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Update on Plant-Microbe Interactions

Elicitation of Plant Hypersensitive Response by Bacteria[1]

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Cell death caused by pathogen infection has been of great interest to plant biologists for many years because of its frequent association with plant resistance. There appear to be two types of plant cell death associated with pathogen infection: a rapid, hypersensitive cell death localized at the site of infection during an incompatible interaction between a resistant plant and an avirulent pathogen, and a slow, "normosensitive" plant cell death that spreads beyond the site of infection during some compatible interactions involving a susceptible plant and a virulent, necrogenic pathogen. Hypersensitive cell death is accompanied by the induction of multifaceted defense responses, including production of active oxygen species and antimicrobial compounds (phytoalexins), rapid cross-linking of cell-wall proteins, and, ultimately, resistance to pathogens (Dixon et al., 1994; Goodman and Novacky, 1994). Consequently, hypersensitive cell death is considered to be a sacrifice of locally infected tissue (sometimes only one or a few cells) to protect against the spread of the pathogen into healthy plant tissues. In contrast, the slow, normosensitive plant cell death does not effectively prevent pathogen multiplication or spread and is therefore not associated with local resistance.

It is interesting that both hypersensitive and normosensitive cell death can lead to a systemic, broad-spectrum resistance response throughout the plant called SAR (Ryals et al., 1994). SAR is effective against subsequent infection by the same or different pathogens. It has long been observed that diverse plant pathogens, from multicellular organisms such as fungi and worms to simple parasites such as viruses, can cause superficially similar hypersensitive cell death in resistant plants (Goodman and Novacky, 1994). Therefore, hypersensitive cell death has been considered to be a conserved mechanism in higher plants for rapidly self-eliminating cells doomed to die, and, in the process of doing so, activating other local and systemic resistance responses either causally or simultaneously. In the past few years, steady progress has been made in understanding the mechanism by which pathogens elicit hypersensitive cell death and the mechanism of signal perception and transduction in the plant cell during hypersensitive cell death. In this *Update* I discuss how bacteria elicit hypersensitive cell death.

WHAT IS THE HYPERSENSITIVE RESPONSE?

Stakman (1915) is generally considered to be the first to use the term "hypersensitive reaction" (HR) to describe rapid host cell death in resistant plants (oat, wheat, and barley) upon infection by the fungus *Puccinia graminis*. Stakman observed that the more resistant a cultivar, the more rapid the death of a limited number of host cells in the vicinity of the invading fungal hyphae. Recently, the term "hypersensitive response" has been more frequently used, but there has been some controversy over the scope of the definition of HR. Is HR merely a cell death response or does it encompass associated resistance responses? Because hypersensitive cell death alone may or may not be sufficient to restrict pathogen infection, it is important to define HR clearly. The original definition of HR by Stakman clearly equated HR with the abnormally rapid death of host cells attacked by fungal hyphae (Stakman, 1915). Therefore, in this *Update* I will restrict the use of the term HR to hypersensitive cell death.

Klement et al. (1964) discovered the ability of pathogenic bacteria to elicit HR almost 50 years after Stakman's discovery of the response. The key to their discovery was the use of a novel inoculation technique for introducing bacteria into plant leaves. They used syringes to infiltrate large numbers ($>10^6$ cells/mL) of an avirulent bacterium (*Pseudomonas syringae* pv *syringae*) into the intercellular space of leaves of a nonhost plant (tobacco) and observed the appearance of rapid, localized hypersensitive necrosis due to the death of most of the plant cells in the infiltrated leaf tissue. A saprophytic bacterium (*Pseudomonas fluorescens*) did not elicit any HR, whereas a virulent bacterium (*P. syringae* pv *tabaci*) caused a slowly spreading tissue necrosis (Klement et al., 1964). Klement et al.'s seminal finding has spurred numerous investigations into the mechanism of bacterial elicitation of the HR. Biochemical, physiological, and microscopic studies were undertaken from the 1960s to the early 1980s, and revealed several important characteristics of the HR: (a) Active bacterial metabolism is required for HR elicitation. In other words, elicitors of HR are not preformed, but are produced after infiltration of bacteria into the plant apoplast. More interestingly, the requirement for active bacterial metabolism is only temporary (30 min to 4 h) (Klement and Goodman, 1967; Roebuck et al., 1978), suggesting that once HR elici-

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Abbreviations: HR, hypersensitive response; SAR, systemic acquired resistance.

tors are produced, living bacteria are no longer needed. (b) Elicitation of the HR requires close contact between bacteria and plant cells. Prevention of such close contact by infiltrating bacteria in diluted agar, which precludes the attachment of bacteria to plant cells, inhibits HR elicitation (Stall and Cook, 1979). (c) In at least some plant-bacterial combinations, one bacterium is sufficient to trigger the death of a plant cell (Turner and Novacky, 1974). (d) Finally, rigorous biochemical studies have been unsuccessful in the search for bacterial elicitors of HR; thus, it appears that HR elicitors are either extremely labile or are presented to the plant cell only on contact (Klement, 1982). Molecular genetic studies undertaken in recent years have provided clear explanations for most of the above observations, as discussed below.

DISCOVERY OF *HRP* GENES

In the early 1980s a number of researchers started to use transposon-mediated mutagenesis to reveal bacterial genes that play important roles in various plant-bacterial interactions. Lindgren et al. (1986) identified clusters of bacterial genes, known as *hrp* (for hypersensitive reaction and pathogenicity) genes, in the bean pathogen *P. syringae* pv *phaseolicola*. Transposon-induced mutations in *hrp* genes were found to abolish the ability of *P. syringae* to elicit the HR in nonhost plants or to cause disease in host plants (Lindgren et al., 1986). *hrp* mutants behave very much like bacteria that have no apparent interactions with plants, such as *Escherichia coli* and *P. fluorescens*. The identification of *hrp* genes suggested that the molecular mechanism(s) underlying bacterial pathogenicity and bacterial elicitation of plant disease resistance may involve the same bacterial genes.

hrp genes have subsequently been isolated from many plant pathogenic bacteria, characterized most extensively from *P. syringae* pv *syringae*, *P. syringae* pv *phaseolicola*, *Pseudomonas solanacearum* (which causes wilt in many solanaceous plants), *Xanthomonas campestris* pv *vesicatoria* (which causes bacterial spot on tomato and pepper), and *Erwinia amylovora* (which causes fire blight on rosaceous plants) (Fenselau and Bonas, 1995; Huang et al., 1995; Van Gijsegem et al., 1995; Bogdanove et al., 1996b). Surprisingly, the cloned *hrp* clusters from *P. syringae* pv *syringae* 61 and *E. amylovora* 321 enabled nonpathogens (e.g. *E. coli* or *P. fluorescens*) to elicit the HR in plants (Huang et al., 1988; Beer et al., 1990). The functional cloning of these two *hrp* clusters in *E. coli* revealed that the minimum number of genes required for elicitation of the HR by plant pathogenic bacteria are carried on a DNA fragment of about 25 to 30 kb in length, a very small portion of the bacterial genome (which is normally about 4000–5000 kb).

DNA-DNA hybridization studies indicate that at least some *hrp* genes are similar among necrogenic bacteria belonging to different genera (*P. syringae*, *E. amylovora*, *Erwinia stewartii*, *P. solanacearum*, and *X. campestris*). Recent DNA sequence studies confirm that many *hrp* genes cloned from diverse plant-pathogenic bacteria are homologous (Fenselau and Bonas, 1995; Huang et al., 1995; Van Gijsegem et al., 1995; Bogdanove et al., 1996b). Thus, *hrp* genes appear to be universal among diverse necrosis-causing, gram-negative bacterial pathogens of plants. In the following section, I shall use the *P. syringae hrp* gene cluster as an example for discussing the biochemical functions of *hrp* genes.

BIOCHEMICAL FUNCTIONS OF *HRP* GENES

The biochemical functions of *hrp* genes have remained a puzzle until quite recently. DNA sequencing has played a major role in the determination of many *hrp* gene functions. As will be discussed below, many *hrp* genes have striking similarities with genes of known function. Figure 1 shows the gene organization and likely functions of *hrp* genes of *P. syringae* pv *syringae* Pss61 (Huang et al., 1995). There are at least 25 *hrp* genes in this bacterium. Based on DNA sequence similarity to other known genes and subsequent biochemical and molecular characterization, we now know that *hrp* genes have at least two biochemical functions: gene regulation and protein secretion.

Three *P. syringae hrp* Gene Products Regulate the Expression of Other Genes

hrp genes are either not expressed or are expressed at very low levels when bacteria are grown in nutrient-rich medium, whereas they are highly expressed when bacteria enter the intercellular space (apoplast) of plant tissues (Rahme et al.,

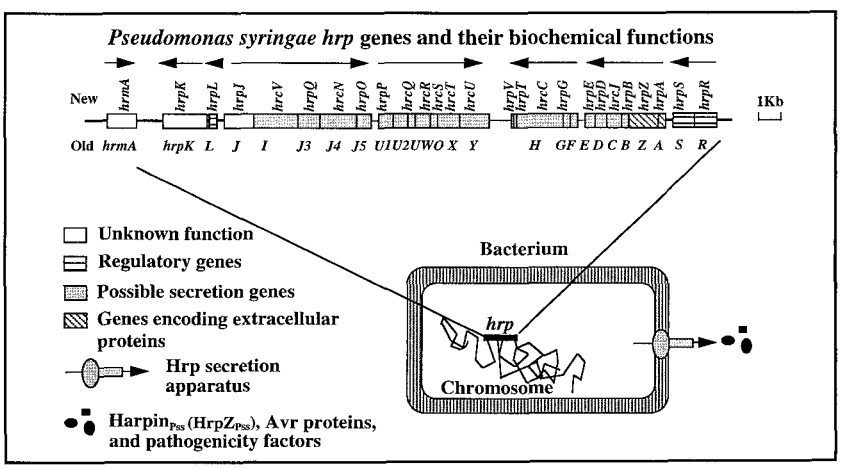

Figure 1. *hrp* genes of *P. syringae* and their functions. New, Proposed new nomenclature for *P. syringae hrp* genes (Bogdanove et al., 1996a). Old, Current nomenclature of *P. syringae hrp* genes (Huang et al., 1995). Arrows indicate transcription direction of each gene operon.

1992; Xiao et al., 1992). Unlike viruses, nematodes, and many fungi, plant-pathogenic bacteria do not invade living plant cells. Therefore, signal exchanges between plant cells and bacteria must occur in (or through) the apoplast outside of the plant cell. A number of researchers have observed that induction of *P. syringae hrp* genes could be achieved by using artificial minimal medium lacking complex nitrogen nutrients, indicating that a lack of nutrients in the plant apoplast may be the signal for the induction of *hrp* genes (Rahme et al., 1992; Xiao et al., 1992; Arlat et al., 1994; Bogdanove et al., 1996b). Specific compounds (e.g. Suc and sulfur-containing amino acids) present in the plant apoplast may also serve as signals for the induction of *X. campestris vesicatoria hrp* genes (Shulte and Bonas, 1992). The induction of *hrp* genes in the nutrient-poor plant apoplast or in artificial minimal medium indicates that *hrp* genes may be involved in releasing nutrients from plant cells.

How do bacteria sense the plant apoplast environment? It was found that at least 3 of the 25 *P. syringae hrp* gene products are involved in the detection of the apoplast environment: HrpL, HrpS, and HrpR (Fig. 1). The *hrpS* and *hrpR* genes are among the first two *hrp* genes to be expressed once bacteria enter plant tissues. It has been hypothesized that the HrpS and HrpR proteins, once produced, bind to the promoter sequence of the *hrpL* gene to induce the production of the HrpL protein, an alternative sigma factor (Xiao et al., 1994). Once the HrpL protein is produced, it activates promoters of other *hrp* genes and some bacterial avirulence (*avr*) genes, which determine gene-for-gene interactions between bacteria and plants (Xiao et al., 1994). HrpS and HrpR are similar in sequence to a family of bacterial proteins that regulates genes involved in diverse metabolic functions, including those involved in nutrient transport and metabolism (Grimm and Panopoulos, 1989). The sequence similarity of *hrpS* and *hrpR* with gene regulators involved in nutrition appears to support the hypothesis that *hrp* genes are involved in obtaining nutrients in the plant apoplast.

An *hrpS* homolog has been found in a very different bacterium, *E. amylovora* (S.V. Beer, personal communication). In *P. solanacearum* a different *hrp* gene (*hrpB*) was found to be involved in the detection of the plant apoplast (Genin et al., 1992). Thus, different bacteria may or may not use the same mechanism to detect the apparently similar environment in the plant apoplast.

Many *hrp* Gene Products Are Components of a Protein Secretion Apparatus

One surprising finding from the sequence analysis was that many *hrp* genes show striking similarities with genes involved in the secretion of proteinaceous virulence factors in human and animal pathogenic bacteria (Fenselau and Bonas, 1995; Huang et al., 1995; Van Gijsegem et al., 1995; Bogdanove et al., 1996b). Most plant-pathogenic bacteria that cause necrosis are gram-negative, and therefore have two cell membranes enveloping the cytoplasm. These bacteria are known to make several types of protein secretion apparatus. For example, *Erwinia chrysanthemi*, a soft-rot-causing bacterium, makes one type (type I) of secretion apparatus for proteases and another (type II) for plant cell-wall-degrading enzymes (Salmond, 1994). Both types of secretion apparatus are widely conserved among many other bacteria, including human pathogens such as *E. coli* and *Pseudomonas aeruginosa* (Salmond, 1994). The *hrp* genes were found to specify a third type (type III), the Hrp secretion apparatus, which appears to be similar to the those discovered in several human-pathogenic bacteria, including *Yersinia* spp. (Fenselau and Bonas, 1995; Huang et al., 1995; Van Gijsegem et al., 1995; Bogdanove et al., 1996b). It is interesting that, although the regulatory *hrp* genes in different bacteria may be different (*hrpS*, *hrpR*, and *hrpL* in *P. syringae* versus *hrpB* in *P. solanacearum*), most *hrp* genes involved in the assembly of the Hrp secretion apparatus are similar among diverse plant pathogenic bacteria. This suggests that although different bacteria may detect the plant apoplast environment in their own unique ways, they nevertheless produce a similar type of protein-secretion apparatus.

BACTERIAL HR ELICITORS

The discovery of the novel Hrp secretion apparatus raised an immediate question: what are the proteins that traverse it? Since *hrp* genes are essential for bacteria, both to elicit the plant HR and to cause disease, it was expected that some of the proteins that traverse the Hrp secretion apparatus may be elicitors of plant HR and that others may be involved in causing necrosis during pathogenesis. Wei et al. (1992) first provided evidence that one of the *E. amylovora hrp* genes (*hrpN*) encodes a proteinaceous elicitor, harpin, which elicits HR necrosis when injected into the apoplast of appropriate plants. Although no *hrpN* gene homolog could be found in *P. syringae*, another proteinaceous HR elicitor, harpin$_{Pss}$, was identified and was shown to be encoded by a different *hrp* gene, *hrpZ* (He et al., 1993). Furthermore, harpin$_{Pss}$ was the first extracellular protein shown to be secreted via the Hrp secretion apparatus (He et al., 1993). Recently, harpin$_{Pss}$ was shown to trigger SAR (Strobel et al., 1996) and plant HR-associated genes (Gopalan et al., 1996). A third bacterial protein elicitor of the HR was identified in *P. solanacearum*, and was named PopA1 (Arlat et al., 1994). The *E. amylovora* harpin, *P. syringae* pv *syringae* 61 harpin$_{Pss}$, and *P. solanacearum* PopA1, although largely dissimilar in primary sequences, share similar properties that may be important in their HR elicitor activities. They are all heat-stable, Gly-rich, and hydrophilic. *E. amylovora* harpin and *P. syringae* pv *syringae* harpin$_{Pss}$ appear to elicit HR irrespective of plant genotypes (Wei et al., 1992; He et al., 1993), whereas *P. solanacearum* PopA1 seems to exhibit a degree of specificity in HR elicitation in cultivars of petunia (Arlat et al., 1994). Mutations in the harpin-encoding gene (*hrpN*) eliminated the HR-eliciting activity of *E. amylovora* in tobacco leaves, suggesting that harpin may be the only HR elicitor produced by *E. amylovora*. In contrast, mutations in the harpin$_{Pss}$-encoding gene (*hrpZ*) only reduced the HR-eliciting activity of *P. syringae* pv *syringae* (Alfano et al, 1996), and *popA1* mutants of *P. solanacearum* elicited a normal HR in tobacco leaves (Arlat et al., 1994), indicating that the latter two bacteria produce other HR elicitors that also traverse the Hrp secretion apparatus.

Avr proteins are candidates for being the "other HR elicitors." *avr* genes mediate the elicitation HR/resistance

only in plants carrying matching plant resistance genes (Keen, 1990; Dangl, 1995). They control the specificity of plant-bacterial interactions, and most *avr* genes are cloned based on this property. For example, *P. syringae* pv *glycinea* *avrB* was cloned based on its ability to convert a virulent (causing disease) strain of *P. syringae* pv *glycinea* into an avirulent (eliciting HR) strain on the soybean cvs Norchief and Harosoy, both of which carry the *RPG1* resistance gene (Keen, 1990). *avrB* was later found to trigger HR in *Arabidopsis thaliana* Columbia, which harbors the plant resistance gene *RPM1* (Bisgrove et al., 1994). *RPG1* and *RPM1* may be the same gene or two different genes possessing similar specificity in the recognition of Avr signals. It appears that during plant-bacterial coevolution, bacteria and plants accumulate a reservoir of *avr* genes and disease-resistance genes, respectively. Whenever a matching bacterial *avr* gene and a corresponding plant disease-resistance gene are present, the interaction becomes incompatible. More than 30 bacterial *avr* genes have been cloned (Dangl, 1995; D.W. Gabriel, personal communication). The function of *avr* genes is strictly dependent on *hrp* genes (Huynh et al., 1989; Gopalan et al., 1996; Pirhonen et al., 1996). Since many Hrp proteins are components of the Hrp secretion apparatus, the simplest explanation is that Avr proteins, like harpins, are secreted via the Hrp secretion apparatus to the plant apoplast. However, when purified Avr proteins were infiltrated into the apoplast of a plant carrying the corresponding plant resistance, no HR was observed (Keen, 1990; Gopalan et al., 1996).

In 1990, *avrD* of *P. syringae* pv *tomato* was found to encode a bacterial cytoplasmic enzyme that uses apparently common bacterial metabolites to produce low-molecular-weight HR elicitors called syringolides (Keen et al., 1990). The mode of action of AvrD appears to be unique, because none of the other *avr* genes has been demonstrated to mediate the production of low-molecular-weight elicitors. It is interesting that like all other bacterial *avr* genes, *avrD* does not function in *hrp* mutants despite the production of syringolides (Keen et al., 1990).

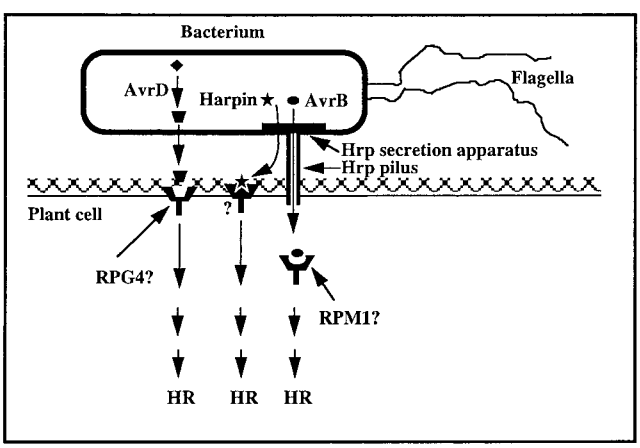

Figure 2. A working model for the delivery of HR elicitors by *P. syringae* and, perhaps, other pathogenic bacteria. AvrD protein is a cytoplasmic enzyme that uses a bacterial metabolite to produce a low-molecular-weight elicitor, which is then diffused to the surface of (or into) the plant cell and interacts with a plant plasmalemma-bound or cytoplasmic receptor (RPG4?). Harpin is secreted via the Hrp secretion apparatus into the plant apoplast and interacts with a hypothetical receptor in the plant plasma membrane. AvrB protein is secreted from the bacterial cytoplasm through the extended Hrp secretion apparatus, including the Hrp pilus, into the plant cell and interacts with a cytoplasmic receptor (RPM1?). RPG4 and RPM1 are plant resistance gene products (receptors?) involved in the signaling pathways of AvrD and AvrB, respectively.

Recently, animal pathogenic bacteria possessing a type-III protein secretion apparatus have been shown to directly inject virulence factors (e.g. protein phosphatase) into host cells (Persson et al., 1995). To test whether Avr proteins are also injected into the plant cytoplasm, *avrB* of *P. syringae* pv *glycinea* was expressed in *A. thaliana* Columbia harboring the corresponding resistance gene *RPM1* (Gopalan et al., 1996). Transgenic Arabidopsis plants expressing AvrB were found to exhibit a systemic HR, leading to seedling death in an *RPM1*-dependent manner (Gopalan et al., 1996). This observation provides strong evidence for direct injection of some Avr proteins or their enzymatic products into the plant cell. Transferring AvrB signal directly into the plant cell is also consistent with the cytoplasmic location hypothesized for Rpm1, a putative AvrB receptor (Grant et al., 1995). It is likely that the original functions of HrpZ and many Avr proteins are to promote parasitism, but the evolving plant surveillance has recognized Avr and HrpZ proteins as elicitors of plant defense responses (Fig. 2).

PERSPECTIVES

Thirty years of research on the mechanism of bacterial elicitation of the HR has generated some of the most intriguing findings in the field of plant-pathogen interactions. It is remarkable that seemingly simple bacteria have evolved such elaborate sensory and protein-delivery systems in adapting to the environment and to physical structures of plant cells, from which the ultimate goal of the infecting bacteria is to get nutrients. It is equally remarkable that the plant has evolved highly sophisticated surveillance systems to recognize bacterial factors as triggers of a cell suicidal program (HR) and other defense responses for combating infecting bacteria. It is hoped that future research will elucidate the mechanism by which bacteria take up nutrients from the plant cell during pathogenesis and how the HR cell death program is actually executed.

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