Cryptic Virulence and Avirulence Alleles Revealed by Controlled Sexual Recombination in Pea Aphids

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ABSTRACT Although aphids are worldwide crop pests, little is known about aphid effector genes underlying virulence and avirulence. Here we show that controlling the genetics of both aphid and host can reveal novel recombinant genotypes with previously undetected allelic variation in both virulence and avirulence functions. Clonal F₁ progeny populations were derived from reciprocal crosses and self-matings between two parental genotypes of pea aphid (*Acyrtosiphon pisum*) differing in virulence on a *Medicago truncatula* host carrying the *RAP1* and *RAP2* resistance genes. These populations showed Mendelian segregation consistent with aphid performance being controlled largely by a dominant virulence allele derived from only one parent. Altered segregation ratios on near-isogenic host genotypes differing in the region carrying *RAP1* were indicative of additional heritable functions likely related to avirulence genes originating from both parents. Unexpectedly, some virulent F₁ progeny were recovered from selfing of an avirulent parent, suggesting a reservoir of cryptic alleles. Host chlorosis was associated with virulence, whereas necrotic hypersensitive-like response was not. No maternal inheritance was found for any of these characteristics, ruling out sex-linked, cytoplasmic, and endosymbiotic factors. Our results demonstrate the tractability of dissecting the genetic basis of pest-host resistance mechanisms and indicate that the annual sexual cycle in aphids may lead to frequent novel genotypes with both increased and decreased virulence. Availability of genomes for both pest and host can facilitate definition of cognate gene-for-gene relationships, potentially leading to selection of crop genotypes with multiple resistance traits.

KEYWORDS aphid; virulence; avirulence; F₁ hybrid; near-isogenic line; *R* gene; gene-for-gene interaction; effector

ABOUT 250 of the 4400 known species of aphids feed on agricultural crops (Oerke *et al.* 1994; Blackman and Eastop 2000). Given their wide host ranges and high fecundity, aphids can have dramatic negative impacts on host plants (Dixon 1987). Aphids feed specifically from phloem sieve elements, causing damage directly by ingestion of plant nutrients and by acting as vectors of many major plant viruses (Ng and Perry 2004). In many instances, aphids may manipulate or evade host defenses by secretion of saliva into phloem sieve elements and by minimizing cellular damage by moving their stylets along intercellular paths (Miles 1999; Will *et al.* 2007).

Aphids have unusual reproductive cycles, typically with numerous asexual parthenogenetic generations followed by one sexual phase in winter (Via 1992). Novel genetic diversity

may be subsequently stably propagated through asexual parthenogenetic lineages (Blackman 1985). Aphid populations harbor a considerable amount of genetic variability (Via 1989, 1991a; Black *et al.* 1992), and cyclic parthenogens can evolve nearly as fast as species with sexual reproduction each generation (Lynch and Gabriel 1983). The genetic variation arising from mutation and recombination enables evolution of virulent biotypes and specialized host races (Gould 1983; Via 1991a; Puterka and Peters 1989, 1990; Sandstrom 1994) and potentially can lead to speciation.

In Europe and central Asia, the pea aphid (*Acyrtosiphum pisum*) appears to be a complex of races and subspecies with different host ranges and preferences (Müller 1980; Müller and Steiner 1985). Although pea aphids feed exclusively on plants of the Fabaceae (legume) family, genetically distinct host races are specialized to exploit particular host species (Peccoud *et al.* 2009). Populations of pea aphid on alfalfa (*Medicago sativa*) and red clover (*Trifolium pratense*) exhibit strong local adaptation (Via 1991a) with clear preferences for and increased fitness on these species (Caillaud and Via 2000; Via *et al.* 2000). Although such sympatric populations

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doi: 10.1534/genetics.114.173088

Manuscript received October 3, 2014; accepted for publication December 9, 2014; published Early Online December 17, 2014.

Supporting information is available online at <http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.173088/-DC1>.

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could interbreed because of the approximately 10% inter-host migration in the field (Via 1999), F₁ hybrids had lower fitness on both hosts compared with the specialized parents (Via *et al.* 2000). Similarly, strong species-level host adaptation was detected in pea aphid populations sampled from eight members of the Fabaceae (Ferrari *et al.* 2008). Attempts to condition pea aphid clones on alternative hosts over several generations are typically unsuccessful, further evidence that host adaptations in this species are genetically based (Via 1991b).

Concepts of virulence and resistance for insect-plant interactions have frequently drawn on knowledge of pathogen-plant systems. In the latter, incompatibility is often associated with interactions between products of plant resistance genes (*R* genes) and pathogen avirulence genes (*avr* genes), providing the basis for the classic gene-for-gene model (Flor 1955). For several crops, quantitative trait loci (QTL) or single dominant *R* genes have been identified that reduce aphid performance (Smith and Boyko 2007). Genes conferring resistance to the Russian wheat aphid (*Diuraphis noxia*) have been reported in barley, rye, and wheat (Smith 1999) and to the greenbug (*Schizaphis graminum*) in sorghum (Teetes *et al.* 1999). *R* genes (*TTR*, *AKR*, and *APR*) have been identified in the barrel medic (*M. truncatula*) that are effective against spotted the alfalfa aphid (*Therioaphis trifolii*), the blue-green aphid (*A. kondoi*), and the pea aphid (*A. pisum*), respectively (Klingler *et al.* 2005, 2007; Gao *et al.* 2007; Guo *et al.* 2009). Unusually, the *R* gene *Mi-1.2* from tomato confers resistance to multiple taxonomically divergent invertebrate pests: root-knot nematodes (*Meloidogyne* spp.), potato aphid (*Macrosiphum euphorbiae*), and sweet potato whitefly (*Bemisia tabaci*) (Milligan *et al.* 1998; Nombela *et al.* 2003). Although many pest resistance loci have been identified, the *Mi-1.2* gene of tomato (Rossi *et al.* 1998) and the *Vat* gene of melon (Dogimont *et al.* 2014) are presently the only two reported cloned *R* genes against insects, and both encode proteins in the nucleotide-binding-leucine-rich repeat (NB-LRR) *R* gene family. Aphid *R* genes appear to conform broadly to predictions of the gene-for-gene model, but crucially, none of the cognate aphid *avr* genes have yet been defined (Jaouannet *et al.* 2014).

Uncovering the genetic basis of virulence in aphids against plants is vital both to the development of durable resistant varieties and to understanding mechanisms of aphid speciation. Although early genetic experiments often were limited by unreliable egg hatching following controlled crosses, improved techniques have facilitated studies of inheritance of virulence and other factors (Via 1992). For example, greenbug inheritance studies analyzing feeding lesions on wheat carrying resistance genes *Gb2* and *Gb3* revealed that virulence is recessively controlled by duplicate genes and a dominant epistatic modifier gene (Puterka and Peters 1989). Similar interactions were evident in a study of three greenbug biotypes on three sorghum genotypes carrying resistance introgressed from different germ plasm sources (Puterka and Peters 1995). Based on reciprocal matings and test crosses, hessian fly (*Mayetiola destructor*) resistance in wheat plants carrying the *H9* gene was shown to

relate to a virulence trait controlled by a single recessive sex-linked gene *vH9* (Formisoh *et al.* 1996). In the brown planthopper (*Nilaparvata lugens* Stål), inheritance of virulence in terms of honeydew excretion and weight gain was similarly sex linked (Fang *et al.* 2005).

However, quantitative data directly assessing the genetic basis of aphid performance, based on the ability to survive and reproduce on particular host genotypes, are generally lacking. In this study, we report the inheritance of aphid virulence and avirulence functions using the pea aphid-*M. truncatula* model. The *RAP1* gene in *M. truncatula* confers race-specific resistance to pea aphid clone PS01 but is ineffective against clone LL01 (Stewart *et al.* 2009). Similar to LL01, another clone, N116, was virulent on *RAP1* genotypes and on a wide range of other natural and cultivated *M. truncatula* genotypes (Kanvil *et al.* 2014). In contrast, PS01 was avirulent on the majority of *M. truncatula* genotypes. The divergent performance of these clones enabled exploration of inheritance of aphid virulence. A series of F₁ progenies derived from selfing and reciprocal crossing of clones N116 and PS01 were assessed on resistant and susceptible *M. truncatula* genotypes. The resistant host was Jemalong A17, the reference genotype for the *M. truncatula* genome sequence (Young *et al.* 2011). The susceptible host was DZA315.16, an Algerian ecotype. Jemalong A17 and DZA315.16 are highly polymorphic and have been used as parents to generate recombinant inbred lines (RILs) developed from an F₂ mapping population (Thoquet *et al.* 2002; Torregrosa *et al.* 2004). Mapping of QTL from this population identified two loci (*RAP1* and *RAP2*) conferring pea aphid resistance and a third independent locus controlling a hypersensitive-like (HR-like) response that results in aphid-induced lesions (*AIL*) (Stewart *et al.* 2009). From the RILs, near-isogenic lines (NILs) were developed that differed at the *RAP1* genomic region, enabling further testing of virulence-resistance relationships. In several of the F₁ aphid populations, we found clear Mendelian segregation of virulence/avirulence. Phenotypes of some self progeny were dissimilar to those of their parents, indicating that recombination resulting from a single round of mating can reveal cryptic alleles that increase or decrease fitness on host plants.

Materials and Methods

M. truncatula

Seeds of *M. truncatula* genotypes Jemalong A17 (hereafter called A17) and DZA315.16 were supplied by the South Australian Research and Development Institute Genetic Resource Centre. Seeds of NILs derived from a mapping population from a cross between A17 and DZA315.16 were generated by Stewart *et al.* (2009). Seeds were scarified with sandpaper and soaked in distilled water for 3–4 hr at room temperature and then incubated overnight on damp filter paper in petri dishes in darkness at 4°. After germination for a further 24 hr in the dark at room temperature, the

seeds were planted in compost (4:1 organic compost Levington's F2 with sand:perlite) and grown in a growth room [21.5°, 16-hr day length, 180 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ light from Starcoat T5 high-output fluorescent tubes (GE Lighting, Budapest, Hungary)]. Plants used in experiments were 3 weeks old with four to five trifoliate leaves.

Aphids

Pea aphid (*A. pisum*) clones were from sources in the United Kingdom (Kanvil *et al.* 2014). Clone PS01 was collected from *Vicia faba* by Glen Powell, and clone N116 was originally collected from *M. sativa* by Julia Ferrari. These clones had contrasting behaviors when tested on a panel of diverse *M. truncatula* genotypes (Kanvil *et al.* 2014). Clone N116 was highly virulent on most hosts, whereas PS01 was avirulent on the reference genotype A17 but virulent on DZA315.16. Clone PS01 was previously shown to be avirulent on hosts carrying the *RAP1* resistance gene, whereas *RAP1* was ineffective against N116 (Stewart *et al.* 2009; Stewart 2010). PS01 and N116 were confirmed as genetically divergent, belonging to *Pisum sativum/V. faba* and *M. sativa* host race groups, respectively (Kanvil *et al.* 2014).

Asexual clones were kept as colonies on broad bean (*V. faba* var. *minor*) under constant 19° temperature, with long days (LDs; 16-hr day, 8-hr night) provided by fluorescent lighting, as described earlier. Aphids were restricted to single plants by perforated plastic bag enclosures. To obtain 1- to 2-day-old nymphs, adult aphids were transferred to new *V. faba* seedlings under glass tubes (2.3 cm diameter and 5 cm high) using a fine brush and allowed to reproduce for 24 hr before they were removed. The nymphs produced remained on *V. faba* enclosed in perforated bags for an appropriate number of days before each experiment.

Induction of aphid sexual forms

For the induction of sexual forms, parthenogenetic fourth-instar nymphs from LD stock cultures were transferred to *V. faba* seedlings and exposed to short-day conditions (12.5 hr light, 11.5 hr dark) at 14° until they became adult and produced nymphs. Adults then were removed, and nymphs were enclosed under perforated bags until adult. From fourth-instar nymphs to the development of sexuparae took 19 days. Subsequently, adult sexuparae were transferred to new *V. faba* seedlings under glass tubes and kept under 16-hr LDs at constant 19° to produce sexual offspring (males and oviparae).

Mating and raising of F₁ progeny

Six adult oviparae and four adult males derived from each parental clone (N116 and PS01) were maintained on *V. faba* seedlings enclosed under a plastic tube (2.5 cm diameter and 9.5 cm high) with a perforated plastic sheet glued at the top to prevent condensation. Four mating combinations were conducted to generate F₁ progeny clones: reciprocal crosses were made between clones N116 and PS01, and parental clones also were selfed. All mating groups were transferred to new plants weekly, and eggs were promptly

removed from foliage before they desiccated, as described by Via (1992). Each leaf was soaked in water to loosen the adhesive attachment of the eggs, and then the eggs were floated off with the aid of a monofilament loop and placed onto wet filter paper in a petri dish. To prevent fungal growth during the long, cold treatment, the eggs were surface sterilized by flooding the petri dishes with a 1:10 dilution of bleach (sodium hypochlorite 5.25% w/v stock solution) for 10 min. The bleach solution was removed, and the eggs were rinsed three times with distilled water. Petri dishes were sealed with Parafilm and incubated at 4° for 105 days (Via 1992).

Following cold treatment, each petri dish was supplied with leaflets of *V. faba* and/or *M. truncatula* genotype DZA315.16 for the F₁ hatchlings to feed on. The dishes then were replaced into cold treatment for a further 5–10 days. Newly hatched fundatrices were transferred daily to pots containing *V. faba* and DZA315.16 plants and raised under LD conditions until they became adult and began reproducing clonally. All progeny clones were reared on *V. faba* for 10 generations before evaluation for virulence. The F₁ progeny clones from interclonal and intracolonial crosses started reproducing on the ninth and eleventh days after birth, respectively. Between 25 and 28 F₁ clones were assessed for each of the four crosses made.

Screening progeny and parental clones

Parental and progeny clones were evaluated for virulence on two different pairs of *M. truncatula* host genotypes. The first comparison was between Jemalong A17 and DZA315.16. The second comparison was on a pair of NILs derived from a mapping population from a cross between A17 and DZA315.16 (LR4 population) (Julier *et al.* 2007). The resistant NIL (rNIL) line 174_5.13 carries the A17 (PS01 resistant) genotype at *RAP1* and the DZA315.16 genotype at *RAP2* and *AIL*, whereas the susceptible NIL (sNIL) line 174_9.10 carries the DZA315.16 allele at all three loci. For each aphid clone, 10 replicate plants of each *M. truncatula* genotype were each infested with five 1- to 2-day-old nymphs. After 8 days for interclonal crosses and 12 days for intracolonial self matings, the number of aphids surviving and the number of nymphs produced were recorded.

Previous work (Kanvil *et al.* 2014) showed that aphid clone N116 causes interveinal chlorosis on *M. truncatula*, whereas PS01 does not. Plants were therefore scored for interveinal chlorosis at 8 or 12 days for cross and self clones, respectively. Infested plants also were scored at these times for the presence of aphid-induced necrotic HR symptoms, defined as brown or whitish spots or patches on the leaves (Kanvil *et al.* 2014).

Statistical analysis and testing of inheritance models

Aphid performance data were analyzed in the statistical program R using the Generalized Linear Model (GLM) simplification with a binomial error distribution for survival and quasi-Poisson error distribution for reproduction. Model simplification involved stepwise grouping of clones with similar performance and led to the minimal adequate model (Crawley 2005). Progeny clones that grouped with the virulent parent N116 in both

survival and reproduction were categorized as virulent (Vir), whereas clones that behaved like the avirulent parent PS01 on hosts carrying *RAP1* were classed as avirulent (Avr). Clones with intermediate performance, significantly different from both parents in survival and/or reproduction, were categorized as intermediates (Int). The observed segregation ratios were tested against hypothesized inheritance models using chi-square analysis with a minimum significance level of $P = 0.05$.

Results

Pea aphid progeny performance on Jemalong A17 and DZA315.16

F₁ reciprocal cross and self progeny derived from the highly divergent PS01 and N116 parental pea aphid clones were first evaluated for survival and reproduction on the *M. truncatula* host genotypes Jemalong A17 and DZA315.16. PS01 is avirulent on A17, which carries the *RAP1* resistance gene, but virulent on DZA315.16, which lacks functional *RAP1*. Parental N116 is virulent on both these hosts (Kanvil *et al.* 2014). On DZA315.16, all F₁ clones derived from self and cross progeny were classed as virulent (Vir) and showed high survival and reproduction, except for one PS01 self clone that was weakly virulent (Supporting Information, Figure S1). No clones were avirulent (Avr) on DZA315.16. In contrast, on A17, the PS01♀ × N116♂ and reciprocal F₁ progeny clone sets both showed 1:1 (Vir:Avr) segregation (Figures 1, A and B, and 2, A and B; Table 1; $\chi^2 = 0.02$, $P > 0.05$), which suggested that virulence/avirulence is inherited as alleles of a single Mendelian gene, with one parent being heterozygous and the other homozygous recessive. On A17, all progeny clones classed as Avr failed to reproduce (Figure 2, A and B), and most died by 8 days after infestation. Across both cross-progeny sets, all clones could be categorized as Vir or Avr, with no clones displaying intermediate performance.

Analysis of clones derived from self progeny of each parent allowed further testing of inheritance of virulence, especially to resolve whether the dominant allele in the interclonal cross populations represented a Vir or an Avr function and from which parent this allele was inherited. Progeny from selfing of N116 showed a segregation ratio consistent with a single-gene model for a heterozygous parent in which virulence is dominant (Vir:Avr 3:1; $\chi^2 = 0.76$, $P = 0.38$; parental genotype designated *Vv*) that acts to suppress resistance carried by A17 (Figure 1C). In contrast, all progeny clones from selfing PS01 displayed avirulent phenotypes on A17 (0:1 Vir:Avr) (Figure 1D), which indicated that the parental PS01 clone is most likely homozygous recessive (*vv*) for this virulence gene. No Int phenotypes were observed in either of the self-progeny clone sets when tested on A17, and low survival of Avr clones was tightly associated with zero or very low reproduction (Figure 2, C and D). All the virulence data from the progeny sets on A17 were therefore consistent with virulence (*V*) being dominantly inherited from N116 (Table 1). The very similar results of the reciprocal crosses further suggested that virulence is not a sex-linked or maternally inherited trait in these aphid populations

and therefore is not likely to relate to cytoplasmic factors or heritable endosymbionts.

Aphid progeny performance on NILs

The host genotypes A17 and DZA315.16 probably differ at many other loci that influence aphid resistance, in addition to the few mapped QTL for these genotypes: *RAP1*, *RAP2*, and *A1L*. Altering the allelic composition of the host plants is therefore likely to provide additional insights into resistance and virulence/avirulence genes of host and aphid, respectively. On this basis, we formulated and tested two interdependent hypotheses: (1) that host genes other than *RAP1* might have an impact on the performance of the different aphid F₁ populations and (2) that multiple *avr* genes, in addition to the virulence factor *V* defined earlier, will affect compatibility on different host genotypes. We took advantage of a set of NILs derived from a Jemalong A17 × DZA315.16 cross that differ at the *RAP1* locus, with rNIL denoting the PS01-resistant line carrying *RAP1* and sNIL denoting the susceptible line lacking this allele. The NILs both lack the *RAP2* resistance allele, leading to the prediction that some aphid genotypes would be more successful on the rNIL carrying only *RAP1* than on the A17 parent that carries both *RAP1* and *RAP2*.

Progeny clones from each of the F₁ populations were reevaluated alongside their parental clones for survival and reproduction on the NIL pair. As expected from the absence of *RAP1*, all parental and progeny clones were classed as virulent on the sNIL, which is consistent with results on the susceptible DZA315.16 host, although a few F₁ clones displayed partial suppression of survival and/or reproduction (Figure S1 and Figure S2). On the rNIL host, each set of progeny clones displayed overall performance (Figure 3 and Figure 4) that differed substantially from the responses observed on A17. Across both sets of PS01 × N116 cross-progeny clones, virulence, on average, was either unchanged or somewhat increased on rNIL compared with on A17 plants, in keeping with the predicted consequences of a lack of *RAP2*. Only one clone (PN23) showed significantly decreased virulence on rNIL compared with A17 plants. These data sets could be interpreted as continuous distributions from fully virulent to fully avirulent, with several individual F₁ clones from the N116 × PS01 crosses classed as Int phenotypes on rNIL plants because they were significantly different from both parents (Figures 3 and 4). Contrary to the prediction that absence of *RAP2* in the rNIL plants would lead to increased virulence, three of the self progeny clones from the virulent N116 parent were less virulent on rNIL than on A17 plants (Figure 3C and Figure 4C). Conversely, and in contrast to their complete avirulence on A17 plants and the avirulence of the parental clone, three PS01 self-progeny clones became virulent on rNIL plants (Figure 3D and Figure 4D).

Aphid-induced host chlorosis

Induced chlorosis symptoms varied substantially among the aphid-host combinations, indicative of a range of

Figure 1 Survival of F₁ pea aphid clones on Jemalong A17 host plants. (A) PS01♀ × N116♂ progeny; (B) N116♀ × PS01♂ progeny; (C) N116 self progeny; (D) PS01 self progeny. Data are mean (± SE) adult aphids surviving per plant 8 days after infestation with five adults. *N* = 10 plants per clone × host combination. Dotted horizontal lines indicate lower and upper boundaries for virulent and avirulent categories, respectively.

genotype × genotype interactions. In general, chlorosis was induced at much higher frequencies from virulent interactions than from avirulent ones (Table 2). As found by Kanvil *et al.* (2014), parental PS01 infestations were achlorotic on all hosts, as were PS01 self progeny (Table S1D), suggesting

that PS01 does not carry the gene(s) necessary for induction of chlorosis. In contrast, parental N116 induced interveinal chlorosis on all hosts, and N116 self progeny showed segregation of chlorosis on A17 plants (23:5 chlorotic:achlorotic) and on rNIL plants (20:8) (Table S1C). These instances of

Figure 2 Reproduction of F_1 pea aphid clones on Jemalong A17 host plants. (A) PS01♀ $\times$ N116♂ progeny; (B) N116♀ $\times$ PS01♂ progeny; (C) N116 self progeny; (D) PS01 self progeny. Data are mean ($\pm$ SE) nymphs produced per plant 8 days after infestation with five adults. $N = 10$ plants per clone $\times$ host combination.

chlorosis matched exactly to each and every virulent clone-host combination. However, the five N116 and PS01 self-progeny clones that failed to induce chlorosis on both A17 and rNIL plants were all virulent on DZA315.16 and sNIL hosts.

Chlorosis symptoms also segregated for N116 $\times$ PS01 reciprocal-cross progenies, with trends somewhat similar

to those found with self progeny (Table 2). With one exception (clone NP8 on rNIL), all instances of strong chlorosis were associated with virulent phenotypes (Table S1, A and B). Most achlorotic A17 and rNIL hosts were associated with avirulent aphids, but, as with the F_1 self progeny, several virulent clone-host combinations also were achlorotic. Such

Table 1 Virulence segregation ratios and single-gene inheritance model for pea aphid F₁ progeny clones evaluated on *M. truncatula* Jemalong A17

Parental clones	Proposed female genotype	Proposed male genotype	Progeny clones (n)	Observed (Vir:Avr)	Expected (Vir:Avr)	χ^2	P
N116♀ × PS01♂	Vv	vv	27	14:13	1:1	0.04	NS
PS01♀ × N116♂	vv	Vv	25	12:13	1:1	0.04	NS
N116♀ × N116♂	Vv	Vv	28	23:5	3:1	0.76	NS
PS01♀ × PS01♂	vv	vv	28	0:28	0:1	NA	—

NA, not applicable as observed and expected ratios are in complete agreement; NS, not significant.

instances were noted across all the tested host genotypes, including DZA315.16 and sNIL. Together the data suggest that host chlorosis is not an essential response during compatible (virulent) interactions but nonetheless occurs more frequently in response to virulent aphids. The reason for such a correlation may be that sustained feeding is required to initiate chlorosis responses, and this would not generally occur in incompatible interactions. Consistent with this, *RAP1*-dependent resistance is very strong, leading to rapid death of avirulent aphid genotypes.

Aphid-induced host necrosis

Necrotic lesions similar to the classic HR in response to PS01 aphid infestation were previously mapped to the *AIL* QTL (Stewart *et al.* 2009), with *AIL* being inherited from A17. Clone N116 also induces necrosis on A17 (Kanvil *et al.* 2014). The F₁ progeny populations from PS01 selfing and both PS01 × N116 crosses all induced necrosis on A17 host plants but almost never on DZA315.16 (Table S1). Because the PS01 × N116 clones segregated 1:1 for virulence on A17, it is clear that necrosis is not a necessary element of host resistance, confirming previous data of Stewart *et al.* (2009). Further evidence for the disconnect between necrosis and resistance came from the NILs, which lack the functional *AIL* allele: here the full spectrum of resistance was found despite the complete absence of induced necrotic symptoms from any aphid clones on either rNIL or sNIL hosts.

Parental N116 aphids induced necrosis on A17 and not on DZA315.16 but were fully virulent on both hosts. The vast majority of N116 self progeny induced necrosis on A17 with only a few weak or asymptomatic responses (Table S1C), suggesting that there may be segregation for this trait. Although the N116 self-progeny populations segregated 23:5 for virulence:avirulence on A17 (Table 1), there was no correlation between virulence and induction of necrosis (Table S1C), indicating that there is no causative relationship and that the underlying loci are unlinked.

Discussion

In this work, inheritance of virulence and avirulence functions in the pea aphid was revealed from testing progeny of controlled crossing of aphid parents on contrasting parental *M. truncatula* genotypes and derived NILs differing in pea aphid resistance. Compared with the many reports on pest

and disease resistance genes in host plants and on virulence and avirulence genes in pathogens, there are few studies directly addressing the Mendelian genetics of aphids. Difficulties and limitations include the rarity of visually scored mutations in aphids, the technical challenges of conducting controlled crosses and hatching of eggs after long diapauses, and the effort required to maintain multiple live clonal progeny populations. Much of the work on aphid genetics has focused on body color and wing development traits, both of which have been reported to have single-gene inheritance (Hales *et al.* 1997). Here we extended knowledge of aphid genetics using the pea aphid model to analyze agronomically important virulence and avirulence characters.

The value of this study is enhanced by simultaneously controlling the genotypes of both aphid and host plant. The design provided a platform from which to reveal novel genotype × genotype interactions that occur during aphid attack. Although backcross and F₂ populations would allow further interpretations, substantial genetic power was gained from F₁ aphid clones. The reciprocal-cross and self-progeny sets enabled deductions about virulence and avirulence loci that differ between the two aphid parents. Given the relatively small F₁ population sizes and the current lack of F₂ progeny, no attempt has been made here to assess the possible linkage and epistasis effects that lead to the high complexity of virulence and resistance phenotype data. Despite this complexity, as pointed out by Puterka and Peters (1989), clear underlying Mendelian segregations are readily detectable, with resolution of additional loci and interactions limited by the tractability of very large-scale controlled genetics experiments on aphids.

A single dominant virulence gene is sufficient to account for pea aphid performance on Jemalong A17 hosts

The genetic interactions uncovered here between pea aphid and *Medicago* appear to conform broadly to the gene-for-gene model originally proposed for plant-pathogen relationships (Flor 1955). The *RAP1*-dependent resistance of A17 plants to PS01 aphids is strong and qualitative, resulting in rapid aphid death, whereas the compatible interaction of clone N116 with A17 plants is characterized by high rates of aphid survival and rapid population increases. The DZA315.16 host lacks *RAP1* and is fully susceptible to both these clones. Parental aphids therefore were readily classified as avirulent or virulent on each host.

Figure 3 Survival of F₁ pea aphid clones on resistant NIL host plants. (A) PS01♀ × N116♂ progeny; (B) N116♀ × PS01♂ progeny; (C) N116 self progeny; (D) PS01 self progeny. Data are mean (± SE) adult aphids surviving per plant 8 days after infestation with five adults. *N* = 10 plants per clone × host combination. Dotted horizontal lines indicate lower and upper boundaries for virulent and avirulent categories, respectively.

All individual clones from the four F₁ aphid populations derived from selfing and crossing PS01 and N116 parents likewise showed categorical performance as either fully virulent or fully avirulent on A17, with no intermediate progeny clones. Scoring of these F₁ populations on A17 indicated clear Mendelian segregation for a single locus (*V*), inherited

as a dominant allele from the heterozygous N116 parent, that was required for virulence on plants carrying *RAP1*. This single-gene model can explain the 1:1 segregation seen in N116 × PS01 and reciprocal crosses and the 3:1 segregation of clones derived from selfing N116 (*Vv*). By deduction, PS01 is homozygous recessive (*vv*) at this locus,

Figure 4 Reproduction of F₁ pea aphid clones on resistant NIL host plants. (A) PS01♀ × N116♂ progeny; (B) N116♀ × PS01♂ progeny; (C) N116 self progeny; (D) PS01 self progeny. Data are mean (± SE) nymphs produced per plant 8 days after infestation with five adults. *N* = 10 plants per clone × host combination.

consistent with its self progeny being 100% avirulent on A17 host plants. However, because all cross and self progeny were virulent on DZA315.16, it is clear that *V* is not an essential prerequisite for virulence on all *M. truncatula* hosts. Previous QTL mapping indicated that a large propor-

tion of the PS01 resistance resides at the *RAP1* locus carried by A17 (Stewart *et al.* 2009), and we therefore concluded that the dominant virulence allele *V* is required for suppression of *RAP1*-dependent resistance. On this basis, *V* and *RAP1* likely represent a gene-for-gene interaction. In

Table 2 Aphid-induced host chlorosis

Host genotype:	Jemalong A17	rNIL	DZA315.16	sNIL
	Plants with chlorosis (mean % $\pm$ SE)			
N116 $\times$ PS01 and reciprocal progeny				
All clones	17.7 $\pm$ 4.1	47.0 $\pm$ 6.7	71.2 $\pm$ 5.7	68.3 $\pm$ 5.7
Virulent	31.2 $\pm$ 7.2	88.7 $\pm$ 5.7	71.2 $\pm$ 5.7	68.3 $\pm$ 5.7
Avirulent	4.2 $\pm$ 1.6	11.0 $\pm$ 6.5	No avirulent phenotypes	
N116 self progeny				
All clones	82.1 $\pm$ 7.4	71.4 $\pm$ 8.7	82.1 $\pm$ 7.4	82.1 $\pm$ 7.4
Virulent	100 $\pm$ 0	100 $\pm$ 0	82.1 $\pm$ 7.4	82.1 $\pm$ 7.4
Avirulent	0 $\pm$ 0	0 $\pm$ 0	No avirulent phenotypes	

Note: F₁ aphid progeny sets were tested on four host genotypes.

the A17 background, the contribution of a second but weaker resistance QTL, *RAP2* (Stewart *et al.* 2009), was harder to ascertain because of the apparent strong effects of *RAP1*.

Differential F₁ aphid performance profiles on resistant NIL hosts carrying *RAP1* compared with Jemalong A17

Resistance in the rNIL derived from the A17 $\times$ DZA315.16 cross is also largely attributable to the *RAP1* major QTL, and most clones had similar performance when compared on A17 vs. rNIL host genotypes. However, in contrast to the clear categorical segregations on A17, several F₁ aphid clones on the rNIL were less readily classified as virulent or avirulent, indicating that the single virulence gene *V* was inadequate to explain all the inheritance patterns. The range of performance scored across the four F₁ populations on rNIL host plants suggested that more than one virulence or avirulence gene was segregating and determining compatibility in the aphid progeny clone populations. For the F₁ reciprocal-cross progeny sets on the rNIL host, performance could be interpreted as a continuum from fully virulent to avirulent, suggesting polygenic inheritance. In contrast, both F₁ self-progeny sets were mostly categorized as virulent or avirulent with few intermediates, implicating regulation by a small number of genes. However, partly because of the small population sizes derived from each cross, it was not possible to confidently assign alternative genetic models. It may be that one avirulence gene interacts with *RAP1*, present in both A17 and rNIL, whereas another interacts with *RAP2*, present in A17 but not in rNIL. In the absence of the corresponding host gene, avirulence functions instead may result in increased virulence, with evolutionary implications discussed further later. Briggs (1965) reported the presence of independent genes in the rubus aphid (*Amphorophora rubi*) that conferred virulence to two resistance sources in raspberry. Similarly, greenbug virulence that overcomes resistance genes *Gb2* and *Gb3* in wheat was conditioned by duplicate genes and a dominant modifier gene epistatic to one of the duplicate genes (Puterka and Peters 1989).

Given that aphid performance differed on A17 and rNIL hosts that both carried *RAP1*, it can be deduced that additional host genes in *M. truncatula* interact with one or more aphid avirulence or virulence gene products. Although

the NIL pairs are derivatives of highly inbred F₆ RILs, with approximately 97% of loci homozygous, the residual proportion of segregating loci may harbor significant resistance functions that act alongside *RAP1*. The generally greater virulence seen on the rNIL host compared with A17 may be explained in part by absence of genes such as *RAP2* in rNIL but presence in A17 hosts. A second possibility is that some alleles derived from the susceptible DZA315.16 parent suppress resistance to certain aphid genotypes. Such susceptibility genes would need to override the very strong resistance conferred by *RAP1*. In other insect-plant systems, dominant host susceptibility loci have been reported recently that enable compatibility with Russian wheat aphid or hessian fly (Liu *et al.* 2013; Anderson *et al.* 2014).

Additional explanations need to be advanced for the few clones that show opposite changes *i.e.*, decreased virulence on the rNIL host compared with A17. The enhanced resistance shown by rNIL hosts to these clones could relate to genes from DZA315.16 that interact with *RAP1*-dependent resistance processes. For example, DZA315.16 might carry a weak allele of a further resistance gene that on its own is not effective against pea aphids but when recombined with *RAP1* could condition enhanced resistance to a subset of otherwise virulent F₁ aphid genotypes.

Evolution of virulence

Although pea aphids typically show a monoecious habit and feed exclusively on plants of the Fabaceae (legume) family, distinct biotypes have been identified that are specialized to exploit particular host species (Peccoud *et al.* 2009). It has been reported that much of the evolution of novel virulence phenotypes either may result from sexual recombination of preexisting genetic diversity within sympatric or isolated aphid populations or is discovered through unveiling of virulence genes when aphids are presented with new, naturally evolved or agriculturally developed plant genotypes (Puterka and Peters 1990). This study provides strong evidence for the former through the novel virulent and avirulent genotypes derived from self-mating of avirulent and virulent parents, respectively. Evidence for the latter phenomenon derives from the differential performance on hosts sharing a common *RAP1* genotype but varying in other elements of their genetic background.

Because even isolated clonal populations have high heterozygosity, annual sexual selfing will frequently alter virulence on the original host and may enable colonization of an alternate host. In turn, the new aphid clone will have the potential to hybridize with other genotypes already resident on and adapted to that host. Peccoud *et al.* (2009) defined 11 pea aphid biotypes showing species-level adaptation, detected in populations drawn from geographically separated regions across Europe. Molecular marker data indicated that ~10% of populations were migrants (*i.e.*, genotypes collected from an abnormal host species), and elements of all 11 biotype profiles also were found in F₁ hybrid individuals. Our study indicates that in addition to species-level host adaptation, finer levels of intraspecific adaptation are readily detected through recombinants derived from controlled matings and from comparisons of performance on different defined host genotypes within a species. Similarly, Kanvil *et al.* (2014) detected great variation in pea aphid biotype adaptation on a diverse range of *M. truncatula* hosts.

Variation in aphid fitness owing to the presence of different combinations of virulence and avirulence alleles is greatly affected by the complement of *R* alleles in the test hosts. Thus, in the absence of a particular functional cognate *R* allele, erstwhile *avr* alleles may act as virulence factors. Indeed, it is generally accepted that most *avr* gene products detected through *R* gene mechanisms will have initially imparted a selective advantage due to virulence functions; otherwise, they would likely have been subject to negative selection. Subsequently, these same virulence alleles may confer avirulence functions when confronted with a host evolved or bred to carry cognate *R* alleles, consistent with classic gene-for-gene resistance interactions. The large number of possible aphid-host genotype $\times$ genotype combinations likely will lead to great variation in selection pressures, both positive and negative, on particular alleles.

Low virulence of hybrid aphids potentially also could be due to intrinsic unfitness, defined by Peccoud *et al.* (2014) as poor performance on a known highly compatible host. For pea aphid, *V. faba* represents such a universal host. In our study, we found little evidence for reduced hybrid fitness on *V. faba* compared with parental genotypes, consistent with the findings of Peccoud *et al.* (2014) across a wide range of F₁ populations derived from crosses between and within species-level biotypes. Nonetheless, we cannot exclude the possibility that at the initial stage of F₁ clonal colony establishment, some fundatrices may have failed to thrive.

Conclusions

Genetic recombination resulting from both selfing and crossing generated novel virulent and avirulent aphid phenotypes that frequently differed from the parental clones. Notably, some individual progeny clones derived from selfing of the avirulent parental clone PS01 were unexpectedly found to be capable of overcoming the

resistance present in the rNIL but not in its A17 parent. Extrapolating this result to field situations, it can be predicted that resistance based on a highly effective *R* gene such as *RAP1* could break down after just one cycle of sexual recombination, even within an isolated pea aphid population. Latent gain of virulence of this type has implications both in modeling how fast single-gene resistance might be overcome and in the design of crop breeding strategies. Similar cryptic virulence alleles in field-collected aphids would not be detected in conventional screening on host genotypes but can be predicted to exist at high frequencies in both isolated and mixed aphid populations. Conversely, novel avirulent biotypes were found among the self progeny derived from the normally virulent N116 parent. We therefore advocate that crop-selection strategies ideally should incorporate controlled genetics of both aphid and host, initially informed by screening multiple aphid biotypes on a wide range of host genotypes (Kanvil *et al.* 2014).

The F₁ aphid populations reported here represent a resource for future analysis of virulence and avirulence functions. F₂ and backcross populations are likely to reveal additional genetic factors and allow refinement of inheritance models. The observed segregations of aphid virulence, coupled with previous QTL analysis of host genotypes (Stewart *et al.* 2009), indicate that these interactions broadly fit the gene-for-gene model, suggesting that the aphid gene products have analogous functions to pathogen effectors. Given the very different lifestyles of insects and pathogens, aphid effectors may differ from those expressed by pathogens, but the molecular basis of aphid avirulence and virulence gene functions remains largely unknown. However, the few *R* genes cloned to date that act against aphids (Rossi *et al.* 1998; Kaloshian 2004) are in the same NB-LRR family as the majority of known *R* genes that act against pathogens. Some common or conserved mechanisms thus can be predicted. For *Medicago*, although resistance QTL such as *RAP1* map to NB-LRR-rich genomic regions (Kamphuis *et al.* 2013), the underlying genes have not yet been isolated. This work provides a genetic platform from which such studies can readily proceed, aided by the availability of genome sequences for both species (International Aphid Genomics Consortium 2010; Young *et al.* 2011) and by recent development of aphid mutagenesis methods (Tagu *et al.* 2014). Definition of cognate effector-*R*-gene relationships will greatly facilitate the design of rational crop-improvement strategies that provide durable multiple-resistance functions against a wide spectrum of pest genotypes.

Acknowledgments

We are grateful for valuable assistance from Martin Selby, Sophie Stewart, and Simon Hodge. S.K. was supported by a Beit Scientific Research Fellowship at Imperial College London. Experiments were designed by S.K. and G.P.; research was conducted by S.K.; data were analyzed by

S.K., C.M.C., G.P., and C.G.N.T. The paper was written by S.K., G.P., and C.G.N.T. All authors approved the final version.

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Communicating editor: B. P. Lazzaro

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Cryptic Virulence and Avirulence Alleles Revealed by Controlled Sexual Recombination in Pea Aphids

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Supplementary Materials

Table S1. Host necrosis and chlorosis phenotypes in response to infestation by F1 pea aphid clones. (A) N116♀ × PS01♂; (B) PS01♀ × N116♂; (C) N116 self; (D) PS01 self. Data are percent of plants showing symptoms 8 d after infestation. N=10 plants each infested with 5 aphids. A17, Jemalong A17; DZA, DZA315.16; rNIL, resistant NIL; sNIL, susceptible NIL; Vir, virulent; Avr, avirulent; Int, intermediate.

A. N116♀ × PS01♂

Clone	Virulence		Necrosis (%)		Chlorosis (%)			
	A17	rNIL	A17	DZA	A17	rNIL	DZA	sNIL
NP10	Vir	Vir	100	0	100	100	100	100
NP28	Vir	Vir	100	0	100	100	0	100
NP4	Vir	Vir	100	0	70	100	100	100
NP7	Vir	Vir	100	0	50	100	100	100
NP14	Vir	Vir	100	0	40	100	100	100
NP3	Vir	Vir	100	0	40	100	100	100
NP12	Vir	Vir	100	0	20	100	100	90
NP9	Vir	Vir	100	0	10	100	100	100
NP25	Vir	Vir	100	0	0	100	40	90
NP18	Vir	Vir	100	0	0	100	0	100
NP5	Vir	Vir	100	20	0	0	0	0
NP17	Vir	Int	60	0	80	60	0	0
NP23	Vir	Avr	100	0	100	10	0	0
NP21	Vir	*	100	0	20	*	100	*
NP2	Avr	Vir	100	0	0	90	0	100
NP16	Avr	Int	100	0	0	0	30	0
NP13	Avr	Int	100	0	0	0	0	0
NP22	Avr	Avr	100	0	20	0	20	100
NP8	Avr	Avr	100	0	10	100	100	100
NP24	Avr	Avr	100	0	10	0	70	100
NP1	Avr	Avr	80	0	0	20	0	100
NP6	Avr	Avr	100	0	0	0	100	100
NP20	Avr	Avr	100	0	0	0	90	90
NP27	Avr	Avr	100	0	0	0	90	90
NP19	Avr	Avr	100	0	0	0	0	30
NP15	Avr	Avr	100	0	0	0	0	0
NP26	Avr	*	100	0	0	*	100	*

*Clones lost, test on NILs not done

B. PS01♀ × N116♂

Clone	Virulence		Necrosis (%)		Chlorosis (%)			
	A17	rNIL	A17	DZA	A17	rNIL	DZA	sNIL
PN17	Vir	Vir	100	0	90	100	100	40
PN14	Vir	Vir	100	0	50	100	100	0
PN8	Vir	Vir	90	0	10	90	100	80
PN25	Vir	Vir	90	0	10	90	100	70
PN1	Vir	Vir	60	0	0	100	100	100
PN15	Vir	Vir	100	0	0	100	70	100
PN3	Vir	Vir	100	30	0	100	70	80
PN20	Vir	Vir	100	0	0	80	100	100
PN21	Vir	Vir	100	0	0	10	100	100
PN12	Vir	Vir	100	0	0	10	100	60
PN22	Vir	Vir	100	0	0	0	100	30
PN26	Vir	Int	100	0	20	0	100	100
PN27	Avr	Vir	100	0	0	0	0	100
PN18	Avr	Vir	100	0	10	0	100	100
PN19	Avr	Int	100	0	0	10	90	20
PN5	Avr	Int	90	0	0	0	100	0
PN9	Avr	Avr	100	0	30	0	100	100
PN16	Avr	Avr	90	0	20	0	100	40
PN6	Avr	Avr	100	0	10	0	100	100
PN28	Avr	Avr	80	0	10	0	100	80
PN11	Avr	Avr	90	0	0	0	100	60
PN2	Avr	Avr	80	0	0	0	100	0
PN23	Avr	Avr	100	0	0	0	90	0
PN7	Avr	Avr	100	0	0	0	80	100
PN24	Avr	Avr	100	0	0	0	60	100

C. N116♀ × N116♂

Clone	Virulence		Necrosis (%)		Chlorosis (%)			
	A17	rNIL	A17	DZA	A17	rNIL	DZA	sNIL
NN1	Vir	Vir	100	0	100	100	100	100
NN2	Vir	Vir	100	0	100	100	100	100
NN3	Vir	Vir	100	0	100	100	100	100
NN8	Vir	Vir	100	0	100	100	100	100
NN9	Vir	Vir	100	0	100	100	100	100
NN10	Vir	Vir	0	0	100	100	100	100
NN13	Vir	Vir	100	0	100	100	100	100
NN15	Vir	Vir	100	0	100	100	100	100
NN16	Vir	Vir	100	0	100	100	100	100
NN17	Vir	Vir	100	0	100	100	100	100
NN18	Vir	Vir	0	0	100	100	100	100
NN19	Vir	Vir	100	0	100	100	100	100
NN20	Vir	Vir	100	0	100	100	100	100
NN21	Vir	Vir	100	0	100	100	100	100
NN22	Vir	Vir	30	0	100	100	100	100
NN23	Vir	Vir	100	0	100	100	100	100
NN24	Vir	Vir	100	0	100	100	100	100
NN25	Vir	Vir	50	0	100	100	100	100
NN27	Vir	Vir	100	0	100	100	100	100
NN30	Vir	Vir	100	0	100	100	100	100
NN6	Vir	Avr	100	0	100	0	100	100
NN14	Vir	Avr	100	0	100	0	100	100
NN29	Vir	Avr	100	0	100	0	100	100
NN4	Avr	Avr	100	0	0	0	0	0
NN5	Avr	Avr	100	0	0	0	0	0
NN7	Avr	Avr	100	0	0	0	0	0
NN11	Avr	Avr	100	0	0	0	0	0
NN12	Avr	Avr	100	0	0	0	0	0

D. PS01♀ × PS01♂

Clone	Virulence		Necrosis (%)		Chlorosis (%)			
	A17	rNIL	A17	DZA	A17	rNIL	DZA	sNIL
PP9	Avr	Vlr	100	0	0	0	0	0
PP10	Avr	Vir	100	0	0	0	0	0
PP27	Avr	Vir	100	0	0	0	0	0
PP1	Avr	Avr	100	0	0	0	0	0
PP2	Avr	Avr	100	0	0	0	0	0
PP3	Avr	Avr	100	20	0	0	0	0
PP4	Avr	Avr	100	0	0	0	0	0
PP5	Avr	Avr	100	0	0	0	0	0
PP6	Avr	Avr	100	0	0	0	0	0
PP7	Avr	Avr	100	0	0	0	0	0
PP8	Avr	Avr	100	0	0	0	0	0
PP11	Avr	Avr	100	0	0	0	0	0
PP12	Avr	Avr	100	0	0	0	0	0
PP13	Avr	Avr	100	0	0	0	0	0
PP14	Avr	Avr	100	0	0	0	0	0
PP15	Avr	Avr	100	0	0	0	0	0
PP16	Avr	Avr	100	0	0	0	0	0
PP17	Avr	Avr	100	0	0	0	0	0
PP18	Avr	Avr	100	0	0	0	0	0
PP19	Avr	Avr	100	0	0	0	0	0
PP20	Avr	Avr	100	0	0	0	0	0
PP21	Avr	Avr	100	0	0	0	0	0
PP22	Avr	Avr	100	0	0	0	0	0
PP23	Avr	Avr	100	0	0	0	0	0
PP24	Avr	Avr	100	0	0	0	0	0
PP25	Avr	Avr	100	0	0	0	0	0
PP26	Avr	Avr	100	0	0	0	0	0
PP28	Avr	Avr	100	0	0	0	0	0

Figure S1. Survival of F1 pea aphid clones on DZA315.16 and susceptible NIL host plants. (A) PS01♀ × N116♂ progeny; (B) N116♀ × PS01♂ progeny (C) N116 self progeny; (D) PS01 self progeny. Black bars, DZA315.16 host plants; grey bars, sNIL host plants. Data are mean ($\pm$ s.e) adult aphids surviving per plant, 8 d after infestation with five adults. N = 10 plants per clone x host combination.

Figure S2. Reproduction of F1 pea aphid clones on DZA315.16 and susceptible NIL host plants. (A) PS01♀ × N116♂ progeny; (B) N116♀ × PS01♂ progeny; (C) N116 self progeny; (D) PS01 self progeny. Black bars, DZA315.16 host plants; grey bars, sNIL host plants. Data are mean ($\pm$ s.e) nymphs produced per plant, 8 d after infestation with five adults. N = 10 plants per clone x host combination.