VALQUÍRIA JOANA MEDINA PINHEIRO

RESPOSTA DE SOJA À HERBIVORIA POR *Anticarsia gemmatalis* POR MODULAÇÃO DA EXPRESSÃO GÊNICA

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Bioquímica Aplicada, para obtenção do título de *Magister Scientiae*.

Orientador: Humberto Josué de Oliveira Ramos

Coorientadores: Maria Goreti de Almeida Oliveira
Camilo Elber Vital

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RESUMO

PINHEIRO, Valquíria Joana Medina, M.Sc., Universidade Federal de Viçosa, fevereiro de 2020. **Resposta de soja à herbivoria por *Anticarsia gemmatalis* por modulação da expressão gênica**. Orientador: Humberto Josué de Oliveira Ramos. Coorientadores: Maria Goreti de Almeida Oliveira e Camilo Elber Vital.

A soja é um dos grãos mais importantes da economia no Brasil, o maior produtor mundial. No entanto, as lavouras sofrem frequentemente danos causados por pragas, como a lagarta da soja (*Anticarsia gemmatalis*), que também ataca outras culturas. Alguns cultivares que demonstram resistência ao ataque de insetos têm sido utilizadas para decifrar os mecanismos através da avaliação de compostos de defesa. A produção de inibidores de protease, como a família Kunitz (SBTI) e Bowman-Birk (BBI), combinada com compostos fenólicos, podem ser os principais agentes que afetam a digestibilidade das lagartas. Este estudo teve como objetivo avaliar a expressão de genes, o perfil proteico e a atividade enzimática de lipoxigenases e inibidores de protease em resposta ao ataque de lagarta. Os resultados mostraram que essas propriedades parecem ser constitutivas e induzidas, como revelados por níveis mais altos de expressão de inibidores de protease no genótipo considerado resistente mesmo antes da herbivoria e por maior atividade e maior aumento da expressão de genes de lipoxigenases após a herbivoria neste genótipo em comparação ao sensível. Além disso, proteínas comumente responsivas a estresses bióticos e abióticos e ainda não caracterizadas para interações planta-inseto foram positivamente reguladas no genótipo IAC 17 resistente. Receptores transmembranas e fatores transcricionais, detectados como positivamente regulados após herbivoria, podem estar envolvidos na defesa. Da mesma forma, a biossíntese de moléculas de defesa atuando por antibiose pode ser modulada pelas proteínas de ligação ao RNA que controlam o metabolismo do cloroplasto.

Palavras-chave: Pragas. Resposta. Proteínas. Resistência.

ABSTRACT

PINHEIRO, Valquíria Joana Medina, M.Sc., Universidade Federal de Viçosa, February, 2020. **Soybean response to *Anticarsia gemmatalis* herbivory by gene expression modulating.** Advisor: Humberto Josué de Oliveira Ramos. Co-advisors: Maria Goreti de Almeida Oliveira and Camilo Elber Vital.

Soy is one of the most important grains in the economy in Brazil, the largest producer in the world. However, crops often suffer damage from pests, such as the soybean caterpillar (Anticarsia gemmatalis), which also attacks other crops. Some cultivars that demonstrate resistance to insect attack have been used to decipher the mechanisms through the evaluation of defense compounds. The production of protease inhibitors, such as the Kunitz family (SBTI) and Bowman-Birk (BBI), combined with phenolic compounds, may be the main agents that affect the digestibility of caterpillars. This study aimed to evaluate gene expression, protein profile and enzymatic activity of lipoxygenases and protease inhibitors in response to caterpillar attack. The results showed that these properties appear to be constitutive and induced, as revealed by higher levels of expression of protease inhibitors in the genotype considered resistant even before herbivory and by greater activity and greater expression of lipoxygenase genes after herbivory in this genotype compared to the sensitive. In addition, proteins commonly responsive to biotic and abiotic stresses and not yet characterized for plant-insect interactions have been positively regulated in the resistant IAC 17 genotype. Transmembrane receptors and transcriptional factors, detected as positively regulated after herbivory, may be involved in defense. Likewise, the biosynthesis of defense molecules acting by antibiosis can be modulated by RNA-binding proteins that control chloroplast metabolism.

Keywords: Pests. Response. Proteins. Resistance.

SUMÁRIO

INTRODUÇÃO GERAL

Citada pela primeira vez por volta de 2838 AC, a soja surgiu na China como planta rasteira e evoluiu a partir do cruzamento natural entre espécies que foram domesticadas e melhoradas por cientistas. Até o século XV não havia ainda saído deste país, mas no século XX a soja já interessava a empresas mundiais devido ao seu alto teor de óleo e proteínas (Agrawal *et al*., 2008). No Brasil, o grão passou a ser de interesse econômico no final da década de 60 e, em 1970, seu preço ficou altíssimo, desde então o país investe em tecnologia e melhoramento para a produção de soja, processo liderado pela Empresa Brasileira de Pesquisa Agropecuária (Embrapa).

Atualmente o Brasil ocupa a posição de maior produtor mundial de soja, ultrapassando os Estados Unidos. A maior parte da produção brasileira é exportada, o que também faz do Brasil o maior exportador mundial de soja, uma contribuição de grande importância para o PIB- Produto Interno Bruto brasileiro, associada ao agronegócio. Apenas 3,5% da produção de soja vai para a indústria alimentícia no Brasil e, a nível mundial, 6% da produção é utilizada diretamente na alimentação. A maior parte da safra mundial é usada como proteína nas rações de bovinos, suínos e aves, devido ao alto teor de proteínas no grão de soja, cerca de 40%. Já o teor de óleo não é tão alto (18%), porém graças a produção em larga escala, o óleo de soja é o segundo óleo mais consumido no Brasil, depois do dendê (Embrapa Soja, 2019).

Apesar de todos os dados favoráveis à produção de soja no Brasil, assim como boas condições climáticas e território, a safra brasileira ainda pode sofrer prejuízos devido a estresses bióticos e abióticos na plantação, entre eles podemos citar as oscilações climáticas e os ataques por insetos (Sinclair *et al*., 1997).

Uma grande variedade de insetos constitui as pragas da soja, que incluem desfolhadores (Lepidoptera, Coleoptera, Orthoptera), sugadores (Hemiptera) e comedores de sementes (picadores) (Coleoptera, Diptera) (Singh, 2010). Mais de 300 espécies de patógenos atacam a soja em todo o mundo, mas relativamente poucos causam perdas econômicas significativas (Hartman *et al*., 1999). No entanto, o Brasil já enfrentou prejuízos de produção por ataque de insetos (Ramos, 2013).

A lagarta-da-soja (*Anticarsia gemmatalis*, Lepidoptera: Noctuidae) é uma espécie de ocorrência tropical e subtropical e restrita aos continentes americanos, presente em várias culturas, principalmente na soja, desde as regiões Norte e Central da Argentina até o Sudeste e Estados do Golfo do México nos Estados Unidos (Ford *et al*., 1975; Herzog;

Todd, 1980; Sinclair *et al*., 1997). A lagarta-da-soja é uma praga desfolhadora, consome folhas durante todos os seus instares larvais, levando à desfolhação completa das plantas diminuindo assim sua produtividade e é considerada a principal praga desfolhadora da soja nos EUA, México, Colômbia, Venezuela, Brasil e Argentina (Hoffmann-Campo *et al*., 2000). No Brasil, nas regiões ao Norte do Paraná, a lagarta-da-soja infesta as lavouras a partir de novembro e, no Sul do País, a partir de dezembro a janeiro, podendo causar desfolhamento de até 100%.

A cultura de soja está sujeita ao ataque de insetos desde a germinação até a colheita. O controle de pragas pode ser feito por meio de várias práticas que exigem monitoramento constante da lavoura para evitar a disseminação do inseto. Uma forma de controle, chamada controle biológico, consiste em inserir na lavoura predadores que eliminem a praga sem causar prejuízos à lavoura, prática que, em algumas situações, pode afetar o equilíbrio ambiental (Embrapa Soja, 2013). Porém, o método mais utilizado ainda é o uso de inseticidas, que ocorre de forma indiscriminada e causa prejuízos tanto ao meio ambiente quanto à saúde humana (Lopes & Alburquerque, 2018). O Manejo Integrado de Pragas, é uma técnica defendida pela Embrapa, baseada na amostragem de pragas e monitoramento da lavoura para uso sustentável de inseticidas, mas caiu em desuso por volta de 1980. Diante disso, a pesquisa sobre compostos naturais como alternativa aos pesticidas sintéticos torna-se cada vez mais importante pois, por se tratarem de moléculas orgânicas que fazem parte da defesa natural da planta, poderiam reduzir os danos ambientais (O'Neill *et al*., 2010; Borges *et al*., 2011; Bueno *et al*., 2013).

As plantas e os insetos coexistem há aproximadamente 400 milhões de anos e nesse tempo ambos desenvolveram estratégias contra o sistema de defesa um do outro. As plantas possuem um sistema de defesa que tem a capacidade de reconhecer moléculas ou sinais de células danificadas e assim ativa sua resposta imune contra os herbívoros (Howe; Jander, 2008).

A defesa das plantas pode operar por vias diretas ou indiretas (Dudareva *et al*., 2006). Na via direta, a defesa é mediada por aspectos morfológicos das plantas (por exemplo, pêlos, tricomas, espinhos e folhas espessas e cerosas) que afetam a preferência do herbívoro ou sua sobrevivência e processos reprodutivos (Dudareva *et al*., 2006; Howe & Jander *et al.*, 2008; Arimura *et al*., 2009), e/ou pela produção de compostos tóxicos como terpenóides, alcalóides, antocianinas, fenóis, quinonas e inibidores de proteases, que podem ter efeito anti-nutritivos para o inseto ou agir como repelentes (War *et al*., 2012). Na via indireta, a proteção se dá pela liberação de compostos

voláteis para atrair inimigos naturais dos herbívoros. Assim, a planta oferece abrigo e alimento para o predador, enquanto ele a protege contra os patógenos (Arimura *et al*., 2009).

De forma geral, a defesa pode ser classificada como tolerância, antixenose e antibiose (Smith, 2005; War *et al*., 2012). Tolerância é o meio de resistência no qual a planta é capaz de suportar e recuperar-se das injúrias causadas por um inseto, ao passo que seu índice de produtividade seja igual ao de outras plantas não atacadas. (Smith & Clement, 2012; WAR *et al*., 2012). A tolerância é constante objeto de estudos atuais que visam à identificação de genótipos de soja com tolerância a insetos praga. Antixenose ou não-preferência é a defesa que afeta o comportamento dos insetos, já que as características fenotípicas da planta hospedeira faz com que ela seja não preferida para alimentação, ovoposição ou abrigo. A antibiose é a defesa que afeta parâmetros biológico dos insetos, com efeitos de mortalidade na fase imatura, menor crescimento e peso, deformações e aumento no ciclo de vida do inseto. Para isso, a planta produz metabólitos secundários que diminuem o estresse ocasionado por fatores bióticos e abióticos (Taiz & Zeiger, 2009).

Ambas defesas, diretas ou indiretas, podem estar presentes na planta constitutivamente ou serem induzidas após o ataque do herbívoro. A resposta induzida nas plantas é um importante componente no controle de pragas para a agricultura e tem sido estudada para o controle de insetos herbívoros (Sharma, 2009). Nas últimas décadas, houve um progresso considerável no estudo das respostas induzidas em plantas contra diferentes estresses e se tornou um tópico importante na biologia evolutiva e ecologia. Embora as respostas induzidas tenham alguns custos metabólicos, estes são importantes a medida que visam aliviar o estresse de forma imediata, pois a maioria desses compostos químicos são produzidos em resposta ao ataque de herbívoros (Miranda *et al*., 2007). As defesas induzidas tornam as plantas fenotipicamente adaptáveis e assim, diminuem as chances de os insetos se adaptarem os produtos químicos induzidos.

Muitos estudos têm mostrado que o reconhecimento do patógeno pela planta leva à indução de resistência a partir da produção de compostos voláteis após o ataque herbívoro, que funcionam como sinalizadores celulares (Moraes *et al.*, 2005). Esses compostos também atuam na interação planta-planta à medida que “preparam” as plantas vizinhas para um possível futuro ataque do inseto (Ton *et al*., 2005). Existem muitos compostos químicos envolvidos nas interações entre plantas. Estes incluem voláteis de folhas verdes [(E)-2-hexenal (Mirabella *et al.*, 2008) (Z)-3-hexen-1-ol (Ruther & Kçeier,

2005) e acetato de (Z)-3-hexen-1-ila](Frost *et al*., 2008), terpenos, como mirceno e misturas de ocimeno [(E)-β-ocimeno, (Z)-β-ocimeno e allo-ocimeno](Godard *et al*., 2008) e formas voláteis de fitohormônios como jasmonato de metila (MeJA)(Karban & Maron, 2002), salicilato de metila (MeSA)(Shulaev *et al*., 1997) e etileno (ET)](O'Donnell *et al*., 1996).

Os terpenos são formados por meio das vias do acetato-mevalonato e do metileritriol fosfato, a partir da união de unidades pentacarbonadas chamadas isopreno, que são agrupadas de acordo com o número dessas unidades na molécula: hemiterpenoides, monoterpenoides, sesquiterpenoides, diterpenoides, triterpenoides e carotenoides (Taiz & Zeiger, 2009).

Os voláteis de folhas verdes são emitidos principalmente quando as folhas são danificadas mecanicamente, são compostos derivados da via da enzima lipoxigenase (rota dos octadecanoides) e podem constituir mais de 50% da emissão de partes danificadas em algumas espécies de planta (Holopainen, 2004). Os compostos voláteis induzem a ativação de vias metabólicas orquestradas e organizadas de defesa da planta. Num primeiro passo, as respostas são desencadeadas por elicitores derivados do herbívoro. A emissão dos voláteis induzidos após o ataque dos herbívoros não pode ser limitada pelo dano mecânico, mas sim quando os elicitores isolados das secreções orais do inseto entram em contato com áreas danificadas da planta (Mattiacci *et al*., 1995). Após a herbivoria ocorre uma despolarização do potencial da membrana e um influxo de cálcio (Ca2+). O dano mecânico produz acúmulo de espécies de oxigênio reativas como superóxido (O2-) e peróxido de hidrogênio (H2O2) além do óxido nítrico, que possuem funções regulatórias de defesa (Wu *et al*., 2004), mas não resulta na síntese *de novo* de voláteis. Porém, após o dano mecânico contínuo, ocorre acúmulo de transcrições de uma MAP quinase a qual parece estar envolvida na síntese do ácido jasmônico, e resulta na produção de voláteis que se assemelham àqueles induzidos após a herbivoria (Mithöfer *et al*., 2005).

Alguns mecanismos de defesa descritos incluem também os metabólitos secundários, compostos que não afetam o desenvolvimento e crescimento da planta, mas reduzem a palatabilidade dos tecidos onde são produzidos (Howe & Jander, 2008) e podem ser sintetizados apenas em resposta a estímulos (Dewick, 2009). Os metabólitos secundários são formados em quatro principais vias a partir de compostos provenientes do metabolismo primário: a via do acetato-malonato, via do acetato-mevalonato, via do metileritriol fosfato e a via do ácido chiquímico. Essas vias produzem terpenoides,

substâncias graxas, compostos fenólicos e compostos nitrogenados. Os metabólitos secundários podem ser constitutivos, armazenados na forma inativa, ou induzidos em resposta ao ataque de insetos ou micróbios. Os inativos são conhecidos como fitoanticipinas e os ativos como fitoalexinas. As fitoanticipinas são ativadas principalmente pela β-glucosidase durante a herbivoria, que por sua vez medeia a liberação de vários metabolitos biocidas da aglicona (Morant *et al*., 2008). Alguns exemplos de fitoanticipinas são glucosinolatos que são hidrolisados por mirosinases (β-tioglucósido gluco-hidrolases endógenas) durante a ruptura do tecido. As fitoalexinas incluem os compostos isoflavonóides, terpenóides, alcalóides e outros que influenciam o desempenho e a sobrevivência dos herbívoros (Walling, 2000).

Dentre os metabólitos secundários, os compostos fenólicos constituem um extenso grupo de substâncias que possuem um anel aromático contendo pelo menos uma hidroxila. Estes compostos incluem: fenóis simples e outros glicosilados, ácidos fenol-carboxílicos, derivados dos ácidos benzóico e cinâmico, a-pirones (cumarinas e isocumarinas), ligninas, flavonóides (flavononas, antocianinas e catequinas) e quinonas (Stangarlin *et al*., 2011). Compostos fenólicos são conhecidos como substâncias fungitóxicas, antibacterianas e antiviróticas (Lo *et al*., 1996). Os fenólicos participam na redução de radicais livres, incluindo espécies reativas de oxigênio (ROS) ânions superóxidos e radicais hidróxidos (Ton *et al*., 2005) o que ativa uma cascata de reações que leva a ativação de enzimas de defesa e assim, contribuem para a defesa da planta quando elas estão submetidas a estresse oxidativo (Bhonwong *et al*., 2009).

Os flavonoides e isoflavonoides são sintetizados a partir da via dos fenilpropanoides, onde a enzima principal é a fenilalanina amônio liase (PAL). Esses compostos estão agrupados em várias classes que incluem, as antocianinas, flavonas, flavonoles, flavanonas, dihidroflavonoles, chalconas, auronas, flavan e proantocianinas (Ton *et al*., 2005). Os flavonoides protegem as plantas influenciando o comportamento dos herbívoros, crescimento e desenvolvimento e alguns possuem propriedades anti-nutritivas em insetos (Simmonds *et al*., 1990), além de eliminarem ROS e reduzirem sua formação atuando como quelante de metais. Alguns dos mais representativos dos flavonoides conhecidos com efeito nocivo em pragas são a rutina e a quercetina, caraterizados em plantas de tabaco (Mallikarjuna *et al*., 2004). Flavonoides como quercetina e kaempferol têm sido associados a propriedades antioxidantes na soja (O'Neill *et al*., 2010). Piubelli e colaboradores (2005), caracterizaram flavonoides em diferentes variedades de soja consideradas como resistentes e aqueles que mostraram relação com a

defesa foram a rutina, genistina e daidzeina que, adicionados à dieta da *A. gemmatalis*, ocasionaram atraso no desenvolvimento.

Muitos hormônios também têm sido associados à resposta de defesa à herbivoria, na comunicação celular e/ou entre plantas (War *et al*., 2012). Fito-hormônios como ácido Jasmónico (JA), ácido salicílico (AS) e etileno (ET) atuam mediando vias de transdução de sinais (Gill *et al*., 2010; Shivaji *et al*., 2010). Estes hormônios podem agir individualmente, sinergicamente ou antagonicamente, de acordo com o tipo de ataque, proporcionando à planta um alto potencial de regulação e pronta adaptação aos estímulos ambientais (Verhage *et al*., 2010). Hormônios como ácido abscísico (ABA), auxinas, giberelinas e brassinosteroides também têm emergido como focos na luta contra as pragas, podendo agir sinergicamente com AS-AJ-ET no sistema de sinalização de defesa das plantas (Wang *et al*., 2007; Howe & Jander, 2008; Vleesschauwer *et al*., 2010).

O ácido jasmônico é a molécula mais importante na regulação dos compostos voláteis induzidos após o ataque dos herbívoros e está conectado à preparação das plantas a futuros ataques (Ton *et al*., 2005), ativando a expressão das vias de defesa diretas e indiretas (Usha *et al*., 2010; Shivaji *et al.*, 2010; War *et al*., 2011). O papel do ácido jasmônico na defesa das plantas foi reconhecido em 1992 por Farmer e Ryan, quando demonstraram pela primeira vez que o jasmonato de metila induz a atividade de inibidores de proteases, que são moléculas que funcionam como compostos de defesa direta. Algumas das vias de defesa induzidas pelos jasmonatos incluem enzimas antioxidativas, inibidores de proteases (IPs), compostos voláteis, produção de alcaloides, formação de tricomas e secreção de néctar extra floral, todas com efeito nocivo para os insetos (Dickens, 2006; Mao *et al*., 2007; Pauwels *et al*., 2009). Os genes de defesa são ativados, por sua vez, pelas oxilipinas pentacíclicas, presentes durante o processo de transdução dos sinais químicos (Gardner, 1995).

O ácido salicílico é um regulador de crescimento que funciona como sinalizador, ativando as respostas das plantas após o ataque de patógenos. O acúmulo do AS nos tecidos das plantas induz a resistência sistêmica adquirida (RSA) (Walling, 2000) e está estreitamente relacionado com a produção de espécies de oxigênio reativo e indução do H_2O_2, compostos que são altamente nocivos para o sistema digestivo de insetos e conduz a retardo do desenvolvimento e crescimento (Peng *et al*., 2004; Maffei *et al*., 2007). O sinal do AS também pode levar a liberação de compostos voláteis que atraem os inimigos naturais das pragas (Boer *et al*., 2004). Além disso, o AS está envolvido na regulação da atividade de várias enzimas como peroxidase (POD), polifenol oxidase (PPO) superóxido

dismutase (SOD), fenilalanina amônia liase (PAL), entre outras, as quais fazem parte dos componentes da defesa induzida na planta (War *et al*., 2011; Zhao *et al*., 2013).

Outro importante hormônio envolvido nas respostas induzidas das plantas é o etileno (Mithöfer *et al*., 2005). Em *Arabidopsis*, o ET e o J agem juntos na indução da expressão de defensinas e genes PR que codificam proteínas de defesa localizadas em vacúolos (Penninckx *et al*., 1998) e agem em sequência para induzir a tolerância sistêmica, chamada de resistência sistêmica induzida (RSI) (Walling, 2000). A produção do etileno em culturas com células de tomate foi comprovada após dano e ação de elicitores como a sistemina, fragmentos oligogalacturonídeos e AJ. Além disso, o etileno pode potencializar a ação do AJ em resposta ao dano. A biossíntese do ET, assim como a do AJ, é estimulada após o dano mecânico (Watanabe *et al*., 1994).

A interrelação entre plantas e insetos é ecologicamente importante para ambos. Os insetos sempre procuram plantas com características favoráveis ao seu desenvolvimento, reprodução e ovoposição e com capacidade para alimentá-los, pois qualquer desequilíbrio na digestão e utilização de proteínas vegetais pelos insetos resulta em efeitos drásticos na fisiologia deles. A alteração da expressão gênica sob estresse, como o ataque de insetos, altera qualitativa e quantativamente o perfil proteico da planta. Dentre as proteínas de defesa presentes em plantas, destacam-se as lectinas, as polifenoloxidases (Constabel & Ryan, 1998), peroxidases, fenilalanina-amônia-liase, lipoxigenases, proteínas relacionadas à patogênese (PR) (Linthorst, 1991; De Wit, 2007), e os inibidores de proteases serínicas e cisteínicas (Siqueira *et al*., 2002) sendo estas melhor descritas quanto a suas propriedades de defesa vegetal (Chen, 2008).

As lectinas são glicoproteínas de ligação à carboidratos que se encontram em todas as plantas na natureza e têm atividade inseticida contra uma grande variedade de pragas (Chakraborti *et al*., 2009; Vandenborre *et al*., 2011). Elas agem como antinutritivos e / ou substâncias tóxicas por ligação de grupos glicosil do revestimento da membrana do trato digestivo, levando a uma série de reações sistêmicas prejudiciais. As lectinas são estáveis em uma ampla faixa de pH e danificam as membranas epiteliais luminais, interferindo assim na digestão e absorção de nutrientes. Assim, as lectinas podem perturbar o metabolismo de lipídeos, carboidratos e proteínas causando atrofia em tecidos importantes, o que altera o estado hormonal e atrapalha o desenvolvimento dos insetos (Chakraborti *et al*., 2009; Vandenborre *et al*., 2011).

O estado oxidativo está associado a resposta da planta hospedeira, que resulta na produção de espécies reativas de oxigênios que, por sua vez, são eliminados por enzimas

antioxidantes. As peroxidases constituem um grupo dessas enzimas, que produzem fenoxi e outros radicais oxidativos em associações com fenóis para deter a alimentação dos insetos e produzem toxinas que podem reduzir a digestibilidade das plantas, causando deficiência de nutrientes no inseto.

Da mesma forma que muitas peroxidases, as polifenoloxidases têm concentrações elevadas em tecidos infectados e têm grande importância nos mecanismos de defesa das plantas (Agrios, 2005). Tais enzimas promovem a degradação oxidativa de compostos fenólicos próximos ao local da lesão provocada pelo patógeno, resultando no aparecimento de substâncias escuras provenientes da polimerização oxidativa das quinonas (Macheix *et al*., 1986, Bindschedler *et al*., 2002). Polifenoloxidases são enzimas que catalisam a oxidação dependente de oxigênio de monofenóis (ex. tirosinases) ou orto-difenóis (ex. catecolases, lacases) a orto-diquinonas (Steffens *et al*., 1994), enquanto as peroxidases são enzimas que agem de forma parecida com polifenoloxidases, mas possuem como substrato principal o peróxido de hidrogênio, além de outra molécula doadora de elétrons, como o fenol ou outros compostos orgânicos (Takahama & Oniki, 2000).

Outro grupo fundamental de enzimas na defesa da planta são as lipoxigenases (LOX), que cumprem função antioxidativa relacionada com a defesa das plantas submetidas a diferentes tipos de estresse. As LOX catalisam a hidroxiperoxidação de ácidos graxos insaturados e produzem ácidos graxos hidroperoxidos (Bruinsma *et al*., 2009), que são degradados a compostos instáveis e reativos. Estes compostos interagem com proteínas, resultando em ligações cruzadas entre proteínas e danificando aminoácidos. Porém a maior importância das lipoxigenases é que atuam pela oxidação do ácido linoleico na via de sinalização do AJ, que é responsável pela produção de enzimas oxidativas e inibidores de protease. Além disso as LOX estão envolvidas na formação de compostos voláteis que atraem os inimigos naturais dos insetos praga (Bruinsma *et al*., 2009).

O termo "proteínas relacionadas à patogênese" (PR) se refere a um grupo de proteínas induzidas em uma planta em resposta a doenças fúngicas, bacterianas e virais, bem como a alguns produtos químicos. Alguns membros da família de proteínas PR foram encontradas sob indução por tratamentos com ácido poliacrílico derivado de aminoácios (Asselin & Cote, 1985), ácido salicílico (Uknes *et al*., 1992) e em condições de distúrbio no equilíbrio entre os fito-hormônios auxinas e citocininas (Mohnen *et al*., 1985). Algumas proteínas PR são induzidas por estresse osmótico e salino com possível

envolvimento do ácido abiscísico (Nelson *et al*.,1992) e outro fito-hormônio de estresse, sendo indutor potente dessas proteínas é o etileno (Van Loon, 1977). As proteínas PR também são sintetizadas constitutivamente em híbridos interespecíficos que apresentam resistência constitutiva à infecção viral (Ahl & Gianinazzi, 1982). A indução das proteínas PR é mediada pela ação de substâncias sinalizadoras que são classificadas em dois tipos, conforme sua origem: elicitores endógenos, da própria planta; e elicitores exógenos, do patógeno (Wyatt *et al*., 1991; Repka, 1996; Christensen *et al*., 2002). As proteínas PR são encontradas nas plantas como isoenzimas ou isoformas, apresentando entre si certas características físico-químicas diferentes, particularmente em relação ao caráter ácido ou básico e são divididas em vários grupos (Okushima *et al*., 2000). A classificação atual divide as proteínas PR em 17 classes, PR-1 – PR-17 e quase a metade delas são conhecidas por conter alérgenos. A numeração é baseada na mobilidade relativa em eletroforese em gel não desnaturante. Essas proteínas são reguladas por estresse, mas sua função exata ainda é incerta (Sels *et al*., 2008). Os genes que codificam as proteínas PR expressam-se através de várias vias de sinalização. PR-1 e PR-5 são dependentes de SA. O gene PR-4 é induzido por AS, AJ e etileno; O gene PR-12 (gene de *Arabidopsis defensin*) é induzido pelo etileno e AJ, mas não responde ao SA (Dzhavakhiya *et al*., 2007). A proteína PR-10 arquetípica é a Bet v 1, o principal alérgeno da *Betula verrucosa*, a bétula branca. As PR-10 têm uma ampla distribuição no reino vegetal. Proteínas PR-10 semelhantes (homólogas Bet v 1) são encontradas em muitos alimentos, incluindo avelã (*Corylus avellana*, Cor a 1), pêssego (*Prunus persica*, Pru p 1), aipo (*Apium graveolens*, Api g 1), soja (*Glycine max*, Gly m 4), maçã (*Malus domestica*, Mal d 1) e amendoim (Ara h 8) (Crowell *et al*., 1992; Breda *et al*., 1996; Sikorski *et al*., 1999).

Por fim, os inibidores de proteases (IPs) abrangem uma das classes defensivas mais abundantes de proteínas nas plantas. (Lawrence & Koundal, 2002; Dunse *et al*., 2010). Nos insetos, as enzimas digestivas são produzidas pelas células epiteliais do intestino médio e secretadas no lúmen, através do qual passa o bolo alimentar e portanto, a hidrólise eficiente de proteínas vegetais para obter aminoácidos essenciais é crucial para a sua sobrevivência (Terra, 1994). Nas plantas, os inibidores de protease são expressos constitutivamente ou induzidos por feridas, tornando-os uma importante estratégia de defesa natural contra insetos fitófagos. O efeito conhecido dos inibidores de protease é a inibição de enzimas digestivas de proteínas no intestino dos insetos, o que resulta em deficiência de aminoácidos por absorção (Gatehouse, 2011) ou por um mecanismo de *feedback*, que resulta em aumento da produção de proteases para compensar a perda da

atividade, causando perda de aminoácidos essenciais (Broadway & Duffey, 1986). De ambas as formas, o estresse nutricional imposto retarda o desenvolvimento dos insetos.

O grupo de inibidores de proteinases serínicas está entre os genes de defesa mais expressos em resposta a herbivoria e inclui os inibidores do tipo Kunitz e do tipo Bowman–Birk (Bode & Huber, 1992). A maioria dos inibidores de protease Kunitiz (KPIs) são positivamente regulados em resposta ao ataque dos insetos, porém, seu grau de indução varia conforme a interação planta-inseto. Além disso, alguns insetos podem apresentar uma resposta constitutiva ou induzida a inibidores de protease, por produção de proteases insensíveis aos inibidores ou por inativação de inibidores ingeridos, resultando em danos ainda maiores às plantas (Zhu-Salzman *et al*., 2008).

O mecanismo de defesa das plantas ao ataque de insetos é complexo e envolve vários fatores e um exemplo disso é a variedade de compostos químicos envolvidos na resposta a herbivoria, relatados ou ainda não descritos. Vários estudos têm sido executados a fim de elucidar os mecanismos de defesa da soja em diferentes níveis: expressão gênica, metabólitos, biossíntese de fito-hormônios e outros (Ludwig, 2000; War *et al.*, 2011; War *et al.*, 2012; Jian-Zhong *et al.*, 2015). Por meio do melhoramento genético, espécies de soja com diferentes níveis de resistência têm sido desenvolvidas no Brasil, porém as informações a respeito da interação molecular entre a soja e *Anticarsia gemmatalis*, principalmente aos níveis de expressão gênica (transcriptoma e proteoma) ainda são escassas.

Desta forma, neste estudo foram investigadas moléculas que podem estar envolvidas na defesa da planta, por análises da modulação da expressão gênica de lipoxigenases e inibidores de protease, bem como suas atividades enzimáticas *in vitro* e a modulação do perfil proteico em genótipos de soja contrastantes para resistência à *Anticarsia gemmatalis*, em resposta à herbivoria.

HIPÓTESE

Diversas proteínas de defesa, assim como enzimas e inibidores de protease envolvidas no mecanismo de resistência de soja contra *Anticarsia gemmatalis* podem ser sintetizadas constitutivamente e/ou por indução pelo ataque da lagarta. A análise do perfil de expressão gênica pode revelar diferenças nas vias de defesa de genótipos suscetíveis e resistentes.

OBJETIVOS

OBJETIVO GERAL

Avaliar os perfis de expressão gênica de enzimas e proteínas relacionadas com o mecanismo de resistência de genótipos de soja à *Anticarsia gemmatalis*.

OBJETIVOS ESPECÍFICOS

- Analisar as proteínas abundantes nas variedades de soja contrastantes para a resistência sem infestação e com infestação da *Anticarsia gemmatalis*.
- Avaliar a modulação da expressão gênica de Lipoxigenases e do inibidor de protease Bowman-Birk nas variedades de soja após a infestação com *Anticarsia gemmatalis.*
- Comparar a atividade enzimática total de lipoxigenases nas variedades de soja após a infestação com *Anticarsia gemmatalis.*
- Comparar a atividade de inibidores de protease totais nas variedades de soja após a infestação com *Anticarsia gemmatalis.*

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Soybean response to *Anticarsia gemmatalis* herbivory by gene expression modulating.

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Resumo

A soja é um dos grãos mais importantes da economia no Brasil, o maior produtor mundial. No entanto, as lavouras sofrem frequentemente danos causados por pragas, como a lagarta da soja (*Anticarsia gemmatalis*), que também ataca outras culturas. Alguns cultivares que demonstram resistência ao ataque de insetos têm sido utilizadas para decifrar os mecanismos através da avaliação de compostos de defesa. A produção de inibidores de protease, como a família Kunitz (SBTI) e Bowman-Birk (BBI), combinada com compostos fenólicos, podem ser os principais agentes que afetam a digestibilidade das lagartas. Este estudo teve como objetivo avaliar a expressão de genes, o perfil proteico e a atividade enzimática de lipoxigenases e inibidores de protease em resposta ao ataque de lagarta. Os resultados mostraram que essas propriedades parecem ser constitutivas e induzidas, como revelados por níveis mais altos de expressão de inibidores de protease no genótipo considerado resistente mesmo antes da herbivoria e por maior atividade e maior aumento da expressão de genes de lipoxigenases após a herbivoria neste genótipo em comparação ao sensível. Além disso, proteínas comumente responsivas a estresses bióticos e abióticos e ainda não caracterizadas para interações planta-inseto foram positivamente reguladas no genótipo IAC 17 resistente. Receptores transmembranas e fatores transcricionais, detectados como positivamente regulados após herbivoria, podem estar envolvidos na defesa. Da mesma forma, a biossíntese de moléculas de defesa atuando por antibiose pode ser modulada pelas proteínas de ligação ao RNA que controlam o metabolismo do cloroplasto.

Palavras-chave: pragas, resposta, proteínas, resistência.

Abstract

Soy is one of the most important grains in the economy in Brazil, the largest producer in the world. However, crops often suffer damage from pests, such as the soybean caterpillar (*Anticarsia gemmatalis*), which also attacks other crops. Some cultivars that demonstrate resistance to insect attack have been used to decipher the mechanisms through the evaluation of defense compounds. The production of protease inhibitors, such as the Kunitz family (SBTI) and Bowman-Birk (BBI), combined with phenolic compounds, may be the main agents that affect the digestibility of caterpillars. This study aimed to evaluate gene expression, protein profile and enzymatic activity of lipoxygenases and protease inhibitors in response to caterpillar attack. The results showed that these properties appear to be constitutive and induced, as revealed by higher levels of expression of protease inhibitors in the genotype considered resistant even before herbivory and by greater activity and greater expression of lipoxygenase genes after herbivory in this genotype compared to the sensitive. In addition, proteins commonly responsive to biotic and abiotic stresses and not yet characterized for plant-insect interactions have been positively regulated in the resistant IAC 17 genotype. Transmembrane receptors and transcriptional factors, detected as positively regulated after herbivory, may be involved in defense. Likewise, the biosynthesis of defense molecules acting by antibiosis can be modulated by RNA-binding proteins that control chloroplast metabolism.

Keywords: pests, response, proteins, resistance.

1.1 Introduction

Soybean is a crop of great economic and social importance worldwide thanks to its high nutritional value and is used for human food and feed production. In Brazil, the importance of soy is due to the country is currently the world's largest producer and exporter of grain (EMBRAPA). *Anticarsia gemmatalis* Hübner, 1818 (Lepidoptera: Noctuidae) (known as soybean caterpillar) is the most common defoliator of soybeans in Brazil, and can lead to 100% plant defoliation. (Hoffmann-Campo *et al*., 2000). The control of crop pests can be done by biological control that consists of inserting predators in the crop that eliminate the pest without causing damage to the crop, a practice that in some situations may affect the environmental balance. However, the most used method today is the use of insecticides, which occurs in a indiscriminated way and causes damage to both the environment and human health (Lopes & Alburquerque, 2018).

Plants have defense mechanisms against insect attack developed by the need for survival during evolution. These mechanisms involve the recognition of pathogen-activated molecules and activation of signal transduction pathways that result in the expression of defense genes (Rojo *et al*., 2003). Several defense mechanisms have been described in the literature. Studies shows that in a first step, plant recognition of the pathogen leads to induction of resistance from the production of volatile compounds after herbivorous attack, which function as cellular signalers (Moraes *et al*., 2005) and also act on plant-plant interaction as they “prepare” neighboring plants for a possible future insect attack (Ton *et al*., 2005). Signal transduction pathways are controlled by the hormones jasmonic acid (JA), salicylic acid (SA), ethylene (ET), abscisic acid (ABA), auxins, gibberellins and brassinosteroids (Gill *et al*., 2010; Shivaji, 2010). They can act individually, synergistically or antagonistically, depending on the type of attack, which provides the plant with regulation and prompt adaptation to environmental stimulus (Verhage *et al*., 2010). The defense pathways also produce secondary metabolites in response to herbivory, such as flavonoids and isoflavones that protect the plant from oxidative stress caused by pathogens, pests and abiotic factors. (Dakora & Phillips, 1996).

In search of higher yields in soy production, genetic improvement processes have been used to develop more productive crops, with tolerance to the main diseases that attack crops. The genetic improvement of soy is generally a multi-stage procedure (Almeida & Kiihl, 1998). First, segregating populations are developed that meet the objectives of the breeding program. Then, the selection process is carried out for several generations until a certain degree of genetic homozygosity is obtained. Finally, the most

advanced populations are selected to evaluate productivity and production stability in various environments, in order to identify the adaptation and stability of production in the interaction of the genotype with the environment (Almeida *et al*., 1999). In Brazil, there are some genetic improvement programs that work in the production of soybean genotypes resistant to pests. Laboratory studies have demonstrated the antibiosis and antixenosis activities of certain soybean genotypes against some insects. For example, the genotypes "L 1-1-01", "IAC 23" and "IAC 74-2832" are resistant to *Piezodorus guildinii* (green bug); the "IAC 19" and "Conquista" genotypes are resistant to *Bemisia tabaci* (whitefly) and the "IAC 17" genotype is resistant to *Euschistus heros* (brown bug) and *Bemisia tabaci* (Souza *et al*., 2015). A varying degree of resistance to sucking and defoliating insects has also been reported by Lourenção *et al*. (1989) in the strains PI 274453, IAC73-2218, IAC78-2318, IAC80-596-2 and IAC80-4228. Resistance to *Anticarsia gemmatalis* has been shown in some soybean genotypes, such as "IAC 17" and "IAC 24" (Fugi *et al*., 2005; Gómez *et al*., 2018; Gómez *et al*., 2020 under publishing). However, studies relating the profile of soybean gene expression and the defense mechanisms against herbivory by *A. gemmatalis* are still scarce (Zavala *et al*., 2001; Matos, *et al.*, 2002; Fugi *et al.*, 2005).

The attack of insects qualitatively and quantitatively alters the protein profile of the plant. In a recent study, the protein profile of soybean genotypes susceptible and resistant to *Lamprosema indicate* was analyzed. After infestation with the caterpillar, 31 differentially abundant proteins (DEPs) were identified in the resistant genotype. The bioinformatic analysis results showed that most of the DEPs were associated with ribosome, linoleic acid metabolism, flavonoid biosynthesis, phenylpropanoid biosynthesis, peroxisome, stilbenoid, diarylheptanoid and gingerol biosynthesis, glutathione metabolism, plant hormone signal transduction, and flavone and flavonol biosynthesis, as well other resistance related metabolic pathways. (Zeng *et al*., 2017). Fan and contributors also analyzed changes in the soy gene expression profiles after infestation by cotton worm herbivory (*Prodenia litura*, Fabricius, 1775) and identified, eleven up-regulated genes, mainly involved in physiological processes, including reative oxygen removal, defense signal transduction, and metabolism regulation (Fan *et al*., 2012). However, few studys have been performed for soybean under interaction with *A. gemmatalis* despite some works have been proposed a resistance mechanism (Zavala *et al*., 2001; Paixão *et al*., 2016; Gómez *et al*., 2018; Gómez et al., 2020).

Defensive proteins against pathogens includes some structural proteins, secondary metabolism enzymes and pathogenesis-related proteins (PR proteins), responsible for the largest quantitative changes in soluble protein contents during defense responses (Stintizi *et al*., 1993). Lipoxygenases form a group of fundamental defense enzymes for various types of stress. Lipoxygenase pathways are involved in the formation of volatile compounds and in the production of jasmonic acid (JA) as one of its intermediates. Compounds derived from LOX activity are precursors of jasmonic acid, which would in turn activate transcription of genes encoding for protease inhibitors (PIs) (Farmer & Ryan, 1992). Protease inhibitors most commonly present in soybean are Kunitz and Bowman-Birk types trypsin inhibitors (Liener & Kakade, 1980). PIs are expressed constitutively or induced by wounds (Gatehouse, 2011). PIs play an important role in defending against pathogens because they inhibit the digestive enzymes of insects causing nutritional stress and consequent developmental delay (Broadway & Duffey, 1986; Gatehouse, 2011). Plant defense proteins also include some transcription factors, which are essential for signal transduction because they activate the transcription of defense genes in response to hormones such as AS and ABA. (Liu & Lam, 1994; Zhang *et al*., 2003; Thurow *et al*., 2005).

Recently, was observed that leaf extracts from a resistant soybean reduces the survival and digestibility of *Anticarsia gemmatalis* caterpillars, in studyperformed on the IAC 17 (resistant) and UFV 105 AP (susceptible) genotypes (Gómez *et al*., 2018; Gómez *et al*., 2020). Metabolic analyzes by LC / MS showed also highly distinct genotypes with a greater amount of glycoconjugate flavonoids in the resistant genotype, suggesting characteristics of constitutive resistance. The integration of metabolic data, gene expression and regulatory cascades is important to reconstruct the defense pathways to better elucidate the mechanisms of resistance detected.

Thus, this study evaluated the proteomic profiles and the expression and activity of some key genes between soybean plants contrasting for resistance to infestation by *Anticarsia gemmatalis.* The biochemicals and molecular mechanisms of defense against the herbivory were proposed which are mediated by antibiosis. Some genes and cascades could be under control of the transmembrane receptors and transcriptional factors were identified as potential genes controlling the resistance genes. In addition, proteins not yet described in the literature may be part of soybean's resistance mechanisms against the herbivore.

1.2 Materials and methods

1.2.1 Soybean genotypes, plant growth and treatments

The cultivar UFV TN 105 AP, named “105 AP”, sensitive to *A. gemmatalis*, was supplied by the Federal University of Viçosa, Minas Gerais (Brazil), originated from the backcrossing of cultivars BARC-8 (Nunes, 2011, Moraes *et al*., 2006) and Monarch to eliminate the gene coding for some lipoxygenases families, presents susceptibility to soybean caterpillar and late growth pattern (Vasconcelos, 2010). The IAC 17 cultivar is descendant from crossings of D 72-9601-1 x ‘IAC 8’ by the Agronomic Institute of Campinas, São Paulo, (Brazil), is considered as resistant genotype that presents early cycle pattern (Matos, *et al.*, 2002; Fugi *et al.*, 2005; Paixão *et al.*, 2016).

The soybean plants were grown and kept in a greenhouse at temperatures between 22.0 and 35.0°C until reaching stages between V4 and V6 (Fehr & Caveness, 1977). For the experiments, 3 pools of 14 plants were submitted to defoliation by neonate *A. gemmatalis* caterpillars for 90h. Thus, the biochemical analyses were performed using 3 distinct pools, resulting in 3 biological replicates. These procedures were performed on both plant cultivars.

After the infestation period, the injured leaves and healthy leaves not exposed to infestation of each genotype were collected and stored at -80 °C. The treatments were designated as: 105 AP-NI for leaf extract from the genotype UFV 105 AP without caterpillar infestation; 105 AP-I for extract from the genotype UFV 105 AP exposed to infestation for 90 h; IAC 17-NI for extract from the genotype IAC 17 without infestation; IAC 17-I for extract from the genotype IAC 17 exposed to infestation for 90 h.

1.2.3 Protein extraction and fractionation with PEG 15%

The soybean leaf proteins were extracted following the method described by Aryal et al. (2012), with some modifications (Lima *et al*., 2019). The leaves were macerated in liquid nitrogen until powder was obtained. 3 g of powder were weighed for each aliquot and resuspended in 10 ml extraction buffer [Tris-HCl 0.5M (pH 8.0), 20mM MgCl2, 2% Triton X-100 and 2% β-mercaptoethanol, protease inhibitor cocktail 0.2% (P-9599, Sigma Aldrich), cocktail phosphatase inhibitor (P-0044, Sigma Aldrich) 1%)]. The samples were agitated in vortex for 30s, then the solution was filtered and the filtrate collected. Subsequently, 10 ml of 30% polyethylene glycol PEG 4000-containing extraction buffer was added to the filtrate. This solution was agitated in vortex for 30s, kept on ice for 30 min and centrifuged 3200 x g for 20 min at 4°C. Following, the supernatant obtained containing the low-density proteins was collected and precipitated

with one volume of 20% trichloroacetic acid in acetone for 16 hs at -20°C. The solution was centrifuged again at 6000 xg for 30 min at 4°C and the supernatant was discarded. The pellet was resuspended and twice washed with 80% acetone on ice and centrifuged at 6000 x g for 10 min at 4°C. Finally, the pellet was washed with 70% ethanol and let to dry at room temperature. The protein extract obtained was solubilized in solubilization buffer composed of 7M urea, 2M thiourea and 2% CHAPS. The solution was solubilized using the UltraSonic Processor (Model GE 50) for cycles of 5 to 10 s until complete pellet solubilization and the samples were stored at -80°C. The protein concentration in samples was determined using bovine serum albumin (BSA) as standard and the analyses were performed at 595 nm (Bradford, 1976).

1.2.4 Protein separation by two-dimensional electrophoresis (2DE)

Isoelectric focusing (IEF) was performed on 24 cm IPG strip pH 3-10 (GE Healthcare). The strips were previously rehydrated for 14-20 h with 450 µl rehydration buffer (7M urea, 2M thiourea, 2% CHAPS, bromophenol blue 0.002%, 2% IPG-buffer, 0.2% DTT) with the addition of DeStreak 50% reagent (GE Healthcare). The amount of protein in each sample was 1.3 mg. Proteins were separated into the first dimension using the IPGphor3 focusing system (GE Healthcare).

After focusing, were added to the strips the equilibration buffer (6 M urea, 50 mM Tris – HCl (pH 8.8), 30% glycerol and 4% SDS) initially containing 1% (w/v) DTT for 15 min followed by 2.5% (w/v) iodoacetamide for 15 min at room temperature. The IPG strips were placed on 12% polyacrylamide gel and sealed with 0.5% agarose. The gels were electrophoresed in the EthanDalt Six system (GE Healthcare) at 20 mA / gel for 10 h. At the end of the electrophoresis the gels were fixed for 16 hours in 40% ethanol and 10% acetic acid solution.

To visualize the protein spots, the gels were submerged in Coomassie brilliant blue solution for 24-48h, and were washed with ultrapure water to eliminate excess coloring solution. The gels were stored in 5% acetic acid solution and were scanned with ImageScanner III (GE Healthcare), with image calibration by Labscan software (GE Healthcare). The protein profile of both infested and uninfested soybean genotypes were compared by ImageMaster2D Platinum 7 software (GE Healthcare) to determine protein abundance in the spots. Spots that had ratio values above 1.0 and ANOVA with p-values below 0.05 were considered as differentially abundant. Three biological replicates were used for each treatment. The differential abundance of proteins in the spots using the criteria above were converted to fold-change and classified as up- or down-regulated.

1.2.5 Gel protein digestion and mass spectrometry for identification

The protein spots of the gels were cut and previously discolored in 50mM ammonia bicarbonate/ 50% methanol solution, followed by dehydration with acetonitrile. Proteins were then reduced with 200mM DTT solution prepared in 100mM ammonium bicarbonate for 30 min at 56 °C and alkylation with 200mM iodoacetamide solution in 100mM ammonium bicarbonate for 30 min at room temperature. The pieces of gels were washed with 100mM ammonium bicarbonate solution, dehydrated with acetonitrile and dried by vacuum centrifugation. For proteolytic digestion, the spots were rehydrated with 20ng/µL trypsin solution for 20h at 37 °C. Digested peptides were extracted for three cycles using the extraction buffer (50% Acetonitrile, 5% formic acid) and dried by vacuum centrifugation.

Peptides were dissolved in 0.1% (v/v) formic acid and analyzed by LC-MS using a nanoUPLC (nanoACQUITY-Waters) system containing a capillary column C18 BEH130 1.7 uM -100 nm × 100 mm, operating at a flow rate of 0.5 uL/min. The eluted peptides were automatically injected into a mass spectrometer ION TRAP (Amazon-Bruker), online using a nanoESI ionization needle. The scanning of the ions from the mass spectrometer was performed at between 300 and 1500 m/z in positive mode and data were acquired for 70 min in each LC-MS/MS analysis. The mass spectrometer was operated in the auto-MSn mode. Data acquisition of the LC-MS instrument was managed by Hystar software (Bruker) and the spectra were processed with the Data Analysis software (Bruker) using the default settings for proteomics.

The spectra were analyzed by PEAKS 7.0 program with a local Client license, connected to a remote server to identify proteins. The parameters used in the program were: a protein list obtained from *Phytozome* (https://phytozome.jgi.doe.gov/pz/portal.html), containing all the proteins described for soybean as database, methionine oxidation as a variable modification of cysteine carbamidomethylation as a fixed modification, one missed cleavage, charge states of 2+, 3+, 4+, trypsin-like cleavage enzyme and mass error of 0.15Da. For protein identification, we considered a false discovery rate (FDR) less than 1.0% along with at least three unique peptides.

After identification, the proteins were grouped by Biological process using the ClueGo 2.5.4 app from Cytoscape 3.6.1 platform (https://cytoscape.org/) with the following parameters: *Arabdopisis thaliana* organism; GO_BiologicalProcess-Custom-GOA_27.03.2019 with “all evidences”; specificity level 3-8 with a minimum three genes/term; kappa score 0.4; statistical options were two-sided hypergeometric test and

pV correction Bonferroni step down and selected ontologies reference set; and GO term grouping with leading group term based on highest significance and kappa score. Finally, these results were confirmed by the platform STRING 11.0 (https://string-db.org/) using the setting organism *Arabdopisis thaliana* with medium confidence (0.4).

1.2.6 RNA extraction, cDNA synthesis and expression analysis by qRT-PCR

The leaves were pulverized with liquid nitrogen and macerated. Total RNA was extracted from control and infested plant leaf tissues using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA quality was examined in agarose gel that was stained with 0,1 μg/mL ethidium bromide (EtBr) and quantified using a Thermo Scientific NanoDrop 2000c. A total of 4 μg of RNA was used for cDNA synthesis with the SuperScript III kit (Invitrogen) following the manufacturer's instructions. Gene expression was evaluated using an ABI 7500 fast thermal cycler (Applied Biosystems, Foster City, CA, USA) and Fast Master SYBR Green Master Mix (Thermo Fisher Scientific). The amplification reactions were performed with the cycling conditions: 15 s at 95 ° C, 40 cycles at 95 ° C for 3 s; 30 s at 60 ° C and final denaturation at 95 ° C for 20 s, followed by a melting curve. RT-qPCR-specific primers were designed using Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), with a melt temperature (T) of 59 to 61 ° C, a length of 18 to 23 bp, an amplifier product size of 120 to 150 bp, and a 40 to 60% GC content. Three biological and three technical repetitions were performed for each gene. Gene expression was quantified using ΔCT to compare the difference between treatments and $2^{-\Delta\Delta CT}$ for genotypes difference. The sequence of the primers used are in the **Figure S1**.

1.2.7 Determination of lipoxygenases activity and protease inhibitors

The leaves were weighed, pulverized with liquid nitrogen and macerated. For the lipoxygenase activity experiments, the powder was homogenized with 50 mM sodium phosphate buffer, pH 6.5, containing 1% polyvinylpolypyrrolidone (PVPP) and 1 mM phenylmethylsulfonyl fluoride (PMSF) in the ratio of 1 g of leaf per 5 mL of buffer. The extract was centrifuged at 17,200 *g* for 30 min at 4 °C and the supernatant was collected. The lipoxygenase activity on linoleic acid was measured by the formation of the conjugated double-bond system of hydroperoxide, detected at 234 nm. The mixture for the reactions consisted of 10 μL of the supernatant (plant extract), 4 μL of 10 mM sodium linoleate and 1000 μL of 50 mM sodium phosphate buffer, pH 6.5. The activity was measured after 2 min of reaction at 234 nm. The speed of the reaction was measured using the molar extinction coefficient of 25.000 M-1 cm^{-1} (Lowenstein, 1981).

For the protease inhibitors experiments, the powder was homogenized with extraction buffer 0.1 M Tris-HCl buffer pH 8.2 containing 20 mM $CaCl_2$ in the ratio of 1 g of leaf per 5 mL of buffer, centrifuged at 17,200 *g* for 30 min at 4 °C. The protease inhibitors were determined by measuring the inhibition of purified trypsin when mixed with the supernatant (plant extract) and using 1.2 mM L-BApNA as substrate. Two controls were used to determinate the total inhibitor activity: the trypsin control consisted of 50 µL of trypsin 4.7 x 10^{-5} M, 300 µL of substrate and 500 µL of extraction buffer, and the substrate control consisted of 50 µL of plant extract, 300 µL of substrate and 500 µL of extraction buffer. The absorbance (410 nm) was measured after 2.5 min. The test tubes with 50 µL of trypsin, 50 µL of plant extract and 450 µL of extraction buffer were incubated at ambient temperature for 5 min, then was added 300 µL of substrate and the absorbance (410 nm) was measured after 30 s. The inhibitor activity was calculated adding the trypsin and substrate controls and subtracting the absorbance from the test tube from this value. The results were converted to mg of trypsin inhibited per gram of protein, according to the equation A x B/(C x 1000 x P), where: A is the absorbance at 410 nm of the test calculated as described; B is the sample dilution; P is the protein concentration of the plant extracts (g.mL-1); and C is the trypsin factor, *i.e.*, the product of the action of 1 µg of active trypsin using L-BApNA as substrate will result in absorbance of 0.019 at 410 nm (Kakade *et al*., 1974). The protein concentration from leaves was determined using bovine serum albumin (BSA) as standard and the analyses were performed at 595 nm (Bradford, 1976).

Three biological and three technical repetitions were performed for each analysis. Data from lipoxygenases and protease inhibitors activity were subjected to *t* Student's test at 5 % significance. The software used was GraphPad Prism, version 6.

1.3 Results

1.3.1 Responsive proteins for both genotypes after infestation with *Anticarsia gemmatalis*

The total proteins extracted from the IAC 17 and 105 AP genotypes was analyzed in both caterpillar infestation control (0h) and 90h treatments, classified according to the increase or decrease of abundance (up- and down-regulated, respectively). The ClueGO platform was used to identify biological processes (according to Gene Ontology) in which they participate. For the 105 AP genotype, 93 total proteins were identified, involved in three main cellular processes: carbohydrate catabolic process, photosynthesis and defense

response to fungus, incompatible interaction. In the IAC 17 genotype, 136 proteins, involved in carbon fixation processes, response to high light intensity, 'de novo' protein folding, serine family amino acid metabolic process, response to cadmium ion, cofactor catabolic process, cell redox homeostasis, regulation of cell death and response to cytokinin and some proteins had their biological processes identified by the platform as "unknown" (**Fig. S2**).

As the genotypes have different genetic backgrounds, contrasts were performed for each cultivar in infested (I) and non-infested (NI) conditions. Afterwards, the responsive protein profiles were analyzed to characterize the molecular response. Twenty and two proteins with differential abundance were observed for both genotypes (**Fig. 1**). The biological process classification by Gene Ontology grouped proteins involved in photosynthesis-light reaction and photosynthetic electron transport chain (biological process) (**Fig. S2**). The biological process was confirmed by the STRING platform database and were grouped into eight major biological processes shown in **Fig 1**. From 22 proteins proteins with differential abundance, 17 spots showed similar behavior among genotypes regarding increase or decrease of abundance in the presence of caterpillar, but some proteins were found in more than one spot, with different isoelectric points, indicating the presence of isoforms or post-translational modifications, with different abundance modulation in the same genotype.

Among the responsive proteins (**Fig. 1**) some isoforms showed abundance patterns highly distinct between the genotypes. For example, to the photosystem II subunit P-1 proteins, (Glyma.02G282500.1.p and Glyma.08G304200.1.p) which are part of the photosynthesis protein complexes, decreased in 105 AP and IAC 17, respectively. While for the photosystem II subunit O-2 (Glyma.16G143600.1.p, Glyma.02G061100.1.p), some spots were also down-regulated in both sensitive and resistant genotype, but also showed others two up-regulated isoforms in IAC 17; photosystem II subunit O-2 Glyma.01G180800.1.p was identified in IAC 17 with two up-regulated and one down isoforms, and cyclophilin 38 (Glyma.13G169500.1.p) with two isoforms decreased in 105 AP, while in IAC 17, one isoform decreased and the other increased, indicating the presence of post-translational modifications.

PDI-like proteins 1-1 Glyma.04G247900.3.p showed up-regulation in 105 AP and down-regulation in IAC 17; PDI-like 1-2 Glyma.04G247900.1.p was present only in IAC 17 up-regulated; and Glyma.06G114800.1.p presented up-regulated in IAC 17 but two isoforms in 105 AP, one up and one down. The PDI-like proteins, which was most

abundant in the resistant than susceptible genotype, could be functions as chaperone (Wilkinson & Gilbert, 2005), isomerase (Meng *et al*., 2015), and also regulates programmed cell death time (PCD) of endothelial cells, monitoring and inhibiting cysteine proteases during traffic to vacuoles (Ondzighi *et al*., 2008).

Two isoforms coding for lipoxygenases 1 (Glyma.15G026500.1.p and Glyma.07G006900.1.p) were found up-regulated in IAC 17 and one (Glyma.07G007000.2.p) slightly increased in 105 AP. Lipoxygenases activity is commonly related to JA response because is a key step in the biosynthesis of this phytohormone. In IAC 17, Kunitz family trypsin and protease inhibitor protein (Glyma.08G341400.1.p) was found as two isoforms highly abundant relative to control, and one (Glyma.09G155500.1.p) with slightly decreased abundance; in 105 AP, one Kunitz inhibitor (Glyma.12G234800.1.p) was identified with highly reduced abundance in the presence of the caterpillar.

Proteins identified in both treatments also include members of the Bet-v-1 family, which generally function through binding ligands such as cytokines, brassinolids and secondary metabolites and trigger downstream signal transduction, being divided into 11 subfamilies including MLP, PR-10 (Pathogenesis related 10), cytokinin receptors and cyclase-like polyketide (Radauer *et al*., 2008). MLP-like protein 423 (Glyma.07G243900.1.p) was identified highly abundant IAC 17 in the presence of the caterpillar, as Glyma.07G243500.2.p too, which maintained abundance levels at 105 AP between control and treatment.

Some proteins involved in the photosynthetic metabolism were responsive for caterpillar's infestation (**Figure 1**). Interesting, were observed also isoforms of chloroplast RNA-binding protein 31B (Glyma.05G043200.1.p) up and down-regulated in IAC 17 and up-regulated in 105 AP. Chloroplast RNA-binding proteins (RBPs) are central regulatory factors controlling posttranscriptional RNA metabolism during plant growth, development, and stress responses. Also regulatory factor 31-kDa RNA binding protein (Glyma.06G175400.1.p) was found down-regulated in both genotypes.

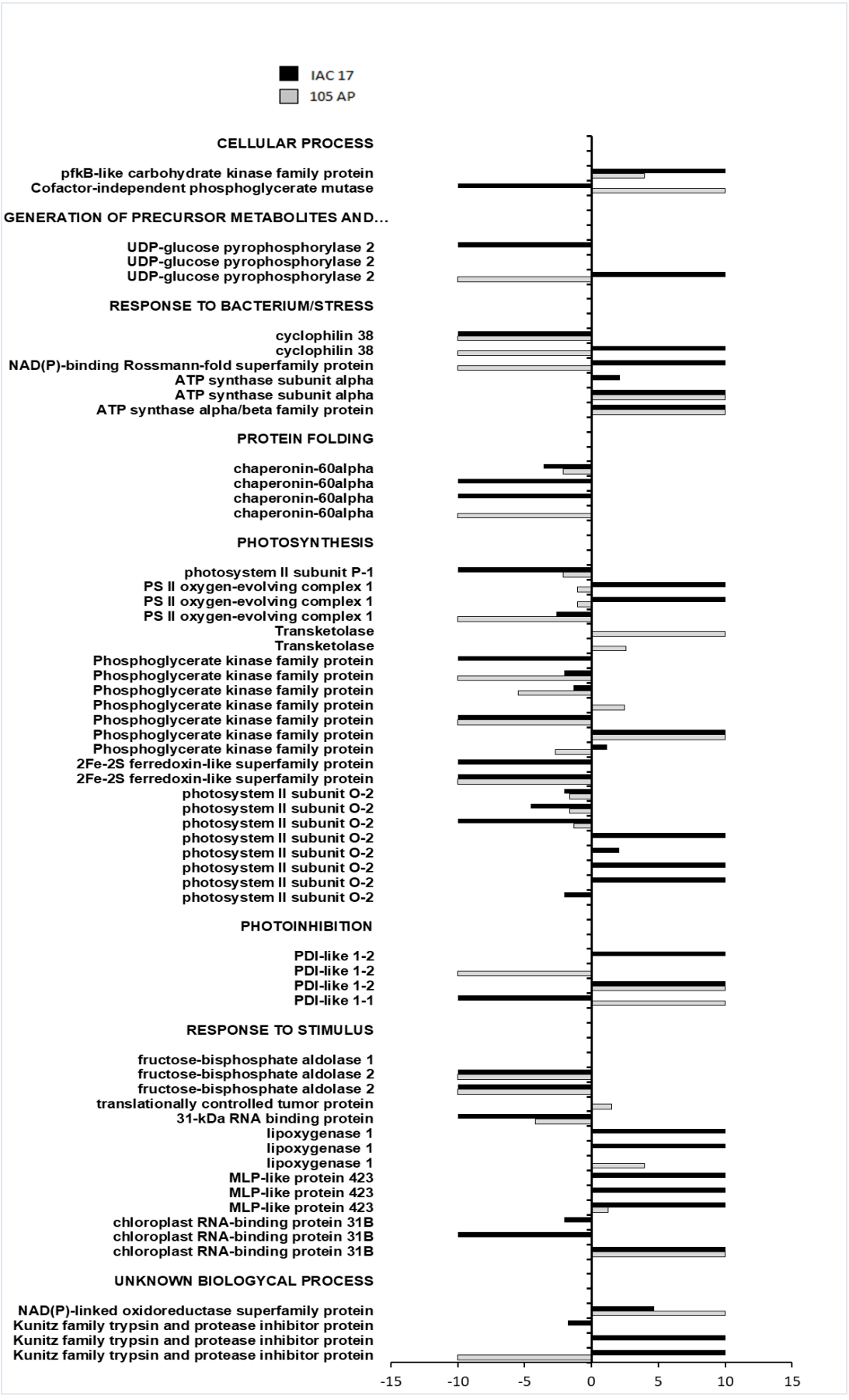

Fig. 1: Protein profile of soybean leaves from both 105 AP and IAC 17 genotypes in response to infestation by *Anticarsia gemmatalis* carterpillars. Values for protein spots when present only in the infested (I) treatments are shown as 10.00 and those present only in the non-infested (NI) treatments are shown as -10.00; abundant isoforms represented in only one of the genotypes (IAC 17 or 105 AP) are shown as 0 for the genotype where it was absent.

1.3.2 Responsive proteins specifically abundant for each genotype

To compare the differential protein abundance between IAC 17 and 105 AP, genotype specific proteins were separately analyzed by Cytoscape. While in the susceptible genotype most of the abundant proteins are involved in metabolic process hexose, the resistant genotype showed mostly proteins that participate in the carbon fixation and posttranslational protein folding processes, as shown in the **Fig. S2**. Then the proteins and their biological processes confirmed by STRING were listed in the **Figures 2 and 3**.

Of the all identified proteins, 53 spots were responsive only in the susceptible genotype 105 AP **(Fig. 2)**, including those of which isoforms are found. Some stimulus response proteins were found up-regulated in the 105 AP after treatment with the caterpillar, including beta-1,3-glucanase 1 (Glyma.03G132700.1.p), class-10 pathogenesis-related protein 1. (Glyma.09G040500.1.p), granulin repeat cysteine protease family protein (Glyma.17G239000.1.p) two isoforms, one up and one down-regulated, voltage dependent anion channel 1 (Glyma.08G296700.1.p) and polyketide cyclase / dehydrase and lipid transport superfamily protein (Glyma.01G009200.1.p). The beta-1,3-glucanase 1 enzyme belongs to the PR-2 group of PR (pathogen-related) proteins, which are induced by pathogen infection, contribute to destabilize its cell wall and are involved in the release of immune elicitors that participate in defense response (Leubner-Metzger & Meins, 1999). But most of the proteins classified as stimulus response proteins had their abundance reduced after treatment, such as as fibrillin (Glyma.09G008100.1.p, Glyma.15G112500.1.p) and chloroplast heat shock protein 70-2 (Glyma.16G002500) proteins. 1.p). Pathogen-specific regulatory patterns suggest that fibrillins are involved in resistance to plant diseases and/or are modulated by pathogens to promote disease.

Also mostly of the classified proteins for photosynthesis processes in the susceptible genotype showed reduced abundance, including photosystem II reaction center PsbP family protein (Glyma.03G230300.1.p), carbonic anhydrase 1 and glyceraldehyde 3-phosphate dehydrogenase A subunit 2 (Glyma.16G044900.1.p). Of the group of RBPs involved in metabolic processes found in both genotypes, the chloroplast RNA binding (Glyma.03G230000.1.p) had its abundance highly reduced in the sensitive genotype.

Contrary to what was observed in the sensitive genotype, most of the 76 proteins responsive only in the resistant genotype IAC 17 had their abundance increased after the treatment **(Fig. 3)**. Some of the up-regulated proteins in the presence of the caterpillar are disease resistance-responsive (dirigent-like protein) family (Glyma.11G150300.1.p), pathogen defense-related proteins PF00407 - pathogenesis-related protein Bet v I family (Bet_v_1) (Glyma.17G030300.1.p), NAD(P)-binding Rossmann-fold superfamily protein (Glyma.11G164700.2.p) and the transcription factors SCARECROW-like 14 (Glyma.11G138600.1.p) and AP2 / B3-like transcriptional factor family protein (NGA3) (Glyma.16G017100.1.p). Many of the stimulus response proteins also had their abundance increased, as transmembrane kinase 1 (Glyma.14G212100.1.p) and chloroplast RNA-binding protein 29 (Glyma.10G058500.1.p). In contrast to the 105 AP genotype, in the resistant genotype the heat shock protein 70 (Glyma.11G140500.1.p) and mitochondrial HSP 70 2 (Glyma.13G254900.1.p) were positively modulated after caterpillar exposure. HSPs play a crucial role in protecting plants against biotic and abiotic stresses by reestablishing cellular homeostasis and interacting with a wide range of co-chaperones and proteases (Sun *et al*., 2002; Wang *et al*., 2004).

Interesting, some of proteins up-regulated in IAC 17 are involved in cascades of signal perception and gene expression regulation, such as the Cysteine-rich RLK (RECEPTOR-like protein kinase) 3 (Glyma.11G205400.1.p). Receptor-like kinases (RLKs) are conserved upstream signaling molecules that regulate several biological processes, including plant development and stress adaptation (Osakabe, 2013).

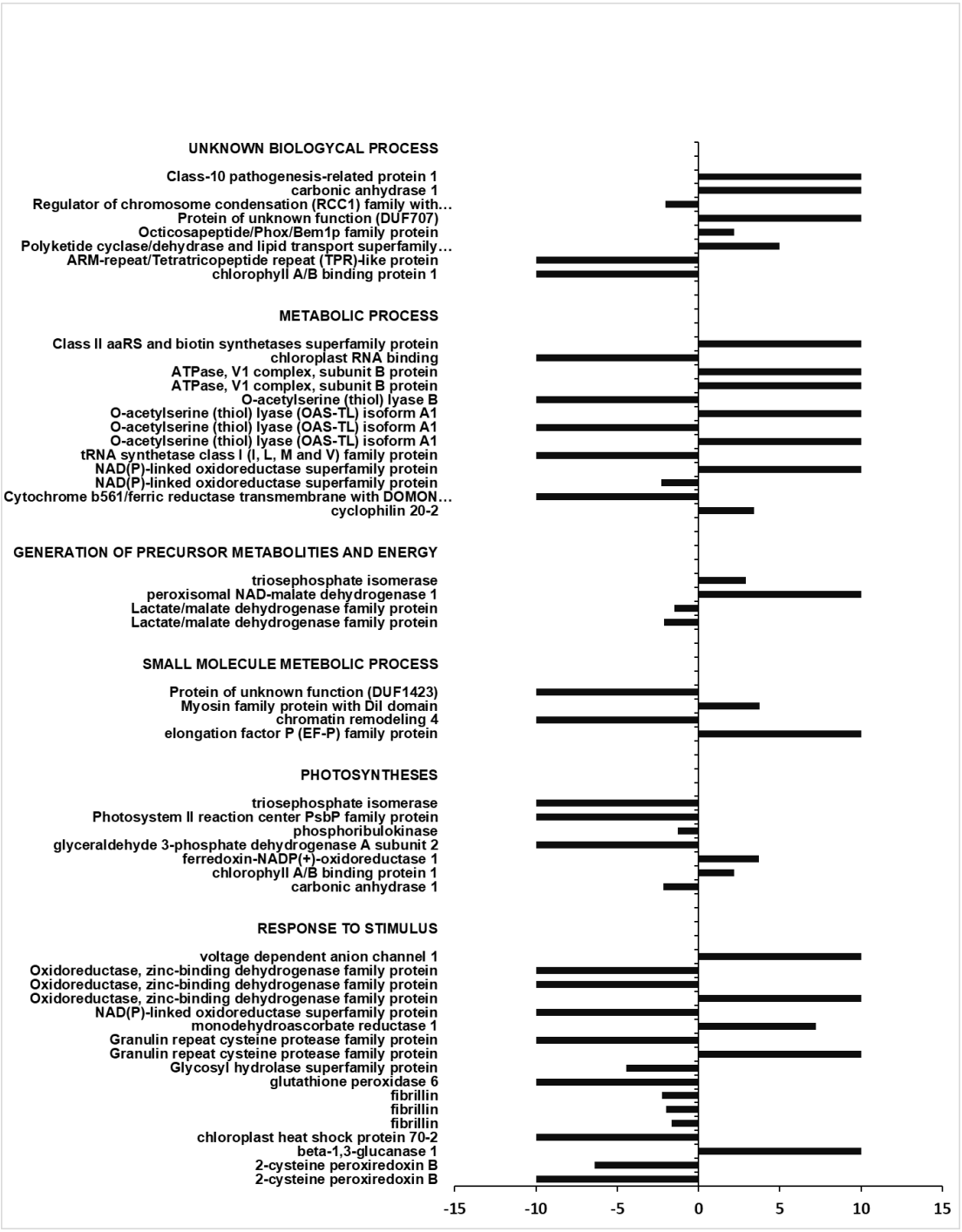

Figure 2: Protein profile of soybean leaves identified specifically from 105 AP genotype in response to infestation by *Anticarsia gemmatalis* carterpillars. Values for protein spots when present only in the infested (I) and non-infested (NI) treatments are shown as 10.00 or -10.00, respectively.

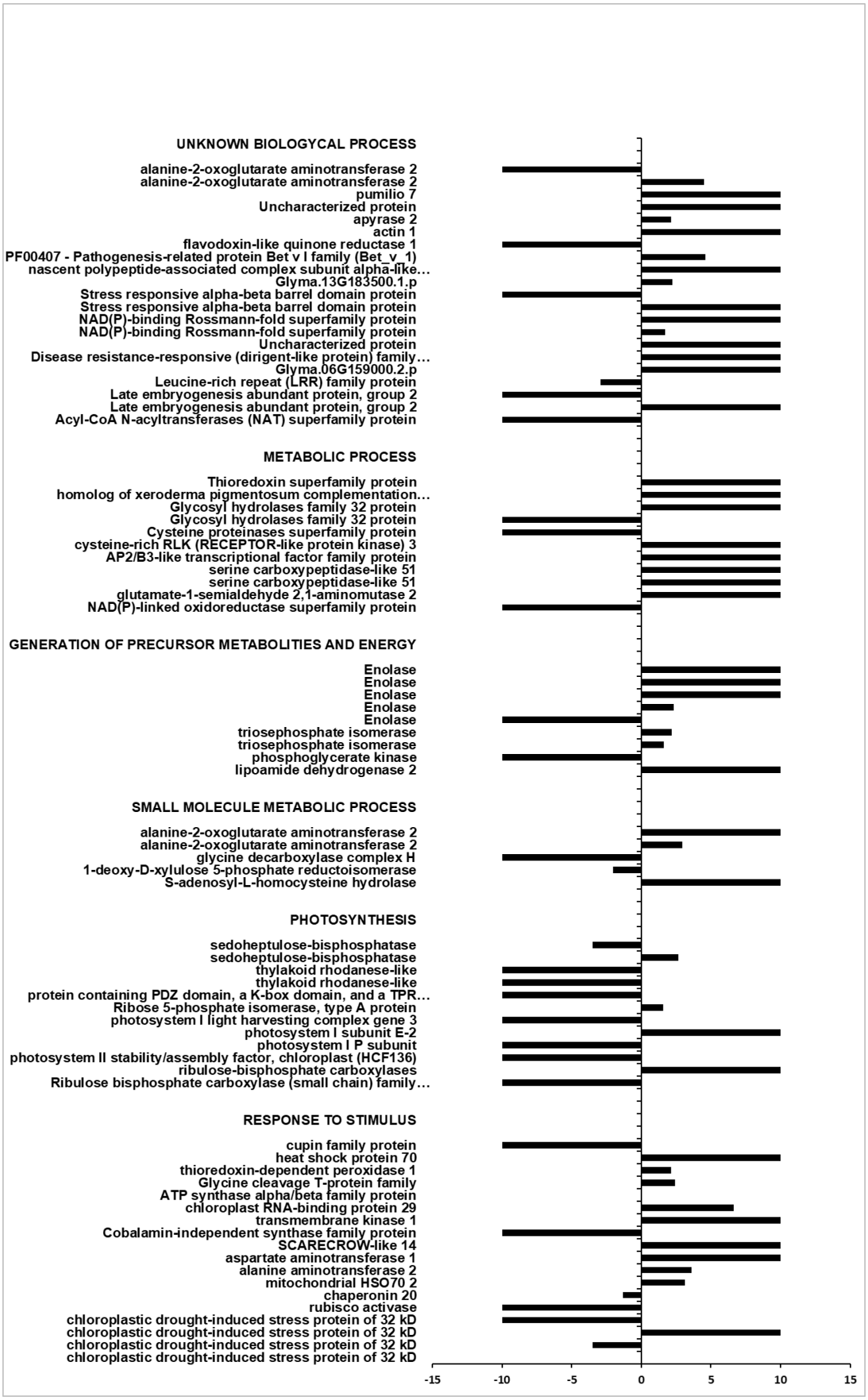

Figure 3: Protein profile of soybean leaves identified specifically from IAC 17 genotype in response to infestation by *Anticarsia gemmatalis* carterpillars. Values for protein spots when present only in the infested (I) and non-infested (NI) treatments are shown as 10.00 or -10.00, respectively.

1.3.3 Gene expression by RT-PCR

The expression of Lipoxygenase and Bowman-Birk serine protease inhibitor genes that are comment involved in the caterpillar's response was evaluated. The ΔCT value was calculated to compare gene expression between treatments and the $2^{-\Delta\Delta CT}$ value was calculated to analyze de relative quantification between the genotypes after treatments (**Fig. 4**). The mRNA expression levels for Lipoxygenase were higher in the sensitive genotype UFV 105 (**Fig. 4B**) lower CT values), however the magnitude of increased in response to the caterpillar infestation were higher for IAC 17 (**Fig. 4A**), indicating that in the absence of the insect stimulus the sensitive genotype shown higher expression. Otherwise, for Bowman-Birk protease inhibitor gene showed highest up regulation of the expression after infestation (**Fig. 4C** and **4D**), however the higher levels were detected for the UFV 105 sensitive genotype. When comparing the mRNA levels before infestation, the resistant IAC 17 genotype showed higher relative abundance (**Fig. 4D**), thus the expression was higher even in absence of the caterpillar stimulus.

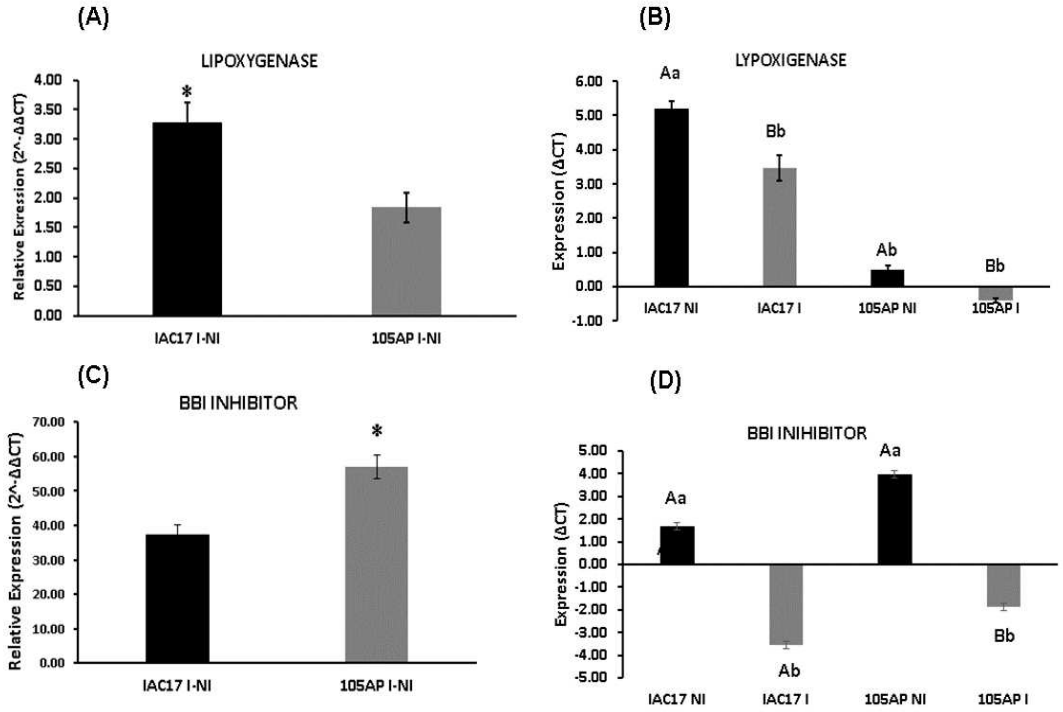

Figure 4: Gene expression analysis of the Lipoxygenase and Bowman-Birk protease inhibitor genes by qRT-PCR before and after infestation by *Anticarsia gemmatalis*. In **(A)** and **(C)** Expression values were obtained using the method of (ΔCT) and the endogenous control. In **(B)** and **(D)** Relative Expression using the method $2^{-\Delta\Delta CT}$ fold change. Bars (mean ± SE; n = 3) with the same lowercase letters indicate no significant difference between control and infested treatments (Student's test: $P < 0.05$).

1.3.4 Leaf lipoxygenase and protease inhibitor activity before and after *Anticarsia gemmatalis* infestation

In addition of the gene expression levels, the lipoxygenase and protease inhibitor activity were also evaluated (**Fig. 5**). Likewise the genes expression analyzes, the

lipoxygenase activities were increased after caterpillar treatment in both genotypes, however was slightly higher in resistant IAC 17 (**Fig. 5A**). Thus, indicating that some regulatory process could be active to allowed a higher protein activity in the IAC 17, even with a lower transcription of the lipoxygenase gene (**Fig. 4B**).

For protease inhibitory activity, likewise the gene expression analysis, the levels were higher in the resistant genotype IAC 17, especially in the presence of the caterpillar infestation. But for the sensitive genotype the inhibitory activity did not accompany the increase in gene expression of inhibitors after treatments. Thus, for the protease inhibitors the regulatory mechanism increases the activities or others genes coding for protease inhibitors were also expressed in IAC 17 or the regulatory mechanism decreases inhibitory activity due to post-transcriptional changes in the sensitive genotype.

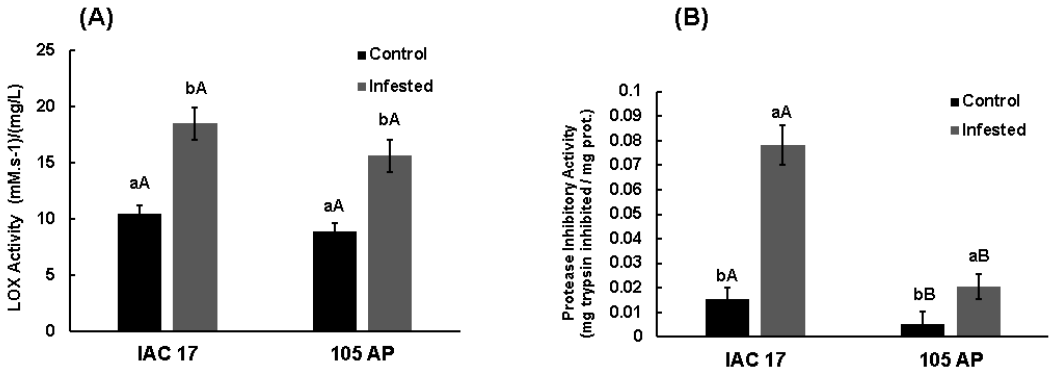

Figure 5: Leaf lipoxygenase and protease inhibitor activity before and after *Anticarsia gemmatalis* infestation. Bars (mean ± SE; n = 3) with the same lowercase letters indicate no significant difference between control and infested treatments and those followed by the same capital letters indicate no significant difference among genotypes within the same treatment (Studant's test: $P < 0.05$).

1.4 Discussion

Insect attach has caused losses in agricultural production (Oerke, 2006) especially in Brazil due the existence of an extensive soybean planting areas. Thus, the use of insecticides for pest control has been necessary (Bueno, *et al*., 2013). The productivity growth of this crop also has been limited by biotic stresses such as insect pests. *Anticarsia gemmatalis* Hubner 1818 (Lepidoptera: Noctuidae) is important soybean pests (Hoffmann-Campo *et al.*, 2000; da Silva *et al.*, 2012) which led to the development of resistant varieties by genetic breeding (Lourenção *et al.*, 1989; Miranda *et al.*, 2003a; Miranda, *et al.*, 2003b; Oliveira, et al., 2014), including IAC 100 (Rossetto *et al.*, 1987), IAC 17 and IAC 19 (Valle & Lourenção, 2002), IAC 23 and IAC 24 (Miranda *et al.*, 2003a; Miranda *et al.*, 2003b). The cultivars IAC 17 present higher relative levels of

resistance to *A. gemmatalis.* Thus, the molecular mechanisms of the resistance in this genotype have been evaluated by dysregulated metabolite abundances (da Silva *et al*., 2007; Gómez *et al*., 2018; Gómez *et al*., 2020 under publishing) and in the present study at the level of the gene expression.

The plant gene expression under of the biotic stress by insect have been studied and characterized, describing list of the responsive proteins (Jones *et al.*, 2006; Fan *et al*., 2012; Jing *et al*., 2015; Van Holle *et al.*, 2016). However, we focused on the expressions that were distinct between the genotypes which could be related with the insect pest response and to the mechanisms of resistance proposed for the interaction of soybean with *A. gemmatalis* (Gómez et al., 2018; Gómez et al., 2020). The global analysis of the protein abundance of both genotypes indicated that some molecular and physiological processes were commons, however others were specifically for IAC 17. In fact, the response could be specific for different insect and caterpillars. Chang and Harman (2017) identified significant single nucleotide (SNP) polymorphic markers in soybean for important insect pest, including for *A. gemmatalis,* and showed that the mechanism of tolerance can involve specific genes and thus could also be related to distinct mechanisms. This study highlighted several leucine-rich repeat-containing genes that have been related with signal transduction to response to pathogens (Tang *et al*., 2017). Interesting, the abundance of a Cysteine-rich RLK protein 3 and of a transmembrane kinase 1, that also encoded for receptor-like protein kinase, were up-regulated specifically for the resistant genotype IAC 17. Thus, alteration on the cascades of signal perception and transduction could be the most important genetic determinants for a resistance to insect pest. RLKs are conserved upstream signaling molecules that regulate a number of biological processes, including plant development and stress adaptation (Osakabe *et al*., 2013; Quezada *et al*., 2019). A scarecrow like-3 protein was up-regulated in the resistant genotype IAC 17 and can act as transcription factors and are member of GRAS gene family (Cenci & Rouard 2017). The GRAS gene also participates in phytohormone signaling pathways such as gibberellin acid (GA), jasmonic acid (JA) and brassinosteroid (BR) signal transduction. AtSCL3 (scarecrow like 3) is a positive regulator of the GA response. AtRGL3 (RGA-like 3) regulates jasmonic acid (JA) signaling, thus, scarecrow like 3 presence could be important for phytohormonal balance in the resistant IAC 17. Also AP2/B3-like transcriptional factor was found up-regulated in IAC 17 after caterpillar infestation.

Likewise, some isoforms for MPL proteins were differently abundant under caterpillar infection. MLP proteins belong to the Bet v 1 family and also are involved in

the phytohormonal cascades, function through binding ligands, such as cytokinins, brassinolides and secondary metabolites and trigger downstream signal transduction (Wang *et al*., 2016). Physiological and biochemical analyzes performed by WANG *et al*. 2016 indicated that MLP43 was involved in stress responses by ABA and drought in Arabidopsis through modulation of the primary metabolic profile and gene expression. Experiments with induction of MLP in Arabidopsis also showed that the total flavonoid content in plants was double in treatment compared to control, suggesting that MLP overexpression is involved in the change in plant flavonoid concentration, secondary metabolites (Chen & Dai, 2010) and recently, it has been suggested that abundance of MLP-like protein may increase Coumestrol (CMS) isoflavone accumulation in response to stress conditions (HA *et al*., 2019). Levels of specific flavonoids were detected in highest concentration in the in the resistant IAC 17 genotype after treatment with the caterpillar, thus MPL-like protein could be involved in the regulation of production de secondary metabolites against caterpillar infestation.

Recent evidence demonstrates a realignment and an up-regulation of primary metabolic pathways following both pathogen and herbivore attack (and it has been proposed that such realignment is necessary to drive the synthesis of secondary metabolites, such as phytohormones, phenolic compound and ROS. Insect herbivory also has a profound impact on photosynthesis both through physiological mechanisms and shifts in the transcriptional profile of genes associated with photosynthetic reactions (Kerchev et al., 2011). However, in the majority of cases defense responses are associated with a reduction in photosynthesis, thus the chloroplast metabolism is affected. The abundance of some RNA binding proteins (RBPs) were modified under insect infestation and could be important for post-transcriptional regulation of chloroplast genes (Lee and Kang 2016). Furthermore, many proteins coding for photosynthetic apparatus were down-regulated in both genotypes, including isoforms for he Photosystem II subunit P-1 and Photosystem II subunit O-2 proteins. The Cyclophilin 38, a member of the immunophilins family, is a cellular receptor with various functions such as foldases, chaperones and scaffolding facilitators (Buchanan, 2005), and participate in the synthesis and maintenance of photosystems I and II. This negative modulation of those proteins may reflect the damage to the photosynthesis process caused by the attack of caterpillar, mostly in the susceptible genotype. Same modulation in both genotypes was also found to Fructose biphosphate aldolase (Glyma.11G111400.1.p), a key enzyme in the glycolysis and gluconeogenesis pathways (Gross, *et al*., 1999). Fibrillins are regulated by elicitor molecules and the plant's defense signaling molecule system, a short polypeptide that

stimulates local and systemic defense responses to herbivorous insects (Pearce *et al*., 1991). In potatoes, fibrillin protein C40.4 is specifically associated with photopolymerization complex II (PSII) (which has been down-regulated at 105 AP I), with a presumed role in modulating photosynthetic efficiency (Monte & Ludevid, 1999). Posttranslational control of fibrillin accumulation by abscisic acid (ABA) (also down-regulated at 105 AP I) has been indicated by studies with ABA-deficient tomatoes that failed to accumulate fibrillin despite normal transcription levels (Gillet *et al*., 1998). In Arabidopsis, ABA treatment stimulates the accumulation of FBN1a protein, which in turn protects photosystem II (PSII) from photoinhibition. The regulation of fibrillin gene expression by ABA is consistent with the role of fibrillins in plant stress tolerance because ABA is involved in plant acclimatization to numerous stresses (Yang *et al*., 2006). Also down-regulated after treatment, chloroplast heat shock protein 70 is a class of highly conserved chaperone proteins in all organisms, which facilitates folding and assembly of other proteins, thus preventing folding or protein aggregation, and plays a role in translocating proteins across membranes while maintaining proteins to an extent and conformation competent for translocation (Brodsky, 1996). Schroda *et al*., (1999) presented direct evidence that the stromal HSP70B protein is involved in resistance to photoinhibition: overexpression of HSP70B reduces photoinactivation of PSII and enhancements recovery, whereas under expression causes the opposite effect. They propose that HSP70B acts by both preventing the destruction of inactivated reaction centers and promoting the synthesis of new centers.

Lipoxygenases were expected to be highly expressed in the treatments, since they are important enzymes in defense against pathogens as they are required in the synthesis of jasmone acid (JA) (Vick & Zimmerman, 1987), participate in ABA synthesis (Creelman *et al*., 1992) and cell membrane degradation during hypersensitive resistance response (Croft *et al*., 1990). Indeed, our experiments showed the increase of lipoxygenases after infestation in resistant and susceptible genotypes, but this increase was more pronounced in resistant IAC 17. Changes were observed in the level of transcription, by qRT-PCR, as well as for post-transcriptional modification as evidenced for four isoforms up-regulated. Despite differences in the gene expression, LOX activity was just slightly higher at IAC 17. In fact, the jasmonic acid levels evaluated by LC/MS increased under infestation, however were similar for both the genotypes (Gómez et al., 2020 under publishing).

During the response to the attack of pathogens, the plant produces molecules and toxins that interact directly with the insects, causing damage to their development in the response called antibiosis (Boiça Júnior *et al*., 2015). These include Kunitz family protease inhibitors (SBTI) and Bowman-Birk (BBI) serine protease inhibitor, trypsin inhibitors that prevent the absorption of proteins in the pathogen's gut. At transcriptional level, BBI inhibitor found highly up-regulated in both genotypes, with mRNA levels higher in the IAC 17. Otherwise, different protein isoforms were observed some detected only for resistant genotype IAC 17. This post-transcriptional regulation could be justifying the higher levels of protease activity verified for IAC 17 or the expression or presence in the leaves of the others protease inhibitors. These results are consistent with the study of survival and of the gut proteolytic activity carried out by Gómez et al., (2019), which showed that caterpillars of *Anticarsia gemmatalis* fed elicited IAC 17 extract had higher reductions of proteolytic capacity of the intestinal system. Thus, protease inhibitors could be contributing for resistance in the IAC genotype, however in not directly controlled by jasmonic acid levels.

Specific protein abundance for IAC 17 was detected for the disease resistance-responsive (dirigent-like protein) Family, derived from the Latin word *dirigere* (*to align or guide*), were first found in *Forsythia intermedia* by Davin *et al*. (1997). The dirigent gene family is present in numerous species, including lichens, ferns, gymnosperms, and angiosperms (Ralph *et al*., 2006, 2007; Wu *et al*., 2009) and many dirigent genes are inducible by different types of abiotic and biotic stress factors, including wounding (Ralph *et al*., 2007), dehydration, low temperature, abscisic acid (ABA) (Wu *et al*., 2009), H2O2, NaCl, and polyethylene glycol (Magalhaes *et al*., 2010). The apparent up-regulation of dirigent genes in response to attacks by fungi (Zhu *et al*., 2007; Reboledo *et al*., 2015) and insects (Ralph *et al*., 2007) are of interest. The predominant roles of the dirigent gene family are in defense responses, fiber biosynthesis (Davin & Lewis, 2003; Ralph *et al*., 2007; Berim *et al*., 2008), and secondary metabolism as they have contribution to lignan and lignin formation. This participation is very important to plant defense, since lignans may have defensive functions against insects or pathogens (Ralph *et al*., 2007).

1.5 Conclusions

Plants respond to herbivory through various morphological, biochemicals and molecular mechanisms of defense against the herbivores, which are mediated both by

direct and indirect defenses. Recent studies have indicated that the soybean genotype IAC 17 produce some defensive compounds constitutively (Gómez *et al*., 2018) and others in response to plant damage that could be related to resistance mechanism to *A. gematalis*, affecting feeding, growth, and survival (Gómez *et al*., 2020 under publishing). Production of protease inhibitors evaluated in this study, such as Kunitz family (SBTI) and Bowman-Birk (BBI), combining with phenolic compounds could be main agents affecting intestine cell structure and digestibility of the caterpillars. Furthermore, these proprieties appear as constitutive characteristics justified by higher levels of expression for protease inhibitors genes and bioactive flavonoids (Gómez *et al*., 2018, Gómez *et al*., 2020 under publishing) in IAC 17, even in absence of caterpillar. Despite of up regulations of several protein after the caterpillar infestation, many of them not were yet characterized for plant-insect interactions and others belong proteins commonly responsive to biotic and abiotic stresses.

The regulatory cascades controlling the resistance mechanism for IAC 17 genotype not involve jasmonic acid levels and production via LOX pathway as verified by (Gómez, 2018) and confirmed in this study from enzymatic assays. Thus, cascades could be under control of the transmembrane receptors and transcriptional factors, detected as up-regulated in this genotype, controlling the resistance genes. Likewise, the control of the key factors for biosynthesis compounds acting by antibiosis directly against *A. gemmatalis* could be also involve RNA binding proteins, acting to modulated the chloroplast metabolism to enable production of defensives molecules.

Finally, this study showed that the soy defense mechanism to herbivory by *A. gemmatalis* presents constitutive and induced characteristics, shown by the differences in the levels of gene expression and enzymatic activity of protease inhibitors and lipoxygenases, respectively, between genotypes, in addition to modulation in the abundance of proteins in response to herbivory.

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Supplementary Figure Legends

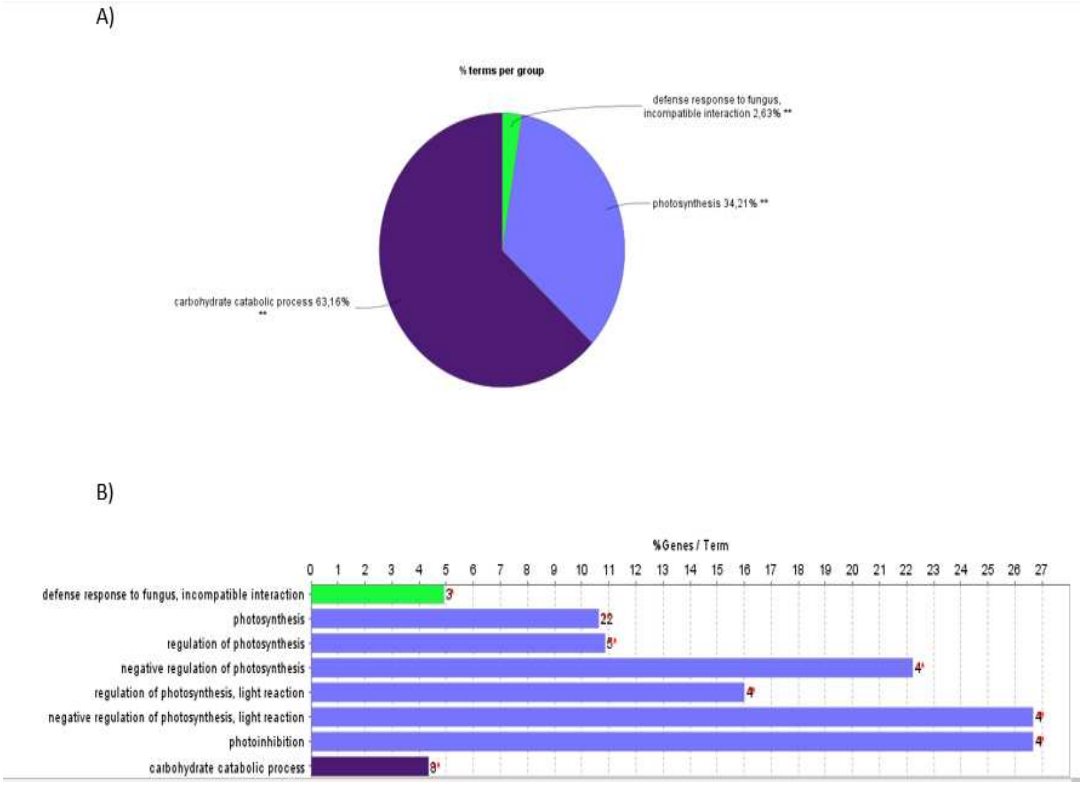
1. **Primers used for RT-qPCR.**
2. **Total identified proteins grouped according to the biological processes in which they participate. A, B) UFV 105 AP; C, D) IAC 17; E) Proteins commonly identified in both genotypes.**
3. **Grouping of proteins differentially abundant in the genotypes by biological process. A) proteins abundant in IAC 17. B) Proteins abundant in UFV 105 AP**

Supplementary Figures

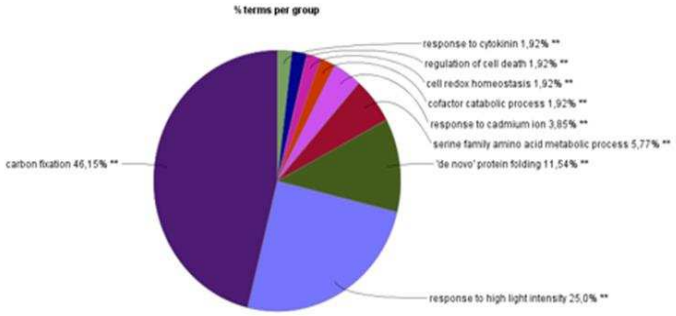
Supplementary figure 1

Primer name	Forward sequence	Reverse sequence
qRT_mrRNA18S	AAACGGCTACCACATCCAAG	CCTTCAATGGATCCATCGTTA
qRT_UNK2	GCCTCTGGATACCTGCTCAAG	ACCTCCTCCTCAAACTCCTCTG
qRT_ LIPOXJ	ATGGGGGCTTAATCCTGAAC	GGGTCTCAAACTTGGGTGTG
qRT_ BIRK	AGAAGGTGGCATTGGTGAAG	TAATTGTTGGGGAGCACCTG

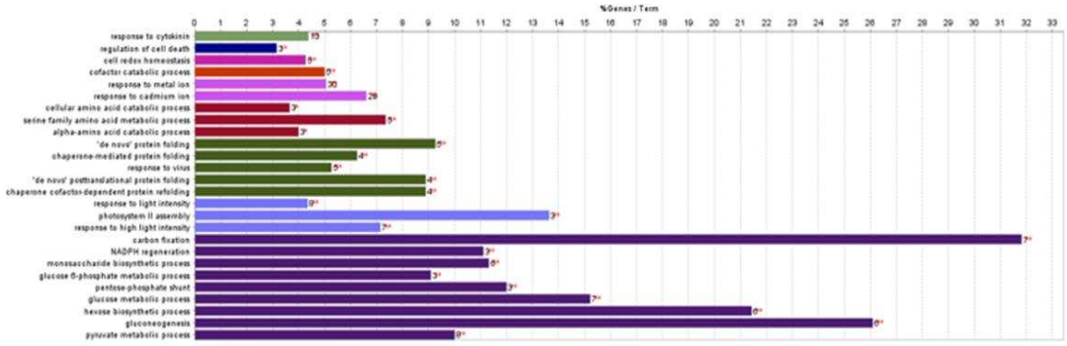
Supplementary figure 2.



C)


% terms per group
response to cytokinin 1,92% **
regulation of cell death 1,92% **
cell redox homeostasis 1,92% **
cofactor catabolic process 1,92% **
response to cadmium ion 3,85% **
serine family amino acid metabolic process 5,77% **
'de novo' protein folding 11,54% **
carbon fixation 46,15% **
response to high light intensity 25,0% **

D)


%Genes / Term
response to cytokinin
regulation of cell death
cell redox homeostasis
cofactor catabolic process
response to metal ion
response to cadmium ion
cellular amino acid catabolic process
serine family amino acid metabolic process
alpha-amino acid catabolic process
'de novo' protein folding
chaperone-mediated protein folding
response to virus
'de novo' posttranslational protein folding
chaperone cofactor-dependent protein refolding
response to light intensity
photosystem II assembly
response to high light intensity
carbon fixation
NADPH regeneration
monosaccharide biosynthetic process
glucose 6-phosphate metabolic process
pentose-phosphate shunt
glucose metabolic process
hexose biosynthetic process
gluconeogenesis
pyruvate metabolic process

Supplementary figure 3