Universidade Estadual de Londrina

VALÉRIA STEFANIA LOPES CAITAR

“ESTUDOS MOLECULARES DO PATOSISTEMA *Glycine max-Pratylenchus brachyurus*: DE ESTRATÉGIAS DE INFECÇÃO DO PATÓGENO E DE DEFESA DO HOSPEDEIRO, À INTERAÇÃO PROTEÍNA-PROTEÍNA”

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Orientadora: Dra. Francismar Corrêa Marcelino Guimarães
Co-orientador no exterior: Dr. Tarek Hewezi

Londrina
2018

Ficha de identificação da obra elaborada pelo autor, através do Programa de GeraçãoAutomática do Sistema de Bibliotecas da UEL

Dados Internacionais de Catalogação-na-Publicação (CIP)

Lopes-Caitar, Valéria Stefania.

ESTUDOS MOLECULARES DO PATOSISTEMA *Glycine max-Pratylenchus brachyurus*: de estratégias de infecção do patógeno e de defesa do hospedeiro, à interação proteína-proteína / Valéria Stefania Lopes Caitar. – Londrina, 2018.
108 f. : il.

Orientador: Dra. Francismar Corrêa Marcelino-Guimarães.
Co-orientadores: Dr. Tarek Hewezi.
Tese (Doutorado em Genética e Biologia Molecular) - Universidade Estadual de Londrina, Centro de Ciências Biológicas, Programa de Pós-Graduação em Genética e Biologia Molecular, 2018.
Inclui bibliografia.

1. nematóide de lesão radicular - Tese. 2. bases moleculares de defesa do hospedeiro - Tese. 3. genes diferencialmente expressos - Tese. 4. genes efetores - Tese. I. Marcelino-Guimarães, Dra. Francismar Corrêa. II. Hewezi, Dr. Tarek. III. Universidade Estadual de Londrina. Centro de Ciências Biológicas. Programa de Pós-Graduação em Genética e Biologia Molecular. IV. Título.

“Permitida a cópia total ou parcial deste documento, desde que citada a fonte – O autor”

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Londrina, 01 de março de 2018

DEDICO

À minha amada avó, meu maior exemplo; e à minha já tão amada filha, Lara, que esta a caminho.

AGRADEÇO

Primeiramente a Deus, por minha vida, por minha saúde e por ter conduzido meus caminhos até aqui, dando-me força e coragem para sempre continuar lutando; e pelas pessoas e oportunidades maravilhosas que sempre me apresentou;

À Universidade Estadual de Londrina e ao programa de pós-graduação em Genética e Biologia Molecular e seu corpo docente e funcionários, pela ótima formação que me proporcionaram.

Ao Centro Nacional de Pesquisa em Soja (Embrapa Soja) e à Universidade do Tennessee, por me proporcionarem as condições e estrutura necessárias para a realização deste trabalho;

À Capes, pelo apoio financeiro imprescindível e pela bolsa concedida, tanto no Brasil quanto no período que estive no exterior.

À coordenadora do curso de pós-graduação Profa. Dra. Silvia Helena Sofia e a ex-coordenadora Profa. Dra. Ana Lúcia Dias, por todo auxílio prestado e respeito durante essa caminhada.

A todos os membros da banca examinadora de defesa de doutorado, por disponibilizarem seu tempo e compartilharem parte de seu conhecimento comigo;

À minha orientadora, Dra. Francismar Corrêa Marcelino Guimarães, por ter me recebido no laboratório de Biotecnologia Vegetal desde a iniciação científica, pela orientação em todos os trabalhos, pelas críticas e por me ajudar a superar meus próprios limites; pessoa essa a quem eu admiro, não apenas por sua vida acadêmica, mas como ser humano;

Ao Prof. Dr. Luiz Filipe Protasio Pereira, por ter me ajudado no início do doutorado, me disponibilizando uma vaga. Sem ele, eu não poderia ter iniciado meu doutorado; também por seu exemplo de excelente profissional;

À Profa. Dra. Mayra Costa da Cruz Gallo de Carvalho, que sempre, mesmo estando em Bandeirantes, me ajuda com todas suas ideias, dicas e me inspira a realizar um bom trabalho de pesquisa. Não poderia me esquecer de você;

Ao meu co-orientador no exterior, Dr. Tarek Hewezi, por me permitir desenvolver parte de minhas análises em seu laboratório; e por ter me concedido a oportunidade de permanecer 6 meses extras, em Knoxville, com auxílio financeiro da Universidade do Tennessee (UT).

Ao Dr. Scott Senseman, CEO do departamento de Plant Sciences da UT, e sua querida esposa Laura Boyd Senseman, por sua atenção, preocupação e excelentes conversas sobre suas experiências no Brasil;

À todos os funcionários da Embrapa Soja, pelo apoio e auxílio na realização desse trabalho; em especial aos funcionários do Laboratório de Biotecnologia Vegetal, Ana, Adriana, Cesar Silveira, Danielle Cristina Gregório da Silva, Marcia Kamogae Kuwahara, Silvana Rochenbach Marin e Vera Lúcia Pierotte pela atenção e amizade;

Aos meus amigos do Laboratório de Biotecnologia Vegetal – Embrapa Soja, por tantas vezes terem me ajudado a superar as dificuldades e tornarem agradável e divertida a rotina de laboratório.

À Ph.D. Family Core, Aditi Rambani, Cheryl Dalid-Gaspar, Christos (Chris Johnson), Denis Osipov, and Manuela Picariello, for being such an amazing friends. Thank you so much for everything you are for me. I love you all.

À Camila Figueiredo e à Daniela Tessaro, minhas amigas queridas no Brasil e em qualquer lugar fora dele;

A minha AMADA FAMÍLIA:

Ao meu marido, Lucas Panicio Caitar, por ter me ajudado em todos os momentos, mesmo nos mais difíceis; por ter suportado a distância e nunca ter me deixado desistir; por ter preenchendo minha vida com tão grande amor, paciência, compreensão e confiança; por ser sempre companheiro tão cheio de alegria e esperança; por ter me ensinado a ser uma pessoa melhor todos os dias; e por ter cuidado tão bem do nosso Eddinho enquanto estive fora;

Aos meus pais, Stéfano Lopes e Sueli Ruiz Lopes (*in memorian*), pelo amor incondicional e todo o apoio;

Meu irmão, Alex Stéfano Lopes, pela força e boas conversas a respeito de nossos futuros;

Meus avós, Antônio Lopes Hernandez (*in memorian*) e Hortência Paranzini Hernandez, pelo amor, educação, cuidados e preocupação com meu futuro;

Meu querido tio, Mauridez Lopes (Tio Tuta), pelo exemplo pureza de coração;

Aos meus sogros, José Melero Caitar e Aparecida Josefina Panicio Caitar, pelo apoio, caronas e entregas na secretaria de pós-graduação do CCE.

Aos meus tios e tias pelos bons conselhos e apoio.

CAITAR, Valéria Stefania Lopes. "**Estudos moleculares do patosistema *Glycine max*-*Pratylenchus brachyurus*:** de estratégias de infecção do patógeno e de defesa do hospedeiro, à interação proteína-proteína". 2018. 108 f. Tese (Doutorado em Genética e Biologia Molecular) – Universidade Estadual de Londrina, Londrina, Paraná, Brasil.

RESUMO

O nematóide de lesão radicular (root-lesion nematode – RLN), *Pratylenchus* spp., é uma praga devastadora que afeta a soja e várias outras culturas em todo o mundo. Até hoje, apenas um gene maior de resistência a *Pratylenchus* spp. foi identificado em trigo e alguns QTLs em outras espécies de plantas; no entanto, nenhum gene de resistência foi mapeado na soja e seu sistema de defesa permanece em grande parte desconhecido. Os estudos do transcriptoma para melhor compreender os mecanismos moleculares de defesa da planta ou de ataque dos nematóides dentro do patosistema podem ser uma excelente abordagem, uma vez que permite identificar novos genes chave no processo e acessar o perfil global de expressão dos mesmos. Neste estudo, realizamos um RNA-seq de genótipos de soja moderadamente resistente (BRSGO Chapadões) e um genótipo suscetível (TMG 115RR), mais especificamente de raízes infectadas por *Pratylenchus brachyurus* às 24, 48, 96 e 192 horas. Os dados permitiram acessar informações sobre a resposta de defesa da planta e os mecanismos de infecção do patógeno. A comparação entre as respostas de ambos os genótipos de soja demonstrou que o primeiro tempo de infecção, 24h, foi o que mais diferenciou os a resposta dos genótipos, tanto em número como em composição de genes diferencialmente expressos (*Differential Expressed Genes* – DEGs). Também foi identificado um elevado número de genes/rotas metabólicas induzidos e relacionados à defesa em BRS, incluindo genes da via de fenilpropanoides-estilbenos. Através de nossa abordagem para elucidar a resposta da soja ao RLN, encontramos genes em BRS envolvidos no reconhecimento de patógenos, como relacionados à ativação de canais de cálcio, quitinases e envolvidos com regulação transcricional, como genes *Myb*. Várias vias metabólicas apresentaram alterações em TMG, especialmente relacionadas a arquitetura de parede celular e seu remodelamento. O transcriptoma do patógeno durante a interação resultou na predição de um conjunto de proteínas secretadas, o "secretoma", de 115 proteínas. Vinte preditos como secretados foram analisados por hibridização *in situ*, o que resultou na identificação de oito genes marcados como expressos nas glândulas esofágicas de *Pratylenchus*, sendo agora considerados como genes candidatos a efetores e, portanto, elementos-chave relacionados ao parasitismo. Dentre os genes identificados nas glândulas esofágicas, alguns apresentam similaridade de sequência aos efetores de nematóides já descritos na literatura, como proteína *14-3-3b*, SEC-2 e transtiretina. Os ensaios de interação proteína-proteína pelo método de duplo híbrido em levedura mostraram que o candidato efetor, PB6584, interage com diferentes proteínas de *Arabidopsis*. Essas proteínas de interação podem estar relacionadas à defesa do hospedeiro, como uma invertase e proteína da família de endopeptidase da serina subtilisina, sugerindo que o ataque do nematóide pode afetar a sinalização de defesa e o reforço de parede celular. Este é o primeiro estudo sobre a interação molecular de soja - *Pratylenchus brachyurus* e os resultados, tanto das estratégias de resposta à planta como de ataque do nematóide, podem ser um importante passo para desenvolver resistência/tolerância em culturas e ferramentas para o controle de patógenos no campo.

Palavras-chave: Nematóide de lesão radicular. Suceptibilidade e resistência moderada da soja. Bases moleculares de defesa do hospedeiro. Transcriptoma. Candidatos a genes efetores.

CAITAR, Valéria Stefania Lopes. **“Molecular Studies of *Glycine max-Pratylenchus brachyurus patosystem***: from pathogen infection strategies and host defense to protein-protein interaction”. 2018. 108 ps. Dissertation (Ph.D. in Genetics and Molecular Biology) – Universidade Estadual de Londrina, Londrina, Parana, Brazil.

ABSTRACT

Root-lesion nematode (RLN), *Pratylenchus* spp., it is a devastating pest that affects soybean and several other crops worldwide. As migratory nematodes, they can migrate from root to root, according to environmental conditions. To date, one resistance gene to *Pratylenchus* spp. was identified in wheat and QTLs in other plant species; however, no resistance gene have been mapped in soybean and its molecular mechanism of defense remains largely unknown. Transcriptome studies for better understand the molecular mechanisms of plant defense or nematode attack in this pathosytem can be a good approach once allows identifying new genes and to accesses the global expression profile. In this study, we conducted a RNA-seq analysis of a moderately resistant (BRSGO Chapadões) and a susceptible genotype (TMG 115RR) of *Glycine max*, from root tissue infected with *Pratylenchus brachyurus* at 24, 48, 96, and 192 hours. The obtained data allowed to either approach plant denfense response or pathogen infection mechanisms. The comparison between the responses of both soybean genotypes demonstrated the first time of infection, 24h, as the most different between genotypes in number and composition of DEGs. We also identified a high number of defense-related genes/metabolic pathways were induced in BRS, including genes of phenylpropanoids-Stilbenes pathway. Several metabolic pathways showed changes in TMG, especially for cell wall architecture and remodeling. Following our approaches to elucidate soybean response to RLN, we found genes in BRS involved in pathogen recognition, calcium activated channel, *chitinases* and *Myb*-involved transcriptional regulation. Results from the pathogen transcriptome during the interaction showed a predicted secretome of 115 proteins. Twenty of those were analyzed by *in situ* hybridization and eight were tagged in the esophageal glands, being now considered as key elements for the parasitism role and effector candidates gene. Some of those similar in sequence to known nematode effectors from literature, as *14-3-3b*, *SEC*-2 protein, and transthyretin-like (TTL) protein. Protein-protein interaction assays by yeast two-hybrid showed that putative effector candidate, *PB6584*, interacts with different *Arabidopsis* proteins. These interactor proteins may be related to host defense, such as an invertase, and subtilisin-like serine endopeptidase family protein, suggesting the nematode infection approach may affect the defense signaling and cell wall reinforcement. This is first study of soybean - *Pratylenchus brachyurus* molecular interaction, and the results from both plant response and nematode infection strategies can be a step forward to develop new resistance/tolerance in crops and tools for pathogen control in the field.

Keywords: Root-lesion nematode. Soybean moderate resistance and susceptibility. Molecular basis of host defense. Transcriptome. Putative effector candidate genes.

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1. INTRODUÇÃO

Os nematóides das “lesões radiculares” (root-lesion nematodes – RLN - *Pratylenchus* spp.) representam um problema para diversos sistemas de produção, causando impacto econômico mundial 100 doenças (MACGUIDWIN; BENDER, 2016; MAY et al., 2016). Na Austrália, Israel, Estados Unidos e México, as perdas em rendimento causando por RLN chegam todo ano a 85%, 70%, 50% e 37%, respectivamente (SMILEY, 2010). No que se refere à cultura da soja brasileira, tal patógeno vem se destacando a cada safra, principalmente em regiões de solos arenosos, onde se concentram grandes áreas dessa cultura, como por exemplo, na região central do Brasil. No estado do Mato Grosso, análises frequentes do solo apontam populações de *Pratylenchus brachyurus* extremamente elevadas, com mais de 1000 indivíduos por grama de raízes de soja, causando reduções de produtividade nestas áreas de até 50% (RIBEIRO, 2010; SILVA et al., 2015).

Pratylenchus spp., é um gênero polífago, que apresenta hábitos migratórios, ou seja, não forma sítio de alimentação, sendo capaz de infectar as plantas tanto nos estágios juvenis como no adulto, o que tem contribuído para seu sucesso em termos de adaptabilidade e dispersão (YU et al., 2012). Plantas atacadas por RLN normalmente apresentam redução no crescimento das raízes, com o aparecimento de lesões necróticas e consequente queda na capacidade de absorção de água e nutrientes (MAY et al., 2016).

O uso de nematicidas, cultivares resistentes e a rotação de culturas se destacam como as principais medidas de controle de fitonematóides. Contudo, poucas fontes de resistência foram identificadas até o momento para RLN (RIOS et al., 2016; WILLIAMS et al., 2002). Os nematicidas apresentam baixa eficiência, são nocivos ao meio ambiente e oneram a produção (NEVES, 2007). Estratégias de manejo apresentam baixa adoção dos produtores, por serem antieconômicas, uma vez que este patógeno é capaz de infectar várias espécies cultivadas, como o milho, milheto, algodão etc. (GOULART, 2008). Por estas razões, as estratégias biotecnológicas estão sendo usadas para o entendimento desse patosistema e na tentativa de desenvolver medidas de controle do patógeno e genótipos mais resistentes ou tolerantes ao parasita.

Até o momento, sabe-se que o parasitismo estabelecido pelos nematóides resulta em uma alteração dramática da expressão de genes do

hospedeiro, observada principalmente em raízes de plantas infectadas (FOSU-NYARKO; JONES, 2016; GHEYSEN; FENOLL, 2002). Além de afetar as respostas de defesa do hospedeiro, esses parasitas causam a reorganização do citoesqueleto (ENGLER et al., 2010), a regulação das vias de sinalização e metabólicas, o saldo de fitormônios (GUTIERREZ et al., 2009; HABASH et al., 2017) e a reprogramação do desenvolvimento celular (HASSAN; BEHM; MATHESIUS, 2010).

Todas essas alterações no hospedeiro são moduladas por genes chamados de efetores, ou proteínas do parasitismo, secretadas pelo patógeno para que esse seja capaz de estabelecer o parasitismo (MITCHUM et al., 2013; QUENTIN; ABAD; FAVERY, 2013). A via de liberação mais conhecida dessas moléculas para o meio celular do hospedeiro por nematóides é pelo estilete (HAEGEMAN et al., 2012). Os efetores liberados via estilete são normalmente expressos nas glândulas esofágicas destes fitoparasitas (HEWEZI; BAUM, 2013). Contudo, acredita-se que essa não seja a única via de liberação de efetores pelos nematóides. Outras vias de secreção de efetores são a cutícula, e hipoderme, o sistema excretor e as glândulas retais (TRUONGA et al., 2015).

Muitos genes efetores já foram identificados, mas sua maioria em nematóides sedentários, como *Heterodera glycines* e *Meloidogyne* sp. (NOON et al., 2012; TRUONGA et al., 2015). Os estudos para a identificação de efetores de nematóides migradores ainda são incipientes, considerando a importância em danos provocados (JONES; FOSU-NYARKO, 2014).

Dado ao exposto, a identificação de proteínas efetores de *Pratylenchus* spp. e as proteínas de interação do hospedeiro apresenta-se como o melhor cenário para elucidarmos essa relação de parasitismo (BRAUN et al., 2013; JONES; FOSU-NYARKO, 2014). Para tal, análises de bioinformática para a predição e analise de expressão de genes candidatos a efetores (DAVIS et al., 2008; ELLING et al., 2009), análises de detecção *in situ* da expressão de genes secretados (BELLAFIORE et al., 2008) e análises de interação proteína-proteína vem sendo amplamente aplicadas (GUPTA et al., 2015). Essas informações auxiliarão no desenvolvimento de novas alternativas de controle de reprodução do patógeno e resistência das plantas à infecção, minimizando, ou mesmo, impedindo os prejuízos causados pelo patógeno.

2. OBJETIVO

2.1. Objetivo Geral

O objetivo deste estudo é elucidar os mecanismos moleculares presentes no patosistema soja e o nematóide de lesão radicular, *Glycine max-Pratylenchus brachyurus*, desde genes e vias metabólicas de defesa da planta, a identificação de efetores do patógeno e suas estratégias de infecção; até o nível de interação entre as proteínas do patógeno e do hospedeiro.

2.2. Objetivos Específicos

- ✓ Caracterizar o perfil transcricional de raízes de soja ao longo da interação compatível e incompatível com *P. brachyurus*;

- ✓ Identificar genes e vias metabólicas associados às respostas de defesa da soja ao patógeno;

- ✓ Caracterizar o transcriptoma de *P. brachyurus* e identificar o conjunto de genes por este secretados, estabelecendo o "secretoma" do patógeno;

- ✓ Identificar os genes do patógeno relacionados a interação e sucesso da infecção no hospedeiro, e elencar possíveis candidatos aos chamados genes efetores;

- ✓ Identificar proteínas do hospedeiro (soja) que sejam alvos das proteínas candidatas a efetores secretadas pelo nematóide durante o processo de infecção na planta.

3. REVISÃO BIBLIOGRÁFICA

3.1. SOJA

A soja é uma leguminosa pertencente à divisão Magnoliophyta, classe Magnoliopsida, subclasse Rosidae, ordem Fabales, família Fabaceae, subfamília Faboideae, gênero Glycine L. e espécie *Glycine max* (L.) Merril. Domesticada há cerca de 7.000 – 9.000 anos na Ásia, a soja tem como seu ancestral *Gycine soja* (KIM et al., 2012). Sua importância no Brasil e no mundo, tem alicerce nos inúmeros subprodutos oriundos da soja, dentre os quais o óleo, amplamente utilizado no consumo humano, e o farelo, utilizado para o consumo animal, são os principais (HARTMAN; WEST; HERMAN, 2011). O óleo, oriundo da primeira etapa de processamento dos grãos da soja, o esmagamento, pode ser do tipo comestível, empregado na fabricação de margarinas, óleos de cozinha, maionese, gorduras vegetais e produtos farmacêuticos, ou utilizado na indústria como biocombustível (OLIVEIRA; REYS, 2009).

Essa é uma das commodites de exportação de grande importância para o Brasil. Desde sua introdução em território nacional, essa cultura tem sido a protagonista no aumento de área e produção de grãos, partindo de 9,8 milhões de toneladas, em 1977/78 e atingindo a estimativa de 111.558,6 milhões toneladas em 34.991,4 mil hectares 2017/18 (CONAB: COMPANHIA NACIONAL DE ABASTECIMENTO, 2017)

No âmbito socioeconômico, a cadeia produtiva da soja gera ao país mais de cinco milhões de empregos e participa com pelo menos metade dos 23% a 24% do PIB (Produto Interno Bruto) brasileiro gerado pela agronegócio (MAPA, 2017; MIRANDA, 2014; SILVA; LIMA; BATISTA, 2011). Atualmente, o país ocupa lugar de destaque no cenário mundial de produção da soja, como grande exportador e o segundo maior produtor, com cerca de 30% da produção mundial (ROSA; BERGAMIN; MAKIYA, 2014). Na safra 2017/18, a estimativa é que o agronegócio da soja participe com 50,77% da produção total de grãos do país (CONAB: COMPANHIA NACIONAL DE ABASTECIMENTO, 2017) .

Junto à expansão da cultura no mundo tem-se notado o aumento dos fatores que afetam a produção, destacando-se os edafoclimáticos e os sanitários, como o ataque de pragas e doenças, sendo este último o principal

fator que limita a obtenção de altos rendimentos. Já foram descritas mais de 100 doenças diferentes provocadas por fitopatógenos (fungos, nematóides, bactérias e vírus (HENNING, 2009; HIRAKURI; LAZZAROTTO, 2014). Cerca de 15% a 20% das perdas anuais de produção na soja são devido às doenças, sendo algumas capazes de ocasionar perdas de quase 100% (HENNING, 2009). Dentre estas, as causadas pelo ataque de nematóides, pertencentes a diferentes gêneros, tais como *Meloidogyne*, *Heterodera*, *Pratylenchus, Rotylenchus* e outros, contribuem para a queda do rendimento da sojicultura, especialmente nas regiões tropicais e subtropicais (DIAS et al., 2010; VICTOR; ALVES, 2015).

Estima-se que o parasitismo estabelecido pelos nematóides no mundo leve a perdas de 10 a 15% na produção de importantes culturas anualmente, o que em termos econômicos pode significar a cerca de US$ 80 bilhões em prejuízos (LIMA et al., 2017). Esses fitopatógenos causam não somente danos diretos às plantas, reduzindo o crescimento e/ou tornando-as improdutivas, mas também indiretos pela sua interação com outros organismos, como fungos e bactérias que tem sua entrada facilitada pelo sistema de raízes (BERNARD; EGNIN; BONSI, 2017).

3.2. NEMATÓIDES PARASITAS DE PLANTAS

Os nematóides, também conhecidos como "vermes", pertencem ao filo Nematoda. Eles representam os helmintos de morfologia arredondada não segmentada e com simetria bilateral (RAHMAN; MANAF, 2014). As evidências evolutivas mostram que o filo Nematoda surgiu antes do Cambriano (~ 700-650 Ma), o que contribuiu para a sua ampla diversificação, estimadas em até um milhão de espécies (DE LEY, 2006). Esses organismos apresentam uma excelente adaptabilidade a muitos nichos ecológicos, que vão desde regiões polares geladas até desertos, até águas salgadas e doces, o que os colocam como um grupo evolutivamente bem-sucedido (MCSORLEY, 2003). A grande maioria dos nematóides são de vida livre, mas alguns outros precisam de um hospedeiro, como animais ou plantas, para completar seu ciclo de vida (SOMMER; STREIT, 2011).

Ao longo de milhões de anos, a associação de plantas e nematóides resultou na evolução do fitoparasitismo (BERNARD; EGNIN; BONSI, 2017). Os

nematóides parasitas de plantas são considerados “inimigos invisíveis”, porque os sintomas por seu ataque nas partes aéreas da planta podem ser normalmente associados/confundidos com formas de estresses abióticos, como falta de nitrogênio ou água. Além disso, a falta de sintomas específicos, torna difícil a identificação da presença dos fitonematóides por serem invisíveis a olho nu e por estarem dispersos no solo, principalmente quando de habito migrador (JONES; FOSU-NYARKO, 2014). Os danos causados pelos nematóides parasitas de plantas podem depender muito do tipo de cultura atacada, de seu estágio de desenvolvimento, e das condições edafoclimáticas (PALOMARES-RIUS; KIKUCHI, 2013; SINGH et al., 2013).

As espécies de nematóides parasitas de plantas podem ser divididas, com base nos seus estilos de vida, em divesos grupos: i) ectoparasitas: nematóides remanescentes do solo e que não penetram nos tecidos da planta; ii) endoparasitas: nematóides que penetram completamente nos tecidos radiculares; e iii) semi- endoparasitas: nematóides que penetram nas raízes com apenas a parte anterior de seus corpos, enquanto a posterior se mantem para a face do solo. Essa divisão fica ainda mais complicada com a presença de estilos de migração ou ciclos de vida sedentária. Os endo- ou ectoparasitas sedentários, como os gêneros *Heterodera* spp (nematóide de cisto) e *Meloidogyne* spp (nematóide de galha), que passam parte de seu ciclo de vida dentro dos tecidos do hospedeiro e são altamente seletivos para a seleção do mesmo, até os endo- ou ectoparasitas migratórios, que tem ampla gama de hospedeiros, como é o caso do gênero *Pratylenchus* spp. (DECRAEMER; HUNT, 2006; ELLING, 2006).

São mais 100 espécies descritas dde nematóides parasitas de planta, envolvendo cerca de 50 gêneros (LIMA et al., 2017). Alguns gêneros de nematóides são de maior importância como parasitas de plantas/culturas, como *Meloidogyne*, *Globodera*, *Heterodera*, *Pratylenchus*, *Ditylenchus*, *Aphelenchoides*, *Bursaphelenchus*, *Xiphinama* e *Trochodorus* sp. (PALOMARES-RIUS; KIKUCHI, 2013).

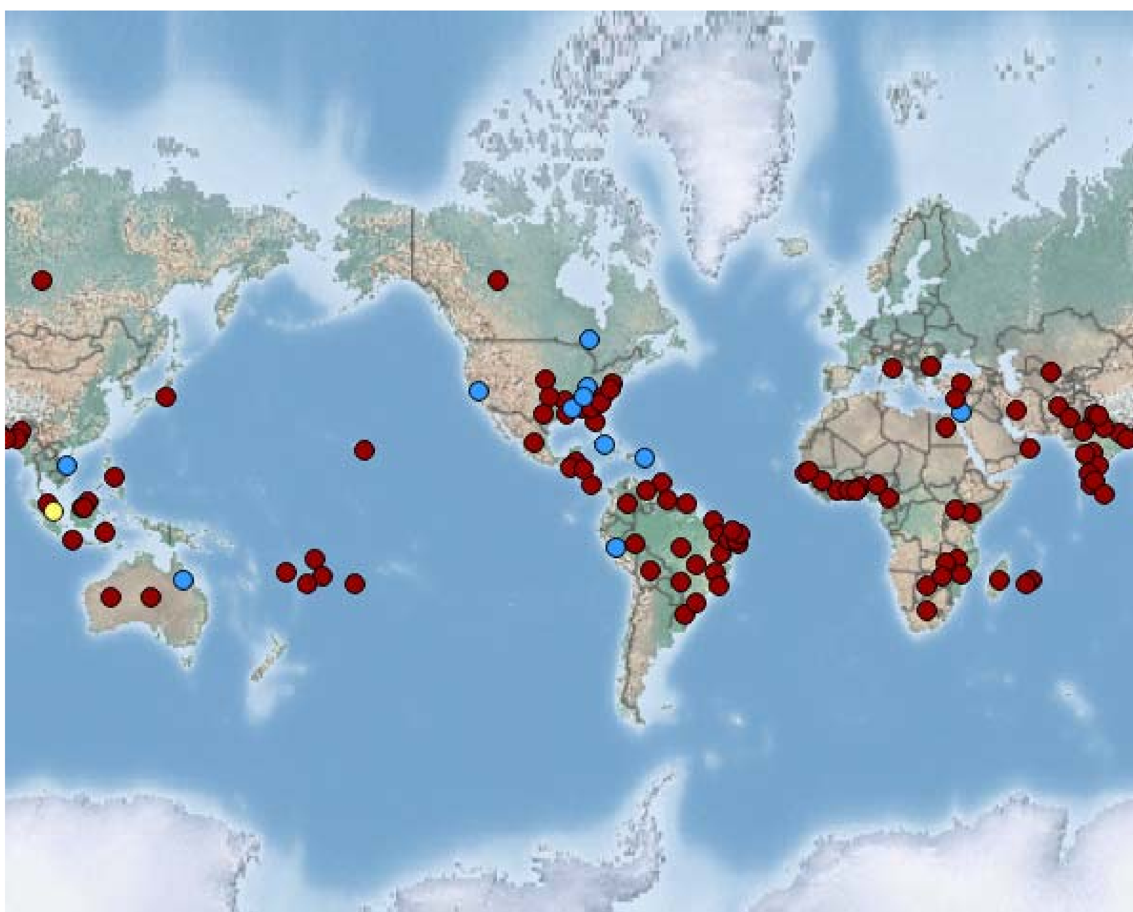
3.2.1. Aspectos Gerais Relacionados ao Gênero *Pratylenchulus* – *P. brachyurus*

Pratylenchus pratensis foi a primeira espécie do gênero descrita em 1880 (DAVIS; MACGUIDWIN, 2000). Atualmente, mais de 75 espécies do gênero *Pratylenchus* estão distribuídas por todo o mundo (ARAYA et al., 2016). Também conhecidos como nematóides de lesão radicular, estes parasitas são biotróficos obrigatórios, enquadrado no grupo dos endoparasitos migradores. Diferente dos nematóides sedentários, os de hábitos migradores não se instalam em um local da raiz, nem desenvolvem um sítio de alimentação, podendo, inclusive, retornar ao solo (GOULART, 2008). Essa disparidade de hábitos entre gêneros de fitonematodes, ocorreu provavelmente de diversos eventos evolutivos independentes dentro do filo de Nematoda (BIRD et al., 2015; QUIST; SMANT; HELDER, 2015).

O comportamento migrador permite que os nematóides do gênero *Pratylenchus* se movimentem ativamente de raíz em raíz, penetrando no tecido do hospedeiro até o córtex da raíz, onde se alimentam principalmente do citoplasma das células corticais. O ataque desses nematóides produz galerias nos tecidos do hospedeiro devido ao movimento livre através da raiz e alimentação, resultando em manchas escuras ou lesões. Devido à sintomatologia nas raízes, os nematóides do gênero *Pratylenchus* são chamados “nematóides das lesões radiculares”.

Descrita originalmente em 1929 no Hawaii, *P. brachyurus* apresenta de 0,3 a 0,45 mm de comprimento nos estádios juvenis e de 0,5 a 0,7 mm nos adultos (MARCHIORATO, 2008). O ciclo de vida é simples e relativamente rápido, cerca de 3 a 4 semanas, quando em condições ambientais adequadas, principalmente de temperatura e umidade, permanecendo a maior parte desse tempo no interior da raiz. A espécie, *P. brachyurus*, reproduz-se por partenogênese, uma fêmea produz em média de 80 a 150 ovos durante toda a vida, os quais podem ser depositados no interior das raízes ou no solo próximo à superfície das raízes (FREITAS, 2013). Dos ovos eclodem juvenis de segundo estádio que, tendo a oportunidade de atacar plantas suscetíveis, irão desenvolver-se em juvenis de terceiro e quarto estádios transformando-se, por fim, em fêmeas adultas. Normalmente ocorrem várias gerações em uma única safra da cultura hospedeira (GOULART, 2008).

P. brachyurus é um organismo polífago, ou seja, é capaz de infectar um grande número de espécies vegetais, sugerindo um parasitismo menos especializado (mais primitivo) em relação a outros fitonematóides. Dentre as culturas afetadas por *P. brachyurus*, estão o milho, cana de açúcar, batata, algodão, quiabo, pessegueiro, entre outras (FLIS et al., 2018). A soja também é afetada, e a maioria das cultivares possibilita a multiplicação do nematóide em suas raízes. Por essa razão, esse nematóide representa atualmente um problema para diversos sistemas de produção, causando impacto econômico mundial (GOULART, 2008; HANDOO; CARTA; SKANTAR, 2008). A dimensão



desua dispersão pelo globo vem intensificando este problema (Fig.1).

Figura 1: Mapeamento da distribuição de nematóides do gênero *Pratylenchus brachyrus*. Os pontos coloridos representam o *status* das populações; pontos azuis marcam regiões onde esse nematóide foi reportardo como amplamente distribuído; os pontos em vermenlho, onde já foram localizados; e os pontos em amarela, onde já ocorreram alguns relatos de sua presença (CABI, 2018).

O parasitismo por *Pratylenchus brachyurus* figura até o momento em segundo lugar em termos de importância para a cultura da soja, o que torna as

atuais e crescentes pesquisas referentes a tal grupo de nematóides essenciais (FERRAZ, 2006). Recentemente, a ocorrência de *P. brachyurus* tem ganhado importância, tanto pelos danos à cultura quanto pela ampla disseminação e alta incidência do patógeno em áreas produtoras do Estado de Mato Grosso, região importante no cultivo da soja. Análises freqüentes do solo apontam populações de *P. brachyurus* extremamente elevadas, com mais de 1000 indivíduos por grama de raízes de soja, causando reduções de produtividade nestas áreas de até 30% (RIBEIRO, 2010).

As injúrias causadas por esse patógeno levam a planta ao estresse hídrico e também desencadeiam sintomas de deficiências minerais, com conseqüente subdesenvolvimento (DIAS et al., 2010). Em áreas atacadas por *Pratylenchus brachyurus*, a presença de reboleiras chama bastante a atenção, onde as plantas ficam menores, mas continuam verdes.

São muitos esforços para identificar, no banco de germoplasma de culturas agrícolas, genótipos resistentes aos nematóides parasitas de plantas. Para a soja, poucas cultivares resistentes ou tolerantes a *P. brachyurus* foram relatadas até o momento, devido principalmente a essa interação ser menos complexas, não havendo a necessidade de formação de um sitio de alimentação, como ocorre com os nematóides de cisto (*H. glycines*) e de galhas (*Meloidogyne* spp), as chances de se encontrar fontes de resistência são menores (GOULART, 2008; RIOS et al., 2016).

3.3. INTERAÇÃO PLANTA-PATÓGENO A NÍVEL MOLECULAR

As interações planta-patógeno são alvos frequentes de pesquisa, uma vez que a ação dos patógenos é responsável por perdas econômicas relvante nas mais importantes culturas (IGLESIAS et al., 2013). De acordo com as terminologias das interações planta-patógeno, a maioria das plantas são imunes aos patógenos (COOK, 1998).

Plantas imunes não são danificadas pelo patógeno, pois apresentam uma condição que leva à incapacidade desses se desenvolverem e se reproduzirem no interior dos tecidos vegetais, normalmente por mecanismo de bloqueio que se manifesta já no início da penetração, inibindo-os. Outros termos, como a resistência e a suscetibilidade de plantas referem-se à habilidade de suprimir o desenvolvimento e a reprodução do patógeno. Plantas

altamente resistentes possibilitam taxas de reprodução muito restritas dos parasitas, podendo ser vertical (raça-específica) ou horizontal (raça inespecífica) em relação ao patógeno, ao passo que as suscetíveis permitem abundante reprodução (VALE; PARLEVLIET; ZAMBOLIM, 2001).

Outra definição importante é a de tolerância. Plantas tolerantes a certas espécies de nematóides sofrem pouca ou nenhuma injúria, mesmo sob alta infecção com desenvolvimento da doença, contudo, sem perdas significativas na produção. Plantas intolerantes, por sua vez, sofrem danos severos. A fisiologia da tolerância não é bem compreendida e pode ser mediada por mono ou poligenes (DEVELEY-RIVIÈRE; GALIANA, 2007; VALE; PARLEVLIET; ZAMBOLIM, 2001). Contudo, sabe-se que atributos de resistência e tolerância são independentes, não devem ser confundidos, e podem ser herdados de maneira separada e diferenciada. Assim, plantas resistentes podem ser intolerantes, sofrendo injúria, mesmo sob baixa infecção (DEVELEY-RIVIÈRE; GALIANA, 2007).

Partindo desses termos, dois diferentes tipos de interação molecular planta-patógeno podem ser descritos, a interação compatível e a incompatível. Esta última ocorre, segundo FLOR (1971), quando a planta, evolutivamente, desenvolve mecanismos bioquímicos de defesa (como a síntese de metabólitos secundários e fitormônios), pela presença de genes de resistência (R) capazes de reconhecer o produto de um gene de avirulência (*avr*), ou elicitor, que está presente no patógeno (mecanismo de resistência gene-a-gene) (DALIO et al., 2014; PINTO; RIBEIRO; OLIVEIRA, 2011). Em termos moleculares, a resistência ou suceptibilidade das plantas envolve normalmente duas vias reconhecimento do patógeno, PTI (PAMP-triggered immunity (ou MAMP e DAMP) e ETI (effector-triggered immunity) (BOYD et al., 2013). Enquanto na interação compatível não há o reconhecimento da ação desempenhada pelo patógeno e, consequentemente, não há resposta da planta.

Na interação incompatível, a planta não desenvolve a doença, mas muitas vezes desenvolve uma resposta de hipersensibilidade (HR), desencadeando a morte celular programada, bloqueando a reprodução e dispersão do patógeno para as demais células. Seguida a HR, há ativação de mecanismos de defesa sistêmicos, também denominado Resistência Sistêmica Adquirida (SAR), capaz de prontamente ativar mecanismos de defesa em

tecidos distantes ao local de infecção, na presença do patógeno. Ambas as respostas local e sistêmica são dependentes da ativação de diferentes rotas de defesa, mediadas por genes já identificados (DALIO et al., 2014; PINTO; RIBEIRO; OLIVEIRA, 2011).

O marco inicial da análise genética da resistência ocorreu 35 anos após as descobertas de Mendel, sobre as unidades de herança transmitidas para as gerações, os genes. A resistência das plantas, como no caso aos nematóides, também pode ser caracterizada pelo número de genes que controlam a característica, podendo ser monogênica, (gene maior, ou ainda qualitativa) mediada por um único gene capaz de conferir a resposta de resistência, assim a planta apresenta ou não a doença (um único gene – *Meloidogyne incógnita*, em tomate), oligogênica (dois a três genes – *Heterodera glycines*, em soja) ou ainda resistência poligênica, também chamada de quantitativa (vários genes), gerando um gradiente em relação ao grau da doença, com genótipos apresentando reações intermediárias (moderadas) entre os extremos, suscetibilidade e resistência. O modo como a resistência a nematóide é herdade é importante na definição da estratégia a ser adotada para incorporá-la em cultivares comerciais de soja (FERRAZ, et. al., 2001;, 1995).

3.3.1. Estudo de patosistemas e análises de interação proteína – proteína

Diferentemente das abordagens de transcriptômica e genômica, a maioria dos métodos para os estudos do “interactoma”, fator de avirulencia/proteína vegetal de reconhecimento, requerem a abordagem proteômica com a expressão de proteínas fusionadas, utilizando ORFs clonadas. Para facilitar os estudos, as abordagens de alto rendimento com base na clonagem recombinatória têm sido desenvolvidas e permitem a clonagem simultânea de centenas de proteínas (BRAUN et al., 2013). Várias coleções de ORFs, algumas com um enfoque biológico particular, têm sido construídas para *Arabidopsis* (por exemplo, WEISTE et al., 2007; YAMADA et al., 2003). No total, ORFs para quase 15.000 *loci* estão agora disponíveis como clones entrada Gateway® no *Arabidopsis* Biological Resource Center (BRAUN et al., 2013). A disponibilidade de bibliotecas de ORF eliminou um importante gargalo do mapeamento de interactomas e vem permitindo aos pesquisadores

concentrar-se em experimentos para o mapeamento de interações reais ou melhor conhecidas.

A investigação imparcial e sistemática das interações entre proteínas da planta e do patógeno fornece a oportunidade de descobrir os recursos de conectividade de proteínas que podem levar a novos *insights*, além da descoberta de novas relações bioquímicas entre duas proteínas de interesse. Ensaios de duplo-hibridos em levedura (Yeast 2-hibrid - Y2H) têm sido amplamente utilizada em experimentos de mapeamento de interação para proteínas animais e de plantas em escalas individuais, módulos biológicos, famílias de proteínas e/ou proteomas inteiros (BRAUN et al., 2013).

O sistema de duplo-híbrido foi descrito pela primeira vez por Fields e Song em 1989 que, baseados em resultados anteriores, descreveram um sistema genético para detecção direta de interações proteína-proteína em leveduras *Saccharomyces cerevisiae* (RAO et al., 2014). Estes autores desenvolveram uma forma de monitorar interações protéicas baseadas nas propriedades modulares da proteína GAL4, encontrada em leveduras. Essa proteína é um ativador transcricional que ativa a transcrição na presença de galactose, por meio de uma ligação a uma sequência específica de DNA (*upstream activation domain* - UAS). A GAL4 apresenta dois domínios distintos: um domínio localizado na porção N-terminal, que desempenha uma função de ligação ao DNA (DNA binding domain - BD); e um domínio localizado na porção C- terminal, que é responsável pela ativação da transcrição (*activation domain* - AD). Ambos os domínios são capazes de manter suas funções de modo independente um do outro, mas não ativam a expressão do gene repórter, quando não há interação entre proteínas (BRÜCKNER et al., 2009).

A técnica foi desenvolvida a partir da idéia de fusionar duas proteínas de interesse aos domínios da proteína GAL4. Para que o sistema Y2H seja eficiente, duas construções devem ser realizadas: uma chamada de “isca”, onde uma proteína é ligada ao DBD) de Gal4, e uma chamada de “presa”, onde a outra proteína de interesse é ligada ao AD da Gal4. Após a interação entre isca e presa, o fator de transcrição Gal4 é reconstituído permitindo a transcrição de um gene repórter. Apesar de proporcionar a observação de interações protéicas *in vivo*, o sistema Y2H exige um minucioso planejamento e cuidados redobrados no desenvolvimento dos experimentos, uma vez que

organismos vivos apresentam uma maior complexidade. Assim, para a eficiência da técnica, a aplicação de métodos de controle de expressão e especificidade é indispensável, e, além disso, o gene repórter selecionado (muitas vezes mais de um gene repórter é utilizado) precisa codificar uma proteína cuja função providencie uma leitura simples e adequada.

Falsos negativos e falsos positivos constituem a maior limitação dos sistemas Y2H. Os falsos negativos são interações proteína-proteína que não podem ser detectadas devido às limitações do método de leitura, ou devido às modificações pós-traducionais de proteínas de eucariotos superiores quando analisadas em leveduras, podendo desenvolver uma conformação tridimensional diferente de seu estado natural (MACDONALD, 2001). Por outro lado, quando interações físicas são detectadas, porém não são reprodutíveis em sistemas independentes, ou não podem ser confirmadas por outros testes *in vitro*, elas podem caracterizar um resultado falso positivo. Nestes casos, estas interações ocorrem por um motivo diferente do esperado e podem, por sua vez, ter origem em várias causas como, por exemplo, a superexpressão da isca e/ou da presa e a ocorrência de dobramentos incorretos das proteínas avaliadas permitindo interações inespecíficas. Assim, de modo geral, estima-se que a taxa de falso positivo no sistema Y2H em larga escala seja de 25 a 45% dos resultados (BRÜCKNER et al., 2009).

Assim, além do planejamento prévio de estratégias de seleção e de todos os cuidados na execução do experimento, depois de identificar as interações é comum a utilização de outras técnicas de análise de complexos protéicos que permitam a validação das interações encontradas através da metodologia de Y2H. Várias tecnologias foram desenvolvidas para a identificação e análise de interações proteína-proteína *in vitro* e *in vivo*, cada uma com suas vantagens e limitações. Dentre as técnicas já aplicadas, podemos destacar a estratégia de complementação bimolecular por fluorescência (BiFC).

Inicialmente descrita para detectar a interação e localização subcelular de fatores de transcrição bZIP e de fatores de transcrição da família “Rel” em células de mamíferos, a técnica de BiFC, também conhecida como “YFP dividido”, baseia-se na observação de que as subunidades N- e C-terminais de GFP (ou seus derivados, por exemplo, YFP) não reconstituem

espontaneamente um fluoróforo funcional. No entanto, uma vez fundidos à proteínas que interagem entre si, as subunidades não-funcionais do fluoróforo são postas em contato próximo, de tal maneira a permitir o redobramento da proteína, tornando-a funcional e restaurando a fluorescência (complementação de fluorescência)(HU; CHINENOV; KERPPOLA, 2002).

Assim, a interação entre as proteínas pode ser facilmente monitorada através da emissão de fluorescência após excitação com um comprimento de onda adequado. Algumas características tornam o GFP e suas variantes, genes repórters especialmente atrativos, pelo fato de que não necessitam de reagente exógeno para detecção da proteína uma vez que ocorra a interação, além de serem conhecidos por sua funcionalidade em praticamente todos os tipos celulares e independente da estrutura subcelular na qual sejam testados (BHAT; LAHAYE; PANSTRUGA, 2006).

Muitos outros trabalhos vem empregando essa técnica para identificação da interação de genes efetores de nematóides e seus respectivos interactores na planta hospedeira (GUPTA et al., 2015). Como um exemplo da utilização da técnica de BiFC para a validação de resultados obtidos através do sistema de duplo híbrido para um efetor de nematóide, podemos citar o trabalho de HEWEZI et al. (2015), no qual além da aplicação da técnica de Y2H para a varredura de uma biblioteca de cDNA de *Heterodera glycines*, também utilizaram ensaios de BiFC confirmando as interações observadas entre a proteína *10A07* e os fatores de interação do hospedeiro, *Arabidopsis*, *IPK* e *IAA7*. Os resultados possibilitaram a identificação de proteínas do hospedeiro bem como sua localização celular.

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To be submitted to Scientific Reports

5. ARTIGO 1: RNA-seq Profiling Reveals Soybean Compatible and Incompatible Interaction and its slightly differences in response against *Pratylenchus brachyurus*

Root-lesion nematode (RLN) causes a serious damage in soybean production, as well as many other crops worldwide. No resistance gene against RLN has been identified in soybean so far and its molecular mechanism of defense remains largely unknown. We conducted a global RNA-seq comparison between moderately resistant (BRSGO Chapadões) and a susceptible genotype (TMG 115RR) of *Glycine max*, from root tissue infected at 24, 48, 96, and 192 hours, to understand its regulatory network in *Pratylenchus brachyurus* (*PB*) defense. The total number of differentially expressed genes (DEGs) in BRS (637) was closer to TMG (628), but with a very different composition. The main differentiated time between genotypes was at 24h, presenting 170 DEGs exclusive in BRS. A high number of defense-related genes/pathways were induced in BRS, including genes of phenylpropanoids-Stilbenes pathway. Several metabolic pathways showed changes in TMG, especially for cell wall architecture and remodeling. Following our approaches to better explain soybean response to RLN, we found genes in BRS involved in pathogen recognition, calcium activated channel, *chitinases* and *Myb*-involved transcriptional regulation. Our results revealed calmodulins, jasmonic acid (JA)/ethylene (ET), the MAPK signaling cascade in a potential defense regulatory network, even more evident in BRS, and provided insights into soybean-RLN interaction.

Keywords: migratory nematode; molecular basis of host defense; differentially mapped genes, and differentially expressed genes.

Background

Plant-parasitic nematodes (PPN) are an issue for the whole world agricultural yields, causing $157 billion dollars loss [1]. Few years back, *Meloydogyne* sp. genera was considered, the most important nematode threat in USA, South America and South Asia's major crops, followed by *Heterodera* and *Globodera* species [2]. This scenario has changed and the root-lesion nematode (RLN; *Pratylenchus* species) is now widespread, being reported in all Southeastern and Central United States, South America, Africa, South Asia, Western Australia and even in Europe [3]. RLN composes an important group of root migratory endoparasites with a broad host variety, which makes its control challenging. Once the eggs are hatched, approximately one week after its deposition, all four juvenile and adult stages are capable of infecting and feeding from plant roots. *P. brachyurus* female reproduces by parthenogenesis, laying an average of one egg per day, with a life cycle that ranges from 45 to 65

days, depending on the environment temperature (Collins and Wilkinson 2015). Unlike the sedentary nematodes, RLN attack do not produce a feeding site or host cell differentiation, but creates galleries in host tissues by their moving and feeding in the plant root cortex, which results in necrotic spots or lesions. *Pratylenchus sp.* host resistance does not suppress the attack or penetration but migration, juvenile maturation, and reproduction [4].

Plant - *Pratylenchus* sp. interaction is considered evolutionarily new in comparison of those displayed by cyst or root knot nematodes, especially due to its migratory behavior [5]. In addition, most of plant resistance genes identified to date are related to sedentary nematodes, probably due to their biotrophic behavior and intimate relationship with the host [6,7]. For instance, *Mi* gene in tomato and *N* gene in pepper are related to root-knot nematode (RKN), *Meloidogyne* spp. [8]. Paal and co-workers (2004) described the gene *Gro*1-4 in tomato as able to confer resistance to *Globodera rostochiensis* (pathotype Ro1). *Rhg1* is another natural resistance gene, very well known in soybean, against soybean cyst nematode (SCN), *Heterodera glycines* [10]. *Prunus* spp., the stone fruit, shows some genes that confer resistance to *Meloidogyne incognita* and *Meloidogyne arenaria*, such as *Ma* and *Rjap* (Prunus cerasifera - plums), *RMia* (*Prunus persica* - peach) and *RMja* (Prunus dulcis – almonds) [11,12]. Other resistance genes include *Gpa*2 from *Solanum tuberosum* resistant to potato cyst nematode (PCN), *Globodera pallida*; *rkn*1 from *Gossypium hirsutum* resistant to *M. incognita*, and *Hs*1pro−1 from *Beta procumbens* against beet cyst nematode, *H. schachtii* [13–15].

Resistance and tolerance in plants to RLN has been identified in a few soybean genotypes, but a resistance gene has only been reported in wheat [16]. A single gene conferring resistance to *P. neglectus and P. thornei* has been mapped in wheat chromosome 7AL [17]. In addition, a major resistance quantitative trait locus (QTL) was identified on chromosome 6DS [18]. Based on the screened-out barley population, five major QTLs (Pne3H-1, Pne3H-2, Pne5H, Pne6H and Pne7H) mapped on four linkage groups (3H, 5H, 6H and 7H), were associated with *P. neglectus* resistance [19]. A highly significant QTL, *QPnToIMI.1*, was identified as related with resistance to *Pratylenchus neglectus* in the legume *Medicago littoralis* [20].

Plants have a complex mechanism of defense against pathogen attack, involving structural and chemical barriers and the induction of defense-related genes expression, such as pathogen-related proteins (PR proteins) [21]. PR proteins are a component of Pathogen-Associated Molecular Pattern (PAMP)-triggered immunity (PTI), the first line of defense in plants; it signals for systemic acquired resistance (SAR) or can directly fight against pathogenic invasion. Once a pathogen is able to disrupt host PTI, with an effector molecule, resistant plants (present R genes) are able to initiate their defense response by ETI (Effector-triggered Immunity) recognition [22].

Genome wide approaches have become a promising tool to elucidate global gene change profiles in plant pathology [23]. However, until now, there was no transcriptome study about soybean response to any migratory nematode, all those studies have been done with sedentary species; and few used RNA-seq analyses of other plant species in response to migratory nematodes. Rice response against the migratory root rot nematode (RRN), *Hirschmanniella oryzae*, comprising induction of programmed cell death and oxidative stress, and obstructs the normal metabolic activity of the root [24]. Furthermore, in Kyndt and co-workers [25] study, RNA-seq analysis at two time points (3 and 7 days after infection - dai), showed hormonal signalling pathway, as jasmonate (JA) and ethylene (ET) induction. *Boehmeria nivea* (L.), ramie, showed 137 significantly differentially expressed genes, such as those for protease inhibitors, pathogenesis-related proteins (PRs), cell wall reinforcement, and transcription factors in response to *P. coffeae* infection [26]. Multi-layered defense mechanisms were induced in a transcriptome analysis of oak tree against *P. penetrans*, comprising reactive oxygen species formation, hormone signaling (e.g. jasmonic acid synthesis), and proteins involved in the shikimate pathway [27].

We aimed to identify the key genes and/or important signaling pathways involved in soybean defense against *Pratylenchus brachyurus*. By conducting an RNA-seq, based on transcriptome comparison between mod. resistante (BRS) and suceptible (TMG) genotypes of *G. max*, we provided an inedited information and insights on the molecular mechanisms of RLN moderate resistance and susceptibility in soybean.

Materials and Methods

Plant material. Two *G. max* genotypes, cv BRSGO Chapadões (designated as BRS in this study) and cv TMG 115 RR (designated as TMG in this study), from the Embrapa Soja Active Germplasm Bank (AGB) were used in the experiment. In a recent study, BRS was identified as moderately resistant, and TMG showed high susceptibility to *Pratylenchus brachyurus* infection [16].

Nematode infection assay and sample preparation. Seeds of each genotype were planted in a 5-gallon tray filled with sterile sand (Nematology Greenhouse - Embrapa Soja, Londrina, PR, Brazil). After achieved five days of emerge, each healthy seedling was transplanted into a cone-tainer filled with sterile sand. All seeding-containing cones were pre-arranged in a cone-tainer support using a randomized complete block design.

The *Pratylenchus brachyurus* nematodes were reared on soybean cv. TMG 115 RR in Nematology Greenhouse for more than two years. Nematodes were harvested from stock roots by blending 20 seconds in water and sieving the solution through nested 200- and 500-μm sieves. The collected content from 500-μm sieve were incubated in a hatch chamber for 3 days at 26 ºC, to ensure the infective forms inoculation. The infective form of *P. brachyurus* was collected, and three days after transplantation, the healthy and uniform seedlings were inoculated with 500 infective forms each. In parallel, seedlings inoculated with water were used as the controls, or non-infected (mock).

To capture transcript variation responses to *PB* in *G. max*, we collected root samples for infected and control plants, respectively, at 24, 48, 96, and 192 hpi for transcriptome quantification. Briefly, roots were excised from both inoculated and non-inoculated controls at 24, 48, 96, and 192 hpi, washed, flash frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. Three uniform individuals plants were pooled as one biological replicate, and three replicates were collected for each genotype at each time point, generating 48 independently samples.

Library construction, sequencing and mapping of RNA-seq reads.

The frozen samples were crushed with a pestle and mortar and total RNA was extracted using the TRIzol® reagents (Invitrogen), according to the

manufacturer's instructions. DNAse I kit (Invitrogen) was used to remove the DNA contamination. Total RNA integrity and concentration were assessed using 1% agarose gel electrophoreses and NanoDrop spectrophotometer 1000 (Uniscience NanoDrop Technologies), respectively (Fig. S1). Following, the same amount of total RNA from each biological repeat were pooled together for one library construction. The cDNA synthesis was performed by Super Script TM III Kit (Invitrogen) applying Oligo-dT primers, according to the manufacturer's instructions and stored the cDNA at -20°C. The quality of the cDNA and the presence of no genomic DNA were checked by performing a PCR reaction using primers designed to two different exons of the β-actin gene (forward_ 5'-CCCCTCAACCCAAAGGTCAACAG-3' and reverse_ 5'-GGAATCTCTCTGCCCCAATTGTG-3') (Fig. S2).

The RNA TruSeqTM SBS Kit v5-GA sample prep kit (Illumina, San Diego, CA) was used to prepare the RNA-Seq library following the manufacturer's instructions. The final 16 libraries were distributed into four lanes on a flow cell for sequencing on an Illumina HiSeq 2000, utilizing a 101-bp read length with v4 sequencing chemistry (Illumina, San Diego, CA, USA). The RNA-seq was performed by FASTERIS Biotechnology company (Geneva, Switzerland).

The initial base calling, adaptors trimming, quality filtering of the reads generated with the Illumina analysis pipeline (Fastq format) were performed using Trimmomatic 0.36 software [28] and checked by FastQC software [29]. High-quality mRNA-Seq reads were aligned to the *Glycine max* reference genome (*Glycine max* Wm82.a2.v1 – Phytozome v12.1) [30] using GFF coordinates and the STAR RNA-seq aligner [31], that is capable to identify alternative splicing junctions, with default parameters. Gene splicing variants were not considered. BAM files were separated into unique mapped reads, multiple mapped reads, and unmapped reads. HTSeq-count program was used to count mapped reads [32]. Since mapping included both unique and multiple reads, we counted multiple reads mapped just for those genes that presents, at least, one unique read mapped.

Differential mapping and expression analyzes. The RNA-Seq method generates absolute information, rather than relative gene expression

measurements, overcoming many of the inherent limitations of microarray technique [33].

The counting data was used to perform differential mapping analysis (differential mapped genes – DGMs), which consisted in the comparisons of presence/absences on mapping results between genotypes and time point, as described below:

- Genes that were mapped in, at least, seven BRS libraries and none of TMG, and *vice versa* (showing genotype specific root expression; differential mapped genes (DGGs)-genotype exclusive genes);
    - BRS_DGGs – exclusive responsive (mapped in, at least, seven BRS libraries (inoc and mock) and none of eithy libraries in TMG);
    - TMG_DGGs – exclusive responsive (mapped in, at least, seven TMG libraries (inoc and mock) and none of eithy libraries in BRS);

- Genes that were mapped in, at least, three BRS inoculated libraries and none of non-inoculated BRS or TMG, and vice versa (showing resistant genotype specific expression; DRGs-exclusive responsive genes);
    - BRS_DRG – exclusive responsive (mapped in, at least, three BRS inoc libraries and none of four BRS mock libraries or any TMG library);
    - TMG_DRG – exclusive responsive (mapped in, at least, three TMG inoc libraries and none of four TMG mock libraries or any BRS library);

All Venn assemblies analysis were performed using VennPainter [34]. The counting data was also applied to calculate transcripts per million (TPM) [35]. The TPM data worked as input for DESeq2 - R package to perform the differential expression analysis between infected and non-infected by time and genotype [36,37]. The ratio of expression (fold-change) was performed by dividing values of gene expression (TPM) under nematode infection by non-infected samples at

each time point and genotype. To avoid false positives and reliably identify the most significant changes in gene expression, the p-value threshold of < 5% and an absolute fold-change (FC) ≤ -1.6 or ≥ 1.6 were used to determine differentially expressed genes (DEGs).

Also, we checked genes that were not significantly differential expressed but displayed repressed profile (FC ≤ -2) in BRS and induced (FC ≥ 2) in TMG, and vice versa, (showing opposite expression pattern gene expression between different phenotype/genotype; opposite expression pattern genes).

Hierarchical clustering analyzes - HCA. As stated before, genes can change their expression profile over time-treatment, being activated/deactivated at the first moment, and switch in the next or the last one. All these changes allow a better understanding on how the plant defense response works; however, as it is always about large set of genes, being able to see all this information is challenging. Indeed, HCA is the tool to work with RNA-seq, being a useful method for analyzing gene expression data with an agglomerative (bottom-up) approach [38]. The clustering results subdivide a set of items (the genes), placing the similar ones into the same cluster, which allows a better exploration and interpretation of the data of thousands of individual genes [39].

Hierarchical clustering (HCL) of DEGs was performed using the Gene Cluster v3.0 and Java Treeview (EisenLab). HCL was applied with Pearson correlation, uncentered similarity metric distance and a complete linkage as clustering method.

Gene Ontology (GO) enrichment and gene pathway analysis. A GO Singular Enrichment Analysis (SEA) was applied to detect significantly enriched or depleted categories, for each set from DGGs by comparing the whole soybean genome (*Glycine max* Wm82.a2.v1) GO using the AgriGO tool v2.0 [40]. SEA parameters consist: Fisher’s test as statistical method; significance level of an adjusted p-value < 0.1; and the Plant GO Slim database.

In order to have an overall view of the ontology categories related to the nematode infection, we performed the parametric analysis of gene set enrichment (PAGE) analysis with the time-treatments of both genotypes. This

analysis consists in cross-comparisons of multiple data sets and the background genome GO annotation, taking in account the number of genes in a certain category but also its expression values [41]. The Parametric Analysis of Gene Set Enrichment (PAGE) was also performed using DEGs sets of all genotype/time-treatment, with expression data, at once, applying Hochberg FDR as statistical method; significance level of an adjusted p-value < 0.1; and the Plant GO Slim database [40].

In addition, MapMan [42], v3.6.0RC1 (2013), was used to map the expressed genes onto metabolic pathways. As MapMan mapping and pathways background are still in the previous version of *Glycine max* genome, we used synonymous information, available in Phytozome, to find the corresponded Gene ID and run this analysis. For this reason, some genes did not present its synonymous and 8 of BRS024 genes could not be included in MapMan analyses; the numbers were 2 genes (BRS048), 3 (BRS096), 15 (BRS192), 9 (TMG024), 6 (TMG048), 3 (TMG096), and 15 (TMG192).

RNA-seq validation experiment by real time RT-qPCR. A new experiment was installed following parameters previously described in the RNA-seq material obtainment. After total RNA extraction, DNAse I treatment, and cDNA synthesis, qPCR assays were carried out.

Reverse transcriptional quantitative PCR (RT-qPCR) was performed using the cDNA product corresponding to 25 ng of total RNA in a 9.4 uL reaction volume and 384-well plate using the Maxima SYBR Green/ROX qPCR Master Mix (2×) (Thermo, USA) on a detection system (ABI 7900HT [Life Technologies, Grand Island, New York, USA]). Dissociation curves were obtained to guarantee the absence of nonspecific amplification. The data were collected in the log phase, and the results were analyzed with the Sequence Detection System v2.4 Standard (Perkin Elmer, Waltham, Massachusetts, U.S).

The efficiency of each primer pair used in this study was tested by amplification of a pool of all samples above mentioned to run RT-qPCR reactions. The $E = [10^{-1}/\text{slope}]^{-1}$ formula was employed to calculate the reaction efficiency and to adjust the final primer concentration. The calibration curve was established based on the Ct and the log of five cDNA serial dilutions [1:10]. All efficiencies tested were up to 0.9%.

Seven soybean reference genes 18S RNAr (*Glyma13G12030* – Phytozome v1.0), *β-actin* (*Glyma.15G05570*), *elongation factor 1-alpha* (*Elf1-α*) (*Glyma.19G07240*), *elongation factor 1-beta* (*Elf1-β*) (*Glyma.02G44460*), *legume lectin* (*Lec*) (*Glyma.02G01590*), *tubulin alpha* (*Tua*) (*Glyma.08G12140*), and *tubulin beta* (Tub) (*Glyma.20G27280*) were tested by RT-qPCR analyses, using all the BRS and TMG *P. brachyurus* infected and non-infected samples from this study. Ct values of each reference gene were analyzed using the RefFinder tool [43]. This program uses the comparative delta-Ct method, BestKeep, GeNorm, and Normfinder algorithms, and various statistics methods, to identify the stable reference genes. Finally, ReFinder also generates a comprehensive ranking, based on the geometric mean of classification data from the four different algorithms, to identify the best reference genes.

To validate the RNAseq data, 20 genes DEGs or DMGs were selected and primers were designed using Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi). Primers were efficiency tested as described before (Tab S1). Ct values from selected genes were first normalized by *TUA* and *ELF1*-β housekeeping genes; the relative expression results were obtained with REST 2009. The final relative quantification of each gene compared with the control conditions was estimated considering the RQ obtained in each biological replicate, represented by each independent experiment, with three replicates each. Significant differences were determined based on estimates of the standard deviation (SD) and with REST software version 2.0.7 (2009) ($p < 0.05$) (http://gene-quantification.eu/chapter-3-pfaffl.pdf).

Results

Illumina sequencing allowed results of DEGs and DMGs. To reveal the RLN infection-associated transcriptional responses in soybean moderately resistant and susceptible genotypes, both RLN-infected and non-infected samples at four different time points were subjected to total RNA extraction and RNA-seq analysis. The Illumina HiSeq2000 high-throughput sequencing generated 154.44 and 124.49 million single-end reads (101 bp) for all BRS and TMG treatments, respectively (Tab S2). All of the raw data will be deposited in the NCBI's Short Read Archive (SRA) database.

To guarantee better results for the following mapping and assembly, the raw reads were further trimmed, filtered, and the average of 8.17% of reads were discarded from each library.

The total length of mapped reads was over 1.4 billion bases, representing nearly 10-fold coverage of the soybean-annotated transcriptome. Among all 56,044-soybean genes (Phytozome V12.1), 47,543 genes (without redundancy) had their expression detected when all libraries mapping reads results were combined. The average of mapped genes by genotype was 46,384 (86.76% of all soybean genes) for BRS, and 46.021 (82.11%) for TMG.

Set comprising DEGs in response to PB was different between genotypes. While the majority of mRNA is expressed at low levels, a small proportion of mRNA is highly expressed/repressed under some specific conditions [44]. By performing RNA-seq of samples under *P. brachyurus* infection and non-infection conditions, we were able to access the DEGs for BRS and TMG, during the timing course. We found 1150 genes (without redundance between genotypes and all time-treatments) that were differentially expressed in soybean plants in response to *Pratylenchus brachyurus*. Results displayed significant changes in mRNA levels, in BRS were 637 DEGs and in TMG were 628, without redundant in all time-treatments.

The number of DEGs was largest at 192 h in both genotypes (BRS192 n = 332 and TMG192 n = 307), when compared to the other time-treatments. For both sets, BRS192 and TMG192, there were a balance between up and down-regulated genes. Unlike, TMG024 and TMG096 displayed a high percentage of up-regulated genes, 71.42% and 80.85%, related to total, respectively. The number of DEGs per time-treatments and distribution in up- and down-regulated is shown in the figure 1 and table S3.

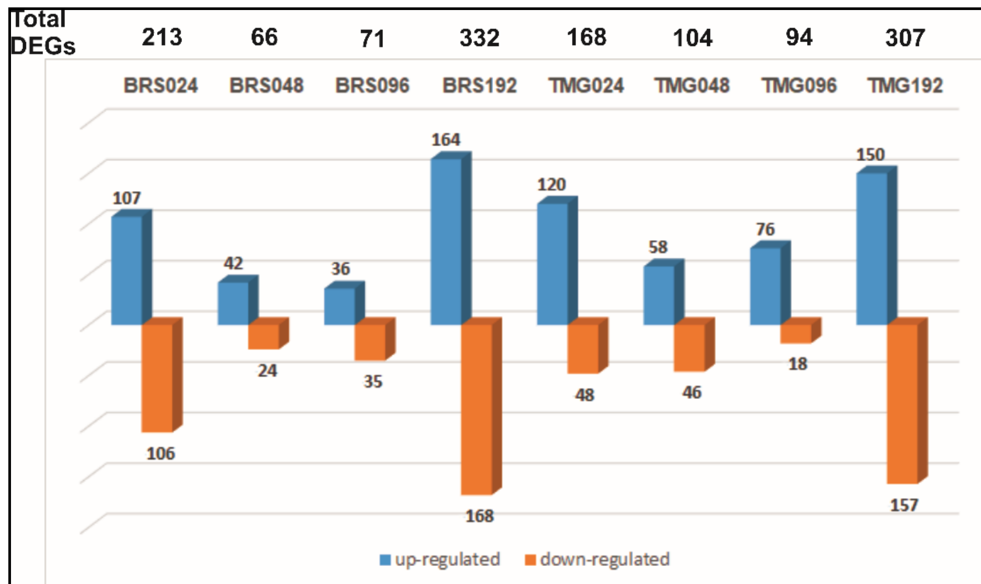

Figure 1. Total DEGs per time-treatments. Blue bars represents the number of up-regulated genes, and orange bars the down-regulated genes.

Volcano plots were used to visualize the distribution of DEGs (Figure S3). The relationship among DEGs in BRS and TMG in all time-treatments datasets was visualized in a Venn diagram (Fig. S4; Tab. S4); additionally, the relationship among DEGs all time-treatments datasets by genotype was visualized also in Venn diagram (Fig. S5A and B; Tab. S4). A very low number of DEGs were observed as related between genotypes.

By applying the hierarchical clustering method, we could display the arrangement and have an overview of the transcriptome changes to *P. brachyurus* infection in both genotypes (Fig. 2 and Fig. S6A). The treatments arrangement and the heat map showed a consistent result and how the majority of these DEGs behaved similarly in both BRS and TMG.

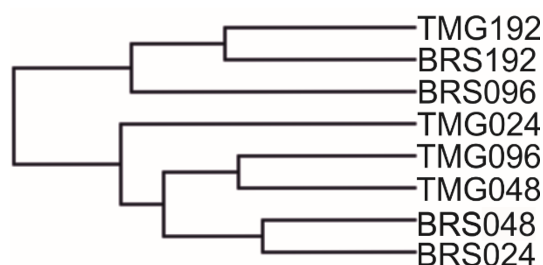

Figure 2. Hierarchical clustering displays the treatments arrangement in both genotypes.

Among the time treatment, 96h is the time point in which the genotypes were most differentiated (Fig. 2). The expression of the set of genes at last time of infection treatment (192 h) was closely related between genotypes. The first two times from BRS were placed together, while TMG024 was turned away from any other time-treatment and genotype. TMG048 and TMG096 were placed together, right close to the first times of BRS. There is a division among the first and last time treatments.

To gain insights into the biological processes associated with resistant responses to RLN *P. brachyurus*, we performed PAGE GO analysis to identify terms/categories significantly over-represented for up- and down-regulated among DEGs (Fig. S7; and Tab S5). As shown in Fig. 3, the transcription regulator activity (see category number 3) showed an early activation at 48 h in BRS, and it were increased at 192 h, while this activation started only at 192 h in TMG. A similar profile was shown by the chromatin binding category in those treatments and genotype.

GO Information					CM							
No	☐ GO Term	Onto	Number	Description	1	2	3	4	5	6	7	8
1	☐ GO:0005618	C	12	cell wall								
2	☐ GO:0003682	F	10	chromatin binding								
3	☐ GO:0030528	F	23	transcription regulator activity								
4	☐ GO:0005515	F	71	protein binding								
5	☐ GO:0000166	F	94	nucleotide binding								
6	☐ GO:0005215	F	47	transporter activity								
7	☐ GO:0017111	F	24	nucleoside-triphosphatase activity								
8	☐ GO:0016740	F	106	transferase activity								
9	☐ GO:0003824	F	388	catalytic activity								
10	☐ GO:0005488	F	354	binding								
11	☐ GO:0043229	C	59	intracellular organelle								
12	☐ GO:0009579	C	24	thylakoid								
13	☐ GO:0006350	P	37	transcription								
14	☐ GO:0050789	P	41	regulation of biological process								
15	☐ GO:0009058	P	109	biosynthetic process								
16	☐ GO:0003677	F	37	DNA binding								
17	☐ GO:0016301	F	43	kinase activity								
18	☐ GO:0050896	P	60	response to stimulus								
19	☐ GO:0009607	P	14	response to biotic stimulus								

Figure 3. Page GO map of selected categories that over-represented for up- and down-regulated among DEGs. It shows ontology categories affected by the nematode infection in all time-treatments of both genotypes. CM at the right upper part identifies BRS024 (1), BRS048 (2), BRS096 (3), BRS192 (4), TMG024 (5), TMG048 (6), TMG096 (7), and TMG192 (8). The color scale shows the categories regulation profile, and ranges from dark blue (strongly negative related) to dark red (highly positive regulated). For full information, see figure S7).

Many important categories changes, such as transferase activity, catalytic activity, and binding, were noticed in BRS at 96 h, and then were kept, or even intensified, at 192 h (Fig.3). Thylakoid and photosynthesis, a biological

process category, comprises a similar set of DEGs and started to decrease its activity at 96 h, and were still down at 192 h (Fig. 3; and Fig. S7; Tab. S5). Many cellular component categories followed a similar profile of activity, as cytoplasm and cell part that were shutted down (Fig. S7).

The opposite profile, an activation, was displayed for molecular functional categories, such as transcription, regulation of biological process, biosynthetic process, DNA binding, nucleotide binging, and kinase activity was positive regulated in BRS, especially at 192 h treatment. Interesting, most of these categories were also activated in TMG192, however, in a lower intensity.

Additionally, most significant changes in response to nematode were in biological process and molecular function categories in BRS, especially at 192 h, while cellular components was dropped. This organization was not so evident to TMG genotype at any time. The response to stress or stimulus categories (numbers 65, 66, and 67) was first activated only in TMG024 then dropped in all further time-treatments (Fig.3).

Selected sets of DEGs from GO categories and metabolic pathways known in plant defense. From both mapped metabolic pathways, and in GO categories results, we could select those set of genes more pronounced to be related to *PB* soybean response. Interesting, 28 receptor-like kinases (RLKs) encoding genes were related to both genotypes response to *PB* as being differential expressed at some point (Fig 4). Among RLKs, there were many leucine rich repeat (LRR) ones, domain of unknown function (DuF), and wall-associated kinases (WAKs). A very high number of kinases (18) and three mitogen-activated protein kinases (MAPKs) were also found in this study.

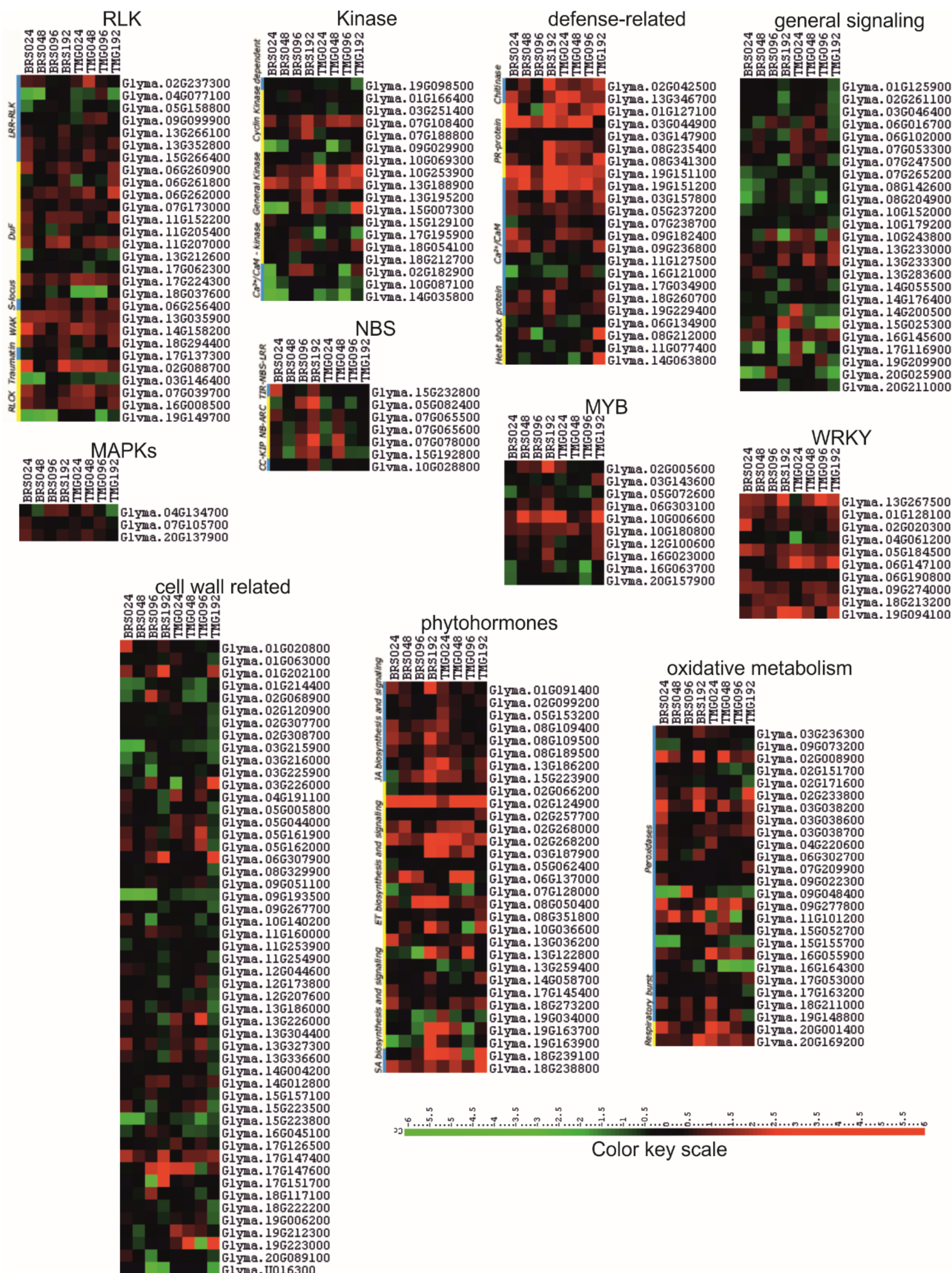

Figure 4. Heat map of the DEGs involved in various defense-related pathways. DEGs encoding RLK, Kinases, MAPKs, NBS, defense-related proteins, general signaling, MYB, WRKY, cell wall related proteins, phytohormones, and proteins related to oxidative metabolism.

Among nucleotide binding-site (NBS) encoding genes, there were a TOLL/interleukin-1 (TIR) and a coiled-coil (CC) domain proteins (Fig. 4). Defense related genes, as chitinases and PR-proteins, were strongly induced but Calcium/Calmodulin proteins and heat shock proteins had a more variety response. Transcription factor (TF) class was mostly induced and comprising ten encoding genes for each MYB and WRKY, the most related TF in plant biotic stresses (Fig. 4). Cell wall related genes were highly repressed and represented an important number in both genotypes. However, it is important to observe that the differential expression patterns were strongly repressed at the first two time-treatments in BRS, 24 and 48 h, and the last one in TMG, 192 h (Fig. 3, Fig. 4, and Fig. S7).

The set of DEGs mapped into main phytohormone metabolic pathways related to biotic stress displayed ethylene (ET) and jasmonate (JA) as most important signals (Fig. 4 and Fig. S7; and Tab. S6). JA was mostly induced in both genotypes, however ET showed more diverse response between BRS and TMG. The jasmonate pathway was induced in TMG024, BRS048 and BRS192 (Fig. S8). Indeed, it seems that jasmonate pathway induction is more pronounced in BRS than TMG, by the number of genes 3 and 1, respectively. Salicylate pathway was activated just at 192h in BRS and TMG. JA and Ethylene were the most hormone pathway represented in number of genes.

The results also presented 24 peroxidases related to *PB* soybean defense, followed by two respiratory burst genes, all related to oxidative metabolic pathways. Respiratory burst pathway is not present in TMG and slightly showed by BRS genotype. BRS displayed the same DEG down-regulated at 24 and 48 h, and one up-regulated at 192h (Fig. 4; Fig. S8; and Tab S6).

Opposite expression pattern or co-expression of DEGs show the slightly differences in response to PB. A relatively small number of DEGs showed opposite expression profiles (see methods) in both genotypes upon infection. These dramatic changes in expression between BRS and TMG can be a very important clue about its type of molecular response. Therefore, DEGs can be induced in BRS and repressed in TMG, or vice and versa, and give us some insights.

Among induced and repressed genes in BRS and TMG, respectively, five were annotated and/or showed similarity with very well known disease resistance genes, containing leucine-rich repeats (LRR) (Fig. 5). The gene set with an opposite profile comprising genes encoding proteins described as related to plant-microbe interaction. The inverse set, the repressed and induced in BRS and TMG, respectively, displays a remorin protein, an AMP-binding protein, and an inhibitor of apoptosis.

A gene that encodes for a pectinesterase, showed an induction in both genotypes during the first contact with the pathogen, but switched its expression profile from induced to repressed in the susceptible genotype over the period of infection (Fig. 5).

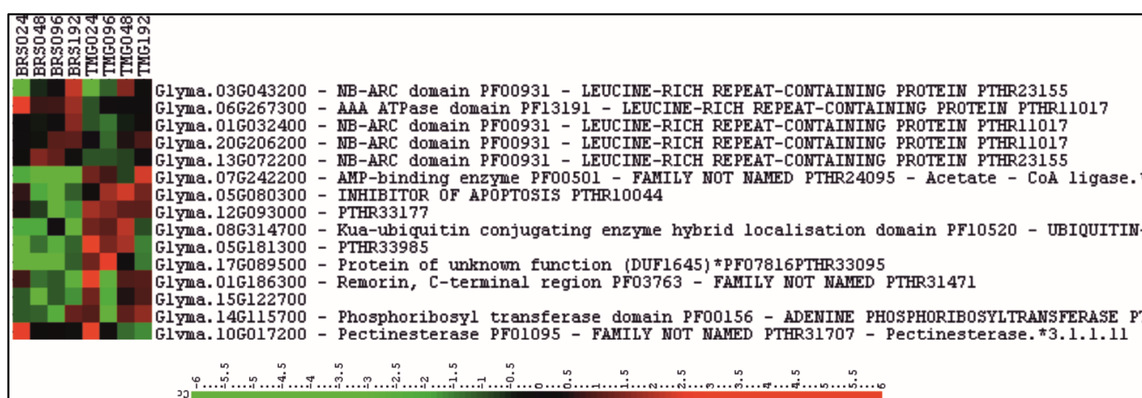

Figure 5. Heatmap (hierarquical clustered) of opposite expression pattern genes. The cluster on the top shows genes that were induced in BRS and repressed in TMG. The cluster above shows the opposite profile. The color key scale shows the $\log_2$ fold change: green = repressed; and red = induced.

The whole gene expression profile showed that there is a core of soybean response, no matter the genotype. There were large clusters of co-expressed genes fully activated; those that showed repression at all time-treatments, also some with repression, just at 192h, in both genotypes (Fig. S6A). However, slight changes were defined as related to different responses between genotypes, and can be seen by identified gene clusters (Fig. S6B). Seven different clusters were selected to better explain these differences. Cluster number 3 was comprised of 49 genes strongly down-regulated in TMG024 and kept it until 192 h, when switched to a slight activation. BRS showed no notable changes for these genes in any time-treatment from response to nematode (Fig. S6B). The expression profile is also interesting due to the opposite coordination during the time-treatments in between genotypes.

BRS drove from a repressed gene expression at 48h and switched to an induction, mostly significant; in 96 and 192 h. TMG showed activation at 48 h and dropped the expression at 96 and 192 h (Fig. S6B).

A new approach, differential mapped genes - DMG, might help understanding soybean response to PB. The RNA-Seq method generates absolute information, rather than relative gene expression measurements, overcoming many of the inherent limitations of microarray technique. The Illumina sequencing of 16 cDNA libraries, eight for each genotype (BRS and TMG), allowed us identify differential mapped genes by genotype.

All the 16 libraries mapped the same 44,862 genes; however, some genes were mapped in most time-treatments, just in one genotype (exclusive), and never in the other one (see methods). There were 99 DGGs in BRS and 71 DGGs in TMG (Fig. 6 and Fig. S9). Among 99 DGGs in BRS, there was enriched ontology categories comprising nucleotide binding, cell cycle and defense response (Fig. S10). There DGGs in BRS showed an important number related to defense response, such as calcium-activated channel, two MYB FT, five RLK, two chitinases, a Rubisco assembly process-RbcX protein, and 12 leucine-rich repeat-containing protein (Tab. S7).

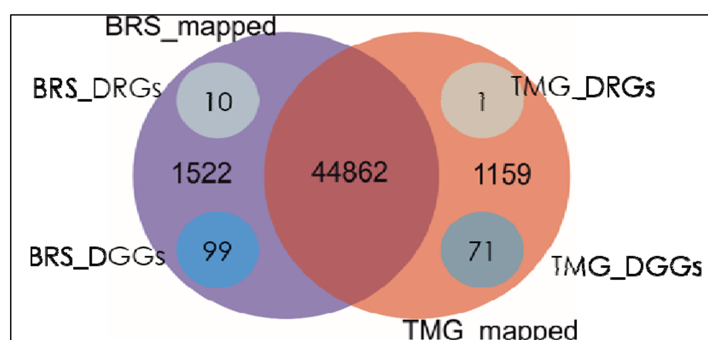

Figure 6. Distribution of differential mapped genes. 99 DGGs were exclusive per genotype in BRS and 71 in TMG. Ten DRGs were exclusive responsive por genotype in BRS and one in TMG.

A different set of ontologies was observed in the 71 DGGs from TMG. The most represented GO categories were biological process:secondary metabolic process and four subcategories of cellular component (Fig. S10). Unlike, just few encoding genes related to defense response, as only two leucine-rich repeat were found, but three different ankyrin- protein kinases.

The set of ten DRGs in BRS comprising cytochromes P450, described as involved in the biosynthetic pathway of major phytoalexins, a kinesin motor domain, a plastocyanin and two leucine-rich repeat-containing protein. A single DRG, *Glyma.18G194100*, was observed in TMG. According to PFAM annotation, it is a gene that encodes for a 3'-5' exonuclease.

A deep looking in all analyses results shows important genes involved in PB resistance. Our results of comparative transcriptome analyses of responses to *P. brachyurus* between BRS and TMG indicated that soybean defense requires enrollment of a variety of protein families involved in recognition of virulent proteins, signaling by phytohormone-mediated, transcriptional regulation by WRKY and MBY transcription factors (TFs), cell wall remodeling and associated signaling, chitinases, photosynthesis pathway and other defense-related proteins (Fig. 4; and Fig. S6-8).

RLKs, LRRs, and kinases, play important roles in defense against various stresses [45]. Among these proteins, a TIR-NBS-LRR (*Glyma.15G232800*), and protein like kinase – DuF domain (*Glyma.11g205400*) were differential co-expressed at 24 h in BRS (Fig. 4; and Fig. S6 – Cluster 2). *Glyma.07G039700* that encodes for an interleukin-1 receptor-associated kinase 4 (IRAK4), showed to be differentially expressed in BRS024, BRS048 and TMG192 (Fig. 4). Three Calcium^{2+}/Calmudulin depended kinases were strongly down-regulated at 24h in BRS and at 96h in TMG (Fig. 4). Five more encoding genes for NBS-LRR, a protein family known to be involved in effector triggered immunity (ETI), were activated at 96 and 192 h in BRS, especially *Glyma.07G078000* and *Glyma.05G082400* (Fig. 4). A RLK encoding gene, *Glyma.18G037600*, was strongly repressed in TMG024, TMG048, and TMG096.

In our study, the set of DEGs indicates that the biosynthesis of jasmonic acid (JA) and ethylene (ET), and their mediated defense signaling were affected by *P. brachyurus* infection (Fig. 4; and Fig. S8). From eight genes in JA pathway, four of it, *Glyma.08G109400*, *Glyma.01G091400*, *Glyma.05G153200*, and *Glyma.08G109500*, encode for chalcone synthase proteins. Twenty-one genes are related to ethylene signaling pathway. Even with two genes,

Glyma.18G239100 and *Glyma.18G238800*, salicylic acid pathway showed to be induced in both genotypes.

A calmodulin binding protein, MILDEW RESISTANCE LOCUS O (Mlo protein) (*Glyma.16g145600*), known to mediate susceptibility to powdery mildew fungi in plants, was down-regulated in BRS192, and up-regulated in TM192 (Kuhn et al. 2017) (Fig. S6 – cluster 6). Besides *Glyma.13G327800*, also another Mlo protein, was down-regulated at 192 h in both genotypes (Fig S6. – cluster 8).

Chitinases were represented by *Glyma.13G346700* and *Glyma.02G042500* as differential expressed, mostly activated in both genotype/time-treatments. In addition, two DGGs in BRS encodes for chitinases *Glyma.03G024500* and *Glyma.03G025000*. *Glyma.13G346700*, cited earlier, was also co-expressed with *Glyma.19G245400* that encodes for a chitin recognition protein (Fig. S6B).

Other genes that encode for proteins already related to plant defense against pathogens include a transketolase pyrimidine binding domain (*Glyma.08G277000*), differentially expressed in BRS048, BRS096, and BRS192 (Fig. 1), TMG showed a pectinesterase (*Glyma.03G215900*) strongly differential repressed at 48 and 192 h. Another pectinesterase (*Glyma.10G017200*) was found in the opposite pattern expression results (Fig. 5) and a BRS DRG, *Glyma.17G177100* that encodes for kinesin motor domain (Tab S7).

Results from RT-qPCR showed a high correlation with RNA-seq data. In order to perform an accurate and reliable validation of our RNA-seq data, we evaluated the stability in expression of seven soybean reference genes. All the algorithms used to evaluate the most stable genes, placed 18s followed by *Lec* as least options for use among those genes we tested (Tab S8). Mostly, *Tub*, *Tua*, and *Elf1-B* were classified as good reference genes by the four algorithms applied. Genes that displayed the most stable expression, and so the best reference genes to be applied in this study, were *Tub*, *Tua*, and *Elf1-B*, in this order (Fig. S11).

In general, linear equation demonstrated a good correlation between data sets. By plotting the RNA-seq expression data of 16 genes, in 30 random

genotype and time-treatment samples, with its results from RT-qPCR, we found a correlation of R^2=0.81 (Fig. 6; and Tab. S9).

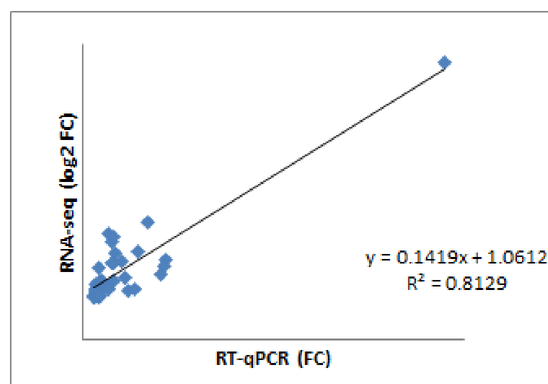

Figure 6. Validation of gene expression. Relative expression of 16 genes displays a high correlation between RNA-seq expression data and RT-qPCR. The *y*-axis represents the gene expression from RNA-seg data in log2 of the fold-change and the *x*-axis represents the gene expression in fold-change from RT-qPCR.

We also checked the correlation by treatment. Each time-treatment was analyzed by seven random primers selected from the set designed for this study. The highest correlation was found in BRS192 (R^2=0.95), while the lowest was in BRS048 (R^2=0.74) (Fig. S12; and Tab. S10). Except BRS048 and TMG024, with R^2=0.78, all the other treatment showed correlation higher than $R^2 > 0.8$.

Discussion

Consistent transcriptomic profiling strategies in understanding of soybean responses to P. brachyurus infection. An extensive gene expression change has been reported upon nematode infection, no matter what a compatible or incompatible interaction [46]; and the elucidation of these changes can lead to the development of new crop technologies. In studies related to plant parasites, RNA-seq approach usually takes different disease responsive genotypes in a contrast analyses. In this sense, the host genes can be specific expressed by a genotype and, even if in a small presence, can produce the defense response against parasites [47]. For these reasons, we chose to investigate several selected time points of *P. brachyurus* infection, in two opposite phenotype profiles, by sample pooling three biological replicates to obtain a representative deep sequencing analysis. The results among samples display how the sequencing presents consistent results about soybean

response to *P. brachyurus*, due to the slight differences observed between genotypes, but especially among the four different time-treatments. There were also two diferent steps of response after contact with the pathogen, an early and a later response in both genotypes. In fact, when biological effect shows a strong signal, like these found here, it is possible to detect differentially expressed genes, even in unreplicated RNA-seq experiments [48].

Indeed, these slight but strong differences were found and are important for a better understanding about compatible and incompatible responses. Instead of just differentially expressed, we also showed a new approach: the differentially mapped genes by genotype. In fact, mostly RNA-seq studies are focused on finding out just which genes are differentially expressed by comparing treatments/conditions and control samples. However, an important group of genes have been left behind: the specific genotype expressed ones. As a result, our study produced a large amount of genome-wide gene expression data in *G. max*, after *PB* infection, and provided a different approach that may lead to a better understanding of plant responses to migratory nematode infection.

In addition, Khang and Lau (2015) stated that the qPCR validation is possible, which was shown by our gene expression validation results, of a R^2= 0.81, from a new sampling experiment. A close correlation result between RNA-seq assays and RT-qPCR was present in soybean by Rodrigues et al. (2015) and Tian et al. (2017a). In addition, it is worth pointing out that RT-qPCR validations applying the same RNA samples assayed in the RNA-seq analysis only validate the technology (Fang and Cui 2011). A new experiment is important to validate the biological conclusion about the treatments/conditions from RNA-seq experiments (Allison et al. 2006). Based on that, we established our conclusions about the differential expression in this study.

Photosynthesis depletion is a stress evidence and 192 h the time for response. A set of 24 genes involved in the photosynthesis was repressed in TMG at 24h; however, in an uncoordinated activity, due to its activation at 48h. While, the same set of genes, were activated in resistant genotype at 96 and 192 h (Fig 4). The repression of DEGs related to the photosynthesis category was a determinant to conclude that BRS had a strong defense response against

P. brachyurus infection. This response was particularly evident due the presence of seven genes coding for proteins in photosystem I (PSI) and six for photosystem II (PSII) reaction centers. Downregulation of proteins from photosystem I (PSI) and six for photosystem II (PSII) reaction centers, ATP synthase and several elements of the light-harvesting complex (LHCII) was associated with biotic damage [49,50]. Tabaco plants infected by *Pseudomonas syringae* showed a decrease of maximal quantum yield of PSII (Fv/Fm) and the density of QA-reducing PSII reaction centers, indicating that the photosystems reaction centers were severely damaged after biotic stress [50].It has been suggested that the energy saved by downregulation of primary metabolism, as photosynthesis in plants, is redirected and used for defense responses [49,51]. A depletion in photosynthesis transcripts were reported in incompatible interaction between oak - *Pratylenchus penetrans* [27].

PTI is pronounced but ETI might also play a soybean defense against P. brachyurus infection. Although extensive studies have been conducted to characterize the role of PTI response in various models of plant-pathogen interactions, nematode-induced PTI responses in plants still need further investigation [52]. The high number of RLKs, Kinase, PR-proteins and phenylpropanoid associated response seen in this study, suggests PTI in soybean defense response against *P. brachyurus* [53]. At the same time, we showed LRR proteins that have been also widely associated to effector-triggered immunity (ETI) in nematode infection by several plant resistant genes already described, such as *Mi*-1, *Hero*, *Gpa*2, and *Gro*1) [54]. In a comparative analysis of resistant and susceptible *G. soja* transcriptome upon SCN infection, Zhang and co-workers (2017) found that both ETI and PTI are important in soybean defense against *H. glycines* infection.

The DRG *Glyma.*17G177100 that encodes for a kinesin motor domain is certainly an interesting aspect when considering a possible ETI. Kinesins are a class of microtubule-based molecular motors and facilitate cell directional transport, generally toward the cell membrane [56]. Bürstenbinder et al. (2013) and Abel et al. (2013) suggested that the interaction of an *Arabdopsis IQD*1 with multiple *CaM* facilitates cellular transport of specific cargo along microtubular tracks via kinesin motor proteins proteins in vitro, which implicates it in the

regulation of defense metabolism. Nine kinesins motor domain proteins were identified as potentially related to herbivore resistance in OPR3 overexpression in rice [59]; and in maize against *Colletotrichum graminicola* [60]. Nevertheless, after a comparative analysis of plant immune receptor architectures targeted by pathogens, a Kinesin motor domain of *Arabidopsis thaliana* was identified as a host interactor of HARXL73 pathogen effectors [61].

Calmodulin binding protein signaling might be related to G. max response against PB. As stated in the results, a *Mlo* encode gene (*Glyma.*16g145600) was differentially up-regulated in TMG192 and down-regulated in BRS192, right together with another Mlo (*Glyma.*13G327800). *Mlo* is one of the best understood of all plant genes involved in disease [62]. The classic case in barley, *Mlo* gene encodes a transmembrane protein, which belongs to the TM class proteins and negatively regulates penetration resistance to powdery mildew [63,64]. In one of the many studies about *Mlo,* the loss of its function in *Arabidopsis thaliana* mutation plants were reported to confer a durable and broad-spectrum resistance against powdery mildew fungi [65]. The authors also showed an increased and accelerated accumulation of many defense-related transcripts in *Arabidopsis mlo* mutants upon *Golovinomyces orontii* infection. Last but not least, it seems that the defense response against *Golovinomyces orontii* was related to a non-canonical activation of a jasmonic acid/ethylene-pathway. *Arabidopsis thaliana* MLO2 was found to be the target of the *Pseudomonas syringae* effector HopZ2 [66]. In another study, two *Mlo* were found up-regulated in susceptible cotton, *Gossypium Hirsutum*, plants against the *Rotylenchulus reniformis*, a sedentary semi-endoparasite nematode also known as reniform nematode [67].

Defense related phytohormones JA and ET signaling in soybean defense response to PB infection. Induced plant defense toward phytophagous organisms rely on such hormones pathways as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) the plant [68]. SA is usually associated with mediating induction of systemic acquired resistance (SAR) in defense response to pathogens, while the JA and ET pathways are induced in plants toward insect feeding [69]. Root-lesion nematode behavior and its recent

interaction with soybean can be considered similar to which is seen in plant-herbivory interactions [70]. However, little is known about underground plant defense and signaling systems in root herbivores, JA has been always associated in studies as one of the plant defense response role [71]. Root-lesion nematodes, *Pratylenchus penetrans*, seems to do not affect isoflavonoids in alfalfa roots, but they have been responsible for inducing mRNAs for phenylpropanoid, among other important ones to plant defense [72]. In fact, the transcriptome comparison analyses of rice in response to root-knot nematode, *Meloidogyne gramini*cola (sedentary) and root-rot nematode, *Hirschmanniella oryzae* (migratory), showed jasmonate pathway being repressed and induced, respectively [24]. In our results, we described JA as induced in BRS earlier than in TMG, plus the four chalcone synthase were DEG at 192 in BRS. Chalcone synthase proteins are directly related to the phenylpropanoids-Stilbenes pathway. The differential up-regulation of a gene that encodes for a transketolase enzyme, in three different time-treatments in BRS, also implicates over the presence of the phenylpropanoids-Stilbenes pathway in its response to root-lesion nematode [73]. A transketolase was found as repressed in susceptible orange infected with *Candidatus liberibacter asiaticus* (CaLas), which causes Huanglongbing disease [74].

Myb transcription factors have been described as regulators for plant responses to biotic stress, also regulating phenylpropanoid metabolism; and our results showed two of those in BRS “exclusive genes” [75,76].

Cell wall related proteins and a special pectinesterase have been repeatedly related to soybean response against nematodes. Clevenger et al. (2017) showed how GO terms of cell wall biogenesis were down-regulated in response to *Meloidogyne arenaria* treatment in susceptible genotype of peanut, *Arachis hypogaea*. In a compatible interaction between soybean and SCN, several genes coding for enzymes with functions related to cell wall architecture and remodeling showed differential methylation followed by gene expression changes [78]. Pectinesterases are also cell-wall-associated enzyme, which facilitates pectin de-esterification, cell wall modification, and subsequent breakdown [79]. The repression of a pectinesterase in TMG, one of the opposite expression pattern from our results, is also a fact described in soybean roots

during a compatible interaction with cyst nematodes [80,81]. However, the most interesting fact about this gene, *Glyma.*10G17200, is that it was described by Pham et al. (2013) as related to root-knot nematode resistance. After a fine mapping and identification of candidate genes that controls the resistance to southern root-knot nematode in PI 96354, the authors found an indel of 6 bps at -598 to -592 in this pectinesterase gene. This mutation, potentially at the ROOTMOTIFTAPOX1 (motif found both in promoters of rolD) or SEF1MOTIF (soybean embryo factor 1 motif) in DNA sequence, may change the expression level and affect function of this gene leading to the Mi resistance in PI 96354 [82].

Chitinases might play an important role in BRS moderately resistance. The induced co-expression of chitinases in BRS at 192 h, and in BRS DMGs, might be an attempt to affect *P. brachyurus* reproduction. Chitinases have been widely reported in plant response against nematode infection. An overexpression of a fungal chitinase (PjCHI-1) in tomato, under the control of a synthetic promoter, pMSPOA, had negative effects on *M. incognita* reproduction [83]. Root-lesion nematodes, *Pratylenchus penetrans*, seems to induce mRNAs encoding for chitinase as well (Baldridge et al. 1998). In fact, chitinases have been reported in response to root-lesion nematodes, and its expression right together with a chitin recognition protein gives it even more sense. The nematode eggshell shows three layers, an outermost vitelline layer, a middle chitinous layer composed of a protein matrix embedded with chitin microfibrils, and an inner lipid layer [84]. The chitins layer protects the lipid and vitelline layers, and provides structural strength to the eggshell [85]. A summary of all suggested gene pathways in soybean related to defense response against *P. brachyurus* infection can be found in figure 7.

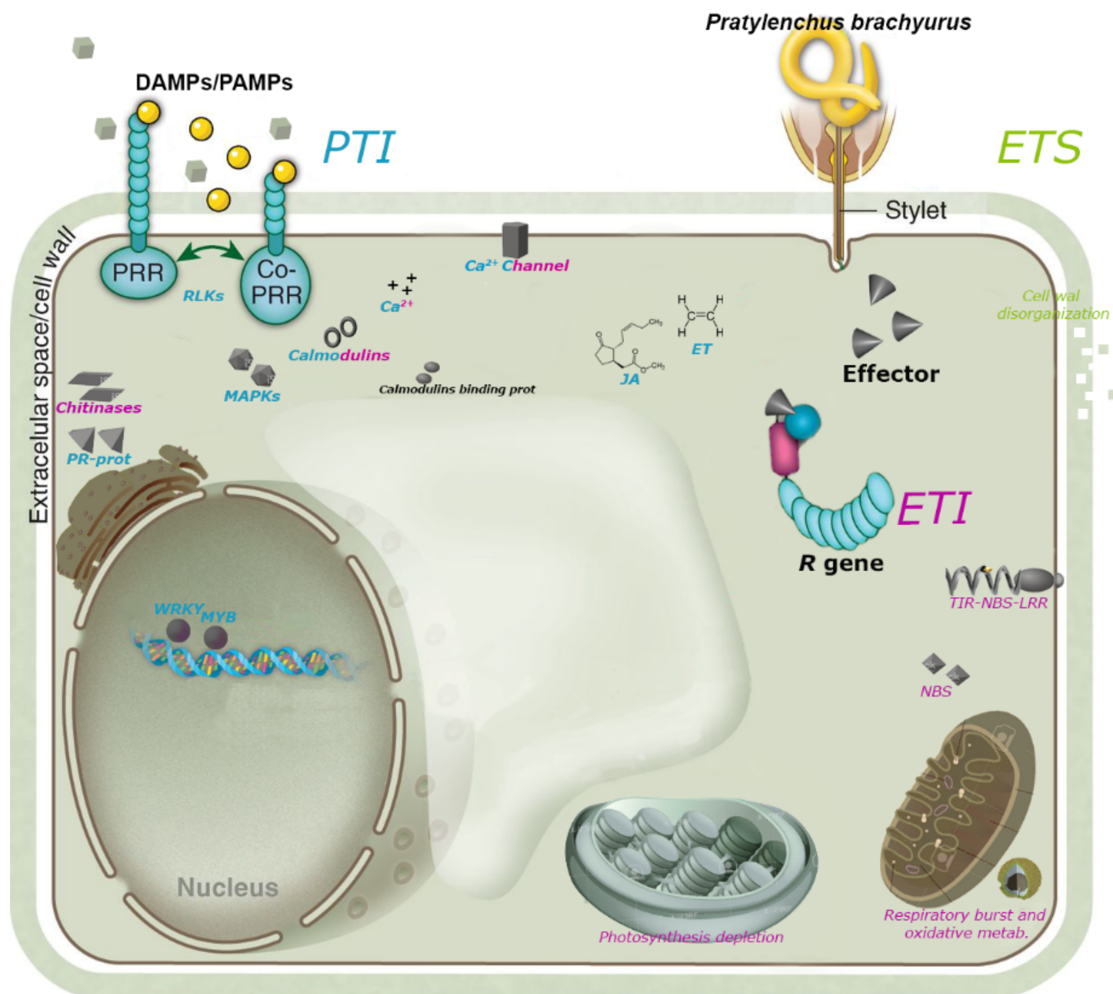

Figure 7. A proposed regulatory model to illustrate the defense reponse to PB infection in *Glycine max.* (modified from [86])

Set of DMGs in BRS seems a primed state of response. By the gene annotation, we found an interesting set of genes related to defense response in DGGs and DRGs in BRS that could not be noticed in TMG. Among those, a high number of genes that encodes for LRRs, calcium-activated channel protein, seven RLKs, *Myb* transcription factors, cyclin kinase dependent, and chitinases. Hence, it led us to questioning. Does BRS genotype presents a primed state of defense? The primed state of defense is a physiological state in which a plant is conditioned to keep, or super activate,

defenses even before getting in contact to the stress [87,88]. Priming results in a faster and stronger induction of plant defense responses, enhancing resistance against pathogen, unlike to what is found in unprimed plants exposed to the same stress [89].

Although the molecular bases of plant-priming response is still not very well known, it seems that can be even inheritable. The transgenerational priming was described in *Arabidopsis thaliana* progeny plants, which had been earlier infected with *Pseudomonas syringae*, showed enhanced resistance [90]. Furthermore, *A. thaliana* and tomato (*Solanum lycopersicum*) progenies were found to produce a priming of jasmonic acid-dependent defense response to herbivory or mechanical damage [91].

In addition, the arrays cluttering results showed BRS in an earlier defense response followed by TMG; it may be possibly related to our hypothesis of primed state. Studies have demonstrated how faster the plant resistant genotype responds against pathogens in comparison to the susceptible ones (Vieira Dos Santos and Rey 2006; Xin et al. 2012; Cregeen et al. 2015; Lee and Yeom 2015; Wu et al. 2017)

Conclusion

- Through our approach, we were able to reveal the main responsive profile of a moderately resistant and susceptible soybean genotype upon root- lesion nematode infection;
- Genotypes response against *P. brachyurus* was better distinguished at 96h, which 71 and 94 DEGs were shown in BRS and TMG, respectively, but showing a very different set content and other genes in opposite expression profile. However, for both genotypes, the major gene expression changes were at 192h with 332 and 307 DEGs in BRS and TMG, respectively ;
- Evidences suggested PTI and ETI could be working simultaneously in soybean response to *P. brachyurus*;
- Differential expression analyses and comparison between genotypes demonstrated the changes in phytohormones pathway, especially for JA and ET, and cell wall genes during interaction with this pathogen;

- The pointed out DEGs and DMGs, especially those that encodes for LRRs, kinases, Myb transcription factors, chitinases, and pectinesterase, are promising in terms of further investigations by genome-wide association study (GWAS) and epigenome-wide association studies (EWAS) to better the hypothesis of priming stated of defense;
- Our results about the molecular basis and mechanisms of soybean response against *Pratylenchus brachyurus*, represent a step ahead to the development of new strategies and technologies that could either control or prevent the damage caused by the root-lesion nematode on soybean crops.

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Suplemental material

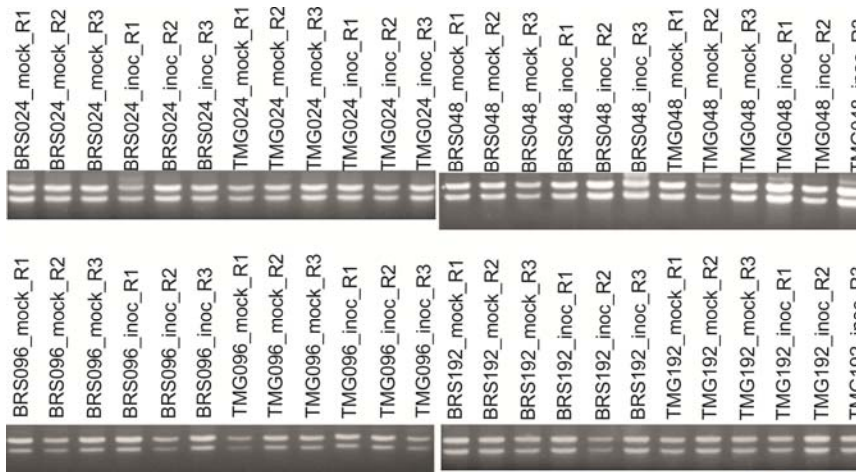


Figure S1. RNA extracted integrity result. Electrophoresis on 1% agarose gel to analyze the total RNA integrity samples extracted from BRS and TMG genotypes with infected and non-infected samples at 24, 48, 96 and 192 hours after infection.

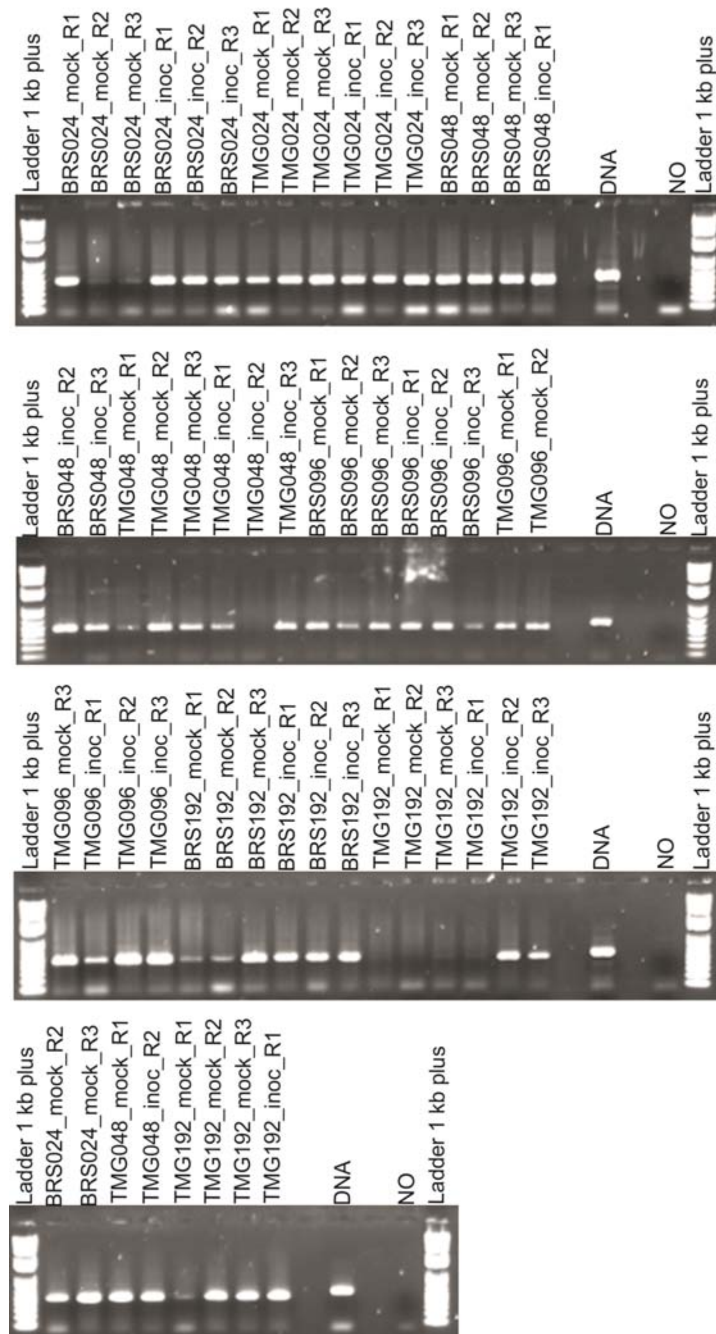


Figure S2. cDNA samples quality check. Electrophoresis on 1.2% agarose gel of PCR products using primers set for β-actin gene with synthesized cDNA samples as template. The expected amplicon size has 520 bp when genomic DNA is the template and 440 bp when RNA is the template.

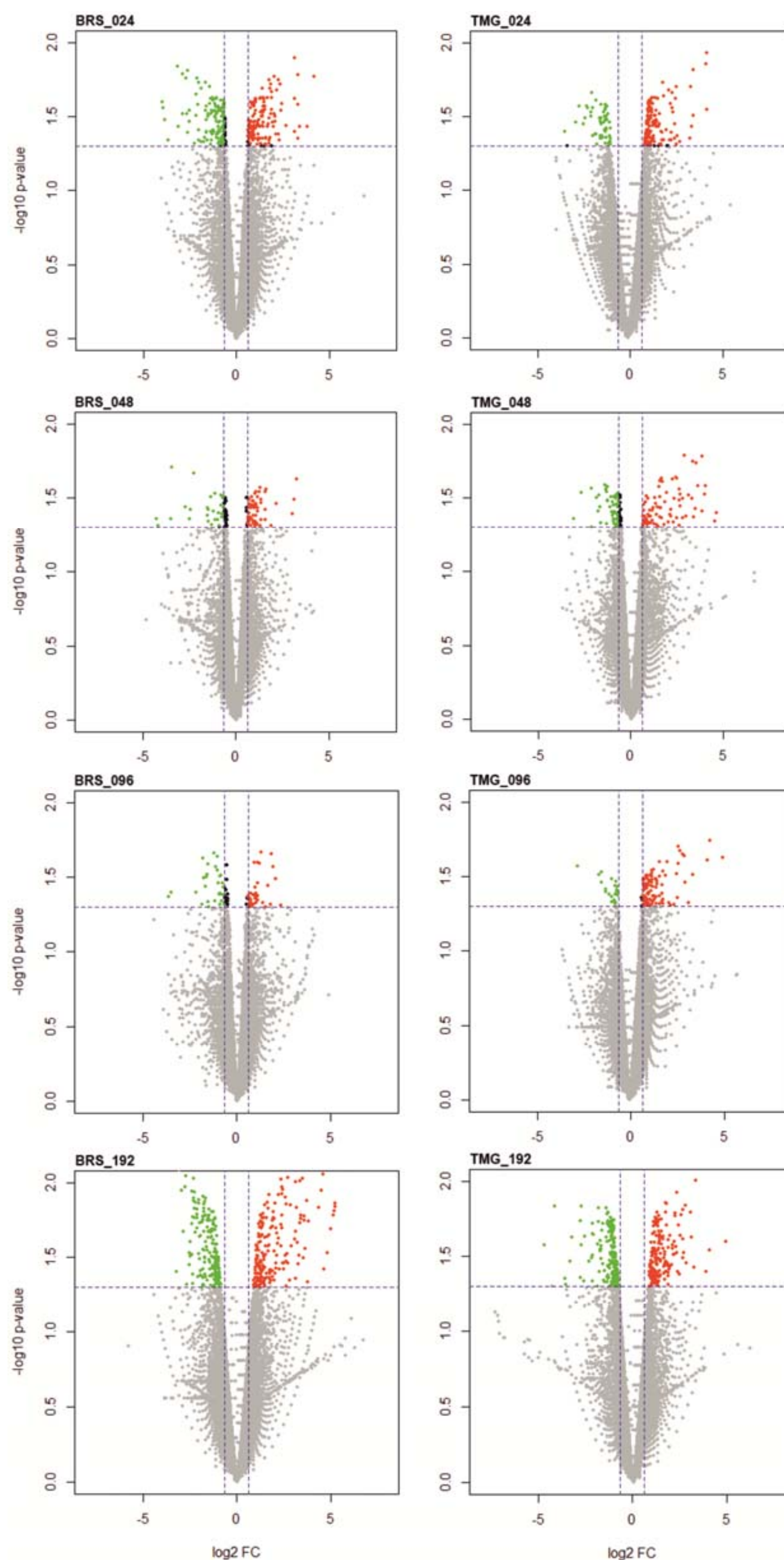


Figure S3. Volcano plots. Expression data were plotted on a log2 FC scale (x-axis) *versus* a -log10 transformation of the p-value (y-axis). Datasets were filtered to remove genes with low expression levels (blue lines from -0.63 to 0.63 on the x-axis), and a significance cut off ($p < 0.05$) was applied on the y-axis. BRS024 (Total number of DEG (n) = 213; 107 up (red spots) and 106 down (green spots)); BRS048 (n = 66; 42 up and 24 down); BRS096 (n = 71; 36 up and 35 down); BRS192 (n = 332; 164 up and 168 down); TMG024 (n = 168; 120 up and 48 down); TMG048 (n =104; 58 up and 46 down); TMG096 (n = 94; 76 up and 18 down); and TMG192 (n = 307; 150 up and 157 down).

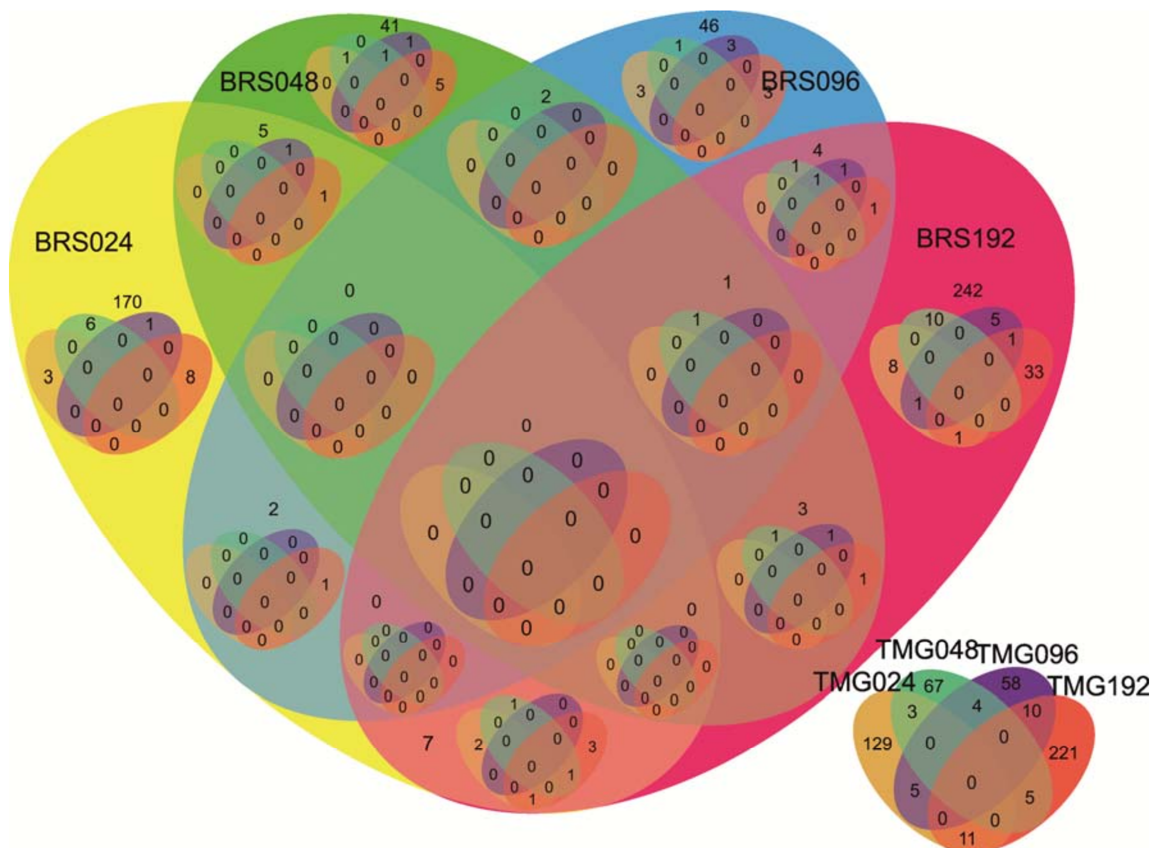


Figure S4. Venn diagram displays the relationship among DEGs, under nematode infection, from each genotype and time-treatment. A total number of 170 DEGs were exclusive in BRS024; 41 DEGs exclusive in BRS048; 46 DEGs exclusive in BRS096; 242 DEGs exclusive in BRS192; 129 DEGs exclusive in TMG024; 67 DEGs exclusive in TMG048; 58 DEGs exclusive in TMG096; and 221 DEGs exclusive in TMG192.

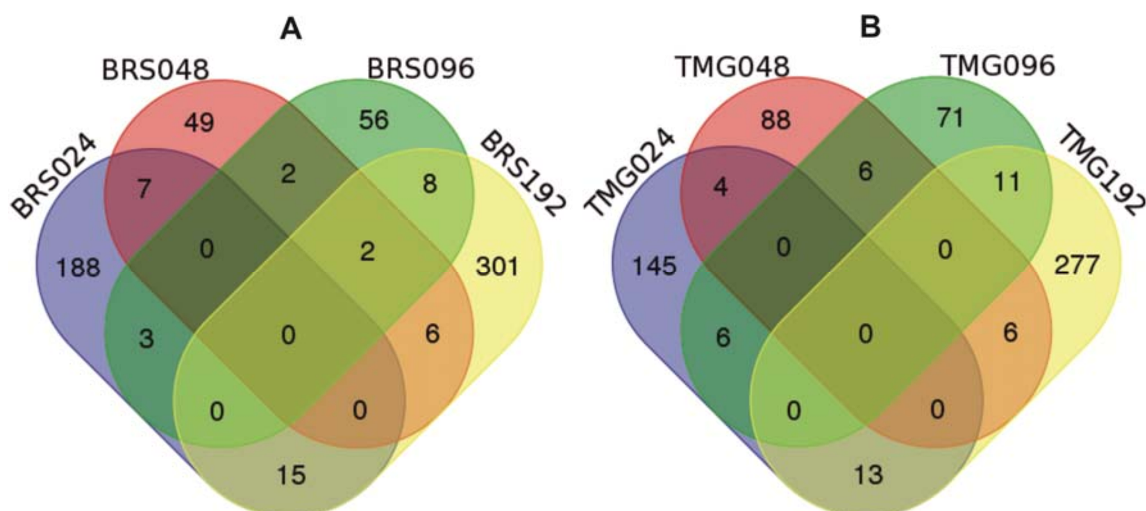
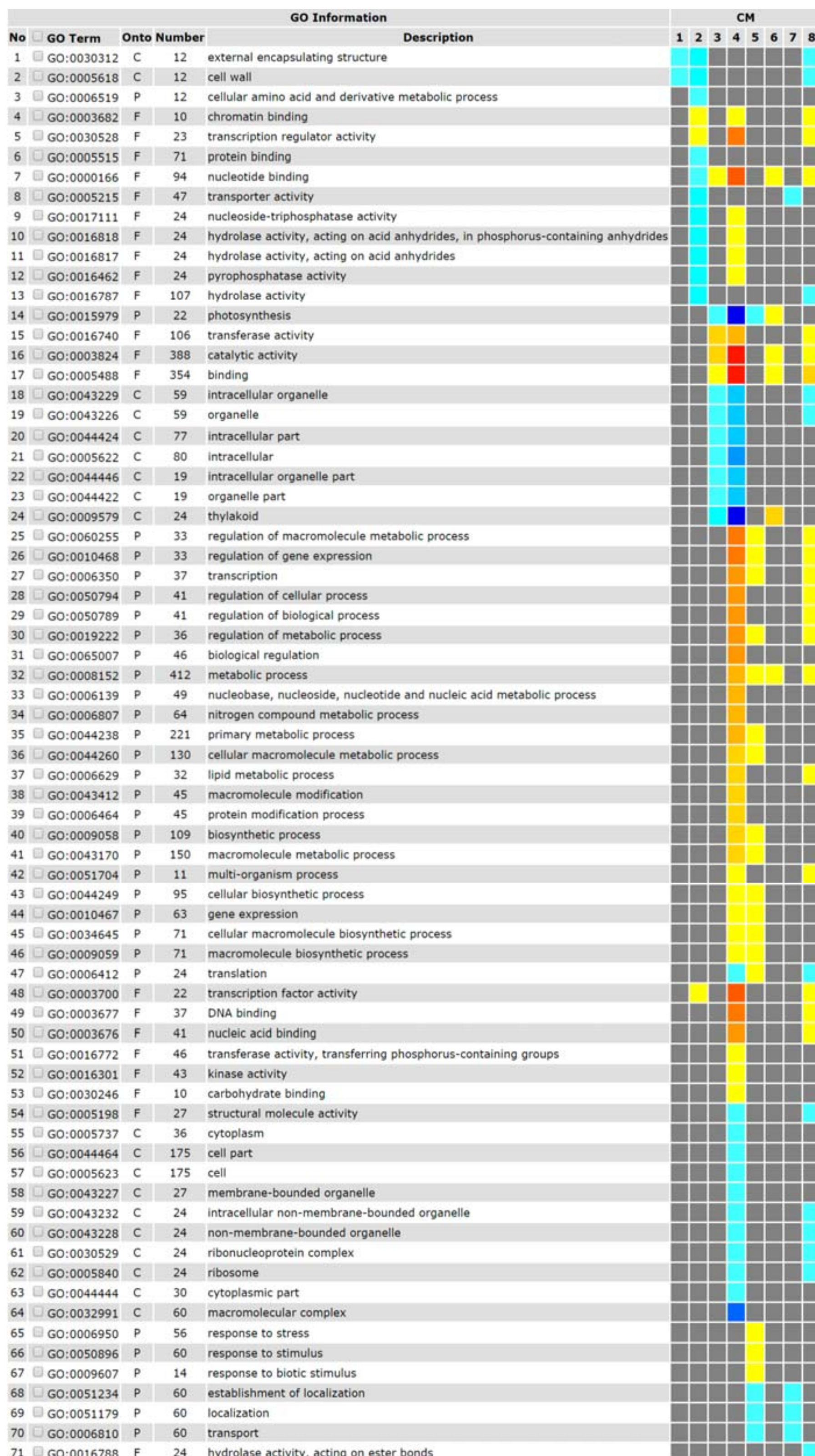


Figure S5. Venn diagram displays the relationship among DEGs in four time-treatments in BRS and TMG. A. Venn diagram shows 188 DEGs were exclusive expressed in BRS024, 49 in BRS048, 56 in BRS096, and 301 in BRS192. B. Venn diagram shows 145 DEGs were exclusive expressed in TMG024, 88 in TMG048, 72 in TMG096, and 277 in TMG192.

Figure S6. Heatmap (hierarquical clustered) of DEGs from this study. A.; shows the whole expression profile of BRS and TMG in response to *P. brachuyrus*. B.; shows the selected clusters (yellow screens in A.) related to the differences of genotype responses. The yellow squares, highlighted in yellow in B., indicate the treatment of

statistical significance for gene expression. The color key scale shows the log2 fold change: green = down-regulated and red = up-regulated.



No	GO Term	Onto	Number	Description
1	GO:0030312	C	12	external encapsulating structure
2	GO:0005618	C	12	cell wall
3	GO:0006519	P	12	cellular amino acid and derivative metabolic process
4	GO:0003682	F	10	chromatin binding
5	GO:0030528	F	23	transcription regulator activity
6	GO:0005515	F	71	protein binding
7	GO:0000166	F	94	nucleotide binding
8	GO:0005215	F	47	transporter activity
9	GO:0017111	F	24	nucleoside-triphosphatase activity
10	GO:0016818	F	24	hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides
11	GO:0016817	F	24	hydrolase activity, acting on acid anhydrides
12	GO:0016462	F	24	pyrophosphatase activity
13	GO:0016787	F	107	hydrolase activity
14	GO:0015979	P	22	photosynthesis
15	GO:0016740	F	106	transferase activity
16	GO:0003824	F	388	catalytic activity
17	GO:0005488	F	354	binding
18	GO:0043229	C	59	intracellular organelle
19	GO:0043226	C	59	organelle
20	GO:0044424	C	77	intracellular part
21	GO:0005622	C	80	intracellular
22	GO:0044446	C	19	intracellular organelle part
23	GO:0044422	C	19	organelle part
24	GO:0009579	C	24	thylakoid
25	GO:0060255	P	33	regulation of macromolecule metabolic process
26	GO:0010468	P	33	regulation of gene expression
27	GO:0006350	P	37	transcription
28	GO:0050794	P	41	regulation of cellular process
29	GO:0050789	P	41	regulation of biological process
30	GO:0019222	P	36	regulation of metabolic process
31	GO:0065007	P	46	biological regulation
32	GO:0008152	P	412	metabolic process
33	GO:0006139	P	49	nucleobase, nucleoside, nucleotide and nucleic acid metabolic process
34	GO:0006807	P	64	nitrogen compound metabolic process
35	GO:0044238	P	221	primary metabolic process
36	GO:0044260	P	130	cellular macromolecule metabolic process
37	GO:0006629	P	32	lipid metabolic process
38	GO:0043412	P	45	macromolecule modification
39	GO:0006464	P	45	protein modification process
40	GO:0009058	P	109	biosynthetic process
41	GO:0043170	P	150	macromolecule metabolic process
42	GO:0051704	P	11	multi-organism process
43	GO:0044249	P	95	cellular biosynthetic process
44	GO:0010467	P	63	gene expression
45	GO:0034645	P	71	cellular macromolecule biosynthetic process
46	GO:0009059	P	71	macromolecule biosynthetic process
47	GO:0006412	P	24	translation
48	GO:0003700	F	22	transcription factor activity
49	GO:0003677	F	37	DNA binding
50	GO:0003676	F	41	nucleic acid binding
51	GO:0016772	F	46	transferase activity, transferring phosphorus-containing groups
52	GO:0016301	F	43	kinase activity
53	GO:0030246	F	10	carbohydrate binding
54	GO:0005198	F	27	structural molecule activity
55	GO:0005737	C	36	cytoplasm
56	GO:0044464	C	175	cell part
57	GO:0005623	C	175	cell
58	GO:0043227	C	27	membrane-bounded organelle
59	GO:0043232	C	24	intracellular non-membrane-bounded organelle
60	GO:0043228	C	24	non-membrane-bounded organelle
61	GO:0030529	C	24	ribonucleoprotein complex
62	GO:0005840	C	24	ribosome
63	GO:0044444	C	30	cytoplasmic part
64	GO:0032991	C	60	macromolecular complex
65	GO:0006950	P	56	response to stress
66	GO:0050896	P	60	response to stimulus
67	GO:0009607	P	14	response to biotic stimulus
68	GO:0051234	P	60	establishment of localization
69	GO:0051179	P	60	localization
70	GO:0006810	P	60	transport
71	GO:0016788	F	24	hydrolase activity, acting on ester bonds

Figure S7. Page GO map of all significantly over-represented for up- and down-regulated among DEGs. It shows ontology categories affected by the nematode infection in all time-treatments of both genotypes. CM at the right upper part identifies

BRS024 (1), BRS048 (2), BRS096 (3), BRS192 (4), TMG024 (5), TMG048 (6), TMG096 (7), and TMG192 (8). The color scale shows the categories regulation profile, and ranges from dark blue (strongly negative related) to dark red (highly positive regulated).

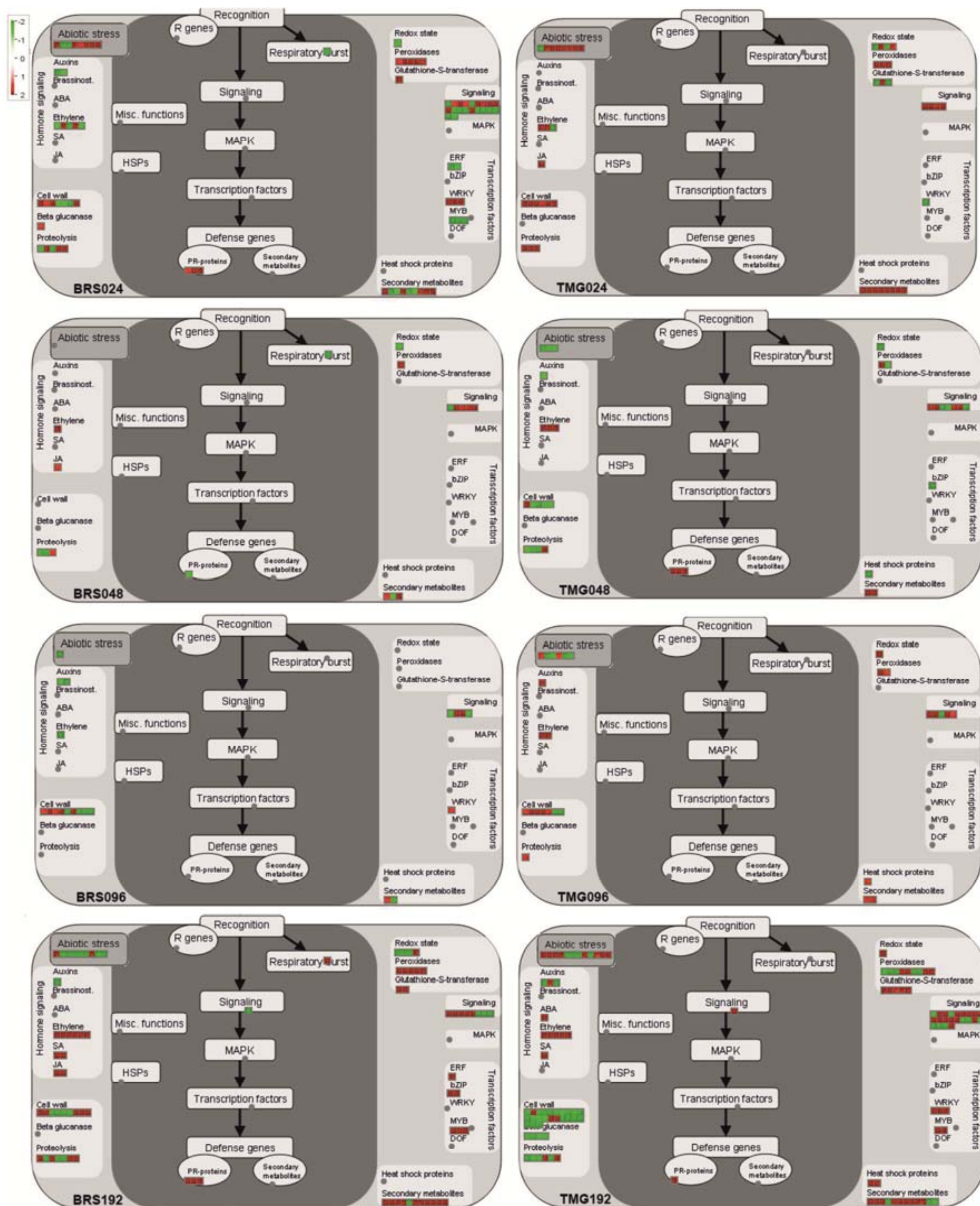


Figure S8. Functional roles triggered in moderately resistant and susceptible soybean genotypes under *P. brachyurus* infection in four different time-points. Genes that were differentially expressed in response to nematode infection were mapped to specific stress-related pathways. The color scale shows the log_2 fold change: red = up-regulated and green = down-regulated.

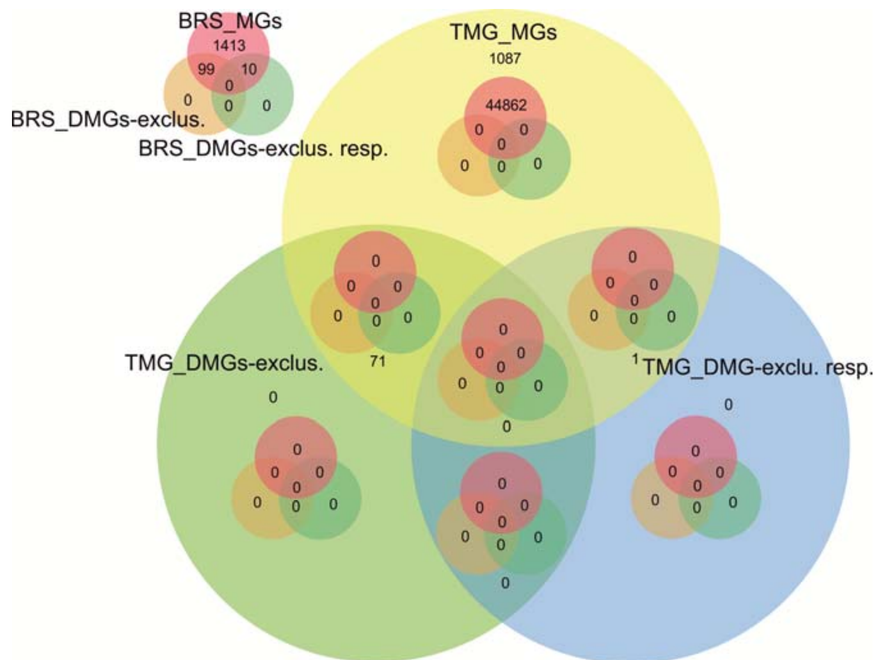


Figure S9. Venn diagram shows the total number of DGGs and DRGs TMG and BRS genes mapped on soybean genome. 44,862 genes were mapped in, at least, one of eight BRS libraries, but also in, at least, one of eight TMG libraries. 1,047 is the total number of genes mapped in one or more TMG libraries, while 1,413 is the total number of genes mapped in one or more BRS libraries. The red squares highlights those DGGs, which means those genes mapped in, at least, seven libraries in one genotype and none of the other. Red arrows are pointing the DRGs.

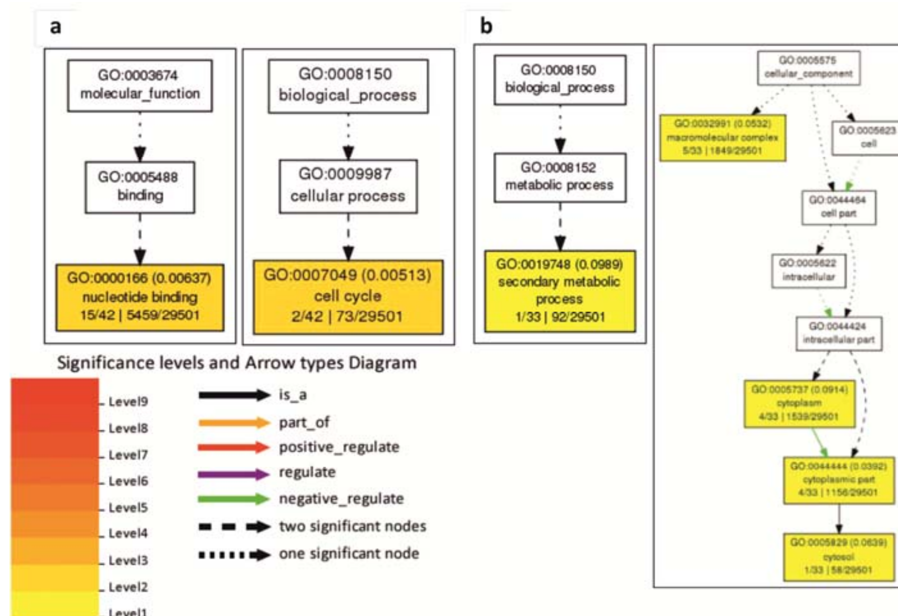


Figure S10. GO categories observed for the genotype exclusive genes. a. Graphics shows results for BRS; 15 out of 42 genes classified in nucleotide binding GO category; and two out of 42 genes classified as cell cycle category. b. Graphics shows results for TMG; one out of 33 genes classified in secondary metabolic process GO category; 5 out of 33 genes classified in macromolecular complex; and four in cytoplasm-cellular component category.

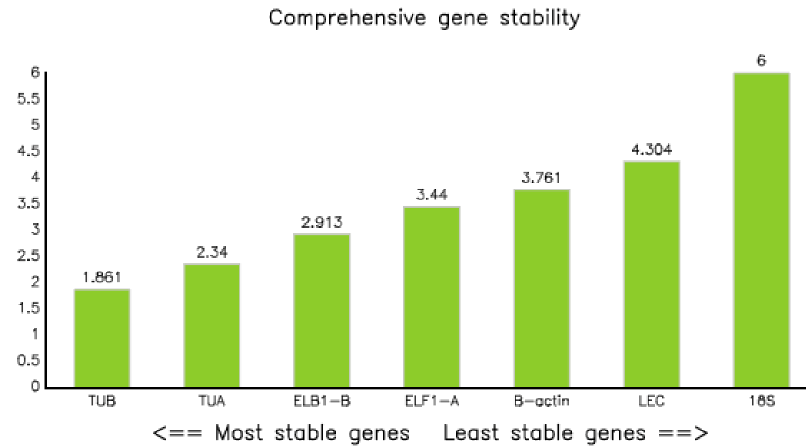


Figure S11. Graphic shows the comprehensive ranking, based on the geometric mean of classification, generated by ReFinder. TUB was ranked as the most stable reference gene, followed by TUA and ELB1-β.

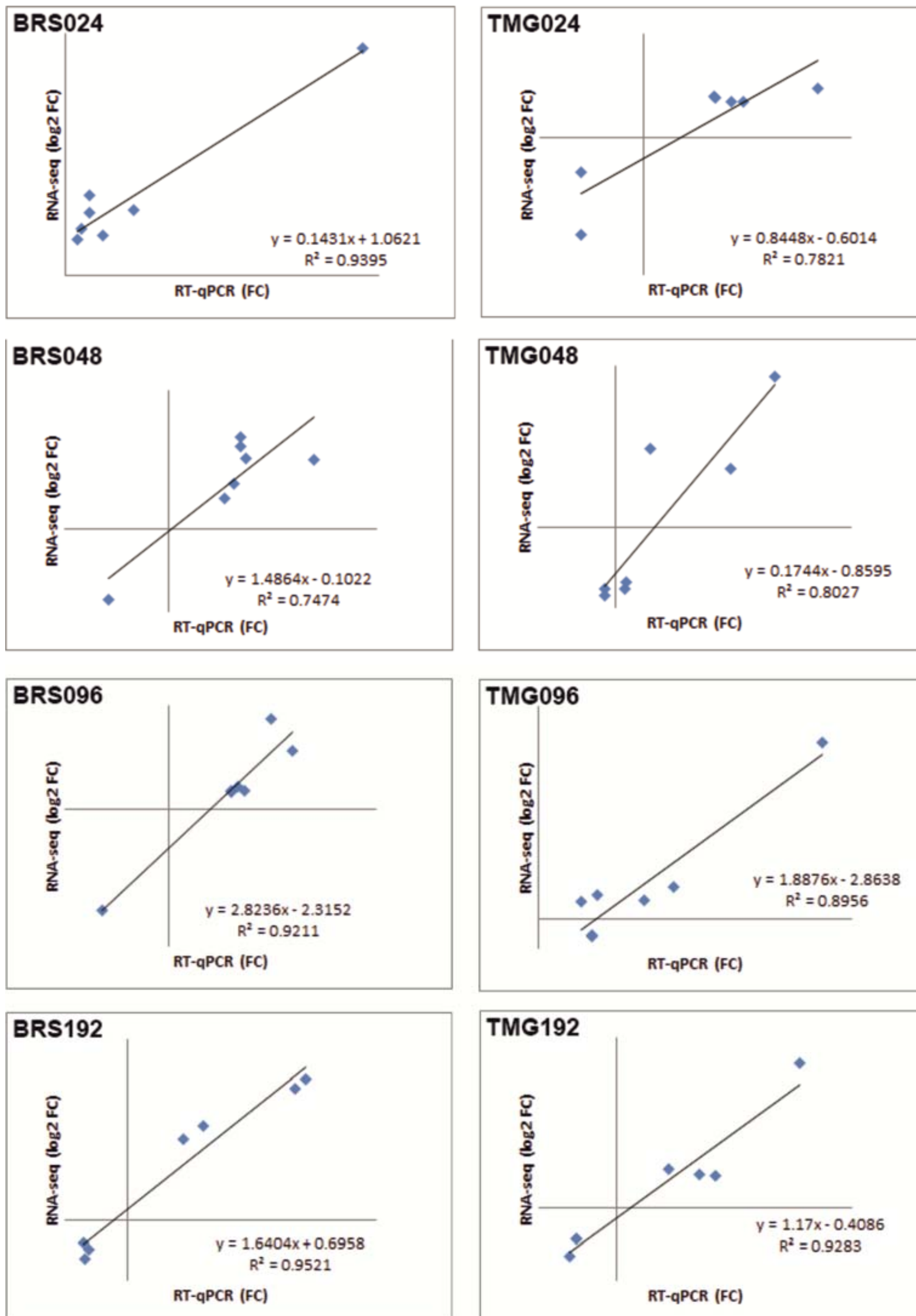


Figure S12. Validation of gene expression *per* time-treatment. Relative expression selected of seven random genes displays a high correlation between RNA-seq expression data and RT-qPCR.

To be submitted to New Phytologist

6. ARTIGO 2: Mining for *Pratylenchus brachyurus* Putative Genes Involved in Parasitism and their Host Targets

Root-lesion nematodes (RLN), *Pratylenchus* spp., are parasites that can migrate from root to root, according to the environmental conditions. The devastation these pests can cause affects several crops worldwide, including soybean. The nematode effector proteins secreted by the pathogen, *in planta,* are believed to be key elements to their infection successes. Many nematode effectors have been described from a number of sedentary plant-parasitic nematodes. However, effector repertoire of the migratory nematodes, including the root-lesion nematode *Pratylenchus brachyurus*, remains largely unknown. In this study, we used *P. brachyurus* transcripts to identify the secretome set of 115 genes and putative effector candidates. We selected a set of 20 genes for *in situ* hybridization analyses and identified eight genes expressed in esophageal glands and seven in the intestine of the nematode. Some of those from esophageal glands are annotated as *14-3-3b*, *SEC*-2 protein, and transthyretin-like (TTL) protein, as previously described effector proteins in other plant-nematode interaction. Yeast two-hybrid assay showed that putative effector candidate, *PB6584*, interacts with different *Arabdopisis* proteins related to host defense, such as an invertase, and subtilisin-like serine endopeptidase family protein. This interaction demonstrates that the nematode attack may affect the signaling and cell wall reinforcement. This is the first study on *Pratylenchus brachyurus* effector genes and the results showed promising putative effector candidates that can be used to controll the pathogen in the field.

Keywords: transcriptome, root-lesion nematode, secretome, effector candidate genes, and protein-protein interaction

Background

A worldwide plant pathogen, root-lesion nematodes (RLN, *Pratylenchus* species) are an important threat to global agriculture, causing massive yield losses in many crops (Yu *et al.*, 2012; Jones & Fosu-Nyarko, 2014). Indeed, *Pratylenchus* sp. is ranked as the third most damaging nematode, right after *Meloidogyne* sp. and *Heterodera* sp/*Globodera* sp.. However, the ranking could reflect that infestation by sedentary endoparasites is much easier to recognize than that for the migratory, as *Pratylenchus* sp. (Jones *et al.*, 2016).

As all phytonematodes, *P. brachyurus* presents a stylet, which is used to puncture, penetrate cell walls and suck the host cells they feed. This endoparasite feeds on the plant cortex in the host root tissue which, combined with the freely movement through the root, creates dark spots or lesions. The migratory behavior increases the damages: after injuring a host, it switches to a

healthy one, restarting the process. In addition, those *Pratylenchus* species with asexual modes of reproduction are becoming particularly more devastating agricultural pests than their sexually-reproducing nematodes (Blanc-Mathieu *et al.*, 2017). Although *P. brachyurus* presents both genders, males are rare and its reproduction mode is mostly by mitotic parthenogenesis (Inomoto & Oliveira, 2008).

Secretions of the esophageal glands are injected also via stylet into the host apoplast (Davis *et al.*, 2008) and cytoplasm (Wang *et al.*, 2010), and are enriched of effectors or parasitism proteins. These secreted effector proteins play a number of roles, including suppression of host defenses and inhibition of an specific signaling, mimic an host protein, enabling migration in plant tissue and its digestion of ingested cytoplasm (Hamamouch *et al.*, 2012; Kiyohara & Sawa, 2012; Hewezi *et al.*, 2015; Jones *et al.*, 2016). The success of the infection depends on the production of these proteins in order to increase the possibility of survival and reproduction within the host environment.

The elucidation of effectors molecular function and their host targets is imperative to develop new strategies for controlling nematodes and other important phytopathogens (Quentin *et al.*, 2013). Despite significant advances in recent years, the molecular mechanisms driving the interaction between plants and RLN remains poorly understood.

The application of high-throughput sequencing technologies for transcriptomic studies has provided new opportunities for better understanding plant–nematode interactions (Hekman *et al.*, 2015). A high number of nematode transcriptomes have been released, which allowed the prediction of putative secreted proteins, combined with *in situ* hybridization to identify transcripts expressed in the esophageal glands, and in doing so, select putative effector candidate genes. The access to nematode transcripts also allows analysis of sequence similarity and common features with effectors already characterized in other nematodes.

Recently, Fosu-Nyarko and coworkers (Fosu-Nyarko *et al.*, 2016a) performed an analysis of the transcriptome of beet cyst nematode, *H. schachtii*, looking for effector candidates. After sequence comparison analysis of effectors already described with other plant parasitic nematodes, it was found 30 *H. schachtii* similar transcripts. Among these identified transcripts are included new

effector sequences for this species, as a calreticulin, *14-3-3* protein and RBP-1 protein. The *14-3-3* protein family is highly conserved in eukaryotic and specific proteins involved in several cellular and biochemical processes through interaction with phosphorylated target proteins. As described in other phytonematodes, *14-3-3* proteins can play several roles in the host-parasite communication.

Thorpe and coworkers (Thorpe *et al.*, 2014) performed a genomic characterization for *Globodera pallida* effectors identification, and were able to identified 128 orthologues of effectors from other nematodes, as well as 117 novel effector candidates for this potato cyst nematode. Additionally, Eves-van den Akker and coworkers (2014) provided the identification and characterization of a new group of hyper-variable extracellular effectors termed HYP, in this potato nematode. HYP expression was identified in secretory cells of the nematodes, by *in situ hybridization*, and the protein was also detected in apoplasm host cells, by immunochemistry. In addition, the HYP silencing, by RNAi *in planta*, resulted in a reduction nematode infection. Other identified effectors, includes the *H. schachtii 4F01,* that mimics a plant annexin to alters the host defenses (Patel *et al.*, 2010), *Hs*10A06 and *Hs*10A07 related to host defense suppression in *H. schachtii* (Hewezi *et al.*, 2010, 2015), a venom allergen protein VAP1 from *G. rostochiensis* (Lozano-torres *et al.*, 2014), and a calreticulin *Mi*CRT, also related to defense suppression in *M. incognita* (Jaouannet *et al.*, 2012).

Indeed, most of the studies have been focused on sedentary plant-parasitic nematode species. Only recently transcriptome analyses of migratory nematodes have been conducted to identify their effector genes, including *Pratylenchus coffeae* (Haegeman *et al.*, 2011), *P. thornei* (Nicol *et al.*, 2012), *P. penetrans* (Vieira *et al.*, 2015), and *P. zeae* (Fosu-Nyarko *et al.*, 2016b). Li et al. (Li *et al.*, 2015a) performed studies with tomatoes and described a nematode calreticulin, *Rs-crt*, in the migratory nematode *Radopholus similis*. After juvenile treatment with *Rs-crt* dsRNA (double strand RNA) for 36 h, *Radopholus similis* individuals were affected in reproductive capability and pathogenicity. Also, transgenic tomatoes expressing RNAi for *Rs-crt* showed significantly improved resistant phenotype to *R. similis*.

Herein we aimed to present the first *Pratylenchus brachyurus* transcriptome expressed during interaction with soybean plants generated by Illumina sequencing and *de novo* assembly. We developed a pipeline for secretome prediction of *Pratylenchus brachyurus,* followed by *in situ* hybridization to identify genes expressed in secretory organs and tissues, and putative effectors candidates. For a further investigation of the parasitic role in the infection, a yeast-2-hibridization assay was performed, from an *Arabidopsis* roots cDNA library under nematode infection (*Meloidogyne incognita*), in order to identify host interactor proteins of a putative effectors candidate gene, PB6584. This study aimed to identify potential *Pratylenchus brachyrus* secreted genes and validated selected putative effector candidates. Addictionally, targeted host proteins were putative effector candidates highlighting targeted host proteins for *PB6584* candidate. Moreover, it is valuable information for better understanding molecular roles in the pathosystem: soybean – *P. brachyurus*.

This study provides a set of potential *Pratylenchus brachyrus* secreted genes and the validation of a set of putative effector candidates, highlighting targeted host proteins for *PB6584* candidate. Moreover, it is valuable information for better understanding molecular roles in the pathosystem: soybean – *P. brachyurus*.

Materials and Methods

Biological material and infection assays. Two *G. max* genotypes, cv BRSGO Chapadões and cv TMG 115 RR, resistant and susceptible to *P. brachyurus*, respectively (Rios *et al.*, 2016), obtained from the Embrapa Soja Active Germplasm Bank (AGB), were grown into cone-tainares, filled with sterile sand, and inoculated 500 infective forms of *Pratylenchus brachyurus*. Infected roots were collected, by genotype, at 24, 48, 96, and 192 hpi, in three biological replicates each, for RNA-seq analyses.

RNA-seq nematode reads filtering and transcriptome assembly. After RNA extraction with TRIzol® reagents (Invitrogen), and library prep with RNA TruSeq^{TM} SBS Kit v5-GA sample prep kit (Illumina, San Diego, CA), samples were sequenced on an Illumina Hi-Seq 2000 utilizing a 101-bp read

length with v4 sequencing chemistry (Illumina, San Diego, CA, USA). The RNA-seq was performed by FASTERIS Biotechnology company (Geneva, Switzerland). Reads were trimmed using Trimmomatic 0.36 software (Bolger *et al.*, 2014), and filtered against *Glycine max*, *Escherichia coli*, and *Bradyrhizobium* sp. using STAR RNA-seq aligner (Dobin *et al.*, 2013). The filtered reads, those that had no mapping against the filtering organisms above cited, were applied on a *de novo* assembly of *P. brachyurus* transcriptome with Trinity software (Grabherr *et al.*, 2011) and quality tested with TransRate software (Smith-Unna *et al.*, 2016).

Prediction of P. brachyurus secretome. The contigs obtained from de novo assembly were mining for secreted genes. Both, ab initio and extrinsic approaches were combined to identify the putative secreted genes. In the *ab initio* prediction, the ORFs translated from the consensus sequences were searched for putative signal peptides for protein secretion, the presence of trans-membrane motifs, and other features that can classify secreted proteins, as followed: Phobius software (Käll *et al.*, 2007) – positive (putative presence of signal peptide and negative for transmembrane domain); Signal P software (Bendtsen *et al.*, 2004) – positive (putative presence for signal peptide); Target P software (Emanuelsson *et al.*, 2000) – positive (putative secreted protein); WolfPsort II software (Horton *et al.*, 2007) – positive (putative exported protein); Predotar software (Liu *et al.*, 2013)– positive (putative addressed to the endoplasmic reticulum); TMHMM software (Krogh *et al.*, 2001) – negative (absence of transmembrane domain); MITO software (Claros, 1995) - negative (not addressed to mitochondria), and; PSI software (Liu *et al.*, 2013) - negative (not addressed to peroxissomal). The combination of all the computational predictors, by a cluster analyses using VennPainter software (Lin *et al.*, 2016a), increased the confidence of the selection.

The extrinsic approach was based on a comparison of putative encoded amino acid sequence with previously described secreted proteins or effectors from plant- and animal- parasitic nematodes protein sequence databases (Borodovsky *et al.*, 1994). If a putative ORF product shows significant sequence similarity to one or more proteins in the database, it is almost certain that the ORF in question is a real gene (Borodovsky *et al.*, 1994). This strategy was

used to avoid discarding erroneously sequences that could be secreted proteins but, as a result of the sequencing method, were not complete and might have lost the signal peptide. All sequences used as secreted data set are available in the supplemental file 1. The cut-off was set for percentage alignment coverage > 20; e-valeu < e^{-10}; percentage of identities > 33; and percentage of positive matches > 51. Sequences that showed similarity to *Caenorhabditis elegans* genes, a non-pathogenic nematode, according to the established cut-off, were excluded.

Localization of the transcript by in situ hybridization. Primers set for 45 predicted secreted candidate genes with amplicons of ~200 bp in length were designed to perform the probe synthesis (Tab S1). From those, 23 presented amplicons in the PCR reactions when using a cDNA pool generated from *P. brachyurus*. These amplicons were used as the template in a unidirectional PCR, to produce sense and antisense DIG labelled probes with Roche kit. In situ hybridizations were performed using all infective forms of *P. brachyurus* nematodes isolated from in vitro culture in alfalfa. We used 3,000 individuals, in a mix of infective forms, for hybridization with each secreted gene candidate. These nematodes were fixed by overnight incubation in 10% deionized formaldehyde before permeabilization by chopping with a razor blade on a glass slide. The chopped nematodes were then digested in 0.1% (w/v) proteinase K solution (AM2546, ThermoFisher, Waltham, MA, USA). The tissues were hybridized with a DIG-labelled specific probes overnight at 42°C. Probes hybridizing to the nematode were detected using alkaline phosphatase conjugated antidigoxigenin antibody (diluted 1:100) and substrate, and examples were observed and photographed under an EVOS FL Auto Imaging System Microscope (Thermo Fisher Scientific).

Yeast Two-Hybrid Screen. Yeast two-hybrid screening was performed as described in the BD Matchmaker Library Construction and Screening Kits user manual (Clontech). The coding sequence of *PB6584* coding sequence was amplified and fused to the GAL4 DNA binding domain of pGBKT7 vector to generate pGBKT7-*PB6584* and then introduced into *Saccharomyces cerevisiae* strain Y187 to generate the bait strain. The

pGBKT7-*PB6584* into *S. cerevisiae* was tested for toxicity and self-activation before the Y2H screening.

The *Arabidopsis* cDNA library prepared from RNA isolated from roots of ecotype Col 0 at 3 days after Meloidogyne incognita infection was generated in *S. cerevisiae* strain AH109 as a fusion to the GAL4 activation domain of pGADT7-Rec2 vector (Dr. Tarek Hewezi's lab at UT). Screens for interacting proteins were conducted using the mating method and subsequent analyses were performed according to Clontech protocols.

Expression profiling of P. brachyurus effector candidates and its interactors during the course of infection. A new experiment was installed following parameters previously described for the RNA-seq material, with inclusion of six different time points of collected samples (12, 24, 48, 72, 96, and 192 hours after infection), and the presence of a non-inoculated treatment for all time-points. Total RNA was extracted with TriZol (Invitrogen) according to the Invitrogen protocol, after DNAse I treatment (Invitrogen), total RNA concentration was measured with a NanoDrop 2000 spectrophotometer (NanoDrop Products, Wilmington, DE, USA). We subjected samples to reverse transcription with the SuperScript III kit (Invitrogen). The primers for reverse transcriptional quantitative PCR (RT-qPCR) were designed with Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) and its efficiency tested (Tab S2). The efficiency of each primer pair used in this study was tested by amplification of a pool of all samples above mentioned to run qPCR reactions. The E = [10-1/slope]-1 formula was employed to calculate the reaction efficiency and to adjust the final primer concentration. The calibration curve was established based on the Ct and the log of five cDNA serial dilutions [1:10].

For each qPCR reaction, cDNA was diluted to 1:10 and 3 uL of the dilution was used. The qPCR was performed in a 9.4 uL reaction volume and 384-well plate using the Maxima SYBR Green/ROX qPCR Master Mix (2×) (Thermo, USA) on a detection system (ABI 7900HT [Life Technologies, Grand Island, New York, USA. Thermocycling was performed as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 56°C for 30 s and 72°C for 30 s. The dissociation curve of the final products was examined to check that there

was only one amplification product. We performed qPCR on triplicate samples of each cDNA from three independent biological replicates.

Ct values from selected genes were first normalized by 18S rRNA *P. brachyurus* gene and TUA and ELF1-B soybean housekeeping genes for effector candidates and interactors, respectively. The effector expression quantification we used random normalization to obtain the final ΔCt mean for each gene in genotype and time-treatment. The relative expression results of soybean potential interactors were obtained with REST 2009. The final relative quantification of each gene compared with the control conditions was estimated considering the RQ obtained in each biological replicate, represented by each independent experiment, with three replicates each. Significant differences were determined based on estimates of the standard deviation (SD) and with REST software version 2.0.7 (2009) ($p < 0.05$) (http://gene-quantification.eu/chapter-3-pfaffl.pdf).

Results

Pratylenchus brachyurus first secretome was predicted. The assembly of all samples reads from this study resulted in a set 8,331 contigs, representing the first *Pratylenchus brachyurus* transcriptome available. All of the raw data were deposited in the NCBI's Short Read Archive (SRA) database under accession number SRRXXXXX. The transcripts maximum length reached 2,150 pb and the number of predited ORFs were 8,310 with size ranging from 14 to 688 amino acids. Among the sequences with ORFs predicted, 4,969 were associated with gene ontology (GO) terms, 3,371 had no hits in the GenBank nonredundant (NR) database and the majority of contigs was similar to hypothetical or secreted proteins of other phytonematode.

A combination of two approaches based *extrinsic* and *ab initio* prediction was used to predict the *P. brachyurus* secretome. This analysis generated a list of 115 putative secreted proteins from the *P. brachyurus* transcriptome (Fig. 1; Tab S3).

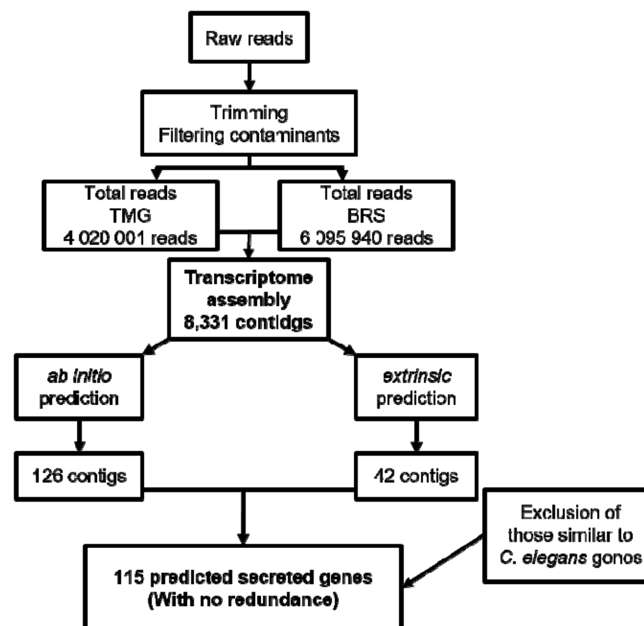


Fig. 1 Diagram of the filtering pipeline method for prediction of secreted genes from the *Pratylenchus brachyurus* transcriptome.

The ab initio prediction identified a total of 126 secreted protein candidates with signal peptides and probably not retained in the cell (Tab S4). The *extrinsic* approach by sequence similarities with secreted and effector proteins, already described in plant and animal-nematodes parasites, enabled the direct identification of 42 contigs (Tab S5). After removing the redundant, 130 contigs composed the secretome list, which 15 sequences were then removed due to their high similarity with *C. elegans genes*, a free-living nematode. The predicted secretome corresponds to 1.38% of the total of the 8,331 *P. brachyurus* contigs expressed *in planta*.

Expressed genes were detected in the esophageal glands, intestine, and sexual glands. In situ hybridization was used to investigate the spatial

expression patterns of the 23 putatively secreted proteins in mixed life stage nematodes. Eight genes were tagged as expressed in glands cells of *P. brachyurus*; three tags in the dorsal gland, one in both, dorsal and subventral glands, and four in the subventral glands (Fig. 2). The gland cell genes were similar in sequence to a SEC-2 protein (*PB0713*), a cellulase-8 (*PB3189*), a transthyretin-like (TTL) protein (*PB1304)*, and a beta-1,4-endoglucanase (PB1953) expressed in the subventral gland cells. Genes similar in sequence to a *14-3-3b* protein (*PB6584*), a LGMN legumain enzyme (*PB6638*) were expressed in the dorsal gland cell. A pioneer gene (unknown) (*PB1365*) was tagged to the dorsal gland and intestine, whereas a hypothetical protein (*PB5635*) shewed signal in the dorsal and subventral glands. All the eight gland cell-localized sequences represent new effectors candidates that could be delivered into the host through the stylet during infection.

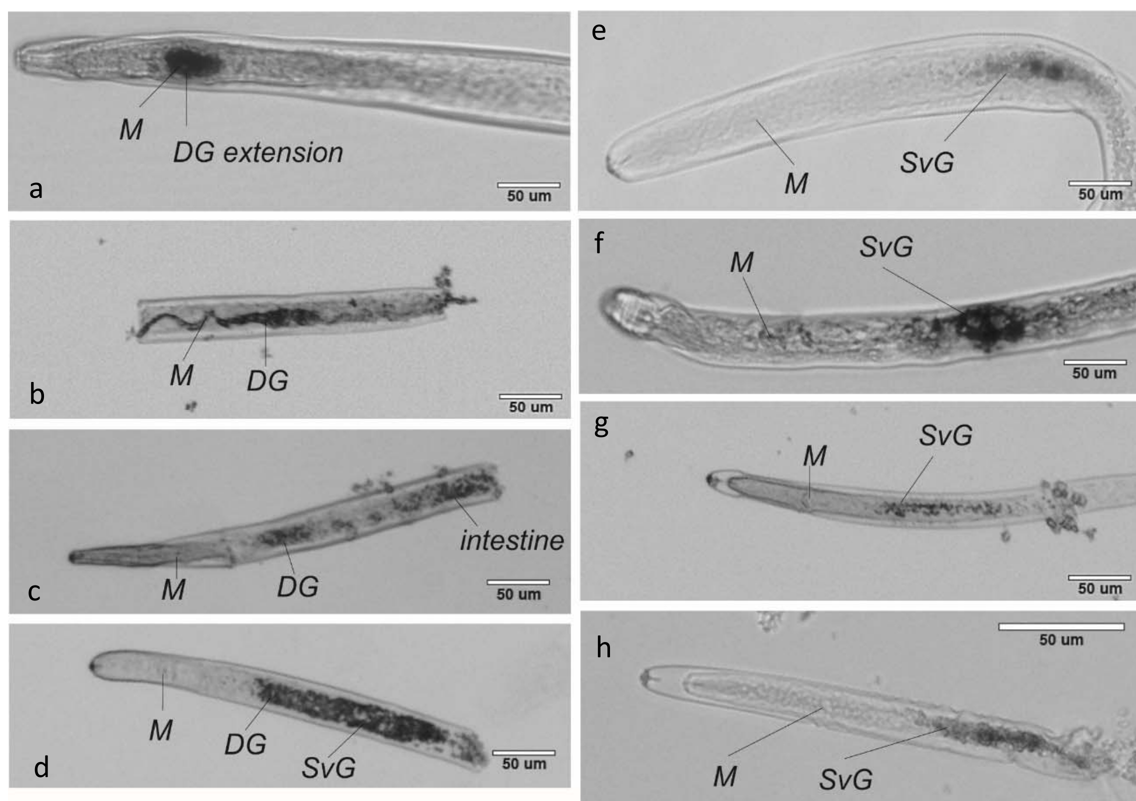


Fig. 2 Localization of candidate effectors expression in the pharyngeal gland cells by in situ hybridization. (a) *PB6584* (anti-sense). (b*) PB6638* (anti-sense). (c) PB1365 (sense). (d) *PB5635* (anti-sense). (e) *PB3189* (anti-sense). (f) *PB0713* (anti-sense). (g) *PB1953* (anti-sense). (h) *PB1304* (sense). M, median bulb; DG, dorsal gland cell; M, metacorpus; and SvG, subventral glands.

Other seven genes showed hybridization signal in the intestine (Fig. S1), whereas one gene was expressed in both, intestine and sexual gland, one gene

gave the signal in the sexual gland, and seven genes gave no *in situ* hybridization signal (not shown). All probes that showed signal had its opposite probes, sense or anti-sense, with no signal.

Genes tagged with expression in the intestine or sexual glands showed similarity and were annotated in a variety of molecular functions. *PB0692* and *PB6909* were annotated as cathepsin L-like proteinase, *PB0386* as a calreticulin, *PB1253* with a putative activity of phospholipid peroxidase, *PB4520* a DNA-binding transcriptional regulator, and *PB7572* an oxygenase dependent ethylene. The other genes, *PB7000* and PB5635 are non-characterized or predicted proteins. In addition, the results of seven and eight genes, out of 23 tested, tagged in the intestine and esophageous glands, respectively, corroborating the high confidence of secretome prediction pipeline applied in this study.

Expression profile of selected genes shows activation during infection. The expression profile of three selected effector candidates genes, *PB0713*, *PB6584*, and *PB6638*, was associated upon the interaction with a *Pratylenchus brachyurus* moderately resistant soybean genotype, BRSGO Chapadões, and a susceptible genotype, TMG 115 RR. Six different time points of the interaction were evaluated, 12, 24, 48, 72, 96, and 192 hours after the infection. All three genes expression profile showed to be different depending on the soybean genotype they were in contact with. *PB0713* was highly activated at 12h when interacting with the moderately resistant genotype, whereas compared to TMG interaction at the same time point (Fig. 3a). From 24h ahead *PB0173* was activated in TMG interaction, whereas in the BRS integration it presented an oscillation, like a strategy of “intermittent attack-recovery”. Likely, both other genes, *PB6584* and PB6638, showed activation in BRS then in TMG at 12h. However *PB6584*, in TMG interaction, increased its expression at 24 and 48h, a strong depletion was detected at 72 and 96h (Fig. 3b). PB6638 showed an expression profile very similar to what was seen for *PB6584*. In TMG interaction, there was activation at the early contact time points, 24h and 48h, followed by depletion at the next two time points and a back activation in 192h (Fig. 3c).

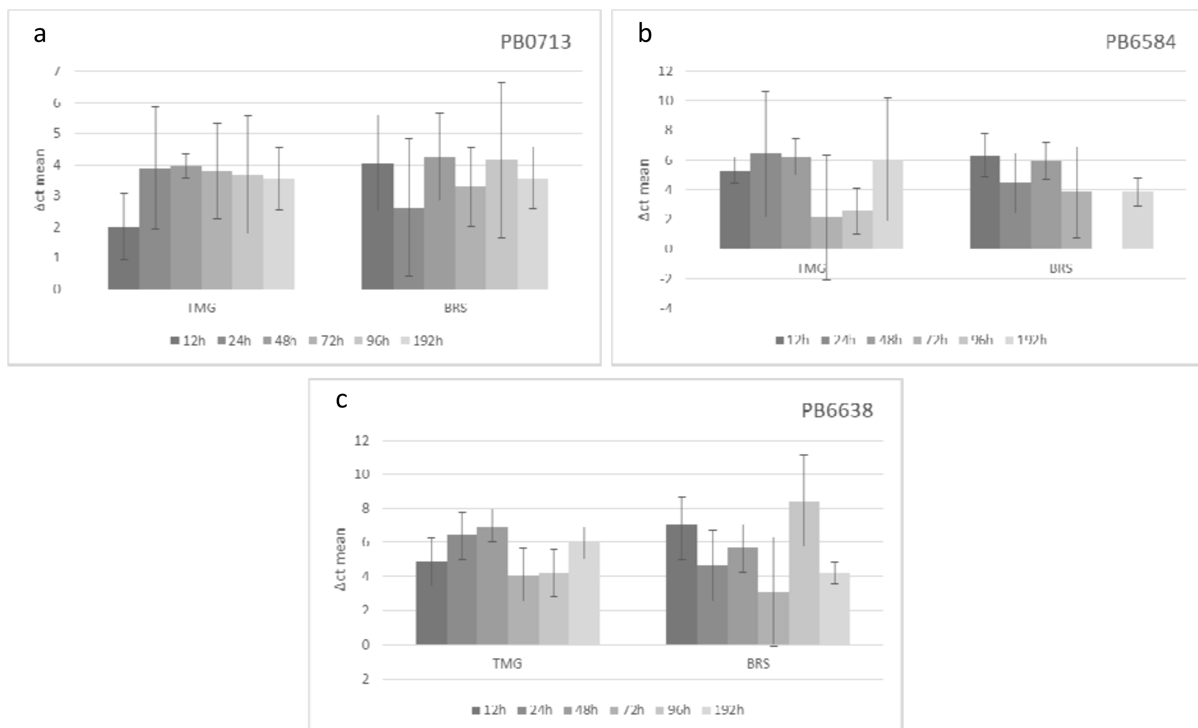


Fig. 3 Expression profile of three expressed esophageous genes from *P. brachyurus*, detected by ISH, during the interaction with a susceptible and moderately resistant soybean genotypes, TMG and BRS, respectively (see methods). a. *PB0713*, a SEC-2 protein previously tagged in the subventral gland; b. *PB6584*, a *14-3-3b* protein detected in the dorsal gland extension; and c. *PB6638*, a LGMN legumain enzyme detected in the dorsal gland.

Even in BRS, *PB6584* and *PB6638* seems to have a similar expression profile. The only exceptional time point is at 96h, which no expression could be detected for *PB6584*, but a very high activation occurred for *PB6638*. It is important to point out that, in interaction with BRS, the expression profile of all the three genes showed a type of strategy that we named here as “intermittent attack-recovery”.

PB6584 effector candidate physically interacts with three host proteins. To search for host proteins that physically interact with *PB6584*, we conducted yeast two-hybrid (Y2H) screens with the full-length *PB6584* coding sequence as bait. After screening more than 15 million yeast colonies from one prey libraries prepared from *Arabidopsis* roots inoculated with *M. incognita* (see methods), we identified sequences corresponding to a subtilisin-like serine endopeptidase family protein (*AT1G20160*), a hyaluronan domain protein (*AT5G47210*), and a cytosolic invertase (*AT1G35580*) as interactors. Out of five positive clones were obtained in the initial Y2H screen, two cDNA clones

sequences corresponded to the last 99 amino acids in the *AT1G20160* C terminus protein region; the sequence alignment shows the transduction at the frame +3, from the second ATG upstream of the prey vector sequence (Fig S2).

AT1G35580 was represented by two independent cDNA clones. The Y2H.3 clone presents a sequence corresponding to the N terminus region of *AT1G35580* protein, from the 39th to the 198th amino acid (Fig S3). The second clone corresponding to the *AT1G35580* cDNA sequence, Y2H.4, aligned in the C terminus region; the transduction frame is the first ATG upstream of the prey vector sequence. The last clone out of five from the Y2H screen, Y2H.5, corresponded to 114 amino acids in the N terminus region of *AT5G47210* protein. All clones showed very specific interactions with bait vector since it was presented in the early stages of the screening (Fig S4).

The soybean closest homologs genes to *Arabidopsis* interactor ones, corresponding to *Glyma.*03G110300 for *AT5G47210* and shows 58.5% of sequence similarity. *AT1G35580* has its soybean homologous as *Glyma.*20G177200 with 92.7% of the full-length gene sequence similarity. Last, but not least, *Glyma.*04G022300 is the corresponding soybean homologous gene for *AT1G20160* with 69.3% of sequence similarity.

PB6584's interactors expression profile. The corresponding soybean homologous to the *Arabidopsis* interactors, found in ours results, was accessed to obtain its relative expression during the infection course. There was a very distinct expression profile for all three genes, depending on the genotype. Apparently, TMG did not show any significant changes, however genes were mostly down-regulated in comparison with the control non-inoculated samples. Instead, BRS presented mostly up-regulated and statistically significant at 48 and 72h for all the three genes (Fig. 4).

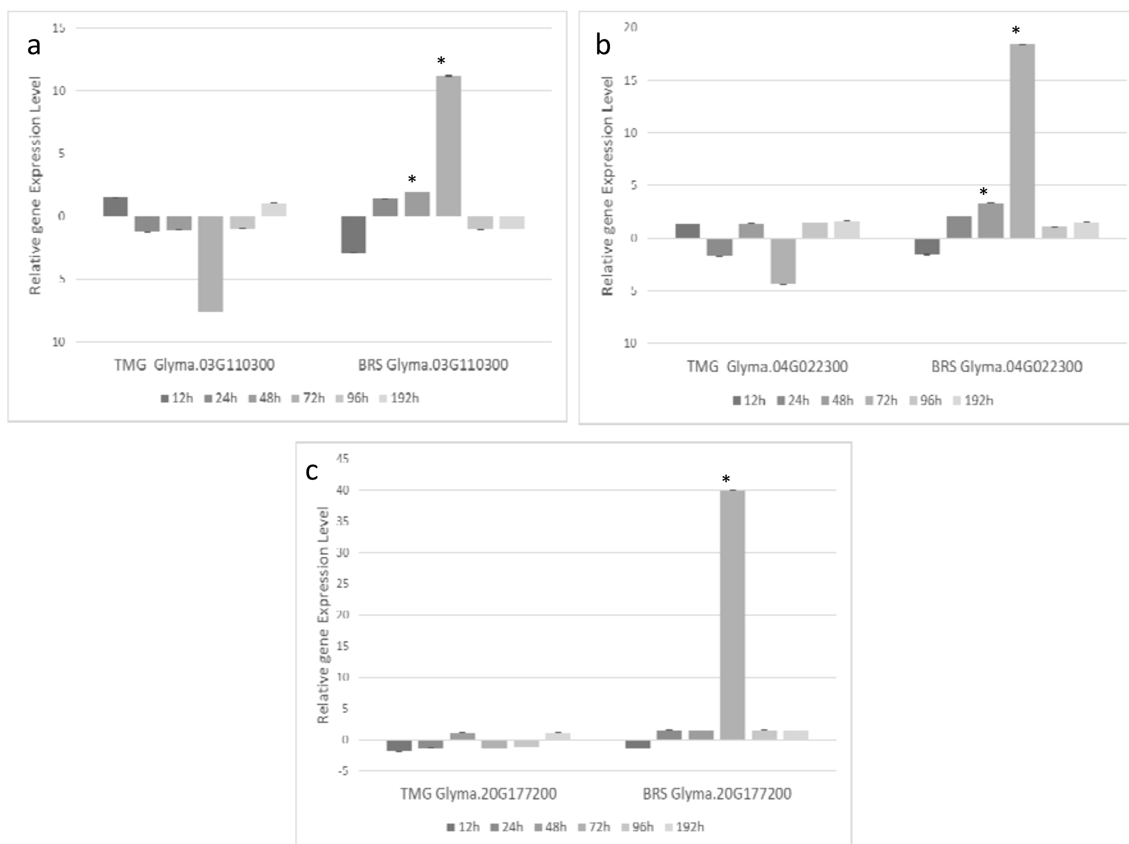


Fig. 4 Relative expression profile of soybean genes *Glyma.*03G110300, *Glyma.*04G022300, and *Glyma.*20G177200 in the course of *Pratylenchus brachyurus* infection. Six different time-points were analyzed, 12, 24, 48, 72, 96, and 192 hours after infection. These three soybean genes are the corresponding homologous to those *Arabidopsis* interactors found in the Y2H assays with PB6584, effector candidate gene. a. *Glyma.*03G110300 (*AT5G47210*), a hyaluronan domain protein; b. *Glyma.*04G022300 (AT1G20160), a subtilisin-like serine endopeptidase family protein; and c. *Glyma.*20G177200 (*AT1G35580*), a cytosolic invertase.

The very similar expression profile of the genotypes for all the homologues genes in soybean *Glyma.*03G110300, *Glyma.*04G022300, and *Glyma.*20G177200 is another evidence that they might be affected by the interaction with PB6584.

Discussion

High-throughput sequencing provides new opportunities for studying plant–nematode interactions. This study presented the first *Pratylechus brachyurus* transcriptome for the scientific community. Due to high-throughput sequencing technologies diffusion many nematode transcriptomes data have been released, allowing the prediction of secretomes and new effector candidates genes (Nguyen *et al.*, 2018). The most recent nematodes

transcriptome sequenced was from *M. incognita* in various stages of development (Choi *et al.*, 2017) and *Heterodera avenae* during their early contact with wheat roots (Chen *et al.*, 2017), as the same method as performed here in our study. Among other plant-parasitic nematode transcriptomes we can cite the study performed with *M. chitwoodi* (Roze *et al.*, 2007), which resulted in an assembly of 5,880 contigs and the identification of 398 secretome members. Kumar and coworker (2014) also identified 1,471 contigs presenting signal peptides but no a trans-membrane helix (TMH) among a total of 27,765 contigs from *Heterodera avenae*. Six different life-stages transcriptome from *Globodera pallida* resulted in a total of 16,419 transcripts predicted and 117 proteins identified as potential novel effectors (Cotton *et al.*, 2014). Additionally, *Heterodera avenae* in a incompatible interaction with *Aegilops variabilis*, a plant species closely related to wheat, was sequenced and 681 putative genes of this parasite were found (Zheng *et al.*, 2015). According to Zhang and cowokers (2015), 56 putative effectors were identified among the primary set of *H. avenae* transcripts. A new *H. avenae* transcriptome analyses identified 351 sequences, among 10.811 contigs, similar to currently known effector genes (Chen *et al.*, 2017; Yang *et al.*, 2017). Other sedentary nematodes transcriptome are *Heterodera schachtii*, *M. incognita*, and *M. enterolobii* (Fosu-Nyarko *et al.*, 2016a; Li *et al.*, 2016; Nguyen *et al.*, 2018)

Among the migratory nematodes, the transcriptome analysis from *Ditylenchus destuctor* generated a total of 9800 ESTs, which 391 were included in the secretome set and 22 sequences were similar to reported effectors, like *1,4*-beta-glucan, pectate lyase and expansin proteins (Peng *et al.*, 2013). A total of 23,715 transcripts were obtained in a de novo assembly of *P. penetrans* and 1,660 transcripts composed the putative secreted proteins set (Vieira *et al.*, 2015). Based on initial analyses of *Pratylenchus coffea*, a 19.76 Mb genome size was obtained and a total of 6,712 encoding sequences were predicted (Burke *et al.*, 2015). *Pratylenchus* sp. presented the smallest genome and number of encoding genes found in the metazoan subkingdom (Burke *et al.*, 2015). Likewise, our study revealed a total of 8,310 ORFs (open reading frames) composing the *P. brachyurus* transcriptome expressed in soybean roots. This is a close number of transcripts considering *P. brachyurus* and *Pratylenchus coffea* belogs to the same genera. The transcriptome assembly of

Pratylenchus thornei presented a total of 6,989 contigs (Nicol *et al.*, 2012), and a *de novo* sequencing of the *P. zeae* transcriptome, using 454 FLX, generated an assembly of 10,63 contigs (Fosu-Nyarko *et al.*, 2016b). These study also presented many transcripts similar to those encoding for known secretory–excretory from sedentary nematode products already described in literature, as *Meloidogyne incognita* and *Heterodera glycines*. Other migratory transcriptome studies include species *Aphelenchoides ritzemabosi*, *Bursaphelenchus xylophilus*, *Hirschmanniella oryzae*, and *R. similis* (Jacob *et al.*, 2008; Beeler, 2013; Bauters *et al.*, 2014; Xiang *et al.*, 2016; Espada *et al.*, 2016).

According to Hwa (2015) and Truonga and coworker (2015), parasitism genes are expressed in organs and tissues capable of releasing molecules to the interface of host and parasite. Other than the classical esophageal glands, the sources are chemosensory amphids, the hypodermis, the cuticle, the excretory system and the rectal glands (Truonga *et al.*, 2015). However, after all has been stated earlier, it is important to state that the term effector should be used more cautiously. In some studies, the term “effector” gene has been already used only because the gene was seen as secreted by *in situ* hybridization assays. In fact, all effectors are secreted, but not all secreted protein are effectors. In our results, among the predicted secretome obtained in this study, probes for 23 different genes were tested, seven being found as expressed in the intestine and eight in the esophageous glands. These results suggest proteins may have been secreted outside of the nematode body, classifying them now as putative effector candidate genes, and validating our *in silico* analysis or revealing a high efficient procedure for effector prediction presented in this study.

Esophagel gland expressed genes from P. brachyurus are putative effector candidate genes. Most of the nematode effector genes have been related to their expression signal from the esophageous glands (Noon *et al.*, 2012; Jones *et al.*, 2016). Among our effector candidate genes detected in the esophageal glands, we identified the SEC-2 protein, *PB0713*, previously suggested to reduced host defense response (Robertson *et al.*, 2000). A SEC protein was first isolated and localized on the *Globodera* spp surface cuticle (gp-sec-2 and gp-sec-3) (Curtis, 2007). This protein was also described as genes

expressed in the *Meloidogyne incognita* subventral gland (Bellafiore *et al.*, 2008). The transcriptome analysis of *Heterodera schachtii* infective stage reported the presence of Sec-2 gene transcripts (Fosu-Nyarko *et al.*, 2016a). Sec-2 gene was among several *Ditylenchus destructor* ESTs that are homologues of other reported effectors (Peng *et al.*, 2013). The Sec-2 was already found in other migratory nematodes species of *Pratylenchus thornei*, *Pratylenchus coffeae*, *Pratylenchus zeae*, and *Radopholus similis* transcriptomes (Jones *et al.*, 2016; Fosu-Nyarko *et al.*, 2016b) and has been found in the subventral gland of *Pratylenchus brachyurus* during soybean interaction in this study.

Nematode transthyretin-like gene was also predicted as a potential effector from different nematodes species, however the gene has still no functional evidence available. The transthyretin-like gene expression at parasitic stages was found in nematodes transcriptome, as *Pratylenchus coffeae*, *Pratylenchus zeae, Radopholus similis*, and *Heterodera schachtii* (Jacob *et al.*, 2007; Jones *et al.*, 2016; Fosu-Nyarko *et al.*, 2016a,b). The protein was also described as genes expressed in the *Meloidogyne incognita* subventral gland (Bellafiore *et al.*, 2008). Lin and coworkers (2016a), reported the cloning and characterization of the gene encoding a transthyretin-like protein, *MjTTL*5, from *M. javanica*. The results of this study, supports our findings related to the gene expression, *PB1304*, in the subventral gland of *P. brachyurus*. More importantly, the studies of *MjTTL*5 presented several lines of evidence that this gene is effector for nematode parasitism (Lin *et al.*, 2016b).

Celullases and its variations, like beta-endoglucanases, has also been a subject of discussion regarding phytonematodes. Although cellulose is a major food source for many animal species, most omnivores and herbivores, such as ruminants and birds, live in symbiosis with cellulolytic microorganisms since they do not produce cellulases themselves. However, it is clear that the phytonematodes are animals that present cellulases genes. Smant and coworkers (1998) first identified and isolated an endogenous b-1,4-endoglucanase genes expressed in the esophageal glands of *Globodera rostochiensis* and *Heterodera glycines* of plant-parasitic cyst nematodes. The authors also assigned the nematode cellulase activity as a facilitator for the pathogen migration through plant roots by the hydrolysis of beta-1,4-glucans

and partial cell wall degradation. A beta-1,4-endoglucanases from *M. incognita* and *M. javanica* (*Mj-eng*-3) was identified as expressed in the subventral gland by ISH (Dautova *et al.*, 2003; Huang *et al.*, 2004; Hu *et al.*, 2013). Including the *P. brachyurus* results, *PB1953* and *PB3189*, presented in this study, many other nematode species have been shown cellulases genes, as *P. zeae*, *P. coffeae*, *M. hapla*, *R. similis*, *D. destructor*, *H. schachtii*, *H. avenae* (*Ha-eng*-2, *Ha-eng*-3), and *Rotylenchulus reniformis* (*Rr-eng*-1) (Ubben *et al.*, 2010; Peng *et al.*, 2013; Long *et al.*, 2013; Jones *et al.*, 2016; Fosu-Nyarko *et al.*, 2016a,b). In planta *Mj-eng*-3 gene silencing, by RNAi assay in transgenic tabacco roots, Hu and coworkers (2013) showed a significant reduction in *M. javanica* virulence, affecting the number of individuals and galls.

Our results in *P. brachyurus* corroborates the studies performed by Jaubert et al. 2004 and Vieira et al. 2012, which *14-3-3b* gene expression was detected in the dorsal gland in *Meloidogyne incognita*. The *14-3-3b* protein molecular function it is to mediate protein-protein interactions through post-translational modification via phosphorylation; however its role in the nematode parasitism success is still unknown (Keller & Radwan, 2015; Jones *et al.*, 2016). There is evidence that places *14-3-3b* protein as an important effector gene for many nematode species. Peng and coworkers (2013), as stated before, found several homologues of other reported effectors among *Dytylenchus destructor* ESTs, including a *14-3-3b* protein tagged in the dorsal gland. Chen and coworkers (2017) found a *14-3-3b* encode transcript among the *H. avenae* ESTs during early interaction with wheat. The infective stage transcriptome also revealed *14-3-3b* expression in *H. schachtii*, *Pratylenchus zeae*, and *Pratylenchus penetrans* (Vieira *et al.*, 2015; Fosu-Nyarko *et al.*, 2016a,b)

Previous to our results on *PB6638*, the only nematode LGMN legumain was described in a transcriptomic analysis of *Angiostrongylus cantonensis* (Yu *et al.*, 2017). LGMN legumain enzyme was originally identified in the vacuoles of legume seeds; however recently, it has been widely studied in human and animal cancer field (Zhen *et al.*, 2015; Yao *et al.*, 2017; Qi *et al.*, 2017). In plants and animals, LGMN legumain proteins activity has been related to the hydrolysis of proteins and small molecule.

Expressed genes in the intestine and sexual glands might be involved in the parasitism. As stated before, the intestine can also secrete proteins out of the nematodes body that may play a role in the parasitism success (Truonga *et al.*, 2015; Jones *et al.*, 2016). Based on the annotation of the intestine addressed genes in this study, some discussions are attempting to understand its activity in relation to *P. brachyurus* parasitism.

Nematode cathepsin L-like proteases have been extensively studied in recent years, and in fact, two *P. brachyurus* cathepsin L-like proteinases encode genes, *PB0692* and *PB6909*, were detected in the nematode intestine. These proteins are required for a wide range of physiological processes, particularly crucial for nematode digestion of host tissues and evasion of the host immune response. The characterization of an intestinal cathepsin L protease from the plant-parasitic nematode *Meloidogyne incognita* showed it is directly related to the parasitic aspects of the parasitism relationship, and/or evasion of primary host plant defense systems (Neveu *et al.*, 2003). Another study demonstrated the effects of knocking-down a *Meloidogyne incognita* intestinal enzyme cathepsin L protease, by either RNA interference or a chemical inhibitor, reducing the feeding efficiency and the parasitism success (Shingles *et al.*, 2007; Dutta *et al.*, 2015).

Similar results were already found in other phytonematode species. The expression analysis of a *Heterodera glycines* cathepsin L protease suggests it plays a role into parasitic stages and nematode life cycle (Thakur *et al.*, 2014). By *in situ* hybridization assays, it was found that a Radopholus similis cathepsin protease, *Rs-cb*-1, is expressed in esophageal glands, intestines and gonads of females, testes of males, juveniles and eggs (Li *et al.*, 2015b), and a Rs-scp-1, in the procorpus, esophageal glands and intestines of females and in the esophageal glands and intestines of juveniles (Huang *et al.*, 2017). By applying in planta RNAi, Li and coworker (2015b) confirmed that *Rs-cb*-1 expression in nematodes were significantly suppressed and the resistance to *R. similis* was significantly improved in T2 generation of transgenic tobacco plants expressing *Rs-cb*-1 dsRNA. Another result showed RNAi significantly suppressed the expression level of *Rs-cps* (Wang *et al.*, 2016) and *Rs-scp*-1 (Huang *et al.*, 2017), which strongly affected the reproductive capability and pathogenicity of *R. similis*.

Other genes expressed in the nematode intestine have also been target of studies. Likewise, we reported a *P. brachyurus* calreticulin protein, *PB0386*, expressed in the intestine, a *Radopholus similis* calreticulin protein, *Rs*-CRT, was found as expressed in the esophageal glands and gonads of females, the gonads of males, the intestines of juveniles and the eggs (Li *et al.*, 2015a). This study also reported the significantly effect on reproductive capability and pathogenicity of *R. si*milis after treatment with Rs-crt dsRNA for 36 h. Another secreted *Meloidogyne incognita* calreticulin protein, *Mi*-CRT, showed to play an important role in the infection success in *Arabidopsis thaliana*. The study performed by Jaouannet and coworkes (2012) showed the *M. incognita Mi-Crt* gene knockdown, by RNA interference, affected the ability of the nematodes to infect plants. In addition, when the gene was overexpressed in *Arabidopsis* raised the susceptibility upon nematode infection.

Different strategies should always be considered in the attempt to control the attack and proliferation of phytonematodes, as *PB7000* reproduction related genes. Fu and coworkers (2014) targeted a gene expressed in the sexual gland, Bx-Prx, of the pine wilt nematode, *Bursaphelenchus xylophilus*. A peroxiredoxin, Bx-Prx, silenced by RNAi, affected the reproduction and pathogenicity of the nematode.

Interactors can play different but essential roles related to infection process. Successful host-parasite interactions require coordinated molecular activity in both the host and pathogen (Hewezi *et al.*, 2015). Effectors work in the host biological system by inhibiting an important defense gene or enzyme activity, a specific signaling, or even by mimicking a host protein (Hamamouch *et al.*, 2012; Kiyohara & Sawa, 2012; Hewezi *et al.*, 2015).

There are some evidences that proteases, as subtilisin-like serine endopeptidase, or subtilases, may play a critical role in plant immunity and can also be host targets of nematode effectors (Baek & Choi, 2008). A tomato subtilase was the first report of this interaction. *P69*B, an apoplastic subtilase known as a PR-protein (PR-7 class) in tomato, is induced upon infection of *Pseudomonas syringe*, *P. infestans*, whereas *P69*C, another tomato subtilase, under salicylic acid treatment (Tornero *et al.*, 1996a; Zhao *et al.*, 2003; Tian *et al.*, 2004). In addition, there is a hypothesis about tomato *P69*C being

responsible for processing of a LRR (leucine-rich repeat protein) under *Citrus exocortis* infection (Tornero *et al.*, 1996b). By performing the proteolysis of a common expressed LRR, the activity of the *P69*C in tomato starts a signaling cascade for defense response. In *Arabidopsis*, the encode gene for an extracelluler subtilase, SBT3.3, showed a fast expression induction under *Pseudomonas syringae* infection (Ramírez *et al.*, 2013).

A recent study in cotton, *Gossypium babardense*, identified a subtilase gene (*GbSbt*1) as responsive/induced in defense to *Verticillium dahlia*, causal agent of verticillium wilt disease (Duan *et al.*, 2016). In the referenced study, the knocking-down of *GbSbt*1 expression, through virus-induced gene silencing, reduced the tolerance of a cotton resistant genotype. In addition, the overexpression of *GbSbt*1 in *Arabidopsis* plants enhanced the resistance to *Fusarium oxysporum* and *V. dahlia* (Duan *et al.*, 2016). Ekchaweng and coworkers (2017) investigated the activity of a rubber tree (*Hevea brasiliensis*) subtilisin-like serine protease in the leaf fall and black stripe diseases, caused by *Phytophthora palmivora*. Transient expression of the subtilases, *HbSap*, in *Nicotina benthamiana* leaves enhanced resistance to *P. palmivora*, suggesting that this gene plays an important role in plant defense. Furthermore, the co-immunoprecipitation (co-IP) assay demonstrated that the *P. palmivora* Kazal-like extracellular protease inhibitor 10 (PpEPI10) specifically interacted and inhibited *Hb*SPA (Ekchaweng *et al.*, 2017). Our findings represent evidence that a *P. brachyurus* gene, *PB6584*, is also an effector candidate gene targeting a host subtilisin-like serine endopeptidase. Figueiredo and coworker (2014) highlighted the plant subtilisin-like proteins as promising genomic tools to plant disease resistance due to its involvement in both, plant immunity and priming events.

Vertebrate, bacterial and plant hyaluronan molecules have identical chemical structure. In mammals, hyaluronan molecules are also involved in wound healing (Heldin *et al.*, 2009); however, its roles in plant metabolism remains unknown.

Plant invertase enzymes have been reported to be affected during plant-pathogen interactions (Tauzin & Giardina, 2014). Theses enzymes are responsible for catalyze the sucrose hydrolysis, shifting the apoplastic hexoses, and a host cytosolic invertase showed also to be target by *PB6584* effector

candidate. Among other molecular functions, the sucrose product acts as defense signaling molecules, by its involvement in the jasmonate and other hormone pathways (Heil *et al.*, 2012). Bonfig and coworkers (2010) demonstrated the posttranslational activity inhibition of an *Arabidopsis* extracellular invertase, by infiltration of acarbose in leaves, increased the susceptibility to *P. syringae*. Some evidences suggests that hexoses, resulted from invertase activity, are responsible for the cell wall reinforcement, creating a physical barrier against invading pathogens (Luna *et al.*, 2011). In a recent study, the RNAi suppression of a cytosolic invertase (CIN) activity by 38–55%, in a wood aspen hybrid (*Populus tremula* x *tremuloides*), led to a 9–13% reduction in crystalline cellulose (Rende *et al.*, 2017). The reduction of CIN activity decreased the amount of the cellulose biosynthesis precursor UDP-glucose in developing wood, affecting the cellulose deposition. These results suggest that CIN activity has an important role in the cellulose biosynthesis of trees by suppling the substrate.

Conclusion

This study allowed the prediction of *P. brachyurus* secreted genes set, the "secretome". This set of genes can be better investigated in future studies of new effectors responsible for the success of nemaotde parasitism. The predicted secretome initial analyzes resulted in the identification of 16 genes expressed in secretion pathways. Eight genes were identified, by the *in situ* hybridization method, being expressed in the esophageal glands of the pathogen, classical pathway of effector gene secretion. Seven genes had their expression identified in the parasite intestinal tract, and may play an important role in the mechanisms of parasitism, serving as a target for further studies. Interaction analyzes of a *P. brachyurus 14-3-3b* gene, *PB6584* - effector candidate, resulted in the identification of three different host genes, encoders of proteins possibly related to plant defense pathways. Among these host genes, there is a protease and an invertase, genes previously related to host response in studies with nematodes. The findings of our study allowed the better understanding on how the migratory phytonematodes, especially *Pratylenchus brachyurus*, approach to stablish a successful parasitic relation with the host.

Finally, the relevance of all these significant findings from this study can be applied as an important tool in the engineering development of new resistant genotype.

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Suplemental material

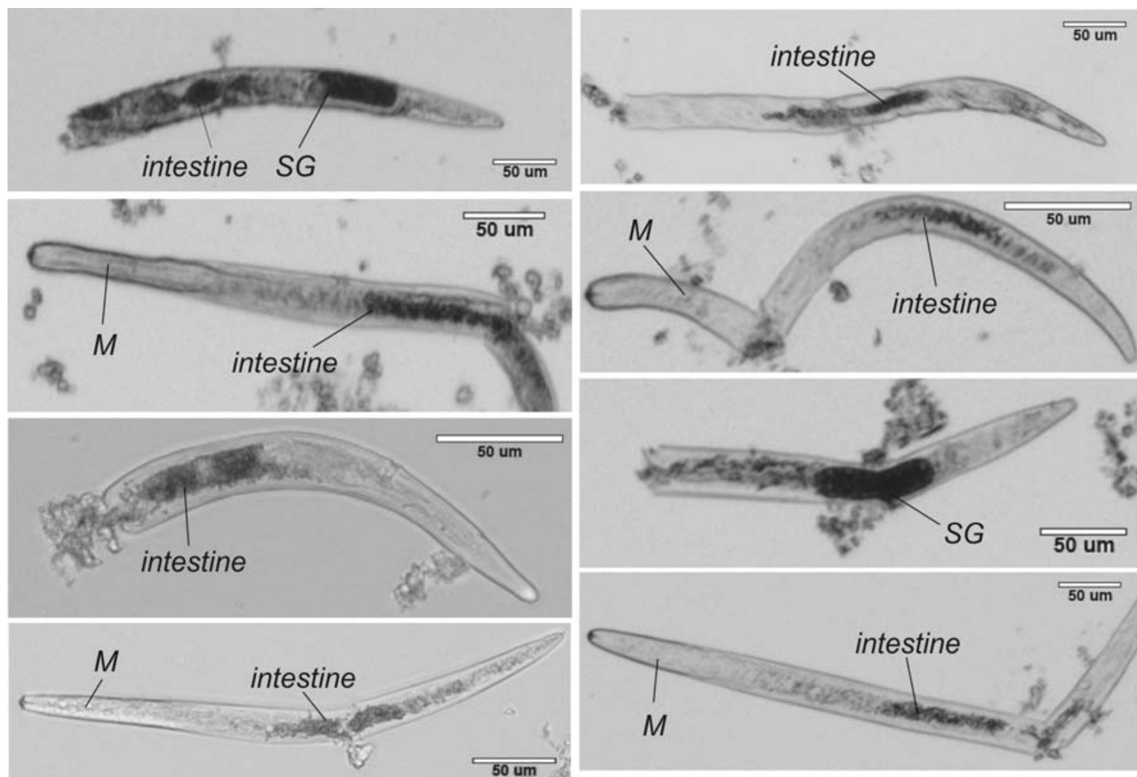

Fig. S1 Localization of *P. brachyurus* genes expression in the intestine and sexual gland by *in situ* hybridization. (a) *PB0279* (sense). (b) *PB0386* (anti-sense). (c) *PB1253* (sense). (d) PB0692 (anti-sense). (e) *PB4520* (anti-sense). (f) *PB6909* (anti-sense). (g) *PB7000* (sense). (h) *PB7572* (sense). M, metacorpus; and SG, sexual gland.

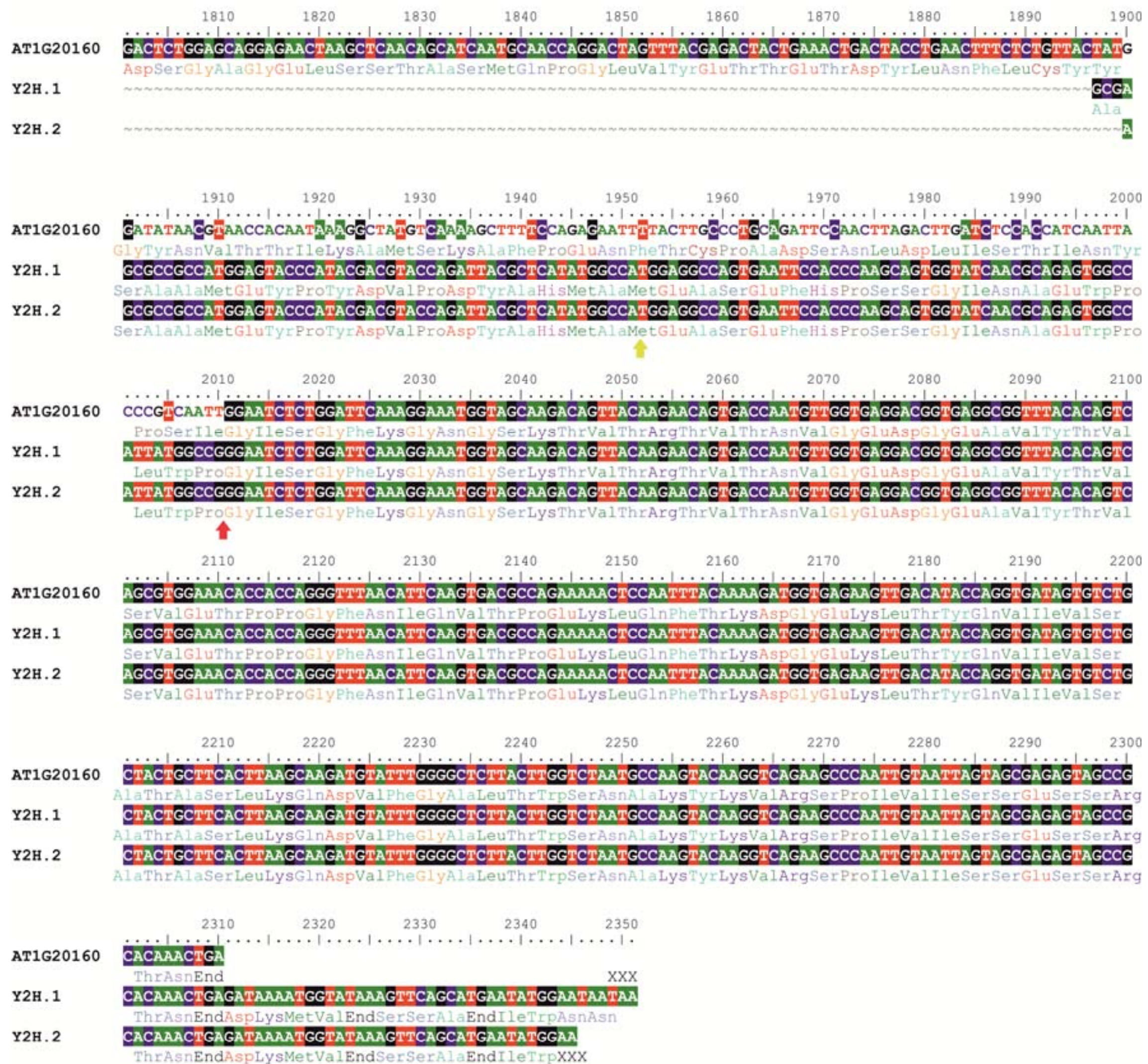

Fig. S2 Alignment of the corresponding *PB6584* interactor gene of *Arabidopsis*, *AT1G20160* and the sequences obtained from the Y2H screen, colonies Y2H.1 and Y2H.2. The red arrow shows the start of cDNA gene sequence, and the yellow arrow indicates the start codon (methionine) from the vector sequence.

Fig. S3 (Fist part) Alignment of the corresponding *PB6584* interactor gene of *Arabidopsis*, Y2H.4. The red arrow shows the start of cDNA gene sequence.

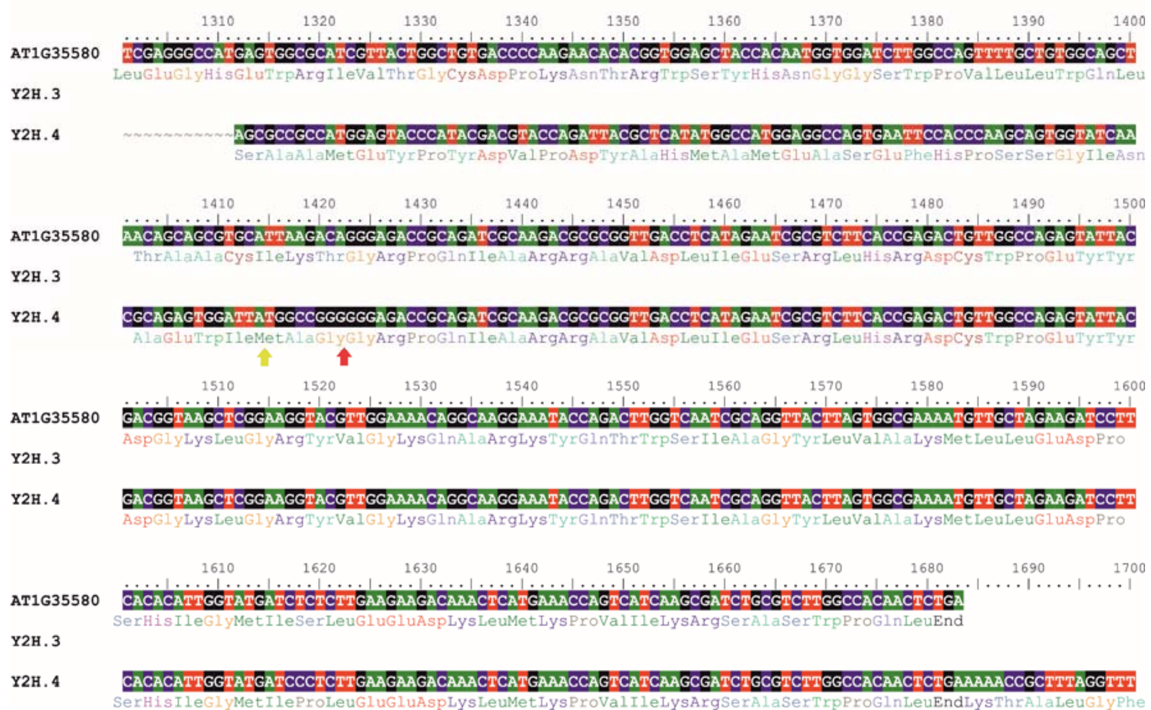

Fig. S3 (Second part) Alignment of the corresponding *PB6584* interactor gene of *Arabidopsis*, Y2H.4. The red arrow shows the start of cDNA gene sequence.

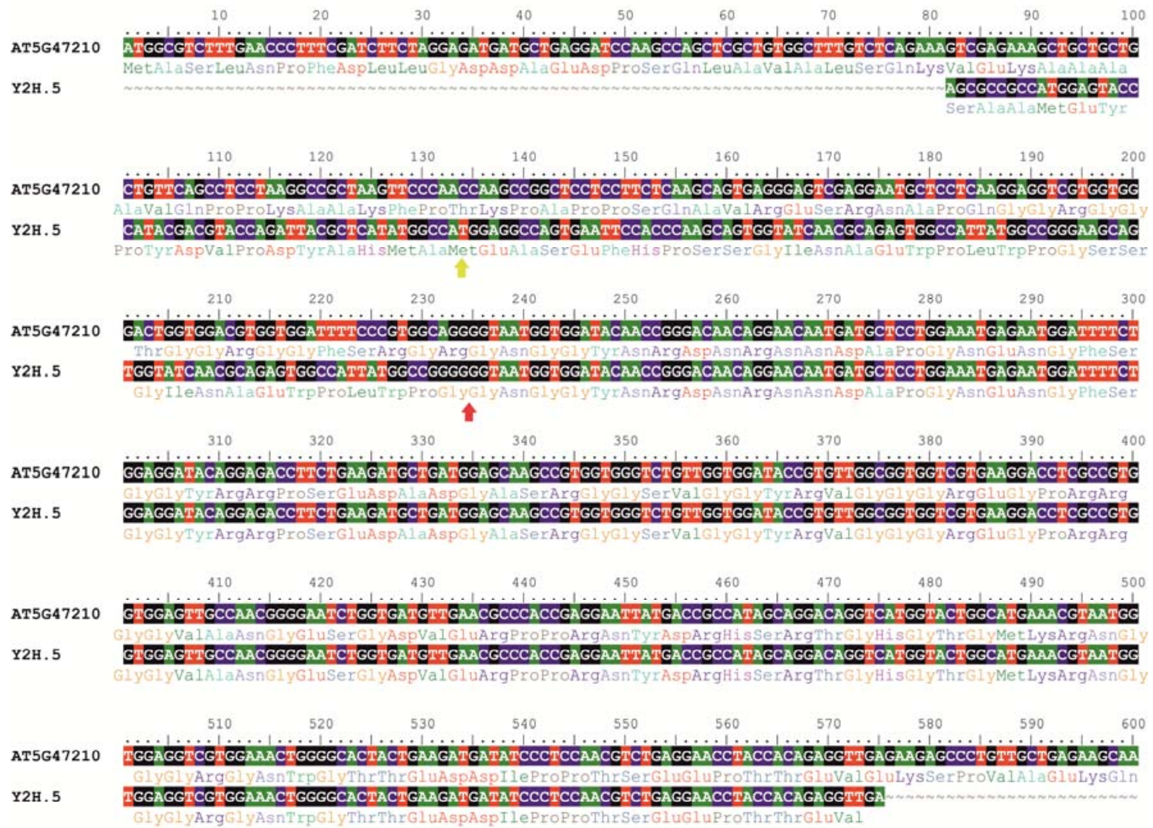

Fig. S4 Alignment of the corresponding *PB6584* interactor gene of *Arabidopsis*, *AT5G47210* and the sequences obtained from the Y2H screen, colony Y2H.5. The red arrow shows the start of cDNA gene sequence, and the yellow arrow indicates the start codon (methionine) from the vector sequence.

7. CONSIDERAÇÕES FINAIS

Os resultados obtidos nesse estudo auxiliam no melhor entendimento dos mecanismos moleculares envolvidos no patosistema soja – *Pratylenchus brachyurus*.

Quanto aos resultados relacionados as respostas de defesa da planta, podemos concluir que nossa abordagem foi capaz de revelar o perfil responsivo cerne dos genótipos de soja com fenótipos moderadamente resistente e suscetível à infecção por nematóide de lesão radicular. A resposta contra *P. brachyurus* no tratamento de 96h foi a que melhor distinguiu os dois diferentes genótipos. No entanto, para ambos os genótipos, as principais alterações de expressão gênica foram às 192h.

A dupla abordagem por analise de expressão diferencial e a nova analise de genes diferencialmente mapeados, evidenciou a presença de genes na resposta de defesa, como os codificadores para LRRs, quinases, fatores de transcrição de *Myb*, quitinases e pectinesterase. Além disso, mostrou as alterações nas vias de biossíntese de fitohormônios, especialmente do jasmonato e etileno, de genes da parede celular. Essas evidências sugerem que tanto PTI como ETI podem funcionar simultaneamente na resposta da soja a *P. brachyurus*. Os genes identificados são promissores para futuras análises, como em estudos genoma-associativo (GWAS) e epigenoma-associativo (EWAS), bem como para melhor direcionar os estudos relacionados a hipótese de estado “*primed*” de defesa.

Em relação aos mecanismos de ataque do patógenos e genes envolvidos no parasitismo, nossa abordagem possibilitou a identificação de um conjunto predito de genes secretados, o “secretoma” de *P. brachyurus*. Esse conjunto de genes pode ainda ser melhor explorado na busca por novos efetores responsáveis pelo sucesso do parasitismo por *P. brachyurus*.

As análises iniciais do “secretoma” do nematóide de lesão radicular resultou na identificação de 16 genes expressos em vias de secreção. Oito genes foram identificados, pelo método de hibridização *in situ*, sendo expressos nas glândulas esofágicas do patógeno, via clássica de secreção de genes efetores. Sete genes tiveram sua expressão identificada nas vias intestinais do parasita, e podem ainda ter uma importante função nos mecanismos de parasitismo, servindo como alvo de futuros estudos.

As analises de interação de um gene *14-3-3b* de *P. brachyurus*, PB6584 - candidato a efetor, resultaram na identificação de três diferentes genes, codificadores de proteínas possivelmente relacionadas às vias de defesa da planta. Dentre esses genes do hospedeiro estão uma protease e uma invertase citosólica, genes já relacionados em estudos anteriores à reposta contra nematóides.

Tais informações são de grande valia para o desenvolvimento de genótipos de soja resistentes ou tolerantes, ainda muito escassos, e para a criação de novas estratégias para o controle ou prevenção de dano causado por fitopatógeno. Além disso, o conhecimento gerado nas análises da interação desse nematóide migrador com a soja pode ser extrapolado em futuras análises com outros hospedeiros e outros nematóides de mesmo comportamento.