Research Note

Hypersensitive Response Elicited by *Erwinia amylovora* Harpin Requires Active Plant Metabolism

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Biochemical processes involved in hypersensitive plant cell death were explored with purified harpin and inhibitors of eukaryotic metabolic processes. α-Amanitin, cycloheximide, vanadate, lanthanum, and WIN50805 prevented or delayed the HR elicited by harpin in tobacco leaves; k-252a and staurosporine had no effect. In contrast, the K^+/H^+ exchange response of tobacco cell cultures elicited by harpin was inhibited by only lanthanum and k-252a.

Additional keywords: hrp genes, plant resistance, signal transduction.

hrp genes control the ability of plant pathogenic bacteria both to elicit the hypersensitive response (HR) in nonhost plants and to cause disease in host plants (reviewed by Willis *et al.* 1991). One of the *Erwinia amylovora hrp* genes (*hrpN*) encodes a proteinaceous HR elicitor named harpin (Wei *et al.* 1992). Purified harpin is biologically active and, by itself, can trigger the HR in a variety of different nonhost plants (Wei *et al.* 1992). Mutations in *hrpN* abolish the ability of *E. amylovora* to elicit the HR in nonhost plants or to produce symptoms in host plants (Wei *et al.* 1992). These data suggest that harpin is both necessary and sufficient for elicitation of the HR by *E. amylovora*. Harpin also elicited the H^+ influx/K^+ efflux response (XR), which is an early physiological response associated with HR (Atkinson *et al.* 1985; Baker *et al.* 1993). The current study addresses whether plant metabolism is involved in the development of the HR elicited by *E. amylovora* harpin.

We first constructed pCPP2139 for overexpression of harpin. The vector pINIII^{113}-A2 (Masui *et al.* 1983) was modified by inserting the *Xba*I-*Hin*dIII portion of the pBluescript SK multiple cloning site into *Xba*I/*Hin*dIII digested pINIII^{113}-A2. The resulting plasmid was designated pCPP50. The 1.3-kb *Hin*dIII fragment carrying the *hrpN* gene of *E. amylovora* was then ligated into pCPP50. *E. coli* DH5α transformants containing the recombinant plasmids were grown in terrific broth (Sambrook *et al.* 1989) in the presence of 0.5 mM isopropyl-β-D-thiogalactopyranoside and were screened for production of harpin on SDS-10% polyacrylamide gels. The plasmid that contained the *hrpN* gene under the control of the *lpp/lac* promoters was identified and was designated pCPP2139.

Harpin was then prepared from DH5α(pCPP2139) using the following procedure. Bacteria were grown overnight at 30° C in 200 ml of terrific broth with 0.5 mM isopropyl-β-D-thiogalactopyranoside. Cells were collected by centrifugation at 15,000 *g* for 10 min, washed once with 50 ml of ice-cold 5 mM 2-(4-morpholino)-ethane sulfonic acid (MES) buffer (pH 5.5), sonicated in 10 ml of the same buffer containing 1 mM phenylmethylsulfonyl fluoride as described before (Wei *et al.* 1992). The sonicate was centrifuged at 15,000 *g* for 30 min, and the pellet was discarded. The supernatant was heated in boiling water for 10 min. The precipitates containing denatured proteins were removed by centrifugation at 15,000 *g* for 30 min. The supernatant was passed through Sephadex G-25 spin columns equilibrated with 5 mM MES buffer (pH 5.5), aliquoted, and stored at –80° C. Protein prepared similarly from DH5α cells (without pCPP2139) was used as a control in assays for the XR.

To test the involvement of various plant metabolic processes in the HR, we infiltrated the intercellular space of tobacco leaves with 1×10^7 cells/ml *E. coli* DH5α(pCPP430), which produces harpin in culture (Beer *et al.* 1990) and one of four compounds: WIN50805 (2 μM), α-amanitin (200 μg/ml), lanthanum (1 mM), and vanadate (50 μM). All four compounds affected (prevented or delayed) the development of the HR (data not shown). We then tested the effect of these and other metabolic inhibitors on the HR elicited by harpin. Most compounds tested were mixed with harpin in water just before infiltration. Because α-amanitin was expected to have a low permeability through the plant plasma membrane, it was first infiltrated into tobacco leaf panels. After the water-soaked appearance disappeared (in about 1 hr), harpin was infiltrated into the same panel. The following five compounds inhibited the harpin-elicited HR in tobacco leaves: vanadate, α-amanitin, cycloheximide, lanthanum, and cobalt (Table 1, Fig. 1). WIN50805 delayed the HR elicited by harpin, whereas k-252a and staurosporine had no effect. Inhibitors alone at the same tested concentrations had no apparent effects on tobacco leaves in the first 48 hr. However, chlorosis was seen in the infiltrated areas after 3–4 days (Fig. 1). To ascertain whether the inhibition was due to interference of the com-

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pounds with the harpin activity, rather than plant metabolism, we incubated harpin with each of the compounds for 30 min, followed by dialysis to remove the compounds prior to infiltration. Typical HR symptoms were observed in all cases, suggesting that none of the compounds directly inactivated harpin.

We next tested whether these HR inhibitors also inhibit the XR. Consistent with our previous observations (Wei *et al.* 1992), harpin elicited a typical XR, as indicated by rapid pH increase of the assay medium containing tobacco cells (Fig. 2). We then added each of the following six compounds individually to the assay mixture: α-amanitin, cycloheximide, k-252a, lanthanum chloride, cobalt chloride, or sodium vanadate. As shown in Figure 2, α-amanitin, cobalt, and vanadate, which inhibited the HR, did not inhibit the XR. Cycloheximide had a partial inhibitory effect only after 2 hr. In contrast, k-252a, which did not inhibit the HR, strongly inhibited the XR. The only compound that inhibited both the HR and XR was lanthanum (Fig. 2). However, we could not test whether death of the cultured tobacco cells can also be prevented by

Table 1. Effect of metabolic inhibitors on the harpin-elicited HR in tobacco leaves[a]

Chemical	Concentration	Probable targets	Inhibition of HR[b]
WIN50805	2.0×10^{-6} M	Y-kinases	+
k-252a	2.2×10^{-6} M	S/T kinases	−
Staurosporine	2.2×10^{-6} M	S/T kinases	−
$LaCl_3$	1.0×10^{-3} M	Ca^{2+}-channels	++
$CoCl_2$	5.0×10^{-4} M	Ca^{2+}-channels	++
α-Amanitin	2.2×10^{-4} M	RNA polymerase II	++
Cycloheximide	7.1×10^{-5} M	80S ribosome	++
Na_3VO_4	5.0×10^{-5} M	ATPases/Y-phosphatase	++

[a]Panels of tobacco leaves were infiltrated with harpin (40 μg/ml) and one of the compounds listed in the table. All compounds except α-amanitin were mixed with harpin in water just before infiltration. α-Amanitin was infiltrated into tobacco leaf panels 1 hr before infiltration of harpin into the same panel. The HR was measured as visible tissue necrosis. The HR appeared within 12 hr when only harpin was infiltrated into leaves. Abbreviations: Y, tyrosine; S, serine; T, threonine.
[b]−, No inhibition; +, the HR was delayed 3 to 6 hr; ++, no HR.

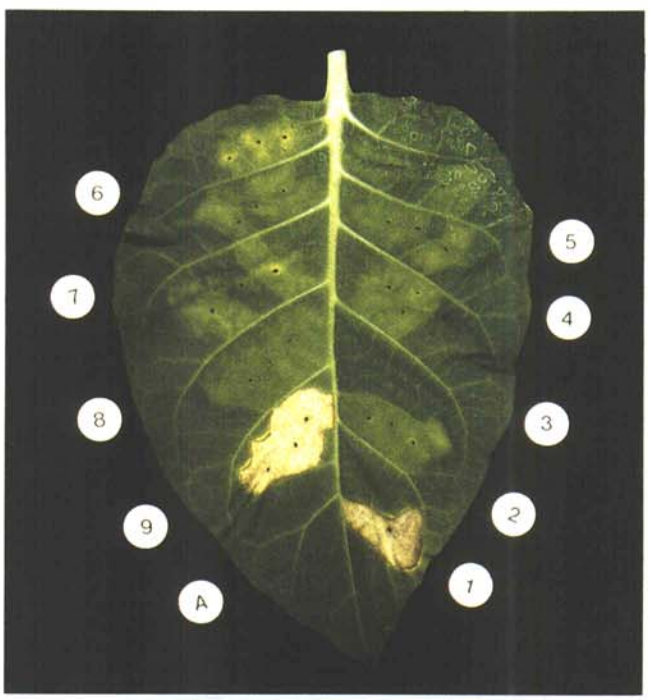

Fig. 1. Inhibition of the harpin-elicited HR in tobacco leaves by metabolic inhibitors. The leaf was infiltrated with 2.2×10^{-6} M k-252a (panel 1), 2.2×10^{-4} M α-amanitin (panels 2 and 6), 7.1×10^{-5} M cycloheximide (panels 3 and 7), 5×10^{-5} M sodium vanadate (panels 4 and 8), and 1×10^{-3} M lanthanum chloride (panels 5 and 9) with (panels 1–5) or without (panels 6–9) 40 μg/ml harpin. Except for α-amanitin (which was infiltrated 1 hr before the infiltration of harpin in the same panel), the inhibitors were coinfiltrated with harpin. WIN50805, k-252a, and staurosporine were dissolved in 0.01% DMSO. All other compounds were dissolved in water. Panel A was infiltrated with harpin alone. The leaf was photographed four days after infiltration. The same results were observed when each of the compounds was infiltrated with harpin in separate leaves.

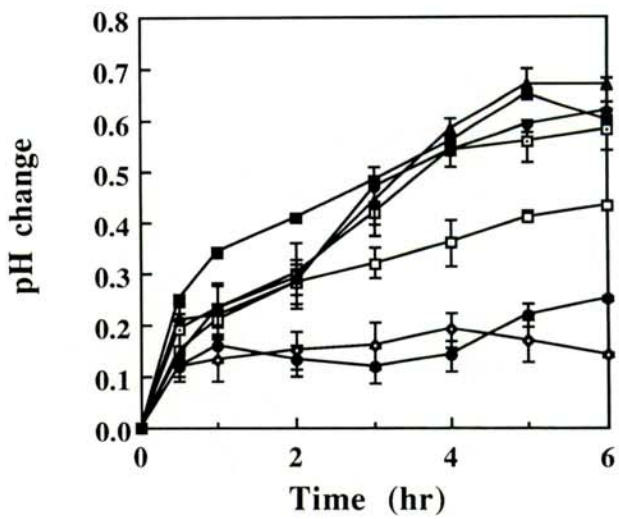

Fig. 2. Effects of metabolic inhibitors on the XR of tobacco cell suspension cultures elicited by harpin. Tobacco cells (NT1) were supplied by the Cornell University Plant Science Center Cell Culture and Transformation Facility. Assays were performed as described previously (Atkinson *et al.* 1985), except for the following modifications: 1) The XR assay medium was buffered with 0.5 mM MES (pH 6.0) and contained 0.05 g/ml washed tobacco cells. 2) Assays (2 ml final volume for each treatment) were done in 24-well microtiter plates. Inhibitors first were incubated with tobacco cells for 25 min, and then harpin (final concentration of 40 μg/ml) or the equivalent volume of the control preparation was added just before the first measurement. The pH of the assay mixture was measured hourly for 6 hr. Lines from top to bottom: filled triangle, cobalt chloride + harpin; filled diamond, vanadate chloride + harpin; filled square, α-amanitin + harpin; stippled square, harpin alone; open square, cycloheximide + harpin; filled circle, lanthanum chloride + harpin, open diamond, k-252a + harpin. Concentrations of harpin and inhibitors are the same as for Figure 1. Each datum point was derived by subtracting the reading of the harpin treatment from the reading of the control treatment. The results presented (mean and standard errors) were calculated from four independent experiments.

the compounds that inhibited the HR because a single addition of harpin even at 200 μg/ml (fivefold higher concentration) did not result in a significantly higher level of cell death compared with the controls without harpin, as indicated by cell browning and Evans blue staining of cells, under the conditions in which the XR was consistently observed.

The concept that the plant HR is an active plant metabolic process previously was supported by three types of physiological evidence. First, HR development is profoundly influenced by environmental factors (light, temperature, relative humidity) that affect plant metabolic processes (Sequeira 1979). Second, the HR always is associated with active plant resistance against avirulent pathogens. Third, the HR elicited by avirulent pathogens can be prevented by certain metabolic inhibitors. Yoshikawa *et al.* (1978) and Keen *et al.* (1981) showed that blasticidin S, a protein synthesis inhibitor in soybean, prevents the HR caused by isolates of *Phytophthora megasperma* var. *sojae* or *Pseudomonas syringae* pv. *glycinea* that are incompatible with certain soybean cultivars. Atkinson *et al.* (1990) showed that the HR elicited by *P. s.* pv. *syringae* was inhibited by Ca^{2+}-channel blockers. Our use of purified harpin precluded possible effects of inhibitors on the pathogen and provided direct evidence that the HR is indeed an active plant metabolic process.

In a recent study, we showed that α-amanitin, vanadate, lanthanum, and cycloheximide also prevented the HR elicited by the *P. s.* pv. *syringae* HR elicitor ($harpin_{Pss}$), which is different in the primary structure from the *E. amylovora* harpin (He *et al.* 1993). This suggests that the HR elicited by different harpins involves the same biochemical processes. Atkinson *et al.* (1985) reported that the pH increase (the result of H^+ influx) in the XR assay medium was coupled with K^+ efflux during the HR. Although we did not investigate effects of HR inhibitors on HR-associated K^+ efflux in this study, Baker *et al.* (1993) have shown that the pH increase in the XR assay medium caused by *E. amylovora* harpin is accompanied by K^+ efflux. It is possible that the XR is the result of changes in the permeability of the plant plasma membrane that occur during early stages in the development of the HR, but it plays no role in subsequent steps leading to the HR. These ion fluxes may be a more general plant plasma membrane response to environmental insults because chemical stimuli that do not appear to trigger the HR can also elicit the XR (Felix *et al.* 1991). These data indicate that the K^+/H^+ exchange response may not be directly involved in the development of the HR. However, our results with harpin must be interpreted cautiously. XR assays were done with suspension-cultured cells, while HR assays were done with whole plants. The uptake and metabolism of added inhibitors might be different under these conditions. It is also possible that harpin is not perceived in the same way by suspension culture cells and by cells in a leaf. The fact that harpin does not appear to significantly cause death of suspension cells makes the possibility more likely. In light of this observation, the use of suspension culture cells to study the HR needs to be reevaluated.

It is noteworthy that lanthanum, but not cobalt or vanadate inhibited the XR elicited by harpin. Atkinson *et al.* (1989 and 1990) showed that all three compounds inhibited the XR elicited by live bacteria. The simplest explanation for this discrepancy is that these compounds inhibit the production or deployment of harpin by living bacteria. It is also likely that the amount of exogenous harpin applied in our experiments is substantially higher than that delivered by living bacteria. However, both vanadate and cobalt effectively inhibited elicitation of the HR by harpin in tobacco leaves. Furthermore, we have no explanation for the difference between the two calcium channel blockers, cobalt and lanthanum, in inhibiting the XR.

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