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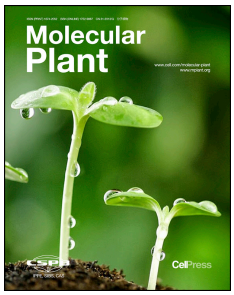

The cellulose synthases are cargo of the TPLATE adaptor complex

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Letter to the Editor

The cellulose synthases are cargo of the TPLATE adaptor complex

Dear Editor,

Clathrin mediated endocytosis (CME) is an evolutionary conserved mechanism by which plasma membrane (PM)-based cargo proteins are recognized by adaptor protein complexes and internalized. Apart from the canonical adaptor complex, AP-2, plant cells rely on the TPLATE complex (TPC) to execute CME (Gadeyne et al. 2014). The TPC is an octameric protein complex, consisting of TPLATE, TASH3, LOLITA, TWD40-1, TWD40-2, TML, AtEH1 and AtEH2 (Gadeyne et al. 2014). As the complex components are not conserved in yeast and animal cells CME initiation appears to be regulated differently in plants (Hirst et al. 2014). This raises important evolutionary questions concerning CME and cargo recognition across eukaryotic Kingdoms (Zhang et al. 2015). Based on *in silico* analysis TASH3, TPLATE, TML and LOLITA might have functions related to AP-2A, AP-2B, AP-2M and AP-2S, respectively (Hirst et al 2014; Zhang et al. 2015), which may include cargo recognition and clathrin recruitment to the PM. Indeed, similar to Arabidopsis AP2M and AP2S (Fan et al. 2013), TPLATE and TML are required for clathrin recruitment to the PM, even after AP-2 depletion (Wang et al. 2016). Therefore, we hypothesized that these proteins are involved in cargo recognition in plants.

Loss of TPC function results in male sterility (Gadeyne et al. 2014), similar to loss-of-function mutants of cellulose synthesis (Persson et al. 2007). CME has been reported to mediate cellulose synthase (CESA) complexes (CSCs) internalization in elongating hypocotyl cells (Bashline et al., 2013, 2015); supported by live cell imaging data (Supplemental Figure 1; (Miart et al. 2014). Since cellulose synthesis is essential for plant growth and the CSCs are unique to plants, it might be expected that the CESAs are TPC cargo, which could explain why the TPC is maintained in plants but not in animals and yeasts. To test this, we performed co-immunoprecipitation (Co-IP) experiments, which revealed that CESA6, a component of the primary wall CSC, interacts with both TML and TPLATE (Figure 1A, upper panel). We corroborated this finding by bimolecular

fluorescence complementation (BiFC) between the Arabidopsis primary wall CESAs, i.e. CESA1, 3 and 6 and TPLATE (Figure 1A bottom panel and Supplemental Figure 2). To confirm that the detected fluorescent signals were not due to either over-expression or random collisions of the split FP halves, we co-expressed each of the primary CESAs with AtEH1, another subunit of the TPC. Here, we did not observe any detectable fluorescent signal from the BiFC assays (Figure 1A bottom panel and Supplemental Figure 2A and C), confirming that the TPLATE-CESA interactions are specific in our BiFC system and suggesting a lack of direct interaction between AtEH1 and the CESAs, while being connected through TPLATE. Notably, the CESA-TPLATE interactions correlated with a change in CESA localization patterns; from a homogeneous and diffuse distribution to a speckled pattern (Figure 1A bottom panel and Supplemental Figure 2A), possibly indicating internalized CSCs. To test this hypothesis, we repeated the BiFC assay between CESA6 and TPLATE in the presence of the clathrin marker CLC2-mCh (Supplemental Figure 2B and D). Here, the BiFC signal of the CESA-TPLATE co-localized with the clathrin marker CLC2-mCh (Supplemental Figure 2B). As expected from the multiple roles of clathrin, we also observed mCherry fluorescence at cellular compartments not marked by TPLATE-CESA interactions.

Defects in CESA internalization are likely to result in severe cellulose deficiency. Accordingly, lines altered in *TML* expression, pESTR:*amiR-TML* (Gadeyne et al. 2014), displayed defects in hypocotyl and root elongation (Figures 1B-upper panels), concomitant with cell swelling (Supplemental Figure 3C), similar to what is found in seedlings impaired in primary wall cellulose synthesis (McFarlane et al. 2014). Furthermore, we observed a strong reduction in cellulose content and an increase in sugars corresponding to non-cellulosic polysaccharides, mainly those of pectin-related monosaccharides (e.g. uronic acids) compared to control seedlings (Figures 1B-bottom panel and Table S1). Plants impaired in two other main steps of CME: vesicle assembly by clathrin triskelia (pINTAM>>RFP-HUB) and vesicle scission (*drp1a-2/rsw9-2*), showed similar phenotypes and cell wall composition to the *amiR*:*TML* line (Supplemental Figure 3 and Table S1). Interestingly, a null mutant for a subunit of the other plant early adaptor complex, the canonical AP-2 (*ap2m-1*), showed increase in hypocotyl length, did not display defects in cell swelling and its sugar composition was comparable to wild-type seedlings (Supplemental Figure 3, Table S1; Bashline et al., 2015).

Defects in CESA internalization should impact CESA dynamics at the PM. Live cell imaging

revealed an increase in FP-CESA6 density at the PM in *amiR*-TML seedlings compared to their control (0.9 ± 0.1 foci/μm^2 in control cells versus 1.6 ± 0.1 foci/μm^2 in *amiR-TML* cells; Figure 1C left and upper-right panels). The increase in FP-CESA6 density could be due to either an enhanced delivery or a reduced internalization rate of the CSCs to and from the PM, respectively. To differentiate between these processes, we calculated the CESA delivery rate (Gutierrez et al. 2009), and found no differences in CESA delivery rate to the PM between the lines (Figure 1C upper-right panel). The increased CESA6 density at the PM should therefore reflect a reduction in the internalization rate of CSC when TML activity is impaired. Moreover, the reduction of CESA6 internalization correlated with an increased amount of slow moving CESA particles at the PM (indicated by less tilted traces in kymographs; Figure 1C left panel). The *drp1a-2* mutant showed similar CSC homeostasis at the PM as observed for *amiR-TML*; i.e., increased CESA6 density and slow-moving particles (Supplemental Figure 4). As CESA movement is related to its activity this explains the reduced cellulose content in the TML and DRP1A knockdown/knock-out lines. As reported previously (Bashline et al., 2015), we did not observe any differences in the CSC speed at the PM between *ap2m-1* and wild-type cells (Supplemental Figure 4) and nor did we observe any significant differences in FP-CESA6 density at the PM in *ap2m-1* as compared to wild-type (Supplemental Figure 4B). While these data contrast with previous reports (Bashline et al. 2013; 2015), they do explain the absence of cellulose and anisotropic cell growth reduction in the *ap2m-1* mutant as compared to the wild-type (Supplemental Figure 4; Bashline et al., 2015). Our results therefore indicate that the TPC is key in the regulation of CSC trafficking and, hence, cellulose synthesis in Arabidopsis seedlings.

With the aim of exploring whether CESAs are also internalized via TPC-related CME in other plant species, we performed split-Luciferase assays combining the rice TPC proteins *Os*TML and *Os*TPLATE2, with *Os*CESA8 and *Os*CESA4, which are primary and secondary wall rice CESAs, respectively. We could detect clear luciferase signals from these assays, which indicate that the rice CSCs also interact with TPC components (Figure 1D). In addition, we performed immuno-precipitation (IP) using an *Os*CESA4 antibody coupled with mass spectrometry (MS) to identify *Os*CESA4 interactors in rice plants. Notably, we found several TPC subunits, including *Os*TPLATE2, *Os*TML, *Os*TWD40-1, *Os*EH2, *Os*TASH3, and *Os*DRP2B/BC3, as well as other cellulose-related proteins in the pellet (Table S2). Our mass spec results did not identify AP-2

subunits in the pull-downs of any of our IP replicates. Hence, CSCs might also be CME cargo recruited by the TPC early adaptor in rice.

In vivo evidence for cargo-CME co-internalization, and re-localization of cargo by the CME components via BiFC, has not been reported in plants. In addition, we were able to identify a plant CME cargo by IP-MS, which is rare in plant biology. Hence, our results provide a foundation for further elaborations of CME-mediated events in plant cells. Our *in vivo* interaction data between TPLATE and TML with CSC, both in Arabidopsis and rice, indicate that TPLATE and TML might recognize the CSC for its internalization. TML has a muHD domain that is also present in APμ subunits (e.g. AP-2M in the AP-2 complex) and in the muniscins (Hirst et al. 2014; Zhang et al. 2015), so it might be anticipated that TML can recognize cargo. Notably, some protein domains suggest a relationship between TPLATE and AP-2B, which does not include any cargo-recognition motif. Our *in vivo* interaction results between TPLATE and CSC suggest that the TPLATE subunit of the TPC may have a cargo-binding capacity in plant cells.

Our results indicate that the TPC is the main early adaptor that recognizes CSC for its internalization via clathrin. The data presented in the current work are in agreement with the fact that the TWD40-2 subunit of the TPC contributes to cellulose synthesis (Bashline et al. 2015), supporting a prominent role of the TPC in this process. AP2-M has been shown to bind to the central cytosolic domain of the primary CESAs (Bashline et al. 2013), which contain putative AP-2M binding domains (YxxΦ) in a yeast assay. Therefore, the CSC might be recognized by both plant TPC and AP-2 early adaptors, possibly at different regions of its cytosolic domains. Notably, TPC and AP-2 co-localize in approximately 50% of all CME events, suggesting a complementary role of these CME adaptors (Gadeyne et al. 2014), plausibly relating to particular cargo or certain growth circumstances. This might be the case under certain stress conditions, such as those experienced by the plant when the TPC is not fully active (Barth and Holstein 2004; Bashline et al. 2015).

In summary, although it has been shown that several cargo proteins showed defective internalization upon depletion of TPC subunits (Gadeyne et al., 2014), our data represent the CSC as the first identified TPC-cargo interaction in seed plants, recognized by TML and TPLATE subunits, adding insights into the evolution of cargo recognition in plant CME.

Figure Legend

Figure 1. TPLATE and TML interact with CESAs and influence cellulose synthesis

A. Arabidopsis primary wall CESAs interact with TML and TPLATE *in planta*. (**Upper panel**) Co-immunoprecipitation (Co-IP) shows that CESA6 interacts with the TPC subunits. (**Upper-left)** 10 µL of total extract of proteins Input (I), Non-bound (Nb) and Bound (B) fractions. (**Upper-right)** 25 µL of Bound fractions. The experiment was performed twice with similar results. (**Bottom panel**) Bimolecular Fluorescence Complementation (BiFC) assays showing interaction of Arabidopsis primary wall CESAs and TPC subunits in *N. benthamiana* epidermal leaf cells. The N-terminal (YN) or C-terminal (YC) part of VENUS was fused in frame with CESA6, TPLATE and *At*EH1. Construct combinations are indicated in each figure panel. As a transformation control, the nuclear marker CFP-N7 (cyan) was included in all experiments.

B. TML is essential for cell elongation and cellulose synthesis in Arabidopsis. (Upper-left panel) Representative images of 5-day-old etiolated seedlings expressing pESTR:*amiR-TML* (*amiR-TML*) and their control grown on either EtOH- or on 5 µM beta-estradiol (in EtOH)-containing medium. Scale bars= 1cm. **(Upper-right panel)** Hypocotyl length of lines shown in (Upper left). Data represent the average (±SE) of n = 3 biological replicates, each containing 20-30 seedlings. **(Bottom panel)** Cellulose and uronic acid (UA) content of the lines shown in (Upper panels), represented as µg of glucose (D-Glc) or µg of glucuronic acid (GlcA) per mg of dried alcohol insoluble residue (AIR), respectively. Data represent the average (±SE) of n = 3 biological replicates, each with three technical repetitions. Student's t test; P-value **<0.01, *< 0.05.

C. **Impaired TML function alters the density and dynamics of plasma membrane localized CESAs. (Left panel)** Representative images from movies obtained with a spinning disk confocal microscope of tdTomato-CESA6 in *amiR-TML* background and its control at the plasmam membrane (PM) in hypocotyl cells of 3-day-old etiolated seedlings. PM particle density shown in single-frame images (particles highlighted in magenta). Time averaged projections of frames acquired over 5 min, and corresponding kymographs showing CESA6 trajectories and movement, respectively. Scale bars= 5µm. **(Upper-right panels)** Quantification of tdTomato-CESA6 density and delivery rate at the PM in photo-bleached areas of cells imaged with a spinning disk confocal microscope. Data represent average (±SE) of at least 6 cells per treatment or genotype; Student's t test P-value *<0.05; **<0.01. **(Bottom-right panel)** Histogram of CSC speeds at the PM in tdTomato-CESA6 expressing seedlings

shown in (Left panel). Mean (±SE) speeds for each line or treatment are given in parentheses. $n \geq$ 589 particles; ≥ 6 cells and ≥ 6 seedlings were tracked. A graph showing the percentage of slow (0-200nm/min) and fast (>200nm/min) moving CSC is included in each panel.

D. Rice primary and secondary wall CESAs can interact with TML and TPLATE2 *in planta*. Split-luciferase complementation assay showing that the primary cell wall *Os*CESA8 can interact with *Os*TML (**Upper panel**) and *Os*TPLATE2 (**Middle panel**). The secondary wall *Os*CESA4 can interact with *Os*TML (**Bottom panel**). Scale bars = 1cm

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Author contributions

CSR, YZ and SP managed the project and wrote the article; CSR, YS, CK, DZ, GSA, LT, AI, ERL, and MF carried out experiments; CSR, YZ, SP, AN, AB, and TU acquired funding and provided supervision; KS, IW and DVD provided additional data; CSR, SP, DVD and AB reviewed and edited the manuscript. .

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Supplemental Information

Document S1. Materials and Methods. Supplemental Figures 1-4, Supplemental Tables 1-3, and Supplemental References.

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