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Cellulose Biosynthesis in Oomycetes

Johanna Fugelstad

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School of Biotechnology
Royal Institute of Technology
AlbaNova University Center
106 91 Stockholm
Sweden

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Abstract

Oomycetes have long been considered as a separate class within the kingdom Fungi, but they are in fact closer to brown algae. They are currently classified in the Stramenopile eukaryotic kingdom, which includes heterokont algae and water molds. The major cell wall polysaccharides in Oomycetes are β-(1→3) and β-(1→6)-glucans, as well as cellulose, which has never been reported in any fungal species. Chitin - the major cell wall polysaccharide in fungi - occurs in minor amounts in the walls of some Oomycetes. Some Oomycete species are pathogens of great economical importance. For example, species of the genus *Phytophthora* are well studied plant pathogens that cause considerable economical losses in agriculture. Saprolegniosis, a fish disease caused by species from the genus *Saprolegnia*, is a major problem in the aquaculture industry and represents a threat to populations of salmonids in natural habitats. Currently, there are no chemicals available that are at the same time efficient Oomycete inhibitors, environmentally friendly and safe for human consumption of treated fishes. The biosynthesis of cellulose in Oomycetes is poorly understood, even though this biochemical pathway represents a potential target for new Oomycete inhibitors. In this work, cellulose biosynthesis was investigated in two selected Oomycetes, the plant pathogen *Phytophthora infestans* and the fish pathogen *Saprolegnia monoica*.

A new Oomycete *CesA* gene family was identified. It contains four homologues designated as *CesA1, CesA2, CesA3* and *CesA4*. The gene products of *CesA1, 2* and *4* contain Pleckstrin Homology domains located at the N-terminus. This represents a novel feature, unique to the Oomycete *CesA* genes. *CesA3* is the dominantly expressed *CesA* homologue in the mycelium of both *S. monoica* and *P. infestans*, while *CesA1* and *CesA2* are up-regulated in virulent life stages of *P. infestans*. *CesA4* was expressed only in minute amounts in all investigated types of cells. Gene silencing by RNA interference of the whole *CesA* gene family in *P. infestans* lead to decreased amounts of cellulose in the cell wall. The inhibitors of cellulose synthesis DCB and Congo Red had an up-regulating effect on *SmCesA* gene expression, which was accompanied by an increased β-glucan synthase activity *in vitro*. In addition, these inhibitors slowed down the growth of the mycelium from *S. monoica*. Zoospores from *P. infestans* treated with DCB were unable to infect potato leaves and showed aberrant cell wall morphologies similar to those obtained by silencing the *CesA* gene family.

Altogether these results show that at least some of the *CesA1-4* genes are involved in cellulose biosynthesis and that the synthesis of cellulose is crucial for infection of potato by *P. infestans*.

Keywords: cellulose biosynthesis; cellulose synthase genes; Oomycetes; *Phytophthora infestans*; *Saprolegnia monoica*.

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Oomyceter antogs länge tillhöra en separat klass i svamparnas utvecklingslinje, men är faktiskt närmare släkt med brunalger. Numera är de klassificerade i stramenopilernas utvecklingslinje, vilket inkluderar heterokonta alger och vattenmögel. De dominerande cellväggs-polysackariderna i Oomyceterna är β-(1→3)- och β-(1→6)-glukaner, tillsammans med cellulosa, som aldrig har återfunnits i cellväggar från någon svampart. Kitin, den dominerande cellväggs-polysackariden hos svampar, förekommer i små mängder i cellväggen hos några Oomyceter. Vissa Oomycetarter är patogena och är av ekonomisk betydelse. Exempelvis är arter från släktet *Phytophthora* välstuderade växtpatogener och orsakar påtagliga ekonomiska förluster inom jordbruket. Saprolegniosis, en fisksjukdom orsakad av arter från släktet *Saprolegnia*, är ett stort problem inom fiskodlingsindustrin och utgör ett hot mot populationer av laxfiskar i naturliga habitat. För närvarande så finns inga kemikalier att tillgå som effektivt bekämpar Oomyceter och samtidigt är miljövänliga samt säkra för mänsklig konsumtion av behandlade fiskar och grödor. Det är oklart hur Oomyceter syntetiserar cellulosa, även om denna biokemiska reaktionskedja är ett möjligt angreppsmål för utvecklandet av bekämpningsmedel. I det här arbetet har cellulosans biosyntes studerats i två utvalda Oomyceter, växtpatogenen *Phytophthora infestans* och fiskpatogenen *Saprolegnia monoica*.

En ny *cellulosa syntas* (*CesA*)-genfamilj har identifierats i Oomyceter. Den innehåller fyra homologer som har kallats *CesA1, CesA2, CesA3* och *CesA4*. Genprodukterna från *CesA1*, *2* och *4* innehåller en Pleckstrin Homolog-domän lokaliserad i N-terminalen. Detta är ett nytt kännetecken, unikt för Oomycet *CesA*-gener. *CesA3* är den mest uttryckta *CesA*-homologen i mycelium från både *S. monoica* och *P. infestans*, medan *CesA1* och *CesA2* uppregleras i *P. infestans* virulenta livsstadier. *CesA4* uttrycktes relativt i mycket låga nivåer i alla undersökta celltyper. Tystande av hela *CesA*-genfamiljen i *P. infestans* med hjälp av RNA-interferens, ledde till minskad mängd cellulosa i cellväggen. Cellulosasyntesinhibitorerna DCB och Kongorött hade en uppreglerande effekt på *SmCesA*-genuttryck, vilket medföljdes av en ökad β-glukansyntesaktivitet *in vitro*. Dessutom så minskade tillväxten av *S. monoica*-mycel behandlat med inhibitorerna. Zoosporer från *P. infestans*, som behandlats med DCB, hade onormal cellväggsmorfologi och var oförmögna att infektera potatisblad. Sammataget visar dessa resultat att åtminstone några av *CesA1-4*-generna är involverade i cellulosans biosyntes samt att syntes av cellulosa är nödvändigt för att *P. infestans* ska kunna infektera potatis.

Nyckelord: cellulosa biosyntes; cellulosa syntas-gener; Oomyceter, *Phytophthora infestans*; *Saprolegnia monoica*.

LIST OF PUBLICATIONS

Paper I

Grenville-Briggs L. J., Anderson V. L., **Fugelstad J**., Avrova A. O., Bouzenzana J., Williams A., Wawra S., Whisson S. C., Birch P. R., Bulone V. and van West P., Cellulose synthesis in *Phytophthora infestans* is required for normal appressorium formation and successful infection of potato, 2008, Plant Cell 20(3):720-38

Paper II

Fugelstad J., Bouzenzana J., Djerbi S., Ezcurra I., Teeri T., Arvestad L. and Bulone V., A novel family of *cellulose synthase* genes from the Oomycete *Saprolegnia monoica*: functional characterization using cellulose synthesis inhibitors, 2008, Manuscript

Paper I: Johanna Fugelstad's contribution is the biochemical characterization of the cell wall, participation in the co-localization studies using antibodies and some of the writing.

Paper II: Johanna Fugelstad did most of the experimental work and the writing with assistance from Dr. Lars Arvestad for the bioinformatic sections.

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1. INTRODUCTION

1.1 Introduction to Oomycetes

1.1.1 General characteristics of Oomycetes

Oomycetes were previously classified in the Fungi kingdom, but in the last decades it has been shown that they in fact belong to the Stramenopiles and are related to heterokont algae, such as for instance brown algae (Kumar and Rzhetsky, 1996; Paquin et al., 1997; Baldauf et al., 2000). The growth of Oomycetes resembles the growth of fungal cells that form coenocytic hyphae. The cell wall of Oomycetes, as opposed to that of fungi, contains cellulose. Most stages in the Oomycete life cycles are diploid (Hardham, 2007). Oomycetes have evolved a saphrophytic and sometimes pathogenic life style. Some species combine both life styles, and have adapted to many types of host organisms such as for instance plants, nematodes, vertebrates and crustaceans (Table 1). Oomycetes can be biotrophs, hemibiotrophs or necrotrophs. Biotrophic species obtain their nutrients from living tissues while hemibiotrophs first feed on living tissues and kill their host at a later stage. Necrotrophs live exlusively on dead host tissues. The plant pathogenic Oomycetes have been most characterized while much less is known about the animal pathogens. *Phytophthora infestans,* the cause of potato blight, belongs to the well studied plant pathogenic genus *Phytophthora* (Agrios, 2005). Among animal pathogens, species from the order Saprolegniales cause most harm by infecting fishes and crustaceans in the aquaculture industry (Meyer, 1991). The phylogenetic relationship between Oomycetes shows that animal pathogens are found in several genera, except the Peronosporales, which contains strictly plant pathogens (Phillips et al., 2008). *P. infestans* and *Saprolegnia monoica,* were chosen as representative model organisms for the investigation presented in this work due to their economical importance and impact as plant and animal pathogens, respectively. However, the economical and biological importance of other Oomycetes should be stressed, although they are not included in the scope of this study.

Table 1. Examples of pathogenic species from different orders of Oomycetes, their specific host and corresponding diseases.

Order	**Representative species of different genera**	**Host and disease**
Saprolegniales	*Saprolegnia monoica*	Cause of Saprolegniosis in fish
	Saprolegnia parasitica	Cause of Saprolegniosis in fish
	Achlya bisexualis	Cause of ulcerative mycosis in fish
	Aphanomyces astaci	Cause of plague in crayfish
	Aphanomyces euteiches	Cause of root rot disease in pea
Lagenidiales	*Lagenidium giganteum*	Infects mosquito larvae
Pythiales	*Pythium insidiosum*	Cause of skin lesions in mammals
	Pythium oligandrum	Infects fungi
Peronosporales	*Hyaloperonospora parasitica*	Cause of downy mildew in plants
	Phytophthora infestans	Cause of late blight in potato
	Phytophthora sojae	Cause of stem and root rot in soybean
	Albugo candida	Cause of white rust in plants

1.1.2 Plant pathogens of the genus *Phytophthora*

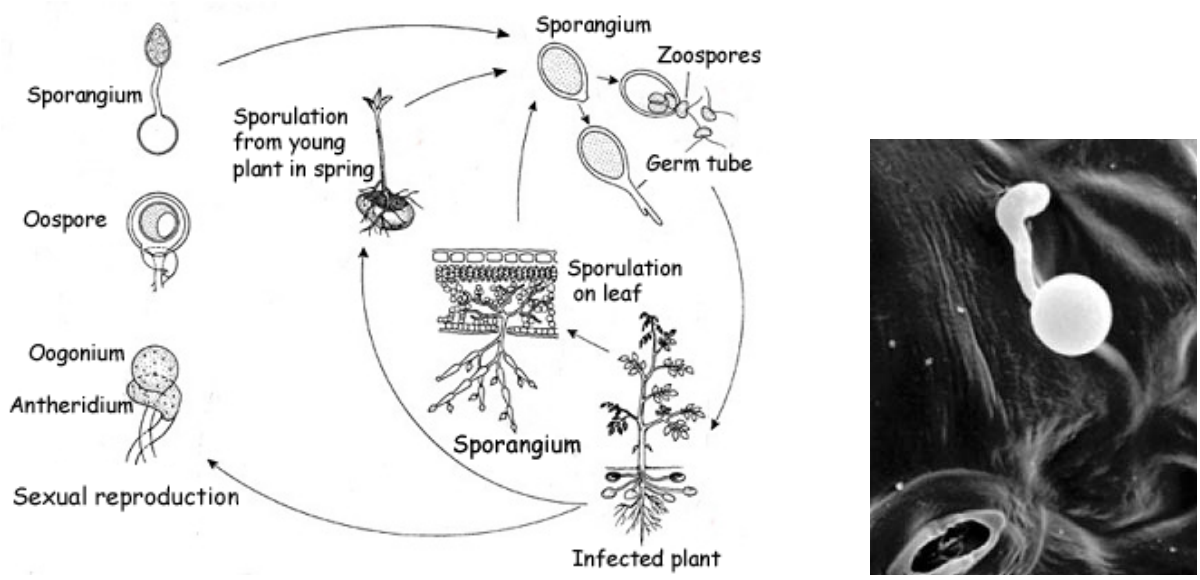
The plant pathogenic genus *Phytopthora* includes hemibiotrophs (*e.g. P. infestans, P. sojae*) and necrotrophs (*e.g. P. cinnamomi*). *Phytophthora* species have had a severe impact on agriculture and have served as model organisms to study plant pathology (Hardham, 2007). A well known and typical example is *P. infestans,* the cause of late blight in potato (Figure 1). This microorganism can infect a variety of plant species other than potato, such as tomato and sojae. It probably has its origin in Mexico (Andrivon, 1996) and followed the spread of potato all over the world (Schumann, 1991). *P. infestans* can infect the whole plant, tubers, roots, or leaves, leading to the death of the plant.

Figure 1. Late blight on a potato leaf caused by *P. infestans*. Photo from paper I.

Life cycle and reproduction of Phytophthora species

The life cycle of Oomycetes includes sexual and asexual reproduction, with flagellated free swimming spores characteristic of Stramenopiles (Figure 2 A). The mycelium of *P. infestans* produces branched sporangiophores containing lemon-shaped asexual sporangia at their tips. When the sporangium bursts it releases motile zoospores with two flagella, which is typical for heterokonts. The role of the zoospores is transmission of the pathogen from host to host. They are also essential for targeting the site of infection (Walker and West, 2007). The host attracts the zoospores by chemotactic and electrotactic stimuli (Tyler, 2002). Spores can also be carried to adjacent places by the wind, explaining their fast dissemination. However, the spores need humid conditions to be able to infect their hosts (Hardham, 2007; Walker and West, 2007). The zoospores lack cell walls and maintain their volume and homeostasis with the help of a contractile vacuole (Mitchell and Hardham, 1999). Upon contact with a host, the zoospores become immotile and encyst, which leads to the formation of a cell wall. A germ tube protrudes from the cyst forming an appressorium that resembles a swollen tip of the germ tube. The appressorium penetrates the host tissue directly or enters via stomata in the leaf (Figure 2 B) and form an infection vesicle. The mycelium then grows from the infection vesicle in between the plant cells, sometimes with haustoria, which is a mycelium that has penetrated the host cell wall and invaginated the plasma membrane (Grenville-Briggs and Van West, 2005). Older infected plant cells die while the mycelium continues to spread into the host tissue (Agrios, 2005; Hardham, 2007).

The sexual reproduction of Oomycetes results in thick walled resistant oospores that can survive for 3-4 years outside a host (Figure 2 A). Oospores are formed when the male reproductive organ, the antheridium, fertilizes the female reproductive organ, the oogonium. For oospore formation in *P. infestans* to take place, strains of two mating types are needed, A1 and A2. It was not until recently that the A2 mating type emerged from Mexico and spread in the rest of the world, thereby making possible the sexual reproduction of *Phytophthora*. This has lead to the emergence of more virulent strains because of genetic recombinations of pathogenic characteristics of the mating strains (Agrios, 2005).



A **B**

Figure 2. A) Life cycle of *P. infestans* with sexual and asexual sporulation (adapted from Schumann, 1991). B) Germinated cyst of *P. infestans* with its appressorium on a potato leaf with a stomata in the left bottom corner (from paper I).

Genomic data of Phytophthora species

More knowledge on the molecular events occurring during the life cycle of Oomycetes is needed to develop tools that allow the control of their dissemination and infection processes. Recently, the genomes of three economically important plant pathogens, namely *Phytophthora infestans*, *Phytophthora sojae* and *Phytophthora ramorum* have been fully sequenced by the Broad Institute (http://www.broad.mit.edu/) and the Joint Genome Institute

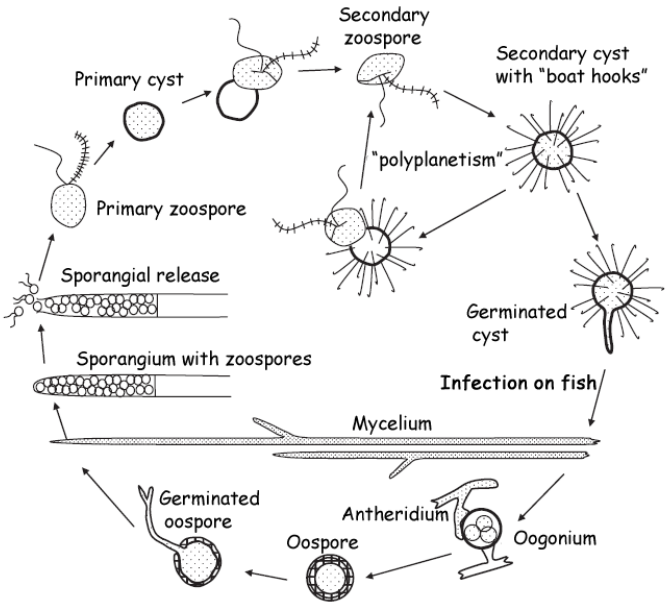
(http://www.jgi.doe.gov/), opening new opportunities to characterize these virulent species (Tyler et al., 2006b). An EST database is also available on several species, including members of the *Saprolegniales* such as *S. parasitica* (Oomycete Genomic Database, www.oomycete.org; Gajendran et al., 2006).

1.1.3 Fish pathogens of the genus *Saprolegnia*

Members of the genus *Saprolegnia* cause the group of diseases called Saprolegniosis on fish or fish eggs. Saprolegniosis are characterized by visible white or gray patches of filamentous mycelium on the fish (Lartseva, 1986). The infection starts by the invasion of the epidermal tissue and can spread over the entire body (Figure 3 A). *Saprolegnia* is considered an opportunistic pathogen which has a saprophytic and necrotrophic life style (Neish, 1977; West, 2006). There is no efficient treatment of Saprolegniosis available, except from good fish management during aquaculture. Stress seems to be a great risk factor for Saprolegniosis (Willoughby and Pickering, 1977; Meyer, 1991).

Life cycle and reproduction of Saprolegnia species

The life cycle of *Saprolegnia* species is similar to that of other Oomycetes, with the ability to produce asexual and sexual spores. The asexual reproduction in *Saprolegnia* includes a special feature called polyplanetism. The asexual spores release primary motile zoospores that within a short time encyst and release a secondary zoospore (Figure 3 B). The evolution of secondary zoospores favours multiple attempts to locate a suitable host. In particular, these spores are motile for a longer period than primary zoospores and possess hairs, also called boat hooks, which are believed to increase attachment efficiency. These hairs are unique to the genus *Saprolegnia* (West, 2006).



A **B**

Figure 3. A) Wild salmon infected by *S. parasitica* with white infected lesions on the skin. B) Life cycle of *S. parasitica* (pictures adapted from van West, 2006).

Genomic data of Saprolegnia species

There is no genome sequenced from any *Saprolegnia* species, but an EST library has been generated with more than 1510 ESTs sequenced from a mycelial cDNA library of *S. parasitica* (Torto-Alalibo et al., 2005). The average amino acid identity between *S. parasitic*a and the three sequenced *Phytophthora* genomes, based on the alignment of 18 deduced sequences of conserved proteins, was of 77 % compared to 93 % among the *Phytophthora* species.

1.1.4 Economical importance of Oomycetes

More than 100 years after the infamous Irish famine caused by the potato late blight, Oomycetes still represent an important problem in agriculture. The losses due to plant pathogenic *Phytophthora* species have constantly increased since the dissemination of the second mating type that lead to genetic recombinations and the emergence of more virulent strains of *P. infestans* (Fry and Goodwin, 1997). The spread of Oomycetes to new habitats has lead to new diseases and devastation of crops. One example is *P. ramorum*, the agent of Sudden Oak Death, killing coastal oaks in California (Rizzo et al., 2002). There is currently no efficient and environmentally friendly drug that inhibits infections by plant pathogenic

Oomycetes. Not even anti-fungal drugs allow the control of the disease caused by Oomycetes (Hardham, 2007).

The growing business of aquaculture is also seriously affected by Oomycetes, particularly by different species of the order *Saprolegniales*. Outbreaks of Saprolegniosis are responsible for losses of up to 50 % (Meyer, 1991). Malachite green, used until recently to control *Saprolegnia*, was banned world wide in 2002 because of its carcinogenic and toxicological effect. Currently, there are no drugs available that are at the same time efficient and safe for the fish host and for human consumption of treated fishes (West, 2006).

1.2 Introduction to Cellulose Biosynthesis

1.2.1 Cellulose, an important carbohydrate

Cellulose has been used as a chemical raw material for about 150 years and, historically, it has been utilized by humans since the time of Egyptian pharaohs in the form of papyrus. Half of the total annual biomass produced by algae, plants and some bacteria is composed of cellulose, making it the most abundant macromolecule on earth and pointing out the biological importance of cellulose. Cellulose is the building material for the cell walls of plants, but also in other organisms such as algae and tunicates, providing mechanical support and protection. In plants, cellulose is deposited into a primary cell wall together with hemicelluloses, pectins and structural proteins. Woody tissues contain a thick secondary cell wall made of crystalline cellulose. The biological importance of cellulose for bacteria and other organisms are discussed later.

Cellulose is currently considered as an alternative source of energy and chemicals to fossil fuels. Cellulose also represents an abundant raw material that can potentially satisfy the increasing demand for environmentally friendly and biocompatible products, and it is already used in fabrics and paper. The biosynthesis of cellulose has been exploited for the development of new biomaterials. A recent typical example is the use of bacterial cellulose for the formation of artificial blood vessels and wound dressings (Klemm et al., 2005; Bodin et al., 2007). However, plants remain the main source of cellulose. For instance, the cotton fiber produces virtually pure cellulose. In most plant tissues however, cellulose exists in the form of a composite together with hemicelluloses, pectins, structural proteins and, most often,

phenolic compounds (lignin). Cellulose and its derivatives, *e.g.* nitrocellulose, cellulose acetate, methyl cellulose, carboxymethyl cellulose *etc*, have numerous industrial applications, for instance in the form of coatings, pharmaceuticals, cosmetics and adhesives (Klemm et al., 2005).

1.2.2 Cellulose structure

Cellulose is a linear homopolymer of D-glucose residues linked through (1→ 4)-β linkages. The D-glucose residues are in the $^4C_1$ chair conformation. The cellulose molecule has a chemical polarity: one end of the β-glucan chain carries a free hemiacetal group with reducing properties and is referred to as the reducing end. The anomeric carbon of the D-glucopyranose unit at the other end is involved in a glycosidic bond with the penultimate residue, and therefore has non-reducing properties (Figure 4). Since the cellulose molecule is composed by (1→ 4)-β linkages, glucose moieties are inverted almost 180° relative to their adjacent moieties. For this reason, cellobiose is sometimes considered as the repeating unit of cellulose. The degree of polymerization (DP) of cellulose varies depending on the origin of the polymer and the extraction protocol. In wood pulp, the DP ranges typically between 300 and 1700 whereas in cotton and fibres from other plants DP values are usually in the range 800-10 000 depending on the treatment (Klemm et al., 2005).

The formation of crystalline cellulose is dependent on interactions between the free hydroxyl groups of the molecule forming intra- and intermolecular bonds. The different forms of cellulose define four types depending on the unit cell parameter of the crystal: cellulose I, II, III and IV. The different forms can be identified by their characteristic ray diffraction patterns. Cellulose I occurs in two distinct allomorphs designated Iα and Iβ, which can both exist in a given sample. The ratio of the Iα and Iβ allomorphs depends on the origin of the cellulose: cellulose Iα is most abundant in bacteria and certain algae while the cellulose of plants mainly consists of the Iβ allomorph (Atalla and Vanderhart, 1984). Cellulose II can be formed irreversibly from cellulose I by two different processes: mercerization or regeneration. Mercerization includes treatment with aqueous sodium hydroxide and recrystallization of the cellulose. Regeneration involves dissolution of the cellulose, *e.g.* in N-methylmorpholine-N-oxide, and subsequent precipitation and crystallization, as for example in the process of making film or rayon fibres (reviewed by Klemm et al., 2005). Cellulose II is more thermodynamically stable than cellulose I. In cellulose I, the chains have a parallel orientation

as opposed to cellulose II, which is antiparallel. Cellulose III can be prepared from cellulose I or II by different chemical treatments and cellulose IV is obtainable by heating cellulose III in glycerol (Pérez and Mazeau, 2005). Cellulose I, also called native cellulose, and cellulose IV are the only forms encountered in nature. Cellulose IV is considered as a disorganized form of cellulose I and occurs in plant primary walls and walls of some microorganisms, *e.g.* Oomycetes (Chanzy et al., 1979; Bulone et al., 1992; Helbert et al., 1997).

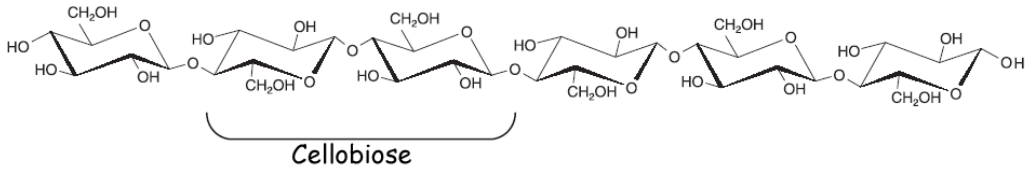


Figure 4. The cellulose chain is composed of D-glucose residues linked through (1→4)-β linkages, with a reducing end and a non-reducing end, referring to the different chemical reactivity of the chain ends. The repeating unit is sometimes considered to be cellobiose, because of the a 2-fold screw axis of the glucan chain with 180° rotation between each glucose monomer (adapted from Taylor, 2008).

1.2.3 Cellulose synthases and cellulose biosynthesis in different organisms

Classification of CesAs

Glycosyltransferases are classified into distinct sequence-based families (Coutinho et al., 2003) described in the carbohydrate-active enzyme database (CAZy) (www.cazy.org; Coutinho and Henrissat, 1999), which currently comprises 91 families of glycosyltransferases (GTs). Cellulose synthases (CesAs) belong to GT family 2 (GT2), one of the largest of the GT families in CAZy. GT2s use an inverting mechanism (see below, *Inverting mechanism of GT2s*) and include both processive and non-processive enzymes. Processive GTs transfer multiple sugar residues to the growing polymer whereas non-processive GTs transfer a single sugar residue to the acceptor (Saxena et al., 1995). CesA is a processive GT, which is related to other processive GT2s such as the chitin synthase and hyaluronan synthase by sequence similarity and probably partially shared structure (Charnock et al., 2001).

Conserved motifs of CesAs and terminal complexes responsible for cellulose biosynthesis

Processive GTs belonging to family 2 contain two conserved domains, A and B (Figure 5). Non-processive GTs from the same family contain only domain A, suggesting that the conserved aspartic acid and the QXXRW motif of domain B are involved in the processivity of the enzyme. Mutagenesis of the two conserved aspartic acid residues in domain A and that in domain B as well as mutagenesis of the Q, R and W residues in the QXXRW motif in CesA from the bacterium *Gluconoacetobacter xylinus* (formerly *Acetobacter xylinum*), has shown that these amino acids are necessary for enzyme activity (Saxena and Brown, 1997; Saxena et al., 2001). All known *CesA* genes encode putative transmembrane proteins, usually with six or more predicted transmembrane helices, two located at the N-terminal end and the remaining ones at the C-terminus.

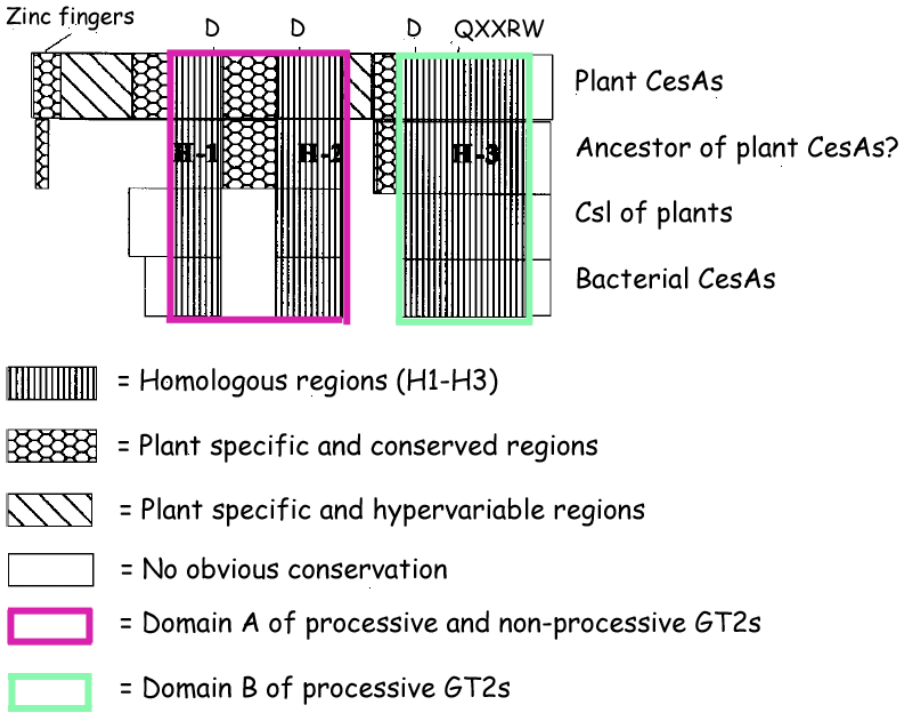
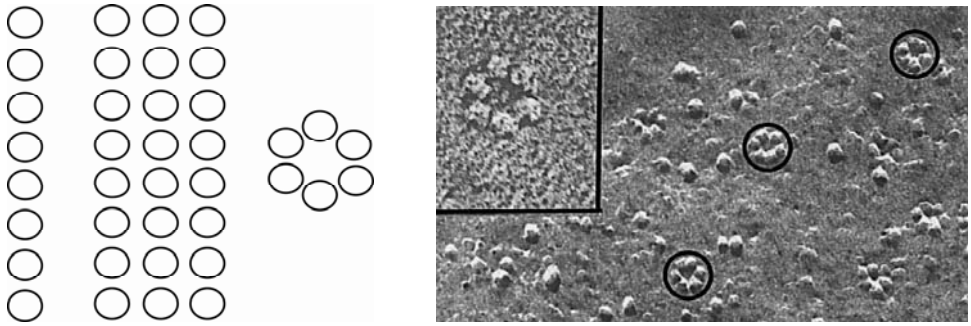


Figure 5. Domain arrangement of CesAs from plants and bacteria. CesA like proteins (Csls) occur in plants and share the homologous regions of CesAs (*Cellulose biosynthesis in plants*, page 16). See text for further explanation (figure adapted from Delmer, 1999).

Cellulose synthesis takes place in the plasma membrane in so-called terminal complexes. In bacteria, tunicates and most algae, the terminal complexes are organized as linear rows of catalytic subunits, single or multiple, depending on the species considered (Figure 6 A;

Brown, 1996). In plants and some algae, the terminal complexes are organized in hexagonal structures called rosettes (Figure 6 B).



A **B**

Figure 6. Schematic representation of terminal complexes. A) The terminal complexes are linearly organized in single rows in bacteria and multiple rows in most algae. In plants (and some algae) the terminal complexes are organized as hexagonal structures designated as rosettes (after Brown, 1996). B) Freeze-fracture of the plasma membrane from a tracheary element of Zinnia elegans. 222 000 times magnification of main micrograph, 504 545 times magnification of inset (adapted from Delmer, 1999).

Inverting mechanism of GT2s

There is no crystal structure available for any processive glycosyltransferase from the GT2 family and the reaction mechanism of CesA is poorly understood. The inverting mechanism is a one-step reaction where the configuration of the anomeric carbon is inverted with respect to the sugar donor. CesAs and other GTs using nucleotide-sugars as donors form β-linkages and use an α-linked donor substrate, such as for instance uridine-diphosphate-glucose (UDP-glucose). The reaction involves a nucleophilic substitution at the anomeric carbon of the nucleotide-sugar (Figure 7). According to the scheme, a catalytic residue, most likely a carboxylate, is acting as a base by deprotonating a hydroxyl group from the non-reducing end of the acceptor, thereby activating it (Saxena et al., 1995; Charnock et al., 2001). A second carboxylate is thought to be involved in the co-ordination of a metal ion, *e.g.* Mg^{2+}, and the nucleotide-sugar (Figure 7). The activated acceptor thereafter attacks the anomeric carbon of the co-ordinated nucleotide-sugar, forming a β-glycoside bond and releasing the nucleotide moiety of the donor. There is only one published three-dimensional structure determined from the GT2 family, the structure of the bacterial nucleotide-sugar transferase SpsA from *Bacillus*

subtilis (Charnock and Davies, 1999). The sugar donor of SpsA is not known but the structure has been used to discuss the possible organization of the catalytic site of CesA (Charnock et al., 2001). The other mechanism for glycosyl transfer is the retaining mechanism, where the configuration of the anomeric carbon of the sugar donor is retained. This involves a glycosyl-enzyme intermediate and the subsequent addition of the acceptor (Sinnott, 1990; Davies and Henrissat, 1995; Saxena et al., 1995; Lairson et al., 2008). A typical example of a GT using this mechanism is glycogen synthase, which forms an α-bond from a UDP-glucose molecule in which the glucose is in the α-configuration.

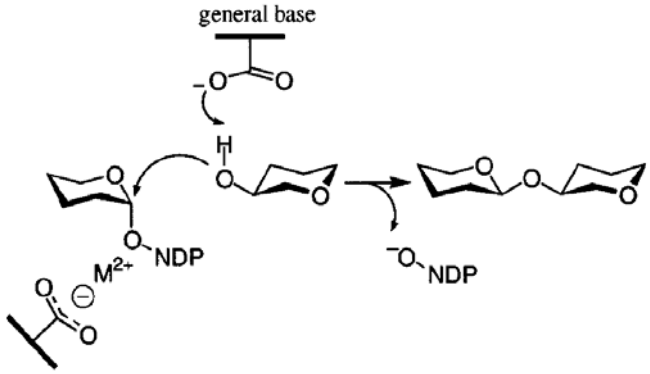


Figure 7. Proposed mechanism of inverting GTs: a nucleophilic substitution at the anomeric carbon of the nucleotide-sugar leads to the formation of a β-linkage from an α-linked donor. A carboxylate is thought to co-ordinate a metal ion associated with the nucleotide-sugar (after Charnock et al., 2001).

Major dilemmas of the cellulose synthesis process

Whether the chain elongation of cellulose takes place at the reducing or non-reducing end is debated. Microdiffraction-tilting using the electron microscope of stained reduced ends of *G. xylinum* cellulose, showed that the reducing ends of the growing cellulose chain points away from the bacterium (Koyama et al., 1997). This provides some evidence that the cellulose molecule is elongated from the non-reducing ends of the growing chains in *G. xylinum* . Other experimental data, based on the use of ^{14}C-pulse and chase reactions with radioactive D-glucose and UDP-glucose support an elongation from the reducing end (Han and Robyt, 1998). In the case of other GT2s, like the hyaluronan and chitin synthases, there is strong experimental evidence that the elongation of the chains occur from the non-reducing ends (DeAngelis, 1999; Imai et al., 2003).

Cellulose synthesis in *Agrobacterium tumefaciens* requires a sugar lipid-linked intermediate (Matthysse et al., 1995), and some observations support the involvement of lipid-linked intermediates also in cellulose synthesis in *G. xylinum* (Han and Robyt, 1998). In plants, a sitosterol-β-glucoside has been suggested to function as a primer for cellulose synthesis (Peng et al., 2002), but the role of a possible primer in cellulose synthesis needs to be further investigated in eukaryotes (Figure 8 page 18).

Cellulose biosynthesis in Bacteria

Bacterial cell walls are composed of peptidoglycan and lack cellulose. Thus, bacterial cellulose exerts other functions than building the cell wall. Cellulose is always produced by bacteria as an extracellular polysaccharide that is either secreted as ribbons composed of packed microfibrils *(e.g. G. xylinum and A. tumefaciens; Ross et al., 1991)*, or inserted into slime tubes, sheats or extracellular slimy materials (e.g. cyanobacteria; Nobles et al., 2001) or biofilms (*Pseudomonas* species and *Escherichia coli*; Ude et al., 2006; Matthysse et al., 2008). The cellulose ribbons produced by *G. xylinum* are used as a raft by this aerobic bacterium to float at the water-air interface. *A. tumefaciens* uses cellulose to attach to the host (Matthysse et al., 1981) and this has also been reported for *E. coli* (Matthysse et al., 2008).

Bacteria have been the preferred model system to study cellulose biosynthesis and the most studied bacterium has been *G. xylinum* (reviewed by Römling, 2002). In *G. xylinum*, cellulose is synthesized by linear terminal complexes and the cellulose chains are secreted outside the cells, forming bundles that assemble into a loose ribbon of fibrils containing approximately 1000 glucan chains. UDP-glucose is the substrate for cellulose synthesis in *G. xylinum* (Glaser, 1958). Cyclic diguanylic acid (c-di-GMP) and Mg^{2+} ions regulate the rate of synthesis, while lipids and protein-linked cellodextrins have been proposed to act as intermediates (Swissa et al., 1980). The catalytic subunit of the bacterial CesA was identified by photolabelling with the photoaffinity probe 5-azido-UDP-glucose, which labelled a UDP-glucose-binding polypeptide of 83 kDa (Lin et al., 1990). The genes involved in cellulose biosynthesis were first identified in *G. xylinum*. Cellulose biosynthesis is controlled by an operon containing four genes including that of the CesA catalytic subunit. The operon is designated as *bcs* or as *acs* depending on the authors (hereafter the name *bcs* is used), and accordingly the CesA catalytic subunit corresponds to *bcsA* or *acsA* gene (Saxena et al., 1990; Wong et al., 1990). The *bcsB* gene encodes a polypeptide probably involved in binding c-di-GMP, and thus functions as a regulator of cellulose synthesis (Mayer et al., 1991). Mutational

studies propose that *bcsD* is involved in the crystallization of cellulose during cellulose biosynthesis (Saxena et al., 1994) while the function of *bcsC* remains unknown. The 3D structure of the bcsD protein was recently obtained (Hu et al., 2008). A cellulase from the CAZy glycoside hydrolase family 8, located upstream of the *bcs* operon, is necessary for cellulose biosynthesis in *G. xylinum* (Standal et al., 1994). There is limited information about the cellulose synthesis in other bacteria than *G. xylinum* and *A. tumafaciens*.

Cellulose biosynthesis in plants

It was not until 1996 that the first plant *CesA* gene was identified and sequenced in cotton (*Gossypium hirsutum*) (Pear et al., 1996). It was demonstrated that a recombinantly expressed fragment of *GhCesA*, the first *CesA* homologue identified in plants, binds UDP-glucose, which is also the substrate of plant CesAs. Radial swelling mutants in *Arabidopsis thaliana* (*rsw*) exhibit a reduced cellulose synthesis, accumulation of noncrystalline (1→4)-β glucan, disassembly of the CesA rosettes, and widespread morphological abnormalities (Baskin et al., 1992; Arioli et al., 1998). The function of CesA in plants was confirmed by the complementation of a *rsw A. thaliana* mutant with the wild type allele, a homologue of *GhCesA* (Arioli et al., 1998).

Freeze-fracture experiments combined with immunogold labelling, demonstrated for the first time the occurrence of the CesA proteins in the rosettes (Kimura et al., 1999). A rosette consists of six globules, and each globule is typically described as containing six CesA catalytic subunits so that the whole CesA complex produces cellulose elementary microfibrils composed of 36 chains (Figure 8; Delmer, 1999). However, the actual number of catalytic subunits in a rosette has never been experimentally determined. Plant CesAs have longer N-terminal ends than bacterial CesAs (Figure 5). Their N-terminal ends include a conserved zinc finger domain and a RING-finger motif, which contains four repeated CXXC motifs (Figure 5). The latter have been shown to be involved in the homo and hetero dimerization of cotton CesAs *in vitro* and in a yeast two hybrid system (Kurek et al., 2002). It has therefore been proposed that the zinc finger domains are responsible for the rosette assembly.

In addition to CesAs, plants contain so-called CesA like proteins (Csl). These proteins belong to the GT2 family and share the conserved D, D, D, QXXRW motifs with CesAs. However, they don't contain the RING-finger motif of CesAs (Figure 5). Csl proteins are predicted to be involved in the synthesis of hemicelluloses. However, only few Csl proteins have been

functionally characterized. CslF synthesize the monocot specific β-(1→3, 1→4)-glucan (Burton et al., 2006b). Experimental data indicate that proteins of the CslA family synthesize β-mannans (Dhugga et al., 2004; Liepman et al., 2005; Burton et al., 2006a). A heterologously expressed CslC has been shown to synthesize β-(1→4)-glucan *in vitro* and is probably involved in the formation of the xyloglucan backbone (Cocuron et al., 2007).

Plant *CesAs* have numerous homologues in all plant species: 10 have been identified in the small annual herb *A. thaliana* while 18 occur in the perennial tree *Populus trichocarpa* (Djerbi et al., 2005). It has been shown that some CesAs are specifically involved in primary cell wall biosynthesis while others are secondary cell wall specific. These conclusions arise from observations in *A. thaliana* mutants and expression profiling of *CesAs* in other plants (Djerbi et al., 2004; Suzuki et al., 2006). Three unique primary cell wall associated AtCesAs, AtCesA1, AtCesA3 and AtCesA6, are needed to form a functioning complex, AtCesA5 and AtCesA2 are partially redundant with AtCesA6 (Persson et al., 2007). In addition, AtCesA1, AtCesA3, and AtCesA6 co-immunoprecipitate *in vitro* (Desprez et al., 2007). These proteins can form homodimers and heterodimers with each other *in vivo*, as shown with bimolecular fluorescence complementation in *Nicotiana benthamiana* leaves by the use of *A. tumefaciens* infiltration of YFP partial chimeric CesA proteins (Desprez et al., 2007). AtCesA8, AtCesA7 and AtCesA4 are specific to secondary cell walls. They co-purify and co-immunoprecipitate *in vitro* and AtCesA4 is needed for complex formation (Taylor et al., 2000; Taylor et al., 2003).

Other genes involved in cellulose biosynthesis are A. thaliana genes coding for Korrigan, KOBITO and COBRA (Nicol et al., 1998; Schindelman et al., 2001; Pagant et al., 2002). A protein similar to Korrigan also occurs in poplar and is up-regulated during secondary cell wall biosynthesis (Rudsander et al., 2003). However, not much is known about the function of any of these proteins, except that Korrigan is a glycosyl hydrolase family 9 membrane-bound β-1,4-endoglucanase necessary for cellulose biosynthesis in *Arabidopsis*. The involvement of sucrose synthase and cortical microtubules in cellulose biosynthesis, the crystallization process of cellulose, the lipid composition surrounding the CesA complex and the mode of translocation of the cellulose chains across the membrane are some of the major unanswered questions related to cellulose biosynthesis (Figure 8) in addition to those mentioned in *Major dilemmas of the cellulose synthesis process* (page 13).

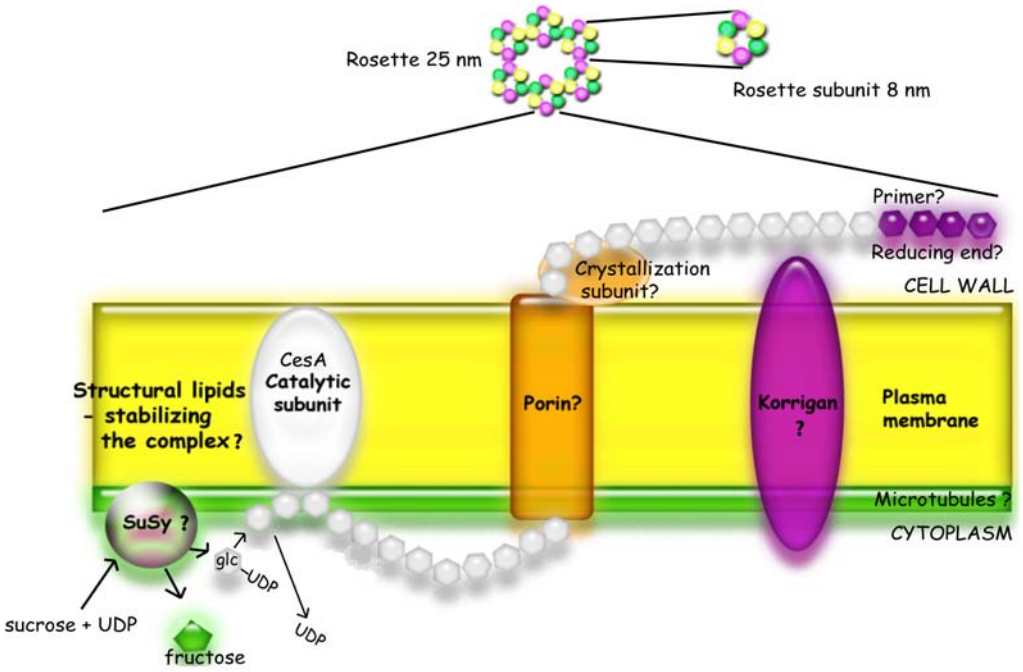


Figure 8. Hypothetical model for cellulose biosynthesis in plants (after Bulone, 2006). The model shows a CesA catalytic subunit in the plasma membrane. Several key aspects regarding the cellulose biosynthesis process are poorly understood and indicated with question marks in the figure: the role of the endoglucanase Korrigan and its physical association to the complex, the involvement of a primer, the direction of chain elongation and the mode of translocation of the nascent cellulose chain across the plasma membrane, the role of sucrose synthase (SuSy), the involvement and importance of structural lipids in the plasma membrane, the occurrence of crystallization subunits, the role of the cytoskeleton, the stoichiometry and the mode of regulation of subunits.

Cellulose biosynthesis in Oomycetes

Little is known about the mechanisms of cellulose synthesis in Oomycetes. The cell walls of some Oomycetes contain chitin, like fungal cell walls, but in a much smaller proportion (<1 % of the total cell wall carbohydrates; Lin and Aronson, 1970; Aronson and Lin, 1978; Bulone et al., 1992) The major cell wall polysaccharides in Oomycetes are β-(1→3)-glucan and β-(1→6)-glucan as well as cellulose, which has never been reported in any fungal species (Bartnicki-Garcia, 1968). The Oomycete cellulose is microfibrillar, of low crystallinity, and corresponds to cellulose IV (Bulone et al., 1992; Helbert et al., 1997). Since little sequence

information from Oomycetes has been available until recently, genes encoding the CesA catalytic subunit in these microorganisms remain unknown. No CesA terminal complex has been observed in Oomycetes as opposed to those visualized in plants, bacteria and algae. The exact mode of assembly of the carbohydrate components in the cell walls of Oomycetes is not known, even though it is believed that the cell walls consist of an inner layer of cellulose microfibrils doubled by an amorphous matrix composed essentially of β-glucans (Bartnicki-Garcia, 1968). Cellulose in Oomycetes has most likely the same scaffolding function as chitin in fungal cell walls.

Cellulose biosynthesis in other organisms

The identification of the *CesA* genes, and sometimes their functional characterization, has been carried out in various other organisms that synthesize cellulose, such as algae like *Valonia* and *Microdictyon* (Brown, 1985), slime molds of the genus *Dictyostelium* (Blanton et al., 2000) and amoebas of the genus *Acanthamoeba* (Anderson et al., 2005). Cellulose synthesis also occurs in a group of marine animals, the tunicates, and the corresponding *CesA* genes have been identified and functionally characterized (Matthysse et al., 2004; Nakashima et al., 2004). Tunicate CesAs are fusion proteins consisting of a cellulase domain and a CesA domain, which is a unique feature in the CesA superfamily (Nakashima et al., 2004).

1.2.4 Biochemical approaches to study cellulose synthesis *in vitro*

Cellulose synthesis has been most studied *in vitro* in the bacteria *G. xylinum* and *A. tumefaciens. In vitro* synthesis of cellulose from UDP-glucose using cell free extracts of *G. xylinum* as a source of enzyme was reported for the first time in the 1950's (Glaser, 1958). Although the genes encoding plant and other CesAs have been identified 10 to 20 years ago, the characterization of the corresponding activities *in vitro* has been difficult, essentially because of the high instability of the CesA complex in cell-free extracts. In addition, *in vitro* synthesis of cellulose using plant enzymes most often leads to very low yields of cellulose while β-(1→3)-glucan is a major *in vitro* product, thereby complicating cellulose characterization. Plant β-(1→3)-glucan, also called callose, is normally produced in small amount by plant cells during cell division, formation of phloem sieve plates, in pollen mother cell walls and pollen tubes (Stone and Clarke, 1992). The production of callose is also triggered by different types of stress, for instance wounding or infection (Stone and Clarke, 1992). It has been suggested that the same enzyme could be responsible for the synthesis of

both callose and cellulose in plants (Delmer, 1999). However, homologues of the yeast FKS protein, a putative β-(1→3)-glucan synthase catalytic subunit (Douglas et al., 1994), have been found in pollen tubes of *Nicotiana alata* (Brownfield et al., 2007) in barley (*Hordeum vulgare*) (Li et al., 2003), *A. thaliana* (Hong et al., 2001) and cotton (Cui et al., 2001). These proteins belong to GT family 48 and do not share the conserved D, D, D, QXXRW motifs with CesAs. In addition, GT2s resembling CesAs have been shown to synthesize β-(1→3)-glucan in *Agrobacterium* (Stasinopoulos et al., 1999). The callose produced normally during cytokinesis could be synthesized by a different enzyme than that responsible of the callose formed upon wounding or *in vitro* extraction of membrane proteins (Saxena and Brown, 2000). Many attempts to improve the yields of *in vitro* synthesis of cellulose have been made, including separation techniques such as flotation in glycerol gradients of the protein extracts and the use of different detergents. Some improvements have been achieved with larger proportions of cellulose produced in the case of the cotton and the blackberry cellulose synthesis systems (Kudlicka et al., 1996; Lai-Kee-Him et al., 2002).

Membrane preparations and detergent extracts of membranes from *S. monoica* have been used as a source of enzyme for *in vitro* synthesis of β-(1→4) and β-(1→3)-glucans in the presence of UDP-glucose. The products synthesized *in vitro* have typically been identified using specific cellulases and β-(1→3)-glucanases (Fèvre and Rougier, 1981; Bulone et al., 1990). The data showed that cellulose and β-(1→3)-glucan are co-synthesized, but the proportion of cellulose is higher when the assay is carried out in the presence of lower substrate concentrations (10 μM instead of 1mM) and $MgCl_2$. Microfibrillar glucans have been observed by transmission electron microscopy after *in vitro* synthesis together with associated globular structures that possibly correspond to the glucan synthase complexes (Figure 9, Fèvre and Rougier, 1981).

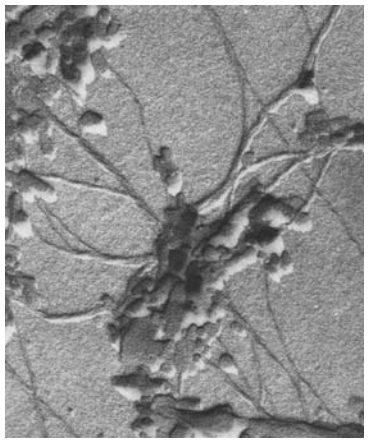


Figure 9. Electron micrograph of glucan microfibrils synthesized *in vitro* by membrane preparations from *S. monoica*; incubated in absence of $MgCl_2$ and in the presence of high substrate concentrations (1 mM) (× 70 000). The bound globular structures may correspond to trapped enzyme complexes (from Fèvre and Rougier, 1981).

Attempts have been made to isolate the β-(1→3) and β-(1→4)-glucan synthases from *S. monoica*, by combining detergent extraction of membrane-bound proteins with subsequent purification by ultracentrifugation on a glycerol gradient and product entrapment (Bulone et al., 1990). The enzymes could be separated into two fractions with proteins of 34, 48 and 50 kDa characteristic of the β-(1→3)-glucan synthase and proteins of 55-60 kDa for the CesA activity. This has lead to the recent identification of the 34 kDa protein as a β-(1→3)-glucan synthase activator that belong to the annexin family (Bouzenzana et al., 2006). It remains however to identify the catalytic subunits of the cellulose and β-(1→3)-glucan synthases in Oomycetes.

1.2.5 Inhibitors of cellulose biosynthesis

There are many compounds that specifically inhibit cellulose biosynthesis. Some cellulose synthesis inhibitors have been used to study cellulose biosynthesis, for instance 2,6-dichlorobenzonitrile (DCB), isoxaben, triazole carboxamides, quinclorac and thiazolidinone (reviewed in Sabba and Vaughn, 1999). For example, in *A. thaliana*, mutants that exhibit resistance to the cellulose synthesis inhibitors isoxaben and thiazolidinone have been analyzed and their resistance was correlated to modifications of the *AtCesA3* gene (Scheible et al., 2001).

DCB

DCB (Figure 10), is a specific but reversible inhibitor of cellulose biosynthesis *in vivo* at micromolar concentrations (Hogetsu et al., 1974; Sabba and Vaughn, 1999) but not *in vitro* (Blaschek et al., 1985). DCB most likely acts in an indirect way, as it does not bind to the catalytic subunit of CesA (Delmer et al., 1987). DCB is affecting cell wall formation and cytokinesis but not nuclear division (Meyer and Hert, 1978). It specifically inhibits cellulose biosynthesis in plants (Montezinos and Delmer, 1980), which is accompanied by a reduced cellulose content in the cell walls of the treated plants (Shedletzky et al., 1990; Shedletzky et al., 1992). DCB has been shown to down regulate the transcription of a gene from glycoside hydrolase family 9 in poplar leaves, leading to lower protein expression levels (Ohmiya et al., 2003). The activity of the sucrose synthase promoter in maize was also inhibited in protoplasts treated with DCB (Maas et al., 1990). DCB causes accumulation of CesA proteins in tobacco cells and *Arabidopsi*s seedlings, as well as of rosettes in the plasma membrane of cells from wheat root (Herth, 1987; Nakagawa and Sakurai, 1998; DeBolt et al., 2007). These observations indicate that DCB can act, most likely indirectly, on the transcription levels of genes involved in cell wall formation.

The protist pathogen *Acanthamoeba* causes blinding keratitis as well as fatal granulomatous encephalitis (reviewed in Niederkorn et al., 1999). The inhibition of this pathogen is difficult as it easily switches to its cyst form, making it insensitive to anti-amoebic drugs. However, the cyst cell wall is composed of cellulose and it was recently reported that micromolar concentrations of DCB prevent encystment (Dudley et al., 2007), possibly by interfering with the cellulose biosynthesis machinery.

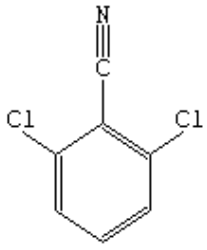


Figure 10. Structure of DCB (adapted from www.wikipedia.org).

Congo Red

Congo Red (Figure 11) is a dye that interacts strongly with β-glucans (Wood, 1980). The mycelium from *S. monoica* cultivated in the presence of Congo Red exhibits a reduced growth rate, an altered morphology and an increase in corporation of glucose into cell wall

carbohydrates (Nodet et al., 1990 a). In addition, Congo Red is acting as an inhibitor *in vitro* of the β-(1→4)-glucan synthase activity at high concentrations (higher than 50 μM) and β-(1→3)-glucan synthase activity at lower concentrations (starting at 25 μM; Nodet et al., 1990 b). Thus, Congo Red affects cellulose biosynthesis in Oomycetes, but its molecular mode of action remains unclear.

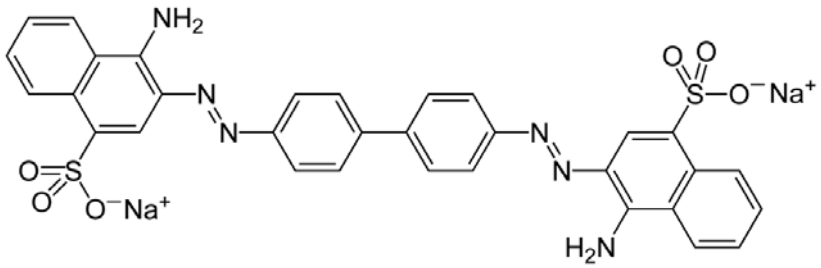


Figure 11. Structure of Congo Red (adapted from www.wikipedia.org).

2. PRESENT INVESTIGATION

2.1. Aim of the present investigation

As stressed in the introduction, Oomycetes represent an important economical problem in the aquaculture and agriculture industries worldwide. New targets for anti-Oomycete compounds must be identified, since no efficient and environmentally friendly inhibitors are currently available. The composition of Oomycete cell walls has been investigated to a great extent and it is one of the features that distinguish the Oomycetes from fungi. However, the enzymes responsible for the biosynthesis of cell wall carbohydrates have hardly been investigated, although they represent potential targets of Oomycete inhibitors. The aim of the work presented in this thesis was to characterize the enzymes responsible for cellulose synthesis in Oomycetes, using *P. infestans* and *S. monoica* as model systems. These two Oomycetes are representative of plant and animal pathogen Oomycetes, respectively.

2.2 Materials and methods

All materials and methods are described in the attached publications.

2.3 Results and discussion

2.3.1 Characterization of a novel family of *CesA* genes in Oomycetes (Papers I and II)

Genomic data on Oomycetes have been available only since the end of 2006. In particular, an EST database and three Oomycete draft genomes have been published recently (Gajendran et al., 2006; Tyler et al., 2006a). BLAST searches in the *Saprolegnia* EST database with plant and bacterial CesA sequences allowed us to identify an EST sequence (ID = SPM13D7) that contained part of the putative processive glycosyltransferase motif (D, QXXRW). The most significant E values after BLAST analysis were of 6×10^{-14} with the *G. xylinus CesA* sequence (accession number AB010645) and 10^{-3} with *AtCesA4* (accession number AAO15532). To amplify the full length gene corresponding to the identified EST from the species *S. monoica* (*SmCesA*), degenerated primers were designed and 3' and 5' RACE (rapid amplification of cDNA ends) were performed (Figure 12).

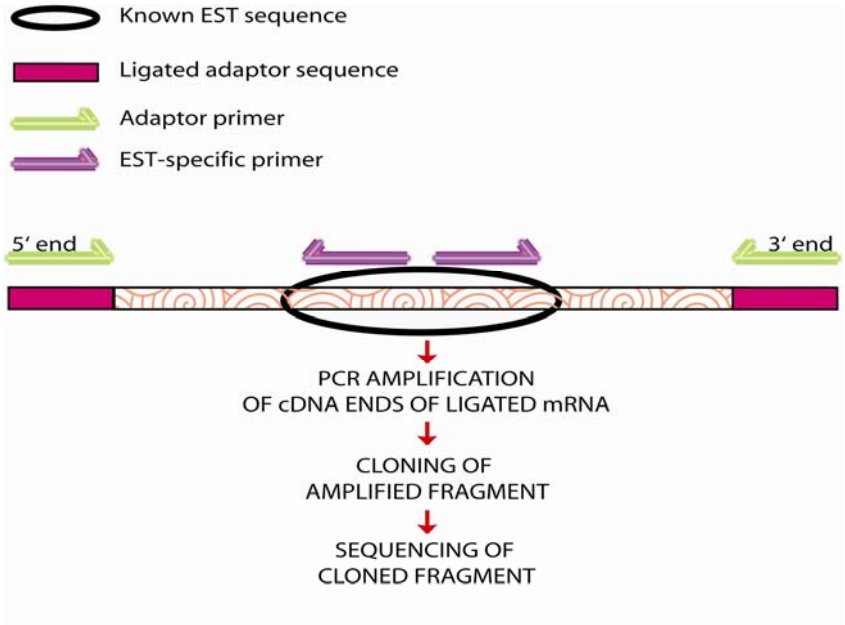


Figure 12. Principle of RACE: primers specific to internal parts of an EST sequence are used together with primers that match sequences ligated to the ends of the target mRNA sequence (5'cap and the 3' poly A-tail). The mRNA ends are then amplified, cloned and sequenced, providing full length sequence information.

When the full length sequence of the first *SmCesA* gene was obtained, it was used to search the recently sequenced *Phytophthora* genomes for homologous gene models. Four gene models were found in each of the three sequenced genomes. The genes were named *CesA1*, *CesA2*, *CesA3* and *CesA4*, and the *SmCesA* gene we identified first corresponded to *CesA3* of *Phytophthora* species. A Southern Blot analysis (Southern, 1975) was made using genomic DNA from *S. monoica* to determine the number of *CesA* homologues present in this species. According to the Southern Blot analysis, three homologues were present (Figure 1 in paper II). Degenerated primers were designed to amplify the full length sequences of the putative two other *SmCesAs*. RACE amplification of the partial sequence corresponding to *SmCesA2* (~90 % of full length) and *SmCesA4* (~30 % of full length) was achieved. The data of the Southern Blot analysis and RACE suggested that *S. monoica* does not have the *CesA1* gene homologue identified in the *Phytophthora* genomes. A phylogenetic analysis with MrBayes (Ronquist and Huelsenbeck, 2003) supported the hypothesis that the split of an ancestral gene into *CesA1* and *CesA2* in *Phytophthora* was a recent event which had not occurred in *S. monoica* (Figure 2 D in paper II). All the *S .monoica* and *Phytophthora* genes identified share

the conserved motifs common to GT2 processive glycosyltransferases, *i.e.* two conserved D residues in domain A, one D residue and the QXXRW motif in domain B. An alignment of a part of the B domain is shown in Figure 1 A in paper II. Orthologous Oomycete *CesA* genes share a greater similarity to one another than paralogues do. The Oomycete *CesA1, 2, 3* and *4* genes constitute a new group of genes in the *CesA* superfamily, with some unique features (Figure 1 in paper I and Figure 2 B in paper II). In particular, the N-terminal ends of CesA1, 2, and 4 from *Phytophthora* and CesA2, and 4 from *S.monoica* contain a Pleckstrin Homology domain (cd00821, http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) believed to be involved in signaling, binding to lipids or attachment to membranes according to the Pfam database (http://pfam.sanger.ac.uk/). This is a remarkable feature since no other known *CesA* contains such domains. The Oomycete *CesA3* genes do not contain a Pleckstrin Homology domain but they exhibit an extended N-terminus with an extra cluster of seven predicted transmembrane helices. Additionally, Oomycete CesA3s share strictly conserved putative zinc-binding domains at the N-terminus, with two CXXC motifs (E value = 1.15×10^{3}. The search was made with SMART, a sensitive search combining Hidden Markov Models, alignments and thresholds; http://smart.embl-heidelberg.de/. However, the latter zinc-binding domains are not similar to those present in plant CesAs (Figure 2 C and 2 D in paper II).

2.3.2 Expression pattern and localization of Oomycete *CesA*s (Paper I and II)

Analyses of the expression levels of the putative *CesAs* from *S. monoica* and *P. infestans* were performed at different life cycle stages, by using quantitative Real Time PCR with SYBR Green (Materials and Methods of papers I and II). *PiCesA3* was the dominantly expressed homologue in the mycelium from *P. infestans*. *PiCesA1* and *PiCesA2* were up-regulated during cyst and appressorium formation, but *PiCesA3* was still the dominantly expressed gene (Figure 5 in paper I). *PiCesA4* was expressed only in minute amounts in the mycelium, appressorium and cyst (Figure 5 in paper I). Transcription levels of *SmCesA* homologues were investigated in the mycelium (Figure 13). The expression pattern of *S. monoica CesAs* in the mycelium follows that of *P. infestans*, with *SmCesA3* being the dominantly expressed gene and *SmCesA2* and *4* being expressed at much lower levels compared to *SmCesA3*.

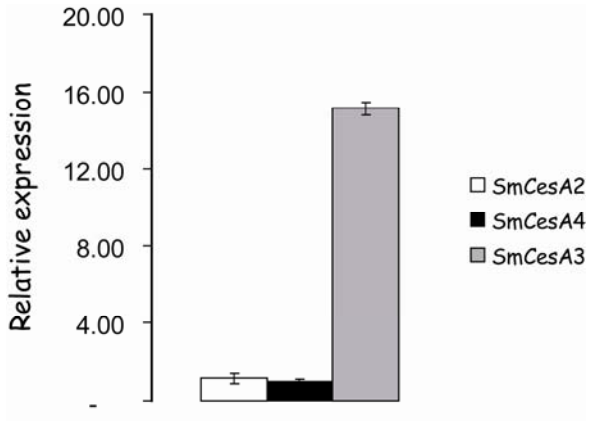


Figure 13. *SmCesA3* is the most abundantly expressed *CesA* gene in the mycelium of *S. monoica*. The analysis was made by quantitative Real Time PCR using SYBR Green, as described in paper II. The expression was normalised to the endogenous *18S RNA* gene, which is constitutively expressed. The levels of expression were calibrated relative to the expression of *SmCesA4* which was arbitrarily set to 1.

Immunolocalization studies in *P. infestans* showed that PiCesAs are localized in the growing tip and infection-like vesicles of appressoria and cysts (Figure 9 in paper I). Antibodies produced against synthetic peptides corresponding to the amino acid sequence of SmCesA3 were used. The peptides were localized in the conserved CesA C-terminal region, which allowed detection of PiCesAs. However, since a conserved region was used, the antibodies do not allow distinction of the different CesA homologues. The antibodies recognized CesA in purified plasma membrane fractions of *P. infestans* and *S. monoica*, but gave no positive signal in cytosolic fractions. This Western Blot analysis confirms the localization of the target CesA proteins in the plasma membranes of both species (Pages 2 and 3 of Supplementary material of paper I).

2.3.3 Effect of silencing *PiCesAs* on appressoria formation (Paper I)

Generation of gene knockouts is a significant technical challenge due to the diploidy of Oomycetes and stable transformation is of low efficiency. Thus, to circumvent these problems, functional characterization of the *PiCesA* genes was performed using gene silencing by the RNA-interference method recently established in *P. infestans* (Whisson et al., 2005). The gene encoding GFP (green fluorescent protein) was introduced in the p34GFN vector (Si-Ammour et al., 2003) and used as a non-endogenous negative control. Lines of *P. infestans* were silenced for all four *CesA* homologues simultaneously, by transforming the

strains with dsRNA. The target sequences are specific to each gene and correspond to the globular regions of the proteins. The expression levels of *CesA* genes in the transformed lines were analyzed by Real Time PCR to confirm gene silencing. Lines with different levels of silencing were obtained giving moderate to severe phenotypes. The phenotypes of the silenced appressoria were further investigated. Normally, at least 60 % of wild-type *P. infestans* cysts produce appressoria *in vitro*. The number of appressoria produced *in vitro* was determined for each individual silenced line. The control lines with the p34GFN vector germinated and at least 60 % of the corresponding cysts formed normal appressoria, which indicates that the control lines behave as the wild-type line. Control lines formed infection-like vesicle structures while silenced lines did not. Some silenced lines produced either typical or aberrant appressoria, which ruptured at their tip, indicating a less resistant cell wall. Most of the individual lines had a clear reduction in the formation of normal appressoria. In addition, a larger proportion of cysts produced germ tubes exhibiting abnormal appresorium-like structures. The severity of the alteration of normal appresorium development and the number of aberrant appressoria produced correlated with the levels of the silencing of *CesA1-4* genes in each silenced line (Figures 6 and 7 in paper I).

2.3.4 Effect of silencing *PiCesA*s on the cellulose content in cell walls (Paper I)

The involvement of the *PiCesA* genes in cellulose synthesis *in vivo* was analyzed in a total of 22 lines. Appressoria corresponding to several of these lines were pooled to obtain three different groups as follows: six non-silenced lines showing a normal phenotype formed group one, eight silenced lines with a severe phenotype represented group two and group three consisted of eight lines with a more moderate phenotype. The three pools were freeze dried and the cellulose extracted from the corresponding cell walls (see Materials and Methods in paper I). Enzymatic hydrolysis of the extracted cellulose was performed using a mixture of three recombinant specific cellulases from *Thermofibida fusca* expressed in *E. coli* (Cel6A, Cel6B and Cel9A; generous gift from Professor David B. Wilson, Cornell University, USA) to determine the cellulose content in the cell walls. An endo β-(1→3)-glucanase from *Trichoderma* (Megazyme) was also used to detect the possible occurrence of β-(1→3)-glucans in the extracted material. Well-characterized polysaccharides were used to verify enzyme specificity as described in Materials and Methods and Supplemental Material of paper I. The kinetics of released reducing sugars was followed during 72 hours hydrolysis, using an assay adapted from Nelson-Somogyi (Nelson, 1944). The kinetics of hydrolysis of the samples from the pooled lines exhibiting severe and moderate phenotypes reached a

plateau after 72 hours, suggesting a complete hydrolysis of the total amount of cellulose originally present in the samples. In the case of the non-silenced lines used as a control sample, the release of reducing sugars continued to increase after 72 hours hydrolysis, indicating that hydrolysis in those samples had not reached completion (Figure 8 in paper I). The amount of reducing sugars released from the extracted wall material of the control line was approximately twice as high as in the samples corresponding to the silenced lines with severe and moderate phenotypes after 72 hours hydrolysis. Taken together, these results show that the cellulose content of the silenced lines is decreased compared to that of the control lines. Therefore, it can be concluded that at least some of the *CesA1-4* genes are involved in cellulose biosynthesis (Figure 8 in paper I).

2.3.5 Effect of DCB treatment on appressoria formation *in vitro* (Paper I)

Silencing of *PiCesAs* reduced the formation of appressoria and infection vesicle-like structures, and was accompanied by the formation of aberrant appressorial structures. *P. infestans* requires a functional appressorium for a successful infection of potato. Thus, the effect of the cellulose synthesis inhibitor DCB was tested on the formation of appressoria and infection of potato. 40 μM and 100 μM of DCB had no significant effect on *P. infestans* growth, but the highest concentration of inhibitor resulted in a severe reduction of zoospore release and in the complete inhibition of cyst germination. At 40 μM DCB, a rupture of the appressoria was observed at the growing tip. In addition, the morphologies of the DCB-treated appressoria and germ tubes were identical to those observed for *CesA*-silenced lines (Figure 6 in paper I). The cell walls of the wild type were uniform when observed by transmission electron microscopy whereas the morphology and structures of the cell walls of the silenced and DCB-treated lines were altered (Figure 11 in paper I). These results indicate that the treatment of *P. infestan*s with DCB leads to similar appressorial and germ tube phenotypes as those observed for the *PiCesAs* silenced lines.

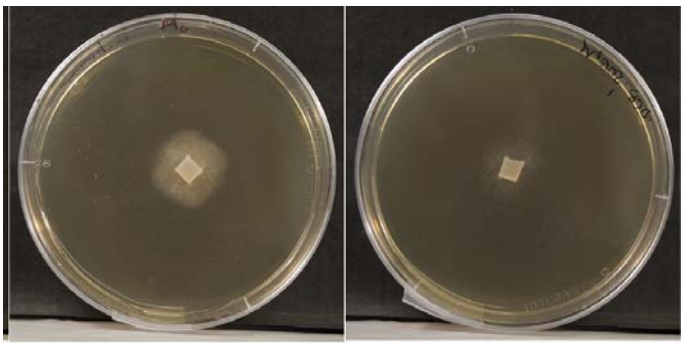
2.3.6 Cellulose biosynthesis is required for infection of potato by *P. infestans* (Paper I)

Since cellulose synthesis is required for the formation of appressoria and normal germ tubes, it may also be needed for the infection process of the host tissues. Normally, zoospores of wild type *P. infestans* encyst and germinate upon contact with the host tissues. Germ tubes are usually short and produce swollen appressoria at their tips. Leaves are subsequently infected by penetration of the appressoria between epidermal cells or through stomata. An infection vesicle is then produced in the epidermal cell adjacent to the site of penetration. The need for

cellulose biosynthesis in the infection process was tested by treating zoospores with 40 μM DCB and inoculating potato leaflets. The progress of the disease was recorded six days after inoculation. The DCB-treated zoospores were found to be completely non-pathogenic, whereas leaflets inoculated with untreated zoospores exhibited lesions and severe necrosis (Figure 12 E in paper I). Scanning electron microscopy observations 16 hours after inoculation showed that the control zoospores had encysted, produced many appressoria and appeared to have penetrated the host. The DCB treated zoospores had also encysted but produced long germ tubes and aberrant appressoria-like structures similar to those observed *in vitro* (Figure 12 A-D in paper I).

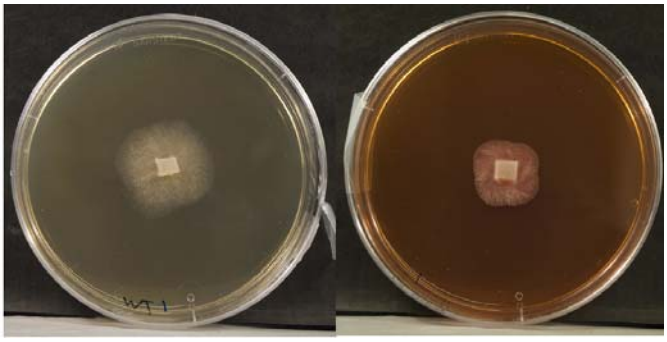
2.3.7 Effect of cellulose synthesis inhibitors on mycelial growth (Paper II)

DCB treatment did not show any effect on mycelial growth in *P. infestans*, but the growth of *S. monoica* mycelium decreased in the presence of DCB and the mycelium was thinner (Figure 14 and Figure 3 in paper II). Congo Red, an inhibitor of β-glucan synthesis, also affected the growth of the mycelium of *S. monoica*, as reported earlier (Nodet et al., 1990 a) (Figure 15 and figure 3 in paper II). Altogether, these data suggest that cellulose biosynthesis is more important for mycelial growth in *S. monoica* than in *P. infestans*.



A **B**

Figure 14. Growth of *S. monoica* mycelium in the presence of DCB (Machlis solid medium). A) A control plate containing 1% methanol (solvent of DCB). B) Plate containing 200 μM DCB in 1% methanol; the colony is smaller and thinner than in the control in Figure A.



A **B**

Figure 15. Growth of *S. monoica* mycelium in the presence of Congo Red (Machlis solid medium). A) Control plate without inhibitor. B) Plate containing 100 μM Congo Red; the colony is smaller than in the control in Figure A and exhibits a denser morphology.

2.3.8 Effect of DCB and Congo Red on the expression levels of *SmCesAs* (Paper II)

The effect of DCB and Congo Red on the transcription of the *SmCesA* genes was investigated by cultivating the mycelium of *S. monoica* in the presence of 200 μM DCB, 100 μM Congo Red, 1 % methanol or water. RNA was extracted at 0.5, 2, 4 and 8 hours after cultivation. The levels of expression of the *SmCesA* genes were analyzed by quantitative Real Time PCR (se Material and Methods in paper II) and normalized with respect to the constitutive expression of the *18S RNA* gene. The relative levels of expression were obtained by dividing all expression values by the value corresponding with the corresponding control at 0.5 hour. The levels of up-regulation of *SmCesAs* by DCB varied during the time course, with the highest effect (approximately 3 times up-regulation) observed for *SmCesA2* after 8 hours when compared with the control sample at the same time point (Figure 4 A in paper II). Each *CesA* gene showed a decreased level of expression after 4 hours of growth in the presence of DCB or 1% methanol. This effect was somewhat reflected in the Congo Red treated samples. It might be due to transcriptional fluctuations during the growth of *S. monoica*. The *SmCesA* genes are up-regulated by Congo Red with a maximum of 5 times increase in the level of expression for *SmCesA3* after 8 hours growth (compared to the control sample at the same time point). Taken together, these results indicate that cell wall biosynthesis or, alternatively the cell wall structure, is disturbed by DCB and Congo Red, which leads to an increase in the transcriptional levels of *SmCesAs*.

2.3.9 *In vitro* glucan synthase activities are increased when *S. monoica* is grown in the presence of DCB or Congo Red (Paper II)

The effect of the inhibitors on β-glucan synthesis was also tested by assaying enzymatic activities in membranes prepared from the mycelium of *S. monoica* grown in the presence of 200 μM DCB, 1 % methanol or 100 μM Congo Red. Total membranes were extracted after four days of cultivation and 500 μg protein was used in *in vitro* assays of glucan synthases. The incorporation of [^{14}C]glucose from UDP-[^{14}C]glucose in ethanol-insoluble β-glucans was measured (Figures 5 A and B in paper II). Product characterization was performed using specific β-glucanases (see Materials and Methods in paper II). The synthesized β-glucans consisted of a mixture of β-(1→3)-glucan and cellulose (Figure 5 C in paper II). The amount of [^{14}C]glucose incorporated was twice as high in the case of the enzyme extracted from the DCB-treated mycelium compared to the control grown in the presence of 1 % methanol. The levels of activity were approximately 30 % higher for the Congo Red sample compared to the enzymatic sample from the untreated mycelium. Since DCB has no effect on *in vitro* synthesis of β-glucans (Blaschek et al., 1985), these data suggest an increase in the amount of expressed glucan synthases (possibly CesAs) or in their specific activity. Congo Red is an inhibitor of glucan synthesis *in vitro* (Nodet et al., 1990 b). Carry over of Congo Red during the membrane extraction may have partially inhibited the *in vitro* synthesis of β-glucans thereby leading to an underestimation of the actual levels of activity measured *in vitro* using enzymatic samples prepared from the Congo Red treated mycelium. These results are in good agreement with the observed increased expression of the *SmCesA* genes when *S. monoica* is grown in the presence of DCB or Congo Red.

2.4 Conclusions and perspectives

Here we report the isolation and characterization of the first Oomycete *CesA* genes in the plant pathogen *P. infestans* and the fish pathogen *S. monoica*. The small *CesA* gene family found in Oomycetes constitutes a new group in the *CesA* superfamily. All the identified genes share the common motifs of CesAs from other organisms and other processive enzymes from the GT2 family. But in addition to the D,D,D and QXXRW motifs, some genes, namely *CesA1,2* and *4* from *Phytopthora* and *CesA2* and *4* from *S. monoica*, also contain unique and novel features. The N-terminal Pleckstrin Homology domain, possibly involved in the trafficking of the CesA molecules, is the most important distinguishing feature. In addition,

the Oomycete *CesA3* genes contain an unique putative zinc-finger domain. A possible function for this highly conserved domain among the orthologues could be an involvement in the dimerization of the CesAs by analogy with the ring-finger motif found in plant CesAs. However, this remains to be demonstrated. The possibility of using cellulose biosynthesis as a target for the development of new Oomycete inhibitors is another subject that needs to be further explored. We have shown that cellulose biosynthesis in Oomycetes is a promising target for such growth inhibitors, since this biochemical process is important for the virulence of *P. infestans*. Its role in the infection process has not been studied in *S. monoica*, but high sequence similarity between the orthologous *CesA* genes of *P. infestans* and *S. monoica* suggests a similar function.

Despite the progress made in our work on the identification of *CesA* genes, numerous questions remain to be addressed regarding cellulose biosynthesis in Oomycetes. For instance, it has not been demonstrated whether terminal complexes exist in Oomycetes and if they are organized as rosettes or linear complexes. The eventual mode of interaction of the different *CesA* gene products is another open question, as well as the possible role of their different N-terminal in the formation of the cellulose synthase machinery. The involvement of specific Oomycete CesAs in specific life stages, as suggested by our preliminary expression analyses, needs to be further investigated. In our future work, we will focus on resolving some of these remaining issues. GFP-fusion proteins or otherwise tagged fusion proteins will be prepared for the transformation of the strains. This will help understanding the localization of the catalytic subunits, their maturation process and mode of association, possibly as terminal complexes. To investigate the potential interaction of the different CesAs, co-localization studies with the CesA homologues fused to different types of fluorescent proteins, *e.g.* GFP, YFP, CFP, or bimolecular fluorescent complementation will be used with subsequent visualisation by confocal microscopy. These methods can also be applied for investigating the role of the different N-terminal domains in localization and protein-protein interaction, like homodimerization or heterodimerization, by the construction of truncated proteins and domain swapping between homologues.

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