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The Active Oxygen Response of Cell Suspensions to Incompatible Bacteria Is Not Sufficient to Cause Hypersensitive Cell Death[1]

Judith A. Glazener[2], Elizabeth W. Orlandi, and C. Jacyn Baker*

Molecular Plant Pathology Laboratory, United States Department of Agriculture, Agricultural Reseach Service, Beltsville, Maryland 20705-2350 (J.A.G., C.J.B.); and Department of Botany, University of Maryland, College Park, Maryland 20742–5815 (E.W.O.)

The inoculation of tobacco (*Nicotiana tabacum* L.) suspension cells with bacterial pathogens that elicit the hypersensitive response (HR) in leaves has been shown to elicit production of active oxygen. This response occurs in two phases, the second of which occurs 1 to 3 h after bacterial addition and is unique to HR-causing interactions. The relationship between the phase II active oxygen response and the HR was characterized using *Pseudomonas syringae* pv *syringae* and *P. fluorescens* (pHIR11), which contains a cosmid clone of the *hrp/hrm* region from *P. syringae* pv *syringae*. Tn*phoA* mutations in complementation groups II through XIII of the *hrp* cluster blocked the phase II active oxygen response, whereas mutations in the group I *hrmA* locus did not affect phase II. Despite the normal active oxygen response, bacteria with mutations in the *hrmA* region did not cause the HR in intact tobacco leaves nor did they induce hypersensitive cell death in cell suspensions. The data indicate that the bacteria do not require the *hrmA* region to elicit active oxygen production, but a full and intact *hrp/hrm* region is required to elicit hypersensitive cell death. Therefore, the phase II active oxygen response does not directly cause hypersensitive cell death nor is the response itself sufficient to trigger the HR.

Our laboratory has been interested in plant recognition mechanisms involved in triggering plant defenses, specifically the HR. The first step in doing this was to identify the earliest possible plant responses unique to incompatible interactions that result in HR. Using cell suspensions of tobacco (*Nicotiana tabacum* L.) and soybean, we have identified two plant responses that precede cell death by several hours in incompatible interactions (Baker et al., 1991, 1993a; Orlandi et al., 1992). The XR involves a net uptake of H^+ and efflux of K^+ from the plant cell (Atkinson et al., 1985). The AO response involves two distinct phases of AO production: phase I is the immediate production of AO after the addition of compatible, incompatible, and saprophytic bacteria; phase II is elicited 1 to 3 h later only by incompatible bacteria and occurs concurrently with the XR (Keppler et al., 1989; Baker et al., 1991, 1993b; Orlandi et al., 1992; Levine et al., 1994). The correlation among phase II AO production, the XR, and hypersensitive cell death has been consistent in several plant/bacteria systems tested (for a review, see Baker and Orlandi, 1995).

The *hrp/hrm* genes of *P.s.s.* have been shown to govern the triggering of the HR and XR in tobacco. Transfer of the cosmid pHIR11, which contains the *P.s.s. hrp/hrm* gene cluster, into *P.f.* enabled the nonpathogen to elicit the HR and the XR (Huang et al., 1988). Tn*phoA* mutagenesis of pHIR11 revealed 13 complementation groups (Huang et al., 1991). Mutations in 12 of the complementation groups resulted in the loss of the ability to elicit both the HR and the XR. It now appears that these 12 complementation groups compose the core *hrp* region responsible for producing and exporting harpin$_{Pss}$, which may be at least partially responsible for these responses (He et al., 1993). We report here that the *hrp* cluster of *P.s.s.* encodes for the ability to elicit phase II of the AO response that occurs simultaneously with the XR; however, the entire *hrp/hrm* region is required for the development of the HR. The data indicate that the *hrp*-elicited XR and AO responses are not sufficient to trigger hypersensitive cell death during incompatible plant/*P.s.s.* interactions.

MATERIALS AND METHODS

Bacteria, Plasmids, and Growth Conditions

P.s.s. 61, which induces an HR in tobacco, and *P.f.* 55, a saprophyte, were obtained from M. Sasser (University of Delaware, Newark). *P.f.*(pHIR11) contains the *hrp/hrm* gene cluster from *P.s.s.* A series of Tn*phoA* mutants in each of the complementation groups for the *hrp* cluster of either *P.s.s.* or *P.f.*(pHIR11) and *P.f.*(pCPP30) containing the vector plasmid alone were obtained from Alan Collmer (Cornell University, Ithaca, NY) and Steve Hutcheson (University of Maryland, College Park). Bacteria were maintained with appropriate antibiotics (Huang et al., 1991) and pre-

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[2] Present address: Kevic Company, Inc., 8401 Colesville Rd., Silver Spring, MD 20910.

* Corresponding author; fax 1–301–504–5617; e-mail cbaker@asrr.arsusda.gov.

Abbreviations: AO, active oxygen; cfu, colony-forming units; HR, hypersensitive response; LDC, luminol-dependent chemiluminescence; *P.f.*, *Pseudomonas fluorescens*; *P.s.s.*, *Pseudomonas syringae* pv *syringae*; XR, K^+/H^+ exchange response.

pared for assays as previously described (Baker et al., 1991).

Monitoring pH and AO Production in Plant Suspension Cells

Suspension cells were derived from pith callus of tobacco (*Nicotiana tabacum* L var Hicks) as previously described (Atkinson et al., 1985). Cultures were maintained and prepared for assay as described by Baker et al. (1991) prior to treatment with bacteria. Changes in pH were monitored by a calomel electrode (Orion Research, Boston, MA)[3], and AO production was monitored using a 1251 luminometer (LKB, Turku, Finland) and LDC as described by Orlandi et al. (1992). The LDC assay measures H_2O_2 as an estimate of total AO concentration (Glazener et al., 1991). In all pH and AO assays a final bacterial concentration of 10^7 cfu/mL was used. This was necessary to avoid the increase in H_2O_2-scavenging activity seen with higher inoculum concentrations (Baker et al., 1995). This concentration assured a substantial phase II AO response but resulted in a greatly diminished phase I in cells treated with *P.s.s.*

Evans Blue Assay for Suspension Cell Death

Suspension cells were monitored for cell death using Evans blue. Evans blue is excluded from intact viable cells and is used to estimate cell death either by microscopic examination or using the spectrophotometric procedure described by Baker and Mock (1994). Briefly, aliquots of treated suspension cells were stained with Evans blue. Cells were then washed to remove excess stain, transferred to 1.5-mL Eppendorf tubes, ground with a microsample pestle in the presence of 0.5% SDS to release trapped stain, and centrifuged to pellet cellular debris. The A_{600} of the supernatant was used to monitor cell death.

RESULTS AND DISCUSSION

As previously reported, suspension cells incubated with incompatible bacteria produce a two-phased AO response during the first few hours after treatment (Keppler et al., 1989; Baker et al., 1991, 1993b; Orlandi et al., 1992; Levine et al., 1994). Figure 1 shows a strong XR and phase II AO response in tobacco suspension cells treated with the incompatible pathogen *P.s.s.* at a concentration of 10^7 cfu/mL. The phase I AO response was insignificant at this bacterial concentration, probably because of an insufficient concentration of preformed phase I elicitor (E.W. Orlandi and C.J. Baker, unpublished results). In contrast, the phase I response induced by *P.f.* was very pronounced, but the saprophyte did not elicit the XR or the phase II AO response. Mobilization of cosmid pHIR11 containing the *P.s.s. hrp/hrm* gene cluster into *P.f.*(pHIR11) conferred the ability to induce the XR and the phase II AO response (Fig. 1). This indicates that the gene cluster contains sufficient

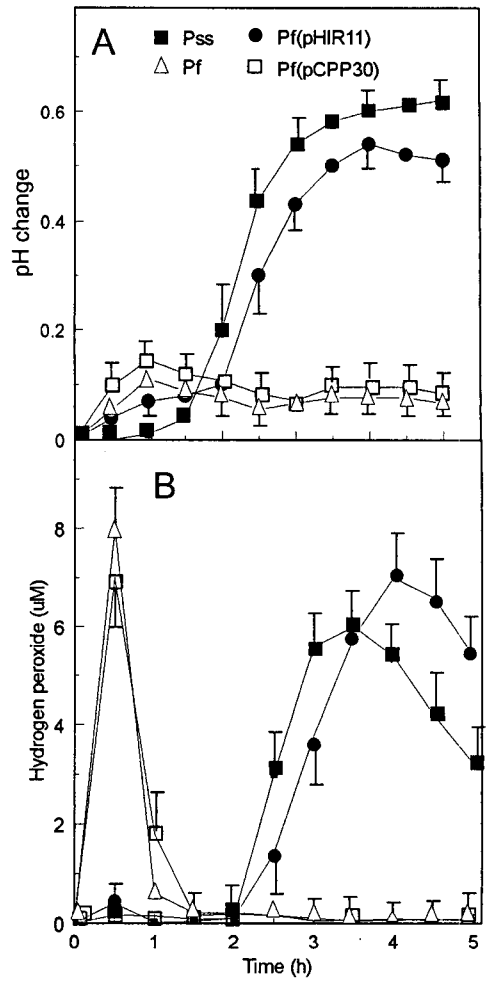

Figure 1. Changes in pH (A) and AO production (B) of tobacco suspension cells incubated with 1×10^7 cfu/mL *P.s.s.*, *P.f.*, *P.f.*(pHIR11) harboring the *hrp* cluster from *P.s.s.*, and *P.f.*(pCPP30), which contains only the vector plasmid. AO production is expressed as micromolar H_2O_2 and was monitored using the LDC assay as described in "Materials and Methods." SE bars are depicted for all data points.

genetic information to enable nonpathogenic bacteria to elicit the phase II AO response and the XR in addition to the HR as previously demonstrated by Huang et al. (1991). It is interesting that phase I AO production was greatly reduced in *P.f.*(pHIR11) at this concentration compared to *P.f.* or *P.f.*(pCPP30) (Fig. 1B). At present it is not clear by what mechanism pHIR11 might reduce phase I AO production; however, preliminary studies indicate that *P.f.*(pHIR11) produces less of the putative phase I elicitor than does *P.f.* (E.W. Orlandi and C.J. Baker, unpublished results).

Huang et al. (1991) identified 13 complementation groups within the *P.s.s. hrp/hrm* gene cluster. They reported that Tn*phoA* mutagenesis in groups II through XIII of *P.s.s.* resulted in mutants unable to elicit either the HR in plants or the XR in suspension cells. Similarly, *P.f.*(pHIR11) with mutations in these complementation groups was unable to elicit the HR. In the current study, mutations within these complementation groups rendered *P.s.s.* and

[3] Mention of a trade name, proprietary product, or vendor does not constitute a guarantee of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other vendors that may also be suitable.

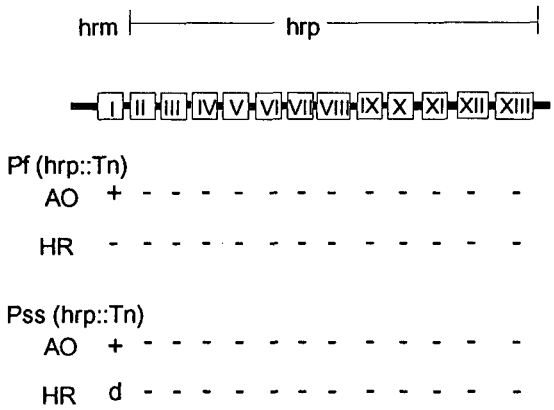

Figure 2. Correlation between HR in tobacco leaves and phase II AO production by *P.s.s.* or *P.f.*(pHIR11) with insertion mutations in different complementation groups. AO production was monitored in suspension cells inoculated with 1×10^7 cfu/mL bacteria using the LDC assay described in "Materials and Methods." HR development was monitored in intact leaves inoculated with 1×10^8 cfu/mL bacteria. +, A typical phase II AO response; −, the absence of either phase II AO or the development of HR symptoms; d, a nontypical HR consisting of spotty necrosis confined to the area of infiltration but not evident until 2 to 3 d after infiltration.

P.f.(pHIR11) unable to elicit the phase II AO response (Fig. 2). Therefore, it appears that complementation groups II through XIII must all be functional to obtain the phase II AO response as well as the XR and HR.

However, mutations in complementation group I, the *hrm*A region, of *P.s.s.* appeared to suppress but not eliminate plant responses to the bacterium: elicitation of necrosis was delayed 1 to 2 d in tobacco leaves and the XR in suspension cells was reduced (Huang et al., 1991). The same *hrm*A mutation in *P.f.*(pHIR11) completely prohibited the development of the HR in leaves. In the current study, we compared the *hrm*A mutants *P.s.s.* 2070 and *P.f.*(pCPP2071) for their ability to elicit these responses in suspension cells. The *hrm*A mutants retained the ability to elicit the XR and phase II AO response (Fig. 3) comparable to that seen in response to bacteria with an intact *hrm*A region (Fig. 1). Despite the substantial XR and phase II AO response, however, suspension cells treated with these mutants did not undergo rapid cell death (Fig. 4). As found by Huang et al. (1991), when inoculated into tobacco leaves *P.s.s.* 2070 did not cause the typical HR but resulted in a confined area of spotty necrosis 2 to 3 d after infiltration. This response has been noted as "delayed" (Fig. 2) because it does not resemble a typical HR in which infiltrated leaf tissue is completely collapsed and necrotic within 10 to 18 h after infiltration. Leaf panels infiltrated with *P.f.*(pCPP2071) did not develop symptoms (Fig. 2).

The relationship between the two early recognition responses, the XR and phase II AO response, and the development of the HR has been of interest for several years. Atkinson et al. (1985) first described the XR in incompatible plant/bacteria interactions. Additional studies using Tn*5* mutants of *P.s.s.* found that mutants that lost the ability to induce the HR in plants also lost the ability to induce the XR (Baker et al., 1987). An apparent breakdown in the correlation between the XR and HR was noted by Baker et al. (1990) when tobacco leaves pretreated with heat-killed bacteria or polygalacturonic acid underwent an XR but did not display any hypersensitive cell death in response to *P.s.s.* This suggested that, although there were no reported instances in which there was not a substantial XR prior to the HR, the XR itself did not cause cell death. It was hypothesized that there were other necessary processes between the XR and the subsequent HR that could be inhibited, effectively blocking the development of the HR. The current study suggests that the same may be true of the phase II AO response: the phase II AO response consistently precedes hypersensitive cell death, but plant cells that produce this AO response do not necessarily develop the HR.

This is contradictory to a recent report by Levine et al. (1994) that suggested that the AO response to incompatible bacteria is itself sufficient to trigger hypersensitive cell death in soybean suspension cells. In their study, the ad-

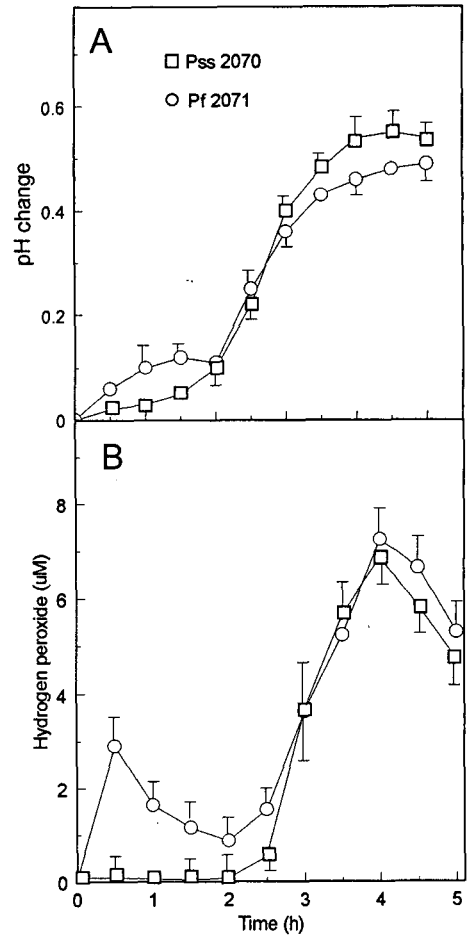

Figure 3. Changes in extracellular pH (A) and AO production (B) in tobacco suspension cells inoculated with 1×10^7 cfu/mL of the *hrm*A mutants *P.s.s.* 2070 and *P.f.*(pCPP2071). AO production is expressed as micromolar H_2O_2 and was monitored using the LDC assay as described in "Materials and Methods." SE bars are depicted for all data points.

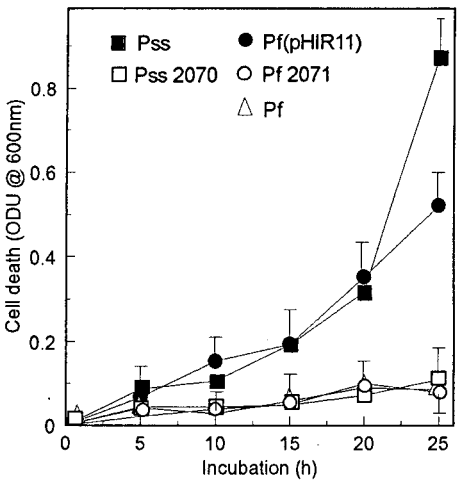

Figure 4. Cell death of tobacco cell suspensions incubated with 1×10^7 cfu/mL *P.s.s.*, *P.f.*, or *P.f.*(pHIR11) as well as *hrm*A mutants *P.s.s.* 2070 and *P.f.*(pCPP2071). Cell death was determined spectrophotometrically using the Evans blue assay described in "Materials and Methods." ODU, Optical density units.

dition of 4 to 8 mM exogenous H_2O_2 was required to elicit cell death comparable to that seen with the addition of incompatible bacteria. These concentrations are far greater than the micromolar amounts monitored in tobacco and soybean suspension cells in this (Figs. 1B and 3B) and previous studies (Legendre et al., 1993; Baker et al., 1995). Legendre et al. (1993) estimated that a 1-mL suspension of optimally responding soybean suspension cells produced 120 μM H_2O_2 during a 15-min period following treatment with a saturating concentration of polygalacturonide elicitor. They estimated that as much as 75% of this amount may be consumed or detoxified by other reactions and would not be measurable. Allowing for estimated rates of AO-scavenging activity in cell suspensions, Baker et al. (1995) estimated a peak rate of H_2O_2 production to be 6 and 12 μM min^{-1} in soybean and tobacco cells, respectively, after elicitation with incompatible bacteria. Therefore, although the study of Levine et al. (1994) indicates that H_2O_2 supplied in sufficient amounts may trigger cell death, the relatively small concentrations of AO produced during the first few hours of an incompatible plant/bacteria interaction do not appear to be sufficient to be the sole trigger of the HR.

The current study confirms earlier reports that the *P.s.s. hrp/hrm* cluster is essential to development of the HR in tobacco leaves. Previous studies have indicated that proteinaceous products of *hrp* genes, the harpins, may be responsible for elicitation of the HR in incompatible reactions. $Harpin_{Ea}$ from *Erwinia amylovora* has been demonstrated to elicit the HR as well as the XR and AO response (Wei et al., 1992; Baker et al., 1993b). $Harpin_{Pss}$, the proteinaceous product of the *P. syringae hrp*Z gene in complementation group XII, has been shown to elicit the HR in tobacco plants (He et al., 1993) and to elicit AO production in suspension cells (E. W. Orlandi and C.J. Baker, unpublished results), although it differs substantially in primary structure from $harpin_{Ea}$. Products of several of the other *hrp* complementation groups are likely to be involved in the secretion of *hrp* proteins (Huang et al., 1992, 1995). Mutations in these groups, therefore, interfere with secretion of *hrp* products, such as $harpin_{Pss}$ (Huang et al., 1995), and with development of the HR, as shown in the current study.

However, if the production of $harpin_{Pss}$ is sufficient for the elicitation of the HR, XR, and phase II AO response, it is difficult to understand why *P.f.*(pHIR11) *hrm*A mutants, which apparently produce sufficient harpin to elicit substantial XR and AO responses, do not cause hypersensitive cell death. One possibility is that $harpin_{pss}$ is just one of two or more triggers/elicitors responsible for the HR during plant/bacteria interactions. Therefore, the addition of $harpin_{Pss}$ at nonbiological levels may result in cell death (He et al., 1994), but the triggering of the HR during incompatible plant/bacteria interactions may not be solely the result of $harpin_{Pss}$ production. A recent study by Alfano et al. (1996) has confirmed that the *hrm*A mutants *P.f.*(pCPP2071) and *P.f.*(pCPP2070) secrete $harpin_{pss}$ at levels comparable to *P.f.*(pHIR11) but do not elicit the HR. Using functionally nonpolar *hrp*Z deletion mutants, this group also demonstrated that *hrp*Z is necessary for saprophytic bacteria carrying pHIR11 to elicit the HR in tobacco leaves but that it is not sufficient for it. The authors proposed that *hrm*A may have a function similar to an *avr* gene and that the *hrm*A product interacts with $harpin_{Pss}$, or acts independently as a necessary second signal, to induce the HR.

In this and previous studies, induction of the XR and the phase II AO response consistently preceded development of the HR in incompatible plant/pathogen reactions. However, studies with *hrm*A mutants demonstrate that hypersensitive cell death does not always follow induction of the XR and phase II AO responses. Although these two responses indicate that the plant cell has recognized the bacteria as an incompatible pathogen, it is likely that other cellular processes must be triggered after recognition to result in hypersensitive cell death. Although artificially high levels of $harpin_{Pss}$ and H_2O_2 have both been demonstrated to elicit hypersensitive cell death, the current study indicates that the HR that results from incompatible plant/bacteria interactions is likely to be the result of two or more triggers/elicitors acting in concert.

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