as the phytomer matures. d) PCA of the same data in (c) highlighted by the NP and Pulvinus (Pulv) tissues. NP and Pulv tissues cluster more closely together than other tissues and progress toward the mature phytomer cluster mentioned in (c) . e) PCA of the same data in (c) highlighted by the WB and Internode (Int) tissues. Young WB tissues cluster more closely together than other tissue types, and the WB(P6) is the closest in the

SAM section (A). X and Y axis are the two principal components and % represents the percentage of variation attributable to that component.

3.3.5. Tissue Specificity of Developing White Band Tissues

Because internodes develop over time as they transition from mostly dividing to mostly maturing, we next wanted to know how the White Band tissue also developed as internodes progress. To accomplish this, we compared the Tissue Specificity (Tau) of genes expressed specifically in early White Band tissues to that of a developing series of White Bands (WB(P6,7,8,13)) (Fig. 3.4). Mature WBs had the most specific genes with 3041 and a strong enrichment in secondary cell wall biogenesis. The second most genes specific to a WB tissue occurred in WB(P6) with 946. WB(P6) had enrichment in microtubule-based movement, cell cycle processes, and chromosome organization. Both cell cycle and chromosome organization genes are known to be involved in meristematic areas. Additionally, many genes with high specificity in younger WBs also have high expression that decreases as WB matures. For example, Cyclin-Dependent Kinase B2 (CDKB2, Sobic.007G207700), has high expression in the WB(P6) which decreases as the WB ages (Fig 3.4, left graph). CDKB2 is involved in the regulation of the G2/M transition of the cell cycle and is required for the organization of the SAM (Fabian et al. 2000; Andersen et al. 2008). The converse is also true: genes with high specificity in mature WBs also tend to have higher expression in mature WBs. Beta-1,4-xylosyltransferase IRX10 (Sobic.003G296400.1) is involved in secondary cell wall biogenesis (A.-M. Wu et al. 2009) and internode maturation has low expression in young WBs with increasing expression as WBs mature (Fig 3.4, right graph). These

findings help support the theory that cell division and secondary cell wall formation (maturation) are inversely proportional (Kebrom, McKinley, and Mullet 2017). These data also suggest that the WB remains active for a certain amount of time before lowering its activity to some basal level in mature internodes.

Figure 3.4 White Band developmental progression reveals declining cell division and increasing maturation a) Diagram showing the tissues compared using Tissue Specificity (Tau>0.7) and how many genes were specific to each WB. b) Gene Ontology (GO) Enrichment for games specific to young (WB(P6)) and old (WB(P13)) WB tissues. c) Example gene, CDKB2 (cyclin dependent kinase) showing developmental repression as the WB tissue ages. d) Example gene, IRX10/GUT1 (secondary cell wall) showing developmental activation as the WB tissue ages.

3.3.6. Disruption of the WB causes broad range of phenotypes

After determining the progression of WB development, and some special characteristics of other tissues within the phytomer, we next wanted to find mutants that contained poorly resolved WB tissues. Standard Yellow Milo (SYM), Dwarf Yellow Milo (DYM), and Double Dwarf Yellow Milo (DDYM), are three near isogenic grain sorghums from the Yellow Milo family that contain only one mutation between the progressive genotypes (Quinby 1974). These mutations occur in the dwarfing family of genes, Dw1 and Dw2. SYM and DYM differ only at the Dw1 locus which is involved in brassinosteroid signaling, internode growth, and cell proliferation (J. Hilley et al. 2016; K. Hirano et al. 2017; Yamaguchi et al. 2016). DYM and DDYM differ only at the Dw2 locus (J. L. Hilley et al. 2017). These genotypes were used previously to determine that Dw2 was involved in internode growth, cell proliferation, lipid signaling, and regulation of the endomembrane system (Oliver et al. 2021). Furthermore, Dw2 was implicated in ROS signaling as Dw2 mutants are particularly sensitive to treatment with ROS inducing chemicals (Chapter 2). Dw2 mutants also have a "White Band" phenotype as few internodes have a visible WB, implicating WB formation in healthy internode development and elongation (Fig 3.5a). his becomes more apparent as plants are left to dry, as the WB tissue remains clearly demarcated within DYM internodes, but does not appear in DDYM internodes (Fig3.5b, insets). Additionally, cell morphology differences between DYM and DDYM reported previously (Oliver 2020) match WB morphologies seen in Figure 3.1, suggesting that the separation between the Pulvinus, NP, and Internode remains poorly defined in dw2 plants. The WB may act as a definitive

boundary between tissues within the internode, and boundary layers are known to control organogenesis events such as phyllotaxy (Reinhardt et al. 2003). DYM and DDYM plants differ in their phyllotaxis (Fig 3.5c,d), with DYM plants showing clear alternate patterns and the majority of DDYM plants showing a decussate pattern.

To determine differences in gene expression in WB mutants, we compared the full wt genotype (SYM) to the WB mutants (DDYM) in an actively growing internode (WB(P7)) (Fig 3.5e,f,g). Since DDYM plants have poorly visible WBs, an average distance from the nodal plane to the top of the Pulvinus was taken in both genotypes with clear WBs. This allowed us to take a "WB Enriched" section in mutants with poorly resolved WBs. Enrichment in SYM of DE genes contained transcription related and gene silencing categories. However, enrichment in genes with greater expression in wb mutants (DDYM) contained secondary cell wall biogenesis, lignin biogenesis, and hydrogen peroxide catabolism (Fig 3.5e). Additionally, the putative boundary layer gene discovered with the Tau calculations has low expression across the internode and in the WB enriched tissue of the mutant (Fig 3.5f). Lignin biosynthetic pathway enzymes, such as CCoAMT1, also have very high expression in young internodes (Fig 3.5g).

We next wanted to test if maturation gene expression changes discovered in DE analysis led to practical increases in secondary cell wall characteristics in early internodes of the mutant. To test this, we stained young internodes that are usually defined by low levels of maturation for lignin. WB mutant plants had lignin diffuse throughout the pith of internode tissue, whereas wt only had strong lignification around the rind and within vascular bundles (Fig 3.5h,i). This difference led to an almost 4x

greater difference in total lignin fluorescence between samples (Fig 3.5j). Taken together, these data suggest that disruption of the WB transition from node to internode, possibly through the delocalized and/or low expression of the LBD gene, leads to early lignification/ maturation of internodes, which can arrest growth and shorten internode growth.

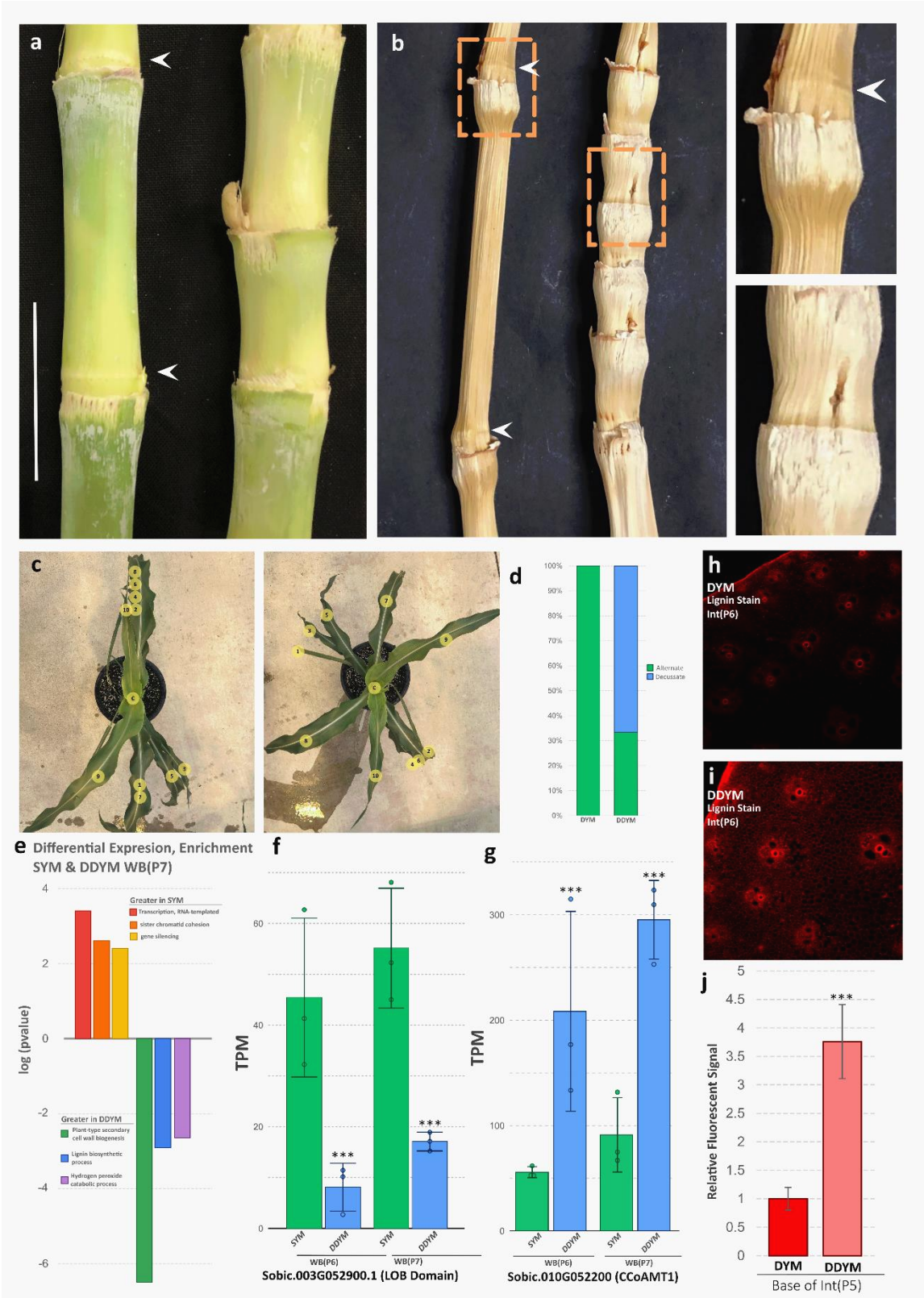

a
b
c
d
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Alternate
Decussate
DYM
DDYM
h
DYM
Lignin Stain
Int(P6)
i
DDYM
Lignin Stain
Int(P6)
e Differential Expresion, Enrichment
SYM & DDYM WB(P7)
Greater in SYM
Transcription, RNA-templated
sister chromatid cohesion
gene silencing
Greater in DDYM
Plant-type secondary cell wall biogenesis
Lignin biosynthetic process
Hydrogen peroxide catabolic process
log (pvalue)
f
TPM
***
SYM
DDYM
WB(P6)
WB(P7)
Sobic.003G052900.1 (LOB Domain)
g
TPM
***
Sobic.010G052200 (CCoAMT1)
j
Relative Fluorescent Signal
***
DYM
DDYM
Base of Int(P5)

Figure 3.5 Disruptions in the WB cause altered phyllotaxy, and short internodes with early maturation a) Two genotypes DYM and DDYM (left, right) that show a WB phenotype. Arrowheads show the WB tissue in mature DYM internodes that are less visible in DDYM internodes. b) Dried stem tissues of DYM and DDYM (left, right) internodes where the WB tissue has remained visible. Arrowheads mark the WB in dried DYM internodes that are less visible in dried DDYM internodes. Orange boxes are the insets to the right with DYM on the top and DDYM on the bottom. c,d) DDYM plants have decussate phyllotaxy. Circles mark the leaf number with C being the center of the whirl. More than 60% of DDYM plants show decussate phyllotaxy. 15 plants of each genotype were used. e) GO Enrichment of differentially expressed genes of a WB wt genotype (SYM) and the WB mutant genotype (DDYM) through the WB tissue. Wild type WBs have an increase in transcription, and gene silencing, whereas WB mutants have an increase in secondary cell wall biogenesis, lignin (maturation), and hydrogen peroxide catabolism. f) Example gene, the LOB domain boundary layer maker showing high expression in WB(P6,7) in the wtWB tissue, with very low expression in the mutant WB tissue. g) Example gene, CCoAMT1 a lignin biosynthetic gene, showing increased expression in WB(P6,7) suggesting early lignification and maturation in WB mutants. h,i,j) WB mutants lignify earlier in development as seen by staining of Int(P5) (h,i) for lignin, and by an overall increase in lignin stain fluorescence (j).

3.4. Discussion

Understanding plant architecture, development, and organogenesis is fundamental to the targeted plant design that is the future of plant science. In particular, stem growth and development is critical as it provides structural integrity, a highway for long distance transportation, and has large capacity for storing excess secondary metabolites, sugars, and specialty bioproducts (Mullet et al. 2014). The stems of bioenergy grasses, such as Sorghum, show promise in accomplishing many of these goals, and this work provides insight into the growth and development of nodes and internodes. As discussed below, the stems of Sorghum contain highly specialized tissues across growing phytomers that contribute to the complexity and uniqueness of monocot stems.

Cell morphologies help distinguish different tissue types, and here we show that the Sorghum stem has a number of different morphologies. First is the Pulvinus and the Nodal Plexus (NP), which both contain disorganized and random cellular shapes. Next, are the neat and uniform cell morphologies of the internode which contain organized cell files. Lastly, is the transition between the random and neat morphologies that occurs at the “White Band" that resides just above the Pulvinus. Cellular shape is known to be controlled by the cytoskeleton, and current growth models imply that random orientations of cytoskeletons at the base of internodes leads to the random morphologies documented there (Knöller et al. 2010). Cells of the internode have reorganized their skeleton to produce the uniform shapes seen in that tissue. Our data also supports this model. There is clear enrichment of cytoskeletal proteins at the WB tissue, which suggests that the reorganization of the cytoskeleton happens at the WB. However, our data also indicates that the bulk of cell division in later internodes is happening at the WB, which could suggest that this remobilization is a consequence of greater cell division.

Furthermore, we show a clear similarity between the NP and the Pulvinus that are not limited to cell shapes. Indeed, NP and Pulvinus tissue cluster together in PCA, mature earlier than internodes, and have relatively few DE genes compared to one another. Our data also clearly shows that the generation of “internodal” tissue happens later (around P(5,6)) in the growth of Sorghum stems. These two pieces of evidence suggest that the NP and Pulvinus form very early in development and that internode growth is a part of that developmental progression, and is facilitated in part by the

maturation of the WB. Delocalized cell division can generate NP and Pulvinus tissues in the apex where the early WB and the Disc of Insertion (DOI) formulate the outer boundaries, along with other known and unknown boundary layer markers. As the stem continues to grow, the WB matures and activates organized cell division at the internode which gives rise to the uniform cell morphology.

The presence of the LBD transcription factor specific to the WB suggest that the WB has true boundary layer characteristics. The closest maize homolog of Sobic.003G052900 is the gene Ramosa 2 (Ra2). Ra2 is expressed in axillary meristems and mutants have altered spikelet numbers and ear formation, but interestingly no difference in height between genotypes was reported. In situ analysis of the axillary inflorescence meristems places expression at the base of multiple axillary meristems in Sorghum and other grasses (Bortiri et al. 2006). Our in situ analysis is still being completed but should show an accumulation of transcript in the WB tissue of growing internodes. Overall, these data suggest that SbRa2 plays a role in the establishment and maturation of the WB tissue that divides the Pulvinus from the Internode. Additionally, localized expression would provide evidence for the WB being a true boundary layer and not simply a transition zone from one tissue to the next.

We have also provided evidence that disruptions in the WB leads to short internodes that mature earlier. Additionally, WB disruption can cause conventional boundary layer phenotypes like altered phyllotaxy, also suggesting the WB might be a true boundary layer. With no doubt there are other WB mutants, but the one discussed here is mutated at the Dw2 locus. Since the AGCVIII kinase Dw2 has been implicated in

cell proliferation, cytoskeletal remobilization, lipid signaling, and endomembrane regulation, this implies that WB formation utilizes some or all of these processes as well (Oliver et al. 2021). The putative substrate of Dw2 kinase activity is PLD Delta, which is involved in Phosphatidic Acid signaling and is activated by hydrogen peroxide (H_2O_2). PLD Delta knockouts are highly sensitive to H_2O_2 and ROS, and are more susceptible to H_2O_2-induced cell death. (W. Zhang et al. 2003). In maize, Abphyl2, a glutaredoxin involved in ROS detoxification, is known to regulate meristem size and phyllotaxy (Yang et al. 2015). Likewise, Dw2 mutants are sensitive to ROS and show highly proliferative nodal roots and severely stunted internodes when ROS are induced (Chapter 2). This Dw2 mutant sensitivity to ROS could come from the mutant's inability to activate the PLD Delta signaling pathway through phosphorylation to detoxify ROS accumulation. These data suggest that ROS signaling in particular has a large impact on the maturation of the WB, and therefore the development of true internodal tissue. Hypothetically, this makes evolutionary sense, as the WB is involved in internode growth. If a growing internode is exposed to light, ROS can accumulate through increased photosynthesis, disrupts the WB, and internode growth can be shut down as the plant does not need to elongate quickly to outcompete neighbors.

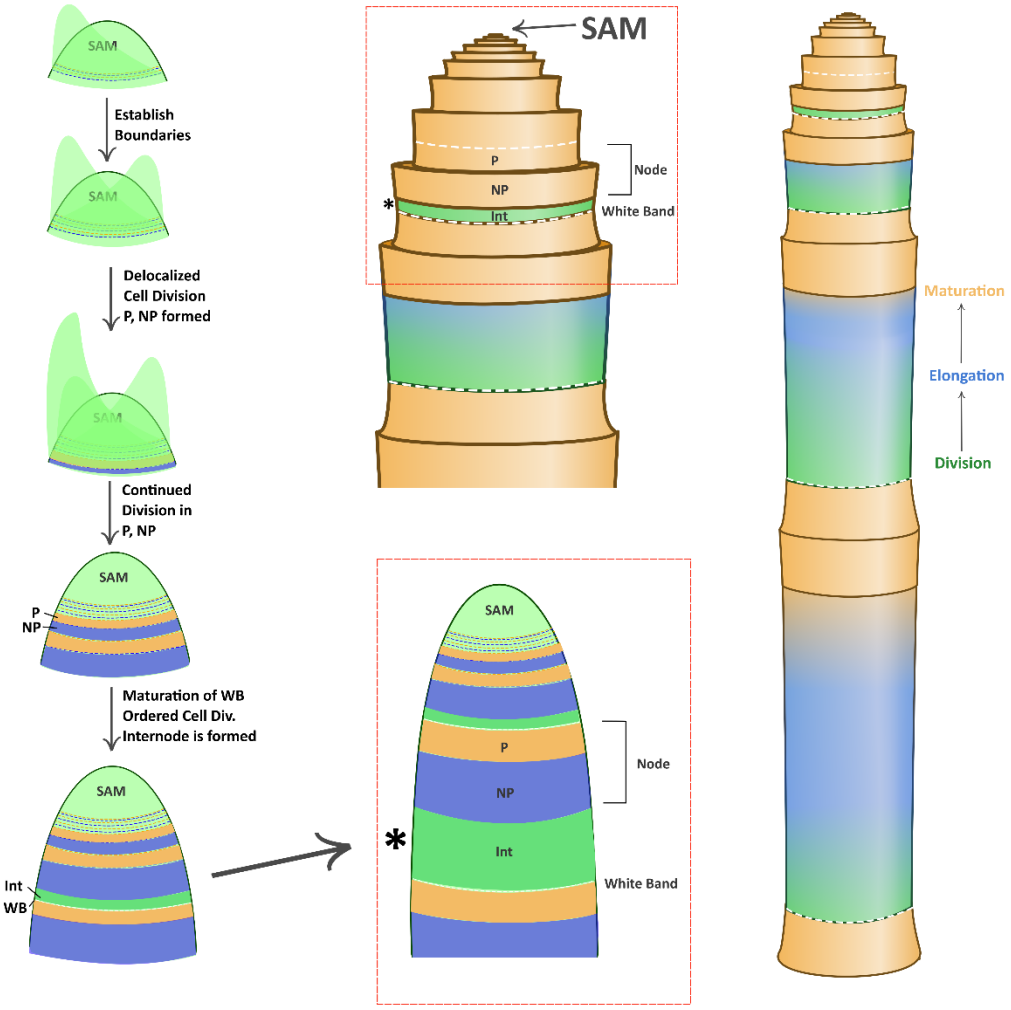
The Pulvinus is another tissue of interest within the monocot stem. Traditionally, pulvinuses have been used to study gravitropism, as the tissue swells in response to plants being turned on their sides. This swelling allows for the already grown internodes to achieve curvature to bend upwards away from gravity (Clore 2013). Many scientists have called the Pulvinus the Intercalary Meristem (IM) throughout the years. Indeed, this

is the area of the stem in which tillers and nodal roots emerge, so meristematic activity must be present to some degree (Kebrom, McKinley, and Mullet 2017). However, our data indicates that this tissue is not heavily involved in internodal growth, as very little cell division occurs there in even young internodes. Instead, our data suggests that this tissue remains transcriptionally flexible, potentially to respond to different conditions sensed within the internode from hormones or other signals. Wholly, these data suggest that the IM may be subdivided into different meristems (the Pulvinus, Nodal Root Meristems, Tiller Meristems, and the WB), similar to the Central Zone, Peripheral Zone, and Rib Meristems that make up the SAM (McKim 2019). It is also possible that the Pulvinus ground tissue itself does not possess meristematic activity after it is initially formed; the broader Pulvinus simply contains dividing cells that are localized to the Tiller and Nodal Root meristems. In any case, subdivisions of the meristems allow for more specialized function in plant organogenesis and architecture. These specializations provide an adaptive advantage to grasses that contain an IM, as individual phytomers can respond to any given environmental, biotic, or abiotic stresses with little influence on the whole plant.

The identification of the WB tissue, and the further characterization of the surrounding tissues, presents interesting possibilities for stem development in monocots. For example, current convention places the stem unit of a monocot phytomer from Nodal Plane to Nodal Plane (Knöller et al. 2010). This has been the historical way to define nodes and internodes as they look at first glance to be discriminate segments of plant stem. While this thinking may be correct, this model would place the WB directly

in between the NP and Pulvinus of the same phytomer. If the WB is a true boundary layer, this would imply that this phytomer unit is discontiguous; the cell division that happens at the WB "splits" the NP from the Pulvinus of the same phytomer as internodal growth beings. This would place the NP and Pulvinus on opposite sides of the same phytomer. As such, we have dubbed this the "Split Node Model" of internodal growth.

There is another, equally plausible model that our data supports. It is possible that the NP and Pulvinus remain intact as adjoining tissues and are bordered on either side by the flanking WBs. In this model, the NP and Pulvinus are the oldest portion of a given phytomer and division that occurs at the WB happens below the NP. This would push the adjoining NP-Pulvinus upward as cell division occurs at the base WB. This model implies that the phytomer is not from Nodal Plane to Nodal Plane, but is instead from White Band to White Band with no WB boundary layer separating the tissues. As such, this model has been dubbed the "Contiguous Phytomer Model" of internodal growth. Throughout our analysis, we have tried to find evidence that supports either the "Split Node" or "Contiguous Phytomer" models. However, we have not been able to make a clear distinction between the two; some of our evidence supports one, some the other, but most is ambiguous. For the sake of brevity and convention, all analyses here have used the "Split Node" model as it is the closest to the current accepted model. We strongly encourage our colleagues and peers to use our data and their own to continue refining our collective knowledge on internodal growth in grasses.


SAM
Establish Boundaries
SAM
Delocalized Cell Division P, NP formed
SAM
Continued Division in P, NP
SAM
P
NP
Maturation of WB Ordered Cell Div. Internode is formed
SAM
Int
WB
SAM
P
NP
Int
Node
White Band
SAM
P
NP
Int
Node
White Band
Maturation
Elongation
Division

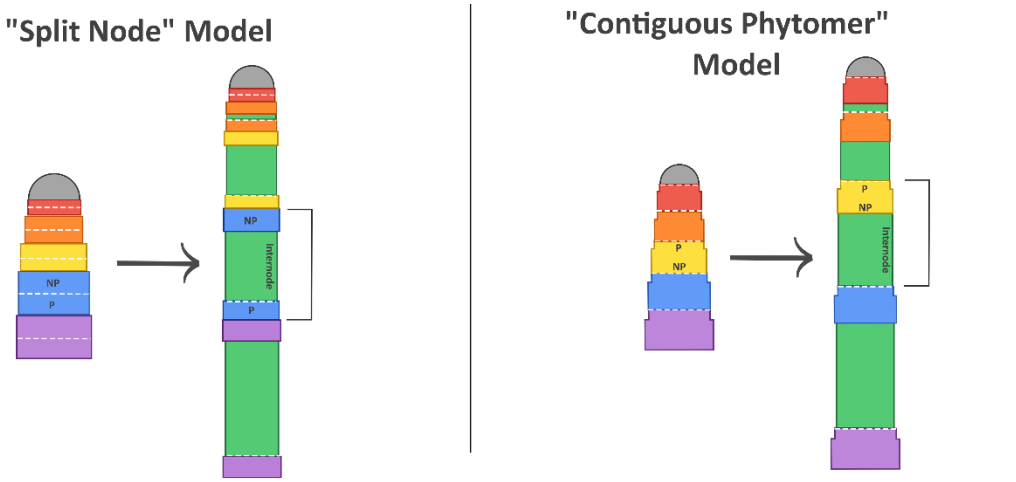

"Split Node" Model
NP
P
NP
Internode
P
"Contiguous Phytomer" Model
P
NP
P
NP
Internode

Figure 3.6 Internodal Growth Model a) Progression of Nodal Plexus (NP) and Pulvinus (P) formation in the early apex of Sorghum. Boundaries are established, delocalized cell division creates the P and the NP until the White Band (WB) matures to activate organized cell division which creates the internode. b) Further grown apex which has fully mature WB tissues and true internodal cells. Red box indicates the similarity between diagrams with the (*) representing the same internode tissue. c) Diagram of stem of *Sorghum bicolor* showing the different zones in the internode tissue, along with the P and NP. d) "Split Node" Model of internodal growth. Stem phytomer segments are from Nodal Plane to Nodal Plane. As the WB matures, the NP and P of the same phytomer are separated (NP moving up, P remaining relatively stationary) by cell division occurring at the WB. Tillers and nodal roots are at the lower portion of the phytomer. e) "Contiguous Phytomer" Model of internodal growth. Stem phytomer segments are from WB to WB. As the WB matures, the NP and P remain joined and are pushed upward as cell division occurs at the base WB. Tillers and nodal roots are at the upper portion of the phytomer.

In summation, we have shown that the stems of *Sorghum bicolor* are made up of at least four independent tissue types: the Pulvinus (hormone signaling, nodal root, tiller formation), the Nodal Plexus (transport and vein anastomosis from the leaf sheath), the Internode (secondary growth, bulk stem tissue), and the WB (area of cell division, transition/boundary from Pulvinus to Internode). Additionally, we have shown that disruptions in the WB cause altered phyllotaxy, and short internodes with early maturation markers and is possibly regulated by ROS signaling. We have proposed two competing models, the "Split Node" and the "Contiguous Phytomer", for internodal growth in *Sorghum bicolor*, which likely remain conserved in all grasses. This work begins illuminating many of the large standing questions in the field of monocot stem biology and poses many interesting questions yet to be answered.

3.5. Materials and Methods

3.5.1. Plant Material, Growth Conditions, Staining, and Paint Tracking

All genotypes were grown in long day (14h/10h day/night) greenhouses during 2018-2019. Plants were grown in five-gallon pots and fertilized with osmocote. Genotypes included in this paper: SYM, DYM, DDYM (RNAseq, Basic Fuchsin lignin staining, Calcofluor White staining, plant images), and Wray (MicroCT). Cell staining of internodes was performed by using Calcofluor White (1:20 in PBS 5 minutes, 2x wash in water) and imaged on a Zeiss Axioplan 2 microscope. Lignin staining of internodes was performed by using Basic Fuchsin (1:20 in PBS for 10 minutes, 2x wash in water) and imaged on an Olympus FV1000 Laser Confocal microscope. Staining images were processed using NIH's ImageJ. Paint tracking was accomplished by cutting a small window in the leaf sheath of growing internodes and applying small dots of paint. Painted window was then covered with foil and allowed to grow for 4 days before imaging. All images were taken with Apple's iPhone 8 and remain unprocessed other than cropping.

3.5.2. MicroCT

MicroCT imaging was performed as previously described in (Tsuda et al. 2017). In brief, stem samples were harvested from ~60 day old Wray plants and fixed in FAA (50% ethanol, 5% glacial acetic acid, 10% formaldehyde) for 1 week. After fixing, samples were stored in 70% ethanol in the fridge. Before imaging, samples were soaked in a 0.3% phosphotungstic acid in 70% ethanol solution. Texas A&M Cardiovascular Pathology Lab performed microCT on provided samples. The reconstruction was done by soaking the specimen in Oxilan (iodine-derivative) (50% Oxilan, 50% DI water) for 7

days, not scanned in contrast solution, as it produced the best contrast. Images were then reconstructed using the 3d Slicer program by North Star Imaging Inc. provided by the TAMU Cardiovascular Pathology Lab.

3.5.3. Sample Collection and RNA extraction

Plants were grown as previously described. For tissue collection, plants were cut down, leaves/leaf sheaths stripped, and a clean razor blade was used to section stem tissue into the four sections: Nodal Plexus, Internode, White Band, and Pulvinus. This was done for three genotypes, SYM, DYM, and DDYM, across four phytomers (P6,7,8, 13). In addition, young stem sections that were too small to divide into individual tissues (apex, A, and P5) were taken as bulk samples. Three replicates of each genotype were collected. Tissues were placed into Whirl Pack (Sigma WPB01018WA) bags and submerged in liquid nitrogen. Tissues were then manually processed by liquid nitrogen cooled mortar and pestle, and ground into a fine powder. Tissue sections were frozen -80 until processed for RNA extraction. For RNA extraction, Zymo Direct-zol RNA extraction (Zymogen R2051) kits were used with no modifications to the protocol. RNA was stored in -80 freezers until sent to the Joint Genome Institute (JGI) for sequencing.

3.5.4. RNAseq, Differential Expression, and PCA

The 151bp reads were aligned to the *Sorghum bicolor* V3.1 genome using the HISAT2 aligner (D. Kim, Langmead, and Salzberg 2015; McCormick et al. 2018). Expression was quantified using the StringTie version 1.3 software (Pertea et al. 2015). Analysis of gene expression was performed on TPM normalized data. The prepDE.py script was used to convert nucleotide coverage data generated by StringTie into reads

that could be used by differential expression statistical packages that use conventional raw reads. Differential expression and the Benjamini-Hochberg adjusted p-value were calculated using the EdgeR package with an FDR cutoff of 0.05. PCA was performed by using base R functions of the entire dataset of Sorghum genes from the SYM RNAseq. Genes with 0 TPM across all conditions were removed from the PCA before calculated.

4. CONCLUSIONS

4.1. Major Conclusions from the characterization of Dw2

Plant specific ACG VIII kinases regulate growth in response to many different conditions, and Dw2 is one of 21 kinases in this family encoded by the sorghum genome. While many of these kinase family members are known to control IAA transport by phosphorylating PIN proteins, we found no evidence of Dw2 differentially phosphorylating auxin transporters. This suggests that the main effect of Dw2 on internode growth is through some other mechanism than IAA transport through the tissues. Mutation of Dw2 was shown to cause downregulation of cell proliferation, cell, and vascular bundle morphology, changes in protein phosphorylation, inhibition of endomembrane activity, and altered accumulation of cell wall polysaccharides. Highlighted below are the major conclusions derived from my dissertation work regarding Dw2 signaling and internode growth.

When Dw2 is mutated, the internode length is shortened. Internodes can be shorter due to shorter cells or fewer cells, and Dw2 was shown to influence cell number and not maximum cell length. Furthermore, the cell proliferation controlled by Dw2 was shown to only occur in older internodes (such as Int(P5,6)). This suggests that Dw2 mediated cell proliferation is activated as the internode develops. Since the closest *Arabidopsis* homolog of Dw2 (KIPK) has only a minor root phenotype, these findings indicate a specialized role for Dw2 in grasses. Mutant Dw2 plants were shown to have altered cell morphologies in the internode, along with malformed vascular bundles.

Proteomic and phosphoproteomic experiments were done to compare wild type and mutant Dw2 internodes. These experiments provided early essential information on connecting the growth of the internode to the endomembrane system. Additionally, this database provides one of the first instances of phosphoproteomics in grasses, especially in the stem. Findings here can be used by our group or others to further understand the intricacies of stem monocot regulation by phosphorylation and provides a foundation from which many more scientific endeavors can be launched.

Many additional phenotypes brought upon by Dw2 were also described in this dissertation. One in particular is the putative regulation of the endomembrane system by Dw2 signaling. Here, we show that mutant Dw2 plants have wavy/tangled root hair morphology, irregular cell shapes and vascular bundles in fully elongated internodes, inhibition of endocytosis/endomembrane activity, and modified localization of two endomembrane trafficked polysaccharides, MLG and Heteroxylan. All of these phenotypes are known to be controlled by the endomembrane/lipid signaling system to varying degrees. Furthermore, the discovery of PLD Delta as the putative substrate of Dw2's kinase activity also fits these observations. While not fully confirmed, the Future Directions chapter of this dissertation provides a series of experiments that could be done to provide direct evidence for Dw2's phosphorylation of PLD Delta. Dw2 mutant plants are also sensitive to ROS, which implicates photosynthesis and ROS detoxification in the elongation of internodes.

Another major finding of this work is that large phenotypes (like height differences) usually brought upon by the alteration of physiological sources and sinks

can be regulated by intracellular sources and sinks, namely the endomembrane system. Dw2 wild type and mutant plants differ in biomass accumulation by almost 3 fold due to their differences in internode length. Since this work showed that Dw2 signaling was controlling the endomembrane system, this implies that the endomembrane system can be used to increase biomass accumulation and coordinate growth. In essence, this work connects macroscopic sources and sinks to the microscopic source and sink, and suggests that the transport between them is limiting in C4 grasses like *Sorghum*. The applications of this are further explained in the Future Directions section of this dissertation.

4.2. Major Conclusions from Tissue Identification

Organogenesis is critical for understanding how plants grow and respond to their environment. Using the knowledge accumulated from centuries of observation and research, careful dissection and characterization of growing monocot tissues have been discovered. One tissue whose growth is less understood is the stem of monocots, which is important for height, biomass accumulation, structural integrity, and long distance transport in monocot plants. This dissertation characterized the stem tissue with transcriptomic and morphological descriptions which further our knowledge of grass stem growth and development. Highlighted below are some of the major conclusions from this work.

Cell morphologies are important for distinguishing different tissue types, and the *Sorghum* stem has many different cell morphologies. The Pulvinus (Pulv) and Nodal Plexus (NP) both contain disorganized and random cell shapes while the internode has

neat uniform cells. The transition between the Pulvinus and the Internode (Int) occurs at the “White Band” (WB) that resides just above the Pulvinus, and the morphologies also transition from the random to the orderly at the White Band.

Transcriptomics of the different tissues (NP, Pulvinus, WB, Int) within the *Sorghum* stem were done to further differentiate the tissues that reside there. This is the first instance of the *Sorghum* stem being divided this way for further analysis and is, to the best of my knowledge, the first time any grass stem has been subdivided into these specific tissues. The information found through this was informative and useful to the entire field of grass biology. This experiment provided evidence that the cell division is occurring just above the WB of the stem, as tissue accumulates between the NP and Pulv. This is a clear distinction of the Intercalary Meristem (IM) known to exist in grasses and provides its exact location. Furthermore, the WB has been suggested to be a putative boundary layer that separates the Node from the Internode (specifically the Pulv from the Int). Additionally, this experiment provided evidence that each subtissue within the *Sorghum* stem is highly specialized with unique functions.

The specific expression of a boundary layer marker gene Ramosa 2 and the different cell morphologies that exist there, suggest that the WB has true boundary layer characteristics. This was further supported by showing a “White Band mutant” that has a poorly resolved WB tissue that results in early maturation of the internode and arrested growth. The WB mutant is the same genotype used to determine the function of Dw2 previously discussed in this dissertation. The identification of *dw2* plants as WB mutants suggests that the boundary between the Node and the Internode requires coordination

between ROS, endomembrane system, and cytoskeletal functions and is, in part, facilitated by Dw2 signaling. However, the existence of the WB as a boundary layer has not been confirmed. The future directions section of this dissertation discusses potential experiments to be done that will provide additional evidence for the WB as a true boundary layer.

Taken together, the information provided in Chapter 3 of this dissertation allowed for the proposal of new models of internode development and growth. In early development, boundaries are established, and delocalized cell division occurs. This causes the formation of the Pulvinus and the Nodal Plexus, both of which were shown to have irregular cell morphologies. Next, the WB is established, which activates organized cell division in the intercalary meristem, which causes the neat and uniform cell morphologies observed there. Transcriptomic evidence suggests that this transition from random to uniform cell morphologies is influenced by cytoskeletal dynamics. Preliminary evidence also suggests hormone signaling plays a key role in the development of different stem tissues. All of this information was incorporated into two different models of internodal growth. The first suggests that the formation of the WB causes cell division, which splits the two pieces of the node (the Pulvinus and the Nodal Plexus) apart from one another. This model is dubbed the “Split Node” model and places the NP and Pulv on opposite ends of the same phytomer. The second suggests that the formation of the WB occurs at the base of the internode, and the cell division there pushes the NP and Pulv upwards. This model is dubbed the “Contiguous Phytomer” model as it places the NP and Pulv directly next to one another on the same phytomer

(refer to figures in chapters for more information). The future directions of this dissertation outline experiments that can be performed to distinguish between the two models.

This dissertation provides key knowledge in the understanding of the growth and development of the stems of *Sorghum bicolor*, which is likely conserved in all grasses. By utilizing the findings here, new and exciting avenues of developmental biology, monocot-dicot evolutionary divergence, and plant design can be ventured.

5. FUTURE DIRECTIONS

5.1. Overview

Grasses are critical to the health and economy of the global population. They are responsible for feeding and fueling the next generation and have been manipulated for centuries to accomplish those goals. The C4 grass *Sorghum bicolor* was initially developed as a food and forage crop and more recently genotypes have been used to create bioenergy sorghum hybrids. Most notably is the design of Sorghum as a dedicated bioenergy crop to combat greenhouse gas emissions through the conversion of biomass into bioenergy. Sorghum is particularly ideal for this role due to its relatively small and tractable genome, diverse germplasm, high water and nitrogen use efficiencies, and its ability to thrive on marginal land (Mullet et al. 2014). Additionally, sorghum plants reach several meters in height and accumulate over 80% of the total biomass within its stem (Olson et al. 2012). Continued design is crucial for the deployment of bioenergy *Sorghum* into wide scale use, and thus understanding the stem biology is vital for future design efforts. As discussed in this dissertation, some knowledge gaps within grass stem biology have been elucidated, such as the role of Dw2 within internode elongation, and subdivision of the stem organ into discriminate tissues, but much is left to discover. Highlighted below are further experiments, directions, and long-standing goals of elucidating mechanisms of stem development and strategic design of *Sorghum* as a dedicated bioenergy grass.

5.2. Determining Dw2 signaling pathway

Internode growth is one of the defining features of grass stem biology, and the plant height that is critical to biomass accumulation is a function of internode number and length (Mullet et al. 2014). Internode length is controlled by the dwarfing genes, and of the current three that have been identified, only two had molecular functions associated with them (Dw1, brassinosteroids, Dw3, auxin pump) (J. Hilley et al. 2016; Yamaguchi et al. 2016; K. Hirano et al. 2017; Multani et al. 2003). Dw2 was identified as an AGCVIII Kinase, but no detailed knowledge existed of its physiological and molecular functions (J. L. Hilley et al. 2017). As such, the molecular mechanisms behind Dw2 were explored in Chapter 2 of this thesis. In brief, mutant Dw2 plants had lower cell proliferation in the Intercalary Meristem of growing internodes as well as altered root hair and vascular bundle morphology. Phosphoproteomic evidence suggested that Dw2 signaling was influencing the lipid signaling and endomembrane pathways and this was further supported through reduced uptake of the endocytic tracking dye FM4-64 and altered deposition of polysaccharides trafficked through the endomembrane system in mutant plants (Oliver et al. 2021). While these findings are important for deciphering the molecular pathways required for internode growth, there is still much work to do to define the exact role of Dw2 during Sorghum development.

5.2.1. Confirmation of PLD Delta phosphorylation by Dw2

The direct substrates of Dw2 kinase activity were not definitively discovered, but there are three pieces of evidence that strongly support the main target substrate to be Phospholipase D Delta: 1) Six replicates were used in the phosphoproteomic experiment

when comparing wild type and mutant Dw2 plants; all five of six wt plants had phosphorylation of PLD delta at S684 whereas this phosphorylation event was not detected in any replicates of the mutant, 2) treatment of growing internodes with a PLD inhibitor, n-butanol, caused wt internodes to phenocopy mutant internodes, and 3) Dw2 mutant plants were highly susceptible to reactive oxygen species, which PLD delta activity is known to detoxify (W. Zhang et al. 2003). This provides preliminary evidence to determine Dw2's effect on PLD delta activity through phosphorylation.

Phospholipases are responsible for cleaving head groups off of phospholipids in cellular membranes, generating phosphatidic acid (PA) and the free head group (Pappan et al. 1998). PA is widely known as a signaling molecule that controls membrane fusion, endomembrane activity, and stress responses (Pleskot et al. 2013; Xing et al. 2021). Because PLD delta is the suspected substrate of Dw2 kinase activity, and this hypothesis is supported by the rest of the molecular evidence, direct phosphorylation of PLD delta by Dw2 should be verified more directly. The traditional biochemical method for achieving this goal would be to purify Dw2 and PLD delta and run one of the many commercially available kinase assays that detect phosphorylation. However, this is complicated by two important factors: PLD delta is tightly associated with the plasma membrane (C. Wang and Wang 2001), and AGCVIII kinases (of which Dw2 is a family member) often have to be activated by phosphorylation from other kinases, particularly Phosphoinositol Dependent Kinase 1 (PDK1) (Rademacher and Offringa 2012).

Membrane associated proteins are often biochemically purified and retain catalytic activity, and PLD delta has been previously purified (C. Wang and Wang

2001). In addition to measuring activity, PLD delta was discovered to be dose dependently activated by oleate, a property not associated with other PLD family members. The effects of site directed mutagenesis were also examined. Mutations in the oleate binding site completely diminished the catalytic activity, whereas mutations in another regulatory site for phosphatidylinositol 4,5 bisphosphate, PIP2, had a weaker effect on activity. When the Arabidopsis PLD delta is compared to the *sb*PLD delta, the phosphorylated Serine residue believed to be the site of Dw2 phosphorylation does not exist. S684 that is the putative substrate of Dw2 resides just after a PPSNGS* insertion that is not present in the Arabidopsis homolog (Fig 5.1). Knowing that Arabidopsis Dw2 homolog (KIPK) knockouts have only a minor root phenotype and no observable shoot phenotypes (T. V. Humphrey et al. 2015), the existence of this different motif in *sb*PLD delta could be the reason a more dramatic phenotype is seen in *Sorghum* versus Arabidopsis; Sorghum evolved a new motif that can be subjected to phosphorylative regulation that is important to the growth of grass stems.

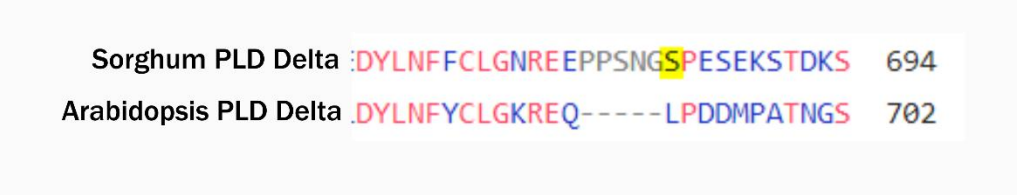


Figure 5.1 Alignment of Sorghum PLD Delta and Arabidopsis PLD Delta. Putative Dw2 target Serine is highlighted in yellow.

While purifying the membrane bound PLD delta has been shown to be relatively straightforward, purifying a catalytically active AGCVIII kinase is more nuanced. As mentioned previously, PDK1 is known as a "master regulator" of AGC kinases, as most

family members need to be phosphorylated by PDK1 in order to become active (Mora et al. 2004). This provides a complication to traditional bacterial purification procedures for Dw2. Arabidopsis KIPK has been shown to autophosphorylate, but neither the location of the phosphorylated residue nor the catalytic activity was measured (Zegzouti et al. 2006)**.** To circumvent this problem, active kinases can be purified using other methods, including expression and purification from insect cells and wheat germ (Wall et al. 2003; Sonkoly, Bardóczy, and Mészáros 2011). However, because the substrate of Dw2 is currently unknown and only hypothesized to be PLD delta, testing catalytic activity is challenging. If a negative kinase result is achieved through incubation of PLD delta and Dw2 (which would suggest no phosphorylation), it is difficult, if not impossible, to determine if that result came from no phosphorylation event or if Dw2 was simply purified in an inactive form.

5.2.2. Analog Sensitive Kinases to determine substrate targets of Dw2

For the reasons mentioned above, finding kinase substrate targets is incredibly difficult. However, there are newer methods for determining kinase substrates in the entire "kinome". One such method is through the use of so-called "Analog Sensitive" (AS) kinases. AS kinase technology uses a chemical-genetic approach to find kinase substrates of any engineered kinase (Lopez, Kliegman, and Shokat 2014). The premise is simple: a large hydrophobic "gatekeeper" residue in the ATP binding pocket of the target kinases is mutated to a small amino acid, which increases the size of the pocket. The newly enlarged pocket allows for only the mutated kinases to accept bulky ATP analogs such as N6-benzyladenosine-5'O-triphosphate (6-Bn-ATP). Additionally, these

AS kinases can also be specifically inhibited by bulky analogs of conventional kinase inhibitors (Shogren-Knaak, Alaimo, and Shokat 2001). AS kinases have also been shown to utilize γ-S varieties of the bulky ATP analogs, which leave the substrate with a thiophosphorylated residue that can be detected through thiophosphate ester-specific antibodies or captured an analyzed via HPLC-MS/MS (Allen et al. 2007; Carlson and White 2012). These innovations allow for genetically coded kinases that are specific to ATP analogs to find substrates of the target kinase. Such methods have been employed *in vivo* in many species, including plants (Harashima et al. 2016).

Using AS kinases for Dw2 substrate identification is appealing for two reasons: 1) it avoids the need to purify an active kinase that has no way to measure activity 2) the absence of the target phosphorylation sequence in the Arabidopsis PLD suggests that Dw2 and the Arabidopsis homolog have other substrates besides PLD Delta. Dw2 is amenable to this technology as well; the gatekeeper residue is M501 with a conditional mutation that further increases pocket size available at S566. If one or both of these residues are mutated (such as M501-G and S566-A), the ATP binding pocket should be increased to facilitate ATP analog binding and catalysis. Introducing a transgenic copy of Dw2 with gatekeeper mutations, and supplementing with the γ-S version of the bulky ATP analog, would allow for the capture, fractionation, and identification of all Dw2 substrates, including PLD delta.

One potential downside of these experiments is that reagents are expensive. The ATP analogs can be purchased, but the γ-S moieties usually have to be specially synthesized. Dw2 is expressed early in development, but wt and mutant plants can only

be differentiated at around ~60 days old when the plants have already reached about a meter in height (J. L. Hilley et al. 2017). This makes treating whole growing plants impractical and costly. However, by placing AS-Dw2 under a constitutively active promoter, like 35S, expression will increase in seedlings and should reduce the amount of material needed to perform this experiment. Growing AS-Dw2 seedlings on agar supplemented with the thiolated ATP analog will allow them to grow, uptake the substrate, and thiophosphorylate any targets of Dw2 activity (as demonstrated in (Harashima et al. 2016)). This method is not foolproof, however, as misexpressing Dw2 too early in the growth might cause problems with development or could lead to target substrates not being available for Dw2 activity due to not having coincident existence. If seedlings are able to grow, but concerns arise about missing potential Dw2 substrates, the window-cutting technique employed in both chapters of this thesis could be utilized. Dw2 is known to increase cell proliferation in growing internodes ((Oliver et al. 2021), Chapter 2) and is therefore an excellent tissue to treat with ATP analogs in AS-Dw2 plants. By exposing the young internode by cutting into the leaf sheath, and treating the growing tissue directly with the ATP analog (with some sort of carrier like lanolin paste or light detergent), the ATP analog is delivered to the exact tissue at the exact developmental stage required for optimal detection. After allowing the tissue to grow for approximately two days, the specific treated section could be excised, proteins extracted, and thiophosphorylated targets of Dw2 captured and identified (Blethrow et al. 2007). This technique would not only potentially confirm PLD delta as a target of Dw2, but would capture any protein substrates of Dw2 activity. Additionally, this would further

solidify AS kinase technique in the identification of novel kinase substrates *in vivo* and *in planta.*

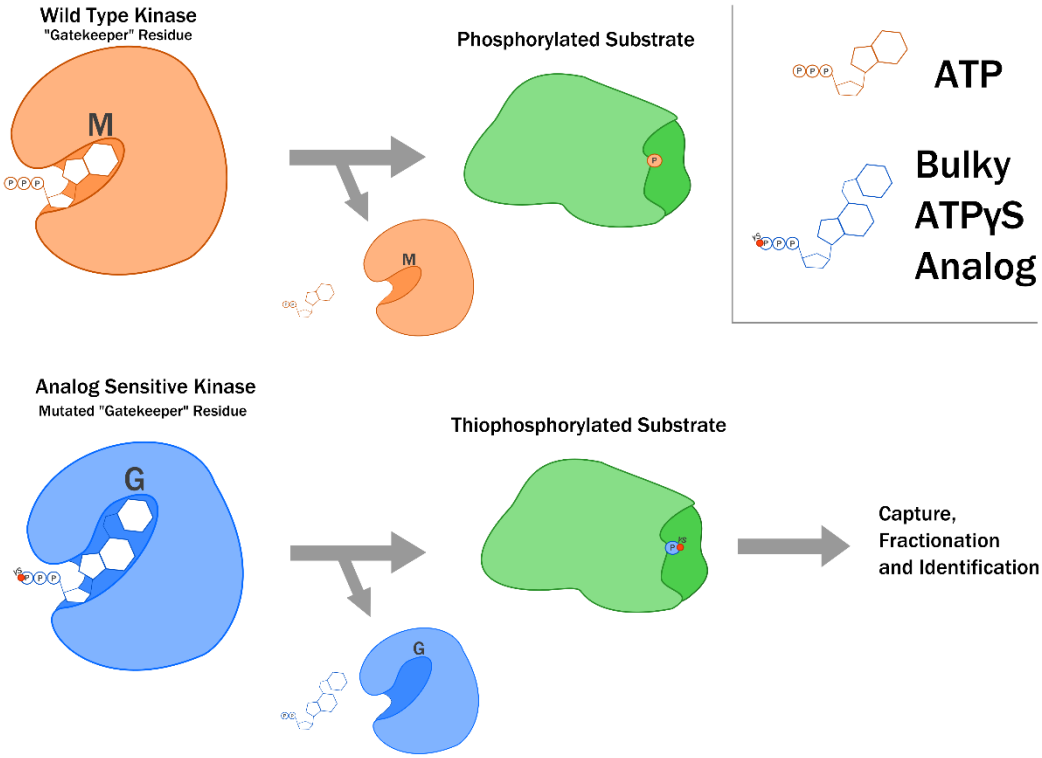


Figure 5.2 Analog Sensitive Kinases to determine substrate targets of Dw2. Wild Type kinases are mutated at a "Gatekeeper" residue (pictures here as M) to a smaller amino acid (G). This opens the ATP binding pocket enough to allow for the binding and hydrolysis of bulky ATP analogs. Gamma S varieties leave the substrate thiophosphorylated, which can be captured and identified through a variety of methods.

5.2.3. Elucidating up and downstream signaling pathway of Dw2

Once potential Dw2 substrates are identified, new realms of examining the Dw2 signaling pathway can be ventured. Many of these substrates could be redundant from other AGCVIII family members, which would give insight into evolutionary redundancies in plant growth and the AGC family more broadly (Hirt, Garcia, and Oelmüller 2011; Rademacher and Offringa 2012). Conversely, completely novel targets could be discovered to further our knowledge of plant signal transduction in general. However, since the putative substrate identified was PLD delta from our previous phosphoproteomic experiments, the next step would have to be identifying the effect of phosphorylation on PLD delta function. As previously mentioned, the phosphorylated serine on PLD delta projected to be the target of Dw2 is not present on the Arabidopsis PLD delta. However, the rest of PLD delta aligns well with the Arabidopsis homolog, suggesting that the two have similar functions. Arabidopsis PLD delta has been implicated in a variety of stress responses, including drought, freezing tolerance, salinity, and immunity and has been shown to interact with microtubules and the plasma membrane (W. Zhang et al. 2003; Pinosa et al. 2013; Q. Zhang et al. 2018; Weiqi Li et al. 2004; Angelini et al. 2018; Gardiner et al. 2001; C. Wang and Wang 2001). One example of stress known to be mediated by PLD delta is the response to Reactive Oxygen Species (ROS) (W. Zhang et al. 2003). PA derived from PLD delta was shown to attenuate H_2O_2 induced cell death, and knockouts of PLD were highly susceptible to ROS toxicity. Mutant Dw2 plants are also sensitive to ROS, and treated mutants arrest

internode growth and overproliferate their nodal roots (Chapter 2). This suggests a positive correlation between Dw2 phosphorylation, PLD activity, and ROS detoxification, and provides preliminary evidence that phosphorylation of PLD delta at S684 increases catalytic activity. However, when PA levels were measured in internodal tissue of growing wt and mutant plants, no difference in total PA concentrations were observed. These pieces of contradictory evidence require more direct means of examination. As previously discussed, purifying an active Dw2 is difficult, which means phosphorylating PLD in vitro for kinetics is as well. Phosphomimics are widely used to determine regulatory roles and biological relevance of phosphorylation events and work by substituting the phosphorylated residue with a negatively charged amino acid such as D or E. (Dissmeyer and Schnittger 2011). By purifying a PLD delta S684D mutant, and running conventional kinetics by measuring the rate of PA concentration increase, more knowledge can be gained about how Dw2 phosphorylation regulates the catalytic activity of PLD delta.

There are two likely possibilities from the outcome of the phosphomimic experiments using the PLD delta S684D mutant. The first would suggest that phosphorylation at S684 by Dw2 upregulates or downregulates the catalytic activity of PLD Delta. The second would suggest that phosphorylation by Dw2 has no effect on the activity of PLD delta, so the phosphorylation event has some other role. Finding no difference in internode PA concentrations supports the second outcome more strongly, but is not incongruous with the first. Dw2 could indeed be changing PLD Delta activity, but it does not alter the total amount of PA within the entire internode. Sorghum has at

least nine phospholipases that are expressed in the stem throughout all stages of development and their activity could more than make up the total PA concentration lost by the inactivity of unphosphorylated PLD Delta. Other PLD family members, such as PLD alpha, are known to localize to the cell plate during division, which increases the local concentration of PA (Novák et al. 2018). PLD delta could also be increasing local concentrations of PA under any of the environmental conditions previously listed. Genetically encoded PA biosensors that change color due to PA concentrations can be transgenically introduced to plants and observed under laser confocal microscopy (Wenyu Li et al. 2019). Upon examination of PA biosensor transgenic Dw2 +/- internode or root tissues, it can be determined if local concentrations of PA change due to Dw2 activity. If phosphorylation was found to not have an effect on catalytic activity, it could influence the subcellular localization of PLD delta instead. Tagging PLD delta with GFP or using immunolocalizations for PLD delta in Dw2 wt and mutant plants could determine subcellular localization by microscopy. Additionally, subfractionation of organelles by sucrose gradient and ultracentrifugation followed by Western blotting of PLD delta under Dw2 +/- conditions could help determine the localization of PLD Delta when regulated by Dw2 phosphorylation.

Another potential avenue for determining the Dw2 signaling pathway as it relates to PLD Delta is to screen conditions under which Dw2 wt and mutant plants have different phenotypes. As previously mentioned, PLD delta is involved in a variety of biological responses, and knockouts are sensitive to many different conditions. This suggests that mutants with perturbations in PLD Delta signaling propagation (such as

Dw2 mutants) could have similar sensitivities. For example, PLD delta has been shown to be activated by oleate, and if treatment of Dw2 mutant plants with oleate rescues the phenotype, then it would suggest that Dw2 regulation of PLD delta works in parallel to PLD delta regulation by oleate. Furthermore, if Dw2 mutants plants can be rescued with PA supplementation, this would suggest that PLD delta activity is regulated by phosphorylation. Since there are so many different roles attributed to PLD delta signaling, there are just as many physiological and environmental treatment conditions (drought, inhibitor, salinity) that can be utilized to narrow down the effect of Dw2 signaling on Sorghum biology.

While many of these proposed experiments so far work downstream of Dw2 signaling, there are just as many questions about what is working upstream from Dw2. PDK1 is known to activate many AGC kinases, and is itself activated by inositol phosphate (IP) concentrations (Mora et al. 2004). Additionally, Dw2 contains the C terminal FxxF motif present in AGC kinases that mediates PDK1 binding for kinase activation. Previous studies have suggested that the Arabidopsis Dw2 (KIPK) can autophosphorylate which implies autoactivation (Zegzouti et al. 2006), but this has never been directly demonstrated. Potential experiments include introducing transgenic plants that have a mutated FxxF motifs, which should abolish PDK1's ability to activate Dw2 in trans. If transgenic plants phenocopy mutant plants, then it would strongly suggest that PDK1 is required for Dw2 activation. Similarly, since IPs are known to activate PDK1, treatment of growing Dw2 internodes with various IPs might stimulate growth even further. Finally, elucidating the IP pathway in the growth of Sorghum would

connect sugar and lipid signaling directly, which would provide valuable information in plant biology as a field.

5.2.4. Impact of Dw2 characterization on the design of bioenergy grasses

Determining the role of Dw2 signaling is important for basic biology, but if the goal is designing a more efficient bioenergy crop, the findings here are more valuable for what they imply than what they describe. As discussed previously, source and sink relationships define plant growth. Most source/sink scientists are focused on entire tissues that represent source production or sink storage, such as the leaves or grain. Such organs represent *physiological* sources and sinks. Specifically, Dw2 was shown to control cell proliferation, vascular bundle morphology, internode growth, and biomass accumulation by *regulating the endomembrane system*. With the Golgi acting as a source for membranous material, membrane proteins, and trafficked macromolecules, and recipient membranes and compartments acting as sinks, the endomembrane system constitutes a veritable *molecular* source and sink. With biomass accumulation being controlled by endomembrane activity, this effectively links the macroscopic source and sink to the microscopic source and sink. This work, in part, suggests that yield limitations in C4 bioenergy crops might not be limited by source availability or sink strength, but instead be limited by the *transport* that happens between source and sinks, particularly molecular source and sinks. Additionally, because Dw2 is a signaling kinase that itself must be activated, this suggests that biomass accumulation could be similarly activated under a chosen condition. These open a whole new area of design that can be

exploited for increasing biomass yield that will be discussed in depth in a later section of this chapter.

5.3. A Refined Model of Internodal Growth

Grass stems are divided into segments of plant material called internodes, with flanking nodes on either side. This is one of the most obvious characteristics of grass biology and has been studied for many years. Despite this, relatively little is known about node/internode development. This is especially true when compared to the breadth of knowledge discovered about Shoot Apical Meristem organization and leaf biogenesis (Bilsborough et al. 2011; Richardson and Hake 2018). As such, Chapter 3 of this thesis set out to better define sub tissues within the grass stem. This project was spawned through careful observation of plants used for this dissertation's second chapter; wt and mutant Dw2 plants had vastly different internode growth and morphologies, and preliminary evidence there suggested tissue growth was being defined by something more than Dw2 signaling (Oliver et al. 2021). As a result, Chapter 3 suggests that the grass stem is divided into four distinct tissue subtypes: 1) the Nodal Plexus, which is involved in transport between organs, 2) the Internode, which enables variation in plant height, provides structural support and is the bulk of biomass tissue within grass stems 3) the Pulvinus, which is involved in hormone signaling and the site of nodal root buds and 4) the so-called “White Band” which provides a boundary between the Pulvinus and the Internode tissue. These findings are important to understanding organogenesis in grasses, and to help further elucidate the evolutionary divergence of monocots from dicots. The data derived for this project was incorporated into two competing models of

internode growth, dubbed the “Split Node” and the “Contiguous Phytomer”. If deeper understanding of monocot stem biology is to be understood, these models must be further dissected.

5.3.1. Boundary Layer Confirmation of “White Band” Tissue

While the evidence presented in Chapter 3 strongly suggests the existence of a boundary layer that separates internode and pulvinus tissues, further research will be to confirm its existence. Boundary layers are key regulators of plant architecture, organogenesis, and morphology, and are controlled by a variety of factors, including hormone gradients and transcription factors (Bell et al. 2012; Scofield et al. 2018). For example, Cupped Cotyledon (CUC) family transcription factors accumulation in boundary layers that separate the Shoot Apical Meristem from leaf primordia (Aida et al. 1997; Richardson and Hake 2018). Other transcription factors, such as the *KNOTTED-LIKE HOMEOBOX* (KNOX) genes, positive regulators of meristems, are downregulated by auxin maxima which allows for differentiation and new organ formation (Veit et al. 1994; Hay, Barkoulas, and Tsiantis 2006). Additionally, boundary layers are characterized by low cell division and parallel microtubules (Hamant et al. 2008). Because boundary layers and their characteristics are well studied, it provides a classic approach to determining the authenticity of the White Band as a bonafide boundary layer.

Although not definitive, there is evidence that suggests the White Band tissue is a true boundary layer. These include observations such as expression of boundary layer marker genes and drastic changes in cell morphology upon reaching the White Band

tissue. However, as previously stated, boundary layers are characterized by having low areas of cell division. This is directly contradictory to the observed increase in cell division at the White Band tissue discussed in Chapter 3. However, boundary layers are, by definition, one or two cell layers thick, particularly in the apex, and are flanked on one side by areas of rapid cell division (Richardson and Hake 2018). Because the White Band tissue was hand sectioned away from the rest of the stem tissue, it is easy to conceive that more tissue than exact boundary layer was collected. This would lead to contamination of RNA that does not come from the boundary layer itself, but is actually from the adjoining area of rapid cell division (the internode). This contamination can be resolved through increased resolution of collection of the White Band and there are many techniques that can achieve this. For example, *in situ* hybridization of target genes and Laser Assisted Microdissection (LAM) coupled with RNAseq (read below for more detail) are both able to demonstrate the existence of the boundary layer with more accuracy and precision. By employing one or both of these methods, we can confirm the existence of boundary layer marker genes within the White Band tissue.

Boundary layers are also controlled by hormone gradients including auxin and brassinosteroids (Benková et al. 2003; Bell et al. 2012). This leads to a relatively straightforward set of experiments to ascertain the existence of the White Band as a true boundary. By simply quantifying phytohormone concentrations throughout the growing internode using any one of the well defined methods (L. Wang et al. 2020), gradients can be discovered and correlated to known gradients that establish boundary layers. Additionally, boundaries serve as a division between tissues, and as such, transport

through them is limited (Barton 2010). This phenomenon could be exploited by tracking radiolabeled phytohormones throughout the plant. For example, treating leaves with radiolabeled auxin would cause the signal to move down from the leaves and into the stem, where sections of the stem can be harvested and radioactivity can be measured (demonstrated in (Multani et al. 2003)). This type of experiment would help differentiate the two models that were proposed in Chapter 3. If young, treated leaves transported their radiolabeled auxin to the stem, and that label accumulated in adjoining Nodal Plexus and Pulvinus, that would support the "Contiguous Phytomer" model. However, if the radiolabeled auxin accumulated on either side of the growing internode, this would suggest that the "Split Node" model was correct. Finally, if the radioactive signal was diffuse throughout the tissue, this would suggest that the White Band is not a boundary layer, as the auxin could move freely about the phytomer.

One defining feature of boundary layers is the cellular shape, which is brought upon by the organization of the cytoskeleton (Hamant et al. 2008). The cellular morphologies described in Chapter 2 already suggest that the cytoskeleton below and above the White Band is different, and RNAseq data show many things involved in cytoskeletal reorganization and structure expressed at the White Band. Additionally, a cytoskeleton reorganization model has been proposed by previous groups when describing internodal growth (Knöller et al. 2010). All of this evidence together provides support for determining the role of cytoskeletal dynamics in internodal growth. This can be achieved through immunolocalizations of microtubules below, at, and above the White Band. If the White Band is a true boundary layer, microtubules should organize in

parallel rows at and above the boundary. Below the White Band, microtubules should be randomly arrayed, which would give rise to their random morphologies. Additionally, treatment with drugs that disrupt the microtubule structure should cause visible White Band phenotypes in plants. These experiments will help provide the necessary information to better understand the role of the cytoskeleton in internodal growth and should help determine if the White Band has true boundary layer properties.

5.3.2. Laser Assisted Microdissection (LAM) of the Sorghum apex

Because the Shoot Apical Meristem is very small and contains many boundary layers and discriminate tissues, physically taking specific sections for further analysis is practically impossible (Richardson and Hake 2018). One exciting new area of developmental biology is the single cell specific RNAseq that is capable due to laser microdissection (Sakai et al. 2018; Kivivirta et al. 2019). In this technique, tissue sections are harvested, fixed, and cryoprotected with a sucrose solution. After that, they are embedded in a freeze resistant resin before being trimmed and sectioned using a cryotome, which achieves thin tissue sections that are adhered to microscope slides. These first steps are similar to embedding needed for *in situ* and immunolocalizations. However, after cryosectioning, these samples are taken to a laser dissecting microscope where the tissue of interest is ablated off of the slide, thus removing it from the surrounding tissues. After removing the tissue, RNA can be extracted and sequenced using any RNAseq system (Kivivirta et al. 2019). Some variations of LAM use embedment in typical paraffin before sectioning as well (Sakai et al. 2018). This technique allows for very small sections to be taken and allows for single cell and tissue

resolution of RNAseq profiles, and is perfectly suited for deciphering the transcriptome of the developing *Sorghum* apex.

By using LAM coupled with RNAseq, the *Sorghum* apex can be more carefully dissected and analyzed. Additionally, since grass nodes/internodes are essentially clonally related phytomeric units (McKim 2019, 2020), a developmental series can be taken within a single fixing and embedment procedure, similarly to the series of phytomeric units taken in Chapter 3. A developmental series across the growing apex is one of the best ways to determine the progression of internode growth, as tissue specific marker genes can be discovered, and their expression tracked over time. This is particularly important when determining between the two models proposed in Chapter 3, as marker genes associated with the same phytomer unit would either appear on one side (Contiguous Phytomer) or flanking each side (Split Node) of the developing internode. Additionally, this type of experiment would give a multitude of other information on how and when internodes develop and grow and could lead to more discoveries on physiological differences between monocots and dicots. Finally, this technique could be used in actively growing and mature internodes to increase the resolution of RNAseq profiling seen in Chapter 3 of this dissertation. LAM is very new and expensive, which means that undertaking a task like this would be high risk and high reward. Additionally, since tissues are sectioned to approximately 8μm thick, the RNAseq profile is essentially two dimensional. However, by increasing the amount of sections taken *through* (epidermis to epidermis) the section can get a complete profile of the growing tissue's transcriptome.

5.3.3. Genetic screen for more White Band mutant Sorghum

Plants with boundary layer mutations tend to have striking phenotypes. For example, the *Knotted-1* (Kn1) transcription factor helps define the boundary between leaf sheath and leaf blade. Overexpression of this Kn1 leads to a gain of function mutation that causes wrinkling (knots) in the leaf blade. When further characterized, it was shown that because the boundary between the leaf sheath and blade had been disrupted, leaf blades began to exhibit leaf sheath morphologies (wrinkling and knots) (Vollbrecht et al. 1991). Another example is the Cupped Cotyledon mutants (CUC1,2). Normally, dicots form two separate cotyledons from their embryo, but *cuc1,2* mutants formed a singular cotyledon in a cup shape forming from fused cotyledons (Aida et al. 1997). The phenotype of the White Band mutant discussed in Chapter 2, however, is less obvious. As described previously, Dw2 mutant plants have short internodes with altered cellular morphology (J. L. Hilley et al. 2017; Oliver et al. 2021). These short internodes also have altered phyllotaxy, and a poorly resolved White Band tissue that results in precocious maturation, shown by increases in expression of maturation marker genes and increased lignin fluorescence in young internodes. With the discovery of Dw2 mutant plants also having increased sensitivity to ROS generating treatments, these data suggest that the White Band formation is critical to internode growth and elongation, and that the endomembrane system and ROS signaling play a role in the boundary layer development. Disruption of White Band formation leads to poorly resolved tissues within the internode, causing early lignification and arrested growth. Taken together, these data provide evidence that the formation of a healthy White Band is critical to

biomass accumulation in *Sorghum*.

While Dw2 mutants are the only characterized White Band mutants currently discussed, there are bound to be others within the Sorghum germplasm. By identifying other mutants with altered White Band formation, further insights into internode growth and development can be achieved. The phenotype associated with the White Band is thus far phyllotaxy and short internodes. This provides easy screening techniques for determining White Band mutants. Additionally, since the Sorghum germplasm is broad, varied genotypes can be planted and screened for height and phyllotaxy. Interestingly, Dwarfing genes seem to play some role in White Band formation (as demonstrated by Dw2), but no White Band phenotype has been recorded with other Dwarfing mutants. Furthermore, not all Dwarfing genes are created equal in the formation of the White Band; Dwarf Yellow Milo (which have a mutated Dw1) show healthy White Bands and robust internode growth (J. L. Hilley et al. 2017; Oliver et al. 2021). Due to the relationship between plant height and breeding, there are many short genotypes already characterized, such as the elite Grain Sorghum BTx623. It is currently unknown whether or not BTx623 has a White Band phenotype, but the genotype has a functional copy of Dw2 (Paterson et al. 2009). This would suggest that if BTx623 has short internodes due to White Band disruptions, a novel mechanism that does not include Dw2 directly would be the cause. Finally, mutagenesis screens could be employed on genotypes with clearly resolved White Bands, screened for height and phyllotaxy, and characterized using RNAseq and other methods mentioned throughout this dissertation. This type of

experiment would allow new insights into the growth and development of nodes and internodes in grasses, which is important for biomass accumulation and yield.

5.3.4. Impact of stem tissue identification on the design of bioenergy grasses

As inculcated many times throughout this dissertation, the stem of bioenergy Sorghum contains the most biomass for conversion into biofuels. Because Sorghum has such strong promise in the development and deployment of bioenergy grasses, understanding stem biology is significant for the future design. Additionally, since the stem is divided into discrete tissues, this presents interesting opportunities for bioaccumulation within those tissues; through careful tissue specific engineering, designed Sorghum could produce specialty bioproducts in each of the three main tissues independently, which could bypass intrinsic regulatory mechanisms that prohibit the accumulation of certain molecules within the entire plant. Furthermore, the identification of the White Band, and its underlying role in internode development and biomass accumulation could be utilized in the future to perturb the phyllochron of developing plants. That is, designed plants could create phytomers more quickly than mundane plants, which allows for increased biomass production through more internode development. Because the White Band plays a direct role in the development of internodes, perturbations or precocious inducement of White Band boundary layer could increase the rate of production of internodal tissue. Finally, this work is fundamental to the understanding of grass biology and presents interesting questions on their relationship to dicots and the evolution of nodes and internodes.

5.4. The Future of designed (bioenergy) crops

Designing plants is an integral part of our culture, society, and civilization. From the first domesticated crops and stone tools to the elite hybrid cultivars used today, humans have used a variety of technologies to increase yield and productivity. As science progresses, more feasible and futuristic design strategies become possible. This is particularly evident in the growing acceptance of synthetic biology (synbio) within science as a whole, and shows promise in advancing the agricultural sector as well (Wurtzel 2019). Like all science, synthetic biology is only limited by the imagination of the designers, but provides a particular flair of creativity, innovation, and foresight. This allows for plant synthetic biologists to come up with seemingly impossible designs to accomplish their goals, and synbio is likely a huge part of agricultural advancement in the future. This section will address some of the biggest scientific hurdles to introducing synbio into plant design, as well as some potential design projects that can be employed using synthetic biology technology.

5.4.1. Overcoming the transgenic bottleneck

Synthetic biology, by definition, requires introducing new genes into plants to create transgenics. Currently, producing transgenic plants in all but a handful of species is time intensive and inefficient (Ahmad et al. 2017; Binns 1990; Atkins and Voytas 2020). This creates a significant bottleneck when trying to increase the throughput and production of genetically engineered plants, particularly if a non-model species (like *Sorghum*) is used. Genetic material being delivered into tissues is one of the more common complications in transgenic production, as many plants are not susceptible to the typical *Agrobacterium* infections or gene gun methods (Lacroix and Citovsky 2019;

Ahmad et al. 2017). To address this problem, there have been some advances to genetic delivery mechanisms throughout the years. Plant viruses evolved to deliver their genetic material to plants over millennia, and they have been engineered with desired traits for expression in plant systems, with Geninivirsuses being one of the more studied viral delivery methods (Baltes, Gil-Humanes, and Voytas 2017). However, engineered strains are largely limited by host range and cargo size, as a small genome size is required for cell-to-cell movement through the plasmodesmata (Gilbertson et al. 2003). Another highly encouraging advancement is through the use of carbon nanoparticles as transgene delivery systems (Demirer et al. 2019; Doyle et al. 2019). In this technology, genetic material is attached to the carbon nanoparticle using electrostatic interactions and introduced into the plant material (usually leaves), where it is taken up and the delivered DNA is transiently expressed. This has been achieved in a plant species independent manner, including in many difficult to transform crops such as wheat and Sorghum (Demirer et al. 2019; Doyle et al. 2019). While only transient expression has been achieved using carbon nanoparticle delivery, this opens the possibility to future stable genome editing using this type of technology.

Once the genetic material has been delivered to the cell, the next steps depend on the goal of the designer. Generally, these fall into two categories, targeted mutagenesis and gene targeting (Paszkowski et al. 1988; Atkins and Voytas 2020) For targeted mutagenesis, the goal is to introduce single nucleotide polymorphisms (SNPs) or small insertions or deletions (indels) at a target sequence which causes a mutation to knock out the gene. In part due to the high selectivity of CRISPR/Cas9 to induce double strand

breaks at specific locations, introducing indels are much more common, and are the result of repair mechanisms through non-homologous end-joining (NHEJ) or microhomolgy-mediated end-joining (MMEJ) (Mara et al. 2019). In contrast to mutagenesis, gene targeting is precise, homology-dependent, and utilizes donor DNA to create targeted modifications at a specific site through homologous recombination (HR) pathways (Puchta 1998). As a result, gene targeting is often used to introduce novel DNA sequences into the genome and much work has been done to optimize donor DNA design and methods of delivery (Atkins and Voytas 2020; T.-K. Huang and Puchta 2019).

After delivery and incorporation of target genetic modifications, the next step is to regenerate full plants from the edited tissues. This is the most time consuming and difficult process due to many species' recalcitrance to tissue culture (Altpeter et al. 2016). To circumvent this problem, novel methods of inducing ectopic meristems have recently been developed (Lowe et al. 2016; Maher et al. 2020). In this innovation, somatic cells are incubated with the delivery agent carrying the target modification (usually *Agrobacterium* or modified viruses) as well as genes for the coexpression of developmental genes known to induce and maintain meristem formation, *BABYBOOM (BBM)* and *WUSCHEL (WUS).* In some cases, a cytokinin biosynthetic gene is also co-delivered to keep regulatory feedback from shutting down ectopic meristem formation (Maher et al. 2020). The inclusion of these developmental regulators causes meristems to form at the site of delivery, which in turn increases the likelihood of the targeted gene to be introduced into actively dividing cells that will ultimately become seed (Lowe et al.

2016; Maher et al. 2020). Utilizing this method prevents the need to culture tissues for large amounts of labor and time, as high transgenic efficiency can be recovered from the seeds produced from edited ectopic meristems (Maher et al. 2020).

One currently unexplored avenue of transgenic production technology is the combination of ectopic meristem formation and carbon nanoparticle delivery methods. By linking these two techniques, edited plants can arise from any treated species, as the carbon nanoparticles are not limited by virus hosts and *Agrobacterium* infection. Additionally, Sorghum plants, and most grasses, form tillers at the base of their internodes that develop into fully formed shoots upon their activation (such as decapitation of the main plant apex) (Kebrom and Richards 2013). This naturally occurring process provides scientists easy access to meristems that are already poised to form full plants. By taking carbon nanoparticles laced with target genomic editing sequences, material for coexpression of ectopic meristem formation, and treating tillers with the cocktail, newly edited plants can theoretically be achieved in a fraction of the time. Additionally, *every* internode in Sorghum produces a tiller that is independent of all other tillers. This means that multiple constructs can be introduced onto the same plant; if an Sorghum plant has 30 internodes, you could, in theory, produce 30 separate transgenic plants from the tiller formed at each internode using this method. Additionally, since the edits are occurring in meristematic tissue, the tillers themselves form seeds which can be easily screened transgene traits. Minor success has been achieved by utilizing this technique in our lab, but results have been inconsistent and unable to be replicated. However, this type of innovation is what is needed to break

through the transgenic bottleneck for the rapid design of Sorghum (and other crops) for specialized uses.

5.4.2. Revisiting the Source and Sink: Future Design Possibilities of Sorghum

Once the transgenic bottleneck has been addressed through experiments mentioned above or through other innovations, it opens a vast area of synthetic biology capable of revolutionizing agriculture. However, as alluded to throughout this document, future design strategies of crops will likely revolve around the same thing they always have: the source and sink relationship. It still behooves scientists to work within this realm of development as tweaking existing structures and relationships is more straightforward than *de novo* plant design. This does not imply facile progress as many scientific hurdles relating to plant synthetic biology remain. However, by utilizing principles that have already been exploited for centuries, scientists can make meaningful strides in designing the plants of the future.

5.4.2.1. On designing the transport between Source and Sink

One such area of Source and Sink design is the regulation of the transport between them briefly mentioned in a previous section. The work highlighted in this dissertation demonstrated the importance of a signaling kinase (Dw2) that regulates the endomembrane system that also directly controls biomass accumulation and growth (J. L. Hilley et al. 2017; Oliver et al. 2021). These findings lead to the hypothesis that simply by controlling the rate or efficiency of the transport between Source and Sink tissues or organelles increases the size and biomass of plants. For a bioenergy grass, such as Sorghum, bigger is better; the larger the plant size, the more biological material

available for conversion into bioenergy. The connection between the endomembrane system and plant size has always been well known; one of the most important exchange factors that regulates the endosomal cycling of auxin efflux proteins (PIN family) is named *GNOM* (from the German for "gnome") because of tiny, malformed plants that grow when mutated (Geldner et al. 2003).

Another example of the correlation between intracellular transport and plant size comes from Tominaga *et al*. in 2013. In this work, plants with engineered motor proteins grew larger due to increased cytoplasmic streaming (Tominaga et al. 2013). While designing Sorghum with an altered rate of transport between Source and Sink organelles has many different areas of exploration, this work provides one of the best examples for proof of concept. The design principles are easy to follow: create a synthetic myosin that moves faster than the wild type myosin within the plant. To achieve this, Tominaga and his colleagues looked at a species of algae that contains large single cell structures, *Chara corallina*. One reason for the algae's large cells is due to their myosin motor proteins that have unique interaction interfaces with actin and increased ATPase and ADP dissociation properties (Higashi-Fujime et al. 1995; Ito et al. 2003, 2007). All of these properties together create the fastest known motor protein to date, which moves at approximately 70 μm × s−1 (Ito 2007). Introducing synthetic myosins using the motor domain from *Chara* and the neck and tail section from *Arabidopsis* into plants increased dry weight by over 44% (Tominaga et al. 2013).

The work done by Tominaga *et al.* presents an interesting possibility for the future of controlled plant size through perturbed Source to Sink interactions, and has

many applications for the design of bioenergy Sorghum. The first and most obvious step would be to replicate the introduction of the synthetic myosin into Sorghum to examine its effect on biomass accumulation. Next would be to take two approaches to further design strategies: 1) scour literature and nature for other types of fast motor proteins 2) use directed evolution to increase the speed of the *Chara* myosin motor. Because evolution has had millenia to provide selective pressure on the most advantageous systems, it stands to reason that other, very fast, motors exist within nature. *Chara* plants grow underwater, where it is hypothesized that cell sizes could grow large due to a lack of gravitational restraints (Tominaga et al. 2013). There are a myriad of other underwater plant species that grow quite large, including other algae species. By examining the morphology and myosin sequences/proteins and comparing them to the known *Chara* myosin, faster motors can be naturally discovered and implemented. Additionally, California Red Wood trees are some of the largest organisms on the planet, and their roots must absorb and transport nutrients to growing leaves over 75 meters high. It is possible, particularly in the phloem, that there are robust transport and motor protein mechanisms that can be used as a base for new designs. Moreover, directed evolution on motor proteins is not a novel idea (Goodman, Derr, and Reck-Peterson 2012; Linke et al. 2020), and principles established previously can be used to further design motor proteins for bioenergy grasses. Finally, plants, like animals, do not only contain myosins, but other motor proteins like kinesins and possibly dyneins ((King 2002; Nebenführ and Dixit 2018). By introducing faster versions of these motors, plant size and biomass could be increased even further. It is important, however, to acknowledge that large plant sizes

do not always work in the field, as evidenced by introduction of Dwarf plants to reduce lodging mentioned previously in this work. However, further design strategies, both molecular (such as engineering stronger lignified stems and deeper root systems) and agricultural (such as wind blocks and planting orientation), can be introduced to minimize these risks.

While increasing the rate of transport to and from molecular Sources and Sinks is one beneficial area of design, there are other methods utilizing the endomembrane system that could increase the productivity of Sorghum as a bioenergy grass. One such example is to bioaccumulate specialty bioproducts, such as sugars, within certain tissues (Liu et al. 2021; M. Fan et al. 2018). In these examples, important enzymes, such as sucrose isomerase, are overexpressed and fused to vacuolar targeting domains. This allows for the directed accumulation of specific sugars within the plants' largest molecular sink, the vacuole (Liu et al. 2021). Strategies like this can also be used to accumulate other sugars, such as Mixed-Linkage Glucans (M. Fan et al. 2018; S. Kim et al. 2018). Increasing the concentration of such sugars leads to easier bioprocessing down the conversion pipeline, and changing Sorghum stem composition in particular is important for the continued design of bioenergy Sorghum (Mullet et al. 2014).

5.4.2.2. On the strategic deployment of nutrients to increase Source supply

Increasing source supply is one of the key features of increasing yield (Chang and Zhu 2017), and was highlighted most definitely within the Green Revolution, which increased source supply by proper use of fertilizers and by reducing competition from pests. These innovations and others lead to the agricultural productivity surge that

currently supports the modern population (Hazell and Fan 2009). Despite advancements in treatment decisions, at any point during its lifetime, a plant could be lacking an essential nutrient required for more efficient growth. Suboptimal nutrition is a constant concern for horticulturists and farmers alike who must essentially guess at when to water and fertilize their crops based on their experience. This can lead to harmful fertilizer runoff and eutrophication of water bodies. Safe practices such as adopting nutrient management techniques, planting field buffers, and using proper drainage techniques can mitigate damage, but such practices are not sufficient (United States EPA Nutrient Pollution; USDA Natural Resource Conservation Service). As such, new technologies are needed to address this problem. One such solution is to develop plants that can act as "Sentinels" which relay meaningful information about nutrient conditions to the farmers for more accurate nutrient deployment strategies. Plants respond to stress in a myriad of ways and systemically send information in order to combat the problem. Most of this information is only useful endogenously and is lost to the caretaker until the plant becomes symptomatic (i.e. yellowing of leaves and wilting), at which point rescuing the plant becomes much more difficult. The key to a good "Sentinel" plant is to exploit these signals in such a way that early detection of nutrient deficiencies becomes readily identifiable; stated more simply, to create a "talking" plant. In order to design a "talking" plant, there are two main components required in the design: a sensory component and the signal component. Ideally, the signal would effectively make the leaves, and their engineered signal, an early-response proxy for soil conditions that allows for the plant's caretaker to alleviate stresses before they become too problematic This can be achieved

through the use of synthetic biology in conjunction with transcriptomic discovery, as expanded upon below. By quickly determining if a plant is healthy or starving, better nutritional delivery can be achieved, greater yield can be accomplished, and the uncertainty of optimal care can be alleviated.

The sensory component is already present in plants in the form of gene expression changes as a plant must alter the expression of its genes during stressful events in an attempt to survive. These genes are under the control of promoters that alter their activity based on perceived conditions. One of the most intuitive examples is the expression of the two types of nitrogen transporters in *Arabidopsis*, which vary according to nitrate levels within the soil (Okamoto et al. 2003). Under abundant nitrogen, the NRT1 family (low affinity transporter) have high expression levels and under low nitrogen conditions, the NRT2 family (high affinity transporters) have high expression levels (Okamoto et al. 2003). This sensitivity makes these nitrate transporters (and their promoters) sensors for soil conditions and can be utilized as the sensory component of the two component system. In addition, there are other genes that will have altered expression during a given set of conditions. By performing RNAseq of various tissues (roots, young leaves, old leaves, etc) under a set of conditions, like nitrogen or phosphorus stress, genes with specific and significant activation can be discovered. Furthermore, by utilizing the stress specific promoters of these genes they become the necessary sensor component.

The second part of the systems is the signal component that relays information activated by the sensor. In plants, there are many examples of mobile RNA sequences

that systemically move throughout the tissues. One such example is encoded by the *Flowering Locus T* (*FT)* gene which is transcribed in leaf tissue and moves to the shoot apical meristem to induce flowering (Notaguchi, Higashiyama, and Suzuki 2015; Jackson and Hong 2012). Additionally, the FT RNA has been shown to promote mobility of other mRNAs when fused together, such as GFP and sgRNAs for Cas9 editing (Luo, Huang, and Yu 2018; Ellison et al. 2020). However, the entire FT mRNA is not required for mobility; more specifically, a cis element within the FT mRNA can be fused to transcripts for increased cell to cell mobility (C. Li et al. 2009). Herein lies the necessary pieces for a robust signaling component: placing Mobile element Fluorescent Protein (MFP) fusions under the control of condition specific promoters. Upon the plant recognizing a stress and activating a subset of genes, such as the activation of the NRT2 transporters during low nitrogen, the MFP transcripts are produced and move endogenously to the apex where they are expressed in the young leaves and measured using many types of spectroscopy (UV/Vis, RAMAN, etc.) on drones or handheld devices. This type of system accomplishes two things: 1) it effectively serves as a proxy for soil conditions based on the expression of genes known to be activated during a stress event and 2) it concentrates the signal into the youngest leaves (the apex) which could allow for more easy detection and less signal dilution. Additionally, this system could be used for any sort of readout transcript (pigment accumulation, leaf crinkling); fluorescent proteins are simply straightforward to measure, to quantify, and are not endogenous to the plant for ease of identification. Furthermore, this technology could be developed for any given condition that the scientist and farmer want to detect early, such

as any nutrient, drought, or pest stresses. Early detection brought upon by this technology, or technology like this, can be crucial to making more strategic caretaking decisions that will increase the yield and overall health of the plant.

The age-old endeavor of plant design can be revolutionized by synthetic biology, and above are just two examples. However, synbio is truly limited by the creativity of scientists. There are a multitude of other ideas that can be implemented such as designing plants for radioactive bioremediation, soil desalination/detoxification, or developing a line of Sorghum plants that can be planted as grain but genetically activated to energy depending on the farmers' desires. All of these technologies will play a critical role in the development of plants as tools for varied use, and one of the first steps is the design of Sorghum as a dedicated bioenergy grass.

5.5. Conclusion

With the looming threat of global population increases and climate change that will require a doubling of agricultural productivity coupled with increasing global energy demand, sustainable solutions must be developed (Foley et al. 2011). One promising solution is using designed crops that accomplish a variety of goals, including increases in grain yield and bioenergy supplementation. The C4 grass *Sorghum bicolor* in particular holds potential for becoming a dedicated bioenergy crop, but further design strategies must be employed to increase its productivity. In this dissertation, knowledge gaps about *Sorghum bicolor* growth and development have been elucidated, and design strategies for the future of crop science have been discussed. We as a species have never shied away from problems on the horizon and solving the energy and food crisis is no

different. Through the use of technologies discussed here, and others brought upon by teams of dedicated scientists, challenges will be met and overcome. Furthermore, the principles underlined here can be applied to all plants but have specifically highlighted the necessity of "watching grass grow".

5.6. Epilogue and Musings of a Young Scientist: The Fourth Agricultural Revolution – Overview

With the increase in population expected to reach 10 billion by 2050 agricultural productivity must be increased by at least 50% to meet the new demand (Foley et al. 2011). This provides a troublesome timeline to meet those food production goals that is exacerbated by global politics and climate change. However, as the "Population Bomb" of the early 1900s was met with increased innovation and global cooperativity to usher in the Third Agricultural Revolution (Green Revolution) (Ehrlich 1971), so too will the explosion of this century. Accomplishing this feat will require new and innovative technologies as well as a change in global mindset on food production. In essence, a "Fourth Agricultural Revolution" must begin. In this brief epilogue, an argument will be made that we are already at the cusp of this Fourth Revolution that is being brought upon by three main technologies: Precision agriculture, "-omics" based science, and Genetic Engineering/Synthetic biology. Furthermore, by combining these technologies together, looming global agricultural productivity problems can be met, surpassed, and redistributed to defuse the 21st century's "Population Bomb".

5.6.1. Precision Agriculture

Precision Agriculture is a management technique used by farmers to give precise treatment decisions based on broad imaging technologies, like satellite imagery. Implementing this technique can increase yield of crops and diminish the variability of yield outcomes (Yost et al. 2017). Using this method, scientists can scan entire fields with unpersoned aerial vehicles, like drones, to determine exactly where crops are struggling to grow and can allocate precise resources to accommodate (Aubert, Schroeder, and Grimaudo 2012). Additionally, the use of robotic farming, including GPS tractors and large scale and sophisticated planting/harvesting equipment, continues to increase the practical productivity of the agriculture market by decreasing required manpower. However, there is a large learning curve for implementing and managing the correct Precision Agricultural principles, which can influence overall quality of output (Pathak, Brown, and Best 2019). Education initiatives and case-by-case consulting can help farmers acquire the minimum knowledge required to appropriately select the proper technology to implement (Higgins et al. 2017). Furthermore, as technology increases, so goes precision agriculture. Imaging and drone technologies in particular are subject to frequent innovation, and introducing the highest quality robotics can increase agricultural productivity by alleviating the problem of imprecise resource allocation.

5.6.2. Omics Based Science

Being able to examine wholescale metabolites, proteins, and genetic material in an unbiased way (defined using the suffix "-omics") has already revolutionized science (Mathé et al. 2018). This is especially true for agricultural and plant-based research (Van Emon 2016). While Precision Agriculture looks to increase the productivity of

previously defined crop species, omics can be used to cultivate and domesticate new crop species or further optimize already existing ones. Additionally, there are multiple levels of omics based science that can be employed based on the researcher's desires or goals. One example is genomics, which examines the DNA of a given species or individual to learn the sequence, evolutionary history, or genetic syntenies. These can be useful in determining potentially new crop species to develop with unique traits and a high-quality genome is required for further design strategies using Genetic Engineering and Synthetic Biology. Another, newer, "omics" field is that of "Phenomics". Phenomics is similar to Precision Agriculture in that they both use high quality and high throughput imaging systems to accomplish their objectives. In phenomics, however, plants are scanned and processed for phenotypic variance (where the "pheno" comes from). This allows for scientists and breeders to access high quality information on phenotypic relationships between their crosses or designs, and therefore increases the productivity of the breeding or engineering pipeline (Houle, Govindaraju, and Omholt 2010). Other types of omics are also useful, as demonstrated in this dissertation. Transcriptomics, or RNAseq, is useful to determine gene expression changes based on conditions or mutations (Chapter 2,3) and can give valuable insight into the development and growth of crop species. The future of omics based science is likely going to be "multi-omics" as described here and elsewhere (McLoughlin et al. 2018). In this type of experiment, tissue samples are divided into groups and processed in different omics platforms, like proteomics, phosphoproteomics, metabolomics, and transcriptomics. This overcomes an important caveat to a single omics based technique: just because something is

transcribed (expressed), does not mean it is translated, and just because something is translated does not mean the resulting protein is active. By combining omics together into wholescale multi-omics based experiments, vast quantities of information can be obtained about the conditions or mutants that are being explored.

5.6.3. Genetic Engineering (GE) and Synthetic Biology (SynBio)

The information obtained from omics based technology are critical for understanding the underlying mechanisms behind plant growth and development. These mechanisms can be utilized or exploited to increase yield through a variety of ways, but the most obvious is through genetic engineering or synthetic biology, as discussed at length in the Future Directions section of this dissertation. In brief, synbio or GE can allow for the design of specialized plants that increase yield under a variety of conditions, including salinity tolerance, growth on marginal land, vertical farming, and nutrient deficiency. This technology can exploit or optimize already existing mechanisms for increasing yield, while also allowing for increased innovation from biotechnology and scientific imagination.

5.6.4. Placing the pieces together

Like any good, complicated problem, no one solution is sufficient. However, complex solutions are often spawned from the combination of various ideologies and strategies. Indeed, the Green Revolution would not have been possible without dwarf plants, targeted pesticide use, and broad scale implementation of farming techniques and seed. The Fourth Agricultural Revolution will be no different. Synthetic biology can create biological sensors for nutrient limitations that can be scanned by Precision

Agricultural equipment. Genomics of rare plants with increased nutrient efficiency can be utilized to grant their efficiency to rice or corn. High throughput phenotyping can be used to examine all of these new traits. Endless combinations of the technologies briefly described above, and others not mentioned, will be employed to solve the caloric problem created by the rapidly growing population. Given proper treatment, resource allocation, attention, and action, combined with the resilience and determination of the human race, current problems with global agricultural productivity will be thought of as nothing more than a bump on the road to the future of our species.

REFERENCES

Ahmad, Malik M., Athar Ali, Saba Siddiqui, Kamaluddin, and Malik Zainul Abdin. 2017. "Methods in Transgenic Technology." In *Plant Biotechnology: Principles and Applications*, 93–115. Singapore: Springer Singapore.

Aida, M., T. Ishida, H. Fukaki, H. Fujisawa, and M. Tasaka. 1997. "Genes Involved in Organ Separation in Arabidopsis: An Analysis of the Cup-Shaped Cotyledon Mutant." *The Plant Cell* 9 (6): 841–57.

Allen, Jasmina J., Manqing Li, Craig S. Brinkworth, Jennifer L. Paulson, Dan Wang, Anette Hübner, Wen-Hai Chou, et al. 2007. "A Semisynthetic Epitope for Kinase Substrates." *Nature Methods* 4 (6): 511–16.

Altpeter, Fredy, Nathan M. Springer, Laura E. Bartley, Ann Blechl, Thomas P. Brutnell, Vitaly Citovsky, Liza Conrad, et al. 2016. "Advancing Crop Transformation in the Era of Genome Editing." *The Plant Cell*, June, tpc.00196.2016.

Ammar, Mohamed-Raafet, Yann Humeau, André Hanauer, Bernard Nieswandt, Marie-France Bader, and Nicolas Vitale. 2013. "The Coffin-Lowry Syndrome-Associated Protein RSK2 Regulates Neurite Outgrowth through Phosphorylation of Phospholipase D1 (PLD1) and Synthesis of Phosphatidic Acid." *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 33 (50): 19470–79.

Andersen, Stig Uggerhøj, Sabine Buechel, Zhong Zhao, Karin Ljung, Ondrej Novák, Wolfgang Busch, Christoph Schuster, and Jan U. Lohmann. 2008. "Requirement of B2-Type Cyclin-Dependent Kinases for Meristem Integrity in Arabidopsis Thaliana." *The Plant Cell* 20 (1): 88–100.

Angelini, Jindřiška, Stanislav Vosolsobě, Petr Skůpa, Angela Yeuan Yen Ho, Erica Bellinvia, Olga Valentová, and Jan Marc. 2018. "Phospholipase Dδ Assists to Cortical Microtubule Recovery after Salt Stress." *Protoplasma* 255 (4): 1195–1204.

Atkins, Paul Ap, and Daniel F. Voytas. 2020. "Overcoming Bottlenecks in Plant Gene Editing." *Current Opinion in Plant Biology* 54 (April): 79–84.

Aubert, Benoit A., Andreas Schroeder, and Jonathan Grimaudo. 2012. "IT as Enabler of Sustainable Farming: An Empirical Analysis of Farmers' Adoption Decision of Precision Agriculture Technology." *Decision Support Systems* 54 (1): 510–20.

Avila, Luis M., Diego Cerrudo, Clarence Swanton, and Lewis Lukens. 2016. "Brevis Plant1, a Putative Inositol Polyphosphate 5-Phosphatase, Is Required for Internode Elongation in Maize." *Journal of Experimental Botany* 67 (5): 1577–88.

Baligar, V. C., N. K. Fageria, and Z. L. He. 2001. "Nutrient Use Efficiency in Plants." *Communications in Soil Science and Plant Analysis* 32 (7–8): 921–50.

Baltes, Nicholas J., Javier Gil-Humanes, and Daniel F. Voytas. 2017. "Genome Engineering and Agriculture: Opportunities and Challenges." *Progress in Molecular Biology and Translational Science* 149 (May): 1–26.

Barbosa, Inês Cr, and Claus Schwechheimer. 2014. “Dynamic Control of Auxin Transport-Dependent Growth by AGCVIII Protein Kinases.” *Current Opinion in Plant Biology* 22 (December): 108–15.

Barton, M. K. 2010. “Twenty Years on: The Inner Workings of the Shoot Apical Meristem, a Developmental Dynamo.” *Developmental Biology* 341 (1): 95–113.

Bell, Elizabeth M., Wan-Ching Lin, Aman Y. Husbands, Lifeng Yu, Venkateswari Jaganatha, Barbara Jablonska, Amanda Mangeon, Michael M. Neff, Thomas Girke, and Patricia S. Springer. 2012. “Arabidopsis Lateral Organ Boundaries Negatively Regulates Brassinosteroid Accumulation to Limit Growth in Organ Boundaries.” *Proceedings of the National Academy of Sciences of the United States of America* 109 (51): 21146–51.

Benková, Eva, Marta Michniewicz, Michael Sauer, Thomas Teichmann, Daniela Seifertová, Gerd Jürgens, and Jirí Friml. 2003. “Local, Efflux-Dependent Auxin Gradients as a Common Module for Plant Organ Formation.” *Cell* 115 (5): 591–602.

Berry, P. M., and J. Spink. 2012. “Predicting Yield Losses Caused by Lodging in Wheat.” *Field Crops Research* 137 (October): 19–26.

Bhandari, Deepali, Jinzhong Zhang, Shekar Menon, Christopher Lord, Shuliang Chen, Jared R. Helm, Kevin Thorsen, Kevin D. Corbett, Jesse C. Hay, and Susan Ferro-Novick. 2013. “Sit4p/PP6 Regulates ER-to-Golgi Traffic by Controlling the Dephosphorylation of COPII Coat Subunits.” *Molecular Biology of the Cell* 24 (17): 2727–38.

Billot, Claire, Punna Ramu, Sophie Bouchet, Jacques Chantereau, Monique Deu, Laetitia Gardes, Jean-Louis Noyer, et al. 2013. “Massive Sorghum Collection Genotyped with SSR Markers to Enhance Use of Global Genetic Resources.” *PloS One* 8 (4): e59714.

Bilsborough, Gemma D., Adam Runions, Michalis Barkoulas, Huw W. Jenkins, Alice Hasson, Carla Galinha, Patrick Laufs, Angela Hay, Przemyslaw Prusinkiewicz, and Miltos Tsiantis. 2011. “Model for the Regulation of Arabidopsis Thaliana Leaf Margin Development.” *Proceedings of the National Academy of Sciences of the United States of America* 108 (8): 3424–29.

Binns, Andrew N. 1990. “Agrobacterium-Mediated Gene Delivery and the Biology of Hostrange Limitations.” *Physiologia Plantarum* 79 (1): 135–39.

Blethrow, Justin D., Chao Tang, Changhui Deng, and Andrew N. Krutchinsky. 2007. “Modular Mass Spectrometric Tool for Analysis of Composition and Phosphorylation of Protein Complexes.” *PloS One* 2 (4): e358.

Borlaug, Norman E. 1958. “Ii. The Impact of Agricultural Research on Mexican Wheat Production.” *Transactions of the New York Academy of Sciences* 20 (3 Series II): 278–95.

Bortiri, Esteban, George Chuck, Erik Vollbrecht, Torbert Rocheford, Rob Martienssen, and Sarah Hake. 2006. “Ramosa2 Encodes a LATERAL ORGAN BOUNDARY Domain Protein That Determines the Fate of Stem Cells in Branch Meristems of Maize.” *The Plant Cell* 18 (3): 574–85.

Buschmann, Henrik, Jacqueline Dols, Sarah Kopischke, Eduardo J. Peña, Miguel A. Andrade-Navarro, Manfred Heinlein, Daniel B. Szymanski, Sabine Zachgo, John H. Doonan, and Clive W. Lloyd. 2015. "Arabidopsis KCBP Interacts with AIR9 but Stays in the Cortical Division Zone throughout Mitosis via Its MyTH4-FERM Domain." *Journal of Cell Science* 128 (11): 2033–46.

Carlson, Scott M., and Forest M. White. 2012. "Labeling and Identification of Direct Kinase Substrates." *Science Signaling* 5 (227): l3.

Carraro, Nicola, and Wendy Ann Peer. 2016. "Immunolocalization of PIN and ABCB Transporters in Plants." *Methods in Molecular Biology (Clifton, N.J.)* 1398: 55–67.

Casto, Anna L., Brian A. McKinley, Ka Man Jasmine Yu, William L. Rooney, and John E. Mullet. 2018. "Sorghum Stem Aerenchyma Formation Is Regulated by SbNAC_D during Internode Development." *Plant Direct* 2 (11): e00085.

Chang, Tian-Gen, and Xin-Guang Zhu. 2017. "Source–Sink Interaction: A Century Old Concept under the Light of Modern Molecular Systems Biology." *Journal of Experimental Botany* 68 (16): 4417–31.

Chevalier, David, Martine Batoux, Lynette Fulton, Karen Pfister, Ram Kishor Yadav, Maja Schellenberg, and Kay Schneitz. 2005. "STRUBBELIG Defines a Receptor Kinase-Mediated Signaling Pathway Regulating Organ Development in Arabidopsis." *Proceedings of the National Academy of Sciences of the United States of America* 102 (25): 9074–79.

Christie, John M., Noriyuki Suetsugu, Stuart Sullivan, and Masamitsu Wada. 2018. "Shining Light on the Function of NPH3/RPT2-like Proteins in Phototropin Signaling." *Plant Physiology* 176 (2): 1015–24.

Clore, Amy M. 2013. "Cereal Grass Pulvini: Agronomically Significant Models for Studying Gravitropism Signaling and Tissue Polarity." *American Journal of Botany* 100 (1): 101–10.

Day, I. S., C. Miller, M. Golovkin, and A. S. Reddy. 2000. "Interaction of a Kinesin-like Calmodulin-Binding Protein with a Protein Kinase." *The Journal of Biological Chemistry* 275 (18): 13737–45.

Demirer, Gozde S., Huan Zhang, Natalie S. Goh, Eduardo González-Grandío, and Markita P. Landry. 2019. "Carbon Nanotube-Mediated DNA Delivery without Transgene Integration in Intact Plants." *Nature Protocols* 14 (10): 2954–71.

Diamond, Jared. 2002. "Evolution, Consequences and Future of Plant and Animal Domestication." *Nature* 418 (6898): 700–707.

Dill, A., H. S. Jung, and T. P. Sun. 2001. "The DELLA Motif Is Essential for Gibberellin-Induced Degradation of RGA." *Proceedings of the National Academy of Sciences of the United States of America* 98 (24): 14162–67.

Dissmeyer, Nico, and Arp Schnittger. 2011. "Use of Phospho-Site Substitutions to Analyze the Biological Relevance of Phosphorylation Events in Regulatory Networks." *Methods in Molecular Biology (Clifton, N.J.)* 779: 93–138.

Doyle, Cara, Katie Higginbottom, Thomas A. Swift, Mark Winfield, Christopher Bellas, David Benito-Alifonso, Taryn Fletcher, M. Carmen Galan, Keith Edwards, and

Heather M. Whitney. 2019. “A Simple Method for Spray-on Gene Editing in Planta.” *BioRxiv*. bioRxiv. https://doi.org/10.1101/805036.
Dubots, Emmanuelle, Magali Audry, Yoshiki Yamaryo, Olivier Bastien, Hiroyuki Ohta, Christelle Breton, Eric Maréchal, and Maryse A. Block. 2010. “Activation of the Chloroplast Monogalactosyldiacylglycerol Synthase MGD1 by Phosphatidic Acid and Phosphatidylglycerol.” *The Journal of Biological Chemistry* 285 (9): 6003–11.
Ehrlich, Paul R. 1971. *The Population Bomb*. London, England: Macmillan.
Ellison, Evan E., Ugrappa Nagalakshmi, Maria Elena Gamo, Pin-Jui Huang, Savithramma Dinesh-Kumar, and Daniel F. Voytas. 2020. “Multiplexed Heritable Gene Editing Using RNA Viruses and Mobile Single Guide RNAs.” *Nature Plants* 6 (6): 620–24.
Eng, Ryan Christopher, and Geoffrey O. Wasteneys. 2014. “The Microtubule Plus-End Tracking Protein ARMADILLO-REPEAT KINESIN1 Promotes Microtubule Catastrophe in Arabidopsis.” *The Plant Cell* 26 (8): 3372–86.
Fabian, T., R. Lorbiecke, M. Umeda, and M. Sauter. 2000. “The Cell Cycle Genes CycA1;1 and Cdc2Os-3 Are Coordinately Regulated by Gibberellin in Planta.” *Planta* 211 (3): 376–83.
Fan, Lusheng, Ruili Li, Jianwei Pan, Zhaojun Ding, and Jinxing Lin. 2015. “Endocytosis and Its Regulation in Plants.” *Trends in Plant Science* 20 (6): 388–97.
Fan, Mingzhu, Klaus Herburger, Jacob K. Jensen, Starla Zemelis-Durfee, Federica Brandizzi, Stephen C. Fry, and Curtis G. Wilkerson. 2018. “A Trihelix Family Transcription Factor Is Associated with Key Genes in Mixed-Linkage Glucan Accumulation.” *Plant Physiology* 178 (3): 1207–21.
Foley, Jonathan A., Navin Ramankutty, Kate A. Brauman, Emily S. Cassidy, James S. Gerber, Matt Johnston, Nathaniel D. Mueller, et al. 2011. “Solutions for a Cultivated Planet.” *Nature* 478 (7369): 337–42.
Fristedt, Rikard, Wioleta Wasilewska, Elzbieta Romanowska, and Alexander V. Vener. 2012. “Differential Phosphorylation of Thylakoid Proteins in Mesophyll and Bundle Sheath Chloroplasts from Maize Plants Grown under Low or High Light.” *Proteomics* 12 (18): 2852–61.
Gardiner, John C., John D. I. Harper, Nimalika D. Weerakoon, David A. Collings, Sian Ritchie, Simon Gilroy, Richard J. Cyr, and Jan Marc. 2001. “A 90-KD Phospholipase D from Tobacco Binds to Microtubules and the Plasma Membrane.” *The Plant Cell* 13 (9): 2143.
Geldner, Niko, Nadine Anders, Hanno Wolters, Jutta Keicher, Wolfgang Kornberger, Philippe Muller, Alain Delbarre, Takashi Ueda, Akihiko Nakano, and Gerd Jürgens. 2003. “The Arabidopsis GNOM ARF-GEF Mediates Endosomal Recycling, Auxin Transport, and Auxin-Dependent Plant Growth.” *Cell* 112 (2): 219–30.
Gilbertson, Robert L., Mysore Sudarshana, Hao Jiang, Maria R. Rojas, and William J. Lucas. 2003. “Limitations on Geminivirus Genome Size Imposed by Plasmodesmata and Virus-Encoded Movement Protein: Insights into DNA Trafficking.” *The Plant Cell* 15 (11): 2578–91.

Goodman, Brian S., Nathan D. Derr, and Samara L. Reck-Peterson. 2012. “Engineered, Harnessed, and Hijacked: Synthetic Uses for Cytoskeletal Systems.” *Trends in Cell Biology* 22 (12): 644–52.

Graham, Doyce, and K. J. Lessman. 1966. “Effect of Height on Yield and Yield Components of Two Isogenic Lines of Sorghum Vulgare Pers. 1.” *Crop Science* 6 (4): 372–74.

Hamant, Olivier, Marcus G. Heisler, Henrik Jönsson, Pawel Krupinski, Magalie Uyttewaal, Plamen Bokov, Francis Corson, et al. 2008. “Developmental Patterning by Mechanical Signals in Arabidopsis.” *Science (New York, N.Y.)* 322 (5908): 1650–55.

Harashima, Hirofumi, Nico Dissmeyer, Philippe Hammann, Yuko Nomura, Katharina Kramer, Hirofumi Nakagami, and Arp Schnittger. 2016. “Modulation of Plant Growth in Vivo and Identification of Kinase Substrates Using an Analog-Sensitive Variant of CYCLIN-DEPENDENT KINASE A;1.” *BMC Plant Biology* 16 (1): 209.

Harlan, J. R., and J. M. J. Wet. 1972. “A Simplified Classification of Cultivated Sorghum 1.” *Crop Science* 12 (2): 172–76.

Hay, Angela, Michalis Barkoulas, and Miltos Tsiantis. 2006. “ASYMMETRIC LEAVES1 and Auxin Activities Converge to Repress BREVIPEDICELLUS Expression and Promote Leaf Development in Arabidopsis.” *Development (Cambridge, England)* 133 (20): 3955–61.

Hazell, P., and S. Fan. 2009. “Balancing Regional Development Priorities to Achieve Sustainable and Equitable Agricultural Growth.” In *Tradeoffs or Synergies? Agricultural Intensification, Economic Development and the Environment*, 151–70. Wallingford: CABI.

Henrichs, Sina, Bangjun Wang, Yoichiro Fukao, Jinsheng Zhu, Laurence Charrier, Aurélien Bailly, Sophie C. Oehring, et al. 2012. “Regulation of ABCB1/PGP1-Catalysed Auxin Transport by Linker Phosphorylation.” *The EMBO Journal* 31 (13): 2965–80.

Higashi-Fujime, S., R. Ishikawa, H. Iwasawa, O. Kagami, E. Kurimoto, K. Kohama, and T. Hozumi. 1995. “The Fastest Actin-Based Motor Protein from the Green Algae, Chara, and Its Distinct Mode of Interaction with Actin.” *FEBS Letters* 375 (1–2): 151–54.

Higgins, Vaughan, Melanie Bryant, Andrea Howell, and Jane Battersby. 2017. “Ordering Adoption: Materiality, Knowledge and Farmer Engagement with Precision Agriculture Technologies.” *Journal of Rural Studies* 55 (October): 193–202.

Hilley, Josie L., Brock D. Weers, Sandra K. Truong, Ryan F. McCormick, Ashley J. Mattison, Brian A. McKinley, Daryl T. Morishige, and John E. Mullet. 2017. “Sorghum Dw2 Encodes a Protein Kinase Regulator of Stem Internode Length.” *Scientific Reports* 7 (1): 4616.

Hilley, Josie, Sandra Truong, Sara Olson, Daryl Morishige, and John Mullet. 2016. “Identification of Dw1, a Regulator of Sorghum Stem Internode Length.” *PloS One* 11 (3): e0151271.

Hillier, Jonathan, Carly Whittaker, Gordon Dailey, Matthew Aylott, Eric Casella, Goetz M. Richter, Andrew Riche, Richard Murphy, Gail Taylor, and Pete Smith. 2009. "Greenhouse Gas Emissions from Four Bioenergy Crops in England and Wales: Integrating Spatial Estimates of Yield and Soil Carbon Balance in Life Cycle Analyses." *Global Change Biology. Bioenergy* 1 (4): 267–81.

Hirano, Ko, Mayuko Kawamura, Satoko Araki-Nakamura, Haruka Fujimoto, Kozue Ohmae-Shinohara, Miki Yamaguchi, Akihiro Fujii, Hiroaki Sasaki, Shigemitsu Kasuga, and Takashi Sazuka. 2017. "Sorghum DW1 Positively Regulates Brassinosteroid Signaling by Inhibiting the Nuclear Localization of BRASSINOSTEROID INSENSITIVE 2." *Scientific Reports* 7 (1). https://doi.org/10.1038/s41598-017-00096-w.

Hirano, Tomoko, Hiroki Konno, Seiji Takeda, Liam Dolan, Mariko Kato, Takashi Aoyama, Takumi Higaki, Hisako Takigawa-Imamura, and Masa H. Sato. 2018. "PtdIns(3,5)P2 Mediates Root Hair Shank Hardening in Arabidopsis." *Nature Plants* 4 (11): 888–97.

Hirt, Heribert, Ana V. Garcia, and Ralf Oelmüller. 2011. "AGC Kinases in Plant Development and Defense." *Plant Signaling & Behavior* 6 (7): 1030–33.

Ho, Angela Y. Y., David A. Day, Melissa H. Brown, and Jan Marc. 2009. "Arabidopsis Phospholipase Dδ as an Initiator of Cytoskeleton-Mediated Signalling to Fundamental Cellular Processes." *Functional Plant Biology: FPB* 36 (2): 190–98.

Hong, Yueyun, Jian Zhao, Liang Guo, Sang-Chul Kim, Xianjun Deng, Geliang Wang, Gaoyang Zhang, Maoyin Li, and Xuemin Wang. 2016. "Plant Phospholipases D and C and Their Diverse Functions in Stress Responses." *Progress in Lipid Research* 62 (April): 55–74.

Houle, David, Diddahally R. Govindaraju, and Stig Omholt. 2010. "Phenomics: The next Challenge." *Nature Reviews. Genetics* 11 (12): 855–66.

Huang, Jin, Ratna Ghosh, and Vytas A. Bankaitis. 2016. "Sec14-like Phosphatidylinositol Transfer Proteins and the Biological Landscape of Phosphoinositide Signaling in Plants." *Biochimica et Biophysica Acta* 1861 (9 Pt B): 1352–64.

Huang, Teng-Kuei, and Holger Puchta. 2019. "CRISPR/Cas-Mediated Gene Targeting in Plants: Finally a Turn for the Better for Homologous Recombination." *Plant Cell Reports* 38 (4): 443–53.

Humphrey, Sean J., Ozge Karayel, David E. James, and Matthias Mann. 2018. "High-Throughput and High-Sensitivity Phosphoproteomics with the EasyPhos Platform." *Nature Protocols* 13 (9): 1897–1916.

Humphrey, Tania V., Katrina E. Haasen, May Grace Aldea-Brydges, He Sun, Yara Zayed, Emily Indriolo, and Daphne R. Goring. 2015. "PERK-KIPK-KCBP Signalling Negatively Regulates Root Growth in Arabidopsis Thaliana." *Journal of Experimental Botany* 66 (1): 71–83.

International Brachypodium Initiative. 2010. "Genome Sequencing and Analysis of the Model Grass Brachypodium Distachyon." *Nature* 463 (7282): 763–68.

Ito, Kohji, Mitsuo Ikebe, Taku Kashiyama, Toshifumi Mogami, Takahide Kon, and Keiichi Yamamoto. 2007. “Kinetic Mechanism of the Fastest Motor Protein, Chara Myosin.” *The Journal of Biological Chemistry* 282 (27): 19534–45.

Ito, Kohji, Taku Kashiyama, Kiyo Shimada, Akira Yamaguchi, Jun ya Awata, You Hachikubo, Dietmar J. Manstein, and Keiichi Yamamoto. 2003. “Recombinant Motor Domain Constructs of Chara Corallina Myosin Display Fast Motility and High ATPase Activity.” *Biochemical and Biophysical Research Communications* 312 (4): 958–64.

Jackson, Stephen D., and Yiguo Hong. 2012. “Systemic Movement of FT MRNA and a Possible Role in Floral Induction.” *Frontiers in Plant Science* 3 (June): 127.

Johnston, Robyn, Samuel Leiboff, and Michael J. Scanlon. 2015. “Ontogeny of the Sheathing Leaf Base in Maize (Zea Mays).” *The New Phytologist* 205 (1): 306–15.

Joo, Junghee H., Shiyu Wang, J. G. Chen, A. M. Jones, and Nina V. Fedoroff. 2005. “Different Signaling and Cell Death Roles of Heterotrimeric G Protein Alpha and Beta Subunits in the Arabidopsis Oxidative Stress Response to Ozone.” *The Plant Cell* 17 (3): 957–70.

Jordan, W. R., P. J. Shouse, A. Blum, F. R. Miller, and R. L. Monk. 1984. “Environmental Physiology of Sorghum. II. Epicuticular Wax Load and Cuticular Transpiration 1.” *Crop Science* 24 (6): 1168–73.

Kang, Byung-Ho, and L. Andrew Staehelin. 2008. “ER-to-Golgi Transport by COPII Vesicles in Arabidopsis Involves a Ribosome-Excluding Scaffold That Is Transferred with the Vesicles to the Golgi Matrix.” *Protoplasma* 234 (1–4): 51–64.

Karlgren, Anna, Jenny Carlsson, Niclas Gyllenstrand, Ulf Lagercrantz, and Jens F. Sundström. 2009. “Non-Radioactive in Situ Hybridization Protocol Applicable for Norway Spruce and a Range of Plant Species.” *Journal of Visualized Experiments: JoVE*, no. 26 (April). https://doi.org/10.3791/1205.

Kasai, Koji, Junpei Takano, Kyoko Miwa, Atsushi Toyoda, and Toru Fujiwara. 2011. “High Boron-Induced Ubiquitination Regulates Vacuolar Sorting of the BOR1 Borate Transporter in Arabidopsis Thaliana.” *The Journal of Biological Chemistry* 286 (8): 6175–83.

Kebrom, Tesfamichael H., Brian McKinley, and John E. Mullet. 2017. “Dynamics of Gene Expression during Development and Expansion of Vegetative Stem Internodes of Bioenergy Sorghum.” *Biotechnology for Biofuels* 10 (1): 159.

Kebrom, Tesfamichael H., and Richard A. Richards. 2013. “Physiological Perspectives of Reduced Tillering and Stunting in the Tiller Inhibition (Tin) Mutant of Wheat.” *Functional Plant Biology: FPB* 40 (10): 977–85.

Kellogg, E. A. 2001. “Evolutionary History of the Grasses.” *Plant Physiology* 125 (3): 1198–1205.

Kido, Natsumi, Ryusuke Yokoyama, Tsuyoshi Yamamoto, Jun Furukawa, Hiroaki Iwai, Shinobu Satoh, and Kazuhiko Nishitani. 2015. “The Matrix Polysaccharide (1;3,1;4)-β-D-Glucan Is Involved in Silicon-Dependent Strengthening of Rice Cell Wall.” *Plant & Cell Physiology* 56 (8): 1679.

Kim, Daehwan, Ben Langmead, and Steven L. Salzberg. 2015. “HISAT: A Fast Spliced Aligner with Low Memory Requirements.” *Nature Methods* 12 (4): 357–60.
Kim, Sang-jin, Starla Zemelis-Durfee, Jacob Krüger Jensen, Curtis G. Wilkerson, Kenneth Keegstra, and Federica Brandizzi. 2018. “In the Grass Species Brachypodium Distachyon , the Production of Mixed-linkage (1,3;1,4)-β-glucan (MLG) Occurs in the Golgi Apparatus.” *The Plant Journal: For Cell and Molecular Biology* 93 (6): 1062–75.
King, Stephen M. 2002. “Dyneins Motor on in Plants.” *Traffic (Copenhagen, Denmark)* 3 (12): 930–31.
Kirchhelle, Charlotte, Cheung-Ming Chow, Camille Foucart, Helia Neto, York-Dieter Stierhof, Monika Kalde, Carol Walton, et al. 2016. “The Specification of Geometric Edges by a Plant Rab GTPase Is an Essential Cell-Patterning Principle during Organogenesis in Arabidopsis.” *Developmental Cell* 36 (4): 386–400.
Kivivirta, Kimmo, Denise Herbert, Matthias Lange, Knut Beuerlein, Janine Altmüller, and Annette Becker. 2019. “A Protocol for Laser Microdissection (LMD) Followed by Transcriptome Analysis of Plant Reproductive Tissue in Phylogenetically Distant Angiosperms.” *Plant Methods* 15 (1): 151.
Knöller, Anne Sophie, Joshua J. Blakeslee, Elizabeth L. Richards, Wendy Ann Peer, and Angus S. Murphy. 2010. “Brachytic2/ZmABCB1 Functions in IAA Export from Intercalary Meristems.” *Journal of Experimental Botany* 61 (13): 3689–96.
Koizumi, Koji, Shuang Wu, Aurora MacRae-Crerar, and Kimberly L. Gallagher. 2011. “An Essential Protein That Interacts with Endosomes and Promotes Movement of the SHORT-ROOT Transcription Factor.” *Current Biology: CB* 21 (18): 1559–64.
Kulak, Nils A., Garwin Pichler, Igor Paron, Nagarjuna Nagaraj, and Matthias Mann. 2014. “Minimal, Encapsulated Proteomic-Sample Processing Applied to Copy-Number Estimation in Eukaryotic Cells.” *Nature Methods* 11 (3): 319–24.
Lacroix, Benoît, and Vitaly Citovsky. 2019. “Pathways of DNA Transfer to Plants from Agrobacterium Tumefaciens and Related Bacterial Species.” *Annual Review of Phytopathology* 57 (1): 231–51.
Lam, Bernard C-H, Tammy L. Sage, Fabrizio Bianchi, and Eduardo Blumwald. 2001. “Role of SH3 Domain-Containing Proteins in Clathrin-Mediated Vesicle Trafficking in Arabidopsis.” *The Plant Cell* 13 (11): 2499.
Lam, Sheung Kwan, Yi Cai, Yu Chung Tse, Juan Wang, Angus Ho Yin Law, Peter Pimpl, Ho Yin Edwin Chan, Jun Xia, and Liwen Jiang. 2009. “BFA-Induced Compartments from the Golgi Apparatus and Trans-Golgi Network/Early Endosome Are Distinct in Plant Cells.” *The Plant Journal: For Cell and Molecular Biology* 60 (5): 865–81.
Łangowski, Łukasz, Kamil Růžička, Satoshi Naramoto, Jürgen Kleine-Vehn, and Jiří Friml. 2010. “Trafficking to the Outer Polar Domain Defines the Root-Soil Interface.” *Current Biology: CB* 20 (10): 904–8.
Latijnhouwers, Maita, Trudi Gillespie, Petra Boevink, Verena Kriechbaumer, Chris Hawes, and Claudine M. Carvalho. 2007. “Localization and Domain

Characterization of Arabidopsis Golgin Candidates." *Journal of Experimental Botany* 58 (15–16): 4373–86.
Lehmann, Caroline E. R., Daniel M. Griffith, Kimberley J. Simpson, T. Michael Anderson, Sally Archibald, David J. Beerling, William J. Bond, et al. 2019. "Functional Diversification Enabled Grassy Biomes to Fill Global Climate Space." *BioRxiv*. bioRxiv. https://doi.org/10.1101/583625.
Levey, Tatjana, Peter Westhoff, and Karin Meierhoff. 2014. "Expression of a Nuclear-Encoded PsbH Gene Complements the Plastidic RNA Processing Defect in the PSII Mutant Hcf107 in Arabidopsis Thaliana." *The Plant Journal: For Cell and Molecular Biology* 80 (2): 292–304.
Li, Chunyang, Ke Zhang, Xianwu Zeng, Stephen Jackson, Yu Zhou, and Yiguo Hong. 2009. "A Cis Element within Flowering Locus T MRNA Determines Its Mobility and Facilitates Trafficking of Heterologous Viral RNA." *Journal of Virology* 83 (8): 3540–48.
Li, Weiqi, Maoyin Li, Wenhua Zhang, Ruth Welti, and Xuemin Wang. 2004. "The Plasma Membrane-Bound Phospholipase Ddelta Enhances Freezing Tolerance in Arabidopsis Thaliana." *Nature Biotechnology* 22 (4): 427–33.
Li, Wenyu, Tengzhao Song, Lukas Wallrad, Jörg Kudla, Xuemin Wang, and Wenhua Zhang. 2019. "Tissue-Specific Accumulation of PH-Sensing Phosphatidic Acid Determines Plant Stress Tolerance." *Nature Plants* 5 (9): 1012–21.
Lingle, Sarah E. 1987. "Sucrose Metabolism in the Primary Culm of Sweet Sorghum during Development 1." *Crop Science* 27 (6): 1214–19.
Linke, Heiner, Birte Höcker, Ken'ya Furuta, Nancy R. Forde, and Paul M. G. Curmi. 2020. "Synthetic Biology Approaches to Dissecting Linear Motor Protein Function: Towards the Design and Synthesis of Artificial Autonomous Protein Walkers." *Biophysical Reviews* 12 (4): 1041–54.
Liu, Guoquan, Yan Zhang, Hao Gong, Shan Li, Yunrong Pan, Christopher Davis, Hai-Chun Jing, Luguang Wu, and Ian D. Godwin. 2021. "Stem Vacuole-Targetted Sucrose Isomerase Enhances Sugar Content in Sorghum." *Biotechnology for Biofuels* 14 (1): 53.
Longoni, Paolo, Iga Samol, and Michel Goldschmidt-Clermont. 2019. "The Kinase STATE TRANSITION 8 Phosphorylates Light Harvesting Complex II and Contributes to Light Acclimation in Arabidopsis Thaliana." *Frontiers in Plant Science* 10 (September): 1156.
Lopez, Michael S., Joseph I. Kliegman, and Kevan M. Shokat. 2014. "The Logic and Design of Analog-Sensitive Kinases and Their Small Molecule Inhibitors." *Methods in Enzymology* 548: 189–213.
Lowe, Keith, Emily Wu, Ning Wang, George Hoerster, Craig Hastings, Myeong-Je Cho, Chris Scelonge, et al. 2016. "Morphogenic Regulators Baby Boom and Wuschel Improve Monocot Transformation." *The Plant Cell* 28 (9): 1998–2015.
Lunn, J. E., and T. ap Rees. 1990. "Apparent Equilibrium Constant and Mass-Action Ratio for Sucrose-Phosphate Synthase in Seeds of Pisum Sativum." *The Biochemical Journal* 267 (3): 739–43.

Lunn, John E., and Elspeth MacRae. 2003. “New Complexities in the Synthesis of Sucrose.” *Current Opinion in Plant Biology* 6 (3): 208–14.
Luo, Kai-Ren, Nien-Chen Huang, and Tien-Shin Yu. 2018. “Selective Targeting of Mobile MRNAs to Plasmodesmata for Cell-to-Cell Movement.” *Plant Physiology* 177 (2): 604–14.
Mace, Emma S., Shuaishuai Tai, Edward K. Gilding, Yanhong Li, Peter J. Prentis, Lianle Bian, Bradley C. Campbell, et al. 2013. “Whole-Genome Sequencing Reveals Untapped Genetic Potential in Africa’s Indigenous Cereal Crop Sorghum.” *Nature Communications* 4 (1): 2320.
Maher, Michael F., Ryan A. Nasti, Macy Vollbrecht, Colby G. Starker, Matthew D. Clark, and Daniel F. Voytas. 2020. “Plant Gene Editing through de Novo Induction of Meristems.” *Nature Biotechnology* 38 (1): 84–89.
Maloney, Victoria J., Ji-Young Park, Faride Unda, and Shawn D. Mansfield. 2015. “Sucrose Phosphate Synthase and Sucrose Phosphate Phosphatase Interact in Planta and Promote Plant Growth and Biomass Accumulation.” *Journal of Experimental Botany* 66 (14): 4383–94.
Mara, Kostlend, Florence Charlot, Anouchka Guyon-Debast, Didier G. Schaefer, Cécile Collonnier, Mathilde Grelon, and Fabien Nogué. 2019. “POLQ Plays a Key Role in the Repair of CRISPR/Cas9-Induced Double-Stranded Breaks in the Moss Physcomitrella Patens.” *The New Phytologist* 222 (3): 1380–91.
Marc, J., D. E. Sharkey, N. A. Durso, M. Zhang, and R. J. Cyr. 1996. “Isolation of a 90-KD Microtubule-Associated Protein from Tobacco Membranes.” *The Plant Cell* 8 (11): 2127–38.
Mathé, Ewy, John L. Hays, Daniel G. Stover, and James L. Chen. 2018. “The Omics Revolution Continues: The Maturation of High-Throughput Biological Data Sources.” *Yearbook of Medical Informatics* 27 (1): 211–22.
McBee, George G., and F. R. Miller. 1982. “Carbohydrates in Sorghum Culms as Influenced by Cultivars, Spacing, and Maturity over a Diurnal Period 1.” *Crop Science* 22 (2): 381–85.
McCormick, Ryan F., Sandra K. Truong, Avinash Sreedasyam, Jerry Jenkins, Shengqiang Shu, David Sims, Megan Kennedy, et al. 2018. “The Sorghum Bicolor Reference Genome: Improved Assembly, Gene Annotations, a Transcriptome Atlas, and Signatures of Genome Organization.” *The Plant Journal: For Cell and Molecular Biology* 93 (2): 338–54.
McKim, Sarah M. 2019. “How Plants Grow Up.” *Journal of Integrative Plant Biology* 61 (3): 257–77.
———. 2020. “Moving on up - Controlling Internode Growth.” *The New Phytologist* 226 (3): 672–78.
McKinley, Brian A., Anna L. Casto, William L. Rooney, and John E. Mullet. 2018. “Developmental Dynamics of Stem Starch Accumulation in Sorghum Bicolor.” *Plant Direct* 2 (8): e00074.
McLoughlin, Fionn, Robert C. Augustine, Richard S. Marshall, Faqiang Li, Liam D. Kirkpatrick, Marisa S. Otegui, and Richard D. Vierstra. 2018. “Maize Multi-

Omics Reveal Roles for Autophagic Recycling in Proteome Remodelling and Lipid Turnover." *Nature Plants* 4 (12): 1056–70.
Meyer, Rachel S., Ashley E. DuVal, and Helen R. Jensen. 2012. "Patterns and Processes in Crop Domestication: An Historical Review and Quantitative Analysis of 203 Global Food Crops." *The New Phytologist* 196 (1): 29–48.
Milla, Rubén, Jesús M. Bastida, Martin M. Turcotte, Glynis Jones, Cyrille Violle, Colin P. Osborne, Julia Chacón-Labella, et al. 2018. "Phylogenetic Patterns and Phenotypic Profiles of the Species of Plants and Mammals Farmed for Food." *Nature Ecology & Evolution* 2 (11): 1808–17.
Monna, Lisa, Noriyuki Kitazawa, Rika Yoshino, Junko Suzuki, Haruka Masuda, Yumiko Maehara, Masao Tanji, Mizuho Sato, Shinobu Nasu, and Yuzo Minobe. 2002. "Positional Cloning of Rice Semidwarfing Gene, Sd-1: Rice 'Green Revolution Gene' Encodes a Mutant Enzyme Involved in Gibberellin Synthesis." *DNA Research: An International Journal for Rapid Publication of Reports on Genes and Genomes* 9 (1): 11–17.
Mora, Alfonso, David Komander, Daan M. F. van Aalten, and Dario R. Alessi. 2004. "PDK1, the Master Regulator of AGC Kinase Signal Transduction." *Seminars in Cell & Developmental Biology* 15 (2): 161–70.
Morishige, Daryl T., Patricia E. Klein, Josie L. Hilley, Sayed Mohammad Ebrahim Sahraeian, Arun Sharma, and John E. Mullet. 2013. "Digital Genotyping of Sorghum - a Diverse Plant Species with a Large Repeat-Rich Genome." *BMC Genomics* 14 (July): 448.
Mullet, John, Daryl Morishige, Ryan McCormick, Sandra Truong, Josie Hilley, Brian McKinley, Robert Anderson, Sara N. Olson, and William Rooney. 2014. "Energy Sorghum--a Genetic Model for the Design of C4 Grass Bioenergy Crops." *Journal of Experimental Botany* 65 (13): 3479–89.
Multani, Dilbag S., Steven P. Briggs, Mark A. Chamberlin, Joshua J. Blakeslee, Angus S. Murphy, and Gurmukh S. Johal. 2003. "Loss of an MDR Transporter in Compact Stalks of Maize Br2 and Sorghum Dw3 Mutants." *Science (New York, N.Y.)* 302 (5642): 81–84.
Munnik, T., S. A. Arisz, T. De Vrije, and A. Musgrave. 1995. "G Protein Activation Stimulates Phospholipase D Signaling in Plants." *The Plant Cell* 7 (12): 2197–2210.
Nebenführ, Andreas, and Ram Dixit. 2018. "Kinesins and Myosins: Molecular Motors That Coordinate Cellular Functions in Plants." *Annual Review of Plant Biology* 69 (April): 329–61.
Noh, B., A. S. Murphy, and E. P. Spalding. 2001. "Multidrug Resistance-like Genes of Arabidopsis Required for Auxin Transport and Auxin-Mediated Development." *The Plant Cell* 13 (11): 2441–54.
Notaguchi, Michitaka, Tetsuya Higashiyama, and Takamasa Suzuki. 2015. "Identification of MRNAs That Move over Long Distances Using an RNA-Seq Analysis of Arabidopsis/Nicotiana Benthamiana Heterografts." *Plant & Cell Physiology* 56 (2): 311–21.

Novák, Dominik, Pavol Vadovič, Miroslav Ovečka, Olga Šamajová, George Komis, Jean Colcombet, and Jozef Šamaj. 2018. “Gene Expression Pattern and Protein Localization of Arabidopsis Phospholipase D Alpha 1 Revealed by Advanced Light-Sheet and Super-Resolution Microscopy.” *Frontiers in Plant Science* 9 (March). https://doi.org/10.3389/fpls.2018.00371.

Oikawa, Ai, Christian Have Lund, Yumiko Sakuragi, and Henrik V. Scheller. 2013. “Golgi-Localized Enzyme Complexes for Plant Cell Wall Biosynthesis.” *Trends in Plant Science* 18 (1): 49–58.

Okamoto, Miwa, Kensuke Okada, Toshihiro Watanabe, and Noriharu Ae. 2003. “Growth Responses of Cereal Crops to Organic Nitrogen in the Field.” *Soil Science and Plant Nutrition* 49 (3): 445–52.

Oliver, Joel, Mingzhu Fan, Brian McKinley, Starla Zemelis-Durfee, Federica Brandizzi, Curtis Wilkerson, and John E. Mullet. 2021. “The AGCVIII Kinase Dw2 Modulates Cell Proliferation, Endomembrane Trafficking, and MLG/Xylan Cell Wall Localization in Elongating Stem Internodes of Sorghum Bicolor.” *The Plant Journal: For Cell and Molecular Biology* 105 (4): 1053–71.

Olson, Sara N., Kimberley Ritter, William Rooney, Armen Kemanian, Bruce A. McCarl, Yuquan Zhang, Susan Hall, Dan Packer, and John Mullet. 2012. “High Biomass Yield Energy Sorghum: Developing a Genetic Model for C4 Grass Bioenergy Crops.” *Biofuels, Bioproducts & Biorefining : Biofpr* 6 (6): 640–55.

Overton, Mark. 1996. “Agricultural Output and Productivity, 1500–1850.” In *Agricultural Revolution in England*, 63–132. Cambridge: Cambridge University Press.

Pappan, K., S. Austin-Brown, K. D. Chapman, and X. Wang. 1998. “Substrate Selectivities and Lipid Modulation of Plant Phospholipase D Alpha, -Beta, and -Gamma.” *Archives of Biochemistry and Biophysics* 353 (1): 131–40.

Paszkowski, J., M. Baur, A. Bogucki, and I. Potrykus. 1988. “Gene Targeting in Plants.” *The EMBO Journal* 7 (13): 4021–26.

Paterson, Andrew H., John E. Bowers, Rémy Bruggmann, Inna Dubchak, Jane Grimwood, Heidrun Gundlach, Georg Haberer, et al. 2009. “The Sorghum Bicolor Genome and the Diversification of Grasses.” *Nature* 457 (7229): 551–56.

Pathak, Hari Sharan, Philip Brown, and Talitha Best. 2019. “A Systematic Literature Review of the Factors Affecting the Precision Agriculture Adoption Process.” *Precision Agriculture* 20 (6): 1292–1316.

Paul, M. J., and C. H. Foyer. 2001. “Sink Regulation of Photosynthesis.” *Journal of Experimental Botany* 52 (360): 1383–1400.

Peng, J., D. E. Richards, N. M. Hartley, G. P. Murphy, K. M. Devos, J. E. Flintham, J. Beales, et al. 1999. “‘Green Revolution’ Genes Encode Mutant Gibberellin Response Modulators.” *Nature* 400 (6741): 256–61.

Pertea, Mihaela, Geo M. Pertea, Corina M. Antonescu, Tsung-Cheng Chang, Joshua T. Mendell, and Steven L. Salzberg. 2015. “StringTie Enables Improved Reconstruction of a Transcriptome from RNA-Seq Reads.” *Nature Biotechnology* 33 (3): 290–95.

Peterman, T. Kaye, Yamini M. Ohol, Lisa J. McReynolds, and Elizabeth J. Luna. 2004. "Patellin1, a Novel Sec14-like Protein, Localizes to the Cell Plate and Binds Phosphoinositides." *Plant Physiology* 136 (2): 3080–94; discussion 3001-2.

Pfeiffer, Brian K., Dennis Pietsch, Ronnie W. Schnell, and William L. Rooney. 2019. "Long-Term Selection in Hybrid Sorghum Breeding Programs." *Crop Science* 59 (1): 150–64.

Pinosa, Francesco, Nathalie Buhot, Mark Kwaaitaal, Per Fahlberg, Hans Thordal-Christensen, Mats Ellerström, and Mats X. Andersson. 2013. "Arabidopsis Phospholipase Dδ Is Involved in Basal Defense and Nonhost Resistance to Powdery Mildew Fungi." *Plant Physiology* 163 (2): 896–906.

Pleskot, Roman, Jiejie Li, Viktor Zárský, Martin Potocký, and Christopher J. Staiger. 2013. "Regulation of Cytoskeletal Dynamics by Phospholipase D and Phosphatidic Acid." *Trends in Plant Science* 18 (9): 496–504.

Puchta, Holger. 1998. "Repair of Genomic Double-Strand Breaks in Somatic Plant Cells by One-Sided Invasion of Homologous Sequences." *The Plant Journal: For Cell and Molecular Biology* 13 (3): 331–39.

Purugganan, Michael D., and Dorian Q. Fuller. 2009. "The Nature of Selection during Plant Domestication." *Nature* 457 (7231): 843–48.

Quinby, J. R. 1974. "The Genetic Control of Flowering and Growth in Sorghum." In *Advances in Agronomy*, 125–62. Advances in Agronomy. Elsevier.

Rademacher, Eike H., and Remko Offringa. 2012. "Evolutionary Adaptations of Plant AGC Kinases: From Light Signaling to Cell Polarity Regulation." *Frontiers in Plant Science* 3 (November): 250.

Ranathunge, Kosala, Ashraf El-Kereamy, Satinder Gidda, Yong-Mei Bi, and Steven J. Rothstein. 2014. "AMT1;1 Transgenic Rice Plants with Enhanced NH4(+) Permeability Show Superior Growth and Higher Yield under Optimal and Suboptimal NH4(+) Conditions." *Journal of Experimental Botany* 65 (4): 965–79.

Rappsilber, Juri, Matthias Mann, and Yasushi Ishihama. 2007. "Protocol for Micro-Purification, Enrichment, Pre-Fractionation and Storage of Peptides for Proteomics Using StageTips." *Nature Protocols* 2 (8): 1896–1906.

Rayburn, A. Lane, Joseph Crawford, Charlotte M. Rayburn, and John A. Juvik. 2009. "Genome Size of Three Miscanthus Species." *Plant Molecular Biology Reporter* 27 (2): 184–88.

Reinhardt, Didier, Eva-Rachele Pesce, Pia Stieger, Therese Mandel, Kurt Baltensperger, Malcolm Bennett, Jan Traas, Jirí Friml, and Cris Kuhlemeier. 2003. "Regulation of Phyllotaxis by Polar Auxin Transport." *Nature* 426 (6964): 255–60.

Richardson, Annis E., and Sarah Hake. 2018. "Drawing a Line: Grasses and Boundaries." *Plants* 8 (1). https://doi.org/10.3390/plants8010004.

Rigal, Adeline, Siamsa M. Doyle, and Stéphanie Robert. 2015. "Live Cell Imaging of FM4-64, a Tool for Tracing the Endocytic Pathways in Arabidopsis Root Cells." *Methods in Molecular Biology (Clifton, N.J.)* 1242: 93–103.

Rooney, W. L. 2004. “Sorghum Improvement—Integrating Traditional and New Technology to Produce Improved Genotypes.” In *Advances in Agronomy*, 37–109. Advances in Agronomy. Elsevier.
Rooney, W. L., and S. Aydin. 1999. “Genetic Control of a Photoperiod-sensitive Response in Sorghum Bicolor (L.) Moench.” *Crop Science* 39 (2): 397–400.
Rooney, William L., Jürg Blumenthal, Brent Bean, and John E. Mullet. 2007. “Designing Sorghum as a Dedicated Bioenergy Feedstock.” *Biofuels, Bioproducts & Biorefining : Biofpr* 1 (2): 147–57.
Sakai, Kaori, Ludivine Taconnat, Nero Borrega, Jennifer Yansouni, Véronique Brunaud, Christine Paysant-Le Roux, Etienne Delannoy, et al. 2018. “Combining Laser-Assisted Microdissection (LAM) and RNA-Seq Allows to Perform a Comprehensive Transcriptomic Analysis of Epidermal Cells of Arabidopsis Embryo.” *Plant Methods* 14 (1). https://doi.org/10.1186/s13007-018-0275-x.
Samaj, Jozef, Jens Müller, Martina Beck, Nils Böhm, and Diedrik Menzel. 2006. “Vesicular Trafficking, Cytoskeleton and Signalling in Root Hairs and Pollen Tubes.” *Trends in Plant Science* 11 (12): 594–600.
Sasaki, A., M. Ashikari, M. Ueguchi-Tanaka, H. Itoh, A. Nishimura, D. Swapan, K. Ishiyama, et al. 2002. “Green Revolution: A Mutant Gibberellin-Synthesis Gene in Rice.” *Nature* 416 (6882): 701–2.
Schmer, M. R., K. P. Vogel, R. B. Mitchell, and R. K. Perrin. 2008. “Net Energy of Cellulosic Ethanol from Switchgrass.” *Proceedings of the National Academy of Sciences of the United States of America* 105 (2): 464–69.
Scofield, Simon, Alexander Murison, Angharad Jones, John Fozard, Mitsuhiro Aida, Leah R. Band, Malcolm Bennett, and James A. H. Murray. 2018. “Coordination of Meristem and Boundary Functions by Transcription Factors in the SHOOT MERISTEMLESS Regulatory Network.” *Development (Cambridge, England)* 145 (9). https://doi.org/10.1242/dev.157081.
Shane, M. 2000. “The Vascular System of Maize Stems Revisited: Implications for Water Transport and Xylem Safety.” *Annals of Botany* 86 (2): 245–58.
Sharman, B. C. 1942. “Shoot Apex in Grasses and Cereals.” *Nature* 149 (3768): 82–83.
Shogren-Knaak, M. A., P. J. Alaimo, and K. M. Shokat. 2001. “Recent Advances in Chemical Approaches to the Study of Biological Systems.” *Annual Review of Cell and Developmental Biology* 17 (1): 405–33.
Smith, C. Wayne, and Richard A. Frederiksen, eds. 2000. *Sorghum*. Wiley Series in Crop Science. Nashville, TN: John Wiley & Sons.
Smith, Millicent R., Idupulapati M. Rao, and Andrew Merchant. 2018. “Source-Sink Relationships in Crop Plants and Their Influence on Yield Development and Nutritional Quality.” *Frontiers in Plant Science* 9 (December): 1889.
Somerville, Chris, Heather Youngs, Caroline Taylor, Sarah C. Davis, and Stephen P. Long. 2010. “Feedstocks for Lignocellulosic Biofuels.” *Science (New York, N.Y.)* 329 (5993): 790–92.
Sonkoly, Boglárka, Viola Bardóczy, and Tamás Mészáros. 2011. “Expression and Purification of Active Protein Kinases from Wheat Germ Extracts.” *Methods in Molecular Biology (Clifton, N.J.)* 779: 55–63.

Spielmeyer, Wolfgang, Marc H. Ellis, and Peter M. Chandler. 2002. “Semidwarf (Sd-1), ‘Green Revolution’ Rice, Contains a Defective Gibberellin 20-Oxidase Gene.” *Proceedings of the National Academy of Sciences of the United States of America* 99 (13): 9043–48.

Stefano, Giovanni, Luciana Renna, Clarissa Wormsbaecher, Jessie Gamble, Krzysztof Zienkiewicz, and Federica Brandizzi. 2018. “Plant Endocytosis Requires the ER Membrane-Anchored Proteins VAP27-1 and VAP27-3.” *Cell Reports* 23 (8): 2299–2307.

Sturm, A. 1999. “Invertases. Primary Structures, Functions, and Roles in Plant Development and Sucrose Partitioning.” *Plant Physiology* 121 (1): 1–8.

Sugimoto-Shirasu, Keiko, Gethin R. Roberts, Nicola J. Stacey, Maureen C. McCann, Anthony Maxwell, and Keith Roberts. 2005. “RHL1 Is an Essential Component of the Plant DNA Topoisomerase VI Complex and Is Required for Ploidy-Dependent Cell Growth.” *Proceedings of the National Academy of Sciences of the United States of America* 102 (51): 18736–41.

Sutton, A., D. Immanuel, and K. T. Arndt. 1991. “The SIT4 Protein Phosphatase Functions in Late G1 for Progression into S Phase.” *Molecular and Cellular Biology* 11 (4): 2133–48.

Tahara, H., E. Yokota, H. Igarashi, H. Orii, M. Yao, S. Sonobe, T. Hashimoto, P. J. Hussey, and T. Shimmen. 2007. “Clathrin Is Involved in Organization of Mitotic Spindle and Phragmoplast as Well as in Endocytosis in Tobacco Cell Cultures.” *Protoplasma* 230 (1–2): 1–11.

Takano, Junpei, Mayuki Tanaka, Atsushi Toyoda, Kyoko Miwa, Koji Kasai, Kentaro Fuji, Hitoshi Onouchi, Satoshi Naito, and Toru Fujiwara. 2010. “Polar Localization and Degradation of Arabidopsis Boron Transporters through Distinct Trafficking Pathways.” *Proceedings of the National Academy of Sciences of the United States of America* 107 (11): 5220–25.

Tian, Feng, De-Chang Yang, Yu-Qi Meng, Jinpu Jin, and Ge Gao. 2020. “PlantRegMap: Charting Functional Regulatory Maps in Plants.” *Nucleic Acids Research* 48 (D1): D1104–13.

Tian, Juan, Libo Han, Zhidi Feng, Guangda Wang, Weiwei Liu, Yinping Ma, Yanjun Yu, and Zhaosheng Kong. 2015. “Orchestration of Microtubules and the Actin Cytoskeleton in Trichome Cell Shape Determination by a Plant-Unique Kinesin.” *ELife* 4 (August). https://doi.org/10.7554/eLife.09351.

Tominaga, Motoki, Atsushi Kimura, Etsuo Yokota, Takeshi Haraguchi, Teruo Shimmen, Keiichi Yamamoto, Akihiko Nakano, and Kohji Ito. 2013. “Cytoplasmic Streaming Velocity as a Plant Size Determinant.” *Developmental Cell* 27 (3): 345–52.

Tsuda, Katsutoshi, Maria-Jazmin Abraham-Juarez, Akiteru Maeno, Zhaobin Dong, Dale Aromdee, Robert Meeley, Toshihiko Shiroishi, Ken-Ichi Nonomura, and Sarah Hake. 2017. “KNOTTED1 Cofactors, BLH12 and BLH14, Regulate Internode Patterning and Vein Anastomosis in Maize.” *The Plant Cell* 29 (5): 1105–18.

Vaddepalli, Prasad, Lynette Fulton, Martine Batoux, Ram Kishor Yadav, and Kay Schneitz. 2011. “Structure-Function Analysis of STRUBBELIG, an Arabidopsis

Atypical Receptor-like Kinase Involved in Tissue Morphogenesis." *PloS One* 6 (5): e19730.
Van Emon, Jeanette M. 2016. "The Omics Revolution in Agricultural Research." *Journal of Agricultural and Food Chemistry* 64 (1): 36–44.
Veit, B., D. Jackson, R. Kerstetter, L. Smith, E. Vollbrecht, J. Yamaguchi, and Sarah Hake. 1994. "Genetic and Molecular Analyses of Pattern Formation Genes in Maize." In *Plant Molecular Biology*, 7–16. Berlin, Heidelberg: Springer Berlin Heidelberg.
Vinogradova, Maia V., Galina G. Malanina, Anireddy S. N. Reddy, and Robert J. Fletterick. 2009. "Structure of the Complex of a Mitotic Kinesin with Its Calcium Binding Regulator." *Proceedings of the National Academy of Sciences of the United States of America* 106 (20): 8175–79.
Vollbrecht, E., B. Veit, N. Sinha, and S. Hake. 1991. "The Developmental Gene Knotted-1 Is a Member of a Maize Homeobox Gene Family." *Nature* 350 (6315): 241–43.
Vos, Jan W., Farida Safadi, Anireddy S. N. Reddy, and Peter K. Hepler. 2000. "The Kinesin-like Calmodulin Binding Protein Is Differentially Involved in Cell Division." *The Plant Cell* 12 (6): 979.
Wall, Michael E., Sharron H. Francis, Jackie D. Corbin, Kennard Grimes, Robyn Richie-Jannetta, Jun Kotera, Brian A. Macdonald, Rowena R. Gibson, and Jill Trewhella. 2003. "Mechanisms Associated with CGMP Binding and Activation of CGMP-Dependent Protein Kinase." *Proceedings of the National Academy of Sciences of the United States of America* 100 (5): 2380–85.
Wang, Cunxi, and Xuemin Wang. 2001. "A Novel Phospholipase D of Arabidopsis That Is Activated by Oleic Acid and Associated with the Plasma Membrane." *Plant Physiology* 127 (3): 1102–12.
Wang, Fay, John Flanagan, Nan Su, Li-Chong Wang, Son Bui, Allissa Nielson, Xingyong Wu, Hong-Thuy Vo, Xiao-Jun Ma, and Yuling Luo. 2012. "RNAscope: A Novel in Situ RNA Analysis Platform for Formalin-Fixed, Paraffin-Embedded Tissues." *The Journal of Molecular Diagnostics: JMD* 14 (1): 22–29.
Wang, Fei, Yifen Shang, Baofang Fan, Jing-Quan Yu, and Zhixiang Chen. 2014. "Arabidopsis LIP5, a Positive Regulator of Multivesicular Body Biogenesis, Is a Critical Target of Pathogen-Responsive MAPK Cascade in Plant Basal Defense." *PLoS Pathogens* 10 (7): e1004243.
Wang, Liyuan, Yilin Zou, Han Yeong Kaw, Gang Wang, Huaze Sun, Long Cai, Chengyu Li, Long-Yue Meng, and Donghao Li. 2020. "Recent Developments and Emerging Trends of Mass Spectrometric Methods in Plant Hormone Analysis: A Review." *Plant Methods* 16 (1): 54.
Wang, Xuemin. 2005. "Regulatory Functions of Phospholipase D and Phosphatidic Acid in Plant Growth, Development, and Stress Responses." *Plant Physiology* 139 (2): 566–73.
Willige, Björn C., Eri Ogiso-Tanaka, Melina Zourelidou, and Claus Schwechheimer. 2012. "WAG2 Represses Apical Hook Opening Downstream from Gibberellin

and PHYTOCHROME INTERACTING FACTOR 5." *Development (Cambridge, England)* 139 (21): 4020–28.
Wu, Ai-Min, Christophe Rihouey, Martial Seveno, Emma Hörnblad, Sunil Kumar Singh, Toshiro Matsunaga, Tadashi Ishii, Patrice Lerouge, and Alan Marchant. 2009. "The Arabidopsis IRX10 and IRX10-LIKE Glycosyltransferases Are Critical for Glucuronoxylan Biosynthesis during Secondary Cell Wall Formation." *The Plant Journal: For Cell and Molecular Biology* 57 (4): 718–31.
Wu, Jiandong, Xiaoyun Tan, Chengyun Wu, Kun Cao, Yan Li, and Yiqun Bao. 2013. "Regulation of Cytokinesis by Exocyst Subunit SEC6 and KEULE in Arabidopsis Thaliana." *Molecular Plant* 6 (6): 1863–76.
Xia, Jingnu, Yunjun Zhao, Payne Burks, Markus Pauly, and Patrick J. Brown. 2018. "A Sorghum NAC Gene Is Associated with Variation in Biomass Properties and Yield Potential." *Plant Direct* 2 (7): e00070.
Xiao, Yao, and Remko Offringa. 2020. "PDK1 Regulates Auxin Transport and Arabidopsis Vascular Development through AGC1 Kinase PAX." *Nature Plants* 6 (5): 544–55.
Xing, Jingjing, Liang Zhang, Zhikun Duan, and Jinxing Lin. 2021. "Coordination of Phospholipid-Based Signaling and Membrane Trafficking in Plant Immunity." *Trends in Plant Science* 26 (4): 407–20.
Xu, Changzheng, Huanhuan Tai, Muhammad Saleem, Yvonne Ludwig, Christine Majer, Kenneth W. Berendzen, Kerstin A. Nagel, et al. 2015. "Cooperative Action of the Paralogous Maize Lateral Organ Boundaries (LOB) Domain Proteins RTCS and RTCL in Shoot-Borne Root Formation." *The New Phytologist* 207 (4): 1123–33.
Yamaguchi, Miki, Haruka Fujimoto, Ko Hirano, Satoko Araki-Nakamura, Kozue Ohmae-Shinohara, Akihiro Fujii, Masako Tsunashima, et al. 2016. "Sorghum Dw1, an Agronomically Important Gene for Lodging Resistance, Encodes a Novel Protein Involved in Cell Proliferation." *Scientific Reports* 6 (1). https://doi.org/10.1038/srep28366.
Yanai, Itai, Hila Benjamin, Michael Shmoish, Vered Chalifa-Caspi, Maxim Shklar, Ron Ophir, Arren Bar-Even, et al. 2005. "Genome-Wide Midrange Transcription Profiles Reveal Expression Level Relationships in Human Tissue Specification." *Bioinformatics (Oxford, England)* 21 (5): 650–59.
Yang, Fang, Huyen Thanh Bui, Michael Pautler, Victor Llaca, Robyn Johnston, Byeong-Ha Lee, Allison Kolbe, Hajime Sakai, and David Jackson. 2015. "A Maize Glutaredoxin Gene, Abphyl2, Regulates Shoot Meristem Size and Phyllotaxy." *The Plant Cell* 27 (1): 121–31.
Yost, M. A., N. R. Kitchen, K. A. Sudduth, E. J. Sadler, S. T. Drummond, and M. R. Volkmann. 2017. "Long-Term Impact of a Precision Agriculture System on Grain Crop Production." *Precision Agriculture* 18 (5): 823–42.
Zeder, Melinda A., Eve Emshwiller, Bruce D. Smith, and Daniel G. Bradley. 2006. "Documenting Domestication: The Intersection of Genetics and Archaeology." *Trends in Genetics: TIG* 22 (3): 139–55.

Zegzouti, Hicham, Wei Li, Todd C. Lorenz, Mingtang Xie, C. Thomas Payne, Kelly Smith, Scott Glenny, Gregory S. Payne, and Sioux K. Christensen. 2006. "Structural and Functional Insights into the Regulation of Arabidopsis AGC VIIIa Kinases." *The Journal of Biological Chemistry* 281 (46): 35520–30.

Zhang, Li-Min, Chuan-Yuan Leng, Hong Luo, Xiao-Yuan Wu, Zhi-Quan Liu, Yu-Miao Zhang, Hong Zhang, et al. 2018. "Sweet Sorghum Originated through Selection of Dry, a Plant-Specific NAC Transcription Factor Gene." *The Plant Cell* 30 (10): 2286–2307.

Zhang, Qiong, Robert Berkey, Joshua J. Blakeslee, Jinshan Lin, Xianfeng Ma, Harlan King, Anna Liddle, et al. 2018. "Arabidopsis Phospholipase Dα1 and Dδ Oppositely Modulate EDS1- and SA-Independent Basal Resistance against Adapted Powdery Mildew." *Journal of Experimental Botany* 69 (15): 3675–88.

Zhang, Wenhua, Cunxi Wang, Chunbo Qin, Tara Wood, Gudrun Olafsdottir, Ruth Welti, and Xuemin Wang. 2003. "The Oleate-Stimulated Phospholipase D, PLDdelta, and Phosphatidic Acid Decrease H2O2-Induced Cell Death in Arabidopsis." *The Plant Cell* 15 (10): 2285–95.

Zhou, Huapeng, Hongqin Duan, Yunhong Liu, Xia Sun, Jinfeng Zhao, and Honghui Lin. 2019. "Patellin Protein Family Functions in Plant Development and Stress Response." *Journal of Plant Physiology* 234–235 (March): 94–97.

Zwiewka, Marta, Elena Feraru, Barbara Möller, Inhwan Hwang, Mugurel I. Feraru, Jürgen Kleine-Vehn, Dolf Weijers, and Jiří Friml. 2011. "The AP-3 Adaptor Complex Is Required for Vacuolar Function in Arabidopsis." *Cell Research* 21 (12): 1711–22.