Utilizing electron microscopy (EM) to study the subcellular localization and functions of *Carbohydrate partitioning defective33* in maize

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Introduction

Photosynthesis is the major biochemical process underlying plant growth and development. During photosynthesis, plants use light energy from the sun to convert carbon dioxide and water into chemical energy stored as sugar (e.g., sucrose). After production in the leaves, sucrose is transported to distant parts of the plant (e.g. roots, developing leaves, flowers, and fruits), where it is broken down and used to fuel growth. This process, known as carbohydrate partitioning, is critically important to plant growth and agricultural yields, yet we know little about the genes involved [1].

Our research group is interested in studying the genes that control sugar movement in maize (*Zea mays*). We discovered a novel gene, C*arbohydrate partitioning defective33* (*Cpd33*), which regulates sugar movement in maize. Maize plants lacking the *Cpd33* gene function (*cpd33* mutant plants) had yellow leaves, indicative of stress, and accumulated significantly more sugar in their leaves compared to normal plants. Furthermore, we tracked sugar movement in *cpd33* mutant plants using radioactively labeled sucrose and found that *cpd33* plants transported less sugar from their leaves than wild-type plants. In plants, direct cell-to-cell movement is facilitated by plasmodesmata (PD) channels, which allow small, soluble molecules, such as sugars, amino acids, and hormones, to diffuse between neighboring cells. Based on the subcellular localization of CPD33 and the labeled sucrose transport assays, we hypothesized that the *cpd33* mutant might affect vein ultrastructure and/or the structure and frequency of PD in veins. Previous studies of other carbohydrate partitioning defective mutants (e.g. *sxd1* mutant and the *tdy2* mutant) indicated that structural alterations of the PD inhibited sugar transport in the leaf, and may account for the abnormal accumulation of sugar [2,3]. To determine whether the inhibition of sugar movement in *cpd33* mutant plants is caused by structural perturbations of plasmodesmata, we examined the leaf tissues of *cpd33* mutant plants using TEM.

Methodology

Fresh maize leaves were cut to 1 mm by 1 mm pieces and immediately submerged in fixative solution (2% formaldehyde and 2% glutaraldehyde in 0.1M Na-Cacodylate buffer). The fixed samples were then high pressure frozen with HPF Compact 02 (Engineering Office M. Wohlwend GmbH) and placed in a Leica AFS Freeze substitution machine. The samples were incubated at -90°C for 19 h in acetone containing 0.1% tannic acid and 0.5 % glutaraldehyde. After three exchanges with cold acetone for 1 h each, the samples were placed in acetone containing 2% osmium tetroxide and 0.1% uranyl acetate, and the AFS procedure was started with the following schedule: -90°C for 13 h, + 5°C/h for 14 h, -20°C for 16 h, + 10°C/h for 2.4 h, 4°C for 1 h. The samples were then placed at room temperature, rinsed in acetone, incubated in

50% Epon for 4 h, 67% Epon for 48 h, and pure Epon for 7 h. After Epon polymerization at 60°C, 75 nm thin sections were cut using a diamond knife, mounted on copper grids, and imaged using a JEOL JEM-1400 TEM at 80 kV equipped with a Gatan Ultrascan 1000 CCD camera.

Samples were collected from six *cpd33* mutant plants and five wild-type plants at five-weeks old. A total of 156 minor veins from wild-type leaves and 118 minor veins from *cpd33* mutant leaves were analyzed.

Results

We used TEM to inspect wild-type and *cpd33* mutant leaves to investigate the ultrastructure of the minor veins. In wild-type leaves, the bundle sheath cells (BS) accumulated starch in the chloroplasts, but the mesophyll (M) cells lacked starch (Fig. 1A) In contrast, both the BS and M cells in *cpd33* mutant leaves over-accumulated starch (Fig. 1B). To test whether *cpd33* mutants had altered PD ultrastructure and/or frequency, we performed TEM and examined a total of 156 minor veins from wild-type leaves and 118 minor veins from *cpd33* mutant leaves. We analyzed a total of 713 wild type and 641 *cpd33* cell interfaces. The number of PD analyzed for wild type and *cpd33* mutants were 1098 and 1086, respectively.

The ultrastructure and frequency of PD between M – BS cells, BS – vascular parenchyma cells (VP), neighboring VP cells, and VP cells – thick-walled sieve element of *cpd33* mutant leaves were all similar to those of wild-type leaves (Fig. 1C-H and Table 1). However, although the frequency of PD at the CC-SE interface is normally low in wild-type plants (0.42 PD per interface) [4,5], we found that this frequency was significantly further reduced to 0.21 PD per interface in *cpd33* mutant minor veins (Table 1). The PD observed at the CC-SE interface had similar ultrastructure in both *cpd33* and wild-type veins, with no occlusions or structural aberrations (Fig. 1 I, J). However, we note that there was tremendous variability in the frequency of observed PD at this specific cellular interface. Nonetheless, these data suggest that the reduction in PD frequency between the CC and SE in the minor veins of *cpd33* mutant leaves may account for the carbohydrate hyperaccumulation phenotype.

Publication

The result of this project has been published under the title:

Tran, T.M., McCubbin, T.J., Bihmidine, S., Julius, B.T., Baker, R.F., Schauflinger, M., Weil, C., Springer, N., Chomet, P., Wagner, R. and Woessner, J. **(**2019) Maize *Carbohydrate partitioning defective33* Encodes an MCTP protein and functions in sucrose export from leaves. Molecular plant.

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sucrose-export-defective (SXD-1) mutant maize is limited by callose deposition at plasmodesmata in bundle sheath—vascular parenchyma interface. *Protoplasma*, *214*, 65–72.

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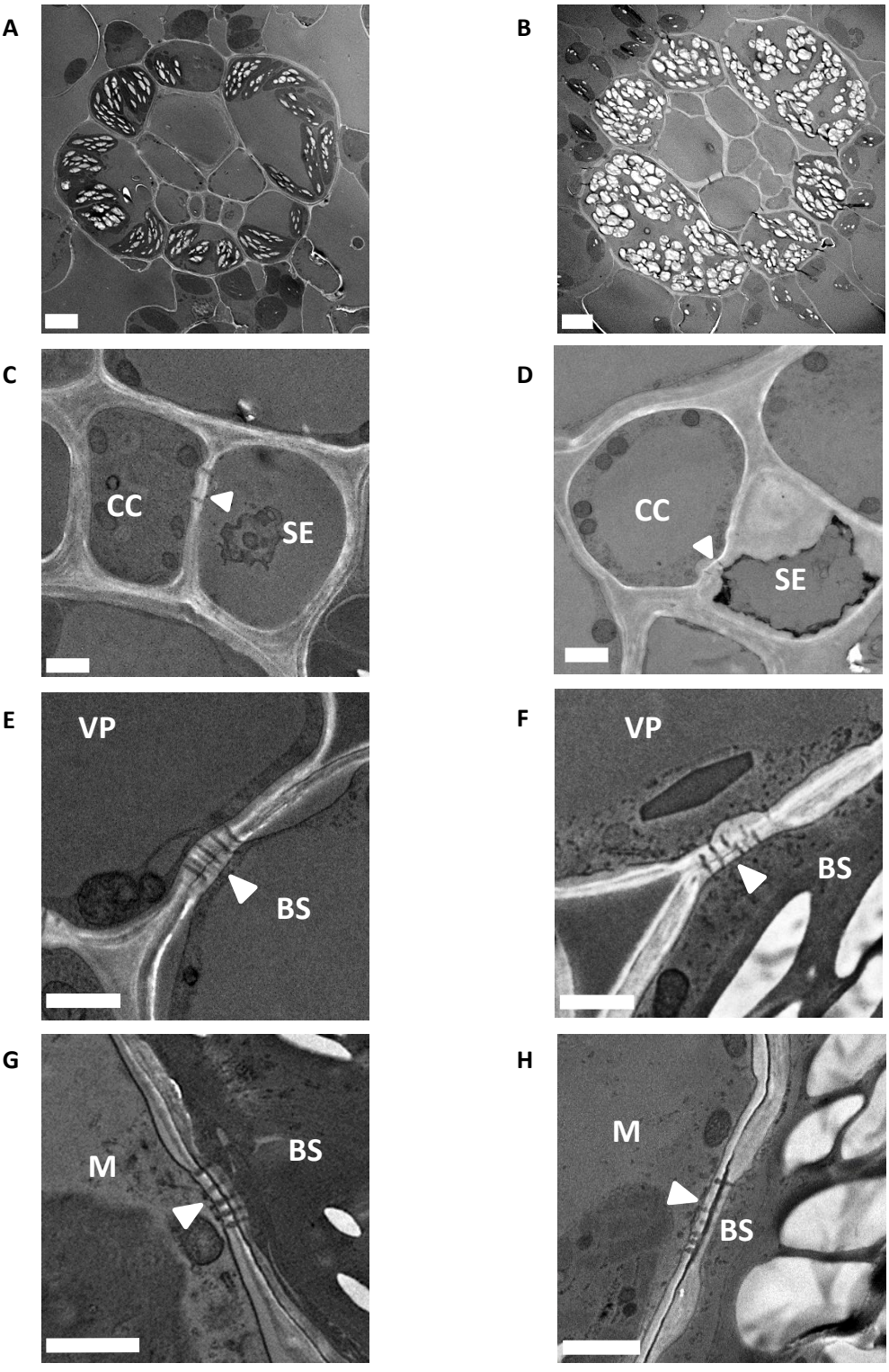
A
B
C
CC
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D
CC
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Figure 1. *cpd33* mutant leaves have normal vein structure, normal plasmodesmata (PD) structure and frequency. A minor vein of wild-types (A) and *cpd33* mutant (B) leaves, cpd33 leaves hyperaccumulated starch in bundle sheath cells (BS) and mesophyll cells (M). PD (white arrow) between companion cell (CC) and sieve element (SE) of WT (C) and *cpd33* mutant leaves (D). PD between vascular parenchyma cell (VP) and bundle sheath cell of WT (E) and *cpd33* mutant leaves (F). PD between mesophyll cell (M) and bundle sheath cell of WT (G) and *cpd33* mutant leaves (H). Scale bars = 5 μm (A and B), 1 μm (C to H).

Table 1. Number of PD at each cell interface expressed per minor vein and per cell-cell junction between different cell types in the leaves of *cpd33* mutant and wild-type (WT) plants. The values represent means ± Stder. Significant differences ($P < 0.05$) within a column determined by ANOVA are indicated by an *

Cell interface	M – BS	BS - VP		VP -VP		CC – SE (thin walled)		VP - Thick-walled SE	
	PD per vein	PD per vein	PD per cell interface	PD per vein	PD per cell interface	PD per vein	PD per cell interface	PD per vein	PD per cell interface
WT	28.91 ± 3.48	9.72 ± 1.41	4.96 ± 0.51	1.68 ± 0.36	0.79 ± 0.16	0.52 ± 0.08	0.42 ± 0.06	0.45 ± 0.08	0.26 ± 0.04
cpd33	20.86 ± 2.08	11.30 ± 1.11	5.35 ± 0.48	1.32 ± 0.23	0.58 ± 0.10	0.24* ± 0.06	0.21* ± 0.05	0.44 ± 0.06	0.28 ± 0.05