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Rapid Communication

Harpin, An Elicitor of the Hypersensitive Response in Tobacco Caused by *Erwinia amylovora*, Elicits Active Oxygen Production in Suspension Cells[1]

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Active oxygen (AO) production and a K^+/H^+ exchange response (XR) are two concurrent early events associated with incompatible plant-bacteria interactions that result in a hypersensitive response (HR). Recently, a protein, termed harpin, produced by *Erwinia amylovora* has been reported to be the elicitor responsible for the HR caused by this pathogen. Although both the bacterium and harpin are reported to induce XR in tobacco (*Nicotiana tabacum*) cell suspensions, there have been no reports regarding the concurrent production of AO in this system. Here we report that *E. amylovora* stimulates the AO response, whereas an *E. amylovora* mutant that does not produce harpin does not elicit the AO response. In addition, a cell-free preparation of harpin induces AO production. This study indicates that harpin may be the bacterial elicitor of the XR and AO responses during the development of *E. amylovora*-induced HR.

Bacterial induction of the HR in soybean (*Glycine max*) and tobacco (*Nicotiana tabacum*) plants is preceded by elicitation of two early plant cell responses: the XR (Atkinson et al., 1985; Baker et al., 1991; Orlandi et al., 1992) and the AO response (Adám et al., 1989; Keppler et al., 1989; Baker et al., 1991; Glazener et al., 1991a). The XR appears to be an ion-exchange response involving an uptake of H^+ from the extracellular space and an efflux of K^+ from the plant cell. The AO response in incompatible interactions consists of two phases. Phase I AO production is nonspecific and occurs immediately after addition of most saprophytic and pathogenic bacteria. The phase II AO response occurs concurrently with the XR and is specifically stimulated only by incompatible (HR-causing) bacteria. The *hrp* cluster of *Pseudomonas syringae* pv *syringae*, which is responsible for the nonhost HR caused by this pathogen (Huang et al., 1991), was shown to be responsible for eliciting the XR and phase II AO production in tobacco suspension cells (Glazener et al., 1991a).

Recently, a protein termed harpin from *Erwinia amylovora* has been reported to cause the HR associated with this bacterium (Wei et al., 1992a). The *hrp*N gene, which encodes this protein, is located within the *hrp* gene cluster, which enables *E. amylovora* (*Ea*321) to elicit the HR on tobacco. The Tn10 mutant, *Ea*321K49, which does not produce harpin in culture, also lacks the ability to cause the HR.

Both *E. amylovora* and harpin reportedly stimulate the XR in tobacco suspension cells (Wei et al., 1992a) but have not been investigated as elicitors of AO production. Because of recent reports demonstrating involvement of AO in bacterial HR (Adám et al., 1989; Glazener et al., 1991a; Orlandi et al., 1992), and defense-related responses such as lignification (Peng and Kúc, 1992), phytoalexin production (Apostol et al., 1989), lipid peroxidation (Rogers et al., 1988), and oxidative cross-linking of cell-wall structural proteins (Bradley et al., 1992), it is important to know if harpin also stimulates AO production. Here we report that *E. amylovora* and an *Escherichia coli* clone expressing harpin induce phase II AO production in tobacco suspension cells similar to that of other HR-inducing bacteria. We also show that a cell-free preparation of harpin induces AO production from tobacco cells.

MATERIALS AND METHODS

The bacterial strains used in this study were obtained from Steve Beer (Cornell University) and were maintained as previously described (Wei et al., 1992a). Strains *Erwinia amylovora* 321 (*Ea*321) and *Escherichia coli* strain DH5α(pCPP430) (*Ec*DH5α[pCPP430]), the latter of which contains the *hrp* cluster from *Ea*321, both produce harpin in culture and produce an incompatible response (HR) on tobacco (*Nicotiana tabacum* var Hicks) leaves. *E. amylovora* strain 321K49 (*Ea*321K49) and *E. coli* strain DH5α(pCCP430K49) (*Ec*DH5α[pCCP430K49]) have mutations in the *hrp* gene cluster and do not produce harpin or a HR response on tobacco leaves.

Bacteria were grown on agar plates for approximately 18 h prior to inoculation of tobacco cell suspensions. Bacterial cells were washed in deionized water, collected by centrifugation, and resuspended in deionized water for a final concentration of 10^8 cfu/mL. The cell-free preparation of harpin

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Abbreviations: AO, active oxygen; cfu, colony-forming unit; HR, hypersensitive response; LDC, luminol-dependent chemiluminescence; XR, K^+/H^+ exchange response.

was a gift from Steve Beer and was prepared by sonication of *E. coli* strains expressing harpin as described by Wei et al. (1992a).

Tobacco cell suspension cultures were derived from pith callus and grown as previously described (Atkinson et al., 1985). Cells used in assays were collected, washed, and resuspended in assay medium (175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, and 0.5 mM Mes, pH 6.1) as previously described (Baker et al., 1991). Changes in extracellular pH were measured using a pH electrode and changes in extracellular $[K^+]$ were determined using ion chromatography as previously described (Baker et al., 1991). AO production by cell suspensions treated with either bacterial strains or the harpin preparation were measured using LDC as previously described (Glazener et al., 1991b; Orlandi et al., 1992). K252a (Fluka Chemical) or $LaCl_3$ (Sigma Chemical) was added to washed cell suspensions for final concentrations of 1.6 or 250 μM, respectively. All experiments represent the averaged results from at least two experiments with two replicates each.

RESULTS AND DISCUSSION

Inoculation of tobacco suspension cells with either *Ea*321 or *Ec*DH5α(pCPP430), which produce harpin, or *Ea*321K49 or *Ec*DH5α(pCCP430K49), which do not produce harpin, caused an immediate increase in phase I AO production (Fig. 1) that lasted about 0.5 h. Phase II AO production, however, occurred only after treatment with the bacterial strains that produce harpin. This phase II AO production began after a lag of approximately 2 h for *Ea*321 (1 h for *Ec*DH5 α[pCPP430]) and was sustained for several hours (Fig. 1). This is similar to previously described systems in tobacco and soybean (Baker et al., 1991; Orlandi, 1992), where both incompatible and compatible bacteria stimulated the phase I AO response but only incompatible bacteria caused the phase II AO response.

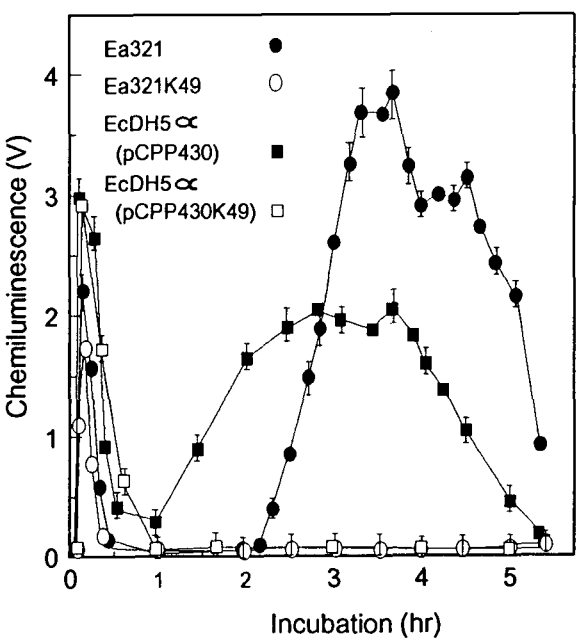

Figure 1. AO levels in tobacco cell suspensions incubated with 10^8 cfu/mL of *E. amylovora* and *E. coli* strains. LDC was monitored as previously described (Glazener et al., 1991b; Orlandi et al., 1992). Data are expressed as volts (V) of response. (The bacterial strains that cause HR on tobacco leaves are represented by the closed symbols.)

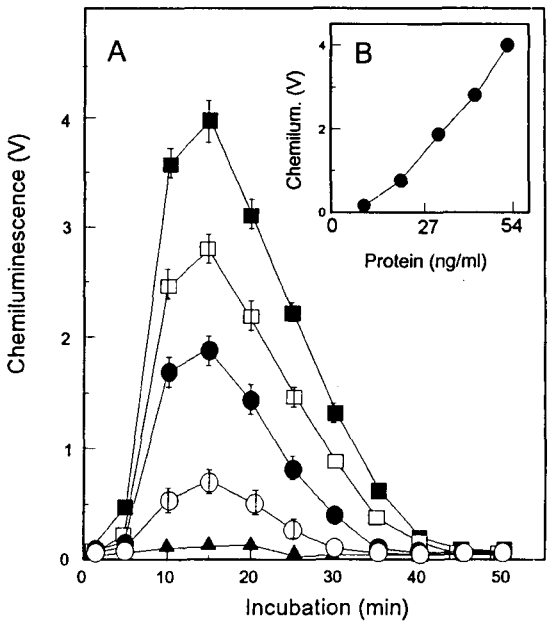

Figure 2. Changes in AO levels of tobacco cell suspensions incubated with varying concentrations of harpin (■, 0.54; □, 0.0443; ●, 0.033; ○, 0.021; ▲, 0.011 μg of protein/mL). LDC was monitored as previously described (Glazener et al., 1991b; Orlandi et al., 1992). Data are represented as volts (V) of response.

Addition of a cell-free preparation of harpin to suspension cells resulted in an increase in AO production that was proportional to the amount of harpin added (Fig. 2B). After 30 to 45 min, the AO levels returned to baseline (Fig. 2A). Predigestion of the harpin preparation with protease, as described by Wei et al. (1992a), completely abolished its ability to induce AO production.

The induction of the phase II AO response by only the harpin-producing bacterial strains indicates that harpin is a likely elicitor of *Erwinia*-induced phase II AO production. However, the relatively short-lived AO production induced by harpin is more characteristic of a bacterial phase I AO response (Fig. 2A). This apparent inconsistency may be explained by results reported by Wei et al. (1992b). In that study, *E. amylovora hrp* gene expression was found to increase beginning at 2 h and continuing for up to 6 h after the bacteria were added to tobacco cell suspensions. This indicates that plant cells inoculated with *Ea*321 are likely to be exposed to continuous and increasing levels of harpin during the same time period in which phase II AO increases are measured. This continuous and increasing exposure is very different from the single harpin addition represented in Figure 2A. Furthermore, the time course of *hrp* gene expression

(Wei et al., 1992b) and the elicitation of phase I AO by nonharpin-producing bacterial strains seem to indicate that harpin is not the bacterial elicitor of this earlier AO response.

As in previous studies with *Pseudomonas* spp. in tobacco and soybean (Baker et al., 1991; Orlandi et al., 1992), *Ea*321 induced extracellular pH and [K^+] increases after a lag of approximately 2 h (Fig. 3, A and B), whereas *Ea*321K49 did not induce corresponding changes. *Ec*DH5α(pCPP430) also elicited an XR whereas the nonharpin-producing *E. coli* strains did not (data not shown). Treatment of suspension cells with harpin elicited an immediate increase in pH and [K^+] that appeared to level off within approximately 2 h. The timing of these XR responses corresponds well with the AO responses induced by treatment with the bacteria and harpin.

The XR and AO responses elicited by incompatible bacteria require a sustained influx of Ca^{2+} (Atkinson et al., 1990; E.W. Orlandi, personal communication). Preliminary studies indicate that both responses may also require protein kinase-mediated protein phosphorylation (M. M. Atkinson, unpublished data; E.W. Orlandi, personal communication). To gain insight as to whether elicitation of the AO response by harpin might have similar requirements, $LaCl_3$, a Ca^{2+} channel blocker, or K252a, a protein kinase inhibitor, was added to suspension cells prior to treatment with the cell-free preparation of harpin. Both inhibitors completely blocked the production of AO (Fig. 4). In these preliminary studies it is interesting to note that addition of the inhibitors 10 min after harpin addition had little effect on AO production. This suggests that the elicitation of AO production by the single addition of harpin is probably finished within 10 min.

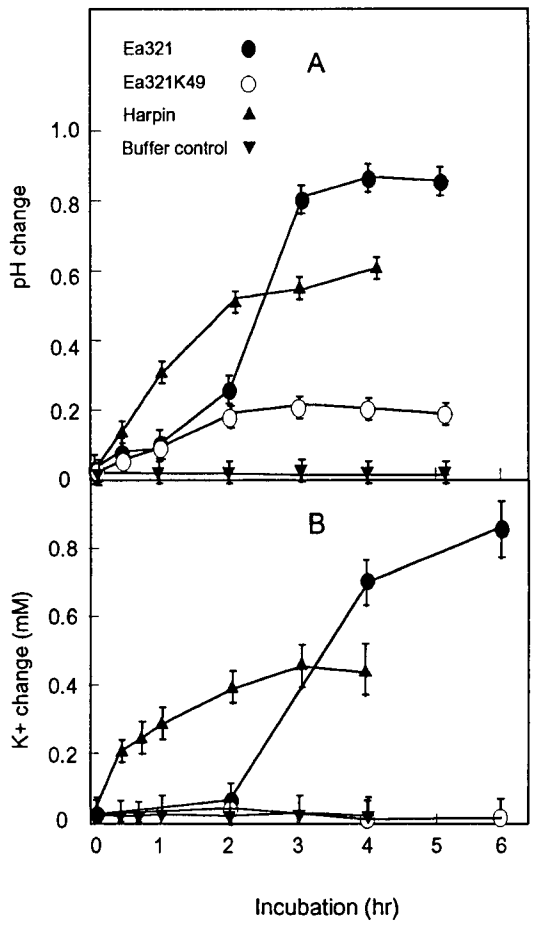

Figure 3. Changes in extracellular pH and [K^+] in tobacco cell suspensions treated with 10^8 cfu/mL of *E. amylovora* 321 or *E. amylovora* 321K49, or 4.1 μg of protein/mL of cell-free harpin preparation. Measurements were taken using a pH electrode and ion chromatography as previously described (Baker et al., 1991).

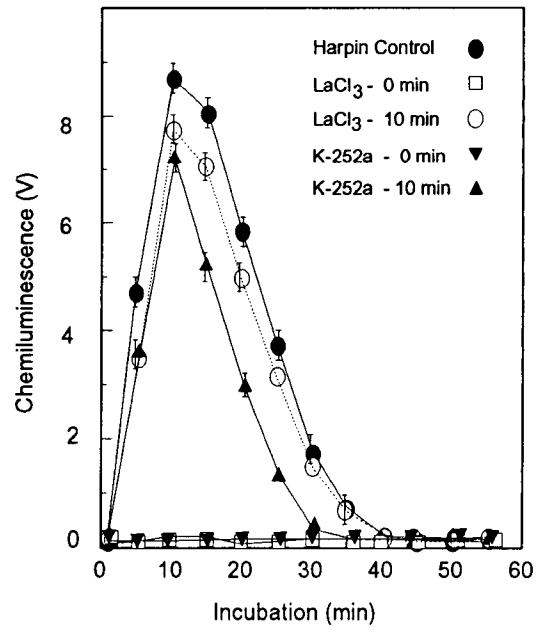

Figure 4. AO production in tobacco cell suspensions treated with 0.05 μg of protein/mL of harpin alone or with harpin and K-252a (1.6 μM final concentration) or $LaCl_3$ (250 μM final concentration). Inhibitors were added immediately prior to harpin addition (0 min) or 10 min after harpin addition (10 min). LDC was monitored as previously described (Glazener et al., 1991b; Orlandi et al., 1992). Data are represented as volts (V) of response.

The results from the current study indicate that a specific product of the *hrp* cluster of *E. amylovora* is required to cause the phase II AO production associated with the XR and HR. Harpin is the first product of a gene in an *hrp* cluster demonstrated to be an elicitor of AO. Although several fungal and plant cell-wall elicitors have been demonstrated to induce AO (Lindner et al., 1988; Apostol et al., 1989; Schwacke and Hager, 1992), harpin is the first bacterial protein associated with AO production. Based on this report and that of Wei et al. (1992a), harpin should prove to be a useful cell-free tool to further investigate plant responses to incompatible bacteria.

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