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

Jaap Timmers [a,b,c], Samantha Vernhettes [d], Thierry Desprez [d], Jean-Paul Vincken [b,1], Richard G.F. Visser [b], Luisa M. Trindade [b,*]

[a] CRC-Wageningen, Wageningen University and Research Centre, 6703 HD Wageningen, The Netherlands
[b] Wageningen UR Plant Breeding, Wageningen University and Research Centre, 6708 PD Wageningen, The Netherlands
[c] Graduate School Experimental Plant Sciences, Wageningen University, The Netherlands
[d] Laboratoire de biologie Cellulaire, Institute Jean-Pierre Bourgin, INRA, Rte de Saint Cyr, Versailles, 78026 cedex, France

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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

* Corresponding author. Fax: +31 317 483457.
E-mail address: Luisa.Trindade@wur.nl (L.M. Trindade).
[1] Present address: Laboratory of Food Chemistry, Wageningen University, 6700 EV Wageningen, The Netherlands.

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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

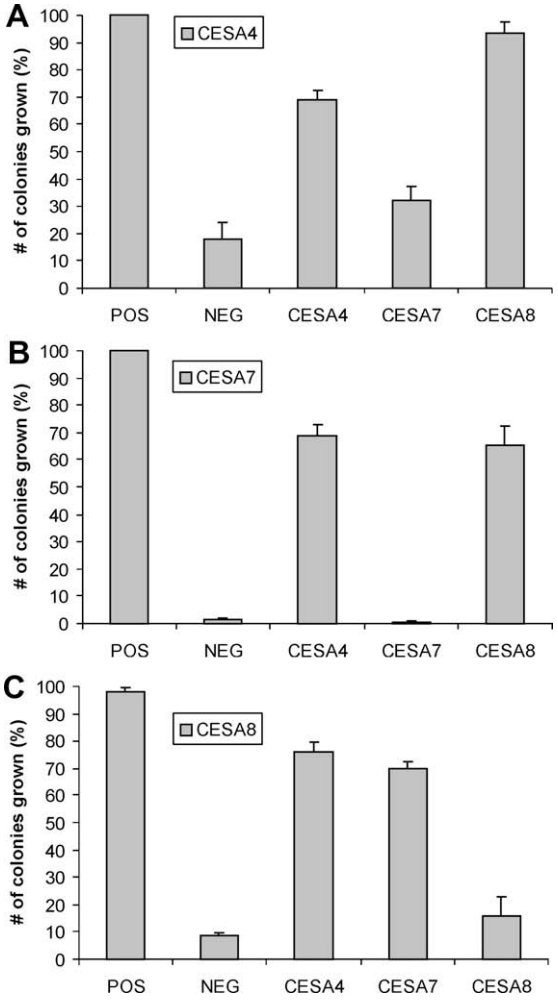

Fig. 1. Interactions between the secondary CESA visualized by yeast growth. Yeast expressing CESA 4 (A), CESA7 (B) or CESA8 (C) as bait with the ALG5 protein fused to NubI and NubG, pos. and neg. control respectively, and different CESA proteins NubG fused proteins, as indicated. The percentage of colonies that show visible growth after 5 days at 30 °C on selective medium is shown. Standard deviation is visualized by the error bar.

YFP/N-CESA4/YFP/C-CESA7, YFP/N-CESA7/YFP/C-CESA4 (Fig. 2F), YFP/N-CESA4/YFP/C-CESA8 (Fig. 2G), YFP/N-CESA8/YFP/C-CESA4,

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                  * *           * *   * *       * *
CESA7wt           CEICGDQIGLTVEGDLFVACNECGFPACRPCYEYERREGTQNCPQC
CESA7C37A         AEICGDQIGLTVEGDLFVACNECGFPACRPCYEYERREGTQNCPQC
CESA7C56A         CEICGDQIGLTVEGDLFVAANECGFPACRPCYEYERREGTQNCPQC
CESA7C64A         CEICGDQIGLTVEGDLFVACNECGFPAARPCYEYERREGTQNCPQC
CESA7C79A         CEICGDQIGLTVEGDLFVACNECGFPACRPCYEYERREGTQNAPQC
CESA7C37A+C56A    AEICGDQIGLTVEGDLFVAANECGFPACRPCYEYERREGTQNCPQC
CESA7C37A+C64A    AEICGDQIGLTVEGDLFVACNECGFPAARPCYEYERREGTQNCPQC
CESA7C37A+C79A    AEICGDQIGLTVEGDLFVACNECGFPACRPCYEYERREGTQNAPQC
CESA7C56A+C64A    CEICGDQIGLTVEGDLFVAANECGFPAARPCYEYERREGTQNCPQC
CESA7C56A+C79A    CEICGDQIGLTVEGDLFVAANECGFPACRPCYEYERREGTQNAPQC
CESA7C64A+C79A    CEICGDQIGLTVEGDLFVACNECGFPAARPCYEYERREGTQNAPQC
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Fig. 3. Sequence alignment of the CESA7 RING-motif indicating the substitutions. The alignment of the RING-motif of CESA7 (AA38 until AA79). * indicate the essential cysteines, the substitutions (C→A) are highlighted (grey) in the different mutated proteins.

YFP/N-CESA7/YFP/C-CESA8 (Fig. 2H), or YFP/N-CESA8/YFP/C-CESA7 (all interactions are shown in Fig. S2 of the Supplementary data). To prevent false positives all fusion proteins were tested for interaction with the negative control, the aquaporin PIP2-1 protein, and all combinations showed no interaction. Although not all combinations were able to restore the YFP fluorescence, results indicated that all isoforms can interact with each other. Some combinations gave a weak signal, indicating that this dimerization is less efficient, particularly the combination of CESA7 and CESA8 even lacked fluorescence above the threshold in some of the repeat experiments. Also homodimerization of the CESA proteins was tested. A strong signal was found for the combination YFP/N-CESA4/YFP/C-CESA4 (Fig. 2B) whereas YFP/N-CESA7/YFP/C-CESA7 (Fig. 2C) was unable to restore YFP fluorescence, and YFP/N-CESA8/YFP/C-CESA8 (Fig. 2D) only gave a very weak fluorescence signal. Whenever CESA8 was expressed the signal was weaker and punctuate structures appear.

3.3. Role of RING-finger in CESA interactions

The N-terminal region of each CESA protein contains a double zinc-finger motif ($CX_2CX_{12}FXACX_2CX_2PXCX_2CXEX_5GX_3CX_2C$) highly homologous to the RING-finger domain. RING-fingers have been implicated in mediating protein-protein interactions, in a redox regulated bridging between cysteine residues [4]. Protein–protein interaction studies with only these zinc-finger domains, showed that they were able to interact with themselves and with the zinc-fingers of other family members [14]. In order to get more insight in the mechanism of interaction between the CESA proteins, the RING-finger motif of CESA7 was mutagenized and

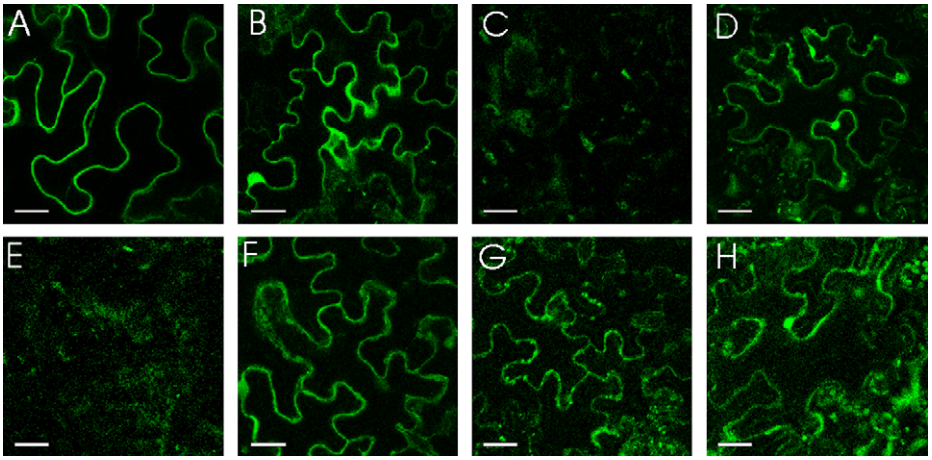

Fig. 2. BiFC in *N. benthamiana* shows *in vivo* dimerization between the secondary CESAs. Positive controls PIP/PIP (A), negative control CESA7/PIP (E), homodimerization of CESA4 (B), CESA7 (C) and CESA8 (D) and the different heterodimerizations YFP/N-CESA7/YPF/C-CESA4 (F), YFP/N-CESA8/YFP/C-CESA4 (G), and YFP/N-CESA7/YFP/C-CESA8 (H). Scale bar = 100 μm.

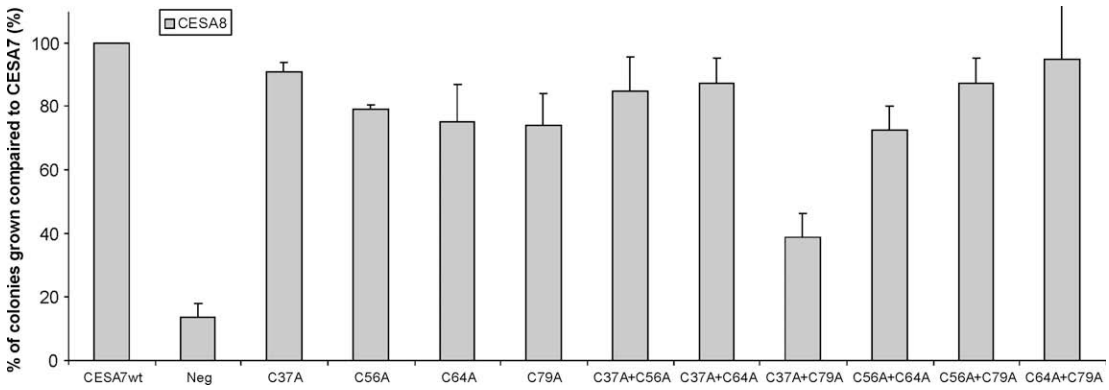

Fig. 4. Effects of the substitution mutations on the interactions between CESA7 and CESA8. Yeast expressing CESA8 as bait with the wild type CESA7 (CESA7wt), NubG-ALG5 (Neg), and the different mutated CESA7 proteins as prey, that show visible growth after 5 days at 30 °C on selective medium as a percentage of the interaction with wild type CESA7 protein.

cysteines (C) at different positions were substituted by alanines (A) using site-directed mutagenesis. Also combinations of the substitutions were made (Fig. 3). The interactions between the different mutated CESA7 proteins and the other secondary CESAs were tested with the MbYTH. The interaction between CESA8 and CESA7 decreased only slightly when one cysteine was mutated (C37A, C56A, C64A, or C79A). Double substitution affected the interaction more in only one combination (C37A + C79A), however the interaction was not abolished (Fig. 4). The interaction between CESA4 and CESA7 also decreased upon introduction of mutations (Fig. SI).

4. Discussion

Different models for the structure of the CESA complex are possible, but the rosette structure model, proposed by Scheible and co-workers [7] and modified by Doblin and co-workers [15], is the most widely accepted. In this model three types of CESAs (α1, α2, and β) are assembled hexagonally in different proportions one α1, two α2, and three β isoforms (Fig. 5A). Three types of protein–protein interactions were proposed: α2–β and α1–β to form each subunit, and α2–α2 between subunits to form rosettes, therefore type-α1 can only bind type-β, type-α2 can bind types α2 and β, whereas type-β can bind to types α1 and α2. Although more complex variants of this model are also possible, less complex models seem unlikely, based on simple geometric considerations [7].

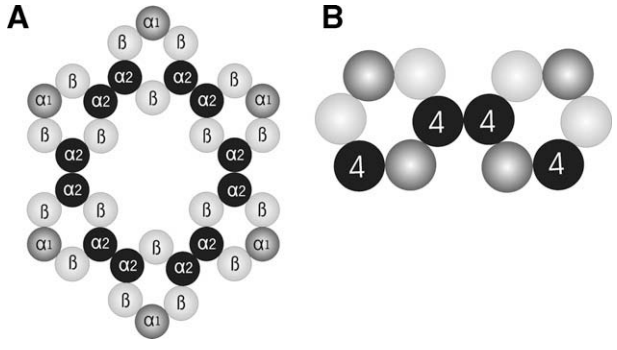

Fig. 5. Proposed models for the structure of the rosette. (A) Six subunits, containing six CESA polypeptides, interact to form a rosette as suggested by Doblin and co-workers. (B) The modified model based upon the results described in this work between the different isoforms, CESA4 (4) interacts with all isoforms, the homodimerization links the subunits together, the two other position are filled by CESA7 and CESA8.

The interaction in yeast using the MbYTH method were confirmed with in planta studies by the BiFC assays, except for the combination YFP/N-CESA7 and YFP/C-CESA8, which might be due to specific interference of the interaction-site of CESA7, and the ability of the CESA8 protein to form homodimers, there a very weak but detectable signal was observed in planta. The reasons for this discrepancy are not clear but it might be that this weak signal is not caused by a direct interaction but by a bridging endogenous protein of *N. benhtamiana* that brings CESA8's in the vicinity of each other resulting in the assembly of the YFP. Furthermore punctuated structures were found in all CESA8 interactions indicate towards a different role for CESA8 compared to the others. In a recent publication pull down experiments indicate that in absence of one CESA the remaining isoforms form mono- and di-mers [3], which confirms the results described in this article. In a dual tagging assay a 240 kDa band was found which might be assign to CESA7 homodimer. As the authors mention, it is difficult to find solid prove that this band is indeed a CESA7 homodimer as the three CESA proteins possess very similar molecular masses [3]. In the MbYTH system expression of CESA7 alone is not sufficient for the formation of a dimer, a result confirmed with the BiFC system by heterologous expression in *N. bentamiana*.

If our results are projected onto the rosette model of Scheible and co-workers, CESA4 self-interacts and therefore it has to occupy position α2. However CESA7(α1) and CESA8(β) both interact with the other two CESA of the rosette, but not with themselves, indicating that next to the three proposed types of protein–protein interactions (α2–β, α1–β, α2–α2) there is also interaction between α1 and α2. We adapted the model with these findings by replacing one β position by a α1 position (Fig. 5B). This results in a complex in which the subunits contain the same number of proteins of each of the isoforms and the homomeric interaction is responsible for the interaction between two subunits. Although the subunits themselves are not symmetric the overall complex is (Fig. 5B). The stoichiometry of the model is that each individual CESA protein is present in the same number, which is supported by the co-expression and regression score between the different secondary *CESA* genes [16]. However to date this has not been confirmed at protein level. The specificity of the interaction suggests a non-random incorporation of CESA proteins into the rosettes, and might hint towards a specific function of each of the CESA proteins in the rosette.

The RING-finger motif was the best candidate to facilitate the interactions between the CESAs, as it has been shown that the RING-finger domains themselves can interact with each other when expressed separately [14]. It was found that a mutation in the RING-motif abolishes the interaction between two

RING-fingers. When this motif is indeed essential for interaction between two CESA proteins, mutations in the RING-finger of CESA7 should abolish the interactions found between CESA7 and CESA4 and CESA8. Although some of the single mutations in the zinc-finger resulted in a decrease in interaction, the interaction itself was still observed and certainly not completely abolished. Combinations of cysteine substitutions did not result in a further decrease of the interaction (Fig. 4), suggesting that other domains than the RING-finger are involved in the interaction. Domain swapping experiments of Wang et al. [6], showed that the catalytic and/or C-terminal domains were the most important for entering the specific site in the complex, which is consistent with our results.

Our results do not exclude involvement of the RING-motif in protein interaction. The localization of the RING-motif at the cytoplasmic face of the plasma membrane suggests a role in recruiting proteins other than CESA. It has also been speculated that the RING-motifs under reduced conditions promote their own degradation [17]. The introduced mutations within this motif reduce the zinc coordination to zinc-fingers, and may therefore lead to the degradation of the subsequent CESA protein. This might be a reason for decreased interaction between the mutated CESA7 proteins and the other CESA isoforms.

In conclusion, our screens have revealed that the CESA proteins involved in secondary cell wall synthesis specifically interact with each other. The interactions found result in an adapted version of the model for the rosette composition in which the homodimerization of CESA4 links the subunits to form the complete rosette. Interaction studies also indicated that the RING-motif is not essential for the interaction between different CESA proteins. More research will be required to understand the architecture of the rosette and the domains involved in the specific interaction between the different CESA within the complex.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.02.035.

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