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Leprose dos citros: um modelo para o estudo da interação planta/ácaro/vírus

Citrus leprosis: a model for the study of the plant/mite/virus interaction

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Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Genética e Biologia Molecular, na Área de Genética Vegetal e Melhoramento.

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RESUMO

A leprose dos citros, causada pelo citrus leprosis virus C (CiLV-C) e transmitida pelo ácaro *Brevipalpus yothersi* é a principal doença viral dos citros no Brasil. Além dos prejuízos econômicos à cultura, a leprose desperta interesse científico por ser uma doença atípica na qual o vírus permanece restrito nos tecidos vegetais ao redor do ponto de alimentação do vetor, assemelhando-se a uma reação de incompatibilidade do tipo hipersensibilidade (HR). Com este trabalho, objetivou-se revelar os mecanismos moleculares envolvidos na interação planta/CiLV-C/*B. yothersi*, ainda amplamente desconhecidos. Para o estudo da interação planta/ácaro foram analisados o transcriptoma de *Arabidopsis thaliana* em resposta ao *B. yothersi* via RNA-Seq, os níveis de ácido salicílico (SA) e jasmônico (JA) através de LC-MS/MS, e a relevância das vias hormonais com o uso de plantas mutantes de Arabidopsis. As plantas selvagens infestadas induziram ambas as vias de SA e JA, enquanto os processos envolvidos no seu crescimento e desenvolvimento foram reprimidos. A oviposição dos ácaros fui reduzida nas mutantes *sid2* e *npr1*, sugerindo que o *B. yothersi* manipula a planta tornando-a mais suscetível à colonização. Para o estudo da interação planta/vírus foram analisados os principais eventos da replicação do CiLV-C através da quantificação da relação RNA1/*p29*sgRNA, a resposta transcricional de Arabidopsis infectada com CiLV-C via RNA-Seq, a presença de células mortas e a produção de espécies reativas de oxigênio (ROS) em tecidos afetados através de ensaios histoquímicos, e a expressão transiente das proteínas virais em *Nicotiana benthamiana*. Em resposta à infecção pelo CiLV-C as plantas apresentaram uma explosão de ROS e uma indução de genes associados ao crescimento celular, à via do SA, e aos processos de morte celular e HR; enquanto reprimiram a via de JA e o metabolismo primário. A expressão da proteína viral P61 mimetizou respostas que ocorrem durante a infecção viral, indicando-a como uma elicitora do sistema imune vegetal. O CiLV-C favoreceu a oviposição do ácaro vetor nas folhas infectadas, sugerindo que o vírus contribui com a infestação do seu vetor. Finalmente, a expressão de alguns genes modulados durante a interação Arabidopsis/CiLV-C/*B. yothersi* foi confirmada em plantas de laranja-doce (*Citrus sinensis*), validando o uso da planta-modelo no estudo do patossistema da leprose. Foi demonstrado ainda que outros vírus transmitidos por *Brevipalpus* (VTBs) são capazes de infectar Arabidopsis, o que permite o seu uso como hospedeira alternativa em estudos de interação com VTBs filogeneticamente distantes ao CiLV-C. Os resultados apresentados, além de proporcionar uma melhor compreensão dos processos fisiopatológicos da leprose, a longo prazo poderão contribuir para o estabelecimento de estratégias de controle mais sustentáveis da doença.

ABSTRACT

Citrus leprosis is caused by citrus leprosis virus C (CiLV-C), transmitted by *Brevipalpus yothersi* mites, and is the main viral disease of citrus in Brazil. Differently from other plant viruses, CiLV-C is unable to accomplish systemic infection in its hosts, remaining restricted to cells around the inoculation sites, where symptoms of viral infection develop. Phenotypically, these features resemble the outcome a hypersensitivity response (HR). In this study, we attempted to unravel the molecular mechanisms involved in the plant/CiLV-C/*B. yothersi* interaction, which are still poorly understood. To disentangle the plant/mite interaction, we analyzed the *Arabidopsis thaliana* transcriptome in response to *B. yothersi* by RNA-Seq, the levels of salicylic acid (SA) and jasmonic acid (JA) in infested plants by LC-MS/MS, and the role of the hormonal pathways during mite infestation using Arabidopsis mutant plants. Infested wild type plants induced both SA e JA pathways, whilst processes involved in plant growth and defense were repressed. *sid2* e *npr1* mutant plants reduced the oviposition of mites, suggesting that *B. yothersi* manipulate plant response to render it more susceptible to its colonization. To uncover plant/virus interaction, we identified the main events of viral replication by quantifying the CiLV-C RNA1/*p29*sgRNA ratio during the infection, the transcriptional Arabidopsis response to CiLV-C using RNA-Seq, the presence of dead cells and reactive oxygen species (ROS) in infected tissues through histochemical assays, and the elicitor activity of viral proteins by transient expression in *Nicotiana benthamiana*. Plants responded to CiLV-C with a ROS burst and the induction of genes related to cell growth, SA pathway, cell death and HR. Conversely, infected plants repressed the SA pathway and the primary metabolism. The expression of the P61 viral protein mimicked responses observed during CiLV-C infection, indicating P61 as a viral component that elicits the plant immune system. CiLV-C presence favored mite oviposition in virus-infected leaves, suggesting that the virus benefit mite infestation. Finally, the expression of selected genes modulated during the interaction with Arabidopsis was confirmed in sweet orange (*Citrus sinensis*), validating the use of this model plant in the study of citrus leprosis. We also showed that other *Brevipalpus*-transmitted viruses (BTVs) infect Arabidopsis, which thus can be used as an alternative host in the studies of plant interaction with CiLV-C-phylogenetically distant BTVs. The results presented here provided a better understanding of the processes developed during citrus leprosis disease and will be helpful to the establishment of sustainable strategies of the disease control.

SUMÁRIO

INTRODUÇÃO GERAL

A citricultura é um dos principais setores do agronegócio brasileiro. O país destaca-se no cenário mundial como o maior produtor e exportador de suco de laranja concentrado congelado e não concentrado (FAO, 2017). No ano de 2017, foram produzidas mais de 18 milhões de toneladas de laranja em uma área de cerca de 630 mil ha, sendo o Estado de São Paulo responsável por 77% da produção nacional (IBGE, 2017). Apesar do seu excelente desenvolvimento como agronegócio, a citricultura brasileira enfrenta problemas de ordem fitossanitária. Neste contexto, destaca-se a leprose dos citros como a principal doença viral que atinge aos citros no país (Freitas-Astúa et al., 2016).

A leprose típica e prevalente é causada pelo citrus leprosis virus C (CiLV-C) e transmitida por ácaros do gênero *Brevipalpus* (Acari: *Tenuipalpidae*). Sintomas típicos incluem lesões cloróticas ou necróticas localizadas na região de alimentação do ácaro em folhas, frutos e ramos. Plantas severamente atacadas podem apresentar desfolha, seca de ramos e queda prematura de frutos, promovendo redução drástica na produção (Bastianel et al., 2010). A forma mais efetiva do manejo da doença é o controle químico do ácaro vetor, o que tem demandado gastos anuais de até U$ 80 milhões à indústria citrícola brasileira (Bastianel et al., 2010). A importância da doença tem aumentado tanto em nível nacional, estando presente em todos os Estados produtores de citros do país, quanto mundial, uma vez que já foi relatada na maioria dos países da América do Sul e Central e, mais recentemente, no México (Castillo et al., 2011). A chegada da leprose ao hemisfério Norte tem preocupado países onde a citricultura é uma atividade econômica relevante, como os EUA, fazendo com que a doença seja considerada re-emergente (Roy et al., 2015).

Citrus leprosis C cilevirus é a especie tipo do gênero *Cilevirus* (Locali-Fabris et al., 2012). Os vírus dessa espécie apresentam um genoma bipartido formado por dois RNAs fita simples de sentido positivo [(+) ssRNA] que codificam seis ORFs. O RNA1 (8745 nt) apresenta duas ORFs, correspondentes à replicase (RNA-dependent RNA polymerase, RdRp) e à putativa capa proteica (P29) do vírus (Locali-Fabris et al., 2006). O RNA2 (4986 nt) apresenta quatro ORFs, codificando a putativa proteína de movimento (*movement protein*, *MP*) e as proteínas P15, P61 e P24 com funções desconhecida (Locali-Fabris et al., 2006). Apesar de as proteínas P61 e P24 apresentarem homologia distante com proteínas estruturais dos vírus específicos de insetos da família *Negeviridae* (Kuchibhatla et al., 2014), metade dos genes do CiLV-C são

considerados "órfãos" (Tautz and Domazet-Lošo, 2011) por não apresentarem homólogos em outras espécies de vírus.

Ácaros do gênero *Brevipalpus* são pestes cosmopolitas e polífagas que atacam, além de citros, centenas de espécies de plantas de famílias distintas, incluindo culturas economicamente importantes e inúmeras plantas ornamentais (Childers et al., 2003; Kitajima et al., 2010). Apesar de causarem dano direto a algumas espécies de plantas, os impactos negativos da infestação por ácaros *Brevipalpus* são associados à sua capacidade de transmitir várias espécies de fitovírus. Os chamados vírus transmitidos por *Brevipalpus* (VTBs) induzem sintomas localizados semelhantes aos causados pelo CiLV-C e incluem espécies dos gêneros *Cilevirus* e *Dichorhavirus* (Freitas-Astúa et al., 2018). Entre os ácaros vetores de VTBs, o *B. yothersi* transmite tanto cilevirus como dichorhavirus, sendo o principal vetor do CiLV-C e a espécie mais comum nos pomares brasileiros (Chabi-Jesus et al., 2018; Ramos-González et al., 2016; Sánchez-Velázquez et al., 2015). Além da sua relevância para a agricultura, os ácaros *Brevipalpus* apresentam uma biologia atípica. Muitas espécies do gênero são haploides durante todo o seu ciclo de vida, uma característica única entre organismos superiores, e suas populações são formadas essencialmente por fêmeas devido à presença de uma bactéria endossimbionte do gênero *Cardinium* (Weeks et al., 2001).

Diferentemente de outros vírus de plantas, o CiLV-C é incapaz de realizar movimento sistêmico em todas as suas hospedeiras conhecidas. Naturalmente, o CiLV-C infecta várias espécies do gênero *Citrus*, sendo as laranjas doces (*C. sinensis*) altamente suscetíveis, e outras espécies vegetais distintas como a rutácea *Swinglea glutinosa* (León et al., 2008) e a trapoeraba (*Commelina benghalensis*) (Nunes et al., 2012). Experimentalmente, o vírus pode ser transmitido para ao menos cerca de 50 espécies vegetais pertencentes a famílias distintas, dentre elas as plantas-modelo *Nicotiana benthamiana* e *Arabidopsis thaliana* (Arena et al., 2013; Garita et al., 2014). Apesar da ampla gama de hospedeiras, invariavelmente o CiLV-C permanece restrito às células ao redor do sítio de inoculação do ácaro vetor, onde os sintomas da infecção viral se manifestam sob a forma de lesões locais. Fenotipicamente, estes sintomas se assemelham ao resultado de uma resposta de hipersensibilidade (*hypersensitive response*, HR), uma forma de resistência caracterizada pela morte de células hospedeiras e pela restrição do patógeno no sítio de inoculação durante uma interação incompatível.

Na natureza, as plantas interagem frequentemente com patógenos e herbívoros com estratégias de infecção e alimentação distintas. Para se proteger contra essa ampla gama de agressoras, as plantas apresentam barreiras pré-formadas (como cera ou tricomas) que previnem ou atenuam o ataque por grande parte dos organismos fitopatogênicos e herbívoros. Muitos

agressores, no entanto, são capazes de superar tais barreiras, levando as plantas a recrutarem defesas induzidas para limitar o ataque dos mesmos. A primeira linha do sistema de defesa induzida é baseada em receptores de reconhecimento de padrões (*pattern recognition receptors*, PRRs) que reconhecem moléculas conservadas associadas aos patógenos/microrganismos (*pathogen/ microbe-associated molecular patterns*, PAMPs/ MAMPs), herbívoros (*herbivore-associated molecular patterns,* HAMPs) ou a danos à célula hospedeira (*damage-associated molecular patterns,* DAMPs), ativando a chamada imunidade desencadeada por padrões (*pattern-triggered immunity*, PTI). Patógenos e herbívoros bem-sucedidos produzem proteínas efetoras, adquiridas durante a co-evolução com suas plantas hospedeiras, capazes de suprimir a PTI, levando a uma susceptibilidade desencadeada por efetor (*effector-triggered susceptibility*, ETS). Como contra-ataque, algumas plantas apresentam genes de resistência (R) que codificam proteínas capazes de reconhecer direta ou indiretamente os efetores, ativando uma segunda linha de defesa denominada imunidade desencadeada por efetor (*effector-triggered immunity*, ETI) (Hogenhout and Bos, 2011; Jones and Dangl, 2006).

A natureza das defesas ativadas durante a PTI ou ETI mostram grande sobreposição (Thomma et al., 2011). Defesas contra patógenos incluem a fortificação da parede celular através da síntese de calose, o acúmulo de metabólitos secundários antimicrobianos como as fitoalexinas, e a indução de proteínas de defesa relacionadas à patogênese (*pathogenesis-related*, PR) como as quitinases e glucanases que degradam paredes celulares. Defesas contra herbívoros podem ser indiretas, como a liberação de voláteis que atraem predadores, ou diretas, que incluem proteínas de defesa e metabólitos secundários com efeitos tóxicos ou inibitórios à alimentação como os glucosinolatos (Wu and Baldwin, 2010). Além disso, as alterações metabólicas induzidas durante a ativação das defesas podem induzir uma explosão de espécies reativas de oxigênio (*reactive oxygen species,* ROS) que pode culminar no desenvolvimento de uma HR (Xia et al., 2015).

A reprogramação transcricional que resulta nas respostas de defesa é mediada pela ação de vias interconectadas dependentes de hormônios, sendo os principais os ácidos salicílico (SA) e jasmônico (JA). A via do SA media defesas contra patógenos biotróficos e tipicamente antagoniza as respostas mediadas por JA, uma estratégia da planta para alocar seus recursos de acordo com a natureza do ataque (Pieterse et al., 2012). A via do JA, por sua vez, é subdividida em dois ramos que se antagonizam entre si para modular a resposta a agressores específicos. O ramo mediado por fatores de transcrição ERF (*ethylene responsive factor*) é regulado em conjunto com etileno (ET) e atua na defesa contra patógenos necrotróficos. O ramo mediado

por fatores de transcrição MYC é modulado por ácido abscísico (ABA) e condiciona a resistência a herbívoros (Pieterse et al., 2012).

Quando se trata de resposta a vírus, as defesas mediadas por vias clássicas de sinalização são conectadas à principal defesa antiviral, o silenciamento de RNA (Hunter et al., 2013; Pumplin and Voinnet, 2013). O mecanismo de silenciamento é disparado pela presença de RNAs dupla-fita (dsRNA), presentes como os intermediários replicativos dos vírus ou em genomas que apresentam estructuras secondarias. Tais moléculas são consideradas PAMPs específicos de vírus e portanto denominadas padrões moleculares associados à virus (*virus-associated molecular pattern*, VAMPs). Os VAMPs são reconhecidos por nucleases *Dicer-like* (DCL) e processados pelas mesmas em pequenas moléculas de RNA interferente (*small interferent RNAs*, siRNAs). Uma fita guia dos siRNAs é incorporada à complexos de silenciamento induzidos por RNA (*RNA-induced silencing complex*, RISC), os quais contém como componente central uma proteína *Argonauta* (AGO), que direcionam a degradação específica ou silenciamento do genoma viral mediado por homologia de sequências (Parent et al., 2012). Como contra-ataque a um mecanismo de defesa tão eficiente, inúmeros fitovírus apresentam efetores que atuam como supressores de silenciamento de RNA (*viral suppressors of RNA silencing*, VSRs). Em um paralelo claro com o esquema clássico de resistência por PTI e ETI, alguns VSRs podem ser reconhecidos por proteínas R de plantas, desencadeando respostas típicas de ETI, incluindo a HR (Pumplin and Voinnet, 2013; Sansregret et al., 2013).

Apesar da singularidade e importância econômica da leprose, o conhecimento acerca dos mecanismos moleculares envolvidos na interação planta-patógeno-vetor é limitado (Freitas-astúa et al., 2007; Marques et al., 2010), havendo uma escassez de informações necessárias a melhor compreensão do patossistema. O estudo da interação dos componentes do patossistema com plantas de citros apresenta complexidades inerentes como a dificuldade de se transmitir o vírus mecanicamente (sendo necessária a transmissão experimental mediante o uso de ácaros virulíferos), a característica infecção localizada e a demora no surgimento dos sintomas (que podem levar de 25 a 60 dias após a infestação com ácaros virulíferos em plantas de citros). Em função destas particularidades, variáveis como as taxas de aquisição e inoculação do vírus pelo vetor, o número de partículas virais encontradas no mesmo, e a movimentação do ácaro pela planta hospedeira afetam a consistência dos dados nos estudos que envolvem indivíduos distintos. Além disso, a análise de dados é limitada em função do restrito conhecimento sobre o genoma da laranja, sequenciado há poucos anos (Xu et al., 2013), e sobre as interações envolvendo citros e seus patógenos, problemas inerentes ao uso de organismos não-modelo.

A complexidade dos estudos de interação envolvendo a leprose dos citros pode ser parcialmente superada com o uso de hospedeiros experimentais apropriados. Neste sentido, a planta-modelo *Arabidopsis thaliana* (Arabidopsis) surge como uma alternativa ideal, destacando-se por apresentar um genoma curado de alta qualidade, um banco completo de mutantes e ser utilizada em inúmeros estudos de interação, tornando-se útil para fins de comparação ou inferências dos resultados obtidos com outros patossistemas (Nishimura & Dangl, 2010). Em relação ao estudo da leprose especificamente, existe ainda a vantagem adicional do período de apenas 7 a 10 dias para a manifestação de sintomas causados pela infecção do CiLV-C, o que reduz o problema de variação e representa um ganho significativo de tempo na avaliação dos experimentos (Arena et al., 2013). Apesar da ampla utilização de Arabidopsis para o estudo de diferentes aspectos da biologia vegetal, a espécie mais utilizada como planta-modelo na fitovirologia é a *Nicotiana benthamina*, devido principalmente ao grande número de vírus capazes de infecta-la. A grande vantagem de *N. benthamiana*, no entanto, é sua alta receptividade à expressão transiente via agroinfiltração, permitindo o estudo funcional de genes de maneira facilitada (Goodin et al., 2008).

Diante do exposto, com o presente trabalho objetivou-se um estudo holístico da interação molecular planta/CiLV-C/*B. yothersi*, com o intuito de elucidar os mecanismos envolvidos no desenvolvimento da doença. Previamente, havia sido feita uma análise pontual da expressão de genes marcadores de vias de defesa em Arabidopsis infestadas com ácaros avirulíferos e virulíferos (Arena, 2014). Os dados obtidos foram complementados com ensaios histoquímicos de tecidos infectados, com a validação da expressão de genes selecionados em plantas de laranja-doce, e com análises da modulação do comportamento do ácaro vetor em plantas infectadas pelo CiLV-C, dando origem a um primeiro modelo da interação planta-patógeno-vetor (Capítulo 1, Arena et al., 2016). Para aprofundar o conhecimento acerca da interação planta/CiLV-C, foram identificados os principais eventos da replicação viral através da quantificação do vírus ao longo da infecção, a resposta transcricional global de Arabidopsis ao CiLV-C com o uso de RNA-Seq, e a atividade elicitora das proteínas virais por meio da expressão transiente em *N. benthamiana* (Capítulo 2). Visando uma melhor compreensão da interação planta/*Brevipalpus*, foram analisados o transcriptoma de Arabidopsis em resposta ao *B. yothersi* através de RNA-Seq, os níveis de hormônios de defesa em plantas infestadas, e a relevância das vias hormonais na interação através do uso de plantas mutantes de Arabidopsis (Capítulo 3, Arena et al., 2018). Finalmente, foi avaliada a capacidade de outros BTVs infectarem Arabidopsis, com o intuito de futuramente se utilizar a planta-modelo como hospedeira alternativa em estudos de interação com vírus relacionados ao CiLV-C, o que

estenderia o conhecimento acerca do desenvolvimento de doenças transmitidas por ácaros *Brevipalpus* (Capítulo 4, Arena et al., 2017). Os resultados apresentados no presente trabalho serão essenciais à compreensão dos mecanismos envolvidos no desenvolvimento da leprose dos citros e, a longo prazo, poderão contribuir para o estabelecimento de estratégias de controle mais sustentáveis.

A ordem dos capítulos apresentados nesta Tese foi definida em função da cronologia dos eventos que levaram à elucidação da interação planta/*Brevipalpus*/CiLV-C. O Capítulo 1 corresponde a um artigo publicado em 2016 contendo aspectos gerais da interação entre os três componentes do patossistema e apresenta o primeiro modelo da interação. Visando aprofundar e complementar o dito modelo, objetivou-se uma análise simultânea do transcriptoma de plantas de *Arabidopsis thaliana* em resposta ao ácaro e ao vírus em um único experimento composto por plantas infestadas com ácaros virulíferos, com ácaros avirulíferos e não-infestadas. Para se definir os tempos da interação que seriam mais informativos para a coleta do material vegetal, foi realizado um experimento de avaliação da cinética de acúmulo do CiLV-C ao longo da sua infecção em plantas, o qual permitiu identificar os principais momentos da replicação viral. Os resultados da cinética de multiplicação do CiLV-C em *A. thaliana* foram comparados ao transcriptoma de plantas infectadas com o CiLV-C e estão apresentados no Capítulo 2 (manuscrito ainda em fase de elaboração). O transcriptoma de plantas infestadas apenas com ácaros *Brevipalpus* é apresentado no Capítulo 3 (trabalho já publicado em 2018). A interação planta/CiLV-C (Capítulo 2) foi apresentada anteriormente à interação planta/*Brevipalpus* (Capítulo 3) pois na primeira ficaram definidos os tempos de avaliação dos experimentos da análise transcriptômica. Por fim, o uso de Arabidopsis como planta modelo para outros vírus transmitidos por *Brevipalpus* (VTBs) está registrado no Capítulo 4 (trabalho já publicado em 2017), que abre uma perspectiva futura de estudos de interação envolvendo *A. thaliana* e VTBs além do CiLV-C.

CAPÍTULO 1

Paper publicado na revista *Frontiers in Plant Science* em 2016:

Citrus leprosis virus C infection results in hypersensitive-like response, suppression of the JA/ET plant defense pathway and promotion of the colonization of its mite vector

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Keywords: cilevirus, herbivory, plant-virus-vector interaction, *Brevipalpus* mites, Arabidopsis, *Citrus sinensis*, hormonal crosstalk, RNA silencing

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Citrus leprosis virus C Infection Results in Hypersensitive-Like Response, Suppression of the JA/ET Plant Defense Pathway and Promotion of the Colonization of Its Mite Vector

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Leprosis is a serious disease of citrus caused by *Citrus leprosis virus C* (CiLV-C, genus *Cilevirus*) whose transmission is mediated by false spider mites of the genus *Brevipalpus*. CiLV-C infection does not systemically spread in any of its known host plants, thus remaining restricted to local lesions around the feeding sites of viruliferous mites. To get insight into this unusual pathosystem, we evaluated the expression profiles of genes involved in defense mechanisms of *Arabidopsis thaliana* and *Citrus sinensis* upon infestation with non-viruliferous and viruliferous mites by using reverse-transcription qPCR. These results were analyzed together with the production of reactive oxygen species (ROS) and the appearance of dead cells as assessed by histochemical assays. After interaction with non-viruliferous mites, plants locally accumulated ROS and triggered the salicylic acid (SA) and jasmonate/ethylene (JA/ET) pathways. ERF branch of the JA/ET pathways was highly activated. In contrast, JA pathway genes were markedly suppressed upon the CiLV-C infection mediated by viruliferous mites. Viral infection also intensified the ROS burst and cell death, and enhanced the expression of genes involved in the RNA silencing mechanism and SA pathway. After 13 days of infestation of two sets of Arabidopsis plants with non-viruliferous and viruliferous mites, the number of mites in the CiLV-C infected Arabidopsis plants was significantly higher than in those infested with the non-viruliferous ones. Oviposition of the viruliferous mites occurred preferentially in the CiLV-C infected leaves. Based on these results, we postulated the first model of plant/*Brevipalpus* mite/cilevirus interaction in which cells surrounding the feeding sites of viruliferous mites typify the outcome of a hypersensitive-like response, whereas viral infection induces changes in the behavior of its vector.

Keywords: cilevirus, herbivory, plant-virus-vector interaction, *Brevipalpus* mites, Arabidopsis, *Citrus sinensis*, hormonal crosstalk, RNA silencing

INTRODUCTION

Most of the known plant viruses systemically infect their main plant hosts. Distinctively, *Citrus leprosis virus C* (CiLV-C), the causal agent of citrus leprosis, is unable to move long distances within any of its almost 50 natural or experimental host species belonging to at least 28 distant plant families (León et al., 2008; Nunes et al., 2012a,b; Arena et al., 2013; Garita et al., 2013, 2014). CiLV-C infection invariably produces chlorotic or necrotic lesions around the feeding sites of its mite vector. However, albeit it shows localized symptoms, citrus leprosis threatens citrus production in the Americas (Roy et al., 2015). Indeed, it is regarded as the most important viral disease affecting citrus in Brazil, the leading sweet orange producer in the world (Bastianel et al., 2010).

CiLV-C is the type member of the genus *Cilevirus* (Locali-Fabris et al., 2011). Virions are enveloped rod-like particles with 50–55 × 120–130 nm and its genome consists of two positive (+) sense single-stranded RNA molecules that contain 5′ cap structures and 3′ poly(A) tails. ORFs in the RNA1 (8745 nt) encode the RNA-dependent RNA polymerase and the putative 29 kDa coat protein. RNA2 contains four ORFs encoding the putative movement protein (MP), which shows conserved motifs of the plant virus MPs of the 30K superfamily, and the P15, P61, and P24 proteins with unknown functions (Locali-Fabris et al., 2006; Pascon et al., 2006). However, P61 and P24 display distant homology with structural proteins of the insect-specific negeviruses (Kuchibhatla et al., 2014).

False spider mites of the genus *Brevipalpus* (Acari: *Tenuipalpidae*) are polyphagous and cosmopolitan pests that colonize, in addition to citrus, several economically important crops and ornamentals plants (Childers et al., 2003a; Kitajima et al., 2010). In order to feed, false spider mites pierce and conceivably inject saliva into the plant mesophyll cells using their interlocked stylet, and after withdrawing it, they suck out the overflowed cell content of punctuated cells through the preoral cavity (Alberti and Kitajima, 2014). *Brevipalpus* spp. are haploid during their entire life-cycle, reproduce through thelytokous parthenogenesis and their adult populations are essentially females due to the presence of the endosymbiont bacterium *Cardinium* sp. (Weeks et al., 2001). Within the group of *Brevipalpus* species that transmit plant viruses, *B. yothersi* (synonym *B. phoenicis* Geijskes citrus type) is the main vector of the cileviruses (Beard et al., 2015; Sánchez-Velázquez et al., 2015; Ramos-González et al., 2016). Once acquired during any of the active phases of these mites, CiLV-C is persistently transmitted to distant parts within the same plant and to new ones, but not to their offspring (Bastianel et al., 2010; Kitajima and Alberti, 2014). CiLV-C replication in *B. yothersi* has been suggested (Roy et al., 2015).

Plants are recurrently invaded by attackers with distinct infecting or feeding strategies. Upon detection of the attacker-associated ligands, i.e., pathogen-associated molecular patterns (PAMP) and/or effectors, the plant immune system triggers a spectrum of dynamic responses to arrest the colonization process (Thomma et al., 2011; Cui et al., 2015). Phytohormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) mediate a transcriptional reprogramming, tuning up defense responses that are modulated by the nature of the injury (Arimura et al., 2011; Pieterse et al., 2012; Alazem and Lin, 2015). The SA signaling pathway is primarily induced in response to biotrophic pathogens and piercing-sucking herbivores that cause minimal tissue damage. The JA pathway is subdivided in two antagonistic and interconnected branches that are activated in response to distinct stimuli. The ethylene responsive factor-branch (ERF-branch) is regulated by ERF transcription factors and synergistically cross-communicates with the ET pathway in response to necrotrophic invaders. On the contrary, MYC-branch, controlled by MYC transcription factors, is independent of ET and mediates defense against herbivores whose action greatly disrupts tissue integrity. Typically, SA antagonizes the JA/ET pathways, a plant strategy to efficiently allocate its resources according to the nature of the attack (Pieterse et al., 2012).

Plant defense against viruses mainly involves the RNA silencing machinery (Mandadi and Scholthof, 2013; Pumplin and Voinnet, 2013). Double stranded RNA (dsRNA) replication intermediates or structured RNA genomes from viruses can be considered a special case of PAMP, i.e., VAMP (virus-associated molecular pattern). VAMPs are recognized by Dicer-like nucleases (DCL), which further process them into virus-derived siRNAs. A guide strand of these molecules and an AGO protein are assembled into the RNA-induced silencing complex (RISC), which direct specific silencing of the homologous viral genome (Parent et al., 2012). Albeit previously considered an independent mechanism of antiviral resistance, the current understanding is that RNA silencing is connected with the hormonal pathways through the SA- and JA-induced RNA-dependent RNA polymerase 1 protein (RDR1) (Hunter et al., 2013).

In addition to hormones, plant response also involves the generation of reactive oxygen species (ROS). ROS may act as signal transduction molecules in the host, as toxic compounds against the attackers or as a blocking agent to their colonization by promoting the reinforcement of the plant cell walls (Foyer and Noctor, 2013; Camejo et al., 2016; Gilroy et al., 2016). Moreover, ROS accumulation may lead to the activation of the hypersensitive response (HR) (Foyer and Noctor, 2005; Xia et al., 2015). ROS and SA pathways are interconnected and mutually enhanced, promoting a self-amplifying feedback loop that drives HR (Xia et al., 2015). Hypersensitive-like response can also be observed as an outcome of plant-attacker interactions where the typically involved effectors and ligands are missing, suggesting the participation of yet unknown host proteins (Li et al., 2010).

Plant defenses are constantly challenged, and even hijacked, by the mechanisms of virulence from evolved attackers. Hormonal cross-talking that usually occurs during the plant immune response can be wielded by the attackers for their own benefit (Pieterse et al., 2012). In contact with plants, some insect eggs induce high level of SA, leading to a strong reduction of the JA-mediated defenses and, hence, decreasing their interference on the future larva feeding (Little et al., 2007; Bruessow et al., 2010; Gouhier-Darimont et al., 2013; Hilfiker et al., 2014). Extracts from these eggs reduce the MYC

protein levels in a SA-dependent manner (Schmiesing et al., 2016). Moreover, insects and mites secrete proteins in their saliva which modulate, and even suppress, the plant defense response (Kant et al., 2015; Villarroel et al., 2016). In the saliva, proteins from arthropod-associated microorganisms, i.e., endosymbiont bacteria and viruses, are also responsible for plant defense elicitation (Chaudhary et al., 2014; Jaouannet et al., 2015). Similarly, plant viruses encode suppressors to efficiently overcome the RNA silencing (Li and Ding, 2006). Virus derived proteins have also been implicated in mutual benefit to pathogens and their vectors (Casteel and Falk, 2016).

Despite the uniqueness of the citrus leprosis pathosystem, its molecular aspects are poorly understood. To extend our comprehension of the mechanisms underlying it, the current study evaluated the model plant *Arabidopsis thaliana* by profiling the mRNA expression of marker genes of the main defense pathways during the course of its interaction with both non-viruliferous and CiLV-C viruliferous *Brevipalpus* mites using RT-qPCR. Moreover, we searched for evidence of an HR by performing histological tests on the injured plant tissues. Finally, we evaluated the feasibility of using Arabidopsis as a model host for citrus leprosis studies by comparing the transcript profiles of some selected Arabidopsis genes with their homologs of *Citrus sinensis*. In addition to the first evidence on how plants respond to both *Brevipalpus* feeding and CiLV-C infection, we present evidence of changes in the behavior of the vector mites in CiLV-C infected plants. Additionally, we provide data about CiLV-C inoculation efficiency by the mite and indicate the plant reference genes to be used in the current and future transcriptomic studies involving the citrus leprosis pathosystem.

RESULTS

An Inoculation Access Period of 6 h Results in 100% of CiLV-C-Infected Arabidopsis plants by Viruliferous *Brevipalpus yothersi* Mites

Since the successful inoculation of CiLV-C is only achieved using viruliferous mites, the analysis of the transcriptional profiles of plant genes involved in the interaction with this virus can only be inferred by comparing the profiles of plants infested with viruliferous and non-viruliferous mites. However, as upon infestation the mites do not feed in a synchronous manner, the time of CiLV-C inoculation may vary among a set of infested plants. To reduce the impact of phased out infections over the analysis of the expression profiles of genes implicated in the earlier steps of the interaction, we conducted a preliminary experiment to determine the minimal feeding period necessary to achieve 100% of infection of plants infested with viruliferous mites. Seven to ten days after infestation (dai), Arabidopsis plants inoculated with viruliferous mites either for 4 or 6 h showed the typical symptoms of CiLV-C infection; i.e., chlorotic spots in green leaves and green islands in yellow senescent ones (Arena et al., 2013) (symptoms as in **Figure 1**). However, the number of symptomatic plants differed between the two treatments. In the 4 h treatment only 30% of the plants displayed symptoms compared to 100% in the 6 h treatment (**Table 1**). Presence of CiLV-C in all symptomatic plants was confirmed by RT-PCR (data not shown). Thus, from these analyses we determined that 6 h was an appropriate time to initiate the evaluation of the Arabidopsis responses to CiLV-C infection.

FIGURE 1 | Phenotypes of *Arabidopsis thaliana* (left) and *Citrus sinensis* **(right)** plants infested with non-viruliferous and CiLV-C viruliferous *Brevipalpus yothersi* mites during the time course experiments. Pictures from Arabidopsis and *C. sinensis* were taken at 8 and 25 days after infestation, respectively. Infested leaves are indicated by asterisks (*).

TABLE 1 | CiLV-C inoculation efficiency of *Arabidopsis thaliana* with five viruliferous *Brevipalpus* mites after different inoculation access periods (IAP).

IAP	Test-plants (n)	Symptomatic plants (n)	PCR-positive plants (n)[b]	Inoculation efficiency (%)
4 h	10	3	3	30
6 h	10	10	10	100
Control[a]	10	10	10	100

[a] *Inoculation positive control, where CiLV-C viruliferous mites were kept onto plants throughout the experiment.*

[b] *Plants were evaluated by RT-PCR using primer pairs designed for a region within the CiLV-C MP gene (Locali et al., 2003).*

F-BOX, *SAND* and *TIP41* Are Suitable Reference Genes for Transcript Normalization during Arabidopsis/Mite/Cilevirus Interaction

A time course experiment to evaluate Arabidopsis interaction with non-viruliferous and viruliferous mites was set up. Six candidate reference genes (*EF1A, F-BOX, GAPDH, PPR, SAND,* and *TIP41*) for transcript normalization were evaluated by assessing their expression levels. Quantification cycle (Cq)-values ranged from 23.98 to 36.01 (**Table 2**), and only those whose transcripts accumulated in moderate quantities ($15 < Cq < 30$, Wan et al., 2010) were further selected (i.e., *EF1A, F-BOX, GAPDH, SAND,* and *TIP41*). Statistical analysis using

TABLE 2 | *Arabidopsis thaliana* candidate reference genes ranked according to their expression stability.

Gene	Locus	Mean Cq (±*SD*)[a]	Stability value (*M*)[b]	Ranking[b]
FBOX	AT5G15710	30.46 ± 0.29	0.18	1
TIP41	AT4G34270	28.15 ± 0.31	0.18	1
SAND	AT2G28390	28.30 ± 0.26	0.19	2
EF1α	AT5G60390	23.98 ± 0.38	0.22	3
GAPDH	AT1G13440	30.19 ± 0.71	0.34	4
PPR[c]	AT5G55840	36.01 ± 0.41	-	-

[a] *Quantification cycle (Cq) and standard deviation (SD) values were obtained from RT-qPCR of 27 samples (three technical replicates of nine biological samples across three experimental conditions: plants infested with viruliferous mites, non-viruliferous mites or not infested).*

[b] *M-values, calculated by geNorm (Vandesompele et al., 2002) based on RT-qPCR data, increase from the most stable pair of genes to the least stable.*

[c] *PPR gene was excluded from analyses due to its low expression level (Cq-value > 35).*

geNorm (Vandesompele et al., 2002) revealed that the *F-BOX*, *SAND,* and *TIP41* genes presented the lowest *M*-values, which correspond to the highest expression stabilities. Since data normalization is preferably done using at least three reference genes (Vandesompele et al., 2002), these were selected for the RT-qPCR analysis conducted in this work.

Infestation with Non-viruliferous Mites Induces the SA Pathway and a Response against Necrotrophic Rather than Herbivorous Attackers in Arabidopsis

Expression of several genes involved in SA response (*ICS1, EDS5*, *NPR1, WRKY70* and *PR5*), JA/ET pathways (*ETR1, EIN2*, *MYC2*, and *PDF1.2*) and gene silencing mechanism (*AGO2*), in addition to marker genes for HR (*NHL10*) were induced as soon as at 6 h after infestation (hai) (**Figure 2**), indicating a rapid and combined response of the plants to the mites.

Genes of the SA pathway remained induced throughout the time course, i.e., *ICS1*: encoding the enzyme responsible for main SA biosynthesis in defense responses, *EDS5:* the transporter of SA from the chloroplast to cytoplasm, *NPR1*: the main regulator of SA-responsive genes and the transcription factor (TF) *WRKY70*. While statistical analysis indicated an induction of the defense gene *PR5* as early as 6 hai, the expression of this gene as well as that of *PR1* were noticeably up-regulated at 8 dai (**Figure 2**).

The expression of most of the evaluated genes of the JA/ET pathways was also induced at least at one time point with the exception of *JAR1*, whose relative expression was not altered at the earlier times of the infestation and was reduced at 8 dai (**Figure 2**). Up-regulation of the ethylene receptor gene (*ETR1*) was observed at 6, 12 hai and 8 dai; while induction of the main positive regulator downstream of the ethylene perception (*EIN2*) was detected at 6 hai and 8 dai. Up-regulation of the TF *MYC2* was detected at 6 hai, but its relative expression was gradually reduced at the following two time points and increased again at 8 dai. The relative expression of the genes *PDF1.2* and *VSP2* increased during the interaction, although at different levels and time points. Induction of *PDF1.2* was noteworthy since its transcript levels were 500-fold higher than in not infested plants (**Figure 2**).

Expression of genes of the RNA silencing mechanism was also modulated in response to mite feeding (**Figure 2**). Induction was verified for *RDR1* (12 hai), *AGO2* (6 hai) and *DCL2* (12 hai). We also observed repression of *AGO1* at 12 hai, of *HEN1* and *RDR6* at 12 hai and 8 dai, and of *DCL4* at 6, 12 hai and 8 dpi.

RBOHD gene, which encodes a NADPH oxidase enzyme responsible for ROS production, was highly induced at 8 dai, whereas the HR specific gene *NHL10* was found up-regulated throughout the time points assessed, except at 24 hai (**Figure 2**).

Arabidopsis Infestation with CiLV-C Viruliferous *B. yothersi* Mites Enhances SA Response and Suppresses the JA/ET Pathway

CiLV-C loads during the time course experiment were assessed using a RT-qPCR assay established during this work. Virus was detected in all plants infested with viruliferous mites, whereas its absence was confirmed in plants either infested with non-viruliferous mites or non-infested. In the subset of plants collected at 12 and 24 hai, virus titers were ca. 2-fold higher ($p < 0.05$) than in plants collected at 6 hai, whereas at the time of symptom appearance (8 dai), the viral load increased 2000-fold (**Figure 3A**).

Overall, the expression of the SA pathway related genes were higher in plants infested with viruliferous mites than in those infested with non-viruliferous ones (**Figure 2**). For instance, the expression of genes coding for proteins acting upstream of the pathway (*ICS1* and *EDS5*) was triggered in higher levels as early as 6 hai, whereas those encoding downstream proteins were gradually up-regulated. Induction of the TF *TGA3* and the defense protein *PR5* were observed in samples collected at 12 hai. In the case of *WRKY70*, *NPR1,* and *PR1*, maximum relative expressions were detected at the latest stage of the infection (8 dai), when the highest viral loads were reached (**Figure 3A**).

In contrast to the general activation of genes of the SA pathway, genes of the JA/ET pathways displayed distinct expression patterns (**Figure 2**). Upon infection, genes encoding the receptors for JA (*JAR1*) and ET (*ETR1*) were up-regulated, although the expression levels of downstream genes of this pathway were reduced. *EIN2* and *MYC2* were repressed at 6 and 12 hai, respectively, whereas the relative expression of the pathway outcome genes *PDF1.2* and *VSP2* showed a marked reduction at the end of the evaluation.

Core genes of the RNA silencing mechanism showed distinct expression profiles in plants infested with non-viruliferous compared to those infested with viruliferous mites (**Figure 2**). *RDR6* and *HEN1* were induced earlier (6 and 12 hai, respectively) than *AGO2* (8 dai), whereas the Dicer-like nucleases genes were suppressed during the infection. The lowest relative expressions of *DCL2* and *DCL4* were detected at 12 hai and 8 dai, respectively.

The expression patterns of the marker genes of the ROS burst and HR were up-regulated in response to virus infection.

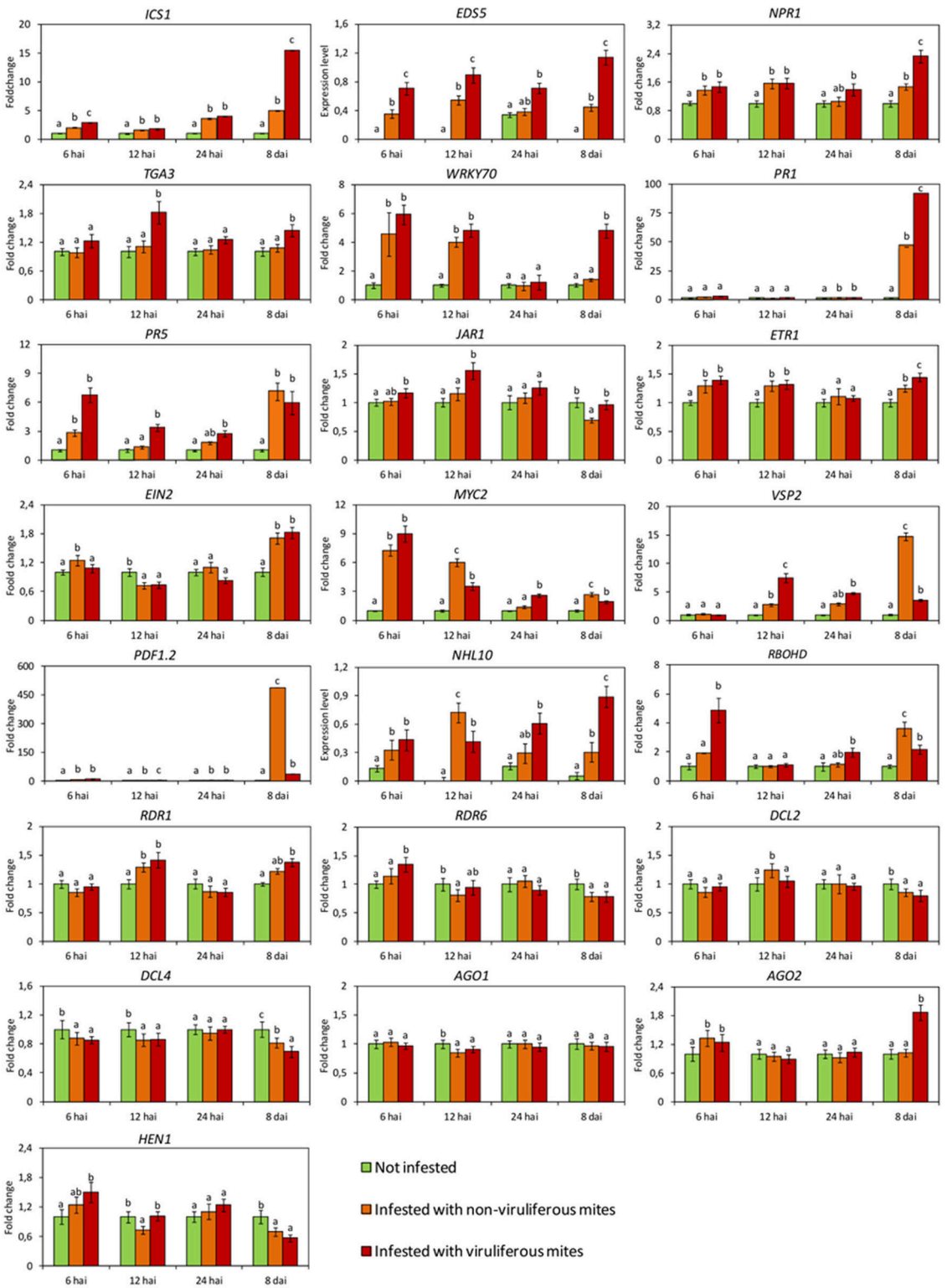

FIGURE 2 | Expression profiles of *Arabidopsis thaliana* genes involved in the main plant defense pathways assessed by RT-qPCR. In green, plants kept without mites. In orange, plants infested with non-viruliferous mites. In red, plants infested with CiLV-C viruliferous mites. Data are presented as fold change values in comparison with not infested plants (with fold change value set to 1) or expression levels when transcripts were not detected in not infested plants. Values represent the mean of 10 biological replicates for each set. Error bars represent standard errors. Different letters correspond to different expression levels between treatments within the same time point (ANOVA and Tukey's HSD *post-hoc* test, $\alpha < 0.05$). hai, hours after infestation; dai, days after infestation.

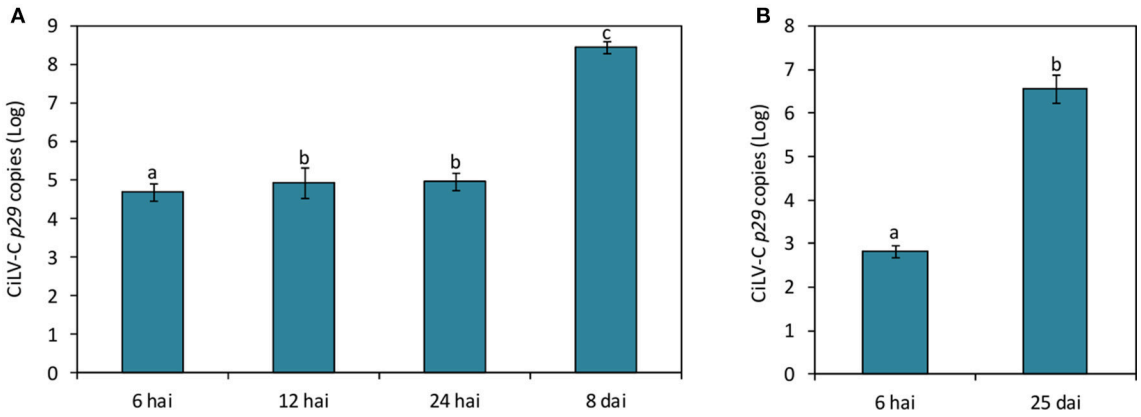

FIGURE 3 | CiLV-C loads on *Arabidopsis thaliana* (A) and *Citrus sinensis* **(B)** plants infested with viruliferous *Brevipalpus yothersi* mites throughout the time course of the interaction. Data are presented as absolute number of copies (log) of the CiLV-C *p29* gene normalized against *SAND* genes corresponding to each plant species. Values represent the means of 10 biological replicates for each time point. Standard errors are represented on each bars. Different letters correspond to different number of copies between the time points assessed (ANOVA and Tukey's HSD *post-hoc* test, $\alpha < 0.05$).

Induction of *RBOHD* was observed as early as 6 hai, whereas induction of *NHL10* was detected at 8 dai.

Sweet Orange Plant Response to Citrus Leprosis Mirrors Major Hallmarks of the Arabidopsis/*Brevipalpus* Mite/CiLV-C Interaction

The expression profiles of some key genes involved in the defense response of Arabidopsis to *Brevipalpus* mites and CiLV-C (*PR1*, *MYC2*, *AGO2,* and *WRKY70*) were also evaluated in infested sweet orange plants.

In citrus plants infested with viruliferous mites, the virus was detected by RT-qPCR as early as 6 hai (**Figure 3B**). Viral loads increased around 10^4-fold after 25 days of infection. Symptoms were firstly observed at 17 dai, when small chlorotic spots started to develop in some plants. Typical chlorotic and necrotic lesions of citrus leprosis only were observed at 25 dai (**Figure 1**).

Differences in gene expression were not observed between not infested plants and those infested with non-viruliferous mites at 6 hai. At this time, only the expression of *WRK70* and *AGO2* were induced in response to the infestation with viruliferous mites (**Figure 4**). However, at 25 dai all genes had their expression altered. Particularly, high relative expression of *AGO2* was observed on plants infested with viruliferous mites, suggesting a RNA silencing response specific to CiLV-C infection. The SA-dependent *PR1* gene was induced both in plants infested with non-viruliferous and viruliferous mites, but in the latter treatment its relative expression was much higher, probably indicating an exacerbated response to the viral infection progress. By contrast, the expression of the JA/ET-related TF *MYC2* was induced only in plants infested with non-viruliferous mites, suggesting that its expression is reduced to basal levels upon infection by CiLV-C. The expression of *WRKY70,* the TF responsible for the SA-JA cross-talk, was induced as a result of mite feeding, but it was higher in plants infected with CiLV-C.

CiLV-C Infection Intensifies the Oxidative Stress and Cell Death in Arabidopsis Plants

ROS production was detected using the assay based on the oxidation of 3,3′-diaminobenzidine (DAB), which turns brown in the presence of hydrogen peroxide (H_2O_2). Discrete brownish spots were detected in leaves collected as early as 6 and 12 hai in plants of both mite-infested treatments, and their frequency increased at 24 hai comprising up to 1.5 and 1.9% of the stained tissue area in plants infested with non-viruliferous and viruliferous mites, respectively (**Figures 5A,B**). DAB stained area concentrated in the leaf midribs, which correspond to preferred mites feeding regions. At 8 dai, the number of spots increased and they were observed alongside the leaves as well (**Figure 5C**). Brownish areas were noticeably larger and more frequently seen in virus-infected leaves, representing approximately 25.6% of the leaf area. In plants infested with non-viruliferous mites the stained tissue only represented 4.5% of the leaf. H_2O_2 production was not observed in non-infested plants.

In infested plants, dead cells were detected since 6 hai and they increased in number over time as revealed by trypan blue dying (**Figure 5D**). Foci of dead cells were mostly confined to the leaf main veins and chiefly corresponded to individual cells during the early times of the interaction (6–24 hai). Leaves of plants infested with viruliferous mites presented a higher frequency of blue spots and these were larger as they likely involved greater number of cells. As observed in the accumulation of H_2O_2, spots of dead cells were scattered throughout the virus-infected leaves at 8 dai. No stain was detected in leaves of the healthy control plants.

CiLV-C Infection in Arabidopsis Modules the Behavior of *Brevipalpus* Mites

To evaluate the putative role of CiLV-C infection over the behavior of *Brevipalpus* mites, two sets of healthy Arabidopsis plants were infested with two populations of either viruliferous or non-viruliferous adult mites. Typical localized symptoms

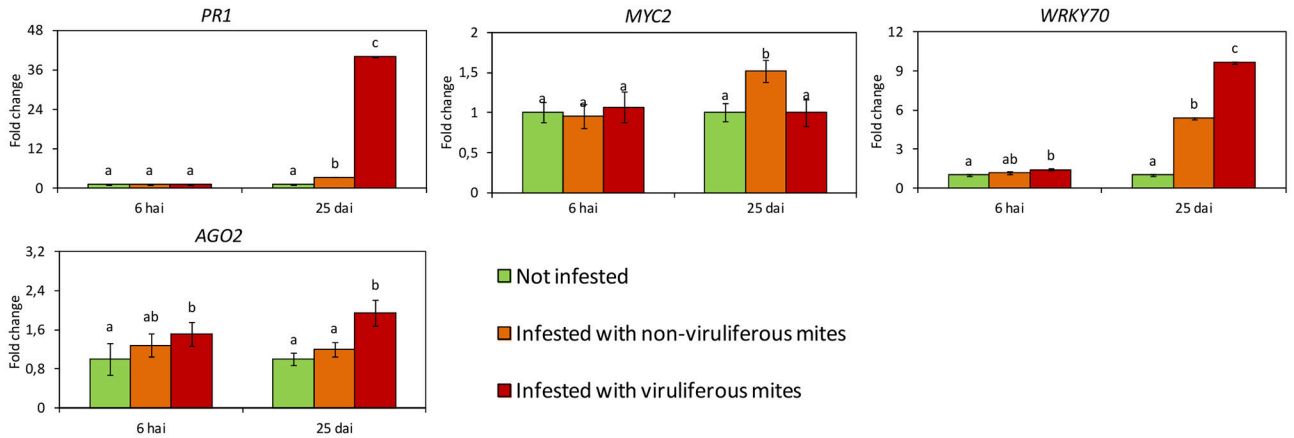

FIGURE 4 | Expression profiles of *Citrus sinensis* genes involved in the main plant defense pathways assessed by RT-qPCR. In green, plants kept without mites. In orange, plants infested with non-viruliferous mites. In red, plants infested with CiLV-C viruliferous mites. Data are presented as fold change values in comparison with not infested plants (with fold change value set to 1). Values represent the mean of 20 biological replicates for each set. Error bars represent standard errors. Different letters correspond to different expression levels between treatments within the same time point (ANOVA and Tukey's HSD *post-hoc* test, $\alpha < 0.05$). hai, hours after infestation; dai, days after infestation.

started to appear at 7 days after infestation with viruliferous mites only. After 13 days of infestation, the number of mites per plant was higher in those infested with viruliferous ones ($p < 0.01$) (Supplementary Table 2, **Figure 6**). While no significant differences in mite numbers were observed between primarily and secondary infested leaves in plants that received viruliferous mites ($p > 0.05$), the non-viruliferous mites were unevenly distributed throughout the infested plants, with higher population densities found in the secondary infested leaves ($p < 0.05$). The total number of mite eggs counted in the two sets of plants did not differ significantly ($p > 0.05$). However, the number of eggs in the primarily infested leaves was superior to that in the secondary infested leaves ($p < 0.01$) for those plants infested with viruliferous mites, whereas no such differences were observed for plants infested with non-viruliferous mites ($p > 0.05$).

DISCUSSION

In this work we provide insights into plant response during citrus leprosis infection, an emergent viral disease that threatens citrus production in the Americas (Roy et al., 2015). Remarkably, this disease reveals an atypical pathosystem in which its etiological agent, CiLV-C, does not systemically spread in the host plants and half of the genes encoded by its genome are considered as orphans since they have no homologs in other viral species (Locali-Fabris et al., 2006; Tautz and Domazet-Lošo, 2011). Additionally, *Brevipalpus yothersi* mites, the CiLV-C vector, also attack hundreds of plant species of very distinct families, have worldwide distribution and exhibit an unusual biology (Weeks et al., 2001; Childers et al., 2003b).

To shed light on citrus leprosis pathosystem, we evaluated the transcriptional profiles of marker genes of Arabidopsis defense pathways, ROS production and the occurrence of cell death upon infestation with either non-viruliferous or CiLV-C viruliferous mites. Moreover, we extended these analyses to sweet orange in order to validate the use of Arabidopsis as a model plant for the study of citrus leprosis.

As a result of the interaction of Arabidopsis with non-viruliferous mites, small areas likely involving a few dead cells proliferated in infested leaves while increased amounts of H_2O_2 were detected within early stages of the interaction. Such a pattern of dead cell patches may be a direct consequence of mite feeding and resembles that observed during interaction of the mite *Tetranychus urticae* with Arabidopsis and bean plants in which only one cell is targeted by the mite's stylet (Bensoussan et al., 2016). *Brevipalpus* activity also elicited the expression of SA pathway genes (biosynthesis, signaling and response), and the JA/ET responsive genes *PDF1.2* and *VSP2*. Notably, the expression level of *PDF1.2* was much higher than that of *VSP2,* ca. 500- and 15-fold, respectively, in comparison to the not infested treatment at 8 dai.

The switching between strong and mild expression levels of the JA-responsive genes was previously described as an herbivore strategy to rewire the plant response in its favor (Verhage et al., 2011). Based on this, our data supports the hypothesis that *B. yothersi* mites might manipulate plant defenses to their own benefit. Typically, plant response to herbivory and tissue damage triggers the JA signaling pathway, which controls two major acting branches (Wu and Baldwin, 2010; Arimura et al., 2011; Erb et al., 2012). Activation of each branch is mediated by several TFs usually represented by MYC2 and ERF1. Whereas the ERF-branch induces the expression of genes such as *PDF1.2* to counteract necrotrophic pathogens, the MYC-branch up-regulates genes such as *VSP2*, which encodes a phosphatase with anti-herbivory activity (Verhage et al., 2011; Kazan and Manners, 2013). TFs from the MYC-branch also interact with TFs of the ERF-branch, and vice versa, repressing each other (Song et al., 2014). Under herbivory attack, wild-type Arabidopsis plants preferably activate the MYC branch; however,

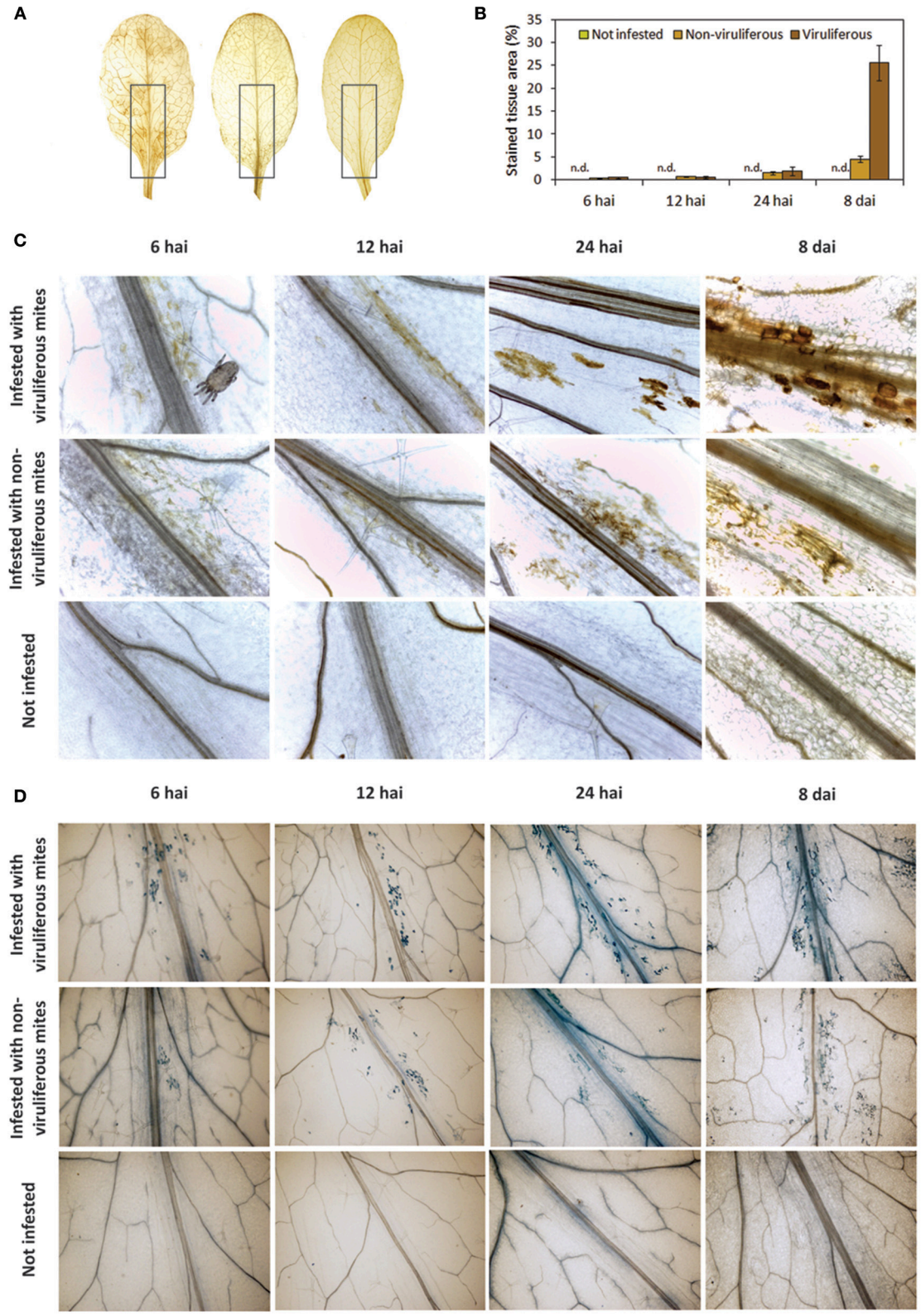

FIGURE 5 | Accumulation of reactive oxygen species (ROS) and cell death in *Arabidopsis thaliana* plants infested with non-viruliferous and CiLV-C viruliferous *Brevipalpus yothersi* mites at 6 hours after the infestation (hai), 12 hai, 24 hai, and 8 days after the infestation (dai). (A) Detection of hydrogen

(Continued)

FIGURE 5 | Continued
peroxide by 3,3′-diaminobenzidine (DAB) staining at 8 dai on leaves infested with viruliferous mites, non-viruliferous mites and not infested, respectively. Highlights on leaves midribs represent the regions where mites concentrate, used to quantification of stained area. **(B)** Quantitative measure of DAB brownish areas on highlighted regions along the course of the infestation. Values represent the means of 10 biological replicates for each set. Error bars represent standard errors. **(C)** Detail of ROS detection in leaves stained with DAB. **(D)** Detail of cell death in leaves stained with trypan blue.

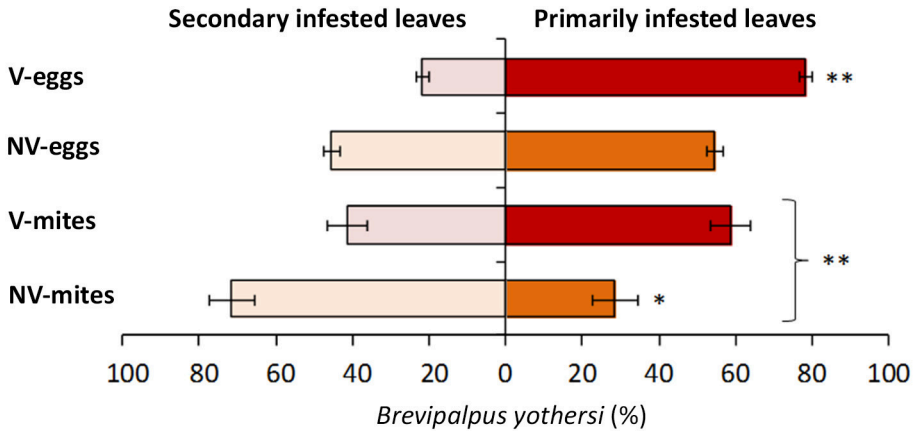

FIGURE 6 | Distribution of *Brevipalpus yothersi* adult mites and eggs in *Arabidopsis thaliana*. Non viruliferous (NV) or CiLV-C viruliferous (V) mites were deposited in three leaves of each plant and were counted at 13 days after the infestation. Data are presented as the average percentages of mites or eggs in the primarily infested leaves (where mites were initially deposited) and secondary infested leaves (due to mite migration to contiguous leaves). Error bars represent standard errors. Statistically significant differences at $p < 0.05$ (*) or < 0.01 (**) are indicated. Bracket indicates the comparison between the number of NV and V mites.

activation of the ERF-branch occurs, for instance, in plants impaired in the MYC-branch, as in the case of Arabidopsis *jar1-1* mutants (Verhage et al., 2011). In the Arabidopsis-*Brevipalpus* interaction assessed in this study, the down-regulation of *JAR1* was accompanied by an increased expression of *PDF1.2* detected at the end of the time course. Therefore, repression of *JAR1* could redirect the JA pathway toward *PDF1.2* thus reducing the anti-herbivory defense conferred by the MYC-branch.

Arabidopsis infestation with *Brevipalpus* also triggered SA signaling leading to the up-regulation of *PR* responsive genes within 24 hai. This has been observed in responses to certain arthropods that cause mild tissue injuries such as the piercing-sucking insects like aphids (Zarate et al., 2007; Arimura et al., 2011). By exploiting the natural cross-talk between the SA-JA/ET signaling pathways, these herbivores suppress JA mediated defenses favoring their own performance (Zarate et al., 2007; Hogenhout and Bos, 2011; Zhang et al., 2013). When induced, SA down-regulates the transcription of JA responsive genes from both ERF and MYC branches through a mechanism mediated by NPR1 and WRKY70 (Caarls et al., 2015). However, differently than in insects, in plant-mite interactions, both SA and JA/ET signaling pathways are simultaneously induced and apparently do not antagonize each other (Zhurov et al., 2014). Although this could be the case of *Brevipalpus*-Arabidopsis interaction, where both *VSP2* and *PDF1.2* were induced at most of the analyzed time points, we cannot exclude that during this interaction SA and JA may actually antagonize each other, although partially, and the JA response we report here is intermediate. Such a relationship was also suggested during plant interaction with *Tetranychus* mites (Alba et al., 2015).

Modulation of plant response by herbivores has been shown to occur by effector proteins present in their saliva (Hogenhout and Bos, 2011). Piercing-sucking insects puncture and deliver effectors to suppress plant defenses and establish compatible interactions. Application of oral secretions of *Pieris rapae* caterpillars on Arabidopsis leaves, for example, activates the ERF-branch, suggesting that compounds in the saliva divert plant response favoring the herbivore (Verhage et al., 2011). Analogous to aphids, spider mites also suppress plant defenses (Sarmento et al., 2011; Alba et al., 2015) through delivery of effectors via their saliva (Villarroel et al., 2016). *Tetranychus evansi* suppresses defense routes in tomato, reducing deterrent compounds to even lower levels than constitutive ones expressed in healthy plants (Sarmento et al., 2011). *Brevipalpus* feeding involves the piercing of plant tissues, and likely, the injection of saliva inside the host cells through a tube formed between its interlocked stylets (Alberti and Kitajima, 2014). Therefore, our results indicating the induction of signaling pathways associated to herbivore manipulation in Arabidopsis suggest that *Brevipalpus* might also inject effectors in their hosts through the saliva. Since herbivore host range is likely limited by its ability to suppress effectual plant defenses (Hogenhout and Bos, 2011), it is suggested that generalists are more suppressive of plant defense than specialists (Ali and Agrawal, 2012). *Brevipalpus* mites are extreme generalists, colonizing a wide range of hosts that includes more than 900 plant species spanning more than a 100 plant families (Childers et al., 2003b). In this regard, the

repertoire of immune response suppression strategies observed during the interaction with Arabidopsis consistently supports the polyphagia of *Brevipalpus* mites.

CiLV-C infection in Arabidopsis triggered SA-dependent genes, a common response of plants to biotrophic pathogens (Pieterse et al., 2009). From a general point of view, the patterns of expression of the SA pathway genes resembled those observed after infestation with non-viruliferous mites, though fold changes of some genes such as *NPR1* and *WRKY70* were higher in the presence of the virus. This activation likely reaches the threshold required to trigger the down-regulation of JA/ET responsive genes. In the presence of CiLV-C, *PDF1.2* and *VSP2* were drastically repressed (13- and 4-fold lower, respectively) at latest stages of the infection when compared to plants infested with non-viruliferous mites, suggesting the occurrence of SA-JA antagonism.

Infestation of Arabidopsis with CiLV-C viruliferous mites typically caused viral infection in the leaves where mites were deposited (primarily infestation). Viral infection as consequence of a secondary infestation due to mite migration between contiguous leaves were barely detected at 8 dai, as observed in **Figure 1**, or even after 13 dai, when the final evaluation was performed. Results obtained in this work indicated that infected leaves become more attractive for the mites. In absence of viral infection, mites were preferentially found in the secondary infested leaves. This behavior seem to be logic considering that the area for secondary infestation is the largest, comprising more and newer leaves available for mite colonization. In contrast, in plants infected by viruliferous mites, no differences were observed in their distribution, and leaves showing typical CiLV-C localized symptoms harbored the largest number of eggs, indicating the preference for oviposition in infected leaves. Moreover, density of mites (mite/plant) reached higher values in the infected Arabidopsis plants. Interestingly, CiLV-C infected sweet oranges plants seem to be also beneficial for *Brevipalpus* mite population. In a multifactorial experiment addressed to evaluate the influence of citrus rootstocks on the relationship between the mite *Brevipalpus* sp. and citrus leprosis disease (Andrade et al., 2013), mite density was significantly higher in CiLV-C infected plants.

Recent studies suggest that some virus-infected plants are more attractive for the viral vector settlements and/or more beneficial to the vector development (Mauck et al., 2012; Casteel et al., 2014; Prager et al., 2015; Su et al., 2015; Casteel and Falk, 2016). Viruses that depend on vectors to move from infected to healthy host plants use this strategy as an effective mean to improve their transmissibility (Belliure et al., 2004; Abe et al., 2012). For instance, infection by *Tomato spotted wilt virus* (TSWV) induces the SA-mediated pathway, which decreases JA-regulated defenses leading to enhanced attractiveness of plants to its vector, the thrips *Frankliniella occidentalis* (Abe et al., 2012). SA pathway induction by CiLV-C and reduction of JA/ET mediated response reported here, in addition to other unknown mechanisms, may benefit *Brevipalpus* infestation. This issue is of special significance in the case of the conspicuous nature of the localized colonization of CiLV-C, because relationship with its vector is not only essential for plant to plant transmission, but also to the infection of other parts within the same plant. On the other hand, CiLV-C may act as a helper (effector-like) factor of mite infestation to suppress the plant defenses. However, whether the viral infection mediated by viruliferous mites indeed may help the mite fitness to plant colonization is yet unclear and has been addressed in ongoing experiments.

During Arabidopsis interaction with both non-viruliferous and viruliferous *Brevipalpus*, fold changes of core genes of the RNA silencing machinery showed low variation and the expression profiles were kept mostly invariable, except in the cases of *RDR6* and *AGO2*, whose expressions were enhanced in response to virus infection at 6 hai and 8 dai, respectively. RISC activity against plant viruses preferentially involves AGO1 and, in case of its suppression, the cell switches to a second layer of defense mediated by AGO2 (Harvey et al., 2011). Increased levels of *AGO2* during virus infection have been observed in Arabidopsis during *Potato virus X* (PVX), *Cucumber mosaic virus* (CMV), *Turnip crinkle virus* (TCV), *Tobacco rattle virus* (TRV), and *Turnip mosaic virus* (TuMV) infections (Carbonell and Carrington, 2015). Control of *AGO2* mRNA levels is mediated by the microRNA miR403 via AGO1 (Harvey et al., 2011). Inactivation of AGO1 by some virus suppressor of RNA silencing (VSRs), e.g., the p38 protein from TCV, leads to the expression of AGO2. TCV-p38 VSR activity is exerted by a WG motif that acts as a hook for AGO1 protein, disabling RISC (Azevedo et al., 2010). Interestingly, a WG/GW motif is also present in the COOH terminal domain of the CiLV-C RdRp protein, suggesting that the activation of AGO2 reported here could result from the hijacking of AGO1 in an analogous manner as the one described for TCV. Further experiments need to be carried out to test the putative VSR activity derived from CiLV-C proteins.

Plant gene expression during CiLV-C resembles that observed in the course of a plant-virus interaction, where SA pathway and RNA silencing are activated (Mandadi and Scholthof, 2013). The current understanding is that both defense responses act coordinately to counteract viral infection (Alamillo et al., 2006; Lewsey et al., 2010; Jovel et al., 2011; Hunter et al., 2013). SA-mediated defense interacts with RNA silencing through ICS1- and NPR1-dependent up-regulation of RDR1, which participates in the generation of secondary siRNA (Hunter et al., 2013). Evidence of interplay between both defenses against viral infections are increasing in the literature, e.g., *Plum pox virus* (PPV) infection of *Nicotiana tabacum* results in the SA-mediated potentiation of RNA silencing, thus inhibiting PPV systemic movement (Alamillo et al., 2006). In sour orange plants (*Citrus aurantium*), *Citrus tristeza virus* (CTV) accumulation and spread are enhanced when the genes *RDR1*, *NPR1,* or *DCL2-DCL4* are silenced (Gómez-Muñoz et al., 2016). For the CiLV-C/Arabidopsis interaction, the induction of *RDR1* corroborates with this mechanism and indicates that the early activation of SA in response to mite feeding could pre-induce resistance to CiLV-C replication.

As observed during the interaction of Arabidopsis with non-viruliferous *Brevipalpus*, infestation with CiLV-C viruliferous mites also elicited the production of H_2O_2. However, in this case both the intensity of histochemical signals and fold changes of *RBOHD* were higher than in the former case, further confirming

our conclusion that infection by the virus amplifies the host responses. Moreover, in these plants, the frequency of patches of dead cells was also higher, indicating that the plant actively recognizes the presence of CiLV-C. The oxidative burst both activates cell death at the infection site during HR and signals defense pathways beyond the infection site (Levine et al., 1994; O'Brien et al., 2012). Thus, the observed cell death during CiLV-C infection may be a consequence of HR which on its turn would limit the systemic colonization of the host. Indeed, characteristic necrotic symptoms of CiLV-C infection resemble the outcome of HR-like, although the evolution up to necrosis shows an atypically slower progression in sweet orange than that expected in case of a typical hypersensitive reaction.

Induction of HR in cells of the vascular tissue of sweet orange leaves was previously proposed as the cause for the non-systemic spread of CiLV-C (Marques et al., 2007, 2010). In this regard, caution has to be taken to interpret these and our own results. Despite the strict relation between cell death and pathogen restriction, evidence that HR limits viral colonization remains controversial (Coll et al., 2011). Moreover, effectiveness and contribution of MP and other CiLV-C-encoded proteins in the movement of this virus remain unknown. However, regardless of whether or not cell death is responsible for restricting CiLV-C systemic movement, the presence of dead cells in association with ROS production are strong evidence of HR-like response elicitation in lesions surrounding the feeding sites of viruliferous mites.

In line with the integration of the RNA silencing with the classic frame of resistance by PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (Pumplin and Voinnet, 2013; Sansregret et al., 2013), it is tempting to speculate that the host plants could recognize CiLV-C during activation or suppression of RNA silencing and summon a classic defense response. Recognition of CiLV-C could be from dsRNA produced by virus replication or secondary siRNA derived from RISC, triggering PTI defenses. Otherwise, plant could trigger an ETI response through R-mediated recognition of the putative VSR. In both cases, activation of the SA may convey to an HR-like response, restricting the virus near the inoculation site by the mite vector. Altogether, sequences of facts here reported during Arabidopsis/CiLV-C interaction, e.g., expression of HR-related genes, activation of effective defense pathways against viral infections, ROS burst sustained along the infection, localized cell death and pathogen restriction at the infection site, suggest that CiLV-C symptoms are the outcome of a HR-like resistance. Consequently, citrus leprosis should be considered the result of an incompatible rather than a compatible interaction.

Finally, responses of sweet orange to the *Brevipalpus* feeding and CiLV-C infection mirrored those detected in Arabidopsis. Our analysis revealed the activation of SA- and JA/ET-dependent defenses against mite feeding. Upon viral infection on viruliferous mites-infected plants, we observed induction of SA pathway and AGO2-mediated RNA silencing and reduction of the JA/ET defenses. However, the defense response was not established at 6 hai, which indicates a later response of *C. sinensis*. Time of symptoms appearance is likely an indicative of this phenomenon: lesions developed from 17 to 25 dai on the woody perennial *C. sinensis*, while in the herbaceous Arabidopsis this period was reduced to 7 to 10 dai. Although with a slower progression, the natural host developed a similar pattern of response than Arabidopsis, which validates this species as a model to be used in interaction studies of citrus leprosis disease.

With the results presented here, we propose the first model of Arabidopsis/*B. yothersi*/CiLV-C interaction (**Figure 7**). This model represents a starting point to understand the processes leading to the development of citrus leprosis and, possibly, other diseases caused by non-systemic viruses also transmitted by these vectors. Future studies shall aim at complementing the model especially with regards to the lacking components and to the contribution of each hormonal pathway to such conspicuous interaction.

MATERIALS AND METHODS

Plant Material

Seeds of the *Arabidopsis thaliana* ecotype Columbia (Col-0) were obtained from the Arabidopsis Biological Resource Center (ABRC). Seeds were sown in sterilized soil in 100 mL pots and incubated during 4 days at 4°C in a dark chamber. After this, plantlets were transferred to a controlled growth chamber (Adaptis AR A1000, Conviron, Winnipeg, Canada) set at 22 ± 2°C and with a 12 h light/dark cycle where they were kept throughout the experiments. *Citrus sinensis* L. Osbeck cv. Pera plants were grown from seeds under greenhouse conditions.

Mite (*Brevipalpus yothersi*) Rearing and Infestation

A population of non-viruliferous mites was obtained from a single female collected from a citrus orchard in the State of Bahia, Brazil, and further confirmed as *B. yothersi* using phase contrast microscopy as reported elsewhere (Beard et al., 2015). Mites were reared onto unripe fruits of leprosis-immune "Tahiti" acid lime (*Citrus latifolia* Tanaka). All fruits were previously cleaned, dried and partially submerged in liquid paraffin to prevent desiccation. Mites were transferred to an area of approximately 4 cm in diameter of the fruit surrounded by a barrier of the pest adhesive Biostop gum (Biocontrole, Indaiatuba, Brazil) prepared with a wet mixture of wheat flour, plaster, and fine sand (1:1:2) (Rodrigues et al., 2007). Viruliferous mites were obtained by rearing the non-viruliferous mites from "Tahiti" acid lime as described above on sweet oranges fruits collected from a citrus grove with high incidence of leprosis caused by the CiLV-C strain SJP (Ramos-González et al., 2016) in São José do Rio Preto, State of São Paulo, Brazil. Mites from both viruliferous and non-viruliferous populations were reared for several generations and periodically evaluated for the presence of CiLV-C by RT-PCR using primer pairs designed for a region within the CiLV-C *MP* gene (Locali et al., 2003).

Assessment of CiLV-C Inoculation Access Period by Viruliferous *B. yothersi* Mites

To define the period needed to achieve 100% of CiLV-C infection of plants after infestation with viruliferous *B. yothersi* mites, five viruliferous mites were transferred to each of 10 Arabidopsis

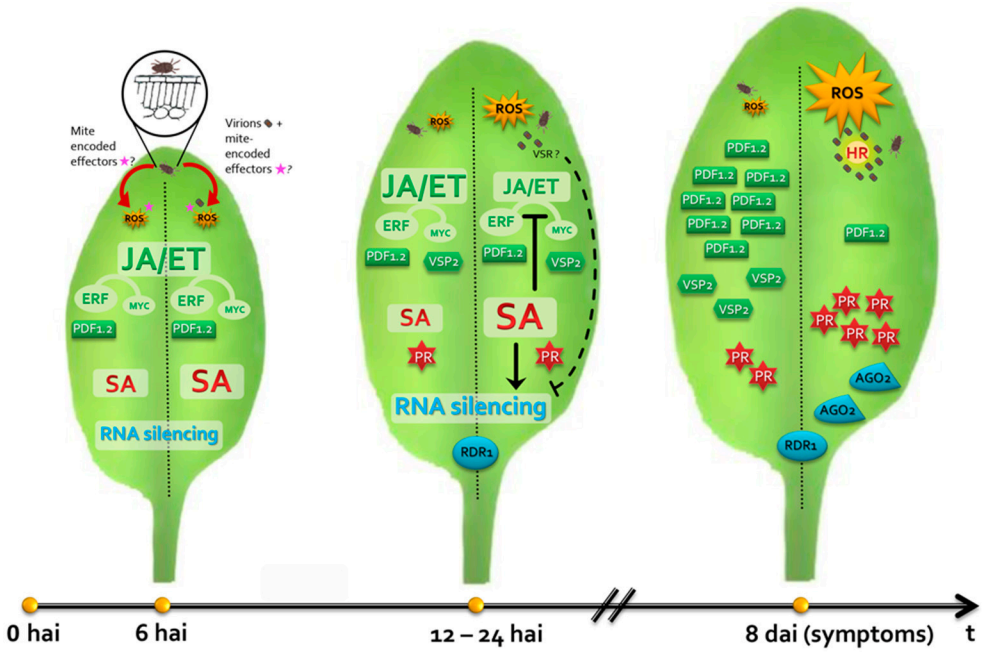

FIGURE 7 | Model representing the interaction of *Arabidopsis thaliana* plants/*Brevipalpus* mites/CiLV-C. Left and right leaf halves show hallmarks during the plant interaction with non-viruliferous and viruliferous mites, respectively. During the feeding of non-viruliferous mites, saliva and putative mite-encoded effectors are delivered into the mesophyll cells targeted by the mite's stylet. Reactive oxygen species (ROS) burst, localized cell death and SA and JA/ET pathways are triggered. Mite induces the expression of the ERF-branch responsive gene *PDF1.2* rather than the MYC-branch responsive gene *VSP2*, which might restrain plant response against herbivores. In plant infested by CiLV-C viruliferous mites, virions reach mesophyll cells mixed with the salivary flow. CiLV-C multiplies in the initially infected cell and locally moves to the neighboring cells. Mite feeding and the increasing viral loads generate a stronger stimulus which intensifies the ROS production, the number of dead cells around the mite feeding site and the SA-mediated response. Increased activity of the SA pathway likely promotes the inhibition of the JA/ET pathways, leading the downregulation of *PDF1.2* and *VSP2,* which probably improve the mite performance. Both non-viruliferous and viruliferous mite infestations induce expression profile changes in the core genes of the gene silencing mechanism. Enhanced SA signaling activity in viral presence may contribute to higher *RDR1* expression. A putative virus suppressor of RNA silencing (VSR) may target and inactivate AGO1, leading to the up-regulation of *AGO2*, which addresses a second antiviral defense line of the RNA silencing. CiLV-C remains restricted at cells surrounding the inoculation site and chlorosis symptoms develop seven to 10 days after inoculation, probably as a result of a hypersensitive-like response (HR) as consequence of an incompatible interaction.

plants, where they were kept for 4 or 6 h. After the inoculation period, mites were removed using a small brush and plants were maintained in a controlled chamber until the development of symptoms. As positive controls, 10 plants were kept infested with viruliferous mites throughout the experiment. The inoculation periods were predetermined considering available data of CiLV-C infection of common bean (*Phaseolus vulgaris*) plants using viruliferous *B. yothersi* (Garita, 2013). Plants were evaluated daily for symptoms and CiLV-C infection was evaluated by RT-PCR (Locali et al., 2003) at the end of the experiment.

Time-Course Gene Expression Analysis on Arabidopsis

A time course gene expression analysis was conducted on plants infested with viruliferous or non-viruliferous mites and on non-infested control plants at 6, 12, and 24 h after infestation (hai) and at 8 days after infestation (dai), when symptoms were visible. Four-week-old *A. thaliana* Col-0 plants were grouped in sets of 20 individuals and were assigned to each treatment. For infestation, five (viruliferous or non-viruliferous) mites were transferred to each of three expanded rosette leaves per plant using a small brush and a stereoscopic microscope. Infested or control leaves were collected at each time-point; leaf samples from two plants were pooled, totaling 10 biological replicates per treatment per time point. Once collected, leaves were flash-frozen in liquid N_2 and stored at −80°C until RNA extraction.

Time-Course Gene Expression Analysis on *C. sinensis*

The time course experiment on sweet orange (*C. sinensis*) seedlings was established with the same infestation treatments and two time points after infestation: 6 hai and 25 dai, corresponding to 100% inoculate and 100% symptomatic plants, respectively. Selection of 6 hai as the first time for evaluation was based on previous results of inoculation access period in Arabidopsis obtained in this work, and those described elsewhere using common bean plants (Garita et al., 2013). Twenty plants per treatment were assayed. Fifteen mites were transferred to one leaf per plant, which was previously coated with a wet mixture of wheat flour, plaster and fine sand (1:1:2) as described above (**Figure 1**) (Rodrigues et al., 2007). Each collected leaf represented an

independent biological replicate, totaling 20 biological replicates per treatment per time point. Once collected, leaves were flash-frozen in liquid N_2 and stored at −80°C until RNA extraction.

RNA Extraction and cDNA Synthesis

Plant RNA was purified from approximately 100 mg of leaves using the RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands). Residual plant DNA was removed by RNAse free DNAse (Qiagen, Venlo, Netherlands) during RNA extraction. RNA quantification and A_{260}/A_{280} ratios were estimated using the NanoDrop ND-8000 micro-spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA integrity was evaluated in 1.2% agarose gels and the removal of genomic DNA was confirmed by reverse transcription (RT-) PCR assays using the RNA as template. cDNA corresponding to each sample (500 ng of total RNA) was generated using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) as described by the manufacturer. cDNA solutions were diluted 25-fold in RNAse-free water for subsequent analysis by qPCR.

CiLV-C Detection and Quantification

CiLV-C loads were assessed by RT-qPCR using a TaqMan® probe complementary to the viral *p29* ORF (G.D. Arena, P.L. Ramos-González, M.A. Machado, J. Freitas-Astúa, *unpublished data*). Reaction mixes were prepared as recommended by the TaqMan® Fast Universal PCR MasterMix 2X kit manufacturer (Thermo Scientific, Waltham, MA, USA) and the amplifications were carried out in a 7500 Fast Real-Time PCR System device (Thermo Scientific, Waltham, MA, USA). Each sample was analyzed in triplicate and three template-free controls were performed for each primer pair. Cq-values were compared with a standard curve to determine absolute quantities of CiLV-C *p29* molecules. Absolute *p29* quantities in infected *A. thaliana* and *C. sinensis* were normalized using the expression levels of the species-specific *SAND* genes as references. Average of the *p29* quantities of each time point were statistically compared with one-way ANOVA and Tukey's HSD (honest significant difference) tests ($\alpha < 0.05$).

Reference Genes Evaluation

Six candidate genes were selected for expression stability analyses based on previous data obtained from *A. thaliana* subjected to different stresses (Czechowski et al., 2005) or virus infection (Lilly et al., 2011) or considering the expression profile of their *Citrus* spp. homolog during the infection with CiLV-C (Mafra et al., 2012). The candidates *EF1A, F-BOX, GAPDH, PPR, SAND,* and *TIP41* were amplified using the primer pairs listed in Supplementary Table 1. Expression levels were assessed by RT-qPCR in Arabidopsis plants infested by either viruliferous or non-viruliferous mites and in non-infested plants. Cq-values and relative quantities were determined as described below for gene expression analysis. Cq-values were imported into geNorm software (Vandesompele et al., 2002) for stability analysis and further selection of the reference genes with the lowest *M*-values.

Gene Expression Analysis

Transcript levels of defense-related genes were assessed by RT-qPCR. All primer sequences were obtained from the literature (Supplementary Table 1) and validated by PCR and RT-qPCR. RT-qPCR mixes were prepared for a final volume of 25 uL with 10 uL of GoTaq qPCR Master Mix (Promega, Madison, WI, USA), 120 or 150 nM of each gene-specific primer pair and 3 uL of the diluted cDNA. Amplifications were performed in a 7500 Fast Real-Time PCR System (Thermo Scientific, Waltham, MA, USA) device, using the standard thermal profile: 95°C for 20 s followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. Each sample was analyzed in triplicate. To confirm the absence of genomic DNA and unspecific reactions, the dissociation curves of each reaction was inspected and three template-free controls were included for each primer pair. After this, primer efficiency and quantification cycle values were determined for individual RT-qPCR using the algorithm of the Real-time PCR Miner (Zhao and Fernald, 2005). Gene expression analyses were performed according the ΔCq model using multiple reference genes (Hellemans et al., 2007). The efficiency value (E) of each primer pair was estimated as the arithmetic mean of values from all samples. The Cq-value of each sample, expressed as the mean of the three technical replicates, was converted into relative quantities (RQ) using the function $RQ=E^{\Delta Cq}$, where ΔCq is the difference between the lowest Cq-value across all samples for the evaluated gene and the Cq-value of a given sample. A normalization factor (NF) for each sample was calculated by the geometric mean of the RQ-values of the three reference genes. Normalized-relative quantity (NRQ) of each sample was calculated as the ratio of the sample RQ and the appropriate NF. Individual fold change values were determined by dividing the sample NRQ by the mean NRQ of samples of the calibrator, that is, non-infested control plants; this procedure renders a mean fold change value of 1 for the set of plants in the non-infested treatment. For statistical analysis, one-way ANOVA and Tukey's HSD test ($\alpha < 0.05$) were used to compare the mean expression levels of the treatments. Statistical analysis was individually done for each gene at each time-point. For the *EDS5* and *NHL10* genes, the number of transcripts in mite-free plants was lower than the detection limit of the assay. In those cases, Cq-values were not determined and the data of mite-infested plants were considered as highly significant.

Histochemical Detection of H_2O_2 and Cell Death

Histochemical analyses of Arabidopsis plants were carried out in a time course manner with the same infestation treatments and time points of the gene expression analysis. Ten plants per treatment combination were assayed. Five mites were transferred to each of two expanded rosette leaves per plant. Each infested leaf of a plant was assayed for H_2O_2 production or cell death. Dead cells were visualized by staining with trypan blue (Martinez de Ilarduya et al., 2003). Infested or control leaves were boiled in the presence of lactophenol-trypan blue for 2 min and incubated overnight at room temperature. Excess dye was removed with chloral hydrate (2.5 g/mL) for 4 days. H_2O_2 was detected by

staining with DAB (Martinez de Ilarduya et al., 2003). Leaves were vacuum infiltrated for seconds and incubated for 5 h in the dark to allow the infiltration of DAB. Tissues were boiled in a mix of ethanol:acetic acid (3:1) for 15 min. Samples were examined under bright-field light using an Olympus MVX10 (Olympus, Tokyo, Japan) microscope and images were capture with an Olympus DP71 (Olympus, Tokyo, Japan) camera. Brownish areas were measured with the QUANT software (Vale et al., 2003).

Biology Behavior of Viruliferous and Non-viruliferous Mites in Wild Type Arabidopsis Plants

Two groups of 15 and 11 healthy Arabidopsis Col-0 plants were infested with CiLV-C viruliferous or non-viruliferous *Brevipalpus yothersi* mites, respectively. Five mites were transferred to each of three expanded rosette leaves of each plant (primary infestation), completing fifteen mites per plant. Sources of mites and their manipulation were described above. After 13 days of infestation, plant leaves were carefully detached, and the number of *Brevipalpus* eggs and adults were counted. The number of eggs and mites found in the primary infested leaves were compared to those found in the rest of the leaves (secondary infestation) using the Mann Whitney non-parametric test implemented in R. Furthermore, the same data were compared using t-test, with similar results. For the parametric test, data were normalized following ln(x) transformation. Presence or absence of CiLV-C in leaf samples was confirmed by RT-qPCR as described above.

AUTHOR CONTRIBUTIONS

GA, PR, and JF conceived and designed the experiments; GA, PR, and MN performed the experiments; GA, PR, MR, LC, and JF analyzed the data; EK, MR, MM, and JF contributed with reagents/materials/analysis tools; GA, PR, and JF wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01757/full#supplementary-material

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CAPÍTULO 2

Hypersensitive response and SA-related defenses are triggered by citrus leprosis virus C infection and mimicked by the expression of the P61 viral protein

Keywords: plant-virus interaction, hypersensitive response, defense pathways, salicylic acid, jasmonic acid, cross-talk, RNA-Seq

Abstract

Citrus leprosis virus (CiLV-C) is an atypical virus that does not spread systemically in any of its known plant hosts. Upon inoculation with CiLV-C viruliferous *Brevipalpus* mites, only localized lesions are observed, and viral infection remains restricted to cells around the inoculation sites, a phenotype that resembles the outcome of a hypersensitive response (HR). Here we aim to expand the limited knowledge of the molecular mechanisms underlying plant/CiLV-C interaction. To unravel the kinetics of CiLV-C accumulation, we developed RT-qPCR assays to detect viral genome and the *p29*-subgenomic RNA along the course of the infection in Arabidopsis plants. CiLV-C loads increase continuously during infection with three distinct replication steps. To better understand the global plant response to CiLV-C, we used RNA-Seq to evaluate the transcriptome of infected plants. Plant transcriptome is progressively reprogrammed in parallel to increasing viral titer. Gene ontology enrichment analysis revealed that plant responds to CiLV-C presence with the induction of cell growth-related processes at early stages of the infection, and the triggering of processes related to the SA-mediated pathway, ROS burst and HR at the pre-symptomatic stage. Conversely, infected plants down-regulates JA and ET-mediated pathways and processes involved in the primary metabolism that includes photosynthesis. The regulation of co-expressed genes by specific classes of transcription factors support the induction of cell growth and HR/SA-mediated defenses, and repression of JA pathway. To clarify the role of CiLV-C proteins in eliciting plant responses, we expressed them individually in *Nicotiana benthamiana*. *Agrobacterium*-mediated transient expression of the CiLV-C p61 protein consistently leads to a ROS burst, increased expression of SA- and HR-related genes, induced levels of SA, reduced levels of JA, and cell death. Mimicry of responses typically observed during CiLV-C-plant interaction indicates p61 as the

putative viral effector to be neutralized by plant defenses and the determinant of the HR-like symptoms associated to the viral infection. Our results support the theory that the symptoms of CiLV-C infection might be outcome of an incompatible rather than a compatible interaction.

1. Introduction

Citrus leprosis virus C (CiLV-C) is the prevalent causal agent of citrus leprosis, a re-emerging viral disease affecting citrus orchards. Citrus leprosis is considered the most important viral disease of citrus industry in Brazil, the world leader in sweet orange production, costing an annual investment of millions of dollars for the chemical control of the mite vector *Brevipalpus yothersi* (Bastianel et al., 2010). Endemic in the Americas, the virus has spread throughout the South and Central America and already reached the north of the continent (Castillo et al., 2011), threatening nearby citrus producers such as USA. Members of the species *Citrus leprosis C cilevirus*, the type of the genus *Cilevirus*, have a bipartite genome of two positive (+) sense single-stranded RNA molecules encoding six proteins. RNA1 (8745 nt) harbors two open reading frames (ORFs) encoding the RNA-dependent RNA polymerase (RDRP) and the putative coat protein (P29). RNA2 (4986 nt) presents four ORFs encoding the putative movement protein (MP) and the P15, P61, and P24 proteins with unknown functions (Locali-Fabris et al., 2006; Pascon et al., 2006). Although P61 and P24 shows distant homology with structural proteins of the insect-specific negeviruses (Kuchibhatla et al., 2014), half of the CiLV-C genes are considered as orphans (Tautz and Domazet-Lošo, 2011) since they have no homologues in other viral species. CiLV-C RNA1 generates one sub-genomic RNA (sgRNA) of 0.7 kb for the expression of *p29* gene, and RNA2 generates three co-terminal sgRNAs of 3 kb, 1.5 kb and 0.6 kb corresponding to *p61*, *mp* and *p24* genes (Pascon et al., 2006).

Plant cells supporting successful replication of viruses are the source for local infections through contiguous cells, invasion of the vascular system and tissue systemic infections. Differently from other plant viruses, CiLV-C is unable to accomplish systemic infection in any of its natural or experimental host species belonging to distant plant families (Arena et al., 2013; Garita et al., 2014). CiLV-C particles are found mainly in the plant parenchyma and epidermal cells but rarely in vascular cells (Rodrigues et al., 2003), which may partially explain the virus inability to perform long-distance movement. Invariably, CiLV-C remains restricted to cells around the inoculation sites by their mite vector, where symptoms of viral infection are manifested as localized chlorotic or necrotic lesions. Phenotypically, these symptoms resemble the outcome a hypersensitivity response (HR), a cell death resistance process accompanied by

the pathogen restriction at the inoculation site during an incompatible interaction (Arena et al., 2016).

Plant defense mechanisms against pathogens are based primary on cell surface receptors that recognize pathogen/microbe-associated molecular patterns (P/MAMPs) and mount the so-called pattern-triggered immunity (PTI). Successful pathogens produce effectors that counteract PTI but are subject to the direct or indirect recognition by plant resistance (R) proteins that trigger an effector-triggered immunity (ETI) (Jones and Dangl, 2006). Metabolic changes induced during plant defense can result on a burst of reactive oxygen species (ROS) that may culminate in a HR (Xia et al., 2015). The transcriptional reprogramming that results in the defense responses is mediated by the action of interconnected phytohormonal-dependent pathways and directed according to the nature of the injury. Typically, the salicylic acid (SA) pathway confers resistance to biotrophic pathogens and antagonizes the jasmonate/ethylene (JA/ET) pathways that in turn induce defenses against herbivores and necrotrophic pathogens (Pieterse et al., 2012). Hormonal-mediated pathogen resistance is also connected with the main antiviral defense, the RNA silencing. Plant viruses carry effectors that acts as viral suppressors of RNA silencing (VSR) which can be perceived by plant R proteins and trigger typical outputs of ETI, including HR (Pumplin and Voinnet, 2013).

Several viral-plant proteins interacting partners have been identified as elicitors of hormonal defense responses. Activation of such responses can trigger HR and prevent viral infection. For instance, the P0 protein from poleroviruses elicits an HR that is associated to the *Nicotiana glutinosa* gene *RPO1*, a likely immune receptor of P0 (Wang et al., 2015). Likewise, the P38 protein from turnip crinkle virus is the avirulence factor recognized by the Arabidopsis R protein *HRT*, which activates an HR-mediated resistance (Cooley et al., 2000; Pumplin and Voinnet, 2013). Interestingly, both P0 and P38 also acts as viral suppressor of RNA silencing (VSR), supporting the connection between RNA silencing and classical mechanism of resistance against pathogens. Furthermore, viral proteins can modulate hormonal pathways to establish mutualism with its vector (Casteel and Falk, 2016). For example, the NIA-Pro protein from turnip mosaic virus (TuMV) interferes with ET-mediated responses, resulting in defense suppression and consequent enhanced performance of its vector, the aphid *Myzus persicae* (Casteel et al., 2015). Viruses that depend on vectors to move from infected to healthy host plants use this strategy of decrease anti-herbivory defense as an effective mean to improve their transmissibility (Abe et al., 2012).

Despite the economic importance and uniqueness of CiLV-C, little is known about the molecular mechanisms during plant infections by this virus. A previous study conducted in *Arabidopsis thaliana* revealed that CiLV-C infection triggers ROS burst and cell death, induces the classical antiviral mechanisms of RNA silencing and SA pathway, suppress the JA-dependent response, and favor the colonization of the mite vector. A model of the interaction using these information was depicted (Arena et al., 2016), but it is still a preliminary draft where many underlying mechanisms of CiLV-C infection remain to be understood. To assess the kinetics of CiLV-C accumulation, we developed specific RT-qPCR detecting viral genome and *p29*-subgenomic RNA and quantified viral loads along the course of the infection. To unravel the global plant response to CiLV-C, we evaluated the transcriptome of infected Arabidopsis plants by RNA-Seq, whereas the role of CiLV-C proteins in eliciting plant responses were assessed by expressing them individually in *Nicotiana benthamiana* plants. Current work expands our knowledge of the plant/CiLV-C interaction and contributes to the identification of both plant and viral proteins involved in the development of citrus leprosis disease.

2. Results

2.1 CiLV-C titer increases continuously through first ten days of leaf infection, a period during which three distinct replication steps are clearly distinguished

The kinetics of accumulation of CiLV-C in Arabidopsis infected leaves was evaluated by RT-qPCR to identify the major steps of viral multiplication and gene expression during the infection. TaqMan-based assays were developed for the quantitative detection of sequences within two ORFs from the CiLV-C RNA1: *p29*, which codes for the putative capsid protein and is transcribed in a subgenomic RNA, and *RdRp*, coding for the viral replicase which is directly translated to protein from the genomic RNA. To assess the amount of both genomic and subgenomic RNA molecules, amplicons corresponding to each region were cloned in plasmids that were further 10-fold serially-diluted to generate standard curves (Fig. 1A, B). Regression analysis of these standard curves demonstrated the high quality of the assays (R^2 = 1 and 0.999 for *p29* and *RdRp*, respectively), high amplification efficiencies (E = 0.96 and 0.92 for *p29* and *RdRp*, respectively), high sensitivity (detecting up to 10^2 molecules) and covered a wide dynamic range (six log units of concentration) (Fig. 1A, B).

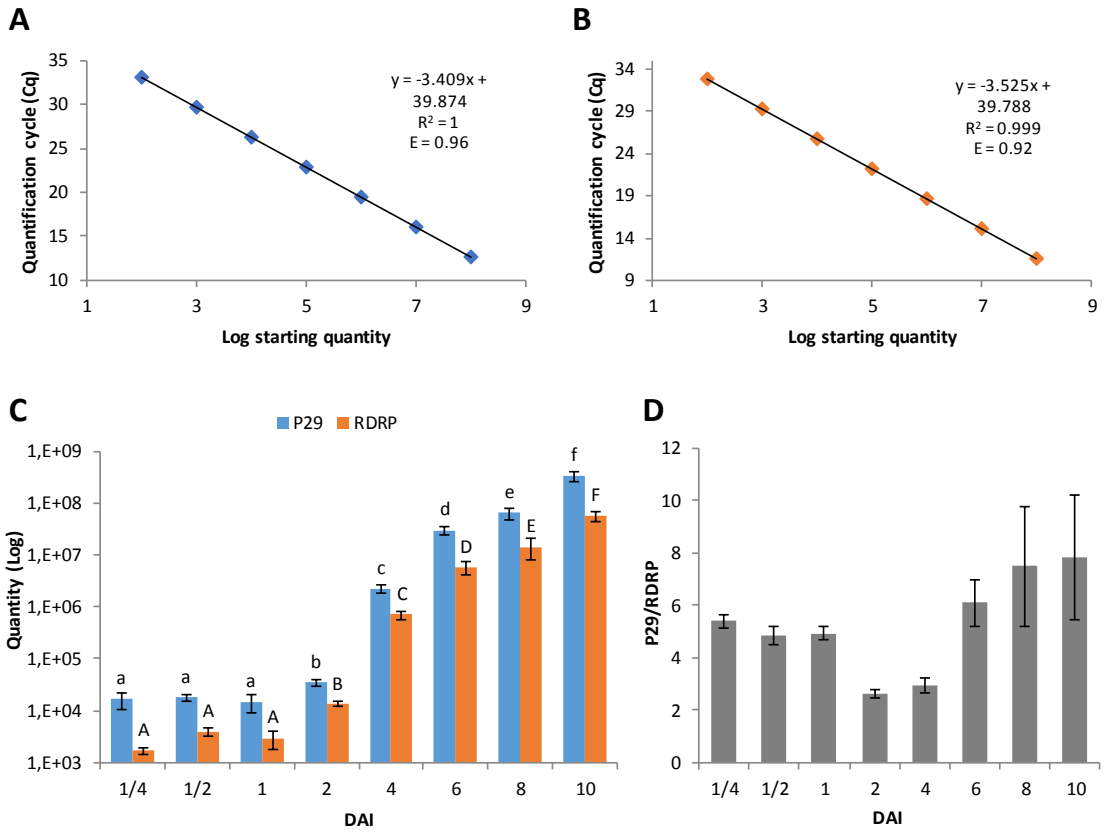

Figure 1. Accumulation of CiLV-C *p29* and *RdRp* RNA molecules through the course of viral infection in *Arabidopsis thaliana*. Standard curves were obtained from serial dilutions of plasmids containing sequences within *p29* (A) and *RdRp* (B) genes. Absolute quantities of both molecules (C) and the *p29/RdRp* ratio (D) were determined eight time points after infestation with viruliferous *Brevipalpus yothersi* mites. Different letters correspond to different number of copies between the time points assessed (ANOVA and Student´s t-test, $\alpha < 0.05$). E = efficiency, DAI: days after the infestation.

To quantify the CiLV-C loads along the course of the viral infection in Arabidopsis, a time course experiment was set up. Plants were infested with *B. yothersi* viruliferous mites for ¼, ½, 1, 2, 4, 6, 8 e 10 days. Symptoms of CiLV-C infection appeared at 7 days after the infestation (dai) in 100% of the infested plants that were kept until the two latest time points. Using the assays and standard curves described above, the absolute quantities of *p29* and *RdRp* molecules were determined (Fig. 1C). All test-samples were positive in both analyses, confirming CiLV-C infection. Level of both molecules kept invariable during the first 24 hours after the infestation (hai). After this point, they increased continuously until the last time point [Tukey's *honest significant difference* (HSD) test, $\alpha \leq 0.05$]. The highest difference between sequential time points was obtained from 2 to 4 days after the infestation (dai), when *p29* and *RdRp* increased 66- and 53-fold, respectively (Fig. 1C).

To better understand the replication process, the ratio *p29*/*RdRp* at each evaluated time point was calculated (Fig. 1D). The number of *p29* molecules was higher than *RdRp* across the whole experiment (Fig. 1C, D). Higher accumulation of *p29* was expected as the assay detects both genomic and sub-genomic RNAs, whilst *RdRp* assay quantifies only the genomic RNA1. Within 2-4 dai, the ratio reached the lowest level and after this period the differences between sub-genomic and genomic RNAs increased to a level much higher than those observed during the first 24 hai (Fig. 1D). The ratio observed at two days matched with increased amount of *RdRp* which may be considered a peak of genome accumulation before the burst of *p29* mRNA transcription. This experiment allowed us to define three main steps during CiLV-C replication in Arabidopsis: i) 0-24 hai, ii) 2-4 dai, and iii) 6-10 dai.

The results obtained from the kinetics of CiLV-C accumulation were considered to select appropriate time-points for the further evaluation of plant response to viral infection. The first time points of these steps that also represent the main events of viral infection were selected. They were as follow: 6 hai, which correspond to the minimum inoculation access period required by mites to obtain 100% of infected plants by viruliferous mites (Arena et al., 2016), 2 dai, the first moment during the infection when a significant increase in viral genome after initial inoculation is obtained, and 6 dai, the pre-symptomatic stage when transcriptional events likely take place to culminate in the disease phenotype.

2.2 CiLV-C infection triggers significant transcriptome changes proportional to increasing viral loads

The global response of Arabidopsis along the course of CiLV-C infection was assessed by RNA-Seq. Plants infested with viruliferous mites (CiLV-C-infected) were compared with those infested with non-viruliferous ones (mock) at 6 hours after infestation (hai), 2 days after infestation (dai) and 6 dai. Illumina sequencing generated roughly 924 million paired-end reads, with an average of 38.5 million per library and higher number of reads from mock treatment (Supplementary Table 1, Fig. 2A). Overall, 93.5% of the reads aligned in the *A. thaliana* reference genome, with a 90.6% average of uniquely mapped reads (Supplementary Table 1, Fig. 2B). The CiLV-C infected samples from 6 dai had the lowest percentage of unmapped reads probably due to the higher virus titer and consequently a higher number of reads mapping to the virus genome (Fig. 2B).

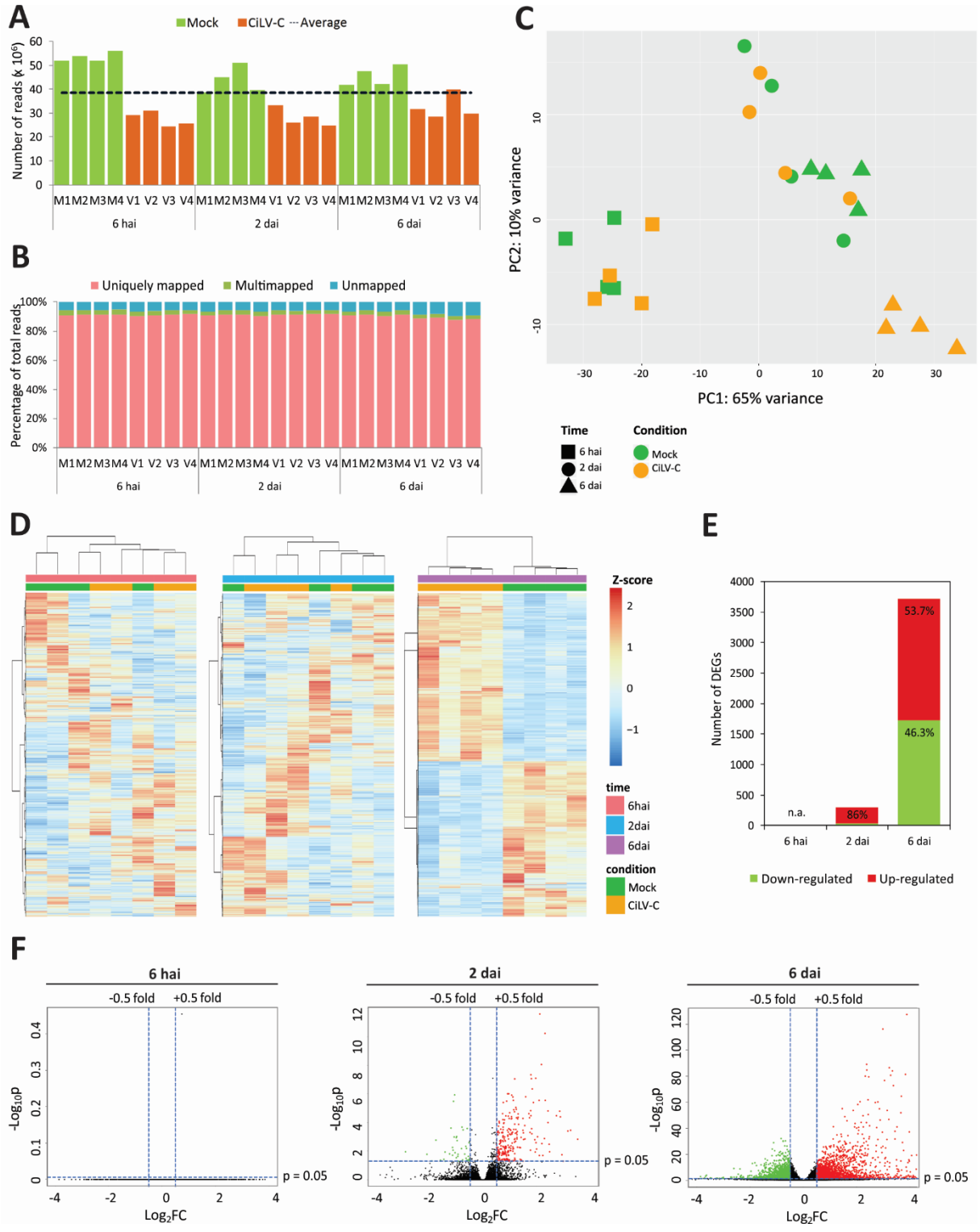

Figure 2. Overview of *Arabidopsis thaliana* transcriptome upon CiLV-C infection. A) Number of *paired-end* reads generated for each library by Illumina HiSeq sequencing. M: mock-infected (plants infested with non-viruliferous mites), V: virus-infected (plants infested with CiLV-C viruliferous mites). Dashed line represents the average of *paired-end* reads from all 24 libraries. B) Proportion of uniquely mapped, multi-mapped and unmapped reads obtained for each library. Reads were mapped in the *Arabidopsis thaliana* (TAIR 10) genome using *TopHat2*. M: mock-infected, V: virus-infected plants. C) Principal component analysis of normalized count data from all samples. D) Hierarchical clustering analysis of normalized count data z-scores

exhibited by differentially expressed genes (DEGs) of each sample within each time point. E) Numbers of up- and down-regulated DEGs in CiLV-C infected plants in comparison to mock control at each time point. DEGs were identified using *DESeq2* and defined by $\log_2$ fold-change ≥ 0.5 and false discovery rate (FDR)-corrected *p*-value ≤ 0.05. F) Volcano-plots of $-\log_{10}$p and $\log_2$FC exhibited by each gene in CiLV-C infected plants compared to mock control at each time point. Up- and down-regulated genes are presented in red and green, respectively. FC: fold-change, p: FDR-corrected *p*-value, hai: hours after infestation, dai: days after infestation.

The main sources of variability within samples were assessed by principal component analysis (PCA) using the normalized count data (Fig. 2C). The first component, which accounts for 64% of the variance, separated the samples by both time after infestation and virus treatment and reflects the intensity of stimuli. Mock samples from different time points grouped separately, due to the differential expression proportional to longer mite feeding period (Arena et al., 2018). At 6 hai, where the lowest virus titer was detected, all samples grouped together regardless the virus presence/absence. Similar profile with a single group of infected and control samples was obtained for samples from 2 dai, where the virus titer is slightly higher than at 6 hai. Distinct expression profiles between infected and control samples might be masked by the massive transcriptome changes imposed by feeding of non-viruliferous mites (Arena et al., 2018). At 6 dai, where the highest virus titer was reached, infected and mock treatment formed two separated groups. Hierarchical clustering of samples within each time point (Fig. 2 D) confirmed the clusterization profile obtained by PCA. Although samples from both treatments were grouped together at 6 hai or 2 dai, mock and virus-infected samples were clearly separated at 6 dai.

Differentially expressed genes (DEGs) in virus infected plants compared with mock were detected within each time point using the negative binomial-based DESeq2 package and *False Discovery Rate* (FDR)-correction of *p*-values for multiple comparisons. Overall, 3892 DEGs ($\alpha \leq 0.05$) were detected (Supplementary Table 2). No gene was differentially expressed at 6 hai (Fig. 2E, F), in agreement with the same expression profile displayed by mock and virus-infected plants (Fig. 2C, D). The number of DEGs progressively raised along the course of the infection (Fig. 2E, F) in parallel to increasing virus titer (Fig. 1C). At 2 dai, 294 DEGs were obtained, of which the majority (253 DEGs, ≅ 86%) was up-regulated (Fig. 2E). The striking largest number of DEGs throughout the interaction was detected at 6 dai, when CiLV-C infection deregulated 3717 genes, evenly distributed in 1995 (≅ 53.7%) up- and 1722 (≅ 46.3%) down-regulated DEGs (Fig. 2E). Analysis performed here show that CiLV-C infection

triggers a significant reprogramming on infected plants that is proportional to the course of viral multiplication.

2.3 CiLV-C infection induces cell growth and HR-related processes, and represses the plant primary metabolism and JA/ET-mediated responses

Gene ontology (GO) enrichment analyses were performed with the independent sets of up- and down-regulated DEGs from each time point to identify the most relevant biological processes (BPs) disturbed during CiLV-C infection (Supplementary Table 2). DEGs and BPs that were shared by or exclusive to experimental sets are presented (Fig. 3A, B). Using the Cytoscape app BinGO, the enriched BPs were visualized as networks where color and size of the nodes identify *p*-values and number of DEGs from each ontology, respectively.

The GO enrichment analysis revealed 49 and 5 over-represented BPs (hypergeometric test, $\alpha \leq 0.001$) in the sets of DEGs that were up- and down-regulated at 2 dai, respectively. Even though most of the DEGs identified at 2 dai were exclusively induced at this time point (146 DEGs, Fig. 3A), the majority of enriched BPs obtained from the set of up-regulated genes at 2 dai overlapped between induced sets of both time points (28 BPs, Fig. 3B). This suggests that several processes triggered soon at 2 dai are still modulated at 6 dai, and the discrepancy in the number of exclusive and shared DEGs probably reflects the occurrence of early and late responses of the same process. BPs enriched in both up-regulated DEGs from 2 and 6 dai included general terms of plant response to stimuli such as “defense response”, “regulation of response to stress” and “regulation of response to stimulus” (Supplementary Table 3). Likewise, enriched BPs that were up-regulated at 2 dai and down-regulated at 6 dai mainly referred to broad ontologies such as “response to hormone”, “response to endogenous stimulus” and “signaling” (Supplementary Table 3). On the other hand, 16 and 5 BPs were exclusive to up-regulated and down-regulated DEGs from 2 dai, respectively (Fig. 3B). The BPs uniquely induced at 2 dai were predominantly related to cell growth, e.g. “cell growth”, “cellular developmental process”, “cell wall organization”, “cell morphogenesis” and “cell differentiation” (Supplementary Table 3). Of the five BPs uniquely repressed at 2 dai, three were associated to the ethylene pathway: “ethylene-activated signaling pathway”, “cellular response to ethylene stimulus” and “response to ethylene” (Supplementary Table 3).

Most of BPs were over-represented in the sets with higher number of genes, i.e. the ones modulated at 6 dai (Fig. 3A). GO enrichment analysis disclosed 124 and 114 enriched BPs (hypergeometric test, $\alpha \leq 0.001$) in the groups of DEGs that were up- and down-regulated at 6

dai, respectively (Supplementary Table 3). Apart from a few broad-term processes common to the DEGs induced at 2 dai, the vast majority of the BPs enriched at 6 dai were exclusive to this time point (Fig. 3B). Within these unique categories, only 4 were shared between 6 dai up- and down regulated-cluster, revealing that induced and repressed genes at 6 dai are mostly not involved in the same process and that distinct pathways are differentially modulated in the pre-symptomatic stage (Fig 3B).

The cluster of up-regulated DEGs at 6 dai was enriched in 92 exclusive categories (Fig. 3B) and revealed a massive modulation of the plant immune system (Fig. 3C, Supplementary Table 3). BP categories were mainly clustered in two groups comprising response to stimulus and biological regulation (Fig. 3C). Both groups were preponderantly represented by processes associated with response to stress and defense. The group of BPs centralized in response to stimulus was branched in stress-related nodes that included “response to biotic stimulus” (linked to the subcategories of response to bacteria, fungus, oomycetes and host defenses), “response to abiotic stimulus” (represented by subcategories of response to osmotic stress and oxygen levels) and “response to oxidative stress” (specified from “response to ROS” to “response to hydrogen peroxide”) (Fig. 3C). A defense-related branch from response to stimulus group displayed general ontologies (e.g. “immune response” and “defense response”) and it was typified by HR-related BPs such as “plant-type HR”, “defense response, incompatible interaction”, “systemic acquired resistance” and “programmed cell death” (Fig. 3C). BP group centralized in biological regulation was branched to a major subgroup comprising the ontologies related to the regulation of both of response to stress and defense (Fig. 3C). Categories from that subgroup included general terms such as “regulation of defense response” and “regulation of response to stress”, and more specific ones e.g. “positive regulation of response to biotic stimulus” and “regulation of systemic acquired resistance”. Another branch from biological regulation group (“regulation of cellular processes”) displayed cell death- and SA-related responses including “regulation of cell death” and “regulation of SA biosynthetic and metabolic process”, respectively (Fig. 3C). A small cluster associated to the senescence process were also present in the up-regulated network (“aging”, “plant organ senescence” and “leaf senescence”) (Fig. 3C). Finally, the major hormonal-mediated pathway enriched in the up-regulated network was the SA pathway, represented by the categories “response to SA”, “cellular response to SA stimulus” and “SA mediated pathway” (Fig. 3C).

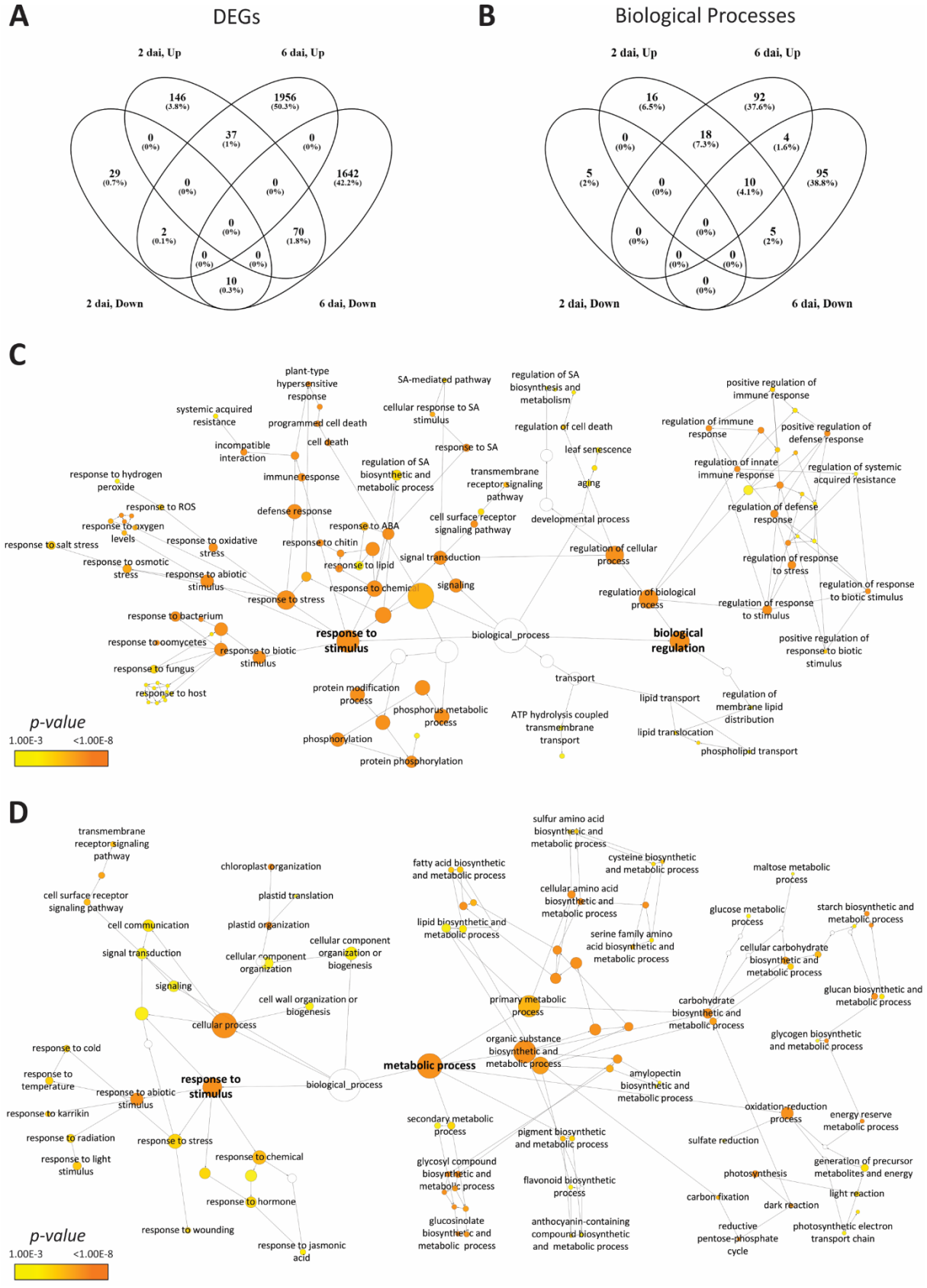

Figure 3. Main responses affected by CiLV-C infection in *Arabidopsis thaliana* plants. A) Venn diagram of up- and down-regulated genes identified within the set of differentially expressed genes (DEGs) from each time point. DEGs were identified using *DESeq2* and defined

by $\log_2$ fold-change ≥ 0.5 and false discovery rate (FDR)-corrected *p*-value ≤ 0.05. B) Venn diagram of overrepresented biological processes (BPs) from each set of up- and down-regulated DEGs identified at each time point. Overrepresented BPs were identified based on a hypergeometric test with FDR-adjusted *p*-values ≤ 0.001. C and D) Networks of enriched BPs from up-regulated (C) and down-regulated (D) DEGs identified at 6 dai, generated using the app BinGO in Cytoscape. Size of the nodes correlates with the number of DEGs. Color of the nodes reveals *p*-values of enriched categories. Names of some BPs were simplified for clarity; full names are displayed in Supplementary Table 3. dai: days after the infestation with viruliferous *Brevipalpus yothersi* mites; ROS: reactive oxygen species, SA: salicylic acid, JA: jasmonic acid, ABA: abscisic acid.

The cluster of down-regulated DEGs at 6 dai was enriched in 95 unique categories (Fig. 3B). Most of the GOs clustered in a major group of metabolic processes harboring mainly the primary metabolism (Fig. 3D). Primary metabolism subgroup included BPs associated to the metabolism of: i) lipids, such as "lipid biosynthetic and metabolic process" and "fatty acid biosynthetic and metabolic process"; ii) amino acids, whose categories included sulfur, cysteine and serine amino acid biosynthetic and metabolic process; and iii) carbohydrate, with numerous broad terms (e.g. "cellular carbohydrate biosynthetic and metabolic process") and specific BPs associated to biosynthesis and metabolism of glucan, starch, glycogen and maltose. Carbohydrate-related processes were connected to a cluster of photosynthesis-related categories such as "photosynthesis, light and dark reaction", "carbon fixation" and "generation of precursor metabolites and energy". Secondary metabolism formed a small branch comprising BPs directed to the biosynthesis and metabolism of glucosinolates (Fig. 3D). Following metabolism group, the down-regulated GO network presented a group centralized in response to stimulus (Fig. 3D). Along with many general terms shared with the up-regulated network, response to stimulus group revealed response to distinct abiotic stimulus (light, radiation and temperature), response to wounding, and JA as the only enriched hormonal pathway within down-regulated processes. Another small group from the network was centralized in "cellular component organization or biogenesis", with ontologies related to chloroplast and cell wall organization/biogenesis (Fig. 3D).

Overall, the GO enrichment analysis showed that early plant responses to CiLV-C infection involves a transient induction of cell growth-related processes, a transient repression of ET-responsive genes, and a stable modulation of defense and stress-related responses that is maintained up to later stages of the infection. At the pre-symptomatic stage, infected plants trigger processes related to the SA-mediated pathway, response to ROS and HR, all of which are present during incompatible interactions. Conversely, infected plants down-regulates

processes involved in the primary metabolism, JA-mediated pathway and synthesis of glucosinolates.

2.4 Regulation of co-expressed genes by specific classes of transcription factors correlates with the modulation of stress defense responses

To unravel the regulation of the transcriptional reprogramming upon viral infection, the classes of TFs associated with co-expressed DEGs were identified. First, we identified the up- and down-regulated DEGs coding for TFs on each time point and their corresponding families (Fig. 4A, B, Supplementary Table 4). Over-representation of specific families from each co-expressed set was assessed with a hypergeometric test ($\alpha \leq 0.001$). Within the set of up-regulated DEGs at 2 dai, 29 (11.5%) TFs from 15 different families were identified. From those, only two families were over-represented: MYB (6 genes, *p*-value = 1.39E-03) and WRKY (4 genes, *p*-value = 4.46E-03) (Fig. 4A), both typically involved in plant defense responses to stresses (Dubos et al., 2010; Phukan et al., 2016). From the down-regulated genes at the same time point, only 6 TFs (7.3%) comprising 3 different families were detected, as expected due to the reduced number of DEGs with such expression pattern. The only over-represented family was AP2/ERF (3 genes, *p*-value = 1.16E-03) (Fig. 4A), whose members are known to act as regulators of the ERF-branch of the JA/ET-mediated pathway (Pieterse et al., 2012). At 6 dai, 134 TFs (6.7%) from 22 families were up-regulated. Only 3 of those families were over-represented, of which the largest and most significant were WRKY (28 genes, *p*-value = 3.67E-14) and NAC (20 genes, *p*-value = 1.59E-05) (Fig. 4B). Similar to WRKY, NAC TFs are also intimately associated with immune responses and specifically to increased resistance against pathogens, including the triggering of HR against viruses (Nuruzzaman et al., 2013; Olsen et al., 2005). Within the set of down-regulated DEGs at 6 dai, 122 TFs (7.1%) evenly distributed in 32 families were identified. Similar to the up-regulated set, only 3 classes of TFs were enriched, and the largest and most significant ones were MYB (17 genes, *p*-value = 3.50E-03) and AP2/ERF (16 genes, *p*-value = 1.31E-02) (Fig. 4B).

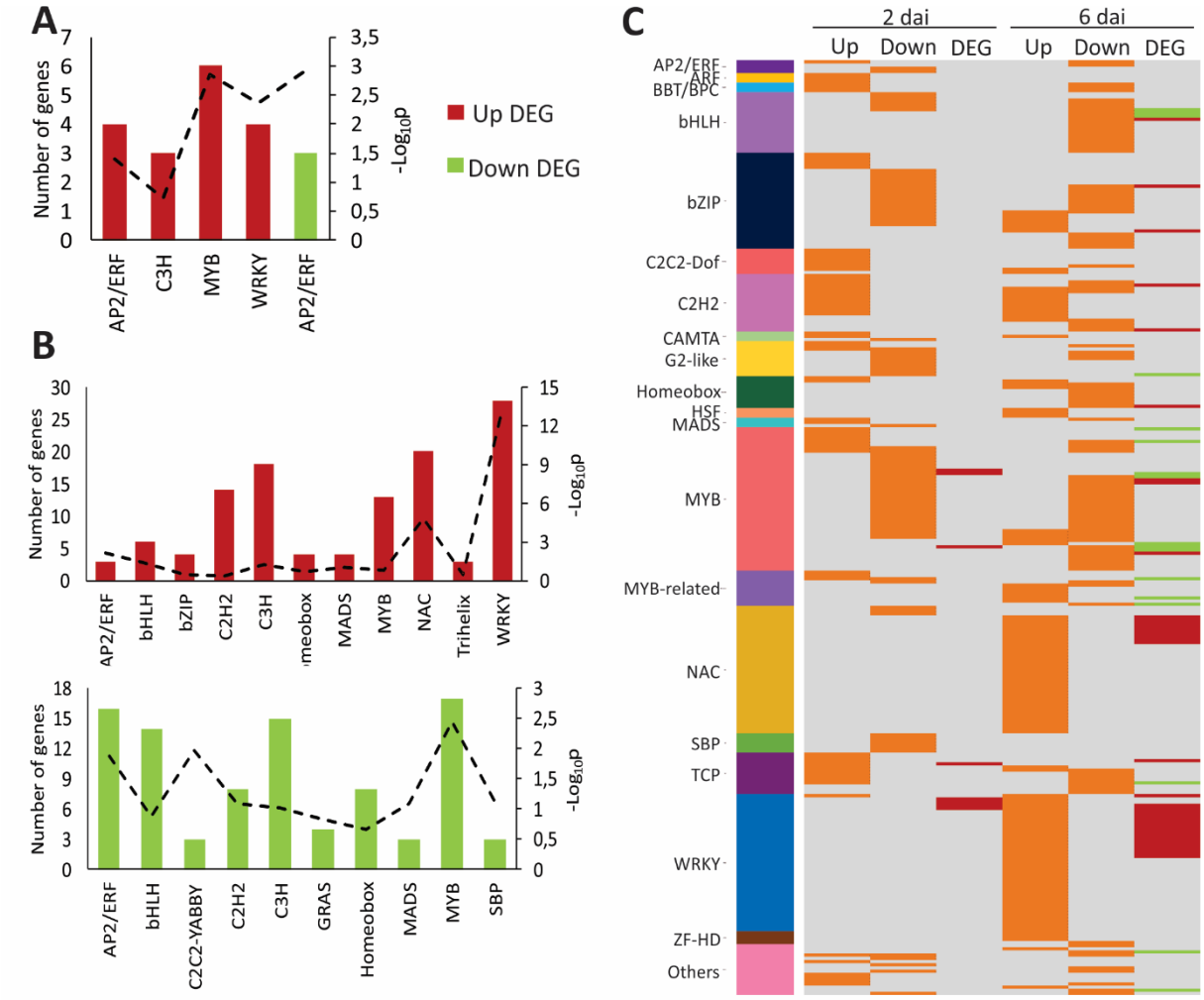

Figure 4. Enriched transcription factors (TFs) and TF targets during CiLV-C infection in *Arabidopsis thaliana*. A and B) Number of up- and down-regulated genes coding for TFs from each TF family identified within the set of DEGs at 2 dai (A) or 6 dai (B). Families encompassing two or less TFs were omitted. Up- and down-regulated DEGs are presented in red and green, respectively. Levels of enrichment ($-\text{Log}_{10}\,p$, with p: *p*-value) of each family (hypergeometric test, $\alpha \leq 0.001$) are presented by a dashed line with its corresponding values in the secondary axis. C) TFs with enriched targets within each set of up- and down-regulated DEG at 2 and 6 dai, identified by TF enrichment tool. TFs are grouped according to their families. Each line identifies one TF. Orange lines correspond to TFs with enriched targets within each set. Red and green lines represent up- and down-regulated DEGs, respectively, encoding TFs at each time point. Grey lines indicate absence of enriched targets for a given TF- and/or TF not differentially expressed. Families encompassing two or less TFs were grouped in "Others". DEG: differentially expressed gene; dai: days after the infestation with viruliferous *Brevipalpus yothersi* mites.

In another approach, we search for TFs with over-represented targets within each set of DEGs by using the TF enrichment tool (Jin et al., 2016). Potential targets were identified based on cis-regulatory elements in the promoters of the test genes and regulatory interactions described at the literature (Jin et al., 2016) (Fig. 4C, Supplementary Table 5). The largest

families with potential targets within DEGs induced at 2 dai were the growth-related TCP (Manassero et al., 2013) and stress-related C2H2 (Kiełbowicz-Matuk, 2012), represented by 10 and 13 TFs, respectively. On the other hand, the families MYB and bZIP – with 29 and 18 TFs, respectively – were the ones that presented the highest numbers of TFs with potential targets in the set of down-regulated genes at 2 dai (Fig. 4C). Interestingly, the analysis of 6 dai sets revealed once again WRKY (43 TFs) and NAC (37 TFs) as the largest families with targets within up-regulated DEGs (Fig. 4C), supporting the involvement of both TF classes in controlling the induction of those genes. Within the down-regulated genes at 6 dai, potential targets were mainly associated to TFs from bHLH (Fig. 4C), which includes the regulators of the MYC-branch of the JA/ABA-mediated pathway (Pieterse et al., 2012), and MYB classes, represented by 33 and 17 genes, respectively. No members of WRKY and NAC families had targets enriched in the down-regulated DEGs from 6 dai, neither TFs from bHLH family presented potential targets within the up-regulated genes at the same time point (Fig. 4C), highlighting the specificity of induced and repressed responses during the pre-symptomatic stage.

These analyses showed that expression of up-regulated genes is mainly driven by TF classes WRKY and NAC, followed by TCP and C2H2, whilst down-regulated genes are potentially controlled by TFs from AP2/ERF, bHLH and bZIP families. MYB TFs regulate both induced and repressed responses. WRKY is highlighted as the only family with both modulated TFs and targets exclusively within up-regulated genes. Results support the modulation of stress responses upon CiLV-C infection, being consistent with the specific induction of cell growth and HR/SA-mediated defenses, and repression of both ET and ABA branches of the JA pathway.

2.5 Genes related to HR are induced at the pre-symptomatic stage

Due to the over-representation of HR-related processes within up-regulated GO network and the HR-like phenotype induced by CiLV-C infection (Arena et al., 2016), the DEGs associated to HR or to the mechanisms underlying the development of such resistance response were thoroughly reviewed. Hierarchical clusters were generated with data from genes included in the categories “response to SA”, “response to ROS”, “cell death”, and “plant-type HR” (Fig. 5A). Without exception, all DEGs belonging to these categories were up-regulated at the 6 dai. Induction of the whole set of genes directly or indirectly related to HR at the pre-symptomatic stage agrees with the theory that symptoms of viral infection result as consequence of a HR.

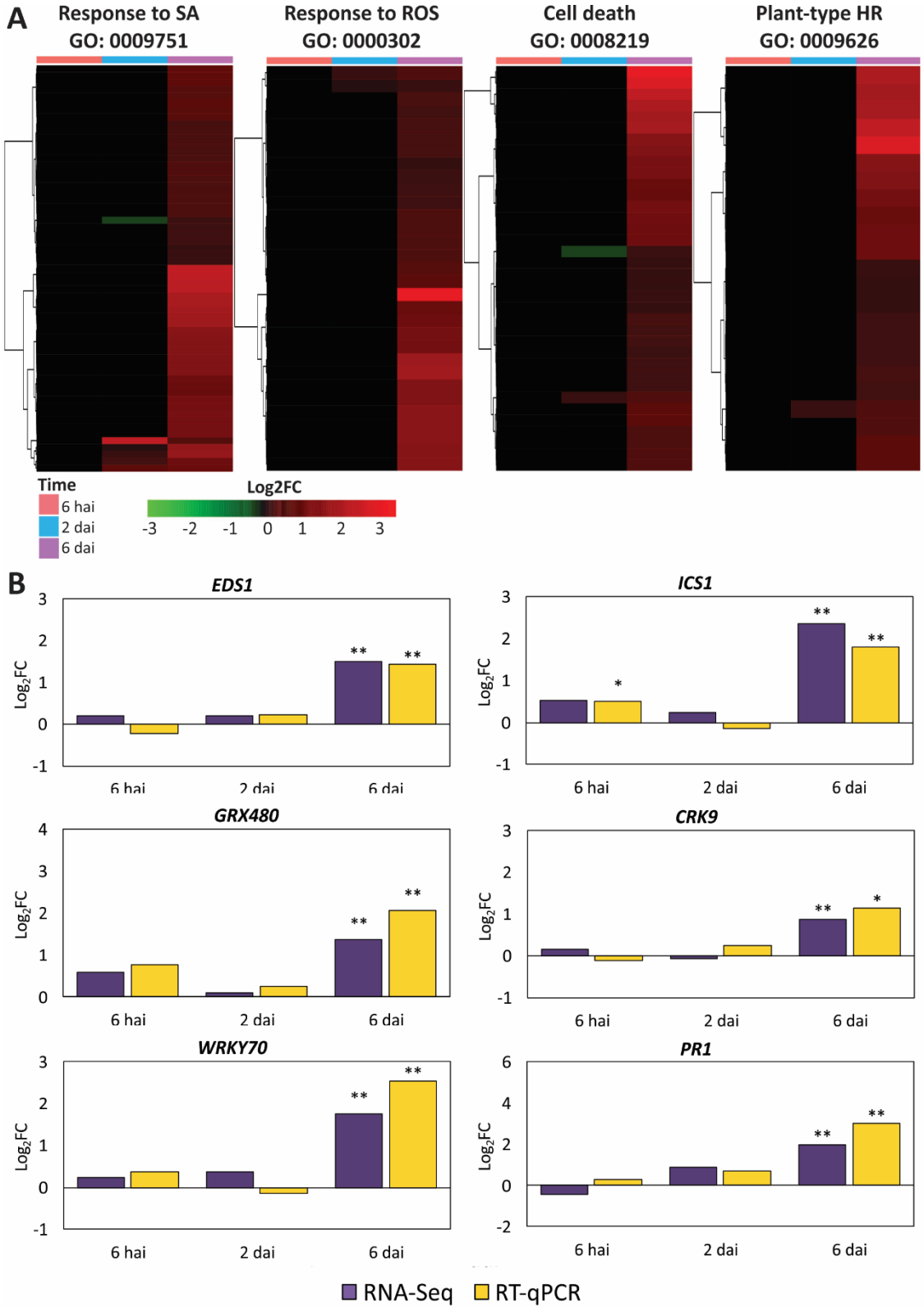

Figure 5. Hypersensitive response (HR)-related genes during plant infection by CiLV-C. A) Hierarchical clustering analysis of $\log_2$ fold-change (FC) exhibited by differentially expressed genes (DEGs) involved in HR-related biological processes. B) Expression profile of selected *Arabidopsis thaliana* genes in virus-infected plants, quantified by RNA-Seq and RT-qPCR. Data are presented as $\log_2$FC values in comparison with mock-infected plants (with $\log_2$FC set to zero). Statistically significant differences of virus-infested versus mock control at *p*-values ≤

0.01 (**) and ≤ 0.05 (*) are indicated. hai: hours after infestation, dai: days after infestation. GO: gene ontology term; SA: salicylic acid; ROS: reactive oxygen species, HR: hypersensitive response; hai: hours after infestation, dai: days after infestation.

To validate the RNA-Seq data and support the involvement of the SA pathway and HR in response to CiLV-C infection, the expression of selected DEGs was assessed by RT-qPCR (Fig. 5B). Six SA- and HR-related genes up-regulated at the pre-symptomatic stage were selected: the signaling component *EDS1* (*enhanced disease susceptibility 1*), the SA biosynthetic enzyme *ICS1* (*isochorismate synthase 1*), the regulator *GRX480* (*glutaredoxin 480*), the receptor-like kinase (RLK) *CRK9* (*cysteine-rich RLK 9*), the transcription factor *WRKY70* (*WRKY DNA-binding protein 70*) and the defense protein *PR1* (*pathogenesis-related protein 1*). Expression profiles of those genes were assessed in a new experiment with plants infested with non-viruliferous (mock) and CiLV-C viruliferous mites at 6 hai, 2 dai and 6 dai (Fig. 5B). All the evaluated genes were induced at 6 dai, in consistency with the RNA-Seq data, supporting the results described in this work.

2.6 Expression of CiLV-C P61 protein triggers HR and mimic plant responses to viral infection

To unravel the role of CiLV-C-encoded proteins in triggering the identified plant responses, the six viral ORFs were cloned in expression vectors and they were individually transiently expressed into *Nicotiana benthamiana* leaves. The elicitor activity of CiLV-C ORFs was elucidated by: i) visual phenotypic observation, ii) histochemical detection of H_2O_2, the main ROS detected during plant-pathogen interactions, iii) evaluation of the expression profile of the SA- and HR-related genes *PR1a, PR1b, PR2, HIN1* and *HSR203J*, and iv) quantification of the SA and JA hormonal content.

While the other CiLV-C proteins did not produce any altered phenotype, the *Agrobacterium*-mediated transient expression of P61 consistently induced cell death on the infiltrated areas at 3 day after infiltration, which clearly contrasted with the healthy phenotype observed in leaves infiltrated with the empty vector (Fig. 6A). Histochemical analysis of the P61 infiltrated leaves revealed the production and accumulation of large amount of H_2O_2 (Fig. 6B). RT-qPCR assays showed that, although plants reacted to the infection by *A. tumefaciens* containing the empty vector, the presence of P61 clearly up-regulated the expression of all evaluated genes (Fig. 6C). Quantification of defense hormones by LC-MS/MS revealed that SA

levels were almost 7-fold higher on plants expressing this viral protein relative to GFP, used as negative control, while JA levels were more that 4-fold lower on the same comparison (Fig. 6D). Mimicry of responses typically observed during CiLV-C-plant interaction put forward elements, indicating P61 as the putative viral effector to be neutralized by plant defenses.

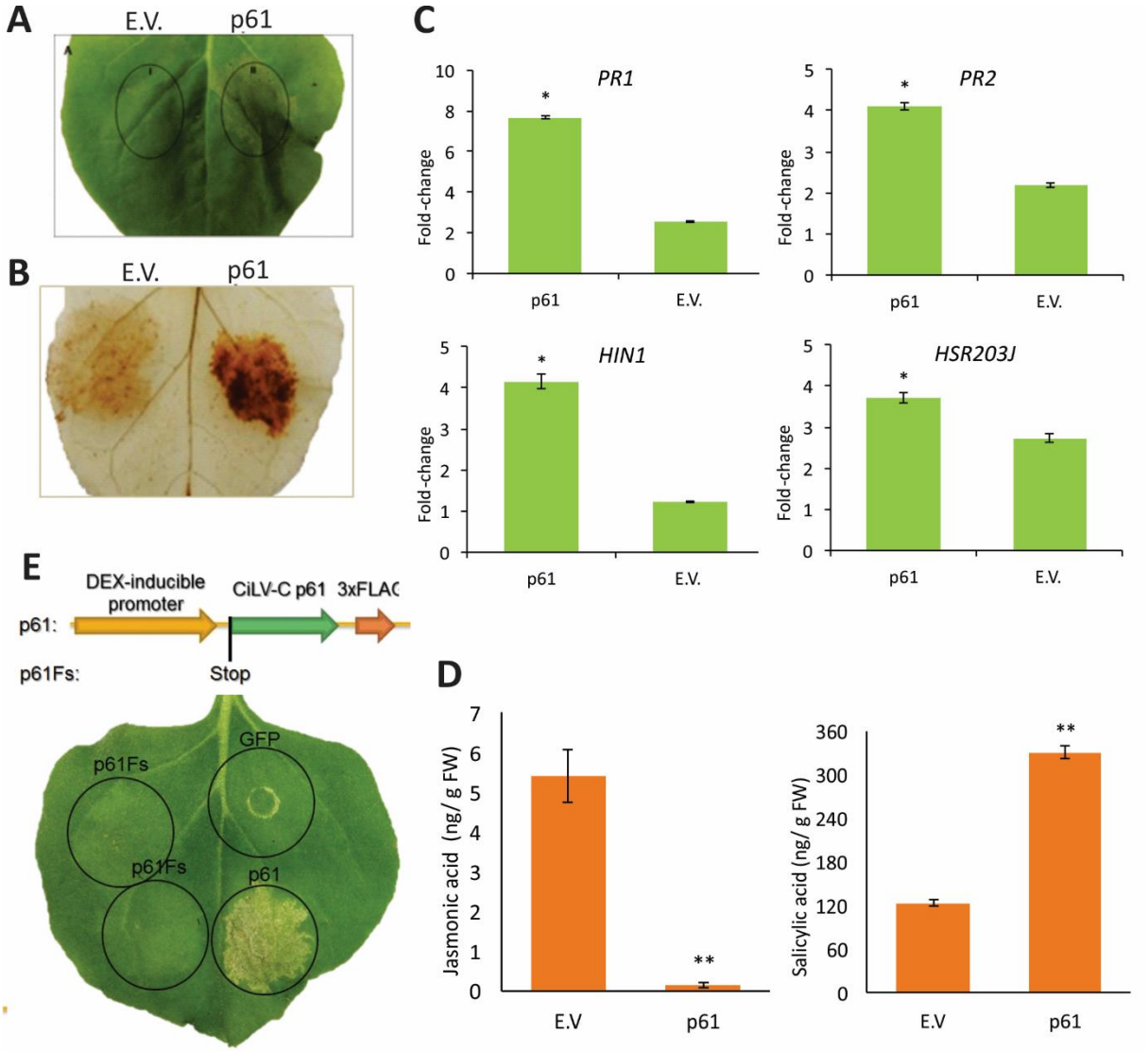

Figure 6. Elicitor activity of CiLV-C P61 protein. The viral protein was transiently expressed in *Nicotiana benthamiana* leaves using *Agrobacterium*-mediated infiltration. Injuries (A), ROS production (B), expression of SA- and HR-related genes (C), and levels of jasmonic acid and salicylic acid (D) were assessed at 3 days after the infiltration. To verify the elicitor activity of the p61 RNA versus protein, a frameshift mutant was produced (D). E.V: empty vector. Statistically significant differences of plants infiltrated with p61 versus empty vector (E.V.) at *p*-values ≤ 0.01 (**) and ≤ 0.05 (*) are indicated.

The above findings showed that the presence of the p61 gene elicits plant defense responses that might culminate in HR. In other plant-virus systems, typical PAMPs (*pathogen-associated molecular patterns*) that elicits the plant immune system are dsRNAs produced during virus replication (Pumplin and Voinnet, 2013). To verify whether the HR is triggered by the P61 protein or the RNA sequence from it is translated, a frameshift mutant was produced to retain the entire sequence of the p61 ORF but prevent the production of the protein (Fig. 6E). The construct had an indel of two nucleotides (T and A) downstream the start codon of the p61 ORF, resulting in a shift of the ORF frame and the generation of a stop codon immediately after the first codon. The clones carrying the genes for the p61 frameshift mutant, the p61 wild type and that encoding the GFP gene were agroinfiltrated in different spots throughout the same *N. benthamiana* leaf to compare the cell death phenotype (Fig. 6E). At 3 dai, the cell death was observed on areas infiltrated with the p61 wild type. No HR was observed in the areas infiltrated with GFP or the p61 frameshift mutant. Expression of the p61 with the frameshift mutation did not affect the synthesis of the p61 RNA but blocks the production of the protein. This result demonstrate that the HR phenotype is triggered by P61 and not the p61 mRNA.

3. Discussion

In this study, we attempted to unravel the molecular mechanisms underlying plant interaction with the cilevirus CiLV-C, a virus atypically unable to accomplish systemic infection in any of its known hosts. We characterized the major events during CiLV-C replication in plant leaves by quantifying viral genomic and subgenomic RNAs, the global plant transcriptional response to CiLV-C infection using RNA-Seq, and the CiLV-C elicitor that triggers plant responses by transient expression of viral proteins.

The kinetics of production and expression of viral RNAs in Arabidopsis revealed three major steps of CiLV-C infection. The earliest stage occurs from the CiLV-C inoculation by viruliferous mites until 24 hai and is characterized by a low replication rate or likely, a replication restricted to a few cells. During such stage of reduced number of viral molecules, plant transcriptional response to CiLV-C infection is undetectable at least until 6 hai. From 2 to 4 dai, the replication step with a high replication rate before the increase in sub-genomic expression takes place, and a moderate number of plant transcriptional responses to viral infection are detected. The latest stage ranges from 6 to 10 dai and it is marked by an increased accumulation of *p29* sub-genomic RNA that might correlate with the expression of late proteins.

During the last step, CiLV-C titers reach the highest levels, a massive transcriptome reprogramming at the pre-symptomatic stage is observed, and symptoms of viral infected arise.

The transcriptome analysis of Arabidopsis plants along the course of infestation with CiLV-C revealed that early transcriptome changes to CiLV-C infection involves the up-regulation of general responses to stresses that are maintained later in the interaction, and the transient induction of cell growth-related processes. Induction of genes related to cell growth might be related to the development of hyperplasia or hypertrophy, typical histological changes promoted by phytopathogens, including plant viruses (Hull, 2009). Interestingly, anatomical studies revealed that both abnormal cell division and enlargement develop in parenchyma cells from local lesions caused by CiLV-C in sweet orange (Marques et al., 2007). Hyperplasia and hypertrophy are also noticeable in plant tissues infected by leprosis-related *Brevipalpus*-transmitted dichorhaviruses (Marques et al., 2010), indicating that up-regulation of cell growth responses might be a pattern from plant infection by BTVs.

Plant infection by CiLV-C down-regulated the inter-connected ET and JA pathways at 2 and 6 dai, respectively. The later stage was also marked by a down-regulation of genes involved in the biosynthesis of glucosinolates, a well-known defense against herbivores typically modulated by JA. Concomitant induction of SA and reduction of JA at CiLV-C infected plants are likely a consequence of the well-known SA-JA antagonism, as previously proposed (Arena et al., 2016). The repression of anti-herbivory defenses upon virus infection has been described as a viral strategy to encourage transmission (Mauck et al., 2018). Several reports describe the ability of different viruses in manipulating plant phenotypes to increase vector fitness or attraction. For example, infection by the potyvirus TuMV alters ET responses, suppressing callose defenses and improving fecundity of its aphid vector, *Myzus persicae* (Casteel et al., 2015). By exploiting the natural SA-JA antagonism, the tosposvirus tomato spotted wilt virus triggers the SA-mediated pathway to reduce JA-mediated defenses, becoming more attractive to the thrips vector *Frankliniella occidentalis* (Abe et al., 2012). Similarly, the induction of SA and reduction of JA pathway and related defenses upon CiLV-C infection might account for an improvement in *Brevipalpus* vector performance. In agreement with this hypothesis, CiLV-C infected Arabidopsis leaves are preferred for mite colonization and oviposition (Arena et al., 2016) and mite density is higher is CiLV-C infected sweet orange trees in contrast to healthy ones in field conditions (Andrade et al., 2013). Furthermore, assays with Arabidopsis mutants revealed a lower oviposition of *B. yothersi* mites in plants compromised in SA signaling (Arena et al., 2018), pointing to a role of SA response in improve

mite performance. CiLV-C/*Brevipalpus* relationship might be mutually beneficial, with the mite performing better on plants with boosted SA response and likely suppressed anti-herbivore defenses, and the virus presenting improved transmission as a consequence of higher vector fitness.

Along with the reduction of the JA pathway and glucosinolates production, the pre-symptomatic stage was marked by a repression of the plant primary metabolism. Down-regulated processes included the metabolism of lipids, amino acids and carbohydrates and photosynthesis. In other plant-virus systems, the infected cells supporting viral replication are transformed into metabolic sinks, triggering a massive reprogramming of the plant primary metabolism (Llave, 2016). Viruses typically interfere with lipid and fatty acid metabolism to promote changes in the membranes required for the formation of viral replication complexes (Llave, 2016). Reduced expression of several sterol or fatty acid biosynthesis genes have been shown to restrain other viruses replication (Llave, 2016) and might similarly affect CiLV-C infection in Arabidopsis. Altered metabolism of amino acids and carbohydrates have been observed in distinct viral infections, but their precise roles have not been fully elucidated and vary profoundly between distinct plant-virus interactions (Llave, 2016). Inhibition of photosynthesis is a general rule in different plant-virus interactions and is usually associated with changes in the chloroplasts and the development of chlorosis and necrosis, some of the most common viral symptoms. Chloroplasts are recognized as prime targets for plant viruses, undergoing massive structural and functional damage during viral infection (Bhattacharyya and Chakraborty, 2018). Moreover, chloroplast factors might participate in essential stages of viral infection such as replication and movement and as such are targeted by viruses to help fulfill their infection cycle (Zhao et al., 2016). On the other hand, chloroplasts are crucial for the plant defense against viruses, acting as the sites for the biosynthesis of defense hormones and HR-related ROS, which may compromise the organelle photosynthetic efficiency in a trade-off process (Bhattacharyya and Chakraborty, 2018; Zhao et al., 2016). In either way, plant viruses commonly disturb chloroplast structure and functions, leading to reduced photosynthetic activity and development of chlorotic or even necrotic symptoms as a result of cell death (Bhattacharyya and Chakraborty, 2018; Zhao et al., 2016). Similarly, the chlorotic and necrotic lesions produced by CiLV-C might reflect chloroplast alterations and reduced photosynthetic activity, both supported by transcriptomic data.

Even though plant genes were modulated at an early stage of the CiLV-C infection, the strikingly majority of the differentially expressed genes were identified at the later time point.

The biological processes identified at the pre-symptomatic stage might culminate in the development of the disease symptoms. Our results showed that such phase of viral infection is mainly typified by the up-regulation of the plant immune system. A large number of up-regulated genes are related to SA-mediated pathway, response to ROS, cell death and HR. Likewise, even though SA and ROS are not exclusively associated to HR, both are required for the development of the resistance response ensuing from incompatible interactions (Xia et al., 2015). Previous histochemical analysis revealed the accumulation of ROS and dead cells in CiLV-C infected tissues, confirming the molecular responses verified here (Arena et al., 2016). In all its recognized natural or experimental hosts, CiLV-C remains restricted to cells around the inoculation sites by the mite vector, where symptoms of viral infection are manifested as localized chlorotic and necrotic lesions (Bastianel et al., 2010). Phenotypically, these symptoms resemble the outcome of a HR, a cell death resistance process accompanied by the pathogen restriction at the inoculation site during an incompatible interaction. Transcriptome changes associated to the induction of HR support the theory that the lesions caused by citrus leprosis may be a consequence of an incompatible rather than a compatible interaction (Arena et al., 2016). In general, the development of an HR resistance is associated with the recognition of the viral protein by corresponding plant resistance (R) proteins in a host-specific manner. It is noteworthy that, differently from other HR-inducing plant-virus systems, HR-like associated phenotypes (cell death and virus restriction) developed during CiLV-C interaction are not associated to a specific plant species but occurs over the spectrum of CiLV-C hosts identified so far.

Finally, we showed that the transient expression by agroinfiltration of the CiLV-C P61 protein reproduces phenotypes observed during plant interaction with CiLV-C. ROS burst, cell death, increased expression of SA- and HR-related genes, higher levels of SA and lower levels of JA are triggered by the transient expression of P61 but not its mRNA. Several viral proteins that mimicry responses of virus infection have been identified. The production of characteristic HR necrotic lesions can be triggered by the transient expression of the p50 helicase domain from tobacco mosaic virus (TMV) replicase (Les Erickson et al., 1999) and the silencing suppressor P0 from poleroviruses (Wang et al., 2015) in resistant *Nicotiana glutinosa* genotypes, for example. The chlorotic symptoms induced by cucumber mosaic virus in tobacco is determined by the interaction of the viral capsid protein (CP) with a chloroplast ferredoxin protein (Qiu et al., 2018). The βC1 protein from the betasatellite of tomato yellow leaf curl China virus interacts with the *MYC2* transcription factor resulting in decreased levels of JA-

responsive genes and consequent enhanced performance of its vector, the whitefly *Bemisia tabaci* (Li et al., 2014). Induction of HR-related defenses and phenotype induced by CiLV-C P61 indicates this protein in the center of processes that triggers the plant immune system, leading to the development of typical HR lesions. Increases SA and reduced JA levels suggest that P61, without any other viral factor, might trigger the cross-talk between the hormonal pathways and might influence vector performance upon CiLV-C infection. Ongoing studies for the identification of plant proteins that interact with P61 will shed light on the mechanism of CiLV-C recognition by the host and development of virus-associated phenotypes.

Comprehensively, we showed that CiLV-C titer progressively increase in infected cells with distinct steps of replication, and we provided evidences based on the host transcriptome reprogramming that support the current understanding that the symptoms of CiLV-C infection results from an HR resistance. Futhermore, we identified a CiLV-C protein that elicits the same immune responses triggered by viral infection and that might determine the development of the HR-like symptoms. Our results improve the previously proposed model on the plant response to the components of the citrus leprosis pathosystem (Arena et al., 2016). In practical terms, we provide assays for the quantitative detection of CiLV-C in infected samples and extensive transcriptome data that can be further explored to unravel distinct mechanisms of plant gene expression regulation by CiLV-C infection.

4. Materials and Methods

4.1 Plant material

Seeds from *Arabidopsis thaliana* ecotype Columbia (Col-0) were obtained from the Arabidopsis Biological Resource Center (ABRC, http://www.arabidopsis.org). *Arabidopsis thaliana* and *N. benthamiana* plants were grown in a controlled growth chamber Adaptis AR A1000 (Conviron, Winnipeg, Canada) at 23 ± 2 °C and a 12 h photoperiod. Four-week-old plants were used in the experiments.

4.2 Mite (*Brevipalpus yothersi*) rearing

Population of mites were initially obtained from a single female collected from a citrus orchard and identified as *B. yothersi* using phase contrast microscopy as reported elsewhere (Beard et al., 2015). Non-viruliferous mites were reared onto unripe fruits of 'Tahiti' acid lime (*Citrus latifolia* Tanaka), a genotype immune to citrus leprosis virus C. Viruliferous mites were

obtained by transferring the non-viruliferous mites from the acid lime to sweet oranges fruits with citrus leprosis symptoms infected with CiLV-C strain SJP (Ramos-González et al., 2016). Fruits were prepared as described before (Rodrigues et al., 2007). Mites were reared for several generations and were evaluated for the presence of CiLV-C by RT-PCR (Locali et al., 2003) before their use in the experiments.

4.3 Kinetics of CiLV-C accumulation

Quantification of CiLV-C loads were performed in Arabidopsis plants infested with *B. yothersi* viruliferous mites at ¼, ½, 1, 2, 4, 6, 8 e 10 dai. Arabidopsis plants were infested with 15 mites (five per each of three rosette leaves), transferred with a brush under a stereoscopic microscope. Each time point had 10 biological replicates. Harvested leaves were flash-frozen in liquid N_2 and stored at -80 °C until RNA extraction. Plant RNA was purified with the RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands), RNA concentration and purity (A_{260}/A_{280}) was determined in NanoDrop ND-8000 micro-spectrophotometer (Thermo Scientific, Waltham, USA), and cDNA was synthesized using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). CiLV-C presence was confirmed by RT-PCR (Locali et al., 2003). TaqMan - Minor Groove Binding (MGB) assays were developed to detect CiLV-C *p29* and *RdRp* ORFs and Arabidopsis *SAND* reference gene. It should be noted that primers and probes for detection of CiLV-C *p29* ORF target the *p29*-sgRNA and the viral- and anti- viral RNA1 molecules of CiLV-C, meanwhile those specific for *RdRp* ORF only target the viral- and anti- viral RNA1 molecules. In this later case, results were considered the indicator of viral genome accumulation. Both primers and probes were designed using Primer-Express software (Thermo Scientific, Waltham, MA, USA) and their sequences are available at Supplementary Table 6. Amplicons generated from a virus-infected sample were purified and cloned in pGEM-T-Easy vector (Promega, Madison, WI, USA). Two clones per amplicon were sequenced in both directions and consensus sequences were 100% identical to the target sequences from the CiLV-C SJP genome. Eight 10-fold dilutions (10^8 to 10^1 molecules) from each plasmid were prepared to generate standard curves. Standards and cDNAs from infected samples were assayed by RT-qPCR. Reaction mixes were prepared with the TaqMan® Fast Universal PCR MasterMix 2X kit, as recommended by the manufacturer (Thermo Scientific, Waltham, MA, USA). Amplifications were carried out in a 7500 Fast Real-Time PCR System device (Thermo Scientific, Waltham, MA, USA). Samples were analyzed in triplicates and no-template controls were included to check for contaminations. *Cycle quantification* (Cq) values from infected samples were compared with the standard curves to determine absolute quantities

of CiLV-C *p29* and *RdRp* molecules. Quantities of each molecule at different time points were statistically compared using one-way ANOVA and Tukey's HSD (*honest significant difference*) test ($\alpha \leq 0.05$).

4.4 RNA-Seq time-course experiment

Arabidopsis plants were infested with 15 non-viruliferous or viruliferous mites (five mites per each of three rosette leaves). Infested leaves were collected at 6 hours after infestation (hai), and 2 and 6 days after the infestation (dai). Sixteen plants were infested per treatment per time point and leaves from two plants were pooled, totaling eight biological replicates. Other set of plants was kept with viruliferous mites for eight days, when symptoms were visible, to confirm virus infection. Harvested leaves were flash-frozen in liquid N_2 and stored at -80 °C until RNA extraction. Plant RNA was purified with the RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands) and treated with RNAse-free DNAse (Qiagen, Venlo, Netherlands) for removal of plant DNA. RNA purity (A_{260}/A_{280} ~ 2.0) and integrity (RIN > 8) were confirmed in NanoDrop ND-8000 micro-spectrophotometer (Thermo Scientific, Waltham, USA), and Bioanalyzer 2100 (Agilent technologies, Santa Clara, USA), respectively. CiLV-C presence in plants infested with viruliferous and absence in the ones infested with non-viruliferous mites were confirmed by RT-PCR (Locali et al., 2003). RNA extracts from two samples (100ng/uL each) were pooled, totaling four replicates per treatment (CiLV-C and mock) per time point for the RNA-Seq. cDNA libraries were prepared with Illumina TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, USA). Sequencing was performed in an Illumina HiSeq 2500 system (Illumina, San Diego, USA) using HiSeq SBS v4 High Output Kit (Illumina, San Diego, USA). Paired-end reads of 2x125 bp were generated.

4.5 Bioinformatics analysis of RNA-Seq data

RNA-Seq data were analyzed following the pipeline from Anders et al. (2013) with some modifications, as described before (Arena et al., 2018).Biological variability of the samples was assessed by principal component analysis (PCA) and hierarchical clustering (using *Euclidean* distance metric and *Ward's* clustering method). Differentially expressed genes (DEGs) between CiLV-C and mock treatments were identified at each time point using the package DESeq2 (Love et al., 2014). *False Discovery Rate* (FDR) correction for multiple comparisons was applied. DEGs with corrected *p*-values ≤ 0.05 and fold-change (log2) threshold of 0.5 were classified as differentially expressed. GO Enrichment Analysis was

performed on DEGs to elucidate mechanisms potentially involved in the CiLV-C infection and symptoms development. A gene set was defined as all DEGs identified at each set (2 dai/ up-regulated, 2 dai/ down-regulated, 6 dai/ up-regulated, and 6 dai/ down-regulated) and the universe comprised all genes of the *A. thaliana* TAIR10 genome. Overrepresented BPs were identified by a hypergeometric test (FDR-adjusted *p*-values ≤ 0.001). GO networks were generated in Cytoscape using the app BinGO (Maere et al., 2005).

4.6 Identification of enriched transcription factors

Enriched TFs were assessed on up and down-regulated DEGs from 2 and 6 dai using two approaches. First, genes coding for TFs within DEGs were identified by searching on PlantTFDB database (Jin et al., 2017) and over-represented TF families on each set of genes were assessed using a hypergeometric test ($\alpha \leq 0.001$). Second, individual TFs with targets enriched within DEGs were identified using the TF enrichment tool (Jin et al., 2017), based on the presence of *cis*-regulatory elements in the sequences of the DEGs assessed and literature mining. TFs with enriched targets were further grouped according to their families.

4.7 Validation of gene expression data by RT-qPCR

A new time course experiment was set with Arabidopsis Col-0 plants infested with viruliferous and non-viruliferous mites at 6 hai, 2 dai and 6 dai. For each time point, plants were grouped in sets of 16 individuals assigned to each treatment (CiLV-C and mock). Plants were infested with 15 mites (5 mites per each of 3 rosette leaves). Infested leaves were collected at each time-point and leaves from two plants were pooled, totaling eight biological replicates per treatment per time point. Harvested leaves were flash-frozen in liquid N_2 until RNA extraction. Plant RNA was purified with the RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands) and treated using RNAse-free DNAse (Qiagen, Venlo, Netherlands). RNA concentration and purity were assessed using NanoDrop ND-8000 microspectrophotometer (Thermo Scientific, Waltham, USA), and RNA quality was confirmed in 1.2% agarose gels. cDNA was generated using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) as described by the manufacturer. qPCR were prepared with 3 ng of cDNA, 6.5 uL of GoTaq qPCR Master Mix (Promega, Madison, WI, USA) and 120 nM of each gene-specific primer pair. Primer sequences are available at Supplementary Table 7. Each cDNA sample was analyzed in triplicate and melting curves were included. Primer pairs efficiencies and Cq values were determined for individual reaction using Real-time PCR Miner (Zhao and Fernald, 2005).

Gene expression analyses were performed using the ΔCq model with efficiency correction and multiple reference genes (Hellemans et al., 2007) as previously described (Arena et al., 2016). Difference between infected and mock samples within each time point were assessed using Student's t-test ($\alpha \leq 0.05$).

4.8 Cloning of CiLV-C ORFs in expression vectors

CiLV-C ORFs were amplified from pre-generated clones using a HiFi polymerase (Thermo Scientific, Waltham, USA) and specific primers were designed to add restriction sites to the ends of each amplicon (Supplementary Table 8). The amplicons were digested and cloned in an intermediary vector based on the backbone of the pUC19 cloning vector (New England Biolabs). Each transcriptional unit was constituted by the 35s cauliflower mosaic virus promoter to constitutive expression of the gene, the Ω fragment from TMV to enhancement of the translation, the CiLV-C ORF, and the *Nopaline synthase* terminator. After assembled, each transcription unit was transferred from the intermediary vector to a pCambia 2300 binary vector. Final constructions were digested with the endonucleases XbaI and XhoI for validation of their identity. After the identification of P61 elicitor activity (methods described below), new expression clones were assembled to express this protein fusioned to a 3xFLAG C-terminal tag, under the control of a dexamethasone (DEX)-inducible promoter. Somewhat similar cloning procedures were performed to construct GFP-expressing clones as negative controls. The Gateway system (Thermo Scientific, Waltham, USA) was used. Specific primers (Supplementary Table 8) were designed to include four Gs and the 25pb of the attB regions in the 5` end (for efficient Gateway cloning), remove the stop codon (for fusion with the 3xFLAG), and add additional nucleotides (to maintain the proper reading frame with the FLAG tag). Genes were amplified using the *Phusion High-Fidelity DNA Polymerase* (New England Biolabs). The amplicons were purified and cloned in the donor vector pDONR207 (Thermo Scientific, Waltham, USA). Entry clones were purified and sequenced. Once confirmed, the transcriptional units were transferred by recombination to a Gateway-compatible version of the pTA7001 destination vector (Aoyama et al., 1997; Li et al., 2013), with a C-terminal 3xFLAG (DYKDHDGDYKDHDIDYKDDDDK) and a DEX-inducible promoter. Expression clones were purified and sequenced to confirm their identity. To generate p61 construct containing the frameshift mutant, the *p61* gene was amplified from the previously constructed vector using a forward primer (Supplementary Table 8) designed to introduce two extra nucleotides following the start codon. The amplicons were cloned in the pDONR207 and the transcriptional units

were further transferred to the Gateway-compatible version of the pTA7001 destination vector. Expression clones were purified and confirmed by sequencing.

4.9 Transient expression assays in *Nicotiana benthamiana* leaves

Once verified, plasmids were inserted into the *Agrobacterium tumefaciens* strain GV3101. Recombinant *A. tumefaciens* was cultivated overnight in 5 mL of Luria-Bertani (LB) medium containing kanamycin (30 µg/mL) and rifampicin (50 µg/mL). Fresh media (Kn, Rif and 150 µM acetosyringone) were inoculated with 1/10 (v/v) of the pre-inoculum until reaching a 0.8-1 OD_{600} nm. Cells were harvested by centrifugation and further incubated in the infiltration buffer (10 mM MES, pH 5.6, 10 mM $MgCl_2$, 150 µM acetosyringone) in the dark for four hours. Infiltrations were carried out using a syringe in leaves of four to six leaf stage plants. In parallel, *N. benthamiana* plants were infiltrated with the empty vectors and GFP-expressing clones (negative controls) and the infiltration buffer (blank). When pTA7001 expression clones were used, infiltrated leaves were sprayed with 30uM DEX and collected at 1, 2 and 3 days after induction for protein detection. Cells were lysed in presence of NP40-based buffer (150mM NaCl, 1% NP40, 50mM Tris-HCl), diluted in a protein disruption buffer (136mM DTT, 192mM Tris, 45mg/mL SDS, 50ug/mL bromophenol blue, 10M urea), heated at 95°C and loaded in SDS-PAGE gels. The fusioned proteins were assessed by Western Blot probed with anti-DYKDDDDK-HRP conjugate (Miltenyi Biotec) at a concentration of 1:2000. Bands of the expected size (~30kD for GFP-3xFLAG and ~63kD for p61-3xFLAG) were observed. Leaves infiltrated with the construction for the expression of P61, empty vector or the expression of GFP were collected for histochemical detection of H_2O_2, expression analysis of marker genes, and quantification of defense hormones. H_2O_2, were visualized by leaf staining with Diaminobenzidine (DAB) as reported elsewhere (Ilarduya et al, 2003). The expression profiles of SA- and HR-related genes were assessed by RT-qPCR as described above (topic 4.7), with primer pairs available at Supplementary Table 7. The SA and JA contents were quantified by LC-MS/MS as described before (Arena et al., 2018).

CAPÍTULO 3

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Making a better home: modulation of plant defensive response by *Brevipalpus* mites

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Keywords: plant-herbivore interaction, plant hormones, defense pathways, salicylic acid, jasmonic acid, cross-talk, Arabidopsis, RNA-Seq

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Making a Better Home: Modulation of Plant Defensive Response by *Brevipalpus* Mites

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False-spider mites of the genus *Brevipalpus* are highly polyphagous pests that attack hundreds of plant species of distinct families worldwide. Besides causing direct damage, these mites may also act as vectors of many plant viruses that threaten high-value ornamental plants like orchids and economically important crops such as citrus and coffee. To better understand the molecular mechanisms behind plant-mite interaction we used an RNA-Seq approach to assess the global response of *Arabidopsis thaliana* (Arabidopsis) plants along the course of the infestation with *Brevipalpus yothersi*, the main vector species within the genus. Mite infestation triggered a drastic transcriptome reprogramming soon at the beginning of the interaction and throughout the time course, deregulating 1755, 3069 and 2680 genes at 6 hours after infestation (hai), 2 days after infestation (dai), and 6 dai, respectively. Gene set enrichment analysis revealed a clear modulation of processes related to the plant immune system. Co-expressed genes correlated with specific classes of transcription factors regulating defense pathways and developmental processes. Up-regulation of defensive responses correlated with the down-regulation of growth-related processes, suggesting the triggering of the growth-defense crosstalk to optimize plant fitness. Biological processes (BPs) enriched at all time points were markedly related to defense against herbivores and other biotic stresses involving the defense hormones salicylic acid (SA) and jasmonic acid (JA). Levels of both hormones were higher in plants challenged with mites than in the non-infested ones, supporting the simultaneous induction of genes from both pathways. To further clarify the functional relevance of the plant hormonal pathways on the interaction, we evaluated the mite performance on Arabidopsis mutants impaired in SA- or JA-mediated response. Mite oviposition was lower on mutants defective in SA biosynthesis (*sid2*) and signaling (*npr1*), showing a function for SA pathway in improving the mite reproduction, an unusual mechanism compared to closely-related spider mites. Here we provide the first report on the global and dynamic plant transcriptome triggered by

Brevipalpus feeding, extending our knowledge on plant-mite interaction. Furthermore, our results suggest that *Brevipalpus* mites manipulate the plant defensive response to render the plant more susceptible to their colonization by inducing the SA-mediated pathway.

Keywords: plant–herbivore interaction, plant hormones, defense pathways, salicylic acid, jasmonic acid, cross-talk, *Tetranychus*, RNA-Seq

INTRODUCTION

Plants are frequently threatened by arthropods herbivores from different feeding guilds causing variable tissue injuries. Chewers consume a significant amount of plant tissue thus promoting extensive damage, while sap-suckers and cell-content-feeders pierce to ingest plant fluids, inflicting minimal physical damage. To further enhance self-protection against attackers, plants display receptors that recognize conserved molecules associated with herbivores (herbivore-associated molecular patterns – HAMPs) or even self-molecules released after cell damage inflicted by the attack (damage-associated molecular patterns – DAMPS) and mount appropriate defense response. Some adapted herbivores have evolved the ability to counteract plant defenses by producing effectors that disrupt plant signaling and induce effector-triggered susceptibility (Hogenhout and Bos, 2011; Ferrari et al., 2013; Pel and Pieterse, 2013). The plant counterattack involves resistance proteins (R proteins) which directly bind the effectors, or the plant proteins they modify, and elicit a second layer of the immune response. The outcome of induced defenses includes the production of toxins that interfere with herbivore feeding, growth, reproduction or fecundity and/or volatile compounds that attract natural enemies of the attacker (Pieterse et al., 2012).

Upon recognition a cascade of phytohormone-dependent signals, modulated by the nature of the damage, orchestrates specific plant defense responses. Generally, arthropods such as chewing insects that greatly damage the plant tissue integrity trigger the jasmonic acid (JA) pathway, whilst herbivores causing minimal tissue disruption, i.e., piercing-sucking arthropods induce salicylic acid (SA) mediated response (Arimura et al., 2011). The SA pathway is typically associated with resistance against biotrophic pathogens and can often antagonize JA-mediated defenses. Ethylene (ET) and abscisic acid (ABA) also control plant responses to herbivore through the modulation of JA signaling branches. ABA regulates the MYC transcription factor branch (MYC-branch) acting in the defenses against herbivores, whereas ET regulates the ethylene responsive factor branch (ERF-branch) to defend against necrotrophic invaders. The ET- and ABA-regulated branches antagonize each other to fine tune JA pathway against the specific invader (Pieterse et al., 2012).

Herbivores can take advantage of the natural cross-talk between hormonal pathways to circumvent plant defenses. *Bemisia tabaci* activates SA responses to suppress effective JA defenses and improve whitefly performance (Zarate et al., 2007; Zhang et al., 2013). Likewise, some insect eggs induce high levels of SA that leads to reduced protein levels of MYC2, subsequent suppression of JA defenses, and the enhancement of larvae performance (Bruessow et al., 2010; Schmiesing et al., 2016). The ERF-MYC branch antagonism is also occasionally exploited by herbivores. Oral secretions of *Pieris rapae* activates the ERF-branch to rewire JA signaling toward the insect preferred branch (Verhage et al., 2011). Beyond through cross-talk, other herbivores are capable of directly suppressing several defense pathways. The mite *Tetranychus evansi* repress both JA and SA signaling in tomato, dramatically reducing the levels of defense compounds (Sarmento et al., 2011; Alba et al., 2015).

Manipulation of plant defenses by herbivores has been shown to frequently occur through saliva-contained effectors. Salivary proteins able to modulate defenses and improve herbivore performance have been identified in insects (Hogenhout and Bos, 2011) and mites (Villarroel et al., 2016). Moreover, proteins from arthropod-associated microorganisms such as endosymbiont bacteria (Casteel et al., 2012; Chung et al., 2013) and viruses (Casteel et al., 2014; Li et al., 2014) may also be present in the saliva and modulate plant defenses to promote herbivore performance.

Current understanding of the mechanisms involved in plant response to herbivores comes mainly from studies of plant–insect interactions. Relatively little is known about molecular responses to other arthropods as mites, most of them focusing on the two-spotted spider mite *T. urticae* (Rioja et al., 2017). False spider mites of the genus *Brevipalpus* (Acari: Tenuipalpidae) are economically important phytophagous mites that attack hundreds of plant species of very distinct families, including large-scale plantations of high-value crops and several ornamental plants (Childers et al., 2003; Kitajima et al., 2010). Besides causing direct damage to some plant species, the negative impacts of infestation are often exacerbated by their ability to vector numerous plant-infecting viruses, the so-called *Brevipalpus*-transmitted viruses (BTVs) (Kitajima and Alberti, 2014). *Brevipalpus yothersi* vectors both cileviruses and tentative dichorhaviruses (Ramos-González et al., 2016; Chabi-Jesus et al., 2018) being the main vector of citrus leprosis virus C (CiLV-C), the prevalent virus causing citrus leprosis disease. Chemical control of *B. yothersi* mites costs millions of dollars each year in Brazil, the world leading producer of sweet orange juice, frozen concentrated orange juice (FCOJ) and not-from-concentrate orange juice (NFC) (Bastianel et al., 2010). The cosmopolitan distribution of *Brevipalpus* spp. poses a major threat to the worldwide citrus industry and to other crops such as coffee (Rodrigues and Childers, 2013; Beard et al., 2015). In addition to their agricultural relevance, *Brevipalpus* mites are also prominent because of their unusual biology. Several species of the genus

are haploid during their entire life cycle, an exclusive feature amongst higher organisms, and are essentially female due to the presence of the endosymbiont bacterium *Cardinium* sp. (Weeks et al., 2001).

Despite the economic and biological significance, many aspects of the *Brevipalpus* mite-plant interaction remain largely unknown. A previous study showed that plants respond to the presence of *B. yothersi* non-viruliferous mites with a ROS burst and induction of specific genes from SA and JA-dependent pathways (Arena et al., 2016). Upon infestation with CiLV-C viruliferous mites, both SA- and JA-responsive genes are reduced, and mite behavior is affected (Arena et al., 2016). To achieve a wider understanding of the molecular mechanisms behind plant-mite interaction, we used an RNA-Seq approach assessing the global response of *Arabidopsis thaliana* plants along the course of the infestation with *B. yothersi.* Transcriptome analysis was complemented with the measuring of the SA and JA hormone levels in plants upon mite feeding. Finally, to further clarify the functional relevance of hormone-triggered plant defense on the interaction, we evaluated the *B. yothersi* oviposition on Arabidopsis mutants impaired in SA or JA-mediated response. Current work provides a comprehensive picture of the plant response to *Brevipalpus* mite feeding.

RESULTS

Brevipalpus Mites Elicit a Significant Transcriptome Reprogramming on Infested Plants

A time-course RNA-Seq experiment was set up to assess the global response of *A. thaliana* plants along the course of infestation with non-viruliferous *B. yothersi* mites. The transcriptome of infested plants was compared with that from non-infested ones (control) at 6 h after infestation (hai), 2 and 6 days after infestation (dai). Overall, 995 million paired-end reads were obtained by Illumina sequencing, with an average of 41.5 million per library and higher average number of reads in samples from the infested treatment (**Supplementary Table S1** and **Figure 1A**). Roughly 94% of the reads mapped against the *A. thaliana* reference genome, with a 91% average of uniquely mapped reads (**Supplementary Table S1** and **Figure 1B**).

Biological variability between samples was verified by principal component analysis (PCA) using the normalized count data (**Figure 1C**). Infested and control samples grouped separately, suggesting a globally distinct expression profile, as expected. Even though a classification of the first principal components as treatment or time of infestation was not clear, the first component (PC1), which accounts for 52% of the variance, apparently separated the samples by the intensity of stimuli. Except for two out of four control samples at 6 dai, all control samples grouped together with those of mite-infested treatments from 6 hai, whose plants were stimulated by a short period of mite feeding. Samples from plants challenged by longer mite feeding period (2 and 6 dai) grouped separately. Hierarchical clustering of samples within each time point confirmed a clear separation between mite-infested and non-infested treatments over the course of the experiment (**Figure 1D**).

By using the negative binomial-based DESeq2 package and FDR correction of *p*-values for multiple comparisons, 5005 differentially expressed genes (DEGs, $\alpha \leq 0.05$) were detected (**Supplementary Table S2**). Mite infestation deregulated 1755, 3069 and 2680 genes at 6 hai, 2 dai, and 6 dai, respectively (**Figure 1E**). At the earliest stage of the interaction (6 hai), the majority of the DEGs was up-regulated. The number of down-regulated genes progressively increased during the interaction reaching its highest rating at 6 dai (**Figures 1E,F**). Analysis performed here show an intense modulation of the plant transcriptome in response to *Brevipalpus* mite infestation.

To validate the RNA-Seq data, 10 genes were selected for Real Time RT-qPCR analysis. Expression of these genes was assessed in a new experiment with mite-infested and non-infested Arabidopsis plants at 6 hai, 2 dai, and 6 dai (**Supplementary Figure S1**). Altogether, expression profiles of selected genes obtained by RT-qPCR were consistent with those obtained by the RNA-Seq, supporting the results described in this work. Additionally, some of these genes had an expression profile similar to that revealed by a qPCR-driven analysis during a comparable experiment previously described (Arena et al., 2016).

The Plant Immune System Is Modulated by *Brevipalpus* Mite Infestation

Gene ontology (GO) enrichment analysis was performed with all 5005 DEGs to identify the most relevant biological processes (BPs), molecular functions (MFs) and cellular components (CC) disturbed during *Brevipalpus* mite-plant interaction. This study identified 264 BPs, 83 MFs and 78 CCs that were over-represented (hypergeometric test, $\alpha \leq 0.001$) in the list of DEGs (**Supplementary Table S3**). Enriched BPs were further visualized as a network using the app BinGO from Cytoscape, where color and size of the nodes identify *p*-values and number of DEGs from each category, respectively (**Supplementary Figure S2**).

The GO network revealed a striking deregulation of plant defensive responses. BP categories were clustered in two major groups comprising metabolism and response to stimuli. BP-metabolism was sub-clustered into two branches separately harboring the primary and secondary metabolisms. Secondary metabolism group was represented by processes related to the biosynthesis and metabolism of "flavonoids," "glucosinolates," "toxins," and "camalexins," which are known to exert anti-herbivory roles and be induced by SA or JA. Primary metabolism included categories associated to the metabolism of: (i) "aminoacids" and "proteins," connected to processes involved in the control of gene expression (such as "protein modification," "phosphorylation," and "transcription"); (ii) "organic acid," whose sub-categories included the "SA metabolism" and "JA biosynthesis and metabolism"; and (iii) "carbohydrates," edged to several photosynthesis-associated categories and processes related to "cell wall modification" such as "callose deposition," a well-known defense against herbivores (Jander, 2014).

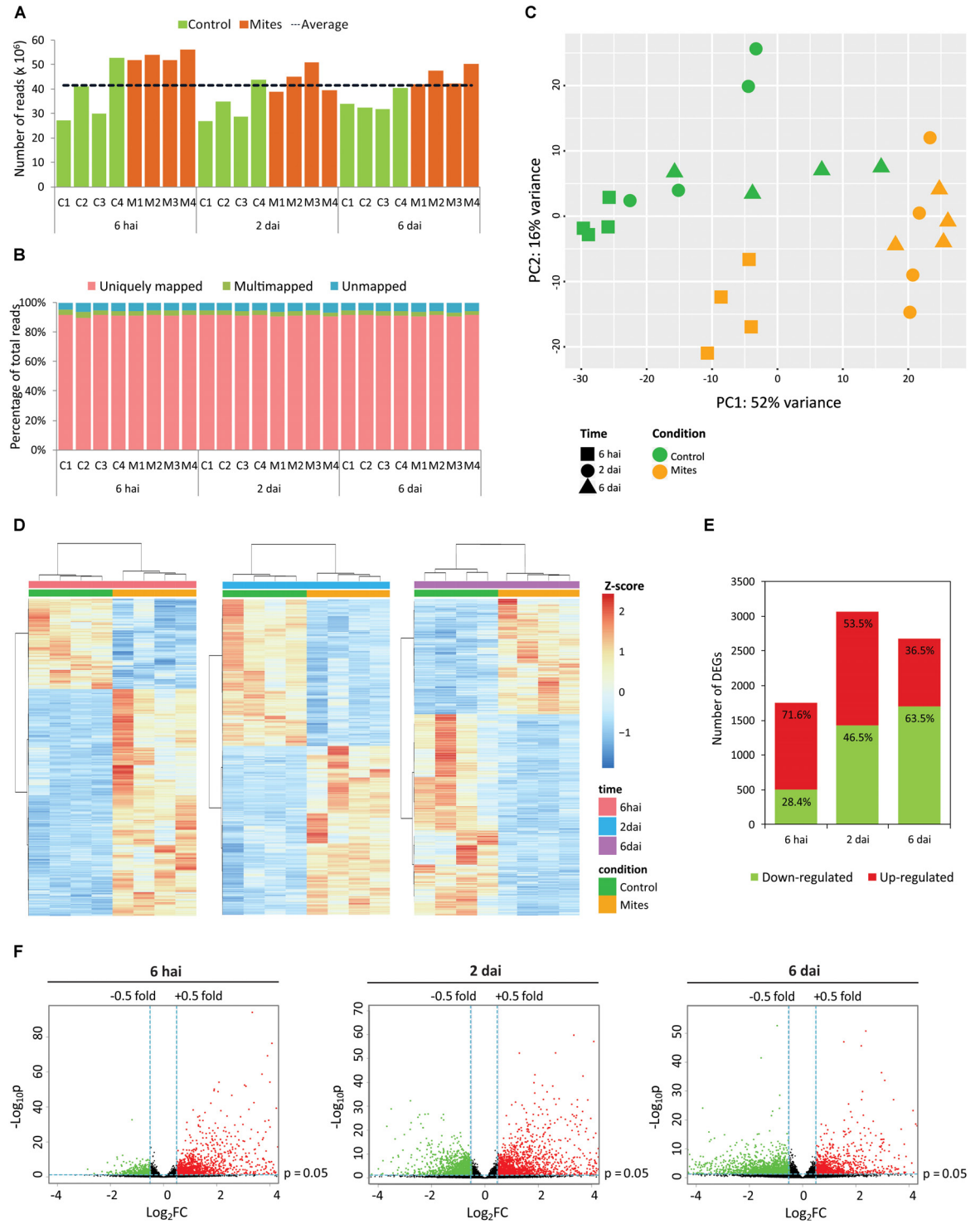


FIGURE 1 | Overview of *Arabidopsis thaliana* transcriptome upon infestation by *Brevipalpus* mites. **(A)** Number of *paired-end* reads generated for each library by Illumina HiSeq sequencing. C, control (non-infested plants); M, mite-infested-plants. Dashed line represents the average of *paired-end* reads from all 24 libraries. **(B)** Proportion of uniquely mapped, multi-mapped and unmapped reads obtained for each library. Reads were mapped in the *A. thaliana* (TAIR 10) genome using *TopHat2*. C, control; M, mite-infected plants. **(C)** Principal component analysis of normalized count data from all samples. **(D)** Hierarchical clustering analysis of normalized count data *z*-scores exhibited by differentially expressed genes (DEGs) of each sample within each time point. **(E)** Numbers of up- and down-regulated DEGs in mite-infested plants in comparison to non-infested control at each time point. DEGs were identified using *DESeq2* and defined by $\log_2$ fold-change ≥ 0.5 and false discovery rate (FDR)-corrected *p*-value ≤ 0.05. **(F)** Volcano-plots of $-\log_{10}$p and $\log_2$FC exhibited by each gene in mite-infested plants compared to non-infested control at each time point. Up- and down-regulated genes are presented in red and green, respectively. FC, fold-change; p, FDR-corrected *p*-value, hai, hours after infestation; dai, days after infestation.

Biological processes cluster centralized in "response to stimulus" was fully represented by processes associated with defense responses. Nodes from the cluster included response to "stress," "abiotic," and "biotic" stimulus (linked to "defense response" and to the subcategories of response to "wounding," "insects," and other pathogens). A large "response to hormone" branch displayed all main hormone-mediated pathways, including SA and JA, but also abscisic acid (ABA), ethylene (ET), auxins (IAA), cytokinins (CK) and gibberellins (GA). Other nodes included in the "response to stimuli" group were "response to chitin," commonly triggered in plants colonized by chitin-rich organisms such as arthropods and fungi (Libault et al., 2007), and "oxidative stress," typically induced in plant-biotic interactions (Arena et al., 2016; Camejo et al., 2016). Several processes related to defense response were also present in "biological regulation" nodes, such as "regulation of hormone levels," "defense response," "immune response," and, "JA pathway."

Specific and Common Transcriptomic Changes Occur at Different Time Points After *Brevipalpus* Mite Infestation of Plants

A comparison of the DEGs deregulated across the experiment revealed both common and specific changes at each time points (**Figure 2A**). Few DEGs were common to all time points, whereas the highest percentage of them were found to be exclusively modulated at 2 or 6 dai suggesting intense reprogramming steps of plant transcriptome throughout the course of the *Brevipalpus* mite infestation (**Figure 2A**).

Most of the BPs (84 terms) over-represented during mite infestation overlap at all time points (**Figure 2B** and **Supplementary Table S4**). These processes included most of the general terms of plant response to stresses and hormones, indicating a continuous and lasting reprogramming of the plant immune system since the beginning of the interaction until at least 6 dai. Several categories were common between 6 hai and 2 dai (75 terms), and 2 dai and 6 dai (20 terms), but no biological process was shared between the first and the last evaluated time points (**Figure 2B**).

Even though processes related to plant defense responses were markedly enriched over the time course of the experiment, time point-specific ontologies were also identified. From all the over-represented BP categories, 49, 47, and 24 were uniquely identified at 6 hai, 2 dai, and 6 dai, respectively (**Figure 2B** and **Supplementary Table S4**). Hormone biosynthesis ("salicylic acid biosynthetic process," "oxylipin biosynthetic process"), early signaling ("activation of MAPK activity"), and structural defenses ("callose deposition," "cell wall thickening," and "lignin biosynthetic process") were processes exclusively enriched at 6 hai (**Figure 2C**). At 2 dai, unique categories were related to the metabolism of defense-related secondary metabolites ("indole glucosinolate metabolic process," "pigment," and "flavonoid biosynthetic and metabolic process"), photosynthesis and oxidative stress ("photosynthesis," "carbon fixation," "photosynthetic electron transport," "response to light intensity," "response to oxidative stress") (**Figure 2C**). Exclusive ontologies that came up with the late infestation state (6 dai) were detoxification processes ("cellular detoxification," "cellular response to toxic substance"), and other associated to cell wall components and structure ("plant-type cell wall organization," "cell wall loosening," and "pectin," "galacturonan," "glucan," "carbohydrate," and "polysaccharide" metabolic process) (**Figure 2C**).

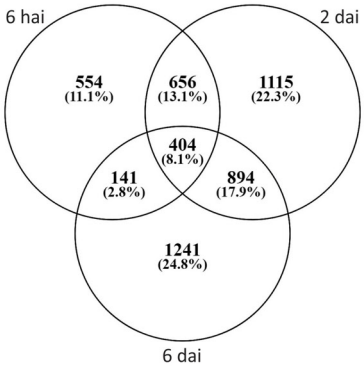
Brevipalpus Mite Infestation Induces Plant Defensive Responses and Represses the Plant Growth-Related Processes

The vast majority of DEGs detected in more than one time point were strictly kept up- or down-regulated. Among the 5005 DEGs identified throughout the analysis, only 201 of them (4%) shift their expression patterns across the experiment (**Supplementary Table S2**). In agreement with this, results of the hierarchical clustering analysis revealed two major clusters of DEGs, which mainly encompassed 2762 and 2243 up-regulated and down-regulated genes, respectively (**Figure 3A**).

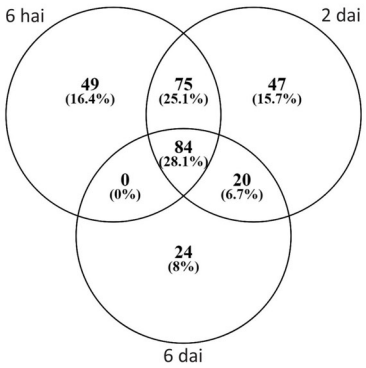
Gene ontology enrichment analysis separately performed with DEGs within each of the predefined clusters identified only 31 common categories between the up- and down-regulated groups (**Figure 3B** and **Supplementary Table S5**). These categories represented higher GO levels and included general BPs such as "regulation of biological quality," "response to stimulus," "metabolic process," "signal transduction," among others. BPs such as "response to hormones" and "hormone-mediated signaling pathway" were also shared between the up- and down-regulated clusters but GO-terms identifying a particular hormonal pathway were always detected in just one of the two groups.

The up-regulated cluster was enriched in 264 exclusive BPs (**Figure 3C** and **Supplementary Table S5**). Network topology was similar to that obtained using all the DEGs (**Supplementary Figure S2**), with two major clusters centralized in metabolic processes and response to stimuli. GO terms within metabolic process cluster involved several BPs related to secondary metabolism, whilst response to stimuli cluster presented terms associated to stress and defense and hormonal pathways. Over-represented categories were massively typified by defensive responses. Besides broad immune system-related terms (e.g., "immune response"), other common categories displayed by the general network (**Supplementary Figure S2**) included responses to hormones, oxidative stress and the production of secondary metabolites, e.g., glucosinolates, flavonoids, and camalexin. Only SA, JA, ET, and ABA-mediated hormonal pathways were represented in the up-regulated cluster network. Induced GO network also included other over-represented processes that were unidentified in the general network (**Supplementary Figure S2**). Among these are included: "response to herbivore," "response to virus," "multi-organism process," "modification of morphology/physiology of other organism," "lignin biosynthetic and metabolic process," "defense response by callose deposition," and "phytoalexin biosynthetic and metabolic process."

A



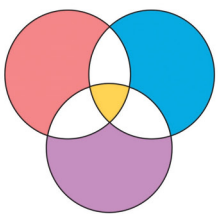
B



C

6 hai	p-value
cellular response to salicylic acid stimulus	3,93E-05
regulation of multi-organism process	9,29E-05
response to endoplasmic reticulum stress	1,25E-04
response to heat	1,26E-04
cell death	1,41E-04
cellular response to salicylic acid stimulus	2,24E-04
salicylic acid biosynthetic process	2,86E-04
defense response by cell wall thickening	2,86E-04
defense response by callose deposition in cell wall	3,01E-04
lignin biosynthetic process	4,25E-04
auxin metabolic process	5,02E-04
oxylipin biosynthetic process	5,16E-04
salicylic acid mediated signaling pathway	6,64E-04
defense response by callose deposition	6,90E-04
oxylipin metabolic process	7,35E-04
activation of MAPK activity	7,61E-04
negative regulation of RNA biosynthetic process	7,61E-04
response to molecule of bacterial origin	9,04E-04
modulation by symbiont of host innate immune response	9,68E-04
activation of protein kinase activity	9,84E-04

2 dai	p-value
cell surface receptor signaling pathway	2,31E-11
fatty acid metabolic process	2,27E-07
flavonoid metabolic process	4,51E-07
flavonoid biosynthetic process	1,32E-06
pigment metabolic process	1,50E-06
reductive pentose-phosphate cycle	2,50E-06
photosynthesis, dark reaction	5,68E-06
carbon fixation	8,55E-06
photosynthetic electron transport in photosystem I	1,38E-05
response to hydrogen peroxide	2,17E-05
transmembrane receptor protein kinase signaling pathway	3,03E-05
pigment biosynthetic process	3,79E-05
response to light intensity	6,39E-05
photosynthetic electron transport chain	7,75E-05
hormone metabolic process	1,46E-04
response to high light intensity	2,85E-04
regulation of stomatal movement	3,92E-04
fatty acid derivative metabolic process	6,67E-04
hormone transport	7,00E-04
indole glucosinolate metabolic process	8,20E-04



6 dai	p-value
polysaccharide metabolic process	5,49E-08
plant-type cell wall organization	2,06E-06
carbohydrate catabolic process	1,57E-05
cellular oxidant detoxification	3,90E-05
cellular detoxification	4,37E-05
cellular response to toxic substance	4,89E-05
oligopeptide transport	8,13E-05
plant-type cell wall loosening	2,10E-04
steroid metabolic process	5,14E-04
response to gibberellin	5,48E-04
lipid biosynthetic process	5,67E-04
response to low light intensity stimulus	5,97E-04
plant-type cell wall organization or biogenesis	6,33E-04
pectin metabolic process	6,35E-04
syncytium formation	6,38E-04
sterol biosynthetic process	6,38E-04
galacturonan metabolic process	6,97E-04
carbohydrate biosynthetic process	7,48E-04
cellular glucan metabolic process	9,93E-04
glucan metabolic process	9,93E-04

6 hai x 2 dai x 6 dai	p-values at respective times		
response to stimulus	2,49E-75	1,69E-98	6,89E-50
response to chemical	4,46E-58	9,46E-70	1,81E-38
response to stress	2,84E-57	1,85E-52	4,57E-19
defense response	1,07E-42	3,09E-40	9,84E-12
response to wounding	3,02E-42	1,18E-33	3,91E-06
response to external biotic stimulus	7,21E-37	6,48E-39	7,52E-10
response to biotic stimulus	1,59E-36	6,48E-39	1,60E-09
response to jasmonic acid	1,66E-33	2,76E-33	1,22E-10
response to hormone	5,57E-33	1,39E-50	1,82E-29
response to bacterium	1,08E-32	1,12E-33	4,78E-06
response to chitin	9,52E-24	1,64E-27	1,72E-09
cell communication	1,09E-23	1,22E-32	1,35E-18
signaling	9,18E-22	1,12E-33	3,81E-18
hormone-mediated signaling pathway	1,71E-16	4,00E-21	4,66E-13
biological regulation	2,68E-14	1,59E-16	4,66E-13
response to abiotic stimulus	1,54E-13	3,28E-32	6,55E-20
secondary metabolic process	8,94E-13	9,29E-14	3,81E-04
response to salicylic acid	1,35E-11	3,60E-11	2,23E-07
response to ethylene	1,45E-08	1,88E-10	2,48E-08
response to abscisic acid	6,36E-04	4,67E-09	1,36E-04

FIGURE 2 | Transcriptomic changes at different time points after *Brevipalpus* mite infestation of *A. thaliana* plants. **(A)** Venn diagram of DEGs in mite-infested plants compared to non-infested control at each time point. DEGs were identified using *DESeq2* and defined by $\log_2$ fold-change ≥ 0.5 and FDR-corrected *p*-value ≤ 0.05. **(B)** Venn diagram of overrepresented BPs of DEGs at each time point. Overrepresented BPs were identified for each time point based on a hypergeometric test with FDR-adjusted *p*-values ≤ 0.001. **(C)** Lists of overrepresented BPs exclusive to each experimental time point (6 hai, 2 dai or 6 dai) and those commons between them (6 hai × 2 dai × 6 dai). BPs corresponding *p*-values obtained in the Gene ontology (GO) enrichment analysis are included in the right column of the tables. Twenty BPs of each list are presented in each table. Complete lists of exclusive and common BPs are available in **Supplementary Table S4**.

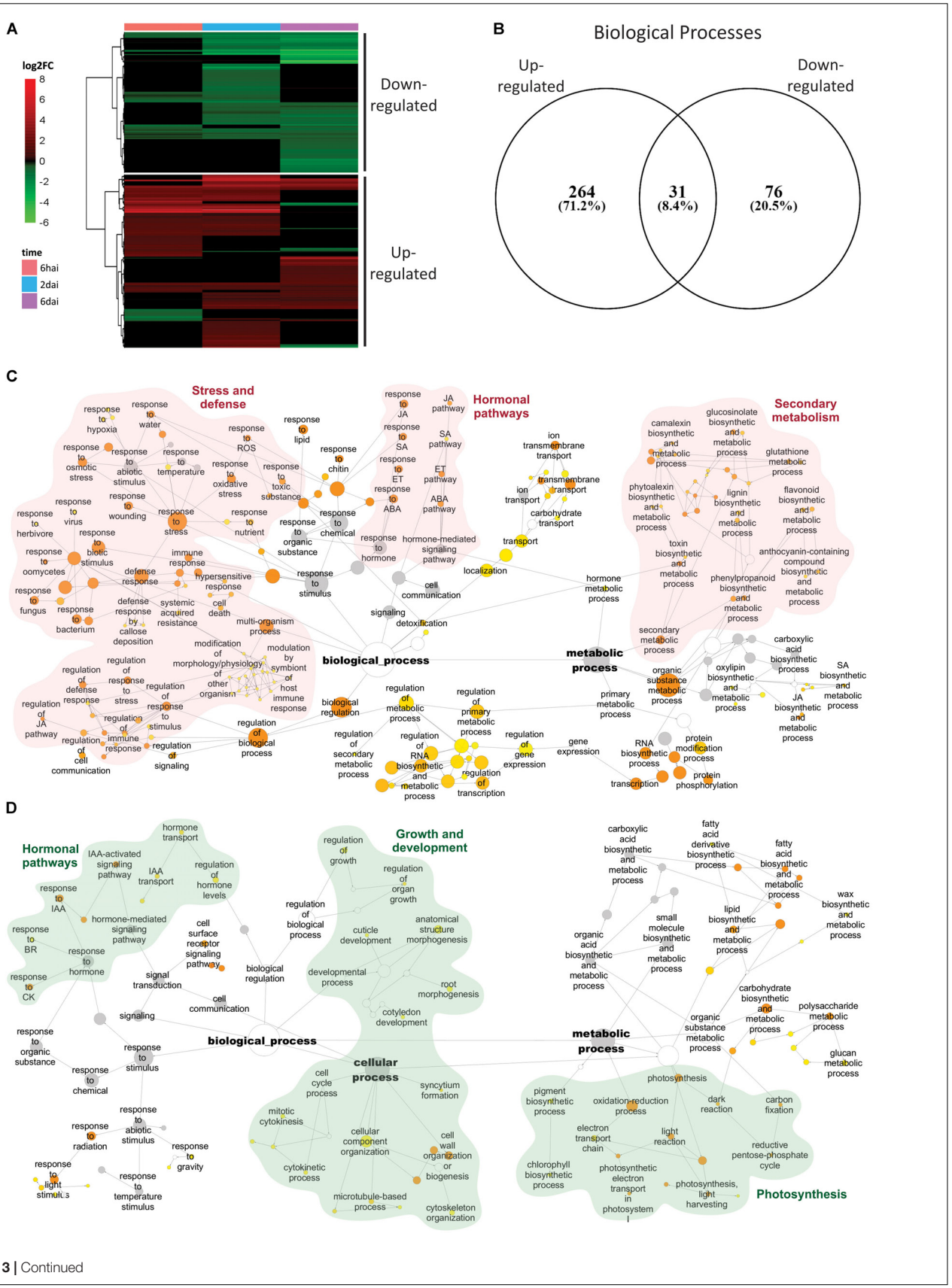


FIGURE 3 | Continued

FIGURE 3 | Induced and repressed responses on *A. thaliana* infested by *Brevipalpus* mites. **(A)** Hierarchical clustering analysis of $\log_2$ FC exhibited by DEGs on mite-infested plants compared to non-infested control. DEGs were identified using *DESeq2* and defined by $\log_2$ fold-change ≥ 0.5 and FDR-corrected p-value ≤ 0.05. hai, hours after infestation; dai, days after infestation. **(B)** Venn diagram of overrepresented BPs of DEGs at each cluster composed by up- and down-regulated genes. Overrepresented BPs were identified for each cluster based on a hypergeometric test with FDR-adjusted p-values ≤ 0.001. **(C,D)** Networks of enriched BPs from clusters of up-regulated **(C)** and down-regulated **(D)** DEGs, generated using the app BinGO in Cytoscape. Size of the nodes correlates with the number of DEGs. Color of the nodes reveals p-values of enriched categories. Nodes in gray represent categories that were shared between clusters of up- and down-regulated genes. Names of some BPs were simplified for clarity; full names are displayed in **Supplementary Table S5**. ROS, reactive oxygen species; SA, salicylic acid; JA, jasmonic acid; ET, ethylene; ABA, abscisic acid; IAA, auxin; CK, cytokinin; BR, brassinosteroid.

The down-regulated cluster was enriched in 76 exclusive BPs, which were predominantly associated with the plant growth and development (**Figure 3D** and **Supplementary Table S5**). Over-represented terms included broad categories, for instance "developmental process" and "regulation of growth," and also those directly related to plant growth such as "cytokinetic process," "cell cycle process," "mitotic cytokinesis," or indirectly related to growth such as "cell wall organization or biogenesis" and "cytoskeleton organization". Among the enriched BPs there were also processes associated to morphogenesis and development of specific plant components such as "root morphogenesis," "cuticle development," and "cotyledon development." The other major class of over-represented BPs uniquely detected in the down-regulated cluster comprised photosynthesis-related processes such as "photosynthesis," "electron transport chain," "carbon fixation," "photosynthesis, dark and light reaction," "light harvesting," "photosynthetic electron transport," and "chlorophyll biosynthetic process." Finally, the only hormones represented in the down-regulated cluster network were the major growth regulators IAA, CK and brassinosteroids (BR).

Co-expression of Genes Correlates With Classes of Transcription Factors (TFs) Involved in SA, JA and Developmental Processes

Since transcriptional reprogramming is mainly controlled by TFs, the regulation of the expression dynamics of DEGs by specific classes of TFs was tested by two different approaches.

First, over-represented TF families were searched based on up- and down-regulated DEGs that encode TFs (**Figure 4A** and **Supplementary Table S6**). Within the cluster of up-regulated DEGs, 254 (9.2%) TFs from 30 different families were identified. From those, 16 over-represented families were detected (hypergeometric test, $\alpha \leq 0.001$). The largest and most significant of them were the WRKY (33 genes, p-value = 2.47E-33) and the AP2/ERF (40 genes, p-value = 8.46E-34), known to act as regulators of SA pathway and ERF-branch of the JA pathway, respectively. From the analysis using the cluster of down-regulated DEGs, 141 (6.3%) TFs belonging to 30 families were detected. Twenty-three of these families were also found in the cluster of up-regulated DEGs. TFs were evenly distributed among 18 over-represented families (hypergeometric test, $\alpha \leq 0.001$), with lower significance (higher p-values). The largest and most significantly over-represented families were bHLH (22 genes, p-value = 3.21E-25), which comprises the regulators of the MYC-branch of the JA pathway, and C3H family (17 genes, p-value = 1.46E-17).

Second, TF families that potentially regulate the expression of the DEGs were searched based on over-represented target genes within the DEGs (**Figure 4B** and **Supplementary Table S7**). Enriched target genes and their corresponding TFs were identified by using a TF enrichment tool that takes advantage of previously identified *cis*-regulatory elements and regulatory interactions from literature mining (Jin et al., 2017). As a result, WRKY was the largest identified family with potential targets within the up-regulated DEGs. Twenty-one out of its 42 TF members were also induced during *Brevipalpus* mite-plant interactions. Targets from WRKY TFs were exclusively enriched in the up-regulated cluster. The next largest families with targets within the induced DEGs were MYB, bZIP, and bHLH, with 29, 26, and 24 TFs, respectively. Targets for MYB, bZIP, and bHLH, however, were not exclusively enriched in the analysis of the cluster of up-regulated DEGs. These families represented by 34, 24, and 14 TFs, respectively, were also among the largest with potential targets within down-regulated DEGs. MYC2, the marker TF from the MYC-branch of the JA pathway, was one of the bHLH TF with targets exclusively enriched in the down-regulated cluster. Notably, the analysis of the down-regulated cluster also revealed the TCP family, which is typically involved in the control of plant development. This family involved 15 and 4 TFs that potentially regulate targets within the assortment of repressed and induced DEGs, respectively.

Overall, the analysis of co-expressed genes with its corresponding TFs showed a correlation of up- and down-regulated genes with TFs that regulates SA, JA and developmental processes. The SA-related WRKY family was the largest one with target genes exclusively enriched in the up-regulated cluster and most of its TF members were also up-regulated. The analysis of enriched TFs settles the involvement of plant hormonal pathways and developmental processes in the plant response to *Brevipalpus* mites, with highlight on the participation of the SA pathway solely on the up-regulated responses.

Focus on Defense Pathways: SA- and JA-Mediated Responses Are Induced in *Brevipalpus* Mite-Infested Plants

Over-represented genes from GO-terms associated with SA and JA-dependent responses were thoroughly reviewed to confirm their induced status. Data from genes included in the categories "response to SA" and "SA metabolic process,"

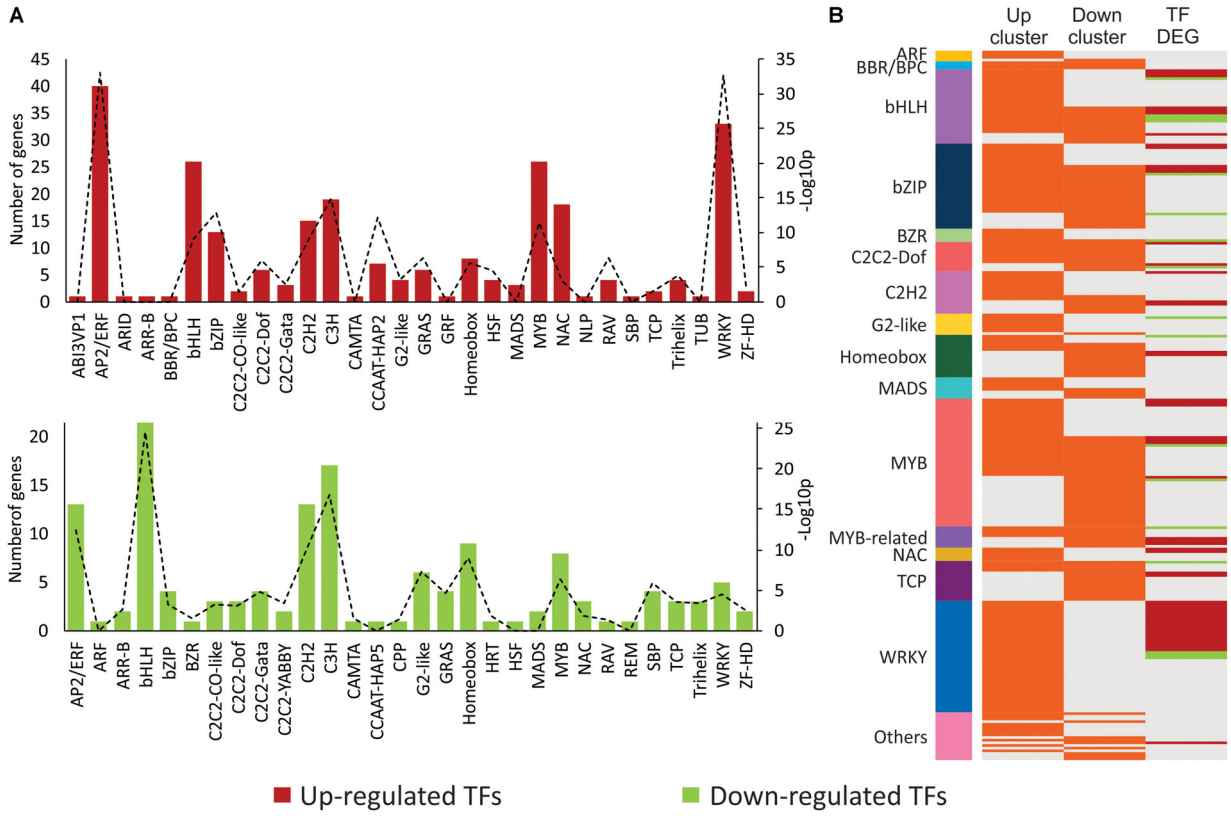


FIGURE 4 | Enriched transcription factors (TFs) and TF targets in clusters of mite-responsive co-expressed genes. **(A)** Number of DEGs coding for TFs within each TF family identified in the clusters of up- and down-regulated DEGs. Up- and down-regulated DEGs are presented in red and green, respectively. Levels of enrichment ($-\text{Log}_{10}\ p$, with p: p-value) of each family (hypergeometric test, $\alpha \leq 0.001$) are presented by a dashed line with its corresponding values in the secondary axis. **(B)** TFs with enriched targets within each cluster of up- and down-regulated DEGs, identified by TF enrichment tool. TFs are grouped according to their families. Each line identifies one TF. In the first and second row ("Up" and "Down" clusters, respectively), orange lines correspond to TFs with enriched targets within each cluster. In the third row ("TF DEGs"), red and green lines represent up- and down-regulated differentially expressed genes, respectively, encoding TFs. Gray lines indicate absence of enriched targets for a given TF- and/or TF not differentially expressed. Families encompassing two or less TFs were grouped in "Others."

or "response to JA," "regulation of JA-mediated pathway," and "JA metabolic process" were processed by a Hierarchical cluster analysis.

The SA-dependent pathway was represented by 103 DEGs (**Supplementary Table S8**). Eighty-one of these DEGs were up-regulated in at least one of the experimental time points. Some of these genes were induced at either early or late stages of the response, but they were not down-regulated in any of the other analyzed time points (**Figures 5A,B**). Examples of these expression patterns are the genes coding for the signaling protein for SA activation *EDS1* (*enhanced disease susceptibility 5*) and the SA-biosynthetic enzyme *ICS1* (*isochorismate synthase 1*) that were up-regulated at the beginning of the interaction, whilst the SA-responsive proteins *PR1* (*pathogenesis-related protein 1*) and *GLIP1* were induced at later time points. Moreover, the expression profile analysis of some DEGs revealed the quick regulation of some SA-responsive genes since the initial steps of the plant-mite interaction. For instances, *PR2/BGL2* (*pathogenesis-related protein 2/beta-1,3-glucanase 2*) was up-regulated as soon as 6 hai and remained activated at least till 2 dai. Among the induced genes associated to the SA pathway there were also several signaling kinases such as the receptor-like kinase (RLK) *CRK9* (*cysteine-rich RLK 9*), the *wall-associated kinases WAK1* and *WAKL10*, and the *L-type lectin receptor kinase LCRK-S.2, LCRK-IV.1* and *LCRK-IX.2*. Other up-regulated DEGs from this cluster were genes encoding the SA biosynthetic enzymes *ICS2* and *PAL1* (*Phenyl ammonia lyase 1*), the transporter of SA from chloroplast to cytoplasm *EDS5* (*enhanced disease susceptibility 5*), the regulator of SA responses *GRX480* (*glutaredoxin 480*), the methyl-salicylate (MeSA) esterase proteins *MES7* and *MES9* (*methyl esterase 7* and *9*), the defense protein *PR5*, and several TFS from WRKY and MYB families.

Another small cluster of SA-related genes comprised a group of 22 DEGs that were mainly down-regulated, particularly at the two latest time points of the experiment (**Figure 5A**). This cluster was largely formed by TFs that are also responsive to JA. Repressed TFs included members of the ERF/AP2, MYB and GRAS/DELLA families. Other repressed genes beyond TFs were *UGT1* (*UDP-glucose transferase 1*), involved in the callose formation, and the

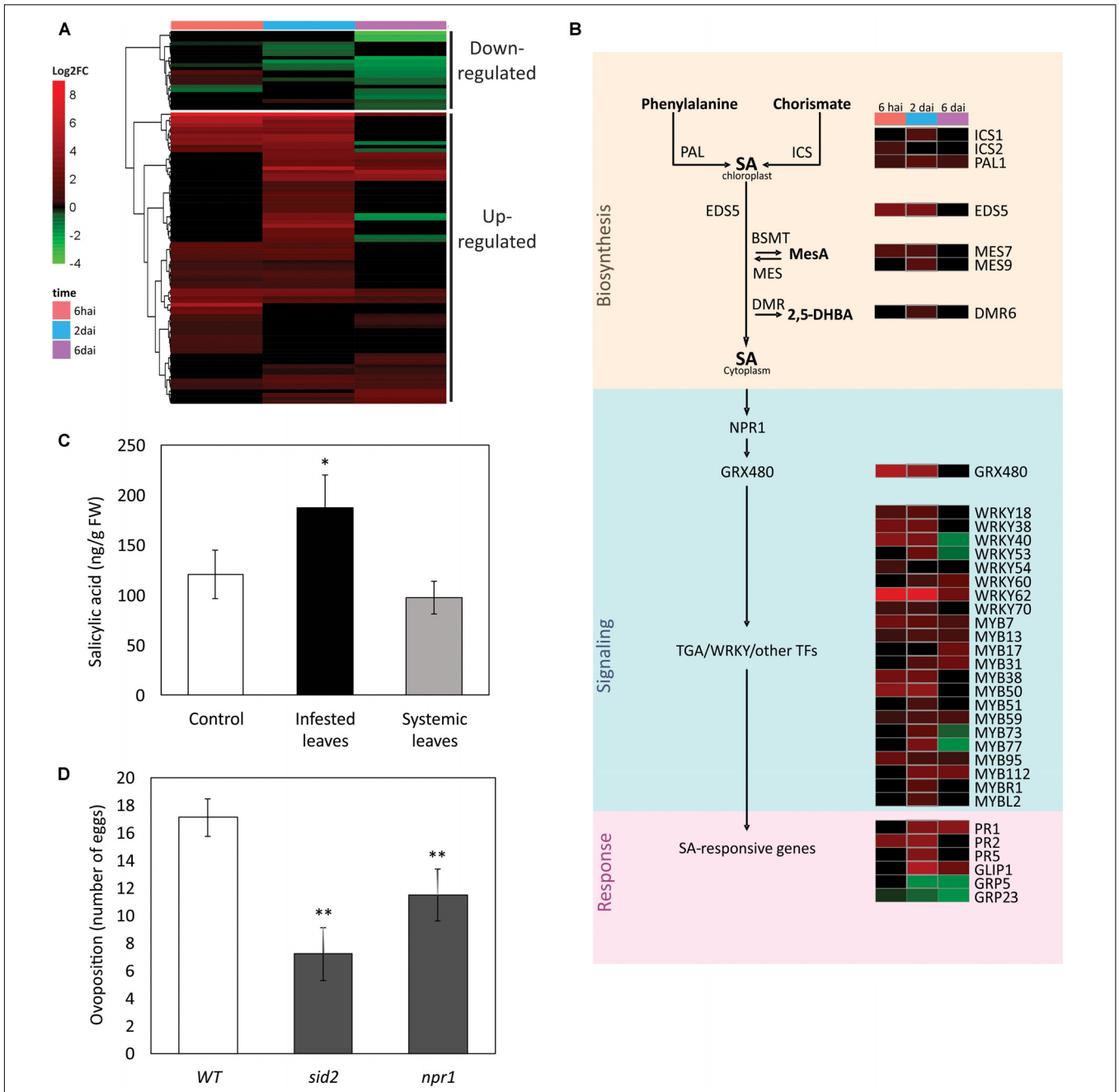


FIGURE 5 | Salicylic acid (SA) pathway in the *A. thaliana* response to *Brevipalpus* mites. **(A)** Hierarchical clustering analysis of log_2 FC exhibited by DEGs involved in the SA pathway. hai, hours after infestation; dai, days after infestation. **(B)** Schematic representation of SA pathway, where a sub-set of DEGs is presented. Colors identifies log_2FC of DEGs at each experimental time point, according to the scale presented in **(A)**. **(C)** SA levels in infested and systemic leaves of mite-infested plants, and in non-infested control plants. Hormone levels were quantified by LC-MS/MS at 6 dai. Error bars represent standard errors. Statistically significant difference at p-value ≤ 0.05 (*) is indicated. FW, fresh weight. **(D)** Mite performance in Arabidopsis mutants compromised in the SA pathway. Data represent the average number of eggs deposited after 4 days of infestation with five *Brevipalpus yothersi* mites/plant. Error bars represent standard errors. Statistically significant differences at p-values ≤ 0.01 (**) are indicated. WT, wild type.

genes encoding the *GRP23* and *GRP5* proteins *(glycine-rich proteins 23 and 5*), which are components of the plant cell wall.

The JA-mediated pathway was composed by 137 DEGs that, similarly to what was observed in the SA-pathway analysis, were mainly up-regulated (**Supplementary Table S8**) (**Figures 6A,B**). DEGs were subdivided in three clusters: two larger groups formed by 60 and 54 highly and mildly up-regulated genes, respectively, and a small one composed by 23 genes that were mostly down-regulated (**Figure 6A**).

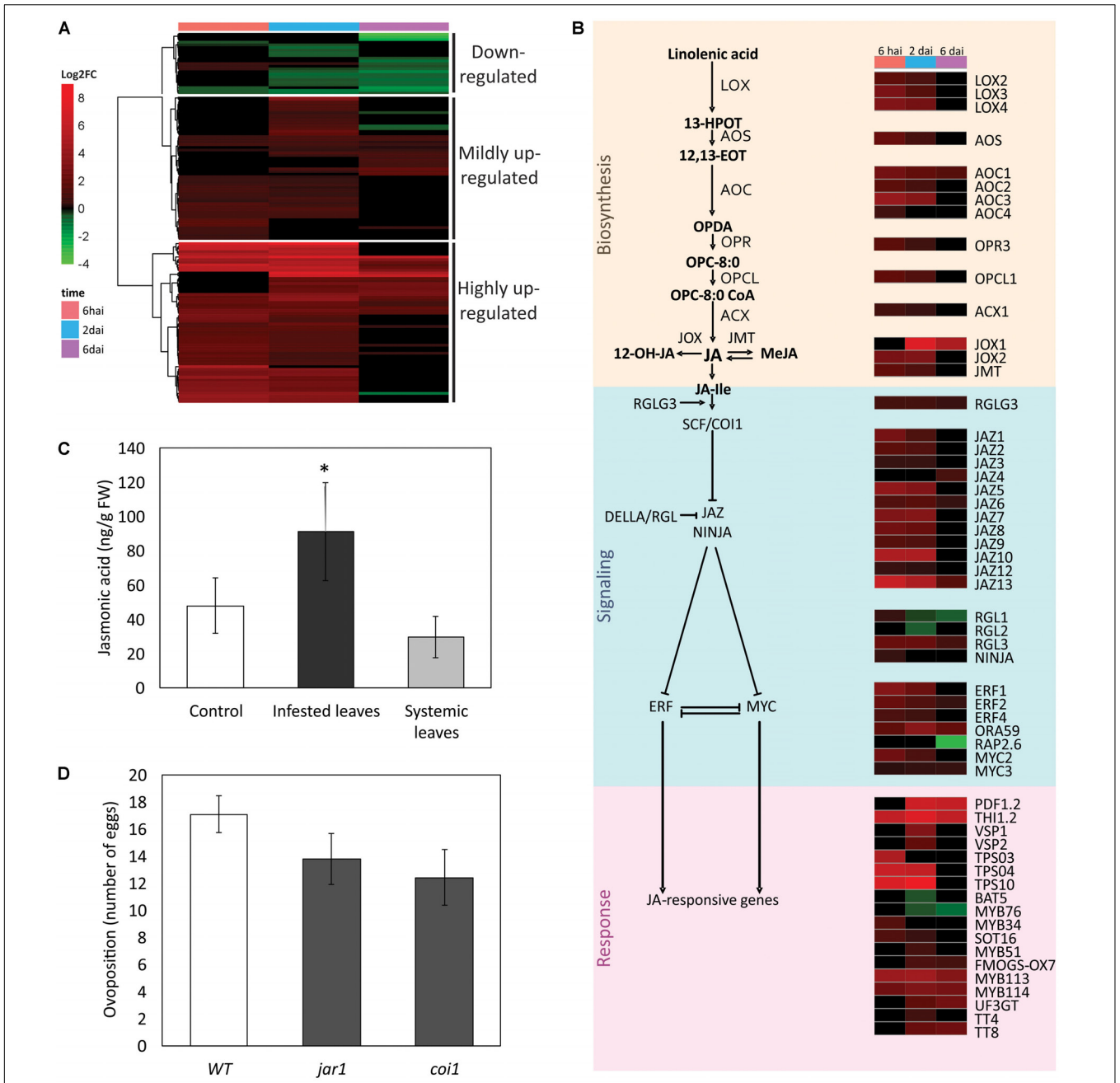


FIGURE 6 | Jasmonic acid (JA) pathway in the *A. thaliana* response to *Brevipalpus* mites. **(A)** Hierarchical clustering analysis of $\log_2$ FC exhibited by DEGs involved in the JA pathway. hai, hours after infestation; dai, days after infestation. **(B)** Schematic representation of JA pathway showing a sub-set of DEGs. Colors identifies $\log_2$FC of DEGs at each time point, according to the scale presented in **(A)**. **(C)** JA levels in infested and systemic leaves of mite-infested plants, and in non-infested control plants. Hormone levels were quantified by LC-MS/MS at 6 dai. Error bars represent standard errors. Statistically significant differences at p-value ≤ 0.05 (*) is indicated. FW, fresh weight. **(D)** Mite performance in Arabidopsis mutants compromised in the JA pathway. Data represent the average number of eggs deposited after 4 days of infestation with five *Brevipalpus yothersi* mites/plant. Error bars represent standard errors. WT, wild type.

Highly induced JA-related genes (**Supplementary Table S8**) at the beginning of the infestation code for proteins acting upstream on the JA pathway such as the DAMP receptor *PEPR2* (*PEP1 receptor 2*) and the JA-biosynthetic and modifying enzymes *AOS* (*allene oxide synthase*), *AOC2* and *AOC3* (*allene oxide cyclase 2* and *3*), *LOX2, LOX3,* and *LOX4* (*lipoxygenase 2*, *3,* and *4*), *OPCL1* (*OPC-8:0 CoA ligase 1*) and *JMT* (*JA carboxyl methyltransferase*) (**Supplementary Table S8**). DEGs induced at the two first experimental time points also included terpene synthases (*TPS03*, *TPS04,* and *TPS10*), several JAZ (*jasmonate-zim-domain*) proteins (*JAZ1, JAZ2, JAZ5, JAZ7, JAZ8, JAZ9/TIFY7,* and *JAZ10*) and the TFs *MYC2* and *ERF1*.

Genes with high expression at later time points encode proteins directly involved in defense such as the marker responsive protein of the ERF-branch *PDF1.2* (*plant defensin 1.2*), and proteins involved in the anthocyanin biosynthesis such as *UF3GT* (*UDP-glucose:flavonoid 3-o-glucosyltransferase*). Other highly up-regulated DEGs included MYB TFs, the DELLA protein *RGL3* that contributes to JA/ET-mediated defenses, the ERF-branch TFs *ERF2* and *ORA59* (*octadecanoid-responsive Arabidopsis AP2/ERF59*), the responsive gene *THI2.1* (*thionin 2.1*), and the JA oxidases *JOX1* and *JOX2* that down-regulate plant immunity by hydroxylation and inactivation of JA.

Genes from the JA pathway that were mildly induced (**Supplementary Table S8**) at the first time points included those coding for the JA-biosynthetic enzymes *ACX1* (*acyl-CoA oxidase 1*) and *AOC4*, the TF *WRKY70* that acts on the SA-JA antagonism, the negative regulators of JA-responsive genes *NINJA* (*novel interactor of JAZ*) and *JAZ3*, and the proteins *MYB34* and *SOT16* (*sulfotransferase 16*) involved in the synthesis of glucosinolates. Later in the infestation, the group of mildly induced genes included those coding for the proteins *MYB75/PAP1* and *TT4* that act in the biosynthetic pathway of anthocyanin, the *MYB51* and *FMOGS-OX7* proteins involved in the synthesis of glucosinolates, and the responsive marker genes of the MYC-branch *VSP1* and *VSP2* (*vegetative storage proteins 1* and *2*), which directly act during the anti-herbivory defense. Two essential modulator genes of the JA signaling, i.e., *RGLG3* (ring domain ligase 3) and the *MYC3* TF were induced at all the assessed time points although at low expression levels. *RGLG3* encodes for a RING-ubiquitin ligase acting upstream of JA-Ile recognition and MYC3 operates together with MYC2 coordinating the expression of responsive genes from the MYC-branch.

The down-regulated JA cluster comprised DEGs that were mainly repressed at 2 and 6 dai (**Supplementary Table S8**). Some of them encode TF commonly acting in the SA-pathway such as members of the families: ERF (*RAP2.6* and *DREB26*), MYB (*MYBS1*, *MYB28*, *MYB29,* and *MYB16*), and GRAS/DELLA (*RGL1* and *RGL2*). Other repressed genes were those encoding the *BAT5* (*bile acid transporter 5*) protein and the *MYB76* TF, both required for the biosynthesis of glucosinolates, the DAMP receptor *PEPR1*, and the JA-repressed protein *AGP31* (arabinogalactan protein 31).

SA and JA Levels Increase in Mite-Infested Plants

Both SA and JA biosynthetic and responsive genes were induced in *Brevipalpus* mite-infested plants. To confirm the consistency of the observed molecular data, the profiles of the SA and JA hormones were determined in Arabidopsis plants challenged with *Brevipalpus* mites. SA (**Figure 5C**) and JA (**Figure 6C**) levels were 1.5- and 2.8-fold higher, respectively, on infested leaves when compared to the control ones (Student's *t*-test, $\alpha \leq 0.05$).

Salicylic acid and JA levels were also verified in systemic leaves of mite-infested plants. No difference was observed between the levels of both hormones in systemic and non-infested control leaves, suggesting a local rather than a systemic response to *Brevipalpus* mite infestation.

Brevipalpus Mites Have a Decreased Performance on Plants Impaired in SA Responses

Salicylic acid- and JA-mediated pathways were clearly induced upon *Brevipalpus* mite infestation. To further explain the functional relevance of the plant hormonal pathways on the interaction, the performance of *B. yothersi* mites was evaluated on Arabidopsis plants impaired in SA or JA-mediated response. Mite oviposition was assessed on mutants defective in SA biosynthesis (*salicylic acid induction deficient2, sid2*) and signaling (*non-expressor of pathogenesis-related protein1, npr1*), and JA signaling (*jasmonate resistant1, jar1* and *coronatine-insensitive1, coi1*). Plants were infested with adult female mites and the number of laid eggs was counted after 6 days.

The number of eggs per plant was 2.4- and 1.5-fold lower on SA-mutants *sid2* and *npr1*, respectively, when compared to the mite's performance in the infested wild-type *A. thaliana* Col-0 plants used as control (Student's *t*-test, $\alpha \leq 0.05$) (**Figure 5D**). No difference was observed between the number of eggs on the mutants affected in the JA pathway (*jar1* and *coi1*) and the wild-type control (Student's *t*-test, $\alpha \leq 0.05$) (**Figure 6D**). These results point to a role of SA-mediated response promoting the *Brevipalpus* mite colonization.

DISCUSSION

False-spider mites of the genus *Brevipalpus* are serious and cosmopolite phytophagous pests with a unique biology (Weeks et al., 2001). They directly provoke injuries in some plant species, but the major consequence of their feeding behavior ensues from the transmission of several cile- and dichorha- viruses that infect economically important crops (Kitajima and Alberti, 2014). Almost 10 species of *Brevipalpus* mites are known to act as virus vector, but, among them, mites of the species *B. yothersi* stands out due to their involvement in transmission of viruses causing citrus leprosis, a severe disease that threatens the citrus industry in the Americas (Beard et al., 2015; Ramos-González et al., 2016). To disentangle the *Brevipalpus*-mite interaction, in the current paper we provide data that extensively describe the response of Arabidopsis plants during their colonization by *Brevipalpus* mites. Changes in the plant transcriptome profile are complemented with the analysis of the accumulation of defense hormones and the results are discussed emphasizing the role of particular plant defense genes during the *Brevipalpus* infestation process.

Our results showed that mite infestation clearly triggers the plant immune system. Processes related to the response to herbivory and other biotic stresses dominate a large number of the over-represented GO categories among all DEGs. Most of the BPs were common between all the time points, although specific changes were also identified. Plant response during the initial 6 h included the induction of genes involved in the hormone

biosynthesis and signaling, consistent with an early recognition of the mite feeding. Transcriptome changes were followed by the up-regulation of a wide range of genes involved in defense and synthesis of secondary metabolites at 2 and 6 dai. The major dissimilarity was between the first and last time points, which do not share any BPs except the ones that were present throughout the infestation. GO enrichment analysis revealed that defense responses were up-regulated and mainly involved the SA- and JA-mediated pathways. Deeper analysis on SA/JA-related DEGs and quantification of hormone contents confirmed that these pathways were distinctly induced upon the infestation by *Brevipalpus* mites. On mite-infested plants, genes involved in the biosynthesis, signaling, and response of the SA and JA pathways were mostly up-regulated, and SA and JA hormone levels were increased. In this regard, simultaneous induction of SA and JA plant response to *B. yothersi* infestation follows a similar pattern to those observed during plant colonization by several spider mites (Kant et al., 2004; Zhurov et al., 2014; Alba et al., 2015; Rioja et al., 2017). Induced defense-related processes also included a clear transcriptional response to oxidative stress. Previous histochemical analysis of infested tissues revealed the production of ROS upon mite feeding (Arena et al., 2016). Induction of ROS production and related transcripts also resembled plant response to spider mite feeding and, in both cases, the role of ROS signaling remains to be determined (Agut et al., 2018).

Gene ontology enrichment analysis revealed an extensive genetic expression adjustment throughout the JA pathway including the hormonal biosynthesis and metabolism, and downstream regulation and response. Mite presence induced the DAMP-receptor PEPR2, suggesting the perception capacity of damaged tissues by Arabidopsis plants. Although minimal, mite feeding causes tissue disruption on infested leaves (Arena et al., 2016). Individual or very few dead cells are observed after mite feeding activity, probably as consequence of punctures by the mite stylets. Upon recognition, JA-biosynthetic enzymes such as AOC, AOSs, and LOXs were up-regulated. Higher JA content in mite-infested leaves confirmed activity of this biosynthetic pathway. Downstream of JA production, several signaling proteins and regulators were induced, including many TFs from MYB, AP2/ERF, and bHLH families. Downstream responses were represented by an array of up-regulated transcripts involved in the production of terpenes, anthocyanin, and glucosinolates. Since induced JA responses to *Brevipalpus* mites are similar to the ones that mediate Arabidopsis response to spider mites (Zhurov et al., 2014), our results indicate a conservation of mite-induced JA regulatory mechanisms. Moreover, several negative regulators of JA response were induced on plants infested by *B. yothersi*, including genes encoding NINJA and numerous JAZ proteins, which interact to repress the TFs that regulates the expression of JA-responsive genes (Wager and Browse, 2012), and JA oxidases, which down-regulates downstream responses by hydroxylation and inactivation of JA (Caarls et al., 2017). In this context, the induced JA pathway might be attenuated, and consequently, the observed data reflect a somewhat mitigated rather than a fully-induced JA-mediated response.

Even though the JA pathway was largely induced upon mite infestation, distinct activation profiles of JA branches were observed. First, TFs from the ERF- and MYC-branches were differentially regulated. AP2/ERF family with TFs that control the ERF-branch was the largest and most enriched family within up-regulated DEGs, whilst bHLH family that includes the TFs that regulates the MYC-branch was the largest and most enriched one within down-regulated DEGs. Particularly, MYC2 that is the major regulator of the MYC-branch responsive genes, was induced, although its target genes were enriched within the cluster of down-regulated genes. Second, the expression levels of defensive genes from the ERF-branch were much higher than that from genes of the MYC-branch. The gene encoding the ERF-responsive anti-microbial protein PDF1.2 figures among the most highly up-regulated DEGs (e.g., FC = 94-fold at 2 dai), whilst those coding for the MYC-responsive anti-herbivory proteins VSP2 and VSP1 were only mildly or not induced at the same experimental time points (e.g., FC = 4- and 10-fold, respectively, at 2 dai). The preferential activation of the ERF-branch over the MYC-branch was described as an herbivore strategy to induce a harmless response in expense of a harmful defense (Verhage et al., 2011; Pieterse et al., 2012). The strongest activation of the ERF-branch reported here corroborates a previous study proposing that *Brevipalpus* mites might mitigate effective defenses by manipulating the plant resistance mechanisms toward herbivore preferred JA responses (Arena et al., 2016). However, further analysis of ERF and MYC mutants are required to clarify the actual role of each one the JA branches in plant response to *Brevipalpus* mites.

Within the induced hormonal pathways in response to *Brevipalpus* infestation, the SA-mediated pathway plays a conspicuous role. On these plants SA levels were elevated, the vast majority of SA-related genes were up-regulated, and the SA-related WRKY TFs as well as their target genes were exclusively over-represented in the cluster of up-regulated genes. Induction of SA response has been associated with stealthy arthropods such as piercing-sucking insects (Nguyen et al., 2016; Patton et al., 2017). Likewise, *Brevipalpus* mite feeding behavior causes minimal tissue disruption. During feeding, mites pierce epidermal cells using interlocked stylets, sometimes through leaf stomata, and suck out overflowed cell content (Kitajima and Alberti, 2014). Activation of the SA pathway by *Brevipalpus* mites agrees with the common pattern observed for herbivores causing little overt tissue damage (Arimura et al., 2011).

Interestingly, an increasing number of evidence indicate that activation of SA pathway favors herbivore performance rather than acts as an effective defense against herbivory. For instance, *Bemisia tabaci* nymphs performs better in the *cpr6* mutants pre-activated for SA-mediated defenses (Zhang et al., 2013), and SA exogenous application render Arabidopsis plants more attractive to thrips (Abe et al., 2012). Using Arabidopsis mutants, we found that the performance of *Brevipalpus* mites is compromised in plants with lower SA content (*sid2*, mutant for ICS1) and defective SA signaling (*npr1*). In comparison with wild-type plants, the number of laid eggs was 2.4- and 1.5-fold lower on *sid2* and *npr1* mutant plants, respectively. Whilst SA levels during

plant-biotic stresses is mainly produced through ICS1-mediated isochorismate pathway (Wildermuth et al., 2001), responses downstream SA accumulation are branched in NPR1-dependent and -independent genes (Uquillas et al., 2004; Shearer et al., 2012). The milder phenotype from *npr1* in comparison with *sid2* might be related to intact NPR1-independent responses. Influence of SA response in *Brevipalpus* mites seems contrary to its role against spider mites. Even though a few reports showed no influence of SA against *Tetranychus* species (Zhurov et al., 2014), a recent study showed that *T. urticae* mites have an increased performance on SA-deficient NahG tomato plants (Villarroel et al., 2016). Lower oviposition of *B. yothersi* mites in either the SA -synthesis or -signaling impaired plants suggests the manipulation by *Brevipalpus* mites of the SA pathway aiming the promotion of host colonization. The positive influence of the SA pathway over *B. yothersi* also has implications for the role of the mite as a vector. We previously reported that infestation with CiLV-C-carrying *B. yothersi* induces even stronger SA response than that reached during non-viruliferous mites feeding (Arena et al., 2016). Higher up-regulation of SA pathway in response to viral infection might further enhance mite colonization and, probably, contribute to the viral transmission.

Salicylic acid-mediated improvement of herbivore performance is usually associated with the antagonistic interactions between the SA and JA signaling pathways (Bruessow et al., 2010; Thaler et al., 2012; Zhang et al., 2013; Caarls et al., 2015). Some arthropods induce SA as a strategy to repress JA effective defenses exploiting the natural cross-talk between signaling pathways. JA defenses, and specifically the production of indole glucosinolates, are central to Arabidopsis defense against *Tetranychus* species (Rioja et al., 2017). Higher reproduction rate of *Brevipalpus* mite due to the induction of the SA pathway could be associated with the reduction on the JA pathway, as previously suggested (Arena et al., 2016). However, our current results show that *Brevipalpus* mite oviposition was not increased in Arabidopsis mutants impaired in JA-responses (*jar1* and *coi1*), therefore, the role of JA pathway against *Brevipalpus* mite colonization is not as obvious as against spider mites, or it does not directly affect oviposition. Molecularly, our results might suggest that the induction of SA antagonizes a set of JA responses which are independent of JAR1 and COI1, or that the SA pathway might improve mite performance by mechanisms alternative to the SA–JA crosstalk. It is noteworthy that upon mite infestation both *JAR1* and *COI1* genes were not induced (**Supplementary Table S2**), consequently, at least at transcriptional level, evidence of involvement of these two gene in response against *Brevipalpus* mite colonization was not revealed. Furthermore, it is possible that the JA pathway influences other aspects rather than mite oviposition such as host preference or mite development. The deeper analysis of other JA mutants and features of mite behavior will help to disentangle the role of the JA on mite infestation.

Some arthropod herbivores are capable of manipulating host responses to circumvent defenses (Stahl et al., 2018). Even though most of the known examples of defense suppression by herbivores involves plant–insect interactions, some cases of suppressive mites have been described (Agut et al., 2018). Defense counteraction has been shown to occur by secreted proteins, called effectors, injected into host cells through herbivores' saliva to interfere with plant responses (Hogenhout and Bos, 2011). Effectors from *Tetranychus* saliva that suppress harmful defenses and increase spider mite performance were recently described (Villarroel et al., 2016). *Brevipalpus* mites likely inject saliva inside host cells through a tube formed between its interlocked stylets (Kitajima and Alberti, 2014). The ability of *B. yothersi* mites to manage the plant response favoring their own performance suggests that, similarly as spider mites do, *Brevipalpus* mites might also deliver saliva-borne effector proteins into plant cells. It is noteworthy, however, that mites from *Brevipalpus* genus employ such a distinct strategy of modulation of plant responses compared to closely-related spider mites. Even though feeding by both *Brevipalpus* and *Tetranychus* mites induce SA and JA pathways simultaneously, the effectiveness of such responses diverges between the two systems. Whilst JA pathway defend plants against *Tetranychus* mites (Zhurov et al., 2014), SA pathway has been described as neutral or detrimental to spider mite species (Villarroel et al., 2016). On the contrary, adverse effect of JA responses to counteract *Brevipalpus* mite infestation was not revealed in the present study, but SA pathway has a positive effect over the colonization of these false-spider mites, pointing to a unique response within described plant-mite interactions.

Polyphagous arthropods likely posses a larger collection of salivary proteins due to their exposure to a wide range of host plants with distinct morphology and physiology (Vandermoten et al., 2014). Large groups of proteins families identified in *T. urticae* saliva were proposed to facilitate the expansion of the host range of these highly polyphagous mites (Jonckheere et al., 2016). Like *T. urticae*, *Brevipalpus* mites infest a wide range of hosts that includes almost a thousand of plant species in more than a hundred of different families (Childers et al., 2003). Collectively, our results suggest that *Brevipalpus* mites manipulate the plant defensive response to render the plant more susceptible to the colonization by inducing the SA-mediated pathway, a mechanism unusual to spider mite species. Mite's ability to modulate the plant physiology in their favor might support the high polyphagous nature of false-spider mites.

Independent GO enrichment analysis from up- and down-regulated DEGs revealed not only the up-regulation of defensive responses, but also the repression of plant growth-related processes. Defensive responses have been long considered to impose a cost that results in reduced plant growth and reproduction (Züst and Agrawal, 2017). Growth-defense trade-off comes from a reallocation of resources to optimize fitness when plants are exposed to environmental changes. Upon herbivory, the plant metabolism is frequently reconfigured. While the secondary metabolism is enhanced to produce defenses, the primary metabolism is suppressed. For instance, induction of JA pathway by *Manduca sexta* results in down-regulation of photosynthesis in *Nicotiana attenuata* (Halitschke et al., 2011). Plant growth genes repressed during the elicitation

of defenses comprise, among others, those associated with cell wall (e.g., expansins), cell division (e.g., cyclins), and DNA replication and photosynthesis (such as components of the light-harvesting complex, photosystem subunits, electron transport chain, chlorophyll biosynthesis, etc.) (Attaran et al., 2014). On *Brevipalpus* mite-infested plants, several of those growth-related genes were down-regulated. Over-represented GO terms within repressed DEGs included processes associated to the cell wall, morphogenesis of cell components, cell division, and photosynthesis.

The plant growth-defense trade-off is modulated through the interplay between defense hormonal pathways mediated by SA and JA and the hormones that act as the major plant growth regulators, i.e., IAA, BR, and GA. Some molecular players that regulate the trade-off have been identified (Huot et al., 2014; Lozano-Durán and Zipfel, 2015; Campos et al., 2016; Züst and Agrawal, 2017). DELLA proteins are key negative regulators of GA signaling that inactivates growth-promoting phytochrome-interacting factors (PIFs). Upon GA elicitation, DELLA proteins are degraded, releasing PIFs and allowing them to activate expression of growth-promoting genes (Huot et al., 2014; Züst and Agrawal, 2017). DELLA and JAZ proteins interact, inhibiting each other actions over the repression of growth and defense-related genes (Yang et al., 2012). The degradation of JAZ proteins triggered by JA accumulation releases DELLA from JAZ binding, thereby strengthens the suppression of PIFs and plant growth (Yang et al., 2012; Züst and Agrawal, 2017). Likewise, overexpression of the DELLA protein RGL3 reduces GA-mediated growth while increases MYC2-dependent expression of JA-responsive genes (Wild et al., 2012). Our results indicate that markers from the molecular mechanism behind the trade-off, such as RGL3, were induced. The SA- and JA-dependent defense responses were up-regulated and IAA-, BR-, and GA-mediated growth processes were downregulated, suggesting that the growth-defense trade-off occurs during Arabidopsis-*Brevipalpus* interaction.

Results obtained here extend our previously proposed model on the Arabidopsis response to non-viruliferous *Brevipalpus* mites (Arena et al., 2016). Beyond the responses focused here, the large-scale transcriptome we obtained will provide a valuable resource to further explore unknown molecular components involved in plant interaction with false-spider mites.

MATERIALS AND METHODS

Plant Material

Wild-type A. thaliana ecotype Columbia (Col-0) was obtained from the Arabidopsis Biological Resource Center[1]. Arabidopsis mutants in the Col-0 background (sid2-1, npr1, and jar1) were obtained from Georg Jander. The Arabidopsis mutant coi1-16 was obtained from Kirk Overmyer. Plants were grown in controlled growth chambers (Conviron, Winnipeg, Canada) at 23 ± 2°C and a 12 h light/dark photoperiod. Four-week-old plants were used in the experiments.

[1]http://www.arabidopsis.org

Mite Rearing

Non-viruliferous mites were initially obtained from citrus orchards and further confirmed as *B. yothersi* using phase contrast microscopy as reported elsewhere (Beard et al., 2015). Mites were reared onto fruits of 'Tahiti' acid lime (*Citrus latifolia* Tanaka), a genotype immune to citrus leprosis virus C, as previously described (Arena et al., 2016). Mites were reared for several generations and were confirmed as non-viruliferous by RT-PCR using primers for CiLV-C (Locali et al., 2003) before their use in the experiments.

RNA-Seq Experiment

A time course experiment was conducted on plants infested with non-viruliferous mites and on non-infested control plants at 6 h after infestation (hai), 2 and 6 dai. For each time point, Arabidopsis Col-0 plants were grouped in sets of 16 individuals assigned to each treatment (infested and control). Plants from both treatments were kept at the same growth chamber. Plants from the infested treatment were challenged with 15 mites (5 mites per each of 3 rosette leaves), transferred with a small brush under a stereoscopic microscope. Mites were not caged. Infested or control leaves were collected at each time-point. From mite-infested plants, only the leaves where mites were originally deposited were collected. Leaves from two plants were pooled, totaling eight biological replicates per treatment per time point, flash-frozen in liquid N_2 and stored at −80°C until RNA extraction. Plant RNA was purified using the RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands) and treated RNAse-free DNAse (Qiagen, Venlo, Netherlands) for removal of residual plant DNA. RNA quality was assessed in Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, United States). All samples had an RNA integrity number (RIN) above eight and were considered suitable for RNA-Seq. RNA extracts from two samples (100 ng/μL each) were pooled in a single sample, totaling four replicates per treatment per time point for library construction and independent sequencing. cDNA libraries were prepared using Illumina TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, United States). Sequencing was performed with HiSeq SBS v4 High Output Kit (Illumina, San Diego, CA, United States) in an Illumina HiSeq 2500 system (Illumina, San Diego, CA, United States) and generated 2 × 125 bp paired-end reads.

Bioinformatics Analysis of RNA-Seq Data

RNA-Seq data were analyzed using R and Bioconductor according to Anders et al. (2013) with some modifications. Quality of the sequences was confirmed using ShortRead (Morgan et al., 2009) and FASTQC. Reads were mapped to the *A. thaliana* TAIR10 genome using TopHat2 (Kim et al., 2013). The number of reads per gene was counted with HTSeq (Anders et al., 2015) and normalized by size factors obtained from the negative binomial-based DESeq2 package (Love et al., 2014). After normalization, clusterization profiles of the samples were assessed by hierarchical clustering (with Euclidean distance metric and Ward's clustering method) and principal component analysis (PCA). Differentially expressed

genes (DEGs) between infested and control treatments were identified at each time point using likelihood ratio tests after negative binominal fittings using the package DESeq2 (Love et al., 2014). Genes with False Discovery Rate (FDR)-corrected p-values ≤ 0.05 and fold-change ($\log_2$) threshold of 0.5 were classified as differentially expressed. To identify mechanisms potentially involved in the plant response to mite feeding, GO Enrichment Analysis was performed. A gene set was defined as all DEGs (unless otherwise noted) and the universe comprised all genes of the *A. thaliana* TAIR10 genome expressed in at least one of the observed conditions. Overrepresented BPs, MFs, and CCs were identified based on a hypergeometric test with FDR-adjusted p-values ≤ 0.001. GO networks were generated using the app BinGO in Cytoscape (Maere et al., 2005).

Identification of Enriched Transcription Factors

A hierarchical clustering was performed with all DEGs to identify up- and down- regulated clusters, using Euclidean distance metric and Ward's clustering method. Two approaches were used to identify enriched TFs on each cluster. First, we searched for genes coding for TFs within DEGs using PlantTFDB database (Jin et al., 2017). Over-represented TFs on each cluster were identified using a hypergeometric test ($\alpha \leq 0.001$). Second, we searched for enriched TF targets using the TF enrichment tool, based on previously identified *cis*-regulatory elements and regulatory interactions from literature mining (Jin et al., 2017).

Validation of Gene Expression Data by RT-qPCR

Another time course experiment was set with plant infested with non-viruliferous mites and non-infested control plants at 6 hai, 2 and 6 dai. For each time point, Arabidopsis Col-0 plants were grouped in sets of 16 individuals assigned to each treatment (infested and control). Plants from the infested treatment were challenged with 15 mites (5 mites per each of 3 rosette leaves). Infested or control leaves were collected at each time-point. Leaves from two plants were pooled, totaling eight biological replicates per treatment per time point, and flash-frozen in liquid N_2. Plant RNA was purified using the RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands) and treated with RNAse-free DNAse (Qiagen, Venlo, Netherlands). RNA concentration was assessed using NanoDrop ND-8000 microspectrophotometer (Thermo Scientific, Waltham, MA, United States) and RNA quality was verified in 1.2% agarose gels. cDNA were generated for each RNA sample (500 ng) using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, United States) as described by the manufacturer. RT-qPCR were prepared with 6.5 μL of GoTaq qPCR Master Mix (Promega, Madison, WI, United States), 120 nM of each gene-specific primer pair and 3 ng of cDNA. Primer sequences are available on **Supplementary Table S9**. Reactions were performed in a 7500 Fast Real-Time PCR System (Thermo Scientific, Waltham, MA, United States) device, using the standard settings. Each sample was analyzed in triplicate and melting curves were included to confirm the absence of genomic DNA and unspecific reactions. Quantification cycle (*C*q) values and primer pairs efficiencies were determined for each individual reaction using Real-time PCR Miner (Zhao and Fernald, 2005). Gene expression analyses were performed according the ΔCq model using multiple reference genes (Hellemans et al., 2007) as previously described (Arena et al., 2016). Statistical significances between infested and control samples within each time point were assessed using Student's t-test ($\alpha \leq 0.05$).

Quantification of Hormone Levels

Four-week-old Arabidopsis Col-0 plants were infested with 10 mites (two leaves with five mites each) or kept without mites. Infested leaves were collected after 6 days. Leaves from two plants were pooled together in one sample, totaling six replicates per treatment. Harvested leaves were weighted, flash frozen in liquid nitrogen and ground in a paint shaker. The SA and JA contents at local and systemic leaves of mite-infested plants were compared with those from the non-infested control as previously described (Casteel et al., 2015). For analysis, 5 μL of each extract were analyzed on a triple-quadrupole liquid chromatography-tandem mass spectrometry system (Agilent 6420A triple-quadrupole with Infinity II HPLC). Extracts were separated on a Zorbax Extend-C18 HPLC column (Agilent, 3.5 μm, 150 mm × 3.00 mm) using 0.1% formic acid in water and 0.1% formic acid in acetonitrile. Statistical significance was assessed using Student's t-test ($\alpha \leq 0.05$).

Mite Performance in Arabidopsis

The mite performance was evaluated on Arabidopsis mutants impaired in SA- (*sid2* and *npr1*) or JA- (*jar1* and *coi1*) mediated response. Plants were infested with five female adult *B. yothersi* mites in a single leaf, caged to prevent escape, and a completely randomized design was set. After 4 days of infestation, plant leaves were carefully detached, and the number of mite eggs was counted. Data from each mutant genotype was compared to the wild-type plants using Student's t-test ($\alpha < 0.05$).

RNA-Seq Raw Data

The RNA-Seq raw data are available at sequence read archive (SRA) with the ID SRP144249.

AUTHOR CONTRIBUTIONS

GA, PR-G, CC, JF-A, and MM conceived and designed the experiments. GA, PR-G, and LR performed the experiments. GA, PR-G, and MR-A analyzed the data. MR-A, CC, MM, and JF-A contributed with reagents, materials, and analysis tools. GA, PR-G, and JF-A wrote the paper. All authors discussed the results and reviewed the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01147/full#supplementary-material

FIGURE S1 | RT-qPCR validation of the RNA-Seq data. Expression profile of selected *Arabidopsis thaliana* genes was analyzed in *Brevipalpus yothersi* mite-infested plants by RNA-Seq and RT-qPCR. Data are presented as $\log_2$ fold-change (FC) values in comparison with non-infested plants (with $\log_2$FC set to zero). Statistically significant differences of mite-infested versus non-infested control at p-values ≤ 0.01 (**) and ≤ 0.05 (*) are indicated. hai, hours after infestation; dai, days after infestation. Note that the gene coding for the SA-responsive protein *PR1* was up-regulated at 2 and 6 dai, following the same profile obtained by RNA-Seq. The SA-related genes *GRX480*, *WRKY70,* and *PR2* were also identified as induced by RT-qPCR at the beginning of the interaction, but statistical significance was not confirmed in the later time points. Expression of the JA-responsive TFs *MYC2* and *ORA59* was also validated by RT-qPCR. Even though *MYC2* transcripts were significantly higher at 6 dai by RT-qPCR but not RNA-Seq, up-regulation of this TF at 6 hai and 2 dai was identified using both techniques. Likewise, *ORA59* gene was up-regulated at 6 hai and 6 dai by both RNA-Seq and RT-qPCR. RT-qPCR results also confirmed the mild induction of the MYC-branch responsive gene *VSP2* at 2 dai, and the high induction of the ERF-branch marker gene *PDF1.2* at 2 and 6 dai. The growth-related gene *EXP3* was down-regulated at 6 hai and 6 dai, while the negative regulator *RGL3* was induced at the same time points, according the RNA-Seq data.

FIGURE S2 | Main biological processes (BPs) affected by *Brevipalpus* mites in *Arabidopsis thaliana* plants. Overrepresented BPs were identified based on a hypergeometric test with false discovery rate (FDR)-adjusted p-values ≤ 0.001. Gene ontology (GO) networks were generated using the app BinGO in Cytoscape. Color and size of the nodes identify the number and p-values of differentially expressed genes (DEGs) from each category. Names of some BPs were simplified for clarity; full names are shown on **Supplementary Table S3**. ROS, reactive oxygen species; SA, salicylic acid; JA, jasmonic acid; ET, ethylene; ABA, abscisic acid; IAA, auxin; CK, cytokinin; GA, gibberellic acid.

TABLE S1 | Sequencing and alignment statistics for all *Arabidopsis thaliana* samples. hai, hours after infestation; dai, days after infestation.

TABLE S2 | Total differentially expressed genes (DEGs) in *A. thaliana* plants infested with *Brevipalpus yothersi* mites at 6 hours after infestation (hai), 2 days after infestation (dai) and 6 dai. FC, fold-change; FDR, False Discovery Rate-corrected p-values. Gene symbols and descriptions were retrieved from ThaleMine (https://apps.araport.org/thalemine).

TABLE S3 | Enriched Biological processes (BPs), molecular functions (MFs), and cellular components (CCs) in the set of DEGs of *A. thaliana* plants infested with *B. yothersi* mites. Overrepresented BPs, MFs, and CCs were identified based on a hypergeometric test with False Discovery Rate (FDR)-adjusted p-values ≤ 0.001. GO, Gene ontology; FDR, FDR-corrected p-values.

TABLE S4 | Enriched BPs at each time point assessed after the infestation of *A. thaliana* plants with *B. yothersi* mites. Overrepresented BPs were identified based on a hypergeometric test with FDR-adjusted p-values ≤ 0.001. GO, Gene ontology; FDR, FDR-corrected p-values; hai, hours after the infestation; dai, days after the infestation.

TABLE S5 | Enriched BPs at each cluster formed by DEGs that were mainly up- or down-regulated during the *A. thaliana* interaction with *B. yothersi* mites. Clusters were defined after a hierarchical clustering analysis of all DEGs. Overrepresented BPs were identified based on a hypergeometric test with FDR-adjusted p-values ≤ 0.001. GO, Gene ontology; FDR, FDR-corrected p-values.

TABLE S6 | Differentially expressed genes (DEGs) coding for TF at each cluster formed by DEGs that were mainly up- or down-regulated during the *A. thaliana* interaction with *B. yothersi* mites. Clusters were defined after a hierarchical clustering analysis of all DEGs.

TABLE S7 | Transcription factors (TFs) with enriched targets within each cluster formed by DEGs that were mainly up- or down-regulated during the *A. thaliana* interaction with *B. yothersi* mites. TFs with enriched targets were identified by TF enrichment tool (Jin et al., 2017). Up- and down-regulated clusters were defined after a hierarchical clustering analysis of all DEGs.

TABLE S8 | Differentially expressed genes (DEGs) involved in salicylic acid (SA) and jasmonic acid (JA) pathways, identified in *A. thaliana* plants infested with *B. yothersi* mites at 6 hai, 2 dai and 6 dai. FC, fold-change; FDR, False Discovery Rate-corrected p-values. Gene symbols and descriptions were retrieved from ThaleMine (https://apps.araport.org/thalemine).

TABLE S9 | Selected genes and corresponding primer pairs of *A. thaliana* used for gene expression analyses by RT-qPCR.

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CAPÍTULO 4

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Arabidopsis thaliana as a model host for *Brevipalpus* mite-transmitted viruses

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Keywords: *Clerodendrum* chlorotic spot virus; *Cilevirus*; *Solanum violaefolium* ringspot virus; Coffee ringspot virus; *Dichorhavirus*

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Note

Arabidopsis thaliana as a model host for *Brevipalpus* mite-transmitted viruses

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ABSTRACT: *Brevipalpus*-transmitted viruses (BTV) are a taxonomically diverse group of plant viruses which severely affect a number of major crops. Members of the group can be subclassified into cytoplasmic (BTV-C) or nuclear type (BTV-N) according to the accumulation sites of virions in the infected plant cells. Both types of BTV produce only local infections near the point of inoculation by viruliferous mites. Features of BTV-plant interactions such as the failure of systemic spread in their natural hosts are poorly understood. In this study we evaluated *Arabidopsis thaliana*, a model plant commonly used for the study of plant-virus interactions, as an alternative host for BTV. Infection of Arabidopsis with the BTV-N *Coffee ringspot virus* and *Clerodendrum* chlorotic spot virus, and the BTV-C *Solanum violaefolium* ringspot virus, were mediated by viruliferous *Brevipalpus* mites collected in the wild. Upon infestation, local lesions appeared in 7 to 10 days on leaves of, at least, 80 % of the assayed plants. Presence of viral particles and characteristic cytopathic effects were detected by transmission electron microscopy (TEM) and the viral identities confirmed by specific reverse-transcriptase polymerase chain reaction (RT-PCR) and further amplicon sequencing. The high infection rate and reproducibility of symptoms of the three different viruses assayed validate *A. thaliana* as a feasible alternative experimental host for BTV.
Keywords: *Clerodendrum* chlorotic spot virus, *Cilevirus*, *Solanum violaefolium* ringspot virus, *Coffee ringspot virus*, *Dichorhavirus*

Introduction

Plant viruses vectored by species of false spider mites of the genus *Brevipalpus* [Tenuipalpidae] are known as *Brevipalpus*-transmitted viruses (BTV) (Kitajima et al., 2014). As a group, BTV affect, at least, 40 plant species ranging from major crops such as citrus and coffee, to economically important cultivated plants such as orchids and passion fruit, and other less significant ornamentals (Bastianel et al., 2010; Kitajima et al., 2010; Ramalho et al., 2014). The BTV-caused citrus leprosis is the main viral disease affecting citrus production in Brazil and is acknowledged to be re-emergent in the Americas (Roy et al., 2015).

BTV are classified as cytoplasmic (BTV-C) or nuclear (BTV-N) according to their replication and accumulation in plant cells. Taxonomically, BTV-C belong to at least one genus, *Cilevirus* [bipartite (+) single-stranded (ss) RNA] (Locali-Fabris et al., 2012), and possibly to *Higrevirus* [tripartite (+) ss RNA] (Melzer et al., 2012). BTV-N are classified into the recently created genus *Dichorhavirus* [bipartite, (-) ssRNA] (Afonso et al., 2016; Dietzgen et al., 2014). Overall, BTV are intriguingly atypical since differently from other plant-virus systems in nature, viral long distance movement is not accomplished in any of their known hosts.

Although BTV were first identified in the early 20th century, molecular information concerning plant-BTV interaction remains barely known. Natural hosts of BTV show large, complex (e.g. citrus, coffee, orchids) (Cai et al., 2015; Xu et al., 2013; Wu et al., 2014; Denoeud et al., 2014) or unknown genomes (e.g. passion fruit) and may require customized installation for their growth and reproduction of BTV-caused diseases. Complexity of the research on the molecular processes involved in plant-BTV pathosystems may be partly bypassed by using appropriate experimental host systems. *Arabidopsis thaliana* appears as the primary alternative host model for plant-pathogen interaction studies, benefiting from a high-quality curated genome and several resources for reverse genetics approaches (Nishimura and Dangl, 2010).

Recently, we reported Arabidopsis as experimental host for *Citrus leprosis virus C* (CiLV-C), a BTV-C (Arena et al., 2013; Ramos-González et al., 2016). In this work, Arabidopsis plants were assessed for their capacity to host a wider range of BTV including another BTV-C, *Solanum violaefolium* ringspot virus (SvRSV), and two BTV-N, *Coffee ringspot virus* (CoRSV) and *Clerodendrum* chlorotic spot virus (ClCSV) (Kitajima et al., 2010).

Materials and Methods

Brevipalpus spp. mites were collected from *Solanum violaefolium*, *Coffea arabica* and *Clerodendrum speciosum* infected with SvRSV, CoRSV and ClCSV, respectively. Plants from wild type *A. thaliana* ecotype Columbia (Col-0) were obtained from the Arabidopsis Biological Resource Center (ABRC) and grown at 22 ± 2 °C with a 12-h light cycle in an environmental-controlled growth chamber Adaptis AR A1000 (Winnipeg, Canada).

Five adult mites collected in plants infected with SvRSV, CoRSV or ClCSV were transferred to each four-week-old Arabidopsis plant using a small brush. Mites were left on the inoculated leaves and plants were kept under controlled conditions throughout the experiment. Plants were evaluated daily for the development of symptoms.

Viral presence was detected through transmission electron microscopy (TEM) and RT-PCR. To detect cytopathic effects caused by BTV-C and -N, lesioned tissues were processed, fixed in Karnovsky reagent, embedded in Spurr's epoxy resin and examined by TEM (Kitajima et al., 2001). Leaves were assayed by RT-PCR using specific primers for SvSRV (Ferreira et al., 2007), CoRSV (Kitajima et al., 2011) and ClCSV. Total RNA from independent plants inoculated with each virus was extracted using TRIZOL® Reagent (Thermo Scientific, WI, USA). RNA quantification and A260/A280 ratios were estimated using the micro-spectrophotometer NanoDrop ND-8000 (Thermo Scientific). RNA integrity was evaluated in a 1.2 % agarose gel. cDNA corresponding to each sample were generated using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) as described by the manufacturer. PCRs were performed with 2 uL cDNA, 1 X reaction buffer, 1.8 mM $MgCl_2$, 0.2 mM dNTP mix, 1 U Taq DNA polymerase (Thermo Scientific), 200 nM of specific primers (Table 1) and sterile Milli-Q water for a final volume of 25 uL. cDNA from healthy plants were used as negative controls. PCR products were visualized in 1 % (w/v) agarose gel stained with ethidium bromide.

To confirm viral identity, the amplicons obtained from three plants infected with each BTV were purified using Promega Wizard SV and PCR clean-up system and cloned into pGEM-T-Easy vector (Promega, WI, USA). Ligation products were introduced into *Escherichia coli* DH5α by transformation. Plasmids from white colonies were purified using the Promega Wizard® Plus SV Minipreps DNA Purification System and after a digestion with *Eco*RI restriction enzyme, those containing appropriate fragments were selected. Three individual clones corresponding to each PCR fragment were sequenced in both directions using the BigDye Terminator Cycle Sequencing kit (Thermo Scientific) in an ABI Prism 3730 automated sequencer (Applied Biosystems, CA, USA). Contigs were assembled and compared to GenBank accessions using BLAST search (http://www.ncbi.nlm.nih.gov).

Results and Discussion

During the last few decades, *A. thaliana* has become the major experimental model for plant biology, including plant-pathogen interactions (Nishimura and Dangl, 2010). Besides the unique characteristics of the plant such as short generation time and small size (allowing for rapid growth and analysis of a large number of individuals in a minimum of space), its genome is compact and completely curated, and a wide mutant collection is available. Much of the current knowledge about the mechanisms underlying plant disease resistance and susceptibility has been discovered studying Arabidopsis pathology and then translated to natural host systems (Nishimura and Dangl, 2010).

We have previously showed that CiLV-C is able to infect Arabidopsis, inducing localized chlorotic symptoms upon infestation with *Brevipalpus* viruliferous mites (Arena et al., 2013; Ramos-González et al., 2016). In Arabidopsis, symptoms of CiLV-C infection occur in approximately 1/3 of the time they appear in sweet orange (*Citrus sinensis*), the virus' natural host. Shorter incubation time represents a significant gain on experiments evaluating plant-virus interaction. In this study, we tested the susceptibility of Arabidopsis to SvRSV, a BTV-C and putative member of *Cilevirus*, CoRSV and ClCSV, two BTV-N and possible members of the genus *Dichorhavirus*.

After 7 to 10 days of infestation with *Brevipalpus* spp. viruliferous mites, localized lesions appeared on the inoculated leaves of more than 80 % of the plants in the sets corresponding to each evaluated virus (16 symptomatic /20 infested plants for CoRSV; 4/5 for ClCSV and 4/5 for SvRSV). Arabidopsis plants infected with CoRSV exhibited brown patches not observable in the leaves infected either with ClCSV or SvRSV. However, chlorotic spots on green leaves and green isolated islands on yellow senescent leaves were observed as a common response to the three inoculated viruses (Figure 1A, B, C). This pattern of symptoms resembles that previously described for Arabidopsis infected with CiLV-C (Arena et al., 2013) and also those occurring during infections of SvRSV, CoRSV and ClCSV in their natural hosts (Figure 1D, E and F). However, in natural hosts a necrotic area usually developed in the center of the chlorotic spots. Necrosis was not visible in any of the Arabidopsis plants infected with BTV assayed in this work. Probably, accelerated senescence that takes place on the Arabidopsis infected leaves hampers observation of such

Table 1 – List of primers used to detect *Brevipalpus*-transmitted viruses (BTV).

BTV	Primer sequence (5'–3')	Target	Amplicon size (bp)	Reference
CoRSV	GGACCATGAGACAGGAGGTG	ORF RdRp RNA2	389	Kitajima et al. (2011)
	CTCTGCCAGTCCTCAATGTG			
SvRSV	TGTCGAACTTTGGTATGAGTCG	ORF RdRp RNA1	596	Ferreira et al. (2007)
	CCGGTTCGTCAAATAACTCC			
ClCSV	ATATCACCGTTTAAGCAAGC	ORF RdRp RNA2	638	Unpublished
	TCCTTGTTACAACTCCTTGC			

CoRSV: *Coffee ringspot virus*; SvRSV: *Solanum violaefolium* ringspot virus; ClCSV: *Clerodendrum* chlorotic spot virus.

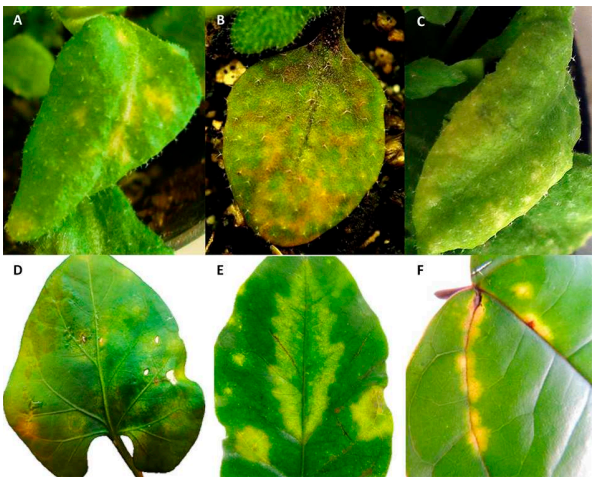

Figure 1 – Symptoms of *Brevipalpus*-transmitted viruses (BTV) infection on model and natural hosts. In the upper line, *Arabidopsis thaliana* leaves exhibiting chlorotic spots after infection of *Solanum violaefolium* ringspot virus (SvRSV) (A), *Coffee ringspot virus* (CoRSV) (B) and *Clerodendrum* chlorotic spot virus (ClCSV) (C) by *Brevipalpus* mite - mediated transmission. In the bottom line, leaves of *Solanum violaefolium*, *Coffea arabica* and *Clerodendrum speciosum* infected with SvRSV (D), CoRSV (E) and ClCSV (F), respectively.

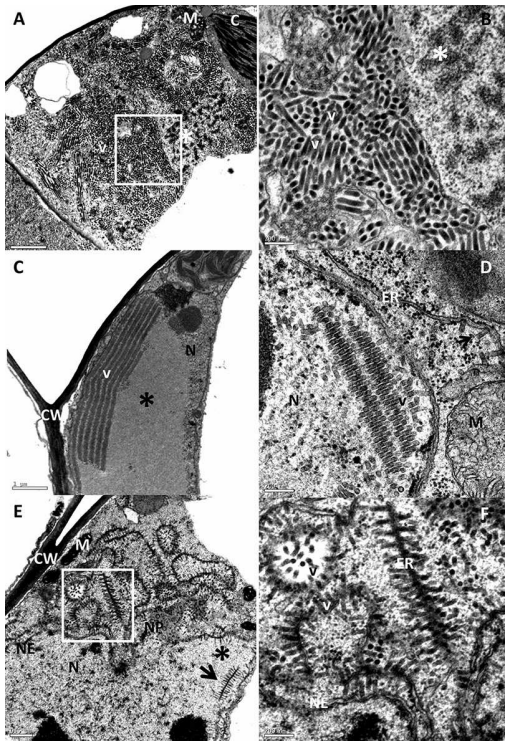

Figure 2 – Micrographs of sections of leaf lesions in *Arabidopsis thaliana* infected by *Solanum violaefolium* ringspot virus (SvRSV) (A and B), *Coffee ringspot virus* (CoRSV) (C and D) and *Clerodendrum* chlorotic spot virus (ClCSV) (E and F). Bacilliform particles (v) and cytoplasmic viroplasma (*) are present in cells infected by SvRSV, while rod-like particles (v) and nuclear viroplasma (*) can be seen in those infected by CoRSV and ClCSV. C: chloroplast; CW: cell wall; M: mitochondrion; NE: nuclear envelope; ER: endoplasmic reticulum.

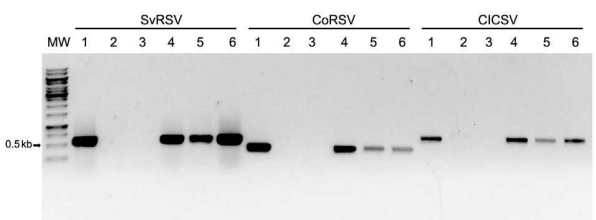

Figure 3 – Agarose gel (1 %) profile of reverse-transcriptase polymerase chain reaction (RT-PCR) products obtained from plants infected with *Solanum violaefolium* ringspot virus (SvRSV), *Coffee ringspot virus* (CoRSV) and *Clerodendrum* chlorotic spot virus (ClCSV). MW: molecular weight marker (GeneRuler 1 kb DNA Ladder, Thermo Scientific); 1: natural hosts infected with each corresponding virus; 2: healthy *Arabidopsis thaliana*; 3: blank; 4-6: *A. thaliana* infected with each specific virus. Sizes of the expected amplicons for SvRSV, CoRSV and ClCSV are 596, 389 and 638 bp, respectively.

symptom. Furthermore, as expected, no disease symptoms were observed in the cauline systemic leaves of Arabidopsis, attesting to the typical non-systemic pattern of BTV infection under natural conditions.

Transmission electron microscopy analyses of plants inoculated with SvRSV allowed detecting enveloped bacilliform particles of ca. 50 × 100 nm in large pockets of the endoplasmic reticulum of cells of chlorotic lesions. Dense viroplasms were also seen in the cytoplasm of these cells (Figure 2A, B). In contrast, in leaf lesions from plants infected with CoRSV and ClCSV, non-enveloped bacilliform, rod-like virions of ca. 40 × 110 nm were observed in both the nucleoplasm and cytoplasm of palisade parenchyma cells. Most particles were arranged perpendicularly to the membranes of the endoplasmic reticulum and the nuclear envelope. Electron lucent viroplasms were detected in the nucleus of CoRSV and ClCSV infected cells, although they were more easily observed in the former case (Figure 2C, D, E, F). In general, virions and cytopathic effects seen in Arabidopsis were identical to those previously described in the natural hosts of SvRSV, CoRSV and ClCSV (Kitajima et al., 2014).

To confirm the identity of viruses present in symptomatic Arabidopsis, RT-PCR tests using specific primers were conducted. As expected, amplicons of approx. 600, 400 and 600 bp were obtained in samples from SvRSV, CoRSV and ClCSV, respectively (Figure 3). Consensi sequences of SvRSV and CoRSV amplicons showed identity values as high as 99 % with sequences obtained from the cognate viruses in the naturally infected hosts available under GenBank accessions number DQ514336 (*Solanum violaefolium* ringspot virus replicase-associated protein) and GQ979998 (*Coffee ringspot virus* isolate Cordeiropolis RNA-dependent RNA polymerase gene). For ClCSV, the

amplicons showed 100 % with the viral sequence in the Clerodendrum plant used as the viral source. However, these sequences exhibited only 69 % of identity with the sequence previously described for ClCSV isolate ESALQ_Piracicaba (HQ853700) (Kitajima et al., 2008), suggesting a wider diversity of BTV-N infecting Clerodendrum plants.

In conclusion, the appearance of localized symptoms in the infected plants, the visualization of typical BTV particles and viroplasms in infected cells, and the confirmation of the identity of the viruses at nucleotide sequence level validated *A. thaliana* as alternative host for both BTV-C and -N. High susceptibility of this plant to mite mediated transmission of BTV and its reduced time for symptom appearance will likely boost research on understanding the interactions involving this peculiar group of plant viruses and their hosts.

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DISCUSSÃO GERAL

A leprose dos citros destaca-se como uma doença re-emergente das Américas e de ampla importância econômica no Brasil, onde é considerada a principal doença viral dos citros (Bastianel et al., 2010; Roy et al., 2015). Além de sua relevância econômica, a leprose se revela como uma doença atípica. O seu agente causal, o vírus da leprose dos citros (citrus leprosis virus, CiLV-C), é incapaz de invadir sistemicamente todas as plantas hospedeiras conhecidas e apresenta um genoma formado em grande parte por genes "órfãos" que não apresentam homólogos em outras espécies de vírus (Locali-Fabris et al., 2006; Tautz and Domazet-Lošo, 2011). A leprose tem como vetor ácaros do gênero *Brevipalpus* (principalmente *B. yothersi*), os quais são extremamente polífagos, cosmopolitas e apresentam uma biologia única (Kitajima & Alberti, 2014; Weeks et al., 2001).

Com o presente trabalho, objetivou-se compreender os mecanismos moleculares envolvidos na interação planta/CiLV-C/*B. yothersi*, ainda amplamente desconhecidos. Dados prévios indicavam a indução de genes marcadores de vias hormonais em resposta a ambos ácaro e vírus em plantas de Arabidopsis (Arena, 2014). Para o estudo da interação planta/vírus, foram analisados: i) a presença de células mortas e espécies reativas de oxigênio (ROS) em ensaios histoquímicos, visando apoiar o envolvimento de uma resposta de hipersensibilidade (HR); ii) a cinética de acúmulo de CiLV-C ao longo da infecção através de RT-qPCR, para se identificar os principais eventos da replicação viral; iii) o transcriptoma de Arabidopsis infectadas com o CiLV-C via RNA-Seq, de forma a se obter a resposta global de plantas na presença do vírus; e iv) a expressão transiente das proteínas virais em *Nicotiana benthamiana* mediante agroinfiltração, para se identificar aquelas com atividade elicitora. Para o estudo da interação planta/ácaro, foram avaliados: i) o transcriptoma de Arabidopsis infestadas com *B. yothersi* através de RNA-Seq, com o intuito de se identificar a resposta global da planta à ácaro; ii) os níveis de ácido salicílico (SA) e jasmônico (JA) em plantas infestadas via LC-MS/MS, para apoiar a ativação de ambas as vias em resposta ao ácaro; e iii) a performance do *B. yothersi* em mutantes de Arabidopsis, visando revelar o papel das vias moduladas na infestação. Além disso, a análise de alguns genes marcadores da interação com ácaros *B. yothersi* avirulíferos e virulíferos para o CiLV-C foi extendida para plantas de *Citrus sinensis* (laranja-doce), com o intuito de validar os dados obtidos em Arabidopsis na principal hospedeira da doença. Foi ainda

avaliada a influência do CiLV-C na colonização do *B. yothersi*, mostrando a capacidade do vírus em modular o comportamento do ácaro vetor.

Os resultados obtidos permitiram a estruturação de um modelo da interação planta/patógeno/vetor (Fig 1). Durante o processo de alimentação, ácaros *B. yothersi* avirulíferos perfuram células do mesófilo com seus estiletes, através dos quais ocorre a injeção de saliva, possivelmente contendo efetores associados ao ácaro. Em resposta à interação com o ácaro, ocorre um acúmulo de SA, levando à indução de genes responsivos da via (ex.: *PR1*), o que favorece a performance do ácaro. Em paralelo, há um acúmulo de JA, associado à expressão de genes de defesa como aqueles envolvidos na síntese de glucosinolatos. Embora ambos os ramos da via de JA sejam ativados, ocorre uma indução preferencial do ramo ERF (modulado por etileno, que tem por gene marcador o *PDF1.2*) em relação ao MYC (regulado por ácido abscícico e associado a indução de genes como o *VSP2*). Além disso, ocorre uma produção de espécies reativas de oxigênio (ROS) restrita a poucas células e uma modulação de poucos genes envolvidos no silenciamento de RNA. Em contrapartida, a infestação por ácaros reprime processos associados ao crescimento e desenvolvimento da planta, incluindo a expressão de genes de síntese de hormônios de crescimento (auxinas, citoquininas e brassinosteroides), possivelmente em função do antagonismo entre defesa e crescimento vegetal. Em plantas infestadas com ácaros virulíferos, o CiLV-C atinge as células do mesófilo juntamente com a saliva do ácaro, se multiplica e se move localmente para células restritas ao redor do sítio de inoculação. A presença do vírus intensifica a produção de ROS e a resposta mediada por SA, além de ativar uma série de genes associados a morte celular e a uma resposta de hipersensibilidade (HR). Estas respostas são também induzidas pela expressão transiente da proteína viral P61, sugerindo esta proteína como o componente do CiLV-C responsável pela elicitação do sistema imune da planta. A infecção pelo CiLV-C causa ainda uma indução de genes associados ao crescimento celular, os quais podem ser os responsáveis pela hiperplasia e hipertrofia observada em células infectadas. Em contrapartida, ocorre uma repressão dos genes responsivos a JA, possivelmente em função de um antagonismo exercido pela indução elevada da via de SA, incluindo aqueles envolvidos na síntese de glucosinolatos, o que hipoteticamente favoreceria a performance do ácaro. A infecção pelo CiLV-C reduz também processos envolvidos no metabolismo primário da planta hospedeira, incluindo a fotossíntese, o que pode estar relacionado ao desenvolvimento dos sintomas de clorose. Por fim, é possível que o CiLV-C codifique um supressor viral de silenciamento de RNA (VSR) que inativa a proteína AGO1, levando à uma indução de *AGO2* e a consequente ativação de uma segunda linha de defesa do

silenciamento de RNA que contribui para a redução da multiplicação do vírus. Como resultado da ativação de mecanismos de defesa, o CiLV-C permanece restrito a células ao redor do sítio de inoculação e os sintomas de morte celular se desenvolvem, provavelmente como resultado de uma HR manifestada durante uma interação incompatível.

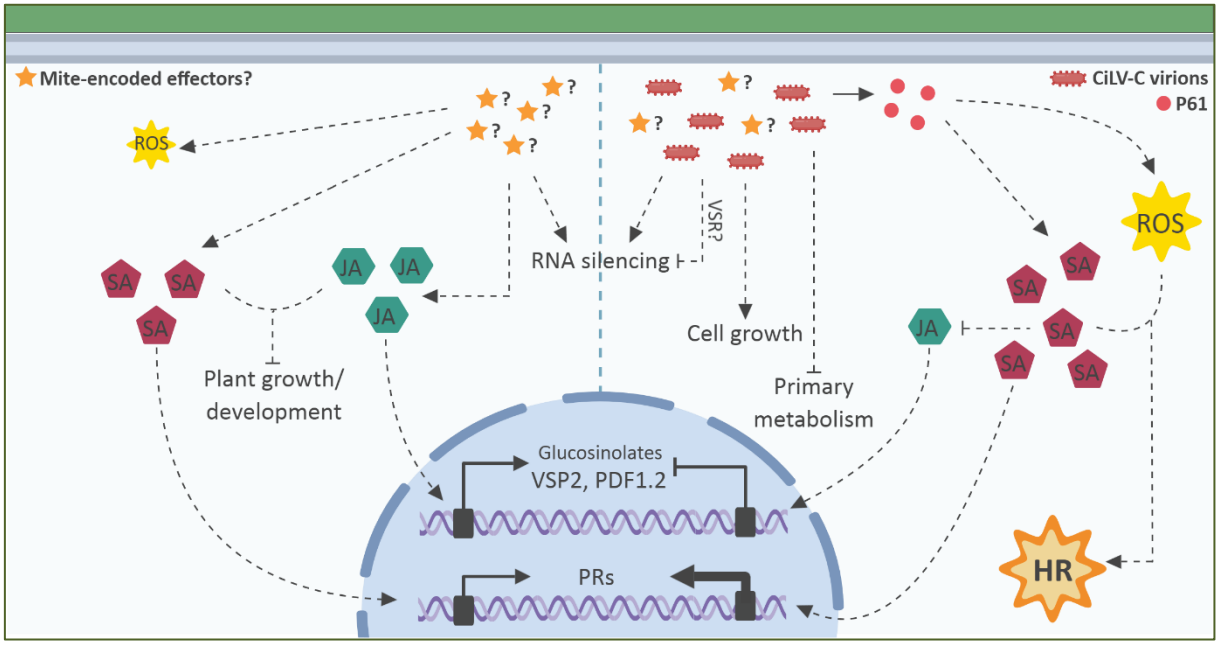

Figura 1. Modelo da interação planta/CiLV-C/Brevipalpus yothersi. Os lados esquerdo e direito da célula representam os eventos que ocorrem durante a interação da planta com o ácaro e o vírus, respectivamente. Os detalhes encontram-se no texto. HR: resposta de hipersensibilidade, SA: ácido salicílico, JA: ácido jasmônico, ROS: espécies reativas de oxigênio, VSR: supressor viral de silenciamento de RNA.

Espera-se que trabalhos posteriores complementem o modelo obtido, adicionando elementos do papel da via de JA e dos glucosinolatos na infestação por ácaros, os efetores codificados pelo ácaro que elicitam as respostas na planta hospedeira, o papel da via de SA e HR na infecção pelo vírus, e as proteínas da planta hospedeira que reconhecem a P61. Para complementar os dados da interação planta/ácaro, já estão em andamento análises da performance do ácaro em mutantes de Arabidopsis para ambos os ramos da via de JA (*myc2, vsp2, ora59, pdf1.2, jaz3*) e para a síntese de glucosinolatos (*qKO, atr1D, cyp79b2/79b3*), que servirão para esclarecer a participação de ambos na infestação. Visando a identificação dos componentes do ácaro responsáveis por elicitar as respostas na planta hospedeira, foram realizadas análises *in silico* para a predição de efetores candidatos, os quais serão clonados e expressos em *N. benthamiana* para elucidação de sua capacidade elicitora. Para maior

compreensão da interação planta/vírus, estão sendo analisadas plantas de Arabidopsis mutantes para a via de SA (*sid2*, *npr1*, *wrky70*) e para genes associados a HR (*nhl10*, *crk13*, *rbohD/rbohF*). A ação da P61 na infecção está sendo explorado através da expressão da proteína P61 em plantas transgênicas NahG de *N. benthamiana*, comprometida no acúmulo de SA, e da geração de plantas transgênicas de Arabidopsis expressando a proteína viral. Neste mesmo contexto, foram ainda realizados ensaios de co-imunoprecipitação seguida de espectrometria de massa em *N. benthamiana* expressando a P61, cujos resultados estão em análise, para se revelar as proteínas vegetais que interagem com a proteína viral e desencadeiam a ativação do sistema imune e o desenvolvimento da HR.

Os dados apresentados neste trabalho proporcionam, portanto, uma melhor compreensão dos processos patofisiológicos que se desenvolvem durante a leprose dos citros e, possivelmente, de outras doenças não-sistêmicas transmitidas por ácaros *Brevipalpus* (Freitas-Astúa et al., 2018). A possibilidade de se utilizar Arabidopsis como hospedeira alternativa para outros VTBs, também descrita neste trabalho, facilitará o estudo dos mecanismos envolvidos na infecção por tais vírus e sua comparação com os identificados durante a interação com CiLV-C. Em termos práticos, o presente estudo forneceu ainda dados úteis para estudos futuros envolvendo os componentes do patossistema leprose, como ensaios TaqMan para a detecção quantitativa do RNA genômico e subgenômico do CiLV-C através de RT-qPCR, e o transcriptoma completo de plantas infestadas por ácaros aviruliferos e virulíferos para a exploração de outros mecanismos não abordados neste trabalho. A longo prazo, os resultados obtidos aqui e nos experimentos em andamento serão essenciais ao estabelecimento de estratégias de controle mais sustentáveis, a exemplo do silenciamento de genes da planta, vírus e ácaro envolvidos no desenvolvimento da doença.

CONCLUSÕES GERAIS

1. Plantas de Arabidopsis infestadas com *Brevipalpus yothersi*, em comparação a plantas não infestadas, apresentam um acúmulo de espécies reativas de oxigênio (ROS) restrito à poucas células infestadas, acumulam maiores níveis de ácidos salicílico (SA) e jasmônico (JA) e induzem a expressão de genes associados a ambas as vias hormonais, além de processos envolvidos na síntese de compostos de defesa como os glucosinolatos. Em contrapartida, processos envolvidos no crescimento e desenvolvimento da planta são reprimidos.

2. A ativação de genes envolvidos na biossíntese e sinalização da via de SA contribui para a performance de *B. yothersi*, apontando para um papel da via em favorecer a infestação do ácaro.

3. O título de CiLV-C aumenta continuamente durante os primeiros dez dias de infecção em folhas de Arabidopsis, um período durante o qual três etapas de replicação viral são identificadas.

4. Plantas de Arabidopsis infestadas com ácaros virulíferos para o CiLV-C, em comparação a plantas infestadas com ácaros avirulíferos, intensificam a explosão de ROS e a ativação da via de SA, e induzem a expressão de genes associados ao crescimento celular, à morte celular e à resposta de hipersensibilidade (HR). Em contraste, plantas infectadas reprimem a via de JA, os processos associados à síntese de glucosinolatos e o metabolismo primário.

5. A expressão transiente da proteína P61 do CiLV-C mimetiza respostas observadas durante a interação com o vírus, como o acúmulo de ROS, a indução de genes associados à via de SA e à HR e a morte celular.

6. Os vírus transmitidos por *Brevipalpus* (VTBs) clerodendrum chlorotic spot virus, coffee ringspot virus e solanum violaefolium ringspot virus são capazes de infectar experimentalmente plantas de Arabidopsis, o que valida o uso desta planta-modelo como hospedeira alternativa nos estudos de interação com VTBs filogeneticamente divergentes ao CiLV-C.

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ANEXO 1

SECRETARIA DE AGRICULTURA E ABASTECIMENTO
AGÊNCIA PAULISTA DE TECNOLOGIA DO AGRONEGÓCIO
INSTITUTO AGRONÔMICO
CENTRO APTA CITROS ´SYLVIO MOREIRA´

DECLARAÇÃO DE BIOÉTICA

Eu, Raquel Luciana Boscariol Camargo, presidente da CIBio do Centro de Citricultura Sylvio Moreira - IAC, o qual possui CQB nº 417/16, declaro que o projeto de tese "Leprose dos citros: um modelo para o estudo da interação planta/ácaro/vírus", desenvolvido pelo aluna de doutorado Gabriella Dias Arena, foi desenvolvido dentro das normas de bioética e biossegurança determinadas por essa comissão.

Cordeirópolis, 10 de outubro de 2018.

Dra. Raquel Luciana Boscariol Camargo
Presidente da CIBio do Centro de Citricultura - IAC

ANEXO 2

Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **"Leprose dos citros: um modelo para o estudo da interação planta/ácaro/vírus"**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 31 de agosto de 2018

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