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Interactions between membrane-bound cellulose synthases involved in the synthesis of the secondary cell wall

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ABSTRACT

It has not yet been reported how the secondary CESA (cellulose synthase) proteins are organized in the rosette structure. A membrane-based yeast two-hybrid (MbYTH) approach was used to analyze the interactions between the CESA proteins involved in secondary cell wall synthesis of *Arabidopsis* and the findings were confirmed in planta by bimolecular fluorescence complementation (BiFC) assay. Results indicated that although all CESA proteins can interact with each other, only CESA4 is able to form homodimers. A model is proposed for the secondary rosette structure. The RING-motif proved not to be essential for the interaction between the CESA proteins.

Structured summary:
MINT-6951243: *PIP2-1* (uniprotkb:P43286) *physically interacts* (MI:0218) *with PIP2-1* (uniprotkb:P43286) *by bimolecular fluorescence complementation* (MI:0809)
MINT-6950816: *CESA4* (uniprotkb:Q84JA6) *physically interacts* (MI:0218) *with CESA4* (uniprotkb:Q84JA6) by *membrane bound complementation assay* (MI:0230)
MINT-6951056, MINT-6951071, MINT-6951088, MINT-6951103: *CESA7* (uniprotkb:Q9SWW6) *physically interacts* (MI:0218) with *CESA4* (uniprotkb:Q84JA6) by *bimolecular fluorescence complementation* (MI:0809)
MINT-6950949, MINT-6950990: *CESA4* (uniprotkb:Q84JA6) *physically interacts* (MI:0218) with *CESA8* (uniprotkb:Q8LPK5) by *membrane bound complementation assay* (MI:0230)
MINT-6950909, MINT-6951030: *CESA4* (uniprotkb:Q8LPK5) *physically interacts* (MI:0218) with *CESA7* (uniprotkb:Q9SWW6) by *membrane bound complementation assay* (MI:0230)
MINT-6951042: *CESA4* (uniprotkb:Q84JA6) *physically interacts* (MI:0218) with *CESA4* (uniprotkb:Q84JA6) by *bimolecular fluorescence complementation* (MI:0809)
MINT-6951004, MINT-6951016: *CESA8* (uniprotkb:Q8LPK5) *physically interacts* (MI:0218) with *CESA7* (uniprotkb:Q9SWW6) by *membrane bound complementation assay* (MI:0230)
MINT-6951217, MINT-6951230: *CESA4* (uniprotkb:Q84JA6) *physically interacts* (MI:0218) with *CESA8* (uniprotkb:Q8LPK5) by *bimolecular fluorescence complementation* (MI:0809)
MINT-6951120, MINT-6951140, MINT-6951156, MINT-6951170, MINT-6951185: *CESA8* (uniprotkb:Q8LPK5) *physically interacts* (MI:0218) *with CESA7* (uniprotkb:Q9SWW6) by *bimolecular fluorescence complementation* (MI:0809)
MINT-6951199: *CESA8* (uniprotkb:Q8LPK5) *physically interacts* (MI:0218) *with CESA8* (uniprotkb:Q8LPK5) by *bimolecular fluorescence complementation* (MI:0809)

1. Introduction

Cellulose synthases (CESAs) are components of membrane-localized complexes (rosettes), and catalyze cellulose fibers elongation. Three CESA family members (#4, #7, and #8) have shown to be required for the formation of a rosette protein complex

Abbreviations: MbYTH, membrane-based yeast two hybrid; CESA, cellulose synthase; BiFC, bimolecular fluorescence complementation; TMD, transmembrane domain; TF, transcription factor; YFP, yellow fluorescent protein

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involved in secondary cell wall cellulose biosynthesis in *Arabidopsis thaliana* [1], hereafter referred to as the secondary CESA proteins. The secondary CESAs are not functionally redundant and gene expression suggest that *CESA4*, *CESA7* and *CESA8* are the only CESAs involved in cellulose synthesis in the secondary cell wall [2]. Immuno-precipitation experiments showed that these CESA proteins co-precipitate [1,3]. Although, this is a step towards the clarification of the CESA protein complex, the specific composition and structure of the rosette complex remain elusive.

All CESAs contain eight transmembrane domains (TMDs) and two putative N-terminal zinc-fingers. These zinc-fingers are thought to mediate protein–protein interactions between the CESAs [4]. However, the disrupted-rosette phenotype of the *rsw1* mutant (V549A) [5] and domain swapping experiments [6] suggest that also other regions of the CESA protein play a role in rosette assembly. The most accepted model of the rosette has been proposed by Scheible and co-workers [7] in which the rosette structure has six symmetrically arranged subunits that in turn consist of six CESA proteins. However, there is no experimental evidence as to how the different CESA proteins are arranged within the complex or the subunits.

To form such a regular structure, the interactions between the CESA proteins are expected to be highly specific. To get more insight into the different interactions, a method to detect one-to-one protein interactions of membrane-bound proteins is essential. The split-ubiquitin membrane-based yeast two-hybrid system (MbYTH) allows the screening for interaction between the different membrane-bound CESA isoforms in yeast [8].

The bimolecular fluorescence complementation (BiFC) assay [9] was implemented to confirm the interactions in living plant cells. This technique provided evidence that the primary CESA proteins can interact in vivo, and therefore are present in the same complex [10]. In this report the possible interactions between the secondary CESA proteins is discussed, and a model for the rosette organization is proposed. Finally the role of the RING-finger motif in protein interaction is discussed.

2. Materials and methods

2.1. Membrane-based yeast two-hybrid (MbYTH) screen

Yeast strain NMY51 (Dualsystems Biotech AG) was transformed according to the protocol (DUALmembrane Kit 1). Interactions were quantified by 100 colonies spotted on SD medium (lacking Leucine, Tryptophan, Histidine and Adenine) containing the appropriate concentration of 3-ammonium-triazole (130 mM, 10 mM, and 75 mM, for baits CESA4, CESA7, and CESA8, respectively) and grown at 30 °C for five days, the number of spots grown was scored. Detection β-galactosidase activity was performed with the filter-lift assay [11]. Experiments have been done twice to confirm results.

2.2. Constructs for the MbYTH system

The full-length cDNAs were obtained from the Riken Bioresource Center [12,13] *ATCESA4* (RAFL15-30-K05), *ATCESA7* (RAFL09-35-F05), and *ATCESA8* (RAFL09-65-M12). Restriction sites were generated by PCR with primers as indicated in Supplementary data (Table SI). The resulting PCR-products were digested and ligated in the pTFB1 vector (Bait) and the pADSL-Nx vector (Prey) (Dualsystems Biotech AG). Bait and prey expression is regulated by the TEF1 and ADH1 promoter, respectively. The sequences of the inserts were confirmed by Sanger sequence analysis. Experiments have been done four times to confirm results.

2.3. Site directed mutagenesis

The QuikChange Multi site-Directed MutagenesisKit from Stratagene (200514) was used to introduce point mutations into the RING-motif of CESA7 using primers CESA7C37, CESA7C56, CESA7C64, and CesA7C79 (Table SI) to introduce mutations C37A, C56A, C64A, C79A, respectively.

2.4. Bimolecular fluorescence complementation screen

Genes were cloned in the pBIFP-2 and pBIFP-3 plasmids and regulated by the constitutive 35S promoter [9]. The sequence of the primers used are in Table SI. Leaves of 3-week-old tobacco (*Nicotiana benthamiana*) plants were transformed by infiltration [10]. YFP (yellow fluorescent protein) fluorescence was detected 3 days after infiltration by using the 514-nm laser line of a SP2 AOBS confocal laser scanning microscope (Leica, Solms, Germany) equipped with an argon laser. To check the YFP reconstitution, spectral analysis was performed with the 496-nm laser line.

3. Results

3.1. Interactions between the secondary CESA proteins

The regular structure of the rosette suggests that the assembly of this complex is highly regulated. In order to understand these complexes, the first step is the identification of specific interaction between the building-blocks of the complex, the different CESAs. The membrane-based yeast two-hybrid (MbYTH) method was used to identify the interactions between membrane-bound CESAs as it avoids the need to co-purify membrane proteins present in the same complex. In this system the protein of interest (bait) is fused to Cub-transcription factor (TF) and expressed in yeast together with another protein (prey) fused to NubG [8]. Upon interaction between the bait and prey, the Cub-TF and NubG reconstitute and the TF is released by a protease so it can activate reporter gene expression. As the interaction is detected by a protease, the location of interaction is therefore not restricted to the nucleus but might also occur at the plasma membrane [8].

The selection with two different auxotrophic markers increased the reliability of the system dramatically in that the prey had to circumvent two different pathways to auto-activate the system, as well as a colorimetric marker. The screening was optimized for each bait by addition of appropriate amounts of inhibitor (3-AT) to the selected medium so that growth of the yeast expressing a bait protein and the positive or negative control were significantly different, to rule out auto activation and to make it possible to screen for interactions between different proteins.

All possible combinations of fusion proteins were grown on selective medium to determine their interactions. Fig. 1A shows the results of the interactions when CESA4 was used as bait, indicating strong interaction with itself and CESA8, and a weaker yet still significant interaction with CESA7. When CESA7 was used as the bait, strong interactions were detected with CESA4 and CESA8, however, CESA7 did not homodimerize (Fig. 1B). Similar results were obtained with CESA8 as a bait; CESA8 interacted with the other CESAs, but was unable to homodimerize (Fig. 1C).

3.2. Identification of CESA interactions in planta

The interactions were also tested in planta using BiFC assays. In this system a YFP fragment, either YFP/N or YPF/C, was linked to the N-terminal part of the secondary CESA proteins and transiently expressed in *N. benthamiana*. To determine whether heterodimers could be formed, two different CESA proteins were co-expressed