

How do pathogens evolve novel virulence activities?

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Abstract

We consider the state of knowledge on pathogen evolution of novel virulence activities, broadly defined as anything that increases pathogen fitness with the consequence of causing disease in either the qualitative or quantitative senses, including adaptation of pathogens to host immunity and physiology, host species, genotypes, or tissues, or the environment. The evolution of novel virulence activities as an adaptive trait is based on the selection exerted by hosts on variants that have been generated *de novo* or arrived from elsewhere. In addition, the biotic and abiotic environment a pathogen experiences beyond the host may influence pathogen virulence activities. We consider pathogen evolution in the context of host-pathogen evolution, host range expansion, and external factors that can mediate pathogen evolution. We then discuss the mechanisms by which pathogens generate and recombine the genetic variation that leads to novel virulence activities, including DNA point mutation, transposable element activity, gene duplication and neofunctionalization, and genetic exchange. In summary, if there is an (epi)genetic mechanism that can create variation in the genome, it will be used by pathogens to evolve virulence factors. Our knowledge of virulence evolution has been biased by pathogen evolution in response to major gene resistance, leaving other virulence activities underexplored. Understanding the key driving forces that give rise to novel virulence activities, and the integration of evolutionary concepts and methods with mechanistic research on plant–microbe interactions, can help inform crop protection.

Box 1: Definitions of terms.

Effector: secreted protein or other molecule that contributes to pathogen fitness in its host(s).

Virulence activities: anything that enables pathogens to infect and multiply (i.e. increases pathogen fitness), with the consequence of causing disease in either the qualitative or quantitative senses.

Virulence factors: genes that directly contribute to a pathogen's ability to infect and multiply within the host

Virulence-related factors: genes that indirectly contribute to pathogen fitness.

Introduction

When the IS-MPMI community voted on the Top 10 questions in Molecular Plant-Microbe Interactions in 2019, the 9th ranked question was, “How do pathogens evolve novel virulence activities?” (Harris et al. 2020). Virulence, as defined below, is fundamental to plant-pathogen interactions, and the study of mechanisms of virulence is a central research theme in the MPMI community. The evolution of novel virulence activities is also critical to the application of foundational MPMI research because pathogen evolution often triggers the emergence and re-emergence of economically and ecologically damaging plant diseases. This review comes at a time when we have access to affordable, and portable, genome and/or transcriptome sequencing for any pathogen. Thus, we are on the cusp of feasibly exploring this question across many pathosystems, and potentially in near-real time. The era of big data and new innovations in artificial intelligence will also contribute to new understanding of known virulence mechanisms and will likely reveal novel virulence activities. In this review, we consider how far we have come in answering this question, compelling open unknowns and directions for future research.

What are virulence activities and how do they evolve?

What is virulence? There has been confusion around the term virulence among plant pathologists since van der Plank (van der Plank 1968) used it to describe the capacity of a pathogen to infect a particular host genotype, while in animal pathology and evolutionary biology virulence is usually related to the harm that infection causes to the host (Sacristán and García-Arenal 2008). The American Phytopathological Society (D’Arcy, Eastburn, and Schumann 2001) adopted the conventions of using pathogenicity as a qualitative description of the ability of a pathogen to cause disease

(similar to van der Planck), and virulence as a quantitative measure of the degree of pathogenicity (similar to the meaning of virulence in other disciplines). Causing disease is a definitive property of pathogens, and it is frequently assumed to be a consequence of pathogen fitness. In this review, we consider **virulence activities** to be anything that enables pathogens to infect and multiply (i.e. increases pathogen fitness), with the consequence of causing disease in either the qualitative or quantitative senses (Figure 1). Therefore, novel virulence activities can include the adaptation of pathogens to: host immunity and physiology; host species, genotypes, or tissues; the environment; and may include production of more efficient reproductive structures or competing/cooperating better with other microorganisms in the ecosystem (Figure 1). These novel virulence activities may evolve by acquisition or adaptation of **virulence factors** (i.e. genes that contribute to a pathogen's ability to infect and multiply within the host) or **virulence-related factors** (i.e. genes indirectly involved in virulence activities). The evolution of novel virulence activities as an adaptive trait is based on the selection exerted on variants that have been generated *de novo* or arrived from elsewhere. In this review we will first address the selective factors that affect virulence activities and then the mechanisms that generate diversity in pathogen populations.

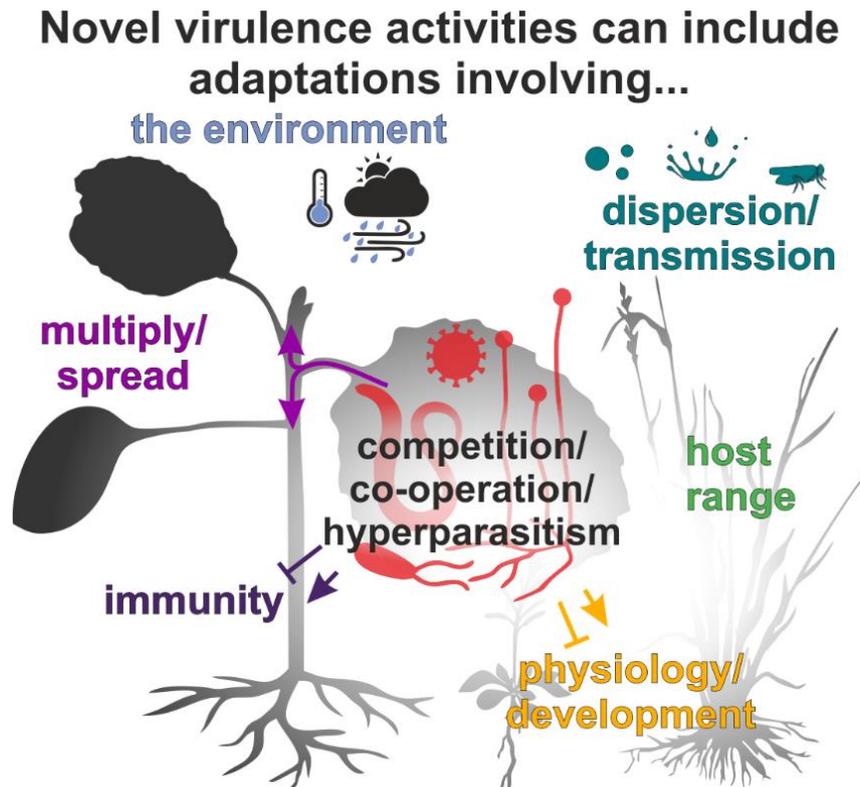


Figure 1. A spectrum of novel virulence activities. Novel virulence activities can include adaptations that alter how pathogens interact with the host immune system (dark purple) or with host physiology and development (orange), the ability to multiply/spread within the host (light purple), how they disperse and are transmitted to other hosts (turquoise), their host range (including host expansions and host jumps, green), how they interact with the environment (blue), and how they interact with other pathogenic and non-pathogenic microorganisms (red).

Selective factors for virulence activities

Pathogens fitness relies on the ability to multiply in a host and transmit to other hosts. Therefore, the host is certainly the main driver for the evolution of virulence activities. Pathogens virulence activities are continually evolving to retain the ability to infect and multiply in the host, which in turn evolves new ways (or is bred) to detect and combat

pathogen attack. Moreover, novel virulence activities may allow the pathogen to acquire new hosts, expanding its host range, or change host specificity leading to host jumps. Besides the host, environmental factors a pathogen experiences during its life cycle may influence the direction of evolution and thus lead to new virulence activities. Non-host factors may be abiotic and biotic. They can condition the capacity of the pathogen to survive outside the host and transmit to other hosts, and also modulate the ability of the pathogen to interact with the host.

Host-pathogen coevolution: the gene-for-gene interaction and beyond

Plants impose physical and biological barriers that pathogens must breach to infect and multiply. For example, plants recognize Microbe-Associated Molecular Patterns (MAMPS) and trigger immune responses that pathogens must overcome by the use of **effectors**: secreted proteins or other molecules that contribute to pathogen fitness in the host (J. D. G. Jones and Dangl 2006). A plant resistance gene (*R* gene) may recognise an effector (termed then an avirulence gene or avr gene), often on a gene-for-gene basis, and trigger localised cell death and thereby blocking the *in planta* spread of the pathogen. In such a case, resistance is dominant, and the host selects for pathogen alleles that avoid this response, including “loss-of-function” (Dodds and Rathjen 2010). It should be emphasised that in this context “loss-of-function” mutations in avr genes refers to alleles that evade immune receptors, although may maintain their virulence function. Novel virulence activities can also be “gain-of-function” events, for example another effector that subverts this response, or alters plant physiology in some other way that benefits the pathogen. Plants in turn can evolve novel *R* gene specificities in a co-evolutionary process that can be tracked by patterns of variation generated by diversifying or balancing selection in pathogen and plant genomes (Van

der Hoorn, De Wit, and Joosten 2002). Screening genomes for signatures of selection has revealed previously unidentified effectors (Vries, Stukenbrock, and Rose 2020), and population studies of gene or allele frequencies have uncovered virulence factors with important but quantitative effects (Abrahamian et al. 2018; Cooke et al. 2012). The gene for gene model of interaction (J. D. G. Jones 2019) has greatly contributed to the understanding of the evolution of pathogenicity of biotrophs and hemibiotrophs (M. C. McDonald and Solomon 2018). Maintenance of allelic variation under this model assumes a cost of virulence activities in the absence of selection, although the impact of the costs, and how to measure them, are not yet fully resolved (Milgroom 2015; Brown and Tellier 2011).

Beyond suppressing the plant immune system, pathogens manipulate plant biology for their growth and transmission. For example, hemi/biotrophic pathogens may deliver effectors to alter the function of non-immunity related host genes (so called S genes (Cox et al. 2017)). In Potyvirus and Sobemovirus, the virus-encoded protein covalently linked to the 5' terminus of the genomic RNA (VPg) must interact correctly with host eukaryotic translation initiation factor (eIF) (Hébrard et al. 2010; Robaglia and Caranta 2006). Resistance is attained by recessive alleles of the host target that are not recognised by the pathogen virulence factors. In this case, host-pathogen coevolution follows the Matching-Allele or inverted gene-for-gene model, in which different variants in pathogen virulence factors evolve to match the corresponding host alleles (Sacristán and García-Arenal 2008). This model is also applicable to the evolution of some necrotrophic pathogens, whereby cell death favours the pathogen, and is stimulated either by triggering *R* gene mediated reactions or by the gain-of-function of a secreted toxin (Stukenbrock and McDonald 2009).

There are thus two main drivers of effector evolution: evasion of host immunity and adaptation to newly evolved or existing host factors. While much of our knowledge has been directed to understanding the former, there is still much to unveil about the later. Also, while gene for gene and matching allele interactions are reasonably numerous and well characterized because they are relatively simple, increasing evidence points to interactions being more complex than previously described (Petit-Houdenot and Fudal 2017). Furthermore, the precise function of effectors and their host targets are still unknown in many cases (Büttner 2016; Camborde et al. 2019; P. Li and Day 2019; Lo Presti et al. 2015). It is also not clear the extent to which non-proteinaceous molecules (nucleic acids, pathogen-derived small molecules, metabolites, and lipids) contribute to virulence activities and whether they are encompassed by these evolutionary models (Leisner and Schoelz 2018; Frantzeskakis et al. 2020).

The infection cycle and its trade-offs

Most of the knowledge about the pathogen interaction with the plant is based on the first steps that lead to infection and have the relatively simple models of single gene interactions explained above. However, the process of pathogen multiplication in the host is complex and the host may exert selection for different pathogen alleles, or combinations thereof, at various stages of infection, growth, replication, and transmission. For example, comparison of different strains of the oat crown rust fungus on different varieties of its host revealed that both pathogen and host genotype significantly affected total spore production, with pathogen genotype having the strongest effect on the early stage of infection efficiency, and host genotype most strongly affecting the later life-history stages of latent period and sporulation capacity (Bruns et al., 2012). The virulence activities across a pathogen life cycle, far from being

independent of each other, are frequently interconnected. There are several examples that show the links between virulence factors that promote pathogens growth within the plant and its transmission to other hosts. Pth effectors in the citrus canker pathogen, *Xanthomonas citri*, cause rapid growth of the bacteria upon infection and are responsible for producing pustules that facilitate transmission by wind and rain (Brunings and Gabriel 2003; Graham et al. 2004). In *Xylella fastidiosa*, biofilm formation increases virulence by restricting water flow in the xylem and is also required for insect transmission (Killiny et al. 2013). Virulence activities may also be a by-product of selection for pathogen survival outside of its host(s) (e.g. biofilm and toxin production (Morris et al. 2009; Kettles et al. 2018)) or a combination of factors. For example, melanin protects fungi from environmental stress and is also required for appressoria to penetrate the host in some pathogenic fungi (Henson, Butler, and Day 1999), while the transcription factor Amr1, which induces melanin biosynthesis in *Alternaria brassicicola*, negatively affects virulence (Cho et al. 2012). Thus, the complex relationships among traits affecting different stages in the infection cycle may result in trade-offs between virulence activities, and these can vary among pathogen genotypes and ecological/host contexts (Bruns, Carson, and May 2012; Kirchner and Roy 2000; Meyer, Stewart, and Clement 2010; Pariaud et al. 2009).

One of the most generally assumed of such trade-offs is that between pathogen virulence and transmission (Anderson and May 1982; Ewald 1983). Examples above illustrate how pathogens may need to attain a within host multiplication level that allows successful transmission, but abundant multiplication can cause so much host harm that it limits available time for transmission. Therefore, models of virulence evolution predict an optimal virulence level that maximizes pathogen growth *and* transmission (Anderson and May 1982; Ewald 1983). Thus, the tradeoffs in virulence activities

within and outside the host may result in virulence (ie. host harm) not being maximized when pathogen fitness is maximized (Alizon et al. 2009). In an extreme case, virulence is reduced in pathogens that are vertically transmitted through the seed to allow the plant to complete the reproductive cycle. *Cucumber mosaic virus* (CMV) virulence was significantly reduced after five generations of vertical transmission through seeds in a serial-passage experiment in *Arabidopsis thaliana*, whereas virulence did not change in viruses transmitted horizontally by mechanical transmission (Cobos et al. 2019; Pagán et al. 2014). Some fungal endophytes of the genera *Epichloe* and *Neotyphodium* that lack sexual reproduction transmit mostly vertically through seed without any harm to the plant, in contrast with sexually reproducing strains that castrate plant flowers with their reproductive structures (Schardl 1996). Adaptation to different hosts may also impose trade offs in the evolution of virulence activities (Bera, Fraile, and García-Arenal 2018; Dutta et al. 2020; Huang et al. 2019; Sacristán et al. 2005). It is assumed that the ability of generalist pathogens to exploit multiple different hosts entails costs, and host specialization may result in more fecund pathogen phenotypes (Barrett and Heil 2012; Elena, Fraile, and García-Arenal 2014). Thus, trade-offs in the adaptation to different hosts may force generalist pathogens towards host specialization, and it is generally assumed that this is the fate of host pathogen coevolution (Burdon and Thrall 2009; Brown and Tellier 2011). However, the molecular mechanisms associated with adaptive evolution of host-specific lineages of a multihost plant pathogen remain mostly poorly understood (Bedhomme, Hillung, and Elena 2015). Trade-offs between virulence and transmission, or in the breadth of host adaptation, may have consequences in the severity of diseases in agroecosystems (Milgroom 2015; B. A. McDonald and Stukenbrock 2016). Agricultural conditions may increase the probabilities of between-host transmission and host specialization

because of large, dense and genetically uniform monocultures, favouring pathogens that reproduce quickly and, thus, selecting for higher virulence. However, experimental data about trade-offs in the evolution of virulence activities are often contradictory (Sacristán and García-Arenal 2008; Remold 2012). Also, there is little known about the molecular mechanisms that hold back pathogen virulence (Sanfaçon 2020; Tanaka et al. 2006).

Adaptation to new hosts

One of the most relevant consequences of novel virulence activities is the ability to infect new hosts, which ultimately leads to the emergence of new diseases (M. McLeish et al. 2017; Woolhouse and Gowtage-Sequeria 2005). We have some knowledge about the mechanisms for overcoming the defences and exploiting host resources by pathogens, but we still do not know the key mechanisms that lead to the adaptation to a new host and, thus, determine pathogen host range (R. A. C. Jones 2009; Morris and Moury 2019). Pathogen host range may vary from one or very few to several hundreds of different plant species (specialists vs. generalists pathogens), although this may be difficult to determine, depending on the definition of host and adaptation to host (Morris and Moury 2019; Sacristán and García-Arenal 2008). Determinants of host range may be both intrinsic (availability of virulence factors that allow the interaction with different hosts) and extrinsic (e.g., exposure of plants to microorganisms and environmental conditions favourable for infection) (M. McLeish et al. 2017; Morris and Moury 2019). Extrinsic determinants include ecological factors such as host population structure and diversity, epidemiological such as vector availability and dynamics, or even stochastic events (Brown and Tellier 2011; M. J. McLeish, Fraile, and García-Arenal 2018). Host range evolution has mostly been

studied by focusing on the intrinsic, genetic factors, but studies on the role of extrinsic factors are starting to gain importance (M. McLeish et al. 2017).

Adaptation of pathogens to new hosts may lead to host range expansions and host jumps, the last when the ability to infect a new host leads to genetic differentiation of pathogen populations on different hosts and finally pathogen speciation (Thines 2019). It is broadly observed that pathogens tend to infect plants that are closely related, rendering the phylogenetic distance between plant taxa as an important predictor of the risk of a new host acquisition (Gilbert et al. 2012; Schulze-Lefert and Panstruga 2011). However, there are also many examples of related plant pathogens that are able to infect distant hosts, so other factors such as host geographical, ecological or physiological distance may play a role (M. McLeish et al. 2017; Morris and Moury 2019; Thines 2019). This is the case of new host acquisitions by indigenous pathogens when a host is introduced in a new area, such as the case of *Cocoa swollen shoot virus*, which was a pathogen of the native forest tree *Cola chlamydantha* before cocoa was introduced in West Africa (Thresh 1982). Also important is the role of bridge hosts in facilitating host jumps and range expansions. For example, widespread deployment of *rwt3* wheat in Brasil, followed by the loss of function of *PWT3*, is proposed to be at the origin of the host jump to common wheat of *Lolium* pathotypes of *Magnaporthe oryzae* (Inoue et al. 2017).

The first step in the ability to infect a new host should be overcoming so-called nonhost resistance (i.e., resistance shown by an entire plant species against all known genetic variants (or isolates) of a specific parasite or pathogen (Antonovics et al. 2013). This type of broad and durable resistance is one of the most aspired aims in crop protection, and indeed MPMI top 10 question #6 is about its molecular basis (Panstruga and

Moscou 2020; Harris et al. 2020). Since nonhost resistance is mechanistically indistinguishable from other types of plant immunity, virulence factors for the adaptation to new hosts may not be very different from those deployed against host resistance (Panstruga and Moscou 2020). Indeed, there is convincing evidence pointing to the role of effectors in shaping host range and leading to new host acquisitions both by gain or loss of functions (J. Li, Cornelissen, and Rep 2020; Morris and Moury 2019; Frantzeskakis et al. 2020). However, nonhost resistance may be one of several hurdles that microorganisms need to overcome to infect and cause disease in a new host, so mechanisms to acquire novel host virulence activities should frequently be more complicated than just acquiring one or few virulence factors (Morris and Moury, 2019; Thordal-Christensen, 2003). For example, *Fusarium oxysporum* f. sp. radicum (Foc) causes disease in cucurbits whereas *F. oxysporum* f. sp. melonis (Fom) is limited to melon. Li et al (J. Li et al. 2020) found that Fom can colonize root xylem of other cucurbits but cannot reach the stem like Foc. A roughly 300 kb region in a pathogenicity chromosome appears to be responsible for this ability to colonise the stem of cucurbits. In phytopathogenic bacteria, such as *Pseudomonas* and *Xanthomonas*, host jumps are associated with acquisition of new genes and alleles, but generally require more than the transfer of single effectors (Timilsina et al. 2020; Dillon et al. 2019). Which genes are important and why, and why some effectors make a relatively greater contribution to overcome nonhost resistance and exploit a new host are open questions.

Selection beyond the host

Abiotic and biotic factors other than the host also affect pathogen virulence activities and thereby fitness (Morris and Moury 2019). Temperature, for example, is a key driver

of ecological specialization of pathogens (Chaloner, Gurr, and Bebber 2020) and can entail trade-offs with components of pathogen fitness (Chen et al. 2017; L. Yang et al. 2018). Ecological complexity also affects evolutionary trajectories of pathogens, and more research using multihost and multipathogen systems is needed to understand the consequences for virulence activities (M. McLeish et al. 2017). Next generation sequencing studies have revealed the enormous diversity of microorganisms that coexist within a plant, showing that co-infection is the rule, rather than the exception, and highlighting the relevance of microbe-microbe interactions in the phytobiome (Hassani, Durán, and Hacquard 2018; Snelders et al. 2018; Teixeira et al. 2019). Indeed, plant pathogens do not exist, or infect, in isolation: they are consumed by animals, are hosts to other pathogens and parasites, and may cooperate (Wheeler et al. 2019) or compete (Snelders et al. 2018) with the many other microbes in their hosts (Tollenaere, Susi, and Laine 2016). These synergistic or antagonistic interactions can increase or decrease disease severity (Mascia and Gallitelli 2016). For example, *Rhizoctonia solani* AG 2-2IIIB hosts an *Enterobacter* sp. that is required for virulence and toxin production on turfgrass (Obasa et al. 2017), while *Cryphonectria parasitica* shows hypovirulence when infected by a mycovirus (Choi and Nuss 1992). Complex biotic interactions may also confuse identification of the “causal” agent (Wheeler et al. 2019). Both biotic and abiotic factors are of considerable importance to understanding pathogen evolution and disease emergence in light of climate change, globalization and other anthropogenic habitat modifications (Bebber 2015; Velásquez, Castroverde, and He 2018). Other Top 10 Questions in MPMI: How does abiotic stress, such as climate change, influence plant-microbe interactions? (Question #2), How do microbe-microbe interactions affect plant-microbe interactions? (Question #4) and How do

observations of binary plant-microbe interactions hold in an ecological context? (Question #10), bring forth these important points (Harris et al., 2020).

Genetic mechanisms underpinning the evolution of novel virulence activities

Selection acts on genetic variation. The ultimate source of epi/genetic variation is DNA mutation that generates novel coding sequences or changes gene regulation. Sexual or asexual genetic exchange between closely or distantly related individuals shuffles genetic variation, resulting in further novel phenotypes. This section reviews the various mechanisms by which pathogens generate and recombine variants, and the virulence activities that result.

Mutation and mutation rate bias

Mutation is defined as any heritable change in the genome of an organism that can occur at the level of one or a few nucleotides up to larger scales of genes or segments of chromosomes. Depending on the mechanism of pathogenesis and host resistance, a single nucleotide mutation can give rise to a virulent pathogen genotype (Bartoli, Roux, and Lamichhane 2016; Grandaubert, Dutheil, and Stukenbrock 2019; Rouxel and Balesdent 2017; Pagán and García-Arenal 2019). Allelic series at effector loci can also give rise to qualitative differences in direct recognition specificity by the corresponding host *R* genes (e.g the *AvrL567* locus in flax rust (Dodds et al. 2006; Ravensdale et al. 2012)). Loss-of-function mutations such as premature stop codons or deletions impair recognition by host surveillance systems of effector functions (Möller and Stukenbrock 2017). Avoiding recognition may also allow pathogens to overcome non-host resistance (Thines 2019) and acquire the ability to infect new hosts

(e.g. loss of PWT3 Avr enabled the emergence of the rice blast fungus as a novel pathogen of wheat (Inoue et al. 2017)).

Although mutation rate is generally thought of as an intrinsic property of each organism (Drake et al., 1998), evidence is emerging that mutation rate bias may be adaptive (Grey Monroe et al. 2020), with some regions of the genome more prone to mutations than others. In *M. grisea*, gain or loss of effector genes is often associated with the unstable telomeric regions of the chromosome (Yoshida et al. 2009). Genomes may be compartmentalized into gene-rich, slow-evolving regions and repeat-rich, gene-sparse regions that exhibit high variability, such as the so-called two-speed genomes of *Phytophthora* spp. (Raffaele et al. 2010; Dong, Raffaele, and Kamoun 2015). Compartmentalisation of virulence factors in the genome stands to increase adaptive potential by allowing rapid evolution while minimizing deleterious effects of mutation on housekeeping genes in gene rich regions. Although by no means universal, similar patterns of compartmentalising effectors to relatively gene-sparse regions of the genome were found in plant-pathogenic fungi (*Leptosphaeria maculans* (Rouxel et al. 2011)), *Zymoseptoria tritici* (Stukenbrock et al. 2010) and *Colletotrichum higginsianum* (Tsushima et al. 2019), bacteria (Rohmer, Guttman, and Dangl 2004), and nematodes (Eves-van den Akker et al. 2016). It is not clear in all cases that mutation rates are higher in these compartments, rather their evolution may be the result of the absence of purifying selection. This pattern is not evident in viruses where mutations in overlapping coding regions and multifunctional proteins may have pleiotropic effects that lead to costs of virulence (García-Arenal and McDonald 2003). In general, empirical estimates of mutation rate for most pathogens remain scarce and the underlying mechanisms unclear (Baer, Miyamoto, and Denver 2007; Tenailon, Denamur, and Matic 2004).

Transposable elements

Gene sparse regions of the genome are often occupied by transposable elements. Transposable elements are powerful mutators in prokaryotic and eukaryotic genomes (Möller and Stukenbrock 2017), and may have consequences in surrounding genes such as deletion, epigenetic silencing, duplication, and recombination that may provide variation on which natural selection can act (Gijzen, Ishmael, and Shrestha 2014; Seidl and Thomma 2017). For example, the insertion of TEs in the sequences of avirulence genes resulted in gain of virulence due to the lack of recognition by the host in *Magnaporthe grisea* (Fudal et al. 2005; P. K. Singh et al. 2019), and gain and loss of genes linked to transposable elements has been related with the ability to infect different hosts (M. C. McDonald et al. 2019; Yoshida et al. 2016). Indeed, “pathogenicity islands” described in prokaryotes (Kim and Alfano 2002) and some eukaryotes (Eves-van den Akker et al. 2016) are groups of clustered genes involved in pathogenicity (e.g. effectors) that can undergo rapid changes and are frequently flanked by transposable elements. In some fungi, close proximity of virulence factors to transposable elements can increase the local mutation rate through repeat-induced point (RIP) mutations. In *L. maculans*, effector alleles with premature stop codons or non-synonymous RIP mutations evolved to evade plant resistance mediated by immune receptors in just a few years (Rouxel et al. 2011). Finally, in some cases, transposable elements have also been neofunctionalized as virulence factors themselves. *Botrytis cinerea* uses TE derived sRNA to hijack host RNA interference pathways and suppress plant immunity (Weiberg et al. 2013). In barley powdery mildew, Nottenstainer et al. (Nottenstainer et al. 2018) demonstrated that the ROPIP1 effector protein derives from a SINE-related transposable element. In most cases, we do not know why some genomes have many more transposable elements than others.

In powdery mildew fungi, extraordinary proliferation of transposable elements result in some of the largest genomes in the ascomycete fungi, perhaps due to the absence of the Repeat-Induced Point mutation (RIP) pathway that is otherwise conserved in all related ascomycetes (Spanu et al. 2010).

Duplication and neofunctionalization

Duplication and neofunctionalization play important roles in shaping plant-pathogen genomes in general, and effector repertoires in particular. The effector repertoires of plant pathogens can number in the hundreds or thousands, yet their evolutionary origins are often unclear (Badet and Croll 2020). Exceptions include horizontal gene transfer (discussed below), and effector gene birth by gene duplication and neofunctionalization. This includes the “weaponization” of transposable elements (discussed above) and endogenous housekeeping functions as effectors. For example, two large-scale gene multiplication events of the housekeeping glutathione synthetase (GS) resulted in the GS-like effectors of plant-parasitic nematodes. New GS-like paralogues acquired canonical features of an effector (e.g. DOG box promoter motif, dorsal gland cell expression, and signal peptide), and the encoded proteins are translocated into host cells (Lilley et al. 2018). Remarkably, the crystal structures of GS-like effectors suggest novel GS paralogues were not just redeployed, but also repurposed to carry out a novel biochemical reaction *in planta*. In addition to explaining the origins of some effectors, the process of gene duplication and neofunctionalization contributes to expansion of certain effectors families, regardless of origin. There are several examples of large effector families, with a diversity of functions, linked to a single origin. For example the WY fold of RXLR effectors is conserved across plant pathogenic oomycete species (Win et al. 2012), the repeat variable di-residues are

characteristic of TAL effectors in *Xanthomonas* bacterial pathogens (Timilsina et al. 2020), and the SPRY domain containing proteins, which are linked to a single origin in animals but deployed as effectors by pathogenic nematodes and ultimately recognized by plants (Sacco et al. 2009).

Beyond the duplication of single effectors or effector gene families, fungal and oomycete pathogens have outstanding chromosome plasticity, with frequent chromosomal aberrations such as aneuploidy or copy number variations resulting from chromosome loss or gain (Covo 2020). Strains of the needle blight pathogen *Dothistroma septosporum* are aneuploids, with chromosome duplications that result in increased gene copy numbers, which correlate with increased production of the toxin dothistromin (Bradshaw et al. 2019). Host induced aneuploidy has been proposed for the oomycete *Phytophthora ramorum*, the causal agent of Sudden Oak Death (Kasuga et al. 2016) and successful strains of the late blight pathogen *P. infestans* exhibit triploidy and aneuploidy (Knaus et al. 2020), although the mechanisms underlying this chromosomal variation, and the specific impact on virulence, remain unclear.

Genetic exchange within species and kingdoms

Genetic exchange (sexual and parasexual) and recombination can give rise to novel virulence activities. Recombination can generate novel combinations of virulence factors and purge deleterious alleles, and thus, has the potential to produce novel virulent phenotypes more rapidly than mutation alone (Milgroom 2015; Grandaubert, Dutheil, and Stukenbrock 2019). For example, the virulent blue13 lineage of *P. infestans* emerged from a sexually recombining population in northern Europe (Cooke

et al. 2012). Recombination can also occur in the absence of sex. Although less common, evidence for mitotic crossing over has been shown among clonal lineages in fungi and oomycetes (Milgroom 2015). Mitotic recombination has been associated with genotypic diversity in *P. ramorum* and *P. capsici* (Dale et al. 2019; Lamour et al. 2012). Parasexuality in ascomycetes (due to hyphal fusion or anastomosis) and somatic hybridization in dikaryotic basidiomycetes (asexual karyogamy) are mechanisms of asexual exchange of nuclei and recombination (Glass and Dementhon 2006; Park and Wellings 2012). Both phenomena have been demonstrated in the laboratory for many species, but its relevance in nature remains unclear. Anastomosis is the most likely mechanism behind the transfer of accessory chromosomes and horizontal gene transfer (HGT) between different fungal lineages or species (Soanes and Richards 2014). Presence/absence of accessory/dispensable chromosomes have been associated with host specialization (Mirocha et al. 1992; Temporini and VanEtten 2004). Indeed, the transfer of accessory chromosomes to non-pathogenic isolates can be sufficient to generate a virulent pathogen (J. Li et al. 2020). The acquisition of novel virulence activities through HGT has been documented in several cases (Soanes and Richards 2014).

Hybridization, defined here as the combining of two phylogenetically distinct genomes, can result in novel genotypes. Hybrids may be reproductively isolated, or continued backcrossing with parental genotypes can result in the introgression of regions of one parental genome into the other parental genome. Multiple examples are found in fungi, oomycetes, and nematodes (Eves-van den Akker and Jones 2018; Feurtey and Stukenbrock 2018). For example, analysis of *Z. tritici* genomes showed evidence of repeated introgression from other *Zymoseptoria* species, including regions containing effectors (Feurtey et al. 2019). A similar pattern of introgression was found among

host-specific races of the oomycete *Albugo candida* (McMullan et al. 2015). By contrast, *Z. pseudotritici* appears to have originated from a single hybridization event and has remained reproductively isolated from its parental species, as evidenced by its clonal population and the mosaic structure of its genome (Stukenbrock et al. 2012).

Intra- and interspecific hybridization events can expand the host range of hybrid offspring and therefore must confer novel virulence activities. Several *Phytophthora* hybrids show enhanced virulence (Brasier et al. 2004) or expanded host range (Jafari et al. 2020). *Blumeria graminis* f. sp. *triticales* is a hybrid of wheat pathogen *B. graminis* f. sp. *tritici* and rye pathogen *B. graminis* f. sp. *secalis* (Menardo et al. 2016). The hybrid *B. g. triticales* can infect the artificial wheat-rye hybrid triticales, whereas the parental pathogens cannot. It is not yet clear how hybridization changed this host-pathogen interaction. Wheat stem rust caused by *Puccinia graminis* f. sp. *tritici* was controlled for more than 30 years thanks to wheat varieties carrying stem rust resistance gene Sr31, until Ug99 overcame Sr31 resistance and rapidly expanded in East and South Africa and the Near East (R. P. Singh et al. 2011). Genomic evidence suggests that Ug99 emerged by somatic hybridization and nuclear exchange between two dikaryons followed by the loss of function of an effector (F. Li et al. 2019). Some plant-parasitic nematodes in the genus *Meloidogyne* have a polyploid origin consistent with hybridisation (allopolyploidy (Trudgill and Blok 2001)), with a further study suggesting some species (e.g. *M. incognita*) result from multiple, additive, hybridization events (Lunt et al. 2014). These hybrids are parthenogenetic, yet can overcome host resistance in the absence of sex (Castagnone-Sereno 2006), and have a broader host range, wider geographical distribution, and greater agricultural impact than their sexual relatives (Blanc-Mathieu et al. 2017). The extraordinarily broad host range of the asexual hybrid *M. incognita* (several thousand hosts) includes representatives from

most orders of flowering plants (Eves-van den Akker and Jones 2018). While there are now numerous examples of hybrid plant pathogens (Depotter et al. 2016), the specific genetic mechanisms that increase virulence or host range of hybrids are largely unknown. Gain of effector function, or at least gain of genetic capital for neofunctionalization (as discussed above), is plausible but by no means a satisfactory explanation for some of the more dramatic increases in host range.

Recombination in virus populations (ie. the exchange of genomic fragments between genotypes) can occur between genotypes of the same or of different virus species (Pagán and García-Arenal 2019), and the emergence of new viral lineages with enhanced virulence and enlarged/different host ranges is in many cases explained by recombination events (Monci et al. 2002; Ruiz et al. 2018; van der Walt et al. 2009). The contribution of recombination to the generation of virus genetic diversity is comparable to that of mutation, despite the exclusive clonal multiplication of these pathogens. In RNA viruses, recombination is due to copy choice template switching during RNA replication, while in DNA viruses the mechanisms are less understood and may involve double-strand break repair events on dsDNA intermediates using host cell factors. In all viral types, recombination breakpoints within coding regions are rare, probably because these are selected against (Lefeuvre et al. 2009). Genetic exchange in virus populations may also result from reassortment of genomic segments in viruses with segmented or multipartite genomes, in a process that is also known as pseudorecombination (Varsani et al. 2018). Multipartite genomes are frequent among plant viruses, despite the cost for transmission if most segments must be transmitted between hosts to cause new infections (Zwart and Elena 2020). Reassortment is strongly related with virus evolution and speciation and it is seen as an advantage for multipartite viruses because of the possibilities of generating fitter variants (Roossinck

2005). However, there are constraints in the generation of reassortants during co-infection, such as incompatible RNA–RNA or protein–RNA interactions from different viral strains, that may prevent them from prevailing in the population (Bonnet et al. 2005; S. M. McDonald et al. 2016).

Bacterial plant pathogens are efficient in acquiring existing virulence factors via HGT. Emerging strains of *Pseudomonas syringae* on cucurbits show convergent acquisition of hrp/hrc genes and effectors, among other genes, across phylogenetically distinct groups (Newberry et al. 2019). These genes appear to have been horizontally transferred in integrated conjugative elements and on plasmids. Similar patterns of convergent host specificity in phylogenetically distinct lineages via horizontal gene transfer of hrp/hrc gene clusters have been reported for *Acidovorax avenae* (Zeng et al. 2017). In *Xanthomonas*, host range expansion of *X. perforans* from tomato to pepper is associated with extensive recombination from sister taxon and pepper pathogen *X. euvesicatoria* (Schwartz et al. 2015; Timilsina et al. 2020; Newberry et al. 2019), both members of the *X. euvesicatoria* species complex (Barak et al. 2016). I

Genetic exchange between kingdoms

Since the first discovery of a cellulase in an animal (Smant et al. 1998), HGT events from bacteria and fungi to plant-parasitic nematodes have been recognized as surprisingly numerous ((conservatively 0.6% of genes (Eves-van den Akker et al. 2016)) and an important catalyst for the evolution of parasitism (reviewed in (Haegeman, Jones, and Danchin 2011)). Interesting examples include pseudo-convergent transfer of specific functions, for example a GH45 cellulase from fungi to *Bursaphelenchus xylophilus* (Kikuchi et al. 2004) and the GH5 cellulase from a bacteria to various Heteroderidae (Danchin et al. 2010; Eves-van den Akker et al.

2016; Cotton et al. 2014). Genome wide analyses have expanded the complement beyond “effector proteins” to metabolism, vitamin biosynthesis, etc (e.g. (Eves-van den Akker et al. 2016; Moran and Jarvik 2010)). Despite apparent widespread transfer from bacteria and some eukaryotes to nematodes, there is a conspicuous absence of transfer from oomycetes, viruses, and plants (although examples of the latter exist in plant-parasitic plants (Z. Yang et al. 2019)).

Fungi and oomycetes are similarly recipients of horizontal transferred genes from other kingdoms that are involved in virulence activities, such as a subtilisin serine protease–encoding gene from a plant donor to an ancestor of *Colletotrichum* fungi (Jaramillo et al. 2013), a glucan glucosyltransferase–encoding gene from bacteria that probably allows vascular fungi to survive in the high osmotic conditions of plant’s xylem (Klosterman et al. 2011) and the necrosis- and ethylene inducing peptide 1 (NEP1)-like proteins (NLPs) that oomycetes have probably acquired from ascomycetes (Richards et al. 2011). In general, we have well characterised examples of genetic transfer events that give rise to virulence activities but it's not clear how often these phenomena occur in the field, and whether novel virulence more often evolves *de novo* or via the transfer of existing virulence factors from some other system.

Concluding remarks

We return to the question “How do pathogens evolve novel virulence activities?” This review tells us that if there is an epigenetic/genetic mechanism that can create variation in the genome (characterised or otherwise) it will be used by pathogens to evolve virulence factors. We may think that we know quite a lot about how pathogens evolve virulence but there are many unexplored areas/questions (Box 2). It seems the most common form of evolution of virulence is the classical loss of an avr, but this may

be due to a bias in research rather than a reflection of reality. There are many other virulence activities during the life stages of the pathogen that are underexplored, as well as the effects of actors other than the host (ie. biotic and abiotic factors in the ecosystem) on the evolution of pathogen virulence activities. More studies are needed to disentangle the involvement of virulence factors in pathogen life-stages other than infection, and the implications in coevolutionary processes and adaptation to new hosts (Morris and Moury 2019).

Understanding the key driving forces that give rise to novel virulence activities in agricultural and wild settings may help inform crop protection. For example, given the diversity of mechanisms, and the veritable certainty of the evolution of pathogens, perhaps the focus should be on not just the stacking of several arbitrary sources of resistance, but rather the stacking of sources of resistance that require very different mechanisms of evolution to overcome? In order to approach these strategies, the integration of evolutionary concepts and methods with mechanistic research on plant–microbe interactions is necessary (Upson et al. 2018).

Virulence activities are just one of the many strategies microorganisms deploy to maximize fitness in their interaction with plants. The range of interactions between plants and microorganisms extends from pathogenic to beneficial with multiple forms in between (Hardoim et al., 2015). Quite often, genotypes exhibit phenotypic plasticity or evolutionary tradeoffs mediated by the biotic interactions, the abiotic environment, or a combination thereof (Hacquard et al. 2016; Muñoz-Barrios et al. 2020; Hardoim et al. 2015; Brader et al. 2017). Pathogens and beneficial microorganisms share many of the tools and mechanisms in their interaction with the host ((Paszkowski 2006) and see also Top 10 MPMI Question #1 “How do plants engage with beneficial

microorganisms while at the same time restricting pathogens? (Harris et al. 2020). The question “how do pathogens evolve novel virulence activities” leads to the more general “why do microorganisms evolve virulence activities”? There are probably as many answers to these questions as there are evolutionary pathways for microorganisms to become virulent.

Box 2. Some outstanding questions/topics in relation to how pathogens evolve novel virulence activities.

Effectors

- While much of our knowledge has been directed to understanding effector evolution to evade host immunity, there is still much to learn about effector adaptation to newly evolved or existing host factors.
- The precise function of effectors and their host targets are still unknown in many cases. Why do some effectors make a relatively greater contribution to novel virulence activities?
- To what extent do non-proteinaceous molecules (nucleic acids, pathogen-derived small molecules, metabolites, and lipids) contribute to virulence activities and are they encompassed by existing evolutionary models?

Models of plant-pathogen interaction

- It is generally assumed that there is a cost of virulence activities in the absence of selection, although the impact of the costs, and how to measure them, are not yet fully resolved.
- While gene for gene and matching allele interactions are reasonably numerous and well characterized because they are relatively simple, increasing evidence points to interactions being more complex than previously described.

Pathogenic traits, life cycle, and trade-offs

- What is the involvement of virulence factors in pathogen life-stages other than infection?
- Experimental data about trade-offs in the evolution of virulence activities are often contradictory.
- Little is known about the molecular mechanisms that restrict pathogen virulence.
- The molecular mechanisms associated with adaptive evolution of host-specific lineages of a multihost plant pathogen remain mostly poorly understood. We still do not know most of the key steps that lead to the adaptation to a new host and, thus, determine pathogen host range.
- Ecological complexity also affects evolutionary trajectories of pathogens, and more research using multihost and multipathogen systems is needed to understand the consequences for virulence activities.

Mechanisms for acquiring novel virulence activities

- In general, empirical estimates of mutation rate for most pathogens remain scarce and the underlying mechanisms unclear.
- What are the mechanisms underlying chromosomal variation and what is its specific impact on virulence?
- What is the relevance in nature of parasexuality and how often do genetic transfer events that give rise to novel virulence activities occur in the field?
- The underlying mechanisms of HGT between different species and kingdoms and are mostly unknown.
- What are the specific genetic mechanisms that increase virulence or host range of hybrids?

- Does novel virulence more often evolve de novo or via the transfer of existing virulence factors from some other system?

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