J Chem Ecol (2009) 35:824–832
DOI 10.1007/s10886-009-9653-1

Stigmasterol and Cholesterol Regulate the Expression of Elicitin Genes in *Phytophthora sojae*

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Received: 5 February 2009 / Revised: 21 May 2009 / Accepted: 11 June 2009 / Published online: 9 July 2009
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Abstract Sterol acquisition by soilborne plant pathogens of the genus *Phytophthora* is presumed to involve extracellular proteins belonging to class-I elicitins. However, little is known about the relationship between sterol availability and elicitin secretion. The objective of this study was to determine the expression of class-I elicitin genes in *Phytophthora sojae* when grown in a medium containing stigmasterol or cholesterol. *P. sojae* growth was stimulated by nanomolar concentrations of stigmasterol and cholesterol, which also resulted in the down-regulation of its elicitin genes over time when expression profiles were monitored using real time Reverse Transcription Polymerase Chain Reaction (RT-PCR). The down-regulation of elicitin genes in response to the two sterols also coincided with a reduction in the amount of elicitins detected in spent filtrates. Our study is the first to show the influence of sterols on elicitin gene expression in *Phytophthora*, which is important with respect to the ecology of elicitin secretion as sterol carrier proteins in the environment.

Keywords Elicitin · Extracellular proteins · *Phytophthora* · Plant pathogens · Sterol carrier protein

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Introduction

Oomycetes are a diverse group of eukaryotic organisms that include the genus *Phytophthora*, many species of which are destructive plant pathogens in agricultural and forest ecosystems (van West et al. 2003). Members of *Phytophthora* all lack sterol biosynthetic pathways and acquire sterols exogenously from their environment to support their growth and sexual reproduction (Hendrix 1975; Elliot and Knights 1981; Nes and Stafford 1983; Ponchet et al. 1999; Marshall et al. 2001). It is speculated that these organisms acquire sterols from the environment via extracellular proteins belonging to class-I of elicitins (Ponchet et al. 1999). These are small (10 kDa) hydrophilic secreted proteins that were classified as sterol carrier proteins because some exhibited sterol and lipid carrier activity *in vitro* (Mikes et al. 1997, 1998; Vauthrin et al. 1999; Osman et al. 2001a, b).

Elicitins are proteins unique to the genus *Phytophthora,,* and are encoded by a large multi-gene family that is divided into at least eight different classes based on sequence homology and protein motif diversity (Jiang et al. 2006a, b). Class-I elicitins are the best characterized, and are considered structurally simple when compared to other elicitin classes (Jiang et al. 2006b). They encode only a conserved 98 amino acid sequence (known as the elicitin domain) and a signal peptide that is post translationally removed (Ponchet et al. 1999; Jiang et al. 2006b), whereas other classes may also encode a variable c-terminal domain that may contain a membrane-spanning domain (Jiang et al. 2006a, b). Elicitin genes are highly conserved across the entire genus of *Phytophthora* (Kamoun et al. 1997a, b; Qutob et al. 2003; Jiang et al. 2006a, b); all species of *Phytophthora* studied so far secrete elicitins abundantly into

their growth medium (Ponchet et al. 1999), suggesting a significant role for elicitins in the biology of these organisms, presumably in sterol uptake.

Class I elicitins became of interest when it was shown that these proteins induced a plant hypersensitive response (HR) when infiltrated on tobacco leaves (Ponchet et al. 1999). Consequently, further research was done to establish whether elicitin genes are avirulence genes that determine host range (Ponchet et al. 1999; Kamoun 2001). As a result, studies on elicitins have focused largely on elicitor activity on plants (e.g., Baillieul et al. 2003), characterization of protein structure (Lascombe et al. 2004), and discovery of new elicitins secreted by *Phytophthora* spp. (Churngchow and Rattarasarn 2000). However, there are no studies on the significance of elicitin secretion with respect to sterol availability and *Phytophthora* ecology. In soil, sterols are described as labile organic compounds (Bull et al. 2000); *in vitro* studies show that *Phytophthora* spp. assimilate exogenously supplied sterols into their membranes (Elliot and Knights 1981), but the relationship between sterol availability and elicitin secretion has not been established. Therefore, the objective of this study was to examine the expression profile of class-I elicitin genes in the model oomycete and pathogen of soybean, *Phytophthora sojae*, when two sterols of different origin, stigmasterol (plant) and cholesterol (animal), are supplemented in its growth medium.

Methods and Materials

The *Phytophthora sojae* culture (race 1) was obtained from Dr. Dorrance (OARDC, Wooster OH, USA). Cultures were maintained on non-clarified V8 agar (18% v/v V8 agar juice, 0.3% $CaCO_3$, and 2% agar) at 25°C in the dark. For growth response bioassays and gene expression experiments, plugs (6 mm or 10 mm diam) were transferred from the edge of actively growing *P. sojae* into a defined minimal medium (Wu et al. 2003) modified to contain glucose (10 g/l). The growth medium was solidified with 2% w/v agar (growth response experiments) or kept in liquid state (gene expression experiments). Cultures were incubated in the dark at 25°C until terminated.

Biological Assays Stigmasterol and cholesterol (95–99% purity) (Sigma-Aldrich, St. Louis, MO USA) were added to the growth medium at a final concentration of 1,000 nM, 100 nM, or 10 nM with chloroform (final 0.01% v/v) as the solvent. For growth response bioassays, single plugs (6 mm diam) were transferred from the edge of actively growing *P. sojae* on V8 agar to the center of experimental agar plates, and incubated in the dark at 25°C. Colony diameter was measured over time with a metric ruler. For gene expression and protein profile experiments, single plugs (10 mm diam) were inoculated into Erlenmeyer flasks (50 ml) that contained experimental media (20 ml). *P. sojae* mycelia were harvested over time by vacuum filtration through pre-weighted nylon membranes (0.2 µm), and replicates were either processed immediately for RNA extraction or dried in an oven overnight (45°C) for dry biomass measurements. All experiments were repeated independently twice. Experiments included three biological replicates. For gene expression experiments, RT-PCR data was generated from three technical replicates of two biological replicates.

Primer Specificity for Elicitin Amplification P. sojae contains at least nine elicitin genes (Qutob et al. 2003), two of which are class-I and named *SOJA* and *SOJB* (referred to as *sojein* in Mao and Tyler 1996 and Becker et al. 2000). The letters A and B following *SOJ* further classify the elicitins according to their isoelectric (pI) points as either acidic (A, pI<5) or basic (B, pI>5), depending on the total number of Lys residues present in the elicitin domain (Ponchet et al. 1999; Qutob et al. 2003). Moreover, four isotypes of the *SOJA* gene are present, (originally named sojein 1–4 in Mao and Tyler 1996 and Becker et al. 2000) that differ from each other by a few amino acid substitutions within the elicitin domain, which results in a slightly different amino acid content. Primers for the *SOJA* and *SOJB* elicitin genes were designed from their cDNA sequences available on the National Center for Biotechnology Information (NCBI) web-site (Table 1), whereas primers for the internal control Actin gene (*ActA*) were designed from sequences of the gene available for *P. sojae* on the US Department of Energy's Joint Genome Initiative (JGI) web-site (Table 1).

Designing primers that are specific for each individual *SOJA* isotype is difficult because differences in the nucleotide sequences are minor. Instead, we designed primers to regions that are homologous to the elicitin domain in all four *SOJA* isotypes, which would amplify a 298 bp fragment of the expressed genes. Elicitin genes lack introns (Mao and Tyler 1996; Ponchet et al. 1999), which allowed us to use genomic DNA from *P. sojae* as a template to test the specificity our elicitin primers. The elicitin domain of *SOJA* is highly conserved and contains nucleotide sequences that are similar to *SOJB* and other elicitin classes present in *P. sojae*. To determine if our *SOJA* primers would amplify other elictin genes having homologous sequences, we cloned the PCR products into *E.coli* and submitted ten individual colonies selected at random for sequencing. Using BLAST, nine of the sequenced clones were identified as *Sojein2* (*SOJA-2*), and one was identified as *Sojein3* (*SOJA-3*). For *SOJB*, primers were designed to amplify a 120 bp fragment of the

Table 1 Oligonucleotide primers used in real-time RT-PCR expression analysis and amplicon size

Gene	GenBank accession	Amplicon size (bp)	Forward and reverse primers
SOJA	AJ007858 (*sojein1*), AJ007859 (*sojein2*), AJ007860 (*sojein3*), AJ007861 (*sojein4*)	298	5′ acc acg tgc acc tcg tcg cag 3′
			5′ tta cag cga cgc gca cgt gga 3′
SOJB	AY183409	120	5′tct aag cgc gtc ctc cag ctc 3′
			5′ctc aca act tag tcc tcg gtt gat ggc 3′
ActA	estExt_fgenesh1_pm.c_490003[a]	264	5′gta ctg caa cat cgt gct gtc g 3′
			5′ tta gaa gca ctt gcg gtg cac g 3′

[a] Expressed sequence tag available from the US Department of Energy's Joint Genome Initiative (JGI)

3′untranslated region (UTR) of the gene because the 3′ UTR region is reported to be variable in elicitin genes (Jiang et al. 2006a, b).

Cloning of SOJA Elicitin Genes The *SOJA* elicitin genes were amplified from genomic DNA of *P. sojae* by regular PCR using the following primers; 5′ATCGAATTC ACCACGTGCACCTCG 3′ (forward) and 5′ AAT CTC GAG TTA CAG CGA CGC GCA CGT GGA CGA GAA 3′ (reverse). These primers are similar to the ones used for gene expression assays, but include *Eco*RI (forward primer) and *Xho*I (reverse primer) sites for cloning and sequencing to confirm the specificity of amplification. The underlined sequences correspond to the elicitin gene flanked by a restriction enzyme site. The cycle conditions were 94°C for 2 min, followed by 35 cycles of 92°C for 1 min, 56°C for 1 min, 72°C for 30 s, and a final extension at 72°C for 5 min. The amplicons were cut with *Eco*RI and *Xho*I, purified on 1% agarose, and ligated into a PGEX-4T-1 vector (GE Health Care, Piscataway, NJ, USA). *E. coli* DH5α was transformed with the construct, and ten individual clones from each amplification reaction were sequenced at the Robarts Research Sequencing facility (The University of Western Ontario, London, ON, Canada).

RNA Extraction and cDNA Synthesis RNA was extracted from mycelia of *P. sojae* using the RNeasy Plant Mini Kit (Qiagen cat # 74904) according to the manufacturer's protocol. The concentration of RNA was determined by using the Quant-iT Tm Ribogreen RNA assay kit (Invitrogen cat# R11490) via a NanoDrop ND-3300 Flurospectrometer. Equivalent amounts of starting RNA (approximately 0.3 μg) from each sample were used as template for first strand cDNA synthesis with the QuantiTect Reverse transcription kit (Qiagen cat# 205311). A preliminary step to remove any contaminating genomic DNA prior to cDNA synthesis is included in the QuantiTect Reverse transcription kit.

RT-PCR Amplification and Analysis Amplification of cDNA was carried out in a final volume of 25 μl that contained 3 μl of a 10-fold dilution of the first strand cDNA, and 0.25 μM primers (*SOJA*) or 0.5 μM primers (*SOJB* or *ActA*) and 12.5 μl iQ™ SYBR Green super mix (Bio-Rad cat# 170-88805). Reactions were performed with a iQ5 multicolor real-time thermal cycler (iQ™ 5 optical module, Bio-Rad, Hercules, CA, USA) for 2 min at 94°C followed by 40 cycles of 94°C for 40 s, annealing at 67°C (*SOJ*A and *SOJ*B) or 59°C (*Act*A) for 40 s, and extension at 72°C for 30 s. The $2^{-\Delta\Delta C}$T method for relative quantification of gene expression was validated as described in (Livak and Schmittgen 2001) prior to the evaluation of elicitin gene expression. Untreated controls (*P. sojae* only receiving delivery solvent) served as calibrators for the treatments, whereas *Act* A served as an internal control to normalize for any variation in the amount of RNA that was initially added to the reverse transcription reaction. Amplification of specific transcripts was confirmed by melting curve analysis provided by the LightCycler instrument and length of PCR products on agarose gels (1.5%). The amplified products also were confirmed at least once by sequencing at the Plant Microbe Genetics facility (The Ohio State University, Columbus, OH, USA).

Protein Analysis The Bradford protein assay (Bradford 1976) was used to quantify the total amount of protein in the spent filtrates of *P. sojae* using γ-globulin as standard. Spent filtrates from replicates of each treatment subsequently were pooled into one sample, and each pooled sample (15 μl) was analyzed on 4–12% gradient Bis-Tris sodium dodecyl sulfate polyacrylamide gel (Invitrogen cat# NP0322BOX) with MES as the running buffer (Invitrogen cat# NP0002) using the XCell SureLock Tm Mini cell system (Invitrogen, Carlsbad, CA, USA) at 200 V. Protein gels were silver stained with the SilverXpress silver staining kit (Invitrogen cat# LCG100) according to the manufacturer's protocol.

For 2D-PAGE analysis, proteins present in 5 ml of spent filtrates from day 13 of controls were precipitated from solution with four volumes of cold acetone (−20°C) according to a protocol published in a technical resource (PIERCE -TR00490.0, Rockford, IL, USA) and dissolved in 4.4% (w/v) CHAPS (200 μl) and urea solution (8 M). The entire volume was used to rehydrate a 3–10 pH IPG strip via a passive rehydration step (1 h) followed by an active rehydration step at 50 V (14 h). The rehydration step was followed by focusing the proteins on the IPG strip overnight at 60,000 vhr. Following focusing, the strips were equilibrated in urea (6 M), Tris buffer (0.375 M; pH8.8 for 25 min), and then in SDS(2% w/v), glycerol (20% w/v), DTT (2% w/v) for 25 min. The equilibrated IPG strips subsequently were run onto pre-cast criterion gels (10–20%) in TGS buffer at 200 V for 60 min, then fixed in methanol (10%) and acetic acid (7%) for 30 min prior to being stained overnight with Sypro Ruby (Bio-Rad cat# 170-3126) according to the manufacturers protocol. Three putative elicitin bands were excised from the gel, placed into water and submitted to the Mass Spectrometry and Proteomics Facilty (The Ohio State University, Columbus, OH, USA) for digestion and analysis by capillary-liquid chromatography-nanospray tandem mass spectrometry (Nano-LC/MS/MS) prior to identification using MASCOT (Matrix Science version 2.2.1, Boston, MA, USA), a search engine that uses MS data to identify proteins from primary sequence databases such as NCBI.

Statistical Analysis Colony diameter measurements, gene expression assay, biomass measurements, and extracellular protein assays from experiments were analyzed by factorial *ANOVA* followed by a *Bonferroni* or *LSD pairwise* comparison of means (α=0.05) using Statistix^{TM} analytical software (P.O. Box 12185, Tallahassee, FL, USA).

Results

Response of P. sojae Sterols Stigmasterol is structurally similar to cholesterol, differing only by the presence of an additional 24-ethyl group and a *trans*-22-double bond (Fig. 1). The sensitivity of *P. sojae* to different concentrations of stigmasterol and cholesterol was determined by dose response growth assays (Fig. 2). Although the pathogen did grow in the absence of sterols, supplementation of growth medium with sterols enhanced the growth rate, as evident by an enhanced stimulation of its mycelium (Fig. 2).

Concentrations of stigmasterol or cholesterol ranging from 1 μM to 1,000 μM stimulated *P. sojae* mycelia equally well (data not shown). However, we were interested in determining the lowest concentration threshold required to stimulate *P. sojae* growth. Interestingly, *P. sojae* appears to be 10-fold more sensitive to stigmasterol than to cholesterol, as 10 nM of stigmasterol was the lowest concentration required to cause a significant (P<0.001) growth difference compared to controls (Fig. 2) vs. 100 nM needed for cholesterol to cause a similar response (Fig. 2).

Transcripts for both *SOJA* and *SOJB* elicitin genes were detected from cDNA generated from *P. sojae* mRNA, indicating both isoforms were expressed by the organism (Fig. 3). This was also confirmed by 2D-PAGE analysis of spent filtrates from control cultures (Fig. 4), which showed three protein bands with a molecular weight of approximately 10 kDa and pI's in the range of 3, 6, and 9. These proteins were confirmed to be elicitins of *P. sojae* after tryptic digestion and analysis of the individual bands using LC/ MS-MS) (data not shown).

By using a dilution series of cDNA from control cultures, we optimized PCR conditions to obtain equivalent amplification efficiencies for the target genes and the internal control gene used to normalize treatments. RT-PCR conditions for amplification of the targets (*SOJA* and *SOJB*) and internal reference gene (*ActA*) were optimized to yield respective amplification efficiencies of 104, 94, and 99% for the *SOJA, SOJB,* and *ActA* transcripts respectively. The amplification efficiency values for target genes fall within approximately 5% of the E value of the internal reference gene. This allowed the use of the $2_T^{-\Delta\Delta C}$ method (Livak and Schmittgen 2001) for the relative expression analysis of elicitin genes. Running the qPCR products on a 1.5% agarose gel showed the presence of a single band at approximately 300 bp, 250 bp, and 100 bp for *SOJ*A, *Act*A, and *SOJ*B, respectively (data not shown). The identity of the qPCR bands was confirmed by sequencing, and the characteristic melting temperatures for *SOJ*A, *SOJ*B, and *Act*A qPCR products were 90.5°C, 80.5°C, and 88.5°C, respectively.

Elicitin Gene Expression in Response to Stigmasterol and Cholesterol The class-I elicitin genes, *SOJA and SOJB*, are constitutively expressed in *P. sojae*, as transcripts for these genes were present in sterol untreated control samples. However, supplementing the growth medium of *P. sojae* with cholesterol or stigmasterol resulted in a differential expression profile of class-I elicitin genes compared to control samples. Generally, class-I elicitin genes became significantly (P<0.001) down-regulated over time compared to untreated controls (Fig. 3), but there appears to be a temporal delay in the down-regulation of *SOJA* and *SOJB* elicitin genes in response to cholesterol when compared to stigmasterol (Fig. 3). Specifically, elicitin genes became significantly (P<0.001) down-

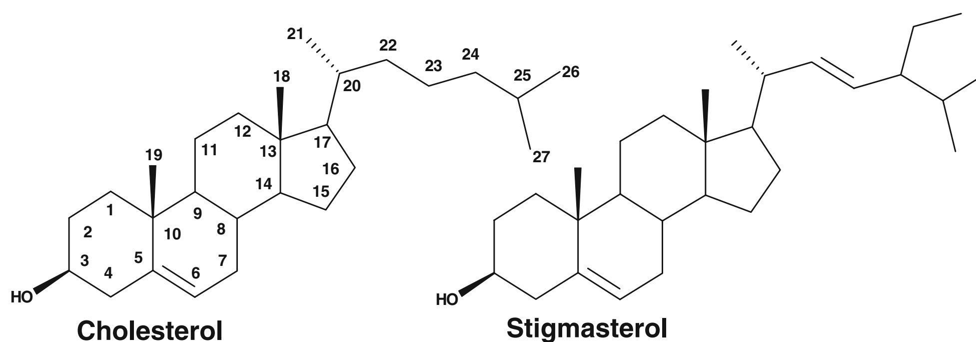

Fig. 1 Chemical structure of the sterols used to supplement *Phytophthora sojae* growth medium. Both sterols are structurally similar except that stigmasterol contains a trans-22 double bond and a 24-ethyl group in the side chain

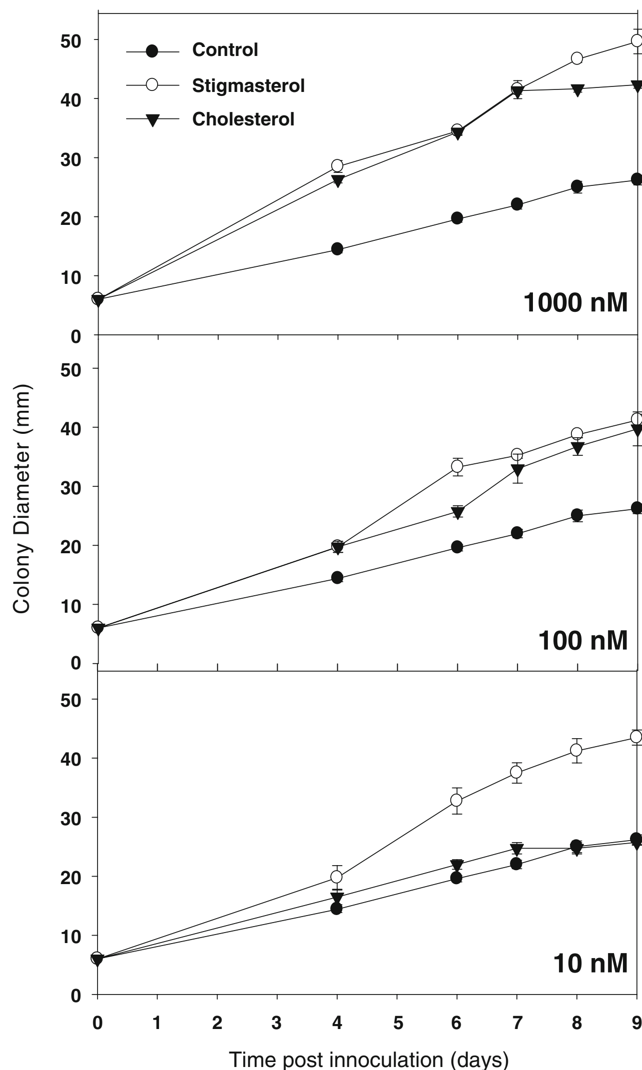

Fig. 2 *Phytophthora sojae* growth measurement (hyphal extension) over time after its growth medium was supplemented with a log-based gradient concentration of 1,000 nM, 100 nM, or 10 nM of either stigmasterol or cholesterol. Data plots represent three replicates ± SE from one experiment. ANOVA followed by a bonferroni pair-wise comparison of means identified significant differences (α=0.05, P< 0.001) in colony diameter measurements between the 10 nM stigmasterol treatment and control, and between the 10 nM stigmasterol and 10 nM cholesterol treatments. No significant difference was identified between the 10 nM cholesterol treatment and the control

regulated on day-9 in response to stigmasterol (Fig. 3), whereas a similar magnitude in elicitin down-regulation occurred on day-13 for cholesterol (Fig. 3).

Expression of class-I elicitin genes appears to be controlled by both negative and positive feedback mechanisms that are dependent on environmental sterol concentrations. An example of negative feedback that is dependent on sterol concentration is observed in the *SOJA* expression profile in response to stigmasterol (Fig. 3). On day-3, *SOJA* genes were down-regulated significantly in response to 1,000 nM of stigmasterol, whereas the down-regulation event was delayed and only observed on day-9 for the lower stigmasterol concentrations of 10 nM and 100 nM (Fig. 3). An example of positive feedback is observed on day-3 in the *SOJA* and *SOJB* expression profile in response to cholesterol (Fig. 3), and *SOJB* expression in response to stigmasterol (Fig. 3), in which the genes were significantly (P<0.001) up-regulated compared to untreated controls.

Extracellular Proteins in Spent Filtrates of P. sojae The total amount of secreted proteins in relation to dry biomass of *P. sojae* was determined over time (Table 2). Extracellular protein profiles were examined by 1D-PAGE analysis (Fig. 5). The goal was to determine if the down-regulation in *SOJA* and *SOJB* genes in response to sterols coincided with a reduced amount of secreted elicitin proteins in the spent filtrates of *P. sojae*. Elicitins are reported to be the most abundant proteins in filtrates of *Phytophthora* spp. (Ponchet et al. 1999). This is consistent with our results for *P. sojae*, as the only protein detected in spent filtrates was a single 10 kDa band that was confirmed as an elicitin of *P. sojae* by LC/MS-MS.

The amount of protein in the spent filtrates of control cultures continued to increase and reached a maximum concentration of 5 μg ml^{-1} on Day-13 (Table 2). The detection limit of the silver stain kit is 0.7 ng band^{-1}. Therefore, the concentration of proteins in spent filtrates prior to day 13 (1–2 μg ml^{-1}) was likely below the detection limit of the silver stain. The 10 kDa band detected from control filtrates on Day-13 cultures was absent in spent filtrates of the 1,000 nM cholesterol and stigmasterol

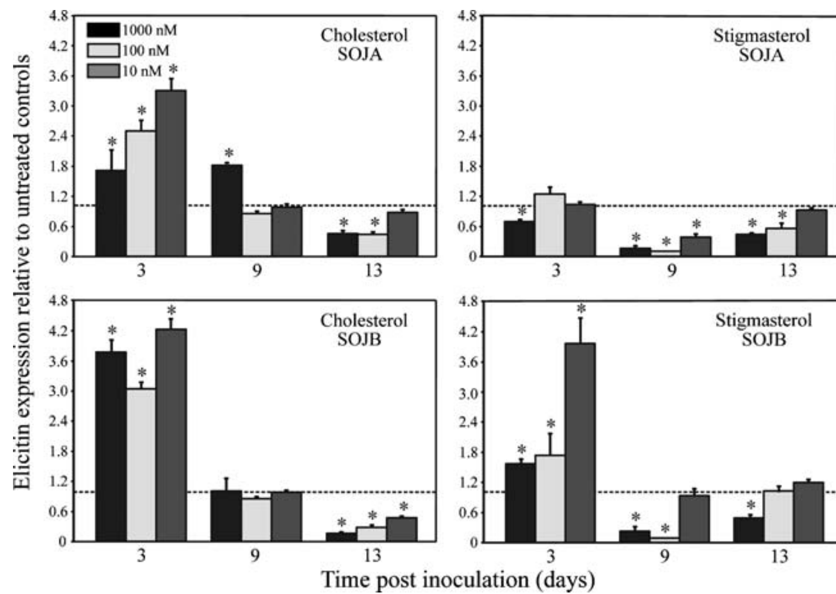

Fig. 3 *Phytophthora sojae* expression of class-I elicitin *SOJA and SOJB* genes was monitored over time in response to a log-based concentration gradient of 1,000–10 nM of cholesterol or stigmasterol using real-time RT-PCR. The y-axis shows expression relative to untreated controls (*P. sojae* not receiving sterol) after normalization to the internal control gene *Act*A(a value of 1.0 indicates no difference between treatment and control, a value greater than 1.0 indicates up-regulation of elicitin genes, and a value less than 1.0 indicates a down-regulation of elicitin genes). Bar graphs represent means of three technical replicates obtained from two biological replicates ± SE from two independent experiments. Bar graphs with a (*) indicate values significantly different from untreated controls after ANOVA followed by an LSD pair-wise comparison of means (α=0.05, P<0.001)

treatments (Fig. 5a) even though there was approximately 1.5 times more biomass in the same volume of experimental media compared to controls (Table 2). The reduced amount of proteins in the sterol treated samples (Fig. 5a) is supported by an analysis of the total amount of protein produced per biomass of *P. sojae* (Table 2). On

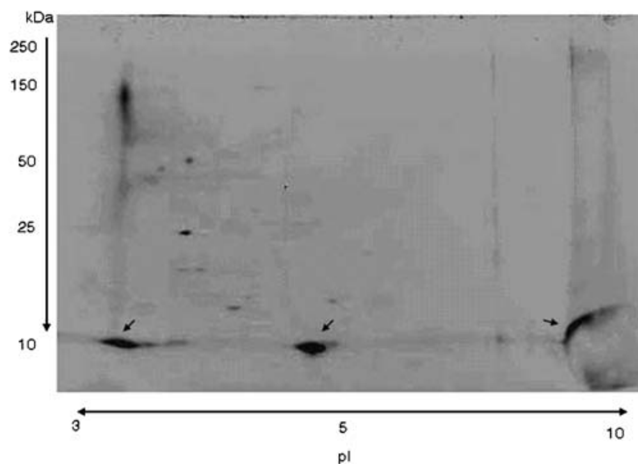

Fig. 4 Two-dimensional SDS-PAGE analysis of *Phytophthora sojae* extracellular proteins (25 µg) present in filtrates after 13 days of growth in a minimal medium in the absence of sterols. Proteins were separated according to their pI (x-axis) and size (y-axis). Arrows point to three putative elicitin isoforms with approximate pI values of 3, 5, and 9, and a molecular weight of 10 kDa. The identity of the proteins was later confirmed to be *P. sojae* elicitins by LC/MS/MS

Day-13, only 0.2 µg mg^{-1} protein was produced in the presence of 1,000 nM stigmasterol and 1.1 µg mg^{-1} for the same concentration of cholesterol compared to a higher amount (i.e., 4.2 µg mg^{-1}) produced by control cultures (Table 2).

The 10 kDa elicitin band was not detected in *P. sojae* spent filtrates treated with stigmasterol over the entire concentration range of 1,000 nM to 10 nM (Fig. 5), whereas it was detected when a cholesterol concentration of 100 nM (Fig. 5b) or 10 nM was used (Fig. 5c). Moreover, the staining intensity of the 10 kDa protein band reached levels similar to controls at the lowest cholesterol concentration (i.e., 10 nM) (Fig. 5c). This also is supported by the lack of differences in the amount of proteins produced per biomass between control cultures and those treated with 10 nM cholesterol (Table 2). This observation is in agreement with the biological dose response assays we conducted that showed the growth of *P. sojae* is more sensitive to stigmasterol compared to cholesterol (Fig. 2).

Discussion

Phytophthora sojae is a hemibiotroph that can survive in the soil as a saprotroph or as a heterotroph in the soybean host (Tyler 2007). In soil, the concentration of sterols varies between 0.2 ppm to 30 ppm and depends on the presence of

Table 2 Total proteins in spent filtrates of *phytophthora sojae* in response to stigmasterol and cholesterol over time

Treatment	Biomass (mg)	Secreted protein (μgml^{-1})	Protein/Biomass** (μgmg^{-1})
Sample Day 3			
Control	6.5 a	1.1	3.4 a
Stigmasterol 1,000 nM	9.4 b	ND*	ND
Stigmasterol 100 nM	7.7 a,b	ND	ND
Stigmasterol 10 nM	8.1 a,b	ND	ND
Cholesterol 1,000 nM	7.7 a,b	ND	ND
Cholesterol 100 nM	7.3 a,b	ND	ND
Cholesterol 10 nM	6.9 a,b	0.5	1.2 b
Sample Day 9			
Control	12.3 a	2.1	3.4 a
Stigmasterol 1,000 nM	18.4 b	ND	ND
Stigmasterol 100 nM	16.5 b	1.4	1.7 a
Stigmasterol 10 nM	17.5 b	1.9	2.3 a
Cholesterol 1,000 nM	17.1 b	ND	ND
Cholesterol 100 nM	18.4 b	2.1	2.3 a
Cholesterol 10 nM	15.4 a,b	2.5	3.3 a
Sample Day 13			
Control	24.3 a	5.0	4.2 a
Stigmasterol 1,000 nM	43.2 b	0.4	0.2 b
Stigmasterol 100 nM	34.1 b	2.3	1.4 b,c
Stigmasterol 10 nM	32.9 b	2.9	1.8 b,c
Cholesterol 1,000 nM	29.2 a,b	1.6	1.1 b,c,d
Cholesterol 100 nM	30.4 a,b	3.3	2.2 c,d
Cholesterol 10 nM	26.6 a	4.3	3.3 a,d

Values followed by the same letter are not significantly different from each other using ANOVA followed by a Bonferroni comparison of means ($P < 0.05$)

*ND indicates no protein detected

**Protein per biomass was calculated according to 20 ml of total experimental volume

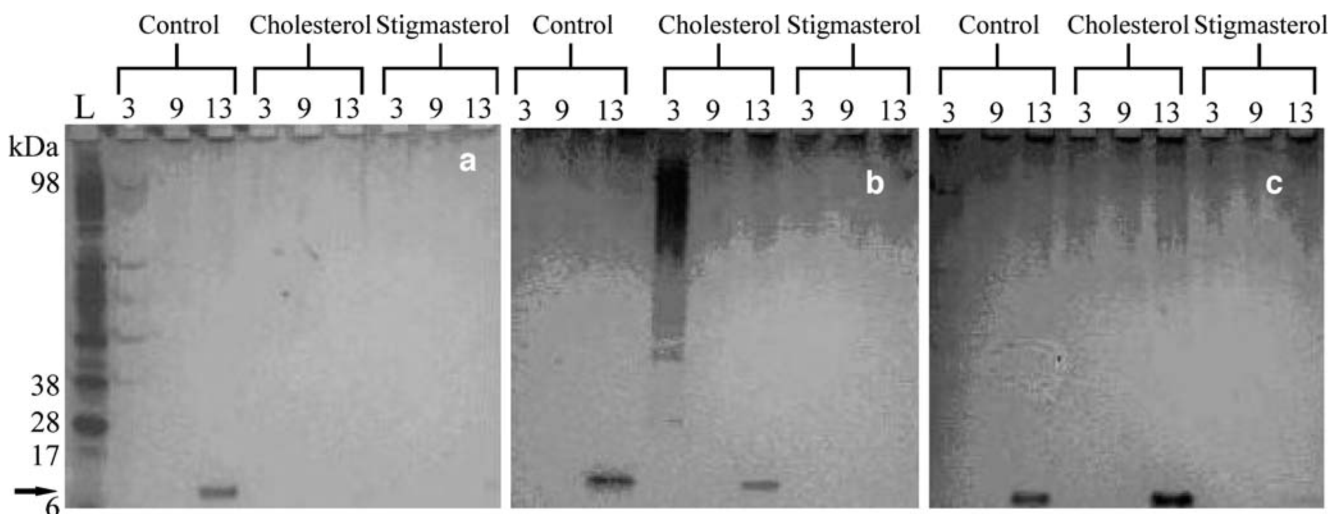

Fig. 5 Extracellular proteins present in filtrates of *Phytophthora sojae* grown in a minimal medium after 3 days, 9 days, or 13 days of growth in the absence of sterol (control), in the presence of 1,000 nM (**A**), 100 nM (**B**), or 10 nM (**C**) of either cholesterol or stigmasterol. The letter L shows standard protein markers with an approximate size range of 98 kDa to 6 kDa (SeeBlue Plus2 pre-stained standard, Invitrogen), whereas the numbers 3, 9, and 13 indicate days post inoculation of *P. sojae* into the experimental media. Spent filtrates (15 μl) containing a maximum amount of 75 ng total protein (e.g., control day 13) were separated on a 4–12% MES-SDS-PAGE gel and stained with silver nitrate. The arrows point to putative elicitin bands with a molecular size of approximately 10 kDa

soil organisms, plant matter, and organic fertilizer inputs (Puglisi et al. 2003). In soybean, sterol concentrations depend on the physiological stage of plant development, and vary between approximately 0.0002–0.005% of plant weight (Fenner et al. 1986). In this study, we evaluated mycelial extension and class-I elicitin gene expression of *P. sojae* in response to varying concentrations of stigmasterol and cholesterol. Mycelial extension of *P. sojae* was stimulated at nanomolar concentrations in response to both sterols, but was 10-times more sensitive to stigmasterol than to cholesterol. These results are significant and show that *P. sojae* (1) is exquisitely sensitive to the presence of sterols, responding to them at very low concentrations, and (2) apparently can discriminate between sterols with small structural differences. Our results are different from other sterol studies with *Phytophthora* (Wood and Gottlieb 1978; Nes and Stafford 1983; Marshall et al. 2001) because we utilized sterol supplement concentrations that are approximately 250 or 2,500 times lower than the lowest concentration of sterol supplements used in other studies to examine a physiological response. The ability of *P. sojae* to respond to sterols at nanomolar concentrations indicates that it is efficient in utilizing sterols, even at low concentrations in the environment.

Studies involving *P. parasitica* (pepper pathogen) (Colas et al. 2001) and *P. infestans* (potato pathogen) (Kamoun et al. 1997b) reported that the expression of elicitin genes in these organisms was down-regulated during plant infection. Colas et al. (2001) and Kamoun et al. (1997b) did not consider sterols as potential regulators of elicitin gene expression in *P. parasitica* or *P. infestans*. In our study, class-I elicitin gene expression in *P. sojae* appears to be controlled by both negative and positive feedback mechanisms that are dependent on sterol concentration and structure. Generally, a reduction in elicitin biosynthesis was observed when either stigmasterol or cholesterol was included in the growth medium of *P. sojae*. However, differences in the expression profile of *SOJA* and *SOJB* elicitin genes was observed in response to each sterol, which may be related to different binding efficiencies for these two sterols with elicitins encoded by the *SOJA* and *SOJB* genes.

Overall, cholesterol was less effective than stigmasterol in down-regulating elicitin expression since the kinetics of down-regulation by cholesterol were slower (Fig. 3). Moreover, the up-regulation of class-I elicitin genes in response to cholesterol on day-3 (Fig. 3) may have occurred to compensate for the slow kinetics of any of (1) elicitin binding with cholesterol, (2) elicitin-sterol complex binding to receptors on *P. sojae* membranes, or (3) metabolism of sterol once incorporated into cells. Following the same argument, the up-regulation of *SOJB,* but not *SOJA* elicitin genes, in response to stigmasterol may have occurred because the basic class-I elicitins encoded by the *SOJB* gene are less efficient in stigmasterol uptake than the acidic class-I elicitin proteins. A different study that involved the soilborne pathogen *P. cactorum* showed that the organism incorporated sitosterol into its mycelium more rapidly than it did cholesterol when a mixture of these two sterols was present in the growth medium (Elliot and Knights 1981), i.e., a mechanism involving elicitins maybe at play. Interestingly, a common observation made between this study and that of Elliot and Knights (1981) is both *P. sojae* and *P. cactorum* are plant pathogens that appear to process plant sterols (stigmasterol and sitosterol) more efficiently over cholesterol —a predominantly animal sterol. It maybe possible that *Phytophthora* plant pathogens have evolved to preferentially utilize sterols commonly present in their host.

In summary, our study shows that class-I elicitin genes of *P. sojae* are regulated by nanomolar concentrations of sterols, and that an apparent differential in temporal gene regulation occurs in response to small structural differences in sterol type (stigmasterol vs. cholesterol). Nonetheless, these results indicate that elicitins play a role in sterol uptake in *P. sojae*. It remains to be determined whether sterols act as diffusible factors that connect elicitin genes to the environment, or whether down-stream mechanisms with putative *P. sojae* elicitin receptors on membranes are involved.

Acknowledgements This work was supported by an Alumni Grant for Graduate Research and Scholarship from the Ohio State University (first author) and the USDA National Needs Graduate Fellowship Program. We appreciate the generosity of Dr. Anne Dorrance for providing the *Phytophthora sojae* isolate. The first author appreciates advice received from Dr. Terry Graham.

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