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Induced expression of defence-related genes in barley is specific to aphid genotype

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Understanding the mechanisms by which plants respond to attack is of great ecological and economic importance. When phloem-feeding insects feed they can influence the expression of defence-related genes in the plant. While it is well-documented that the genotype of the feeding insect can influence plant fitness traits, thus far the effect of insect genotype on the induction of defence-related genes in the plant has had relatively little attention. To investigate the molecular specificity of plant–insect interactions, the model plant *Hordeum vulgare* was exposed to four different genotypes of the aphid *Sitobion avenae*. When the plants were previously exposed to a specific aphid genotype, the population growth of other aphid genotypes was reduced. A global gene expression study of the barley genome showed that these effects can occur indirectly through physiological changes in the plant. We found 1018 transcripts to be differentially induced by different aphid genotypes, with some specific to one aphid genotype. This work identifies core and genotype-specific plant response genes to aphids and supports the notion that the genotypic composition of the herbivore population can trigger the transcription of different defence-related genes in the host plant, thus affecting the population structure of these herbivores and potentially the wider ecological community. © 2015 The Authors. *Biological Journal of the Linnean Society* published by John Wiley & Sons Ltd on behalf of Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **117**, 672–685.

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INTRODUCTION

The interaction between plants and insects has been a fundamental aspect of many ecosystems for at least the last 350 Myr (Gatehouse, 2002). Herbivorous insect species diversity is high, driven by both the associated community of competitors, predators and parasitoids (Bernays & Graham, 1988), and host plant chemical diversity, including defence strategies (Futuyma & Agrawal, 2009). Plants are estimated to have evolved over 500 000 secondary metabolites (Mendelsohn & Balick, 1995) that act to reduce insect fitness (e.g. reproductive success) and have been described as a plant's chemical weapon system against herbivory (Wu & Baldwin, 2010). These defences create strong selective pressures on the feeding insects for adaptation to overcome them. Herbivorous insects almost always induce the expression of defence-related genes in a plant when they feed (Howe & Jander, 2008; Smith & Clement, 2012; Jaouannet *et al.*, 2014), but many herbivores have also evolved the ability to suppress defence-related genes and thus avoid the detrimental affects (Zhu-Salzman, Bi & Liu, 2005; Peccoud *et al.*, 2010), e.g. in aphids (Zhu-Salzman *et al.*, 2004; Thompson & Goggin, 2006; Will *et al.*, 2007; Elzinga & Jander, 2013), whitefly (Zarate, Kempema & Walling, 2007) and *Helicoverpa zea* caterpillars (Musser *et al.*, 2005).

One important group of herbivorous insects are aphids, which feed on the phloem sap of a plant using highly specialized mouthparts (stylet). These mouthparts minimize the physical damage to plant cells by

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allowing the aphid to navigate between the cells when probing for the phloem sieve-tubes, where feeding occurs (Powell, Tosh & Hardie, 2006). As an aphid probes between the plant cells, it can induce not only a wounding defence response (i.e. induced response pathways) in the plant but also responses similar to those associated with pathogens, such as bacteria and fungi (i.e. induced systemic and systemic acquired resistance pathways) (Walling, 2000; Thompson & Goggin, 2006; Howe & Jander, 2008; Jaouannet *et al.*, 2014). Aphid feeding can also induce the production of nutrients in the plant and thus manipulate the plant into creating a more favourable environment (Couldridge *et al.*, 2007). This may be achieved either through the induction of genes involved in nutrient production, e.g. carbon assimilation (Thompson & Goggin, 2006), or by inducing the reallocation of resources, such as mannitol (Divol *et al.*, 2005), nitrogen (Thompson & Goggin, 2006) or by altering the amino-acid composition of the phloem sap (Telang *et al.*, 1999). Several gene expression studies have demonstrated that phloem-feeding aphids alter the expression of a wide variety of genes in a plant, including genes related to mechanical wounding, pathogenesis, metabolism, oxidative stress, signalling, cell wall modification, senescence and insect digestion (Moran *et al.*, 2002; Voelckel, Weisser & Baldwin, 2004; Zhu-Salzman *et al.*, 2004; De Vos *et al.*, 2005; Divol *et al.*, 2005; Couldridge *et al.*, 2007; Kusnierczyk *et al.*, 2008; Delp *et al.*, 2009; Smith *et al.*, 2010; Liu *et al.*, 2011; Marimuthu & Smith, 2012; Jaouannet *et al.*, 2014). Among the induced up-regulated genes are those that code for volatile chemicals, which a plant releases after experiencing damage by herbivores to attract parasitoids (Du *et al.*, 1998) and predators to the plant (Takabayashi & Dicke, 1996). Some aphids can also suppress the expression of plant genes through the release of salivary enzymes and other compounds into the phloem sieve tube (Miles, 1999; Prado & Tjallingii, 2007; Elzinga & Jander, 2013; Furch, van Bel & Will, 2015). Suppression of other genes that are only indirectly involved in the resistance of the plant to insect feeding is also known to occur; for example, Sorghum greenbug feeding can induce the down-regulation of photosynthetic genes as the energy used in this pathway is reallocated elsewhere (Zhu-Salzman *et al.*, 2005). These genome-based interactions between plant and insect could lead to coevolution between the interacting species, although it should be noted that the presence of an interaction does not infer coevolution (Janzen, 1980).

Different attackers (i.e. different herbivore species, species morphs or developmental stages) are expected to induce different responses in the plant, often due to feeding behaviour, and this results in some individuals inducing much stronger defence responses than others (Agrawal, 2000). Pre-exposure of a plant to aphids (*Macrosiphum euphorbiae*) confers resistance in tomato plants to subsequent colonization of whitefly (*Bemisia tabaci*), but whitefly infestation does not confer plant resistance to the aphids (Nombela *et al.*, 2009). In fact, Nombela *et al.* (2009) found that for one aphid clone, pre-infestation by the whitefly actually increased aphid numbers. On peach (*Prunus persica*), pre-infestation by aphids (*Myzus persicae*) could induce susceptibility and resistance in the plant dependent on plant genotype (Sauge *et al.*, 2006). Hays *et al.* (1999) first showed that these interactions could occur at the intraspecific level. They found that pre-conditioning of a plant with greenbug (*Schizaphis graminum*) biotype E could reduce the performance of biotype H, whereas pre-conditioning the plant with biotype H had no effect. This was only found to occur in plants containing a resistant gene that conferred resistance to biotype E but not biotype H, and shows that there is variation across greenbug biotypes in the ability to induce specific defence responses in plants (Hays *et al.*, 1999). More recent work, has shown that the pattern of gene expression can differ between different *Diuraphis noxia* aphid biotypes on wheat carrying a gene conferring resistance to one aphid biotype but not another (Zaayman, Lapitan & Botha, 2009; Liu *et al.*, 2011). Gene-for-gene resistance has been found to explain particular aphid–plant resistance interactions (Glazebrook, 2001; Dogimont *et al.*, 2010; Sauge *et al.*, 2011). For example, *Mi*-mediated resistance of tomato plants to the potato aphid (*Macrosiphum euphorbiae*) is specific to one aphid clone, which means there is genetic variation for effectiveness of plant resistance (Rossi *et al.*, 1998). In addition, the gene *Sd1* confers resistance of apple plants to two biotypes of the aphid *Dysaphis devecta*, however, this gene does not confer resistance to a third, rare, aphid biotype indicating again that there is intraspecific variation in the aphids for avoidance of plant defences (Roche *et al.*, 1997).

In evolutionary terms, it may be expected that such rare biotypes would increase in frequency compared with those that are selected against, through the reduced effects of plant resistance. However, the complexity of plant–insect interactions does not necessarily mean that this scenario will occur, as there are many other factors reducing the frequency of rare biotypes. Recently, it has been shown that different cereal aphid (*Sitobion avenae*) genotypes preferentially colonize different barley plants (Zytynska & Preziosi, 2011) and this can be influenced by competition among aphid genotypes (Zytynska & Preziosi, 2013). This indicates that genotypic interactions between and within interacting species can influence the final population size and community structure of the insects on the plants.

In this paper, a single genotype of barley (*Hordeum vulgare*) that is susceptible to aphid feeding is used to investigate whether pre-conditioning a plant with different *Sitobion avenae* aphid genotypes influences the reproductive potential of other aphid genotypes. This study confirmed that indirect interactions occur between the aphid genotypes via insect-induced physiological changes in the plant, and that not all the variation can be explained by direct aphid competition. In a second experiment, it is shown that the different aphids induce genotype-specific expression profiles in the plant by measuring the global gene expression in the barley genotype.

MATERIAL AND METHODS

Aphid and barley study system

A single barley (*Hordeum vulgare* L.; Poaceae) genotype, Steptoe, was used for all experiments (seed originally obtained from P. Hayes, Oregon State University). This barley genotype is a parent variety used alongside Morex to create a double-haploid F1 mapping population. Experimental barley seeds were harvested from plants grown in a common glasshouse environment. Barley seeds used in this experiment were taken from a single harvest to minimize differences from seed stocks grown in different years. Aphids will readily feed on this barley genotype, which confers average performance to all our aphid genotypes when compared with five other barley genotypes (Zytynska & Preziosi, 2011). The presence of specific aphid resistance genes is unknown, but a QTL study using the Steptoe/Morex double-haploid mapping population did not find a QTL associated with resistance in Steptoe lines, but rather in the Morex lines (Moharramipour *et al.*, 1998). The longest leaf of the plants was measured as 25.2 ± 0.13 cm (mean $\pm$ SE) after 7 days and 65.1 ± 0.24 cm after 26 days' growth.

Four aphid (S*itobion avenae* F.) genotypes were used (CLO7, DAV95, H1 and HF92a), originally obtained from Rothamsted Research, UK. These aphids were originally collected from UK field sites and genotypes were identified using microsatellite markers (Rothamsted Insect Survey, Rothamsted Research, Harpenden). They are also known to differ in their reproductive rate (performance) and preference across different barley genotypes (Zytynska & Preziosi, 2011). The aphid lines have been kept in asexual reproduction (clonal lines) for the previous 4 years within the current research group (and as long as 15–20 years in Rothamsted for HF92a and DAV95 clones), and were reared on a generic batch of *H. vulgare* seed. The stock aphids were kept at low population levels (~20 aphids) to minimize variation within the clones and experimental populations were reared from five founder aphids to maintain pure clonal experimental populations.

Analysis of aphid population growth rate on pre-conditioned (aphid pre-infected) plants

Experimental design

In this experiment, plants were first exposed to one of the four aphid genotypes (hereafter called the pre-conditioning genotype). Our hypothesis for this experiment is that pre-conditioning of a plant by one aphid genotype can reduce the performance of subsequent colonizing aphid genotypes. After 5 days these pre-conditioning aphids were removed and either the same (control treatment) or another aphid genotype was introduced to the plant (post-conditioning aphids). The experimental design was fully factorial, with all aphid genotypes being reared on plants that had been pre-conditioned by all other genotypes, including its own (16 treatments). Twenty repeats were made for each treatment.

Experimental set-up

The barley seeds were germinated by placing the seeds between two layers of filter paper, in a petri dish, moistened with sterilized distilled water. The petri dishes containing the seeds were placed in a dark growth chamber at 21 °C for 5 days. The barley seedlings were then transplanted into 10 cm diameter pots (one seedling per pot) containing John Innes Compost No. 3. After 7 days, five aphids (3^{rd} and 4^{th} instar) were placed on a plant and each pot was covered using a plastic tube with a mesh top and mesh window, to isolate each plant from the others. The pre-conditioning aphids were left on the plants for 5 days, after which all aphids were carefully removed with a fine paintbrush and the plant checked carefully to ensure it was free of aphids. The plants had just grown a third leaf when the pre-conditioning aphids were introduced and over the 5 days of aphid exposure these leaves grew in size but there was little new growth. Two adult aphids (post-conditioning aphids, either of the same or different genotype) were then placed on the plants and allowed to feed and reproduce on both the older and newer leaf tissue. The experiment was maintained at The Firs Botanical Grounds, The University of Manchester in a glasshouse at 18–25 °C, 16:8 light:dark regime. Total aphid number was counted 14 days after the second aphids were introduced to the plants. Since equal numbers of aphids were introduced to the plants for a specific number of days, the final aphid number can also be considered a population growth rate, comparable between aphid genotypes.

Data analysis

The data were analysed using a generalized linear model in R with quasipoisson error distribution (R Core Development Team, 2015) to determine the influence of the pre-conditioning aphid genotype on the number of post-exposure aphids. *Post-hoc* contrasts were used to show the particular aphid genotypes that reduced aphid number compared with those plants with the same aphid. Pearson correlations were used to assess the association between the number of differentially regulated genes in the next experiment with aphid number in the current experiment.

Global gene expression of barely plants exposed to different aphid genotypes

Experimental design

In this experiment, barley plants from a single genotype were exposed to four different aphid genotypes (CLO7, DAV95, H1 and HF92a) and there was a 'no-aphid' control, with 15 repeats per treatment. The aphids remained on the plant for 5 days after which the plant leaf material was harvested, the RNA extracted and plant gene expression analysed using microarrays. The RNA was pooled from five plants within the same treatment, per microarray.

Experimental set-up

The barley seeds were sterilized by soaking in 10% NaOCl for 10 min on a rotary shaker and then washed six times in sterilized distilled water. The seeds were germinated by placing between two layer of filter paper moistened with sterilized distilled water and placing in a dark growth chamber at 21 °C for 5 days. The seedlings were transplanted into 10 cm pots filled with autoclaved horticultural grade sand, watered with sterilized distilled water. The plants were watered and fertilized with 40 ml of autoclaved Hoagland solution (Hoagland & Arnon, 1950) on the second, fourth and sixth day. The experiment was undertaken in an experimental growth chamber at 21 °C, 16:8 light:dark regime. Each pot contained one seedling and was covered using a plastic tube with a mesh top and mesh window. After 2 days, five 2nd or 3rd instar aphids were introduced to the plants. In order to control for density effects across aphid genotypes, the plants were checked daily to ensure no-aphid reproduction had occurred. Five days after aphid infestation, the plants were harvested and the leaf material immediately submersed in liquid nitrogen. The aphids were on the plants for the same amount of time that the pre-conditioning aphid genotype was allowed on the plant in the first experiment. The leaf material was sampled from the leaf that hosted the greatest number of aphids. It must be noted that after 7 days' growth for these plants there were only two leaves and the majority of aphids were located on the older leaf tissue. RNA was extracted from 100 mg leaf material using a Qiagen RNeasy (Qiagen, Hilden, Germany) kit, following the guidelines in the manual. One extraction was made per plant and the RNA quality was checked to ensure successful extractions. For each treatment (CLO7, DAV95, H1, HF92a, No Aphids), the RNA from five of the 15 plants was randomly pooled resulting in three biological repeats for each of the treatments (three repeats of five pooled plant samples). Each sample was hybridized to a microarray chip (GeneChip Barley1 22k genome array; Affymetrix, Santa Clara, CA, USA) at the Genomic Technologies Core Facility at The University of Manchester. A total of 15 microarray hybridizations were performed. One microarray chip was discarded due to poor quality, which resulted in three replicates for each treatment except for the 'no-aphid' treatment. Within this paper, the main comparisons are made between the aphid genotype treatments, which all had three replicates. Throughout the paper, an increase in transcript abundance is noted as up-regulation and a decrease in transcript abundance as down-regulation. When one aphid genotype induced a response in the plant that was not induced by another aphid genotype, this is described this as differential expression/regulation.

Data analysis

Microarray data were analysed using the puma package implemented in Bioconductor (Pearson *et al.*, 2009). This software uses a Bayesian approach (multi-mgMOS method; Liu *et al.*, 2006) to associate credibility intervals with expression levels. Therefore, it does not calculate *P*-values, but rather the probability of a positive log ratio (PPLR), which gives a ranking of the significance of differential expression of transcripts but does not actually calculate a false-discovery rate (Liu *et al.*, 2006). Fold changes (FC), i.e. the ratio between expression levels, were calculated from the multi-mgMOS normalized expression data. A transcript was considered differentially expressed if the expression level (averaged across replicates) between treatments was different by a two-fold expression amount, and if the PPLR value was within the 15% tail of the distribution. A previous paper has used similar FC levels, with slightly more generous PPLR levels of 20% at each tail (Packham *et al.*, 2009). This means our criteria were FC $\leq$ 0.5 and PPLR $\geq$ 0.85 for down-regulated transcripts, or FC $\geq$ 2 and PPLR $\leq$ 0.15 for up-regulated transcripts. Information concerning target description, gene symbol, gene title, pathway, Gene

Ontology (GO) biological process term, GO molecular function and GO cellular component term was looked up for each probe in the NetAffx Analysis Center database (https://www.affymetrix.com). In addition, the transcripts were also annotated using the Barley1.77 BEST BLASTX (www.harvest-web.org) and UniProt (www.uniprot.org).

Validation of microarray results using real-time PCR

cDNA was transcribed from 1 μg of total RNA using the QuantiTect Reverse Transcription Kit (Qiagen catalogue no. 205311) according to the manufacturer's instructions. The expression levels of a selection of contigs (Contig6344_at, Contig25242_at, ContigD10057_at, Contig5788_at, Contig1583_at, D10057_AT; Supporting Information, Table S1) across all aphid treatments were determined by quantitative real-time PCR using the Quantitect real-time PCR kit (Qiagen catalogue no. 204143). The PCR reactions were performed in triplicate (Naseeb & Delneri, 2012) and relative fold change in expression was calculated according to the C_t method (Schmittgen & Livak, 2008), using the contig HV09A09u_s (housekeeping gene) as a reference. The expression levels of these contigs from the microarray and the real-time PCR, across different aphid treatments, was compared using a paired *t*-test to show that there was a high degree of association between the methods ($t = 4.27$, d.f. = 9, $P = 0.002$). The strong positive correlation between the samples using the two methods also shows this high degree of association ($r = 0.978$, $P < 0.0001$; Supporting Information, Fig. S1), thus validating the microarray results.

RESULTS

Aphid pre-conditioned plants affect the population growth rate of other aphid genotypes

Here, aphid population growth rate was measured on plants that had been pre-conditioned by itself or another aphid genotype. Final aphid number was influenced by the focal aphid genotype (post-conditioning aphid: $F_{3,309} = 8.68$, $P < 0.0001$) and this was mediated by the genotype of the pre-conditioning aphid genotype (Interaction effect: $F_{9,300} = 3.36$, $P < 0.001$; Fig. 1). Thus, the effect of post-conditioning aphid genotype was dependent on the genotype of the pre-conditioning aphid. For example, for the CLO7 aphid genotype, the number of aphids on the plants was reduced when the plants were pre-conditioned with aphid genotypes DAV95 ($t_{75} = -2.47$, $P = 0.016$) and H1 ($t_{75} = -3.51$, $P < 0.001$) and the number of DAV95 aphids was reduced when plants were pre-conditioned with aphid genotypes CLO7 ($t_{76} = -2.25$, $P = 0.027$) and HF92a ($t_{76} = -1.87$, $P = 0.066$). In some cases, the reduction in performance was approaching 50%, e.g. CLO7 aphids on plants pre-conditioned by H1 aphids (Fig. 1).

Aphid genotypes induced differential gene expression in plants

The global expression profile of the barley genome exposed to different aphid genotypes was investigated using microarrays. Out of 22 740 transcripts, the expression of 1018 genes were altered by at least one aphid genotype compared with another. Transcripts were considered significantly up-regulated when the FC ≥ 2 and PPLR value ≤ 0.15 and significantly down-regulated when the FC ≤ 0.5 and PPLR ≥ 0.85.

Of the 1018 transcripts, where the expression was altered due to the exposure to different aphid genotypes, 356 had annotation information. From these, 101 are related to known defence responses in plants, the rest were categorized as nucleotide binding, metabolic processes, oxidation-reduction processes and transport, among others (see Supporting Information, Table S2 for the full list of 356 annotated contigs). Due to the already large number of differentially expressed genes categorized as known plant defence responses, these are the primary focus of this paper.

The main pathways found to be differentially induced by different aphid genotypes were the well-known phloem-feeding insect-inducing jasmonate, ethylene and abscisic acid pathways. In addition, other differentially expressed genes belonged to the thionin, hordein, cytochrome P450, Lipoxygenase and ubiquitin groups (Table 1). Aphid genotypes DAV95 and CLO7 induced the greatest number of up-regulated transcripts compared with the other genotypes, with H1 upregulating very few (Fig. 2). We found 15 transcripts to be up-regulated (and nine down-regulated) by only one aphid genotype compared with the others. The majority of these were up-regulated by DAV95 aphids due to a strong genotype-specific induction of thionin related transcripts (Table 1; Fig. 3), and down-regulated by H1 due to a reduced induction of jasmonate-related transcripts (Table 1; Fig. 3). HF92a aphids did not induce differential expression of any transcripts compared with all other aphid genotypes (Table 1).

Relationship between aphid-induced transcriptome profile in *H. vulgare* and secondary aphid colonizations

The amount of transcriptome changes induced by a single aphid genotype in barley was not associated

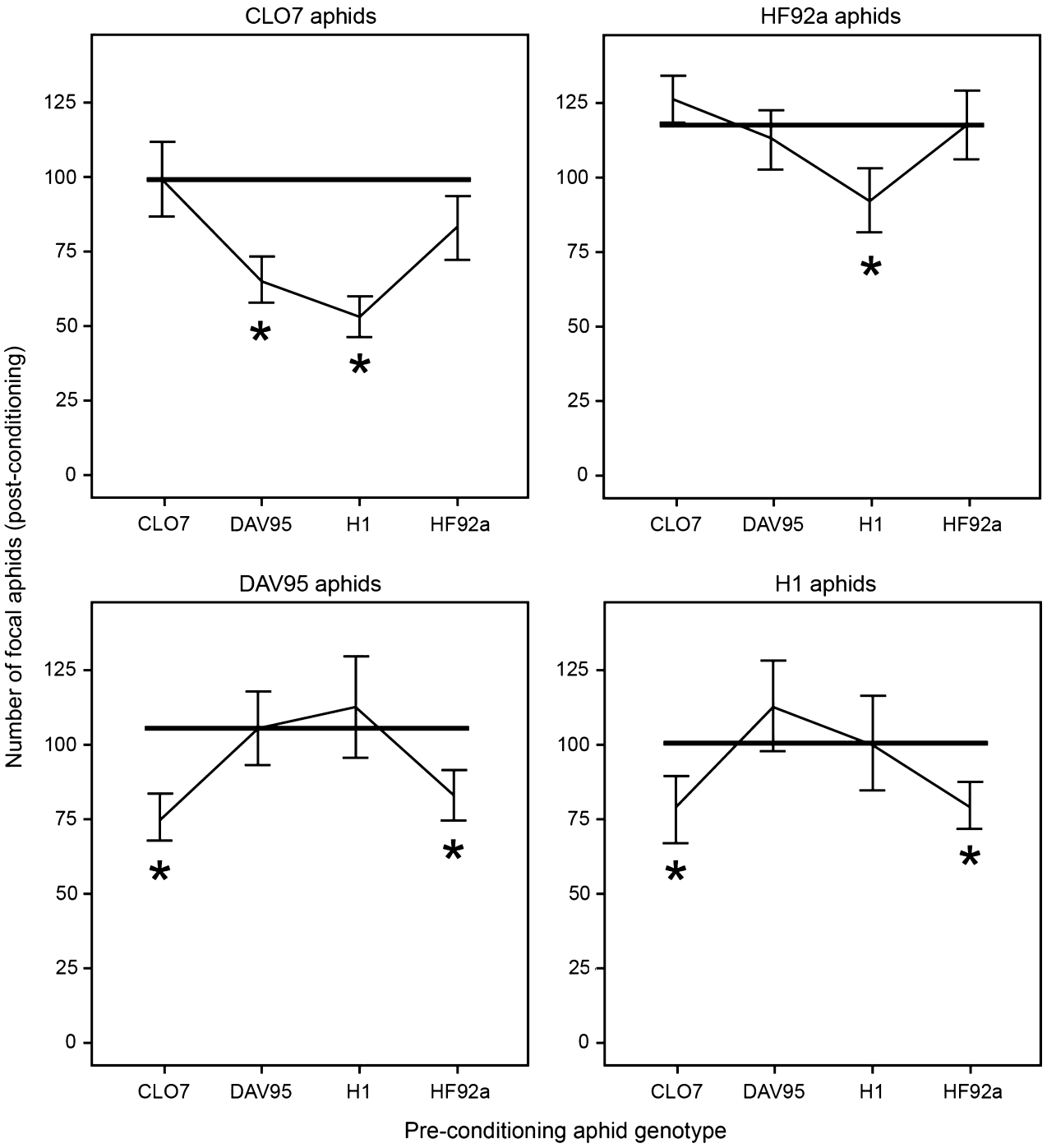

Figure 1. The number of focal aphids from the post-conditioning genotype (each panel), when Steptoe barley has been pre-conditioned with another aphid genotype (bottom axis). Error bars represent ± 1 SE. *$P < 0.05$ indicates significant deviation from the number of aphids when pre- and post-conditioning aphid are the same (black horizontal bars).

with the reduced population growth rate of subsequent aphid populations colonizing this plant genotype. There was no correlation between the number of post-conditioning aphid genotypes reported in experiment 1, and the number of up-regulated (Pearson's $r = -0.08$, $P = 0.15$) or down-regulated (Pearson's $r = -0.07$, $P = 0.21$) transcripts in the plant induced by the pre-conditioning aphid genotype in experiment 2. This result suggests that specific gene function, rather than the global amount of

Table 1 . Summary of known defence-related transcripts that are up-regulated in the plant due to exposure to specific aphid genotypes. Values show significant fold changes in expression between the first aphid genotype (top row) compared with the second aphid genotype (second row), for all genotype comparisons

		CLO7			DAV95			H1			HF92a		
Annotation	Contig	DAV95	H1	HF92a	CLO7	H1	HF92a	CLO7	DAV95	HF92a	CLO7	DAV95	H1
Jasmonate													
Jasmonate induced protein	rbags15p13_s_at		3.62										3.70
Jasmonate induced protein	Contig2899_at		6.23	2.76		3.08							
Jasmonate induced protein	Contig2899_s_at	2.02	3.59										2.67
Jasmonate induced protein	Contig2900_at		4.46			2.69							3.90
Jasmonate induced protein	Contig6155_at		2.57										
Jasmonate induced protein	HX05K09r_at		2.27			2.44							2.22
Jasmonate induced protein	Contig1675_s_at		2.41			2.37							2.06
Jasmonate induced protein	Contig1679_s_at		7.07			4.43							6.15
Jasmonate induced protein	Contig1686_at		7.23			4.75							3.91
Jasmonate induced protein	Contig7886_at		5.56	4.75		3.97	3.39						
Jasmonate induced protein	Contig7887_at		2.51	3.99		2.40	3.81						
Abscisic acid													
Abscisic acid stimulus response	Contig7589_at			2.68									
Abscisic acid-induced protein ABA7	Contig6276_s_at						3.09						
Thionin													
Alpha-hordothionin	Contig254_x_at				2.25			2.35					
Alpha-hordothionin	Contig796_at				2.49	2.53	2.30						
Alpha-hordothionin	HB29O17r_x_at				3.58	2.78	3.38						
Thionin	Contig1570_s_at		15.01			21.61							11.79
Thionin	Contig1579_s_at				2.41	2.02	2.60						
Thionin precursor	Contig2653_s_at				6.53		2.27						
Thionin precursor	Contig1582_x_at				2.09	5.68	3.18						
Thionin precursor	Contig1583_at				4.93	5.08	2.49						
Thionin precursor, leaf	Contig1580_x_at		6.35	2.32		6.66	2.43						
Thionin, putative	rbags16e08_at					2.13							
Gamma-thionin-like protein	Contig22143_at				3.76	2.19					4.25		2.48
Hordein													
Gamma-hordein 1 precursor	HVSMEi0004O10r2_at				3.47	4.27	3.27						
Hordein	HB26H24r_x_at				2.88	2.76	3.57						
Gamma-hordein 3	EBed07_SQ003_I06_at	3.35	3.44	2.67									

Table 1. *Continued*

Annotation	Contig	CLO7			DAV95			H1			HF92a		
		DAV95	H1	HF92a	CLO7	H1	HF92a	CLO7	DAV95	HF92a	CLO7	DAV95	H1
Cytochrome P450													
Cytochrome P450	HVSMEf0019N09r2_at				4.71						4.60		
Cytochrome P450, putative	Contig14804_at				2.07			2.66		2.58			
Cytochrome P450, putative	Contig9308_s_at	7.41	4.38										
Cytochrome P450-like protein	HVSMEn0022N15r2_at				3.24	2.06	3.00						
Putative cytochrome P450	Contig12509_at					2.78	4.21						
Lipoxygenase													
Lipoxygenase 2 (LoxC)	HI02E21u_s_at	5.15	8.38									6.34	10.32
Lipoxygenase 2 (LoxC)	HY03N19u_s_at		6.34			3.61							6.59
Ubiquitin related													
Ubiquitin conjugating enzyme	HVSMEm0014A16r2_s_at	2.13							2.10				
Ubiquitin precursor	Contig1106_s_at								2.14				
Ubiquitin-dependent protein	Contig10844_at	4.00		2.27									
Ubiquitin-dependent protein	Contig13732_at	2.82		2.28									
Ubiquitin-dependent protein	Contig2526_at	2.09											
Ubiquitin-dependent protein	Contig4905_at	2.90											
Response to wounding													
Response to wounding	Contig20686_at				2.13		2.28						
Response to wounding	HF18A22r_s_at								2.21				
Response to wounding	EBem06_SQ003_C15_at										3.67		
Probable wound-induced protein	Contig3514_at				3.01	2.44	2.26						

Up-regulated transcripts shown only, number is the fold change between the comparisons. All given fold change values had significant PPLR values. For a full list of differentially expressed transcripts (see Supporting Information, Table S1).

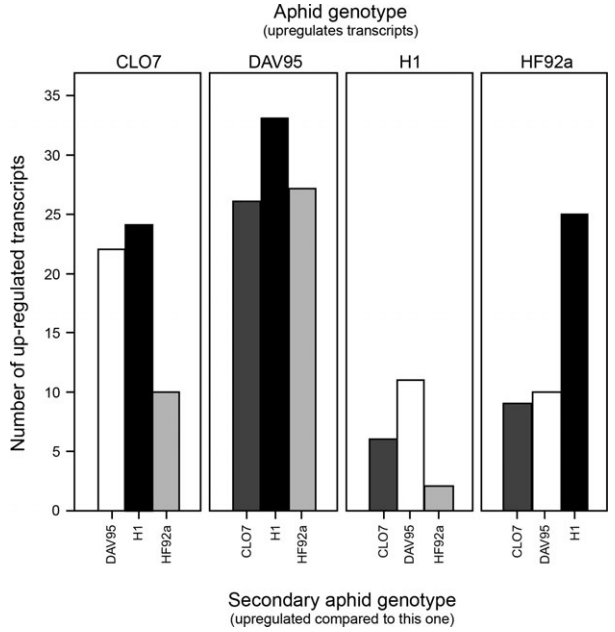

Figure 2. The number of transcripts up-regulated by one aphid genotype compared with all others. Columns show the aphid genotype that has up-regulated the transcript, compared with the other aphid genotype (each individual bar).

transcriptome changes, is more important to prevent further aphid colonization.

DISCUSSION

These results show that aphid population growth rate on a single plant genotype (Steptoe; *H. vulgare*) can be reduced when the plant is pre-conditioned with a different aphid genotype (dependent on the identity of the other aphid genotype). These results concur with Hays *et al.* (1999), who showed that pre-conditioning of a plant with one aphid biotype affects the probing and feeding behaviour of a second aphid biotype. In the present study, not every aphid genotype influenced the population growth rate of all other aphid genotypes, indicating there is variation for these traits depending on the genotype of both aphids. By carefully removing all aphids from the plants before introducing the second genotype it was shown that the effect of one aphid on another in a single plant genotype is likely to occur via physiological changes in the plant, such as induced defence response. The second part of this study showed that gene expression within this single plant genotype was altered due to exposure to different aphid genotypes. This experiment produced evidence to show that the different aphid genotypes induced differential expression of plant defence-related genes and this could explain the different effects the aphid genotypes can have on one another.

We have studied these four aphid genotypes in a number of papers providing us with multiple data sets on the performance of these aphid genotypes on previously uninfested plants (Supporting Information, Fig. S2). Two of these papers focus on the effect of plant genotype and the presence of other aphid genotypes on the performance and preference of the focal aphid genotype (Zytynska & Preziosi, 2011, 2013), while the others consider the effect of rhizobacteria (Tétard-Jones *et al.*, 2007) and a hemiparasitic plant (Zytynska *et al.*, 2014) on these interactions, and the effect of these plant-aphid genetic interactions on parasitoid wasps (Zytynska *et al.*, 2010). The different plant genotypes used vary with regards to the traits of interest when they were bred (e.g. Steptoe is high yielding, Morex and BCD47 bred for malting and Baronesse used for feed). In addition, the two OWB genotypes were selectively bred for dominant and recessive characteristics of 12 morphological traits (Costa *et al.*, 2001). Overall, each aphid actually exhibits average performance on our focal plant genotype (Steptoe) when compared with the other barley genotypes tested (Zytynska & Preziosi, 2011); however, it is likely that H1 and DAV95 are more affected by plant defences than the other aphid genotypes, since they show a generally reduced performance compared with CLO7 and HF92a (Supporting Information, Fig. S2). Despite the average reproductive rate of DAV95 on Steptoe and potential reduced tolerance to plant defences, DAV95 actually shows active choice towards this barley in a choice experiment (Zytynska & Preziosi, 2011); however, when in competition with CLO7 or HF92a this choice behaviour is reduced (Zytynska & Preziosi, 2013). The results here suggest that these effects may be mediated by differential induction of plant defences that lead to the environment of the host-plant changing due to the identity of the aphid genotypes feeding on it. Our current results show that pre-conditioning of a plant with an aphid genotype does not significantly increase the population of growth of another, indicating little facilitation between aphid genotypes.

In both current experiments, the plants were exposed to five aphids of the pre-conditioning genotype for 7 days before either extracting the RNA or adding the second aphid genotype. Thus, our measure of gene expression corresponds to the time point when the post-conditioning aphids were introduced to the plants. Often studies on insect feeding effects on plant gene expression have a duration of 3 days or less (Thompson & Goggin, 2006), but others have found many genes to be still induced at day 4 (Moran

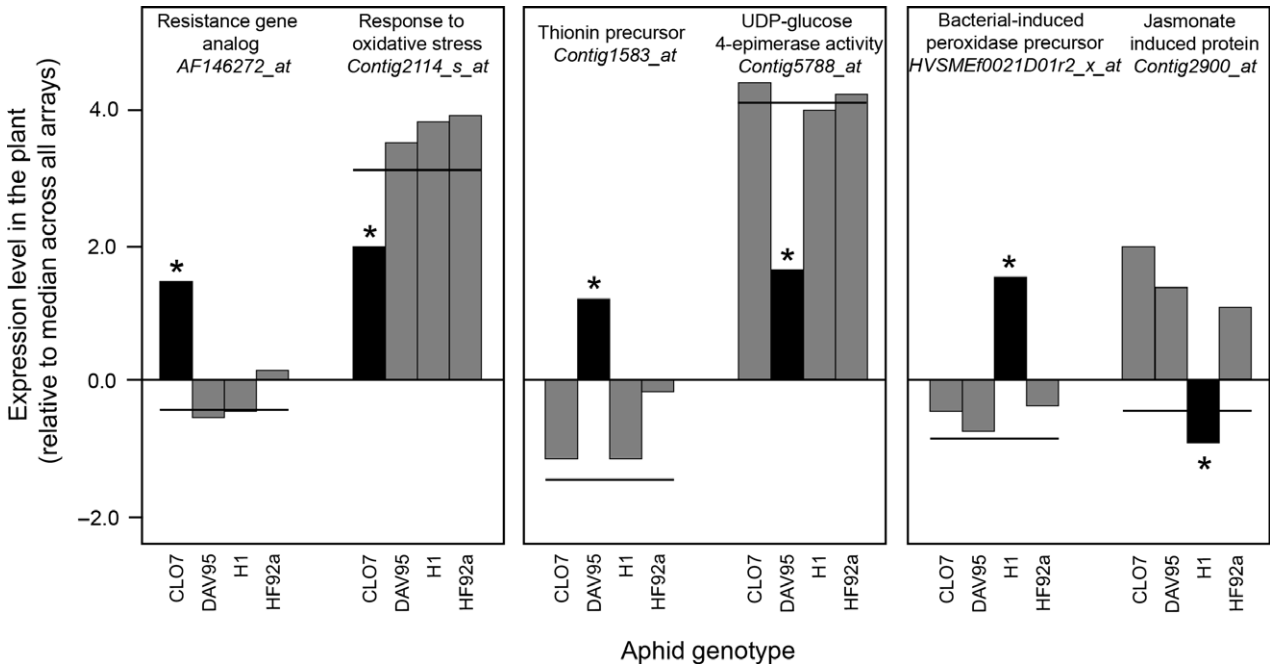

Figure 3. Example data showing four transcripts with increased level of expression and four with decreased level of expression due to the exposure to specific aphid genotypes. The box on the left shows the genotype-specific transcripts up- or down-regulated by CLO7, the middle for DAV95 and the right-hand box for H1 aphids. HF92a aphids did not induce any genotype-specific transcripts. The black horizontal lines represent the expression level in control plants ('no aphids'). Note the values relate to microarray data. Expression levels are normalized data showing level relative to the median across all arrays.

et al., 2002) and late-response genes expressed at day 7 that were not present at day 3 (Divol *et al.*, 2005). Previous work by Delp *et al.* (2009) found the strongest induction of aphid resistance genes in barley plant on day 4 of a time course experiment, with RNA collected every 24 h from 0 to 4 days. As there is no single time point at which to reliably detect all expressed genes, either a general time point (e.g. day 5 as in this experiment) must be chosen or a time-series can be conducted, where samples could be pooled for analysis. Further, recruitment of basal defences by susceptible plants may occur later than for resistant plants and thus a longer time-span maximises the number of expressed genes in the samples (Fu & Dong, 2013). Other environmental conditions (e.g. soil substrate) did vary between the two experiments due to using a more controlled environment for the gene expression experiment where high quality RNA was required. However, these differences highlight the more general biological effects of the results, which are thus likely to be independent from specific environmental conditions.

Of all the aphid genotypes used, DAV95 aphids induced the greatest number of up- and down-regulated transcripts in the plants, compared with the other genotypes. In particular, DAV95 aphids induced greater expression of thionin transcripts in the plant, which are related to plant defence (Stec, 2006; Mehrabi, Åhman & Jonsson, 2014). Specifically, thionins are antimicrobial peptides that are toxic to bacteria, fungi, yeasts and mammalian cell types *in vitro*, and they have been most well studied in plant defence to pathogens (Ji *et al.*, 2015). Aphids require a primary bacterial symbiont for nutritional resources and, very speculatively, thionins could disrupt this pathway and thus have an impact on aphid performance. However, DAV95 aphids only reduced the population size of CLO7 aphids, thus it is possible that CLO7 aphids are susceptible to thionin-based defences, whereas the other aphid genotypes are less affected. Variation in traits involved in the suppression of plant defences, e.g. salivary enzymes (Miles, 1999; Prado & Tjallingii, 2007; Elzinga & Jander, 2013; Furch *et al.*, 2015), or in the relationship between aphids and its primary symbiont could explain differences among aphid genotypes. Further experiments producing empirical data would be needed to support this. Plants exposed to H1 showed the smallest number of up-regulated transcripts compared with the other genotypes, with significant reduced induction of several jasmonate induced proteins. H1 aphids also induced a genotype-specific

up-regulation of a bacterial-induced peroxidise precursor, and DAV95 aphids induced a probable wound-induced protein. Aphids are often considered to induce a plant defence response more similar to that of bacteria and pathogens than a mechanical wounding response, as their feeding methods minimize tissue damage (Walling, 2000). Indeed, along with a reduced induction of jasmonate-related proteins by H1, this may show that it does not cause so much mechanical damage as the other genotype, specifically compared with DAV95 (Moran *et al.*, 2002). There was no single aphid genotype that induced a response in the plant that reduced the population growth rate of all other genotypes, and this can be explained in two different ways. The first is that the genes of interest are those only induced by the pre-conditioning aphid genotype, compared with the aphid genotype or genotypes that also show reduced population growth rate. Alternatively, an aphid genotype increases the expression of a gene in the plant relative to all other aphid genotypes, but one (or more) of the aphid genotypes has tolerance to the related defence in the plant. This would mean that those aphids without a reduced population growth rate are exhibiting tolerance to this induced defence or perhaps in turn, they suppress these themselves.

Specific gene pathways are well known to be involved in plant defence against herbivores and lead to reduction in aphid colonization through active preference for control plants rather than those with induced defences, for example the jasmonate and salicylate pathways (Walling, 2000; Goggin, 2007). Recent work on these pathways show that for *S. avenae* aphids on wheat, methyl-jasmonate likely acts as a feeding deterrent in the mesophyll, whereas salicylic acid acts at the level of the phloem (Cao, Wang & Liu, 2014). There was no association between the numbers of transcripts up- or down-regulated in the plant and the number of aphids in our experiments and this suggests that gene function, rather than the number of expressed genes, is of greater importance. Several other studies have identified many different genes that are induced in a plant due to aphid feeding (Moran *et al.*, 2002; Voelckel *et al.*, 2004; Zhu-Salzman *et al.*, 2004; De Vos *et al.*, 2005; Divol *et al.*, 2005; Couldridge *et al.*, 2007; Kusnierczyk *et al.*, 2008; Delp *et al.*, 2009). The use of microarray data can, however, sometimes mislead the functional understanding of the traits involved, as annotations are described as opposed to traits (Kant & Baldwin, 2007). Thus, the results presented in this paper are only a step forward in understanding how within-species genetic variation in an insect species can alter gene expression within a single genotype of plant. These results were validated using real-time PCR and this technique could further be used to identify specific candidate genes involved in plant defence against particular aphid genotypes, along with more recent techniques such as RNA sequencing (Ozsolak & Milos, 2010).

The differences described here between aphid genotypes are expected to be subtle, for example, through the behaviour of feeding, such as probing or stylet insertion, and potentially due to genetic differences in saliva enzymes (Miles, 1999; Howe & Jander, 2008; Elzinga & Jander, 2013). Intraspecific variation in aphids for the effectiveness of plant resistance has been previously shown to involve gene-for-gene interactions (Roche *et al.*, 1997; Rossi *et al.*, 1998), where resistance is not conferred in the plant to all aphid biotypes studied. Here, we show the wide range of possible genes involved in these interactions and the potential impacts at the population level. If an aphid genotype induces a defence response in the plant that cannot be tolerated by other genotypes, then the first will increase in abundance as it outcompetes the others. Therefore, the genes related to plant defence avoidance or suppression in the aphids will also increase in frequency. Non-random associations of aphid genotypes on different plant genotypes (Zytynska & Preziosi, 2011, 2013) can create differentiation between populations, particularly if aphid preference is to some extent positively correlated with performance. This may be further enhanced in agricultural species through the planting of large areas with a single crop variety, and lead to the spread of common clones, e.g. *S. avenae* clone 53 that was found in all field sites by Llewellyn *et al.* (2004). Such common clones could also have further economic impacts by evolving other traits such as insecticide resistance (Foster *et al.*, 2014). However, conversely, genetic polymorphism in the aphid population could be maintained through these competitive interactions between aphid genotypes, especially when no single aphid genotype is superior relative to all others, even on a single plant genotype in a genotypically diverse aphid population.

In conclusion, this paper shows that the expression of plant defence-related genes (in a single plant genotype) induced by phloem-feeding insects is dependent on the genotype of the feeding insect. It also demonstrates that pre-conditioning of a plant with one insect genotype can reduce the fitness of another genotype, via physiological changes in the plant. Interactions via plant-induced resistance and susceptibility within a multi-genotypic aphid population could lead to evolutionary changes through altering the competitive ability of each aphid genotype (Zytynska & Preziosi, 2013). These differences could also have community-wide effects, with the defence responses induced in a plant by one insect

species affecting another herbivore species that happens to be feeding on the plant (Van Zandt & Agrawal, 2004; Chen, 2008; Broekgaarden *et al.*, 2010). Lastly, understanding how both resistant and susceptible genotypes of crop plants deal with attack from multiple insect genotypes could uncover mechanisms to be exploited within sustainable agricultural pest management.

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AUTHOR CONTRIBUTIONS

SEZ and RFP designed the experiment. SEZ performed the experiments. VJ analysed the microarray data, and SN and DD performed the rt-PCR. SEZ and RFP wrote the first draft of the manuscript with substantial comments from all other authors.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Correlation of microarray and RT-PCR data.
Figure S2. Meta-analysis of aphid performance on Steptoe.
Table S1. RT-PCR primer details.
Table S2. Microarray data for annotated transcripts with differential expression across aphid genotypes.

SHARED DATA

The primary microarray datasets will be made publicly available through PLEXdb: gene expression resources for plants and plant pathogens Dash *et al*. (2012). Accession number BB107.