



Transient Expression of Whitefly Effectors in *Nicotiana benthamiana* Leaves Activates Systemic Immunity Against the Leaf Pathogen *Pseudomonas syringae* and Soil-Borne Pathogen *Ralstonia solanacearum*

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Infestation of plants with the phloem-feeding whitefly *Bemisia tabaci* modulates root microbiota and both local and systemic immunity against microbial pathogens. Specifically, aboveground whitefly infestation suppresses pathogen propagation and symptom development caused by the soil-borne pathogens *Agrobacterium tumefaciens* and *Ralstonia solanacearum* in the root system through systemic signal transduction. Therefore, we hypothesized that secreted protein(s)/non-protein factors from whitefly saliva (referred to as candidate effectors) might function as insect determinants that activate systemic acquired resistance (SAR) in the host plant. Here, we intensively screened a cDNA library constructed from mRNA from whitefly feeding on *Nicotiana benthamiana* leaves and selected three candidate effectors 2G4, 2G5, and 6A10, that appear to reduce disease development caused by the aboveground pathogen *Pseudomonas syringae* pv. *tabaci* and the soil-borne pathogen *R. solanacearum*. Transient expression of the three candidate effector cDNAs in leaves primed the expression of SAR marker genes *NbPR1a* and *NbPR2* in local and systemic leaves against *P. syringae* pv. *tabaci*, while leaf infiltration with 2G4 or 6A10 cDNA elicited strong defense priming of SAR markers following drench application of *R. solanacearum* on plant roots. *In silico* and qRT-PCR analyses revealed the presence of 2G5 and 6A10 transcripts in insect salivary glands. This is the first report of whitefly effectors that prime SAR against aboveground and belowground bacterial pathogens.

Keywords: whitefly, effector, *Nicotiana benthamiana*, *Ralstonia solanacearum*, transient expression

INTRODUCTION

Plants are constantly exposed to diverse insect pests and microbial pathogens (Agrios, 2005). To protect from these enemies, immune responses, including chemical and physical defense mechanisms, are activated in local and systemic plant tissues (Koornneef and Pieterse, 2008; Dangl et al., 2013). Plants have developed a sophisticated immune system against insect herbivory. Compared with our understanding of plant defensive responses against chewing insects, little is known about plant responses to phloem-feeding insects in the order Hemiptera (Van Oosten et al., 2008; Walling, 2008; Rodriguez-Medina et al., 2011; van Dam and Heil, 2011; Louis and Shah, 2013; Pitino and Hogenhout, 2013; Rao et al., 2013; VanDoorn et al., 2015). The detailed mechanisms of plant responses to this group of insects, such as aphids and whitefly (*Bemisia tabaci* Genn.), have only recently begun to be uncovered due to the small size and limited genetic and physiological information about these insects (Louis and Shah, 2013; Pitino and Hogenhout, 2013; VanDoorn et al., 2015).

For instance, aboveground (leaf) whitefly infestation increases plant immunity against soil-borne plant pathogens, indicating that systemic plant signaling is activated and translocated from leaf to root. Infestation of pepper leaves by whitefly increases systemic resistance against the soil-borne pathogen *Ralstonia solanacearum* (Yang et al., 2011; Lee et al., 2012). Further investigation revealed that infestation with this insect leads to the recruitment of beneficial rhizosphere bacterial species, which act as a biological trigger to elicit plant systemic defense responses against subsequent whitefly attack (Murphy et al., 2003). More recently, whitefly infestation was found to reduce *Agrobacterium tumefaciens* mediated crown gall formation on stems and roots (Song et al., 2015). Transcriptome and virus-induced gene silencing analyses demonstrated that whitefly-induced salicylic acid (SA) signaling attenuates *Agrobacterium* T-DNA transformation and gall formation. Root exudates that were collected from tobacco contained approximately 2.5-fold higher SA levels when whiteflies had infested leaf tissues (aboveground) compared to the uninfested control. Intriguingly, whitefly-elicited plant immunity in pepper activates both SA- and jasmonic acid (JA)-related gene expression in aboveground and belowground tissue, indicating that SA- and JA-dependent pathways are activated from leaf to root in response to whitefly feeding on leaves (Park and Ryu, 2014). Further investigation involving the fine-tuning of these signaling pathways following whitefly infestation using virus-induced gene knockdown of SA- and JA-responsive and biosynthesis genes revealed that SA is a major player in whitefly feeding-dependent signaling (Lazebnik et al., 2014; Song et al., 2015).

The whitefly determinant that confers resistance against soil borne pathogens in systemic plant tissues is still unknown. Our understanding of insect-mediated changes in the activation of plant immune responses is limited due to the lack of information on whitefly determinants that suppress or induce plant immune responses. To fill this knowledge gap, most studies on insect factors that modulate plant immunity have focused on the suppression of insect resistance rather than the induction of

plant resistance responses such as systemic acquired resistance (SAR) (Kempema et al., 2007; Cooper et al., 2010; Su et al., 2012, 2015). The effector proteins from pathogenic bacteria and fungi induce and suppress plant immunity via a well-known process described by the zigzag theory (Jones and Dangl, 2006). Effectors are a group of proteins that translocate from microbes such as bacteria, fungi, and nematodes to host plants and animals (Elzinga and Jander, 2013). The major function of effectors is to modulate host immune responses through interactions with their counterpart proteins in the host plant. The outcomes of these interactions include effector-triggered susceptibility (ETS) and effector-triggered immunity (ETI) or immune reactions in the target plant, which occur in a protein-dependent manner.

Unlike microbial effectors, insect effectors have not been intensively studied. Recent studies explored effector proteins, primarily from sucking insects, and their role in plant immunity (Elzinga and Jander, 2013). Hemipteran and dipteran insect species, including phloem-feeding aphids and whiteflies, secrete certain proteins and translocate them into the cytosol of the host cell through their stylets (Kaloshian and Walling, 2015). These effectors play important roles in suppressing plant defense responses and helping the insect overcome plant immunity. The aphid effectors *Coo2* and *Armet*, which were identified through transcriptome analysis of aphid glands, increase insect survival and host colonization (Mutti et al., 2006; Wang et al., 2015a). Another aphid effector, SHP (structure sheath protein), is primarily expressed in saliva and functions as a virulence factor. Interestingly SHP does not share any sequence homology with proteins from other insects, suggesting that it would be a good target for RNA interference-mediated insect control in SHP dsRNA-overexpressing transgenic plants (Abdellatif et al., 2015; Will and Vilcinskis, 2015). ACE2 (angiotensin-converting enzyme 2), SSGPs (secreted salivary gland proteins), and Mp10, Mp55, Me10, and Me23 are also candidates for this technique (Elzinga and Jander, 2013; Wang et al., 2015b; Zhao et al., 2015). However, the functions and molecular roles of effectors from whitefly have only recently been explored. Whitefly saliva is thought to contain proteins that modulate plant defense responses and facilitate feeding. Secreted whitefly laccase 1 (LAC1) and small RNAs have been identified and are thought to help the insect overcome plant immunity responses (van Kleeff et al., 2016; Yang et al., 2017).

In the current study, to extend our understanding of plant-microbe-insect tritrophic interactions, we focused on the following: (1) establishing a high-throughput screening system to screen whitefly effectors that elicit plant immune responses against aboveground virulent pathogens, (2) evaluating aboveground effector-mediated plant SAR against soil-borne pathogens, and (3) characterizing the identified effectors and confirming expression in the salivary gland. We used whitefly as a model insect and two microbial pathogens as model pathogens, including *Pseudomonas syringae* on local and systemic leaves (aboveground) and the soil-borne pathogen *Ralstonia solanacearum* on the plant root system (belowground).

The objective of the current study was to identify candidate whitefly effectors that activate plant immunity, as revealed

by the suppression of symptom development caused by virulent *Pseudomonas syringae* pv. *tabaci* or attenuation of the hypersensitive response (HR, a plant programmed cell death response) caused by avirulent *Pseudomonas syringae* pv. *syringae*. We hypothesized that pre-infiltration of *Nicotiana benthamiana* leaves with candidate whitefly effectors would delay or totally suppress lesion formation caused by the two *P. syringae* pathovars in the overlapping regions of leaves after cross-infiltration. Finally, we validated three putative effectors identified from high-throughput screening of an *Agrobacterium tumefaciens*-mediated transient expression system as candidate effectors that elicit plant systemic immunity (SAR) against a soil-borne pathogen *Ralstonia solanacearum*, prime plant SAR marker gene expression on root and confirmed their localization *in silico*. This study represents the first demonstration of whitefly effectors that trigger SAR against aboveground and belowground bacterial pathogens.

MATERIALS AND METHODS

Insect Culture and Plant Growth Conditions

Whiteflies (*Bemisia tabaci*) were grown and maintained in the KRIBB Greenhouse Facility in Daejeon, South Korea, as described previously (Yang et al., 2011; Park and Ryu, 2014). The whitefly was identified as Q biotype (data now shown). *N. benthamiana* was used as the model system, as described previously (Song et al., 2016). *N. benthamiana* seeds were surface-sterilized with 6% sodium hypochlorite, washed four times with sterile distilled water, and incubated in a growth chamber at $25 \pm 2^\circ\text{C}$ under fluorescent lights (light: dark 12: 12h; c. 7000 lux light intensity). Seedlings were individually grown in plastic pots 9 cm in diameter at $25 \pm 2^\circ\text{C}$ under fluorescent lights in a controlled environment growth room (12 h/12h day/night cycle, 7000 lux light intensity). Three-week-old *N. benthamiana* plants were used in the experiments. The experiments were repeated three times with 10 replications (one plant per replication and three leaves per plant).

Whitefly cDNA Library Construction

Whiteflies were collected from *N. benthamiana* leaves at mid-day and quickly ground in liquid nitrogen for RNA isolation using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and RNase-free DNase I (Promega). Purified total RNA was reverse-transcribed to cDNA using a Cloneminer II cDNA Library Construction Kit (Invitrogen) following the manufacturer's instructions. The cDNA was inserted into the pDONR222 Gateway vector. The initial titer of the library was determined through colony counts via plating on LB plates containing kanamycin (50 $\mu\text{g}/\text{mL}$). The average insert size was analyzed by restriction enzyme digestion with BsrG1 (New England Biolabs). The initial cDNA fragments were transferred to the pK7WG2 vector using LR recombinase (Invitrogen), and transformed into *A. tumefaciens* LBA4404 cells by electroporation and used for transgene expression in *N. benthamiana*.

5' Rapid Amplification of cDNA Ends (RACE)

Sense cDNA was synthesized via 5' rapid amplification of cDNA ends (RACE) adapter (5'-GCUGAUGGCGAUGAAUGA ACACUGCGUUUGCUGGCCUUUGAUGAAA-3') ligation with a First choice RLM-RACE Kit (Ambion). Antisense cDNA was synthesized by PCR using the 5' RACE adaptor outer primer following the manufacturer's instructions. The amplified double-stranded cDNA was cloned into the pGEM-T Easy vector (Promega).

Intensive Whitefly Effector Screening via a Cross-Infiltration Assay

Agrobacterium-mediated gene transfer was conducted as described elsewhere with minor modifications (Win et al., 2011). The *A. tumefaciens* GV2260 culture was pelleted by centrifugation for 5 min at 4,000 rpm at room temperature (RT), and the cell pellet was washed three times with distilled water. The cells were re-suspended in Agro-induction medium (10 mM MgCl_2 , 150 μM acetosyringone, pH 5.6). The concentration of the suspension was adjusted to $\text{OD}_{600} = 0.1$ prior to infiltration. To identify effectors from a total cDNA library prepared from whitefly during plant infestation, we reasoned that *Agrobacterium tumefaciens*-mediated transient transformation should delay or totally suppress lesion formation caused by *Pseudomonas syringae* pv. *tabaci* (*Pta*) and *P. syringae* pv. *syringae* (*Psy*) in the overlapping regions of leaves after cross-infiltration. Agro-infiltration assays were performed on the middle leaves of 3-week-old *N. benthamiana* plants. The two *P. syringae* pathovars (*Pta* and *Psy*) were selected on solid King's B medium containing 100 $\mu\text{g}/\text{mL}$ rifampicin at 30°C for 2 days, scraped off the plates, and re-suspended in 10 mM MgCl_2 (King and Zeevaart, 1974; Song et al., 2015). The negative control was empty vector (pK7WG2). For *Pta* symptom evaluation, leaves were infiltrated with *Pta* ($\text{OD}_{600} = 0.01$) 3 days after agro-infiltration. To visualize HR symptoms, leaves were infiltrated with *Psy* ($\text{OD}_{600} = 0.1$) 3 days after agro-infiltration. The HR is normally apparent 24 h after infiltration.

P. syringae pv. *tabaci* Pathogenesis Assay

To investigate the impact of the effectors in detail, symptom development and bacterial numbers in local and systemic leaves at day were measured on days 0, 3, and 5 after pathogen challenge (Figure 2A). The foliar parts of 3-week-old *N. benthamiana* seedlings were infiltrated with 2 mL of a 10^6 – 10^7 cfu/mL suspension of *A. tumefaciens*. The positive control was 0.5 mM BTH (Syngenta, Durham, NC, USA), which elicits SAR to bacterial pathogens. The negative control was empty vector. *Pta* was selected on solid King's B medium containing 100 $\mu\text{g}/\text{mL}$ rifampicin at 30°C for 2 days, scraped off the plates, and re-suspended in 10 mM MgCl_2 (King and Zeevaart, 1974; Song et al., 2015). For *Pta* symptom evaluation, leaves were infiltrated with *Pta* ($\text{OD}_{600} = 0.01$) at 3 days after agro-infiltration. To investigate the population size of *Pta* in leaves, *Pta* cells were counted at 0, 3, and 5 days after pathogen inoculation. Leaf discs (1 cm diameter) were ground in 10 mM MgCl_2 , and serial dilutions of

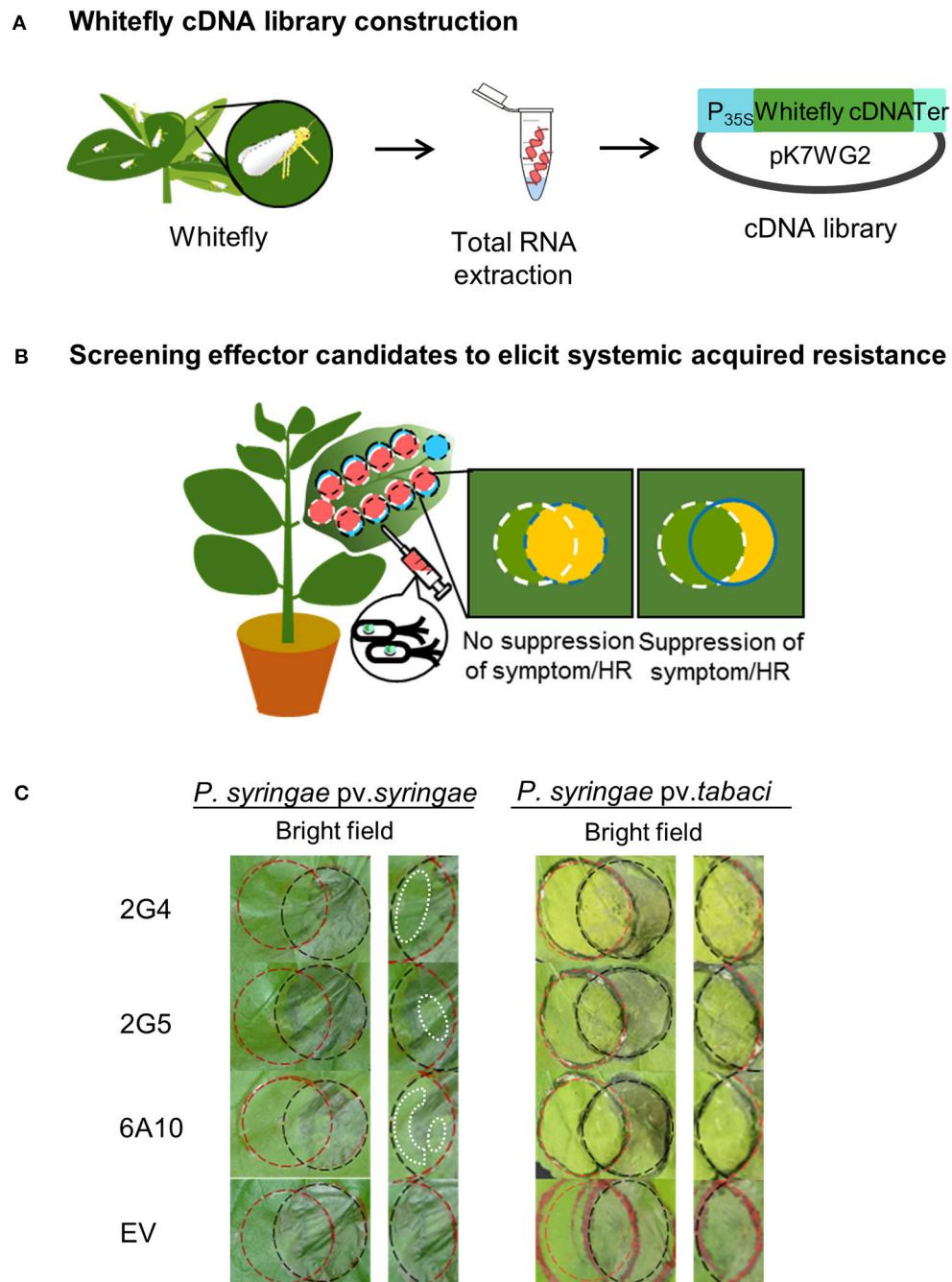


FIGURE 1 | Screening system to identify candidate whitefly effectors that induce plant immunity. **(A)** Whitefly cDNA library construction. Extraction of total RNA from whitefly during infestation of *Nicotiana benthamiana* leaves. The cDNA library from whitefly mRNA was constructed in *Agrobacterium tumefaciens* using a binary vector system. **(B)** Screening candidate effectors that elicit systemic acquired resistance against virulent *Pseudomonas syringae* pv. *tabaci* and avirulent (a non-host pathogen) *Pseudomonas syringae* pv. *syringae*. To identify whitefly effectors that activate plant pathogen immunity, cross-circle infiltration was performed with cDNA clones and pathogens, and the suppression of disease symptoms caused by *Pta* and the hypersensitive response (HR) caused by *Psy* at 1–5 days after pathogen infiltration were evaluated. **(C)** Suppression of the HR and symptoms by whitefly cDNA clones 2G4, 2G5, and 6A10. The white dotted lines in the left panel indicate the suppressed HR. The empty vector (EV) treatment showed no inhibition of the HR and symptom development in the intersecting area. The experiment was repeated three times with similar results.

bacterial solution were spread onto selection medium (King's B agar medium containing 100 $\mu\text{g}/\text{mL}$ rifampicin) and incubated for 2 days in a 30°C growth chamber.

***R. solanacearum* Pathogenesis Assay**

Ralstonia solanacearum was grown on Casamino acid-Peptone-Glucose (CPG) at 30°C for 1 day. The *R. solanacearum* culture was pelleted at RT for 5 min at 4,000 rpm and re-suspended in 10 mM MgCl_2 (Song et al., 2015). A freshly prepared 50 mL aliquot of *R. solanacearum* suspension at $\text{OD}_{600} = 1$ was used to drench the roots of *N. benthamiana* seedlings at 3 days after leaf infiltration with whitefly cDNA clones 2G4, 2G5, and 6A10 and empty vector (EV) (Chandrasekaran et al., 2016). The severity of *R. solanacearum* symptoms was scored on a scale of 0–10 as follows: 0, no leaves wilted; 1, 1–5% of leaves wilted; 2, 6–20% of leaves wilted; 3, 21–35% of leaves wilted; 4, 36–50% of leaves wilted; 5, 51–65% of leaves wilted; 6, 66–80% of leaves wilted; 7, 81–95% of leaves wilted; 8, 96–100% of leaves wilted but stems intact; 9, 96–100% of leaves wilted and stems broken; and 10, 100% of leaves wilted and stems broken (Song et al., 2016; **Figure 4A**). The total number of *R. solanacearum* cells in the rhizosphere was counted at 10 days after drench application. Whole roots were collected from each plant without soil particles, placed in a flask containing 200 mL of sterilized distilled water, and incubated with shaking for 30 min at 30°C. The liquid from the flask was serially diluted and spread onto CPG agar plates. To test the effect of the candidate whitefly effectors on plant growth rates, root weights were measured 10 days after pathogen challenge as described previously (Yang et al., 2009). The experiment was repeated three times with 10 replications. Preparation of graphs were performed using the R studio (R-Studio, Boston, MA, USA).

GUS Staining

Histochemical GUS staining was performed as described previously (Brown et al., 2003). Three plants treated with candidate effectors were collected for GUS staining on days 0, 3, 5, and 7. The positive control was 0.5 mM BTH (Syngentay Crop Protection Inc., Greensboro, NC, USA), and the negative control was empty vector. Leaves were immersed in staining solution (2 mM X-Gluc in N,N-dimethyl formamide, 100 mM NaH_2PO_4 , 10 mM Na_2EDTA , 0.5 mM ferrocyanide, and 0.1% Triton X-100, pH 7.0) and incubated at 37°C overnight in the dark. The leaves were cleared of chlorophyll by treating them with 70% ethanol after staining at RT for 24 h. Stained samples were observed and photographed with a digital camera (Sony, Park Ridge, NJ, USA).

Extraction of Plant RNA, cDNA Synthesis, and Quantitative RT-PCR

For the leaf pathogen (*P. syringae*) experiment, following agro-infiltration, leaf tissue was collected 0, 12, and 24 h after agro-infiltration and used for total RNA isolation. Following inoculation with *Pta*, the leaf tissue was harvested at 0, 24, and 48 h after inoculation and used for total RNA isolation. Following agro-infiltration, root tissue was collected 0, 1, and 2 days after agro-infiltration and used for total RNA isolation. For the soil-borne pathogen, following inoculation with *R. solanacearum*,

root tissue was harvested at 0, 1, and 2 days after inoculation and used for total RNA isolation. To validate candidate effector production in whitefly, whitefly adults were collected from *N. benthamiana* leaves at mid-day and used for total RNA isolation. Total RNA was isolated from tobacco leaf tissues using Trizol (Invitrogen) according to the manufacturer's instructions and as described in our previous study (Lee et al., 2012). First-strand cDNA synthesis was performed with 1 μg of DNase-treated total RNA, oligo-dT primers, and Moloney murine leukemia virus reverse transcriptase (Enzymomics, Daejeon, Korea). The qRT-PCR reaction mixtures consisted of cDNA, iQTM SYBR[®] Green Supermix (Bio-Rad Inc., Hercules, CA, USA), and 10 pM each primer. The cycling parameters were as follows: initial polymerase activation for 3 min at 95°C, followed by 45 cycles of 30 s at 95°C, 60 s at 60°C, and 30 s at 72°C. Relative transcript levels were calculated using the $2^{-\Delta\Delta\text{CT}}$ method. The reference genes were *NbACT* mRNA (GenBank accession no. U60489) in tobacco plants, and *BtACT* mRNA (GenBank accession no. AF071908) in whitefly. For convenient comparisons, the expression levels were presented as fold change relative to those of the control (where empty vector or LacI is equal to 1). Gene specific primer sequences are listed in Table S1.

RNA Isolation, De Novo Assembly and Relative Expression Levels

Whiteflies (*B. tabaci* MEAM1_UvA) were reared on cucumber plants (*Cumumis sativus*, Ventura, RijkZwaan, the Netherlands) in a climatized chamber (Snijders, Tilburg, the Netherlands; 28°C, 16H light 150 $\mu\text{E m}^{-2}\text{s}^{-1}$, RH75%) as previously described (Bleeker et al., 2011).

The samples for RNA sequencing were obtained as follows: whitefly eggs and nymphs (1st, 2nd, and 3rd instar) were removed, between 10 a.m. and 6 p.m., from the cucumber leaf using an insect pin and transferred directly into 100% acetone for storage at RT. For whole body ($n = 1$), thorax ($n = 2$), and abdomen ($n = 1$) samples the whiteflies were collected at 9 AM by aspiration and transferred to 100% acetone at RT. The whiteflies were halved with a surgical knife while submerged in 100% acetone to obtain the thorax and abdomen samples. Salivary glands ($n = 1$) and midguts ($n = 1$) were dissected from adult whiteflies as described previously (Kliot et al., 2014).

RNA isolation was performed using the RNeasy mini kit from Qiagen (www.qiagen.com) according to manufacturer's protocol. RNA integrity was examined using the 2200 TapeStation System with Agilent RNA ScreenTapes (Agilent). RNA with RIN values greater than 7.4 were used for Illumina RNA sequencing (www.illumina.com, HiSeq 2000) except for salivary glands (RIN 5.4) and midgut (RIN 5.8). The Illumina reads were cleaned from adapter and ambiguous sequences by Trimmomatic 0.32 software (usadellab.org) (Bolger et al., 2014). The clean reads of thorax, abdomen, eggs and whole body (± 20 million per sample) were used for *de novo* assembly using Trinity software (r20140313) [https://github.com/trinityrnaseq/trinityrnaseq/wiki; (Grabherr et al., 2011; Haas et al., 2013)] using the default settings. Trinity (r20140313) was used to realign reads to the *de novo* transcriptome contigs and to calculate the

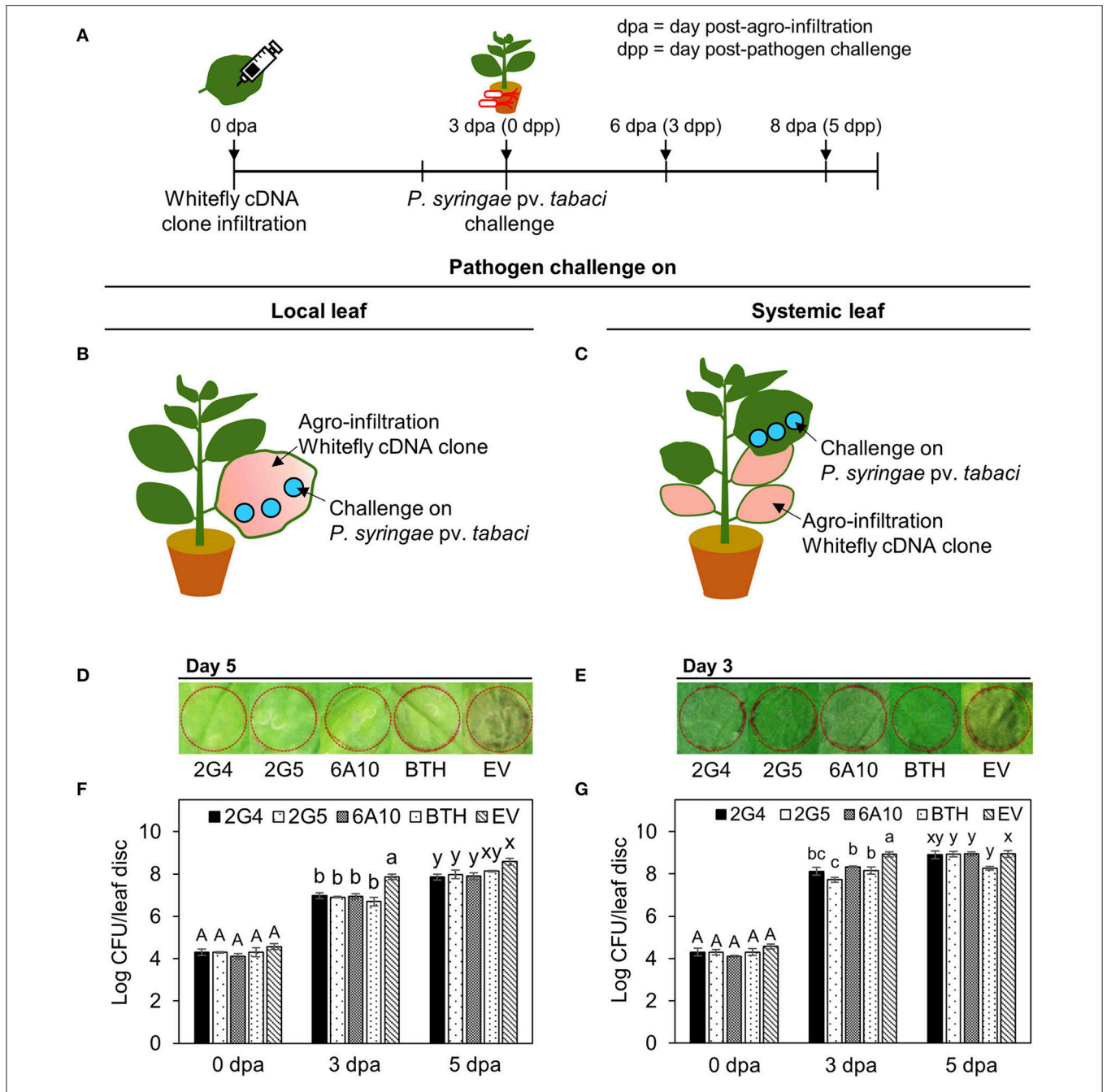


FIGURE 2 | Systemic acquired resistance against *P. syringae pv. tabaci* elicited by candidate whitefly effectors on local and systemic tobacco leaves. **(A)** Schematic diagram of the experimental design for investigating SAR against *Pta* after leaf infiltration with whitefly cDNA clones 2G4, 2G5, and 6A10. **(B)** SAR against *Pta* in a local tobacco leaf. A suspension of *Pta* at $OD_{600} = 0.001$ was infiltrated into a tobacco leaf that had been pre-infiltrated with whitefly cDNA clones 2G4, 2G5, and 6A10 throughout the leaf at 3 days before pathogen challenge. **(C)** SAR against *Pta* on a systemic tobacco leaf. A suspension of *Pta* at $OD_{600} = 0.001$ was infiltrated into a whole tobacco leaf that had been pre-infiltrated with whitefly cDNA clones 2G4, 2G5, and 6A10 at 3 days before pathogen challenge using a needleless syringe. **(D)** Disease symptom development at 5 days after pathogen challenge on local leaf. **(E)** Disease symptom development at 3 days after pathogen challenge on systemic leaf. **(F)** Bacterial population size of local leaf were measured at 0, 3, and 5 days after *Pta* infiltration with a needleless syringe. **(G)** Bacterial population size of systemic leaf were measured at 0, 3, and 5 days after *Pta* infiltration with a needleless syringe. Bars represent the mean value \pm SEM ($N = 10$). Infiltration with 1 mM BTH and *Agrobacterium* empty vector (EV) suspension was used as a positive and negative control, respectively. Different letters (a, b, and c; x and y) within day indicate statistically significant differences ($P < 0.05$).

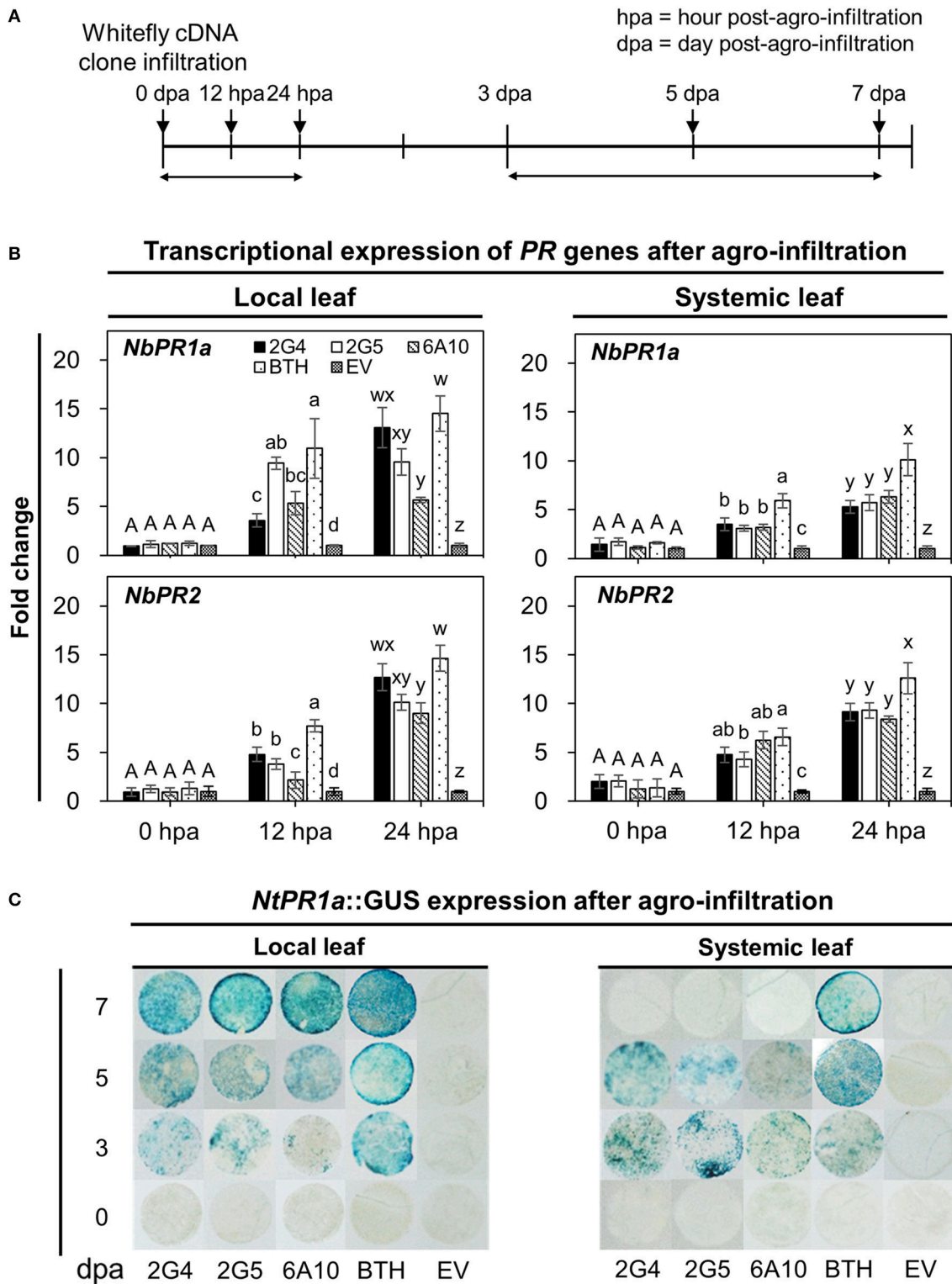
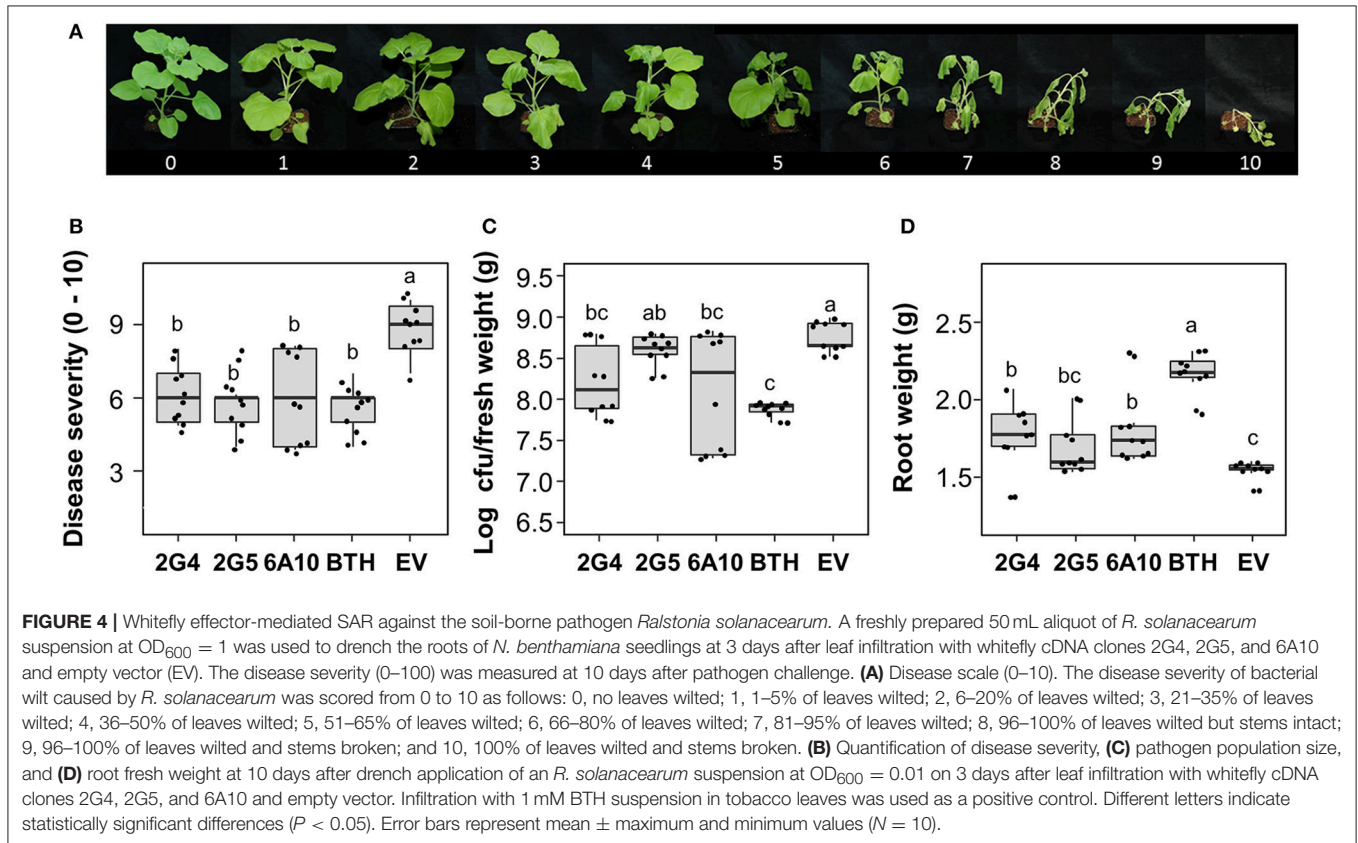


FIGURE 3 | Whitefly effector-mediated early and late expression of SAR marker genes in *N. benthamiana*. **(A)** Schematic diagram of the experimental design for investigating the expression of marker genes (*NbPR1a*, *NbPR2*) and the staining of leaf disc from *NtPR1a::GUS* transgenic tobacco after leaf infiltration with whitefly cDNA clones 2G4, 2G5, and 6A10 and empty vector (EV). The samples used for the early gene expression were local and systemic leaves after whitefly cDNA clone leaf-infiltration at 0, 12, and 24 h post-*Agrobacterium* infiltration (hpa). The samples for GUS staining to late expression of a SAR marker gene were leaf disc collected at 3, 5, and 7 days post-*Agrobacterium* infiltration (dpa). **(B)** Quantification of defense priming of SAR marker genes *NbPR1a* and *NbPR2* in plants at 0, 12, and 24 h

(Continued)

FIGURE 3 | after inoculation with whitefly cDNA clones 2G4, 2G5, and 6A10 on local (left panel) and systemic (right panel) leaves by qRT-PCR. Transcription is shown relative to empty vector (expression level = 1) with the *NbActin* gene as an internal reference. Bars represent the mean value \pm SEM ($N = 10$). Infiltration with a suspension of 1 mM BTH and *Agrobacterium* with empty vector (EV) was used as a positive and negative control, respectively. Different letters (a, b, c, and d; w, x, y, and z) within hpa indicate statistically significant differences ($P < 0.05$). **(C)** *NtPR1a* gene expression pattern following infiltration with candidate effectors using *NtPR1a::GUS* transgenic *Nicotiana tabacum* plants. GUS staining was conducted by incubating leaf tissues ($N = 30$) in X-gluc solution at 0, 3, 5, and 7 days post-*Agrobacterium* infiltration with whitefly cDNA clones 2G4, 2G5, and 6A10 and empty vector. Infiltration with 1 mM BTH suspension was used as a positive control.



fragments per kilobase transcript length per million fragments mapped (FPKM) using RSEM (<http://deweylab.github.io/RSEM>; Li and Dewey, 2011), after which an normalization (Trimmed Mean of M) was performed across all whitefly samples using the `abundance_estimates_to_matrix.pl` script (Li and Dewey, 2011; Haas et al., 2013). The RNA-seq data are deposited at the European Nucleotide Archive (<https://www.ebi.ac.uk/ena>) which is a mirror site of NCBI (the project number: PRJEB26594).

Statistical Analysis

Data were subjected to analysis of variance using JMP software ver. 4.0 (SAS Institute Inc., Cary, NC, USA; www.sas.com). The significance of biological or chemical treatment effects was determined by the magnitude of the F -value at $P = 0.05$. When a significant F -value was obtained for treatments, separation of means was accomplished using Fisher's protected least significant difference (LSD) test at $P < 0.05$. The results of repeated trials of each experiment outlined above were similar. Hence, one representative trial of each experiment is reported.

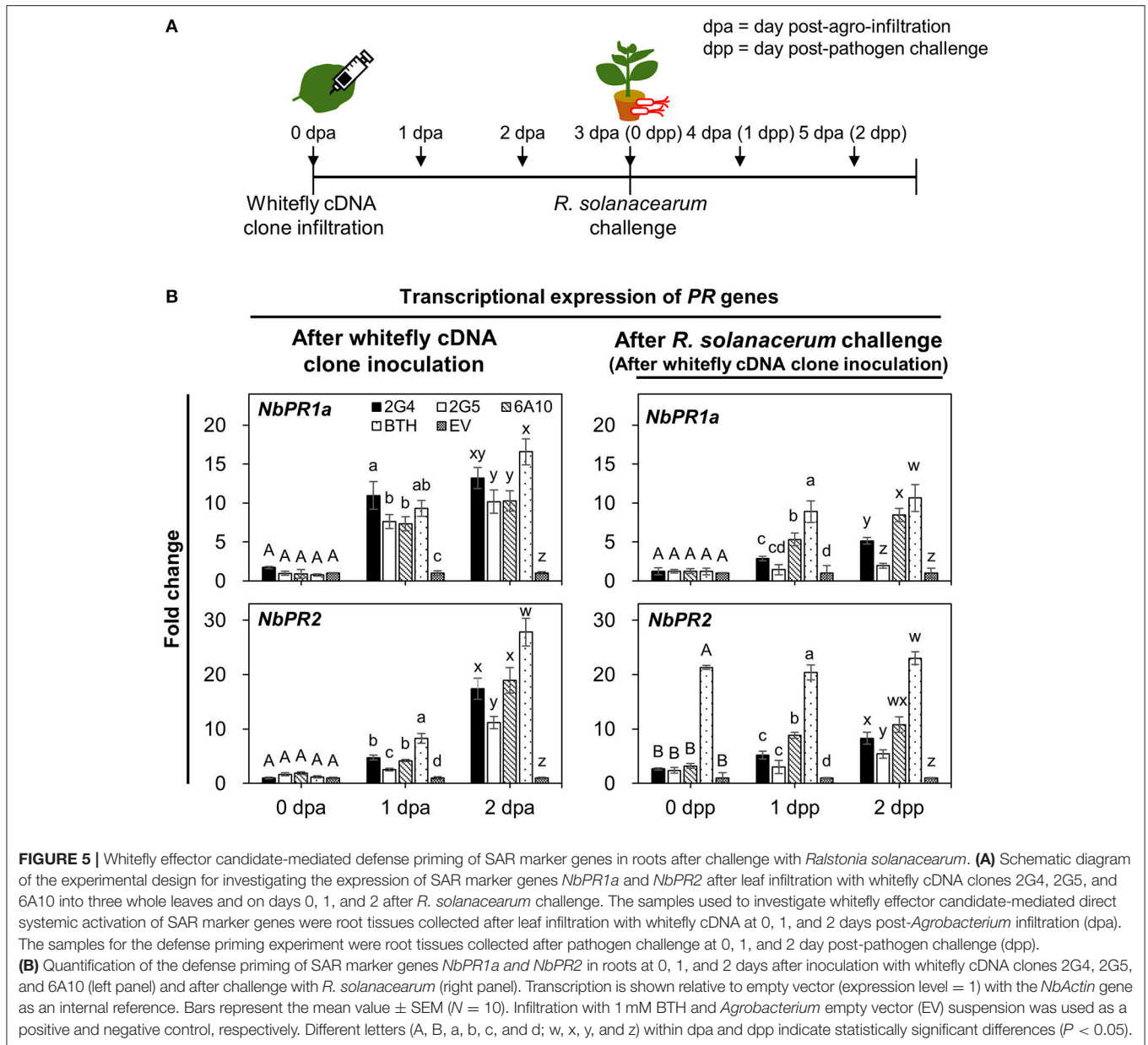
RESULTS

High-Throughput System Design and Identification of Potential Effectors

We developed a new screening method to assess the attenuation or suppression of the HR or symptom development caused two *P. syringae* pathovars (*Pta* and *Psy*) in the overlapping regions of *N. benthamiana* leaves after cross-infiltration with candidate whitefly effectors. In the first screening with using the two *P. syringae* pathovars in the overlapping regions of leaves after cross-infiltration, we selected 24 and 9 clones after *Pta* and *Psy* infiltration, respectively (Figures 1A,B). Of the 893 clones in the cDNA library, we ultimately selected three cDNA clones, 2G4, 2G5, and 6A10, due to their clear suppressive effects on lesion formation (Table S2).

Effector-Mediated Plant Immunity Against the Aboveground Pathogen *P. syringae* pv. *tabaci*

We evaluated whether the three candidate effectors would elicit plant immunity in local or systemic tissues of *N. benthamiana*.

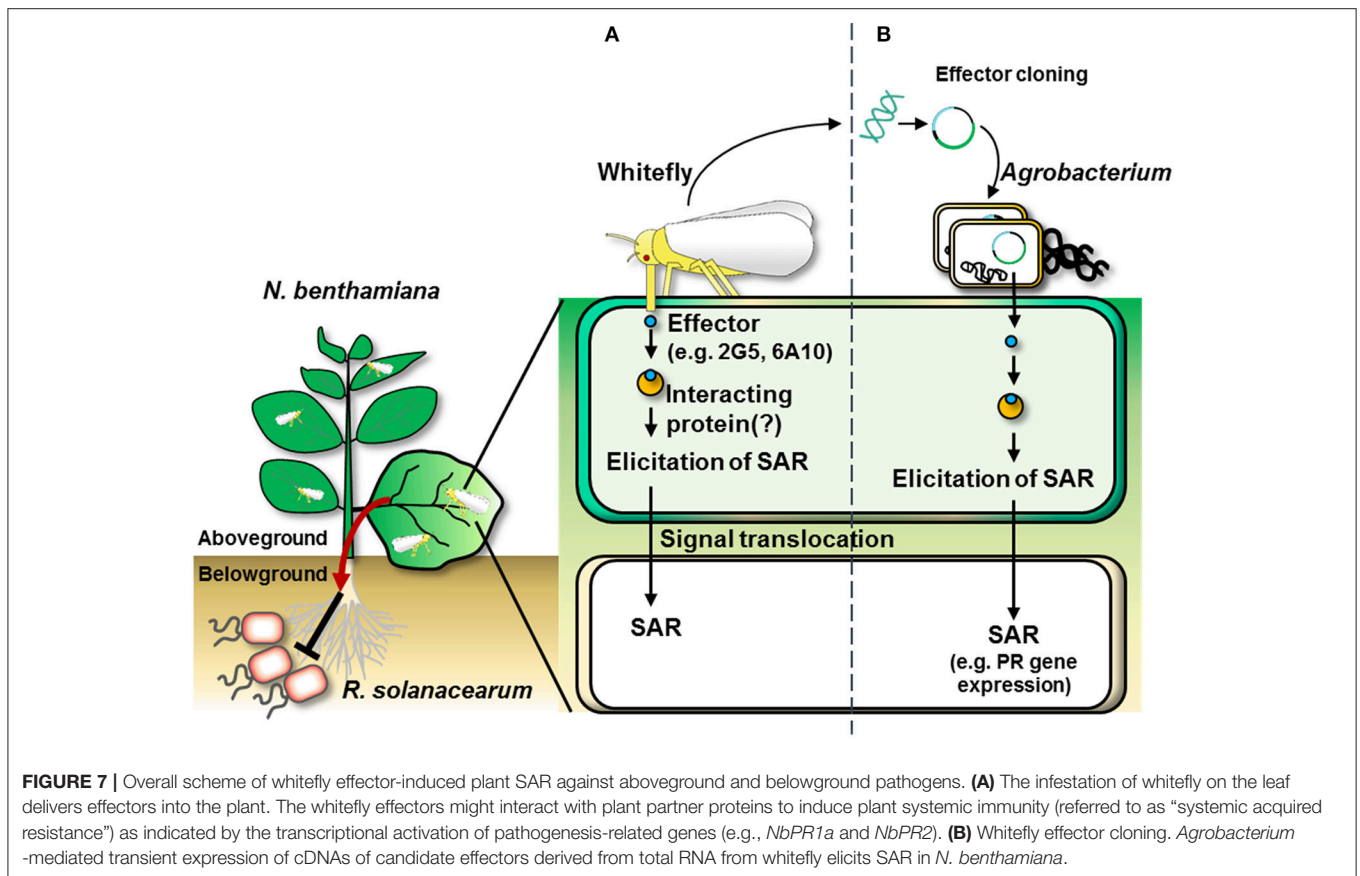
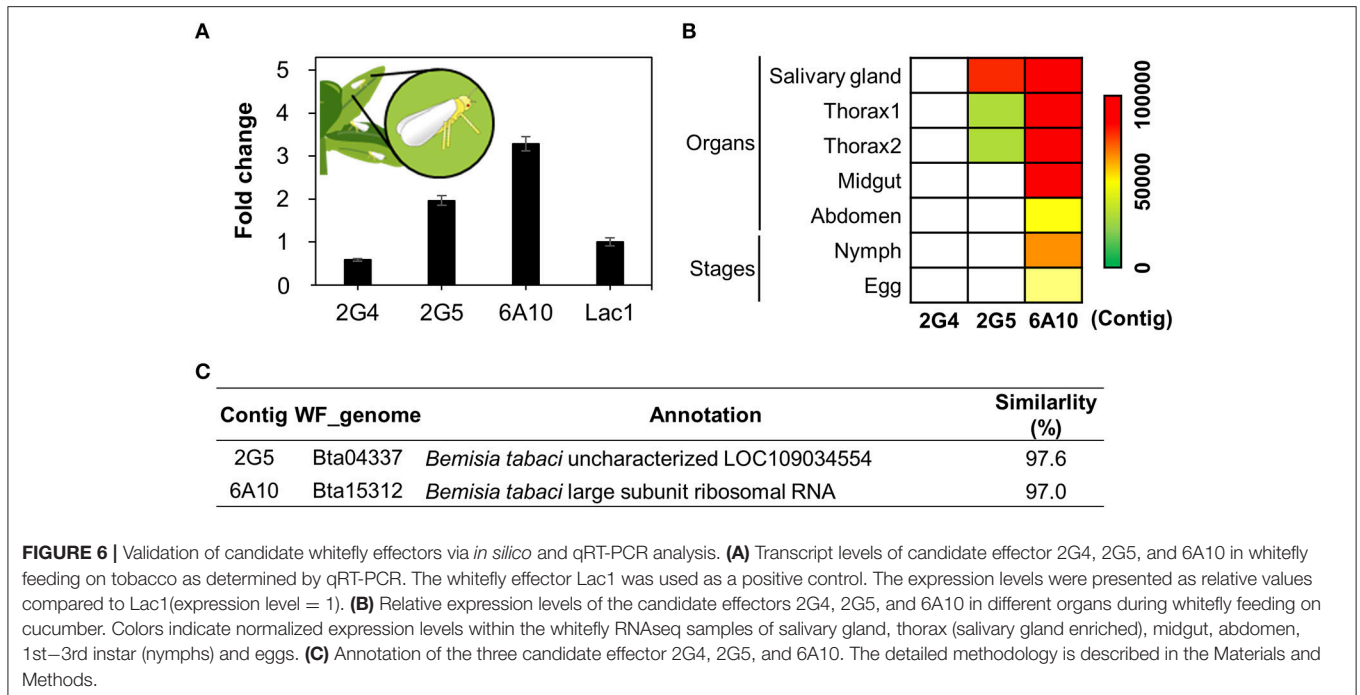


We infiltrated each whitefly cDNA clone into one half of a leaf and the vector control into the other half. At 3 days after infiltration, we challenged the plants with *Pta* and measured bacterial numbers on days 0, 3, and 5. First, we confirmed the inhibition of symptom development by *Pta* using the overlay method after infiltration of the candidate effectors into whole leaves (Figures 2B,C). Plants pretreated with the three candidate whitefly effectors, 2G4, 2G5, and 6A10, showed significantly ($P < 0.05$) fewer (10-fold) bacteria number on days 3 and 5 than the empty vector control in local leaves (Figure 2F). On day 5 after pathogen challenge in leaves infiltrated with the three candidate effectors, the number of bacteria was not statistically different among systemic leaves while they differed compared to empty vector control on day 3 (Figure 2G), and the number of bacteria was similar to that of the BTH-pretreated positive control in local

leaves (Figures 2F,G). The symptom in local and systemic leaves at day 5 after pathogen challenge are presented (Figures 2D,E).

Candidate Whitefly Effector-Elicited SAR Marker Gene Expression

We evaluated the short- and long-term elicitation of SAR by quantifying the expression of SAR marker genes *NbPR1a* and *NbPR2* at 0, 12, and 24 h and 0, 3, 5, and 7 days after infiltration with the candidate effector cDNAs (Figures 3A,B). To quantify the early expression of SAR marker genes, we evaluated the effects of pretreatment with the three candidate effectors, 2G4, 2G5, and 6A10, which induced the early expression of marker genes both locally and systemically (Figure 3B). Of the three clones, 2G4 had the strongest effects at 24 h to a level similar with that of positive control BTH treatment in local leaves (Figure 3B, left panel),



while all three clones increased *NbPR2* expression, and clones 2G4 and 6A10 more strongly increased *NbPR1a* expression in systemic leaves compared with the control (**Figure 3B**, right panel). At the same time, the transcriptional level of *NbPR1a* and *NbPR2* on the local leaf infiltrated with clone 2G5 was similar with 6A10 but higher than empty vector control (**Figure 3B**, left panel).

Next, to quantify the long-term expression of SAR marker genes, we evaluated their expression at 0, 3, 5, and 7 days after agro-infiltration. The activation of local and systemic plant immune responses was confirmed by examining the expression of *NtPR1a::GUS*, a representative SAR biomarker gene for plant immunity in tobacco (**Figure 3C**). *GUS* expression in tobacco leaves infiltrated with the three candidate effectors was first detected on day 3 and reached a maximized level on day 7 in local leaves, whereas the expression of this gene changed little in leaves pretreated with 1 mM BTH on days 3 to 7 (**Figure 3C**, left panel). The systemic expression of *NtPR1a* induced by the three whitefly effectors was detected only on day 3 and 5 but not on day 7 (**Figure 3C**, right panel). Maximum expression of *NtPR1a* in the positive BTH-treated control was detected on day 7 in local leaves and day 5 in systemic leaves (**Figure 3C**).

Whitefly Effector Expression in Aboveground Plant Tissues Activates Immunity Against the Soil-Borne Pathogen *R. solanacearum*

One important characteristic of plant immunity is “defense priming” (Song et al., 2015). Strong defense priming is generally detected at an early time point after pathogen challenge. To evaluate candidate effector-mediated SAR and defense priming against the soil-borne pathogen *R. solanacearum*, we measured bacterial wilt symptoms at 10 days after drench application of a 10^8 cfu/mL *R. solanacearum* suspension at 3 days after leaf infiltration with the three candidate effector cDNAs. Disease severity and the pathogen population were significantly reduced by 2G4 and 6A10 cDNA treatment (**Figures 4B,C**). We detected a 24, 12, and 27% reduction in symptom development in tobacco plants treated by leaf infiltration with 2G4, 2G5, and 6A10 cDNA, respectively (**Figure 4B**). The root fresh weight was 20% higher in plants pre-infiltrated with 6A10 than in the control (**Figure 4D**). By contrast, the bacterial number and root fresh weight in plants treated with 2G5 cDNA did not differ from those in the control (**Figures 4C,D**). However, the bacterial number in root system of plants treated with 2G4 cDNA was statistically lower than control treatment (**Figure 4C**).

To obtain further confirmation of candidate effector-mediated SAR against *R. solanacearum*, we performed two qRT-PCR experiments to evaluate the transcriptional expression of SAR marker genes *NbPR1a* and *NbPR2* in roots on days 0, 1, and 2 after cDNA infiltration and pathogen challenge (**Figures 5A,B**). First, we evaluated transcript levels of the two SAR marker genes after cDNA infiltration without pathogen challenge (**Figure 5B**, left panel). Compared with the control, the expression of *NbPR1a* was significantly different under all treatments on day 1. No significant difference was detected on day 2 across 2G4 and 6A10

treatments (**Figure 5B**, left and above panel). The maximum expression of *NbPR2* was detected in 2G4-, 2G5-, and 6A10-treated plants at 2 days (2 dpa) after leaf infiltration than in the control (**Figure 5B**, left panel). The expression of these genes was not different compared to control at the pathogen inoculation time point (3 dpa and 0 dpp) when *R. solanacearum* drench-applied 3 d after clone infiltration on the leaves (0 d for pathogen challenge in the roots) (**Figure 5B**, right panel).

Second, to evaluate the defense priming of SAR marker genes *NbPR1a* and *NbPR2*, we measured their expression at 0, 1, and 2 days after drench application of *R. solanacearum* on roots (**Figure 5B**, right panel). In plants subjected to leaf infiltration with 2G4 and 6A10 cDNA, *NbPR1a* and *NbPR2* were upregulated compared with the control at 2 days after pathogen challenge (**Figure 5B**, right panel). By contrast, pre-infiltration with 2G5 cDNA did not prime the expression of the two marker genes. The positive control treatment, 1 mM BTH, significantly increased *NbPR1a* and *NbPR2* expression after both direct infiltration and pathogen drench treatment (**Figure 5B**, right panel).

In Silico Analysis of Effector Expression in Whitefly

When whiteflies feed on phloem, they first produce saliva (Jiang et al., 1999; Jiang and Walker, 2003). This saliva, like the saliva of other herbivores, is thought to contain effector molecules produced by the salivary glands (Bos et al., 2010; Villarreal et al., 2016). From the *de novo* assembled RNA-seq data, the contig N50 number, median contig length, average contig length, and total assembled bases were 2445, 448, 1089.85, and 94604597 respectively. To validate candidate effector production in whitefly, we first performed qRT-PCR analysis to measure the expression levels of the three candidate genes from whitefly fed on tobacco (**Figure 6A**). All three candidates were expressed in whole whitefly adults (**Figure 6A**). Second, to confirm expression of the whitefly effectors in salivary glands, we generated RNA sequencing (RNA seq) libraries from whitefly salivary glands, thorax (salivary gland enriched), midgut, abdomen (midgut enriched), nymphs, and eggs from whiteflies collected from cucumber (**Figure 6B**).

Transcripts of 6A10 and 2G5 were detected in both whitefly salivary gland and thorax tissue, indicating that they might be transferred in to plant tissue (**Figure 6B**). The 2G5 effector shows high expression in salivary glands and thorax compared to midgut, abdomen, nymph and egg. However, 2G4 expression is overall low (**Figures 6A,B**). These data indicate that 6A10 and 2G5 are indeed expressed in salivary glands, pointing to the possibility that they are transferred into plant tissue (**Figure 6B**). However, we did not detect any mRNA of 2G4 in the whitefly organs nor nymph/egg (**Figure 6B**). The candidate effector cDNAs encode proteins annotated as follows: 2G5, an unknown protein, and 6A10, large subunit ribosomal RNA (**Figure 6A**). The expression levels of these genes did not significantly differ from that of the positive control, *Lac1* (**Figure 6A**), encoding a recently identified whitefly effector (Yang et al., 2017). Collectively, of the three clones, only clones 2G5 and 6A10 represent solid candidate whitefly effectors.

DISCUSSION

Our study demonstrates that transient expression of putative whitefly effector cDNA induces plant systemic resistance against the soil-borne pathogen *R. solanacearum*, as well as the air-borne (aboveground) pathogen *P. syringae* pv. *tabaci* (*Pta*), in *N. benthamiana*. These findings add to our previous finding that whitefly infestation elicits plant immunity in the root system through signal transduction from aboveground to belowground plant parts (Yang et al., 2011). Crosstalk between hormone signaling pathways is often detected in plants infested by chewing and phloem-sucking insects. In contrast to JA/ET-dependent signaling elicited by chewing insects, the infestation of *Arabidopsis* with sucking insects such as whitefly increases the expression of marker genes for the SA-response pathway (Park and Ryu, 2014).

In this study, we designed a new high-throughput screening protocol for isolating putative whitefly effectors that are translocated to the plant and activate systemic plant immunity. We performed *Agrobacterium*-mediated transient transformation of *N. benthamiana* leaf tissues with cDNA prepared from total RNA extracted from whitefly during infestation at mid-day (Figure 1A). Cross-inoculation with whitefly cDNA and virulent/avirulent pathogens allowed us to detect the induction of plant resistance responses (Figures 1B,C). The three selected candidate effectors suppressed avirulent pathogen-mediated HR responses and virulent pathogen-mediated symptom development. Indeed, we previously demonstrated bacterial effector-induced suppression of both the HR and pathogen-mediated symptom development, and transcriptome analysis revealed that whitefly infestation induced the expression of a large portion of a set of genes for plant immunity in leaves and roots (Park and Ryu, 2014). However, the identity of the determinants from whitefly that elicit SA signaling from leaf to root was previously unclear.

Our current results describing SA-responsive gene expression induced by candidate effectors in leaves are in agreement with previous investigations of SA marker genes in pepper and tobacco (Yang et al., 2011; Song et al., 2015). The induction of SA-responsive genes detected in the current study corresponded to the induction of plant immunity against *Pta* (Figure 2). More interestingly, the strength of whitefly effector-mediated SAR was more obvious in local leaves (transiently expressing elicitor) than in systemic (distal) leaves. The infiltration of all three candidate effectors was sufficient to attenuate pathogen growth in the intracellular spaces of local but not systemic leaves on day 5, but bacterial numbers were reduced on day 3 in both local and systemic leaves (Figure 2). The results of defense-related gene expression analysis support the differential induction of SAR in local vs. systemic leaves (Figure 3). The defense priming of SA-responsive biomarker genes *NbPR1a* and *NbPR2* was weaker in systemic vs. local leaves (Figure 3A). More importantly, *NbPR1a* expression was not detected systemically at 7 days after cDNA infiltration in the face of pathogen challenge (Figure 3B).

As expected, systemic plant immunity was induced by transient expression of the candidate whitefly effectors. Of the three candidates, 2G5 and 6A10 cDNA suppressed bacterial wilt

symptom caused by *R. solanacearum* (Figure 4B). This decrease in 2G5 treatment severity could not be explained by defense priming of SA-responsive genes *NbPR1a* and *NbPR2* (Figure 5B). Pre-infiltration with 2G5 cDNA did not prime the response of the two SAR marker genes to *R. solanacearum* challenge at 1 dpp in roots (Figure 5B). These results might be due to the weak activation of defense priming by clone 2G5 (Figure 5B). The induction of SAR by clone 2G5 cannot be dependent of SA signaling but can be dependent some other defense signaling such as jasmonic acid or ethylene signaling (Hase et al., 2008; Baichoo and Jaufeerally-Fakim, 2017; Liu et al., 2017). In contrast, the pre-infiltration with 6A10 cDNA elicited defense priming in similar level of BTH treatment used as a positive control (Figure 5B). A previous study also showed strong defense priming when plants activated the SAR response (Song et al., 2015). Overall, these results represent the first demonstration that a single whitefly effector elicits SAR against aboveground and belowground microbial pathogens through systemic signal transduction.

The candidate effector cDNAs are annotated as encoding large subunit ribosomal RNA (6A10) and an unknown protein (2G5). Bioinformatics analysis revealed the presence of 2G5 and 6A10 in whitefly salivary glands. The identification of 6A10 as a large subunit ribosomal RNA of *B. tabaci* deserves further study. There are many examples of the secretion of effector proteins that modulate plant immunity in saliva from hemipterans including aphids and whitefly (Atkins et al., 2011; Will et al., 2013; Sharma et al., 2014; Su et al., 2015; Peng et al., 2016; Villarreal et al., 2016). For instance, the effector proteins C002, Mp1, and Mp2 from aphid promote fecundity, whereas Mp10 and Mp42 decrease fecundity (Bos et al., 2010; Pitino and Hogenhout, 2013).

We do not fully understand how whitefly rRNAs such as 6A10 are translocated to plant cells to elicit SAR. Besides protein effectors, non-protein salivary factors can also act as effectors (Su et al., 2015). Bacterial rRNAs and plant DNA were recently shown to elicit SAR (Bhat and Ryu, 2016; Lee et al., 2016). Destroying the structure of bacterial rRNA via sonication and RNase treatment greatly reduced its effect on inducing SAR, indicating that certain (structural or sequence) signatures of rRNA are required for full SAR elicitation. While this signature has not been identified, bacterial rRNA was successfully detected in plant cells. It appears that the plant recognizes whitefly rRNA as a non-self-molecular pattern. Also, three small RNAs from whitefly were detected in tomato leaf tissue through sequencing tomato phloem RNA from whitefly-infested plants and the nymphs themselves, and the translocation of these RNAs was confirmed using stem-loop qRT-PCR (van Kleeff et al., 2016). The translocation of sRNA has also been observed during plant-fungus interactions. The gray mold fungal pathogen *Botrytis cinerea* delivers its sRNA and suppresses host defense responses through silencing host mRNAs related to defense signaling (Weiberg et al., 2013). Like non-coding sRNAs that function as effectors from whitefly, the non-coding rRNA identified in the current study appears to function as a trigger of SAR, a notion that is currently under investigation.

In conclusion, we revealed a new function for whitefly effectors, i.e., eliciting systemic immunity from aboveground to belowground plant parts. Adding to our previous discovery

of whitefly-mediated SAR against aboveground and soil-borne plant pathogens, in the current study, we demonstrated that treatment with candidate whitefly effectors alone was sufficient to elicit plant immunity against microbial pathogens in local and systemic tissues (Figures 7A,B). *In silico* and qRT-PCR analyses confirmed that the candidate effectors were expressed. Both 2G5 and 6A10 were expressed in salivary glands and could be translocated into the host plant, resulting in defense priming of SAR-related marker genes, even in distal tissues. To our knowledge, this is the first report of whitefly effector-mediated induction of plant systemic immunity.

AUTHOR CONTRIBUTIONS

C-MR designed the study. H-RL, SL, and SP performed experiments. PvK performed the RNA-seq sampling and bioinformatic analysis. RC supervised PvK. C-MR contributed to scientific discussions that guided the project's direction. C-MR and H-RL wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/feco.2018.00090/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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