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Studying Plant-Pathogen Interactions in the Genomics Era: Beyond Molecular Koch's Postulates to Systems Biology

David J. Schneider[1,2] and Alan Collmer[2]

[1]U.S. Department of Agriculture, Agricultural Research Service, Robert W. Holley Center for Agriculture and Health, Ithaca, New York 14853; email: Dave.Schneider@ars.usda.gov

[2]Department of Plant Pathology and Plant-Microbe Biology, Cornell University, Ithaca, New York 14853; email: arc2@cornell.edu

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Abstract

Molecular factors enabling microbial pathogens to cause plant diseases have been sought with increasing efficacy over three research eras that successively introduced the tools of disease physiology, single-gene molecular genetics, and genomics. From this work emerged a unified model of the interactions of biotrophic and hemibiotrophic pathogens, which posits that successful pathogens typically defeat two levels of plant defense by translocating cytoplasmic effectors that suppress the first defense (surface arrayed against microbial signatures) while evading the second defense (internally arrayed against effectors). As is predicted from this model and confirmed by sequence pattern–driven discovery of large repertoires of cytoplasmic effectors in the genomes of many pathogens, the coevolution of (hemi)biotrophic pathogens and their hosts has generated pathosystems featuring extreme complexity and apparent robustness. These findings highlight the need for a fourth research era of systems biology in which virulence factors are studied as pathosystem components, and pathosystems are studied for their emergent properties.

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Effector: all pathogen proteins and small molecules that alter host-cell structure and function (36)

INTRODUCTION

> What makes an evening primrose open when it does? Why does salt water fail to satisfy thirst? What is the description of aging in biochemical terms?...They are all problems which involve dealing simultaneously with a *sizeable number of factors which are interrelated into an organic whole*...Science must, over the next 50 years, learn to deal with these problems of organized complexity.
>
> Warren Weaver, Rockefeller Foundation, 1948

The interactions of plants and microbial pathogens are among the most complex phenomena in biology. Plant diseases collectively involve multiple microbial kingdoms, diverse host and tissue specificities, a myriad of symptoms, and a potentially unlimited variety of pathogen molecules that can interact with targets in virtually any cellular component of any plant. Since the early 1900s plant pathologists have been using ever more powerful tools to seek and rigorously evaluate determinative pathogen molecules that can explain the development of plant diseases. However, the notion of a determinative virulence factor for many diseases is now challenged by recent discoveries that pathogen genomes contain bewilderingly complex repertoires of candidate virulence genes and by the overwhelming scale of the molecular invasion of host cells by pathogens. Furthermore, we now have the outlines of a unified model for plant-pathogen interactions, and evolutionary expansion and internal redundancy in several virulence factor classes appear to be natural outcomes of this model. Genomics has released the genie of complexity, and consequently, the value of different kinds of information about virulence factors is changing. As we explain below, fulfilling molecular Koch's postulates for a candidate virulence factor may be less useful than efforts aimed at understanding the factor's role(s) in systems-oriented models of pathogenesis. Although we use the term virulence factor throughout this article, our thesis is that the meaning of "factor" is increasingly becoming synonymous with "system component."

To develop this thesis, we address historical changes in the methods for finding and validating virulence factors using the example of a few well-studied factors. We summarize the recently developed unified model of plant-pathogen interactions, emphasizing the genesis of complexity (and robustness) in some pathosystems. We then explore the systems properties of the type III effector repertoires of *Pseudomonas syringae* strains, asking how such effectors operate together to promote pathogen growth. With these examples brought to the fore, we revisit the challenge of applying molecular Koch's postulates and of categorizing virulence factors and multiple classes of effectors. Finally, we address new ways to gather, report, and visualize data about virulence system components that more efficiently yield systems-level knowledge with greater explanatory power and practical utility.

HOW PHYTOPATHOGEN VIRULENCE FACTORS HAVE BEEN FOUND AND VALIDATED

We begin with a brief historical overview because it is useful to see how the technical limitations of each era functioned as filters on what types of virulence factors could be identified and because seeing the trajectory of our progress may help us anticipate future challenges. The specific virulence factors we illustrate are intended to provide a common starting point for readers with diverse backgrounds, for example, plant pathologists seeking broad explanations for why pathogens so easily overcome crop resistance, molecular biologists grappling with the lack of a demonstrable role in virulence for pathogen molecules known to have host targets, and genomicists trying to put in biological context the many expanded gene classes that mediate host-pathogen interactions.

One concept that must be explained at the outset is that pathogens fall broadly into two classes regarding their interactions with plants: necrotrophs rapidly kill host tissue

and often have wide host ranges, whereas (hemi)biotrophs have a nutritional relationship with living plant cells (105). (Some biotrophs induce substantial host cell death later in the infection and are known as hemibiotrophs. We use the term biotroph broadly here and differentiate hemibiotrophs and strict biotrophs only as needed.) Many biotrophs show a high degree of host specificity, and they can be controlled by the introduction, through plant breeding, of major resistance (*R*) genes (42, 78). Importantly, plant defenses against biotrophs and necrotrophs are distinct and in competition with each other (29, 96).

Overview of the Three Eras in Molecular Plant Pathology Research

The search for the molecular basis for plant-microbe interactions can be broadly divided into three eras defined by available tools. The first was the era of disease physiology, which extended from the early 1900s until the mid-1980s. The starting point for the success stories from this era of "grind and find" research typically was a cell-free extract with biological activity. By the 1970s, advances in biochemistry enabled purified virulence molecules to be isolated from these extracts and studied in detail for their effects on plants. The second era was the era of molecular genetics focused on one or a few genes, which extended from the mid 1980s to 2000 for bacterial pathogens, with slight lags for pathogenic fungi, oomycetes, and nematodes (we do not address viruses). The starting point of success stories in the "screen for gene" era typically was a pathogen with a strong virulence-related phenotype resulting from an insertion-marked mutation or a heterologously expressed gene. The third era, the genomics era, began in 2000 with the sequencing of the complete genome of the bacterial pathogen *Xylella fastidiosa* (94). As discussed below, the typical success stories (so far) from the "patterns that matter" era involve a validated virulence factor that was initially identified as a candidate by sequence patterns associated with its gene. The methods of each era continue to be improved and used in the succeeding eras as parts of an ever-expanding toolkit.

Systems Historically Used to Validate Virulence Factors

The tools of each era also affect the rigor of the validation tests for candidate virulence factors. The following criteria have been used over all three eras to implicate and validate candidate virulence factors in phytopathogens:

Capability, Time, and Place

1. Factor is produced by the pathogen.
2. Factor is produced during infection.
3. Factor is delivered to a location in the host appropriate for its proposed function.

Sufficiency

4. Experiments involving exogenous application of the purified factor indicate that the factor is sufficient for the proposed virulence function.
5. Gain-of-function (GOF) experiments involving heterologous gene expression in a related nonpathogen or a less virulent pathogen, or in transformed plants, indicate that the factor is sufficient for the proposed virulence function.

Necessity

6. Loss-of-function (LOF) experiments involving a biochemical inhibitor indicate that the factor is necessary for the proposed virulence function.
7. LOF experiments involving a mutant indicate that the factor is necessary for the proposed virulence function.

The first three criteria provided circumstantial evidence implicating individual factors in the disease physiology era, and variations of these criteria are providing strategies for comprehensive identification of candidate virulence factors now in the genomics era. Similarly, sufficiency tests involving biochemically fractionated candidate virulence factors are now commonly replaced with high-throughput assays involving panels of cloned candidate virulence

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CWDE: cell wall–degrading enzyme

HST: host-selective toxin

genes. Although the circumstantial evidence from sufficiency tests is useful, LOF tests are needed to rigorously validate a candidate virulence factor.

The following molecular Koch's postulates were formulated by Stanley Falkow in 1988 when the era of single-gene molecular genetics was also empowering research in human and animal pathogenic microbiology (25):

1. The phenotype or property under investigation should be associated with pathogenic members of a genus or pathogenic strains of a species.
2. Specific inactivation of the gene(s) associated with the suspected virulence trait should lead to a measurable loss in pathogenicity or virulence.
3. Reversion or allelic replacement of the mutated gene should lead to restoration of pathogenicity.

As noted by Falkow in a personal recollection 15 years later, these postulates and expanded versions "served their function at the time as a working hypothesis for the study of the genetic and molecular basis of pathogenicity" (25). For the purposes of this review, the term "molecular Koch's postulates" conveniently evokes rigorous validation tests based on loss of gene function and measurable reduction in virulence. We discuss below these postulates and lessons from their application, but our purpose here is not to refine any scheme of tests that a factor must pass before it can be called a virulence factor. Rather, it is to foster a broader look at host-microbe interactions as molecular systems.

Finding and Validating Virulence Factors in the Disease Physiology Era

The pathogen stars of this era were the necrotrophs that, in culture, abundantly produced plant cell wall–degrading enzymes (CWDEs), cutinases, host-selective toxins (HSTs), or enzymes that detoxify phytoalexins (low molecular weight antimicrobial compounds produced by plants in response to pathogens). These virulence factors could be purified from biologically active cell-free extracts and shown to be sufficient for key abilities of the pathogen: tissue maceration and cell killing (pectolytic CWDEs) (5), degradation of the plant cuticle (cutinase) (80), host-specific cell killing and/or induced susceptibility (HSTs) (117, 125), and tolerance of phytoalexins (e.g., pisatin demethylase) (66). Particularly broad attention was given to pectolytic CWDEs because they are produced in abundance by many necrotrophic bacteria, fungi, and oomycetes (17). Although studies using isolated pectic enzymes elucidated their lethal effects on plant cell walls (5, 99), they did not reveal whether the enzymes were necessary for virulence or how the deployment of these destructive factors was integrated into the development of the pathogen-host interaction.

Pathogen genetics in this era was limited to fungal pathogens. Notably, genetic studies with fungal necrotrophs provided correlative support for the role of the *Nectria haematococca* pisatin demethylase and the *Cochliobolus* spp. HSTs (104, 114, 129). Regarding the latter, crosses involving *Cochliobolus victoriae* and *Cochliobolus carbonum*, differing in their production of the HSTs victorin (oat) and HC toxin (maize), respectively, produced progeny with a perfect correlation between the plant specificity of the HST and the pathogenicity of the fungus producing it (90). These experiments provided elegant validation of the determinative role of the two HSTs in pathogenesis, but as we explain below, the tools of molecular genetics were needed to understand how these HSTs functioned by exploiting vulnerabilities in plant defense systems.

In contrast to these limited successes with necrotrophs, the molecular basis for biotroph pathogenesis remained a mystery during this era. Notably, Flor's gene-for-gene hypothesis, which was based on genetic studies of flax cultivars and the flax rust *Melampsora lini*, predicted that pathogens paradoxically carry many genes conditioning avirulence (27). It took the tools of molecular genetics to reveal that the products of such avirulence genes were effector proteins that are central to biotrophic pathogenesis.

Finding and Validating Virulence Factors in the Era of Single-Gene Molecular Genetics

The next era enabled homology-driven reverse genetics to more rigorously test the roles of the necrotrophic bacterial and fungal virulence factors found in the physiological era. With the exception of the HSTs, the vast majority of these factors failed molecular Koch's postulates. Multiple pectate lyase genes in *Erwinia chrysanthemi* (now *Dickeya dadantii*) were deleted, but mutants retained a residual capacity to cause disease (84). Multiple CWDE genes in *C. carbonum* were mutated, but only a global regulatory mutant unable to express all such enzymes was reduced in virulence (106). An *N. hematococca* cutinase thought to be essential for pathogenesis based on inhibitor studies was found to contribute only quantitatively to disease (64, 85, 97). Similarly, the *N. hematococca* pisatin demethylase *pda1* gene was found to be encoded on a dispensable chromosome that segregated with multiple virulence genes in genetic crosses (68), and a targeted disruption of *pda1* quantitatively reduced virulence in pisatin-producing pea plants (118). To further complicate the picture, some pathogens were found to produce, in planta, cutinases and pectic enzymes different from those found in culture and studied in the era of disease physiology (7, 45, 128). However, each one of these factors, as they variously failed molecular Koch's postulates tests, revealed larger pathogen systems involved in plant cell wall and cutin degradation, phytoalexin tolerance, and the modular inheritance of virulence.

Regarding the biotrophs in the molecular genetics era, forward genetic screens involving libraries of heterologously expressed genes from a related pathogen enabled the discovery of a novel class of virulence factors. These were the avirulence proteins that are now seen to be cytoplasmic effectors (CEs) delivered into host cells by many bacterial, fungal, oomycete, and nematode pathogens. In bacteria, these genes were found primarily by the avirulence phenotype their GOF expression conferred on otherwise virulent pathogens in pathosystems involving pathogen races and host cultivars interacting in a gene-for-gene manner (47, 98, 122).

In contrast, forward genetic screens for reduced virulence of transposon-tagged bacterial mutants largely yielded genes encoding protein secretion systems and global regulators. For example, pioneering screens for mutants in the genera *Pseudomonas* and *Ralstonia* yielded *hrp* (hypersensitive response and pathogenicity) mutants, now known to be deficient in the type III secretion system (T3SS), which delivers CEs into host cells (12, 60). Importantly, CE genes were not found in these screens, and reverse-genetic tests involving CEs previously found through their avirulence phenotypes confirmed that their virulence-promoting phenotypes were typically too weak to be detected in a large-scale, forward-genetic screen (62). This virulence system architecture characterized by nonredundant protein secretion systems and global regulators deploying large sets of redundant factors that make individually minor contributions to virulence appears to be widespread among the proteobacterial necrotrophic and biotrophic pathogens.

Cytoplasmic effector (CE): protein translocated into plant cells by a pathogen

Finding and Validating Candidate Virulence Factors in the Genomics Era

The era of genomics for phytopathogens was initiated with the sequencing of a strain of *X. fastidiosa* that causes citrus variegated chlorosis (94). As a bacterium with fastidious nutritional requirements, *X. fastidiosa* had not been amenable to the tools of the disease physiology and molecular genetic eras. The genome sequence converted this black box to a series of testable hypotheses (in the form of candidate virulence factors) based on homology with known virulence genes in other pathogens (for example, CWDEs) and unusually amplified gene classes (for example, iron uptake systems that could account for the chlorosis). We now have complete genome sequences for many of the important phytopathogenic bacteria, fungi, and oomycetes (**http://cpgr.plantbiology.msu.edu**). Each one of these

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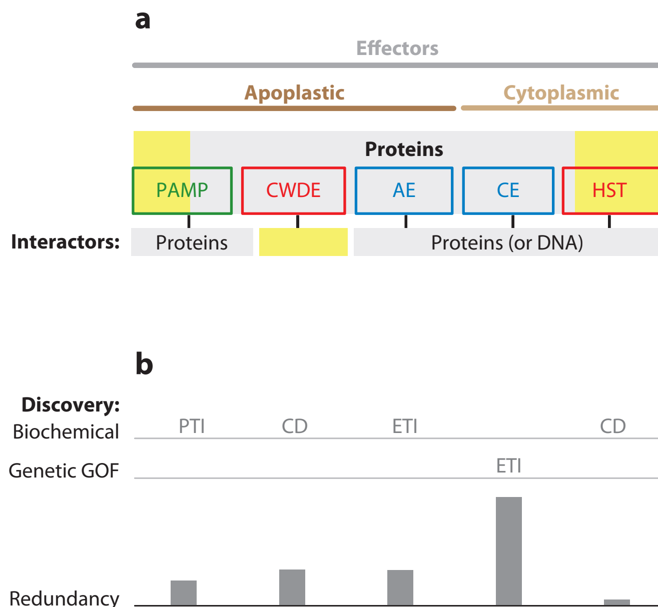

Figure 1

Overview of the discovery, function, and relative internal redundancy of representative effector classes. (*a*) Box border colors denote whether the primary activity of effectors in that class is inducing PTI (*green*), promoting necrotrophic pathogenesis (*red*), or promoting biotrophic pathogenesis (*blue*). Box shading indicates whether the effector and interacting host molecules are proteins (*gray*) or other classes (*yellow*), such as peptidoglycan [a pathogen-associated molecular pattern (PAMP)], plant cell wall polysaccharides [cell wall–degrading enzyme (CWDE) targets], or nonribosomal peptides and polyketides [many host-selective toxins (HSTs)]. (*b*) The pioneer representatives for each class were discovered by biochemical or genetic gain-of-function (GOF) tests for plant responses associated with PAMP-triggered immunity (PTI), effector-triggered immunity (ETI), or various forms of necrotroph-induced cell death (CD), including that associated with tissue maceration in the case of CWDE. Relative internal redundancy is indicated schematically. Note that no pioneer for these effector classes was found through a mutant phenotype, and the most highly expanded class of effectors (CE) involves pathogen proteins that largely function through interactions with plant proteins within plant cells. There are exceptions to this general pattern. For example, some fungal pectic enzymes also interact with plant polygalacturonase inhibitor proteins (26), the *Pyrenophora tritici-repentis* HST is a protein (15), and some effector proteins may have activities in both the apoplast and host cytoplasm.

sequences has generated new lists of virulence candidates based on homology, pathogen-specific paralog amplification, linkage with regions that are variable and/or enriched in known virulence genes, and other criteria, as exemplified by work with bacterial genomes (18, 59, 71, 110, 116). Comprehensive repertoires of candidate bacterial CE genes have been identified through patterns associated with promoters and with targeting-associated regions in CE proteins (57, 116). Thus, the criteria of "right time and place" used to implicate individual virulence factors in previous eras has been systematically applied to find all candidates for this class of virulence factor. Similarly, in oomycetes, candidate CE genes have been comprehensively identified though the combination of protein sequence patterns associated with secretion from the pathogen and then translocation into host cells (83). These CE candidates have been validated by various translocation tests (24, 88, 115, 123). As a result of this powerful engine of discovery, many hundreds of candidate CE genes that would have previously escaped detection because of weak virulence phenotype are now known. Furthermore, with next-generation sequencing we can begin to address the concept of super repertoires for the pan-genome of various species, and we are likely to expand our inventory of phytopathogen CEs into the thousands.

A UNIFIED MODEL OF PLANT-PATHOGEN INTERACTIONS: FROM COMPONENTS TO SYSTEMS

In this section, we place the virulence factors introduced above in the context of a model of pathogenesis that unifies many observations with biotrophic pathogens and provides a framework for exploring the contrasting strategies of necrotrophic pathogens. **Figure 1** summarizes the discovery and functions of representative virulence factors and other effectors, as broadly defined (36). The model for biotroph-plant interactions emerged from a combination of molecular genetic and biochemical studies of a few exemplary molecules (1, 32, 42, 131), as discussed below. Our brief description of this model is intended to highlight different systems properties of plant interactions with biotrophs and necrotrophs, and point to the origins of system complexity.

Biotrophs: Pathogenesis Mediated by Cytoplasmic Effector Proteins

We begin with the case of bacteria in the genera *Pseudomonas*, *Xanthomonas*, and *Ralstonia* because studies with these pathogens nucleated the current model for biotroph pathogenesis mediated by CEs. A priori, one might have postulated that bacterial pathogens would have evolved to simply evade defensive recognition in their parasitic niche in the apoplast and that CEs would primarily promote nutrient release. Instead, the primary role of CEs is to suppress plant defense in association with the following chain of events in which pathogen molecules (shaded) and plant molecules (unshaded) mediate endless counterattacks (1, 11, 30, 42, 131):

1. Bacteria (commonly with robust abilities to thrive on plant surfaces or away from the plant) swim through stomates and wounds into their parasitic niche in the apoplast and, in so doing, present plants with pathogen (or microbe)-associated molecular patterns (PAMPs or MAMPs), such as flagellin, LPS, peptidoglycan, and elongation factor TU (EF-Tu).
2. These common microbial features are directly recognized by surface-arrayed pattern recognition receptor-like kinases and elicit PAMP-triggered immunity (PTI).
3. Pathogens overcome PTI by translocating suppressive CEs.
4. Plants may recognize the activity of one or more of these effectors inside their cells via resistance (R) proteins. In most cases, the R proteins [typically nucleotide-binding leucine-rich repeat (NB-LRR) proteins] recognize the activity of the effector on a guardee or decoy plant target. Known susceptibility targets, guardees, and/or decoys include both proteins and DNA sequences. The resulting effector-triggered immunity (ETI) typically elicits localized programmed cell death (PCD) and qualitative resistance.
5. Pathogens can overcome ETI through mutations in genes encoding effectors that are betraying them or by deploying an effector that suppresses the ETI elicited by one or more effectors. Evasion of ETI through mutation is a successful strategy because CE repertoires are highly redundant and any individual effector is typically dispensable.
6. Plants evolve or acquire through recombination new R proteins that recognize the activity of an effector that has ETI-suppressive activity or potentially any effector that is prevalent in the pathogen population. Steps 3 to 6 can be repeated indefinitely.

Plants deploy hundreds of pattern recognition receptors and R proteins in this two-layered, outside-inside defense against bacteria and other biotrophic pathogens in interactions following the CE/PTI/ETI model (2). Individual pathogens in the genera *Pseudomonas*, *Xanthomonas*, and *Ralstonia* typically deploy 15 CEs to 50 CEs (20, 44, 79). Fungi, nematodes, and oomycetes also appear to have large CE repertoires, with *Phytophthora infestans* possibly delivering more than 700 such effectors (34). *R* genes represent the most polymorphic class of genes in natural populations of *Arabidopsis thaliana* (16), and genomics has revealed that pathogen CE gene repertoires are also highly polymorphic (3, 34).

Two variations in the ETI aspect of the CE/PTI/ETI model that involve fungal pathogens are noteworthy. First, *Cladosporium fulvum* is unusual among biotrophic fungi in that it remains entirely intercellular in its host (tomato) leaves and relies on apoplastic effector (AE) proteins to interdict extracellular components of the PTI system (100). These AEs are under ETI surveillance by transmembrane R protein sentinels with extracellular LRR domains (41). Second, the flax rust pathogen *M. lini* (an obligate biotroph) translocates CEs, such as AvrL567 variants, that are directly recognized by cognate cytoplasmic NB-LRR R proteins (23). That is, the flax R proteins appear to monitor the structure rather than the activity of their cognate CEs. The implications of these variations are discussed below.

PAMP: pathogen-associated molecular pattern

PTI: PAMP-triggered immunity

ETI: effector-triggered immunity

Apoplastic effector (AE): protein secreted by pathogen into the apoplast, characteristically to suppress host defenses

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Regarding the development of the CE/PTI/ETI model of biotroph-plant interactions, it is noteworthy that PTI was the final piece of the puzzle to fall into place. The ability of general elicitors to elicit local induced resistance had been known since the disease physiology era (91), but the PTI concept crystallized with the discovery that bacterial flagellin contained a 22-amino-acid region that functioned as a PAMP and was recognized by the *Arabidopsis* receptor-like kinase FLS2 (14, 31, 33). Whereas research with CE/ETI components had been driven by the strong phenotypes associated with gene-for-gene interactions, the lack of such phenotypes associated with polymorphisms in PAMP/PTI components hid the system from geneticists. Rather, a biochemical approach was used to discover flg22 and thereby convert the local induced resistance phenomenon to the PTI molecular model (32).

It is also worth noting the value of biochemical study of the *P. syringae* pv. *tomato* CE AvrPto and the tomato resistance protein Pto in founding the ETI model. *Pto* was the first ETI resistance gene to be cloned (65). AvrPto directly interacts with the Pto serine/threonine kinase, but ETI activation requires the Prf NB-LRR (87, 103). The crystal structure of the AvrPto-Pto complex reveals AvrPto to be an inhibitor of Pto kinase activity (127). AvrPto also directly interacts with the FLS2-BAK1 coreceptor complex to inhibit its kinase activity and suppress PTI (92, 126). Since Pto has not been shown to have a role in PTI, the current model is that Pto is a decoy kinase whose perturbation by AvrPto is detected by Prf, thus eliciting ETI (131). Therefore, R protein sentinels may recognize the activity of CEs through their effects on PTI decoys or PTI guardees, which integrate the dual activities of many CEs in both PTI and ETI.

Necrotrophs: Pathogenesis Mediated by Plant Cell Wall–Degrading Enzymes, Host-Selective Toxins, and Potentially Many Other Factors

Turning to the necrotrophic pathogens, we will briefly describe three pathosystems that illustrate the greater diversity in the pathogenic strategies of these pathogens. First, the pectolytic CWDEs of the soft-rot enterobacteria represent an amplified class of effectors that act in the apoplast according to the following abbreviated interaction scenario, with the pathogen attack shaded and the plant response unshaded throughout this section (22, 73, 109, 110):

1. Pectic enzymes secreted by the pathogen type II pathway cleave internal glycosidic linkages in structurally important pectic polymers in the middle lamella and primary cell walls in dicot tissues, resulting in tissue maceration and plant cell death.
2. Plants activate defenses in response to oligogalacturonate products of pathogen enzyme activity.
3. Pathogens deploy pectic enzymes, along with defense suppressors, only when a quorum for successful pectolytic attack is achieved.

Pectic enzymes are produced in the late stage of complex pathogenesis that can also involve latent infections with low levels of bacteria (109). Although the pectic enzyme repertoires of necrotrophs and the CE repertoires of biotrophic bacteria are similarly amplified and possess internal redundancies (8), they appear fundamentally different from the perspective of interaction systems. For example, there is no evidence that individual pectic enzymes are under immune surveillance, and their repertoires are conserved in three *Pectobacterium* species (28). Furthermore, the soft-rot enterobacteria typically have broad host ranges, and there is no known *R* gene resistance against them. Thus, disease incidence is influenced more by environmental conditions than by host genotype. Because plants can detect the oligomeric products of pectic enzymes as damage-associated molecular patterns (DAMPs) (11, 22), a key aspect of soft-rot enterobacterial virulence appears to be activation of pectic enzyme gene expression only when a bacterial quorum has been sensed (43, 77), and coordinated

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expression of the T3SS may further aid the defeat of DAMP-triggered immunity (61).

Turning to the necrotrophic fungal pathogens, we find in the genus *Cochliobolus* a genomic amplification of nonribosomal peptide synthetases and polyketide synthases, some of which produce HSTs (48, 52). An interesting aspect of diseases involving *Cochliobolus* HSTs is the sudden appearance of races producing them in cereal crops through inadvertent breeding for susceptibility (129). One example of this involves *C. carbonum* race 1, which produces HC-toxin, a cyclic tetrapeptide HST that mediates the following interaction sequence involving a lethal leaf spot and ear mold in maize (40, 67, 74, 81):

1. HC-toxin is produced by race 1 and has the capacity to inhibit histone deacetylase activity, thus inhibiting defense gene expression and promoting disease.
2. HC-toxin reductase, encoded by *Hm1*, is present in most plants and detoxifies HC-toxin, thus conferring resistance.
3. A rare *hm1* mutant occurring in a maize breeding program is sensitive to HC-toxin and is aggressively attacked by *C. carbonum* race 1.
4. Susceptible *hm1* plants are removed from the breeding program, thus preventing disease outbreak.

Given that HC-toxin can inhibit histone deacetylase activity in all plants and HC-toxin reductase is produced by virtually all cereals, it appears that *C. carbonum* race 1 causes an ancient disease that was largely extinguished by the ancient evolution of HC-toxin reductase (40, 95).

C. victoriae, which produces victorin, a cyclized pentapeptide HST, yields another lesson with major implications regarding the challenge that plants face in defending themselves against both necrotrophs and biotrophs (124, 129). The rise and fall of *C. victoriae* as an agronomic problem can be illustrated as follows:

1. Oats carrying the crown rust (*Puccinia coronata*) resistance gene *Pc-2* derived from cultivar Victoria become widely planted in the 1940s.
2. *C. victoriae* produces victorin, which elicits *Pc-2*-dependent PCD resulting in pathogen growth and the Victoria blight of oats epidemic.
3. Oats carrying *Pc-2* are removed from agricultural use, thus extinguishing the disease outbreak.

Recent studies involving *Arabidopsis*, which is more genetically tractable than allohexaploid oat, have revealed that victorin-induced PCD requires an NB-LRR and thioredoxin h5 (63, 101), which also mediates redox changes activating the master defense regulator NPR1 (102). The observations with oat and *Arabidopsis* collectively point toward victorin subverting ETI defenses against biotrophs, such as *P. coronata*, to promote susceptibility to a necrotroph.

Revisiting Models of Biotrophic and Necrotrophic Pathogenesis from a Systems Perspective

Three systems properties of CE/PTI/ETI interactions are noteworthy: (*a*) system incompatibilities at the ETI level (CE recognized by sentinel) trump everything else in the interaction and result in resistance; (*b*) ETI involves interactions between CEs and host sentinels that are either proteins or DNA sequences, which enables rapid coevolution by both partners; and (*c*) the resulting arms race of surveillance and evasion at the ETI level may produce large repertoires of interacting proteins and robust pathosystems (42). Interestingly, the *C. fulvum*–tomato interaction appears to have independently evolved systems properties similar to those of tomato–*P. syringae* interactions but with AE proteins and extracellular ETI sentinels (100). Remarkably, these similarities extend to indirect ETI recognition involving decoy proteins, which indicates the broad applicability of the decoy extension of the PTI/ETI model (113). On the other hand, the contrasting direct interactions of an allelic series of CE and R proteins in the *M. lini*–flax pathosystem makes the search for

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the susceptibility targets of these CEs a high priority because the findings could yield a new model for the CE/PTI/ETI system operating in obligate biotrophic pathogens.

In contrast to the biotrophs, the interactions of necrotrophs with plants are not dominated by CEs or similar AEs, and the outcomes of these interactions are not determined by ETI. Rather, the necrotroph diseases described above involve interactions mediated by classes of molecules that are more slowly evolving, for example, pectic polysaccharides in the plant cell wall and products of fungal nonribosomal peptide synthetases. In general, these pathosystems appear more fragile than biotroph pathosystems, with disease outbreaks resulting from jackpot environmental conditions in the case of soft-rot enterobacteria or jackpot host genotypes in the case of *Cochliobolus* spp. This observation leads to two major systems-level questions: (*a*) What enables these pathogens to lurk in plant casinos and, in the case of *Cochliobolus* spp., prolifically coin new gambling chips in the form of novel nonribosomal peptides and polyketides? (*b*) Given the differing and conflicting defenses of plants against biotrophs and necrotrophs, why don't necrotrophs generally subvert the ETI system as *C. victoriae* appears to do?

Pseudomonas syringae Cytoplasmic Effector Protein Repertoires as a Test Case for Systems-Level Approaches

As described above, a key aspect of the CE repertoires of most biotrophic pathogens is internal redundancy, which permits individual CEs to be lost with minimal virulence penalty when the local host plant population acquires a corresponding new *R* gene. This phenomenon renders *R* gene–mediated resistance unstable in the field for many important crop pathosystems, and it explains why many CEs fail molecular Koch's postulates tests. The CE repertoire of *P. syringae* pv. *tomato* DC3000 has been particularly well characterized, and 28 CEs appear to be actively deployed (with several others encoded by apparent pseudogenes or more weakly expressed genes) (20, 57). Genome sequencing and comprehensive analysis of CE repertoires in other pathovars of *P. syringae* and another strain of pathovar *tomato* have revealed that the repertories are surprisingly different, even for pathogens of the same host (3, 57). DC3000 is a pathogen of tomato and the model plants *A. thaliana* and *Nicotiana benthamiana* (if the *hopQ1-1* CE gene, which acts as an avirulence determinant in *N. benthamiana*, is deleted), and a growing collection of combinatorial CE gene polymutants is available for this pathogen (49, 121). Thus, DC3000 is an ideal pathogen for exploring the potential operation of CE repertoires as systems with emergent properties (see sidebar, Emergent Properties).

Such emergent properties could arise from interplay among CEs and structured redundancies in CE repertoires. As an example of interplay, loss of *virPphA* (*hopAB1*) from *P. syringae* pv. *phaseolicola* 1449B results in avirulence in bean, the normal host, because of failure to suppress ETI triggered by another effector in the 1449B repertoire (39). As an example of redundancy, deletion of individual CE genes or many combinations of CE genes can have little effect on reducing DC3000 growth in *N. benthamiana* or other hosts (121). However, deletions involving certain other combinations yield strong reductions in growth. For example, deletion of both *avrPto* and *avrPtoB* or of *avrE*, *hopM1*, and *hopR1* strongly reduce growth in tomato and *N. benthamiana* (49, 55). Importantly, deletion from DC3000 of *fliC*, which encodes flagellin, the major PAMP detected by *N. benthamiana*, restores growth to the Δ*avrPto*Δ*avrPtoB*

EMERGENT PROPERTIES

"It is thus likely that over the coming years and decades biological sciences will be increasingly focused on the systems properties of cellular and tissue functions. . .These properties are sometimes referred to as 'emergent' properties since they emerge from the whole and are not properties of individual parts" (72).

"The scientific meaning of emergent, or at least the one I use, assumes that, while the whole may not be the simple sum of its separate parts, its behavior can, at least in principle, be understood from the nature of its parts plus the knowledge of how these parts interact" (19).

mutant but not to the Δ*avrE*Δ*hopM1*Δ*hopR1* mutant (49). Furthermore, AvrPto and AvrPtoB both target PAMP coreceptors (92, 126), whereas HopM1 and possibly AvrE and HopR1 disrupt vesicle trafficking associated with antimicrobial deployment (35, 70). These observations suggest that at least a part of the internal redundancy in the DC3000 CE repertoire is structured around redundant effector groups (REGs) that target a particular process in PTI, as depicted in **Figure 2**.

Plant defenses have a similar internal redundancy, requiring polymutants for strong phenotypes and gaining system robustness through democratic signaling networks (111). Further support for redundancy and system robustness comes from the finding that forward genetic screens for *Arabidopsis* mutants impaired in perception of the EF-Tu PAMP yielded endoplasmic reticulum quality control factors involved in the processing of EFR (the cognate pattern recognition receptor for EF-Tu) rather than downstream signaling components (54, 86). Coevolution with pathogen CE repertoires is likely to be a factor driving such defense redundancy. Disentangling the complexities in pathogen CE repertoires and plant PTI components will be an important challenge in the next decade, as will be discussed in a later section.

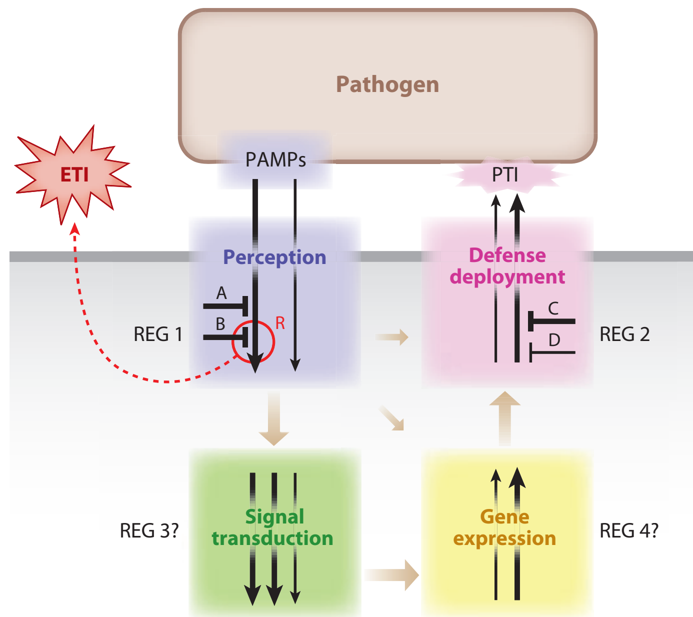

Figure 2

Model for the possible role of redundant effector groups (REGs) in assuring that key processes in pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) are blocked despite mutational loss of any cytoplasmic effector in the repertoire. A REG comprises effectors that function redundantly to block one of the depicted high-level processes leading to PTI (49). Effectors A and B redundantly block PAMP perception. Thus, if effector B is detected by an R protein sentinel, the effector gene can be jettisoned from the pathogen population with little virulence penalty. REG 2 is depicted as blocking vesicle trafficking, a central process in antimicrobial deployment. Plants appear to use signaling bypasses and various forms of internal redundancy in each high-level process to achieve PTI robustness. Thus, loss of single pathogen effectors or single plant defense components has minimal effect on PTI system performance in a typical biotroph-plant interaction (49, 111). Key questions to address with disassembled effector repertoires include whether there are REGs targeting other PTI processes, and whether any minimal subset of effectors that can restore strong pathogen growth does so by completely blocking a single high-level PTI process (for example, through deployment of an additional member of REG 1 to block all PAMP perception) or by attacking multiple high-level processes. Note that pathosystems with reduced redundancy may allow single effectors to have a stronger role. Thus, the absence of alternative deployable defenses in the host and other REG 2 effectors in the pathogen could endow effector C with a strong phenotype.

THE CHALLENGE OF DEFINING VIRULENCE FACTORS AND EFFECTORS

In previous sections, we have used the example of CEs to illustrate the need to understand virulence factors as components of complex systems. Here, we address the problems this complexity creates when we try to refer to effectors and other virulence factors unambiguously in a few words. Attempts to differentiate virulence and pathogenicity factors have a long history in plant pathology. Indeed, a primary goal of the disease physiology and early molecular genetics eras was to identify first the pathogenicity factors that were *qualitatively* essential for pathogenesis and then the virulence factors that contributed *quantitatively* to pathogen growth and symptom development (93). As molecular genetic tools became available, molecular Koch's postulates could be used to differentiate and validate pathogenicity and virulence factors based on the strength of mutant virulence defects.

REG: redundant effector group

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An alternative approach to classifying virulence genes was proposed in 2001 when Wassenaar & Gaastra (119) addressed the challenge of where to draw the line in the annotation of virulence genes. They proposed three classes based on broad functions rather than degree of contribution to virulence: (*a*) true virulence genes code for factors or enzymes producing factors that are involved in interactions with the host and are directly responsible for the pathological damage during infection; (*b*) virulence-associated genes encode factors that are involved in the deployment (regulation, secretion, processing) of the products of the true virulence genes; and (*c*) virulence lifestyle genes encode factors promoting host colonization or tolerance of host defenses, such as reactive oxygen and phytoalexins.

More recently, Hogenhout and coworkers (36) proposed the broad definition of effectors that we have used here. This definition also includes PAMPs. This use of effector has the advantage of a single term intuitively and broadly encompassing the pathogen molecules that directly interact with host targets, whether promoting disease or eliciting defense. We think this inclusive definition will better serve the development of systems-level approaches, although its application often requires delineating various subclasses of effectors, as we have done here for CWDEs, AEs, CEs, and HSTs.

All of these terms for virulence factors (36, 119) can be integrated in the following general statements about pathogen genes involved in host interactions:

1. The true virulence genes encode effectors, which often make only quantitative contributions to virulence.
2. Virulence-associated genes direct the deployment of effectors and the development of eukaryotic pathogen specialized infection structures (e.g., appressoria), and these are often qualitatively needed for pathogenicity.
3. Virulence lifestyle genes encode antimicrobial tolerance factors that are not considered effectors (for example, those involved in tolerance to reactive oxygen species and phytoalexins) and other factors promoting pathogen colonization of host tissues, and these factors often make only quantitative contributions to virulence.

The term virulence-related would encompass all of these genes as well as many others that are hard to classify because they serve basic cellular functions that are particularly important during pathogenesis, such as the *P. syringae* pv. *tomato* DC3000 DsbA periplasmic protein disulfide isomerase (46). The quantitative contribution to virulence of many virulence lifestyle genes may result from functional redundancies, as with the effectors.

We must emphasize that these statements describe typical cases, and there are informative exceptions to each statement. For example, one type III effector, DspE, is qualitatively essential for *Erwinia amylovora* pathogenicity (10, 27a), whereas the T3SS pathway itself contributes only quantitatively to virulence in the soft-rot enterobacteria (6, 37, 82). Exceptional pathosystems can be experimentally useful for studying the function of individual virulence factors, and they yield insights into the virulence system of which the effector is a component.

The *E. amylovora* case is particularly illustrative in this regard because DspE is a homolog of the *P. syringae* pv. *tomato* DC3000 AvrE effector, which was described above as being a member of a redundant effector group in DC3000. AvrE can restore partial virulence to an *E. amylovora dspE* mutant (10), which suggests that the primary difference between these effectors is the system of which they are components. In fact, redundancy in T3SS translocon components and effectors is greater in *P. syringae* than in *E. amylovora* despite many overall similarities in the two systems (9, 50). The differing strengths of *avrE* and *dspE* mutant phenotypes raise fundamental questions about the comparative evolution of *P. syringae* and *E. amylovora* virulence systems.

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Because the identification of many CEs in the genomics era is based solely on sequence patterns and validation tests involving translocation rather than virulence, the majority of recently identified effectors have not been shown to make any contributions to virulence (20, 44, 79). Furthermore, CE gene repertoires are likely to contain several genes that are inactive even though they encode bona fide CEs. The *P. syringae* pv. *tomato* DC3000 HopAI1 phosphothreonine lyase is an example of this (53, 89, 115, 130). Given the value of comprehensiveness for systems analysis of effector repertoires, it seems more useful to include the *hopAI1* gene (with an appropriate footnote) than to drop it from consideration. For example, its apparent inactivation may be found to have some significance from a systems perspective.

In summary, whether a given factor qualitatively promotes pathogenicity or quantitatively promotes virulence is likely to be more a property of the system than the factor. During the current genomics-driven discovery phase of research, a relatively relaxed and inclusive use of the terms virulence-related, virulence factor, and effector in peer-reviewed journal articles and informal discussion seems appropriate given the utility of complete lists of candidate factors and the thicket of exceptions that would arise with any attempt at more stringent definitions. Furthermore, systematic efforts to move validated effectors into the confirmed virulence factor category by applying molecular Koch's postulates based on subtle virulence phenotypes may return little insight for the effort. Analyses based on combinatorial genetic dissection of pathosystems, as illustrated above, are more likely to yield strong virulence phenotypes and insights into the functions of effectors within systems. Furthermore, as we discuss next, gene ontology (GO) terms provide a parallel system for systematically accumulating information about virulence factors that possesses the rigor lacked by our advocated relaxed use of virulence factor and effector in broad discussions of virulence.

DEVELOPING COMMUNITY RESOURCES FOR STUDYING PLANT-PATHOGEN INTERACTION SYSTEMS

GO: Gene Ontology

Our hypothesis that virulence factors function as components of complex host-pathogen interaction systems with emergent properties leads to three strategic questions. What is the most efficient way to gather information on an individual factor that reveals its function in the system? How does the operation of the factor fit into conceptual models of plant-pathogen interaction, such as the PTI/ETI model? How can we most efficiently discover emergent properties in pathosystems that may predict additional system components, refine interaction models, and lead to new disease management strategies? To address these questions, we see the need for two complementary knowledge bases. The first is reductionist, factor-centric, components-biology knowledge captured with GO annotation. The universal nature of GO terms will facilitate comparisons with other virulence factors in diverse systems, but GO terms by their nature are not system specific. In contrast, the second knowledge base will be pathosystem specific and systems biology oriented (72), with a focus on how each factor functions in the context of other virulence factors in that system (see sidebar, Systems Biology). It is also important to note here that continued development of mechanistic models, such as those embodied in the guard and decoy hypotheses, will aid both the factor-centric and pathosystem-centric approaches. As models become validated, they can be integrated into GO, and similarly, the models provide a framework to guide the probing of individual pathosystems.

SYSTEMS BIOLOGY

"Systems biology. . .investigates the behavior and relationships of all of the elements in a particular biological system while it is functioning. These data can then be integrated, graphically displayed, and ultimately modeled computationally" (38).

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Using Gene Ontology to Capture Components Biology Advances

The GO Consortium was founded in 1998 through collaboration of three model eukaryotic genome communities and provides a system for all biologists to annotate genes using a universal vocabulary and standardized evidence codes (4). The Plant-Associated Microbe Gene Ontology (PAMGO) interest group, involving genome projects addressing phytopathogenic bacteria, fungi, oomycetes, and nematodes, was formed in 2004 and has worked with the GO Consortium to generate more than 800 new GO terms (107). GO involves three ontologies that separately address biological process, molecular function, and cellular components. Many of the new terms address biological processes associated with host-microbe interactions. As a result of PAMGO efforts, a dual taxon capability now permits capturing the NCBI taxon IDs of the microbe producing a factor and the host in which the factor acts (107). GO terms capture information on a factor using a hierarchy-like structure that can accommodate increasingly granular information about the biological function, biochemical activity (including interaction with a host factor), and location of the factor (including the cellular location in the host). Given that the terms are universal and not system-specific, they foster broad comparisons. With wider GO annotation of pathogen genomes, we will have a better ability to compare the repertoires and functions of CEs produced by diverse pathogens attacking diverse hosts, including both plants and animals. Recent issues of *BMC Microbiology* and *Trends in Microbiology* are focused on PAMGO and include several effector-related articles (13, 56, 58, 108).

It is helpful here to compare the use of terms in peer-reviewed journal articles and GO. A journal article will typically provide context and clarifications that diminish potential confusion arising from nonuniform use of terms such as virulence factor and effector. However, this nonuniformity thwarts the utility of such information for comparative genomics and systems biology. In contrast, gene annotations lack such context and therefore must capture functional data with a universal vocabulary. GO addresses this problem and in so doing makes functional annotation machine readable. Hence, investment in ongoing GO annotation should have a multiplier effect on the value of new data about CEs and other virulence factors. Similarly, ongoing work will be needed to update and expand GO terms that capture our growing understanding of the biological processes underlying host-microbe interactions. Brett Tyler, who led the PAMGO effort, has suggested that journals and granting agencies encourage researchers to capture experimental results in GO annotation as part of the publication process (112).

Pathosystem-Specific Resources to Advance Systems Biology

We anticipate two phases in the development of resources for exploring systems-level properties of plant-pathogen interactions. These resources will generally support an established framework for systems biology (see sidebar, Framework for Systems Biology) (38). The first phase will be centered on individual pathosystems and their research communities and will provide initial models and pilot tests for standardized assays and associated data types. These tests include assays for interaction processes such as PAMP perception, signal transduction, and the shift in hemibiotroph pathogenesis to the lesion formation phase. Informal, yet increasingly refined, models of plant-pathogen interactions are likely to yield better markers for specific subprocesses. These informal

A FRAMEWORK FOR SYSTEMS BIOLOGY

1. Define all of the components of the system.
2. Systematically perturb and monitor components of the system.
3. Reconcile the experimentally observed responses with those predicted by the model.
4. Design and perform new perturbation experiments to distinguish between multiple or competing model hypotheses.

models and standardized assays can then guide the development of the second phase, which will integrate data from many pathosystems and will necessarily involve further standardization and formal structure. The primary aim of the first phase will be to help laboratory biologists explore the emergent properties of virulence systems and integrate these findings with molecular-level interaction models. Importantly, the extensive datasets resulting from systems-level approaches can enhance hypothesis formulation in components biology (69).

The starter kits for research communities in the first phase will likely include (using CE repertoires as an example) (*a*) pathosystems involving genetically tractable partners that allow parallel disassembly of CE repertoires and defense components and therefore genetics-squared study of the interaction (76); (*b*) complete inventories of CEs and interacting host proteins (with all relevant genes GO annotated); (*c*) community standards for assay procedures and data types; (*d*) community Web resources for collecting all data in a machine-readable form and enabling researchers to generate tables and matrices (genotype x system performance) to aid visualization of patterns; and (*e*) an interaction model as a framework to guide hypothesis formulation and testing as well as integration of new system components knowledge. Visual models presented in publications ranging from comprehensive review articles to *Cell* SnapShots, will continue to be important (42, 75). However, it seems inevitable that Web-based resources and the tools of the second phase will be needed to adequately display the full complexity and dynamic nature of interaction models.

The second phase in plant-pathogen systems biology will support computational analysis of emergent behaviors and will require the development and adoption of universal standards for data types, formal concept analysis, and mechanistic dynamical models. Visually oriented tools for the construction and display of complex models will almost certainly play a central role. The initiatives that have led to the Systems Biology Graphical Notation (51) and associated software systems are forerunners of the kind of conceptual and computational infrastructure that will be needed. Computational modeling will have far more power to discern emergent properties, predict exploitable vulnerabilities, and permit more rigorous analysis of plant-pathogen interaction systems in the larger context of biological systems with properties of robustness (21). Whereas first-phase efforts can be nucleated by various pathosystem research communities using existing resources with minimal external support, the development of the second phase will require significant coordination and external support.

CONCLUSIONS AND CHALLENGES

We began this review with a quote from Warren Weaver (120), a pioneer in mathematical communication theory and a visionary director of the Rockefeller Foundation's Division of Natural Sciences and Agriculture. Now, more than half a century later, Weaver's challenge is being met with the tools of systems biology and applied to levels of complexity from the molecular to the ecological. Focusing on plant disease here, we have explained that many pathogen virulence factors appear to function as components of complex systems. By using a systems perspective we can better understand the basis for discovering the known classes of virulence factors over three eras of advancing technology, and we can see that the rising trajectory of discovery leads to a fourth research era that will be integrated with systems biology.

We can also see that the coevolution of pathogen effector repertoires and host defense systems, particularly in the context of the CE/PTI/ETI model, has produced redundancy on both sides of the interaction that diminishes the role of individual factors while increasing system robustness. By studying and disassembling complete virulence factor repertoires, we can better reveal hidden functions of individual factors and features of the system that confer robustness. Such systems-level knowledge may

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offer practical benefits in guiding the breeding of durable crop resistance and in enabling better reading of threat potentials in the genome sequences of emerging pathogens.

The primary means for advancing our understanding of pathogenesis until now has been peer-reviewed articles that provide molecular Koch's postulates validation and new insights into the molecular function of a virulence factor. However, this model does not scale well with the extensive interplay that virulence factors are now seen to have with other factors in their pathosystem and with the large numbers of such factors. Given the challenge of complexity, the most efficient progress is likely to be made by applying a balance of reductionist (components biology) and integrative (systems biology) approaches and by developing ways for components biology to more easily facilitate systems biology. This could be done by components biologists consistently integrating major results with systems-based models of interactions, capturing new knowledge in GO annotation, and contributing relevant data to systems-oriented databases. This transformation in the way in which knowledge grows could be fostered through modest investments in infrastructure for model pathosystems, support for Web-based resources that foster community development of unified interaction models, new journal policies encouraging GO annotation, and curatorial resources to aid such annotation. Rewards for such an approach could include better prioritization of components biology research, more rapid development of predictive models of plant-pathogen interactions, and new ways to manage plant diseases.

SUMMARY POINTS

1. Microbial pathogens disarm and parasitize plants largely through secreted virulence molecules, but identifying and validating these factors is often thwarted by high levels of functional redundancy.
2. The search for virulence factors can be broadly divided into three eras defined by available tools: disease physiology and biochemistry ("grind and find"), single-gene molecular genetics ("screen for gene"), and genomics ("patterns that matter"). Consideration of the nature of the factors found in each successive era yields broad lessons about pathogen-plant interactions.
3. The myriad and phylogenetically diverse pathogens of plants can be broadly divided into two classes based on their nutritional relationship with host tissues, with biotrophs and necrotrophs growing in living and rapidly killed tissue, respectively.
4. According to a recently developed, unified model, the primary virulence factors for biotrophic pathogens are proteins, collectively referred to as cytoplasmic effectors, which are delivered by the pathogen into host cells to defeat defense mechanisms triggered by host cell–surface associated receptors while evading or defeating internally arrayed anti-effector detection agents such as R-proteins.
5. Coevolutionary arms races between pathogens and plants have generated large repertoires of cytoplasmic effectors that are collectively essential but individually dispensable and plant defenses that possess parallel redundancies.
6. Cytoplasmic effectors and other virulence factors increasingly will be studied as virulence system components, with knowledge about the system being as important as knowledge about the component. Thus, understanding the complex ensemble of molecular interactions underlying pathogen-plant interactions will require increasing integration with the tools of systems biology in a fourth era of research.

FUTURE ISSUES

1. How can we encourage a research community that traditionally disseminates components biology advances through journal articles to make this information machine readable and therefore accessible for systems biologists?
2. What is the best way for a diverse research community working with many pathosystems to develop tools to support computational approaches to the study of emergent properties in those systems and to enhance our ability as biologists to comprehend and communicate these findings?
3. How do we enhance the use of systems-level knowledge in pathogen genomics, for example, to predict the threat potential of emerging pathogens or rapidly develop science-based response plans?
4. What types of information will most rapidly reveal emergent properties in the cytoplasmic effector repertoires of model pathogenic bacteria, fungi, oomycetes, and nematodes that may predict broadly important phenomena, such as the basis for pathogen host specificity and plant nonhost resistance?
5. What are the best targets in agriculture for the application of systems-level approaches to enhance crop health and productivity?

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