Micro-Review

First insights into the genes that control plant–bacterial interactions

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SUMMARY

The events leading up to the cloning of the first bacterial avirulence gene, *avrA*, from *Pseudomonas syringae* pv. *glycinea* are described. The cloning of this gene marked the beginning of the molecular analyses of bacterial effectors and has paved the way for determining of the role of bacterial effectors in pathogen virulence and the triggering of plant innate immunity.

It is hard to believe that it has been nearly 25 years since the first ‘avirulence gene’ was cloned from the phytopathogenic bacterium *Pseudomonas syringae* pv. *glycinea* (*Psg*) in our laboratory. In looking back at the events that led to the cloning of the first avirulence gene, I recall several important biological observations and technological advances that had just become available, allowing our experiments to be successful. Firstly, earlier work performed by the late Noel Keen at the University of California, Riverside had established the soybean–*Psg* pathosystem to study host–pathogen specificity. The important aspect of this work was the development of a facile and robust system to score the phenotype of resistant and susceptible interactions of *Psg* infections on a differential set of soybean cultivars. The resistant reaction was scored as a hypersensitive cell death reaction (HR) that occurred after 24 h, whereas the susceptible reaction was scored as a water-soaked lesion that usually occurred 48–72 h after inoculation. Secondly, the adoption of cosmid cloning to generate DNA libraries of bacteria that could be propagated in *Escherichia coli* and easily conjugated to a recipient host bacterium was another important step forward in the study of bacterial genetics (Friedman *et al.*, 1982). The culmination of these events set the stage for the cloning of the first bacterial avirulence gene—*avrA* (Staskawicz *et al.*, 1984).

Depicted in Table 1 are the inoculation phenotypes of two races of *Psg* on two separate cultivars of soybean. The reactions of *Psg*

race 5 and 6 are reciprocal on the cultivars Harosoy and Norchief. Thus, the strategy employed to clone *avrA* was to construct a cosmid library of *Psg6* DNA in *E. coli* and transfer this library into *Psg5* via triparental matings. The exconjugants could then be inoculated onto the two soybean cultivars and scored for either a resistant or susceptible reaction. In this manner, we could determine whether resistance or susceptibility could be transferred from *Psg6* to *Psg5*. Initial experiments showed that, if we tried to conjugate the entire library ‘en masse’ to *Psg5* and then score *Psg5* exconjugants for an altered reaction on either Harosoy or Norchief, we were unsuccessful. Unfortunately, after we inoculated over 1000 exconjugants that had arisen from the ‘en masse’ conjugations, no change in host reaction was observed. We finally determined that the majority of the exconjugants contained only small inserts, as these clones conjugated at a higher frequency. We next decided to embark on a more ‘brute force’ approach by performing 680 individual conjugations with *Psg5* as recipient. A single cosmid clone Pg6L3 was identified that enabled *Psg5* to induce an HR on the previously susceptible Norchief cultivar (Staskawicz *et al.*, 1984). No exconjugants were recovered that converted *Psg5* to a susceptible reaction on Norchief. Thus, we concluded that avirulence was dominant in the merodiploids that we had constructed. To further characterize the cosmid clone that specified a resistance reaction on Harosoy, transposon mutagenesis was employed to identify which fragments contained the effector activity, allowing *avrA* to be cloned.

Although we were able to clone *avrA*, we still did not have a deep insight into the mechanisms by which the AvrA protein elicited immune responses in resistant soybean cultivars. We naively conjugated the active *avrA* clones into both *Pseudomonas fluorescens* and *E. coli* and inoculated the cultivar Harosoy. These exconjugants did not elicit HR, suggesting that *avrA* was either not expressed or required other factors for its activity. In addition, neither cell extracts nor cell-free culture supernatants of *avrA*-expressing *Psg5* strains triggered HR when inoculated into Harosoy. These experiments led us to ponder how *avrA* operated to induce a race-specific HR when expressed in the normally virulent race *Psg5*.

The next important series of experiments that began to shed light on the mechanisms involved in how *avrA* controlled the

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Table 1 Disease Phenotypes of *P.s. glycinea* on Soybean Cultivars.

	Harosoy	Norchief
Psg6	—	+
Psg5	+	—
Psg5 (avrA)	—	—

induction of race-specific resistance came from the laboratory of Nick Panopoulos at UC Berkeley. The identification and characterization of the *Hrp* (hypersensitive reaction and pathogenicity) gene cluster of *P. syringae* pv. *phaseolicola* in 1986 was a major advancement towards a molecular understanding of the genes controlling both bacterial resistance and susceptibility (Lindgren *et al.*, 1986). The first documentation of a molecular relationship between avirulence genes and *Hrp* genes was revealed in 1989 in a paper from our laboratory (Huynh *et al.*, 1989). Although we still did not know that the *Hrp* genes encoded the type 3 secretion system (T3SS) or that avirulence genes encoded effector proteins that were delivered to plant cells, we made the observation that a second *Psg* avirulence gene (*avrB*) was dependent on several *Hrp* genes for transcriptional activation. This was later confirmed and extended when Roger Innes published the identification of the *Hrp* box (GGAACCNA-N14-CCACNNA) (Innes *et al.*, 1993), which is located in the promoter region of several *Hrp* gene operons and avirulence genes. The *Hrp* box was found to play a critical role in the co-ordinate regulation of these genes in response to changes in the availability of nutrients in the plant apoplast. Such conditions could be mimicked when strains were cultured in a low-pH minimal medium with sucrose as a carbon source. This paved the way for future studies aimed at elucidating the biochemical function of HRP and avirulence effector proteins in culture.

It was not until the equivalent *Hrp* loci of *Xanthomonas campestris* pv. *vesicatoria* and *Ralstonia solanacearum* were sequenced and found to show significant sequence similarity to the T3SS of mammalian pathogens that we realized that the original gene we had cloned 8 years earlier was most likely delivered directly into the plant cell via a specialized protein secretion apparatus (Fenselau *et al.*, 1992; Gough *et al.*, 1992). This revelation immediately explained why the original experiments of putting avirulence genes into either *P. fluorescens* or *E. coli* did not elicit an HR. It also explained why the cell-free supernatants from *Psg* expressing *avrA* did not elicit a race-specific HR.

The question that was probably most frequently asked after the initial cloning of the *avrA* gene was: 'Why would a pathogen contain a gene that triggers host immunity, and what selective advantage would it have in maintaining it?' Our first clue that so-called 'avirulence genes' had a dual role in both avirulence and virulence came from work showing that mutations in the *avrBs2* gene of *X. campestris* pv. *vesicatoria* resulted in a loss of

virulence in pepper plants which did not contain the cognate *Bs2* resistance gene (Kearney and Staskawicz, 1990; Swords *et al.*, 1996). These genetic experiments suggested why *avrBs2* had a selective advantage and why it is so widely maintained in many different *X. campestris* pathovars.

Once it was established that phytopathogenic bacteria contained a T3SS and delivered avirulence proteins (now commonly referred to as effector proteins) to plant cells, one could postulate that avirulence genes either modulated or suppressed plant innate immunity, causing plants to evolve a sophisticated, multilayer surveillance system to detect and respond to T3SS-delivered effector proteins. The ensuing years have seen great advances in our molecular understanding of the role of effector proteins in modulating plant innate immunity in both resistant and susceptible plant interactions (reviewed in this issue). Future challenges include the identification and biochemical characterization of host virulence targets and the elucidation of the precise cellular events involved in effector-triggered resistance.

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