



Analysis of a cellulose synthase catalytic subunit from the oomycete pathogen of crops *Phytophthora capsici*

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Abstract *Phytophthora capsici* Leonian is an important oomycete pathogen of crop vegetables, causing significant economic losses each year. Its cell wall, rich in cellulose, is vital for cellular integrity and for interactions with the host organisms. Predicted cellulose synthase (CesA) proteins are expected to catalyze the polymerization of cellulose, but this has not been biochemically demonstrated in an oomycete. Here, we present the properties of the four newly identified CesA proteins from *P. capsici* and compare

their domain organization with that of CesAs from other lineages. Using a newly constructed glucosyltransferase-deficient variant of *Saccharomyces cerevisiae* with low residual background activity, we have achieved successful heterologous expression and biochemical characterization of a CesA protein from *P. capsici* (*PcCesA1*). Our results demonstrate that the individual *PcCesA1* enzyme produces cellobiose as the major reaction product. Co-immunoprecipitation studies and activity assays revealed that several *PcCesA* proteins interact together to form a complex whose multiproteic nature is most likely required for cellulose microfibril formation. In addition to providing important insights into cellulose synthesis in the

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oomycetes, our data may assist the longer term identification of cell wall biosynthesis inhibitors to control infection by pathogenic oomycetes.

Keywords Cellulose synthase · Oomycete · Crop pathogen · *Phytophthora capsici* · Cell wall biosynthesis

Introduction

Phytophthora capsici Leonian (Leonian 1922) has a devastating epidemiology (Hausbeck and Lamour 2004; Lamour et al. 2012) and ranks among the top ten pathogenic oomycetes in terms of global incidence, the range of plant species affected, the extent of damage to the host plant caused by infection and the resulting economic losses (Silvar et al. 2006; Kamoun et al. 2015). Under favourable conditions, *P. capsici* can infect more than 26 families of plants, including cucurbitaceae, solanaceae, and leguminosae, causing root, crown and fruit rot in susceptible host plants, many of which are important food and feed crops (Erwin and Ribeiro 1996; Granke et al. 2012). Significant global economic losses are therefore attributed to *P. capsici*-related diseases in crop vegetables every year (Erwin and Ribeiro 1996; Hausbeck and Lamour 2004). Efficient control methods are urgently required (Ristaino and Johnston 1999; Granke et al. 2012), but are often limited in their efficacy, partly due to the heterothallic nature of this pathogen, often allowing the production of oospores which can survive in the soil for years (Erwin and Ribeiro 1996).

The oomycete cell wall plays important roles in various biological processes, including the maintenance of cell shape, and is a significant store of catalytic and non-catalytic proteins, which are vital for interactions with the host organism. Analysis of cell wall-associated proteins identified from the *P. capsici* sister species *Phytophthora ramorum* suggests that this class of proteins are vital for pathogenicity (Meijer et al. 2006). New pharmacological interventions which disrupt the cell wall may therefore be useful means of controlling oomycete growth and infection (Grenville-Briggs et al. 2008; Blum et al. 2010).

The oomycete cell wall generally consists of cellulose and other β -glucans (Aronson et al. 1967;

Mélida et al. 2013). Cellulose from *Saprolegnia* and *Phytophthora* species occurs as a network of non-oriented narrow microfibrils of 4–5 nm in width that are of low crystallinity, similar to cellulose I β from plant primary cell walls (Bulone et al. 1992; Helbert et al. 1997). In *Phytophthora* spp., cellulose typically represents 32–35% of the cell wall content (Mélida et al. 2013). Cellulose synthases (CesAs) are the enzymes responsible for the biosynthesis of cellulose. They contain multiple transmembrane domains and belong to glycosyltransferase family 2 (GT2) (Lombard et al. 2014). GT2 proteins are repetitive or ‘processive’ enzymes that add monosaccharides from nucleotide-activated sugar donors (e.g., uridine-diphosphate-glucose (UDP-Glc)) to growing polysaccharide chains, with net inversion of the anomeric configuration with respect to the donor sugar (Campbell et al. 1997; Lairson et al. 2008). *CesA* genes have been identified in bacteria (Wong et al. 1990; Omadjela et al. 2013), higher plants (Pear et al. 1996; Taylor et al. 2000; Watanabe et al. 2015), cyanobacteria (Nobles et al. 2001; Zhao et al. 2015), algae (Katsaros et al. 1996; Collén et al. 2013; Brawley et al. 2017), and oomycetes (Grenville-Briggs et al. 2008; Fugelstad et al. 2009), but very few *CesA* proteins have been successfully purified and biochemically characterized. The only examples of *CesA* proteins for which the catalytic activity has been demonstrated *in vitro* are the bacterial *CesA* from *Rhodobacter sphaeroides* (Omadjela et al. 2013), *CesA8* from hybrid aspen (Purushotham et al. 2016) and *CesA5* from the moss *Physcomitrella patens* (Cho et al. 2017). In all cases recombinant forms of the proteins were reconstituted in artificial membrane-mimicking systems. However, the mechanism of the *R. sphaeroides* *CesA* has been characterized in much more detail, largely through the determination of the structure of the enzyme, which provided unprecedented mechanistic insights into β -glucan polymerization and translocation across the plasma membrane (Morgan et al. 2013). While this bacterial system has a catalytic core and cellulose-translocating channel formed by interactions between just two protein molecules (Morgan et al. 2013), higher plants utilise more intricate cellulose synthase complexes (CSCs) that can be visualized by electron microscopy as discrete structures designated as ‘rosettes’ (reviewed in Guerriero et al. 2010b). Organisms with high numbers of *CesAs* may utilize different isoforms for cellulose biosynthesis in specific

tissues and during specific developmental stages. In *Arabidopsis thaliana*, *AtCesA1*, 3, and 6 form a complex required for biosynthesis of cellulose in primary cell walls (Desprez et al. 2007), while *AtCesA4*, 7, and 8 assemble as CSCs to synthesize cellulose in secondary cell walls (Atanassov et al. 2009).

Similar to the situation in plants, multiple *CesA* genes are often found in oomycete genomes. For example, four such genes have been identified in the genomes of *Phytophthora infestans* (Grenville-Briggs et al. 2008) and *P. capsici* (<https://genome.jgi.doe.gov/Phyca11/Phyca11.home.html>). Compared to the great advances in the study of *CesA*s in plants and bacteria, there is a dearth of equivalent insights for the oomycetes. Previous studies on *P. infestans* using gene silencing by RNA interference and the cellulose synthesis inhibitor 2,6-dichlorobenzonitrile suggested that *CesA* proteins are involved in cellulose biosynthesis, and that this process is vital for infection of host plants (Grenville-Briggs et al. 2008). However, as this study involved the simultaneous silencing of all four *CesA* genes identified in *P. infestans*, no detailed information on the function and activity of each individual gene and their products is available. Similarly, no investigation has been conducted on oomycete *CesA* proteins to determine whether they form complexes similar to the type of CSC described in other classes of organisms. Instead, past studies have focused on biological aspects of cellulose biosynthesis, such as the importance of *CesA* genes in plant infection, or the bioinformatics analysis of their domain organization (Grenville-Briggs et al. 2008; Fugelstad et al. 2009). These studies revealed that the predicted C-terminal domain organization of oomycete *CesA* proteins is generally similar to that of their counterparts from organisms from other taxonomic groups (Fig. 1). The N-terminal ends of the proteins, however, are more divergent. With the exception of *CesA3*, oomycete *CesA*s contain a predicted pleckstrin homology (PH) domain at their N-terminal end that has been shown to be potentially involved in their regulation, trafficking and/or targeting to the plasma membrane (Fugelstad et al. 2012).

Here, we report the first evidence for the interaction of multiple *CesA* subunits in an oomycete using immunoprecipitation experiments, and successfully produce and purify an individual *CesA* protein (*PcCesA1*) in recombinant form with demonstrable

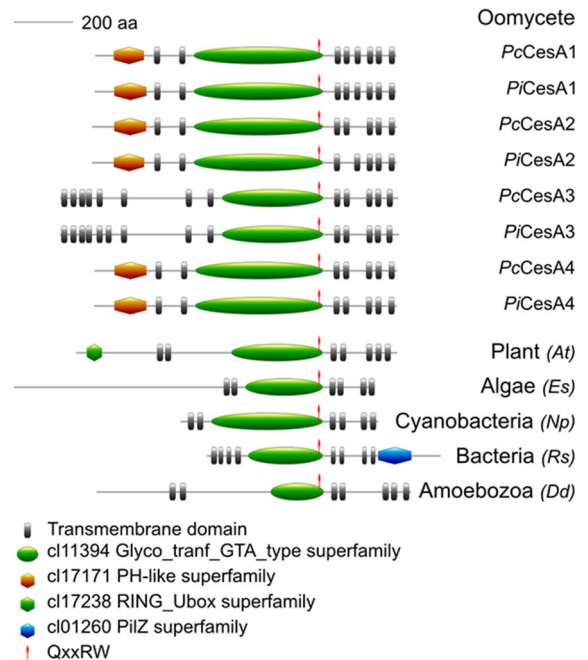


Fig. 1 Predicted domain organization of *PcCesA* proteins relative to *CesA*s from members of other taxonomic groups. *Pc*, *Phytophthora capsici*; *Pi*, *Phytophthora infestans*; *At*, *Arabidopsis thaliana*; *Es*, *Ectocarpus siliculosus*; *Np*, *Nostoc punctiforme*; *Rs*, *Rhodobacter sphaeroides*; *Ds*, *Dictyostelium discoideum*

catalytic activity *in vitro*. We also present an analysis of the glucan synthase activity occurring in the mycelium of *P. capsici*, and show that a complex of proteins is most likely required for the production of polymeric β -1,4-linked glucans. We additionally provide an analysis of the domain organization of the *P. capsici* *CesA* sequences and equivalent sequences from other taxonomic groups, giving new insights into the structural relationship between *CesA*s in the oomycetes and other kingdoms of life.

Materials and methods

Domain organization of putative cellulose synthases from *P. capsici*

The domain organization of *CesA* proteins from *P. capsici* (*PcCesA1*, *PcCesA2*, *PcCesA3* and *PcCesA4*) was analyzed and compared to that of *CesA*s representative of distinct lineages, namely *CesA*s from the plant *Arabidopsis thaliana* (*At*), the alga *Ectocarpus*

siliculosus (*Es*), the bacterium *Rhodobacter sphaeroides* (*Rs*), the cyanobacterium *Nostoc punctiforme* (*Np*) and the Amoebozoia *Dictyostelium discoideum* (*Dd*). The sequences of the following proteins were retrieved from the UniProt Database (<https://www.uniprot.org>) and the *P. capsici* genome (<https://genome.jgi.doe.gov/Phyca11/Phyca11.home.html>)

(UniProt and *P. capsici* ‘Phyca’ genome entries in parentheses): *PcCesA3* (H6U2P7), *PiCesA1* (A5A5Z3), *PiCesA2* (A5A5Z4), *PiCesA3* (A5A5Z5), *PiCesA4* (A5A5Z6), *AtCesA1* (O48946), *EsCesA* (D7FP53), *NpCesA* (B2IZN9) *RsBcsA* (Q3J125), *DdCesA* (Q9U720), *PcCesA1* (Phyca11_509423), *PcCesA2* (Phyca11_509421), and *PcCesA4* (Phyca11_107079). Transmembrane domains were predicted using the TOPCONS2 program (<https://topcons.cbr.su.se>) (Tsirigos et al. 2015) for all sequences, except for *RsBcsA*, for which the topology and crystal structure of the protein is available (Morgan et al. 2013). Putative protein domains were predicted using the NCBI CDD program (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) (Marchler-Bauer et al. 2011). Schematic diagrams representing the domain organization of each protein (Fig. 1) were created using Prosite MyDomains (<https://prosite.expasy.org/cgi-bin/prosite/mydomains/>).

Expression analysis of four *CesA* genes in the mycelium of *P. capsici*

P. capsici strain Hd11, originally isolated from a pepper field (Pang et al. 2013), was cultured in Potato Dextrose Broth (PDB) for three days at 25 °C in the dark. An RNeasy[®] kit (Qiagen, Hilden, Sweden) and a TURBO DNA-free[™] kit (Ambion, USA) were used respectively to extract RNA from the mycelium and remove contaminating DNA. The Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Lithuania) was used to synthesize the first strand cDNA from 1 µg total RNA.

For expression analysis of the *P. capsici* *CesA* genes, specific primer pairs (Table S1 in Supplementary Material) were designed using NCBI Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast>). The 40S ribosomal protein S3A, WS21 (Yan and Liou 2006) and mago nashi RNA-binding protein homologue (Vetukuri et al. 2011) were selected as housekeeping genes for normalization of transcript abundance, after their stability was determined using

geNorm (D’haene et al. 2012). Quantitative RT-PCR analysis for each *CesA* gene was performed using a CFX96 real-time PCR detection system (Bio-Rad) following the manufacturer’s instructions and relative expression levels were determined using Biogazelle’s qbase^{PLUS} (version 3.0).

Preparation and immunochemical analyses of membrane proteins

P. capsici was cultured in PDB medium, and the mycelium was collected after 7 days of growth at 25 °C in the dark. Microsomal membranes were obtained by differential centrifugation and membrane proteins were extracted with 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) as described previously (Fugelstad et al. 2009; Guerriero et al. 2010a). The resulting CHAPS extract was used as a source of protein for subsequent Western blot and immunoprecipitation experiments, and cellulose synthase activity assays.

Monoclonal antibodies were raised in mice against peptides unique to each of the four *PcCesA* proteins by the company Abmart (Shanghai, China). The specific sequences used were SAMDIRQPGE (*PcCesA1*), NKGILETAGD (*PcCesA2*), AVLSNVAFTSLQDGG (*PcCesA3*), and ANRQPPGLGA (*PcCesA4*). Antibody specificity was verified by dot blot analysis. For this purpose, 20 µg of each peptide was adsorbed onto nitrocellulose membranes (Millipore; 0.45 µm) which were subsequently blocked at room temperature in Tris Buffer Saline (TBS pH 7.4; 25 mM Tris; 150 mM NaCl) containing 5% non-fat milk and 0.05% Tween 20 (TBST). Membranes were incubated overnight at 4 °C under gentle agitation with one of the four monoclonal antibodies used at a 1:500 dilution. After six washes of 10 min each in TBST, a solution of anti-mouse IgG coupled to horseradish peroxidase (1:2000 dilution in TBST; Life Technologies) was incubated with the membranes for 2 h at room temperature under gentle agitation. The luminal-based Amersham ECL[™] Prime Western Blotting Detection Reagent (GE Healthcare) and a CCD imager (Intelligent Dark Box II, FuJIFILM LAS-1000) were used to visualize and record images of the membranes, respectively. Western blot analyses were conducted using 0.45-µm polyvinylidene difluoride (PVDF) membranes (Bio-Rad) and CHAPS-extracted microsomal proteins as antigens (20 µg protein per lane on 10% SDS-PAGE

gels). All incubations were performed in the same conditions as the dot blot experiments described above, except for the use of more concentrated solutions of anti-CesA antibodies (1:500 dilution in TBST).

Immunoprecipitation experiments were performed by incubating 500 μl CHAPS-extracted proteins (1 mg mL⁻¹) overnight at 4 °C with the anti-CesA1 monoclonal antibody (1:500 dilution). Protein G coupled to Sepharose[®] 4B (Sigma, cat# P-3296) was used to capture the protein/antibody complexes following the manufacturer's instructions. The pellets containing the immunoprecipitated proteins were heated in 2 \times SDS buffer at 95 °C for 7 min and Western blot analyses were performed on the released proteins using each anti-CesA antibody as described above. The proteins recognized by the different antibodies were excised and subjected to trypsin proteolysis and LC–MS/MS analysis as described earlier (Srivastava et al. 2016).

Generation of a yeast glucan synthase mutant for expression of *PcCesA1*

To minimize the background levels of glucosyltransferase (GlcT) activity in the yeast expression host, a deletion strain of *Saccharomyces cerevisiae* with reduced glucan synthase activity (LoGSA) was generated. All yeast strains (Table S2 in Supplementary Material) were grown at 30 °C on YPD or synthetic complete (SC) medium lacking individual amino acids. Plasmids pRS315 (Sikorski and Hieter 1989), pRS317 (Sikorski and Boeke 1991), and pRS413 (Mumberg et al. 1994) harboring the auxotrophic marker genes *LEU2*, *LYS2*, or *HIS3*, respectively, served as templates for gene amplification. Gene replacement cassettes (GRCs) with overhangs for homologous recombination directly up- and downstream of the coding sequence of the target gene were amplified by PCR (Phusion High-Fidelity DNA Polymerase; New England Biolabs, UK) from plasmids harboring the auxotrophic marker gene of choice. All PCR products were purified (QIAquick PCR Purification Kit; Qiagen, Sollentuna, Sweden) and their size was verified by agarose gel electrophoresis. For replacement of the glycogen synthase (*GSY2*) and endopeptidase (*PEP4*) genes, linear DNA fragments with 45–48 bp-long homologous flanks were generated in a single PCR step using primers containing

homologous ends (Table S3 in Supplementary Material). Replacement of the glycogen synthase gene *GSY1* by GRCs with short flanks was not successful. Therefore, DNA fragments with about 1 kb flanks were generated in two subsequent PCR steps (Wach 1996). First, homologous flanks up- and downstream of the *GSY1* coding sequence were amplified from extracted yeast genomic DNA (ZR Fungal/Bacterial DNA MiniPrep Kit; Zymo Research, Freiburg, Germany) with primer pairs (*GSY1_grcF* and *GSY1_seqF* or *GSY1_grcR* and *GSY1_seqR*, respectively; see Table S3 in Supplementary Material) containing short stretches homologous to *HIS3*. The corresponding purified PCR fragments were used as primers for amplification of the full GRC from plasmid pRS413.

For construction of the *S. cerevisiae* LoGSA strain, a *FKS1*-deficient *S. cerevisiae* strain (ATCC # 4015251) was used for replacements of *GSY2*, *PEP4*, and *GSY1* in consecutive rounds of transformation of chemically-competent *S. cerevisiae* by homologous recombination, following the lithium acetate/single-stranded carrier DNA/polyethylene glycol method (Gietz and Woods 2002) modified by Drew et al. (2008). All steps were carried out at 4 °C. Briefly, the yeast strain to be transformed was grown in 50 ml YPD until an OD (600 nm) of 0.6 was reached and the cells were harvested by centrifugation at 3000g for 5 min. After a wash with sterile water and two washes with 100 mM lithium acetate, 50 μl of competent cells was mixed with 240 μl of 50% (w/w) polyethylene glycol 3350, 50 μl of 2 mg mL⁻¹ ssDNA, and 4 or 2 μg of GRC for deletion of *GSY2* and *PEP4* or *GSY1*, respectively. After a 30-min incubation, cells were heat-shocked at 42 °C for 25 min and plated out on SC medium lacking the individual amino acids for appropriate selection of transformants. Agar plates were incubated at 30 °C for 48 h and genomic DNA was extracted (ZR Fungal/Bacterial DNA MiniPrep Kit) from random colonies incubated in 5 ml selective SC medium at 30 °C for another 48 h. Replacement of target genes and integrity of dysfunctional genomic loci for auxotrophic marker genes were verified by PCR (see Table S3 in Supplementary Material for primers) and agarose gel electrophoresis (Figure S1 in Supplementary Material).

Heterologous expression and purification of *PcCesA1*

The expression of *PcCesA1* in the LoGSA yeast strain was performed as described previously (Drew et al. 2008). The *PcCesA1* gene was cloned into the pDDGFP-2 yEGFP-His₈-containing vector (Newstead et al. 2007) which was transformed into the LoGSA yeast cells by heat shock. Colonies were screened for galactose-induced over-expression of the *PcCesA1* protein fused to GFP by whole-cell (FLUOstar OPTIMA microplate reader; BMG Labtech) and in-gel fluorescence (Newstead et al. 2007; Drew et al. 2008). The transformant showing the highest level of protein expression was chosen for upscaling recombinant protein production and the presence of the correct sequence of the *PcCesA1*-GFP fusion gene in the selected cells was confirmed after DNA extraction (GeneJET Plasmid Miniprep Kit, ThermoFisher Scientific, Lithuania) and sequencing. Additionally, the fluorescent protein band showing the expected apparent molecular mass after in-gel fluorescence analysis was subjected to partial proteolysis in the presence of trypsin and LC-MS/MS analysis as described earlier (Srivastava et al. 2016). Microsomal proteins were extracted using 0.5% CHAPS as briefly described above and elsewhere (Fugelstad et al. 2009; Guerriero et al. 2010b).

The Ni-NTA His-Bind[®] resin from Novagen (Germany) was used to purify the recombinant *PcCesA1* protein by immobilized metal affinity chromatography (IMAC), using its fused His₈ sequence as an affinity tag, as per the manufacturer's instructions. Briefly, the detergent-solubilized protein was incubated with the resin at 4 °C for 1 h, and the resin was washed in phosphate buffer saline (PBS) pH 7.4 containing 150 mM NaCl, 30 mM imidazole and 10% glycerol. For protein elution, the imidazole concentration was increased to 180 mM and an Amicon Ultra-15 50 K Centrifugal Filter Device (Millipore) was subsequently used to concentrate the purified recombinant *PcCesA1* protein.

β-Glucan synthase assays

Radiometric assays were performed essentially as described earlier (Fugelstad et al. 2009; Brown et al. 2012) to quantify the incorporation of glucose (Glc) from UDP-Glc into water-soluble and insoluble

glucans produced by three types of protein samples: microsomal membranes produced by the yeast strains used and generated here; CHAPS-extracts of microsomal membranes from *P. capsici*; and recombinant *PcCesA1* protein. CHAPS was used as a detergent to extract glucan synthases from microsomal membranes and solubilize the recombinant *PcCesA1* protein prior to activity assays. CHAPS was retained as a detergent over digitonin, which is also commonly used for the extraction of glucan synthases from biological membranes (Brown et al. 2012), as the levels of activity measured in CHAPS extracts were twice as high compared to digitonin (data not shown).

To confirm that the deletion of the *GSY1* and *GSY2* genes in the original BY4742 and FKS1-deficient (ATCC # 4015251) yeast strains led to a nearly complete abolition of glucan synthase activities *in vitro* in the LoGSA strain, the assays were performed in conditions where most glucan synthase activities are readily detectable, i.e. in the presence of 1 mM UDP-Glc concentrations (Brown et al. 2012). For this purpose, 150 µg of microsomal proteins prepared from the different yeast strains were incubated in 100 mM MOPS buffer pH 7 containing 1 mM UDP-glucose, 0.4 µM UDP-[¹⁴C-U]glucose (250 mCi/mmol, Perkin Elmer, Boston, USA), 8 mM CaCl₂ and 5% glycerol (total volume of reaction mixtures: 200 µL). After 2 h incubation at room temperature, the reactions were terminated by addition of 400 µL of 100% ethanol, or 300 µL of chloroform and 100 µL of water, for quantification of radioactive insoluble and soluble reaction products, respectively (Brown et al. 2012). The radioactive insoluble products were recovered on Whatman GF/C glass-fiber filters, which were successively washed with 4 mL of 66% (vol/vol) ethanol, 4 mL of water and again 4 mL of 66% ethanol. This step allows the elimination of the excess of ¹⁴C-labelled substrate and the radioactivity retained of the filters is a measure of the catalytic activity responsible for the formation of ethanol-insoluble polysaccharides (Brown et al. 2012). For determination of the amount of water-soluble products formed *in vitro*, the samples recovered after the addition of chloroform and water were deposited on Dowex anion-exchange mini-columns prepared in micropipette tips, as described in Brown et al. (2012). During this step the excess of radioactive substrate binds to the Dowex matrix through its charged phosphate groups, whereas the neutral water-

soluble oligosaccharides formed *in vitro* are readily eluted. The eluates containing the radioactive products were subsequently mixed with 4 mL of Ultima Gold scintillation reagent for aqueous solutions and analyzed in a liquid scintillation counter as detailed in Brown et al. (2012).

Assays conditions that favor the formation of β -1,4-glucans (cellooligosaccharides or cellulose) by oomycete cell-free protein preparations (Bulone et al. 1990; Fugelstad et al. 2009) were used to detect cellulose synthase activity in CHAPS-extracts of microsomal membranes from *P. capsici* and to demonstrate that the purified recombinant *PcCesA1* protein is able to catalyze the formation of β -1,4-glucans. The reaction mixtures had a final volume of 200 μ L and contained 50 μ L of enzyme preparation, 7.5 mM Pipes/Tris pH 6.0, 1 μ M UDP-[14 C-U]glucose (302 mCi/mmol; Perkin Elmer, Boston, USA), and 10 mM MgCl₂. After incubation at room temperature for 1 h, the reactions were stopped as indicated above and the radioactive insoluble and soluble products were separated from the excess of radioactive substrate and quantified by liquid scintillation (see previous paragraph) (Bulone et al. 1990; Fugelstad et al. 2009; Brown et al. 2012).

Characterization of products formed in glucan synthase assays

Soluble reaction products synthesized *in vitro* were present in low concentration and as complex mixtures containing proteins, CHAPS, glycerol and the reagents required for glucan synthase assays. As this impeded direct subsequent characterization, oligosaccharides were purified by chromatography on 1-mL graphite columns (HyperSep Hypercarb SPE Cartridges, ThermoFisher Scientific). The latter were successively pre-equilibrated in 50% acetonitrile/0.1% trifluoroacetic acid (TFA), washed with five volumes of distilled water, loaded with the samples and washed again with five volumes of distilled water. Oligosaccharides were eluted with 1 volume of 25% acetonitrile/0.05% TFA, dried under vacuum, and resuspended in a minimum volume of water (50–200 μ L) for subsequent analyses by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), and thin layer chromatography (TLC).

HPAEC-PAD analysis was performed on a Dionex ICS-3000 HPLC system fitted with a Dionex Carbopac

PA1 column, using 4 solvents: water (solvent A), 300 mM NaOH (solvent B), 200 mM NaOH/170 mM sodium acetate (solvent C), and 1 M sodium acetate (solvent D). The following programme was used for the separation of the longest oligosaccharides: 5 min pre-equilibration with 33% B followed by 15 min with 33% B/10% D; 1 min with 33% B/20% D; 1 min with 75% B/25% D; and 3 min with 33% B/3% D. Shorter oligosaccharides were better separated using the following conditions: 35 min with 33% B; 1 min with 33% B/20% D and 9 min with 33% B. Oligosaccharides were identified by comparing their retention times with those of known standards (Figure S2 in Supplementary Material).

For TLC analysis, the samples (2 μ L) were spotted onto silica gel-60 plates (Merck) and developed using butan-1-ol/acetic acid/water (2:1:1 by volume) up to the full height of the plates (20 cm). Upon completion of the runs, the plates were successively dried, dipped into 8% H₂SO₄ in ethanol and heated with a heat gun until spots became visible. Radiolabelled products formed in the presence of UDP-[14 C-U]glucose were quantified after TLC separation by cutting each lane of the dried plates into 1-cm high rectangles and measuring the corresponding radioactivity by liquid scintillation (Brown et al. 2012).

Results

Analysis of domain organization of *PcCesA* proteins

The general domain organization of the predicted *CesAs* from *P. capsici* is similar to that of *CesAs* from other oomycetes and organisms from other taxonomic groups, as illustrated in Fig. 1 with some representatives from major lineages. Indeed, all *CesA* proteins possess the typical signatures of a glycosyltransferase A-type of fold and Pfam03552 (cellulose synthase) domain, multiple transmembrane helices at both the *N*- and *C*-terminal ends of the proteins, three catalytic aspartic acids, and the QXXRW motif (Grenville-Briggs et al. 2008; Fugelstad et al. 2009) present in most processive glycosyltransferases (Campbell et al. 1997) (Fig. 1). Consistent with previous studies of oomycete *CesAs* (Grenville-Briggs et al. 2008; Fugelstad et al. 2009), preliminary analysis of the predicted *CesA* sequences from *P. capsici* revealed that all four

proteins are more similar to orthologous proteins from other oomycete species than they are to each other. For example, *PcCesA1* shares higher similarity with *CesA1* proteins from other oomycetes than with *PcCesA2*, *PcCesA3*, or *PcCesA4*. Additionally, among all known oomycete *CesA*s, *CesA1*, 2, and 4 exhibit a higher degree of similarity with each other than with *CesA3* proteins. Our data also indicate that the oomycete *CesA3* proteins, including *PcCesA3*, lack the PH domain present at the *N*-terminal end of the other oomycete *CesA* proteins (Fig. 1). PH domains are small α -helical modules which interact with lipids, and are likely involved in regulation, targeting, or trafficking of polysaccharide synthases to the site of cell wall biosynthesis (Fugelstad et al. 2012). The PH domain is also not present in non-oomycete *CesA*s (Fig. 1). However, some *CesA* proteins from other lineages contain domains that are not encountered in their oomycete counterparts. This is the case for example of the RING finger domains present in plant *CesA*s and involved in *CesA* dimerization (Kurek et al. 2002), and the PilZ domain present in some bacterial *CesA*s and involved in the binding of the activator cyclic di-guanylic acid (Morgan et al. 2014) (Fig. 1). The next sections of this report focus on the use of biochemical approaches to better understand the properties of the poorly characterized *CesA* proteins from oomycetes, using *P. capsici* as a model organism.

Interactions between *CesA* proteins in *P. capsici* mycelial membranes

All four identified *CesA* genes were confirmed to be expressed in the mycelium of *P. capsici*, as determined by quantification of RNA transcript abundance, with *PcCesA3* presenting a higher level of expression than the other three genes (Fig. 2). In addition, Western blot analysis confirmed all four *CesA* proteins are expressed in the mycelium as a single protein band was apparent after separate incubations of CHAPS-extracted microsomal proteins with anti-*PcCesA1*, anti-*PcCesA2*, anti-*PcCesA3*, and anti-*PcCesA4* specific antibodies (Fig. 3A). Specificity of each of the antibodies was confirmed by dot blot analysis using peptides corresponding to each of the four *CesA* proteins (Figure S3A in Supplementary Material). Immunoprecipitation of detergent-solubilized *P. capsici* mycelial membrane proteins using anti-*PcCesA1*

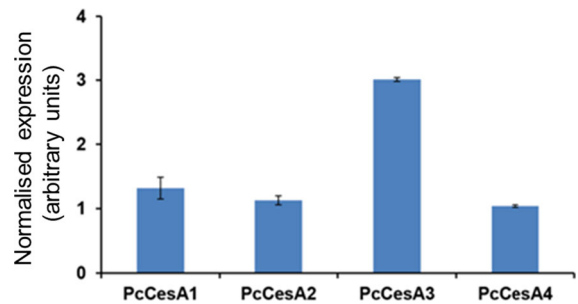


Fig. 2 Analysis of *CesA* gene expression in the mycelium of *P. capsici*. Gene expression levels were measured by qPCR and normalized using the reference genes for the 40S ribosomal protein S3A (WS21) and mago nashi RNA-binding protein homologue. Error bars represent the standard deviations obtained from expression analyses conducted on three independent mycelial cultures

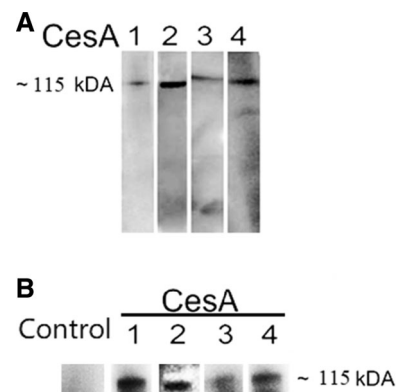


Fig. 3 Western blot and immunoprecipitation analyses of the four *CesA* proteins from *P. capsici*. **A** Western blot analysis using anti-*CesA* antibodies specific for *PcCesA1* (lane 1), *PcCesA2* (lane 2), *PcCesA3* (lane 3) and *PcCesA4* (lane 4). Each antibody was used separately to detect its target proteins in CHAPS-extracts of mycelial microsomal membranes. **B** Western blot analysis performed on the proteins immunoprecipitated by the anti-*PcCesA1* antibody. The different lanes were probed using the anti-*PcCesA1*, anti-*PcCesA2*, anti-*PcCesA3* and anti-*PcCesA4* antibodies as indicated at the top of the figure. Control: negative control performed in the absence of primary antibody

antibodies showed that *PcCesA1*, *PcCesA2*, and *PcCesA4* proteins interact in the membrane, whereas the Western blot signal obtained for *PcCesA3* in the immunoprecipitate suggests a much weaker interaction or no interaction at all of this protein with the other *CesA*s (Fig. 3B). As expected the negative control performed in the absence of primary antibody did not give any signal at the expected molecular

weight of the Cesa proteins (115 kDa) (Fig. 3B, Control lane). A high background was observed in these Western blots at lower molecular weights, as visible on the full membranes presented in Figure S3B in Supplementary Material. This is due to the fact that the heavy and light chains of the mouse primary anti-*PcCesA1* antibodies are abundant in the immunoprecipitate and heterogenous in molecular weight due to glycosylation, hence the high signal obtained from these proteins across a large range of apparent molecular weights after incubation of the blots with the secondary anti-mouse IgG antibodies conjugated to horseradish peroxidase. The absence of a 115-kDa band in the negative control confirms the specificity of the immunoprecipitation reaction for the Cesa proteins. Consistent with these results, *PcCesA1*, *PcCesA2*, and *PcCesA4* proteins, but not *PcCesA3*, could be identified among the immunoprecipitated proteins by LC–MS/MS analysis (Figure S4 in Supplementary Material).

Demonstration of the β -1,4-glucosyltransferase activity of *PcCesA1*

CHAPS extracts prepared from a mycelial microsomal fraction of *P. capsici* were first used as a source of protein to test for glucan synthase activity *in vitro*. The radiometric assays revealed that the enzyme preparation is able to incorporate Glc from UDP-[U- 14 C]Glc into water-soluble reaction products (2321 ± 106 pmol Glc incorporated into soluble β -glucans per mg protein), but the level of insoluble glucan formed is comparatively low, almost negligible (49 ± 2 pmol Glc incorporated per mg protein) (Fig. 4). These data suggest that the ‘native’ enzymes present in the detergent extracts are essentially unable to form polymeric products *in vitro*, reflecting the possible loss of a factor essential for chain elongation during detergent extraction or the disruption of the integrity of the enzyme complex expected to be required for the formation of insoluble polymeric glucan chains. Biochemical characterization of heterologously expressed glucosyltransferase (GlcT) enzymes in *S. cerevisiae* is hampered by the presence of endogenous β -1,3-glucan synthases (Fks1p (Douglas et al. 1994; Castro et al. 1995; El-Sherbeini and Clemas 1995) and Fks2p (Inoue et al. 1995; Mazur et al. 1995) and glycogen synthases (Gsy1p (Farkas et al. 1990) and Gsy2p (Farkas et al. 1991)), which

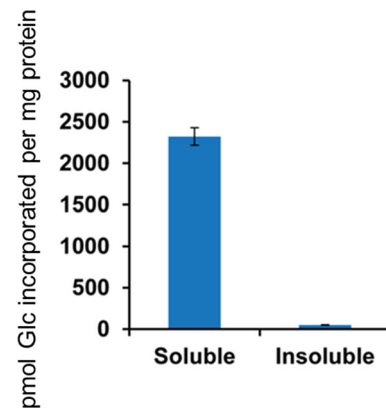


Fig. 4 Cellulose synthase activity detected in detergent extracts of the mycelial microsomal membranes from *P. capsici*. The data are expressed as pmol of Glc incorporated into water-soluble and insoluble glucans per mg of protein (see Methodology for details on experimental conditions). Experiments were performed three times and the error bars represent means \pm standard errors

utilise UDP-Glc as a substrate, masking the activity of recombinant GlcT enzymes. We therefore developed a new yeast strain designated as LoGSA (for ‘low glucan synthase activity’) that lacks Fks1p, Gsy1p, and Gsy2p (Figure S1 in Supplementary Material), and therefore has significantly reduced GlcT activity (Figure S5 in Supplementary Material). The *FKS2* gene was left intact as the contribution of Fks2p to β -1,3-glucan synthase activity is minor if not stimulated by calcium ions (Mazur et al. 1995), and disruption of *FKS2* in cells lacking *FKS1* is lethal (Inoue et al. 1995; Mazur et al. 1995). To improve the stability and yield of heterologously expressed proteins we further engineered the yeast strain to be protease-deficient by replacement of the *PEP4* gene (Drew et al. 2008). In this way the activity of proteinase A, the major vacuolar endopeptidase required for *in vivo* maturation of other vacuolar hydrolases (Jones et al. 1982; Ammerer et al. 1986; Woolford et al. 1986) is abolished.

Attempts to express Cesa1 and Cesa3 proteins from *P. capsici* in the yeast LoGSA strain were successful only in the case of *PcCesA1*, as evidenced by in-gel fluorescence analysis of crude yeast membrane samples from the cultures that over-express the protein (Fig. 5). A fluorescent band corresponding to the size of *PcCesA1* (around 115 kDa) was confirmed to be the *PcCesA1* protein by LC–MS/MS analysis,

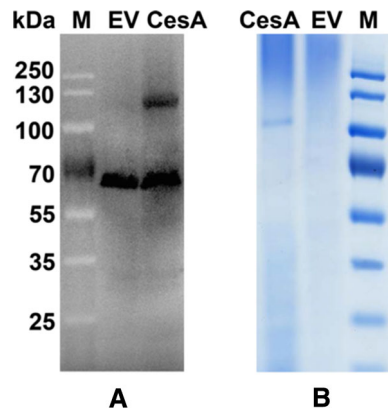


Fig. 5 Over-expression in yeast and purification of *PcCesA1*. **A** SDS-PAGE in-gel fluorescence of crude yeast membrane proteins isolated from cells transformed with the empty expression vector (EV), the vector carrying the *PcCesA1* gene fused with GFP and the His₈ tag (CesA). **B** SDS-PAGE analysis of the fraction obtained after purification of *PcCesA1* by IMAC (lane marked CesA) and corresponding EV control (Coomassie blue staining). M: molecular weight markers

with nearly 15% sequence coverage (Figure S6 in Supplementary Material). DNA sequencing additionally confirmed that the gene encoding *PcCesA1* was present without error in the over-expressing transformant. After purification by IMAC, the target band corresponding to *PcCesA1* was purified to homogeneity as judged by Coomassie blue staining (Fig. 5).

Radiometric assays of cellulose synthase activity were performed on microsomal fractions from the *S. cerevisiae* transformant that over-expresses *PcCesA1* prior to the extraction of the recombinant enzyme with CHAPS. These assays showed no significant difference compared to the empty vector control, suggesting that the recombinant protein has limited or no access to the substrate when it is embedded in the yeast membrane. This contrasts with the radiometric activity assays conducted on CHAPS-extracts of the microsomal fractions from the yeast transformant, which showed an incorporation of 271 ± 30 pmol Glc into β -glucans per mg protein. This activity was more than 13-fold higher than the activity detected from membrane proteins isolated from the empty vector control yeast strain, which showed only the low residual activity (20 ± 0.5 pmol Glc incorporated per mg protein) of the LoGSA expression system (Fig. 6A). No insoluble product could be recovered from these reactions, indicating that only short-chain β -glucans were synthesized. Recombinant *PcCesA1* was

relatively stable in solution, with maximum Glc incorporation achieved in less than 1 h. Activity was lost significantly if the protein was frozen or stored at $+4$ °C. Replicate assays from separate expression experiments confirmed the repeatability of activity measurements shown in Fig. 6B. HPAEC-PAD analysis showed that the major product of the reaction was cellobiose (Fig. 6C). Radiometric TLC analysis suggested the additional presence of small amounts of ^{14}C -glucose and ^{14}C -cellotriose (Figure S7 in Supplementary Material), but these were not of sufficient concentration to be detectable by HPAEC-PAD, and may appear due to the rather diffuse separation achievable by TLC. The addition of Glc in the reaction mixture was accompanied by a moderate gain of activity detectable by radiometric assay (329 ± 9 pmol Glc incorporated into β -glucans per mg protein) (Fig. 6A) and TLC analysis indicated a slightly elevated proportion of ^{14}C -cellotriose (Figure S7 in Supplementary Material) compared to the reaction assay performed in the absence of Glc, but this again could not be confirmed by HPAEC-PAD. After IMAC purification (Fig. 5), the *PcCesA1* protein was unstable and showed reduced activity (125 ± 7 pmol Glc incorporated per mg protein; Fig. 6B), and TLC analysis confirmed the same reaction products as for the crude CHAPS-extracted microsomal fraction (Figure S7 in Supplementary Material, right panel).

Discussion

Silencing of the whole *CesA* gene family in *P. infestans* using an RNA interference method previously caused a more than 50% reduction in cellulose content in the cell walls of silenced transformants, and cell wall disruption of the infectious appressoria (Grenville-Briggs et al. 2008). These data suggested that *PiCesAs* are specifically involved in cellulose biosynthesis, with particular importance during plant infection. However, the function of individual *CesA* proteins, and detailed knowledge of the cellulose biosynthetic process in oomycetes, remains unknown.

Our results demonstrate conclusively that four *CesA* proteins are expressed in the mycelium of *P. capsici* at both the RNA and protein levels (Fig. 2; Fig. 3A). At the time of writing, there are no reports in the literature demonstrating evidence for a multi *CesA*

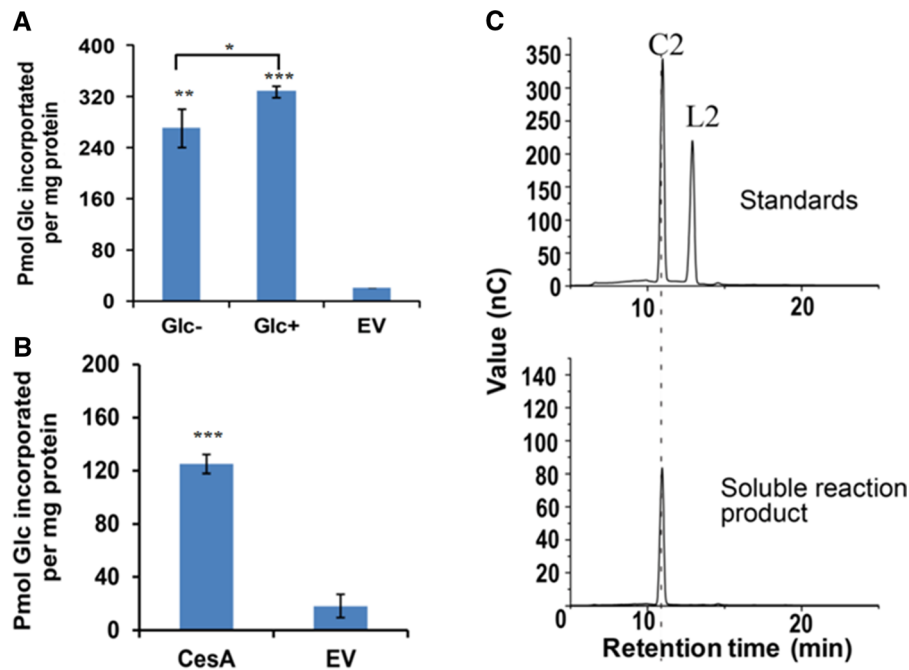


Fig. 6 Cellulose synthase assays on recombinant *PcCesA1* fractions from yeast and analysis of the water-soluble products formed *in vitro* by the purified protein. **A** Radiometric quantification of Glc incorporation into soluble β -glucan products by CHAPS-extracts of yeast microsomal membranes (LoGSA strain) containing the recombinant *PcCesA1*. Glc-, reaction performed in the absence of added glucose. Glc+, reaction performed in the presence of 250 μ M glucose in the assay. **B** As in **A**, but using the IMAC-purified *PcCesA1* fraction (no Glc added in the assay). In figures **A** and **B**, statistical

analyses were performed using Student's *t*-test for unpaired data or one-way ANOVA analysis based on three independent experiments. **C** HPAEC-PAD chromatogram showing the presence of one major reaction product, which exhibits an identical retention time as cellobiose [C2: cellobiose, L2: laminaribiose (β -1,3-linked disaccharide of Glc residues)]. Glc and additional standards of the celooligosaccharide and laminarioligosaccharide series with degrees of polymerization up to six were also used. The corresponding chromatograms are presented in Figure S2 (Supplementary Material)

protein complex in oomycetes. Immunoprecipitation analysis using anti-*PcCesA1* antibodies revealed that at least *PcCesA1*, *PcCesA2*, and *PcCesA4* are linked together. The weak protein band observed in Western blots using anti-*Cesa3* antibodies (Fig. 3B), and an inability to detect any *PcCesA3* protein in the immunoprecipitate by MS (Figure S4 in Supplementary Material), suggests that *PcCesA3* may also have a weak interaction with the complex or that it may not be part of it. The typical roles of PH domains in protein trafficking to membranes could explain a weaker interaction of *PcCesA3* with the membrane-bound *Cesa* complex (Fugelstad et al. 2012). Further, and as discussed in more detail below, *PcCesA1* alone produces only short celooligosaccharides *in vitro* (Fig. 6), suggesting that the aforementioned *Cesa* complex is required to form the type of cellulose microfibrils typically observed in oomycete cell walls (Bulone et al. 1992; Helbert et al. 1997).

Cesa proteins have multiple transmembrane domains, which has been a significant hindrance to *in vitro* expression and biochemical characterization as heterologously expressed membrane proteins, once solubilized for experimental use, are often unstable. The successful expression of a recombinant form of the hybrid aspen *PttCesA8* (Purushotham et al. 2016) and the moss *PpCESA5* (Cho et al. 2017) proteins in yeast are significant achievements toward the full characterization of plant *Cesa*s, but as yet there is no comparable example of heterologous expression of an oomycete *Cesa*. Further hampering efforts to biochemically characterize over-expressed *Cesa* enzymes is the presence in the *S. cerevisiae* expression strain of GlcT enzymes, which can use UDP-Glc as substrate, causing a high background in glucan synthase activity assays. Measurement of enzymatic activity from an introduced GlcT is confounded by these endogenous activities. To overcome this

problem we have engineered a new yeast expression strain (LoGSA) that lacks Fks1, Gsy1, and Gsy2 activities (Figures S1 and S5 in Supplementary Material), greatly facilitating biochemical characterization of putative GlcT enzymes through heterologous expression in this strain.

Using this LoGSA strain, we attempted to express recombinant forms of the individual Cesa proteins from *P. capsici*, in particular *PcCesA3* as the most highly expressed of all *PcCesA* genes (Fig. 2) and *PcCesA1*, the most highly expressed of the PH domain-containing CesaAs. Attempts to over-express *PcCesA3* were not successful, possibly due to the absence of a PH domain in this enzyme. Indeed, previous biochemical studies of the PH domain from the *Saprolegnia monoica* Cesa2 suggest a function in regulation, trafficking and/or targeting of the enzyme to the site of cellulose biosynthesis (Fugelstad et al. 2012). The lack of a PH domain in *PcCesA3* may therefore be a factor in the lack of expression in our system. However, we were able to successfully and consistently over-express *PcCesA1*, which could be purified and retained enzymatic activity. The amount of pure recombinant protein and level of activity were not sufficient though to allow determination of the enzyme kinetic parameters.

PcCesA1 belongs to the GT2 family and is therefore predicted to be an inverting enzyme (Campbell et al. 1997; Lairson et al. 2008). The recombinant protein produced cellobiose as the main reaction product, in the conditions utilized here (Fig. 6; Figure S7 in Supplementary Material). With the addition of glucose in the assay as a possible acceptor molecule, we observed a minor increase in activity (Fig. 6) but no significant change in the profile of the reaction products (Figure S7 in Supplementary Material). This suggests that the added glucose monosaccharide is not able to act as an acceptor or primer to the GlcT reaction catalyzed by *PcCesA1*. HPAEC-PAD data show the presence of a small amount of glucose in the microsomal membrane preparation containing the recombinant *PcCesA1* (not shown), which likely interferes with the glucose added in the GlcT *in vitro* reaction. The minor activity increase resulting from the inclusion of additional glucose indicates that the glucose arising from the microsomal membrane preparation is almost sufficient for the enzyme to achieve maximum activity. Our *in vitro* results further show that the enzyme is not capable of adding

additional glucose residues to cellobiose or cellotriose once synthesized, which may suggest that the yeast membrane environment is sub-optimal for *PcCesA1* activity, due to a lack of necessary protein–protein interactions, or other conditions which are not ideal for enzyme activity. Alternatively, it may be that the interaction of multiple Cesa proteins in a complex is required for the production of cellulose, perhaps primed by the production of cellobiose. Indeed, we have shown that *PcCesA1* interacts with at least two other Cesa proteins in a complex which may also include other as yet unidentified proteins that may be essential for polymerization of longer cellulose chains of the type found in oomycete cell walls (Bulone et al. 1992; Helbert et al. 1997).

It is tempting to extrapolate from these findings that *PcCesA1* produces di- and trisaccharides to initiate the synthesis of cellulose, and that this short glucan is elongated by other associated enzymes. This would echo the mechanism of cellulose biosynthesis in plants, where at least three types of Cesa proteins are involved in membrane-bound complexes to produce cellulose microfibrils *in vivo* (Doblin et al. 2002; Desprez et al. 2007; Guerriero et al. 2010b). The data, however, contrast with the *in vitro* formation of cellulose microfibrils by single catalytic subunits of plant CesaAs expressed recombinantly and reconstituted in artificial systems (Purusotham et al. 2016; Cho et al. 2017). It can be speculated that the multiple Cesa proteins of *P. capsici* interact in a similar manner as the plant enzymes do *in vivo*, although no complexes of similar size as the plant cellulose synthase rosettes have been observed in oomycete membranes.

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Author contributions VB and XL conceived the study. ZP performed most experimental work. SMDM, SK and PO generated the *S. cerevisiae* LoGSA strain. VS performed mass spectrometry analyses and LSM characterized enzyme reaction products. ZP, VB, LSM and VS analyzed the data, with input

from the other authors. ZP, LSM and VB wrote the manuscript, with input and critical editing by all other authors.

Availability of data and materials The deletion strain of *S. cerevisiae* with reduced glucan synthase activity (LoGSA) generated in this work will be made available to other research groups upon request to the corresponding author (V. Bulone; email: bulone@kth.se).

Code availability Not applicable.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest associated with the work presented.

Ethics approval Not applicable.

Human and animal rights participants Not applicable.

Informed consent Not applicable.

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