Running title: Mixed CESA complexes are functional in planta

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Research Area: Cell Biology
Title: Complexes with mixed primary and secondary cellulose synthases are functional in Arabidopsis thaliana plants

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Footnotes:

A.C, Y.G. and C.S were supported, in part, by the U.S. Department of Energy (grant no. DE-FG02-09ER16008) and the Energy Biosciences Institute.

S.L., L.L., and Y.G. were funded, in part, by startup funds from Pennsylvania State University, Department of Biochemistry & Molecular Biology and The Center for LignoCellulose Structure and Formation, an Energy Frontier Research Center funded by the U.S. Department of Energy, Office of Science under Award Number DE-SC0001090.

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Abstract:

In higher plants cellulose is synthesized by so called rosette protein complexes with cellulose synthases (CESAs) as catalytic subunits of the complex. The CESAs are divided into two distinct families, three of which are thought to be specialized for the primary cell wall and three for the secondary cell wall. In this article the potential of primary and secondary CESAs forming a functional rosette complex has been investigated. The membrane-based yeast two-hybrid (MbYTH) and biomolecular fluorescence (BiFC) systems were used to assess the interactions between 3 primary (CESA1, CESA3, CESA6) and 3 secondary (CESA4, CESA7, CESA8) Arabidopsis thaliana CESAs. The results showed that all primary CESAs can physically interact both in vitro and in planta with all secondary CESAs. Although CESAs are broadly capable of interacting in pairwise combinations they are not all able to form functional complexes in planta. Analysis of transgenic lines showed that CESA7 can partially rescue defects in the primary cell wall biosynthesis in a cesa3 mutant (je5). GFP-CESA protein fusions revealed that when CESA3 was replaced by CESA7 in the primary rosette the velocity of the mixed complexes was slightly faster than the native primary complexes. CESA1 in turn can partly rescue defects in secondary cell wall biosynthesis in a cesa8ko mutant, resulting in an increase of cellulose content relative to the cesa8ko. These results demonstrate that sufficient parallels exist between the primary and secondary complexes for cross-functionality and opens the possibility that mixed complexes of primary and secondary CESAs may occur at particular times.
Introduction:

Cellulose is the most abundant component of the biosphere, with more than $10^{11}$ tons estimated to be synthesized each year (Brown, 2004). This linear β-1,4 glucan polymer is synthesized by the membrane-embedded cellulose synthase (CESA) which is represented by 10 isoforms in Arabidopsis (Doblin et al., 2002; Somerville 2006). In higher plants, CESA proteins form a rosette complex 25nm in diameter in the plasma membrane, proposed to consist of 36 CESA subunits (Taylor et al., 2003; Scheible et al., 2001; Muller et al., 1980; Gidding et al., 1980; Kimura et al., 1995). Genetic evidence shows that at least three isoforms are involved in the synthesis of primary walls in growing cells, CESA1, 3, 6, and three other isoforms are involved in the deposition of secondary walls in xylem cells, CESA4, 7, 8 (Fagard et al., 2000; Scheible et al., 2001; Desprez et al., 2002; Ellis et al., 2002; Taylor et al., 2000; Persson et al., 2007; Desprez et al., 2007). Double and triple mutants and co-immunoprecipitation analysis in Arabidopsis demonstrates that the remaining CESA proteins namely CESA2, 5 and 9 are partially redundant with CESA6 (Persson et al., 2007; Desprez et al., 2007), suggesting specialized functions for CESAs in certain developmental or environmental conditions (Mutwil et al., 2008).

Phylogenetic analysis revealed six distinct CESA clades found in seed plants, each corresponding to one of the six required components of the primary and secondary cellulose synthase complexes in Arabidopsis (Holland et al., 2000; Samuga and Joshi, 2002; Tanaka et al., 2003; Burton et al., 2004; Nairn and Haselkorn, 2005; Djerbi et al., 2005; Ranik and Myburg, 2006; Suzuki et al., 2006; and Kumar et al., 2009; Carroll and Specht, 2011). The interaction between the different CESA proteins in the primary and secondary rosette has been characterized previously by co-immunoprecipitation and yeast two-hybrid methods, showing interaction patterns with similarities between primary and secondary CESAs (Taylor et al., 2000; Desprez et al., 2007; Wang et al., 2008; Timmers et al., 2009; Attanasov et al., 2009). These results, suggest that despite the ancient divergence of the families, the complexes may have retained the same positioning of the CESAs in the complex with respect to each other.

The primary and secondary cell wall are formed at different developmental stages. The primary cell wall is synthesized during cell division, and expansion, while the secondary cell wall is deposited after the expansion phase. Primary CESAs do not appear to be coordinately expressed with secondary CESAs (Persson et al., 2005). The primary CESAs are thought to be expressed from the initial stages of cell formation till soon after the end of cell expansion, while the secondary CESA genes are assumed to be expressed from the last stages of cell expansion till cell death. Thus there may be a limited period of time when both primary and secondary CESA genes are co-expressed.
GFP-labeled CESA complexes are seen by confocal microscopy as particles in the plasma membrane which move in linear tracks organized by cortical microtubules (Paredes et al., 2006). Fluorescently labeled CESAs are also seen in Golgi bodies and in small microtubule associated compartments called SMaCCs (or MASCs), which are implicated in trafficking CESA from the Golgi to the plasma membrane (Gutierrez et al., 2009; Crowell et al., 2009). Although the association of CESA complexes with microtubules appears to be mediated by the cellulose synthase interactive protein 1 (CSI1, Li et al., 2012), the timing and mechanism of CESA complex assembly remains an open question.

The localization of cellulose synthases is critical to their function. Cellulose is presumably only synthesized at the plasma membrane. Signal from GFP-labeled complexes at the membrane is rapidly lost following osmotic or mechanical shock, and chemical inhibition through a number of inhibitors such as isoxaben (Gutierrez et al., 2009; Crowell et al., 2009). The timing of CESA complex assembly remains uncertain. Freeze fracture images establish it at the membrane (Kimura et al., 1999). The only TEM images of immunolabeled CESA within the Golgi do not show apparent complexes at the stage of localization to the Trans-Golgi network (Crowell et al., 2009).

In this report we demonstrate limited interchangeability between primary and secondary CESAs, which suggests retention of CESA positioning in the rosette complex and similarities in function across primary and secondary CESA complexes. The parallels between the primary and secondary CESA complexes were investigated by introducing primary CESA proteins in the secondary rosette and vice versa. The interactions between both primary and secondary CESA proteins in Arabidopsis were probed using the split-ubiquitin membrane based yeast two-hybrid and bimolecular fluorescence, and revealed they are able to interact and form both homo and heterodimers. Through a series of promoter exchanges, we demonstrate specific secondary CESA constructs are able to partially rescue mutants of certain primary CESAs and incorporate into the complex at the plasma membrane in these mutants. The functional incorporation of specific primary CESAs into the secondary walls are also shown. The incompleteness of the rescue suggests the development of some specialization in the function or regulation of CESA families. These results may also suggest that the synthesis of cellulose during the transition between the primary and secondary cell wall may involve the action of mixed primary-secondary complexes.

Results:

Primary CESAs interact with secondary CESAs in vitro

All possible combinations of one-to-one interactions between the primary CESAs (CESA1, CESA3 and CESA6) and secondary CESAs (CESA4, CESA7 and CESA8) were assessed
using the split-ubiquitin yeast two-hybrid system (MbYTH, Dualsystems Biotech AG). Upon testing the interactions between the three primary CESA isoforms the results show that all the primary CESAs were able to form both homodimers and heterodimers with all the other primary CESA isoforms (Figure 1), confirming previous reports using BiFC analysis (Desprez et al., 2007). These protein interactions were carried out with each of the primary CESAs as bait and as prey and both sets of experiments showed the same results (Figure 1). The lack of growth in the negative controls indicated that the interactions were specific as an unrelated protein expressed as prey and an empty prey vector (pADSL-Nx) were not able to activate the system.

In a second step the interactions were determined between three members of the primary CESAs (CESA1, CESA3, CESA6) and the secondary CESAs (CESA4, CESA7, CESA8) using the same MbYTH system. Though with different interaction strength, the six primary and secondary CESAs all had the ability to form heterodimers in all possible combinations (Figure 1).

**Primary and secondary CESAs can be part of the same complex in planta**

The bimolecular fluorescence (BiFC) technique offers the possibility of analyzing protein interactions in living plant cells (Walter et al., 2004). To analyze the interaction between the three primary CESAs (CESA1, CESA3 and CESA6) and the secondary CESAs in planta, the BiFC assays were used and the results are shown in Figure 2. It was observed that YFP fluorescence was reconstituted for all of the combinations, indicating that all isoforms from the primary CESAs (CESA1, CESA3, CESA6) can interact with that of the secondary CESAs (CESA4, CESA7, CESA8). The intensity of the YFP signals was not the same for all combinations. Upon interaction of CESA3 and CESA7 a weaker signal was observed which may indicate that dimerization is less stable. All the pair-wise CESA combinations were carried out with each of the CESA’s fused with the N- and C- terminus of the YFP and both sets of experiments showed the same results.

**CESA7 can partially rescue the defects in the cesa3 mutant je5**

To determine whether CESAs from the secondary complex could enter and function in the primary complex, a series of promoter-swap constructs was generated. Combinations of each of the primary promoters placed upstream of each of the secondary CESA coding sequences, both with and without an N-terminal GFP. We named these constructs PX-CY based on the promoter and coding sequence used. A construct containing the CESA1 promoter is P1, while one containing the coding sequence of CESA4 is C4, giving the combination of the two the name P1C4. If GFP is N-terminally fused, we place the character “G” before the coding sequence. The fusions with GFP (P1-G-C4, P1-G-C7, P1-G-C8, P3-G-C4, P3-G-C7, P3-G-C8, P6-G-C4, P6-G-C7, and P6-G-C8) and without GFP (P1C4, P1C7, P1C8, P3C4, P3C7, P3C8, P6C4, P6C7, and P6C8) were transformed into the mutant lines corresponding to the
promoter used. The CESA1 promoter constructs were transformed into the temperature
sensitive (ts) cesa1 mutant rsw1-1 (lines P1-G-CY; c1ts), CESA3 promoter constructs were
transformed into the weak cesa3 mutant je5 (P3-G-CY; c3w), and CESA6 promoter
constructs were transformed into the cesa6 null line prc (P6-G-CY; c6ko). In addition, the
weak cesa3 mutant je5 was transformed with the P3-G-C3 construct (Figure 3).
A partial rescue in the P3-G-C7 (c3w) lines was observed (Figure 4B). Etiolated seedlings of
P3-G-C7 in je5 were not significantly different in hypocotyl length from Colombia plants or
from P3-G-C3 (c3w) plants up to 2.5 days of growth. After 2.5 days, however, P3-G-C7 (c3w)
does not elongate as rapidly as Colombia or P3-G-C3 (c3w) (Figure 4A). The CESA7 rescue
of the cesa3 primary cell wall mutants without GFP was also incomplete (Figure S1). No
rescue was apparent for either the P6-G-C7 (c6ko) or P1-G-C7 (c1ts). The CESA4 and
CESA8 constructs did not rescue any of the primary cell wall mutants, either with (data not
shown) or without the N-terminal GFP (Figure S1).
RT-PCR analysis of GFP transcript revealed that expression of the CESA7 gene in the P3-G-
C7 (c3w) mutant was similar to the expression of CESA3 gene in the rescues c3w mutant
(P3-G-C3), and shown in Supplementary Figure S2.

Mixed rosette complexes behave differently from primary rosettes

Spinning disk confocal microscopy analysis in 2.5 day old P3-G-C7 (c3w) and P3-G-C3 (c3w)
etiolated seedlings revealed GFP signal in Golgi bodies and in membrane particles (Figure
5A,B Movie S1, S2). The velocity distributions of both P3-G-C7 (c3w) and P3-G-C3 (c3w)
were calculated by tracking individual particles in time-lapse movies. This revealed that
membrane particles were approximately half as abundant in P3-G-C7 (c3w) compared to P3-
G-C3 (c3w). Interestingly, complexes in the P3-G-C7 (c3w) line migrated about 30% faster
than P3-G-C3 (c3w) complexes, a difference that is significant at p < 0.001 in a two-tailed T-
test (Figure 5C). This phenomenon was consistently observed across biological replicates (26
GFP-CESA3 control and 15 P3-G-C7 (c3w) plants) acquired across 7 different days, tracking
around 40,000 GFP-CESA3 and 11,000 GFP-CESA7 labeled complexes (Figure 5D, 5E and
Table 1). The number of plasma-membrane localized particles decreased for both P3-G-C3
(c3w) and P3-G-C7 (c3w) lines after 3.5 days of etiolation, but the decrease in particle
number was far more pronounced in P3-G-C7 (c3w), making it difficult to track enough
particles for an adequate characterization of particle velocity in P3-G-C7 (c3w) after 2.5 days
of growth. Using the total distance travelled by all CESA complexes observed in cells of the
P3-G-C7 (c3w) and GFP-CESA3 lines, we estimated the relative cellulose produced in those
lines over the course of the movies. Tracked complexes in GFP-CESA3 travelled an average
of 572252 pixels (77.3 mm) per cell, compared to 421133 pixels (56.9 mm) in P3-G-C7 (c3w).
This estimated that the cellulose content of the P3-G-C7 (c3w) at around 26% lower than the
content in the GFP-CESA3 control. Chemical determination of the cellulose content showed
similar results of lower cellulose content in P3-G-C7 mutant relative to the GFP-CESA3 control (data not shown).

In P1-G-C4 (c1ts) and P1-G-C8 (c1ts) plants, confocal microscopy revealed fluorescence in Golgi bodies, but no membrane complexes were detected (Movie S3). Additionally, small fluorescent bodies were faintly visible in focal planes at or near the plasma membrane which did not behave like linearly moving complexes, and whose behavior resembled previously reported sub-populations of SMaCCs (Movie S4). In P1-G-C7 (c1ts), the GFP-CESA7 signal in SMaCCs was more apparent when plants were grown at the restrictive temperature of 30°C (Movie S4). To determine whether the failure of GFP-CESA7 to reach membrane complexes was due to the compromised CESA6 and CESA1 proteins in these mutant lines, or due to competition from the WT CESA3, the P3-G-C7 construct was transformed into WT, generating the line P3-G-C7 (WT). These lines did not have any noticeable phenotype (Figure S1), indicating that the incompleteness of the rescue in P3-G-C7 (c3w) was most likely not due to a dominant-negative effect of CESA7 expression. P3-G-C7 (WT) plants had strong GFP-CESA7 fluorescence in Golgi bodies but no signal from membrane complexes (Movie S5). The same fluorescence patterns were observed when GFP-CESA7 was transformed into either prc or rsw1-1 and fluorescence was strongly visible in Golgi bodies but not visible in membrane complexes (data not shown). These lines retained their phenotypes: prc was radially swollen and dwarfed, as was rsw1-1 when grown at the restrictive temperature. This is consistent with the hypothesis that GFP-CESA7 is excluded from membrane complexes in the presence of a WT copy of CESA3, as both prc and rsw1-1 retain WT copies of CESA3. One cannot exclude the possibility that the GFP-CESA7 containing rosettes are somehow blocked in the transport to the plasma membrane.

Primary CESA1 substitutes CESA8 in secondary walls

The expression profile comparison between primary CESA1 and secondary CESA8 indicates that secondary CESA8 are more stringently controlled; therefore the promoter of CESA7 was chosen to be used in the promoter swap constructs. The null mutants of CESA4, CESA7, and CESA8 (cesa4ko, cesa7ko, cesa8ko) were identified by PCR identification of the T-DNA flanking regions (primers in Table S1). All the secondary promoter swap constructs (P7C1, 7C3) were transformed into cesa4ko, cesa7ko, cesa8ko. Among all the possible combinations, only P7C1 partially complemented the cesa8ko phenotype. The leaf morphology of cesa4ko, cesa7ko, and cesa8kos were indistinguishable from each other, all displaying dark green and reduced leaf size (Figure S3). The leaf of P7C1 (cesa8ko) was reverted to almost its wild type size. However, the margin of leaf was not as even as those of wild type (data not shown). The adult homozygous plants of cesa4ko, cesa7ko, and cesa8ko were dwarfed, mainly due to shorter internodes (Figure 6). In addition, cesa4ko, cesa7ko, and
cesa8ko were almost completely sterile. P7C1 (cesa8ko) partially recovered the elongation defect in internodes and these recoveries were more obvious in the main stem. In addition, P7C1 plants were fully fertile. A deficiency in secondary cell wall cellulose deposition leads to collapsed xylem cells, as shown in irx1, irx3, or irx5 plants (Taylor, 2000). Examination of the stem sections from cesa8ko showed its collapsed xylem phenotype. The xylem cells in P7C1 (cesa8ko) showed a similar phenotype to the wild type, indicating that P7C1 complemented the collapsed xylem phenotype in cesa8ko (Figure 7A-C). In both stems and leaves, the cellulose content in cesa4ko, cesa7ko, and cesa8ko were reduced confirming Taylor’s (2000) results. Lesions in either IRX1, IRX3, or IRX5 plants resulted in a decrease in cellulose of more than 70% in stems (Taylor, 2000). Correlating with the morphological recovery, the cellulose content of P7C1 (cesa8ko) was increased in both stems and leaves (Figure 7B), indicating that P7C1 functionally incorporated into the secondary CESA complexes.

Discussion

Several studies have shown absolute requirement of six unique CESA proteins, AtCESA1, AtCESA3 and AtCESA6-like, which form primary complexes (Desprez et al., 2002; 2007), and AtCESA4, AtCESA7 and AtCESA8 which form secondary complexes (Taylor et al., 2008, Timmers et al., 2009) for normal deposition of cellulose in the primary and secondary cell wall, respectively. Phylogenetic analysis reveals that these unique components, in the primary and secondary cell wall, represent distinct gene families which diverged early in the evolution of land plants (Holland et al., 2000; Samuga and Joshi, 2002; Tanaka et al., 2003; Burton et al., 2004; Nairn and Haselkorn, 2005; Djerbi et al., 2005; Ranik and Myburg, 2006; Suzuki et al., 2006; and Kumar et al., 2009; Carroll and Specht, 2011).

Primary and secondary CESAs can be part of the same protein complex

The yeast two-hybrid and bimolecular fluorescence results indicated that the CESAs can broadly interact with each other and that this interaction can be observed both in vitro and in planta. In contrast to the secondary cell wall, all primary wall CESA’s are able to homodimerize supporting previous BiFC data (Desprez et al., 2007). This result suggests that there is more flexibility in the positioning of the individual CESAs in the primary rosette complex than in the secondary complex, where only CESA4 is able to form homodimers (Timmers et al., 2009).

Previous reports have shown that primary and secondary CESAs are mainly expressed at different developmental stages in plants (Persson et al., 2005). Detailed gene expression analysis of single cells in Arabidopsis roots confirmed these results however they also revealed that primary and secondary CESAs can be co-expressed in specific cell types at certain time points (Birnbaum et al., 2003). The ability of primary and secondary CESAs to interact in all combination indicates that these CESAs have the potential to be part of the same rosette complex provide they are co-localizing.
Although the existence of CESA mixed complexes has not been possible to resolve in vivo with the methods currently available, there are several reports supporting the idea that primary and secondary wall formation are interrelated. Over expression of a mutant allele of the Arabidopsis CESA7 gene, named fra5, resulted in changes in cellulose synthesis during primary wall formation (reduced thickness of the cell wall and cell elongation) as well as causing a dominant-negative effect on cellulose synthesis during secondary wall formation (Zhong et al., 2003) as was also suggested in the case of the widely recognized secondary wall-specific AtCESA7 (MUR10) being required for normal primary cell wall carbohydrate composition in mature leaves, normal plant growth, hypocotyl strength and fertility (Bosca et al., 2010). Another study shows that despite CESA9 having already been classified as a primary cell wall CESA (Persson et al., 2007, Desprez et al., 2007) a non-redundant role was shown in secondary cell wall thickening in seed coat (Stork, 2010). The rice brittle culm mutant bc11 has shown both altered primary (increased callose, pectic arabinan and xylan) and secondary (brittleness of the culm, abnormal secondary structure, decreased wall thickness and reduced cellulose content) wall composition (Zhang et al 2009) further supporting the possibility of cross-talk and overlapping functions between the primary and secondary CESAs. In addition, the putative ability of primary and secondary CESAs to change roles through evolution appears more dynamic than was once believed. Recent results have shown that the secondary complexes produce secondary thickenings of cotton fibers, while the primary complexes have acquired this role in the analogous Arabidopsis structure of trichomes (Betancur 2010).

**CESA7 partially rescues the defects in the primary cesa3 mutant (je5)**

Although the MbYTH and BiFC revealed that all primary and secondary CESAs can be part of the same protein complex, mutant complementation analysis revealed that the mixed complex where CESA3 has been replaced by CESA7 is functional in the primary cell wall and can partially rescue the cesa3 knockout mutant. At the same time, CESA7 could not rescue cesa1 or cesa6 mutants, indicating that the rescue occurs due to CESA7’s ability to substitute for CESA3. The exclusion of GFP-CESA7 from the plasma membrane of WT-CESA3 plants suggests that WT-CESA3 outcompetes CESA7 for inclusion in the cellulose synthase complex, indicating that there has been a small degree of shift in the interactions required to place a protein into the complex at the CESA3 position. Shifts in the affinity of CESA-CESA interactions over time could also explain the inability of CESA4 and CESA8 to rescue any of the primary cesa mutants. One interpretation of these results is that individual isoforms within the CESA-complex can be thought of as having assigned “positions.” These positions could be consistent and distinct spatial locations in the structure of the complex, or they could instead arise more loosely from stronger interaction affinities between CESA classes during assembly of the complex. From these results, it appears the CESA3 and CESA7 can gain access to the same position in the complex.
Another possible explanation is that CESA7 incorporates into the complex as efficiently as CESA3, but the transfer of these complexes to the membrane is deficient. This could occur because the process responsible for transport or fusion involves a check on the integrity of the complex. CESA7-containing complexes are slightly deficient in this check, not so much that they cannot be transferred, but enough that CESA3-containing complexes outcompete for access to the transfer process and saturate transfer to the membrane.

Since the je5 line is a weak allele of CESA3, we also cannot exclude the possibility that some mutant copies of CESA3 are able to help CESA7 enter the complex or otherwise facilitate complex formation.

The faster movement of GFP-CESA7 raises a number of questions. The general explanation would be that activity at the CESA3/CESA7 position is the rate limiting process for complex mobility. A biochemical perspective may provide a better general explanation. The process of cellulose synthesis may be rate-limited by steps in addition to catalysis, for example, the nascent cellulose chain may have to crystallize before synthesis can continue. If the substitution of CESA7 for CESA3 in the complex changes some property of the cellulose produced, this could produce an effect that could propagate through to the complex as a whole. Mutations in CESA1 and CESA3 were recently described which caused the complex to move faster and also altered cellulose crystallinity (Harris et al., 2012). It is also possible that the faster rate may reflect a compensatory mechanism to the lower abundance of complexes visible in the GFP-CESA7 line. Substantially higher rates of CESA compartment movement have been reported previously (Wightman et al. 2009).

**CESA 1 partially rescues the defects of cesa8 knock out.**

Lesions in the secondary CESAs, CESA4, CESA7 and CESA8, result in deficiency in the deposition of cellulose in secondary cell walls and in collapsed xylem cells. These mutants are also known as irregular xylem mutants, *irx5* (CESA4), *irx3* (CESA7) and *irx1* (CESA8). Reverse genetic approaches have identified additional alleles of the *irx* mutants that were used in this study including *irx1-5, irx3-4, and irx5-4* (Brown et al., 2005). In addition to defects in xylem cells, these T-DNA mutants also display defects in overall morphology, such as dwarf statue, slow growth, dark green and reduced leaf size, short siliques, and reduced fertility (Brown et al., 2005). CESA1 was able to completely rescue the collapsed xylem cells in *irx1-5* mutant, which is consistent with the recovered cellulose content. In terms of overall morphology, CESA1 was also able to rescue defects in leaf color and fertility in the *irx1-5* mutant. CESA1 was partially able to recover the overall statue of the plant in *irx1-5*. The non-redundant phenotype of secondary *cesa* mutants supports the hypothesis that CESA4, CESA7, CESA8 comprise the secondary cellulose synthase complex. CESA1 apparently is able to take over the role of secondary CESA when it is expressed in the secondary cell walls.

**C-terminus sequence separates primary and secondary CESAs into three groups**
In order to further analyse the similarities and differences between the primary and secondary CESAs comprising the complex, the site of the C-terminus rsw5 mutation implicated in disrupting the incorporation of CESA3 into the primary cellulose synthase complex were compared (Wang et al. 2006; Carroll and Specht, 2011). The C-terminus is a putatively cytosolic region of approximately 20 amino acids which follows the 8th transmembrane domain. The C-terminal region contains two strongly conserved cysteines, and we speculate that the formation of disulfide bonds between the C-terminus of one CESA to one of the other cysteine rich regions in another CESA might help mediate complex assembly. Chimeric CESA and CESA/CSLD proteins exchanging the N-terminal region (Wang et al. 2006) and catalytic domain (Park et al. 2011) have both retained the identity of the genetic position or localization of the C-terminal domain. This site was absolutely conserved in CESA families 3, 4, 6, and 7, but not in CESA families 1 and 8, with CESA families 3 and 7 showing more similarity to each other than with the other CESAs (Carroll and Specht, 2011). These observations are in agreement with the rescues of the primary and secondary knock out mutants, where CESA7 can partially rescue the defects in the cesa3 mutant and CESA1 can partially rescue the cesa8 mutant. These results, and the fact that most primary and secondary CESA proteins are not able to rescue CESAs, demonstrate that additional selectivity exists within the plant cell, either through directed assembly or competition for interacting partners. This also supports the possibility that CESAs have distinct functions in the rosette, either structurally and/or enzymatically related.

Materials and Methods:

Constructs for the split-ubiquitin membrane-based yeast two-hybrid

The full-length cDNAs were obtained from the Riken Bioresource center (Seki et al., 1998; Seki et al., 2002) AtCESA1 (RAFL09-89-G08), AtCESA3 (RAFL05-19-M03), and AtCESA6 (RAFL05-02-P19), AtCESA4 (RAFL15-30-K05), AtCESA7 (RAFL09-35-F05), and AtCESA8 (RAFL09-65-M12) (Timmers et al., 2009). The cDNA’s of the CESA genes were amplified by PCR using the Phusion DNA Polymerase (Finnzymes, Helsinki, Finland) with the primers in Supplementary Table S1. The resulting PCR-products were digested and ligated into the pTFB1 vector (Bait) and the pADSL-Nx vector (Prey) (Dualsystems Biotech AG). Bait and prey expression was regulated by the TEF1 and ADH1 promoter, respectively. The sequences of the inserts were confirmed by Sanger sequence analysis. Both the bait and prey protein were fused N-terminally to the Cub-TF reporter cassette of the vector pTFB1 and NubG cassette of the vector pADSL-Nx respectively.

The split-ubiquitin membrane-based yeast two hybrid screen (MbYTH)
The interactions between the CESA proteins were assayed using the split-ubiquitin membrane-based yeast two-hybrid (Johnsson and Varshavsky, 1994; Reinders et al., 2002) with the yeast strain NYM51 in the Split Ubiquitin System kit (Dualsystems Biotech AG). The assays were performed according to supplier instructions (DUAL membrane Kit 1). This system (Stagljar et al., 1998; Stagljar and Heesen, 2000) was used to detect interaction between the CESAs, in which each CESA was fused to the Cub-coding sequence of vector pTFB1 (bait), the Cub-Transcription factor (TF) and the NubG-coding sequence of vector pADSL-Nx (prey, FetChko and Stagljar, 2004). The yeast resident ER protein ALG5 fused to NubG was used as a negative control. Co-expression of the bait proteins with prey protein ALG5-NubG should not result in an interaction, and therefore not in activation of the system, as it is not involved in the pathways of interested. As a positive control the ALG5 protein was fused to the wild-type ubiquitin domain. In contrast to the I13G mutant (NubG), the wild-type N-terminal ubiquitin domain (NubI) can readily interact with the C-terminal ubiquitin domain. Thus the co-expression of the bait, containing the CUB, with a prey fused to the NubI will lead to an interaction and therefore may be used to test for bait expression and accessibility without the need for the fused proteins to interact. Interactions were quantified by 100 colonies spotted on SD medium (lacking Leucine, Tryptophan, Histidine and Adenine) containing the appropriate concentration of 3-ammonium-triazole (3-AT), as reported in Timmers et al. (2009), and grown at 30°C for five days, the number of spots grown was scored. The bait was also screened using the inhibitor (3-AT) in the selection medium to rule out auto activation. Detection of β-galactosidase activity was performed with the filter-lift assay (Breeden and Nasmyth, 1985). All experiments have been performed in quadruplicate of independent biological replicates. Having two different auxotrophic markers for selection increased the reliability of the system in that the prey had to circumvent two different pathways to auto-activate the system, as well as a colorimetric marker.

**Constructs for split-YFP**

The full-length cDNA of the CESA genes were generated through Phusion DNA Polymerase (Finnzymes, Helsinki, Finland) with suitable primers (Desprez 2007; Timmers, 2009; Table S1). Coding sequences of the CESAs were cloned into the gateway-compatible destination vectors pBiFc-2 and pBiFc-3 plasmids regulated by the constitutive 35S promoter (Hu et al., 2002). The N-terminal and C-Terminal fragments of YFP were both fused to the N-terminus of the coding sequences of the CESAs. As a positive control, the aquaporin PIP2-1 (Boursiac et al., 2005; Desprez et al., 2007) was used, as aquaporins are known to form homotetramers in the plasma membrane (Murata et al., 2000). As a negative control, PIP2-1 chimera was co-expressed with the corresponding CESA constructs.

**Split-YFP screen**
The Bimolecular Fluorescence Complementation screen (BiFC) was used to analyze in planta the interaction between the different CESA proteins. All possible combinations between the three primary and three secondary CESAs were analysed with this method: YFP/N-CESA1/YFP/C-CESA4, YFP/N-CESA1/YFP/C-CESA7, YFP/N-CESA1/YFP/C-CESA8, YFP/N-CESA3/YFP/C-CESA4, YFP/N-CESA3/YFP/C-CESA7, YFP/N-CESA3/YFP/C-CESA8, YFP/N-CESA6/YFP/C-CESA4, YFP/N-CESA6/YFP/C-CESA7, YFP/N-CESA6/YFP/C-CESA8. These interaction were also tested in the reverse combination, thus with both C- and N-terminus of the YFP. Leaves of 3-week-old tobacco (Nicotiana benthamiana) plants were infiltrated following transformation with Agrobacterium tumefaciens strain GV3101pMP90 (Koncz and Schell, 1986) by transient co-expression of the desired protein pairs (Desprez et al., 2007). YFP fluorescence was detected 3 days after infiltration using the 514-nm laser line of a SP2 AOBS CLSM (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. All experiments were carried out in triplicates.

Promoter swap constructs

Using the same full length cDNA genes previously indicated, the coding sequence for each of CESAs (CESA4, 7, and 8) was amplified using Phusion DNA Polymerase (Finnzymes, Helsinki, Finland) with primers suitable for the gateway BP cloning reaction. These were inserted into pDONR207 through a BP reaction. CesA7 was amplified with Phusion DNA Polymerase, an adenine overhang added through 30 minute incubation with Taq-polymerase at 72°C, and inserted into the PCR8 TOPO vector from Invitrogen. All three DONR vectors were inserted into destination vectors carrying the 2kb upstream promoter region of each of the primary CESAs and the coding sequence from GFP immediately prior to the attR recombination sites (Desprez et al. 2007). The final vectors were sequenced over the entire length of their CesA coding region to confirm that no point mutations were present and to confirm that the GFP-CESA fusion was in-frame. These constructs were made with N-terminal GFP fusions as well as untagged versions of the constructs. This resulted in two sets of 9 constructs which were termed PX-G-CY for the fusion of the promoter for CesA X to the GFP-fused coding sequence of CesA Y (P1-G-C4, P1-G-C7, P1-G-C8, P3-G-C3, P3-G-C4, P3-G-C7, P3-G-C8, P6-G-C4, P6-G-C7, and P6-G-C8) and PX-CY for the untagged construct (P1C4, P1C7, P1C8, P3C4, P3C7, P3C8, P6C4, P6C7, and P6C8) to designate the promoter (P) driving the coding sequence (C) in each construct. CESA1 promoter constructs were transformed into the temperature sensitive cesa1 mutant rswt-1 (lines P1-G-CY(c1ts), CESA3 promoter constructs were transformed into the weak cesa3 mutant je5 (P3-G-CY(c3w), and CESA6 promoter constructs were transformed into the CESA6 null line prc P6-G-CY(c6ko) through the floral dip method (Clough and Bent 1998). These constructs and lines are illustrated in Figure 1.
Fourteen transgenic P3-G-C7 and 27 P6-G-C7 lines were identified by genotyping. Ten lines from each type of transformant were investigated for the presence of fluorescence with 7 P3-G-C7 lines and 10 P6-G-C7 lines having visible fluorescence. Two lines of each were selected for further in-depth analysis. We identified 30 transgenic lines for each other construct by genotyping, all were investigated for fluorescence, with only a few lines found to display weak fluorescence for each construct.

The CESA7 promoter was amplified using primers indicated in Supplementary Table S1. The amplified CESA7 promoter was inserted into PCR8 TOPO (Invitrogen). Sequence confirmed PCR8-pCESA7 was digested using Sma I/Xba I and inserted into pGW2 vector (Nakagawa et al. 2007) to replace the 35S promoter. The full-length cDNAs of CESA1 and CESA3 were PCR-amplified and cloned into pDONR-zeo using primers in Supplementary Table S1. CESA1 and CESA3 were then inserted to destination vectors containing the 2kb CESA7 promoter using LR clonase II (Invitrogen).

**Isolation of T-DNA insertion line**

The identification of secondary *cesa* knockout lines from the SIGNAL collection (http://signal.salk.edu/cgi-bin/tdnaexpress) was based on a combination of database searches and PCR amplification of T-DNA flanking regions. For T-DNA lines identified from the SIGNAL collection, seeds were obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, Columbus, OH). PCR reactions were carried out to identify single plants for the T-DNA insertion. Primers used for T-DNA genotyping of CESA alleles were listed in Supplementary Table S1.

The secondary *cesa* *Arabidopsis thaliana* homozygous mutants used in this study included irx1-5, irx3-4, and irx5-4 (Brown et al., 2005).

**Plant growth conditions**

*Arabidopsis thaliana* Columbia (Col-0) seeds and various mutant lines were sterilized and germinated on MS plates (1/2 × MS salts, 0.8% agar, 0.05 % MES, pH 5.7). Seedlings were then grown vertically on the agar at 22°C under continuous light for 5 days before being transferred to pots in a green house at 22°C under 16-hr light and 8hr dark.

**RT-PCR analysis**

Total RNA was isolated from Arabidopsis seedlings using the RNAeasy Mini Kit (QIAGEN). Reverse transcription and PCR amplification were performed. For GFP amplification, 30 cycles of PCR amplification (94°C for 30 sec, 54°C for 30 sec, and 72°C for 1 min) were performed using the primers shown above. As PCR amplification and loading controls, the same template cDNA was amplified using primers for the constitutive ACTIN2 (*ACT2*) gene. The primers used for RT-PCR analysis were: 5’GFP: ATGGTGAGCAAGGGCGAGGA, 3’GFP: TACAGCTCGTCCATGCCGTGA, 5’ACT2: ATGGCTGAGGCTGATGATAT, 3’ACT2: TTAGAAACATTTTCTGTGAAC.
Cellulose measurement

Rosette leaves or stems were harvested and grounded in liquid nitrogen. After overnight extraction in 80% ethanol at 65°C in water bath, tissues were exchanged with acetone. Dry cell wall materials were ball-milled to fine powder. Cellulose was measured as described by Updegraff (1969). Data were collected from five technical replicates for each tissue sample. Experiments were repeated twice.

Xylem staining

Stems from Arabidopsis were hand cut by a razor blade and stained in 0.02% toluidine blue O as previously described (Persson et al. 2005). Stem sections were rinsed, mounted in water, and viewed with a compound microscope (Leitz DMRB, Leica, Deerfield, IL). Around 5 individual plants were examined for each line.

Confocal microscopy

For analyses of GFP-CESA proteins expressed in the promoter swap lines, seeds were germinated on MS agar plates and grown vertically in darkness for 3 d at 22°C. Seedlings were mounted between two cover-slips in water. Imaging was performed on a Yokogawa CSUX1 spinning disk system featuring the DM6000 Leica motorized microscope and a Leica 100×/1.4 NA oil objective. GFP was excited at 488 nm, and a band-pass filter (520/50 nm) was used for emission filtering. Image analysis was performed using Metamorph (Molecular Devices) and Imaris (Bitplane) software.

Movies were collected on 7 separate days, without a consistent pattern as to which lines were imaged first. Movies were taken at ambient temperatures. On none of the 7 days did the average recorded GFP-CESA3 control velocity exceed the velocity of the P3C7 lines recorded on that day. The lower number of P3-G-C7 movies compared to GFP-CESA3 occurs because the weaker signal makes it more difficult to maintain the focal plane appropriately. An approximately equal number of acquisitions were attempted for each, with poor focal quality movies discarded during post-processing.

Image analysis was performed with ImageJ (Magelhaes et al. 2004) and Imaris software. Movies were first contrast enhanced in ImageJ and a walking average of 4 frames taken using the kymograph plugin for ImageJ. These steps were performed to improve the accuracy of automated particle recognition performed in subsequent steps. These images were then opened in Imaris 6.2.1 then switched from Z-series to time series. The voxel size was set to 135 nm/voxel based on measurements from the scope and the time interval to 5 seconds. The particle recognition algorithm in Imaris was performed with a spot size of 250 nm. High
intensity signal was filtered to eliminate Golgi signal. Following this the connected components program was run, which determines particle identity over several frames and converts a particles’ movement into tracks. All tracks present for less than 60 seconds (12 frames) were discarded. The displacement and duration of the remaining tracks were exported to a spread sheet and their average velocity, distribution of velocities, and any directional bias were calculated.

Acknowledgements

We gratefully acknowledge the assistance of Thierry Desprez (Laboratoire de Biologie Cellulaire, Institute Jean-Pierre Bourgin, INRA, France) with the BiFC experiments and also the assistance of Kian Hématy for his assistance with the conceptual design of the experiments.
Supplementary Movies

Movie S1: GFP-CESA3 particles observed at the plasma membrane of a P3-G-C3 (c3w) plant

Complexes containing GFP-CESA3 are visible as small, distinct puncta moving in linear trajectories at the plasma membrane. Large, bright Golgi bodies containing GFP-CESA3 are seen moving rapidly from cytoplasmic streaming and coming into and out of the focal plane near the membrane. Movie is 5 minutes in length, with one frame taken every 5 seconds.

Movie S2: GFP-CESA7 particles observed at the plasma membrane of a P3-G-C7 (c3w) plant

Complexes containing GFP-CESA7 are visible as small, distinct puncta moving in linear trajectories at the plasma membrane. Large, bright Golgi bodies containing GFP-CESA7 are seen moving rapidly from cytoplasmic streaming and coming into and out of the focal plane near the membrane. Movie is 5 minutes in length, with one frame taken every 5 seconds.

Movie S3: Incorporation of CesA4 and CesA8 protein into complexes is minimal

GFP-CESA8 signal is observed weakly in Golgi bodies and SmaCCs in P1-G-C8 (c1ts) plants grown at the restrictive temperature of 30° C. This movie is only 70 seconds in length due to the difficulty in determining the focal plane at the membrane, as images acquired live are much dimmer than the enhance contrast images shown. One frame taken every 5 seconds.

Movie S4: GFP-CESA7 is observed in Golgi bodies and SmaCCs of a P1-G-C7 (c1ts) plant imaged at the restrictive temperature of 30° C

GFP-CESA7 signal is observed in small, distinct puncta near the plasma membrane, showing behaviors of being stationary at the membrane, moving rapidly in linear tracks, or random, erratic motion characteristic of SmaCCs. Signal is also visible in Golgi bodies. Movie is 5 minutes in length, with one frame taken every 5 seconds.

Movie S5: GFP-CESA7 is observed in Golgi bodies of a P3-G-C7 (WT) plant

GFP-CESA7 signal is observed strongly in large Golgi bodies undergoing cytoplasmic streaming. Puncta moving in linear tracks with a regular velocity are not observed, indicating that the presence of a WT CesA3 allele causes GFP-CESA7 to be excluded from membrane particles. Movie is 5 minutes in length, with one frame taken every 5 seconds.
References:


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Ranik M, Myburg AA (2006) Six new cellulose synthase genes from Eucalyptus are associated with primary and secondary cell wall biosynthesis. Tree Physiology 26: 545-556


Wang J, Howles PA, Cork AH, Birch RJ, Williamson RE (2006) Chimeric proteins suggest that the catalytic and/or C-terminal domains give CesA1 and CesA3 access to their specific sites in the cellulose synthase of primary walls. Plant physiology 142: 685-695


Figure 1: Interactions between the primary and secondary CESA visualized by yeast growth. In y axes the percentage of colonies that show visible growth after 5 days at 30°C on selective medium is represented. Yeast expressing CESA1, CESA3, CESA6, CESA4, CESA7 and CESA8 as bait with N-terminal fusions of Nub and Cub to a CESA and with the ALG5 protein fused to NubI as positive control (AI) and NubG as negative control (DL) and an empty prey vector as another negative control (Nx) and different CESA proteins fused to NubG, as prey. Standard deviation is indicated by the error bar.

Figure 2: Bimolecular fluorescence (BiFC) analysis of the one-to-one interactions between the different primary and secondary CESA’s proteins. The proteins were transiently expressed in Nicotiana benthamiana leaf epidermal cells. (A) Positive Control YN-PIP/YC-PIP, (B) Negative Control YN-PIP/YC-CESA7, (C) YFP/N-CESA1/YFP/C-CESA4, (D) YFP/N-CESA1/YFP/C-CESA7, (E) YFP/N-CESA1/YFP/C-CESA8, (F) YFP/N-CESA3/YFP/C-CESA4, (G) YFP/N-CESA3/YFP/C-CESA7, (H) YFP/N-CESA3/YFP/C-CESA8, (I) YFP/N-CESA6/YFP/C-CESA4, (J) YFP/N-CESA6/YFP/C-CESA7, (K) YFP/N-CESA6/YFP/C-CESA8, (L) YFP/N-CESA8/YFP/C-CESA6. Scale bar =100μm.

Figure 3: Promoter swap constructs generated and transformed into plants. Arrows indicate promoter regions, the presence of the star-like symbol indicates that the coding sequence of GFP is N-terminally fused in frame to the coding sequence of one of the secondary CESAs, indicated as a labeled rectangular box. Primary and secondary promoter and coding sequences are colored based on grouping of their sequence similarity at the C-terminus.

Figure 4: GFP-CESA7 is able to partially rescue the cesa3 mutant je5. (A) A growth curve of hypocotyl elongation after various periods of etiolation revealed that early in growth the rescue of GFP-CESA7 is more complete, with elongation slowing after 3.5 days. Error bars represent standard deviation, asterisks indicate significant difference from WT control at p < 0.001 (B) At 5.5 days after germination in dark grown conditions, the GFP-CESA7 containing line P3C7 in the je5 background is able to partially rescue the je5 phenotype of reduced hypocotyl elongation.

Figure 5: GFP-CESA7 incorporates into CESA complexes in the je5 background. (A) GFP-CESA7 containing puncta are visible at the plasma membrane focal plane, and are arranged in linear tracks. Slightly out of focus Golgi bodies containing GFP-CESA7 near the membrane are also visible as large, circular areas of fluorescence. (B) A time projection of a 3 minute movie shows the motion of individual CESAs along tracks in the membrane. (C) The distribution of particle velocity indicates that GFP-CESA7 containing complexes have a faster average velocity than those observed in GFP-CESA3 containing complexes. (D) The average velocity in the 34 GFP-CESA3 and 15 P3-G-C7 are represented as box plots to show day to
day variability. The whiskers show one standard deviation from the mean, while the lines of
the box indicate the first quartile, the median, and the third quartile. (E) Kymograph of GFP-
CESA7 particle movement in a track. Bar length indicates 1 µm.

Figure 6: Whole plant morphology of secondary cesa mutants and various transgenic
lines. Whole-plant morphology of various transgenic lines in secondary cesa mutants. From
the left to right, wild type (WT), cesa4ko, cesa7ko, cesa8ko, P7C3 in cesa4ko (P7C3-4),
P7C3 in cesa7ko (P7C3-7), P7C3 in cesa8ko (P7C3-8), P7C1 in cesa4ko (P7C1-4), P7C1 in
cesa7ko (P7C1-7), P7C1 in cesa8ko (P7C1-8).

Figure 7: P7C1 complements morphological and molecular defect in cesa8ko. (A) Cross
sections of stem vascular bundles. Stem sections were stained with toluidine blue O. WT. (B)
cesa8ko. (C) P7C1 in cesa8ko (P7C1-8). Arrows indicate collapsed xylem vessels. Bar = 50
mM. (D) Cellulose content in leaf or stem from wild type and various transgenic lines in
secondary cesa mutants. Error bars represent SE, n = 5.

Supplementary Figure S1. Rescues from secondary swap constructs without N-
terminus GFP fusion. (A) The temperature sensitive CESA1 mutant rsw1-1 was transformed
with the swap constructs. No difference from WT (Col) was seen at the permissive
temperature. (B) No rescue was observed at the restrictive temperature. (C) The weak
CESA3 mutant, je5 is partially rescued by the CESA7 construct, but neither CESA4 nor
CESA8 constructs can rescue je5. There is no dominant negative effect seen in the plants
with a WT copy of CESA3. (D) The CESA6 null mutant prc1-1 is not rescued by any of the
swap constructs. All pictures were taken 3.5 days after germination.

Supplementary Figure S2: RT-PCR analysis of GFP expression in P3-G-C7 plants.
Accumulation of GFP transcript in control (P3-G-P3) and in P3-G-C7 plants. The actin gene
act2 was used as an internal control.

Supplementary Figure S3. Leaf morphology of secondary cesa mutants and various
transgenic lines. A From the left to right, wild type (WT), cesa4ko, cesa7ko, cesa8ko, P7C3
in cesa4ko (P7C3-4), P7C3 in cesa7ko (P7C3-7), P7C3 in cesa8ko (P7C3-8), P7C1 in
cesa4ko (P7C1-4), P7C1 in cesa7ko (P7C1-7), P7C1 in cesa8ko (P7C1-8). Bar = 10 mm. (B)
Measurement of leaf length.

Table legends

Table 1: CesA complexes containing GFP-CESA7 are less abundant than complexes
containing GFP-CESA3 in the je5 background. Movies were selected in which the
membrane of a single cell spans the field of view. 3 minute movies were taken with frames
captured at 2 second time intervals. Particles tracked for longer than 30 seconds were counted. The difference between P3-G-C7 and GFP-CESA3 is significant at $p < 0.003$.

Supplementary Table S1. DNA primers used in the study.
MbYTH interactions between primary and secondary CESAs

% of colonies

BAIT

CESA1
CESA3
CESA6
CESA7
CESA8

PREY

Al
DL
NX
CESA1
CESA3
CESA6
CESA7
CESA8
Secondary to Primary Swaps:

P1-G-C4:

P-CESA1 → CESA4 cds

P1-G-C7:

P-CESA1 → CESA7 cds

P1-G-C8:

P-CESA1 → CESA8 cds

P1-C4:

P-CESA1 → CESA4 cds

P1-C7:

P-CESA1 → CESA7 cds

P1-C8:

P-CESA1 → CESA8 cds

Transformed into: \textit{rsw1-1}

Transformed into: \textit{je5}, WT

Transformed into: \textit{prc1-1}

Control FP fusions:

P-CESA3 → CESA3 cds in \textit{je5}

P-CESA6 → CESA6 cds in \textit{prc}

Primary to Secondary Swaps:

P7-C1:

P-CESA7 → CESA1 cds

P7-C3:

P-CESA7 → CESA3 cds

Transformed into: CESA4ko, CESA7ko, and CESA8ko
Table 1: CesA complexes containing GFP-CESA7 are less abundant than complexes containing GFP-CESA3 in the je5 background. Movies were selected in which the membrane of a single cell spans the field of view. 3 minute movies were taken with frames captured at 2 second time intervals. Particles tracked for longer than 30 seconds were counted. The difference between P3-G-C7 and GFP-CESA3 is significant at $p < 0.003$.

<table>
<thead>
<tr>
<th>Line</th>
<th>Number of movies</th>
<th>Particles per Cell, S.D</th>
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</thead>
<tbody>
<tr>
<td>GFP-CESA3</td>
<td>34</td>
<td>2255 ± 1145</td>
</tr>
<tr>
<td>P3-G-C7</td>
<td>15</td>
<td>1294 ± 629</td>
</tr>
</tbody>
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