ELUCIDATION OF THE GENETIC CONTROL OF CARBOHYDRATE PARTITIONING IN

Zea mays

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Zea mays

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ABSTRACT

Carbohydrate partitioning, the process of transporting carbohydrates from sites of synthesis in photosynthetic tissue to developing sink tissues, is crucial for the growth, development, and yield in plants and crops. Anatomical, physiological, and biochemical studies have shed light on this process; however, there is not much known in regards to the genetic control of carbohydrate partitioning. The work described here uses forward and reverse genetic methods to further elucidate the genetic control of carbohydrate partitioning in *Zea mays* (maize).

Chapter 1 reviews what is currently known regarding the function of sugar transporters in the phloem loading and unloading pathways. Additionally, the importance and role of carbohydrate partitioning during abiotic and biotic stress responses is discussed.

Chapters 2-4 describe the characterization and cloning of the allelic recessive mutants *carbohydrate partitioning defective28* (*cpd28*) and *carbohydrate partitioning defective47* (*cpd47*) (Chapter 2) and the semi-dominant gain-of-function mutant *Carbohydrate partitioning defective1* (*Cpd1*) (Chapters 3 and 4). All three mutant's hyper-accumulate carbohydrates in their mature leaves due to decreased sucrose export. The *cpd28* and *cpd47* mutations decrease the amount of crystalline cellulose and likely impair primary cell wall development. The *Cpd1* mutation results in the unregulated deposition of the callose in the phloem of leaf and root tissue.

Chapter 5 summarizes the Sugars Will Eventually Exit Tissue (SWEET) transporter family and their function across a number of plant species. In this review I was responsible for the generation of a 15 angiosperm protein phylogenetic analysis describing the relationship and structure found in the transporter family.

Chapter 6 builds upon the phylogentic study performed in Chapter 5 and identifies and characterizes the function of the *SWEET13a*, *SWEET13b*, and *SWEET13c* transporters in the phloem loading pathway in maize utilizing CRISPR/Cas9 mutagenesis.

Chapter 7 summarizes the discoveries made in the previous chapters, their contribution to the field, and discusses future research directions.

Appendix A describes a protocol I optimized for the fixation, embedding, sectioning, Laser Capture Microdissection, and RNA-sequencing of desired cell groups in mature maize leaf tissue. Appendix B describes the characterization and cloning of the recessive mutant *carbohydrate partitioning defective33* (*cpd33*) and its role in plasmodesmatal function. I contributed to the cloning of *cpd33*. Appendix C analyzes the expression levels and potential roles of *SWEET* and *TST* transporters in sweet and grain sorghum. I was responsible for the expression analysis of the *SWEET* genes discussed in this publication.

CHAPTER 1: Sugar Transporters in Plants: New Insights and Discoveries

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ABSTRACT

Carbohydrate partitioning is the process of carbon assimilation and distribution from source tissues, such as leaves, to sink tissues, such as stems, roots, and seeds. Sucrose, the primary carbohydrate transported long-distance in many plant species, is loaded into the phloem and unloaded into distal sink tissues. However, many factors, both genetic and environmental, influence sucrose metabolism and transport. Therefore, understanding the function and regulation of sugar transporters and sucrose metabolic enzymes is key to improving agriculture. In this review, we highlight recent findings that 1) address the path of phloem loading of sucrose in rice and maize leaves; 2) discuss the phloem unloading pathways in stems and roots and the sugar transporters putatively involved; 3) describe how

heat and drought stress impact carbohydrate partitioning and phloem transport; 4) illuminate how plant pathogens hijack sugar transporters to obtain carbohydrates for pathogen survival, and how the plant employs sugar transporters to defend against pathogens; and 5) discuss novel roles for sugar transporters in plant biology. These exciting discoveries and insights provide valuable knowledge that will ultimately help mitigate the impending societal challenges due to global climate change and a growing population by improving crop yield and enhancing renewable energy production.

Abbreviations:

CC, companion cell

CF, 5,6-Carboxyfluorescein

CP, carbohydrate partitioning

CWIN, cell wall invertase

GA, gibberellins

GFP, green fluorescent protein

GUS, beta-glucuronidase

H⁺-PPase, proton pyrophosphatase

INV, invertase

LeHT1, *Solanum lycopersicum* hexose transporter1

Lr67res, *Lr67* resistant allele

Lr67sus, *Lr67* susceptible allele

MP, movement protein

PD, plasmodesmata

PM, plasma membrane

PMF, proton motive force

R, TYLCV-resistant tomato inbred line

Ri, *LeHT1* silenced resistant lines

RFP, red fluorescent protein

S, TYLCV-susceptible tomato inbred line

SE, sieve elements

SE-CCC, sieve element – companion cell complexes

SPS, sucrose phosphate synthase

STPs, sugar transport proteins

SUS, sucrose synthase

SUT, Sucrose Transporters

TYLCV, *Tomato yellow leaf curl virus*

TMT, tonoplast monosaccharide transporter

TST, tonoplast sugar transporter

YFP, yellow fluorescent protein

Xoo, *Xanthomonas oryzae* pv. *Oryzae*

Key words: Phloem, Stress tolerance, Sucrose, Sugar transporters, SUTs, SWEETs

Introduction

All living organisms require organic carbon to generate the metabolites needed to survive and grow. Many eukaryotes, humans included, are unable to generate their own carbon supply. As such, humans rely on plants as the ultimate source of fixed carbon because plants are able to 1) assimilate atmospheric carbon dioxide into sugars, 2) generate compounds that our bodies can easily metabolize (i.e., nutrients), and 3) store these compounds in organs readily harvested in mass quantities. Therefore, it is vital to understand how plants control carbon assimilation and long-distance transport of carbohydrates to continue increasing crop yields to sustain the growing human population (Godfray et al. 2010).

Carbohydrate partitioning (CP), the process of assimilating, transporting, and distributing sugars from source leaves (net exporting) to sink tissues (net importing), such as flowers, stems, and roots, is fundamental to plant growth and development (Bihmidine et al. 2013; Braun and Slewinski 2009; Yadav et al. 2015). Additionally, this process is crucial for plant tolerance to abiotic and biotic stress (Lemoine et al. 2013; Ludewig and Flügge 2013). Thus, understanding CP and its genetic regulation are essential to enabling breakthroughs to increase crop yield and stress tolerance. While selective breeding has led to great progress modifying CP in crop species such as maize (*Zea mays*), barley (*Hordeum vulgare*), and potato (*Solanum tuberosum*), much remains to be discovered of the underlying genes and control mechanisms.

Numerous reviews discussing CP and sugar metabolism have recently been published (Braun et al. 2014; Chen et al. 2015; Eom et al. 2015; Lemoine et al. 2013;

Li et al. 2017a; Ruan 2014). As humans obtain >60% of their daily calories from cereals (Alexandratos and Bruinsma 2012), in this review we will emphasize recent progress in understanding CP in grasses, which has been less studied, as well as exciting discoveries from other plants. We will also discuss current results illuminating the path for phloem loading of sucrose in maize and rice (*Oryza sativa*) leaves. Additionally, we will describe recent findings on sugar unloading in vegetative sinks, which are important both for the development of biofuel plants for renewable energy, and for engineering enhanced abiotic stress tolerance in crop plants. Finally, we also focus on sugar transport and metabolism under biotic and abiotic stress, specifically drought and heat stress, and discuss questions that should form the basis for future research.

Roles for rice sugar transporters and phloem loading: Apoplastic vs. symplasmic?

In most annual plants, sucrose is the predominant carbohydrate transported long-distance through the phloem sieve tubes, which are constituted of sieve elements (SE) connected end-to-end (Sleewinski and Braun 2010; Zimmermann and Ziegler 1975). During maturation, SE undergo limited autolysis, degrading most of the organelles, and become reliant on companion cells (CC) for metabolic support (Esau 1977). These two cell types are referred to as SE-CC complexes (SE-CCC). In leaves of many annual, herbaceous plants, including most crop species, the SE-CCC share very few plasmodesmata (PD) with adjacent cells (Evert et al. 1978; Gamalei 1989). Hence, sucrose synthesized in the photosynthetic cells (e.g., mesophyll cells) is

ultimately exported to the apoplast (cell wall), likely by SWEET efflux proteins, prior to entry into the SE-CCC via Sucrose Transporters (abbreviated as SUT or SUC). Furthermore, the sucrose content is higher inside the SE-CCC than in surrounding cells, necessitating the active pumping of sucrose against its concentration gradient to accumulate within the SE-CCC (Evert et al. 1978; Geiger et al. 1973). This process is referred to as apoplastic phloem loading of sucrose. However, some plants, in particular trees, have high PD connectivity between the SE-CCC and adjacent cells, as well as a lower concentration of sucrose (or the corresponding transported carbohydrate, e.g., sugar alcohols) in the SE-CCC than mesophyll cells. Hence, sucrose moves down a concentration gradient and enters the phloem passively (without an energy-requiring step), in a process termed symplasmic phloem loading (Turgeon and Medville 1998). For more information on the different strategies used by plants for phloem loading, please see (Liesche 2017; Rennie and Turgeon 2009; Slewinski and Braun 2010).

SUTs are H⁺/sucrose symporters that utilize the proton motive force (PMF) present across the plasma membrane (PM) of the SE-CCC to load sucrose against its concentration gradient into the phloem (Carpaneto et al. 2005; Chandran et al. 2003; Lalonde et al. 2004; Reinders et al. 2012). Multiple *Sut* genes have been identified in the genomes of both apoplastic and symplasmic phloem loading species (Aoki et al. 2003; Ayre 2011; Lalonde et al. 2004; Peng et al. 2014; Zhang et al. 2014). To examine the phylogenetic relationships among the SUTs, with a particular focus on grasses, Braun and Slewinski (2009) constructed a phylogenetic tree consisting of SUTs from available monocot and select eudicot genomes. These

analyses identified five distinct clades or groups. Group 1 consists entirely of monocot SUT sequences. Group 2 contains only eudicot SUT sequences, several of which are known to be responsible for apoplastic phloem loading of sucrose in leaves, including *StSUT1* from potato (Lemoine et al. 1996) and *AtSUC2* from *Arabidopsis thaliana* (Gottwald et al. 2000; Srivastava et al. 2008). Group 3 consists of both monocot and eudicot SUTs, but their functions are unclear. Group 4 also contains SUTs from both monocots and eudicots. Members of this group have been localized to the tonoplast and are proposed to function in the efflux of sucrose out of the vacuolar lumen into the cytoplasm (Reinders et al. 2012). Group 5 consists entirely of monocot SUTs whose functions are unknown (please see (Braun and Slewinski 2009) for a more detailed discussion of the different SUT groups).

Maize has long been a model organism for studying phloem transport (Hofstra and Nelson 1969), and is characterized as an apoplastic phloem loading species (Evert et al. 1978; Gamalei 1989; Heyser 1980; Slewinski et al. 2012). Evidence supporting this hypothesis includes: 1) ultrastructural studies demonstrating few PD between the SE-CCC and adjoining cells (Evert et al. 1978), 2) the ability of SE to directly accumulate ^{14}C -labeled sucrose from the apoplasm (Fritz et al. 1983), 3) a significant increase in the pH of vascular bundles fed sucrose through the transpiration stream (Heyser 1980), and 4) the inhibition of sucrose export from the leaf upon exposure to para-chloro-mercuribenzenesulfonate (Thompson and Dale 1981), an inhibitor of SUT function. Through molecular expression analyses and biochemical studies, *ZmSut1* was identified as a candidate for apoplastic phloem loading of sucrose in maize leaves (Aoki et al. 1999; Carpaneto et al. 2005). Direct

evidence supporting this hypothesis came from the evaluation of a transposable element insertion allele of *ZmSut1*. Utilizing the *zmsut1* mutant, Slewinski et al. (2009, 2010) demonstrated that *ZmSut1* functions to load sucrose into the phloem from the apoplasm. Plants defective in *ZmSut1* display stunted plant growth, leaf chlorosis, failure to transport radioactively labeled sucrose in source leaves, and sugar and starch accumulation within the leaf (Slewinski et al. 2009). Furthermore, research examining the cellular and subcellular localization of the ZmSUT1 protein in maize, utilizing transgenic fluorescent protein reporter lines, found ZmSUT1 localized to the PM of CC (Baker et al. 2016). Hence, ZmSUT1 functions to load sucrose into the SE-CCC in maize leaves.

The rice ortholog of *ZmSut1*, *OsSut1*, was first characterized by Hirose et al. (1997), who hypothesized *OsSut1* played a role in sucrose movement out of tissue stores and into developing tissues (e.g., developing seeds). To test this hypothesis, Scofield et al. (2002) examined *OsSut1* function using antisense RNA suppression lines. In some of the transgenic lines, strong, yet incomplete, reduction of the *OsSut1* RNA was achieved. However, reduced grain fill and delayed germination were the only observed differences relative to wild-type siblings. Scofield et al. (2002) hypothesized that the observed reduction in grain filling was either directly due to OsSUT1 function being impaired in the grain, or indirectly due to reduced sucrose transport out of the leaf. Interestingly, in germinating seeds, an *OsSut1* promoter: beta-glucuronidase (GUS) reporter gene was expressed in the phloem of the scutellum, and the authors proposed that OsSUT1 functions to remobilize seed reserves (Scofield et al. 2007a). Thus, the delayed germination phenotype in the

ossut1 antisense lines reported by Scofield et al. (2002) may result from limited phloem loading of endosperm-derived sucrose into scutellar veins (Matsukura et al. 2000), supporting the hypothesis that OsSUT1 functions to load sucrose into the phloem in rice, at least in germinating seedlings. In parallel, Ishimaru et al. (2001) used a similar antisense RNA approach to examine the role of *OsSut1* in phloem loading. Again, this strategy resulted in a strong but incomplete knock-down of gene expression. Ishimaru and colleagues observed results similar to those of Scofield et al. (2002), with no change in plant height, leaf photosynthesis, or leaf sugar and starch accumulation. Interestingly, Scofield et al. (2007b) determined that the *OsSut1* promoter GUS reporter gene was expressed in the phloem, and that the OsSUT1 protein immunolocalized to the SE-CCC of vegetative tissues, suggesting it might function to load sucrose into the phloem from the apoplasm. From these results, Ishimaru et al. (2001) and Scofield et al. (2002) suggested the lack of a visible plant phenotype in the antisense RNA lines might be due to 1) the ability of the transgenic plants to apoplasmically load sucrose into the phloem as a result of incomplete suppression, or 2) transport from the apoplasm is not the predominant form of sucrose entry into the phloem of rice leaves (Ishimaru et al. 2001; Scofield et al. 2002).

To resolve any potential uncertainties from the antisense lines being incomplete knockout mutations, Eom et al. (2012, 2016) characterized an *OsSut1* null allele caused by a *Tos17* retrotransposable element insertion into the coding region. Unfortunately, this disruption caused an impairment in pollen tube germination, resulting in the inability to recover homozygous mutant plants through self-

pollination (Hirose et al. 2010). To circumvent this limitation, Eom et al. (2012, 2016) performed anther tissue culture to regenerate homozygous *ossut1* mutant individuals. The mutant exhibited an absence of phenotypes usually associated with an impaired ability to load sucrose into the phloem. For example, loss-of-function of the *OsSut1* gene did not impact plant growth, leaf chlorophyll levels, photosynthetic rate, carbohydrate contents in leaves, or time to flowering. Based on these results, the authors suggested rice may utilize symplasmic phloem loading. They also raised the possibility another member of the *Sut* gene family may be responsible for the apoplastic phloem loading of sucrose in rice.

There are five *Sut* genes in the rice genome (Aoki et al. 2003). To address their ability to load sucrose into the phloem from the apoplasm, Eom et al. (2016) designed a transgenic complementation experiment to assess whether any rice *Sut* gene could rescue the phenotype of the Arabidopsis *atsuc2* mutant. *atsuc2* mutant plants grow poorly, hyperaccumulate carbohydrates in their leaves, and do not set seed due to the attenuation of sucrose import into the SE-CCC (Gottwald et al. 2000; Srivastava et al. 2008). Only the *OsSut1* cDNA driven by the *AtSUC2* promoter complemented the *atsuc2* mutant phenotype, restoring plant growth and leaf carbohydrate levels back to those of wild type. These data suggest that of the rice SUT family members, only OsSUT1 might function to apoplastically load sucrose into the phloem. It is curious as to why OsSUT1 performs this function when heterologously expressed in the Arabidopsis phloem, but does not seem to do so endogenously in rice, although as mentioned above, OsSUT1 is expressed in the

phloem and the ultrastructure of rice leaf minor veins suggests a symplasmic discontinuity for sucrose to enter the SE-CCC (Braun et al. 2014; Kaneko et al. 1980). Further independent evidence supporting the hypothesis that rice uses an apoplasmic phloem loading mechanism derives from research examining the localization of a proton pyrophosphatase (H^+ -PPase) and sucrose synthase (SUS) proteins in rice veins (Regmi et al. 2016). As described above, SUTs function by utilizing the PMF present across a membrane to energize the co-transport of a sucrose molecule and a H^+ against a sucrose concentration gradient. The PMF is generated by the cleavage of sucrose into fructose and UDP-glucose by SUS, with additional biochemical reactions generating ATP. The ATP in turn is catabolized by a H^+ -ATPase, providing the energy to pump a H^+ across the PM to generate the PMF (Geigenberger et al. 1993; Lerchl et al. 1995). In Arabidopsis, a phloem-expressed, PM-localized H^+ -PPase has been proposed to function as a synthase to generate pyrophosphate to drive sucrose catabolism and the resultant ATP production, which is required to sustain the PMF (Gaxiola et al. 2012; Pizzio et al. 2015). Subsequently, Regmi et al. (2016) hypothesized that if rice imports sucrose into the phloem from the apoplasm, then a SUS and a H^+ -PPase would be expected to localize to the PM of CC and/or SE. Conversely, if rice uses symplasmic phloem loading, then the H^+ -PPase would likely be predominantly localized to the tonoplast, as found in sink tissues (Langhans et al. 2001; Nakanishi and Maeshima 1998; Pizzio et al. 2015; Segami et al. 2014). Utilizing polyclonal antibodies raised against the H^+ -PPase or SUS, Regmi et al. (2016) determined that the H^+ -PPase localized to the PM of the phloem SE-CCC, and that SUS was located in close proximity to the PM in these cells.

These results suggest that the necessary components to support sucrose transport into the phloem from the apoplasm are present in the SE-CCC of rice minor veins. Therefore, these data are consistent with the hypothesis that rice uses apoplasmic phloem loading rather than symplasmic loading. If so, might another *Sut* gene mediate apoplasmic phloem loading?

Investigations of OsSUT2, a member of the group 4 SUTs, suggested the possibility of an alternative method of sucrose loading. Members of this group of SUTs localize to the tonoplast and are proposed to move sucrose out of the vacuolar lumen into the cytoplasm (Reinders et al. 2008; Reinders et al. 2012; Schneider et al. 2012). When the function of *OsSut2* was disrupted by a T-DNA insertion, the mutant plants, referred to as *ossut2*, displayed a reduction in plant height, tiller number, and seed weight, as well as an increase in the amount of sucrose, glucose, and fructose present in mature leaves (Eom et al. 2011). Sugar export from the leaf was also reported to be significantly reduced in *ossut2* mutants, suggesting *OsSut2* influences sucrose movement into the phloem (Eom et al. 2012). Further, when the expression of an *OsSut2* promoter reporter was examined, the gene was found to be expressed in mesophyll and bundle sheath cells, but not in the veins. Since *ossut1* mutants failed to display phenotypes indicative of the inability to load sucrose into the phloem, but *ossut2* mutants exhibited some of these phenotypes, Eom et al. (2012) hypothesized that rice may use a modified form of symplasmic phloem loading mediated by OsSUT2. If correct, it would indicate that rice utilizes a different mechanism for loading sucrose into the phloem compared to closely related grasses, including wheat (*Triticum aestivum*), barley, maize, sugarcane (*Saccharum* sp), and

sorghum (*Sorghum bicolor*), which have all been proposed to use apoplastic phloem loading (Bihmidine et al. 2015; Evert et al. 1978; Evert et al. 1996; Robinson-Beers and Evert 1991; Thompson and Dale 1981).

This intriguing hypothesis motivated Leach et al. (2017) to investigate the function of the single group 4 gene in maize, *ZmSut2*. *ZmSUT2* is orthologous to *OsSUT2*, with the two proteins sharing 91% amino acid identity, and both proteins residing on the tonoplast. The authors characterized homozygous *zmsut2* mutant plants resulting from transposable element insertions in the protein-coding region to determine the biological function of the gene. Sequence and expression analyses revealed both mutations were likely null alleles. When grown in the field, *zmsut2* homozygous mutant plants displayed a reduction in plant height and reproductive structures, i.e., ear and tassel length, as well as an increase in sugars and starch in leaves compared to wild-type siblings. Another interesting aspect of the *zmsut2* mutant phenotype is that with increasing planting density, both ear length and total kernel number progressively decreased, whereas kernel weight remained relatively unchanged (Leach et al. 2017). These data indicate *ZmSut2* is important for maintaining crop productivity, especially under higher planting densities associated with commercial seed production. Additionally, Leach et al. (2017) used radioactively labeled sucrose to examine whether *ZmSUT2* played a direct role in phloem loading of sucrose in leaves. These experiments demonstrated that sucrose loading and transport was unaffected by the mutation. Collectively, these data suggest that the phenotypes observed in *zmsut2* mutants, which are highly similar to those observed in *ossut2* mutants, are due to the inability to transport sucrose out of the vacuole in

photosynthetic cells, rather than an inability to load sucrose into the phloem SE-CCC.

If the hypothesis that rice uses symplasmic phloem loading is in doubt, might there be another explanation for why *ossut1* mutants fail to produce a phloem-loading-related phenotype? One potential reason may be the growth conditions under which these mutant plants were characterized (summarized in Table S1.1). To our knowledge, all of the characterized antisense RNA *ossut1* transgenic lines reported were cultivated in greenhouses without supplemental lighting under natural conditions (in the articles from (Ishimaru et al. 2001) and (Scofield et al. 2002) the light levels were not reported), whereas the *ossut1 Tos17* insertional mutants were grown in a growth chamber under $300 \text{ mmol m}^{-2} \text{ s}^{-1}$ of light (Eom et al. 2016). These growing conditions might have been insufficient to trigger a phloem-loading-related defect. It has been demonstrated that when *Arabidopsis* plants carrying homozygous null mutations in *AtSUC2* are grown under low light levels, they are able to complete their lifecycle and produce seed (Srivastava et al. 2009). Similarly, we observed that *zmsut1* mutant plants grown in a greenhouse during the winter months supplemented with incandescent lighting ($400\text{-}900 \text{ mmol m}^{-2} \text{ s}^{-1}$) grow normally, reach reproductive maturity at similar times to wild-type siblings, produce fertile pollen and ears, and make viable seed upon self-fertilization (Fig. 1). This is in stark contrast to the phenotype observed in *zmsut1* mutant plants grown in a greenhouse during the spring through fall seasons or in the summer field under high light ($\sim 2000 \text{ mmol m}^{-2} \text{ s}^{-1}$) conditions (Baker et al. 2016; Slewinski et al. 2010; Slewinski et al. 2009). Under these conditions, the mutant plants display stunted

growth, leaf chlorosis, and failure to produce fertile reproductive structures. Hence, consistent with rice vein anatomy (Braun et al. 2014; Kaneko et al. 1980), the ability of *OsSut1* to complement the *atsuc2* phloem-loading-deficiency in Arabidopsis (Eom et al. 2016), and the presence of a H⁺-PPase and SUS at the PM of rice CC (Regmi et al. 2016), we hypothesize that OsSUT1 functions to load sucrose from the apoplasm into the phloem in rice leaves, and that this function may have been overlooked due to the permissive plant growth conditions used. Future work should reexamine the relationship between plant growth and productivity under high light, OsSUT1 function, and the mechanism of sucrose phloem loading in rice leaves.

New insights into sugar transporter functions in vegetative sinks

Grass stems have emerged as important sources of structural and non-structural carbohydrates (e.g., sucrose) for fiber and fuel production (Braun et al. 2014; Byrt et al. 2011; Slewinski 2012). Thus, a greater understanding of the mechanisms through which sucrose is concentrated in storage stems is needed to facilitate crop improvement (Bihmidine et al. 2013). Immature internodes contain a developmental gradient, with meristematic and elongating tissues located toward the base, and maturing tissue toward the apex (Esau 1977). Elongating tissues are considered utilization rather than storage sinks (Ho 1988), as sucrose is rapidly cleaved to hexoses and metabolized to facilitate growth. Most sucrose accumulation occurs in mature, fully elongated internodes, and, within mature stems, sucrose accumulation tends to be greatest in the basal internodes and lowest in the apical internodes (Bihmidine et al. 2015; Hoffmann-Thoma et al. 1996; Rae et al. 2005). In

sweet sorghum varieties, significant sucrose accumulation in the stem occurs from the time of panicle elongation within the flag leaf sheath (termed booting) until physiological maturity (Bihmidine et al. 2015; Hoffmann-Thoma et al. 1996; Lingle 1987; Milne et al. 2015). However, cessation of internode elongation rather than reproductive development is thought to be the primary driver of sucrose storage (Hoffmann-Thoma et al. 1996; Lingle and Smith 1991).

Within this developmental context, biochemical and histological techniques have been utilized to infer the primary path of phloem unloading in developing and mature internodes (Fig. 2). In sweet sorghum and sugarcane, developing internodes are characterized by a high ratio of hexoses to sucrose (Hoffmann-Thoma et al. 1996; Milne et al. 2015; Rose and Botha 2000). The high hexose concentration could result from a predominantly apoplastic phloem unloading pathway, with sucrose cleavage by cell wall invertase (CWIN) and cellular uptake by hexose transporters (Ruan 2014). However, asymmetrically radiolabeled sucrose transport studies indicate that although CWIN activity is present in the apoplasm, the majority of translocated sucrose is taken into parenchyma cells intact in both developing and mature internodes (Lingle 1989; Tarpley and Vietor 2007; Thom and Maretzki 1992). Furthermore, high expression and biochemical activity of SUS is observed in developing internodes of multiple species, suggesting that a substantial portion of sucrose catabolism occurs in the cytosol (Fig. 2A) (Hoffmann-Thoma et al. 1996; Lingle and Smith 1991; Martin et al. 2016). Collectively, these data suggest that either apoplastic unloading of sucrose and post-phloem uptake without cleavage occurs, or that a symplasmic phloem unloading path from the SE-CCC to the storage

parenchyma exists. To examine the path of sucrose unloading in stems, the movement of small fluorescent dyes has been investigated. 5,6-Carboxyfluorescein (CF), a phloem-transported, membrane-impermeable dye, was reported to accumulate within vascular parenchyma and surrounding storage parenchyma cells of maturing internodes of sugarcane, supporting symplasmic phloem unloading into stems (Fig. 2B) (Rae et al. 2005; Walsh et al. 2005). Another fluorescent dye, 8-acetoxypyrene-1,3,6-trisulfonic acid, applied to the base of the stem via the transpiration stream at the booting stage, was reported to accumulate in storage parenchyma cells adjacent to the vasculature of a maturing sorghum internode. The authors interpreted this accumulation as support for symplasmic phloem unloading (Milne et al. 2015). In contrast, Bihmidine et al. (2015) found that after the loading of CF into mature source leaves of intact plants at anthesis, the dye was confined to the phloem of mature stem tissue in both a grain and a sweet sorghum cultivar. Hence, at this developmental stage, a symplasmic discontinuity exists between the SE-CCC and adjacent cells of mature internodes, indicating sorghum apoplasmically unloads sucrose in ripening stem tissues (Bihmidine et al. 2015). These conflicting results may be explained by multiple factors, including differences in cultivars analyzed, plant growth conditions, life stages examined, internodes sampled, and dye-feeding protocols. Nonetheless, sugar transporters may facilitate apoplasmic phloem unloading during specific phases of development, and more generally, mediate sucrose retrieval from the phloem and storage parenchyma apoplasm. Although some of the dye tracer studies discussed above are consistent with apoplasmic phloem unloading in stems, the transporters that facilitate the release of

sucrose into the SE apoplasm remain uncharacterized. It is hypothesized that the SWEET transporter family performs this function. These transporters are proposed to function as passive uniporters that efflux sucrose and/or hexoses down their concentration gradient across a cell membrane (Baker et al. 2012; Braun 2012; Chen 2014; Chen et al. 2015; Eom et al. 2015; Latorraca et al. 2017). Phylogenetic analysis of this gene family in plants identified four distinct clades, with the clades roughly predicting which sugars are transported. Clades I, II, and IV predominantly transport hexoses, whereas clade III transports sucrose, although exceptions are known (Le Hir et al. 2015). Additionally, the clade IV SWEETs are typically localized to the tonoplast (Chardon et al. 2013; Klemens et al. 2013). Clade III SWEET transporters have been identified as candidates for the facilitated diffusion of sucrose into the apoplasm in multiple tissues (Chen et al. 2012; Le Hir et al. 2015). In a grain sorghum RNAseq expression atlas, three paralogously duplicated genes, *SbSWEET13a-c*, are the most strongly expressed clade III SWEETs in both leaves and stems, with *SbSWEET13a* predominating (Makita et al. 2015). These expression patterns were independently validated for mature stems from both a grain and a sweet sorghum cultivar (Bihmidine et al. 2016). Likewise, the closely related *SvSWEET13b* gene was strongly expressed in mature tissue in a recent stem developmental transcriptome of green millet (*Setaria viridis*), suggesting apoplasmic phloem unloading of sucrose (Fig. 2B) (Martin et al. 2016; McGaughey et al. 2016). Interestingly, although *SvSWEET* expression is generally low in developing stems, suggesting predominantly symplasmic phloem unloading (Martin et al. 2016), at least one clade III SWEET (*SvSWEET15*) is strongly expressed in elongating tissue

(Fig. 2A) (McGaughey et al. 2016). Thus, recent expression data indicates the possibility of apoplastic unloading in both developing and mature stems. It will be interesting to assess whether these patterns of *SWEET* expression are conserved across multiple grass species, particularly for developing stems. However, pending localization of the *SWEET* proteins to the PM of SE-CCC, interpretation of *SWEET* gene expression as evidence of apoplastic unloading of sucrose must be done with caution in light of other potential functions of these transporters in stem tissues (see below).

In addition to their putative role in phloem unloading, *SWEET*s may also localize to the PM of storage parenchyma cells and promote facilitated diffusion of sucrose into the apoplasm of mature stems. In millet, *SvSWEET13b* is co-expressed with both the SUT transporters *SvSut1* and *SvSut5* and several aquaporin genes (Fig. 2B) (McGaughey et al. 2016). The authors proposed that *SvSWEET13b* and several PM-localized (*SvPIP*, *SvNIP*) and tonoplast-localized (*SvTIP*) aquaporins may function to partition sucrose and water between vacuoles, cytosol, and the storage parenchyma apoplasm for cell turgor adjustment and recycling to the vasculature, respectively (McGaughey et al. 2016; Moore and Cosgrove 1991). Tissue and cell localization data are required to resolve these potential roles for *SWEET*s in maturing stems.

As the majority of apoplasmically unloaded sucrose enters parenchyma cells without cleavage by *CWIN* (see above), PM-localized SUTs are promising candidates for sucrose uptake in stems. The genomes of both sweet and grain sorghum cultivars contain six *SbSut* genes, all of which mediate active sucrose uptake when heterologously expressed in yeast (Bihmidine et al. 2015; Braun and Slewinski

2009; Milne et al. 2013). The suite of SUTs expressed in internodes varies with genetic background and stem development. For example, although the PM-localized *SbSut5* is weakly expressed in fully elongated, mature sorghum internodes, *Sut5*, along with *Sut1*, predominates in the maturing region of developing internodes in both sorghum and green millet (Bihmidine et al. 2015; Martin et al. 2016; Milne et al. 2013; Milne et al. 2017). *SbSut1* and the tonoplast-localized *SbSut2* are broadly expressed in mature stems of both grain and sweet sorghum cultivars, but the relative expression of other *SbSuts* is variable (Bihmidine et al. 2015; Milne et al. 2017; Qazi et al. 2012). Thus, which SUTs are expressed at maturity may vary between internodes at differing stages of ripening, although genetic and environmental effects may also contribute. Interestingly, *SbSut* RNA expression levels were not different between grain and sweet sorghum cultivars within mature internodes, even though the stem solute levels varied approximately 24-fold (Bihmidine et al. 2015). In contrast, *SbSut2* and *SbSut4* expression was elevated in source leaves of a sweet sorghum relative to a grain sorghum (Bihmidine et al. 2015; Milne et al. 2013). Therefore, although the level of *Sut* expression is unlikely to be rate-limiting for sucrose accumulation within stems, it may be a critical aspect of source responses to altered sink strength, as reported for model eudicots (Ainsworth and Bush 2011).

To determine where SbSUT proteins are expressed during sorghum internode development and maturation, Milne and colleagues (2017) utilized a polyclonal antibody raised against a peptide from a conserved sequence of the potato StSUT1 protein. The antibody cross-reacted with multiple SbSUT proteins, so it was not

possible to determine which SUT was detected. The authors reported that SbSUTs are localized to SE, but not CC, in both developing and mature regions of a sorghum stem, and to storage parenchyma cells during a limited developmental window between the cessation of cell elongation and the maturation of storage parenchyma cell walls (Milne et al. 2017). These results are in agreement with the phloem SE specificity of wheat TaSUT1 and rice OsSUT1 proteins in SE-CCC in mature stems (Aoki et al. 2004; Scofield et al. 2007b). However, they contrast with sugarcane ShSUT1 localization data, in which the protein immunolocalized to mestome sheath and vascular parenchyma cells, but not to the phloem (Rae et al. 2005). Symplasmic phloem unloading was reported for all of these mature tissues (Aoki et al. 2004; Milne et al. 2015; Rae et al. 2005), and both the phloem- and peripheral vasculature-localized SUTs are predicted to function in retrieval of leaked sucrose from the apoplasm (Fig. 2B). In contrast, dye tracers fed through the xylem of developing internodes at the seven leaf stage diffuse freely through the apoplasm to the storage parenchyma cells, indicating a potential route for apoplasmic unloading (Fig. 2A) (Milne et al. 2015). Thus, it is possible that SUTs also mediate direct uptake of sucrose from the apoplasm into storage parenchyma cells in addition their proposed role in retrieval of leaked sucrose in the phloem (Bihmidine et al. 2015; Milne et al. 2017).

To untangle the different possible roles and to decipher the biological functions of each *Sut* gene during development, genetic studies will be necessary. However, pleiotropy is likely, especially for SUTs that contribute to both apoplasmic phloem loading of sucrose in source leaves and retrieval in sink tissues, as hypothesized for

SbSUT1 (Bihmidine et al. 2015; Milne et al. 2013; Milne et al. 2017). For example, tissue-specific elimination of gene expression will be necessary to evaluate the function of the maize *ZmSut1* gene in sink organs, as null mutants exhibit a severely stunted phenotype due to attenuated phloem loading in leaves (Slewinski et al. 2009). Cell-specific promoters have yet to be identified for most grass sink organs. Hence, in the short term, a combination of RNA *in situ* hybridization and fluorescent reporter gene constructs may provide the best insights into the tissue specificity of individual SUTs in grass stems. This approach was recently utilized to evaluate *ZmSut1* expression in sink tissues using a promoter: red fluorescent protein (RFP) reporter gene and a tagged protein construct (Baker et al. 2016). Both the yellow fluorescent protein (YFP)-tagged ZmSUT1 transgene and a previously reported green fluorescent protein (GFP)-tagged AtSUC2 translational fusion protein partially complemented their respective mutants, suggesting that these SUT-fluorescent protein fusions are functional *in planta* (Baker et al. 2016; Srivastava et al. 2008). In contrast to the reported phloem and transient storage parenchyma cell immunolocalization of SbSUTs discussed earlier (Milne et al. 2017), the *ZmSut1* promoter: RFP reporter gene and ZmSUT1-YFP expression patterns suggest ubiquitous expression of *ZmSut1* in vascular and storage parenchyma cells of both developing and mature stems, albeit at reduced levels in mature tissue (Baker et al. 2016). Unlike sugarcane and sweet sorghum, extensive lignification and suberization of parenchyma cell walls has not been reported for mature maize internodes (Fig. 2B) (Jacobsen et al. 1992; Milne et al. 2015; Welbaum et al. 1992). Thus, it is plausible that the high level of *ZmSut1* expression observed by Baker and

colleagues is necessary to retrieve leaked sucrose that would otherwise escape into the transpiration stream.

Although some portion of sucrose is released into the storage parenchyma apoplast to regulate cell turgor, the majority of post-SE sucrose transport is directed to the vacuole (Lingle 1989; Moore and Cosgrove 1991). Active sucrose transport across the tonoplast membrane is a key control point for sucrose partitioning in multiple organisms and tissues (Hedrich et al. 2015). For example, in *Arabidopsis* leaves, active transport into vacuoles is partially mediated by members of the tonoplast sugar transporter family (TST), a small group of sugar: H⁺ antiporters capable of translocating both sucrose and its catabolites glucose and fructose across the vacuolar membrane (Schneider et al. 2012; Wingenter et al. 2010; Wormit et al. 2006). The TST family, previously called tonoplast monosaccharide transporters (TMTs), has been defined in several grass species, including sugarcane, rice, sorghum, and green millet (Bihmidine et al. 2016; Casu et al. 2015; Cho et al. 2010; Martin et al. 2016). In sorghum, both *SbTST1* and *SbTST2* were strongly expressed in leaves and mature internodes from booting through anthesis, (Bihmidine et al. 2015; Bihmidine et al. 2016; Milne et al. 2017). Furthermore, expression of both *SbTST* genes was significantly enriched in sweet relative to grain sorghum stems (Bihmidine et al. 2016). Green millet stem was reported to accumulate comparable solute levels (percent Brix) to mid-range sweet sorghums, and is thus a promising rapid-cycling model system for stem sucrose accumulation (Martin et al. 2016). Interestingly, *SvTMT1* and *SvTMT2* expression increased with tissue maturation in developing stems (Martin et al. 2016). *SvTMT2*

expression was correlated with two other putative tonoplast-localized sugar transporters, *SvSUT4* and *SvSWEET16* (Martin et al. 2016; McGaughey et al. 2016). Thus, similar molecular players are likely involved in partitioning sucrose to terminal storage sinks in multiple Panicoid grass species.

Although these expression data strongly implicate TSTs in concentrating sucrose within grass stems, they are also strongly expressed in leaf tissues of sweet sorghums, and the relative contributions of TST expression within these two tissues to stem sucrose concentration remains to be determined (Bihmidine et al. 2016). In *Arabidopsis* leaves, *AtTST* over-expression putatively enhanced source strength by sequestering glucose within the vacuole, which led to upregulation of *AtSUC2* transcripts (Wingenter et al. 2010). Interestingly, expression of *SbSWEET13a*, a candidate for apoplasmic phloem loading of sucrose, was enhanced in sweet relative to grain sorghum leaves (Bihmidine et al. 2016). Thus, the relationship between vacuolar sugar sequestration and enhanced expression of phloem loading sugar transporters may be broadly conserved between *Arabidopsis* and grasses.

Similar sugar transporters are likely involved in carbohydrate partitioning in roots

The implication of sorghum and millet TSTs in sucrose accumulation indicates intriguing commonalities in CP strategies between monocot stems and eudicot taproots. Expression of a sugar beet (*Beta vulgaris*) TST gene, *BvTST2.1*, was recently shown to correlate with the phase of rapid sucrose accumulation in high-accumulating taproots versus fodder cultivars (Jung et al. 2015). In tobacco

(*Nicotiana tabacum*) leaf vacuoles, BvTST2.1 exhibited sucrose: H⁺ antiport capacity, with preferential uptake of sucrose over glucose, whereas the related BvTST1 protein exhibited promiscuous uptake of both sugars (Jung et al., 2015). Hence, TST expression is strongly correlated with root and stem sucrose accumulation in multiple crop species (Bihmidine et al. 2016; Casu et al. 2015; Jung et al. 2015).

The potential for crop improvement via optimization of root sugar transporter expression extends beyond storage taproots, particularly under abiotic stresses such as drought. Drought is the primary limiting factor for agricultural production and a major contributor to global food insecurity (Boyer et al. 2013; Boyer 1982). Classical drought stress phenotypes, including an elevated root: shoot biomass ratio and the inhibition of leaf expansion are well-documented in multiple species (Comas et al. 2013; Lemoine et al. 2013). A recent metabolic flux analysis in *Arabidopsis* leaves indicated that leaf expansion is inhibited more strongly than photosynthesis under water deficit, and that elevated root: shoot biomass partitioning may occur due to a larger pool of carbon available for export to the roots (Hummel et al. 2010). However, the sugar transporters mediating enhanced carbon flux to roots are not known.

An exciting advance in identifying candidate sugar transporters that may function to enhance carbon delivery to roots under drought was recently reported by Durand et al. (2016). Using soil-based rhizobox growth systems, Durand and colleagues combined classical physiological phenotyping, imaging of root system architecture, and candidate gene discovery in adult *Arabidopsis* plants. Intriguingly, Durand et al. (2016) observed elevated expression of all five clade III *SWEET* genes (*AtSWEET11-*

AtSWEET15) under drought, and suggested that the increased *SWEET* expression potentially results in enhanced efflux of sucrose to the apoplasm. Dye tracer and chemical inhibitor studies indicate that symplasmic continuity between the SE-CCC and surrounding tissues ceases within millimeters of the root apex (Hukin et al. 2002; Wright and Oparka 1994); hence, apoplastic phloem unloading predominates over most of the root length. Durand et al. (2016) also found that *AtSUC2* was strongly upregulated in roots experiencing drought, potentially to retrieve sucrose lost to the apoplasm. Future research will need to address whether the elevated transcript accumulation of *AtSWEET* and *AtSUC2* genes leads to a corresponding increase in sugar transport, and whether the tissue localization of *SWEET* proteins is consistent with a role in apoplastic phloem unloading.

Interestingly, expression of *AtSUC1*, the predominant *Sut* gene expressed in well-watered roots, declined under water deficit (Durand et al. 2016). *AtSUC1* is strongly expressed in growing root tips (Sivitz et al. 2007), and Durand and colleagues suggested that attenuated expression of this *SUC* gene may contribute to the observed reduction in root elongation. During drought, rooting depth is an important parameter for water acquisition (Lynch 2013), and so identification of sugar transporters that promote elongation maintenance in growing root tips may also contribute to crop improvement. In a recent proteomic analysis of maize root tips experiencing strong water deficit, ZmSUT2 was correlated with elongation maintenance (Voothuluru et al. 2016). Although the function of ZmSUT2 in root drought response remains to be characterized, it may contribute to remobilization of vacuolar reserves for metabolism or for osmotic adjustment. As ZmSUT2 is

localized to the tonoplast and enriched within the symplasmic unloading zone of the root tip, it is unlikely to contribute directly to phloem unloading (Baker et al. 2016; Hukin et al. 2002; Leach et al. 2017). In total, these results suggest that sugar transporters contribute broadly to drought adaptation in both apoplasmically and symplasmically unloading root tissues and are promising targets for future crop improvement.

Turning up the heat: How heat stress affects carbohydrate partitioning

Along with drought, high temperature is one of the main constraints to crop production worldwide, and the two stresses frequently co-occur (Mittler 2006; Teixeira et al. 2013). Heat stress directly impacts carbon utilization and distribution in plants. Recent studies revealed that photosynthesis, carbon fixation, and sucrose metabolism were inhibited under heat stress, while respiration increased (Kaushal et al. 2013; Zhou et al. 2017). Thus, under high temperature, photosynthetic efficiency decreases while other physiological processes which demand carbon, increase, thereby shifting whole-plant carbon supply and demand.

Most reports examining the effects of heat stress on sugar metabolism and transport have investigated source leaves of apoplasmic sucrose phloem loading species (Kaushal et al. 2013; Ribeiro et al. 2014; Sun et al. 2016; Vasseur et al. 2011; Xu et al. 2013; Zhou et al. 2017). From these studies, soluble carbohydrate levels in source leaves generally decline when plants are exposed to high temperatures over the short term (typically less than seven days). For example, under heat stress,

photosynthesis decreased, resulting in lower sugar and starch content in the leaves of *Arabidopsis*, thermal bentgrass (*Agrostis scabra*), castor bean (*Ricinus communis*), and chickpea (*Cicer arietinum*) (Kaushal et al. 2013; Ribeiro et al. 2014; Vasseur et al. 2011; Xu et al. 2013). Similarly, in maize, the sucrose levels in the leaves of three inbred lines decreased after a seven day exposure to heat stress (Sun et al. 2016). However, Vasseur et al. (2011) found that in *Arabidopsis*, elevated CO₂ concentration and high light could enhance photosynthetic rates, increase the sugar contents in leaves, and reverse the negative effects caused by heat stress. These results suggest that maintenance of photosynthesis and carbohydrate assimilation is a key trait for plants to adapt to high temperature. Consequently, tolerance to heat stress is frequently associated with maintaining sugar content in source leaves (Vasseur et al. 2011; Zhou et al. 2017). For example, in tomato (*Solanum lycopersicum*), the fructose and sucrose contents in mature leaves of a heat tolerant genotype were significantly higher compared to a sensitive genotype under heat stress (Zhou et al. 2017).

In addition to a negative effect on carbohydrate assimilation, heat stress also reduces sugar export from leaves. Suwa et al. (2010) reported that the ¹³C export rate from maize ear leaves decreased after 14 days of high temperature treatment, which suggests that sucrose loading into the phloem was inhibited by heat stress. Interestingly, Zhang et al. (2012) showed that the starch content in mesophyll cells of tomato leaves initially decreased during the first five days of heat stress, but was significantly higher than that of controls after 10 days of heat stress, and that the starch content in leaves progressively increased during this period. In these

apoplastic phloem loading species (i.e., maize, tomato) SWEETs are proposed to efflux sucrose into the phloem apoplasm, and SUTs to import sucrose into the SE-CCC (Braun et al. 2014; Osorio et al. 2014). Collectively, these observations suggest that in the long term (>7 days), phloem sucrose export by SWEETs and/or import by SUTs was inhibited in heat-stressed leaves, which subsequently resulted in sugar and starch accumulation.

Interestingly, the transcripts of several genes encoding sugar transporters were differentially expressed in response to heat stress (Jian et al. 2016; Qin et al. 2008). Qin et al. (2008) found that in wheat leaves, microarray analysis indicated that 22 sugar transporters were responsive to heat stress; three of them were upregulated, while nineteen of them were downregulated (Qin et al. 2008). In addition, Jian et al. (2016) examined the expression of oilseed rape (*Brassica napus*) *BnSUC* and *BnSWEET* genes under heat stress. However, rather than downregulated, they found that 11 of the 16 selected *BnSUC* and *BnSWEET* genes were initially upregulated in leaves after three hours of heat treatment. Subsequently, after 6-24 hours of heat stress, their levels declined, and only *BnSUC1-2* was differentially expressed (Jian et al. 2016). According to these authors, *BnSUC1-2* is a close homolog of *AtSUC1* and *AtSUC2* and belongs to the group 2 *Suts*, some of which function in phloem loading. Unfortunately, neither the sugar and starch levels in the leaves nor the carbon export rate of the heat-stressed Brassica plants were reported, so it was not clear if the gene expression changes correlated with altered carbohydrate contents or phloem loading.

In addition to the changes in *Sut* and *SWEET* gene expression described above, heat stress has also been reported to negatively impact sugar transport through sieve tubes. Xu and Huang (2000) provided $^{14}\text{CO}_2$ to castor bean seedlings and found that the proportion of newly photosynthesized ^{14}C allocated to the roots was significantly reduced under heat stress. Similarly, Ribeiro et al. (2014) showed that in creeping bentgrass (*Agrostis palustris*), the root to shoot biomass ratio was decreased under heat stress, which also suggested that carbon translocation between shoot and root was reduced. However, in these experiments ample water was provided to the heat-stressed plants to avoid also inducing drought-stress, which typically increases the root: shoot ratio. As these two stresses frequently co-occur, it will be important in future studies to characterize how the plants respond to multiple stresses. Another possible explanation for the observation of reduced carbohydrate translocation to distal sink tissues would be physical blockage of the phloem under heat stress. Furch et al. (2007) showed in *Vicia faba* leaves that soon after heat shock (by burning the leaf tips), callose was deposited over the sieve pores physically blocked phloem transport several cm proximal from the site of heat-stress. Callose is a beta-1,3-glucan polymer, and the deposition of callose has been described as a common response to abiotic stresses (Maeda et al. 2014; Peuke et al. 2006), biotic stresses (Hofmann et al. 2010; Nishimura et al. 2003), and wounding (Jacobs et al. 2003). Additionally, Furch et al. (2007) observed that localized heat shock caused forisomes to change conformation and occlude sieve plates. Forisomes are specific proteins that occur in the sieve tubes of the Fabaceae that can change shape from an elongated, low-volume conformation into a spherical,

high-volume conformation. This rapid switch in conformation has also been observed under other stress conditions, such as wounding and freezing (Hafke et al. 2013; Knoblauch et al. 2001; Thorpe et al. 2010). All together, these data suggest that under heat stress, carbohydrate assimilation is initially reduced and sugar abundance in leaves declines, which may change the activity of sugar transporters (Kühn 2012). Over the longer term, the reduced activity of sugar transporters caused by heat stress would alter sugar export into the phloem. Thus, strategies to maintain photosynthesis and sucrose transport through the phloem under heat stress may result in better yielding crops.

In addition to affecting sugar transport, heat stress also impacts sugar metabolism. In plants, sucrose metabolism is directly controlled by sucrose phosphate synthase (SPS), SUS, and invertase (INV) (Koch 2004; Ruan 2014). SPS is the principal enzyme involved in sucrose synthesis and catalyzes the synthesis of sucrose-6-phosphate from UDP-glucose and fructose-6-phosphate. SUS reversibly converts sucrose into UDP-glucose and fructose in the presence of UDP. INV irreversibly hydrolyzes sucrose into glucose and fructose, and there are distinct isoforms localized to different cellular compartments (cell wall, cytoplasm, and vacuole). Studies have linked these three classes of sucrose metabolic enzymes to a plant's response to heat stress (Durand et al. 2012; Kaushal et al. 2013; Li et al. 2012b). Kaushal et al. (2013) reported that in heat tolerant chickpea genotypes, under heat-stress conditions, the activities of SPS and SUS were 40–43% higher, and the activity of vacuolar INV and CWIN were 17–23% higher relative to sensitive genotypes. However, the activities of these enzymes were significantly reduced in leaves of

both genotypes relative to the unstressed controls. Similarly, Durand et al. (2012) performed proteomic analyses on cambial tissues of young poplar (*Populus tremula x alba*) plants under heat stress, and determined that it strongly reduced the abundance of seven SUS isoforms. A link between the activity of these sucrose metabolic enzymes and sugar unloading in sink tissues has also been reported in tomato. Li et al. (2012b) showed that a heat tolerant tomato line exhibited increased activity of CWIN and a faster import rate of sucrose into young fruits compared to a heat sensitive variety. Recently, Palmer et al. (2015) found that apoplastic phloem unloading is the dominant pathway in tomato fruit during the cell expansion stage. These authors also described that a tomato CWIN functioned in the cell walls of SE and increased in activity during the ovary-to-fruit transition, thereby facilitating phloem unloading into the fruit. After sucrose cleavage by CWIN in the apoplasm, the hexoses are influxed across the PM of sink cells by hexose transporters (LeHT1,2,3) (McCurdy et al. 2010). Therefore, under heat stress in tomato fruits, the increased activity of CWIN would result in an increase in the efflux of sucrose into the apoplasm via sucrose effluxers (potentially by SWEETs), followed by the influx of hexoses into the sink cells via hexose transporters. Please see Ruan (2014) for additional discussion of how high CWIN activity correlates with sucrose import capacity in sink tissues under heat stress. Collectively, these data support the involvement of sugar transporters and sucrose metabolic enzymes in the plant's response to heat stress; however, additional research is required to understand how and when these genes are functioning.

In summary, heat stress negatively affects multiple aspects of CP, including assimilation and metabolism, phloem loading and unloading by sugar transporters, and long-distance translocation, which ultimately impacts plant growth and productivity. Recent studies highlight opportunities to develop heat-tolerant crops better able to withstand rising temperatures expected with global climate change. For example, by manipulating the activities of sugar transporters or sugar metabolic enzymes, it should be possible to enhance sugar loading and unloading from the phloem under heat stress, thus enhancing crop yield.

The invisible war: Conflict between pathogens and plants for sugar

In addition to abiotic stress, pathogens present significant challenges to world agriculture. Every year billions of dollars are lost due to crop diseases caused by bacterial, fungal, oomycete, and viral pathogens (Chakraborty and Newton 2011; Oerke 2006; Strange and Scott 2005). Different pathogens broadly utilize three strategies to acquire the necessary nutrients for survival and replication from plant cells: 1) manipulating host proteins to export nutrients to the apoplast where they reside (biotrophic), 2) causing the host cell to undergo programmed cell death and feeding on the remaining nutrients (necrotrophic), or 3) a combination of the two approaches (hemibiotrophic). While there are a variety of nutrients that a pathogen requires from its host, some of the most important are carbohydrates, predominantly in the form of glucose or fructose, but studies have found specific pathogens also uptake sucrose (Talbot 2010). Therefore, invisible wars are constantly being waged between plants and pathogens over these essential sugars.

Here, we highlight recent papers discussing how these wars are fought, the roles of sugar transporters on the front lines, and the potential for tipping the scales to favor plant resistance against pathogens.

Several studies in rice illuminate the ability of pathogens to hijack the regulation of specific members of the SWEET transporter family in order to acquire carbohydrates. Specifically, *OsSWEET11*, *OsSWEET12*, or *OsSWEET14* are upregulated depending on the particular strain of *Xanthomonas oryzae pv. oryzae* (*Xoo*), with different strains presenting different bacterial effector proteins that target promoter elements in these genes (Antony et al. 2010; Chen et al. 2010; Chu et al. 2006; Yang et al. 2006). These three genes are all clade III SWEETs, which are thought to predominantly transport sucrose (Eom et al. 2015). Their transcriptional induction by the pathogen likely results in the host cell effluxing sucrose into the apoplast for uptake by the pathogen (Baker et al. 2012; Chen et al. 2010). Through targeted modification of the effector binding sites in the promoter of *OsSWEET14*, Li et al. (2012a) successfully inhibited effector-mediated upregulation while conserving protein function. This prevented co-option of the *SWEET* gene, which is required to promote *Xoo* pathogenicity, and created a novel allele of *OsSWEET14* that will be useful to breed resistant varieties of rice. Additionally, Streubel et al. (2013) demonstrated through the use of artificial effectors that the remaining clade III *OsSWEET* genes, *OsSWEET13* and *OsSWEET15*, are possible targets for pathogen manipulation. Interestingly, the strategy to upregulate *SWEET* gene expression to enable pathogenicity is not unique to *Xoo* and rice. Studies have also found that *SWEET* genes are upregulated upon pathogen attack in cassava (*Manihot esculenta*),

Arabidopsis, citrus (*Citrus paradisi* and *Citrus sinensis*), grapevine (*Vitis vinifera*), and sweet potato (*Ipomoea batatas*) (Chen et al. 2010; Chong et al. 2014; Cohn et al. 2014; Hu et al. 2014; Li et al. 2017b). Therefore, this transporter family seems to be a common target of bacterial, fungal, and oomycete pathogens regardless of their feeding strategy. For additional discussions of the interplay between *SWEET* genes and pathogen interactions, please see Chandran (2015).

While the pathogen-host interactions described above resulted in strong upregulation of *SWEET* genes, a recent study by Asai et al. (2015) provides evidence for both positive and negative expression changes of *SWEET* genes upon pathogen attack. Gray mold (*Botrytis cinerea*) was found to upregulate the tomato *SWEET15* gene and, counterintuitively, downregulate numerous other members of the *SWEET* family across the four different clades. Little discussion exists in the literature regarding the significance of downregulation of *SWEET* genes during pathogen attack. However, *SWEET* downregulation may result in the disruption of various sugar signaling pathways, which has been shown to occur upon pathogen attack (Berger et al. 2007; Sade et al. 2013). This in turn may result in a more beneficial environment for pathogen survival and replication. Further investigation into differential *SWEET* gene expression during pathogenesis is needed to gain a better understanding of the physiology underlying pathogen-host interactions.

In addition to the SWEETs, hexose transporters are also exploited by pathogens to acquire carbohydrates. A recent study in wheat identified the *Lr67* locus, which confers partial resistance to various leaf rust diseases (Moore et al. 2015). The *Lr67* gene encodes a member of the sugar transporter (STP) gene family, an orthologue of

the Arabidopsis *AtSTP13* gene (see below). The sequence of the susceptible wheat allele is conserved among other plant STP13-like members, suggesting that the resistance phenotype is gained as a result of two non-synonymous single nucleotide polymorphisms. Both the resistant (*Lr67res*) and susceptible (*Lr67sus*) alleles are upregulated when plants are challenged by pathogens. Interestingly, the protein encoded by the resistant *Lr67* allele lacks glucose transport activity *in vitro* whereas the susceptible protein is capable of glucose transport. Additionally, the *Lr67res* and *Lr67sus* proteins localize to the PM and form homo- and heterodimers with homeologous glucose transporters, resulting in a dominant-negative effect from the *Lr67res* allele. Intriguingly, even though the *Lr67res* protein lacks glucose transport activity, grain yield is not affected in plants carrying this allele. Therefore, the *Lr67res* allele should be highly valuable in developing wheat cultivars resistant to leaf rusts. However, the *Lr67* locus is physically located close to the *RhtD1b* semi-dwarf gene, which is tightly linked to the susceptible allele of *Lr67*, resulting in possible selection against the resistant allele in wheat germplasms used today. The identification of the *Lr67* resistant allele presents a remarkable opportunity for conventional breeding or transgenic solutions to increase wheat yield by disabling various fungi's ability to access host sugars.

While the sugar transporters described above are targets for pathogen manipulation to access carbohydrates, other plant sugar transporters were recently discovered that tip the scales in favor of the plant by providing an important layer of defense. Yamada et al. (2016) reported that hexose uptake from the apoplasm to the cytoplasm by the Arabidopsis *AtSTP13* protein increased in response to flg22

detection (Fig. 3A, B). Flg22 is a 22 amino acid peptide derived from the bacterial flagellin protein that is recognized by the plant FLS2 protein, a leucine rich repeat receptor kinase (LRR-RK), located on the PM, and upon binding activates plant defense pathways (Felix et al. 1999). Additionally, the AtSTP13 protein physically interacts with FLS2 and the LRR-RK co-receptor protein BAK1, which are crucial components in plant defense response pathways (Chinchilla et al. 2007). When FLS2 binds flg22, the AtSTP13 protein is phosphorylated by BAK1 (Fig. 3C). This in turn increases AtSTP13 hexose uptake from the apoplast into the cytoplasm, sequestering sugars away from the pathogen. Furthermore, after binding flg22, FLS2 signaling upregulates *AtSTP13* transcription, resulting in more mRNA and subsequently protein, thereby increasing uptake of hexoses from the apoplast (Fig. 3C). While the regulation of sugar transporter activity by protein phosphorylation has been previously suggested (Niittyla et al. 2007; Ransom-Hodgkins et al. 2003), there has been no direct evidence for this type of regulation being important for protein function until now. Much more work is needed to understand how post-translational modifications of sugar transporters controls their activity and subcellular localization (Krügel et al. 2008; Slewinski and Braun 2010), but the exciting findings by Yamada et al. (2016) suggest that this is a fruitful topic for future studies.

How wide spread might the interaction of a receptor complex against pathogens functioning to regulate a sugar transporter be? There is little precedent for it in the literature; however, it has been observed in rice that several cell wall-associated receptor-like kinases (WAKs) are either up- or downregulated upon rice blast

fungus infection, and that overexpression or knock-out of these genes results in a decrease of disease symptoms (Delteil et al. 2016). Additionally, the rice disease resistance locus *Xa21* encodes an LRR-RLK that results in significantly decreased bacterial load and reduced lesions in plants inoculated with Xoo (Song et al. 1995). Whether these receptor protein kinases physically interact with sugar transporters to limit pathogen invasion is not yet known, but is a tantalizing hypothesis. If confirmed, this paradigm could be a novel method of regulating sugar transporter activity to increase resistance against pathogens.

Additional evidence of host sugar transporters functioning in plant defense against viruses has been reported in tomato. *Tomato yellow leaf curl virus* (TYLCV) is a devastating disease resulting in significant crop losses each year (Moriones and Navas-Castillo 2000). Two inbred tomato lines, one resistant (R) and one susceptible (S) to TYLCV, were used to identify genes potentially responsible for resistance to the virus. Eybishtz et al. (2010) found that among other genes the hexose transporter *LeHT1* is strictly expressed in R lines. To test whether this gene plays a role in TYLCV resistance, the gene was silenced using Tobacco rattle virus-induced gene silencing. Interestingly, *LeHT1* silenced resistant lines (Ri) became susceptible to TYLCV infection, but not to the extent observed in S lines lacking *LeHT1* expression. In Ri and S lines, the virus exhibited increased mobility as it was found in and between leaf veins compared to in R plants, where the virus was scattered throughout the mesophyll and absent in the main vascular system. Additionally, Ri plants underwent programmed cell death after inoculation with the virus, a response that was not observed in R or S lines. It is interesting to speculate

about the possible function of this hexose transporter in defense against TYLCV, since it would not be necessary to sequester sugars from a virus, as would be the case for a cellular pathogen (bacterial, fungal, or oomycete) infection. In their discussion, Eybishtz et al. (2010) suggested that silencing *LeHT1* could increase PD permeability and thereby increase TYLCV mobility. Thus, it may be that the LeHT1 protein is involved in maintaining sugar levels and hexose: sucrose ratios required for appropriate cellular signaling pathways, which may influence plasmodesmatal permeability. In support of this hypothesis, Sade et al. (2013) found that the transcriptional profile of Ri plants was more similar to that of R than S plants. However, when comparing sugar profiles, the Ri plants were found to be more similar to S plants, which exhibited changes in the mono: disaccharide ratio upon challenge with TYLCV. Additionally, previous work indicated that the movement protein (MP) from the tobacco mosaic virus increases PD pore size, allowing for increased virus mobility (Wolf et al. 1989). This plasmodesmatal expansion also occurs in transgenic potato plants overexpressing the MP, and results in increased carbon export from leaves (Olesinski et al. 1996). Interestingly, the source of the resistance allele against TYLCV was from a wild tomato species, *Solanum habrochaites*, which may have decreased PD permeability due to *LeHT1* expression. This in turn may have limited carbohydrate export from leaves, resulting in smaller fruits and less yield. Therefore, *LeHT1*-mediated resistance could have been selected against in domesticated tomato lines to increase fruit yield. It will be interesting to test PD permeability, and carbohydrate and virus mobility in regards to the presence and absence of *LeHT1* expression. If this hypothesis is correct, it may be

possible to engineer viral resistance in domesticated tomatoes without sacrificing yield by incorporating a *LeHT1* transgene that is specifically expressed upon viral detection.

The studies described above outline the importance of plant sugar transporters involved in both pathogenesis and plant defense. Up to this point, this review has focused on the functions of host sugar transporters; however, pathogen sugar transporters also play crucial roles in the battle over sugar. For example, the corn smut fungus (*Ustilago maydis*), encodes a novel, high-affinity sucrose transporter, UmSRT1, which is more efficient in sucrose uptake than the host ZmSUT1 protein expressed by the plant cell (Wahl et al. 2010). Upon deletion of *UmSrt1*, pathogen virulence was greatly decreased, signifying the essential function of this transporter for pathogen survival and replication. Additionally, Wittek et al. (2017) recently provided evidence that UmSRT1 also has a higher affinity than ZmSUT1 for protons. Hence, UmSRT1 is able to function at a higher pH and membrane potential than ZmSUT1. Through mathematical modeling of both transporters kinetic and biophysical parameters, the authors found that upon infection apoplastic sucrose levels drop due to UmSRT1 uptake. As a result, the sucrose concentration gradient is expected to increase between the phloem SE-CCC and the apoplasm. In Wittek et al. (2017) models, this results in ZmSUT1 switching modes from importing sucrose into the phloem, to exporting sucrose out of it and into the apoplasm. Therefore, in a biochemical arms race between UmSRT1 and ZmSUT1, the pathogen outcompetes the host cell for apoplastic sucrose as well as tricking the host into provisioning the fungus with sucrose from the phloem. An important consideration for future

iterations of the Wittek et al. (2017) model is that the functions of SWEET transporters, which may also contribute sucrose to the apoplast, were not considered. Hence, it will be interesting to see if reversal of ZmSUT1 transport direction is expected when additional sucrose transporters are taken into account. Finally, it should also be noted that while we have limited our discussion to pathogenic relationships, sugar transporters also play key roles in symbiotic relationships between plants and microorganisms (please see (Bitterlich et al. 2014; Garcia et al. 2016; Manck-Götzenberger and Requena 2016; Sugiyama et al. 2016) for discussion). The exciting recent progress in understanding the functions of sugar transporters in mediating the interactions between pathogens and plants demonstrates that the balance is tipping in our favor, but much more work is needed to protect crop yields from pathogen loss.

Beyond sugar: Additional roles for sugar transporters in plant biology

A recent exciting development in the study of *SWEET* genes is a report that certain members of the family may function not only as sugar transporters, but also as plant hormone transporters (Kanno et al. 2016). This study found that Arabidopsis clade III AtSWEET13 and AtSWEET14 proteins are capable of transporting multiple forms of gibberellins (GA), and that the *atsweet13; atsweet14* double mutant plants exhibited phenotypes related to GA responses in reproductive organs and seedlings. Specifically, delayed anther dehiscence was observed in the double mutants and was restored upon exogenous GA application. This rescue, along with an observed decrease in exogenous GA transport from roots to shoots in the double mutant

plants, supports the conclusion that these SWEETs have GA transport function *in planta*. The authors suggested a specific role of GA transport in or out of the vascular tissue facilitating GA signaling during various developmental processes. While this is the first report describing SWEETs transporting a plant hormone, there is evidence in the literature suggesting that SWEETs may also transport ions such as copper (Lopes and Araus 2008; Redondo-Nieto et al. 2012; Yuan et al. 2010; Yuan and Wang 2013; Zhao et al. 2009). Therefore, based on these studies, it is tempting to speculate that in addition to sugars, members of the SWEET family may transport other plant hormones, signaling molecules, or metabolites. If true, it may help explain the significant increase in the number of *SWEET* genes present in plant genomes relative to animals (typically ~20 or more *SWEET* genes in plant genomes compared to a single gene in animals), as well as the redundancy observed in terms of tissue expression and affinity for sugars (Eom et al. 2015). Future work is needed to verify these additional transport functions and their biological significance.

Conclusions and future directions

This review highlights recent discoveries about sugar transporters and sucrose metabolism under optimal as well as abiotic and biotic stress conditions. Understanding the regulation of sucrose transport in crops such as maize and rice is essential to increase yield. For example, it is unresolved if rice utilizes an apoplasmic or symplasmic phloem loading pathway to export sucrose from mature source leaves. With initiatives to convert rice from a C_3 to a C_4 photosynthetic species, which are expected to improve CO_2 fixation and potentially increase yields

by 50% (Mitchell and Sheehy 2006; Zhu et al. 2010), determining the sucrose transport pathway is essential (Baker et al. 2016). Additionally, further knowledge of the phloem unloading mechanisms underpinning food, feed, fiber, and biofuel production is crucial for overcoming challenges facing humanity, such as global climate change, energy insecurity, and feeding an increasing population.

Mounting evidence indicates that sugar transporters play important roles in plant responses to different abiotic and biotic stresses. Little is known about the function of sugar transporters under heat and drought stress (Durand et al. 2016; Durand et al. 2012; Jian et al. 2016; Qin et al. 2008). With the temperature on Earth predicted to increase with the rising CO₂ levels, plants will likely experience higher heat stress as well as drought stress, reducing crop yields due to decreased CP (Boyer et al. 2013; Boyer 1982; Teixeira et al. 2013; Wahid et al. 2007). Thus, more research is needed to understand how sucrose metabolism and sugar transport can be altered to generate plants better adapted to these and other stresses to stabilize and enhance crop yields. While we do not yet have a complete understanding of how photoassimilate transport is modified by drought or heat stress, promising new approaches to studying this long-standing question, such as dynamic radiotracer imaging techniques using positron emission tomography (PET) combined with a phloem transported radiolabeled sugar, could be a powerful tool in deciphering how photoassimilate transport changes under abiotic and biotic stresses (Ferrieri et al. 2012; Hubeau and Steppe 2015; Rotsch et al. 2015; Tran et al. 2017).

In addition to increased abiotic stress due to climate change, plants face additional challenges from pathogens. It is now apparent that many pathogens utilize a

common strategy of usurping host sugar transporters to obtain carbohydrate resources, whether as hexoses or sucrose, and/or alter cellular sugar homeostasis. Through genome-engineering approaches, sugar transporters that are targeted by pathogens can be modified, either through fine-tuning transcriptional control (Li et al. 2012a; Wolt et al. 2016) or protein function, resulting in resistance to the pathogen without impacting yield. Future efforts to prevent disease should focus on determining the mechanisms of how pathogens commandeer plant sugar transporters during pathogenesis. By translating this knowledge to crops, whether through traditional breeding methods, such as with the *Lr67* locus in wheat, or utilizing gene editing technology, as demonstrated with the OsSWEET members in rice, we can tip the scales of this invisible war in the favor of the plants and in turn humanity.

Many new and exciting breakthroughs have been made in recent years regarding sugar transporters and their functions. These discoveries hold great potential for future innovations and further understanding of the regulation of CP in plants. For example, the function of the *SWEET* gene family as sugar transporters was only first characterized in 2010 (Chen et al. 2010). Knowledge of the diverse roles played by these transporters has since exploded, implicating SWEETs as crucial for phloem loading of sucrose, reproductive organ development, seed filling, senescence, and disease resistance. These and other recent findings, suggesting that SWEETs may transport hormones and additional compounds other than sugars, underscore the importance of understanding each gene's function. In addition, the SUTs, TSTs, STPs, and additional sugar transporter families not discussed above, including those yet to

be discovered, may hold the keys to engineering stress tolerance and yield improvements. As research in this field progresses, more exciting discoveries can be expected as sugar is central to every aspect of plant growth, development, and crop yield.

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TABLES

Table S1.1

Growing conditions reported in characterizing rice *ossut* mutants

Reference	Nature of <i>ossut</i> mutant	Growing conditions reported
<i>ossut1</i>		
Ishimaru et al. (2001)	cv. Nipponbare, <i>ossut1</i> antisense RNA transgenic plants	T ₀ and T ₁ seedlings grown under natural daylight in a glasshouse maintained at 28C by day and 24C by night. No light levels or daylength reported. No mention of supplemental lighting was provided.
Scofield et al. (2002)	cv. Taipei 309 <i>ossut1</i> antisense RNA transgenic plants	T ₀ and T ₁ plants grown in a glasshouse under natural light with a daytime temperature of 25C and nighttime temperature of 22C. No light levels or daylength reported. No mention of supplemental lighting was provided.
Hirose et al. (2010)	cv. Nipponbare, <i>Tos17</i> retrotransposon element insertion into <i>ossut1</i>	No homozygous mutant individuals produced, so none characterized.
Eom et al. (2012)	cv. Nipponbare, <i>Tos17</i> retrotransposon element insertion into <i>ossut1</i> exon	Photo of <i>ossut1</i> mutant plant grown in a pot, but no growing conditions given.
Eom et al. (2016)	cv. Nipponbare, <i>Tos17</i> retrotransposon element insertion into <i>ossut1</i> exon	Plants grown in an environmental chamber under a 14/10 hour light/dark cycle, 24- 28C, 70-80% relative humidity, and 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.
<i>ossut2</i>		
Eom et al. (2011)	cv. Hwayoung <i>ossut2</i> T-	Plants grown in a

	DNA insertion	greenhouse at 30C during the day and 20C at night in a light/dark cycle of 14 h/ 10 h. No light levels or mention of supplemental lighting included. Additionally, plants were grown in an experimental field plot (rice paddy field) at Kyung Hee University under natural environmental conditions during the summer. No light levels or daylength reported.
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FIGURES

Figure 1.1

ZmSut1 wild-type (WT) and homozygous *zmsut1* mutant plants grown in a greenhouse during the winter months with incandescent lighting reaching a maximum of $900 \text{ mmol m}^{-2} \text{ s}^{-1}$ of light. Under these conditions, *zmsut1* mutant plants

grow similarly to wild type (A) and are able to produce ears with viable seeds after self-fertilization (B).

Key:

Figure 1.2

Model of sugar transporter functions in grass stems. (A) In developing stems, sucrose is unloaded from the SE-CCC symplasmically via PD (dashed line) into storage parenchyma cells. SUT protein localization indicates possible roles in sucrose retrieval into the SE-CCC and uptake into parenchyma cells. SWEET expression is generally low in developing stems. Sucrose is catabolized in the cytosol by SUS to facilitate growth and development. (B) In mature stems, both symplasmic (dashed line) and apoplastic phloem unloading of sucrose (SWEET and SUT transporters) have been proposed. Within the vasculature, SUT proteins may also retrieve sucrose into the SE-CCC. Unlike developing stems, SUS activity is low, and most sucrose is effluxed into and out of storage parenchyma cell vacuoles by TST and SUT proteins for storage. In addition to their proposed roles in apoplastic phloem unloading, SUT and SWEET proteins may mediate leakage and retrieval of sucrose into the storage parenchyma apoplasm to adjust cell turgor. Aquaporins (AQP) located on the PM (PIPs and NIPs) and tonoplast (TIPs) are also proposed to regulate cell turgor. Diffusion of leaked sucrose from the storage parenchyma apoplasm back to the SE-CCC is putatively prevented by cell wall suberization and/or lignification (thick black line).

Figure 1.3

Model of AtSTP13 function upon pathogen attack and flg22 detection in Arabidopsis leaves. (A) AtSTP13 is located on the PM and imports glucose and fructose from the apoplast into the cytosol. (B) When present, pathogens feed on apoplastic sugars and inject effector proteins into the host cell to increase export of cytosolic sugars into the apoplast. (C) Upon FLS2 and BAK1 binding of the pathogen-derived flg22 peptide, AtSTP13 is phosphorylated, resulting in increased uptake of glucose and fructose into the cytosol. In addition, *AtSTP13* transcription is upregulated, resulting in increased abundance of AtSTP13 protein. This in turn results in less sugars in the apoplast for the pathogen to feed upon, and decreased pathogenicity.

CHAPTER 2: *Brittle Stalk 2 Like 3*, a member of the COBRA family, functions in cell wall formation and carbohydrate partitioning in *Zea mays*

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ABSTRACT

Carbohydrate partitioning, the process of transporting carbohydrates from sites of synthesis in photosynthetic source tissues to non-photosynthetic sink tissues, is required for proper plant growth and development. Many questions concerning the genetic control of carbohydrate partitioning remain unanswered. The maize (*Zea mays*) recessive *carbohydrate partitioning defective28* (*cpd28*) and *carbohydrate partitioning defective47* (*cpd47*) mutants were identified from EMS-mutagenized populations based on phenotypes associated with increased carbohydrate accumulation, such as leaf chlorosis, decreased plant vigor, and accumulation of starch and soluble sugars in the source leaves. The two mutant lines were determined to be allelic following genetic complementation tests. Transport studies with ¹⁴C-sucrose found drastically decreased export from mature leaves in *cpd28* and *cpd47* mutants relative to wild-type siblings, resulting in a 4-15x increase in

soluble sugars and starch in the mutant leaves. Using a whole genome sequencing approach, we identified the causative mutation for each allele in the *Brittle Stalk2-Like3* (*BK2L3*) gene, a member of the COBRA family involved in cell wall development across monocots and eudicots. Interestingly, none of the previously characterized COBRA genes are reported to affect carbohydrate partitioning. In agreement with other characterized COBRA members, the BK2L3 protein localizes to the plasma membrane, and both mutant alleles condition a dwarf phenotype in dark-grown shoots and primary roots. Likewise, both alleles exhibit a significant crystalline cellulose deficiency in mature source leaves. Therefore, *BK2L3* functions in tissue growth and cell wall development, and unexpectedly impacts carbohydrate partitioning. This work demonstrates an unprecedented connection between cellulose crystallinity and whole-plant carbohydrate partitioning.

INTRODUCTION

Many products used in society, from clothing to food to alternative energy sources, are harvested from various crops and vegetation. These resources are the result of carbohydrate partitioning, the transport of carbohydrates synthesized in photosynthetic source tissues (leaves) to various non-photosynthetic sink tissues (e.g., roots, reproductive organs, stem) for proper growth, development, and yield (Lalonde et al., 2003; Slewinski and Braun, 2010; Braun et al., 2014; Yadav et al., 2015; Julius et al., 2017). In maize and other crops, sucrose (Suc) is synthesized in the mesophyll and/or bundle sheath cells of source leaves and must be transported to the phloem, the vascular tissue responsible for long-distance transport (Heyser et

al., 1978; Ohshima et al., 1990; van Bel, 2003; Braun and Slewinski, 2009; Ayre, 2011). The phloem is composed of three cell types: phloem parenchyma cells, companion cells (CC), and sieve elements (SE). The latter two cell types are found in complexes (CC-SE complexes) as the SE are enucleate and rely on associated CC for metabolic support (Esau, 1977; van Bel and Knoblauch, 2000). Suc is transported from the mesophyll and bundle sheath cells to the phloem parenchyma cells via cell wall spanning cytoplasmic connections called plasmodesmata (PD) (Evert, 1982). In maize, the phloem parenchyma and CC-SE complexes are largely symplasmically isolated from each other due to the rare occurrence of PD at this interface (Evert et al., 1978). Therefore, Suc is effluxed into the apoplastic cell wall space and imported into the CC by SWEET proteins and the SUCROSE TRANSPORT1 (SUT1) protein, respectively (Slewinski et al., 2009; Baker et al., 2012; Braun, 2012; Chen et al., 2012; Baker et al., 2016; Bezruczyk et al., 2018). Suc undergoes symplastic transport via PD from the CC into the SE (Evert, 1982). In plants, SE are connected end-to-end by porous sieve plates forming a sieve tube, a transport path throughout the plant allowing for assimilate export from source leaf tissue to various sink organs (Chen and Kim, 2009; Xie et al., 2011).

There have been a number of mutants described that exhibit alterations in carbohydrate partitioning. In maize these mutants exhibit a suite of phenotypes such as decreased plant growth, chlorotic leaves, and reduced fertility, all associated with hyperaccumulation of carbohydrates. The mutants can be separated into three groups: 1) *psychedelic* (*psc*), *tie-dyed1* (*tdy1*), and *tdy2*, in which the causes of inhibited carbohydrate transport are unknown, yet the *tyd* loci are speculated to

function in CC-SE PD transport (Braun et al., 2006; Baker and Braun, 2007, 2008; Ma et al., 2008; Slewinski et al., 2008; Ma et al., 2009; Slewinski and Braun, 2010; Slewinski et al., 2012; Baker et al., 2013), 2) *sucrose transporter1* (*sut1*), *sucrose transporter2* (*sut2*), and the triple mutant *SWEET13a, b, and c*, which function as a Suc transporters (Carpaneto et al., 2005; Slewinski et al., 2010; Baker et al., 2016; Leach et al., 2017; Bezruczyk et al., 2018), and 3) *sucrose export defective1* (*sxd1*) and *carbohydrate partitioning defective1* (*Cpd1*), which result in ectopic callose deposits that block Suc movement at various points in its transport pathway (Russin et al., 1996; Julius et al., 2018). Therefore, many genes with various functions influence carbohydrate partitioning in maize.

Plant cells are surrounded by the cell wall, a heterogeneous polysaccharide matrix, which is crucial for structural support, plant growth, cell differentiation, cell-to-cell signaling, water and sugar movement, and defense against pathogens (Cosgrove, 2005). Additionally, there have been significant differences found between the composition of eudicot, noncommelinoid monocot, and gymnosperm (Type I) compared with grass (Type II) cell walls (Carpita, 1996). Both Type I and Type II cell walls contain cellulose, however in Type I walls, cellulose is found in a network of xyloglucan, pectin and structural proteins, while in Type II walls, the cellulose is networked with glucuronoarabinoxylans, hydroxycinnamates, and low levels of pectin and structural proteins. Cellulose is a major component of both primary cell walls (PCW), which control the anisotropic growth of cells, and secondary cell walls (SCW), which are deposited upon the completion of cell elongation and are crucial for structural support of the plant tissue (Carpita, 1996).

It has been shown that cellulose is synthesized at the plasma membrane (PM) by cellulose synthase complexes (Bashline et al., 2014; McFarlane et al., 2014). These complexes are composed of several cellulose synthase proteins (CESA), which have sub-functionalized for the deposition of cellulose in either the primary or SCW. Additional components separate from the CESAs are also required for proper cellulose synthesis and organization, such as the COBRA proteins (Schindelman et al., 2001).

The COBRA family was first identified in *Arabidopsis thaliana* with the characterization of the *cobra* mutant that exhibited decreased and abnormal root growth (Benfey et al., 1993). Several additional gene family members have been characterized in Arabidopsis, maize, rice (*Oryza sativa*), cotton (*Gypsum hirsutum*), and tomato (*Solanum lycopersicum*) affecting plant height, leaf and stem brittleness, and the development of root hairs, seed coat, pollen, and cotton fiber cells. In all cases, mutant alleles exhibited decreased crystalline cellulose content, while overexpression lines in tomato exhibited increased crystalline cellulose levels (Hauser et al., 1995; Schindelman et al., 2001; Roudier et al., 2002; Li et al., 2003; Roudier et al., 2005; Ching et al., 2006; Brady et al., 2007; Sindhu et al., 2007; Hochholdinger et al., 2008; Dai et al., 2009; Sato et al., 2010; Dai et al., 2011; Cao et al., 2012; Li et al., 2013; Liu et al., 2013; Ben-Tov et al., 2015; Niu et al., 2015). COBRA proteins contain four conserved domains: an N-terminal protein targeting domain, a carbohydrate-binding motif (CBM), a conserved CCVS domain of unknown function, and a hydrophobic C-terminal tail domain. Both the N-terminal domain and the hydrophobic C-terminal domain are cleaved as post-translational

modifications, and a glycosylphosphatidylinositol (GPI) anchor is attached at the C-terminal end of the protein (Roudier et al., 2002; Brady et al., 2007). The GPI anchor integrates into the PM with the remaining peptide sequence located in the cell wall space (Eisenhaber et al., 2003). The CBM domain binds cellulose, and disruption of this domain inhibits proper function of COBRA family members (Sato et al., 2010). There has been no reported carbohydrate partitioning defect in characterized COBRA mutations.

In this paper we determined that the allelic *carbohydrate partitioning defective28* (*cpd28*) and *carbohydrate partitioning defective47* (*cpd47*) mutations occur in the gene *Brittle stalk2-like3* (*BK2L3*). *BK2L3* is a member of the maize COBRA gene family, and is involved in cell wall development and carbohydrate partitioning. This novel finding demonstrates a surprising connection linking the importance of proper cell wall structure with whole-plant carbohydrate partitioning.

MATERIALS AND METHODS

Plant materials and growth conditions

Maize (*Zea mays* L.) plants used for the photosynthetic and gas exchange analyses, microscopy, and soluble sugar and starch quantifications were grown in the field at the University of Missouri South Farm Agricultural Experiment Station in Columbia, MO. The *cpd28* and *cpd47* mutations were generated through EMS mutagenesis of B73 by Patrick Schnable and Nathan Springer, respectively. A third allele with a

Uniform *Mu* transposon insertion was ordered from the Maize Genetics Cooperative Stock Center (University of Illinois).

Plants used for the carbon-14 (^{14}C)-Suc transport assays and microscopy were grown in a greenhouse supplemented with high-pressure sodium lighting ($1600 \text{ mmols m}^{-2} \text{ s}^{-1}$) under 16-hour light and 8-hour darkness. The plants were kept at a day- and night-time temperature of 30°C and 24°C , respectively. Additionally, etiolated plants used for microscopy and height measurements were grown for 13-15 days under 24-hour dark conditions at 22°C . Genomic DNA was extracted from plants and sequenced to genotype *cpd28* and *cpd47* homozygous mutants, heterozygotes, and homozygous wild-type individuals. A dCAPS assay was developed to allow for easier genotyping of both *cpd28* and *cpd47* mutant alleles. After PCR amplification, DNA from individuals segregating the *cpd28* allele were digested with either *HaeIII* or *AluI* restriction enzymes, where the wild-type allele is cut with *HaeIII* and the *cpd28* mutant allele is cut with *AluI*. Families segregating the *cpd47* allele were PCR amplified and digested with *HinfI* and the wild-type allele is cut. All primer sequences can be found in Supp. Table 1.

Nicotiana benthamiana plants used for transient protein expression were grown in a growth chamber under 12 h light and 12 h dark conditions at $18\text{-}25^{\circ}\text{C}$ and 55-65% relative humidity for 4-5 weeks (Tran and Braun, 2017).

Starch staining

Leaves were cleared and stained with iodine-potassium iodide as previously described (Baker and Braun, 2007).

¹⁴C-Suc transport studies

¹⁴C-Suc was purchased from PerkinElmer (USA) and transport assays were performed on 3-5 week old plants as described previously (Tran et al., 2019). N = 8 plants for *cpd28*, *cpd47*, and wild-type sibling plants, respectively.

Photosynthesis and gas exchange measurements

Gas exchange and photosynthesis measurements were performed on mature source leaves between 9:00 AM and 12:00 PM using a portable infrared gas exchange system (LI-6400XT, LI-COR Inc., Lincoln, NE, USA) as described (Bihmidine et al., 2015). Net photosynthesis (A_{net} , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) and stomatal conductance (g_s , $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) rates were measured at a photon flux density of $2,000 \mu\text{mol m}^{-2} \text{ s}^{-1}$ and ambient CO_2 concentration of $400 \mu\text{mol mol}^{-1}$. Five biological samples each were measured for the wild-type, *cpd28*, and *cpd47* plants.

Microscopy

All images were taken using the same microscope and camera settings using a Nikon Eclipse 80i epifluorescent microscope equipped with a 100-W mercury bulb and a DXM1200F camera. Mature and etiolated leaf tissue free-hand cross-sections were generated and imaged as previously described (Baker et al., 2016). Callose was visualized under UV excitation by staining mature leaf sections with 0.1% (w/v) aniline blue in 0.15 M potassium phosphate buffer (pH 8.2) for 5 minutes. Lignin was visualized by staining leaves with phloroglucinol-HCl stain for 7 minutes before

mounting and imaging. Unstained leaves were sectioned and mounted in water and lignin auto-fluorescence was visualized under the 100-W mercury bulb and a FITC filter.

Mapping and whole genome sequencing

Mapping population generation, bulked segregant analysis (BSA), fine mapping, and whole genome sequencing was performed as described previously (Settles et al., 2014; Tran et al., 2019). The fine mapping population consisted of 127 mutant and 298 wild-type plants. Sequences of mapping markers can be found in Supp. Table 2.1.

Root growth assay

Seedling root growth was assayed using the growth conditions described in Sharp et al., (1988) and Voothuluru et al., (2016)(Sharp et al., 1988; Voothuluru et al., 2016). Briefly, 100-150 segregating seeds from a self-fertilized heterozygote were surface-sterilized in 1% NaClO for 15 min, rinsed under running water for 20 min, and treated with 0.1% myclobutanil (Chemsico Inc., St. Louis, MO, USA) for 20 min. Seeds were imbibed for 24 h in 1 mM CaSO₄ at room temperature with constant aeration, and germinated for 48 h on germination paper moistened with 1 mM CaSO₄ at 29°C in darkness. Germinated seedlings with primary root lengths between 0.5-2 cm were sown in Plexiglas boxes containing well-watered vermiculite moistened to drip-point with 1 mM CaSO₄, and grown for 48 h under non-transpiring conditions at 29°C in darkness. Seedlings were removed from the

growth medium, primary root axial lengths were measured, and shoot tissue was harvested for genotyping. Seedling roots grew vertically without contacting the bottom of the container for the duration of the growth experiment.

Cell Wall Isolation & Compositional Analyses

Cell walls were isolated from six biological replicates each for mutant and wild-type sibling plants as described in Mertz et al., (2012) (Mertz et al., 2012). Briefly, 200-500 mg frozen leaf tissue from field-grown immature sink and mature source leaves harvested at 5:30 AM were weighed, ground with a mortar and pestle under liquid nitrogen, and stored at -80°C until all samples were processed. Frozen powder was suspended in ~10 mL of 50 mM Tris-HCl, pH 7.2 containing 1% v/v SDS, and homogenized in a 30 mL glass-glass Duall tissue homogenizer (DWK Life Sciences, Rockwood, TN). The homogenate was pelleted at 2000 g for 5 minutes and the supernatant was replaced with fresh buffer and heated to 65°C for 20 minutes to extract soluble proteins. Walls were pelleted and washed 3x each with warm (50°C) dH₂O, warm 50% ethanol, and room temperature dH₂O. Isolated walls were resuspended in dH₂O with 1-2 drops of toluene to prevent microbial growth and stored at 4°C.

For quantification of TFA-labile cell wall sugars, 1 mL aliquots of wall suspension were treated with 2 U porcine α -amylase/mg carbohydrate in 10 mM Tris-Malate, pH 6.9 as described in Pettolino et al., (2012) to remove starch that would confound the analysis of cell wall Glc moieties (Pettolino et al., 2012). 1-2 mg of destarched,

lyophilized wall material were hydrolyzed in 2 M TFA containing 500 nmol/ μ L *myo*-inositol for 90 minutes at 120°C, reduced with NaBH₄, and converted to alditol acetates as described in Gibeaut and Carpita, (1991) (Gibeaut and Carpita, 1991). Derivatized sugars were separated and quantified by gas chromatography-mass spectrometry using the parameters described in Mertz et al., (2012) (Mertz et al., 2012).

For quantification of crystalline cellulose by the phenol-sulfuric assay, TFA-insoluble pellets of 4-6 mg lyophilized wall material were recovered following hydrolysis and washed 3x with dH₂O in a 5 mL conical Reacti-Vial (Pierce). Triplicate 200 μ L aliquots of suspended pellets from each sample were combined with 5% v/v phenol and concentrated sulfuric acid, developed for 2 h, and analyzed spectrophotometrically for Glc equivalents at 500, 510, and 520 nm according to Dubois et al., (1956) (Dubois et al., 1956).

Soluble sugar and starch quantification

Mature source leaf tissue was harvested from field-grown plants and immediately placed in liquid nitrogen before being stored at -80°C until measurement. Samples were harvested at 5:30 AM. Five biological samples were measured for each genotype. Soluble sugar and starch samples were extracted according to Leach and Braun (2016) (Leach and Braun, 2016). Samples were quantified using high-performance anion exchange chromatography against known standards according to Leach et al. (2017).

Subcellular localization assays

The coding sequence for the pH insensitive Citrine fluorescent protein was inserted in the coding sequence of the BK2L3 protein (Zm00001d034049) immediately after the N-terminal secretory peptide domain and synthesized in the pDONOR221 vector (Life Technologies, USA) (Tian et al., 2004). The BK2L3-Citrine construct was recombined into the pEarleyGate100 vector generating the p35S::BK2L3-Citrine translation fusion protein. *Agrobacterium tumefaciens* transformation and tobacco leaf infiltration was performed as described in (Tran et al., 2019).

RESULTS

***cpd28* and *cpd47* mutants are dwarfed and exhibit reduced photosynthesis**

The *cpd28* and *cpd47* mutants were identified from an EMS-mutagenized population based on a suite of phenotypes, including reduced plant height, chlorosis, and anthocyanin accumulation in the leaves (Figures 2.1 and S2.1). Additionally, the chlorosis and anthocyanin accumulation develops in a basipetal pattern as the leaf is exposed to sunlight (Figures 2.1B-C and S2.1B-C). Based on Mendelian segregation ratios both *cpd28* and *cpd47* are recessive mutants segregating in a 1:3 ratio for mutant to wild-type plants. Due to the similarity in phenotype between the two mutants, heterozygous plants carrying either allele were crossed together, and the resulting offspring segregated the phenotype in a 1:3 mutant to wild-type ratio, supporting that the two mutants are allelic.

Due to the chlorosis in the mutant leaves, measurements were taken to determine if there is a difference in photosynthesis between the wild-type and mutant siblings. Both the photosynthetic rate and stomatal conductance were dramatically decreased in the *cpd28* and *cpd47* individuals relative to wild type (Figure S2.2).

***cpd28* and *cpd47* mutants hyperaccumulate carbohydrates in their leaves**

The decreased plant height, leaf chlorosis, anthocyanin accumulation, and reduced gas exchange in the *cpd28* and *cpd47* mutants are similar to phenotypes observed in other mutants exhibiting defects in carbon export from their leaves. Therefore, mature *cpd28*, *cpd47*, and wild-type sibling leaves were harvested at end of night, cleared, and stained with iodine potassium iodide (IKI) for the presence of starch. Both mutant alleles exhibited hyperaccumulation of starch relative to their wild-type siblings (Figures 2.2A-B and S2.3A-B).

Additionally, starch and the soluble sugars Suc, Glc, and Fru content in *cpd28*, *cpd47*, and wild-type mature leaves were quantified. In agreement with the starch staining results, each mutant exhibited increased levels of starch and all three soluble sugars in their leaves relative to wild-type samples (Figures 2.2C and S2.3C). Taken together, these results demonstrate that *cpd28* and *cpd47* mutants have increased carbohydrate accumulation in their leaves, which we hypothesize is due to decreased Suc export from source leaves.

Mutant leaves exhibit decreased Suc export

To test the above hypothesis, transport studies were conducted in which radiolabeled ^{14}C -Suc was applied to cut tips of source leaves from 5-week-old *cpd28* and *cpd47* mutant plants and their wild-type siblings. After application of the radiolabeled Suc, the plants were allowed to transpire for 1 h before the leaves were harvested for imaging. Figure 3 shows ^{14}C -Suc is transported to the base of the mature wild-type leaf. However, in *cpd28* and *cpd47* individuals, the ^{14}C -Suc is severely limited in its transport, and no signal is seen in the basal half of the leaves (Figures 2.3 and S2.4). To determine the extent of the decreased transport, the images were quantified, and there was a significant reduction in the amount of ^{14}C -Suc detected from 10 cm from the application site and further proximal in each mutant allele compared to wild-type siblings (Figures 2.3C and S2.4C). These data suggest that the hyperaccumulation of carbohydrates in the *cpd28* and *cpd47* mature leaves is a result of decreased Suc export.

Carbohydrate accumulation is associated with lignin deposition in phloem

cpd28 and *cpd47* mutant veins were analyzed to determine if there were any abnormalities in regards to anatomy or ectopic deposition of callose or lignin potentially causing the inhibited transport of Suc as seen in the *Cpd1* mutant (Julius et al., 2018). Cross sections were made near the tip of mature leaves displaying anthocyanin accumulation in *cpd28* and *cpd47* individuals and comparable tissues from wild-type plants. The cross-sections were stained with aniline blue, and, interestingly, some callose deposition was seen in the phloem of both mutants but not in wild type (Figure 2.4A-C). Additional cross-sections were made and stained

with phloroglucinol-HCl, which results in a pink hue in cell walls containing lignin (Ruzin, 1999). Some faint pink coloration in the phloem was observed in both mutant samples while absent in wild-type sections (Figure 2.4D-F). To confirm these results cross-sections were observed under UV light with a FITC filter, under which lignin will autofluoresce bright yellow-green (Bihmidine et al., 2015). Consistent with the phloroglucinol-HCl staining results, *cpd28* and *cpd47* plants exhibited ectopic lignin autofluorescence in their phloem, while none was observed in the phloem of wild-type samples (Figure 2.4G-I). Therefore, we conclude both mutants exhibit ectopic deposition of callose and lignin in their phloem; however, it is not known if these deposits are the cause of the decreased Suc export in the mature leaves. Alternatively, the callose and/or lignin deposits may be downstream effects of Suc hyperaccumulation.

To address this question, *cpd28*, *cpd47*, and their wild-type siblings were grown for two weeks under etiolated conditions, which precludes carbohydrate hyperaccumulation. Cross-sections were made in the tips of fully expanded leaves and observed under UV light with a FITC filter to check for the presence of lignin autofluorescence in the phloem. Interestingly, under these conditions no lignin autofluorescence was observed in the phloem of either mutant line or their wild-type siblings (Figure 2.5A, B, E, F). Additionally, cross-sections stained with aniline blue exhibited no ectopic callose deposits in the phloem (Figure 2.5C, D, G, H). Therefore, we conclude that the ectopic lignin and callose deposition observed in the phloem of the two mutants grown in field conditions are likely downstream

effects of the hyperaccumulation of carbohydrates, and not the cause of the increased carbohydrate levels.

***cpd28* and *cpd47* exhibit allelic differences in regards to growth**

Interestingly, while performing the etiolated growth experiments on *cpd28* and *cpd47* mutants, we observed that the *cpd28* mutant plants exhibited a severe growth defect compared to wild-type siblings, whereas this phenotype was not immediately obvious in *cpd47* mutants (Figure 2.6A-B). However, upon measurement of the etiolated *cpd28* and *cpd47* shoots, both mutants were significantly shorter than wild type (Figure 2.6C). Additionally, the root system in *cpd28* mutant plants appeared smaller than wild-type roots (Figure 2.6A). Therefore, segregating families for *cpd28*, *cpd47*, and their wild-type siblings were imbibed and germinated on germination paper to eliminate possible differences in germination rate. Germinated plants of similar size were transferred to transparent containers filled with saturated vermiculite. The plants were grown for two days before they were excavated to measure root lengths and genotyped. In agreement with the etiolated shoot data, *cpd28* mutant plants had an average root length ~6 cm shorter than their wild-type siblings, and *cpd47* plants had an average root length ~2.5 cm shorter than their wild-type siblings (Figure 2.6D). Therefore, it appears that both *cpd28* and *cpd47* mutants have growth defects in addition to their carbohydrate partitioning phenotype, with *cpd28* being the more severe allele.

cpd28* and *cpd47* mutations occur in *Brittle Stalk2-like3

To understand the molecular mechanism for the inhibited carbohydrate transport and dwarf phenotype, we employed a Bulk Segregant Analysis (BSA) to map the mutations to the tip of the long arm of chromosome one. The region was further narrowed with polymorphic PCR markers DEL.22259 and DEL.22319 to a ~750 kb region, containing 17 predicted protein-encoding genes (Settles et al., 2014). To identify the causative mutations for each allele, a whole genome sequencing approach was utilized with DNA isolated from independent pools of >40 mutant plants for each allele. *cpd28* and *cpd47* DNA sequence was aligned with the B73 reference sequence in the region of interest to identify any mutations in the 17 genes. Finally, a comparison of identified mutations between the *cpd28* and the *cpd47* sequences resulted in the identification of a single gene, *Brittle Stalk2-like3* (*BK2L3*), with unique mutations in both *cpd28* and *cpd47*.

BK2L3 is one of nine members of the maize COBRA gene family (Brady et al., 2007). The *cpd28* mutation is a single nucleotide polymorphism (SNP) resulting in a non-synonymous (missense) amino acid substitution in the first amino acid, changing it from Methionine to Isoleucine, and therefore resulting in the loss of the start codon with the next start codon located 30 amino acids downstream out of frame, likely resulting in a non-functional protein, if translated. The *cpd47* mutation is a SNP resulting in a missense mutation in amino acid 103, changing an Aspartic Acid to Asparagine (Figure 2.7A). It is important to note that this Aspartic Acid residue is conserved in six of the nine maize COBRA family members, and results in an additional predicted N-glycosylation site at the end of the CBM domain, likely impairing proper protein folding and/or function (Figures 2.7B and S2.5).

Therefore, we conclude that the *cpd28* allele is likely a null allele, and *cpd47* is a hypomorphic allele. This hypothesis is supported by the differences in the etiolated growth phenotype reported between the two mutants, where *cpd28* mutants have a more severe growth defect, and *cpd47* mutants exhibit a less severe growth defect. A third independent mutant allele containing a transposon insertion in the 5'UTR of *BK2L3* was confirmed through genetic complementation testing by starch staining heteroallelic plants crossed by *cpd28* (Figures 2.7 and S2.6). Collectively, these data indicate that mutations in the *BK2L3* gene underlie the *cpd28* and *cpd47* mutant phenotypes.

BK2L3* is closely related to *AtCOBRA* and *OsBC1L4

There have been several members of the COBRA family characterized in Arabidopsis, cotton, tomato, rice, and maize. Phylogenetic analysis separates the protein family into five clades based upon the length of peptide sequences and function. COBRA proteins in Clades I, II, and III typically consist of ~400-450 amino acids, while members in Clades IV and V consist of ~675 amino acids (Figures S2.5 and 2.7). Clade I includes *OsBC1L4* and *AtCOBRA*, which function in the development of PCWs, and each exhibits a dwarf phenotype when mutated (Schindelman et al., 2001; Roudier et al., 2005; Dai et al., 2009; Dai et al., 2011). Similarly, *ZmBK2L3* is located in Clade I, and etiolated *cpd28* and *cpd47* mutant seedlings also exhibit diminished growth phenotypes. Interestingly, *AtCOBRA*, *OsBC1L4*, and *ZmBK2L3* are co-expressed with PCW CESAs (Dhugga, 2001; Appenzeller et al., 2004; Brady et al., 2007). Clade II contains the COBRA proteins

OsBC1, ZmBK2, and AtCOBL4, which have all been characterized as playing important roles in SCW development (Li et al., 2003; Brown et al., 2005; Ching et al., 2006; Sindhu et al., 2007; Liu et al., 2013). Non-structural carbohydrate levels were quantified in the maize *bk2* mutant leaves, but no differences were found in soluble sugars relative to wild type, although starch levels were decreased (Figure S2.8). Hence, these data may suggest that BK2L3 functions in PCW rather than SCW formation.

***cpd28* and *cpd47* exhibit crystalline cellulose deficiencies in mature leaves**

To evaluate whether the classical cobra mutant cell wall phenotype underlies the observed carbohydrate partitioning defective phenotype in *cpd28* and *cpd47*, cellulosic and non-cellulosic cell wall sugars in immature sink and mature source leaves of both mutant plants and their wild-type siblings were quantified. Cell walls were isolated and digested in 2 M trifluoroacetic acid (TFA) to separate TFA-labile sugar moieties derived from pectins, arabinoxylans, mixed-linkage glucans from the amorphous cellulose located in the insoluble pellet comprised of recalcitrant crystalline cellulose and lignin polymers. Immature sink leaf cell wall composition was broadly similar between mutant and wild-type siblings for both mutants (Fig. 2.8A and C). However, multiple alterations in cell wall composition were apparent between mature mutant and wild-type leaves. A substantial reduction in the relative proportion of crystalline cellulose per unit dry mass was seen for both *cpd28* and *cpd47* mutants relative to wild type (Figure 2.8B). Interestingly, a stronger proportional reduction was observed for *cpd28* than *cpd47* (34% and 19%,

respectively), consistent with the above growth data suggesting that *cpd28* is the more severe allele. Minor, but consistent, pleiotropic decreases in the proportions of xylose and galactose, and a minor increase in arabinose, were apparent for both mutant tissues, suggesting slight changes to arabinoxylan and pectin composition (Figure 2.8D). Mature leaf tissue of both mutants and wild-type siblings exhibited the expected proportional increase in xylose and decrease in Glc moieties relative to immature sink leaves (Figure 2.8C-D). Thus, the inability of the mature mutant leaves to sustain wild-type levels of carbohydrate transport is not due to a failed transition from immature sink to mature source tissue.

BK2L3 localizes to the plasma membrane

Previous studies localized COBRA proteins to the PM, anchored there via their GPI attachment (Roudier et al., 2005; Dai et al., 2011). To determine whether BK2L3 also localized to the PM, the yellow fluorescent protein Citrine was translationally fused to BK2L3 and, because of the expected post-translational protein processing at both the N- and C-termini, positioned immediately after the predicted N-terminal secretory peptide in the BK2L3 protein, upstream of the remaining peptide sequence. *Nicotiana benthamiana* leaves were co-infiltrated with p35S::BK2L3-Citrine and p35S::PIP2a-CFP, a tagged aquaporin protein that localizes to the PM (Nelson et al., 2007). The fluorescent signals of the BK2L3-Citrine and PIP2a-CFP proteins overlapped, indicating that BK2L3 localizes to the PM (Figure 2.9).

DISCUSSION

The *cpd28* and *cpd47* mutant alleles were identified from EMS-mutagenized populations and display a suite of phenotypes similar to other previously characterized mutants inhibited in their carbohydrate transport, such as decreased plant height and vigor, anthocyanin accumulation, and chlorosis of mature source leaves (Riesmeier et al., 1994; Russin et al., 1996; Bürkle et al., 1998; Braun et al., 2006; Hackel et al., 2006; Baker and Braun, 2008; Srivastava et al., 2008; Slewinski et al., 2009; Slewinski and Braun, 2010; Bezruczyk et al., 2018; Julius et al., 2018). Starch and soluble sugar quantifications and ^{14}C -Suc transport studies confirmed *cpd28* and *cpd47* mutants hyperaccumulate carbohydrates due to decreased Suc export from the mature source leaves. The observed chlorosis and reduced photosynthetic measurements in the mutant plants likely result from high Glc and/or Suc levels in the leaves, which are known to downregulate photosynthetic genes (Rolland et al., 2006). *cpd28* and *cpd47* mutants also exhibited dwarf phenotypes when grown in dark conditions, with *cpd28* being more severe than *cpd47*. A similar difference in severity was observed in regards to crystalline cellulose content in the immature and mature leaves. Concurring with the differences in phenotype severity, the *cpd28* mutation results in the loss of the start codon in the *Brittle Stalk2-Like3* gene. However, the *cpd47* mutation results in a missense mutation leading to an Asparagine to Aspartic acid change immediately following the CBM domain, and resulting in an additional predicted N-glycosylation site. It has been shown that COBRA proteins are N-glycosylated, and in rice the CBM domain is crucial for proper function of the COBRA gene *BC1* (Sato et al., 2010; Liu et

al., 2013). Therefore, it is plausible that the *cpd47* mutation is a hypomorphic allele due to an impaired CBM domain.

All characterized COBRA family members function in cellulose biosynthesis, however, they can be separated into five phylogenetic clades, which correlates with specific functions, such as primary or SCW formation, or root hair, pollen, or fruit/seed development (Figure S2.7). *BK2* was the first COBRA member to be characterized in maize, and was reported to function in SCW development (Ching et al., 2006; Sindhu et al., 2007). The predominant phenotype of *bk2* mutants is a loss of tissue flexibility, resulting in brittle plants that snap under slight pressure. *bk2* plants reach normal height, and no anthocyanin accumulation or chlorosis is observed in the mature leaves (Ching et al., 2006; Sindhu et al., 2007). Additionally, *bk2* plants do not hyperaccumulate starch or soluble sugars in their mature source leaves (Figure S2.8). The *bk2* brittle phenotype is not observed in *cpd28* and *cpd47* mutants. Instead, they are dwarfed compared to wild-type siblings. This suggests that the *BK2L3* gene functions in PCW development, as improper formation of PCWs would inhibit elongation of growing tissues. This was reported in both *Atcobra* and the *brittle culm1-like4* (*bc1l4*) mutant in rice, which both exhibit dwarf phenotypes and are closely related to *ZmBK2L3* (Figure S2.7). Additionally, specific *CESA* genes responsible for PCW growth are co-expressed with *BK2L3* in maize RNA-seq data sets (Dhugga, 2001; Appenzeller et al., 2004; Brady et al., 2007), suggesting a role for *BK2L3* in PCW formation.

Interestingly, of the currently characterized COBRA genes, none have been reported to have a carbohydrate partitioning defect. However, in addition to the

dwarfed phenotype and decreased culm cellulose, *Osbc114* mutants exhibited increased levels of starch in their stems relative to wild-type siblings. The authors suggested the starch accumulation was due to the inability to incorporate sugars into cell wall components, resulting in the unincorporated Glc being converted to starch (Dai et al., 2011). Additionally, in the *Arabidopsis* null mutant *cell elongation defect1* (corresponding to the *Atcobra-5* and *Atcobra-6* alleles) anthocyanin accumulated in aerial tissues, and callose deposition was observed in mutant leaves (Ko et al., 2006). These phenotypes are similar to those observed in *cpd28* and *cpd47* mutants in maize. While there were no reports of increased carbohydrate levels in the *cobra* mutants, defense-related genes were upregulated in *cob-5* and *cob-6* compared to wild-type siblings (Ko et al., 2006). It has been reported in other carbohydrate accumulating mutants that increased carbohydrates appear to prime the defense response system (Gebauer et al., 2017; Julius et al., 2018). Hence, it will be interesting to determine in future studies if either *Atcobra* or *Osbc114* mutants accumulate carbohydrates in their leaves when grown under high light conditions.

Based on publicly available maize gene expression data (Li et al., 2010; Wang et al., 2014; Stelpflug et al., 2016), *BK2L3* is broadly expressed across multiple tissue types, and shows peak expression early in organ development, consistent with a role in PCW biosynthesis. The expression profile of *BK2L3* RNA in a developing maize leaf is consistent with expression during both proto- and metaploem development, but not SCW development in the xylem (Evert et al., 1996; Li et al., 2010; Wang et al., 2014). The large proportional decrease in crystalline cellulose content in mature, fully penetrant source tissue occurs after growth and

development have ceased, and is likely a pleiotropic effect similar to that reported by Dai and colleagues (2011) (Dai et al., 2011). Therefore, we hypothesize that defects in PCW development in *bk2l3* mutants (i.e., *cpd28* and *cpd47*) result in decreased Suc export, and subsequent build-up of carbohydrates in the mature leaves. Speculatively, this could be due to improperly formed PD connections along the symplastic transport pathway in the mature leaf. Brecknock *et al.* (2011) showed spokes connecting the cell wall to the PM in the PD in the alga *Chara*, and enzymatic degradation revealed cellulose-pectin networks were crucial for this structure (Brecknock et al., 2011). If similar connections are present in angiosperm PD, alterations of cellulose crystallinity and cell wall integrity could possibly impair symplastic transport. Alternatively, it is possible improper formation of the SE cell wall impairs bulk, long-distance phloem sap transport. In the grasses *Triticum aestivum* and *Aegilops comosa*, the innermost, load-bearing layer of both proto- and metaphloem is comprised of dense parallel arrays of radially oriented cellulose microfibrils (Eleftheriou and Tsekos, 1982; Eleftheriou, 1987). If the correct formation of this load bearing wall were impaired, the cells may not appropriately respond to the high turgor pressure required in the mature source phloem needed to drive bulk flow to various sink tissue phloem (Lalonde et al., 2003; Patrick, 2012). To test these and other hypotheses, future ultrastructural studies will determine whether BK2L3 is required for ordered cellulose microfibril deposition and cell wall thickening on a cell-specific basis, with particular attention to the cells of the Suc export pathway in developing leaves.

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TABLES

Table S2.1

Mapping and genotyping primer sequences

Primer Name	Primer Sequence 5'-3'
DEL.22259 R4	GAAGCATATGTGCGGAGAATACC
DEL.22259 F4	AGCCAAGGACTTTCTGCTTTC
DEL.22243 R1	GACTCCAATTGTGGGAATGCT
DEL.22243 F1	TCAGGAAACAGTGAAGTCCCTA
DEL.22319 F1	GTCTTGCCATAATCCATCATCCTC
DEL.22319 R1	GTTGAACATCCCGTCCCTATTG
cpd47 dCAPS <i>Hinf</i> I F	AGTCCAGTCGACCTGTACAATA
cpd47 dCAPS <i>Hinf</i> I R	GGGCTGCTCTTGAACTTTGAGGAGT
cpd28 dCAPS <i>Hae</i> III F	TGCTGCTGCTTCCGCTGCAGTAAA
cpd28 dCAPS <i>Hae</i> III R	ACGCGACGGATCTGCCGCTCGCGGC

FIGURES

Figure 2.1

cpd28 plants are dwarfed relative to wild-type siblings, and exhibit basipetal accumulation of anthocyanin in their leaves. (A) Field grown wild-type (left) and *cpd28* mutant (right) plants. (B-C) A gradient of *cpd28* (B) and wild-type (C) leaves from oldest (left) to youngest (right). The mutant leaves accumulate anthocyanins in a basipetal fashion when exposed to sunlight.

Figure 2.2

cpd28 mature leaves hyperaccumulate starch and soluble sugars. (A-B) Wild type (WT, top) and *cpd28* (bottom) mature leaves before (A) and after (B) IKI stain. (C) Soluble sugar and starch quantification in WT and *cpd28* mature leaves. Error bars are mean $\pm$ StdEr. Asterisks represent a significant difference at $P \leq 0.05$ using Student's *t*-test.

Figure 2.3

Export of C^{14} -Suc is reduced in *cpd28* mutant source leaves. (A) Dried wild-type (WT, top) and *cpd28* leaves (bottom). (B) Autoradiograph showing C^{14} -Suc distribution in WT (top) and *cpd28* (bottom) leaves. (C) Quantification of C^{14} -Suc distribution in WT (white bars) and *cpd28* (black bars) leaves. Translocation was measured according to signal intensity at each distance (in cm) from the application site. The graph represents means of intensity $\pm$ StdEr. Asterisks represent a significant difference at $P \leq 0.05$ using Student's *t*-test.

Figure 2.4

cpd28 and *cpd47* mutants exhibit ectopic callose and lignin deposits in phloem of mature leaves. Cross-sections of mature wild-type (A,D,G), *cpd28* (B,E,H), and *cpd47* (C,F,I) mutant leaves were stained with aniline blue (A,B,C), phloroglucinol-HCl (D,E,F), or unstained (G,H,I). (A-C) Cross-sections stained with aniline blue were imaged under UV light to identify the presence of ectopic callose deposits in the phloem (white arrowheads). (D-F) Cross-sections stained with phloroglucinol-HCL were imaged under bright-field. (G-I) Autofluorescence of lignin in the phloem (white arrows) of *cpd28* and *cpd47* mutants was visualized under UV light with an FTIC filter. Scale bars = 50 μ m

Figure 2.5

No ectopic callose or lignin was seen in the phloem of immature leaves of *cpd28* and *cpd47* mutants. Cross-sections from etiolated 2-week old wild-type (A,C,E,G), *cpd28* (B and D), and *cpd47* (F and H) mutant leaves were imaged under UV light with an FTIC filter for lignin autofluorescence (A,B,E,F), or stained with aniline blue for detection of callose (C,D,G,H). Scale bars = 50 μm.

Figure 2.6

cpd28 and *cpd47* mutants exhibit dwarf phenotypes under etiolated conditions. (A-B) Two week-old etiolated wild-type (WT, left) and *cpd28* (A) or *cpd47* (B) mutant (right) seedlings. (C) Height measurements for *cpd28* and *cpd47* mutants and WT sibling shoots. (D) Length measurements for *cpd28* and *cpd47* mutant and WT sibling primary roots. Error bars are mean $\pm$ StdEr. Asterisks represent a significant difference at $P \leq 0.05$ using Student's *t*-test.

Figure 2.7

The causative mutations for the *cpd28* and *cpd47* alleles were identified in the *Brittle Stalk 2-like3* gene. (A) The *cpd28* mutation results in loss of the start codon. The *cpd47* mutation results in a missense mutation changing an Aspartic Acid to Asparagine. A third allele contains a transposon insertion in the 5'UTR. (B) The NetNGlyc 1.0 program was used to predict N-glycosylation sites in the wild-type (left) and *cpd47* (right) BK2L3 protein sequences. The *cpd47* mutation results in an additional predicted N-glycosylation site (blue box).

Figure 2.8

cpd28 and *cpd47* mutants exhibit decreased crystalline cellulose, and altered pectin and hemicellulose content in mature leaves. (A-D) Measurement of crystalline cellulose (A and B), and pectin and hemicellulose (C and D) in wild-type, *cpd28*, and *cpd47* mutant immature sink leaves (A and C) and mature source leaves (B and D). Error bars are mean $\pm$ StdEr. Asterisks represent a significant difference at $P \leq 0.05$ using Student's *t*-test.

Figure 2.9

BK2L3 localizes to the plasma membrane. (A-B) Localization of p35S:ZmBK2L3-Citrine (A) and the PM-localized p35S:AtPIP2a-CFP (B) in *Nicotiana benthamiana* leaf epidermal cells. (C) Bright-field image of *N. benthamiana* epidermal cells. (D) Overlay of p35S:ZmBK2L3-Citrine, p35S:AtPIP2a-CFP, and bright-field images. Scale bar = 30 μ m

Figure S2.1

cpd47 mutant plants are dwarfed relative to wild-type siblings and exhibit basipetal accumulation of anthocyanin in their leaves. (A) Field grown wild-type (left) and

cpd47 mutant (right) plants. (B-C) A gradient of *cpd47* (B) and wild-type (C) leaves from oldest (left) to youngest (right). The mutant leaves accumulate anthocyanins in a basipetal fashion when exposed to sunlight.

Figure S2.2

cpd28 and *cpd47* mature leaves exhibit decreased photosynthesis and stomatal conductance. (A) Net photosynthetic rate measured in *cpd28* and *cpd47* mutant leaves (black bars) and wild-type (WT) siblings (white bars). (B) Stomatal conductance measured in *cpd28* and *cpd47* mutant leaves (black bars) and WT siblings (white bars). Error bars are means $\pm$ StdEr. Asterisks represent a significant difference at $P \leq 0.05$ using Student's *t*-test.

Figure S2.3

cpd47 mature leaves hyperaccumulate starch and soluble sugars. (A-B) Wild-type (WT, top) and *cpd47* mutant (bottom) leaves before (A) and after (B) IKI stain. (C) Soluble sugar and starch quantification in WT and *cpd47* mature leaves. Error bars are mean ± StdEr. Asterisks represent a significant difference at $P \leq 0.05$ using Student's *t*-test.

Figure S2.4

Export of C^{14} -Suc is reduced in *cpd47* mutant source leaves. (A) Dried wild-type (WT, top) and *cpd47* (bottom) leaves. (B) Autoradiograph showing C^{14} -Suc distribution in WT (top) and *cpd47* (bottom) leaves. (C) Quantification of C^{14} -Suc distribution in WT (white bars) and *cpd47* (black bars) leaves. Translocation was measured according to signal intensity at each distance (in cm) from the application site. The graph represents means of intensity $\pm$ StdEr. Asterisks represent a significant difference at $P \leq 0.05$ using Student's *t*-test.


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2m8K219 -----MGRFVFLILMCCSGSRF-----
2m8K217 -----MEPRFVLLALAVAAALSVVA-----
2m8K216 -----LKVGGGALLALAVAAALSVVA-----
2m8K216 MAPPPLPAPFVAASVALLAVAPSSSLTRPS-----
2m8K213 MAAS-----GRSVACCAALAAALLSAPT-----
2m8K214 MAAS-----GRSIACFAAVLAAALLSAPT-----
2m8K215 MVMAAPVFLPAPFVAASVALLAVAPSSSLTRPS-----
2m8K213 -MAGSVAFHVVLLGLLAGLAAQRAATTA-----
2m8K218 MAMFVVV-VMAAMPFIFLVLAHTASAQPD-----
2m8K219 -----AGCGILLTYTLQRIKIRPVAAPSGQPSFSASATVVAAGTRPLRANALLTFVHGEILVSDG-----

2m8K219 -----TGATDPI-----DP-----
2m8K217 -----TDPL-----DP-----
2m8K216 -----VATDPL-----DP-----
2m8K216 -----GATDPL-----DP-----
2m8K213 -----TEATDEL-----DP-----
2m8K214 -----TEATDEL-----DP-----

2m8K215 AVLLDGSCLPAPVGG-----ATFAGTTPMADLLSITETAGEPSLIESKIRITGTGTFQVWAPGRMPDKTINLTPVGFRCFAPNHDV-----HYCCCV-----KDRFPAKKAASR-----
2m8K213 AVLTGGGCLPAPVGG-----TSFGTTPHDLTFIATAGDLSQIQASVGITVTLFAGPGFVPLPTALSLDP-----ATACPAATNVTARV-----LSTCCVLTFRKAAKAAIDAMTTPD-----
2m8K218 AVLTSGAALPAPVGG-----ATFAGTTPMADLLSITETAGEPSLIESKIRITGTGTFQVWAPGRMPDKTINLTPVGFRCFAPNHDV-----HYCCCV-----KDRFPAKKAASR-----

2m8K219 -----NGSITIVNDPQSLVAGHTPYT-----VWVSIHNYQMYRHIER-PGNRLSWKAGKEVINSTTGATTE-----QDCSRVSGGSGPRCCQKQFVMDLPGTPVYHQ-----
2m8K217 -----NGSITIKNDIMNTPDQ-----YV-----AVVTINSPQTYRQITA-PGNTVGTWKAKEVINSMVGAQATE-----QDCSR-PKANI-PRCCRTQAVVDLLPGTPVYHQ-----
2m8K216 -----NGSITIKNDIVISNTPDQ-----YV-----AMVTHMSPQMYRHIMA-PONTLWKAKEVINSTTGATTE-----QDCSR-PKGGI-PRCCRTQAVVDLLPGTPVYHQ-----
2m8K213 -----NGSITIKNDIMNTPDQ-----YV-----AVVSLAYTQVYRHIAQPPGNRLWKAKEVINSMVGAQATE-----QDCSR-PKASVLPCCRRDQEVVDLLPGTPVYHQ-----
2m8K214 -----NGSITIKNDIMNTPDQ-----YV-----AVVTHMSPQMYRHIMA-PONTLWKAKEVINSMVGAQATE-----QDCSR-PKGGI-PRCCRTQAVVDLLPGTPVYHQ-----
2m8K215 -----YQTRHADLTFADVLQANTH-----YQ-----VQVTIDMSPIRLN-----NHLTWKAGKEVINSMVGAQATE-----QDCSR-PKGGI-PRCCRTQAVVDLLPGTPVYHQ-----
2m8K213 KDFPFGTGDVITVDVLQAPPS-----YL-----ALVTLEHAKLGRLE-----NHLTWKAGKEVINSMVGAQATE-----QDCSR-PKGGI-PRCCRTQAVVDLLPGTPVYHQ-----
2m8K218 -----FLPRTGDVITVDVLQAPPS-----YL-----ALVTLEHAKLGRLE-----NHLTWKAGKEVINSMVGAQATE-----QDCSR-PKGGI-PRCCRTQAVVDLLPGTPVYHQ-----

2m8K219 VAN-----CCRGVLSLVSGLTSAAPFQMVGFALARDSGGKEPKFPQFDMY-----PGVTCGNATTVADTRIRVIN-----NRYVQALLTNGVTCYSYQASAAAPSCVSMFTFYSE-----
2m8K217 IAN-----CCRGVLSLVSGLTSAAPFQMVGFALARDSGGKEPKFPQFDMY-----PGVTCGNATTVADTRIRVIN-----NRYVQALLTNGVTCYSYQASAAAPSCVSMFTFYSE-----
2m8K216 IAN-----CCRGVLSLVSGLTSAAPFQMVGFALARDSGGKEPKFPQFDMY-----PGVTCGNATTVADTRIRVIN-----NRYVQALLTNGVTCYSYQASAAAPSCVSMFTFYSE-----
2m8K213 IAN-----CCRGVLSLVSGLTSAAPFQMVGFALARDSGGKEPKFPQFDMY-----PGVTCGNATTVADTRIRVIN-----NRYVQALLTNGVTCYSYQASAAAPSCVSMFTFYSE-----
2m8K214 IAN-----CCRGVLSLVSGLTSAAPFQMVGFALARDSGGKEPKFPQFDMY-----PGVTCGNATTVADTRIRVIN-----NRYVQALLTNGVTCYSYQASAAAPSCVSMFTFYSE-----
2m8K215 VGNLPCCGKSGTLLPPTMDPQKRAMFQMVYH-----LPPDLNRTALYFPQNKISG-----KLNDQYACGFPVRSQCFDPTGLMSTTFAVAMNQVACNITRP-KRRASKCVSFSAYYND-----
2m8K213 IGRINDCCRGSTILPKSMDAQSKAFQMVYH-----LPPDLNRTALYFPQNKISG-----KLNDQYACGFPVRSQCFDPTGLMSTTFAVAMNQVACNITRP-KRRASKCVSFSAYYND-----
2m8K218 IGRINDCCRGSTILPKSMDAQSKAFQMVYH-----LPPDLNRTALYFPQNKISG-----KLNDQYACGFPVRSQCFDPTGLMSTTFAVAMNQVACNITRP-KRRASKCVSFSAYYND-----

2m8K219 TIVPCRCRCGCGGSGPSP-----CCVSVDTQCCPMLPAP-----GDCEPSSAPVW-----CSHMCPIRVHNVHVSRYKRYVHVVSVHNSYLSANSTON-SLVLR-----
2m8K217 TIVPCRCRCGCGGSGPSP-----CCVSVDTQCCPMLPAP-----GDCEPSSAPVW-----CSHMCPIRVHNVHVSRYKRYVHVVSVHNSYLSANSTON-SLVLR-----
2m8K216 TIVPCRCRCGCGGSGPSP-----CCVSVDTQCCPMLPAP-----GDCEPSSAPVW-----CSHMCPIRVHNVHVSRYKRYVHVVSVHNSYLSANSTON-SLVLR-----
2m8K213 TIVPCRCRCGCGGSGPSP-----CCVSVDTQCCPMLPAP-----GDCEPSSAPVW-----CSHMCPIRVHNVHVSRYKRYVHVVSVHNSYLSANSTON-SLVLR-----
2m8K214 TIVPCRCRCGCGGSGPSP-----CCVSVDTQCCPMLPAP-----GDCEPSSAPVW-----CSHMCPIRVHNVHVSRYKRYVHVVSVHNSYLSANSTON-SLVLR-----
2m8K215 SVVPCRCRCGCGGSGPSP-----CCVSVDTQCCPMLPAP-----GDCEPSSAPVW-----CSHMCPIRVHNVHVSRYKRYVHVVSVHNSYLSANSTON-SLVLR-----
2m8K218 SVVPCRCRCGCGGSGPSP-----CCVSVDTQCCPMLPAP-----GDCEPSSAPVW-----CSHMCPIRVHNVHVSRYKRYVHVVSVHNSYLSANSTON-SLVLR-----

2m8K219 PHLRSITQLPFPFNYRLVET-GAYSDTGMFN-----GLRYTHMLQ-----D-----GNVQSEML-EKES-DFTYSGGNAFPRSVTFMGQCVMPADQVYPLPHASAL-----GNF-----
2m8K217 PHLRSITQLPFPFNYRLVET-GAYSDTGMFN-----GLRYTHMLQ-----D-----GNVQSEML-EKES-DFTYSGGNAFPRSVTFMGQCVMPADQVYPLPHASAL-----GNF-----
2m8K216 PHLRSITQLPFPFNYRLVET-GAYSDTGMFN-----GLRYTHMLQ-----D-----GNVQSEML-EKES-DFTYSGGNAFPRSVTFMGQCVMPADQVYPLPHASAL-----GNF-----
2m8K213 PHLRSITQLPFPFNYRLVET-GAYSDTGMFN-----GLRYTHMLQ-----D-----GNVQSEML-EKES-DFTYSGGNAFPRSVTFMGQCVMPADQVYPLPHASAL-----GNF-----
2m8K214 PHLRSITQLPFPFNYRLVET-GAYSDTGMFN-----GLRYTHMLQ-----D-----GNVQSEML-EKES-DFTYSGGNAFPRSVTFMGQCVMPADQVYPLPHASAL-----GNF-----
2m8K215 PHLRSITQLPFPFNYRLVET-GAYSDTGMFN-----GLRYTHMLQ-----D-----GNVQSEML-EKES-DFTYSGGNAFPRSVTFMGQCVMPADQVYPLPHASAL-----GNF-----
2m8K218 PHLRSITQLPFPFNYRLVET-GAYSDTGMFN-----GLRYTHMLQ-----D-----GNVQSEML-EKES-DFTYSGGNAFPRSVTFMGQCVMPADQVYPLPHASAL-----GNF-----

2m8K219 CFIILLFPFVV-----
2m8K217 AVVVLGMIYA-----
2m8K216 AFILLLLIYA-----
2m8K215 AANTAFAVLS-----
2m8K213 VFWVALAALAYA-----
2m8K214 IFWVALAALAYA-----
2m8K215 ANAAALVMIVALVDSCL-----
2m8K213 PFLVASAFLILQ-----
2m8K218 AFMLILL-----

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Figure S2.5

The *cpd47* mutation (yellow) occurs at the end of the CBD (green). A multiple sequence alignment was performed on the protein sequences of the COBRA family members in *Zea mays*.

Figure S2.6

Mature leaves from *cpd28/mu1037292* heteroallelic plants hyperaccumulate starch.

(A-B) Wild-type and *cpd28/mu1037292* mutant leaves before (A) and after (B) IKI staining.

Figure S2.7

A phylogeny of the COBRA family protein sequences in *Arabidopsis thaliana* (At), *Zea mays* (Zm), *Oryza sativa* (Os), *Solanum lycopersicum* (Sl), and *Gossypium raimondi* (Gr). The COBRA members separate into five distinct clades. ZmBK2L3 is highlighted by a yellow star, while black stars represent previously characterized COBRA family members.

Figure S2.8

Soluble sugar and starch quantification in wild-type and *bk2* mature leaves. Error bars are mean $\pm$ StdEr. Asterisks represent a significant difference at $P \leq 0.05$ using Student's *t*-test.

CHAPTER 3: Maize *Carbohydrate partitioning defective1* impacts carbohydrate distribution, callose accumulation, and phloem function

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exchange measurements. GJ participated in the design of the study and helped critically revise the manuscript. DMB participated in the design of the study, and helped draft and critically revise the manuscript.

HIGHLIGHT

The maize *Carbohydrate partitioning defective1* mutant causes ectopic callose formation in sieve elements, which partially inhibits carbohydrate partitioning and is associated with hyperactive defense responses under insect pest exposure.

ABSTRACT

Plants synthesize carbohydrates in photosynthetic tissues, with the majority of plants transporting sucrose to non-photosynthetic tissues to sustain growth and development. While the anatomical, biochemical, and physiological processes regulating sucrose long-distance transport are well characterized, little is known concerning the genes controlling whole-plant carbohydrate partitioning. To identify loci influencing carbon export from leaves, we screened mutagenized maize plants for phenotypes associated with reduced carbohydrate transport, including chlorosis and excessive starch and soluble sugars in leaves. *Carbohydrate partitioning defective1* (*Cpd1*) was identified as a semi-dominant mutant exhibiting these phenotypes. Phloem transport experiments suggested that the hyperaccumulation of starch and soluble sugars in the *Cpd1/+* mutant leaves was due to inhibited sucrose export. Interestingly, ectopic callose deposits were observed in the phloem of mutant leaves, and probably underlie the decreased transport. In addition to the

carbohydrate hyperaccumulation phenotype, *Cpd1/+* mutants overaccumulate benzoxazinoid defense compounds and exhibit increased tolerance when attacked by aphids. However, double mutant studies between *Cpd1/+* and benzoxazinoid-less plants indicate that the ectopic callose and carbon hyperaccumulation are independent of benzoxazinoid production. Based on the formation of callose occlusions in the developing phloem, we hypothesize that the *cpd1* gene functions early in phloem development, thereby impacting whole-plant carbohydrate partitioning.

Keywords: Benzoxazinoid, callose, chlorosis, *Cpd1*, lignin, maize, phloem, starch, [¹⁴C]sucrose, sugars.

Abbreviations: BS, bundle sheath; *bx1*, *benzoxazinoneless1*; *bx2*, *benzoxazinoneless2*; CC, companion cell; CF, carboxyfluorescein; CFDA, carboxyfluorescein diacetate; *Cpd1*, *Carbohydrate partitioning defective1*; DIMBOA, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one; DIMBOA-Glc, glucosylated-DIMBOA; EOD, end of day; EON, end of night; HPAE, high-performance anion exchange; M, mesophyll; NSC, non-structural carbohydrate; PD, plasmodesma; PP, phloem parenchyma; SE, sieve element

INTRODUCTION

The transport of carbohydrates from photosynthetic source tissues (leaves) to non-photosynthetic sink tissues (e.g. roots, reproductive organs, and stem) is crucial for plant growth, development, and yield (Lalonde et al., 2003; Slewinski and Braun,

2010a; Braun et al., 2014; Yadav et al., 2015; Julius et al., 2017). In the majority of crop species, including maize (*Zea mays*), sucrose is the predominant (or sole) carbohydrate transported long distance for delivery to different sink tissues (Heyser et al., 1978; Ohshima et al., 1990). This process, termed whole-plant carbohydrate partitioning, occurs in the phloem tissue of the veins (van Bel, 2003; Ayre, 2011). Phloem tissue is composed of three cell types: phloem parenchyma (PP) cells, companion cells (CCs), and sieve elements (SEs); however, the SEs are enucleate and depend on neighboring CCs for metabolic support (van Bel and Knoblauch, 2000). Therefore, these two cell types function as a unit and are referred to as the CC/SE complex (Esau, 1977). There are few plasmodesmatal connections between the PP cells and the CC/SE complexes in maize leaves (Evert et al., 1978). Therefore, sucrose must be exported across the plasma membrane into the cell wall space, or apoplasm, before being imported into the CCs (Slewinski et al., 2009, 2010; Baker et al., 2012, 2016; Braun, 2012; Chen et al., 2012). Sucrose then moves cell to cell symplasmically through plasmodesmata (PDs) into the SEs, which are aligned end to end to form sieve tubes that act as a conduit for long-distance sucrose transport from source tissues to the sinks (Evert, 1982). The SE lumens are connected at the end walls by large arrays of PDs (i.e. sieve plates) that modulate the bulk flow of sieve tube sap by selective deposition or degradation of callose, a β -1,3-linked glycan, within the pores of the PDs (Chen and Kim, 2009; Xie et al., 2011). Callose deposits are known to block cell to cell movement of solutes, hormones, and proteins, and therefore the control of callose deposition is a crucial mechanism of phloem sap transport, resource allocation, and plant development (van Bel, 2003;

Chen and Kim, 2009; Guseman et al., 2010; Barratt et al., 2011; Vatén et al., 2011; Xie et al., 2011; Piršelová and Matušíková, 2013; Han et al., 2014; Cui and Lee, 2016).

Plant pests and pathogens target the long-distance transport systems within veins to acquire resources and to spread to different tissues throughout the plant (Samuel, 1934; Carrington et al., 1996; Oparka and Cruz, 2000; Bové and Garnier, 2003; Will and van Bel, 2006; Sun et al., 2013). To counteract this attack, plants have evolved chemical defense responses not only to limit pathogen feeding and nutrient acquisition, but also to modulate the transport process to limit the spread of infectious agents from the site of entry (Aist, 1976; Iglesias and Meins, 2000; Hopkins et al., 2009; Luna et al., 2011). One such family of compounds is the benzoxazinoids, which are predominantly found in grasses, such as maize and wheat (*Triticum aestivum*) (Zúñiga et al., 1983; Frey et al., 2009; Ahmad et al., 2011). In response to chemical signals associated with insect feeding or microbial attack, ectopic callose is deposited over the sieve plate pores to limit phloem transport (Iglesias and Meins, 2000; van Bel, 2003; Will and van Bel, 2006; Luna et al., 2011; Koh et al., 2012).

Toward identifying genes that regulate carbohydrate allocation and ultimately influence crop productivity in maize, we screened mutagenized populations for mutants with visible phenotypes associated with carbohydrate hyperaccumulation in leaves, such as decreased plant growth, chlorotic leaves, and reduced fertility. Five maize mutants have been reported to express these phenotypes and found to function in carbohydrate partitioning: *sucrose export defective1* (*sxd1*), *sucrose transporter1* (*sut1*), *psychedelic* (*psc*), and the *tie-dyed1* (*tdy1*) and *tdy2* mutants

(Russin et al., 1996; Braun et al., 2006; Baker and Braun, 2008; Braun and Slewinski, 2009; Slewinski et al., 2009; Slewinski and Braun, 2010b; Baker et al., 2013).

Interestingly, the *sxd1* mutant exhibits ectopic callose deposits over the PDs between the bundle sheath (BS) and PP cells of leaf minor veins (Botha et al., 2000). Occlusions in these PDs were suggested to inhibit symplasmic transport of sucrose, and thus sucrose export from the mature leaf. In contrast, *psc*, *sut1*, *tdy1*, or *tdy2* mutants have not been reported to exhibit ectopic callose deposition in leaf veins (Baker and Braun, 2008; Ma et al., 2008; Slewinski et al., 2010, 2012).

In addition to the above-mentioned maize mutants with defects in carbon export from leaves, we have identified many additional loci with similar overall phenotypes. Here, we characterize the maize mutant *Carbohydrate partitioning defective1* (*Cpd1*). Besides reduced plant growth, chlorotic leaves, and overaccumulation of non-structural carbohydrates (NSCs) in leaves, the mutant displays several additional, unique phenotypes that shed light on its function.

MATERIALS AND METHODS

Plant materials and growth conditions

To screen for putative cpd mutants, mutagenized maize populations were evaluated for families segregating small, slow-growing, chlorotic plants, and, in conducive genetic backgrounds, anthocyanins in the leaves. A confirmatory screen was performed on the identified mutant lines to test whether the mutants overaccumulated starch and, if so, they have been included in our cpd mutant collection. Over multiple years of screening, and with additional alleles donated

from collaborators, we have identified nearly 100 mutations that give varying levels of the cpd suite of phenotypes. In this report, we characterize *Cpd1*, which was generated by Gerry Neuffer (University of Missouri) using ethyl methanesulfonate mutagenesis of maize pollen (Neuffer and Coe, 1978).

Maize plants used for the morphometrics, photosynthetic and gas exchange analyses, microscopy, and soluble sugar and starch quantifications were grown in the field at the University of Missouri South Farm Agricultural Experiment Station in Columbia, MO. *Cpd1*/+ backcrossed into the inbred line B73 at least four times was used for all experiments. The *benzoxazinoneless1* (*bx1*) and *bx2* mutations, which have been previously described by Tzin et al. (2017), were also utilized. For the morphometric studies, two families segregating for the *Cpd1*/+ mutation in a 1:1 mutant:wild-type ratio were analyzed. Sample sizes for days to anthesis and days to silking measurements in the wild type and *Cpd1*/+ mutants were n=40 and n=43; ear length measurements were n=39 and n=41; 100 kernel weight measurements were n=36 and n=37; kernel number measurements were n=17 and n=17; and plant height measurements were n=39 and n=44, respectively.

Plants used for the carboxyfluorescein diacetate (CFDA) and [¹⁴C]sucrose transport assays, innate benzoxazinoid measurements, and microscopy were grown in a greenhouse supplemented with high-pressure sodium lighting (1600 $\mu\text{mol m}^{-2} \text{s}^{-1}$) under 16 h light and 8 h darkness. The plants were kept at a daytime and night-time temperature of 30 °C and 24 °C, respectively. Additionally, etiolated plants were grown for 13–15 d under 24 h dark conditions at 22 °C. Plants were PCR-genotyped to identify *Cpd1* homozygous mutants, heterozygotes, and homozygous wild-type

individuals using the primer sequences Forward, 5'-ttgccaaaggtcagtttagatgat-3'; and Reverse, 5'-aaattggctggttaactgacagaa-3'.

Maize plants used for the aphid and caterpillar assays and the post-aphid benzoxazinoid measurements were sown in plastic pots (~200 cm³) filled with moistened growth medium [produced by mixing 0.16 m³ Metro-Mix 360 (Scotts), 0.45 kg of finely ground lime, 0.45 kg of Peters Unimix (Scotts), 68 kg of Turface MVP (Profile Products), 23 kg of coarse quartz sand, and 0.018 m³ pasteurized field soil]. Plants were grown for 2 weeks in growth chambers under a 16 h light/8 h dark photoperiod and 180 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light at constant 23 °C and 60% humidity.

Starch staining

Leaves were cleared and stained with iodine–potassium iodide as previously described (Baker and Braun, 2007).

[¹⁴C]Sucrose and CF transport studies

Carboxyfluorescein (CF) and [¹⁴C]sucrose transport assays were conducted as previously described (Ma et al., 2009; Slewinski et al., 2009). For each experiment, three independent plants of each genotype, *Cpd1*/+ and the wild type, were analyzed, and the experiments were repeated three times (n=9 of each genotype analyzed). For the *Cpd1*/+ mutants, leaves that were fully chlorotic were abraded near the tip to apply the dye or radiotracer. In both experiments, leaves were harvested after 1 h of transport time, and cross-sectional images taken for the CF studies were located ~15 cm proximal to the application site, eliminating diffusion

as a possible explanation for transport. Representative images of the results are shown.

Photosynthesis and gas exchange measurements

Gas exchange, and photosynthetic rate and capacity measurements were taken on mature source leaves between 09.00 h and 24.00 h using a portable infrared gas exchange system (LI-6400XT, LI-COR Inc., Lincoln, NE, USA) as described (Bihmidine et al., 2015; Huang et al., 2009). Chlorophyll content was measured using a Minolta SPAD-502 meter (Spectrum Technologies, Plainfield, IL, USA) (Ma et al., 2008). Net photosynthesis (A_{net} , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) and stomatal conductance (g_s , $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) rates were measured at a photon flux density of $2000 \mu\text{mol m}^{-2} \text{ s}^{-1}$ and ambient CO_2 concentration of $400 \mu\text{mol mol}^{-1}$. The maximum photochemical efficiency of PSII (F_v/F_m) was determined on dark-adapted leaves using a leaf fluorometer attached to the LI-6400XT infrared gas analyzer at an ambient CO_2 concentration of $400 \mu\text{mol mol}^{-1}$. For the *Cpd1*/+ mutant leaves, the regions analyzed for the F_v/F_m experiment contained a mixture of both chlorotic margins expressing anthocyanins and greener tissue closer to the mid-rib. Five biological samples were measured for both the wild type and *Cpd1*/+ mutants.

Light and fluorescence microscopy

Microscopy images were taken using a Nikon Eclipse 80i epifluorescent microscope equipped with a 100 W mercury bulb and a DXM1200F camera, except for the aniline blue staining of the immature leaf gradient and the etiolated seedling images

in which LED lights (Lumencore Sola SMII light engine) replaced the 100 W mercury bulb. Mature leaf tissue free-hand cross-sections were generated and imaged as previously described (Baker et al., 2016). Callose was visualized under UV excitation by staining mature leaf sections with 0.005% (w/v) aniline blue in 0.15 M potassium phosphate buffer (pH 8.2) (Ruzin, 1999). Lignin was visualized by staining leaf sections with Maule stain (0.5% KMnO₄) for 10 min. The leaf sections were subsequently rinsed in water, de-stained in 10% HCl for 5 min, and again rinsed in water before mounting in concentrated NH₄OH and imaging them (Chapple et al., 1992).

Soluble sugar and starch quantification

Mature source leaf tissue was harvested from field-grown plants and immediately placed in liquid nitrogen before being stored at -80 °C until measurement. End of day (EOD) samples were harvested at 16.30 h, and end of the night (EON) samples were harvested at 05.30 h. Five biological samples were measured for each genotype and time point. Soluble sugar and starch samples were extracted according to Leach and Braun (2016). Samples were quantified using high-performance anion exchange (HPAE) chromatography against known standards according to Leach et al. (2017).

Aphid bioassays

Corn leaf aphids (*Rhopalosiphum maidis*) were reared on the maize line B73 as previously described (Meihls et al., 2013). For aphid bioassays, 10 adult aphids were

confined on 2-week-old seedling plants using micro-perforated polypropylene bags. Five days after the infestation, the aphid progeny were counted and the number of nymphs produced per adult per day was calculated. Sample sizes for wild-type and *Cpd1*/+ mutant plants were n=17 and n=9, respectively.

Caterpillar bioassays

Eggs of three lepidopteran species, *Spodoptera exigua* (beet armyworm), *Spodoptera eridania* (southern armyworm), and *Spodoptera frugiperda* (fall armyworm), were purchased from Benzon Research Inc. (Carlisle, PA, USA). Spodoptera eggs were hatched in a 29 °C incubator (~48 h). First-instar larvae (one larva per plant) were confined on 2-week-old seedling plants using micro-perforated polypropylene bags. Ten days after infestation, the larvae were collected, lyophilized, and weighed. Sample sizes for wild-type and *Cpd1*/+ mutant plants were, for beet armyworm n=4 and n=3; for southern armyworm, n=5 and n=5; and for fall armyworm, n=10 and n=5, respectively.

Benzoxazinoid measurements

For measurement of leaf benzoxazinoid content post-aphid treatment, maize leaf tips (~5 cm) of the third and fourth leaves were independently collected from plants that had been fed upon by aphids for 5 d. Similar tissue samples were taken from individuals not exposed to aphids for innate benzoxazinoid measurements. Metabolite extraction and quantification with LC-MS analysis were performed as previously described (Handrick et al., 2016). Sample sizes for post-aphid treatment

for wild-type and *Cpd1*/+ mutant plants were n=17 and n=9, respectively. Sample sizes for innate benzoxazinoid measurements for wild-type and *Cpd1*/+ mutant plants were n=8 and n=7, respectively.

RESULTS

The *Cpd1* mutation results in shorter plants with pigmented leaves

The Mendelian segregation ratio of the *Cpd1* mutant indicates that it is conditioned by a semi-dominant mutation (Fig. 3.1A; Table S3.1). The homozygous mutant exhibits delayed growth in the shoot and root, resulting in a stunted appearance (Fig. 3.1A). Additionally, the shoot exhibits a highly variable continuum of light green to chlorotic leaves, which rapidly progresses to necrosis, initiating from the tip and progressing basipetally. Ultimately, the homozygous mutant is seedling lethal and survives only 2 weeks after planting. Heterozygote mutants (*Cpd1*/+) grow to maturity, but exhibit regions of chlorosis and anthocyanin accumulation in the source leaves as early as 2 weeks after germination. *Cpd1*/+ mutants also display decreased plant stature and shorter ear length compared with wild-type siblings (Fig. 3.1A–C). Additionally, *Cpd1*/+ mutant plants are developmentally delayed, requiring more time to reach anthesis and silking; however, the number of kernels per ear is not significantly different from that of the wild type, although kernel weight was significantly reduced in the mutant (Table 3.1). For all of our analyses, the *Cpd1* mutation was backcrossed four or more times to the B73 inbred line, thereby greatly reducing the likelihood that other mutations are segregating in the background.

To characterize when the defect first appeared in *Cpd1*/+ mutant leaves, we examined mutant leaves of field-grown plants from their initial emergence from the whorl to monitor phenotypic progression. The *Cpd1*/+ mutant phenotype first manifests in the tip of leaves as slightly chlorotic regions as the leaves emerge from the whorl, with new chlorotic regions continuing to appear in a basipetal fashion as leaf emergence continues (Fig. 3.2). While the surrounding leaf tissue appears to undergo normal greening, the chlorotic regions progressively increase in severity over time, and anthocyanin accumulation typically occurs as chlorosis increases. Once formed, the chlorotic regions do not appear to increase in size and do not revert to dark green tissue.

***Cpd1*/+ mutant leaves accumulate excess starch and sugars**

Due to the similarity of the *Cpd1*/+ mutant phenotype to *sxd1*, *sut1*, *psc*, and the *tdy* mutants, which all hyperaccumulate starch and sugars in their leaves, *Cpd1*/+ mutant and wild-type leaves were analyzed with potassium iodine staining to visualize starch accumulation. In this assay, regions of the leaves containing abundant starch appear black/brown, whereas those with little starch appear a pale tan color. No starch accumulation was detected in the wild-type leaves collected near dawn, indicative that the transitory stored starch was depleted over the course of the night due to remobilization (Fig. 3.3A). However, the *Cpd1*/+ mutant leaves displayed intense starch accumulation in the chlorotic regions (Fig. 3.3B). To extend these results, free-hand cross-sections of these stained leaves were microscopically analyzed to determine in which cells the starch accumulated. In wild-type plants, no

starch granules were present in the chloroplasts of BS and mesophyll (M) cells, consistent with the remobilization of starch over the course of the night (Fig. 3.3C) (Rhoades and Carvalho, 1944). However, in *Cpd1*/+ mutants, both BS and M cells contained abundant starch, with the BS cells showing substantial staining (Fig. 3.3D). These results demonstrate that the mutant leaves hyperaccumulate starch, suggesting that *Cpd1*/+ mutants are severely limited in the ability to remobilize stored carbon.

Given the elevated starch levels in *Cpd1*/+ mutant leaves, we quantitatively assessed the levels of NSCs in mutant and wild-type leaves. Tissue samples were harvested at the EOD and EON from mature source leaves of field-grown plants when the mutants were beginning to display anthocyanin accumulation at the edge of the blade. Mutant leaf tissues contained ~2- to 4-fold the amount of soluble sugars and ~2-fold the amount of starch compared with their wild-type siblings in the EOD samples (Fig. 3.3E, F). However, the *Cpd1*/+ mutants contained ~14- to 28-fold the amount of soluble sugars and ~25-fold greater starch levels than their wild-type siblings in the EON samples. This large increase in soluble sugar and starch levels in the *Cpd1*/+ mutant leaves further suggests that the mutant is unable to export sucrose effectively from the source leaves.

Sugars can act as potent signals to down-regulate photosynthesis (Sheen, 1990, 1994; Goldschmidt and Huber, 1992; Krapp and Stitt, 1995; Koch, 1996; Jeannette et al., 2000; Rolland et al., 2006; Ruan, 2014). Therefore, we anticipated that the marked excess of sucrose and glucose in the mutant leaves would serve as sugar signals to repress photosynthesis. To test this idea, leaf photosynthetic capacity and

gas exchange rates were measured for both mutant and wild-type plants. In comparison with the wild type, the chlorotic regions of *Cpd1*/+ mutant plants displayed a 50% decrease in leaf chlorophyll concentration, a 75% decrease in photosynthetic rate, and a 10% decrease in the maximum quantum efficiency of PSII photochemistry (Fig. S3.1A–C). The *Cpd1*/+ mutant also showed a 68% decrease in stomatal conductance (Fig. S3.1D). However, there was no difference between wild-type and mutant plants in the number of stomata on either leaf epidermal surface, indicating that the decreased gas exchange was not due to fewer stomata in the mutant leaves (data not shown). In summary, the data are consistent with the high sugar levels leading to feedback inhibition of photosynthesis and thus substantially decreased photosynthetic capacity in the mutants as compared with wild-type plants.

Sucrose transport is inhibited in *Cpd1*/+ mutant leaves

To test directly whether phloem transport of sucrose is altered in the mutant, ¹⁴C-labeled sucrose was applied to an abraded region at the tip of a mature source leaf of an intact plant to observe its transport in both wild-type and *Cpd1*/+ mutant leaves. As expected, in the wild-type leaf, the [¹⁴C]sucrose was readily transported from the tip to the base of the leaf (Fig. 3.4A, B). However, in the *Cpd1*/+ mutant leaf, little to no transport of the [¹⁴C]sucrose occurred, with the majority of the label remaining at the application site (Fig. 3.4C, D).

To confirm these results, a phloem-mobile tracer, CFDA, was similarly applied to mature source leaves of both wild-type and *Cpd1*/+ mutant plants. CFDA is cell

permeable and, once inside the cell, is cleaved to form CF. CF is a fluorescent, charged molecule unable to move across cell membranes, and is transported through sieve tubes in a similar manner to sucrose (Grignon et al., 1989; Ma et al., 2009; Bihmidine et al., 2015; Baker et al., 2016). Leaf cross-sections were taken 15 cm proximal from the CFDA application site to determine if CF was transported through the phloem. In agreement with the [^{14}C]sucrose transport data, CF was not present in the phloem of *Cpd1/+* mutant leaf veins (Fig. 3.4G, H). These samples appeared similar to negative control sections to which no CF was applied and which were used to visualize tissue autofluorescence (Fig. 3.4I, J). However, CF fluorescence was clearly seen in the wild-type phloem (Fig. 3.4E, F). Thus, the *Cpd1/+* mutant is unable to transport sucrose and CF effectively through the phloem of mature source leaves, consistent with the hypothesis that sucrose export is partially inhibited. Furthermore, this transport failure could explain the hyperaccumulation of NSCs, leaf chlorosis, and reduction in photosynthetic performance in the mutant leaves.

***Cpd1/+* mutants exhibit ectopic callose and lignin deposition in the phloem**

Wild-type and *Cpd1/+* mutant leaves were examined by light microscopy to determine whether anatomical differences could account for the inhibited phloem transport. No differences were observed in vein number or size, cell number or size, or overall leaf architecture in the mutants. Intriguingly, examination of cross-sections from mature *Cpd1/+* mutant chlorotic leaves beginning to accumulate anthocyanin revealed visible blockages in the phloem under both bright-field and

UV illumination (Fig. 3.5B, D). No such blockages were observed in wild-type leaves sampled from the equivalent region (Fig. 3.5A, C). Histochemical analyses were performed on the leaf samples to determine the chemical nature of the blockages. Staining of the *Cpd1*/+ mutant leaf sections with aniline blue, a dye that binds callose and fluoresces blue–white under UV light (Ruzin, 1999), showed greatly increased callose deposition in the phloem tissue in both the lateral and minor veins (Fig. 3.5F, H, J). These deposits were absent from the phloem tissue of wild-type samples (Fig. 3.5E, G, I). Based on the cell positions and sizes, these deposits appeared to be present in the SEs, but not the CCs or PP cells, although we cannot rule out the possibility that these other cell types also accumulate ectopic callose (Fig. 3.5F). Interestingly, in most veins analyzed, both callose-occluded and unoccluded sieve tubes were found (Fig. 3.5F, J). Further examination of the *Cpd1*/+ mutant leaves with Maule reagent, which stains lignin red, revealed ectopic lignin in the cell walls of the phloem tissue in regions with severe chlorosis and anthocyanin accumulation (Fig. 3.5M, N). Lignin was never observed in the phloem of the wild-type veins (Fig. 3.5K, L).

The above results afford a possible explanation for the hyperaccumulation of NSCs in *Cpd1*/+ mutant leaves; that is, the ectopic callose in some sieve tubes impairs phloem transport and reduces sucrose export, resulting in sugar and starch build-up in mutant leaves. However, an alternative possibility is that NSC accumulation or exposure to light results in the callose deposition. To distinguish between these hypotheses, we analyzed etiolated *Cpd1/Cpd1* homozygotes, *Cpd1*/+ heterozygotes, and wild-type seedlings resulting from the self-fertilization of a heterozygote plant,

since dark-grown seedlings are not expected to have high levels of NSCs in leaves. Fully expanded second leaves from etiolated seedlings were collected and starch stained. As predicted, none of the mutant or wild-type seedlings accumulated any detectable starch (Fig. 3.6A). Leaf samples were then sectioned and stained with aniline blue to determine whether callose deposits were present in the phloem tissue independent of starch hyperaccumulation. As expected, the wild-type plants did not have callose deposits in their phloem tissue (Fig. 3.6B). However, both the heterozygous and homozygous *Cpd1* mutant plants exhibited callose deposits in the lateral and minor veins, with the homozygous mutants having more numerous and severe deposits than heterozygous plants (Fig. 3.6C, D). These results support the hypothesis that the callose deposits inhibit phloem transport in the *Cpd1* mutant and precede the NSC accumulation in leaves and the other phenotypes.

Callose deposition in *Cpd1*/+ mutant plants occurs early in phloem development

Callose deposition and degradation are crucial steps in the development of mature SEs and their sieve plates (Esau et al., 1962; Northcote and Wooding, 1966). Therefore, we hypothesized that misregulation of this process during vein development could result in the ectopic callose seen in the *Cpd1* mutant plants. To determine the approximate developmental time at which the excess callose forms in *Cpd1*/+ mutant veins, immature leaves were harvested when their tips were just emerging from the whorl and were green, but their middle and bases were still within the whorl and pale yellow-green or yellow-white, respectively. Aniline blue

staining revealed that the tips of the *Cpd1/+* mutant leaves contained high levels of callose in most minor veins and lateral veins, with no callose detected in corresponding wild-type samples (Fig. 3.7A, B). Similar results were seen in sections taken from the middle of the leaves (Fig. 3.7C, D). Interestingly, in cross-sections taken from the leaf base, very few callose deposits were present in the *Cpd1/+* mutant veins, and the majority were located in the lateral veins, as minor veins had just initiated development at this stage (Fig. 3.7E, F). At this point in leaf vein development, the metaphloem in the lateral vein has recently formed after the collapse of the protophloem (Evert et al., 1996). No ectopic lignin was observed in wild-type or *Cpd1/+* immature leaves using Maule reagent (data not shown). These results indicate that the callose deposits are occurring during the maturation of the phloem SEs.

***Cpd1/+* mutants exhibit biotic stress responses**

Callose deposition is often a defense response to pest or pathogen attack to limit nutrient loss. Therefore, we initially hypothesized that the callose deposition in the *Cpd1/+* mutant might be due to activation of defense response pathways. To test this hypothesis, caterpillar-feeding experiments were performed to determine whether *Cpd1/+* mutant plants exhibited enhanced defense against insects. Three species of Lepidoptera, the generalists beet armyworm (*Spodoptera exigua*) and southern armyworm (*Spodoptera eridania*), and the monocot-feeding specialist fall armyworm (*Spodoptera frugiperda*) were separately placed on wild-type and *Cpd1/+* mutant 2-week-old seedlings and allowed to feed for 10 d. Interestingly, the

fall armyworms that fed on the *Cpd1*/+ plants were significantly larger than those that fed on the wild-type plants at the end of the 10 d feeding period (Fig. 3.8A). The opposite effect was observed for the southern armyworm, as the insects feeding on the wild-type plants gained more weight (Fig. 3.8A). This trend also held for the beet armyworm. However, the average caterpillar weights of the two treatments were not significantly different for the beet armyworm (Fig. 3.8A).

Additionally, an aphid feeding experiment was conducted to test whether the *Cpd1*/+ mutant showed increased resistance against piercing/sucking pests, such as corn leaf aphids (*Rhopalosiphum maidis*), which are specialized for feeding on grasses. Adult aphids were placed on 2-week-old wild-type or *Cpd1*/+ mutant plants, and after 5 d their progeny were counted. Those feeding on the wild-type plants produced ~2-fold more nymphs per adult per day compared with those placed on *Cpd1*/+ mutant plants (Fig. 3.8B). These data indicate that aphid fecundity was reduced on *Cpd1*/+ mutant plants.

Callose deposition in maize leaves has been reported to be triggered by increased levels of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), a member of the benzoxazinoid defense metabolite family, in the leaf apoplasm (Ahmad et al., 2011). To determine if the above results are due to increased defense metabolite levels, the wild-type and *Cpd1*/+ mutant leaves from the aphid feeding experiment were analyzed to determine whether the *Cpd1*/+ mutant leaves contained elevated DIMBOA levels. Indeed, we found that the *Cpd1*/+ mutant plants contained ~2- to 3-fold higher levels of both DIMBOA and glucosylated-DIMBOA (DIMBOA-Glc) (Fig. 3.9A). As there may be some degradation of DIMBOA-Glc to DIMBOA during the

extraction process, the reported DIMBOA abundance may be higher than what is actually in the leaves. These results are in agreement with the hypothesis that increased DIMBOA levels trigger callose deposition in the phloem tissue, resulting in the decreased carbohydrate transport and the mutant phenotype. Thus, the ectopic callose in *Cpd1/+* mutant leaves is associated with a poorer ability of the aphids to reproduce and with decreased growth of caterpillar herbivores, with the exception of the fall armyworm. This was probably due to this species' ability to inactivate grass-specific metabolites, such as benzoxazinoids, in their gut (Glauser et al., 2011; Maag et al., 2014), which allowed the caterpillar to feed on the excess carbohydrates in the mutant leaves.

DIMBOA-deficient mutants fail to rescue the *Cpd1/+* mutant phenotype

Bx1 and *Bx2* encode enzymes that catalyze the first and second steps, respectively, in the synthesis of a variety of benzoxazinoid defense compounds, and *bx1/bx1* and *bx2/bx2* mutants lack benzoxazinoids (Frey et al., 2009; Tzin et al., 2017). As DIMBOA has been shown to trigger callose formation (Ahmad et al., 2011; Meihls et al., 2013), we addressed whether increased benzoxazinoids were the cause of the ectopic callose deposition observed in *Cpd1/+* mutant individuals, by generating F2 families segregating *Cpd1/+* and *bx1/bx1* or *bx2/bx2* mutants. If benzoxazinoids are required to induce callose deposition in *Cpd1/+* mutants, it was expected that *Cpd1/+; bx1/bx1* and *Cpd1/+; bx2/bx2* double mutant plants would not synthesize DIMBOA, and would therefore have reduced callose accumulation in the phloem. If correct, we predicted that this would alleviate the block in phloem transport and

rescue the *Cpd1*/+ mutant phenotype. However, upon examination, both *Cpd1*/+; *bx1/bx1* and *Cpd1*/+; *bx2/bx2* double mutant plants exhibited the canonical *Cpd1*/+ mutant phenotypes of anthocyanin and starch accumulation in mature leaves, as well as the ectopic callose deposition in the phloem (Fig. 3.10A, B, E; Fig. S3.2). *bx1/bx1* and *bx2/bx2* single mutants did not show these phenotypes (Fig. 3.10C–F; Fig. S3.2). In addition, innate DIMBOA and DIMBOA-Glc levels were measured in *Cpd1*/+ leaf tissue free from biotic attack, and did not differ from wild-type samples (Fig. 3.9B). Therefore, while *Cpd1*/+ mutant individuals exhibit high levels of benzoxazinoids upon aphid feeding, we conclude that the ectopic callose deposition and resulting inhibited sucrose transport are independent of benzoxazinoid levels.

DISCUSSION

We identified the *Cpd1*/+ mutant by its leaf chlorosis and anthocyanin accumulation, decreased plant stature, and delayed growth and development. This suite of visible phenotypes is shared with other previously studied carbohydrate partitioning-defective mutants, including *sxd1*, *sut1*, *tdy1*, *tdy2*, and the *psc* loci from maize, as well as some sucrose transporter mutants in Arabidopsis, tobacco (*Nicotiana tabacum*), tomato (*Solanum lycopersicum*), and potato (*Solanum tuberosum*) (Riesmeier et al., 1994; Russin et al., 1996; Bürkle et al., 1998; Braun et al., 2006; Hackel et al., 2006; Baker and Braun, 2008; Srivastava et al., 2008; Slewinski et al., 2009; Slewinski and Braun, 2010b). These mutants all have defects in sucrose export and/or phloem transport in leaves. Therefore, we hypothesized that the *Cpd1* mutation impairs carbohydrate partitioning, potentially from an inability to export

sucrose from its source leaves. The sugar and starch overaccumulation, decreased rates of photosynthesis, and reduced [^{14}C]sucrose and CF transport in mutant leaves lend support to this hypothesis.

In further agreement with this hypothesis, ectopic callose and lignin deposition were found in some SEs in the phloem of *Cpd1* mutants. None of the other characterized maize carbohydrate hyperaccumulation mutants exhibit ectopic callose in their sieve tubes. Lignin deposits were found only in the distal tip regions of strongly expressing *Cpd1* mutant leaves, but not earlier in development, and do not seem to be the cause of the hyperaccumulation of carbohydrates. However, the observation of callose deposits in young, immature, and etiolated leaf tissue indicated that the callose deposition preceded the increased starch and soluble sugar accumulation and was independent of excess sugar or light signaling pathways. Additionally, we determined that the ectopic callose deposition in the phloem occurred very early during the development of mutant leaves (Figs 3.6, 3.7). However, in order for *Cpd1*/+ mutant plants to survive, unoccluded SEs must also form normally to transport photosynthate throughout the plant. This suggests that the ectopic callose depositions observed in *Cpd1*/+ mutant veins are not due to a systemic defect in phloem development, but instead must be dependent on a variable, local signal acting early in vein development.

Callose is a structural polymer in the cell wall that is formed through β -1,3 linkages of glucose molecules by callose synthases (Chen and Kim, 2009). It plays an important role in the formation of sieve pores located at the connecting ends of SEs (Barratt et al., 2011; Xie et al., 2011). The exact size and number of pores in the sieve

plate directly impact the flow of phloem sap throughout the plant. For example, the *Arabidopsis Glucan Synthase7 (gsl7)* callose synthase mutant results in the loss of callose lining the sieve plate pores, resulting in improperly formed sieve plates and a decreased ability to transport photosynthate to the flowering stem (Barratt et al., 2011). Furthermore, this mutation also resulted in elevated levels of starch and anthocyanin accumulation in leaves compared with wild-type plants, a phenotype shared with the *Cpd1/+* mutant. However, in contrast to the *gsl7* mutant, the *Cpd1/+* mutation resulted in increased levels of callose deposition in the SEs, thereby limiting phloem transport of sucrose and presumably other molecules, such as hormones, RNAs, and proteins.

Intriguingly, there have been several mutants reported to show decreased export of photosynthate from the source leaves as a result of callose deposition in the vasculature. Callose deposition specifically at the BS cell–PP cell PDs was found in the maize *sxd1* mutant, a defect that probably resulted in decreased symplasmic transport and therefore hyperaccumulation of NSCs in the leaves (Russin et al., 1996; Botha et al., 2000; Provencher et al., 2001). Similar phenotypes were found in terms of the leaf NSC accumulation and callose deposition in potato plants with reduced expression of the *sxd1* ortholog (Hofius et al., 2004). Additionally, studies of the *sxd1* ortholog in *Arabidopsis*, *vitamin E1*, found similar NSC hyperaccumulation in mutant leaves, but only under low temperature conditions (Maeda et al., 2006). It was determined that all three orthologous genes function in tocopherol synthesis (Sattler et al., 2003; Hofius et al., 2004; Maeda et al., 2006). The reduced plant growth, NSC accumulation in leaves, ectopic callose deposition in veins, and reduced

carbon exported from leaves of plants grown only at low temperatures was even more pronounced by a mutation in an upstream step of tocopherol biosynthesis encoded by the *VTE2* gene of *Arabidopsis* (Maeda et al., 2006). However, in this case, callose deposition first occurred in a site-specific pattern in the phloem. Specifically, callose deposition was first observed at the transfer cell wall ingrowths at the boundary between the PP transfer cells and CC/SE complexes, and progressed to encircle the PP cells, including the PD connections with adjoining cells. Therefore, lack of tocopherols appears to induce callose deposition in the phloem tissue, specifically in PP cells, but not in the SEs. In contrast, in the *Cpd1*/+ mutant, accumulation of ectopic callose occurred in the phloem SEs but was undetectable in the CCs and PP cells. However, the presence of callose in these two cell types cannot be precluded due to the limitations of resolution with light microscopy, and electron microscopy studies will be needed to resolve the cell type specificity of the ectopic callose. Nonetheless, due to the observable difference in cell-specific callose deposition, we do not favor the hypothesis that *Cpd1* is involved in tocopherol production.

We had initially hypothesized that the *Cpd1*/+ mutation led to a constitutive defense response, which included ectopic callose plugging of the SEs, as has been shown to occur during pathogen attack, but subsequent findings led us to revise this idea. Interestingly, in citrus (*Poncirus trifoliata*) trees, citrus greening disease, which is caused by *Candidatus Liberibacter asiaticus* infection, results in callose deposition over the PD pore units between the CCs and SEs, as well as in the SEs themselves, blocking phloem transport (Koh et al., 2012). A similar defense response to

pathogens and hemipteran pests has been reported in maize, rice (*Oryza sativa*), tobacco, and *Arabidopsis* (Will and van Bel, 2006; Yun et al., 2006; Hao et al., 2008; Luna et al., 2011; Tzin et al., 2017). Ahmad et al. (2011) showed that the accumulation of benzoxazinoid compounds in the apoplasm of maize leaves results in increased callose deposition. While the cellular localization of the callose deposits was not determined, this theoretically could result in a blockage of sucrose transport if localized to the phloem. If so, this would effectively cut off the food supply and reduce infection by pests and pathogens attacking the plant. Therefore, we hypothesized that the excess callose deposition in the SEs of *Cpd1/+* mutant leaves could be due to increased benzoxazinoid levels.

Consistent with this idea, DIMBOA and its inactive form DIMBOA-Glc were present in 2- to 3-fold higher amounts in the *Cpd1/+* mutant after aphid feeding compared with wild-type plants. The results of the caterpillar and aphid feeding experiments further supported our initial hypothesis that a hyperactive defense response in the *Cpd1/+* mutant could result in its inability to transport phloem sap properly. Both of these results potentially contribute to the reduced fecundity of aphids feeding on the mutant leaves. Moreover, whereas beet armyworms and southern armyworms prefer to feed on various eudicot vegetable crops, fall armyworm feed primarily on monocots. The fall armyworm is not only more specialized for feeding on maize than the other two tested caterpillar species, but also has a specific detoxification mechanism for benzoxazinoids (which are induced more in *Cpd1/+* than in control plants by aphid feeding, Fig. 3.9) (Ali and Agrawal, 2012; Maag et al., 2014). Since fall armyworms are relatively impervious to maize chemical defenses, this could

explain why they grow better on *Cpd1*/+ mutant leaves, but the other two caterpillar species do not.

To test directly whether DIMBOA is required for the *Cpd1*/+ mutant phenotype, double mutants with stocks devoid of benzoxazinoids were created. However, genetic tests of the causative role for benzoxazinoids in underlying the *Cpd1*/+ mutant phenotype failed: *Cpd1*/+; *bx1*/*bx1* and *Cpd1*/+; *bx2*/*bx2* double mutant plants exhibited ectopic callose depositions similar to the *Cpd1*/+ single mutant individuals, demonstrating that the ectopic callose deposition observed in *Cpd1*/+ mutant veins is independent of benzoxazinoid levels. Additionally, innate DIMBOA levels in *Cpd1*/+ mutant plants were similar to those of wild-type individuals. Based on these data, we do not favor our initial hypothesis presented above for *Cpd1* functioning to induce a hyperactive defense response, and further revise it to account for the *Cpd1*/+; *bx* double mutant studies such that it is independent of benzoxazinoid accumulation. However, a potentially more parsimonious hypothesis is that the increased tolerance to insect feeding and enhanced DIMBOA levels post-aphid treatment seen in *Cpd1*/+ mutant plants is not due to a hyperactive defense response, but instead is due to priming of the defense response caused by an abundance of carbohydrates (Gebauer et al., 2017). In this case, the *Cpd1*/+ mutant plant would be able to synthesize an abundance of defense compounds quickly compared with wild-type siblings.

An alternative hypothesis that we favor to explain the *Cpd1*/+ ectopic callose is that it results from a defect in sieve plate development during SE differentiation and maturation. During sieve plate development, callose is deposited around PDs in the

end walls of the maturing SEs, signifying the location of future pores. Later, the callose surrounding the PD/pore is degraded up to the point where it lines the pore (Esau et al., 1962; Northcote and Wooding, 1966). The proper formation and function of these pores ensure phloem sap transport through the sieve tubes at maturity (Barratt et al., 2011). If this process is misregulated in the *Cpd1*/+ mutant, it could result in either excessive deposition of callose or a failure to initiate callose degradation, with either scenario resulting in callose occlusion of the SEs. In support of this hypothesis, callose deposition was first observed very early during the development of the SEs in the lateral veins (Fig. 3.7) and to increase in severity over time.

As *Cpd1*/+ is a semi-dominant mutation, it is interesting to speculate whether the *Cpd1* mutation is a gain- or loss-of-function mutation, and what the biological role of the wild-type *cpd1* gene may be. For example, it is possible that *Cpd1* is a gain-of-function mutation, and results either directly or indirectly in the constitutive deposition of callose in the SEs (Vatén et al., 2011). On the other hand, *Cpd1* could be a haploinsufficient loss-of-function mutation, with the wild-type gene encoding a regulator of callose synthesis or an upstream component of this pathway. Under this scenario, insufficient *cpd1* gene product would be produced and not function to repress callose deposition, resulting in the occluded SEs. Further investigation is required to identify and characterize the function of the wild-type *cpd1* gene to understand how it controls phloem development and callose synthesis/degradation, and ultimately carbohydrate accumulation in leaves. Genetic fine-mapping and whole-genome sequencing approaches have delimited the region containing the

cpd1 locus to contain 10 predicted ORFs. None of these is predicted to have any biological functions in callose synthesis or degradation, tocopherol biosynthesis, PD development or function, production of defense metabolites, sucrose transport, starch metabolism, or vein development. Because *Cpd1* probably resulted from a single base pair change, is a semi-dominant mutation, and is represented by a single mutant allele, transgenic approaches to test these candidate genes have been initiated to identify the gene and causative mutation, but these experiments will take a considerable time. However, our characterization of *Cpd1* has uncovered a genetic lesion influencing callose accumulation in developing phloem SEs, and possibly the CCs and PP cells in maize leaves, which to our knowledge is a novel phenotype. We note that the ectopic callose occurs in only some phloem sieve tubes, suggesting that the mutation sensitizes some but not other developing phloem cells to deposit callose. We do not understand the factors underlying this deposition, but it does not appear to be related to plant age or size, growth environment (etiolated versus mature field grown leaves), phloem position with the vein, vein size, or vein length (lateral versus minor veins). However, it is more severe and frequent in the homozygous mutant individuals compared with the heterozygotes, consistent with the mutant allele conferring the phenotype in a dose-dependent manner.

Understanding the function of *cpd1* will potentially have broad applications in unraveling the intersections among the induction of callose synthesis, sieve plate development, phloem function, plant defense priming, and carbohydrate partitioning, and it could provide knowledge valuable to generate crops with increased carbohydrate transport and yield.

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TABLES

Table 3.1

Cpd1/+ mutants show reduced height and yield, and delayed flowering

	<i>Cpd1/+</i> (mean $\pm$ SE)	Wild type (mean $\pm$ SE)
Plant height (cm)	214.89 $\pm$ 1.60*	223.03 $\pm$ 1.39
Days to anthesis	61.00 $\pm$ 0.24*	57.63 $\pm$ 0.20
Days to silking	62.25 $\pm$ 0.23*	58.72 $\pm$ 0.19
Ear length (cm)	121.83 $\pm$ 2.42*	148.89 $\pm$ 1.52
Weight of 100 kernels (g)	22.99 $\pm$ 0.36*	26.00 $\pm$ 0.26
Kernel number per ear	330.71 $\pm$ 20.23	348.82 $\pm$ 14.09

An asterisk signifies significantly different means between *Cpd1/+* mutant and wild-type plants at $P \leq 0.05$, using a two-tailed Student's *t*-test.

Table S3.1

χ^2 table for 1:1 segregation of *Cpd1*/+: wild-type families

Family No.	Observed <i>Cpd1</i> /+	Total Plants in Family	Expected <i>Cpd1</i> /+	p-value
1	2	6	3	
2	13	25	12.5	
3	10	22	11	
4	9	12	6	
5	5	14	7	
6	8	15	7.5	
7	11	19	9.5	
8	10	18	9	
9	6	14	7	
10	7	16	8	
T total	81	161	81.5	0.9574

FIGURES

Figure 3.1

Cpd1 mutants have diminished plant stature and reduced ear size relative to their wild-type siblings. (A) Two-week-old sibling plants: wild-type on the left, heterozygous *Cpd1*/+ mutant in the middle, and homozygous *Cpd1* mutant on the right. (B) A wild-type plant (left) at maturity and its *Cpd1*/+ mutant sibling (right) at the same age exhibiting diminished stature and delayed development. (C) A wild-type ear (left) and *Cpd1*/+ mutant ear (right) displaying the reduced ear size of the mutant.

Figure 3.2

Developmental time course of the *Cpd1/+* mutant leaf chlorosis phenotype. The leftmost leaf image (A) was photographed beginning 5 d post-emergence of the tip of the leaf from the whorl. Day 1 was the first day that the border between emergent pale-chlorotic (top) and normal regions (bottom) was evident (arrow), and was also the day that the region containing this border had emerged. The chlorotic sectoring pattern became progressively more visible, but remained unaltered (did not spread) over the life span of the leaf (B–D). Minor differences in the size of sectors between images are due to further emergence from the whorl and slight daily differences

(e.g. angle) in the photographing of the leaf. A wild-type leaf is shown on the far right for comparison (E).

Figure 3.3

Non-structural carbohydrate quantification and starch staining of *Cpd1*/+ mutant and wild-type sibling leaves. (A and B) Wild-type (WT) (A) and *Cpd1*/+ mutant (B) mature leaf photographs before (left) and after starch staining (right). (C and D) Cross-sections of starch-stained leaves in WT (C) and *Cpd1*/+ mutant mature leaves (D) at end of the night (EON). The arrow indicates starch accumulation in a M cell plastid. Scale bar=50 μ m. (E and F) Sucrose, glucose, and fructose (E), and starch (F) content in WT and *Cpd1*/+ mutant sibling leaves at end of the day (EOD) and EON time points. Black bars=WT EOD; gray bars=*Cpd1*/+ mutant EOD; black and white

striped bars=WT EON; gray and white striped bars=*Cpd1*/*+* mutant EON samples.

Values are means $\pm$ SE, and significantly different values at $P \leq 0.05$ were determined using a two-tailed Student's *t*-test, and are indicated with different letters.

Figure 3.4

Cpd1/+ mutant leaves exhibit decreased sucrose and carboxyfluorescein (CF) phloem transport. (A–D) ^{14}C -Labeled sucrose transport in wild-type (A, B) and *Cpd1/+* mutant (C, D) sibling leaves. The black arrow represents the abraded site on the leaf where the ^{14}C]sucrose was applied. (A, C) Leaf photographs. (B, D) Autoradiograms of ^{14}C signal. (E–J) Cross-sections of a lateral vein in wild-type (E, F, I, J) and *Cpd1/+* mutant (G, H) sibling leaves with (F, H) and without (J) CF application examined under UV (E, G, I) or blue light (F, H, J). CF is abundant in the

phloem of the wild-type vein (F, arrow), but not detected in the phloem of the *Cpd1*/⁺ mutant vein (H). Scale bar=50 μ m.

Figure 3.5

The phloem of *Cpd1/+* mutant leaves contains ectopic callose and lignin deposition.

(A, C, E, G, I, K, and L) Images of wild-type leaf sections; (B, D, F, H, J, M, and N) images of *Cpd1/+* mutant leaf sections. (A, B, K–N) Bright-field images; (C and D) UV autofluorescence, (E–J) aniline blue staining of callose under UV illumination.

Callose deposition in cross-sections of *Cpd1/+* mutant minor (B, D, F) and lateral veins (J) observed under bright-field (B) and UV light (without and with aniline blue staining, D and F). The inset in (F) shows a close-up of the phloem region containing ectopic callose. Asterisks signify CCs (inset F). Paradermal longitudinal sections of

wild-type (G) and *Cpd1/+* mutant (H) veins show that callose deposits along the length of the sieve tube are only observed in the mutant (arrowheads). In addition to callose staining by aniline blue (white arrowhead), non-aniline-blue-positive staining material can also be observed in the mutant *Cpd1/+* vein (black arrows in J). Ectopic lignin deposition in the *Cpd1/+* mutant phloem was revealed by Maule staining in both minor and lateral veins in chlorotic tissue (M and N). Lignin was only observed in the xylem cell walls of wild-type tissue (K and L). Scale bars in A–F=25 µm; in G, H, K, M=100 µm; in I, J, L, N=50 µm.

Figure 3.6

Ectopic callose is present in *Cpd1* mutant veins prior to starch accumulation. (A) Starch-stained second leaves of etiolated wild-type (top), *Cpd1/+* heterozygote (middle), and *Cpd1/Cpd1* homozygous mutant (bottom) siblings. Staples were used for structural support as the leaves were fragile. (B–D) Aniline blue-stained cross-sections of etiolated juvenile leaf tissue from wild-type (B), *Cpd1/+* heterozygote (C), and *Cpd1/Cpd1* homozygous mutant plants (D). Arrowheads indicate excess callose deposits. Scale bars=100 μm .

Figure 3.7

Cross-sections of expanding immature wild-type and *Cpd1/+* mutant leaves stained with aniline blue and imaged under UV light. (A, C, and E) Images from different regions (tip, middle, and base, respectively) of the same wild-type leaf. (B, D, and F) Images from these three regions from the same *Cpd1/+* mutant leaf. (A and B) Cross-sections from the tip of emerging immature leaves, which were becoming exposed to sunlight. (C and D) Cross-sections from the middle region of the immature leaves, which were still embedded within the whorl. (E and F) Cross-sections from the base

of the immature leaves, which were embedded within the whorl. Arrowheads indicate excess callose deposits. Scale bars=50 μm .

Figure 3.8

Chewing and non-chewing pest resistance in *Cpd1/+* mutant plants compared with wild-type (WT) siblings. (A) Caterpillar weight after 10 d of feeding on *Cpd1/+* mutant (gray bars) and WT (black bars) siblings. (B) Number of aphid progeny produced per adult aphid after 5 d feeding on *Cpd1/+* mutant (gray bars) and wild-type (black bars) plants. Values are means $\pm$ SE, and an asterisk indicates significantly different means at $P \leq 0.05$, using a two-tailed Student's *t*-test.

Figure 3.9

Benzoxazinoid (BX) levels in juvenile wild-type (WT) and *Cpd1/+* mutant leaves after *R. maidis* treatment (A) and plants not exposed to *R. maidis* (B). DIMBOA and DIMBOA-Glc levels were measured in the third and fourth leaves of the WT (black bars) and the *Cpd1/+* mutant (gray bars). Values are means $\pm$ SE, and an asterisk indicates significantly different means at $P \leq 0.05$, using a two-tailed Student's *t*-test.

Figure 3.10

Cpd1/+; bx1/bx1 double mutant plants exhibit the *Cpd1/+* mutant leaf phenotype of anthocyanin (A) and starch accumulation (B) relative to *+/+; bx1/bx1* (C and D) leaves. Additionally, *Cpd1/+; bx1/bx1* double mutants exhibit ectopic callose deposition in the phloem (arrowhead in E) compared with *+/+; bx1/bx1* controls (F). Scale bars=50 μ m.

Figure S3.1

Differences in photosynthetic capacity and gas exchange measurements between wild-type (WT) and chlorotic regions of *Cpd1/+* mutant sibling leaves.

Figure S3.2

Cpd1/+; *bx2*/*bx2* double mutant plants exhibit the *Cpd1*/+ mutant leaf phenotype relative to +/+; *bx2*/*bx2* leaves.

CHAPTER 4: The neomorphic *Carbohydrate partitioning defective1* mutant exhibits polarly localized ectopic callose deposition in root phloem in *Zea mays*

INTRODUCTION

In previous chapters I have discussed the importance of the carbohydrate partitioning pathway, as well as highlighting the transport steps involved in phloem loading and whole plant transport. However, not much has been discussed about the various sink tissues in maize. A major carbohydrate sink present throughout the majority of the plant's life is the root system. The maize root system is composed of two categories of roots: the embryonic and post-embryonic roots (Atkinson *et al.*, 2014, Bray & Topp, 2018, Hochholdinger & Tuberosa, 2009, Hochholdinger *et al.*, 2004, Hochholdinger *et al.*, 2018). The embryonic root system includes the primary and seminal roots that develop during embryogenesis (Feldman, 1994, Kiesselbach, 1949). The post-embryonic root system is developed after germination and is composed of nodal shoot-borne roots and all lateral roots, including those located on the embryonic roots (Bodner *et al.*, 2013, Bray & Topp, 2018, Hochholdinger *et al.*, 2018, Yu *et al.*, 2016).

To date, there have only been a handful of maize genes identified to play a role in root development (Hochholdinger & Tuberosa, 2009, Hochholdinger *et al.*, 2018, Yu *et al.*, 2016). Interestingly, several of these genes function in auxin signaling, a plant

hormone crucial for proper growth and development (Hochholdinger *et al.*, 2018, Taramino *et al.*, 2007, von Behrens *et al.*, 2011, Xu *et al.*, 2015). Auxin is primarily generated in the shoot tissue and transported long distance through the phloem to the root tip. AUX and PIN proteins function in the root to direct polar transport to obtain appropriate auxin maxima and minima for lateral root primordia formation, root tip growth, and appropriate gravitropism response (Baker, 2000, Bhalerao *et al.*, 2002, Friml & Palme, 2002, Ljung *et al.*, 2001, Marchant *et al.*, 2002, McSteen, 2010, Morris & Thomas, 1978, Normanly *et al.*, 1993, Novoplansky *et al.*, 1989, Nowacki & Bandurski, 1980, Woll *et al.*, 2005). Lateral root primordia (LRP) develop from pericycle cells where auxin maxima are generated after they have left the meristematic zone (Alarcón *et al.*, 2016, Song *et al.*, 2009, Xu *et al.*, 2005, Yu *et al.*, 2016, Zhuang *et al.*, 2006). In the maize mutant *rootless with undetectable meristems 1* (*rum1*) neither lateral roots or seminal roots form due to a mutation in a *Aux/IAA* gene required for auxin signaling (Taramino *et al.*, 2011, Woll *et al.*, 2005).

Additionally, it was demonstrated in arabidopsis that other compounds such as nitrate and sucrose are crucial for lateral root development (Bhalerao *et al.*, 2002, Drew, 1975, Zhang & Forde, 2000, Zhang *et al.*, 1999).

Callose is β -1,3-linked glucan and is a structural component necessary for plasmodesmata and sieve plate formation highlighted in the previous chapter. During sieve plate formation, PD connect SE at their end walls and identify the location of the future pores in the mature sieve plate. Callose is deposited around the PD and fills the sieve plate space, and is then degraded to the point it simply lines the pores of the mature sieve plate (Barratt *et al.*, 2011, Esau *et al.*, 1962,

Northcote & Wooding, 1966). This ensures proper sieve plate formation and pore size, required for appropriate phloem sap transport through the mature sieve tubes (Barratt *et al.*, 2011, Vatén *et al.*, 2011). Additionally, callose is involved in the development of lateral roots. During lateral root development callose is heavily deposited in the cells surrounding the LRP, including the nearby phloem and xylem. As the LRP matures and the lateral root emerges through the cortex cells, the callose is degraded (Benitez-Alfonso *et al.*, 2013). Therefore, callose is a crucial compound during the development of phloem and lateral roots.

The previous chapter discussed the characterization of the semi-dominant *Carbohydrate partitioning defective1* (*Cpd1*) mutant. In summary, the *Cpd1* mutant exhibits decreased sucrose export from mature leaves due to ectopic callose plugs in its sieve elements that form early in meta-phloem development. Here, we identify that the semi-dominant *Carbohydrate partitioning defective1* is a gain-of-function mutant, and begin the work to clone the causative mutation through transgenic studies. Additionally, *Cpd1* accumulates ectopic callose deposits in the phloem of mutant roots. Interestingly, in heterozygote individuals the callose accumulates in a polarly localized pattern at sites of lateral root formation. These findings continue to shed light on the function of the native *Cpd1* gene.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The *Cpd1* mutant was generated by Gerry Neuffer (University of Missouri) via ethyl methanesulfonate mutagenesis of maize pollen (Neuffer & Coe, 1978). Plants used

for mapping and dosage studies were grown at the University of Missouri South Farm Agricultural Experiment Station in Columbia, MO. Hyperploid TB-7Sc seed was provided by Dr. James Birchler, and plants were crossed as males with *Cpd1/+* females (Beckett, 1978).

Plants used for root images and callose staining were grown in a greenhouse supplemented with high-pressure sodium lighting ($1600 \text{ mmols m}^{-2} \text{ s}^{-1}$) under 16-hour light and 8-hour darkness. The plants were kept at a daytime and night-time temperature of 30°C and 24°C, respectively. *Cpd1* plants were genotyped as described previously (Julius *et al.*, 2018). The *vanishingtassel2* and *barren inflorescence2* mutants, previously described by were utilized for aniline blue staining (McSteen *et al.*, 2007, Phillips *et al.*, 2011).

Nicotiana benthamiana plants used for transient protein expression were grown in growth tents under 12h light and 12hr dark conditions at 18-25°C and 55-65% relative humidity for 4-5 weeks.

Light and fluorescence microscopy

All images taken of *Cpd1* roots and aniline blue staining were performed as described previously (Julius *et al.*, 2018).

Roots of plants expressing the *Cpd1* and wild-type phenotypes were harvested after 3-4 weeks. Fluorescent in situ hybridization was performed as described previously (Albert *et al.*, 2019).

***Cpd1* mapping and whole genome sequencing**

The *Cpd1* mutant was backcrossed to the inbred line B73 to generate mapping populations. Bulk Segregant Analysis (BSA), fine mapping, and was performed as previously described (Tran *et al.*, *in prep*). The fine-mapping population consisted of 821 mutant individuals.

Construct Design

The mutated coding sequences for the three candidate *Cpd1* genes, hereby noted as Cpd1-2, Cpd1-6, and Cpd1-9, identified through whole genome sequencing were synthesized with 1-1.5kb upstream sequence to capture their native promoters (Life Technologies, USA). These constructs were cloned into the pZY101 plasmid to enable stable maize transformation (Zeng *et al.*, 2004). The native promoter sequences were recombined into the gateway vector pBGWFS7 to generate the pCpd1::eGFP:GUS reporter constructs for each of the three candidate genes (Karimi *et al.*, 2002). All six constructs were transformed into the AGL1 *Agrobacterium tumefaciens* strain for stable transformation into maize. The mutated coding sequences of the three *Cpd1* candidates used in the transgenic studies were recombined into the pEarleyGate104 vector, generating p35S::eYFP:Cpd1 candidate translation fusion proteins for subcellular localization assays (Earley *et al.*, 2006).

Subcellular localization assays

Agrobacterium tumefaciens transformation and tobacco leaf infiltration was performed as described in Tran *et al.*, (*in prep*).

RESULTS

***Cpd1* roots accumulate ectopic callose in their phloem**

While it was shown that the *Cpd1* leaves accumulated callose in their phloem, it was unknown if this phenomenon occurred elsewhere in the plant (Julius *et al.*, 2018). To answer this question two-week old *Cpd1* homozygous, heterozygous, and wild-type plants were harvested and root cross sections were stained with aniline blue (Figure 4.1A-J). As expected, wild-type roots did not exhibit ectopic callose deposition in the phloem (Figure 4.1A-D). However, callose accumulation was seen in all phloem in *Cpd1/Cpd1* homozygous mutants (Figure 4.1E-F). Surprisingly, there was no ectopic callose deposits seen throughout most of the *Cpd1/+* heterozygous root, except at lateral root primordia (Figure 4.1G-I). Callose deposition was seen strictly in the phloem at the LRP site, and not in the neighboring phloem cells (Figure 4.1J). Since auxin plays a major role in LRP formation, *vanishing tassel2* and *barren inflorescence2* mutants, which have impaired auxin signaling, were grown for two weeks and stained with aniline blue (McSteen *et al.*, 2007, Phillips *et al.*, 2011). No callose accumulation seen in these mutants suggesting that the callose deposition is not due to improper auxin transport and biosynthesis (Figure 4.1 K-N). Additionally, homozygous mutants exhibited a reduced root system both in size and post-embryonic root development. Nodal roots were reduced in size relative to wild-type and heterozygous individuals, and lateral roots failed to develop (Julius *et al.*, 2018, Chapter 3). Therefore, the

Cpd1 mutation results in callose accumulation in the phloem of root and shoot, and the native *Cpd1* gene appears to be involved in phloem development of lateral roots.

***Cpd1* is a gain-of-function mutant**

Dosage studies were performed to gain a better understanding of how the mutation results in ectopic callose deposition in the phloem. B-A translocation lines, where the short arm of chromosome 7 was translocated to the B chromosome, were crossed with *Cpd1*/+ individuals. Mutant offspring containing the *Cpd1* allele and two, one, or zero dosages of the wild-type allele, respectively, were generated. Root apical meristems of *Cpd1* expressing plants were digested and fluorescent in situ hybridization experiments were performed with a whole chromosome probe binding to chromosome 7 sequence (Albert *et al.*, 2019). Karyotypes were generated from these individuals and hyperploid plants containing a 1:2 ratio of *Cpd1*:wild-type alleles were expressing the heterozygous mutant phenotype (Figure 4.2). This suggests that the *Cpd1* mutation is a gain-of-function mutation as plants with a 1:2 dosage of *Cpd1* to wild-type alleles exhibit the same phenotype as plants with a 1:1 dosage.

Mapping and cloning the *Cpd1* mutation

A mapping population between B73 and Mo17 was generated to identify the causative mutation for the *Cpd1* phenotype. Bulk Segregant Analysis narrowed the region of interest to the short arm of chromosome 7. Fine-mapping using insertion and deletion markers between the two inbreds narrowed the region

further to 8 candidate genes. Whole genome sequencing was performed on homozygous *Cpd1* and inbred Mo17 plants. Only 3 of the 8 candidate genes contained unique, missense mutations in their coding region after comparing the homozygous *Cpd1* sequence to the Mo17 and B73 reference sequence. Due to the *Cpd1* mutation resulting in a gain-of-function mutant and the lack of a second allele, a transgenic strategy was utilized to identify which of the three candidate genes contained the causative mutation. The mutated coding sequences were independently transformed into maize driven by their native promoters to phenocopy the *Cpd1* mutant. Additionally, each of the three candidate's native promoters were cloned into a reporter construct to drive expression of eGFP:GUS. This accomplishes 1) demonstration of functionality of the native promoter and 2) characterization of the tissue level expression of each candidate gene. Preliminary results from single event T0 plants show that the Cpd1-6 and Cpd1-9 reporter constructs have sporadic GUS expression in mature leaf veins and primary and nodal roots (Figure 4.3). Additionally, two Cpd1-6 gene T0 and one Cpd1-2 gene events do not exhibit chlorosis or anthocyanin production in mature leaves; however, introgression into B73 is required before ruling out these mutations as the cause of the *Cpd1* phenotype. Transgenic studies are still in progress to generate T0 plants containing the Cpd1-2 reporter construct and the Cpd1-9 mutant sequence construct.

Subcellular localization of Cpd1

Subcellular localization studies are underway for all three candidate genes to gain a better understanding of their function. The mutated cDNA sequence used for the transgenic studies were cloned into the p104 Earleygate vector resulting in a *pro35S::eYFP-Cpd1* candidate protein fusion. Each construct was then independently transformed into agrobacterium and infiltrated into *Nicotiana benthamiana* leaves. Preliminary data suggests that Cpd1-2 localizes to the cytoplasm. Cpd1-6 localizes to the nucleus and potentially vesicles as concentrated punctae are visible throughout the cell. Finally, Cpd1-9 localizes to the nucleus and endoplasmic reticulum (Figure 4.4). Further studies with positive markers for these locations are required to confirm the candidate gene's localization.

DISCUSSION

The semi-dominant *Carbohydrate partitioning defective1* mutant exhibits inhibited carbohydrate partitioning due to ectopic callose deposition in the leaf phloem (Julius *et al.*, 2018, Chapter 3). Here we have shown that both the heterozygote and homozygote *Cpd1* mutants accumulate callose in the root phloem; however, they differ in their severity and tissue location of callose accumulation. The homozygous mutant exhibits callose deposition in nearly every sieve element in the root, in agreement with the severe callose accumulation in almost every vein in the leaf. These mutants rarely survive longer than two weeks, which is likely due to the complete blockage of the phloem throughout the plant inhibiting the movement of phloem sap, containing auxin and sucrose along with other important compounds,

from source to sink tissue (Bhalerao *et al.*, 2002, Friml & Palme, 2002, Julius *et al.*, 2018). This explains the drastically decreased root mass and architecture in the *Cpd1* homozygous mutant, where there is very little post-embryonic root development.

Interestingly, in the heterozygous *Cpd1* mutant callose deposition is observed strictly at the sites of lateral root formation. This polar localization of callose deposition is not observed in the heterozygous leaves. While we currently do not understand the cause of this polar callose deposition in heterozygous roots, or callose deposition in any of the phloem tissue, here it may be linked to the development of new phloem tissue in the emerging lateral root. Benitez-Alfonso *et al.* has shown that during normal lateral root development in *Arabidopsis*, callose is deposited in all surrounding cells during stage III and stage IV LRP development (Benitez-Alfonso *et al.*, 2013). Upon maturation of the LRP the accumulated callose is degraded resulting in a newly formed lateral root (Benitez-Alfonso *et al.*, 2013). Therefore, it is possible that the *Cpd1* gene functions in the development and maturation of phloem, specifically proper development of sieve elements and sieve plates. This would explain the polarly localized callose deposition in the heterozygote mutant, as well as the excessive callose deposition in the homozygote mutant. As highlighted previously, during phloem development, protophloem is first generated as a preliminary transport tissue. As the organ matures and elongates, the protophloem is degraded and replaced by metaphloem (Evert *et al.*, 1996). Therefore, protophloem generated during embryogenesis and organogenesis throughout the plant's life cycle may be properly formed in the *Cpd1* mutant

allowing for proper phloem sap transport during germination and mobilization of kernel reserves. However, upon protophloem degradation the *Cpd1* mutant undergoes improper metaphloem development. During the development of metaphloem and lateral roots, callose is deposited throughout the cells, followed by controlled degradation leaving the mature sieve plate (Benitez-Alfonso *et al.*, 2013, Evert *et al.*, 1996, Northcote & Wooding, 1966). However, in the *Cpd1* mutant, it is possible that there is improper regulation of the initial callose deposition leading to the plugged phloem that is observed in the mutant plants. A second possibility is the callose originally deposited is not properly degraded and therefore the mature sieve plate or late stage LRP is never formed and the phloem remains plugged.

Additionally, there must be a signaling threshold required for this process, as the callose accumulation in heterozygote mutant leaves is not present in every vein.

Subcellular localization of the Cpd1 protein is required to understand its function.

Therefore, a $\text{pro}^{35\text{S}}::\text{eYFP}:\text{Cpd1}$ construct was generated for each of the three candidate genes. All three candidates exhibit varying localization patterns. The location of Cpd1-2 and Cpd1-6 proteins are somewhat surprising given that each protein contains nine transmembrane domains and were expected to localize to a membrane. The Cpd1-9 protein exhibited strong localization to the nucleus and endoplasmic reticulum in tobacco epidermal cells. This result supports database annotations of this protein as a nucleoredoxin. However, additional experiments with positive markers for these organelles as well as those seen with Cpd1-2, and Cpd1-6 proteins are required to confirm these preliminary results. Subcellular

localization assays must also be performed with C-terminal YFP fusions to test if the protein's proper localization is disrupted with either an N- or C-terminal tag.

It was unknown the *Cpd1* mutation is a gain- or loss-of-function mutation, which is crucial to understand the function of the native *Cpd1* gene. Utilizing the TB-7Sc B-A translocation line, dosage experiments identified the *Cpd1* mutation as a gain-of-function mutation. Therefore, if *Cpd1* is involved in the development and maturation of metaphloem, its specific function could be in the deposition of callose, or the inhibition of callose degradation, both of which would lead to the over-accumulation of callose and plugging of the phloem. Further insight will be provided upon cloning the causative mutation for the *Cpd1* phenotype. Toward this goal, transgenic experiments are underway to phenocopy the *Cpd1* mutation through independent insertion of the mutated sequence of three candidate genes. Transgenic experiments are also underway to identify the tissue level expression of the native candidate genes. These studies are currently underway and future analysis will be required to answer these questions.

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FIGURES

Figure 4.1

Transverse sections of 2-week old wild-type (A-D), *Cpd1/+* (E-H), *Cpd1/Cpd1* (I-J), *bif2* (K-L), and *vt2* (M-N) primary roots stained with aniline blue visualized by bright-field (A, C, E, G, I, K, and M) or UV light (B, D, F, H, J, L, and N). Lateral roots can be seen emerging through the cortex (C-D, G-H, K-N). Callose accumulation occurs in the phloem of *Cpd1* mutants (arrowheads). Scale bars = 100 μ m.

Figure 4.2

(A) A karyotype image of a hyperloid *Cpd1*^{+/+/+} with the short arm of chromosome 7 truncated from one A chromosome (yellow arrowhead) and present on two B chromosomes (white arrowheads). Chromosome 7 probe results in the red fluorescence of chromosome 7 sequence. (B) Transverse section of *Cpd1*^{+/+/+} stained with aniline blue and callose accumulation seen in the phloem (white arrowheads). (C) The hyperploid *Cpd1*^{+/+/+} plant exhibits anthocyanin accumulation and chlorosis in mature leaves similar to *Cpd1*^{+/+} plants. Scale bars = 5 μ m in A and 100 μ m in B.

Figure 4.3

(A-B) $\text{proCpd1-6::eYFP:GUS}$ and (C-D) $\text{proCpd1-9::eYFP:GUS}$ T_0 mature leaf (A and C) and root (B and D) tissue stained for GUS expression (blue).

Figure 4.4

Subcellular localization of the three *Cpd1* candidate proteins with N-terminal eYFP fusions expressed in *Nicotiana benthamiana* epidermal pavement cells. A.

pro35S::eYFP:Cpd1-2. B. *pro35S::eYFP:Cpd1-6*. C. *pro35S::eYFP:Cpd1-9*.

CHAPTER 5: SWEETs, transporters for intra- and intercellular sugar translocation

Note: The information in this appendix was published under the title:

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Contributions: BTJ wrote the second paragraph of the section: Identification of SWEETs and generated the 15 angiosperm SWEET protein phylogeny.

Key words: glucose, fructose, hexose, sucrose, transporter

ABSTRACT

Three families of transporters have been identified as key players in intercellular transport of sugars: MSTs (monosaccharide transporters), SUTs (sucrose transporters) and SWEETs (hexose and sucrose transporters). MSTs and SUTs fall into the major facilitator superfamily; SWEETs constitute a structurally different class of transporters with only seven transmembrane spanning domains. The predicted topology of SWEETs is supported by crystal structures of bacterial homologs (SemiSWEETs). Plant genomes contain ~5-47 paralogs that serve distinct

physiological roles. In *Arabidopsis*, AtSWEET8 and 13 feed the pollen; SWEET11 and 12 provide sucrose to the SUTs for phloem loading; AtSWEET11, 12 and 15 have distinct roles in seed filling; AtSWEET16 and 17 are vacuolar hexose transporters; and SWEET9 is essential for nectar secretion. The remaining family members await characterization, and could play roles in the gametophyte as well as other important roles in sugar transport in the plant. In rice and cassava, and possibly other systems, sucrose transporting SWEETs play central roles in pathogen resistance. Notably, the human genome also contains a glucose transporting isoform. Further analysis promises new insights into mechanism and regulation of assimilate allocation and a new potential for increasing crop yield.

Introduction

Historically, humans relied primarily on bio-based economies. However, during the industrial revolution a shift occurred towards a fossil fuel economy, most notably in energy, as well as industrial scale production of synthetic chemicals and materials. There is an ongoing discussion regarding a return to an industrial scale bio-based economy. However, such concepts, while at first sight potential innovative drivers of the economy, with the added value of apparent sustainability, also bear major risks. Feeding a world population of 6–9 billion people is already a challenge and has major ecological impacts, thus if industry begins to compete for land and crop production, humans will need new ways to ensure efficient food production and secure biodiversity. In that sense, biofuels were a test case – probably for complex reasons, including the not yet fully developed science, only a major success in Brazil.

Yet it initiated a new thinking – the production of high value products from photosynthesis to support the development of other bio-based products – that is returns on high value products could generate enough income to make biofuels competitive and to ultimately transition from the fossil fuel to a bio-based economy. Since today's agriculture is not sustainable, and since productivity is already limiting, scientists and breeders need to find creative new ways to massively increase productivity. Potential targets for crop yield engineering have been carefully explored (Zhu *et al.*, 2010). There is no doubt that carbon allocation in plants must be a central player. Yet this fundamental process is still poorly understood; therefore rational engineering is not yet feasible and demands further research.

Sucrose is produced in photosynthetically active tissues of the leaf and stem, is actively loaded into the phloem, from which it is withdrawn at the various tissues and cells that depend on external sugar supply. At the same time, concentration of sucrose as the dominant osmoticum in the phloem sap, is thought to create the driving force for translocation of all other solutes in the phloem. A bit more than 20 years ago, the first sucrose transporter gene responsible for phloem loading was identified (Riesmeier *et al.*, 1992), a significant step towards the understanding of sugar translocation. More recently, the lessons learned from model species are starting to be reconciled and implemented in crops such as corn (Slewinski *et al.*, 2009). Yet the story is incomplete: sucrose transporters (SUTs) function as sucrose/proton symporters, thus under standard conditions (high negative

membrane potential and acidified cell wall), SUTs primarily import sucrose from the cell wall into the cells responsible for long distance translocation. However, sucrose is synthesized from photosynthetically derived precursors in mesophyll cells. Thus at some point sucrose must be released into the cell wall, either directly from mesophyll or from bundle sheath cells, or if sucrose can move from cell to cell via the conspicuous plasmodesmata, release would be expected to occur from cells inside the phloem, such as the phloem parenchyma. Similarly, the sugar efflux transporters for nectar secretion, efflux from seed coat, tapetum, and transmitting tract, the transporters supplying symbiotic and pathogenic microorganisms and the effluxers from the root epidermis, are missing. A unique new family of sugar transporters, the SWEETs, could fill this gap at many of the expected sites. This review focuses on SWEETs from *Arabidopsis* since the physiological function of these transporters has been studied in most detail in this reference organism, and we highlight a few other SWEETs, e.g. from rice and cassava in the context of their role in pathogen susceptibility.

Identification of SWEETs

SWEETs were identified by coexpression of membrane proteins with no assigned function with genetically-encoded fluorescent sugar sensors in human HEK293T cells. HEK293T cells are characterized by low endogenous glucose uptake activity and do not show significant sucrose uptake (Takanaga *et al.*, 2008, Chen *et al.*, 2010, Sosso *et al.*, 2013). With the help of a FRET glucose sensor we performed a screen for glucose transporters at neutral pH, expecting that this would enable us to

identify potential pH-independent uniporters. In this way, we succeeded in identifying members (Clade I and II) of the SWEET family, which contain 17 members that fall into four phylogenetic clades in *Arabidopsis* (Figure 5.1 and Interactive Phylogenetic Tree online) (Chen *et al.*, 2010). We used an analogous approach to identify novel sucrose transporters, that is, we expressed a FRET sucrose sensor in HEK293T cells and confirmed the absence of sucrose uptake in the human cells. We then performed a screen of unknown membrane proteins including SWEET homologs, and to our surprise found that clade III SWEETs (SWEET9–15) are able to transport sucrose (Chen *et al.*, 2012, Lin *et al.*, 2014). We note that, although SWEET do not appear to show strong pH-dependencies, and although they can function in both uptake and efflux assays, formally a uniport function has not been demonstrated.

Based on the available sequenced genomes annotations (14 April 2015), within Angiosperms, we observed that the number of SWEETs can vary from as low as eight in *Amborella trichopoda*, the earliest diverging Angiosperm, which is not a member of the monocots or dicots, to as high as 47 in *Eucalyptus grandis*, a dicotyledonous tree. Intriguingly, in comparison, the *Chlamydomonas reinhardtii* genome contains five SWEETs, and the *Physcomitrella patens* genome contains six SWEETs, indicating that there has been a great expansion in SWEET genes in both monocot and dicot lineages (Chen *et al.*, 2010). Membership of a SWEET in a clade does not seem to predict the physiological process the protein is involved in, for example, AtSWEET5, AtSWEET8 and AtSWEET13 are vital for pollen nutrition, yet

they are found in either clades II or III. Of the SWEETs that have been studied so far, placement of a SWEET in a clade appears to correlate well with the relative selectivity towards mono- versus di-saccharides (Clade I and II prefer hexoses, clade III are efficient sucrose transporters). As stated earlier, all of the clade III SWEETs in *Arabidopsis* transport sucrose. Also, both clade IV SWEETs in *Arabidopsis* (AtSWEET16-17) are located on the tonoplast membrane and likely transport fructose. We propose that SWEET genes identified in the future be named according to their phylogenetic relationships to the founding members of the family in *Arabidopsis* and rice (Chen *et al.*, 2010), i.e., SWEETs 1-3 in clade I, SWEETs 4-8 in clade II, SWEETs 9-15 in Clade III, and SWEETs 16-17 in clade IV. If due to genome duplication there are multiple genes that are co-orthologous to the closest *Arabidopsis* or rice gene, we suggest a letter designation follow the gene name to distinguish closely related genes, e.g., Eucalyptus EgSWEET15a-f, rather than each gene being numbered sequentially.

SWEET structure

SWEETs are different from all other known sugar transporters in that they are heptahelical proteins composed of a tandem repeat of three transmembrane domains (TMs), connected by a linker-inversion TM (Chen *et al.*, 2010). SWEET homologs exist also in animals and human genomes. Eukaryotic SWEETs most likely evolved from ancestral prokaryotic homologs that are only ~100 amino acids in length and contain only three TMs (SemiSWEETs) (Sosso *et al.*, 2013, Xuan *et al.*, 2013). *Bradyrhizobium* BjSemiSWEET1 mediates sucrose transport, whereas

LbSemiSWEET mediates glucose transport. Atomic structures for three SemiSWEETs have been obtained in outside open and occluded states (Xu *et al.*, 2014, Wang *et al.*, 2014). Split ubiquitin yeast two-hybrid and split green fluorescent protein (GFP) assays indicated that *Arabidopsis* SWEETs can oligomerize (Xuan *et al.*, 2013). Transport-deficient mutants of AtSWEET1 were able to suppress the activity of coexpressed wild-type AtSWEET1, potentially indicating that SWEET oligomerization is necessary for function (Xuan *et al.*, 2013). The atomic structures are highly valuable for studying the transport mechanism as well as the regulation of SWEETs.

Clade III SWEET9 is essential for nectar secretion

Nectaries are responsible for producing nectar, which serves as an attractant and reward for pollinators. Although the function and composition of nectar are well characterized, the mechanisms driving nectar secretion have not been determined. Pre-dating the discovery of the function of SWEETs, one of the paralogs had been identified as a nectary-specific gene. Nec1 (as the *Petunia* homolog of SWEET9 was originally named) is expressed in nectaries and its developmental regulation correlates inversely with nectarial starch content, implying its role in sugar secretion in nectaries (Ge *et al.*, 2000). Silencing of the clade III SWEET homolog Nec1 from *Petunia* triggered male sterility, but a nectar phenotype was not reported (Ge *et al.*, 2001). Once the SWEETs had been identified, SWEET9 paralogs were identified as nectary-specific in *Arabidopsis*, *Brassica* and *Nicotiana* (Lin *et al.*, 2014). Furthermore, SWEET9 was found to function as a sucrose transporter and be

essential for nectar production. These data are consistent with a model in which sucrose synthesized in the nectary parenchyma is secreted by SWEET9 into the apoplasm, where sucrose is partially hydrolyzed by cell wall invertase to produce a mixture of sucrose, glucose and fructose. Thus SWEET9 appears to be responsible for secreting sucrose from the nectarial cells (Figure 5.2A).

Clade III SWEETs in phloem loading

Phloem loading is a multi-step process: sucrose is produced in mesophyll cells and traffics between cells possibly through plasmodesmata towards the phloem. Since SUTs load the sieve element companion cell complex with sucrose from the cell wall space, sucrose exits the symplasm with the help of sucrose transporting SWEETs. SWEET11 and 12 were found to be expressed in leaves, and subject to diurnal/circadian regulation (Chen *et al.*, 2012, Haydon *et al.*, 2011). Both proteins, as judged from analyses of GUS reporter fusions, were detected in the phloem. AtSWEET11-GFP was found to localize to the plasma membrane of cells inside the phloem, most likely phloem parenchyma (Chen *et al.*, 2012), restricting sucrose flux to the interface between the phloem parenchyma and the sieve element companion cell complex deep inside the leaf. Analysis of *atsweet11;12* double mutants provided strong evidence for a key role in sucrose translocation out of the leaf; in particular the double mutants accumulated starch in leaves, and radiotracer efflux from petioles was reduced (Chen *et al.*, 2012) (Figure 5.2B). Interestingly, AtSWEET13, the closest homolog of SWEET11/12, was upregulated in the *atsweet11;12* mutant, implying regulatory systems that adjust SWEET transcript levels. Key future

questions are whether SWEETs play similar roles in crop plants, how their activities are coordinated with sucrose anabolism and catabolism and SUT transport activity. The apparent restriction of apoplastic transfer to the interface between phloem parenchyma and the sieve element companion cell complex may indicate that apoplastic sucrose levels in other parts of the leaf are low. It may thus be necessary to reevaluate the hypothesis that sugars accumulate to millimolar levels in the leaf apoplasm, which is mainly based on apoplastic wash fluids, a technology that measures solute efflux into sucrose-free medium and averages across the whole leaf. Cell surface-targeted FRET sucrose sensors may be a suitable tool for monitoring sucrose, such as in the apoplasm surrounding epidermal and mesophyll cells (Okumoto *et al.*, 2012).

Role of SWEETs during senescence

Hexoses, in particular galactose, fructose and glucose, accumulate in senescent leaves, and it has been speculated that SWEET members may also function in remobilization of carbohydrate during senescence (Seo *et al.*, 2011). The expression of the *AtSWEET15* (SAG29) gene is highly induced in leaves during senescence. *Arabidopsis* plants overexpressing *AtSWEET15* showed accelerated senescence and salt hypersensitivity while *atsweet15* mutants were less sensitive to high salinity. However, translational fusion with GUS under control of its own promoter indicate that *AtSWEET15* protein does not accumulate to high levels during senescence (Chen *et al.*, 2015). This apparent difference may be attributable to overall changes in RNA turnover or to post-translational regulation. Similarly, overexpression of

OsSWEET5, which can transport galactose, in rice causes growth retardation and early senescence (Zhou *et al.*, 2014). We observed that overexpression of other SWEETs (e.g. AtSWEET11 and 12) had similar phenotypic effects of growth retardation (Chen and Frommer unpublished; Figure 5.3). The negative impact of SWEET ectopic expression on plant growth may not be surprising; sugar translocation is limited to a subset of cell types (e.g. phloem parenchyma in the leaf) and polarized. Ectopic expression is expected to lead to a loss in the directionality of sugar flux, and to general leakage of sucrose from cells, thereby reducing phloem loading and causing rather non-specific effects. Therefore, a potential role in reallocation of carbon under conditions of salinity may be indicated, but additional work is required to evaluate the role of SWEETs in leaves for recapturing of carbon during stress or senescence.

Clade III SWEETs in seed filling

Based on the finding that SWEETs act as cellular efflux transporters in phloem parenchyma and nectaries, we hypothesized that they may also contribute to assimilate transfer from maternal seed coat to developing embryo. GFP fusions show that AtSWEET11, 12 and 15 are expressed in seed coat and endosperm. Analysis of the triple knockout mutant showed a severe delay in embryo development and a wrinkled seed phenotype at maturity due to lower starch and lipid content and a smaller embryo (Chen *et al.*, 2015) (Figure 5.2C). These findings are exciting because the identification of SWEETs involved in seed coat efflux opens up the possibility of identifying the mechanisms that coordinate the delivery of

sucrose from leaves with the needs of the developing seed. Extensive studies of the regulation of post-phloem unloading into seeds by osmotic gradients and sucrose provide guidance for further analysis (Patrick & Stoddard, 2010). A generalized model of phloem unloading is presented in Figure 5.4.

SWEETs in pollen nutrition

Large-scale gene expression analyses indicate that at least four AtSWEETs may play roles in nurturing the gametophyte (Bock *et al.*, 2006). AtSWEET5 (*VEX1*) is expressed in mature, hydrated and germinating pollen and was found specifically in the vegetative cell of pollen grains, which may supply the generative cell with sugars (Engel *et al.*, 2005). Mutation of the hexose transporting clade II AtSWEET8 (RPG1) caused male sterility (Chen *et al.*, 2010, Guan *et al.*, 2008). More recent work showed that AtSWEET13 (RPG2) also plays a role in pollen nutrition (Sun *et al.*, 2013). In rice, OsSWEET5 is also expressed in anthers.

SWEET recruitment by pathogens

Apparently, sucrose efflux by SWEETs for phloem loading is restricted to an interface of a few microns deep inside the leaf. One would therefore assume that the apoplastic space (e.g. between epidermal or mesophyll cells) contains only low sugar levels. Consequentially, the large majority of the apoplastic space in a leaf may not serve as a good environment for rapid growth of pathogens. Thus pathogens may have to find ways to increase apoplastic sugar levels in their vicinity. One such example is the direct induction of rice SWEETs by TAL effectors (transcriptional activator-like proteins) from *Xanthomonas oryzae*. Five out of the

21 SWEET genes in the rice genome have been shown to support pathogen growth (Streubel *et al.*, 2013). This group of SWEETs all belong to clade III (OsSWEET11–15), and almost all were tested and function as sucrose transporters (Chen *et al.*, 2012, Zhou *et al.*, 2015, and Qu, Sosso, Chen & Frommer, unpublished). Interestingly, two of the SWEETs had been identified as recessive blight resistance loci, named *xa13* and *xa25*; *xa13* corresponds to OsSWEET11 (Yang *et al.*, 2006) and *xa25* to its close homolog OsSWEET13 (Liu *et al.*, 2011). Furthermore, OsSWEET14 is targeted by various *X. oryzae* pv. *oryzae* (Xoo) strains (Antony *et al.*, 2010). Interestingly, OsSWEET12 and 15 have not been found to be targets of Xoo pathovars so far, indicating that the survey of strains is not broad enough to identify pathovars that hijack these transporter genes, that the pathogens have difficulties targeting these genes, or that induction of these proteins has negative side effects. One possible explanation why only sucrose transporting SWEETs can support pathogen growth may be that, in contrast to sucrose pools, hexose pools in the cytosol are limiting, therefore activation of a hexose transporting SWEET may be insufficient to sustain growth of the pathogen population. The understanding of these mechanisms will help engineering broad-spectrum resistance by genomic editing of the binding sites of the bacterial TAL effectors using either CRISPR/Cas9 or TALEN technologies in rice (Jiang *et al.*, 2013, Li *et al.*, 2012).

Recessive resistance in rice involving SWEETs had originally been seen as unique; however, recently SWEETs have been shown to be induced by TAL effectors from the Cassava blight pathogen *X. axonopodis* pv. *Manihotis* (Cohn *et al.*, 2014) –

interestingly, two clade III SWEETs were also induced (MeSWEET10 and 15). Mutation of the TAL effector responsible for induction of SWEET10 led to resistance. It is noteworthy that in Cassava blight, invertases are also upregulated, enabling the pathogen to acquire hexoses.

The expression of AtSWEETs from clades other than clade III are induced by *Pseudomonas syringae* pv. *tomato* strain DC3000, *Golovinomyces cichoracearum* and/or *Botrytis cinerea* (Chen *et al.*, 2010). Glucose transporter VvSWEET4 from *Vitis vinifera* is involved in the interaction with *B. cinerea*, and the mutants of the orthologous AtSWEET4 are less susceptible to *B. cinerea* (Chong *et al.*, 2014), indicating a role in facilitating glucose efflux into cell wall space to enhance fungal growth. Expression of CsSWEET1 from citrus is stimulated in a TAL effector dependent manner upon the infection of *X. citri* (Hu *et al.*, 2014). Notably, the founding member of the SWEET family, MtN3, was identified as a nodulation-induced gene in *Medicago truncatula* (Gamas *et al.*, 1996). Further work will be required to determine whether SWEETs play a role in feeding the symbiotic *Rhizobium* (Udvardi *et al.*, 1990). SWEETs have been found to be induced in many systems: for example the infection of wheat by *Puccinia* (Yu *et al.*, 2010). It is thus tempting to speculate that SWEETs serve as host susceptibility factors for a wide variety of pathogens including oomycetes and fungi as well as different types of symbionts including mycorrhiza and the microbiota in the rhizosphere.

SWEETs and copper transport

OsSWEET11 was found to interact with two copper transporters homologs of the COPT family (Yuan *et al.*, 2010). Data from Wang's lab indicated that OsSWEETs are involved in copper transport. Specifically, they observed that the rice copper transporters COPT1 and COPT5 interacted with OsSWEET11, and that coexpression of all three proteins was necessary to restore copper uptake deficiency of a copper transport-deficient yeast mutant (MPY17 [ctr1Δctr3Δ]). However, copper uptake was not directly measured. The growth phenotype mutant is conditional and is not observed in the presence of glucose. An alternative interpretation of the results may be that COPT1 and 5 mediate low level copper uptake while OsSWEET11 mediates uptake of contaminant sugars from the medium (i.e. the agar), thus partially relieving the dependency of mutant growth on copper. At higher copper levels (20 μM), COPT1 and 5 complement MPY17 (Yuan *et al.*, 2011), potentially supporting the proposed mechanism. Alternatively, OsSWEET11 might affect plasma membrane targeting efficacy, thereby increasing copper transport activity. It will be important to further characterize this phenomenon *in planta*.

Vacuolar SWEETs

The world's sugar supply comes from the vacuoles of sugarcane stems and sugar beet taproots. It is thus of critical importance to understand the interplay of metabolism and transport across the tonoplast. While 10 years ago little was known, now a large number of different sugar transporters on the tonoplast have been identified: proton cotransporters, likely for export of sugars (VGT1 and SUT4), and proton antiporters (TMT) and potential uniporters of the ERD-like family (Poschet

et al., 2011, Neuhaus 2007, Schulz *et al.*, 2011) (Figure 5.5). Most recently, homologs of the TMTs were identified as responsible for sucrose accumulation in sugarbeet (Jung *et al.*, 2015). Interestingly, clade IV SWEETs have been shown to also function as vacuolar hexose transporters (Guo *et al.*, 2014, Klemens *et al.*, 2013, Chardon *et al.*, 2013). Both AtSWEET 16 and 17 localize to the tonoplast, and AtSWEET17 is a key determinant of leaf fructose content. Despite this massive gain in knowledge, it remains a mystery how this complex suite of transporters is coordinated and how it supports transient accumulation of sugars, and what the key differences are between vacuoles that store low and high levels of sugars. An overview over activities and function of the various SWEETs has been published recently (Chen *et al.*, 2015).

Regulation of SWEETs

If SWEETs function as uniporters, which has not yet been formally demonstrated, one might speculate that it would be important to tightly regulate their activity to prevent uncontrolled leakage. One could even envisage that their activity is feedback-regulated by their substrates, coordinated with the activity of their partners, the SUTs in the adjacent cells or regulated by turgor pressure (Patrick & Stoddard 2010). Post-translational regulatory mechanisms are indicated based on the finding that serine residues in the C-terminus of AtSWEET11 were phosphorylated in response to sucrose addition to seedlings grown in liquid culture (Niittylae *et al.*, 2007). However, because the ectopic transcriptional induction by pathogens leads to sugar secretion, and AtSWEET9 overexpression led to an

increase in nectar volume, post-transcriptional control systems may not be essential for SWEET activity. The typically very long cytosolic C-terminus of the SWEETs may serve as a docking platform for protein interactions, however different from the AMTs, for which the C-terminus is highly conserved and serves as an allosteric trans-activation domain (Loqué *et al.*, 2007). SWEET C-termini show much less conservation and are characterized by extensive length variability.

SWEETs in the animal kingdom

Excitingly, SWEETs have also been identified in humans, and we showed that both a *Caenorhabditis elegans* and the human homolog mediate glucose transport (Chen *et al.*, 2010). In *C. elegans*, one of the seven SWEETs, Swt-1, was identified in a screen for mutants that accumulate lipids using Nile red staining, which could be a secondary effect of glucose accumulation (Ashrafi *et al.*, 2003). Mutation of the sea squirt homolog CiSWEET1/Ci-RGA led to early developmental defects, underlining the importance of SWEETs in metazoan (Hamada *et al.*, 2005). In *Drosophila*, SWEETs had been identified as genes expressed in embryonic salivary glands (Artero *et al.*, 1998). The human SWEET1 shows ubiquitous expression with highest expression in oviduct, epididymis and intestine as well as in β -cell lines. HsSWEET1 mainly localized to the Golgi of HEK293T cells (Chen *et al.*, 2010). Based on the localization and weak glucose efflux activity, we hypothesize that HsSWEET1 may play a role in the vesicular glucose efflux from β -cells, the intestine and liver cells (Chen *et al.*, 2010).

Summary and outlook

The identification of the large SWEET family as sugar transporters has led to the discovery of many elusive transport steps, including nectar secretion, phloem loading and post-phloem unloading as well as novel vacuolar transporters. Moreover, the human homologs likely play important roles in human physiology. The discovery of SWEETs also enabled the identification of bacterial SemiSWEETs. Atomic resolution structures of the SemiSWEETs have provided insights into the transport mechanism and will enable detailed structure–function analyses. Importantly, SWEETs play key roles in pathogen susceptibility and promise new ways to engineer robust broad-spectrum pathogen resistance. Future studies will be directed towards understanding the regulatory networks that integrate SWEET activity with metabolism and other transporters. Genetically encoded fluorescent sugar sensors targeted to various cellular compartments promise to provide insights into the distribution and the fluxes of sugars which in combination with the analysis of sugar transporter will help us better understand the important fundamentals of phloem loading and unloading in model and crop species. Understanding and fine tuning the fluxes between cells in leaves and seeds will likely be key for engineering crop yield potential.

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FIGURES

Figure 5.1

A phylogenetic tree of SWEET proteins from fifteen plant taxa focusing on the Angiosperms. Accessions were identified through BLAST on Phytozome, Ensembl Plants, NCBI, and CoGe. The SWEET lists were filtered to only include proteins containing two triple-helix-bundle repeats (MtN3 domains). An alignment was generated using default parameters on MUSCLE, and was then curated using Gblocks to exclude non-informative sites (Edgar 2004, Castresana 2000). The

maximum likelihood method PhyML was used to generate the phylogeny (Tamura *et al.*, 2013, Guindon *et al.*, 2010). CrSWEET1 from *Chlamydomonas reinhardtii* was used as an outgroup. See the Figure S5.1 and the Interactive Phylogenetic Tree online for individual protein names and bootstrap values.

Figure 5.2

SWEETs in sugar secretion. **(A)** Sugars can be exported from cells, for example in the nectary, by SWEETs. **(B)** SWEETs for the secretion of sucrose from the phloem parenchyma for phloem loading. Cell A secretes sucrose, which is subsequently

loaded (against a concentration gradient) into the sieve element companion cell complex (cell B) via a sucrose/H⁺ symporter SUT. Secondary active sucrose uptake is energized by the H⁺/ATPase. **(C)** SWEETs in secretion of sucrose from the seed coat to supply the developing endosperm and the embryo. Sucrose secreted from cell A is either taken up by SUTs or SWEETs into cell B, or after cleavage by a cell wall invertase (Inv-CW) taken up via clade I/II SWEETs or by monosaccharide transporters, e.g. of the STP family of hexose/H⁺ symporters. Note that formal uniport, as indicated by the arrows has not been formally demonstrated for the SWEETs.

Figure 5.3

Negative impact of mutation of ectopic expression of clade III SWEETs. Both the *sweet11;12* double mutant (Chen *et al.*, 2012) and overexpressor lines for *SWEET11* and *SWEET12* driven by the CaMV 35S promoter (Chen and Frommer, unpublished) show reduced growth.

Figure 5.4

Generic model for principal transport steps and pathways for phloem unloading in various tissues. Sucrose arriving at the phloem endings is either moving symplasmically via plasmodesmata or unloaded (from phloem or after moving symplasmically from post-phloem cells) via a transporter (PT); it can be taken up by sink cells using either a sucrose importer (U1), or by a hexose importer (U2) after cleavage by an extracellular invertase (Inv-CW). Imported sucrose can be catabolized to hexoses by a cytosolic invertase (Inv-N) or sucrose synthetase. Subsequently sucrose or hexoses can be transferred to the vacuole by sucrose or

hexose transporters on the tonoplast (V1, V2). The model represents a simplification of the phloem and postphloem unloading pathways.

Figure 5.5

Transporters for vacuolar uptake and efflux of hexoses and sucrose. Three types of sugar transporters have been identified on the tonoplast: proton symporters (SUT4 for sucrose and VGT1 for hexoses), proton antiporters (TMT for monosaccharides and TST for sucrose) and potential uniporters (ERD-like transporters and SWEETs) for hexoses.

Group IV

Group III

Group I

Figure S5.1.

Molecular Phylogenetic analysis by Maximum Likelihood method

Phylogenetic trees of the four clades represented in Figure 1. **(A)** Clade I **(B)** Clade II **(C)** Clade III **(D)** Clade IV. Fifteen plant taxa were included in the analysis: *Chlamydomonas reinhardtii* (Cr), *Amborella trichopoda* (Ambo), *Vitis vinifera* (Vv), *Arabidopsis thaliana* (At), *Medicago truncatula* (Mt), *Coffea canephora* (Cc), *Theobroma cacao* (Tc), *Eucalyptus grandis* (Eg), *Musa acuminata* (Ma), *Phoenix dactylifera* (Pd), *Zea mays*

(Zm), *Sorghum bicolor* (Sb), *Oryza sativa japonica* (Os), *Brachypodium distachyon* (Bd), and *Setaria italic* (Si). The evolutionary history was inferred by using the Maximum Likelihood method based on the Le and Gascuel (2008) model (Le & Gascuel 2008). The tree with the highest log likelihood (-25412.4774) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a LG model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories (+G, parameter = 1.3093)). The analysis involved 315 amino acid sequences. There were a total of 126 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013). Bootstrap values are located at each node and were calculated from 1000 replicates (Felsenstein 1985). SWEETs manually annotated are denoted with an * and may be pseudogenes.

CHAPTER 6: SWEET13a, SWEET13b, and SWEET13c function as sucrose effluxers during phloem loading in *Zea mays*

INTRODUCTION

I have highlighted in previous chapters the apoplastic phloem loading pathway which requires the transporter gene families *Sucrose transporter (SUT)* and *SWEETs* (Chapters 1-3 and Chapter). There have been a number of studies identifying *Sucrose transport1* as the protein responsible for import of sucrose into the companion cell in maize (Aoki *et al.*, 1999, Baker *et al.*, 2016, Carpaneto *et al.*, 2005, Slewinski *et al.*, 2010, Slewinski *et al.*, 2009). However, until recently only a single study in *Arabidopsis thaliana* has suggested that members of the *Sweet* family, *AtSweet11* and *AtSWEET12*, are responsible for the export of sucrose into the apoplast (Chen *et al.*, 2012). Recently, Bezruczyk *et al.* identified the *SWEET13a*, *SWEET13b*, and *SWEET13c* genes as the sucrose exporters functioning in the apoplastic phloem loading pathway (Bezruczyk *et al.*, 2018). It is also important to note that some plants, specifically trees, undergo symplasmic phloem loading which has been described previously (Chapter 1).

Here I confirm the SWEET transporters functioning in the phloem-loading pathway in maize through CRISPR-Cas9 mutagenesis. Additionally, quadruple mutants were generated that knock-out all known SUT and SWEET transporters involved in

phloem loading in maize. Surprisingly, these mutant individuals survive when grown in low light conditions, suggesting that there are additional transporters during phloem loading in maize, or maize employs the symplasmic phloem loading pathway to a higher degree than previously thought.

MATERIALS AND METHODS

Plant materials and growth conditions

T₀ maize plants were grown in the Sears greenhouse at the University of Missouri, and supplemented with high-pressure sodium lighting (1600 mmols m⁻² s⁻¹) under 16-hour light and 8-hour darkness. The plants were kept at a day-time temperature of 30°C and a night-time temperature of 24°C. Plants were also grown in field conditions at the University of Missouri South Farm Agricultural Experiment Station in Columbia, MO. The maize *sut1-m1* mutant described by Slewinski et al (2009) was genotyped as described previously (Slewinski *et al.*, 2009, Tran *et al.*, 2017).

Quadruple mutant *sut1-m1;sweet13a;sweet13b;sweet13c* plants were grown at the Botany greenhouse at the University of Missouri with no supplemental lighting from the months of November to February of 2019.

Expression data mining

Expression data for the tip and base of developing maize leaves was mined from publicly available RNA-seq data on maizegdb for the *ZmSWEET11a-15b* genes (Sekhon *et al.*, 2012, Sekhon *et al.*, 2011, Stelpflug *et al.*, 2016).

CRISPR-Cas9 sgRNA design

A multiple sequence alignment was performed with the coding sequencing of the Clade III maize *SWEET* genes using MUSCLE (Edgar, 2004). Conserved 5'-NGG-3' sites between the *SWEET13a*, *SWEET13b*, and *SWEET13c* genes to identify potential single guide RNA's (sgRNA) for CRISPR-Cas9 mutagenesis. Several sites were identified and narrowed to two 20 bp sgRNA's used for mutagenesis designated sgRNA1 and sgRNA2. The sgRNA1 sequence was completely conserved in all three genes. The predicted cut site was located in the recognition site for the *Taq*I restriction enzyme in the 5' region of the coding sequences. The sgRNA2 sequence was conserved between *SWEET13a* and *SWEET13c*, and contained a single mismatch in the 5' region on of *SWEET13b*. The predicted Cas9 cut site for sgRNA2 was located in the recognition site of the *Fat*I restriction enzyme, and was located in the middle of the coding sequences. sgRNA1 and sgRNA2 were cloned into the pBUE411 plasmid optimized for CRISPR-Cas9 mutagenesis in maize (Xing *et al.*, 2014). The design of the sgRNA's to have the predicted Cas9 cut sites occur in restriction sites allowed for identification of mutant alleles with >5bp insertion/deletion mutations through restriction fragment length polymorphism (RFLP) assays. Sanger sequencing was performed on T₀ plants to identify the specific mutations generated. T₀ plants containing either the sgRNA1-Cas9 or the sgRNA2-Cas9 construct were backcrossed to B73 inbred line for two generations before outcrossing to *sut1-m1* heterozygous plants in the B73 background. Field grown plants were screened for the sgRNA1-Cas9 and sgRNA2-Cas9 construct via Cas9 specific primers. All primers can be found in Table S6.1.

Starch Staining

Mature leaves of T₀ sgRNA1 and sgRNA2 plants were cleared and stained with iodine-potassium iodide as previously described (Baker & Braun, 2007).

Soluble sugar and starch quantification

Mature source leaf tissue was harvested from T₀ greenhouse grown plants at the end of night and immediately placed in liquid nitrogen followed by storage at -80°C until processing. Four, six, and three biological samples were measured for sgRNA1, sgRNA2, and empty vector control plants that also underwent *Agrobacterium tumefaciens* transformation and regeneration, respectively. Soluble sugar and starch sample extraction was performed as previously described (Leach & Braun, 2016). Samples were quantified via high-performance anion exchange (HPAE) chromatography against known standards (Leach *et al.*, 2017).

RESULTS

The maize SWEET gene family contains 21 members involved in a variety of transport functions. A 15-angiosperm phylogeny identified the Clade III maize *SWEETs* as the most closely related family members to the *Arabidopsis thaliana* *SWEET11* and *SWEET12* known to be involved in the apoplastic phloem loading pathway (Eom *et al.*, 2015)(Chapter 5). Since the maize leaf transitions from sink to source in a basi-petal fashion, *SWEETs* involved in apoplastic phloem loading would likely be expressed in the leaf tip at higher levels than in the base of a developing

leaf. Therefore, expression data for these tissues was mined from existing RNA-seq datasets for the Clade III SWEETs (Sekhon *et al.*, 2012, Sekhon *et al.*, 2011, Stelpflug *et al.*, 2016). Three of the eleven clade III SWEETs exhibit this expression pattern: *SWEET13a*, *SWEET13b*, and *SWEET13c* (Figure 6.1).

CRISPR-Cas9 mutagenesis was utilized to generate knockout alleles for each of the three SWEETs simultaneously with a one sgRNA. Two sgRNA-Cas9 constructs, sgRNA1 and sgRNA2, were independently transformed via *Agrobacterium tumefaciens* into maize Hi II embryos. Transformed callus tissue was sampled to screen for the presence of insertion/deletion mutations in the three *SWEET13* genes via RFLP assays for both sgRNA's. Interestingly, the two sgRNA's exhibited different efficiencies of mutagenesis with sgRNA1 generating mutations in more callus samples than sgRNA2 (Table 6.1). Mutated callus samples were selected for plant regeneration to increase the likelihood of obtaining T₀ plants with mutations of the *SWEET13* genes present in the germ-line.

T₀ plants containing the sgRNA1-Cas9 construct were severely delayed in their growth and exhibited chlorosis and anthocyanin accumulation in their mature leaves, and decreased plant vigor (Figure 6.2). However, they did grow to maturity and produce viable tassels and ears, albeit smaller than control plants. Sequencing confirmed these plants were *sweet13a;sweet13b;sweet13c* triple mutants. However, T₀ plants containing sgRNA2-Cas9 construct developed normally and did not display any chlorosis or anthocyanin accumulation (Figure 6.2). Sanger sequencing confirmed these plants were *sweet13a;sweet13c* double mutants, and did not result

in a mutation in *SWEET13b*. This is likely due to the mismatch in the target sequence between this gene and the sgRNA2 sequence.

Starch staining was performed on sgRNA1 and sgRNA2 T₀ leaves, and as expected, the sgRNA1 leaves exhibited starch hyper-accumulation in their leaves relative to control plants, while sgRNA2 leaves did not (Figure 6.3). Starch and soluble sugars were quantified and sgRNA1 plants contained significantly increased levels of sucrose, glucose, fructose, and starch relative to T₀ control plants (Figure 6.3). Interestingly, sgRNA2 plants had normal levels of glucose, fructose, and starch; however, they did exhibit a significant increase in sucrose levels relative to other T₀ control plants (Figure 6.3). The previous results strongly support the hypothesis that the *SWEET13a*, *SWEET13b*, and *SWEET13c* genes function as the sucrose exporters during phloem loading in maize.

sweet13a;sweet13b;sweet13c triple mutant plants containing the sgRNA1-Cas9 construct were outcrossed to *sut1-m1* heterozygote plants, and the resulting offspring were outcrossed to *sut1-m1/+* plants to generate *sweet13a;sweet13b;sweet13c;sut1-m1* quadruple mutants. Surprisingly, the quadruple mutants survived when grown in low light conditions; however, they are drastically reduced in their growth and development relative to wild-type, *sut1-m1*, and *sweet13a;sweet13b;sweet13c* plants (Figure 6.4). This observation suggests that there is novel unknown mechanism for phloem loading in mature maize leaves, such as the existence of additional transporters functioning in phloem loading, or a more predominant role for symplasmic phloem loading.

DISCUSSION

We hypothesized that members of the maize clade III *SWEET* transporter family function as sucrose exporters in the apoplastic phloem loading pathway due to their phylogenetic relationship with *Arabidopsis thaliana* *SWEET*s functioning in this capacity (Chapter 5). We confirmed this hypothesis by identifying and generating triple mutants of the *SWEET13a*, *SWEET13b*, and *SWEET13c* transporters.

Two sgRNA's were independently used to generate mutations in the *SWEET13* genes, which interestingly, we observed differing mutations frequencies between the two sgRNA's. The sgRNA1 sequence was completely conserved between all three *SWEET13* genes, while the sgRNA2 was conserved between *SWEET13a* and *SWEET13c* but contained a single mismatch at the 5' region of the sequence in *SWEET13b*. This likely explains the failure to generate mutations in the *SWEET13b* gene during callus screening. Additionally, there was decreased mutation frequency for both *SWEET13a* and *SWEET13c* in sgRNA2 samples relative to sgRNA1 samples even though both sgRNA's were conserved in these two genes. We hypothesize this could be due to the availability of the genomic DNA sequence to the Cas9 machinery *in vivo*. Steric hindrance by nucleosomes may not allow for the Cas9 machinery to access the sgRNA2 target sequence as easily as the sgRNA1 target sequence. Several recent studies have demonstrated this to be the case, and the importance of chromatin state when targeting sequence for CRISPR-Cas9 editing (Chen *et al.*, 2016, Chen *et al.*, 2017, Daer *et al.*, 2016, Hinz *et al.*, 2015, Horlbeck *et al.*, 2016, Isaac *et al.*, 2016, Verkuijl & Rots, 2019).

The *sweet13a;sweet13b;sweet13c* triple mutant T₀ plants exhibited the expected carbohydrate accumulation and visible plant phenotypes if these genes are responsible for sucrose export in the apoplastic phloem loading pathway. Bezruczyk et al independently confirmed these results (Bezruczyk *et al.*, 2018). Interestingly, while the *sweet13a;sweet13c* double mutant did not display any visible phenotypes or exhibit starch, glucose, or fructose accumulation, these plants did exhibit higher levels of sucrose. Therefore, the *SWEET13* genes are functionally redundant as a triple mutant is required to obtain the full carbohydrate partitioning defect. However, it appears that plants without all three functional proteins can still adequately load sucrose into the phloem, but appear to do so at decreased rates resulting in sucrose accumulation in the double mutant leaves.

The sgRNA1-Cas9 construct was outcrossed to B73 and subsequent generations were planted in the field under high light conditions. Under these conditions, *sut1-m1* mutants fail to survive longer than several weeks (Baker *et al.*, 2016).

Surprisingly, the *sweet13a,b,c* triple mutant plants were not only able to survive in the field, but also produced viable tassels and ears, albeit these reproductive structures were much smaller than wild-type siblings. Additionally, both the *sweet13a;sweet13b;sweet13c* triple mutant and the *sut1-m1* mutant phenotypes are less severe when grown under greenhouse conditions, and produce viable ears and tassels. Taken together, these observations suggest there may be additional transport pathways in maize phloem loading.

sut1;sweet13a;sweet13b;sweet13c quadruple mutant plants were generated to knock-out the known transporters in the apoplasmic phloem loading pathway in

maize. Again, these mutant plants were able to survive when grown in low light, short day conditions. However, the quadruple mutants were shorter than *sweet13a;sweet13b;sweet13c* triple mutants, *sut1-m1* single mutants, and wild-type plants, suggesting an additive effect on the quadruple mutant. Interestingly, the gradient observed in figure 6.4 may provide insights into the overall importance of phloem loading, retrieval, and unloading in the maize plant. *SWEET13a-c* are expressed in the mature phloem loading tip, as well as in the stele of maize roots (Stelplflug *et al.*, 2016). This suggests they are involved in both phloem loading and unloading. It has been shown that *SUT1* is not involved in phloem unloading, but is crucial for retrieval of leaked sucrose throughout the phloem transport pathway (Baker *et al.*, 2016). Therefore, we can conclude that in the quadruple mutants, phloem loading, retrieval, and unloading are impaired. However, in the *sweet13a-c* triple mutant phloem loading and unloading are impaired while retrieval of sucrose from the apoplast is still functional. It is possible that the triple mutant plants grow taller and aren't as developmentally delayed as the quadruple mutant for this reason. Sucrose likely builds to high levels in the mesophyll, bundle sheath, and phloem parenchyma cells resulting in leakage into the apoplast. The escaped sucrose is imported by *sut1* into these cells, as well as the companion cell. Therefore, there is still a small level of phloem loading occurring yet likely not as efficient as wild-type plants.

Additionally, in the *sut1* mutant, phloem loading and retrieval are impaired while unloading is still functional. Therefore it is interesting to speculate why these mutants do not display as severe of height and development defects as the triple or

quadruple mutants. One hypothesis is increased expression of *SWEET* and/or *SUT* genes in maize results in sucrose uptake into the companion cell from the apoplast. However, Bezruczyk et al did not observe increased expression of any clade III *SWEET*s in their *sweet13a;sweet13b;sweet13c* triple mutant plants so it is unlikely that any *SWEET*s or *SUT*s compensate for the lack of *sut1* (Bezruczyk et al., 2018). Alternatively, it is possible that maize utilizes both a symplasmic and apoplastic transport pathway, where the apoplastic pathway is the predominant route. This would allow for some phloem loading to occur, and due to the normal phloem unloading in the *sut1* mutant, allow for these plants to grow taller and display a weaker phenotype than the triple or quadruple mutant.

This model would explain the differences seen in field conditions where *sut1* displays a severe, seedling lethal phenotype as it relies strictly on symplasmic phloem loading compared to the *sweet13a-c* triple mutant, where leakage and phloem loading still occur and symplasmic unloading is functional. Therefore, it may be that crops such as maize required the adoption of apoplastic phloem loading to accommodate high levels of photosynthate production and transport, which symplasmic transport could not maintain without stress and ultimately death overcoming the plant.

Much of this is speculation based on preliminary genetic experiments and must be validated with future studies. RNA-seq analysis on the mature leaf tissue of the quadruple mutant, triple mutant, single mutant, and wild-type plants will be required to detect increased transcript levels in plants with knocked-out or decreased apoplastic phloem loading relative to wild-type plants. Additionally,

CFDA transport studies on the mutant gradient may provide insight concerning potential changes in symplasmic continuity between the phloem and surrounding cells. These are simply two potential future experiments that will shed additional light on the phloem loading pathway in maize, and potentially other apoplastic phloem loading plants.

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TABLES

Table 6.1

CRISPR-Cas9 mutation frequencies based on RFLP callus assays

	sgRNA1	sgRNA2
<i>SWEET13a</i>	38%	6%
<i>SWEET13b</i>	19%	0%
<i>SWEET13c</i>	29%	24%

Mutation frequencies are shown for sgRNA1 and sgRNA2 for *SWEET13a*, *SWEET13b*, and *SWEET13c*. The percentages are based on restriction fragment length polymorphism (RFLP) assays performed on gDNA isolated from positively selected callus tissue grown on biolaphos plates.

Table S6.1

Genotyping primer sequences

Primer Name	Primer Sequence 5'-3'
13a_RFLP_F1	TCGTATAACAGACCGACGTT
13a_RFLP_R1	GTTTTATACGCGTGCTCAGG
13b_RFLP_F1	GATTGATCCTTGCGCCA
13b_RFLP_R1	AACACTATAGATGCTACGGT
13c_RFLP_F1	TTGAAATTATGCAGACCGAC
13c_RFLP_R1	AGATGCATGGTGCTGCT
ZmCas9-F1	GCCATCCTCCTGTCTGATATT
ZmCas9-R1	GTTGTCCAGGAAGTCCTTATCC

FIGURES

Figure 6.1

RNA-seq expression data was mined from the maizegdb expression atlas for the clade III maize SWEETs in a developing leaf. The tip of the leaf represents photosynthetic source tissue and the base represents non-photosynthetic sink tissue.

Figure 6.2

A. sgRNA1 T₀ plants exhibited delayed growth compared to sgRNA2 T₀ plants. B. sgRNA1 plants exhibited chlorosis and anthocyanin accumulation in the margins of mature leaves.

Figure 6.3

sgRNA1 T_0 plants hyper-accumulate starch and soluble sugars relative to sgRNA2 and control T_0 plants. A-C Starch staining of control (A), sgRNA2 (B), and sgRNA1 (C) mature leaves harvested at the end of night. Starch, sucrose, glucose, and fructose content in sgRNA1, sgRNA2, and control leaves at the end of night. Values are means $\pm$ SE and significantly different values at $P \leq 0.05$ are signified by different letters.

Figure 6.4

The *sweet13a,b,c;sut1* quadruple mutant (far left) is developmentally delayed and exhibits decreased height relative to its *sweet13a,b,c* triple mutant (second from left), *sut1* single mutant (second from right), and wild-type (far right) siblings.

Plants were imaged at 7wk (A), 12wk (B), 14wk (C), and 15wk (D) after planting

CHAPTER 7: Conclusions and future directions

The studies presented in the previous chapters contribute to the field's current understanding of the genes regulating maize carbohydrate partitioning. Forward genetic studies on the maize mutants *carbohydrate partitioning defective28* (*cpd28*) and *carbohydrate partitioning defective47* (*cpd47*) identified the COBRA family member *Brittle Stalk2-Like3*, and demonstrated its role in primary cell wall development and carbohydrate partitioning. To date there has been no report of a COBRA family member influencing carbohydrate partitioning and the *cpd28* and *cpd47* mutations provide a model into the mechanism in which improper cell wall development results in decreased sucrose transport. Additionally, characterization of the *Carbohydrate partitioning defective1* maize mutant has demonstrated the importance of regulation of callose deposition during sieve plate formation. Identification of the causative mutation will provide insight into the genes regulating callose deposition and/or degradation during sieve plate and lateral root formation. Finally, using CRISPR/Cas9 mutagenesis I demonstrated the *SWEET13a*, *SWEET13b*, and *SWEET13c* genes function in sucrose efflux in apoplastic phloem loading in the mature maize leaf. In the following section I provide potential directions where this research can be built upon to further understand the genetic control of carbohydrate partitioning.

Analysis of the relationship between cell wall development and carbohydrate partitioning

The maize mutants *Cpd1*, *cpd28*, and *cpd47* disrupt the proper development of cell wall composition, whether that be decreased cellulose in cell walls or the unregulated deposition of callose in sieve elements. These mutants provide an interesting connection between cell wall development and whole plant carbohydrate partitioning. However, further studies are required to fully understand this relationship.

Ongoing transgenic experiments to clone the causative mutation resulting in the *Cpd1* phenotype will provide insight into the specific mechanism resulting in the increased callose deposition. The mutation has been mapped to three candidate genes: two of unknown function and a nucleoredoxin with preliminary results suggesting localization to the nucleus, ER, and cytoplasm (Chapter 4). While we cannot speculate as to the function of the unknown proteins, it is interesting to speculate how a nucleoredoxin is regulating callose deposition in the sieve elements, specifically in the development and maturation of sieve plates (described in Chapters 3 and 4).

Nucleoredoxins are members of the redoxin superfamily of oxidoreductases involved in the redox regulation of a variety of enzymes and cellular processes, including development (Delorme-Hinoux *et al.*, 2016). Benitez-Alfonso *et al.* identified the thioredoxin *GFP Arrested Trafficking1* (*GAT1*), which regulated callose deposition at plasmodesmatal connections in *Arabidopsis thaliana* (Benitez-Alfonso *et al.*, 2009). It is possible the mutated nucleoredoxin results in constitutive redox

signaling resulting in the continuous deposition of callose at the sieve plate.

Alternatively, it is possible that the mutation results in the constitutive down-regulation of the callose degradation machinery leading to continued build-up of callose in the sieve element. These hypotheses are all speculation and completion of the transgenic analyses described in Chapter 4 is required. Regardless, identification of the *Cpd1* mutation and the mechanism by which callose deposition is regulated provides novel insights concerning the genetic control of sieve plate and lateral root formation.

I have presented strong evidence that decreased cellulose content, likely in the primary cell walls, in *cpd28* and *cpd47* results in the decreased export of sucrose from mature leaves (Chapter 2). The mechanism by which this occurs is currently unknown; however, detailed microscopic analysis may provide an answer. Electron microscopy will examine the plasmodesmatal connections between cells in the mature leaf tissue. Altered PD function and/or frequency is likely the cause for decreased sucrose export in the *carbohydrate partitioning defective33* maize mutant (see Appendix B). Therefore, it is possible decreased cellulose content alters development of the primary cell wall, impairing PD development in *cpd28* and *cpd47*. In support of this hypothesis, cellulose and pectin are involved in the stabilization of the plasma membrane with the cell wall in PD (Brecknock *et al.*, 2011). Observation for any abnormalities in the cell wall of phloem tissue in the mutant relative to wild-type samples will also be possible with electron microscopy. It is possible that the structural integrity of the sieve element cell wall is impaired, and no longer able to withstand the high turgor pressure required for bulk flow of

phloem sap (Lalonde *et al.*, 2003, Patrick, 2012). This hypothesis will be tested using Atomic Force Microscopy (AFM) to probe the phloem cell walls of mutant and wild type individuals to test their structural integrity (Torode *et al.*, 2018). *cpd28* and *cpd47* provide an exciting model to probe the relationship between cell wall development and whole plant carbohydrate partitioning. It will be interesting to perform carbohydrate partitioning studies on previously described mutants defective in primary cell wall development to determine if they exhibit previously unreported defects, or is this connection specific to *cpd28*, *cpd47*, and the COBRA gene *Brittle Stalk2-Like3*.

The use of CRISPR/Cas9 technology to further dissect the phloem loading, transport, and unloading pathways

I hypothesized the SWEET13a, SWEET13b, and SWEET13c transporters function as the sucrose effluxer during apoplastic phloem loading in maize based on phylogenetic (Chapter 5) and gene expression (Chapter 6) analyses. I confirmed this hypothesis through CRISPR/Cas9 mutagenesis resulting in a triple *sweet13a*, *sweet13b*, and *sweet13c* knockout (Chapter 6), and these results were independently confirmed by Bezruczyk *et al.* (Bezruczyk *et al.*, 2018). Interestingly, the triple *sweet13* mutant survived suggesting the potential for an additional phloem loading mechanism in maize. To further test this idea I generated the *sweet13a*; *sweet13b*; *sweet13c*; *sut1* quadruple mutant. Surprisingly, when grown under low light conditions the plants survive and reach reproductive maturity though all known sucrose transporters involved in apoplastic phloem loading were knocked out.

Therefore, this quadruple mutant provides a model to further study the potential role of symplasmic phloem loading in maize, and/or the presence of unknown or compensating sucrose transporters.

Phloem specific RNA-sequencing analysis (protocol for this outlined in Appendix A) is required from the series of mutants (quadruple *sweet13; sut1*, triple *sweet13*, and single *sut1* mutants) as well as wild-type siblings to test the hypothesis that other transporters are functioning in the pathway. Additionally, dye tracer studies may provide further insight into the potential of increased symplasmic transport.

Carboxyfluorescein Diacetate (CFDA) is a non-fluorescent compound that is cleaved upon entering a cell, resulting in fluorescent CF that is limited to symplastic transport (Wright & Oparka, 1996). Careful application of CFDA to the mesophyll and/or bundle sheath cells followed by observation for presence/absence of the fluorescent CF in the phloem basal to the application site could provide evidence of increased symplasmic transport in mutant plants. Electron microscopy could determine if there are differences in the plasmodesmatal structure or frequency, similar to studies proposed for *cpd28* and *cpd47*, between the Companion Cell and Phloem Parenchyma in the quadruple mutant.

The quadruple *sweet13; sut1*, triple *sweet13*, and single *sut1* mutant series provides additional genetic resources to further probe the phloem loading, transport, and unloading pathways as discussed in Chapter 6. Additionally, CRISPR/Cas9 can be adapted to specifically mutate desired genes in a tissue specific pattern to separate confounding factors and pleiotropic phenotypes. For example, the *SWEET13* genes function in phloem loading, but likely are involved in phloem unloading in the root

based on their expression profiles (Sekhon *et al.*, 2011, Stelpflug *et al.*, 2016).

Therefore, an either root or shoot apical meristem specific promoter driving Cas9 expression will generate root or shoot specific mutations. This strategy would theoretically result in a chimeric plant with functional SWEET13 transporters in either the root or shoot while knocking out the transporters in the opposite tissue, allowing for the specific study of *SWEET13* function in either phloem loading or unloading. It is easy to imagine this system being deployed to drive tissue specific mutagenesis, removing pleiotropic effects common to many previously characterized mutants in maize and other systems. The current limitation to this strategy is the lack of known tissue specific promoters for the many tissue and cell groups in maize; however this likely is a short -term limitation as more detailed RNA-seq expression analyses are routinely performed.

In summary, this thesis research has contributed novel knowledge and genetic tools advancing the current understanding of carbohydrate partitioning in maize. It is expected that further discoveries and innovations continue to build on this foundation. Ultimately, the knowledge obtained here, and in the future, will directly contribute to increased plant yield and resistance to abiotic and biotic stresses, increasing our ability to feed the growing population.

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APPENDIX A: A Protocol for Freeze-Substitution Acetone Fixation and Polyester Wax Embedding of Mature Maize Leaf Tissue for Laser Capture Microdissection and RNA-Sequencing

INTRODUCTION

Vascular tissues, or veins, are responsible for the long distance transport of water, minerals, carbohydrates, hormones, and a number of other compounds required for proper growth and development. Veins are composed of two groups of cells, the xylem and the phloem. Xylem tissue is responsible for the transport of water and minerals taken in by the roots to the various shoot tissues in the plant. The phloem is responsible for the long distance transport of carbohydrates, hormones, and other compounds from source tissues, such as mature photosynthetic leaves, to developing sink tissues, such as roots, reproductive organs, and developing leaves. Maize leaves exhibit Kranz anatomy where the vascular tissues are embedded in a concentric ring of bundle sheath cells, surrounded by mesophyll cells (Esau, 1977). As a result, the vascular tissues are difficult to access as they are embedded in the middle of the leaf.

In mature maize leaves sucrose is synthesized in the mesophyll and bundle sheath cells, and must be mobilized into the Companion Cell-Sieve Element (CC-SE)

complex for long-distance transport to various sink tissues (Lunn & Furbank, 1999, Slewinski & Braun, 2010). This is accomplished via the apoplastic phloem loading pathway highlighted in previous chapters. Due to the unique function of phloem in transport of carbohydrates and other compounds, as well as the unique CC-SE complex, there are likely genes that exhibit phloem specific, or phloem enriched expression patterns. One such example in *Arabidopsis thaliana* is the *AtSUC2* gene, which is strictly expressed in phloem tissue (Stadler & Sauer, 1996, Truernit & Sauer, 1995). It is crucial to develop a method in which phloem tissue can be specifically isolated from surrounding cells while maintaining RNA integrity to identify genes specifically expressed in the phloem.

Mammalian studies have long shown that Laser Capture Microdissection (LCM) results in the isolation of specific cells from sectioned tissue (Emmert-Buck *et al.*, 1996, Simone *et al.*, 1998). This technique has been coupled with RNA extraction and sequencing to generate cell-type specific RNA datasets in a variety of plant tissues (Chavan *et al.*, 2018, Kerk *et al.*, 2003, Li *et al.*, 2010, Nakazono *et al.*, 2003, Ohtsu *et al.*, 2007, Tang *et al.*, 2006). However, these protocols are not often performed on mature leaves due to the difficulty of fixative penetration and issues with RNA degradation. LCM was performed on maize leaf tissue to identify genes enriched in the vasculature by isolating vascular bundles, including the bundle sheath cells, from surrounding mesophyll and epidermal tissue (Nakazono *et al.*, 2003). However, the authors were unable to separate the xylem, phloem, and bundle sheath cells to identify phloem specific or enriched genes, likely due to the fractioning cells and tissue sections during cryofixation (Kerk *et al.*, 2003). Li *et al.*

also describe a method in which they fixed and embedded tissue from maize leaf without cryofixation and performed LCM-RNA-seq (Li *et al.*, 2010). However, I was unable to obtain high quality RNA in the mature maize leaf tissue using this protocol. An adaptation to the Li et al protocol for RNA fixation in mature leaves is reported in Protocol 1 with an added freeze substitution step followed by embedding the tissue in polyester wax (steedman's wax). The embedded tissue was then sectioned utilizing a previously described protocol (Chavan *et al.*, 2018). Intact, high quality RNA was extracted from tissue sections with the Arcturus Paradise Plus RNA extraction and isolation kit. This method will result in the clean capture of desired cells in mature maize leaf tissue through LCM.

PROTOCOL 1

In order to perform LCM, tissue must first be fixed to maintain the histology and RNA quality of the tissue. Once fixed the desired tissue must be embedded in a solid media, often paraffin or steedman wax blocks, followed by sectioning to generate samples to view under the microscope. The tissue sections then undergo a dewaxing step before the desired cells can be captured via LCM and their RNA extracted. This results in a lengthy period of time from initial tissue harvest to RNA extraction, requiring adequate fixation of the tissue. In the following protocol the tissue is fixed with -80°C acetone. Acetone is used as the preferred fixative as it has a low freezing temperature (-95°C) and is still liquid at -80°C, and the low temperature inhibits RNase activity. The acetone is moved from -80°C to 4°C resulting in slow warming of the tissue, and penetration of the acetone into the leaf tissue without RNA

degradation. Additionally, fixation with pure acetone results in improved RNA extraction in later steps relative to cross-linking fixatives such as formaldehyde-acetic acid-ethanol (FAA) (Nakazono *et al.*, 2003).

Following fixation, the samples undergo a series of ethanol dehydration steps followed by infiltration with paraffin or polyester wax. Polyester wax is used here due to its lower melting temperature (38°C-40°C) relative to paraffin (60°C), reducing the possibility of RNA degradation during the embedding and dewaxing steps (Chavan *et al.*, 2018, Evers *et al.*, 2011). Embedded tissue blocks can be stored for several months at -20°C before and after sectioning, allowing for multiple uses of the same tissue (Chavan *et al.*, 2018, Kerk *et al.*, 2003).

Materials

Diethyl pyrocarbonate (DEPC)-treated water

RNase AWAY (Thermo Fisher Scientific)

Mature maize leaf tissue

ACS Certified Acetone (Fisher Scientific)

Polyester Wax (Electron Microscopy Sciences)

1:1 EtOH/polyester wax solution

200 proof Ethanol (EtOH) (Decon Laboratories)

70% EtOH/30% DEPC solution

95% EtOH/5% DEPC solution

Autoclave

Oven

Slimsette plastic biopsy cassettes (Thermo Scientific)

Aluminum foil

Forceps (4.5 in)

Glass plate

Pencil

Single-edge razor blades

Sealed chamber and vacuum pump

2 250mL glass beakers

1 500mL glass beaker

1 2000mL glass beaker

1 250mL glass graduated cylinder

40°C incubator

-80°C freezer

Drierite

Sealable plastic bag

Kimwipes

70mm diameter aluminum weighing dish (Ted Pella, Inc.)

Preparation

1. Wrap all metal and glassware in aluminum foil and bake in oven overnight to sterilize and confirm all are RNase free. Store at room temp until ready for use.

2. Pour acetone into both 250mL beakers and cover with aluminum foil. Place in -80°C freezer overnight, or until ready for use.

Tissue fixation

1. Spray benchtop, razors, forceps, slimsette cassette, and anything else that may come into contact with the leaf tissue with RNase Away.
2. Bring one 250mL beaker with -80°C acetone out of -80°C freezer and place in vacuum container surrounded by ice.
3. Cut piece of tissue from one half of the maize leaf, excluding the mid-vein. Immediately dip cut tissue in cold acetone and section on RNase-free glass into pieces 1mm in length or less. (Figure A.1)
 - a. This is crucial as tissue sections > 1mm will not fix as well and have decreased RNA quality at the end of the protocol.
4. Place tissue sections into slimsette plastic biopsy cassette and place cassette in acetone. (Figure A.1B)
 - a. Steps 2-4 should be completed as quickly as possible to reduce chance of RNA degradation and should take no longer than 5 minutes total.
5. Place vacuum container filled with ice and 250mL beaker of cold acetone in 4°C and pull vacuum for 45 minutes.
 - a. At the end of the 45 minutes there should no longer be air bubbles coming from tissues. If this is not the case then continue to pull the vacuum for 15 min or until air bubbles cease.

6. Remove the cassette containing the leaf sections with RNase-free forcep and transfer to fresh 250mL acetone in -80°C freezer.
7. Store samples in acetone in -80°C freezer overnight, or indefinitely until ready to proceed with tissue embedding.

Tissue embedding

1. Place 500-1000g polyester wax into RNase-free 2000mL beaker and place in 40°C incubator the day before beginning tissue embedding protocol.
 - a. Once wax is completely melted make 1:1 EtOH:polyester wax solution in 500 mL RNase-free beaker to provide enough time for solution to become homogenous before use. Store in 40°C incubator.
2. Remove samples submerged in acetone from -80°C freezer and place in 4°C for 30 minutes.
3. Replace acetone with cold 70% EtOH and incubate at 4°C for 30 minutes
 - a. Use 200 proof EtOH and DEPC water to make ethanol solutions.
4. Replace 70% EtOH with 95% EtOH and incubate at 4°C for 30 minutes.
5. Replace 95% EtOH with 100% EtOH and incubate at 4°C for 30 minutes.
6. Repeat previous step 2X.
7. Replace 100% EtOH with 1:1 EtOH:polyester wax solution and place in 40°C incubator overnight.
8. The next day replace 1:1 EtOH:polyester wax solution with pure melted polyester wax and incubate for 2 hours at 40°C.
9. Repeat the previous step 2x.

10. Before embedding, be sure that wax in 2000mL beaker and the wax samples are submerged in are completely clear to ensure easier embedding. (FIGURE A.2)
 - a. If the wax is cloudy then increase the temperature by 1-2°C and give time for wax to clear.
11. Pour clear polyester wax into the RNase-free 70mm diameter aluminum tray and quickly place leaf sections flat on the bottom of the tray before the wax begins to solidify. (Figure A.3)
 - a. It is crucial to quickly place the tissue so that upon solidification the tissue can be seen in the wax block in preparation for microtome sectioning.
 - b. 4 sections can be placed per aluminum tray distributed evenly apart from each other in 4 quadrants to allow for block preparation for microtome sectioning.
 - c. Put remaining melted polyester wax back into 40°C incubator for use during block mounting.
12. Allow to wax to solidify at room temperature before placing in labeled Ziploc bag containing Drierite. Place samples in 4°C for up to 3 months.
13. Place plastic cassettes into -20°C freezer for use during block mounting for microtome sectioning.
14. Remove block from aluminum tray and split into four equal quadrants, with each quadrant containing a leaf section.

- a. Be sure to perform this on an RNase-free surface using RNase-free razors
15. Trim each quadrant to a rectangle roughly 1.5-2x the width of the leaf tissue with the tissue in the center. Also trim the block so that the leaf tissue is roughly 1-3mm from the front of the block and roughly 1.5-2 cm from the back. (Figure A.3)
- a. Be careful while trimming the wax as it is likely to shatter if not done slowly and a little bit at a time.
 - b. Ensure that the front and back edges of the block are as flat as possible to assist during block mounting.
16. Remove plastic cassette from -20°C freezer and pour melted polyester wax onto face of cassette.
17. Quickly place one of the wax blocks onto the pool of polyester wax with the back edge contacting the plastic cassette.
18. Allow the wax to solidify and reinforce the attachment of the wax block to the cassette by applying additional melted polyester wax on all four sides of the wax block. (Figure A.3)
19. Store mounted blocks in Ziploc bag with Drierite at 4°C until microtome sectioning.

Slide preparation

Before proceeding with laser capture microdissection (LCM) the tissue must be sectioned and placed onto polyethylene naphthalate (PEN) membrane slides.

Chavan et al describe a method in Basic Protocol 2 to section and adhere tissue samples to PEN slides while minimizing RNA degradation (Chavan *et al.*, 2018).

10mm thick sections were used in this study on maize leaf tissue.

Laser Capture Microdissection

Laser capture microdissection was performed using Applied Biosystems® ArcturusXT system in the Cytology Core at the University of Missouri – Columbia on test sections to confirm phloem bundles can be cleanly captured without contamination from surrounding cells (Figure A.4). Please see the manufacturer’s user guide for details on running the system. The protocols described above are also applicable when using other LCM systems.

RNA extraction and isolation

RNA extraction from the captured tissue will be performed with the Arcturus Paradise Plus RNA Extraction and Isolation Kit (ThermoFisher). Alternatively, before performing LCM, RNA extraction of whole tissue scrapes (protocol included in kit) can be performed to verify that the tissue was fixed and embedded properly and the RNA is of high quality. This is a recommended checkpoint to test before continuing with LCM. RNA was extracted via the tissue scrape protocol from mature maize leaf tissue fixed, embedded, and sectioned by the protocols stated above, and was tested on the Advanced Analytical Fragment Analyzer at the DNA Core at the University of Missouri – Columbia. The RNA was of sufficient quality (RQN score of 6.1) to perform RNA-seq.

COMMENTARY

LCM-RNA-seq has been utilized a number of times in plant and animal systems. However, in all cases, these tissues are 1) easily penetrable by fixative solutions, 2) the vascular bundles are unable to be separated due to fractioning with cryofixation, or 3) utilize equipment unavailable at many institutions. Therefore, it is difficult to obtain high quality, intact RNA for LCM-RNAseq in mature maize leaf tissue. Li et al published a procedure for LCM-RNA-seq in developing maize leaves; however, using this protocol on mature maize leaf tissue did not result in high quality, intact RNA (Li *et al.*, 2010). Therefore, adaptations were made to include a freeze substitution step to improve RNA fixation and quality described in Protocol 1. Additionally, tissue was embedded in polyester wax due to its lower melting temperature, thereby decreasing the risk of RNA degradation. This protocol followed by the Chavan et al sectioning protocol, LCM, and RNA extraction will result in the generation of cell type specific RNA-seq data sets in maize leaf and vasculature (Chavan *et al.*, 2018). This in turn will lead to the identification of novel genes responsible for the development of xylem, phloem, bundle sheath cells, and any other desired group of cells in maize leaf as well as other tissues. Development of new tools, such as phloem specific markers, will be possible, significantly contributing to future maize research. Additionally, this protocol can be adapted for use in any application where tissue must be fixed and embedded while maintaining RNA quality, such as RNA in situ hybridization experiments.

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FIGURES

Figure A.1

Mature leaf with tissue excised for sectioning and acetone fixation (left). Excised tissue sectioned for extraction and placed in slimsette plastic biopsy cassette for submersion in -80°C acetone (right).

Figure A.2

70mm diameter aluminum tray (left) for tissue embedding. Completely melted polyester wax in RNase-free beaker for tissue embedding.

Figure A.3

Wax block removed from 70mm diameter aluminum tray (left). The block was trimmed around the tissue resulting in a small block (middle). The trimmed block was mounted to a slimsette plastic biopsy cassette and attached with solidified polyester wax (right).

Figure A.4

Mature maize leaf tissue before (top) and after (bottom) laser capture microdissection of the phloem bundle (red arrow) of a lateral vein.

APPENDIX B: Maize *Carbohydrate partitioning defective33* localizes to plasmodesmata and functions in sucrose export from leaves

Note: The information in this chapter was submitted to publish under the title:

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Contributions: TMT and DMB participated in the design of the study and drafted and critically revised the manuscript. TMT performed the phylogenetic analysis, starch staining, soluble sugars and starch measurements, measurements of photosynthesis, gas exchange and chlorophyll content, radioactively labeled sucrose transport assays, morphology and histochemistry analyses, DANS assays, and statistical analyses. TLS, SB, CW, NS, and RFB isolated the mutants, performed genetic complementation assays, and performed initial mapping studies. TMT, PC, RW, JP, KG, and DMB conducted the fine-mapping. TMT and BTJ isolated the gene. TMT and TM conducted the subcellular localization analyses and BiFC assays.

ABSTRACT

To sustain plant growth, development, and ultimately crop yield, sucrose must be

transported from its site of synthesis in the leaves to distant parts of the plant, such as seeds and roots. To identify genes that regulate sucrose accumulation and transport in maize (*Zea mays*), we isolated *carbohydrate partitioning defective33* (*cpd33*), a recessive mutant that accumulated excess starch and soluble sugars in the mature leaves. The *cpd33* mutants also exhibited chlorosis in the leaf blades, greatly diminished plant growth, and reduced fertility. We cloned the *Cpd33* gene and confirmed its identity by characterizing six independent mutant alleles. The *Cpd33* gene encodes a protein containing multiple C2 domains and transmembrane regions. Subcellular localization experiments showed the CPD33 protein localized to the plasmodesmata (PD) and the plasma membrane. We also found that a loss-of-function-mutant of the CPD33 ortholog in Arabidopsis, QUIRKY, had a similar carbohydrate hyperaccumulation phenotype. Radioactively labeled sucrose transport assays showed that sucrose export was significantly lower in *cpd33* mutant leaves relative to wild-type leaves. However, leaf architecture, vein anatomy, and PD transport in the adaxial-abaxial direction were unaffected in *cpd33* mutant leaves. Collectively, our results suggest that CPD33/QKY functions to promote sucrose export into the phloem.

Keywords: carbohydrate accumulation, maize, phloem, sucrose, *carbohydrate partitioning defective33*, *cpd33*

INTRODUCTION

Whole-plant carbohydrate partitioning is the process by which sugars are transported from their synthesis site (source leaves) to distal parts of the plant (sink tissues). In maize (*Zea mays*), sucrose is the soluble carbohydrate that is transported long-distance through the phloem (Zimmermann, 1975; Julius et al., 2017). Phloem loading of sucrose in maize source leaves includes both symplastic and apoplastic steps (Slewinski and Braun, 2010a). Sucrose is synthesized in mesophyll cells and then moves symplasmically through plasmodesmata (PD) into the bundle sheath cells and phloem parenchyma cells (Evert et al., 1978; Braun and Slewinski, 2009). From there, it is effluxed into the apoplast via SWEET sucrose transporters prior to import into the companion cell–sieve element complex by SUCROSE TRANSPORTER1 (ZmSUT1) (Evert et al., 1978; Aoki et al., 1999; Carpaneto et al., 2005; Braun and Slewinski, 2009; Braun et al., 2014; Eom et al., 2015; Baker et al., 2016; Bezruczyk et al., 2018). After being loaded into the phloem, sucrose is translocated to sink tissues (Bihmidine et al., 2013; Julius et al., 2017).

Symplastic transport is mediated by plant specific structures called PD, plasma membrane (PM)–lined cytoplasmic channels that connect adjacent cells. These structures are essential for plant growth and development, transporting nutrients, metabolites, and signaling macromolecules cell-to-cell (Tilsner et al., 2016). PD permeability is controlled by some PD-associated proteins and the deposition and degradation of callose, a β -1,3-glucan polymer, around the neck of PD (Thomas et al., 2008; Lee et al., 2011; Han et al., 2014; Kitagawa and Jackson, 2017). Modifications in PD frequency or structure can influence symplastic transport (Provencher et al.,

2001; Kobayashi et al., 2007; Chen and Kim, 2009; Burch-Smith et al., 2011; Xu et al., 2012; Han et al., 2014).

To date, six maize mutants that function in carbohydrate partitioning have been reported: *sucrose export defective1 (sxd1)*, *tie-dyed1 (tdy1)*, *tie-dyed2 (tdy2)*, *psychedelic (psc)*, *sucrose transporter1 (sut1)*, and *Carbohydrate partitioning defective1 (Cpd1)* (Russin et al., 1996; Botha et al., 2000; Braun et al., 2006; Slewinski et al., 2009; Slewinski and Braun, 2010b; Slewinski et al., 2012; Baker et al., 2016; Julius et al., 2018). Of these six mutants, four show impairment of the PD-mediated symplastic transport steps involved in trafficking sucrose to the phloem. The first gene, *Sxd1*, encodes tocopherol cyclase, an enzyme that functions in the vitamin E biosynthesis pathway. The *sxd1* mutant causes vitamin E deficiency, resulting in ectopic callose deposition, blocking PD at the interface of the bundle sheath and vascular parenchyma cells, and inhibiting sucrose export from the leaf (Russin et al., 1996; Botha et al., 2000; Provencher et al., 2001). The connection between interrupted tocopherol cyclase activity and callose deposition is unclear, but the role of tocopherols in phloem loading is conserved among different plant species (Sattler et al., 2003; Hofius et al., 2004; Maeda et al., 2014). The second gene, *Tdy2*, encodes a callose synthase and is highly expressed in the vascular tissue of developing leaves. In the *tdy2* mutant, symplastic sucrose transport from the companion cell into the sieve element is reduced (Slewinski et al., 2012). The third gene, *Tdy1*, encodes a novel transmembrane protein with unknown function (Slewinski et al., 2008; Ma et al., 2009); however, genetic and transmission electron

microscopy studies suggest it functions in the same pathway as *Tdy2* and separate from *Sxd1* (Baker and Braun, 2008; Ma et al., 2008; Baker et al., 2013). In addition, *Cpd1*, a semi-dominant mutant, has ectopic callose deposits in the sieve elements, which likely inhibit sucrose symplastic transport through sieve plates (modified PD); the gene encoding *Cpd1* is unknown (Julius et al., 2018).

In this report, we characterized *carbohydrate partitioning defective33* (*cpd33*), a novel maize mutant that hyperaccumulates carbohydrates in its leaves. We cloned the gene responsible for the *cpd33* mutant phenotype and found that *Cpd33* encodes a multiple C2 domain and transmembrane region protein, localizes to the PD and PM, and potentially functions to promote sucrose export into the phloem.

RESULTS

***cpd33* mutant leaves hyperaccumulate carbohydrates**

The *cpd33* mutant was isolated from an ethyl methanesulfonate (EMS)-mutagenized population (Table S1) (Liu et al., 2010; Julius et al., 2018). Outcrossing experiments and segregation ratios in the F1 and F2 generations showed that the *cpd33* mutant was recessive. In addition, we identified five other alleles of *cpd33* (*cpd36*, *cpd52*, *cpd63*, *cpd82*, *cpd93*) based on their similar mutant phenotypes (Fig. S1) and genetic complementation testing.

The *cpd33* mutant leaves are chlorotic and show anthocyanin accumulation, especially at the tip of the leaf blade (Fig. 1A). To test if these leaf phenotypes were associated with carbohydrate hyperaccumulation, the leaves were collected at the end of the night, cleared of photosynthetic pigments, and stained for starch using iodine-potassium iodide (IKI) (Ruzin, 1999). The *cpd33* mutant leaves stained a dark brown color, indicating high starch content, whereas the wild-type leaves contained little starch (Fig. 1B).

To test if soluble sugars as well as starch hyperaccumulated in *cpd33* mutant leaves, we quantified the sugar content of mature leaves from 8-week-old field grown plants collected at the end of the night. Sucrose, glucose, fructose, and starch contents were significantly higher in mature leaves from *cpd33* mutants than in wild-type leaves (Fig. 1 C, D), consistent with the starch staining results. In addition, histological analyses showed the bundle-sheath cells of *cpd33* mutant leaves contained abundant starch compared to the wild-type leaves (Fig. 1 E, F). Hence, *cpd33* mutants hyperaccumulate both starch and soluble sugars.

***cpd33* mutant plants exhibit reduced photosynthesis, growth, and reproductive development**

The *cpd33* mutant exhibits reduced plant height (Fig. S1, S2B), stunted tassel development (Fig. S2A), and fails to produce ears. Because excess carbohydrate accumulation in leaves can inhibit photosynthesis (Rolland et al., 2006), we measured photosynthesis and chlorophyll content of *cpd33* mutant and wild-type

leaves. Compared to wild type, *cpd33* mutants displayed reduced chlorophyll content, photosynthetic rate, stomatal conductance, and maximum photochemical efficiency of photosystem II (Fig. S2 C-F). Therefore, the data indicate that hyperaccumulation of carbohydrates in *cpd33* mutant leaves is associated with reduced photosynthetic traits, plant growth, and development.

Sucrose export is reduced in *cpd33* mutant leaves

cpd33 mutant leaves contained high levels of sucrose and showed the build-up of starch in bundle sheath cells (Fig. 1F), similar to what was previously observed in other mutants with perturbed sucrose export (Russin et al., 1996; Braun et al., 2006; Slewinski and Braun, 2010b; Julius et al., 2018). We tested whether *cpd33* mutant leaves can export sucrose directly using radioactively labeled ^{14}C -sucrose or 1'-[^{18}F]fluoro-1'-deoxysucrose (1'- ^{18}F -sucrose) in sucrose transport assays (Tran et al., 2017). The labeled sucrose was applied to the tip of a mature leaf on an intact plant and allowed to transport for 1 h. The leaf was then excised and autoradiographed using phosphor plates. Wild-type leaves showed considerable ^{14}C -sucrose transport down the length of the blade, whereas *cpd33* mutant leaves displayed greatly reduced capacity to transport the labeled sucrose (Fig. 2). We obtained similar results with either ^{14}C -sucrose or 1'- ^{18}F -sucrose (Fig. S3). These data indicate that sucrose export is substantially inhibited in *cpd33* mutant source leaves.

***cpd33* mutant leaves have normal vein structure**

To examine whether vein structural differences might account for the reduction in sucrose export, we examined cross-sections of wild-type and *cpd33* mutant leaves under a microscope by bright-field and blue-light illumination. *cpd33* mutant leaves exhibited a strong reduction in chlorophyll abundance and autofluorescence compared to wild-type leaves, but displayed normal anatomy (Fig. 3A-D). A previous study of another carbohydrate partitioning defective mutant, *Cpd1*, showed that reduced sucrose export was associated with ectopic callose and lignin deposits in the phloem (Julius et al., 2018). To test whether the reduction in phloem translocation in *cpd33* mutant leaves was related to ectopic callose deposits, we stained cross-sections of *cpd33* and wild-type leaves with aniline blue, which binds callose. However, we did not observe any abnormal callose deposition in the veins of *cpd33* leaves (Fig. 3E, F). In addition, we examined lignin deposition in the veins of *cpd33* and wild-type leaves by staining with phloroglucinol-HCl. While we found lignin associated with the metaxylem and sclerenchyma cells (Fig. 3G, H), we did not observe any ectopic lignin deposition in *cpd33* mutant veins. Together, the anatomical and histochemical results demonstrated that the failure to export sucrose from *cpd33* mutant leaves is not associated with abnormal vein structure, at least as detectable by light microscopy.

***Cpd33* encodes a protein containing multiple C2 domains and transmembrane regions**

To understand the molecular function of *Cpd33*, we cloned the gene responsible for the mutant phenotype. The *cpd33* locus was fine-mapped to a 2.5 Mbp region on the

long arm of chromosome 8, between umc2555, a simple sequence repeat (SSR) marker, and Del.139787, a polymorphic deletion marker (Fig. 4A) (Settles et al., 2014). The fine-mapped region contained 65 predicted protein-coding genes. To identify the candidate gene, we employed a whole genome sequencing approach using genomic DNA isolated separately from two pools of >50 mutant plants for both *cpd33* and *cpd36*. By comparing the sequence reads obtained from *cpd33* and *cpd36* with the B73 reference genome sequence (Schnable et al., 2009), we identified a gene within the mapping interval, *GRMZM5G852378*, which contained a single independent nucleotide change in both *cpd33* and *cpd36* mutant DNA. The nucleotide change in *cpd33* caused an amino acid substitution from arginine to cysteine, and the nucleotide change in *cpd36* caused a premature stop codon. This candidate gene was cloned from the four additional mutant *cpd33* alleles by PCR and sequenced. The DNA sequencing data identified a distinct nonsense mutation in all four alleles, confirming that the causative gene was cloned (Fig. 4 B, C).

CPD33 is an ortholog of Arabidopsis QUIRKY

The *Cpd33* gene contains no introns (Fig. 4B). RNA-sequencing analyses indicated that *Cpd33* is expressed at very low levels throughout the plant, with relatively higher levels in roots and leaves (maximum expression value of <40 FPKM detected) (Fig. S4) (Stelpflug et al., 2016). Protein prediction software revealed that the CPD33 protein is 1084 amino acids long, and contains four C2 domains and two transmembrane regions, which are embedded in a phosphoribosyltransferase C-terminal region (PRTC domain) (Fig. 4C). C2 domains bind phospholipids in a Ca^{2+} -

dependent manner and have been shown to associate with cell membranes (Nalefski and Falke, 1996; Liu et al., 2017). BLAST and phylogenetic analyses showed that the protein encoded by *Cpd33* belongs to the multiple C2 domain and transmembrane region proteins (MCTPs) family (Shin et al., 2005; Fulton et al., 2009; Vaddepalli et al., 2014). We identified 16 homologs from maize, 16 homologs from *Arabidopsis thaliana*, 11 homologs from rice (*Oryza sativa*), 16 homologs from *Medicago truncatula*, and 21 homologs from *Populus trichocarpa*. All MCTPs have three to four C2 domains and a PRTC domain. Based on the phylogenetic analysis, the MCTPs proteins could be classified into seven clades (Fig. 5). We found that clade V homologs were specific for eudicots. CPD33 belongs to clade VII and is an ortholog of the Arabidopsis QUIRKY (AtQKY) protein. The CPD33 and QKY amino acid sequences share 65% similarity. Previous studies suggested that AtQKY interacts with the receptor-like kinase STRUBBELIG at PD to regulate tissue morphogenesis and vesicle trafficking (Vaddepalli et al., 2014). Within the Arabidopsis MCTPs family, FT-INTERACTING PROTEIN1 (FTIP1) was the first protein to be characterized (Liu et al., 2012). FTIP1 is required for the FLOWERING LOCUS T protein to transit through the phloem, and thereby regulates the transition to flowering in Arabidopsis (Liu et al., 2012). To our knowledge, CPD33 is the initial member of the MCTP family to be characterized in maize.

Arabidopsis *qky* mutant leaves accumulate high starch levels

The QKY loss-of-function mutant, *qky*, showed defects in plant growth, outer integument development, floral organ shape, and stem twisting (Fulton et al., 2009;

Vaddepalli et al., 2014). However, these studies did not discuss whether *qky* mutants accumulate carbohydrates in the leaves. We therefore performed starch staining to investigate whether carbohydrates hyperaccumulate in *qky* mutant leaves. We found that *qky* mutant plants grown under long day conditions had high starch accumulation in the mature leaves at the end of day and after two hours into the dark period, but interestingly, were similar to the wild-type leaves at the end of night (Fig. 6). These results suggest that Arabidopsis QKY functions similarly to CPD33 to promote sucrose export from the leaves.

CPD33 localizes to the PM and PD

Based on the subcellular localization of QKY, we predicted that CPD33 would also localize to PD. Vaddepalli et al. (2014) reported that expressing eGFP fused to the N-terminus, but not the C-terminus, of QKY complemented Arabidopsis *qky* mutants. Thus, to address the subcellular localization of CPD33, we made an N-terminal translational fusion of YFP to CPD33, under the control of the Cauliflower Mosaic Virus 35S promoter (p35S::YFP:CPD33), and transiently expressed it in tobacco (*Nicotiana benthamiana*) leaves using *Agrobacterium*-mediated transformation. The YFP signal of the fusion protein appeared to be unevenly distributed along the cell walls of the epidermal cells (Fig. 7B and E). The uneven and punctate distribution of YFP suggested that CPD33 was associated with PD. To test if CPD33 localized to PD, we transiently coexpressed p35S::YFP:CPD33 with an Arabidopsis PLASMODESMATA LOCATED PROTEIN1a:CFP (PDL1a:CFP) fusion (Thomas et al., 2008). We found that the YFP signal co-localized with the CFP signal, indicating that

CPD33 localized to PD (Fig. 7A-C). Additional evidence to support the localization of YFP:CPD33 to the PD came from staining the YFP:CPD33-expressing tissue with aniline blue, which marked callose associated with the neck of PD (Fig. 7D-F). Hence, similar to AtQKY, CPD33 localizes to PD and the PM.

As an additional localization assay, we stably expressed the p35S::YFP:CPD33 construct in Arabidopsis Col-O. The YFP signal was distributed in the PM of developing root tips (Fig. S5). However, we were not able to detect the YFP signal in mature leaves of the Arabidopsis transgenic lines (data not shown). These results suggest that the YFP:CPD33 protein was unable to accumulate in shoots of stable transgenic Arabidopsis plants, which was similar to previous results for eGFP:QKY transgenic Arabidopsis (Vaddepalli et al., 2014). Altogether, the localization assays indicate that the CPD33 protein localizes at PD and the PM.

CPD33 forms homodimers

Previous studies reported that AtQKY forms heterodimers with the receptor-like kinase STRUBBELIG and the PB1-domain containing protein PAL OF QUIRKY (Trehin et al., 2013; Vaddepalli et al., 2014). In addition, there are other C2 domain containing proteins, such as the animal protein synaptotagmin, which have also been reported to homo- or heterodimerize (Schauder et al., 2014). Thus, dimer formation seems common to C2 domain containing proteins. However, it is not known whether the proteins in the plant MCTPs family form homodimers. To investigate this possibility, we employed a bimolecular fluorescence

complementation (BiFC) assay using the pDOE system in tobacco leaves (Gookin and Assmann, 2014). In the following two transformation experiments, the C-terminal half of YFP was fused to the N-terminus of the CPD33 protein, and the N-terminal half of YFP was fused to either the N- or C-terminus of CPD33, respectively (Fig. 7G, H). We detected a YFP signal on the PM when the N-terminus of YFP was fused to the N-terminus of CPD33 and was co-expressed with the C-terminus of YFP fused to the N-terminus of CPD33 (Fig. 7G). These results indicate that CPD33 is capable of forming homodimers. However, we failed to detect a YFP signal when the N-terminal half of YFP was fused onto the C-terminus of CPD33 and was co-expressed with the C-terminal half of YFP fused with the N-terminus of CPD33 (Fig. 7H). This result suggests that CPD33 is a transmembrane protein, with the N- and C-terminal protein tails located on different sides of a membrane. However, we cannot rule out the possibility that no BiFC signal was detected because the C-terminal half of YFP fused to the C-terminus of CPD33 interfered with CPD33 protein localization or folding.

The PD permeability of photosynthetic cells was not affected in *cpd33* mutants

The radioactive sucrose transport and the sugar quantitation assays indicate that sucrose export was reduced in *cpd33* mutant source leaves. In addition, the subcellular localization analyses demonstrated that CPD33 localized to PD. Based on these data, we hypothesized that the *cpd33* mutant affected symplastic transport by changing PD permeability. To test this hypothesis, we examined the impact of the *cpd33* mutant on PD permeability in the adaxial-abaxial leaf axis by performing a

Drop-ANd-See (DANS) assay (Cui et al., 2015). The DANS assay uses carboxyfluorescein diacetate succinimidyl ester dye (CFDA), an uncharged, membrane permeable, non-fluorescent dye. When CFDA is applied to the surface of an abraded leaf, it is loaded into the adaxial epidermal cells, the acetate groups are cleaved by non-specific cellular esterase enzymes, and it is converted into carboxyfluorescein (CF). CF is polar, fluorescent and membrane-impermeable (Slewinski et al., 2012; Bihmidine et al., 2015). CF can diffuse across the leaves *via* PD into the mesophyll cells, bundle sheath cells, and subsequently into the abaxial epidermal cells. The extent of the dye movement was imaged by fluorescent microscopy and quantified as a measurement of PD permeability (Wright and Oparka, 1996; Cui et al., 2015). PD permeability was measured by the signal intensity of the fluorescent dye, and was expressed as the ratio of the signal intensity on the abaxial surface divided by the signal intensity on the adaxial surface of the leaf. The DANS assay showed that the ratio of CF diffused from the adaxial to abaxial surfaces of *cpd33* mutant leaves was comparable to wild type and the *sut1* mutants (Fig. 8). These data indicate that the PD permeability of the photosynthetic cells was not affected in *cpd33* mutants.

DISCUSSION

In this study, we described a new maize mutant, *cpd33*, which hyperaccumulates carbohydrates in the leaves, resulting in photosynthetic down-regulation and

subsequent negative effects on plant growth and development. Genome sequencing revealed that the *cpd33* mutation has a nucleotide change resulting in an amino acid substitution in *GRMZM5G852378*, and that the allelic *cpd36* mutation has a nucleotide change causing a premature stop codon in the same gene. Furthermore, sequencing of PCR-amplified products from four other mutant alleles showed that all have nucleotide changes that result in a premature stop codon in this gene. These findings confirm that we cloned the correct sequence. Although the six different mutations affect different domains of CPD33, they all result in similar mutant phenotypes. These data suggest all six mutant alleles perturb CPD33 function to the same degree and represent null mutations.

Previous studies on other carbohydrate partitioning defective mutants have suggested abnormal vein structure can inhibit sucrose export (Botha et al., 2000; Julius et al., 2018). For example, *sxd1* mutants show ectopic callose deposition between the bundle-sheath and phloem parenchyma cells (Botha et al., 2000), whereas *Cpd1* mutants display callose and lignin deposits in the sieve elements (Julius et al., 2018). Similar to results for *sxd1* and *Cpd1*, radioactively labeled sucrose transport assays demonstrated sucrose export was partially inhibited in the phloem of *cpd33* mutant leaves. However, we found that *cpd33* mutant leaves appear normal, and did not accumulate ectopic callose or lignin in their veins. Similarly, *tdy2*, another mutant with a carbohydrate hyperaccumulation phenotype and reduced sucrose transport in the leaves, did not show abnormal accumulation of callose or lignin (Baker and Braun, 2008; Slewinski et al., 2012). The *tdy2* mutant

causes defects in the functional development of the phloem, which blocks cell-to-cell sucrose movement through PD from companion cells to sieve elements (Slewinski et al., 2012; Baker et al., 2013). From our light and fluorescent microscopy studies, we did not find any changes in vein anatomy in *cpd33* mutant leaves; hence, we do not think that the carbohydrate hyperaccumulation observed in *cpd33* mutant leaves is caused by abnormal vein structure.

Phylogenetic analysis revealed that CPD33 is orthologous to the Arabidopsis membrane-bound multiple C2 domain protein QKY. Consistent with this idea, we find that QKY plays a role in carbohydrate partitioning, based on starch staining of *qky* mutants. While our results suggest that carbohydrate export was slower in *qky* mutant leaves, interestingly, the starch content of *qky* mutant leaves was similar to those in the wild-type leaves at the end of the night, in contrast to what we observed with *cpd33* mutants in maize. There are several possible explanations for the low starch content in *qky* mutant leaves at the end of the night. First, Arabidopsis is a C3 plant and has a lower photosynthetic efficiency, resulting in lower carbohydrate assimilation, than maize, a C4 plant. Therefore, less starch will accumulate in an Arabidopsis leaf compared to a maize leaf. In addition, the Arabidopsis plants were grown in a growth chamber where the light intensity was much lower compared to the light intensity of the field during the summer where the maize plants were grown (maximum of 200 PAR compared to 2000 PAR). Nevertheless, the similar phenotypes of *qky* and *cpd33* mutants indicate that CPD33 and QKY have similar functions with respect to sucrose export.

QKY has been reported to mediate tissue morphogenesis in Arabidopsis and to localize to PD (Vaddepalli et al., 2014). Similarly, *cpd33* mutants had reduced plant height, shorter leaves, and diminished reproductive structures, and the CPD33 protein also localized to the PM and PD. As PD are responsible for cell-to-cell transport, it is possible that CPD33/QKY might function to limit the diffusion of sucrose (and other molecules) through PD. To test this hypothesis, we performed a DANS assay to examine the permeability of PD in *cpd33* mutant leaves. In this assay, we compared the PD permeability of *cpd33* to *sut1* mutants and to wild-type plants. Maize SUT1 functions in loading sucrose from the apoplast into companion cells, and in retrieving sucrose from the apoplast during phloem translocation (Slewinski et al., 2009; Baker et al., 2016). *sut1* mutants are assumed to have normal PD structure. Contrary to our expectation, the DANS assay results showed that the diffusion of CF was not altered in *cpd33* mutants. This result might be explained by the maize companion cell-sieve element complex being nearly symplastically isolated from the surrounding cells (Evert et al., 1978); therefore, if CF was loaded into epidermal cells it would not effectively enter into the phloem. The DANS assay predominantly examines the permeability of PD between epidermal, mesophyll, bundle sheath, and vascular parenchyma cells, but not between companion cells and sieve elements. Hence, the data suggest that the defect in the *cpd33* mutant may affect symplastic transport between companion cells and sieve elements.

A previous study of the expression of the *AtQKY* gene using a β -glucuronidase (GUS) reporter showed that QKY was expressed in the vascular tissues of rosette leaves,

cotyledons, and roots (Liu et al., 2017). Similarly, Arabidopsis FTIP1, another paralog of CPD33, also showed an expression pattern specifically in the companion cell - sieve element complex (Liu et al., 2012). The RNA-sequencing data revealed that *Cpd33* is expressed at low levels throughout the plant, suggesting that *Cpd33* expression could be cell type-specific (Stelpflug et al., 2016). Therefore, considering the similar carbohydrate accumulation phenotypes of the *cpd33* and *qky* mutants, and the similarities in protein structure and subcellular localization of CPD33 and QKY, it is tempting to speculate that CPD33 might also be expressed and function specifically in vascular tissues. Future research will characterize the cellular localization of CPD33 to test this hypothesis.

Protein structure prediction suggested that CPD33 is a multiple C2 domain and transmembrane region containing protein. Other C2 domain containing proteins, such as the animal extended synaptotagmin (E-SYT) protein, have been reported to homodimerize. Human E-SYT2, a transmembrane protein, has two C2 domains and a synaptotagmin-like mitochondrial-lipid-binding protein domain. E-SYT2 was reported to mediate the exchange of lipids between the endoplasmic reticulum and PM, and homodimerization is important for its function (Schauder et al., 2014). Our BiFC experiments revealed that CPD33 is also able to form homodimers, which to our knowledge is the initial documentation of this result for a plant family member. In addition, the CPD33 protein is predicted to contain two transmembrane regions in the PRTC domain. Our BiFC results also suggested that CPD33 is a transmembrane protein, with the N- and C-terminal protein tails located on

different sides of a membrane. Considering the structural similarity of CPD33 and SYTs, it will be interesting to determine the role of homodimerization in CPD33 function.

In summary, we identified the *Cpd33* gene that when mutated is responsible for a carbohydrate accumulation mutant phenotype. We also found evidence to suggest that sucrose transport is perturbed in *cpd33* mutants, even though the leaf architecture and vein anatomy appears normal in the mutants. From dye transport assays, we found no changes in PD transport in the adaxial-abaxial direction in *cpd33* mutants, suggesting there were no restrictions in the movement through PD along this leaf axis. Taken together, our data lead us to hypothesize that CPD33/QKY may promote sucrose symplastic movement between companion cells and sieve elements in the phloem. It will be interesting to determine whether future efforts to enhance the expression and function of *Cpd33* will increase the transport of sucrose (and potentially other molecules) through PD into the phloem translocation stream. Determining how *Cpd33* controls carbohydrate partitioning could open new avenues for improving sucrose distribution and enhancing crop yields.

METHODS

Plant materials and growth condition

The *cpd33*, *cpd36*, *cpd52*, *cpd63*, *cpd83*, and *cpd93* mutants were identified from carbohydrate partitioning defective mutant populations, generated by pollen EMS mutagenesis of B73 (Table S1) (Liu et al., 2010; Julius et al., 2018). The mutations were introgressed into the Mo17 and B73 inbred backgrounds two or three times, followed by self-fertilization to create segregating families used for mapping and phenotypic characterization (see below). For morphology studies, starch staining, and sugar analyses, the maize plants were grown during the summer at the South Farm Research Center at the University of Missouri. For radioactively labeled tracer transport and DANS assays, the plants were grown in the Sears Greenhouse at the University of Missouri. For the radioactively labeled sucrose transport assays, the plants were transferred into a growth chamber at least 24 h prior to the assays as described (Tran et al., 2017).

Arabidopsis thaliana plants were grown in a growth chamber (Tran and Braun, 2017) under short days (10 h light/14 h dark) for three weeks and then under long days (14 h light/10 h dark) at 18–25°C and 55–65% relative humidity. Col-0 and T-DNA insertional mutants in *qky* (SALK_043901) were obtained from the Arabidopsis Biological Resource Center (ABRC, USA).

For transient protein expression, *Nicotiana benthamiana* plants were grown in the growth chamber under long days (14 h light/10 h dark) at 18–25°C and 55–65% relative humidity for 4–5 weeks.

Mapping

Bulked segregant analysis (BSA) mapping populations were constructed by backcrossing *cpd33*, *cpd36*, and *cpd52* mutants to the inbred line Mo17 for two generations, followed by self-fertilization to create segregating populations (BC2F2). We collected genomic DNA samples from twenty *cpd33*, eighteen *cpd36*, twenty *cpd52*, and thirty wild-type siblings each for BSA mapping. Genomic DNA was isolated from two pools corresponding to the mutant and wild-type siblings, respectively, and hybridized to Illumina SNP50 chips to initially map the mutants to a chromosomal region. This map position was refined by seed chipping and PCR screening individual kernels from segregating BC2F2 and BC3F2 families for recombinant chromosomes. These individuals were used for further fine mapping. For fine mapping, we used BC2F2 and BC3F2 populations segregating for *cpd33*, *cpd36*, and *cpd52*. The fine mapping population consisted of 907 mutant plants and 49 wild-type plants. Nearby SSR and insertion-deletion polymorphism (IDP) markers were obtained from the Maize Genome Database (<http://www.maizegdb.org>). After the public SSR and IDP markers within the genetic interval defined by IDP7320 and UMC2555 were exhausted, we developed new insertion-deletion (Indel) PCR markers between these two flanking markers as described (Settles et al., 2014). Table S2 lists the primer sequences of markers used for fine mapping. DNA extraction was performed as described (Leach et al., 2016). PCR conditions were 94°C for 2 min, 94°C for 45 s, 60°C to 65°C for 1 min, and 72°C for 1 min with 40 cycles. Amplified fragments were visualized by electrophoresis on 2% agarose gels (IDP markers) or 5% super-fine resolution agarose gels (SSR and

Indel markers) and stained with 1% SYBR™ Safe DNA Gel Stain (ThermoFisher, USA). Recombinants were scored visually from the gel images.

Whole genome sequencing was performed on two alleles: *cpd33* and *cpd36*. DNA samples were collected from more than 50 *cpd33* mutant plants and more than 50 *cpd36* mutant plants, purified using the DNeasy Plant Mini Kit (Qiagen, USA), and sequenced at the DNA Core Facility at the University of Missouri. Paired-end sequencing was performed with an Illumina Hiseq 2000 (Illumina, USA). The data was uploaded and analyzed by the PGen workflow available at <http://soykb.org/Pegasus/index.php> of Soybean Knowledge Base (SoyKB) as described (Liu et al., 2016). The reads were aligned against the B73 reference genome using the Burrows-Wheeler Aligner, then single nucleotide polymorphisms (SNP) and Indels were called and filtered by the Haplotype caller algorithm from the Genome Analysis Toolkit 3.0. These results identified a single candidate gene containing a SNP in both mutant alleles.

To confirm the candidate gene, we amplified the candidate sequence from all six alleles by PCR (Table S2). The PCR products were purified by Diffinity RapidTip (Diffinity Genomic, USA) and sequenced (GENEWIZ Inc., USA). The PCR product sequences were assembled and aligned against the *Cpd33* gene sequence from B73 using DNASTAR's Lasergene sequence analysis software (DNASTAR Inc., USA).

Phylogenetic analysis

To identify homologs of CPD33, BLASTP searches using the full-length CPD33 protein sequence were performed against the sequenced genomes of maize, rice, *Arabidopsis thaliana*, *Medicago truncatula*, and *Populus trichocarpa* in Phytozome version 12 (<http://www.phytozome.net/>) databases. The protein sequences were aligned by MUSCLE (Edgar, 2004). The alignment results were refined by Gblocks (Talavera and Castresana, 2007). The unrooted phylogenetic trees were constructed using the maximum likelihood method in MEGA version 6 (Hall, 2013). Default settings were used. The reliability of the trees was estimated with 1000 bootstrap iterations.

Starch staining

Starch staining was performed as previously described (Ruzin, 1999). For maize leaves, the *cpd33* mutant and wild-type leaves were collected at 5 AM, boiled with 95% ethanol to clear the photosynthetic pigments, and stained with 1% IKI. *Arabidopsis* leaves were harvested at the end of the day, at 2 hours into the night period, or at the end of the night, cleared with 95% ethanol, and stained with 1% IKI.

Soluble sugar and starch measurements

Mature leaves of 8-week-old maize plants were harvested at the end of the night (5 AM). Soluble sugars and starch were extracted as described (Leach and Braun, 2016). The extracts were analyzed using high-performance anion-exchange (HPAE) chromatography (ICS-5000, Thermo-Fisher Scientific, USA) as previously described

(Leach et al., 2017). A total of n=18 *cpd33* mutant plants and n=18 wild-type plants was used.

Photosynthesis, gas exchange and chlorophyll content measurements

Gas exchange measurements were taken on fully expanded maize source leaves grown in the field using a portable gas exchange system (LI-6400XT, LI-COR Inc., USA) as described (Huang et al., 2009; Bihmidine et al., 2015). Photosynthesis rate, stomatal conductance, and transpiration rate were measured at a photon flux density of 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and ambient CO_2 concentration of 400 $\mu\text{mol mol}^{-1}$. The measurements were performed 8 weeks after planting on n=17 *cpd33* mutant plants and n= 19 wild-type plants.

Leaf photochemical efficiency was expressed as leaf chlorophyll fluorescence (F_v/F_m). The F_v/F_m ratio was measured with dark-adapted leaves using the FlourPen FP100 chlorophyll fluorescence meter (Photon System Instruments, Czech Republic) on n=12 *cpd33* mutant plants and n= 12 wild-type plants.

Leaf chlorophyll content was measured using a chlorophyll meter (SPAD-502, Konica Minolta, Japan) on n=21 *cpd33* mutant plants and n= 25 wild-type plants.

Radioactively labeled sucrose transport assays

^{14}C -sucrose was purchased from PerkinElmer (USA), while $1'\text{-}^{18}\text{F}$ -sucrose was radiosynthesized as described (Ying et al., 2013; Rotsch et al., 2015). Radioactively labeled sucrose transport assays were performed as previously described on 4-to-5-

week-old plants (Slewinski et al., 2009; Tran et al., 2017). For these experiments we used *cpd33* mutant source leaves prior to anthocyanin accumulation. Briefly, 2.54 cm of the tip of mature leaf six was cut and then dipped into 2 ml of 1 mM unlabeled sucrose spiked with 200 μ Ci of ^{14}C -sucrose or $1'\text{-}^{18}\text{F}$ -sucrose. After 3 minutes, the leaf was removed from the sucrose solution and cleaned twice with wet followed by dry Kimwipes. The leaf was then allowed to translocate the labeled sucrose for one hour. Afterwards, a 25.4 cm segment (measured from the cut site) was excised from the leaf, taped on filter paper, and exposed. For the ^{14}C -sucrose experiments, the leaves were first dried at 80°C on a gel drier and then exposed to a phosphor plate for 10 days. We used n=8 plants for both *cpd33* mutant and the wild type. If ^{18}F -sucrose was used for the transport assays, the leaf was exposed to a phosphor plate for 60 min. We used n=2 for both *cpd33* mutant and the wild type. The phosphor plates were scanned with a GE Typhoon FLA 9000 scanner (GE Healthcare, USA). The laser was set at 635 nm, and pixel size was 100 μm . Image data were quantified using GE ImageQuant TL Toolbox program 7.0.

Morphology and histochemistry analyses

Free-hand sections from fresh leaves were used to examine chlorophyll content, autofluorescence, and vein structure under bright-field and blue-light illumination as described (Ruzin, 1999; Slewinski et al., 2010; Julius et al., 2018). For callose staining, fresh leaf tissues were incubated in Aniline blue solution (0.05% in 0.1 M K_2HPO_4 buffer, pH 9) for 10 min, then free-hand sectioned and observed under UV illumination. For lignin staining, free-hand sections from fresh leaves were

incubated with phloroglucinol (0.0068 M in 13.7% HCl) for 30 min prior to observation under bright-light illumination. The samples were examined with a Nikon Eclipse 80i epifluorescence microscope (Nikon Instruments Inc., USA) as described (Baker et al., 2016).

Subcellular localization analyses and BiFC assay

The coding sequence for the CPD33 protein (GRMZM5G852378_T01) followed by a linker of 10 alanine residues was synthesized in the pDONOR221 vector (Life Technologies, USA). The CPD33 coding sequence was recombined into the pEarleyGate104 vector (Earley et al., 2006) to generate the p35S::YFP:CPD33 translational fusion protein clone.

The coding sequence of AtPDL1a in pDONR221 (CIW00441) was obtained from the Arabidopsis Biological Resource Center (ABRC, USA) and recombined into the pEarleyGate102 vector (Earley et al., 2006) to generate the p35S::PDL1a:CFP translational fusion protein clone.

Binary vectors pDOE-07 and pDOE-08 were used for BiFC (Gookin and Assmann, 2014). The full-length coding sequence of the CPD33 protein was amplified by using the primers listed in Table S2 and cloned into pDOE7 and pDOE8.

The constructs were transformed into *Agrobacterium tumefaciens* GV3101. Agroinfiltration in tobacco leaves was performed as described (Sparkes et al., 2006). Infiltrated tobacco leaves were tested 3 days after inoculation. Plasmodesmal

callose staining was performed by staining the mature leaves with 0.1% aniline blue solution containing 1 M glycine (pH 9.5) for 15 min prior to confocal imaging.

The p35S::YFP:CPD33 construct was stably transformed into *Arabidopsis* Col-0 by the floral-dip method (Zhang et al., 2006). T1 plants were selected with Basta and used for subcellular localization. A Leica TCS SP8 confocal microscope system (Leica Microsystems, Germany) was used to collect confocal images. The microscope and camera settings used were as previously described (Baker et al., 2016).

DANS assay

We modified the DANS dye-loading assay (Cui et al., 2015) to analyze cell-to-cell CF movement in maize leaves. The adaxial surface of leaf five from three-week-old maize plants was gently abraded with a 5-mm wide strip of very fine grain sand paper (2000 grain) to remove the cuticle. To minimize dye loss to the xylem or phloem, a 1 μ l droplet of CFDA solution (1 mM) was loaded between two major veins on the adaxial surface of the gently abraded region. After 5 min, the leaf segment was excised, and the dye movement was observed under a Leica M205 FA stereoscope with an eGFP filter (Leica Microsystems, USA). ImageJ was used to quantify the region containing the dye by counting the color pixels.

Statistical analyses

Means and standard errors (SE) were calculated using Microsoft Excel. Differences in sugar and chlorophyll content, plant height, photosynthetic parameters, and ^{14}C

intensity were assessed using the Student's *t*-test embedded in the Microsoft Excel program, at the $P \leq 0.05$ level. For the DANS assay, differences in the dye intensity ratio were assessed using Tukey's Studentized Range (HSD) Test using the Proc ANOVA package in SAS (v 9.4) (SAS Institute, NC, USA), at the $P \leq 0.05$ level.

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TABLES

Table SB.1

Source of *cpd33* mutant alleles

Allele	Stock	Source
<i>cpd33</i>	NM830	N. Springer
<i>cpd36</i>	04IAB73PS025C6	C. Weil/ T.L. Slewinski
<i>cpd52</i>	NM2113	N. Springer
<i>cpd63</i>	NM2139	N. Springer
<i>cpd82</i>	NM1630	N. Springer
<i>cpd93</i>	04IAB73PS140A3	C. Weil/ T.L. Slewinski

All alleles originated from pollen EMS mutagenesis of the B73 inbred line.

Table SB.2

List of primers used

Primer	Primer sequence	Used for
DEL.139787-F	CTCTTCTGTCAGCTTCAAA	Mapping
DEL.139787-R	TGCATCAGGTAATGAGAAC	Mapping
INS.111854-F	CAACAGGTTGCGCCAGTTA	Mapping
INS.111854-R	GCCCTTCTATGCTGGTTT	Mapping
INS.111846-F	CGAGAGCACATGGTATGC	Mapping
INS.111846-R	CACTTGAACGGCTAGGATAC	Mapping
INS.111884-F	CCTCTCCCTGTACCTCATTA	Mapping
INS.111884-R	TTAGGGCTAAAGTAAGCATCTC	Mapping
INS.111891-F	CAGATGTCCCACTCCTTTC	Mapping
INS.111891-R	CGGCTCCTTCAGTGATTT	Mapping
INS.111892-F	CGGACCAAACAGACCAAAT	Mapping
INS.111892-R	GCTGTTGTAGGCCCATATT	Mapping
INS.111893-F	GGGAGTTTATATGATGACGA	Mapping
INS.111893-R	GTCTTCTGCTGTTGTAGG	Mapping
DEL.139847-F	GGCCGCCTTGAATGAAT	Mapping
DEL.139847-R	GTAGCTGAAGACGAGAAGAAC	Mapping
umc2555-F	GCGACATACTAGTTTAGTGCCGCT	Mapping/

		Genotyping
umc2555-R	GTACGTGACTGCTGTCTCCCTCA	Mapping/ Genotyping
GRMZM5G852378-cF1	CGGATCTGTGAGCAGAGCAA	Cloning
GRMZM5G852378-cR1	AATGGAACCAGGTGTTCGCC	Cloning
GRMZM5G852378-cF2	GAGGTCACAGGGATAGTCGC	Cloning
GRMZM5G852378-cR2	CTCTGCTCTCCCCAAAAGCA	Cloning
GRMZM5G852378_sF1	CTGAGATTGAGATGTGACG	Sequencing
GRMZM5G852378_sF2	GGAGAATAATCAGGGTGTG	Sequencing
GRMZM5G852378_sF6	CCTTGATCATGGACCGATCCTC	Sequencing
GRMZM5G852378_sF8	TCGTACGACGACGATTGCAC	Sequencing
AscI-CPD33	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCG <u>GCGCGCC</u> gCTGCTGGTGGTGGCGGTG	BiFC
CPD33-SpeI	GGGGACCACTTTGTACAAGAAAGCTGGGTAACT <u>AGTT</u> gcAGCAGCGGCGGCAGC	BiFC
SnaBI-CPD33	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCT <u>ACGTAGCGCTGCTGGTGGTGGCGGTG</u>	BiFC
CPD33-AaTII	GGGGACCACTTTGTACAAGAAAGCTGGGTAGA <u>CGTCGGTG</u> CAGCAGCGGCGGCAGC	BiFC

FIGURES

Figure B.1

cpd33 mutant leaves hyperaccumulate starch and soluble sugars. (A) Wild-type (WT) and *cpd33* mutant leaves. The WT leaf has a uniform green coloration, whereas the *cpd33* leaf displays chlorosis and anthocyanin accumulation. (B) WT and *cpd33* mutant leaves harvested at the end of the night, cleared of chlorophyll, and stained with IKI, showing that the WT leaf contains little starch, whereas the *cpd33* leaf

contains abundant starch. (C, D) Carbohydrate levels in WT and *cpd33* mutant mature leaves. Leaf 14 was collected at 5 AM from 8-week-old plants. White columns represent WT values, black columns represent *cpd33* mutant values. Each value is the mean of samples $\pm$ SE; n=18 *cpd33* mutant plants and n=18 WT plants. (C) Sucrose. (D) Glucose, fructose and starch. An asterisk represents a significant difference at $P \leq 0.05$. Free-hand cross-sections of (E) WT and (F) *cpd33* mutant leaves harvested after the dark period and stained with IKI. *cpd33* mutants hyperaccumulate starch in the bundle-sheath cells, whereas WT tissues contain no starch. Scale bars = 100 μ m.

Figure B.2

Export of ^{14}C -sucrose is reduced in *cpd33* mutant source leaves. (A) Dried wild-type (WT) and *cpd33* leaves. (B) Autoradiography showing ^{14}C -sucrose distribution in WT and *cpd33* leaves. (C) Quantification of ^{14}C -sucrose distribution in WT and *cpd33* leaves. Translocation was measured according to signal intensity at each distance

(in cm) from the application site. The graph represents means of intensity $\pm$ SE. An asterisk represents a significant difference at $P \leq 0.05$ using Student's *t*-test.

Figure B.3

cpd33 mutant leaves have normal vein structure. (A, B) Bright-field, and (C, D) blue-light illumination show reduced chlorophyll abundance and fluorescence, respectively, in *cpd33* leaf cross-sections as compared to those of wild type (WT). No

vein patterning defects were evident. (E, F) Aniline blue staining of callose (bright blue-white) under UV illumination. (G, H) Phloroglucinol-HCl staining of lignin (purple and red) under bright field. Scale bars = 100 μm (A to D), 50 μm (E to H).

Figure B.4

Cpd33 encodes a protein with multiple C2 domains and predicted transmembrane regions. (A) The fine-mapping of *cpd33*. (B) Structure of *Cpd33* cDNA showing the position of six *cpd33* allelic mutations (*cpd33*, *cpd36*, *cpd52*, *cpd63*, *cpd82*, and *cpd93*) (C) Structure of the CPD33 protein showing the four predicted C2 domains (white boxes), the PRTC domain (blue box), and the two putative transmembrane regions (red bars). The mutation underlying each mutant allele is noted beneath the

allelic designation. Individual letters represent amino acids, numbers denote the amino acid positions, and asterisks indicate a stop codon.

Figure B.5

Phylogenetic tree showing predicted relationships between multiple C2 domain and transmembrane region proteins from maize, rice, *Arabidopsis thaliana*, *Medicago truncatula*, and *Populus trichocarpa*. The phylogenetic tree was created using the maximum likelihood method. The sequences fall into seven clades. Clade V (orange) contains only sequences from eudicots, while the other clades contain sequences from both monocot and eudicot species. CPD33 (highlighted in red) belongs to clade VII and is an ortholog of *Arabidopsis* QKY (highlighted in blue). FTIP1 (clade I) and QKY (both highlighted in blue) have been characterized in *Arabidopsis*.

Figure B.6

qky leaves accumulate starch compared with wild-type (WT) leaves. Clearing and subsequent IKI staining of wild type (WT, top) and *qky* mutant (bottom) leaves reveal the latter have greater starch accumulation both at the end of day and at 2 hours into the dark period, but not at the end of the night.

Figure B.7

CPD33 is localized to the plasma membrane and plasmodesmata (PD). (A to C) Confocal images of tobacco leaf epidermal cells co-expressing (A) CFP fused to AtPDL1a, a PD marker (blue), (B) YFP fused to CPD33 (yellow), and (C) merged image showing co-localization. (D to F) Confocal images of (D) aniline blue staining of callose (blue) around the PD, (E) YFP fused to CPD33 (yellow), and (F) merged image showing co-localization. In (C) and (F), white arrowheads show co-localization. (G) and (H) CPD33 is a transmembrane protein that forms homodimers as assessed by BiFC in tobacco leaf epidermal cells. (G) NVen210:CPD33 coexpressed with CVen210:CPD33 produces a strong YFP signal (yellow indicating reconstitution of mVenus). (H) CPD33:NVen210 coexpressed with CVen210:CPD33 produces no BiFC signal. In (G) and (H) successful transformation is evident from the expression of the cotransformed mTq2 Golgi marker (cyan color). Scale bars = 20 μm .

Figure B.8

The PD permeability of photosynthetic cells is not affected in *cpd33* mutants. (A) DANS assays on wild-type (WT), *cpd33*, and *sut1* mutant leaves, showing that cell-to-cell movement of CF is unaffected in *cpd33*. Top images show dye absorption into the adaxial surface, whereas the bottom ones show dye diffusion into the epidermal cells of the abaxial surface 5 minutes after loading CFDA onto the adaxial epidermis. (B) Quantification of PD permeability in WT, *cpd33*, and *sut1* leaves. PD permeability was measured as the signal intensity of fluorescent dye, and is expressed as the ratio of the signal intensity of the abaxial surface to that of the adaxial surface. The graph shows the ratio averages of n=26 WT, n=18 *cpd33* mutant, and n= 9 *sut1* mutant samples. Error bars represent the SE. There were no significant differences at the $P \leq 0.05$ level. Scale bars = 1 mm.

Figure SB.1

Whole-plant comparisons of six *cpd33* allelic mutants (*cpd33*, *cpd36*, *cpd52*, *cpd63*, *cpd82*, *cpd93*) and their wild-type (WT) siblings grown in the field.

Figure SB.2

cpd33 mutants display stunted reproductive tissues and reduced plant growth. (A) A *cpd33* tassel (right) showing diminished growth compared to a wild-type (WT) tassel (left). (B) Average heights of WT siblings and *cpd33* mutant plants. (C-F) Chlorophyll content (SPAD value) and photosynthetic parameters of WT siblings and *cpd33* mutant plants, net photosynthesis (Anet), stomatal conductance (g_s), maximum photochemical efficiency of photosystem II (Fv/Fm). White columns represent WT sibling plants and black columns represent *cpd33* mutant plants. Data represent means of samples $\pm$ SE. For Anet and g_s , $n=17$ *cpd33* mutant plants and $n=19$ WT plants. For plant height and chlorophyll content, $n=21$ *cpd33* mutant plants and $n=25$ WT plants. For Fv/Fm, $n=12$ *cpd33* mutant plants and $n=12$ WT plants. An asterisk represents a significant difference at $P \leq 0.05$ using Student's *t*-test.

Figure SB.3 Export of $1'\text{-}^{18}\text{F}$ -sucrose is reduced in *cpd33* mutant source leaves. (A) Fresh leaves of wild type (WT) and *cpd33*. (B) Autoradiography showing $1'\text{-}^{18}\text{F}$ -sucrose distribution in WT and *cpd33* leaves.

Figure SB.4

Levels of *Cpd33* transcripts in various tissues as determined by RNA-sequencing (adapted from Stelpflug et al., 2016).

Figure SB.5

Confocal images of Arabidopsis developing root cells expressing YFP fused to CPD33. (A) YFP:CPD33 signal (yellow). (B) Bright field. (C) Merged image. Scale bars = 30 μm .

APPENDIX C: *Tonoplast Sugar Transporters (SbTSTs)*

putatively control sucrose accumulation in sweet sorghum stems

Note: The information in this appendix was published under the title:

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Keywords: *Sorghum bicolor*, *TSTs*, *SWEETs*, Sucrose, Carbohydrate partitioning

Abbreviations: *TSTs*, *Tonoplast Sugar Transporters*; *SUTs*, *Sucrose Transporters*; qRT-PCR, quantitative reverse-transcription polymerase chain reaction

Abstract

Carbohydrates are differentially partitioned in sweet versus grain sorghums. While the latter preferentially accumulate starch in the grain, the former primarily store large amounts of sucrose in the stem. Previous work determined that neither sucrose metabolizing enzymes nor changes in *Sucrose transporter (SUT)* gene expression accounted for the carbohydrate partitioning differences. Recently, two additional classes of sucrose transport proteins, Tonoplast Sugar Transporters

(TSTs) and SWEETs, were identified; thus, we examined whether their expression tracked sucrose accumulation in sweet sorghum stems. We determined two *TSTs* were differentially expressed in sweet vs. grain sorghum stems, likely underlying the massive difference in sucrose accumulation. A model illustrating potential roles for different classes of sugar transport proteins in sorghum sugar partitioning is discussed.

Sucrose harvested from plants represents a multi-billion dollar (US) annual industry, with great interest in expanding production for food and biofuel uses (Dweikat *et al.*, 2012, Slewinski 2012, Braun *et al.*, 2014, Ruan 2014, McGrath & Townsend 2015). Multiple crops have been independently bred to store high concentrations of sucrose in terminal storage organs, namely, the taproots of sugar beet (*Beta vulgaris* L.), and the stems of sweet sorghum (*Sorghum bicolor* L. Moench) and sugarcane (*Saccharum officinarum* L.) (McGrath & Townsend 2015, Rooney *et al.*, 2007, Wang *et al.*, 2013, Bihmidine *et al.*, 2013, Calviño & Messing 2012). However, the sucrose contents of these crops appear to be approaching maximal levels attainable from breeding efforts (McCormick *et al.*, 2009, Patrick *et al.*, 2013); therefore, new approaches are needed to increase sucrose accumulation in storage organs. Hence, characterizing the genes that function in sucrose transport and storage will reveal potential new targets for future manipulations to enhance crop yields (Braun *et al.*, 2014, Ruan 2014, Jung *et al.*, 2015, Braun & Slewinski 2009, Ma *et al.*, 2009, Slewinski *et al.*, 2012, Slewinski & Braun 2010).

Different sorghum genotypes have been selectively bred to store carbohydrates in contrasting storage organs: sweet sorghums accumulate large quantities of soluble sugars, mostly sucrose, in stem tissues, whereas grain sorghums primarily store carbohydrates as starch in the seeds (Dweikat *et al.*, 2012, Slewinski 2012, Rooney *et al.*, 2007, Murray *et al.*, 2008, Sukumaran *et al.*, 2012). The molecular basis for the difference in carbohydrate partitioning between these sorghum types is unknown. Previous research found that sucrose accumulation within sweet sorghum stems was not correlated with the activities of enzymes involved in sucrose metabolism, invoking sucrose transport proteins as potentially controlling sucrose content (Hoffmann-Thoma *et al.*, 1996). Transport experiments using asymmetrically radiolabeled sucrose determined that sucrose movement into stems likely included an apoplastic transport step (Tarpley & Vietor 2007). Subsequent dye transport studies suggested the phloem tissues within sorghum stems are symplasmically isolated from surrounding tissues, supporting that sucrose phloem unloading occurs apoplastically, and thus requires sucrose transport proteins (Bihmidine *et al.*, 2015). However, other studies support a possible symplasmic transport route from phloem sieve elements to storage parenchyma cells in mature stems (Milne *et al.*, 2015). Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analyses indicated that *Sucrose Transporters (SUTs)*, which function as H⁺/sucrose symporters to transport sucrose across membranes, were not differentially expressed in the stem of a flowering sweet sorghum line, UNL71-2011, a sweet sorghum derived from cultivar Wray, in comparison to a similarly staged grain sorghum line, UNL3016, selected from cultivar Macia (Bihmidine *et al.*, 2015). These

data suggest other types of sucrose transport proteins may underlie sucrose accumulation within sorghum stem tissues.

Two additional distinct classes of sucrose transport proteins have recently been described. SWEETs are a family of sugar transport proteins, with different family members preferentially transporting hexoses or sucrose (Baker *et al.*, 2012, Eom *et al.*, 2015, Braun 2012, Chen *et al.*, 2014, Chen *et al.*, 2012, Feng & Frommer 2015). Clade III SWEET proteins, which localized to the plasma membrane, have been proposed to function as uniporters that facilitate the transport of sucrose down a concentration gradient. A different class of sugar transport proteins, Tonoplast Sugar Transporters (TSTs, also known as Tonoplast Monosaccharide Transporters), is located on the tonoplast and function as H⁺/sucrose antiporters to transport sucrose into the vacuole (Wormit *et al.*, 2006, Martinoia *et al.*, 2012, Schulz *et al.*, 2011, Wingenter *et al.*, 2010). Recently, a TST was shown to be responsible for sucrose accumulation within the sugar beet taproot (Jung *et al.*, 2015). Whether *TST* or *SWEET* genes have also been selected during the domestication of other major sucrose storage crops, such as sweet sorghum or sugarcane stem tissues, is not known. Since *SbSUT* genes were not differentially expressed between sweet and grain sorghum stem tissues (Bihmidine *et al.*, 2015), we decided to examine the expression of other predicted sucrose transport proteins, specifically, the clade III *SbSWEET* and the *SbTST* genes. For these studies, we compared gene expression between the sweet sorghum line UNL71-2011 at anthesis, when sugars are actively accumulating in the stem, with the equally staged grain sorghum line UNL3016, with

low stem sugar content, to determine if any *SbSWEET* or *SbTST* genes are associated with sucrose accumulation in stem tissues. These lines are herein simply referred to as sweet and grain sorghums for clarity. As a point of reference, we found that the total solute levels, consisting primarily of sucrose, increased approximately 24-fold in sweet sorghum stems compared with grain sorghum stems during the ripening process from anthesis to physiological maturity (Bihmidine *et al.*, 2015).

Bioinformatic analyses were used to identify *SbTST* and *SbSWEET* genes in the sorghum genome. Three *SbTST* genes and 20 *SbSWEET* genes were identified (Eom *et al.*, 2015). We next analyzed a sorghum gene expression database to determine which of these genes were expressed in leaf and stem tissues (Table 1) (Makita *et al.*, 2015). *SbSWEET13A* was the most strongly expressed clade III gene within these tissues. Its expression was more than 10-fold higher than other clade III sweet genes; *SbSWEET13B* and *SbSWEET13C* had lower, but appreciable, expression relative to *SbSWEET13A*. The other clade III *SbSWEET* genes likely to transport sucrose were all very lowly or not detectably expressed (Table 1). Therefore, we selected the three *SbSWEET13* genes and all *SbTST* genes for further expression analyses. Gene-specific qRT-PCR primer sets were validated for each gene (Table 2). The qRT-PCR experiments and statistical analyses were performed as previously described (Bihmidine *et al.*, 2015). In examining mature leaf and ripening stem tissues of both grain and sweet sorghum, we determined that *SbTST1*, *SbTST2*, and *SbSWEET13A* were reliably expressed, whereas *SbTST3*, *SbSWEET13B*, and *SbSWEET13C* were expressed at a much lower level (Figures 1-2). *SbSWEET13B*

expression was at least 33-fold less than *SbSWEET13A* in all tissues examined. Similarly, *SbSWEET13C* was expressed at least 20-fold less than *SbSWEET13A* in leaves of both cultivars. *SbSWEET13C* was expressed approximately 6.7- and 29-fold lower than *SbSWEET13A* in grain and sweet sorghum stems, respectively; however, *SbSWEET13C* exhibited lower expression in sweet sorghum stems compared to grain sorghum stems, which is the opposite of what was hypothesized if the gene functioned to promote sucrose accumulation in sweet sorghum stems. Based on these results, we conclude that *SbTST3*, *SbSWEET13B*, and *SbSWEET13C* are minimally expressed in mature leaf and ripening stem tissues and therefore do not likely contribute substantially to sucrose accumulation within these tissues.

Based on the previous results, we examined *SbTST1*, *SbTST2*, and *SbSWEET13A* to determine if they are differentially expressed between sweet and grain sorghum leaf and stem tissues. In mature leaves, *SbTST1* and *SbTST2* showed ~3.5-fold and ~7.4-fold higher expression levels in sweet sorghum relative to grain sorghum ($p \leq 0.05$; Figure 3A, B). Within stem tissues at anthesis, *SbTST1* and *SbTST2* showed significantly higher expression levels in sweet sorghum compared to grain sorghum (~2.6- and ~4.4-fold, respectively) (Figure 3A, B). *SbSWEET13A* showed reduced expression in sweet compared to grain sorghum leaves and comparable expression in stem tissues of both genotypes (Figure 3C). These data indicate *SbTST1* and *SbTST2* are significantly more highly expressed in leaves and stem tissues of sweet sorghum than in grain sorghum, and that *SbSWEET13A* expression was reduced in sweet sorghum leaves compared to grain sorghum but not differently expressed in

stem tissues. Thus, these data suggest that differential expression of *SbTST1* and *SbTST2* genes, but not *SbSWEET13A* may play an important role in sugar accumulation in sweet sorghum stems. To our knowledge, no previous reports have shown the differential expression of *SbTSTs* associated with sugar accumulation in the stems of sweet vs. grain sorghum.

From our expression studies, we developed a model of the various sucrose transporter protein functions to explain the basis of sugar accumulation within sorghum leaf and stem tissues and to stimulate new directions in research (Figure 4). Within leaves, *SbSUT2* and *SbSUT4*, but not *SbSUT1*, were more highly expressed in sweet sorghum than in grain sorghum, suggesting that *SbSUT4* may function to import sucrose into cells, and *SbSUT2* may function to export transitory stored sucrose from the vacuole. *SbSUT1* function is likely conserved between grain and sweet sorghum, and based on orthology with the maize (*Zea mays*) *ZmSUT1* gene, it likely functions in sucrose phloem loading in leaves (Slewinski *et al.*, 2010, Slewinski *et al.*, 2009, Rotsch *et al.*, 2015). *SbSWEET13A* showed reduced expression in sweet compared with grain sorghum leaves, whereas *SbTST1* and *SbTST2* were both more highly expressed in sweet sorghum leaves, suggesting that they may function to import sugars into the vacuole for temporary storage during daylight. In stem tissues, none of the *SbSUT* or *SbSWEET13A* genes were differentially expressed, suggesting they do not account for the differences in sugar accumulation. However, both *SbTST1* and *SbTST2* were highly significantly expressed in sweet sorghum stems, suggesting these genes function to import sucrose for storage in the

vacuole within stem parenchyma cells. Based on these results, we hypothesize the ~24-fold increase in total stem solutes observed in sweet sorghum compared with grain sorghum is predominantly due to the significantly higher expression of *SbTST1* and *SbTST2* in sweet sorghum tissues.

In summary, based on both our previous and current results, we determined *SbTST1* and *SbTST2*, but probably not *SbSUTs* or *SbSWEETs*, are likely responsible for the substantial sugar accumulation in sweet sorghum stems. Testing of this hypothesis will potentially require characterizing loss-of-function mutations in both genes, since they were found to be partially functionally redundant in *Arabidopsis thaliana*. These efforts are currently underway. Furthermore, these data suggest *TSTs* have been the target of selection for sugar accumulation in both the sweet sorghum stem and the sugar beet taproot. It will be interesting to determine whether *TSTs* have similarly been selected within sugarcane stem tissues. If so, it would indicate *TSTs* have been convergently selected during domestication of the world's three major sucrose storage crops. Our findings tantalizingly suggest *SbTST1* and *SbTST2* are candidate genes for the control of sucrose accumulation in sweet sorghum stems. Hence, modifying the expression or function of *TSTs* through genetic engineering or selective breeding, could potentially achieve greater sucrose accumulation and therefore enhancement of crop yields in sugar-storing organs, which would lead to direct benefits for food and fuel production.

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TABLES

Table C.1

The expression level of the *SbTST* and *SbSWEET* genes by RNA-seq in the leaf and stem tissues. The numbers represent the average expression obtained from the FPKM (fragments per kilobase of transcript per million mapped reads) plots.

Gene name	Phytozome reference no.	Gene ID.	Leaf	Stem
<u><i>SbTST1</i></u>	Sobic.001G312900	Sb01G030430	105	149
<u><i>SbTST2</i></u>	Sobic.004G099300	Sb04G008150	110	130
<u><i>SbTST3</i></u>	Sobic.010G276100	Sb10g031000	Not detected	Not detected
<i>SbSWEET11A^{III}</i>	Sobic.007G191200	Sb07g026040	Not detected	10
<i>SbSWEET11B^{III}</i>	Sobic.002G259300	Sb02g029430	Not detected	Not detected
<i>SbSWEET12^{III}</i>	Sobic.001G373600	Sb01g035490	Not detected	Not detected
<u><i>SbSWEET13A^{III}</i></u>	Sobic.008G094000	Sb08g013620	2250	200
<u><i>SbSWEET13B^{III}</i></u>	Sobic.008G094300	Sb08g013840	28	2
<u><i>SbSWEET13C^{III}</i></u>	Sobic.008G094400	Sb08g014040	120	20

<i>SbSWEET14^{III}</i>	Sobic.005G123500	Sb05g018110	1	3
<i>SbSWEET15^{III}</i>	Sobic.004G157100	Sb04g021000	Not detected	1
<i>SbSWEET16^{IV}</i>	Sobic.001G377600	Sb01g035840	Not detected	Not detected
<i>SbSWEET1A^I</i>	Sobic.003G377700	Sb03g041740	100	135
<i>SbSWEET1B^I</i>	Sobic.009G143500	Sb09g020860	82	2
<i>SbSWEET2A^I</i>	Sobic.003G182800	Sb03g024250	15	8
<i>SbSWEET2B^I</i>	Sobic.003G269300	Sb03g032190	75	4
<i>SbSWEET3A^I</i>	Sobic.009G080900	Sb09g006950	135	300
<i>SbSWEET3B^I</i>	Sobic.003G015200	Sb03g001520	Not detected	1
<i>SbSWEET4A^{II}</i>	Sobic.004G136600	Sb04g015420	85	35
<i>SbSWEET4B^{II}</i>	Sobic.004G133500	Sb04g012910	20	30
<i>SbSWEET4C^{II}</i>	Sobic.004G133600	Sb04g012920	Not detected	Not detected
<i>SbSWEET5^{II}</i>	Sobic.009G252000	Sb09g030270	Not detected	Not detected
<i>SbSWEET6^{II}</i>	Sobic.003G213000	Sb03g027260	30	10

The numbers are estimated from the FPKM plots obtained from the MOROKOSHI sorghum transcriptome database (<http://sorghum.riken.jp/morokoshi>).³³ The

different upper-case roman letter superscripts indicate the clade to which each *SbSWEET* gene belongs. Not detected means that the gene had no detectable RNAseq counts in the corresponding tissue. The underlined genes are those selected for this study.

Table C.2

List of primers used.

Primer name	Primer sequence (5' - 3')	Product size (bp)
SbTST1-F	GATGGGCTGACCTGTTTG	175
SbTST1-R	GCAGAAGATGCGCTAAGG	175
SbTST2-F	TTGGAGGTTGGAGGAGAC	150
SbTST2-R	CTTGGAAGGTCGAGCAATC	150
SbTST3-F	CTGTTGCTTCGTCATGGG	146
SbTST3-R	TGACAGGAAGAGAGTAGGTG	146
SbSWEET13A-F	CGCTCACTACTGCTAAGTATTAT	96
SbSWEET13A-R	ACAGTAGTCTGGGATCGATTA	96
SbSWEET13B-F	CATGAGTCGAGTCCGAATG	116
SbSWEET13B-R	AGCTACGGTTGGATAAACG	116
SbSWEET13C-F	ACCCGTTTATCCAACCCTTAG	87
SbSWEET13C-R	TGAAATTCCTGCCTGGTTACA	87
Luciferase-F	CCAGGGATTTTCAGTGGATGT	183

Luciferase-R	AATCTGACGCAGGCAGTTCT	183
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FIGURES

Figure C.1

Expression levels of *SbTST2* and *SbTST3* relative to *SbTST1* in grain and sweet sorghum mature leaves and stems. A, B show grain sorghum (black bars), and C, D show sweet sorghum (white bars); A, C are mature leaf tissues, and B, D are flowering stems. Values are means $\pm$ standard error of N = 5 plants, and an asterisk

indicates significantly different means between the two genes at $p \leq 0.05$. Relative gene expression is shown compared to exogenously added *Luciferase* RNA as a normalization control.

Figure C.2

Expression levels of *SbSWEET13B* and *SbSWEET13C* relative to *SbSWEET13A* in grain and sweet sorghum mature leaves and stems. A, B show grain sorghum (black bars), and C, D show sweet sorghum (white bars); A, C are mature leaf

tissues, and **B**, **D** are flowering stems. Values are means $\pm$ standard error of N = 5 plants, and an asterisk indicates significantly different means between the two genes at $p \leq 0.05$. Relative gene expression is shown compared to exogenously added *Luciferase* RNA as a normalization control.

Figure C.3

Expression levels of *SbTST1*, *SbTST2*, and *SbSWEET13A* in leaves and stems of sweet sorghum relative to grain sorghum. Expression levels are shown for *SbTST1* (A), *SbTST2* (B), and *SbSWEET13A* (C). An asterisk indicates significantly different means between the two lines at $p \leq 0.05$ of $N = 5$ plants.

Figure C.4

A model illustrates the roles for different sucrose transport proteins in sucrose movement across cellular membranes in sorghum leaf (green shaded background) and stem (blue shaded) tissues in grain sorghum (left) vs. sweet sorghum (right). The vacuole is shown in grey. SPC = stem parenchyma cell. SUT proteins are shown by a blue circle, with an arrow indicating the direction of sucrose movement, and the numbers correspond to SbSUT1, SbSUT2, or SbSUT4. Purple diamond with an arrow refers to a TST protein located on the tonoplast, and

the numbers represent SbTST1 or SbTST2. The green boxes labeled 13A correspond to SbSWEET13A. The increased size of the shapes indicates increased expression of the corresponding gene in sweet (UNL 71-2011) vs. grain (UNL 3016) sorghum tissue.

VITA

Benjamin Thomas Julius was born September 22nd, 1992 in Saint Louis, MO to Kevin and Diane Julius. Upon graduation from Saint Mary's Catholic High School, Ben attended Truman State University and joined the research group of Dr. Diane Janick-Buckner and Dr. Brent Buckner to understand the genetic control of maize leaf development. Ben graduated from Truman State University with a B.S. in Biology in 2014 and moved to Columbia, MO to join Dr. David Braun focusing on the genetic control of carbohydrate partitioning in maize. Ben completed his doctorate degree in Biological Sciences in May 2019, during which he was the first author of one research article and one review paper; he also co-authored one research article and one review paper.