**Identification and Characterization of Rust Effectors that Affect Host Immunity**

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**Project Goals: An analysis of the *Melampsora larici-populina* genome reveals a large number of genes encoding candidate secreted effector proteins (CSEPs) that may play many roles in host infection. A screen was developed in tobacco leaves and poplar protoplasts for CSEPs that affect host immunity. Two subsets of effectors were targeted for analysis: one with significant homology to CSEPs in other fungal pathogens of plants, and another family, containing unique members with no similarity to any proteins in sequence databases. The results suggest that homologous CSEPs target conserved host factors and immune mechanisms to inflict disease. A platform was developed to further explore structure-function relationships within CSEP families. Gateway cloning of effector genes into a bacterial expression vector facilitated purification, and the use of fusions with a form of maltose binding protein modified for enhanced crystallization may greatly facilitate structure analysis.**

Abstract: The genomes of fungal pathogens of plants contain genes for small, cysteine-rich secreted proteins that are specifically up-regulated for expression during infection, signifying a key role in host colonization and disease. Computational analysis of the 1,524 candidate secreted effector proteins (CSEPs) in *Melampsora larici-populina* shows that they belong to 807 structural families, including 600 single-member families, another 206 families that contain 2-36 members, and one family that has undergone a remarkable expansion to 117 members. To reveal the molecular interactions of poplar host factors and rust effectors a screen was developed to identify CSEPs that can suppress host immunity in tobacco and poplar. Two subsets of *Melampsora* CSEPs were targeted for analysis. The first group contained 160 *Melampsora larici-populina* CSEPs belonging to 67 structural families, all of which share significant homology among pathogenic rust, *Septoria* or powdery mildew fungi. The other subset was the unique 117-member family, whose members show virtually no similarity to any protein in sequence databases. Bootstrapped phylogenetic trees of this particular family were constructed using the maximum likelihood approach for both the DNA and protein sequences under best-fitted evolutionary models. The trees were used to identify family members common to branches on both trees that most likely diverged recently. This approach identified a modest subset of 10-15 relatively-distinct members for an analysis of their effect on host immunity.

The two subsets of CSEPs were expressed transiently in tobacco along with well-known 'autoactive' domains of *R* genes or an *R* gene-*AvrP* pair that promotes a hypersensitive response (HR) or HR-like cell death. Attenuation (or enhancement) of

salicylate levels stemming from changes in HR was measured quantitatively using LC-MS to assess the impact of *Melampsora larici-populina* CSEPs on mounting an immune response. For the first group, the screen led to the identification of 72 CSEPs that affect host immunity. These 'reactive' CSEPs belong to 49 of the 67 structural families, which suggests that homologous pathogen effectors may disrupt conserved plant immune responses and trigger disease. Transient expression of the reactive CSEPs in poplar protoplasts is being used to confirm the results in tobacco, and if validated, will enable tobacco-based large-scale screens for additional *Melampsora larici-populina* CSEPs that disrupt host factors and immunity, providing a foundation for engineering durable rust resistance in *Populus* spp. Transient expression of the second CSEP group will reveal the impact of the relatively unique members on host immunity, and shed light on how pathogen gene expansion and amplification can overcome host resistance.

The use of a recombination-based (Gateway) cloning system has enabled the facile transfer of CSEP genes from binary vectors for functional studies in plants to bacterial vectors for expression and purification. Production of *Melampsora larici-populina* CSEPs as fusions to a form of maltose binding protein that has been modified for enhanced solubility, affinity purification and containing amino acid substitutions that enhance crystallization has been piloted on three candidates. Initial results suggest promise of this approach for large-scale investigation of structure-function relationships among effector families important for plant-pathogen interactions.

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