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Plant Immunity

Methods and Protocols

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Preface

Examples of How New Experimental Technologies Have Enabled Landmark Advances in Understanding of Plant Immunity Over the Last Half-Century

This volume of Methods in Molecular Biology was designed to emphasize emerging technologies that can be applied to outstanding questions in plant immunity. The content is complementary to another recent, excellent volume in the series with a similar focus (1). Below, I provide a brief historical overview highlighting major conceptual advances in molecular plant–microbe interactions that would not have been possible without exploitation of new technologies. Additionally, I outline current conceptual challenges in our field that can be addressed with methods described in this volume. Finally, I speculate on technological advances in the near term that enable deeper understanding of plant immunity and support rational strategies for durable disease control.

As all readers of this volume know, much effort has been invested in understanding the molecular mechanisms through which plants and microbes interact. Much of the progress in this field has been fueled by timely, thoughtful exploitation of new methodologies. For example, H.H. Flor's use of classical genetics clearly demonstrated that the outcome of encounters between flax and flax rust can be dictated by single genes on both sides of the interaction (2). Equally important, his methodology revealed striking specificity in these interactions, which led to formulation of the seminal "gene-for-gene" model. This genetics-driven model provided a conceptual framework for the plant immunity that proved generally applicable and remains relevant today (3, 4).

Subsequent emergence of molecular biology tools enabled the gene-for-gene model to be elaborated in molecular terms. For example, gene cloning technologies were used to isolate avirulence (*avr*) genes, resistance (*R*) genes, and additional components of pathogenicity and immunity. Molecular approaches, along with judicious biochemistry, provided for critical examination of "receptor-ligand" models that predicted direct interaction between the products of *R* and *avr* genes (e.g., (5)). Three important themes emerged from these efforts: First, the majority of plant resistance genes encode proteins from a single superfamily, defined by a nucleotide-binding site and leucine-rich repeats (NB-LRR) (6). Second, pathogen Avr proteins are, in many cases, translocated into plant cells where they act as effectors to reprogram plant cells for susceptibility (7). Third, NB-LRR proteins often do not interact directly with corresponding Avr proteins but instead monitor guardees or decoys that are modified by the Avr protein (8–10). In addition, experiments with pathogen "elicitor" molecules revealed a second branch of the plant immune system, which directly recognizes pathogen-associated molecular patterns (PAMPs) that are evolutionarily conserved among diverse pathogens (11). The two branches of the plant immune system have been connected by recent models predicting that pathogen effectors may have evolved to interfere with PAMP-triggered immunity (12). NB-LRR receptors thereby provide a second line of defense by recognizing the molecular signatures of

effector activity. In sum, the adoption of molecular biology methods led to major advances in understanding of plant immunity that could not have been anticipated by models based (however logically) on genetic data alone.

At present, "omics" tools are being used to build on molecular advances and provide new insights. For example, it is now possible to survey a plant genome and identify all of the potential immune receptors using queries based on conserved motifs (e.g., (13)). From this, it has become clear that plants maintain hundreds of probable immune receptors, which in many cases appear to be evolving dynamically to cope with ever-changing pathogen populations (14). From an applied perspective, these inventories can greatly accelerate the process of resistance gene identification (e.g., for cloning and/or marker-assisted breeding of *R* genes from wild relatives into crops).

Similar advances are underway in pathogen genomics. For example, molecular signatures are being developed for pathogen effector proteins that enable comprehensive effector gene inventories to be predicted *in silico*. Genome level comparisons have revealed that pathogen genomes contain dozens (in prokaryotes) to hundreds (in eukaryotes) of effector genes (15, 16). Like the cognate surveillance genes in plants, these genes are often variable and subject to rapid turnover. Large-scale characterization of effector functions is a major focus of effort in the field of plant immunity that is discussed further below.

Transcript profiling is also impacting understanding of plant immunity. For example, early experiments with microarrays revealed massive transcriptional changes that accompany the activation of the immune system and illuminated molecular distinctions between different resistance mechanisms (e.g., (17)). Subsequent studies that combine transcript profiling with immune response mutants have provided insight into the structure of the defense hierarchy and have identified previously unknown components of the network (e.g., (18)). Analyses of transcript profiles have also provided important insights into the molecular mechanisms through which pathogens manipulate the environment inside plant tissue (e.g., (19)).

At present, it is inarguable that our current, exciting level of understanding of plant immunity (and pathogen evasion thereof) owes much to the timely adoption of new methodologies in genetics and molecular biology, as well as genomics. However, we remain far from a complete understanding of how the plant immune system functions, or how its functionality is perturbed by adapted pathogens. Many questions remain that will require new methodologies to be developed, optimized, and widely adopted. This volume of Methods in Molecular Biology was designed to emphasize emerging technologies that can be applied to outstanding questions in plant immunity.

For example, although NB-LRR immune surveillance proteins have now been known of for 1.5 decades, we still do not understand exactly how they function, and it is not clear whether all NB-LRR proteins function in a similar manner (20, 21). Moreover, we still lack a complete inventory of downstream signaling components, and we do not understand how these components interact. Methods that can be applied for new insights into molecular functionality of NB-LRR proteins and other immune signaling components are described in Chaps. 1–4. Chapter 1 addresses the understudied question of exactly where in the cell NB-LRR proteins exercise their functions of surveillance and downstream activation. In particular, the approaches therein can be applied to study dynamic relocalization of NB-LRRs in response to pathogen invasion (e.g., (22)). Chapter 2 describes a very innovative "fragment complementation" approach for understanding the functions of intramolecular interactions between different NB-LRR functional domains (e.g., (23)). Chapters 3 and 4 provide new protocols for the oft-vexing

process of purifying low-abundance protein complexes. These protocols were developed with the immediate goal of identifying the components within immune surveillance complexes (e.g., (24, 25)), but are also potentially applicable to any protein expressed *in planta*.

Chapters 5–7 have similarly broad applicability. Chapter 5 describes chromatin immunoprecipitation, which is being widely used to characterize protein–DNA interactions in vivo and identify targets of transcription factors in a variety of organisms (e.g., (26)). This chapter was written in reference to WRKY transcription factors, which are ubiquitous in plants and are key regulators of immunity and other plant processes. The procedures could be adapted for other plant proteins (e.g., NB-LRR proteins that function inside the nucleus) or for pathogen effector proteins that mimic plant transcription factors. Chapter 6 provides new information on an inducible system for plant transgene expression that is frequently used in studies of plant immunity (e.g., for expressing effector proteins *in planta* (27)). This chapter helps researchers maximize the versatility of this system and clearly understand its limitations. Chapter 7 describes a creative method for detection and quantification of alternatively spliced transcripts (28). Alternative splicing is important for the regulation of some NB-LRR resistance gene regulation and is currently understudied with respect to immune system function (29).

Chapters 8–17 describe methods used to identify and functionally characterize pathogen effector proteins. As mentioned above, pathogen genomics have revealed a plethora of candidate effectors. Understanding how they function is one of the most active areas in plant–pathogen research at present (15, 16). One emergent generality is that almost all types of pathogens deploy moderate to large batallions of secreted effectors, many of which operate inside plant cells. Chapters 9 and 10 provide approaches to isolate plant cells that are in intimate contact with fungi and nematodes, respectively. These cells can serve as sources for cDNA libraries that are enriched for transcripts encoding effectors (e.g., (30, 31)). This is a proven approach toward effector gene discovery for pathogens with no reference genome sequence.

The bacterium *Pseudomonas syringae* has been at the forefront of effector characterization, and Chap. 10 describes methods whereby single or multiple gene knockouts can be constructed. This approach is vital to establish loss-of-function phenotypes, deconvolute the redundancy in effector repertoires, and evaluate the contribution of effectors to bacterial host range (e.g., (32)). In the eukaryotic kingdom, oomycetes from the *Phytophthora* genus have been at the forefront of effector identification; however, transformation of *Phytophthora* is often challenging even for experienced labs (33). Chapter 11 provides procedures for transformation of *P. capcisi*, which appears more amenable to genetic manipulation and can infect *N. benthamiana* and defense-compromised *Arabidopsis* mutants. Chapter 12 describes procedures pertaining to a second oomycete, *Hyaloperonsopora arabidopsidis*, that has long been used as a model pathogen of *Arabidopsis* and is becoming even more widely used for oomycete comparative genomes and investigation of oomycete effector proteins (34).

Bacteria deploy dozens of effectors, and oomycetes, fungi, and nematodes likely produce many-fold more (15, 16). To facilitate functional characterization of large collections of effectors, several high-throughput assays have been recently developed. Two such assays, presented in Chaps. 13 and 14, can be used to estimate immune-suppressive capacity of effectors from almost any pathogen (e.g., (35–37)). Chapter 15 describes a transient expression system optimized for protein complex purification, similar to Chaps. 3 and 4, that can be applied at medium-throughput to identify plant proteins which interact with pathogen effectors (or other types of protein interactions *in planta*). Chapters 16 and 17 describe approaches for visualizing subcellular localization

of effectors in plant cells, which is of key importance for understanding effector function (e.g., (38)).

In the final chapters, the focus returns to the plant at a fine spatial scale. One aspect of plant–pathogen interactions that has not been adequately addressed relates to spatial differences in the molecular responses of plant cells in different locations of the infected organ, relative to pathogen infection structures. Chapters 18–20 provide information on laser microdissection, which is one of the most promising technologies for addressing questions relating to spatio-temporal differences in different cell types in infected organs (e.g., (39)). Finally, Chap. 21 zooms in even further (completing a spatial circle with Chap. 1) to describe exciting approaches to visualize subcellular dynamics in infected cells (e.g., (40)). This is undoubtedly one of the major emerging areas in plant–microbe interactions in the upcoming years (41).

The authors of these chapters sincerely hope that our contributions are of use, and we wish readers the best of success in applying these methods to their favorite pathosystems. We also look forward to the next volume(s) in this series that address plant–microbe interactions. Perhaps the next volume describes new technologies for structural studies of immune receptor complexes, along with advanced proteomic and metabolomic surveys of infected tissue at fine spatial scales. A major challenge will be to integrate data from disparate approaches, with the potential payoff being holistic models of infected cells, tissues, and organs. It would be particularly valuable to understand regulatory connections between immunity and other plant processes that might predict undesirable side effects of engineered resistance strategies (e.g., yield loss, reduced resistance to abiotic stress). It is exciting to imagine that such depth of understanding might even prompt a subsequent Methods volume focusing on "translational" approaches; for example, bioinformatic approaches to efficiently identify durable resistance genes for breeding or transgenics, or even surveillance genes that are custom-designed to detect PAMPs or indispensable pathogen effectors. Is this far-fetched? Perhaps…but if we were plant breeders in the 1950s could we have anticipated the depth of understanding that has already been achieved in only five short decades?

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