WHITEHEAD INSTITUTE

Whitehead Institute
for Biomedical Research
Nine Cambridge Center
Cambridge, Massachusetts 02142-1479
617.258.5000 / 617.258.6294 fax
www.whitehead.mit.edu

Michael J. Axtell, Ph.D.
Postdoctoral Fellow
617-258-8346
axtell@wi.mit.edu

September 19, 2005

Yves Brun, Ph.D.
Biology Department
Indiana University
Jordan Hall 142
1001 East 3rd St.
Bloomington, IN 47405-7005

Dear Dr. Brun:

I am writing to apply for the faculty position in systems biology advertised in the 2 September issue of Science. My research focus is the use of *Arabidopsis* and other model organisms to elucidate the function of deeply conserved non-coding small RNAs, termed microRNAs, in plant development. I have broad experience in plant molecular biology, with expertise in *Arabidopsis* molecular genetics, protein biochemistry, genomics, and computational biology. Both my graduate studies of *Arabidopsis* disease resistance and my postdoctoral studies of plant microRNAs have made important and original contributions to plant biology, and I believe that my research will fit well within the mission of the Biocomplexity Institute and the Department of Biology at Indiana University.

My dissertation research with Brian Staskawicz (University of California, Berkeley) elucidated the molecular basis for the specific recognition of a bacterial plant pathogen by *Arabidopsis*. This work, one of the first clear examples of indirect pathogen recognition by plants, has proven to be seminal in the field. As a postdoctoral fellow, my interest in the emerging field of microRNAs led me to pursue research with David Bartel (Whitehead Institute/MIT/HHMI). I have designed, tested, and implemented the first custom microarray for the detection of plant microRNAs, with which I generated a microRNA expression profile of the normal development of *Arabidopsis*. I also discovered that many *Arabidopsis* miRNAs were also evident in RNA samples from basal plants such as ferns and mosses and that in every case examined the targets of these conserved microRNAs in basal plants were identical to those in *Arabidopsis*. Thus, many of the same microRNA-target interactions have been shaping plant development since the last common ancestor of mosses and flowering plants. This research, published in *The Plant Cell*, was selected as a "Research Highlight" in the 26 May issue of *Nature*.

Recently, I have been cloning and characterizing microRNAs from the basal plants *Selaginella moellendorffii* (a lycopod) and *Physcomitrella patens* (a moss). The complete genome sequence for both of these plants is currently being assembled, and *P. patens* has the additional advantage of being a genetically tractable organism where gene knockouts and replacements are feasible due to a high frequency of homologous recombination of foreign DNA. To date, I have discovered at least 20 novel

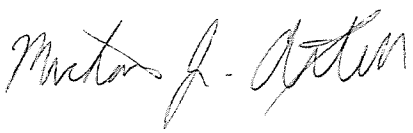
miRNA families expressed by *P. patens*, as well as six other families that are identical to microRNAs in flowering plants. Experiments to study the function of the deeply conserved microRNAs using reverse genetics in *P. patens* are underway. I am truly excited by the potential of studying the evolution of conserved microRNA-regulated transcriptional networks in both *Arabidopsis* and *P. patens*, with the long-term goal of utilizing the powerful genetic and genomic techniques applicable to both of these "post-genomic" organisms to gain a deep understanding of land plant development. My research is inherently interdisciplinary, leveraging molecular biology, genomics, and computational biology, and I believe it will benefit from the diverse group of scientists at the Biocomplexity Institute.

I am committed to the academic ideals of rigorous basic research and scholarship, and I look forward to the opportunity to be a mentor to young scientists. I am enthusiastic about teaching the subjects of my expertise, including molecular biology, plant biology, genetics, and genomics, to all levels of students from introductory undergraduate courses to advanced seminars for graduate students. I am excited by the opportunity to join the faculty of excellent biologists at Indiana University.

In support of my application, I have requested that four letters of recommendation be delivered to you from Drs. David Bartel, Brian Staskawicz, Barbara Baker, and David Baulcombe. In addition, I have enclosed two reprints representative of my research to date.

Thank you very much for your consideration. I look forward to your response.

Sincerely,



Michael J. Axtell

Enclosures:
- *Curriculum vitae*
- Research Overview and Teaching Interests
- Reference contact information
- Representative Publications: "Antiquity of microRNAs and their targets in land plants" and "Initiation of RPS2-specified disease resistance in Arabidopsis is coupled to the AvrRpt2-directed elimination of RIN4"

Michael J. Axtell

I. Dissertation Research: Molecular Mechanisms of Pathogen Recognition by *Arabidopsis*

A. Background

Plants have evolved a sophisticated immune system that is capable of sensitive perception of diverse pathogens including viruses, bacteria, fungi, insect, and nematodes. Plant immunity to many pathogens has long been known to be the result of single plant "Resistance" (R) genes. By definition, a plant R gene is necessary for the immune perception only of pathogens that express a particular gene, historically denoted an "Avirulence" (avr) gene. The simplest model to account for this genetic phenomenon is that plant R genes encode specific receptor proteins, each of which evolved to uniquely recognize a particular pathogen protein. Cloning of multiple R genes has shown that most encode proteins with domains consistent with a receptor function. The proteins encoded by the corresponding pathogen avr genes are structurally and functionally diverse, and most are thought to function as virulence factors that have evolved to modulate host physiology to the pathogen's advantage during infection. While a direct ligand-receptor model, with plant R proteins specifically recognizing and responding to a specific pathogen protein, would seem to be the simplest explanation for this type of immune perception, my doctoral dissertation research showed that a more subtle and elegant indirect method of R protein-mediated pathogen perception is utilized by plants.

B. Indirect Recognition of a Bacterial Pathogen by *Arabidopsis*

In *Arabidopsis*, the R protein RPS2 specifically triggers disease resistance against strains of the bacterium *Pseudomonas syringae* that express the AvrRpt2 protein. During an infection, AvrRpt2 is one of several proteins that are injected into host cells by *P. syringae* and, if the host lacks RPS2, acts as a virulence factor that promotes pathogen growth. I showed that one effect of AvrRpt2 in *Arabidopsis* cells was the degradation of a plant protein called RIN4. Using biochemical and genetic techniques, I demonstrated that the RPS2 R protein was physically coupled to the RIN4 protein, and initiated immune function not as a result of AvrRpt2's presence *per se*, but rather as a consequence of the AvrRpt2 triggered degradation of RIN4 (*1*). I then discovered that AvrRpt2 was a novel type of cysteine protease, and demonstrated that the probable catalytic amino acid residues of AvrRpt2 were required for the degradation of RIN4, the subsequent triggering of recognition by RPS2, and the ability of AvrRpt2 to promote pathogen virulence in plants that lacked RPS2 (*2*). Thus, my research demonstrated a novel, indirect mode of pathogen recognition where plant immune "receptors" are really monitoring for the modulation of host physiology caused by pathogen virulence proteins, rather than for the individual pathogen proteins themselves. This paradigm of indirect pathogen recognition has since been shown to occur in many R protein-mediated recognition events, and is thought to be a common molecular mechanism by which plants specifically respond to pathogen attack.

II. Post-Doctoral Research: Expression and Conservation of Plant microRNAs

A. Background

The discovery of RNA interference (RNAi) is one of the first major breakthroughs of 21st century molecular biology. All RNAi-related processes involve a small RNA (typically 21 to 24 nucleotides) that functions to target selected messenger RNAs (mRNAs) for repression, either through endonucleolytic mRNA cleavage, or by otherwise preventing translation of the mRNA. Small RNAs achieve target specificity by Watson-Crick complementarity with their targets. There are two types of

small RNAs, short interfering RNAs (siRNAs) and microRNAs (miRNAs), which differ in biogenesis and target types but not in their molecular modes of target repression. siRNAs are processed from long perfectly double-stranded RNA templates and primarily target the transcripts from which they derive. In contrast, miRNAs are processed from a tightly defined region of a non-coding RNA transcript that is capable of folding into a characteristic imperfect stem-loop structure and target other transcripts in *trans* (*3*). In plants, siRNAs typically arise from invading viruses, introduced transgenes, or heterochromatic regions of the genome such as transposons, peri-centromeres, and inverted repeats. In contrast, miRNAs generally arise from euchromatic genes that, similar to protein-coding genes, are differentially expressed in response to developmental and environmental cues.

The finding that *Arabidopsis* expresses a diversity of miRNAs was closely followed by the even more exciting discovery that the targets of these miRNAs can be readily identified by computation based upon high complementarity between the miRNAs and targets (*4*). An overwhelming majority of the *Arabidopsis* miRNA targets described to date are mRNAs coding for master developmental regulatory transcription factors or F-box proteins (*5*). Mutations in genes critical for miRNA biogenesis as well as individual miRNAs and their targets cause massive defects in *Arabidopsis* development, demonstrating that miRNA-mediated post-transcriptional control of critical developmental targets is essential to the morphogenesis of flowering plants (*6*). Many of the miRNAs and targets discovered in the dicot *Arabidopsis* are also evident within the monocot *Oryza sativa* (rice) genome, indicating that these regulatory units have been conserved between widely separated lineages of flowering plants (*7*).

B. Expression Profiling of Plant microRNAs

The observation that many plant miRNA targets appear to play a role in development suggests that temporal and spatial control of miRNA expression is critical for their function. To analyze miRNA expression patterns in *Arabidopsis* I designed and implemented the firsrt custom microarray capable of detecting plant miRNAs. I then used this array to determine the expression profiles of *Arabidopsis* miRNAs in various organs and developmental stages of wild-type *Arabidopsis*. The results demonstrated that many miRNAs are specifically enriched or depleted in tissue-specific manners, consistent with the observation that they are involved in developmental control. I then compared the accumulation of target mRNAs to the accumulation of the cognate miRNAs in the same tissues by making use of publicly available mRNA expression array data. I found that there was a significant negative correlation between the abundance of a miRNA and its targets within tissues, supporting the hypothesis that plant miRNAs frequently act to clear given cells or tissues of target mRNAs (*8*).

C. Detection of deeply conserved plant miRNAs and targets

The fact that the majority of plant miRNAs and their targets were known to be present in both *Arabidopsis* and rice led me to wonder how deeply conserved these regulatory circuits were. I used the miRNA microarray to assay RNA samples collected from diverse plants representative of major land plant lineages and found that a great number of flowering plant miRNAs were readily detectable in basal plants such as gymnosperms, ferns and mosses (Figure 1). I then devised a novel and empirical strategy to determine the targets of the conserved miRNAs in basal plants. I found that in all cases in gymnosperms, ferns, and mosses that the basal plant targets were identical to the targets of the same miRNAs in flowering plants, directly demonstrating that many individual miRNA-target relationships have remained constant throughout the evolution of land plants (*8*).

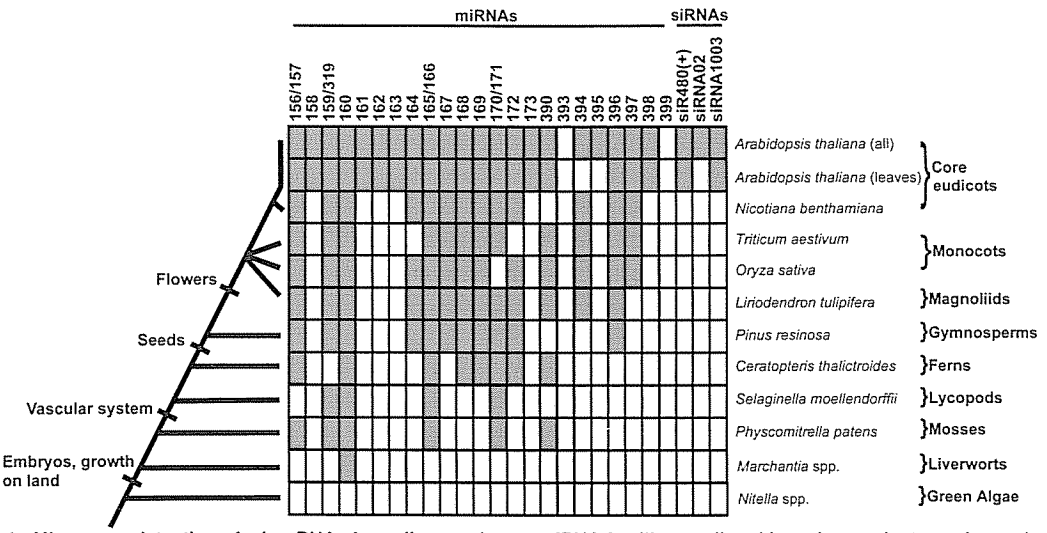


Figure 1: Microarray detection of microRNAs from diverse plants. miRNA families are listed by column, plant specimens by row. Gray squares indicate detection, white indicates indicate not detected. Cladogram on left represents approximate relationships between sampled plants, and indicates major innovations during land plant evolution. Figure was updated from (*8*) to reflect recent experiments.

D. Cloning and characterization of basal plant miRNAs

My discovery that many plant miRNAs and their targets are evolutionarily ancient has prompted me to perform experiments upon model non-flowering plants. There are two basal plant genome projects that are scheduled to be completed soon: The primitive vascular plant *Selaginella moellendorffii* (a lycopod), and the moss *Physcomitrella patens*. *P. patens* is especially attractive as a non-flowering plant model organism since transgenic plants can be easily generated by homologous recombination. I have cloned several thousand small RNAs from both *S. moellendorffii* and *P. patens*. Using the whole-genome shotgun traces that are currently being released to the public in combination with custom software that I developed, I have mapped these cloned small RNAs onto the unassembled genomes. Analyses of these data are ongoing, but to date I have identified the 6 deeply conserved miRNAs indicated by my earlier microarray analyses, as well as at least 20 novel miRNA families in *P. patens* (Figure 2). This discovery alone nearly doubles the known number of plant miRNA families, and I am very excited in my current project of devising computational and empirical strategies to identify the targets of the novel miRNAs.

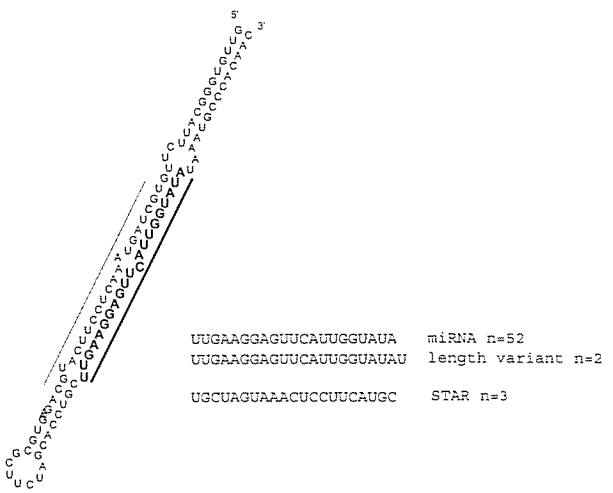


Figure 2: An example of one of at least 20 novel *P. patens* microRNAs found by cloning and comparison to whole-genome shotgun traces. A trace containing a match to the cloned small RNAs was folded using RNAfold. The miRNA (bold, highlighted by thick line) is found within a typical imperfect stem-loop structure, and was isolated 52 times, in addition to a length variant cloned twice. The "STAR" species, a typical low-abundance species arising from the opposite side of the stem-loop during miRNA processing, was cloned three times (thin line).

III. Planned Research: Conservation and divergence of microRNA-controlled regulatory networks.

A. Functions of deeply conserved miRNA-target interactions in the moss *Physcomitrella patens*

The generation of transgenic *P. patens* via homologous recombination is a technically simple process. I plan to exploit this property to investigate the phenotypic consequences of disrupting conserved miRNAs and their targets in moss. I will both delete and over-express each of the *P. patens* miRNAs that are conserved in *Arabidopsis* and assess the mutants for developmental abnormalities. As discussed above, most of the conserved plant miRNAs control the expression of master developmental regulators in both organisms, so the most likely outcome of the disruption of *P. patens* miRNAs would seem to be morphological alterations. I will also generate and characterize *Arabidopsis* plants with similar genetic lesions in the conserved miRNAs. Comparison of the phenotypes of mutant *Arabidopsis* and *P. patens* plants that both contain the same perturbations of a conserved miRNA regulatory interaction will offer unique insight into how the evolution of plant development has utilized ancient genetic control switches in the two lineages.

In parallel to the disruption of moss miRNAs, transgenic *P. patens* plants containing miRNA-resistant targets of the conserved miRNAs will be generated. Transgenic plants will contain multiple third codon mutations within the miRNA complementary site that eliminate miRNA targeting but that do not change the amino acid sequence of the target gene product. In *Arabidopsis*, this approach has created dominant gain of function mutants that result from the escape of the target transcript from the normal miRNA-mediated control. The phenotypes of such mutant moss plants will be compared to the phenotypes of *Arabidopsis* transgenics containing the homologous miRNA-resistant transgenes. I will also characterize the temporal and spatial expression patterns of deeply conserved plant miRNAs and their targets by generating transgenic moss plants with reporter gene fusions. Reporters will be generated for both the miRNA loci and their targets. The standard reporter genes β-Glucuronidase (GUS) and green fluorescent protein (GFP) have been shown to function in *P. patens* and will be used for these experiments. Again, comparison between the expression patterns of conserved miRNAs and their targets in *Arabidopsis* and *P. patens* will illuminate differences and similarities in the uses of the conserved regulatory interactions in flowering plants and moss.

B. Targets and functions of novel, moss-specific microRNAs

To date, my cloning of *P. patens* small RNAs and subsequent computational and experimental analyses have led to the discovery of 20 novel moss miRNA families. Based on both RNA blots against diverse plant species and database analysis, these novel miRNAs appear to be specific for the bryophyte lineage. As discussed above, a majority of the regulatory targets of higher plant miRNAs are transcription factors involved in developmental control. It will be very interesting to determine whether the targets of the newly discovered, bryophyte-specific miRNA families are also predominantly involved in developmental control, or whether miRNAs in this lineage have evolved to control the expression of other types of genes.

Higher plant miRNA targets are readily predicted using genomic information based on the presence of highly complementary sequence motifs. However, this strategy is at present not feasible for *P. patens*, since the genome has not yet been assembled from the primary whole-genome shotgun sequence reads. Therefore, I plan to computationally predict the targets of the novel, moss-specific miRNAs when the first annotated *P. patens* genome assembly becomes

available. I will also empirically determine the targets of the novel moss miRNAs by taking advantage of the fact that when messenger RNAs are cleaved as a result of miRNA targeting they leave behind a characteristic cleavage fragment. I have developed a novel two-step RT-PCR technique which has been shown to allow the discovery of these cleavage fragments even in the absence of any pre-existing genomic information. Through this combination of computational and empirical strategies I will be able to answer the question of what biological functions the novel, bryophyte-specific miRNAs are controlling. Following the successful identification of the targets of these novel miRNAs I expect to experimentally probe their function using similar genetic strategies as were outlined for the deeply conserved miRNAs above.

C. Evolution of miRNA-controlled transcriptional networks

One of the major contributions of molecular genetics to the understanding of multicellular development has been the discovery that the genes having macroscopic effects upon morphology most frequently are transcriptional regulators responsible for lineage- or location-specific control of gene expression. I believe a quantitative understanding of development will require detailed knowledge of the transcriptional programs controlled by such master regulators. I have discovered that several master regulators have been conserved and controlled by the same miRNAs across vast evolutionary distances. Therefore, my long-term goal is to determine the extent to which gene expression programs controlled by deeply conserved miRNA-target modules have also been conserved, versus the extent to which such conserved modules have been recruited to control different transcriptional responses in divergent plant lineages.

In initial experiments, transgenic *Arabidopsis* and moss plants expressing miRNA-resistant target genes under their native control elements will be produced and used for mRNA expression profiling experiments. Transcripts whose accumulation changes in response to the loss of miRNA-mediated control will be compared between the two organisms. Similarly, transgenic *Arabidopsis* and *P. patens* will be generated with inducible promoter-driven expression of conserved miRNAs. Comparative expression profiling of induced plants will reveal the direct transcriptional responses to the miRNA in both organisms. Comparative genomics and bioinformatic analysis of these expression profiling datasets from two disparate plants will provide a unique picture of the evolution of development-related transcriptional networks. Downstream genes discovered in this manner will then themselves become the subject of study in both organisms. The use of powerful genetic techniques in two distantly related plants to study deeply conserved regulatory units will provide major insights into the molecular basis for plant development and morphology.

IV. Teaching interests and philosophy

I decided to become a biologist as a direct result of my undergraduate experience in the Ithaca College Biology Department. During these years, the excellent mentorship of several faculty members provided me with the intellectual nourishment that lead me to pursue graduate study at the University of California after receiving my Bachelor's degree. Many of my courses had a lab component in which the primary professor interacted with the students on a personal basis. The single largest benefit of this time was my involvement in a multi-year independent research study that provided me with my first experience as an experimental biologist. My teaching philosophy emulates the personal and hands-on style that had such a profound impact on my development as a young scientist, and encourages interactive discussions and learning by experimentation.

As a graduate student, I was a teaching assistant in two undergraduate courses: An elective for non-majors entitled "The Secret Life of Plants" and an Introductory course on biochemistry and molecular biology for majors, "Biology 1A". In both instances, I was responsible for weekly discussions and one-on-one discussions with students. Additionally, in Biology 1A, I supervised a weekly lab component to the course. I believe that these experiences had a positive impact on the undergraduates in the courses, and that I encouraged an interactive approach to learning. Also during my dissertation studies, I worked with an undergraduate researcher by training her in basic molecular biology techniques and experimental design. It was very fulfilling to help develop an interest in basic research in a promising young student. This student has since gone on to pursue a Ph.D. at the University of Chicago.

I am interested in teaching the areas of my expertise at all levels. I am well qualified to teach introductory and advanced genetics, genomics, molecular biology, and all areas of plant physiology and biology. My research interests are in the biological roles of RNA interference (RNAi) and microRNAs, and I am especially enthusiastic about the possibility of teaching an advanced topics course (or sub-section of a broader course) on this fascinating and rapidly evolving topic. I am also very interested in serving as coordinator for an journal club class. I believe that critical reading and discussion of the primary literature is a valuable experience for students at both the advanced undergraduate and graduate levels. Perhaps most importantly, I look forward to the opportunity to involve all of my students in cutting-edge biological research. I firmly believe that experimentation and observation are critical aspects of learning in the biological sciences.

V. Selected References

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