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Begomovirus–whitefly mutualism is achieved through repression of plant defences by a virus pathogenicity factor

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Abstract

Plant-mediated interactions between herbivorous arthropods and pathogens transmitted by herbivores are important determinants of the population dynamics of both types of organisms in the field. The role of plant defence in mediating these types of tripartite interactions have been recognized but rarely examined especially at the physiological and molecular levels. Our previous work shows that a worldwide invasive whitefly can establish mutualism with the begomovirus *Tomato yellow leaf curl China virus* (TYLCCNV) via crop plants. Here, we show that TYLCCNV and betasatellite co-infection suppresses jasmonic acid defences in the plant. Impairing or enhancing defences mediated by jasmonic acid in the plant enhances or depresses the performance of the whitefly. We further demonstrate that the pathogenicity factor βC1 encoded in the betasatellite is responsible for the initiation of suppression on plant defences and contributes to the realization of the virus–vector mutualism. By integrating ecological, mechanistic and molecular approaches, our study reveals a major mechanism of the plant-mediated mutualism between a virus and its vector. As the test plant is an important economic crop, the results also have substantial implications for developing novel strategies for management of crop viruses and the insect vectors associated with them.

Keywords: *Begomovirus*, *Bemisia tabaci*, jasmonic acid, pathogenicity factor, virus–vector–plant interactions

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Introduction

Plants in managed and natural ecosystems are frequently attacked by both insect pests and plant pathogens transmitted by the insects. Plant-mediated interactions between pathogens and insect vectors are known to exert important influences on the abundance of herbivores and the epidemiology of plant diseases (Colvin *et al.* 2006; Stout *et al.* 2006; Mauck *et al.* 2010). These tripartite interactions of plant–pathogen–vector relationships are complex and are often assumed to be mediated by plant defences (Belliure *et al.* 2005; Colvin *et al.* 2006; Stout *et al.* 2006; Jiu *et al.* 2007). In the past, ecological and molecular approaches often have been used independently to study tripartite interactions (Paul *et al.* 2000; Hatcher *et al.* 2004). Although in recent years molecular biologists and ecologists have joined forces to reveal the interactions between plants and herbivores as well as those between plants and pathogens (Glazebrook 2005; Kaloshian & Walling 2005; Bruce & Pickett 2007; Kempema *et al.* 2007; Spoel & Dong 2008), we are still at the pioneering phase in unravelling the plant-mediated interactions between pathogenic microorganisms and herbivorous arthropods, especially when the herbivores are also pathogen vectors (Stout *et al.* 2006; Pieterse & Dicke 2007).

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Salicylic acid (SA) and jasmonic acid (JA) are two important components of signal transduction cascades regulating plant defence responses against biotic stresses. In general, SA mediates the plant resistance to biotrophic pathogens, whereas JA controls the plant resistance to nectrotrophic pathogens, tissue-damaging insects and some phloem feeding insects (Felton & Korth 2000; Bruce & Pickett 2007; Kempema *et al.* 2007; Pieterse & Dicke 2007; Zarate *et al.* 2007). Crosstalk between SA and JA signalling pathways may mediate the reciprocal effects of induced plant defences on pathogens and herbivores (Preston *et al.* 1999; Thaler *et al.* 1999, 2010; Felton & Korth 2000; Glazebrook 2005; Koornneef & Pieterse 2008). However, this crosstalk does not necessarily always occur during plant–pathogen–herbivore interactions. For instance, TMV induces SA but does not affect JA in tomato plants, and its infection results in increased susceptibility to a caterpillar *Spodoptera exigua* and resistance to an aphid *Myzus persicae* (Thaler *et al.* 2010). Despite these advances, our knowledge about the role of SA and JA defence pathways in regulating plant–pathogen–herbivore interactions is limited, especially when the herbivore also serves as the pathogen vector.

The *Begomovirus* genus in the family *Geminiviridae* contains the largest and most economically important group of plant viruses in tropical and subtropical agroecosystems worldwide (Moffat 1999; Mansoor *et al.* 2003). Many begomoviruses have bipartite genomes, referred to as DNA-A and DNA-B, but some species have only a single genomic component that resembles DNA-A, and an increasing number of monopartite begomoviruses have been found to be associated with betasatellites (formerly DNAβ). In many cases, betasatellites are required for symptom induction in helper begomoviruses–infected hosts (Mansoor *et al.* 2003; Cui *et al.* 2004). More than 200 species of begomoviruses can be exclusively transmitted by the whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) (Hogenhout *et al.* 2008; Nawaz-ul-Rehman & Fauquet 2009), which is a cryptic species complex composed of at least 28 morphologically indistinguishable species (De Barro *et al.* 2011; Hu *et al.* 2011). In this whitefly species complex, the Middle East-Asia Minor 1 species, commonly referred to as the B 'biotype' (herein we use 'B whitefly' to refer to this species to facilitate linking of this study with literature), has risen to international prominence since the 1980s because of its global spreading (Brown *et al.* 1995; Oliveira *et al.* 2001; Liu *et al.* 2007). The B whitefly causes severe crop losses through direct feeding as well as transmission of begomoviruses (Brown *et al.* 1995; Oliveira *et al.* 2001; Varma & Malathi 2003; Colvin *et al.* 2006; Jiu *et al.* 2007). Thus, plant–begomovirus–whitefly interactions have received increasing attention in recent years, and they may serve as a model for investigating plant–pathogen–vector interactions (Colvin *et al.* 2006; Jiu *et al.* 2007; Hogenhout *et al.* 2008; Liu *et al.* 2009, 2010).

Begomoviruses and whiteflies often establish mutualism via their shared host plants, and this indirect mutualism may contribute to the invasion of whiteflies and disease pandemics of plant viruses (Banks *et al.* 2001; McKenzie 2002; McKenzie *et al.* 2002; Colvin *et al.* 2006; Jiu *et al.* 2007; Liu *et al.* 2010). For example, population increases of B whitefly are accelerated on tobacco plants infected with *Tomato yellow leaf curl China virus* (TYLCCNV) (Jiu *et al.* 2007). However, the physiological and molecular mechanisms underlying this mutualism remain largely unknown, although recent studies using the model plant *Arabidopsis* have offered some hints that plant-mediated SA and JA defence pathways may be involved (Zarate *et al.* 2007; Yang *et al.* 2008).

Here, we attempt to examine mechanisms of the plant-mediated mutualism between B whiteflies and the plant pathogen TYLCCNV via an integration of ecological, mechanistic and molecular approaches. We found that the betasatellite of TYLCCNV (TYLCCNB) plays an essential role in initiating the interaction and that co-infection of TYLCCNV and its betasatellite can repress tobacco JA-mediated defences against the whitefly, thereby accelerating its population increase. Further, we revealed that the βC1 protein encoded by TYLCCNB suppresses JA responses and contributes to the improved performance of whiteflies on virus-infected plants.

Materials and methods

Whiteflies and plants

The B whitefly (mtCO1 GenBank accession no: GQ332577) of the *B. tabaci* species complex was maintained on cotton plants (*Gossypium hirsutum* cv. ZheMian 1793) in climate chambers at 27 ± 1 °C, a photoperiod of 14 h light–10 h darkness and 70 ± 10% relative humidity. The purity of the culture was monitored every 3–5 generations using the random amplified polymorphic DNA polymerase chain reaction technique combined with the sequencing of mtCO1 gene, which has been used widely to differentiate *B. tabaci* genetic groups (De Barro *et al.* 2011). Cotton and tobacco plants (*Nicotiana tabacum* cv. NC89) were grown in a greenhouse under natural lighting and controlled temperature at 25 ± 3 °C. Cotton plants were cultivated to the 6–7th true-leaf stage for rearing whiteflies, and tobacco plants were cultivated to the 2–3rd true-leaf stage for virus inoculation and 6–7th true-leaf stage for further experiments.

Construction and inoculation of infectious clones of TYLCCNV, TYLCCNB and TYLCCNB-M

Infectious clones of TYLCCNV, TYLCCNB and TYLCCNB-M were constructed as described previously (Cui *et al.* 2004). To obtain virus-infected tobacco, plants at the 2–3rd true-leaf stage were inoculated with TYLCCNV, TYLCCNV+TYLCCNB or TYLCCNV+TYLCCNB-M by agroinoculation as described previously (Cui *et al.* 2004). Control plants were mock-inoculated with the *Agrobacterium tumefaciens* strain EHA105. All plants were grown in a greenhouse under natural lighting and controlled temperature at 25 ± 3 °C. Virus infection of test plants was judged by the typical symptomatology and further confirmed by PCR using the procedure described previously (Qian & Zhou 2005). Inoculated plants were used for further experiments when they reached the 6–7th true-leaf stage (approximately 25 days after agroinoculation of the virus).

RNA isolation and quantitative real-time PCR (qRT–PCR) analysis

Leaves from three replicate plants in each treatment were sampled. Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and treated with RNase-free DNaseI (Takara, Dalian, China). RNA purity and quantity were determined using Nanodrop (Nanodrop Technologies). First-strand cDNA was synthesized from total RNA using the oligo(dT) primer and reverse transcriptase (Takara). qRT–PCRs were carried out in a LightCycler® 480 real-time PCR instrument (Roche, Rotkreuz, Switzerlanad) using the LightCycler® 480 SYBR Green I Master kit (Roche) as described previously (Huang *et al.* 2009). Three replicates for each treatment were conducted, and three technical qRT–PCR replicates were analysed for each biological replicate. The GAPDH gene was used as reference. Primers for qRT–PCRs are shown in Table S1.

SA and JA analysis

Twenty-five days after tobacco plants were inoculated with EHA105, TYLCCNV, TYLCCNV+TYLCCNB or TYLCCNV+TYLCCNB-M, leaves in three replicate plants for each treatment were sampled. The leaf materials from each plant were flash-frozen in liquid N_2, weighed and stored at −80 °C until SA and JA analysis. SA and JA were extracted for GC-MS analysis using labelled internal standards (328 ng D3-JA, kindly supplied by Ian T. Baldwin, Max Planck Institute of Chemical Ecology, Jena, Germany, and 345 ng D6-SA, Cambridge Isotope Laboratory, Cambridge, MA, USA) as described previously (Heidel & Baldwin 2004; Lou & Baldwin 2006).

VIGS silencing assays

Approximately 300-bp DNA fragments of the target genes were amplified from tobacco plant cDNA with specific primer pairs shown in Table S1. The resulting PCR products were cloned into *Xba*I-*BamH*I-digested pBIN2mDNA1 plasmid to generate the gene-silencing vector 2mDNA1-target genes (Huang *et al.* 2009), which were mobilized into *A. tumefaciens* strain EHA105 by electroporation. Tobacco curly shoot virus (TbCSV) was used as a helper virus in the VIGS assay, and *A. tumefaciens* cultures carrying TbCSV and 2mDNA1-target genes (OD600 = 0.6 for each construct) were co-infiltrated into tobacco plants. Control plants were inoculated with *A. tumefaciens* cultures carrying TbCSV and 2mDNA1 without any gene insert (empty vector). All plants were grown in a greenhouse under natural lighting and controlled temperature at 25 ± 3 °C and cultivated to the 6–7th true-leaf stage when used in experiments. Total RNA was isolated in gene-silenced plants approximately 25 days after inoculation, and the efficiency of silencing was measured using qRT–PCR.

Performance of whiteflies on MeJA-treated plants

For each plant, 300 μg of MeJA in 20 μL of lanolin paste was applied to the stem 3 days before plants were exposed to whiteflies following protocols described previously (Lou & Baldwin 2006). Control plants were treated with the lanolin paste alone. Eleven replicates were conducted for each of two treatments, and for each replicate, newly emerged whiteflies adults (10 females and 10 males) were released onto the lower surface of a plant leaf (third leaf from the top) enclosed in a clip cage. We observed adult survival and counted the eggs laid by adults on treated and control plants 1 week after release of whiteflies.

Generation of transgenic tobacco plants

To generate Nt*COI1*-overexpressing tobacco plants, full-length cDNA of Nt*COI1* was amplified by PCR using PrimeSTAR® HS DNA Polymerase (Takara) with the primer pair oe*COI1*F/oe*COI1*R and cloned into a pGEM T-Vector (Promega, Madison, WI, USA) to obtain pGEM-Nt*COI1*. Then, the *Sac*I/*Pst*I fragment of pGEM-Nt*COI1* was inserted into the *Sac*I/*Pst*I site of the binary vector pCHF3 to obtain a pCHF3-*COI1*-overexpression vector in which the Nt*COI1* gene was expressed under the control of the CaMV 35S promoter (Cai *et al.* 2007). The empty vector pCHF3 was used as a control.

To generate Nt*COI1*-RNAi tobacco plants, two 364-bp DNA fragments of Nt*COI1* were amplified from plasmid pGEM-Nt*COI*1 using the primer pairs i*COI1*F1/i*COI1*R1 and i*COI1*F2/i*COI1*R2. The latter DNA fragment was ligated with an intron of the RTM1 gene from *Arabidopsis* using overlap extension PCR. The two different length fragments were cloned into the pMD-18T vector (Takara) to obtain pMD-F-intron and pMD-R. The *Sac*I/*Xho*I fragment of pMD-F-intron was inserted into *Sac*I/*Xho*I-digested pMD-R in the reverse orientation to generate pMD-i*COI1*. The *Sac*I/*Pst*I fragment of pMD-i*COI1* was inserted into the *Sac*I/*Pst*I site of binary vector pCHF3 to obtain pCHF3-*COI1*-RNAi in which the hairpin was expressed to interfere with the expression of the *COI1* gene. The empty vector pCHF3 was used to generate a control.

For the 35S: HA-βC1 construct, the βC1 gene (381 nucleotides) was amplified by PCR from TYLCCNV+TYLCCNB infectious clone with the primer pair C1F/C1R, and then a HA tag was added to the N-terminate of βC1 using overlap extension PCR for convenient detection. After digestion with *BamH*I and *Sal*I, the PCR fragment was cloned between a duplicated 35S promoter and the nopaline synthase terminator in the expression vector pBin438 (Cui *et al*. 2005). The HA tag was also amplified and cloned into pBin438 to obtain a control 35S: HA.

All the expression vectors were sequenced to confirm the fidelity of the inserts and then introduced into *A. tumefaciens* strain EHA105 by electroshock. The derived *A. tumefaciens* strains were then used to transform leaf explants of tobacco plants as described previously (Cui *et al*. 2004). The presence of the *COI1*-overexpressing, *COI1*-RNAi, pCHF3, 35S:HA-βC1 and 35S:HA transgenes in tobacco plants was determined by PCR and Southern blot analysis as described previously (Cui *et al*. 2004). The efficiency of overexpression and RNAi of *COI1* was detected by qRT–PCR. Primers for construction and detection were shown in Table S1. All transgenic tobacco plants were grown in greenhouses under natural lighting and controlled temperature at 25 ± 3 °C and were cultivated to the 6-7th true-leaf stage when used in experiments.

Whitefly population experiments

Five whitefly population experiments were conducted to evaluate the effects of tobacco plant treatments on whitefly population increases. (i) To investigate inoculation of plants with EHA105, TYLCCNV or TYLCCNV+TYLCCNB, eight replicates were used for each of the three treatments. (ii) To investigate whitefly population increases on *COI1*-, *FAD3*-, *BGL2*- and *NPR1*-silenced, and empty vector–inoculated plants, ten replicates were used for each of the five treatments. To examine whether the 2mDNA1 vector could affect whitefly performance, population increases of whiteflies on healthy plants and plants inoculated with empty vector as well as those mock-inoculated with EHA105 were compared, and each treatment was replicated nine times. (iii) To assess whitefly population increases on control pCHF3 transgenic plants, *COI1*-overexpressing transgenic plants and *COI1*-RNAi transgenic plants, eight replicates were used for each of the three treatments. (iv) For whitefly increases on EHA105- and TYLCCNV+TYLCCNB-M-inoculated plants, nine replicates were used for each of the two treatments. (v) For population increases of whiteflies on control 35S:HA transgenic plants, and 35S:HA-βC1 transgenic plants with mild symptoms, six replicates were used for each of two treatments.

To initiate the experiments, ten females and ten males of newly emerged adult whiteflies were released onto one plant in a whitefly-proof, ventilated cage (120 mesh) for each replicate in a climatic room at 27 ± 1 °C, a photoperiod of 14 h light–10 h darkness and 70 ± 10% relative humidity. Plants were sampled on the 28th day after whitefly adult release, and individuals in all developmental stages were counted in each of the replicates.

Data analysis

Data in all figures are shown as mean ± SEM. Statistical significance was evaluated using one-way ANOVA at a 0.05 level followed by Bonferroni tests (for the different developmental stages of whiteflies) or LSD tests (for the gene expression and hormone level). Data in percentages were transformed by arcsine square root before analysis. All data analysis was conducted using the software STATISTICA 6.1 (StatSoft, Inc.).

Results

Betasatellite is necessary for acceleration of whitefly population increase

To determine which viral DNA component accounts for the improved performance of whiteflies on begomovirus-infected tobacco plants (*Nicotiana tabacum* cv. NC89) reported previously (Jiu *et al*. 2007), we compared the population increases of whiteflies on tobacco plants infected with either TYLCCNV or TYLCCNV+TYLCCNB and *Agrobacterium tumefaciens* strain EHA105-infiltrated plants. Four weeks after the release of whiteflies onto the plants, the whitefly (adults and all nymphal stages) numbers per plant on TYLCCNV+TYLCCNB-co-infected plants were significantly higher than those on

TYLCCNV-infected ($F_{1,14}$ = 4.0–13.78, P = 0.0002-0.025) or control plants ($F_{1,14}$ = 3.79–16.58, P = 0.0004–0.028) (Fig. 1). In contrast, no significant differences were observed between TYLCCNV-infected and control plants (Fig. 1; $F_{1,14}$ = 0.009–1.32, P = 0.656–0.948). Thus, TYLCCNV+TYLCCNB co-infection, but not TYLCCNV infection alone, allowed a more rapid whitefly population increase.

Co-infection of betasatellite with TYLCCNV represses JA defence but does not affect SA defence

To find out whether TYLCCNV and TYLCCNB influences the plant defence response, we compared the expression of SA and JA biosynthesis and defence marker genes in *A. tumefaciens* EHA105-infiltrated, TYLCCNV-infected and TYLCCNV+TYLCCNB-co-infected tobacco plants using quantitative real-time PCR (qRT–PCR). The transcript level for the SA-regulated gene *BGL2* was increased ($F_{1,4}$ = 30.75, P = 0.005), but in contrast, the transcripts for JA-biosynthesis genes *FAD3* and *FAD7*, as well as the JA-regulated gene *PDF1.2*, were repressed in TYLCCNV+TYLCCNB-co-infected leaves when compared with EHA105-infiltrated leaves ($F_{1,4}$ = 55.46–110.93, P = 0.0005–0.002) or TYLCCNV-infected leaves ($F_{1,4}$ = 15.96–490.78, P = 0.00003–0.016); and in addition, the transcript levels of all the genes we tested remain unchanged in TYLCCNV-infected leaves when compared with EHA105-infiltrated leaves (Fig. 2a,b; $F_{1,4}$ = 0.15–1.36, P = 0.308–0.716 and $F_{1,4}$ = 0.17–6.81, P = 0.06–0.698 for SA- and JA-pathway genes, respectively). Because the phenylalanine pathway has been demonstrated to be the main route of SA biosynthesis in *N. tabacum* (Ogawa *et al.* 2006), we tested

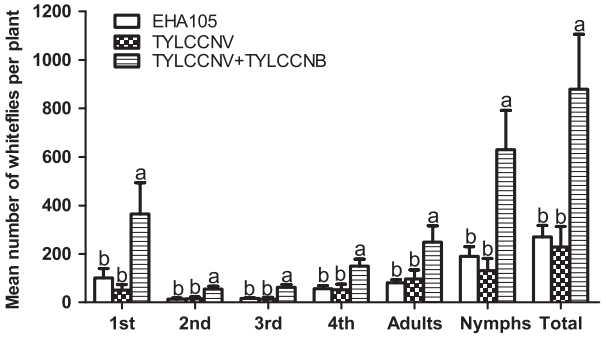

Fig. 1 Role of the TYLCCNV betasatellite in allowing more rapid population increases of whiteflies on tobacco plants. By the 28th day after whitefly adults were released onto the plants, the mean numbers of all individuals and each developmental stage of the whiteflies per plant on EHA105-infiltrated plants, TYLCCNV-infected plants and TYLCCNV+TYLCCNB-co-infected plants are shown. The error bars represent the SEM, and different letters above the bars indicate significant differences (P < 0.05, one-way ANOVA).

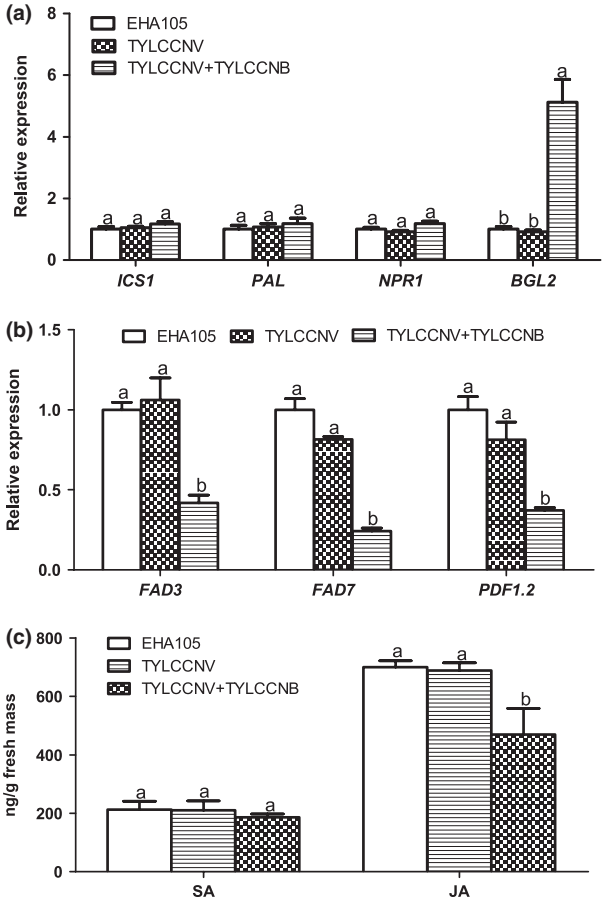

Fig. 2 Effect of TYLCCNV+TYLCCNB co-infection on the Salicylic acid (SA) and jasmonic acid (JA) defence in tobacco plants. (a, b) The transcript levels of SA (a) and JA (b) defence genes in EHA105-infected, TYLCCNV-infected, and TYLCCNV+TYLCCNB-co-infected plants were compared using qRT–PCR. (c) The SA level and JA levels in leaves of EHA105-infiltrated, TYLCCNV-infected and TYLCCNV+TYLCCNB-co-infected plants were analysed using GC-MS. The error bars represent SEM, and different letters above bars indicate significant differences (P < 0.05, one-way ANOVA).

the transcript levels of *PAL*, the key gene in this pathway. The abundance of the gene did not change after co-infection of TYLCCNV+TYLCCNB (Fig. 2a). Hence, the SA levels are unlikely to be altered in TYLCCNV+TYLCCNB-co-infected tobacco plants. In contrast, the transcripts for JA-biosynthesis genes *FAD3* and *FAD7* were down-regulated in TYLCCNV+TYLCCNB-co-infected tobacco plants (Fig. 2b), indicating the likelihood that the JA level is reduced in virus-infected plants.

To examine whether regulation of plant defence genes by virus infection influences the SA and JA production in the leaves, we measured the SA and JA levels in TYLCCNV- and TYLCCNV+TYLCCNB-infected tobacco plants, as well as in control plants, using GC-MS. While SA levels were similar in the three

treatments ($F_{2,6}$ = 0.38, P = 0.705), the JA level was reduced by approximately one-third in TYLCCNV+TYLCCNB-co-infected plants as compared with TYLCCNV-infected plants ($F_{1,4}$ = 5.63, P = 0.03) or controls ($F_{1,8}$ = 6.37, P = 0.025); and in addition, the JA level remained unchanged in TYLCCNV-infected plants when compared with mock-inoculated control plants ($F_{1,8}$ = 0.1, P = 0.891) (Fig. 2c). These data demonstrate that TYLCCNV+TYLCCNB co-infection does not influence the SA titre but represses the JA titre in tobacco plants.

Repression of JA responses allows more rapid whitefly population increases

To test the roles of JA and SA signalling pathways in the tobacco defence against whiteflies, we silenced the JA-defence pathway genes *FAD3* and *COI1* as well as SA defence pathway genes *BGL2* and *NPR1* in tobacco plants using the VIGS silencing vectors 2mDNA1 and examined population increase in whitefly on these silenced plants. The silencing vector itself does not influence the performance of whiteflies on tobacco plants (Fig. S1, Supporting Information; $F_{2,24}$ = 0.23, P = 0.8). By the 28th day after whitefly adults were released onto the plants, the mean numbers of whiteflies per plant were significantly higher on *COI1*- and *FAD3*-silenced plants than on control plants ($F_{1,18}$ = 5.15–14.82, P = 0.000002–0.019 and $F_{1,18}$ = 3.12–9.29, P = 0.002–0.048 for all individuals, adults and several nymphal stages on *COI1*-silenced plants and *FAD3*-silenced plants, respectively). In contrast, the mean numbers of whiteflies at all developmental stages on *BGL2*- and *NPR1*-silenced plants remained unchanged as compared to those on control plants ($F_{1,18}$ = 0.004–0.74, P = 0.489–0.972 and $F_{1,18}$ = 0.000009–2.12, P = 0.255–0.963 for all developmental stages on *BGL2*-silenced plants and *NPR*-silenced plants, respectively) (Fig. 3a). In parallel, we monitored the expression levels of *COI1*, *FAD3*, *BGL2* and *NPR1* transcripts in silenced plants to assess the silencing efficiency. The qRT–PCR analysis showed that transcript levels of these genes are lower in silenced plants compared with those in control plants (Fig. S2a, Supporting Information; $F_{1,4}$ = 41.26–292.82, P = 0.00007–0.003).

Because *COI1* is an important gene involved in JA-defence signalling, we produced *COI1*-RNAi and *COI1*-overexpressing transgenic tobacco plants and assessed whitefly population increases on *COI1* transgenic plants. By the 28th day after whitefly adults were released onto the plants, the mean whitefly number per plant on *COI1*-RNAi plants was significantly higher than that on control plants, and these differences were also evident for the developmental stages from the 2nd instar nymphs to adults ($F_{1,14}$ = 4.13–10.16, P = 0.002–0.033). In contrast, the mean numbers of adults and all individuals of whiteflies per plant on *COI1*-overexpressing plants were significantly lower than those on control plants (Fig. 3b; $F_{1,14}$ = 6.28–12.16, P = 0.003-0.046). In

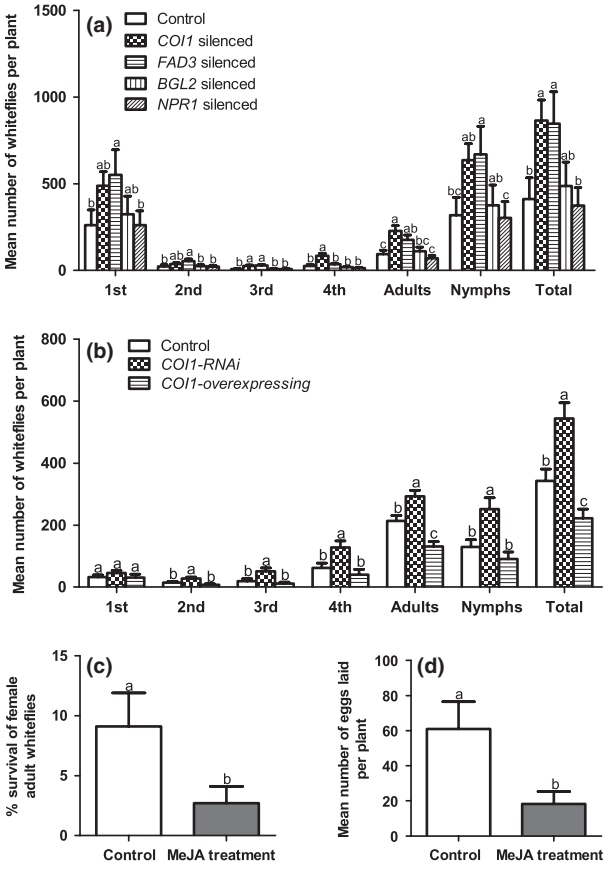

Fig. 3 Effect of regulation of jasmonic acid (JA) and Salicylic acid (SA) defence in tobacco plants on the performance of whiteflies. (a) The population increases of whiteflies on JA- and SA-responsive genes silenced tobacco plants. By the 28th day after whitefly adults were released onto the plants, the mean numbers of all individuals and each developmental stage of the whitefly per plant on *COI1*-, *FAD3*-, *BGL2*- and *NPR1*-silenced tobacco plants as well as 2mDNA1 vector-inoculated control plants were counted and compared. The 2mDNA1 vector does not affect the whitefly performance on tobacco plants (see Fig. S1, Supporting Information). (b) The whitefly population increase on JA-pathway gene *COI1*-RNAi and *COI1*-overexpressing transgenic tobacco plants. By the 28th day after whitefly adults were released onto the plants, the mean numbers of all individuals and developmental stages of the whitefly per plant on *COI1*-RNAi plants, *COI1*-overexpressing plants and control pCHF3 transgenic plants were counted and compared. (c, d) The performance of adult whiteflies on MeJA-treated tobacco plants. One week after whitefly feeding, the percentages of survival of the adults (c) and the number of eggs laid by female whiteflies (d) on MeJA-treated plants and lanolin paste–treated control plants were compared. The error bars represent SEM, and different letters above bars indicate significant differences (P < 0.05, one-way ANOVA).

parallel, we monitored the expression level of *COI1* in the transgenic plants to assess the transgene efficiency. The qRT–PCR analysis showed that *COI1* levels were lower in *COI1*-RNAi tobacco plants ($F_{1,4}$ = 340.06, P < 0.00001) and higher in *COI1*-overexpressing plants ($F_{1,4}$ = 7.87, P = 0.049) when compared with control plants (Fig. S2b, Supporting Information). Collectively, these data demonstrate that repressed JA but not SA responses compromised the resistance of tobacco to whiteflies, suggesting the importance of JA-signalling pathway in tobacco plant defences against whiteflies.

To further confirm the role of JA-regulated defences in whitefly resistance, tobacco plants were treated with MeJA and the performance of adult female whiteflies was examined. One week after whitefly feeding, the percentage of survival and fecundity of adult female whiteflies on MeJA-treated plants was reduced to approximately one-third of that on control plants (Fig. 3c,d; $F_{1,20}$ = 5.23, P = 0.033 and $F_{1,22}$ = 6.26, P = 0.021 for the percentage of survival and fecundity, respectively). These data further confirm the importance of JA-regulated defences in tobacco resistance to the whitefly.

βC1 suppresses JA responses and contributes to whitefly population increase on virus-infected plants

The results obtained above suggest that TYLCCNB has a key role in the regulation of plant defences. Because TYLCCNB only encodes the βC1 protein, we speculated that βC1 may regulate plant defences. To test this hypothesis, the mutant TYLCCNB (TYLCCNB-M), in which the start codon of the βC1 ORF was mutated (Cui *et al.* 2004), was inoculated into tobacco plants together with TYLCCNV. When JA- and SA-regulated transcripts and the levels of JA and SA were analysed, no significant differences were detected in the TYLCCNV+TYLCCNB-M-infected plants when compared with those in the mock-inoculated control plants (Fig. 4a–c; $F_{1,4}$ = 0.78–3.56, P = 0.132–0.427 and $F_{1,4}$ = 0.01–1.14, P = 0.345–0.938 for JA-regulated genes and SA-regulated genes, respectively; $F_{1,8}$ = 0.04, P = 0.845 and $F_{1,8}$ = 0.04, P = 0.847 for JA level and SA level, respectively). These results thus contribute to the notion that βC1 functions in JA suppression.

To confirm the role of βC1 in the regulation of defences, we produced βC1 transgenic tobacco plants and then examined the transcripts of JA- and SA-regulated genes in these transgenic plants. When the transgenic plants were cultivated to the 6–7 leaf stage, the severity of disease symptoms varied. Some transgenic plants had severe symptoms characterized by extensive stunting, leaf cupping and leaf curling, and these symptoms were much more severe than those in wild-type plants infected with TYLCCNV+TYLCCNB. The severe symptoms probably occurred because the CaMV 35S promoter used to generate the βC1

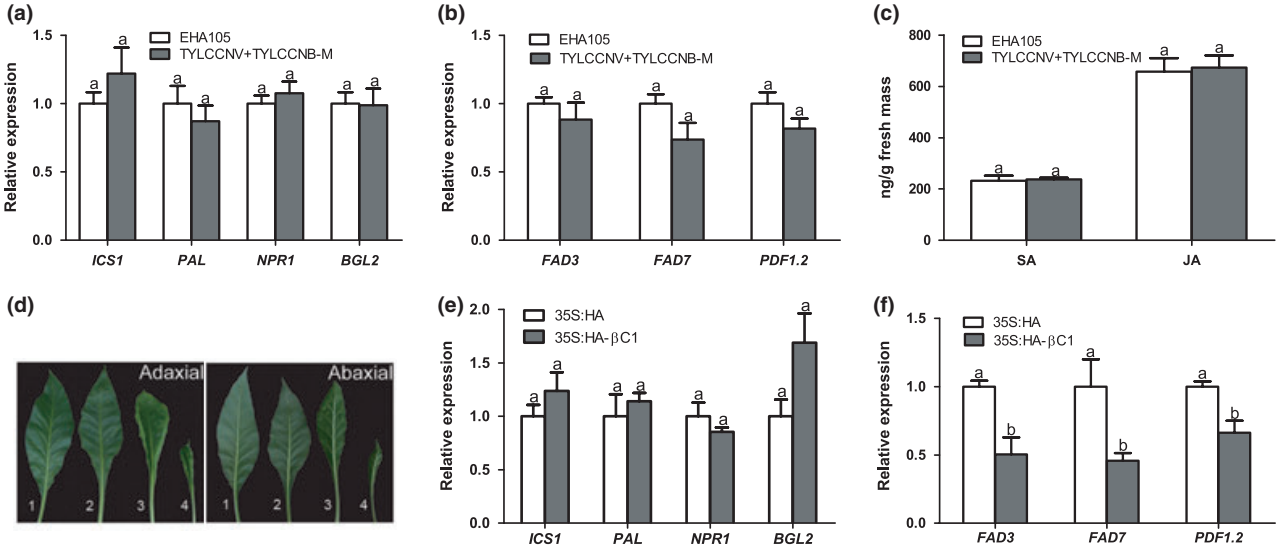

Fig. 4 Effects of βC1 encoded by the betasatellite on the Salicylic acid (SA) and jasmonic acid (JA) defences of tobacco plants. (a–c) The transcript levels of SA (a) and JA (b) defence genes and the SA and JA levels (c) in EHA105-infiltrated plants and plants co-infected by TYLCCNV with mutant TYLCCNB (TYLCCNB-M), in which the start codon of the βC1 ORF was mutated. (d) Comparison of symptoms of transgenic and TYLCCNV+TYLCCNB-inoculated tobacco plants. 35S:HA transgenic plants (1), TYLCCNV+TYLCCNB-inoculated plants (2), and 35S:HA-βC1 transgenic plants with mild (3) or severe (4) symptoms. (e, f) The transcript levels for SA- and JA-defensive genes in the 35S:HA-βC1 transgenic tobacco plants with mild symptoms and the control 35S:HA transgenic plants were compared. The error bars represent SEM, and different letters above bars indicate significant differences (P < 0.05, one-way ANOVA).

transgenic plants is expressed constitutively whereas the βC1 promoter in TYLCCNB is expressed specifically in the phloem (Guan & Zhou 2006). Such extremely severe symptoms may inhibit the feeding and oviposition of whiteflies on the leaf surface. In contrast, other transgenic plants had mild symptoms that were comparable to those of TYLCCNV+TYLCCNB-co-infected plants (Fig. 4d). Therefore, transgenic plants with mild symptoms were used in the following experiments. qRT–PCR analysis showed that the transcript levels of the SA defence pathway genes *ICS1*, *PAL*, *NPR1* and *BGL2* remained unchanged in the βC1 transgenic plants when compared with those in the control plants (Fig. 4e; $F_{1,4}$ = 0.4–4.79, P = 0.094–0.561). In contrast, the transcripts for JA-biosynthesis genes *FAD3* and *FAD7*, as well as JA-regulated gene *PDF1.2*, were down-regulated in the βC1 transgenic plants when compared with those in the control plants (Fig. 4f; $F_{1,4}$ = 6.79–14.13, P = 0.02–0.05). These data demonstrate that βC1 suppresses the JA responses in tobacco plants.

To further evaluate the role of βC1 in affecting whitefly performance, whitefly population increases were analysed on TYLCCNV+TYLCCNB-M-infected tobacco plants. By the 28th day after whitefly adults were released onto the plants, the mean numbers of whiteflies per plant on TYLCCNV- and TYLCCNB-M-infected plants were similar to those on control plants (Fig. 5a; $F_{1,16}$ = 0.0004–2.3, P = 0.15–0.985), indicating that βC1 plays an important role in the acceleration of whitefly population increase shown above.

Further, the performance of the whitefly on βC1-transgenic plants and control plants was studied. From the results of whitefly population increase described above, we knew that the mean numbers of whitefly pupae and/or adults per plant can reflect the differences in whitefly population increase on different plants. Thus, here we only counted the numbers of whitefly pupae and adults on each of the test plants. Due to likely effects caused by the generation of transgenic plants, the mean numbers of whitefly pupae and/or adults on control transgenic plants were lower than those on nontransgenic plants seen earlier. Nevertheless, by the 28th day after whitefly adults were released onto the plants, the mean numbers of whitefly pupae and/or adults per plant on the plants with mild symptoms were significantly higher than those on control plants (Fig. 5b; $F_{1,10}$ = 6.02, P = 0.034; $F_{1,10}$ = 5.58, P = 0.04 and $F_{1,10}$ = 6.63, P = 0.028 for pupae, adults and total numbers of pupae and adults, respectively). Thus, the collective data shown in the experiments above provide persuasive evidence that βC1 contributes to the acceleration of whitefly population increases on TYLCCNV+TYLCCNB-co-infected plants.

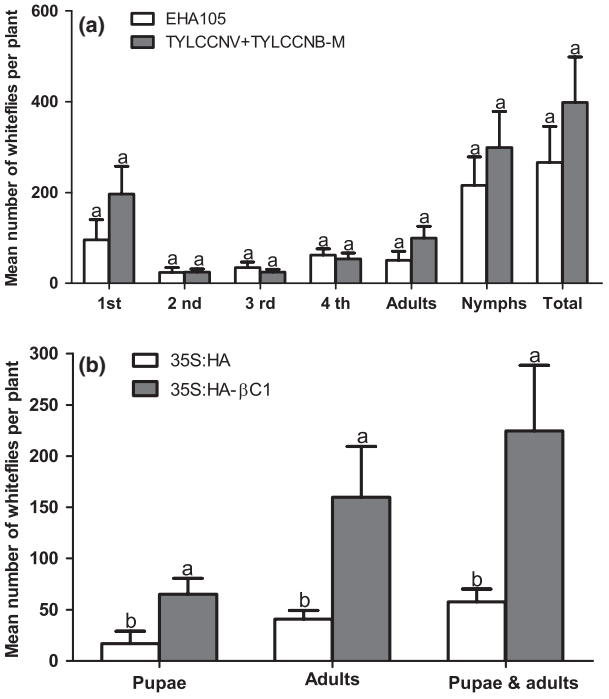

Fig. 5 Effects of βC1 on the performance of whiteflies. (a) Whitefly population increases on tobacco plants co-infected by TYLCCNV and mutant TYLCCNB (TYLCCNB-M). By the 28th day after whitefly adults were released onto the plants, the mean numbers of all individuals and each developmental stage of whiteflies per plant on TYLCCNV+TYLCCNB-M-infected tobacco plants and EHA105-infected plants were counted and compared. (b) Whitefly population increases on βC1 transgenic plants. By the 28th day after whitefly adults were released onto the plants, the mean numbers of pupae and/or whitefly adults per plant on the 35S:HA-βC1 transgenic plants with mild symptoms and on control 35S:HA transgenic plants were counted and compared. The error bars represent SEM, and different letters above bars indicate significant differences (P < 0.05, one-way ANOVA).

Discussion

Plant-mediated mutualism between pathogens and their vectors can contribute significantly to outbreaks of herbivores and epidemics of plant diseases (Belliure *et al.* 2005; Colvin *et al.* 2006; Jiu *et al.* 2007). Our data demonstrate that co-infection of the begomovirus TYLCCNV and its betasatellite can repress JA-regulated defences of tobacco against invasive whiteflies and accelerate population increases of the insects. We further show that the pathogenicity factor βC1 of TYLCCNB is responsible for the suppression of the plant defences and modulates the mutualistic effects during infection. The combined results reveal a major mechanism of the indirect mutualism between TYLCCNV and its vector whitefly at the ecological, physiological and molecular levels. To our knowledge, this is the first demonstration

that integrates ecological, mechanistic and molecular approaches to reveal how a pathogenicity factor of a plant pathogen affects crop plant defences against an insect vector.

Apart from the suppression of JA-mediated defences by TYLCCNV+TYLLCCNB in tobacco plants, TYLCCNV+TYLLCCNB can also repress JA-mediated defences in *Arabidopsis* (Yang *et al.* 2008). Down-regulation of transcripts in JA-pathway has been reported in *Arabidopsis* by infections with begomoviruses such as *Cabbage leaf curl virus* and *Tomato yellow leaf curl Sardinia viru*s (Ascencio-Ibanez *et al.* 2008; Lozano-Durán *et al.* 2011). Thus, it seems likely that begomoviruses affect JA-related defences in many species of plants and that mechanisms similar to those revealed in this study may contribute to increased population densities of B whiteflies on *Tomato mottle virus*-infected plants (McKenzie 2002; McKenzie *et al.* 2002). It is known that the JA pathway regulates the generation of many plant secondary metabolites and defensive proteins that are harmful to herbivores (Pieterse & Dicke 2007). Thus, it would provide further understanding on the mechanisms underlying the begomovirus–whitefly–plant interactions if these plant secondary metabolites or defensive proteins can be identified.

In general, the SA levels remain unchanged in compatible virus–plant interactions; conversely, the SA levels increase during incompatible virus–plant interactions (Malamy *et al.* 1990; Gaffney *et al.* 1993; Ryals *et al.* 1996; Whitham *et al.* 2006). The interaction between TYLCCNV and *N. tabacum* in this study is compatible and indeed TYLCCNV+TYLCCNB co-infection did not induce increased SA levels in the tobacco plants, although co-infection increased the transcript level of the SA-regulated gene *BGL2* probably through regulating other signalling pathways. Thus, the observed suppression of the JA-regulated defences by TYLCCNV+TYLCCNB co-infection is not likely to be caused by induction of SA defences in the tobacco plants. Further, a previous study demonstrated that TYLCCNV+TYLCCNB can suppress selective JA-regulated plant responses through interactions between the pathogenicity factor βC1 and a leaf development regulating factor ASYMMETRIC LEAVES 1 (AS1) in *Arabidopsis* (Yang *et al.* 2008). AS1 acts as a negative regulator of JA response, presumably preventing premature deployment of the defence response and its associated costs. AS1 is orthologous to *NSPHAN* in tobacco plants (Nurmberg *et al.* 2007). Hence, we speculate that TYLCCVB βC1 may bind to *NSPHAN* and then repress the JA-regulated defences in *N. tabacum* through a SA-independent mechanism. Likewise, the whitefly can affect JA-regulated defences in Lima bean rather than through the SA pathway (Zhang *et al.* 2009).

Case studies available so far indicate that mutualistic relationships between plant pathogens and insect vectors are more likely to occur in plants with a low level of constitutive suitability to herbivores before infection with pathogens (Belliure *et al.* 2005; Jiu *et al.* 2007; Liu *et al.* 2010). When these plants are eventually infected by pathogens, repression of plant defences may improve the suitability of plants for herbivore development. This in turn increases the numbers of insect vectors and results in increased acquisition and transmission of pathogens. One may speculate that plants that can support rapid increases in insect vector populations already have repressed or inactive defences. Hence in these cases, infections with plant pathogens may result in only limited effects on the rates of insect multiplication and thus may not have obvious effects on vector and pathogen ecology. For example, B whiteflies exhibit similar rates of population increases on TYLCCNV-infected and healthy tomato plants, which support rapid population increases of the B whiteflies in the absence of begomovirus infections (Liu *et al.* 2009). Experiments to compare these phenomena in other plants may provide a useful test of the generality of this phenomenon.

In summary, our findings reveal that a begomovirus pathogenicity factor can suppress the plant defences and provide a mechanism whereby reduced defences result in increased whitefly vector multiplication. Our study also provides an interesting model to study the plant–pathogen–vector interactions through an integration of ecological, physiological and molecular approaches. The indirect mutualism between a virus and its vectors may potentiate damages to the host plant. The selection pressure from both viruses and their vectors also may accelerate the evolution of plant defences. In the future, efforts will be warranted to determine crosstalk roles that individual defence signalling pathways have on various classes of metabolites or toxic proteins involved in tripartite interactions (Felton & Korth 2000; Stout *et al.* 2006; Bruce & Pickett 2007). Efforts in this direction are likely to provide new insights into how plants fine tune their defences to cope with multiple enemies, and in turn may lead to development of novel strategies for management of insect and microbial pests (Felton & Korth 2000; Stout *et al.* 2006; Dicke *et al.* 2009; Pieterse *et al.* 2009). Moreover, with new information on the transcriptome and genomes of insect vectors (e.g. Wang *et al.* 2010, 2011), novel research approaches may be conducted to examine how insect vectors respond to changes in the physiology of virus-infected plants and to invasion by plant viruses (e.g. Luan *et al.* 2011) and eventually facilitate a more detailed molecular basis for our understanding on plant–pathogen–vector interactions.

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Data accessibility

Oligonucleotides used for qRT–PCR and construction: Table S1 of the Supporting Information. Complete sample data for Figures 1–5 and S1–S2 are uploaded as an Excel file named as 'Appendix S1' in Supporting Information.

Supporting information

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

Table S1 Oligonucleotides used for qRT-PCR and construction.

Fig. S1 Inoculation of 2mDNA1 empty vector does not affect the performance of whiteflies on tobacco plants.

Fig. S2 2mDNA1 vector-induced gene silencing and *COI1* transgenes show high efficiency.

Appendix S1 Complete sample data for Figures 1–5 and S1–S2.

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