

# Structural characterization of a putative chitin synthase gene in *Phytophthora* spp. and analysis of its transcriptional activity during pathogenesis on potato and soybean plants

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**Abstract** Although chitin is a major component of the fungal cell wall, in oomycetes (fungal-like organisms), this compound has only been found in very little amounts, mostly in the cell wall of members of the genera *Achlya* and *Saprolegnia*. In the oomycetes *Phytophthora infestans* and *P. sojae* the presence of chitin has not been demonstrated; however, the gene putatively encoding chitin synthase (CHS), the enzyme that synthesizes chitin, is present in their genomes. The evolutionary significance of the *CHS* gene in *P. infestans* and *P. sojae* genomes is not fully understood and, therefore, further studies are warranted. We have cloned and characterized the putative *CHS* genes from two *Phytophthora* spp. and multiple isolates of *P. infestans* and *P. sojae* and analyzed their phylogenetic relationships. We also conducted CHS inhibition assays and measured *CHS* transcriptional activity in *Phytophthora* spp. during infection of susceptible plants. Results of our investigations suggest that *CHS* contains all the motifs that are typical in *CHS* genes of fungal origin and is expressed, at least at the mRNA level, during in vitro and in planta growth. In infected tissues, the highest levels of expression occurred in the first 12 h post inoculation. In addition, results from our

inhibition experiments appear to suggest that CHS activity is important for *P. infestans* normal vegetative growth. Because of the considerable variation in expression during infection when compared to basal expression observed in in vitro cultures of non-sporulating mycelium, we hypothesize that CHS may have a meaningful role in *Phytophthora* pathogenicity.

**Keywords** Chitin · Chitin synthase · Oomycetes · *Phytophthora* · Potato · Soybean

## Introduction

Although originally classified as a member of the Kingdom Fungi, members of the genus *Phytophthora* belong to the phylum Oomycota (Kong et al. 2009) in the Kingdom Chromista (also known as Stramenopila). While chitin is a distinctive component of the fungal cell wall, in oomycetes this compound has only been found in very little amounts, mostly in the cell wall of *Achlya* spp. and *Saprolegnia* spp. In the oomycetes *Phytophthora infestans* and *P. sojae* the presence of chitin has not been demonstrated; however, the gene putatively encoding chitin synthase (CHS), the enzyme whose primary biological role is participating in the biosynthesis of chitin, is present in their genomes. Because the evolutionary significance of the *CHS* gene in *P. infestans* and *P. sojae* genomes is not fully understood, and in the absence of experimental evidence indicating the presence of chitin in *Phytophthora*, further studies on the oomycete *CHS* gene are warranted.

*Phytophthora infestans*, the causal agent of the devastating disease known as late blight of potato, and *P. sojae*, an equally harmful soil borne pathogen causing stem and root rot in soybeans, possess closer taxonomic affinity to brown

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algal species and diatoms while also displaying characteristics affiliated with true fungi such as filamentous growth patterns (Haas et al. 2009; Tyler et al. 2006). *Phytophthora* spp. are hemibiotrophic pathogens: after the initial biotrophic phase consisting of the first 2–3 days of infection, an additional phase of host plant tissue necrosis occurs, allowing for colonization of host by pathogen (van Damme et al. 2012). During the early stages of the infection process *Phytophthora* produces swollen structures at the tip of the germ tube called appressoria, which secrete adhesive compounds in order to maintain close contact with, penetrate, and (likely in conjunction with the action of cell wall-degrading enzymes), facilitate invasion of the host. After successful penetration of the epidermal cell, the pathogen produces a profusely branched, intercellular mycelium; subsequently, specialized infection structures, called haustoria, develop at the intercellular hyphal tips (Coffey and Wilson 1983). Haustoria penetrate the host mesophyll cell and absorb nutrients from it via intimate contact with its plasma membrane (Panstruga and Dodds 2009).

In *Phytophthora* spp. the cell wall is mainly composed of  $\beta$ -glucans [up to 90%, with a maximum of 25% thought to be cellulose (Bartnicki-Garcia 1966; Bartnicki-Garcia and Wang 1983), although more recent estimates have raised this percentage to 32–35% (Mélida et al. 2013)] and proteins [4–11%, depending on the species in question (Bartnicki-Garcia 1966; Bartnicki-Garcia and Wang 1983; Meijer et al. 2006)]. In *P. infestans*, up to 31 putative proteins have been identified in cell wall preparations from germinating cysts with appressoria, sporulating mycelium, and non-sporulating mycelium using proteomic analyses (Grenville-Briggs et al. 2010). Interestingly, hydroxyproline, an amino acid not present in fungal cell walls, constitutes approximately 5% of the overall amount of amino acids found in the hyphal cell walls of *Phytophthora* spp. (Bartnicki-Garcia 1966; Bartnicki-Garcia and Wang 1983).

Although apparently absent in *Phytophthora* species, chitin is still found in other oomycete water molds such as species belonging to order *Leptomitales* and members of the genus *Achlya* and *Saprolegnia* (Alexopoulos et al. 1996; Dietrich 1973; Reiskind and Mullins 1981). Furthermore, in oomycetes such as *Achlya ambisexualis*, *Aphanomyces euteiches*, and *Saprolegnia monoica* the presence of a gene encoding chitin synthase (CHS), the enzyme that synthesizes chitin, has been demonstrated. In some of these oomycetes, chitin is localized in the hyphal cell wall and occasionally in sporangial structures (Badreddine et al. 2008; Bulone et al. 1992). In these instances, however, chitin only comprises about 2% of cell wall carbohydrates as a whole (Fugelstad et al. 2009). While the presence of chitin in *P. infestans* and *P. sojae* has not been demonstrated, a putative *CHS* gene is indeed present in their genomes (Ospina-Giraldo et al. 2010).

The *CHS* genes were first examined in depth in different filamentous fungi and yeast, with the most notable findings described by Bowen et al. (1992), who identified a highly conserved region in all analyzed *CHS* genes; such region is believed to code for the center of catalytic activity in the CHS protein. Further analyses revealed that the *CHS* genes could be grouped into three different categories (class I–III) based on the amino acid sequence similarity of the proteins they encode, the gaps appearing during multiple sequence analysis, and other features, such as a proline residue occurring after the first gap in all class III CHS amino acid sequences (Bowen et al. 1992). This classification was further expanded to seven classes, grouped in three divisions, based on the CHS amino acid sequence (Chigira et al. 2002; Choquer et al. 2004; Din et al. 1996; Fujiwara et al. 1997; Mandel et al. 2006; Riquelme and Bartnicki-García 2008).

Given the seemingly minor structural role in the oomycete cell wall composition, the evolutionary significance of the putative *CHS* gene in the *P. infestans* and *P. sojae* genomes has not extensively been studied and, therefore, is not completely understood. In the related oomycetes *Aphanomyces euteiches* and *Saprolegnia monoica* chitin has been observed, through lectin labeling (using a wheat germ agglutinin that interacts specifically with chitosaccharides), to be present on the surface of encysted zoospores, germ tubes and the resulting hyphae, with the most intense labeling on the germ tubes and hyphae (Badreddine et al. 2008). Although similar experiments have revealed no significant presence of chitosaccharides in *Phytophthora* spp. cell walls, the possibility that *CHS* glycosyltransferase activity is involved in *Phytophthora* reproduction, host infection, or other processes besides cell wall formation, should not be completely ruled out. Therefore, sequence analysis and expression studies of the *CHS* gene in infected and mycelial tissues will help to gain insight into its additional roles, if any, in *P. infestans* and *P. sojae* infection and reproductive processes. If *CHS* is significantly expressed, further experimentation, such as confirmation of protein synthesis, activity, and localization, can be conducted to determine the suitability of the *CHS* gene as a target for pathogen control measures.

In this paper, we report on the cloning, sequence characterization, and phylogenetic relationships of the putative *CHS* gene in several *Phytophthora* species and multiple races of *P. infestans* and *P. sojae*. We also describe the results of CHS inhibition assays and our quantitative analysis of expression during infection of susceptible plants.

## Methods

### *Glycine max* and *Solanum tuberosum*

Seeds from *Glycine max* cv. Williams were obtained from the USDA Agricultural Research Service in Beltsville, MD. *Solanum tuberosum* seeds of cv. Russet Burbank were obtained from the Netherland Bulb Company (Easton, PA). Plants were grown in Miracle Gro® Potting Soil at 24 °C with a 16 h light:8 h dark cycle and watered daily. Plants were grown for three (potato) and six (soybean) weeks, respectively, before infection assays were conducted.

### Phytophthora isolates

*Phytophthora* spp. isolates were obtained from the USDA Agricultural Research Service in Beltsville, MD. *Phytophthora infestans* isolates ME-06-B-US8, PA-POD Tom/R, MD02-pet1, FL01-2, and Pi-02-007 and *Phytophthora sojae* race 1, race 4 and race 5 isolates were utilized for gene cloning and sequence analysis. Quantification of *CHS* gene expression in real time was conducted with *P. infestans* FL01-2 and *P. sojae* race 5 isolates.

### Growth media

Initial cultures were grown on high-nutrient-content unclarified V8®/Lima plates made from autoclaved baby lima beans, V8® vegetable juice and Difco agar amended with 2.8 g of calcium carbonate (CaCO<sub>3</sub>) with adjusted pH (using either HCl and KOH) to 6.00 to induce sporulation (Miller 1955). Additional cultures were grown on nutrient-limiting Rye B Agar plates using organic rye grain (Shiloh Farms, Sulfur Springs, AR) amended with 15 g Bacto Agar, 20 g sucrose and 0.05 g β-sitosterol (a membrane-growth stimulating steroid) to induce sporulation (Caten and Jinks 1968). Mycelia for DNA and RNA extractions were grown in Pea broth (120 g of frozen peas autoclaved with 2 g CaCO<sub>3</sub> and 0.05 g β-sitosterol in 1L water) at 22 °C in the dark. Isolates on all solid media were kept in the dark at a temperature of 18 °C to induce sporulation while liquid cultures were grown in the dark at room temperature.

### Infection assays

Assays with *P. sojae* were conducted by inserting a 2 mm<sup>2</sup> plug from a two-week old unclarified V8®/Lima Bean agar culture of *P. sojae* race five into a small incision, produced using a sterile scalpel, in the soybean hypocotyl. Infection assays with *P. infestans* were conducted as follows: after two weeks of incubation, unclarified V8®/Lima Bean agar culture plates of *P. infestans* FL01-2 were flooded with 5 ml of ddH<sub>2</sub>O and the mycelium was gently rubbed with a

glass spreader to dislodge sporangia from hyphal tips. The sporangial suspension was transferred to a 15 ml conical centrifuge tube, and set on ice. This procedure was repeated with a second 5-ml aliquot of ddH<sub>2</sub>O and the sporangial suspensions were combined and centrifuged for two minutes at 500 g. Following centrifugation, 5 ml of supernatant was carefully decanted. Sporangium concentration was determined using a hemocytometer and adjusted to 1.5 × 10<sup>4</sup> sporangia/ml. This process was repeated with two more plates. Potato plants were inoculated with approximately 3 × 10<sup>4</sup> sporangia, using a hand-held atomizer. Infected plants were kept at 20 °C with a 16-h photoperiod. Leaf tissue samples were collected at 12, 24, 36, and 48 h post inoculation (hpi).

### Cloning of the *CHS* gene in *P. infestans* and *P. sojae*

Genomic DNA was extracted using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma, St. Louis, MO), following the instructions provided by the supplier. The full-length *CHS* genes in *P. infestans* and *P. sojae* isolates were initially cloned via PCR using primers based on the genomic sequence for the *CHS* gene in *P. infestans* (GenBank accession XM\_002908585.1) and *P. sojae* (GenBank accession XM\_009525864.1). Amplification of the untranslated regions upstream and downstream from the 5' and 3' ends of the gene, respectively, were done by RACE (Invitrogen-Life Technologies, Carlsbad, CA), using specific primers (see below). All PCR products were cloned into pCR®2.1-TOPO® vector (Invitrogen-Life Technologies, Carlsbad, CA). DNA sequencing was conducted by GENEWIZ, Inc. (South Plainfield, NJ).

### Primer design

Primers specific to the mRNA transcripts from the *CHS* gene and the constitutively expressed housekeeping gene actin (GenBank accessions M59715.1 and XP\_009528756.1 for *P. infestans* and *P. sojae*, respectively) were designed using Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and synthesized by IDT (Coralville, IA). Primers designed for use in real-time quantitative PCR (qPCR) had a GC content between 50–60% and expected amplicon size between 60–150bp. See Table S1 in Supplementary materials for all primer sequences.

### Sequence acquisition and analysis

*CHS* sequences from various fungal and oomycete species were obtained using NCBI's BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and FungiDB (<http://fungidb.org/fungidb/?nobounce>). For the sequences obtained

from FungiDB, every possible effort has been made to refer to these sequences by their GenBank accession.version identifier. MEME (Multiple Em for Motif Elicitation) Analysis (<http://meme-suite.org/>) was performed on all sequences to identify conserved motifs within the coding sequence and in the promoter-containing region. NCBI's BLASTp ([https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE\\_TYPE=BlastSearch&LINK\\_LOC=blasthome](https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome)) was used to conduct conserved domain searches on the putative CHS protein sequences. Transmembrane domains were predicted using the TMHMM Server v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>). Finally, the presence of canonical or non-canonical signal peptides was determined using SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>) and SecretomeP 2.0 (<http://www.cbs.dtu.dk/services/SecretomeP/>), respectively.

### Phylogenetic analysis

CHS amino acid and nucleotide sequences were aligned using ClustalW and phylogenetic analyses was conducted using MEGA6 (Tamura et al. 2013). The evolutionary relationships among oomycete CHS sequences and between oomycete and fungal CHS sequences were inferred via phylogenetic trees constructed using the Maximum Likelihood (ML) and Neighbor-joining (NJ) methods. *Phytophthora infestans* and *P. sojae* sequences were analyzed for evidence of selection by using Tajima's Test of Neutrality (Tajima 1989).

Once the phylogenetic tree for the oomycete and fungal CHS protein sequences was constructed, a smaller, DNA-based, evolutionary analysis including the sequences from multiple isolates obtained in our laboratory was conducted, in order to validate the relationships among them and between these and other oomycetes. This smaller group included sequences from two other oomycetes (*A. euteiches* and *S. monoica*), which possess CHS genes with confirmed expression and function. Trees were inferred using the ML and NJ algorithms; the latter was preferred for analysis because of the rapidly increasing number of possible topologies (unrooted trees) that are generated by other algorithms when the number of taxa increase, which makes it very difficult to examine all topologies. In addition, it has been demonstrated that NJ method generates the correct topology more often than others when the number of nucleotides or amino acids used is relatively small (Nei et al. 1998; Takahashi and Nei 2000). A class V CHS DNA sequence from *Fusarium oxysporum* was used as outgroup in the inference of the phylogenetic relationships.

### RNA extraction and mRNA purification

The Qiagen RNeasy® Plant Mini Kit (Qiagen, Gaithersburg, MD) was used to extract total RNA from *Phytophthora* flash-frozen mycelial tissue, using liquid nitrogen and following the manufacturer's protocol. An on-column DNase treatment was included in the protocol. The same method was used to obtain total RNA samples from infected host plant tissues at 12, 24, 36, and 48 hpi. Total RNA was further treated with DNase using the RNase-free DNase set from Qiagen, following the instructions for removal of contaminating DNA in RNA solutions, prior to final RNA cleanup.

### cDNA synthesis and PCR

cDNAs were synthesized using CHS and actin specific primers for *P. infestans* and *P. sojae*. Reverse transcription was carried out using Qiagen's Quantitect Reverse Transcription Kit® (Qiagen, Gaithersburg, MD), which includes an additional DNase treatment step, to eliminate potential contamination with genomic DNA.

### Real-time quantitative PCR (qPCR)

Real-time quantitative polymerase chain reaction (qPCR) was performed with the SYBR® Green PCR kit (Qiagen, Gaithersburg, MD), using the cDNA made from RNA extracted from mycelia and infected tissues. Fold expression was calculated using the constitutively expressed actin gene of *P. infestans* (GenBank accession M59715.1) and *P. sojae* (GenBank accession XP\_009528756.1) as the reference gene (Avrova et al. 2003). Mycelial expression was used as the calibration value to which each experiment was compared and standardized.

qPCR data obtained from three technical replicates were analyzed using the Livak method to calculate fold expression (Livak and Schmittgen 2001). The Livak method compares the critical threshold ( $C_t$ ) cycle values obtained for the target gene in the treated group (infected tissue) and the untreated one (mycelial tissue), to determine the fold expression change based on the difference in  $C_t$  values ( $\Delta C_t$ ). Data are the average of three technical replicates. Data was visualized using the Multiexperiment Viewer application (<http://www.tm4.org/mev.html>), which is part of the microarray software suite TM4 (Saeed et al. 2003).

### CHS inhibition assays

Uncolored V8/Lima agar plates containing 100  $\mu$ M Nikkomycin Z (Sigma–Aldrich, St. Louis, MO), a selective competitive inhibitor of chitin synthase, were inoculated with a 25 mm<sup>2</sup> plug of *P. infestans* and *P. sojae*. Growth

was measured daily from the center of the inoculum to the edge of the growing mycelium.

## Results

### The *CHS* gene in *Phytophthora* spp

We have cloned the putative *P. infestans* *CHS* gene (XM\_002908585.1) and its *P. sojae* ortholog (XM\_009525864.1) from multiple isolates of both species. The coding sequence, which contains no introns, was found to be 2742 bp in both *P. infestans* and *P. sojae*. The 3' untranslated region in the *CHS* mRNA from *P. infestans* consists of 223nt while in *P. sojae* this region is 226 nt long. The 5' untranslated region in the *CHS* mRNA from *P. infestans* is 93 nt long. Despite multiple attempts using different strategies, the 5' untranslated region of the *CHS* gene from *P. sojae* could not be determined.

Neither SignalP nor SecretomeP algorithms suggested the presence of a canonical or non-canonical signal peptide in either of the sequences analyzed. However, seven transmembrane helices (starting on residue 579) were predicted by TMHMM, with at least 159 amino acids expected to be part of the transmembrane helices. All, but one, of the transmembrane domains were predicted to contain 22 amino acids (the fifth domain was predicted to contain 17 amino acids only). No amino acids were expected to be part of a transmembrane helix in the first 60 amino acids of the protein sequence. The total probability that the amino terminus is on the cytoplasmic side of the membrane was 0.0045.

MEME analyses using a 500 bp-fragment directly preceding the translational start site in all *Phytophthora* *CHS* sequences available revealed the existence of three different motifs of 39, 33, and 37 nt in length, respectively (Fig. 1). Motif 1 (39 nt) was present in all sequences and is located closest to the translational start site. Motifs 2 (33 nt) and 3 (37 nt) were found in five of the eight sequences analyzed and, although their locations are not identical in all sequences, the nucleotide distance between them is approximately the same. Furthermore, in relation to the translational start site, motif 2 always precedes motif 3 in the five sequences where these motifs were found (Fig. 1).

Analyses of a dataset composed of 32 fungal and 34 oomycete sequences, including *CHS* sequences from *Aphanomyces euteiches* and *Saprolegnia monoica*, which have been demonstrated to be functional, uncovered the presence of a domain distinctive of the glycosyltransferase superfamily located between amino acid positions 234 and 546 (Fig. 2). This conserved domain (cd04190) is known as the catalytic B domain and typically contains various motifs (LAEDRIL and QRRRW being two of the most

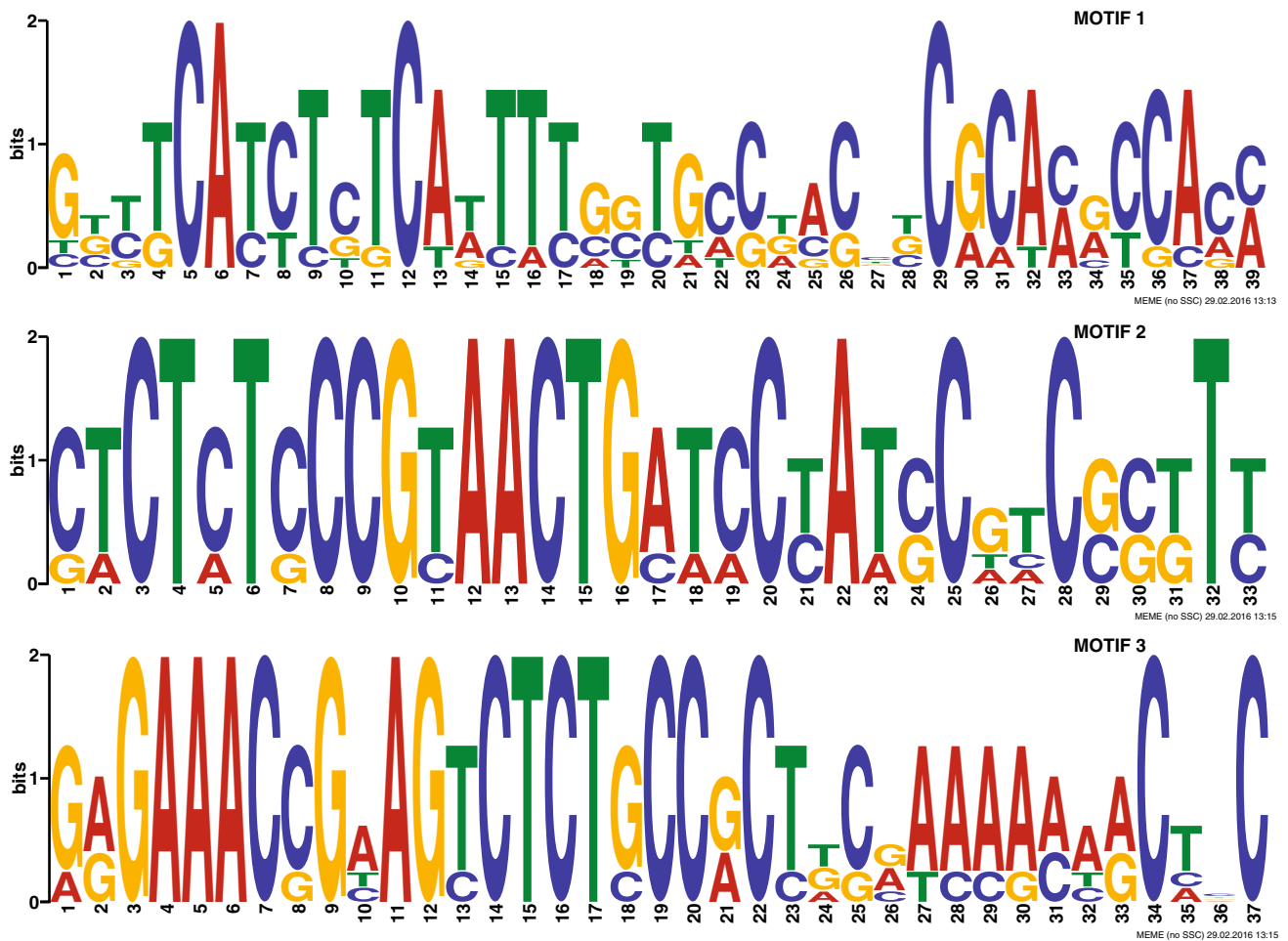
conserved ones), some of which were found in the analyzed sequences. In the Saprolegniales, the QRRRW motif does not have any variations while in some members of the Peronosporales a lysine residue has substituted the arginine residue in the third position of the motif. The domain containing the QRRRW motif is predicted to include the ligand-binding site for the substrate UDP-GlcNAc. It also includes the DXD motif, a metal-ion binding site. Finally, a large group of sequences, including all those belonging to species from the order Saprolegniales and four from species within the Peronosporales (*Pythium aphanidermatum* PAG1 G005394, *Phytophthora ramorum* PSURA 84041, *Phytophthora cinnamomi* PHYCI 219007, and *Phytophthora sojae* XP\_009514470.1, all of which appear to be closely related to the Saprolegniales; Fig. 3), also contained the conserved domain cd: 02656 MIT, which is typical of microtubule interacting and trafficking molecules.

### Phylogenetic analyses

An alignment of 66 *CHS* amino acid sequences from oomycetes and fungi (34 and 32 sequences, respectively), which included representatives from all fungal *CHS* classes described so far, was used to infer the phylogenetic relationships among all sequences using the ML and the NJ methods (Fig. 3). Both approaches yielded very similar results. Analysis of the phylogenetic trees obtained by these methods revealed the presence of two major clades. One of the clades contained exclusively fungal sequences, belonging to *CHS* classes 3–8. The other clade was subdivided in two large sub-clades, the first one formed by all the oomycete sequences while the second one was composed of fungal sequences mostly belonging to *CHS* classes 1 and 2. In addition, this sub-clade also contained four class 3, one class 4 and one class 8 *CHS* sequences.

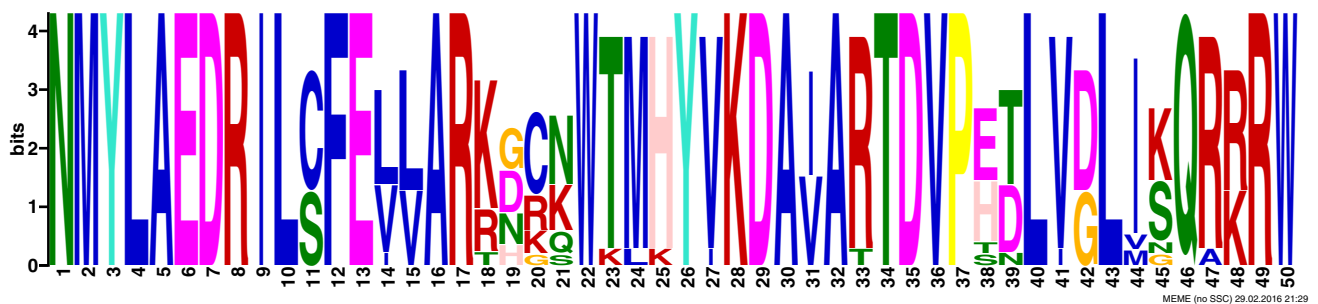
The phylogenetic tree obtained exclusively with 42 oomycete nucleotide sequences, which included information from eight isolates (five *P. infestans* and three *P. sojae*) sequenced in our laboratory, displayed two major clades, one constituted by sequences from the orders Pythiales (*Pythium* spp. and *Phytophthora* spp.) and Peronosporales (*Hyaloperonospora arabidopsidis*, *Plasmopara halstedii*, and *Albugo* spp.) and the other containing all the sequences from the order Saprolegniales (*Saprolegnia* spp. and *Aphanomyces* spp.). This clade, however, also contained a few sequences from *Phytophthora* spp. and *Pythium aphanidermatum* (Fig. 4).

A total of 23 single nucleotide polymorphisms, all of them C:T transitions, were observed within the *P. infestans* sequences obtained from five of our isolates. On the other hand, only 7 substitutions (also transitions) were noticed within the three *CHS* sequences obtained from our *P. sojae* isolates (data not shown). These nucleotide changes



**Fig. 1** Shared motifs found in the *CHS* promoter sequence regions. MEME analysis revealed the existence of three different motifs of 39, 33, and 37 nt in length, respectively, in the promoter region. Motif 1 was present in all sequences and is located closest to the translational start site. Motifs 2 and 3 were found in five of the eight

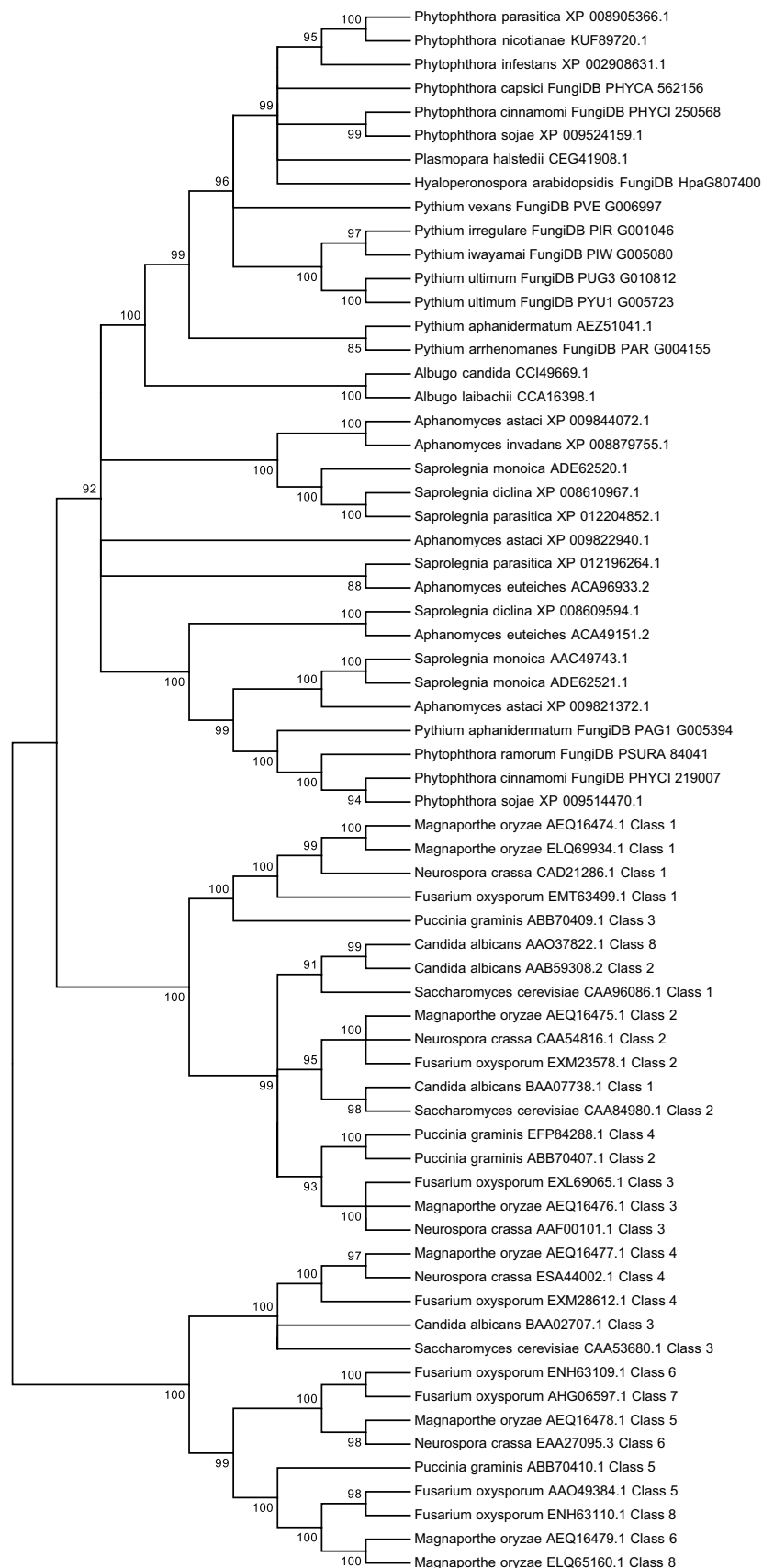
sequences analyzed and, although their locations are not identical in all sequences, the nucleotide distance between them is approximately the same. Size of base indicates the relative level of conservation in the examined sequences

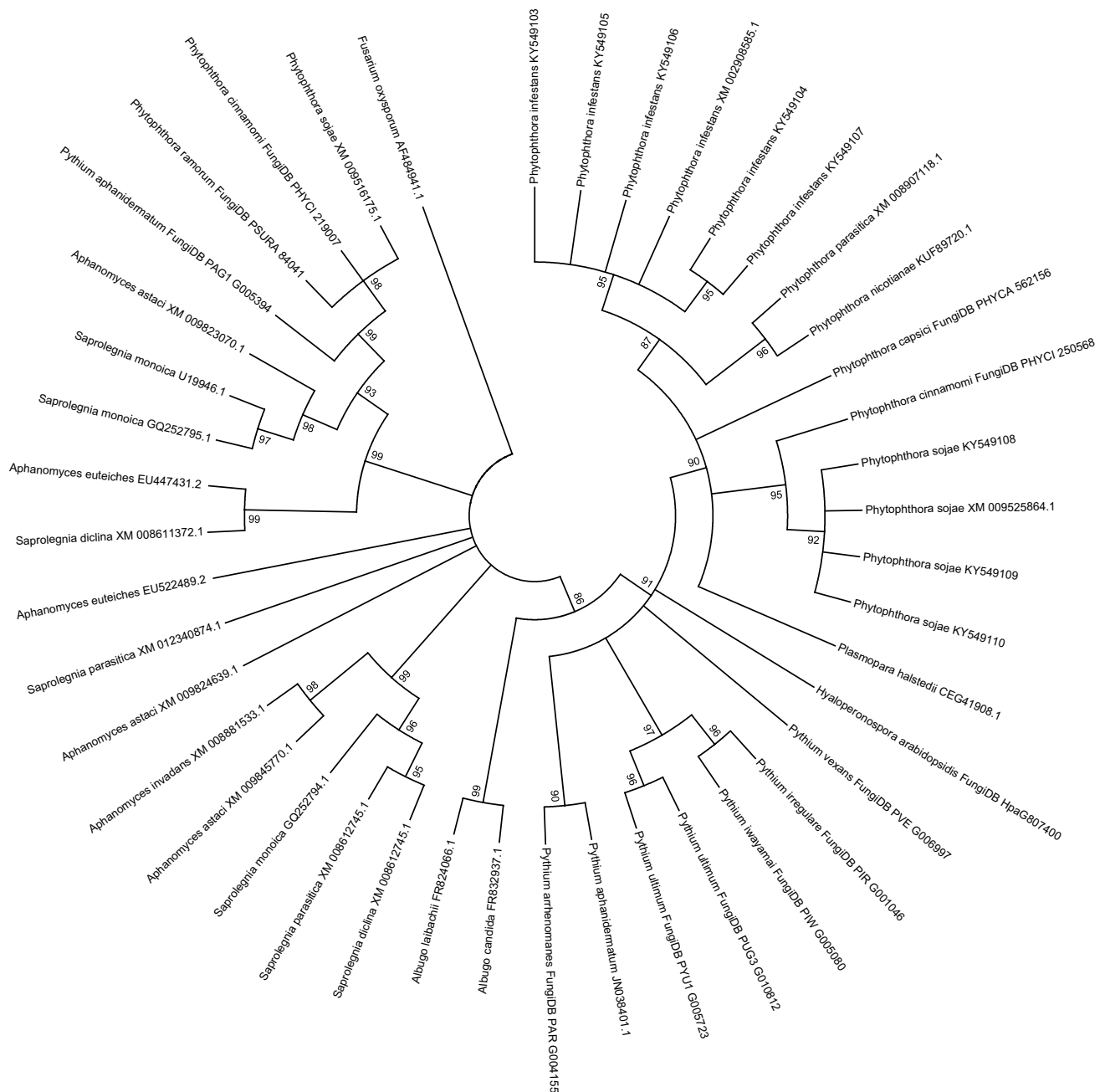


**Fig. 2** Glycosyltransferase superfamily domain. Located between amino acid positions 234 and 546 of the *CHS* protein, this conserved domain is known as the catalytic B domain and typically contains

various motifs. It is predicted to include the ligand-binding site for the substrate UDP-GlcNAc. This domain also includes the DXD motif, a metal-ion binding site

**Fig. 3** Evolutionary relationships of fungal and oomycete CHS proteins. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Branches corresponding to partitions reproduced in less than 80% bootstrap replicates are collapsed. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 66 amino acid sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. The analysis was repeated using the Maximum Likelihood method, with very similar results. In both cases, two major clades were identified: one of them contained exclusively fungal sequences, belonging to CHS classes 3–8, while the other one was formed by all the oomycete sequences and some fungal sequences mostly belonging to CHS classes 1 and 2. There were a total of 563 positions in the final dataset. Evolutionary analyses were conducted in MEGA6





**Fig. 4** Molecular phylogenetic analysis by Maximum Likelihood method of oomycete *CHS* genes. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 80% bootstrap replicates are collapsed. Two major clades were identified, one constituted by sequences from the orders Pythiales and Peronosporales and the other containing all the

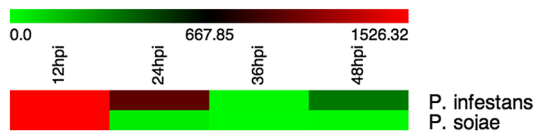
sequences from the order Saprolegniales. The analysis involved 43 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 1929 positions in the final dataset. Evolutionary analyses were conducted in MEGA6

resulted in a total of 12 and 6 non-synonymous substitutions, respectively. No evidence of selection was found after analyzing these *CHS* sequences with Tajima's Test of Neutrality (Tajima's *D* values:  $-0.08$  and  $0.00$ , for *P. infestans* and *P. sojae*, respectively).

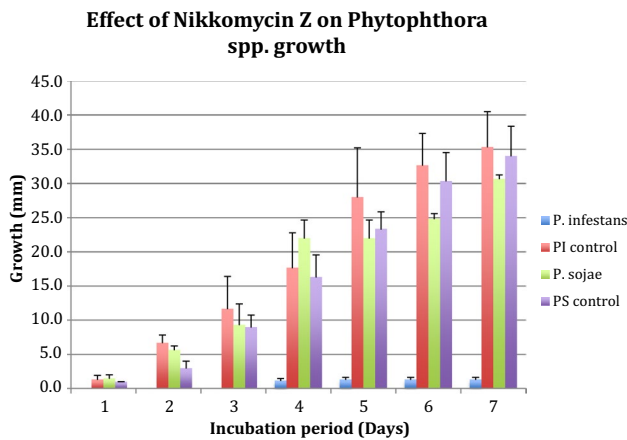
#### Expression of the *CHS* gene in infected tissues

qPCR experiments using RNA extracted at each of the specific time points revealed that the *CHS* gene expression in both *Phytophthora* spp. is highly increased at 12hpi





**Fig. 5** Relative expression of the *CHS* gene in *Phytophthora infestans* and *P. sojae* at four time points post-inoculation obtained by analysis of qPCR data using Livak’s method. Actin was used as a reference gene, and the calibrating sample was mycelial RNA. Red indicates up-regulation, green down-regulation, black neutral, and grey indicates transcription was not detected. Reactions were run in triplicate. The range of fold expression variation is shown at the top



**Fig. 6** Effect of Nikkomycin Z on *Phytophthora* spp. growth in culture. Radial growth on unclarified V8®/Lima agar plates containing 100 μM Nikkomycin Z was measured daily over a 7-day period. Statistical analysis revealed a significant difference in growth between *P. infestans* cultures grown in the presence of Nikkomycin Z and those grown in the absence of this CHS inhibitor ( $p=0.0002$ ). On the other hand, *P. sojae* cultures did not appear to be affected by the presence of Nikkomycin Z in the growth medium ( $p=0.1297$ )

(Fig. 5). The expression levels in *P. infestans* declined over the next 24-h period only to increase slightly again at 48 hpi. In *P. sojae*, a sharp decline in expression was observed at 24 hpi. This low level of expression was maintained until the end of the experiment.

**CHS inhibition assays**

After 7 days, growth of *P. infestans* on unclarified V8®/Lima agar plates containing 100 μM Nikkomycin Z, an inhibitor of CHS activity, was severely restricted as compared to controls (Fig. 6). Statistical analysis indicates there was a significant difference in growth between cultures grown in the presence of Nikkomycin Z and those grown in the absence of this CHS inhibitor ( $p=0.0002$ ). On the other hand, *P. sojae* cultures did not appear to be affected

by the presence of Nikkomycin Z in the growth medium ( $p=0.1297$ ).

**Discussion**

**The putative *Phytophthora* spp. *CHS* gene and its deduced protein contain all the motifs typically found in sequences of fungal origin**

A major structural difference between oomycetes and fungi is the presence of cellulose in the cell wall of the former as opposed to chitin, which is typically found in the cell wall of fungi. Although evidence of the presence of a chitin synthase gene in the animal pathogenic oomycete *Saprolegnia monoica* had been put forward 20 years ago (Mort-Bontemps et al. 1997), the existence of this gene in phytopathogenic oomycetes such as *Phytophthora* was not known. During the annotation processes of several *Phytophthora* genomes carried out in the early to mid-2000’s, one of us (MO-G) found bioinformatic evidence suggesting that the gene was present in the genome of *P. infestans*, *P. ramorum*, and *P. sojae* (Ospina-Giraldo et al. 2010). More recently, further bioinformatic analyses have revealed that this gene also exists in oomycetes such as *Albugo* spp., *Hyaloperonospora arabidopsidis*, *P. capsici*, *P. cinnamomi*, *P. nicotianae*, and *Pythium* spp. among others (unpublished).

Sequence analyses of the putative *CHS* genes discovered in *P. infestans* and *P. sojae* indicate that the encoded protein contains all motifs typically found in CHS proteins from fungal origin. The first motif, which is part of the conserved domain cd04190: Chitin\_synth\_C and is known as the catalytic B domain (Fig. 2), contains the amino acid consensus sequence QRXRW, which is the characteristic signature motif of all fungal CHS proteins with processive capacity (Choquer et al. 2004; Riquelme and Bartnicki-García 2008). This domain is predicted to contain the ligand-binding site for the substrate UDP-GlcNAc. When the analysis was expanded to include all oomycete sequences available, in the majority of them, the domain consisted of the residues QRRRW. All members of the Saprolegniales contained this motif sequence, with the exception of *Albugo* spp., which contained the sequence QRKRW. In the Peronosporales, the third position of the motif was occupied either by arginine or lysine, with the former being most prevalent. This motif, along with pfam01644 (also present in the analyzed sequences) is a member of the superfamily cl11394: Glyco\_tranf\_GTA\_type, which is typically found in glycosyl transferase family 2 (GT-2) proteins. Another conserved sequence found was the DXD motif, which is thought to be involved in the binding of a metal ion cofactor that is used to coordinate the phosphates of the NDP-sugar in the active site. Interestingly, only a group of

sequences (all of the Saprolegniales and four from Peronosporales origin) contained a motif (cd02656: MIT) whose actual molecular function is unclear, but that is characteristic of the so-called microtubule interacting and trafficking molecules (Ciccarelli et al. 2003). The MIT domain is found in nexins, vacuolar sorting proteins, and others. In oomycetes, this domain was first uncovered in *S. monoica* and it was suggested that, given its potential involvement in protein sorting, it might play a role in the delivery of chitin to the apical region during cell wall formation (Guerrero et al. 2010). However, because of the absence of the MIT domain in most of the Peronosporales sequences analyzed in this study, in particular in those organisms believed to contain only one of copy of the gene, it is difficult to clearly ascertain the importance of this domain in cellular physiology.

In both, *P. infestans* and *P. sojae* CHS sequences, SignalP and SecretomeP failed to detect the presence of a signal peptide targeting the putative protein to the cell membrane. Furthermore, the TMHMM analysis predicted that no amino acids would be expected to be part of a transmembrane helix in the first 60 amino acids of the protein sequence, which is consistent with the SignalP and SecretomeP results. However, the bioinformatic evidence favoring the fact that the CHS protein is membrane located is very strong and suggests that the transmembrane domain might be the one targeting the protein to the chitosomes and the secretory pathway, as is typical of most type II and multi-spanning membrane-bound proteins (Brandizzi et al. 2002; Munro 1995). It is important to note, however, that fluorescence experiments have shown that labeled CHS complexes do not appear to move through conventional secretory routes (Riquelme et al. 2007).

Analysis of a 500-bp region upstream of the start codon revealed the existence of three different motifs of 39, 33, and 37 nt in length, respectively (Fig. 1). Motif 1 (39 nt), which was present in all sequences and is located closest to the translational start site, bears partial resemblance to the previously located motif GCTCATTYNCAWTTTT hypothesized to be the transcription start point in oomycetes (McLeod et al. 2004).

### Evolutionary dynamism of the oomycete CHS genes

Evolutionary relationships among a large dataset of CHS sequences from fungal and oomycete origin were determined using Maximum Likelihood and Neighbor-joining algorithms, with almost identical results. Oomycete CHS sequences appear to be more closely related to a clade containing all fungal CHS class 1 and class 2 proteins. This clade also contains four (out of six) class 3 and one (out of four) class 4 CHS sequences (Fig. 3). Therefore, while it is safe to assume that oomycete CHS proteins are likely

part of CHS classes 1, 2, or 3, the phylogenetic analysis does not provide conclusive evidence regarding which specific class oomycete CHS proteins belong to. It does indicate, however, that most class 4, and all class 5, 6, 7, and 8 fungal CHS sequences form a clade of their own and are only distantly related to oomycete CHS sequences. Analysis by Bowen et al. (1992) revealed that similarities among some sequences were consistent with current taxonomic groupings of the respective organisms where these sequences were found. However, it is clear from our analysis that, as the field of known or putative CHS sequences has expanded, classification solely based on sequence analysis has become more difficult. This relative uncertainty of the gene tree contrasts with the robustness of the species phylogenetic tree, in which the placement of several *Phytophthora* spp. has been clearly elucidated and is strongly supported by evolutionary studies based on nuclear and mitochondrial genomes (Blair et al. 2008; Lassiter et al. 2015).

Three *Phytophthora* spp. (*P. cinnamomi*, *P. ramorum*, and *P. sojae*) and one species of *Pythium* (*Py. aphanidermatum*) contain at least two copies of the CHS gene. The placement of these copies in the phylogenetic tree (one in the clade containing sequences from the Pythiales and Peronosporales orders and the other forming part of the Saprolegniales clade; Fig. 4) supports the notion that gene duplication occurred prior to speciation events that gave rise to these species, and that the genes are, most likely, true orthologs (Badreddine et al. 2008). It also indicates, however, that the rate of evolutionary changes in the paralogous copies of these genes is considerable high, as duplicated copies in the same species are placed in fairly distant evolutionary clades. Furthermore, in the case of *P. infestans* the sequence divergence of the duplicated copy seems to be so high that bioinformatic analyses can no longer find this copy in the genome. This accelerated rate of evolutionary changes is noticeable within CHS sequences from isolates of the same *Phytophthora* spp., in which a considerable number of nucleotide substitutions were detected (data not shown). However, although these nucleotide changes resulted in a total of 12 and 6 non-synonymous substitutions in *P. infestans* and *P. sojae* CHS genes, respectively, Tajima's Test of Neutrality conducted with these sequences did not provide evidence of current selection.

Our phylogenetic analysis also shed additional light on the evolutionary placement of the CHS genes from *P. capsici* and *Plasmopara* spp. Previous studies (Badreddine et al. 2008) have suggested that a sequence from *Pl. viticola* did not appear to be related to other oomycete CHS sequences. In our study, the full-length sequence from a related species, *Pl. halstedii*, was unequivocally placed within the *Phytophthora* clade (Fig. 4), suggesting that the discrepancy about the evolutionary location of *Pl. viticola*

*CHS* might be due to the fact that only a fragment of its sequence was used in the phylogenetic analysis described in those studies. Likewise, it has been suggested that the *P. capsici* protein groups together with *S. monoica* CHS and not with *P. infestans* CHS (Guerriero et al. 2010). In our analysis, however, the *P. capsici* CHS appears to be very closely related to the *P. sojae* and *P. infestans* CHS clades.

Although it was not our goal to further dissect the evolutionary relationships among fungal CHS proteins, our phylogenetic analyses revealed that the fungal CHS protein sequences used in our study belong to two clearly distinct evolutionary branches, which are only distantly related; furthermore, one of these branches shares the most recent ancestor with the oomycete sequences (Fig. 3). These results confirm the proposed ancestral gene duplication that might have occurred very early in the evolutionary history of oomycetes and fungi (Riquelme and Bartnicki-García 2008; Roncero 2002), an event likely followed by multiple speciation events that led to the appearance of the species in both groups (see below).

In the absence of protein expression and localization data, the biological significance of the presence of the *CHS* gene in *Phytophthora* spp. remains unclear. Most fungi and some oomycetes belonging to the order Saprolegniales still retain up to five or six copies of the *CHS* gene while *Phytophthora* species only possess one or two copies. If *Phytophthora* spp. have evolved to rely mainly on cellulose for their structural support, the proposition that the gene may be an ancestral relic with a yet to be defined role might be tantalizing.

### The putative *CHS* gene in *Phytophthora* spp. is actively expressed during pathogenesis

Expression analysis of the *CHS* gene in *P. infestans* and *P. sojae* throughout the infection process showed a trend of higher transcriptional activity towards the beginning of infection that tapered off as time progressed (Fig. 5). This is consistent with the natural infection cycle of *P. sojae* and *P. infestans*, which is characterized by an active period of colonization of the host during the first 12–15 hpi and vigorous growth in biomass. For example, at 4 hpi *P. sojae* colonizes three to four cell layers of the root cortex; after 7 hpi hyphae have been found as far as 10 cell layers deep within the root, and after 15 hpi the pathogen, which until now has been growing intercellularly only, starts growing intracellularly, and necrotic host cells begin to appear. At 20 hpi colonization via hyphae and haustoria is in decline and the transition into necrotrophy has already begun, with roots showing considerable damage (Enkerli et al. 1997). The highest fold expression of the *CHS* gene in *Phytophthora* spp. during the time of peak hyphal growth and host plant colonization (12–15 hpi) could correlate chitin

synthesis (or, at least, CHS glycosyltransferase activity) with hyphal formation and/or structural integrity of the cell wall. At 24 hpi, expression of *P. sojae* *CHS* was considerably lower than the peak expression at 12 hpi. By 36 hpi, *CHS* expression decreased dramatically and remained at this low level at 48 hpi (Fig. 5). The reduced *CHS* expression at 48 hpi coincides with the transition observed in *P. sojae* infection cycle, in which by 48 hpi, the organism has entered the necrotrophic phase and is no longer colonizing the host as actively as it did during the biotrophic period (Torto-Alalibo et al. 2007).

In infected potato tissues, the transcriptional activity of the *P. infestans* *CHS* gene was similar to that observed in *P. sojae* infected tissues (Fig. 5). The highest expression level was seen at 12 hpi with a slight decline from 24 to 36 hpi, indicating again a likely decrease in the synthesis of the CHS enzyme as the pathogen progresses to the necrotrophic phase of its hemibiotrophic lifestyle. At 48 hpi the expression increases slightly, a result that is puzzling, since once the necrotrophic stage has been reached, mycelial growth is halted and the pathogen shifts to the production of sporangia, which are released into the atmosphere for aerial dispersion (Fry 2008).

### In *Phytophthora infestans*, CHS activity is required for vegetative growth

Although the *CHS* gene appears to be expressed in *Phytophthora* spp.-infected tissues, the specific role of chitin in the pathogen, if present, is yet unknown. Chitin is found in the cell wall, and has been sporadically located in sporangial structures, of *Saprolegnia* spp. and *Aphanomyces* spp. (Badreddine et al. 2008; Guerriero et al. 2010; Leal-Morales et al. 1997; Mort-Bontemps et al. 1997). However, there has been no success in localizing chitin in the cell walls of *P. sojae* and *P. infestans*, where the polysaccharide is postulated to play a structural role. Moreover, results from our CHS inhibition experiments appear to suggest that CHS glycosyltransferase activity is important for normal vegetative growth, at least in *P. infestans* (Fig. 6). The fact that *P. sojae* growth was less affected by the presence of Nikkomycin Z in the medium might be explained by the existence of an additional copy of the *CHS* gene in its genome, which might not be equally susceptible to Nikkomycin Z inhibition. In *P. sojae*, a second putative *CHS* gene exists (XM\_009516175.1); this gene is only distantly related to XM\_009525864.1, which we believe is the true *P. sojae* ortholog of the *P. infestans* *CHS* gene (XM\_002908585.1). Multiple phylogenetic analyses consistently placed both copies in two unequivocally distinct clades (Fig. 4) and the percentage of identical amino acids between them reaches only 34%. Therefore, it is possible that Nikkomycin Z may not

fully inhibit the protein encoded by one of the *CHS* gene copies (unfortunately, in the absence of *in vivo* protein expression data, it is not possible to determine exactly which copy was inhibited and which one was not. We can only hypothesize that, given the extremely close evolutionary relationship between XM\_009525864.1 and its ortholog in *P. infestans*, both of which are placed in the Pythiales/Peronosporales sub-clade, if the *P. infestans* copy is susceptible to inhibition by Nikkomycin Z, so will be its ortholog in *P. sojae*). This differential response to the presence of Nikkomycin Z in the growth medium by two different species is not unique, and has been previously reported in other oomycetes. For example, it has been found that cells from *S. monoica* are much less susceptible to Nikkomycin Z than those of *S. parasitica*. This difference in susceptibility has been attributed to an increase in the expression of *S. monoica CHS* genes (Guerriero et al. 2010).

In conclusion, the structural characterization of the putative *CHS* gene in *Phytophthora* spp. revealed that it contains all the motifs that are typical in *CHS* genes of fungal origin, including the catalytic B domain and the amino acid consensus sequence QRXRW, which is predicted to include the ligand-binding site for the substrate UDP-GlcNAc, and is the characteristic signature motif of all fungal CHS proteins with processive capacity. Although evolutionarily related to fungal CHS, phylogenetic analyses distinctly placed the oomycete CHS sequences in a clade of their own. In *Phytophthora* spp., the *CHS* gene is expressed, at least at the mRNA level, during *in vitro* and *in planta* growth, with the highest level of expression occurring in the first 12 h post inoculation. Our inhibition experiments suggested that the CHS glycosyltransferase activity (possibly in chitin biosynthesis, although alternative roles for this enzyme in other biosynthetic processes cannot definitely be ruled out) is important for normal vegetative growth. However, because of the considerable variation in expression during infection when compared to basal expression observed in *in vitro* cultures of non-sporulating mycelium, we also hypothesize that CHS may have a meaningful role in *Phytophthora* pathogenicity. Evidently, additional mRNA and protein detection and quantification studies accompanied by biochemical and ultrastructural analyses may shed more light on this and other issues pertaining to the biological significance of the presence of a *CHS* gene in *Phytophthora* spp.

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