An Aphid Facultative Symbiont Suppresses Plant Defense by Manipulating Aphid Gene Expression in Salivary Glands

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April 28, 2020

Abstract

Aphids often carry facultative symbionts to achieve diverse advantages. Serratia symbiotica, one of facultative endosymbionts, increases aphid tolerance to heat. However, whether it benefits aphid colonization on host plants is yet to be determined. In the current study, we found that Acyrthosiphon pisum harboring S. symbiotica had longer feeding duration on Medicago truncatula than Serratia-free aphids. Contrastingly, Serratia-free aphids triggered higher accumulation of ROS, jasmonic acid and salicylic acid responsive genes and cytosolic Ca2+ elevations than Serratia-infected aphids. Transcriptomic analysis of salivary glands indicated that a histidine-rich Ca2+-binding protein-like gene (ApHRC) was expressed more highly in the salivary gland of Serratia-infected aphids than that of Serratia-free aphids. Once ApHRC was silenced, Serratia-infected aphids also displayed shorter phloem-feeding duration and caused Ca2+ elevation and ROS accumulation in plants. Our results suggest that ApHRC, a potential effector up-regulated by S. symbiotica in the salivary glands, evaded plant defense response by suppressing Ca2+ elevation and ROS accumulation, allowing colonization of aphids. This study has provided a revolutionary insight into how facultative symbionts facilitate aphid colonization and adaption to host plants.

Keywords

Acyrthosiphon pisum ; *Serratia symbiotica* ; salivary gland; histidine-rich calcium-binding protein-like; calcium

Introduction

Insects are commonly associated with symbiotic microbes which could bring some advantages to themselves. The pea aphid *Acyrthosiphon pisum* , a model insect for endosymbiont studies, usually hosts one obligate symbiont and several facultative symbionts (Douglas & Prosser, 1992; La Pena, Vandomme, & Frago, 2014). Since phloem sap is a suboptimal diet for aphids due to imbalanced amino acids: carbohydrates proportions, the obligate symbiont, *Buchnera aphidicola* that resides in aphid bacteriocytes offers essential amino acids to aphids (Douglas, 1998). By contrast, facultative symbionts are usually not required for survival and reproduction of aphids but are able to improve their fitness particularly when they are confronted by various biotic and abiotic challenges. Studies indicate that some facultative symbionts increase heat tolerance (Doremus & Olive, 2017), defense against the natural enemies and fungi (Łukasik, Van Asch, Guo, Ferrari, & Godfray, 2013; Oliver, Russell, Moran, & Hunter, 2003), and host plant adaptation of aphids (Leonardo & Muiru, 2003). Previous research shows that an elicitor protein GroEL from *B. aphidicola*triggers plant resistance against aphids (Chaudhary, Atamian, Shen, Briggs, & Kaloshian, 2014). Hence, endosymbionts may modify the components of salivary proteins that modulate aphid feeding on plants. Very little is known, however, how facultative endosymbionts affect aphid feeding by altering aphid salivary proteins.

Aphids use their stylets to obtain nutrients from phloem sieve elements of the host plants (Tjallingii & Esch, 1993). While probing, aphids secrete saliva to inhibit plant defense response caused by wounding due to aphid feeding as well as clogging of the fluid in the phloem tissue usually induced by Ca^{2+} influx (Aidemark, Andersson, Rasmusson, & Widell, 2009). Plants typically initiate rapid cytosolic Ca^{2+} sparks upon aphid probing (Vincent et al., 2017), resulting in the activation of local defenses including reactive oxygen species (ROS) and phytohormone signaling (Tian et al., 2019). ROS-induced cell death prevents the aphid stylet from reaching the phloem (Laitinen et al., 2017) and triggers the salicylic acid (SA) signaling pathway to confer the local plant resistance to aphids (Hogenhout & Bos, 2011; Jaouannet et al., 2014). In addition, Ca^{2+} is also a long-distance signal that can be transmitted from injured cells to uninfected tissues, activating the wound-related systemic jasmonic acid (JA) signaling pathway (Farmer, Gasperini, & Acosta, 2014). Both synergistic and antagonistic JA- and SA-regulated defense have been reported in plant responses to aphids (Moran and Thompson,2001; Moran et al., 2002; De Vos et al., 2005; Mewis et al., 2006; Pegadaraju, 2005). Conversely, Ca^{2+}-binding proteins secreted by aphids into plant tissues have been shown to facilitate aphids feeding. For example, Armet, a salivary protein from the pea aphid sequesters Ca^{2+} to counteract the Ca^{2+}-triggered occlusion in *Vicia fabae* , which prolongs aphid phloem feeding time (Wang et al., 2015). Effectors containing Ca^{2+}-binding domains presumably could efficiently quench the cytosolic Ca^{2+} elevation of host plants, which consequently improves aphid feeding.

The facultative symbiont *Serratia symbiotica* increases heat tolerance of *A. pisum* , and enhances resistance to parasitoids and predators (Costopoulos, Kovacs, Kamins, & Gerardo, 2014; Hopper et al., 2018; Montllor, Maxmen, & Purcell, 2002), but little in known of whether and how *S. symbiotica* benefits aphid feeding. Previous studies showed that pea aphid clones collected from 11 legume plants differed in their facultative symbionts and infectious rate (Frantz, Calcagno, Mieuzet, Plantegenest, & Simon, 2009; Henry et al., 2013; Simon et al., 2003), suggesting that host plant species impact the facultative symbionts associated with aphids. During July-August of 2015-2019, we intended to determine the prevalence of endosymbionts of pea aphids in *Medicago sativa* fields in Yinchuan city, Ningxia province, China, and identified 5 endosymbionts within 154 sampled pea aphids (Fig. S1). *S. symbiotica* had a relatively high infectious rate (85%) in all sampled aphids, we hypothesized that infection of*S. symbiotica* may benefit the aphid growth on *Medicago*plants by facilitating aphid feeding. To test the hypothesis, we monitored aphid feeding behavior, performed aphid salivary gland transcriptomic analysis in the presence and absence of *S. symbiotica* . *Histidine-rich calcium-binding protein-like* (GeneID: 103308203) (*A. pisum HRC* , *ApHRC*) that encodes a salivary protein was up-regulated by *S. symbiotica* . Further investigation indicated that ApHRC suppressed Ca^{2+} elevation, which otherwise would have induced the plant defense response. Our study has demonstrated that *S. symbiotica* infection can modify aphid salivary composition by modulating salivary gland gene expression, which suppressed plant defense and benefited aphid feeding.

Methods

Aphids and Plants. Pea aphids (*A. pisum*) in red color used in experiments were collected from *Medicago sativa* fields in Yinchuan (106.27°N, 38.47°E), Ningxia, China in 2015.*Serratia* -infected aphids were parthenogenetic descendants from a single isolated female. We established *Serratia* -free aphids by injecting ampicillin into *Serratia* -infected aphids. To minimize the effect of the antibiotic on experiments, we reestablished*Serratia* -carrying aphids, namely *Serratia* -rebuilt aphids, by injecting hemolymph of *Serratia* -infected aphids into*Serratia* -free (see *Supplementary Information* for details).*M. truncatula* plants (cv. A17) was kindly provided by Professor Wenhao Zhang, Institute of Botany, Chinese Academy of Sciences, China. Transgenic *Nicotiana benthamiana* overexpressing *GCaMP3*used to detect Ca^{2+} sparks, was kindly provided by Department of Cell & Systems Biology, University of Toronto, Toronto, Canada (Defalco et al., 2017). Details of plant growth conditions were described in *Supplementary Information* .

Aphid Mean Relative Growth Rate (MRGR), Offspring Number and Development Duration. To measure pea aphid MRGR, 10 2^{nd} instar nymphs from*Serratia* -free or *Serratia* -infected aphids were weighed with an automatic electrobalance before and after feeding on *M. truncatula* plants for 5 days. The MRGR was calculated as previously described (Leather & Dixon, 1984): MRGR = (ln W2 - ln W1)/t, where

W1 was the initial weight, W2 was the final weight, and t represented days between weighing, i.e. t=5. Data were collected from 50 aphids.

For reproduction and developmental duration, one adult from*Serratia* -free or *Serratia* -infected aphids was placed on a fifth trifoliate leaf (counting from the base) of 4-week-old *M. truncatula* to reproduce. Newborns were counted and moved to another*M. truncatula* plant . Fifty newborn nymphs were individually recorded every 7 to 9 hours for their developmental status. Data were collected from 50 aphids for both aphid populations.

Quantitative PCR. For gene expression analyses of plants and aphids, total RNA of each sample was isolated from 100 mg leaves of plant fed by aphid for 24 h using the RNA Easy Mini Kit (Qiagen, Valencia, CA, USA), 5 pea aphids using TRNzol A+ (Tiangen, Beijing, China) or 20 salivary glands using the Absolutely RNA Nanoprep Kit (Agilent Technologies Inc., Santa Clara, United States), respectively. RNA (1 μg) was used to synthesize cDNAs (20μl) with FastQuant RT Kit (Tiangen, Beijing, China) for each sample. To detect gene expression, qPCR was performed in a 20 μL reaction volume with 2×SYBR Premix EX TaqTM (Qiagen, USA) master mix using gene-specific primers (Table S4) for phenylalanine ammonia lyase (*PAL*), nonexpresser of PR genes 1 (*NPR1*), pathogenesis-related protein 1 (*PR1*), lipoxygenase 2 (*LOX2*), allene oxide synthase 2 (*AOS2*) in plants and*ApHRC* in aphids and the salivary glands. Reactions were carried out on the Mx 3500P detection system (Stratagene, La Jolla, CA, USA): 3 min at 95 °C; followed by 40 cycles of 30 s at 95 °C, 30 s at 56 °C and 30 s at 72 °C, and finally stay at 72 °C for 3 min. Six biologically replicates were conducted for each treatment and each biological replicate contained three technical repeats.

Measurement of ROS Production. ROS production in *M. truncatula* leaves was monitored using 2'7'-dichlorofluorescin diacetate (DCFH-DA) (Beyotime Institute of Biotechnology, China). Its oxidation by ROS generates fluorescent 2',7'-dichlorofluorescein (DCF). Briefly, adaxial leaves were shaved to remove trichome. They were then soaked in either 1 ml aphid saliva or 1 ml 15% source solution for 6 h in dark, followed by infiltration with 10 μM of DCFH-DA in phosphate buffer (PBS, pH 7.4) for 90 s in 5ml syringes, and 3 washes with phosphate buffer. These leaves then placed in dark for 20 min before fluorescence detection with a Zeiss LSM710 laser confocal microscope (Zeiss, Germany). Leaf cuttings were sandwiched between two microscope coverslips with the adaxial side facing the 488-nm argon laser. Once excited, the fluorescence emissions were detected through wavelength bands 510-550 nm for dichlorofluorescein and 650-750 nm for the red fluorescence for chloroplasts (Lei et al., 2016). Images were analyzed using imaging system software (Zen, Zeiss, Germany).

Expression of *ApHRC* in *M. truncatula*. The full-length cDNA of *ApHRC* was amplified and ligated into a binary vector pBWA(V)HS. The construct was sequence-validated and transformed into *Agrobacterium tumefaciens* strain GV3101. When recombinant*Agrobateria* were grown in liquid LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 50μg/ml kanamycin) at 28 °C to an OD_{600} of 1, cells were collected by centrifugation and resuspended in infiltration buffer (10 mM MES, 10 mM $MgCl_2$, 200 μM acetosyringone, pH5.7). Leaves of 4 week-old *M. truncatula* were infiltrated with *A. tumefaciens* . The uninfiltrated leaf area was harvested after 12 h for RNA isolation and cDNA synthesis. The cDNA samples were subjected to qPCR to detect *ApHRC* expression. Aphid feeding behavior, expression of JA- and SA-pathway genes and H_2O_2 production were recorded 12 h after infiltration.

Fluorescent Signal Analysis. TIFF files were imported into Fiji (Image J) v1.52n (National Institutes of Health, USA). Fluorescence values were analyzed by GFP fluorescence of every photo as a whole. $\Delta F/F_0$ was calculated according to the equation $\Delta F/F_0 = (F_t - F_0)/F_0$, where F_0 was the first fluorescence value of the series photos and F_t was the current fluorescence value.

Results

S. symbiotica **Improves Aphid Feeding** . To determine the effect of *S. symbiotica* on aphid feeding activity, we established*Serratia* -rebuilt aphids by injecting the hemolymph of*Serratia* -infected aphids into *Serratia* -free aphids to exclude antibiotic influence. Electrical penetration graph (EPG) was used to monitor the feeding behavior of pea aphid on *M. truncatula* . *Serratia* -free aphids spent more time secreting saliva

into sieve elements phase (E1 wave) and less time ingesting phloem (E2 wave) than *Serratia* -infected and*Serratia* -rebuilt aphids (Figure 1a and b). The*Serratia* -rebuilt aphids began the first phloem ingestion phase significantly earlier than *Serratia* -free aphids although such difference was not statistically significant between*Serratia* -infected and *Serratia* -free aphids (Figure 1c). Shorter salivary secretion and longer passive feeding suggested that harboring *S. symbiotica* promoted feeding efficiency of pea aphids (Table S1).

Localization of *S. symbiotica* and *B. aphidicola* in Aphids. Fluorescence *in situ* hybridization (FISH) was used to determine the locations of *S. symbiotica* and *B. aphidicola*in pea aphids. As expected, only *B. aphidicola* was detected in bacteriocytes of *Serratia* -free aphids (Figure 1d), Whereas*S. symbiotica* was also seen near bacteriocytes in*Serratia* -infected and rebuilt aphids (Figure 1e and f).

Serratia-**infected Aphids Avoided Triggering Strong Plant Defense.** To determine the effect of *Serratia* -infection on aphid feeding-induced plant defense, we analyzed defense gene expression in*M. truncatula* infested by *Serratia* -free vs.*Serratia* -infected aphids. Compared with *Serratia* -free aphids, *Serratia* -infected aphids triggered lower gene expression levels of *PAL, NPR1* , *PR1* in the SA pathway, as well as*AOS2* and *LOX2* in the JA pathway (Figure 2a-e). Consistently, the EPG experiment indicated that *Serratia* -infected aphids had longer feeding duration than *Serratia* -free aphids.

We then assessed the ROS levels in plants after aphid infestation or saliva infiltration. Upon 6h infestation, *Serratia* -infected aphids triggered less H_2O_2 accumulation in plant leaves than *Serratia* -free aphids (Figure 2f). Furthermore, infiltration of saliva from *Serratia* -free aphids for 6 h led to a stronger fluorescence than from *Serratia* -infected aphid as well as mock-infiltration (Figure 2g). Presumably,*Serratia* -infected aphids would suffer less from ROS defense than*Serratia* -free aphids, in agreement with the observation that*Serratia* -infected aphids spent less time probing relative to*Serratia* -free aphids

Since Ca^{2+} is a ubiquitous signal that activates plant defense, we further studied the effect of aphid saliva on the cytosolic Ca^{2+} dynamics in plants using CaMV35S: GCaMP3 transgenic *N. benthamiana* . Saliva collected from*Serratia* -free and *Serratia* -infected aphids both induced a robust Ca^{2+} signal within the initial 90 s in a 300 s time course whereas 15% sucrose control did not (Figure 2h). After 90 s however, Ca^{2+} signal decreased substantially in plants infiltrated with saliva from *Serratia* -infected aphids but remained high in plants infiltrated with saliva from*Serratia* -free aphids for the remaining time period (Figure 2I; Movie S1-S3). Apparently, saliva of *Serratia* -infected aphids significantly suppressed Ca^{2+} signal.

S. symbiotica **Modulates Gene Expression in Aphid Salivary Glands.** To assess the effect of *S. symbiotica* infection on the gene transcription of salivary glands of pea aphids, the transcriptomic analyses were conducted using salivary glands of *Serratia* -free and *Serratia* -infected aphids. Of the 18,598 annotated genes,*S. symbiotica* significantly down-regulated the expression of 373 genes and up-regulated 347 genes (Figure 3a; Table S7, S8). Among the differentially expressed genes, 17 up-regulated and 37 down-regulated genes were predicted to contain signal peptides (Table S5 and S6). A gene annotated with *ApHRC* was almost 10 folds higher in salivary glands of *Serratia* -infected aphids than that of*Serratia* -free aphids (Figure 3b). The 2.3kbp *ApHRC* encodes a 448 amino acid protein with a predicted signal peptide and two putative Ca^{2+}-binding domains (Figure S2).

ApHRC expressed preferentially in a symmetrically disposed pairs of large secretory cells of the middle-lower cells of principal salivary glands (Figure 3c). Increase expression in the salivary glands and in the aphid body was detected in the presence of *S. symbiotica*(Figure 3d and e).

ApHRC **Facilitated Feeding of *Serratia*-infected Aphids.** To investigate whether *ApHRC* influenced the feeding of*Serratia* -infected aphid, RNAi was performed to silence*ApHRC*. Twenty-four h after ds*HRC* -RNA injection, the*ApHRC* expression level decreased 53% ± 12% in the body and 95% ± 4% in salivary glands (Figure 4a and b). *ApHRC* -silenced*Serratia* -infected aphids displayed prolonged salivary secretion (E1 wave), decreased the phloem ingestion phase (E2 wave) (Figure 4c and d), and increased intracellular punctures (pd wave, i.e. more navigation time to the phloem) (Figure 4e), which inevitably led to reduced feeding efficiency (Table S2).

ApHRC **Suppresses Plant Defense by Eliminating the Ca^{2+} Elevation.** Silencing *ApHRC* in*Serratia* -infected aphids resulted in higher *PAL* and lower*LOX2* expression, but no change in *PR1* , *NPR1* and*AOS2* (Figure 4f-j). Furthermore, after 6 h infestation,*ApHRC*- silenced aphid produced significantly more H_2O_2 and stronger green fluorescence than the ds*GFP* control aphids (Figure 4k and l), suggesting that ApHRC can efficiently suppress plant ROS signals. The Ca^{2+} dynamics resembled that of *Serratia* -free aphids (Figure 4m and n; Movie S4-S6). These results indicated*ApHRC* was able to suppress the plant Ca^{2+}signal during aphid infestation.

Expression of *ApHRC* in Plants Facilitated Feeding of*ApHRC*-silenced *Serratia*-infected Aphids . To further determine the effects of overexpression of *ApHRC* on plant defense and the feeding activity of *ApHRC*- silenced*Serratia* -infected aphids, we transiently expressed the full-length of *ApHRC* in *M. truncatula* by agroinfiltration. Overexpression of *ApHRC* in *M. truncatula* shortened salivary secretion (E1 wave) and elongated the duration of phloem feeding (E2 wave) (Figure 5a and b; Table S3). Induced *PAL* and suppressed *LOX2* expression was also observed (Figure 5c-g). While H_2O_2 was abundant in the plant cells in the vector-infiltrated plants, the *ApHRC* infiltrated plants barely accumulated any (Figure 5h and i). Therefore, the transient expression of *ApHRC* in *M. truncatula* leaves led to the inhibition of plant defense responses.

The Infection of *S. symbiotica* Improves the development of Pea Aphids . To determine the effect of *S. symbiotica*infection on the performance of pea aphids, offspring number, mean relative growth rate (MRGR) and the developmental duration of nymphs were compared between *Serratia* -free aphids and*Serratia* -infected aphids when reared on*M. truncatula* .*S. symbiotica* infection did not significantly affect MRGR and offspring number of pea aphids (Figure 6a and b). By contrast,*Serratia* -infected aphids had significantly shorter 2^{nd} instar and 3^{rd} instar duration than those of *Serratia* -free aphids (Figure 6c). Therefore,*S. symbiotica* infection improved aphid development.

Discussion

The beneficial effects of microbial facultative endosymbionts on aphid hosts are well recognized, especially when aphids are challenged by biotic and abiotic stresses. On the other hand, these endosymbionts usually impair aphid growth and development, or suppress reproduction under normal environment as physiological costs (Chen, Montllor, & Purcell, 2000; Ferrari, Darby, Daniell, Godfray, & Douglas, 2004; Oliver, Moran, & Hunter, 2005). Facultative endosymbionts in pea aphids including *H. defensa* , *Spiroplasma* , *Rickettsia*decrease longevity, fecundity and/or body weight of the aphid (Fukatsu, Tsuchida, Nikoh, & Koga, 2001; Polin, Simon, & Outreman, 2014; Russell, & Moran, 2006). By contrast, *Serratia* -infected aphids developed more rapidly than *Serratia* -free aphids on *M. truncatula* . Similarly, *S. symbiotica* accelerated cedar aphid*Cinara cedri* growth as well by directly providing essential amino acids to host, gradually turning into an obligate endosymbiont (Lamelas et al., 2011). However, few amino acid metabolic genes have been annotated in the genome of *S. symbiotica* in pea aphids to supply nutrition (Manzanomarin, Lamelas, Moya, & Latorre, 2012). Since the field population of pea aphids had a relatively high infectious rate of*S. symbiotica* , we speculated that *S. symbiotica* was likely involved in the early aphid feeding and colonization on *Medicago*plants by supplying sufficient nutrition leading to accelerated population growth (Figure S1). In the current study, we demonstrated that *S. symbiotica* sharply up-regulated *ApHRC* in salivary glands of pea aphids, which efficiently suppressed Ca^{2+} signal and plant defense.

S. symbiotica is usually localized freely within hemolymph or near the bacteriocytes within pea aphids (Moran, Russell, Koga, & Fukatsu, 2005). *S. symbiotica* infection could impair the cellular immunity of pea aphid and decreased indole-3-lactate, an antioxidant, which may down-regulate ROS in aphid hemolymph (Burke, Fiehn, & Moran, 2010). Also, genome sequencing revealed that the IMD pathway along with many immune genes such as those encoding peptidoglycan recognition proteins and AMPs are lost in the pea aphid, possibly facilitating association of aphids with microbial symbionts (Gerardo et al., 2010). Interestingly, *S. symbiotica* increased drastically the expression of *ApHRC* , a gene encoding a presumably secretory protein in salivary glands (Figure 3B). Although little is known concerning its function in aphid immune response to the infection of *S. symbiotica* , we suspected that up-regulation of *ApHRC* possibly suppressed immune

responses of aphid during the infection of *S. symbiotica* via quenching the early elevation of the Ca^{2+} signal as well.

Wounding caused by aphid feeding elicits plant Ca^{2+}influx, a signal that turns on down-stream defenses, such as sieve element occlusion and ROS accumulation to prevent aphid feeding (Sun, Voorrips, Kaauwen, Visser, & Vosman, 2020). However, aphids secret salivary effectors to maintain constant phloem feeding (Mutti et al., 2008). Notably, several effectors have calcium-binding domains, such as Armet, that suppresses plant Ca^{2+} signal to facilitate aphid feeding (Cui et al., 2019). Here, *Serratia* infection aphids, either infested by aphids or infiltrated with saliva, led to weaker ROS and less H_2O_2 concentration in plant leaves, possibly attributing to the inhibition of Ca^{2+} signal via Ca^{2+}-binding in*M. truncatula* (Tian et al., 2019). Consistent with this notion, silencing *ApHRC* significantly decreased the phloem ingestion time of aphids and induced higher ROS in *M. truncatula* , suggesting that *ApHRC* was a *Serratia* -induced salivary effector that improved aphid feeding. In addition to ROS, JA and SA signaling pathways have been widely reported to be involved in plant resistance against aphids (Mohase & Der Westhuizen, 2002; Selig, Keough, Nalam, & Nachappa, 2016; Sun et al., 2020). Since silencing *ApHRC* in the salivary glands or expression of *ApHRC* in plants only affected*LOX2* and *PAL* but was not significant for *AOS2* ,*NPR1* and *PR1* , we conclude that JA and SA signaling pathways are only weakly affected by ApHRC. By contrast, the infestation of *Serratia* -infected aphids triggered weaker JA and SA signaling pathways than *Serratia* -free aphid in terms of all measured marker genes, indicating that some other salivary effectors of*Serratia* -infected aphids down-regulated the JA and SA signaling pathways instead of *ApHRC* . Since a number of differentially expressed genes were affected by *Serratia* infection in our transcriptomic data of salivary glands, their function in suppression of plant defenses may be responsible for the discrepancy between*Serratia* -infection and ApHRC effects in activation of JA and SA signaling pathways. For example, *Serratia* -infected aphid could decrease the expression of a chemosensory protein (CSP) MP10 known to up-regulate JA and SA pathways of the host plant (Rodriguez, Stam, Warbroek, & Bos, 2014). Furthermore, *HRC* was also annotated in several other aphid species including *Myzus persicae* ,*Diuraphis noxia* , and *Rhopalosiphum maidis* , suggesting that the function of *HRC* might be conserved in aphids (Figure S3). In addition, a recent study showed that the mutation of a *HRC* in wheat enhanced the resistance against *Fusarium* head blight (Li et al., 2019). Most likely, *HRC* could down-regulate the Ca^{2+} responses to fungi infection, conferring susceptibility to the fungal diseases in wheat. Suppressing plant defense, as a newly discovered function of *S. symbiotica* , may explain its high prevalence in our field sampling compared with other facultative symbionts (Figure S1).

Growing evidence suggests that microbial mutualistic symbioses of insects could be orally secreted into a plant and directly manipulate the plant defenses (Chung, Rosa, Hoover, Luthe, & Felton, 2013). For example, a psyllid bacterial endosymbiont *Candidatus Liberibacter psyllaurous* could suppress JA and SA defensive signaling pathways of tomato plants (Casteel, Hansen, Walling, & Paine, 2012). The Colorado potato beetle secretes symbiotic bacteria to elicit SA-regulated defense while suppressing the efficient JA signaling pathway (Sorokan, Burkhanova, Benkovskaya, & Maksimov, 2019). Since most of the symbionts reside in bacteriocytes, gut, and hemolymph of insects, it is more common that the endosymbionts modulate the insect-plant interaction by indirectly regulating the transcripts of insect salivary gland genes rather than directly being secreted into the plant. We have shown that, on the basis of the transcriptome of salivary glands, the infection of*S. symbiotica* could modify a number of salivary proteins of pea aphids (Table S5-S8). Our study has revealed a novel strategy employed by aphids where they host microbial facultative symbionts to benefit their own feeding. *Serratia* -infected aphids are thus more likely to be successful in host colonization and population expansion on*Medicago* plants.

Author Contributions

F.G. and Y.S. conceived the original screening and research plans; H.G. and Y.S. supervised the experiments; Q.W. and X.L. performed most of the experiments; Q.W., E.Y. conducted the field sampling; Q.W. and H.G. designed the experiments and analyzed the data; Q.W., Y.S. and K.Z-S. wrote the article with contributions of all the authors.

Acknowledgments

We thank Professor Zi-Hua Zhao for conducted field sampling, and Dr. Jing Gao for sharing RT-qPCR laboratory techniques. This project was supported by the National key R&D Program of China (no. 2017YFD0200400) and the Strategic Priority Research Program of the Chinese Academy of Sciences (no. XDB11050400) and the National Nature Science Fund of China (nos. 31770452 and 31870394).

Declaration of Interests:

The authors declare no competing interests.

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Fig 1. *S. symbiotica* **facilitated pea aphids' feeding on** ***M. truncatula*** **(cv. A17) and its location in aphid body.**

(a-c) EPG results of *Serratia* -: *Serratia* -free,*Serratia* +: *Serratia* -infected, and *Serratia* +r:*Serratia* -rebuilt aphids for 8 h on *M. truncatula* . (a) Time spent on salivary secretion into sieve elements (E1 wave) (n=10). (b) Ingestion time (E2 wave) (n=10). (c) Time to phloem ingestion (n=10). (d-f) Fluorescence in situ hybridization (FISH) to detect *B. aphidicola* and *S. symbiotica* in aphid abdomen. Aphid DNA was stained with 4',6-diamidino-2-phenylindole (blue), *B. aphidicola*DNA was hybridized with Cy5-labeled DNA probe (red), *S. symbiotica* DNA was hybridized with Cy3-labeled DNA probe (green). (d)*B. aphidicola* in *Serratia* -free aphids. (e) *B. aphidicola* and *S. symbiotica* in *Serratia* -infected aphids. (f) *B. aphidicola* and *S. symbiotica* in*Serratia* -rebuilt aphids. The data shown are mean ± standard error (SEM). * indicate significant differences among different treatments at $P < 0.05$ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) one-way ANOVA analysis for EPG data.

Fig 2. ***M. truncatula*** **(cv. A17) defense triggered by aphids' feeding or aphid saliva.**

(a-e) Relative expression levels of *M. truncatula* JA and SA pathway genes induced by aphids. *Serratia* -: *Serratia* -free aphids and *Serratia* +: *Serratia* -infected aphid. (a)*PAL* , phenylalanine ammonia lyase; (b) *NPR1* , nonexpresser of PR genes 1; (c) *PR1* , pathogenesis-related protein 1; (d)*LOX2* , lipoxygenase 2; (e) *AOS2* , allene oxide synthase 2. (n=6). (f) H_2O_2 concentration induced by 10 *Serratia* -infected and *Serratia* -free aphids, respectively for 6 h (n=6). (g) Subcellular localization of fluorescent probes, DCFH-DA, in leaves of *M. truncatula* infiltrate by*Serratia* -free (the middle panel) and *Serratia* -infected aphid saliva (the lower panel). Control (the upper panel): 15% sucrose solution treatment. Bars = 100 μm. (h) Normalized fluorescence ($\Delta F/F_0$) of Ca^{2+} signal measurements every 3 s in *N. benthamiana* detached leaves infiltrated by*Serratia* -: *Serratia* -free, and *Serratia* +:*Serratia* -infected aphids saliva. Control: 15% sucrose solution treatment. ΔF, the difference between measured fluorescence and the fluorescence of the very first picture (F_0) (n=15). Yellow shading indicates significant difference between *Serratia* - and *Serratia* + treatment ($P < 0.05$). (i) Representative fluorescence microscope images showing fluorescence of Ca^{2+} signal in *N. benthamiana* leaves infiltrated by *Serratia* -free (the middle panel) and*Serratia* -infected aphid saliva (the lower panel). Control (the upper panel): 15% sucrose solution treatment. Bars = 100 μm. The data shown are mean ± standard error (SEM). * above the bars indicate significant differences among different treatments at $P < 0.05$ (t-test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Fig 3. The ***ApHRC*** **expression level in salivary glands of pea aphids.**

(a) Heat map of differentially expressed genes (DEGs) in the salivary gland of *Serratia* -free aphid (*Serratia* -) and*Serratia* -infected aphid (*Serratia* +). (b) The relative expression of the top 10 up-regulated candidate secretary saliva proteins in *Serratia* -infected salivary glands transcriptome when compared with *Serratia* -free aphid. Gene in the red box is *ApHRC* . Three biological replications were conducted in the RNA-seq analysis. (c) FISH to detect the *ApHRC* expression level in aphid salivary glands. *ApHRC* mRNA was

hybridized with 5- carboxyfluorescein (5-FAM) in red, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) in blue. PG, principal gland; AG, accessory gland. Bars = 50 μm. (d) *ApHRC* relative expression level in aphid body (n=6). (e) *ApHRC* relative expression level in aphid salivary gland (n=6). The data shown are mean ± standard error (SEM). * above the bars indicate significant differences among different treatments at $P < 0.05$ (t-test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Fig 4. Stimulation of plant defense after *ApHRC* was silenced.

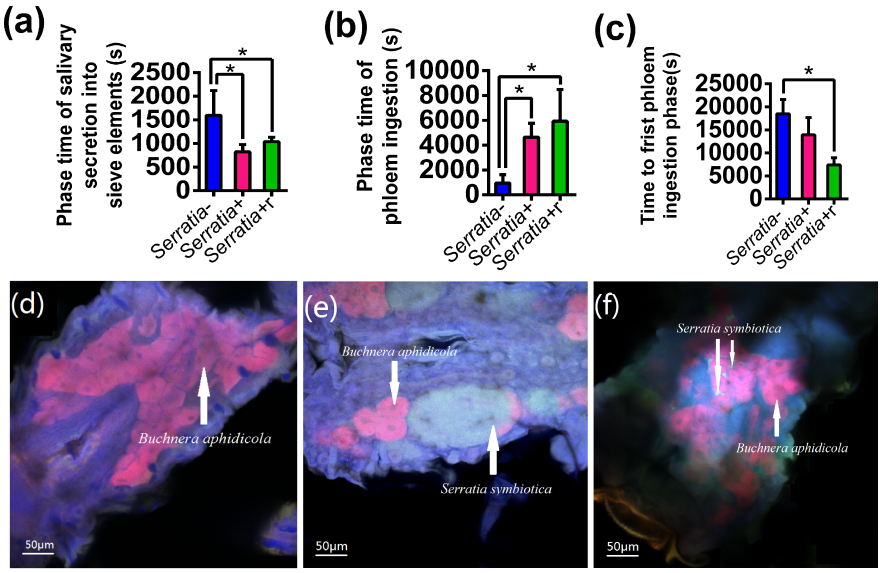
Relative *ApHRC* expression in *Serratia* -infected (a) aphid body and (b) salivary glands 24 h after injection of ds*HRC* -RNA (n=6). (c-e) EPG results of ds*GFP* : *Serratia* -infected aphid injected ds*GFP* as control, and ds*HRC* :*ApHRC* -silenced *Serratia* -infected aphid for 8 h on *M. truncatula.* (c) Time spent on salivary secretion into sieve elements (E1 wave) (n=10). (d) Ingestion time (E2 wave) (n=10). (e) Time of intracellular punctures (pd wave) (n=10). (f-j) Relative expression of*M. truncatula* JA and SA pathway genes induced by ds*HRC*-and ds*GFP* -aphids infestation. (f) *PAL* , phenylalanine ammonia lyase; (g) *NPR1* , nonexpresser of PR genes 1; (h)*PR1* , pathogenesis-related protein 1; (i) *LOX2* , lipoxygenase 2; (j) *AOS2* , allene oxide synthase 2. (n=6). (k) H_2O_2 concentration induced by 10 ds*HRC* and ds*GFP* aphids, respectively for 6h (n=6). (l) Subcellular localization of fluorescent probes, DCFH-DA, in leaves of*M. truncatula* infiltrate by ds*GFP* -aphid (the middle panel) and ds*HRC* aphid saliva (the lower panel). Control (the upper panel): 15% sucrose solution treatment. Bars = 100 μm. (m) Normalized fluorescence ($\Delta F/F_0$) of Ca^{2+} signal measurements every 3 s in *N. benthamiana* detached leaves infiltrated by ds*GFP* -aphid and ds*HRC* -aphid saliva. Control: 15% sucrose solution treatment. ΔF, the difference between measured fluorescence and the fluorescence of the very first picture (F_0) (n=15). Yellow shading indicates significant difference between ds*GFP*- aphids and ds*HRC* -aphid saliva treatment ($P < 0.05$). (n) Representative fluorescence microscope images showing fluorescence of Ca^{2+} signal in *N. benthamiana* leaves infiltrated by ds*GFP*- aphid (the middle panel) and ds*HRC* -aphid saliva (the lower panel). Control (the upper panel): 15% sucrose solution treatment. Bars = 100 μm. The data shown are mean ± standard error (SEM). * above the bars indicate significant differences among different treatments at $P < 0.05$ (t-test, *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$).

Fig 5. ***ApHRC*** **expression promoted aphid performance.**

(a-b) EPG results of *ApHRC* -silenced *Serratia* -infected aphids for 8 h on EV: empty vector, and OE*HRC* : overexpression of*ApHRC M. truncatula* . (a) Time spent on salivary secretion into sieve elements (E1 wave) (n=10). (b) Ingestion time (E2 wave) (n=10). (c-g) Relative expression levels of EV and OE*HRC* *M. truncatula* JA and SA pathway genes induced by *ApHRC* -silenced*Serratia* -infected aphids. (c) *PAL* , phenylalanine ammonia lyase; (d) *NPR1* , nonexpresser of PR genes 1; (e) *PR1* , pathogenesis-related protein 1; (f) *LOX2* , lipoxygenase 2; (g)*AOS2* , allene oxide synthase 2. (h) H_2O_2 concentration of EV and OE*HRC* *M. truncatula* leaves induced by 10*ApHRC* -silenced *Serratia* -infected aphids, respectively for 6 h (n=6). (i) Subcellular localization of fluorescent probes, DCFH-DA, in leaves of EV (the middle panel) and OE*HRC* (the lower panel)*M. truncatula* infiltrate by *ApHRC* -silenced*Serratia* -infected aphids. Control (the upper panel): 15% sucrose solution treatment. Bars = 100 μm. The data shown are mean ± standard error (SEM). * above the bars indicate significant differences among different treatments at $P < 0.05$ (t-test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Fig 6. ***S. symbiotica*** **influence on pea aphid MRGR, fecundity and development time.**

(a) MRGR of *Serratia* -infected (*Serratia* +) and*Serratia* -free (*Serratia* -) aphids (n=50). (b) Fecundity of*Serratia* -infected and *Serratia* -free aphids (n=50). (c) Nymphal duration of *Serratia* -infected and *Serratia* -free aphids (n=50). Data shown are mean ± standard error (SEM). * above the bars indicate significant differences among different treatments at $P < 0.05$ (t-test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).



Serratia-free aphid *Serratia*-infected aphid *Serratia*-rebuilt aphid