RESEARCH PAPER

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

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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.

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 Matusíková, 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 (sutt1), psychedelic (psc), and the tie-dyed1 (tdy1) and tdy2 mutants (Rusin 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, sutt1, 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 gpd 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 gpd 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 gpd 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 Cole, 1978).

Maize plants used for the morphometrics, photosynthetic and gas exchange analyses, microscopy, and soluble sugar and starch quantification 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 μmol m⁻² 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’-ttgccaaaggtcattagttgat-3; and Reverse, 5’-aaggtggctatgacagaa-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 m⁻³ pasteurized field soil. Plants were grown for 2 weeks in growth chambers under a 16 h light/8 h dark photoperiod and 180 μmol m⁻² 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).

[^14]C-Sucrose and CF transport studies
Carboxylfluorescein (CF) and [^14]C-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 9 am and 12 noon 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 (Amax, μmol CO₂ m⁻² s⁻¹) and stomatal conductance (gₛ, mmol H₂O m⁻² s⁻¹) were measured at a photon flux density of 2000 μmol m⁻² s⁻¹ and ambient CO₂ concentration of 400 μmol mol⁻¹. The maximum photochemical efficiency of PSII (Fv/Fm) was determined on dark-adapted leaves using a leaf fluorometer attached to the LI-6400XT infrared gas analyzer at an ambient CO₂ concentration of 400 μmol mol⁻¹. For the Cpd1/+ mutant leaves, the regions analyzed for the Fv/Fm 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 Microscopy images were taken using a Nikon Eclipse 80i epifluorescent microscope equipped with a 100 W mercury bulb and a DXM1200F.

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 immediately after harvest (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 (Meihs 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.

Benzoazoxinoid measurements
For measurement of leaf benzoazoxinoid 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 benzoazoxinoid 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 benzoazoxinoid 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. 1A; Supplementary Table S1 at JXB online). The homozygous mutant exhibits delayed growth in the shoot and root, resulting in a stunted appearance (Fig. 1A). Additionally, the shoot exhibits a highly variable continuum of light green to chlorotic leaves, which rapidly progress to necrosis, initiating from the tip and progressing basipetally. Ultimately, the homozygous mutant is seedling lethal and survives only 2 weeks after planting. Heterozygous 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. 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 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. 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. 3A). However, the Cpd1/+ mutant leaves displayed intense starch accumulation in the chlorotic regions (Fig. 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. 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. 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. 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

Table 1. Cpd1/+ mutants show reduced height and yield, and delayed flowering

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

An asterisk signifies significantly different means between Cpd1/+ mutant and wild-type plants at P≤0.05, using a two-tailed Student’s t-test.
leaves. As expected, in the wild-type leaf, the \([14C]\)sucrose was readily transported from the tip to the base of the leaf (Fig. 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. 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 \([14C]\)sucrose transport data, CF was not present in the phloem of Cpd1/+ mutant leaf veins (Fig. 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. 4I, J). However, CF fluorescence was clearly seen in the wild-type phloem (Fig. 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. 5B, D). No such blockages were observed in wild-type leaves sampled from the equivalent region (Fig. 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. 5F, H, J). These deposits were absent from the phloem tissue of wild-type samples (Fig. 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. 5F). Interestingly, in most veins analyzed, both callose–occluded and unoccluded sieve tubes were found (Fig. 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. 5M, N). Lignin was never observed in the phloem of the wild-type veins (Fig. 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

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**Fig. 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). (This figure is available in colour at JXB online.)
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. 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. 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. 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. 7A, B). Similar results were seen in sections taken from the middle of the leaves (Fig. 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. 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...
Fig. 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. (This figure is available in colour at JXB online.)

Fig. 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) are images from different regions (tip, middle, and base, respectively) of the same wild-type leaf, and (B, D, and F) are 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. (This figure is available in colour at JXB online.)
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. 8A). The opposite effect was observed for the southern armyworm, as the insects feeding on the wild-type plants gained more weight (Fig. 8A). However, the average caterpillar weights of the two treatments were not significantly different for the beet armyworm (Fig. 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. 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. 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.
metabolites, such as benzoxazinoids, in their gut (Glauser et al., 2011; Maag et al., 2014), probably due to this species’ ability to inactivate grass-specific defense compounds, and aphids to reproduce and with decreased growth of caterpillar herbivores, with the exception of the fall armyworm. This was 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 [14C]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 deposition 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

### 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 [14C]sucrose and CF transport in mutant leaves lend support to this hypothesis.

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during the development of mutant leaves (Figs 6, 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 (gs7) 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 gs7 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 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 ends 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 benzoazinoid compounds in the apoplast 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 benzoazinoid 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 benzoazinoids (which are induced more in Cpd1/+ than in control plants by aphid feeding, Fig. 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 benzoazinoids were created. However, genetic tests of the causative role for benzoazinoids 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 benzoazinoid 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 Wouding, 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. 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 haplo-insufficient 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.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Differences in photosynthetic capacity and gas exchange measurements between wild-type (WT) and chlorotic regions of Cpd1/+ mutant sibling leaves.

Fig. S2. Cpd1/+; bx2/bx2 double mutant plants exhibit the Cpd1/+ mutant leaf phenotype relative to +/+; bx2/bx2 leaves.

Table S1. \( \chi^2 \) table for 1:1 segregation of Cpd1/+; wild-type families.

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Author contributions

BTJ conducted morphometric data analysis, light and fluorescence microscopy, starch staining, and soluble sugar and starch quantification, drafted the manuscript, and helped critically revise it. TLS conducted the \([14C]\)sucrose and CFDA transport experiments, and helped critically revise the manuscript. RFB conducted morphometric measurements and their data analysis, light and fluorescence microscopy, starch staining, and the photographic time series of chlorosis development in emerging leaves, and helped critically revise the manuscript. VT and SZ conducted the benzoxazinoid measurements, and VT performed the caterpillar and aphid assays. SB conducted photosynthesis and gas 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.

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