

APPLICATION FOR FEDERAL ASSISTANCE  
SF 424 (R&R)

2. DATE SUBMITTED	Applicant Identifier
3. DATE RECEIVED BY STATE	State Application Identifier
4. Federal Identifier	HL095948

1. \* TYPE OF SUBMISSION  
 Pre-application  Application  Changed/Corrected Application

5. APPLICANT INFORMATION \* Organizational DUNS: 073130411

\* Legal Name: Massachusetts General Hospital (The General Hospital Corp)

Department: Research Management Division:

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\* State: MA: Massachusetts Province:

\* Country: USA: UNITED STATES \* ZIP / Postal Code: 02199

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6. \* EMPLOYER IDENTIFICATION (EIN) or (TIN): 1042697983A1

7. \* TYPE OF APPLICANT: M: Nonprofit with 501C3 IRS Status (Other than Institution of Higher Education)

Other (Specify):

Small Business Organization Type  Women Owned  Socially and Economically Disadvantaged

8. \* TYPE OF APPLICATION:  New  Resubmission  Renewal  Continuation  Revision

If Revision, mark appropriate box(es).  
 A. Increase Award  B. Decrease Award  C. Increase Duration  D. Decrease Duration  
 E. Other (specify):

\* Is this application being submitted to other agencies? Yes  No  What other Agencies?:

9. \* NAME OF FEDERAL AGENCY: National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER: TITLE:

11. \* DESCRIPTIVE TITLE OF APPLICANT'S PROJECT:  
Cross regulation of divergent host responses to viral and bacterial pathogens

12. * AREAS AFFECTED BY PROJECT (cities, counties, states, etc.) N/A	13. PROPOSED PROJECT: * Start Date: 12/01/2009 * Ending Date: 11/30/2011	14. CONGRESSIONAL DISTRICTS OF: a. * Applicant: MA-009 b. * Project: MA-009
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15. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: Dr. \* First Name: Suresh Middle Name:

\* Last Name: Gopalan Suffix: Ph.D.

Position/Title: Assistant in Molecular Biology

\* Organization Name: Massachusetts General Hospital (The General Hospital Corp)

Department: Molecular Biology Division: Genetics

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**RESEARCH & RELATED Other Project Information**1. \* Are Human Subjects Involved?  Yes  No

1.a. If YES to Human Subjects

Is the IRB review Pending?  Yes  NoIRB Approval Date: Exemption Number:  1  2  3  4  5  6Human Subject Assurance Number: 2. \* Are Vertebrate Animals Used?  Yes  No

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending?  Yes  NoIACUC Approval Date: Animal Welfare Assurance Number 3. \* Is proprietary/privileged information included in the application?  Yes  No4.a. \* Does this project have an actual or potential impact on the environment?  Yes  No4.b. If yes, please explain: 4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed?  Yes  No4.d. If yes, please explain: 5.a. \* Does this project involve activities outside the U.S. or partnership with International Collaborators?  Yes  No5.b. If yes, identify countries: 5.c. Optional Explanation: 6. \* **Project Summary/Abstract**    7. \* **Project Narrative**    8. **Bibliography & References Cited**    9. **Facilities & Other Resources**    10. **Equipment**    11. **Other Attachments**    OMB Number: 4040-0001  
Expiration Date: 04/30/2008

The goal of this proposal is to study cross-regulation between divergent host responses during infection by viral and bacterial pathogens sequentially and in combination. The proposal utilizes *Arabidopsis thaliana*, plants in the genus *Nicotiana*, and the nematode *Caenorhabditis elegans* as model hosts that use divergent arms of innate immunity during interactions with RNA viruses and bacterial pathogens. In the host-pathogen combinations proposed, the current known dominant pathogen restriction mechanism of the host engages components of the RNA interference (RNAi) machinery for the viral pathogens and immunity to the other (bacterial) pathogen involves different pathways that result in the synthesis of anti-microbial effectors. The latter class typically involves one or more of conceptually similar pathways conserved in different kingdoms, that include recognition of microbial components by a variety of pattern recognition receptors, often followed by activation of programmed cell death pathways, MAP kinase cascades, and transcription factors that effect immunity.

It is anticipated that these studies will identify novel aspects of interaction between the arms of innate immunity to these pathogen classes and the resultant effect in local and systemic tissues that would otherwise be difficult to dissect. The study should contribute to fine tuning the responses altered during such multi-pathogen infections in addition to having applications in RNAi based therapeutics.

The model hosts will be infected with one class of pathogen and the effect on the other class of pathogen in local and systemic (in the case of the plant models) tissues will be studied. Organismal scale gene expression studies and high-throughput sequencing of small RNA species will be used to identify robust markers of the interactions considered relevant, to aid further analyses and to design high-throughput genetic studies. In the case of *C. elegans*, heterologous viral pathogens are typically used, thus the existence of naturally occurring viral pathogens of this nematode will be explored through deep sequencing of small RNA species to better model these interactions.

The goal of this proposal is to study cross-regulation between divergent host responses during infection by viral and bacterial pathogens sequentially and in combination using invertebrate model hosts with a large number of tools. It is anticipated that these studies will identify novel aspects of interaction that should aid fine tuning the responses altered in such multi-pathogen infections for therapeutic purposes. The ability to fine tune these responses should also aid ongoing attempts to develop therapeutics to a number of diseases using RNA interference (RNAi) mediated mechanism, where there is a high premium to avoid negative modulation by other arms of the innate immune response.

## **Resources:**

**Research facilities organization:** The Department of Molecular Biology is located in the newly constructed (2005) Richard B. Simches Research Center on the Massachusetts General Hospital main campus. The part of the department where I am currently housed (Ausubel lab) is located on Simches 7, which also houses the laboratories of Gary Ruvkun and Joshua Kaplan, both of whom work with *C. elegans*. The Department occupies ~ 44,000 total square feet of laboratory benches, desks, office space and shared facilities.

From a broader perspective, the Simches Research Building is designed around thematic centers that house many laboratories at MGH at the forefront of modern biological research. Thus in addition to Molecular Biology, the Simches building has multi-investigator groups studying systems biology, human genetics, stem cells, chemical genetics, genomics, and computational and integrative biology.

Simches 7 has a full complement of shared general and specific laboratory facilities including instrument rooms, cold rooms, microscope rooms, a walk-in plant growth facility, a chemical storage room, a media preparation facility, a glass washing/sterilization facility, a tissue culture facility, electrophoresis and gel rooms, a dark booth and a specialty dark room as well as laboratory supply storage space. Floor secretarial offices, the offices of the Department's Faculty members, conference rooms (with advanced audio/visual equipment), and "tea rooms" equipped with full kitchen and meeting facilities, are also located on Simches 7.

**Plant and worm resources:** A number of plant walk-in growth facilities and reach-in chambers are available to conduct the *Arabidopsis* and *Nicotiana* based experiments proposed, coordinated by a Greenhouse manager who also maintains the greenhouse (in the Their building that the department used to occupy), which is part of the available resources. Since three labs work with *C. elegans*, there is an extensive maintenance of common resources (e.g., regularly re-stamped RNAi library for rapid cherry picking and testing phenotypes) often coordinated on a rotating basis by postdoctoral members and students, in addition to a variety of worm mutants and reporter strains and constructs that are regularly used. The components of RNAi pathway that are being identified by the laboratory of Gary Ruvkun should also prove very useful for the goals of the proposed project (as a readily available resource when published or accepted for publication or a collaboration when extensive use is warranted, to test hypotheses generated - see enclosed letter). In addition, a biweekly multi-group worm meeting provides a basis to get ideas and progress discussed and evaluated internally on a regular basis.

**Equipment:** Almost all equipment in the Department of Molecular Biology is shared. This includes most modern equipment needed for state-of-the-art cell and molecular biology research. In addition to the equipment and facilities in the Department of Molecular Biology, MGH has a robotic instrumentation facility, an oligonucleotide synthesis facility, and a DNA sequencing facility and more recently added a Solexa sequencing machine to the collection of available resources to be available to through the core. Additional services to all departmental researchers needing peptide synthesis, protein sequencing, and/or amino acid analysis are also available. In addition, many of the data collection for system-wide analyses proposed can be carried out at the core

facilities available through the Harvard University and the Partners Network, usually through prior arrangement and for a charge.

Some equipment available in the Department includes: 2 Beckman L8 ultracentrifuges, 3 Beckman J2-21 high speed centrifuges, a Beckman TL-100 ultracentrifuge, a Beckman J6 low speed centrifuge, a Beckman Biomek robot, 5 -80°C freezers, a regular scintillation counter and a Perkin Elmer luminometer/scintillation counter for high-throughput reads in 96 well plates, 6 thermo-cyclers for PCR reactions, thermal cyclers for real-time quantitative PCR, a Beckman DU-640 spectrophotometer, an FPLC, an HPLC, a GLC, a capillary zone electrophoresis system, a Perkin Elmer fluorescence spectrometer, a Molecular Dynamics Phosphor Imager, a hybridization oven, fraction collectors, bacterial shakers, bacterial and cell culture incubators, inverted and epi fluorescence Zeiss microscopes, a Leica confocal microscope, ultramicrotome, lyophilizer, gel drier, table-top microcentrifuges, electrophoresis equipment, and all the miscellaneous small equipment required for carrying out standard molecular biology experiments.

**Computer and Bioinformatic resources:** The Department of Molecular Biology also has extensive computer facilities with web and database servers and a dedicated computer services core staff who also provide regular computer and network maintenance and consultation, as well as a new Bioinformatics Core Facility consisting of three full-time bioinformaticians. Each member of a laboratory has their own Macintosh G4 notebook or PC computer. In addition, many Macintosh G4, Linux, and PC computers are available at common workstations dispersed throughout the Department.

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**BIOGRAPHICAL SKETCH**

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.  
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Suresh Gopalan, Ph.D.	POSITION TITLE Assistant in Molecular Biology		
eRA COMMONS USER NAME GOPALAN			
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
Birla Institute of Technology and Science, Pilani, India (Dual degree scheme)	B.E. (Hons)	1988	Mechanical Engineering
Birla Institute of Technology and Science, Pilani, India (Dual degree scheme)	M.Sc. (Hons)	1988	Biosciences
Center for Biotechnology, Anna University, Madras, India	Ph.D.	1993	Biotechnology

**A. PROFESSIONAL EXPERIENCE**

- 1987 Summer internship, Cancer Institute, Madras, India.  
**Generation of monoclonal antibodies against the estrogen receptors from the human breast cancer cell line 247D.**
- 1987 Masters thesis, Birla Institute of Technology and Science, India  
**Leukocyte culture for scoring environmental mutagens.**
- 1988 Internship Program part of Bachelors degree, Hindustan Aeronautics Limited, India.  
**Analysis of Low Aspect Ratio Wings**
- 1988 – 1993 Ph. D thesis, Anna University, India (Advisor: Prof. Kunthala Jayaraman)  
**Recombinant DNA approaches and process optimization strategies for enhanced production of the mosquito larvicidal proteins of *Bacillus sphaericus* 1593M**
- 1990 Academic scientist (technology transfer), Hindustan Antibiotics Limited, India.  
**Large scale cultivation of *B. sphaericus* biopesticide in 27 kL reactors.**
- 1991 Exchange Scientist, Swiss Federal Institute of Technology, Zurich  
**Development of a defined medium for the study of growth, sporulation and toxin synthesis in *B. sphaericus*.**
- 1993 - 1995 Postdoctoral Scholar, Dept. Plant Pathology, University of Kentucky, Lexington, KY  
**Plant responses to bacterial hypersensitive response elicitors.** (S. Y. He Lab)
- 1995 - 1998 Postdoctoral Research Associate, MSU-DOE-Plant Research Laboratory, East Lansing, MI  
**Plant perception and signaling during gene-for-gene mediated cell death response (HR) and resistance initiated by bacterial pathogens**(S. Y. He Lab)
- 1999 Research Associate (Instructor), Molecular Genetics and Cell Biology Department, University of Chicago, Chicago, IL.
- 1999 - 2001 Postdoctoral Research Associate, Institute of Biological Chemistry, Washington State University, Pullman, WA  
**Molecular genetic analysis of plant-virus interactions** (J. Carrington Lab)
- Nov'01 - Jan '02 Scientist at Genome Therapeutics Corporation, Waltham, MA (through Aerotek)  
**Mechanism of action of antimicrobials identified by screening chemical libraries and development of 'ready-to-high throughput' cell based assays for the study of biochemical processes**
- Feb'02 – Aug'02 Independent Investigator  
**Detect SNPs (*in silico*), organize and analyze SNPs in Arabidopsis and compare to other plants and organisms.**
- Sep'02–Sep' 06 Independent Investigator &  
Consultant: Massachusetts General Hospital & Harvard University, MA. (L. Rahme Lab)  
Collaborator and Visiting Scientist, MGH & Harvard University (Fred Ausubel Lab)

**Genomic analysis of pathogen (*P. aeruginosa*) and host (mouse and Arabidopsis) processes.**

**Explore newer methods to analyze and utilize large-scale gene expression datasets**

Oct'06-Mar'08 Visiting Scientist,

Mar'08-current Assistant in Molecular Biology, Department of Molecular Biology, Massachusetts General Hospital and Harvard Medical School, Boston, MA. (Fred Ausubel Lab)

**Models and systems to study recalcitrant and emerging infectious diseases**

**HONORS AND FELLOWSHIPS**

1987 **Best lecture of the year**, IDEATION, Birla Institute of Technology and Science, India. "Cancer therapy and Monoclonal Antibodies".

1988 - 1993 Junior and Senior Research Fellowship, Council for Scientific and Industrial Research, and Department of Science and Technology (SERC), India.

1993 – 1994 Post Doctoral Fellowship, Research and Graduate Studies, University of Kentucky, Lexington.

1997 Young Researcher's bursary to participate and present talk entitled "Reversal of the plant hypersensitive response by the growth regulator auxin" in the "Plant Workshop: Leaves". La-Colle-sur-Soup, France.

**SELECTED INVITED PRESENTATIONS**

1) Development of a defined medium by chemostat pulse and shift technique for the cultivation of the biopesticide *B. sphaericus* 1593M. Swiss Federal Institute of Technology (ETH), Honnigerberg, Zurich, Switzerland. 1991.

2) Plant genes induced during the hypersensitive response to incompatible bacterial pathogens. International Laboratory for Tropical Agricultural Biotechnology/The Scripps Research Institute, California. 1995.

3) Genes galore: how do we get to their functions? Seminar at Michigan State University, 1996.

4) *Pseudomonas syringae* pv. *glycinea* AvrB elicits a genotype specific hypersensitive necrosis in transgenic *Arabidopsis thaliana* plants: dependence on RPM1 and a possible second target gene. 7th International Conference on Arabidopsis Research. Norwich, England. 1996.

5) Reversal of the plant hypersensitive response by the growth regulator auxin. The Plant Workshop: Leaves. La-Colle-sur-Soup, France. 1997.

6) *Pseudomonas syringae* avirulence protein AvrB initiates a genotype-specific hypersensitive resistance response inside the plant cell. UNESCO/DBT/SBPL sponsored workshop on Cells and Molecules in Biotechnology. Madras, India. 1997.

7) Identification of putative signaling components in *Pseudomonas syringae* AvrB-mediated plant disease resistance. 9th International Conference on Arabidopsis Research, Madison, Wisconsin. 1998.

8) Genetic Analysis of Plant Susceptibility to Tobacco Etch Virus. New England Arabidopsis Meeting, 2001.

**B. PUBLICATIONS AND PATENT**

1. Rajendra. K.B. and Suresh. G. (1989) Comments on the Lawrence equation for low aspect ratio wings. *J. Aircraft*, 26:883-885.

2. Rajamohan. F., Suresh. G. and Jayaraman. K. (1992) Enhanced expression of a mosquito larvicidal gene(s) from *B. sphaericus* 1593M in *E. coli*. *Curr. Sci.*, 63:205-208.

3. Suresh. G., Radhika. C.G. and Jayaraman. K. (1992) Enhanced expression of a second mosquito larvicidal gene of *B. sphaericus* 1593M in *E. coli*. *Biotech. Lett.*, 14:567-572.

4. Meenakshisundaram. S., Suresh. G (Gopalan)., Fernando. RK., Jenny. K., Sachidanandham. R., and Jayaraman. K. (1997) Metabolic response of *Bacillus sphaericus* 1593M for dual-substrate limitation in continuous and total-cell-retention cultures. *Appl. Microbiol. Biotechnol.* 47:554-559.

5. Collmer. A., Alfano. J. R., Bauer. D. W., Preston. G. M., Loniello. A. O., Conlin. A., Ham. J. H., Huang. H-C., Gopalan. S. and He. S. Y (1996) Secreted proteins, secretion pathways, and the plant pathogenicity of *Erwinia chrysanthemi* and *Pseudomonas syringae*. In: *Biology of Plant-Microbe Interactions*. Eds. G. Stacey., B. Mullin., P. M. Greshoff. International Society for Molecular Plant Microbe Interactions. St. Paul.

159-164.

6. Strobel. N. E., Ji. C, Gopalan. S, J. A. Kuc. and He. S. Y. (1996) Induction of systemic acquired resistance in cucumber by *Pseudomonas syringae* pv. *syringae* 61 HrpZ<sub>P<sub>SS</sub></sub> protein. Plant J. 9:413-439.
7. Gopalan. S. and He. S. Y. (1996) Bacterial genes involved in the elicitation of hypersensitive response and pathogenesis. Plant Disease 80:604-609.
8. Gopalan. S., Wei. W. and He. S. Y. (1996) *hrp* gene-dependent induction of *hin1*: A plant gene activated rapidly by harpins and an *avr* gene-mediated signal. Plant J. 10:591-600.
9. Gopalan. S., Bauer, D. W., Alfano, J. R., Loniello, A. O., He. S. Y. and Collmer, A. (1996) Expression of *Pseudomonas syringae* AvrB in plant cells alleviates its dependence on the Hrp secretion system in eliciting genotype-specific hypersensitive cell death. Plant Cell 8:1095-1105.
10. Gopalan. S. and He. S. Y (1998) Disease resistance: beyond the resistance genes. Trends in Plant Sci. 3:207-208.
11. Yuan. J., Wei. W., Gopalan. S., Hu. W., Jin. Q., Plovanich-Jones. A., Muncie. L. and He. S. Y. (2000) *hrp* genes of *Pseudomonas syringae*. Page 1-20 in Plant-Microbe Interactions, Stacey. G. and Keen. N, eds. APS Press. St. Paul, Minnesota.
12. Dormann, P., Gopalan, S., He, S. Y., Benning, C. (2000) A gene family in *Arabidopsis thaliana* with sequence similarity to *NDR1* and *HIN1*. Plant Physiol. Biochem. 38: 789-796.
13. Gopalan. S (2004) ResurfP: a response surface aided parametric test for identifying differentials in GeneChip based oligonucleotide array experiments. Genome Biol. 5: P14. **(Deposited Research)**
14. Padfield. K. E., Astrakas. L. G., Zhang. Q., Gopalan. S., Mindrinos. M. N., Dai. G., Tompkins. R. G., Rahme. L. G and Tzika. A. A. (2005) Burn Injury Causes Mitochondrial Dysfunction In Skeletal Muscle. Proc. Natl Acad. Sci. USA. 102: 5368-5373.
15. Déziel. E., Gopalan. S., Tampakaki. A., Lépine. F., Padfield. K., Saucier. M., Xiao. G., and Rahme. L.G. (2005) The contribution of MvfR to *Pseudomonas aeruginosa* pathogenesis and quorum sensing circuitry regulation: multiple quorum sensing-regulated genes are modulated without affecting *lasRI*, *rhlRI* or the production of N-acyl-L-homoserine lactones. Mol. Microbiol. 55:998-1014.
16. Astrakas. L. G., Goljer. I., Yasuhara. S., Padfield. K. E., Zhang. Q., Gopalan. S., Mindrinos. M. N., Dai. G., Yu. Y-M., Martyn. J. A., Tompkins. R. G., Rahme. L. G., Tzika. A. A. (2005) Proton NMR spectroscopy shows lipids accumulate in skeletal muscle in response to burn trauma induced apoptosis. FASEB. J. 19:1431-1440. 16. Padfield. K. E., Zhang. Q., Gopalan. S., Mindrinos. M. N., Tzika. A. A., Tompkins. R. G., Rahme. L. G. (2006) Local and Distal Burn Injury Alter Immuno-inflammatory Gene Expression in Skeletal Muscle. J. Trauma 61:280-292.
17. Shang. Y., Li. X., Cui. H., He. P., Thilmony. R., Chintamanani. S., Zwiesler-Vollick. J., Gopalan. S., Tang. X., Zhou. J.M. (2006) RAR1, a central player in plant immunity, is targeted by *Pseudomonas syringae* effector AvrB. Proc. Natl Acad. Sci. USA. 103:19200-19205.
18. Gopalan. S (2007) A multidirectional non-cell autonomous control and a genetic interaction restricting tobacco etch virus susceptibility in Arabidopsis. PLoS ONE 2:e985.
19. Denoux. C., Galletti. R., Mammarella. N., Gopalan, S., Werck, D., De Lorezo. G., Ferrari. S., Ausubel. F.M., Dewdney. J. (2007) Activation of defense response pathways by OGs and Flg22 elicitors in Arabidopsis seedlings (Molecular Plant, accepted)
20. Gopalan. S. (2008) Reversal of an immunity associated plant cell death program by the growth regulator auxin. BMC Research Notes 2008, 1:126.  
Gopalan. S (2007) Reversal of an immunity associated plant cell death program by the growth regulator auxin. Nature Precedings <http://dx.doi.org/10.1038/npre.2007.1347.1> **(Deposited Research)**
21. Gopalan, S., and Ausubel, F. M. (2008) A metasytem of framework model organisms to study emergence of new host-microbe adaptations. PLoS ONE 3:e3891.
22. Methods and systems for high confidence utilization of datasets. **US Patent 7,480,593**. (2009)

## C. RESEARCH SUPPORT

None in the last three years.

It is expected the first year would also include cost of some miscellaneous equipment, including a high quality dissection scope for the *C. elegans* work. Keeping with the spirit of the R21 award to rapidly standardize the system to be applicable for in-depth study, the initial work including Affymetrix chip processing and deep sequencing would be done using core facilities which would cost slightly higher, but would allow the personnel to focus on setting up the biological system in desirable state. Factoring these aspects a higher amount is requested for the first year than the second. It is expected that by the end of the first year robust conditions for studying the interactions between the two arms proposed would be identified and will form the basis for an RO1 grant, that would become effective around the time when this R21 expires. The second year will capitalize on much of the data generated and the system built, finish up link experiments, and be submitted for peer-reviewed publication.

## Cross regulation of divergent host responses to viral and bacterial pathogens

This is a resubmission of application # R21 HL095948. Changes have been made to the Research Plan to address reviewer comments/suggestions on the earlier version of the application. In most instances the changes are indicated within bold square brackets. Deleted sentences are not shown, however, to conform to the page limit. Below is a summary of the responses to the reviews and changes made in the proposal. Some of the major changes include:

- 1) Additional statements highlighting the unique strengths of the experimental system and how it would relate to relevant multi-pathogen infections in higher organisms.
- 2) Previous **AIMS 2-4** are combined into a single aim, **AIM 2**, based on their unity of purpose.

The first of the above changes highlight the need for a model system and the contributions it could make in understanding more complex systems where other arms of immunity etc. (e.g., adaptive immunity) would likely mask cross regulatory interactions, making them more difficult to detect. Since there was no doubt concerning the importance of the problem being addressed or the fact that it is understudied, the importance of having a model system seemed to have been lost in the details of the proposed experiments. The changes made in the revised proposal now clearly highlight the significance of the model.

The combination of all *C. elegans* related aims into one, emphasizes the focus of the proposed goals: to establish models to study cross regulation of host responses during multi-pathogen infections involving certain classes of viral and bacterial pathogens, and using two different model hosts. The use of more than one host and multiple approaches to setup a system for the proposed study, maximizes the chances of success. And as remarked by one of the reviewers, could lead to multiple directions each warranting full fledged study by itself. This possibility is one of the designed objectives, and in that case multiple lines of study will be initiated and pursued to make it a comprehensive multifaceted model to address this important line of investigation in a future proposal. Priority of focus will be based on the relevance to real life situations.

A second major comment was that the end points were not clear. In all cases the assays that would be used are clearly defined (typically well established assays). This is an exploratory grant with multiple possible outcomes. The outcome with respect to any particular combination in terms of cross regulation, as measured by these phenotypes can only be a speculation at this stage, though in two cases the potential to uncover unexpected outcomes have been highlighted. As one reviewer pointed out, one possibility is that different host pathogen combinations proposed might result in very different outcome. This possibility is not a deterrent as this might indicate what is to be expected in a medically relevant setting and guide how one would design the best possible strategy to maximize effort and answer key questions. This is highlighted by newly added statements in the 'Summary and Future Plans' section of 'Research Design'. Over the last decade there have been numerous studies using these model systems that have uncovered many common mechanisms or mechanistic concepts (and in some instances direct orthologous molecules) with more complex organisms. Given the questions and hypotheses raised in the proposal and applications cited, both basic and applied aspects would likely benefit from the wide range of outcomes possible – as outlined in 'Specific Aims' section and other sections in the proposal. In contrast to a statement made in the review, the RFA does not stipulate the use of microbial interactions directly from clinical settings.

As to the statement on non specific host alterations that could lead to alterations in phenotypic outcomes studied, the revised proposal now highlights that unlike systems currently in use, one of the major advantages of the experimental system proposed here is the lack of such gross alterations. The comment on natural viral pathogens has now been incorporated as an alternate possible outcome at the end of AIM 2C, and how such an outcome could be used productively.

Here alterations of specific pathogenic and host damage phenotypes are proposed to be studied, the extensive knowledge base and tools of these model organisms will be used to decipher the mechanism. However, it is predicted that some aspects can be studied more efficiently using molecular markers of this cross regulation to design additional screens. Further, the goal is to get a deeper understanding of signaling processes effecting cross regulation observed in the model(s) selected. Thus AIM 3 (previously AIM 5) proposes to generate system wide transcriptome data (based on maturity of technology) and high throughput sequencing of small RNA (based on relevance to this system) to pave the way towards gaining such an understanding.

## **A. SPECIFIC AIMS**

The goal of this proposal is to study cross-regulation between divergent host immune responses during infection by viral and bacterial pathogens sequentially and in combination. The proposal is based on extensive preliminary data derived from studies of *Arabidopsis thaliana*, plants in the genus *Nicotiana*, and the nematode *Caenorhabditis elegans* as model hosts that use divergent arms of innate immunity during interactions with RNA viruses and bacterial pathogens individually. In these combinations, the current known dominant pathogen restriction mechanisms of these hosts engages components of the RNA interference machinery for viral pathogens. Immunity to the other (bacterial) pathogen involves different pathways that result in the synthesis of anti-microbial effectors and other host strategies. The response to bacterial pathogens typically involves one or more of conceptually similar pathways conserved in different kingdom, that include recognition of microbial components by a variety of pattern recognition receptors, often followed by activation of programmed cell death pathways, kinase cascades, and transcription factors that effect immunity. The use of the term RNA silencing includes homology dependent silencing, typically through small interfering RNAs (siRNA), viz., RNA interference (RNAi), mechanisms involving microRNAs (miRNA) and other small RNA pathways that act in diverse processes including induction of epigenetic states. Many components are shared between these different pathways of RNA silencing.

[The proposal is geared towards development of robust systems and molecular and phenotypic markers of medical and biological relevance to study cross regulatory networks that affect these two arms of immunity (anti-microbial responses and RNA silencing) during multi-pathogen infection involving bacteria and viruses. Keeping with the exploratory spirit of an R21 proposal and to avoid identifying processes that are uniquely applicable to a specific system, the study of multiple pathogens affecting two independent model hosts are proposed to establish the most relevant system(s). The set up and tools will be used in a subsequent full length grant to develop further insight that should aid basic understanding and develop potentially novel therapeutic strategies.]

Some unique advantages of the models used in the experimental system described in this proposal include:

- (i) lack of obscuring signals from many different interactions between innate and adaptive immunity as would be the case when vertebrate whole organismal models are used. Nevertheless, the system will yield relevant cross regulatory information that could be harnessed in medically relevant settings;
- (ii) [combinations of systemic effects of signals mediated by infection of each of these pathogens on the other pathogen and on the complementary arms of immunity can be studied due to the unique advantages of the system and the experimental design;
- (iii) infection of these two hosts using the selected viruses does not cause any visible phenotypic or developmental defects (thus avoiding gross suppression of host immunity or non-specific effect on host physiology);]
- (iv) ability to design large scale and high-throughput experiments and availability of forward and reverse genetic tools;
- (v) study of non-cell autonomous signals and their interactions that cannot be easily studied in cell culture models and single cell organisms;
- (vi) the use of microbes as triggers offers many more advantages than studying these interactions using host mutants in the dominant immune pathways, as it is expected that the net effect of a microbial infection encompasses more aspects than the known individual components and pathways.

In addition to contributing to the less studied, hence less understood, effects on these dominant and divergent arms of immunity effected by these multi-pathogen infections, the study should also contribute to fine tune the responses and the pathways that could have therapeutic benefit in medically relevant settings. The ability to fine tune these responses should also aid the currently envisaged and ongoing attempts to develop therapeutics using RNA silencing mechanisms, where there is a high premium to avoid activation of other arms of the innate immune response that would interfere with some aspects of this goal. Alternatively, it may be desirable in some circumstances to activate the RNAi and other RNA silencing pathways despite the activation of other arms of immunity that normally preclude RNAi.

The three inter-related aims are:

1. **1A:** Study the effect of viral infection (where the current known major host immunity response involves components of RNA silencing machinery) on subsequent infection by bacterial pathogens (where the current known dominant host response involves very different components) in the same tissue, and in uninfected systemic tissues. *Arabidopsis* and *Nicotiana* will be used as model hosts and Tobacco etch virus (TEV) and *Pseudomonas syringae* will be used as initial model pathogens. **1B:** Study the converse effect of the above aim #1A and system, viz., initial bacterial infection followed by subsequent infection of viral pathogen in local and systemic tissue.
2. **2A:** Develop a simple feeding model for an RNA virus that readily infects *C. elegans* and engages the immune pathway involving components of the RNA silencing machinery (TEV and other heterologous viruses from different kingdoms will be used for this purpose). Some preliminary data are included. **2B:** Explore the existence of natural viral pathogens of *C. elegans* by the use of recent advances in extremely high-throughput short read sequencing of small RNAs, to better model these interactions. **2C:** Develop transgenic *C. elegans* expressing transcripts of viruses selected above and/or a pretested virus (Flock House Virus) under different modes of regulated expression (exogenous inducer and stage/tissue specific regulation) with appropriate reporters for monitoring viral infection and engagement of RNA silencing machinery **2D:** Use appropriate model(s) from #2A, #2B and #2C to preinfect *C. elegans* with a viral pathogen and study the effect on subsequent challenge with bacterial pathogens (initially using the human opportunistic pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus*).
3. Initiate experiments to dissect mechanisms and identify robust markers of cross-regulation of host responses during these multi-pathogen interactions using organismal scale studies and using gene expression and deep sequencing technologies.

## **B. BACKGROUND AND SIGNIFICANCE**

The objectives of this proposal are to identify novel responses that result during multi-pathogen infections that are typically resisted by the model hosts through divergent innate mechanisms. The host-pathogen combinations used here avoids the contribution of classically defined adaptive immunity in mammals and the two way interactions involved in the cells mediating innate and the adaptive immunity, which might indeed be useful in other contexts (Medzhitov 2007). The choice to leave out this arm provides distinctive advantages in studying interactions of the two dominant arms of innate immunity (outlined below) and other yet to be recognized signals altered during these host-microbe interactions that modulate the dominant host responses to one of both class of pathogens. In addition, the number of mutants isolated in these dominant immune pathways in these hosts during their interactions with each of these pathogens and the research tools available for these models should prove invaluable to dissect the inferred interactions rapidly.

### **Some prominent examples of host responses to multiple microbes**

It has long been realized that infection of one class of pathogens affect the host response to other classes or in some cases simply infection by different strains of a particular pathogen species can alter the outcome of interactions between the host and microbes. The prominent and most striking examples involve alterations following a viral infection, in most cases increased susceptibility, to subsequent bacterial and other infections. Indeed it is believed that the influenza pandemic of 1918 was so devastating due to simultaneous bacterial pneumonia (probably enhanced by the viral infection) as due to a particularly virulent strain of the virus (Taubenberger and Morens 2006; Morens and Fauci 2007). This theory has recently been reinforced by molecular evidence that the viral accessory protein PB1-F2 of influenza that matches the protein sequence of 1918 pandemic strain led to more severe secondary bacterial pneumonia (McAuley et al. 2007). In some cases, like in the case of HIV, the immune deficiency that ensues makes the patients more susceptible to many pathogens and opportunistic microbes that are normally well resisted by the immune system. Other examples include increased susceptibility to necrotizing fasciitis caused by many bacteria (e.g., *Streptococcus pyogenes*, *Vibrio vulnificus*) in many instances by a previous or existing viral infection, for e.g., by herpes, influenza (Okamoto et al. 2003) and also in the case of patients with HIV. These examples of simultaneous infections causing substantially more damage to the host may be a consequence of the interaction of different of arms of the immune response (further detailed below) that usually includes innate immunity and activation of adaptive immunity. The change in status of the host immune system to a different steady state would alter the outcome of the interaction of the initial pathogen and subsequent simultaneous interaction with other pathogens.

From the perspective of some bacterial interactions, such interactive states are exemplified by the intricate balance of microbes for example in the intestine and the teeth of many hosts communicating by the use of signals that cross regulate among themselves, and regulate local host immune status (Bassler and Losick 2006; Kolter and Greenberg 2006; Dethlefsen et al. 2007; Turnbaugh et al. 2007).

### **Two broadly classified dominant arms of innate immunity:**

#### **1. Innate immunity involving recognition of pathogen components through pattern recognition receptors (PRRs) and activation of anti-microbial effectors**

This best studied arm of immunity that is the dominant pathway in mammals in response to most classes of pathogens uses a set of pattern recognition receptors (PRRs) for initial recognition. Most pathogens are often recognized by PRRs [so termed due to the broad specificity of many of them for conserved and invariant features of many microorganisms - (Janeway 1989)]. In simplified terms, some well studied PRRs include toll like receptors (TLRs) and NOD like receptors (NLRs) both of which are characterized by the presence of leucine rich repeats (LRRs) and use different class of adaptors (CARD domain containing adaptors and TIR domain containing adaptors, respectively – (Akira et al. 2006; Fritz et al. 2006; Meylan et al. 2006; Beutler et al. 2007). These receptors recognize a variety of pathogen components including proteinaceous molecules, lipopolysaccharides, toxins of bacteria, viral proteins and CpG DNA of invading bacteria and single and dsRNA of viruses. The toll gene was cloned in *Drosophila* as a gene required for dorso-ventral patterning, and subsequently shown to play a role immunity (Hashimoto et al. 1988; Lemaitre et al. 1996). Other major classes of receptors include the RLRs that include the RIGI like helicases that recognize a variety of heterologous



single and double stranded DNA and RNA molecules (that include a variety of viruses) and DAIs that recognize double stranded DNA molecules. The third major class of receptors are dectins that recognize glucans and other components of fungi and yeast. The recognition by PRRs is usually accompanied one or more of the following events: engagement of a variety of adaptor molecules, kinase cascades, followed by convergence of many different pathways onto one or few transcriptional regulators (e.g., NFKB, IRFs) that trigger a variety of immune related functions. Another major component of relevance to this proposal is dsRNA dependent protein kinase (PKR), that has been shown to be activated by dsRNAs (Sledz et al. 2003) which triggers a potent interferon (IFN) response that usually precludes RNAi.

Some background in plants and *C. elegans* relevant to this proposal is outlined below. Since their first cloning from *Arabidopsis* (Mindrinos et al., 1993; Bent et al., 1993) it has been realized that plants have a large family of LRR containing proteins identified as genetically recognizing specific and general components of a variety of pathogens, e.g., parts of bacterial flagellin and elongation factor Tu (Gomez-Gomez and Boller 2000; Zipfel et al. 2006). These proteins are termed receptors in the remainder of the proposal, are defined on the basis of their genetic recognition of matching pathogen effectors (though direct interaction is not always demonstrated). Consistent with the intracellular presence of these LRR containing receptors recognizing specific bacterial components, it has been shown that the cognizant bacterial effector proteins are also functional inside the plant cell (as initially demonstrated initially for the effector protein AvrB – (Gopalan et al. 1996), delivered into the host by the type III secretion apparatus that is present in most gram-negative pathogens (reviewed in (Galan and Collmer 1999). Other major classes of pathogen recognition molecules cloned in plants include kinases (Martin et al. 1993) and enzymes that detoxify toxins of pathogens (Johal and Briggs 1992; Scott-Craig et al. 1992). In the case of worms it is still not clear if the only member of the TLR type receptor class, *tol-1*, plays a role in recognizing pathogen components or uses other components or mechanisms to recognize/sense pathogens. In both plants and *C. elegans* MAP kinase cascades (Ligterink et al. 1997; Zhang and Klessig 2001; Asai et al. 2002; Kim et al. 2002), cell death (Klement 1964; Aballay and Ausubel 2001) and transcription factors that trigger immunity (in case of plants, a protein NPR1 that shares many features of NFKB – (Cao et al. 1997; Ryals et al. 1997), and in the case of *C. elegans* a transcription factor DAF-16 that is downstream of, and is negatively regulated by the insulin-like receptor DAF2 (Garsin et al. 2003) that also controls longevity) have been identified. It is likely that both these models will highlight different components and conceptually similar modules that are conserved across different kingdom, including mammals. It is not clear if these conceptually similar arms in different kingdoms arose independently or through convergent evolution (Ausubel 2005).

## 2. RNA silencing and its role in innate immunity to viral pathogens

More recently, over the last decade, it has been recognized in several host organisms that the components of RNA silencing machinery is a major immune mechanism that restrict viral pathogens (Baulcombe 2004; Ding and Voinnet 2007). This often happens through the recognition of dsRNA intermediates through RNA interference (RNAi) to dice up these dsRNA molecules (typically by dicer, a ribonuclease of RNase III family) into small RNAs (typically 21-25 nt long) one strand of which base pairs with target RNA with perfect complementarity and cleaves the latter (typically by a member of Argonaute family), and the cycle further amplified and continued by the small RNAs produced initially. Homology dependent silencing of RNA has been recognized in plants for a long time (Neuhuber et al. 1994; Que and Jorgensen 1998; Lindbo and Dougherty 2005). The components of the pathway were identified rapidly more recently after recognition in other systems that small dsRNA can effectively shut down host transcript function in *C. elegans* and a variety of eukaryotic cells and tissues (Fire et al. 1998; Tuschl et al. 1999; Hammond et al. 2000; Elbashir et al. 2001). Some prominent components of this recognition and dicing into 21-25 nt small RNAs are dicer (a ribonuclease of RNase III family), Argonauts and other adaptor proteins (that form what is commonly termed the RISC - RNA induced silencing complex). Many components of the RNAi machinery are also involved in the recently recognized microRNA (miRNA) pathway - prototypical members *lin-4* and *lin-14* originally recognized in *C. elegans* – (Lee et al. 1993; Wightman et al. 1993). Many components of the RNAi including dicer and members of argonaute family also participate in the processing of miRNAs. miRNAs are derived from small non-coding or sometime intron derived RNAs with secondary structure that forms a hairpin loop that are cleaved into 21-25 nt sized small dsRNAs. Unlike siRNAs, most miRNAs are not completely complementary to their target RNA (though some rules have been deciphered) and the prominent mode of their action is through translational inhibition rather than by cleavage of the target transcript (Bartel 2004; Lewis et al. 2005; Zamore and Haley 2005; Grimson et al. 2007; Neilson et al. 2007; Ruby et al. 2007; Filipowicz et al. 2008). In the case of plants

most miRNAs have been shown to act via target RNA cleavage (Vaucheret 2006) though the prevalence of translational inhibition mechanism has recently been reported (Brodersen et al. 2008). miRNAs have recently been recognized to play a very extensive role in host development and homeostasis. An additional mechanism that is increasingly being recognized is the engagement of host microRNA (miRNA) to restrict viruses and conversely the presence of miRNAs in viral genome that are complementary to host encoded genes and in some instances shown to modulate the outcome of the interaction (Lecellier et al. 2005; Lodish et al. 2008). Thus many aspects of RNA silencing machinery mediated alteration could have an indirect role in modulating many aspects of host physiology including the PRR recognition mediated arm of immunity. Though the above examples and other articles highlight the role of host and viral encoded miRNAs and their role in cross-regulating each other in other kingdoms; the role of RNAi in mammalian immunity is not unequivocal – see for example (Cullen 2006). Even in the case of the dicer-1<sup>-/-</sup> mouse being hypersusceptible to VSV, it has been shown that it was probably due to in the effect on miR24 and miR93 synthesis in the mutant mice (Otsuka et al. 2007). A third aspect that involves components of RNA silencing machinery is their involvement in epigenetic silencing [e.g., (Matzke and Birchler 2005; Irvine et al. 2006; Dunoyer et al. 2007)]. The role for epigenetic state of genes modulated during immune responses by modifying access to transcription factors can easily be inferred, but there are also recent reports of immune response modulating the epigenetic state of some target genes (immune effectors) – [e.g., (Cheng et al. 2008; Dong et al. 2008)] - and the modulation of epigenetic state of such target immune related genes by pathogen effector (Arbibe and Sansonetti 2007; Arbibe 2008).

### **Pathogen effectors mediating alteration of host immunity**

Much of the above background deals with the responses primarily from the perspective of the host and sets the tone for the some kinds of interactions among known immune pathways one would predict to occur. The other side of the effects that could have major impact on the outcome of these interactions arise from the perspective of the pathogen. It is a well accepted fact that pathogen and host undergo constant coevolution in order to benefit their survival. Thus many pathogens have evolved a suite of effectors that affect many aspect of host immunity and homeostasis [e.g., (Bhavsar et al. 2007; Roy and Mocarski 2007) for some recent reviews on this topic]. In addition it has been recognized that viral proteins can modulate (downregulate) the host immunity mediated by the arm involving the RNAi machinery. A number of plant viral proteins have been identified as suppressors of RNA silencing often through binding of siRNA [e.g., (Kasschau and Carrington 1998; Voinnet et al. 1999; Silhavy et al. 2002; Chapman et al. 2004; Lakatos et al. 2006)]. In addition, Tat protein of HIV, NS1 of influenza, E3L of vaccinia that are also antagonist of the interferon response are suppressors of RNA silencing in some cases (Bucher et al. 2003; Li et al. 2004; Bennasser et al. 2005; Lu et al. 2005; Haasnoot et al. 2007), sometimes in a different kingdom. This mechanism could again be an ancient one conserved across different kingdoms or it could have arisen independently due to the propensity of conserved components utilized to recruit related components to carry out functions that result in a similar outcome.

### **Some examples of cross-regulation between these two arms of immunity**

Surprisingly, there are not many reports directly addressing cross-regulation or interactions between these two arms of immunity. Although it has been recognized that the IFN response (typically a hallmark of PRR mediated responses in mammals) seem to preclude RNAi (targeted RNA destruction through siRNAs), it does not answer the question if this response can be circumvented and how. As highlighted above many viral components that suppress interferon response that normally precludes RNAi are also suppressors of RNAi. In the case of Drosophila C Virus (DCV) it has been shown that components involved in RNAi machinery [e.g., Dicer-2 (Galiana-Arnoux et al. 2006) and the major nuclease of RISC, Ago-2 (van Rij et al. 2006)] as well as the JAK-STAT pathway and induction of several anti-microbial peptides (Dostert et al. 2005; Galiana-Arnoux et al. 2006; Tsai et al. 2008) contribute to host defense. The mammalian TRBP (transactivating RNA response-TAR-RNA binding protein) that has been shown to interfere with the induction of the PKR protein kinase mediated interferon response has also been shown to essential for RNA silencing responses via its ability to bind to dicer, thus reinforcing the concept that the cross-regulation that need to be better understood (Haase et al. 2005). In one case, an miRNA upregulated by TLR4 mediated response to LPS has been proposed to be involved in attenuation of TLR4 signaling (Taganov et al. 2007). There are also examples of miRNAs modulating immune cell lineage and development (Lodish et al. 2008). But miRNAs have remarkably wide range of roles in the host homeostasis and development.

[In the case of plants an miRNA induced by a resistance inducing pathogen associated molecular pattern (PAMP) – the peptide flagellin – has been shown to suppress auxin signaling, and stimulating this arm of auxin signaling may promote susceptibility (Navarro et al. 2006). Another recent study reported that preinfection of Arabidopsis with TuMV (Turnip Mosaic virus) makes the plant more susceptible to commonly not infectious type III secretion mutant of the pathogen *Pseudomonas syringae* pv. *tomato*, as well as a non-host bacteria *P. syringae* pv. *phaseolicola* (Navarro et al. 2008). *In this study at these time points no gross host alterations were obvious after viral infection, but it is well established that TuMV causes severe leaf curling and floral development defects at later stages of infection.* Thus a need for a more appropriate system for studying such cross regulation is warranted. Though there is no direct evidence of RNAi acting as a host strategy to restrict viruses in mammals, there are a number of cases where the virus encodes miRNA that can target host genes and examples of viral genome harboring targets for host encoded miRNA. [as exemplified by herpes viruses and HIV –some aspects covered in recent reviews (Cullen 2009a; Cullen 2009b)]. These and other examples highlighted above, clearly indicate that RNA silencing most certainly plays a role in viral pathogen restriction, and any cross regulatory effect from other immune arm or by another pathogen would have an effect on the outcome dependent on RNA silencing. Two other aspects that would support this contention are (i) the well accepted concept of host and microbes continuously coevolving strategies to counter each other, and (ii) shared components between different aspects of RNA silencing including RNAi.]

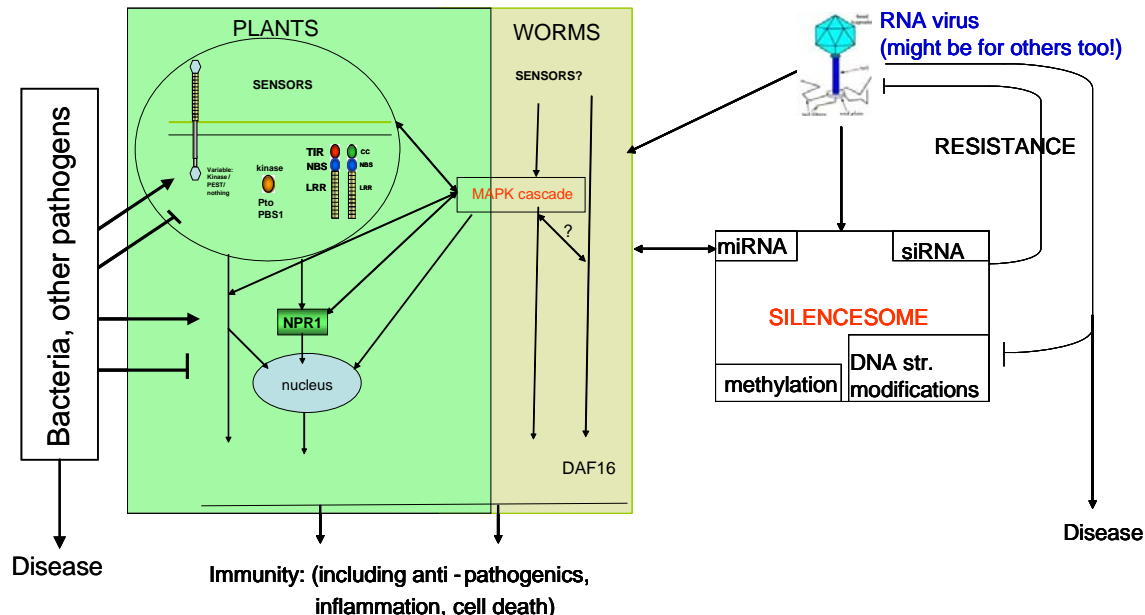
### **Some key questions on the cross-regulation between these two arms of immunity**

The above background highlight a poorly understood field of study that focus on cross-regulation of the two divergent arms of immunity proposed in this study. It is also apparent that many aspects of host immunity could be regulated using such knowledge to better manage increased host damage resulting from multi-pathogen infections. Some important questions that remain to be addressed better include (1) *What are the points where these two arms of immunity intersect to modulate each other directly and through adaptive host state changes?* (2) *Can the PRR mediated responses that preclude RNAi (siRNA mediated degradation of target RNAs) be overcome?* (3) *Since many components of RNA silencing are shared between RNAi, processing and regulation of miRNA mediated silencing, and other processes involving these components how does the current known suppression by viral components alter some but not all pathways?* (4) *Are there RNA or DNA receptors of PRR class for viruses in plants, Drosophila and worms?* (5) *What is the mechanism behind the lack of vertical transmission of viruses in these hosts?*

## D. RESEARCH DESIGN AND METHODS

The broad goal of the study is to set-up systems using model hosts to study cross-regulation between host responses during simultaneous and sequential infection by viral (RNA viruses) and bacterial pathogens. The choices involve host-pathogen combinations where the dominant restriction mechanisms to the viral pathogen involves RNAi and aspects that involve machinery of RNA silencing, and the dominant restriction mechanism of the bacterial pathogen involves the synthesis of known and other yet to be elucidated anti-microbial effectors and host strategies often involving recognition of broadly conserved or specific bacterial components by LRR containing proteins, kinase cascades and some well studied transcription factors. [One unique feature of this model system proposed here is the fact that the host-virus combinations that comprise the bulk of the proposal do not cause any obvious visible phenotype. The broad goals of the proposal are outlined in Fig. 1 below.]

[This is an exploratory and developmental grant and the novel responses observed will be used to develop robust assays and high throughput screens to be used in a future grant. Some preliminary data are included to highlight the fact that some individual experimental components have been established or based on previously well established systems, technical challenges to overcome and some novel outcomes possible besides the direct goals proposed. These data also highlight need for systematic evaluation of the robust range of conditions to study the cross-regulation between host responses to these two classes of pathogens to harness the complete potential of this system.]



**Fig. 1** Simplified schematic depicting the broad goals of this proposal

### AIM 1: STUDY THE EFFECT OF VIRAL INFECTION ON SUBSEQUENT INFECTION BY BACTERIAL PATHOGEN AND VICE-VERSA IN *ARABIDOPSIS* AND *NICOTIANA*.

#### Specific background on the system to be used:

*Arabidopsis* and plants in the genus *Nicotiana* have proven to be extremely beneficial in the study of viral and bacterial pathogens individually. TEV is a monopartite single stranded positive sense RNA virus and can tolerate insertions in its genome (Dolja et al. 1992), thus providing the opportunity to construct viruses with reporters and genes for selection of restrictive and permissive phenotypes (Whitham et al. 1999). This property has been extensively used in the case of TEV to dissect the roles of many aspects of viral infection and interaction with hosts. In the case of TEV it has been shown that their restriction to infected leaves in certain ecotypes of *Arabidopsis* is not dependent on host components involving salicylic acid (a non-classical hormone that plays a major role in many types of resistance in plants), as shown by lack of difference in phenotypes between wild type plants and mutants of components effecting signaling in the pathways mediating this

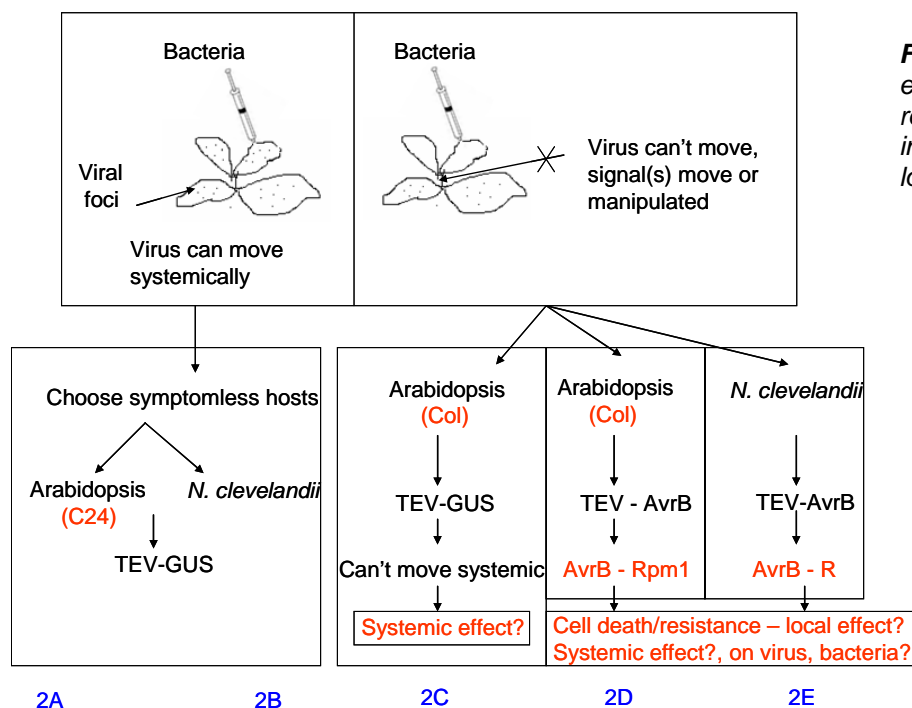
resistance (Mahajan et al. 1998). [An extremely attractive characteristic of TEV-Arabidopsis interaction is the lack of visible pathogenic or any detected developmental phenotypes, for example compared to TuMV that cause extreme developmental phenotypes in Arabidopsis.] RNAi mediated pathways (direct homology dependent silencing in infected cells and systemic signals that effect spread of silencing to other cells) operate to restrict the infection with viruses in both *Nicotiana* and *Arabidopsis* and several mutants in the components of RNA silencing are impaired in this restriction have been identified. TEV also encodes components that mediate suppression of this silencing [e.g., HCPro - (Kasschau and Carrington 1998)].

In the case of bacterial pathogens and these hosts, two possible outcomes and their alteration will be studied during the cross-regulation proposed. One response is resistance, activated by PRRs that have evolved to genetically recognize specific pathogen components (often termed avirulence (Avr) gene products), and characterized by the HR and SAR described earlier. In addition, there are PRRs that recognize general conserved components of bacteria that elicit a resistance response although much weaker and not necessarily involving macroscopic cell death like in the case of HR. When not resisted effectively, the bacterial pathogens (*Pseudomonas syringae* to be used in these studies) multiply, obtain nutrients, suppress host resistance and elicit host damage by interfering with host signaling and structural components and metabolism by a suite of effectors often delivered into the host cell and by the use of toxins. This multiplication of the pathogen also results in death of host cells albeit slowly (thus allowing the bacteria to obtain nutrients and spread beyond the site of infection).

### **AIM 1A: STUDY THE EFFECT OF VIRAL INFECTION ON SUBSEQUENT INFECTION BY BACTERIAL PATHOGEN**

#### **Experimental set-up for effect of existing viral infection to subsequent bacterial infection on local and systemic tissue**

**Fig. 2** below illustrates the various experimental designs to be used to test Aim 1A. [This depiction also shows the unique ability to dissect local and systemic effects (by powerful experimental design) exerted by one or both dominant innate immune responses operative in the host to restrict these pathogen classes.]



**Fig. 2** Schematic representation of the experimental design for testing cross-regulation of host responses during infection by bacterial and viral infection in local and systemic tissue

#### **The assay:**

The assay in broad terms involves infecting *Nicotiana* or *Arabidopsis* plants (the different host choices, their advantages and differences are outlined below) with TEV reporter constructs and subsequently challenging with a bacteria pathogen. The effect of viral infection on subsequent bacterial infection in local (virus infected)

and systemic uninfected parts of virus infected plants on bacterial infections (bacterial growth, and host damage – including programmed cell death as during HR type resistance and host damage – visible and reporter based assays) will be studied. Robust conditions and assays will be established for subsequent dissection of mechanisms using system-wide molecular changes and high-throughput screens for altered phenotypes.

Different pathovars (subclassification based on their pathogenicity based on host range) of *P. syringae* will be used as bacterial pathogens, e.g., *P. s. pv. tabaci* in the case of experiments with *Nicotiana*, and *P. s. pv. tomato* or *P.s. pv. maculicola* on *Arabidopsis* as host.

### **The different combination of host plants and viral combinations for restrictive and permissive viral infections for study of local and systemic effects on bacterial infections of viral infected plants**

The host plants to be used are *Nicotiana clevelandii*, *Nicotiana benthamiana*, and *Arabidopsis thaliana* ecotypes Columbia and C24. *Nicotiana tabacum* cv. *xanthii* will be used for routine propagation and purification of TEV and transgenic TEV constructs using well established protocols. The viruses will typically be infected using abrasion with carborundum manually or using the air-brush inoculation technique (Whitham et. al., 1999).

### **The read-outs for bacterial and viral infection and pathogenesis/resistance and development of additional tools:**

Bacteria will be infiltrated at different doses. Bacterial growth will be followed by measuring *change in colony forming units by standard CFU counting methods as well as by the activity of a luciferase reporter developed recently* (Fan et al. 2008). Host damage will be followed by visible symptoms and a variant read-out based on the activity of a constitutively expressed luciferase reporter (under a CaMV 35S promoter), – in 96 well plates in a luminometer. *The utility of this read-out to quantitatively follow host damage will be standardized as part of this proposal.* The viral infection (effectiveness and load) will typically be measured using reporters (by staining for GUS activity – the *E. coli uidA* gene) and counting the number of foci at a given time point (typically after 3 days in *Arabidopsis* and as early as one day in *Nicotiana*), and rate of expansion of foci as needed. Subsequently GFP reporter viruses and luciferase reporters will be developed for non-destructive and easier quantitation.

### **Use of mutants in currently known dominant innate immune mechanisms to improve robustness:**

The model hosts and the pathogens proposed to be used in for all the aims are based on well studied models where extensive knowledge is available on the dominant innate immune arms individually to a single pathogen. When possible the initial step in each experiment will be conducted using an appropriate mutant (or RNAi treatment in the case of *C. elegans*) attenuated or amplified in the response being studied, as appropriate in addition to the wild type host. Such approaches should be helpful in improving the amplitude and robust range of the phenotype.

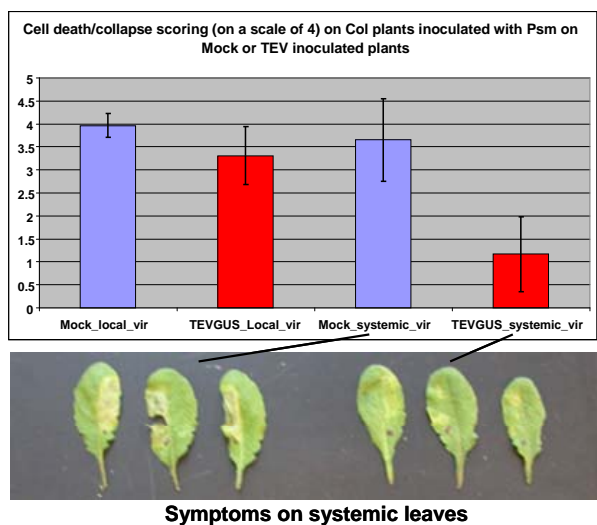
### **1A1) USE OF NICOTINA SPECIES TO STUDY EFFECT OF COINFECTION OF VIRAL AND BACTERIAL PATHOGENS IN THE SAME TISSUE**

*N. clevelandii* has mild to no TEV symptoms on upper uninoculated leaves other than in newly emerging leaves at very late stages of infection, though there is extensive spread of the virus as assayed by GUS activity after infection with TEV-GUS 6-8 days after infection – data not shown). This interaction will be used to assay local effects of simultaneous infections by viral and bacterial pathogens (in this case *P. syringae* pv. *tabaci*, that is virulent on these *Nicotiana* species). The upper leaves of these TEV-GUS inoculated plants will be infiltrated with *P. s. pv. tabaci* at different concentrations (equivalent to block 2B in Fig. 2). If found to be appropriate *N. benthamiana* will also be used. The advantages of using *N. benthamiana* would be its stature i.e., the distance between different leaves due to elongated stem as opposed to *N. clevelandii*. An additional advantage of using *N. benthamiana* and *N. clevelandii* is highlighted in the third approach (**AIM 1A3**) mentioned below. *Nicotiana* species are included in the aims proposed in this study, because it is possible that the effect of the virus in a genus that is a natural host might be different than in *Arabidopsis*.

## 1A2) USE OF NATURAL VARIATION IN LONG DISTANCE MOVEMENT OF TEV IN *ARABIDOPSIS* CULTIVARS

During the initial set up of *Arabidopsis* as a system for infection and study of TEV infection (Mahajan et al. 1998), it was observed that all the cultivars tested formed foci not visible to naked eye (visible by staining for GUS activity when infected with TEV GUS) on the infected leaves, but there were differences in the long distance movement (movement to other part of plants other than infected leaves) between different ecotypes. For example, the commonly used ecotype Columbia (Col) belonged to the restricted category (*equivalent to block 2C in Fig. 2*), whereas the ecotype C24 had GUS activity indicative of viral movement in flower tissue in emerging bolts after about 10 days (*equivalent to block 2A in Fig. 2*). Thus the use of the ecotype Col would provide an opportunity to study the effect of propagating long distance signals resulting from viral infection in systemic tissues, since it is expected that the signals would still move (*equivalent to block 2C in Fig. 2*). In these cases local and systemic tissue would be assayed for differences in bacterial growth (in this case *P. syringae* pv. *tomato* DC3000, and *P. s. pv. maculicola* 4326) and host damage due to bacterial infection, at different time points after infection with virus. A *35S::luc* expressing transgenic in Col background is available for assessing host damage as change in luciferase activity as mentioned above. Both the Col and C24 ecotypes can be used to study coinfection of TEV and *P. syringae* in the same tissue (*equivalent to block 2A in Fig. 2*). The air brush inoculation technique could be used to achieve higher number of viral infection foci.

Preliminary results indicate a potentially promising effect on systemic tissue compared to local tissue in terms of host damage to both resistant (a host-pathogen combination capable of causing HR and associated immunity, more details and experiments below) and virulent bacterial pathogens (**Fig. 3**).



**Fig.3** Symptom of bacterial infection in virus infected and systemic leaves (virus does not spread in the Col ecotype used).

Bacteria *P. s. maculicola* (Psm) was inoculated at ABS 600 of 0.02 on virus inoculated (hand inoculated) or systemic leaves and the symptoms recorded as visual symptoms (on a scale of 4, 4 being the highest damage) at 40 h post bacterial infection (pbi). The leaf pictures shown were photographed about 45 h pbi. Shown are results with virulent Psm. The avirulent combination (Psm expressing *AvrB* had similar difference between local and systemic leaves). The differences were obvious only for several hours after which the symptoms between virus infected and systemic leaves looked similar.

In both these cases, the host damage as visualized by cell death was delayed in systemic tissues. The difference in response shown there seem to be a time difference of few hours. While this result is very promising and unique, it also reveals the need for a systematic analysis of timing and dosage of inoculation of the bacteria, and possibly some experimentation with environmental conditions. It also raises an interesting question that while the two kinds of cell death (host damage) are supposed to be different, the difference observed here seem to be identical, thus raising many interesting conceptual questions on the understanding of the damage or other possible interference to the HR cell death response. [Existence of such common signaling themes between these two types of cell death is also proposed as a plausible explanation for the different effects of the growth regulator auxin on an immunity associated cell death program as well as the ability of exogenous auxin to enhance pathogenesis in compatible interactions (Gopalan 2008). In the context of signaling and changes in host status in systemic tissues by viruses, the currently known long distance signals implicate nucleic acids (Voinnet and Baulcombe 1997) and the involvement of components of miRNA and heterochromatic silencing pathways (Dunoyer et al. 2007). A potentially novel non-cell autonomous signal has also been proposed (Gopalan 2007). A recent report using plants as a model to examine similar effects proposed here observed that miRNA pathways are manipulated by pathogenic bacteria and that preinfection with the virus TuMV makes the plants susceptible to a type III secretion mutant bacteria as well as *E. coli* (Navarro et al. 2008). A major advantage of the system proposed here is the fact that the virus to be used in this study does not cause any phenotypic alterations indicative of gross host status alterations, as opposed to

TuMV that cause severe leaf curling and other floral developmental defects (though it was not apparent in the time point shown in that study.】

**1A3). EFFECT OF VIRAL INFECTION ON SUBSEQUENT INFECTION WITH AVIRULENT BACTERIA THAT IS TYPICALLY RESISTED BY THE HOST THROUGH A RAPID HR CELL DEATH RESPONSE AND ASSOCIATED IMMUNITY**

The above three aims will also be conducted with bacteria that are typically resisted by the host by the rapid programmed cell death (HR) that is associated with immunity. This typically occurs in two ways, one being the gene-for-gene interaction based system (as exemplified by AvrB-Rpm1, and AvrRpt2-RPS2 systems in *Arabidopsis*). Another way for this immune arm to be activated is the so called non-host response, where in a different pathovar of bacteria e.g., *P. s. tabaci* that is virulent on *Nicotiana* is resisted by HR in many other species of plants that are not host, including *Arabidopsis*. Thus these bacteria (either isogenically expressing an Avr protein that causes HR or a non-host HR) will be tested for their response after viral infection in all the combinations proposed above.

**AIM 1B. STUDY THE CONVERSE EFFECT OF THE ABOVE AIM #1A AND SYSTEM, VIZ., INITIAL BACTERIAL INFECTION FOLLOWED BY SUBSEQUENT INFECTION OF VIRAL PATHOGEN IN LOCAL AND SYSTEMIC TISSUE.**

In all the above cases studies addressing the effect of an existing viral infection on subsequent bacterial infection were proposed. Studying the converse should prove to be as interesting and informative towards the goal of understanding the cross-regulation between the host responses to two pathogen classes normally restricted by dominant divergent pathways in these systems. To this effect leaves of *Arabidopsis* or *Nicotiana* would be infiltrated with appropriate doses of the bacterial pathogen to elicit an appropriate response (viz., a virulent infection or an avirulent interaction that typically results in HR). The leaves would then be infected with the viral pathogen (TEV) or its transgenic derivatives mentioned above in systemic tissues. Since bacterial infection in both these modes cause host damage, for studies on determining the effect in local tissue, one half of the leaf would be infiltrated with the bacteria and the other half will be infected with the virus. For experiments involving assay of long distance movement of virus the ecotype C24 will be used in the case of *Arabidopsis* and bacteria (DC3000, or Psm 4326) with and without expression of AvrRpt2 (in a plasmid).

*Depending on the results observed with quantitative differences in the presence of viral foci or spread, virus derived small RNA (vsRNA) will be quantitated.*

**1B2) USE OF TRANSGENIC TEV EXPRESSING AN AVIRULENCE PROTEIN AND ASSESSING ITS EFFECT ON SUBSEQUENT BACTERIAL INFECTION.**

【As introduced earlier, infection with bacterial pathogens used in this study in case of a susceptible interaction or the interaction that result in defense both result in a forms of cell death. This is a serious limitation to study effect on viral infection after prior infection with bacterial pathogen.】 Thus, the third approach would involve the use of transgenic TEV expressing the avirulence protein AvrB (that I had shown works inside of the plant cell to elicit the HR in the presence of corresponding LRR protein that recognize it (RPM1, cloned by (Grant et al. 1995), in addition to having additional RPM1 independent activity in transgenic plants that had not been previously recognized, Gopalan et. al., 1996). 【This approach reflects recapitulating the strong immune response based on genetically well defined and widely studied host and bacterial gene-for-gene interactions.】 Pilot experiments conducted by me on the Col ecotype of *Arabidopsis* several years ago revealed novel responses that are not evident during the other currently used experimental approaches, though there was no HR like foci in the infected leaves. This potentially interesting result needs to be followed up with additional experiments. In addition, this also gives an opportunity to study the systemic (long distance) effects of activation of two arms of innate immunity both expected to transmit different long distance signals (i.e., viral infection transmitting signals relating to RNA silencing, and the HR type response that should also transmit the signals leading to SAR). Interestingly, in a recent report the replication of TuMV (a potyvirus related to TEV but that causes severe developmental defects in *Arabidopsis*) was prevented when it transgenically expressed ATR3 (an effector from oomycete pathogen *Hyaloperenospora parasitica* that causes HR cell death in that ecotype of *Arabidopsis* - Rentel et. al., 2008).



The ecotype Col recognizes AvrB by virtue of the presence of a functional Rpm1 gene that results in genetic recognition and HR cell death and associated immunity (*equivalent to block 2D in Fig. 2*). Similar experiments will be initiated with the avirulence gene AvrRpt2 (Whalen et al. 1991) that is recognized by a different LRR containing R gene RPS2. The advantage of this being that the C24 ecotype (though permits long distance movement of TEV) does not contain a functional Rpm1 gene, but has a functional RPS2 based recognition system for AvrRpt2. Similar experiments would be performed with *Nicotiana* (*equivalent to block 2E in Fig. 2*). **The choice of *Nicotiana clevelandii* and *Nicotiana benthamiana* above is based on the fact that both of them naturally have the capability to recognize and cause HR in response to bacteria harboring AvrB.**

**Expected results and contingency plans:** The above design comprehensively addresses different aspects of the cross-regulatory effect of viral pathogens primarily restricted by RNA silencing in the hosts, and bacterial pathogens restricted by a basal defense and a strong cell death associated immunity against bacterial pathogens in the model plants *Arabidopsis* and *Nicotiana*. [The experiment proposed with TEV-AvrB partially address the inability to test the effect of viral infection subsequent to bacterial infection in local tissue, in addition to addressing one of the initial hypothesis questions “the effect of preexisting defense typifying the PRR based defense on the RNAi/RNA silencing based defense and pathogenesis (and effect on pathogenesis), and how this observed effect can be overcome?”] *An alternate approach to quantitate the effect on virus infection and spread in the presence of a preactivated defense arm that is typically operative to restrict bacterial pathogen is to use the chemical inducer SA or its functional analog isonicotinic acid (INA).* Mutants in dominant innate immune pathways would prove invaluable to infer the contribution of previously known components during these interactions. Besides the dominant host immune mechanisms to these pathogens, these pathogen classes have also evolved a number of ways to hijack and circumvent host pathways to their advantage (either through suppression or subversion of immune mechanisms or through modulation of status, e.g., metabolic status), some aspects of this can be confirmed using pathogen mutants available. In some cases, mechanisms involving non-cell autonomous short and long distance signals will be discerned using single cell systems. In one or a few interesting interactions are identified, organismal scale studies and high-throughput screens (see **AIM 3**) would be set up. A technical contribution would be a comprehensive way to measure many aspect of pathogen load and host damage using simpler modern tools, standardized during the course of this project.

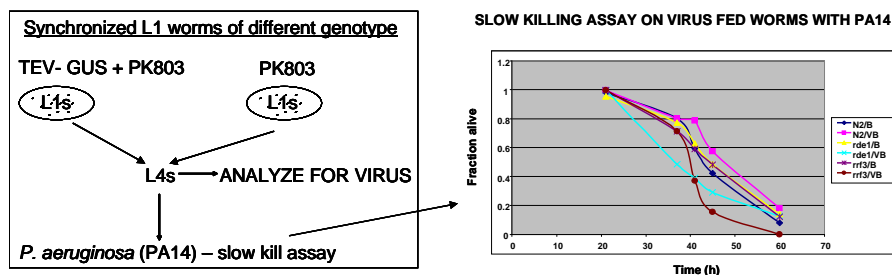
#### **AIM 2A: DEVELOP A SIMPLE FEEDING MODEL FOR AN RNA VIRUS THAT READILY INFECTS *C. elegans* AND ENGAGES THE PATHWAY INVOLVING COMPONENTS OF RNA SILENCING MACHINERY**

In the last decade great progress has been made with *C. elegans* as a pathosystem for understanding a number of aspects of pathogen infection applicable to different kingdom. Three viruses have been shown to infect *C. elegans*, or cells derived from them. They include PEG mediated infection of vaccinia virus – an enveloped DNA virus (Liu et al. 2006), infection of *C. elegans* cells in culture by the negative strand RNA virus, vesicular stomatitis virus – vsv (Schott et al. 2005; Wilkins et al. 2005), and flock house virus (FHV, a positive strand bipartite RNA virus) when transgenically expressed in the worm (Lu et al. 2005). While the vaccinia virus study implicated the involvement of PCD pathways, both the other studies showed that impairment of RNAi machinery components increases the viral titer. In addition as discussed earlier number of viral suppressors have been shown to work in different kingdoms (including *C. elegans*) - e.g., FHV B2 protein (Li et al. 2002). Several aspects of RNA silencing, powerful genetic tools and ability to do rapid high-throughput genome-wide RNAi screen, and a strong community with shared resources (also apply to studies with *Arabidopsis*) make *C. elegans* an attractive system for this work.

Though these approaches demonstrate that *C. elegans* can be infected with a variety of viruses, none of these approaches are natural infection of the intact organism using virions. This probably reflects lack of an uptake mechanism for these viruses in *C. elegans* or that the epithelial cells lining the intestine and the cuticle function as a barrier or mount an immune response that restricts them. One of the goals of this proposal is to try to develop a feeding model for TEV virions. If successful, this would form one uniform component affecting the hosts in two different kingdoms, presenting the possibility to derive conserved and organism specific features.

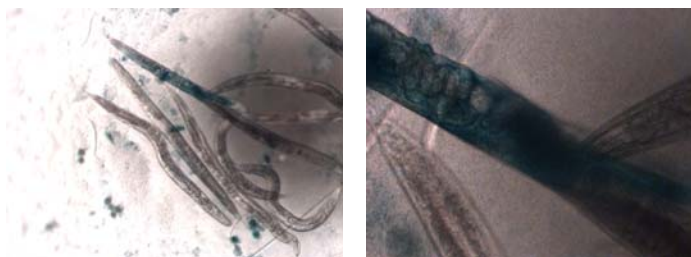
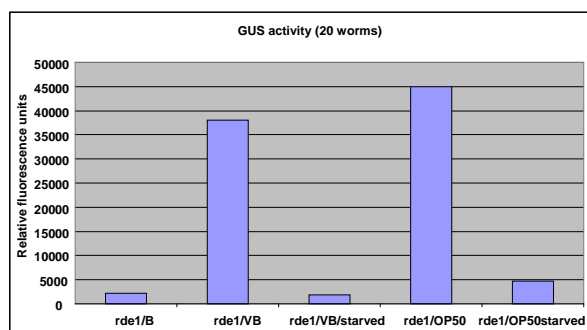
Preliminary experiments were carried out by growing synchronized L1 worms using purified virions of TEV-GUS either mixed with the *uidA* *E.coli* - PK803 (Jefferson et al. 1987) food or incubating the worms for a few hours in purified virions as sole food and then adding *E. coli* food. (**Fig. 4**). The TEV GUS fed worms show

strong GUS activity (**Fig. 5a**) that is abolished by starving the worms on plates without food for 1h (indicating that bulk of the activity is probably in the intestine that is excreted during the starvation step).



**FIG. 4.** Schematic of the assay for multi-pathogen infection using *C. elegans* model. PA14 is used as a representative pathogen to show the initial plan for these experiment.

Representative GUS histochemical staining in **Fig. 5b** and **5c** shows sporadic GUS activity accumulating all over the worm, and more often in the intestinal lumen at earlier time points – data not shown - that shows up as GUS stained contents spilled from the gut. The occasional GUS reporter staining of whole worms do indicate that the virus likely infects and spread in this heterologous host and that establishment of successful infection and site of infection might be a key issue. [A key technical problem revealed by this experiment is the fact that the virus packs GUS and replicative intermediates in its virions under the condition of propagation and purification (data not shown).] Technical approaches to overcome these issues include sequencing the starting plasmid (to rule out inadvertent mutation affecting the stringency of viral packaging) or obtaining another copy of TEV encoding plasmid or TEV from another source, and propagating the virus in different growth conditions.



**Fig. 5 GUS activity in *C. elegans* fed with TEV-GUS virions.**

The left panel (**Fig. 5a**) shows fluorometric GUS activity (using 4-MUG) of 20 worms per condition, read using a 96 well plate reader. B- *E. coli* PK803; VB – is TEV-GUS virus particles mixed with PK803; OP50 is *uidA*<sup>+</sup> *E. coli*. Worms (in this case *rde-1* mutant worms in N2 background) were plated on food combinations listed above in L1 stage and late L4 worms were picked for assay. Panel in the middle (**Fig. 5b**) showing sporadic histochemical GUS staining (with X-gluc as substrate) and staining of spilled contents. Panel on the right (**Fig. 5c**) shown GUS staining all over a worm –which was observed in occasional worms fed with TEV-GUS.

Similar approaches will be attempted with a variety of viruses [that could be used in BL2 or lesser containment] that are widely used in experimental studies (especially the ones that could tolerate foreign proteins that could be used as reporter) from other kingdoms. In the case of plants a major route for entry of TEV virions or naked transcripts is through wounded cells. Many viruses use different approaches to enter their host cells e.g., specific attachment proteins to receptors on target cells, or using receptors with broader specificity like that for phosphatidylserine shown to mediate subsequent uptake through engulfment as in endocytosis of vaccinia virus (Mercer and Helenius 2008) and also thought to be important for uptake of HIV (Fair and Grinstein 2008). In some cases such uptake is also shown to be dependent on the pH as is exemplified by the case for murine hepatitis virus (Eifart et al. 2007). While such physiological conditions can be explored in parallel, more productive approaches would include (i) injecting *in vitro* transcribed viral RNA (that is infective in plants) into the worm with a co-injection marker and look for accumulation of strong GUS activity, (ii) infecting a non-lethal allele of *bli-1* and other *bli* mutants that is known to form blistered cuticles and other cuticular defects, thus providing a natural injury thorough which the virus might have a chance to enter the cells and then replicate. But these approaches would only provide further support for using TEV and any of the other successful viruses in the *C. elegans* model, and not likely to provide a robust model to study the main goals of this proposal. The other two models of infection mentioned above (vsv infection of cells of *C. elegans* or transgenic FHV

expressed under a heat shock promoter) would not be useful for the goals of this proposal either. The former due to the fact that primary the goal here is to use organismal models, and the latter due to the fact that heat shock might cause additional changes in the system status and there is no visual marker.

**Expected results and contingency plan:** This is one of the most rewarding and technically challenging goal, and the most risky. Appropriate condition to propagate the virus to without packing replicative intermediates and translation products, and having a reporter system in the virus or in the worm (like suppression of a GFP marker transgene in the worm) would greatly aid picking infected worms to answer the proposed questions. In addition the feeding mode of infection would also aid to answer some of the other key questions highlighted in the Background and Significance section, through other genetic screens. Even a demonstration that TEV can replicate in the worm would be a significant advance. **[In addition the successful establishment of a TEV infection system in *C. elegans* will prove to be immensely powerful to glean common and different aspects of a single viral pathogen on two very different hosts. An advantage likely unique to this experimental design is the potential to use the same virus on two evolutionary divergent hosts and applying it to answer the same key fundamental questions, that would otherwise prove extremely difficult to design. This might also address questions of cross kingdom adaptations which happen more often with viral pathogens. The contingency plan in case of unusual difficulty in accomplishing this aim is to use FHV that has been shown to replicate in *C. elegans*.]**

**AIM 2B: EXPLORE THE EXISTENCE OF NATURAL VIRAL PATHOGENS OF *C. elegans* BY THE USE OF EXTREMELY HIGH-THROUGHPUT SHORT READ SEQUENCING OF SMALL RNAS**

As mentioned above, there no known natural viral pathogens that is known to infect *C. elegans*. This primarily is due to the fact that there was no easy way until recently to look for viral pathogens from *C. elegans* or other related nematodes isolated from nature. The recent advent of several deep-sequencing technologies and availability of genomes of several organisms have made this an achievable goal. In the case of Drosophila C virus (DCV) requirement of JAK-STAT pathways, induction of the synthesis of anti-microbial peptides, as well as the restriction RNAi mediated mechanisms have been demonstrated in Drosophila. Some host and viral components that inhibit the interferon response in mammals are also shown to inhibit RNAi mediated response, this in addition to cross-kingdom effects of viral RNAi suppressors would indicate there should be continuing evolution of host and viral strategies to outwit each other and partial suppression of one or both arms of immunity. In addition, extensive use of viruses in plants to study pathogenesis and immunity, and the study in other model systems (e.g. *Drosophila*, *C. elegans*) have not yet revealed the presence of a PRR that would act as nucleic acid sensor of viruses. These aspects justify the exploration of viral pathogens in nature that infect *C. elegans* and related nematodes. This would be explored by isolating total DNA, RNA and small RNA species from *C. elegans* and other nematodes isolated from nature (typically in compost pits and rotting fruits). With deep sequencing of small RNAs and comparing to genome databases and mapping to the source genome (simple PCR based extensions using potential sequences that are very deviant from sequenced worm genome) and from cDNAs from the longer RNA population one should be able to identify viruses and assemble them or look for the source in the stored subpopulation of natural isolates. Dr. Marie-Anne Felix (Institut Jacques Monod, France) who focuses on ecological and evolution aspects of nematodes in the environment and who has collected samples from many different locales for that purpose, has kindly agreed to send freshly collected samples of *C. elegans* and *C. briggsae* for this purpose (communication enclosed in Letters of Support).

**Expected results and contingency plan:** The advantage of finding such a natural isolate would be to identify the contribution of RNA silencing based and other arms of immunity operative against the same virus. Evaluation of the current literature suggests that the chances of multiple arms of immunity to viruses in *C. elegans* would be higher for a natural pathogen. **[Alternate possible outcomes include not being able to identify a genuine natural viral pathogen of *C. elegans*, or a more benign adaptation. The latter case will also be interesting due to its potential to aid in study of adaptations leading to persistent latent forms of infection, combining the advantages of the simple organismal model systems.]** Different isolates of *C. elegans* can be explored for their response to explore natural variation in the response and recognition receptors. The only contingency plan for this goal is to sequence deeper as well to include multiple cloning strategies and if necessary, an amplification step. **[This aim is independent of other aims, so can be done in parallel and**

positive results can be incorporated into in a subsequent work and to initiate new directions to address other outstanding questions highlighted earlier, e.g., immune and entry receptors and vertical transmission.]

### **AIM 2C: DEVELOP TRANSGENIC *C. ELEGANS* EXPRESSING TRANSCRIPTS OF VIRUSES UNDER DIFFERENT MODES OF REGULATION WITH APPROPRIATE REPORTERS TO MONITOR VIRAL INFECTION AND ENGAGEMENT OF RNA SILENCING**

Ideally, a simple feeding model with a virus (with an easy to score reporter that would aid picking infected L4s) that engages the host RNA silencing mechanism as proposed in Aim 2 will be available. In parallel an alternative system using TEV (failing which FHV) will be set up. This would involve making transgenic viruses with GFP reporters that are expressed under a tetracycline inducible promoter (or other newly identified inducible promoter shown to work well in *C. elegans*). The use of GFP reporter also provides another advantage in testing the engagement of RNA silencing for example abolishment of GFP reporter in worms expressing a GFP reporter in all cells (like *sur-5::gfp*). [In the case of TEV and/OR another virus chosen, all vectors will be constructed in Gateway compatible manner for two different purposes (i) for introduction of different reporters, and (ii) for exchange of different promoters (e.g., to achieve tissue/stage specific expression) . This would be an invaluable tool to address many key questions raised in the previous sections.] The construction of transgenic worm is a well established procedure, which involves microinjection of the appropriate plasmid and a coinjection marker, that can further be integrated into the genome using ionizing radiation – typically gamma rays, or coinjecting single stranded oligos (Mello et al. 1991; Mello and Fire 1995). Depending on the experiment, initial results and worm background, L4 worms with expression of the virus by observing GFP or silencing of a transgenic GFP will be picked for assays on to assay plates with *Pseudomonas aeruginosa* (PA14) or *Staphylococcus aureus* and the survival followed [as outlined in AIM 2D].

### **AIM 2D: EVALUATION OF THE EFFECT OF VIRAL INFECTION ON SUBSEQUENT INFECTION WITH BACTERIAL PATHOGENS IN *C. elegans***

*C. elegans* has been used to successfully model host-pathogen interactions using a number of pathogens of importance to mammalian health, and some natural pathogens. Some examples include the bacterial pathogens *P. aeruginosa* (Tan et al. 1999), *Salmonella typhimurium* (Aballay et al. 2000; Labrousse et al. 2000), *Yersinia pestis* (Darby et al. 2002), *Enterococcus faecalis*, *Staphylococcus aureus* (Garsin et al. 2001; Sifri et al. 2003), *Microbacterium nematophilum* (Hodgkin et al. 2000). In many cases same virulence factors, mechanisms and regulators of the pathogen that are important for infection in *C. elegans* have been shown to be important for infection in other kingdom e.g., (Tan et al. 1999; Rahme et al. 2000). On the other hand, analysis on the host side has uncovered components that are conceptually similar in other kingdoms, including mammals, e.g., the p38 MAP kinase pathways, and other pathways where equivalent processes affecting immunity to pathogens are not found in the other kingdom e.g., the insulin signaling pathway that involves the FOXO type transcription factor (DAF16) - that is also involved in longevity. More components and their interactions are being identified by a variety of approaches. The methods for infecting worms with a variety of bacterial and fungal pathogens are well standardized and published as a collection recently (Powell and Ausubel 2008). In this aim it is proposed to evaluate the effect of an existing viral infection on subsequent bacterial infection. Typically the bacterial infection involves growing wild type or mutant synchronized worms from an egg prep to L4 on *E. coli* and transferring 20 L4 hermaphrodite worms to appropriate plates containing *P. aeruginosa* (PA14) or *Staphylococcus aureus* (with three or more replicate assays). The difference in effect of prior exposure to viral pathogen compared to normal controls will be assayed. The two bacterial pathogens initially assayed would be *Pseudomonas aeruginosa* (strain PA14) and *Staphylococcus aureus*, gram-negative and gram-positive opportunistic human pathogens, respectively.

**Expected outcome and contingency plan:** As mentioned before, an ideal scenario would be the use of a simple feeding model of viral infection using virions. In the event of failure to achieve that goal since viruses that are known to replicate in worm cells are known this aim is fail proof. The expected mechanistic outcomes are varied, including identification of miRNAs involved that would now easily become apparent due to titration of some components by viral infection (though current results have shown the involvement the homology dependent RNAi arm only, additional insights are expected during the course of this study). In addition a comprehensive RNAi based screen to identify components affecting RNA silencing in *C. elegans* is being undertaken by Gary Ruvkun's lab. The identified components expected to be published should form a good

sub-library to screen for an appropriate cross-regulation phenotype, either through consented use or through a collaboration. Mutants affecting bacterial infection published and being identified would also be used to address their role in the observed difference in phenotype. *Because the current bacterial assays are done starting with L4 stages of the worm, the effect of preexisting bacterial infection on subsequent viral infection would likely be difficult to study.*

### **AIM 3: INITIATE EXPERIMENTS TO DISSECT MECHANISMS AND IDENTIFY ROBUST MARKERS OF INTERACTION BETWEEN THESE TWO DIVERGENT ARMS OF IMMUNITY USING ORGANISMAL SCALE STUDIES AND USING GENE EXPRESSION AND DEEP SEQUENCING TECHNOLOGIES**

[The primary aim of the research direction proposed is to establish a robust system to study cross regulation of host responses to these two classes of pathogens during a multi-pathogen infection and to use it to understand the molecular components and mechanisms effecting them.] Some aspects of the new phenotypes and assays would be directly amenable to high-throughput forward genetic screens. In the case of *C. elegans* a good assay would also be amenable to chemical screens and genome-wide RNAi screens (Kamath and Ahringer 2003; Moy et al. 2006). Thus robust markers that could be utilized for easily scorable reporters would be necessary to design high-throughput screens. For this purpose, organismal scale gene expression studies from the most interesting (and likely to be most informative) phenotype(s) will be generated using Affymetrix gene expression chips. In addition depending on the phenotype and the nature of expected directions, sequencing of small RNAs will be conducted on one of the systems that together with the gene expression data and databases like miRBase should prove complementary and aid forming new hypotheses. These two technologies are chosen to start with, among many organismal level studies of biomolecules due to (i) maturity of gene expression studies, and (ii) complementarity and direct relevance to the dominant arm of immunity to viral pathogens in these model hosts in the case of deep-sequencing of small RNAs.

**Expected results and contingency plans:** This goal is aimed at setting the stage for further analyses of observed interactions that modulate these multi-pathogen interactions, and add some mechanistic insight or hypotheses to the initial publication. It would be of great assistance at many levels for setting up assays and, forward and reverse genetic screens as part of a future grant. Due to the exploratory and developmental nature of the project the exact phenotype, and to which organism these organismal scale studies would be applied would be difficult to predict at this point. There are no contingency plans for this aim.

**SUMMARY AND FUTURE PLANS:** This proposal addresses an important area of cross-regulation of host response to pathogen classes when infected in combination, that are normally restricted by divergent arms of innate immunity. The model hosts and pathogens proposed to be used here provide unique advantages in terms of their mode of restriction, and availability of a number of tools to rapidly dissect molecular mechanisms of the novel responses observed, rapidly. Further the choice also circumvents certain layers of complexity that would be posed by using a mammalian model thus providing the ability to focus primarily on two broad arms of innate immunity. [Removal of this layer of complexity in addition to aiding the study of direct cross regulation proposed here does not diminish the importance in relevant multi-pathogen infections of this nature because initial stages in those instances are likely impacted in a major way by innate immunity and before adaptive immunity is engaged. Further multiple systems and the goal of using the same virus in multiple hosts would help answer unique questions on emergence of adaptations as well as answer simple but profound questions like are we to expect vastly different outcome and cross regulation in different pathogen combinations. If yes, how can we rationally design, evaluate designs and understand mammalian (and agricultural) diseases of practical significance effectively?] The expected contributions to the broad area of host-pathogen interactions include the ability to fine tune such responses during multi-pathogen infections, answering some key mechanistic and evolutionary questions pertaining to host responses to these two classes of pathogen. In addition, the results should have applications in RNAi based therapeutics that are normally precluded in the presence of strong innate immune responses constituting the other arm. This is an exploratory and developmental grant in that sense and the novel responses observed will be used to develop robust assays and high throughput screens to gain deeper mechanistic insight in a future grant.

**LETTERS OF SUPPORT** from **Profs. Frederick M. Ausubel** and **Gary Ruvkun** and email communication with **Dr. Marie-Anne Felix** are enclosed as attachments in **Research Plans** section of PHS398 package.

**Select Agents List**

Not Applicable

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March 9, 2009

Dr. Suresh Gopalan  
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Dear Suresh,

I am very pleased to fully support your revised R21 grant application entitled “Cross regulation of divergent host responses to viral and bacterial pathogens”, in which you are proposing to study the interaction between two divergent arms of the innate immune system: RNA silencing on the one hand, and the activation of immune effectors such as antimicrobial peptides, on the other. Your decision to carry out these studies in *C. elegans* and Arabidopsis makes sense since it takes full advantage of the work that we have carried out on these two model hosts at MGH over the years.

I will be able to provide you appropriate space to carry out the proposed project. This space has been allocated to my laboratory by the Department of Molecular Biology. I am also able to offer you unlimited access to the equipment and infrastructure that is available to all researchers in the Department of Molecular Biology. This is possible because of the manner in which almost all major equipment is shared between laboratories in our Department. In addition, my laboratory is fully equipped to carry out all aspects of modern molecular biology research and you will have unfettered access to this infrastructure as well. Finally, our Department is fully equipped to carry out plant biology research.

As you know, within our Department, my laboratory and Jen Sheen’s laboratory work with Arabidopsis, and my laboratory, Joshua Kaplan’s laboratory and Gary Ruvkun’s laboratory work with *C. elegans*. There is not only a great deal of know how among these laboratories, but also a variety of reagents that will be available to you including an extensive RNAi library for *C. elegans*. Finally, the work in my laboratory is complementary, but not overlapping to what you have proposed in your R21 grant. I will be pleased to make available to you the full resources of my laboratory and I am looking forward to working with you as appropriate on this exciting and important project.

Best wishes,

Fred Ausubel



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Gary Ruvkun, Ph.D.  
Professor of Genetics  
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March 10, 2009

Dr. Suresh Gopalan  
Department of Molecular Biology  
Massachusetts General Hospital

Dear Suresh,

I am writing in support of the R21 grant application that you are submitting to the NIH, proposing to develop models and study cross-regulation of RNA silencing and other innate immune signaling pathways in response to viral and bacterial pathogens using *C. elegans* and plants as model hosts.

From listening to your presentation and the experimental design in our multi-group meeting, I think this is an important area to be addressed and the system has unique advantages. Use of these model systems should highlight some key areas important in understanding these different modes of response during multi-pathogen infections and uncover some fundamental mechanisms regulating them. In reading the latest version of the grant application, I find the depth of knowledge of these systems you have used in designing the experimental system you propose to be very impressive. Further, the statements you have included to address some of the concerns raised by referees based on your previous submission of this application clearly highlight the importance and uniqueness of the system and the design.

Currently we have three faculty (myself, Fred Ausubel and Josh Kaplan) and about thirty students and postdoctoral fellows working on different aspects of *C. elegans* biology. The three labs have developed a lot of specialized worm-related infrastructure and routinely share reporter strains, mutants, and a library of *E. coli* RNAi clones, and other tools. In addition, my laboratory is doing a comprehensive RNAi screen for components affecting RNA silencing in *C. elegans*. We would be glad to provide you with appropriate tools, mutants and experimental assistance, and as needed collaborate with you in this quest to study cross-regulation of innate immune-related pathways.

Regards,

Gary Ruvkun  
Professor of Genetics

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**From:** Marie-Anne Felix [mailto:felix@ijm.jussieu.fr]  
**Sent:** Wednesday, June 04, 2008 7:31 AM  
**To:** gopalan@molbio.mgh.harvard.edu  
**Subject:** Re: Nematodes from environment

Dear Suresh,

This sounds great. i was just discussing this deep sequencing of small RNAs approach with a fly colleague recently (at a demonstration against the plans of the government to reform the research system- a very good scientific meeting place!). This sounds a promising approach to find viruses.

Summer is coming and I will start again sampling *C. elegans* from rotting fruits. What is the substrate you like to have? Worms just coming out from the wild that I would freeze/fix somehow and send you? Or plates with the progeny of these worms?

I see that you don't want to necessarily focus on *C. elegans*?

It is hard for me to tell *C. elegans* from *C. briggsae* when they come out, but for the rest, I usually can tell them apart.

Best,  
Marie-Anne

Dear Dr. Marie-Anne Felix

I am member of Fred Ausubel's laboratory at the Department of Molecular Biology, Massachusetts General Hospital and the Department of Genetics, Harvard Medical School. I know that you are quite involved in ecological and evolution aspects of nematodes by studying isolates from their environment. I am aware of your excellent collaboration with members of this Department (Emily Troemel in the Ausubel lab and a recent collaboration a new postdoc in Gary Ruvkun's laboratory).

One aspect of my research involves the study of cross-regulation of divergent immune responses in *C. elegans* and model plants to viral and bacterial pathogens. During the course of writing a grant I am proposing to explore the existence of natural viral pathogens of *C. elegans* (nematodes in general - that could probably extrapolated to *C. elegans*). I propose to explore this aspect by deep sequencing small RNAs in nematodes isolated from their natural setting and proceeding from there to look for viral pathogens. I was wondering if you would be willing to contribute nematode samples isolated from environment and not propagated much in the laboratory.

I would be willing to discuss additional aspects of this research as needed.

Best Regards.

Suresh

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## Resource Sharing

All 'model organisms' generated by this project will be distributed freely or deposited into a repository/stock center (e.g., Arabidopsis Biological Resource Center, *C. elegans* Genetic Center) making them available to the broader research community, either before or immediately after publication. If we assume responsibility for distributing the newly generated model organisms, we will fill requests in a timely fashion. In addition, we will provide relevant protocols and published genetic and phenotypic data upon request. Other resources generated (e.g., organismal scale gene expression data and high-throughput sequencing data) will be deposited in community repositories in accordance with community standards. Material transfers will be made with Simple Letter Agreement (SLA) that conform to the intent of Uniform Biological Materials Transfer Agreement (UBMTA). Should any intellectual property arise which requires a patent, we will ensure that the technology (materials and data) remains widely available to the research community in accordance with the NIH Principles and Guidelines document and that of Massachusetts General Hospital and Harvard University.