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SPECIAL FEATURE

PLANT-MEDIATED INTERACTIONS BETWEEN ABOVE- AND BELOW-GROUND COMMUNITIES

Whitefly infestation of pepper plants elicits defence responses against bacterial pathogens in leaves and roots and changes the below-ground microflora

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Summary

1. Upon facing biotic stresses, plants orchestrate defence mechanisms via internal and external mechanisms that are mediated by signalling molecules such as salicylic acid, jasmonic acid, ethylene and various other volatile compounds. Although pathogen- and chemical-induced plant resistance has been studied extensively within the same plant compartment, the effects of above-ground (AG) insect-elicited plant defence on the resistance expression in roots and the below-ground (BG) microbial community are not well understood.

2. We assessed the effect of AG whitefly (*Bemisia tabaci*) attack on the elicitation of induced resistance against a leaf pathogen, *Xanthomonas axonopodis* pv. *vesicatoria*, a soil-borne pathogen, *Ralstonia solanacearum*, and on BG modifications of the rhizosphere microflora in peppers (*Capsicum annuum*).

3. Symptom development caused by the two bacterial pathogens on leaves and roots was significantly reduced in whitefly-exposed plants as compared to controls. A combined treatment with benzothiadiazole (BTH) and whitefly caused an additive effect on induced resistance, indicating that whitefly-induced plant defence can utilize salicylic acid (SA)-dependent signalling. To obtain further genetic evidence of this phenomenon, we evaluated the gene expression of *Capsicum annuum pathogenesis-related protein* (*CaPR*) *1*, *CaPR4*, *CaPR10* and *Ca protease inhibitor II*, and observed increased expression after BTH and/or whitefly treatment indicating that AG whitefly infestation elicited SA and jasmonic acid signalling in AG and BG. Since the expression pattern of PR genes in the roots differed, we assessed microbial diversity in plants treated with BTH and/or whitefly.

4. In addition to eliciting BG defence responses, a whitefly infestation of the leaves augmented the population of root-associated Gram-positive bacteria and fungi, which may have positively affected plant growth and induced systemic resistance. Whitefly feeding reduced plant size, which usually occurs as a consequence of the high costs of direct resistance induction.

5. *Synthesis.* Our results demonstrate that whitefly-induced resistance against bacterial pathogens can cross the AG–BG border and may cause further indirect benefits on future plant development, because it can positively affect the association or plant roots with putatively beneficial microorganisms.

Key-words: above-ground, below-ground, plant growth-promoting rhizobacteria, plant–herbivore interactions, *Ralstonia solanacearum,* whitefly, *Xanthomonas axonopodis*

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Introduction

Plants have developed general and specific defence mechanisms to protect themselves from pathogens and insects in nature (Smith, De Moraes & Mescher 2009). Such defence mechanisms can be classified broadly as constitutive and induced resistance (Pieterse *et al*. 2009). Induced resistances can be expressed in response to feeding by herbivores or infection by pathogens and are mainly regulated by salicylic acid (SA)-, jasmonic acid (JA)- and ethylene (ET)-dependent signalling pathways, which are interconnected by complex signalling networks and cross-talk phenomena (Pieterse *et al*. 2009). Once induced, most resistance traits are not only expressed locally but also in more or less distant tissues. This long-distance signalling can be mediated by classical hormones such as JA, SA and their derivatives, which move through the vascular system of the plant, but plants may also use volatile signals to achieve a faster signalling or to circumvent vascular restrictions (Heil & Ton 2008). This volatile-mediated resistance can be directed against herbivores (Heil & Karban 2010) and pathogens (Yi *et al*. 2009).

Considering this multitude of resistance phenomena and their systemic nature, plants require a biologically and environmentally sensitive control of the chemical nature, the levels and the sites of resistance expression. First, resistance expression against herbivores or pathogens may use limited resources and can then cause significant allocation costs (Baldwin 1998; Agrawal 2000; Heil *et al*. 2000; Heil & Baldwin 2002; Cipollini & Heil 2010). Second, resistance to one type of attacker can increase the susceptibility of the plant to another attacker, thereby causing significant ecological costs. For example, JA-mediated responses are usually directed against herbivores and necrotrophic pathogens, whereas SA-mediated systemic acquire resistance (SAR) responses are active against biotrophic pathogens (Stout *et al*. 1999; Bostock 2005). Many scientists observed a trade-off between SA-mediated and JA-mediated resistance responses (Heil & Bostock 2002; Thaler, Fidantsef & Bostock 2002). For example, pathogen-elicited SAR against the anthracnose fungus *Colletotrichum orbiculare* was associated with increased feeding preference by the cucumber beetle, *Diabrotica undecimpunctata*, and an increased reproduction rate of melon aphids, *Aphis gossypii* (Moran 1998). Similarly, tobacco plants challenged with tobacco mosaic virus (TMV) were more susceptible to grazing by tobacco hornworm (*Manduca sexta*) larvae than untreated control plants (Preston *et al*. 1999), and infection with white mould, *Sclerotium rolfsii*, promoted feeding behaviour by *Spodoptera exigua* in peanut plants (Cardoza, Alborn & Tumlinson 2002).

Because most systemic responses are mediated by long-distance signals (Heil & Ton 2008) they can cross the AG–BG border, meaning that interactions of the aerial parts of the plant with its enemies or mutualists can change the resistance status of the roots and *vice versa*. The resulting patterns are highly complex. For example, feeding on roots by herbivores can increase or decrease the resistance level of the aerial parts of the plant and *vice versa* (van Dam & Heil 2011). Furthermore, the resulting effects can cross the border of feeding guilds and phyla, meaning that, for example, AG damage by herbivores can mediate root resistance to microorganisms whereas mutualistic associations with soil microorganisms can interfere with the resistance against leaf feeders (van Dam & Heil 2011). For example, many strains of rhizosphere microbes referred to as plant growth-promoting rhizobacteria/fungi (PGPR/PGPF) exhibit beneficial effects on the plant as they positively affect plant growth and also the resistance level of the AG parts against pathogens – a process known as induced systemic resistance (ISR) (Kloepper, Ryu & Zhang 2004; Kloepper & Ryu 2006; Shoresh, Harman & Mastouri 2010). This ISR appears to depend mainly on JA rather than SA signalling and can therefore also affect the resistance to insects; these results can be highly complex. For example, colonization of *Arabidopsis* roots by *Pseudomonas fluorescens* strain WCS417r did not result in any difference in the mean weight of the specialist herbivore *Pieris rapae* larvae or average pupation time, but significantly reduced the weight gain of the generalist herbivore *Spodoptera exigua* on systemic leaves (Van Oosten *et al*. 2008). The treatment of rice under field conditions with a mixture of three *Pseudomonas fluorescens* strains reduced the damage of the leaffolder, *Cnaphalocrocis medinalis* (Saravanakumar *et al*. 2007).

In summary, the outcomes of AG–BG interactions are highly complex and general patterns are difficult to predict (van Dam & Heil 2011). Generalizations are particularly difficult because the current knowledge is based on a multitude of different study systems and because most studies were conducted exclusively at the level of phenotypes. Our current study focuses, therefore, on one plant species and one AG insect attacker. We used whitefly as the infesting insect, an attacker that is known to elicit SA-dependent resistance responses in plants and that, therefore, is prone to elicit effects on representatives of different phyla. Several recent studies demonstrated enhanced resistance to pathogens following feeding by insects such as whiteflies with a sucking mode of feeding because phloem-feeding insects like whiteflies and aphids can induce biochemical responses similar to those induced by pathogens (Walling 2008). For comparison, we elicited resistance also chemically, applying benzothiazole (BTH), a well-known mimic of SA in plant resistance responses (Bostock 2005). The induction of resistance was confirmed by whitefly infestation-elicited upregulation of transcriptional expression of *Capsicum annuum pathogenesis-related* (*CaPR*)*1* for an SA-responsive signalling pathway (Kim & Hwang 2000; Yang, Yu & Ryu 2009), *CaPR4* for ET/JA-responsive signalling (Park *et al*. 2001; Yang, Yu & Ryu 2009), *CaPR10* for SA/ET/JA-responsive signalling (Park *et al*. 2004), and *Capsicum annuum protease inhibitor II* (*CaPIN II*) for mainly JA-responsive signalling (Shin *et al*. 2001; Song, Kim & Lee 2005). Resistance was induced aboveground by whitefly infestation and/or BTH to study the biological effects on both a shoot- and a root-infecting bacterial pathogen and on potentially mutualistic soil microorganisms as well as the resulting changes in gene expression patterns both in the AG and the BG compartment. Our results suggest

that whitefly-elicited plant defence involves SA-dependent signalling and modulates BG defence responses and the microbial composition of the rhizosphere.

Materials and methods

STUDY ORGANISMS

We used pepper (*Capsicum annuum* L. cv. Bukwang) as the study plant because it interacts with multiple enemies and mutualists representing different guilds and because genetic tools allow the analysis of gene expression patterns under different conditions. Whitefly (*Bemisia tabaci*) that naturally occurred in the greenhouse in Daejeon, South Korea, in 2008–2010 was used as the biological inducer. The whitefly was maintained on tomato plants (*Solanum lycopersicum* cv. Solar set). To investigate for cross-phyla induced resistance against bacteria above and below ground, we challenged *Xanthomonas axonopodis* pv. *vesicatoria* (Yang, Yu & Ryu 2009), a causal pathogen of bacterial spot disease on the leaves, and *Ralstonia solanacearum*, a causal pathogen for bacterial wilt in the roots of various species of the Solanaceae (Lafortune *et al.* 2005) at 1 week after whitefly exposure. For comparing the root colonizing microorganisms in the rhizosphere, each whole root system was cleaned to remove soil particles and then placed in a 250-mL Erlenmeyer flask containing 100 mL of 0.1 M $MgSO_4$, which was vigorously stirred with a shaking incubator at 30 °C for 30 min at 1 week after whitefly exposure. The populations of bacteria and fungi were determined as root colonization capacity by plating on 1/10 strength TSA (tryptic soy broth agar, Bacto™ Tryptic Soy Broth, BD, Sparks, MD, USA) for the selection of Gram-negative, Gram-positive, actinomycetes and total bacteria and on PDA (potato dextrose broth agar, Difco™ Potato Dextrose Broth, BD, Sparks, MD, USA) for fungi population by the dilution plating method and by incubating plates for 3 days at 30 °C. Water treatment was used as negative control. We measured the population density of total bacteria that grew on the 1/10 TSA medium without any antibiotics, Gram-negative bacteria (addition of 10 μg mL^{-1} vancomycin to the media to inhibit growth of Gram-positive bacteria), Gram-positive bacteria (heat treatment at 50 °C for 30 min to kill Gram-negative bacteria and fungal spores) and actinomycetes (heat treatment at 50 °C for 30 min to kill Gram-negative bacteria and fungal spores), and on the PDA medium for fungi growth by addition of 100 μg mL^{-1} rifampicin to the media to inhibit growth of whole prokaryotes. The Gram-positive bacteria and actinomycetes were differentiated according to colony morphology and growth rate. The all colony forming units (cfus) that grew on each semi-selected medium were counted from roots and calculated actual bacteria.

PLANT GROWTH CONDITIONS AND PATHOGEN CHALLENGE

The seeds of *Capsicum annuum* were surface-sterilized with 6% sodium hypochlorite, washed four times with sterile distilled water (SDW) and then maintained at 25 °C for 3 days until germination. The germinated seeds were then planted on natural pepper field soil that composited sand and silt loam soil obtained from the KRIBB greenhouse facility, Daejeon, South Korea. Plants were grown at 25 ± 2 °C under fluorescent light (12 h/12 h day/night cycle, c. 7000 L × light intensity) in a controlled-environment growth room. Two-week-old pepper plants were drenched with either 10 mL solution of 0.5 mM benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (benzothiadiazole = BTH) (Syngenta, Research Triangle Park, NC, USA) or sterile water. Each plant was put into a transparent acrylic plastic cylinder (diameter = 15 cm, height = 50 cm and thickness = 3 mm) open at the top and bottom. Then, the top and bottom of the cylinder were covered with nylon stocking. For the whitefly infection treatment, the top was left uncovered for half of the plants to allow whiteflies to access the plants. Before each pathogen challenge, we determined whitefly numbers at 11 am by taking a picture of the leaf where the whiteflies were placed (Fig. 1). The average whitefly infestation was uniform across the various experiments. After 1 week, all plants were inoculated with *Xanthomonas axonopodis* pv. *vesicatoria* on Luria-Bertani (LB, Duchefa, Haarlem, the Netherlands) agar or with *Ralstonia solanacearum* on PGC medium (10 g peptone, 5 g glucose, 1 g pancreatic digest of casein and 17 g agar per 1 L). For experimental use, bacteria were scraped from plates and resuspended in sterile water. The bacterial suspensions were adjusted to 10^6 cfu mL^{-1} based on optical density and injected into pepper leaves using a 1-mL needle-less syringe (Doo Won Meditec Co., Kim je, South Korea). Disease severity (0–5) was measured 7 days after pathogen challenge as described previously (Yang, Yu & Ryu 2009). This experiment was designed as a completely randomized design with 10 replications and was independently repeated four times. *Ralstonia solanacearum*, a causal pathogen for bacterial wilt on diverse Solanaceae family plants, was drench-applied to the roots of pepper plants at 7 days after whitefly infestation. The disease rate was quantified as follows: 0 = no symptom, 1 = weak chlorosis of the newly developed leaves, 2 = 3–6 top leaves showed chlorosis, 3 = chlorosis was developed downward from the top leaves and crown part (near the soil around the stem) was showed early wilt symptom, 4 = all leaves except the 2–3 lower leaves showed wilt phenotype, 5 = fully developed wilt of the whole plant.

Fig. 1. Representative photograph of whitefly infestation on a pepper leaf. The picture was taken 7 days after whitefly exposure.

WHITEFLY EFFECTS ON PLANT GROWTH

To test the effect of whiteflies and BTH on the growth rates of pepper plants, shoot length, shoot dry weight and root dry weight were measured 7 days after pathogen challenge as described previously (Yang, Yu & Ryu 2009). The experiment was repeated four times with 10 replications.

QUANTITATIVE RT-PCR

To obtain molecular evidence for whitefly-elicited gene expression related to bacterial resistance in pepper, we employed qRT-PCR. The

relative mRNA expression of *CaPR1, CaPR4, CaPR10* and *CaPIN II*, previously reported during incompatible pathogen-elicited SAR and PGPR-elicited ISR, was measured in the leaves and roots (Park *et al.* 2001, 2002, 2004; Yang, Yu & Ryu 2009). Total RNA was isolated from leaf and root tissues treated with whitefly, water, BTH + whitefly and BTH at 1 week after each treatment according to the protocol described by Yang, Yu & Ryu (2009). Total RNA was treated with 1 U RNase-free DNase (Promega, Madison, WI, USA) for 10 min at 37 °C and subjected to a second round of purification using the TRI reagent. First-strand cDNA synthesis was carried out in 1 μg DNase-treated total RNA, oligo-dT primer and Moloney murine leukaemia virus reverse transcriptase (MMLV-RT, Enzynomics, Daejeon, South Korea). All PCRs were carried out according to the manufacturer's instructions. The candidate priming gene was analysed using the following primers: 5′-ACTTGCAATTATGATCCACC-3′ (CaPR1-F) and 5′-ACTCCAGTTACTGCACCATT-3′ (*CaPR1*-R). Additional genes and the primer sets used to detect them are as follows: *CaPR4*, 5′-AACTGGGATTTGAGAACTGCCAGC-3′ and 5′-ATCCAAGGTACATATAGAGCTTCC-3′; *CaPR10*, 5′-ATGTTGAAGGTGATGGTGGTGCTG-3′ and 5′-TCCCTTAGAAGAACTGATACAACC-3′; and *CaPIN*-II, 5′-CTCGGAATTGTGATACAAGA ATTGC-3′ and 5′-AAGGTACGTACGGCTGCTTCTTTAC-3′. As a control, to ensure that equal amounts of RNA were analysed in each experiment, we also analysed *CaActin* using the primers 5′-TTGGACTCTGGTGATGGTGTG-3′ and 5′-AACATGGTTGAGCCACCACTG-3′. Candidate priming genes were amplified from 100 ng of cDNA by PCR using an annealing temperature of 55 °C. Amplified PCR products were separated by 2% agarose gel electrophoresis. A Chromo4 real-time PCR system (BIO-RAD) was used to carry out qRT-PCR. Reaction mixtures (20 μL) contained 10 μL of 2 × Brilliant SYBR Green QPCR master mix (BIO-RAD), cDNA and 100 pm of each primer. The thermocycle parameters were as follows: initial polymerase activation, 10 min at 95 °C, and then 40 cycles of 30 s at 95 °C, 60 s at 55 °C and 30 s at 72 °C. Conditions were determined by comparing threshold values in a series of dilutions of the RT product, followed by a non-RT template control and a non-template control for each primer pair. Relative RNA levels were calibrated and normalized to the level of *CaACT1* mRNA (GenBank accession no. AY572427).

DATA ANALYSIS

Data were subjected to analysis of variance using JMP software ver. 4.0 (SAS Institute Inc., Cary, NC, USA; URL: http://www.sas.com). The significance of direct and indirect 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) at $P = 0.05$. Results of repeated trials of each experiment outlined above were similar. Hence, one representative trial of each experiment is reported.

Results

DEFENCE INDUCTION BY WHITEFLY ATTACK

An initial experiment was conducted in natural soil (silt loam)-grown pepper plants that were freely exposed to the whitefly population in the greenhouse. The average number of whiteflies per leaf was 18 ± 3.3 (Fig. 1). In leaves, symptom development after bacterial inoculation was significantly reduced after whitefly infestation and BTH treatment as compared to the water control (Fig. 2). In water control plants, weak necrosis appeared at 5 days after pathogen challenge on leaves, while plants that received whitefly, BTH, or BTH + whitefly treatment did not show any visible symptoms (Fig. 2a). At day 7, severe necrosis was seen on the leaves of the control plants; leaves inoculated with BTH or subjected to the whitefly alone treatment showed mild necrosis and chlorosis and those subjected to the BTH + whitefly treatment did only show mild chlorosis (Fig. 2a,b). Statistical analysis of the disease severity revealed a significant level of induced resistance after whitefly feeding (ANOVA: numerator d.f. = 3, denominator d.f. = 58, $F = 41.6662, P < 0.0001, n = 10$). *Post hoc* analysis revealed that the greatest resistance against *Xanthomonas axonopodis* pv. *vesicatoria* was observed after BTH + whitefly treatment, indicating an additive resistance effect of both treatments as compared to BTH or whitefly treatment alone (Fig. 2b). The average disease severities were ordered BTH + whitefly < whitefly < BTH < water control (Fig. 2b). The quantification of bacteria showed similar patterns (data not shown).

This whitefly-induced resistance effect crossed the AG–BG border. Whitefly treatment significantly reduced the number of wilted plants in response to challenging the roots with *Ralstonia solanacearum* (disease incidence, data not shown) and disease severity (ANOVA: numerator d.f. = 3, denominator d.f. = 36, $F = 21.546$, $P < 0.0001$, $n = 10$). Leaves of control plants that had developed after the challenging treatment showed chlorosis symptoms that developed downwards to the mature leaves at 7–9 days after pathogen inoculation. The whole plant showed typical wilt symptoms. By contrast, all three resistance-induction treatments (whitefly, BTH and whitefly + BTH) caused significantly lower disease severities than the controls, although no significant differences were observed among the various induction treatments (Fig. 2c).

PLANT GROWTH AFFECTED BY WHITEFLIES

Quantifying shoot length, shoot dry weight and root dry weight 1 week after treatment with whitefly, BTH, or both, revealed that the seedling growth of whitefly-exposed peppers was significantly decreased by 20% as compared to control plants (Fig. 3a,b). However, combined whitefly + 0.5 mM BTH treatment showed the greatest effect on plant growth arrest among the four treatments, reducing growth by as much as 53.8% as compared to whitefly or BTH treatment alone (Fig. 3b). The shoot lengths of pepper seedlings treated with whitefly or BTH alone did not statistically differ from each other, but did significantly differ from control and whitefly + BTH combination treatments (Fig. 3b). The shoot dry weight showed similar patterns, with the exception that BTH-treated plants showed no difference from those subjected to the combination treatment (Fig. 3c). Unexpectedly, the root dry weight of whitefly-exposed seedlings was greater than in other treatment groups (Fig. 3d). The root dry weight of whitefly + BTH combination-treated plants

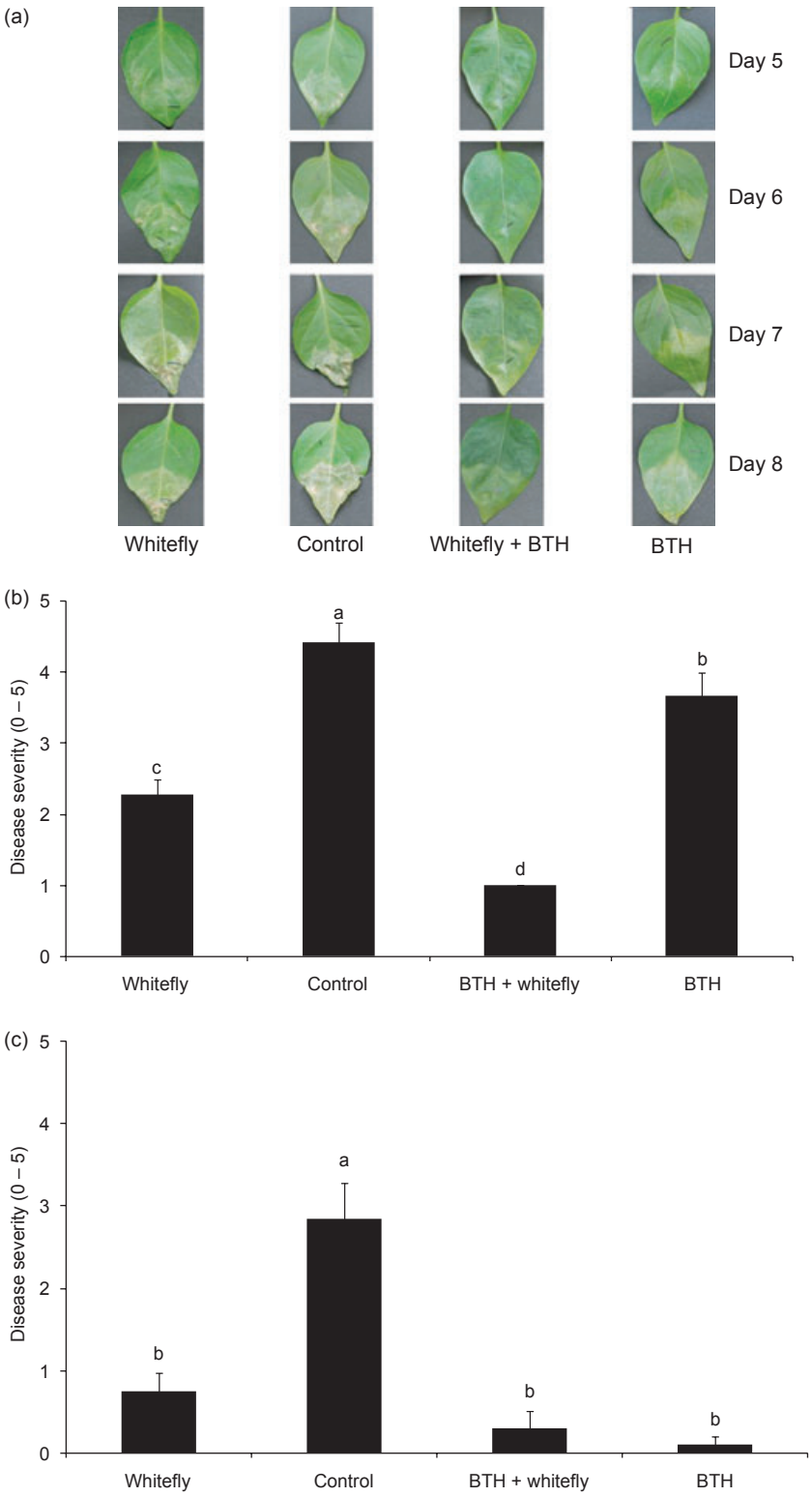

Fig. 2. The induction of systemic resistance by whitefly feeding. (a) Bacterial spot disease symptoms at 5, 6, 7 and 8 days after infiltration of *Xanthomonas axonopodis* pv. *vesicatoria* at 10^6 cfu mL^{-1} (b). The disease severity was measured 7 days after *X. axonopodis* pv. *vesicatoria* challenge. Bars represent mean ± SE, sample size $n = 10$ plants per treatment. Different letters indicate significant differences between treatments ($P < 0.05$ according to the least significant difference (LSD). The experiment was repeated four times with similar results. (c) The disease severity measured at 14 days after drench application of *Ralsonia solanacearum* on the pepper crowns. Bars represent mean ± SE, sample size $n = 10$ plants per treatment. Different letters indicate significant differences between treatments ($P < 0.05$ according to LSD). The experiment was repeated four times with similar results.

did not significantly differ from that of control plants, but was increased by 30% compared to BTH treatment alone. Whitefly infestation increased root dry weight by as much as 1.6- and 3.7-fold, respectively, as compared to combination or BTH treatment (Fig. 3d). The fresh weight of shoots and roots showed similar patterns to those of the dry weight (data not shown).

MOLECULAR EVIDENCE FOR SYSTEMIC DEFENCE RESPONSE

To obtain molecular evidence for a whitefly-infestation-mediated plant defence signalling pathway that confers resistance against bacterial pathogens, we employed qRT-PCR. An increased expression of *CaPR1*, *CaPR4*, *CaPR10* and *CaPIN*

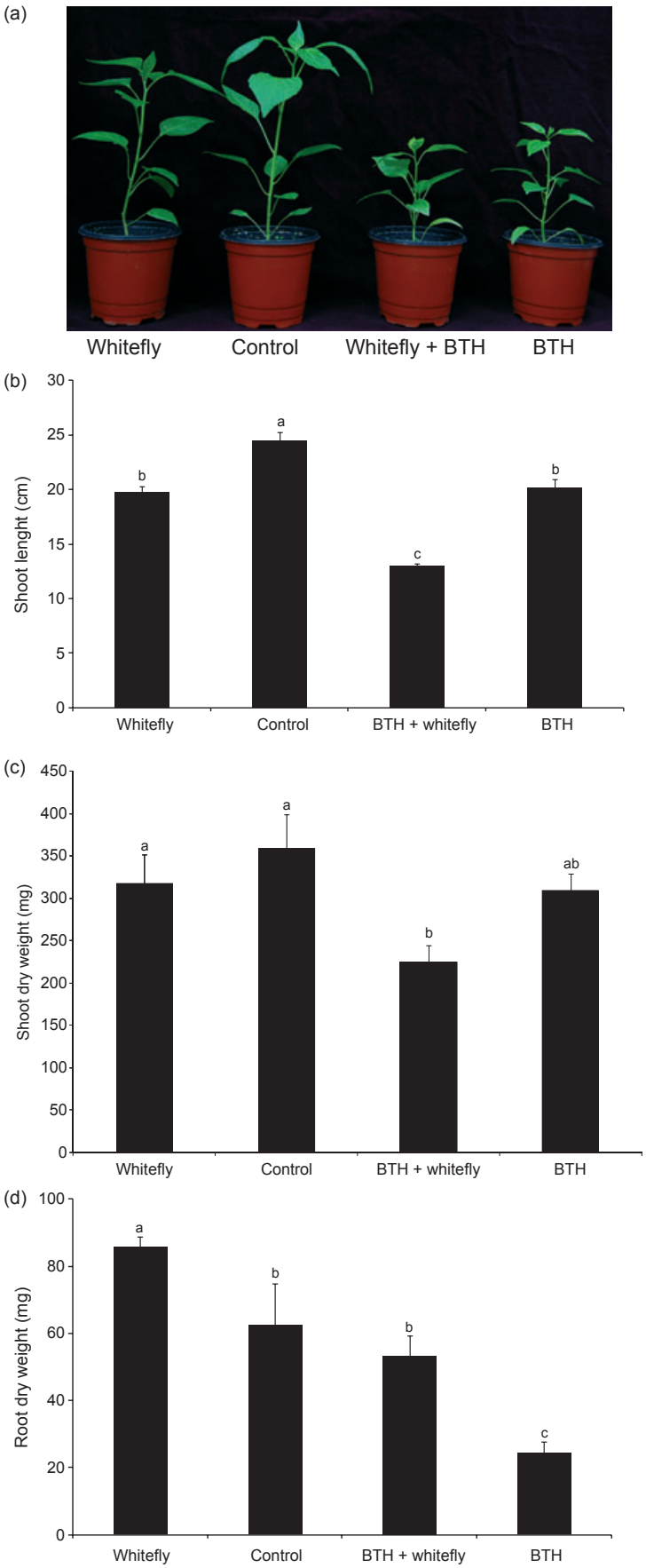

Fig. 3. Effects of whitefly infestation on pepper growth. (a) The representative photo was taken 7 days after whitefly, BTH, water control, and BTH + whitefly combination treatment of plants. Shoot height (b), shoot dry weight (c) and root dry weight (d) were measured at 7 days after whitefly, BTH, water control, and BTH + whitefly combination treatment. Bars represent mean ± SE, sample size $n = 10$ replications per treatment. Different letters indicate significant differences between treatments ($P < 0.05$ according to least significant difference). The experiment was repeated four times with similar results.

II was previously reported under elicitation of an incompatible pathogen-induced SAR condition and pharmaceutically applied defence signal molecules such as SA, JA, ET and abscisic acid (Kim & Hwang 2000; Shin *et al.* 2001; Park *et al.* 2001, 2004; Song *et al.* 2005). The transcriptional expression of three genes *CaPR1*, *CaPR4* and *CaPIN II* in the roots (Fig. 4) was significantly induced by whitefly infestation compared to other treatments, indicating that whitefly infestation significantly activates BG defences. Similarly, in the leaves (the AG parts of the plants), the transcriptional expressions of *CaPR1*, *CaPR10* and *CaPIN II* were significantly upregulated by whitefly infestation (Fig. 4a,b,c,d). These results suggest that AG-feeding by whitefly systemically elicited both SA- and JA/ET-dependent defence signalling pathways. BTH only induced the transcription of *CaPR1* and *CaPR4* below ground and *CaPR1*, *CaPR10* and *CaPIN II* in the leaves (Fig. 4a,c). To further assess the role of whitefly on the induction of defence signalling, we evaluated whether the BHT + whitefly treatment negatively or positively affects defence signalling. The SA- and ET-dependent defence genes together with BTH + whitefly

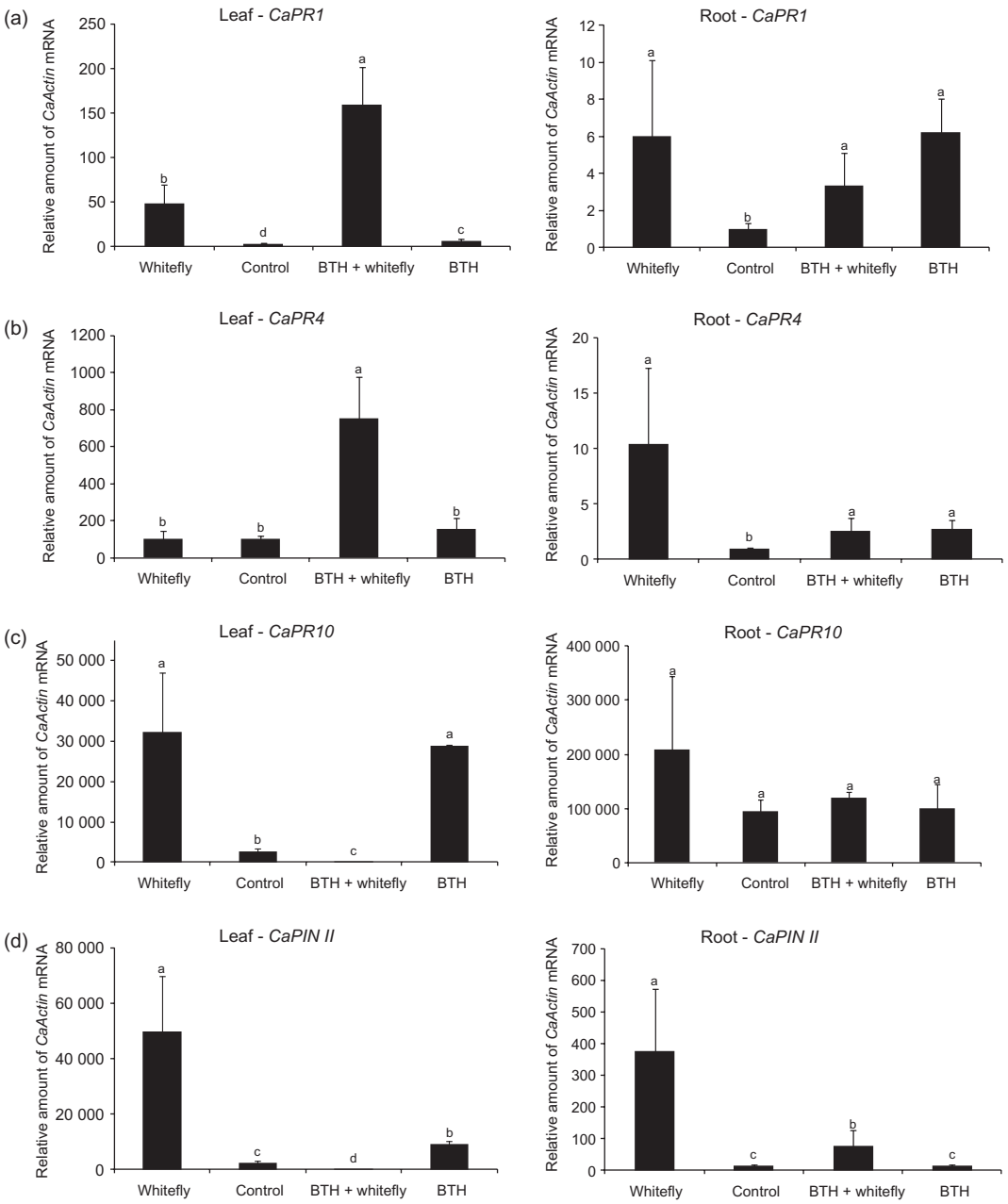

Fig. 4. Induction of *CaPR1*, *CaPR4*, *CaPR10* and *CaPIN II* genes by whitefly-, BTH-, water control-, and whitefly + BTH combination-treated plants. The expression levels of pepper resistance genes *CaPR1* (a), *CaPR4* (b), *CaPR10* (c) and *CaPIN II* (d) were quantified by qRT-PCR. The housekeeping gene *CaActin* was used as a control. Bars represent mean ± SE, sample size $n = 10$ replications per treatment. Different letters indicate significant differences between treatments ($P < 0.05$ according to least significant difference). The experiment was repeated four times with similar results.

combination treatment synergistically activated *CaPR1* and *CaPR4* respectively in the AG parts of the plants (Fig. 4a,b). In contrast, under the same condition the JA response genes *CaPR10* and *CaPIN II* in the leaves were significantly repressed compared with BTH or whitefly-alone treatments (Fig. 4c,d).

CHANGE IN BELOW-GROUND MICROFLORA ELICITED BY WHITEFLY CHALLENGE

The total number of soil-derived bacteria did not differ among treatments, with the exception of the roots of BTH-treated pepper plants, where total bacterial number increased up to twofold. Above-ground whitefly attack of peppers elicited no effect on the Gram-negative bacterial population but did significantly ($P < 0.05$) increase the number of Gram-positive and actinomycete bacteria and fungi (Fig. 5b–e). The number of Gram-negative and Gram-positive bacteria in BTH-treated pepper plants differed from that of water-control-treated plants, while other treatments elicited no difference (Fig. 5b,c). To estimate the additive effect of BTH and whiteflies, the combined BTH + whitefly treatment was assessed. Compared to BTH alone, the combined treatment led to a decreased number of Gram-negative bacteria; other microbial counts did not differ significantly (Fig. 5b).

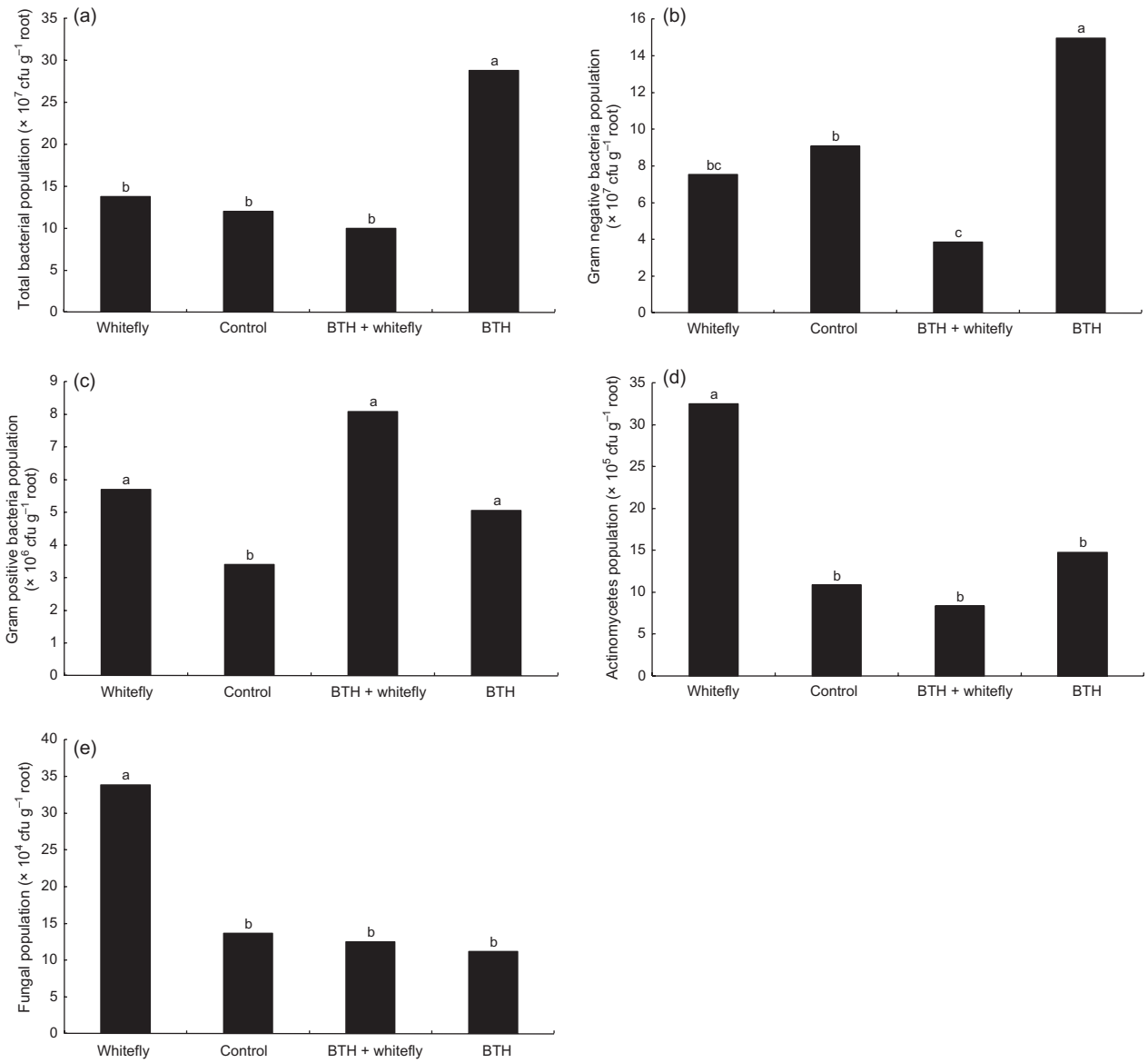

Fig. 5. Population changes in root-colonizing microbes by whitefly infestation on the pepper roots. Plants were either treated directly with 0.5 mM BTH (BTH), exposed to whiteflies (whitefly), received combination treatment (BTH + whitefly), or treated with water (control). The methods used to isolate total bacteria (a) Gram-negative bacteria, (b) Gram-positive bacteria, (c) Actinomycetes, (d) fungi and (e) T-RFLP analysis of soil bacteria by PCR-amplified 16s rRNA gene fragments are provided in the Materials and methods section. Bars represent mean ± SE, sample size $n = 10$ replications per treatment. Different letters indicate significant differences between treatments ($P < 0.05$ according to least significant difference). The experiment was repeated four times with similar results.

Discussion

The communication between AG and BG plant compartments plays a critical role in the management of plant metabolism, which can lead to changes in the defence machinery against insect pests and microbial pathogens. Previously, many studies have focused on the induction of resistance to an insect after AG or BG infestation with the same or a different insect species, and usually studies were conducted within the same compartment, i.e. looking for resistance in leaves after other leaves have been challenged. Studies on rice (Kanno & Fujita 2003; Kanno *et al.* 2005), watermelon (Russo *et al.* 1997) and tomato (Mayer *et al.* 2002) demonstrated that AG insect feeding can also change plant resistance against AG microbial pathogens. Here, we demonstrate that the phenomenon of cross-resistance among phyla can also cross the AG–BG border: insect feeding on leaves induced root resistance to BG pathogens and also altered the populations of root-colonizing (and potentially beneficial) bacteria.

This study demonstrates that feeding on leaves by phloem-feeding insects can induce a systemic resistance against both a leaf bacterial pathogen and a soil-borne bacterial pathogen. A similar study using whitefly as an AG feeding insect to test the alteration of plant defences only observed its effects against AG pathogens (Mayer *et al.* 2002). In our study, whitefly infestation elicited significant systemic resistance against bacterial spot disease caused by *Xanthomonas axonopodis* pv. *vesicatoria* and bacterial wilt by *Ralstonia solanacearum* (Fig. 2). The AG–BG aspect of this result confirms earlier results from other study systems. For example, silverleaf whitefly (*Bemisia argentifolii*)-infested *Brassica* sp. and tomato plants showed altered pathogen resistance and resistance-related protein accumulation in leaves and reduced AG disease incidence of the biotrophic fungus powdery mildew (*Erysiphe cichoracearum*) but did not affect the TMV titre (Mayer *et al.* 2002).

These results do not appear too surprising: the induction of plant resistance by phloem-feeding insects resembles microbial pathogen-elicited SAR more than herbivore-induced resistance, because phloem-feeding insects cause minimal mechanical damage to plants. A biochemical study revealed that whitefly feeding augmented the level of SA-induced pathogenesis-related proteins such as chitinase and peroxidase (Mayer *et al.* 2002; Gorovits *et al.* 2007). Among the genes that previously had been reported as being activated during incompatible pathogen-elicited SAR and PGPR-elicited ISR in pepper (*CaPR1*, *CaPR4*, *CaPR10* and *CaPIN II*), only *CaPR4* expression in the leaves did not differ between whitefly-treated and control plants (Yang, Yu & Ryu 2009; Fig. 4). In our study, whitefly feeding on leaves augmented pepper defence genes *CaPR1*, *CaPR4*, *CaPR10* and *CaPIN II* in roots. The qRT-PCR analysis of these molecular marker genes for SAR in pepper revealed that the *CaPR1* gene was significantly upregulated by whitefly in the above- and below-ground parts: *CaPR1* indicates the induction of SA-dependent signalling. By contrast, *CaPR4*, which reflects ET/JA-dependent signalling, was induced in BG parts of the plant compared to the control treatment and expression of the JA-responsive gene *CaPIN II* in both AG and BG parts of plants was significantly increased by whitefly treatment (Fig. 4b,d). Therefore, induced resistance to whiteflies shares similarities to plant reactions against microbial pathogens via SA and JA/ET-dependent pathways (Walling 2000; Kaloshian & Walling 2005). Whitefly infestation resulted in the induction of resistance against *Xanthomanas axonopodis* pv. *vesicatoria*, which requires the expression of *CaPR1*, *CaPR10* and *CaPIN II*. However, the induction of systemic resistance against *Ralstonia solanacearum* involves the *CaPR1*, *CaPR4* and *CaPIN II* genes. *CaPR1* and *CaPIN II* are common to both pathways and may play a critical role in whitefly-elicited systemic resistance against bacterial pathogens. It is noteworthy that expression of *CaPIN II*, which was reported to be a JA-responsive gene in another model plant, was related to resistance against necrotropic pathogens such as *Alternaria* spp. and *Botrytis* spp. because induction of SA signalling is thought to cause resistance against biotropic and semi-biotropic pathogens such as most bacterial pathogens and oomycetes (Pieterse *et al.* 2009). It is also striking that the combined treatment of whitefly + BTH significantly activated a SA-responsive *CaPR1* gene but repressed a JA-responsive *CaPIN II* suggesting that whitefly infestation additively induce SA signalling, resulting in the inhibition of JA signalling probably by antagonizing defence signalling between SA and JA defence mechanisms (Pieterse *et al.* 2009).

By contrast, although many recent studies have reported the induction of resistance against microbial pathogens following feeding by insects, our study appears the first to clearly demonstrate an AG–BG effect: insect infestation of the leaves caused the induction of systemic resistance against soil-borne pathogens. Moreover, the same infestation treatment changed the microflora in the rhizosphere. Because we observed an increased root biomass of the whitefly-infested plants (Fig. 3d) this effect may be a consequence of a plant tolerance mechanism. When suffering from damage to the AG parts, many plants re-allocate important resources to the roots, which can have positive effects on the associated organisms. Such effects can lead to a facilitation of root feeders after folivory (Kaplan *et al.* 2008) but might also affect mutualistic soil microorganisms (this study). As speculated by van Dam and Heil (2011), effects seen in one compartment after resistance induction in the other compartment can result both from systemic resistance responses and from side-effects of other physiological changes. Our data now demonstrate that both effects can occur in response to the same infestation event.

Our results further indicate that whitefly or BTH treatment reduced plant growth via the strong activation of plant defence genes, leading to allocation costs (Heil & Baldwin 2002). Negative effects of BTH treatment on plant growth rate and other fitness-relevant parameters have been reported from other systems [Heil *et al.* 2000; Yi *et al.* 2009; see Heil & Walters (2009) for a recent review] but were usually not directly correlated with altered gene expression patterns. Our study adds further data to the growing body of evidence for multiple physiological and ecological changes that plants suffer after whitefly infestation. Whiteflies can directly deplete plant reserves, reduce primary production, and cause phytotoxic effects, and

can also cause secondary damage through honeydew excretion that enables sooty mould development, blocks sunlight and reduces photosynthesis in zucchini and cotton (Yee *et al*. 1996; Henneberry *et al*. 1996; Chen *et al*. 2004), indicating that whiteflies may modify basic physiological plant processes. The additive effect on AG plant growth parameters of the BTH + whitefly treatments clearly supported our hypothesis (Fig. 3). For example, *Bemisia tabaci* causes increased stomatal resistance, reduced transpiration and photosynthesis rates, reduced chlorophyll content in tomato leaves and reduced net photosynthesis rates resulting in interference with the accumulation of soluble sugars in infested leaves (Buntin, Gilbertz & Oetting 1993; Lin, Schwartz & Saranga 1999; Lin *et al*. 2000). More intriguingly, whitefly infestation of the leaves led to significant changes in the population dynamics of rhizosphere microbes. In our study, the number of Gram-positive and fungal colonies isolated from whitefly-treated pepper roots was higher than that isolated from the control (Fig. 5c,d). The results of the BTH + whitefly combination treatment suggest that BTH had an additive effect on Gram-positive bacteria but not on fungal population. However, whitefly infestation did not exert any influence on the total and Gram-negative bacteria population. Only a slight, not significant difference was observed between whitefly and control treatments (Fig. 5b). Our results demonstrated that insect attack on AG tissue leads to direct effects on the Gram-positive and fungal microbial flora in the BG compartment.

Many strains of PGPR/PGPF elicited ISR against insect infestation (Mayer *et al*. 2002; Kloepper, Ryu & Zhang 2004; Shoresh *et al*. 2010). We speculate that leaf whitefly infestation may be augmented the secretion of root exudates, which may recruit beneficial Gram-positive bacteria and fungi such as PGPR and PGPF. The patterns in the populations of actinomycetes and fungi coincide with the increased root dry weight seen after whitefly infestation and are therefore likely a consequence of the increased allocation of assimilates to roots, a response that might serve as a tolerance mechanism. It is also noteworthy that BTH drench-application on the soil dramatically increased total and Gram-negative bacterial populations, but not Gram-positive and fungal populations, indicating that BTH and whitefly treatment elicited different changes in the population dynamics of the targeted microbes. Overall, the pattern of Gram-negative bacterial populations was the converse to the Gram-positive populations. Gram-negative bacteria were the lowest and Gram-positive bacteria the highest with combination treatment.

In summary, we report that foliar attack by a sap-sucking insect not only elicited AG resistance against a leaf pathogenic bacterium, *Xanthomonas axonopodis* pv. *vesicatoria*, but also manipulated the composition of the BG microflora and enhanced resistance against the soil-borne pathogenic bacterium, *Ralstonia solanacearum*. Systemic defence signalling apparently involved SA, JA and ET, because qRT-PCR analyses revealed increased expression levels of each hormone responding genes by whitefly infestation on the leaves. Whitefly feeding on the leaves led to significant upregulation of *CaPR1*, *CaPR4*, *CaPR10* and *CaPIN II* genes in the roots, indicating AG-mediated induction of systemic defence genes below ground. In addition to AG whitefly-elicited BG-defence responses, the microflora in the rhizosphere of pepper roots was altered, with increased populations of Gram-positive bacteria and fungi that may positively affect future plant growth and systemic resistance. Our results provide new insights into understanding the molecular basis of plant-mediated AG and BG communication, particularly the increase of BG defence mechanisms and beneficial microorganism populations after AG attack by sucking insects, which may help to prepare the plant for subsequent pathogen or herbivore attack.

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