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ASPECTOS MOLECULARES DA INTERAÇÃO PATOGÊNICA ENTRE *Sclerotinia sclerotiorum* E *Phaseolus vulgaris* L.

MARÍLIA BARROS OLIVEIRA

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MARÍLIA BARROS OLIVEIRA

ASPECTOS MOLECULARES DA INTERAÇÃO PATOGÊNICA ENTRE *Sclerotinia sclerotiorum* E *Phaseolus vulgaris* L.

Tese apresentado ao Programa de Pós-Graduação em Ciências Biológicas do Instituto de Ciências Biológicas da Universidade Federal de Goiás, como requisito parcial para obtenção do título de Doutor em Ciências Biológicas.

Orientadora: Prof. Dra Silvana Petrofeza da Silva

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ATA DA SESSÃO PÚBLICA DE DEFESA DE TESE DE Nº 46

Aos vinte e seis dias do mês de fevereiro do ano de dois mil e dezesseis, às oito horas e trinta minutos, no Anfiteatro do Instituto de Ciências Biológicas III da Universidade Federal de Goiás, reuniram-se os componentes da banca examinadora: Profa. Dra. Silvana Petrofeza da Silva, Prof. Dr. Marcos Gomes Cunha, Profa. Dra. Rosana Pereira Vianello, Prof. Dr. Hyrandir Cabral de Melo, e Prof. Dr. Murillo Lobo Júnior para, em sessão pública presidida pela primeira examinadora citada, procederem à avaliação da defesa de tese intitulada "ASPECTOS MOLECULARES DA INTERAÇÃO PATOGÊNICA ENTRE *Sclerotinia sclerotiorum* E *Phaseolus vulgaris* L.", em nível de Doutorado, área de concentração em Bioquímica e Genética, de autoria de **Marília Barros Oliveira**, discente do Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Goiás. A sessão foi aberta pela presidenta, que fez a apresentação formal dos membros da banca. A palavra, a seguir, foi concedida à autora da tese que, em cerca de 50 minutos, procedeu à apresentação de seu trabalho. Terminada a apresentação, cada membro da banca arguiu a examinada, tendo-se adotado o sistema de diálogo sequencial. Terminada a fase de arguição, procedeu-se à avaliação da tese. Tendo-se em vista o que consta na Resolução nº1340 de 2015 do Conselho de Ensino, Pesquisa, Extensão e Cultura (CEPEC), que regulamenta o Programa de Pós-Graduação em Ciências Biológicas, a tese foi aprovada, considerando-se integralmente cumprido este requisito para fins de obtenção do título de Doutor em Ciências Biológicas pela Universidade Federal de Goiás. A conclusão do curso dar-se-á quando da entrega da versão definitiva da tese na secretaria do programa, com as devidas correções sugeridas pela banca examinadora, no prazo de trinta dias a contar da data da defesa. Cumpridas as formalidades de pauta, às 13 horas e 00 minutos, encerrou-se a sessão de defesa e, para constar, eu, Renato César Rodrigues,

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Assistente em Administração da Secretaria de Pós-graduação do Instituto de Ciências Biológicas da Universidade Federal de Goiás, lavrei a presente ata que, após lida e aprovada, será assinada pelos membros da banca examinadora em três vias de igual teor.

Profa. Dra. Silvana Petrofeza da Silva
Universidade Federal de Goiás

Prof. Dr. Marcos Gomes Cunha
Universidade Federal de Goiás

Profa. Dra. Rosana Pereira Vianello
Embrapa Arroz e Feijão

Prof. Dr. Hyrandir Cabral de Melo
Universidade Federal de Goiás

Prof. Dr. Murillo Lobo Júnior
Embrapa Arroz e Feijão

Aos meus pais, Dioripes e Isvaldeni, que sempre acreditaram que seria possível conseguir um futuro melhor.

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RESUMO

O mofo branco causado pelo fungo *Sclerotinia sclerotiorum* (Lib.) de Bary é uma das doenças mais devastadoras do feijoeiro comum (*Phaseolus vulgaris* L.) e de outras culturas. Este trabalho teve como objetivo obter uma melhor compreeensão do patossistema *S. sclerotiorum-P. vulgaris* identificando os genes potencialmente envolvidos durante essa interação. Duas diferentes abordagens foram utilizadas para avaliar esse patossistema: 1) cultivar suscetível Pérola (BRS) de *P. vulgaris*; e 2) tratamento dessa cultivar com o indutor de resistência metil jasmonato (MeJA). Para as duas situações, a metodologia de biblioteca de cDNA construída a partir da hibridização subtrativa por supressão (HSS) foi usada para identificar genes diferencialmente expressos no patógeno e na planta hospedeira. Na interaçao entre a cultivar suscetível e *S. sclerotiorum* 979 *unigenes* (430 *contigs* e 549 *singletons*) foram identificados, sendo que 29% desses *unigenes* foram categorizados como genes do fungo, relacionados à degradação de parede celular, degradação de proteínas, estresse oxidativo dentre outros. E 58,3 % foram categorizados como genes de plantas relacionados a metabolismo energético, degradação de proteínas e resposta a estresse biótico e abiótico. A seleção dos clones diferenciais das respectivas bibliotecas HSS foi validada por transcrição reversa e PCR quantitativa em Tempo Real (RT-qPCR). Foram validados genes relacionados à patogenicidade e fatores de virulência de *S. sclerotiorum*, como: genes que codificam para enzimas que degradam parede celular da planta (poligalacturonases, celulases, hemicelulases and proteases). E além desses, genes relacionados à defesa da planta hospedeira como: genes relacionados à patogenicidade (*PR1*, *PR2*, e *PR3*), via dos fenilpropanoides (*Isof*, *FPS1* e *4CL*), e genes envolvidos em defesa ou relacionados à estresse (*Lox*, *Hiprp*, *GST*, *POD* e *DOX*). A aplicação exógena de MeJA induziu resistência parcial contra *S. sclerotiorum* em plantas e determinou um consistente aumento na atividade de proteínas relacionadas à patogênese. Além disso, a validação da HSS feita por RT-qPCR demonstrou a expressão diferencial de importantes *unigenes* ao longo do tempo, tais como: proteínas relacionadas à patogênese *PR3* (quitinase classe 1), *PvCallose* (Calose sintase), *PvNBS-LRR* (NBS-LRR resistance-like protein), *PvF-box* (F-box family protein-like) e proteínas inibidoras de poligalacturonases (*Pvpgip1-4*). Os dados obtidos neste estudo permitiram evidenciar alguns dos mecanismos envolvidos na patogenicidade do

fungo, bem como as respostas de defesa produzidas pela planta nas duas condições avaliadas, contribuindo assim para uma melhor compreensão do patossistema.

Palavras-Chave: Expressão gênica, feijão comum, hibridização subtrativa por supressão, mofo branco, transcritos induzidos por estresse.

ABSTRACT

White mold caused by *Sclerotinia sclerotiorum* (Lib.) De Bary is one of the most devastating diseases of common bean (*Phaseolus vulgaris* L.) and others crops. This study aimed to achieving a better understanding of the pathosystem *S. sclerotiorum*-*P. vulgaris* identifying genes potentially involved in this interaction. Two different approaches were used to evaluate this pathosystem: 1) susceptible cultivar Pérola (BRS) of *P. vulgaris*; and 2) treatment of this cultivar with the resistance inducer methyl jasmonate (MeJA). For both situations, a suppression subtractive hybridization (SSH) cDNA library approach was used to identify differentially expressed genes in the pathogen and the host plant. In the interaction between the susceptible cultivar and *S. sclerotiorum* 979 unigenes (430 contigs and 549 singletons) were inentified with 29% of these unigenes were categorized as fungal genes, related to cell wall degradation, proteins degradation, oxidative stress among other. And 58,3% were categorized as plant genes related to energetic metabolism, protein degradation, response to biotic and abiotic stress. The selection of differential clones of their libraries SSH has been validated by reverse transcription and quantitative real-time PCR (RT-qPCR). Were validated genes related to pathogenicity and virulence factors of *S. sclerotiorum*, such as genes encoding enzymes that degrade plant cell walls (polygalacturonases, cellulases, hemicellulases and proteases). And besides these, defense-related genes of the host plant as: pathogenesis-relateds genes (*PR1*, *PR2*, and *PR3*), phenylpropanoid pathway (*Isof*, *FPS1*, and *4CL*), and involved in defense and stress-related categories (*Lox*, *Hiprp*, *GST*, *POD*, and *DOX*). Exogenous MeJA application induced partial resistance agains *S. sclerotiorum* in plants as well as a consistent increase in pathogenesis-related protein activities. Furthermore, the validation of SSH made by RT-qPCR showed differential expression of important unigenes over time, such as: pathogenesis related protein *PR3* (chitinase class I), *PvCallose* (callose synthase), *PvNBS-LRR* (NBS-LRR resistance-like protein), *PvF-box* (F-box family protein-like), and a polygalacturonase inhibitor protein (PGIP). The data obtained in this study have highlighted some of the mechanisms involved in the pathogenicity of the fungus, as well as defense responses produced by the plant in two conditions evaluated, thus contributing to a better understanding of pathosystem.

Keywords: Common bean, gene expression, suppression subtractive hybridization, stress-induced transcripts, white mold.

1. INTRODUÇÃO

O feijão comum (*Phaseolus vulgaris* L.) é uma cultura de grande importância social, sendo a leguminosa mais consumida, fornecendo até 15% do total de calorias diárias e 36% de proteínas totais diária em parte da África e das Américas (FAO).

O Brasil é um dos o maiores produtores de feijão comum do mundo, entretanto essa cultura sofre muitas perdas devido a estresses bióticos e abióticos. Dentre as principais doenças que acometem a cultura do feijoeiro nos dias atuais, está o mofo branco, causado pelo fungo habitante de solo *Sclerotinia sclerotiorum* (Lib.) de Bary. Nas últimas safras, essa doença, tem causado prejuízos significativos aos produtores brasileiros com bastante intensidade em vários estados tais como Paraná, Goiás, Mato Grosso do Sul e Minas Gerais onde a presença da doença foi detectada com frequência na cultura do feijoeiro.

Atualmente os métodos de controle para este patógeno incluem práticas culturais, como a rotação com gramíneas e controle de irrigação, estratégia de escape à doença como uso de cultivares de porte ereto, cobertura do solo, aplicação de fungicidas e controle biológico, que podem reduzir a severidade da doença (Ando et al., 2007; Görgen et al., 2009; Lobo Júnior et., 2009).

As técnicas moleculares têm dado suporte para compreenção dos mecanismos que regem a interação fungo-planta. O controle da expressão gênica ocorre em vários níveis, e compreender como a expressão individual de genes contribui na resposta final em termos celulares, fisiológico e agronômicos pode auxiliar no desenvolvimento de estratégias para o controle de patógenos. Nesse contexto, o estudo da expressão gênica diferencial é de extrema importância, pois possibilita a identificação de genes-chave por meio da comparação da expressão gênica durante o desenvolvimento da planta sob condições normais ou quando submetida a algum tipo de estresse.

A identificação diferencial de genes encontra uma poderosa ferramenta na tecnologia de hibidização subtrativa por supressão (HSS, Diatchenko et al*.,* 1996). As bibliotecas geradas a partir dessa técnica têm enriquecido os bancos de dados e gerado informações importantes a respeito do processo de patogenicidade de muitos fungos. As etiquetas de sequências expressas (ESTs) são sequências parciais de genes transcritos e representam a expressão gênica em diferentes tecidos ou genótipos, muitas vezes dependendo da planta, tratamento e estágio de desenvolvimento em que o mRNA foi extraído.

Vários trabalhos em feijão comum têm-se valido da análise de transcriptoma por HSS para encontrar genes diferencialmente regulados sob estresse, levando à caracterização de pontos chaves nas respostas de defesa. A exemplo disso, algumas bibliotecas de ESTs foram desenvolvidas para identificar as respostas a estresse abiótico (Ramirez at al., 2005; Blair et al., 2011), assim como genes envolvidos durante interação patogênica contra *Colletotrichum lindemuthianum* (Melotto et al., 2005; Oblessuc et al., 2012), *Rhizoctonia solani* (Guerrero-González et al., 2011) e *Uromyces appendiculatus* (Thibivilliers et al., 2009).

Diante desse cenário e buscando encontrar soluções que possam contribuir para a redução dos danos ocorridos pelo mofo branco, o presente trabalho teve como objetivo compreender os mecanismos moleculares do patossistema *P.vulgaris-S. sclerotiorum,* identificando os genes potencialmente envolvidos nessa interação.

1.1. O mofo branco

O Brasil destaca-se mundialmente como um dos maiores produtores de feijão comum, que constitui o alimento protéico básico da dieta do povo brasileiro. Para atender a essa demanda, o feijão comum é plantado durante todo o ano em três etapas: safra das águas, safra da seca e safra irrigada. Na safra 2015, foram produzidos 3,1 milhões de toneladas de feijão comum em uma área de 3 milhões de hectares, com uma produtividade média nacional de 1.020 Kg/ha (Conab 2015).

O Estado de Goiás é o quinto maior estado produtor de feijão, respondendo a cerca de 10% da produção nacional, o que representou 314.800 toneladas, em 2015 (Conab 2015). Esta produção está concentrada nas épocas de semeadura das águas, correspondendo aos meses de outubro a dezembro (40%), e de inverno, abril a julho (49%).

O cultivo do feijão no inverno sob irrigação, em especial com o uso de pivô central, gera um microclima favorável ao desenvolvimento de doenças como, por exemplo, o mofo branco. Essa doença é uma das mais destrutivas do feijoeiro comum, capaz de causar perdas de até 100%, quando medidas preventivas eficazes não são tomadas (Singh e Schwartz, 2010). A doença é causada pelo fungo habitante de solo *Sclerotinia sclerotiorum* (Lib.) de Bary. O primeiro registro dessa doença afetando a cultura de feijoeiro no Brasil foi em 1954 no estado do Rio Grande do Sul. Posteriormente, foi detectado no Paraná na década de 70, e na região dos cerrados na década de 80 (Lobo Júnior, 2011). Mundialmente esse

parasita está distribuído por todos os continentes, sendo relatado na África do Sul, Austrália, Bulgária, Canadá, China, Coréia, Estados Unidos da América, México, Nova Zelândia, Nigéria, Portugal, Romênia, Taiwan e outros (Farr e Rosman, 2010).

Desde os anos 80, com a implementação do sistema de irrigação por pivô central, o mofo branco é um dos fatores limitantes na produção de feijão no cerrado brasileiro (Nasser, et al., 1990). Os sintomas dessa doença geralmente aparecem nas culturas de feijoeiro logo após a época de floração, em reboleiras com alta densidade de plantas. Com o progresso da doença, obsevam-se manchas encharcadas em folhas, caules e vagens para posteiormente chegar numa massa de crescimento micelial que cobre os tecidos. Finalmente os escleródios são formados dentro e fora dos tecidos infectados tornando-os quebradiços (**Figura 1**) (Kimati et al., 2005).

Figura 1. Mofo branco em feijoeiro. (A) vagens afetadas. **(B)** e **(C)** escleródios nos tecidos vegetais. **(D)** Apotécios. Fonte: Embrapa Arroz e Feijão.

1.1.1. O agente causador da doença

Sclerotinia sclerotiorum (Lib.) de Bary é um fungo pertencente ao filo Ascomycota, classe Leotimycetes, ordem Helotiales, família Sclerotiniaceae (Ruggiero et al., 2012). Este fitopatógeno é polífago e um dos mais destrutivos, apresentando baixa especificidade de hospedeiro. Ataca mais de 400 espécies ou variedades de plantas, sendo a maioria plantas herbáceas da subclasse dicotiledônea e de grande importância econômica como: girassol, soja, tomate, amendoim, fumo, entre outras (Boland e Hall, 1994).

Esse patógeno sobrevive principalmente na forma de escleródios que são estruturas compactas, melanizadas podendo sobreviver por longos períodos de tempo sob condições adversas. Essa propriedade é essencial para a sobrevivência, propagação e disseminação do fungo, já que encontrando condições favoráveis como alta umidade e temperatura baixa poderá dar início à sua germinação. O enorme potencial reprodutivo, juntamente com a capacidade de sobrevivência a longo prazo, fazem dos escleródios componentes centrais na epidemiologia de *S. sclerotiorum.* Os escleródios podem germinar de duas formas: carpogênica ou miceliogênica, dependendo das condições ambientais, resultando em duas formas distintas desta doença. A forma miceliogênica produz hifas que podem atacar diretamente os tecidos das plantas. A forma carpogênica produz apotécios e posteriormente ascósporos que infectam partes de plantas hospedeiras (**Figura 2**) (Bardin e Huang, 2001).

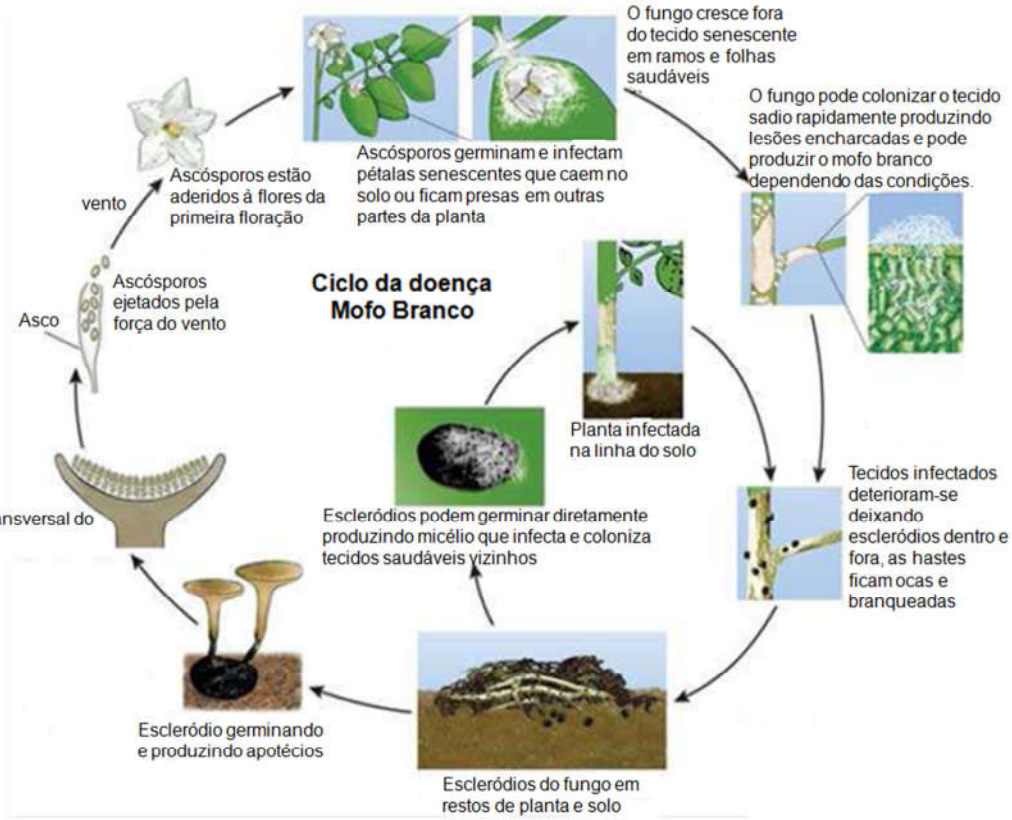

Figura 2: Esquema do ciclo da doença mofo branco. São mostradas as fases carpogênicas e miceliogênicas de propagação. Fonte: Adaptada de www.potatodiseases.org.

De acordo com o estilo de vida, patógenos de plantas podem ser divididos em necrotróficos e biotróficos (Glazebrook, 2005), embora não haja uma fronteira estrita entre estes grupos. Os necrotróficos destroem as células hospedeiras, muitas vezes através da produção de fitotoxinas e se alimentam do seu conteúdo. Os biotróficos penetram ou estabelecem contato com as células hospedeiras, crescem e se reproduzem, nutrindo-se do tecido vivo do hospedeiro. Muitos patógenos apresentam os dois estilos de vida dependendo do estágio do seu ciclo vital, e são chamados hemibiotróficos (Pieterse et al., 2009).

S. sclerotiorum é necrotrófico, capaz de se alimentar de células mortas (Boland e Hall, 1994). Dois principais fatores de patogenicidade, secreção de ácido oxálico e enzimas hidrolíticas, agem em concerto na maceração de tecidos vegetais e geração de necrose. A degradação de componentes da parede celular da planta está ligada à produção de uma ampla e complexa variedade de enzimas líticas, como celulases, hemicelulases, pectinases e proteases. Sequencialmente à secreção pelo fungo, enzimas líticas não só facilitam a penetração, colonização e

maceração, mas também geram uma importante fonte de nutrientes para o patógeno (Bolton et al., 2006).

1.1.2. Interação *S.sclerotiorum*-planta: fatores de patogenicidade

1.1.2.1. Ácido Oxálico

Durante o processo de infecção, o pH neutro ou ligeiramente alcalino do tecido vegetal estimula a síntese de oxalato pelo fungo *S. sclerotiorum*, resultando em uma acidificação do meio extracelular (Rollins e Dickman, 1998). A síntese e a secreção de oxalato têm como determinante o pH do meio via um fator de transcrição conhecido como *PacC* (Rollins e Dickman, 2001), tendo seu homólogo *pac1*, em *S. sclerotiorum.* Esse fator de transcrição é necessário para o seu crescimento em pH neutro, patogenicidade, produção de ácido oxálico e produção normal de escleródios.

S. sclerotiorum, assim como outros fungos, produzem ácido oxálico a partir do oxalato em uma reação catalizada pela enzima oxalato acetilhidrolase (OAH). Análises em nível de transcrição, demonstraram que o gene que codifica para essa enzima é expresso durante o crescimento vegetativo e infecção de *S. sclerotiorum* em *Brassica napus* (Sexton et al., 2006) e em *P. vulgaris* (Oliveira et., 2013). Assim, o acúmulo de ácido oxálico e a consequente redução do pH são descritos como responsáveis pela morfogênese e virulência de *S. sclerotiorum* (Rollins, 2003; Bueno et al., 2012; Liang et al., 2014).

Vários mecanismos descrevem o ácido oxálico como um fator chave na patogênese de *S. sclerotiorum:*

(a) Enzimas hidrolíticas secretadas pelo fungo durante a invasão de tecidos vegetais têm atividade máxima em pH ácido, assim, a redução do pH provocada pela secreção do ácido oxálico favorece a degradação enzimática da parede celular das plantas (Marciano et al., 1983).

(b) Por ser tóxico para as células hospedeiras, devido à sua acidez, o ácido oxálico enfraquece a planta facilitando a invasão de patógenos (Noyes e Hancock 1981).

(c) O ácido oxálico é um quelante de íons Ca^{2+} do apoplasto. Assim, desestabiliza a parede celular, além de comprometer respostas de defesa da planta dependentes de Ca^{2+} (Bateman e Beer, 1965).

(d) Suprime o estresse oxidativo que constitui importante resposta de defesa para muitas espécies de planta (Cessna et al., 2000).

(e) Diminui a afinidade da ligação entre poligalacturonases (PGs) do fungo com inibidores de poligalacturonases do hospedeiro (Favaron et al*.,* 2004).

(f) Dispara a morte celular programada nos tecidos da planta hospedeira (Kim et al., 2008).

Vários trabalhos demonstraram que a secreção de ácido oxálico é determinante para a patogênese em *S. sclerotiorum* (Marciano et al., 1983; Godoy et al., 1990; Rollins e Dickman, 2001; Williams et al., 2011). Mutantes de *S. sclerotiorum* que não produziam ácido oxálico foram incapazes de colonizar tecidos da planta hospedeira (Godoy et al*.,* 1990). Além disso, plantas de soja transgênicas capazes de eliminar oxalato, através da atividade da enzima oxalato oxidase, foram resistentes a este patógeno (Donaldson et al., 2001).

Williams et al., (2011) demonstraram que mutantes deficientes na produção de ácido oxálico, além de não patogênicos, disparavam uma série de respostas de defesa na planta, como respostas de hipersensibilidade, deposição de calose e indução de estresse oxidativo. Por outro lado, *S. sclerotiorum* que secretava ácido oxálico suprimiu todas estas respostas com uma completa ausência de estresse oxidativo durante os estágios iniciais do crescimento do fungo e desenvolvimento da doença. Dessa maneira, foi sugerido que o ácido oxálico, evita o reconhecimento do fungo pela planta.

1.1.2.2. Enzimas que hidrolisam a parede celular da planta hospedeira.

A habilidade de degradar a parede celular da planta é um importante fator de patogenicidade para os fungos necrotróficos. Os carboidratos da parede celular vegetal formam uma rede complexa de diferentes polissacarídeos que são divididos em três categorias: celulose, hemicelulose (incluindo xilana, xiloglicanas, glicogalactonanana, galactana e respectivas cadeias laterais), e pectina (composta de galacturana, ramagalacturama, e suas respectivas dadeias laterais) (Amselem et al., 2011). Esta rede de carboidratos é alvo de enzimas e proteínas auxiliares

necessárias para acessar o tecido interno da planta e degradar os componentes da parede celular para monômeros simples que servem como fontes de carbono.

Várias enzimas que degradam parede celular da planta são produzidas por *S. sclerotiorum* durante a infecção (Marciano et al*.,* 1983 e Riou et al*.,* 1991). Dentre essas enzimas estão incluídas: pectinases, β-1,3-glicanases, glicosidases, celulases, xilanases, cutinases e proteases (Lebeda et al*.,* 2001). O genoma de *S. sclerotiorum* contem, pelo menos, 118 genes que codificam para enzimas associadas com a degradação da parede celular vegetal (Amselem et al., 2011).

A hidrólise da pectina enfraquece a parede celular e facilita a penetração e colonização da planta hospedeira. O fungo *S. sclerotiorum* produz formas variadas de enzimas pectinolíticas: exo e endo-poligalacturonases (PGs), pectina metil esterase, pectina liase e acetil esterase (Bolton et al., 2006). Dentre essas, as PGs têm recebido particular atenção. A atividade total dessas enzimas é aumentada com a liberação de ácido oxálico durante a infecção da planta. Tal efeito é particularmente evidenciado quando o pH está baixo, ideal para a atividade das PGs (Favaron et al., 2004; Oliveira et al., 2013).

As poligalacturonases (EC 3.2.1.15) são as primeiras enzimas a serem secretadas por patógenos necrotróficos durante a degradação da parede celular da planta. Sua contribuição para a patogenicidade foi demonstrada, por exemplo, com a deleção do gene *Bcpg2,* que codifica uma isoforma de PG em *Botrytis cinérea,* resultou na redução da virulência deste fungo em plantas tomate e feijoeiro (Kars et al., 2005).

As endo-poligalacturonases (endo-PGs) atuam clivando as ligações α(1-4) entre resíduos de ácido D-galacturônico em homogalacturona, causando a separação e maceração do tecido do hospedeiro. Já as exopoligalacturonases (exo-PGs) clivam grupos glicosil monoméricos ou diméricos dos polissacarídeos pécticos da parede celular. Um maior interesse é dado às endo-PG as quais são capazes de macerar os tecidos da planta promovendo a liberação de oligossacarídeos que são potentes sinalizadores celulares e podem ativar a resposta de defesa da planta hospedeira (Benito, et al., 1998).

Em *S. sclerotiorum*, a análise de ESTs (Li et al., 2004a) identificou distintos cDNAs (*sspg1, sspg3, sspg5* e *sspg6)* codificando para quatro endo-PGs e duas exo-PGs (*ssxpg1* e *ssxpg2*). Esses genes foram diferencialmente expressos durante a patogênese (Cotton et al*.,* 2002; Kasza et al., 2004; Li et al*.,* 2004b, Oliveira et al.,

2010). Por exemplo, trabalho realizado em hastes de feijoeiro durante infecção de *S. sclerotiorum* demonstrou que os genes *Sspg1* e *Sspg5* foram altamente expressos durante a fase inicial de colonização, enquanto que *Sspg6* foi fracamente expresso durante esse processo (Oliveira et al., 2010).

Após a despolimerização da pectina e o consequente enfraquecimento da parede celular, outros polímeros, como celulose e hemicelulose, são expostos aumentando a acessibilidade dos componentes da parede celular da planta para a degradação por outras enzimas como celulases e hemicelulases (Annis e Goodwin, 1997; Lagaert et al., 2009).

Embora *S. sclerotiorum* tenha certa "preferência" em degradar pectina, já que os compostos pécticos que representam fonte de energia para o patógeno estão mais facilmente disponíveis que os polímeros celulósico e hemicelulósico (Lagaert et al., 2009), o número de enzimas putativas envolvidas na degradação de celulose e hemicelulose é bastante expressivo, 20 e 40 respectivamente (Amselem et al., 2011). Trabalhos têm demonstrado o papel dessas enzimas como importantes fatores de patogenicidade, por exemplo, fungos mutantes para o gene que codifica para β-Xylosidase tiveram a agressividade reduzida em tecidos de canola (Yajima et al., 2009). Além disso, durante a patogênese de *S. sclerotiorum* em plantas de feijoeiro, foram demonstrados a indução de genes que codificam para celulases e hemicelulases e o aumento na atividade dessas enzimas durante o processo de infecção (Oliveira et al., 2013).

As proteases secretadas por *S. sclerotiorum* atuam na degradação da parede celular da planta e na inativação ou inibição de algumas proteínas de resposta de defesa da planta (Bolton et al., 2006). A secreção destas enzimas também está relacionada com a produção de ácido oxálico e a consequente acidificação do meio (Poussereau et al., 2001; Bueno et al., 2012). Duas proteases ácidas, secretadas por *S. sclerotiorum,* foram caracterizadas *in vitro* e durante a interação desse patógeno com plantas de feijoeiro, demonstrando o envolvimento no processo de invasão de *S. sclerotiorum* (Bueno et a., 2012).

1.2. Mecanismos de defesa da planta contra fungo

Plantas possuem mecanismos de defesa contra o ataque de patógenos. Tais mecanismos de defesa podem ser ativados na presença de patógenos ou de substâncias químicas. Os componentes deste sistema de defesa geralmente

incluem polímeros específicos da parede celular e uma mistura de metabólitos secundários, peptídeos e proteínas antimicrobianos (Thomma et al*.,* 1998). A indução de resposta de defesa é ativada pelo reconhecimento por receptores da parede celular da planta a moléculas elicitoras derivadas de microrganismos invasores. Estes eventos de reconhecimento disparam uma cascata de transdução de sinal que resulta, dentre outras coisas, na produção de componentes endógenos de sinalização (Ryals et al*.,* 1996).

As plantas têm, pelo menos, três linhas de indução de defesa contra patógenos: (1) presença de barreiras físicas, como camada de cera e rígida parede celular; (2) produção de metabólitos secundários, como compostos antimicrobianos e espécies reativas de oxigênio (ERO) e (3) expressão de genes associados à defesa e indução de morte celular no local da infecção para evitar que o fungo se espalhe (Mur et al., 2008). No entanto, este tipo de resposta pode favorecer os fungos necrotróficos.

Três distintas, ainda que interconectadas vias de sinalização, mediadas por ácido salicílico (AS), ácido jasmônico (AJ), e etileno (ET) estão envolvidos em respostas de defesa de plantas (Feys e Parker 2000; Pieterse et al., 2009, Zhang et al., 2012). Estas vias de sinalização são diferencialmente reguladas e atuam individualmente, sinergicamente ou de forma antagônica dependendo do patógeno envolvido (Glazebrook 2005; Kachroo e Kachroo 2007). De acordo com o modelo geral, o AS atua como regulador central em defesa contra patógenos hemitróficos ou biotróficos, e a via de sinalização AJ/ET funciona principalmente na resistência contra ataque de patógenos necrotróficos (Glazebrook, 2005; Thomma et al., 1998). Entretanto, foi demonstrado que a via de sinalização de AS é um possível alvo para manipulação por *S. scleortiorum* (Nováková et al., 2014).

O reconhecimento do ataque do patógeno dispara reações de hipersensibilidade (RH) na planta, que resulta na geração de estresse oxidativo e dispara a morte celular. A RH pode ser uma estratégia efetiva de defesa contra fungos biotróficos, entretanto pode ser ineficiente contra patógenos necrotróficos (Greenberg, 1997; Hutcheson, 1998; Govrin e Levine, 2000).

As RH constituem na primeira etapa de respostas de defesa da planta que dispara uma série de outras alterações tais como: produção de proteínas relacionadas à patogenicidade (PRs), principalmente peroxidases, quitinases, β-1,3-glucanases; aumento na expressão de fenilalanina amônia liase (PAL); deposição de

calose e lignina; produção de fitoalexinas e aumento dos níveis de ácido salicílico (Verbene et al., 2000). Estas e outras respostas de defesa da planta ao patógeno serão apresentadas a seguir.

1.2.1. Proteínas relacionadas à patogênese – PRs

As PRs são definidas como proteínas induzidas no hospedeiro em resposta à infecção por um patógeno ou por estímulos abióticos, e podem estar correlacionadas com a resistência não específica do hospedeiro ao patógeno (Stangarlin et al., 2011). Essas proteínas PRs foram detectadas pela primeira vez no início da década de 70 em folhas de fumo (*Nicotina tabacum*) infectadas pelo vírus do mosaico do fumo (TMV) (Van Loon e Van Kammen, 1970). Atualmente são conhecidas 17 famílias de PRs em vegetais, com as mais diferenciadas funções (Gorjanovic, 2009).

O papel das PRs na resistência de plantas contra microrganismos patogênicos pode ser tanto direto como indireto. Uma ação direta, como por exemplo, a inibição do crescimento do patógeno ou da germinação de esporos, representa uma concepção simplificada da defesa de plantas contra a entrada de agentes patogênicos. Neste sentido, em muitos casos as PRs apresentam atividade antimicrobiana *in vitro*. Isoformas básicas de PRs normalmente exercem maior atividade antifúngica, apresentando ação reduzida contra bactérias, insetos, nematóides ou vírus (Stangarlin et al., 2011). Em sua maioria, PRs possuem uma maior importância na ação indireta, ou seja, no processo de indução de resistência, como por exemplo, na ação preventiva contra penetração de patógenos, por ação oxidativa de componentes da parede celular vegetal por meio de peroxidases (PR-9); ou envolvimento na transdução de sinais durante a interação patógeno-hospedeiro, como na ação de oxalato oxidases (PR-15, PR-16) e de proteínas não específicas relacionadas com o transporte de lipídios (PR-14). Um número pequeno de famílias das PRs possui atividade enzimática como é o observado nas glicanases (PR-2), quitinases (PR-3, PR-8, PR-11), peroxidases (PR-9), ribonucleases (PR-10) e (PR-4), ou ação inibitória (PR-6). A atividade inibitória de proteases e α-amilases é atribuída às famílias PR-5, PR-12 e PR-14 (Van Loon et al., 2006; Gorjanovic, 2009; Stangarlin et al., 2011).

A família PR-1 é geralmente o grupo mais abundante de proteínas e é induzido em níveis muito elevados durante a infecção (Van Loon et al., 2006). A proteína PR-1 é geralmente usada como marcadores de resistência sistêmica

adquirida (SAR). Embora sua função não seja bem estabelecida, PR-1 é associada à propriedades antifúngicas como hidrólise da parede celular de fungos (Van Loon et al., 2006). A superexpressão de PR-1 de pimenta em plantas de tabaco transgênico aumentou a resistência a *Phytophthora nicotianae, Ralstonia solanacearum*, and *Pseudomonas syringae* pv. *Tabaci* (Sarowar et al., 2005).

As β-1,3-glicanases (E.C.3.2.1.39), pertencentes à família PR-2, são amplamente distribuída em plantas superiores. São enzimas líticas capazes de degradar os polissacarídeos encontrados nas paredes celulares de muitos fungos (Boava et al., 2009). As glicanases podem atuar diretamente pela inibição do crescimento do patógeno, ou indiretamente auxiliando na geração de moléculas que podem funcionar como elicitores de mecanismos adicionais de defesa (Van Loon et al., 2006).

As quitinases (EC 3.2.1.14; família PR-3) constituem o segundo maior grupo de proteínas antifúngicas. Elas catalisam a clivagem hidrolítica do β-1,4-glicosídeo, ligação presente em biopolímeros de N-acetil-D-glicosamina, principalmente em quitina (Ferreira, 2007). As quitinases podem ser agrupadas em duas categorias: exoquitinases, atuam na extremidade não redutora da cadeia de quitina, e endoquitinases, que hidrolisam as ligações internas (Kasprzewska, 2003). Assim, as quitinases de plantas inibem o crescimento do fungo e geram oligossacarídeos de quitina que atuam como elicitores de defesa (Ferreira 2007).

O ataque de patógenos e diferentes condições de estresse podem ativar a produção de espécies reativas de oxigênio, o que pode danificar o DNA e as proteínas, e severamente comprometer a função da membrana. Em resposta ao aumento produção de radicais de oxigênio, as plantas ativam um sistema antioxidante complexo composto das moléculas capazes de limpar essas espécies, entre elas uma variedade de enzimas. As principais enzimas vegetais envolvidas na desintoxicação de tais radicais são as peroxidases (PR-9) (Hiraga et al., 2001; Almagro et al., 2009).

As peroxidases (E.C. 1.11.1.7) participam de um grande número de processos biológicos, como biossíntese de etileno, lignificação e suberização das células hospedeira durante a defesa contra patógenos, injúria, metabolismo de auxina e resposta a estresse (Cochrame et al., 2000; Hiraga et al., 2001; Almagro et al., 2009). Mudanças na atividade das peroxidases têm sido frequentemente

correlacionadas à resposta de resistência ou suscetibilidade em diferentes patossistemas (Stangarlin et al., 2011).

1.2.2. Fenilalanina amônia-liase

Outra enzima relacionada aos mecanismos de defesa da planta é a fenilalanina amônia-liase (PAL; E.C. 4.3.1.5). A PAL é a primeira enzima envolvida na via de biossíntese dos fenilpropanoides, e é responsável pela produção de compostos fenólicos como flavonoides, fitoalexinas, ligninas e derivados do ácido benzoico (Zhao et al., 2007). Vários estudos demonstraram que os níveis de atividade da PAL têm importantes funções em plantas nas respostas de defesa contra infecção de patógenos. Estudos mostraram que a expressão do gene *PAL* foi induzida pelo ataque do fungo da antracnose em *P. vulgaris* (Cools e Ishii, 2002), e plantas com supressão de PAL foram mais susceptíveis à infecção por fungos (Maher et al., 1994).

1.2.3. Lipoxigenase

A lipoxigenase (LOX; EC 1.13.11.12) é uma enzima da via dos octodecanoides que resulta na produção de ácido jasmônico, que será discutido adiante. LOX cataliza a oxidação de ácidos graxos insaturados produzindo oxilipinas, que têm importante papel na defesa de plantas, já que atuam como moléculas sinalizadoras e/ou compostos protetores (Blée, 2002). A via da LOX é crucial para o processo de peroxidação de lipídeos durante a resposta de defesa de plantas contra patógenos (Hwang e Hwang, 2010). Estudos genéticos revelaram o papel da via da LOX na defesa de tabaco e *Arabidopsis* (Rancé et al., 1998; Hwang e Hwang, 2010; López et al., 2011).

1.2.4. Lignina

A lignina é um polímero complexo depositado na parede celular e responsável pelo enrijecimento da parede e pelo aumento da resistência ao ataque de agentes externos (Strack, 1997). A síntese de lignina é induzida em resposta a danos mecânicos ou injúria, e muitas plantas respondem à invasão de patógenos com a deposição de lignina ou material similar (Vance et al., 1980; Nicholson e Hammerschmidt, 1992; Boudet et al., 1995; Dixon e Paiva, 1995). A lignina desempenha um papel importante na modificação das propriedades mecânicas da

parede celular, aumentando sua rigidez para limitar a difusão de toxinas do patógeno ao hospedeiro, e de nutrientes do hospedeiro ao patógeno, além de limitar a degradação de polissacarídeos por enzimas exógenas (Ride, 1978; Weng e Chapple, 2010).

1.2.5. Fitoalexinas

As fitoalexinas são metabólitos secundários, antimicrobianos, de baixa massa molecular e produzidos pelas plantas em resposta a estresses físicos, químicos ou biológicos. São capazes de impedir ou reduzir a atividade de agentes patogênicos, sendo a taxa de produção/acúmulo dependente dos genótipos do hospedeiro e/ou patógeno (Lo et al., 1996; Pedras et al., 2011). São considerados compostos biocidas, sendo prejudiciais para bactérias, fungos, nematoides, plantas e animais. De forma geral, o modo de ação das fitoalexinas sobre fungos inclui granulação citoplasmática, desorganização dos conteúdos celulares, ruptura da membrana plasmática e inibição de enzimas fúngicas. Esses efeitos refletem-se na inibição da germinação e elongação do tubo germinativo e redução ou inibição do crescimento micelial (Braga, 2008).

As fitoalexinas apresentam grande diversidade e mais de 300 tipos já foram caracterizados entre diferentes classes de compostos químicos como cumarina, diterpeno e flavonóide, entre outras, tendo sido identificadas em mais de 20 famílias de vegetais superiores (Smith, 1996). A indução para produção de fitoalexinas pode ocorrer em resposta à penetração fúngica e pelo tratamento com elicitores abióticos e bióticos, como aqueles obtidos de plantas e de micélio de fungos (Stangarlin et al., 1999; 2008).

1.3. Metil jasmonato (MeJA) como elicitor de respostas de defesa em plantas

Os ataques de patógenos são percebidos pelos chamados elicitores de defesa que são capazes de induzir a resposta natural de defesa da planta. Uma variedade de moléculas pode atuar como elicitores, incluindo oligo e polissacarídeos, proteínas, lipídios e moléculas sintéticas (Boller, 1995; Côté et al., 1998; Garcia-Brugger et al., 2006). Eles são constituintes do patógeno ou secretados por eles, ou eles são liberados da parede celular da planta ou do patógeno por enzimas hidrolíticas do patógeno ou da planta (Garcia-Brugger et al., 2006). A percepção do elicitor pela planta dispara várias vias de sinalização: fluxo de

íons, estresse oxidativo, e síntese de moléculas sinalizadoras como ácido salicílico, ácido jasmônico, e etileno. Genes relacionados à defesa, quando induzidos, levam ao reforçamento da parede celular da planta, acúmulo de compostos antimicrobianos como fitoalexinas, e síntese de proteínas com atividade hidrolítica ou inibitória para microrganismos (Kombrink e Somssich, 1995; Garcia-Brugger et al., 2006; Nováková et al., 2014).

A classe dos jasmonatos, derivados de ácidos graxos tem sido largamente estudadas. Estes compostos estão envolvidos em processos cruciais do desenvolvimento e sobrevivência de plantas, incluindo respostas de defesa, metabolismo secundário, processo reprodutivo, senescência, desenvolvimento de frutos entre outros (Seo et al., 2001; Wasternack et al*.,* 2007).

O ácido jasmônico (AJ), o mais caracterizado membro da família dos jasmonatos, foi identificado e isolado da cultura do fungo *Lasiodiplodia theobromae* (Avanci et al., 2010). O AJ é derivado da via dos octodecanoides, que inclui a peroxidação do ácido linolênico, presente na membrana dos cloroplastos, pela enzima lipoxigenase (Blée, 2002). A formação do ácido linolênico, e consequentemente do AJ, é disparada por estímulos externos como injúria e ataque de patógenos através da via de biossíntese de oxilipinas (Wasternack 2007; Gluaser et al., 2009). O AJ pode ser rapidamente metabolizado para o composto volátil metil jasmonato (MeJA), através da atividade da enzima ácido jasmônico carboxil metiltransferase (Seo et al*.,* 2001).

Os jasmonatos são importantes moléculas sinalizadoras produzidas por plantas que regulam a expressão de genes envolvidos na defesa contra patógenos necrotróficos (Grant Lamb, 2006; Guo et al., 2007, Wang et al., 2012; Zhang et al., 2012). Devido ao seu envolvimento na cascata de transdução de sinais levando a respostas de defesa, o AJ e o MeJA, têm sido utilizados como indutores de mecanismos de defesa em muitos patossistemas (Creelman e Mullet, 1995; Belhadj et al., 2006; Wang et al., 2012; Nováková et al., 2014).

O potencial dos jasmonatos na proteção de plantas contra infecção por patógenos foi mostrado após interação entre batata e *Phytophthora infestans* (Cohen et al., 1993), entre algodão e *Verticillium dahiae* (Li et al., 1996) e entre *Arabidopsis* e *Pythium mastophorum* (Vijayan et al*.,* 1998) e *Alternaria brassiclola* (Pennickx et al*.,* 1996). O ácido jasmônico mostrou ser também um potente elicitor da expressão de inibidores de proteinases em tomate (Farmer e Ryan, 1992).

Trabalho realizado por Seo e colaboradores (2001) mostrou que super-expressão da enzima ácido jasmônico carboxil metiltransferase levou ao acúmulo de MeJA e aumentou a resistência de *Arabidopsis* ao ataque de patógenos. O MeJA também mostrou promover a expressão de genes de resistência em plantas de uva (Belhadj et al., 2006). Tratamentos de folhas com MeJA exógeno elevou o nível de acúmulo de transcritos que codificam para proteínas relacionadas à patogenicidade, como quitinases, inibidores de serinas proteases, PGIPs e β-1,3-glicanases, além de estimular genes envolvidos na biossíntese de fitoalexinas. A capacidade de promover resistência contra o ataque de patógenos foi confirmado pelo aumento da tolerância para o patógeno *Erysiphe necator* (oídeo) após o tratamento com MeJA (Belhadj et al*.,* 2006).

As respostas de defesa das plantas podem ser reguladas, além do ácido jasmônico, por outras moléculas sinalizadoras como o ácido salicílico (SA) e etileno (ET). O ácido salicílico tem seus níveis aumentados na resposta sistêmica adquirida (SAR) que é caracterizada pela ativação de proteínas relacionadas à patogênese (PR), algumas das quais mostraram ter atividade antimicrobiana (Smith et al*.,* 1996). Já o ácido jasmônico e o etileno estão relacionados com a resistência sistêmica induzida (IRS).

A ativação de uma via de defesa em detrimento de outra é determinada pelo tipo de patógeno. Assim, foi demonstrado que patógenos biotróficos são geralmente sensíveis às respostas de defesa mediada pelo AS, enquanto patógenos com estilo de vida necrotrófico são comumente sensíveis às respostas de defesa controladas por AJ e ET (Becker and Spoel, 2006). Entretanto, muitos estudos mostraram que as defesas mediadas por AS são também envolvidas em resistência a patógenos necrotróficos (Ferrari et al., 2003; Guo e Stotz, 2007).

Estudos recentes da interação entre *B. napus* e *S. sclerotiorum*, um típico patógeno necrotrófico, mostraram que as três principais vias de sinalização e defesa (AS, AJ e ET) foram ativadas (Wang et al., 2012; Nováková et al., 2014). Similarmente, a defesa contra *B. cinerea*, outro patógeno necrotrófico, parece ser dependente de AJ, ET e também AS em *Arabidopsis* (Zimmerli et al., 2001; Ferrari et a., 2003) assim como em tomate (Díaz et al., 2002). Porém esses e outros trabalhos demonstraram que as vias de sinalização por ácido salicílico e ácido jasmônico são mutualmente antagonistas, assim, os genes que são ativados pela via do ácido jasmônico são suprimidos com a aplicação exógena de ácido salicílico e *vice versa*

(Thomma et al*.,* 1998; Pieterse et al., 2009; Leon-Reyes et al, 2010; Wang et al., 2012).

Alguns estudos mostram que as plantas, após o reconhecimento do patógeno invasor, são capazes de discriminar com precisão entre diferentes vias de sinalização por meio de interações sinérgicas e antogônicas (Pieterse et al*.,* 2001). O *cross-talk* entre as vias de sinalização AS e AJ/ET otimizam as respostas de defesa contra o ataque de patógenos (Mur et al., 2006; Koornneef e Pieterse, 2008). Juntas, as evidências sugerem que uma forte interação entre patógenos necrotróficos e hospedeiros deve existir antes de matar e ser morto.

2. OBJETIVOS

2.1. Objetivo geral

Investigar os mecanismos moleculares da interação entre *S. sclerotiorum* e plantas de feijoeiro comum (*P. vulgaris*) buscando uma melhor compreensão desse patossistema.

2.2. Objetivos específicos/Metas

2.2.1. Identificar os genes potencialmente envolvidos na interação entre *S. sclerotiorum* e plantas de feijoeiro por meio da técnica de hibridização subtrativa por supressão (HSS).

2.2.2. Avaliar a expressão de genes do fungo relacionados a fatores de patogenicidade durante a interação com *P. vulgaris*.

2.2.3. Avaliar a expressão dos principais genes envolvidos nas respostas de defesa da planta contra *S. sclerotiorum.*

2.2.4. Determinar os níveis de atividade de proteínas relacionadas à patogênese.

2.2.5. Elaborar um modelo que demonstre a interação *S. sclerotiorum* e *P. vulgaris,* a partir de dados moleculares.

3. METODOLOGIA

3.1. Estabelecimento do processo de infecção em plantas de feijoeiro

3.1.1. Material vegetal *Phaseolus vulgaris*

Plantas de feijoeiro comum (*P. vulgaris*), cultivar comercial Pérola (BRS), foram cultivadas em vasos com 2 kg de solo adubado com NPK (4-39-16) (1g/Kg de solo), e mantidas sob condições de luz natural, temperatura de até 24±2ºC, umidade relativa de 60± 5% e irrigação diária em casa de vegetação.

Para as plantas que receberam o indutor de resistência metil jasmonato (MeJA), o tratamento foi feito da seguinte forma: aproximadamente 10-12 dias após a emergência, com as folhas primárias completamente expandida, as plantas foram pulverizadas com uma solução aquosa de 10 µM MeJA (10 mL / planta; 100 plantas por tratamento). Foram feitas quatro aplicações do indutor químico com intervalos de seis dias entre cada aplicação. As plantas controle foram pulverizadas com 10 mL de água destilada.

3.1.2. Patógeno *Sclerotinia sclerotiorum*

O fungo *S. sclerotiorum*, isolado SPS, isolado de plantas de feijoeiro naturalmente infestadas, foi caracterizado no Laboratório de Biologia Molecular de Fungos Filamentosos da Universidade Federal de Goiás (UFG). A manutenção da cultura fúngica foi feita em meio BDA (Batata, Dextrose, Ágar) suplementado de 100 µg/mL de ampicilina.

Para extração de RNA do fungo, 4 *plugs* de micélio/BDA (0,5 cm de diâmetro) crescido por 3 dias em placa de Petri foram adicionados em frascos com meio mínimo (2 g/L NH_4NO_3, 1 g/L KH_2PO_4, 0.1 g/L $MgSO_4.7H_2O$, 0.5 g/L extrato de levedura, 3 g/L DL-ácido málico, 1 g/L NAOH) suplementado com 1% de glicose e crescido sob agitação constante (120 r.p.m.) por 72 horas a 20°C.

3.1.3. Inóculo das plantas

As plantas foram avaliadas em duas condições. Para a condição 1, plantas de feijão em estágio de florescência (R6) foram inoculadas com *plugs* micélio/BDA (0,5 cm de diâmetro) na região axilar. O grupo controle foi composto de plantas

inoculadas apenas com o *plug* de BDA, porém sem micélio. Já para a condição 2, as plantas foram inoculadas 12 h após receber a última aplicação de MeJA, as plantas controle, que receberam somente água, também foram inoculadas. Todas as plantas foram mantidas a 20°C e umidade relativa de 90% para prover condições de infecção adequada. As amostras de tecidos de 10 mm foram coletadas tanto da região de lesão do caule quanto da região sadia nos períodos de 6, 12, 24, 48 e 72 horas após o inóculo (hai). As amostras de tecidos foram armazenadas em nitrogênio líquido para posterior extração de RNA total. O desenho experimental foi completamente randomizado e consistiu de três replicatas biológicas para cada tratamentos. Para cada condição experimental, segmentos de caule (~10 mm) de 20 plantas diferentes formaram cada uma das três replicatas biológicas.

3.2. Construção de banco de cDNA

3.2.1. Extração de RNA

O RNA total foi extraído de tecidos de planta e material fúngico usando o protocolo padrão TRIZOL (Invitrogen Corp., Carlsbad, CA, USA) conforme instruções do fabricante. Hastes de caule e/ou micélio foram triturados até obtenção de um pó fino em nitrogênio líquido e homogenizado com TRIZOL (100 mg de tecido/1 mL de TRIZOL). O RNA total foi quantificado utilizando o ensaio colorimétrico Qubit (Invitrogen Corp., Carlsbad, CA, USA) e a verificação da qualidade foi feita utilizando eletroforese em gel de agarose 0,8% contendo brometo de etídeo (EtBr). Todas as amostras de RNA foram mantidas em *freezer* a -80ºC.

Duas misturas diferentes de RNA foram preparadas como amostras *tester* e *driver* para a construção de cada biblioteca de subtração. **Condição 1:** para a amostra *tester,* igual quantidade de RNA isolado de tecido de hastes de feijoeiro inoculadas a 6, 12, 24, 48 e 72 horas com *S. scleorotiorum* foram misturadas. No caso da amostra *driver,* tecidos de hastes de plantas não inoculadas coletados no mesmo período (6 a 72 h) mais RNA extraído de micélio do fungo foram misturados na razão 2:1. **Condição 2:** para a amostra *tester,* igual quantidade de RNA total isolado de tecido de hastes de plantas tratadas com MeJA e inoculadas por 12, 24, 48 e 72 horas com *S. scleorotiorum* foram misturadas, enquanto que para a amostra *driver,* foram utilizados tecidos de hastes de plantas não tratadas, mas inoculadas, coletados no mesmo período (12 a 72 h).

Para remover a contaminação com DNA genômico, o RNA foi tratado com DNAse I (Invitrogen Corp., Carlsbad, CA, USA) seguida de inativação da enzima (EDTA 2,5 mM, 65°C / 10 min) e precipitação com etanol.

3.2.2. Síntese de DNA complementar (cDNA)

Uma quantia de 1.0 µg de cada mistura de RNA total foi empregada para produzir a dupla fita de cDNA utilizando o conjunto de síntese "Super SMART^{TM} cDNA Synthesis Kit" (Clontech Laboratories Inc., Palo Alto, CA, USA) de acordo com o protocolo do fabricante. O "Kit SMART" possibilita a síntese de cDNA de alta qualidade a partir de nanogramas de RNA total ou poli A+, necessário para a construção de biblioteca de cDNA (Chenchik et al., 1998).

3.2.3. Hibridização subtrativa por supressão (HSS) e clonagem dos produtos de PCR

A HSS foi feita de forma independente para a cada condição (1 e 2) segundo as instruções do manual da Clontech "CLONTECH PCR-Selected cDNA Subtraction Kit User Manual". Os cDNAs dupla fita foram digeridos separadamente com a enzima de restrição *Rsa* I por 1.5 h a 37°C. Em seguida os produtos da digestão enzimática foram purificados com o kit de purificação "PureLink^{TM} PCR Purification Kit" (Invitrogen Corp., Carlsbad, CA, USA). O cDNA *tester* foi dividido em dois *pools* e ligado aos diferentes adaptadores (adaptador 1 e 2). A reação de ligação foi realizada em termociclador a 16°C durante 14 h, sendo então finalizada pela adição de 1µL de EDTA. As amostras foram aquecidas a 72°C por 5 min para inativar a enzima ligase.

As amostras *Tester*1 (ligada ao adaptador 1R) e *Tester* 2 (ligada ao adaptador 2R) foram hibridizadas com a amostra de cDNA *driver* também digerida com *Rsa* I e purificada. A primeira reação de hibridização foi realizada em termociclador a 68°C por 8h. Após esse período, os produtos de reação foram imediatamente utilizados para a segunda reação de hibridização.

Na segunda etapa de hibridização, as duas amostras provenientes da primeira reação de hibridização foram misturadas e uma alícota de cDNA *driver* recém desnaturado foi adicionado à mistura, para propiciar o enriquecimento das sequências diferencialmente expressas. A segunda reação de hibridização foi realizada em termociclador a 68°C por 14 h. Após esse período foi adicionado ao

produto da reação de hibridização, o tampão de diluição seguido de incubação a 68°C por 7 min e em seguida utilizado nas reações de PCR.

A primeira reação de PCR foi realizada por 27 ciclos (94°C, 30 s; 66°C, 30 s; 72°C, 90 s) a segunda etapa de amplificação (*Nested* PCR) foi realizada a partir de 3 µL do produto da primeira reação de PCR por 12 ciclos (94°C, 30 s; 68°C, 30 s; 72°C, 90 s). Os produtos de PCR foram visualizados por eletroforese em gel de agarose 1,5%/EtBr.

Após os dois *rounds* de amplificação por PCR, os cDNAs diferencialmente expressos durante a interação entre plantas de feijoeiro e o fungo *S. sclerotiorum* foram amplificados e enriquecidos. Os produtos de PCR foram purificados utilizando o kit de purificação "PureLink™ PCR Purification Kit" (Invitrogen Corp., Carlsbad, CA, USA) e inserido no vetor de clonagem PCR 4®-TOPO (Invitrogen Corp., Carlsbad, CA, USA). O vetor com o inserto clonado foi utilizado para transformar células de *Escherichia coli* One Shot® Top 10 Eletrocomp™ (Invitrogen Corp., Carlsbad, CA, USA).

As várias etapas da HSS e seu princípio estão representados de forma esquemática na **figura 3**.

Os clones recombinantes foram selecionados e transferidos para meio Luria-Bertani LB líquido suplementado com canamicina 100 µg/mL e cultivadas por 16 h. O DNA plasmidial foi extraído pelo método de minipreparação por lise alcalina (Sambrook e Russel, 2001).

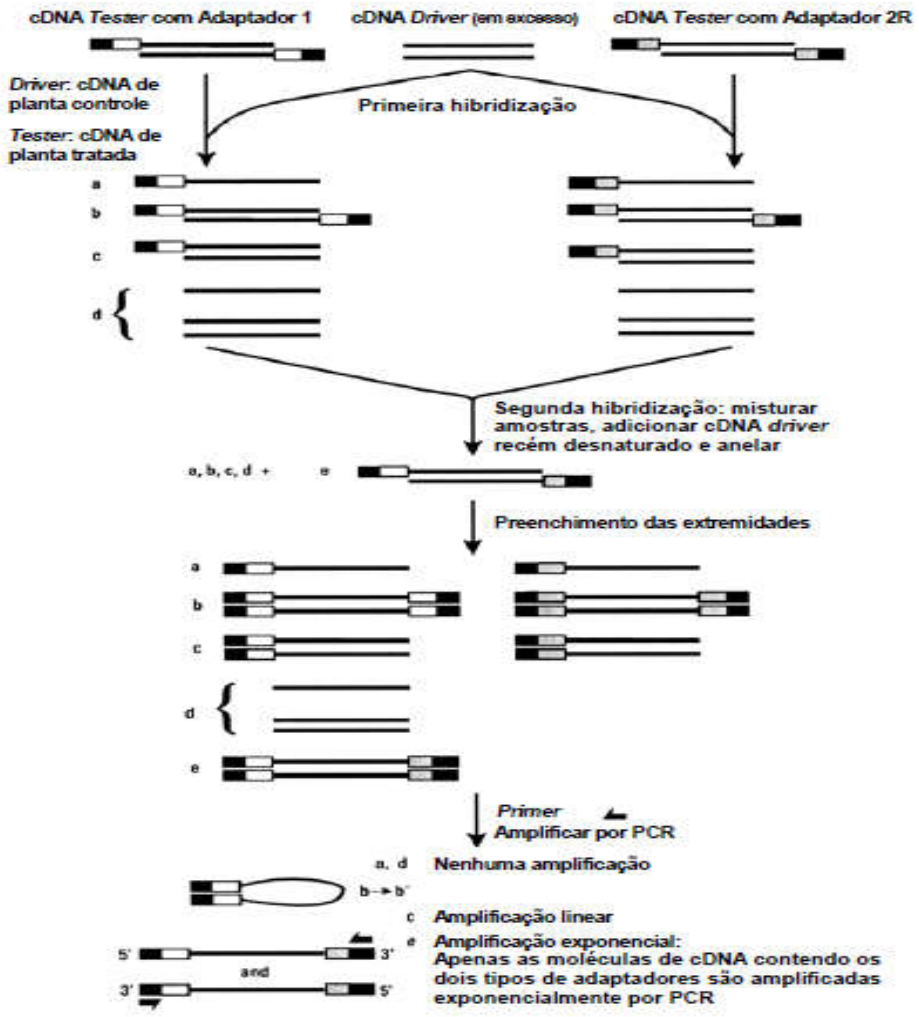

Figura 3: Representação diagramática da hibridização subtrativa por supressão. As linhas sólidas representam os cDNAs *tester* ou *driver*; as caixas sólidas representam a região externa dos adapatadores 1 e 2R que correspondem a sequência de do *primer* utilizado na PCR (*primer* 1); as caixas claras representam a região interna do adaptador 1R e da sequencia *Nested* PCR *primer* 1 correspondente; as caixas sombreadas representam a região interna do adaptador 2R e da sequencia do *Nested* PCR *primer* 2 correspondente.

3.2.4. Sequenciamento da biblioteca subtrativa e análise de bioinformática

As sequências de nucleotídeo de cada inserto foram determinadas por sequenciamento automático em eletroforese capilar, sendo as corridas realizadas em sequenciador de DNA, ABI PRISM 3130 (Applied Biosystems, Carlsbad, CA, USA). As reações de sequenciamento foram feitas utilizando o kit Big Dye Terminator v 3.1 Cycle Sequencing (Applied Biosystems, Carlsbad, CA, USA). O oligonucleotídeo M13-*Forward* foi utilizado para determinar as sequências.

As etiquetas de sequências expressas (ESTs) foram pré-processadas usando os softwares *Phred* (Ewing and Green 1998) e *Cross Match* (www.phrap.org). Somente as sequências com pelo menos 100 nucleotídeos e com valor de qualidade *Phred* maior ou igual a 20 foram mantidos para a análise. A sequência do vetor foi removido das ESTs usando o programa *CrossMatch* (www.phrap.org). As sequências resultantes foram alinhadas para formar os *contigs* usando o programa CAP3. As sequências filtradas foram comparadas com o banco de dados não redundante (nr) do *GenBank* usando o algorítmo BLASTX do Centro Nacional para Informação Tecnológica (http://www.ncbi.nlm.nih.gov). A anotação funcional usando os termos do *Gene Ontology* (http://www.geneontology.org) foi feita usando o software *Blast2GO* (Conesa and Götz 2008). A anotação das vias metabólicas foi feita usando o banco de dados KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg).

3.3. Validação da expressão gênica por transcrição reversa e PCR quantitativa em tempo real (RT-qPCR)

O RNA total de hastes de feijoeiro infectada ou não por *S. sclerotiorum* (condição 1) e plantas tratadas ou não com MeJA e inoculadas com *S. sclerotiorum* (condição 2) foi extraído conforme descrito no item 3.2.1. Igual soma de cada uma das amostras de RNA (1 µg) foi reversamente transcrito usando o *primer* oligo(dT)12-18 e submetido à análise de PCR em tempo real (RT-qPCR).

A análise que RT-qPCR foi realizada utilizando o sistema StepOnePlus™ e "Fast SYBR® Green PCR Master Mix" (Applied Biosystems, Carlsbad, CA, USA). Em 10 µL de reação continha 0.4 µM de cada nucleotídeo, 6 µL de SYBR Green PCR Master mix (2X), e 0.2 µL do cDNA. Após a desnaturação inicial a 95°C por 10 min, as amplificações foram realizadas por 40 ciclos (95°C, 15 s; 60°C, 60 s). Para checar a especificidade do produto de PCR, as curvas de *melting* foram analisadas para cada ponto.

Os níveis de expressão relativa dos genes alvo foram calculados usando o método ΔΔCt (Livak and Schmittgen 2001) e os genes *actin-11* e *gpdh* foram usados como genes de referência para *P. vulgaris* e *S. sclerotiorum* respectivamente (Borges et al., 2012). A amostra de referência foi escolhida para representar a expressão 1X do gene de interesse. Três amostras foram analisadas para cada tratamento. Os valores foram expressos como média ± desvio padrão.

3.4. Determinação de atividade enzimática

Para a extração de proteínas, 0,5 g das amostras de tecido de feijoeiro foram maceradas em nitrogênio líquido com auxílio de almofariz e homogeneizadas em 1 mL de tampão acetato 0,1 M pH 5.2. O homogenato foi centrifugado a 20,000 x g durante 25 min a 4°C. O sobrenadante obtido foi considerado o extrato bruto para a determinação de atividades enzimáticas. As proteínas totais foram detrerminadas usando o kit Quant iT-Protein Assay (Invitrogen, Carlsbad, CA, EUA).

3.4.1. Peroxidases

A atividade das peroxidases foi determinada por espectrofotometria direta a 470 nm pela mensuração da velocidade de oxidação do guaiacol na presença de peróxido de hidrogênio (H_2O_2), como descrito por Malolepsza (2006). A reação consistiu de 2,9 mL de uma solução contendo 250 µL de guaiacol (Sigma-Aldrich St. Louis, MO, EUA) e 306 µL de peróxido de hidrogênio em 100 mL de tampão fosfato 0,01 M (pH 6,0) e 0,1 mL do extrato proteico bruto. A reação branco continha 3 mL de uma solução com 250 µL de guaiacol e 306 µL de peróxido de hidrogênio em 0,1 mL de tampão fosfato 0,01 M, pH 6.0. A atividade da peroxidase foi definida como um aumento de 0,1 unidades de absorvância por minuto. Os resultados foram expressos em unidade enzimática (U.E.)/min/mg de proteína.

3.4.2. Fenilalanina amônia-liases (PAL)

A atividade de PAL foi mensurada pela produção de ácido trans-cinâmico a partir de L-fenilalanina como substrato. A reação consistiu de 100 µL do extrato enzimático, 400 µL de 50 mM de L-fenilalanina em tampão Tris-HCl 25 mM pH 8.8 e 500 uL de tampão 25 mM Tris-HCl que foi incubada durante 2 horas a 40 ºC. A quantidade de ácido trans-cinâmico produzida foi determinada por leitura de absorvância a 290 nm. Os testes foram realizados em triplicata. A atividade da PAL foi definida como a quantidade de enzima que produz aumento na absorção a 290 nm de 0,01 por hora do ensaio padrão. Esta mudança é equivalente a formação de 1 µg de ácido trans-cinâmico por mL de mistura de reação, tal como descrito por (Zucker, 1969)

3.4.3 β -1,3- Glicanase

A atividade de β-1,3- glicanase foi mensurada através da formação de glicose a partir de laminarina como substrato, como descrito por BOLLER (1992). A reação consistiu de 0,05 mL do extrato proteico bruto, 0,1 mL de 0,25 % de laminarina (Sigma - Aldrich , St. Louis , MO , EUA) dissolvida em tampão de acetato de sódio 50 mM, pH 5.2 que foi incubada por 30 minutos a 50ºC. A reação foi parada pela adição de 750 uL de ácido dinitrossalicílico (DNS) e fervida durante 5 minutos. A concentração de açúcar redutor foi determinada por espectrofotometria a 550 nm pelo método de DNS (MILLER, 1959), utilizando glicose como padrão. Uma unidade de atividade enzimática (U) foi definida como a quantidade de enzima necessária para formar 1 µmol de açúcar redutor por minuto de reação.

3.4.4. Quitinase

A atividade de quitinase foi mensurada através da formação de glicose a partir da quitina coloidal (Sigma-Aldrich, St. Louis, MO, EUA) como substrato. A reação consistiu de 50 µL do extrato proteico bruto, 50 µL de 1% de quitina coloidal dissolvida em tampão acetato de sódio 100 mM, pH 5.0, que foi incubada a 40ºC durante 60 minutos. A reação foi parada pela adição de 1 mL de DNS e fervida durante 5 minutos. A concentração de açúcar redutor foi determinada por espectrofotometria a 550 nm pelo método de DNS (MILLER, 1959), utilizando glicose como padrão. Uma unidade de atividade enzimática (U) foi definida como a quantidade de enzima necessária para formar 1 µmol de açúcar redutor por minuto de reação sob as condições descritas.

3.4.5. Poligalacturonase

A atividade das poligalacturonases (PG) foi quantificada registrando o aumento na absorvância a 575 nm causado pela liberação de açúcar redutor do ácido poligalacturônico. A reação continha 25 µL do extrato proteico bruto e 225 µL de 0,5% de ácido poligalacturonico (PGA, Sigma-Aldrich, St. Louis, MO,USA) dissolvido em 0,1 M de tampão acetato de sódio, pH 5.0. Após incubar por 30 min a 50ºC, a reação foi parada pela adição de 750 µL de DNS; então a mistura foi fervida por 5 min e colocada no gelo (Miller, 1959). A atividade enzimática foi expressa em micrograma de açúcar redutor liberado durante 30 min de incubação.

O efeito inibitório das proteínas inibidoras de poligalacturonase (PGIPs) sobre a atividade de poligalacturonase (PG) foi determinado usando como PG,

sobrenadante de cultura de *S. sclerotiorum* crescido em meio mínimo (2 g/L NH_4NO_3, 1 g/L KH_2PO_4, 0.1 g/L $MgSO_4.7H_2O$, 0.5 g/L extrato de levedura, 3 g/L DL-ácido málico, 1 g/L NAOH) suplementado com 1% de pectina cítrica sob agitação por 48 h. Esse material foi misturado na razão 1:10 com extrato do tecido vegetal (fonte de PGIP) obtido em diferentes tempos de coleta. A atividade enzimática foi determinada usando 0,1 M de acetato de sódio, pH 5.0, com 0,5% de ácido poligalacturônico (PGA) como substrato. As condições de reação foram as mesmas utilizadas para a determinação da atividade de PG. As amostras foram analisadas em triplicata para cada tratamento. Os resultados foram expressos como média ± desvio padrão (SD).

3.5. Análises de microscopia de luz

Secções de hastes de plantas de feijão inoculadas com fungo (12-48 hpi) e sem inoculação (controle) foram preparadas para observação histológica utilizando microscopia de luz de acordo com o método descrito por Mendonza et al. (1993). Inicialmente as amostras foram desidratadas com séries gradativas de álcool (80%, 90% e 100%) e em seguida incorporadas numa solução de etanol absoluto e resina (1:1, v/v; Historesin, Leica Microsystems Nußloch GmbH, Heidelberg, Germany) por 4 h, e estocada em resina pura por 24 h. A resina foi polimerizada usando um polimerizador líquido. Após secagem os bloquinhos foram seccionados em micrótomo em fatias de 8 µm. As secções foram coradas com 0.05% de azul de toluidina por 2 h. As secções de tecidos foram fotografadas utilizando microscópio Leica DM500, com câmera Leica ICC50 e o software Leica Application Suite (LAS) EZ V3.0.0 (Leica Microsystems, Switzerland).

4. RESULTADOS E DISCUSSÃO

4.1. CAPÍTULO 1: Analysis of genes that are differentially expressed during *Sclerotinia sclerotiorum–Phaseolus vulgaris* interaction

É crucial entender os mecanismos pelos quais as plantas reconhecem a presença do patógeno, bem como as respostas produzidas pelas células hospedeiras após a infecção do patógeno. O patossistema *S. sclerotiorum-P. vulgaris*, apesar de ser bem estudado em níveis celular e de resistência fisiológica, ainda não existem trabalhos, em nível molecular, que demonstre de forma global as particularidades dessa interação. Nesse sentido, o presente estudo teve como objetivo compreender os mecanismos moleculares que regulam a interação *S. sclerotiorum-P. vulgaris.*

A técnica molecular hibridização subtrativa por supressão (HSS) permite a comparação entre duas populações de mRNA e a obtenção de clones de genes expressos diferencialmente em uma população, após a remoção das sequências comuns por hibridização. O método de HSS utilizado no presente estudo, basou-se na amplificação preferencial, por PCR, de sequências diferencialmente representadas em duas populações de cDNA, enquanto que o fenômeno da supressão impediu a amplificação das sequências comuns. Para isso, foram utilizados RNAs extraídos de hastes de plantas de feijoeiro infectadas e não infectadas pelo fungo.

Neste trabalho, uma biblioteca de cDNA construída a partir da técnica de HSS, permitiu identificar e analisar os genes diferencialmente expressos envolvidos na interação *S. sclerotiorum-P. vulgaris*, tanto no processo de infecção de *S. sclerotiorum* a plantas de feijoeiro como nas respostas de defesa produzidas pela planta.

A partir da análise de expressão de alguns dos genes identificados, foi possível estabelecer um modelo que pode explicar os mecanismos moleculares da interação entre *S. sclerotiorum* e *P. vulgaris* permitindo assim, uma melhor compreenção desse patossistema.

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Analysis of genes that are differentially expressed during the *Sclerotinia sclerotiorum–Phaseolus vulgaris* interaction

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ABSTRACT

The fungus *Sclerotinia sclerotiorum* (Lib.) de Bary, one of the most important plant pathogens, causes white mold on a wide range of crops. Crop yield can be dramatically decreased due to this disease, depending on the plant cultivar and environmental conditions. In this study, a suppression subtractive hybridization (SSH) cDNA library approach was used for the identification of pathogen and plant genes that were differentially expressed during infection of the susceptible cultivar BRS Pérola of *Phaseolus vulgaris* L. A total of 979 unigenes (430 contigs and 549 singletons) were obtained and classified according to their functional categories. The transcriptional profile of 11 fungal genes related to pathogenicity and virulence were evaluated by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR). Additionally, the temporal expression profile obtained by RT-qPCR was evaluated for the following categories of plant defense-related genes: pathogenesis-related genes (*PvPR1*, *PvPR2*, and *PvPR3*), phenylpropanoid pathway genes (*PvIsof*, *PvFPS1*, and *4CL*), and genes involved in defense and stress-related categories (*PvLox*, *PvHiprp*, *PvGST*, *PvPod*, and *PvDox*). Data obtained in this study provide a starting point for achieving a better understanding of the pathosystem *S. sclerotiorum–P. vulgaris.*

Keywords: necrotrophic fungi, common bean, gene expression, *Sclerotinia sclerotiorum*, suppression subtractive hybridization, stress-induced transcripts

INTRODUCTION

Common beans (*Phaseolus vulgaris* L.) are the world's most important grain legume for direct human consumption (Broughton et al., 2003). However, bean production is affected by several diseases, such as white mold, which is one of the most important diseases that affects the quality and yield of crops on a global scale. The pathogen associated with white mold, *Sclerotinia sclerotiorum* (Lib.) de Bary, is a necrotrophic pathogen with worldwide distribution and is known to infect over 400 species of plants (Boland and Hall, 1994). The sclerotia of this pathogen are capable of surviving in the soil for several years and infect the majority of hosts indirectly, i.e. they germinate to produce apothecia, which release ascospores (Bolton et al., 2006). Because of its ability to infect economically important crops causing major losses, *S. sclerotiorum* has been the focus of many research programs. As a consequence, scientists have successfully annotated its genome (Amselem et al., 2011), investigated the fungus at the level of gene expression, and performed proteome-level studies (Yajima and Kav, 2006).

Previous studies on the pathogenicity of *S. sclerotiorum* mainly focused on the secretion of oxalic acid (Lumsden, 1979; Godoy et al., 1990) and hydrolytic enzymes (Marciano et al., 1983; Cotton et al., 2003), which act in concert to macerate plant tissues and generate necrosis. Lytic enzymes, such as cellulases, hemicellulases, pectinases, and proteases, sequentially secreted by the fungus facilitate penetration, colonization, and maceration and also generate an important source of nutrients (Hegedus and Rimmer, 2005; Bolton et al., 2006). However, oxalic acid is likely to have more important roles because it suppresses the oxidative burst and resistance of the host plant and triggers mediated apoptotic-like programmed cell death (PCD) (Kim et al., 2008). In the compatible interactions between *S. sclerotiorum* and its host plant, host cells maintain viability and a suppression of the oxidative burst is observed, which is akin to compatible biotrophic pathogens during the early stage of infection (Kabbage et al., 2013, 2015; Williams et al., 2011). Furthermore, direct acidification within the middle lamella enhances the activity of many cell wall-degrading enzymes, including polygalacturonases (PGs; Riou et al., 1991). Secretion of oxalic acid may help inactivate plant polygalacturonase-inhibiting proteins, thereby allowing the pathogen to overcome this specific host defense response (Favaron et al., 2004). Nonetheless, despite extensive studies on *S. sclerotiorum*, its pathogenic

mechanisms are still incompletely understood because its pathogenesis is very complex (Hegedus and Rimmer, 2005; Amselem et al., 2011; Williams et al., 2011).

Several attempts to genetically study the pathosystem *S. sclerotiorum–P. vulgaris* have been performed to establish methods to detect physiological resistance (Kolkman and Kelly, 2000; Schwartz and Singh, 2013). The infection process and establishment of compatible interactions have also been well characterized at the cytological level (Lumsden, 1979; Lumsden and Wergin, 1980; Tariq and Jeffries, 1986). Differential accumulation of specific defense-related transcripts, such as mRNA for polygalacturonase-inhibiting protein (PGIP) and pathogen related proteins, during the *S. sclerotiorum–P. vulgaris* interaction has also been reported (Oliveira et al., 2010; Kalunke et al., 2011; Oliveira et al., 2013).

Increasing our knowledge of the infection strategies of necrotrophic pathogens, in general, and of *S. sclerotiorum,* specifically, should be helpful in designing novel rational disease control strategies. This study, in which genes differentially expressed in the *S. sclerotiorum–P. vulgaris* interaction were identified and analyzed, provides a starting point for achieving a better understanding of the pathosystem *S. sclerotiorum–P. vulgaris*.

MATERIALS AND METHODS

Plant Material and Pathogen Inoculation

Dry bean plants (*Phaseolus vulgaris* L. cv. BRS Pérola), which are susceptible to *S. sclerotiorum,* were grown in 2-kg plastic pots containing soil fertilized with NPK (4-39-16) (1 g/kg of soil) in a greenhouse at 24 ± 2°C with 60 ± 5% relative humidity and a 16 h/8 h light/dark period.

Sclerotinia sclerotiorum isolate SPS was collected from a naturally infected dry bean plant and grown on Petri dishes containing potato-dextrose agar (PDA) culture medium for 5 days at 20°C, and 3-mm plugs of this culture were used to inoculate the axillary region of dry bean plants at the flowering stage (R6), which is the main stage of infection in field. The control group was composed of a set of plants mock-inoculated with sterile agar plugs. All of the plants were kept at 20°C and 90% relative humidity to provide adequate conditions for infection. Tissue samples were collected from both the necrotic part and the chlorotic area of each lesion within 10 mm of the lesion edge at 6-, 12-, 24-, 48-, and 72-h post inoculation (hpi) and

immediately frozen in liquid nitrogen prior to RNA and protein extraction. The experimental design was completely randomized and consisted of three biological replicates for each of the treatments. For each of the experimental conditions, stem segments (approximately 10 mm) from 20 plants different plants were pooled together to form one of three biological replicates.

RNA Extraction

Total RNA was extracted from the plant tissues (stem segment) and fungal material using the standard Trizol protocol (Invitrogen Corp., Carlsbad, CA, USA). Total RNA samples were examined quantitatively using the Qubit fluorometry assay (Invitrogen Corp., Carlsbad, CA, USA) and qualitatively using agarose gel electrophoresis.

Two different RNA mixtures were prepared as the source of the tester and driver samples for the suppressive subtractive hybridization (SSH) library construction. For the tester samples, equal amounts of the total RNA isolated from the stem tissues inoculated with *S. sclerotiorum* at 6, 12, 24, 48, and 72 hpi were mixed. In the case of the driver samples, RNA from non-inoculated stem tissues harvested at the same times (6 to 72 h) plus RNA extracted from the mycelium of the fungus [grown separately in minimal medium (2 g/L NH_4NO_3, 1 g/L KH_2PO_4, 0.1 g/L $MgSO_4.7H_2O$, 0.5 g/L yeast extract, 3 g/L DL-malic acid, 1 g/L NAOH) supplemented with 1% glucose] were mixed at a 2:1 ratio (estimated based on qPCR experiments – data not shown).

Suppressive Subtractive Hybridization Library Construction

For subtractive hybridization, 1.0 µg of each total RNA mixture was employed to produce double-stranded cDNA using the Super SMART™ cDNA Synthesis Kit (Clontech Laboratories Inc., Palo Alto, CA, USA) according to the manufacturer's instructions. To determine which genes were differentially expressed during the interaction with *S. sclerotiorum*, a forward subtractive cDNA library was constructed using the PCR-Select™ cDNA Subtraction Kit (Clontech Laboratories Inc., Palo Alto, CA, USA) according to the standard protocol provided. The subtracted cDNA population was cloned into a PCR 4®-TOPO vector (Invitrogen Corp., Carlsbad, CA, USA) and used to transform One Shot® Top 10 Eletrocomp™ *Escherichia coli* cells (Invitrogen Corp., Carlsbad, CA, USA). The plasmid DNA was obtained using alkaline lysis. The cloned products were sequenced using a M13 forward primer and the Big

Dye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA). The automated capillary electrophoresis sequencing runs were performed on an ABI prism 3130 analyzer (Applied Biosystems, Carlsbad, CA, USA).

Bioinformatic Analyses of Expressed Sequence Tags (ESTs)

The sequences were pre-processed using Phred (Ewing and Green, 1998) and Cross Match softwares (www.phrap.org). Sequences with at least 100 nucleotides and Phred quality greater than or equal to 20 were considered for further analysis. Cleaned ESTs were assembled into contigs using the CAP3 assembly program and compared against the GenBank non-redundant (nr) database using the BLASTx algorithm available at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Functional annotation using Gene Ontology terms (http://www.geneontology.org) was analyzed employing the Blast2GO program (Conesa and Götz, 2008). KEGG pathway annotation was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.genome.jp/kegg).

Primers were designed using Software Primer Express (Applied Biosystems, Carlsbad, CA, USA) for the amplification of gene fragments that were approximately 80-150 bp in length and with an annealing temperature of 60ºC (**Table 1**). The primer specificity was checked *in silico* against the NCBI database (http://www.ncbi.nlm.nih.gov/) through the Primer-BLAST tool. The selected primers were tested with PCR, and their specificity was checked by nucleotide sequencing on an ABI 3130 sequencer (Applied Biosystems, Carlsbad, CA, USA) using DyeTerminator chemistry4 to confirm their identities.

Reverse Transcriptase – quantitative PCR (RT-qPCR)

RNA was extracted from plant tissues and fungal material using the standard Trizol protocol (Invitrogen Corp., Carlsbad, CA, USA). After DNase I (Invitrogen Corp., Carlsbad, CA, USA) treatment in the presence of RNase inhibitor (Invitrogen Corp., Carlsbad, CA, USA), equal amounts of RNA (1 µg) were reverse-transcribed using oligo(dT)12-18 primer and evaluated with qPCR. RT-qPCR analysis was carried out using the StepOnePlus™ System and Power SYBR® Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) in 10 µL reactions containing 0.4 µM of each oligonucleotide (**Table 1**), 6 µL of SYBR Green PCR Master mix (2×), and

0.2 µL of template cDNA. After initial denaturation at 95°C for 10 min, amplifications were performed for 40 cycles at 95°C for 15 s and at 60°C for 1 min. To check the specificity of the PCR product, the melting curves were analyzed for each data point. The relative expression levels of target genes were calculated using the ΔΔCt method (Livak and Schmittgen, 2001) and the reference genes *actin-11* for *P. vulgaris* and *gpdh* for *S. sclerotiorum*. The reference sample was chosen to represent 1x expression of the gene of interest. Three samples were analyzed for each treatment; values are expressed as means ± standard deviation (SD).

Table 1 Oligonucleotide primer pairs used for RT-qPCR analysis

Gene	Description	Forward/Reverse
Sspg1	Endopolygalacturonase1	F: TCTTGCAGCAGTCGAGAAG
		R: GTGTTGTGTCCGAGGGAGT
Sspg3	Endopolygalacturonase3	F: ACCCACCACTTTGGCTACTG
		R: TGAGACGGTAAGACCCTTG
Sspg6	Endopolygalacturonase6	F: AAGCTTATTGGAATGGGTAT
		R: CTGGAGTTGACGATTTGACTA
SsBgsidase	β-1,3-glucosidase	F: CTGACGGTTCGACCCTGTAT
		R: GTACTCCCAACGGAGAACCA
β-1,4	β-1,4-glucanase	F: CAAGGCAGCTTAACCGCTAC
		R: GATCCATCGGAGTCGAGGTA
acp1	Acid protease	F: GCCACCCAAAACGGAGAAT
		R: GAGGTGAGGACGGAGTTTTGTT
aspS	Aspartyl protease	F: TGCTACTGGGTCCAACATCGT
		R: TGCGCTTGATGCACTTGGT
ScXylo	β-xylosidase	F: CGCTCTTTTTCCCCATACAA
		R: AGCGATGCGTATCTTCGAGT
Cellobio	Cellobiohydrolase	F: ACTCTCTGCCCTGATGCAGT
		R: AGGAGTGTAAGCGGCAGAAA
Oxalo	Oxaloacetate acetylhydrolase	F: CCCAATCGTCGAGGACAAGC
		R: TGCCTGCTCCGGTCATGTAA
ScCap20	hypotetical protein cap 20	F: GAAATTCGGCAAGCCATTTA
		R: TGCCCTTTGAAATCGGATAG
SsGPDH	Glyceraldehyde-3-phosphate	F: TGGCTCCTACTAAAGTTG
	dehydrogenase	R: CAAGCAGTTGGTTGTGCAAG
PvPR1	Pathogenesis-related PR-1	F:AAAGCCAAGAGCGATTCTCTTTTCA
		R:GAACACTCTGATTTGATAACACTTC
PvPR2	B1-3 endoglucanase	F: GAAGATGAGCtCAAAGCTGGTAA

		R: CAAGGATTGGCCAAAAGGTA
PvPR3	Chitinase class I	F: ATTGTTGTGCCAATCCCTTT
		R: CACCGCCATACAGTTCAAAA
PvPAL	Phenylalanine ammonia-lyase	F: GACACACAAGTTGAAGCACCA
		R: TGCAGCTTCTTAGCATCCTTC
PvLOX	Lipoxygenase	F: AGCACTGTGCCTGTTTTCAGT
		R: AACACACGAGAAGATTCAACCA
PvDOX	α-dioxigenase	F: CACAAATCCTCCCAAAATGG
		R: AAAGGTTCACACCATTGATTGG
PvFPS1	Farnesyl pyrophosphate syntase 1	F: CGGAATAGACGTTGAGGAATG
		R: AACACCTACCCGAATTTCTGC
PvIsof	Isoflavonoid glucosyltransferase	F: AGCTGAAACACAGCACCAACT
		R: AACACCATGCTTGGCAAATAG
Pvcallose	Callose synthase-like protein	F: TGGCTTAGTATTCGGATGTACCA
		R: CGTTGTAATGAAAGCGAAGTGT
Pv4CL	4-coumarate CoA-ligase	F: AGGTTGTTGGTGCTGAGAATG
		R: CCAACCAAGTCAAAGATTCCA
PvHPRP	Hypersensitive-induced response protein	F: ATTGCATGGTTCATAGCCAGT
		R: CCTCCACACAAGTATCAAAGGA
PvGST	Glutatione S-transferase	F:AGCTCTTCAAGGACACTGAGCCAA
		R:AAAGGCTGTGGATGCTGCACTAGA
PvPOD	Peroxidase	F: TCCTTTTCAGCACTTTCACT
		R: AGAAAGCAGTGTTCTTGTGG
PvACT11	Actin11	F: TGCATACGTTGGTGATGAGG
		R: AGCCTTGGGGTTAAGAGGAG
M13	Universal forward primer	F: GTAAAACGACGGCCAG

Light Microscopy

Resin sections of stems inoculated with fungal samples (12–48 hpi) and without inoculation (control) were prepared for histological observation using light microscopy according to the method described by Mendoza et al. (1993). Briefly, the samples were dehydrated using a graded alcohol series, embedded in a mixture of absolute ethanol and resin (1:1, v/v; Historesin, Leica Microsystems Nußloch GmbH, Heidelberg, Germany) for 4 h, and stored for 24 h in pure Historesin. The resin was polymerized using a hardener and the samples were sectioned into 8-µm thick slices with a rotary microtome. The sections were stained with 0.05% toluidine blue for 2 min and mounted. Tissue sections were photographed using a Leica DM500

microscope, with a Leica ICC50 camera, and the Leica Application Suite (LAS) EZ V3.0.0 software (Leica Microsystems, Switzerland).

RESULTS

Disease Development: Time Course Analyses of *S. sclerotiorum* Infection

To evaluate the *S. sclerotiorum* behavior in the interaction with *P. vulgaris* cv BRS Pérola, stem tissues of inoculated plants were sampled at 6-, 12-, 24-, 48-, and 72-hours post inoculation (hpi). The pathogen showed an infection capacity at 12 hpi, although the tissue from the inoculated plants remained healthy (**Figure 1A**). Necrotic spots appeared on stem tissues after 24 hpi (**Figure 1B**), and small necrotic areas were clearly visible at 48 hpi (**Figure 1C**). In addition, at 72 hpi, late extensive necrosis was observed in the vascular tissues of inoculated stems (**Figure 1D**).

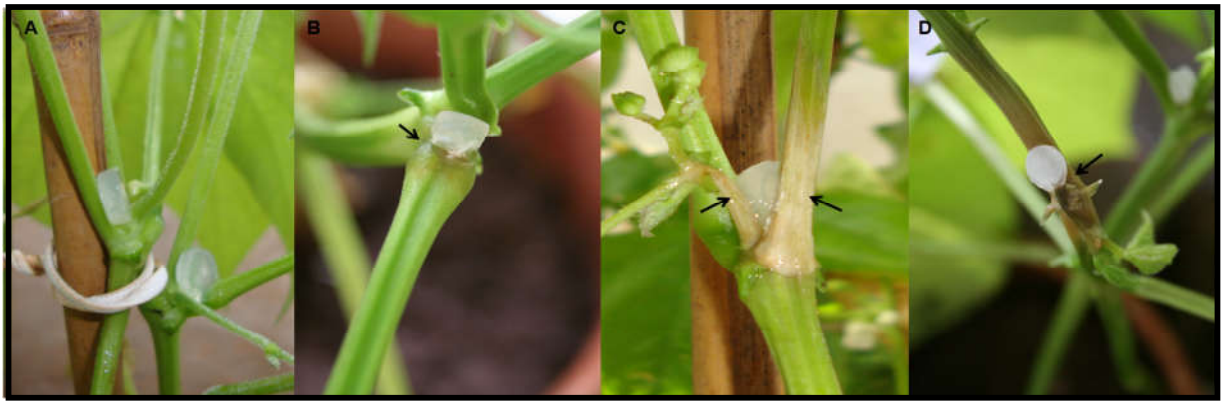

FIGURE 1: Plant disease symptoms observed (arrow) in *Phaseolus vulgaris* stems inoculated with plugs of *Sclerotinia sclerotiorum* PDA culture. (A) 12 hpi. **(B)** 24 hpi. **(C)** 48 hpi. **(D)** 72 hpi.

Functional classification of the significantly differentially expressed genes

Aiming to isolate the pathogen and plant genes that were differentially expressed during the *S. sclerotiorum–P. vulgaris* interaction, the susceptible common bean cultivar BRS Pérola was used, and suppression subtractive hybridization (SSH) was employed for the generation of a cDNA library. The SSH library was constructed using cDNA from stem tissues inoculated with *S. sclerotiorum* as the tester and cDNA from non-inoculated stem tissue plus RNA from the fungus grown in minimal medium as the driver.

A total of 1440 plasmids were sequenced. After removing the vector and adaptor sequences, the cleaned expressed sequence tags (ESTs) were assembled

into contigs using the CAP3 assembly program comprising a total of 979 unigenes (representing 430 contigs and 549 singletons). The putative function of the 979 ESTs was assigned using the BLASTX program of the NCBI non-redundant (nr) database employing the Blast2GO program with an *E-value* cutoff of $<1^{e-06}$ and the Gene Ontology database.

Initially, to identify metabolic processes that are specifically modulated by fungal infection, the 979 unigenes were classified into functional categories (biological processes, cellular components, and molecular functions) according to their putative biological functions reported by the Gene Ontology database (**Figure 2**). Of 979 unigenes derived from the *S. sclerotiorum–P. vulgaris* interaction, 29% were categorized as fungal, and 58.3% as plant genes. Of these, 12,7% had no significant match and therefore could not be classified.

The SSH library was normalized to reduce the redundancy of the most highly expressed genes. Nevertheless, some genes are very highly expressed and thus remain overrepresented in the normalized library. The number of reads/contig and their length distribution are shown in Supplementary Table S1. Most contigs were assembled from two to three reads. As expected, the largest contigs (i.e., those composed of most sequences) are involved in stress response pathways, such as contig 161, which has 28 sequences encoding a binuclear zinc transcription factor (*Colletotrichum gloeosporioides*).

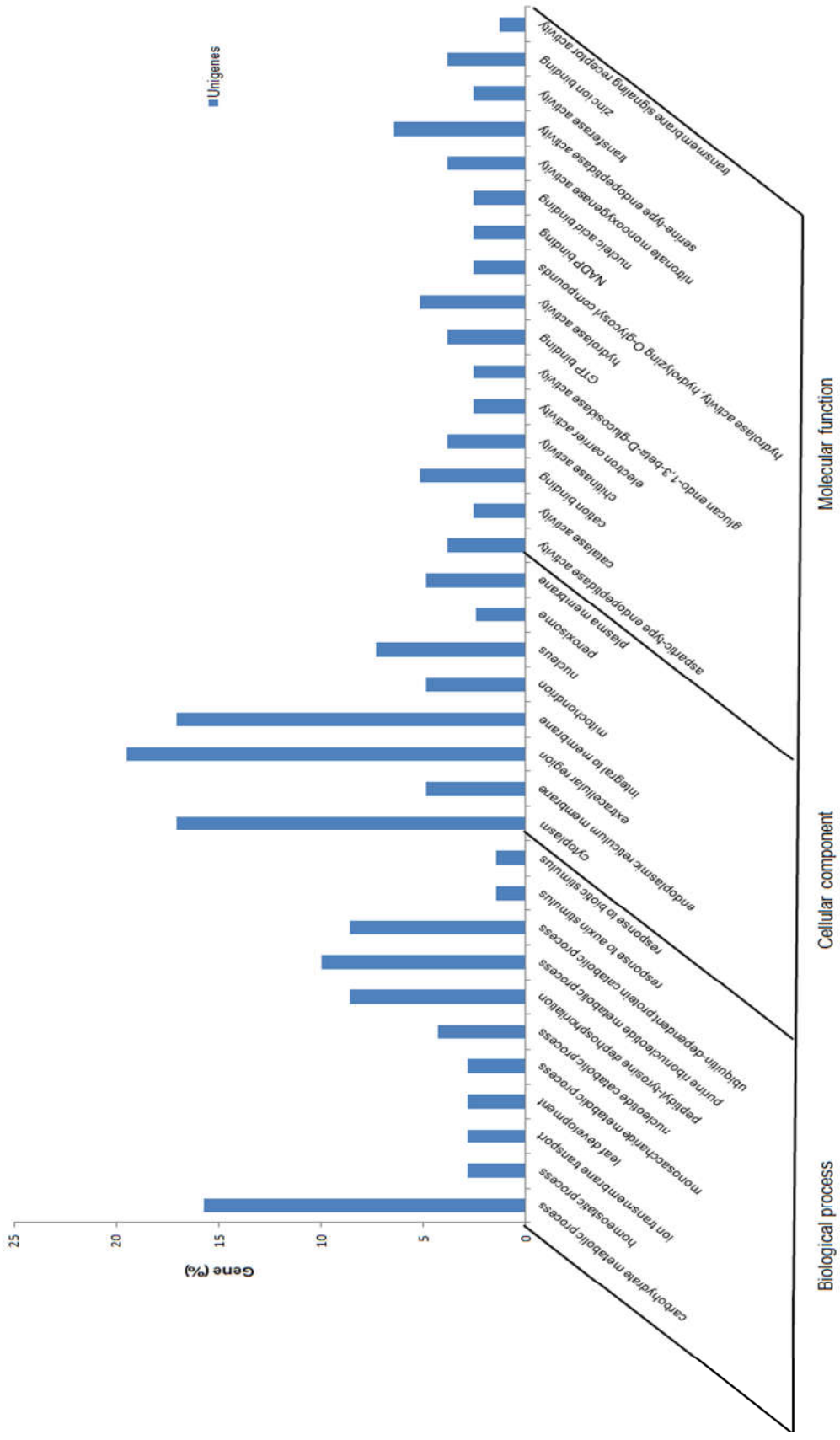
Unigenes
Gene (%)
0
5
10
15
20
25
carbohydrate metabolic process
homeostatic process
ion transmembrane transport
leaf development
monosaccharide metabolic process
nucleoside catabolic process
peptidyl-tyrosine dephosphorylation
purine ribonucleoside metabolic process
response to auxin stimulus
response to biotic stimulus
cytoplasm
endoplasmic reticulum membrane
extracellular region
integral to membrane
mitochondrion
nucleus
peroxisome
plasma membrane
aspartic-type endopeptidase activity
catalase activity
cation binding
chitinase activity
electron carrier activity
glucan endo-1,3-beta-D-glucosidase activity
GTP binding
hydrolase activity
hydrolase activity, hydrolyzing O-glycosyl compounds
NADP binding
nucleic acid binding
nitronate monooxygenase activity
serine-type endopeptidase activity
transferase activity
zinc ion binding
transmembrane signaling receptor activity
Biological process
Cellular component
Molecular function

FIGURE 2: Summary of Gene Ontology annotation as assigned by Blast2GO. Functional annotation of the expressed sequence tags (ESTs) in *Phaseolus vulgaris* infected with *Sclerotinia sclerotiorum* SSH library using gene ontology terms (http://www.geneontology.org) by Blast2GO program. Most consensus sequences were grouped into major functional categories: biological process, cellular component, and molecular function.

Gene Expression Profiles in *S. sclerotiorum* during *P. vulgaris* Infection

A total of 979 unigenes (representing 430 contigs and 549 singletons) were generated from the *S. sclerotiorum–P. vulgaris* interaction and aligned to the *Sclerotinia* genome database (Broad Institute, Cambridge, MA, USA) for the identification and screening of fungal sequences. Preliminary analysis of the fungal unigenes (163 contigs and 120 singletons) revealed a total of 67 unique fungal ESTs in this database. The proteins encoded by this unique EST set were assigned to the following major categories: cell wall degradation, protein degradation, intracellular transport, oxidative stress, among others (**Table 2**). Global analysis detected a small but significant group of genes related to virulence during the penetration stages of development (6 to 72 hpi).

Table 2 | Annotation of *Sclerotinia sclerotiorum* expressed sequence tags (ESTs) (contigs) derived from its interaction with *Phaseolus vulgaris*.

Contig	Accession number[a]	Name and/or putative function	Organism	*E* -value
Cell wall degradation				
Contig 27 - SS_024	SS1G_01493	β-xylosidase [b]	*Sclerotinia sclerotiorum*	3e-15
Contig 45 - SS_041	SS1G_13255.3	β-1,4-glucanase [b]	*Sclerotinia sclerotiorum*	6e-73
Contig 57 - SS_052	SS1G_08474	β-1,3-glucanase precursor	*Sclerotinia sclerotiorum*	6e-05
Contig 98 - SS_091	SS1G_07146.3	cellobiohydrolase [b]	*Sclerotinia sclerotiorum*	2e-40
Contig 124 - SS_116	SS1G_12937	glycosyl hydrolase	*Sclerotinia sclerotiorum*	2e-32
Contig 145 - SS_136	SS1G_07393	β-1,3-glucanase	*Sclerotinia sclerotiorum*	1e-64
Contig 42 - SS_038	SS1G_02789	glucan endo-1,3-β-glucosidase [b]	*Sclerotinia sclerotiorum*	3e-22
Contig 33 - SS_029	SS1G_01005	α-glucosidase putative	*Sclerotinia sclerotiorum*	3e-40
Contig 18 - SS_016	SS1G_12021	β-1,6-glucanase putative	*Sclerotinia sclerotiorum*	5e-18
Protein degradation				
Contig 25 - SS_022	SS1G_0336	serine peptidase putative	*Sclerotinia sclerotiorum*	3e-43
Contig 26 - SS_023	SS1G_06534	serin endopeptidase	*Sclerotinia sclerotiorum*	2e-53
Contig 16 - SS_014	SS1G_03181	aspartyl protease [b]	*Sclerotinia sclerotiorum*	2e-20

Contig 13 - SS_011	SS1G_00624	acid protease [b]	*Sclerotinia sclerotiorum*	8e-15
Contig 62 - SS_057	SS1G_11818	vacuolar protease A	*Sclerotinia sclerotiorum*	4e-53
Contig 74 - SS_068	SS1G_00477	ubiquitin homeostasis protein lub1 (phospholipase)	*Sclerotinia sclerotiorum*	1e-12
Contig 63 - SS_058	SS1G_0770	proteasome component PRE6	*Sclerotinia sclerotiorum*	3e-47
Contig 61 - SS_056	SS1G_01331	translocation protein Sec62 putative	*Sclerotinia sclerotiorum*	1e-22
Intracellular transport				
Contig 40 - SS_036	SS1G_07860	protein SEY1	*Sclerotinia sclerotiorum*	6e-42
Contig 77 - SS_071	SS1G_09620	hypothetical protein (GTPase)	*Sclerotinia sclerotiorum*	2e-04
Contig 103 - SS_096	SS1G_10229	GTP-binding protein EsdC	*Sclerotinia sclerotiorum*	1e-51
Contig 160 - SS_150	SS1G_08784	GTP-binding protein	*Sclerotinia sclerotiorum*	6e-33
Contig 148 - SS_139	SS1G_11149	SEC24 related gene family (GTPase)	*Sclerotinia sclerotiorum*	3e-31
Contig 9 - SS_007	SS1G_02490	DENN domain-containing protein	*Sclerotinia sclerotiorum*	2e-32
Contig 86 - SS_080	SS1G_09635	geranylgeranyl pyrophosphate synthetase (biosynthesis of secondary metabolites)	*Sclerotinia sclerotiorum*	5e-25
Oxidative stress				
Contig 53 - SS_048	SS1G_14466	oxidoreductase, 2-nitropropane dioxygenase family	*Sclerotinia sclerotiorum*	9e-19
Contig 44 = SS_040	SS1G_12928	peroxidase/catalase	*Sclerotinia sclerotiorum*	2e-38
Contig 88 - SS_082	SS1G_10037	cytochrome P450	*Sclerotinia sclerotiorum*	4e-29
Contig 161 - SS_151	SS1G_06754	zinc transcription factor	*Sclerotinia sclerotiorum*	4e-42
Contig 153 - SS_143	SS1G_11235	2-nitropropane dioxygenase	*Sclerotinia sclerotiorum*	2e-44
Others				
Contig 10 - SS_008	SS1G_06319	perilipin MPL1- like protein CAP 20[b]	*Sclerotinia sclerotiorum*	1e-07
Contig 97 - SS_090	SS1G_03197	pH-response regulator protein	*Sclerotinia sclerotiorum*	2e-09
Contig 75 - SS_069	SS1G_10538	ceramidase	*Sclerotinia sclerotiorum*	6e-20
Contig 142 - SS_133	SS1G_00477	phospholipase	*Sclerotinia sclerotiorum*	1e-12

[a]GenBank database (http://www.ncbi.nlm.nih.gov)
[b]Validated by RT-qPCR

To gain a better understanding of the mechanisms of *S. sclerotiorum* pathogenesis, 9 genes from the *S. sclerotiorum–P. vulgaris* SSH library related to plant cell wall degradation were selected for expression patterns analysis: polygalacturonases (*Sspg1, Sspg3*, and *Sspg6*); cellulases (β-1,3-glucosidase, β-1,4-glucanase, and cellobiohydrolase); hemicellulases (β-xylosidase); and proteases (aspartyl protease – *aspS* and acid protease – *acp1*). These genes were evaluated at 6, 12, 24, 48, and 72 hpi by reverse transcription quantitative real-time polymerase

chain reaction (RT-qPCR). The relative expression levels of all genes tested were normalized to the *S. sclerotiorum GPDH* gene.

The polygalacturonase *Sspg1* gene showed higher transcript levels in the late stage of the interaction. *Sspg3* expression was induced at 12 hpi and was reduced at 72 hpi. The *Ssp6* gene showed higher transcript levels at the early stages (6 to12 hpi) after inoculation (**Figures 3A-C**). Previously, we showed that the *Sspg1* and *Sspg5* genes are highly expressed during *S. sclerotiorum* infection of *P. vulgaris* plants and also that the *Sspg3* gene is expressed during the initial phase of colonization, whereas *Sspg6* is weakly expressed during this process (Oliveira et al., 2010).

The genes encoding β-1,3-glucosidase and β-xylosidase were also upregulated during the early stages of the *S. sclerotiorum–P. vulgaris* interaction (**Figures 3D,E**). Conversely, the genes encoding β-1,4-glucanase and cellobiohydrolase showed increased expression levels in the later stages of infection (**Figures 3F-G**). The gene *acp1*, which encodes an acid protease, was highly expressed from the beginning of infection, and presented the highest level of expression at 72 hpi. A similar profile was also observed for the *aspS* gene, which encodes an aspartyl protease (**Figures 3H-I**).

In *S. sclerotiorum*, as well as in other fungi, oxalic acid is produced from oxaloacetate in a reaction catalyzed by oxaloacetate acetylhydrolase (OAH). In this work, *oah* transcripts were detected in the early stage of infection (6 hpi), and its expression was higher in the late stage (72 hpi; **Figure 4A**).

Additionally, 15 unigenes (5 contigs and 10 singlets) that encode perilipin-like protein were detected in the *S. sclerotiorum–P. vulgaris* SSH library. Perilipin-like protein is largely responsible for the regulation of lipid storage and metabolism in filamentous fungi (Duan et al. 2013). Our analysis showed that although this gene was upregulated starting at the early stage of infection, the highest expression level was observed in the final stage (72 hpi) (**Figure 4B**).

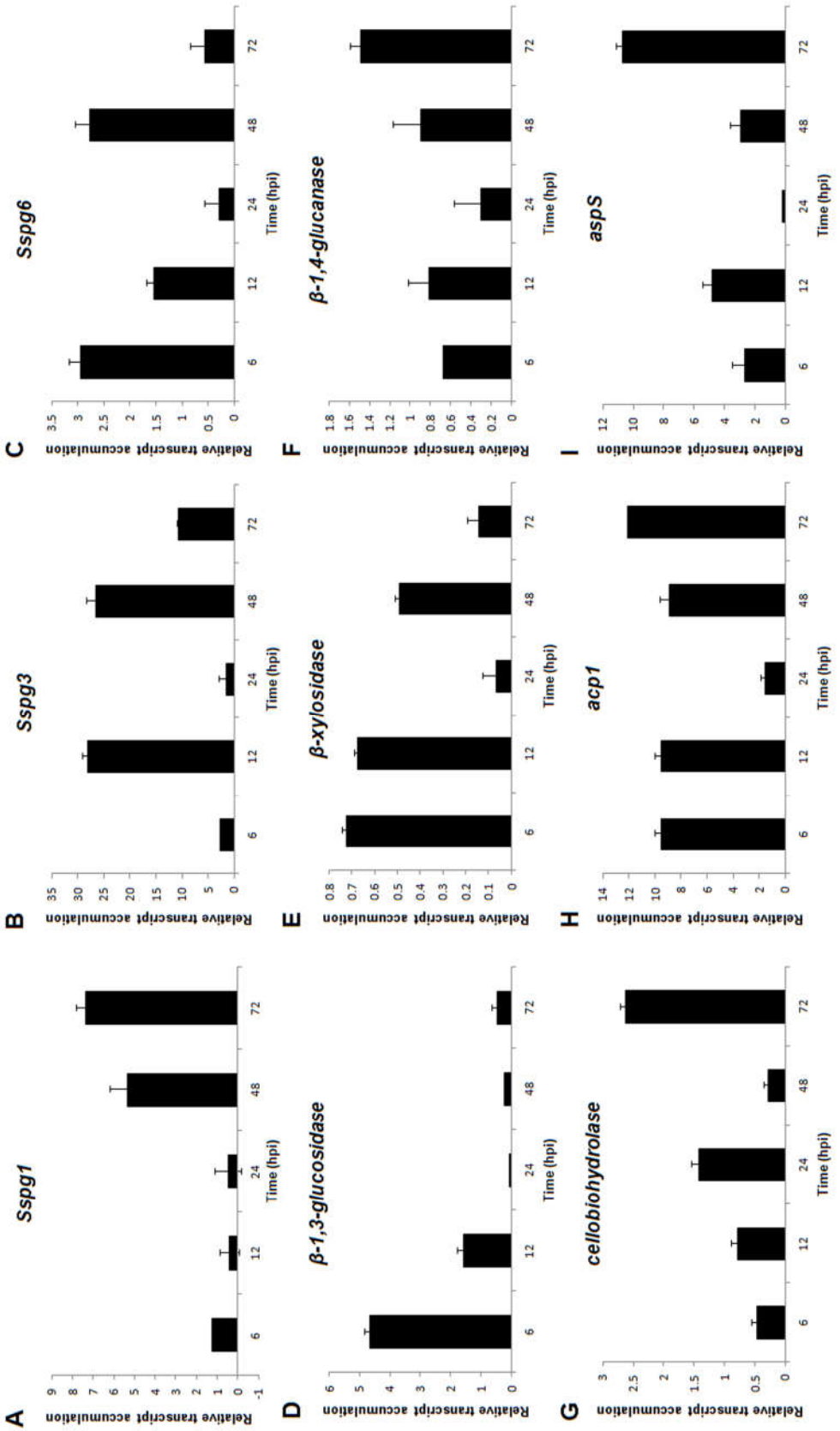
A
Sspg1
B
Sspg3
C
Sspg6
D
β-1,3-glucosidase
E
β-xylosidase
F
β-1,4-glucanase
G
cellobiohydrolase
H
acp1
I
aspS
Relative transcript accumulation
Time (hpi)
6
12
24
48
72

FIGURE 3: Gene expression profiles of *Sclerotinia sclerotiorum* during infection to *Phaseolus vulgaris* L. Expression analyses using RT-qPCR were performed and transcript levels were calculated in triplicate using a comparative method. *GPDH* gene was used as the reference gene in *S. sclerotiorum* and a health plant as the reference sample. Tissue samples were collected at 6, 12, 24, 48, and 72 hpi. Results are reported as means ± standard deviation (SD) of three samples for each treatment. **(A)** *Sspg1*. **(B)** *Sspg3*. **(C)** *Sspg6*. **(D)** *β-1,3-glucosidase*. **(E)** *β-xylosidase*. **(F)** *β-1,4-glucanase*. **(G)** cellobiohydrolase. **(H)** *acp1* (acid protease). **(I)** *aspS* (aspartyl protease).

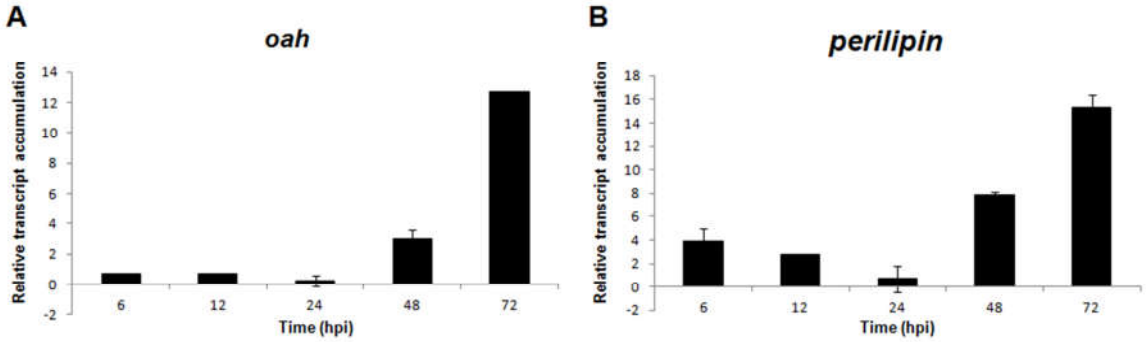

FIGURE 4: Gene expression profiles of *S. sclerotiorum* during infection to *P. vulgaris*. Expression analyses using RT-qPCR were performed and transcript levels were calculated in triplicate using a comparative method. *GPDH* gene was used as the reference gene in *S. sclerotiorum* and a health plant as the reference sample. Tissue samples were collected at 6, 12, 24, 48, and 72 hpi. Results are reported as means ± standard deviation (SD) of three samples for each treatment. **(A)** *oah* (oxaloacetate acetylhydrolase). **(B)** *perilipin*.

Defense Genes Expressed and Regulated Over Time in the Common Bean Stem During Interaction with *S. sclerotiorum*

As a result, the majority of the cDNAs from the SSH library were derived from *P. vulgaris*. In total 570 unigenes (267 contigs and 303 singletons) showed significant similarities to plant sequences, that indicates that 58.3% (979 unigenes) of our database represent common bean genes. The list of *P. vulgaris* the most common contigs/reads and their length distribution are shown in **Table S1** – supplementary material. The proteins encoded by the unique EST set were assigned to five major categories: metabolism, energy, protein degradation, response to biotic and abiotic stimulus, and others. An important group of the identified genes are related to biotic and abiotic stress responses, among these are PR1 (pathogenesis related protein 1), and peroxidase genes. Although most of the defense-related genes were not detected in our EST collection (979 unigenes), primers were designed based on the bean transcriptome sequence (Melotto et al., 2005) to investigate the plant response during the interaction with *S. sclerotiorum.* We searched for ESTs that may play an

important role in the defense response to *S. sclerotiorum* disease and can be used as reference for comparison among different pathosystems.

We evaluated the temporal expression profile of the three main categories of defense-related genes, namely: 1) pathogenesis-related genes *PvPR1* (function unknown), *PvPR2* (β-1,3-endoglucanase), and *PvPR3* (chitinase); 2) phenylpropanoid pathway genes, such as *PvISOF* (isoflavonoid glucosyltransferase), *PvFPS1* (farnesyl pyrophosphate synthetase 1), *PvPAL* (phenylalanine ammonia-lyase), and *Pv4CL* (4-coumarate CoA-ligase), which are involved in phytoalexin biosynthesis; and 3) genes involved in defense and stress-related categories, such as *PvLOX* (lipoxygenase), *PvHIPRP* (hypersensitive-induced response protein), *PvGST* (glutathione S-transferase), *PvPOD* (peroxidase), and *PvDOX* (alpha-dioxygenase). The time-course expression patterns were determined using control and infected samples at 6, 12, 24, 48, and 72 hpi. The relative expression levels of all of the genes were normalized using the *P. vulgaris Actin 11* gene (*Act11*) as the reference gene (Borges et al., 2012a).

Significant fold-change differences in expression levels were observed for all of the selected genes in the infected plant (fungus-inoculated) compared with the mock-inoculated plant. The temporal expression of the pathogenesis-related genes *PvPR1* (function unknown) and *PvPR2* (β-1,3-endoglucanase) showed a similar expression profile. Both genes presented a basal expression during the early stages of infection (6–12 hpi) and a high expression level at 72 hpi; however, at this time, the expression of *PvPR2* was two-fold higher than that of *PvPR1* (**Figures 5A-B**). The expression of the *PvPR3* gene was observed only in the early stages of infection (6–12 hpi), but at lower levels compared with the other *PvPR* genes analyzed (**Figure 5C**).

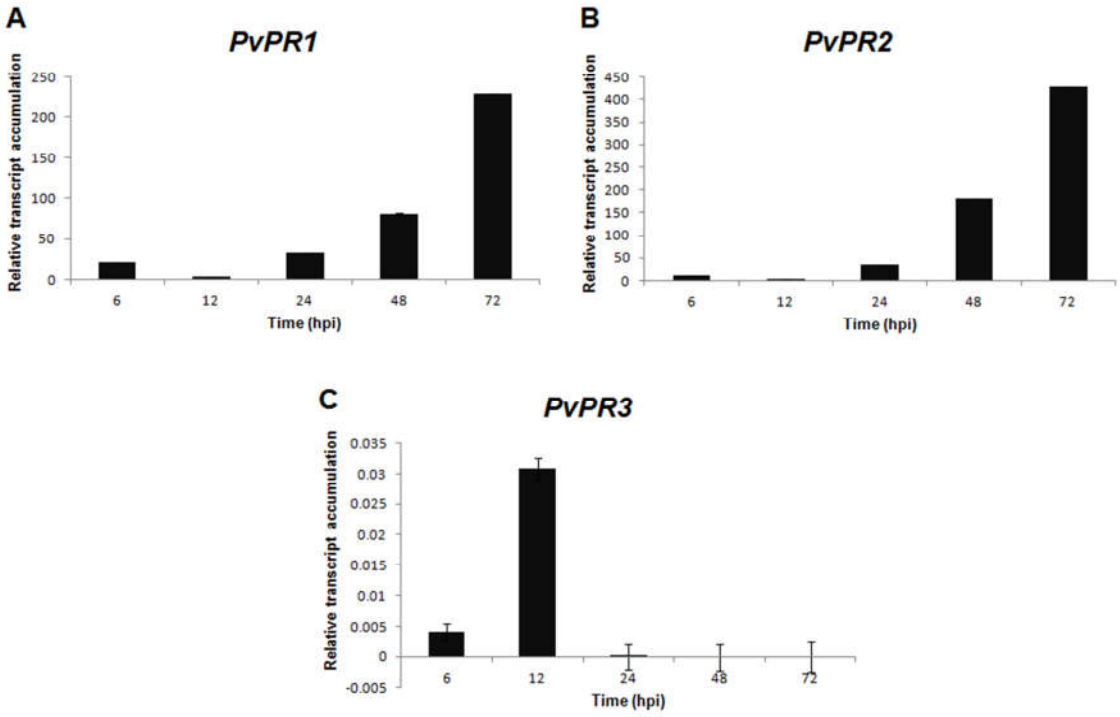

FIGURE 5: Expression pattern of pathogenesis-related genes in *P. vulgaris* in response to *S. sclerotiorum* infection. Expression analyses using RT-qPCR were performed and transcript levels were calculated in triplicate using a comparative method. *Actin-11* gene was used as the reference gene in *P. vulgaris* and a health plant as the reference sample. Tissue samples were collected at 6, 12, 24, 48, and 72 hpi. Results are reported as means ± standard deviation of three sample for each treatment. **(A)** Pv*PR1* (pathogenesis related protein 1). **(B)** *PvPR2* ((pathogenesis related protein 2). **(C)** *PvPR3* ((pathogenesis related protein 3).

The genes involved in the phenylpropanoid pathway, *PvISOF* (isoflavonoid glucosyltransferase), *PvFPS1* (farnesyl pyrophosphate synthetase 1), *PvPAL* (phenylalanine ammonia-lyase), and *Pv4CL* (4-coumarate CoA-ligase), were upregulated in dry bean plants during the interaction with *S. sclerotiorum*. The highest levels of expression were registered for *PvPAL* (a two-fold increase compared with *PvFPS1* and a 10-fold increase compared with *PvISOF* at 48 hpi (**Figures 6A-C**) and *Pv4CL* at 12 hpi (**Figure 6D**).

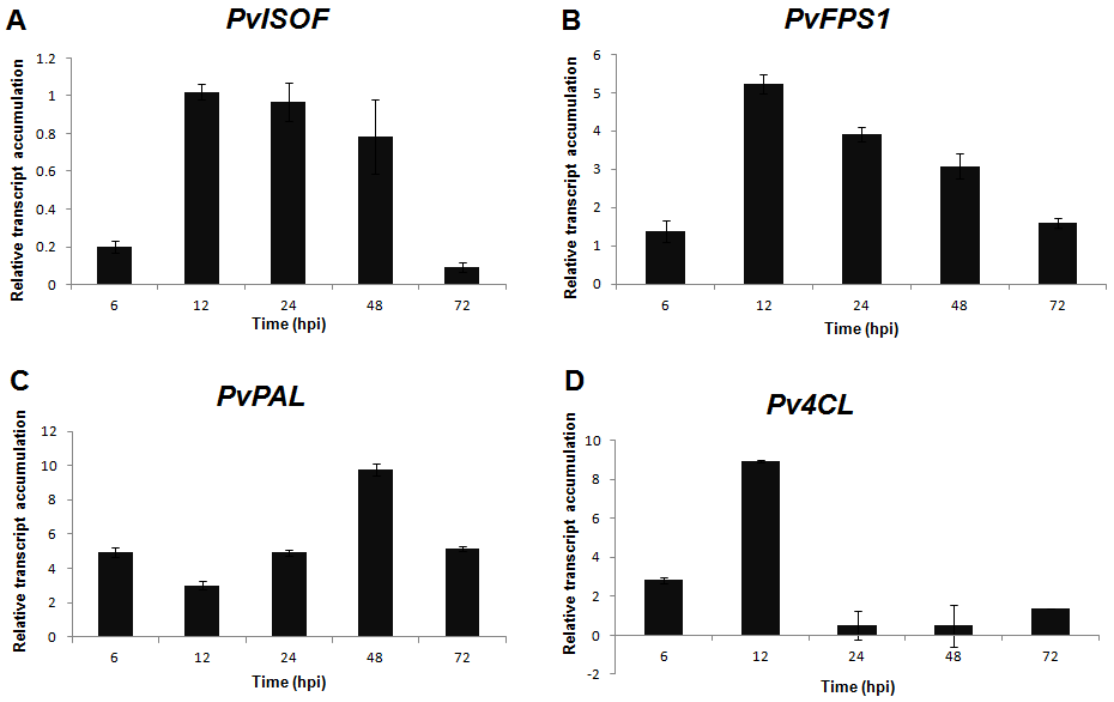

FIGURE 6: Expression pattern of phenylpropanoid pathway genes in *P. vulgaris* in response to *S. sclerotiorum* infection. Expression analyses using RT-qPCR were performed and transcript levels were calculated in triplicate using a comparative method. *Actin-11* gene was used as the reference gene in *P. vulgaris* and a health plant as the reference sample. Tissue samples were collected at 6, 12, 24, 48, and 72 hpi. Results are reported as means ± standard deviation of three sample for each treatment. **(A)** *PvIsof* (isoflavonoid glucosyltransferase). **(B)** *PvFps1* (farnesyl pyrophosphate synthetase 1). **(C)** *PvPAL* (phenylalanine ammonia-lyase). **(D)** *Pv4CL* (4-coumarate CoA-ligase).

Among the genes involved in the defense and stress-related categories, *PvLOX* was activated in the early stages of infection and reached a maximum expression at 12 hpi (**Figure 7A**). The *PvHIPRP* gene, which is related to hypersensitive responses (HR), was induced at the early stages of infection and peaked in expression at 12 hpi (**Figure 7B**). The *PvGST* (glutathione S-transferase) gene presented a progressive increase in expression up to 48 hpi, followed by a decrease at 72 hpi (**Figure 7C**), whereas the *PvPOD* (peroxidase) gene had a maximum expression at 12 hpi (**Figure 7D**). Finally, the *PvDOX* gene, which encodes α-dioxygenase, showed a high level of expression at 72 hpi (**Figure 7E**) in the *S. sclerotiorum–P. vulgaris* pathosystem.

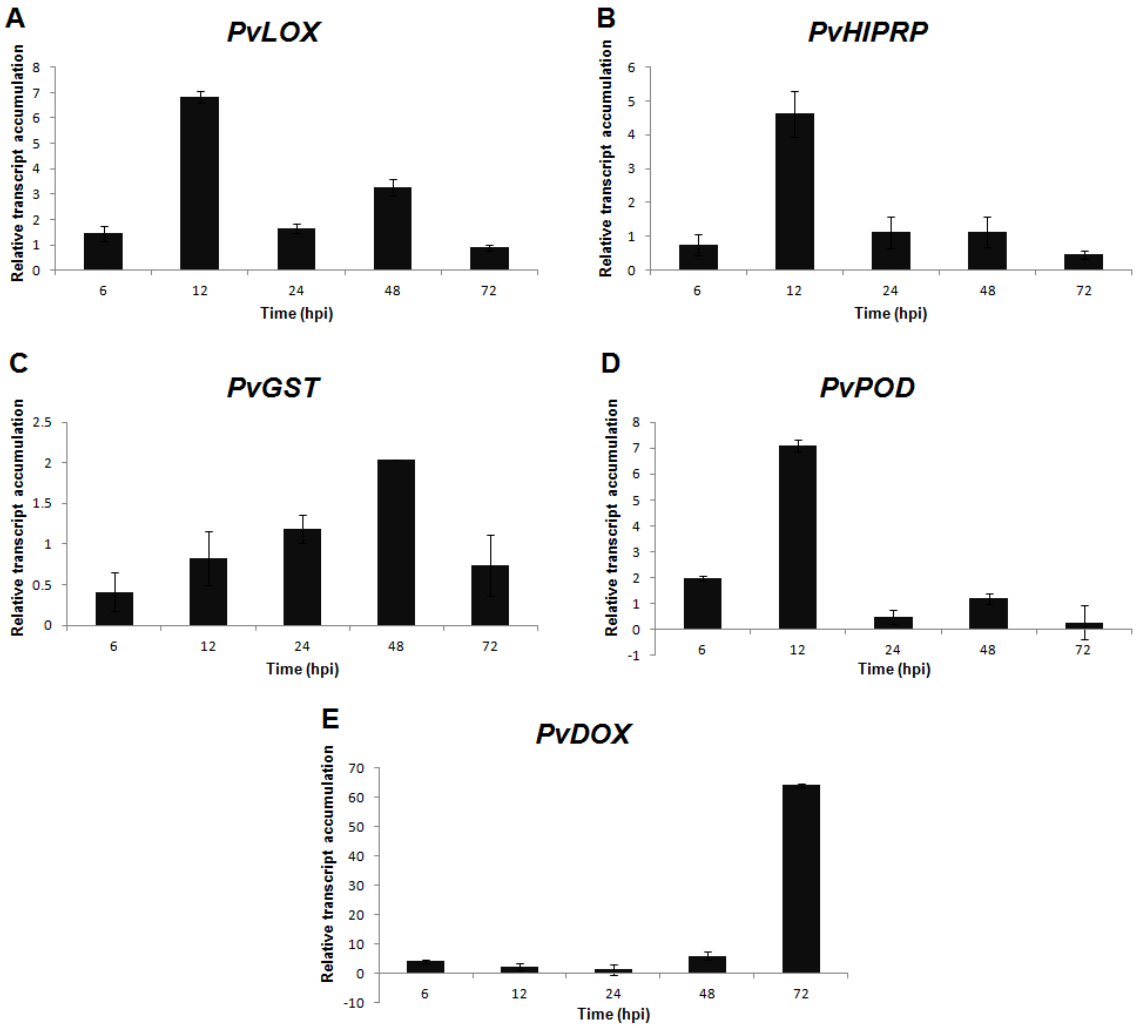

FIGURE 7: Expression pattern of defense and stress related genes in *P. vulgaris* plant in response to *S. sclerotiorum* infection. Expression analyses by RT-qPCR were performed and transcript levels were calculated in triplicate using a comparative method. *Actin-11* gene was used as the reference gene in *P. vulgaris* and a health plant as the reference sample. Tissue samples were collected at 6, 12, 24, 48, and 72 hpi. Results are reported as means ± standard deviation of three samples for each treatment. **(A)** *PvLox* (lipoxygenase). **(B)** *PvHiprp* (hypersensitive-induced response protein). **(C)** *PvGST* (glutathione S-transferase). **(D)** *PvPOD* (peroxidase). **(E)** *PvDOX* (alpha-dioxygenase).

Distinct vascular changes in the stem were noticed in response to *S. sclerotiorum* infection. Histological sections of bean stem showed that under *S. sclerotiorum* infection (12 to 72 hpi – **Figures 8 B** to **D**), bean plants had evident lignification, which was not observed in the (**Figure 8 A**) control (mock-inoculated). The uppermost 3–4 cell layers of the epidermis were stained a greenish–blue color with Aniline blue, indicating an accumulation of phenolic compounds. Thickness of

lignin layer (**E**) was measured using Leica LAS EZ V3.0.0 software (Leica Application Suite, Leica Microsystems, Switzerland).

To assess whether the callose gene (*Pvcallose*) was modulated during the *S. sclerotiorum* invasion of *P. vulgaris* stem tissues, the level of its transcription was evaluated using RT-qPCR. The results indicate that this gene was activated in the early stages of infection (6 hpi) and showed an increased accumulation of transcripts from 12 hpi until 48 hpi (**Figure 8F**).

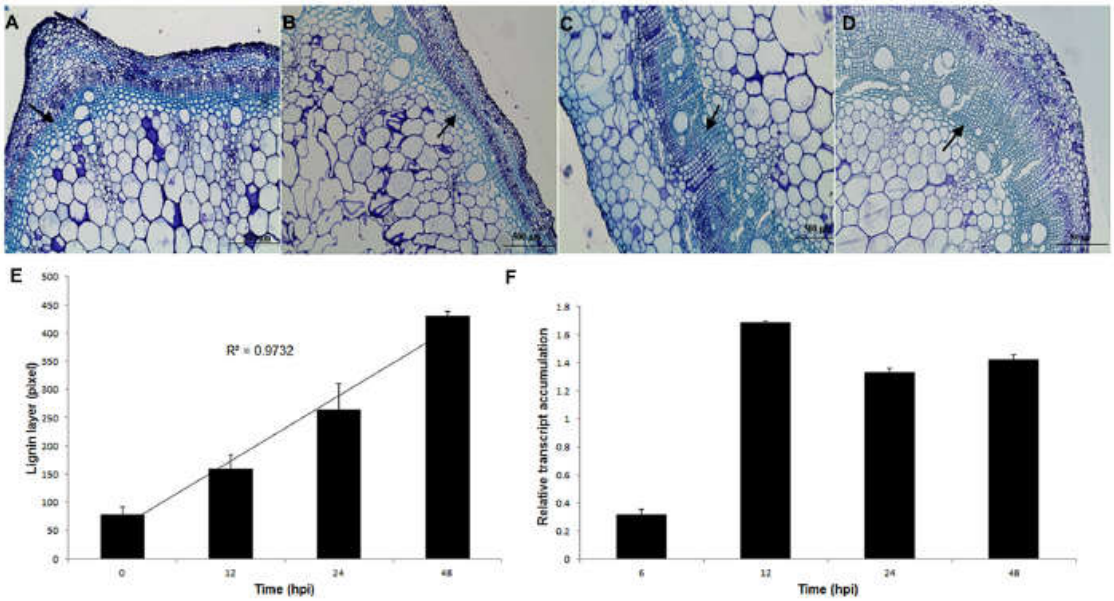

FIGURE 8: Thickening layers of lignin in *P. vulgaris* stems in response to *S. sclerotiorum* infection. (A) Without infection. **(B)** 12 hpi. **(C)** 24 hpi. **(D)** 48 hpi. Bar = 500 µm. **(E)** Thickness of lignin layer (arrows) measured using Leica LAS EZ V3.0.0 software (Leica Application Suite, Leica Microsystems, Switzerland). **(F)** Relative transcript accumulation of *Pvcallose* gene. Expression analyses using RT-qPCR were performed and transcript levels were calculated in triplicate using a comparative method. *Actin-11* gene was used as the reference gene in *P. vulgaris* and a health plant as the reference sample. Results are reported as means ± standard deviation of three experiments.

DISCUSSION

Although *S. sclerotiorum* is considered to be a typical necrotroph, there is evidence that it colonizes plant tissues through multiple phases involving important transcriptional and physiological reprogramming (Kabbage et al., 2013, 2015; Guyon et al., 2014; Hegedus and Rimmer, 2005). In this study, we evaluated the infective process of *S. sclerotiorum* in *P. vulgaris* plants. The gene expression profiles of stem tissues in the inoculation site were examined over 3 days. The steady-state levels of

all of the identified genes were quantified at the time of inoculation (0 hpi), at 2 early time points following inoculation (12 and 24 hpi) and at somewhat later time points (48 and 72 hpi), at which point necrotic lesions develop on the stems and intensive mycelial colonization occurs. From our transcript profile results, we propose the model illustrated in **Figure 9**, which depicts a plausible interpretation of the observed expression patterns of the *S. sclerotiorum–P. vulgaris* interaction.

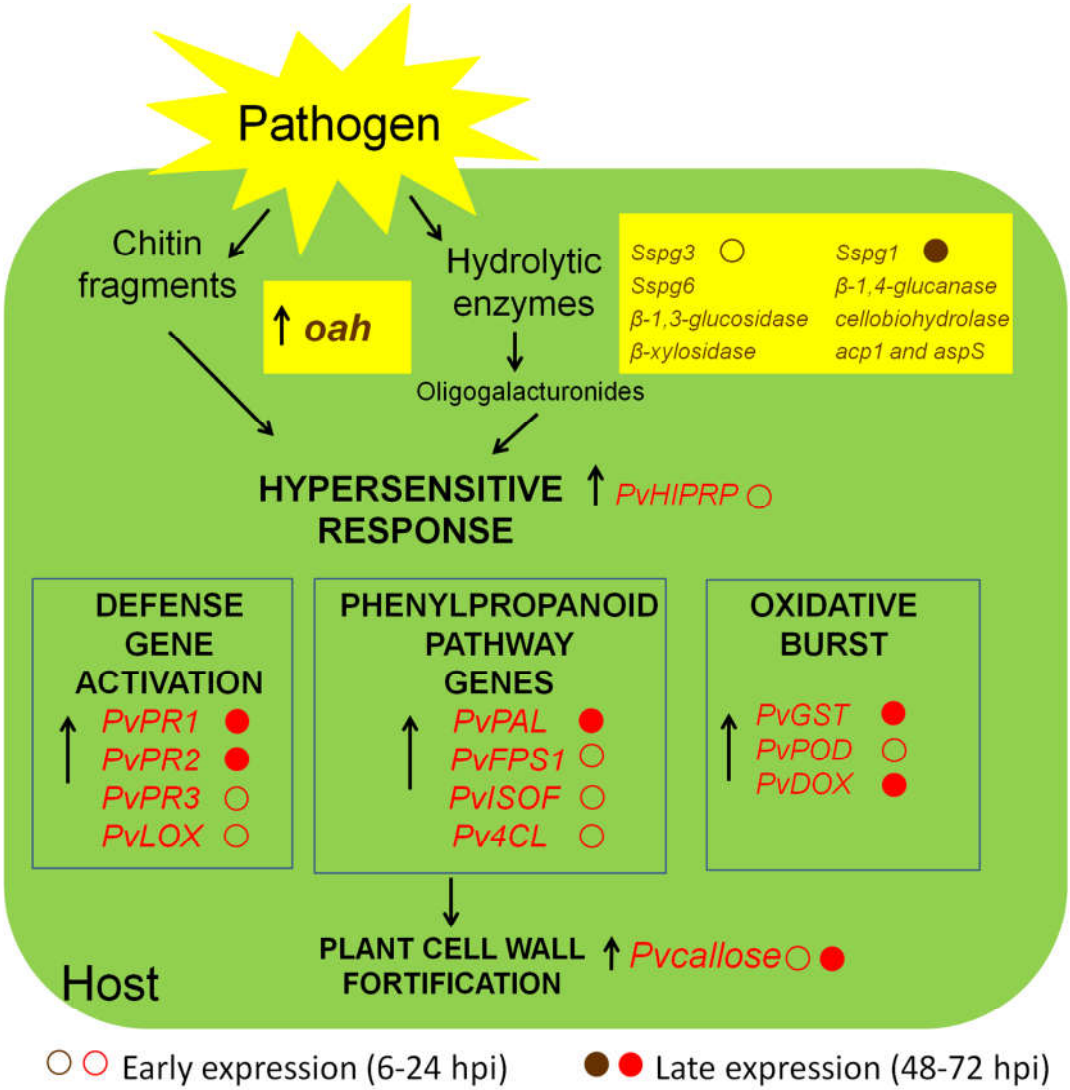

FIGURE 9: Schematic representation of the interaction between *S. sclerotiorum* and *P. vulgaris*. In the beginning of the pathogenic process, the fungus secretes hydrolytic enzymes (polygalacturonases, PGs) and oxalic acid. PGs degrade plant cell walls and release oligogalacturonides (OGs), which are elicitors of defense response. The hypersensitive response (HR) of the plant is used by the fungus as a strategy to promote virulence. Chitin fragments of fungus and OGs released from the plant itself allow the recognition of the presence of the pathogen and activate transcription factors which lead to the expression of genes belonging to different pathways of defense. The arrows indicate genes up-regulated in RTqPCR analysis. In brown, *S. sclerotiorum* genes, and in red, plant response genes.

The recent description of the genome sequences of two fungal necrotrophs, *S. sclerotiorum* and *Botrytis cinerea,* revealed 346 and 367 genes, respectively, encoding putative carbohydrate-active enzymes (CAZymes), with over 100 genes potentially associated with plant cell wall degradation (Amselem, *et al., 2011).* In this work, we observed that many genes encoding cell wall-degrading enzymes: polygalacturonases (*Sspg1, Sspg3*, and *Sspg6*), cellulases (β-1,3-glucosidase, β-1,4-glucanase, and cellobiohydrolase) and hemicellulases (β-xylosidase), were found to be differentially expressed and showed accumulated transcripts in the inoculated sample.

A >2-fold induction in expression was measured at 12 hpi for *Sspg3* (28-fold), *Sspg6*-encoding endo-PGs (3-fold) and β-1,3-glucosidase genes (4.8-fold), even though no necrotic symptoms in the stem are visible at this time. PGs enable a pathogen to invade plant tissues; however, on the other hand, their activity may also trigger plant defense responses. Namely, the degradation of homogalacturonan, the main component of pectin, by polygalacturonases results in the release of oligogalacturonides (OGs), which are capable of inducing plant immune reactions. The inactivation of PG activity by plant PGIPs has been shown to reduce disease development (Powell et al., 2000). More recent attempts to delay disease by expressing PGIPs, which are plant proteins that inhibit fungal endopolygalacturonases, have been successful against neurotropic fungi (Borras-Hidalgo et al., 2012).

The increase in *β-1,4-glucanase* (1.5-fold) and *cellobiohydrolase* (2.8-fold) transcript accumulation coincided with the phase of symptom development (48 – 72 hpi) in which intensive mycelial colonization of the stem occurred and soaked lesions were observed.

Proteases secreted by this fungus can degrade host plant proteins, some of which are involved in defense responses to fungal inoculation, and also seem to be correlated with symptom development (Bolton et al. 2006). During the *S. sclerotiorum–P. vulgaris* interaction the expression of the gene (*acp1*), encoding an acid protease, and the gene *aspS,* encoding an aspartyl protease, was low at the beginning of infection but increased at the stage of spreading necrosis. Proteases are also regarded as antagonists of the antifungal proteins secreted as part of the

defense response by the host. In previous reports, Poussereau et al., (2001) showed that *acp1* and *aspS* were expressed *in planta* during sunflower cotyledon infection.

In this work, oxaloacetate acetylhydrolase (*oah*) transcripts were already detected in the early stages of infection (6 hpi), and its expression was increased at the late stage of infection (72 hpi) (**Figure 4A**). Corroborating this, Liang et al., (2014) recently demonstrated that the Ss-*oah1* gene, which encodes an oxaloacetate acetylhydrolase, is required for oxalic acid accumulation and affects the pH-responsive growth, morphogenesis and virulence of *S. sclerotiorum* on a variety of plant hosts. Recently, oxalic acid was found to create reducing conditions in plant cells ahead of advancing hyphae. It was speculated that reductive conditions dampen the oxidative burst, allowing for precious time for fungal establishment prior to plant recognition (Kim et al., 2008). Also Kabbage et al., (2013, 2015) suggested that *S. sclerotiorum*, a prototypical necrotroph, has a biotrophic phase that occurs during the initial stages of disease establishment. During the early stages, oxalate dampens the host oxidative burst, an early response associated with plant defense, as part of a potential biotrophic interaction, before triggering generation of reactive oxygen species (ROS) at the later stages of infection, culminating in programmed cell death (PCD), the advanced necrotrophic phase of the interaction and disease.

The results presented in this study show that dry bean plants respond to inoculation with *S. sclerotiorum* in the early stages of the process. The *PvHIPRP* gene, related to hypersensitive responses (HR), was induced at the early stages of infection in infected tissue and had its expression peak at 12 hpi (4.8-fold). The importance of the HR for successful infection by necrotrophic pathogens is exemplified by studies showing that plants unable to undergo PCD (Dickman, 2001) or incapable of generating a HR (Govrin and Levine, 2000) are more resistant to such pathogens.

Among the genes involved in the defense and stress-related categories, *LOX* was activated in the early stages of infection (12 hpi – 6.8-fold). Lipoxygenase (LOX) catalyzes the oxidation of unsaturated fatty acids, producing oxylipins, which play a pivotal role in plant defense by acting as signaling molecules and/or protective compounds (Blée, 2002).

Plants that have to cope with oxidative stress can improve their ROS scavenging capacity via the upregulation of related enzymatic activities, such as glutathione S-transferase (Mhamdi et al., 2010), catalase, superoxide dismutase, and

peroxidase (Nanda et al., 2010). In the present study, *PvPOD* (peroxidase) was induced at 6 hpi and peaked in expression at 12 hpi (7.5-fold). The *PvGST* (glutathione S-transferase) and *PvDOX* (α-dioxygenase) genes presented a progressive increase in expression following infectious development. The plants lacking a dioxigenase, DOX2 (α-DOX2-deficient mutant), which catalyzes oxylipin production from fatty acids, were more susceptible to *Botrytis cinerea* (Angulo et al., 2015). During the interaction with *Rhizoctonia solani–P. vulgaris,* this gene was highly expressed, and it was suggested that this enzyme protects plant tissues undergoing excessive necrosis associated with oxidative stress during pathogenesis (Guerrero-González et al., 2011).

Once the plant has identified the attack, it can respond by activating the synthesis of a diverse number of proteins. These proteins include antibiotic proteins, such as pathogenesis-related (PR) proteins. The genes encoding the *PvPR1* and *PvPR2* protein families increased in expression 250-fold and 450-fold at 72 hpi, respectively, following fungal infection. Similarly, Borges et al., (2012b) reported the upregulation of *PvPR1* and a β-1,3-glucanase (*PvPR2*) during the incompatible interaction between the common bean and *Colletotrichum lindemuthianum.* Although this interaction is not the same, we found a similar expression profile in our experiment in which *PvPR1* and *PvPR2* were activated in the later stages of infection.

PR1 proteins have frequently been used as markers for systemic-acquired resistance (SAR) and have been associated with antifungal properties, such as the hydrolysis of fungal cell walls. These signaling events occur normally by hormones such as salicylic acid (SA), ethylene and jasmonic acid (JA), which can activate PR genes (Edreva, 2005; Ferreira et al. 2007; Leon-Reyes *et al., 2009*).

The expression of the *PvPR3* gene (chitinases) was observed only in the early stages of infection (6–12 hpi). *PvPR3,* like *PvPR2* (β-1,3-endoglucanase), may act directly by inhibiting the growth of the pathogen or indirectly by aiding in the generation of signaling molecules that may function as elicitors of further defensive mechanisms (Edreva, 2005; Van Loon et al., 2006; Ferreira et al., 2007).

Apart from inducible proteins, active plant defenses against pathogens also include phenylpropanoid pathway enzymes, which are known to be involved in plant disease resistance. The comparative analysis of the bean plant inoculated with *S. sclerotiorum* and the mock-inoculated plant (without infection) proved that the

PvISOF (isoflavonoid glucosyltransferase), *PvFPS1* (farnesyl pyrophosphate synthetase 1), *PvPAL* (phenylalanine ammonia-lyase), and *Pv4CL* (4-coumarate CoA-ligase) genes, which are involved in the phenylpropanoid pathway, were upregulated during infectious development.

PAL is the primary enzyme involved in the phenylpropanoid biosynthetic pathway and is responsible for the production of phenolic compounds, such as flavonoids, phytoalexins, lignins, and benzoic acid derivatives (Zhao et al., 2007). Several studies have demonstrated that the levels of PAL activity have important functions in the plant defense response to pathogen infection. *PvPAL* gene expression was induced by anthracnose fungal attack in *P. vulgaris* (Borges et al., 2012b), and PAL-suppressed plants were more susceptible to fungal infection (Maher et al., 1994).

In this study, the expression level of the *Pv4CL* gene was transient during the analyzed time period, with the greatest upregulation (9.5-fold) in the early stages (12 hpi) of infection. The 4-coumarate CoA-ligase is the third enzyme of the phenylpropanoid pathway in plants, and leads to the synthesis of lignin, pigments, and many defense molecules (Zabala et al., 2006).

In accordance with these findings, the results obtained here with aniline blue staining showed a progressive thickening of the lignin layers in dry bean plants during *S. sclerotiorum* infection. Certain phenylpropanoid compounds are polymerized to form defensive barriers, such as lignin, which is a crucial component of the plant defense repertoire against abiotic and biotic stress factors (Dixon et al., 2002). It has been strongly demonstrated that lignification is a common phenomenon in the expression of disease resistance in plants. Lignin synthesis is induced in response to mechanical damage or wounding, and many plants respond to invading pathogens with the deposition of lignin and lignin-like material (Vance et al., 1980; Nicholson and Hammerschmidt, 1992; Boudet et al., 1995; Dixon and Paiva, 1995). Additionally, callose is an effective barrier induced at the site of attack during the early stages of pathogen invasion and is an established marker associated with incompatible responses (Williams et al. 2011).

CONCLUSION

This study contributes to a better understanding of the kinetics of induced defenses against a fungal pathogen of the common bean, as well fungal genes related to pathogenicity and virulence, and provides a valuable first step towards the understanding and analysis of the *Phaseolus vulgaris* – *S. sclerotiorum* interaction.

AUTHOR CONTRIBUTIONS

MO carried out the experiments and drafted the manuscript. RA participated in the transcription analysis experiments. MG participated in the design of the study and drafted the manuscript. SP conceived the study and participated in its design and coordination. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Suplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb. 2015.01162

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Supplementary Material

Table S1 Number of reads/contig and their length distribution obtained in the SSH library

Contig	Size (BP)	Accession number	Gene Description	*E* value	Total ESTs in Contig
Fungal					
Contig 1 - SS_001	553	XP_390440.1	glutamine synthetase (*Gibberella zeae*)	8e-61	08
Contig 3 - SS_003	322	XP_001593397.1	perilipin mpl1-like protein (*Metarhizium flavoviride var minus*)	5e-29	04
Contig 5 - SS_004	549	XP_001593397.1	perilipin mpl1-like protein (*Metarhizium flavoviride var minus*)	2e-28	07
Contig 6 - SS_005	229	XP_003717922.1	homoserine dehydrogenase (*Magnaporthe oryzae*)	2e-11	06
Contig 8 - SS_006	861	NP_826813.1	endochitinase (*Streptomyces avermitilis*)	2e-82	02
Contig 9 – SS_007	321	XP_001596270.1	den domain-containing protein (*Sclerotinia sclerotiorum* 1980)	2e-31	02
Contig 10 - SS_008	465	ADH51542.1	perilipin MPL1-like protein CAP20 (*Sclerotinia sclerotiorum* 1980)	1e-07	10
Contig 12 - SS_010	417	BAB69489.1	guanine nucleotide-binding protein beta subunit (*Fusarium oxysporum*)	2e-65	04
Contig 13 - SS_011	658	XP_008088069.1	acid protease (*Sclerotinia sclerotiorum* 1980)	8e-15	02
Contig 14 - SS_012	299	EFY98676.1	sorting nexin-41 (*Metarhizium anisopliae*)	7e-20	02
Contig 16 - SS_014	378	EFY94441.1	aspartyl protease (*Sclerotinia sclerotiorum* 1980)	1e-20	03
Contig 17 - SS_015	331	EJP70905.1	transcription initiation protein (*Beauveria bassiana*)	2e-54	11
Contig 18 - SS_016	728	EHK19451.1	beta-1,6-glucanase putative (*Sclerotinia sclerotiorum* 1980)	5e-18	02
Contig 21 - SS_019	606	EFY99484.1	tripeptidyl peptidase precursor (*Metarhizium anisopliae*)	1e-14	02
Contig 22 - SS_020	348	EFZ04249.1	NAD transhydrogenase (*Metarhizium anisopliae*)	1e-32	02
Contig 23 - SS_021	355	ELQ33320.1	antiviral helicase ski2 (*Magnaporthe oryzae* Y34)	4e-38	02
Contig 25 - SS_022	646	CCT67208.1	serine peptidase putative (*Sclerotinia sclerotiorum* 1980)	3e-43	16
Contig 26 - SS_023	530	XP_001592294.1	serine endopeptidase (*Sclerotinia sclerotiorum* 1980)	2e-53	02
Contig 27 - SS_024	249	EGR50086.1	beta-xylosidase (*Sclerotinia sclerotiorum* 1980)	3e-15	03
Contig 28 - SS_025	696	AAP15044.1	alkaline proteinase (*Trichoderma hamatum*)	9e-144	07
Contig 29 - SS_026	449	EHK26726.1	glycoside hydrolase family 17 partial (*Trichoderma virens*)	1e-11	05
Contig 31 - SS_027	252	EFQ30677.1	catalase hydroperoxidase (*Glomerella graminicola*)	1e-31	02

Contig 33 - SS_029	550	EJP66560.1	α-glucosidase putative (*Sclerotinia sclerotiorum* 1980)	3e-40	02
Contig 34 - SS_030	415	AAP15044.1	alkaline proteinase (*Trichoderma hamatum*)	9e-33	03
Contig 36 - SS_032	335	XP_961711.1	GTP-binding protein gtr1 (*Neurospora crassa)*	6e-46	02
Contig 39 - SS_035	382	XP_007825222.1	oligopeptide transporter (*Metarhizium anisopliae*)	3e-30	02
Contig 40 - SS_036	382	XP_963374.1	protein sey1 (*Sclerotinia sclerotiorum 1980*)	6e-42	09
Contig 41 - SS_037	370	CAE47978.1	oleate delta-12 desaturase (*Aspergillus fumigatus*)	4e-30	05
Contig 42 - SS_038	520	EHK45968.1	glucan endo-1,3-β-glucosidase (*Sclerotinia sclerotiorum* 1980)	3e-22	02
Contig 43 - SS_039	541	CCF38756.1	glycosyl (*Colletotrichum higginsianum*)	1e-51	03
Contig 44 - SS_040	417	EFQ30677.1	peroxidase/ catalase (*Sclerotinia sclerotiorum* 1980)	2e-29	02
Contig 45 - SS_041	804	XP_001591700.1	beta-1,4-glucanase (*Sclerotinia sclerotiorum* 1980)	6e-73	05
Contig 46 - SS_042	229	XP_001275643.1	mitochondrial deoxynucleotide carrier (*Aspergillus clavatus*)	1e-11	02
Contig 47 - SS_043	373	EFZ02693.1	class e vacuolar protein-sorting machinery protein hse1 (*Metarhizium anisopliae*)	8e-28	03
Contig 48 - SS_044	430	XP_001586791.1	sec14 cytosolic factor SS1G_11820 (*Sclerotinia sclerotiorum*)	1e-71	02
Contig 52 - SS_047	371	CCE27416.1	bar domain-containing protein (*Metarhizium acridum*)	2e-26	02
Contig 53 - SS_048	318	ELA31202.1	oxidoreductase 2-nitropropane dioxygenase (*Sclerotinia sclerotiorum* 1980*s*)	9e-19	05
Contig 57 - SS_052	392	AAL84695.1	β-1,3-glucanase precursor (*Sclerotinia sclerotiorum* 1980)	6e-05	02
Contig 58 - SS_053	679	AAN62894.1	cell wall protein (*Saccharomyces cerevisiae*)	3e-13	10
Contig 59 - SS_054	665	ELA27998.1	oligopeptide transporter (*Colletotrichum gloeosporioides*)	1e-72	03
Contig 60 - SS_055	562	CCF44746.1	woronin body major protein (*Colletotrichum higginsianum*)	1e-61	02
Contig 61 - SS_056	257	EGR51636.1	translocation protein sec62 (*Sclerotinia sclerotiorum 1980*)	1e-22	03
Contig 62 - SS_057	481	XP_001586789.1	vacuolar protease A SS1G_11818 (*Sclerotinia sclerotiorum 1980*)	4e-53	06
Contig 63 - SS_058	411	EFY98807.1	proteasome component pre6 (*Sclerotinia sclerotiorum 1980*)	3e-47	03
Contig 64 - SS_059	301	EFY98401.1	mfs multidrug (*Metarhizium anisopliae*)	5e-47	02
Contig 65 - SS_060	395	EJP65591.1	NADH-ubiquinone oxidoreductase (*Beauveria bassiana*)	8e-71	02
Contig 66 - SS_061	502	EFY99256.1	ATP synthase beta chain (*Metarhizium anisopliae*)	3e-102	02
Contig 67 - SS_062	422	BAF37919.1	acetaldehyde dehydrogenase (*Claviceps purpurea*)	4e-68	02
Contig 74 - SS_068	221	SS1G_00477	ubiquitin homeostasis protein lub1 – phospholipase (*Sclerotinia sclerotiorum 1980*)	1e-12	05

Contig 75 - SS_069	331	SS1G_10538	ceramidase (*Sclerotinia sclerotiorum* 1980)	6e-20	08
Contig 76 - SS_070	497	EGR51699.1	predicted protein (*Trichoderma reesei*)	3e-35	02
Contig 77 - SS_071	297	SS1G_09620	hypothetical protein – GTPase (*Sclerotinia sclerotiorum 1980)*	2e-04	08
Contig 78 - SS_072	186	XP_001594760.1	pyruvate kinase (*Sclerotinia sclerotiorum 1980)*	4e-14	02
Contig 82 - SS_076	337	CCF45341.1	26s protease regulatory subunit 4 (*Colletotrichum higginsianum*)	3e-23	02
Contig 84 - SS_078	395	CCF34677.1	zz type zinc finger domain-containing protein (*Colletotrichum gloeosporioides*) (*Sclerotinia sclerotiorum 1980)*	6e-10	03
Contig 86 – SS_80	417	SS1G_14466	geranylgeranyl pyrophosphate synthetase (biosynthesis of secondary metabolites)	5e-25	03
Contig 87 - SS_081	329	EGR44538.1	conidial pigment polyketide synthase alb1 (*Trichoderma reesei*)	2e-20	06
Contig 88 - SS_082	588	CCF46208.1	cytochrome p450 alkane hydroxylase (*Sclerotinia sclerotiorum 1980)*)	4e-29	03
Contig 89 - SS_083	827	KDB11413.1	GTP binding protein (*Villosiclava virens*)	2e-39	02
Contig 90 - SS_084	496	EFQ30677.1	catalase/peroxidase (*Colletotichum sublineola*)	4e-54	04
Contig 91 - SS_085	305	EJP69242.1	ser thr protein phosphatase family (*Beauveria bassiana*)	2e-30	02
Contig 92 - SS_086	554	EHK17607.1	dynamin family protein (*Trichoderma viren*)	2e-28	02
Contig 97 - SS_090	307	EJT81032.1	ph-response regulator protein (*Sclerotinia sclerotiorum* 1980)	2e-09	02
Contig 98 - SS_091	540	SS1G_07146.3	cellobiohydrolase (*Sclerotinia sclerotiorum* 1980)	2e-40	02
Contig 99 - SS_092	740	EHK49081.1	glycoside hydrolase family 18 protein (*Trichoderma atroviride*)	2e-119	02
Contig 101 -SS_094	270	EHK44088.1	phosphoenolpyruvate carboxykinase (*Trichoderma atroviride*)	7e-22	02
Contig 102 - SS_095	383	XP_001272491.1	high affinity methionine permease (*Aspergillus clavatus*)	1e-31	02
Contig 103 - SS_096	778	XP_001588682.1	GTP-binding protein (*Sclerotinia sclerotiorum* 1980)	1e-51	02
Contig 105 - SS_098	502	EHK23397.1	high-affinity glucose transporter (*Trichoderma virens*)	1e-89	02
Contig 106 - SS_099	221	EJP67622.1	homoserine dehydrogenase (*Beauveria bassiana*)	3e-14	04
Contig 108 - SS_101	743	EGR45177.1	glutaryl- dehydrogenase (*Trichoderma reese*)	1e-80	02
Contig 111 - SS_104	336	XP_960423.1	fructose- -bisphosphatase (*Neurospora crassa*)	9e-30	02
Contig 116 - SS_108	398	EHK22417.1	hypothetical protein TRIVIDRAFT (*Trichoderma virens*)	3e-25	02
Contig 117 - SS_109	380	EGR50812.1	glycoside hydrolase family 20 protein (*Trichoderma reesei*)	1e-10	03
Contig 118 - SS_110	580	EFY98486.1	glutamine synthetase (*Metarhizium anisopliae*)	1e-48	03
Contig 119 - SS_111	305	XP_001210923.1	mitochondrial phosphate carrier protein (*Aspergillus terreus*)	1e-17	02

Contig 120 - SS_112	390	EJP65662.1	cytochrome b-c1 complex subunit 2 (*Beauveria bassiana*)	1e-61	02
Contig 121 - SS_113	423	EFY85620.1	eukaryotic translation initiation factor subunit (*Metarhizium acridum*)	6e-34	03
Contig 123 - SS_115	446	EHK50815	glycoside hydrolase family 18 protein Chitinase III (*Trichoderma atroviride)*	5e-23	04
Contg 124 - SS_116	1032	XP_001586359.1	glycosyl hydrolase (*Sclerotinia sclerotiorum* 1980)	2e-32	09
Contig 125 - SS_117	749	CCF36376.1	mac1 interacting protein 1 (*Colletotrichum higginsianum*)	2e-14	02
Contig 126 - SS_118	461	XP_001258000.1	beta-mannosidase (*Neosartorya fischeri*)	3e-33	02
Contig 127 - SS_119	400	EGR52289.1	tyrosine-protein phosphatase (*Trichoderma reesei*)	4e-27	02
Contig 129 - SS_121	582	XP_001593152.1	hypothetical protein SS1G_06074 (*Sclerotinia sclerotiorum* 1980)	2e-14	18
Contig 131 - SS_123	331	EFY85061.1	cystathionine beta-synthase (*Metarhizium acridum*)	1e-36	02
Contig 132 - SS_124	751	XP_002484510.1	hypothetical protein TSTA_040370 (*Talaromyces stipitatus*)	2e-50	13
Contig 135 - SS_127	655	ELA27998.1	oligopeptide transporter (*Colletotrichum gloeosporioides*)	4e-74	07
Contig 139 - SS_131	654	EJP70027.1	major facilitator superfamily transporter (*Beauveria bassiana*)	5e-63	03
Contig 142 – SS_133	336	SS1G_00477	Phospholipase (*Sclerotinia sclerotiorum* 1980)	1e-12	05
Contig 143 - SS_134	312	EJP63000.1	exocyst complex component sec10 (*Beauveria bassiana*)	6e-40	02
Contig 144 - SS_135	553	CAA10978.1	protein disulfide isomerase (*Trichoderma reesei)*	8e-63	02
Contig 145 - SS_136	431	EHK25266.1	beta-1,3-glucanase (*Sclerotinia sclerotiorum* 1980)	1e-64	08
Contig 148 - SS_139	303	EFY92509.1	sec24 related gene family (*Sclerotinia sclerotiorum* 1980)	3e-31	06
Contig 149 -			No significant similarity found		13
Contig 150 - SS_140	317	XP_003652726.1	glycoside hydrolase family 35 protein (*Thielavia terrestris*)	1e-21	02
Contig 153 - SS_143	569	ELA26500.1	2-nitropropane dioxygenase (*Sclerotinia sclerotiorum* 1980)	2e-44	02
Contig 155 - SS_145	316	EHK25869.1	glycoside hydrolase family 2 protein (*Trichoderma virens*)	2e-20	04
Contig 158- SS_148	597	EGX91377.1	cytochrome p450 51b (*Cordyceps militari*)	5e-111	02
Contig 159 - SS_149	388	EFY92417.1	beta-1,3-glucan synthase catalytic subunit (*Metarhizium acridum*)	6e-51	02
Contig 160 - SS_150	290	XP_001577682.1	GTP-binding protein (*Sclerotinia sclerotiorum* 1980)	6e-33	04
Contig 161 - SS_151	425	ELA31513.1	zinc transcription factor (*Sclerotinia sclerotiorum* 1980)	2e-44	28
Contig 163 - SS_153	221	ELA23385.1	polysaccharide deacetylase family protein (*Colletotrichum gloeosporioides*)	6e-22	03

Plant					
Contig 04 - SSPV_004	635	CAA09212.1	RNA helicase [*Arabidopsis thaliana*]	2e-34	3
Contig 02 - SSPV_002	489	AAF19401.1	phosphoenolpyruvate carboxylase kinase [*Glycine max*]	3e-72	13
Contig 03 - SSPV_003	607	AAD34548.1	cystathionine gamma-synthase [*Glycine max*]	1e-14	5
Contig 5 - SSPV_045	568	XP_003520228.1	ferredoxin-nadp reductase [*Glycine max*]	2e-16	3
Contig 06 - SSPV_006	657	AT3G20820	leucine rich repeat protein [*Arabidopsis thaliana*]	4e-47	5
Contig 07 - SSPV_007	489	XP_006601343.1	auxin response factor-like protein [*Glycine max*]	2e-57	7
Contig 08 - SSPV_008	653	CAA47345.1	heat shock protein 70 [*Phaseolus vulgaris*]	1e-94	3
Contig 09 - SSPV_009	556	AAX31514.1	DNA mismatch repair protein [*Phaseolus vulgaris*]	2e-144	4
Contig 10 - SSPV_010	488	ATCG01130	chloroplast chlorophyll a b-binding protein [*Arabidopsis thaliana*]	8e-30	3
Contig 11 - SSPV_011	532	AT4G35770	senescence-associated protein [*Arabidopsis thaliana*]	4e-60	4
Contig 12 - SSPV_012	483	AET79244.1	diphenol oxidase laccase [*Glycine max*]	5e-63	3
Contig 13 - SSPV_013	433	AGV54475.1	Lipoxygenase [*Phaseolus vulgaris*]	5e-22	3
Contig 14 - SSPV_014	501	CAA59482.1	pectin methylesterase [*Phaseolus vulgaris*]	1e-58	3
Contig 15 - SSPV_015	538	BAI68016.**1**	ATP synthase f1 subunit 1 [*O. sativa*]	5 e-70	3
Contig 16 - SSPV_016	665	XP_006583980.1	S-adenosylmethionine synthetase [*Glycine max*]	4 e-79	3
Contig 17 - SSPV_017	590	NP_177796	DNAJ heat shock protein [*Arabidopsis thaliana*]	1e-112	5
Contig 18 - SSPV_018	325	AGV54715.1	xyloglucan endotransglucosylase hydrolase [*Phaseolus vulgaris*]	4e-102	3
Contig 19 - SSPV_019	559	AAP69867	glutathione peroxidase 1 [*Lotus japonicus*]	1e-32	3
Contig 20 - SSPV_020	457	CAB16852	beta-galactosidase like protein [*Arabidopsis thaliana*]	2e-18	4
Contig 21 - SSPV_021	308	P06215	Endochitinase precursor [*Phaseolus vulgaris*]	3e-75	3
Contig 22 - SSPV_022	502	AAK84883	NAC domain protein NAC1 [*Phaseolus vulgaris*]	1e-23	3
Contig 23 - SSPV_023	555	XP_003526970.1	aspartyl aminopeptidase-like [*Gycine max*]	1e-56	6
Contig 24 - SSPV_024	689	AES79588.2	proteasome subunit alpha [*Medicago truncatula*]	9 e-54	9
Contig 25 - SSPV_025	368	AHA84124.1	elongation factor 1 [*Phaseolus vulgaris*]	7 e-37	6
Contig 26 - SSPV_026	601	P06215	class I chitinase [*Phaseolus vulgaris*]	1e-17	3
Contig 27 - SSPV_027	650	AAQ20042	cytochrome P450 [*Medicago truncatula*]	1 e-88	3
Contig 28 - SSPV_028	509	XP_003528630.1	polygalacturonase-like protein [*Gycine max*]	8e-14	3

Contig 29 - SSPV_029	336	AAR26001	endo-1,3-beta-glucanase [*Glycine max*]	1e-34	3
Contig 30 - SSPV_030	439	AAB00098	G-box binding factor [*Gycine max*]	2e-29	3
Contig 31 - SSPV_031	651	AAZ79659	sucrose synthase [Vigna angularis]	1e-28	2
Contig 32 - SSPV_032	408	AAB97165	ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit [*Phaseolus vulgaris*]	2e-31	10
Contig 33 - SSPV_033	532	AAZ79659	Putative cellulose synthase [*Fagus sylvatica*]	1e-10	3
Contig 34 - SSPV_034	568	AAA50172	Chlorophyll a/b-binding protein [*Glycine max*]	5e-19	3
Contig 35 - SSPV_035	640	AFF57838.1	Peroxidase [*Phaseolus vulgaris*]	9e-57	2
Contig 36 - SSPV_036	441	XP_003555942.1	ribose 5-phosphate isomerase [*Glycine max*]	4e-21	3
Contig 37 - SSPV_037	611	AEA47059.1	proteasome subunit alpha (*M. tuncatula)*	1e-42	2
Contig 38 - SSPV_038	341	AAC37357.1	Catalase [*Zea mays*]	7e-69	3
Contig 40 - SSPV_040	489	AGZ15378.1	fructose bisphosphate aldolase [*Phaseolus vulgaris*]	2e-123	3
Contig 41 - SSPV_041	621	AGV54395.1	s-adenosylmethionine decarboxylase [*Phaseolus vulgaris*]	2e-126	3
Contig 42 - SSPV_042	406	CAA40339.1	ribulose- -bisphosphate carboxylase oxygenase small subunit [*Phaseolus vulgaris*]	3e-52	5
Contig 43 - SSPV_043	431	AEE32999.1	26s protease regulatory subunit [*Arabidopsis thaliana*]	3e-42	3
Contig 44 - SSPV_044	549	CAB52473.1	ATP synthase beta subunit [*Arabidopsis thaliana*]	3e-23	5
Contig 45 - SSPV_045	453	AEE34128.1	glutamine amidotransferase subunit [*Glycine max*]	4e-37	2
Contig 46 - SSPV_046	307	AAC78474.1	GDP-mannose pyrophosphorylase [*Arabidopsis thaliana*]	1e-31	2
Contig 48 - SSPV_048	455	NP_001237049.1	ferritin- chloroplastic [*Glycine max*]	1e-47	2
Contig 49 - SSPV_049	297	ACJ61476.1	ubiquitin-protein ligase [*Glycine max*]	1e-59	5
Contig 50 - SSPV_050	346	AAD33696	PR1 a precursor [*Glycine max*]	1e-63	3
Contig 51 - SSPV_051	289	XP_007152897	hypothetical protein/ kinase family protein [*Phaseolus vulgaris*]	3e-60	3

4.2. Capítulo 2: Exogenous application of methyl jasmonate induces a defense response and resistance against *Sclerotinia sclerotiorum* in dry bean plants.

O mofo branco, doença causada por *S. sclerotiorum,* é responsável por perdas consideráveis, tendo se mostrado de difícil controle, sendo que dentre as principais culturas de interesse agrícola a resistência genética ao fungo não foi encontrada até o momento. Assim, o desenvolvimento de estratégias alternativas e ambientalmente viáveis do controle dessa doença é essencial.

O uso de indutores químicos de resistência em plantas tem sido uma alternativa bastante viável, já que a aplicação exógena desses produtos leva ao acúmulo de metabólitos secundários aumentando a resistência das plantas contra patógenos.

Nesse sentido, o presente trabalho teve o objetivo de investigar o papel do indutor de resistência metil jasmonato (MeJa) na defesa de plantas de feijoeiro (*P. vulgaris*) contra *S. sclerotiorum*. Para isso, foi utilizada a metodologia de hibridização subtrativa por supressão (HSS) de cDNA para identificar genes que são diferencialmente expressos durante a interação planta-patógeno após o tratamento com MeJa.

A aplicação foliar de MeJA induziu resistência parcial contra *S. sclerotiorum* em plantas de feijoeiro e determinou aumento consistente na atividade de proteínas relacionadas à patogênese e outros marcadores de resistência indusida.

Nossos resultados fornecem importantes informações sobre os mecanismos moleculares e fisiológicos da indução de resistência por MeJA em *P. vulgaris* durante a interação com *S. sclerotiorum.*

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Molecular Biology

Exogenous application of methyl jasmonate induces a defense response and resistance against *Sclerotinia sclerotiorum* in dry bean plants

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ABSTRACT

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic fungal pathogen that causes a disease known as white mold, which is a major problem for dry bean (*Phaseolus vulgaris* L.) and other crops in many growing areas in Brazil. To investigate the role of methyl jasmonate (MeJA) in defending dry bean plants against *S. sclerotiorum*, we used suppression subtractive hybridization (SSH) of cDNA and identified genes that are differentially expressed during plant–pathogen interactions after treatment. Exogenous MeJA application enhanced resistance to the pathogen, and SSH analyses led to the identification of 94 unigenes, presumably involved in a variety of functions, which were classified into several functional categories, including metabolism, signal transduction, protein biogenesis and degradation, and cell defense and rescue. Using RT-qPCR, some unigenes were found to be differentially expressed in a time-dependent manner in dry bean plants during the interaction with *S. sclerotiorum* after MeJA treatment, including the pathogenesis-related protein *PR3* (chitinase), *PvCallose* (callose synthase), *PvNBS-LRR* (NBS-LRR resistance-like protein), *PvF-box* (F-box family protein-like), and a polygalacturonase inhibitor protein (PGIP). Based on these expression data, the putative roles of differentially expressed genes were discussed in relation to the disease and MeJA resistance induction. Changes in the activity of the pathogenesis-related proteins β-1,3-glucanase, chitinase, phenylalanine ammonia-lyase, and peroxidase in plants after MeJA treatment and following inoculation of the pathogen were also investigated as molecular markers of induced resistance. Foliar application of MeJA induced partial resistance against *S. sclerotiorum* in plants as well as a consistent increase in pathogenesis-related protein activities. Our findings provide new insights into the physiological and molecular mechanisms of resistance induced by MeJA in the *Phaseolus vulgaris*–*S. sclerotiorum* pathosystem.

Keywords: Methyl jasmonate. Dry bean. Pathogenesis-related genes. PGIPs. White mold

Introduction

The necrotrophic fungus *Sclerotinia sclerotiorum* (Lib.) de Bary is capable of infecting at least 408 economically important plant species (Boland and Hall, 1994). In Brazil, this pathogen causes severe damage to dry bean (*Phaseolus vulgaris* L.) crops, resulting in yield losses. The disease in beans, commonly referred to as white mold, is difficult to control due to the persistence of the pathogen as sclerotia in the soil and plant debris (Bolton et al., 2006).

The physiological and molecular mechanisms of resistance to white mold in *P. vulgaris* genotypes remain poorly understood, and thus far, only partial resistance has been reported in this species (Miklas and Grafton, 1992; Kolkman and Kelly, 2002). Moreover, the lack of adapted resistance sources inhibits the progress in breeding cultivars with white mold resistance (Miklas and Grafton, 1992; Kolkman and Kelly, 2002; Miklas et al., 2001; Schwartz and Singh, 2013). Therefore, the development of alternative control strategies is essential. The use of chemical resistance inducers is an additional opportunity for controlling plant diseases within an integrated crop protection system.

Stress in plants often promotes the accumulation of a wide range of secondary metabolites that act to protect the plants. Based on this principle, there are many strategies of treating plants with various chemical resistance inducers to promote crop protection (Wang et al., 2012).

Jasmonates are important signaling molecules produced by plants; jasmonates regulate, either in positive or negative crosstalk with ethylene, subsets of genes involved in defense against necrotrophic microorganisms (Grant and Lamb, 2006; Guo and Stotz, 2007; Kravchuka et al., 2011; Yu et al., 2011; Wang et al., 2012; Zhang et al., 2012). In previous studies, exogenous applications of methyl jasmonate (MeJA) have been shown to induce the accumulation of defense-related genes, including increased activities of pathogenesis-related proteins, as well as cause oxidative bursts, phytoalexin accumulation, lignification, and cell wall stiffening (Durrant and Dong, 2004; Guo and Stotz, 2007; Fujimoto et al., 2011; Wang et al., 2012; Chen et al., 2012).

To date, there is a paucity of detailed published information on MeJA signaling molecules for the interaction of *P. vulgaris–S. sclerotiorum*. Therefore, the main objectives of this study were to investigate the molecular basis of partial resistance

induced by MeJA treatment and to identify the molecular mechanisms of the dry bean (*P. vulgaris* L.) response to *S. sclerotiorum*. To achieve these goals, we performed a suppression subtractive hybridization (SSH) study and compared the gene expression at time points during the period of early infection in a susceptible dry bean genotype. Additionally, using the RT-qPCR approach, we evaluated the temporal expression profile of genes involved in plant defense. The study of the identification and regulation of these genes can provide valuable insights into the resistance induced by MeJA in dry beans against white mold, one of the most devastating diseases for this crop.

Materials and methods

Plant material and pathogen inoculation

Dry bean plants (*Phaseolus vulgaris* L. cv.BRS Pérola) that are susceptible to *S. sclerotiorum* were grown in 2-kg plastic pots filled with soil fertilized with NPK (4-39-16) (1 g/kg soil) in a greenhouse at 24 ± 2 ºC with 60 ± 5% relative humidity and a 16 h/8 h light/dark period. Approximately 10–12 days after seeding, the primary leaves were completely expanded and the plants were sprayed with an aqueous solution of 10 µM MeJA (10 mL/plant; 100 plants/treatment). The inoculation with *S. sclerotiorum* was performed 12 h after the application of the chemical inducer. Control plants were sprayed with 10 mL of distilled water and then were also inoculated with the pathogen.

Sclerotinia sclerotiorum isolate SPS was collected from a naturally infected dry bean plant and then grown on Petri dishes containing potato-dextrose agar (PDA) culture medium for 5 days at 20 ºC, and 5-mm plugs of these cultures were placed in the leaf axils to inoculate dry bean plants. All of the plants were kept at 20 ºC and 90% relative humidity to provide adequate conditions for infection. Tissue samples (hypocotyl segments) were collected at 6, 12, 24, 48, and 72 h post-inoculation (hpi) and immediately frozen in liquid nitrogen prior to RNA and protein extraction. The experimental design was completely randomized, consisting of three biological replicates for each of the treatments. For each of the experimental conditions, hypocotyl segments (approximately 10 mm) from 20 different plants were pooled together to form one of three biological replicates.

RNA extraction and cDNA library synthesis

RNA was extracted from plant tissues (hypocotyl segments) and fungal material using the standard Trizol protocol (Invitrogen Corp., Carlsbad, CA, USA). Total RNA samples were quantitatively examined using the Qubit (Invitrogen Corp., Carlsbad, CA, USA) and qualitatively with agarose gel electrophoresis. To construct the SSH library, two different RNA mixtures were prepared as the source of tester and driver samples. For the tester, equal levels of total RNA were isolated from stem tissues of plants treated with MeJA and inoculated for 12, 24, 48, and 72 h with *S. sclerotiorum* were mixed, whereas for the driver, RNA of non-treated and inoculated plant stem tissues harvested at the same times (12 to 72 h) were used.

SSH, differential screening, and sequencing

For SSH, 1.0 µg of each mixture of total RNA was used to produce double-stranded cDNA using the Super SMART cDNA synthesis kit (Clontech Laboratories, Palo Alto, CA, USA) in accordance with the manufacturer's protocol. A forward-subtracted cDNA library (where infected plants served as the tester and mock plants as the driver) was constructed, cloned using the pCR4-TOPO vector (Invitrogen Corp., Carlsbad, CA, USA), and used to transform One Shot TOP10F electrocompetent *Escherichia coli* cells (Invitrogen Corp., Carlsbad, CA, USA). Plasmid DNA was obtained using the alkaline lysis procedure (Sambrook and Russel, 2001). The cloned products were sequenced using M13 forward primer on an ABI PRISM 3130 DNA automated sequencer (Applied Biosystems, Carlsbad, CA, USA).

Sequencing and bioinformatic analyses of expressed sequence tags (ESTs)

For this purpose, the cloned products were sequenced using an M13 forward primer and the Big Dye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems). Automated capillary electrophoresis sequencing runs were performed on an ABI Prism 3130 (Applied Biosystems, Carlsbad, CA, USA). The sequences obtained were processed using Phred. Only sequences with at least 100 nucleotides and a Phred

quality value ≥ 20 were kept for further analysis. The vector sequence and adaptors were removed using Phred (Ewing and Green, 1998) and Cross Match software (http://www.phap.org/). Cleaned expressed sequence tags (ESTs) were assembled into contigs using the CAP3 assembly program. The sequences were compared with the GenBank non-redundant (nr) database using the BLASTx algorithm from the National Center for Biotechnology Information (http://www.ncbi.nih.gov). Functional annotation by gene ontology terms (http://www.geneontology.org) was analyzed using the Blast2GO program (Conesa and Götz 2008). KEGG pathway annotation was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.genome.jp/kegg).

Reverse Transcriptase – Quantitative PCR (RT-qPCR)

Primers were designed using Software Primer Express (Applied Biosystems, Carlsbad, CA, USA) for amplification of gene fragments of approximately 80-150 bp in length and an annealing temperature of 60ºC (Table S1- supplementary materials). The primer specificity was checked *in silico* against the NCBI database (http://www.ncbi.nlm.nih.gov/) with the Primer-BLAST tool. The selected primers were tested by PCR, and their specificity was checked by nucleotide sequencing on an ABI 3130 sequencer (Applied Biosystems, Carlsbad, CA, USA) using DyeTerminator chemistry to confirm their identities.

The reverse transcriptase – quantitative polymerase chain reaction (RT-qPCR) system using SYBR Green detection (Applied Biosystems, Carlsbad, CA, USA) was employed for gene expression analysis of RNA samples. After treatment with DNase I (Invitrogen Corp., Carlsbad, CA, USA) in the presence of RNase inhibitor (Invitrogen Corp., Carlsbad, CA, USA), equal levels of RNA (1 µg) were reverse transcribed using oligo(dT)12-18 primer and evaluated with qPCR. Amplification assays were performed using a StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) and 10 µL reactions containing 0.4 µM of each primer (Supplementary Material, Table S1), 6 µL of SYBR Green PCR Master mix (2 ×), and 0.2 µL of template cDNA. After initial denaturation at 95 ºC for 10 min, the amplifications were performed with 40 cycles at 95 ºC for 15 s and at 60 ºC for 1 min. To check the specificity of the qPCR assay, the melting curves were analyzed for each point. The relative gene expression was obtained with the formula: fold

induction = $2^{-\Delta\Delta C}{}_{T}$ (Livak and Schmittgen, 2001). The *phaseolus vulgaris actin11* gene was used as the reference gene (Borges et al., 2012a). Samples were analyzed in triplicate for each treatment and values are expressed as the means ± standard deviation (SD).

Determination of the enzyme activity

Tissue samples (hypocotyl segments) collected from plants treated with or without MeJA and infected with *S. sclerotiorum* were macerated in liquid nitrogen and homogenized in 1 M NaCl and 0.1 M sodium acetate buffer, pH 5.2 (3 mL/g tissue). The homogenate was centrifuged at 20.000 × *g* at 4 ºC for 25 min. The supernatant was considered the tissue extract. Total proteins were determined using the Quant-iT Protein Assay kit (Invitrogen Corp., Carlsbad, CA, USA).

Peroxidase (POX) activity was assayed spectrophotometrically with the speed of oxidation of guaiacol in the presence of hydrogen peroxide (H_2O_2), as described by Małolepsza (2006). The reaction mixture consisted of 0.1 mL of tissue extract and 2.9 mL of a solution containing 250 µL of guaiacol (Sigma-Aldrich, St. Louis, MO, USA) and 306 µL of H_2O_2 in 100 mL of 0.01 M phosphate buffer, pH 6.0, whereas the reference cuvette had 3 mL of the same solution. A unit of peroxidase activity was defined as an increase of 0.1 absorbance units per minute.

Phenylalanine ammonia-lyase (PAL) activity was measured using L-phenylalanine as a substrate for determining the level of trans-cinnamic acid produced. To measure the PAL activity, a 100 µL aliquot of the enzyme extract was incubated for 2 h at 40 ºC in 400 µL of 5 mM L-phenylalanine in 25 mM Tris HCl buffer, pH 8.8, and 500 µL of 25 mM Tris HCl buffer. The blank contained 100 µL of 100 mM sodium acetate buffer. The amount of trans-cinnamic acid produced was spectrophotometrically measured at 290 nm. Because one unit of activity is defined as the amount of enzyme that produces an increase in absorption at 290 nm of 0.01 per hour in the standard assay, this change in absorption is equivalent to the formation of approximately 1 µg of trans-cinnamic acid per mL of reaction mixture (Zucker, 1968).

Chitinase (CHIT) activity was determined by measuring the rate of formation of reducing sugar using colloidal chitin (Sigma-Aldrich, St. Louis, MO, USA) as a substrate. A standard curve, constructed by plotting known concentrations of

glucose, was used to calculate the amount of reducing sugar released in the reaction. The reaction mixture consisted of 50 µL of supernatant of macerated tissue extract with 50 µL of 1% colloidal chitin dissolved in 100 mM sodium acetate buffer, pH 5.0. After incubation at 40 ºC for 60 min in water bath, the samples received 1 mL of dinitrosalicylic acid (DNS) and were homogenized under agitation and heated in boiling water bath for 5 min. After cooling on ice, the content of each tube was diluted with 1 mL of distilled water, and the absorbance was measured with a spectrophotometer at 550 nm. The results are expressed in units of activity (U), where one unit of enzyme activity (U) is defined as the amount of enzyme that can release one µmol of reducing sugar/min under the described conditions.

The activity of β-1,3-glucanase (GLU) was determined by measuring the amount of reducing sugar released from laminarin (β-1,3-glucan) (Sigma-Aldrich, St. Louis, MO, USA). The reaction medium consisted of 0.05 mL supernatant and 0.1 mL of substrate solution (0.25% laminarin solution in 50 mM acetate buffer, pH 5.2). After incubation at 50 ºC for 30 min, the concentration of reducing sugar was determined using the DNS method (Miller, 1959).

The polygalacturonase (PG) activity was quantified by recording the increase in absorbance at 575 nm caused by the release of reducing sugar from polygalacturonic acid. The reaction mixture contained 25 µL of macerated tissue extract and 225 µL of 0.5% orange polygalacturonic acid (PGA, Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.1 M sodium acetate buffer, pH 5.0. After 30 min of incubation at 50 ºC, the reaction was stopped by adding 750 µL of DNS reagent; then, the mixture was boiled for 5 min and cooled on ice (Miller, 1959). The enzyme activity was expressed as micrograms of reducing sugar released during the 30-minute incubation. The enzyme and substrate controls were included in all assays.

The inhibitory effect of polygalacturonase-inhibiting proteins (PGIPs) on the activity of polygalacturonase (PG) was assayed using as the PG from the supernatant of *S. sclerotiorum* culture grown on minimal medium (2 g/L NH_4NO_3, 1 g/L KH_2PO_4, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L yeast extract, 3 g/L DL-malic acid, and 1 g/L NAOH) supplemented with 1% citrus pectin under agitation for 48 h. This material was blended in a ratio of 1:10 with tissue extract (source of PGIP) obtained at the different collection times. The enzyme activity was performed using 0.1 M sodium acetate, pH 5.0, with 0.5% polygalacturonic acid (PGA) as a substrate. The reaction conditions were the same as those used to evaluate the PG activity. The samples

were analyzed in triplicate for each treatment. The results are expressed as the mean ± standard deviation (SD).

Results and discussion

The initial purpose of this study was to test if exogenous application of MeJA could induce dry bean resistance against *S. sclerotiorum*. Plants of the susceptible dry bean cultivar Pérola treated with or without MeJA were inoculated with *S. sclerotiorum* by placing BDA plugs in the leaf axils.

The induction of a defense response against *S. sclerotiorum* was evidenced by the decrease in infection in the susceptible cultivar of dry bean after the exogenous application of 10 µM MeJA. There was a necrosis point as early as 24 hpi in control plants, and the expansion of the water-soaked lesion led to browning and collapse of the infected hypocotyl tissue at 72 hpi (Fig. S1A, Supplementary Material). However, a significant delay in the symptom development was registered when plants treated with 10 µM MeJA were inoculated with *S. sclerotiorum* because they did not have necrotic points at 72 hpi (Fig. S1B), suggesting that MeJA is involved in the induction of partial resistance of host plants against *S. sclerotiorum* infection.

We investigated whether MeJA directly affects the growth of *S. sclerotiorum.* Mycelial plugs (5 mm) were taken from a uniform mycelial culture and transferred to PDA culture medium supplemented with 10 µM MeJA. Fungal growth was measured every 24 h in orthogonal directions on three Petri dishes. Based on our results, no effects of MeJA were observed on the growth of *S. sclerotiorum* at a concentration of 10 µM. The fungal growth rate with the medium containing MeJA (0.89 ± 0.10 cm/day) was not significantly different from the average growth rate of the control (0.90 ± 0.10 cm/day).

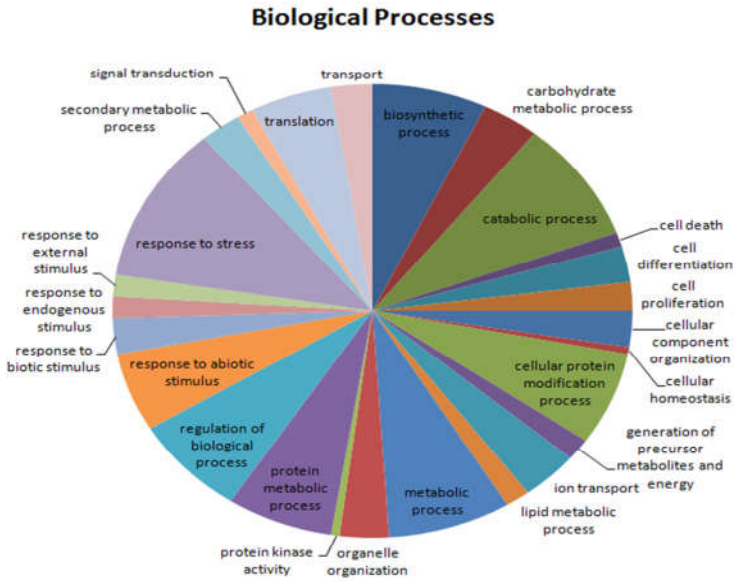
Functional classification of the significantly differentially expressed genes

To investigate the role of MeJA in the partial resistance of dry bean plants against *S. sclerotiorum,* we constructed an SSH library of *P. vulgaris* using two different RNA mixtures, which were prepared as sources of tester and driver samples. For that, equal levels of total RNA isolated from stem tissues of plants

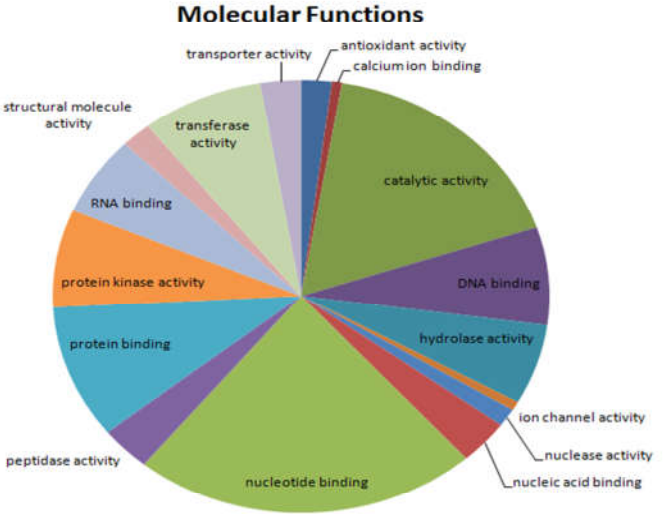
treated with MeJA and inoculated at 12, 24, 48, and 72 h with *S. sclerotiorum* were mixed. For the driver, we used RNA of non-treated and inoculated plant stem tissues, harvested at the same times (12 to 72 h). Preliminary analyses of sequences from the forward library (MeJA–*S. sclerotiorum* inoculation) revealed a total of 207 differentially expressed ESTs.

ESTs were classified into functional categories based on their similarities with known proteins. Functional annotation of the ESTs from the forward library was analyzed using gene ontology terms (http://www.geneontology.org) and the Blast2GO program, revealing that 22% of the ESTs had no match in the public database, whereas 99 unigenes were identified (Table S2, Supplementary Material'). Most of the genes expressed in the forward library are associated with biological processes as follows: 11% involved in response to stress, 8.6% in catabolic processes, 7.1% in the regulation of biological processes, 5.6% in response to abiotic stimulus, 2.5% in response to biotic stimulus, 1% in cell death, and 1% in signal transduction (Fig. 1A). The functional categories that were enriched in the SSH library may give an indication of some important molecular processes that take place during the phases of infection. For example, 19.1% of the genes (11% involved in response to stress, 5.6% in response to abiotic stimulus, and 2.5% in response to biotic stimulus, as already stated), representing a significant percentage of the assessed unigenes, are involved in the response to different types of stress. This finding indicates that plant defenses may be highly active in these initial phases (MeJA treatment) and play a role in minimizing the relative fungal growth. Additionally, based on the molecular functions, 22.5% of the sequences were classified as nucleotide binding, 17.2% as catalytic activity, and 9.9% as protein binding (Fig. 1B), whereas 15.9% presented as relevant to nuclear function and 10% plasma membrane (Fig. 1C). Together, these results suggest that the induction of partial resistance in dry bean plants with an exogenous MeJA supply occurs through transcriptional reprogramming, both in signaling and metabolism, during their early response to *S. sclerotiorum*.

A


Biological Processes
signal transduction
transport
carbohydrate metabolic process
secondary metabolic process
translation
biosynthetic process
catabolic process
cell death
cell differentiation
cell proliferation
cellular component organization
cellular homeostasis
response to external stimulus
response to endogenous stimulus
response to biotic stimulus
response to stress
response to abiotic stimulus
cellular protein modification process
generation of precursor metabolites and energy
ion transport
lipid metabolic process
regulation of biological process
protein metabolic process
metabolic process
protein kinase activity
organelle organization

B


Molecular Functions
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antioxidant activity
calcium ion binding
structural molecule activity
transferase activity
catalytic activity
RNA binding
DNA binding
protein kinase activity
protein binding
hydrolase activity
ion channel activity
nuclease activity
peptidase activity
nucleotide binding
nucleic acid binding

C

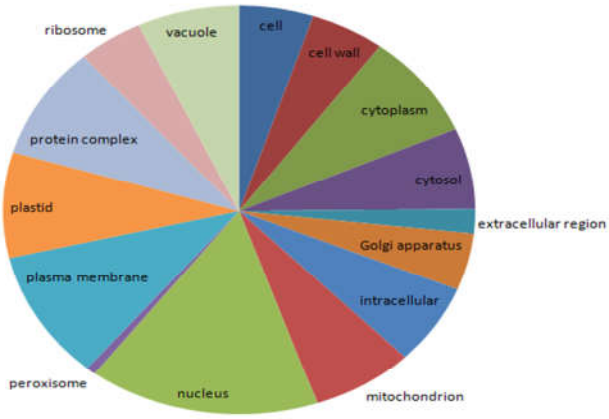
Cellular Components
ribosome
vacuole
cell
cell wall
cytoplasm
protein complex
cytosol
plastid
extracellular region
Golgi apparatus
plasma membrane
intracellular
peroxisome
nucleus
mitochondrion

Fig. 1. Summary of the Gene Ontology annotation as assigned by BLAST2GO. Gene Ontology classification of ESTs from *P. vulgaris* SSH library according to: (A) biological processes; (B) molecular functions; (C) cellular components.

Confirmation of differential gene expression by quantitative PCR

Time-course gene expression analysis was performed for categories of defense-related genes to validate the SSH screening (Table S1). Through qRT-PCR quantification of samples collected from plants treated with MeJA and infected with *S. sclerotiorum* (MeJA treatment) and plants treated with water and infected with *S. sclerotiorum* (Control), the selected transcripts were differentially expressed in different developmental infection stages of the pathogen at 0, 12, 24, 48, and 72 hpi. As a result, MeJA is an effective elicitor of systemically induced defense mechanisms against *S. sclerotiorum* in dry bean plants. Exogenously applied MeJA via foliar application resulted in strong induced defense in the systemic tissue (hypocotyl), exemplified by the strong upregulation of *PvChit1* (chitinase). Additionally, a systemic upregulation of *PvCallose* (Callose synthase), *PvNBS-LRR* (NBS-LRR resistance-like protein), and *PvF-box* (F-box family protein-like) genes was also observed (Fig. 2).

The *PR3* or *PvChit1* (chitinase) gene was significantly upregulated during *S. sclerotiorum* infection (6–72 hpi) in plants treated with MeJA (Fig. 2A). This may contribute to the increased resistance, whereas the over-expression of chitinase in transgenic plants has been reported to reduce or retard the development of disease after fungal attack in studies involving resistance to *Botrytis cinerea* (Kishimoto et al., 2002), *Magnaporthe grisea*, and *Rhizoctonia solani* (Li et al., 2009).

Similarly, the deposition of callose, a (1,3)-β-glucan cell wall polymer, can also play an essential role in the defense response to invading pathogens. This is because penetration resistance is based on the transport of callose synthase to the site of attempted fungal penetration and the subsequent formation of enlarged callose deposits (Naumann et al., 2013). In this study, we observed that the *PvCallose* (callose synthase) gene was more strongly induced after MeJA treatment at 6 hpi compared with the control (infected plant) (Fig. 2B). That may in turn have contributed to enhancing dry bean resistance to *S. sclerotiorum* in the early stage of infection; however, we did not directly test that association.

Our analysis also showed that among all of the upregulated genes after foliar hormone application, the *PvNBS-LRR* (NBS-LRR resistance-like protein) gene was

significantly enriched (Fig. 2C). This finding suggests that the signal perception was extensively activated early in plants infected with *S. sclerotiorum* after MeJA treatment.

The F-box protein family plays a crucial role in plant growth and development as well as in their response to biotic and abiotic stresses (Jia et al., 2013). The observed expression profile (Fig. 2D) suggests that the dry bean F-box gene (*PvF-box*) had temporal expression patterns after MeJA treatment. In *Arabidopsis*, *COI1*, an F-box protein, is an important factor in the jasmonic acid (JA) signal response and is required for JA-dependent responses (Sheard et al., 2010). Following the production of JA at the site of tissue damage and transmission of the long distance signal, the activation of defense responses in systemic tissues requires COI1 (Li et al., 2002).

Similarly, we also observed a systemic upregulation of the *PvHIPRP* (hypersensitive-induced response protein) mRNA levels in the stem after foliar applications of MeJA during the early steps of defense perception signaling (6 hpi; Fig. 2E). This result agrees with the previous finding of Garg et al. (2008), who reported that resistant *Brassica napus* genotypes frequently respond with a hypersensitive reaction when infected with *S. sclerotiorum*, as evidenced by the localized necrosis of the palisade mesophyll cells near the site of infection at 4 and 6 days post-inoculation. Additionally, hypersensitive-induced response (HIR1) genes have already been shown to induce the expression of defense-related genes as well as to confer enhanced resistance to bacterial pathogens (*Pseudomonas syringae* and *Xanthomonas campestris*) and an oomycete (*Hyaloperonospora parasitica*) in transgenic *Arabidopsis* plants (Zhou et al., 2010).

We specifically demonstrated that *PvPR1* and *PvPR2*, members of the PR gene superfamily, were strongly induced in *P. vulgaris* plants treated with water in response to *S. sclerotiorum* infection. In contrast, treatment with MeJA suppressed PvPR1 and PvPR2 gene expression, resulting in the downregulation of these genes in response to infection with the necrotrophic fungus *S. sclerotiorum* (Fig. 2 F and H). PR1 proteins have frequently been used as markers for systemic-acquired resistance (SAR), and at the time of pathogen attack, *PR1* genes are transcriptionally regulated by either the SA (*van Loon et al., 2006; Wang et al., 2012; Borges et al, 2012b)* or ET/JA-signaling pathways (Mei et al., 2006; Mitsuhara et al., 2008; Yu et al., 2011), which have antagonistic relationships.

Additionally, other genes, including those involved in the phenylpropanoid pathway such as *PAL* (phenylalanine ammonia-lyase gene), which are also involved in phytoalexin biosynthesis, as well as genes involved in defense- and stress-related categories such as *LOX* (lipoxygenases), were up-regulated in plants treated with water in response to infection with *S. sclerotiorum* (Fig. 2G and H). Activation of these genes (*PvPal* and *PvLox*) was also repressed by treatment with MeJA, suggesting that the JA biosynthesis pathway may be a target for SA-mediated antagonism. Comparing all observations of defense genes, we conclude that the foliar application of MeJA has a strong potentiating effect on systemic defense pathways in dry bean plants. Induced defense responses in plants are extremely complex and more than one type of defense can simultaneously be engaged. There is extensive cross-talk between SA and JA/ET signaling. Most of this cross-talk consists of mutual repression, although some genes can be induced by both exogenous SA and JA administration (Grant and Lamb, 2006; Guo and Stotz, 2007; Yu et al., 2011; Zhang et al., 2012). The molecular mechanisms responsible for the negative cross-talk between JA and SA signaling are not well understood. A recent report by Wang et al. (2012) suggests that defense against *S. sclerotiorum* in oilseed rape is associated with the sequential activation of SA and JA signaling. The authors identified a set of SA and JA signaling marker genes in *B. napus* and used them to monitor the signaling responses of the plant to *S. sclerotiorum* infection by examining their temporal expression profiles. SA signaling was activated within 12 hpi and then followed by JA signaling, which was activated at approximately 24 hpi. Moreover, SA–JA crosstalk genes were activated during this process.

We evaluated the potential side effects of foliar treatment with 10 µM MeJA on plant development (leaf area, chlorophyll content, stem length, flower development, total nitrogen content and dry mass of the aerial part, root volume and dry mass), but no observable phenotypic effects on plant growth or development were detected (data not shown).

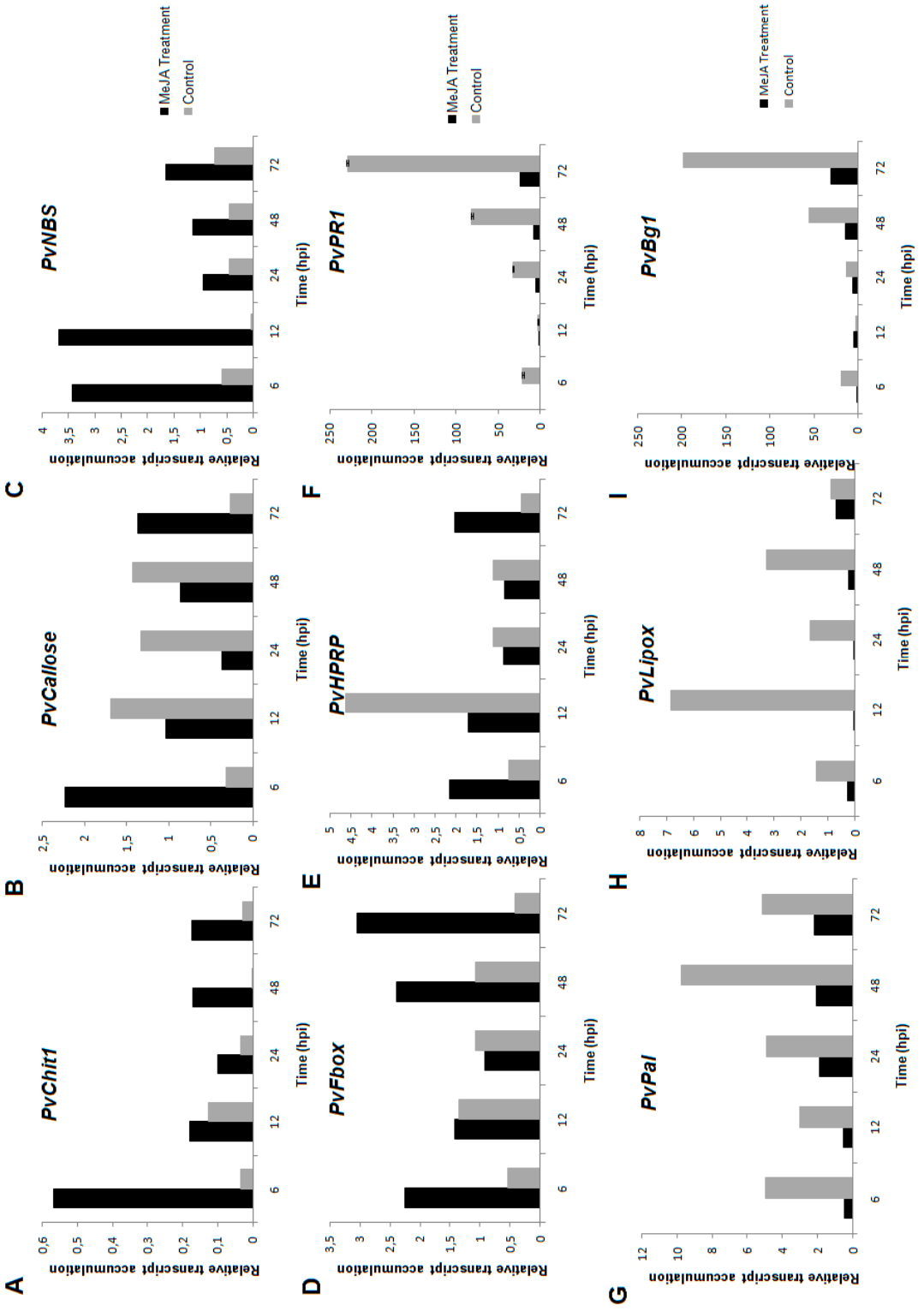

Fig. 2. Patterns of significantly differentially expressed genes in a time-dependent manner in dry bean (*P. vulgaris* L.) stem during the interaction with *S. sclerotiorum* and in response to treatment with 10 µM methyl jasmonate (MeJA). Expression analyses using RT-qPCR were performed and transcript levels were calculated in triplicate using a comparative method. *Actin-11* gene was used as the reference gene in *P. vulgaris* and a health plant as the

reference sample. Tissue samples (hypocotyl segments) of plants treated with MeJA and infected with *S. sclerotiorum* (MeJA treatment) and plants treated with water and infected with *S. sclerotiorum* (Control) were collected at 6, 12, 24, 48, and 72 hpi. Results are reported as means ± standard deviation (SD) of three experiments. (A) *PvChit1* (*PvPR3*-chitinase class 1); (B) PvCallose (callose synthase); (C) *PvNBS-LRR* (NBS-LRR resistance-like protein); (D) *PvFbox* (F-box family protein-like); (E) *PvHIPRP* (hypersensitive-induced response protein) genes; (F) *PvPR1*; (G) *PvPR2*; (H) *PvPAL* (phenylalanine ammonia-lyase); and (I) *PvLox* (lipoxygenase).

Enzyme assay: peroxidase (POX), phenylalanine ammonia lyase (PAL), β-1,3-glucanase (GLU), and chitinase (CHIT)

With the aim of comparing the temporal kinetics of the defense-related enzymes POX, PAL, CHIT, and GLU in plants treated with MeJA and infected with *S. sclerotiorum* (MeJA treatment) as well as in plants treated with water and infected with *S. sclerotiorum* (Control), we conducted experiments on the proteins extracted from dry bean hypocotyl segments at different times after inoculation with the pathogen.

Although partial resistance to *S. sclerotiorum* was observed in the plants after MeJA exogenous treatment, a small and non-significant difference in POX activity was observed compared with the control. The POX activity in treated plants was close to or below the activity registered in control plants until 48 hpi, which was followed by an increase at 72 hpi in control plants (Fig. 3A).

The PAL activity in treated plants peaked at approximately 6 hpi, indicating an early induction of this enzyme after MeJA treatment (Fig. 3B). However, from 12 hpi until 24 hpi, the PAL activity was markedly reduced. In sharp contrast, control plants displayed low PAL activity, which increased from 24 to 72 hpi (Fig. 3B).

The CHIT activity increased in treated plants from 24 to 72 hpi, indicating a later induction of this enzyme in partially resistant plants. In contrast, control plants displayed little activity of this enzyme from 6 until 72 hpi (Fig. 3C).

After pathogen colonization and detection by plants, GLU activity was markedly increased from 24 to 72 hpi (phase of invasive growth of the pathogen and progression of the necrotic zone) in control plants as a consequence of *S. sclerotiorum* infection (Fig. 3D).

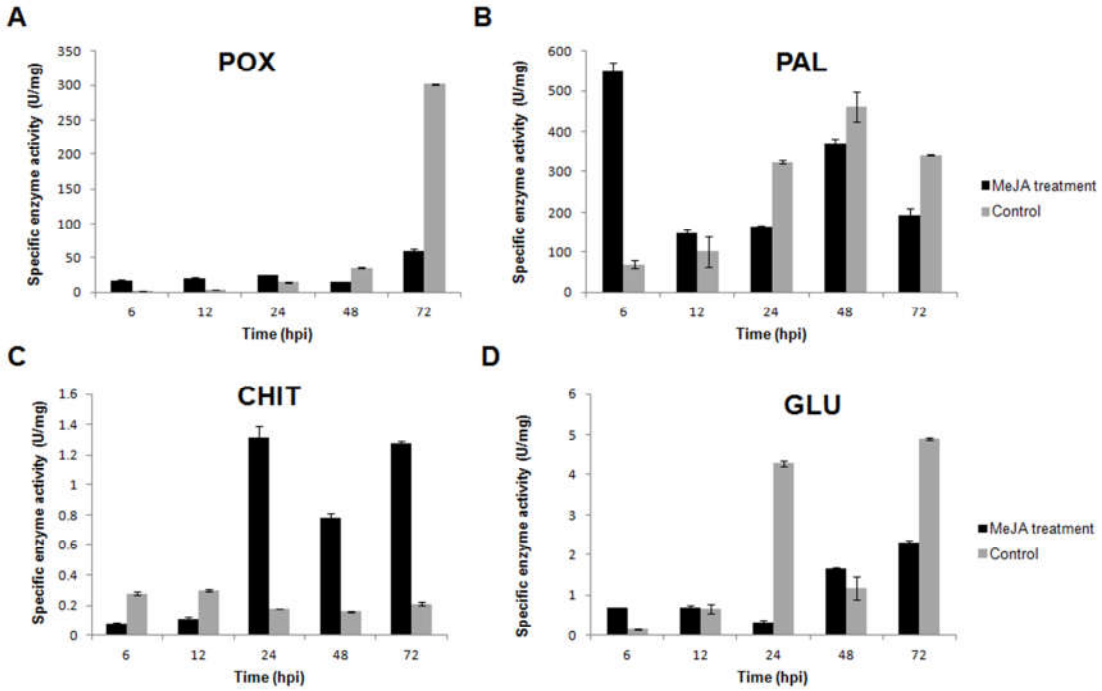

Fig. 3. Enzyme assay. The tests were performed in triplicate and the results are expressed in terms of activity unit per microgram of protein (U/mg), as described in the Materials and methods section. Three samples were analyzed for each treatment and the results are reported as means ± standard deviation (SD). (A) Peroxidase (POX); (B) phenylalanine ammonia-lyase (PAL); (C) chitinase (CHIT);. and (D) β-1,3-glucanase (GLU).

Polygalacturonase-inhibiting proteins (PGIPs)

A family of at least four *pgip* genes has been identified in the *P. vulgaris* genome (D'Ovidio et al., 2004), and the accumulation of *pgip* transcripts is induced by pathogen infection in bean plants (Bergmann et al., 1994; Nuss et al., 1996; De Lorenzo et al., 2001; Guerrero-González et al., 2011). In particular, in the *S. sclerotiorum-P. vulgaris* pathosystem, Kalunke et al. (2011) showed by qRT-PCR analyses that the transcript levels of *Pvpgip1*, *Pvpgip2* and *Pvpgip3* increase significantly following fungal infection, whereas *Pvpgip4* remains unchanged. We have previously reported a variation in the expression pattern of Pv*pgip* genes (Pv*pgip1*, Pv*pgip2*, Pv*pgip3* and Pv*pgip4*) following infection of common bean (*P. vulgaris* L. cv BRS Pérola) hypocotyls with the necrotrophic fungal pathogen *S. sclerotiorum (Oliveira et al., 2010).* In this work, our results clearly show that the *Pvpgip1*, *Pvpgip2*, and *Pvpgip3* transcript levels are strongly increased in plants treated with MeJA (observed in Fig. 4A-C, respectively). The *Pvpgip*1 gene showed a

more prompt induction following *S. sclerotiorum* infection, with a maximum 2.4-fold increase at 6 hpi and decrease at 72 hpi. *Pvpgip2* transcripts were detected 6 hpi and increased progressively, peaking at 48 hpi (3.5-fold). The *Pvpgip3* gene was poorly expressed at 6-48 hpi, but it was strongly induced at 72 hpi (34-fold).

Although *Pvpgip4* is reportedly not induced during any of the bean plant interactions with the fungal pathogens *Botrytis cinerea*, *S. sclerotiorum* and *Colletotrichum lindemuthianum* (Kalunke et al. 2011) or by stress signals such as wounding, salicylic acid, glucan and oligogalacturonides (D'Ovidio et al., 2004), we demonstrated by qRT-PCR analysis that this gene was positively regulated in *P. vulgaris* hypocotyls in response to infection with the necrotrophic fungus *S. sclerotiorum* (Fig. 4D). The *Pvpgip4* gene level had a 40-fold (0.6- to 25-fold compared to a healthy plant reference sample) increase in its expression in plants treated with water and infected with *S. sclerotiorum* (Control) compared to plants treated with MeJA and infected with *S. sclerotiorum* (MeJA treatment) (Fig. 4D).

In particular, *Pvpgip2* reached its highest accumulation (3.5-fold) in the earlier stage of infection (6 – 48 hpi) in plants that were pretreated with MeJA and infected with *S. sclerotiorum* (MeJA treatment). PvPGIP2 is a stronger inhibitor of the PG activity of *B. cinerea* (D'Ovidio et al., 2004; Manfredini et al., 2006), and it efficiently inhibits the two PGs secreted by *S. sclerotiorum* during host infection (Sella et al., 2005; Farina et al., 2009). The *Pvpgip2* gene responds promptly to several stress signals such as wounding, salicylic acid, glucan and oligogalacturonides (D'Ovidio et al., 2004). According Kalunke et al. (2011), the finding that *Pvpgip2* is the only bean member that is clearly expressed in all analyzed tissues as well as the one that undergoes the earlier and stronger transcript accumulation following *S. sclerotiorum*, *B. cinerea* or *C. lindemuthianum* infection reinforces the notion that *Pvpgip2* plays a primary role in host defense against fungal pathogens.

The *Pvpgip1* gene was the one that underwent earlier (6 hpi) and stronger transcript accumulation in plants that were pretreated with MeJA and infected with *S. sclerotiorum* (MeJA treatment). Moreover, the overexpression of PvPGIP1 does not enhance the disease resistance of transgenic tomato plants against the pathogenic fungi *Fusarium oxysporum* f. sp. *lycopersici*, *B. cinerea*, and *Alternaria solani* (Desiderio et al., 1997).

Additionally, recent work (Borges et al., 2012b) has shown that the Polygalacturonase-inhibiting protein homologues (PGIa and PGIb) were responsive

to the incompatible interaction between common bean and *C. lindemuthianum*, but with different expression levels among the tissues analyzed. In leaves, PGI expression was negatively regulated, whereas the transcripts accumulated mainly in epicotyls (in the later period - 96 hpi) and hypocotyls (in earlier periods of interaction - 24 and 48 hpi).

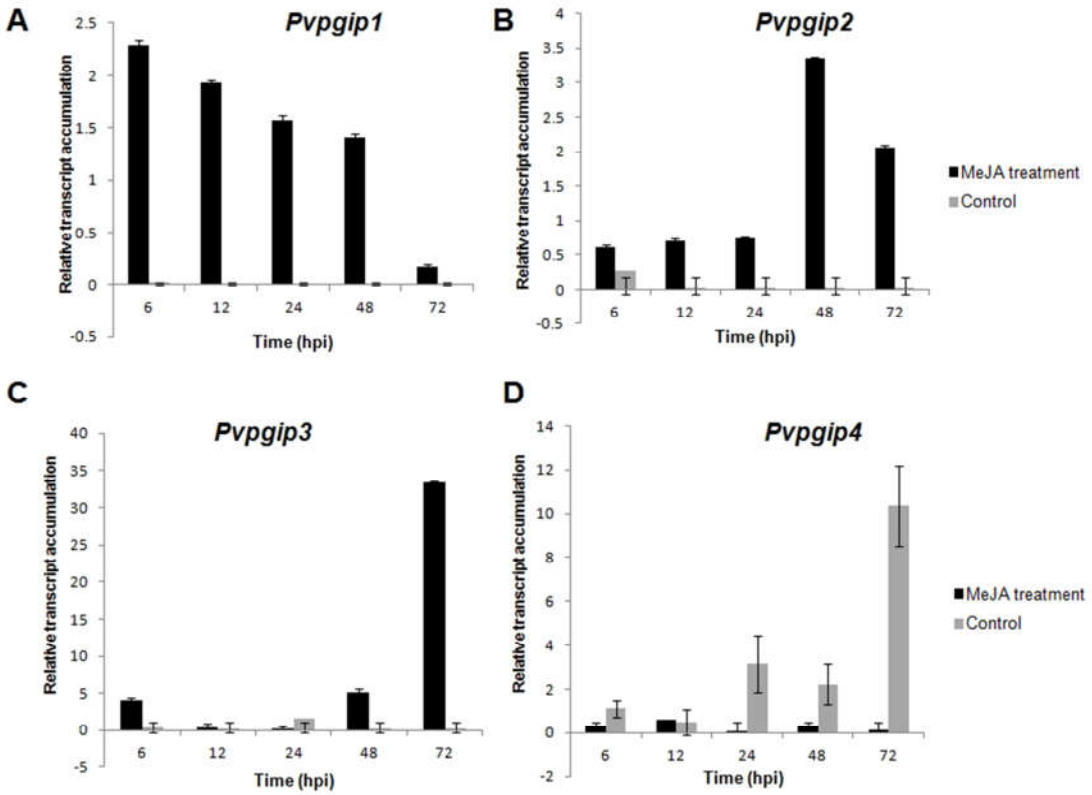

Fig. 4. Expression analysis of *Pvpgip* genes of *P. vulgaris* L. in response to *S. sclerotiorum* infection and foliar treatment with 10 µM methyl jasmonate (MeJA). Expression analyses using RT-qPCR were performed and transcript levels were calculated from triplicate data using a comparative method. *Actin-11* gene was used as the reference gene in *P. vulgaris* and a health plant as the reference sample. Tissue samples were collected at 6, 12, 24, 48, and 72 h post-inoculation (hpi). Results are reported as means ± standard deviation (SD) of three experiments. (A) *Pvpgip1*; (B) *Pvpgip2*; (C) *Pvpgip3*; and (D) *Pvpgip4*.

Polygalacturonase (PG) activity and inhibition assay

Considering the accumulation of *Pvpgip* transcripts observed in our experiments, and to further explore the role of PGIP in the defense against *S. sclerotiorum*, PG assays were performed to determine the differences in enzyme activity during the infection stages of *S. sclerotiorum* in plants that were or were not treated with MeJA.

We did not expect to detect high PG activity levels in the tissue of plants treated with MeJA and inoculated with *S. sclerotiorum* because a significant delay in the development of symptoms was observed. Nonetheless, the PG activity was significantly increased following fungal infection in plants that were treated with water and infected with *S. sclerotiorum* (Control) as well as in plants that were pretreated with MeJA and infected with *S. sclerotiorum* (MeJA treatment) (Fig. 5A).

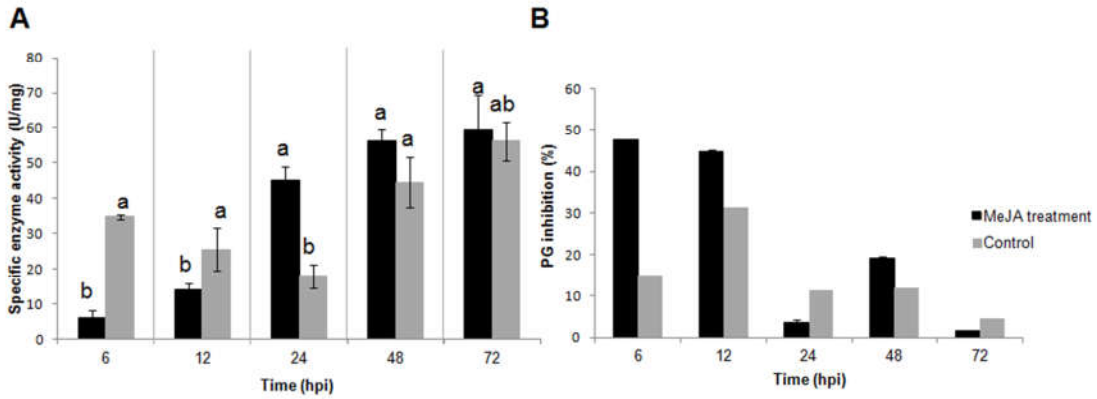

Fig. 5. (A) Polygalacturonase activity in *P. vulgaris* L. plants: the tests were performed in triplicate and the results are expressed in terms of activity unit per microgram of protein (U/mg), as described in the Materials and methods section. Three samples were analyzed for each treatment and the results are reported as means ± standard deviation (SD). Different letters on bars indicate statistical significance at 1% probability ($p < 0.01$). (B) Polygalacturonase activity inhibition assay: the percentage of inhibition was compared with the control. The test was carried out in cell free supernatant of *S. sclerotiorum* culture grown on minimal medium and in tissue extract obtained in different treatments as described in the Materials and methods section. For the control group, we measured only the activity of the culture supernatant. The enzyme activity was measured using polygalacturonic acid as substrate based on the release of reducing sugar. The results are reported as means ± standard deviation (SD) of three experiments.

The inhibition test was performed to confirm the inhibitory effect of *P. vulgaris* PGIPs on the *S. sclerotiorum* PG activity during the infective process. This test was performed using the cell-free supernatant of the *S. sclerotiorum* culture grown on minimal medium supplemented with 1% citrus pectin as the source of PG and the tissue extract obtained at different stages of *S. sclerotiorum* infection in plants that were or were not treated with MeJA as the source of PGIPs.

All evaluated treatments were positive for PG inhibitory activity, and a larger proportion of the PG activity from the pectin grown fungus was inhibited (Fig. 5B). The highest percentage of inhibition was observed in the initial stage of the infection process (6–12 hpi) (Fig. 5B). One possible explanation is that there is a lower

pathogen level in the initial stage of infection and consequently a lower level of secreted PG. Another important factor is that in different stages of infection, *S. sclerotiorum* may induce the expression of different proportions of PG isozymes or different endoPG:exoPG ratios (Sella et al., 2005; Oliveira et al., 2010). EndoPGs are likely to exhibit variation in their susceptibility to PGIP, whereas exoPGs are likely to be unaffected (Cervone et al., 1990; Desiderio et al., 1997; Berger et al., 2000).

These findings suggest that the bean *PvPgip* family is expressed and undergoes stronger transcript accumulation upon MeJA pretreatment following pathogen infection, especially *Pvpgip1* and *Pvpgip2*, which further reinforces the notion that PvPGIP has a primary role in counteracting the colonization of the host tissue by fungal pathogens.

The importance of PGIPs in plant defense has been corroborated by in vivo studies. Both a significant increase in the PG-inhibitory activity and a decrease in the susceptibility to *B. cinerea* have been found in transgenic tomato (Powell et al., 2000) and grapevine (Aguero et al., 2005) plants that overexpress a pear pgip. Additionally, *Arabidopsis* and wheat are protected by the transgenic expression of a bean PGIP in greenhouse trials against the fungi *Fusarium graminearum* and *Bipolaris sorokiniana* (Janni et al., 2008; Ferrari et al., 2012). Tobacco plants that overexpress a bean pgip (Pvpgip2) have protection against a fungal pathogen *Rhizoctonia solani* and two oomycetes (*Phytophthora parasitica* var. *nicotianae* and *Peronospora hyoscyami* f. sp. *tabacina*) (Borras-Hidalgo et al., 2012).

Conclusion

It is of practical interest to determine whether elicitor molecules released during the early stages of the plant–pathogen interaction could be directly applied to plants to suppress the effects of fungal diseases. We showed that the application of exogenous MeJA substantially protected dry bean plants from the necrotrophic pathogen *S. sclerotiorum*, reducing the number and size of lesions and, consequently, the incidence of the disease.

These results indicate that MeJA application induces systemic defenses in dry bean plants by increasing the levels of transcripts that encode proteins related to pathogenicity, such as chitinases, callose synthase, NBS-LRR resistance-like protein, F-box family protein-like, and polygalacturonase inhibitor proteins (*Pvpgip1*

to *3*). Although this study does not fully address the detailed defense signaling events, it offers molecular insight into the *S. sclerotiorum–P. vulgaris* pathosystem.

Conflict of interest

The authors confirm that the content of this article has no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.jplph.2015.04.006.

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Supplementary Material

Table S1 Oligonucleotide primer pairs used for RT-qPCR analysis

Gene	Description	Forward/Reverse
PvPR1	Pathogenesis-related PR-1	F: AAAGCCAAGAGCGATTCTCTTTTCA
		R: GAACACTCTGATTTGATAACACTTC
PvPR3	Chitinase class I	F: ATTGTTGTGCCAATCCCTTT
		R: CACCGCCATACAGTTCAAAA
PvLipox	Lipoxygenase	F: AGCACTGTGCCTGTTTTCAGT
		R: AACACACGAGAAGATTCAACCA
Pvcallose	Callose synthase-like protein	F: TGGCTTAGTATTCGGATGTACCA
		R: CGTTGTAATGAAAGCGAAGTGT
PvPAL	Phenylalanine ammonia-lyase	F: GACACACAAGTTGAAGCACCA
		R: TGCAGCTTCTTAGCATCCTTC
PvNBS-LRR	NBS-LRR resistance-like protein	F: TGGGAAGTTTGTTCAGAGTGC
		R: CAGTCACTGAAAGGGAACCAG
PvF-box	F-box family protein-like	F: AGTCAAGGAAACAACCAGCAA
		R: CTCTAGCAAACAACCCCCTTC
PvHPRP	Hypersensitive-induced response protein	F: ATTGCATGGTTCATAGCCAGT
		R: CCTCCACACAAGTATCAAAGGA

PvBG1	Beta1,3 glucanase1	F: AGCAGCTCTGCAAGCACTCA R: ACGAGCAGTGTCGGCATTG
PvPGIP1	Polygalacturonase-inhibiting protein1	F: CGCCTCACCGGGAAGAT ACGAGCAGTGTCGGCATTG
PvPGIP2	Polygalacturonase-inhibiting protein2	F: TTCGACGGCAACCGAATC R: TGGTCATCGACGTAAACAGCTT
PvPGIP3	Polygalacturonase-inhibiting protein3	F: TCCTTCCCGAAGCATTTCAC R: GCGTCGCCGGTATATTGC
PvPGIP4	Polygalacturonase-inhibiting protein4	F: TCCTTCCCGAAGCATTTCAC R: GCCAGCGTCGTCGGAATAT
PvACT11	Actin11	F: TGCATACGTTGGTGATGAGG R: AGCCTTGGGGTTAAGAGGAG

Table S2 List of unigenes obtained from suppression subtractive hybridization (SSH) derived from *Phaseolus vulgaris* L. cultivar Pérola treated with MeJA during *Sclerotinia sclerotiorum* infection.

Contig Seq. Name	Size (BP)	Blast matching acession nº	Gene Description	E-value	Total ESTs in Contig
Contig 4 SSJA_001	427	XP_002303842.1	probable ccr4-associated factor 1 (*V. vinifer*a)	4e-38	2
Contig 11 SSJA_002	832	XP_003551762.1	microtubule-associated protein *(G. Max*)	1e-69	3
Contig 12 SSJA_003	469	XP_003605980.1	dnaj protein homolog (*M. truncatula)*	4e-15	2
Contig 13 SSJA_004	345	ENH80648.1	nitrogen permease reactivator protein (*C. orbiculare*)	9e-29	2
Contig 15 SSJA_005	441	EFY85599.1	woronin body major protein (*M. acridum*)	1e-20	2

Contig 17 SSJA_006	489	XP_003526928.1	auxin response factor-like protein (*G. max)*	2e-57	2
Contig 19 SSJA_007	541	EFY98486.1	glutamine synthetase (*M. anisopliae)*	1e-67	2
Contig 22 SSJA_008	807	EFY98486.1	glutamine synthetase (*M. anisopliae)*	1e-121	5
Contig 24 SSJA_009	435	EMT63714.1	outer mitochondrial membrane protein porin (*F.oxysporum)*	1e-64	2
Contig 26 SSJA_010	621	XP_003602710.1	cleft lip and palate transmembrane protein (*M. trunculata)*	6e-66	2
Contig 27 SSJA_011	483	XP_003551448.1	Laccase (*G. max)*	5e-63	2
Contig 28 SSJA_012	537	P41380.1	eukaryotic initiation factor (*G. max*	1e-39	2
Contig 30 SSJA_013	208	EFZ01018.1	c2h2 finger domain protein (*M. anisople)*	1 e-17	2
Contig 32 SSJA_014	538	YP_002000594.1	atp synthase f1 subunit 1 (*O. sativa)*	5 e-70	3
Contig 33 SSJA_015	665	XP_003557087.1	s-adenosylmethionine synthetase (*G. max)*	4 e-84	2
Contig 34 SSJA_016	767	EMT60609.1	serine threonine-protein kinase (*F. oxysporum)*	4 e-136	19
Contig 35 SSJA_017	675	XP_003520270.1	v-type proton atpase 16 kda proteolipid 9*G. max)*	2e-68	2
Contig 37 SSJA_018	186	NP_565463.1	50s ribosomal protein l4 (*A. thaliana)*	1e-24	2
Contig 39 SSJA_019	382	YP_004222824.1	atpase subunit 1 (*V. radiata)*	3e-64	2
Contig 41 SSJA_020	433	EHK48933.1	ef hand domain protein (*T. atroviride)*	2 e-32	4
Contig 42 SSJA_021	131	XP_001527437.1	hygromycin resistance kinase (*L. elongisporus)*	1 e_10	2
Contig 43 SSJA_022	392	ENH80648.1	nitrogen permease reactivator protein (*C. oebiculare)*	1 e-29	8
Contig 44 SSJA_023	317	XP_003531232.1	dead-box atp-dependent rna helicase (*G. max)*	4 e-43	2
Contig 45 SSJA_024	689	XP_003589251.1	nbs-lrr resistance protein (*M. truncatula)*	9 e-54	2
Contig 46 SSJA_025	368	XP_003629279.1	proteasome subunit alpha (*M. truncatula)*	7 e-37	6
Contig 48 SSJA_026	598	CAA48579.1	carrier protein mitochondrial (*V. vinifera)*	7 e-52	2
Contig 49 SSJA_027	650	ABS10822.1	elongation factor 1 (*P. vulgaris)*	1 e-88	2
Contig 52 SSJA_028	388	XP_002314835.1	atp binding (*R. communis)*	1e-29	2
Contig 54 SSJA_029	651	XP_003614400.1	cytochrome p45 (*M. truncatula)*	1e-54	2
Contig 56 SSJA_030	607	NP_564907.1	copine (calcium-dependent phospholipid-binding protein) (*A. thaliana)*	2e-71	2

Contig 57 SSJA_031	904	EFZ02537.1	rna binding effector protein scp160 (*M. anisopliae)*	3e-136	5
Contig 59 SSJA_032	1072	EMT70299.1	phenylalanyl-trna synthetase (*F. oxysporum*	2e-174	3
Contig 63 SSJA_033	515	XP_002526287.1	atp binding (*R. cummunis)*	7e-18	2
Contig 64 SSJA_034	640	NP_001237601.1	Peroxidase (*G. max)*	9e-57	2
Contig 65 SSJA_035	591	XP_003531382.1	60s ribosomal protein	7e-86	2
Contig 66 SSJA_036	611	XP_003629279.1	proteasome subunit alpha (*M. tuncatula)*	1e-42	2
Contig 67 SSJA_037	639	EFZ00983.1	phenylalanyl-trna synthetase (*M. anisople)*	7e-69	3
Singlets					
SSJA_038	413	NP_195870.1	hsp70 protein (*A. thaliana)*	4e-37	
SSJA_039	307	EFZ02321.1	gdp-mannose pyrophosphorylase (*M. anisopliae)*	1e-31	
SSJA_040	199	XP_004148778.1	trans-2-enoyl-mitochondrial-like (*C. sativus)*	1e-15	
SSJA_041	385	NP_198206.1	luminal binding protein (*A. thaliana*)	3e-50	
SSJA_042	466	NP_198206.1	luminal binding protein (*A. thaliana*)	9e-42	
SSJA_043	469	XP_003537870.1	adomet-dependent rrna methyltransferase (*G. max)*	5e-43	
SSJA_044	333	XP_003553509.1	pre-mrna-processing factor 39-like (*G. max)*	1e-44	
SSJA_045	501	XP_003630584.1	auxin response factor-like protein (*M. truncatula)*	1e-55	
SSJA_046	484	XP_003521310.1	pre-mrna-processing factor 39-like (*G. max)*	7e-84	
SSJA_047	578	XP_003520756.1	receptor-like serine threonine-protein kinase (*G. max)*	8e-52	
SSJA_048	452	NP_186827.2	protein tplate-like (*A. thaliana)*	1e-49	
SSJA_049	505	CAC16168.1	hsp70 protein (*Z. mays*)	7e-52	
SSJA_050	439	ENH80648.1	nitrogen permease reactivator protein (*C. orbiculare*)	1e-29	
SSJA_051	514	CAD59767.1	reverse transcriptase (*C. arieticum)*	1e-12	
SSJA_052	540	XP_003553280.1	ubiquitin-protein ligase (*G. max)*	1e-100	
SSJA_053	468	XP_003619702.1	ubiquitin-like protein 9*M. tuncatula*)	5e-87	
SSJA_054	447	XP_003535516.1	nucleic acid binding (*G. max*)	1 e-27	
SSJA_055	406	XP_003599101.1	trna (cytidine -2 -o)-methyltransferase-like	3e-71	

			(*M. truncatula*)	
SSJA_056	268	XP_003591150.1	translationally controlled tumor protein (*M. truncatula*)	3e-31
SSJA_057	334	XP_965487.2	transcriptional activator hac1 (*N. crassa)*	2e-23
SSJA_058	453	NP_001237601.1	peroxidase (*G. max*	3e-68
SSJA_059	419	EHK24137.1	glycoside hydrolase (*T. virens*)	1e-49
SSJA_060	499	XP_003625373.1	pre-mrna-processing factor (*M. truncatula)*	6e-76
SSJA_061	289	XP_003599101.1	trna (cytidine -2 -o)-methyltransferase (*M. truncatula)*	5e-52
SSJA_062	482	XP_003520172.1	proteasome subunit alpha (*G. max)*	8e-20
SSJA_063	568	XP_003593130.1	histidine decarboxylase (*M. truncatula)*	3e-110
SSJA_064	543	XP_003593129.1	histidine decarboxylase (*M. truncatula*	9e-101
SSJA_065	585	XP_003555130.1	ubiquitin-protein ligase (*G. max)*	6e-99
SSJA_066	522	NP_001238183.1	Peroxidase (*G. max*)	1e-80
SSJA_067	497	XP_003630584.1	auxin response factor (*M. truncatula*	9e-56
SSJA_068	547	XP_003526983.1	rich repeat receptor-like serine threonine tyrosine-protein kinase (*G. max*)	1e-63
SSJA_069	565	CAD59767.1	reverse transcriptase (*C. arietinum*)	1e-12
SSJA_070	570	XP_003626788.1	1-aminocyclopropane-1-carboxylate oxidase (*M. truncatula)*	1e-49
SSJA_071	594	AFN84621.1	ferritin- chloroplastic (*G. max*)	2e-32
SSJA_072	593	CAD59768.1	reverse transcriptase (*C. arietinum*)	1e-14
SSJA_073	519	CBL62363.1	Beta-tubulin (*C. sativa*)	6e-14
SSJA_074	592	ABB18117.1	proteasome subunit alpha type-7-like (*N. benthamiana*)	2e-35
SSJA_075	560	EHK22206.1	ca2+ h+ antiporter (*T. virens*)	9e-40
SSJA_076	562	XP_003540370.1	carrier protein mitochondrial (*G. max)*	2e-52
SSJA_077	551	XP_003543079.1	v-type proton atpase 16 kda proteolipid (*G. max*)	1e-54
SSJA_078	482	AFN88217.1	anthocyanidin 3-o-glucosyltransferase (*P. vulgaris)*	2e-53
SSJA_079	565	NP_001237358.1	biotin carboxylase precursor (*G. max)*	1e-59
SSJA_080	569	AAK32919.1	tubulin beta-2 beta-3 chain (*M.truncatula*)	4e-108
SSJA_081	536	XP_003614400.1	cytochrome p450 (*M.truncatula*)	6e-55
SSJA_082	517	CAD59768.1	reverse transcriptase (*C. arietinum*)	7e-15

SSJA_083	571	CAD59768.1	reverse transcriptase (*C. arietinum*)	1e-14
SSJA_084	597	XP_003533706.1	alpha peptide from puc9 partial (*G. max*)	2e-11
SSJA_085	555	ACU23780.1	60s ribosomal protein (*G. max*)	6e-86
SSJA_086	555	XP_003534114.1	glutamate decarboxylase (*G. max*)	5e-32
SSJA_087	560	XP_004134855.1	proteasome subunit alpha type-7-like (*C. sativus*)	1e-43
SSJA_088	591	XP_003606980.1	bzip transcription factor (*M. truncatula*)	2e-25
SSJA_089	454	XP_003589665.1	u-box domain-containing protein *M. truncatula*)	4e-31
SSJA_090	518	EMT70299.1	phenylalanyl-trna synthetase beta chain (*F. oxysporum*)	4e-71
SSJA_091	502	ELA28342.1	nitrogen permease reactivator protein (*C. gloesosporides*)	6e-25
SSJA_092	422	XP_003714560.1	t-complex protein 1 subunit delta (*M. oryzae*)	1e-27
SSJA_093	556	CAD59768.1	reverse transcriptase (*C. arietinum*)	8e-15
SSJA_094	499	XP_004142669.1	nadh dehydrogenase (*C. sativus*)	1e-29

Fig. S1: Effects of foliar application of 10 µM methyl jasmonate (MeJA) on *Sclerotinia sclerotiorum* infection in the stem of dry bean (*P. vulgaris* L.) plants. Plants in the treated group were sprayed with 10 mL of aqueous solution of 10 µM MeJA and inoculated with *S. sclerotiorum* 12 h after this application. Plants in the control group were sprayed with 10 mL of distilled water and also inoculated with the pathogen, as described in the Materials and methods section. (A) Control plants at 72 hours post-inoculation (hpi). (B) Treated plants at 72 hpi.

5. CONCLUSÕES

Neste estudo foi avaliado os aspectos moleculares do patossistema *S. sclerotiorum-P. vulgaris* em duas situações: planta suscetível e planta suscetível com resistência induzida. Os resultados levaram às seguintes conclusões:

- Durante a interação com a cultivar suscetível, o fungo secreta ácido oxálico e enzimas hidrolíticas, que juntos permitem a colonização do tecido hospedeiro.
- Fragmentos de oligossacarídeos liberados pela degradação da parede celular da planta, e fragmentos de quitina do fungo, atuam como elicitores das respostas de defesa da planta.
- A planta, para contrapor ao ataque do patógeno, dispara as respostas de hipersensibilidade que leva à ativação de genes de defesa tais como: genes relacionados à patogênese; genes que codificam enzimas da via dos fenilpropanoides; e genes relacionados ao estresse oxidativo.
- A aplicação foliar de MeJA induziu resistência parcial contra *S. sclerotiorum* em plantas de feijoeiro suscetíveis, fator este substanciado pela redução da lesão em plantas infectadas previamente tratadas com MeJA.
- O tratamento com MeJa induziu aumento nos níveis de transcritos de vários marcadores moleculares de defesa incluindo, proteínas relacionadas à patogênese, *PvPR3*, *PvCallose*, *PvNBS-LRR*, *PvF-box* e inibidores de poligalacturonases (*Pvpgip1-3*).

6. PERSPECTIVAS

A identificação de genes diferencialmente expressos durante a interação *S. sclerotiorum-P. vulgaris* constitui o primeiro passo para a compreensão desse patossistema em nível molecular. A partir dos resultados obtidos neste trabalho, surgem novas perspectivas que visam ao entendimento dos mecanismos que regem esse modelo de interação, tais como:

- Ampliar os dados do transcriptoma a partir da análise de RNA-seq, visando identificar genes envolvidos no processo de patogênese, além de microRNAs que regulam as respostas durante a interação fungo-planta.
- Análise comparativa do transcriptoma de cultivares suscetível e parcialmente resistente, durante a interação com *S. sclerotiorum* visando à identificação das bases moleculares da resistência parcial.
- Validar a função dos principais genes identificados no processo de interação *S.sclerotiorum-P. vulgaris* por meio de técnicas moleculares, por exemplo:
    - Uso de técnicas como RNA interferente (RNAi) ou *knockout* para verificar o papel de determinados genes no processo de patogenicidade do fungo.
    - Transformação de plantas, por exemplo, com genes que codificam enzimas que degradam ácido oxálico, visando a obtenção de plantas resistentes a *S. sclerotiorum.*

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