Molecular characterization of cellulose synthase (*CesA*) genes and impact of mutations on fungicide resistance in oomycetes

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SUMMARY

Many oomycetes are important pathogens of plants (e.g. arable crops, fruit and forest trees), animals or microbes and, upon attacking their hosts, cause considerable economic damage mainly in agriculture and aquaculture. To minimize yield losses efficient disease control, primarily relying on the application of anti-oomycete compounds with a single-site mode of action, is very important. Some anti-oomycete compounds specifically inhibit mitochondrial respiration, RNA synthesis, microtubule organization or cell wall synthesis. The oomycete cell wall is mainly composed of β-1,3-, β-1,6 glucans and cellulose that provide rigidity to the cell. The synthesis of cellulose was recently shown in *Phytophthora infestans* to be the target for mandipropamid (MPD), which belongs to the oomycete specific carboxylic acid amide (CAA) fungicides controlling members of the *Peronosporales*. However, there are several oomycetes that the CAAs cannot control (e.g. the entire genus *Pythium*), even though cellulose is an important cell wall component of all oomycetes. Furthermore, the mode of action of CAA fungicides is highly specific, thus the resistance risk is assumed to be moderate to high. Consequently, resistant individuals were recovered in *Plasmopara viticola* and *Pseudoperonospora cubensis* populations, but the underlying mechanism of resistance remained unknown. This thesis aims to identify and characterize the cellulose synthase (*CesA*) genes of various oomycete species and thereby to investigate the mechanism(s) of resistance in sensitive species, i.e. species normally affected by CAAs, and tolerance in insensitive species, i.e. species unaffected by CAA fungicides.

A family of four cellulose synthase genes was identified and fully sequenced in the downy mildew pathogens *P. viticola* and *P. cubensis*. Phylogenetic analyses of the four genes revealed their close relatedness to cellulose synthase genes of *Phytophthora* spp. and the red algae *Porphyra yezoensis*. Sequencing of the *CesA* genes in CAA-resistant and -sensitive field isolates of *P. viticola* and *P. cubensis* uncovered single nucleotide polymorphisms (SNPs) affecting the amino acid structure of CesA proteins. Inheritance of resistance in *P. viticola* was confirmed to be correlated with one recessive SNP located in the *CesA3* gene. This SNP led to an exchange from a highly conserved glycine (encoded by GGC) to serine (AGC) at position 1105 (G1105S), whereas in *P. cubensis*, mutations causing amino acid substitutions from glycine (GGG) to valine (GTG) or tryptophane (TGG) (G1105V, G1105W) occurred in the same codon. The results demonstrate for the first time that SNPs in codon 1105, when present in both alleles, lead to amino acid exchanges in the CesA3 enzyme causing inheritable and stable

resistance to all CAA fungicides.

New insights into the molecular basis of CAA tolerance in *Pythiales* were provided by characterizing five genes putatively involved in carbohydrate synthesis of the root rot and damping off causative agent *Pythium aphanidermatum*. Using the CODEHOP PCR strategy, one chitin synthase gene, *PaChs*, and four cellulose synthase genes, *PaCesA1* to *PaCesA4*, out of which *PaCesA3* encodes the MPD target enzyme, were amplified and fully sequenced. These genes were individually upregulated during encystment, germination of cystospores and mycelial growth indicating their relevance for cell wall formation. However, almost no change in *PaCesA* or *PaChs* expression was observed when mycelium was treated with MPD concentrations slightly affecting mycelial growth. Detailed analyses of the putative target site in PaCesA3 revealed a specific amino acid configuration (L1109) also present in CAA resistant *P. infestans* mutants. The affected amino acid residue is located only four amino acids downstream of the G1105 residue, where amino acid exchanges cause inheritable resistance to CAAs in *P. viticola* and *P. cubensis* field isolates. This implies that MPD tolerance in *P. aphanidermatum*, and most likely in other *Pythium* species, is based on the leucine configuration at position 1109 which may affect the binding of CAAs to the enzyme.

To further examine the results obtained with *P. aphanidermatum*, the *CesA3* gene structure as well as the sensitivity to CAAs of 25 species representing the *Albuginales*, *Leptomitales*, *Peronosporales*, *Pythiales*, *Rhipidiales* and *Saprolegniales* was investigated. Molecular characterization of the putative target site in CesA3 revealed a conserved glycine at position 1105 (G1105) in all oomycete species. However, at position 1109 the *Peronosporales* displayed the amino acid valine, whereas all species from the other orders showed either leucine or methionine at this position. The observed amino acid configurations (L1109 and M1109) correlated with MPD tolerance, suggesting that amino acid changes at position 1109 may also affect CAA efficacy in sensitive species. In addition, the full-length nucleotide sequence of the *CesA3* gene was used to study phylogenetic relatedness among oomycetes originating from the six distinct orders. The phylogenetic tree constructed with the *CesA3* gene sequence was largely in agreement with trees based on other markers (e.g. cox2, SSU, LSU rDNA), implying that this gene represents a promising tool to reconstruct an overall picture of the oomycete phylogeny.

The presented insights into the molecular mechanism(s) of CAA resistance and tolerance significantly contribute to a sound assessment of resistance risk when CAAs are used to control oomycetes. In addition, the results open up novel tools for basic investigations on cellulose biosynthesis in oomycetes.

GENERAL INTRODUCTION

1. INTRODUCTION TO OOMYCETES

The oomycetes, also known as "water molds", form a diverse group of fungus-like microorganisms. Currently, at least 800 oomycete species are known, but many more are likely to be discovered. Even though the oomycetes share many characteristics with true fungi (e.g. apical hyphal growth, absorptive mode of nutrition, reproduction by formation of spores) they do not belong to the fungal kingdom. Molecular phylogeny placed the oomycetes in a separate kingdom: either in the kingdom Stramenopila or the stramenopile lineage belonging to the "super-kingdom" Chromalvelates (Baldauf et al., 2000); or in the kingdom Chromista (Cavalier-Smith and Chao, 2006). Molecular data has demonstrated that their closest relatives are heterokont algae (Ben Ali et al., 2001), even though the lifestyle of oomycetes is completely different. In contrast to their relatives, the oomycetes have lost plastids and absorb nutrients from surrounding water or soil or invade other organisms to feed.

Oomycetes have a global distribution and survive in diverse environments. Apart from the saprophytic species that feed on dead material, many oomycetes are important parasites of plants, animals or microbes. By attacking their hosts, which include trees, many crop plants, fish, crustaceans, amphibians and occasionally humans, (Mendoza and Newton, 2005; Phillips et al., 2008) they have a huge impact on the environment as well as on human welfare.

1.1. Origin and phylogeny of oomycetes

Oomycetes belong to the kingdom Chromista / Stramenopila that encompasses the three phyla Bigyra, Ochrophyta, and Pseudofungi (Cavalier-Smith and Chao, 2006) (Fig. 1). According to Bakes et al. (2011), the oomycetes have been placed in the phylum Pseudofungi, together with the uniflagellate *Hyphochrytrids*, the flagellate parasitoid *Pirsonia*, and the free-living bacteriotrophic marine zooflagellate *Developayella* (Fig. 1). However, this new classification is still under debate and in many textbooks and reviews, the oomycetes are given their own phylum status.

Recent genomic studies have shown that the oomycetes have their evolutionary roots in the sea, evolving from simple holocarpic marine parasites that are supposed to have a common biflagellate photosynthetic ancestor (Bakes et al., 2011). Thus it is not surprising that the earliest diverging oomycete clade includes the two genera *Eurychasma* (Sekimoto et al., 2008) and *Haptoglossa* (Hakariya et al., 2007), both

representing marine, obligate parasites of brown seaweeds (Gachon et al., 2009), rotifers and nematodes (Newell et al., 1977) (Fig. 2). Some of these "basal oomycetes" might have migrated along with their nematode hosts from the sea to the land occupying new habitats (Beakes and Sekimoto, 2009). These higher evolved oomycetes, also called "crown oomycetes" (Beakes and Sekimoto, 2009) live predominantly in a terrestrial (the peronosporalean lineage) or freshwater (the saprolegnian lineage) environment and have the ability to form oospores as a result of sexual reproduction (Bakes et al., 2011). Currently, the group of "crown oomycetes" is considered to consist of six different orders of which the saprophytic *Leptomitales* and *Rhipidiales* are the most basal orders based on analysis with molecular markers such as *cox2* (Hudspeth et al., 2000), partial 28 rRNA (Bakes et al., 2011; Petersen and Rosendahl, 2000) and SSU rRNA (Lara and Belbahri, 2011). According to Beakes and Sekimoto (2009), it is suggested that the freshwater *Saprolegniales* evolved from the *Leptomitales*, and that the *Rhipidiales* gave rise to the terrestrial *Pythiales*, *Albuginales* and *Peronosporales* (Fig. 2).

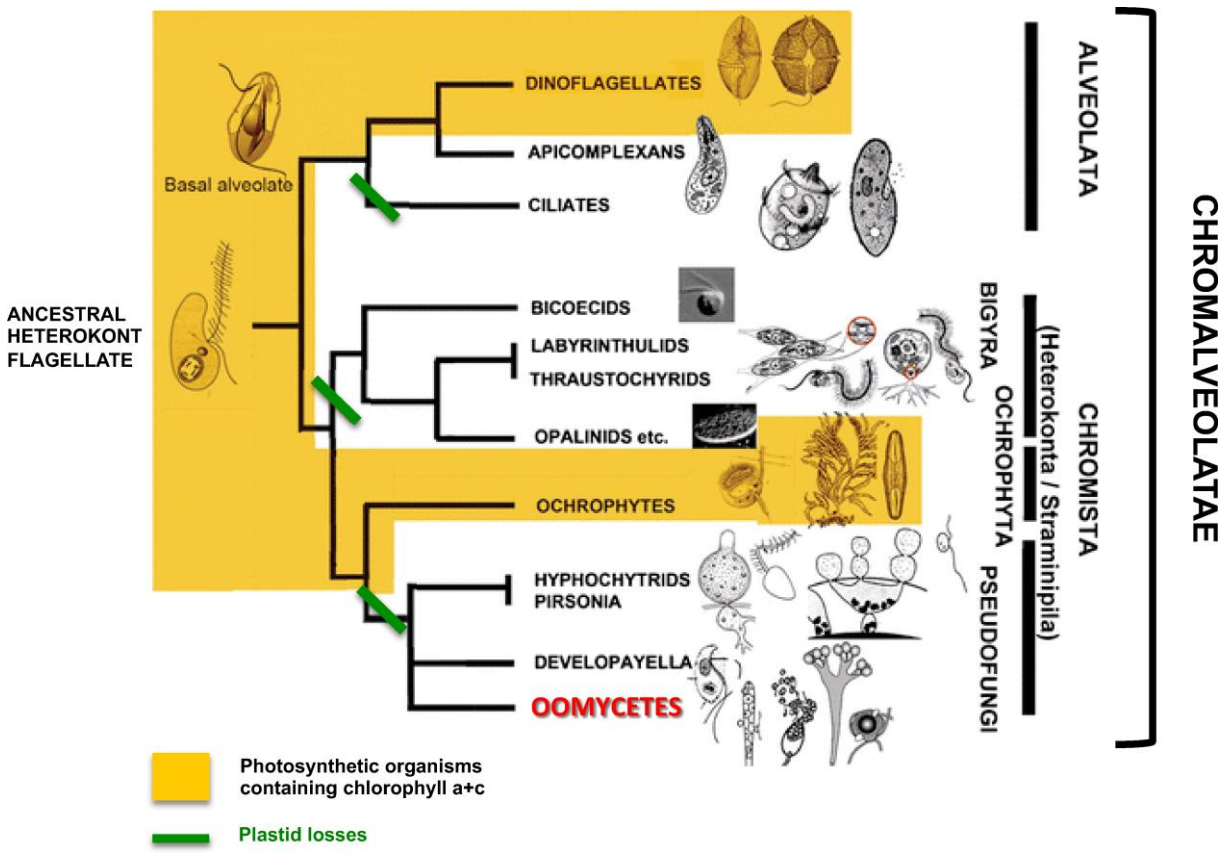

Fig. 1. Phylogenetic tree showing the likely phylogenetic relationships between the diverse members of the "cromalveolate" superkingdom. The tree is adapted from Beakes et al., 2011.

However, even though huge progress was made during the last years, mainly based on the integration of molecular data, for understanding the phylogenetic relatedness of the oomycetes, their evolutionary patterns are still under debate. The use of further molecular markers that are suitable to resolve the deep phylogeny of this group will probably lead to further revisions in taxonomy.

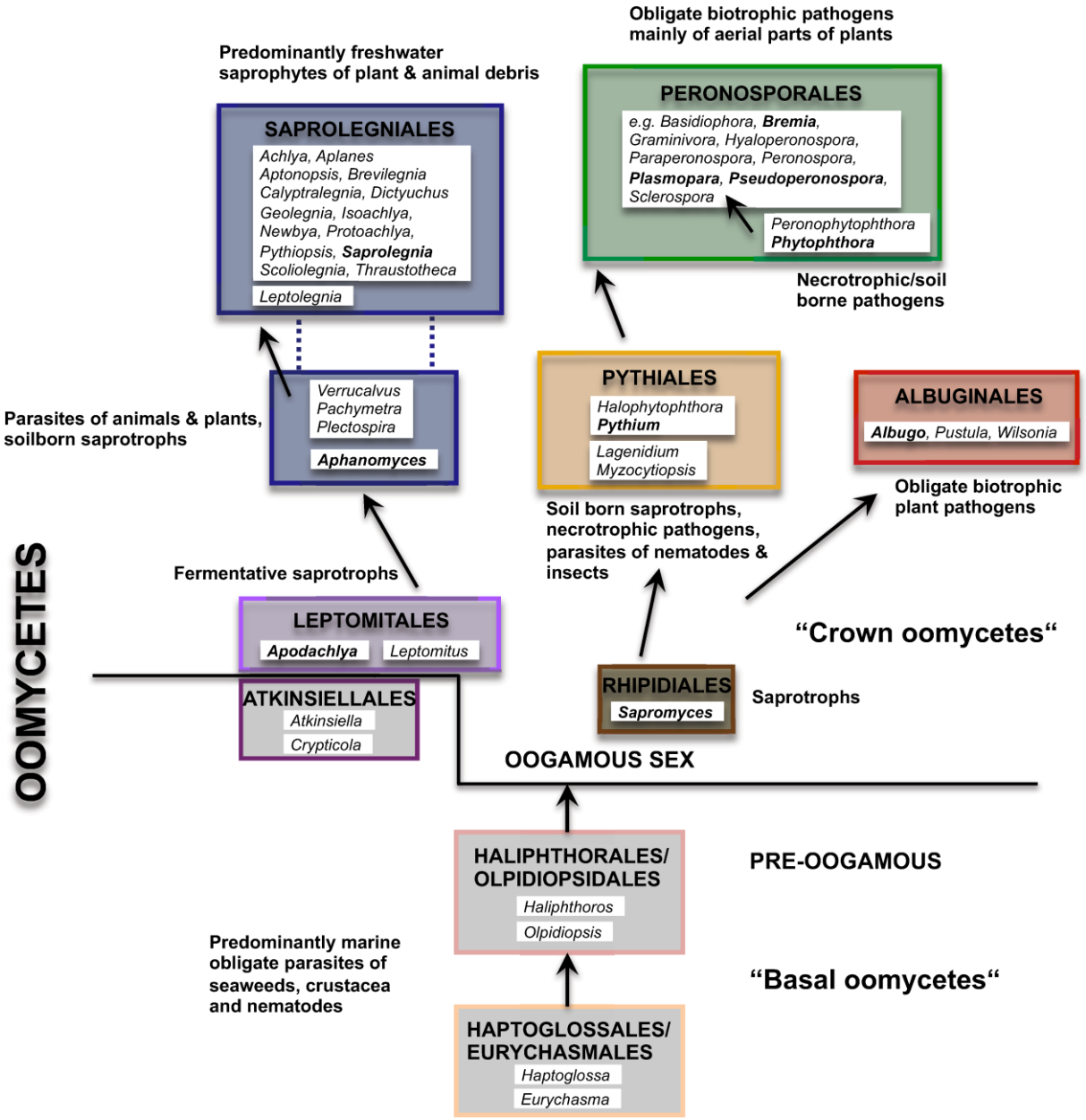

Fig. 2. Putative phylogenetic relationships between the main orders within the oomycetes, based on molecular sequence data (Cox2, SSU, and LSU rDNA). Some ecological and morphological characteristics of species are indicated. Modified from Beakes and Sekimoto, 2009.

1.2. Oomycete characteristics

Most oomycetes have similar biological characteristics as the true fungi, such as absorptive mode of nutrition, filamentous growth, formation of asexual and sexual spores and specialized infection structures (Latijnhouwers et al., 2003; Richards et al., 2006). However, the oomycetes are characterized by a number of biological attributes that clearly distinguish them from true fungi and several other eukaryotic microorganisms: (1) oomycete cells are diploid at the vegetative stage with meiosis preceding gamete formation; (2) oomycetes use the β-1,3 glucan mycolaminarin as energy storage, that is also found in kelps and diatoms (Kamoun, 2003); (3) hyphae of oomycetes are coenocytic with few or no septa; (4) many oomycetes are (partially) sterol auxotrophs, i.e. their membranes contain lipids with long-chain fatty acids presumably replacing sterols in the membrane (Latijnhouwers et al., 2003); (5) asexual reproduction mainly occurs by formation of motile, biflagellate zoospores which are able to swim in water and in some cases by non-motile sporangia; (6) the oomycete cell wall is mainly composed of β-1,3-glucan polymers and cellulose and contains (unlike the fungal cell wall) little or no chitin (Bartnicki-Garcia, 1968; Bulone et al., 1992; Helbert et al., 1997).

Apart from these cytological and biochemical features, oomycetes also share some unique genomic characteristics. Recent genome analyses of oomycete species such as *Phytophthora infestans*, *P. ramorum* and *P. sojae* (Haas et al., 2009; Tyler et al., 2006) indicated that the genome of oomycetes is characterized by an abundance of repetitive sequences (Lamour et al., 2007). However, only few genome sequences, mainly of species belonging to the genus *Phytophthora,* have been published so far, thus, it remains to be investigated if these repetitive elements represent a general oomycete feature. Based on studies that were performed with *Phytophthora* species, there is also evidence, that the transcriptional machinery in oomycetes is somehow unique. Several attempts to induce gene expression under promoters from non-oomycete species failed, suggesting that the transcriptional mechanism in oomycetes is different from other eukaryotes (Judelson and Michelmore, 1991; Judelson et al., 1992). Unlike to other eukaryotes, the transcriptional start sites of many oomycete genes are usually located only 50 to 100 bp upstream of the start codon (Pieterse et al., 1994), suggesting that oomycetes bear highly compact transcripts with very short untranslated regions (Kamoun, 2003). A further general feature of oomycete genes seems to be the low

number of introns. Analysis of *Phytophthora* sequences revealed an average of 1.5 introns per gene ranging from 26 to 172 bp (Kamoun, 2003).

Meanwhile additional oomycete genomes have been or are in progress to be sequenced such as for the species *Hyaloperonospora arabidopsidis* (Baxter et al., 2010), *Pythium ultimum* (Levesque et al., 2010), *Saprolegnia parasitica* (http://www.broadinstitute.org/annotation/genome/Saprolegnia_parasitica/MultiHome.html) and *Albugo laibachii* (Kemen et al., 2011). The gained sequence information will certainly provide further knowledge about the unique molecular events taking place in these organisms.

1.3. Important plant and animal pathogenic oomycetes

Many oomycetes are responsible for massive destruction and huge losses in agriculture and aquaculture owing to their ability to infect a range of plants and animals (Phillips et al., 2008). Within the plant-pathogenic oomycetes that cause devastating diseases in numerous crops, ornamental and native plants, species of the genus *Phytophthora* are probably the most devastating pathogens of dicotyledonous plants (Erwin and Ribeiro, 1996). By attacking important crop species such as potato, tomato, pepper, and soybean, as well as native plants such as fruit and forest trees, *Phytophthora* spp. can cause enormous economic damage (Fry and Goodwin, 1997; Rizzo et al., 2005).

The most extensively studied and best-known species is *P*. *infestans*, the causal agent of potato late blight (Fig. 3A). Introduced from Mexico to Europe in the mid-nineteenth century, it was responsible for the potato blight famine leading to the death and displacement of millions of people (Andrivon, 1996). Today, *P. infestans* remains a devastating pathogen, causing annual losses exceeding $5 billion in potato production worldwide (Haverkort et al., 2008).

However, important plant pathogenic oomycetes also occur outside the genus *Phytophthora*. Within the order *Peronosporales*, there are several obligate biotrophic species (e.g. *Plasmopara viticola*, *Bremia lactucae*, *Pseudoperonospora cubensis*) which cause downy mildew on several crops (Fig. 3B, C). The genus *Albugo,* also containing strictly biotrophic species, causes white blister rust on several *Brassica* species (Fig. 3D) leading to one of the most destructive foliar diseases of horseradish (Gilijamse et al., 2004). Another important plant pathogenic genus is represented by *Pythium* that includes more than 100 species, which are mainly occupying water and soil habitats (Fry

and Grünwald, 2010). *Pythium* species are also known for causing damping-off and seed rot disease in young plant seedlings (Fig. 3E). Attacked seedlings are often completely destroyed by the pathogen, leading to significant yield reductions (Martin and Loper, 1999). Further species causing seedling and root-rot diseases on many legumes can be found in the genus *Aphanomyces* (Fig. 3F). *A. euteiches* affects several legumes and is considered to be the most limiting factor in pea production, notably in Europe (Levenfors et al., 2003).

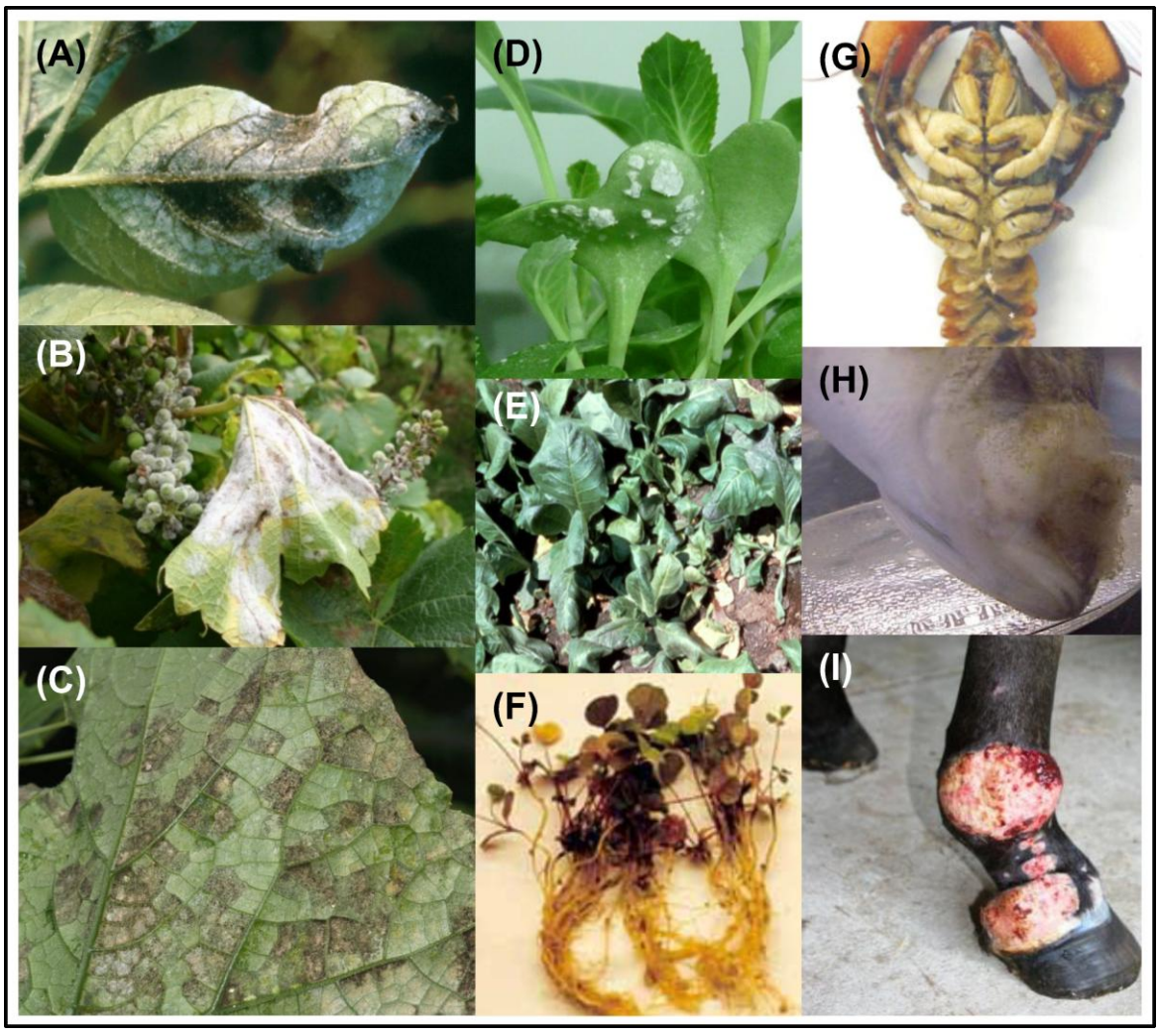

Fig. 3. Diversity of symptoms caused by oomycetes on their plant and animal hosts.
(**A**) Necrotrophic growth of *Phytophthora infestans* on potato (image by R. James). (**B**) Heavy sporulation of *Plasmopara viticola* on grape leaves and berries (image by F. Huggenberger). (**C**) Cucumber leaf infected with *Pseudoperonospora cubensis.* (**D**) Infection of cabbage seedlings by *Albugo candida* forming white pustules. (**E**) *Pythium* damping off on Tobacco seedlings (image by R. Reynolds). (**F**) Root-rot of alfalfa plants caused by *Aphanomyces euteiches* (image by D. Malvick). (**G**) *Aphanomyces astaci* infection in the subabdominal cuticle of crayfish (image by D. Uribendo). (**H**) Mycelial growth of *Saprolegnia parasitica* on perch (image by T. Volk). (**I**) Horse leg infected with *Pythium insidiosum* causing the chronic disease Pythiosis.

Animal-pathogenic species that are responsible for devastating diseases in aquaculture and natural ecosystems mainly occur in the order *Saprolegniales*. *Aphanomyces astaci* causes crayfish plague (Fig. 3G) leading to high mortalities in several European *Astacus* species (Phillips et al., 2008). Members of the genus *Saprolegnia* are known to cause serious infections of various freshwater fish (Fig. 3H). *S. parasitica* is a huge problem in salmon farms because of lacking control strategies; it has also been implicated in the decline of wild salmon populations around the world (van West, 2006). Many *Pythium* species are important plant pathogens; however, this genus contains one species, *P. insidiosum*, which is able to infect mammals, such as horses, dogs, cats and even humans (Fig. 3I). The disease, called pythiosis is extremely difficult to control due to the inefficiency of antifungals, and often requires amputation of the infected tissue (Phillips et al., 2008). It is quite evident that the oomycetes have a severe impact on agriculture, aquaculture and natural ecosystems, thus influencing not only the environment but also human welfare. Hence, there is a strong demand for appropriate control strategies to combat these organisms.

2. CONTROL OF OOMYCETES

Efficient disease control of oomycetes in crop plants has been a major challenge in the past and still is today. Nowadays, control of oomycete diseases is either achieved by breeding for resistant cultivars or chemical control. Identification of inheritable traits for resistance either through major gene resistance (Wastie, 1991) or quantitative resistance (Clement et al., 2003) led to specific plant breeding programs. The implementation of resistant cultivars allowed at least in some crops (e.g. lettuce, melon) partial control of oomycete diseases (Barnes and Epps, 1954; Belbahri et al., 2001; Yamamizo et al., 2006). However, resistance to oomycetes of most cultivars relies on major gene resistance that has a history of being rapidly overcome by the pathogen. As an example, *P. infestans* is known to frequently change Avr proteins. Consequently, the new versions of the Avr proteins are no longer recognised by the deployed resistance in the plant (Fry, 2008). Furthermore, breeding for resistance is limited to crop plants with known resistance traits. Therefore the breeding approach has its limitations, especially for disease control in tree species and animals and is therefore not feasible or appropriate as a short term disease control measure (Whisson et al., 2011).

Today, the most efficient control measure relies on chemical control. However, many conventional fungicides that are successfully applied against "true fungi" do not control oomycete diseases. This is not surprising since oomycetes have quite unique biochemical characteristics and are phylogenetically distinct from true fungi (Bakes et al., 2011).

2.1. Anti-oomycete compounds

One of the first chemical compound used for oomycete disease control was Bordeaux mixture, a copper based preparation, introduced by Millardet in 1885 to combat the downy mildew pathogen *Plasmopara viticola* (Viennot-Bourgin). About 50 years later, organic compounds, such as dithiocarbamates and phthalimides, both having a multi-site mode of action became very important tools for disease control incited by oomycetes and true fungi (Kuck and Gisi, 2008). In the late 1970s, compounds having a specific single-site mode of action were introduced to the marked. Among these were the oomycete specific phenylamides, such as metalaxyl-M (Mefenoxam), that are proposed to inhibit the RNA polymerase I complex (Davidse et al., 1983; Wollgiehn et al., 1984). However, the precise subunit of this multisubunit complex that is targeted by phenylamides remains unknown (Whisson et al., 2011). Further compounds for oomycete control such as Cymoxanil and Fosetyl-Al were developed by DuPont and Rhone-Poulenc respectively, and launched in 1977 (Hillebrand and Zundel, 2008). Despite extensive research on the biochemical mode of action, the specific target for both compounds has not yet been fully elucidated (Griffith et al., 1992; Ziogas and Davidse, 1987).

During the last decade, additional compounds were developed and introduced to the marked such as Cyazofamid that specifically inhibits electron transport in oomycete mitochondria resulting from binding at the Qi site of cytochrome bc1 (Mitani et al., 2001); the microtubule inhibitor Zoxamide (Young and Slawecki, 2001); the carboxylic acid amides (CAAs); as well as Fluopicolide that is suggested to target spectrin-like proteins of oomycetes (Toquin et al., 2008). According to the Fungicide Resistance Action Committee (FRAC), there are currently nine specific modes of action available for oomycete control (Kuck and Gisi, 2008).

2.2. Carboxylic acid amide (CAA) fungicides

The chemical group of carboxylic acid amide (CAA) fungicides was officially announced by the Fungicide Resistance Action Committee (FRAC) in 2005. Designated as group number 40 (FRAC code list), it includes the three sub-classes cinnamic acid amides (dimethomorph, flumorph), valinamide carbamates (benthiavalicarb, iprovalicarb, valifenalate) and mandelic acid amides (mandipropamid) (Fig. 4). Even though the chemical structures of the CAAs varies significantly, they have been grouped together based on their common cross resistance pattern in *P. viticola* (Gisi et al., 2008).

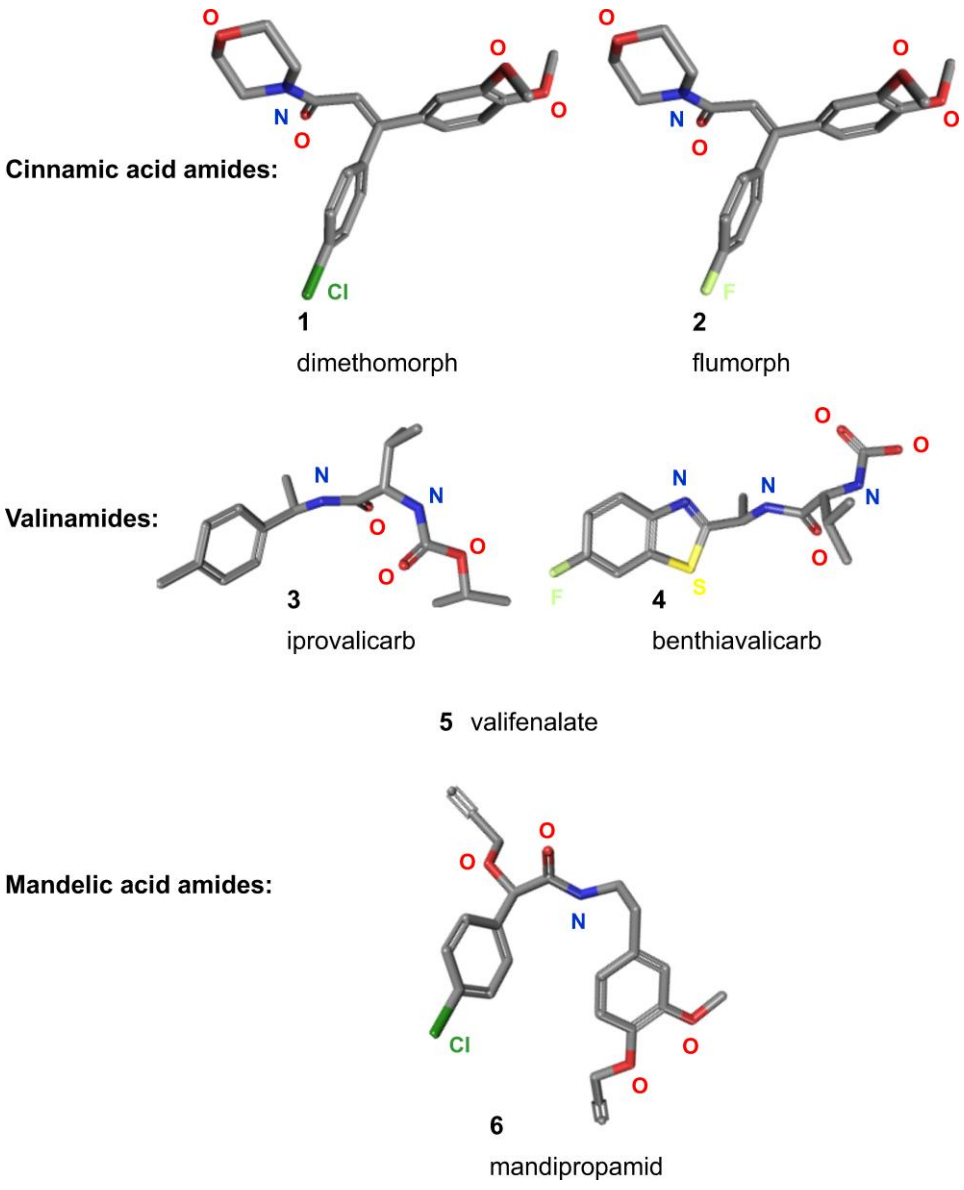

Fig. 4. Carboxylic acid amide (CAA) fungicides launched into the market (status 2011). The chemical structures were obtained from the PubChem database at www.ncbi.nlm.nih.gov/pccompound. The 3-D structure of the compound valifenalate is not yet available and is therefore not shown.

Historically, dimethomorph (1) was the first member of the CAA class to be introduced in the early 1980s by Celamerck, now BASF (Albert et al., 1988). It was followed by the Bayer product iprovalicarb (3) in 1998 (Stenzel et al., 1998), flumorph (2) introduced by Shenyang in 2000 (Liu and Liu, 2001), benthiavalicarb (4) commercialized since 2003 by Kumiai-Ihara (Miyake et al., 2003) and the Syngenta compound mandipropamid (6) in 2005 (Huggenberger et al., 2005). The last member of this class, valifenalate (5), was launched in Italy and France in 2009 by the company Isagro (Walter, 2011).

The biological activity of CAAs is restricted to the *Peronosporales* that includes various *Phytophthora* species (e.g. *P. infestans* causing potato late blight) and downy mildew pathogens such as *Plasmopara viticola*, *Bremia lactucae*, and *Pseudoperonospora cubensis* (Cohen et al., 2008; Gisi et al., 2007; Kim et al., 2009; Lebeda and Cohen, 2011). However, there is no control of *Pythiales* or any species of true fungi. CAAs mainly inhibit cystospore and sporangia germination, but also the growth of germ tubes and mycelia, all stages that rely on an intact cell wall (Cohen and Gisi, 2007; Knauf-Beiter and Hermann, 2005). In contrast, they have no effect on zoospore release, motility and encystment (Cohen and Gisi, 2007). When applied preventively, CAAs exhibit the strongest activity. Limited curative effects were also reported based on translaminar movements of the compounds (Gisi et al., 2008).

Cytological and biochemical studies performed with dimethomorph, iprovalicarb and benthiavalicarb indicated that CAAs somehow inhibit processes involved in cell wall biosynthesis or deposition (Delvos, 2009; Kuhn et al., 1991; Mehl, 2006; Miyake et al., 2005). Other studies reported on alterations of microtubule organization upon flumorph treatment in *P. cubensis* (Zhu et al., 2007a) or alterations in phospholipid synthesis suggesting that lecithin biosynthesis may be the target of CAA fungicides (Griffiths et al., 2003). Despite the discrepancy of these biochemical results, all observations had one common basis: alterations of processes that are involved or associated with cell wall synthesis. In 2010, a mode of action (MoA) study performed with mandipropamid (MPD) and *P. infestans* provided further evidence that inhibition of cell wall synthesis is the main target of this fungicide class. For the first time the biochemical mode of action of a CAA fungicide could be linked to alterations in the putative target enzyme, cellulose synthase 3 (Blum et al., 2010), which finally led to the classification of the CAAs as cellulose synthesis inhibitors (H5, FRAC code list).

2.3. Mode of action of mandipropamid

Even though many attempts were undertaken to elucidate the mode of action of any CAA fungicide, the precise target remained unknown until the year 2010. The break-trough was finally achieved by a study performed with mandipropamid (MPD): Blum et al. (2010) successfully utilized a combination of biochemical and genetic techniques to identify the molecular target of MPD in the oomycete *P. infestans.* Although several studies reported on difficulties in generating resistant mutants to CAA fungicides (Chabane et al., 1993; Rubin et al., 2011; Stein and Kirk, 2004), Blum et al. (2010) were able to produce resistant mutants displaying stable resistance to MPD and other CAA fungicides. For the first time, protoplasts of *P. infestans* were successfully mutagenized using a high dose of the chemical mutagen ethylmethane-sulfonate (EMS) leading to resistant *P. infestans* mutants.

Since treatment of germinating cystospores with low MPD concentrations (nM range) led to swelling symptoms at the growing tip (Blum et al., 2010; Cohen and Gisi, 2007), typical for cell wall synthesis inhibitors, the authors hypothesized that the primary biochemical effect was inhibition of synthesis or deposition of the cell wall. These findings were supported by studies with ^{14}C-labelled MPD showing that the compound did not enter the cell but affects the cell wall (Blum et al., 2010). Furthermore, in germinating cystospores, ^{14}C-glucose incorporation into cellulose was perturbed in the presence of MPD indicating that cellulose synthesis may be the primary target of MPD. Hence, the authors sequenced putative target genes in the MPD resistant *P. infestans* mutants, encoding a variety of cell wall proteins, among those four cellulose synthase (*CesA*) genes, that were previously identified by Grenville-Briggs et al. (2008). Sequence analysis of these genes in the resistant *P. infestans* mutants revealed two point mutations in the cellulose synthase 3 (*PiCesA3*) gene (Blum et al., 2010) known to be involved in cellulose biosynthesis (Grenville-Briggs et al., 2008). Both mutations in the *PiCesA3* gene were located at the very same position and resulted in change of a glycine to alanine or valine (G1105A, G1105V) at the C-terminal end of the protein. The final proof for the observed resistance was given by back transformation studies. For this purpose, the ability to transform *P. infestans* was exploited to introduce copies of the mutated allele into a sensitive wild-type isolate. Indeed, transformed strains overexpressing the mutated allele were resistant to MPD, demonstrating that (I) the mutations in *PiCesA3* were responsible for the observed resistant phenotype in the

artificial mutants; (II) MPD most likely targets the CesA3 enzyme in *P. infestans* (Blum et al., 2010).

2.4. Fungicide resistance

Resistance (lack of sensitivity) to fungicides in plant pathogens is one of the major causes affecting proper disease control. Development of fungicide resistance is determined by multiple factors such as the mode of action of the compound, the biology of the pathogen, fungicide use pattern and the cropping system. With the introduction of site-specific fungicides such as benzimidazoles and phenylamides in the late 1970s, the problem of fungicide resistance became apparent. A few years after the introduction of these new fungicides, some pathogen species were able to readily overcome the specific mechanism of inhibition, leading to failures in disease control (Reuveni et al., 1980; Smith, 1988). Since the registration of the first site-specific fungicides, today many compounds have been developed also having a single-site mode of action, thus being at increased risk of resistance problems. In general, a pathogen species develops resistance to a fungicide by genetic adjustments (mutations) that result in reduced sensitivity to the fungicide. Resistance often relies on single- or multiple-gene mutations. Single-gene mutations conferring resistance to site-specific fungicides are more likely to occur than mutations in multiple genes necessary to confer resistance to multi-site inhibitors. Several mechanisms have been described conferring resistance to fungicides: mutations and alteration in the target site, which is the most common mechanism; reduced fungicide uptake and active export of the fungicide, mediated by efflux pumps; degradation of the fungicide (Deising et al., 2008; Ma and Michailides, 2005).

Repeated use of a fungicide exerts a selection of resistant individuals (carrying mutations) in a population, initially occurring at very low frequencies. As a consequence of this selection process, the frequency of resistant individuals increases leading to failures in disease control. The shift in decreased sensitivity can occur at different rates, depending on the number of genes involved in the resistance. Resistance based on single-gene mutations may evolve rapidly leading to a population that is predominantly resistant and disease control is lost abruptly. In contrast, when multiple genes are involved, the sensitivity shift progresses much slower, leading to a reduced sensitivity of the entire population and a gradual loss in disease control. For CAA fungicides that have a single-site mode of action, resistance development strongly depends on the biology of

the pathogen. In *Phytophthora* species (mainly *P. infestans*) up to know, resistance to CAAs has never been detected in field populations, even though dimethomorph was used since more than 15 years (Gisi et al., 2008). However, in the downy mildew pathogen *P. viticola*, CAA resistant isolates were detected in 1994, shortly after the introduction of dimethomorph in France (Chabane et al., 1993) and increased in frequency since then (Gisi et al., 2007). CAA resistant isolates were also reported to occur in *P. cubensis* populations (Olaya et al., 2009; Zhu et al., 2007b). In *Peronophythora litchii* resistant isolates were recovered after a selection process on CAA amended agar plates, indicating that resistant isolates may also occur in *P. litchii* populations, but at very low rates (Wang et al., 2010).

Based on segregation studies, performed with *P. viticola*, resistance to CAAs was shown to be controlled by one or two nuclear recessive genes (Gisi et al., 2007). Nevertheless, the molecular mechanism conferring CAA resistance remained unknown at that time. New insights into the mode of action of mandipropamid (MPD) have provided a key element for further investigations on the resistance mechanism to CAA fungicides. Since MPD inhibits cellulose synthesis by targeting the cellulose synthase 3 (CesA3) enzyme in *P. infestans* (Blum et al., 2010a), cellulose synthase genes (especially *CesA3*) of oomycetes, were of major interest for this thesis.

3. INTRODUCTION TO CELLULOSE

Cellulose is the world's most abundant natural, renewable macromolecule and has been widely used for centuries in all kinds of practical applications. Globally, each year between 10^{10} and 10^{11} t of cellulose are synthesized and thereafter decomposed, pointing out the biological importance of this polymer (Hon, 1994). Especially in plants, but also in other organisms, such as algae, oomycetes and tunicates, cellulose plays an essential role for cell volume regulation, determination of cell size and shape, as well as for mechanical protection from environmental influences (Bessueille and Bulone, 2008). Synthesized at the cell surface, it is organized in crystalline microfibrils that provide unique strength and stiffness. Cellulose and its derivates, e.g. cellulose acetate, nitrocellulose, methylcellulose, are used for paper production, in the textile industry, for the preparations of films, pharmaceuticals and cosmetics (Engelhardt, 1996; Klemm et al., 2005). Besides these traditional sectors, cellulose became more and more important for bioethanol production. Nowadays, it is considered that bioethanol has the potential

to significantly reduce the use of fossil fuels for transportation (Ragauskas et al., 2006). For several decades, the process of cellulose biosynthesis has been extensively studied, but despite the progress made during the past years, mainly due to the identification of cellulose synthase encoding genes, the corresponding molecular mechanisms are still not well understood. Since there is not much known about cellulose biosynthesis in oomycetes, most information of the following section concerning cellulose structure, synthesis and its catalytic enzymes derives from plants, algae and other organisms.

3.1. Cellulose structure

Cellulose is a homopolymer, comprised of hydrogen-bonded, β-1,4-linked D-glucose residues. The successive glucose moieties are rotated 180°, forming a flat ribbon in which cellobiose is the structural repeating unit (Fig. 5) (Somerville, 2006).

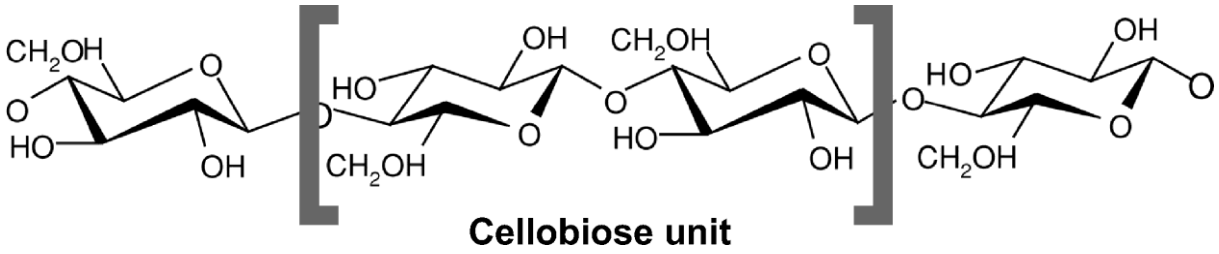

Fig. 5. Cellulose chain fragment composed of D-glucose residues. The alternating sugar residues are linked through β-1,4-linkages, showing a 180° rotation between each monomer. Cellobiose is considered to be the repeating unit.

Hydrogen bonds and Van der Waals forces hold the cellulose chains in a crystalline structure to form microfibrils (Nishiyama et al., 2002). The length of these microfibrils strongly depends on the degree of polymerization (DP) of the D-glucose chains and its source (e.g. 800-10000 for plant celluloses) (Klemm et al., 2005). Cellulose occurs in six different polymorphs (I, II, III_1, III_{11}, IV_1 and IV_{11}) that can be interconverted by different mechanisms (OSullivan, 1997). Native cellulose, also called cellulose I, is by far the most abundant natural form of the polymer, it occurs either as I_α or I_β allomorph, depending on the origin of cellulose (Atalla and Vanderhart, 1984; Brown et al., 1996). Cellulose I_α exists as a single-chain triclinic unit cell and is mainly found in bacteria and marine green algae, whereas cellulose I_β has a two-chain monoclinic unit cell and is present mainly in plants and tunicates (Finkenstadt and Millane, 1998; Sugiyama et al., 1994). In both allomorphs of cellulose I, the β-1,4-linked glucose chains are oriented in parallel (Kuga and Brown, 1988), as opposed to cellulose

II that consists of antiparallel chains (KroonBatenburg and Kroon, 1997). Type II is only found in very few organisms such as algae and bacteria (Delmer, 1999) and can be artificially produced from cellulose I either by regeneration (solubilisation in a solvent followed by reprecipitation in water) or mercerization (sodium hydroxide treatment) leading to a thermodynamically more stable polymer that is characterized by an additional hydrogen bond per glucose residue (Klemm et al., 2005). By treating cellulose I or II with liquid ammonia or some amines, Cellulose III_1 and III_{11} can be formed (Sarko et al., 1976). Further heating of celluloses III_1 and III_{11} to 206°C in glycerol leads to formation of cellulose IV_1 and IV_{11} (OSullivan, 1997).

According to X-ray diffractometry and fourier transform infra-red (FT-IR) analyses, cellulose in oomycete cell walls occurs either in the form of IV_1 or low crystalline I_β form (Helbert et al., 1997). Like in plant cell walls, cellulose of oomycetes is organized in fibrils that are composed of a bundle of individual and very fine microfibrils (~5nm in width) (Helbert et al., 1997). It is suggested that these cable-like structures have the same scaffolding function in the cell wall of oomycetes as chitin has in the wall of true fungi. However, the mode of assembly of these microfibrils and other carbohydrate components (β-1,3-glucans in the amorphous matrix of the cell wall) remains unknown.

3.2. Enzymatic complexes responsible for cellulose formation

The enzyme complexes, also called terminal complexes (TCs), responsible for cellulose synthesis, are located in the plasma membrane associated with the ends of cellulose microfibrils and were first observed by freeze fracture electron microscopy, in the green algae *Oocystis apiculata* (Brown and Montezinos, 1976). The organization of TCs is categorized in two main types: In land plants and charophycean green algae, TCs are organized in hexagonal structures (rosettes) (Fig. 6A), whereas linear TCs, either organized as single or multiple rows, are found in prokaryotes, red algae, brown algae, ulvophycean green algae, slime molds and tunicates (Fig. 6B) (Tsekos, 1999). In land plants, it is suggested that each individual globule of a rosette is composed of six cellulose synthase catalytic subunits, enabling the simultaneous elongation of 36 glucan chains finally co-crystalling in microfibrils (Fig. 6A) (Delmer, 1999). In oomycetes, the freeze fracture technique has not (yet) been used to visualize the cellulose synthesizing

enzyme complexes. Therefore, it remains to be investigated whether the TCs are organized in rosettes (hexagonal) or in linear forms. However, based on the close phylogenetic relatedness of oomycetes to brown algae, it is tempting to speculate that oomycete TCs may be organized in single linear rows as it has been shown for some brown algae (Katsaros et al., 1996; Reiss et al., 1996). Even though many studies suggested that TCs are implicated in cellulose microfibril assembly, it was not until 1999, that Kimura et al. have demonstrated by immunogold labelling, that the rosette TCs of vascular plants are composed of a single catalytic unit called cellulose synthase (CesA). However, the actual number of active catalytic subunits in individual TCs is still not known.

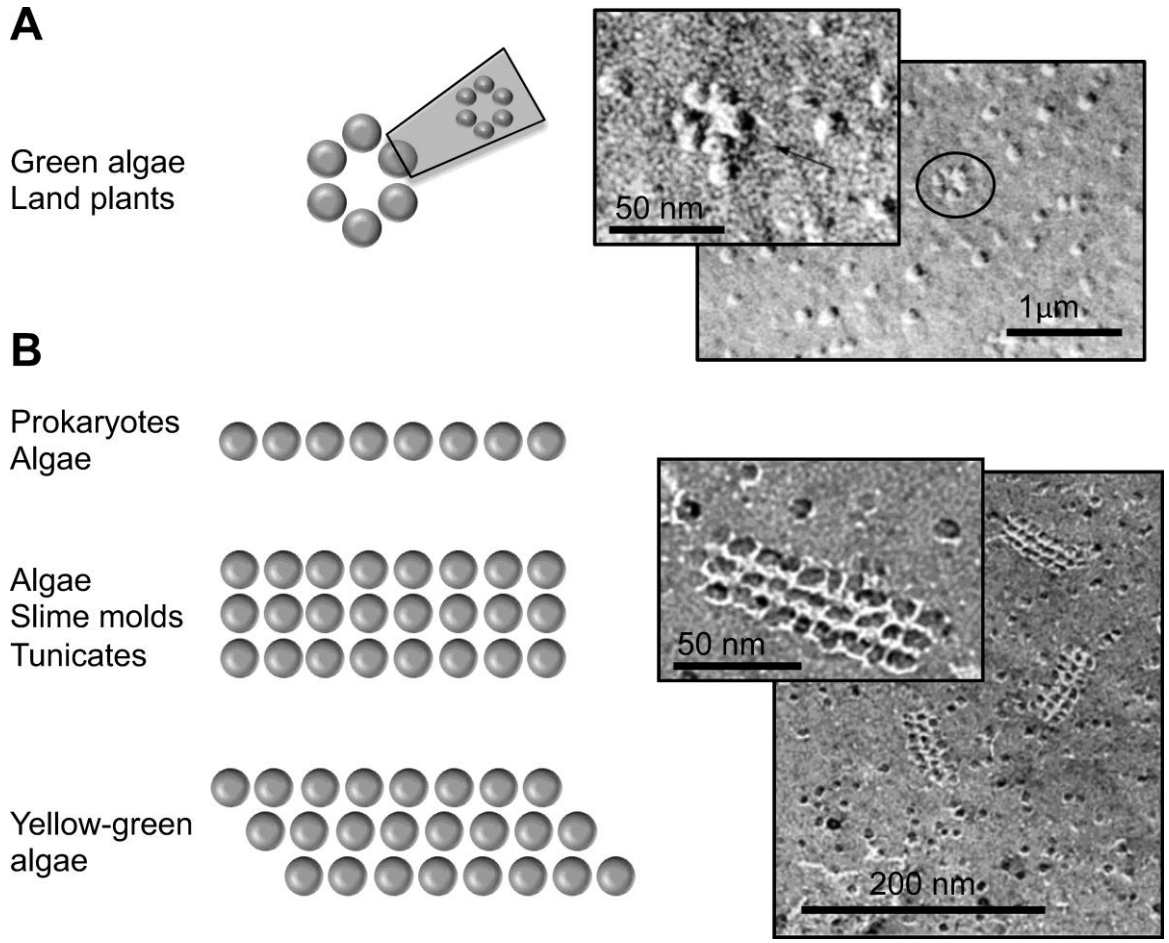

Fig. 6. Terminal complexes (TCs) originating from species of different kingdoms.
(**A**) The TCs of land plants and some green algae are organized as hexamers, also called rosettes. The image on the right shows the P-face of the plasma membrane including clusters of rosettes (circle) in *Gossypium hirsutum.* The insert displays the 6-fold symmetry of a single particle rosette developing in vitro. Adapted from Kimura et al., 1999. (**B**) TC organization in single linear or multiple linear rows as found mostly in bacteria, algae and animals. The image on the right shows the P-fracture face of the plasma membrane in the algae *Phaeothamnion confervicola*. TCs consist of three linear rows of particles as shown in the insert. Adapted from Okuda et al., 2004.

3.3. CesA proteins – the catalytic subunits for cellulose synthesis

CesA proteins are ancient enzymes (Nobles et al., 2001) and their encoding genes are represented in various organisms. The deduced amino acid sequences of CesA proteins from plants, bacteria, algae, slime molds, tunicates and oomycetes vary significantly in size (~650 aa to ~1300 aa, Fig. 7). However, they share some common features such as the four catalytic motifs containing the signature D, DxD, D, Q/RxxRW (Fig. 7) that is highly conserved in all known CesAs and in most processive glycosyltransferases (Saxena et al., 1995). The importance of these motifs for CesA enzyme activity was demonstrated by mutagenizing the conserved aspartic acid residues (D, DxD, D) as well as the Q, R, and W residues in the QxxRW motif from the bacterium *Gluconacetobacter xylinus* (Saxena and Brown, 1997; Saxena and Brown, 2001). All CesAs are characterized by putative transmembrane (TM) helixes that are suggested to play a role in anchoring the enzyme in the plasma membrane (Delmer et al., 1999). In plants, the CesAs are predicted to contain up to eight TM helices, two located near the N-terminus, and the other six near the C-terminal end (Fig. 7) (Somerville, 2006). Additional TM domains are found in eukaryotic and bacterial CesAs (Fig. 7). Many CesAs can be classified as multi-domain enzymes: Apart from the cellulose synthase domain, the N-terminal end of plant CesAs contains a specific zinc-finger (ZnF) domain (Fig. 7), which is characterized by four repeated CXXC motifs (Somerville, 2006). These motifs have been shown to play a relevant role for homo and hetero dimerization of cotton CesAs *in vitro* (Kurek et al., 2002). The CesAs of red algae display an additional glycogen-binding domain at the N-terminus (Fig. 7) (Matthews et al., 2010) and the tunicate CesAs contain a glycosylhydrolase domain at the C-terminal end (Fig. 7) (Nakashima et al., 2004). In oomycetes, a Pleckstrin homology (PH) domain was identified in CesA isoforms 1, 2 and 4 at the N-terminus. (Fig. 7) (Fugelstad et al., 2009; Grenville-Briggs et al., 2008). This domain seems to be specific for oomycete CesAs and has not been reported in other CesA enzymes. Since PH domains occur in a wide range of proteins involved in signal transduction or as constituents of the cytoskeleton with the ability to bind phosphatidylinositol lipids within biological membranes (Saraste and Hyvonen, 1995), it is suggested that PH domains in CesAs of oomycetes might facilitate the binding of the enzymes to the plasma membrane. This however remains to be demonstrated.

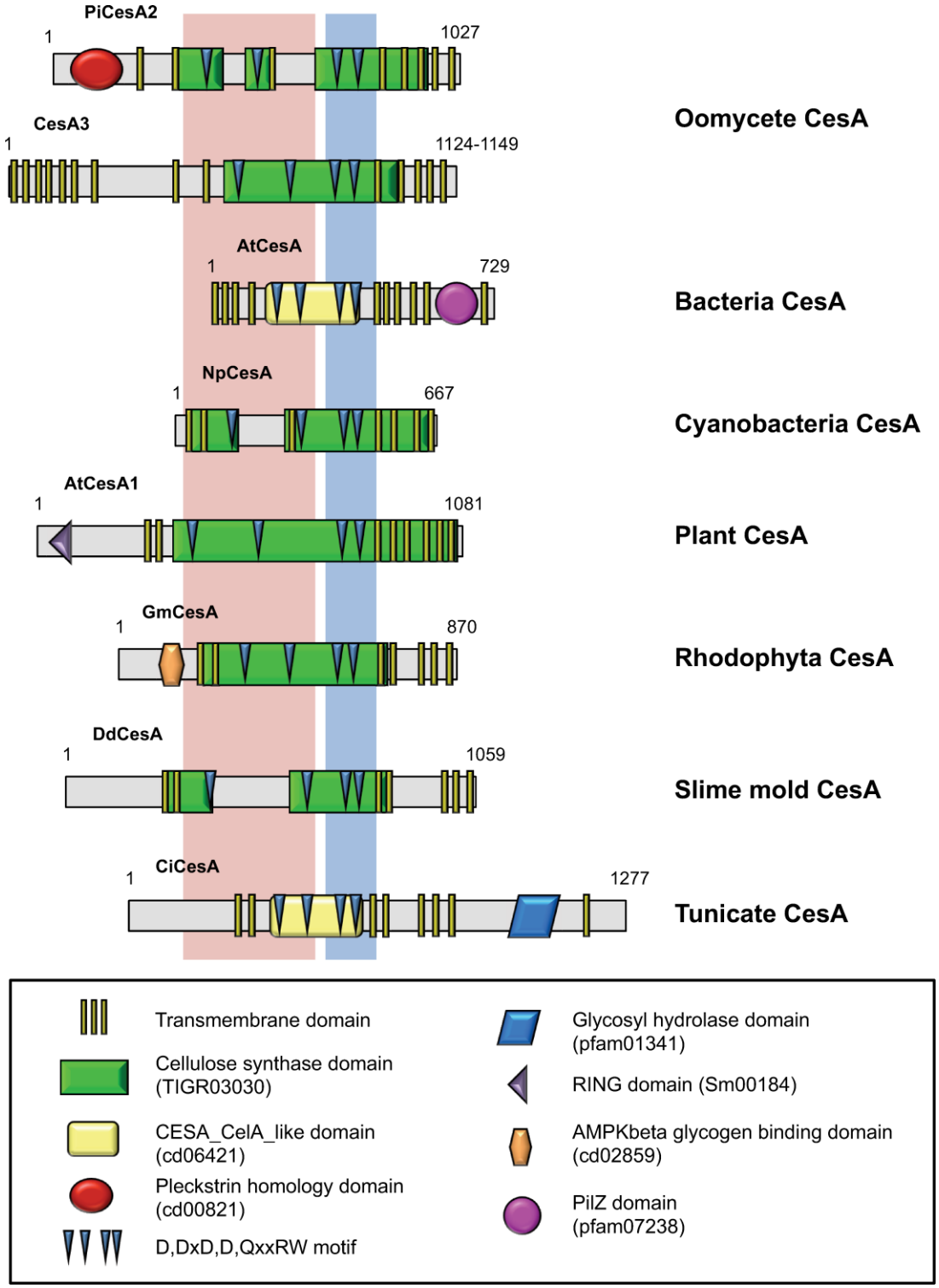

Fig. 7. Domain organization of the cellulose synthases (CesAs) of oomycetes, bacteria, plants, algae and animals. Total length of deduced amino acid sequences is given above each protein. Sequences were aligned according to the position of the QxxRW motif (triangle). The proteins contain regions typical for cellulose synthases and cellulose synthase like enzymes. Several transmembrane (TM) domains are also present. Motifs (D, DxD) present in processive and non-processive glycosyltransferases are highlighted in red. The motif (D, QxxRW) of processive glycosyltransferases is shaded in blue. Full names of the species are: *Phytophthora infestans* (Pi), *Agrobacterium tumefaciens* (At), *Nostoc punctiforme* (Np), *Arabidopsis thaliana* (At), *Griffithsia monilis* (GM), *Dictyostelium discoideum* (Dd) and *Ciona intestinalis* (Ci).

3.4. CesA genes encoding putative cellulose synthases

By complementation of a cellulose deficient mutant, the first gene encoding the putative catalytic subunit of a cellulose synthase (CesA) was identified in the bacterium *Gluconacetobacter xylinus* (Wong et al., 1990). Few years later, Matthysse et al. (1995) reported on the identification of *CesA* genes for cellulose biosynthesis in *Agrobacterium tumefaciens*. In plants, genes encoding cellulose synthases were first discovered in *Gossypium hirsutum* (Pear et al., 1996). These findings prompted many researchers in the plant cell wall community to identify genes that encode the machinery of cell wall polysaccharide synthesis. In 2000, already 1250 *CesA* and *CesA*-like sequences, from 29 different plant species were deposited in GenBank (Richmond and Somerville, 2000) and many additional *CesA* sequences have been deposited since then. *Arabidopsis thaliana* for example, has ten *CesA* genes, which serve distinct functions in primary and secondary cell wall syntheses (Fagard et al., 2000; Holland et al., 2000; Scheible et al., 2001; Taylor et al., 1999). Further plant *CesA* genes were isolated from maize (Appenzeller et al., 2004), rice (Vergara and Carpita, 2001), barley (Burton et al., 2004), poplar (Djerbi et al., 2005), and pine (Nairn and Haselkorn, 2005). Apart from higher plants, *CesAs* also exist in algae (Roberts and Roberts, 2004; Roberts and Roberts, 2009), slime molds (Blanton et al., 2000), tunicates (Matthysse et al., 2004; Sagane et al., 2010) and other eukaryotes. The number of *CesA* genes can vary between the different kingdoms and even between species of the same phylum.

Even though oomycetes are known to contain cellulose in the cell wall since a long time, the genes encoding the putative cellulose synthases, responsible for cellulose production, have not been identified till recently. Full genome sequencing of oomycetes species (Haas et al., 2009; Tyler et al., 2006) finally enabled the identification of four putative *CesA* genes. These four genes were functionally characterized by RNA interference showing their direct involvement in cellulose biosynthesis in *P. infestans* (Greenville-Briggs et al., 2008). Expression analysis of this gene family in *P. infestans* demonstrated that it plays a significant role in germination and appressoria formation, both processes that depend on a strong and intact cell wall (Grenville-Briggs et al., 2008). Further papers were published recently, describing the *CesA* genes in *Saprolegnia monoica* (Fugelstad et al., 2009) and *Pythium ultimum* (Levesque et al., 2010). At the present time, it seems that oomycetes contain up to 4 different *CesA* genes (CesA1 to 4). However, in some species of the *Saprolegniales* only three *CesAs* (*CesA2*, *CesA3* and

CesA4) were identified (Fugelstad et al., 2009). Recent genome analysis of *Saprolegnia parasitica* revealed even the occurrence of *CesA* paralogs. In *S. parasitica,* there are three paralogs of *CesA2* and one paralog of *CesA3*, whereas CesA4 seems to be present as a single copy gene (http://www.broadinstitute.org/annotation/genome/ Saprolegnia_parasitica /MultiHome.html). Nevertheless, the *CesA* gene doubling or tripling may be rather specific for *S. parasitica* since only four distinct copies were identified in the genome of *Phytophthora* or *Pythium* species (Haas et al., 2009; Levesque et al., 2010; Tyler et al., 2006).

3.5. Cellulose biosynthesis in higher plants

Most of our current knowledge about the molecular mechanisms of cellulose formation is based on studies with *Arabidopsis thaliana*. So far, there is only little information available about cellulose biosynthesis in other organisms. Also in oomycetes, the process of cellulose formation is poorly understood. In *A. thaliana* the terminal complexes (TCs) are organized as hexamers (rosettes) and each of the six subunits is suggested to be composed of six cellulose synthase catalytic subunits, enabling the elongation of 36 glucan chains (Fig. 8) (Delmer, 1999). Even though the definite composition of a rosette is still not fully clear, it has been demonstrated that three unique CesAs (AtCesA1, AtCesA3 and AtCesA6) are needed to form a functional complex for primary cell wall synthesis (Persson et al., 2007). The interaction (formation of homo- and heterodimers) of the individual CesAs was shown by bimolecular fluorescence complementation (BiFC), using CesAs with partial yellow fluorescent proteins (YFP) (Desprez et al., 2007). Similarly, AtCesA4, AtCesA7 and AtCesA8 seem to form a complex that is responsible for secondary cell wall synthesis (Taylor et al., 2003; Taylor et al., 2000). It is suggested that the zinc finger domain of plant CesAs plays an important role for the interaction between CesAs, since these domains were reported to be involved in homo- and hetero-dimerization of CesAs *in vitro* (Kurek et al., 2002).

Besides the CesA proteins that are directly involved in cellulose biosynthesis, there are additional proteins like KORRIGAN, KOBITO1, CSI1 and COBRA that have been claimed as playing a role for cellulose synthesis (Gu et al., 2010; Nicol et al., 1998; Pagant et al., 2002; Schindelman et al., 2001). KORRIGAN for example, is a membrane bound β-1,4 endoglucanase that might contribute directly to cellulose synthesis in *A. thaliana* by

mediating the transfer of glucose residues to a growing β-glucan chain (Fig. 8) (Sato et al., 2001).

Further, it has been shown in bean that the cellulose synthesis complex is associated with a sucrose synthase like-protein (Fujii et al., 2010). Sucrose synthases (SuSy) are known to catalyse the formation of UDP-glucose from sucrose whereas the former is the substrate for cellulose synthesis (Babb and Haigler, 2001). However, since *A. thaliana* mutants missing any functioning sucrose synthase showed normal cellulose formation (Barratt et al., 2009), the specific role of this enzyme for cellulose biosynthesis remains to be investigated.

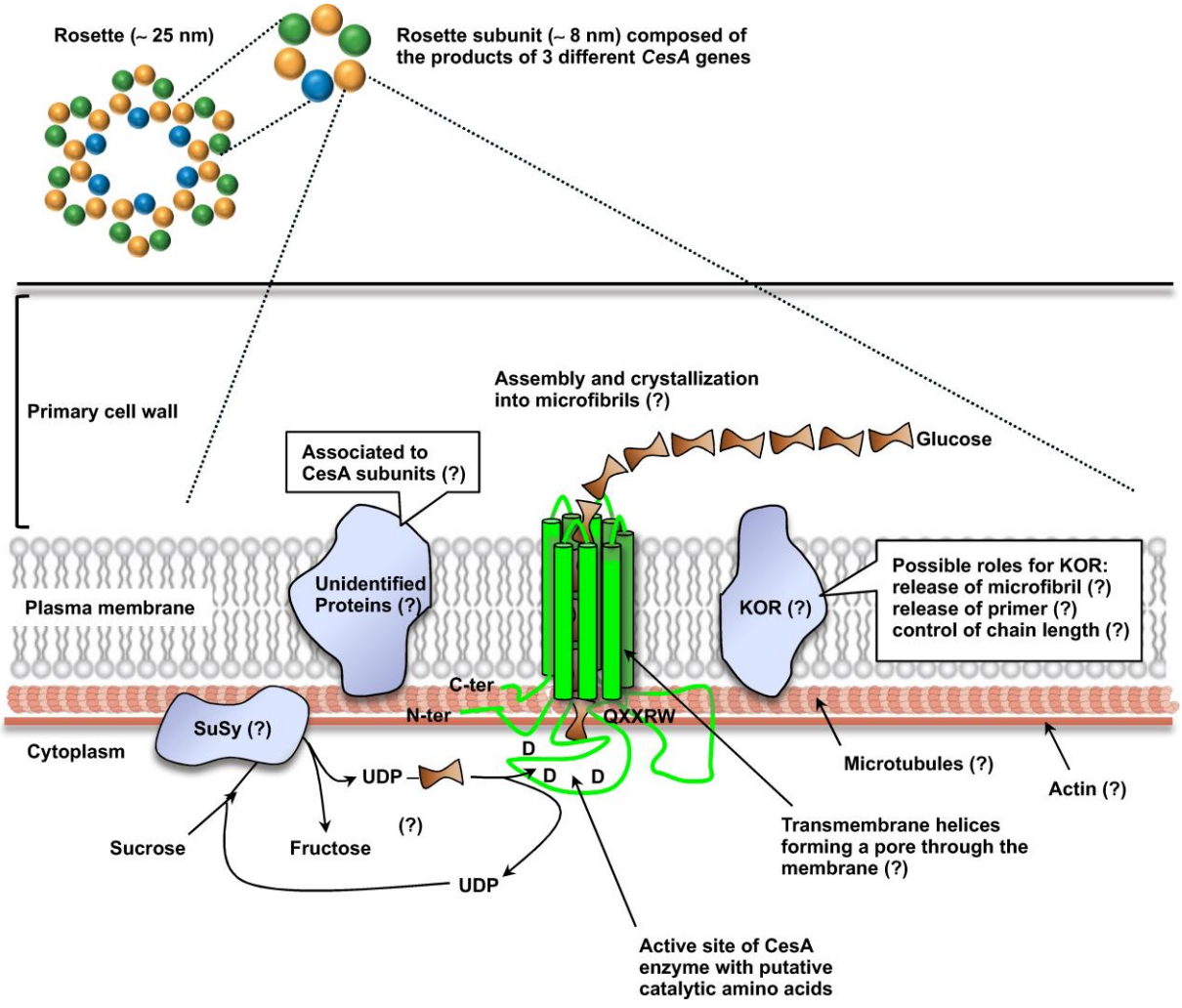

Fig. 8. Hypothetical model of cellulose biosynthesis in higher plants. In the top left corner, the diagram represents a rosette (top-view), with its hypothetical organization composed of 36 catalytic subunits. The CesA enzyme (green), representing one of the 6 catalytic subunits is characterized by 8 putative transmembrane helices. Its catalytic subunit including the D,DxD,D,QxxRW motif, which is assumed to be responsible for the binding and polymerization of UDP-glucose, faces the cytoplasmic side of the plasma membrane. In addition, cytoskeleton, KORRIGAN, Sucrose synthase (SuSy) and additional proteins that may also be involved in cellulose biosynthesis are indicated. Modified from Guerriero et al., 2010.

Since long time, the role of the cytoskeleton for cellulose biosynthesis is under debate. In 1974, Heath postulated that cellulose microfibrils are either directly or indirectly associated with cortical microtubules, thus imposing their own orientation on them (Heath, 1974). It was not until 2006, that Paredez et al. (2006) demonstrated by YFP labelling of cellulose synthases in *A. thaliana*, that the complexes responsible for cellulose formation move within the plasma membrane along linear tracks that are aligned with cortical microtubules (Fig. 8). This key finding provided evidence that cortical microtubules govern cellulose deposition by defining the rails of cellulose synthase complexes (CSCs). However, even though it appears that the microtubules influence the rotary movements of CesA trajectories, the linear movement of CSCs at the plasma membrane seems to be independent from microtubules, since taxol (microtubule stabilizing drug) treatment did not affect CSC movement (Chan et al., 2007). On the contrary, it is rather the polymerization of cellulose itself that propels the CSCs in the plasma membrane (Endler and Persson, 2011). But vice versa, cellulose synthesis activity has a direct influence on microtubule organization (Paredez et al., 2008). Treatment of *A. thaliana* cells with the cellulose inhibitor isoxaben that targets the CesA3 and CesA6 subunit (Scheible et al., 2001) disturbed microtubule organization (Paredez et al., 2008), indicating that there is a close connection between the cellulose synthesis machinery and microtubules. Also, the actin cytoskeleton plays a central role for cellulose synthesis by distributing the CesA complexes in the cell (Crowell et al., 2009; Gutierrez et al., 2009). It was demonstrated by Gutierrez et al. (2009) that disruption of the actin cytoskeleton caused aggregation and reduced motility of the CesA-containing Golgi bodies, thus leading to an uneven distribution of CSCs at the plasma membrane. In addition, it seems that a microtubule-associated protein, MAP20, also plays an important role, probably as intermediate protein between microtubules and the CSCs, since it has the ability to bind microtubules as well as the cellulose synthase inhibitor 2,6- dichlorobenzonitrile (DCB) *in vitro* (Rajangam et al., 2008). However, the interplay between MAP20 and microtubules remains to be investigated in more detail.

Cellulose synthesis largely contributes to maintain cell wall strength and growth in changing environments. Therefore, this process needs to be tightly regulated in order to quickly respond to changes during the cell cycle, but also to environmental influences. The rapid turnover of celluloses synthases was demonstrated for CesAs in cotton fibres

showing a half-life of only about 30 minutes (Jacob-Wilk et al., 2006). Several aspects may be involved in the regulation process, such as transcriptional regulation, phosphorylation as well as the red/far-red light photoreceptor PHYTOCHROME B (PHYB) (Bischoff et al., 2011; Chen et al., 2010; Zhong et al., 2008). However, information about the interplay of these mechanisms for cellulose synthesis regulation is still rather scarce and needs to be investigated in more detail.

3.6. Cellulose inhibitors and their molecular targets in plants

Cell wall synthesis including the formation of cellulose is a vital process for plants and some microorganisms. Therefore, these processes represent a promising target for the development of herbicides or anti-microbial drugs. In plants, there are several inhibitors that specifically act on cellulose biosynthesis either by directly targeting the catalytic subunit as competitive inhibitor or by indirect inhibition of other components involved in the biosynthetic pathway. Cellulose synthesis inhibitors (CBIs) that directly target the catalytic subunits of CesAs are for example isoxaben, flupoxam, CGA 325'615 or AE F150944, whereas 2,6-dichlorobenzonitril or morlin are considered to inhibit cellulose biosynthesis by a secondary effect.

Isoxaben that specifically inhibits cellulose biosynthesis in higher plants (Heim et al., 1990) targets the AtCesA3 and AtCesA6 subunit in *A. thaliana*. These findings were provided in isoxaben resistant mutants (*ixr1-1, ixr1-2 and ixr2-1*) that displayed mutations at the C-terminal end of AtCesA3 and AtCesA6 either located in a predicted transmembrane domain, or in between, in an extracellular loop (Desprez et al., 2002; Scheible et al., 2001). Since the mutated residues are far downstream from the catalytic site, it is suggested that isoxaben does not directly affect the catalytic activity of the enzyme but rather disturbs CesA complex formation. This is supported by YFP:CesA6 labelling studies showing a rapid loss of CesA6 labelled complexes out of the membrane upon isoxaben treatment, suggesting that isoxaben disrupts the formation of CesA complexes (Paredez et al., 2006).

Flupoxam, an annual broad-leaf herbicide, seems also to directly target CesA subunits of *A. thaliana*, since mutations in AtCesA3 and AtCesA1 were identified that could be linked to resistance against this compound (Robert et al., 2010).

Another CBI that interacts with the CesA subunits is CGA 325'615. This compound is thought to inhibit the formation of crystalline cellulose by disrupting the

CesA rosette architecture possibly by disturbing oxidative conditions that consequently prevent proper dimerization of CesAs (Kurek et al., 2002; Peng et al., 2001). A similar mechanism of cellulose synthesis inhibition was proposed for the compound AE F150944. Biochemical and microscopical analyses in *Zinnia elegans* led to the assumption that the compound may inhibit crystalline cellulose synthesis by destabilizing plasma membrane rosettes (Kiedaisch et al., 2003).

2,6-dichlorobenzonitrile (DCB) is known to specifically inhibit cellulose biosynthesis at micromolar concentrations (Sabba and Vaughn, 1999). However, since DCB does not inhibit the incorporation of UDP-glucose into cellulose nor binds to the catalytic subunits of CesAs (Delmer et al., 1987), it most likely acts in an indirect way. Indeed, Rajangam et al. (2008) identified a microtubule-associated protein called MAP20 that was shown to bind DCB *in vitro*. This protein is upregulated in secondary cell walls and is thought to be responsible for the assembly and stability of microtubules (Wasteneys and Yang, 2004). However, DCB did not affect MAP20 binding to microtubules, indicating that this protein might be associated to CesAs either in a direct or indirect form. Therefore, it is suggested that DCB might disrupt this interaction, consequently separating the CesA complexes from the cortical microtubule network (Rajangam et al., 2008).

The cumarin analogue morlin may inhibit cellulose biosynthesis in an indirect way. In *A. thaliana*, it was shown that morlin acts on the cortical microtubules and alters the movement and distribution of CESAs in the plasma membrane (DeBolt et al., 2007). Even though the direct target for morlin could not be identified, it is speculated that the compound may bind to a structural protein that interacts with both microtubules and the CESA complex (DeBolt et al., 2007).

In conclusion, there are several ways how CBIs interfere with the synthesis of cellulose. Since the CAAs are the first anti-oomycete compounds that inhibit cellulose biosynthesis, it is of major interest to make comparisons between the CBIs of plants and those used for oomycete control. Therefore, the knowledge gained from the mechanisms of action of CBIs used in plants that directly target one of the CesA subunits (e.g. Isoxaben) probably facilitates the understanding by which mechanism the CAA fungicides inhibit cellulose biosynthesis in oomycetes.

4. OUTLINE OF THE THESIS

Plant pathogenic oomycetes can cause huge losses in agriculture. To prevent such losses, disease control measurements are necessary, mainly based on chemical control. However, the development of resistance to anti-oomycete compounds in pathogen populations, can strongly affect disease control. Accurate knowledge about the mechanism conferring resistance is therefore a prerequisite for proper management strategies. Since the mode of action of the carboxylic acid amide (CAA) fungicides, that are frequently used for oomycete control, has been elucidated and shown to be directly linked with the inhibition of cellulose biosynthesis, this thesis aimed to identify the cellulose synthase (CesA) encoding genes of a range of oomycete species and to investigate the molecular mechanism of resistance and tolerance to this fungicide class.

Hence, as first step, the *CesA* genes were amplified and characterized in the oomycete species *Plasmopara viticola* and *Pseudoperonospora cubensis*, both downy mildew pathogens that developed resistance to mandipropamid (MPD) in the field. Molecular analyses were undertaken in both organisms for the identification of the resistance mechanism to MPD (Chapter I, II).

A second focus of this thesis was to investigate the tolerance mechanism of *Pythium* species to mandipropamid and other CAA fungicides. To achieve this task, the *CesA* genes of *Pythium aphanidermatum* were identified, sequenced and characterized by gene expression profiling and inhibitor studies using MPD (Chapter III).

As a third objective, a range of different oomycete species, originating from six distinct orders was tested against the fungicide MPD. By combining their fungicidal response with molecular data of the *CesA3* gene, new insights were obtained explaining why certain oomycetes are insensitive and cannot be controlled by CAA fungicides. In addition, based on the new molecular data (*CesA3* gene), the relatedness of species within the oomycete phylum was studied (Chapter IV).

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CHAPTER I

A single point mutation in the novel *PvCesA3* gene confers resistance to the carboxylic acid amide fungicide mandipropamid in *Plasmopara viticola*

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A single point mutation in the novel *PvCesA3* gene confers resistance to the carboxylic acid amide fungicide mandipropamid in *Plasmopara viticola*

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ABSTRACT

The grapevine downy mildew, *Plasmopara viticola*, is one of the most devastating pathogens in viticulture. Effective control is mainly based on fungicide treatments, although resistance development in this pathogen is reported for a number of fungicides. In this study we describe for the first time the molecular mechanism of resistance to a carboxylic acid amide (CAA) fungicide. We identified a family of four cellulose synthase (*CesA*) genes containing conserved domains that are found in all processive glycosyltransferases. Phylogenetic analysis revealed their close relationship to the cellulose synthases of *Phytophthora* sp. Sequencing of the *CesA* genes in a CAA-resistant and -sensitive field isolate revealed five single nucleotide polymorphisms (SNPs) affecting the amino acid structure of the proteins. SNP inheritance in F_1-, F_2- and F_3-progeny confirmed resistance to be correlated with one single SNP located in *PvCesA3*. Only if present in both alleles, this SNP led to the substitution of a glycine for a serine residue at position 1105 (G1105S) in the deduced amino acid sequence, thus conferring CAA- resistance. Our data demonstrate that the identified genes are putative cellulose synthases and that one recessive mutation in *PvCesA3* causes inheritable resistance to the CAA fungicide mandipropamid.

Keywords: *CesA* genes; Cellulose synthases; Fungicide resistance; Grapevine downy mildew; Inheritance; Single nucleotide polymorphism (SNP)

1. INTRODUCTION

The oomycete phylum, belonging to the kingdom Straminopila, is a large group of organisms composed of more than 800 different species that includes both saprophytes and pathogens of plants, insects, fish, nematodes and vertebrates (Phillips et al., 2008). Phytopathogenic members of the *Peronosporales* within oomycetes are among the most damaging pathogens of agricultural crops (Erwin and Ribeiro, 1996), including many *Phytophthora* species causing blights and root rots and downy mildews such as *Bremia*, *Peronospora*, *Pseudoperonospora and Plasmopara* species (Grenville-Briggs and van West, 2005). *Plasmopara viticola* (Berk. and Curt.) Berl. et de Toni, causal agent of grapevine downy mildew is one of the most devastating grapevine diseases, occurring in over 90 countries (Emmett et al., 1992). Under humid conditions, *P. viticola* releases zoospores, which are able to swim toward host plant stomata (Müller and Sleumer, 1934). When zoospores encounter a stoma, they shed their flagella and encyst. A cell wall is produced during encystment followed by the formation of a germ tube growing into the substomatal cavity, where it dilates into an infection vesicle (Kiefer et al., 2002). Unprotected grapevine tissue is infected within a short period of time, affecting leaves and young berries, leading to yield losses (Blaise and Dietrich, 1996).

To avoid yield losses control measures are required, mainly based on different cultural practices, warning systems, breeding for resistance and chemical control. The use of fungicides remains the most effective management tool against downy mildew of grapes (Gisi, 2002) although resistance has evolved to several classes of fungicides (http://www.frac.info/frac/index.htm). The development of fungicides with a novel mode of action is one of several strategies to overcome resistance problems. In 2005, a new fungicide, a mandelic acid amide derivative, mandipropamid (MPD), was launched for the effective control of oomycete foliar pathogens (Huggenberger et al., 2005). Mandipropamid belongs, together with benthiavalicarb, dimethomorph, flumorph, iprovalicarb and valifenalate, to the class of carboxylic acid amide (CAA) fungicides (FRAC, 2008; Gisi et al., 2007). Resistance in *P. viticola* field populations to CAAs has been reported in France and Germany for almost 10 years, with cross resistance shown between the representatives of this fungicide family (http://www.frac.info/frac/index.htm). Despite several attempts, neither the mode of action (MoA) nor the mode of resistance (MoR) of CAA fungicides has been identified so

far. Several studies proposed alterations of the cell wall structure (Cohen et al., 1995; Cohen and Gisi, 2007; Jende et al., 2002; Reuveni, 2003), changes in phospholipid biosynthesis (Griffiths et al., 2003), and disruption of F-actin (Zhu et al., 2007) as primary mode of action.

Microscopy tests with *P. viticola* indicated, that MPD has no effect on zoospore release from sporangia or zoospore motility, but is highly effective against cyst germination (Knauf-Beiter and Hermann, 2005). A recent study reported four genes to be transcriptionally upregulated during cyst germination and appressoria formation in the related oomycete plant pathogen *P. infestans* (Grenville-Briggs et al., 2008). These genes were shown to be cellulose synthase genes (*CesA*) responsible for cellulose synthesis. Silencing of the entire gene family with RNA interference led to malformation of germ tubes and appressoria-, indicating their involvement in pathogen development and cell wall solidity (Grenville-Briggs et al., 2008). In the cell wall of oomycetes, cellulose is associated with (1→3)-β-d-glucans and (1→6)-β-d-glucans (Bartnicki-Garcia, 1968). The fibrillar structure of cellulose (Helbert et al., 1997) is thought to provide strength and rigidity to the cell wall. Cellulose is found in the cell wall of all major groups of plants (Brown, 1996), the cellular slime mold *Dictyostelium discoideum* (Blanton et al., 2000), tunicates (Kimura and Itoh, 1995), and some prokaryotes (Ross et al., 1991).

The effect of MPD on cellulose biosynthesis has been extensively studied in the related pathogen *P. infestans*: ^{14}C glucose incorporation into cellulose was perturbed in presence of MPD indicating cellulose synthesis to be the main target of MPD (Blum et al., 2010). For many fungicides, resistance is based on single nucleotide polymorphisms (SNPs) in the respective target gene (target-site mutations, point mutations). This has been described for Quinone outside Inhibitors (QoIs), Demethylation Inhibitors (DMIs) and Benzimidazoles (MBCs) (Brasseur et al., 1996; Broomfield and Hargreaves, 1992; Koenraadt et al., 1992). Inheritance of resistance to the CAA fungicide MPD, in the diploid organism *P. viticola*, was reported to be recessive (Blum and Gisi, 2008; Gisi et al., 2007) and was associated with one or two nuclear genes (Gisi et al., 2007). A first attempt to identify mutations in *PvCesA1* conferring resistance to CAAs did not result in any change correlated to resistance (Dubius et al., 2008).

The goals of the present study were the identification of *CesA* genes encoding putative cellulose synthases and the discovery of potential SNPs in these genes

conferring resistance to CAA fungicides in *P. viticola*. We demonstrated that the four identified gene products contain, like the CesA proteins of *Phytophthora* sp., multiple transmembrane domains and conserved motifs also found in processive glycosyltransferases (Saxena et al., 1995). Based on the comparison of the *PvCesA* genes in MPD resistant and sensitive isolates, SNPs leading to amino acid changes in the PvCesA proteins were identified. We then followed by crossing studies the inheritance of these SNPs in F_1-, F_2- and F_3-progeny. Comparison of inherited SNPs with the resistant phenotype revealed one SNP in *PvCesA3* to be linked with MPD resistance. These findings finally enabled us to elucidate the mechanism of resistance to the CAA fungicide MPD in *P. viticola.*

2. MATERIALS AND METHODS

2.1. Isolates and culture conditions

Field isolates of *P. viticola* were collected in 2007 from grape leaves from 15 different sites across European countries (France, 6; Germany, 7; Italy, 4; Portugal, 4; Switzerland, 5; and Spain, 4). Single-sporangiophore isolates were produced from these bulk samples by picking sporangia from a single-sporangiophore using a fine needle. Parental isolates (D05 and CH05.2), F_1- and F_2-progeny isolates were selected from the strain collection derived from previous experiments (Gisi et al., 2007) (details in Supplementary Table 1). Isolate D5, collected in 2001 in Germany, was a P1 mating-type isolate sensitive to MPD, whereas CH05.2, collected in Switzerland, was P2 resistant to MPD. All isolates were propagated and maintained on detached leaves of glasshouse-grown *Vitis vinifera* cv. Gutedel plants.

2.2. Sensitivity assay

To test the sensitivity of F_3-progeny and of field isolates to the fungicide mandipropamid, a sensitivity assay on grape leaf disks was used as described by Gisi et al. (2007). Fungicide dilutions (from a 10,000 mg active ingredient L^{-1} stock solution dissolved in DMSO) were made to give final concentrations of 100, 10, 1, etc. down to 0.01 mg active ingredient L^{-1}. After fungicide application (with Tecan spray equipment, Genesis) the disks were spray-inoculated with a sporangial suspension (50,000 sporangia mL^{-1}). Disease assessment was done after a 6 day incubation period by visual estimation of the percentage leaf disk area infected compared to the untreated control.

For data analysis *agstat* program (Syngenta internal *software*) was used to determine the EC_{50} values.

2.3. Crossing of P. viticola isolates and progeny analyses

The method for crossing *P. viticola* was described in detail by Gisi et al. (2007). For generation of F_3-progeny, two F_2-isolates with defined mating type and sensitivity to mandipropamid (J4, mating type P1 and sensitive to MPD and J26, mating type P2 and resistant to MPD) were crossed according to the protocol cited above with minor modifications. Eight week old leaf disks containing oospores, produced by the crosses, were homogenized in cold distilled water. The homogenate was mixed with perlite in a six well-plate. During an incubation period of 7–10 days at 19 °C, oospores were able to germinate by formation of a macrosporangium releasing zoospores into the substrate, which consequently produced infections in the superimposed leaves. First sporulating spots were observed alongside the veins of the upper leaf surface. Out of those spots, sporangia from one sporangiophore were isolated and transferred to a droplet of water (5 µL) which was placed on young grape leaves. Newly generated single-sporangiophore isolates were cultured and tested for sensitivity to mandipropamid as described in the previous section.

2.4. Bioinformatics

CesA gene orthologs in *P. viticola* were identified using CODEHOP-primers which consist of 11–12 completely degenerate core nucleotides at the 3'-end and a consensus 18–25 nucleotide clamp sequence at the 5'-end. *CesA* specific sequences were designed using the CODEHOP strategy (Rose et al., 1998) based on the multiply-aligned *Phytophthora* CesA sequences recently published (Grenville-Briggs et al., 2008): ABP96902, ABP96906, ABP96910 for CesA1; ABP96903, ABP96907, ABP96911 for CesA2; ABP96904, ABP96908, ABP96912 for CesA3; and ABP96905, ABP96909, APB96913 for CesA4. The multiple alignment was generated using ClustalW (Thompson et al., 1994). Two conserved motifs QXXRW and MSXXX were selected for primer design using the default parameters of the CODEHOP server (http://blocks.fhcrc.org/)

2.5. DNA and RNA extraction

Samples for DNA or RNA extraction were obtained by collecting sporangia from infected grape leaves. Samples were immediately frozen in liquid nitrogen and stored at

−20 °C until further use. DNA extraction was done according to the modified CTAB protocol of Riethmüller et al. (2002). Total RNA was extracted using SV Total RNA Isolation System (Promega), following the manufacturer's protocol. Integrity of the RNA was tested by agarose gel electrophoresis. First-strand cDNA was synthesized from 100 to 300 ng total RNA by oligo(dT) priming using Omniscript RT Kit (Quiagen) following the manufacturer's protocol.

2.6. PCR manipulations

All PCR amplifications were performed in a TGradient Thermocycler (Biometra, Göttingen, Germany); primers were obtained from Microsynth (Balgach, Switzerland). For the CODEHOP approach, PCR reactions were performed on 50 ng genomic DNA in a total volume of 50 µL containing 2 U of AmpliTaq Gold polymerase (Applied Biosystems), 0.2 mM dNTP and 0.5 µM primers in the polymerase manufacturer's buffer. *CesA* specific CODEHOP primers are listed in Table 1. The PCR program was designed according to the CODEHOP server's tips with minor modifications as follows: first 4 min initial denaturation at 94 °C, then 34 cycles of 30 s at 94 °C, 30 s at 63 °C, 45 s at 72 °C and finally a 5 min extension step.

Table 1
CODEHOP primers used for CesA amplification in *P. viticola.*

Primer	Sequence	Protein consensus	Fragment size (bp)	Target gene
Sense				
Pcode1f	[a]G.GTG.GCT.GGA.GCC.ATG.GCG.cag.cgh.aar.cgt.tg[b]	SVAGAMAQRKRW	694	*PvCesA1*
Pcode2f	G.GTG.GCT.GGC.TCG.TTG.GCT.car.cgt.aar.cgy.tg	SVAGSLAQRKRW	694	*PvCesA2*
Pcode3f	G.GTC.GCT.GCT.GCC.ATG.GGT.cag.aag.aar.cgt.tg	TVAAAMGQKKRW	679	*PvCesA3*
Pcode4f	T.GTG.GCT.GCT.GCT.CTG.GCG.car.cgt.aar.cgt.tg	SVAAALAQRKRW	691	*PvCesA4*
Antisense				
Pcode1r	GCT.CCA.GCC.GAA.GTA.CTC.ctg.rat.rct.ca	MSIQEYFGWS	694	*PvCesA1*
Pcode2r	GCT.CCA.GCC.AAA.GTA.CTC.ctg.gat.gct.ca	MSIQEYFGWS	694	*PvCesA2*
Pcode3r	GTC.CAA.GCC.ACA.GTA.CTC.ygt.gat.act.ca	MSITEYCGWD	679	*PvCesA3*
Pcode4r	GCT.CCA.GCC.GAA.GTA.CGT.ctg.cav.rct.ca	MSLQTYFGWS	691	*PvCesA4*

[a] For each primer the consensus clamp (XXX.XXX) is given in upper case.
[b] Degenerate core (xxx.xxx) is given in lower case. y = [C, T], r = [G, A], h = [A, T, C], v = [G, A, C].

Flanking regions of amplified *PvCesA* gene fragments were obtained by Genome walking using DNA Walking SpeedUp kit (Seegene), following manufacturer's instructions. PCR amplifications of the complete open reading frames (ORF) of the four

putative cellulose synthase genes were performed on 50 ng genomic DNA using PCR SuperMix High Fidelity (Invitrogen) and a final primer concentration of 0.2 µM. Sequences of primer Pcesa1f, Pcesa1r used for amplification of *PvCesA1*; Pcesa2f, Pcesa2r for *PvCesA2*; Pcesa3f, Pcesa3r for *PvCesA3*; and Pcesa4f, Pcesa4r for *PvCesA4* are available in Supplementary Table 2.

Verification of intron/exon structure in *PvCesA3* and *PvCesA4* was done using gene specific primers Pcesa3cDNAf, Pcesa3cDNAr, Pcesa4cDNAf and Pcesa4cDNAr (see Supplementary Table 2). Prior to cloning, PCR products were analyzed on 1% agarose gels; fragments of expected size were purified using the NucleoSpin Extract II kit (Macherey–Nagel).

PCR reactions for pyrosequencing were done in a final volume of 50 µL containing 20 ng of extracted gDNA, 1.25 U of GoTaq DNA polymerase (Promega), 0.2 mM dNTP and 0.2 µM each of the forward and reverse primers (one of which biotinylated) in the polymerase manufacturer's buffer. PCR involved denaturation at 94 °C for 4 min, followed by 45 cycles of 30 s at 94 °C, 30 s at 50 °C and 20 s at 72 °C, followed by a final extension at 72 °C for 7 min.

2.7. Cloning, characterization and analysis of the PvCesA genes

Putative *CesA* fragments obtained after CODEHOP and Genomic walking PCR reactions were generally less than 1000 bp in size and were cloned into pCR4-TOPO vector (Invitrogen). Complete *CesA* ORFs, up to 4000 bp in size, were cloned into a pCR-XL-TOPO vector (Invitrogen). Following heat shock transformation of *Escherichia coli* TOPO10 (Invitrogen) cells with the ligation mixture, direct PCR on clones was performed using Taq Polymerase (Invitrogen). PCR products were purified with Sephacryl S-300 (GE Healthcare) before sequencing.

Sequencing reactions were done with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to manufacturer's instructions using M13 or sequence specific primers (Supplementary Table 2). Sequence analysis was performed on a 3130 Genetic Analyzer (Applied Biosystems) and further processed using Lasergene software (DNASTAR, Inc.).

In order to identify correct clones, a BLASTN search was carried out in the NCBI database (http://www.ncbi.nlm.nih.gov/). Prediction of complete *CesA* ORFs was done using the NCBI ORF finder program (http://www.ncbi.nlm.nih.gov/projects/gorf/).

Verification of *PvCesA3* and *PvCesA4* intron/exon structure was performed by comparison of genomic DNA and its corresponding cDNA after reverse transcription. The predicted amino acid sequences were analyzed using the NCBI Conserved Domain Database (http://www.ncbi.nih.gov/Structure/cdd/wrpsb.cgi) (Marchler-Bauer and Bryant, 2004). Prediction of transmembrane domains was done using SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosoui/) (Hirokawa et al., 1998).

2.8. SNP and SSR analysis

To test the genetic uniformity of progeny isolates and to support the success of sexual recombination, two microsatellite (SSR, simple sequence repeat) loci, ISA and CES, each with three alleles (Supplementary Table 1) were used. SSR analysis was performed as described in detail elsewhere (Gobbin et al., 2003).

CesA gene sequences from parental isolate CH05.2 and D05 were aligned using MegAlign (DNASTAR, Inc.) for single nucleotide polymorphism (SNP) identification. Inheritance of identified SNPs leading to amino acid changes was verified by pyrosequencing technology. Specific primers for each SNP (*cesa1–9*, *cesa1–58*, *cesa2–256*, *cesa2–553*, and *cesa3–1105*) were designed (Supplementary Table 2) using PSQ assay design software (Biotage). Pyrosequencing was performed on a PyroMark ID system (Biotage) following manufacturer's instructions; it can be found in detail elsewhere (Arnold et al., 2005).

2.9. Biallelic transcription analysis of the SNP (coding for G1105S) in PvCesA3

Total RNA was extracted from F_1-progeny isolates and reversely transcribed as described in the "DNA and RNA extraction" section. The cDNA was subsequently used for PCR amplification of the *PvCesA3* fragment using gene specific primers Pcesa3cDNAf and Pcesa3-1105r (Supplementary Table 2). All PCR products were purified, sequenced using primer Pcesa3seqf4 and analyzed as described in Section 2.7. Quantification of biallelic transcripts was done by pyrosequencing using Biotage's allele quantification software. Amplification of cDNA was performed as described for the SNP analysis in the previous section.

2.10. Phylogeny

All cellulose synthase sequences used for phylogenetic analysis were obtained from the protein databases at the National Center for Biotechnology Information (NCBI)

(http://www.ncbi.nlm.nih.gov/) (Supplementary Table 3). Alignments were made with ClustalW (Thompson et al., 1994) and phylogenetic dendrograms were constructed using MEGA 4 (http://www.megasoftware.net/) (Tamura et al., 2007), with the minimum evolution algorithms using 1000 bootstrap replications.

3. RESULTS

3.1. Molecular characterization of the PvCesA genes

A preliminary BLASTN search to the NCBI nucleotide database revealed the cloned fragments, resulting from the CODEHOP reactions, to be putative *CesA* (encoding cellulose synthases) genes. Extension of the amplified PCR products (Table 1) by genomic walking resulted in the full-length nucleotide sequence of the putative *CesA* genes of *P. viticola.* Sequences were deposited under GenBank Accession No. GQ258973 (*PvCesA1*), GQ258974 (*PvCesA2*), GQ258975 (*PvCesA3*) and GQ258976 (*PvCesA4*). Overall, sequence identity of *Pv*CesA gene products ranged from 34% to 70% with PvCesA3 to be the most divergent member of the family (Table 2). Comparison of *PvCesA* genes with orthologous *P. infestans CesA* genes, recently published by Grenville-Briggs et al. (2008), showed that they share a greater identity with each other than with their paralogs: For example, *PvCesA3* and *PiCesA3* share 83% identity at the DNA and up to 95% at the amino acid level (Table 2).

Table 2
Amino acid sequence identities of P. viticola (Pv) and P. infestans (Pi) CesA proteins.

Gene	*Plasmopara PvCesA* Sequences				*Phytophthora PiCesA* Sequences			
	PvCesA1	*PvCesA2*	*PvCesA3*	*PvCesA4*	*PiCesA1*	*PiCesA2*	*PiCesA3*	*PiCesA4*
%								
PvCesA1	100[a]	–	–	–	89[b] (81)[c]	71	34	50
PvCesA2	70	100	–	–	71	94 (84)[b]	39	55
PvCesA3	34	38	100	–	35	37	95 (83)	37
PvCesA4	50	54	37	100	52	53	36	92 (81)

[a] Identities are expressed as percentage, based on pairwise protein alignment (for nucleotide calculations the complete open reading frames were used).
[b] Underlined values indicate orthologous *Plasmopara* and *Phytophthora* sequences.
[c] Nucleotide Sequence identity of *CesA* orthologs is given in parenthesis.

Generally, CesA proteins contain a conserved set of motifs (D,D,D,QXXRW) (Fig. 1) that are located in the central globular region of the protein (Saxena and Brown, 1997; Saxena et al., 1995). Domain A contains two conserved Asp residues (D [motif A1],

D [motif A2]), domain B consists of a third conserved Asp residue (D [motif B1]) and three conserved amino acids (QXXRW [motif B2]). Domain A is common to both processive and non-processive enzymes and binds the UDP sugar (Saxena and Brown, 2000), whereas domain B forms the acceptor binding region and completes the single transfer center by provision of the catalytic base (Charnock et al., 2001). The four *CesA* genes of *P. viticola* described in this study are two domain proteins, containing the A and B domains in their predicted amino acid sequences (Fig. 1), suggesting their ability to function as processive glycosyltransferases.

All proteins belonging to the cellulose synthase superfamily are integral membrane proteins with one or more transmembrane domains at their N-terminal end, followed by a large cytoplasmic globular region containing the domain B motifs, and several transmembrane segments at the C-terminal end (Saxena and Brown, 2000). Structural predictions of all four *P. viticola* CesA proteins were performed using SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosoui/) and supported the hypothesis that these proteins are integral membrane proteins containing several transmembrane segments (Fig. 2A).

3.2. Domain structure and classification of cellulose synthases in P. viticola

Conserved domain searches were performed using all four *P. viticola* CesA proteins. Comparison of domains from *Pv*CesA amino acid sequences with *Phytophthora* CesA protein domains showed that the structure among orthologs is very similar (Fig. 2A). Like in all *Phytophthora* CesA proteins, the C-terminal end of each amino acid sequence exhibited similarity to cellulose synthase domains found in plants and bacteria. The N-terminal end of each sequence was more divergent. Either there was similarity to the COG125 glycosyltransferase domain or no predicted structural similarity at all (Fig. 2A). Like other CesA sequences derived from *Peronosporales*, *Pv*CesA1, *Pv*CesA2 and *Pv*CesA4 contained sequence similarity to a Pleckstrin homology (PH) domain toward the N-terminus of the protein. PH domains occur in a wide range of proteins involved in intracellular signalling or as constituents of the cytoskeleton and were not reported for any of the cellulose synthases outside of the *Peronosporales* (Grenville-Briggs et al., 2008). The plant cellulose synthase specific zinc finger (or LIM-like) CxxC motif, that has been suggested to be involved in protein–protein interactions

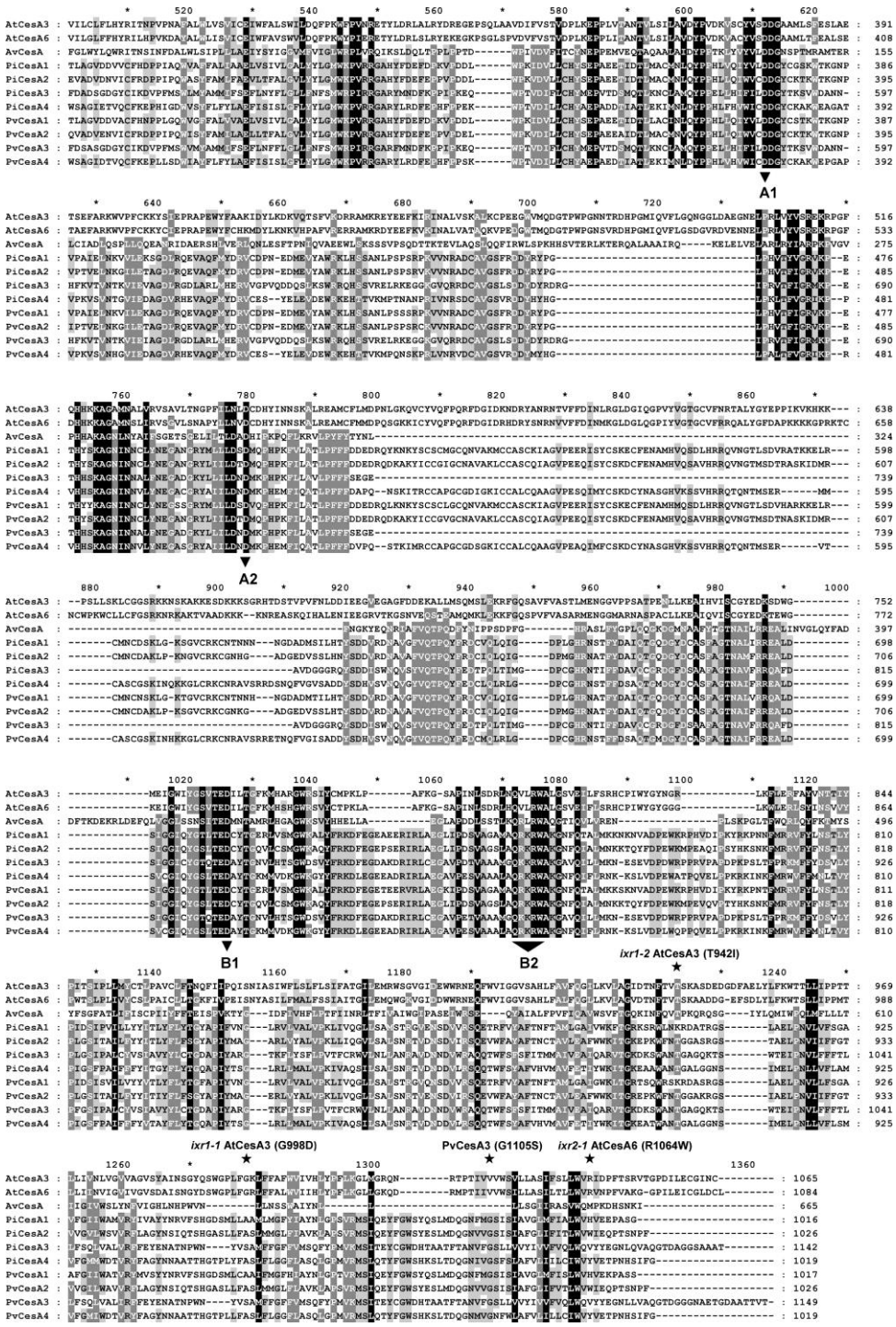

Fig. 1. Comparison of identified CesA sequences of *Plasmopara viticola* with other members of the cellulose synthase superfamily (obtained from the NCBI database). Sequence alignment was made by ClustalW including the deduced amino acid sequences of CesAs from *P. viticola*, CesAs from *Phytophthora infestans*, cellulose synthases from *Arabidopsis thaliana* (*At*CesA3, *At*CesA6) and *Anabaena variabilis* (*Av*CesA). The non-homologous N-terminal regions are not shown. Black, dark-gray and light-gray shading of residues correspond to 100%, 80% and 60% overall similarity between the 11 sequences aligned. Numbers indicate the position of the amino acid residues of each protein. The three Asp (D) residues (A1, A2, and B1) and the QXXRW motif (B2) that are critical for the function of CesAs are marked with triangles. Mutations causing isoxaben resistance in *A. thaliana* and the mutation causing mandipropamid resistance in *P. viticola* are indicated by asterisks above the corresponding residue.

(Delmer, 1999; Kurek et al., 2002), was absent in all of the four *Plasmopara* CesA proteins. Minor differences between *Pv*CesA and *Pi*CesA proteins were found in the number and structure of transmembrane domains (Fig. 2A).

Phylogenetic analysis of *Plasmopara* CesA gene products with published cellulose synthases of various kingdoms revealed that they group as a distinct clade together with the cellulose synthases of *Phytophthora species* and *Saprolegnia monoica*, recently published by Fugelstad et al. (2009). The oomycete clade is split into four subclades, each representing one of the four *CesA* genes that have been identified in oomycetes (Fig. 3).

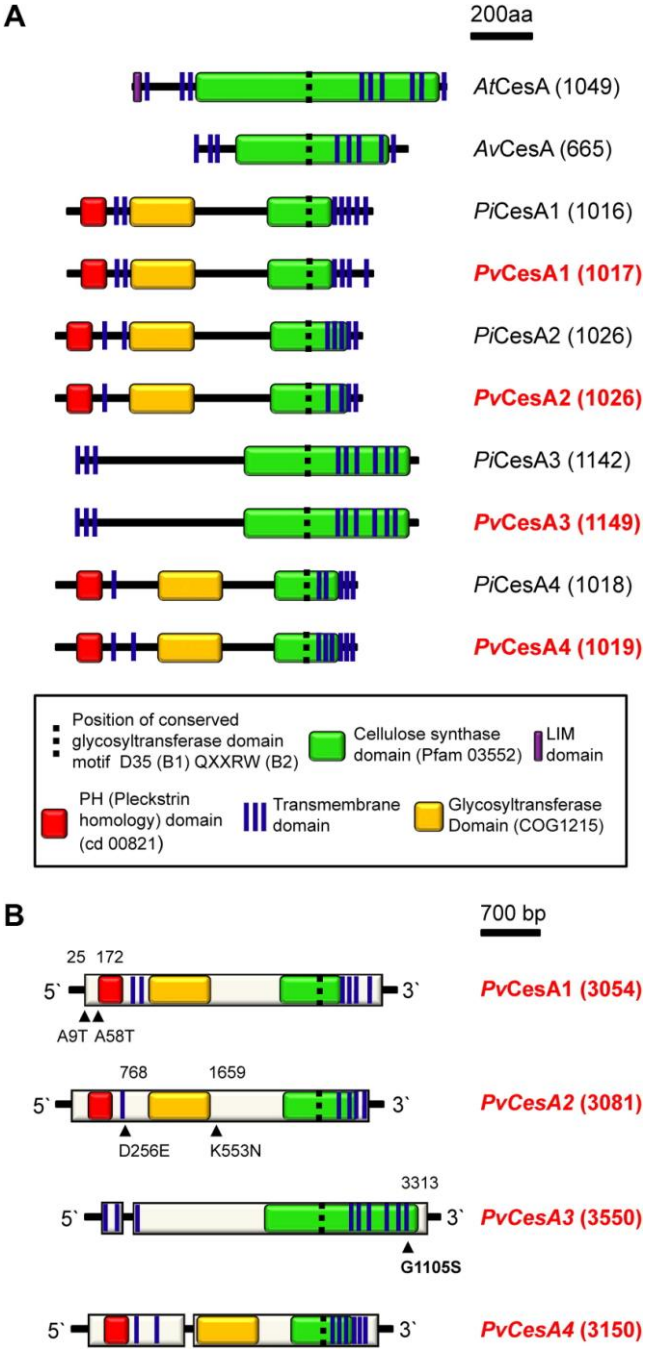

Fig. 2. Sequence analyses of the *PvCesA* genes. (**A**) Domain organization of the cellulose synthases (CesAs). Schematic diagrams were adapted from Grenville-Briggs et al. (2008) and represent domain structures of *P. viticola (Pv)* CesA proteins. Total amino acids (aa) are given in parenthesis after the name of each protein. Sequences are aligned according to the position of the domain B containing the QXXRW (B2) motif (dotted line). The proteins contain regions similar to cellulose synthases and to other glycosyltransferases. Several trans-membrane domains are also predicted. The domain organizations of CesAs from *P. infestans* (*Pi*), *A. thaliana* (*At*) and *A. variabilis* (*Av*) were taken from Grenville-Briggs et al. (2008) and are given for comparison. (**B**) Intron-/exon-structure of *PvCesA* genes. Total length in base pairs (bp), including introns, are given in parentheses after the name of each gene. Nucleotide position of mutations in *PvCesA* genes is given as number. Amino acid changes and position in the peptide sequence is marked by a triangle below each gene.

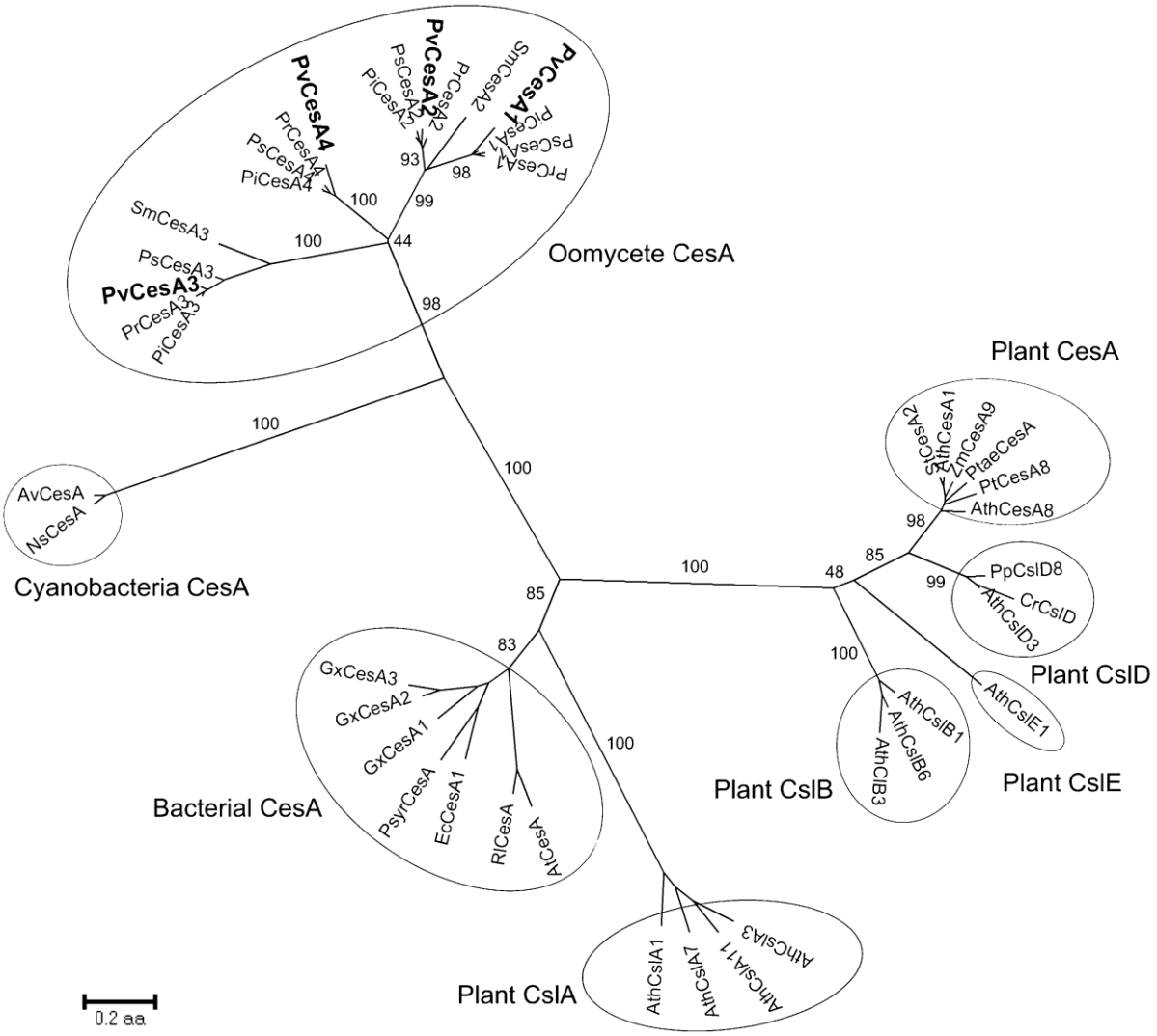

Fig. 3. Unrooted Phylogram of cellulose synthases derived from oomycete sequences and several sequences form Eukaryotes and Prokaryotes. Alignments were made using ClustalW. The phylogram was produced using Mega 4 with a minimum evolution algorithm and 1000 bootstrap values. The analysis is based on the complete amino acid (aa) sequence of cellulose synthases (CesA) and cellulose synthase like (CslA-E) gene products (Supplementary Table 2 for Accession number of each sequence). Scale bar indicates amino acid changes. Bootstrap values are indicated along the relevant branches. Full species names are: Pi, *Phytophthora infestans*; Pr, *Phytophthora ramorum*; Ps, *Phytophthora sojae*; Pv, *Plasmopara viticola*; Sm, *Saprolegnia monoica*; Ath, *Arabidopsis thaliana*; Cr, *Ceratopteris richardii*; Pb, *Physcomitrella patens*; Ptae, *Pinus taeda*; Pt, *Populus tremula*; St, *Solanum tuberosum*; Zm, *Zea mays*; At, *Agrobacterium tumefaciens*; Ec, *Escherichia coli*; Gx, *Gluconacetobacter xylinus*; Ns, *Nostoc sp*; Av, *Anabaena variabilis*; Rl, *Rhizobium leguminosarum*; Psyr, *Pseudomonas syringae*.

3.3. Polymorphisms in CesA gene sequences of P. viticola

The coding region of the four *PvCesA* genes was analyzed and compared on genomic DNA in two isolates (CH05.2, MPD resistant and D05, MPD sensitive); both used for crossing experiments in the study of Gisi et al. (2007). Comparison of all four *CesA* gene sequences in those two isolates revealed 100 single nucleotide polymorphisms

(SNPs) on the genomic level; five of them affected the amino acid sequence of the *Pv*CesA proteins (Table 3). The two isolates showed that their *CesA* sequences differed at about 12 nucleotides per kilobase (kb; nucleotide diversity = 0.012). Nucleotide substitutions from C to T (65%) and A to G (17%), representing transitions, were the most frequent whereas G to T changes were the least frequent (3%) (Table 3).

Table 3
Number and distribution of SNPs in the coding sequence of *CesA* genes in field isolates CH05.2 and D05[a] of *P. viticola.*

Gene	SNP (A/C)	SNP (A/G)	SNP (A/T)	SNP (C/G)	SNP (C/T)	SNP (G/T)	Total SNPs
PvCesA1	1	4 (2)[b]	0	1	14	0	20
PvCesA2	1	6	1	4 (1)	20	1 (1)	33
PvCesA3	1	6 (1)	3	4	22	2	38
PvCesA4	0	1	0	0	8	0	9
Total	3	17	4	9	64	3	100

[a] Allelic differences within an isolate are neglected.
[b] SNPs affecting the amino acid structure of the protein are given in parentheses.

Out of the 20 SNPs in *PvCesA1*, two SNPs affected the amino acid sequence leading to a substitution from GCG to ACG encoding an Ala residue in isolate D05 and a Thr residue in isolate CH05.2 respectively. The first SNP was located in codon 9, the second in codon 58. Both mutations were situated in the N-terminal sequence of the CesA1 protein next to the predicted PH domain (Fig. 2B). In *PvCesA2* two SNPs were detected leading to an amino acid exchange form Asp (isolate D05) to Glu (isolate CH05.2) (codon 256, GAT substituted by GAG) and from Lys (isolate D05) to Asn (isolate CH05.2) (codon 553, AAG substituted by AAC). The encoded amino acid was either located next to the predicted PH (Pleckstrin homology domain) (codon 256), or close to a predicted conserved glycosyltransferase domain (codon 553) (Fig. 2B). The SNP in *PvCesA3* was located in codon 1105 (GGC) encoding Gly in isolate D05; it was substituted to AGC encoding Ser in isolate CH05.2. The translated amino acid of codon 1105 was located in the last of the 9 predicted transmembrane domains spanning the cellulose synthase domain (Pfam 03552) (Fig. 2B).

3.4. Identification and inheritance of mutations in PvCesA3 and other PvCesA genes

To verify if resistance to MPD is correlated with one or more of the five identified amino acid changes located in PvCesA1, PvCesA2 and PvCesA3, inheritance of their

corresponding SNPs over three generations was investigated. Parental isolates (CH05.2, D05), some samples of F_1- and F_2-progeny isolates produced in the study of Gisi et al. (2007), as well as F_3-progeny isolates originating from this study were included (see Supplementary Table 1). The two microsatellite markers ISA and CES were used to discriminate F_1- and F_2-progeny isolates from each other (showing the same SNP constellation) as well as from their parents (data shown in Supplementary Table 1). Crossing of a resistant isolate with a sensitive isolate was shown to result in a sensitive F_1-progeny (Blum and Gisi, 2008). Molecular characterization of F_1-progeny isolates resulting from the cross D05 × CH05, revealed each of the five SNPs identified in *PvCesA* genes to be heterozygous (Table 4).

Crossing of sensitive F_1-progeny isolates led to segregation of resistance in F_2-progeny. None of them displayed intermediate resistance, assuming that resistance to mandipropamid is linked with one or two recessive alleles. SNP pattern of several F_2-progeny isolates was analyzed by pyrosequencing. The SNP in *PvCesA3* located in codon 1105, encoding the amino acid glycine (GGC) or serine (AGC), showed perfect correlation with the resistant phenotype (Table 4). Only F_2-progeny isolates with substitutions in both codons (=homozygous) displayed resistance (EC_{50} > 100 mg L^{-1}). All resistant F_2-progeny isolates had the amino acid substitution Gly1105 to Ser1105 (G1105S). None of the resistant isolates was heterozygous, concerning the SNP in *PvCesA3* (Table 4), suggesting the mutation causing nucleotide substitution Guanine to Adenine in codon 1105 to be recessive.

Analysis of the SNP in codon 1105 of *PvCesA3* at the cDNA level showed that both alleles were transcribed (i.e. biallelic expression) in heterozygous F_1-progeny isolates (Supplementary Fig. 1). The presence of both alleles in an equal ratio (Supplementary Fig. 1) suggests that no allele specific discrimination occurred during transcription.

Other SNPs located in *PvCesA1* and *PvCesA2* did not show any correlation with resistance. Isolate J12 and K20, both sensitive to MPD, displayed exactly the same SNP constellation like the resistant isolate CH05.2 The only difference was found in *PvCesA3* at codon 1105. Whereas isolate J12 displayed the codons for Gly1105 and Ser1105, isolate K20 only showed the codon for Gly1105 (Table 4). This strongly supports, that only the mutation (G1105S) in *Pv*CesA3 has an influence on mandipropamid sensitivity.

A further crossing of a sensitive F_2-progeny isolate (J4) with a resistant F_2-progeny isolate (J26), both with defined SNP pattern (Table 4), was implemented to

Table 4

Amino acid substitutions in CesA gene products of 41 *P. viticola* single sporangiophore isolates and correlation to MPD sensitivity

Generation	Isolate	PvCesA1		PvCesA1		PvCesA2		PvCesA2		PvCesA3		Sensitivity[a]
		Ala9[b]	Thr9	Ala58	Thr58	Asp246	Glu246	Lys553	Asn553	Gly1105	*Ser1105*[f]	
P	**D05**[c]	[GCG][d]		[GCG]		[GAT]		[AAA]		[GGC]		s
P	**CH05.2**		[AGC]		[ACG]		[GAG]		[AAC]		*[AGC]*	**r**
F_1	**II8**, **II10**, **II25**, **II26**, **II29**	[(G/A)CG)][e]		[(G/A)CG]		[GA(T/G)]		[AA(A/C)]		[(G/A)GC]		s
F_2	G1, G18, J2, **J4**, J6, J10, J19, K4, K25	[(G/A)CG)]		[(G/A)CG]		[GA(T/G)]		[AA(A/C)]		[(G/A)GC]		s
F_2	J13	[GCG]		[GCG]		[GAT]		[AAA]		[GGC]		s
F_2	J20, J22, K10, K16	[GCG]		[GCG]		[GAT]		[AAA]		[(G/A)GC]		s
F_2	J12		[AGC]		[ACG]		[GAG]		[AAC]	[(G/A)GC]		s
F_2	K20		[AGC]		[ACG]		[GAG]		[AAC]	[GGC]		s
F_2	J17, K23, K27	[(G/A)CG)]		[(G/A)CG]		[GA(T/G)]		[AA(A/C)]		[GGC]		s
F_2	G20, J7, J18, **J26**	[(G/A)CG)]		[(G/A)CG]		[GA(T/G)]		[AA(A/C)]			*[AGC]*	**r**
F_2	K29	[GCG]		[GCG]		[GAT]		[AAA]			*[AGC]*	**r**
F_3	JJ1	[GCG]		[GCG]		[GAT]		[AAA]		[(G/A)GC]		s
F_3	JJ4, JJ7, JJ9, JJ10	[(G/A)CG)]		[(G/A)CG]		[GA(T/G)]		[AA(A/C)]		[(G/A)GC]		s
F_3	JJ3		[AGC]		[ACG]		[GAG]		[AAC]	[(G/A)GC]		s
F_3	JJ2, JJ5, JJ8	[(G/A)CG)]		[(G/A)CG]		[GA(T/G)]		[AA(A/C)]			*[AGC]*	**r**
F_3	JJ6		[AGC]		[ACG]		[GAG]		[AAC]		*[AGC]*	**r**

[a]Sensitivity: s = EC_{50} < 3.5 mg L^{-1}; r = EC_{50} > 100 mg L^{-1}

[b]Numbers represent amino acid position in corresponding CesA peptide sequence

[c]Isolates used for crossing experiments are written in bold (cross II = D05 x CH05.2; cross J = II8 x II29; cross G = II8 x II26; cross K = II10 x II 25; cross JJ = J4 x J26)

[d]Codons for amino acid residues are in parentheses

[e]Heterozygous codons representing both alleles are in double parentheses

[f]The amino acid correlating with resistance to MPD is written in italic

confirm the current findings. Out of this crossing, 10 F_3-isolates could be generated, four of them resistant and six sensitive to MPD (for EC_{50} values see Supplementary Table 1). Resistant isolates showed Ser1105 instead of Gly1105 in the gene product of *PvCesA3*, whereas sensitive isolates displayed glycine and serine (encoded by [G(A)GC]) at position 1105. Due to heterozygosity of parental isolate J4 for the SNP in *PvCesA3*, F_3-progeny isolates were either heterozygous and MPD sensitive or homozygous and MPD resistant. As shown for isolate JJ3, SNPs located in *PvCesA1* and *PvCesA2* had no influence on MPD sensitivity (Table 4).

3.5. Occurrence of G1105S in field populations of P. viticola

Out of several *P. viticola* field isolates that were collected during the year 2007 within the framework of the Syngenta sensitivity monitoring, 30 isolates, originating from six European countries, were selected for further investigation of the mutation (G1105S in the *Pv*CesA3 gene product), that was suggested to cause MPD resistance. Single-sporangiophore isolates were raised and analyzed. Sequencing of the 21 sensitive (EC_{50} < 2 mg L^{-1}) isolates revealed Gly1105 encoded by codon (GGC) or Gly1105 and Ser1105 encoded by [(G/A)GC], whereas the nine resistant isolates (EC_{50} > 100 mg L^{-1}) showed Ser1105 encoded by codon (AGC) (Table 5.) Despite the small field isolate data set, all resistant isolates investigated so far displayed an amino acid substitution at position 1105 in the gene product of *PvCesA3* indicating a strong correlation between this amino acid exchange and the resistant phenotype.

4. DISCUSSION

In the present study, we identified four *PvCesA* genes encoding putative cellulose synthases in *P. viticola*. By comparing the *CesA* gene sequences in MPD resistant and sensitive isolates, we have investigated the mechanism of resistance to the CAA fungicide mandipropamid (MPD). Inheritance studies of identified SNPs affecting the amino acid sequence of the PvCesA proteins showed that resistance to MPD co-segregated with a recessive mutation in *PvCesA3*. Our results strongly suggest that this mutation causing a glycine to serine substitution at position 1105 (G1105S) in the deduced amino acid sequence, confers resistance to MPD in *P. viticola.*

Table 5
Single sporangiophore field isolates from 2007 sorted according to decreasing sensitivity (increasing EC_{50})

Isolate	Country	Region	Year	Amino acid at position 1105 in *Pv*CesA3	EC_{50} (mg L^{-1})
E06	Spain	Pontevedra	2007	Gly [GGC][b]	0.13[a]
E20	Spain	Pontevedra	2007	Gly [GGC]	0.16
CH02	Switzerland	Zürich	2007	Gly [GGC]	0.25
E03	Spain	Pontevedra	2007	Gly [GGC]	0.37
P12	Portugal	Braga	2007	Gly [GGC]	0.45
F01	France	Loire-Atlantiqe	2007	Gly [GGC]	0.46
CH06	Switzerland	Vaud	2007	Gly / Ser [(G/A)GC]	0.47
D11	Germany	Rheinland-Pfalz	2007	Gly [GGC]	0.53
D14	Germany	Rheinland-Pfalz	2007	Gly [GGC]	0.54
D02	Germany	Baden-Württemberg	2007	Gly [GGC]	0.58
IT15	Italy	Toscana	2007	Gly [GGC]	0.61
IT07	Italy	Veneto	2007	Gly [GGC]	0.64
P14	Portugal	Braga	2007	Gly [GGC]	0.65
D09	Germany	Baden-Württemberg	2007	Gly [GGC]	0.71
P17	Portugal	Torres Vedras	2007	Gly [GGC]	0.74
F62	France	Haut-Rhin	2007	Gly / Ser [(G/A)GC]	0.81
CH08	Switzerland	Vaud	2007	Gly [GGC]	0.88
F29	France	Gironde	2007	Gly [GGC]	0.92
E16	Spain	Pontevedra	2007	Gly [GGC]	1.02
P02	Portugal	Alijó	2007	Gly [GGC]	1.04
F39	France	Marne	2007	Gly [GGC]	1.23
D01	Germany	Rheinland-Pfalz	2007	Ser [AGC]	>100
D5	Germany	Baden-Württemberg	2007	Ser [AGC]	>100
D10	Germany	Baden-Württemberg	2007	Ser [AGC]	>100
CH04	Switzerland	Aargau	2007	Ser [AGC]	>100
CH07	Switzerland	Vaud	2007	Ser [AGC]	>100
F21	France	Champagne-Ardenne	2007	Ser [AGC]	>100
F56	France	Loire-Atlantique	2007	Ser [AGC]	>100
IT12	Italy	Trentino-Alto Adige	2007	Ser [AGC]	>100
IT13	Italy	Trentino-Alto Adige	2007	Ser [AGC]	>100

[a]Sensitivity values derived from leaf disc assays.
[b]Codon is given in parenthesis.

4.1. PvCesA genes encode putative cellulose synthases

In oomycetes, cellulose is the fibrillar component of cell walls, it is always associated with other polysaccharides, such as β-1,3 – and β-1–6 glucans (Helbert et al., 1997). In *P. infestans*, cellulose synthesis was reported to be essential for host infection (Grenville-Briggs et al., 2008). Four *CesA* genes, representing a novel class of cellulose synthases, transcriptionally upregulated during cyst germination and appressoria formation, were identified using the published genome sequence of *P. infestans* (Grenville-Briggs et al., 2008).

In this study, we used the CODEHOP strategy (Rose et al., 1998) to identify *CesA* orthologs in *P. viticola*. The *Plasmopara* genome seems to contain four *CesA* genes. The existence of additional *CesA* genes is rather unlikely because only four distinct *CesA* genes are present in the genome of the closely related species *P. infestans*, *P. ramorum* and *P. sojae* (Grenville-Briggs et al., 2008). Comparison with orthologous *P. infestans CesA* sequences revealed a higher percentage of identity than between paralogs of *P. viticola*. This suggests that the *CesA* genes already diverged from each other in a common ancestor of the Peronosporales.

All *Pv*CesA gene products contain the D,D,D,QXXRW motif, that has been proposed to define the nucleotide sugar-binding domain and the catalytic site of these enzymes necessary to act as processive glycosyltransferases in plants (Charnock et al., 2001). As plant CesAs and Csls, putative membrane proteins, the *P. viticola* proteins are characterized by four to six transmembrane domains in the carboxyl terminal region of the protein and one to three transmembrane domains in the amino terminal region.

The presence of a PH (Pleckstrin homology) domain in cellulose synthases was reported to be specific to oomycetes (Grenville-Briggs et al., 2008). In *P. viticola*, a PH domain exists in PvCesA1, PvCesA2 and PvCesA4, located at the N-terminal region of the protein (Fig. 2A). PH domains occur in a wide range of proteins involved in signal transduction or as constituents of the cytoskeleton with the ability to bind phosphatidylinositol lipids within biological membranes (Saraste and Hyvonen, 1995). Although their function in oomycete CesA proteins remains unclear, we suggest that PH domains recruit the CesA enzymes to the plasma membrane, where they are situated as shown by immunolocalization (Grenville-Briggs et al., 2008).

In oomycetes, very little is known about cellulose biosynthesis and the interaction of the involved enzymes. Transient silencing of the four *CesA* genes in *P. infestans*, using dsRNA, affected the cellulose content in cell walls, indicating their relevance for cellulose biosynthesis (Grenville-Briggs et al., 2008). Expression studies of *CesA* genes in the life cycle of *P. infestans* showed that *PiCesA3* is the most highly upregulated gene of this family during mycelium growth, cyst germination and appressoria formation (Grenville-Briggs et al., 2008). Although simultaneous but only partial silencing of the whole *PiCesA* gene family was successful, it is still unclear if *PiCesA3* plays a superior role in cellulose synthesis and if redundancy between different *PiCesAs* exists.

In *P. viticola* we have no direct evidence that *PvCesAs* encode gene products involved in cellulose synthesis. However, their high percentage of identity to *PiCesAs* is consistent with this hypothesis. Although we did not focus on gene expression, we can assume that the function of *PvCesA3* and its ortholog *PiCesA3* is the same. Their gene products show up to 95% identity, and domain organization as well as number and position of transmembrane domains are very similar.

In our phylogenetic analysis the oomycete CesA proteins grouped together in a distinct clade, separate from either bacterial, cyanobacterial or plant cellulose synthases. Based on our phylogenetic tree, the CesA gene products of oomycetes show the closest relationship to cyanobacterial cellulose synthases (Fig. 3). This finding is consistent with the results obtained by Grenville-Briggs et al. (2008) and Fugelstad et al. (2009). In *P. viticola* and other members of the oomycetes it remains to be explored how CesA proteins interact together during cellulose biosynthesis. Cyanobacterial cellulose synthases are proposed to assemble in junctional pore complexes (JPs), forming a ring around the filament (Nobles et al., 2001), whereas in plants, terminal rosette complexes (TCs) were shown to be a prerequisite for cellulose biosynthesis (Kurek et al., 2002).

4.2. Molecular mechanism conferring MPD resistance

Based on high genotypic diversity, large populations and its combined reproduction systems (both sexual and asexual), *P. viticola* is suggested to have a high evolutionary potential (Gobbin et al., 2006) leading to fast environmental adaption. Therefore, this pathogen is considered to be a high risk pathogen in terms of likelihood of resistance evolution (http://www.frac.info/frac/index.html). Thus, it is not surprising, that resistance in *P. viticola* field populations evolved in France shortly after the introduction of dimethomorph, one of the first CAA fungicides introduced to the market. Generally, resistance to fungicides is based mainly on changes in the target enzyme (affecting affinity to the inhibitor), but also altered influx/efflux balances were reported (Sierotzki and Gisi, 2003). Although the mechanism of resistance is not necessarily associated with the primary site and biochemical mode of action of a compound, it can provide evidence to determine the site of action. In this study, we describe for the first time the molecular mechanism of resistance to CAA fungicides. Based on our findings, there is strong evidence that resistance to MPD is caused by a recessive mutation in the *PvCesA3* gene that is probably involved in cellulose

biosynthesis. Due to the perfect correlation of the identified homozygous mutation in *PvCesA3* with the resistant phenotype, we suggest that the putative target for CAAs is cell wall assembly as proposed by several authors (Cohen et al., 1995; Cohen and Gisi, 2007; Jende et al., 2002; Reuveni, 2003), rather than lipid biosynthesis (Griffiths et al., 2003) or disruption of F-actin (Zhu et al., 2007). Our assumption is supported by a current study within *P. infestans* in which a combination of biochemical and genetic techniques was used to elucidate the mode of action of the CAA fungicide mandipropamid: Generation of artificial mutants by ethyl methanesulfonate (EMS) mutagenesis and characterization of *PiCesA* genes, revealed mutations at position 1105 in PiCesA3 affecting sensitivity to MPD. Transformation of a sensitive isolate with the mutated allele and subsequent overexpression led to resistant individuals indicating cellulose synthesis to be the main target of MPD (Blum et al., 2010). In combination with the reverse genetic approach in *P. infestans*, the G1105 mutation that naturally occurs in MPD resistant *P. viticola* field isolates indicates that resistance to MPD is linked to a mutation in the target site.

In *Arabidopsis thaliana*, inheritance of resistance to the herbicide isoxaben, a cellulose biosynthesis inhibitor (Heim et al., 1990), was extensively studied. Crossing of an isoxaben resistant with a sensitive plant (both homozygous) led to F_1-progeny with reduced sensitivity to isoxaben (Heim et al., 1989) indicating a semi dominant nature of isoxaben resistance. More than a decade later, mutations in *AtCesA3* and *AtCesA6*, encoding cellulose synthases, were identified conferring a high degree of isoxaben resistance (Desprez et al., 2002; Scheible et al., 2001). Remarkable similarity exists between the mutations conferring isoxaben resistance in *A. thaliana* and the mutation conferring MPD resistance in *P. viticola*. All mutations occur in a highly conserved region of the enzyme near to the carboxyl terminus that is well separated from the proposed active site. The *ixr-1-1* mutation in *A. thaliana* leads to replacement of a conserved glycine at position 998 in the AtCesA3 protein (Scheible et al., 2001), whereas in *P. viticola*, the mutation leads to replacement of a glycine with a serine residue at position 1105. Glycine at this position is conserved among all CesA3 sequences that were identified in *Peronosporales* (Supplementary Fig. 2). In *A. thaliana*, two of the mutated residues are located near to the center (*ixr1-1*) and at the C-terminal end (*ixr2-1*) of a predicted transmembrane helix, whereas another is located in a short extracellular loop that connects two proposed transmembrane helices (*ixr1-2*) (Delmer, 1999; Desprez et

al., 2002; Scheible et al., 2001). The mutated residue in *P. viticola* is also located near to the center of a predicted transmembrane helix (Supplementary Fig. 2). ClustalW sequence alignment of AtCesA3 and AtCesA6 with PvCesA3 indicates that the mutations causing isoxaben resistance (*ixr-1-1*, *ixr-2-1* and *ixr-1-2*) are separated less than 100 amino acids from the G1105S mutation (Fig. 1). In *A. thaliana*, it has been proposed that these regions, being in or around predicted transmembrane helices, are critical for the formation of a proposed pore (Delmer, 1999) secreting glucan chains, and that isoxaben may bind to these regions and disrupt pore formation. Conceivably, mutations that prevent herbicide binding might allow proper pore formation in the presence of the herbicide (Scheible et al., 2001).

In oomycetes much less is known about cellulose biosynthesis and suggestions for a comprehensive three-dimensional structure of the cellulose synthase complex are lacking. Therefore, the consequences of the G1105S mutation in the PvCesA3 enzyme remain speculative. The exchange of a glycine residue with the slightly bigger serine residue is favoured in membrane proteins, generally without affecting enzyme functions (Betts and Russell, 2003). Because the mutated amino acid is far downstream the proposed active site residues (D,D,D,QXXRW), it is unlikely that MPD directly interferes with the catalytic site. The following suggestions can be given for explaining the mechanism of resistance: (i) the amino acid exchange might fully prevent fungicide binding and subsequent pore disruption as proposed for isoxaben in *A. thaliana*; (ii) conformation changes caused by serine might block MPD on the way from outside of the enzyme to the binding site; (iii) the amino acid substitution might alter the conformation of the binding site of CesA3, which in turn might reduce the affinity to MPD.

4.3. Inheritance of G1105S and correlation with MPD resistance

Fungicide resistance is genetically determined and its inheritance in *P. viticola* has been proposed to be either cytoplasmatic (e.g. for QoIs) or nuclear (e.g. for Phenylamides or CAAs) (Blum and Gisi, 2008; and Gisi et al., 2007). Due to the diploid nature of *P. viticola*, mutations in the coding sequence of genes are not necessary linked with alterations in the phenotype. Thus, mutations can be dominant, semidominant or recessive. The latter has been proposed for the inheritance of CAA resistance and its respective gene(s) in *P. viticola* (Gisi et al., 2007). In this study, we were able to link the recessive nature of CAA resistance with a recessive mutation in the *PvCesA3* gene. To our

knowledge this is the first report that describes inheritance of a phenotypic trait linked to a recessive SNP in an oomycete pathogen.

In the current work, we sequenced all four *CesA* genes of the parental isolates used previously by Gisi et al. (2007). Comparison of the *CesA* gene sequences revealed 100 single nucleotide polymorphisms (SNPs) resulting in a nucleotide diversity of 0.012 per kilobase. Making a projection of this nucleotide diversity on the entire genome, it would be nearly twice as big in *P. viticola* as in *A. thaliana* (Nordborg et al., 2005; Schmid et al., 2005). Of the five SNPs, leading to amino acid changes, only the recessive mutation G1105S in PvCesA3 correlated with resistance (Table 4); recessiveness of the mutation could be followed in F_1-, F_2- and F_3-progeny (Fig. 4). Although F_1-isolates displayed both alleles, on genomic DNA level (GGC and AGC, coding for Gly1105 and Ser1105 respectively) (Table 4), their phenotype to MPD was sensitive (EC_{50} < 3.5 mg L^{-1}). Therefore, we assumed transcription of the SNP in heterozygous isolates to be monoallelic, encoding Gly1105 only. Surprisingly, in heterozygous F_1-isolates both alleles of codon 1105 in *PvCesA3* were transcribed (i.e. biallelic expression) (Supplementary Fig. 1). At the current stage it remains unknown if the processed PvCesA3 enzyme is composed of translated sequences from both transcripts, or if the transcript coding for S1105 is discriminated at a later step in enzyme processing. Resistance reappeared in a few F_2- and F_3-progeny isolates (Fig. 4). Indeed, only homozygous F_2- and F_3-progeny isolates, displaying codon AGC in both alleles (coding for S1105), showed the resistant phenotype (Fig. 4).

It is rather unlikely that a second gene is involved in MPD resistance. The reason for the distorted segregation pattern (*r*:*s* = 1:9, rather than 1:3 as expected for one recessive gene) observed in the previous study (Gisi et al., 2007), may be due to the small number of offspring isolates, or the emergence of clonal lines during mating process or progeny generation. In general, we cannot exclude the involvement of additional genes participating in MPD resistance. To fully exclude additional genes, one should investigate segregation of resistance in several crosses with different parental isolates. However, all resistant field-isolates tested, revealed the presence of the G1105S mutation in PvCesA3. In addition, no MPD resistant field isolate has been detected without showing the described mutation in PvCesA3. Therefore, it is rather unlikely that mutations in other genes may also affect MPD sensitivity.

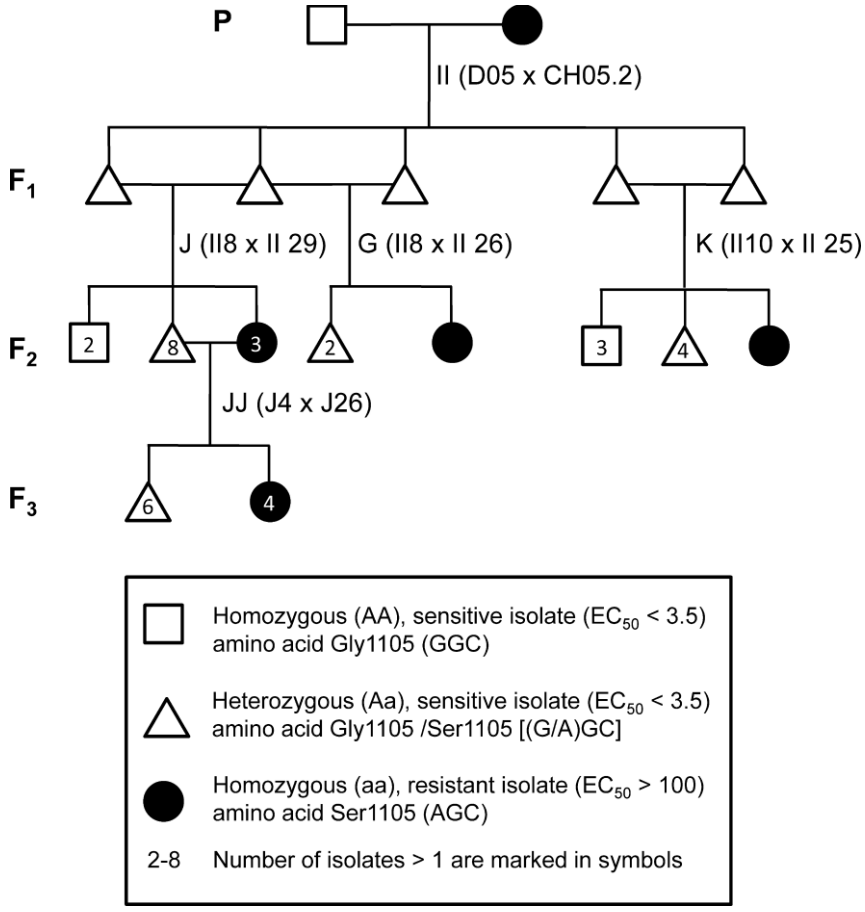

Fig. 4. Cosegregation of the G1105S mutation and MPD sensitivity (*s*/*r*) in crosses of *P. viticola.* The pedigree including 41 isolates reveals the genetic link between MPD resistance and presence of the G1105S mutation. Inheritance of the mutation follows a recessive scheme. Homozygous isolates displaying G1105, marked as empty squares are sensitive (EC_{50} < 3.5 mg L^{-1}). Heterozygous isolates marked as empty triangles are all sensitive (EC_{50} < 3.5 mg L^{-1}). Homozygous isolates carrying S1105, marked as filled circles, are resistant to MPD (EC_{50} > 100 mg L^{-1}). In cross G no homozygous sensitive isolates were found due to the small number of samples.

As MPD belongs to the group of CAA fungicides, the G1105S mutation may affect sensitivity to all members of the CAAs. Cross-resistance among MPD, benthiavalicarb, dimethomorph and iprovalicarb was shown in *P. viticola* field- and F_2-progeny isolates (Gisi et al., 2007). In this study, we sequenced the MPD resistant parental isolate CH05.2 and some resistant F_2-progeny isolates that were shown to exhibit cross-resistance among CAAs (Gisi et al., 2007). All of them displayed Ser1105 in the PvCesA3 gene product indicating that this mutation also causes resistance to other CAAs. Whether the same – or additional mutations are selected in *P. viticola* populations by all members of the CAAs remains to be investigated. Nevertheless, based on our findings resistance to

CAAs is controlled by one recessive nuclear gene. Therefore it is expected that CAA resistance in *P. viticola* can be managed under field conditions if appropriate product use strategies are implemented. In addition to the use of CAAs in mixture with multi-site or other non cross resistant fungicides as recommended by the Fungicide Resistance Action Committee (FRAC), the high-dose/refuge (HDR) strategy (Rausher, 2001) may also be considered to manage and delay the evolution of CAA resistance in *P. viticola*. The HDR strategy assumes that resistant alleles are rare and functionally recessive. It is successfully used to delay *Bacillus thuringiensis* (*Bt*) toxin resistance (associated with one recessive gene (Gahan et al., 2001)). However, due to the large population during epidemics, the implementation and efficiency of this strategy to manage CAA resistance in *P. viticola* remains to be explored.

The identification of the putative resistance mechanism for CAA fungicides in PvCesA3 may provide further insights in the complexity of the cellulose synthesis machinery and the role of the four cellulose synthase isoforms. By confirmation of the proposed target-site mutation, MPD may represent an excellent tool for studying cellulose synthesis in *P. viticola*, and other oomycetes. Moreover, our findings enable the development of molecular diagnostic assays (e.g. Q-PCR, Pyrosequencing) for CAA resistance monitoring in field populations. It will be much easier to detect the early evolution of CAA resistant field isolates and to determine the origin of resistance within *P. viticola* populations (as shown for QoI resistance by Chen et al. (2007)) addressing the basic question whether resistance alleles evolved simultaneously through recurrent mutations and sexual recombination at different spots or originated from only one “hot spot” and were displaced trough effective propagation.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1
Origin of *P. viticola* isolates used in this study. Two Single Sequence Repeat (SSR) loci, ISA and CES, both with 3 alleles were used for genotypical characterization. EC_{50} values, representing the sensitivity to mandipropamid, result from leaf disc assays.

Generation	Isolate	Origin	Year	ISA (SSR)				CES		EC_{50} (mg L^{-1})
				111[a]	133	137	137	169	171	
P	**D05[b]**	Germany	2002	x	x			x	x	0.11 ± 0.10[c]
P	**CH05.2**	Switzerland	2001			x	x			>100[c]
F_1	**II10**	Cross II	2005		x	x	x		x	0.06 ± 0.06[c]
F_1	**II26**	Cross II	2005		x	x	x		x	0.06 ± 0.04[c]
F_1	**II25**	Cross II	2005		x	x	x	x		0.07 ± 0.02[c]
F_1	**II8**	Cross II	2005	x		x	x		x	0.12 ± 0.06[c]
F_1	**II29**	Cross II	2005		x	x	x		x	0.41 ± 0.02[c]
F_2	G1	Cross G	2006	x		x	x		x	1.51[c]
F_2	G18	Cross G	2006		x	x	x		x	3.24[c]
F_2	G20	Cross G	2006	x		x	x		x	>100[c]
F_2	**J4**	Cross J	2006			x	x			0.04 ± 0.01[c]
F_2	J13	Cross J	2006	x		x	x		x	0.16[c]
F_2	J2	Cross J	2006			x	x		x	0.27[c]
F_2	J10	Cross J	2006	x	x		x		x	0.73[c]
F_2	J19	Cross J	2006	x		x	x			0.8[c]
F_2	J12	Cross J	2006	x	x		x		x	0.85[c]
F_2	J17	Cross J	2006	x	x		x		x	0.90[c]
F_2	J6	Cross J	2006			x	x		x	0.91[c]
F_2	J22	Cross J	2006		x	x	x			1.7[c]
F_2	J20	Cross J	2006	x	x				x	1.8[c]
F_2	J7	Cross J	2006			x	x		x	>100[c]
F_2	J18	Cross J	2006			x	x		x	>100[c]
F_2	**J26**	Cross J	2006			x	x		x	>100[c]
F_2	K10	Cross K	2006		x	x	x		x	0.52[c]
F_2	K25	Cross K	2006		x		x	x		1.33[c]
F_2	K27	Cross K	2006			x	x		x	1.41[c]
F_2	K20	Cross K	2006		x	x	x		x	1.46[c]
F_2	K23	Cross K	2006		x	x	x	x		1.76[c]
F_2	K4	Cross K	2006		x	x	x		x	1.91[c]
F_2	K16	Cross K	2006		x		x			2.51[c]
F_2	K29	Cross K	2006			x	x		x	>100
F_3	JJ3	Cross JJ	2008			x	x			0.29[d]

F_3	JJ4	Cross JJ	2008	x	x		0.34[d]
F_3	JJ9	Cross JJ	2008	x	x	x	0.41[d]
F_3	JJ1	Cross JJ	2008	x	x	x	0.89[d]
F_3	JJ10	Cross JJ	2008	x	x	x	0.92[d]
F_3	JJ7	Cross JJ	2008	x	x	x	1.14[d]
F_3	JJ2	Cross JJ	2008	x	x		>100[d]
F_3	JJ4	Cross JJ	2008	x	x		>100[d]
F_3	JJ6	Cross JJ	2008	x	x	x	>100[d]
F_3	JJ8	Cross JJ	2008	x	x	x	>100[d]

[a]Size of loci is given in base pairs (bp).
[b]Isolates used for crossing experiments are written in bold. More information is given in the study of Gisi et al. (2007).
[c]Sensitivity values derived from the study of Gisi et al. (2007). Note that due to limited availability of isolates only a subset could be investigated. Furthermore 6 F_2-progeny isolates were excluded due to strong suspicion of clonal origin, indicated by SSR and SNP analysis.
[d]Sensitivity values derived from this study.

Supplementary Table 2
Oligonucleotide primer pairs, sequencing primers and amplicon sizes used in template preparation for sequencing and pyrosequencing

Gene	Primer Name	Primer Sequence (5'-3')	Application	Amplicon Size (bp)
PvCesA1	Pcesa1f	GCCATTCGTCTTCATTTGCTCTGAAGG	PCR	3279
	Pcesa1r	CCATATTCATTACGATATACGGCTTAAAAGGC	PCR	
	Pcesa1seqr	ACCCAAAGATCTCGATGATG	Sequencing	
	Pcesa1seqf1	ACCGAGGATTTGAAGTCATG	Sequencing	
	Pcesa1seqf2	CGACACGTTATTGGCGTG	Sequencing	
	Pcesa1seqf3	GGAGGAGCGTATTAGCTAC	Sequencing	
	Pcesa1seqf4	GGCAAAGGGTAACTTTCAGAC	Sequencing	
	Pcesa1-9f	ACCTTTTTCAGGCAACCATGTCTA[a]	PCR (Pyroseq)	88
	Pcesa1-9r	GAAGGGGTTGCGAACTTTGA	PCR (Pyroseq)	
	Pcesa1-9seq	TTGGAGGCATCCCCG	Sequencing	
	Pcesa1-58f	AGCCTGGCGAGACTGAGA[a]	PCR (Pyroseq)	183
	Pcesa1-58r	AGTAGCGACGTTTCCAGCTCT	PCR (Pyroseq)	
	Pcesa1-58seq	ATGACATTTTCTTGCAAC	Sequencing	
PvCesA2	Pcesa2f	AGTCCAAAGCACACTCACGAAGC	PCR	3213
	Pcesa2r	TTGTAAGTGGTATCCTAAGTAGTAGCC	PCR	
	Pcesa2seqr	GATACT TGCGGACGT TTG	Sequencing	
	Pcesa2seqf1	ATGCTGATCTGGTAGTGG	Sequencing	
	Pcesa2seqf2	CCCAAGGTCGACATCTTG	Sequencing	
	Pcesa2seqf3	GTGTGCCAGAAGAGCAG	Sequencing	
	Pcesa2seqf4	GAGTGGAAGATGCCAGAG	Sequencing	
	Pcesa2-256f	CCGGTTTTTCTACTTTCTCTTCG	PCR (Pyroseq)	240
	Pcesa2-256r	TCGCGAAAGCAAATCACG[a]	PCR (Pyroseq)	
	Pcesa2-256seq	TACGCTGACGTCCCT	Sequencing	

	Pcesa2-553f	TGTCGGTTGCAATGCTGTC	PCR (Pyroseq)	106
	Pcesa2-553r	GGCATTCTCAAAGCAATCCT[a]	PCR (Pyroseq)	
	Pcesa2-553seq	TTGCAATGCTGTCGC	Sequencing	
PvCesA3	Pcesa3f	AACCCGACCAGGGAGAAACCGAGCGATCC	PCR	3839
	Pcesa3r	GCTACATAGGCTACACTACCGCTTCTTGCTGC	PCR	
	Pcesa3seqr1	GCAATGACAAACTCTTGACG	Sequencing	
	Pcesa3seqr2	GCAATCGAGTAGGTCGAC	Sequencing	
	Pcesa3seqf1	GCATTCGCTTTATGGACTC	Sequencing	
	Pcesa3seqf2	TGTCTAGCGATGCAGTAC	Sequencing	
	Pcesa3seqf3	TACGTACAGACTCCACAATAC	Sequencing	
	Pcesa3seqf4	CTACTCTTTCTTGCCTGTG	Sequencing	
	Pcesa3cDNAf	CGTCATCGCGTCCGTCATAGG	PCR (cDNA)	531
	Pcesa3cDNAr	GAAAGCACGGCACTCGAGTCG	PCR (cDNA)	
	Pcesa3cDNAseq	GCAATGACAAACTCTTGACG	Sequencing	
	Pcesa3-1105f	ACCCCATGGTCAAGATGAGTATC[a]	PCR (Pyroseq)	137
	Pcesa3-1105r	CTCGTAATAGACTTGCCACAGCT	PCR (Pyroseq)	
	Pcesa3-1105seq	AGACAACCAGCAACG	Sequencing	
PvCesA4	Pcesa4f	TACCGTAAGGGTACGACTGTCTGTACC	PCR	3280
	Pcesa4r	CTCAAGCATGGCAAGGTTCTTTGTCG	PCR	
	Pcesa4seqr	AAGTCCTGGTCCGTCTCG	Sequencing	
	Pcesa4seqf1	TCGATTGCCGCAAGTAC	Sequencing	
	Pcesa4seqf2	GCTCGGGTTCCTCTATTAC	Sequencing	
	Pcesa4seqf3	GAGAAGATCATGAACCTCGA	Sequencing	
	Pcesa4seqf4	ATCATGCGCTGTTGTGC	Sequencing	
	Pcesa4seqf5	TCGCAAAGACCTCGAAG	Sequencing	
	Pcesa4seqf6	CGTCATGGCGGTGTTTG	Sequencing	
	Pcesa4cDNAf	CGACGAGATTCAGCTCTTGTACG	PCR (cDNA)	566
	Pcesa4cDNAr	GCCATTCGTCCACTTCGAGTTCG	PCR (cDNA)	
	Pcesa4cDNAseq	GCTCGGGTTCCTCTATTAC	Sequencing	

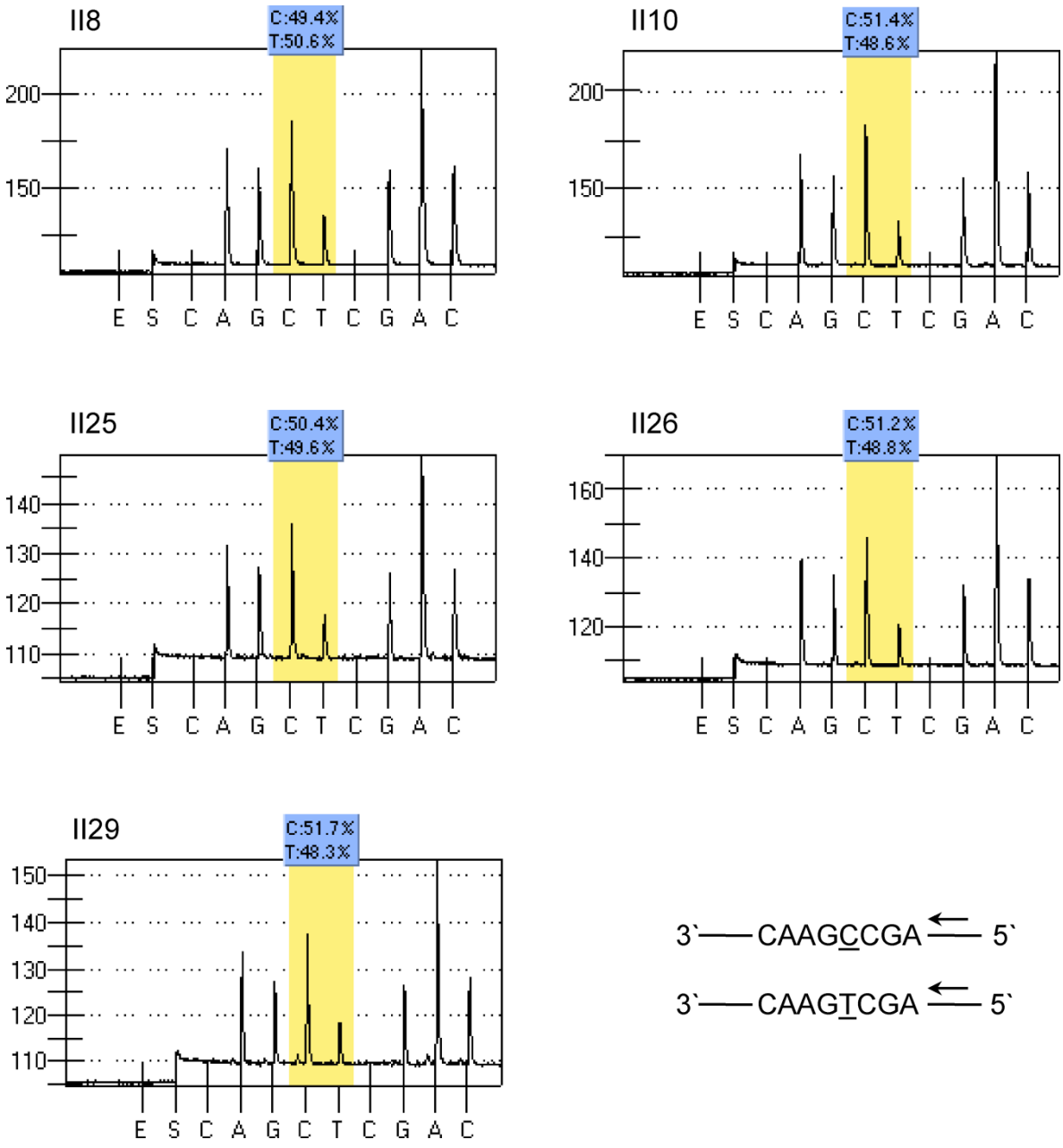
[a]Primer is 5'-biotinylated.

Supplementary Table 3
Sequences of cellulose synthase and cellulose synthase-like gene products used for ClustalW alignment

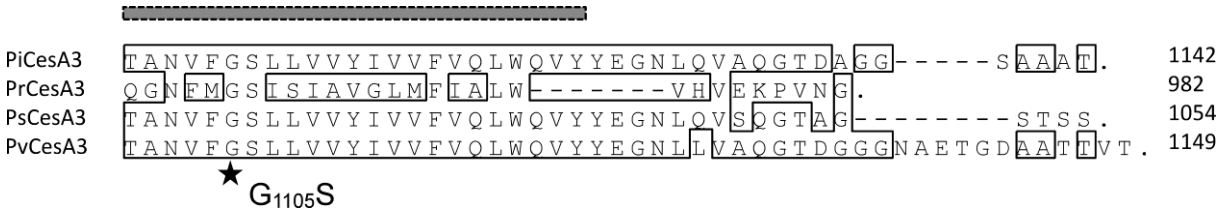
Sequence input	Type	Organism	Accession Number[a]
PiCesA1	Cellulose synthase	*Phytophthora infestans*	**ABP96902**
PiCesA2	Cellulose synthase	*Phytophthora infestans*	**ABP96903**
PiCesA3	Cellulose synthase	*Phytophthora infestans*	**ABP96904**
PiCesA4	Cellulose synthase	*Phytophthora infestans*	**ABP96905**
PrCesA1	Cellulose synthase	*Phytophthora ramorum*	**ABP96910**
PrCesA2	Cellulose synthase	*Phytophthora ramorum*	**ABP96911**
PrCesA3	Cellulose synthase	*Phytophthora ramorum*	**ABP96912**
PrCesA4	Cellulose synthase	*Phytophthora ramorum*	**ABP96913**
PsCesA1	Cellulose synthase	*Phytophthora sojae*	**ABP96906**

PsCesA2	Cellulose synthase	*Phytophthora sojae*	**ABP96907**
PsCesA3	Cellulose synthase	*Phytophthora sojae*	**ABP96908**
PsCesA4	Cellulose synthase	*Phytophthora sojae*	**ABP96909**
PvCesA1	Cellulose synthase	*Plasmopara viticola*	**GQ258973**
PvCesA2	Cellulose synthase	*Plasmopara viticola*	**GQ258974**
PvCesA3	Cellulose synthase	*Plasmopara viticola*	**GQ258975**
PvCesA4	Cellulose synthase	*Plasmopara viticola*	**GQ258976**
SmCesA2	Cellulose synthase	*Saprolegnia monoica*	**ACX56230**
SmCesA3	Cellulose synthase	*Saprolegnia monoica*	**ACX56231**
NsCesA	Cellulose synthase	*Nostoc sp.*	**NP_487797**
AvCesA	Cellulose synthase	*Anabaena variabilis*	**YP_322086**
AthCesA1	Cellulose synthase	*Arabidopsis thaliana*	**NP_194967**
AthCesA8	Cellulose synthase	*Arabidopsis thaliana*	**NP_567564**
PbCesA	Cellulose synthase	*Physcomitrella patens*	**AAT48368**
PtCesA8	Cellulose synthase	*Populus tremula*	**AAT09898**
PtaeCesA	Cellulose synthase	*Pinus taeda*	**AAX18647**
StCesA2	Cellulose synthase	*Solanum tuberosum*	**AAP97497**
ZmCesA9	Cellulose synthase	*Zea mays*	**NP_001104959**
AtCesA	Cellulose synthase	*Agrobacterium tumefaciens*	**NP_357298**
EcCesA1	Cellulose synthase	*Escherichia coli*	**YP_002409927**
GxCesA1	Cellulose synthase	*Gluconacetobacter xylinus*	**P21877**
GxCesA2	Cellulose synthase	*Gluconacetobacter xylinus*	**Q59167**
GxCesA3	Cellulose synthase	*Gluconacetobacter xylinus*	**Q9WX75**
RlCesA	Cellulose synthase	*Rhizobium leguminosarum*	**ZP_02294314**
PsyrCesA	Cellulose synthase-like A	*Pseudomonas syringae*	**ZP_03395445**
AthCslA1	Cellulose synthase-like A	*Arabidopsis thaliana*	**NP_193392**
AthCslA3	Cellulose synthase-like A	*Arabidopsis thaliana*	**NP_850952**
AthCslA7	Cellulose synthase-like A	*Arabidopsis thaliana*	**NP_565813**
AthCslA11	Cellulose synthase-like A	*Arabidopsis thaliana*	**NP_197123**
AthCslB1	Cellulose synthase-like B	*Arabidopsis thaliana*	**NP_180820**
AthCslB3	Cellulose synthase-like B	*Arabidopsis thaliana*	**NP_850190**
AthCslB6	Cellulose synthase-like B	*Arabidopsis thaliana*	**NP_193267**
AthCslD3	Cellulose synthase-like D	*Arabidopsis thaliana*	**NP_186955**
AthCslE1	Cellulose synthase-like E	*Arabidopsis thaliana*	**NP_175981**
CrCslD	Cellulose synthase-like D	*Ceratopteris richardii*	**AAT48374**
PpCslD8	Cellulose synthase-like D	*Physcomitrella patens*	**XP_001764498**

[a]All sequences were obtained from the NCBI database



Suppl. Fig.1. Pyrograms of the coding SNP in the *PvCesA3* gene (codon 1105) after amplification of cDNA from heterozygous F_1-progeny isolates. Expression of alleles for each isolate is given as percentage above of the corresponding pyrogram. The SNP site (G/A), corresponding to amino acid glycine (GGC) or serine (AGC) is highlighted. Due to assay design reasons the antisense strand was taken as template. Therefore the program shows (C/T) instead of (G/A). The Sequence including the SNP is written on the right side of the last pyrogram. Isolate names are given on the top-left side of the pyrograms. Primer position is shown by arrows. The underlined nucleotides indicate the SNP site (G/A), corresponding to amino acid glycine (GGC) or serine (AGC).



Suppl. Fig.2. Multiple alignment of the C-terminal amino acid sequences of four CesA3s from *P. infestans* (Pi), *P. sojae (*Ps*)*, *P. ramorum* (Pr) and *P. viticola* (Pv). Numbers indicate the positions of the last amino acid present in the corresponding sequence. Hatched bar indicates the last predicted transmembrane domain in *P. viticola*. Boxed residues are identical for the isoforms. The position of the G1105S mutation in PvCesA3 is indicated by an asterisk below the alignment.

CHAPTER II

Resistance mechanism to carboxylic acid amide (CAA) fungicides in the cucurbit downy mildew pathogen *Pseudoperonospora cubensis*

Chapter corresponds to the publication: Blum, M., Waldner, M., Olaya, G., Cohen, Y., Gisi, U., and Sierotzki, H. 2011. Resistance mechanism to carboxylic acid amide (CAA) fungicides in the cucurbit downy mildew pathogen Pseudoperonospora cubensis. Pest Manag. Sci. 67, 1211-1214.

Resistance mechanism to carboxylic acid amide (CAA) fungicides in the cucurbit downy mildew pathogen *Pseudoperonospora cubensis*

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ABSTRACT

BACKGROUND: *Pseudoperonospora cubensis,* the causal oomycete agent of cucurbit downy mildew, is responsible for enormous crop losses in many species of *Cucurbitaceae*, particularly in cucumber and melon. Disease control is mainly achieved by combinations of host resistance and fungicide applications. However, since 2004, resistance to downy mildew in cucumber has been overcome by the pathogen, thus driving farmers to only rely on fungicide spray applications, including carboxylic acid amide (CAA) fungicides. Recently, CAA resistant isolates of *P. cubensis* were recovered but the underlying mechanism of resistance was not revealed. The purpose of this study was to identify the molecular mechanism controlling resistance to CAAs in *P. cubensis*.

RESULTS: We characterized the four *CesA* (cellulose synthase) genes responsible for cellulose biosynthesis in *P. cubensis*. Resistant strains showed a mutation in the *CesA3* gene, at position 1105, leading to an amino acid exchange from glycine to valine or tryptophan. Cross resistance tests with different CAAs indicated, that these mutations lead to resistance against all tested CAAs.

CONCLUSION: Point mutations in the *CesA3* gene of *P. cubensis* lead to CAA resistance; accurate monitoring of these mutations amongst *P. cubensis* populations may improve/facilitate adequate recommendation/deployment of fungicides in the field.

Keywords: *CesA* genes; Cellulose synthesis; Fungicide resistance; Mutations; Oomycetes

1. INTRODUCTION

The downy mildew *Pseudoperonospora cubensis* [(Berkeley & M. A. Curtis) Rostovzev] is economically one of the most important plant pathogens in cucurbits (Savory et al., 2011). Its wide host range and global distribution leads to significant yield losses in many countries throughout the world including the USA, Europe, China, Japan and Israel (Thomas, 1996). Efficient control of downy mildew is mainly based on resistant cultivars and application of systemic, single-site mode of action fungicides. However, *P. cubensis* is a high-risk pathogen in terms of developing fungicide resistance (FRAC, 2010), and host resistance has been overcome by the pathogen since the year 2004 (Holmes and Thomas, 2006). Thus, there is an urgent need for appropriate control strategies, including chemical control. Carboxylic acid amide (CAA) fungicides, targeting cellulose biosynthesis in oomycetes (Blum et al., 2010a), are widely used to control downy mildews (Gisi, 2002) However, CAA resistant isolates have been detected in *Plasmopara viticola* populations for more than 10 years, and since the year 2007, in the USA, resistant isolates were also detected in *P. cubensis* populations (Lebeda and Cohen, 2011; Olaya et al., 2009; Zhu et al., 2008). Whereas in *P. viticola*, the underlying resistance mechanism has been elucidated and linked to a single point mutation in the *CesA3* gene (Blum et al., 2010b), this was not yet determined for *P. cubensis*.

This work describes the molecular mechanism conferring resistance to CAA fungicides in *P. cubensis*. We identified the four putative *CesA* genes in *P. cubensis*. The *CesA3* gene of CAA resistant and sensitive isolates was sequenced and mutations identified at position 1105 of this gene, conferring CAA resistance.

2. MATERIALS AND METHODS

2.1. Isolates and culture conditions

P. cubensis field isolates used in this study originated from the Czech Republic, Israel, Turkey, Switzerland and the United States. Samples were isolated from different hosts between the years 1982 and 2010 (Table 1). Isolates were maintained and propagated in a growth chamber (70% relative humidity, 20°C, 14h light/day) on detached cucumber leaves in moisten Petri dishes.

Table 1
Sensitivity of *P. cubensis* field isolates to CAA fungicides in correlation to mutations at position 1105 in the *PcCesA3* gene.

Isolate	Origin	Year	Host	EC_{50} BENT (mg L^{-1})[a]	EC_{50} DMM (mg L^{-1})	EC_{50} MPD (mg L^{-1})	Amino acid at pos. 1105 in *PcCesA3*
C-1	S. Carolina	1982	Cantaloupe	1	2.64	0.66	Gly [GGG][b]
CH01	Switzerland	2009	Cucumber	0.93	0.67	0.87	Gly [GGG]
21	Israel	2009	Cucumber	0.85	2.85	0.27	Gly [GGG]
2	Turkey	2009	Cucumber	1.88	2.7	0.54	Gly [GGG]
73	Czechia	2003	Cucumber	2.17	4.02	1.25	Gly [GGG]
26	Israel	2008	Cucumber	>100	>100	>100	Val [G**T**G]
Biu	Israel	2010	Cucumber	>100	>100	>100	Val [G**T**G]
163	Florida	2008	Watermelon	>100	>100	>100	Trp [**T**GG]
164	Florida	2008	Zucchini	>100	>100	>100	Trp [**T**GG]
171	Florida	2008	Cantaloupe	>100	>100	>100	Trp [**T**GG]
279	N. Carolina	2008	Cucumber	>100	>100	>100	Trp [**T**GG]
299	Michigan	2008	Cucumber	>100	>100	>100	Trp [**T**GG]

[a]EC_{50} values result from leaf disc assays
[b]Codon at position 1105 is given in parenthesis

2.2. Sensitivity assay and cross resistance tests

Sensitivity of *P. cubensis* isolates to different CAAs (Benthiavalicarb, Dimethomorph and Mandipropamid) was assessed using a leaf disc bioassay (Gisi et al., 2007). Disease assessment was done after a 6 day incubation period by visual estimation of the percentage leaf disc area infected compared to the untreated inoculated control. Data were analyzed and EC_{50} values were determined with the *agstat* program (Syngenta internal software).

2.3. Bioinformatics

For the identification of the *CesA* gene orthologs in *P. cubensis*, *CesA* specific consensus primers were designed based on the multiply-aligned *Phytophthora* and *Plasmopara* CesA sequences (Blum et al., 2010b, Grenville-Briggs et al., 2008). The multiple alignment was generated using ClustalW (Thompson et al., 2002).

2.4. PCR manipulations

DNA was extracted using the CTAB method as previously described (Blum et al., 2010b). PCR for *CesA* gene fishing experiments was performed with 50 ng genomic DNA

in a total volume of 50 µl containing 1.5 U of GoTaq Polymerase (Promega, Madison, WI), 0.2 mM dNTPs and 0.4 µM primers (supplied by Microsynth, Balgach, CH) in the polymerase manufacturer's buffer. *CesA* specific consensus primers are listed in Table 2. The PCR program was as follows: first 4 min initial denaturation at 94°C, then 34 cycles of 30 sec at 94°C, 30 sec at 50-60°C, 2 min at 72°C and a final 5 min extension step. Flanking regions of amplified *PcCesA* gene fragments were obtained by Genome walking using DNA Walking SpeedUp kit (Seegene, Rockville, MD), following manufacturer's instructions.

Table 2
Consensus primers for *CesA* amplification in *P. cubensis* designed according to conserved amino acid motifs in CesA sequences.

Primer	Sequence (5'–3')	Protein consensus	Amplicon size (bp)	Target Gene
Sense				
CesA1f	AAGGCCAAGATCTACTACTTCCACTCG	KAKIYYFHS	2708	*PcCesA1*
CesA2f	ACGGACGAGGAGTACCGCATGTGG	TDEEYRMW	2503	*PcCesA2*
CesA3f	TGTACCGAGTGTGGTGAGGCGATGC	FCTECGEAMP	2224	*PcCesA3*
CesA4f	CCAGGACTTTACCATGTGGTTCGACG	DQDFTMWFD	2550	*PcCesA4*
Antisense				
CesA1r	ACCCACAGCGCAATGAACATGAGG	GLMFIALW	2708	*PcCesA1*
CesA2r	CGTTGCCCTGGTCCATCAGACTCTC	ESLMDQGNV	2503	*PcCesA2*
CesA3r	ACGTCTTCTGACCGGCACCAGTG	NTGAGQKT	2224	*PcCesA3*
CesA4r	GTTGCCCTGGTCCGTCATACTCTTGTGG	SHKSMTDQGN	2550	*PcCesA4*

2.5. Cloning, characterization and analysis of the PcCesA genes

Putative *CesA* PCR fragments were cloned into a pCR4-TOPO vector (Invitrogen, Carlsbad, CA) and further processed following manufacturer's instructions. Sequencing reactions were done with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions, using gene specific primers (Supplementary Table 1). A BLASTN search was carried out in the NCBI database (http://www.ncbi.nlm.nih.gov/) to identify correct clones. Prediction of complete *CesA* open reading frames (ORFs) was done using the NCBI ORF finder program (http://www.ncbi.nlm.nih.gov/projects/gorf/). The predicted amino acid sequences were analyzed using the NCBI Conserved Domain Database (http://www.ncbi.nih.gov/Structure/cdd/wrpsb.cgi) (Marchler-Bauer and Bryant, 2004).

Prediction of transmembrane domains was done using Tmbase (http://www.ch.embnet.org).

2.6. Phylogeny

Multiple sequence alignments were made with ClustalW and phylogenetic dendrograms were constructed using MEGA 5 (http://www.megasoftware.net/) (Tamura et al., 2011). The dendrogram is based on the full CesA protein sequences that were obtained from NCBI (http://www.ncbi.nlm.nih.gov/) (Accession No. in Supplementary Table 2). Phylogeny was constructed with the Maximum Likelihood algorithm using 500 bootstrap replications.

3. RESULTS AND DISCUSSION

The full-length nucleotide sequence of the four putative *PcCesA* genes was deposited under GenBank Acession No. JF799096 (*PcCesA1*), JF799097 (*PcCesA2*), JF799098 (*PcCesA3*), JF799099 (*PcCesA4*). There was no intron in the ORF of *PcCesA1* and *PcCesA2*, whereas *PcCesA3* and *PcCesA4* were each interrupted by one single intron. All four putative CesAs contained the conserved D,D,D,QXXRW motif that is present in most processive glycosyltransferases and all known *CesAs* (Campbell et al., 1997) (Fig. 1A). The predicted protein products displayed several transmembrane domains as described for CesAs in *P. infestans*, *P. viticola* and *Saprolegnia monoica* (Grenville-Briggs et al., 2008; Fugelstad et al., 2009; Blum et al., 2010b). However, the predicted TM subunit of a Periplasmic Binding Protein in the CesA3 protein of *P. cubensis* seems to be a new feature for oomycete CesAs (Fig. 1 A).

Phylogenetic analysis of Oomycete CesAs confirmed the existence of subclades for each of the four different Oomycete *CesA* genes (Fig. 1B). The four CesA sequences of *P. cubensis* clustered together with other orthologs of the *Peronosporales* (Fig. 1B).

The tested isolates could be clearly allocated into either CAA resistant (EC_{50} >100 mg L^{-1}) or sensitive (EC_{50} < 3 mg L^{-1}) isolates. The resistance factor observed in CAA resistant-compared to sensitive isolates was more than 100, despite differences of intrinsic activities of the CAAs used. Such high resistance factors are often associated with an alteration of the target site (Sierotzki and Gisi, 2003). Sequencing the *PcCesA3* gene, encoding the putative target protein of CAAs (Blum et al., 2010a), revealed mutations at position 1105 (Fig. 1A). CAA resistant isolates displayed the amino acid valine, encoded by GTG, or tryptophan, encoded by TGG, respectively (Table 1). Sensitive

isolates showed the amino acid glycine encoded by GGG (Table 1). All isolates having the G1105V or G1105W mutation were cross-resistant to the CAAs tested.

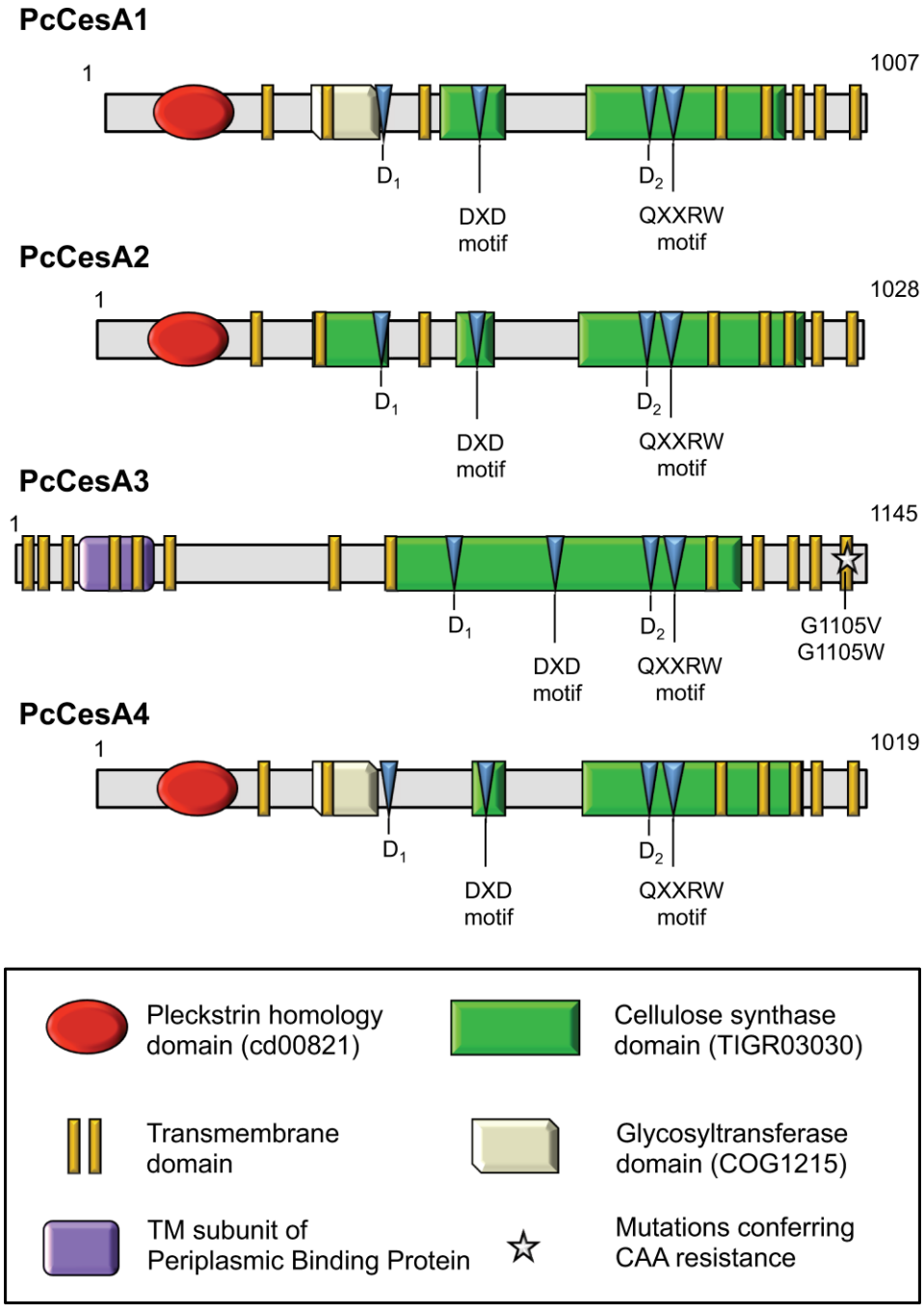
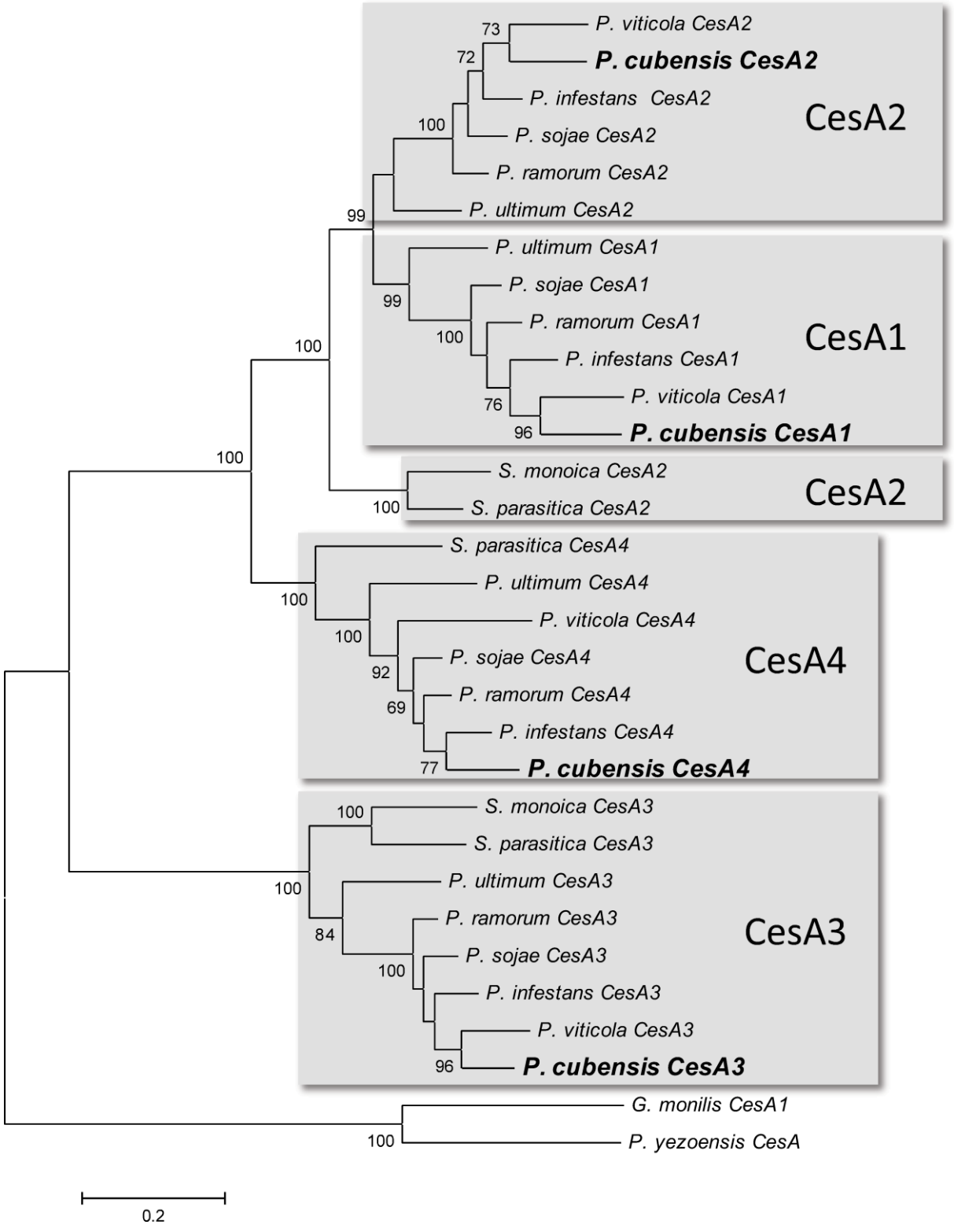


Fig.1. Sequence analysis of the *PcCesA* genes. (**A**) Domain comparison of the cellulose synthases (CesAs) from *P. cubensis*. Total amino acids (aa) are given on the upper right side of each protein. The proteins contain several transmembrane (TM) domains and regions similar to cellulose synthases and other glycosyltransferases. CesA1, CesA2 and CesA4 include a characteristic Pleckstrin Homology (PH) domain. A new feature for oomycetes CesA proteins is the predicted TM subunit of a Periplasmic Binding Protein in PcCesA3. The mutation (marked by an asterisk) conferring resistance to CAAs is located in the last predicted TM domain in PcCesA3.



(**B**) Phylogenetic analysis of the *PcCesA* genes based on Maximum likelihood algorithm. Branch lengths are proportional to the number of amino acid replacements, as indicated by the scale bar. Bootstrap values are indicated when >60. CesA sequences of *Porphyra yezoensis* and *Griffithsia monilis* were chosen as outgroups. The analysis is based on the complete amino acid sequence of cellulose synthase gene products (Supplementary Table 2 for Accession numbers).

In contrast, isolates displaying G1105 were fully sensitive to all CAAs included in this study (Table 1). Our results clearly show that CAA fungicides fully control sensitive *P. cubensis* populations. However, in populations containing resistant individuals with either the V1105 or W1105 mutation, the use of CAAs should be restricted as it is the case for *P. viticola* (see CAA FRAC recommendations, http://www.frac.info). So far, in *P. cubensis* populations, little is known about the distribution and frequency of isolates carrying the V1105 or W1105 mutation. Revealing the mechanism, responsible for resistance to CAAs, will now enable the development of highly sensitive molecular monitoring methods based on Q-PCR, Pyrosequencing or PCR-RFLP. The latter method has already been developed for the detection of the *PvCesA3* gene allele conferring resistance to mandipropamid in *P. viticola* populations (Aoki et al., 2011). In *P. cubensis* the PCR-RFLP method needs to be developed and validated first in order to detect the *PcCesA3* alleles in populations. However, it remains to be investigated if PCR-RFLP or rather the much more sensitive Q-PCR or Pyrosequencing will be the method of choice for accurate monitoring CAA resistance in *P. cubensis* populations, and therefore improving disease management in the field.

ACKNOWLEDGMENT

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SUPPLEMENTARY MATERIAL

Supplementary Table 1
Oligonucleotide primer pairs and sequencing primers used in template preparation for sequencing the *PcCesA* genes

Gene	Primer Name	Primer Sequence (5‘-3‘)	Application
PcCesA1	Pcesa1f	GAGACTCTTGGGCGAGGCACAGC	PCR
	Pcesa1r	GCGTTTGACTCGCCATCTTCTACTGTCC	PCR
	Pcesa1seqf	GCCAAGATCTACTATTTTCACT	Sequencing
	Pcesa1seqf1	TTG ACT TTA GCG GGT GTT GA	Sequencing
	Pcesa1seqf2	GATTGCCTCACGTGACTTT	Sequencing
	Pcesa1seqf3	CCGCAATACTTTCGCGA	Sequencing
	Pcesa1seqf4	ATTGTGCAGGGTGTGATGT	Sequencing
PcCesA2	Pcesa2f	CACACTCACACACACGTAGTCCCTATCG	PCR
	Pcesa2r	AGTCGCTAAAGACCGACCCTGACTGG	PCR
	Pcesa2seqr	CAATCAGGAACAAGAAGTAGA	Sequencing
	Pcesa2seqf	GGACCGTTACACAAGAGCT	Sequencing
	Pcesa2seqf1	TGGGTGCTCTGTCCTGA	Sequencing
	Pcesa2seqf3	CAATCGATACCGCTGATG	Sequencing
	Pcesa2seqf4	CTT GCA GGC TAC AAT AGC A	Sequencing
PcCesA3	Pcesa3f	CTTCAGCTGTCCTCCTATCGTTCTTCTCG	PCR
	Pcesa3r	AGAGATCAACTGCGTCTTTATGACAGCTCG	PCR
	Pcesa3seqr	TGC TGT CGG ATG TAG CCT T	Sequencing
	Pcesa3seqf	AGACGACGGAGCTGGA	Sequencing
	Pcesa3seqf1	GGTGAGGCGATGCCTAC	Sequencing
	Pcesa3seqf2	TGATGATCTTCAG	Sequencing
	Pcesa3seqf3	GCATCACTCCAAGGCTG	Sequencing
	Pcesa3seqf4	CTACGACTCGGTGCTGTA	Sequencing
PcCesA4	Pcesa4f	AGTGTCTGTGATTCCGCACGTTTGAGTATCC	PCR
	Pcesa4r	GGAGGTCCTCAAAAGGAGAGAGAAAGCG	PCR
	Pcesa4seqf	GCTACTTTGCCCTCGAAG	Sequencing
	Pcesa4seqf1	GGTATCGAGCCCTGTGA	Sequencing
	Pcesa4seqf2	GGTGATGTCCGTCACGAG	Sequencing
	Pcesa4seqf3	GTAACCAGTTCGTGGGTATTTC	Sequencing
	Pcesa4seqf4	ACGTCTGCTTATGGCTCT	Sequencing

Supplementary Table 2
Sequences of cellulose synthase gene products used for ClustalW alignment

Sequence input	Type	Organism	Accession Number
PiCesA1	Cellulose synthase	*Phytophthora infestans*	**ABP96902** [a]
PiCesA2	Cellulose synthase	*Phytophthora infestans*	**ABP96903** [a]
PiCesA3	Cellulose synthase	*Phytophthora infestans*	**ABP96904** [a]
PiCesA4	Cellulose synthase	*Phytophthora infestans*	**ABP96905** [a]
PrCesA1	Cellulose synthase	*Phytophthora ramorum*	**ABP96910** [a]
PrCesA2	Cellulose synthase	*Phytophthora ramorum*	**ABP96911** [a]
PrCesA3	Cellulose synthase	*Phytophthora ramorum*	**ABP96912** [a]
PrCesA4	Cellulose synthase	*Phytophthora ramorum*	**ABP96913** [a]
PsCesA1	Cellulose synthase	*Phytophthora sojae*	**ABP96906** [a]
PsCesA2	Cellulose synthase	*Phytophthora sojae*	**ABP96907** [a]
PsCesA3	Cellulose synthase	*Phytophthora sojae*	**ABP96908** [a]
PsCesA4	Cellulose synthase	*Phytophthora sojae*	**ABP96909** [a]
PvCesA1	Cellulose synthase	*Plasmopara viticola*	**ADD84670** [a]
PvCesA2	Cellulose synthase	*Plasmopara viticola*	**ADD84671** [a]
PvCesA3	Cellulose synthase	*Plasmopara viticola*	**ADD84672** [a]
PvCesA4	Cellulose synthase	*Plasmopara viticola*	**ADD84673** [a]
PcCesA1	Cellulose synthase	*Pseudoperonospora cubenis*	**JF799096** [b]
PcCesA2	Cellulose synthase	*Pseudoperonospora cubenis*	**JF799097** [b]
PcCesA3	Cellulose synthase	*Pseudoperonospora cubenis*	**JF799098** [b]
PcCesA4	Cellulose synthase	*Pseudoperonospora cubenis*	**JF799099** [b]
PuCesA1	Cellulose synthase	*Pythium ultimum*	**PYU1_T002607** [c]
PuCesA2	Cellulose synthase	*Pythium ultimum*	**PYU1_T002606** [c]
PuCesA3	Cellulose synthase	*Pythium ultimum*	**PYU1_T006539** [c]
PuCesA4	Cellulose synthase	*Pythium ultimum*	**PYU1_T006496** [c]
SmCesA2	Cellulose synthase	*Saprolegnia monoica*	**ACX56230** [a]
SmCesA3	Cellulose synthase	*Saprolegnia monoica*	**ACX56231** [a]
SpCesA2	Cellulose synthase	*Saprolegnia parasictica*	**SPRG_06590T0** [d]
SpCesA3	Cellulose synthase	*Saprolegnia parasitica*	**SPRG_06051T0** [d]
SpCesA4	Cellulose synthase	*Saprolegnia parasitica*	**SPRG_08607T0** [d]
PyCesA	Cellulose synthase	*Porphyra yezoensis*	**ABX71734** [a]
GmCesA1	Cellulose synhtase	*Griffithsia monilis*	**ADK77974**

[a]Sequences obtained from the NCBI database
[b]Sequences produced in this study
[c]Sequences obtained from: The *Pythium ultimum* Whole Genome Sequencing Project (http://pythium.plantbiology.msu.edu/project_info.html)
[d]Sequences obtained from: *Saprolegnia parasitica* Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/)

CHAPTER III

Insights into the molecular mechanism of tolerance to carboxylic acid amide (CAA) fungicides in *Pythium aphanidermatum*

Chapter corresponds to the publication: Blum, M., Gisi, U., 2012. Insights into the molecular mechanism of tolerance to carboxylic acid amide (CAA) fungicides in *Pythium aphanidermatum.* Pest Manag. Sci. (in press).

Insights into the molecular mechanism of tolerance to carboxylic acid amide (CAA) fungicides in *Pythium aphanidermatum*

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ABSTRACT

BACKGROUND: Tolerance to the oomycete specific carboxylic acid amide (CAA) fungicides is a poorly understood mechanism in *Pythium* species. Here we used the root rot and damping off causative agent, *Pythium aphanidermatum* and the CAA fungicide mandipropamid (MPD), to investigate the molecular basis of CAA tolerance.

RESULTS: Five genes putatively involved in carbohydrate synthesis were identified and characterized: one chitin synthase gene, *PaChs*, and four cellulose synthase genes *PaCesA1* to *PaCesA4*, of which *PaCesA3* encodes the MPD target enzyme. These genes were differentially expressed throughout the life cycle of *P. aphanidermatum*. Mycelium treated with MPD concentrations slightly affecting mycelial growth did not cause a change in *PaCesA3* expression nor a strong upregulation of *PaCesA* homologues. The high tolerance level of *P. aphanidermatum* and the lack of *PaCesA* upregulation imply that MPD tolerance is the result of a specific amino acid configuration in the cellulose synthase 3 (CesA3) target enzyme. Indeed *P. aphanidermatum* displays the amino acid L1109 that is also associated with MPD resistance in artificial mutants of *Phytophthora* species.

CONCLUSION: We conclude that MPD tolerance in *P. aphanidermatum* is not caused by compensatory mechanisms, but most likely by an inherent target site configuration in PaCesA3 hindering MPD binding to the enzyme pocket.

Keywords: Cellulose synthesis; *CesA* genes; Chitin synthesis; Mandipropamid; *Pythiales*; Target site configuration

1. INTRODUCTION

Species of the genus *Pythium* form one of the most important groups of seedling pathogens causing damping-off and seed rot (Martin and Looper, 1999). Under wet soil conditions and favourable temperatures, severe disease outbreaks can occur which are difficult to control. Disease management is mainly achieved by soil sterilization, composting, biological control and fungicide application (Martin and Looper, 1999). Today, only few fungicides are registered for *Pythium* control including mefenoxam (Subdue Maxx®), propamocarb (Banol®), cyazofamid (Segway®) and fluopicolide (Stellar®). Their molecular mode of action is still not fully elucidated and it is so far unknown how these fungicides act on *Pythium* species. Several other oomycete specific fungicides, among these the carboxylic acid amides (CAAs), are commercially available, showing activity against the *Peronosporales* but not the *Pythiales*. CAAs are widely used to control downy mildews and late blight caused by members of the *Peronosporales* (Gisi and Sierotzki, 2008). However, biological studies revealed that members of the genus *Pythium* are tolerant to CAAs (Albert et al., 1988; Kuhn et al., 1991; Stenzel et al., 1998), but the underlying mechanism is not well understood. To elucidate the underlying mechanism of CAA tolerance in *Pythiales*, the present study focused on the soil-borne zoosporic plant pathogen *Pythium aphanidermatum* (Edson) Fritzp.

P. aphanidermatum has a cosmopolitan distribution and a broad host range, infecting many vegetables, fruits, grass and ornamental crops (Moorman et al., 2002). It is homothallic, reproducing sexually by formation of oospores, and asexually by production of motile biflagellate zoospores (Estradagarcia et al., 1990). These wall-less cells are attracted to roots, adhere and encyst forming germ tubes that subsequently penetrate the host plant to cause disease (Longman and Callow, 1987). The fungicide mandipropamid (MPD), member of the CAAs (FRAC, 2011), is highly active against cystospore germination and subsequent germ tube elongation in *Peronosporales* (Cohen and Gisi, 2007; Cohen et al., 2008; Knauf-Beiter and Hermann, 2005).

However, no such activity is reported against *P. aphanidermatum* or any other *Pythium* species. Thus, the characterization of the genes involved in cystospore formation and subsequent germ tube elongation may provide information on how this pathogen counteracts fungicide activity.

During the last few years, further progress was made in understanding the mode of action of CAAs: studies conducted with MPD demonstrated that the fungicide inhibits

cellulose biosynthesis by targeting the cellulose synthase 3 (CesA3) enzyme in *P. infestans* (Blum et al., 2010a). The proposed mode of action was confirmed by induced mutations in the *CesA3* gene, encoding the target enzyme of MPD, that led to complete resistance against CAAs in the downy mildew pathogens *Plasmopara viticola* and *Pseudoperonospora cubensis* (Blum et al., 2010b; Blum et al., 2011; Sierotzki et al., 2011). The results confirmed that cell wall polysaccharide synthases represent a novel fungicide target in oomycetes. Taking advantage of these new findings, they can now be used to further study the underlying mechanism of tolerance to CAA fungicides in *Pythium* species.

The cell wall of *Pythium* and all other oomycetes is composed mainly of cellulose, β-(1→3) and β-(1→6)-glucans (Bartnicki-Garcia, 1968). Unlike fungal cell walls of true fungi, chitin or soluble chito-oligosaccharides are either absent or present only in minor amounts (Badreddine et al., 2008; Bartnicki-Garcia, 1968; Bulone et al., 1992; Cherif et al., 1993; Guerriero et al., 2010). Cellulose is considered to act in a similar way as chitin does in true fungi: its fibrillar structure provides rigidity and strength to the cell wall (Farkas, 1976). In oomycetes, four *CesA* genes encoding the catalytic subunits of cellulose synthase, the membrane-bound complex responsible for cellulose synthesis, were identified in the genome of *Phytophthora infestans*, *Phytophthora ramorum* and *Phytophthora sojae* (Haas et al., 2009; Tyler et al., 2006). These four *CesA* genes were functionally characterized by gene silencing using RNA interference in *P. infestans*, revealing their involvement in cellulose biosynthesis (Grenville-Briggs et al., 2008). Cellulose is also the main component in the cell wall of *Pythium ultimum* (Cherif et al., 1993). Genome analysis of *P. ultimum* demonstrated that it contains, similar to *Phytophthora* and *Plasmopara*, four *CesA* genes encoding putative cellulose synthases (Levesque et al., 2010). Mandipropamid is classified as a cellulose biosynthesis inhibitor (FRAC, 2011), which specifically targets the CesA3 enzyme. Therefore it is surprising that CAAs are hardly active against members of the genus *Pythium*. Till now, the involvement of the four *CesA* homologues was described in specific life stages only for *P. infestans* (Grenville-Briggs et al., 2008). For *Pythium* species no such data is available and it is presently unclear whether the *CesA3* gene is of similar importance for this genus.

To gain basic information about the molecular mechanism of MPD tolerance in *Pythium* species, the present study aimed (i) to identify and characterize the *CesA* genes,

in particular *CesA3* encoding the MPD target enzyme in *P. aphanidermatum*; (ii) to determine the inherent tolerance level to the fungicide mandipropamid by mycelial growth- and cystospore germination assays; (iii) to investigate *CesA* expression levels in different life cycle stages providing basic insights about the relevance of these genes for the development of this organism; (iv) to determine, using gene expression studies, whether the observed tolerance is due to overexpression of the target enzyme, or compensation mechanisms either caused by *CesA* homologs or a *Chs* gene encoding a putative chitin synthase; and (v) to analyze whether the amino acid configuration at the PaCesA3 target site differs from MPD sensitive oomycetes possibly leading to fungicide tolerance.

2. MATERIALS AND METHODS

2.1. Strain and culture conditions

The *Pythium aphanidermatum* (Edson) Fitzp. strain 620 originated from Vero Beach, FL 32976, USA and was originally isolated from turf grass in 1999. This strain was used for all experiments performed in this study, unless otherwise stated and is stored in the Syngenta culture collection. The strain was maintained on 10% V8 agar media [200 ml V8 juice (Campbell foods, Puurs, BEL), 3 g $CaCO_3$, Agar Bacteriological Nr.1 (Oxoid, Hampshire, UK), 3 mg cholesterol and 800 ml de-ionised water] and incubated in petri dishes (9 cm) in the dark at 26 °C.

2.2. Sample preparation for RNA extraction

Non-sporulating mycelium of *P. aphanidermatum* was grown on V-8 agar plates for three days before harvesting and subsequent freezing in liquid nitrogen. The different life cycle stages (zoospores, cystospores and germlings) were prepared as follows: To induce zoospore formation, *P. aphanidermatum* mycelium was treated as previously described (Nelson and Craft, 1989). Twenty agar plugs of growing cultures (five days old) were placed in petri dishes (9 cm) and leached for two consecutive 10-min periods in 20 ml of buffer (pH 5.8) containing 0.01 M $Ca(NO_3)_2 \cdot 4H_2O$, 0.004 M $MgSO_4 \cdot 7H_2O$, and 0.005 M KNO_3. After each 10-min leaching period, buffer was replaced and agar plugs were incubated in the buffer for 1 h. Then, the buffer was replaced by 20 ml distilled H_2O, and agar plugs were incubated over night at 26 °C in the dark. Released zoospores were filtered through Whatman No. 1 paper (GE Healthcare) to remove

hyphal debris and collected in 50 ml polypropylene centrifuge tubes. Zoospores were collected by centrifugation at 2000 Rpm for 5 min at 20 °C, frozen in liquid nitrogen, and stored at -80 °C until required for RNA extraction. To prepare cystospores, the zoospore suspension was agitated with a Vortex mixer (MS1 Minishaker, IKA-Works Inc, Wilmington, NC) for 1 min to induce encysting (Gornhardt et al., 2000) . Within 5 min, zoospores lost their motility and cystospores were synchronously formed which were then immediately collected as described above. For preparation of germlings, zoospores were treated as described above to induce synchronized cystospore formation. Cystospores were then incubated at room temperature in distilled H_2O for 4 h to allow germination to occur. Germination rate of cystospores was verified by microscopy and proved to be higher than 90 %. After 4h, germlings were collected and stored as described above.

To assess the effect of the two cellulose inhibitors mandipropamid (MPD, supplied by Syngenta Crop Protection AG, Stein, Switzerland) and 2,6-Dichlorobenzonitrile (DCB, supplied by Sigma-Aldrich, Germany) on *CesA* and *Chs* gene expression, mycelium was prepared as follows: 25 ml of liquid V8 medium was poured into Erlenmeyer flasks (125 ml) and inoculated with 3 agar plugs (5 mm diameter) covered with 3 day old mycelium. Cultures were incubated for 24 h in the dark at 26 °C prior to the addition of MPD (100µM) or DCB (200µM) dissolved in 0.5 % DMSO, which was also included in the study as untreated control. Mycelium was harvested at 0.5, 2, 4 and 8 hours and immediately frozen in liquid nitrogen.

2.3. Effect of cellulose inhibitors on mycelial growth and germ tube elongation

The effect of MPD and DCB on mycelial growth was tested by inoculating V8 agar plates (14 cm) in triplicate, amended either with 10 µM MPD, 100 µM MPD or 200 µM DCB (dissolved in 0.5% DMSO), with a V8 agar plug (5 mm diameter) covered with 3 day old mycelium. Plates were incubated for three days in the dark at 26 °C, radial hyphal growth was measured at time intervals of 12 hours and compared to the 0.5% DMSO control.

Cystospores were produced as described in section *2.2* and then treated immediately with MPD (final concentrations 0.5, 5, 25, 50, 121 µM) or DCB (5, 50, 100, 250, 500 µM), respectively. Triplicate samples (50 µl) were transferred on microscopic slides and incubated in plastic boxes (20 x 20 cm) at room temperature for 4 h. Germ

tube length of 30 germlings per sample was visualized by a Axiolab light microscope (Zeiss, Feldbach, SUI) and measured using AxioVison imaging software (Zeiss).

2.4. DNA and RNA extraction

For extraction of genomic DNA, mycelium was harvested from cultures grown on V8 agar, immediately frozen in liquid nitrogen and stored at -80 °C until further use. DNA was isolated following a modified CTAB protocol (Riethmüller et al., 2002). Total RNA from individual life cycle stages (mycelium, zoospores, cystospores and germlings) and mycelium from liquid cultures treated with cellulose inhibitors, was extracted from frozen samples using E.Z.N.A™ Fungal RNA Kit (Omega Bio-Tek, Georgia, USA) following the manufacturer's protocol. Prior to cDNA synthesis, RNA samples were DNAse treated using Turbo DNA free Kit (Ambion, Texas, USA) according to the manufacturer's protocol. Integrity and quality/quantity of extracted RNA was tested by gel electrophoresis and by spectrophotometry using a NanoDrop® ND-2000 spectrophotometer (Thermo Scientific, USA).

2.5. Bioinformatics

To identify the *CesA* gene orthologs in *P. aphanidermatum*, consensus primers were designed (Table 1) based on the multiply-aligned *Phytophthora*, *Plasmopara*, *Pythium* and *Saprolegnia* CesA sequences. (GenBank accession number ABP96902, ABP96906, ABP96910, ADD84670, PYU1 T002607 for CesA1; ABP96903, ABP96907, ABP96911, ADD84671, PYU1 T002606, ACX56230 for CesA2; ABP96904, ABP96908, ABP96912, ADD84672, ACX56231, PYU1 T006539 for CesA3; and ABP96905, ABP96909, APB96913, ADD84673, PYU1 T006496 , ACX56232 for CesA4). The multiple alignment was generated using ClustalW (Thompson et al., 1994). Sequence alignment revealed conserved amino acid motifs (Table 1) for each of the four *CesA* genes, which were consequently chosen for primer design. Consensus primers for the *Chs* gene (Table 1) encoding a putative chitin synthase were designed as described above using CHS sequences from *Phytophthora infestans*, *Phytophthora ramorum*, *Phytophthora sojae* and *Pythium ultimum*, respectively (GenBank accession number XP002908631.1, Pr 77229, Ps_143614, PYU1_T005734).

2.6. PCR amplification

All PCR amplifications were performed with a TGradient Thermocycler (Biometra, Göttingen, GER) and synthetic oligonucleotide primers (Microsynth, Balgach, CH). PCR reactions using *Chs* and *CesA* specific consensus primers (listed in Table 1) were performed on 50 ng genomic DNA in a total volume of 30 µl containing 1.5 U of GoTaq® polymerase (Promega, Madison WI), 0.2 mM dNTP and 0.4 µM primers in the polymerase manufacturer's buffer. The PCR program was as follows: first 3 min initial denaturation at 94°C, then 35 cycles of 30 sec at 94°C, 30 sec at 50-62°C (temperature gradient), 2.5 min at 72°C and finally a 7 min extension step.

Table1

Consensus primers for *CesA* and *Chs* gene amplification in *Pythium aphanidermatum* designed according to conserved amino acid motifs in CesA and CHS sequences.

Primer	Sequence (5′–3′)	Protein consensus	Amplicon size (bp)	Target Gene
Sense				
CesA1f	GAGAAGGCCAAGATCTACTACTTCCA	EKAKIYYFH	2660	*PaCesA1*
CesA2f	GAGTACCGCATGTGGTTCCAGGGC	EYRMWFQG	2462	*PaCesA2*
CesA3f	GCGCGTCAGGAGTTTGTGATCGC	ARQEFVIA	2895	*PaCesA3*
CesA4f	CACGGATGGATGCACAAGCAGGG	HGWMHKQG	2745	*PaCesA4*
Chsf	ATCTGTATCACCATCTACAACGAAGGTCC	ICITIYNEGP	1536	*PaChs*
Antisense				
CesA1r	CAGCCGAAGTACTCCTGCGTGGT	TTQEYFGW	2660	*PaCesA1*
CesA2r	CTCCAGCCAAAGTACTCCTGGATACT	SIQEYFGWS	2462	*PaCesA2*
CesA3r	CCCAACCACAGTACTCCGTGATACTCATC	KMSITEYCGW	2895	*PaCesA3*
CesA4r	ACTCTTGTGGCTCCAGCCAAAGTACG	TYFGWSHKS	2745	*PaCesA4*
Chsr	CTTGGTGCCCCACGACAAGTCCTG	QDLSWGTK	1536	*PaChs*

Flanking regions of amplified *PaChs* and *PaCesA* gene fragments were obtained by Genome walking using DNA Walking SpeedUp Premix Kit II (Seegene, Rockville, MD), following manufacturer's instructions.

PCR amplifications of the complete open reading frames (ORFs) of the four putative cellulose synthase genes and the putative chitin synthase gene, were performed on 50 ng genomic DNA using PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA) and a final primer concentration of 0.2 µM. Supplementary Table 1 lists sequences of primer Pcesa1f, Pcesa1r used for amplification of *PaCesA1*; Pcesa2f, Pcesa2r for *PaCesA2*; Pcesa3f, Pcesa3r for *PaCesA3*; Pcesa4f, Pcesa4r for *PaCesA4*; and Pchsf, Pchsr for *PaChs.*

Prior to cloning, PCR products were analyzed on 1% agarose gels and fragments of expected size were purified using the NucleoSpin Extract II kit (Macherey-Nagel).

2.7. Cloning, characterization and analysis of the PaCesA genes

PCR reactions performed for *CesA* genes using consensus primers led to specific fragments for *CesA1*, *CesA2*, *CesA3* and *CesA4*. All gene fragments were all approximately 2.5 kb in size. For the *Chs* gene a single 1.5 kb fragment was amplified. Full-length nucleotide sequences were obtained by genome walking and named *PaCesA1*, *PaCesA2*, *PaCesA3 PaCesA4* and *PaChs*, respectively. Complete *CesA* and *Chs* open reading frames (ORFs), up to 4000 bp in size, were cloned into a pCR-XL-TOPO vector (Invitrogen) and further processed as described previously by Blum et al. (2010b).

Sequencing reactions were done with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Forster City, CA) according to the manufacturer's instructions using M13 (included in the TOPO cloning kit, Invitrogen) or sequence specific primers (Supplementary Table 1). Sequence analysis was performed on a 3130 Genetic Analyzer (Applied Biosystems) and further processed using Lasergene software (DNASTAR, Inc.).

Identification of correct clones was performed by doing a BLASTN search in the NCBI (http://www.ncbi.nlm.nih.gov/) and Joint Genome Institute (http://genome.jgi-psf.org) databases. Prediction of complete *CesA* and *Chs* ORFs was performed with the NCBI ORF finder program (http://www.ncbi.nlm.nih.gov/projects/gorf/). Verification of intron/exon structure in *PaCesA3* was done by comparison of genomic DNA and its corresponding cDNA after reverse transcription. Putative domains of the predicted amino acid sequences were identified using the NCBI Conserved Domain Database (http://www.ncbi.nih.gov/Structure/cdd/wrpsb.cgi) (Marchler-Bauer et al., 2011). Prediction of transmembrane domains was done with the Tmbase program (http://www.ch.embnet.org/software/TMPRED_form.html). The diagram in Fig. 5B representing the predicted transmembrane topology at the C-terminus of PaCesA3 was drawn using TMRPres2D software (http://biophysics.biol.uoa.gr/TMRPres2D/) (Spyropoulos et al., 2004).

2.8. Expression analysis of PaChs and PaCesA genes in different life cycle stages and effect of cellulose inhibitors on gene expression

Total RNA for cDNA synthesis was obtained as described in section *2.4*. cDNA was synthesized from DNA-free RNA (200ng/µl) using OmniScipt® Reverse Transciptase (Quiagen, Hilden, GER) according to the manufacturer's instructions. cDNA was diluted 1:2, and 1 µL was used as template for quantitative Real-Time (qRT-PCR) reactions containing TaqMan® Gene Expression Master Mix (Applied Biosystems), probe (0.25µM) and gene specific primers (0.9µM), in a total volume of 20 µL. All reactions were performed in an ABI Prism® HT-7900 Sequence detection system (Applied Biosystems) with the following conditions: initial hold at 50 °C for 2 min, followed by 10 min at 95 °C, then 40 cycles of 15 sec at 95 °C and 1 min at 60 °C.

qRT-PCR analyses were normalized against the *actA* gene which was previously used as a suitable control gene in *P.* infestans (Avrova et al., 2003; Grenville-Briggs et al., 2008). The *actA* gene showed stable and constitutive expression in all growth stages (mycelium, zoospores, cystospores and germlings) as well as under treatment conditions with cellulose inhibitors in *P. aphanidermatum*, and was therefore chosen as endogenous control. Since the *actA* gene sequence of *P. aphanidermatum* was unknown, we first amplified it by designing consensus primers (Pactaconf and Pactaconr, Supplementary Table.1) according to conserved motifs in *Phytophthora* actA sequences, as described in section *2.5*. 3' and 5'-ends were isolated by genome walking as previously described in section *2.6*. The full-length nucleotide sequence was deposited under GenBank accession no. JN038402.

For the endogenous control gene (*actA*) and for each of the five target genes (*PaCesA1*, *PaCesA2*, *PaCesA3*, *PaCesA4* and *PaChs*) transcript specific primers and TaqMan® probes (Table 2) were designed using Applied Biosystems' primer design pipeline. Amplification efficiency of primers for all five target genes and the endogenous control were tested using a 5-log dilution range (with 5 concentrations) as described in Applied Biosystems User Bulletin 2#. Since amplification efficiency for all assays were equivalent and close to one (0.93 – 1.02) the comparative Ct method ($\Delta\Delta C_t$) was used to calculate relative expression levels (Schmittgen and Livak, 2008). Expression of all five target genes during different life cycle stages was compared with the level of their expression in a calibrator sample, which was cDNA from zoospores. The expression of

PaCesA1 in cDNA of zoospores was assigned the value of 1.0 to allow comparisons to be made between the genes.

For expression studies using cellulose inhibitors, the expression of each gene was calculated relative to the 0.5% DMSO control calibrator sample (after 0.5 h), which was given the arbitrary value 1.0. All qRT-PCR assays, either for the expression of genes throughout the life cycle or during inhibitor assay experiments, were performed on three independent biological samples each with three technical replicates.

Table 2
Primers and probes (TaqMan) used for quantitative Real-Time PCR experiments

Name	Type	Sequences (5`-3`)	Position	Amplicon size (bp)	Gene
RtActAf	Forward	CGTCGCCCTTGACTTCGA	654-672	79	*ActA*
PActA	Probe	CAGCGGCGGTCTTG	681-698		
RtActAr	Reverse	GTCAGGAAGCTCGTACGACTT	712-732		
RtCesA1f	Forward	CACAGGACACGTGGTTCGT	2714-2792	79	*PaCesA1*
PCesA1	Probe	ACGCAGCGAACACAG	2755-2770		
RtCesA1r	Reverse	CGGCCCGTGATCTTCCA	2776-2792		
RtCesA2f	Forward	CGTACCGTCGAGAACAACGA	2604-2624	54	*PaCesA2*
PCesA2	Probe	CATCGTGCGTTCCC	2625-2638		
RtCesA2r	Reverse	GTACGCGAACCAGGTCTCTT	2639-2658		
RtCesA3f	Forward	CCGTGCCGTCGACAGT	2925-2940	65	*PaCesA3*
PCesA3	Probe	CACGCCACACATCGTT	2941-2956		
RtCesA3r	Reverse	TGATGAATGCGTACGAGAACCA	2968-2989		
RtCesA4f	Forward	CCTTCCCTGCCATCTTCTTCTT	2480-2501	93	*PaCesA4*
PCesA4	Probe	CAACGCCCCAATCTA	2532-2546		
RtCesA4r	Reverse	CCATGAGCAGACGCAAACC	2554-2572		
RtChsf	Forward	CCATCCTCACAGTTGGAGTTTACTT	2018-2042	79	*PaChs*
PChs	Probe	CTACACGGCGAAGTAC	2056-2071		
RtChs	Reverse	TGTGTAAACGTGAGCATGATGTGAT	2072-2096		

2.9. Phylogeny

For phylogenetic analyses, several full-length cellulose synthase (CesA) and chitin synthase (CHS) protein sequences of different oomycete species were used (Accession no. in Supplementary Table 2; Supplementary Table 3). Cellulose synthases were obtained by performing a Blastp search with the PiCesA sequences (Accession no. in

Supplementary Table 2) against the non-redundant protein databases from the National Center for Biotechnology (http://blast.ncbi.nlm.nih.gov/), against the *S. parasitica* database at the Broad Institute (http://www.broadinsitute.org/) and against the *P. ultimum* genome database (http://pythium.plantbiology.msu.edu/project_info.html). Identification of chitin synthases was done using the PiCHS sequence (Accession no. XP002908631.1) for Blastp searches against the database of the Department of Energy (DOE) Joint Genome Institute (http://genome.jgi-psf.org) and other databases mentioned above. The Phylogenetic tree containing the CesA sequences was rooted with CesA sequences from the red algae *Griffithsia monilis* and *Porphyra yezoensis* (Accession no. ADK77974, ABX71734). Rooting of the tree containing the CHS sequences was performed with the CHS1 sequence of *Neurospora crassa* (Accession no. M73437). Protein sequence alignments were made with ClustalW 1.38 (Thompson et al., 1994), and phylogenetic analyses were performed with MEGA 5 software (http://www.megasoftware.net/) (Tamura et al., 2011). Trees were constructed with the Maximum likelihood algorithm using the WAG amino acid substitution model (Whelan and Goldman, 2001). The robustness of the resulting branching pattern was tested by 500 bootstrap replications.

3. RESULTS

3.1. Identification of the PaChs and PaCesA genes

In the present study, we identified and characterized the full-length sequence of four *CesA* genes in *P. aphanidermatum*. In addition we also isolated the full-length sequence of a *Chs* gene encoding a putative chitin synthase. Blast searches performed in oomycete genome databases (http://www.broadinstitute.org/, http://www.jgi.doe.gov/, http://pythium.plantbiology.msu.edu/) using the *PiChs* gene sequence from *P. infestans*, revealed the presence of two different *Chs* genes, named *Chs1* and *Chs2*. While the *Saprolegniales* contain both, the *Chs1* and the *Chs2* gene (Badreddine et al., 2008; Guerriero et al., 2010), the *Peronosporales* and the *Pythiales* have according to Blast searches either only the *Chs1* or both (*Chs1* and *Chs2*) genes.

A BlastN search to the NCBI nucleotide database (http://blast.ncbi.nlm.nih.gov/) with the fragments obtained from PCR reactions using *CesA* or *Chs* specific consensus primers (Table 1), led to the identification of four cellulose synthase (*CesA*) genes, named *PaCesA1*, *PaCesA2*, *PaCesA3*, *PaCesA4* and one chitin synthase (*Chs*) gene, named

PaChs in *P. aphanidermatum*. Full-length nucleotide sequences were obtained by genome walking, cloned, fully sequenced and submitted to the GenBank database under accession numbers JN038397 (*PaCesA1*), JN038398 (*PaCesA2*), JN038399 (*PaCesA3*), JN038400 (*PaCesA4*) and JN038401 (*PaChs*).

3.2. Analysis of the PaChs and PaCesA genes

Except for *PaCesA3* including a 76 bp intron near the 5' end, all five genes were intronless. The *PaCesA* genes encoded polypeptides of 1025-1137 amino acids (aa) in length, whereas the translated product of *PaChs* consisted of 901 aa (Fig. 1A). Overall, sequence identity of *PaCesA* gene products ranged from 34% to 72%, with *PaCesA3* being the most divergent member of the family (Table 3). CesA orthologues showed greater identity than paralogues did (Table3), which is in agreement with previous studies (Blum et al., 2010b; Grenville-Briggs et al., 2008; Fugelstad et al., 2009). Compared to their *Phytophthora* and *Pythium* orthologues, sequence identity varied from 74% for CesA1 up to 83% for CesA2 (Table 3). Sequence identity of PaCesAs with *P. ultimum* orthologues was generally higher than with the more distantly related orthologues of *P. infestans* (Table 3). Amino acid sequences deduced from the full-length *PaCesA* genes shared the conserved D,DXD,D,QXXRW motifs (Supplementary Figure 1A) observed in orthologous oomycete CesA sequences (Blum et al., 2010b; Blum et al., 2011; Grenville-Briggs et al., 2008; Fugelstad et al., 2009). This motif is present in most processive glycosyltransferases and occurs in all known CesAs (Campbell et al., 1997). Proteins belonging to the cellulose synthase family are integral transmembrane (TM) proteins. Structural prediction of all four PaCesA proteins with Tmpred software (http://www.ch.embnet.org/software/TMPRED_form.html) revealed the presence of two or more TM domains at the N-terminal end and several TM segments at the C-terminus (Fig. 1A). Interestingly, six additional TM domains were predicted at the N-terminal end of PaCesA3, which seem to be unique for oomycete CesA3 proteins. While in PaCesA1, PaCesA2 and PaCesA4 a Pleckstrin homology (PH) domain (cd00821, http://www. Ncbi.nlm.nih.gov) was present at the N-terminus, this domain did not occur in PaCesA3 (Fig. 1A). In contrast, a CesA3 specific domain was present in *P. aphanidermatum* (Supplementary Fig. 1C), that has previously been identified in CesA3 of *S. monoica* (Fugelstad et al., 2009). This domain contains two CxxC motifs that are characteristic for Ring-Finger domains in plant cellulose synthases (Kurek et al., 2002).

Table 3
Sequence identity at the amino acid level of CesA and CHS proteins in *P. aphanidermatum*, *P. infestans* and *P. ultimum.*

Protein	*Pythium aphanidermatum* (Pa) CesAs				*Phytophthora infestans* (Pi) CesAs/CHS					*Pythium ultimum* (Pu) CesAs/CHS				
	PaCesA1	PaCesA2	PaCesA3	PaCesA4	PiCesA1	PiCesA2	PiCesA3	PiCesA4	PiCHS	PuCesA1	PuCesA2	PuCesA3	PuCesA4	PuCHS
%														
PaCesA1	100[a]	-	-	-	**74[b]**	70	33	50	-	**77**	71	33	51	-
PaCesA2	72	100	-	-	73	**80**	36	53	-	74	**83**	36	53	-
PaCesA3	34	36	100	-	36	37	**78**	37	-	35	36	**82**	37	-
PaCesA4	49	53	38	100	50	52	38	**79**	-	51	52	38	**82**	**-**
PaCHS	-[c]	-	-	-	-	-	-	**-**	**73**	-	-	-	**-**	**74**

[a]Identities are expressed as percentage, based on pairwise protein alignment.
[b]Values highlighted in bold indicate orthologous sequences between *P. aphanidermatum*, *P. infestans* and *P. ultimum*.
[c]Sequence alignments of CHS with CesA sequences were poor, thus identities are not shown.

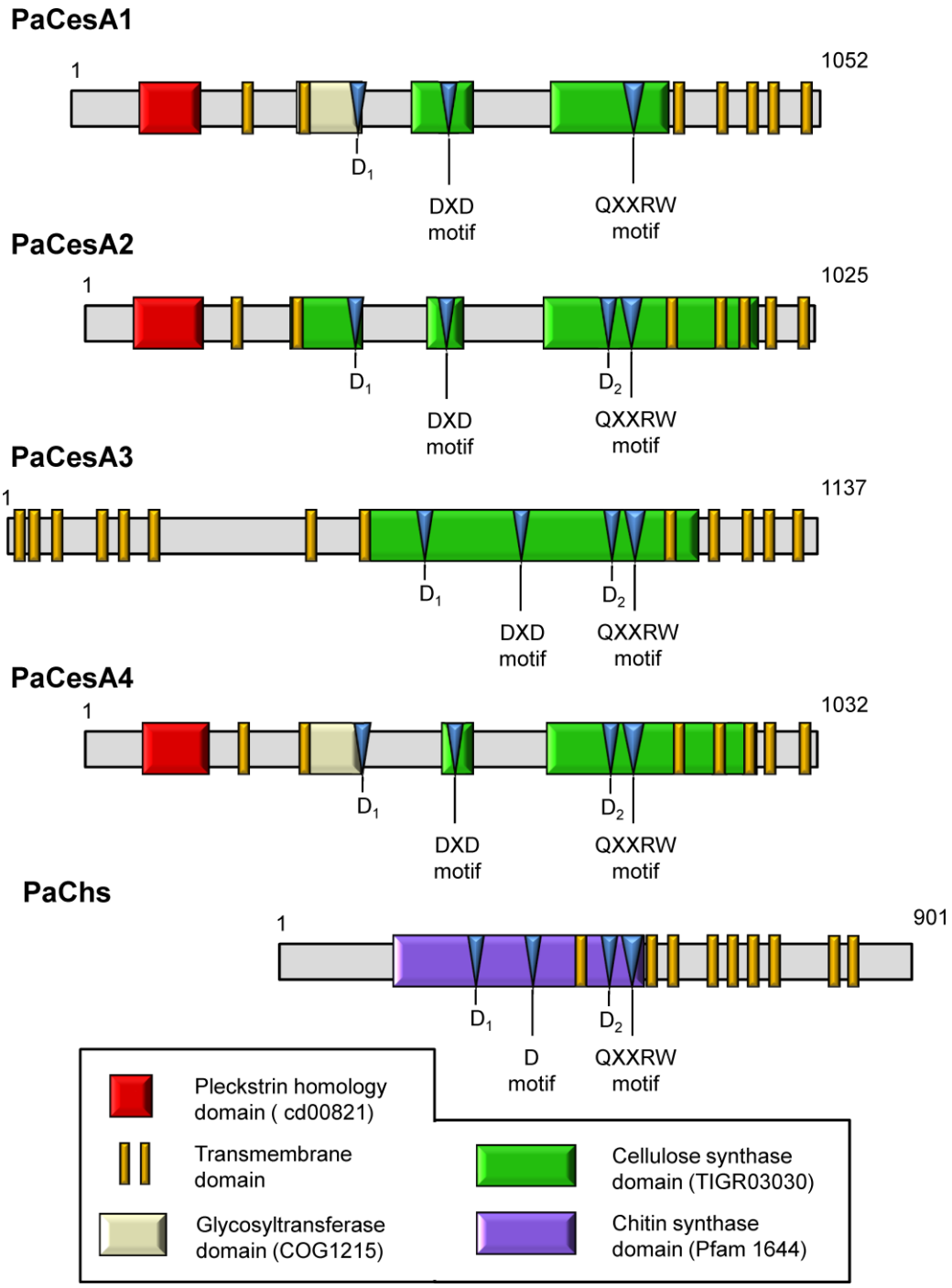
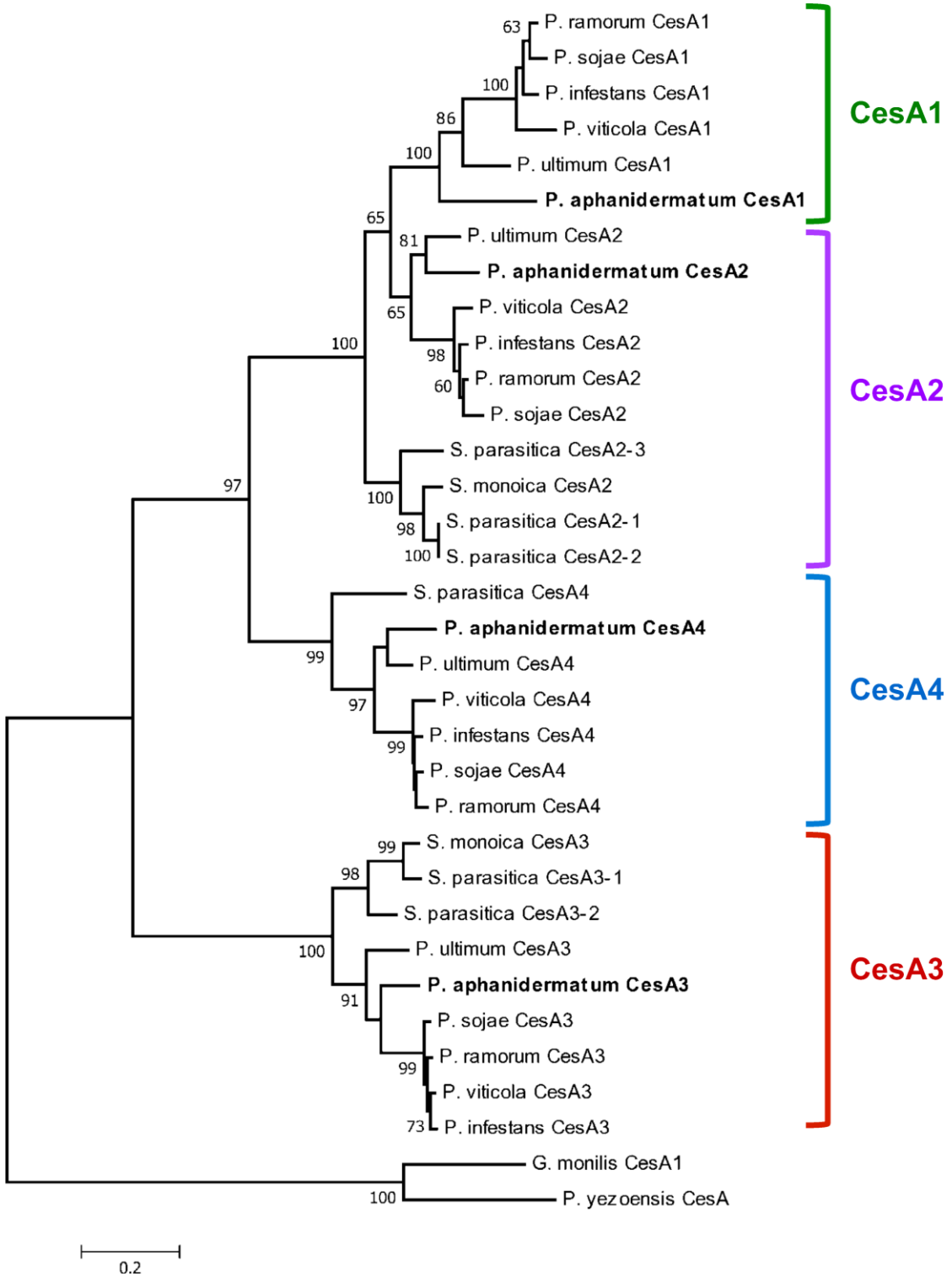
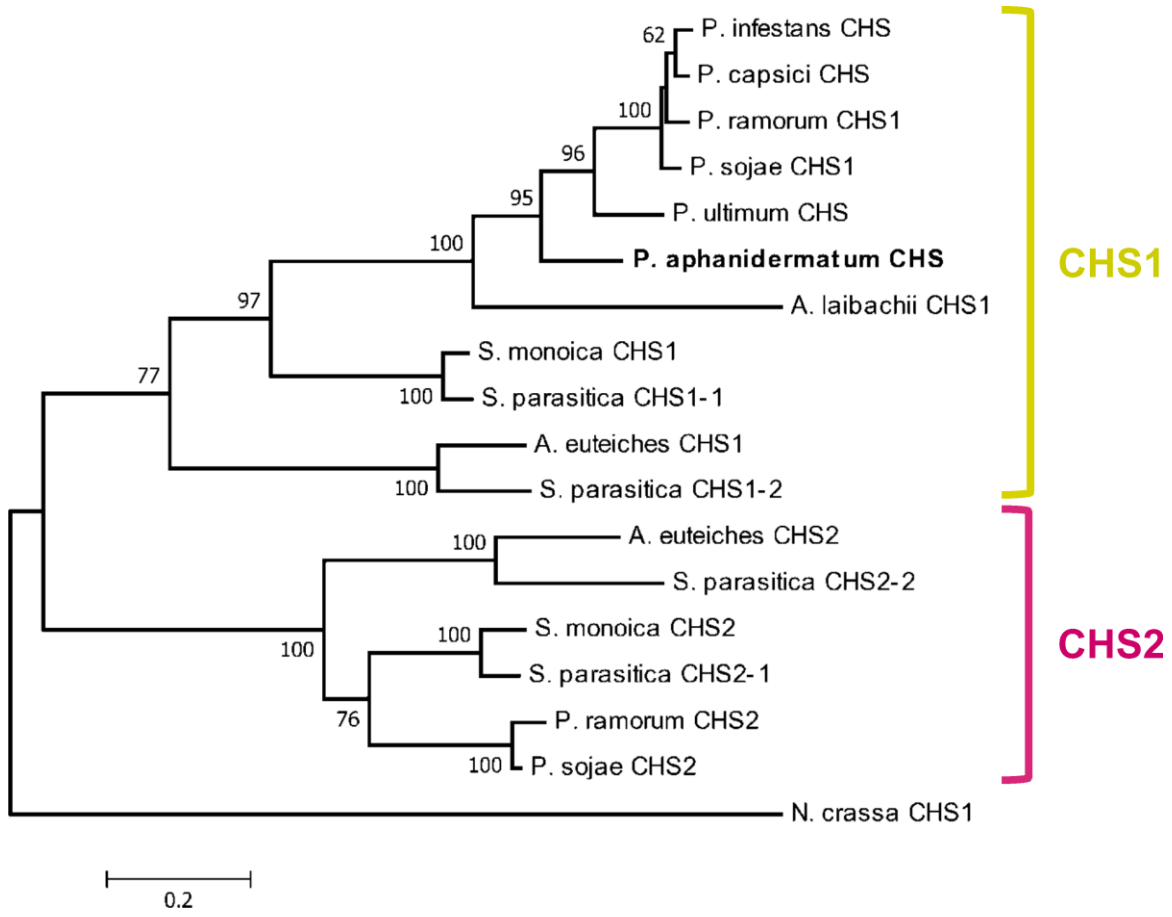


Fig. 1. Sequence analyses of the *PaChs* and *PaCesA* genes in *P. aphanidermatum*.
(**A**) Domain organization of the putative chitin (CHS) and cellulose synthases (CesAs). Sequences are aligned according to the position of the QXXRW motif. A CesA specific Pleckstrin homology domain is predicted at the N-terminus of PaCesA1, PaCesA2 and PaCesA4. The N-terminal end of PaCesA3 is characterized by 6 additional transmembrane (TM) domains. CesA proteins contain domains similar to cellulose synthases. The predicted chitin synthase domain in the CHS sequence is followed by several TM domains.



(**B**) Phylogenetic relationships between *P. aphanidermatum* CesAs (indicated in bold) and CesAs originating from related members of *Peronosporales*, *Pythiales* and *Saprolegniales*. The phylogram was produced using Mega 5 with a Maximum Likelihood algorithm and 500 bootstrap replicates. The analysis is based on the complete CesA amino acid (aa) sequence of various oomycetes (Accession numbers in Supplementary Table 2). Rooting of the tree was performed with CesAs of the two red algae *Griffithsia monilis* and *Porphyra yezoensis* (Accession no. ADK77974, ABX71734). Bootstrap values are shown if > 60. Scale bar indicates the relative length of each branch proportional to the number of amino acid changes.



(**C**) Phylogenetic analyses of *P. aphanidermatum* PaCHS (indicated in bold) with chitin synthases (CHS) originating from related members of *Albuginales*, *Peronosporales*, *Pythiales* and *Saprolegniales*. The analysis is based on the complete CHS amino acid (aa) sequence of various oomycetes (Accession numbers in Supplementary Table 3). The tree was rooted with the CHS sequence of *Neurospora crassa* (Accession no. M73437).

Phylogenetic analysis with other members of the oomycete CesA family revealed the formation of five different clades (Fig. 1B). Each clade represents one of the four oomycete *CesA* genes. The four PaCesA sequences of *P. aphanidermatum* each clustered together with their CesA orthologues originating from *Pythiales* and *Peronosporales* in one distinct clade (Fig. 1B). The CesA2 sequences of *Saprolegniales* acted as outgroup for both CesA1 and CesA2.

The putative chitin synthase encoded by *PaChs* showed up to 74% identity at the amino acid level with its orthologous sequences from *Phytophthora* and *Pythium* (Table 3), but was more dissimilar to members of the CHS2 type (data not shown). In PaCHS, a catalytic chitin synthase domain (pfam 01644) and several CHS specific motifs (motifs a – e in Supplementary Fig. 1B) were present that are characteristic for yeast and fungal CHS proteins (Ruiz and Ortiz, 2010). In addition, the PaCHS protein contained the D,D,D,QXXRW signature (Fig. 1A, Supplementary Fig1B), present in most processive

glycosyltransferases (Campbell et al., 1997), indicating that PaCHS could probably act as N-acetylglucosaminyltransferase. Structural prediction with TMpred software revealed the presence of nine TM domains, eight of which located towards the C- terminus next to the chitin synthase pfam domain 01644 (Fig. 1A).

Phylogenetic analysis with other CHS sequences revealed the formation of two clusters which could be divided in CHS1 and CHS2 (Fig. 1C). The PaCHS protein grouped in the "CHS1" cluster, in a distinct clade, together with CHS1 and CHS sequences from *Peronosporales*, *Pythiales* and *Albuginales*. The CHS1 sequences of the *Saprolegniales* also grouped in the CHS1 cluster, but in separate clades (Fig. 1C). The second cluster "CHS2" included CHS2 sequences of *Saprolegniales* and of *Peronosporales*.

3.3. Expression of the PaChs and PaCesA genes in different life cycle stages

The expression profiles of the four cellulose synthase (*CesA*) genes, in particular *PaCesA3*, and the chitin synthase (*Chs*) gene were assessed in the life cycle of *P. aphanidermatum* by quantitative Real-Time PCR (qRT-PCR) using gene specific TaqMan probes. The expression of each gene was compared to the level of *PaCesA1* expression in zoospores. *PaCesA1* and *PaCesA2* were upregulated during cystospore formation (~1.6-fold, and ~1.7-fold, respectively). In zoospores and germlings these genes were similarly expressed, but both less pronounced than in cystospores (Fig. 2). Both transcripts were downregulated in mycelium (~2-fold). *PaCesA3* appeared to be upregulated in cystospores, germlings and mycelium (~2.4-fold, ~1.4-fold, and ~2.8 fold, respectively). Out of the four *PaCesAs*, *PaCesA3* was the most dominantly expressed gene in mycelium compared to *PaCesA1*, *PaCesA2* and *PaCesA4* (~4-fold, ~2.9-fold and ~5-fold, respectively) (Fig. 2). *PaCesA4* was expressed at comparatively low levels and seemed to be slightly upregulated only in mycelium (~2-fold).

The expression pattern of the *PaChs* gene differed from the *PaCesA* genes. *PaChs* was upregulated in zoospores and cystospores, but hardly expressed in germlings and mycelium. Transcript level in zoospores and cystospores was increased ~46 fold and ~81 fold, respectively, compared to mycelium (Fig. 2). Due to the low expression in germlings and mycelium, the *PaChs* gene seems to be less important at these stages than *PaCesAs*. Compared to *PaCesA3* the expression of the *PaChs* gene was ~200-fold lower in germlings and ~390-fold lower in mycelium. Generally, results obtained by qRT-PCR

demonstrated that the *PaCesA* genes were expressed during different stages in the life cycle of *P. aphanidermatum*.

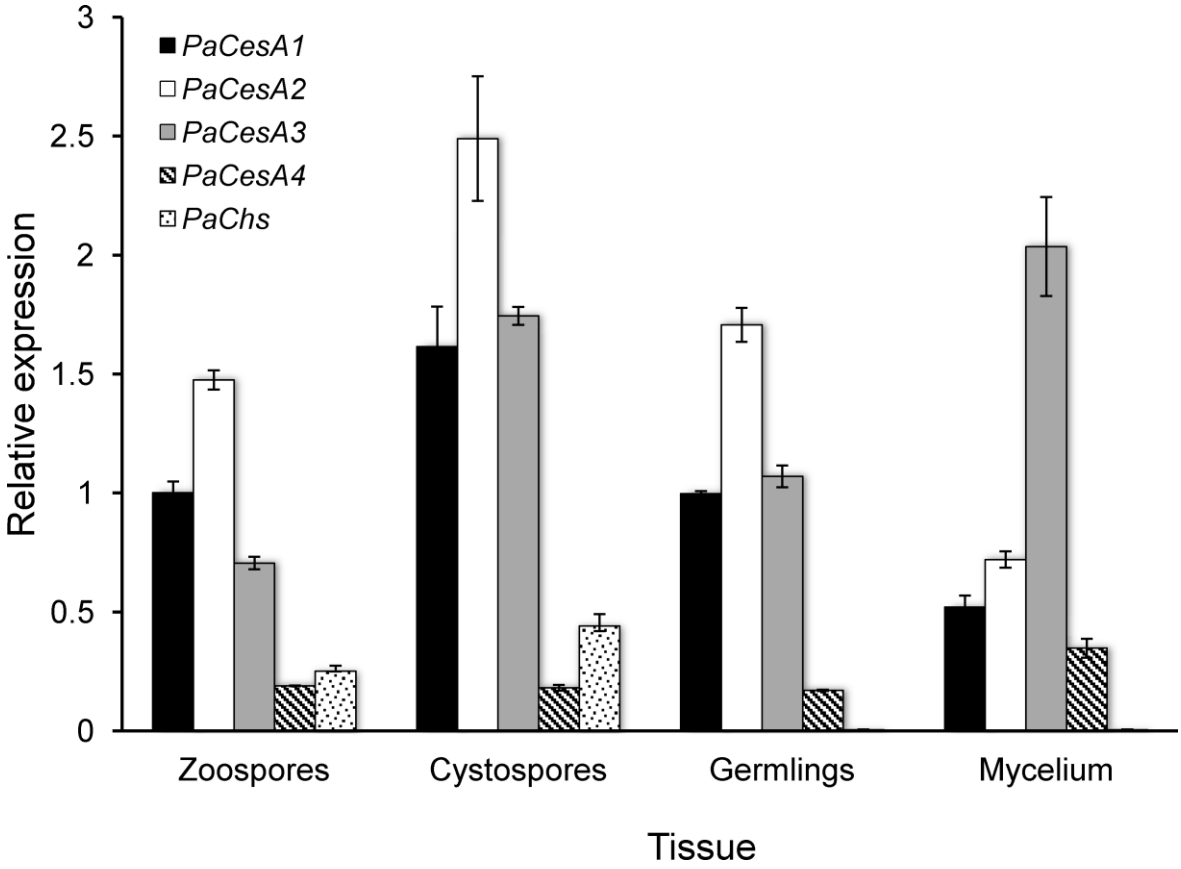


Fig. 2. Gene expression of chitin synthase (*Chs*) and cellulose synthase (*CesA*) genes in different life cycle stages of *P. aphanidermatum*. Expression of each sample is relative to the expression of *PaCesA1* in zoospores, which have been assigned the value of 1. Normalization was performed using the constitutively expressed *actA* gene (JN038402) as a reference. Each value represents the mean of three independent biological samples each with three technical replicas. Bars represent Standard errors.

3.4. Effect of the cellulose inhibitors MPD and DCB on mycelial growth and germ tube elongation

The fungicide mandipropamid (MPD) and the herbicide 2,6 Dichlorobenzonitrile (DCB) are both described as cellulose synthesis inhibitors acting at micromolar levels (Blum et al., 2010a; Montezinos and Delmer, 1980). After a three day incubation period, mycelial growth of *P. aphanidermatum* was hardly affected in presence of 10 µM MPD. However, at 100 µM, mycelial growth was reduced by ~34% compared to the untreated control (0.5% DMSO) (Fig. 3A). Unlike *S. monoica*, *P. aphanidermatum* is highly tolerant to DCB. Mycelium grown on V8 agar plates amended with 200 µM DCB was reduced by ~12% (Fig. 3A), compared to ~40% as observed in *S. monoica* (Fugelstad et al., 2009).

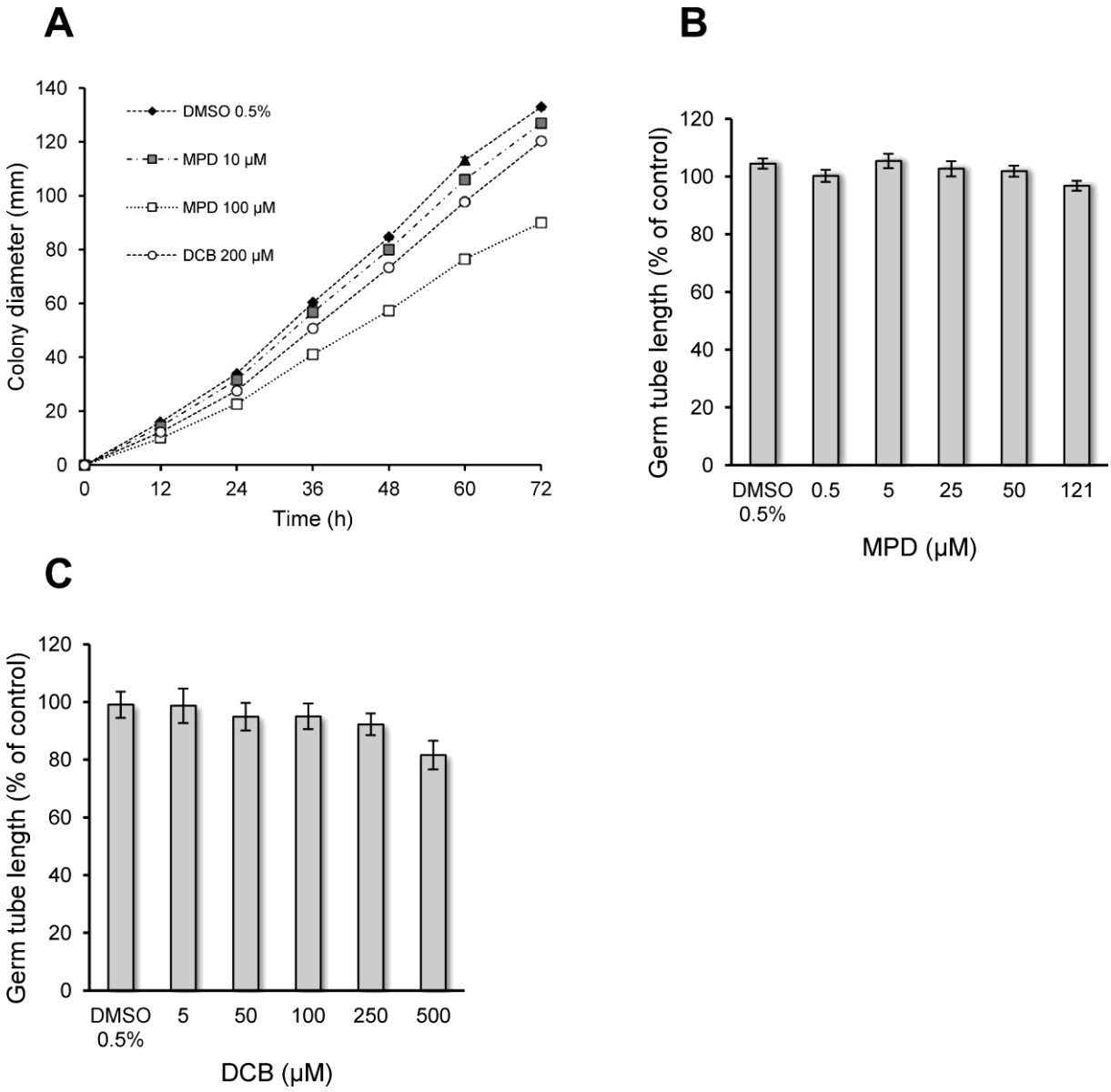


Fig. 3. Effect of cellulose inhibitors on radial growth and germ tube elongation in *P. aphanidermatum*. (**A**) Radial growth (colony diameter, mm) on V8 agar media either amended with 10 µM mandipropamid (MPD), 100 µM MPD, or 200 µM 2,6-dichlorobenzonitrile (DCB) and an untreated control with 0.5% dimethylsulfoxide (DMSO). Data are mean of three biological replicates. Standard errors are too small to show. (**B**) Germ tube length of germlings in relation to untreated controls incubated for 4h at room temperature in 0.5% DMSO or at different MPD concentrations (0.5 to 121 µM). Bars represent Standard errors calculated for three biological replicates each containing 30 germ tube measurements. (**C**) Germ tube length of germlings in relation to untreated controls incubated for 4h at room temperature in 0.5% DMSO or at different DCB concentrations (5 to 500 µM). Error bars represent Standard errors calculated for three biological replicates each containing 30 germ tube measurements.

To test the effect of MPD on germ tube elongation in *P. aphanidermatum*, synchronized encystment of zoospores was induced by vortexing and germ tube length measured after a 4h incubation period. Germ tube growth was hardly affected by any MPD concentration tested. Even the highest possible concentration (121 µM, limit of solubility) did not inhibit germ tube growth (Fig. 3B).

Concentrations of 40-100 µM DCB provoked a severe reduction of zoospore release and complete inhibition of cystospore germination in *P. infestans* (Grenville-Briggs et al., 2008), therefore, similar effects were expected for *P. aphanidermatum*. However, 100 µM DCB had no effect on zoospore release, nor on encystment (data not shown). Germ tube elongation was only slightly affected at very high DCB concentrations (>100 µM). Treatment of germinating cystospores with 500 µM DCB led to a reduction in germ tube length by ~18% (Fig.3C.)

3.5. Effect of MPD and DCB on PaChs and PaCesA gene expression

To test whether *P. aphanidermatum* is able to counteract inhibitor action by overexpressing the *PaCesA* genes or the *PaChs* gene, mycelium was challenged with inhibitor dosages that visually affected mycelial growth (see methods).

Even though the expression of the *PaCesA* genes was rather constant over time in the untreated control samples (0.5% DMSO), slight fluctuations were observed for the *PaChs* gene in untreated controls (Fig. 4). Expression of the *PaCesA3* gene, encoding the MPD target enzyme, remained nearly unchanged after MPD treatment at all time points (Fig. 4), suggesting that tolerance to MPD does not result from target enzyme overexpression. Transcript levels of *PaCesA4* and *PaChs* were also nearly unaffected by MPD (Fig. 4) indicating that there is no cell salvage mechanism in *P. aphanidermatum* enabling the formation of chitin instead of cellulose. However, MPD provoked a slight upregulation of *PaCesA1* and *PaCesA2.* Upregulation for *PaCesA1* (~1.6-fold) and *PaCesA2* (~2-fold) was highest after 8h (compared to the DMSO control) (Fig.4).

In *S. monoica* DCB induced a general upregulation of *CesA* genes (Fugelstad et al., 2009). In *P. aphanidermatum*, however, DCB led to a slight downregulation of all four *CesA* genes during the first three time points (0.5, 2 and 4 h) (Fig. 4). After 8 h, expression levels of DCB treated samples equalled those of untreated controls (Fig. 4). *PaChs* transcript levels were rather constant over the 8h time period without generally exceeding the levels of the DMSO control (Fig. 4). Thus, DCB did not affect *PaChs* gene expression.

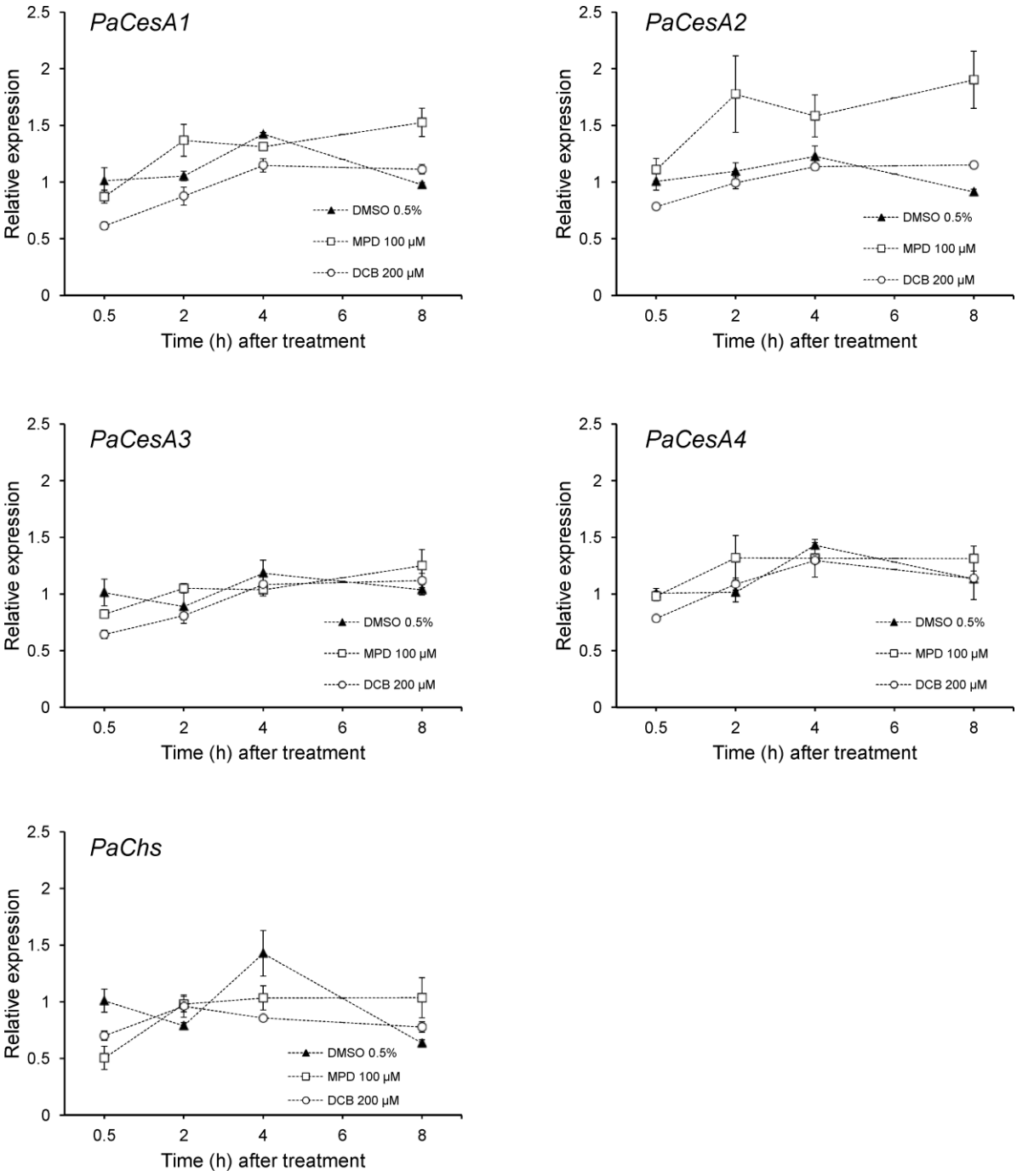


Fig. 4. Effect of the cellulose inhibitors DCB and MPD on *PaChs* and *PaCesA* gene expression in *P. aphanidermatum*. Expression was determined by qRT-PCR. For normalization the constitutively expressed *actA* gene was used. Expression levels were measured at 0.5, 2, 4 and 8 hours and calibrated to the 0.5 h control (0.5% DMSO) expression value for each gene. Values represent mean of three independent samples, each with three technical replicas. Bars represent Standard errors.

3.6. Amino acid configuration in the cellulose synthase 3 (CesA3) target site of MPD

Reduced fungicide activity is in many cases caused by mutations in the drug target enzyme (Leroux and Walker, 2010; Sierotzki and Gisi, 2003). In *Peronosporales*, mutations were detected in the CesA3 enzyme leading to resistance towards MPD (Fig 5A) (Blum et al., 2010a; Blum et al., 2010b; Blum et al., 2011; Sierotzki et al., 2011; Chen et al., 2011). Analysis of CesA3 sequences from *Peronosporales* and *Pythiales* revealed that the region where these mutations occur is highly conserved between the two orders. In both orders, a predicted transmembrane (TM) domain (17 to 28 amino acids in length, Fig. 5A) characterizes the region.

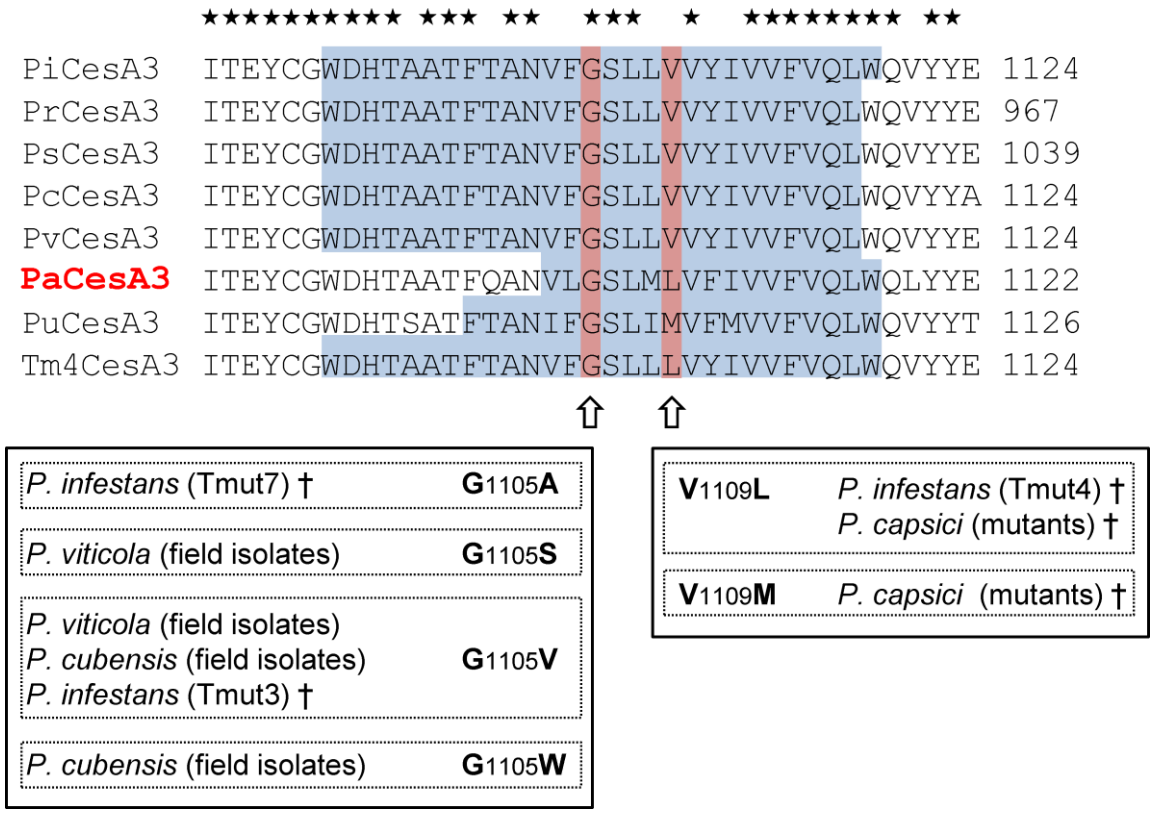
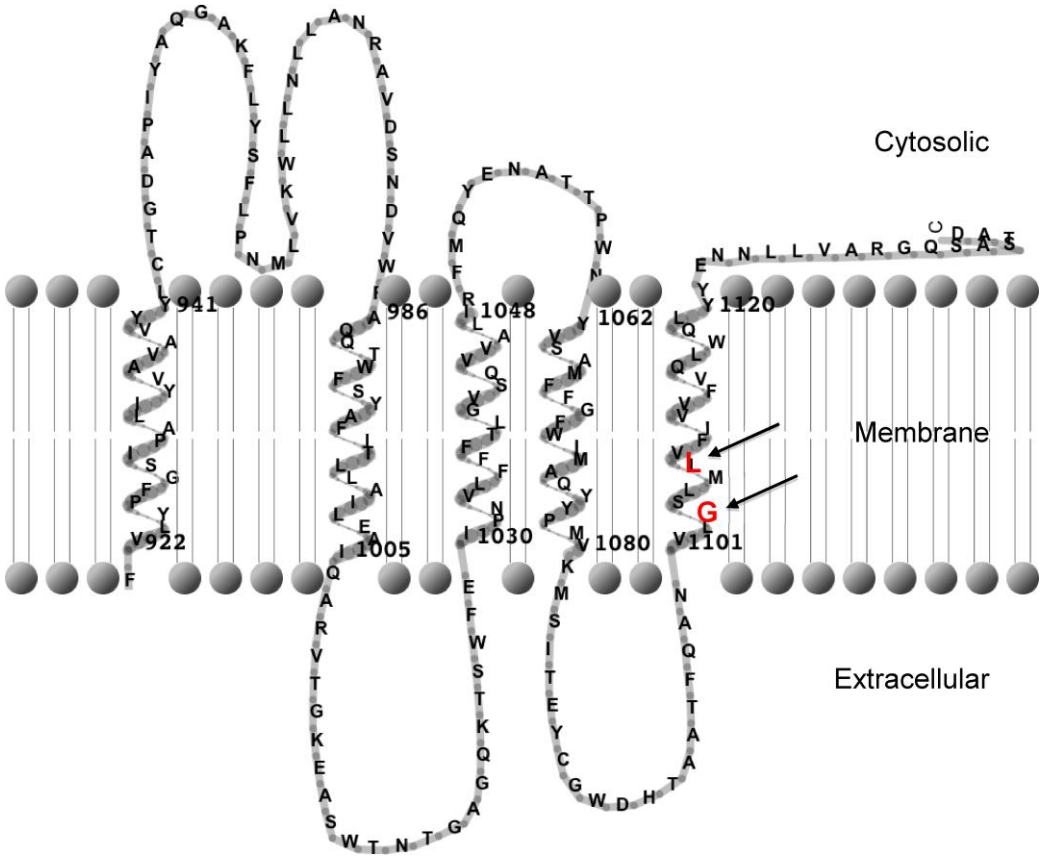


Fig. 5. Analyses of CesA3 sequences comprising the MPD target site.
(**A**) Multiple alignment of the C-terminal amino acid sequences of CesA3s from *Phytophthora infestans* (Pi), *Phytophthora sojae (*Ps*)*, *Phytophthora ramorum* (Pr), *Plasmopara viticola* (Pv), *Pseudoperonospora cubensis* (Pc), *Pythium aphanidermatum* (Pa), *Pythium ultimum* (Pu) and the artificially generated *P. infestans* mutant Tmut4. Numbers indicate the positions of the last amino acid present in the corresponding sequence. The last predicted transmembrane (TM) domain of each sequence is shaded in blue. The amino acid positions responsible for MPD resistance/tolerance are highlighted in red. The different amino acid substitutions leading to MPD resistance at position 1105 and 1109 in field isolates and in artificial mutants (†) are shown below the alignment. Identical residues are marked by asterisks.

Like the CAA sensitive *Peronosporales*, both *P. aphanidermatum* and *P. ultimum,* displayed the strictly conserved amino acid glycine at position 1105 (Fig. 5A). A substitution of this glycine residue confers resistance to CAA fungicides in *Peronosporales* (Blum et al., 2010a; Blum et al., 2010b; Blum et al., 2011; Sierotzki et al., 2011; Chen et al., 2011). However, at position 1108, 1109 and 1111, both *Pythium* species revealed amino acid configurations that differ from *Peronosporales* (Fig. 5A). The configuration at position 1109 is of special interest because a leucine in *P. aphanidermatum* and a methionine in *P. ultimum* occur instead of a valine as in *Peronosporales* (Fig. 5A). Similar to *P. aphanidermatum* the artificially mutated (CAA resistant) *P. infestans* isolate, Tmut4, displays the amino acid L1109 instead of V1109 (Fig. 5A). According to the hypothetical transmembrane 2-D topology model that was created using TMRPres2D software (Spyropoulos et al., 2004), L1109 is located close to the G1105 residue, in the same predicted alpha-helix forming the last TM domain of oomycete CesA3 proteins (Fig. 5A; Fig. 5B).



(**B**) 2-D topology model of the C-terminal end of PaCesA3 in *P. aphanidermatum*. Several transmembrane (TM) domains are predicted at the C-terminus. The conserved amino acid glycine and the amino acid leucine probably leading to MPD tolerance (marked by arrows) are located in the last out of the 13 predicted TM domains.

4. DISCUSSION

Tolerance to carboxylic acid amide fungicides has been described in several *Pythium* species (Albert et al., 1988; Gisi and Sierotzki, 2008; Kuhn et al., 1991), but little is known about its molecular mechanism. A previous study demonstrated that the CAA fungicide MPD targets the CesA3 enzyme in *P. infestans* (Blum et al., 2010a), which is involved in cellulose biosynthesis (Grenville-Briggs et al., 2008). The present study identified and characterized five genes (*PaCesA1* to *PaCesA4* and *PaChs*) putatively involved in carbohydrate synthesis to investigate the underlying tolerance mechanism to MPD in *P. aphanidermatum*. The results demonstrate that *P. aphanidermatum* contains at least four cellulose synthase (*CesA)* genes and at least one chitin synthase (*Chs*) gene. So far, four orthologous *CesA* sequences have been identified in *Phytophthora*, *Plasmopara, Pseudoperonospra* and *Pythium* species (Blum et al., 2010b; Blum et al., 2011; Grenville-Briggs et al., 2008; Levesque et al., 2010). In contrast, species of the genus *Saprolegnia* contain either three (*S. monoica*) or six (*S. parasitica*) *CesA* genes with *CesA1* being absent (Fugelstad et al., 2009). However, in contrast to *S. parasitica*, evidence for the existence of additional *CesA* paralogues in *P. aphanidermatum* is lacking. Various primers matching conserved CesA motifs failed to amplify paralogues of the four *CesA* genes (data not shown). Therefore it appears that *P. aphanidermatum* only has four *CesA* genes like species of the *Peronosporales* and *Pythiales* order. The four *PaCesA* genes in *P. aphanidermatum* are highly identical at the amino acid level to its *Phytophthora* orthologues (≥ 74%, Table 3) and share a similar domain structure, indicating that they are probably involved in cellulose biosynthesis as previously demonstrated for the orthologous *CesA* genes in *P.* infestans (Grenville-Briggs et al., 2008). The translated amino acid sequence of the identified *PaChs* gene displays conserved motifs (Supplementary Fig. 1B) also present in fungal chitin synthases (Ruiz and Ortiz, 2010) thus, it is likely that *PaChs* is involved in chitin synthesis of *P. aphanidermatum*. Oomycete cell walls generally contain little or no chitin (Badreddine et al., 2008; Bartnicki-Garcia, 1968; Cherif et al., 1993). However, in *S. monoica* the *Chs* genes are involved in chitin synthesis and probably play an important role for cell wall integrity (Guerriero et al., 2010). Even though both *Peronosporales* and *Pythiales* also contain *Chs* genes, it remains to be demonstrated whether they are directly involved in

the synthesis of chitin or other GlcNAc-based carbohydrates and if these components play a similar role in the cell wall as in *S. monoica*.

Cell wall synthesis in oomycetes is a well-regulated process and specific gene expression is closely related to the corresponding stage in the life cycle (Judelson et al., 2008), as shown for the *CesA* genes in *P. infestans* (Grenville-Briggs et al., 2008). However, no such data is available for *Pythium* species and it is unknown if the expression pattern of *PaCesA* genes is similar to the *CesAs* of *P. infestans*. The present study demonstrated that all four *PaCesA* genes are expressed during different stages in the life cycle of *P. aphanidermatum* (Fig. 2). Similar to *Phytophthora* and *Saprolegnia* species (Grenville-Briggs et al., 2008; Fugelstad et al., 2009) the expression of the *PaCesA3* gene in mycelium is the highest among *CesA* homologues in *P. aphanidermatum*, suggesting that it may play an important role for cell wall formation in *Pythium* species. Due to its higher expression in mycelium but lower expression levels in cystospores and germlings, compared to its homologues *PaCesA1* and *PaCesA2,* one could speculate that multiple CesA3 subunits may form a complex that is predominantly used for cellulose synthesis during mycelial growth, whereas CesA1 and CesA2 may form a complex that is mainly involved in cellulose synthesis during cystospore cell wall formation and germination. However, since it is unknown whether the oomycete CesA complex is composed of the four different CesAs (CesA1 to 4) or whether each CesA isoform itself is able to form a multi subunit complex, it remains to be demonstrated if the composition of the CesA complex varies during the different stages in the life cycle. Even though the gene expression experiments of the current study suggest that the MPD target enzyme (CesA3) is likely to be present in all life cycle stages of *P. aphanidermatum*, thus being de facto a potential target for MPD, it remains unknown whether the mRNA level actually reflect protein levels.

Expression profiles obtained for *PaChs* encoding a putative chitin synthase demonstrated that this gene is expressed particularly in zoospores and cystospores, but hardly in germlings and mycelium. Therefore, chitin or other GlcNac-based carbohydrates may be important for cell wall formation especially during encystment of zoospores but not in mycelium. Based on the low expression of *PaChs* in mycelium, chitin is unlikely to be a major component in the cell wall of *P. aphanidermatum* contrary to *P. ultimum* (Cherif et al., 1993).

Our data reveal that *P. aphanidermatum* is highly tolerant to both cellulose inhibitors used in this study. Mycelial growth was completely inhibited by MPD concentrations of 0.35 µM in *P. infestans* (Blum et al., 2010a). However, in *P. aphanidermatum*, concentrations of up to 100 µM provoked only a slight growth reduction by 34% (Fig. 3A). Thus, we can conclude that (i) mycelium of *P. aphanidermatum* is able to grow at ~300-fold the minimum inhibitory dose of MPD that was determined for *P. infestans*; (ii) MPD, causing slight growth reduction, does reach the PaCesA3 target enzyme but fails somehow to significantly inhibit enzyme activity.

Cystospore germination is the most susceptible stage to CAAs in *Peronosporales* (Cohen and Gisi, 2007; Cohen et al., 2008; Kim et al., 2009; Knauf-Beiter and Hermann, 2005), but is hardly affected in *P. aphanidermatum*. Thus *PaCesA3* (unlike *PiCesA3*) may not be as important for cystospore germination in *P. aphanidermatum* as indicated by the *CesA* gene expression profiles (Fig. 2). Concentrations of 200 µM DCB only partially affected mycelial growth and cystospore germination (Fig. 3A, Fig. 3C) in *P. aphanidermatum*. Similarly the mycelium of *P.* infestans was hardly affected by DCB (Grenville-Briggs et al., 2008), whereas mycelial growth of *S. monoica* was strongly affected by 200 µM DCB (Fugelstad et al., 2009).

It is unlikely that MPD tolerance is the result of cell wall salvage mechanisms leading to compensatory formation of chitin or other GlcNAc-based carbohydrates in the cell wall of *P. aphanidermatum*, because MPD had hardly any effect on *PaChs* gene expression. However, in several fungal species, cell wall salvage upon echinocandin treatment is an important switch mechanism resulting in chitin instead of β-(1,3)-glucan synthesis, ultimately leading to enhanced drug tolerance (Stevens et al., 2006; Walker et al., 2010). Although *PaCesA3* gene expression was not affected by MPD treatment (Fig. 4), other CesAs might compensated for the reduced CesA3 activity. Indeed, we observed a slight upregulation of *PaCesA1* and *PaCesA2*. However, as long as the specific role for each of the four *CesAs* is not determined, it is unclear whether *PaCesA1* or *PaCesA2* can compensate for *PaCesA3*.

MPD neither induced *PaCesA3* expression nor strongly upregulated *PaCesA* homologues, thus an alteration in the CesA3 target enzyme is postulated that may prevent proper binding of the inhibitor molecule. This hypothesis is supported by the observed magnitude of tolerance to MPD in *P. aphanidermatum*. Sensitivity shifts of about 10 to 100 fold are often the result of target gene overexpression or altered efflux

mediated by upregulation of efflux pumps (ABC transporters) (Cannon et al., 2009; Hamomoto et al., 2000; Judelson and Senthil, 2006), whereas mutations in the target site most likely cause sensitivity shifts of up to 1000 fold (Ma ZH and Michailides, 2005; Deising et al., 2008).

Target site mutations at position 1105 in CesA3 causing high levels of CAA resistance were described in field isolates of *P. viticola* and *P. cubensis* (Blum et al., 2010b; Blum et al., 2011; Sierotzki et al., 2011) In the same codon, an additional mutation (G1105A) was artificially generated by EMS mutagenesis in *P. infestans* (Blum et al., 2010a), and by UV mutagenesis in *Phytophthora capsici* (Chen et al., 2011). Further mutations leading to a substitution of the conserved valine by leucine or methionine at position 1109 (V1109L, V1109M) were induced artificially by UV and EMS mutagenesis in *P. infestans* (Syngenta internal data), *P. capsici* and *Phytophthora melonis* isolates (Chen et al., 2011), all of them conferring CAA resistance. These mutations all occur in a highly conserved region at the C-terminus in a putative TM domain (Fig. 5A). Since Gly 1105 seems to be conserved in *Pythium* species (Fig. 5A), other amino acid changes may be the cause for MPD tolerance in *P. aphanidermatum*. Interestingly, like in the artificially mutated (CAA resistant) *P. infestans* isolate, Tmut4, the amino acid leucine is present at position 1109 in *P. aphanidermatum* (Fig. 5A), suggesting that this amino acid configuration may be responsible for MPD tolerance. Sequence analysis of the CesA3 target site in two other *P. aphanidermatum* isolates (126, 186) also revealed a leucine residue at position 1109 (data not shown). Therefore, contrary to Peronosporales that contain the amino acid valine at this position, L1109 is obviously the inherent amino acid configuration in *P. aphanidermatum*. However, only a single point mutation in codon 1109 (CTG) is required to change this leucine residue to methionine, valine, glutamine, arginine or proline. Whereas methionine and valine naturally occur at position 1109 in CesA3, it is unknown whether the enzyme may also tolerate glutamine, arginine or proline since their chemical properties are quite different. Currently it remains unknown if additional amino acid positions (like 1108 or 1111) could also be responsible for MPD tolerance in *P. aphanidermatum*. However, so far, CAA resistant strains of sensitive species either carried mutations at position 1105 or 1109, but not at any other position, implying that these are the most prominent positions affecting sensitivity to CAA fungicides. An additional aspect that has not been covered in this study concerns the tertiary structure of the CesA3 enzyme that might also affect

fungicide binding. Even though the sequence identity of PaCesA3 is rather high compared to CesA3 sequences of the CAA sensitive *Peronosporales*, it remains unclear, whether the transmembrane topology or the folding of the protein might be different in *P. aphanidermatum*.

In plants, several attempts to characterize intact CesA complexes failed due to their instability upon extraction from the plasma membrane (Delmer, 1999). As a consequence, the three-dimensional structure of any cellulose synthase remains unknown. The absence of a three-dimensional CesA enzyme model in oomycetes prohibits predicting the influence of amino acid changes at positions 1105 and 1109 on the structural integrity of the protein. Thus, we only can speculate that alterations at position 1109 may cause changes in the three-dimensional structure of the target site possibly leading to reduced fungicide binding and MPD tolerance in *P. aphanidermatum*. However, the present study does not provide direct proof of concept for MPD tolerance caused by the L1109 configuration in PaCesA3. Site directed mutagenesis to induce the (MPD sensitive) V1109 configuration, followed by gene replacement, could be an accurate way to address our hypothesis. However, such experiments are extremely challenging in a diploid organism such as *Pythium* in which homologous recombination does not seem to occur (Latijnhouwers et al., 2003). Alternatively, the *CesA3* gene of various oomycetes should be sequenced and their response to MPD be tested, to confirm whether a substitution of V1109 by L1109 or M1109 always correlates with MPD tolerance (investigations in progress). Nevertheless, according to the data obtained in this study and according to the mutagenesis studies performed in *P. capsici* and *P. melonis* (Chen et al., 2011) we consider it worthwhile to establish molecular tests (e.g. Q-PCR, Pyrosequencing or PCR-RFLP) that cover in addition to position 1105 (Aoki et al., 2011), also position 1109 in CesA3. This certainly will help to monitor CAA resistance in field populations and consequently improves disease management.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1

Oligonucleotide primer pairs, sequencing primers, and amplicon sizes used in template preparation for sequencing and DNA walking

Gene	Primer Name	Primer Sequence (5'-3')	Application	Amplicon Size (bp)
PaCesA1	Pcesa1f	GTTGACTTCTAGCGAGTCGTCATGTTCGG	PCR	3345
	Pcesa1r	GCTCGCTACCACGAATGTCTAGATAAGC	PCR	
	Pcesa1seqr	AATGGCATCCTCAACACCT	Sequencing	
	Pcesa1seqf1	GATCTTGTCGTTGGTGCT	Sequencing	
	Pcesa1seqf2	AAGGTCGATGTTCTCATCTG	Sequencing	
	Pcesa1seqf3	GTGTTCCTGAAGAACGCA	Sequencing	
	Pcesa1seqf4	AAGTTGCCATCCCGAAGTA	Sequencing	
	Pcesa1Tsp1f	CGCATGGGTGTTGTTCTCATTCC	DNA walking	
	Pcesa1Tsp2f	CAACCGTGGTGTCAAGAGTCTTGATGC	DNA walking	
	Pcesa1Tsp3f	TCTGCTGTGTTCGCTGCGTTCTGG	DNA walking	
	Pcesa1Tsp1r	GGCGAGAAGTATCCACTGAACG	DNA walking	
	Pcesa1Tsp2r	ATGTCAATTCGTTTGGTGTCCGCTCAGC	DNA walking	
	Pcesa1Tsp3r	TCCAAATGGCACGGCTGAATACCGACG	DNA walking	
PaCesA2	Pcesa2f	AGTTCGGTCGAGTACACACATCCACCATGG	PCR	3296
	Pcesa2r	CGAAGCTATCGTAGTAAATGCCTCCATCTTG	PCR	
	Pcesa2seqr	ACCAGAAAATGACACCGAGA	Sequencing	
	Pcesa2seqf1	GAACATGCCATCATCTCCA	Sequencing	
	Pcesa2seqf2	CAAGGGCATCCTCGAGA	Sequencing	
	Pcesa2seqf3	CGCTGTGTGAACTGCGA	Sequencing	
	Pcesa2seqf4	GGTACGCTCTACCCACTT	Sequencing	
	Pcesa2Tsp1f	TACTCATCCAAGGCATGCTATCG	DNA Walking	
	Pcesa2Tsp2f	ACCGTACCGTCGAGAACAACGACATCG	DNA Walking	
	Pcesa2Tsp3f	CGTCCTGGAGGCTCTCTGGTGGAAGC	DNA Walking	
	Pcesa2Tsp1r	GATCAATGATGTTTGCTGGATGGTCG	DNA Walking	
	Pcesa2Tsp2r	AGCCCGACGACAAACCAGAAAATGACACC	DNA Walking	
	Pcesa2Tsp3r	GAAGGGTATCACGACGTACCGCATCC	DNA Walking	
PaCesA3	Pcesa3f	GCGCTGGTGAAGGAAGGACGGACACC	PCR	3701
	Pcesa3r	ATGGGCCGAGACAGACATGGTTACAAGGAG	PCR	
	Pcesa3seqr1	TGCCACCATCCTGGAAG	Sequencing	
	Pcesa3seqf1	TCATCATTTTCGTGCTCGTC	Sequencing	
	Pcesa3seqf2	CGCTTTATGGATGGTGGT	Sequencing	
	Pcesa3seqf3	CTCGTGCACATCTACATTC	Sequencing	
	Pcesa3seqf4	CTTTCGCCGGTACCAAC	Sequencing	
	Pcesa3seqf5	TACGCATTCATCACGCT	Sequencing	
	Pcesa3Tsp1f	CTCTTTCTTGCCGAACATGCTTG	DNA Walking	
	Pcesa3Tsp2f	CTCGTACGCTTTCATCACGCTGCTGG	DNA Walking	

	Pcesa3Tsp3f	GCAAGGAAGCGTCGTGGACCAACACTGG	DNA Walking	
	Pcesa3Tsp1r	GACTGCCGACACGGTTGTAGC	DNA Walking	
	Pcesa3Tsp2r	CGCATGGCGAGTTGGTTCAAGACACC	DNA Walking	
	Pcesa3Tsp3r	CCTGGGCCGAGCTGTCAATGTTGTCG	DNA Walking	
PaCesA4	Pcesa4f	CGCGTTTCCCCTTGGGACCGATATTGG	PCR	3189
	Pcesa4r	AGGAACAGAGTCAACGTGAGGCAGCAGG	PCR	
	Pcesa4seqr	ATACCCGCGAGTTCGAT	Sequencing	
	Pcesa4seqf1	CCGAGAACGACTTTACCATG	Sequencing	
	Pcesa4seqf2	GCTACTGCAAGTCCAAGTG	Sequencing	
	Pcesa4seqf3	CGCCAAGGTTAATGCCA	Sequencing	
	Pcesa4seqf4	CCTTCCCTGCCATCTTCT	Sequencing	
	Pcesa4Tsp1f	CAGTCAGCAGACATGGTTCTCG	DNA Walking	
	Pcesa4Tsp2f	GTAACAGCAGCATGGAGATTCCCAACATC	DNA Walking	
	Pcesa4Tsp3f	CTCTGGGCCATTGTGCGTTTCTTCACG	DNA Walking	
	Pcesa4Tsp1r	GCTTGGGGCCACACTTGATACC	DNA Walking	
	Pcesa4Tsp2r	GTCCGCTGTCTTGTACTTGATGTCACACG	DNA Walking	
	Pcesa4Tsp3r	CTTGACAACGTTGCCTGTGCCCGAGC	DNA Walking	
PaChs	Pchsf	CTTTGTCACTCGCCTTCTCTTCGTCG	PCR	3075
	Pchsr	GCA GAA CGC GGT GAA GTC GTG TCC	PCR	
	Pchsseqr	CAT TGC GTA TCC ACC TTC A	Sequencing	
	Pchsseqf1	CGAAGTGTTCAGGACTTG	Sequencing	
	Pchsseqf2	ACCCAGAGGACGATTATTC	Sequencing	
	Pchsseqf3	CATGTACCTTGCAGAAGAC	Sequencing	
	Pchsseqf4	CACGGCGAAGTACATCA	Sequencing	
	PchsTsp1f	GTACCTCTTCGGATTGCTGATGC	DNA Walking	
	PchsTsp2f	GTCATCATCGGGCTTGGCAACAATCC	DNA Walking	
	PchsTsp3f	CTACTTCGTCAGCAGTGTGTTCGCTG	DNA Walking	
	PchsTsp1r	TCCGCTCAAACATGTGGCATTGG	DNA Walking	
	PchsTsp2r	CCAGCATCTTCGGGTTGATCTGTTCG	DNA Walking	
	PchsTsp3r	TTGATCTGTTCGCGGCCGTCAGC	DNA Walking	
ActA	Pactaconf	AACTGGGACGACATGGAGAAGATCTGG	PCR	841
	Pactaconr	AGATCCACATCTGCTGGAACGTCG	PCR	
	PactaTsp1f	GTATCCACGACTGCACGTTCC	DNA Walking	
	PactaTsp2f	GCAACATCGTGCTCTCGGGTGGTACC	DNA Walking	
	PactaTsp3f	CTTACGGCCCTTGCCCCATCGACC	DNA Walking	
	PactaTsp1r	CGTGTGCGAGACACCATCACC	DNA Walking	
	PactaTsp2r	CGTACAGCGAAAGCACGGCCTGG	DNA Walking	
	PactaTsp3r	ATCTGCGTCATACGCTCACGGTTGG	DNA Walking	

Supplementary Table 2

Sequences of cellulose synthase gene products used for ClustalW alignment

Sequence input	Type	Organism	Accession Number
PiCesA1	Cellulose synthase 1	*Phytophthora infestans*	**ABP96902** [a]
PiCesA2	Cellulose synthase 2	*Phytophthora infestans*	**ABP96903** [a]
PiCesA3	Cellulose synthase 3	*Phytophthora infestans*	**ABP96904** [a]
PiCesA4	Cellulose synthase 4	*Phytophthora infestans*	**ABP96905** [a]
PrCesA1	Cellulose synthase 1	*Phytophthora ramorum*	**ABP96910** [a]
PrCesA2	Cellulose synthase 2	*Phytophthora ramorum*	**ABP96911** [a]
PrCesA3	Cellulose synthase 3	*Phytophthora ramorum*	**ABP96912** [a]
PrCesA4	Cellulose synthase 4	*Phytophthora ramorum*	**ABP96913** [a]
PsCesA1	Cellulose synthase 1	*Phytophthora sojae*	**ABP96906** [a]
PsCesA2	Cellulose synthase 2	*Phytophthora sojae*	**ABP96907** [a]
PsCesA3	Cellulose synthase 3	*Phytophthora sojae*	**ABP96908** [a]
PsCesA4	Cellulose synthase 4	*Phytophthora sojae*	**ABP96909** [a]
PvCesA1	Cellulose synthase 1	*Plasmopara viticola*	**ADD84670** [a]
PvCesA2	Cellulose synthase 2	*Plasmopara viticola*	**ADD84671** [a]
PvCesA3	Cellulose synthase 3	*Plasmopara viticola*	**ADD84672** [a]
PvCesA4	Cellulose synthase 4	*Plasmopara viticola*	**ADD84673** [a]
PaCesA1	Cellulose synthase 1	*Pythium aphanidermatum*	**JN038397** [b]
PaCesA2	Cellulose synthase 2	*Pythium aphanidermatum*	**JN038398** [b]
PaCesA3	Cellulose synthase 3	*Pythium aphanidermatum*	**JN038399** [b]
PaCesA4	Cellulose synthase 4	*Pythium aphanidermatum*	**JN038400** [b]
PuCesA1	Cellulose synthase 1	*Pythium ultimum*	**PYU1_T002607** [c]
PuCesA2	Cellulose synthase 2	*Pythium ultimum*	**PYU1_T002606** [c]
PuCesA3	Cellulose synthase 3	*Pythium ultimum*	**PYU1_T006539** [c]
PuCesA4	Cellulose synthase 4	*Pythium ultimum*	**PYU1_T006496** [c]
SmCesA2	Cellulose synthase 2	*Saprolegnia monoica*	**ACX56230** [a]
SmCesA3	Cellulose synthase 3	*Saprolegnia monoica*	**ACX56231** [a]
SmCesA4	Cellulose synthase 4	*Saprolegnia monoica*	**ACX56232** [a]
SpCesA2-1	Cellulose synthase 2	*Saprolegnia parasictica*	**SPRG_06590** [d]
SpCesA2-2	Cellulose synthase 2	*Saprolegnia parasitica*	**SPRG_05124** [d]
SpCesA2-3	Cellulose synthase 2	*Saprolegnia parasitica*	**SPRG_06591** [d]
SpCesA3-1	Cellulose synthase 3	*Saprolegnia parasitica*	**SPRG_06052.2** [d]
SpCesA3-2	Cellulose synthase 3	*Saprolegnia parasitica*	**SPRG_06051.2** [d]
SpCesA4	Cellulose synthase 4	*Saprolegnia parasitica*	**SPRG_08607.2** [d]
PyCesA	Cellulose synthase	*Porphyra yezoensis*	**ABX71734** [a]
GmCesA1	Cellulose synhtase 1	*Griffithsia monilis*	**ADK77974** [a]

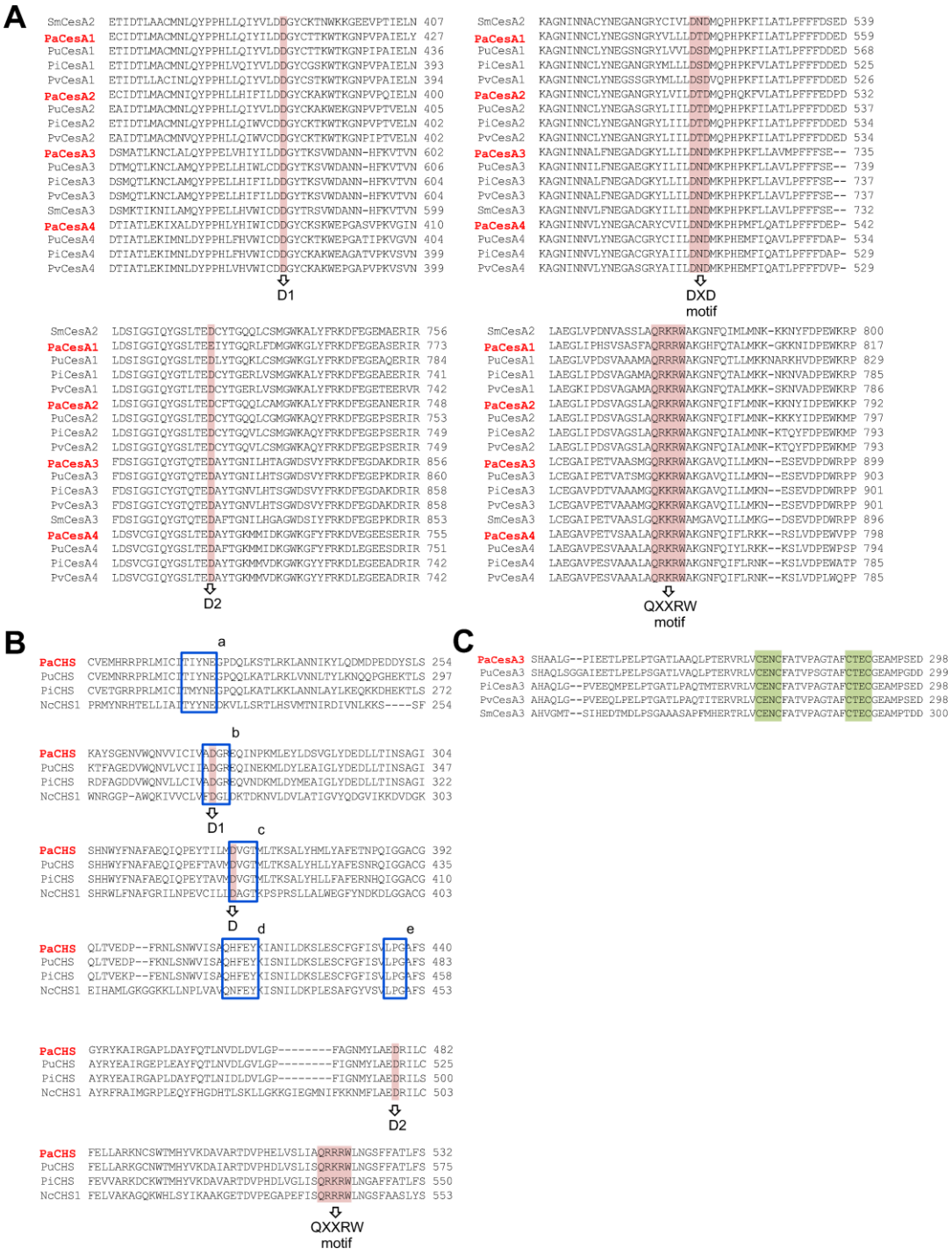
[a]Sequences obtained from the NCBI database
[b]Sequences produced in this study
[c]Sequences obtained from: The *Pythium ultimum* Whole Genome Sequencing Project (http://pythium.plantbiology.msu.edu/project_info.html)
[d]Sequences obtained from: *Saprolegnia parasitica* Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/)

Supplementary Table 3

Sequences of chitin synthase gene products used for ClustalW alignment

Sequence input	Type	Organism	Accession Number
AlCHS1	Chitin synthase 1	*Albugo laibachii*	**CCA16398.1** [a]
AeCHS1	Chitin synthase 1	*Aphanomyces euteiches*	**EU522489** [a]
AeCHS2	Chitin synthase 2	*Aphanomyces euteiches*	**EU447431** [a]
NcCHS1	Chitin synthase 1	*Neurospora crassa*	**M73437** [a]
PiCHS	Chitin synthase	*Phytophthora infestans*	**XP002908631.1** [a]
PcCHS	Chitin synthase	*Phytophthora capsici*	**unknown**
PrCHS1	Chitin synthase 1	*Phytophthora ramorum*	**Pr_77229** [b]
PrCHS2	Chitin synthase 2	*Phytophthora ramorum*	**Pr_84041** [b]
PsCHS1	Chitin synthase 1	*Phytophthora sojae*	**Ps_143614** [b]
PsCHS2	Chitin synthase 2	*Phytophthora sojae*	**Ps_155246** [b]
PaCHS	Chitin synthase	*Pythium aphanidermatum*	**JN038401** [c]
PuCHS	Chitin synthase	*Pythium ultimum*	**PYU1_T005734** [d]
SmCHS1	Chitin synthase 1	*Saprolegnia monoica*	**GQ252794** [a]
SmCHS2	Chitin synthase 2	*Saprolegnia monoica*	**GQ252795** [a]
SpCHS1-1	Chitin synthase 1	*Saprolegnia parasitica*	**SPRG_09812** [e]
SpCHS1-2	Chitin synthase 1	*Saprolegnia parasitica*	**SPRG_02074** [e]
SpCHS2-1	Chitin synthase 2	*Saprolegnia parasitica*	**SPRG_04151.2** [e]
SpCHS2-2	Chitin synthase 2	*Saprolegnia parasitica*	**SPRG_02554.1** [e]

[a]Sequences obtained from the NCBI database
[b]Sequences obtained from the Department of Energy (DOE) Joint Genome Institute (http://genome.jgi-psf.org)
[c]Sequences produced in this study
[d]Sequences obtained from: The *Pythium ultimum* Whole Genome Sequencing Project (http://pythium.plantbiology.msu.edu/project_info.html)
[e]Sequences obtained from: *Saprolegnia parasitica* Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/)



Supplementary Fig. 1. Multiple alignment of segments from oomycete CesA and CHS sequences.
(**A**) Sequence alignment of *Pythium aphanidermatum* (Pa) CesA sequences (highlighted in red) with CesAs of *Phytophthora infestans* (Pi), *Plasmopara viticola* (Pv), *Pythium ultimum* (Pu) and *Saprolegnia monoica* (Sm). Conserved D,D,D,QXXRW motifs that are present in most processive glycosyltransferases are shaded in red. Numbers indicate the position of the amino acid residues of each protein. (**B**) Sequence alignment of the *Pythium aphanidermatum* (Pa) CHS sequence (highlighted in red) with CHS sequences of *Phytophthora infestans* (Pi), *Pythium ultimum* (Pu) and *Neurospora crassa* (Nc) containing the conserved D,D,D,QXXRW motifs (shaded in red). Conserved motifs (a to e) present in yeast and fungal CHS sequences are boxed. (**C**) Alignment of the CesA3 specific domain located at the N-terminal end of oomycete CesA3 sequences. The two CxxC motifs also present in plant ring finger domains are shaded in green.

CHAPTER IV

The cellulose synthase 3 (*CesA3*) gene of oomycetes: structure, phylogeny and influence on sensitivity to carboxylic acid amide (CAA) fungicides

Chapter corresponds to the publication: Blum, M., Gamper, H.A., Waldner, M., Sierotzki, H., Gisi, U., 2012. The cellulose synthase 3 (CesA3) gene of oomycetes: structure, phylogeny and influence on sensitivity to carboxylic acid amide (CAA) fungicides. Fungal Biol. (in press).

The cellulose synthase 3 (*CesA3*) gene of oomycetes: structure, phylogeny and influence on sensitivity to carboxylic acid amide (CAA) fungicides

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ABSTRACT

Proper disease control is very important to minimize yield losses caused by oomycetes in many crops. Today, oomycete control is partially achieved by breeding for resistance, but mainly by application of single-site mode of action fungicides including the carboxylic acid amides (CAAs). Despite having mostly specific targets, fungicidal activity can differ even in species belonging to the same phylum but the underlying mechanisms are often poorly understood. In an attempt to elucidate the phylogenetic basis and underlying molecular mechanism of sensitivity and tolerance to CAAs, the cellulose synthase 3 (*CesA3*) gene was isolated and characterized, encoding the target site of this fungicide class. The *CesA3* gene was present in all 25 species included in this study representing the orders *Albuginales*, *Leptomitales*, *Peronosporales*, *Pythiales*, *Rhipidiales* and *Saprolegniales*, and based on phylogenetic analyses, enabled good resolution of all the different taxonomic orders. Sensitivity assays using the CAA fungicide mandipropamid (MPD) demonstrated that only species belonging to the *Peronosporales* were inhibited by the fungicide. Molecular data provided evidence, that the observed difference in sensitivity to CAAs between *Peronosporales* and CAA tolerant species is most likely caused by an inherent amino acid configuration at position 1109 in CesA3 possibly affecting fungicide binding. The present study not only succeeded in linking CAA sensitivity of various oomycetes to the inherent CesA3 target site configuration, but could also relate it to the broader phylogenetic context.

Keywords: Cellulose synthesis; Mandipropamid; Phylogenetics; Target site

1. INTRODUCTION

Cellulose, a linear homopolymer of β-(1→4) linked D-glucose residues, is the most abundant biopolymer on earth holding a variety of important biological and functional roles. Beside the plant kingdom, where it provides a key role in structural support for the cell wall and growth (Smith and Oppenheimer, 2005), cellulose was discovered in prokaryotes, rhodophyta, amoebozoa, tunicates and oomycetes (Bartnicki-Garcia, 1968; Blanton et al., 2000; Brown et al., 1993; Roberts and Roberts, 2009; Sagane et al., 2010).

In the oomycete cell wall, cellulose represents the main microfibrillar component and is associated with β-(1→3) and β-(1→6) glucans (Bartnicki-Garcia, 1968; Helbert et al., 1997). Cellulose synthesis is essential for the production and expansion of a new cell wall during cystospore germination and appressoria formation in the late blight pathogen *Phytophthora infestans* (Grenville-Briggs et al., 2008). In oomycetes and plants, the machinery responsible for cellulose formation is a membrane bound enzyme complex (Grenville-Briggs et al., 2008; Paredez et al., 2006) probably composed of several distinct cellulose synthase (CesA) subunits (Desprez et al., 2007; Persson et al., 2007). In oomycetes, up to four CesA encoding genes have been identified in *Peronosporales*, *Pythiales* and *Saprolegniales* (Blum and Gisi, 2012; Blum et al., 2010b; Blum et al., 2011; Fugelstad et al., 2009; Grenville-Briggs et al., 2008; Levesque et al., 2010); and their direct involvement in cellulose biosynthesis was demonstrated using RNA interference in *P. infestans* (Grenville-Briggs et al., 2008). Out of the four *CesAs*, the *CesA3* gene was most strongly expressed during mycelial growth of *Phytophthora*, *Saprolegnia* and *Pythium* species (Blum and Gisi, 2012, Grenville-Briggs et al., 2008, Fugelstad et al., 2009) indicating that it is of particular importance for the formation of cellulose in oomycetes.

Since cellulose synthesis is a vital process for oomycetes, it also represents a potential fungicide target. Indeed, members of the carboxylic acid amides (CAAs) successfully inhibit cellulose biosynthesis in *Peronosporales* (Gisi et al., 2012) by targeting the cellulose synthase 3 (CesA3) enzyme (Blum et al., 2010a). However, single amino acid exchanges in CesA3 have been detected, conferring resistance to CAA fungicides in different plant pathogens of the *Peronosporales* such as *P. infestans* (Blum et al., 2010a), *Phytophthora capsici*, *Phytophthora melonis* (Chen et al., 2011), *Plasmopara viticola* (Blum et al., 2010b), and *Pseudoperonospora cubensis* (Blum et al.,

2011). In most cases, resistance was based on a single point mutation in the *CesA3* gene, leading to a change at amino acid position 1105 from a conserved glycine to either alanine, serine, valine or tryptophan (G1105A, G1105S, G1105V, G1105W) (Blum et al., 2010a; Blum et al., 2010b; Blum et al., 2011; Sierotzki et al., 2011). In artificially generated mutants of *P. infestans*, *P. capsici* and *P. melonis*, a change from valine to leucine or methionine at position 1109 (V1109L, V1109M) also confers resistance to CAAs (Chen et al., 2011, Syngenta internal data). Further, the amino acid configuration L1109 was observed in the root rot and damping-off causative agent *Pythium aphanidermatum* that is generally tolerant to CAA fungicides. (Blum and Gisi, 2011).

Activity of CAAs has been extensively studied in *Peronosporales* (e.g. *P. infestans*, *P. capsici*, *Bremia lactucae*, *P. cubensis, P. viticola;* (Cohen and Gisi, 2007; Cohen et al., 2008; Gisi et al., 2007; Kim et al., 2009; Lu et al., 2010; Zhu et al., 2008) and to some extent in *Pythiales* (Albert et al., 1988; Blum and Gisi, 2011; Stenzel et al., 1998). However, comprehensive data about CAA sensitivity of oomycete species outside *Peronosporales* or *Pythiales* is lacking. In those species, little is also known about the existence of the *CesA3* gene and its corresponding protein structure the configuration of which is suggested to affect sensitivity to CAAs.

So far, the *CesA3* gene has been described only in few oomycetes belonging to the *Peronosporales*, *Pythiales* and *Saprolegniales* (Blum and Gisi, 2012; Blum et al., 2010b; Blum et al., 2011; Fugelstad et al., 2009; Grenville-Briggs et al., 2008; Levesque et al., 2010), thus, the present study aimed to isolate and characterize the *CesA3* gene in species from all six "crown" oomycete orders (Beakes and Sekimoto, 2009). We identified the *CesA3* gene in 14 different species of 11 genera, belonging to the six orders *Albuginales*, *Leptomitales*, *Peronosporales*, *Pythiales*, *Rhipidiales* and *Saprolegniales.* The *CesA3* gene sequences were used to investigate whether species of different phylogenetic rank differed in their amino acid configurations at positions 1105 and 1109, which are known to mediate tolerance to CAA fungicides. Since no sensitivity data were available for most of the analysed oomycetes, the CAA fungicide mandipropamid (MPD) was used to determine the inhibitory spectrum and activity against 21 of the species. The taxonomically broad set of 25 species was chosen, to finally also determine the phylogenetic position at which sensitivity or tolerance to CAAs evolved.

2. MATERIALS AND METHODS

2.1. Strains and culture conditions

A total of 25 different oomycete species originating from the six "crown" oomycete orders *Albuginales, Leptomitales, Peronosporales, Pythiales, Rhipidiales* and *Saprolegniales* were included in this study. The sensitivity to the fungicide mandipropamid was assessed in 21 of the 25 species and the *CesA3* gene was newly identified and sequenced in 14 species. In 11 species the *CesA3* sequence was accessed from public databases. The strains are either held in the Syngenta culture collection, or accessible via the Centralbureau voor Schimmelculure (CBS) database (Table 1). Strains of non-obligate biotrophic species (*Phytophthora*, *Pythium*, *Aphanomyces*, *Apodachlya*, *Sapromyces* and *Saprolegnia*) were cultured on potato dextrose (PDA) or Rye dextrose (RDA) media. Biotrophic species (*Albugo*, *Bremia*, *Plasmopara* and *Pseudoperonospora*) were maintained on cotyledons or adult leafs of the corresponding host plant. The *Albugo candida* strain ZUM3352 was cultured on white cabbage seedlings Cilion F1 hybrid. The *Plasmopara viticola* strain D05 was propagated on *Vitis vinifera* cv. Gutedel leaves. The *Bremia lactucae* strain HS1 was maintained on *Lactuca sativa* cv. Crispa cotyledons. The *Pseudoperonospora cubensis* strain CH01 was propagated on *Cucumis sativus* cv. Delikatesse leaves.

2.2. Fungicide sensitivity assays

Technical-grade mandipropamid (MPD) was provided by Syngenta Crop Protection AG (Stein, Switzerland). A stock solution (10'000 mg l^{-1}) of the active ingredient (a.i.) was prepared in dimethyl sulfoxide (DMSO). For mycelial growth assays, PDA and RDA plates were amended with different MPD concentrations. Non-fungicide amended plates containing the same DMSO concentrations (< 1 % by volume) were used as control. Mycelial plugs (7 mm in diam.) were cut from the margins of growing colonies (five d old) and transferred onto PDA or RDA plates amended with 1, 10 or 100 mg l^{-1} MPD. Isolates were tested on triplicate plates and incubated at 18 to 25 °C in the dark for 2 to 10 days. After incubation the diameter of each colony was measured, averaged for each treatment and expressed as the percentage of growth inhibition. EC_{50} (effective concentration to obtain 50 % growth inhibition) was calculated by regressing percentage of growth inhibition against the logarithmic values of fungicide concentration using Agstat software (supplied by Syngenta).

Table 1

List of species used in this study and GenBank entries. Accession numbers for newly generated *CesA3* sequences are highlighted in bold.

Species	Order	Isolate ID	Source	*CesA3* identifier	*CesA3* accession No.
Albugo candida	*Albuginales*	ZUM3352[a]	*Brassica oleracea*	*AcanCesA3*	**JF960416**[c]
Albugo laibachii	*Albuginales*	Nc14[a]	*Arabidopsis thaliana*	*AlaiCesA3*	FR824299.1[d]
Apodachlya brachynema	*Leptomitales*	CBS 184.82[b]	-	*AbraCesA3*	**JN561769**[c]
Bremia lactucae	*Peronosporales*	HS1[b]	*Lactuca sativa*	*BlacCesA3*	**JN561771**[c]
Hyaloperonospora arabidopsidis	*Peronosporales*	Emoy2	*Arabidopsis thaliana*	*HaraCesA3*	810051[e]
Phytophthora capsici	*Peronosporales*	188[a]	*Capsicum annum*	*PcapCesA3*	**JN561772**[c]
Phytophthora cinnamomi	*Peronosporales*	273[a]	*Erica gracilis*	*PcinCesA3*	**JN561773**[c]
Phytophthora cactorum	*Peronosporales*	119[a]	*Fragaria ananassa*	*PcacCesA3*	**JN561774**[c]
Phytophthora infestans	*Peronosporales*	96[a]	*Solanum tuberosum*	*PinfCesA3*	EF563995.1[d]
Phytophthora palmivora	*Peronosporales*	224[a]	*Cocos nucifera*	*PpalCesA3*	**JN561775**[c]
Phytophthora sojae	*Peronosporales*	CBS 125701	*Glycine max*	*PsojCesA3*	EF563999.1[d]
Phytophthora ramorum	*Peronosporales*	102	*Quercus agrifolia*	*PramCesA3*	EF564003.1[d]
Plasmopara viticola	*Peronosporales*	D05[a]	*Vitis vinifera*	*PvitCesA3*	GQ258975.1[d]
Pseudoperonospora cubensis	*Peronosporales*	CH01[a]	*Cucumis sativus*	*PcubCesA3*	JF799098.1[d]
Pythium aphanidermatum	*Pythiales*	620[a]	Turfgrass	*PaphCesA3*	JN038400[d]
Pythium arrhenomanes	*Pythiales*	411[a]	*Beta altissima*	*ParrCesA3*	**JN561776**[c]
Pythium coloratum	*Pythiales*	336[a]	Soil	*PcolCesA3*	**JN561777**[c]
Pythium irregulare	*Pythiales*	73[a]	*Pisum sativum*	*PirrCesA3*	**JN561778**[c]
Pythium iwayamai	*Pythiales*	948[a]	Soil under *Pinus* sp.	*PiwaCesA3*	**JN561779**[c]
Pythium ultimum	*Pythiales*	71[a]	*Cuccumis sativus*	*PultCesA3*	PYU1_T006539[f]
Pythium violae	*Pythiales*	339[a]	*Daucus carota*	*PvioCesA3*	**JN561780**[c]
Sapromyces elongatus	*Rhipidiales*	CBS 213.82[b]	-	*SeloCesA3*	**JN561781**[c]
Aphanomyces euteiches	*Saprolegniales*	CBS 154.73[b]	*Pisum sativum*	*AeutCesA3*	**JN561770**[c]
Saprolegnia monoica	*Saprolegniales*	CBS 539.67[b]	Water	*SmonCesA3*	FJ775603.1[d]
Saprolegnia parasitica	*Saprolegniales*	CBS 223.65[b]	*Esox lucius*	*SparCesA3*	SPRG_06052.2[g]

[a]Isolates held at the culture collection of Syngenta Crop Proteciton, Stein, Switzerland
[b]Isolates held at the CBS, Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands
[c]Sequence produced in this study
[d]Sequence obtained from the NCBI database
[e]Sequence obtained from: *Hyaloperonospora arabidopsidis* Sequencing Project, VBI Microbial database(VMD) (http://www.vmd.vbi.vt.edu/)
[f]Sequence obtained from: The *Pythium ultimum* Whole Genome Sequencing Project
[g]Sequence obtained from: *Saprolegnia parasitica* Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/)

For the biotrophic oomycetes, in planta sensitivity assays were conducted using 7 to 10-d-old cotyledons or leaf discs excised from three-week-old plants. Sensitivity to MPD of *P. viticola* and *P. cubensis* strains was examined using a leaf-disc assay previously described by (Gisi et al., 2007). Sensitivity of *A. candida* was tested by inoculating 10-d-old white cabbage (*Brassica oleracea* cv. Cilion F1 hybrid) cotyledons. Zoospore

suspensions were prepared in distilled H_2O as previously described by Gilijamse et al. (2004). Suspensions containing 30'000 zoospores ml^{-1} were mixed with MPD to reach final concentrations of 0, 1, 10 and 100 mg l^{-1}. Cotyledons were inoculated on the upper surface with 2-3 droplets (10 µl) containing zoospores and MPD or DMSO (as controls). After inoculation, cotyledons were immediately transferred in a non-transparent plastic box and incubated in a growth chamber (18° C, in the dark) for 12 h. Thereafter cotyledons were exposed to 14h light/-d at 70 % relative humidity and 20 °C until white pustules were formed.

To assess the sensitivity of *B. lactucae* to MPD, sporangia were collected from freshly sporulating lettuce leaves and their density was adjusted to 20'000 ml^{-1} in distilled H_2O. Sporangia suspensions were mixed with MPD as described above and then transferred (2-5 droplets of 10 µl) onto the upper surface of lettuce (*L. sativa* cv. Capitata) cotyledons. Thereafter, they were placed in a dew chamber (18°C, in the dark) for 20 h and then transferred to a growth chamber at 20°C (14 h light/d, 100 µE m^{-2} s^{-1}). At 5 d post-inoculation (dpi) plants were placed in Perspex boxes for two days to induce sporulation of the pathogen. Disease assessment was done after a 7-d incubation period by visual estimation of the percentage cotyledons infected compared to the untreated DMSO control. The EC_{50} values were determined using Agstat software.

2.3. DNA and RNA extraction

For extraction of genomic DNA (gDNA) or total RNA, mycelium or spores were harvested from agar plates or infected plant leaves, immediately frozen in liquid nitrogen and stored at -80 °C until further use. DNA was isolated following the modified CTAB protocol of Riethmüller et al. (2002). Total RNA was extracted using the E.Z.N.A™ Fungal RNA Kit (Omega Bio-Tek, Georgia, USA) following the manufacturer's protocol. Prior to cDNA synthesis, RNA samples were DNAse treated using Turbo DNA free Kit (Ambion, Texas, USA) according to the manufacturer's protocol. Integrity of extracted RNA was tested by gel electrophoresis and quality/quantity was measured using a NanoDrop® ND-2000 spectrophotometer (Thermo Scientific, USA). First-strand cDNA was synthesized from 200 ng total RNA by oligo(dT) priming using Omniscript RT Kit® (Quiagen, Hilden, GER) following the manufacturer's protocol.

2.4. Primer design

Different primer design strategies were chosen for the identification of the *CesA3* gene. The primers PhytCesA3f and PhytCesA3r (Table 2) used for the amplification of *CesA3* in *Phytophthora* species were designed according to conserved nucleotide sequences flanking the open reading frames (ORFs) of the *CesA3* gene. A 5 kb fragment (including the *CesA3* gene) from the *Phytophthora infestans*, *Phytophthora ramorum* and *Phytophthora sojae* genome was accessed from the Broad Institute (http://www.broadinstitute.org/annotation/genome/phytophthora_infestans/MultiHome.html) or the JGI genome database (http://genome.jgi-psf.org/) and aligned using ClustalW (Thompson et al., 2002). Two nucleotide motifs in the 5' and 3'-untranslated region (UTR) were highly conserved and consequently chosen for primer design.

The *CesA3* gene of *Pythium* species was identified using the primers PytCesA3f and PytCesA3r (Table 2). Alignment of the previously identified CesA3 sequences of *Pythium ultimum* (PYU1_T006539) and *Pythium aphanidermatum* (JN038399) using ClustalW revealed the highly conserved amino acid motifs SLAFGGWS and YPMVKMSITE, respectively. Based on those motifs, degenerated primers were designed.

To identify the *CesA3* gene of species from the *Albuginales*, *Leptomitales*, *Peronosporales* (*Bremia* spp.), *Rhipidiales* and *Saprolegniales*, *CesA3* specific primers were designed using the CODEHOP strategy (Rose et al., 2003) based on the multiply aligned CesA3 sequences originating from *P. infestans* (ABP96904), *P. viticola* (ADD84672), *P. cubensis* (JF799098), *Pythium aphanidermatum* (JN038399), *Pythium ultimum* (PYU1_T006539) and *Saprolegnia monoica* (ACX56231). Two conserved motifs VAAAMGQKKRW and MSITEYCGWD in the C-terminal region of the proteins were selected for designing the CODEHOP primers CodeCesA3f and CodeCesA3r (Table 2) by using the default parameters of the COEDHOP server (http://blocks.fhcrc.org/codehop.html).

2.5. Amplification of CesA3 gene fragments

All PCR reactions were performed in a TGradient Thermocylcer (Biometra, Göttingen, GER) and primers were obtained from Microsynth (Balgach, CH). Unless otherwise stated, gDNA of 14 different oomycete species was used as template for *CesA3* gene fishing experiments using the primers listed in Table 2. PCR amplifications of the *CesA3* gene in *Phytophthora* and *Pythium* species was performed on 50 ng gDNA in a

total volume of 30 µl containing 1.25 U of GoTaq polymerase (Promega, Madison, WI), 0.2 mM dNTPs and 0.2 mM primers (PhytCesA3f and PhytCesA3r for *Phytophthora* species; PytCesA3f and PytCesA3r for *Pythium* species) in the polymerase manufacturers buffer. The PCR program was as follows: first 4 min initial denaturation at 94 °C, then 36 cycles of 30 s at 94 °C, 30 s at 50 to 60 °C (gradient temperature), 3 min at 72 °C and finally a 5 min extension step.

PCR reactions using the CODEHOP approach were performed on 50 ng gDNA, in a total volume of 50 µl containing 2 U of AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA), 0.2 mM dNTPs and 0.5 mM primers (CodeCesA3f, CodeCesA3r) in the manufacturer's buffer. The PCR program was performed according to the CODEHOP server's tips with minor modifications: first 4 min initial denaturation at 94 °C, then 34 cycles of 30 s at 94 °C, 30 s at 55 to 63 °C, 45 s at 72 °C and finally a 5 min extension step.

Table 2

CODEHOP and degenerated primers used for *CesA3* gene amplification in oomycetes.

Primer	Sequence (5'–3')	Protein consensus	Target Organisms
Sense			
PhytCesA3f	CTCGGCAGCAGACCAAAGCG	Non coding	*Phytophthora*
PytCesA3f	TCGCTSGCSTTYGGCGGYTGGTC	SLAFGGWS	*Pythium*
CodeCesA3f	[a]GGTCGCTGCTGCCATGGGTcarmrbaarcgytg[b]	VAAAMGQKKRW	*Albugo, Aphanomyces, Apodachlya, Bremia, Sapromyces*
Antisense			
PhytCesA3r	GCYTATGTAGCGAATCAGCKGTGC	Non coding	*Phytophthora*
PytCesA3r	TCSGTGATRCTCATYTTVACCATSGGG	YPMVKMSITE	*Pythium*
CodeCesA3r	GTCCCAGCCACAGTACTCngwratrctca	MSITEYCGWD	*Albugo, Aphanomyces, Apodachlya, Bremia, Sapromyces*

[a]Consensus clamp (XXX.XXX) is given in uppercase
[b]Degenerate core (xxx.xxx) is given in lower case. b = [G,C,T]; m = [A, C]; n = [A, C, G, T]; r = [G, A]; w = [A, T], y = [C, T]

2.6. PCR amplification by genome walking

The primers used for *CesA3* amplification in *Phytophthora* species produced the full-length *CesA3* gene sequence, thus no further PCR manipulations were necessary. However, in *Pythium* and other oomycetes, *CesA3* sequences were only partially amplified due to restrictions of conserved motifs for primer design. Flanking regions (5'

and 3' ends) were obtained by genome walking using the DNA Walking SpeedUp kit I and II (Seegene, Rockville, MD) following manufacturer's instructions. *CesA3* gene fragments (700-1200 bp) obtained by genome walking reactions were purified by Sephacryl S-300 (GE Healthcare, Uppsala, SE), directly sequenced and subsequently used for primer design of further walking primers. Walking reactions for each *CesA3* gene were repeated till the full-length open reading frame (ORF) was reached. The technique was successfully used for 14 oomycete *CesA3* sequences except for the sequence of *Sapromyces elongatus*. For this species a ~ 80 bp fragment at the 5' end, including the start codon, could not be amplified due to the high AT content (>60 %) in this region.

2.7. Amplification, cloning and sequencing of the complete CesA3 ORFs

The full-length *CesA3* gene of 14 oomycetes species was amplified from 50 ng gDNA using PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA) and a final primer concentration of 0.2 µM to confirm their complete sequence. The primers used for this purpose are listed in Supplementary Table 1. Before cloning, PCR products were analyzed on 1 % agarose gels and fragments of expected size were purified using the NuceloSpin Extract II kit (Macherey-Nage, Düren, GER).

Complete *CesA3* ORFs, up to 4000 bp in size, were cloned into a pCR-XL-TOPO vector (Invitrogen) and subsequently used for heat shock transformation of *Escherichia coli* TOPO10 (Invitrogen) cells. Direct PCR on colonies was performed using Taq Polymerase (Invitrogen) to amplify correct inserts. Thereafter fragments of expected size were purified with Sephacryl S-300 (GE Healthcare) and sequenced.

Sequencing reactions were done with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions using M13 or sequence specific primers (Supplementary Table 1). Sequencing was carried out on a 3130 Genetic Analyzer (Applied Biosystems) according to the instructions provided with the instrument.

2.8. Sequence analysis

The *CesA3* sequences were edited using SeqMan and EditSeq software (DNASTAR, Inc). Translation of nucleotide sequences into protein sequences revealed that *CesA3* genes of the 14 species contained at least one intron at the 5' end. Intron/exon structure was verified by comparing the full-length gDNA *CesA3* sequence

with its corresponding cDNA after reverse transcription. Correct start and stop codons were identified either by using the NCBI ORF finder program (http://www.ncbi.nlm.nih.gov/projects/gorf/) or by comparison with existing predicted *CesA3* gene models from oomycete genome sequencing projects (www.broadinstitute.org/; www.jgi.doe.gov/; http://pythium.plantbiology.msu.edu; http://www.vmd.vbi.vt.edu/).

The conserved domains of the CesA3 sequences and their paralogues from various kingdoms were analysed using the NCBI CD search program (Marchler-Bauer et al., 2011) at (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Diagrams of CesA sequence motifs were initially constructed using DomainDraw (Fink and Hamilton, 2007) and modified thereafter. Transmembrane domains were predicted using the TMpred program at the European molecular biology network (http://www.ch.embnet.org/software/TMPRED_form.html). Sequence alignments of amino acid sequences comprising the MPD target site were made by ClustalW using the software MegAlign (DNASTAR, Inc). Percentage identity of oomycete *CesA3* orthologues was calculated from pairwise sequence comparison using the Martinez/Needleman-Wusch method (for nucleotides) and the Lipman-Pearson alignment (for amino acids) in the MegAlign program (DNASTAR, Inc.).

2.9. Phylogenetic analysis

The 14 new *CesA3* cDNA sequences of this study were aligned with 11 *CesA3* sequences from public databases (see Table 1 for accession numbers), using ClustalW as implemented in MEGA vers. 5.02 (Tamura et al., 2011). The multiple sequence alignment was manually optimized and ambiguous sites excluded in MacClade vers. 4.08 (Maddison and Maddision, 2011), yielding 2962 aligned nucleotide positions for the phylogenetic analyses, of which 1811 contributed parsimony-informative character states. The model by Tamura and Nei (1993) was determined as the optimal model of sequence evolution in MEGA, using the implemented model evaluation and choice based on the Bayesian Information Criterion (BIC). Maximum Likelihood (ML), Maximum Parsimony (MP), and Neighbor Joining (NJ) phylogenetic analyses were all carried out in MEGA, where the sequence dataset was also 5000 times bootstrapped for inferring the statistical branch support. A Bayesian tree inference was run in MrBayes vers. 3.1.2 (Huelsenbeck and Ronquist, 2001) on the CIPRES Science Gateway vers. 3.1

(http://www.phylo.org/sub_sections/portal/), employing the TeraGrid computer cluster (Miller et al., 2010) with two runs of four incrementally heated Markov Chain Monte Carlo (MCMC) iterations over 4 million generations, starting from random trees and the sequence evolution model determined in MrModeltest vers. 2.3 (Nylander, 2004). Tree space was sampled every 100 generation resulting in a total of 40,000 trees from which the last 30,000 were used to compute a 50% majority rule consensus tree. Tracer vers. 1.5 (Rambaut and Drummond, 2007) was used to graphically verify that searches converged before trees were used for calculating the posterior probabilities of branch support. The multiple alignment and the phylograms with associated branch support values from the various analyses were deposited in TreeBASE (Sanderson et al., 1994) under accession number S12300. Branch support values of ML, Bayesian, and MP character-based, and NJ distance-based analyses were mounted on the ML phylogram in TreeGraph2 vers. 2.0.47-206 beta (Stöver and Müller, 2010), where the tree was also partially edited. Any conflicts in tree topology among different algorithms of phylogenetic inference are indicated with brackets as used by the TreeGraph2 vers. 2.0.47-206 beta software. Missing clades in one of the analyses are indicated with dashes instead of a branch support value

3. RESULTS

3.1. Isolation of the CesA3 genes

Degenerated primers (Table 2) based on conserved regions of the deduced amino acid sequences of published oomycete *CesA3* genes (Table 1) were used to amplify *CesA3* gene fragments from various oomycetes belonging to the *Albuginales*, *Leptomitales*, *Peronosporales*, *Pythiales*, *Rhipidiales* and *Saprolegniales*. Primer pair PhytCesA3f-r (Table 2) amplified a fragment of approximately 4000 bp in the four *Phytophthora* species: *P. capsici*, *P. cactorum*, *P. cinnamomi* and *P. palmivora*. Partial sequencing of obtained fragments and subsequent BlastN analysis to the NCBI GenBank revealed a high similarity to the *CesA3* orthologues from *P. infestans*, *P. sojae* and *P. ramorum*. The information obtained by sequence comparison led to the identification of start and stop codons in frame at the N-terminal and C-terminal ends. Species-specific primers (Supplementary Table 1) flanking the complete *CesA3* ORFs amplified the full-length gene. Translation of the nucleotide sequences into protein sequences using EditSeq showed that all four sequences were interrupted by one single intron (98 to 136 bp in

size) located in the 5' region, starting at nucleotide position 144 (Supplementary Fig. 2). Sequences that included putative introns were compared between amplified gene fragments obtained from gDNA and cDNA, respectively, and confirmed the predicted intron-exon boundaries.

Primer pair PytCesA3f-r (Table 2) used for the amplification of the *CesA3* gene in the five *Pythium* species: *P. arrhenomanes*, *P. coloratum*, *P. irregulare*, *P. iwayamai* and *P. violae* produced gene fragments of approximately 3000 bp in size. BlastN analyses with other *CesA3* orthologues demonstrated that at both ends (5' and 3') a 300 bp fragment was missing. Missing ends were isolated by genome walking and the newly obtained sequences were used for the design of species-specific primers (Supplementary Table 1). Analysis of translated sequences revealed in all five *Pythium* species the presence of a single intron (73 to 113 bp) located in the 5' region (Supplementary Fig. 2).

The CODEHOP primer pair CodeCesA3f-r (Table 1) amplified a specific fragment of approximately 680 bp in the following species: *Albugo candida*, *Aphanomyces euteiches*, *Apodachlya brachynema*, *Bremia lactucae* and *Sapromyces elongatus*. Direct sequencing of the obtained fragments and subsequent BlastN analyses at the NCBI database showed the best hit for each sequence with orthologue *CesA3* sequences deposited in the GenBank. Since only partial sequences were obtained, genome walking was performed (see methods 2.6) until the full length ORF was reached. Similar to *Phytophthora* and *Pythium* species, a single intron was present in the 5' region of the *CesA3* gene of *A. candida*, *A. euteiches* and *B. lactucae* starting at nucleotide position 138, 141 and 144, respectively (Supplementary Fig. 2). The sequences of *A. brachynema* and *S. elongatus* were interrupted by two or three introns, respectively, all of which located in the 5' region (Supplementary Fig. 2). The full length *CesA3* sequences of 13 species and the partial *CesA3* sequence from *S. elongatus* were deposited in the NCBI database under accession numbers: JF960416 (*AcCesA3*, *A. candida*); JN561770 (*AeCesA3*, *A. euteiches*); JN561769 (*AbCesA3*, *A. brachynema*); JN561771 (*BlCesA3*, *B. lactucae*); JN561772 (*PcacCesA3*, *P. cactorum*); JN561773 (*PcapCesA3*, *P. capsici*); JN561774 (*PcinCesA3*, *P. cinnamomi*); JN561775 (*PpCesA3*, *P. palmivora*); JN561776 (*ParrCesA3*, *P. arrhenomanes*); JN561777 (*PcolCesA3*, *P. coloratum*); JN561778 (*PirrCesA3*, *P. irregulare*); JN561779 (*PiwaCesA3*, *P. iwayamai*); JN561780 (*PvioCesA3*, *P. violae*); JN561781 (*SeCesA3*, *S. elongatus*).

3.2. CesA3 sequence analyses

The *CesA3* nucleotide sequences (cDNA) and their predicted protein sequences of 14 oomycete species were compared to 11 published *CesA3* orthologues by using ClustalW. Sequence comparisons at the nucleotide level confirmed that the *CesA3* gene of congeneric species shared the greatest identity (up to 94 % in the genus *Phythophtora*, 87 % in *Pythium* and 86 % in *Albugo*). However, *CesA3* nucleotide identity of members belonging to the genus *Saprolegnia* only reached 74 % (Supplementary Fig 1), showing variation in the broadness of genus definitions. .

The amino acid sequences deduced from the full-length *CesA3* genes that have been identified in this study, revealed predicted protein lengths of 1124 amino acids for the *Saprolegniales* and up to 1149 for the *Peronosporales*. Structural prediction of conserved domains with the NCBI CD search program (Marchler-Bauer et al., 2011) showed that the overall structure of oomycete CesA3 sequences was highly conserved. Figure 1 provides a representative scheme showing general domain structures of oomycete CesA3 sequences. Newly identified CesA3 sequences and the corresponding gene models accessed from the oomycete genome databases, all shared the conserved D,DXD,D,QXXRW motif (Supplementary Fig. 3A) located in a predicted cellulose synthase domain (TIGR03030) (Fig. 1). Sequence comparison with other CesAs from various kingdoms revealed that this motif is also present in CesAs of plants, bacteria, cyanobacteria rhodophyta, slime molds and tunicates (Fig. 1).

The conserved Pleckstrin Homology (PH) domain (cd00821, http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) at the N-terminus that has been identified in oomycete homologues of CesA1, CesA2 and CesA4 (Grenville-Briggs et al., 2008; Fugelstad et al., 2009) was absent in all CesA3 sequences (Fig. 1). However, instead of the PH domain, a CesA3 specific N-terminal domain containing two CxxC motifs occurred (Supplementary Fig. 3B). These motifs are characteristic for Ring-Finger domains in plant cellulose synthases (Kurek et al., 2002). As predicted by Tmpred, all CesA3 sequences contained 13 to 14 transmembrane domains, seven of which located at the N terminus (Fig. 1).

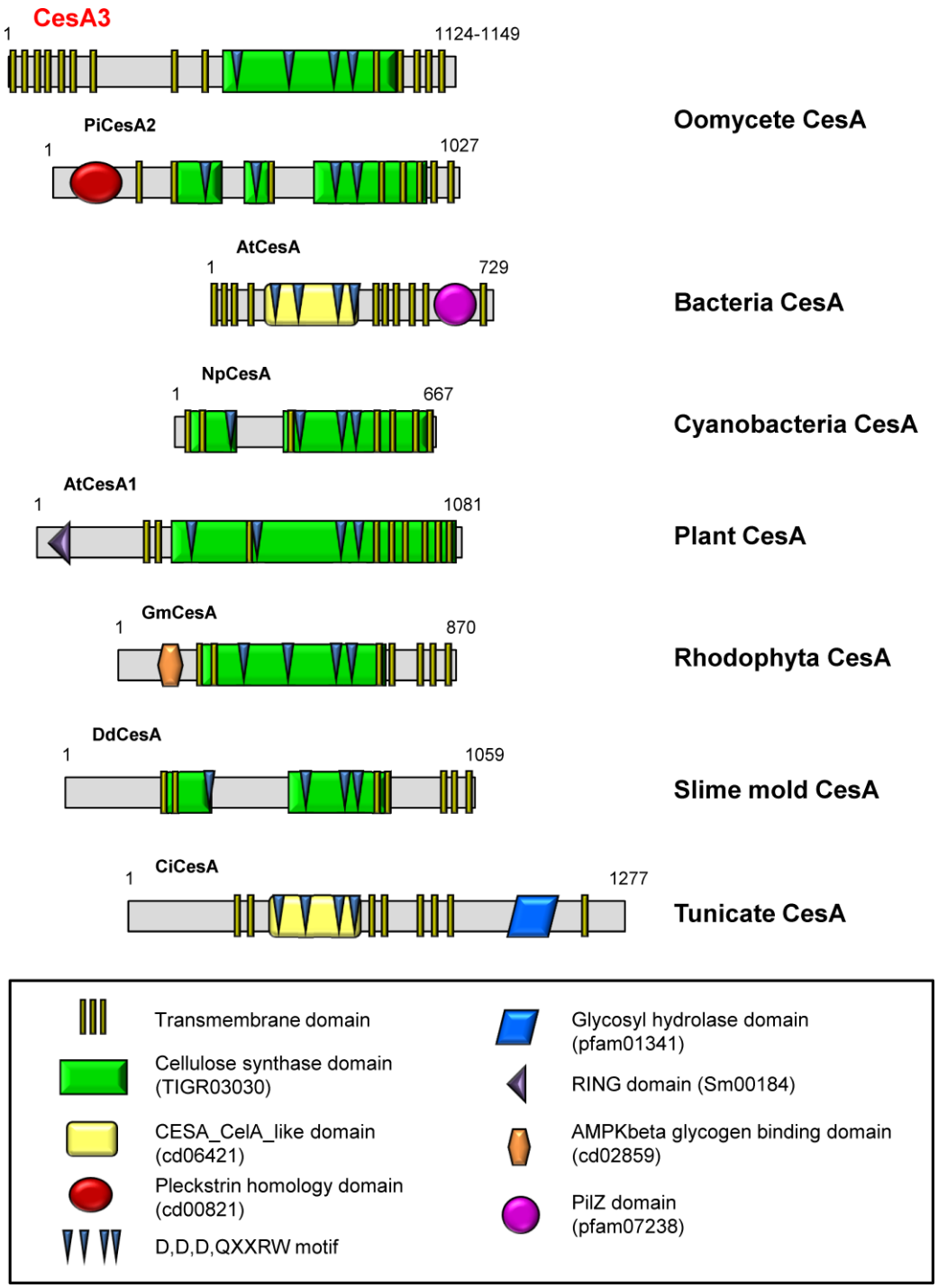

Fig. 1. Domain organization of the cellulose synthases (CesAs) of oomycetes in comparison to CesAs of members of other kingdoms. The schematic diagram shown for an oomycete CesA3 sequence (first row), represents the general domain structure of all CesA3 sequences identified in this study. Total length of deduced amino acid sequences are given above each protein. Sequences were aligned according to the position of the QXXRW motif (triangle). The proteins contain regions typical for cellulose synthases and cellulose synthase like enzymes. Several transmembrane (TM) domains are also present. Domain organizations of CesAs of *Phytophthora infestans* (Pi, EEY56433.1), *Agrobacterium tumefaciens* (At, NP_357298.1), *Nostoc punctiforme* (Np, YP_001865112.1), *Arabidopsis thaliana* (At, NP_194967.1), *Griffithsia monilis* (GM, ADK77974.1), *Dictyostelium discoideum* (Dd, XP_646256.1) and *Ciona intestinalis* (Ci, NP_001041448.1) are given for comparison.

3.3. Phylogenetic analysis of the oomycete CesA3 gene

Character- and distance-based phylogenetic analyses with four alternative algorithms yielded phylograms of a largely congruent topology and also comparable branch support at lower taxonomic ranks. All recognized orders to which the 25 analyzed oomycete species belong, were phylogenetically well supported, except *Pythiales*.

Maximum Likelihood (ML) analysis of the nearly full-length coding nucleotide sequence of the *CesA*3 gene provided a best tree with a log-likelihood of -38159.34. The average log-likelihood of the two Bayesian Analysis (BA) runs was -43,536.70 and Maximum Parsimony (MP) analysis gave a tree reflecting 8852 evolutionary steps. Neighbor-Joining (NJ) distance clustering yielded a tree of a total branch length of 3.58219845. The ML phylogram, with the posterior probability and bootstrap branch support values of all these tree inference algorithms is shown in Fig. 2.

The *CesA3* gene tree divided the 25 analyzed oomycete species into two main groups, namely a peronosporalean and saprolegnian lineage, often referred to as galaxies (Sparrow, 1976; Dick, 2001) (Fig. 2). In the saprolegnian galaxy, the two *Saprolegnia* spp. separated well from *A*. *euteiches*, which itself was separated from *A*. *brachynema,* representing the *Leptomitales.* The peronosporalean galaxy in the tree comprises species of the *Peronosporales*, *Pythiales*, *Albuginales* and *Rhipidiales* and is reasonably well resolved from the saprolegnian galaxy, which includes species of the *Leptomitales* and *Saprolegniales* and is supported by a bootstrap value of 67% in the ML and a posterior probability of 1.00 in the Bayesian tree. However, the clades representing these two main lineages received only weak branch support in the MP and none in the NJ analyses (Fig. 2). *Sapromyces elongatus*, representing *Rhipidiales,* together with the two *Albugo* spp. of the completely resolved *Albuginales* form the most basal clade of the peronosporalean galaxy, supported by 71 % in ML bootstrapping, a probability of 1.00 in BA, and 99 % in MP bootstrapping. The two orders *Pythiales* and *Peronosporales* of the peronosporalean galaxy were well separated from the other oomycetes, but the seven *Pythium* species fell into two distinct and well-supported clades, rendering the order *Pythiales* paraphyletic (Fig. 2). The clade representing the order *Peronosporales* with four downy mildews (*B. lactucae*, *H. arabidopsidis*, *P. viticola*, *P. cubensis*), and several *Phytophthora* species, received maximum branch support in the ML and MP trees (Fig. 2). Monophyly of the clade formed by the four representatives of

the downy mildews was confirmed by high branch support values in the ML (98 %) and BA (1.00) trees, but not in the MP and NJ trees (Fig. 2).

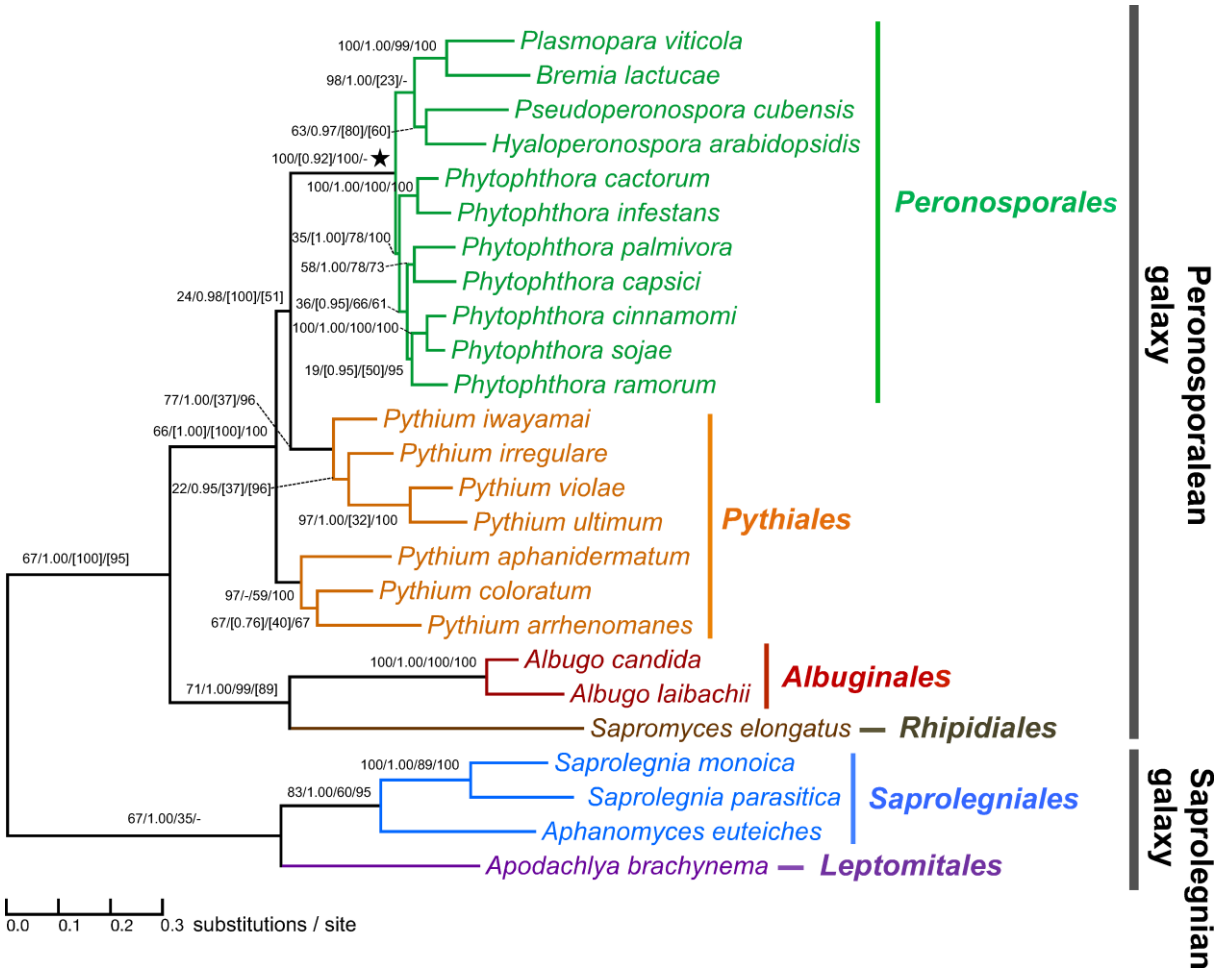

Fig. 2. Phylogram of the cellulose synthase 3 (*CesA3*) gene of members of the phylum *Oomycota*. The asterisk indicates the phylogenetic position at which sensitivity to carboxylic acid amide (CAA) appears to have evolved. The phylogram shows the topology and branch lengths of the maximum likelihood tree (ML, log likelihood: -38159.34). Branch support values originate (in sequence from left to right) from ML bootstrap, Bayesian posterior probability, maximum parsimony, and neighbor joining bootstrap analyses. Phylogenetic analyses were carried out on the coding nucleotide sequences of the entire *CesA3* gene (2962 sites), only excluding sites that were not shared among all 25 taxa. The scale bar indicates the number of inferred substitutions per site. See Table 1 for the respective sequence accession numbers and strain identifiers and Materials and Methods for further details, relating to the phylogenetic analyses.

3.4. Sensitivity of oomycetes to mandipropamid

The sensitivity of 21 different species from six distinct orders to MPD was investigated. Overall, the 21 tested oomycete species could be allocated into two distinct groups: one group consisted of MPD sensitive (EC_{50} < 1 mg l^{-1}) and the other of MPD tolerant (EC_{50} > 25 mg l^{-1}) species (Table 3).

MPD specifically inhibited mycelial growth and sporulation of members belonging to the *Peronosporales* with lowest EC_{50} values for *Phytophthora* species (0.01 to 0.05 mg l^{-1}) and somewhat higher for biotrophic species (0.11 to 0.87 mg l^{-1}) (Table 3). Outside the *Peronosporales* MPD was mostly inactive (EC_{50} > 25 mg l^{-1}). Within the MPD tolerant species, the fungicide slightly affected representatives of the *Leptomitales* and Saprolegniales (EC_{50} values 27 to 32 mg l^{-1}). *Sapromyces elongatus*, representing *Rhipidiales* and *A. candida* representing *Albuginales* were even "less responsive" (EC_{50} 70 to > 100 mg l^{-1}). Except for *P. arrhenomanes* (EC_{50} 70 mg l^{-1}), members of the order *Pythiales* showed no response to MPD (EC_{50} > 200 mg l^{-1}). The results show that only species belonging to the *Peronosporales* are highly sensitive to the fungicide MPD. All other species included in this study did not respond to MPD regardless of their differential taxonomic origin.

3.5. Analysis of the MPD target site in CesA3

Since changes in the amino acid sequence of the CesA3 target enzyme at position 1105 and 1109 can affect CAA fungicide activity (Blum and Gisi, 2012; Blum et al., 2011; Chen et al., 2011; Sierotzki et al., 2010), the CesA3 sequences of 21 oomycete species that were tested in this study and four additional sequences (accessed from the NCBI database; Accession No. in Table 1) were aligned to analyze whether specific amino acid configurations in the putative target site correlated to fungicidal response. In all oomycetes, a predicted transmembrane domain (ranging from 17 to 26 amino acids) characterized the target site region in CesA3 (Fig. 3). The region containing the putative target site was highly conserved between the six different orders. Out of 36 amino acids, 18 residues were fully identical (Fig. 3). The CesA3 sequences of the 11 *Peronosporales* were even more conserved showing only two variable amino acids. All 25 species displayed the residue glycine at position 1105 (G1105), the substitution of which with various amino acids (Fig. 3) is known to confer CAA resistance in *Peronosporales* (Blum *et al*. 2010b; Sierotzki *et al*. 2011). However, out of the 35 amino acids flanking the G1105 residue, there were only two amino acid positions (1109, 1111) that differed between the MPD sensitive *Peronosporales* and the five MPD tolerant orders.

Table 3

Sensitivity to the fungicide mandipropamid and amino acid configuration at position 1105 and 1109 in the cellulose synthase3 (CesA3) enzyme of representative members originating from the six "crown" oomycete orders

Order	Species	Strain	Amino acid in CesA3		EC_{50} MPD (mg l^{-1})[b]
			1105[a]	1109	
Albuginales	*Albugo candida*	Zum3352	Gly [GGA]	Leu [TTA]	>100[c]
	Albugo laibachii	Nc14	Gly [GGG]	Leu [TTA]	-
Leptomitales	*Apodachlya brachynema*	CBS 184.82	Gly [GGG]	Leu [CTG]	26[d]
Peronosporales	*Bremia lactucae*	HS1	Gly [GGC]	Val [GTC]	0.66[c]
	Hyaloperonospora arabidopsidis	Emoy2	Gly [GGT]	Val [GTG]	-
	Phytophthora cactorum	119	Gly [GGC]	Val [GTG]	0.06[d]
	Phytophthora capsici	188	Gly [GGC]	Val [GTG]	0.03[d]
	Phytophthora cinamomi	273	Gly [GGC]	Val [GTG]	0.05[d]
	Phytophthora infestans	96	Gly [GGC]	Val [GTG]	0.01[d]
	Phytophthora palmivora	224	Gly [GGT]	Val [GTG]	0.02[d]
	Phytophthora sojae	CBS 125701	Gly [GGC]	Val [GTG]	0.02[d]
	Phytophthora ramorum	Pr-102	Gly [GGC]	Val [GTG]	-
	Plasmopara viticola	D05	Gly [GGC]	Val [GTT]	0.11[c]
	Pseudoperonospora cubensis	CH01	Gly [GGG]	Val [GTG]	0.87[c]
Pythiales	*Pythium aphanidermatum*	620	Gly [GGC]	Leu [CTG]	230[d]
	Pythium arrhenomanes	411	Gly [GGC]	Leu [CTC]	70[d]
	Pythium coloratum	336	Gly [GGC]	Leu [CTG]	>300[d]
	Pythium irregulare	73	Gly [GGC]	Met [ATG]	>300[d]
	Pythium iwayamai	948	Gly [GGC]	Met [ATG]	>300[d]
	Pythium ultimum	71	Gly [GGT]	Met [ATG]	>300[d]
	Pythium violae	339	Gly [GGT]	Met [ATG]	>300[d]
Rhipidiales	*Sapromyces elongatus*	CBS 213.82	Gly [GGA]	Leu [CTT]	70[d]
Saprolegniales	*Aphanomyces euteiches*	CBS 154.73	Gly [GGT]	Leu [TTG]	232[d]
	Saprolegnia monoica	CBS 539.67	Gly [GGC]	Leu [CTC]	-
	Saprolegnia parasitica	CBS 223.65	Gly [GGC]	Leu [CTC]	32[d]

[a]Codon is given in parenthesis
[b]EC_{50} = effective concentration for 50% growth inhibition.
[c]EC_{50} values result from in planta tests.
[d]EC_{50} values result from mycelial growth tests performed on agar plates.

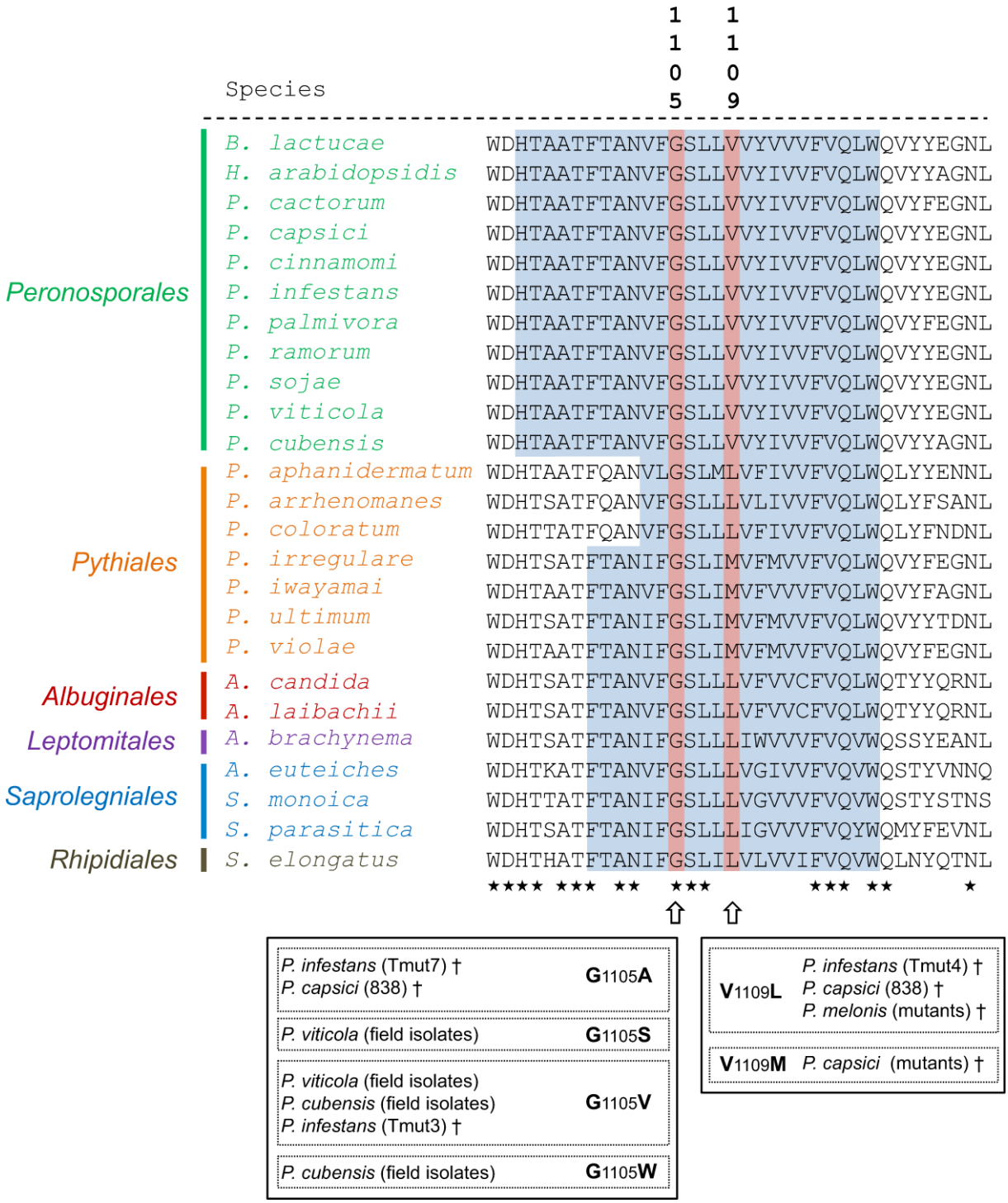

Fig. 3. Comparison of the putative target site of carboxylic acid amide (CAA) fungicides in the cellulose synthase 3 (CesA3) sequences of 25 oomycetes. Each sequence is characterized by a predicted transmembrane (TM) domain (shaded). The amino acid positions 1105 and 1109 responsible for resistance and tolerance to mandipropamid (MPD) are shown on top of the alignment. Strictly conserved residues are marked by asterisks. Amino acid substitutions leading to MPD resistance in field isolates and artificial mutants (†) are listed below the alignment.

In all species belonging to the *Peronosporales* the inherent amino acid residue at position 1109 was valine (V1109), whereas species of the *Albuginales*, *Leptomitales*, *Rhipidiales*, *Saprolegniales* and *Pythiales* displayed the amino acid leucine (L1109) or in some cases (*Pythiales*) also methionine (M1109) (Fig. 3). At position 1111, the amino acid tyrosine (*Peronosporales*), was replaced by phenylalanine (*Albuginales*, *Pythiales*), leucine (*Pythiales*, *Rhipidiales*), tryptophan (*Leptomitales*) or glycine (*Saprolegniales*).

An exchange of the conserved V1109 residue with L1109 or M1109 led to CAA resistance in artificial mutants of *P. infestans*, *P. capsici* and *P. melonis* (Chen et al., 2011), thus it is likely that the inherent amino acid configuration (L1109, M1109) in *Albuginales*, *Pythiales*, *Leptomitales*, *Rhipidiales* and *Saprolegniales*, is the cause for tolerance to MPD in these orders. Species with the L1109 residue displayed EC_{50} values of > 25 to < 300 mg L^{-1} (with the exception of *P. coloratum*), while the M1109 configuration in the four *Pythium* species caused EC_{50} values of > 300 mg L^{-1} (Table 3).

4. DISCUSSION

Cellulose is the main component of oomycete cell walls (Bartnicki-Garcia, 1968), the synthesis of which represents a very sensitive fungicide target as shown for *Peronosporales* (e.g. *Phytophthora, Plasmopara*, *Pseudoperonospora*, *Bremia*) (FRAC). The present study comprehensively analyzed the *CesA3* gene that is involved in cellulose biosynthesis (Grenville-Briggs et al., 2008) and that encodes the target protein of carboxylic acid amide (CAA) fungicides (Blum et al., 2010a). Since in *Peronosporales* mutations in the CesA3 enzyme at position 1105 and 1109 directly affect sensitivity to CAAs (Blum et al., 2010b; Blum et al., 2011; Chen et al., 2011; Sierotzki et al., 2011), this study focused on the influence of specific amino acid configurations at these positions on sensitivity to the CAA fungicide mandipropamid in oomycetes. We isolated the *CesA3* gene of various oomycetes representing the six "crown" orders (Beakes and Sekimoto, 2009) containing pathogens of plants (*Peronosporales*, *Albuginales*, *Pythiales*, *Saprolegniales*), pathogens of fish (*Saprolegniales*) and saprophytic species (*Leptomitales*, *Rhipidiales*). By designing degenerated primers we were able to identify the *CesA3* gene in all representatives of the six orders, suggesting that it may play an important role in cellulose biosynthesis of all six oomycete orders.

Sequence comparison at the nucleotide and amino acid level demonstrated that the *CesA3* orthologues are highly conserved, especially among species of the same order

(Supplementary Fig. 1), implying that the *CesA3* gene may have been acquired from a common ancestor and then subsequently evolved within the different orders, which is in agreement with the results obtained in phylogenetic analysis of this gene. Even though minor differences exist in the gene structure of oomycetes (number of introns, Supplementary Fig. 2), the overall domain organization of the deduced amino acid sequences is highly conserved. For example all CesA3 sequences contained a predicted cellulose synthase domain (TIGR03030) in the C-terminal region of the enzyme including the conserved D, DXD, D, QXXRW motif (Fig. 1, Supplementary Fig. 3A). This motif has been proposed to define the nucleotide sugar-binding domain and the catalytic site of the enzyme necessary to act as processive glycosyltransferase (Charnock et al., 2001) and occurs in CesAs of various kingdoms (Fig. 1).

Similar to other CesAs, putative membrane proteins, the CesA3 sequence is characterized by several transmembrane (TM) domains, suggesting that the protein is localized in the plasma membrane, as it has been shown by immunolocalization studies in *Phytophthora infestans* (Grenville-Briggs et al., 2008). Unlike other CesAs, the CesA3 sequence contains only one predicted domain representing a putative cellulose synthase (TIGR03030) (Fig. 1). This feature clearly distinguishes the CesA3 sequence from its CesA homologues: Additionally in CesA1, CesA2 and CesA4 a predicted PH domain (Fig. 2) exists in *Peronosporales*, *Pythiales* and *Saprolegniales* (Blum and Gisi, 2012; Blum et al., 2010b; Blum et al., 2011; Fugelstad et al., 2009; Grenville-Briggs et al., 2008). However, instead of this PH domain, several TM domains and two specific motifs (CxxC) are located at the N-terminal end in CesA3 (Supplementary Fig. 3B). These motifs are characteristics of ring finger domains in plants and are postulated to be involved in the dimerization of CesA subunits (Kurek et al., 2002; Richmond and Somerville, 2000). Therefore, it is likely that in oomycetes, the CesA3 enzyme consists of two subunits that may need to dimerize to be functional.

Several previous studies reconstructed taxonomically broad phylogenies of the oomycetes, however, mostly based on sequences of the nuclear ribosomal small subunit RNA gene (SSU rRNA) (Bakes et al., 2011; Lara and Belbahri 2011), the nuclear ribosomal large subunit RNA gene (LSU rDNA) (Petersen and Rosendahl 2000; Riethmüller et al., 2002), the ribosomal internal transcribed spacer region (ITS) in between of these two genes (Cooke et al., 2000; Robideau et al., 2011; Voglmayr, 2003), the mitochondrial *cox2* gene (Hakariya et al., 2007; Hudspeth et al., 2000; Thines et al.,

2008), the cytochrome c oxidase subunit I (COI) (Robideau et al., 2011), or the cytochrome b (*cyt b*) gene (Giresse et al., 2010). Here, we used the full-length nucleotide sequences of the cellulose synthase 3 (*CesA3*) gene of 25 species, representing 11 genera and six orders within *Oomycota* to illustrate where in the phylogeny sensitivity or tolerance to CAA fungicides evolved. Characterization of the target codons of CAAs, associated with fungicide resistance/tolerance (Table 3) suggests that the CesA3 amino acid sequence responsible for CAA sensitivity must have arisen late in the phylogeny of *Oomycota*, when the *Peronosporales* clade emerged (Fig. 2).

Phylogenetic grouping of the analysed species in the *CesA3* gene tree is generally in good agreement with that known from phylogenies based on other markers, such as *cox2*, ITS, LSU, and SSU (Beakes et al., 2011; Hudspeth et al., 2000; Lara and Belbahri 2011; Petersen and Rosendahl 2000; Riethmüller et al., 2002; Robideau et al., 2011), implying that the *CesA3* gene may be a useful addition to the current set of molecular markers used in the phylogenetic reconstruction of the evolutionary relationships among oomycetes. In fact, the phylogenetic information contained in the *CesA3* gene proved useful to putatively resolve the phylogenetic position of *Sapromyces elongatus*, representing *Rhipidiales*. Besides considerable phylogenetic distance, branch support from all four statistical analyses, separated *Rhipidiales* from *Albuginales* and the remainder of the *Oomycota*. In particular, the two members of the *Albuginales* separated well from *S. elongatus,* a result that was also obtained by analysis of amino acid sequences of the *cox2* gene (Sekimoto et al., 2008). In our *CesA3* based analyses, this clustering is highly supported, not only in the MP and NJ trees, which could be due to long-branch attraction (Bergsten, 2005), but also in the ML and BA trees. According to the present analyses, *Rhipidiales* are clearly members of the peronosporalean galaxy, which is a finding that agrees with what has been indicated in previous studies (Beakes and Sekimoto, 2009; Hudspeth et al., 2003; Sekimoto et al., 2008; Thines et al., 2008), but not yet with comparably high confidence.

Albuginales initially thought to be related to the downy mildews (Beakes, 1989), are meanwhile recognized to take a phylogenetic sister position to the lineage comprising members of the orders *Peronosporales* and *Pythiales* (Hudspeth et al., 2003; Riethmüller et al., 2002; Voglmayr and Riethmuller, 2006), mainly owing to information from nucleotide sequences. The *CesA3* gene tree confirmed the basal position of the two *Albugo* species in the peronosporalean lineage and justifies at least an ordinal taxonomic

status. It remains to be determined by sequencing the *CesA3* gene of further members of the *Rhipidiales* and *Albuginales* whether it is, indeed, justified to raise a subclass *Albuginomycetidae* (Thines and Spring, 2005).

Member species of the genus *Pythium* cluster also in the *CesA3* gene tree in paraphyletic positions to the *Peronosporales*, which is in agreement with previous phylogenetic reconstructions (Levesque and De Cock, 2004; Uzuhashi et al., 2010; Voglmayr, 2003). Confirming the findings by Göker et al. (2003), the genus *Phytophthora*, clustered sister to the clade with the downy mildews, the two main groups of important plant pathogens in the order *Peronosporales*. The monophyly of this order is supported by the considerable phylogenetic distance and maximal branch support values in the ML and MP trees. However, the *CesA3* gene tree resulting from the ML analysis, resolves the genus *Phytophthora* as monophyletic, which is in agreement with the findings of Kroon et al. (2004) but contradictory to other studies that resolved *Phytophthora* as clearly paraphyletic (Cooke et al., 2000; Göker et al., 2007; Runge et al., 2011; Vogelmayr, 2003). Yet only shown with significant support by Göker *et al*. (2007), the present analyses on the *CesA3* gene sequences support the monophyly of the downy mildews (Fig. 2), which was mentioned to be uncertain by Thines et al. (2009).

The current study showed that the *CesA3* gene is present in a broad range of phylogenetically distinct oomycetes. As a consequence the CesA3 enzyme should generally be a suitable target for CAA fungicides in all oomycetes. Several studies reported the high activity of CAAs against *Peronosporales* (Cohen and Gisi, 2007; Cohen et al., 2008; Gisi et al., 2007; Kim et al., 2009; Lu et al., 2010; Zhu et al., 2008) but only few studies addressed the insensitivity of the *Pythiales* against this fungicide class (Albert et al., 1988; Blum and Gisi, 2012; Stenzel et al., 1998). It is surprising that the CAAs were never tested against representatives of the animal parasitic order *Saprolegniales*, members of which can cause enormous losses in aquaculture (Phillips et al., 2008; van West, 2006), or against plant parasitic *Albuginales* causing white blister rust. As shown in our study, species of these orders all contain the *CesA3* gene. However, according to the data obtained in the sensitivity tests, the response to the CAA fungicide mandipropamid was generally low in *Saprolegniales* and *Albuginales* (Table 3). Indeed, only species of the *Peronosporales* order were highly sensitive to MPD, whereas all other orders including the saprophytic *Rhipidiales* and *Leptomitales* were mostly unaffected by the fungicide (Table 3). Interestingly, representatives of the *Pythiales* that showed

sistergroup relationship to the *Peronosporales* (Fig. 2), were least sensitive among all tested species (Table 3).

It is unlikely that the observed insensitivity of species outside *Peronosporales* is the result of specific barriers that hinder the molecule from reaching the target site, because uptake studies with ^{14}C-labelled MPD showed that it acts on the cell wall without entering the cell (Blum et al., 2010a). A much more fundamental consideration concerns the alteration of the target site itself. Several studies demonstrated that specific mutations in the C-terminal end of the CesA3 target protein lead to fungicide resistance (Blum et al., 2010b; Blum et al., 2011; Chen et al., 2011; Sierotzki et al., 2011). The MPD tolerant species tested in this study showed EC_{50} values similar to CAA resistant field strains of sensitive species displaying mutations (Blum et al., 2010b; Blum et al., 2011). Thus, CAA tolerant oomycetes most probably have an inherent amino acid configuration at the target site that somehow inhibits proper binding of the molecule. At the most prominent position (1105) that was observed to mutate in resistant field isolates, there was no change (glycine 1105, Fig 3). However, at position 1109, the amino acid configuration of *Peronosporales* (V1109) deviated from other oomycetes (L1109, M1109). Additionally, at position 1111, the amino acid tyrosine (*Peronosporales*), was replaced by phenylalanine (*Albuginales*, *Pythiales*), leucine (*Pythiales*, *Rhipidiales*), tryptophan (*Leptomitales*) or glycine (*Saprolegniales*). These differences may be explained by evolutionary processes, since the *CesA3* sequence of *Peronosporales* seems to diverge late in the peronosporalean galaxy (Fig. 2). Currently there is no direct evidence that CAA tolerance is linked with alterations at amino acid position 1109 or 1111. However, since an exchange from valine to leucine or methionine (V1109L, V1109M) conferred CAA resistance in mutated *Phytophthora* species (Chen et al., 2011) we postulate that the amino acid configuration at position 1109 directly affects fungicide efficiency. Up to now, mutations at position 1111 of sensitive species, conferring resistance to CAAs, were not reported. Therefore it remains to be investigated if position 1111 may also play a role for the interaction with the fungicide. The absence of a three-dimensional CesA enzyme model in oomycetes prohibits predicting the influence of specific amino acid changes on the structural integrity of the protein. Thus, we can only speculate that alterations at position 1109 (or 1111) may cause changes in the three-dimensional structure of the target site possibly leading to reduced fungicide binding and MPD tolerance in these oomycetes.

The present study provided new information on the lack of activity of the CAA fungicide MPD against oomycetes other than *Peronosporales*. According to our data, there is strong evidence that the inherent target site configuration in CesA3 determines whether a certain species can be inhibited by the fungicide or not. Even though all species outside *Peronosporales* tested in this study were insensitive to MPD, it might be worthwhile to consider synthesizing molecules with the same mode of action but modified binding properties. However, if this approach is feasible depends largely on the advances on modelling the binding pocket of these inhibitors in CesA3.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1

Oligonucleotide primer pairs and sequencing primers used in template preparation for sequencing of the oomycete *CesA3* genes

Gene	Primer Name	Primer Sequence (5'-3')	Application	Amplicon Size (bp)
AcanCesA3	Ac_cesa3f	AATATTGCCATCGAACCCTCTGTCTGGC	PCR	3723
	Ac_cesa3r	CTTCCGCCCTGAATGCATTACCTGC	PCR	
	Ac_cesa3seqr1	TGAGTCGTACATAATGCC T	Sequencing	
	Ac_cesa3seqf1	CATGGATGACTTCAGATGGT	Sequencing	
	Ac_cesa3seqf2	ATGACAGTATGGCTCGTCCA	Sequencing	
	Ac_cesa3seqf3	TGAACACAAAAGTGATCGAGAT	Sequencing	
	Ac_cesa3seqf4	GACTCTATCGGTGGTATTCAG	Sequencing	
	Ac_cesa3seqf5	TGCTGTTGATAACAATGACGT	Sequencing	
AeutCesA3	Ae_cesa3f	TAGAGAGGGCAGAAAGCCAGCCAG	PCR	3769
	Ae_cesa3r	CAGGAATTCAAGGCATCGTGTGTTGCC	PCR	
	Ae_cesa3seqr1	TTCATGCATTGAGGTCATG	Sequencing	
	Ae_cesa3seqf2	TACAACAAGGTTGGTTCCCA	Sequencing	
	Ae_cesa3seqf3	CCGTTGTTGCCTTGCTC	Sequencing	
	Ae_cesa3seqf4	AGGATGGCAGCAAGGGA	Sequencing	
	Ae_cesa3seqf5	TCCGCAAGGATTTCGAAG	Sequencing	
	Ae_cesa3seqf6	CTCCTTGTTTGTGAGTCAGT	Sequencing	
AbraCesA3	Ab_cesa3f	CCATCGAGACAACAGCACTTTTAATCACC	PCR	3613
	Ab_cesa3r	ATCCTTTTATGCAGTTGTTGTAGACGACG	PCR	
	Ab_cesa3seqr1	CCAAATAACTAGCATCAGCA	Sequencing	
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	Ab_cesa3seqf2	CAATGAAGCTCGGTCAATC	Sequencing	
	Ab_cesa3seqf3	GTAGATACCATGAAAACCCTGA	Sequencing	
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	Ab_cesa3seqf5	TTGCCAGTCATGCTTGCT	Sequencing	
BlacCesA3	Bl_cesa3f	GGATACTCGTGAGAACACCGTCACTCG	PCR	3700
	Bl_cesa3r	CGTACATGGCTACACTTTGGCTACACTTCC	PCR	
	Bl_cesa3seqr1	CCAGATCATAAACATGACCGT	Sequencing	
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	Bl_cesa3seqf2	GTCGTTTCGATCGTTTGCCA	Sequencing	
	Bl_cesa3seqf3	CTGGGATGCTAATAATCACT	Sequencing	
	Bl_cesa3seqf4	CTTTTGCCGGCACGAAC	Sequencing	
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	Pc_cesa3r	GCACGACTTCGGGTACAGCTGATTCG	PCR	
	Pc_cesa3seqr1	CACGCGGTTGTAGCACAT	Sequencing	
	Pc_cesa3seqf1	TCAAGTACATCATCTTCTCGG	Sequencing	
	Pc_cesa3seqf2	GCATTCGCTTCATGGACT	Sequencing	
	Pc_cesa3seqf3	CGACATCTTCCTGTGTCACT	Sequencing	

	Pc_cesa3seqf4	TGCTGCCGTTCTTCTTCT	Sequencing	
	Pc_cesa3seqf5	GTTCCTGTACTCTTTCTTGCC	Sequencing	
PcacCesA3	Pcac_cesa3f	CTCGGCAGCAGACCAAAGCG	PCR	3678
	Pcac_cesa3r	GCACAGCTGATTCGCTACATAGGC	PCR	
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	Pcac_cesa3seqf4	TGCTGCCGTTCTTCTTCT	Sequencing	
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	Pcin_cesa3seqf4	TGCTGCCGTTCTTCTTCT	Sequencing	
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PpalCesA3	Pp_cesa3f	CTCGGCAGCAGACCAAAGCG	PCR	3715
	Pp_cesa3r	GCACCGCTGATTCGCTACATAGGC	PCR	
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	Pc_cesa3r	CCAAACGCCGTTTGCACGAAGACG	PCR	
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	Pc_cesa3seqf2	GATGAGCATGAGCATGAAG	Sequencing	
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	Pi_cesa3r	GCTCTTGTGTTGTCTTCTGTCGATCGC	PCR	
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PiwaCesA3	Piw_cesa3f	TGTATCGCGTCCGCACTCATTCG	PCR	3939
	Piw_cesa3r	TTCACTACCCAACATCACGCAGCAGG	PCR	
	Piw_cesa3seqr1	ATGGAGAGCGTGTCGTT	Sequencing	
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	Piw_cesa3seqf3	AGAGCTGCTGCACATCT	Sequencing	
	Piw_cesa3seqf4	AGACGCCGCAGTACTTC	Sequencing	
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PultCesA3	Pu_cesa3f	GATAGCTTCGCGAAGTTTCCCAGG	PCR	3886
	Pu_cesa3r	CGTGTGTGTAGTGAGAAAGCGAAGC	PCR	
	Pu_cesa3seqr1	AAGCAGCAGAGCCACCA	Sequencing	
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	Pu_cesa3seqf2	TGGTCGTTCCATTCGCTT	Sequencing	
	Pu_cesa3seqf3	GTCACTACACGGAACCTGT	Sequencing	
	Pu_cesa3seqf4	TACGTGCAAACACCGCA	Sequencing	
	Pu_cesa3seqf5	GCTCCGATTTATGCCAAG	Sequencing	
PvioCesA3	Pv_cesa3f	TCGCAGATCCGCAGAAACCAGAAGC	PCR	4075
	Pv_cesa3r	TATGTTTGCCTGAAACCGCGTTGCTACC	PCR	
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	Pv_cesa3seqf3	TGTTCTTGTGTCACTACACG	Sequencing	
	Pv_cesa3seqf4	TCGGATGATATCTCGTGGA	Sequencing	
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	Se_cesar	CCACTGAGAGCAGCAAAATATAGCAGG	PCR	
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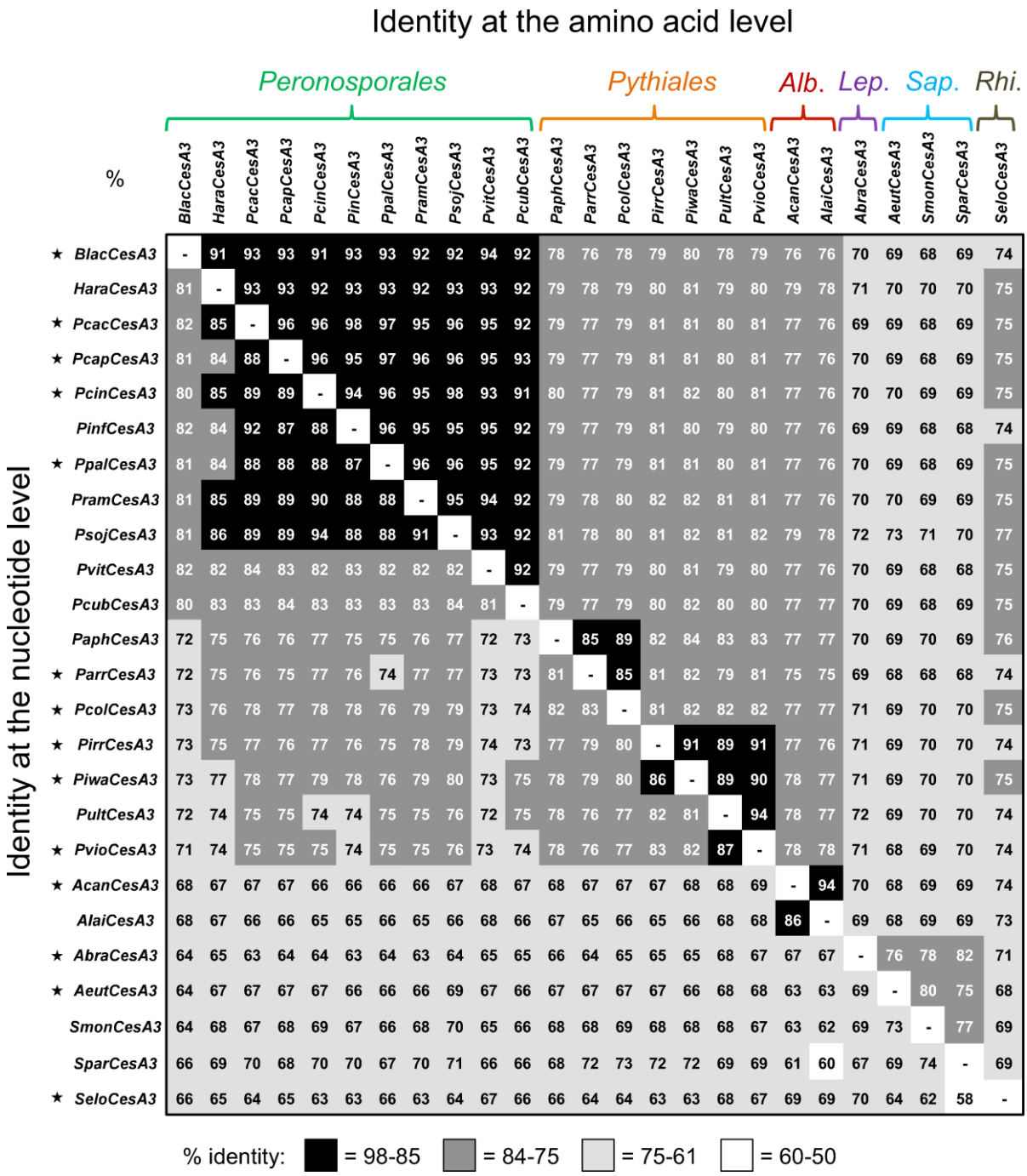
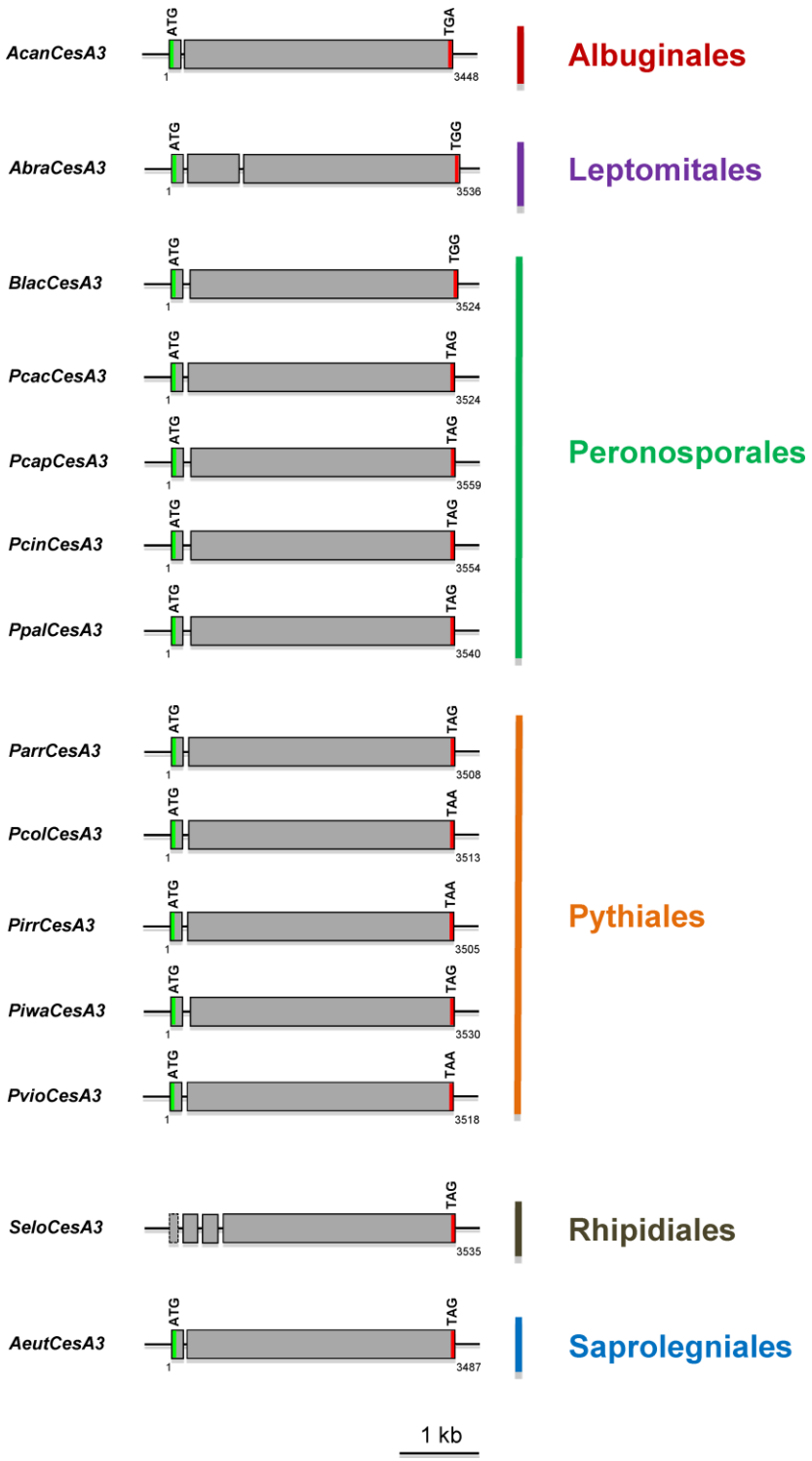
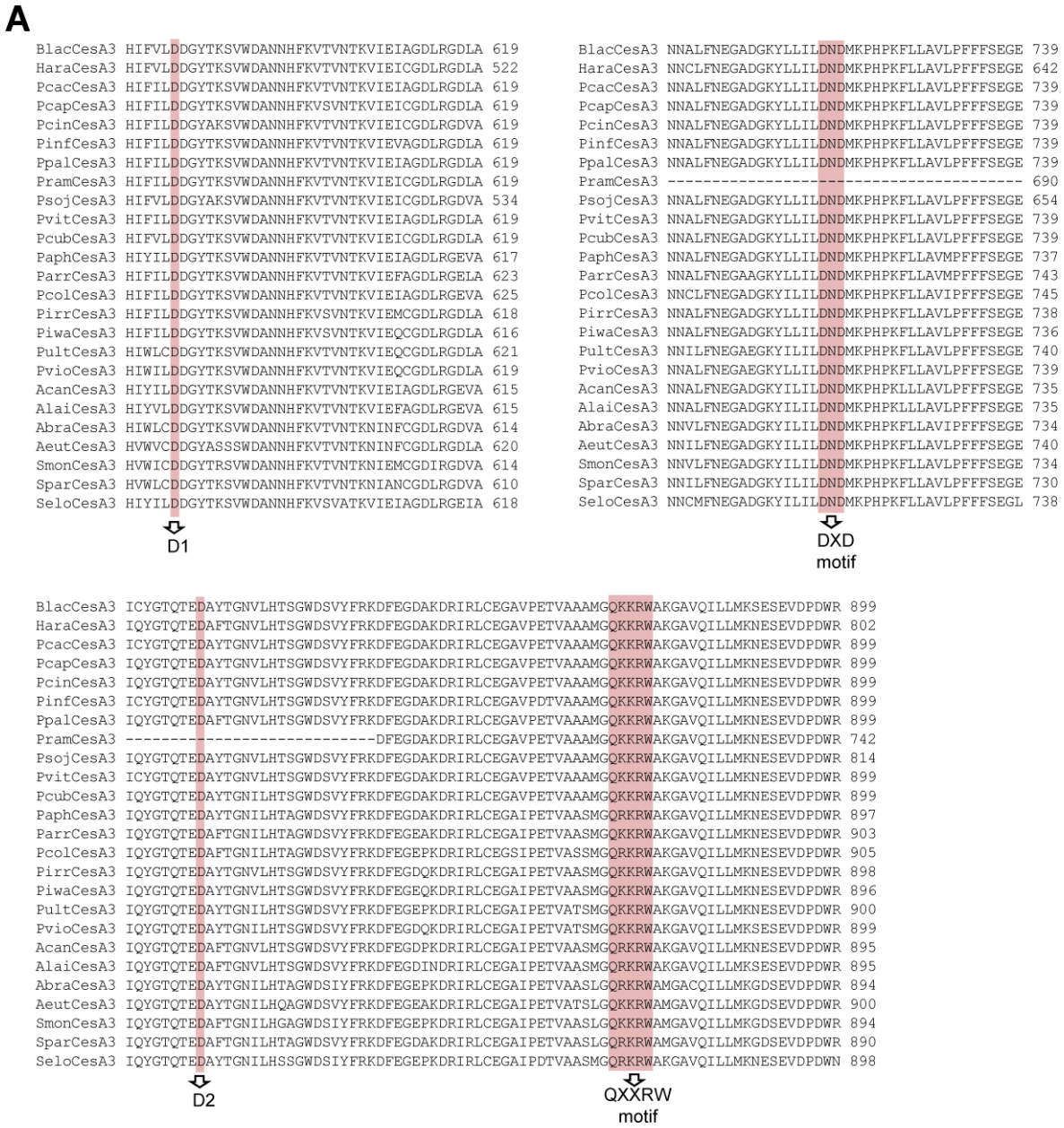
	Se_cesa3seqf1	TTGTTTGGGCTGGTGTTG	Sequencing	
	Se_cesa3seqf2	TGCTGTTACTGGTTTATGGT	Sequencing	
	Se_cesa3seqf3	TGGTCGTATGAAACCAGAAGT	Sequencing	
	Se_cesa3seqf4	CACCTCGTGTACCTGCT	Sequencing	



Supplementary Fig. 1. Identity of nucleotide and amino acid cellulose synthase 3 (*CesA3*) sequences of various oomycetes. Percentage identity for isolates of 25 different species belonging to the six "crown" orders *Albuginales* (*Alb.*), *Leptomitales* (*Lep.*), *Peronosporales*, *Pythiales*, *Rhipidiales* (*Rhi.*) and *Saprolegniales* (*Sap.*) are shown. Cell shading indicates percentage identity. Sequences with an asterisk were determined in this study. Abbreviations of species names are as follows: *Acan* (*Albugo candida)*, *Alai* (*Albugo laibachii)*, *Aeut* (*Aphanomyces euteiches*), *Abra* (*Apodachlya brachynema*), *Blac* (*Bremia lactucae*), *Hara* (*Hyaloperonospora arabidopsidis*), *Pcac* (*Phytophthora cactorum*), *Pcap* (*Phytophthora capsici*), *Pcin* (*Phytophthora cinnamomi*), *Pinf* (*Phytophthora infestans*), *Ppal* (*Phytophthora palmivora*), *Pram* (*Phytophthora ramorum*), *Psoj* (*Phytophthora sojae*), *Pvit* (*Plasmopara viticola*), *Pcub* (*Pseudoperonospora cubensis*), *Paph* (*Pythium aphanidermatum*), *Parr* (*Pythium arrhenomanes*), *Pcol* (*Pythium coloratum*), *Pirr* (*Pythium irregulare*), Piwa (*Pythium iwayamai*), *Pult* (*Pythium ultimum*), *Pvio* (*Pythium violae*), *Smon* (*Saprolegnia monoica*), Spar (*Saprolegnia parasitica*), *Selo* (*Sapromyces elongatus)*.



Supplementary Fig. 2. Sequence analyses of *CesA3* genes identified in this study. Start and stop codons are indicated in green and red, respectively. Gaps interrupting exon sequences (grey) represent introns. Total length in base pairs (bp), including introns, are given below each gene sequence. Full names of the species from which the *CesA3* genes were isolated are: *Albugo candida* (Acan), *Aphanomyces euteiches* (Aeut), *Apodachlya brachynema* (Abra), *Bremia lactucae* (Blac), *Phytophthora cactorum* (Pcac), *Phytophthora capsici* (Pcap), *Phytophthora cinnamomi* (Pcin), *Phytophthora palmivora* (Ppal), *Pythium arrhenomanes* (Parr), *Pythium coloratum* (Pcol), *Pythium irregulare* (Pirr), *Pythium iwayamai* (Piwa), *Pythium violae* (Pvio), *Sapromyces elongatus* (Selo).



Supplementary Fig. 3. Multiple alignment of segments from oomycete CesA3 sequences.
(**A**) Alignment of CesA3 sequences originating from 25 oomycete species. Conserved D,DXD,D,QXXRW motifs that are present in most processive glycosyltransferases are shaded in red. Numbers indicate the position of the amino acid residues of each protein. Full species names are: *Albugo candida* (*AcanCesA3*), *Albugo laibachii* (*AlaiCesA3*), *Aphanomyces euteiches* (*AeutCesA3*), *Apodachlya brachynema* (*AbraCesA3*), *Bremia lactucae* (*BlacCesA3*), *Hyaloperonospora arabidopsidis* (*HaraCesA3*), *Phytophthora cactorum* (*PcacCesA3*), *Phytophthora capsici* (*PcapCesA3*), *Phytophthora cinnamomi* (*PcinCesA3*), *Phytophthora infestans* (*PinfCesA3*), *Phytophthora palmivora* (*PpalCesA3*), *Phytophthora ramorum* (*PramCesA3*), *Phytophthora sojae* (*PsojCesA3*), *Plasmopara viticola* (*PvitCesA3*), *Pseudoperonospora cubensis* (*PcubCesA3*), *Pythium aphanidermatum* (*PaphCesA3*), *Pythium arrhenomanes* (*ParrCesA3*), *Pythium coloratum* (*PcolCesA3*), *Pythium irregulare* (*PirrCesA3*), *Pythium iwayamai* (*PiwaCesA3*), *Pythium ultimum* (*PultCesA3*), *Pythium violae* (*PvioCesA3*), *Saprolegnia monoica* (*SmonCesA3*), *Saprolegnia parasitica* (*SparCesA3*), *Sapromyces elongatus* (*SeloCesA3*).

B

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BlacCesA3 LVCYNRVGTHAQMG--PVDEQLPELPTGATLPAQTITERVRLVCENCFATVPAGTAFCTECGEAMPSED- 298
HaraCesA3 LMCYNRVGAHAQLG--PVEEQLPELPTGATLPAQTLTERVRLVCENCFATVPSGTAFCTECGEAMPSED- 213
PcacCesA3 LMCYNRVGAHAQLG--PVEEQMPELPTGATLPAQTMTERVRLVCENCFATVPAGTAFCTECGEAMPSED- 298
PcapCesA3 LMCYNRVGAHAQLG--PVEEQMPELPTGATLPAQAMTERVRLVCENCFATVPSGTAFCTECGEAMPSDD- 298
PcinCesA3 LMCYNRVGAHAQLG--PVEEQMPELPTGATLPAQTMNERVRLVCENCFATVPSGTAFCTECGEAMPSDD- 298
PinfCesA3 LMCYNRVGAHAQLG--PVEEQMPELPTGATLPAQTMTERVRLVCENCFATVPAGTAFCTECGEAMPSED- 298
PpalCesA3 LMCYNRVGAHAQLG--PVEEQMPELPTGATLPAQAMTERVRLVCENCFATVPSGTAFCTECGEAMPSED- 298
PramCesA3 LMCYNRVGAHAQLG--PVEEQMPELPTGATLPAQAMTERVRLVCENCFATVPSGTAFCTECGEAMPSDD- 298
PsojCesA3 LMCYNRVGAHAQLG--PVEEQMPELPTGATLPAQTMNERVRLVCENCFATVPSGTAFCTECGEAMPSDE- 213
PvitCesA3 LLCYNRVGAHAQLG--PVEEQLPELPTGATLPAQTITERVRLVCENCFATVPAGTAFCTECGEAMPSED- 298
PcubCesA3 LGCYNRVGTHAQLG--PVEEQLPELPTGATLPAQVPTERVRLVCENCFATVPSGTAFCTECGEAMPTDD- 298
PaphCesA3 LVCYNRVGSHAALG--PIEETLPELPTGATLAAQLPTERVRLVCENCFATVPAGTAFCTECGEAMPSED- 298
ParrCesA3 LICYNRVGSHANLG--PIEENLPELPTGATLAAQLPAERVRLVCENCFATVPAGTAFCTECGEAMPGDD- 297
PcolCesA3 LICYNRVGAHAALG--PIEENLPELPTGATLNAQMATERVRLVCENCFATVPAGTAFCTECGEAMPGDD- 299
PirrCesA3 LICYNRVGSHAQLGG-PIEENLPELPTGATLVAQHATERVRLVCENCFATVPTGTAFCTECGEAMPGDD- 298
PiwaCesA3 LICYNRVGSHAQLGG-PIEENLPELPTGATLAAQTSTERVRLVCENCFATVPSGTAFCTECGEAMPTDD- 297
PultCesA3 LACYNRVGSHAQLSGGAIEETLPELPSGATLVAQLPTERVRLVCENCFATVPSGTAFCTECGEAMPGDD- 299
PvioCesA3 LVCYNRVGSHAQLSG-PIEENLPELPTGATLVAQMPTERVRLVCENCFATVPSGTAFCTECGEAMPGDD- 298
AcanCesA3 MGCYNKVGKRASFAA-AMQENLPELPVGSTLSAQNSAERVRLVCENCFATVPTGTAFCTECGEAMPSED- 297
AlaiCesA3 MGCYNKVGNRASFAG-AMQENLPELPVGSTLQAQNAVERVRLVCENCFATVPTGTAFCTECGEAMPSDD- 297
AbraCesA3 LLCYNKVGGHVP-LQGSLEEDLPDLPRGAAASAPFQNENTRLVCENCFATVPTGTAFCTECGEAMPSGE- 299
AeutCesA3 LICYNKVGSHAGMGMTSMHEETLELPSGAAASAPFMHERTRLVCENCFATVPAGTAFCTECGEAMPGDD- 306
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SparCesA3 LFCYNKAGAHLP-MNGVLQEDLPELPPGAAAGAPHLNERNRLVCENCFATVPQGTAFCTECGEAMQLNDD 299
SeloCesA3 LGCYNRVGAHADLGN-PVNESLPELPTGAALDAQLPHENVRLVCENCFATVPAGTAFCTECGEAMPSDD- 300
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(**B**) Alignment of the CesA3 specific domains located at the N-terminal end of oomycete CesA3 sequences. The two CxxC motifs also present in plant ring finger domains are shaded in green.

GENERAL DISCUSSION

1. RATIONALE AND MAIN FINDINGS

Many oomycetes are important pathogens of plants and can cause massive yield losses having a huge impact on agriculture. Today, oomycete control is mainly achieved by the use of site-specific inhibitors that are safe for non-target organisms and the environment, but at risk of resistance development. The most common resistance mechanisms are alterations in the target site of the compound. Since carboxylic acid amides (CAAs) are known to inhibit cellulose biosynthesis by targeting the cellulose synthase 3 (CesA3) protein in *Phytophthora infestans* (Blum et al., 2010a), the *CesA* gene family of several oomycete species was identified and characterized in this thesis.

Identification and molecular characterization of the *CesA* genes in CAA resistant field isolates of *Plasmopara viticola* and *Pseudoperonospora cubensis* revealed single nucleotide polymorphisms (SNPs) in *CesA3* leading to amino acid changes in the encoded protein (Chapters I/II). Inheritance studies in *P. viticola* demonstrated that the mutations were recessive and directly linked to resistance against CAAs (Chapter I). Furthermore, expression studies confirmed that the *CesA3* gene was expressed in different life cycle stages of *Pythium aphanidermatum,* but showed no upregulation upon CAA treatment. In *P. aphanidermatum* the inherent target site configuration in CesA3 may be linked to intrinsic tolerance towards CAAs (Chapter III).

The inclusion of various oomycete species from six different orders confirmed that the *CesA3* gene was always present and also represents a potential marker for studying phylogenetic relatedness among oomycetes. Moreover, the inherent target site configuration in CesA3 causes tolerance to CAAs in *Pythium* species, but most likely also in species of the *Albuginales*, *Leptomitales*, *Rhipidiales* and *Saprolegniales*. Based on our current knowledge carboxylic acid amides exclusively control species of the *Peronosporales* (Chapter IV).

The results of this thesis have already been discussed in the individual chapters, but open up novel questions and topics that will be reviewed in more detail including: (I) effect of mutations on CesA3 enzyme configuration and inhibitor binding; (II) occurrence of resistance to CAA fungicides; (III) recessive nature of CAA resistance; (IV) implications and future research topics; (V) perspectives.

2. EFFECT OF MUTATIONS ON CESA3 ENZYME CONFIGURATION AND INHIBITOR BINDING

Resistance to fungicides can be caused by various mechanisms (Fluit et al., 2001; Gisi et al., 2000; Ma and Michailides, 2005; McGrath, 2001) that include (I) alteration of the target site affecting compound binding to the enzyme; (II) overexpression of the target gene; (III) active efflux or reduced uptake of the compound; (IV) metabolic breakdown; (V) activation of an alternative enzyme substituting the target.

Previously it has been demonstrated in *P. infestans* that mandipropamid (member of the CAAs) affects the CesA3 protein, since artificial mutants displayed mutations at codon 1105 in *CesA3* causing resistance to MPD and other CAAs (Blum et al., 2010a). In *P. viticola* and *P. cubensis* natural (field) resistance to carboxylic acid amides (CAAs) is based on single point mutations at codon 1105 in the *CesA3* gene resulting in amino acid changes at the putative CAA-target site (Chapters I/II). The involvement of the mutations in CAA resistance has been confirmed by inheritance studies in *P. viticola* field isolates (Blum et al., 2010b). Furthermore, the tolerance mechanism to CAAs of *Pythium* and other oomycete species is most likely caused by specific configurations at position 1109 in CesA3 (Chapters III/IV). The correlation between the mutation in the *CesA3* gene and CAA resistance has been experimentally demonstrated (Blum et al., 2010b), but it is still not known how the resulting amino acid changes in CesA3 affect binding and interaction of CAA compounds with its putative target. The absence of a co-crystal structure (CesA3 enzyme with CAA molecules) makes it impossible to predict whether the binding pocket is located in the region around amino acid position 1105/1109 or somewhere else in the enzyme. It can be speculated whether (I) the corresponding amino acids at position 1105/1109 may alter the binding pocket of the enzyme (either directly or indirectly) thus influencing the binding of the molecules to the target by increasing the binding energy between the two; (II) the amino acid configuration at position 1105/1109 may cause a change in the membrane topology of the protein, which may also affect inhibitor binding; (III) changes at position 1105/1109 may lead to direct or indirect steric hindrance preventing the compound from reaching the actual binding site.

The amino acid residues causing CAA resistance and tolerance are localized in a highly conserved region at the C-terminal end of the CesA3 protein. Both residues are

located near the centre of a predicted transmembrane (TM) helix (Blum et al., 2010a; Blum et al., 2010b; Blum et al., 2011) that is suggested to be embedded in the plasma membrane according to a hypothetical 2-D topology model (Blum and Gisi, 2012). In the absence of a comprehensive three-dimensional model for any CesA3 enzyme, it remains unclear if these amino acid residues are hidden in the plasma membrane or if they are somehow exposed to the surface therefore being accessible for the CAA toxophore. In *Arabidopsis thaliana*, similar mutations in AtCesA3 (*ixr1–1*, *ixr1-2*) and ACesA6 (*ixr2-1*) were observed conferring resistance to the herbicide isoxaben, a well known cellulose biosynthesis inhibitor (Coriocostet et al., 1991). Interestingly, these mutations also occur in a highly conserved region near the carboxyl terminus (Desprez et al., 2002; Scheible et al., 2001). In *AtCesA3* they replace glycine 998 with aspartic acid, and threonine 942 with isoleucine, respectively and are located near the centre of a predicted TM helix (*ixr1-1*), or in a short extracellular loop (*ixr1-2*) connecting two proposed transmembrane helices (Scheible et al., 2001). In AtCesA6 (*ixr2-1*), the mutation causes the amino acid change R1064W, which is located next to a predicted TM domain delimiting the C-terminal end of the predicted TM anchor (Desprez et al., 2002). Furthermore, the type of amino acid changes leading to resistance is quite similar as in oomycetes. In both *Arabidopsis* and in oomycetes, the amino acids causing resistance and tolerance are generally bigger than the inherent amino acids in sensitive enzymes, suggesting that the altered residues may hinder inhibitor binding to the enzyme by a steric effect.

The similarity of amino acid changes causing CAA resistance and tolerance in oomycetes, and resistance to isoxaben in *A. thaliana*, may suggest that these inhibitors interfere with cellulose biosynthesis in a similar manner. Mandipropamid and isoxaben are both extremely active, with EC_{50} values in the nanomolar range (Cohen et al., 2008; Heim et al., 1989); they both specifically inhibit glucose incorporation into the (acid insoluble) cellulosic cell wall fraction (Blum et al., 2010a; Heim et al., 1990b). However, it is rather unlikely that the compounds inhibit cellulose synthesis by direct interference with the catalytic site, since (in both cases) the mutated residues are remote from the presumed active site residues (D,DXD,D,QxxRW) which are located at the cytosolic globular region of the enzyme (Williamson et al., 2002). Therefore, the compounds are assumed to disturb other processes such as the extrusion of glucan chains through a proposed pore that may be formed by transmembrane domains (Sandhu et al., 2009).

Both inhibitors may plug up the pore possibly inhibiting the formation of glucan chains and subsequent assembly in cellulose microfibrills. Mutations in these regions might prevent such a blockade by affecting proper binding of the molecules to the pore. Alternatively, pore-formation itself may be affected. Assuming that the region being in or around proposed transmembrane helices are critical for forming a pore, the inhibitors may bind to these regions and disrupt pore formation.

Another hypothesis for CAA and isoxaben activity may be their interference with the interaction between different CesA subunits. One could speculate that the regions being in or around predicted transmembrane helices may be involved in CesA complex formation. Thus, CAAs and isoxaben may both destabilize the CesA complex by binding to these specific regions consequently inhibiting proper formation of the cellulose synthase complex. Destabilization of the CesA complex seems to be one of the most plausible mechanisms of action and is also experimentally supported in *A. thaliana*: Different responses upon treatment with cellulose inhibitors have been shown for yellow fluorescent protein (YFP) labelled AtCesA6 that is suggested to be the direct target of isoxaben (Desprez et al., 2002): The herbicide 2,6-dichlorbenzonitril that inhibits cellulose synthesis by targeting the MAP20 protein (Rajangam et al., 2008), led to an accumulation and immobilization of YFP:CesA6 particles at the plasma membrane (DeBolt et al., 2007). In contrast, isoxaben treatment resulted in rapid clearing of YFP:CesA6-labeled complexes from the plasma membrane (Pardez et al., 2006). This loss may be explained by the disruption of the CesA enzyme complex and subsequent recycling of the CesA6 subunits in the cytosol. Thus, amino acid changes in CesA3 of oomycetes as well as in AtCesA3 and AtCesA6 of *A. thaliana* may block disruption of the CesA complexes. However, the YFP labelling technique has not yet been used in oomycetes, thus it remains unknown whether the CesA3 complex may disappear from the plasma membrane upon CAA treatment.

3. OCCURRENCE OF RESISTANCE TO CAA FUNGICIDES

Carboxylic acid amide fungicides are used for oomycete control since more than 20 years (Gisi et al., 2008). Despite a relatively strong selection pressure in the field, only few resistance problems were reported, so far mainly for the downy mildew pathogens *P. viticola* and *P. cubensis* (FRAC; Gisi et al., 2007; Zhu et al., 2007). Single point mutations in the cellulose synthase 3 (*CesA3*) gene of *P. viticola* and *P. cubensis*

field isolates lead to CAA resistance (Blum et al., 2010b; Blum et al., 2011), but it is largely unknown as why other oomycetes, especially the genus *Phytophthora* (mainly *P. infestans*) did not develop resistance in the field, although CAAs have been frequently used for disease control since several years (FRAC). Based on genomic data, it is known that *Phytophthora* species possess four *CesA* genes (Grenville-Briggs et al., 2008), that display a rather high sequence identity (up to 85%) to *CesA* orthologues from other *Peronosporales* (e.g. *P. viticola* and *P. cubensis*). Alignments made with CesA3 sequences of different species from the *Peronosporales* revealed that the putative target site in CesA3 is also highly conserved between *Phytophthora* and other *Peronosporales*, such as *Plasmopara*, *Bremia* or *Pseudoperonospora*.

Despite these similarities on the genomic level, resistance to CAAs did so far not occur in *Phytophthora* spp. field populations (Cohen et al., 2007; Jang et al., 2009; Keinath, 2007; Olaya et al., 2008). This is somehow surprising, since artificial mutants were generated successfully in *Phytophthora capsici*, *P. infestans* and *Phytophthora melonis* using UV or EMS mutagenesis revealing mutations in *CesA3* (Blum et al., 2010a; Chen et al., 2011). These mutations were located at the very same position as those identified in resistant field isolates of *P. viticola* and *P. cubensis* (Blum et al., 2010b; Blum et al., 2011). The base triplet (GGC) encoding the amino acid Gly1105 is exactly the same in *P. infestans* as in *P. viticola*. Thus, the same point mutations could theoretically arise in *P. infestans* leading to serine 1105 (AGC) or valine 1105 (GTC) causing resistance to CAAs.

One might speculate that in *Phytophthora* species special mechanisms (e.g. improved DNA mismatch repair (MMR) (Li, 2008), mitotic gene conversion (Chamnanpunt et al., 2001) may exist specifically preventing mutations at this locus. Vice versa, there is also the possibility that *P. viticola* and *P. cubensis* may have specific systems such as the repeat induced-point mutation (RIP) genome defence system (Montiel et al., 2006), that favours the induction and frequency of mutations, but that such systems are lacking in *Phythophtora* species. Consequently, mutations in *P. viticola* and *P. cubensis* would develop much faster than in *Phytophthora* spp. Indeed, *CesA3* sequence analysis of *P. viticola* field isolates revealed an increased number of single nucleotide polymorphisms (SNPs) compared to *P. infestans* and *P. capsici*. However, this finding could not be confirmed in *P. cubensis* isolates. In contrast, the *CesA3* sequence of *P. cubensis* isolates was highly conserved and revealed fewer SNPs than the *CesA3* gene

of *P. infestans* and *P. capsici* isolates (unpublished data). Therefore, the lack of appearance of CAA resistance may not simply be explained by mechanisms preventing or increasing mutations in *CesA3*.

In *P. capsici,* stable CAA resistant mutants have quite easily been generated in the lab by UV or EMS mutagenesis (Chen et al., 2011; Lu et al., 2010; Meng et al., 2011). It is not known if such mutations may also exist in the heterozygote state in field populations. The crucial question may not be why these mutations do not occur, but rather why the resistant phenotype does not emerge in field populations. The mutations may somehow affect fitness, consequently being of disadvantage for the mutated individuals. However, since there is currently no evidence that these mutations affect enzyme function in *P. viticola* or *P. cubensis*, a negative effect in *Phytophthora* species is questionable but cannot be ruled out. Another explanation may be given by the recessive nature of CAA resistance, first described by Gisi et al. (2007). In this thesis, it was demonstrated that heterozygous *P. viticola* isolates, (only one allele mutated in *CesA3*) were still fully sensitive to CAAs, whereas homozygous isolates displaying mutations in both alleles were resistant. In diploid organisms, spontaneous mutations, e.g. induced by UV-light, failures during DNA replication, or by the RIP system (Montiel et al., 2006) affect in most cases only one of the two alleles. The probability that both alleles of a certain gene mutate at exactly the same position (=homozygous mutation) is generally lower than for the induction of heterozygous mutations (Greene et al., 2003). However, homozygous isolates can result by sexual recombination of two heterozygous isolates, each having one mutated allele (mutation at the very same position). Such isolates, resistant to CAAs, were detected in *P. viticola* and *P. cubensis* populations (Blum et al., 2010b; Blum et al., 2011). The crucial point here is that sexual recombination largely increases the probability that heterozygous individuals become homozygous. Considering biological processes in *Plasmopara*, *Pseudoperonospora and Phytophthora,* one may find an answer why resistance to CAAs did rapidly develop in *Plasmopara* and *Pseudoperonospora*, but not in *Phytophthora* species: Simply because the mutations in *CesA3* do not switch from the heterozygous into the homozygous state in *Phytophthora*.

In *P. cubensis* and especially *P. viticola,* oospores (resulting from sexual recombination (Cohen et al., 2011; Wong et al., 2001) are an important inoculum source for primary downy mildew infections (Cohen and Rubin, 2011; Kennelly et al., 2007) and also play a key role during the development of downy mildew epidemics (at least in

P. viticola populations) (Gobbin et al., 2005; Rossi et al., 2009). In contrast, the role of oospores in the epidemiology of *P. infestans* may be of minor importance. Indeed, even though several studies reported the occurrence of oospores in *P. infestans* populations (Andersson et al., 1998; Smirnov et al., 2008; Turkensteen et al., 2000), their influence on population structure seems to be rather limited, since most populations remain dominated by asexual reproduction, at least in Europe and the U.S.A. (Fry et al., 2009; Gisi et al., 2011). Contrary, analyses of *P. capsici* populations in the U.S.A. showed a high genotypic diversity (due to sexual recombination) at the beginning of the growing season. Nevertheless, genotypic diversity was shown to decrease and clonal lineages significantly increased within the growing season (Lamour, 2009). This indicates that sexual reproduction may be of limited importance in the epidemiology also for this pathogen.

In the case of *P. infestans* there might be an additional explanation as why resistance to CAAs did not develop. Several studies reported on the occurrence of *P. infestans* isolates deviating from the expected ploidy (2n) (Catal et al., 2010; Daggett et al., 1995; Hamed, 2011; van der Lee et al., 2004). So far, it is unknown if aberrant ploidy levels (aneuploid, triploid, trisomic) have an effect on the inheritance of mutations, especially when the mutations have a recessive nature. Differences in ploidy may represent an additional bottleneck for the establishment of mutations leading to resistance in a population.

Since up to now, no resistance to CAAs was reported in *Phythophtora*, it is expected that mutations in the homozygous state in *CesA3* do not occur in any field population. However, the presence of isolates displaying the mutation in a heterozygous state should be considered as a potential risk for resistance evolution. Therefore, special attention should be taken for sexually reproducing populations in *P. infestans*. In such cases, it may be worthwhile to consider adapting resistance management strategies (FRAC recommendations) by using CAAs in mixture with other compounds having a different mode of action to minimize the selection pressure.

4. RECESSIVE NATURE OF CAA RESISTANCE

Oomycetes carry two copies of each chromosome in their cells. Due to this diploid nature, mutations in the coding sequence of genes are not necessary linked to alterations in the phenotype. This is especially valid for recessive mutations. The

recessiveness of CAA resistance in *P. viticola* was linked to a single, recessive point mutation in the *PvCesA3* gene. Thus, both alleles must be mutated for expression of CAA resistance (Blum et al., 2010b, Blum et al., 2011). However, it is still unknown why isolates displaying mutations in *CesA3* in the heterozygote state remain fully sensitive to CAAs. The recessiveness of CAA resistance is somehow surprising since resistance to the herbicide isoxaben, that is also caused by mutations at the CesA target site, is semi-dominant (heterozygotes show a 10-fold higher IC_{50} value than wt homozygotes) (Heim et al., 1990a). Such a difference in sensitivity could not be observed for heterozygous *P. viticola* isolates when tested by leaf disc assays (Blum et al., 2010b).

In general, the mechanisms making a mutation recessive or dominant are poorly understood. Some studies reported on genomic imprinting (methylation of alleles preventing transcription) (Bartolomei, 2009), others described suppression of transcription by RNA interference (RNAi) (Hammond et al., 2001) to be the cause for the dominance of one allele over the other. However, none of these mechanisms seem to be applicable to the recessive point mutation conferring CAA resistance: Transcript analysis of *P. viticola* isolates displaying the G1105S mutation in the heterozygote state, showed that both alleles (AGC/GGC) were transcribed, probably both serving as template for translation into CesA3 proteins either containing glycine1105 (G1105) or serine1105 (S1105). So far, it is unknown whether both transcripts in heterozygous individuals are translated to the enzyme, or if the mutated version is discriminated at a later step in enzyme processing. Nevertheless, since the mutation seems not to affect enzyme function, CesA3 proteins carrying S1105 might not be discriminated in the final enzyme complex of heterozygous individuals.

In plants, algae, bacteria and tunicates the CesA enzyme is organized in a multi-subunit manner (Tsekos, 1999); it is assumed that oomycete CesAs are organized in a similar way. In brown algae, CesA complexes are organized in single linear rows comprising of 10 to 100 CesA subunits (Michel et al., 2010). Due to the close relatedness of oomycetes to brown algae (Baldauf et al., 2000), one could suggest that the oomycete CesA complex may have a similar organization and stoichiometry. Thus, in heterozygous isolates both forms of the enzyme (mutated and wild type) may be present as a mixture in the final enzyme complex. Such chimeric enzyme complexes could still be fully sensitive to the inhibitor, probably because CAA binding to the sensitive subunits is strong enough to destroy the CesA complex structure completely. This hypothesis is

confirmed by observations made in *A. thaliana*: Scheible et al. (2001) demonstrated that *ixr1* homozygotes transformed with the wild-type gene showed a lower resistance to isoxaben than nontransformed *ixr1* controls. Similarly, transformants of *P. infestans* overexpressing the mutated allele, but still carrying the wild type allele, did display lower resistance to mandipropamid than a homozygous mutant (Blum et al., 2010a). These findings suggest that the presence of even a few CAA-sensitive subunits within an otherwise resistant complex may cause disruption of the entire complex by CAA compounds. To conclude, the recessiveness of CAA resistance is most probably based on mixed CesA complex structure and on the specific mode of interaction between inhibitor and enzyme, rather than its control on the genetic level.

5. IMPLICATIONS AND FUTURE RESEARCH TOPICS

Until recently, the detection of CAA resistance in field populations relied on biological tests that required the isolation of the pathogen and subsequent testing on CAA amended media or plant leaves treated with CAAs. These methods are labour-intensive and time-consuming especially when testing large numbers of isolates. The elucidation of the molecular mechanism of resistance to carboxylic acid amides provides new opportunities for the rapid detection of CAA resistant genotypes in oomycete populations. Currently, there are several molecular techniques such as PCR-restriction fragment length polymorphism (PCR-RFLP) (Furuya et al., 2009; Sierotzki et al., 2000), allele-specific real-time PCR (Fraaije et al., 2002; Malandrakis et al., 2011), and pyrosequencing (Wiederhold et al., 2008) that have been successfully used to detect single point mutations causing resistance to fungicides in several pathogens. These techniques have meanwhile been adapted to monitor the mutations causing CAA resistance in *P. viticola* populations (Aoki et al., 2011; Sierotzki et al., 2011). Similar assays based on real-time PCR and pyrosequencing technology are under development for other oomycetes that display resistance to CAAs in the field (e.g. *P. cubensis*), or for those that are at risk of resistance development (e.g. *P. infestans*, Syngenta internal data). These highly sensitive methods will improve the detection of CAA resistance alleles, even when occurring at very low frequencies. Moreover, they can be used to study the evolution of CAA resistance in populations and provide an important tool for further resistance risk assessment and evaluation of resistance management strategies.

So far, three amino acid changes (G1105S, G1105V, G1105W) in CesA3 leading to CAA resistance have been detected in field populations (Sierotzki et al., 2011). Nevertheless, one should also consider developing molecular assays for position 1109. Like for the amino acid exchange at position 1105, only a single point mutation, at the first codon position is needed to change the codon for valine 1109 (GTG, GTT, GTA, GTC) to leucine (CTG, CTT, CTA, CTC, TTA, TTG) or methionine (ATG). Sensitive species displaying V1109 may adopt the L1109 or M1109 configuration that is responsible for natural tolerance to carboxylic acid amides in many oomycetes.

Still, due to diploidy of oomycetes and the recessive nature of the mutations causing CAA resistance, molecular approaches have their limitations. A major drawback of current molecular techniques is the inability to distinguish sensitive isolates carrying the mutation in the heterozygous state from bulk samples containing mixtures of homozygous isolates with either the resistant or sensitive allele. Therefore, a new method has to be developed to distinguish homo- from heterozygous bulk isolates. As long as this issue of homozygous vs. heterozygous samples is not solved, biological tests may still be needed for monitoring CAA resistance in field populations.

Another research topic that arises from the current findings of this thesis concerns the specific function of the CesA enzymes during cellulose biosynthesis. Since carboxylic acid amides act directly on the CesA3 enzyme, mandipropamid (and other CAAs) may represent useful tools for future mechanistic studies on enzyme action and cellulose biosynthesis in general. One could also use these inhibitors to investigate whether redundancy exist between the different CesAs (e.g. if CesA1, CesA2 and CesA4 may be able to compensate for the reduced CesA3 activity when targeted by CAAs). In general, having these highly specific inhibitors in hand, it might be possible to elucidate the specific role of the *CesA3* isoform during cellulose biosynthesis in oomycetes.

Furthermore, the *CesA3* gene represents a potential marker for unravelling phylogenetic relationships among oomycetes. To date oomycete phylogeny is largely based on highly variable markers such as ITS (Cooke et al., 2000; Robideau et al., 2011; Vogelmayr, 2003), *cox2* (Hudspeth et al., 2000;) or partial 28S rRNA (Petersen and Rosendahl, 2000; Riethmüller et al., 2002) sequences. Even though such markers are particularly useful for discrimination of closely related taxa at the species to genus level, they might be too variable for inferring an overall picture of the oomycete phylogeny. Recently the whole oomycete phylogeny was reconstructed on complete sequences of

the SSU rRNA (Lara and Belbahri, 2011), a slow-evolving and stable gene that is one of the most commonly used markers in phylogenetic studies. According to our data, the *CesA3* gene of oomycetes is like the SSU rRNA gene, a stable and conserved gene that is likely to be present in all oomycetes. Thus, it might also be useful to reconstruct an overall picture of the oomycete phylogeny and to resolve phylogenetic relationships at genus or even species level. However, since only species of the six "crown" oomycete orders were included in our study, more species, also from the "lower" oomycetes should be included to test its reliability as molecular marker for phylogenetic analyses in oomycetes.

6. PERSPECTIVES

The identification and characterization of the cellulose synthase (*CesA*) gene family in various oomycetes enabled to investigate the molecular mechanism of resistance and tolerance to carboxylic acid amide (CAA) fungicides. However, there are still a lot of open questions to be addressed for future research. The resistance mechanism in sensitive species has been successfully supported in inheritance studies, carried out with *P. viticola*, by a single point mutation at codon 1105 in the *CesA3* gene (Chapter I). On the other hand, the direct involvement of the amino acid configuration at position 1109 in conferring tolerance to CAAs in *Pythium* and other oomycete species has not yet been fully confirmed. However, the amino acid configuration leucine or methionine at position 1109 is claimed to be responsible for CAA tolerance because of the correlation between the observed phenotype and genotype. Proof of concept could be achieved by co-segregation studies (as done for the G1105S mutation in *P. viticola*), site-directed mutagenesis followed by gene replacement (even though this is not yet feasible in oomycetes) or transformation studies (as done with *P. infestans*) (Blum et al., 2010a). Moreover, the effect of these mutations on enzyme configuration and inhibitor binding is not known. For further evaluating the influence of these mutations on CAA binding, a crystal structure of the CesA3 enzyme is of uttermost importance. However, up to now a crystal structure has only been reported for the bacterial cellulose synthase (Bcs) complex (Mazur and Zimmer, 2011), but not for any plant, algal or oomycete CesA, probably due to difficulties in extracting the intact CesA complex from the plasma membrane.

Another area of research refers to the specific function of individual *CesA* genes and their encoded products. Currently, the knowledge about cellulose biosynthesis in oomycetes is quite limited and the enzymes involved in this process are poorly characterized. Meanwhile, two studies have been published dealing with the functional characterization of *CesA* genes and the encoded enzymes, showing that they are directly involved in cellulose biosynthesis (Grenville-Briggs et al., 2008; Fugelstad et al., 2009). However, the role of the individual CesA enzymes during cellulose biosynthesis is still not clear. Based on *CesA* expression studies performed in *P. infestans* (Grenville-Briggs et al., 2008) and *P. aphanidermatum* (Chapter III of this thesis), the *CesA3* gene seems to play a very important role during cystospore germination and mycelial growth. Nonetheless, it is largely unknown whether the CesA3 isoform is more important for cellulose synthesis than the other CesAs. It also remains to be investigated whether or how the different CesAs interact during cellulose biosynthesis and if redundancy exists between them. Furthermore, the assembly, stoichiometry and the final structure (rosette vs. linear) of the active CesA enzyme complex is unknown and needs to be elucidated before models can be proposed on how cellulose synthesis proceeds in oomycetes.

Based on the full length *CesA3* sequence of various oomycetes the relatedness within "crown" oomycetes has been studied. The results suggest that the *CesA3* sequence represents a promising tool for reconstructing a phylogeny at phylum (oomycetes) and order level. Since the gene is suggested to be present as a single copy in most genomes (so far only in *Saprolegnia parasitica* a paralogue of *CesA3* has been detected) and due to its high degree of sequence conservation as well as its universal distribution among oomycetes, the *CesA3* gene might also be suitable for inferring phylogenetic relationships at genus and species level. However, since no species of the "lower" oomycetes were included in our analyses, further studies should be carried out to thoroughly evaluate the potential of this marker for reconstructing evolutionary trends in the oomycete phylum.

7. CONCLUSIONS

In this PhD thesis the cellulose synthase (*CesA*) gene family of several oomycetes was identified and characterized. In *P. viticola*, *P. cubensis* and *P. aphanidermatum*, the CesA3 gene product differed in structure and predicted domains from its isoforms CesA1, CesA2 and CesA4. Due to the high degree of sequence conservation as well as its universal distribution among oomycetes, the *CesA3* gene was successfully used for unravelling phylogenetic relationships among oomycetes and clustering the different genera in existing orders. The obtained tree topology was largely in agreement with those inferred by other markers such as ITS, *cox2*, SSU rRNA and partial 28S rRNA, but further analyses are required to validate whether the *CesA3* gene represents a useful marker for reconstructing the major evolutionary trends among the oomycete phylum.

Furthermore, investigations about the molecular mechanism of resistance to carboxylic acid amides (CAAs) provided strong evidence that in sensitive species mutations at position 1105 in the CesA3 target site lead to resistance to all CAA molecules. Inheritance studies in *P. viticola* demonstrated the recessive nature of CAA resistance and a correlation to single nucleotide polymorphisms (SNPs) causing amino acid changes (G1105S, G1105V). However, at the moment the evolution of the mutations as well as the recessive nature remains unclear and further investigations are needed to fully understand the precise mechanisms of recessiveness.

The underlying mechanism of tolerance to CAAs in *Pythiales* and oomycetes originating from orders other than *Peronosporales* is most likely based on specific amino acid configurations (leucine, methionine) at position 1109 in CesA3 even though direct proof of concept is still lacking. Currently, the absence of a three-dimensional CesA enzyme model in oomycetes prohibits predicting the influence of amino acid changes at positions 1105 and 1109 on the structural integrity of the protein and on inhibitor binding. Thus, the mechanisms how mutations affect inhibitor action remain speculative.

Overall, the findings presented in this PhD thesis support that the highly conserved CesA3 enzyme represents a promising target for carboxylic acid amides at least in *Peronosporales* and may also be suitable for future anti-oomycete drugs to be developed.

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APPENDIX

CURRICULUM VITAE

Mathias Blum
Biologist

Name	Mathias Blum
Date of birth	May 11th, 1982
Citizenship	Swiss
Private address	Haltingerstrasse 99, 4057 Basel, Switzerland

PhD Study

10/2008 – 02/2012	PhD in Plant Sciences Institute of Botany (University of Basel), Syngenta Crop Protection AG
Thesis title	Molecular characterization of cellulose synthase (*CesA*) genes and impact of mutations on fungicide resistance in oomycetes
Supervision	Prof. Dr. Ulrich Gisi and Prof. Dr. Thomas Boller

Presentations

December 2011	BSPP Presidential Meeting, Cambridge, United Kingdom (poster) "*Insights into the molecular mechanism of tolerance to carboxylic acid amide (CAA) fungicides in Pythium aphanidermatum"*
October 2011	The Second International Fungal Cell Wall Meeting, Giens, France (talk) "*The cellulose synthase 3 (CesA3) enzyme: a promising target for anti-oomycete drugs?"*
September 2010	57. Deutsche Pflanzenschutztagung, Berlin, Deutschland (talk) "*Molekularer Wirkungsmechanismus des CAA Fungizides Mandipropamid (MPD)"*
June 2010	SPSW Summer School, Mürren, Switzerland (poster) "*Resistance mechanism to carboxylic acid amide (CAA) fungicides in oomycetes"*
April 2010	16th International Reinhardsbrunn Symposium, Germany (poster) "Comparison of *cellulose synthase 3 (CesA3) gene structure in different oomycetes"*

Awards BSPP Best Student Paper Prize 2010 for the paper: "*Mandipropamid targets the cellulose synthase-like PiCesA3 to inhibit cell wall biosynthesis in the oomycete plant pathogen, Phytophthora infestans"*

Education

10/2002 – 03/2007 Studies of Biological Sciences at University of Basel, Basel, Switzerland

2007: Master in Plant Sciences
"Inheritance of fungicide resistance in Plasmopara viticola"

2005: Bachelor degree in Biological Sciences

08/1993 – 06/2001 High school diploma ("Matura") at Gymnasium Bäumlihof Basel-Stadt (specialisation in Latin).

Work Experience

04/2007 – 09/2008 Post-graduate at Syngenta, fungicide MoA project, Stein

03/2006 – 04/2006 Temporary work at the Pharmaceutical institute, construction of a fungal and plant extract library, Basel-Stadt

11/2005 – 02/2007 Part time work at Viollier, microbiology diagnostics, Allschwil

11/2001 – 04/2002 Temporary work at UBS, database research, Basel-Stadt

References

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LIST OF PUBLICATIONS

1. **Blum, M.,** Gamper, H.A., Waldner, M., Sierotzki, H., Gisi, U.
   The cellulose synthase 3 (CesA3) gene of oomycetes: structure, phylogeny and influence on sensitivity to carboxylic acid amide (CAA) fungicides.

   Fungal Biology, 2012 (in press)

2. **Blum, M.,** Gisi, U.
   Insights into the molecular mechanism of tolerance to carboxylic acid amide (CAA) fungicides in Pythium aphanidermatum.

   Pest Management Science, 2012 (in press)

3. **Blum, M.,** Waldner, M., Olaya, G., Cohen, Y., Gisi, U., Sierotzki, H.
   Resistance mechanism to carboxylic acid amide (CAA) fungicides in the cucurbit downy mildew pathogen Pseudoperonospora cubensis.

   Pest Management Science, 2011 (67, 1211-1214)

4. Rubin, A.E., Werdinger, A.C., **Blum, M.,** Gisi, U., Sierotzki, H., Hermann, D., Cohen, Y.
   EMS and UV irradiation induce unstable resistance against CAA fungicides in Bremia lactucae.

   European Journal of Plant Pathology, 2011 (129, 339-351)

5. **Blum, M.,** Sierotzki, H., Gisi, U.
   Comparison of cellulose synthase 3 (CesA3) gene structure in different oomycetes.

   Modern Fungicides and Antifungal Compounds VI, 2011 (151-154)

6. Sierotzki, H., **Blum, M.,** Olaya, G., Waldner-Zulauf, M., Buitrago, C., Wullschleger, J., Cohen, Y., Gisi, U.
   Sensitivity to CAA fungicides and frequency of mutations in cellulose synthase (CesA3) gene of oomycete pathogen populations.

   Modern Fungicides and Antifungal Compounds VI, 2011 (101-110)

7. **Blum, M.,** Waldner, M., Gisi, U.
   A single point mutation in the novel PvCesA3 gene confers resistance to the carboxylic acid amide fungicide mandipropamid in Plasmopara viticola.

   Fungal Genetics and Biology, 2010 (47, 499-510)

8. **Blum, M.**, Boehler, M., Randall, E., Young, V., Csukai, M., Kraus, S., Moulin, F., Scalliet, G., Avrova, A.O., Whisson, S.C., Fonné-Pfister, R.
   Mandipropamid targets the cellulose synthase-like PiCesA3 to inhibit cell wall biosynthesis in the oomycete plant pathogen, Phytophthora infestans.

   Molecular Plant Pathology, 2010 (11, 227-243)

9. **Blum, M.**, Gisi, U.
   Inheritance of fungicide resistance in Plasmopara viticola.

   Modern Fungicides and Antifungal Compounds V, 2008 (101-104)