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Co-evolutionary interactions between host resistance and pathogen effector genes in flax rust disease

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Summary

Plant-pathogen co-evolutionary selection processes are continuous, complex and occur across many spatial and temporal scales. Comprehensive studies of the flax – flax rust pathosystem has led to the postulation of the gene-for-gene model, a genetic paradigm describing recognition events between host disease resistance proteins, and pathogen effector proteins. Identification of directly interacting fungal effector proteins and plant disease resistance proteins in this pathosystem has facilitated the study of both the physical nature of these interactions and the evolutionary forces that have resulted in a molecular arms race between these organisms. The flax – flax rust pathosystem has also been detailed on the scale of interacting populations, and integration of molecular and population scale datasets represents a unique opportunity to further our understanding of many poorly understood facets of host – pathogen dynamics. In this review, we discuss recent developments and insights in the flax – flax rust pathosystem and their implications for both long-term co-evolutionary dynamics in natural settings, as well as short-term co-evolutionary dynamics in agro-ecosystems.

Keywords

Melampsora lini; *Linum usitatissimum*; *Linum marginale*; TIR-NBS-LRR; effector proteins; virulence; avirulence; co-evolution

Introduction

Interaction with parasites has been postulated to be a major driver of the evolution and maintenance of diversity in both plants and animals. Infection of hosts can lead to reduction of fitness and selection for defense or avoidance mechanisms. Conversely, pathogens are selected to circumvent the continually evolving defenses mounted by their target hosts. However, despite significant advances in our understanding of these interactions at both molecular and population levels, there are still major questions to be resolved regarding the mechanisms of host resistance and pathogen virulence, their variation in space and time and their long-term effect on host-pathogen co-evolution.

The interaction between flax and flax rust has been an important model system for understanding the genetic and molecular basis of host-pathogen interactions in plant diseases as well as for understanding co-evolution processes in natural disease systems. Flax rust, *Melampsora lini*, is a fungal pathogen that infects cultivated flax (*Linum usitatissimum*) as well as a number of related *Linum* species including the native Australian flax *L. marginale* (Barrett *et al.*, 2009; Lawrence *et al.*, 2007). Rust pathogens are obligate biotrophs, which

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depend on living plant tissues for propagation. The invading fungal hyphae form specialised feeding structures called haustoria that extract nutrients from host mesophyll cells.

Working with the cultivated flax - rust system, Flor (1956) defined the gene-for-gene model which has proved widely applicable as the basic genetic paradigm of plant disease resistance. In this model, the outcome of infection is based on the interaction of dominant resistance (*R*) genes in the host and dominant avirulence (*Avr*) genes in the pathogen. This genetic interaction is now understood in terms of effector triggered immunity (Chisholm *et al.*, 2006; Dodds & Rathjen, 2010; Jones & Dangl, 2006), where R proteins constitute the recognition component of the plant immune system and detect the presence of specific pathogen effector (Avr) proteins and trigger a defense response that prevents infection. These host and pathogen genes thus confer 'extended phenotypes' (Dawkins, 1999), that is their effects extend to the phenotype of another organism, implying close co-evolutionary interactions which have been the basis of much theoretical modelling (Sasaki, 2000; Thrall & Burdon, 2002). However there are few experimental systems in which it is possible to evaluate theoretical predictions arising from different co-evolutionary scenarios (e.g. cyclical selection vs. an escalating arms race). Recent work in the flax rust system has now delineated the molecular basis of gene-for-gene resistance and revealed new insights into the evolutionary consequences of gene-for-gene interactions in wild systems. Here we summarize the current state of knowledge of molecular and population level interactions in the flax rust disease system and future directions for understanding how these organisms co-evolve.

MOLECULAR BASIS OF GENE-FOR-GENE INTERACTIONS

R resistance proteins

In cultivated flax, 30 genes that confer resistance to flax rust have been mapped to 5 loci (*K, L, M, N, P*) consisting of closely linked or allelic genes (Islam & Mayo, 1990). A total of 19 *R* genes have been cloned from flax (11 from *L*, 3 from *M*, 3 from *N*, 2 from *P*), all of which encode intracellular Toll interleukin 1 receptor–nucleotide binding site–leucine-rich repeat (TIR-NBS-LRR) class proteins (Anderson *et al.*, 1997; Dodds *et al.*, 2001a, b; Ellis *et al.*, 1999; Lawrence *et al.*, 1995; Lawrence *et al.*, 2010a). The *L* locus consists of a single gene with 13 allelic variants (*L, L1* to *L11*, and *LH*) distinguished by their reaction to rust strains carrying different *Avr* genes. The *N, M* and *P* loci are more complex, containing 4, up to 15, or 6–8 tandemly arranged paralogues respectively.

Most variation between alleles and paralogues at these loci occurs in the LRR domain (Dodds *et al.*, 2001a, b; Ellis *et al.*, 2000; Ellis *et al.*, 1999), and domain swap experiments have confirmed the LRR domain is important for determining R-Avr recognition specificity. For instance chimeric L proteins consisting of the L2 LRR and either L6 or L10 N-termini express L2 recognition specificity (Ellis et al., 1999). In addition, the L6 and L11 proteins differ by 32 amino acid polymorphisms, all in the LRR domain, and a recombinant protein with a chimeric L6/L11 LRR showed a novel recognition specificity (Dodds *et al.*, 2006; Ellis *et al.*, 2007). In general, LRR domains are horseshoe shaped molecules (Figure 1) composed of repeating leucine-rich units of approximately 24 to 30 amino acids (Kobe & Deisenhofer, 1995). Variable residues are exposed on a concave β-sheet surface and available for participation in R-Avr interactions. In fact the different *P* and *P2* specificities are a result of just six amino acid polymorphisms found in the LRR β-sheet region (Dodds *et al.*, 2001a). Collectively, these results indicate that the LRR domain is the major determinant of Avr recognition specificities. Congruently, in the rice-rice blast pathosystem the LRR domain of the rice *R* gene *Pi-ta* was observed to interact directly with the corresponding Avr-Pita effector protein in a yeast-2-hybrid assay (Jia *et al.*, 2000). Likewise domain swaps have shown that pathogen recognition specificity is controlled by the LRR region of the

barley Mla resistance proteins and the Rx/Gpa2 proteins in potato (Rairdan & Moffett, 2006; Shen *et al.*, 2003). Pull-down experiments have shown that the LRR domain of Arabidopsis RPP1 protein associates with the *Hyaloperonospora arabidopsidis* ATR1 effector (Krasileva *et al.*, 2010).

While the LRR domain appears to be the primary mediator of recognition specificity, the TIR domain may also influence this function. TIR-NBS domain swaps between *L10* and *L2* or *L9* determined that *L* protein function requires co-adapted TIR-NBS and LRR regions, raising the possibility that these domains interact with each other (Luck *et al.*, 2000). Indeed positive selection has acted on the L TIR domain, suggesting that polymorphisms in this region are related to the function of the L proteins ((Luck et al., 2000); Ravensdale, unpublished data). Hwang and Williamson (2003) have reported that intramolecular interactions between the coiled coil (CC) and LRR domains of the Mi resistance protein mediated downstream hypersensitive response (HR) signalling in tomato. Similarly, studies of the Rx resistance protein demonstrated interactions between the CC-NBS and LRR domains, and between the CC domain and the NBS-LRR region (Moffett *et al.*, 2002). Importantly, these interactions were disrupted in the presence of the cognate Rx effector ligand. In the case of the tobacco-TMV pathosystem, the p50 fragment of the TMV replicase protein associates indirectly with the TIR domain of the N resistance protein through an intermediate protein (Burch-Smith *et al.*, 2007) but then appears to be recognised by binding directly to the LRR domain (Ueda *et al.*, 2006). Collectively, these studies have led to hypothetical models of R protein function where recognition of cognate effectors causes intramolecular conformational changes within the R protein, resulting in signal transduction (Rafiqi *et al.*, 2009).

Avr effectors

Of the ~30 *Avr* specificities identified in flax rust via genetic studies, genes from four *Avr* families, representing 9 recognition specificities, have been cloned to date: *AvrL567, AvrM, AvrP123* and *AvrP4* (Barrett *et al.*, 2009; Catanzariti *et al.*, 2006; Dodds *et al.*, 2004). These were identified by screens for rust genes expressed during infection (*AvrL567*) or haustorially expressed ESTs encoding secreted proteins (*AvrM, AvrP4* and *AvrP123*) that co-segregate with the *Avr* loci. Avirulence functions were confirmed by *Agrobacterium*-mediated transient expression in flax lines expressing the corresponding *R* genes, which induced HR, whereas expression in flax lines without these resistance genes resulted in no HR. Recently, direct confirmation that these genes are responsible for avirulence was achieved using *Agrobacterium*-mediated transformation of flax rust and RNAi to silence *AvrL567* genes; transgenic rust isolates acquired virulence on flax plants containing *L5, L6* and *L7*, (Lawrence *et al.*, 2010b).

All the *Avr* gene variants encode small secreted proteins (Table 1) that are expressed in haustoria and appear to be translocated into plant cells (Catanzariti *et al.*, 2006;Dodds *et al.*, 2004). They are characterised by high levels of polymorphism associated with differences in recognition specificity. For instance there are 12 variant forms of *AvrL567*, of which seven are recognised by L5, L6 or L7, while the other 5 are virulence alleles (Dodds et al., 2006). Likewise several different alleles of *AvrP123* are differentially recognised by *P, P1, P2* and *P3*, and a recombinant allele showed novel recognition phenotype (Barrett *et al.*, 2009;Dodds & Thrall, 2009). None of the effector families isolated from flax rust share sequence similarity with each other or other currently known proteins, although AvrP123 contains 10 cysteine residues that conform to the consensus spacing of the kazal family of protease inhibitors (Catanzariti *et al.*, 2006). Homologs of *AvrL567, AvrM* and *AvrP4* occur in the poplar rust (*M. larici-populini*) genome (http://genome.jgi-psf.org/Mellp1/Mellp1.home.html), and *AvrP4* homologs occur across 22 *Melampsora* species (Van der Merwe *et al.*, 2009).

The flax R proteins are cytoplasmic and transient expression of *Avr* proteins lacking N-terminal signal peptides results in an *R*-gene dependent HR, which indicates that Avr recognition occurs inside the plant (Catanzariti *et al.*, 2006; Dodds *et al.*, 2004). Thus Avr proteins must be translocated into plant cells during infection and indeed immunolocalisation has detected the AvrM protein inside host cells containing an haustorium (Rafiqi *et al.*, 2010) Similarly, translocation of flax rust effectors appears to be independent of the pathogen, since transient expression of *AvrM* in resistance flax leaves results in an HR regardless of the presence of a signal peptide (Catanzariti et al., 2006). This suggests that plant derived transport machinery may be exploited by these pathogens. Recently Rafiqi *et al.* (2010) demonstrated that the N-terminus regions of AvrM and AvrL567 are sufficient to direct the translocation of secreted GFP fusion proteins. Likewise, translocation of effectors produced by ooymycete pathogens is mediated by a conserved *N*-terminal RxLR motif (Whisson *et al.*, 2007) and can occur independently of the pathogen (Dou *et al.*, 2008).

Molecular basis of Avr-R interactions

The physical nature of Avr-R recognition has the potential to significantly impact the evolution of these proteins. There are two hypothetical models that describe how pathogen effector proteins may interact with plant resistance proteins. The first is based on a direct physical interaction (receptor-ligand) and has been described in the rice – rice blast pathosystem where Pi-ta interacts with Avr-Pita, in *Arabidopsis thaliana – Ralstonia solanacearum* pathosystem where RRS1 interacts with avirulence protein PopP2, and in the tobacco-tobacco mosaic virus pathosystem where N interacts with the TMV replicase protein (Bernoux *et al.*, 2008; Deslandes *et al.*, 2003; Jia *et al.*, 2000; Ueda *et al.*, 2006). Alternatively, R proteins may recognize the presence of Avr proteins indirectly by detecting changes induced in other plant proteins by Avr proteins. In this scenario, R proteins are guarding the targets of Avr proteins. The guard hypothesis has been demonstrated in the *A. thaliana – Pseudomonas syringae* pathosystem where RPM1, RPS2, and RPS5 recognize changes in RIN4 induced by the presence of bacterial effectors (Axtell *et al.*, 2003; Mackey *et al.*, 2002; Mackey *et al.*, 2003; Shao *et al.*, 2003). More recently, the guard model has been modified to include the possibility that guarded host proteins may evolve as decoys, thus functioning only as dedicated effector-detectors (van der Hoorn & Kamoun, 2008). R proteins that function as guards are likely to select against the pathogenicity function of Avr proteins, while R proteins that bind directly to Avr effectors will impose a strong selection pressure on those proteins to evade physical detection. When Avr recognition is not related to effector function it is possible for mutations to occur that disrupt recognition without affecting effector function. Thus, ‘guard’-type R proteins should provide stable, long-term resistance; whereas directly interacting R proteins should accelerate the evolution of new virulence phenotypes (Ellis & Dodds, 2003; van der Hoorn *et al.*, 2002).

The diversification of both *R* and *Avr* allelic variants in the flax rust system is suggestive of a direct protein-protein interaction and indeed yeast-2-hybrid assays have confirmed this (Catanzariti *et al.*, 2010; Dodds *et al.*, 2006). For example, Dodds et al. (2006) co-expressed *L5, L6* and *L6L11RV* (a chimeric gene derived from *L6* and *L11*) with all 12 *AvrL567* variants in the yeast-2-hybrid assay, and found a close correlation between Avr-R interactions in yeast and inductions of HR *in planta*. Likewise M and AvrM interact directly in yeast, and this interaction correlates with the recognition specificities observed *in planta* (Catanzariti et al., 2010). Whereas M is ~80% identical to L5 and L6 at the amino acid level, AvrL567 and AvrM are unrelated. In addition, L5 and L6 are the most sequence-diverged (87 polymorphisms; 59 in the LRR domain) of the L proteins and yet they recognize an overlapping set of AvrL567 proteins, either as a result of convergent evolution, or because conserved sequences found in both L5 and L6 mediate these interactions. Collectively, these

data suggest that the LRR domain is evolutionarily flexible and has evolved to directly recognize a diverse set of Avr effectors.

Protein sequence analysis of flax rust Avr effectors has revealed that these protein families are highly polymorphic, and appear to be under diversifying selection. For example, *AvrM* variants contain 14 polymorphic sites as well as a number of deletions and truncations, and comparison with flanking sequence variation clearly indicated the effects of positive selection (Catanzariti et al., 2006). An initial study of *AvrP4* revealed 7 polymorphisms concentrated in the *C*-terminal region of the protein that appeared to be the result of diversifying selection (Catanzariti et al., 2006), and comparison of AvrP4 homologs across 22 Melampsora species revealed significant positive selection in 15 codons located in the 3' region (Van der Merwe et al., 2009). AvrL567 exhibits high amino acid sequence variability with 27.5% of residues being polymorphic between variants, and DNA sequence analysis has revealed that this locus is also under significant positive, diversifying selection (Dodds et al., 2006). Solution of the crystal structures of AvrL567 revealed that the side chains of all the polymorphic amino acids are exposed on the surface of the molecule (Wang *et al.*, 2007). Mutational analysis confirmed the role of several of these residues in controlling recognition specificity, and it appears that these specificities are mediated by multiple amino acid contacts in a quantitative manner (Wang et al., 2007). Consequently, evolution of virulent forms of AvrL567 could occur in a stepwise manner where single amino acid changes in avirulent forms result in partially virulent forms (i.e. weakly recognized) that would be selectively advantageous to the rust, and subsequent amino acid changes could eventually result in complete virulence.

The *L* locus in flax is also highly polymorphic with 131 sites (30 in the TIR-NBS regions, 101 in the LRR domain) being under significant positive selection (Ravensdale, unpublished). Wang *et al.* (2007) utilized the known structure of internalin A as a template for building a hypothetical structural model for the LRR domains of L5 and L6. These hypothetical L5 and L6 LRR models were then used to develop docking models for AvrL567-A / L5 and L6 interactions. This has resulted in a list of amino acid residues found in the LRRs of L5 and L6 that could be making contact with AvrL567-A. Superimposition of the 101 positively selected LRR residues onto the model of potential interacting residues has highlighted specific regions that may be involved in the interaction (Figure 1). These sites can be evaluated in domain swap and mutation experiments. Since Avr567 interacts with L5 and L6 in yeast, it should be possible to use the yeast-2-hybrid interaction to select for mutagenized L proteins with novel recognition specificities. A similar approach was successful in generating a variant of the potato Rx resistance protein with novel viral coat protein recognition specificities (Farnham & Baulcombe, 2006).

CO-EVOLUTION IN A NATURAL GENE-FOR-GENE SYSTEM

Gene-for-gene resistance in *L. marginale*

In addition to cultivated flax, *M. lini* also infects the related wild flax *L. marginale*, a short-lived perennial herb endemic to Australia. Both molecular and pathogenicity data indicate that the interaction between *M. lini* and wild flax represents a gene-for-gene association that is similar to but evolutionarily differentiated from the interaction between *M. lini* and cultivated flax. Of 15 pathogen isolates sampled from different *L. marginale* populations, only three were able to cause infection on a set of 25 *L. usitatissimum* rust resistance gene differential lines (Lawrence, 1989). In contrast, a set of 46 *L. marginale* lines derived from across the geographic range of the native host, were all susceptible to at least several rust isolates, and 6 lines were susceptible to all isolates tested (Lawrence & Burdon, 1989). Furthermore, on *L. marginale*, isolates of *M. lini* display a variety of infection phenotypes in terms of extent of sporulation and damage to the host plant not seen on *L. usitatissimum*,

causing in some cases the loss of older leaves at the bottom of the stem (Lawrence & Burdon, 1989). This differentiation between rust isolates from cultivated and wild flax is also seen in patterns of DNA variation in pathogen avirulence genes, with distinct *Avr* variants occurring in wild populations (Barrett *et al.*, 2009; Dodds *et al.*, 2006). The large variation in resistance and virulence phenotypes further suggests that *L. marginale* and *M. lini* have coevolved for a long time and therefore *M. lini* is unlikely to be a recent introduction to Australia (Barrett *et al.*, 2008b; Lawrence & Burdon, 1989). As in the cultivated flax system, rust resistance in *L. marginale* results from single dominant genes, with a minimum of 17 different *R* genes or alleles (Burdon, 1994).

Impact of life history on host-pathogen interactions at the population level

Host-pathogen co-evolution is likely to be strongly influenced by life history factors such as environmental conditions, effective population sizes (the number of individuals in a population who contribute offspring to the next generation) and pathogen dispersal mechanisms, which have been characterised extensively in the wild flax pathosystem. *L. marginale* is found across Australia in various habitats, including eucalypt forests and savannah, open alpine areas covered with snow several months a year, coastal sand dunes and along water courses in more arid inland areas (Lawrence & Burdon, 1989). *M. lini* is also found across this extensive geographic and habitat range. Dikaryotic rust urediospores are wind-dispersed and their quick propagation can lead to local epidemics,with up to 8 asexual reproduction cycles in a growing season. In colder and wetter environments (e.g. subalpine regions referred to as the “Mountains”) plants flower in mid- to late-summer before the first autumn frosts induce a large fraction of them to die, causing significant and abrupt crashes in pathogen numbers. Here, plants overwinter as underground rootstocks with or without a few green shoots protected from frost by the surrounding vegetation. These shoots may carry occasional pustules over to the next growing season but there is also a significant probability of local loss of the pathogen. In contrast, in environments with hot and dry summers and mild winters (e.g. drier inland regions referred to as the “Plains”) epidemics start earlier, last longer and are often more severe. The sexual cycle can be initiated late in the season as above-ground shoots die back during the summer drought. This results in the production of sclerotic diploid teliospores that are resistant to environmental extremes. The decline in pathogen numbers is not as drastic as in the Mountains region.

Several lines of evidence indicate that the pathogen has the potential to impose severe selection on the population structure of native hosts. Disease incidence varies in space and time, ranging from virtually non-existent to epidemic levels; such patterns are consistent with varying patterns of co-evolution across landscapes (Burdon & Thompson, 1995). Within local populations, incidence, prevalence and disease severity start low and increase throughout the season, favoured by humid conditions that may result in epidemics with prevalence of ~100% *vs* ~20% for dry years (Jarosz & Burdon, 1992). Host survival rate is not affected by infection during the growing season, but over-winter plant survival may be as low as 20–30% in years of severe epidemics in contrast to 80–90% for a growing season with low pathogen pressure (Jarosz & Burdon, 1992). In epidemic years, disease also affects host population demography and population structure (see below). Younger plants have fewer stems and as result, they are less prone to infections than older (and larger) individuals, increasing their chances of survival over winter in years of severe epidemics (Jarosz & Burdon, 1992). Following severe epidemics, host population size is often greatly reduced. Smaller populations (<100 individuals) are less likely to be infected than medium or large populations (up to 5000 individuals) in years of mild epidemics (Burdon & Jarosz, 1992). This may result from bottlenecks occurring at the end of the growing season. If too few infected hosts support overwintering pathogens, the pathogen may go extinct. Given

reduced pathogen pressure, host population size is likely to increase, rendering the host population more prone to infection.

Patterns of resistance and virulence at multiple geographical scales

Host populations represent discrete groups that are genetically and phenotypically differentiated. For example, extensive inoculation studies have shown that, in the Mountains region, with regard to resistance structure, populations range from being nearly monomorphic to having at least 18 resistance phenotypes (Burdon & Thompson, 1995; Jarosz & Burdon, 1991). The number of resistance phenotypes in a population shows spatial and temporal variation indicating that host populations follow different ecological and evolutionary trajectories despite close geopgraphical distance. This suggests that selection or drift can be stronger than gene flow. Indeed, following severe epidemics, the resistance structure of host populations can be significantly more diverse (Burdon & Thompson, 1995). In turn, among-population variation in host resistance is a major determinant of the severity of pathogen epidemics (Thrall & Burdon, 2000). Taken together, it appears that *M. lini* exerts a mild to very strong selective pressure on populations of *L. marginale*, variable in space and time, shaping host population size, demography and genetic structure. Surprisingly, the predicted effects of selection are not consistentlty detected, e.g. after an epidemic where a significant number of hosts die over winter due to infection, the surviving hosts may not be more resistant to pathotypes from the previous year. Conversely, selection imposed by resistance on pathogen isolates at the local scale may be reduced by the large dispersal capacity of the pathogen. Therefore, selection may only be detected when looking at the metapopulation level, due to pathogen selective pressure competing with selection imposed by other environmental parameters at a local and short-time scale.

Genetic analysis of *M. lini* isolates collected from *L. marginale* populations across Australia has revealed the existence of at least 2 distinct pathogen lineages (termed AA and AB). Lineage AB appears to have originated from hybridization between lineage AA and an extinct or as yet unidentified lineage BB (Barrett *et al.*, 2007). The two lineages AA and AB are for the most part in geographic isolation from each other, with hybrids occurring mostly in areas of cool-temperate climate with annual rainfall above 880mm while non-hybrids are found in hotter drier environments with under 640mm rainfall. No sexual reproduction was observed in the Mountains where hybrids are prevalent compared to extensive sexual reproduction in the Plains where hybrids are present only at low frequencies (Barrett et al., 2007). Accordingly, AB isolates show a fixed pattern of heterozygosity (one A and one B allele at corresponding microsatellite loci). The extent to which differences in pathogen mating system are under genetic *vs*. environmental control is still unclear, although both field surveys and glasshouse inoculation studies indicate that telial formation (the precursor stage for sexual reproduction) is largely genetically determined (Barrett *et al.*, 2008a). High temperatures appear to be the main environmental factor that triggers telial formation in lineage AA (Nemri, unpublished) and current studies aim to assess whether lineage AB isolates are able to proceed beyond telial formation to complete the sexual cycle. The implication is that differences in reproductive strategies and geographic distribution contribute to divergent evolutionary trajectories and could result in the observed regional differences in virulence and diversity. Over time, this could lead to further specialisation on hosts and subsequent host adaptation and eventually pathogen speciation.

Ecological differentiation within the host also occurs. Thus, within the Mountains region two morphologically discrete ecotypes (termed “bog” and “hill”) occur at close geographical distances but in environments that differ, e.g. in terms of soil moisture; (Laine *et al*., unpublished data). Populations of the hill ecotype show distinctly more resistance than neighbouring bog ecotype populations and their associated pathogen populations show correspondingly higher virulence (Thrall *et al.*, 2001) but also have a environment-

independent lower survival rate as shown by transplant studies (Carlsson-Graner *et al.*, 1999). Host differentiation in reproductive strategies is evident at larger geographic scales. Thus, the Plains metapopulation exhibit a significant level of outcrossing whereas plants in the Mountains metapopulation are essentially inbred (Burdon *et al.*, 1999). Such differences in mating system are associated with marked differences in the level and structure of resistance within and among host populations and metapopulations which match observed differences in pathogen mating system, diversity and virulence.

Consistent with these local and regional patterns of differentiation in life-history, phylogeny and variation in resistance and virulence in *L. marginale* and *M. lini*, adaptation between the pathogen and its host (a signature of co-evolution) has also been demonstrated at multiple spatial scales. This includes continental (Burdon *et al.*, 2002; Lawrence & Burdon, 1989), among regions (Plains *vs.* Mountains, (Barrett *et al.*, 2007; Burdon *et al.*, 1999), and within a single metapopulation (bog *vs.* hill, (Carlsson-Graner et al., 1999); or local adaptation (Thrall *et al.*, 2002). For example, at the continental scale, rust isolates from Victoria and southern New South Wales are significantly more virulent on hosts from these regions than isolates from other parts of Australia. Similarly, closely adjacent inbreeding host populations in the Mountains metapopulation were found to be more similar with regard to the relative abundance of particular resistance phenotypes than they were with more distant populations. At very local scales, mating system has also been recently demonstrated to have an impact on the within-population structure of resistance (Nemri, unpublished). It is somewhat puzzling that despite the broad dispersal ability of the pathogen relative to that of the host (as evidenced by the lack of distance-dependent distribution patterns for virulence phenotypes in associated pathogen populations; (Burdon & Jarosz, 1991; Thrall & Burdon, 2003), the most virulent pathotypes do not generally dominate host populations. This contrasts with agricultural crop systems where super-virulent pathogens rise and disperse globally over relatively short timescales (often within a few years). This discrepancy may be explained by an evolutionary trade-off between infection strategies that is suggested by glasshouse inoculation studies revealing a negative relationship between virulence and spore production (Thrall & Burdon, 2003). It is not known whether that negative correlation can be explained by avirulence effectors negatively contributing to pathogen fitness in their virulence form, although preliminary evidence suggests that there is no detectable fitness penalty to silencing *AvrL6* in a host background lacking *L6* (Lawrence et al., 2010b).

Partial infections, i.e. infections in which the pathogen can not achieve maximum spore production, are commonly observed in glasshouse single spore inoculation studies. Analysis of data from multiple populations in the *Linum-Melampsora* system has shown that the number of partial infection phenotypes relative to the total number of resistant responses is likely to be greater when hosts are challenged with sympatric rather than allopatric pathogen isolates (Antonovics et al., submitted). Some host populations have a high prevalence of partial resistance genes compared to the average across the metapopulation, while some pathogen populations contain primarily isolates that are unable to cause a severe infection regardless of host origin (Antonovics et al., submitted). The occurrence of partial infection phenotypes in different host/pathogen population pairs thus depends on the host, the pathogen and the interaction. These differences constitute an important yet so far poorly understood aspect of the co-evolutionary interaction between host and pathogen.

Avr gene diversity in natural populations

M. lini isolates from the wild pathosystem contain homologs of the *AvrL567*, *AvrM*, *AvrP123* and *AvrP4* genes identified from the cultivated pathosystem. However the patterns of selection differ between these genes. A geographically diverse set of isolates all contained the same *AvrM* variant, suggesting that *AvrM* does not contribute to the outcome of the gene-for-gene interaction, probably because there are no *R* genes in the *L. marginale*

populations that recognise this *Avr* gene (Dodds & Thrall, 2009). Some variation has been observed amongst *AvrL567* homologs in rust isolates from *L. marginale*, but the complex nature of this multi-copy locus has made genetic characterisation at the population level more difficult. However, isolates of *M. lini* from *L. marginale* populations show extensive variation and a signature of strong positive selection at the *AvrP4* and *AvrP123* loci (Barrett et al., 2009). Transient expression of these *AvrP123* and *AvrP4* variants in *L. marginale* plants can trigger hypersensitive responses in some host genotypes, suggesting that there is differential recognition of these genes by *R* genes in host populations (Barrett et al., 2009). It is not yet known whether these recognition specificities are mediated by homologs of the *P* genes from *L. usitatissimum*. However, there is among-population variation in the frequency of recognition of *AvrP123* and *AvrP4* variants and also considerable year-to-year variation within populations of *L. marginale* (Ravensdale *et al*., unpublished data). Likewise there is variation in the frequency of different *AvrP123* and *AvrP4* variants between rust populations (Barrett et al., 2009). This suggests the possibility of strong selection acting on the cognate *R* and *Avr* genes in this system. The spatial and temporal variation in the cognate *R* genes could explain the maintenance of observed polymorphism at *AvrP4* and *AvrP123*.

Concluding remarks

The combination of in depth molecular and population level information makes the flax rust disease system a powerful model for understanding host-pathogen co-evolution. Detailed knowledge of the molecular interaction between R and Avr proteins in cultivated flax provides a framework for understanding the selective forces underlying the population level variation that is observed in the wild flax rust interaction. There still remain many challenges to integrating these data. For instance, although we know that *Avr* genes identified from cultivated flax rust also operate in the wild system, we do not yet know whether the corresponding *R* genes in *L. marginale* are also homologs of the cultivated flax *R* genes. It will be important in future to correlate phenotypic analysis of the wild populations with DNA-based studies of the distribution of specific *R* and *Avr* genes. Likewise, much knowledge still needs to be gathered to enable generalisation of findings from this system to other plant-pathogen interactions, including those occurring in agricultural crop systems. For the management of crop diseases, information on dispersal, disease dynamics and the virulence structure of rust populations is crucial. In the long term, determining how the selective pressure imposed by host genetic structure and other life-history components shapes rust adaptation and evolution is also critical (Barrett et al., 2008b). While considerable research effort has focused on major *R* genes, little is known about the epidemiological or evolutionary consequences of gene-for-gene interactions that result in partial infection, even though they have the potential to significantly impact disease dynamics and pathogen adaptation. The integration of molecular understanding of gene-for-gene interactions with population genetics in the flax rust system is now providing a powerful approach to measuring the effects of gene-for-gene co-evolution. However, such studies also need to account for other selective factors in the environment, i.e. life-history, with which they likely interact. A specific experimental challenge will be to find the spatial and temporal scales at which one can verify predictions emerging from the arms-race model in the context of co-evolving metapopulations of hosts and pathogens. This will be necessary, not only to address issues such as the maintenance of host and pathogen diversity, but also to explain the modality of the arms-race and the maintenance of sexual reproduction in the pathogen and outcrossing in the host with respect to the Red Queen hypothesis (Van Valen, 1973).

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Definitions

Avr proteins	Pathogen effectors that are recognised by host R proteins
Effectors	Pathogen proteins that are produced to interfere with host processes and allow disease establishment.
ETI	Effector triggered immunity. An immune response triggered by R-Avr recognition.
Virulence	(as is used in plant pathology) means the capability of causing infection and aggressiveness refers to the reproductive output of the pathogen. Pathogen fitness is referred to as aggressivity.
Positive selection	is a type of directional selection in which one allele is favoured and rises from rare to predominant in a population. Genes under positive selection typically have a high ratio of non-synonymous mutations (Ka/Ks).
Diversifying selection	is characterised by alleles at both extremes of a phenotype spectrum being selected at the same time while individuals with alleles encoding intermediate phenotypes would be selected against.
The co-evolutionary arms-race in host-parasite interactions	is an asymmetrical genetic interaction in which fitness of the host is negatively correlated to fitness of the parasite. To try to increase its fitness, the host evolves defence mechanisms such as resistance that the pathogen has to circumvent by evolving virulence mechanisms such as ETI effectors. This results in the constant accumulation of defense mechanisms on the host side and virulence mechanisms on the pathogen side. At the gene level, it is characterised by the rapid and continuous fixation of novel mutations with advantageous effect.
A metapopulation	is a group of populations located at a geographic distance that permits genetic exchange of propagules (pollen, seeds, spores) between them.

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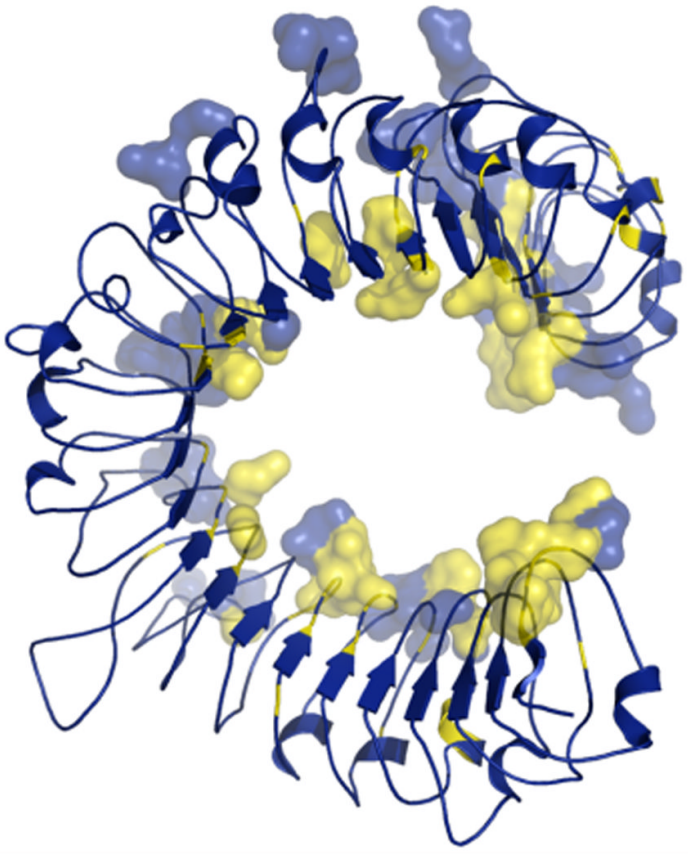

Figure 1.
A structural model of the LRR domain of L5. Residues that may mediate interactions with AvrL567-A have been rendered as 3D surfaces. Residues that are under significant ($p>0.95$) positive selection are coloured yellow

Table 1

Characteristics of cloned effector genes and their protein products from flax rust, *Melampsora lini*

Locus	Mature protein size (aa)	Number of cloned variants:	Cognate R genes	Variation in rusts pathogenic on *L. marginale*
AvrL567	127	12	L5, L6, L7	not polymorphic
AvrM	184–349	6	M	not polymorphic
AvrP123	88–94	6	P, P1, P2, P3	7 alleles
AvrP4	67	3	P4	13 alleles