Aboveground insect infestation attenuates belowground Agrobacterium-mediated genetic transformation

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Introduction

Under natural conditions, plants must constantly respond to diverse biotic stresses. Plants have developed defensive machinery to protect themselves against a variety of invading pathogens and insects (Nimchuk et al., 2003; Hogenhout & Bos, 2011). Perception of pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively) results in rapid activation of defence responses and accumulation of reactive oxygen species in local tissue (Nimchuk et al., 2003; Hogenhout & Bos, 2011). In addition to these rapid responses, the major phytohormones, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), are produced (Glazebrook, 2001; Thomma et al., 2001; Pieterse et al., 2009), which act as key signalling molecules in regulating plant defence. Salicylic acid predominantly participates in plant defence against biotrophic and hemibiotrophic pathogens and sucking insects. It has been demonstrated that SA and its analogues, such as 2,6-dichlororionicotinic acid (INA) and benzo-1,2,3-thiadiazole-7-carbothioic acid S-methyl ester (BTH), activate systemic acquired resistance (SAR) to certain pathogens and insects (Ryals et al., 1996; Achuo et al., 2004; Wang et al., 2007; Lee et al., 2012). On the other hand, accumulation of JA and ET occurs in response to necrotrophic pathogens and wounding or herbivore damage (Howe & Jander, 2008). Although some genes are induced by both SA and JA/ET application, most cases of crosstalk between these two signalling pathways are antagonistic (Schenk et al., 2000; Kunkel & Brooks, 2002; Glazebrook et al., 2003).

Agrobacterium tumefaciens is a soil-borne, Gram-negative bacterium. It causes crown gall disease in a broad range of dicotyledonous plants. Agrobacterium species transfer their DNA into other organisms (Pitzschke & Hirt, 2010); transferred DNA (T-DNA) is subsequently integrated into the host chromosomal DNA resulting in a hyperplastic response, gall formation. This process of T-DNA transport is controlled by proteins encoded by the virulence genes on the bacterial tumour inducing (Ti)

Summary

- Agrobacterium tumefaciens causes crown gall disease. Although Agrobacterium can be popularly used for genetic engineering, the influence of aboveground insect infestation on Agrobacterium induced gall formation has not been investigated.
- Nicotiana benthamiana leaves were exposed to a sucking insect (whitefly) infestation and benzothiadiazole (BTH) for 7 d, and these exposed plants were inoculated with a tumorigenic Agrobacterium strain. We evaluated, both in planta and in vitro, how whitefly infestation affects crown gall disease.
- Whitefly-infested plants exhibited at least a two-fold reduction in gall formation on both stem and crown root. Silencing of isochorismate synthase 1 (ICS1), required for salicylic acid (SA) synthesis, compromised gall formation indicating an involvement of SA in whitefly-derived plant defence against Agrobacterium. Endogenous SA content was augmented in whitefly-infested plants upon Agrobacterium inoculation. In addition, SA concentration was three times higher in root exudates from whitefly-infested plants. As a consequence, Agrobacterium-mediated transformation of roots of whitefly-infested plants was clearly inhibited when compared to control plants. These results suggest that aboveground whitefly infestation elicits systemic defence responses throughout the plant.
- Our findings provide new insights into insect-mediated leaf–root intra-communication and a framework to understand interactions between three organisms: whitefly, N. benthamiana and Agrobacterium.
plasmid and chromosome and includes chv and vir genes (Zhu et al., 2000; Tzfira & Citovsky, 2002; Gelvin, 2003). Phenolic compounds such as acetosyringone, released from wounded plants, activate VirA, which is a membrane-bound sensor, and this activation regulates the intracellular response regulator, VirG (Wolanin et al., 2002). Specific attachment of Agrobacterium depends on the chromosomal genes chvA, chvB and pscA (also known as exoC) which play a role in the synthesis and localization of periplasmic β-1,2 glucan (McCullen & Binns, 2006). After vir gene activation and attachment of Agrobacterium to plant cells, the VirD2 protein along with VirD1 nicks the T-DNA region of the Ti plasmid and VirD2 remains attached to the 5′ end of the T-DNA. VirB complex, which belongs to the class of type IV secretion system (T4SS) and consists of at least 12 proteins, and VirD4 are required for transport of VirD2-T-DNA and several other proteins such as VirE2, VirE3, VirF and VirD5 into the host cell (Vergunst et al., 2005). VirE2 is a single-stranded DNA binding protein and presumably coats the T-DNA in the plant cell, thus forming a VirD2-T-DNA and VirE2 complex (T-complex) (Vergunst et al., 2000; Cascales & Christie, 2003). In addition, two other virulence proteins, VirE3 and VirF, have been shown to play a role in T-DNA transport to nucleus and integration (Lacroix et al., 2005). The ability of Agrobacterium to transport and integrate part of its DNA into a host genome has been widely exploited to express, disrupt or silence genes in plants (Hellens et al., 2000; Ditt et al., 2001).

Plant responses to Agrobacterium infection result in the release of a number of signalling molecules, including plant sugars, phenolic compounds, opines, SA, indole-3-acetic acid (IAA), γ-amino butyric acid (GABA), quorum-sensing signals, ethylene and other unidentified signals (Chevrot et al., 2006; Liu & Nester, 2006; Yuan et al., 2007; Anand et al., 2008; Nonaka et al., 2008). Exogenous SA inhibits induction of vir genes and reduces the virulence of Agrobacterium in Arabidopsis thaliana (Yuan et al., 2007) and Nicotiana benthamiana (Anand et al., 2008). Nicotiana benthamiana plants silenced for genes involved in SA biosynthesis and signalling were more susceptible to Agrobacterium infection (Anand et al., 2008). In addition, exogenous application of BTH to N. benthamiana and tomato impairs the development of crown gall disease caused by Agrobacterium tumorigenic strain A348 (Anand et al., 2008). All of these results suggest that SA plays an important role in plant responses to Agrobacterium infection.

Whitefly (Bemisia tabaci) is a sap-sucking insect widely distributed across the warmer parts of tropical and subtropical regions. Whitefly infestation enhances plant defence responses dependent on the SA and JA/ET-signalling pathways (Kaloshian & Walling, 2005). In addition, a novel signalling pathway not regulated by SA or JA may also exist against whitefly infestation (van de Ven et al., 2000). We have previously shown that whitefly infestation on leaves significantly enhances plant defence responses in both leaves and roots against hemibiotropic bacterial spot disease caused by infection with Xanthomonas axonopodis pv. vesicatoria and bacterial wilt caused by Rastonia solanacearum infection (Yang et al., 2011). Root biomass increased significantly in whitefly-infested plants, although it was reduced in plants treated with BTH (Yang et al., 2011). Transcriptome analysis indicated that whitefly infestation on aboveground parts of pepper plants elicited SA and JA/ET signalling in both aboveground and belowground tissues (Park & Ryu, 2014). In addition, auxin-responsive genes and several transporters (including ATP-binding cassette, peptide, zinc and phosphate transporters) were significantly upregulated in whitefly-infested roots, suggesting the release of auxin and nutrients in root exudate by whitefly-infested plants and implicating the role of upregulated genes in the facilitation of root biomass (Park & Ryu, 2014).

In this study, we investigated whether whitefly infestation in N. benthamiana leaves changed the susceptibility of above- and belowground tissues to Agrobacterium infection. Crown gall disease on plants stem and crown regions was significantly attenuated by whitefly infestation. At least a two-fold reduction in gall weight and increased stem thickness was seen in whitefly-infested plants in comparison with water treatment. Endogenous SA accumulated only in response to Agrobacterium inoculation in whitefly-infested plants. A reverse genetic approach revealed that silencing isochorismate synthase 1 (ICS1) in N. benthamiana plants compromised the protective effect of whitefly infestation against Agrobacterium infection. Agrobacterium-mediated transformation was also inhibited in roots of whitefly-infested plants. In addition, SA concentration was three times higher in root exudates from whitefly-infested plants when compared to control plants. Our results clearly show that leaf infestation by whitefly enhances the ability of the SA-dependent defence-signalling pathway to attenuate Agrobacterium-mediated transformation in plant roots.

Materials and Methods

Agrobacterium growth

An AT-ammonium sulphate-glucose (ANG) medium containing 10.9 g KH2PO4, 0.16 g MgSO4·7H2O, 0.005 g FeSO4·7H2O, 0.011 g CaCl2·2H2O, and 0.002 g MnCl2·4H2O plus 1 g ammonium sulphate and 2 g of glucose per litre was used for Agrobacterium growth. Agrobacterium tumefaciens strain C58 was grown on solid ANG medium containing 100 μg ml⁻¹ rifampicin at 30°C for 2 d, scraped off plates, re-suspended in sterilized distilled water (SDW), and adjusted to proper concentration for further experiments.

Plant materials and treatments

Seeds of Nicotiana benthamiana Domin. were surface-sterilized with 6% sodium hypochlorite, washed four times with SDW and plated on half-strength Murashige and Skoog (0.5 × MS) salts supplemented with 3% sucrose and 0.6% plant agar. Seeds were germinated and incubated in a growth chamber 25 ± 2°C under fluorescent light conditions (light: dark 12:12 h; c. 7000 lux light intensity). Seedlings were transferred to soilless potting medium (Punong, Co. Ltd, Gyeongju, South Korea). Leaves of 3-wk-old plants were separately infested with whitefly (Bemisia tabaci Genn.), or treated with 40 ml of 0.5 mM BTH or 40 ml of...
sterilized water (control) for 7 d in plastic containers (65 x 70 x 80 cm).

After plant treatments with whitefly, BTH and water (control) for 7 d, suspensions of A. tumefaciens C58 (OD<sub>600</sub> = 2) were inoculated by slightly injuring stems with a needle at heights of 2, 4, 6 and 8 cm above soil level as described previously (Anand et al., 2007, 2008). In another independent experiment, the crown region of stems was inoculated with A. tumefaciens C58 (OD<sub>600</sub> = 2) as described above. Gall formation was assessed at 15 and 30 d post inoculation (dpi) on stem or crown regions of plants.

**Virus-induced gene silencing (VIGS)**

For VIGS, 2-wk-old N. benthamiana seedlings were infiltrated with TRV-based VIGS vectors (pTRV2) containing N. benthamiana homologues of the plant defence-related and plant defence hormone signalling genes NbICS1 and NbCOI1 or with a vector control pTRV2::00, as described previously (Anand et al., 2007; Senthil-Kumar & Mysore, 2014). The infiltrated plants were monitored for c. 2 wk. Treatments with whitefly, BTH or water (control), followed by inoculation with A. tumefaciens C58 and assessment of gall formation, were conducted as described above.

**Extraction of plant and bacterial RNA, cDNA synthesis and real-time quantitative PCR**

Stems of N. benthamiana infested with whitefly or treated with water and inoculated with Agrobacterium C58, together with control stems, were frozen in liquid nitrogen. Total RNA was isolated using the RNeasy<sup>®</sup> plus mini kit according to the manufacturer’s protocol (Qiagen). Bacterial RNA was isolated using the same method.

First-strand cDNA was synthesized using 2 µg RNA, oligo dT primer, dNTP, and Moloney murine leukaemia virus reverse transcriptase (M-MLV RT; enzynomics, Daejeon, South Korea). Quantitative reverse transcription polymerase chain reactions (qRT-PCR) were carried out using a Chromo4 real-time PCR system (Bio-Rad). The reaction mixture contained 2× Brilliant SYBR Green qRT-PCR Supermix (Bio-Rad), cDNA, and 0.5 µM of each gene-specific primer.

Sequences were amplified using thermocycle parameters: 10 min at 95°C, followed by 44 cycles of 30 s at 95°C, 30 s at 60°C and 42 s at 72°C. Relative RNA levels were calibrated and normalized relative to the level of NbACT mRNA (GenBank accession no. U60489). The primer sets used in this study are listed in Supporting Information Table S1.

**Quantification of amounts of endogenous plant defence hormones**

Five replicate samples per treatment were analysed to quantify SA and JA concentrations in response to Agrobacterium in stems of whitefly-infested and water-treated plants. The prepared samples were quantified using high performance liquid chromatography mass spectrometry (HPLC-MS) 1100 series mass spectrometer (Agilent, Santa Clara, CA, USA) with a Sunfire™ C18 (2.1 x 10 mm) column (Waters, Milford, MA, USA) at a flow rate of 300 l min<sup>-1</sup>.

The stock solutions of SA and JA were prepared at a concentration of 1 mg ml<sup>-1</sup> in 100% methanol. Calibration curves were based on the comparison between the ratio of SA and JA peak area/internal standard area and the ratio of concentration for SA and JA/internal standard. The limits of detection and quantification were 3 and 10, respectively, which was determined by a signal-to-noise ratio.

**In vitro bioassays**

Nicotiana benthamiana seeds were surface-sterilized and germinated, as described above. Four-day-old seedlings were transferred to plates (60 x 15 mm, SPL) containing 26 ml of 0.5 x MS liquid media. Plates were placed in the plastic container (phytohealth, 103 x 78.6 mm, SPL). Whitefly, BTH and water control treatments were applied to plants as described above. Plant growth was monitored for 7 d after treatments and the weight of shoot and root measured. In addition, root exudates were collected at 7 and 14 d after treatments, when plants were 28 and 35 d old, respectively. For each replicate, containing 16 plants, 0.80 ml of root exudate was collected from plates. No media contamination was observed in the entire experiment.

**Image capture and microscopy**

In order to visualize Agrobacterium transformation at the root surface, seedlings were inoculated with disarmed A. tumefaciens strain GV2260 carrying the binary vector pBISN1-GUS (Narasimhulu et al., 1996), and observed using a Leica stereo-microscope S8APO (Leica, Wetzlar, Germany). Representative pictures were taken using a Hamamatsu digital camera, model Leica DFC295.

**Statistical analysis**

Analysis of variance of experimental datasets was performed using JMP software v5.0 (SAS Institute Inc., Cary, NC, USA). Significant effects of treatment were determined by the magnitude of the F-value (P < 0.05). When a significant F-test was obtained, separation of means was accomplished by Fisher’s protected least significant difference at P = 0.05.

**Results**

Whitefly infestation attenuates gall formation by Agrobacterium in stems

It has been recently reported that SA is a key player in attenuating the severity of crown gall disease in N. benthamiana (Anand et al., 2008) and that aboveground infestation with whitefly enhances plant defences in both aboveground and belowground organs of pepper plants by activating SA-signalling-related gene expression (Yang et al., 2011; Park & Ryu, 2014). This led us to consider
whether whitefly-triggered, SA-dependent signalling negatively modulates gall formation by *Agrobacterium tumefaciens* in *N. benthamiana*. To test this, 3-wk-old *N. benthamiana* plants were infested with whitefly (55 ± 7 insects per leaf) or treated with 0.5 mM BTH as a positive control; water was used as a control treatment. One week after initial treatments, we inoculated *N. benthamiana* stems at four places with suspensions of a tumorigenic *A. tumefaciens* C58 (OD$_{600}$ = 2). These treatments, even after inoculating with *Agrobacterium* for 30 d, did not have any distinguishable effect on plant height and leaf numbers (data not shown). Gall formation was assessed at 15 and 30 dpi.

Strikingly, the size of galls was smaller in whitefly-infested plants than in water-treated control plants (Fig. 1a). A c. 3.5-fold reduction of gall weight was recorded in whitefly-infested plants at both time-points when compared to control plants (Fig. 1b). No obvious physiological difference was observed in stems among different treatments (Fig. S1). To prevent any possible subtle variation in diameter of stems, which might affect gall weight, we further evaluated gall formation using relative thickness. Relative thickness (%) = ((b - a)/a) × 100 (Fig. 1c). In agreement with the gall weight, relative gall thickness at 30 dpi was 35% lower in whitefly-infested plants than in controls (Fig. 1c). To determine if there were site-specific effects of whitefly infestation on *Agrobacterium* infection, relative gall thickness was analysed at four independent heights (2, 4, 6 and 8 cm above soil level) on the stem. Relative gall thickness values were lower in whitefly-infested plants, regardless of inoculation sites, compared with water-treated controls (Fig. 1d), clearly indicating that whitefly infestation negatively affects gall formation by *A. tumefaciens*. Similar results were obtained at 15 dpi (Fig. S2).

Differential expression of SA- and JA-dependent genes and accumulation of endogenous SA and JA in whitefly-infested plants in response to *Agrobacterium*

In order to examine whether *Agrobacterium*-mediated gall formation in whitefly-infested plants was affected by signalling pathways involved in plant defence responses, we measured transcript levels of the SA biosynthetic and signalling-related genes *pathogenesis-related* (*PR*) 1a, 2, phenylalanine ammonia lyase1 (*PAL1*) and *PAL2* (Anand *et al.*, 2008) and the JA biosynthetic and signalling-related genes *lipoxigenase3* (*LOX3*), oxophytodienoate reductase3 (*OPR3*), trypsin protease inhibitor (*TPI*) and threonine deaminase (*TD*) (Stitz *et al.*, 2011). Three-week-old *N. benthamiana* plants were treated with whitefly infestation, BTH or water for 7 d, before being inoculated with *Agrobacterium* for 5 d.

Two genes involved in SA signalling, *PR1a* and *PR2*, showed at least a three-fold increase in induction in whitefly-infested plants (Fig. S3a), but were not significantly activated following inoculation with *Agrobacterium*, compared with water controls (Fig. 2a). By contrast, *PR1a* and *PR2* expression was induced upon *Agrobacterium* inoculation in BTH pre-treated plants (Fig. 2a). Expression levels of the SA-biosynthetic genes *PAL1* and *PAL2* were also reduced in whitefly-infested plants compared with water-treated controls (Fig. 2b). Similar results were obtained at 15 dpi (Fig. S2).

**Fig. 1** Assessment gall formation by *Agrobacterium tumefaciens* in whitefly-infested *Nicotiana benthamiana* stems.

Three-week-old *N. benthamiana* plants were treated with whitefly infestation, 0.5 mM benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) or water (control) for 7 d. Stems were inoculated with *A. tumefaciens* (OD$_{600}$ = 2) at four different places, located at 2 cm increments from soil level to top. (a) A representative picture was taken at 30 d post inoculation (dpi) of *A. tumefaciens*. (b) Individual gall was weighed at 30 dpi. (c) Schematic representative of relative thickness (RT) calculation RT = ((b - a)/a) × 100% is shown in above graphs. Relative thickness was assessed at 30 dpi. (d) Relative thickness measured at different heights of the stem. Significant difference between treatments and water control: *, P = 0.05. Error bars, ± SE of the mean (n = 5).
and PAL2 doubled in plants infested with whitefly (Fig. S3b) and their expression was upregulated 1 d after Agrobacterium inoculation (Agro 1 d) when compared to the control plants (Fig. 2b). However, PAL1 and PAL2 were not upregulated following BTH treatment, but its expression level did increase 5 dpi with Agrobacterium (Fig. 2b). Overall, expression of SA signalling and biosynthetic genes was similarly affected by whitefly infestation and biosynthetic genes remained induced upon Agrobacterium inoculation.

Unlike expression patterns of SA-related genes, expression of JA-related genes was affected in a pathway-specific manner, as expression of JA biosynthetic pathway genes differed from that of genes involved in JA-signalling pathways. For example, TPI and TD were not induced by whitefly
infestation (Fig. S3c), whereas expression of LOX3 and OPR3 approximately doubled in levels at 7 d after whitefly infestation, relative to expression in water-treated control plants (Fig. S3d). The amount of TPI transcript increased in whitefly-infested plants at 1 dpi with Agrobacterium, but there was no increase in the level of LOX3 and OPR3 expression at any time-point after Agrobacterium inoculation (Fig. 2c,d). No significant increase in expression levels of ET-related genes occurred at most of the tested time-points in whitefly-infested plants, before or after Agrobacterium inoculation with the exception of EIN2, which was induced at 5 dpi with Agrobacterium (Fig. S4).

In order to investigate the endogenous plant defence hormones in response to whitefly infestation, we analysed the contents of SA and JA. Notably, the concentration of endogenous SA doubled in 7 d after whitefly infestation, relative to control plants (Fig. S5a). An increase in endogenous SA was also seen at 1 dpi with Agrobacterium, compared with the water-treated controls (Fig. 3a). By contrast, although the JA concentration was slightly increased following whitefly infestation (Fig. S5b), it was drastically declined after Agrobacterium inoculation (Fig. 3b). These results indicate that whitefly-derived defence against Agrobacterium virulence is dependent on the SA-related pathway.

Silencing the SA-biosynthetic gene, ICS1, compromises the plant’s protective effect against Agrobacterium due to whitefly infection

In order to identify plant genes involved in whitefly-induced plant defence-signalling pathways modulating Agrobacterium-mediated gall formation, we used TRV-mediates virus-induced gene silencing (VIGS) (Anand et al., 2007) to silence the N. benthamiana ICS1 (SA biosynthetic gene) and coronatine-insensitive1 (COI1, a JA signalling gene) genes. The ICS1 and COI1 silenced N. benthamiana plants were slightly shorter and taller, respectively, when compared to nonsilenced control plants (data not shown). Plants silenced for these genes were treated with whitefly, BTH and water (control) for 7 d, and then immediately inoculated with Agrobacterium, as described above. Before and after Agrobacterium inoculation no significant difference was observed in the same genotype among each treatment (data not shown). A mock control (no TRV inoculation) for VIGS was included. Gall formation was monitored and found to be attenuated in whitefly-infested nonsilenced control plants (Fig. S6).

Gall weights were significantly lower in whitefly- and BTH-treated plants than in water-treated plants and empty-vector (pTRV2::00) controls (Fig. 4a). When the ICS1 gene was silenced, the gall weights from plants infested with whitefly were similar to gall weights from water-treated controls (Fig. 4b), suggesting the requirement of ICS1 for whitefly-mediated plant protection against Agrobacterium. By contrast, gall formation was still attenuated in COI1-silenced plants infested with whitefly (Fig. 4c), suggesting the nonrequirement of COI1 in whitefly-induced defence signalling. Surprisingly, both gene-silenced plants and empty-vector controls produced similar gall weights when treated with BTH (Fig. 4a–c). Taken together, our data suggest that the whitefly-triggered SA-dependent pathway plays a key role in attenuating Agrobacterium-mediated gall formation in plants.

Whitefly infestation impairs gall formation by Agrobacterium on crown tissue

Having examined whether whitefly infestation attenuated gall formation by Agrobacterium on stems (Fig. 1), we next investigated whether activation of defence responses by whitefly infestation on parts of the plant aboveground systemically and negatively affected the formation of galls by Agrobacterium on belowground parts. Three-week-old N. benthamiana plants were infested with whitefly or treated with BTH or water (control) for 7 d and directly injected with A. tumefaciens C58 (OD₆₀₀ = 2) in belowground crown tissue. Crown galls were harvested at 15 and 30 dpi.

Considerably smaller crown galls occurred on whitefly-infested and BTH-treated plants in comparison with water-treated controls (Fig. 5a). Whitefly infestation had a highly significant effect on crown gall weight at 30 dpi; the mean of gall weight from water-treated controls (Fig. 5a). Whitefly infestation had a highly significant effect on crown gall weight at 30 dpi; the mean of gall weight from water-treated controls (Fig. 5a). Whitefly infestation had a highly significant effect on crown gall weight at 30 dpi; the mean of gall weight from water-treated controls (Fig. 5a). Whitefly infestation had a highly significant effect on crown gall weight at 30 dpi; the mean of gall weight from water-treated controls (Fig. 5a). Whitefly infestation had a highly significant effect on crown gall weight at 30 dpi; the mean of gall weight from water-treated controls (Fig. 5a). Whitefly infestation had a highly significant effect on crown gall weight at 30 dpi; the mean of gall weight from water-treated controls (Fig. 5a).
Our data clearly suggest that aboveground whitefly infestation systemically enhanced the basal level of plant defence responses to Agrobacterium infection in belowground tissue.

Whitefly infestation attenuates Agrobacterium transformation efficiency

In order to address how Agrobacterium gall formation was modulated in whitefly-infested plants, we developed a novel in vitro system, summarized in Fig. 6(a). Nicotiana benthamiana seedlings were grown for 3 wk in liquid culture media before being treated with whitefly or BTH or water (control) for a week. In order to verify the accumulation of total SA in root exudates from whitefly-infested plants, we measured total SA concentration in the root exudates from the rhizosphere in whitefly-infested plants. The results showed that the concentration of SA in root exudates was c. 2.5-fold higher in the rhizosphere of whitefly-infested plants than that of the control plants (Fig. 6b). Because SA was endogenously overproduced in root exudates from whitefly-infested plants, the transformation efficiency of Agrobacterium in roots of plants infested with whitefly was examined. Three-week-old N. benthamiana seedlings were treated with whitefly or BTH or water (control) for 7 d and then inoculated with A. tumefaciens (OD600 = 2) at four individual places on the stem. Plants were incubated separately for 30 d post-inoculation (dpi). Letters above the bars indicate a significant difference (P = 0.05) between treatments. Error bars, ± SE of the mean (n = 4).

Discussion

Before this study, no evidence has been shown regarding insect-elicited manipulation of the Agrobacterium-mediated gall disease in plants. In this study, we intriguingly find several novel aspects: ecological stimulus such as insect infestation manipulates the Agrobacterium-mediated gall formation in plants; higher concentration of salicylic acid (SA) induced by whitefly infestation dramatically contributes to plant immunity against Agrobacterium infection and thus transformation; and our study provides a new insight into a three-way interaction amongst insect (whitefly), plant and pathogen (Agrobacterium).

Although there is considerable evidence to support the effects of SA signalling on plant–microbial interactions, only few studies have clearly demonstrated a direct effect of SA on bacterial, especially Agrobacterium, growth and virulence (Yuan et al., 2007, 2008; Anand et al., 2008). The direct effect of SA might depend on exogenous concentration, type of growth media and growth conditions. For example, SA significantly inhibits Agrobacterium growth in acidic minimal media at very low concentrations (5–8 μM) but does not affect growth under neutral conditions (Yuan et al., 2007). Likewise, Anand et al., found that SA inhibited Agrobacterium growth in minimal media, but that low SA concentrations (5–15 μM) did not impede growth in rich media, whereas much higher concentration (200 μM) did (Anand et al.,...
Recently, exogenously applied SA has been shown to inhibit the vir gene mRNA expression (Yuan et al., 2007; Anand et al., 2008). Now, although our data could not convincingly support the reduction in transcript levels of vir genes in root exudates of whitefly-infested plants, we show that SA is endogenously produced in root exudates from whitefly-infested plants (Fig. 6b). Our results suggest that the SA-mediated defence machinery triggered by aboveground whitefly infestation may be involved in attenuating Agrobacterium-mediated plant transformation (Fig. 6c,d). We note that SA is structurally similar to indole-3-acetic acid (IAA) and the phenolic compound, acetosyringone (Liu & Nester, 2006). We speculate that the increased production of SA after whitefly infestation may suppress the transformation strategy by Agrobacterium via an antagonistic effect on acetosyringone. Furthermore, elevated production of IAA has been observed in Arabidopsis (Efetova et al., 2007) and Ricinus communis (Veselov et al., 2003) in response to Agrobacterium infection. We also observed an elevated level of IAA in whitefly-infested plants after Agrobacterium inoculation; this may also affect Agrobacterium virulence (Fig. S8). Unlike SA, endogenous IAA did not accumulate in whitefly-infested plants, but its concentrations increased dramatically in response to Agrobacterium infection. Therefore, we suggest that SA accumulation triggered by whitefly infestation is central to the subsequent reduction in Agrobacterium-mediated virulence, and that the rise in IAA concentrations following Agrobacterium infection acts in conjunction with SA to attenuate gall formation in plants.

It has also been proposed that herbivore-associated molecular patterns (HAMPs) are applicable to plant–insect interactions. Diverse elicitors and HAMPs have been characterized in insect herbivores, including modified lipids such as fatty acid-amino acid conjugates (FACs; Alborn et al., 1997), glucose oxidase (Diezel et al., 2009), beta-glucosidase (Mattiacci et al., 1995), inceptins (Schmelz et al., 2007) from lepidopterans, and sulphur-containing fatty acid caeliferins from a grasshopper (Alborn et al., 2007). More specifically, infestation by silverleaf whitefly, a phloem-feeding insect, causes an upregulation in SA-signalling but not JA/ET-signalling pathways of Arabidopsis (Zarate et al., 2007). This indicates that a nymph effector may enhance SA production locally and systemically; a precedent for such an idea is the example of insect effectors modulating nicotine production and volatile biosynthesis (Musser et al., 2002; Bede et al., 2006). An intriguing finding of the present study is that we show a correlation between whitefly-triggered endogenous SA accumulation and attenuation of Agrobacterium-mediated crown gall disease. We speculate that HAMPs/elicitors/effectors released by whitefly activate SA production and lead to systemically induced resistance in plants. Accumulation of SA in whitefly-infested plants may result in more rapid recognition of Agrobacterium PAMP after infection, resulting in increased resistance and attenuation of crown gall formation by Agrobacterium.

Although the mechanism by which root exudates are secreted is still poorly understood, our knowledge of multitrophic interactions in the rhizosphere mediated by chemicals secreted from roots has recently advanced (Badri et al., 2008). These chemicals are released in root exudates by plants in response to chemical
and physical stimuli (Gleba et al., 1999). Here, we have focused on potential mechanisms by which root exudates from whitefly-infested plants mediate changes in pathogen virulence. We suggest that whitefly infestation triggers production of endogenous SA followed by increased secretion of phytochemicals via root exudates. This suggestion is supported by the fact that exogenous application of SA, methyl jasmonate (MeJA) and nitric oxide (NO) induces both the accumulation of a wide range of secondary metabolites (Zhao et al., 2005) and an increase in root exudation of phytochemicals (Badri & Vivanco, 2009). For example, exogenous SA and MeJA treatments result in the secretion of indole glucosinolates and camalexin by Arabidopsis plants (Badri et al., 2008). Our results suggest that SA accumulated in root exudates in whitefly-infested plants clearly attenuates Agrobacterium-mediated gall disease.

Earlier studies have shown that whitefly infestation inhibits growth of aerial tissues but results in an increase of root biomass in pepper plants (Yang et al., 2011; Park & Ryu, 2014). These physiological aspects of infestation also occur in N. benthamiana (Y. S. Park et al., unpublished). Surprisingly, growth of aerial tissues is promoted in N. benthamiana plants grown in root exudate from whitefly-infested plants (Y. S. Park et al., unpublished), suggesting that whitefly infestation can regulate plant physiology as well as resistance to the Agrobacterium infection. Further studies are needed to elucidate the full role of root exudates from

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**Fig. 6** Effect of root exudates from whitefly-infested plants on Agrobacterium virulence gene expression and transformation efficiency. (a) Schematic representation of the experimental setup. (b) Three-week-old Nicotiana benthamiana plants grown in 0.1× MS liquid media were treated with whitefly or water (control) for 7 d. Endogenous total salicylic acid (SA) production was measured in root exudates from whitefly-infested plants. (c) Visualization of root segments transformed with A. tumefaciens disarmed strain GV2260 carrying the binary vector pBISN1-gusA. (d) Expression levels of gusA in root segments. Significant difference between treatments: *, P = 0.05. Error bars, ± SE of the mean (n = 5).

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**Fig. 7** A model depicting the mechanism by which a sucking insect (whitefly) infestation on leaves attenuates crown gall disease caused by Agrobacterium tumefaciens. (a) A plant under infestation by a sucking insect whitefly in aboveground tissue. (b) Whitefly-infested plant produces endogenous salicylic acid (SA) and elicits plant systemic defence signalling in above- and below-ground tissues. (c) SA released from root exudates negatively affects the transformation efficiency of A. tumefaciens thus reducing the severity of crown gall disease.
whitefly-infested plants in modulating plant physiology and plant defence responses.

In conclusion, although *Agrobacterium*-mediated transformation of plant host genomes has been extensively studied, our understanding of precise plant host defence responses against *Agrobacterium* is still not complete. Here, we show a novel aspect of plant defence whereby infestation with whitefly modulates the virulence and pathogenicity of *Agrobacterium* infection; the results are summarized in Fig. 7. Our findings therefore suggest that whitefly-induced resistance determinants have potential as bio-control agents, enhancing plant immunity to diseases caused by *Agrobacterium* and perhaps other pathogens.

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References


Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Assessment of stem thickness.

Fig. S2 Gall formation by A. tumefaciens in whitefly-infested N. benthamiana stems at 15 dpi.

Fig. S3 Defence-related gene expression in response to whitefly infestation.

Fig. S4 Expression patterns of ethylene related genes in whitefly-infested plants following A. tumefaciens inoculation.

Fig. S5 Quantification of SA and JA concentrations after whitefly infestation.

Fig. S6 A. tumefaciens-mediated gall formation as no VIGS control in whitefly-infested N. benthamiana stems.

Fig. S7 Examination of crown gall formation by Agrobacterium inoculation at 15 dpi.

Fig. S8 Quantification of endogenous indole-3-acetic acid (IAA) in whitefly-infested plants in response to A. tumefaciens inoculation.

Table S1 Primers used for qRT-PCR analyses

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