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Research on Molecular Plant-Microbe Interactions in Canada

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ABSTRACT

The study of molecular plant-pathogen interactions is a very active field of research in Canadian universities and research centers. This is not surprising given that the Canadian economy is tightly bound to food crops and forest resources for domestic use and export. The goal of this mini-review is to provide an overview of the excellent research that is being done on this topic in Canada. The availability of funding for genome-scale projects on model and non-model crop plants has led the field of plant pathogen interactions into a new era. The different approaches used by researchers in Canada to decipher bacterial and fungal virulence strategies and the research tools currently being used to study the plant immune system will be discussed. Relevant work on *Arabidopsis thaliana* and *Nicotiana benthamiana* is included but most of the work discussed herein is related to non-model species.

Keywords: bacterial effectors, fungal effectors, host defense response, molecular plant-microbe interaction, viral effectors
Abbreviations: Avr, avirulence; **CC-NB-LRR**, coil-coil–nucleotide binding site–leucine-rich repeat; **EST**, express sequence tag; **HR**, hypersensitive response; **NSERC**, Natural Sciences and Engineering Research Council of Canada; **R**, resistance; **TIR-NB-LRR**, Toll/Interleukin1 receptor–nucleotide binding site–leucine-rich repeat; **SA**, salicylic acid; **VIGS**, virus-induce gene silencing

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INTRODUCTION

More than half a century ago Harold Flor postulated the gene-for-gene hypothesis to explain the flax-rust pathosystem (Flor 1942). Although new theories such as the guard hypothesis, the zigzag model and the decoy model propose variations to this hypothesis (Flor 1942; Van der Biezen and Jones 1998; Abramovitch *et al.* 2002; Jones and Dangl 2006; van der Hoorn and Kamoun 2008) the gene-for-gene model remains the core of molecular plant-microbe interactions. In this theory a resistance (R) protein in the plant recognizes a specific effector (also termed avirulence factor; Avr) in the pathogen to trigger a defense response. If one or the other is missing, successful infection occurs. Although the specific interactions between effectors and their cognate R proteins are unique, the mechanisms downstream of R protein activation seem to be quite conserved across species.

Canadian research on molecular plant-pathogen interactions is very active. Since Canada is largely dependent on its plant natural resources, improving the resistance of edible and ligneous crops is of utmost importance and is therefore relatively well funded. Funding opportunities in Canada are either through the Natural Sciences and Engineering Research Council of Canada (NSERC) for basic research, the Ministry of Natural Resources for federally funded research Centers, Genome Canada for large scale genomic projects, and there is also support available from provincial funding agencies. Research on crop or ligneous species in Canada is largely (but not only) done in federally funded agencies. Across Canada, 19 federal research centers conduct research for Agriculture Canada and 5 for the Canadian Forest Services (**Fig. 1**). In addition, universities also support research on molecular plant-microbe interactions using crop and ligneous species as well as more fundamental research that utilizes model species like *Arabidopsis thaliana* and *Nicotiana benthamiana*.

Different avenues can be used by researchers to better understand plant-pathogen interactions and plant resistance, but this review will be limited to the following two approaches: the study of microbial pathogens (bacterial, fungal and viral) and their effectors, and the study of host resistance (signal transduction and biochemistry). In addition to highlighting the work done in Canada on the two latter topics we will discuss the potential application of those research findings as well as forthcoming challenges. We apologize to colleagues whose work could not be included due to our limited expertise and space constraints. Also note that work on symbiosis and biocontrol is not covered by this mini-review.

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Invited Mini-Review

Fig. 1 Representation of the different research centers mentioned in this review administered by Agriculture Canada and the Canadian Forest Service.

MICROBIAL PATHOGENS AND THEIR EFFECTORS

Major progress has been made in the last decade with respect to the characterization and identification of R and Avr genes. This is mainly due to two factors: 1) the use of the very powerful genetic models *Arabidopsis* and *N. benthamiana* for understanding the mechanism of R protein interaction/activation, and 2) the availability of complete genomes of *Arabidopsis*, rice and pathogen species, and the plethora of ESTs available for a wide range of species.

BACTERIAL EFFECTORS

Genomic information is one place to start when searching pathogen genomes for putative effector proteins. It was recently shown that several effectors display conserved motifs necessary for their secretion (Whisson *et al.* 2007; Dou *et al.* 2008). Using these signature motifs to datamine genomes can be very useful, but the number of candidate genes retrieved can be overwhelming (Jiang *et al.* 2008). One of the leading groups studying bacterial effectors and their evolution is that of Dr. David Guttmann at the University of Toronto. Dr. Guttmann's lab has been focusing on the *Pseudomonas syringae* type III secretion system (Guttman *et al.* 2006) and has used host specificity as a means to retrieve host-specific virulence factors from *P. syringae* genomes (Sarkar *et al.* 2006). The lab has extensively studied the evolution of virulence factors (Guttman *et al.* 2006; Ma *et al.* 2006; Stavrinides *et al.* 2006). More recently the lab has demonstrated the differential effect of alleles of HopZ effectors on plant responses (Zhou *et al.* 2009) and performed a detailed functional characterization of HopZ1a and HopZ2 (Lewis *et al.* 2008). Recently appointed, and also working on bacterial effectors at University of Toronto, is Dr. Darrell Desvaux. Dr. Desvaux also contributed to the functional characterization of the HopZ and HopF2Pto effectors (Lewis *et al.* 2008; Wilton *et al.* 2010) and devised a high throughput chemical genomic approach to find molecules affecting the susceptibility of *Arabidopsis* to *P. syringae* infection (Schreiber *et al.* 2008). His lab also uses large scale yeast-two hybrid assays to identify interactions between *P. syringae* effectors and *Arabidopsis* target proteins. As part of his multi-tools approach Dr. Desvaux, in collaboration with Dr. Dinesh Christendat, also aims to obtain crystal structures of *P. syringae* effectors. Such a comprehensive approach should provide novel insight on *P. syringae* effector biology.

FUNGAL EFFECTORS

Fungi cause diseases on a staggering number of Canadian crops. With sequencing prices rapidly decreasing and the emergence of new sequencing technologies, more fungal pathogen genomes are becoming available, thus enabling similar genome mining to what has been done in bacteria. For example, an international sequencing effort in collaboration with the laboratory of Dr. Richard Hamelin from the Canadian Forest Service, has recently led to the release of the first biotrophic tree pathogen genome sequence, *Melampsora laricina-populina*, which is a major threat to poplar (www.jgi.doe.gov/Melampsora). Similarly, Dr. Linda Harris and Dr. Thérèse Ouellet from Agriculture Canada participated in the sequencing of the *Fusarium graminearum* genome, an important cereal pathogen (Cuomo *et al.* 2007). Genome analysis will yield insight into putative secretomes and pathogenicity related factors. In fact, the group of Dr. Hamelin is actively pursuing the secretome analysis for the *Melampsora laricina-populina* genome as part of a collaborative project (R. Hamelin, pers. comm.). Analysis of the *Phytophtora sojae* genome by the laboratory of Dr. Mark Gijzen at the Southern Crop Protection and Food Research Centre on RLXR-effectors led to more insight on their evolutionary nature (Dong *et al.* 2009).

However, since genome analysis can only yield predictions, more accurate data about the secretome of proteins expressed during infection can be obtained through proteomics approaches. The Harris laboratory has used quantitative proteomics to study the proteome of *F. graminearum* grown under mycotoxin-inducing conditions; mycotoxins are known to be produced during pathogen attack (Taylor *et al.* 2008). Earlier, another laboratory at Agriculture Canada had identified a gene involved in butenolide synthesis (a mycotoxin) (Harris *et al.* 2007). The same pathogen was also used in a proteomics study aimed at identifying differentially expressed proteins between uninfected and infected plants (Zhou *et al.* 2006). Such *in planta* experiments provide a "real" snapshot of infection related proteins, however, as can be expected, very few proteins belonging to the pathogen can be identified amidst all the plant proteins. To obtain a higher yield of secreted proteins, these can, in some cases, be extracted from liquid cultures (Yajima and Kav 2006). To gain information about virulence factors, protein profiles of virulent and avirulent isolates can be compared (Cao *et al.* 2009). For a review of the application of proteomics to plant biology, see (Rampitsch and Srinivasan 2006).

Not all effectors can be predicted and predicted effectors often have low-coding potential and thus a high

level of false predictions are observed (Jiang *et al.* 2008). Methods like transcriptomics and proteomics may be limited by the low expression level of effector proteins which may cause them to go undetected in the noise of abundant transcript and proteins. Other methods will be needed to identify putative effectors. Gene disruption is a method of choice to identify genes involved in virulence. For example, the group of Dr. Jim Kronstad at the University of British Columbia has used homologous recombination to delete *Ustilago maydis* genes that are involved in virulence (Boyce *et al.* 2006; Klose and Kronstad 2006). Fungal transformation has been hampered by the fact that a transformation protocol is not available for several pathogens. Recently the pathogen responsible for dutch elm disease, *Ophiostoma novo-ulmi* subsp. *novo-ulmi*, was transformed in the laboratory of Dr. Louis Bernier at Laval University, using a restriction enzyme mediated integration protocol, and fitness-related genes could be identified (Plourde *et al.* 2008). Similarly, the laboratory of Dr. Katherine Dobinson at the Southern Crop Protection and Food Research Centre in London, Ontario, has recently developed a protocol for the site-directed gene disruption of the broad range pathogen *Sclerotinia sclerotiorum* (Liberti *et al.* 2007). Fungal transformation has also been used in functional studies to demonstrate the iron requirement in *F. graminearum* during infection (Greenshields *et al.* 2007). In cases where growth and transformation are difficult, heterologous transformation in an easier-to-work-with specie may be a method of choice (Hu *et al.* 2007a). Stage specific EST libraries such as the ones done for the wheat leaf rust *Puccinia triticina* by Dr. Guus Bakkeren's group at Agriculture Canada can also provide important insight into the transcriptional control of pathogenesis (Hu *et al.* 2007b).

VIRAL EFFECTORS

In addition to fungi and bacteria, plants are also plagued by viruses. Viruses cause severe damage to crops, and to date no chemical method is widely and routinely available to control virus propagation, thus justifying the need for fundamental research in the field of plant-virus interactions. One of the hallmarks of plant defense that is specific to infection by viruses is virus induced gene silencing (VIGS). Now used as a method to silence genes of interest, it is a very powerful mechanism used by the plant to suppress viral propagation. The laboratory of Dr. Hélène Sanfaçon at the Pacific Agri-Food Research Centre has studied the link between virus titer and RNA silencing in *N. benthamiana* following a hypersensitive response (HR) caused by the tomato ringspot virus (ToRSV). Although the plants seem to recover following infection and the silencing machinery is activated, the virus seems to use an undefined system to evade degradation since the level of viral RNA remains unchanged (Jovel *et al.* 2007). In order to replicate virus particles must be targeted to the proper cellular compartment. Dr. D'Ann Rochon, also at the Pacific Agri-Food Research Centre, has studied the Cucumber necrosis virus (CNV) and observed that a 38-amino acid peptide serves as a transit peptide and is necessary and sufficient for the targeting of the virus coat protein to the chloroplast (Xiang *et al.* 2006). Similarly, Dr. Jean-François Laliberté at the Institut Armand Frappier studies the interaction of virus and plant proteins necessary for viral replication. Throughout the years his lab has identified interactions between viral proteins and several plant proteins, namely Heat Shock protein 70 (Dufresne *et al.* 2008a), poly(A)-binding proteins (Dufresne *et al.* 2008b), elongation factor 1A (Thivierge *et al.* 2008) and translation initiation factor 4E (Beauchemin *et al.* 2007). The laboratory of Dr. Aiming Wang at the Southern Crop Protection and Food Research Centre has focussed on the plant response to viral infection and has used microarray technology to identify plant genes that are up- or down-regulated in the Plum pox virus/*Arabidopsis* system. In addition to finding 2013 up- and 1457 downregulated transcripts following infection, they also identified putative defense related orthologs in the natural host *Prunus persica* (Babu *et al.* 2008).

HOST RESISTANCE STRATEGIES

R proteins act at the forefront of resistance signaling pathways in plants to counter the pathogen's arsenal of effectors. Activation of an R protein is achieved either through direct effector recognition or via recognition of the effect of an effector protein. Most studied R proteins contain an N-terminal TIR- or CC-domain followed by an NB-LRR domain. Depending on their N-terminus R proteins will either signal through the EDS1 pathway or the NDR1 pathway. The mechanism of R protein activation is still unknown but major insight was gained by the work of Dr. Peter Moffet, previously at Cornell University and now at Sherbrooke University. In his work Dr. Moffett uses the potato resistance protein Rx which is a CC-NB-LRR protein that recognizes the effector protein coat protein (CP) of the Potato Virus X (PVX) to elicit an HR in *N. benthamiana*. By splitting the Rx protein in two parts, Dr. Moffett could show that when the CC and the NB-LRR were co-expressed (same for the CC-NB and LRR) they could interact and trigger the HR (Moffett *et al.* 2002). Using the same model the group of Dr. Moffett later demonstrated that the CC-domain contains a highly conserved EDVID motif that is important for intramolecular interaction but is dependent on several domains within the rest of the Rx protein (Rairdan *et al.* 2008). Domain swapping revealed that the LRR domain was the recognition site for the PVX elicitor and that the ARC 2 domain (located between the NB and LRR domain) was necessary to maintain the protein in an auto-inhibited state (Rairdan and Moffett 2006).

The identity of proteins acting downstream of R protein activation is still largely unknown but several key players have been identified. Our laboratory has taken advantage of the unique *Arabidopsis* gain-of-function mutant *snc1*. The TIR-NB-LRR mutant *snc1* constitutively expresses defense marker genes, accumulates high levels of salicylic acid (SA) and is more resistant to pathogens. We carried out several genetic screens to search for immune components that function downstream of *snc1*. To date, we have identified 15 complementation groups of *modifier of snc1* (*mos*) mutants and we have cloned 13 of them. One unexpected result of these screens is the high number of mutants defective in nucleocytoplasmic trafficking. Thus far, three nucleocytoplasmic pathways have been shown to affect immunity: Nuclear Localization Signal (NLS)-dependent protein import, Nuclear Export Signal (NES)-dependent protein export and mRNA export (Palma *et al.* 2005; Zhang and Li 2005; Sacco *et al.* 2007; Cheng *et al.* 2009). We also identified a few mutants defective in RNA processing and protein modification (Goritschnig *et al.* 2007, 2008; Monaghan *et al.* 2009). Our findings highlight the fact that signaling downstream of R proteins is largely dependent on nucleocytoplasmic trafficking, RNA processing and post-translational protein modification. For a review on the MOS mutants and comments on MOS7 see (Monaghan *et al.* 2010; Wiermer *et al.* 2010). Many other cellular processes have been shown to be involved in plant responses to pathogens. Dr. Keiko Yoshioka's group at University of Toronto recently revealed the requirement of cyclic nucleotide-gated ion channels for programmed cell death and their capacity to activate defense pathways (Yoshioka *et al.* 2001; Urquhart *et al.* 2007). Once activated, R proteins must send the "message" to the nucleus to initiate a proper defense response. Dr. Kamal Bouarab at Sherbrooke University demonstrated that although mutations in *EDS1* or *SGT1* in *Arabidopsis* cause loss of R protein activation for biotrophic pathogens (Austin *et al.* 2002; Feys *et al.* 2005), the suppression of *EDS1* and *SGT1* can enhance resistance of *N. benthamiana* to necrotrophic pathogen *Botrytis cinerea* (El Oirdi and Bouarab 2007).

Systemic acquired resistance (SAR) occurs downstream of R protein activation and requires the function of NPR1 in

Arabidopsis and *Brassica napus* (Potkayala *et al.* 2008). Significant work on NPR1 and transcription factors involved in SAR in Canada should be credited to the labs of Dr. Charle Despres, Dr. Pierre Fobert and Dr. Normand Brisson at Brock University, the National Research Council Plant Biotechnology Institute and the University of Montréal, respectively. Cellular localization of NPR1 is believed to be controlled by changes in redox potential (for a review see (Fobert and Després 2005). Following infection or SA induction NPR1 monomerizes and is translocated to the nucleus where it can interact directly with the TGA1 transcription factor (Després *et al.* 2003). NPR1 and TGA2 both directly interact with the *PR1* promoter independently of each other and independently of SA (Rochon *et al.* 2006). SAR also depends on Whirly transcription factors but Whirly activation by SA is independent of NPR1 (Desveaux *et al.* 2004). Another plant defense mechanism that protects plants is age-related resistance (ARR). Work on ARR is led by Dr. Robin Cameron at McMaster University. ARR takes place when plants start to flower and enhances resistance to *P. syringae* pathovar *tomato* (*Pst*) in a SA dependent manner. However, ARR to *Hyaloperonospera arabidopsidis* appears to be SA independent indicating that several pathways could contribute to ARR (Rusterucci *et al.* 2005). Using genetic screens Dr. Cameron's group identified several genes involved in ARR, including transcription factors and showed the dependence on EDS1 for successful ARR (Kus *et al.* 2002; Carviel *et al.* 2009). The laboratory of Dr. Kovalchuk from Lethbridge University has made significant breakthrough in showing that the genomic instability of an infected plant could be transmitted to its progeny and that its LRR-containing loci could be hypomethylated in opposition to housekeeping genes that were hypermethylated (Boyko *et al.* 2007).

Research that uses model organisms such as *Arabidopsis* and *N. benthamiana* provides insight into the mechanistics of plant-pathogen interactions. However, most of the time it does not translate into actually improving resistance in the field. Crop plant improvement has changed a lot in the last decades and now relies heavily on mapping using molecular markers to locate and follow the resistance locus in several generations. Fine mapping can help identify actual resistance genes and/or reduce linkage drag. Major strides have been made in Canada to identify loci that confer resistance to crop pathogens. Loci controlling resistance to the wheat diseases *Fusarium* head blight (FHB) and wheat leaf rust have been mapped (Cuthbert *et al.* 2006, 2007; Fofana *et al.* 2008; Hiebert *et al.* 2008). Whether or not pathogens will overcome the addition of "exogenous" R genes to susceptible cultivars is only a matter of time. However, the stacking of multiple R genes to one susceptible cultivar may theoretically be impossible to overcome by the pathogen. With this goal in mind Dr. Hou's group at Agriculture and Agri-Food Canada in Manitoba has used molecular marker-assisted selection to introduce three resistance loci against Soybean mosaic virus in soybean plants (Shi *et al.* 2009).

In trees, such mapping and stacking approaches are made even more tedious and time consuming due to long generation times, small progeny and the need for huge growth space. Until recently the set of tools and sequences available for molecular biology in trees was very limited. Large genomic projects, initiated at both ends of the country led by the groups of Dr. Joerg Bolhmann at UBC and Dr. John MacKay and Dr. Jean Bousquet at Laval University have generated a large amount of ESTs and genomic and transcriptomic tools for spruce, poplar and pine (Pavy *et al.* 2005; Ralph *et al.* 2006; Bérubé *et al.* 2007; Pavy *et al.* 2007; Ralph *et al.* 2008; Shi *et al.* 2009). In addition, an activation-tagged poplar population is now available, although no defense-related mutants have been found in this population so far (Harrison *et al.* 2007). These new tools should facilitate the discovery of defense mechanisms in trees at the molecular level. Applications of these tools permitted microarray studies of the poplar leaf rust by the group of Dr. Peter Constabel and Dr. Armand Séguin both at the Canadian Forest Services (Miranda *et al.* 2007; Azaiez *et al.* 2009). How trees respond to infection has also been assessed using proteomic tools (Islam *et al.* 2008).

DISCUSSION AND FORTHCOMING CHALLENGES

Despite the extensive amount of research that is being done on plant-pathogen interactions in Canada and the world, our plants continue (and will continue) to be plagued by infections. Understanding these infections and the plant response is the initial stage in our quest to keep our plants free from infection. To obtain more resistant plants, a better understanding of both pathogen and plants is required. However, the community often restricts its focus to a limited set of pathosytems. In *Arabidopsis*, one of the best studied of all plants; the extensive biotrophic pathosystems used are *Pseudomonas* and *Hyaloperonospora*. Only very recently have we started to see *Arabidopsis* being used to study necrotrophs, mildews and other pathogens. Now that genomic resources will become available (or obtainable) for more species, perhaps a wider spectrum of pathosystems will enable the discovery of novel defense components. In addition to all the inherent advantages related to the use of *Arabidopsis* as a model system, the high level of collaboration of its research community is certainly one of the driving factors that led to its widespread use. The breadth of resources available through the Arabidopsis Biological Resource Center (ABRC), accessible at very-low cost through the user-friendly TAIR (The Arabidopsis Information Resource) web site, both supported by the National Science Foundation, is unmatched for other plant species. Researchers willing to establish new pathosystems should keep in mind that sharing information and stocks are key to establishing new systems. As more genomes are becoming available users constantly face new interface and different database format. If databases cannot all be integrated into one major database, care should be taken into making databases uniform to facilitate cross-searches by users. In the future, as genome sequencing becomes more affordable to several labs, researchers should make an effort to have the data released in public databases.

More resistant crops are still being obtained through classical breeding with increasing help from molecular markers. However classical genetic approaches are time consuming and face the caveats of linkage drag. Increased resistance using single or multiple transgene insertions is becoming more attractive as pathogens, such as the rust strain Ug99, compatible with most commercially grown wheat cultivars, is threatening the world wheat stock. However, several countries have been very reluctant to commercialize transgenic crops and have established strict regulations that impede research on transgenic plants.

Research on molecular plant-pathogen interactions is extremely active in Canada. Great discoveries have been made in the field of effectors and resistance mechanisms. As the mechanistic details of R protein activation is being unraveled and as more resistance loci are being identified and technological tools are becoming more accessible, plant engineering will become more feasible. We are facing new challenges, however: global climate change may have an unpredictable effect on plant yield, pathogen growth, and plant-pathogen interactions. Using cellulose derived bioethanol as a fuel source may cause price volatility, as monoculture crops are often prone to epidemics. Having both our food stock and fuel stock at the mercy of a pathogen could be quite perilous. It is thus of great importance that research on molecular plant-pathogen interaction in Canada continues to thrive.

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