

Functional analysis of complexes with mixed primary and secondary cellulose synthases

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In higher plants, cellulose is synthesized by cellulose synthase complexes, which contain multiple isoforms of cellulose synthases (CESAs). Among the total 10 *CESA* genes in Arabidopsis, recessive mutations at three of them cause the collapse of mature xylem cells in inflorescence stems of Arabidopsis (*irx1^{cesa8}*, *irx3^{cesa7}* and *irx5^{cesa4}*). These *CESA* genes are considered secondary cell wall CESAs. The others (the function *CESA10* is still unknown) are thought to be specialized for cellulose synthesis in the primary cell wall. A split-ubiquitin membrane yeast two-hybrid system was used to assess interactions among four primary CESAs (*CESA1*, *CESA2*, *CESA3*, *CESA6*) and three secondary CESAs (*CESA4*, *CESA7*, *CESA8*). Our results showed that primary CESAs could physically interact with secondary CESAs in a limited fashion. Analysis of transgenic lines showed that *CESA1* could partially rescue *irx1^{cesa8}* null mutants, resulting in complementation of the plant growth defect, collapsed xylem and cellulose content deficiency. These results suggest that mixed primary and secondary *CESA* complexes are functional using experimental set-ups.

Primary cell walls differ from secondary cell walls in the composition of polysaccharides, the mechanical and structural properties of microfibrils, and their specific roles during plant growth.¹ Primary cell walls are synthesized during cell expansion. Therefore primary cell walls are highly extensible and incorporative. Secondary cell walls are synthesized after the cell ceases its expansion and they provide tensile strength and rigidity, but not extensibility. Although cellulose is a major component of both primary and secondary cell walls, the degree of polymerization, the amount of cellulose relative to other wall polymers and the dimensions of microfibrils differs in primary and secondary walls.² These differences may arise from the composition and/or activity of cellulose synthase complexes (CSCs) and their interactions with cytosolic components.³ Consistent with this idea, CSCs for primary wall cellulose synthesis have different sets of *CESA* proteins from those for secondary wall cellulose synthesis. For primary wall cellulose synthesis, CSCs are composed of *CESA1*, 3, 6 or 6-like primary *CESA* proteins, whereas *CESA4*, 7 and 8 are required to form functional CSCs during secondary wall cellulose synthesis.⁴⁻⁶

In this short communication, pair-wise interactions were tested between four primary CESAs (*CESA1*, 2, 3 and 6) and three secondary CESAs (*CESA4*, 7 and 8) using the split-ubiquitin yeast two-hybrid (Y2H) system.⁷ Each of the primary CESAs investigated were able to form homodimers except for *CESA1* (Fig. 1). For primary CESAs, the following interactions were observed: *CESA1* with *CESA3* and *CESA6*, *CESA3* with *CESA2* and *CESA6*, and *CESA2* with *CESA6*. Two of the three secondary

CESAs, *CESA7* and *CESA8*, were able to form homodimers. Although *CESA4* was not able to form homodimers, it was able to interact with *CESA7* and *CESA8*. *CESA7* and *CESA8* were also able to interact with one another. Interactions were also observed between primary and secondary CESAs. *CESA7* interacted with both *CESA1* and *CESA3*. *CESA4* and *CESA8* were each able to interact with each of the primary CESAs under investigation except for *CESA1*. These results indicate that primary CESAs can physically interact with secondary CESAs in a limited fashion.

To test whether mixed primary and secondary CESAs are functional in planta, we generated a series of promoter-swap constructs. Analysis of the expression profile of primary and secondary *CESA* indicates that secondary CESAs are more tightly controlled.⁸ Since the three secondary *CESA* genes have similar expression profiles (Fig. 2A), the *CESA7* promoter was chosen to drive the expression of primary CESAs (*CESA1* and *CESA3*) in cells synthesizing secondary cell walls. *CESA1* and *CESA3* were chosen for the promoter-swap experiment because these two *CESA* isoforms are believed to be essential for primary CSCs. Two constructs, P7C1 and P7C3 (Fig. 2B, P7 refers to the *CESA7* promoter and C1 and C3 refer to *CESA1* and *CESA3* cDNA, respectively), were transformed into three secondary *CESA* mutants, *irx5^{cesa4}*, *irx3^{cesa7}* and *irx1^{cesa8}*.⁹⁻¹¹ Since these are null mutants, it is possible to determine whether P7C1 or P7C3 can complement the function of any of the individual secondary *CESA* mutants without any interference from the endogenous secondary *CESA* of interest. The growth morphology was first

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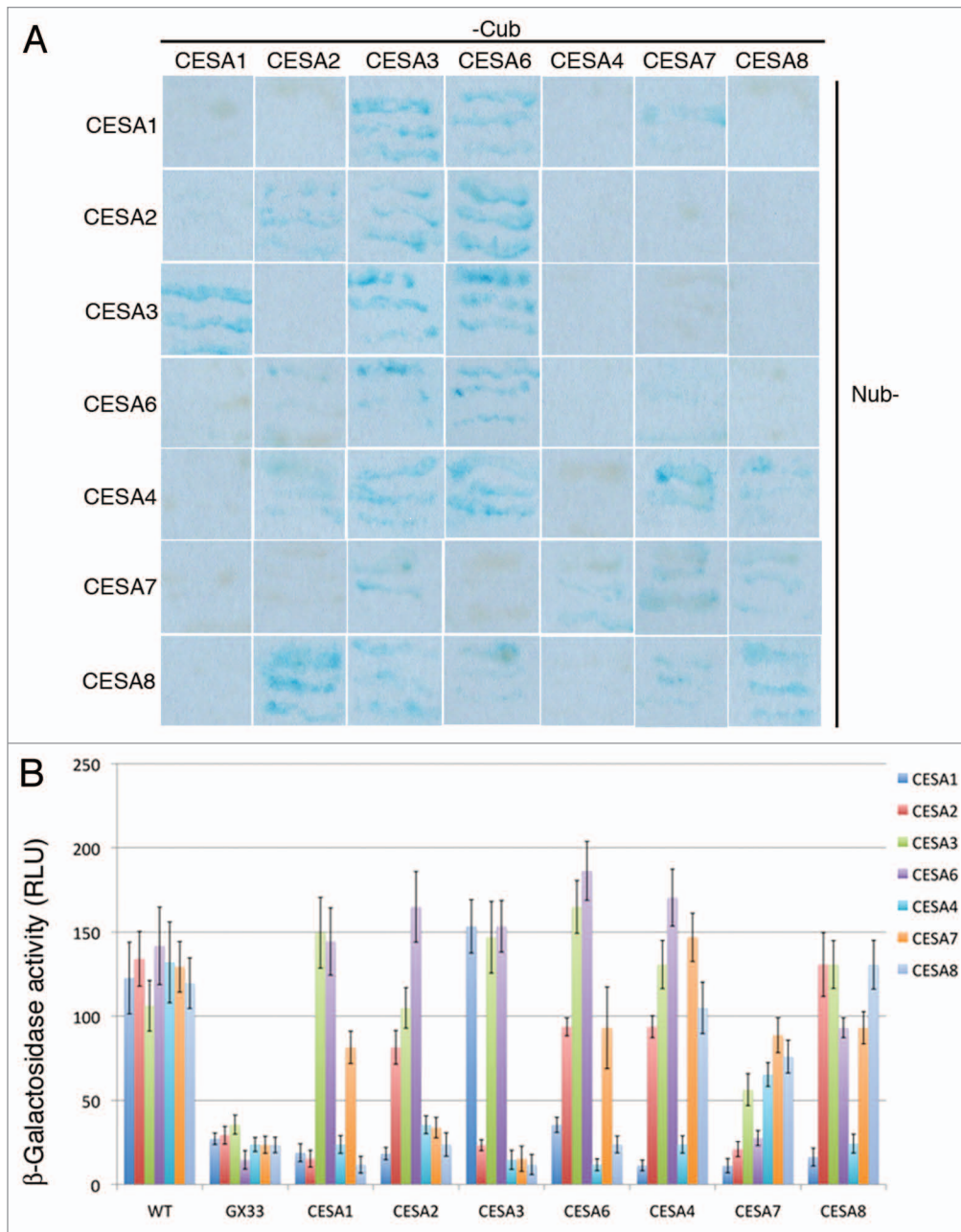


Figure 1. Interactions between CESA isoforms. (A) The growth and β -galactosidase expression of yeast transformed with plasmids containing both N-terminal fusions of Nub to a CESA (indicated by the labels of the top row) and C-terminal fusions of Cub to a CESA (indicated by the labels in the right column). (B) β -galactosidase activity was quantified colorimetrically in colonies expressing pairs of Nub and Cub CESA fusions. Cub CESA fusions were also expressed with WT Nub or mutated Nub (GX33) as a positive and negative control, respectively. The error bars indicate standard deviation.

examined in all transgenic lines. P7C1 partially complemented several *irx1^{cesa8}* growth defects such as dwarf stature, reduced fertility, reduced leaf size and short siliques (Fig. 3A–B). *irx1^{cesa8}* is also known to have defects in xylem cells.^{9,10} Analysis of the cross section of P7C1 *irx1^{cesa8}* stems showed that P7C1 completely rescued the collapsed xylem defect of *irx1^{cesa8}*, indicating P7C1 can incorporate in the secondary CSCs (Fig. 3C–E). The growth defects in the adult stem and leaves of *irx1^{cesa8}* are consistent with reduced cellulose content in these tissues (Fig. 3F). P7C1 recovered the cellulose content deficiency phenotype of *irx1^{cesa8}* whereas the rest

of transgenic lines did not affect the cellulose content in their respective mutant background. Interestingly, the only interaction partner of CESA1 in the split-ubiquitin yeast two-hybrid assay was CESA7, suggesting that CESA1 may substitute for CESA8 in a way that differs from the native functional secondary CSCs. This observation may explain the partial complementation of *irx1^{cesa8}* growth defects by P7C1.

Carroll et al. reported that all primary CESAs (CESA1, CESA3 and CESA6) interacted with secondary CESAs (CESA4, CESA7 and CESA8) in a split-ubiquitin Y2H assay.¹² We cannot

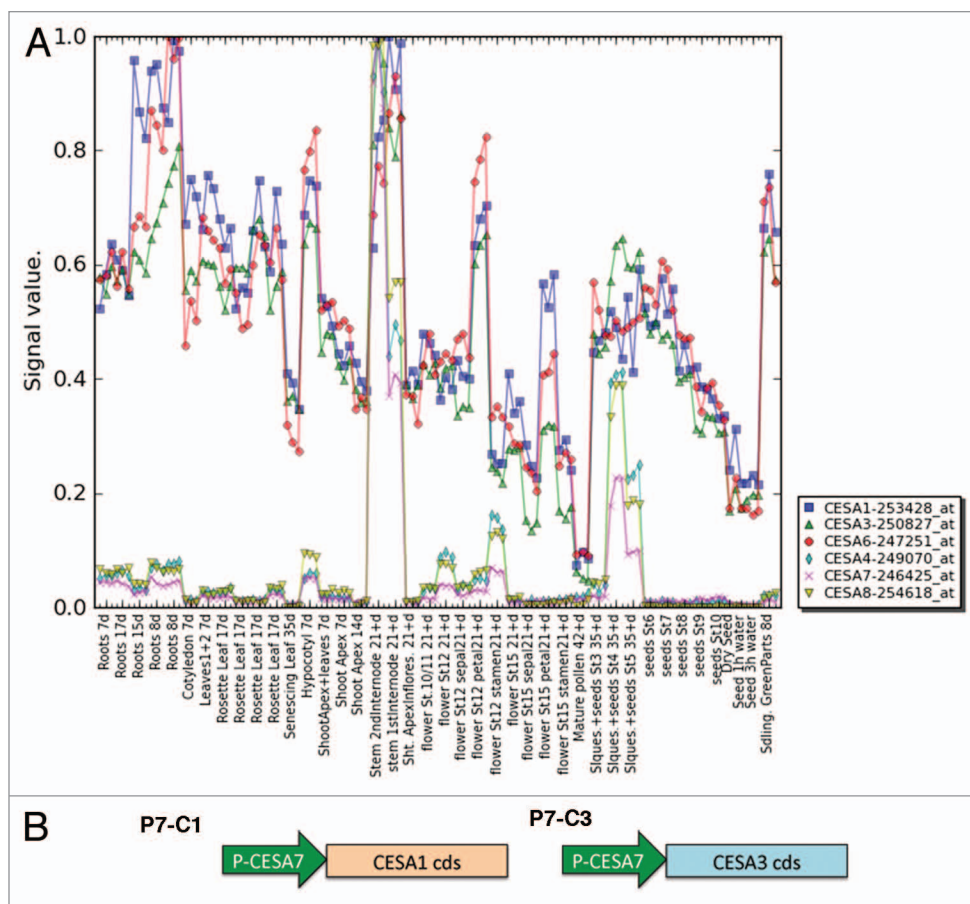


Figure 2. Design for promoter swap constructs. **(A)** The expression of the primary and secondary CESAs during different developmental stages in diverse organs (Schmid et al., 2005). **(B)** Schematic diagram of primary CESAs driven by the secondary CESA7 promoter. Arrows indicate promoter regions. Rectangular boxes indicate the coding sequence of primary CESAs.

confirm this observation with our split-ubiquitin Y2H assay. This discrepancy may arise from the ability of our split-ubiquitin system to optimize the expression of Cub-CESAs under the control of MET25 promoter.⁷ We tested interactions on different methionine concentrations to keep the background to the minimum. Moreover, different from Carroll et al., our system generates a C-terminal fusion of Cub for the bait CESA protein and an N-terminal fusion of Nub for the prey CESA protein. The positions of the fusions could potentially alter the interaction abilities. The split-ubiquitin Y2H is prone to false positives and false negatives, therefore it is crucial to validate all possible fusion combinations in planta. Nevertheless, our results indicate that there are limited cross-interactions between primary and secondary CESAs, suggesting that CESAs have preferential interaction partners in the CSC complex that may impact the assembly or activity of CSCs.

Although many studies have shown unique components in primary (CESA1, 3, 6 and 6-like) and secondary CESA complexes (CESA4, 7 and 8), our results suggest mixed primary and secondary CESA complexes are functional in Arabidopsis. The primary and secondary CESAs are thought to be expressed at different times in plants according to microarray analysis (Fig. 2). However, there may be a limited period of time when

both primary and secondary CESA genes are coexpressed. There are several reports supporting a role of primary CESAs during secondary cell wall formation. For example, the primary CESA clades (CESA1, 3, 5 and 6) are required for secondary wall synthesis in Arabidopsis trichomes.¹³ Another example came from the most recent finding that primary CESAs have function in the formation of secondary cell walls during seed coat development.^{14,15} Specifically, CESA5 is responsible for mucilage attachment and CESA2, 5 and 9 contribute to secondary cell wall biosynthesis in the columella cells.¹⁶ These findings suggest that the distinction between primary and secondary CESAs might not be as strict as initially defined. Moreover, defects in secondary cell wall cellulose biosynthesis showed complex alterations in the primary wall structure in *cesa7^{mur10}* and *cesa4^{brittle cum11}*, suggesting that the formation of primary cell wall and secondary cell wall might not be entirely separated or that an integrity sensing mechanism exists from the secondary wall to the primary wall synthesis and remodeling machinery.^{15,17-19} The molecular details of the primary to secondary cell wall transition and mechanisms that underlie the cross talk between primary and secondary cell wall biosynthesis are largely unknown.

Our results suggest that mixed primary and secondary CSCs are functional using experimental setups. The question remains

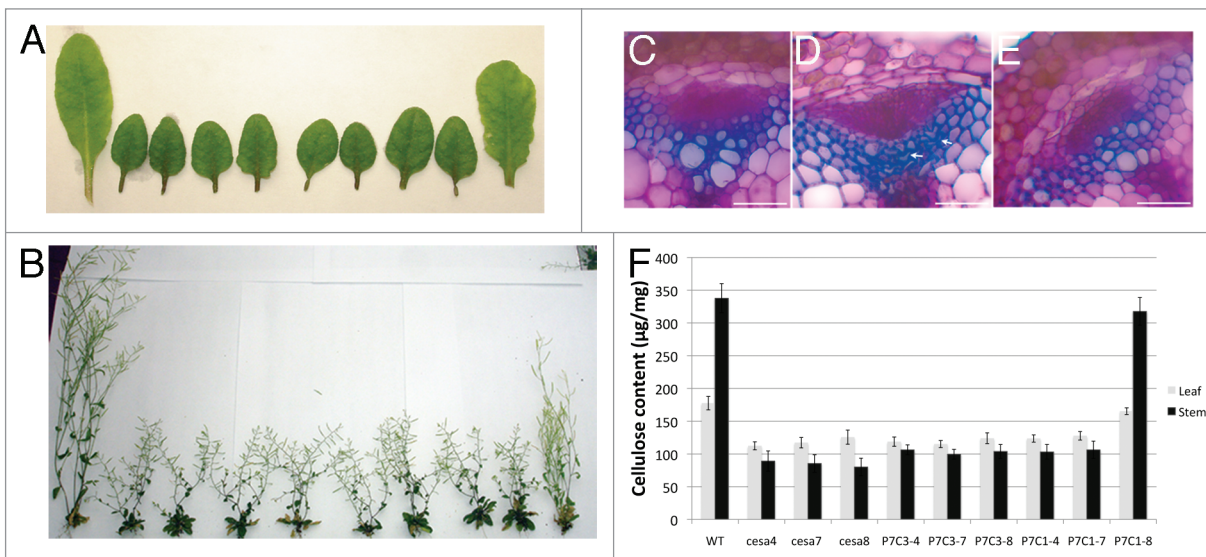


Figure 3. P7C1 partially complements morphological and molecular defect in *irx1^{cesa8}*. (A) Leaf morphology of secondary *cesa* mutants and various promoter-swap transmorphants in secondary *cesa* mutants. From the left to right, wild type (WT), *irx5^{cesa4}*, *irx3^{cesa7}*, *irx1^{cesa8}*, P7C3 in *irx5^{cesa4}* (P7C3-4), P7C3 in *irx3^{cesa7}* (P7C3-7), P7C3 in *irx1^{cesa8}* (P7C3-8), P7C1 in *irx5^{cesa4}* (P7C1-4), P7C1 in *irx3^{cesa7}* (P7C1-7), P7C1 in *irx1^{cesa8}* (P7C1-8). (B) Whole-plant morphology of secondary *cesa* mutants and various promoter-swap transgenic lines. Plants were arranged in the same order as shown in (A). (C–E) Transverse sections stained with toluidine blue were taken from the base of stem. Wild type (C), *irx1^{cesa8}* (D) and P7C1 *irx1^{cesa8}* (E). White arrows indicate the collapsed metaxylem. Bar = 50 µm. (F) Cellulose content in leaf or stem from wild type and various transformants in secondary *cesa* mutants. Error bars represent SE, n = 5. The figure is modified from Carroll et al., 2012 (www.plantphysiol.org, Copyright American Society of Plant Biologists).

if mixed primary and secondary complexes exist in planta. Nevertheless, our findings may provide insight on the organization or position of CESA isoforms within CSCs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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